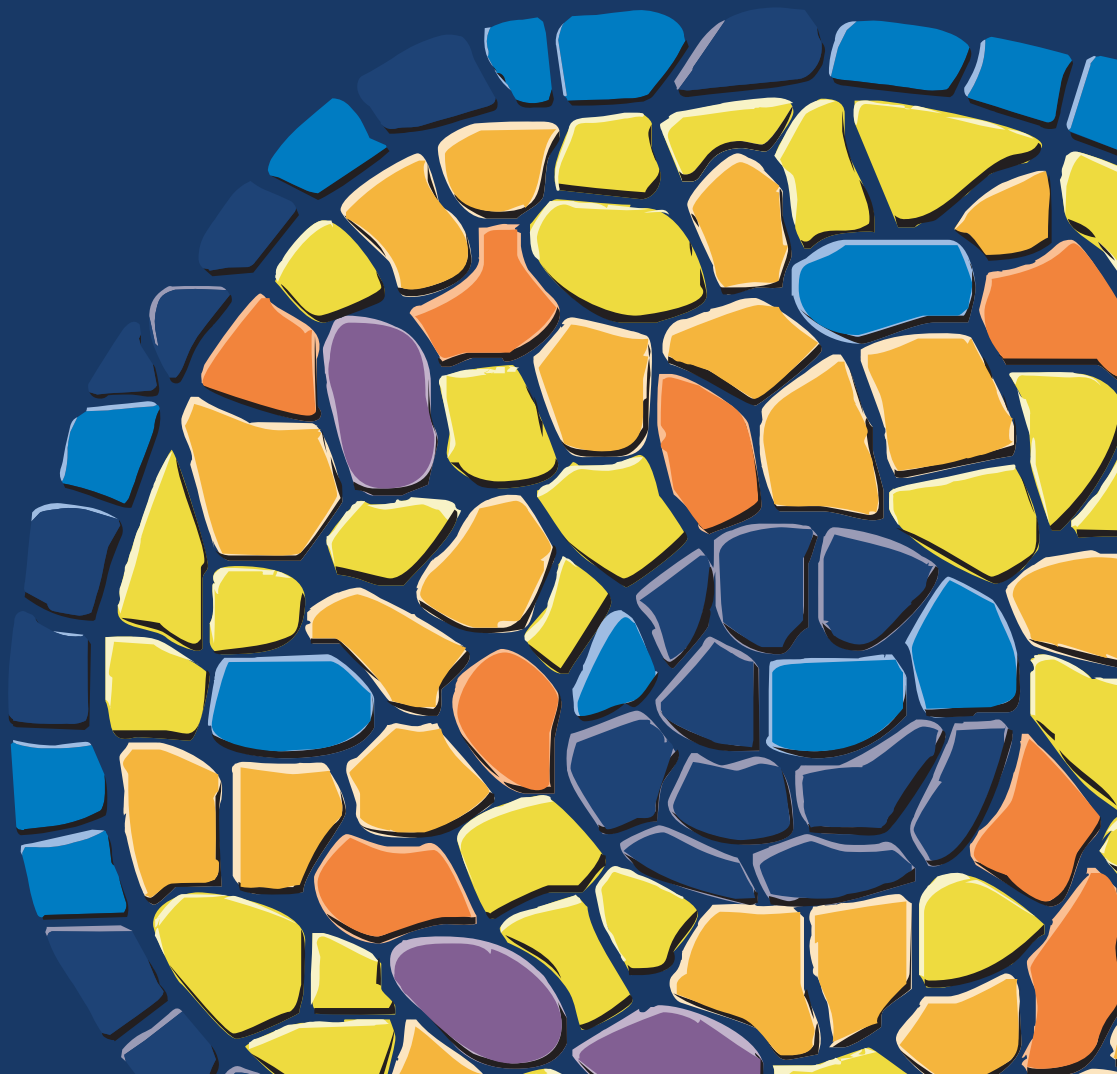
IRB BARCELONA

INSTITUTE FOR RESEARCH IN BIOMEDICINE

2008 Scientific Report



Centres de recerca de Catalunya

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Produced by:
Office of Communications and External Relations
Institute for Research in Biomedicine — IRB Barcelona
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08028 Barcelona, Spain
www.irbbarcelona.org

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Printing:
La Trama

Legal deposit:
B-20007-2009

This document has been printed using environmentally-friendly paper produced from sustainable forestry initiatives.

2008 Scientific Report

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IRB Barcelona: Building on a solid foundation

Not far from the IRB Barcelona premises, construction on the new phase of the Barcelona Science Park (PCB) continues at a steady pace. The PCB, which houses the IRB Barcelona laboratories, is undergoing a massive expansion and is set to grow from 36,800 m^2 to 90,000 m^2 by 2011. The large hole in the ground has been filled and a solid foundation has been laid for the many floors that will house new laboratories and services for the scientific community.

Something similar is happening at IRB Barcelona and 'construction' on the Institute forges ahead apace with that of its host. Three years have passed since IRB Barcelona was officially founded in 2005 and began its operations. The foundations of the Institute have been laid and we are building upon them to consolidate IRB Barcelona as one of the foremost research centres worldwide.

In 2008, IRB Barcelona expanded its faculty with the recruitment of international scientific talent to head new research groups and core facilities. Jens Lüders (Stanford University, USA) joined the Cell and Developmental Biology Programme in January and Xavier Salvatella (University of Cambridge, UK) joined the Chemistry and Molecular Pharmacology Programme in July. Travis Stracker was recruited from the Memorial Sloan Kettering Cancer Center (USA) to lead a group in the Oncology Programme, and will take up his position in early 2009. Additionally, two new core facilities were established in 2008 in support of IRB Barcelona research groups. David Rossell (MD Anderson Cancer Center, USA) joined to head the Bioinformatics and Biostatistics Unit, and Julien Colombelli arrived from the European Molecular Biology Laboratory (Germany) to launch the Advanced Digital Microscopy Core Facility, a joint initiative between IRB Barcelona and the PCB.

At the level of institutional collaborations, the joint programme with the Barcelona Supercomputing Center (BSC), launched in 2007, continues on course with the establishment of the Experimental Bioinformatics Laboratory, a group run jointly by group leaders from IRB Barcelona and the BSC. In addition, researchers from IRB Barcelona and the Institut d'Investigacions

Biomèdiques August Pi i Sunyer (IDIBAPS) have begun to plan regular seminars as part of the newly created Institut d'Investigació Sanitària Clínic-IDIBAPS. The initiative aims to foster collaborations between scientists at basic and clinical research centres in order to promote translational research.

In addition to the core funding provided by the Government of Catalonia (through the Ministry of Innovation, Universities and Business and the Ministry of Health), IRB Barcelona scientists were highly successful in 2008 in increasing research resources obtained through competitive grants and private funding. The European Commission awarded IRB Barcelona the coordination of two European health research projects, on malaria and diabetes, as part of the second call of the VII Framework Programme, and IRB Barcelona researchers participate as partners in a further four projects. Eduard Batlle, coordinator of IRB Barcelona's Oncology Programme, received an ERC Starting Grant for his group's work on colorectal cancer. Substantial grants for research and related activities were also provided by philanthropic entities. The Banco Bilbao Vizcaya Argentaria Foundation extended and fortified their existing collaboration with IRB Barcelona to fund research activities in the Oncology Programme as well as sponsor Barcelona BioMed activities. The Marcelino Botín Foundation continues to support research groups in the Molecular Medicine and Structural and Computational Biology Programmes.

Significant progress has been made in consolidating PhD training activities at IRB Barcelona. The "la Caixa" Foundation chose IRB Barcelona as one of four research institutes in Spain to receive special funding to recruit talented students to join their international PhD programmes. This initiative began with IRB Barcelona's 2008 call for applications and allowed 10 new fellows to join the Institute to complete work toward their doctoral theses. Students at IRB Barcelona continue to profit from close mentoring and have access to a wide variety of scientific activities, services and networks. They have taken steps to form a council that will coordinate activities such as a student-run PhD symposium, which will take place in November 2009.

In 2008 IRB Barcelona launched a call for Interdisciplinary postdocs, offering positions for postdoctoral researchers to work on a project led by groups in two research programmes. The four positions were successfully filled – 3 postdocs took up their positions in 2008, with the fourth to begin in 2009. In recognition that postdocs have specific needs in terms of training and career development support, IRB Barcelona has begun to implement structures and initiatives that go beyond the bench. Courses will be offered to enable postdocs to gain and share specific scientific expertise as well as training in 'soft' areas, such as lab management and grant writing. Presentation skills will also help the transition to more senior positions.

2008 has been a year of consolidation and hard work and significant progress has been made in many areas. As the Institute continues to grow, our scientists and support staff look forward to scientific and organisational challenges to ensure that IRB Barcelona secures a prominent place in the landscape of international biomedical research centres.

Cell and Developmental Biology Programme

FERRAN AZORIN

Chromatin structure and function

Genomic functions take place in chromatin, not in naked DNA. Over the last few years, we have become increasingly aware of the important contribution of chromatin to the regulation of genomic functions. Changes in chromatin structure have been found to play a fundamental role in the regulation of multiple genomic processes, from gene expression to chromosome segregation and the maintenance of genome integrity and stability. Furthermore, increasing evidence indicates that alterations in chromatin structure and function are at the root of many human pathologies, including some types of cancer and neurological disorders. Our current knowledge about the regulation of chromatin functions has benefited from the identification of components and mechanisms that covalently and structurally modify chromatin. These include chromatin assembly and remodelling complexes, histone modifications (*eg*, acetylation, methylation, phosphorylation, ubiquitination, etc.) and the corresponding enzymes (*eg*, HATs, HDACs, HMTs, HDMs, etc.), non-histone proteins that recognise specific histone modifications and contribute to the establishment of distinct functional domains (*eg*, HP1, PC, etc.), histone variants that localise to specific chromosomal locations (*eg*, CenH3/CENP-A, H3.3, H2A.Z, macroH2A, etc.), and non-coding RNAs that modify chromatin structure and regulate gene expression. Our research focuses on the molecular basis of chromatin function and its regulation. More precisely, we seek to elucidate the contribution of chromatin to the regulation of the following: (i) centromere identity and function, (ii) gene expression, and (iii) long-distance genomic interactions.

1a **1b**

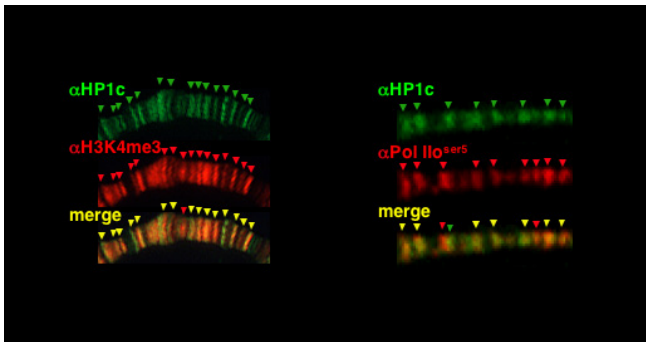

Figure 1. *Drosophila HP1c co-localises with H3K4me3 (a) and the poised form of RNA polymerase II, Pol IIo^{ser5} (b).*

The contribution of HP1 proteins to the regulation of gene expression

The contribution of chromatin to the regulation of gene expression is well established. Most frequently, regulation by chromatin involves the establishment of specific patterns of post-translational histone modifications, which result in the recruitment of regulatory non-histone proteins. Heterochromatin-protein 1 (HP1) is one of the best studied examples, where a regulatory non-histone protein is recruited to chromatin through the recognition of a specific histone modification, di- or tri-methylation of lysine 9 on the histone H3 tail (H3K9me2,3). This interaction, which involves the N-terminal chromo-domain of HP1, is known to play a fundamental role in the formation and maintenance of heterochromatic domains.

With the exception of budding yeast, HP1 is widely conserved in eukaryotes, with most species having several isoforms. HP1 proteins are characterised by a common structural organisation consisting of two conserved domains, the N-terminal chromo-

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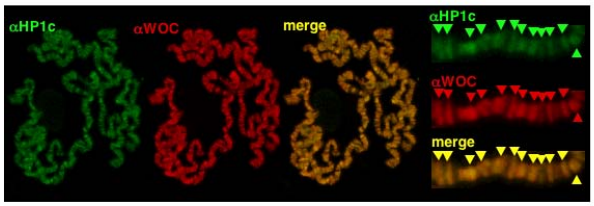
domain and the C-terminal chromo-shadow domain, which are spaced by a variable non-conserved hinge-domain. The existence of multiple isoforms suggests functional specialisation, with distinct isoforms exerting different functions. For instance, in *Drosophila*, three of the five HP1 isoforms (HP1a, HP1b and HP1c) are ubiquitously expressed, while the other two (HP1d/Rhino and HP1e) are predominantly expressed in the germ-line. Moreover, ubiquitously expressed HP1 isoforms show differential chromosomal distributions, as HP1a is mainly associated with heterochromatin, while HP1c is excluded from centromeric heterochromatin and HP1b is found both in euchromatic and heterochromatic domains. A similar situation is observed in mammals, where the localisation patterns of the three HP1 isoforms (HP1α, HP1β and HP1γ) overlap only partially and show differential dynamics during differentiation and cell cycle progression.

The molecular mechanisms that determine the distribution of the various HP1 isoforms and their differential functional properties remain largely unknown. Most of our knowledge about the mechanisms of action of HP1 proteins derives from studies addressing the functional properties of *Drosophila* HP1a or mammalian HP1α. From these studies, a general picture emerges by which, through the chromo-domain, HP1 proteins bind chromatin regions enriched in H3K9me2,3, while through the chromo-shadow domain they recruit different factors, thereby resulting in various functional outcomes, namely heterochromatin assembly and gene silencing. It is uncertain whether this general picture applies to all HP1 proteins and scenarios. In this context, we have reported the functional characterisation of HP1c, a *Drosophila* HP1 protein of largely unknown properties (Font-Burgada *et al*, 2008). Our results show that HP1c extensively co-localises with poised RNA polymerase II and H3K4me3, a modification that correlates with active chromatin

2a



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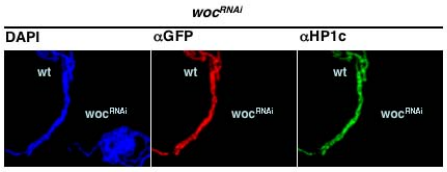
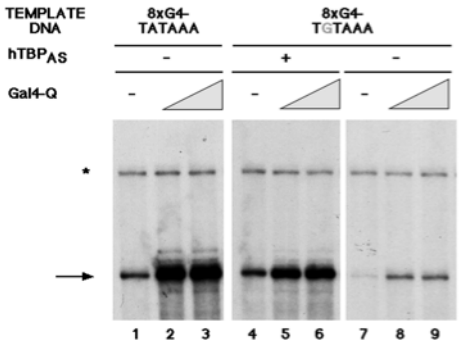


Figure 2. *Drosophila HP1c interacts with the zinc-finger proteins WOC and ROW. (a) HP1c extensively co-localises with WOC (a similar co-localisation is observed with ROW). (b) Binding of HP1c to chromatin is abolished in the absence of ROW (row^{RNAi} chromosomes; similar results are obtained in woc^{RNAi} chromosomes).*

3a



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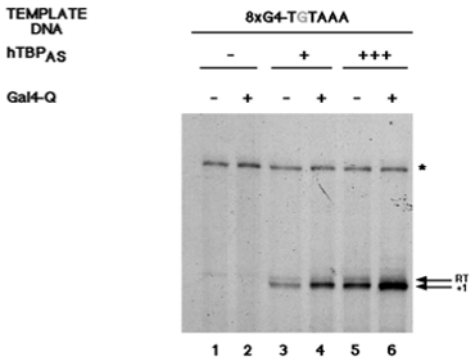


Figure 3. *In vitro transcription experiments showing that the GAGA Q domain, when fused to GAL4, activates transcription despite the presence of a non-functional TATA-box (TGTAAA). When complemented with an altered specificity, TBP (hTBP$_{AS}$) basal transcription is restored and activation is possible even in a heat-inactivated nuclear extract.*

domains, thereby indicating that HP1c contributes to the regulation of gene expression (Figure 1). In fact, HP1c forms a distinct multi-protein complex with two zinc-finger proteins, WOC (without children) and Relative-of-WOC (ROW), both putative transcription factors. HP1c efficiently binds H3K9me2,3 *in vitro*, but its binding to chromatin depends strictly on both WOC and ROW (Figure 2). Moreover, expression profiling indicates that HP1c, WOC and ROW extensively co-operate to regulate gene expression, especially in the context of the nervous system. From this study, which unveils the essential contribution of DNA-binding proteins to HP1c functionality and recruitment, HP1 proteins emerge as an increasingly diverse family of chromatin regulators.

The contribution of the *Drosophila* GAGA factor to the regulation of gene expression

GAGA is a *Drosophila* transcription factor that is involved in many nuclear activities. In transcription experiments performed *in vitro*, we have obtained evidence that GAGA factor enhances transcription by stabilising pre-initiation complex (PIC) and by promoting reinitiation (Vaquero *et al*, 2008). Formation of PIC prior to GAGA addition prevents activation, thereby suggesting that GAGA is required early in the formation of activated complexes. GAGA stimulation of transcription can be attributed in part to the stabilisation of PIC and can occur *in vitro* and *in vivo* even in the absence of a functional TATA box. This finding suggests that GAGA recruits and/or nucleates the general transcription factors (in particular TBP and/or TFIID) to assemble PIC in a similar way as the well-known Gal4VP16 activator acts. All these properties depend on the GAGA C-terminal glutamine-rich domain and, in addition to other roles and previous data, support a role of GAGA as a transcription factor (Figure 3).

GAGA overexpression and depletion experiments performed genome-wide in *Drosophila* S2 cells have shown that GAGA is essentially an activator of transcription (Bernués *et al*, 2007). Despite a relatively short list of genes whose expression is affected by GAGA dosage in these experiments, a large number of uncharacterised genes have been shown to be under its control (~50% of them). Among the known targets, some show a high score and are likely to be direct GAGA targets. While they are currently under assay, the first studied in more detail is a pro-apototic gene (sickle), which we have shown to be responsible for the high lethality observed in two experimental conditions in flies. Moreover, we have demonstrated this lethality to be fully rescued by co-expression of the anti-apoptotic DIAP1. Remarkably, lethality in other conditions cannot be recovered in this way, thereby strongly suggesting that the effects of GAGA are context-dependent. Gene ontology analysis of our results revealed that GAGA is most highly relevant in regulating the expression of genes involved in cell adhesion and development in S2 cells. These findings have been obtained in a cellular system and do not represent the complete picture of GAGA action. Therefore, and also because of the highly significant effect of GAGA on genes involved in development, we are currently performing similar experiments in transgenic flies (Figure 4).

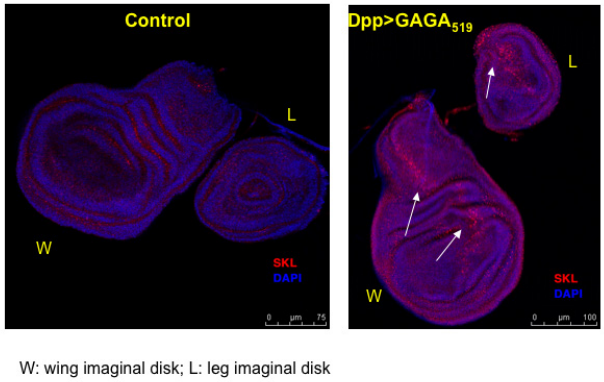
Long-distance genomic interactions: the contribution of CENP-B to the regulation of mating-type switching in fission yeast

In the fission yeast *Schizosaccharomyces pombe*, haploid cells switch mating type by means of a tightly regulated gene conversion event that involves long-distance interactions between an expressed *locus* (*mat1*) with either of two silent donor *loci* (*mat2* and *mat3*), which are located 17 kb and 29 kb away from *mat1*, respectively. Mating-type information is contained in the silent *mat2-P(plus)* and *mat3-M(minus) loci* but is expressed only after translocation to the *mat1 locus*, thereby giving rise to *mat1P* or *mat1M* cells, depending on whether *mat2* or *mat3* information is expressed at *mat1*. Silencing at the *mat2* and *mat3 loci* is mediated by heterochromatin, which, in the mating-type region, extends for a 20-kb long domain.

Mating-type switching initiates during DNA replication with the introduction of a strand-specific single-strand (SSB) imprint at *mat1*, which, in the next round of DNA replication, is converted into a double-strand break (DSB). This DSB is then healed by gene conversion using *mat2* or *mat3* as donors. Donor selection is, however, not random. On the contrary, *mat1P* cells preferentially use *mat3* as donor while *mat1M* cells use *mat2*. Directionality of switching therefore ensures that cells switch to the opposite mating-type with a very high frequency. Directionality of switching is determined by the cell-type specific distribution of the Swi2/Swi5-complex, which promotes recombination. In mat1P cells, Swi2/Swi5 localisation is restricted to a recombination-enhancer (SRE) located adjacently to *mat3* so that under these circumstances only *mat3* is efficiently used as donor. In contrast, in *mat1M* cells, Swi2/Swi5 spreads across the entire mating-type region, reaching the *mat2 locus*, which becomes the preferred donor site as a result of the structural constraints imposed by heterochromatin. The spread of Swi2/Swi5 in *mat1M* cells relies on heterochromatin, as it is abolished by mutations that affect heterochromatin formation. Consequently, in the absence of heterochromatin, *mat3* is used as a donor at a much higher frequency than *mat2* (Figure 5).

However, the mechanisms that regulate the spread of Swi2/Swi5 across heterochromatin are not fully understood. In this context, we have reported on the identification of the first factor required for the heterochromatin-mediated spread of Swi2/Swi5, Abp1 (Aguilar-Arnal *et al*, 2008). We have shown that Abp1 binds at the mating-type *locus* and regulates directionality of switching. In abp1Δ cells, *mat3* is preferentially used as donor as occurs when heterochromatin is disrupted. The deletion of abp1, however, does not affect heterochromatin organisation at the mating-type region. This observation indicates that Abp1 is required for the efficient spread of Swi2/Swi5 across heterochromatin. ChIP-experiments have confirmed this hypothesis.

Abp1 is a member of the CENP-B family of proteins, which were first identified in humans as centromere-associated proteins. CENP-Bs are conserved sequence-specific DNA-binding proteins that derive from pogo-like transposases through independent domestication events. In fact, in *S. pombe*, Cbh1 and Cbh2 are also homologous to CENP-B and, together with Abp1, play redundant functions in the regulation of various nuclear processes. However, the contribution to the regulation of directionality of mating-type switching is specific to Abp1, as deletion of *cbh1* or *cbh2* shows no effects on this process (Aguilar-Arnal *et al*, 2008). Taken together, these observations unveil the diversity of functions regulated by CENP-B proteins, which emerge as general regulators of chromatin structure and function.



W: wing imaginal disk; L: leg imaginal disk

Figure 4. *DppGal4-directed overexpression of $GAGA_{519}$ induces sickle (Skl) misexpression in wing and leg disks that later results in loss of legs and strong lethality. Arrows indicate Skl expression.*

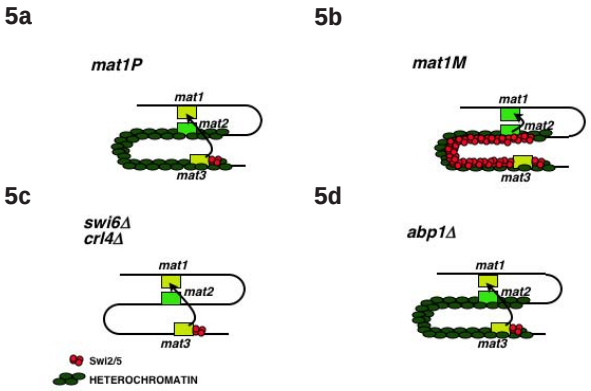


Figure 5. *Abp1 is required for heterochromatin-dependent spread of the Swi2/Swi5 complex. In mat1P cells, the Swi2/Swi5-complex localises to mat3 (a) and only in mat1M cells does it spread to mat2 (b), thereby allowing its use as donor during switching. The spread of Swi2/Swi5 to mat2 is mediated by heterochromatin and is abolished by mutations in heterochromatin assembly factors (ie, swi6Δ, crl4Δ) (c), which prevent the use of mat2 as donor. In abp1Δ cells, heterochromatin organisation of the mating-type locus is preserved but mat2 is not efficiently used as donor, thereby indicating that the spread of Swi2/Swi5 to mat2 is abolished (d).*

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Research networks and grants

Anàlisi estructural i funcional de la cromatina
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00678 (2005-2008)
Principal investigator: Ferran Azorín

Ayuda complementaria al proyecto europeo 'Vectores episomales como sistemas de modificación genética para aplicaciones terapéuticas'
Spanish Ministry of Science and Innovation, BIO2006-26123-E (2007-2009)
Principal investigator: Ferran Azorín

Caracterización biológica de inhibidores de metil transferasas
Spanish Ministry of Science and Innovation, PET2007-0319-02 (2008-2010)
Principal investigator: Ferran Azorín

Cromatina silenciada: análisis de los factores y mecanismos implicados en su formación y mantenimiento
Spanish Ministry of Science and Innovation, BFU2006-01627/BMC (2007-2009)
Principal investigator: Ferran Azorín

Characterisation of the role of histone H1 and its post-translational modifications in the functional regulation of chromatin
European Commission, Marie Curie Action, International Reintegration Grant (2007-2009)
Principal investigator: Ferran Azorín

Epigenética: Mecanismos y enfermedad, Ingenio 2010
Spanish Ministry of Science and Innovation, CSD2006-49 (2006-2011)
Principal investigator: Ferran Azorín

Episomal vectors as gene delivery systems for therapeutic application
European Commission, FP6-2003-LSH-2 (2005-2008)
Principal investigator: Ferran Azorín

Episomal vectors as gene delivery systems for therapeutic application (EPI VECTOR)
European Commission, LSHB-CT-2004-511965 (2005-2008)
Principal investigator: Ferran Azorín

Regulación transcripcional de genes controlados por el factor GAGA: Identificación de nuevos genes diana y de los mecanismos de activación/represión que operan in vivo
Spanish Ministry of Science and Innovation, BFU2007-64395/BMC (2007-2010)
Principal investigator: Ferran Azorín

Collaborations

Analysis of the contribution of chromatin to the stability of episomal vectors
Hans J Lipps, University of Witten (Witten, Germany)

Analysis of the contribution of HP1c, WOC and ROW to the regulation of gene expression in Drosophila
Herbert Auer and David Rossell, IRB Barcelona (Barcelona, Spain)

Analysis of the contribution of multi-KH-domain proteins to RNA editing and heterochromatin organisation
Sergio Pimpinelli, University of Rome (Rome, Italy)

JORDI CASANOVA

Signalling in morphogenesis

Our research focuses on the genetic control of development, and in particular the role of cell communication mechanisms in development in the context of the whole organism. The work of many laboratories has allowed us to begin to elucidate the genetic logic behind development and we are now addressing how these mechanisms impinge on cell behaviour and how changes in individual cells sum up to generate organs and the whole organism. We are analysing these mechanisms in two model systems in *Drosophila*, namely Torso RTK signalling and the formation of the trachea. In particular, we have begun our work at the interphase between development and cell biology using tracheal formation to study how transcription factors and signalling pathways regulate the cellular mechanisms responsible for changes in cell shape and cell behaviour such as migration and invagination.

The major outcomes of our research in 2008 can be broken down into the following sections:

Modulation of intracellular trafficking regulates cell intercalation in the *Drosophila* trachea

Epithelial cells exchange places in a spatially oriented manner by means of intercalation, a fundamental mechanism underlying elongation during morphogenesis (Pilot and Lecuit, 2005). Epithelial cells are tightly coupled through distinct intercellular junctions, including adherens junctions. Whether trafficking-mediated regulation of adhesion through adherens junctions modulates intercalation *in vivo* remains controversial (Pilot and Lecuit, 2005; D'Souza-Schorey, 2005). In *Drosophila melanogaster*, cells in most branches intercalate during tracheal development. However, Wingless (Wg)-promoted expression of the transcription factor Spalt (Sal) in the dorsal trunk inhibits intercalation (Ribeiro *et al*, 2004) by an unknown mechanism.

In collaboration with Marta Llimargas (at IBMB-CSIC), we have examined the role of trafficking in tracheal intercalation and found that it requires endocytosis, whereas it is opposed by *Rab11*-mediated recycling in the dorsal trunk. Subapical Rab11 accumulation is enhanced by *sal* and elevated *Rab11*-mediated recycling occurs in the dorsal trunk, thereby suggesting that upregulation of Rab11 is one way in which *sal* inhibits intercalation. We found that *dRip11*, which regulates Rab11 localisation and function (Ribeiro *et al*, 2004), is regulated by *sal* and can modulate intercalation. Finally, we observed that expression of E-cadherin (DE-cad), an

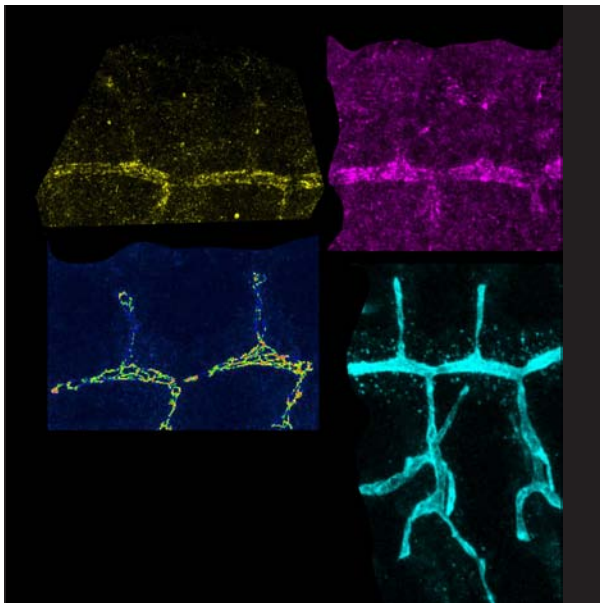

Figure 1. *During Drosophila tracheal development, upregulation of dRip11 (yellow) in the dorsal trunk enhances Rab11 (magenta) accumulation, which increases junctional cadherin expression (shown in rainbow panel). This up-regulation of adhesion prevents cell intercalation in this branch, and thus helps to sculpt the shape of the tracheal network (labelled by Sas, in teal; figure from Dan Shaye).*

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adherens junction component (Oda *et al*, 1994), and Rab11-compartment cargo (Classen *et al*, 2005; Langevin, 2005; Lock *et al*, 2005) are dynamically regulated by trafficking during tracheal development, and that such regulation modulates intercalation. Our work points to a mechanism by which trafficking of adhesion molecules regulates intercalation and shows how this mechanism is modulated *in vivo* to influence cell behaviour (Figure 1).

A functional antagonism between the pgc germ-line repressor and torso in the development of somatic cells

Segregation of the germ-line is a fundamental event during early development (see Strome and Lehmann 2007). In *Drosophila*, germ cells are specified at the posterior pole of the embryo by germplasm, and as zygotic expression is activated germ cells remain transcriptionally silent (Van Doren *et al*, 1998) owing to Polar granule component (Pgc), a small peptide present in germ cells (Martinho *et al*, 2004; Hanyu-Nakamura *et al*, 2008). Somatic cells at both embryonic ends are specified by the Torso (Tor) RTK and in *tor* mutants the somatic cells closest to the germ cells do not cellularise properly (Schüpbach and Wieschaus, 1986; Degelmann *et al*, 1986). In collaboration with Rui Martinho (Gulbenkian Institute) and Ruth Lehman (New York University), we have shown that extra wild-type gene copies of *pgc* cause a similar cellularisation phenotype and that both excessive *pgc* and lack of *tor* are associated with an impairment of transcription in somatic cells. Moreover, lack of *pgc* partially ameliorates the cellularisation defect of *tor* mutants, thus unveiling functional antagonism between *pgc* and *tor* in the specification of germ-line and somatic properties. As transcriptional quiescence is a general feature of germ cells, similar mechanisms might operate in many organisms to "protect" somatic cells that abut germ cells from inappropriately succumbing to such quiescence (Figure 2).

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Shaye DD, Casanova J and Llimargas M. Modulation of intracellular trafficking regulates cell intercalation in the *Drosophila* trachea. *Nat Cell Biol*, **10**(8), 964-70 (2008)

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Research networks and grants

Ajut per a grups de recerca singular
Agency for Administration of University and Research Grants (AGAUR), SGR-2005-00508 (2005-2008)
Principal investigator: Jordi Casanova

Cellular properties and morphogenesis. From genes to shape: analysis of morphogenesis in Drosophila and vertebrates
Spanish Ministry of Science and Innovation, CSD-2007-2008 (2008-2012)
Principal investigator: Jordi Casanova

Mecanismos de señalización celular y morfogénesis en el desarrollo de Drosophila
Spanish Ministry of Science and Innovation, BFU2006-01935/BMC (2006-2009)
Principal investigator: Jordi Casanova

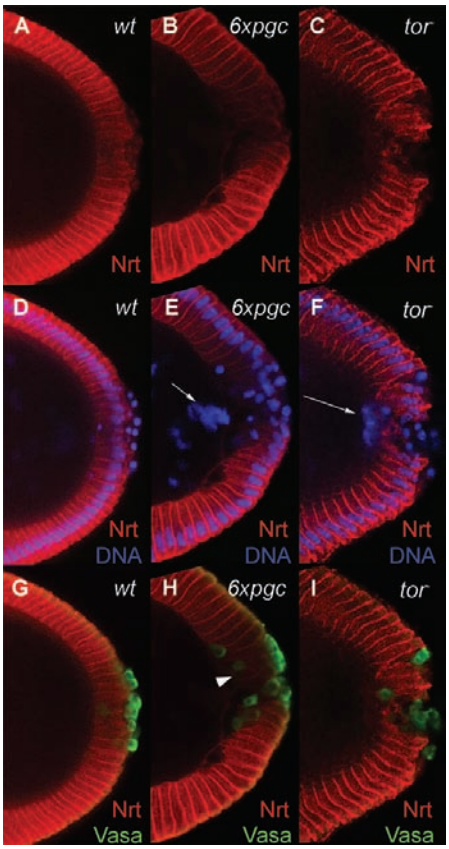

Figure 2. *Posterior poles of wild-type (A,D,G), 6x[pgc] (B,E,H) and tor (C,F,I) embryos. In red, anti-Neurotactin (Nrt) labels somatic but not germ cells; DAPI labels nuclei; in green, anti-Vas labels germ-cells. Groups of nuclei fall into the yolk in 6x[pgc] and tor mutants (arrows in E and F), some cells fail to complete cellularisation as shown by the lack of a basal membrane (ie, see arrowhead in E) and many cells have lost the typical epithelial elongated shape. Occasionally, a few nuclei fall into the yolk in wild-type. In 6x[pgc] (H) and tor (I) embryo, germ cells are found in the 'hole' between the somatic cells (figure from Jose M de las Heras).*

Collaborations

A functional antagonism between the pgc and torso
Ruth Lehmann, New York University (New York, USA)

Intracellular trafficking regulates cell intercalation in the Drosophila trachea
Marta Llimargas, Institut de Biologia Molecular de Barcelona (Barcelona, Spain)

Cell division

Our goal is to elucidate the mechanisms of cell division. We apply a multidisciplinary approach that combines genetics, molecular biology and advanced *in vivo* microscopy. We use *Drosophila* as well as cultured cells derived from vertebrates as model systems. Current on-going projects include the study of the mechanisms of spindle assembly, the characterisation of new centrosomal proteins and the modelling of cancer in *Drosophila* to determine the functional connections between stem cell polarity and tumour growth.

During the last few years we have been exploiting *Drosophila* to study some of the basic principles of cell proliferation and malignant growth (Causinus and González, 2005; Wodarz and González, 2006). This line of research focuses on the role of larval neural stem cells (Neuroblasts: NBs) as the origin of tumours.

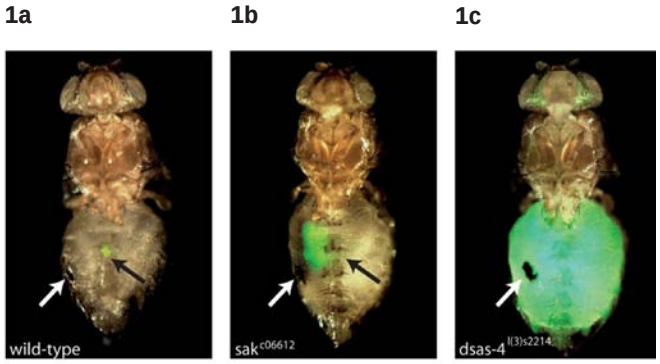

Figure 1. Benign and malignant growth following allograft culture. *(a) A piece of GFP-labelled wild-type larval brain (black arrow) implanted into the abdomen of an adult host (white arrows point to the scar produced by the needle at the point of injection) does not show any significant growth in two weeks. (b) In the same period, a mutant implant of the same size that grows significantly. This type of growth is graded as benign as it does not notably compromise the survival of the host. (c) An implant from a distinct mutant tissue that grows and spreads throughout the entire abdominal cavity (green), severely compromising the viability of the host. Extracted from Castellanos et al, 2008.*

Drosophila as a model for cancer research

The first observations of deadly tumours in *Drosophila* were made almost one hundred years ago, but experiments in this field started in earnest four decades ago. This research effort has led to the identification of dozens of genes whose function is required to prevent tissue overgrowth and which are collectively referred to as *Drosophila* tumour suppressors (TSs). All the TSs identified in *Drosophila* to date are essential for cell differentiation and development. Many of them have homologues in vertebrates, thus opening up the possibility of using this model system to further characterise the pathways in which they operate. Moreover, some of these homologues have been reported to be mutated in human cancers, thus strengthening the relevance of the fly model in cancer research.

The first *Drosophila* TSs were identified *in situ* by observation of the growth of massive neoplasms in mutant third instar larvae (Gateff, 1978). The best established assay to discern between benign and malignant growth in *Drosophila* is to implant the affected tissue in a healthy host. Such an allograft or "dauer" culture is now a standard technique in our laboratory (González, 2007). Upon implantation, wild-type tissue never overgrows, and benign hyperplasias grow slowly, do not invade other tissues, and retain their capacity to differentiate. Malignant neoplasms, in contrast, display autonomous growth, the capacity to migrate to and colonise distant organs, and lethality to the host. Moreover, malignant neoplasms frequently become immortal and can expand limitlessly through successive rounds of implantation into healthy hosts.

Drosophila neural stem cells

Neurons and glia in the developing central nervous system of *Drosophila* are generated by the self-renewing asymmetric division of neural stem cells, called neuroblasts (NBs). Acquisition of

Principal Investigator Cayetano González **Research Associate** Elena Rebollo **Postdoctoral Fellows** Jens Januschke, José Reina, Fabrizio Rossi, Zhanna Shcheprova **PhD Students** Elisabeth Castellanos, Ana Janic, Leire Mendizábal **Research Assistant** Salud Llamazares **Lab Technician** Jan Peter **Lab Manager** Maria Isabel Santori

NB identity imposes a self-renewing asymmetric division mode whereby each of the two daughter cells acquires one of two possible developmental fates: NB or ganglion mother cell (GMC). GMCs can be considered intermediate progenitors -to use the terminology that is common in vertebrates- that divide, normally just once, to generate cells that eventually differentiate into neurons or glia. Therefore, some of the key processes that characterise stem cells occur in *Drosophila* NBs. Thus this model system is probably the best model to study asymmetric division in animal stem cells.

Self-renewing asymmetric division of *Drosophila* NBs relies on the tight coordination of two processes: (I) the differential sorting of the Pins and Par complexes to the apical cortex and the Mira and Pon complexes to the basal cortex and (II) the controlled positioning of the plane of cytokinesis, which leads to the unequal segregation of cortical protein complexes between the daughter cells (reviewed in González, 2008). Clearly, both processes are necessary for self-renewing asymmetric division of these cells, but neither is sufficient.

How polarity is re-established in each cell cycle is still unclear. Polarised cortical markers are not detected after mitosis, and the first sign of them returning to the cortex occurs very late in the cell cycle, when mitosis starts. A tantalizing hypothesis, derived from results obtained recently in our lab, is that polarity information is kept by the asymmetric structure of the NB cytoskeleton (Rebollo *et al*, 2007). Data from live imaging of microtubules and centrosomes in these cells shows that soon after cytokinesis both centrosomes migrate to the nearest cortex, which roughly coincides with the region where apical markers were last localised. A few minutes later, the two centrosomes start to display markedly asymmetric behaviour. One stays fixed at the apical cortex, organising an aster that will be the main

microtubule network during most of the NB interphase. The orientation of the future mitotic spindle and the future localisation of the apical complexes can be accurately predicted from the position of this apical aster, long before any of the known polarised markers can be seen at the apical cortex of the cell (González, 2007). The second centrosome, which has little, if any, PCM and does not display any significant MTOC activity, moves extensively throughout the cytoplasm, mainly in the apical side first, more basally later, until shortly before mitosis when it slows down near the basal cortex, recruits PCM and organises the second mitotic aster. Thus, the structure and function of the two centrosomes of a NB differ greatly. They are also unequal in fate since the apical centrosome remains in the stem cell, while the other centrosome goes into the differentiating daughter cell.

Therefore, while we cannot discard that unknown cues might guide centrosomes to the apical cortex, it is also possible that it is the positioning of the centrosome itself at the apical side of the NB that contributes to triggering the sorting of the apical markers (Januschke and González, 2008). Interestingly, cultured individual NBs show the same asymmetric centrosome behaviour as NBs observed "in toto". This observation therefore strongly suggests that regulation of such stereotyped behaviour does not depend on the crosstalk between NBs and their neighbouring cells (Januschke and González, 2008).

Self-renewing asymmetric division in NBs and tumour suppression

Loss of cell polarity and malignant transformation are tightly correlated in human carcinomas. There are several hypotheses to explain how loss of polarity contributes to neoplastic transformation. Most of these call on models in which changes in cellular architecture impinge directly on the cell cycle either by inhibiting cell proliferation restraints or by enhancing mitogenic pathways. Alternatively, loss of polarity might, if affecting asymmetrically dividing stem cells, impair the fate of the daughter cells, rendering them unable to respond to the mechanisms that control proliferation in the wild-type lineage and initiating tumour growth. The possible functional link between failed NB asymmetry and tumour growth was first proposed after the identification of known TS genes as key regulators of NB asymmetry. However, direct demonstration of this link came from results published by our laboratory showing that pieces of larval brain tissue mutant for any of several elements that regulate NB asymmetry develop as tumours when transplanted to the abdomen of adult hosts (Caussinus and González, 2005; Clevers, 2005). We found that these tumours grow unrestrained and often give rise to the development of tumour colonies dispersed around the body, which kill the implanted hosts in about two weeks. Moreover, these tumours can be re-transplanted into healthy hosts and survive for years, thereby showing that the transformed cells become immortal (Caussinus and González, 2005; Castellanos *et al*, 2008).Therefore, these tumours fulfill the criteria for neoplastic growth: invasiveness and metastasis, lethality to the host, and autonomous, limitless growth.

Subsequent reports from several laboratories have confirmed our results and expanded the number of what is now a long list of genes known to be involved in NB polarity and tumour suppression in these cells, including cell fate determinants, some elements of the apical cortex complexes, and kinases that regulate stem cell polarity like AurA and Polo (Betschinger *et al*, 2006; Lee *et al*, 2006; Wang *et al*, 2006; Wang *et al*, 2007; Bowman *et al*, 2008; Knoblich, 2008; Castellanos *et al*, 2008).

The main conclusion derived from these observations is that NBs can become malignant cells by disrupting their delicately balanced process of self-renewing asymmetric division. This finding provides additional support to the general hypothesis proposing that malfunction of the asymmetric cell division machinery of stem cells contributes to their transformation (For review, see Januschke and González, 2008).

Origin and functional relevance of genome instability in *Drosophila* tumour models

In most solid tumours in humans, malignancy is often correlated with genome instability (GI), defined as quantitative and/or qualitative changes in the genetic material—aneuploidies, polyploidies, deficiencies, translocations, and inversions. This correlation suggests that GI might not merely be a consequence of transformation, but a contributing factor to it. However, causality has not been unequivocally established between GI and tumour progression.

Interestingly, GI is observed in all types of *Drosophila* tumours that arise as a result of the deregulation of the mechanisms that drive asymmetric stem cell division. When grown in allograft culture, all these tumours display significant levels of chromosomal alterations that affect both chromosome integrity and number, regardless of whether the mutation that initiated the tumour causes a certain level of GI or none at all (Castellanos *et al*, 2008; Caussinus and González, 2005). Moreover, we have recently shown that GI is not an efficient tumorigenic condition in *Drosophila* NBs (Castellanos *et al*, 2008), thereby suggesting that GI is a downstream effect of transformation and leaving open the question of whether or not it plays an active role in the progression of these tumours towards malignancy.

SCIENTIFIC OUTPUT

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Research networks and grants

Ajut per a grups de recerca consolidats
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00821 (2006-2008)
Principal investigator: Cayetano González

Ayuda complementaria al proyecto europeo 'An integrative approach to cellular signalling and control processes: bringing computational biology to the bench'
Spanish Ministry of Science and Innovation, BFU2005-24117 (2006-2009)
Principal investigator: Cayetano González

Cancer stem cells and asymmetric division (ONCASYM)
European Commission, STREP LSHC-CT-2006-037398 (2006-2009)
Principal investigator: Cayetano González

Centrosoma 3D: Hacia la comprensión estructural y funcional del centrosoma
Spanish Ministry of Science and Innovation, CSD2006-23 (2006-2011)
Principal investigator: Cayetano González

Identificación mediante análisis genético y farmacológico de proteínas esenciales para prevenir la transformación maligna de células madre en Drosophila
Oncostem Pharma SL, Cibasa (2006-2008)
Principal investigator: Cayetano González

Identification of pathways that are relevant for the malignant transformation of stem cells in Drosophila
Spanish Ministry of Science and Innovation, BFU2006-05813-BMC (2007-2009)
Principal investigator: Cayetano González

Collaborations

Co-evolution of the chaperonin CCT and tubulins from antarctic fishes; United States Antarctic Programme expedition
William Dietrich, Northeastern University (Massachusetts, USA)

Control of asymmetric division in cancer stem cell
Marcos González, University of Geneva (Geneva, Switzerland)

Microtubule organisation

Our overall goal is to reach a comprehensive understanding of how cells generate, maintain, and remodel the microtubule cytoskeleton during cell cycle and cell differentiation. Defects in the structure and function of the microtubule cytoskeleton are linked to cancer and certain developmental disorders. To ensure proper microtubule organisation, cells control where and when microtubules are made. Microtubule organising centres, such as the animal centrosome, participate in this regulation by providing sites that nucleate the polymerisation of new microtubules. These sites are formed by interaction with a large, multi-subunit protein complex, the γ-tubulin ring complex, which functions as microtubule nucleator. Microtubule nucleation sites provide a unique molecular environment, not only for the control of microtubule nucleation but also for regulating microtubule behaviour, and thus are central to our understanding of microtubule organisation. However, the exact molecular composition of microtubule nucleation sites and the spatio-temporal regulation of their assembly are poorly understood. To address this issue, we study centrosomal and non-centrosomal microtubule nucleation pathways in several cell types by identifying the molecular players and by characterising their function and regulation *in vitro* and *in vivo*.

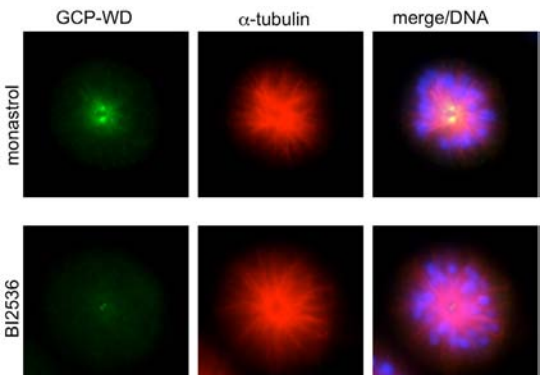


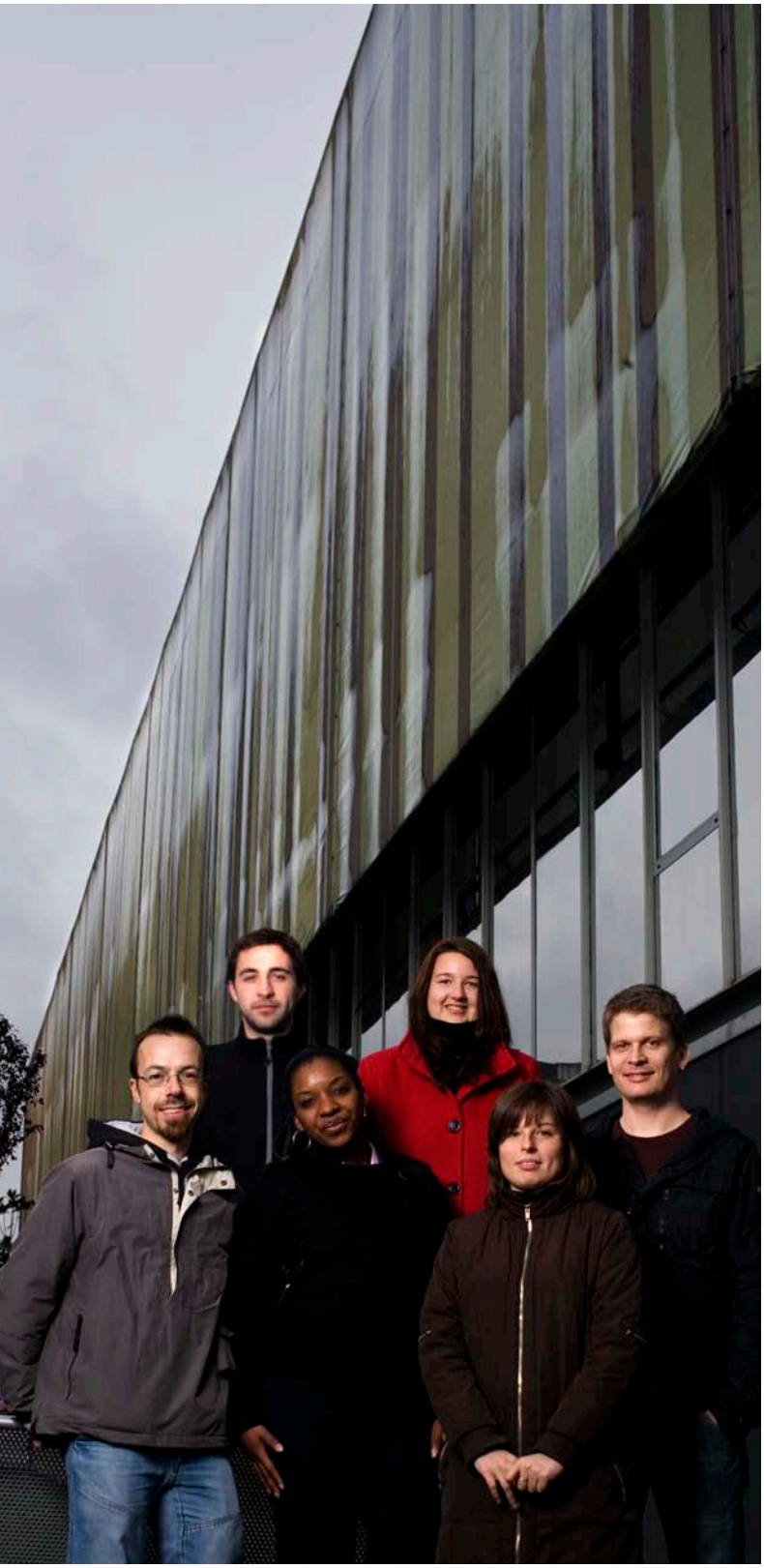
Figure 1. Plk1 activity is required for localisation of the γ-tubulin targeting factor GCP-WD to centrosomes and spindle microtubules. *HeLa cells treated with monastrol or the Plk1 inhibitor BI2536 were fixed and immunostained for GCP-WD and α-tubulin to label microtubules. DAPI was used to label DNA. Both treatments produce monopolar spindles. Monastrol treatment, however, does not affect centrosome accumulation of GCP-WD, whereas BI2536 almost completely blocks recruitment of GCP-WD to the centrosomes.*

In proliferating cells, the major microtubule organising centre (MTOC), the centrosome, organises the mitotic spindle. Numerical and functional centrosome abnormalities including aberrant size and shape, and microtubule (MT) nucleation activity are frequently found in cancer cells. Such centrosomal defects can impair proper spindle assembly and function and result in genomic instability. In addition to centrosomal MT nucleation, our previous work and studies by others show that proper mitotic spindle assembly requires MT nucleation from non-centrosomal sites. Non-centrosomal nucleation pathways are poorly characterised, but might be crucial for rapidly dividing cancer cells. These pathways may therefore provide great potential for future anti-cancer therapies.

Centrosomal microtubule nucleation in mitosis

The centrosome comprises a pair of barrel-shaped centrioles surrounded by a dense proteinaceous matrix, the pericentriolar material (PCM). The nucleation of MT polymerisation occurs within the PCM and requires the recruitment of γ-tubulin ring complexes (γTuRCs) from the cytoplasm. These complexes contain γ-tubulin, a paralogue of α- and β-tubulin that is not incorporated into the MT polymer but functions as MT nucleator. We have previously shown that the interaction of γTuRCs with centrosomes is mediated by a protein named GCP-WD (also

Principal Investigator Jens Lüders **Postdoctoral Fellow** Marco Archinti **Research Assistant** Cristina Lacasa **Visiting Students** Florian Baier (Germany), Sabine Klischies (Germany), Leila Njimoluh (USA)



known as NEDD1) (Lüders *et al*, 2006; Haren *et al*, 2006). When cells prepare for mitosis in late G2 phase of the cell cycle, size and microtubule nucleating activity of the duplicated centrosomes increase. This is accomplished by the recruitment of additional PCM to the centrosomes, including proteins involved in microtubule nucleation and organisation, such as γ-tubulin. This process, also termed centrosome maturation, is critical for the function of centrosomes as MTOCs in mitosis, and depends on the activity of mitotic kinases such as Polo-like kinase 1 (Plk1). Interference with Plk1 function by RNAi or specific inhibitors prevents the recruitment of γ-tubulin to mitotic centrosomes, thereby essentially inactivating their MT nucleation activity, and impairing bipolar spindle formation. Plk1 inhibitors are currently being studied in clinical trials as potential agents for cancer therapy. To date, a Plk1 substrate that controls γ-tubulin recruitment in a phosphorylation-dependent manner has not been identified.

We discovered that Plk1 associates with GCP-WD, the γ-tubulin targeting factor, and Plk1 activity contributes to mitotic phosphorylation of GCP-WD (Haren *et al*, 2009, in preparation). Plk1 depletion or inhibition revealed that accumulation of γ-tubulin at centrosomes is regulated by controlling the levels of centrosomal GCP-WD (Figure 1). Surprisingly, GCP-WD mutants that are defective in Plk1 binding and phosphorylation still accumulate at mitotic centrosomes and recruit γ-tubulin. At present, we are studying whether the Plk1-dependent phosphorylation of GCP-WD serves other functions and whether it affects spindle assembly and function.

Interestingly, our studies revealed that Plk1 also controls the recruitment of other PCM proteins implicated in centrosomal γ-tubulin attachment. Our results support a model in which Plk1-dependent recruitment of γ-tubulin to mitotic centrosomes is regulated upstream of GCP-WD, and involves multiple PCM proteins and potentially multiple Plk1 substrates (Haren *et al*, 2009, in preparation). Our next goal is to identify these substrates and, through phospho-mutant analysis, shed light on the Plk1-dependent pathway that triggers centrosome maturation and γTuRC recruitment.

To study the role of Plk1 and other mitotic kinases in the regulation of microtubule nucleation during spindle formation, we have initiated a collaboration project with Carme Caelles and Joan Roig, researchers in the Molecular Medicine Programme at IRB Barcelona.

In addition to the general importance of this pathway for proliferating cells, two of the proteins involved in γ-tubulin recruitment to centrosomes have recently been implicated in micro-

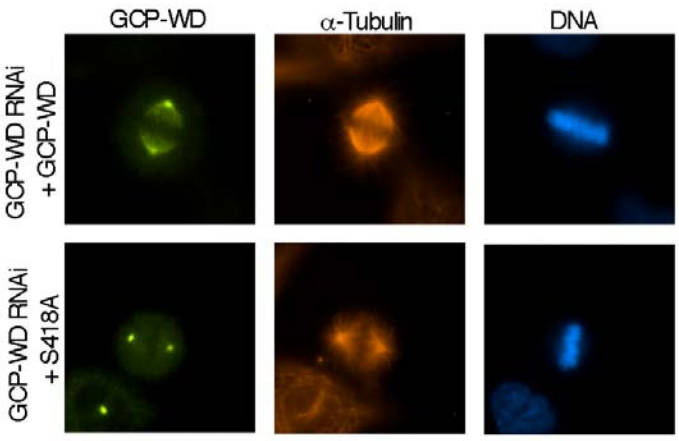


Figure 2. Mitotic phosphorylation of GCP-WD is required for spindle targeting but not for centrosome targeting. *HeLa cells were cotransfected with a plasmid expressing shRNA targeting endogenous GCP-WD and plasmids expressing either RNAi-insensitive GCP-WD-MycHis or the phosphorylation mutant GCP-WD S418A-MycHis. Cells were stained with anti-Myc and anti-α-tubulin antibodies and DAPI to visualise DNA.*

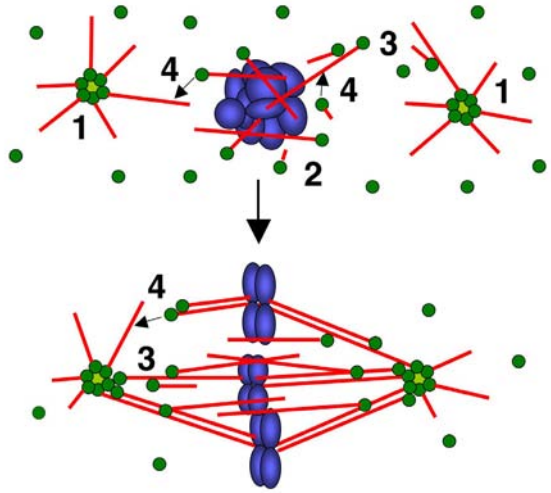


Figure 3. 'Amplification model' for the function of the γTuRC in spindle assembly. *Microtubules are nucleated by γTuRC at the two centrosomes (1) and near the mitotic chromatin (2). A third pathway depends on γTuRC bound to the sides of existing spindle microtubules (3). This pathway 'amplifies' the process of spindle assembly by nucleating additional microtubules where needed, within the forming spindle. Correct orientation is achieved by directional nucleation, or by motor-mediated orientation soon after nucleation. Minus end-bound γTuRC facilitates the capture of free microtubules by directing their ends to the sides of other microtubules (4). γTuRCs are depicted in green, microtubules in red, and chromosomes in blue.*

cephaly, a condition associated with certain neurodevelopmental disorders. Mutations in the genes of Cep215/Cdk5Rap2 and pericentrin cause primary microcephaly and Seckel syndrome, respectively. In both cases, affected individuals have abnormally small brains and show mental retardation. It has been speculated that microcephaly is caused by defects in the proliferation of neuronal progenitor cells, but mechanistic insight is still missing. We hope that our studies will contribute to a better understanding of these diseases.

Non-centrosomal microtubule nucleation in mitosis

γTuRCs typically interact with MT minus ends at centrosomes, where they nucleate and stabilise MTs. However, a large number of γTuRCs are also found in the soluble fraction of the cytoplasm and associated with mitotic spindle microtubules. In mitosis, these γTuRCs participate in non-centrosomal MT nucleation pathways, such as the chromatin-mediated nucleation pathway, which is controlled by the small GTPase Ran. Recently, we discovered that mitotic phosphorylation of GCP-WD at a Cdk1 consensus phosphorylation site targets the protein to a subset of spindle MTs (Figure 2). Mutation of this phosphorylation site interferes specifically with the recruitment of γTuRC to spindle MTs.

The lack of spindle-associated γTuRCs results in defective spindles that assemble less efficiently and have a lower density of MTs, but does not seem to affect centrosomal or chromatin-mediated nucleation (Lüders *et al*, 2006). On the basis of these findings, we proposed that spindle-bound γTuRC is required to nucleate additional MTs within the spindle to allow proper spindle formation. Studies in *Drosophila* have identified components of a protein complex termed augmin, which function upstream of GCP-WD in mediating the association of γTuRCs with mitotic spindle microtubules (Goshima *et al*, 2008). Together, these studies support a model in which γTuRCs are recruited to the sides of pre-existing spindle MTs to nucleate additional MTs, thereby "amplifying" MT nucleation and promoting spindle formation (Lüders and Stearns, 2007; Figure 3).

We have started to characterise this new pathway in human cells. One of our goals is to achieve a molecular understanding of how these novel, non-centrosomal MT nucleation sites are assembled. Using a cell line that stably expresses γ-tubulin fused to photoactivatable GFP, we demonstrated that, compared to the interaction of γ-TuRCs with centrosomes, the interaction of γTuRCs with spindle microtubules is much more dynamic (Archinti, Lacasa and Lüders, in preparation), thereby hindering direct analysis in living cells. We are using bioinformatics, yeast two-hybrid screening, biochemical and RNAi-based approaches to identify the components of this pathway. We have already identified several candidate genes, which we are currently analysing for a role in non-centrosomal MT nucleation within the spindle. In addition, we have generated tools, such as stable cell lines and antibodies, for the analysis of this pathway *in vitro* and *in vivo*.

Microtubule nucleation during differentiation

During the differentiation of muscle cells, myoblasts fuse to form multi-nucleated myotubes. This process involves a reor-

ganisation of the MT network from a radial, centrosome-based MT array to an elongated array composed of parallel MTs in myotubes. Interestingly, centrosomes degenerate during myotube formation and differentiation. Several PCM proteins, including γ-tubulin, redistribute to the surface of the nuclear envelope, which functions as MTOC. Whether the nuclear envelope-associated MTOC is critical for myotube formation or differentiation is not known.

Using lentivirus-mediated infection and expression of shRNA to deplete endogenous GCP-WD in mouse C2C12 cells, an *in vitro* model for muscle cell differentiation, we found that, in contrast to its role in centrosome attachment, the γ-tubulin targeting factor GCP-WD is not required for nuclear envelope localisation of γTuRC. Moreover, a few days after differentiation is initiated, GCP-WD expression is down-regulated, thereby suggesting that its function is not required in differentiated muscle cells. γ-tubulin expression remains relatively constant, suggesting that the γTuRC participates in MT organisation throughout the differentiation process. To address this question, we have also established conditions for efficient RNAi-mediated depletion of γ-tubulin in muscle cells. These studies will provide the first insight into the role of non-centrosomal MT nucleation pathways in the reorganisation of the MT cytoskeleton during cellular differentiation.

SCIENTIFIC OUTPUT

Research networks and grants

Microtubule organizing centers and microtubule nucleation in mitosis (MTOC function)
European Commission, PEOPLE-2007-4-3-IRG (2008-2012)
Principal investigator: Jens Lüders

Collaborations

Recruitment of γ-tubulin complexes to mitotic centrosomes
Andreas Merdes and Laurence Haren, Institut de Sciences et Technologies du Médicament de Toulouse, Centre National de la Recherche Scientifique/Pierre Fabre (Toulouse, France)

Regulation of microtubule nucleation through phosphorylation
Carme Caelles and Joan Roig, Molecular Medicine Programme, IRB Barcelona (Barcelona, Spain)

MARCO MILÁN

Development and growth control

During the development of a given tissue or organ, growth and fate specification are controlled in a coordinated manner by the activity of a discrete number of signalling molecules and their corresponding pathways to give rise to a well-formed structure with a particular size, shape and pattern. The activity of these signalling molecules has to be tightly regulated since deregulation of their activity might cause uncontrolled growth and cancer. Understanding how the activity of these molecules is controlled and how cells of different tissues or organs translate the activity of their pathways into activation or repression of the cell cycle machinery in a context-dependent manner are currently two of the most intriguing questions in developmental and cancer biology. Growth and fate specification must also be tightly coupled to generate a shaped and sized structure. Uncoupling these two processes has disastrous consequences in development and disease. In the last few years, much has been learned about the regulation of growth and fate specification. However, very little is known about how these processes are coupled. In multicellular organisms, stable subdivision into adjacent tissues and organs relies on the acquisition of distinct cell affinities. These are defined either by the differential expression of cell adhesion molecules or by interactions between adjacent cell populations that lead to cell repulsion. Intermingling of cells between adjacent tissues and organs has catastrophic effects on patterning and growth. The wing primordium of *Drosophila*, a highly proliferative epithelium that arises as a group of 30-40 cells in the embryonic ectoderm and proliferates during five days to reach a final size of around 50,000 cells, is a highly suitable model system to analyse these processes at a genetic, cellular and molecular level during development.

Our laboratory works on the following research lines: 1) Regulation of the activity of signalling molecules, 2) Coordinated control of cell cycle progression, tissue growth and fate specification, and 3) Molecular characterisation of cell affinity differences between adjacent tissues. In particular, the following topics have been addressed during 2008:

A wingless and Notch double-repression mechanism regulates G1-S transition in the *Drosophila* wing

The control of tissue growth and patterning is orchestrated in various multicellular tissues by the coordinated activity of the signalling molecules Wnt/Wingless and Notch, and mutations in these pathways can cause cancer. The role of these molecules in the control of cell proliferation and the crosstalk between their corresponding pathways remain poorly understood. Crosstalk between Notch and Wingless (Wg) has been proposed to be responsible for organising the growth and patterning of the *Drosophila* wing primordium.

Héctor Herranz, with the help of Lidia Pérez in the lab, has revised the role of Wg and Notch in the control of cell proliferation and has presented evidence that a Wg and Notch double-repression mechanism controls G1-S transition in the presumptive wing primordium (Figure 1). These molecules exert their function by regulating the expression of the *dMyc* proto-oncogene and the *bantam* micro-RNA, which positively modulate the activity of the E2F transcription factor. Thus, in this cellular context Notch acts as a repressor of cell cycle progression and Wg has a permissive role in alleviating Notch-mediated repression of G1-S progression in wing cells. This work clarifies and simplifies the role of Notch and Wg in cell cycle control in the *Drosophila* wing and provides a suitable model by which to analyse the function of Notch and Wg signalling pathways in the regulation of the cell cycle machinery.

There is evidence in mammals that Notch acts as a tumour suppressor gene or as an oncogene, depending on the cellular context. The cellular context should then modulate the differential response of the cells to changes in Notch activity, in some cells

Principal Investigator Marco Milán **Postdoctoral Fellows** Isabelle Becam, Andrés Dekanty, Héctor Herranz **PhD Students** Lara Barrio, Duarte Mesquita, Neus Rafel, Georgina Sorrosal **MSc Student** Ana Ferreira **Research Assistant** Lidia Pérez **Programme Technician** Ainoa Olza **Visiting Student** Katrin Rein (Estonia)

leading to hyper-proliferation, in others to quiescence. How this context and the differential response are defined at the molecular level remains unclear. *Drosophila*, again, provides a very suitable model system to address this issue. Although our results highlight that Notch is involved in the inhibition of G1-S progression in wing discs, in other developmental contexts Notch exerts a proliferative function. Interestingly, in both cases, Notch acts through Rbf and E2F to positively or negatively control the G1-S transition. We therefore speculate that the molecular context is then defined by the effectors available, like *dMyc* and *bantam*, or by the presence of nuclear factors that act as a switch in the ultimate activation/inactivation of E2F via Rbf.

Notch signalling coordinates tissue growth and wing fate specification in *Drosophila*

During the development of a given organ, tissue growth and fate specification are simultaneously controlled by the activity of a discrete number of signalling molecules. The wing primordium contains the progenitors of both the adult body wall and the wing (Figure 2b). The developmental decision between wing and body wall is made early in development and it is defined by the opposing activities of the two secreted signalling molecules Wg and the EGFR ligand Vein (Vn) in the most ventral and dorsal sides of the wing primordium, respectively (Figure 2a). Notch activity has been proposed to participate in this process since loss of this signalling molecule during this developmental stage leads to the failure to induce wing fate with a concomitant duplication of body wall structures (Figure 2b). With the corresponding wing and body wall molecular markers, Neus Rafel in the lab is further analysing the role of Notch in this process, not only in the adult fly but also in the developing wing primordium. Her data indicate that growth of the wing primordium mediated by the activity of Notch is required for wing fate specification (Figure 2a).

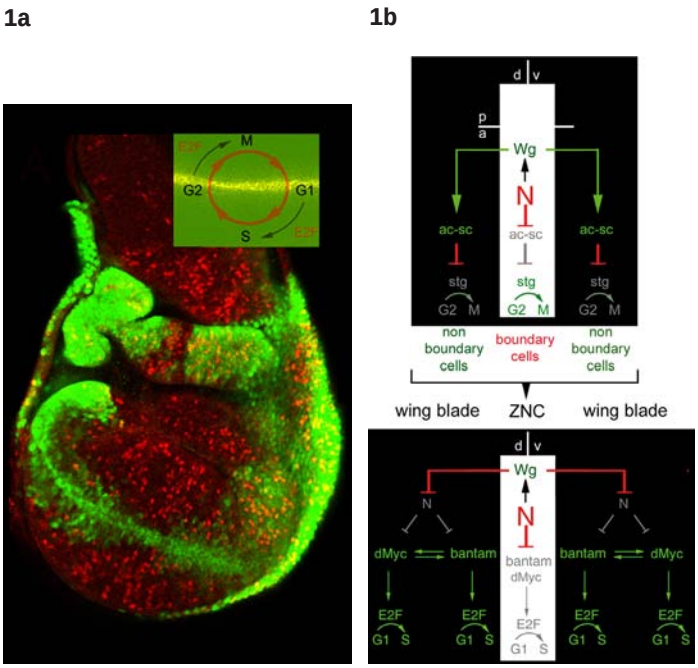


Figure 1. *(a) Wing imaginal disc labelled to visualise cells in S-phase (red) and Notch activation (green). (b) Regulation of G1-S and G2-M transition in the Drosophila wing is controlled by the activity of Notch (N) and Wingless (Wg) through bantam micro-RNA and dMyc proto-oncogene.*

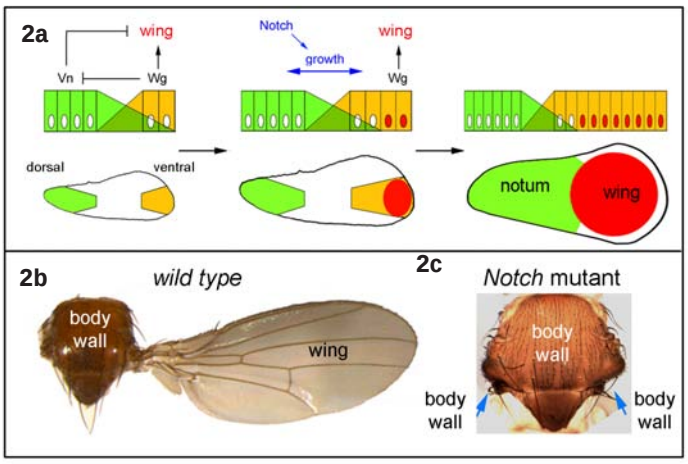


Figure 2. *(a) Subdivision of the wing primordium into wing and body wall is mediated by the opposite activities of the signalling molecules Wingless (Wg) and Vein (Vn), respectively. Growth induced by the Notch activity pulls the Wg and Vn sources apart, thus facilitating wing fate specification. (b, c) Adult wing and body wall of wild-type (a) or mutant fly lacking Notch activity (b). Note in b lack of wings and duplication of body wall structures.*

Expression of Wg in the most ventral part of the wing disc specifies the wing field at the same time as restricting Vn expression to the most dorsal part. Vn is required to block responsiveness of body wall cells to Wg. Thus, the relative concentration of the diffusible proteins Wg and Vn experienced by disc cells directs their wing versus body wall fate. In the early wing primordium, Vn reaches every wing cell, thereby blocking responsiveness to Wg and repressing wing fate specification. Growth induced by Notch activity pulls the sources of Wg and Vn apart, most ventral cells do not sense sufficient Vn levels so Wg is able to induce wing fate. These results highlight a critical role of Notch in linking tissue growth and fate specification in the developing wing primordium.

Growth promoted by Notch has also been shown to be directly involved in the specification of the eye within the *Drosophila* eye-antenna primordium, a process that also depends on the opposing activities of two secreted signalling molecules, in this case Dpp (a TGF-β family member) and Wg. Thus, in both eye and wing primordial, Notch elegantly coordinates tissue growth and eye/wing specification by modulating the response of cells to the activities of signalling molecules. These results also suggest that the same mechanism may be commonly used in animal development to coordinate tissue growth and fate specification.

The evolution of wings was crucial in the process of adaptation, allowing insects to escape predators or colonise new niches. It has recently been shown that loss and recovery of wings has occurred during the course of evolution. This finding would suggest that wing developmental pathways are conserved in wingless insects and are being re-used. On the basis of our results, we speculate that adaptative changes in animal size modulates the cellular response to signalling molecules like Wg, thus helping to drive some of these extraordinary reversible transitions.

A permissive role of Notch in maintaining the DV affinity boundary of the *Drosophila* wing

Cell affinities can contribute to organising cells into tissues and organs. *Drosophila* limbs and the vertebrate central nervous system are subdivided into adjacent populations that do not mix. These cell populations are called compartments. Cell interactions mediated by Notch receptor have been implicated in the specification of compartment boundaries (Figure 3). However, the contribution of Notch to this process remains controversial. The instructive role of Notch and the transcriptional requirement of the pathway have been questioned in the last few years.

Given the central role of Notch in making developmental boundaries in vertebrates and invertebrates, Isabelle Becam is re-evaluating its contribution and its signalling pathway in the maintenance of an affinity difference between dorsal (D) and ventral (V) compartments in the *Drosophila* wing. Her data indicate that unrestricted, low levels of Notch are sufficient for the formation of a stable DV affinity boundary. Cleavage of the Notch protein, release of the intracellular domain and a transcriptional function of Notch via the Suppressor of Hairless

transcription factor is required and sufficient in this process. Isabelle's data support a permissive role of Notch in maintaining the DV affinity boundary. This contrasts with the instructive role of Notch in executing the organising activity of this boundary. The DV compartment boundary organises the growth and pattern of the wing primordium. Restricted and high levels of Notch activity mediate this organising activity in a Su(H)-dependent manner. Unrestricted activation of Notch has disastrous consequences on the growth and patterning of the wing primordium.

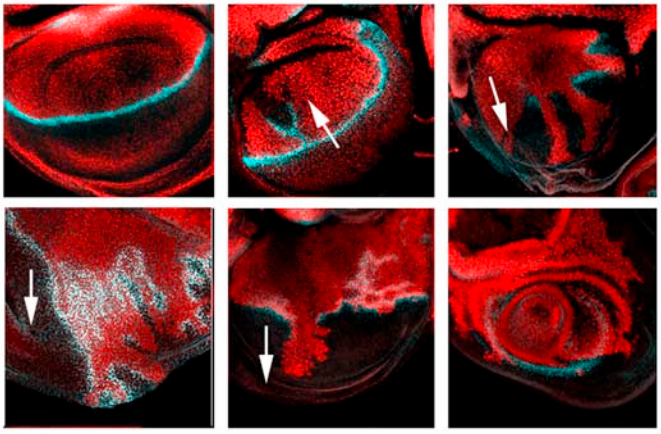


Figure 3. *The interface between dorsal (red) and ventral (black) cells behaves as an affinity boundary but also as an organiser centre. This affinity boundary is violated (see white arrows) when the DV boundary (blue) is not properly established.*

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Ajuts a grups emergents
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00118 (2006-2009)
Principal investigator: Marco Milán

Compartments, organizing molecules and growth control in Drosophila
EMBO Young Investigator Programme (2008-2010)
Principal investigator: Marco Milán

Establishment and maintenance of compartment boundaries in the Drosophila wing imaginal disc
Spanish Ministry of Science and Innovation, BFU2007-64127/BMC (2007-2010)
Principal investigator: Marco Milán

From genes to shape: analysis of morphogenesis in Drosophila and vertebrates
Spanish Ministry of Science and Innovation, CSD2007-00008 (2008-2012)
Principal investigator: Marco Milán

Collaborations

Growth control in the Drosophila wing
Francisco Martín, Centro de Biología Molecular, Universidad Autónoma de Madrid/CSIC (Madrid, Spain)

Awards

EMBO Young Investigator Programme Award, European Molecular Biology Organization (2008-2010)

LLUÍS RIBAS DE POUPLANA

Gene translation laboratory

The gene translation machinery is directly linked to a large number of human diseases. Our general goal is to contribute to the understanding of genetic code biology and its integration within the cellular metabolism. In recent years it has become evident that aminoacyl-tRNA synthetases (ARSs) play central roles in cell cycle regulation, the control of gene expression, cell-cell communication and tissue development. This array of new functions has directly linked ARSs and protein synthesis with regulatory cellular pathways and an increasing number of human diseases. Our current projects and aims are as follows: (i) to study the effect of the accumulation of translation errors on human cells, and identify the mechanisms of control and correction of such problems; (ii) to generate an animal model for mitochondrial disease linked to translation defects and to study the cellular responses to increasing levels of protein synthesis disruption; (iii) to study the role of cytokine-like ARS domains in the infectious cycle of *Entamoeba*; and (iv) to examine the set of ARSs in *Plasmodium falciparum* and identify new inhibitors of these enzymes.

The effect of the accumulation of translation errors on human cells, and identification of the mechanisms of control and correction of such problems

The human genome codes for at least 30,000 proteins, and the machinery required to produce these makes, on average, one error per 1,000 amino acids that are incorporated into polypeptide chains (Edelmann and Gallant, 1977). The capacity of ARSs to specifically recognise their cognate amino acid and tRNA substrates and, in some cases, to correct initial recognition errors via their editing domains, constitutes the main proofreading step in protein synthesis. A complementary check is later performed by elongation factors, which can reject certain misacylated tRNAs (LaRiviere *et al*, 2001).

A decrease in the fidelity levels of the protein synthesis apparatus has been shown to be deleterious and potentially lethal. Several studies have demonstrated that an increase in the rate of error during protein synthesis reduces cell viability (Beebe *et al*, 2003). In mammals, the malfunctioning of some elements of the translation machinery causes disease. For example, a point mutation in the editing domain of alanine-tRNA synthetase (AlaRS) causes cerebellar Purkinje cell loss and ataxia in mice (Lee *et al*, 2006). This mutant AlaRS has lost its capacity to hydrolyse ser-tRNAAla.

We have devised a genetic approach with engineered tRNAs to introduce random and widespread mutations of increasing impact into a metazoan proteome. Using a human cell line and an embryonic vertebrate model, we have studied the cellular reaction to increasing amounts of mutagenic insult and have identified the pathways that react first, and proportionally, to the accumulation of proteome defects. Our results indicate that the endoplasmatic reticulum acts, through the activation of the unfolded protein response (UPR), as the cellular sensor for overall protein quality, reacting steadily to the accumulation of errors that escape the action of upstream editing mechanisms.

We have shown that the UPR acts not only as a regulator of the quality of secreted proteins, but also as a general sensor for the condition of the entire cellular proteome. The UPR reacts earlier than the ubiquitination pathway to errors in protein synthesis and, unlike the proteasome, it is activated differentially depending on the degree of biophysical stress caused by the errors in translation (Geslain *et al*, in preparation).

Comparable to the effect of a mischarging ARS would be the advent of widespread mutations in the genome. It has been amply reported that genomic mutational damage caused by radiation or exposure to DNA-modifying chemicals can cause cell death. However, very little is known about the control mechanisms used by cells to respond to mistranslation events that escape the control mechanisms aforementioned. Consequently, this project aims to characterise, in a quantitative manner, the cellular response to this kind of insult.

Principal Investigator Lluís Ribas de Pouplana **Research Associate** Alfred Cortés **Postdoctoral Fellow** Laia Cubells **PhD Students** Manuel Castro, Valerie Crowley, Yaiza Español, Tanit Guitart, Thomas Jones, Eva Maria Novoa **Master Student** Ana Bernadó **Research Assistant** Núria Rovira **Lab Technician** Noelia Camacho **Visiting Students** Ewa Chudyk (Poland), Assitan Sidibe (France)

Generating a model for mitochondrial disease linked to translation defects and studying the cellular responses to increasing levels of protein synthesis disruption

The mitochondrial translational machinery is responsible for the synthesis of the respiratory chain proteins encoded by the organelle genome. Therefore, defects in the mitochondrial translational apparatus lead to a variety of health disorders, mainly related to mitochondrial metabolism.

More than 100 mutations involving rRNA and tRNA have been found in the human mtDNA. Furthermore, several diseases have been reported to be related to mutations in mt-tRNA modification proteins or in mitochondrial proteins involved in translation. In the last few years a number of mitochondrial ARS mutations have been found to cause central nervous system disorders (Scheper *et al*, 2007; Edvarson *et al*, 2007) or shown to be susceptibility agents for diabetes mellitus ('t Hart *et al*, 2005). It is generally believed that mitochondrial diseases caused by mutations in ARS and tRNAs are caused by the effect of these mutations on the protein synthesis rate of the organelle.

A characteristic of mitochondrial metabolic diseases is their extreme case-to-case variability, a feature attributed to the polyclonal nature of mitochondria. This feature, together with the experimental difficulty of manipulating mitochondria, makes these diseases extremely refractive to research efforts. Thus, the complexity and medical relevance of mitochondrial diseases justifies interest in developing animal models to study the consequences of mitochondrial aminoacylation deficiencies.

In this line of research, we are building an animal model for mitochondrial disease in *Drosophila melanogaster* by generating tissue-specific and gradual deficiencies in mitochondrial serylation in this organism. To achieve this goal, we are generating fly strains in which the nuclear-encoded mitochondrial seryl-tRNA synthetase (DmSRS2) can be silenced by interference RNA (Brand *et al*, 1993). We have observed that the ubiquitous and constitutive silencing of DmSRS2 produces lethality and that tissue-specific silencing leads to a range of phenotypic defects in the fly. We are currently evaluating these defects and studying the stress pathways activated by this insult (Figure 1).

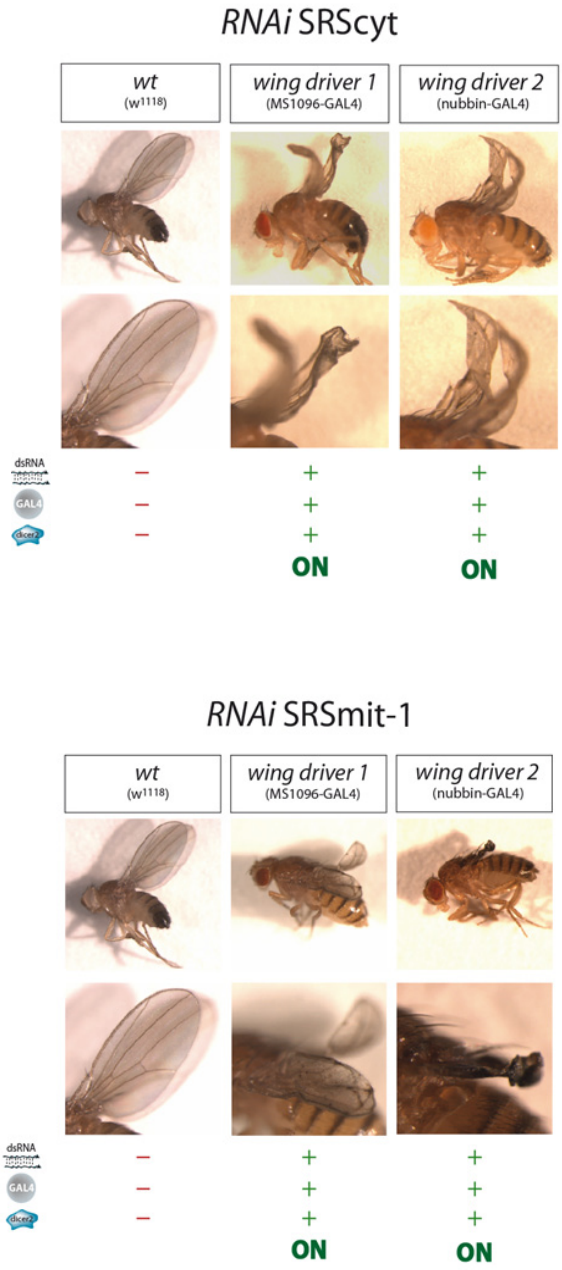

Figure 1. *Effect of silencing of cytosolic (top) and mitochondrial (bottom) SRSs in the Drosophila wing.*

Studying the role of cytokine-like ARS domains in the infectious cycle of *Entamoeba*

ARSs and related proteins carry out additional cellular functions and are implicated in several metabolic pathways, cell signalling mechanisms, and developmental processes (Ribas de Pouplana and Geslain, 2008). AIMP1, the best characterised of these proteins, is a wide acting cytokine that induces inflammatory responses in endothelial cells, apoptosis, and migration of inflammation-related cell types. AIMP1 and derived peptides are active in a large number of cell signalling and developmental pathways (van Horssen *et al*, 2006).

Protozoan parasites have evolved a variety of strategies to evade the immune responses of their hosts. In the best documented cases, these adaptations involve the modification of the parasite's surface to escape the immune response (Cortes, 2007). We have discovered that in several *Entamoeba* species the genes coding for methionyl- and lysyl-tRNA synthetases (EhKRS) contain a C-terminal domain homologous to the human AIMP1 cytokine. *Entamoeba* is the first case in which this domain is found attached to KRS, and it is also the first known case of a genome containing two copies of human-like AIMP1-type domains attached to distinct genes. In this project, we explore the interactions between mammalian inflammatory pathways and the human-like cytokine (EhAIMP) that we have recently discovered in the genus *Entamoeba*.

Kinetic characterisation of the protein showed that the functional role of EhAIMP1 is not related to the aminoacylation reaction of $tRNA^{Lys}$. To test whether EhAIMP1 was capable of mimicking the functions of human AIMP1, we checked its capacity to affect the viability of bovine aorta endothelial cells (BAECs). We showed that EhAIMP1 and EhKRS induced apoptosis in BAECs in a dose-dependent manner at concentrations comparable to those required to exert the same effect with human AIMP1.

We also analysed the TNF-α secretion level after the addition of EhKRS, EhKRSΔ and EhAIMP1 proteins and observed an increase in TNFα release to similar levels as those caused by human AIMP1 (Figure 2). This effect is mainly due to the activity of the EhKRS synthetase domain, although the EhAIMP1 domain also produces a small effect. Inactivated *Entamoeba* proteins do not produce an increase in TNFα secretion. These results support the notion that this protein mimics the role of its human counterpart and that it participates in the interaction of *Entamoeba* with its human hosts during the infection cycle.

Studying the set of ARSs in *P. falciparum* and identifying new inhibitors of these enzymes

This research project is focused on tRNA biology in *P. falciparum* and on the development of a new pharmacological screen for the discovery of *Plasmodium* ARS inhibitors. Protein synthesis in *Plasmodium* remains largely uncharacterised. As an example, the first experimental evidence that protein translation is active in *Plasmodium* apicoplasts was obtained as recently as 2005 (Chaubey *et al*, 2005). Furthermore, it has long been recognised that protein synthesis inhibitors, like doxycycline, are active against *P. falciparum* and are useful drugs in the treatment of malaria (Clyde *et al*, 1971). Thus, the study of components of

the genetic code of *P. falciparum* may provide new and relevant information on the biology of the parasite and, more importantly, may open up new leads for the development of novel anti-malarials.

Genomic data readily allows the identification of the complete set of *P. falciparum* ARS genes, including several duplicated ones that could correspond to enzymes that are active in the apicoplast or the mitochondria of the parasite. At present, the cellular localisation of several of these enzymes is unclear, particularly in the mitochondria where the predicted set of ARS is clearly incomplete.

Preliminary analysis of several *P. falciparum* life cycle inhibitors performed in our lab has shown several types of inhibitory effects, characterised in some cases by a delayed mode of action (Camacho *et al*, in preparation). More specifically, up to three types of inhibitors can be identified on the basis of the delay observed before arrest of the erythrocyte invasion cycle. These delayed effects have been previously linked to disruptions of the apicoplast metabolism (Dahl *et al*, 2007).

A central focus of this research line lies in drug development. Particularly interesting as drug targets are ARSs that are unique to *P. falciparum* and not present in human cells. In this project, we plan to analyse known inhibitors of ARSs for their potential use against *P. falciparum*, construct combinatorial chemistry libraries based on the molecular data obtained by several of the partners involved, and develop a new method for the discovery of new inhibitors based on a positive selection scheme that will accelerate the current methods of drug discovery. In this regard, our lab coordinates an EU consortium of eight laboratories that seeks to elucidate several aspects of gene translation in this parasite and extend this knowledge to the discovery of new inhibitory molecules.

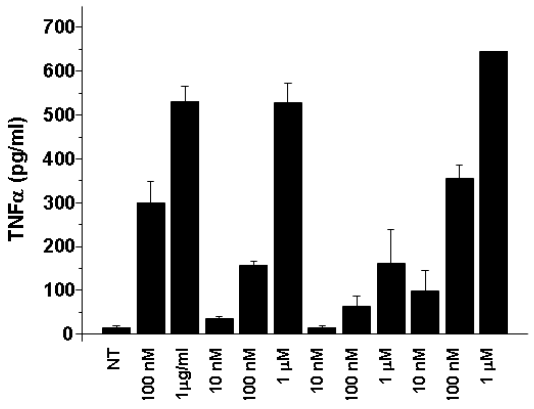

Figure 2. *EhAIMP1 causes an increase in TNFα secretion in human RAW264.7 cells.*

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Research networks and grants

Ayuda para la preparación del proyecto europeo 'Planificación de transmal: una red europea de investigación sobre malaria'
Spanish Ministry of Science and Innovation, SAF2007-29381-E (2008)
Principal investigator: Lluís Ribas de Pouplana

Desarrollo de un nuevo método para la selección de antibióticos
Spanish Ministry of Science and Innovation, BIO2006-01558 (2007-2009)
Principal investigator: Lluís Ribas de Pouplana

Plasmodium falciparum que codifican ligandos para la invasión de eritrocitos y fenotipos asociados al silenciamiento o activación de los mismos
Instituto de Salud Carlos III, PI07891 (2007-2010)
Principal investigator: Lluís Ribas de Pouplana

Support to exceptional research groups
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00350 (2006-2008)
Principal investigator: Lluís Ribas de Pouplana

Targeting protein synthesis in the apicoplast and cytoplasm of plasmodium (MEPHITIS)
European Commission, FP7-HEALTH-2007-B (2008-2011)
Principal investigator: Lluís Ribas de Pouplana (coordinator)

Collaborations

Characterisation of Entamoeba EhAIMP1
Sunghoon Kim, University of Seoul (Seoul, Korea)

Characterisation of Entamoeba EhAIMP1
Antonio Celada, IRB Barcelona (Barcelona, Spain)

Characterisation of Mycoplasma penetrans LysRS
Rebecca Alexander, Wake Forest University (North Carolina, USA)

Discovery of ARS inhibitors
Omnia Molecular SL (Barcelona, Spain)

Evolution of human ARS domains
aTyr Pharma (San Diego, USA)

Generation of animal models of ARS-related diseases
Elena Jordanova, University of Antwerp (Antwerp, Belgium)

Generation of an animal model of cellular mistranslation
Elisa Martí, Institute of Molecular Biology of Barcelona, Spanish National Research Council (IBMB-CSIC) (Barcelona, Spain)

Protein synthesis in Plasmodium
EU-funded consortium of eight laboratories coordinated by our group

EDUARDO SORIANO

Developmental neurobiology and regeneration

Brain development is a complex process that involves several sequential steps: regional determination, specification of neuronal cell types, control of cell migration, guidance and formation of neural connective networks, and activity-dependent synaptic plasticity. Recent research has demonstrated that these steps are exquisitely controlled by a variety of molecular and cellular mechanisms, including the expression of specific transcription factors, the activity of morphogens and growth factors, the expression of guidance molecules and extracellular proteins, and synaptic activity. Our research focuses on the identification of new genes involved in these processes, and the characterisation of the intracellular signalling pathways activated in growth cones in response to extracellular signals. Moreover, it is known that the adult brain does not regenerate, either after lesions or disease-associated cell-death processes. Studies on the mechanisms that govern the normal development and growth of the nervous system are essential to explain the lack of spontaneous brain repair in adult tissue and to design new regenerative approaches to repair brain lesions.

Further roles of netrins and semaphorins in neuronal guidance

We have further investigated the roles of several guidance molecules in the formation of complex brain structures, such as the cerebral cortex and the cerebellum. For instance, elucidating the way in which GABAergic interneurons in the cerebellar cortex migrate or finding the guidance cues that steer them are part of our research efforts. Recent data show that the development of interneurons starts at the cerebellar germinal epithelium on top of the fourth ventricle. These interneurons continue to proliferate in the postnatal cerebellar white matter and later migrate to their final position in the cerebellar cortex. We have demonstrated a chemorepulsive action of Netrin1 on postnatal cerebellar interneurons *in vitro*; we have also reported the expression pattern of Netrin1 and its receptors DCC and Unc5 in the developing cerebellar system. Our expression results corroborate that Netrin1 is involved in the migration of GABAergic interneurons *in vivo*. Moreover, our data point to Bergmann glial fibers as possible tracks for these cells en route to the molecular layer. Finally, experiments using blocking antibodies have allowed us to conclude that DCC, although expressed by postnatal cerebellar interneurons, is not involved in the repulsive response triggered by Netrin1 in these cells (Guijarro *et al*, 2006).

We have also studied the distribution and role of a specific variant of semaphorin Y/6C (Sema6C) in mouse forebrain development and plasticity. Growth cone collapse of entorhinal and pyramidal neurons, as well as activation of glycogen synthase kinase-3 (GSK-3) through depletion of the inactive pool, is induced by a diffusible Sema6C1 form, thereby suggesting that this protein participates in development. We found this isoform to be widely expressed during development, remaining in the adult and showing variations in distribution when the perforant pathway was axotomised. These changes were detected in both the hippocampal and entorhinal cortices. In axotomised animals, the ipsilateral hippocampus hemisphere, but not the contralateral, showed that Sema6C-IR had moved into the stratum lacunosum-moleculare, the medial molecular layer of the dentate gyrus (DG) and the fibers, but not the cell bodies, of the entorhinal cortex (EC). These results indicate a specific role for Sema6C variants in the generation and/or stability of circuits and synapses (Burgaya *et al*, 2006).

The tyrosine kinase ACK1/PYK1 in brain development and plasticity

Cytosolic tyrosine kinases play a critical role both in neural development and in adult brain function and plasticity. We have isolated a cDNA that directs the expression of a 125-kD protein that can be autophosphorylated in tyrosines. This clone corresponds to the mouse homologue of Ack1 (Ack1/

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Pyk1) and is a non-receptor protein tyrosine kinase that comprises a tyrosine kinase core, an SH3 domain, a Cdc42-binding region, a Ralt homology region, and a proline-rich region. The highest levels of Ack1/Pyk1 expression are detected in the brain, particularly in the hippocampus, neocortex, and cerebellum. Electron microscopy studies show that Ack1/Pyk1 protein is expressed both at dendritic spines and presynaptic axon terminals, thereby indicating that this protein is involved in synaptic function. Furthermore, Ack1/Pyk1 mRNA levels are strongly up-regulated by increased neural activity, which points to a role in plasticity. During development, Ack1/Pyk1 is also expressed in the proliferative ventricular zones and in postmitotic migrating and maturing neurons. These results demonstrate that this kinase is up-regulated during development and that it is expressed in proliferative areas and in migratory pathways in the developing brain. In neuronal cultures, Ack1/Pyk1 is detected in developing dendrites and axons, including dendritic tips and growth cones. Moreover, Ack1/Pyk1 colocalises with Cdc42 GTPase in neuronal cultures and co-immunoprecipitates with Cdc42s (Ureña *et al*, 2006; De la Torre *et al*, 2006). Activation of integrins by cell adhesion on fibronectin leads to strong tyrosine phosphorylation and activation of Ack. Upon cell stimulation with EGF or PDGF, Ack is tyrosine-phosphorylated and recruited to activated EGF or PDGF receptors, respectively. Moreover, tyrosine-phosphorylated Ack forms a stable complex with the adapter protein Nck via its SH2 domain (Galisteo *et al*, 2006). Taken together, our findings indicate that Ack1/Pyk1 tyrosine kinase has a functional role as an early transducer of multiple extracellular stimuli, and that it may be involved in adult synaptic function and plasticity and in brain development.

The axonal growth cone: a sophisticated exploring "apparatus" designed to integrate convergent and divergent signalling pathways

During the development of the nervous system, precisely ordered neuronal connections are formed in a stereotyped, stepwise process. Initially, finely orchestrated expression of

axon guidance molecules and their receptors in the projecting and the target area provide positional and directional information for ingrowing axons, which leads to a coarse connection between distinct groups of neurons. Later, activity-dependent processes, including the formation and elimination of new branches, sharpen the projection, resulting in precise point-to-point connections. Throughout this process, the key apparatus of the growing axons is the neuronal growth cone. This cone can be envisaged as an exploring region at the axonal tips which integrates information from the neighbouring 'milieu' to transduce signals that finally may stop or increase axonal growth. In recent years, many signalling pathways that regulate axonal navigation have been identified (*eg*, netrins, semaphorins, ephrins, etc.), each bearing a full complement of receptors and associated intracellular mediators. However, how these signalling pathways, often with opposite effects, interact with each other, the hierarchy among them (if present), or how ligand/receptor complexes talk to other components of cell machinery, like cytoskeletal proteins and proteins regulating membrane trafficking, are not known.

Our research activities explore these issues by means of simple neuronal culture models. For instance, we have recently discovered a protein-to-protein interaction between the DCC guidance receptor and the SNARE proteins Syntaxin 1 and SNAP-25. Furthermore, these SNARE proteins are required for Netrin1/DCC-induced axonal guidance and migration, both *in vitro* and after electroporation in the spinal cord. These data point to a link between guidance receptors and the cell machinery controlling exocytosis and membrane addition (Cotrufo *et al*, in preparation).

Similarly, we explore cross-talk mechanisms between guidance molecule receptor systems. For instance, we have evidence of an interaction between the neurotrophin/trk cascade and the Netrin1/DCC and EphrinA-associated signalling pathways. We have recently shown that activation of EphrinA blocks neurotrophin-induced effects on axonal branching and synapse formation (Marler *et al*, 2008).

Dissecting novel Reelin functions in development and neurodegenerative diseases

Reelin is a glycoprotein that is essential for the correct cytoarchitectonic organisation of the developing central nervous system (CNS). Reelin binds to very low-density lipoprotein receptor and apolipoprotein E receptor 2, thereby inducing mDab1 phosphorylation and activation of the phosphatidylinositide 3 kinase (PI3K) pathway. We have now demonstrated that Reelin activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway, which leads to the phosphorylation of Erk1/2 proteins. The inhibition of Src family kinases (SFK) blocks Reelin-dependent Erk1/2 activation. This has also been shown in neuronal cultures from mDab1-deficient mice. Although rat sarcoma viral oncogene was weakly activated upon treatment with Reelin, pharmacological inhibition of the PI3K pathway blocked Reelin-dependent ERK activation, which indicates cross-talk between the ERK and PI3K pathways. We have shown that blockade of the ERK pathway does not prevent the chain migration of neurons from the subventricular zone (SVZ) but does inhibit the Reelin-dependent detachment of migrating neurons. We have also demonstrated that Reelin induces the transcription of the early growth response 1 transcription factor (Simó *et al*, 2006). In addition, we have shown a novel role of Reelin in the migration of cerebellar granule cells, which is highly dependent upon ERK activation (Simó *et al*, 2007). These findings indicate that Reelin triggers ERK signalling in an SFK/mDab1- and PI3K-dependent manner and that ERK activation is required for Reelin-dependent transcriptional activation, the detachment of forebrain neurons migrating from the SVZ, and the migration of cerebellar granule cells.

The function of Reelin in the adult brain is not understood, although it has been proposed that this protein is involved in signalling pathways linked to neurodegeneration. We have analysed Reelin expression in brains and cerebrospinal fluid (CSF) from patients with Alzheimer's disease (AD) and from non-demented controls. We found a 40% increase in the Reelin protein levels in the cortex, but not in the cerebellum, of AD patients compared with controls. Similar increases were detected at the Reelin mRNA transcriptional level. This expression correlates with parallel increases in CSF but not in plasma samples. We also studied the pattern of Reelin glycosylation by using several lectins and the anti-HNK-1 antibody. Glycosylation differed in plasma and CSF. Furthermore, the pattern of Reelin lectin binding differed between the CSF of controls and AD patients. Our results show that Reelin is up-regulated in the brain and CSF in several neurodegenerative diseases and that CSF and plasma Reelin have distinct cellular origins, thereby supporting the notion that Reelin is involved in the pathogenesis of a number of neurodegenerative diseases (Botella *et al*, 2006). To test this hypothesis, we have generated a conditional transgenic mouse model that overexpresses Reelin in the forebrain. This transgenic mouse line is being crossed with several murine models of AD to ascertain whether the over-activation of the Reelin pathway increases neural degeneration in these mice.

Functions of Nogo-66, MAG and CS in axonal regeneration

Damaged axons do not regenerate after axotomy in the adult mammalian CNS. This may be due to local inhibitory factors at the site of injury, such as the overexpression of chondroitin sulfate (CS) proteoglycans (CSPGs), and the presence of myelin-associated inhibitors. To overcome CSPG- or myelin-induced inhibition, strategies based on extrinsic and intrinsic treatments have been developed. For example, NEP1-40 is a synthetic peptide that promotes axonal regeneration by blocking Nogo-66/NgR interaction, thereby promoting axon regrowth. Myelin-associated glycoprotein (MAG) also contributes to the prevention of axonal regeneration. We have studied the role of MAG, Nogo-66 and CS in the regeneration of cortical connections *in vitro*. We show that MAG expression is regulated in a distinct manner in the EC and the hippocampus in response to axotomy of the perforant pathway. The participation of MAG in preventing axonal regeneration was tested *in vitro*: neuraminidase treatment of axotomised entorhino-hippocampal cultures potentiates axonal regen-

eration (Mingorance *et al*, 2005). We have also examined whether the combination of complementary strategies facilitates axonal regeneration in slice co-cultures. The combination of CS cleavage with ChABC and NEP1-40 strongly facilitates the regrowth of entorhinal axons after axotomy, permitting the re-establishment of synaptic contacts with target cells. However, combined treatments do not improve the regeneration induced by ChABC alone (Mingorance *et al*, 2006). These results demonstrate that MAG, CS and Nogo-66 limit axonal regeneration in the cerebral cortex, and provide insights into the development of new assays and strategies to enhance axon regeneration in injured cortical connections.

Stem cells, neuronal precursor specification, and brain repair

The nervous system is formed by hundreds of types of neurons. The mechanisms by which the different types of neurons are generated and specified remain unclear. We have shown that in the cerebellum the pancreatic transcription factor *Ptf1a* is required for the specific generation of Purkinje cells and interneurons. Moreover, we have reported that granule cell progenitors in the external granule cell layer appear to be unaffected by deletion of *Ptf1a*. Cell lineage analysis in Ptf1a$^{Cre/Cre}$ mice was used to establish that, in the absence of *Ptf1a* expression, E12/E13-proliferating progenitors—normally fated to produce Purkinje cells and interneurons— shift to a granule cell phenotype and aberrantly migrate to the external granule layer. These findings indicate that *Ptf1a* is necessary for the specification and normal production of Purkinje cells and cerebellar interneurons, two essential GABAergic cell types of the cerebellar cortex. We have also established that Ptf1a is required for the suppression of the granule cell specification programme in cerebellar ventricular zone precursors (Pascual *et al*, in preparation). Given the key role of *Ptf1a* in Purkinje cell specification, we are now exploring whether the induced expression of this gene in neuronal stem cells of distinct origin induces their phenotypic differentiation into a Purkinje cell-like phenotype. If so, we will have devised a method to produce Purkinje cells *in vitro*, thereby facilitating cell therapy approaches in murine models of cerebellar ataxia.

The production of neurons is a temporally restricted process that occurs during embryonic life, except in a few brain areas (the hippocampus, cerebellum, and the subventricular zone). In fact, new granule neurons are produced in the DG of rodents and humans throughout adult life. Understanding the mechanisms that control cell proliferation and neuron production in these areas is crucial to devise therapeutic strategies aimed at producing neurons from the natural "niches" that contain neural stem cells. Recent studies have also reported adult neurogenesis in the cerebral cortex of healthy animals and after brain injury. We have analysed whether the absence of the synaptic input from the main hippocampal afferents induces neuronal generation in the hippocampus outside the DG and/or regulates the proliferation of DG neuroprogenitors. We have shown that the denervation of the hippocampus does not induce neurogenesis in hippocampal regions other than the DG. However, neuroprogenitor proliferation in the DG is reduced after fimbria-fornix lesions but not after entorhinal deafferentation. This observation supports the view that neuroprogenitor proliferation and differentiation in the DG are controlled from basal forebrain/septal neurons. We have also studied cell proliferation in the hippocampus of rodents and the intrinsic putative neurogenic potential of EC progenitors. We show that only the DG generates new neurons in the hippocampus. In addition, neurospheres from the EC have the capacity to differentiate into neurons and glia *in vitro* and after transplantation in the adult DG (Fontana *et al*, 2006). In a more recent study, we have identified Netrin1 as a key factor controlling neurogenesis and differentiation of neural stem cells, specifically in the DG (Barallobre *et al*, in preparation) and we are currently focusing our research efforts on elucidating the cellular mechanisms that control symmetrical versus asymmetrical neural cell division.

SCIENTIFIC OUTPUT

Publications

Díaz-Ruiz C, Parlato R, Aguado F, Ureña JM, Burgaya F, Martínez A, Carmona MA, Kreiner G, Bleckmann S, Del Río JA, Schütz G and Soriano E. Regulation of neural migration by the CREB/CREM transcription factors and altered Dab1 levels in CREB/CREM mutants. *Mol Cell Neurosci*, **39**(4), 519-28 (2008)

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Research networks and grants

Ajuts a grups de recerca consolidats
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00830 (2005-2008)
Principal investigator: Eduardo Soriano

Funciones de nuevos genes candidatos y proteínas asociadas a mielina durante el desarrollo y regeneración de las conexiones corticales
Spanish Ministry of Science and Innovation, BFU2006-13651 (2006-2009)
Principal investigator: Eduardo Soriano

Identificació i caracterització d'un nou sistema de senyalització associat a exocitosis i neurotrofines: paper en la generació del dolor
'La MTV3' Foundation, MTV3-071410 (2008-2010)
Principal investigator: Eduardo Soriano

Identificación y caracterización de nuevos genes y vías de señalización implicados en desarrollo cortical
Spanish Ministry of Science and Innovation, SAF2005-00171 (2005-2008)
Principal investigator: Eduardo Soriano

Implicación de las semaforinas transmembranales y sus receptores en plasticidad sináptica y en enfermedades neurales: Estudio celular y análisis de la transducción de señal
Instituto de Salud Carlos III, PI070500 (2008-2010)
Principal investigator: Ferran Burgaya

Papel de la reelina en la formación de conexiones sinápticas in vitro e in vivo y en el desarrollo de enfermedades neurodegenerativas
Instituto de Salud Carlos III, PI070715 (2008-2010)
Principal investigator: Albert Martínez

Papel de la tirosina quinasa Ack1 en la formación de dendritas y axones en neuronas de neocorteza y de cerebelo. Relación con la enfermedad de Alzhéimer y los procesos de 'long-term potentiation'
Instituto de Salud Carlos III, PI070942 (2008-2010)
Principal investigator: Jesús Mariano Ureña

Paper de la poteïna extracel·lular reelin en l'estudi cognitiu i la patogènesi de la malaltia de l'Alzheimer
Caixa Catalunya, Obra Social (2008-2011)
Principal investigator: Eduardo Soriano

Potencial del gen Ptf1a/p48 en la regeneración del cerebelo
'La Caixa' Foundation, BM06-335-0 (2006-2008)
Principal investigator: Eduardo Soriano

Collaborations

Functions of the novel tyrosine kinase Pyk1 in brain development
Joseph Schlessinger, Yale University (Connecticut, USA)

Interactions between Ephrin and Trk signalling pathways in axonal navigation
Uwe Drescher, MRC Developmental Neurobiology (London, UK) and Joan X Comella, University of Lleida (Lleida, Spain)

Role of Alex-3 in mitochondrial biology
Antoni Andreu, Vall d'Hebron Hospital (Barcelona, Spain), José Berciano, University of Santander (Santander, Spain), Ramón Trullás, CSIC-IIBB (Barcelona, Spain), Pablo Villoslada, CIMA (Pamplona, Spain), Jaume Bertranpetit, Pompeu Fabra University (Barcelona, Spain), Martin Kerschensteiner, Ludwig Maximilians University (Munich, Germany)

Role of Alex-3 in Wnt/B-catenin signalling pathway
Eduard Batlle, IRB Barcelona (Barcelona, Spain)

Role of CREB family transcription factors in brain development
Günther Schultz, DKMC (Heidelberg, Germany)

Role of Netrin1 and NogoR in neural development and regeneration
Marc Tessier-Lavigne, Genentech (San Francisco, USA)

Role of Reelin/Dab1 in prionic diseases
Adriano Aguzzi, University of Zurich (Zurich, Switzerland)

Role of Syntaxin1 and Podocalyxins in axonal guidance and brain development
Thomas Südhoff and José Rizo-Rey, Southwestern University (Dallas, USA) and Esther Stoeckli, University of Zurich (Zurich, Switzerland)

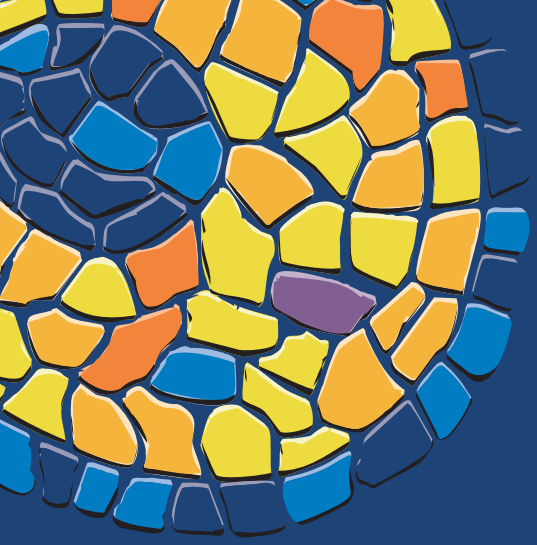
Role of the glycogen synthase enzyme in neuronal function and degeneration
Joan J Guinovart, IRB Barcelona (Barcelona, Spain)

Role of the pdf1 gene in cerebellar development and repair
Paco X Real, UPF/IMIM (Barcelona, Spain)

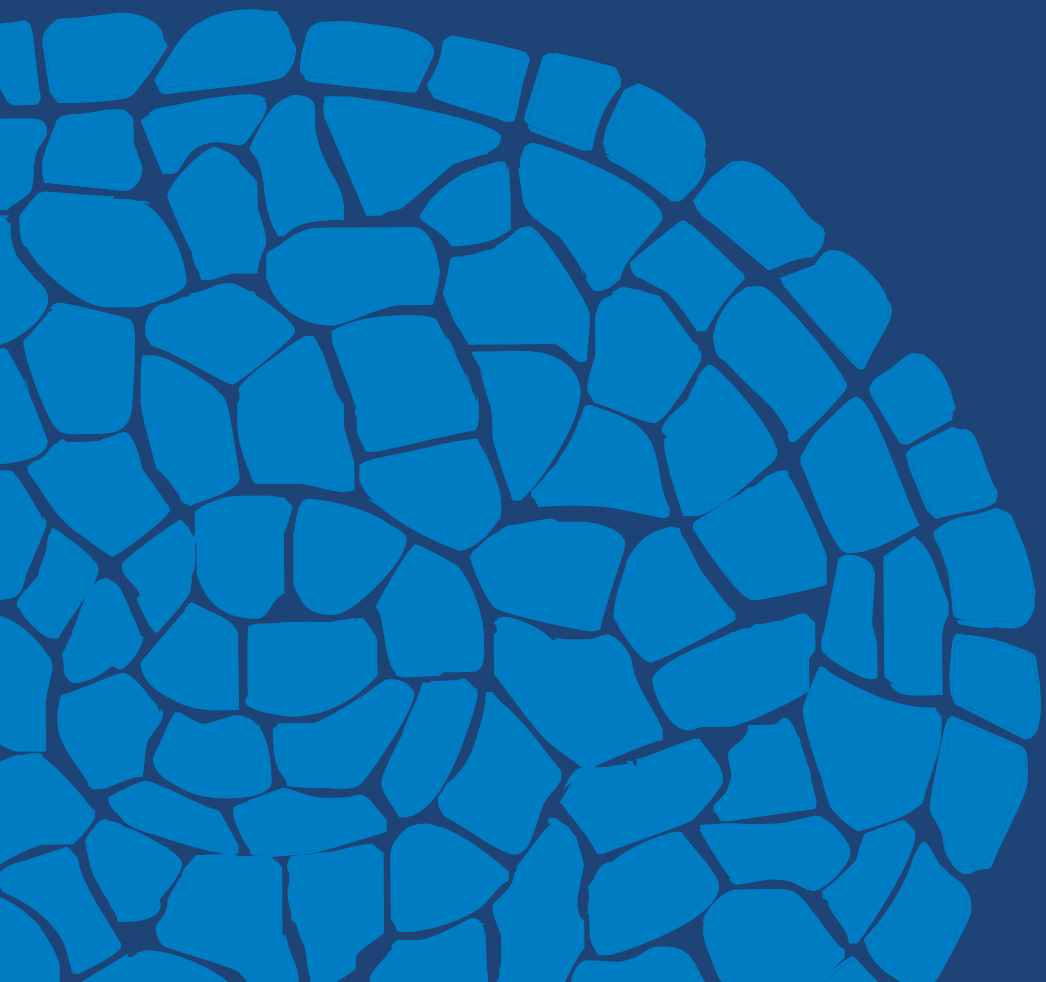
The mlc1 gene in megalencephalic leukodistrophy
Manuel Palacín and Raul Estévez, IRB Barcelona (Barcelona, Spain)

Transmembrane semaphorins and epilepsia epilepsy
Javier de Felipe, Cajal Institute (Madrid, Spain)

Ultrashort lasers, axonal guidance and brain repair
Pablo Loza, Institute of Photonic Sciences (Barcelona, Spain)



Structural and Computational Biology Programme



PATRICK ALOY

Structural bioinformatics and network biology

Proteins are the main perpetrators of most cellular tasks. However, they seldom act alone and most biological processes are carried out by macromolecular assemblies and regulated through a complex network of protein-protein interactions. Thus, modern molecular and cell biology no longer focus on single macromolecules but now look into complexes, pathways or even entire organisms. The many genome-sequencing initiatives have provided a near complete list of the components present in an organism, and post-genomic projects have aimed to catalogue the relationships between them. The emerging field of systems biology is now mainly centered on unravelling these relationships. However, all these interaction maps lack molecular details: they tell us who interacts with whom, but not how. A full understanding of how molecules interact can be attained only from high resolution three-dimensional (3D) structures, since these provide crucial atomic details about binding. These details allow a more rational design of experiments to disrupt an interaction and therefore to perturb any system in which the interaction is involved. Our main scientific interests are in the field of structural bioinformatics, in particular, the use of protein sequences and high-resolution 3D structures to reveal the molecular bases of how macromolecular complexes and cell networks operate.

Incorporating high-throughput proteomics experiments into structural biology pipelines

Recent years have seen the emergence of many large-scale proteomics initiatives that have identified thousands of new protein interactions and macromolecular assemblies. However, unfortunately, only a few of the complexes discovered meet the high-quality standards required to be promptly used in structural studies. This has thus created an increasing gap between the number of known protein interactions and complexes and those for which a high-resolution 3D structure is available. We have developed and validated a computational strategy to distinguish those complexes found in high-throughput affinity purification experiments that stand the best chances to be successfully expressed, purified and crystallised with little further intervention. Our study suggests that there are some 50 complexes recently discovered in yeast that could readily enter the structural biology pipelines. Indeed, we have used our target selection strategy to pick a list of 20 complex candidates whose structure determinations are going to be attempted by groups within 3D Repertoire, a large European integrated project that aims to solve the structures of all amenable protein complexes in yeast at the best possible resolution. The web version of the system is publicly available at http://targetselection.pcb.ub.es

Contextual specificity in peptide-mediated protein interactions

Protein interactions are central to virtually every major cellular function. While large protein-protein interfaces are typical in tightly associated macromolecular complexes, in most signalling events there is a globular domain in one protein that recognises a linear peptide from another, creating a relatively small interface. These interactions are predominantly found in regulatory networks and, due to their transient nature, are much more difficult to handle biochemically. Recently, large-scale experiments for the determination of peptide recognition profiles of interaction domains, and derivation of the corresponding patterns, have been developed, although transient peptide-mediated interactions are still underrepresented in high-throughput experiments. Even though binding is mediated by a small number of contacts formed by the residues in linear motifs, this type of interaction is extremely specific *in vivo*. For instance, it has been shown that the Pbs2 peptide is recognised only by the SH3 domain of Sho1 (its biological partner) and that it does not cross-react with any of the other 26 SH3 domains in yeast, although interactions with SH3 domains from other species are biophysically possible. More recently, another study has also shown that the binding specificity of PDZ domains is

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optimised across the 157 domains contained in the mouse proteome. However, bonds created between residues in linear motifs and globular domains, while sufficient to ensure binding, are too few to explain the high degree of specificity observed *in vivo*. It is thus, as occurs in phosphorylation events, the biological context that will ultimately determine the interaction specificity. This context has several aspects – certain subcellular localisation or expression patterns will determine whether proteins that are potential competitors for an interaction *in vitro* actually meet *in vivo* and thus evolve into niches of molecular recognition that allow them to bind only the desired target domain. Nevertheless, even within a cellular compartment several interaction domains and their complementary ligands are regularly expressed simultaneously, so yet more contextual information is required to achieve the observed specificity. This information is, to a great extent, contained in the residues surrounding the motif.

In the lab, we have systematically identified all instances of peptide-mediated protein interactions of known 3D structure and used them to study the individual contribution of motif and context to the global binding energy. We have found that, on average, the context is responsible for roughly 20% of the binding and plays a crucial role in determining interaction specificity, by either improving the affinity with the native partner or impeding non-native interactions. We have also examined and quantified the topological and energetic variability of interaction interfaces and have found a much higher heterogeneity in the context residues than in the consensus binding motifs (Figure 1). Our analysis partially revealed the molecular mecha-

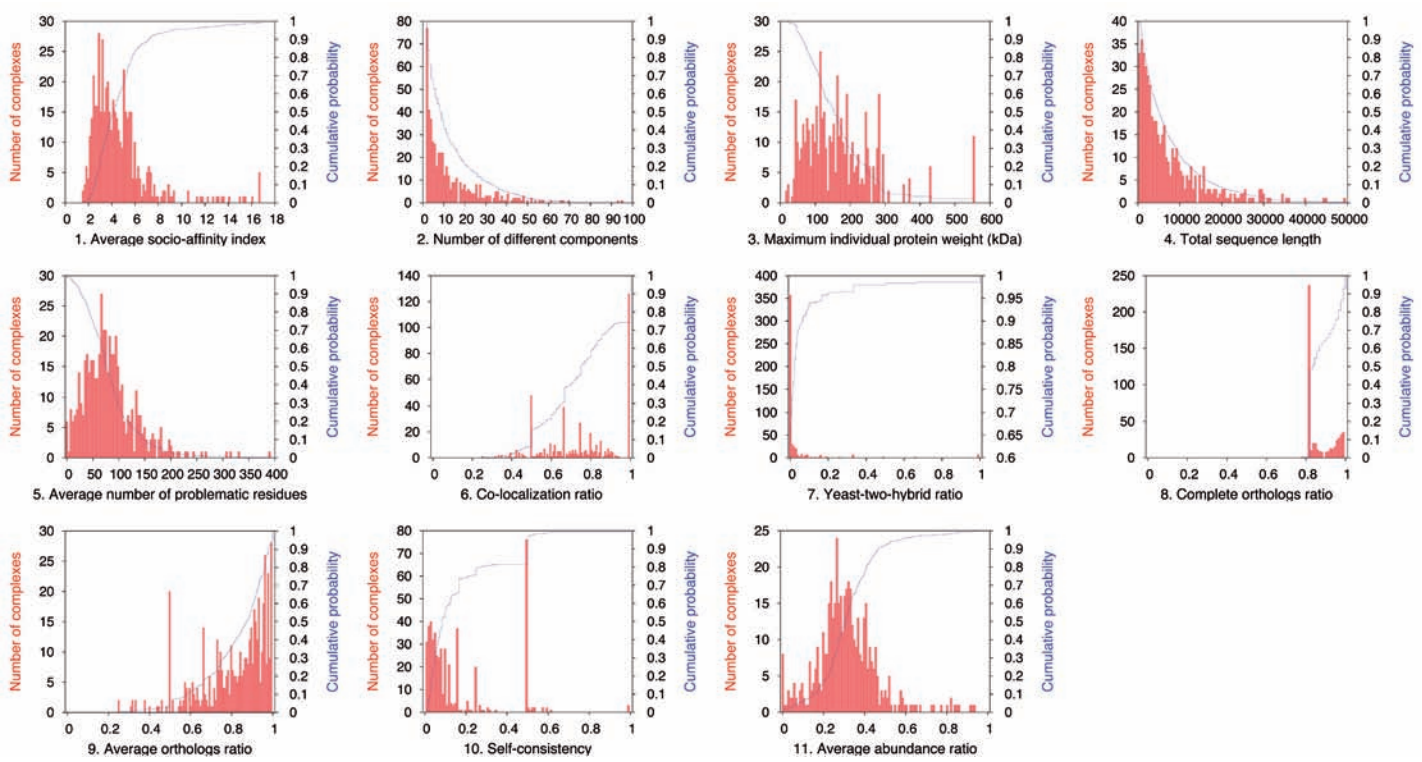

Figure 1. Distributions of partial scores and cumulative probabilities. *The distributions of partial scores for the eleven criteria used in the construction of the final feasibility score are shown in red, and the cumulative probabilities used for the normalisation in blue.*

nisms responsible for the dynamic nature of peptide-mediated interactions, and suggested a global evolutionary mechanism to maximise binding specificity. Finally, we have examined the viability of non-native interactions and highlighted cases of potential cross-reaction that might compensate for individual protein failures and establish backup circuits to increase the robustness of cell networks.

Exploiting gene deletion fitness effects to study the modular architecture of protein complexes under different growth conditions

An understanding of how individual genes contribute towards the fitness of an organism is a fundamental issue in biology. Although recent genome-wide screens have generated abundant data on quantitative fitness for single gene knock-outs, very few studies have systematically integrated other types of biological information to study how and why the deletion of specific genes gives rise to a particular fitness effect. In a recent study, we combined quantitative fitness data for single gene knock-outs in yeast with large-scale interaction discovery experiments to examine the effect of gene deletion on the modular architecture of protein complexes, under a range of growth conditions. Our analysis revealed that genes in complexes show more severe fitness effects upon deletion than other genes (Figure 2). However, in contrast to what has been observed in binary protein-protein interaction networks, we found that this was not related to the number of complexes in which they are present. We also observed that, in general, the modular components of protein complexes (*ie*, core and attachment proteins) are equally relevant for the complex machinery to function. However, when quantifying the importance of core and attachments in single complex variations, or isoforms, we found that this global trend originates from a combination of apparently unrelated factors, thereby indicating the presence of distinct fitness patterns in a single complex across growth conditions. Finally, our study also highlighted some interesting cases of potential functional compensation between protein paralogues and, perhaps, a new piece to fit in the histone-code puzzle.

Towards a molecular characterisation of Alzheimer's disease

In the last century, biomedical sciences were clearly immersed in a conceptual reductionism induced by the success of molecular biology. The development of methods to isolate and study individual cells and molecules has significantly increased our understanding of the nature of life and has led to considerable social advances, including the development of new medicines. Recent years have witnessed how the many genome sequencing projects have provided nearly complete lists of the macromolecules present in an organism, including humans. However, biological systems are often complex in nature, and the knowledge

of the components reveals relatively little about their function and organisation. The scientific community is now aware of the difficulties of predicting the behaviour of an intact organism from the individual actions of its parts in isolation and is rapidly moving to systems approaches, where global properties are also considered. In fact, most follow-up initiatives to the sequencing projects have been directed towards solving the systems' complexity and have focused on unveiling the millions of inter-relationships between macromolecules in an organism or monitoring how they co-ordinately change in response to a particular stimulus (*ie*, disease). Indeed, functional genomics initiatives are already delivering the first drafts of whole organism interactomes, gene expression profiles for many tissues and conditions, and the initial quantifications of metabolites in humans.

Pharmacological sciences have gone through a similar process, with traditional approaches being mostly reduced to the study, at the molecular level, of the target-compound duet. However, the truth is that phenotypic observations (*ie*, disease symptoms) are often the result of an incredibly complex combination of molecular events. This is because virtually every major biological process is not carried out by a single molecule but by large macromolecular assemblies and is often regulated through a complex network of transient interactions. Moreover, since most pathways are interconnected, slight changes in these transient regulatory networks can trigger one process or another, with completely different outcomes.

This reductionism has had striking consequences. For instance, many promising drug candidates have failed the last, and most expensive, sclinical phases because of the poorly understood mechanisms of action of the pathways they target or an inappropriate choice of the animal models, which proved ineffective at predicting off-target effects. It is thus clear that to increase the revenues of drug discovery, we need to improve our knowledge of the molecular mechanisms of disease by considering the full biological context of a drug target and moving beyond individual genes and proteins.

The main goal of our laboratory could be defined as the global molecular characterisation of pathological pathways through a combination of computational biology and interaction discovery techniques, in a real dry-wet cycle, where we use computational modelling to design the experiments needed to complement and complete the initial models (Figure 3). To this end, the recent creation of the Experimental Bioinformatics Lab, a joint initiative between IRB Barcelona and the Barcelona Supercomputing Center (BSC), has been crucial. The power of our approach is that we start from *in silico* modelling and therefore we are not restricted to the study of one or a few patho-physiological pathways. On the contrary, the first steps will involve a global modelling of all the human routes that might arise from known data, which will reveal novel and unexpected connections between them. We will then choose to further study those routes of most relevance from an academic or clinical perspective.

Starting from a set of seed proteins, an initial interactome is built using known protein-protein interactions. The resulting seed interactome or pathway is then extended and validated before putting its components into a spatio-temporal context based on gene expression data. Perturbation of the system finally allows us to unveil relationships between pathway topology and biological activity, with important implications for several kinds of clinical applications.

Indeed, we have already started to implement our approach to study the molecular bases of Alzheimer´s disease, where we have used three high-throughput interaction discovery approaches to screen over three thousand interactions. The initial phases of the project are yielding extremely interesting results (*ie,* we have discovered roughly 300 novel interactions and three potential new seed Alzheimer proteins), which will be pursued in the coming years.

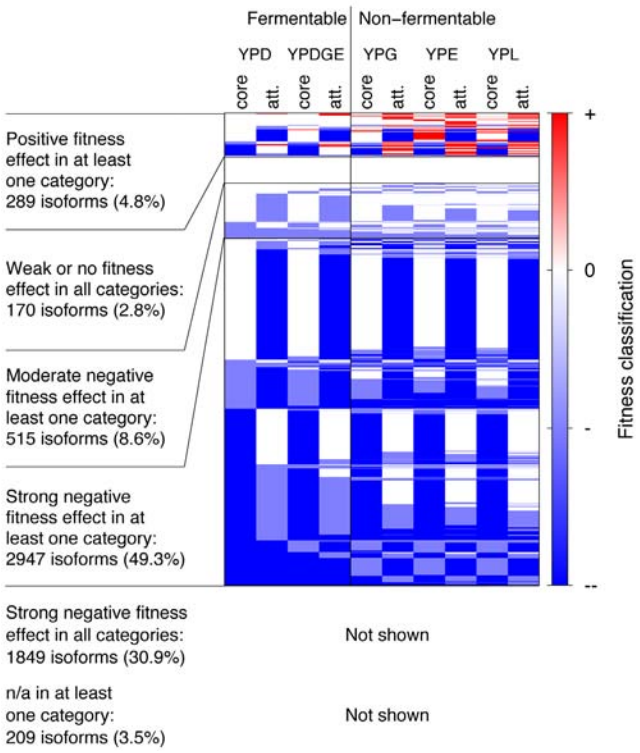

Figure 2. Fitness of the complex core and attachments of single isoforms across distinct growth conditions. *Expected fitness effects upon deletion of a random component of the given core or set of attachment proteins for all 5979 isoforms across the two fermentable and the three non-fermentable media are considered. The fitness values are partitioned into four categories: 'strong negative effect' (--/blue), 'moderate negative effect' (-/light-blue), weak or no effect' (0/white) and 'positive effect' (+/orange). Each line represents the fitness profile of a given isoform, treating the core and the attachments (att.) separately. 'n/a': the expected fitness effect is unknown due to a lack of quantitative fitness information for the genes in the respective core or attachments. When grouping the fitness profiles, we gave priority to n/a, positive, strong negative and moderate negative fitness effect in that order.*

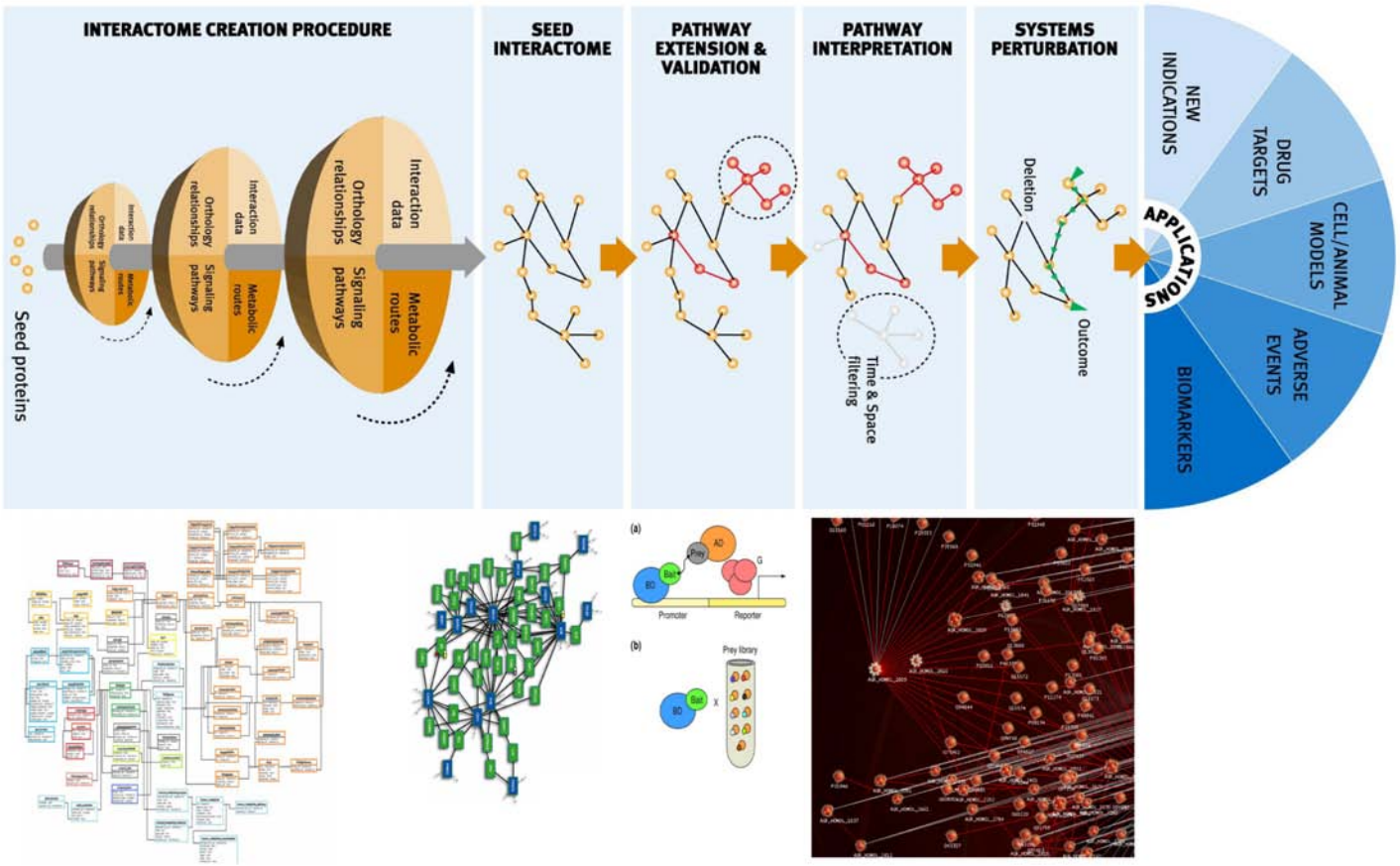

Figure 3. *Global strategy for the molecular characterisation of pathways and potential clinical applications.*

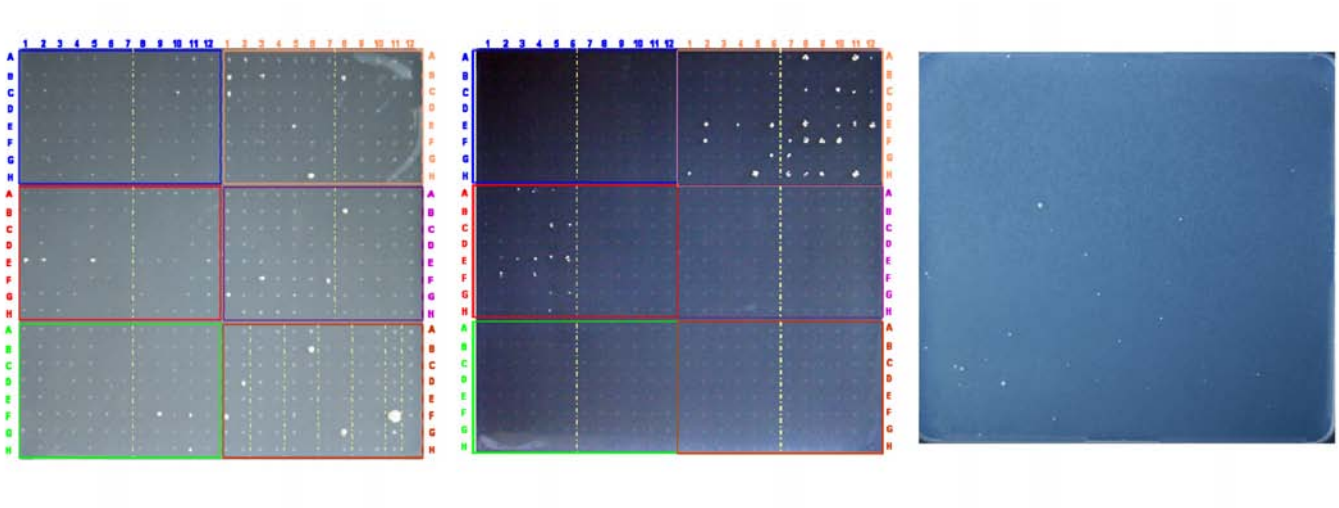

Figure 4. *Positive interactions found in the Alzheimer's interactome identified by co-transformation, mating and adult brain cDNA library two-hybrid screens.*

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Research networks and grants

A multidisciplinary approach to determine the structures of protein complexes in a model organism (3D-Repertoire)
European Commission, LSHG-CT-2005-512028 (2006-2009)
Principal investigator: Patrick Aloy

Aproximación bioinformática al estudio de la especificidad contextual en redes de interacciones entre proteínas y sus posibles aplicaciones biomédicas y biotecnológicas
Spanish Ministry of Science and Innovation, BIO2007-62426 (2007-2010)
Principal investigator: Patrick Aloy

Identificación de dianas secundarias y diseño de fármacos para enfermedades relacionadas con el envejecimiento mediante el análisis estructural y funcional de sus rutas biológicas
Spanish Ministry of Science and Innovation, PSE-010000-2007-1 (2007-2008)
Principal investigator: Patrick Aloy

Collaborations

Identification of genes regulator by FOXM1
Anastassis Perrakis, Nederlands Kanker Instituut (Amsterdam, The Netherlands)

Identification of potential phosphorylation targets for AURORA A kinase in human
Isabelle Vernos, Centre for Genomic Regulation—CRG (Barcelona, Spain)

Modular architecture of protein complexes and gene deletion fitness in yeast
Madan M Babu, MRC Laboratory of Molecular Biology (Cambridge, UK)

Network-based therapeutics
José Manuel Mas, Anaxomics Biotech (Barcelona, Spain)

New inhibitors of protein-protein interactions
Denis Shields, Trinity College (Dublin, Ireland)

Structural characterisation of molecular machines in yeast
Luís Serrano, Centre for Genomic Regulation (Barcelona, Spain)

Structural systems biology
Rob Russell, European Molecular Biology Laboratory (Heidelberg, Germany)

MIQUEL COLL

Structural biology of proteins and nucleic acids, and their complexes

Our group seeks to structurally characterise proteins and nucleic acids, and their complexes with the aim to further our understanding of several essential mechanisms in the cell. Using a number of molecular and structural biology techniques, with an emphasis on X-ray crystallography, we study the regulatory mechanisms of gene expression and the control mechanisms of DNA replication. In addition, we address molecular machines for DNA translocation, such as those involved in horizontal gene transfer in bacteria and DNA packaging in viruses. We also study unique DNA structures, such as DNA junctions.

Transcription regulation

In order to elucidate how transcription is regulated, we have structurally characterised several transcription factors, their complexes with other regulatory proteins, and their DNA binding regions (Badia *et al*, 2006). In one study, we addressed the *E. coli* PhoB transcriptional activator, a response regulator of the two-component signal transduction system that controls the expression of more than 40 genes related to phosphate assimilation.

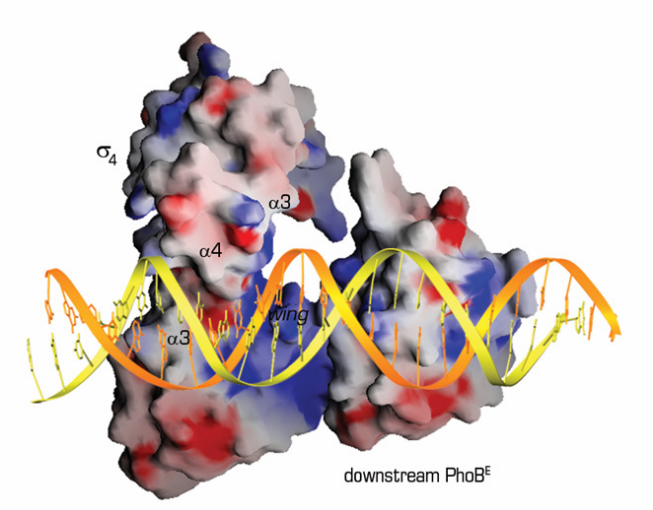

Figure 1. *A transcriptional initiation sub-complex formed by an effector molecule and parts of the RNA polymerase subunits.*

A transcription initiation quaternary sub-complex was prepared and crystallised and its structure determined. This sub-complex includes the transcriptional regulator PhoB, the σ_4 domain of the σ^{70} subunit of the RNA polymerase, the β-flap tip-helix of the RNA polymerase and the pho box promoter DNA region. The structure unveils how the RNA polymerase is recruited to the promoter region and provides clues on how transcription initiates (Gomez-Blanco *et al*, 2009, in preparation; Figure 1). In another study, we analysed proteins of the L-ascorbate *ula* regulon (Garcés *et al*, 2008a, b).

Horizontal gene transfer

Whatever the route used, the horizontal transfer of DNA, a phenomenon that contributes to the rapid evolution of micro-organisms, requires sophisticated multi-protein machinery to enable the long and charged nucleic acid molecule to cross the cell envelope barriers. In bacteria, the main route for cell-to-cell DNA transfer is conjugation, a mechanism responsible for the spread of antibiotic resistance. We have been studying the DNA processing machinery for conjugation in Gram-negative bacteria (Gomis-Rüth and Coll, 2006; Russi *et al*, 2008). Recently, we have extended the analysis to a Gram-positive bacteria conjugative system. The structure of the streptococcal plasmid relaxase MobM has been the first component of this system to be characterised (Russi *et al*, 2009, in preparation). The N-terminal nuclease domain has been determined in complex with a DNA hairpin, showing how the origin of transfer is recognised by the relaxase (Figure 2).

DNA packaging in herpesviruses

Another system for DNA translocation that we have addressed is the DNA packaging machinery of human cytomegalovirus (HCMV). Like all herpesviruses, HCMV replicates its genomic DNA

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into high molecular mass head-to-tail concatemers by a rolling-circle mechanism. The long DNA molecule is then cut into unit-length genomes and each genome is packaged into one viral procapsid. The DNA endonuclease and packaging activities are performed by a complex of proteins called terminase, which, in HCMV, includes UL56 and UL89. In collaboration with Darren Hart (EMBL, Grenoble) and using an ultra-high throughput method, we have screened 18,000 constructs and found a soluble domain that corresponds to the nuclease domain of the UL80 terminase subunit. Its 3D structure has been solved and shows an RNase H fold with a 2 metal-containing active site.

DNA replication control

DNA replication is a key cell event performed by diverse mechanisms in different organisms. Among these mechanisms, rolling circle replication (RCR) is a rapid one, leading to the generation of single or multiple copies of circular DNA or RNA

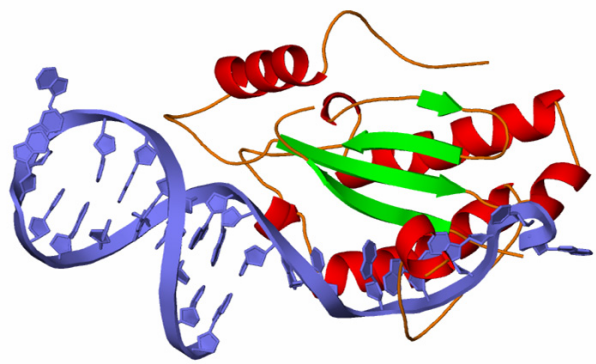

Figure 2. *The MobM relaxase in complex with the origin of transfer DNA hairpin.*

molecules. RCR is always initiated by a triggering reaction that consists of the site-specific cleavage of the parental nucleic acid within the origin of replication region. This cleavage is catalysed by initiator proteins, which thus provide a primer for the DNA or RNA polymerases to start synthesis. We have solved the 3D structure of one of these initiator proteins, the plasmid pmv158 initiator RepB, and have unveiled that, like ring-helicases, it oligomerises as an hexamer with a central channel. The protein presumably encircles one of the DNA strands to confer processivity to the replisome complex (Figure 3; Boer *et al*, 2009).

DNA structure and drug-DNA interactions

We have analysed unique DNA structures, such as the four-way and three-way junctions related to DNA recombination and other processes. Three-way DNA junctions were proved to be the structural targets of novel cytotoxic drugs consisting of supramolecular helicates that perfectly fit in the central cavity of the junction (Oleksy *et al*, 2006). During 2008 we have determined several DNA-helicate complexes (Figure 4) that show both similar and new drug-nucleic acid interactions.

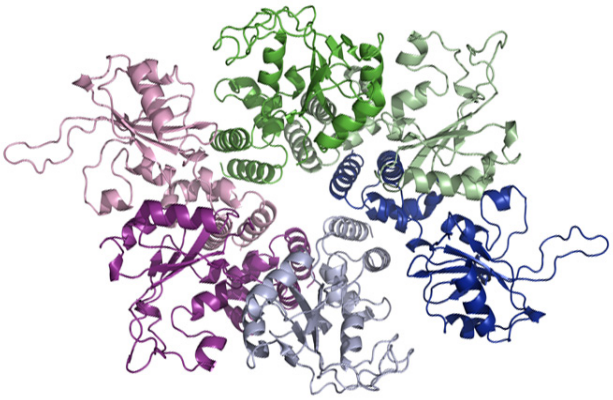

Figure 3. *Hexameric ring structure of replication initiation protein RepB.*

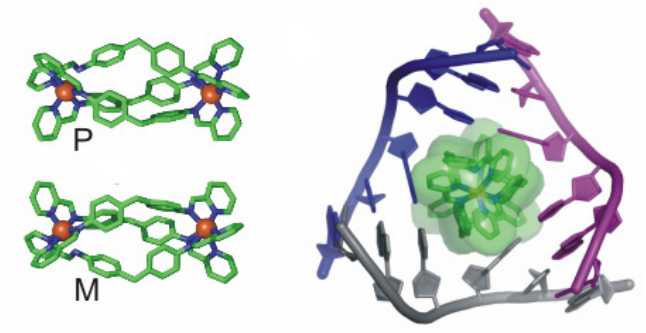

Figure 4. *Supramolecular helicate enantiomers and the binding of one of them to a DNA 3-way junction.*

SCIENTIFIC OUTPUT

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European Commission, LSHG-CT-2005-512028 (2005-2011)
Principal investigator: Miquel Coll

Ajuts a grups reconeguts
Agency for Administration of Universiry and Research Grants (AGAUR), 2005-SGR-00280 (2005-2008)
Principal investigator: Miquel Coll

Ayuda complementaria al proyecto 'Genómica estructural comparativa para enzimas víricos'
Spanish Ministry of Science and Innovation, BFU2005-24122-E (2006-2010)
Principal investigator: Miquel Coll

Ayuda complementaria al proyecto 'Una aproximación multidisciplinaria para determinar las estructuras de los complejos proteicos en un organismo modelo'
Spanish Ministry of Science and Innovation, BFU2005-24123-E (2006-2010)
Principal investigator: Miquel Coll

Caracterización estructural de los inhibidores de metiltransferasas
Spanish Ministry of Science and Innovation, PET2007-0319-03 (2008-2010)
Principal investigator: Miquel Coll

Centrosoma 3D: Hacia la comprensión estructural y funcional del centrosoma
Spanish Ministry of Science and Innovation, Consolider (2006-2011)
Principal investigator: Miquel Coll

Comparative structural genomics of viral enzymes involved in replication (VIZIER)
European Commission, LSH-2003-1121 (2004-2008)
Principal investigator: Miquel Coll

Epigenetic chromatin regulation proteins as targets in anticancer strategy
'La MTV3' Foundation, MTV3-052810 (2006-2008)
Principal investigator: Miquel Coll

Estructura de proteínas y complejos de unión al ADN
Spanish Ministry of Science and Innovation, BFU2005-06758-BMC (2005-2008)
Principal investigator: Miquel Coll

Genius Pharma Consortium
Spanish Ministry of Science and Innovation, CENIT (2006-2009)
Principal investigator: Miquel Coll

Spine2-complexes (S2C)
European Commission, VI-PM-LSHG-6 (2006-2010)
Principal investigator: Miquel Coll

Collaborations

Centrosomal proteins
Cayetano González, IRB Barcelona (Barcelona, Spain)

Chromatin-modifying proteins
Ferran Azorín, IRB Barcelona (Barcelona, Spain)

DNA drugs
Cristina Vicent, Instituto de Química Orgánica General-CSIC (Madrid, Spain)

DNA drugs
Mike Hannon, University of Birmingham (Birmingham, UK)

DNA packaging
José Carrascosa and José María Valpuesta, Centro Nacional de Biotecnología-CSIC (Madrid, Spain)

Histone methyl transferases
Xavier Barril, University of Barcelona (Barcelona, Spain)

HTP protein expression
Darren Hart, European Molecular Biology Laboratory (Grenoble, France)

Plasmid replication and transfer
Manuel Espinosa and Gloria del Solar, Centro de Investigaciones Biológicas-CSIC (Madrid, Spain)

Prolyl oligopeptidases
Ernest Giralt, IRB Barcelona (Barcelona, Spain)

RepB protein motion
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Transcription regulation
Margarita Salas, Centro de Biología Molecular-CSIC (Madrid, Spain); Antonia Herrero, Instituto de Bioquímica Vegetal y Fotosíntesis-CSIC (Seville, Spain); Ramón Díaz, Centro de Investigaciones Biológicas, CSIC (Madrid, Spain); Juan Aguilar and Josefa Badia, University of Barcelona (Barcelona, Spain)

IGNASI FITA

Structural biology and oxidative stress: X-ray crystallography of aggregates and proteins

Our laboratory has a long tradition in structural biology research, using mainly X-ray crystallography. Over the years, we have worked on the structure determination and analysis of a number of biological systems spanning in size from small peptides and oligonucleotides to large molecular aggregates, such as virus antibodies and receptor complexes or vaults. In many cases, we have established fruitful collaborations with groups working on the biological or biomedical aspects of these systems. In addition, the laboratory has focused on a number of biological issues. For example, in recent years, we have been deeply involved in structural and functional studies of proteins related to oxidative stress, using both theoretical (computational) and experimental approaches, in particular X-ray crystallography. We also consider it a priority to work on methodologically challenging problems of structural biology, both for their intrinsic scientific interest and the new avenues they often open but also as a way to maintain and increase the professional skills and specialisation of the laboratory.

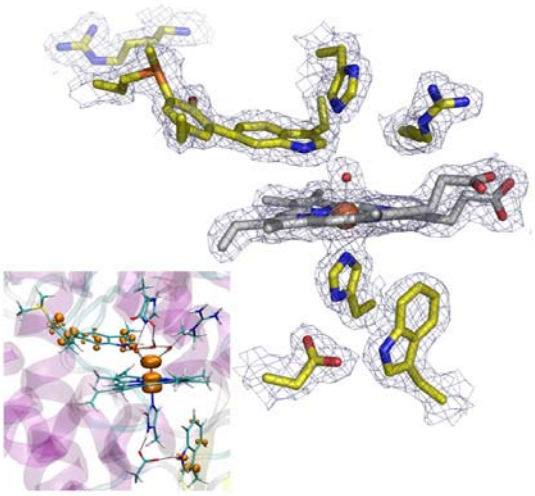

Figure 1. *We have shown that the KatG-specific adduct formed by a Met, a Tyr and a Trp and located at the active site acts as a one-electron donor towards the high-valent iron-oxo porphyrin (as illustrated by the spin density distribution shown in the insert). This result is in line with recent experimental findings suggesting the participation of protein-based radicals in the activation of isoniazid, a front line drug in the treatment of tuberculosis, by KatG.*

Structure determination of oxidative stress systems and other large molecular aggregates

We have continued our research into systems related to oxidative stress. In particular, we have done extensive work on the catalase-peroxidase system in an attempt to clarify the biochemical mechanisms that allow the function of these moonlight enzymes and also because of its crucial role in the activation of isoniazide, one of the main anti-tubercular treatments. This work has been done in close collaboration with Peter C Loewen at the University of Manitoba (Canada) and with Carme Rovira (an ICREA scientist at the Barcelona Science Park). Also in the field of oxidative stress, we have continued our scientific collaboration with Xavier Parés and Jaume Farrés (at the Autonomous University of Barcelona, UAB), concentrating efforts mainly on the human enzymes p53-inducible quinine oxidoreductase (PIG3) and aldo-keto reductase AKR1B10.

Our group has also been involved in the structure determination of a number of large molecular aggregates. In particular, in a collaboration project led by Nuria Verdaguer (Institute of Molecular Biology of Barcelona, IBMB-CSIC), we have determined the structures of the seven N-terminal domains of the major protein from the ribonucleoproteic vault particles at almost atomic resolution, and of the intact vault particles at 8 Å resolution. These results have been submitted for publication and might explain (among other things) the opening mechanisms of these large particles. In a continuing collabo-

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PhD Students David Aparicio, Barbara Calisto, Luca Martinelli **Research Assistant** Rosa Pérez
Lab Technician Mª Queralt Garcia **Visiting Students** Maria Adell

ration we have had for many years with Vicente Rubio (Instituto de Biomedicina de Valencia, IBV-CSIC), we have also contributed to the structure determination and analysis of a number of kinase complexes. The paper in *Current Opinion in Structural Biology* (by Llacer, Fita and Rubio, 2008) reviews some of the most relevant recent results on arginine and nitrogen storage that we (and others) have obtained in these systems.

Deciphering membrane proteins through X-ray crystallography

For the last few years, the laboratory has focused considerable efforts on membrane proteins, one of the main challenges in protein crystallography. Some very promising results have now been obtained thanks to a close collaboration headed by Manuel Palacín (Molecular Medicine Programme, IRB Barcelona) on the structure-function relationship in heteromeric amino acid transporters (HATs). The structural studies of HATs has allowed us to define their oligomeric state, membrane topology and the key residues involved in amino acid transport. The new structural and mechanistic insights into PKC α-C2 domain association (Guerrero-Valero *et al*, *PNAS*, in press) and the prokaryotic secreted lipoxygenases (Carpena *et al*, in preparation) contribute to our knowledge of membrane-related proteins.

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Research networks and grants

Estructura y función de hemo-enzimas
Spanish Ministry of Science and Innovation, BFU2005-08686-C02-01 (2005-2008)
Principal investigator: Ignasi Fita

Unravelling the molecular mechanism of nitrosative stress resistance in tuberculosis
European Commission, HEALTH-F3-2008-223335 (2008-2011)
Principal investigator: Ignasi Fita

Collaborations

Catalytic mechanism and regulation of glycogen synthase
Joan Guinovart, IRB Barcelona (Barcelona, Spain), Miquel Pons, IRB Barcelona (Barcelona, Spain), Joan Carles Ferrer, University of Barcelona (Barcelona, Spain)

Large molecular aggregates
Nuria Verdaguer, Institute of Molecular Biology of Barcelona (Barcelona, Spain)

Oxidative stress-related systems
Peter C Loewen, University of Manitoba (Winnipeg, Canada)

Pathogenicity in Mycoplasms
Enric Querol and Jaume Piñol, Autonomous University of Barcelona (Barcelona, Spain)

Structural and biochemical studies of TREX1 inhibition
Antonio Celada, IRB Barcelona (Barcelona, Spain)

Structural characterisation of enzymatic systems involved in cellular detoxification and regulation
Xavier Parés and Jaume Ferrés, Autonomous University of Barcelona (Barcelona, Spain)

Structure determination and analysis of kinase complexes
Vicente Rubio, Institute of Biomedicine of Valencia (Valencia, Spain)

Structure-function relationship in heteromeric amino acid transporters (HATs)
Manuel Palacin and Modesto Orozco, IRB Barcelona (Barcelona, Spain)

MARIA MACIAS

Protein NMR Group

Intracellular communication is fundamental to the existence and survival of multicellular organisms and defects in this process are often a key feature of diseases. At the molecular level, the formation of complex networks, many of them based on protein-protein interactions performed by domains specialised in the recognition of specific sequence targets, are responsible for information transfer. Our research seeks to provide three-dimensional (3D) structures of biomolecules in solution that can offer a detailed description of biological processes involving protein-ligand interactions. For this purpose, we apply multidimensional nuclear magnetic resonance (NMR) spectroscopy in conjunction with other biophysical techniques. We have also developed several computer tools to speed up NMR data analysis and structure calculation of protein complexes.

Our work has focused on the structural determination of several FF domains. From these results, we have concluded that FF domains share a conserved scaffold with divergent binding capacity.

FF domains -so-called because they flank conserved phenylalanine residues-, are protein-protein interaction modules of about 70 amino acids and are often found in several copies. They are present in three protein families: the splicing factors FBP11, Prp40 and URN1, the transcription factors CA150, and the p190RhoGTPase-related proteins. This simplicity in distribution, however, is contrasted by the difficulty in defining their biological role.

Studies performed to date show that a number of FF domain constructs interact with several kinds of ligands, namely the phosphorylated CTD repeats, the TFII-I transcription factor, the $(D/E)_{2/5}$-F/W/Y-$(D/E)_{2/5}$ consensus sequence, a TPR motif, and with RNA. Indeed, for each FF domain studied there is a ligand which does not contain conserved features when compared to other previously characterised FF domains. We performed systematic comparisons of FF sequences to clarify domain composition and similarity in related sequences. However, these analyses did not provide patterns of conserved residues. On the contrary, they demonstrated only that FF domain sequences are extraordinarily divergent.

To gain insight into the molecular function of FF domains, we selected several divergent domains to determine their structures and also to establish their interaction sites.

Solution structure of the yeast URN1 splicing factor FF domain: Comparative analysis of charge distribution and implications in ligand recognition. FFs and SURPs, two domains with a similar fold

The URN1 yeast-splicing factor contains 465 residues but only two protein domains, a WW and a FF domain, which are separated by a linker of 178 residues. Beyond this FF domain there is another unstructured region of 200 amino acids.

URN1 is described in the Saccharomyces Genome Database (SGD) as a pre-mRNA splicing factor associated with the U2-U5-U6 snRNPs, the RES complex, and the Prp19-associated complex (NTC). A number of URN1 binding partners are also reported in interaction databases, but no specific targets have been described for the URN1FF domain. The solution structure reveals that the domain adopts the classical FF fold, with a distinctive negatively charged patch on its surface.

To examine the FF sequences from a different perspective, we set out to compare the URN1FF structure with all available FF structures. In this comparison, we also included an analysis of electrostatic surfaces. We found that the fold is conserved but the electrostatic distribution is variable, even for domains with similar pKas. This observation suggests that charge distribution on the surface rather than pKa values can be used as a hallmark to classify FF domains (Figure 1).

By comparing structural and biological information from proteins with similar characteristics, we have found a

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parallelism between the FF and SURP domain families. From a structural point of view, both are all-helical domains, have a α1–α2–310–α3 topology and, as shown by our structural analysis, give similar RMSD values when superimposed to each other (Figure 2). From this perspective, we propose that SURP modules are included in the FF fold.

We observed that the main difference between all these structures resides in the orientation of the second helix. The sequence and structural variability observed for α2 may be the consequence of ligand specialisation, thereby supporting the hypothesis that a potential common binding site is located in this region. This specialisation may range from DNA recognition, like in the DEK domain, to protein binding, as shown in SF3a120SURP2 and Prp40FF1 domains. The SURP ligand folds as a well-ordered single helix, which packs in the groove formed between the first two helices and the short 3_{10}. So far, only one helical ligand has been identified as an interacting partner of FF domains, namely the TPR motif, which binds to Prp40FF1. In this case the binding site mapped also involved α2 and 3_{10} helices. This similarity could be a mere coincidence or, on the contrary, sustain the hypothesis that at least some members of each family have evolved to recognise ligands of a similar structure.

Domain phosphorylation by the PDGF-receptor α requires its partial unfolding

The p190 RhoGAP subfamily is formed by two proteins, p190-A and p190-B, and constitutes the unique set of cytoplas-

matic proteins containing FF domains. p190 RhoGAPs are formed by an N-terminal GTPase domain, four consecutive FF domains in the central part of the proteins, and a C-terminal RhoGAP domain. Remarkably, the four FF domains lack one of the characteristic phenylalanine residues that give the name to the domain. These proteins belong to the family of RhoGTPase Activating Proteins (RhoGAPs), which regulate the switch from the active (GTP-bound) to the inactive (GDP-bound) conformation of RhoGTPases.

Furthermore, it has been described that p190-A RhoGAP interacts with the N-terminal of TFII-I via the region containing the FF domains. TFII-I is a signal-induced transcription factor implicated in distinct processes such as the transcriptional regulation of c-fos gene and the G-kinase signal transduction pathway. TFII-I binding to p190-A RhoGAP prevents TFII-I translocation into the nucleus. When Tyr308 (located at the first FF domain) is phosphorylated, the interaction between p190-A RhoGAP and TFII-I is disrupted and TFII-I can enter the nucleus and recover its transcriptional activity. This is the only case reported of FF domain phosphorylation as a way to modify its ligand binding specificity.

Thus, we studied the implications of semi-conserved or non-conserved substitutions in the RhoGAP FF sequences on the 3D fold. For this purpose, we solved the structure of first FF domain of p190-A RhoGAP using multidimensional heteronuclear NMR. All previously characterised FF structures superimposed well. However, the comparison of the RhoGAPFF1 structure with the other FF structures revealed several differences, among these the α1-α2-α3-α4 architecture instead of the α1–α2–3_{10}–α3 classical arrangement.

Furthermore, the structure revealed that Tyr308, described as the phosphorylation target of PDGF-α receptor kinase, participates in the formation of the hydrophobic core of the domain, and consequently, it is placed in an unfavourable position to be phosphorylated.

We thus examined whether Tyr308 is phosphorylated *in vitro*. The phosphorylation of RhoGAPFF1 required a partial unfolding of the domain, which occurred at 37˚C. Furthermore, we observed that a phospho-mimicking Y308D mutant was largely insoluble and precipitated after removal of the fusion tag, thereby indicating that the presence of a charged residue at position 308 is incompatible with the FF fold. The lack of structure probably accounts for the observation that domain phosphorylation prevents binding to the TFII-I transcription factor. Phosphorylation sites in proteins are often found in accessible areas, such as loops or in linkers connecting domains. In contrast, one of the phosphorylation sites of RhoGAP is inaccessible to kinases, as it is buried in the FF1 protein core, and is incompatible with the structure since Tyr308 phosphorylation stabilises the unfolded state. Thus, the inhibitory role that phosphorylation plays in this regulatory process seems to compensate the energetic cost of shifting the folding-unfolding equilibrium towards the unfolded state, in a non-reversible manner.

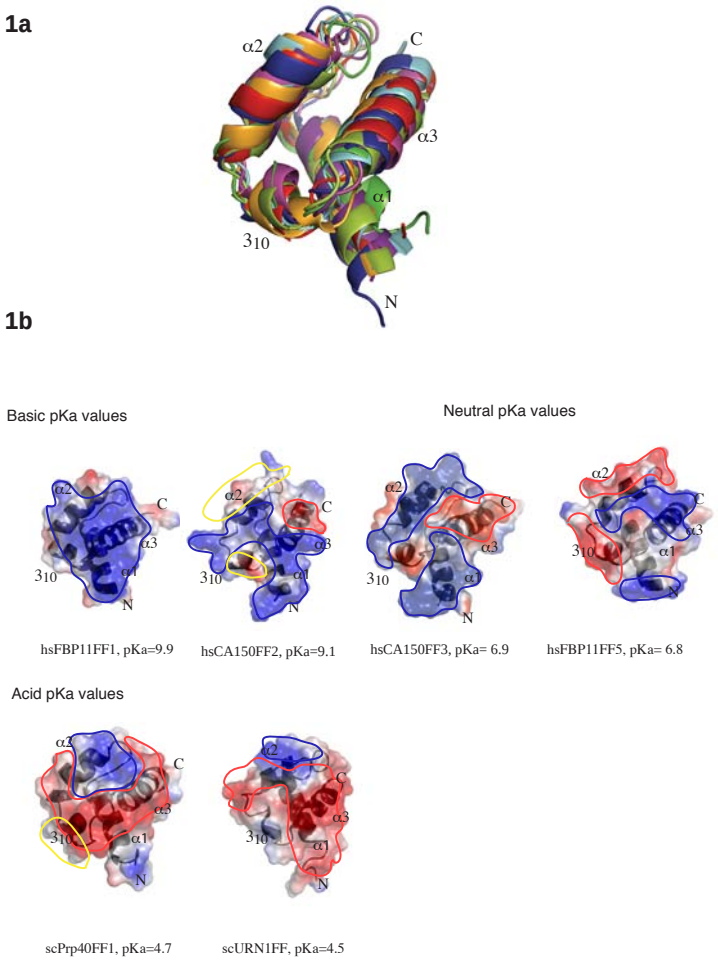

Figure 1. *(a) Five superimposed FF structures. (b) Charge distribution and hydrophobic patches.*

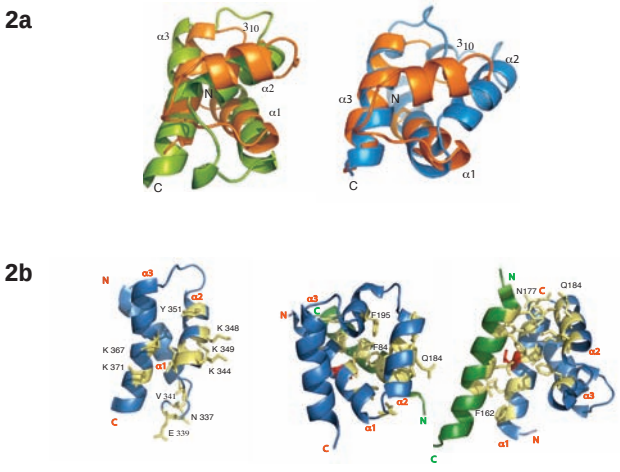

Figure 2. *(a) Structural comparison of FF domains with a DNA binding (left) and a surp domain (right). (b) Binding site comparison of FF and surp domains.*

Publications

Bonet R, Ramirez-Espain X and Macias MJ. Solution structure of the yeast URN1 splicing factor FF domain: comparative analysis of charge distributions in FF domain structures-FFs and SURPs, two domains with a similar fold. *Proteins*, **73**(4), 1001-09 (2008)

Research networks and grants

Determinación de estructuras de dominios FF de proteínas y de sus interacciones mediante la aplicación de la resonancia magnética nuclear multidimensioinal en solución
Spanish Ministry of Science and Innovation, BFU2005-06276 (2005-2008)
Principal investigator: Maria Macias

Collaborations

NMR studies of amino acid modifications in peptides and analogues
Miriam Royo, Barcelona Science Park (Barcelona, Spain)

NMR studies of somatostatin and derivatives
Antoni Riera, IRB Barcelona (Barcelona, Spain)

Protein complexes by NMR
Joan Massagué, Memorial Sloan-Kettering Cancer Center (New York, USA)

Molecular modelling and bioinformatics group

Our long-term objective is to explain the behaviour of living organisms by means of theoretical models, the roots of which are anchored in the basic principles of physics and chemistry. With this general aim, we work with several methodologies, from the mining of biological databases to classical dynamics and quantum chemistry calculations. The use of such diverse techniques allows us to explore a wide range of problems, from drug design to genome analysis. Special emphasis is placed on connecting basic interactions with the global properties of biological systems. In general terms, our work centres on the following three major areas: (i) the study of small model systems, (ii) the analysis of stressed or unusual nucleic acids, and (iii) the dynamics of proteins.

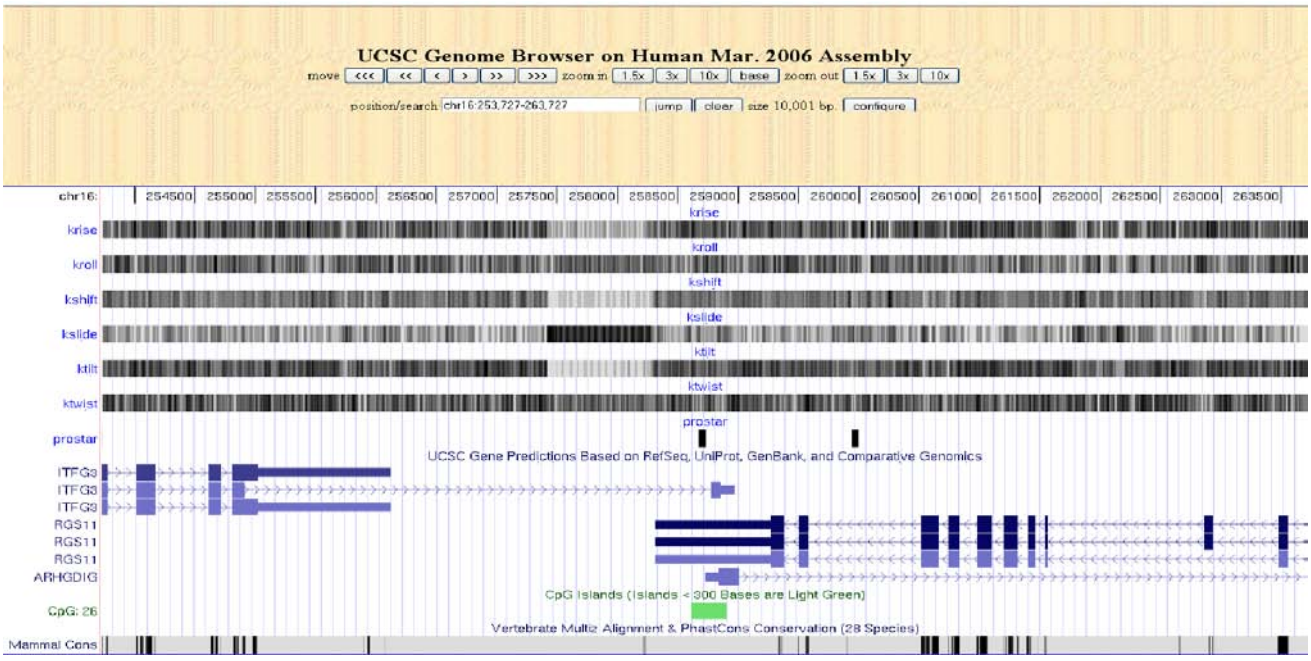

Figure 1. *Example of a classical 2D output of DNAAlive. Top figures correspond to physical properties derived from quasi-harmonic mesoscopic simulations of DNA (parameters obtained from MD simulations). Figures at the bottom correspond to biological annotations on the same genome fragment. Marked in red is a region of unusual physical properties, which is later found to correspond to a transcription start site.*

Principal Investigator Modesto Orozco **Research Associates** Xavier de la Cruz, Agustí Emperador, Josep Lluís Gelpí **Postdoctoral Fellows** Montse Barbany, Oliver Carrillo, Chiara Catellazzi, Marco d'Abramo, Rebeca Garcia, Agnes Noy, Alberto Pérez, Guillem Portella, Nadine Utz **PhD Students** Annalisa Arcella, Ozgen Deniz, Ignacio Faustino, Carles Fenollosa, Josep Ramon Goñi, Adam Hospital, Sergio Lois, Tim Meyer, Jordi Morata, Laura Orellana, Antonella Paladino, David Piedra, David Talavera **Research Assistants** José Antonio Alcántara, Jordi Camps, Chiara Castellazi, Damjan Cicin **Lab Technician** Mª Luz Ruiz **Administrative Assistants** Margarita Pedro, Cristina Villanueva **Visiting Scientists** Andrea Amadei (Italy), Sarah Harris (United Kingdom) **Visiting Students** Ariadna Giró (Spain), Venu Kurella (India), Eva Novoa (Spain), Francesco Raimondi (Italy)

Small model systems

Our group has a long trajectory in the study of small model systems of biological relevance (nucleobase complexes, drugs, isolated complexes of amino acids, stacked or hydrogen bonded complexes, etc.). The study of these simple systems can shed light on the behaviour of much more complex biological molecules. Almost a decade ago, we realised that such studies were simple in the gas phase but very difficult in aqueous solution, thereby hampering the true application of the information obtained to the biological scenario. This led us to develop methods to describe solvent systems, some of which are considered the current state of the art in the field, and to develop approaches for accurate representation of molecular interactions.

As planned, during 2008 we have advanced in the development of methods for the efficient representation of polarisation effects (Soteras *et al*, 2008a; Soteras *et al*, 2008b). We have also considerably improved our MST continuum model, which has now been adapted to provide complete thermodynamics of solvation with high accuracy (Bidon-Chanal *et al*, 2008; Klamtz *et al*, 2008).

In collaboration with colleagues at Minnesota (Xie *et al*, 2008), we have developed a novel method to perform full quantum mechanical calculations in proteins. The method is based on the partition of the entire system in inner (treated as QM clusters) and external (treated classically) fragments. We are now parallelising the code to allow extension of these quantum mechanical/molecular dynamics (QM-MD) calculations to the nanosecond time scale. The potential use of all these new methodologies in the context of drug development and nucleobase design have been explored in detail (Cozzini *et al*, 2008; Vázquez-Mayogoita, 2008) during 2008.

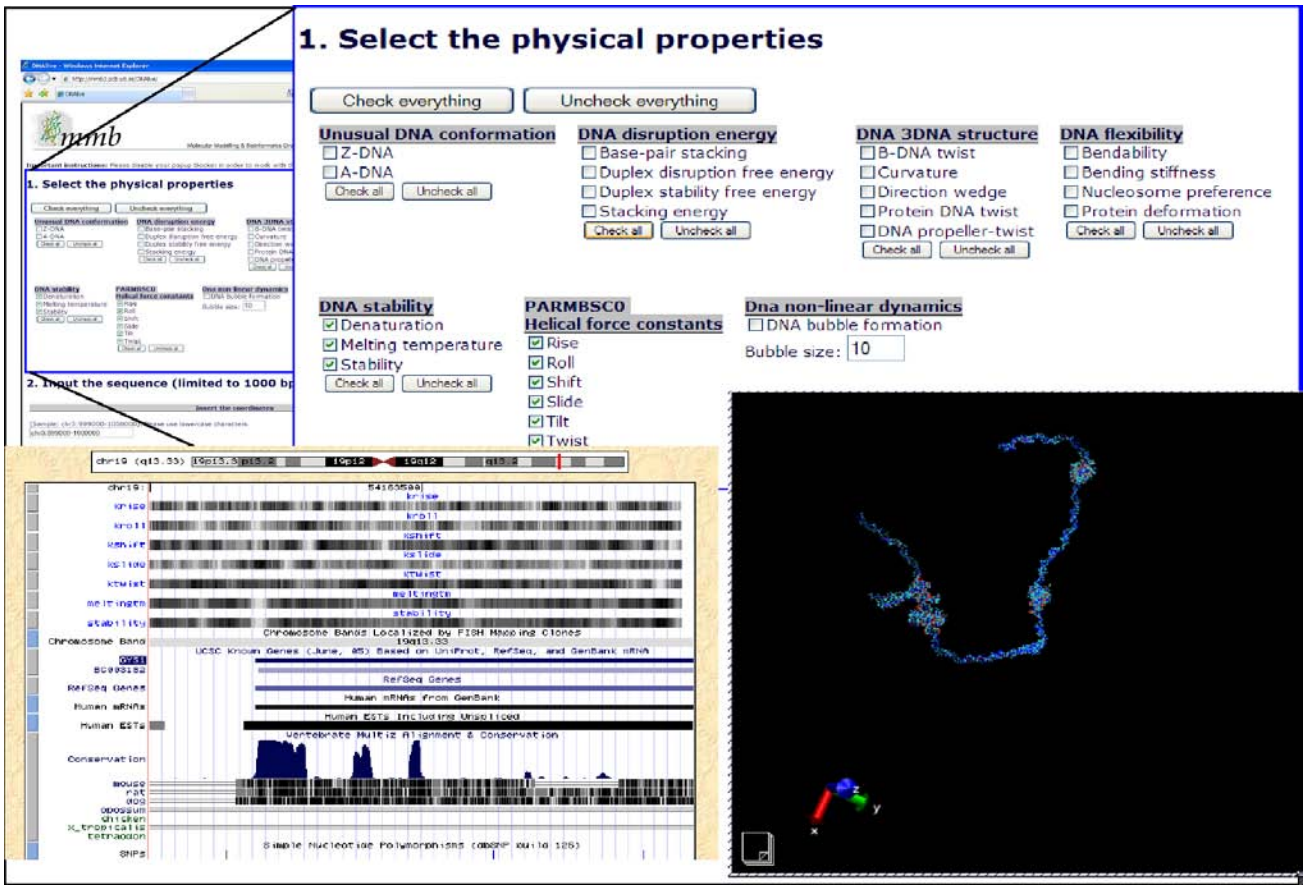

Figure 2. *Example of the possibilities of DNA in its physical description. Bottom left panel corresponds to mesoscopic simulations of the structure and dynamics of the chromatin fiber, including proteins.*

Analysis of stressed or unusual nucleic acids

Major breakthroughs in the field of nucleic acid simulations emerged from the work performed by our group in 2007 related to the development of PARMBSC0 force-field, which, after additional tests (Svozil *et al*, 2008), has become the default force-field for nucleic acid simulations (Orozco *et al*, 2008). Thanks to this refined force-field and to the massive computer power of MareNostrum, we have deciphered the mechanism by which RNAase H differentiates between hybrids to be degraded and homopolymers that must be resistant to the enzyme (Noy *et al*, 2008). Using the same force-field, we have successfully designed modified nucleobases that display a unique effect on G-DNAs, stabilising or destabilising them depending on the position where they are inserted (Gross *et al*, 2008).

Considerable effort has been focused on the characterisation of the equilibrium sequence-dependent stiffness of duplex DNA through PARMBSC0-powered molecular dynamics simulations (Pérez *et al*, 2008). These studies have allowed us to derive a full mesoscopic model of the flexibility of DNA, which can be used to study chromatin deformability (Perez *et al*, 2008, Goñi *et al*, 2008). In this context, the development of DNAlive (Goñi *et al*, 2008) has been a major landmark since it has allowed us for the first time to obtain an integrated view of chromatin by including physical information and biological annotations in a single tool. DNAlive is the perfect link between DNA simulation and genomics (Figures 1-2). We are currently using DNAlive intensively to study several aspects of chromatin structure and dynamics and during 2009 we plan to extend it to account for epigenetic changes.

Protein dynamics

The creation of the MODEL (Molecular Dynamics Extended Library) database has been the focus of a massive amount of work by the group. During 2008 we have finished the first draft of the database and started to work on the derivation of complementary databases, such as that of proteins of pharmacological interest, kinases and protein complexes. We have also almost completed the analysis of the trajectories to decipher the patterns of protein flexibility and their association with solvent structure (Figure 3).

Using our μMODEL reduced set, we have examined the impact of environmental changes on protein structure. Multi-microsecond-long simulations on urea have been performed during 2008 (they will finish in 2009) to illustrate the atomistic mechanism of chemo-thermal unfolding. This year we have completed the mapping of the gas phase proteome (Meyer *et al*, 2008), again using the reduced μMODEL database. This study has provided surprising evidence of the stability of proteins under electrospray-like conditions, thereby opening up the possibility to use gas phase structural information derived from x-free electron laser microscopy to obtain the protein structure in solution (Figure 4).

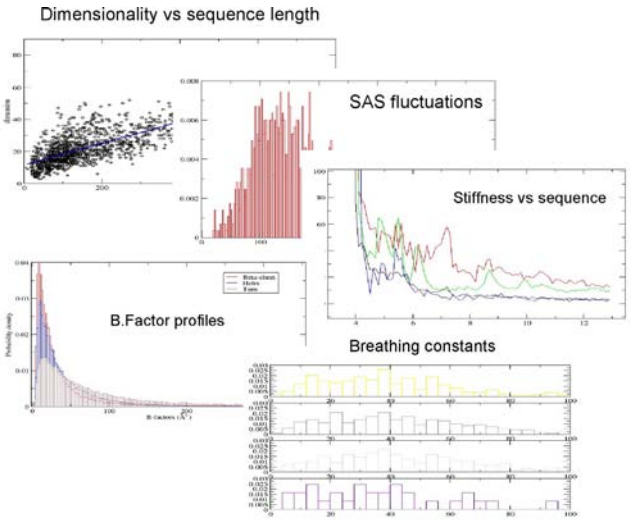

Figure 3. *Examples of dynamic analysis of protein flexibility derived from our MODEL database.*

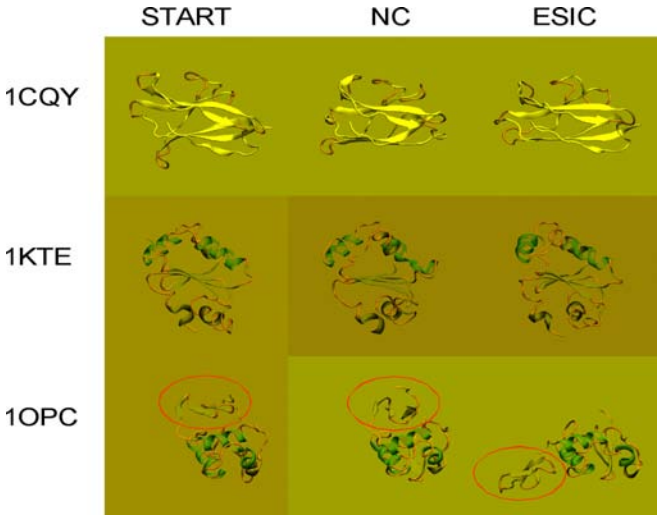

Figure 4. *Structures of 3 ultra-representative proteins in solution (start) and upon ideal (NC) and normal (ESIC) electrospray vaporisation.*

The availability of several terabytes of trajectories in μMODEL has opened up many possibilities for proteome-scale analysis, including systematic exploration of essential dynamic movements in proteins and their connection with function and evolution (Velázquez-Muriel *et al*, 2008). It has also provided the opportunity to study protein channels following the MDGRID methodology developed by our group (Carrillo *et al*, 2008) and to perform genome-scale ensemble docking experiments to identify new drugs.

MD trajectories deposited in μMODEL have helped us to derive new coarse-grained models designed to obtain a fast first-order approximation to protein flexibility using very simple potential functions. During 2008 we have developed and optimised pseudo-harmonic methods based on normal model analysis and Brownian MD (Emperador, 2008a). We have explored the use of ballistic equations for recovering dynamics trajectories using either residue or atom-resolution representations of proteins (Emperador, 2008b). Given their speed, these methods are expected to allow a full-proteome representation of protein flexibility, even in protein mixtures. We are currently finishing the development of an entire platform (named FlexServ) that will automatise these types of studies.

Finally, we should remark that dynamic information, obtained from either MD, coarse-grained methods or even evolutionary methods (Velázquez-Muriel *et al*, 2008) can help to improve the structural representation of proteins obtained for ensemble techniques such as NMR, as demonstrated in the COCO web server developed by our group, in collaboration with colleagues in Nottingham and at the European Bioinformatics Institute (Laughton *et al*, 2008).

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Estudio de formas inusuales o tensionadas del DNA. Implicaciones biotecnológicas y biomédicas
Spanish Ministry of Science and Innovation, BIO2006-01602 (2006-2009)
Principal investigator: Modesto Orozco

European Life Science Infrastructure for Biological Information (ELIXIR)
European Commission, 211601 (2007-2010)
Principal investigator: Modesto Orozco

Molecular recognition
Marcelino Botin Foundation
Principal investigator: Modesto Orozco

Proyecto CONSOLIDER Supercomputación y eCiencia
Spanish Ministry of Science and Innovation, CSD2007-00050 (2007-2012)
Principal investigator: Modesto Orozco

Reconeixement molecular
Generalitat de Catalunya, 2005-SGR0286 (2006-2009)
Principal investigator: Modesto Orozco

Red temática de investigación cooperativa en biomedicina computacional (COMBIOMED)
Instituto de Salud Carlos III, COMBIOMED RD07/0067/0009 (2008-2012)
Principal investigator: Modesto Orozco

Structural bioinformatics
Genoma España, GN4 (2007-2009)
Principal investigator: Modesto Orozco

Other funding sources

Collaboration contracts with Salvat Laboratories, Neuropharma and Palau Pharma-Grupo Uriach

Collaborations

Design of nucleobase derivatives for stabilization of anomalous DNAs
Ramon Eritja, IRB Barcelona-CSIC (Barcelona, Spain)

Development of new tools for computer assisted drug design
Francisco Javier Luque, Faculty of Pharmacy, University of Barcelona (Barcelona, Spain)

Development of strategies for improvement of NMR-samplings
Charles A Laughton, Nottingham University (Nottingham, UK), Williams Vranken, European Bioinformatics Institute, EBI-EMBL (Cambridge, UK)

Force-field refinement for nucleic acid simulations
Thomas Cheatham, University of Utah (Salt Lake City, USA), Jiri Sponer (Brno University (Brno, Czech Republic) and Filip Lankas, Laussanne University (Lausanne, Switzerland)

Introduction of polarisation effects in force-field calculations
Cristophe Chipot, Nancy University (Nancy, France) and Francisco Javier Luque, University of Barcelona (Barcelona, Spain)

Mixed QM-MM methods for protein simulations
Donald Truhlar and J Gao, University of Minnesota (Minnesota, USA)

New algorithms for drug design
Paolo Cozzini, Peruggia University (Peruggia, Italy) and Robert Soliva, PalauPharma-Uriach Group (Barcelona, Spain)

Physical properties of DNA
David Torrents, Barcelona Supercomputing Center (Barcelona, Spain)

SCRF solvation methods
Jacopo Tomasi, Pisa University (Pisa, Italy), Andreas Klamtz, Cosmologic Inc. (Leverkusen, Germany) and Francisco Javier Luque, University of Barcelona (Barcelona, Spain)

Study of DNA-metal complexes
Miguel Fuentes-Cabrera, Oak Ridge National Laboratory (Oak Ridge, USA) and Francisco Javier Luque, University of Barcelona (Barcelona, Spain)

Awards and honours

Distinguished fellow of the Marcelino Botin Foundation
Awardee: Modesto Orozco

MIQUEL PONS

Structure, dynamics and interactions of flexible proteins and ligands

E pur si muove was the dramatic claim of Galileo Galilei after he was forced to retract his ideas of a universe not centred on a static Earth. Dynamic macromolecular structures and interactions are now recognised as key elements of life and the basis of function. Efficient regulation relies on narrow equilibrium conditions that can be shifted in response to stimuli. To maintain responsiveness, the energy changes must be small, *ie*, involving weak and dynamic interactions. Intrinsically unfolded proteins (IUPs) exist in a dynamically exchanging ensemble of multiple conformations, which are easily perturbed by interactions with specific targets. Eukaryote leverage of IUPs results in 30% of their proteins having unfolded regions, in contrast to 4% in eubacteria and 2% in archaea. In this regard, 80% of the proteins associated with human cancer have unfolded regions. A recent report in *Science* demonstrates that IUPs are themselves tightly regulated and describes their central physiological role. Our group addresses the challenge of flexible systems by combining powerful techniques, such as NMR, Small Angle X-ray Scattering (SAXS), and fluorescence spectroscopy with new interpretation paradigms, taking into account the emerging properties of ensembles and networks. Biomedical applications of our research are pursued in the fields of cardiovascular diseases, bacterial pathogenicity, antibiotic resistance, and network pharmacology.

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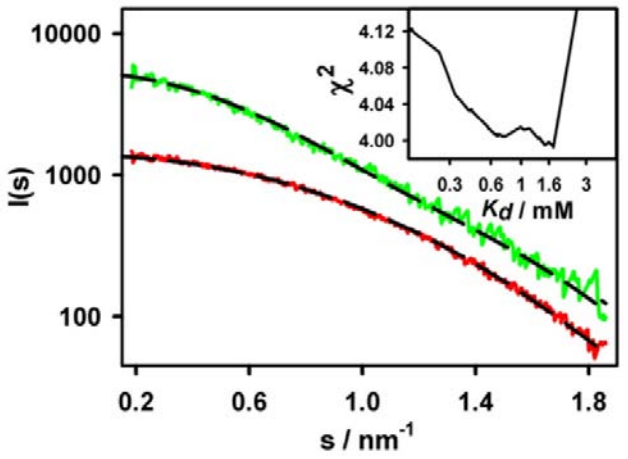

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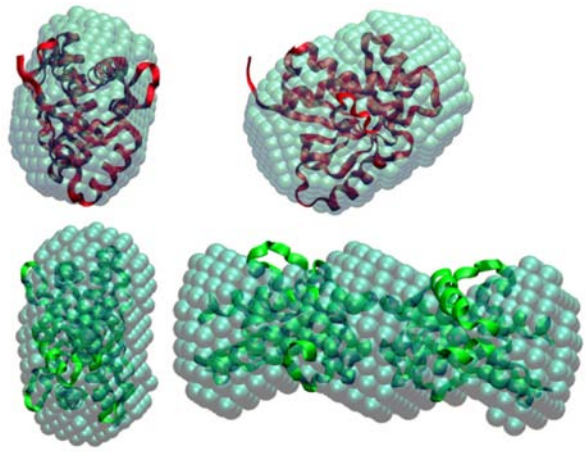

Figure 1. *(a) Pure curves for the major (red) and minor (green) species in the oligomerisation equilibrium of bovine low molecular weight phosphatase. (b) Low resolution structures (cyan spheres) superimposed to the X-ray structures of the monomer (red) and dimer (green). The two sets of images are orthogonal views.*

Principal Investigator Miquel Pons **Research Associates** Pau Bernadó, Jesús García **Postdoctoral Fellows** Cristina Gabellieri, Yolanda Pérez **PhD Students** Jascha Blobel, Giovanni Cincilla, Tiago Cordeiro, Carles Fernández, Arola Fortian, Oriol Marimon **Visiting Students** Catalina Granados (Colombia), Hager Memni (Tunisia)

Nucleoid-associated proteins and horizontal gene transfer

Our group maintains a stable collaboration with Antonio Juarez (University of Barcelona and Institute for Bioengineering of Catalonia) for the structural study of nucleoid-associated proteins of the H-NS and Hha families, and their interaction partners.

H-NS is a general regulator of gene expression in response to environmental challenges, such as changes in osmolarity or temperature. It has also been described as a genome guardian because of its role in providing a stealth effect by facilitating the acquisition of horizontally transferred genes. Horizontal gene transfer is the process of intercellular exchange of DNA fragments, thereby providing the bacteria with new capacities. Transfer can take place between individuals of the same species or between distinct bacterial species. One of the most studied cases of this transfer is the spread of antibiotic resistance to cells that have not previously been exposed to the drug. Mobile genetic elements may occur in autonomously replicating plasmids or be integrated in the bacterial chromosome. Many of the genes involved in pathogenicity are associated with horizontally transferred genes.

H-NS is an abundant protein encoded in the bacterial chromosome of many gram-negative bacteria. Related proteins are also encoded in plasmids associated with bacteria belonging to the enteric group. One of the current challenges is to elucidate the differential regulation of two large groups of genes that are under the control of H-NS but that are repressed under different conditions. On the one hand, there are the 'house-keeping' genes, which ensure the metabolic response to temperature and osmolarity, for example, and on the other hand, the horizontally transferred genes - often associated with pathogenic phenotypes - that are de-repressed during host colonisation. While changes in temperature and osmolarity are among the environmental cues used by bacteria to signal host colonisation, the differentiation between house-keeping and pathogenicity-related genes remains poorly understood. Two recent advances in this regard have been provided by observations made by Antonio Juarez's group: the role of the helper protein Hha and the differential regulation by plasmidic forms of H-NS. In both cases, the gene-pool regulated

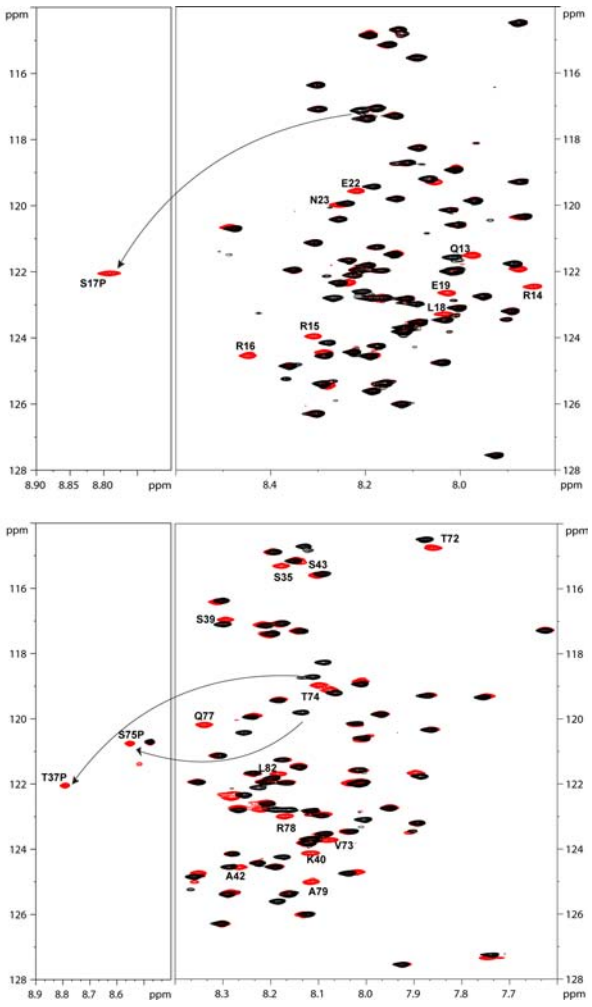

Figure 2. *NMR detection of the specific phosphorylation at the different sites of the unique domain of c-Src.*

by H-NS can be divided in two groups, one containing mostly horizontally transferred genes (Baños *et al*, submitted, *PLoS Genet*, 2008).

Structural studies performed by our group are shedding light on both mechanisms. The active role of Hha is demonstrated by the lack of activity of a Hha mutant that retains the three dimensional (3D) structure and the capacity to bind H-NS of the wild-type (Cordeiro *et al*, 2008). Structural differences have been observed by NMR in the C-terminal domain of plasmidic and chromosomal H-NS (Fernández de Alba *et al*, in preparation). These results are reinforced by complementary work with the C-terminal domain of Ler, which shows high sequence homology with the DNA-binding domain of the two forms of H-NS. After a careful evaluation of a variety of DNA sequences, the 3D structure of DNA-bound Ler is currently being determined by solution NMR by Tiago Cordeiro in collaboration with the Griesinger group in Göttingen (Germany).

Our group is studying several paralogues of Hha, including YmoA, the paralogue of Hha in Yersinia, and Ydgt. YmoA and Ydgt show interactions with other proteins that do not belong to the H-NS family. These intriguing results may indicate moonlighting behaviour of the Hha family of proteins or point to a higher level integration of the H-NS-based regulatory system. Jesús García leads this line of research. Other ongoing collaborations are with Marc Baldus' group in Utrecht, which has already succeeded in the observation and partial assignment of solid-state NMR spectra of full length H-NS. At present, we are comparing the apo- and DNA-bound forms. We are also collaborating with Juan Recio (Barcelona Supercomputing Center) to include Paramagnetic Relaxation Enhancement (PRE) information, as well as the effect of point mutations and NMR chemical shifts to derive a docking model of the Hha-H-NS complex. Additional collaborations are maintained with Modesto Orozco (IRB Barcelona) for the prediction of differential DNA features of HTG and house-keeping genes, and with Fèlix Ritort, in the use of optical tweezers to study DNA-H-NS complexes.

Weak protein-protein oligomers. NMR and SAXS studies

Our group has a long-standing interest in mammalian low molecular weight tyrosine protein phosphatases (lmwPTPs) and their oligomerisation through the active site. We have hypothesised that inactive oligomers are supramolecular pro-enzymes that are maintained in an inactive form until competition with a phosphorylated substrate triggers the release of the active monomer. This hypothetical mechanism would allow signalling pulses to be transmitted, as in the absence of substrate the phosphatase would return to its inactive oligomeric form. The high dissociation constant of the dimer *in vitro* causes two types of problems: a technical one derived from the low concentration of the oligomeric forms *in vitro*, even at high protein concentrations, and a conceptual one, namely how to demonstrate the biological relevance of such a weak interaction mechanism.

During 2008, work in the group carried out under the direct supervision of Pau Bernadó has provided answers to both problems. Concerning the technical issue, we have developed a method to deconvolute SAXS curves recorded at a range of protein concentrations in order to extract the SAXS profiles of the 'pure' species (monomer and dimer; Blobel *et al*, *J Am Chem Soc*, in press, 2008). Low resolution structures of both species can be derived from the pure curves, in perfect agreement with the independently determined X-ray structures, thereby confirming that it is possible to extract structural information from a minor species in an equilibrium mixture (Figure 1).

With regard to the physiological relevance of the weak oligomerisation, we have demonstrated that a low molecular weight phosphatase from *Bacillus subtillis*, initially studied by Changwen Jin (Beijing) by means of NMR, shows a completely analogous behaviour to the eukaryotic form, in spite of non-trivial sequence differences and distinct substrate selectivity (Blobel *et al*, in preparation). To the best of our knowledge, this is the first demonstrated example of the conservation of a weak protein-protein interaction and it reinforces our previous hypothesis of a physiological role for this interaction.

Our group also addresses another protein, STAT5a, involved in signal transduction pathways. This protein has been studied by SAXS in order to differentiate between distinct proposed models for the dimer present in equilibrium with the monomer. Like the low molecular weight phosphatase, STAT5a shows a large dissociation constant. (Bernadó *et al*, in press, 2009).

Intrinsically unfolded proteins: the unique domain of c-Src

The unfolded unique domain of human tyrosine kinase c-Src has become a focal point in our group.

In contrast to the well-folded multidomain core, which is well conserved among diverse family members, the unique domain of the proteins belonging to the Src family differ completely. Although the unique domain is essential for substrate specificity, very few structural studies have been performed as a result of the technical and interpretative difficulties associated with IUPs. The complete NMR assignment of the domain, achieved by Yolanda Pérez using carbon-detected NMR experiments, has allowed us to study the conformational ensembles of the unique domain using PRE of spin-labelled derivatives and Residual Dipolar Couplings (RDC). We have also studied the forms phosphorylated in Ser17, Thr37 and Ser75 (Figure 2).

We have recently started a new study funded by the 'Marató de TV3' Foundation call for research projects on cardiovascular diseases. In this project we examine the unique domains of several members of the Src family of kinases and their possible cardioprotective action. The underlying hypothesis is based on the observation of a cardioprotective interaction between the ND2 mitochondrial protein and the unique domain of c-Src.

New tools for leveraging the power of small molecules in structural biology

The LINGO concept for efficient molecular similarity calculations has been combined with a new clustering method published in *Science* in 2007 by Frey and Dueck. We have developed a new method by which we have been able to derive the intrinsic structure of the NIH PubChem database, which contains 19 million compounds. The method identifies around 50 eigenmolecules, molecules that show very little similarity between them but that, collectively, show similarity to most (99.9%) of the molecules in the database. Although no information on biological function was used for the classification, the clusters derived succeeded in grouping the ligands of most of the 40 targets in the Directory of Useful Decoys (DUD) database (Figure 3; Cincilla *et al*, in preparation).

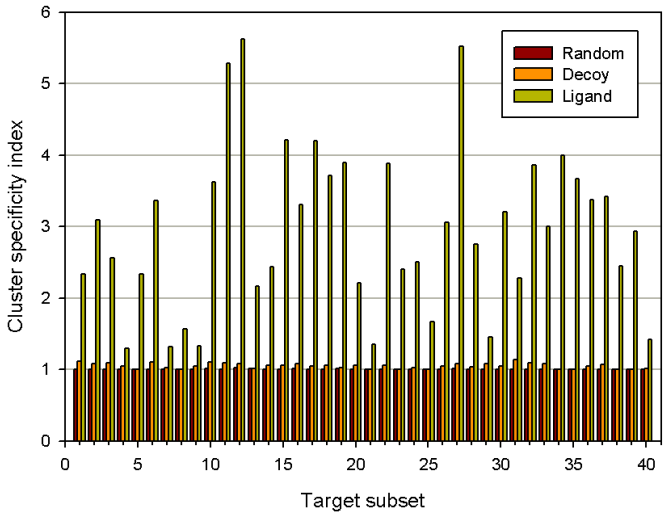

Figure 3. *Ligands of each target are preferentially clustered, in contrast to equivalent sets of random molecules or decoy molecules (inactive molecules sharing low level of properties (molecular weight, atom composition, polarity, etc.) with the ligand set.*

Physicist Cristina Gabellieri has joined the group this year to explore the use of Dynamic Nuclear Polarisation to increase the sensitivity of NMR, especially for small molecules. High nuclear polarisation is transferred from that of unpaired electrons at temperatures close to 1K (-272°C) and the polarised nuclei are rapidly (less than two seconds) transported to a conventional NMR instrument and measured at room temperature. Signal enhancements higher than 2000 can now be easily achieved for slowly relaxing nuclei.

SCIENTIFIC OUTPUT

Publications

Bernadó P and Svergun DI. New perspectives in small-angle scattering to study unstructured states of proteins. In Protein Misfolding (Cian B O'Doherty and Adam C Byrne, ed.), Nova Science Publishers (2008)

Bernadó P, Pérez Y, Svergun DI and Pons M. Structural characterisation of the active and inactive states of Src kinase in solution by small-angle X-ray scattering. *J Mol Biol*, **76**(2), 492-05 (2008)

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Cincilla G, Vidal D and Pons M. An improved scoring function for suboptimal polar ligand complexes. *J Comput Aided Mol Des*, Epub ahead of print (2008)

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Research networks and grants

Acceso externo a la ICTS de Barcelona
Spanish Ministry of Science and Innovation, ICTS2008-27 (2008)
Principal investigator: Miquel Pons

Acción de mejora de la infraestructura científico-técnica singular de RMN de Barcelona
Spanish Ministry of Science and Innovation, ICTS2006-05 (2007-2008)
Principal investigator: Miquel Pons

Dominis únics de quinases de la família Src implicats en malalties cardiovasculars
'La MTV3' Foundation, 81510 (2008-2011)
Principal investigator: Miquel Pons

EMAR-Multidisciplinary frontiers of magnetic resonance support
SPCI2006-A9-0690 (2007-2012)
Principal investigator: Miquel Pons

Collaborations

Bacterial nucleoid-associated proteins
Antonio Juárez, University of Barcelona and Institute for Bioengineering of Catalonia (Barcelona, Spain)

Computational studies in drug design
Michael Thormann, Origenis (Munich, Germany)

Characterisation of unfolded states
Javier Sancho, University of Zaragoza and Institute for Biocomputation and Physics of Complex Systems-BIFI (Zaragoza, Spain)

NMR-based protein-protein docking
Juan Recio, Barcelona Supercomputing Center (Barcelona, Spain)

NMR, Hoffmeister effects and uroporphyrinogen
Oscar Millet, CIC bioGUNE (Bilbao, Spain)

Ribosomal proteins
Mikael Akke, Lund University (Lund, Sweden)

SAXS
Dimitry Svergun, European Molecular Biology Laboratory (Hamburg, Germany)

Solid state NMR studies of H-NS
Marc Baldus, University of Utrecht (Utrecht, The Netherlands)

Target characterisation in drug design
Andrew Marsh, University of Warwick (Coventry, UK)

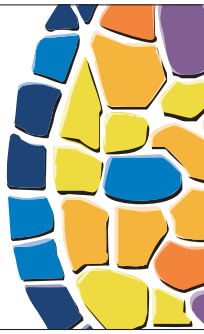
Unfolded proteins and residual dipolar couplings
Martin Blackledge, Institut de Biologie Structurale (Grenoble, France)

Honours

Steering committee member, ISMAR

Board of Trustees member, EUROMAR

Experimental Bioinformatics Laboratory



The Experimental Bioinformatics Lab (EBL) was established in January 2008 as part of the collaborative research programme between IRB Barcelona and the Barcelona Supercomputing Center (BSC). Recent progress in genomics and high-throughput techniques has brought about an explosion of biological data, which in turn has provided a great opportunity to computationally predict the complex biological networks in living organisms with high accuracy. However, the implementation of computational methods in research raises questions about the predictive capacity of computer simulations. The EBL is dedicated to implementing advanced experimental approaches that allow computational biologists in the programme to incorporate experimental measurements into their predictions.

The most important activity at the EBL during 2008 has been the establishment of the laboratory. This facility has been equipped to allow more than 10 people to simultaneously work in the fields of systems biology (protein-protein interaction networks) and genome regulation. The initial set-up of the lab from scratch implied a major financial investment, covered by IRB Barcelona and the BSC, and by grants of the PIs that comprise the programme. As a result, the lab is now fully functional, while some experiments which require highly sophisticated tools (such as electrospray measures or ultra-sequencing) are outsourced to the University of Barcelona and IRB Barcelona research platforms.

Genome-wide approaches to study chromatin modifications

Chromatin modifications have been shown to have a profound impact on the regulation of gene expression.

Determination of triplex structures in vacuum. Supporting the modelling studies currently carried out by Modesto Orozco's group, we have set up, in collaboration with the Mass Spectrometry Core Facility at IRB Barcelona, the first experiments in Spain to measure collision cross sections (CCS) of nucleic acids. The project has been a great challenge due to the lack of expertise in this type of experiment and its technical complexity. Oligonucleotides synthesised by the EBL-member Mari Luz Ruiz, in Ramon Eritja's group, were treated to remove salt, and were then annealed and hybridised in water-ammonium acetate. The resulting triplexes, characterised by DC and UV measures were then vaporised and analysed at the Electrospray Scientific Platform, a technical service run by the University of Barcelona. The spectra of the triplexes are clear and confirm the lack of strand separation predicted by Orozco's simulations (Figure 1). Retardation experiments were done and also found clear signals. We are now finishing the calibration of the equipment using known nucleic acid standards, either purchased or synthesised, and in January 2009 we will have CCS for the triplex structures considered. We hope that these experimental measures will confirm molecular dynamics (MD) predictions that the triplex structure is largely preserved in the gas phase (Arcella *et al*, in preparation).

Genomic profile of nucleosome positioning. The positioning of nucleosomes with respect to DNA can directly influence gene regulation. In recent years, several genome-wide maps of nucleosome positions in yeast, worm and across all human promoters have emerged. While the underlying DNA sequence might have a role in the rotational placement of nucleosomes around the histone core, most nucleosomes depend on exclusion signals that may exert a greater influence in regulating their translational positioning along the genome.

Following the interests of Orozco's group, and in collaboration with Ferran Azorin's group at IRB Barcelona, the EBL is pursuing the mapping of nucleosome positions on a genome-wide scale for a variety of yeast species and humans to study the factors that affect positioning in certain regions of the genome. For these purposes, mononucleosome-sized DNA will be isolated from nuclease-digested chromatin and subsequently analysed by either tiling microarrays containing overlapping probes or by massively parallel sequencing with an Illumina Genome Analyzer using Solexa technology, which will be available at the IRB Barcelona

Laboratory Director Montse Soler **Laboratory Manager** Maica López **Postdoctoral Fellows** Kathryn Collinet, Guillermo Suñé **PhD Students** Clara Berenguer, Özgen Deniz, Elisa Durán **Master Student** Rodrigo Arroyo **Lab Technicians** Chiara Castellazzi, Ricart Lluís, Mari Luz Ruiz

Functional Genomics Core Facility at the beginning of 2009.

Genomic profile of DNA methylation. In mammals, DNA methylation is confined to the addition of a methyl group to the Cytosine in a CpG dinucleotide. *De novo* methylation of promoters that contain CpG islands leads to gene inactivation. Moreover, DNA methylation of transcription-factor-binding sites can influence their binding and, indeed, aberrant DNA methylation is a well-established marker of cancer. Simulations by Orozco's group predict that methylation produces a large change in DNA deformability (Pérez *et al*, in preparation). These results are now being analysed by the EBL team (Chiara Castellazzi and Montse Soler) through cyclation experiments. If theoretical results are confirmed, in a second step we will study the possible connection between CpG methylation and nucleosome position as a mechanism for gene regulation. The theoretical part will require the implementation of methylation-sensitive mesoscopic models of DNA.

On the experimental side, again in collaboration with Azorin's group, our laboratory (EBL members Montse Soler, Özgen Deniz and Kathryn Collinet) will perform nucleosome position experiments in normal and methylase-deficient human cell lines using the methodology described above. We will also analyse nucleosome position in yeast transformed with *de novo* methylases as an alternative model to verify the robustness of our bioinformatics models. Overall, we plan to derive a full integrative model to predict nucleosome positioning and repositioning as a function of gene activity and methylation pattern.

Genome-wide analysis of gene regulation. Deciphering the regulatory networks encoded in the genome is one of the most

interesting and challenging tasks in the post-genome sequencing era. The identification and location of human promoters are valuable in the search for genes in a given region of the genome and for studying conditions under which these genes are active.

Based on the finding that promoter regions display unusual physical properties, computational biologists of the programme (Modesto Orozco's group in collaboration with David Torrents' group at the BSC) have developed a method to identify human promoters and it is especially powerful for the location of unusual promoters. To validate the method, the EBL will measure the regulatory potential of the putative promoter sequences *in vivo* by using a luciferase reporter gene assay in mammalian cells. This project, which has not started yet, will involve work by Elisa Durán, Chiara Castellazzi and Montse Soler in close collaboration with the groups led by David Torrents and Modesto Orozco.

Molecular characterisation of pathological pathways

A deeper knowledge of the molecular bases of human disease will have an important bearing on the discovery of new drug targets and biomarkers, the optimisation of preclinical models and understanding how biological networks change from the healthy state to disease. Following the interest of Patrick Aloy's computational group, a real dry-wet cycle approach is applied to describe pathological pathways at molecular level, using a combination of computational biology and interaction discovery techniques. After computational modelling of pathological pathways, the EBL carries out the experiments required to complement and complete these initial models. Bioinformatics tools will be subsequently applied to integrate the experimental information in order to validate and further expand the protein interaction networks involved in the pathways.

The EBL has implemented an experimental approach using the yeast-two-hybrid system (Y2H) to analyse protein-protein interaction networks at physiological concentrations, thereby providing an efficient *in vivo* screening tool. The positive Y2H pair-wise interactions will be validated in mammalian cells *in vitro* by co-affinity purification and co-immunoprecipitation binding assays.

As a long-term research objective, the EBL will apply fluorescent techniques (FRET) to quantify the protein interactions and the dynamics of single protein molecules within living cells.

The EBL is involved in two ongoing projects related to the molecular mechanisms associated with Alzheimer's disease and colon cancer, respectively.

Alzheimer's disease (AD) interaction network. The members of the EBL involved in this project are Ricart Lluís, Maica

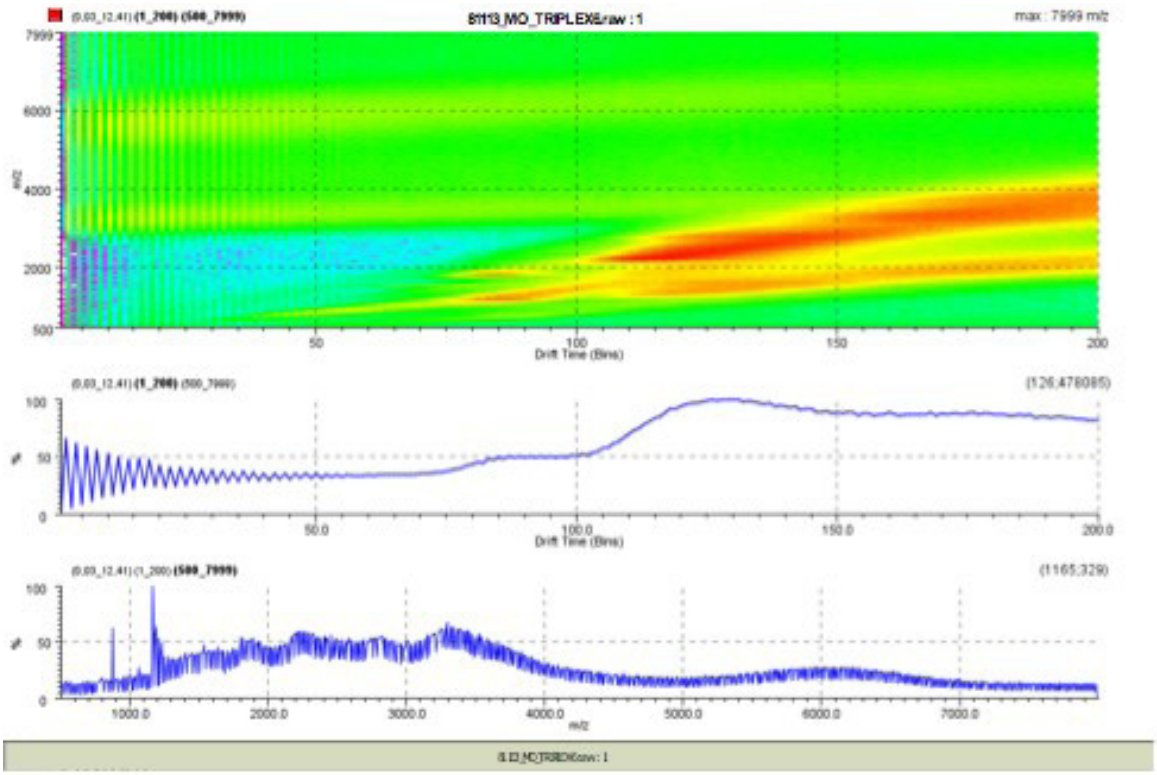


Figure 1. *ESI spectra (plain M/Z spectrum in bottom and Drif times in TOP) of the d(GA)6·d(TC) 6-d(CT) 6 triplex.*

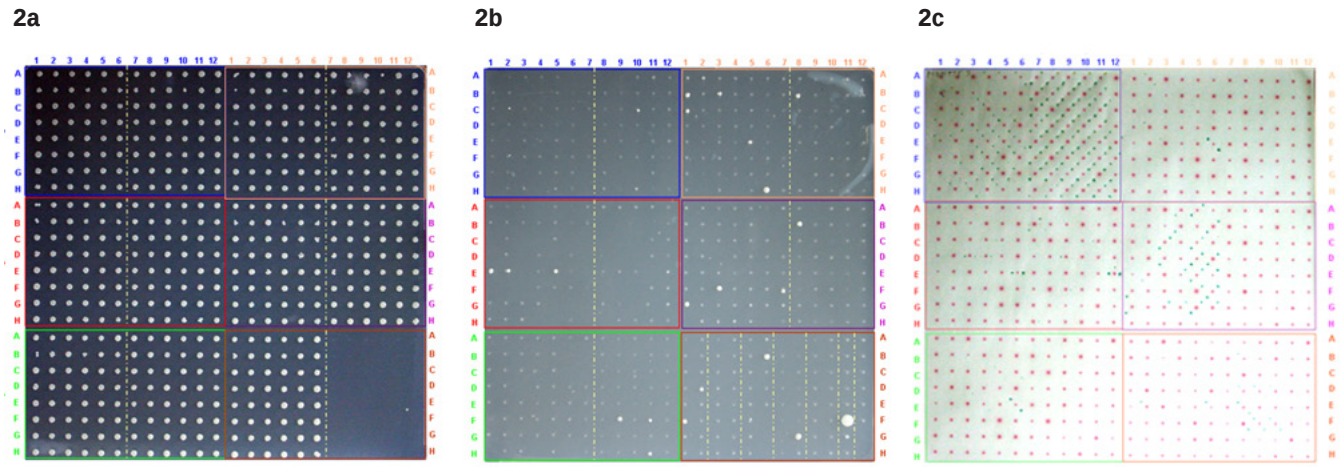


Figure 2. *First Y2H interaction screen for AD. (a) Yeast colonies expressing Y2H plasmids, respectively. (b) Identification of Y2H protein-protein interactions (PPIs) by spotting yeast clones onto selective media agar plates. (c) PPIs identified by lacZ reporter gene assay.*

López and Montse Soler, in collaboration with Patrick Aloy's computational group. An initial list of 55 putative AD-causing genes was provided by this group at the end of April. Experimental design and acquisition of human encoding cDNAs for the genes of interest were ready in early May. The corresponding Y2H clones were generated by the end of June. In collaboration with Ulrich Stelzl's group at the Max Planck Institute of Molecular Genetics Berlin, the first screen was subsequently performed during the following months and its validation has recently been accomplished. The project has already yielded preliminary results: a total of 2,200 pair-wise protein interactions were examined either by plasmid cotransformation or by mating approaches using special yeast strains. We have identified 135 interactions among 53 proteins (Figure 2). Expansion of first round Y2H screen by a library-based approach is currently underway.

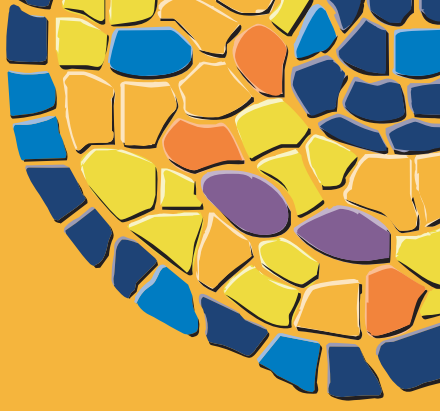
Colon cancer interaction network. EBL members Clara Berenguer and Montse Soler, in collaboration with Patrick Aloy's group and in close contact with Eduard Batlle and Elena Sancho from the Oncology Programme at IRB Barcelona, are now in the process of designing the experimental approach and the acquisition of gene encoding cDNAs. Experiments will be performed in the first half of 2009 following a similar protocol to that described above.

SCIENTIFIC OUTPUT

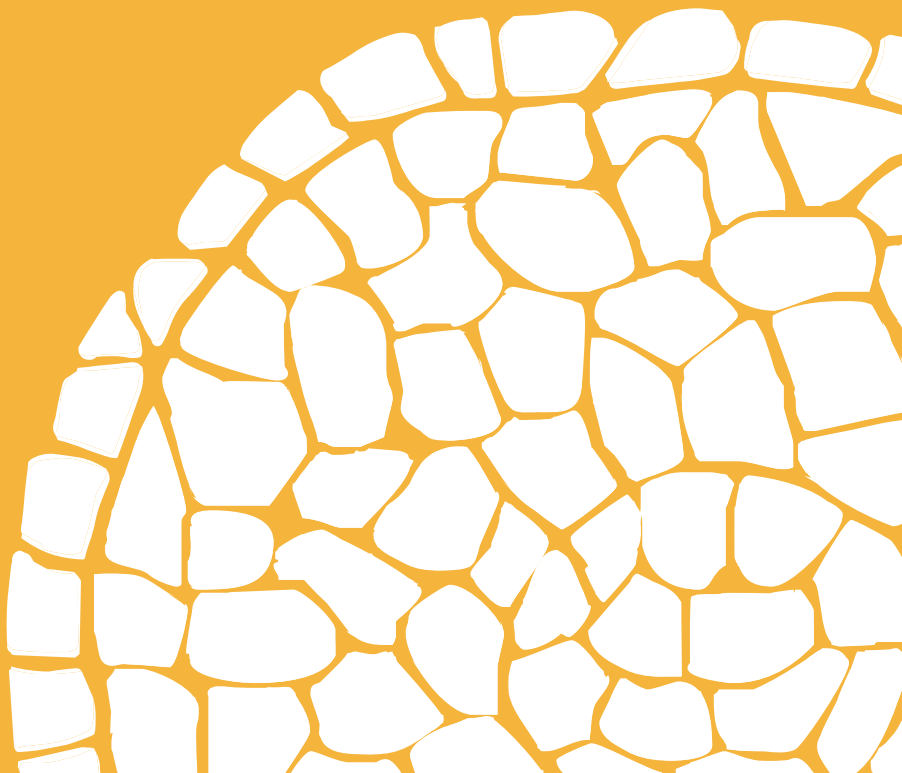
Collaborations

Alzheimer's disease interaction network
Ulrich Stelzl, Max Planck Institute of Molecular Genetics Berlin (Berlin, Germany)

The EBL is fully supported by the structural budget of the IRB Barcelona—Barcelona Supercomputing Centre Joint Research Programme in Computational Biology and by grants of the principal investigators using it.



Molecular Medicine Programme



CARME CAELLES

Cell signalling: Regulation and function

Our group studies the regulatory and cross-talk mechanisms that underlie signal transduction, with the aim to gain a better understanding of physiological and pathological processes and to improve and develop therapeutic tools. We focus on two research lines, the nuclear receptor–MAPK negative cross-talk and the Nercc1/Nek6/7 NIMA-family signalling cassette. In relation to the former, we address a subset of nuclear receptors (NRs), namely the glucocorticoid receptor (GR) and the members of the peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) subfamilies, and the c-Jun N-terminal kinase (JNK) pathway. We are exploring the mechanisms responsible for the inhibition of the JNK pathway by these NRs, and analysing the involvement of this inhibition in mediating the pharmacological actions of the ligands of these NRs, namely anti-inflammatory and/or anti-diabetic activities. In relation to the second research line, we study the regulation and function of the signalling module formed by the NIMA-family kinases Nercc1 (also known as Nek9), Nek6 and Nek7. Previous data showed that this module is activated during mitosis and has a central role in spindle formation and mitotic progression. Our goals are to unravel the mechanism of Nercc1 activation, to identify Nercc1 and Nek6/7 substrates, and to validate these kinases as drug targets and/or prognosis markers in diseases related to cell cycle dysfunction.

Nuclear receptor-MAPK pathway cross-talk: mechanisms and actions

In this research line, we focus on the down-regulation of the JNK pathway by a subset of NRs whose ligands exhibit anti-diabetic and/or anti-inflammatory properties. These are the PPAR and LXR subfamilies, which are involved in the control of glucose and lipid homeostasis, and the GR, respectively.

Compelling evidence has shown that chronic inflammation promotes the development of several pathological conditions such as obesity and type 2 diabetes. The role of pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, in promoting insulin resistance in obesity, a condition linked to the development of type 2 diabetes, was reported many years ago. In recent years, it has been demonstrated that pro-inflammatory signal transduction pathways, in particular JNK, IKK/NFkB and PKCθ, are involved in the obesity-induced progressive loss of insulin sensitivity (revised in Wellen and Hotamisligil, 2005). In particular, JNK phosphorylates the insulin receptor substrate (IRS), abrogating its interaction with the insulin receptor (IR), a mechanism that constitutes a negative feedback loop on insulin signalling. However, under pathological conditions, JNK phosphorylation of the IRS promotes insulin resistance (reviewed in Hotamisligil, 2005). In addition to obesity and type 2 diabetes, recent studies have implicated JNK in other pathological conditions, such as cancer, cardiac hypertrophy and failure, arthritis, asthma and neurodegeneration. Thus, given their potential therapeutic applications, JNK inhibitors have received much research attention (reviewed in Manning and Davis, 2003).

JNK inhibition by PPARs and LXRs. Thiazolidinediones (TZDs) are synthetic PPARγ ligands with insulin-sensitising activity and are thus used in the treatment of type 2 diabetes. In spite of the relevance of these drugs, little is know about their mechanism of action or their molecular target. This knowledge gap thus blunts the rational development of new molecules with improved pharmacological profiles. We have demonstrated that TZDs, through interaction with PPARγ, inhibit JNK pathway activation. Moreover, we have shown that the JNK inhibition by TZD/PPARγ is crucial for the anti-diabetic properties of these drugs since their hypoglycemic activity is abrogated in JNK1-deficient mice (Díaz-Delfín *et al*, 2007).

JNK inhibition by TZDs also occurs in macrophages and correlates with their capacity to repress pro-inflammatory gene expression. In this context, this inhibitory action on the JNK pathway is related to the anti-inflammatory properties of these molecules. In addition, the current state of the art, which agrees that a low-grade chronic inflammatory process is at the origin

Principal Investigator Carme Caelles **Research Associate** Joan Roig **PhD Students** Mª Teresa Bertran, Kader Cavusoglu, Rodrigo Gatica, Jordi Lanuza, Laura Regue, Mariana Vargas **Lab Technician** Cristina Vila **Visiting Student** Giuseppe Pulice

and/or promotes a series of diseases including type 2 diabetes, supports the notion that the anti-inflammatory activity exerted by TZDs has a relevant role in the anti-diabetic action of these compounds. In agreement with this, Mercedes Ricote's group (Hevener *et al*, 2007) has demonstrated that macrophage-specific PPARγ-deficient mice are glucose-intolerant as a result of exacerbated secretion of pro-inflammatory mediators by macrophages. Also, the anti-diabetic action of TZDs is almost abrogated in these animals. In addition to TZDs, we have demonstrated that ligands for other members of the PPAR and LXR subfamilies, which also show anti-inflammatory, and in some cases anti-diabetic activity, also inhibit the activation of the JNK pathway in macrophages.

In our search for a mechanism responsible for the inhibition of JNK by TZDs, we observed an increased expression of the cell cycle regulator p21waf-1 in the adipose tissue of obese mice. This finding led us to study the phenotype of the waf-1 knockout (KO) mouse in the context of obesity and insulin resistance. Waf-1 KO mice are protected from the development of adiposity and insulin resistance when fed a high-fat high-carbohydrate diet (HFD; Figure 1).

JNK inhibition in the anti-inflammatory action of glucocorticoids. We previously reported the inhibition of JNK by glucocorticoids (GCs) and proposed the relevance of this signalling pathway for the anti-inflammatory actions of these drugs. We also described a molecular mechanism responsible for this inhibition (Caelles *et al*, 1997; Bruna *et al*, 2003). In addition to JNK, the IKK/NFκB pathway is also relevant for the inflammatory response, and it is also known to be target of GC action. According to our data, JNK is required for NFκB activation since it increases the $SCF^{\beta TRCP}$ complex, by enhancing βTRCP mRNA stability, which is responsible for IκB ubiquitination and, hence,

degradation. We have demonstrated that JNK inhibition by GCs through this mechanism negatively regulates NFκB activation.

Generation of a transgenic murine model that allows the conditional activation of JNK and its validation as a model to study pancreatic failure. We have generated a transgenic mouse with the aim to study the contribution of JNK activation to the development of insulin resistance and pancreatic β-cell failure. This mouse model harbours a Cre-conditional expression transgene encoding a constitutively activated mutant form of MKK7, a highly specific JNK MAP2K. We have activated the transgene in pancreatic β-islets by crossing parental transgenic mice with a mouse strain that expresses the Cre recombinase specifically in pancreatic β-cells. Cre-mediated recombination of the transgene leads to JNK activation in pancreatic β-cells concomitantly with the development of glucose intolerance and a failure to increase insulinemia in response to hyperglycemia.

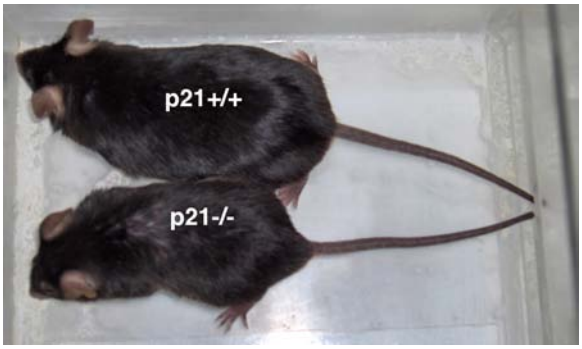


Figure 1. *p21 deficiency protects from HFD-induced adiposity. Picture shows 16-week-old WT (p21+/+) and p21-deficient (p21-/-) mice which had been fed a HFD since they were 4 weeks old.*

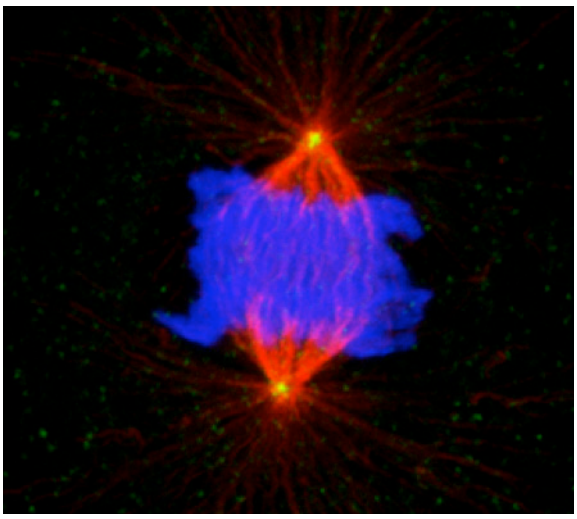


Figure 2. *Phospho-Eg5 immunolocalisation in mitosis. Mitotic XL177 cell fixed and stained with antibodies to XlEg5[Ser1046P] (green), and ß-tubulin (red). DNA is stained with DAPI (blue).*

The signalling module formed by the NIMA family of kinases Nercc1/Nek9-Nek6/7: regulation and function

The NIMA family of protein kinases is named after the product of the nimA gene, a protein kinase from *Aspergillus nidulans* that has a central role in G2/M transition and mitotic progression (O'Connell *et al*, 2003). During evolution, the NIMA family of kinases (Nek1-11 in mammals) has acquired several functions related to the control of centrosome/microtubule systems, including crucial roles during mitotic progression (Quarmby and Mahjoub, 2005; Roig and Avruch, 2006; O'Regan *et al*, 2007). We have previously described that Nercc1 (also known as Nek9) binds and activates two other highly homologous members of the NIMA family, Nek6 and Nek7, and have proposed that they form a signalling module activated at the centrosomes and spindle poles during mitosis (Roig *et al*, 2002; Belham *et al*, 2003; Roig *et al*, 2005). Nercc1, like Nek6 and Nek7, is required for correct spindle formation, chromosome segregation and mitotic progression (Roig *et al*, 2002; Roig *et al*, 2005), although the molecular bases for this action are currently unknown.

To elucidate the cellular role of the Nercc1/Nek6/7 module during mitosis, relate it to other mitotic signalling networks, and identify its signalling targets, our group aims to (i) study how the activity of the upstream kinase, Nercc1/Nek9, is regulated; (ii) determine the structural basis for the mechanism of Nercc1 inhibition/activation; (iii) identify and study Nercc1 and Nek6/7 substrates; (iv) use different systems to interfere with Nercc1 and Nek6/7, in order to gain a broad view of their function at the cellular and organismic level, relating it to the results of point 3; and (v) validate Nercc1 as a drug target and identify chemical inhibitors of the protein kinase with therapeutical value. We are now pursuing the above goals in collaboration with several research groups and a biotechnology company. Our final objective is to produce a complete picture of the position of the Nercc1/Nek6/7 module in relation to the myriad of signalling events that control mitosis and the cell cycle.

During 2008, we have advanced in both our understanding of the activation mechanism of Nercc1 during mitosis, as well as the identification of substrates of the Nercc1/Nek6 module. In addition, we are collaborating with David Reverter (Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona) to produce, crystallise and determine the structure of an inactive form of Nercc1; with Jens Lüders (IRB Barcelona) to elucidate how phosphorylation controls microtubule nucleation in mitosis; and with Isabelle Vernos (CRG, Barcelona) to study the signalling module in the *Xenopus* mitotic egg extract.

We have performed a number of yeast two-hybrid assays using distinct forms of Nercc1/Nek9 as bait, and now have several

candidates for Nercc1 regulators and/or Nercc1/Nek6/7 substrates, 12 of them proteins with a known function during mitosis. At present, we are validating the interactions identified and starting to analyse a selected group of these proteins. Regarding Nercc1/Nek6 substrates, we have identified one of the most important mitotic motors, the kinesin Eg5 (also known as Kif11 and Kinesin-5), as a Nercc1/Nek6-interacting protein, and demonstrated that it is a Nek6 substrate both *in vitro* and *in vivo* (Rapley *et al*, 2008). Thus, Nek6 is constitutively associated with the C-terminal tail of Eg5 and has the capacity to phosphorylate the kinesin at Ser1033 (in human Eg5), a site that is phosphorylated *in vivo* during mitosis. We have produced several antibodies that recognise both human and *Xenopus* Eg5 phosphorylated at the Nek6 site, and have shown that, while during mitosis total Eg5 resides in the cytoplasm and the length of spindle microtubules, the phosphorylated motor is localised at spindle poles and the proximal region of spindle microtubules (but not in astral microtubules, Figure 2). This subcellular location overlaps in part with the activation pattern of Nercc1/Nek6. Our results show for the first time that cells have differentially modified and spatially localised pools of Eg5. This finding may help us to explain how Eg5 performs its multiple cellular roles. We have also demonstrated that phosphorylation of the novel Nek6 site is physiologically important for normal Eg5 function, as mutant forms of the kinesin lacking a phosphorylable Nek6 site rescue Eg5 depletion with only 50% of the efficiency shown by wild-type kinesin. We are currently studying the molecular basis behind these observations.

SCIENTIFIC OUTPUT

Publications

Rapley J, Nicolàs M, Groen A, Regué L, Bertran MT, Caelles C, Avruch J and Roig J. The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. *J Cell Sci*, **121**, 3912-21 (2008)

Valledor AF, Arpa L, Sánchez-Tilló E, Comalada M, Casals C, Xaus J, Caelles C, Lloberas J and Celada A. IFN-γ-mediated inhibition of MAPK phosphatase expression results in prolonged MAPK activity in response to M-CSF and inhibition of proliferation. *Blood*, **112**, 3274-82 (2008)

Valledor AF, Sánchez-Tilo E, Arpa L, Park JM, Caelles C, Lloberas J and Celada A. Selective role of MAPKs during the macrophage response to IFN-γ. *J Immunol*, **180**, 4523-29 (2008)

Research networks and grants

El módulo de señalización Nercc1/Nek6/7; regulación y funciones
Spanish Ministry of Science and Innovation, BFU2008-03441/BMC (2008-2011)
Principal investigator: Joan Roig

Estudio de una nueva via de señalización mitótica compuesta por las NIMA quinasas Nercc1, Nek6 y Nek7. Regulación y funciones
Spanish Ministry of Science and Innovation, BFU2005-05812 (2006-2008)
Principal investigator: Joan Roig

Papel de la c-Jun N-terminal kinase en las acciones fisiológicas y farmacológicas de los glucocorticoides y los ligandos de PPARs y LXRs
Spanish Ministry of Science and Innovation, BFU2007-62087/BMC (2007-2010)
Principal investigator: Carme Caelles

Relación de la expression del receptor de insulina y la activación de la vía PI3K/AKT con la expresión de enzimas glicogénicas y gluconeogénicas en células tubulares de riñón de ratas normales y diabéticas
Fundación Marcelino Botín (2008-2009)
Principal investigator: Carme Caelles

Collaborations

Functional analysis of JNK activation in pancreatic β-cells
Ramón Gomis, IDIBAPS (Barcelona, Spain)

Identification of inhibitors of mitosis
Mercury Therapeutics Inc (Woburn, MA, USA)

MAPK pathways in macrophages
Antonio Celada, IRB Barcelona (Barcelona, Spain)

Regulation of microtubule nucleation through phosphorylation
Jens Lüders, IRB Barcelona (Barcelona, Spain)

Structural basis for the mechanism of Nercc1 autoinhibition
David Reverter, Institute of Biotechnology and Biomedicine, Autonomous University of Barcelona (Barcelona, Spain)

Study of the regulation and function of the Nercc1/Nek6/7 signalling module in the Xenopus *egg extract system*
Isabelle Vernos, Centre for Genomic Regulation (Barcelona, Spain)

The role of JNK in myogenesis
Pura Muñoz-Cánoves, Pompeu Fabra University (Barcelona, Spain)

ANTONIO CELADA

Inflammation and macrophages

Inflammation occurs when the body suffers aggression either by microbes, trauma or a variety of physical agents, such as heat, radiation, etc. Inflammation is also involved in the pathogenesis of chronic diseases of autoimmune origin (*eg*, rheumatoid arthritis) and cancer. In the early stages of inflammation, there is an increase in the size of the vessels around the inflammatory loci and the release of liquids. After, distinct cells reach these loci in a highly specific order: in the first 24 h neutrophils, at 48 h macrophages, and several days later lymphocytes. Neutrophils destroy most types of microbes. In the initial stages of inflammation, macrophages destroy the remaining microbes that escape the neutrophils, remove the apoptotic bodies of dead neutrophils and present antigen to T lymphocytes, thereby initiating the mechanisms of acquired immunity, which ends in the production of antibodies, cytokines and memory cells, the latter a key element for vaccines. Macrophage activity then switches from being pro-inflammatory to anti-inflammatory, whereby they remove all the tissue debris, thus achieving healing (Figure 1). Our project is the continued work of many years devoted to the biology of macrophages and dendritic cells. These cell types play a key role in the innate immune response and form a bridge between innate and acquired immune response. Macrophages are generated in bone marrow and reach all body tissues through the blood. In normal conditions, a few cells are differentiated in response to certain stimuli and become mature cells or tissue-specific cells: dendritic cells, Kupffer cells, microglia, etc., while most are removed by apoptosis. When an inflammatory process occurs, macrophages proliferate, differentiate or become activated under the effect of interleukins or growth factors. When a macrophage becomes activated, it ceases to respond to proliferative stimuli. In certain circumstances, when chronic inflammation is produced, macrophages have a harmful rather than repairing effect and cause lesions. Our group seeks to determine the molecular mechanisms involved in the proliferation, activation, differentiation and apoptosis of macrophages. Improved knowledge of these mechanisms could provide therapeutic targets to modulate the activity of these cells during acute or chronic inflammation.

Signal transduction and gene regulation that mediate proliferation, activation and apoptosis of macrophages

Mitogen-activated protein kinases (MAPKs) are one of the best characterised signal transduction pathways involved in the sequential activation of Ras, Raf-1, mitogen/extracellular signal-regulated kinase (MEK) and the extracellularly regulated kinase (ERK). Activated Raf-1 phosphorylates MEK1 and MEK2, which in turn activate ERK1 and ERK2. In unstimulated cells, ERK1 and ERK2 are found in the cytoplasm and they relocate to the nucleus after being phosphorylated. Once in the nucleus, they phosphorylate a series of transcription factors. These kinases also participate in the synthesis of nucleotides and in protein translation processes, both required for cell proliferation and activation. In macrophages, we have observed that ERK activation is required not only for proliferation but also for lipopolysaccharide (LPS)-mediated activation, although the latter also blocks proliferation. The duration and time of initiation of ERK phosphorylation determines whether the cell proliferates (short phosphorylation) or becomes activated (long phosphorylation). This is explained by the fact that MKP-1, the phosphatase re-

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sponsible for ERK dephosphorylation, is induced rapidly in response to macrophage colony-stimulating factor (M-CSF) or slowly in response to LPS (Cassals *et al*, in press). In both cases, MKP-1 induction is mediated by PKCε and is independent of ERK phosphorylation. Furthermore, IFN-γ, which also inhibits proliferation, blocks the MKP-1 induction caused by M-CSF by elongating ERK phosphorylation (Valledor *et al*, 2008). Inhibition of MKP-1 induction by RNA interference (RNAi) blocks proliferation and elongates ERK activation. IFN-γ signalling also cross-talks with MAP kinases (Valledor *et al*, 2008).

We have also defined all the intracellular pathways required for this induction and the regulation of the MKP-1 gene regulation mediated by phosphorylated c-jun and CREB. We have cloned the MKP-1 promoter and, by means of luciferase mutations and activity assays, we have localised an AP-1/CRE box that is critical for MKP-1 induction by M-CSF and by LPS. By Electrophoretic Mobility Shift Assays and Chromatin Immunoprecipitation, we have determined that this box is bound by Jun and CREB factors. c-Jun and the phosphorylation of CREB are induced by LPS and M-CSF with the same kinetics as MKP-1 (Cassals *et al*, in press; Figure 2).

Our group has devoted many years to the study of the regulation of MHC class II molecules. Peptides derived from processed proteins bind to a cleft in the MHC class II molecule surface and are presented to T lymphocytes. Thus, the expression of MHC class II molecules regulates not only the generation of the T lymphocyte repertoire but also the induction and maintenance of immune response. MHC class gene transcription depends on the interaction and co-operation of several transcription factors which bind to the regulatory elements found in the promoter. However, all the transcription factors described to date show ubiquitous expression, which does not correlate with the differential tissue expression of MHC class II. CIITA (class II transactivator), which does

not bind directly to DNA, has been described and shown to be required for the expression of these genes. We have determined that an AP-1 box acting as an enhancer is responsible for the induction of MHC class II gene expression in B lymphocytes and dendritic cells treated with LPS. Also, the upstream regulatory elements interact with the proximal promoter, thereby blocking the transcription. This loop is open when CIITA is present (Serrat *et al*, in press).

In collaboration with Victor Puntes (Institut Català de Nanotecnologia) and Ernest Giralt (IRB Barcelona), we have found that nanoparticles have the capacity to activate macrophages by interacting with Toll-like receptor 4 (TLR4) (Bastús *et al*, 2009; Figure 3).

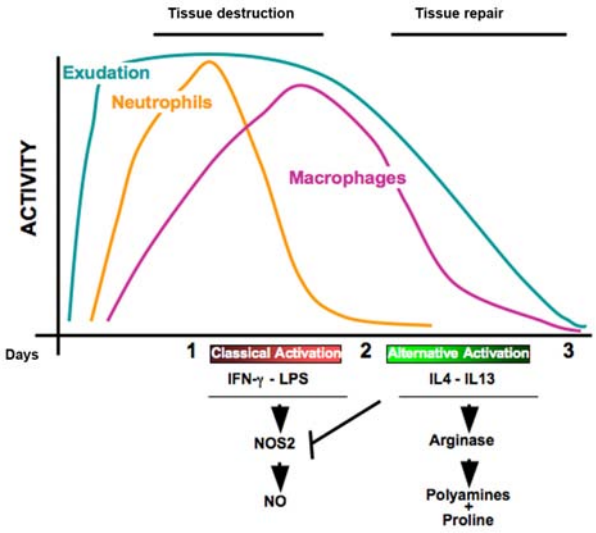

Figure 1. *Dual activity of macrophages at the inflammatory loci.*

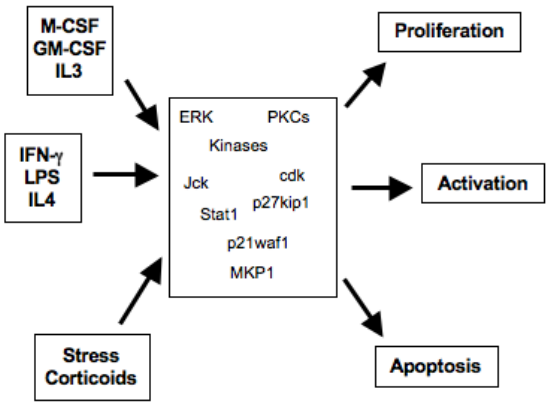

Figure 2. *Group factors, activators or inducers of apoptosis use several signal transduction molecules to achieve proliferation, activation or apoptosis.*

Molecular mechanisms involved in classical and alternative activation of macrophages

Classical or pro-inflammatory activation of macrophages is induced by IFN-γ or LPS while that triggered by IL-4, IL-10 or IL-13 is known as alternative or anti-inflammatory activation. Apart from a series of structural and functional modifications, the main difference between these phenotypes is the biochemical pathway used for processing the amino acid arginine. IFN-γ or LPS induce NOS2 enzyme, thereby producing nitric oxide (NO), which has great destructive power and in the first phases of inflammation kills microorganisms. In anti-inflammatory macrophages, arginase is induced and produces proline and polyamines, which catalyse the reconstitution of the damaged extracellular matrix, an event that occurs during the final phases of inflammation. We have found that activation with IL-4 or IFN-γ blocks proliferation in G1/S. However, while the mechanism behind IL-4 inhibition of the proliferation is $p21^{waf1}$- and Stat6-dependent, the mechanism used by IFN-γ differs (Arpa *et al*, 2009). Also, we have shown that the kinase p38 α serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression (Kim *et al*, 2008).

Role of TREX1 exonuclease in transcription

We have cloned TREX1 exonuclease, a protein that binds to DNA. This enzyme catalyses the digestion of DNA in the 3'->5' direction and shows homology to the TREX2 exonuclease (30%). Genetically modified mice, with a deletion in the TREX1 locus, developed inflammatory myocarditis and had a reduced half life compared to their wild-type counterparts. In humans, mutations in the Trex1 gene have been associated with Aicardi-Goutières Syndrome, a chronic inflammation of the brain, as well as with systemic lupus erythematosus, an autoimmune disease. TREX1 has also been associated with protein members of the SET complex, which digest DNA from cells where apoptosis has been induced by Granzyme A.

In collaboration with IRB Barcelona experts in crystallography (Ignasi Fita) and in NMR (Maria Macías), we have determined the structure of TREX1 alone and its binding to DNA. TREX1 binds preferentially to certain DNA sequences that correlate with exonuclease activity. TREX1 has a proline-rich domain not found in TREX2. This domain allows interaction with SH3 or WW domains, which we have demonstrated by NMR and co-immunoprecipitation. These data, together with the nuclear localisation of the protein, have led us to study whether TREX1 is involved in transcription. In addition, we have identified a new active histidine conserved in DEDDh exonucleases that is required for functional activity (Brucet *et al*, 2008, Figure 4).

Deregulated gene expression in aging

We have been testing the molecular changes that occur in the genome of macrophages during aging. As we culture macrophages alone *in vitro*, we disregard the effects that other cells could exert. In addition, we have recently reported that deacetylase activity is required for granulocyte macrophage-colony-stimulating factor (GM-CSF)-dependent functional response of macrophages and dendritic cell differentiation (Sebastian *et al*, 2008). Since deacetylase activity plays a crucial role in aging in lower

organisms, these results have prompted us to study whether it is involved in macrophage aging. GM-CSF-dependent proliferation is impaired in macrophages from senescence-accelerated mice (Espía *et al*, 2008).

To examine the effect of aging, we cultured bone marrow-derived macrophages from aged mice *in vitro*. In these cells, compared to those of young mice, the telomeres were shorter and GM-CSF- but not macrophage (M)-CSF-dependent proliferation was impaired as a result of decreased phosphorylation of STAT5a. The same defects were found in macrophages from knock-out (KO) mice for telomerase (*terc*$^{-/-}$). Macrophages from aged and *terc*$^{-/-}$ mice showed increased susceptibility to oxidants and an accumulation of intracellular reactive oxygen species. In these macrophages, STAT5a oxidation was reduced, which led to the decreased phosphorylation observed. These results suggest that telomere loss produces enhanced oxidative stress, reduced STAT5a oxidation and phosphorylation and, ultimately, impaired GM-CSF-dependent macrophage proliferation (Sebastian *et al*, in press).

LXR in neuroinflammation and neuronal degeneration

LXRs (liver X receptors, initially discovered in the liver) are members of the nuclear receptor superfamily. Nuclear receptors are ligand-dependent transcription factors that regulate many aspects of development and homeostasis. LXRs are regulated by oxidised forms of cholesterol (oxysterols) and by intermediary products of cholesterol biosynthesis. Together with their heterodimeric partners RXRs (retinoid X receptors), LXRs play crucial roles in the positive regulation of genes involved in lipid homeostasis. Furthermore, LXRs also have the capacity to down-modulate specific programmes of gene expression through a process known as trans-repression. This process translates in the modulation of immune activities.

As a result of their lack of cellular division and their low capacity to recover from injury, neurons are extremely sensitive to inflammatory processes and immune autodestruction. For this reason, intervention of the inflammatory process has recently gained attention as a therapeutic strategy to halt neurodegenerative disorders. LXR activation has been shown to exert anti-inflammatory roles in the central nervous system (CNS), although the mechanisms underlying this process remain to be determined. The primary immune effector cells in the CNS are microglia, which are originally derived from monocytes/macrophages. We have observed that primary microglia express the isoforms LXRα and β, and RXRα and β. In these cells, stimulation with endogenous ligands of LXR resulted in activation of known LXR target genes involved in cholesterol efflux, such as ABCA1 and ABCG1. In the mature brain and under physiological conditions, resting microglia serve the role of immune surveillance and host defence. However, these cells are particularly sensitive to changes in their microenvironment and readily become activated in response to infection or injury.

Most of the factors released by activated microglia are pro-inflammatory and neurotoxic, thereby contributing to the progression of the neurodegenerative disorder. We have been studying the role of LXR in the regulation of microglial activation *in vitro*. Microarray technology has allowed us to establish pro-inflammatory gene subsets that are susceptible to down-modulation by LXR agonists. We are currently comparing the data obtained *in vitro* with *in vivo* models of neuroinflammation.

Furthermore, we have also explored the role of the LXR-RXR pathway on programmed cell death in the CNS. The simultaneous use of LXR and RXR agonists resulted in synergistic effects that promote high expression of genes involved in protection against apoptosis in microglial cells, *eg*, Blc-xL, AIM and NAIP (neuronal apoptosis inhibitory protein). The observation that AIM, an anti-apoptotic factor secreted by macrophages, is also induced in this system leads us to propose that this factor mediates paracrine anti-apoptotic actions on other neighbouring cells in the CNS, *eg*, astrocytes and neurons. We are currently testing these effects using mixed glia-neuronal cultures and pure neuronal systems. Microarray experiments in pure neuronal cultures are also underway in order to determine direct effects

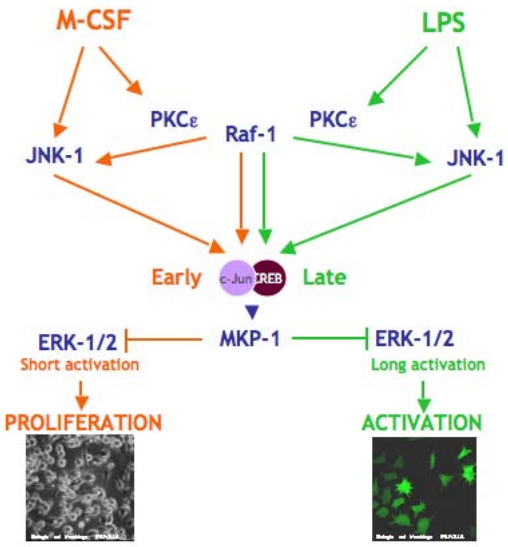

Figure 3. *Signal transduction of M-CSF and LPS inducing the expression of MKP-1.*

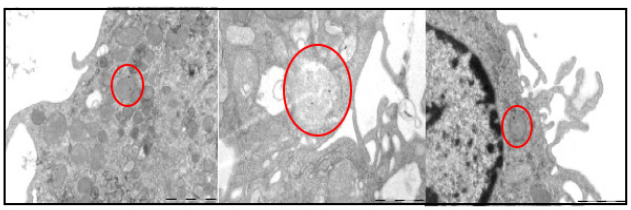

Figure 4. *Nanoparticles ingested by macrophages.*

of LXR agonists on neuronal cells. Our final goal is to establish whether the anti-apoptotic and anti-inflammatory actions of the LXR/RXR pathway can be exploited for the therapeutic intervention of neurodegenerative disorders *in vivo*.

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Anti-inflammatory and anti-apoptotic effects of LXR/RXR agonists in the central nervous system
European Commission, Marie Curie international reintegration grants, 031137 (2006-2008)
Principal investigator: Annabel Fernández

Ayuda para potencializar los grupos de investigación consolidados
Agency for Administration of University and Research Grants (AGAUR), 2005SGR-00910 (2005-2008)
Principal investigator: Antonio Celada

Programas transcripcionales regulados por LXR en microglía y en neuronas: implicaciones en neuroinflamación y neuroprotección
Spanish Ministry of Science and Innovation, SAF2007-63543 (2008-2010)
Principal investigator: Annabel Fernández

Regulation of the expression of genes involved in the proliferation, differentiation, activation and apoptosis of macrophages and dendritic cells
Spanish Ministry of Science and Innovation, BFU2007-63712/BMC (2007-2011)
Principal investigator: Antonio Celada

Other funding sources

Estudio de la capacidad fagocítica de los macrófagos de origen canino
Affinity Petcare SA (2008)
Principal investigator: Jorge Lloberas

Estudio piloto de inmunización
EraBiotech (2007-2008)
Principal investigator: Jorge Lloberas

Collaborations

Alternative activation of macrophages
Manuel Modolell, Max Planck Institute (Freiburg, Germany)

Inflammation and apoptosis
Joan Maña, Ciudad Sanitaria y Universitaria de Bellvitge, (Barcelona, Spain)

Inflammation and dermatology
Jin Mo Park, Massachusetts General Hospital and Harvard Medical School (Boston, USA)

Inflammation in dermatology
Ignacio Umbert, Clínica Corachan (Barcelona, Spain)

Inflammation and neutrophils
Victor Asensi, Hospital General de Asturias (Oviedo, Spain)

Inflammation and polymerases
Antonio Bernard, Centro Nacional Investigaciones Cardiovasculares (Madrid, Spain)

Macrophages and aging
Robert D Schreiber, Washington University (St Louis, MI, USA)

Telomerase and macrophaging
María Blasco, Centro Nacional de Investigaciones Oncológicas (Madrid, Spain)

JOAN J GUINOVART

Study of the regulatory mechanisms of glycogen metabolism and characterisation of therapeutic targets

Our research group specialises in the study of the regulatory mechanisms of glycogen metabolism and the alterations of these processes in disease. We have a long tradition of research into glycogen synthase, the key enzyme in the regulation of glycogen synthesis. To this end, to address biological issues, we combine our knowledge of biochemistry and metabolism and follow a multidisciplinary approach that includes a wide variety of techniques from molecular biology, cell biology, proteomics, RNA silencing, gene transfer, mutant mouse generation and structural biology. Our work has contributed to revitalising this field by making a series of discoveries that have shown that, against general belief, there is still much ground to be covered. Our main achievements during 2008 are summarised below.

Determination of the key phosphorylation sites involved in the modulation of glycogen deposition in liver

Glycogen synthase (GS), the key enzyme that catalyses glycogen synthesis, is regulated by reversible phosphorylation at multiple sites. While much research effort has focused on the identification and functional consequences of the phosphorylation of the almost ubiquitous muscle isoform of GS (MGS), little has been devoted to the liver isoform (LGS). Dephosphorylation is correlated with the activation of GS, but in the particular case of LGS, the key sites involved in its activation have not been identified. In this regard, we have analysed the effect of dephosphorylation at the sites of LGS homologous to those described for MGS. Serine residues at these sites were replaced by non-phosphorylatable alanine residues, singly or in pairs, and the resultant LGS variants were expressed in cultured cells using adenoviral vectors. The sole mutation at site 2 (Ser7) yielded an enzyme that was almost fully active and able to induce glycogen deposition in primary hepatocytes incubated in the absence of glucose, as well as in FTO2B cells, a cell line that does not normally synthesise glycogen. Mutation at site 2 was also sufficient to trigger the aggregation and translocation of LGS from the cytoplasm to the hepatocyte cell cortex in the absence of glucose. However, this redistribution was not observed in hepatocytes incubated without glucose when an additional mutation (E509A), which renders the enzyme inactive, was introduced. This result strongly suggests that LGS translocation is strictly dependent on glycogen synthesis.

In conclusion, site 2 of LGS is the most potent regulatory site of the activity of the enzyme. Given that the LGS activation state is reduced in diabetes mellitus, the development of strategies aiming to increase the phosphorylation of this site may improve the accumulation of glycogen in liver, and thereby contribute to reducing hyperglycemia (Ros *et al*, in press, 2008).

Study of the adaptative regulation of glycogen metabolism in the embryonic liver

Mammalian embryonic livers accumulate glycogen in the absence of glucokinase expression. Glucokinase (hexokinase type IV) is required for the accumulation of glycogen in adult liver, and in embryonic livers hexokinases I and II are the only glucose-phosphorylating enzymes expressed. In adult liver, these two hexokinases would not normally have the capacity to build up enough levels of glucose-6-phosphate to activate LGS. Our results show that embryonic livers express massive levels of both hexokinases I and II, thus allowing the synthesis of sufficient amounts of glucose-6-phosphate to activate LGS and consequently hepatic glycogen synthesis.

Our results provide an explanation for the reorganisation of hexokinase expression in liver during fetal life and after birth. Glycogen plays a key role during embryonic development as it ensures pup survival in the period between birth and first receiving its mother's milk. Pups use glycogen deposits as a ready source of energy at the moment of birth and need to build their glycogen reserves during development in such a way that they do not depend on their mother's feeding state. By a considerable increase in the expression of the high affinity hexokinases I and II, embryos not only ensure

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their capacity to use glucose in all circumstances, but at the same time they produce enough glucose-6-phosphate to ensure hepatic glycogen accumulation even when the mother is fasting and blood glucose levels decrease. By means of this mechanism, embryos safeguard liver glycogen stores, thereby providing a crucial advantage at the moment of birth before their first ingestion of milk, which dramatically induces the expression of glucokinase (Cifuentes *et al*, 2008).

Study of the mechanisms of action of the anti-diabetic and anti-obesity agent sodium tungstate

Our group discovered that tungstate is an oral glucose-lowering and anti-obesity agent This compound has completed Phase I and II of clinical trials. Tungstate normalises carbohydrate metabolism in liver, stimulates insulin secretion, and regenerates pancreatic beta-cells in neonatally streptozotocin-treated diabetic rats. This compound is an efficient anti-diabetic agent in ZDF rats, a genetic model of type 2 diabetes. Our group, in collaboration with those headed by Ramon Gomis (IDIBAPS-Hospital Clínic de Barcelona), Rafael Salto (University of Granada) and Joan Enric Rodríguez Gil (Autonomous University of Barcelona), has devoted much research effort to the study of the effects of tungstate at both the physiological and molecular level.

During 2008, in collaboration with the group led by Joana Mª Planas (University of Barcelona), we have addressed the effects of tungstate treatment on the transport of monosaccharides in the intestine of diabetic animals. This is a crucial aspect to study in diabetes since the first step in the control of glycemia is the regulation of the transit of dietetic sugars from the intestinal lumen through the enterocytes to the bloodstream. To this end, we have analysed the action of this compound on the intestinal expression of Na^+/D-glucose cotransporter (SGLT1) and brush-border membrane

disaccharidase activities. In mammal intestine, D-glucose and D-galactose enter enterocytes through the brush-border membrane, mainly via the Na^+-dependent, high-affinity, low-capacity SGLT1. In diabetic rats, up-regulation of SGLT1 increases the capacity of the intestine to absorb monosaccharides. Our results indicate that tungstate restores the activity of brush-border disaccharidases and the expression and activity of SGLT1 in rat jejunum. These effects limit the entry of sugars into the body, thereby contributing to the anti-diabetic action of tungstate (Miro-Queralt *et al*, 2008).

A second study done in collaboration with Rafael Salto's group (University of Granada) is an extension of joint work between our groups aimed to dissect the action of tungstate on glucose transport in muscle myotubes at the molecular level. Our results show that tungstate treatment enhances glucose uptake in myotubes through an increase in the total amount and translocation of GLUT4 transporter. The effects on glucose uptake were additive to those of insulin. Our results indicate that tungstate exerts its actions through an increase in the transcription of GLUT4 mediated by the myocyte enhancer factor-2 (MEF2). This transcriptional activation is dependent on one of the key molecular actions of tungstate, the activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2). This is the first study to report the activation of GLUT4 transcription by a glucose-lowering compound through an ERK1/2-dependent increase in MEF2 levels (Girón *et al*, 2008).

Study of the regulatory mechanisms of glycogen metabolism through the laforin-malin complex and the consequences of the accumulation of glycogen in neurons

Our group has discovered that neurons have the enzymatic machinery and the capacity to synthesise glycogen but not to degrade it (Vilchez *et al*, 2007). In addition, we have shown that glycogen accumulation in these cells is pro-apoptotic. Neurons keep the glycogen synthesising machinery inactive by a series of well coordinated intracellular mechanisms: (i) confinement of GS (the key enzyme for glycogen synthesis) in the nucleus; (ii) inactivation of GS by phosphorylation; and (iii) controlled degradation of GS and Protein Targeting to Glycogen (PTG), a regulatory subunit of protein phosphatase-1, by a novel regulatory mechanism involving a complex formed by two new players, laforin and malin, and the ubiquitin-proteasome system. Failure to keep GS under control, which results in glycogen synthesis, damages neurons by triggering apoptotic signalling. We are currently dissecting the apoptotic signalling cascade induced by the accumulation of glycogen in neurons and we have made several ultrastructural studies on the effects in neurons. In addition, we are currently generating animal models of gain- and loss-of-function of GS and the associated regulatory proteins in order to provide further insight into the physio-pathological implications of abnormal glycogen accumulation *in vivo*.

Our studies, in collaboration with the groups headed by Pascual Sanz (Instituto de Biomedicina de Valencia, CSIC) and Santiago Rodríguez de Córdoba (Centro de Investigaciones Biológicas, CSIC), have gone further into the characterisation of the mechanisms that regulate the laforin-malin complex. We have shown that the interaction between laforin and malin is a regulated process that is modulated by the AMP-activated protein kinase (AMPK). We provide evidence that the formation of the laforin-malin complex is positively regulated by AMPK. We show that laforin, but not malin, has the capacity to interact physically with the catalytic subunit of AMPK and that AMPK phosphorylates laforin. These data provide evidence of an additional function of AMPK in glycogen metabolism, where its activation is known to lead to an increase in the phosphorylation and inactivation of GS and also to an increase in glucose uptake (Solaz-Fuster *et al*, 2008).

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Ayudas para potenciar y dar soporte a los grupos de investigación
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR057 (2005-2008)
Principal investigator: Joan J Guinovart

Enfermedad de Lafora: papel de laforina y malina
'La Caixa' Foundation, BM06-340-02 (2007-2009)
Principal investigator: Joan J Guinovart

Estudio de las alteraciones en la homeostasis iónica e implicación de las proteínas G en el mecanismo de acción del agente antidiabético tungstato de sodio
Spanish Ministry of Science and Innovation, SAF2007-64722 (2007-2008)
Principal investigator: Joan J Guinovart

Estudio de un nuevo mecanismo de regulación del metabolismo del glucógeno. Análisis de las implicaciones patológicas de la acumulación anómala de polímeros de glucosa
Spanish Ministry of Science and Innovation, BFU2008-00769 (2009-2011)
Principal investigator: Joan J Guinovart

Mejora de la predicción traslacional de los ensayos de seguridad no clínica al hombre
Spanish Ministry of Science and Innovation, Noscira (former Neuropharma), Consorcio Melius, CENIT project (2007-2010)
Principal investigator: Joan J Guinovart

Molecular basis of progressive myoclonus epilepsy of the Lafora type
'MTV3' Foundation, 061930 (2007-2009)
Principal investigator: Joan J Guinovart

Nuevos fármacos y dianas para el tratamiento de la diabetes mellitus
'Marcelino Botín' Foundation (2006-2010)
Principal investigator: Joan J Guinovart

Regulación del metabolismo del glucógeno hepático, muscular y neuronal. Alteraciones en situaciones patológicas
Spanish Ministry of Science and Innovation, BFU2005-2253/BMC (2005-2008)
Principal investigator: Joan J Guinovart

Relación del síndrome diabético con la expresión y localización celular de la fructosa 1,6-Bifosfatasa y la glucógeno sintasa, enzimas claves en la homeostasis de la glucosa
Spanish Agency for International Cooperation, A/6647/06 (2007-2008)
Principal investigator: Joan J Guinovart

Collaborations

Analysis of the 3D structure of glycogen synthase
Joan C Ferrer, University of Barcelona (Barcelona, Spain)

Characterisation of glycogen metabolism in reproductive tissue: analysis of alterations in pathological situations
Joan E Rodríguez-Gil, Autonomous University of Barcelona (Barcelona, Spain)

Characterisation of the anti-diabetic and anti-obesity actions of tungstate
Ramon Gomis, IDIBAPS-Hospital Clínic (Barcelona, Spain)

Determination of the 3D structure of the glycogen synthases
Ignasi Fita, IRB Barcelona (Barcelona, Spain)

Glycogen-induced dysfunctions in pancreas and retina and their involvement in the ethiogenesis of diabetes mellitus
Ramon Gomis, IDIBAPS-Hospital Clínic (Barcelona, Spain) and Rafael Simó, Institut de Recerca Hospital Vall d'Hebrón (Barcelona, Spain)

Histological analysis of the alterations in the neuronal glycogen metabolism in neurological diseases
Teresa Ribalta, Hospital Clínic (Barcelona, Spain)

In silico design of modulators of the glycogen synthase activity
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Laser-induced forward transfer: a direct writing technique for biosensors preparation
José L Morenza, University of Barcelona (Barcelona, Spain)

Mechanism of action of anti-hyperglycaemic compounds and development of in vitro methods for screening their mode of action
Loranne Agius, School of Clinical Medical Sciences-Diabetes, The Medical School (Newcastle upon Tyne, UK)

Molecular basis of Lafora disease
Santiago Rodríguez de Córdoba, Centro de Investigaciones Biológicas-CSIC (Madrid, Spain) and Pascual Sanz, Institute of Biomedicine of Valencia-CSIC (Valencia, Spain)

Molecular dissection of the mechanisms of action of the anti-diabetic agent sodium tungstate in skeletal muscle
Rafael Salto and Mª Dolores Girón, University of Granada (Granada, Spain)

Study of hypoxia and glycogen accumulation
Luis del Peso, Instituto de Investigaciones Biomédicas-CSIC (Madrid, Spain)

Study of the actions of sodium tungstate on the ionic homeostasis
Miguel A Valverde, Pompeu Fabra University (Barcelona, Spain)

Study of the alterations in glycogen metabolism associated with colon cancer
Santiago Ramón y Cajal, Institut de Recerca Hospital Vall d'Hebrón (Barcelona, Spain)

Study of the alterations of glycogen metabolism in animal models with neurological diseases
Martí Pumarola, Autonomous University of Barcelona (Barcelona, Spain)

Study of the molecular targets and biological actions of sodium tungstate
José Ramón Murguia, Universidad Politécnica de Valencia (Valencia, Spain)

Study of the proteomic alterations induced by tungstate treatment of diabetic animals
Carmen Cámara, Universidad Complutense de Madrid (Madrid, Spain)

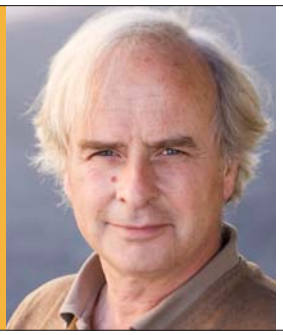
The use of Drosophila melanogaster as a model system for the study of Lafora disease
Marco Milán, IRB Barcelona (Barcelona, Spain)

Awards and honours

Josep Trueta Prize for the best research manuscript, Academy of Medical and Health Sciences of Catalonia and the Balearic islands (2008)
Awardee: Joan J Guinovart

Young Scientific Award (accessit), Promega Biotech Ibérica, XXXI Congress of the Spanish Society for Biochemistry and Molecular Biology (2008)
Awardee: David Vílchez

MANUEL PALACÍN



Amino acid transporters: biochemistry, physiopathology, genetics and structural biology

Our research efforts focus on the molecular bases of renal reabsorption of amino acids, the physiopathology of the inherited aminoacidurias cystinuria and lysinuric protein intolerance (LPI), the structure-function relationship in heteromeric amino acid transporters (HATs), and the study of the multiple functions of heavy chains of HATs. With regards to the molecular bases of renal reabsorption of amino acids, we address the generation and characterisation of mutated mouse models of renal amino acid transporters. In the physiopathology of inherited aminoacidurias, our goals are the following: (i) to develop animal models to study the impact of several renal amino acid transporters on cystinuria, (ii) to identify mechanisms of pathology in this inherited disorder, (iii) to search for new drugs for the treatment of lithiasis in cystinuria, and (iv) to generate and characterise a mouse model for LPI. Finally, our group works towards developing the three-dimensional (3D) structure of HATs, using both human transporters and prokaryotic homologues.

The molecular bases of renal reabsorption of amino acids

Our laboratory has identified and characterised three amino acid transporters involved in the renal reabsorption of amino acids: systems $b^{0,+}$ (heterodimer rBAT-$b^{0,+}$AT), y^+L (heterodimer 4F2hc-y^+LAT1) and exchanger L (heterodimer 4F2hc-LAT2; Figure 1). We have also demonstrated the role of systems $b^{0,+}$ and y^+L in cystinuria and LPI. This has allowed us to propose a mechanism of reabsorption in which these amino acid exchangers participate. This model requires basolateral transporters with a net flux of neutral amino acids. The search for these transporters is done mainly with functional studies of orphan transporters within the described amino acid transporter families. Characterisation of mutated mouse models of LAT2 and EEG1 might shed light on this issue. Moreover, in collaboration with Paolo Gasparini, we are studying whether there is an association between amino acid transporter polymorphisms and renal reabsorption of amino acids in genetically isolated human populations. In this regard, we have identified groups of amino acids with co-variation in urinary excretion. This activity was initiated within the European Union project EUGINDAT (European Union Genomic Initiative on Disorders of Amino Acid Transporters).

Physiopathology of inherited aminoacidurias cystinuria and lysinuric protein intolerance (LPI)

Our laboratory has identified the genes involved in cystinuria (system $b^{0,+}$; heterodimer rBAT-$b^{0,+}$AT) and LPI (system y^+L; heterodimer 4F2hc-y^+LAT1), and within the International Cystinuria

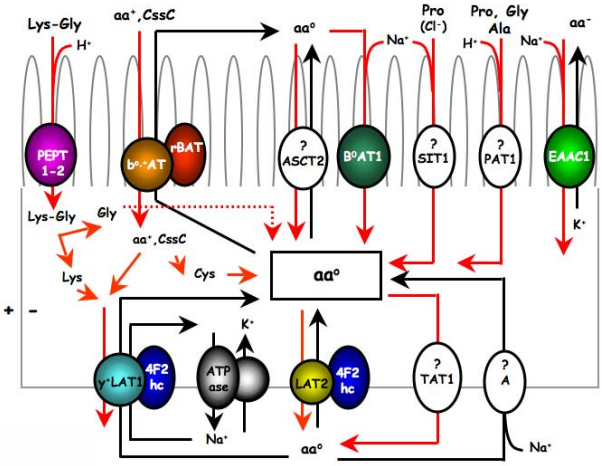


***Figure 1**. Proximal tubule model for amino acid transporters involved in renal and intestinal reabsorption of amino acids. Transporters with a proven role in renal reabsorption or intestinal absorption of amino acids are coloured, whereas those expressed in the plasma membrane of epithelial cells of the proximal convoluted tubule (or of the small intestine) but with no direct experimental evidence supporting their role in reabsorption are shown in white. Amino acid fluxes in the reabsorption direction are in red. PEPT1 and PEPT2 are expressed in the small intestine and kidney respectively. Adapted from Moe et al, 2008.*

Principal Investigator Manuel Palacín **Research Associate** José Luis Vázquez **Postdoctoral Fellows** Paola Bartoccioni, Susana Bodoy, Joana Fort, Lukasz Kowalczyk, Lorena Martin, Albert Rossell, Eva Maria Valencia **PhD Students** Meritxell Costa, Gonzalo Delgado, Mercè Ratera, Laura Rodríguez **Lab Technician** Susanna Bial **Lab Manager** Miriam Alloza **Project Manager** Judith Arrazola

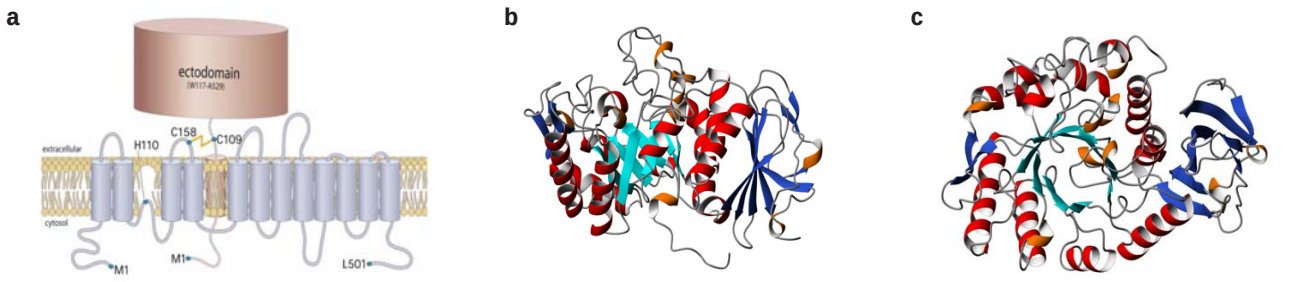


Figure 2. *Structure of 4F2hc-ED. A, HAT schematic representation. 4F2hc (pink) with a bulky N-glycosylated ectodomain (4F2hc-ED, covering residues Trp117 to the C-terminal Ala529) is linked by a conserved disulfide bridge (Cys109 in human 4F2hc) with a light subunit (blue), a 12 trans-membrane-spanning non-glycosylated protein. Lateral (B) and upper (C) views of 4F2hc-ED structure. The N-terminal position corresponds to Cys109. The structure is similar to that of* α*-glycosidases, including two domains: a TIM-barrel (/)8 and a C-terminal domain with eight antiparallel* β*-sheets. Adapted from Fort* et al, *J Biol Chem,* ***282****, 31444-52 (2007).*

Consortium, which we founded, we have identified most of the mutations causing these diseases. We have established a wide genotype-phenotype correlation in cystinuria that has allowed us to propose a new classification of the disease: type A, caused by *SLC3A1* mutations, and type B, caused by *SLC7A9* mutations. The objectives that we are currently pursuing are the following: (i) the identification of molecular mechanisms to explain the distinct phenotypes in cystinuria, using animal and cell models; (ii) the identification of modulator genes of lithiasis in cystinuria, using animal models; (iii) the search for new drugs to treat lithiasis in cystinuria, using our murine cystinuria model *Stones*; and (iv) the identification of the mechanisms that lead to immunological disorders associated with LPI, using a newly generated floxed y^+LAT1 mouse line.

Structure-function relationship in heteromeric amino acid transporters (HATs)

Our laboratory has identified most of the members of the HATs. Moreover, we have approached the structure-function relationships of HATs by defining: the oligomeric state of HATs, the atomic structure of the ectodomain of 4F2hc (CD98hc; in collaboration with IRB Barcelona researcher Ignasi Fita; Figure 2), the light subunit as the catalytic component, the membrane topology of the light subunits, and the key residues for transport. Recently, in collaboration with Dimitrios Fotiadis (EUGINDAT project), we obtained the first projection map of a prokaryotic homologue of the light subunits of HATs at a subnanometer scale (6.5Å). This map revealed striking similarities with unrelated transporters with the so-called 'double inverted repeat' fold (Figure 3). At present, we are conducting 3D crystallisation screenings of several transporter homologues of the light subunits of HATs (APC superfamily) within the European Union project EDICT (European Drug Initiative on Channels and Transporters). Functional studies in parallel seek to identify key residues for amino acid transport function within HATs.

Study of the multiple functions of heavy chains of HATs

One of the heavy subunits of HATs identified, 4F2hc (CD98), is involved in many cellular functions, such as cellular transformation, adhesion and fusion. Very recently, we have developed the 3D structure of the extracellular domain of 4F2hc (PDB 1Y4N and 1Y5Z). This has allowed us to study the role of the extracellular domain of 4F2hc in its multiple functions, including interaction with β1 integrins. Moreover, the recombinant extracellular domain of 4F2hc is a powerful tool for the identification of potential ligands of 4F2hc.

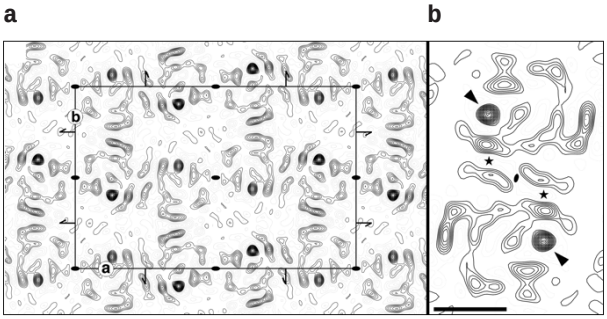


Figure 3. *Projection structure of AdiC-W293L. A, p22121-symmetrised projection map of AdiC-W293L at 6.5Å resolution calculated from five electron micrographs. The black rectangle marks the unit cell (lattice dimensions: a=184Å, b=119Å, γ=90°), which contains four AdiC-W293L dimers (two up- and two down-oriented dimers). B, improved projection map of AdiC-W293L after symmetrisation of one of the four identical dimers in the unit cell exploiting the internal, non-crystallographic 2-fold symmetry axis of the dimer. The only strong density peak in the projection structure of the AdiC-W293L monomers is marked by arrowheads. This projection map shows similarities to the amino acid transporter LeuT (Yamashita et al, Nature, 437, 215-23, 2005), one of the structural paradigms for transporters with the "double inverted repeat" fold. The putative intradimeric contact sites are indicated by stars. The 2-fold axes perpendicular to the membrane plane and the screw axes parallel to the membrane plane are indicated. Solid lines indicate density above the mean, whereas negative contours are shown as light grey lines. The scale bar represents 25Å. Figure adapted from Casagrande et al, 2008.*

SCIENTIFIC OUTPUT

Publications

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Duarri A, Teijido O, López-Hernández T, Scheper GC, Barriere H, Boor I, Aguado F, Zorzano A, Palacín M, Martínez A, Lukacs GL, van der Knaap MS, Nunes V and Estévez R. Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in MLC1 cause folding defects. *Hum Mol Genet*, **17**(23), 3728-39 (2008)

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Research networks and grants

CIBER de enfermedades raras (CIBERER)
Instituto de Salud Carlos III (2007-2010)
Principal investigator Unit 731: Manuel Palacín

European Drug Initiative on Channels and Transporters (EDICT)
European Commission, 7th Framework Programme, 201924 (2008-2012)
Principal investigator: Manuel Palacín

Random approach to build a thermostable polytopic membrane protein for crystallisation
Spanish Ministry of Science and Innovation, BFU2008-04637 (2008-2012)
Principal investigator: José Luis Vázquez-Ibar

Role of 4F2hc in tumorogenesis
'La MTV3' Foundation (2006-2009)
Principal investigator: Manuel Palacín

Transportadores heteroméricos de aminoácidos: estructura, genómica funcional y fisiopatología (cistinuria y lisinuria con intolerancia a proteínas)
Spanish Ministry of Science and Innovation, BFU2006-14600-C02-01 (2006-2009)
Principal investigator: Manuel Palacín

Other funding sources

Support to incentive research activity in the University of Barcelona (2007-2010)

Collaborations

Physiopathology of inherited aminoacidurias cystinuria and lysinuric protein intolerance (LPI)
Virginia Nunes, Bellvitge Institute for Biomedical Research and University of Barcelona (Barcelona, Spain), Gianfranco Sebastio, Federico II University (Naples, Italy) and Josep Chillarón, University of Barcelona (Barcelona, Spain)

Study of the multiple functions of heavy chains of HATs
María Antonia Lizarbe, Universidad Complutense de Madrid (Madrid, Spain), Joaquín Abian, CSIC-Autonomous University of Barcelona (Barcelona, Spain) and Mark H Ginsberg, University of California San Diego (La Jolla, USA)

Structure-function relationship in heteromeric amino acid transporters (HATs)
Dimitrios Fotiadis, University of Bern (Bern, Switzerland), Ignasi Fita, IRB Barcelona (Barcelona, Spain), Modesto Orozco, IRB Barcelona (Barcelona, Spain), Steve Baldwin, Astbury Centre for Structural Molecular Biology, Institute of Membrane and Systems Biology, University of Leeds (Leeds, UK) and Matthias Quick, Weill Medical College, Cornell University (New York, USA)

The molecular bases of renal reabsorption of amino acids
Virginia Nunes, Bellvitge Institute for Biomedical Research and University of Barcelona (Barcelona, Spain), Paolo Gasparini, Institute for Maternal and Child Health IRCCS-Burlo Garofolo (Trieste, Italy)

ANTONIO ZORZANO

Mitochondrial metabolism and insulin resistance

It has been estimated that between 200 and 300 million people worldwide will meet World Health Organisation diagnostic criteria for diabetes mellitus by the end of this decade. This epidemic of predominantly type 2 diabetes has been mediated largely by our shift toward a more sedentary lifestyle, which predisposes us to obesity and insulin resistance. Individuals affected by type 2 diabetes may also exhibit an array of associated undesirable effects, such as hypertension, dyslipidemia and hypercoagulability, which lead to morbidity and mortality from atherosclerotic vascular disease. The co-existence of several of these disorders with insulin resistance constitutes the metabolic syndrome. In Western society, metabolic syndrome diseases are growing at epidemic rates and currently affect approximately 20% of the general population and more than 40% of people over 60 years of age. Recent epidemiological and biological data suggest that the etiology of these diseases shares unexpected and common genetic and biochemical mechanisms.

Insulin resistance is strongly associated with obesity, and several mechanisms mediating this interaction have been identified. A number of circulating hormones, adipocytokines (leptin, adiponectin, resistin, plasminogen activator inhibitor-1, interleukin-6, TNFα, and retinol-binding protein 4), and metabolic fuels, such as non-esterified fatty acids (NEFA), originate in the adipocyte and modulate insulin action. An increased mass of stored triglyceride, especially in visceral or deep subcutaneous adipose depots, leads to large adipocytes that are themselves resistant to the capacity of insulin to suppress lipolysis. This resistance results in increased release and circulating levels of NEFA and glycerol, both of which aggravate insulin resistance in skeletal muscle and liver. When chronic, the increased circulating NEFA and other lipids that occur in obesity lead to ectopic fat storage as triglycerides in muscle and liver. Ectopic lipid accumulation has been implicated in insulin resistance, possibly as a result of triglyceride turnover and the production of fatty acid-derived signalling molecules, or of the activation of deleterious intracellular pathways.

There is growing evidence indicating the existence of cross-talk between mitochondrial function and insulin signalling that may be relevant for the pathogenesis of insulin resistance and the disorders characteristic of the metabolic syndrome. Thus, it has been reported that insulin resistance and diabetes are associated with decreased mitochondrial oxidative capacity and at present, there are several lines of evidence supporting the view that alterations in mitochondrial function cause insulin resistance. Experimental evidence linking deficient mitochondrial activity and insulin resistance comes from studies in animals and in cultured cells. Thus, artificial selection of rats on the basis of low intrinsic exercise capacity for 11 generations has been associated with a high risk of cardiovascular disease or the metabolic syndrome. The decrease in aerobic capacity was associated with a reduction in the amount of oxidative enzymes in skeletal muscle. Studies in knockout mice have also demonstrated that mitochondrial dysfunction caused by long-chain Acyl-CoA dehydrogenase deficiency produces hepatic steatosis and hepatic insulin resistance. Studies in muscle cells in culture have also shown that mitochondrial dysfunction induces aberrant insulin signalling and deficient glucose utilisation. In addition, clinical observations indicate that healthy elderly people have a marked tendency toward insulin resistance, and this resistance is associated with reduced mitochondrial oxidative phosphorylation activity compared with body mass index and activity-matched young individuals. Similarly, insulin-resistant offspring of parents with type 2 diabetes also have impaired mitochondrial function, with mitochondrial adenosine triphosphate (ATP) synthesis being reduced by approximately 30%. These reductions in mitochondrial function were found to be associated with severe muscle insulin resistance and an 80% increase in intramyocellular lipid content. Because these individuals had no abnormalities of systemic or localised rates of lipolysis or plasma concentrations of tumour necrosis factor-α, interleukin-6, resistin, or adiponectin, it is likely that the genetic factor that explains the heritability of type 2 diabetes is connected with the loss of mitochondrial activity in these individuals.

Several mechanisms might contribute to the reduction of mitochondrial activity in insulin-resistant conditions, namely

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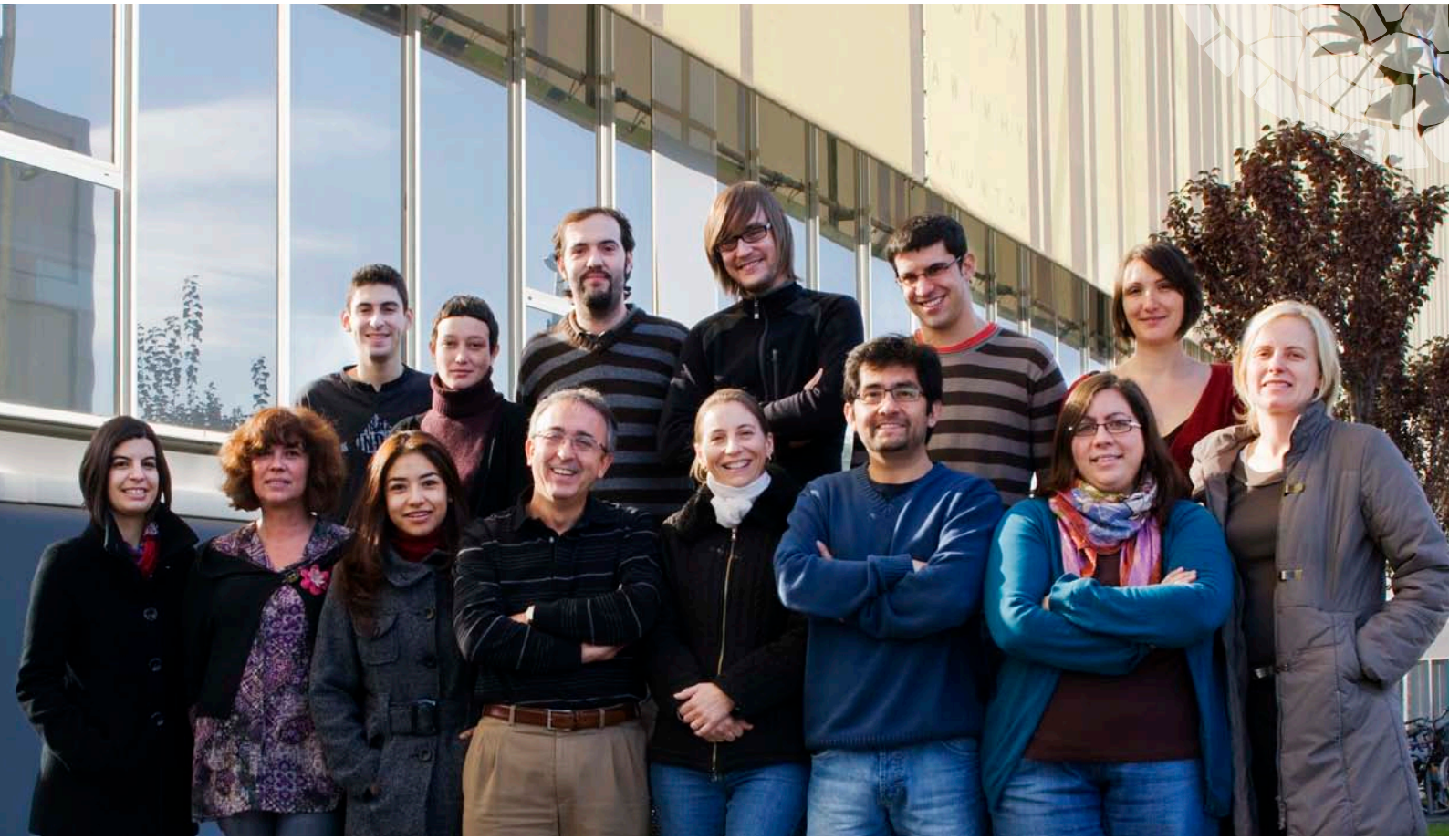

changes in mitochondrial density or in mitochondrial function. As to the regulation of mitochondrial density, key nuclear coregulators that are known to control mitochondrial biogenesis include PPARγ coactivator 1α (PGC-1α) and PGC-1β. Thus, PGC-1α and PGC-1β gene expression were recently found to be decreased in muscle of patients with type 2 diabetes and in non-diabetic subjects with a family history of type 2 diabetes. PGC-1α, like PGC-1β, induces the expression of genes involved in oxidative phosphorylation in both muscle and liver cells.

Mitochondrial dynamics and mitochondrial metabolism

Mitochondria are dynamic organelles whose morphology is regulated by fusion and fission processes. A growing body of evidence shows the relevance of these shaping processes in the control of mitochondrial activity and cell metabolism. Several genes encoding mitochondrial fusion and fission proteins have been recently identified. Mammalian proteins involved in mitochondrial fission are Fission 1 homologue protein (Fis1) and Dynamin-related protein 1 (Drp1). Similarly, Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic Atrophy gene 1 (OPA1) are proteins that participate in mitochondrial fusion in mammals. However, there is no evidence to date that demonstrates the capacity of an upstream or transcriptional regulator to shift the balance between mitochondrial fusion and fission events by selective regulation of these proteins.

Several reports provide evidence that Mfn2 protein elicits pleiotropic effects which may be involved in pathology. For instance, Mfn2 is mutated in Charcot Marie Tooth type 2A neuropathy and, interestingly, some of these mutants cause selective defects in mitochondrial fusion, a reduction in mitochondrial axonal transport or defects in mitochondrial coupling, thereby leading to inefficient mitochondria. Defective Mfn2 may also contribute to

impaired mitochondrial function in the context of obesity and type 2 diabetes. This notion is supported by the observation that muscle Mfn2 expression is reduced in patients with these conditions. In addition, we have previously reported that Mfn2 modulates mitochondrial activity through changes in the electron transport chain (ETC) and that this modulation is independent of its role in mitochondrial morphology.

Peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) α and β are important positive regulators of mitochondrial activity and biogenesis in mouse skeletal muscle. Despite these similarities, PGC-1α and PGC-1β display low overall sequence identity, with the highest percentages found in two particular domains (activation and RNA recognition domains, with identities of 40% and 50% respectively). Furthermore, key mitochondrial processes, such as organelle biogenesis and uncoupling, are differentially regulated by these homologues. For instance, in C2C12 muscle cells, PGC-1α, but not PGC-1β, increases mitochondrial uncoupling, whereas PGC-1β causes a larger increase in mitochondrial volume than PGC-1α under the same conditions. In addition, while PGC-1β expression in distinct tissues is unaffected by physiological processes characterised by increased energy expenditure, such as cold exposure (in brown adipose tissue), fasting (in liver) or exercise (in muscle), PGC-1α is highly regulated at the transcriptional level under similar physiological challenges. These data suggest that PGC-1β regulates basal mitochondrial biogenesis, whereas PGC-1α is involved in regulated mitochondrial activity. In keeping with this view, expression of PGC-1β is higher than that of PGC-1α in primary muscle cells under basal conditions.

The functional independency of these homologues in mitochondrial physiology is further illustrated by the phenotypes of PGC-1α and PGC-1β knockout (KO) mice. In both animal models, a general defect in the ETC system has been described, thereby demonstrating that PGC-1α does not fully compensate the effects of PGC-1β on mitochondria or vice versa. Furthermore, several mitochondrial phenotypes described in the particular case of PGC-1β-ablated mice cannot be completely explained by impairment of the ETC system. For instance, muscle and liver from PGC-1β KO mice show a reduction of mitochondrial volume without changes in mitochondria number. This decreased mitochondrial volume together with impaired ETC gene expression may explain the mitochondrial respiration defect found only in muscle strips and not in isolated mitochondria. In keeping with these data, this reduction in mitochondrial size is absent in PGC-1α KO mice under basal conditions, probably because of normal PGC-1β expression. Of note, despite all these differences, both genes show a diminished expression in the context of type 2 diabetes, thereby suggesting an impairment of mitochondrial effects selectively regulated by each homologue in this disease.

We previously reported that PGC-1α induces Mfn2 transcription and that mitochondrial activity regulated by PGC-1βα partly depends on correct Mfn2 expression. However, effects on mitochondrial fusion were not determined. In the light of these results and the PGC-1β control of basal mitochondrial biogenesis, we have studied whether PGC-1β regulates Mfn2 transcription and, therefore, whether the mitochondrial dynamics balance can be modulated by transcriptional regulation.

PGC-1β induces Mfn2 transcription through ERRα coactivation

We have examined whether PGC-1β regulates the expression of Mfn2 in C2C12 muscle cells. Differentiated C2C12 cells show low levels of PGC-1β mRNA, as assessed by Northern blot and by real-time PCR. To this end, C2C12 myotubes were transduced either with a mouse PGC-1β adenovirus or with a control LacZ adenovirus. Mfn2 mRNA levels doubled in PGC-1β-expressing muscle cells compared to control transduced myotubes. To demonstrate that PGC-1β directly increases Mfn2 transcription, we transfected 10T1/2 mouse fibroblasts or HeLa cells with a construct containing a 2-kb fragment (-1982/+45) of the Mfn2 promoter fused to a luciferase reporter gene, together with an irrelevant vector (Basal) or mouse PGC-1β expression vector. PGC-1β markedly enhanced Mfn2 promoter activity (10.3±0.9- and 4.2±0.6-fold over basal Mfn2 promoter activity in 10T1/2 and HeLa respectively). In a previous study, using electrophoretic mobility shift and chromatin immunoprecipitation assays, we showed that ERRα binds to the Mfn2 promoter between nucleotides -459/-396. This DNA region contains three putative boxes with the capacity to bind nuclear receptors, where box 2 is critical for Mfn2 promoter response to PGC-1α coactivation of ERRα. On the basis of these observations, we determined whether PGC-1β coactivated ERRα through box 2 in a similar way as PGC-1α. We transfected 10T1/2 cells with a construct containing a -459/-352 Mfn2 promoter fragment fused to a luciferase reporter gene or with a mutated version of the same fragment that disrupted box 2. We observed

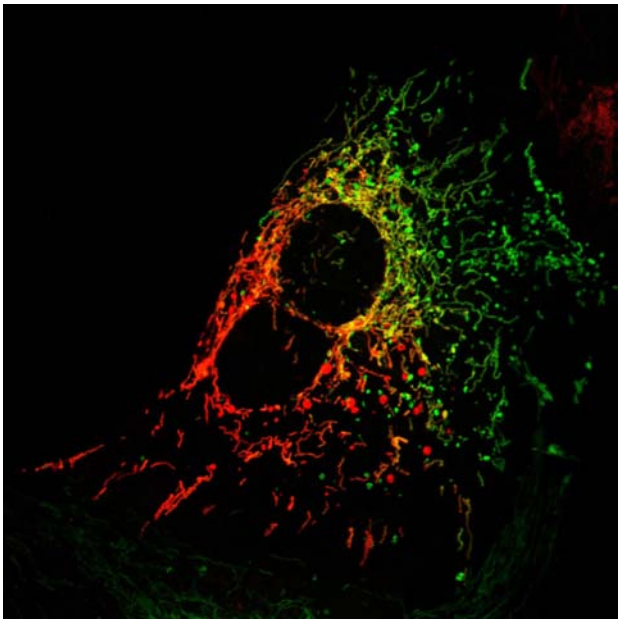

Figure 1. *High rate of mitochondrial fusion in myoblasts after overexpression of PGC-1β. Yellow indicates fusion of green- and red-labelled mitochondria (mitochondrial matrix-targeted green fluorescent protein and red fluorescent protein).*

marked coactivation of ERRα by PGC-1β in the -459/-352 Mfn2 promoter fragment. This effect was completely blunted when box 2 was disrupted (11.9±1.09 vs. 2.8±0.6-fold over basal promoter activity, p=0.001, Figure 1C). Furthermore, cancellation of box 2 markedly reduced the activation driven by PGC-1β (7.0±0.5 vs. 1.9±0.5, p=0.001) or by ERRα (2.2±0.1 vs. 1.6±0.2, p=0.04), although residual activation was still present. Similar results were obtained when the 2- kb Mfn2 promoter was cotransfected with ERRα or PGC-1β.

PGC-1β causes a large induction of Mfn2 protein levels

To determine whether PGC-1β-mediated Mfn2 transcription leads to enhanced Mfn2 protein expression, we transduced C2C12 myotubes with PGC-1β or two distinct control adenoviruses at a range of multiplicities of infection (MOI). Total protein extracts and mitochondrial-enriched fractions were obtained and analysed by Western blot. PGC-1β induced Mfn2 in muscle cells and a direct relationship between PGC-1β adenoviral dose and Mfn2 protein induction was detected. PGC-1β also increased the cellular content of the constitutive mitochondrial protein Porin, used as a measure of mitochondrial mass. Densitometric quantification of Porin induction at MOI 100 showed a 1.8±0.2-fold increase in total lysates and a 1.45±0.003-fold increase in mitochondrial-enriched fractions. Porin induction values in total lysates are consistent with the increase in mitochondrial mass volume reported in PGC-1β-overexpressing C2C12 muscle cells.

On the basis of the effects of PGC-1β on Mfn2 and Porin expression, we also analysed whether this coactivator regulates the expression of other proteins involved in mitochondrial dynamics and in the ETC system. PGC-1β-transduced myotubes induced the expression of Mfn1, OPA1, Drp1 and Fis1 in mitochondrial-enriched extracts (1.7-, 2.2-, 1.4-, or 2.1-fold over basal values, respectively) to a level similar to that detected for Porin (1.45-fold stimulation). Mfn2 displayed a significantly higher increase in expression compared to Porin abundance in mitochondrial-enriched extracts (4.3-fold induction). When data were expressed as protein levels relative to Porin expression, we detected significant stimulation of Mfn2 and Fis1 only in response to PGC-1β overexpression. This superior induction of Mfn2 protein was also detected in C2C12 myoblasts.

We also analysed several subunits of the ETC system in mitochondrial-enriched fractions from C2C12 myotubes transduced at MOI 100. All the subunits of complexes I, II, III, IV and V studied were induced in response to PGC-1β, and the extent of induction was similar to that detected in Porin levels (ranging from 1.3- to 2.1-fold increase).

PGC-1β changes mitochondrial morphology and increases the rate of mitochondrial fusion

As PGC-1β induces Mfn2 expression, we next studied whether this effect was linked to changes in mitochondrial morphology. To this end, we immunofluorescently labelled mitochondria from C2C12 myoblasts transduced with PGC-1β or LacZ adenovirus. PGC-1β caused an increase in the length of mitochondrial tubules in most myoblasts. This increased mitochondrial size was also observed by transmission electron microscopy, which also showed normal cristae morphology. To demonstrate that this increase in mitochondrial length was linked to enhanced mitochondrial fusion, we performed a polyethylene glycol-(PEG)-mediated cell fusion assay using two distinct C2C12 lines stably expressing mitochondrial matrix-targeted GFP (mtGFP) or red fluorescent protein (mtRFP). Four hours after PEG addition and in the presence of cycloheximide, a significantly higher percentage (73% vs. 55% in PGC-1β and control cells, respectively) of polykaryons from C2C12 cells transduced with PGC-1β showed a higher level of mitochondrial matrix content mixing than control LacZ polykaryons (that is to say mtGFP and mtRFP exchange caused by mitochondrial fusion and displayed as yellow mitochondria) (see Figure 1 corresponding to cells after overexpression of PGC-1β). This higher mtGFP and mtRFP mixing indicated an increase in the rate of mitochondrial fusion induced by PGC-1β overexpression. In agreement with previous data, the overexpression of PGC-1β in C2C12 myoblasts under these conditions also increased the mitochondrial membrane potential values by ~50%, as measured by using the fluorescent probe JC-1.

Mfn2 is required for PGC-1β-induced changes in mitochondrial morphology

To study whether the change in mitochondrial morphology induced by PGC-1β was mediated mainly through Mfn2 activity, we used mouse embryonic fibroblasts (MEFs) from wild-type and from Mfn2 or Mfn1 KO mice. Overexpression of PGC-1β led to an increase in mitochondrial length of wild-type and Mfn1 KO MEFs, similarly to what was observed in C2C12 myoblasts. Importantly, PGC-1β gain-of-function was unable to promote mitochondrial elongation in Mfn2 KO MEFs, in conditions in which ETC subunits Cox4 and Uqcrc2 were increased 1.6- and 1.5-fold respectively. These data demonstrate the requirement of Mfn2 expression for PGC-1β-mediated changes in mitochondrial morphology.

PGC-1β KO mice show Mfn2 repression in skeletal muscle and myocardium

We next studied the effects of *in vivo* ablation of PGC-1β on Mfn2 expression in gastrocnemius muscles. Mfn2 protein levels were reduced by approximately 50% in KO mice. The reduction of Mfn2 levels was relatively specific as indicated by the absence of major changes in proteins involved in mitochondrial dynamics, *ie*, Mfn1, OPA1, Drp1 and Fis1. Similar results were obtained using mitochondrial fractions. Reduced Mfn2 protein expression paralleled lower levels of Mfn2 mRNA.

Soleus muscle from PGC-1β KO mice also showed a decrease in complex IV (subunits Cox4 and Cox5b) mRNA levels. This observation led us to study whether the protein levels of Cox4 and other subunits of complexes I, II, III and V were also altered in gastrocnemius muscle of this mouse model. These mice displayed a ~40% reduction in complex V (ATP5a1) and III (Uqcrc2). Complex IV (Cox4) showed a ~25% reduction and complex I (Ndufa9) a ~20% decrease while no changes were observed in complex II protein levels (Sdha).

The heart is one of the organs with the highest expression of PGC-1β. Interestingly, heart lysates from KO mice displayed

a clear reduction in Mfn2 protein levels (WT 1.00±0.06, KO 0.57±0.09, p=0.0015; data not shown), thereby suggesting the impairment of this regulatory pathway also in cardiac muscle.

These studies demonstrate that mitochondrial dynamics balance can be shifted towards fusion by transcriptional regulation. More specifically, we show that PGC-1β is a regulator of mitochondrial fusion through its effects of selectively promoting Mfn2 expression upon coactivation of ERRα. This new role of PGC-1β in mitochondrial physiology has been demonstrated using both *in vitro* (muscle cells) and *in vivo* (PGC-1β-ablated mice) approaches. Firstly, we have shown that PGC-1β overexpression in muscle cells regulates mitochondrial dynamics through a mechanism that involves the preferential induction of Mfn2 expression, among other mitochondrial dynamics effectors such as Mfn1, OPA1, Drp1 and Fis1. Furthermore, PGC-1β gain-of-function results in an elongation of mitochondrial tubules, which is linked to increased mitochondrial fusion. Importantly, the effects of PGC-1β on the promotion of mitochondrial elongation are not observed in Mfn2-ablated cells, thereby demonstrating that Mfn2 activity is essential for PGC-1β-mediated changes in mitochondrial dynamics.

In summary, we provide evidence that the mitochondrial dynamics balance is selectively controlled by a transcriptional regulator, thereby unravelling an upstream mediator of mitochondrial fusion. Furthermore, we also provide evidence of a novel role of PGC-1β in mitochondrial physiology. Given the cross-talk between mitochondrial activity and dynamics, together with reduced Mfn2 and PGC-1β expression in type 2 diabetes, we conclude that the pathway reported here is not only relevant for the thorough explanation of mitochondrial dynamics regulation and the overall mitochondrial effects of PGC-1β but may also provide the basis for the understanding of the alterations of mitochondrial metabolism associated with type 2 diabetes.

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Adipose tissue: a key target for prevention of the metabolic syndrome
European Science Foundation, COST Action BM0602 (2007-2011)
Principal investigator/Member of the management committee: Antonio Zorzano

Ajuts grups reconeguts Generalitat
Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00947 (2005-2008)
Principal investigator: Antonio Zorzano

CIBERDEM-CIBER de Diabetes y Enfermedades Metabólicas Asociadas
Instituto de Salud Carlos III (2007-2011)
Principal investigator/Director of scientific training: Antonio Zorzano

Functional analysis of novel candidate genes of insulin resistance
Spanish Ministry of Science and Innovation, SAF2005-0445 (2005-2008)
Principal investigator: Antonio Zorzano

MITIN-Integration of the system models of mitochondrial function and insulin signalling and its application in the study of complex diseases
European Commission, 7th Framework Programme, FP7-HEALTH-2007-B, 223450 (2008-2011)
Principal investigator: Antonio Zorzano

Collaborations

Early-onset type 2 diabetes and mitochondrial function
John Nolan, St James Hospital, Trinity College Dublin (Dublin, Ireland)

Expression of genes in human adipose tissue
Joan Vendrell, Hospital Joan XXIII (Tarragona, Spain)

Extramitochondrial functions of mitofusin-2
Luca Scorrano, Venetian Institute of Molecular Medicine (Padova, Italy)

Functional analysis of adipose cell proteins
José Manuel Fernández-Real, Trueta Hospital (Girona, Spain)

Mitochondrial dynamics in cardiac cells
Sergio Lavandero, University of Chile (Santiago, Chile)

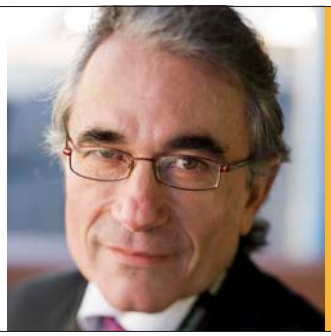
Mitochondrial fusion in muscle cells
Manuel Rojo, Institut de Biochimie et Génétique Cellulaires-IBGC, (Bordeaux, France)

PGC-1β and mitochondrial dynamics
Antonio Vidal-Puig, Cambridge University (Cambridge, UK)

Type 2 diabetes in morbid obesity and mitochondrial function
Geltrude Mingrone, Catholic University, School of Medicine (Rome, Italy)

Chemistry and Molecular Pharmacology Programme

Medicinal chemistry



Using a robust chemical platform, our main goal is to identify compounds, mainly natural products or analogues, with biological/therapeutic activity (drug discovery) and to facilitate their reaching the target (drug delivery systems). Our background in chemistry is complemented by biology and pharmacology, and our research involves the use of techniques such as solid-phase synthesis, combinatorial chemistry and cell biology approaches, and nanotechnologies.

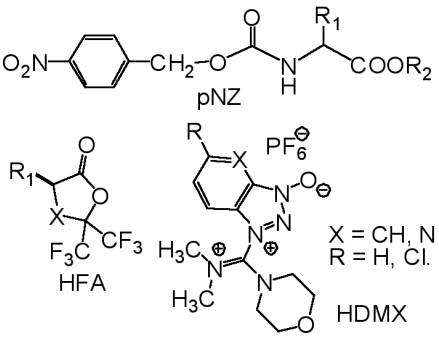


Figure 1. *Structures of amino-protecting group pNZ, bidentate protecting group for a-functionalised carboxylic acids HFA, and new coupling reagent HDMX.*

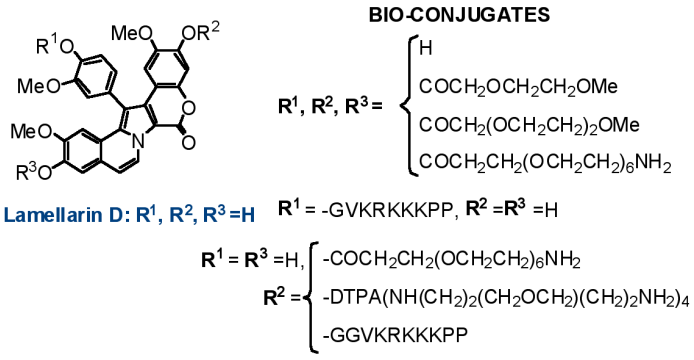


Figure 2. *Structures of Lamellarin D bioconjugates.*

Methodology

ChemMatrix, a superior resin. Our efforts in the development of this resin have allowed us to achieve the best solid support for peptide synthesis. As an example, ChemMatrix has allowed the stepwise synthesis of a Chemokine, a 66-amino acid protein, and industrial processes for T-20 and Zadaxin. Furthermore, we are currently developing a combinatorial approach based on this resin for the purification of proteins.

HFA chemistry, coupling reagents, protecting groups. The bidentate protecting/activating HFA reagent for α-functionalised carboxylic acids has facilitated the preparation of new topological peptides (see siamese peptides) as well as access to relevant building blocks for our research. This chemistry has allowed the study of the effects of amide replacement by esters on protein stability. The development of protecting groups, such as pNZ, which is removed in very mild conditions, as well as a coupling reagent, such as HDMX, has allowed us to prepare complex peptides, such as oxathiocoraline (Figure 1).

Synthesis of natural products. Medicinal chemistry programmes. Lamellarins form a family of more than forty members of marine alkaloids that show relevant bioactivity. Lamellarin D is a cytotoxic agent against various tumour cells, an inhibitor of topoisomerase I, and a potent pro-apoptotic agent.

We recently described the total synthesis of Lamellarin D, the preparation of a library of more than forty analogues and structure-activity relationship (SAR) studies. The preparation of PEG-conjugates to improve the solubility of this compound, studies on cell penetration and the mechanism by which Lamellarin D induces apoptosis have been recently sent for publication. We have also addressed a second generation of bio-conjugates with a nuclear localisation signal peptide and a poly(ethylene glycol)-based dendrimer (Figure 2).

Principal Investigator Fernando Albericio **Research Associate** Mercedes Álvarez **Postdoctoral Fellows** Carles Ayats, Svetlana Savina, Miroslav Sisa, Jan Spengler, Judit Tulla **PhD Students** Tommaso Cupido, Peter Fransen, Yésica García, Delia Hernández, Leticia Hosta, Albert Isidro, Carles Mas, Laia Miret, Marta Pelay, Daniel Pla, Elisabet Prats, Pau Ruiz, Lorena Simón, Ramón Subirós, Gemma Vilar **Research Assistants** Gerardo Acosta, Miriam Góngora, Marta Paradís **Visiting Scientists** Sukhendu Dev (USA), Ayman El-Faham (Egypt), Athanassios Galanis (Greece), Irene Izzo (Italy), Marcelo Kogan (Chile), Aikaterini Zompra (Greece) **Visiting Students** Eva de Mol (Belgium), Michelle Drogat (France), Chiara Falciani (Italy), Simón Guerrero (Chile), Eleonora Marcucci (Italy), Camila Martínez (Argentina), Teresa Oliveira (Portugal)

The use of optical tweezers to study topoisomerase activity and the modification of force hysteresis by the presence of the topoisomerase inhibitor Lamellarin D have been recently developed in collaboration with the Nanotechnology group at the Institute for Bioengineering of Catalonia (IBEC) - Bielefeld Universität.

Dictyodendrins are a family of alkaloids isolated from the sponge *Dictyodendrilla verongiformis* collected off the southern coast of Japan. These alkaloids have a common pyrrolo[2,3-c]carbazole core but differ in their respective substituents at the α position of the pyrrole ring and in their degree of oxidation. We have synthesised the pyrrolo[2,3-c] carbazole. The sequence is based on a Suzuki cross-coupling reaction between a pyrrole fragment and an indole fragment, followed by tandem photochemical 6π-electro-cyclisation/ aromatisation.

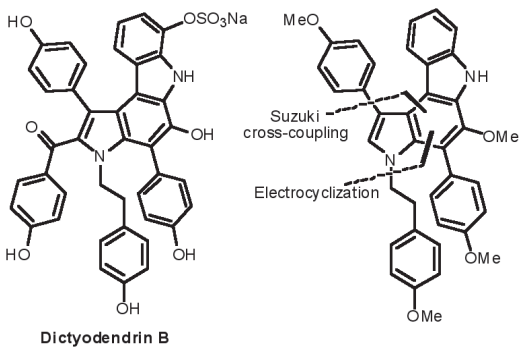


Figure 3. *Studies towards the synthesis of Dictyodendrin B.*

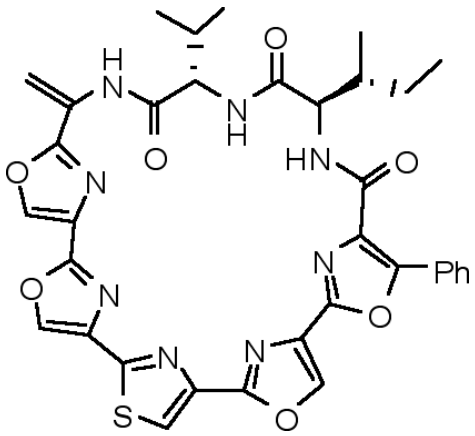


Figure 4. *Structure of Mechercharmycin A.*

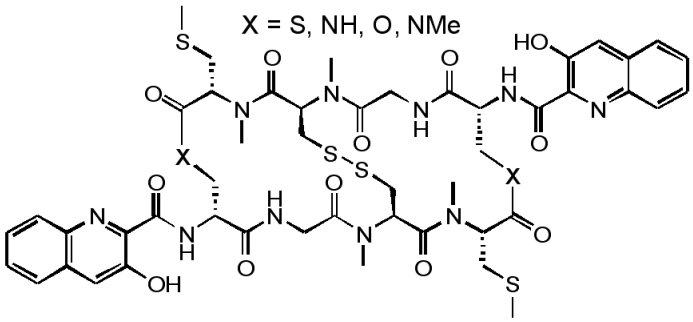


Figure 5. *Structure of thiocoraline and analogues; X=S, thiocoraline; X=O, oxathiocoraline; X=NMe, azathiocoraline.*

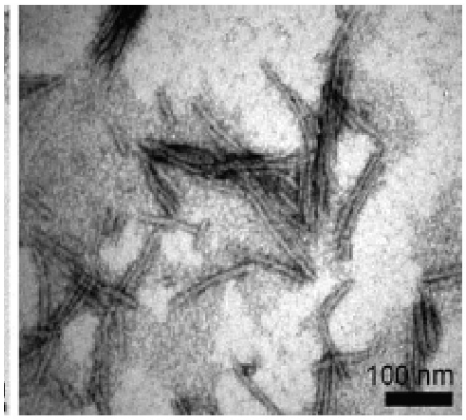


Figure 6. *Transmission electron microscopy (TEM) image of C-16 SAP showing fibril-like nanostructures.*

Mechercharmycin A. We have used a combination of peptide and heterocyclic chemistry for an efficient and versatile convergent synthesis of the natural compound IB-01211. We have explored the synthesis of several analogues of the natural product and performed studies on the macrocyclisation of rigid peptide compounds. A library of derivatives of the natural product, as well as conjugates to improve the solubility, were synthesised. We have also studied cell penetration using confocal microscopy and addressed the mechanism of action of this compound.

Thiocoraline analogues. Taking advantage of the arsenal of protecting groups, coupling reagents, and handles developed, we have synthesised three analogues of the natural thiocoraline (X=S). We have demonstrated that the introduction of N-Me-amides in bridges mimics the thioester bonds without imposing steric hindrance. Bridged-N-Me amides allow conservation of the hydrogen bonding map of the natural product.

N-Me-azathiocoraline displays *in vitro* activity in the same order as the natural product [and superior to oxa (X=O) and aza (X=NH), in this order], and similar behaviour when interacting with DNA. Furthermore, this compound is significantly more stable. This approach will be used to enhance stability in other depsipeptides and side-chain to side-chain cyclic peptides with similar restraints.

Drug delivery systems. Nanotechnologies

New antisepsis agents. Peptides that interact with lipopolysaccharide (LPS) can provide the basis for the development of new therapies. We have prepared, structurally characterised, and biologically evaluated several LPS-neutralising acyl peptides derived from LALF, BPI and SAP. In all cases, peptides bearing long acyl chains showed greater LPS-neutralising activity than the original acetylated peptides. The structural analysis of these peptides revealed that N-acylation with long acyl chains promotes the formation of micellar or fibril-like nanostructures. This observation thus proves a correlation between anti-LPS activity and nanostructure formation.

The results of this study provide useful structural insights for the future design of new acyl peptides that strongly bind LPS and therefore act as antisepsis drugs. Furthermore, this nanostructure-biological activity correlation can be transferred to other therapeutic areas.

Gold nanoparticles (GNPs). The conjugation of Kahalalide F analogues to GNPs improves the capacity of the peptide to target tumoral cell lysosomes (the putative target for Kahalalide F). Using GNPs with high capacity to enter the cell allows higher concentrations of peptide molecules inside the cells, which in turn enhances cytotoxicity, as observed for peptides conjugated to GNP-40. The conjugation of bioactive peptides such as Kahalalide F to GNPs could be used to direct the drug to the desired pharmacological target, thereby widening the therapeutic applications of current cancer therapies. In addition, the peptides conjugated to GNP-40 could be used in treatments that seek to destroy tumoral cells; thus, NIR radiation could be applied to those cells that contain accumulated nanoparticles.

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Research networks and grants

Combinatorial chemistry for drug discovery
Spanish Ministry of Science and Innovation, CTQ2006-03794/BQU (2006-2008)
Principal investigator: Fernando Albericio

Nanobiotechnology for diagnosis and therapy of solid tumours
Spanish Agency for International Cooperation (AECID), A/010915/07 (2008)
Principal investigator: Fernando Albericio

Networking centre on bioengineering, biomaterials and nanomedicine
Instituto de Salud Carlos III, CIBER-BBN-0074 (2006-2009)
Principal investigator: Fernando Albericio

Reagents for the synthesis of peptides with therapeutical activity
Spanish Agency for International Cooperation (AECID), A/9846/07 (2008)
Principal investigator: Fernando Albericio

Sabbatical stay
Agency for Administration of University and Research Grants (AGAUR), PIV-F Albericio (2008-2009)
Principal investigator: Fernando Albericio

Collaborations

Anti-inflamatory compounds
Enrique Pérez-Payá, Instituto Príncipe Felipe (Valencia, Spain)

Anti-leishmania compounds
Luis Rivas, Spanish National Research Council-CSIC (Madrid, Spain)

Antitumoral compounds
Rosa Aligué, Faculty of Medicine, University of Barcelona (Barcelona, Spain) and Ramón Mangues, Institut de Recerca Hospital de Sant Pau (Barcelona, Spain)

Biological evaluation of molecules and ChemBioBank
Mabel Loza, University of Santiago de Compostela (Santiago de Compostela, Spain)

Combinatorial chemistry for purification of proteins
Osvaldo Cascone, University of Buenos Aires (Buenos Aires, Argentina)

Delivery systems for SiRNA
Ramón Eritja, IRB Barcelona/CSIC (Barcelona, Spain)

Dendrimers as drug delivery systems
Simó Schwartz Jr, Institut de Recerca Hospital Universitari Vall d'Hebron (Barcelona, Spain)

Nanoparticles for therapy
Marcelo Kogan, University of Chile (Santiago de Chile, Chile)

Production of libraries and medicinal chemistry program
Almirall (Barcelona, Spain)

Synthesis of natural products of marine origin
PharmaMar (Madrid, Spain) and Instituto Biomar (Leon, Spain)

Synthesis of peptides
Lonza AG (Visp, Switzerland) and Luxembourg Biotech (Rehovot, Israel)

Therapeutic polymers
Maria Jesús Vicent, Instituto Príncipe Felipe (Valencia, Spain)

Other collaborations

Andrés Parra, University of Granada (Granada, Spain)

Fanny Guzmán, Catholic University of Valparaiso (Valparaiso, Chile)

Javier de Mendoza, Institute of Chemical Research of Catalonia (Tarragona, Spain)

John Joule, University of Manchester (Manchester, UK)

José Antonio del Río, Institute for Bioengineering of Catalonia (Barcelona, Spain)

José Luis Mascareñas, University of Santiago de Compostela (Santiago de Compostela, Spain)

Norbert de Kimpe, University of Ghent (Ghent, Belgium)

Ramon Estevez, University of Santiago de Compostela (Santiago de Compostela, Spain)

Roser González, Department of Genetics, University of Barcelona (Barcelona, Spain)

RAMON ERITJA

Synthesis and properties of modified oligonucleotides

Synthetic oligonucleotides are omnipresent in most laboratories as a result of the highly optimised protocols developed for solid-phase synthesis. These protocols make it possible to synthesise a large variety of modified oligonucleotides. Our group studies the methodology used for the synthesis of DNA and RNA derivatives in order to obtain novel compounds with new and/or improved properties. The projects undertaken during this year address the following: (i) the conjugation of small molecules (peptides, lipids, carbohydrates) to DNA and RNA for potential use in DNA/ RNA therapeutics, (ii) the effect of modified bases in the structural and biological properties of oligonucleotides, (iii) the use of modified oligonucleotides in the assembly of nanomaterials and biosensors, and (iv) the preparation of new DNA-binding drugs.

Synthesis of oligonucleotide conjugates carrying small molecules (peptides, lipids, steroids and carbohydates)

The use of synthetic oligonucleotides to control gene expression has triggered the search for new oligonucleotide derivatives with improved therapeutic potential. To enhance the activity of oligonucleotides and their analogues, they have been covalently linked to intercalating, alkylating, photo-crosslinking and radical generating reagents. In addition to increasing the affinity for the target sequence, some of these compounds promoted the uptake of oligonucleotides by cells and improved their resistance to nucleases. Lipid moieties, such as cholesterol, to oligonucleotides enhance the antisense activity of these compounds as well as the silencing properties of small interfering RNA (siRNA). We have prepared several oligonucleotide (DNA and RNA) conjugates carrying lipids, steroids and carbohydrates. At present, we are examining the properties of these new compounds. We are collaborating with several groups in this field. Jose Carlos Perales' group (University of Barcelona) is working on the evaluation of the inhibitory properties of conjugates *in vivo*. The first manuscript derived from this collaboration has recently been published. In this paper we described the synthesis of RNA carrying nucleoplasmin. The resulting siRNA duplexes carrying nucleoplasmin were efficiently delivered to HeLa cells and these conjugates entered the RNAi pathway to silence gene expression as efficiently as unmodified and 3'-cholesterol modified siRNA duplexes (Aviñó *et al*, 2009).

Other collaborations in this field are: (i) the synthesis and evaluation of the properties of oligonucleotide-carbohydrate conjugates, with Juan Carlos Morales (CSIC, Seville); (ii) the synthesis and evaluation of the properties of oligonucleotide-lipid conjugates, with Sylentis SAU; and (iii) the synthesis of oligonucleotide conjugates carrying steroids, which are being used for the development of bioanalytical devices for the detection of steroidal anabolic hormones. This last line of research is funded by the strategic action on nanotechnology and it is being done in collaboration with the groups led by Pilar Marco (CSIC, Barcelona), Josep Samitier (Institute for Bioengineering of Catalonia, Barcelona), Iraida Loinaz (CIDETEC-Centre for Electrochemical Technologies, San Sebastian) and Maria Teresa Martínez (CSIC, Zaragoza).

In addition, we have described the first example of a measurement of carbohydrate-aromatic interactions using a dangling-ended DNA model system (Morales *et al*, 2008). The interactions between carbohydrates and proteins play a fundamental role in living organisms in processes such as apoptosis, bacterial and viral infection, inflammation, and fertilisation. Several non-covalent forces have been proposed to participate in these interactions: CH-π interactions, Van der Waals forces and the hydrophobic effect. Carbohydrate-aromatic interactions have been studied using biological tools, NMR, IR, computational methods and model systems but few experimental data have been reported about this interaction and their quantification has been described only in two specific examples using rigid peptides as scaffolds to hold carbohydrates and aromatic amino acids in close proximity.

The novelty of the experimental design developed by Morales *et al* is the use of the denaturation curves of a DNA duplex to measure this type of interaction. The robustness of the analyti-

Principal Investigator Ramon Eritja **Research Associate** Anna Maria Aviñó **Postdoctoral Fellows** Alejandra Garibotti, Santiago Grijalvo, Sonia Pérez, Álvaro Somoza **PhD Students** Margarita Alvira, Ruben Ferreira, Brendan Manning, Sandra Milena **MSc Student** Najah Mizouri

cal method allowed the study of the impact of hydroxylation, stereochemistry and geometry on the carbohydrate-aromatic interactions. The results of this research were highlighted by Chad Tatko in an editorial of Nature Chemical Biology (2008).

Development of new drugs that bind DNA

Drug development has traditionally focused on active sites of proteins, and on identifying molecules, such as inhibitors, that bind to these sites and directly block interactions with natural substrates. In addition to this direct mode of enzymatic regulation, several drugs that interact with nucleic acids have become important antibiotic, antiviral and anticancer drugs. In collaboration with Fernando Albericio's group (IRB Barcelona), Miriam Royo (Barcelona Science Park) and Crystax Pharmaceuticals, we are designing and synthesising new drugs that interact with DNA. The structure of the first generation of new compounds prepared in this project is shown in Figure 1. Two manuscripts have been published this year in this regard (Aviño *et al*, 2008 and Farrera *et al*, 2008).

Synthesis of oligonucleotides carrying DNA methyltransferase inhibitors

Aberrant DNA methylation is a common finding in cancer. Several drugs that inhibit DNA methylation are active against some malignancies. The cytosine analogues 5-azacytidine and 5-aza-2'-deoxycytidine are the most frequently studied inhibitors of DNA methylation. Zebularine (1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one), another pyrimidine analogue which lacks the 4-amino group of the other cytosine analogues, inhibits DNA methylation and may have activity against cancer. Zebularine has the advantage of being very stable and can be administered orally. However, it has the disadvantage of being a less potent inhibitor of DNA methylation compared to the azacytosine derivatives. It is believed that zebularine is not metabo-

lised to its triphosphate form as efficiently as 5-azacytidine and 5-aza-2'-deoxyctyidine, and therefore it is not efficiently incorporated into DNA. In collaboration with Victor Márquez (NIH-National Institutes of Health, USA) and Allen Yang (USC-University of Southern California, USA), we have prepared oligonucleotides carrying 2'-deoxyzebularine in order to measure the efficiency of incorporation in response to the drug. When zebularine was included in the template strand, dGTP was preferentially incorporated by the Klenow fragment opposite the drug, but dATP and dTTP were incorporated with 8.1% and 5.2% the efficiency of dGTP. In addition, zebularine in the template strand was noted to inhibit DNA extension. Thus dZTP is efficiently incorporated into DNA and acts as a cytosine analogue, which is consistent with its capacity to inhibit DNA methylation (Dowd *et al*, 2007).

In addition, using DNA melting experiments, we determined the stability of duplexes containing 2'-deoxyzebularine paired with the four natural bases. Melting temperatures and thermodynamic data showed the preference of 2'-deoxyzebularine for 2'-deoxyguanosine, which behaves like a 2'-deoxycytidine analogue, forming a less stable base pair as a result of the absence of the amino group at position 4. Moreover, we studied the duplex-hairpin equilibrium of a self-complementary oligonucleotide carrying several natural and non-natural bases including 2'-deoxyzebularine as a central mispair. Depending on the base present in the middle of the sequence, the stability of the bimolecular duplex that modulates the duplex-hairpin equilibrium is affected. Magnesium ions were shown to preferentially stabilise the bimolecular duplex form. The results indicate the importance of the modifications and the role of cations in shifting structural equilibrium (Aviñó *et al*, 2009).

Oligonucleotides and nanotechnology

There is considerable interest in the use of biopolymers (peptides, proteins and nucleic acids) for the assembly of nanomaterials. Also, oligonucleotides linked to nanoparticles are being used to monitor DNA hybridisation as well as to detect a particular nucleic acid sequence of interest. Our group is actively collaborating with several groups in this field. Several projects funded by the European Commission are currently underway. In one of these projects, we have developed a new photolithographic method that uses photolabile DNA hairpins to make patterns on silicon oxide wafers. The method described offers an attractive option for the fabrication of patterned surfaces of potential in electronics and biosensors (Ramos *et al*, 2008 and Manning *et al*, 2009).

In the framework of the strategic action on nanotechnology, we provided Mª Teresa Martínez (CSIC, Zaragoza) with modified oligonucleotides to study DNA hybridisation on carbon nanotube field-effect transistors (CNTFETs) at the Molecular Foundry of the Lawrence Berkeley National Laboratory. Using oligonucleotides, a special polymer developed by Iraida Loinaz at CIDETEC and the facilities at the Molecular Foundry for the fabrication of CNTFETs (Figure 2), Mª Teresa Martínez achieved high precision measurements of DNA hybridisation. The DNA hybridisation produced changes in the I_{ON} current, I_{OFF} current and I_{ON}/I_{OFF} current ratio as well as the V_{tp} and V_{tn}. These findings imply that it was possible to detect the charge transfer inherent to the hybridisation reaction (Martínez *et al*, 2009; Figure 2).

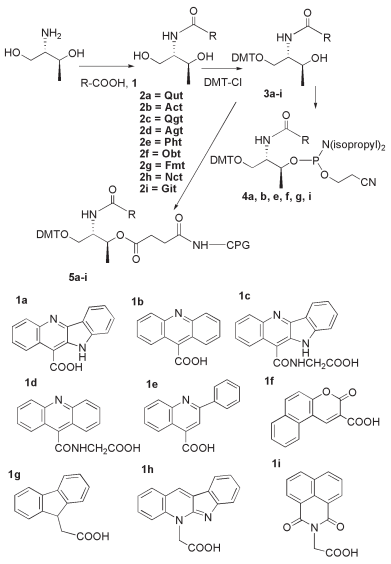

Figure 1. Development of new drugs that bind DNA. *Synthesis scheme of the first generation of compounds prepared in this project (Aviñó et al, 2008).*

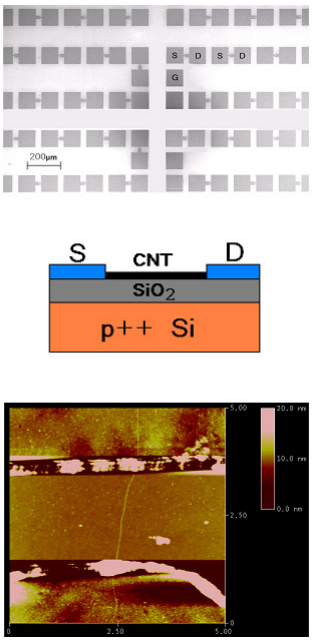

Figure 2. Oligonucleotides from IRB Barcelona spark in the Molecular Foundry. *(Top) Scanning electron micrograph of the array design. S: source. D: drain. G: contact to back gate. 896 pairs of S/D electrodes are packed in 1cm2. (Middle) Diagram of device structure. The heavily doped p-type substrate is used as the gate. (Bottom) 5µm x 5µm AFM picture of a CNTFET device. Vertical scale: 20nm (Martínez et al, 2009).*

G-quadruplex, *i*-motif and other non-canonical DNA structures

DNA shows great flexibility and exhibits large polymorphism depending on sequence, chemical modifications, or alterations in the DNA environment. For this reason, DNA adopts several structures in solution apart from the Watson-Crick double helix, ranging from disordered single strands to higher order structures such as parallel duplexes, triplexes, *i*-motifs or G-quadruplexes.

One of these non-canonical DNA structures is found on G-rich oligonucleotides. These may form intra- or inter-molecular structures involving the formation of G tetrads. These structures are naturally present at the end of the chromosomes or telomeres. Recently, G-quadruplex structures have been found in promoter regions of oncogenes and several authors have suggested that the G-quadruplex regulates the expression of these proteins. Due to the potential biological relevance of the G-quadruplex, we have initiated the study of G-quadruplex structures as well as the complementary C-rich strand, which may also form a quadruplex structure know as an *i*-motif.

In collaboration with Modesto Orozco's group (IRB Barcelona), we described the triplex stabilisation properties of several 8-amino purines. In these studies we showed 8-amino guanine to be a guanine derivative of interest because of its triplex stabilisation properties. Furthermore, we found that 8-amino guanine destabilises quadruplexes when inserted in the thrombin-binding aptamer (TBA). This oligonucleotide forms an intramolecular anti-parallel quadruplex that binds specifically to thrombin at nanomolar concentrations and has anti-coagulant properties of interest.

Preliminary theoretical calculations made by Orozco's group suggested that the quadruplex destabilising properties of 8-amino guanine do not hold for parallel quadruplexes. To clarify this point, we undertook a systematic study of the effect of 8-amino guanine on the parallel quadruplexes TGGGGT and TGGGGGT, introducing this modified base at each G-tetrad. Our results demonstrate that the replacement of guanines by 8-amino guanines enhances the energetics of the quadruplex, as a strong increase in association kinetics was coupled to a similar increase in thermal stability. However, this replacement is not universal, as it is not equally favourable at all positions. Theoretical calculations suggest that nucleobase-ion interactions are at the basis of the different stabilisation properties of 8-amino guanine when it is placed inside or at the extremes of a quadruplex (Gros *et al*, 2008).

In addition and in collaboration with Raimundo Gargallo's group (UB), we provided several TBA derivatives for the study of the interaction of TmPyP4 with TBA by surface plasmon resonance, HPLC and melting curves. The results show the formation of a 1:1 TmPyP4 /TBA complex and logarithm of equilibrium constant equal to 5.7 ± 0.2 (del Toro *et al*, 2008).

Finally, we explored the capacity of circular dichroism (CD) to efficiently classify DNA secondary structures. CD spectroscopy is a simple technique that is sensitive to structural changes but it has not been fully used to classify DNA structures. The application of multivariate data analysis methods to the CD spectra data set proved very useful to classify DNA sequences on the basis of their experimental CD spectra. The three chemometric methods used by Jaumot *et al* 2009 allowed the extraction of information from the data set. To obtain a complete data set, our group contributed to providing a collection of oligonucleotides covering all known DNA secondary structures.

SCIENTIFIC OUTPUT

Publications

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Somoza A. Protecting groups for RNA synthesis: an increasing need for selective preparative methods. *Chem Soc Rev,* **37**(12), 2668-75 (2008)

Somoza A, Silverman AP, Miller RM, Chelliserrykattil J and Kool ET. Steric effects in RNA interference: probing the influence of nucleobase size and shape. *Chem Eur J,* **14**(26), 7978-87 (2008)

Research networks and grants

Ajuts grups reconeguts Generalitat
Agency for Administration of University and Research Grants (AGAUR), 2005SGR-00693 (2006-2008)
Principal investigator: Ramon Eritja

Design and functionality of non-linear electrochemical nanoscale devices (DYNAMO)
European Commission, STREP-NEST-2004-ADV-028669-1 (2007-2009)
Principal investigator: Ramon Eritja

Development of nanobio-analytical platforms based on biomolecular recognition using optical and/or electrical detection (Nanobiomol)
Spanish Ministry of Science and Innovation, NAN2004-09415-C05-03 (2005-2008)
Principal investigator: Ramon Eritja

Inhibitory carbohydrate-ARN conjugates as new antiviral agents by several mechanisms: RNA interference (siARN), antisense, aptamers and ribozymes
Spanish Ministry of Science and Innovation, PIF06-045 (2006-2009)
Principal investigator: Ramon Eritja

Multi-scale formation of functional nanocrystal-molecule assemblies and architectures (FUNMOL)
European Commission, STREP-NMP-2007-213382 (2008-2010)
Principal investigator: Ramon Eritja

Nucleic acids chemistry group (CIBER-BBN)
Instituto de Salud Carlos III, CB06/01/0019-CIBER-BBN (2006-2010)
Principal investigator: Ramon Eritja

Self-assembly guanosine structures for molecular electronic devices
European Commission, COST-action-MP0802 (2008-2011)
Principal investigator: Ramon Eritja

Síntesis racional de moléculas con afinidad al ADN de doble cadena constituidas por diversas unidades activas por el mecanismo de intercalación
PTRI, PTR1995-0976-OP (2006-2008)
Principal investigator: Ramon Eritja

Synthesis and properties of modified oligonucletides of biomedical and structural interest (OMIBE)
Spanish Ministry of Science and Innovation, BFU2007-63287 (2007-2010)
Principal investigator: Ramon Eritja

Other funding sources

Synthesis of RNA interference linked to lipids
Research contract with Sylentis SAU

Collaborations

Characterisation of peptide nanotubes
Juan Granja, University of Santiago de Compostela (Santiago de Compostela, Spain)

Oligonucleotide conjugation to carbon nanotubes
Mª Teresa Martínez, Institute of Carbochemistry, Spanish National Research Council (Zaragoza, Spain)

Research on nanosensors functionalised with oligonucleotides
Josep Samitier, Institute for Bioengineering of Catalonia (Barcelona, Spain)

Synthesis and characterisation of DNA quadruplex structures
Stefania Mazzini, University of Milan (Milan, Italy)

Synthesis and characterisation of oligonucleotides carrying non-natural bases
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Synthesis and evaluation of modified siRNA
José Carlos Perales, University of Barcelona, Bellvitge Campus (Barcelona, Spain)

Synthesis and NMR characterisation of oligonucleotides
Carlos González, Institute of Structure of Matter, CSIC (Madrid, Spain)

Synthesis of new drugs that bind DNA
Juan Aymamí, Crystax Pharmaceuticals, Barcelona Science Park (Barcelona, Spain)

Synthesis of new RNA derivatives
Ana Isabel Jiménez, Sylentis SAU (Madrid, Spain)

Synthesis of oligonucleotide-carbohydrate conjugates
Juan Carlos Morales, Institute of Chemical Research, CSIC (Seville, Spain)

Synthesis of oligonucleotide-steroid conjugates
Mª Pilar Marco, Institute of Chemical and Environmental Research (IIQAB), CSIC (Barcelona, Spain)

Synthesis of active oligonucleotides against AIDS
Mª Ángeles Muñoz Fernández, Gregorio Marañón Hospital (Madrid, Spain)

Synthesis of oligonucleotides carrying DNA-methyltransferase inhibitors and conformationally-restricted nucleosides
Victor Marquez, National Institutes of Health (Frederick, USA)

Synthesis of oligonucleotides with cell penetrating peptides
Fernando Albericio, IRB Barcelona (Barcelona, Spain) and Miriam Royo, Barcelona Science Park (Barcelona, Spain)

Synthesis of oligonucleotides with structural interest
Raimundo Gargallo, University of Barcelona (Barcelona, Spain)

Synthesis of triplex-forming oligonucleotides
Gilles Mirambeau, Hospital Clínic (Barcelona, Spain)

Design, synthesis and structure of peptides and proteins

The design of ligands that recognise predefined 'patches' of the surface of a protein is highly relevant in biomedicine since this will allow future custom-designed modulation of protein-protein interactions. Our lab is working on several systems of therapeutic relevance, including inhibitors of beta-amyloid protein aggregation, 'chemical chaperones' for P53, angiogenesis inhibitors based on the molecular recognition of VEGF, and human brain prolyl oligopeptidase (POP) inhibitors. Although the design of these ligands is hampered by our poor knowledge, at a quantitative level, of non-covalent interactions, we have developed new methodologies that combine the automatic design *in silico* based on evolutionary algorithms with experimental 'validation' using a variety of spectroscopic techniques, in particular nuclear magnetic resonance (NMR; Pellecchia *et al*, 2008). Finally, it is necessary to emphasise the significant role that peptide 'shuttles' will play in the future. These shuttles will allow the new synthetic ligands to overcome physiological barriers, such as the plasmatic membrane or the blood-brain barrier (BBB), and reach their protein target.

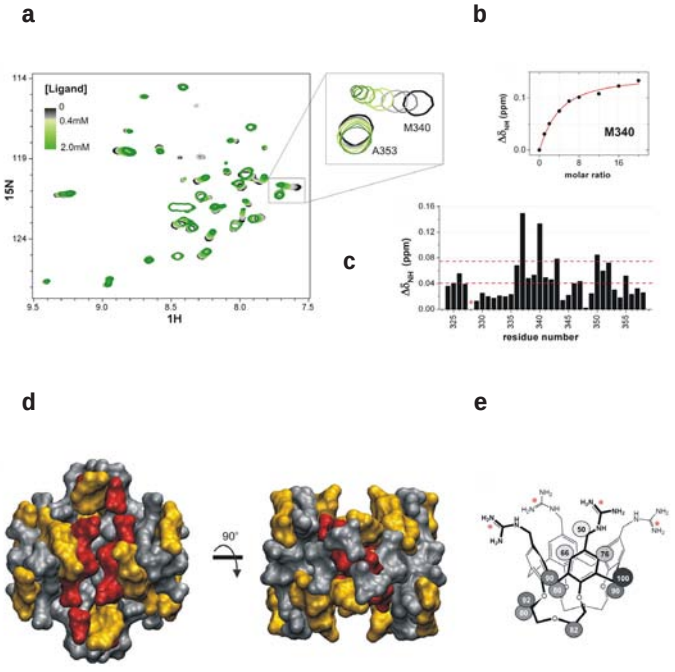

Figure 1. *Interaction of a calyx[4]arene ligand with the tetramerisation domain of protein p53.*

p53: Rescuing narcissistic protein-protein interactions

The stabilisation of protein-protein interactions holds potential as a therapeutic strategy, yet it has gained little attention. Our group has recently reported calixarene molecules that rescue and stabilise the functional tetrameric state of certain mutants of protein p53 (Gordo *et al*, 2008). Protein p53 is a transcription factor that is crucial for cell cycle and genome integrity. It is able to induce both cell arrest when DNA is damaged and the expression of DNA repair machinery. When the damage is irreversible, it triggers apoptosis. In its active form, p53 protein is a tetramer formed by four identical copies of proteins bound together. p53 has four domains with differentiated functions: activation of transcription, DNA binding, tetramerisation and regulation. The tetramerisation domain is responsible for stabilising the tetrameric structure.

More than 50% of cancer patients have mutations in the p53 gene. Although most of these are located in the DNA-binding domain, several mutations are found in the tetramerisation domain, thereby causing destabilisation of the entire protein with the consequent loss of activity. Two well documented examples of this kind of congenital predisposition are pediatric adrenocortical carcinoma and Li-Fraumeni syndrome. Therefore, the design of compounds with the capacity to stabilise the tetramerisation domain of p53 represents a new and attractive strategy for the development of anti-tumour drugs.

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In collaboration with de Mendoza's group (Institute of Chemical Research of Catalonia, Tarragona), we have designed, synthesised and studied a compound with the capacity to interact with the p53 tetramerisation domain. This new compound is a conical calix[4]arene with four cationic guanidiniomethyl groups at the wider edge (upper rim) and hydrophobic loops at the narrower edge (lower rim) that fits nicely and cooperatively into the hydrophobic clefts between two of the monomers at each side of the protein and keeps the tetrameric structure, like molecular templates, by both ion-pair and hydrophobic interactions.

We have used a variety of biophysical tools to thoroughly characterise the interaction between the ligand and wild-type p53. These include 1H saturation transfer difference NMR, advanced mass spectrometry, differential scanning calorimetry, and circular dichroism. More importantly, we have shown how this new rationally designed molecule is capable of holding together the four monomers of the mutated p53-R337H protein, thereby recovering the tetramer integrity as in the wild-type structure. Furthermore, we have found a good agreement between the structure of the complex and the nature of the interactions predicted by molecular dynamics calculations.

Looking to the future, this is the first proof of concept on the way towards the design of a new class of drugs based on the use of small molecules that could act as moulds or tethers to preserve the active form of protein tethers in order to stabilise the native forms of proteins or to recover/rescue disease-related mutated ones.

Jumping hurdles

The adage that 'good fences make good neighbours' is perhaps nowhere better illustrated than in the human body, which

encompasses various gas- and fluid-filled sacs, (*eg*, the lungs, colon, and stomach) separated by specialised tissues (uterus, brain capillaries) that act as barriers, regulating the passage of molecules from one compartment to another (Teixidó *et al*, 2007). Several diseases require treatment of the brain. These include neurodegenerative diseases such as Parkinson's and Alzheimer's, but also central nervous system (CNS) diseases such as schizophrenia, epilepsy and bipolar disorder. Cerebral cancer, HIV and some aspects of obesity can also be included as pharmaceutical targets in the brain. In many cases, there are promising compounds for their treatment. However, because of problems associated with crossing the BBB, more than 98% of these compounds do not reach the drug development stage (Teixidó *et al*, 2008).

Prolyl oligopeptidase (POP) is a cytosolic serine peptidase that hydrolyses proline-containing peptides at the carboxy terminus of proline-residues. POP has been associated with schizophrenia, bipolar affective disorder and related neuropsychiatric disorders and therefore may have important clinical implications. In previous work, we used 19F NMR to search for new POP inhibitors from a library of plant extracts used in traditional Chinese medicine and identified several extracts as powerful inhibitors of this peptidase. We have recently discovered (Tarragó *et al*, 2008) that the alkaloid baicalin, which we isolated as the active component of an extract of *Scutellaria baicalensis* roots, exhibits POP inhibitory activity. Baicalin inhibited POP in a dose-dependent manner. Inhibition experiments using baicalin analogues showed that the sugar moiety was not required for activity. The IC50s of baicalin and its aglycone derivative baicalein were similar, thereby suggesting that the sugar moiety was not involved in the interaction of baicalin with POP. These results were confirmed by saturation transfer difference NMR experiments.

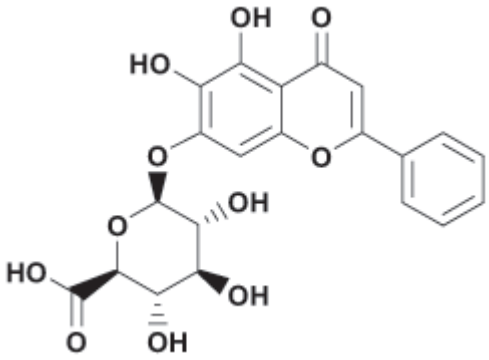

Figure 2. *The chemical structure of baicalin.*

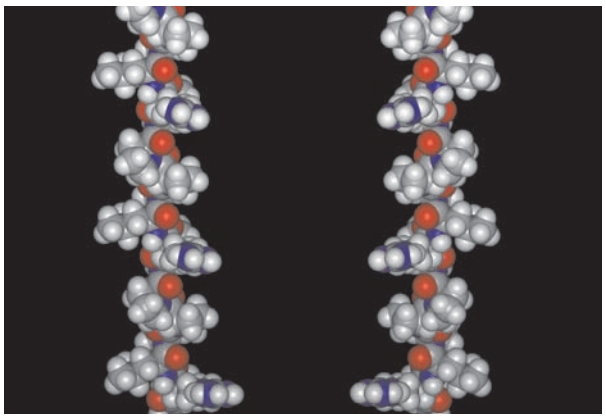

Figure 3. *D-SAP: a new, non-cytotoxic and fully protease-resistant cell-penetrating peptide.*

To further elucidate the absorption and transport mechanisms of baicalin and baicalein, we evaluated their transport *in vitro* through the gastrointestinal tract and the BBB using a parallel artificial membrane permeability assay. The molecule which potentially crosses both barriers was identified as baicalein, the aglycone moiety of baicalin. Our results show that baicalin is a new prodrug with POP inhibitory activity. As baicalin is a natural compound with a long history of safe administration to humans, it is a highly attractive base from which to develop new treatments for schizophrenia, bipolar affective disorder and related neuropsychiatric diseases.

For drug targets located inside the cell, attaining satisfactory intracellular delivery is crucial. Unfortunately, most drug candidates are unable to cross the cytoplasmic membrane alone. Hence, several drug delivery strategies have been proposed, including microinjection, electroporation, liposomal formulation and the use of viral vectors. However, each of these has its respective problems in terms of toxicity and therapeutic feasibility. An alternative delivery strategy is the use of peptide sequences that can translocate across the cytoplasmic membrane (Pujals *et al*, 2008).

Our main contribution to this field during the last five years has been to show that amphipathic Pro-rich peptides are a promising source of cell-penetrating peptides (CPPs). When compared to the principal families of CPPs, they exhibit very low cytotoxicity but much lower uptake. The search for non-cytotoxic derivatives with much more efficient translocation is still underway. In the meantime, we have recently prepared a fully protease-resistant CPP. Proteolytic stability was obtained by chiral inversion of the residues of a known self-assembling CPP from all L-amino acids to all D-amino acids and then assessed against trypsin and human serum. Circular dichroism studies confirmed the enantiomeric structure of the analogue, and transmission electron microscopy (TEM) studies indicated that the new inverso analogue retains the capacity of the original peptide to self-assemble. The results of uptake experiments indicate that the protease-stable (that is, D-amino acid) analogue of the peptide is internalised by cells to the same extent as the protease-susceptible (that is, L-amino acid) parent peptide. The all D-amino analogue has also proven to be non-cytotoxic and successfully distributed among several organs in a preliminary *in vivo* study (Pujals *et al*, 2008).

Publications

Araya E, Olmedo I, Bastus NG, Guerrero S, Puntes V, Giralt E and Kogan MJ. Gold nanoparticles and microwave irradiation inhibit beta-amyloid amyloidogenesis. *Nanoscale Res Lett*, **3**(11), 435-43 (2008)

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Teixidó T and Giralt E. The role of peptides in blood-brain barrier nanotechnology. *J Pept Sci*, **14**(2), 163-73 (2008)

Research networks and grants

Ajuts per potenciar i donar suport als grups de recerca
Generalitat de Catalunya, 2005SGR00663 (2005-2008)
Principal investigator: Ernest Giralt

Design of peptide ligands for protein-surface recognition
Spanish Ministry of Science and Innovation, BIO2005-00295 (2005-2008)
Principal investigator: Ernest Giralt

Design, synthesis and structural studies of new VIH protease dimerisation inhibitors
FIPSE-Foundation for AIDS Research and Prevention in Spain, 36606/06 (2006-2009)
Principal investigator: Ernest Giralt

Estudis estructurals i dinàmics d'espècies oligomèriques i fibril·lars de beta amiloide. Experiment d'intercanvi protodeuteri analitzat per ressonància magnètica
'La MTV3' Foundation, BM05-60-0 (2006-2008)
Principal investigator: Ernest Giralt

Nanotechnologies in biomedicine (Nanobiomed)
Spanish Ministry of Science and Innovation, CONSOLIDER-INGENIO 2010 (2006-2010)
Principal investigator: Ernest Giralt

Novel nanobiomaterial development: Modification of autoaggregation and protein conformation to reduce toxicity
Spanish Agency for International Cooperation (AECID), A/010967/07 (2008)
Principal investigator: Ernest Giralt

Structural and dynamic characterisation of αβ aggregation. Examination of αβ aggregation peptide inhibitors
'La MTV3' Foundation, 092 (2006-2009)
Principal investigator: Ernest Giralt

Structure and dynamics of β-amyloid oligomeric and fibrillar species. Hydrogen/deuterium exchange experiments analysed by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS)
'La Caixa' Foundation, BM05-60-0 (2006-2008)
Principal investigator: Ernest Giralt

Studies of neurosecretion by remote control of exocytosis and endocytosis (OpticalBullet)
European Commission, ERC-StG Starting Grants, ID 210355 (2008-2013)
Principal investigator: Ernest Giralt

Use of peptides for intracellular nanoparticle delivery (NANOFAR)
Spanish Ministry of Science and Innovation, NAN2004-09159-C04-02 (2006-2008)
Principal investigator: Ernest Giralt

Collaborations

Applications of the Suzuki reaction to the synthesis of conformationally constrained peptides
Paul-Lloyd Williams, Organic Chemistry Department, University of Barcelona (Barcelona, Spain)

Cyclodepsipeptides as potential anticancer agents
Ricardo Pérez-Tomas, Bellvitge Hospital, University of Barcelona (Barcelona, Spain)

Design of HIV-1 protease dimerisation inhibitors
Michele Reboud-Ravaux, CNRS-University of Paris (Paris, France)

Design, synthesis and study of P53 ligands
Javier de Mendoza, Institute of Chemical Research of Catalonia (Tarragona, Spain)

Remote manipulation of protein aggregation
Marcelo Kogan, University of Chile (Santiago, Chile)

Synthesis and conformational analysis of cyclodepsipeptides from marine origin
Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Synthesis and structural studies of β-peptides
Rosa Mª Ortuño, Chemistry Department, Autonomous University of Barcelona (Barcelona, Spain)

Awards

Ernest Giralt, appointed member of the Royal Academy of Sciences and Arts of Barcelona (2008)

ANTONI RIERA

Asymmetric synthesis

Our group focuses on the synthesis of biologically active compounds for the various stages of drug development. Several of our projects are devoted to the development of basic synthetic methodology, with a special focus on asymmetric synthesis, while others address the synthesis of compounds of known therapeutic interest, in which emphasis is placed on the reliability, efficiency and scalability of the processes. Finally, other projects underway are related to drug discovery; these aim to prepare chemical libraries for biological screening.

Basic synthetic methodologies

New developments in the Pauson-Khand reaction. The Pauson-Khand reaction (PKR) is one of the most powerful reactions for the preparation of cyclopentanic compounds. The PKR is a cobalt-promoted or catalysed cycloaddition between an alkene and an alkyne, with the insertion of a carbon monoxide molecule to give a cyclopentenone. One of our main targets is to use these cyclopentenones as starting materials for the synthesis of biologically active substances such as carbanucleosides, prostaglandins and phytoprostanes. On route to a new approach for the synthesis of prostaglandins, a few years ago we uncovered a novel photochemical rearrangement (Figure 1). After studying the scope of this new reaction in 2007, this year, in collaboration with Santiago Olivella, we have published a study of the detailed mechanism of this unprecedented reaction (Olivella *et al*, 2008; Figure 2).

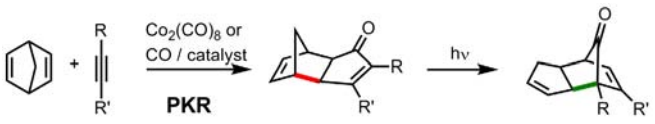


Figure 1. *Pauson-Khand reaction (PKR) and photochemical rearrangement of the PKR adducts.*

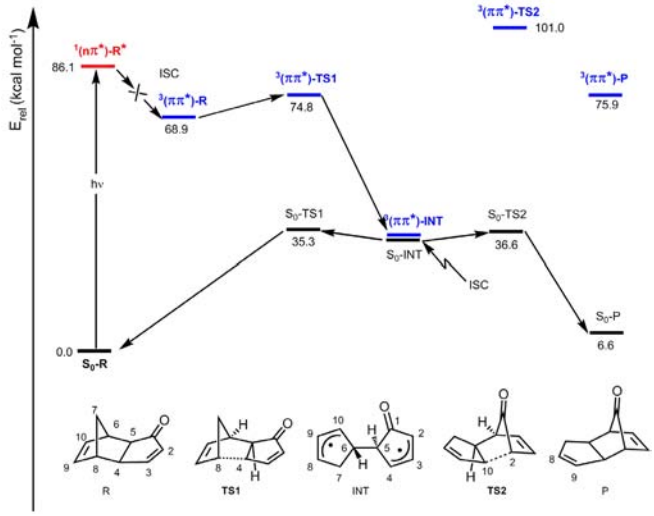
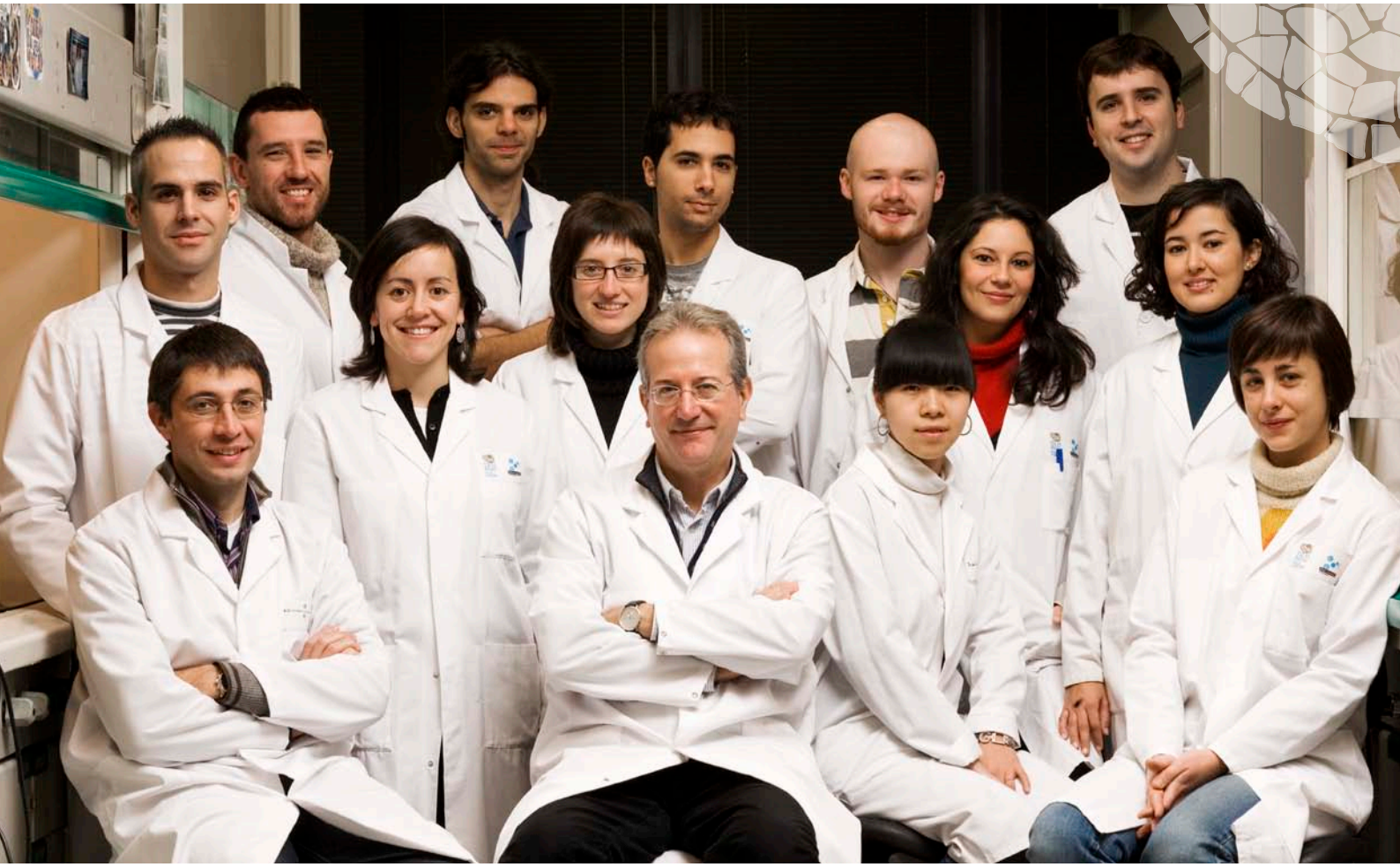


Figure 2. *Calculated mechanism of the photochemical rearrangement showing the relative energy of all intermediates and excited states along the reaction path.*

A new family of ligands for metal-catalysed reactions. To date, the best approach for an asymmetric version of the PKR is to use chiral ligands. In 2007 we designed an original family of hemilabile P,S-ligands with an unprecedented structure showing a backbone constituted by four linked heteroatoms (P-N-S-O). This year we have synthesised non-racemic *N*-phosphino-p-tolylsulfinamide ligands and studied their coordination behaviour towards dicobalthexacarbonyl complexes (Revés *et al*, 2008; Figure 3). We found that the presence of an aryl group on the sulfinamide reduces the hemilabile character of the sulfur-metal bond. Intermolecular PKR of the resulting complexes led to selectivities of up to 94% ee.

Applications of the new *N*-phosphinosulfinamide ligands to other metal-catalysed processes are now being studied in our lab. We have published the synthesis and the main structural features of several rhodium complexes of these ligands (Achard *et al*, 2009). The activity of these rhodium complexes towards cyclotrimerisation reactions is now being evaluated.

Principal Investigator Antoni Riera **Research Associate** Xavier Verdaguer **Postdoctoral Fellows** Thierry Achard, Cati Ferrer **PhD Students** Sean Doran, Yi Ning Ji, Pablo Antonio Martin, María Moreno, Rosario Ramon, Marc Revés, Ana María Vázquez **MSc Students** Thierry Leon, Albert Pesquer **Lab Technician** Ferran Santacana



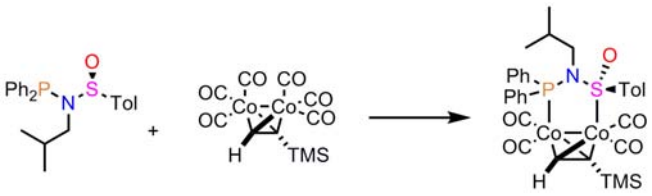


Figure 3. *Reaction of PNSO ligands with dicobalt complexes produces bridged-type complexes in which phosphorous (orange) and sulfur (magenta) are bonded to several cobalt atoms.*

Synthesis of biologically active compounds

Synthesis of five-membered-ring compounds. As mentioned, one of the best ways to prepare five-membered ring compounds is using the PKR. In 2007 we published an efficient enantioselective protocol for the preparation of cycloadduct 1 from trimethylsilylacetylene and norbornadiene. This year we have completed a new enantioselective approach for the synthesis of carbanucleosides starting from this useful Pauson-Khand adduct, thus showing its usefulness as a cyclopentenone synthon. Carbanucleosides are nucleosides in which the furanose ring has been substituted by a cyclopentane. Carbovir and Abacavir (Ziagen) are synthetic cyclopentanic carbanucleosides. They have shown major antiviral and anticancer activities. Due to its toxicity, Carbovir was not developed beyond the preclini-

cal phase; however, Abacavir was approved and launched for the treatment of HIV. In our synthetic approach, the PKR adduct was prepared in enantiomerically pure form using *N*-benzyl-*N*-diphenylphosphino-*tert*-butyl-sulfinamide as a chiral P,S ligand. From PKR adduct (−)-1, both (−)-Carbovir and (−) Abacavir were efficiently prepared in optically pure form (Vazquez *et al*, 2008; Figure 5).

In the field of phytoprostane and prostane synthesis, we have a fruitful collaboration with Paul Evans (Trinity College, Ireland). We have published a full paper in which we describe the use of the PKR adduct (+)-1 as starting material for the preparation of 5-alkylidenecyclopent-2-enones. This approach is exemplified by the short, stereoselective total syntheses of cyclopentenone phytoprostanes such as 13,14-dehydrophytoprostane J_1 (DPPJ1). We have also reported the capacity of this family of synthetic compounds to activate the peroxisome proliferator-activated receptor-γ (Figure 6).

Enantioselective synthesis of amino acids and alkaloids. The enantioselective synthesis of non-natural amino acids is attracting increasing interest due to the growing importance of modified peptides and drugs that contain fragments of amino acid derivatives. Cyclic α-amino acids are also present in many biologically relevant compounds. In particular, hydroxypipecolic acids can be considered as expanded hydroxylated homoprolines or as constrained serine derivatives.

(−)-*Cis*-4-hydroxypipecolic acid (−)-3 (Figure 7), isolated from the leaves of *Calliandra pittieri* and *Strophantus scandeus*, has been identified as a constituent of cyclopeptide antibiotics such as virginiamycin S2. It has also been used as precursor in the preparation of selective *N*-methyl-D-aspartate (NMDA) receptor antagonists and in the synthesis of Palinavir, a potent peptidomimetic-based HIV protease inhibitor.

(−)-*Trans*-3-hydroxypipecolic acid (−)-2 is a non-natural cyclic β-hydroxy-α-amino acid that has been used as a precursor in the synthesis of (−)-swainsonine, a potent and specific inhibitor of α-D-mannosidase (Figure 7). We have described two new enantioselective entries to *cis*-4 and *trans*-3-hydroxypipecolic acids (−)-3 and (−)-2 with complete control of the stereochemistry of both stereogenic centres from enantiomerically enriched 2,3-epoxy-5-hexen-1-ol (−)-4 (Alegret *et al*, 2008). This useful epoxy alcohol is readily available in multigram scale by Sharpless asymmetric epoxidation.

The structural diversity and pharmacological activity associated with alkaloids found in amphibian skin have stimulated research into their synthesis. Many of these compounds have an indolizidine structure. For instance, alkylindolizidine alkaloids, isolated from the skin secretions of certain neotropical frogs of the *Dendrobatidae* family, have been demonstrated to non-competitively block neuromuscular transmission. We have shown the suitability of the same unsaturated epoxide used in the syntheses of pipecolic acids as starting material for a stereocontrolled synthesis of indolizidine alkaloid *trans*-209D. The key intermediate of this synthesis was enantiomerically pure *N*-Boc-baikiain (−)-5, the preparation of which was described by our group some years ago (Figure 7).

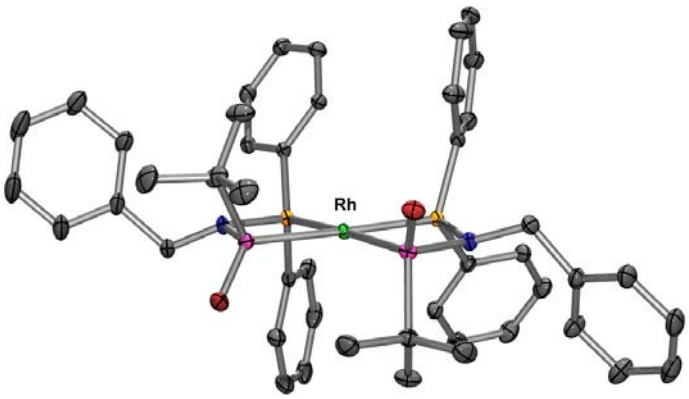


Figure 4. *Crystal structure of a Bis-PNSO-Rhodium complex. The Rhodium (green) centre is coordinated to phosphorous (yellow) and sulfur (magenta).*

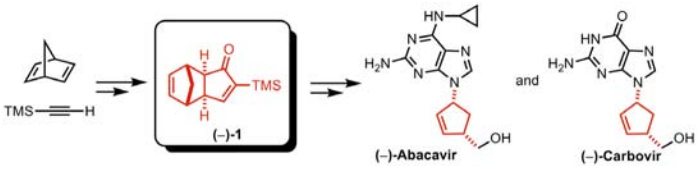


Figure 5. *Scheme of the enantioselective syntheses of Abacavir and Carbovir performed by our group at IRB Barcelona.*

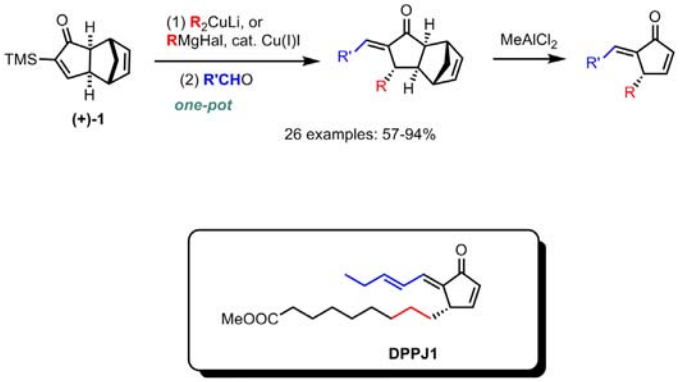


Figure 6. *Enantioselective syntheses of dehydrophytoprostanes developed in collaboration with Paul Evans's group.*

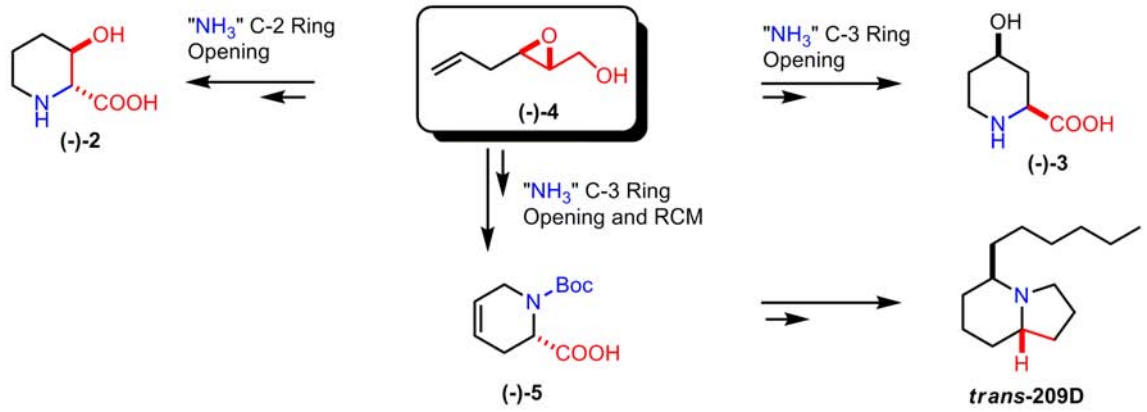


Figure 7. *Enantioselective syntheses of pipecolic acids and alkaloid trans-209D performed by our group.*

SCIENTIFIC OUTPUT

Publications

Alegret C, Ginesta X and Riera A. Asymmetric synthesis of cis-4- and trans-3-hydroxypipecolic acids. *Eur J Org Chem*, **10**, 1789-96 (2008)

Alegret C and Riera A. Enantioselective synthesis of indolizidine alkaloid trans-209D. *J Org Chem*, **73**(21), 8661-64 (2008)

Iqbal M, Duffy P, Evans P, Cloughley G, Allan B, Lledo A, Verdaguer X and Riera A. The conjugate addition-Peterson olefination reaction for the preparation of cross-conjugated cyclopentenone, PPAR-gamma ligands.*Org Biomol Chem*, **6**(24), 4649-61 (2008)

Olivella S, Sole A, Lledo A, Ji Y, Verdaguer X, Suau R and Riera A. Theoretical and experimental studies on the mechanism of norbornadiene Pauson-Khand cycloadducts photorearrangement. Is there a pathway on the excited singlet potential energy surface? *J Am Chem Soc*, **130**(50), 16898-07 (2008)

Revés M, Achard T, Sola J, Riera A and Verdaguer X. N-phosphino-p-tolylsulfinamide ligands: Synthesis, stability, and application to the intermolecular Pauson-Khand reaction. *J Org Chem*, **73**(18), 7080-87 (2008)

Vázquez-Romero A, Rodríguez J, Lledo A, Verdaguer X and Riera A. Enantioselective syntheses of carbanucleosides from the Pauson-Khand adduct of trimethylsilylacetylene and norbornadiene. *Org Lett*, **10**(20), 4509-12 (2008)

Research networks and grants

Identificación de inhibidores específicos de la actividad transcripcional de la beta-catenina en cáncer de colon
'La Caixa' Foundation, BM-05-68-0 (2005-2008)
Principal investigator: Antoni Riera

Identificació d'inhibidors específics de l'activitat transcripcional de la beta-catenina en la progressió tumoral
'La MTV3' Foundation, 050630/31/32 (2006-2008)
Principal investigator: Antoni Riera

Síntesis enantioselectiva de moléculas bioactivas mediante catálisis asimétrica: Reacciones de Pauson-Khand, organocatálisis y oxidaciones de Sharpless
Spanish Ministry of Science and Innovation, CTQ2005-00623/BQU (2006-2008)
Principal investigator: Antoni Riera

Collaborations

Asymmetric catalysis
Miquel Pericàs, Institute of Chemical Research of Catalonia (Barcelona, Spain)

Molecular orbital calculations
Santiago Olivella, Spanish National Research Council (Barcelona, Spain)

NMR studies of peptide structures
Maria Macias, IRB Barcelona (Barcelona, Spain)

Synthesis and biological activity of phytoprostanes
Martin Müller, Julius-von-Sachs-Institut of Biosciences, Universität Würzburg (Würzburg, Germany)

Synthesis and biological activity of phytoprostanes
Paul Evans, Trinity College, University of Dublin (Dublin, Ireland)

Synthesis of peptide analogues
Berta Ponsati, Jimena Fernández-Carneado and Marc Gómez, BCN Peptides SL (Barcelona, Spain)

Synthesis of pharmaceutically active compounds
Llorenç Rafecas, Alex Comely and Nicolas Tesson, Enantia SL (Barcelona, Spain)

Synthesis of specific inhibitors of β-catenin
Antonio Garcia de Herreros, Parc de Recerca Biomèdica de Barcelona and Pompeu Fabra University (Barcelona, Spain)

Synthesis of specific inhibitors of β-catenin
Mireia Duñach, Autonomous University of Barcelona (Barcelona, Spain)

Other funding sources

Two contract research projects with Enantia SL and one with BCN Peptides SA through the 'Bosch i Gimpera' Foundation

MÀRIUS RUBIRALTA

Peptides, bioactive heterocycles and enantiomeric recognition

The preparation of new chemical entities (NCEs) is the first and perhaps the most important step in drug discovery. The methodology to design and prepare such compounds in amounts and purities suitable for practical use is therefore critical for any biomedical project. Our group focuses on the development of novel chemical processes that lead to new types of peptide-like molecules and heterocycles (substructures frequently occurring in drugs and natural products), and on the efficient separation of enantiomers, both for analytical and preparative purposes. With the ultimate goal of preparing these bioactive structures, our approach is based on heterocyclic and peptide synthesis. We work on the description of new multi-component reactions (MCRs) based on heterocycles and also use some synthetic constructs as tools to separate enantiomers and other products of high added value by means of several chromatographic and related technologies. Fundamental procedures and methodological research are also tackled in the latter field.

Peptide-like structures for biomedical use and enantioselective separations

We have designed, synthesised and tested several cilengitide® analogues that display potent anti-angiogenic activity. The design is based on the observation that thiazole amino acids, previously prepared in our group, display cell penetration properties of interest, while inducing the structural features suitable for the recognition sequence of the drug. Therefore, using standard solid-phase peptide sequence, we prepared a number of constructs which kept the RGD sequence and novel thiazole amino acids with 1, 2 and 3 heteroaromatic rings (Figure 1). Some of these compounds, which were tested in collaboration with LEITAT, showed potent anti-angiogenic activity comparable to that of the parent compound. Active research is ongoing to determine their potential application in therapeutics.

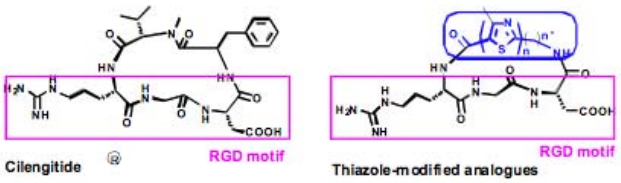


Figure 1. *Anti-angiogenic peptides.*

The capacity of certain synthetic peptides to recognise and stereoselectively differentiate enantiomeric compounds is also one of our focuses of interest. Some polyproline derivatives, previously prepared following solid-phase synthesis, have been bonded to silica gel of distinct formats, either beads or monolithic rods, and their chromatographic behaviour has been studied in response to diverse enantiomeric mixtures. The most outstanding property of these molecules is the increase in the loading capacity attained when the polymeric nature of the polyproline selectors is merged with the monolithic nature of the chromatographic matrix. In such conditions, loading capacity can be 20 times higher than that observed for monomeric chiral selectors containing the

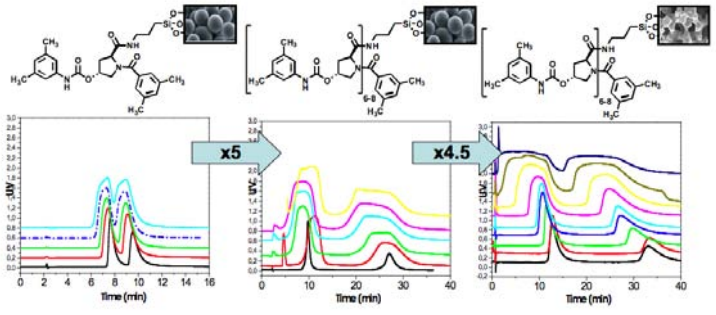


Figure 2. *Loading capacity results.*

Principal Investigator Màrius Rubiralta **Research Associates** Anna Diez, Rodolfo Lavilla, Cristina Minguillón **Postdoctoral Fellows** Mª José Arévalo, Davide Bello, Federica Catti, Nicolas Isambert, Javier Ruiz **PhD Students** Biotza Gutiérrez, Nicola Kielland, Carlos López, Patricia López, Jordi Mas, Anna Pérez, Sara Preciado, Núria Rubio, Miriam Sala, Raquel Sancho, Esther Vicente **Administrative Assistant** Montse Moreno **Visiting Students** Edgar Báguena (Spain), Jan Nubiola (Spain), Alia Pedrosa (Spain), Anna Servat (Spain)

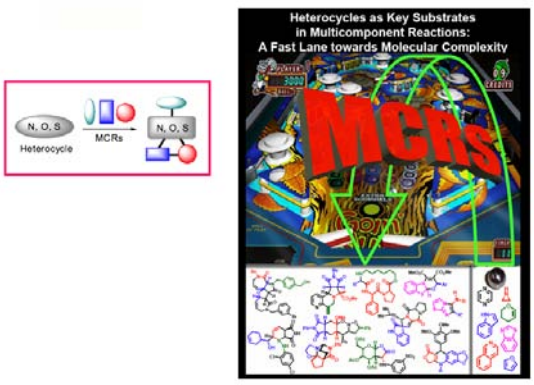


Figure 3. *MCRs based on heterocycles.*

same chiral scaffold (Figure 2). This property is of considerable relevance for the application of these materials to the preparative chromatographic separation of enantiomers. After considering the significance of the bonding sense to the chromatographic matrix (either by the amino or the carboxyl end of the peptide), we have studied the polyproline chiral selectors in solution in order to identify the basis underlying the particular chromatographic behaviour of the chiral chromatographic stationary phases studied. Circular dichroism combined with NMR using diverse solvents allowed us to attribute some of the properties observed to the helicity of these chiral selectors in certain solvents.

Synthesis and reactivity of bioactive heterocycles

We design, test and implement new MCRs involving meaningful heterocycles (pyridines, indoles, oxygen-heterocycles, *etc*). Our main goal is to develop efficient synthetic methodologies for the preparation of bioactive compounds. We have recent-

ly introduced this concept and described the most significant features in an article published in the *Chemistry a European Journal* (Figure 3). The feasibility of the transformations, their capacity to explore wide regions of the chemical space and the availability of the starting materials are the most sought-after properties for these multi-component approaches. These features are critical for the straightforward development of structural activity relationship studies of drug-like compounds, as it takes only one reaction to prepare the new generation of compounds, thus facilitating the hit finding process and also the transition from hits to leads.

We have worked out new multi-component procedures with pyridine derivatives, the most frequently found subunit in drugs, (substituted pyridines, quinolines and other azines, dihydroderivatives, *etc*). During this year, we have developed new processes to address the activation of isocyanides with fluorine-containing anhydrides, which leads to unexpected reactive compounds that display a dipolar structure (Figure 4). In this process up to 7 bonds are formed. Furthermore, given that these entities can be coupled to alcohols and amines and show low toxicity and good cell permeability, preliminary biological studies are now in course. For instance, these dipoles are being evaluated for their potential application in nucleic acid chemistry, in a collaboration project with Ramon Eritja's group (IRB Barcelona).

Regarding the isonitrile reactions, the reported MCR involving dihydropyridines, iodine and isocyanides to yield benzimidazolium salts in one step described last year has found usefulness in biology. In collaboration with Ernest Giralt's group (IRB Barcelona), when screening the library of benzimidazolium salts for inhibitory activity against POP, some compounds qualified as potent and selective agents against this enzyme (Figure 5). This striking result opens new and interesting possibilities to design and prepare novel derivatives with enhanced properties. Research efforts towards the follow-up of this project are ongoing.

In a separate project, although following the same guidelines on synthetic feasibility, we searched for pro-apoptotic agents with potential application in cancer therapy. In collaboration with the groups led by Fernando Albericio (IRB Barcelona) and Joan Gil (University of Barcelona, Bellvitge Institute for Biomedical Research-IDIBELL), we prepared a family of aryl-bisthiazoles in a very short sequence and these compounds were tested against a panel of cancer cell lines. Potent pro-apoptotic activity was detected for some of them. The selected hits were subjected to further studies and were found to be active through a p53-independent mechanism of action (Figure 6). This observation makes these compounds extremely attractive for prospective therapeutic use, as mutations in p53 are present in roughly 50% of all cancers, and these mutations cause many anti-tumoral treatments to fail. The intellectual property rights for these results are currently being addressed through a patent filed by the University of Barcelona's intellectual property office.

The scientific knowledge generated in this research is being applied by pharmaceutical companies through technology transfer agreements via two research contracts developed in our laboratories (collaborations with Almirall and Ferrer Group). In this regard, a paper describing part of this work was published this year (*Synthetic Communications*). Furthermore, we are currently exploring the bioactivity of the scaffolds thus prepared in collaboration with Antoni Riera's group (IRB Barcelona), for targeting β-catenin antagonists, and Pelayo Camps' group (University of Barcelona), for the rapid synthesis of designed acetylcholinesterase inhibitors with anti-Alzheimer's properties. Several compounds displayed potent and selective activities against these targets, and we are now developing structure-activity relationships studies to optimise their biological performance.

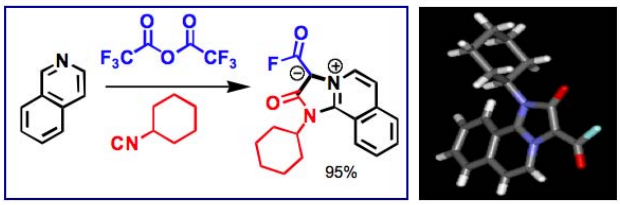


Figure 4. *New MCR based on the activation of isocyanides with TFAA.*

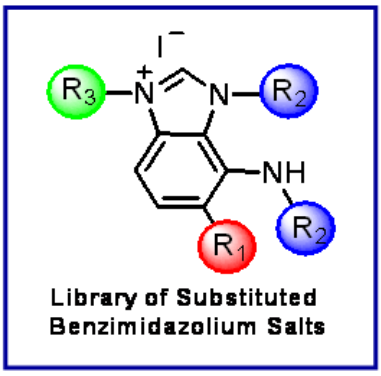


Figure 5. *POP inhibitors based on MCR adducts.*

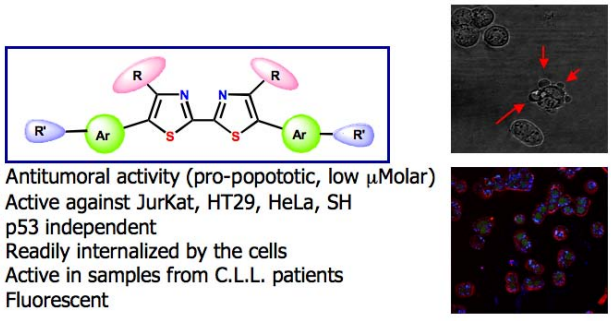


Figure 6. *Pro-apoptotic bis-thiazoles.*

Enantioselective molecular recognition

One of our goals is to demonstrate the applicability of counter current chromatography (CCC) as a preparative tool in the separation of enantiomers. This chromatographic technique involves two liquids and does not need a solid support. To make it enantioselective, a chiral solute must be added to the liquid used as a stationary phase. This solute must be immobilised in the stationary phase and must not be partitioned towards the mobile phase. We have synthesised an analogue of the chiral selector present in the chiral stationary phases commercialised as Whelk-O®. We have introduced a highly lipophilic chain into the structure to prevent partition of the compound to the aqueous mobile phase when it is used as chiral selector in enantioseparation. However, the synthesis provided a racemic mixture that had to be resolved into the enantiomers. This was accomplished using an S-naproxen derivative as chiral selector in CCC. The separation has been optimised, in terms of concentration of the chiral selector in the stationary phase and solvent system used, and scaled up (Figure 7). The results obtained allowed us not only to obtain the desired molecule in its enantiomerically pure form, but also to study the application of the technique. In this context, collaboration with Ian Sutherland at the Brunel Institute of Biotechnology (Brunel University) has proved crucial as he has loaned us a CCC device of low volume. Moreover, one of the members of the group, Núria Rubio, spent several months in that laboratory within the framework of a pre-doctoral stay in which the enantioselectivity of the new chiral selector for diverse chiral drugs was addressed.

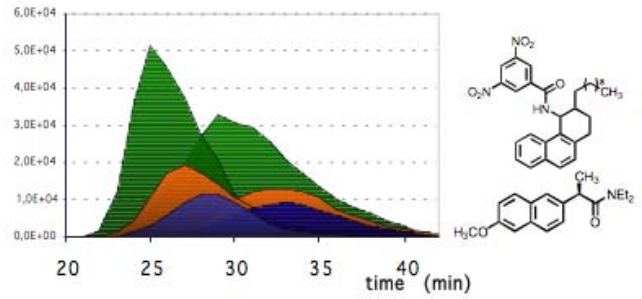


Figure 7. *Preparative separation of enantiomers in CCC.*

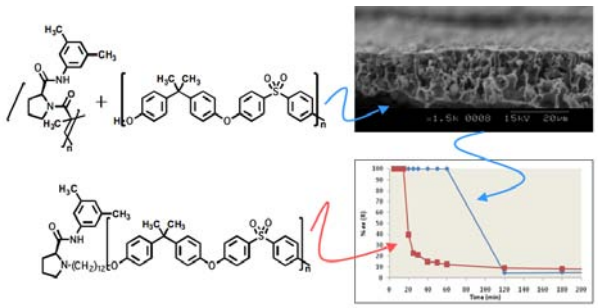


Figure 8. *Enantiomeric excess attained using a composite membrane compared with that obtained using a modified polysulfone.*

L-Proline and (4R)-hydroxy-L-proline derivatives have been studied exhaustively as chiral selectors either in solution (in CCC or bulk liquid membranes) or incorporated into a polymer as chiral carriers. From the application of these derivatives in CCC and using model test compounds as racemates, we have drawn several conclusions that will contribute to our understanding of the technique itself (relationship between concentration of chiral selector in the stationary phase and enantioselectivity or loading capacity, unexpected inversion of elution order with increasing concentration of the chiral selector in the stationary phase, applicability of alternative elution modes). As chiral carriers in membranes, these derivatives showed high enantioselectivity values both in liquid bulk membranes and in diverse formats of solid membranes. The most outstanding result in this context is the maintenance of the high enantiomeric excess for more than one hour in simple transport experiments. This was attained using a composite membrane made of polysulfone, a polymer commonly used in membrane devices, to which microparticles of a polymethacrylate of the chiral selector were added (Figure 8). The studies involving membranes are included in Biotza Gutierrez's PhD thesis, which will be presented in the first semester of 2009.

Our expertise in enantioseparation has allowed us to collaborate with diverse research groups in academia and also in pharmaceutical companies. While collaboration with the former may generate scientific publications (Narcís Avarvari, in press), the latter are often submitted to confidentiality. However, occasionally the collaboration involves other means of knowledge dissemination such as lectures. This year Cristina Minguillón was invited to participate, as an expert in enantioseparation, in a workshop on drug impurities organised by Sigma-Aldrich and addressed to pharmaceutical companies.

SCIENTIFIC OUTPUT

Publications

Arévalo MJ, Kielland N, Masdeu C, Miguel M, Isambert N and Lavilla R. Multicomponent access to functionalized mesoionic structures based on TFAA activation of isocyanides: Novel domino reactions. *Eur J Org Chem*, in press (2008)

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Tarragó T, Masdeu C, Gómez E, Isambert N, Lavilla R and Giralt E. Benzimidazolium salts as small, nonpeptidic and BBB-permeable human prolyl oligopeptidase inhibitors. *ChemMedChem*, **3**(10), 1558-65 (2008)

Research networks and grants

Diseño, síntesis y evaluación biológica de peptidomiméticos de conformación restringida con interés farmacológico
Spanish Ministry of Science and Innovation, CTQ2007-60764 (2007-2009)
Principal investigator: Màrius Rubiralta

Nuevas tecnologías para la separación preparativa de enantiómeros: cromatografía en contracorriente y membranas enantioselectivas
Spanish Ministry of Science and Innovation, CTQ2006-03378/PPQ (2007-2009)
Principal investigator: Cristina Minguillón

Plataforma combiquímica basada en productos naturales: descubrimiento y administración de fármacos
Dirección General de Investigación Científica y Técnica (DGICYT), BQU2006-03794 (2007-2009)
Principal investigator: Rodolfo Lavilla

Química combinatoria per al desenvolupament de nous compostos
Inter-ministerial Council for Research and Technological Innovation (CIRIT), 2005SGR-00662 (2006-2008)
Principal investigator: Rodolfo Lavilla

Other funding sources

Building blocks for lead finding
Almirall Prodesfarma, APF-004 (2007-2009)
Coordinator: Rodolfo Lavilla

Building blocks of interest for the 'Quimiocinas' project
'Bosch Gimpera' Foundation, FBG-302256
Coordinator: Rodolfo Lavilla

Synthesis of bioactive molecules
Ferrer Laboratories, CNV-FERRER-01 (2007-2008)
Coordinator: Rodolfo Lavilla

Collaborations

Applicability of MiniCCC to the separation of enantiomers
Ian Sutherland, Brunel Institute for Bioengineering, Brunel University (Uxbridge, UK)

Development of new inhibitors for Acetylcholinesterase
Pelayo Camps, Department of Pharmacy and Therapeutic Chemistry, University of Barcelona (Barcelona, Spain), Francisco Javier Luque, Faculty of Pharmacy, University of Barcelona (Barcelona, Spain)

Polyproline-derived chiral selectors bonded to monolithic silica gel chromatographic columns
Frantisek Svec, Department of Chemistry, University of California, Berkeley (California, USA)

Preparative enantioseparation of chiral sulfoxides
Narcis Avarvari, Laboratoire Chimie, Ingénierie Moléculaire et Matériaux, Angers University (Angers, France)

Proline derived chiral selectors as monomers in the preparation of monolithic capillary columns and its use in enantioselective electrochromatography
Guillermo Ramis and José Manuel Herrero, Faculty of Chemistry, University of Valencia (Valencia, Spain)

Study of new bis-thiazoles as pro-apoptotic drugs
Joan Gil, Department of Physiological Sciences, University of Barcelona, IDIBELL (Barcelona, Spain)

Synthesis and structure-activity relationships of proline oligopeptidase inhibitors
Ernest Giralt, IRB Barcelona (Barcelona, Spain)

Synthetic methodology. Synthesis of bioactive compounds. Nanotechnology
Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Other collaborations

Amirall Laboratories (Barcelona, Spain)

Ferrer International Group (Sant Cugat, Spain)

Ferrer Laboratories, Ferrer Group (Barcelona, Spain)

LEITAT (Barcelona, Spain)

PharmaMar (Madrid, Spain)

Sigma-Aldrich (Madrid, Spain)

XAVIER SALVATELLA

Early and late stages in protein deposition diseases

A number of prevalent diseases, such as Alzheimer's and Parkinson's disease, involve large conformational transitions in proteins and the formation of amyloid fibrils. In some of these diseases, intrinsically disordered polypeptides form amyloid fibrils in the brains of patients while in others the triggering factor is the partial unfolding of an otherwise globular protein to form aggregation-prone species. Most of these diseases remain incurable as a result of a lack of understanding of the biophysical principles that determine the conformation, interactions and toxicity of partially folded proteins and of the biochemical processes in place for the regulation of their concentration. The Laboratory of Molecular Biophysics seeks to elucidate, at atomic resolution, the mechanism of the key pathogenic processes in protein deposition diseases with the aim to provide opportunities for therapeutic intervention. Key goals of the laboratory include the following: (i) the identification of the residues of the intrinsically disordered amyloid beta (Aβ), which are responsible for the establishment of the intermolecular interactions that lead to oligomer formation in Alzheimer's disease; (ii) the rationalisation of the effect of mutations on the stability of human lysozyme in non-neuropathic systemic amyloidosis; and (iii) the identification of the biophysical properties that underlie oligomer and amyloid fibril cytotoxicity in a number of protein deposition diseases.

Beyond single structures: The simultaneous determination of the structure and the dynamics of proteins

Study of the conformation of the species involved in the transitions associated with protein deposition diseases (partially folded proteins, intrinsically disordered proteins and oligomers) is challenging because these species are structurally heterogeneous, *ie*, they cannot be described by a single, average structure and must instead be described by a native ensemble of structures, which, on average, has properties that are compatible with experimental data.

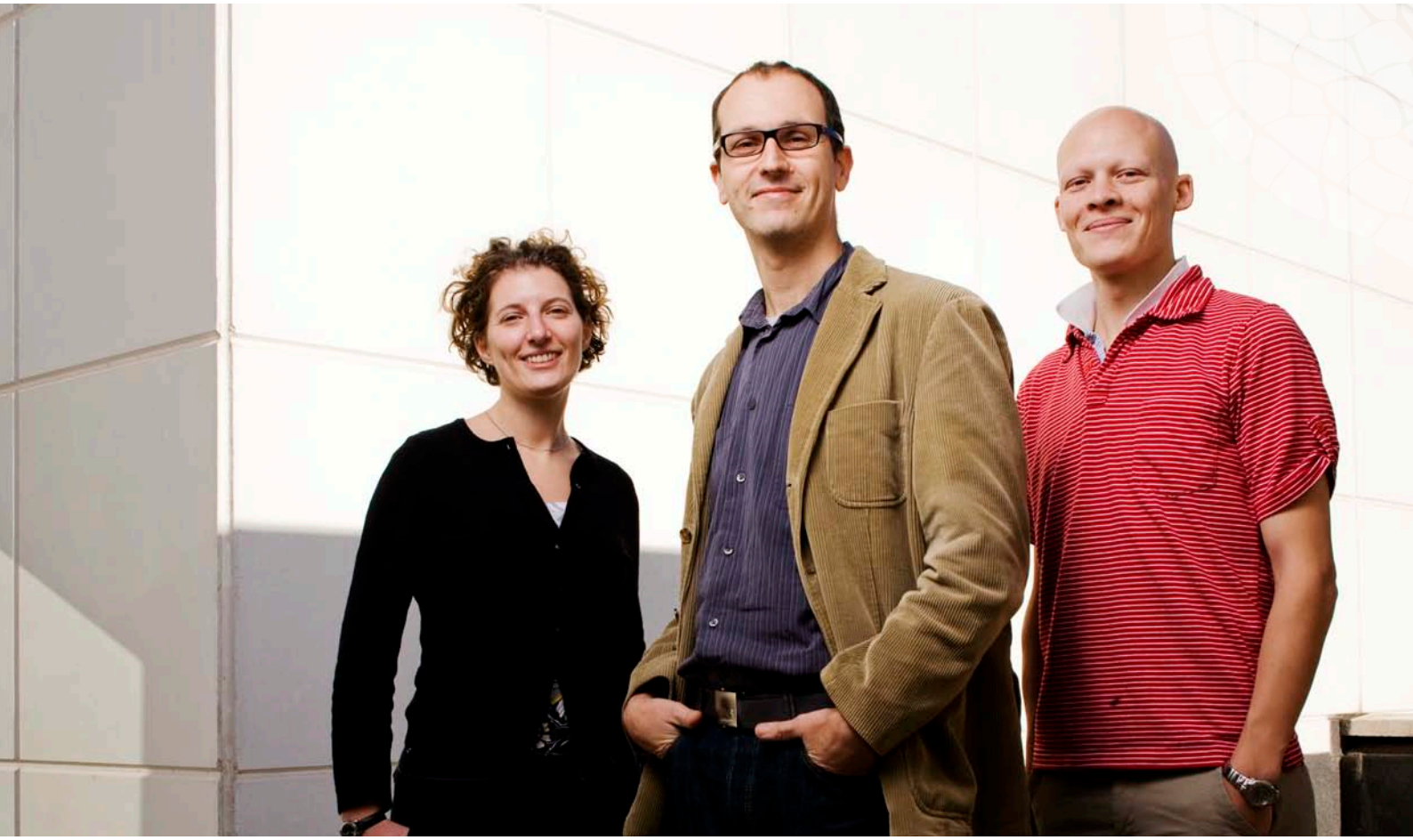
These ensembles can in principle be obtained by using ensemble or time-averaged molecular simulations with constraints derived from Nuclear Magnetic Resonance (NMR) experiments. However, in the first implementations of these approaches it was realised that the NMR data that were then available as restraints, mostly Nuclear Overhauser Effects (NOEs), suffered intrinsic shortcomings that rendered the methods prone to over-fitting (or under-restraining), *ie*, that the geometrical information contained in NOEs was insufficient to allow for the simultaneous determination of the structure of all members of the native ensemble.

Residual Dipolar Couplings (RDCs), which can be measured between pairs of nuclei in proteins that present rotational anisotropy, provide angular information not encoded in conventional NMR restraints and are sensitive to the amplitude of protein motions in the sub-ms time-scale. The availability of such observable NMR makes it possible, for the first time, to determine native ensembles without over-fitting.

During 2008 the Laboratory of Molecular Biophysics has been very active in the development and optimisation of computational methods for the simultaneous determination of the structure and the dynamics of proteins in terms of native ensembles. In collaboration with Griesinger's group (Max Planck Institute for Biophysical Chemistry), which kindly provided us with a very large set of RDCs measured for the protein ubiquitin, we have determined a native ensemble that is in unprecedented agreement with all experimental data available for this protein.

We determine the ensembles using restrained ensemble molecular dynamics simulations, where we simultaneously simulate several (typically 32 or 64) copies of the protein molecule and ensure that at each time-step the simulated ensemble is consistent with the RDCs measured for this protein. We have carried out a thorough validation of this approach by assessing its capac-

Principal Investigator Xavier Salvatella **Postdoctoral Fellows** Robert Brynmor Fenwick, Maria Francesca Mossuto



ity to reproduce the distribution of inter-nuclear distances in a reference ensemble produced using unrestrained simulations. The results show that the agreement is highly satisfactory, *ie*, that the method is successful at capturing the fluctuations and that the resulting ensemble is a relatively accurate sample of the Boltzmann ensemble of the protein (De Simone *et al*, in press, 2008).

Understanding binding allostery and folding cooperativity: Correlated motions in the β-sheet of ubiquitin

The recent availability of native ensembles allows the analysis of dynamic properties that could until now be studied only using theoretical methods. We have used the high-resolution native ensemble that we have determined for ubiquitin to carry out a detailed analysis of the presence of correlated motions in the backbone of this protein. By studying the statistical independ-

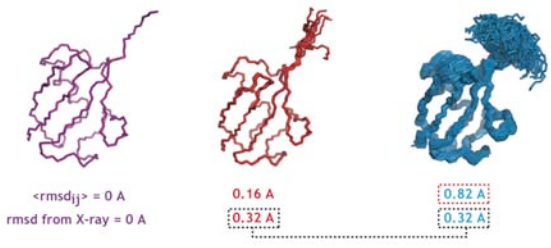


Figure 1. *Comparison of the native ensemble determined for the protein ubiquitin (blue) with the X-ray structure (purple - pdb code 1ubq) and with the conventional NMR structure (red - pdb code 1d3z). The average structure of the native ensemble is in as good agreement with the X-ray structure as the conventional NMR structure.*

ence of the φ and ψ backbone torsions of all possible pairs of residues, we have identified the crankshaft motion that anticorrelates the ψ(i) and φ(i+1) of consecutive residues as a result of the partial double bond character of the amide bond, and a very significant correlation between residues that are far in sequence but connected by hydrogen bonds in the β-sheet.

We have found that the degree of correlation is related to the strength of the hydrogen bonds, as assessed by trans-hydrogen bond scalar couplings measured by NMR, to the stability of the different elements of secondary structure in the partially folded state (the A state) that ubiquitin forms in aqueous methanol, and to the structure of the transition state for folding, as determined by protein engineering methods and expressed in φ-values.

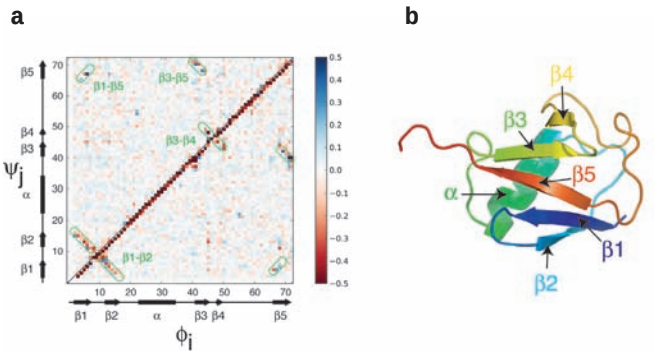


Figure 2. *(a) Plot of the degree of correlation between the fluctuations of the ϕ and ψ torsion angles of the residues of ubiquitin in the sub-ms timescale. Zones in the correlation plot that correspond to pairings of β-strands have been highlighted in green to facilitate comparison with the average solution structure of this protein, presented in (b).*

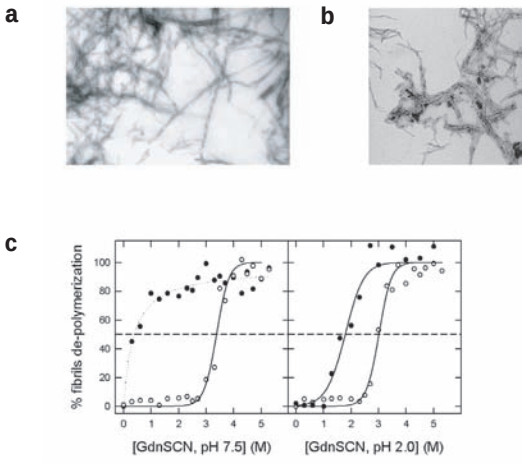


Figure 3. *(a) TEM image of fibrils formed at pH 2, where the structure of lysozyme is unfolded. (b) TEM image of fibrils formed under physiological pH, where the protein is only partially unfolded. (c) Comparison of the stability of the two morphologies: under all conditions, the fibrils formed by physiological, partially folded lysozyme are metastable.*

The results that we have obtained with ubiquitin are relevant because they provide a pathway for the relay of conformational change across the structure of the protein; such a relay is a necessary condition for binding allostery and folding cooperativity but had been very challenging to characterise experimentally until now. We anticipate that the determination of native ensembles and their analysis in terms of correlated motions will provide key insights into these important biological phenomena. Most importantly, we consider this approach a promising avenue for the study of the break-down of folding cooperativity that underlies a number of protein deposition diseases.

Influence of frustration on amyloid formation: Implications for cytotoxicity

Protein sequences have evolved to efficiently fold into the well-packed and uniquely defined structures that characterise native states. Although dynamic, native structures are remarkably resistant to changes of sequence, solution conditions, temperature and concentration. This robustness, summarised in Anfinsen's dogma that the native structure of the protein is determined uniquely by its sequence, is an important property that contributes, as do other relevant biological processes such as proteostasis, to the capacity of organisms to withstand significant changes in environment.

In contrast to native structures, the oligomeric species involved in protein deposition diseases form in a process that can involve the population of a significant large number of long-lived intermediate states and leads to the formation of amyloid fibrils, which are, contrary to native states, not uniquely defined. Indeed, it is possible for the same polypeptide sequence to lead to the formation of stable structurally distinct amyloid fibrils with very different biophysical and biomedical properties. In the Laboratory of Molecular Biophysics we are carrying out a detailed analysis of such polymorphism in the fibrils formed by the human lysozyme protein.

Human lysozyme forms amyloid fibrils in the disease lysozyme non-neuropathic systemic amyloidosis, in which the fibrils accumulate in the spleen and liver of patients that present destabilising mutations in the β domain of this protein. We have found that solution conditions can have a dramatic impact on the properties of the amyloid fibrils. Fibrils formed at low pH and room temperature appear very structured under the microscope, are rich in β secondary structure and show high stability to de-polymerization by chaotropic agents such as guanidinium salts; fibrils formed under physiological conditions and high temperature are much less structured, poor in secondary structure and much less stable to de-polymerisation. We have evidence that this polymorphism is not due to differences in the quaternary structure of the protein in the fibrils but, rather, that it is associated with a significantly different secondary and tertiary structure at the monomer level.

Most importantly, we have found that the fibrils formed at low pH are more stable than those formed under physiological conditions even when conditions are exchanged, thereby providing direct evidence that amyloid formation under physiological conditions is under kinetic control, *ie*, that the amyloid fibrils that form are in a kinetic trap. In addition, we have also carried out a study to determine whether these two morphologies can be made to grow in conditions that differ to those used for their formation. In this regard, we have found that, contrary to what is often observed in intrinsically disordered proteins such as Aβ, there is no propagation of the conformation of the seed.

Our results are compatible with an oligomerisation mechanism that is largely determined by the degree of structure present in the precursor state. In conditions in which the protein is completely unfolded, it can form the amyloid fibrils of highest stability. In contrast, in conditions in which it is partially folded, only a fraction of the sequence is available for the formation of the intermolecular interactions that lead to oligomerisation, thereby yielding fibrils with a fraction of the amyloid structure and a fraction of the stability of the fully formed fibrils.

Conclusion

The approach that we use in the Laboratory of Molecular Biophysics involves the development of computational methods for the analysis of NMR data to provide high-resolution information about the correlated dynamics of proteins and the use of biophysical methods to study the conformational variability in amyloid fibrils. This combination provides a very powerful tool with which to study key conformational transitions in protein diseases.

SCIENTIFIC OUTPUT

Publications

Calloni G, Lendel S, Campioni S, Gianinni S, Gliozzi A, Relini A, Vendruscolo M, Dobson CM, Salvatella X and Chiti F. The structure and dynamics of a partially folded protein are decoupled from its mechanism of aggregation. *J Am Chem Soc*, **130**(39), 13040-50 (2008)

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Research networks and grants

Amyloid hot spots
European Commission, FP7-PEOPLE-IEF-2008-2367 (2008-2010)
Principal investigator: Xavier Salvatella

Collaborations

Determination of native ensembles for Aβ and in Alzheimer's disease and identification of the earliest stages of oligomerisation
Christopher Dobson, Department of Chemistry, University of Cambridge (Cambridge, UK)

Methods for the determination of the structure and dynamics of proteins using chemical shifts
Michele Vendruscolo, Department of Chemistry, University of Cambridge (Cambridge, UK)

Methods for the simultaneous determination of the structure and dynamics of native proteins using Residual Dipolar Couplings. Identification of correlated motions between residues far in sequence in the β-sheet of the protein ubiquitin
Christian Griesinger, Max Planck Institute for Biophysical Chemistry (Göttingen, Germany)

Study of the structural determinants of the cytotoxicity of the oligomers formed by HypF-N, a model protein, in amyloidogenic conditions
Fabrizio Chiti, Department of Biochemical Sciences, University of Florence (Florence, Italy)

Oncology Programme

EDUARD BATLLE

Wnt signalling and EphB-ephrin interactions in intestinal stem cells and CRC progression

A wealth of data has revealed an essential role for Wnt signalling in the maintenance of mammalian intestinal stem cells (ISCs) as well as in colorectal cancer (CRC; reviewed in Van der Flier and Clevers, 2008). Mice engineered to lack Wnt signalling in the intestinal epithelium lose the crypt progenitor compartment. Conversely, constitutive activation of the Wnt pathway results in a massive expansion of crypt progenitor/stem cell numbers and the onset of intestinal tumorigenesis. Most human CRCs are initiated by mutations in the tumour suppressor gene APC, which switches on the Wnt pathway in a constitutive fashion. Remarkably, most Wnt target genes induced by APC mutations in intestinal tumours are physiologically expressed in crypt ISCs and/or in transient amplifying progenitor cells. Recently, the tumour-initiating potential of several crypt cell populations has been assessed (Barker *et al*, 2008). These studies have revealed that deletion of the APC gene in mouse ISCs triggers tumour formation with high efficiency whereas transient amplifying progenitor cells or differentiated cells are relatively resistant to Wnt-driven transformation. Thus, it appears that some specific features of ISCs are required to initiate CRC. Overall, these data have led to the notion that Wnt signalling sustains the expression of the crypt ISC gene programme, which, upon mutational activation of the Wnt pathway, is constitutively imposed on tumour-initiating cells (*ie*, on stem cells and perhaps also on early progenitor cells; Van de Wetering *et al*, 2002; Barker *et al*, 2007; Van der Flier and Clevers, 2008).

The functional analysis of Wnt target genes identified in CRC cell lines revealed that beta-catenin/Tcf dictate different sets of instructions that collectively regulate the biology of crypt progenitor/stem cells and also intestinal tumours. So far, three independent gene modules have been identified (reviewed in Batlle and Clevers, 2006):

- The core module enforces the undifferentiated-proliferative phenotype of crypt progenitor/stem cells and of CRC cells. Blockage of beta catenin/Tcf-mediated transcription in fully malignant CRC cell lines results in cell cycle arrest and differentiation. Strong evidence suggests that c-Myc acts as master regulator within this gene module.

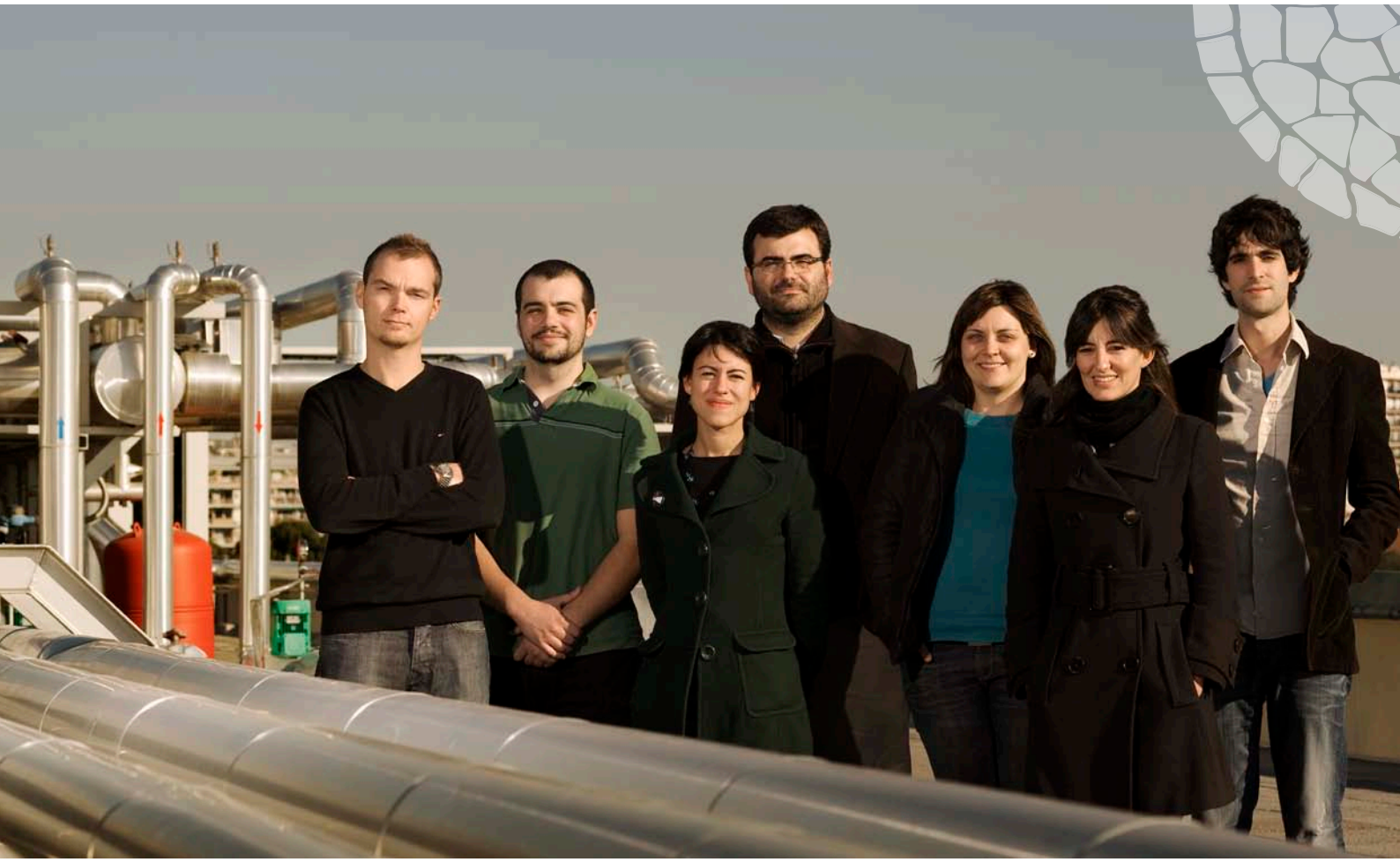
- The second module of the beta-catenin/Tcf programme is required for Paneth cell maturation. Paneth cells are a secretory cell type localised close to the bottom of the crypts that receive physiological Wnt signals.

- The third module controls the positioning of epithelial cells along the crypt axis and regulates their ordered migration. The main effectors of this function are the beta-catenin/Tcf targets EphB2 and EphB3, two members of the Eph family of receptor tyrosine kinases. Eph receptors and their cognate ligands, ephrins, are well-known mediators of cell repulsion during embryo development. We demonstrated that EphB2/-B3-deficient mice show a range of defects in intestinal cell compartmentalisation, including the loss of the tight boundary between the differentiated and proliferative cell compartments, abnormal migration of progenitor cells along the crypt axis and mispositioning of Paneth cells (Batlle *et al*, 2002).

EphB receptors as suppressors of CRC progression

Most early CRC lesions in humans showed homogenous EphB2, EphB3 and EphB4 expression as a result of mutational activation of the Wnt pathway. Strikingly, we observed that the expression of all three EphB receptors is silenced around the adenoma-carcinoma transition in most intestinal tumours. We proved a causal role for EphB silencing in CRC progression by engineering mice in which the *Apc*^{min} mutation was placed in

Principal Investigator Eduard Batlle **Postdoctoral Fellows** Anna Merlos, Guiomar Solanas
PhD Students Francisco Barriga, Carme Cortina, Gavin Whissell **Research Assistants** Sergio Palomo, Nerea Peiró **Lab Technician** Javier Hernando



a genetic background with low EphB activity. In the absence of EphB activity, tumour progression in the large intestine of *Apc*$^{min/+}$ mice is strongly accelerated, resulting in the development of aggressive colorectal adenocarcinoma (Batlle *et al*, 2005). Thus, over several years of malignization, the original tumour-initiating gene programme is refined. While CRC cells retain certain characteristics of progenitor/stem cells, not all the instructions codified by the beta-catenin/Tcf4 programme promote tumorigenesis. Instead, some of these instructions, such as the positional information imposed by EphB receptors, block the acquisition of malignancy beyond the earlier stages.

Over the last three years, our lab has studied the mechanism of EphB-mediated tumour suppression in the intestine. We have generated *in vitro* models that mimic EphB/ephrinB interactions in CRC. We have taken advantage of CRC cell lines that do not express EphB receptors or ephrinB ligands to generate two populations of the same cell line that express either EphB (plus GFP) or ephrinB (plus RFP) molecules. Co-culture of EphB- and ephrinB-expressing cells resulted in cell contact-mediated EphB-ephrinB bi-directional signalling. Analysis of cell dynamics in this *in vitro* model revealed that EphB signalling induces repulsion and compartmentalises the growth of CRC cells by enforcing E-cadherin adhesion.

We also demonstrated that tumour compartmentalisation occurs *in vivo*. In *Apc*$^{Min/+}$ mice, EphB+ tumour cells that form incipient adenomas are in continuous contact with normal intestinal epithelial cells expressing ephrinB ligands. Through the use of mouse models deficient in EphB or ephrinB ligands, we showed that *Apc* mutant tumour founder cells cannot colonise the regions of the normal epithelium that express high levels of ephrinB1 because of EphB repulsive signals. We have proposed

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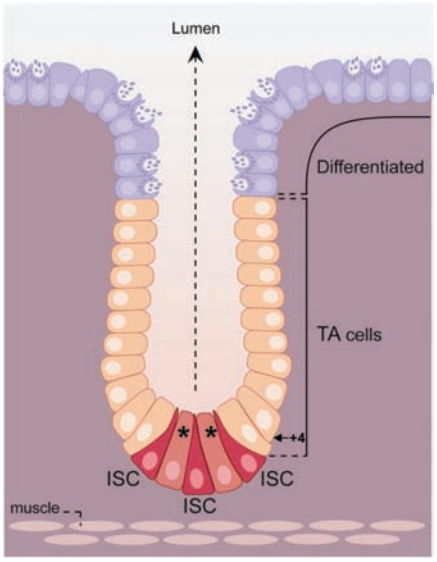

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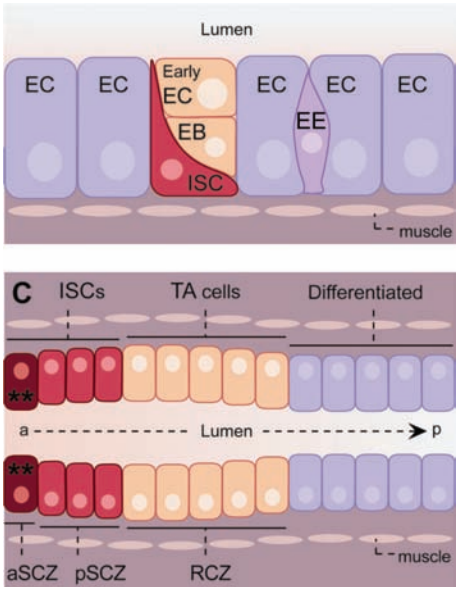

Figure 1. *The mammalian and Drosophila intestinal epithelium. Organisation of a mammalian colon crypt (a), the Drosophila midgut (b) and hindgut (c) epithelium. The colour code is kept in the three schemes to indicate identity between cell types, ISCs being shown in red, progenitor cells in light orange and differentiated cells in blue. Cells intermingled between ISCs in A (labelled with asterisks) represent Paneth cells in the small intestine or other secretory types in the colon which localise at the crypt base. In the fly hindgut, ISCs marked with two asterisks are Wg-secreting cells that localise in the anterior region of the Spindle Zone. Arrow indicates the direction of migration and cell renewal, ie, from the base towards the lumen in crypts, or in an anterior (a) posterior (p) fashion in the hindgut. ISC; intestinal stem cells. TA; transient amplifying. EB; enteroblast. EC; enterocyte. EE; enteroendocrine cells. aSCZ and pSCZ; anterior or posterior Spindle Zone respectively. RCZ; Round Cell Zone.*

that tumour cell compartmentalisation is a general mechanism of tumour suppression in tissues whose architecture is defined by Eph-ephrin interactions. Overall, our observations imply that fully malignant CRC cells bearing multiple mutations in oncogenes and tumour suppressors respect the boundaries imposed by EphB-ephrinB interactions (Cortina *et al*, 2007).

Intestinal stem cells

In mammals, the inner lining of the intestinal tube is a monostratified epithelium folded into millions of invaginations known as crypts (Figure 1a). Each crypt represents a cell production factory that contributes to the constant renewal of the epithelial layer. The tremendous regenerative power of the mammalian intestinal epithelium is reflected by the magnitude of cell production in the crypts: the small intestine of an adult mouse contains roughly a million crypts, each producing around 300 cells per day, which together generate an estimated 300 million cells every day of their life.

This remarkable process is ultimately sustained by a small population of stem cells (4-6 cells) that reside at the base of each crypt. The progeny of mammalian ISCs does not differentiate immediately but rather it is amplified by cell division during a process of continuous upward migration along the crypt axis. Around 150 undifferentiated cycling progenitor cells or Transient Amplifying (TA) cells occupy the crypt length. Progenitor cells divide with fast kinetics (about 1 division every 12 hours). Cell cycle arrest and functional differentiation occur as migrating TA cells reach the upper part of the crypt. Three differentiated cell types populate the intestinal tract; mucosecreting, enteroendocrine and absorptive cells. The small intestine contains an additional secretory cell type, Paneth cells, which localise at the bottom-most positions of the crypt (Figure 1).

The location and the precise identity of mammalian ISCs are controversial issues due to the lack of specific marker genes and assays to study their properties (reviewed in Batlle *et al*, 2008). In a pioneering study, Hans Clevers and colleagues have recently identified bona-fide gastrointestinal stem cells at the bottom-most positions of the stomach, small intestine and colon crypts (Barker *et al*, 2007). ISCs in the intestinal tract can be specifically recognised by the expression of Lgr5, a Wnt-target gene that codifies for an orphan G-protein coupled receptor of unknown function. Lgr5+ ISCs are multipotent, divide approximately once every day and are capable of regenerating the intestinal epithelium for long periods (>12 months).

To assess the gene programmes that operate in ISCs, we have recently developed a method to purify crypt cell populations. To this end, we used EphB2 as a surface marker, a Wnt target gene expressed in gradient from the crypt base to the surface epithelium (Batlle *et al*, 2002). FACS sorting of cells expressing different levels of EphB2 has allowed us to obtain the expression profiles of ISCs (Figure 2), TA cells and differentiated cells. These expression profiles are instrumental tools to understand the biology of the crypt ISCs as well as their role in the initiation and progression of CRC.

In addition to the work on mammalian ISCs, we have recently

started a collaboration project with Jordi Casanovas and Andreu Casali (IRB Barcelona) to analyse adult ISCs in *Drosophila*. The stem cells of the *Drosophila* midgut lie in a basal position relative to the rest of the epithelial cell types and show a wedge-like morphology that resembles that of mouse Lgr5+ cells (Figure 1b). Unlike in mammals, midgut ISCs are the only known cell type in the posterior midgut that proliferates, as their progeny is not further amplified. Upon cell division, the descendants of midgut ISCs regenerate the stem cell pool and/or become quiescent progenitor cells (known as enteroblasts or EB cells), which ultimately differentiate to absorptive (AC) or enteroendocrine (EE) cells (reviewed in Casali and Batlle, 2009).

Recent work has shown that, in a similar fashion to that of the mammalian intestinal epithelium, Wnt and Notch signalling play essential roles in the specification and maintenance of midgut ISCs in *Drosophila* (reviewed in Casali and Batlle, 2009). We are currently examining the extent to which the *Drosophila* midgut intestine represents a good model to study the role of ISCs in intestinal cancer.

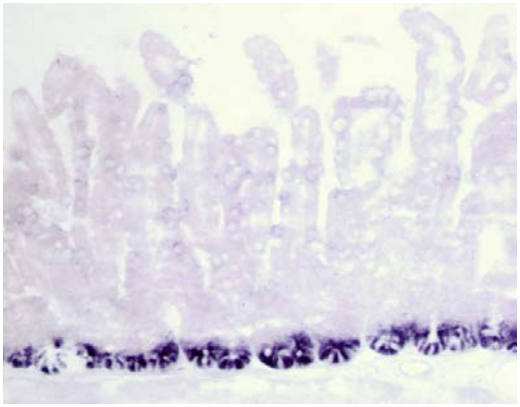

Figure 2. *In situ hybridisation of an ISC marker gene, OLFM4. Cells detected by OLFM4 probe at the crypt base (in blue) correspond to ISCs.*

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Cancer biology
Spanish Ministry of Science and Innovation, CSD2007-00017 (2007-2012)
Principal investigator: Eduard Batlle

CRC programme
ERC Starting Grant (StG), 208488 (2008-2013)
Principal investigator: Eduard Batlle

ELENA SANCHO

Molecular mechanisms involved in the initiation and progression of colorectal cancer

Colorectal cancer (CRC) is the third most common type of cancer and the second cause of death by cancer in the Western world. It causes around 650,000 deaths worldwide per year. Most sporadic colorectal cancers arise from adenomas that initially are benign and occur frequently: approximately 50% of the Western population develops an adenoma by the age of 70. However, the development of a full-blown malignant colorectal tumour is a progressive process that often takes several years. During this period, the progression of the disease appears to follow a precise series of molecular events, requiring the accumulation of mutations in proto-oncogenes and tumour suppressor genes in these initially benign lesions. Access to specimens of CRC at different stages of the malignancy has allowed the analysis of the molecular alterations most frequently associated with each step of the disease (reviewed in Sancho *et al*, 2004). The aim of the research in our laboratory is to decipher the molecular instructions that underlie the signalling pathways that are altered in CRC and that are responsible for the initiation and progression of the disease.

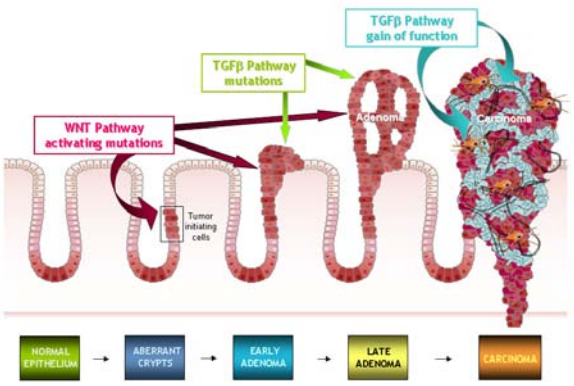

Figure 1. *Genetic alterations frequently associated with CRC progression and main signalling pathways. The accessibility to specimens of CRC at different stages of the malignancy has allowed the analysis of the molecular alterations most frequently associated with each step of the disease. Our research is focused mainly on elucidating the mechanisms of CRC initiation by activating Wnt signalling mutations, and on studying the dual role played by TGF-beta signalling at later stages of the disease. TGF-beta signalling is lost in CRC epithelial cells through the acquisition of mutations as the disease progresses, but there also appears to be a gain of function in the stromal component of the tumour, which we are currently investigating.*

Wnt signalling and the initiation of CRC

Around 70% of sporadic colorectal tumours show bi-allelic inactivation of the APC gene (Adenomatous polyposis Coli). A high percentage of remaining tumours show activating mutations in beta-catenin or axin. These molecules are components of the Wnt signalling pathway. Activating mutations of this signalling pathway are the only known genetic alterations present in early premalignant lesions in the intestine, such as aberrant crypt foci and small adenomas. In various animal models, activating mutations in this pathway effectively initiate tumorigenesis in the intestine in a process characterised by the formation of displastic cripts and adenomas similar to those found in humans. Therefore, it is widely accepted that constitutive activation of Wnt signalling caused by mutations in components of the pathway are responsible for the initiation of CRC (reviewed in Sancho *et al*, 2004; see Figure 1).

Mutations in Wnt signalling components that lead to CRC result in the stabilisation and accumulation of beta-catenin in the nucleus, and as a result in increased transcriptional activation mediated by the beta-catenin/TCF complex. Therefore, the transactivation of beta-catenin/TCF target genes is a primary transforming event in CRC. A few years ago we identified the genetic programme driven by beta-catenin and TCF in CRC cells. Our studies indicated that beta-catenin/TCF target genes are expressed not only in tumours but also in healthy non-transformed intestinal progenitor cells at the bottom of the crypts (van de Wetering *et al*, 2002; see Figure 2).

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PhD Student Elisa Espinet **Research Assistant** Sergio Palomo

Our results, together with those obtained from several animal models in which Wnt signalling was genetically manipulated (Pinto *et al*, 2003; Korineck *et al*, 1998), implied that the stem cell and progenitor compartments were controlled by Wnt signalling. These findings led us to propose that the first step towards malignancy in CRC consists of the acquisition of a crypt progenitor-like phenotype (van de Wetering *et al*, 2002). Our hypothesis has marked a milestone in the field and has completely changed the view on the initiation of CRC. We are currently developing animal models that will formally prove this concept and help to shed light on the mechanisms behind why Wnt signalling mutations are an important pre-requisite for the development of CRC.

Our studies are now oriented towards the identification of the nature of the founding CRC cells and the mechanisms by which they escape cell renewal. During 2008, we have generated several DNA constructs to allow the development of animal models that will be used for this purpose. These include the conditional expression in the intestine of oncogenes involved in CRC combined with colour markers that will help to identify mutant cells. These studies may shed additional light on specific pathways that can be targeted to block CRC progression.

Having identified that the initial event triggering transformation is the blockage of founder tumour cells into a progenitor phenotype, our lab now seeks to identify differences between the true physiological progenitors and initial founder mutant cells. To this end, during 2008, in collaboration with Eduard Batlle's lab (IRB Barcelona), we have developed a protocol which allows the isolation of epithelial cells from the bottom of colonic crypts (*ie*, stem cells and early progenitors) from fresh tissue. This protocol is also applicable to the isolation of tumour cells from early adenomas or displastic crypts. We are currently comparing the genetic profile of physiological progenitors with that of tumour cells from adenomas. We aim to identify tumour-specific molecular targets susceptible to being targeted by the pharmaceutical industry. These will be useful, particularly for patients suffering Familial Adenomatous Polyposis (FAP). These patients inherit a mutation in APC, and as a result of loss of heterozygosity (LOH) they develop hundreds of polyps in the intestinal tract and are therefore predisposed to the development of malignant CRC.

TGF-beta signalling during CRC progression

Our lab also addresses how the acquisition of mutations in other signalling pathways may modulate the initial progenitor phenotype imposed by Wnt signalling to overcome the bottle-

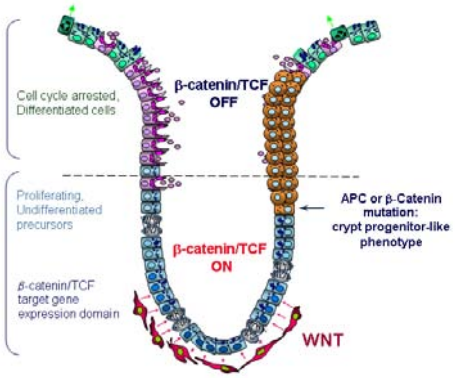

Figure 2. *Schematic representation of a colon crypt in relation to wnt signalling and proposed model for the initiation of CRC. The intestinal epithelium is organised in a series of invaginations called crypts of Lieberkühn. Epithelial cells within these crypts are in constant renewal. This is achieved by a small group of stem cells that reside at the bottom of the intestinal crypts. Stem cells divide asymmetrically to give rise to early progenitors that rapidly divide whilst migrating towards the intestinal lumen. As they do so, they become predetermined towards the differentiation into one of the functional cell types present in the intestine (adsorptive, mucosercreting or enteroendocrine cells). Differentiation takes place in the top part of the crypts. The proliferative compartment of intestinal crypts is maintained by the target gene programme directed by beta-catenin/Tcf in response to wnt signals. When these cells physiologically down-modulate beta-catenin/Tcf activity, they cease to proliferate and differentiate. Cells mutant in components of wnt signalling (APC, beta-catenin, axin) become independent of these signals, exhibit constitutive activation by beta-catenin/Tcf and are blocked in a progenitor proliferative phenotype.*

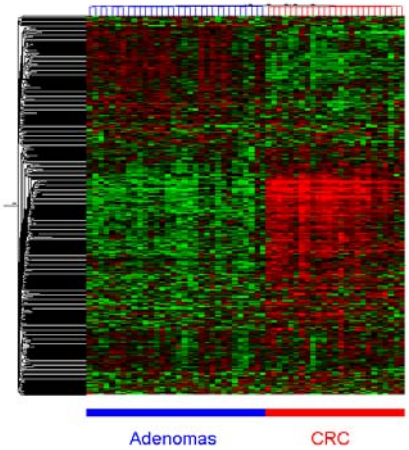

Figure 3. *The TGF-beta responding signature (TBRS) is differentially expressed between adenomas and adenocarcinomas. Unsupervised clustering analysis of a collection of tumours of known transcriptomes on the basis of target genes controlled by TGF-beta signalling.*

necks associated with CRC progression. One of the most prevalent mutations found during CRC progression are those inactivating the TGF-beta signalling pathway (reviewed in Grady and Markowitz, 2003; Figure 1). The TGF-beta pathway is involved in numerous processes in development and homeostasis of adult tissues. TGF-beta ligands activate the signalling pathway by binding to TGF-beta receptor type II homodimers. Ligand-bound receptor II recruits TGF-beta receptor I homodimers, which are subsequently transphoshorylated and thus activated by receptor type II. Phosphorylation of the intracellular mediators smads by activated receptor I allows dimer formation with smad-4 and translocation to the nucleus, where the specific outcome of the signalling will depend on the cell type and the context of the cell itself (reviewed in Shi and Massague, 2003).

Around 80% of all microsatellite instable CRCs contain mutations in type-II TGF-beta receptor (TGFBR2) that impair signalling. In addition, inactivation of downstream TGF-beta pathway effectors, in particular SMAD4 and SMAD2, have also been found in a significant fraction of microsatellite stable CRCs. Overall, the incidence of TGF-beta resistance in CRCs appears to be around 30% (reviewed in Grady and Markowitz, 2003). In addition, virtually all CRC cell lines have lost their TGF-beta response. Modelling CRC progression in mice has revealed that disruption of TGF-beta signalling in the intestinal epithelium does not initiate intestinal tumorigenesis per se (Biswas *et al*, 2004; Munoz *et al*, 2006). However, when the onset of CRC is triggered by deficiency of the tumour suppressor APC, compound Tgfbr2 (Munoz *et al*, 2006) or Smad4 (Takaku *et al*, 2004), null alleles accelerate adenoma to carcinoma progression in the intestinal tract. Collectively, the data described above strongly support the notion that TGF-beta signalling suppresses CRC. This is in accordance with data obtained for solid tumours, such as breast cancer, prostate cancer and skin tumours, among others, which have lead to the general belief that TGF-beta acts as a tumour suppressor in the initial stages of carcinogenesis. However, several studies have suggested additional roles for TGF-beta in CRC progression. The expression of TGF-beta increases in late stage CRCs (Tsushima *et al*, 1996), and TGF-beta serum levels are associated with disease progression and predict recurrence and metastasis in CRC patients (Robson *et al*, 1996; Tsushima *et al*, 2001).

Our lab currently focuses on the role of TGF-beta signalling in CRC progression. For many years, tumorigenesis was studied from the perspective of tumour cells alone. Recently, much attention has been given to the contribution of the stromal component of solid tumours during disease progression. The tumour microenvironment is a complex mixture of cell types that includes fibroblasts, immune cells, blood vessels and a multitude of factors. The control of stromal changes within a developing tumour has become a major topic of research in oncology that has drawn the attention of some of the leading groups in cancer. We are studying the transcriptional events controlled by TGF-beta in CRC cells as well as in stromal cells. We have identified changes in approximately 500 genes in response to TGF-beta in intestinal fibroblasts and have studied the modulation of the stromal TGF-beta-controlled gene programme during CRC progression. Remarkably, the TGF-beta responding signature (TBRS) obtained from fibroblasts is differentially expressed between adenomas and adenocarcinomas, thereby implying that these

genes may contain the information that drives the adenoma/carcinoma transition.

Our lab is devoted to dissecting this information in order to identify TGF-beta genes that play an executive role in the adenoma/carcinoma transition. Overall, we are performing detailed analysis of the observed gain of function in TGF-beta signalling during CRC progression, particularly regarding the stromal component of the tumour (Figure 1). We are currently characterising TGF-beta target genes that show strong classifying capacity between adenomas and carcinomas present within the f-TBRS that could have a potential role in tumorigenesis and metastatic dissemination of CRC. We are approaching this from a multidisciplinary perspective, which includes the development of orthotopic models of colorectal tumours in nude mice to test the role of the TGF-beta-controlled gene signature, and screening for TGF-beta-regulated genes that are relevant for CRC by performing systematic shRNA-mediated down-regulation of genes contained in this signature. Moreover, we are developing animal models that will mimic the initial loss of TGF-beta signalling in CRC epithelial cells as well as a gain of function at later stages of the disease.

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Research networks and grants

Start-up grant for emergent research groups
Agency for Administration of University and Research Grants (AGAUR), 2005SGR 00775 (2006-2009)
Principal investigators: Eduard Batlle and Elena Sancho

Variations in the genetic program under the control of beta-catenin/Tcf during colorectal cancer progression
'La Caixa' Foundation, BM06-241-0 (2007-2009)
Principal investigators: Eduard Batlle and Elena Sancho

Collaborations

TGF-beta target genes in CRC
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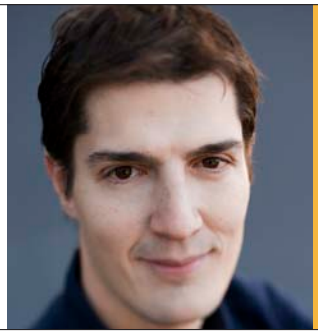
TGF-beta target genes in CRC metastasis
Joan Massagué, Memorial Sloan-Kettering Cancer Center (New York, USA)

Wnt signalling in CRC
Hans Clevers, Hubrecht Laboratory (Utrecht, The Netherlands)

ROGER GOMIS

Tumoral Metastasis Laboratory (MetLab)



Intricate signalling networks control cell division, differentiation, movement, organisation and death. Cancer cells disobey these signals during tumour progression and metastasis, which is the final step in 90% of all fatal solid tumours. Metastasis is therefore a grave public health problem and consequently a field of considerable pharmaceutical interest. A major research focus of our group is to identify and study the genes and functions that allow tumour cells to achieve metastatic colonisation of vital organs.

Growth control and cancer metastasis

Our research focuses on aberrant gene responses that enable invasion and metastasis in tumour cells. We seek to elucidate the mechanisms mediating tissue-specific metastasis, in particular in breast cancer. Metastasis, a complex process caused by elaborate interactions between tumour cells and the surrounding healthy tissues in several vital organs, accounts for 90% of all cancer deaths in patients with solid tumours. The molecular and cellular mechanisms that lead primary tumours to form metastases must be elucidated in order to better address this major life-threatening condition. The identification of metastatic genes and mechanisms is essential to understand the basic biology of this lethal condition and its implications for clinical practice (Fidler, 2003; Gupta and Massagué, 2006). We aim to explain how and why metastasis occurs, the mechanisms that make metastasis a tissue-specific process, the events that allow dormant metastases to become active and lethal many years after removal of a primary tumour and the metastasis-mediating genes that would eventually constitute worthy therapeutic targets.

Our contribution to the field builds on a novel experimental approach based on the use of moderately metastatic cells that are injected into a mouse model for the selection of highly metastatic breast cancer subpopulations. Live animal-imaging techniques are used to track the spread, homing, and outgrowth of the metastatic cells in several organs. After harvesting metastatic lesions and verifying that highly metastatic cells have been selected, we use genome-wide transcriptomic profiling to identify metastasis-linked genes. Gene transfer techniques are then used to assess the contribution of individual genes to various steps (invasion, homing, outgrowth, angiogenesis, and stroma adaptation) of the metastatic process.

Breast cancer is the most frequently diagnosed cancer in women in Europe and the United States, with an estimated 608,380 new cases of invasive disease in 2007 (American Cancer Society, 2007; Ferlay *et al*, 2007). Despite a recent decrease in its incidence rates in Europe and the United States (Ravdin *et al*, 2007), it remains the second leading cause of cancer deaths among women. Most of these patients die as a result of the metastatic spread of the tumour. Our current understanding of the biology of breast cancer is a major barrier to identify novel therapies and improve existing therapies for the treatment and prevention of this disease.

The predisposition of primary tumours to selectively invade different organs has long been recognised (Paget, 1889). Recent work has functionally identified and clinically validated sets of genes whose overexpression in estrogen receptor (ER)-negative breast cancer and prostate cells confers a selective advantage for the colonisation of bones (Kang *et al*, 2003; Lynch *et al*, 2005) and lungs (Minn *et al*, 2005). Moreover, under certain conditions tumour cells cannot grow or survive in the absence of a supportive microenvironment. Indeed, the microenvironment may even drive tumour and metastasis development by selecting for highly invasive and resistant cancer cell phenotypes (Bernards and Weinberg, 2002) and systemically fostering the mobilisation of marrow-derived progenitor cells (Kaplan *et al*, 2005). The capacity to subsequently colonise distant organs depends on the organ-colonising faculties of disseminated tumour cells as well as on certain requirements that may be present in the otherwise restrictive microenvironment of target organs (Gupta and Massagué, 2006). Thus, the various steps of metastasis do not necessarily represent the acquisition of individual specialised mutations but rather the random accumulation of traits that provide the advantage necessary to adapt to the microenvironment of a given organ.

Breast cancer is a remarkably heterogeneous disease, but subsets of tumours show recurrent patterns of transcriptional, genomic, and biological abnormality. Understanding how genes in

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these 'patterns' collectively function in an otherwise heterogeneous biological setting to enable progression and modulate response to therapy is critical to improve management of the disease. In particular, we aim to determine how the ER and HER2 pathways contribute to leading molecular events in breast cancer metastasis. ERα is overexpressed in around 65% of breast cancer cases, referred to as 'ER-positive'. Binding of estrogen to the ER stimulates the proliferation of mammary cells. ER-positive tumour cells are highly dependent on this stimulus to proliferate, and therefore ER is currently used as a therapeutic target (Ali and Coombes, 2002). Approximately 15-20% of breast cancers have an amplification of the *HER2* gene or overexpression of its protein product. HER2 is a cell membrane receptor tyrosine kinase and is normally involved in the epidermal growth factor signal transduction pathway leading to cell growth and proliferation. Overexpression of this receptor in breast cancer is associated with increased disease recurrence and poor prognosis (Slamon *et al*, 2001).

ER- and HER2-positive breast cancer cells preserve, among each subtype, genome-aberration-induced transcriptional changes with high fidelity. The resulting dominant genes will reveal molecular events that predict the metastatic outcome despite substantial genomic, transcriptional, translational, and biological heterogeneity in the overall system. The two tumour subtypes may metastasise to the same secondary organ. However, it is unknown whether the developmental history of a cancer would result in different or common mediators of site-specific metastasis. Predisposing factors related to the cell of origin may engender several rate-limiting barriers during the progression of metastasis. Our work aims to set the stage for a detailed study of mechanisms of metastasis and their potential value as new therapeutic targets. We are screening metastatic cell populations from pleural effusions derived from breast cancer patients in order to identify new metastatic gene signatures. For this purpose, on the basis of collaborations with clinical and basic researchers at the Hospital Clínic, in Barcelona, and the Memorial Sloan-Kettering Cancer Center, in New York, the MetLab team has initiated the isolation of metastatic cells from pleural effusions derived from lung and breast cancer patients. Once injected in mice, these cells are labelled with the GFP-Luciferase-TK protein fusion and visualised by bioluminescent techniques. On the basis of these metastatic cell populations, highly aggressive subpopulations with tropism to specific tissues will be isolated. These subpopulations will be used to identify and validate metastatic gene signatures by means of gene expression profile analyses and biochemical, cellular and molecular biology techniques.

We have also focused our attention on groups of genes that drive metastatic ER-negative cancer cells to one tissue or another. Particularly, we address metastatic suppressor genes and their functions in the metastatic process. We are conducting studies on the group of metastatic suppressor genes required for breast to lung metastasis, identified in Joan Massagué's laboratory (Minn *et al*, 2005) at the Memorial Sloan-Kettering Cancer Center. For this purpose, we are using the MDA-MB-231 breast cancer cell line model and its derivatives #4175 and #1833, which have a strong metastatic capacity to lung and bone.

Finally, our research centres, in part, on recent progress in the analysis of the TGF-beta cytostatic programme and its evasion in metastatic breast cancer. This project seeks to clarify the role of C/EBPβ transcription factor in the TGF-beta cytostatic programme in epithelial cells. Breast cancer cells are refractory to TGFβ-mediated growth arrest, thus leading to further tumour progression and metastasis. The molecular characterisation of TGFβ-mediated cytostasis in keratinocytes has placed C/EBPβ at the heart of this response. Furthermore, deregulation of C/EBPβ mediates evasion of the TGFβ-induced cytostatic effects in metastatic breast cancer cells. We found that the transcription factor C/EBPβ is essential for not only the induction of the cell cycle inhibitor *p15INK4b* by a FoxO-Smad complex but also for the repression of c-MYC by an E2F4/5-Smad complex. Interestingly, the *p15INK4b* and *c-MYC* gene responses, which are central to the TGFβ cytostatic programme, were selectively missing in primary metastatic breast cancer cells from half of the patients with advanced-stage disease that we analysed. Remarkably, this loss coincided with increased expression of the C/EBPβ inhibitory isoform LIP, which has been implicated in tumour progression. By normalising the LIP:LAP ratio, we restored these TGFβ cytostatic gene responses and growth inhibition in primary metastatic cells derived from human patients. Building on this work, we will determine the mechanism by which LIP expression is deregulated in metastatic breast cancer cells. Thus, by using biochemical and molecular biology techniques, primary human breast cancer cell cultures and animal model studies, we will study the molecular mechanisms that lead to the deregulation of the C/EBPβ function and consequent loss of the TGF-beta cytostatic response in cancer cells.

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Papel de C/EBPbeta en los mecanismos moleculares de regulación de la respuesta citostática al TGF-beta; implicaciones fisiológicas y sus alteraciones en el cáncer de mama
Spanish Ministry of Science and Innovation, SAF2007-62691 (2007-2009)
Principal investigator: Roger Gomis

Study of the molecular mechanisms of metastasis of breast cancer to the lung: therapeutic function and potential of metastasis suppressor genes
AECC-Spanish Cancer Association (2008-2010)
Principal investigator: Roger Gomis

Other funding sources

Mechanisms of metastasis
BBVA Foundation

Collaborations

Cristina Nadal, Oncology Service, Hospital Clínic Barcelona (Barcelona, Spain)

Awards

Josep M Sala-Trepat Award for studies in gene transcription mechanisms. Catalan Academy of Sciences

Core Facilities

JULIEN COLOMBELLI

Advanced Digital Microscopy Core Facility

The Advanced Digital Microscopy Facility provides researchers at IRB Barcelona and the Barcelona Science Park (PCB) an open-access system to state-of-the-art light microscopy instruments. In 2009, the Facility plans to introduce new applications and to foster scientific collaborations involving modern imaging techniques. Our primary goal is to set up, maintain and make fully accessible a wide range of complementary techniques, mainly based on fluorescence imaging, and to support researchers during the entire imaging process. The 3D imaging of fixed and living samples is supported through spectral confocal microscopy, and soon additional 3D microscopy will be accessible on several platforms, like spinning disk or multiphoton confocal microscopes, to allow optimisation of specific imaging parameters, for example to limit phototoxicity, enhance fast imaging or improve imaging deeper into samples and organisms. In 2008, the Facility, a joint initiative of IRB Barcelona and the PCB, invested significant efforts to set up the laboratory. Designed to offer ideal conditions in which to perform full microscopy experiments and image analysis, the lab space now offers optimised rooms for microscopes. A cell culture room, a wet bench, and computer workstations for image analysis are expected to become operational in 2009. The Facility also focuses on developing instruments and providing a range of custom systems that combine laser-based imaging and manipulation techniques, like FRAP and laser surgery, in order to offer researchers greater flexibility than that achieved with commercially available applications.

Figure 1. *Layout of the ADM Core Facility located in the Barcelona Science Park. 3D render: Arch M Corda.*

Construction phase and lab

The Advanced Digital Microscopy Core Facility was created in July 2008. From August to December 2008, the new lab space of the Facility was designed and prepared in collaboration with the PCB, as shown in Figure 1. In an area of about 120m^2, the lab includes office and desk space for open-access computer workstations dedicated to image analysis. Five wide dark rooms were optimised to host a total of ten systems in optimum working conditions, including stable temperature and electrical supply, mechanical isolation, complete darkness and a dust-limited environment. A shared lab space provides access to a wet bench and to a dedicated space that hosts two fluorescent stereoscopes optimised for sample preparation, manipulation and high magnification fluorescent imaging. A culture room with a sterile environment is also available for critical experiments.

Services for IRB Barcelona researchers

The Facility offers the following techniques:

Facility Manager Julien Colombelli **Research Officer** Lída Bardia

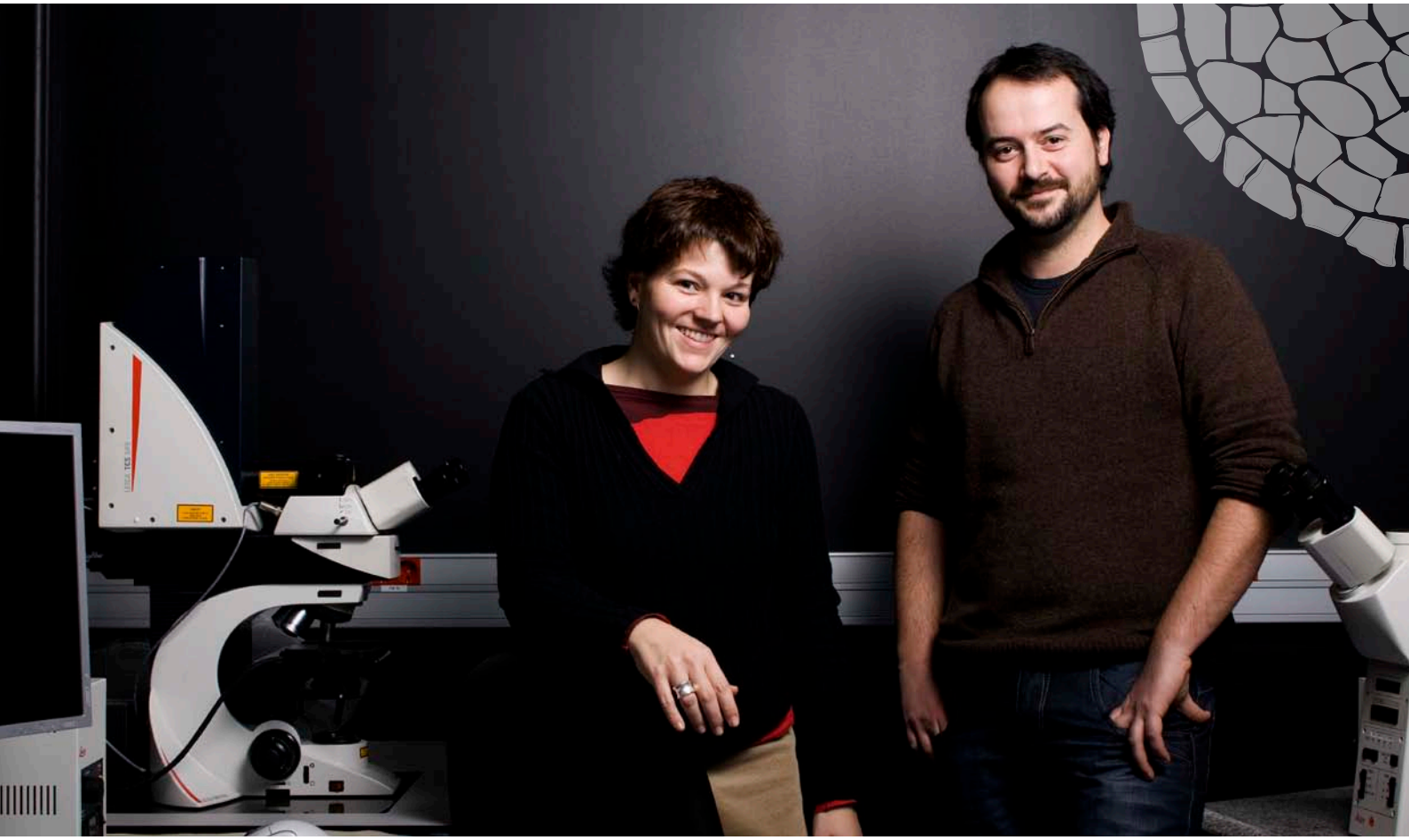

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Figure 2. *Construction work on the Facility in 2008. The room for the spinning disk confocal microscope in August (left) and November (right).*

- **Conventional transmission and fluorescence microscopy.** Bright field, Phase contrast, Differential Interference Contrast (DIC), Dark field and multiple colour fluorescence imaging of fixed samples. Fluorescence stereoscopy for sample manipulation and selection.

- **Spectral confocal microscopy.** 3D, 4D and 5D imaging with optical sectioning, custom spectral detection and resolution, multiple position and incubated environment control for living cells.

- The Facility is currently setting up **image processing workstations** to provide access to high computing power and specialised image analysis software packages to perform 3D image visualisation and quantification, advanced imaging techniques analysis (FRAP, etc.), deconvolution, and general data interpretation and presentation.

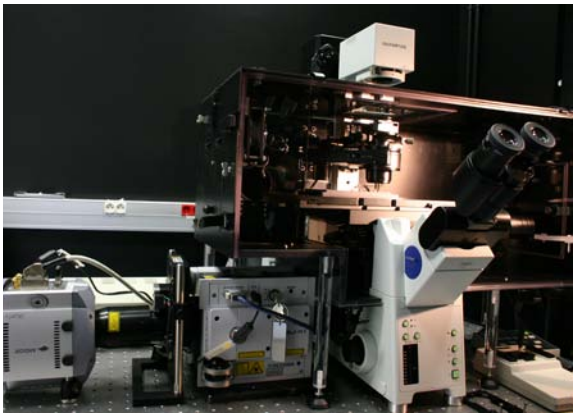


Figure 3. *The new spinning disk confocal microscope from Andor, set up at the Facility. The microscope is aligned on a vibration isolation table and offers an incubation environment for live cell imaging.*

New instrumentation and perspectives

In 2009, staff at the Facility plan to set up new microscopy techniques to enhance certain features of confocal microscopy and to focus on fluorescence laser manipulation.

To increase fluorescence imaging speed, a spinning disk equipped with a sensitive electron-multiplying (EM) camera will be set up. Higher sensitivity will reduce phototoxicity and increase acquisition speed to favour live confocal imaging of fast events. The system also offers multipositioning and temperature control for the simultaneous imaging of multiple samples.

A multiphoton contrast will be set up on the current Leica SP5 spectral confocal in order to achieve deeper imaging with reduced out-of-focus phototoxicity.

A new Olympus MVX10 fluorescence macroscope with motorised components will become available for flexible imaging and manipulation at low magnification.

An Olympus Total Internal Reflection Fluorescence (TIRF) Microscope will be set up to allow high quality imaging at the interface with glass surfaces. TIRF typically provides high contrast fluorescence images of 100nm axial depth to image membrane and cytoskeletal dynamics, single molecules, adhesions, etc. The system is also equipped with multipositioning and incubation chamber to allow high-throughput live data acquisition.

A laser-based manipulation platform is being established to combine techniques like laser nanosurgery, fluorescence recovery after photobleaching (FRAP), fluorescence photoactivation and automated imaging. The platform will be useful in many fields of applications like rapid molecular dynamics, cytoskeletal dynamics, diffusion and transport-based protein exchanges, force and morphogenesis in cells and organisms, DNA damage, neurobiology, etc.

SCIENTIFIC OUTPUT

Collaborations

Development of laser-based microscopy techniques
Ernst Stelzer and Light Microscopy Group, European Molecular Biology Laboratory (Heidelberg, Germany)

DAVID ROSSELL

Biostatistics/Bioinformatics Unit

In the last decade, a number of technologies that generate vast amounts of data have been popularised. For instance, microarrays measure mRNA expression levels for tens of thousands of genes simultaneously, tiling arrays assess enrichment in millions of chromosomal locations, and next generation sequencing technologies deliver hundreds of millions of genomic sequences in a single experiment. Nowadays researchers face not only the challenge of obtaining scientifically relevant data, but also of extracting as much valuable information from them as possible. Statistics is the science that transforms data into information. It provides a disciplined and scientifically sound framework to test scientific hypotheses and to learn about the systems and processes that generate biomedical data. Also, the experimental design theory guides researchers as to the best way to conduct experiments in order to reach their goals. We offer scientists support in the following areas: (i) experimental design (sample size calculation, study design, planning of statistical methodology); (ii) data analysis (clinical or biomedical databases, high-throughput data, *eg*, genomics, proteomics); (iii) statistical methodology; and (iv) software (help in using statistical software, development of software to meet special data analysis or study design needs).

The Biostatistics and Bioinformatics Unit was created in January 2008. By the end of the year the facility was staffed by its manager, David Rossell, and a research officer, Evarist Planet. During 2008, we have been involved in 25 collaborative research projects that have arisen from thirteen groups at IRB Barcelona. In addition, we have provided technical guidance in a number of projects focused on fields such as gene regulation, developmental biology, oncology, bioinformatics and molecular medicine.

Our mission is to offer the IRB Barcelona research community a competitive advantage by increasing both the quality and speed of its research. Quality has been furthered by making available cutting-edge methodology and tailored solutions to specific problems while speed has been increased by developing software tools to facilitate the generation and interpretation of experimental results.

In terms of methodological research, we have developed the GaGa model for differential expression analysis, which has contributed to proving that several chromatin-regulating transcription factors share a common regulatory programme. As another example, we have derived a framework for Bayesian Gene Set Enrichment Analysis, which has facilitated assessment of the biological relevance of findings from gene expression studies. Most of this research has been either published

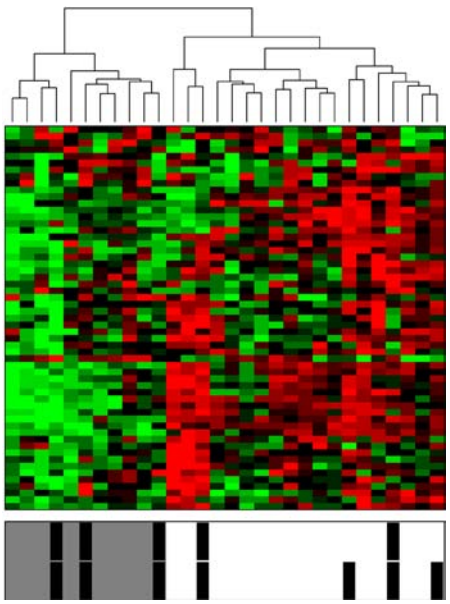
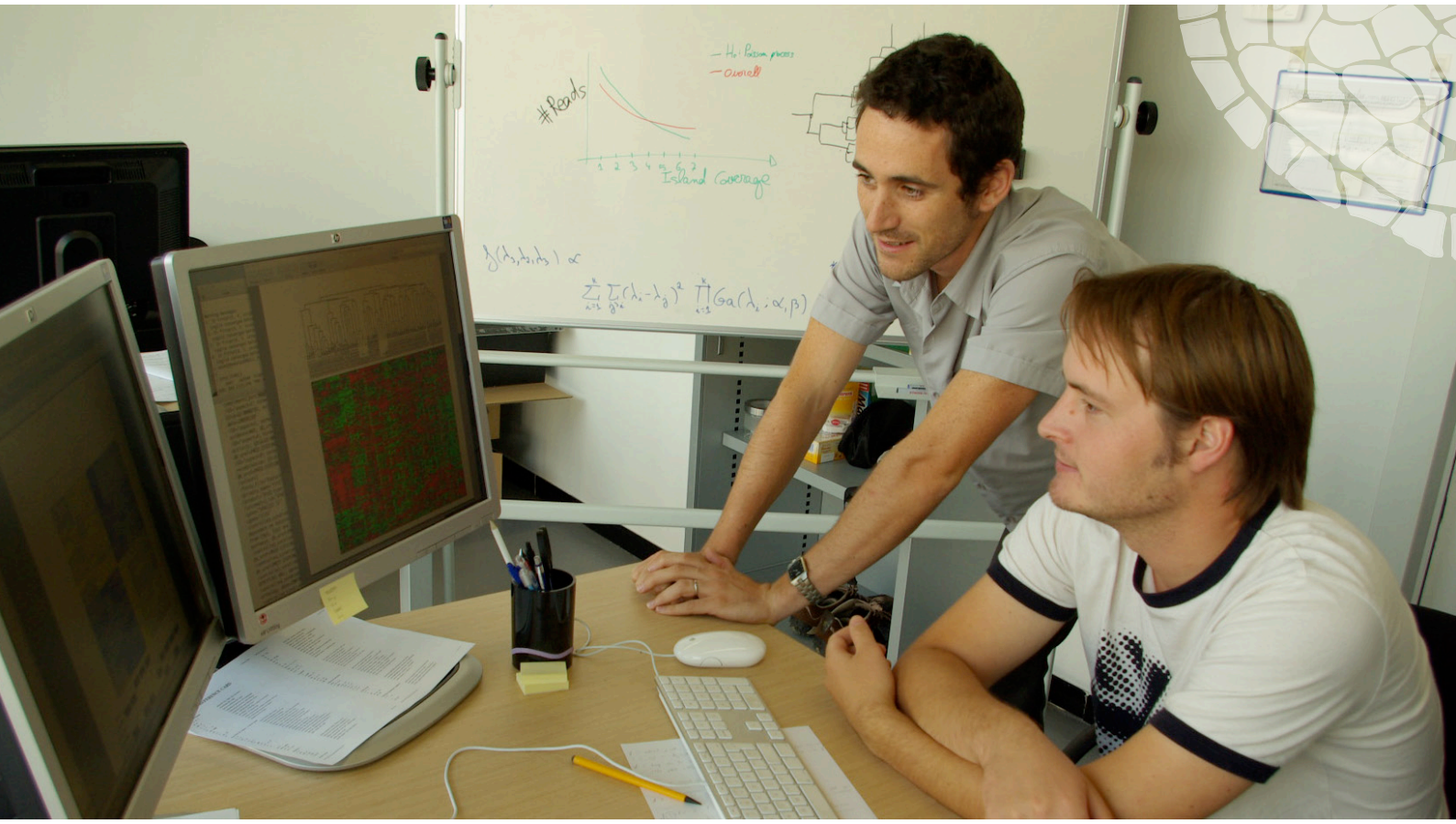


Figure 1. *Hierarchical clustering analysis of gene expression data reveals associations with clinical outcomes.*

Unit Manager David Rossell **Research Officer** Evarist Planet



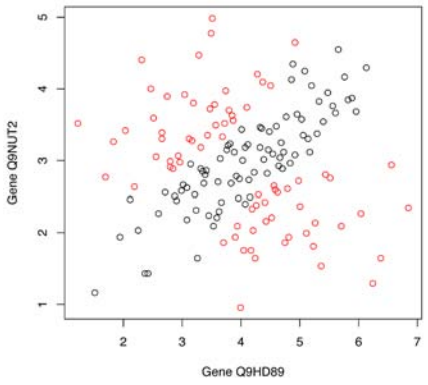


Figure 2. *Expectation-maximisation algorithm reveals correlation between two genes in the presence of noise. Black circles indicate correlated observations, red circles indicate observations arising from noise.*

or submitted for publication in scientific journals, thereby contributing to consolidating IRB Barcelona as a cutting-edge research institution.

In terms of software, we have developed routines to automatically produce reports with hyper-links to a number of on-line databases and resources. This has allowed researchers to obtain, for instance, additional information about specific genes or gene networks with a single click on their computer.

In collaboration with the IT Department, we have also provided a web browser interface which allows researchers to access their results moments after we have produced them. This development circumvents the inherent delay caused by copying large files with results on compact discs and sending them to researchers.

We have collaborated with IRB Barcelona groups in a number of research projects on developmental biology, structural and computational biology, molecular medicine and oncology.

Publications

Font-Burgada J, Rossell D, Auer H and Azorín F. *Drosophila* HP1c isoform interacts with the zinc-finger proteins WOC and relative-of-WOC (ROW) to regulate gene expression. *Genes Dev*, **22**(21), 3007-23 (2008)

Rossell D, Baladandayuthapani V and Johnson VE. Bayes factors based on test statistics under order restrictions. In Bayesian Evaluation of Informative Hypotheses in Psychology (H Hoijtink, I Klugkist, P Boelen, ed.), Springer (2008)

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Collaborations

Paper on Bayes factors
Valen Johnson and Veerabhadran Baladandayuthapani, MD Anderson Cancer Center (Houston, USA), Herbert Hoijtink, Irene Klugkist and Paul A Boelen, Utrecht University (Utrecht, The Netherlands)

Paper on differential expression analysis
Rudy Guerra, Rice University (Houston, USA) and Clayton Scott, University of Michigan (Ann Harbor, USA)

Paper on sequential design for high-throughput experiments
Peter Müller, MD Anderson Cancer Center (Houston, USA)

Functional Genomics Core Facility

During the last decade, molecular biology developed from a gene-by-gene analysis into a more comprehensive approach to study regulatory networks involving dozens to hundreds of interacting partners. For successful performance in this area, researchers require an increasing number of tools to either interrogate or alter genes on a genome-wide level. The Functional Genomics Core Facility provides state-of-the-art genomic tools for researchers at IRB Barcelona and other centres. These tools fall into two categories. The first is the genome-wide analysis of transcription, DNA polymorphisms, and chromatin immunoprecipitation (ChIP-chip). These analyses are performed using microarrays produced by Affymetrix and NimbleGen. For both analytical methods, the Facility provides a complete service, including initial consultation during the design of a project, quality control of starting material, sample and array processing, data analysis in collaboration with statisticians, and data interpretation and validation by real-time-PCR. The second category is the alteration of gene expression. For knock-down of gene expression, the Facility provides a genome-wide human and mouse shRNA library (Sigma), containing approximately 100,000 clones each, covering the majority of all known transcripts. For overexpression, we provide a human open-reading-frame library (Open Biosystems) containing 15,000 clones, covering three quarters of all human genes.

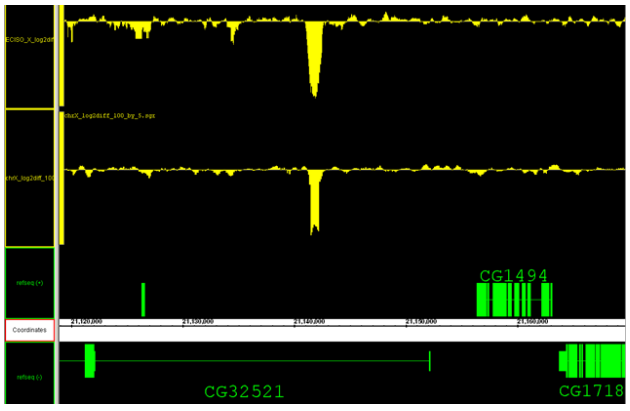


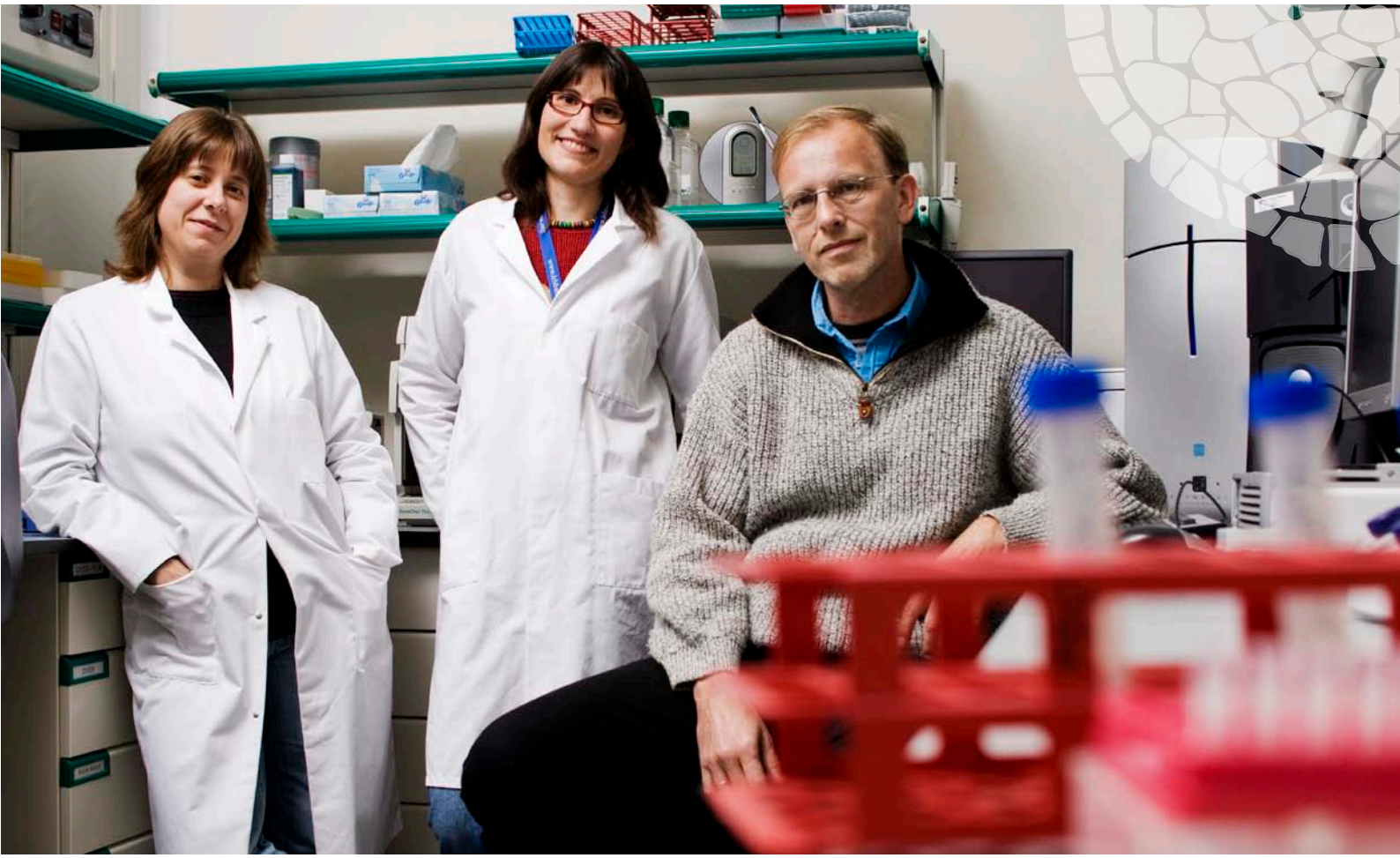
Figure 1. *DNA copy number variation in Drosophila strains. The top line shows a homozygous deletion within the CG32521 gene in a Drosophila strain as measured by Next Generation Sequencing; the line below shows the result of the same chromosomal region measured by tiling arrays.*

During 2008 the Facility performed projects with over 20 research groups from four programmes at IRB Barcelona and from other institutions throughout Barcelona, Catalonia and Spain.

Using products provided by Affymetrix, the Facility performs genome wide expression analysis at the gene and exon levels as well as comparative genome hybridisation (CGH) analysis. These technologies are provided for over 20 organisms including all standard model organisms and humans. For CGH analysis, resolution is further increased by use of tiling arrays. These arrays provide probes tiled across the entire genome without prediction of genes; therefore, this type of array offers the most comprehensive picture of genomic alterations currently available in microarray technology.

Since summer 2008, the Facility also offers services based on NimbleGen microarray products. NimbleGen technology provides longer probes than Affymetrix and therefore higher specificity. In addition, NimbleGen microarray production is extremely flexible and consequently facilitates the design of customised microarrays, even for small projects. This technology is currently used for expression and CGH analysis.

Facility Manager Herbert Auer **Senior Research Officers** Eva González, Silvia Rodríguez



The tools for altering gene expression, namely the shRNA libraries and the open-reading-frame library, contain over 200,000 clones. These are centrally stored and a database has been developed for clone administration. It also provides information about knock-down efficiency and accuracy of clone annotation.

Services for IRB Barcelona researchers

DNA/RNA quantification and quality control
Various analyses are provided for the assessment of purity, integrity and concentration of nucleic acids. These analyses include specific quantification of DNA and RNA using nuclei acid-specific fluorometric assays, spectrophotometric assessment of contamination, quantitative measurements of fractions of RNAs, like small RNAs, mRNA and rRNAs, and the evaluation of RNA integrity.

Expression profiling
Genome-wide analysis of transcripts is provided at three levels of resolution:

- 3' biased arrays containing one probe set per gene; these arrays are available for more than one hundred organisms.
- Exon arrays containing one probe set per exon; these arrays are currently available for human, mouse and rat.
- Tiling arrays interrogating the entire genome at a 35-base-pair resolution; these arrays are currently available for human, mouse, *Drosophila*, *S. cerevisiae* and *S. pombe* from Affymetrix and can be customised via NimbleGen for every sequenced organism.

DNA polymorphism analysis
Genome-wide analysis of DNA polymorphisms comes in two forms:

- For over 20 organisms, DNA copy number variation (CNV) is measured at the resolution of individual genes. For organisms where exon or tiling arrays are available (see above) even higher resolution can be provided. For CNV analysis, arrays can be customised via NimbleGen for every sequenced organism.

- For human DNA, up to one million single nucleotide polymorphisms (SNPs) can be measured in parallel with the same number of CNVs across the genome.

Validation of microarray results by real-time PCR
For real-time PCR validation of microarray data, assays are designed and performed and data are analysed for differential expression.

Alteration of gene expression
Bacterial clones are provided for the knock-down of almost all well characterised human and mouse transcripts. Multiple clones targeting the same transcript are available to assess off-target effects. For overexpression, one open-reading-frame clone is available per human gene. Clones are centrally managed at the Facility and are provided as bacterial stocks. The clone database is available online and clones can be requested electronically.

SCIENTIFIC OUTPUT

Publications

Cuscó I, del Campo M, Vilardell M, González E, Gener B, Galán E, Toledo L and Pérez-Jurado LA. Array-CGH in patients with Kabuki-like phenotype: identification of two patients with complex rearrangements including 2q37 deletions and no other recurrent aberration. *BMC Med Genet*, **11**, 27 (2008)

Font-Burgada J, Rossell D, Auer H and Azorín F. *Drosophila* HP1c isoform interacts with the zinc-finger proteins WOC and Relative-of-WOC to regulate gene expression. *Genes Dev*, **22**(21), 3007-23 (2008)

Rodríguez-Mulero S and Montanya E. Islet graft response to transplantation injury includes up-regulation of protective as well as apoptotic genes. *Cell Transplant*,**17**(9), 1025-34 (2008)

Salgado R, Toll A, Espinet B, González-Roca E, Barranco CL, Serrano S, Solé F and Pujol RM. Analysis of cytogenetic abnormalities in squamous cell carcinoma by array comparative genomic hybridization. *Actas Dermosifiliogr*, **99**, 199-06 (2008)

Singh S, Robinson M, Ismail I, Saha M, Auer H, Kornacker K, Robinson ML, Bates CM and McHugh KM. Transcriptional profiling of the megabladder mouse: a unique model of bladder dysmorphogenesis. *Dev Dyn*, **237**(1), 170-86 (2008)

Collaborations

Allele specific gene expression
Jorge Ferrer, IDIBAPS (Barcelona, Spain)

Alterations of glycogen metabolism in pathological conditions
Joan Guinovart, IRB Barcelona (Barcelona, Spain)

Characterisation of genes implicated in mitochondrial dynamics
Antonio Zorzano, IRB Barcelona (Barcelona, Spain)

Characterisation of nucleosome positioning in S cerevisiae
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Characterisation of yeast mutants used in wineries
Ricardo R Cordero Otero, Rovira i Virgili University (Tarragona, Spain)

Compartment boundary formation: identification of new genes and properties
Marco Milán, IRB Barcelona (Barcelona, Spain)

Copy number variation in metabolic diseases
Luis Castaño, Hospital de Cruces (Basque Country, Spain)

Development and evaluation of next generation sequencing algorithms
Karl Kornacker, Nationwide Children's Hospital, Columbus (Ohio, USA)

Evaluation of miRNA analysis platforms
ABRF Microarray Research Group

Gene expression in S pneumoniae
Adela González de la Campa, Caubet-Cimera Foundation, Joan March Hospital (Bunyola, Spain)

Genomic and transcriptomic characterisation of cancer in Drosophila
Cayetano González, IRB Barcelona (Barcelona, Spain)

Intestinal stem cells and colorectal cancer stem cells
Eduard Batlle, IRB Barcelona (Barcelona, Spain)

Molecular characterisation of mutants relevant to the cerebral cortex
Eduardo Soriano, IRB Barcelona (Barcelona, Spain)

Molecular characterisation of neural development in chicken
Marian Martinez Balbas, IBMB, CSIC (Barcelona, Spain)

Molecular mechanisms causing metastasis
Roger Gomis, IRB Barcelona (Barcelona, Spain)

Roles of the nuclear receptor LXR in macrophage biology
Antonio Celada, IRB Barcelona (Barcelona, Spain)

TGF beta signalling in colorectal cancer
Elena Sancho, IRB Barcelona (Barcelona, Spain)

The cross-talk between GR, PPAR-γ and the JNK/AP-1 pathway
Carme Caelles, IRB Barcelona (Barcelona, Spain)

The role of DNA binding proteins in chromatin structure
Ferran Azorín, IRB Barcelona (Barcelona, Spain)

The role of the GAGA factor in the regulation of chromatin structure and function
Jordi Bernués, IRB Barcelona (Barcelona, Spain)

MARTA VILASECA

Mass Spectrometry Core Facility

Mass Spectrometry (MS) plays a pivotal role in several scientific disciplines. Today it is an integral part of proteomics and drug discovery processes and also provides relevant information about structural biology. The MS Core Facility provides the IRB Barcelona research community with modern chromatographic and spectrometric tools for the identification and characterisation of a broad range of biological species. One of the main objectives of the Facility is to implement intact protein analysis (top-down approach) for their complete characterisation. In this approach, protein ions are introduced into the gas phase by electrospray and are subsequently fragmented in the mass spectrometer, thereby yielding the molecular mass of both the protein and the fragment ions. The top-down approach is successful for targeted studies of single proteins of less than 100 kDa; however, extending the approach to the analysis of the whole proteome still requires improvements in proteome fractionation. The Facility is working on the development of bottom-up proteomic techniques for protein quantitation and for the determination of post-translational modifications. Moreover, the novel ion mobility-MS coupling methodology is being used to study the macromolecular structure and conformation of proteins and nucleic acids. Along the same line, non-covalent protein-protein and protein-ligand interactions can be directly detected and studied, thereby providing clues as to the mechanisms of action of these proteins in biological processes.

General activities over the year

The MS Core Facility was established in 2007 with the preparation of the lab site and the arrival of instruments at the end of the year. During 2008 all instrumentation was set up and the Facility grew from 2 to 3 members of staff with the incorporation of a second research officer. By June the Facility was providing service to groups in 4 out of the 5 Research Programmes at IRB Barcelona. The Facility also supported research groups at the University of Barcelona and the Autonomous University of Barcelona.

In 2008 we implemented the technology necessary to detect intact non-covalent protein-protein, protein-ligand and DNA complexes of moderate size by infusion. The detection of non-covalent ions is done by applying gradual changes in vacuum between the source and the analyser (ion-cooling). For macromolecular structure and conformation studies, we use ion mobility technology coupled to MS. The calibration of drift time data to collision cross sections is done with protein standards of known cross sections.

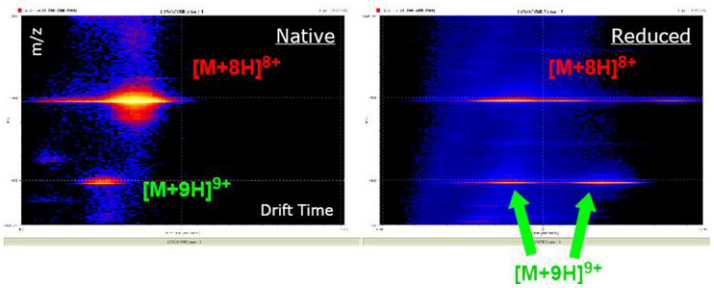


Figure 1. *Example that shows the capacity of Synapt HDMS. This instrument recognises different conformers of a particular charge state. In the mobilogram m/z is represented on the y axis and drift time on the x axis. Note the clear difference between 2 conformers for charge 9 of Hen egg lyzozyme once the native form is reduced.*

Acting Facility Manager Marta Vilaseca **Research Officers** Claudio Diema, Núria Omeñaca

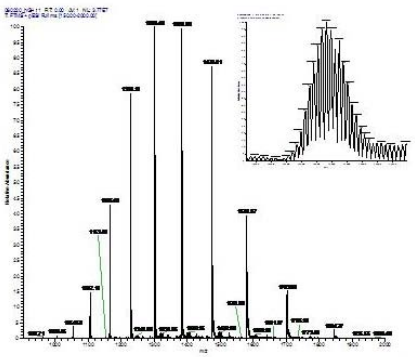


Figure 2. *MS spectra of hGH on LTQ-FT.*

The Facility has set up instrumentation for bottom-up proteomic applications and has begun to implement the techniques for protein quantitation with this approach. Applications of these novel methods are being applied for the identification of biological markers in body fluids.

For the complete characterisation of intact proteins, the above mentioned top-down approach is one of the targeted analyses of the Facility. Using Fourier Transform Mass Spectrometry, accurate data on intact proteins and their fragments ions, formed by Electron Capture Dissociation (ECD) and collision activated dissociation (CAD), facilitate the characterisation of coding polymorphisms (cSNPs), alternative splice events, and post-translational modifications (PTMs) by providing information on 100% of the primary protein structure. This is a challenging approach which we have begun to explore for moderately sized proteins in pure samples as a first step before addressing more complex mixtures.

Services for IRB Barcelona researchers

The services offered include MS, MS/MS and MSn analysis using atmospheric pressure ionization techniques (electrospray and APcI) coupled to LC, nanoLC or infusion inlets. The Facility also provides consultancy services and analytical method development for specific applications, as well as mass spectra data processing. Samples are analysed either directly by the service or by researchers (previously trained by Facility members) who can use mass spectrometers through an open-access system.

Equipment and specialised applications

1-LTQ FT Ultra (ThermoFisher Scientific). Hybrid Mass Spectrometer consisting of a linear Ion Trap, combined with a Fourier Transform Ion Cyclotron Cell. This instrument has exceptional applications for the identification and structural characterisation of small molecules. It permits both bottom-up (proteomics) and top-down approaches for the analysis of intact proteins, including post-transductional modifications (Figure 2).

2-Synapt High Definition MS System (Waters-Micromass). Hybrid QTOF instrument with an incorporated Triwave Cell. This instrument allows tandem MS to be combined with ion mobility, thus permitting the analysis of samples differentiated not only by their mass to charge ratio (like standard analysers) but also by their size and shape. The instrument is used to analyse the macromolecular structure and conformation of intact proteins and to study non-covalent interactions (Figure 3).

3-LCT-Premier XE (Waters-Micromass). Orthogonal acceleration time-of-fight mass spectrometer ideal for analysing high molecular weight compounds. This instrument has been modified to achieve inert conditions inside the ionisation source, to allow amide H/D exchange experiments for the study of the dynamic and structural properties of proteins and their complexes.

Figure 3. *Synapt HDMS Mass Spectrometer.*

The Facility also has 2 LC devices: a) Acquity UPLC (Waters; attached to the LCT-Premier), and b) a quaternary micro LC pump with a micro-autosampler (Thermo) attached to the LTQ-FT and an infusion inlet, Advion Triversa Nanomate, based on chip infusion.

SCIENTIFIC OUTPUT

Collaborations

Cerebrospinal fluid proteomic analysis of patients with Amyotrophic Lateral Sclerosis (ALS)
Jacques Borg, Jean Monet University (Saint-Étienne, France), Joan Guinovart, IRB Barcelona (Barcelona, Spain) and Eliandre de Oliveira, PCB Proteomic Platform (Barcelona, Spain)

DNA non-covalent complexes studies by ion mobility mass spectrometry
Modesto Orozco, IRB Barcelona (Barcelona, Spain) and Ramon Eritja, IRB Barcelona (Barcelona, Spain)

H/D exchange determined by ESI to study molecular recycling in Aβ(1-42) amyloid fibrils
Natàlia Carulla, IRB Barcelona (Barcelona, Spain)

Top-down mass spectrometry
Ernest Giralt, IRB Barcelona (Barcelona, Spain), Michaela Scigelova and Vlad Zabrouski, ThermoFisher Scientific (Bremen, Germany)

Mouse Mutant Core Facility

The purpose of the IRB Barcelona Mouse Mutant Core Facility is to generate murine models of disease and development for researchers at IRB Barcelona and their collaborators. The Facility has been operating since January 2007 and is staffed by scientists with extensive experience in cell culture, embryo manipulation and molecular biology. Genetically modified mice play a vital role in basic and applied biomedical research. The study of normal and abnormal gene expression, of regulatory elements, and of gene mutations relevant for human disease are examples of applications of genetically modified animal models in experimental research. A wide range of modifications can now be made to the mouse genome, including the introduction of simple exogenous transgene DNA, targeted deletions and insertions, conditional sequences, point mutations and other modifications. These mutations are produced using a variety of techniques, most of which involve the manipulation of pre-implantation stage embryos or mouse embryonic stem (ES) cells.

Early in 2008, the Mouse Mutant Core Facility moved into a refurbished, dedicated laboratory space of about 60 m^2, containing a small self-contained tissue culture lab, office and bench space and equipment for carrying out molecular biology work. In addition, we have a dedicated microinjection lab within the SPF facility, which houses two independent microinjection stations, as well as other apparatus required for embryo manipulation and mouse surgery.

In the first year, after setting up the facility in 2007, we generated a number of transgenic founder lines. 2008 has seen the creation of the first of our gene-targeted lines.

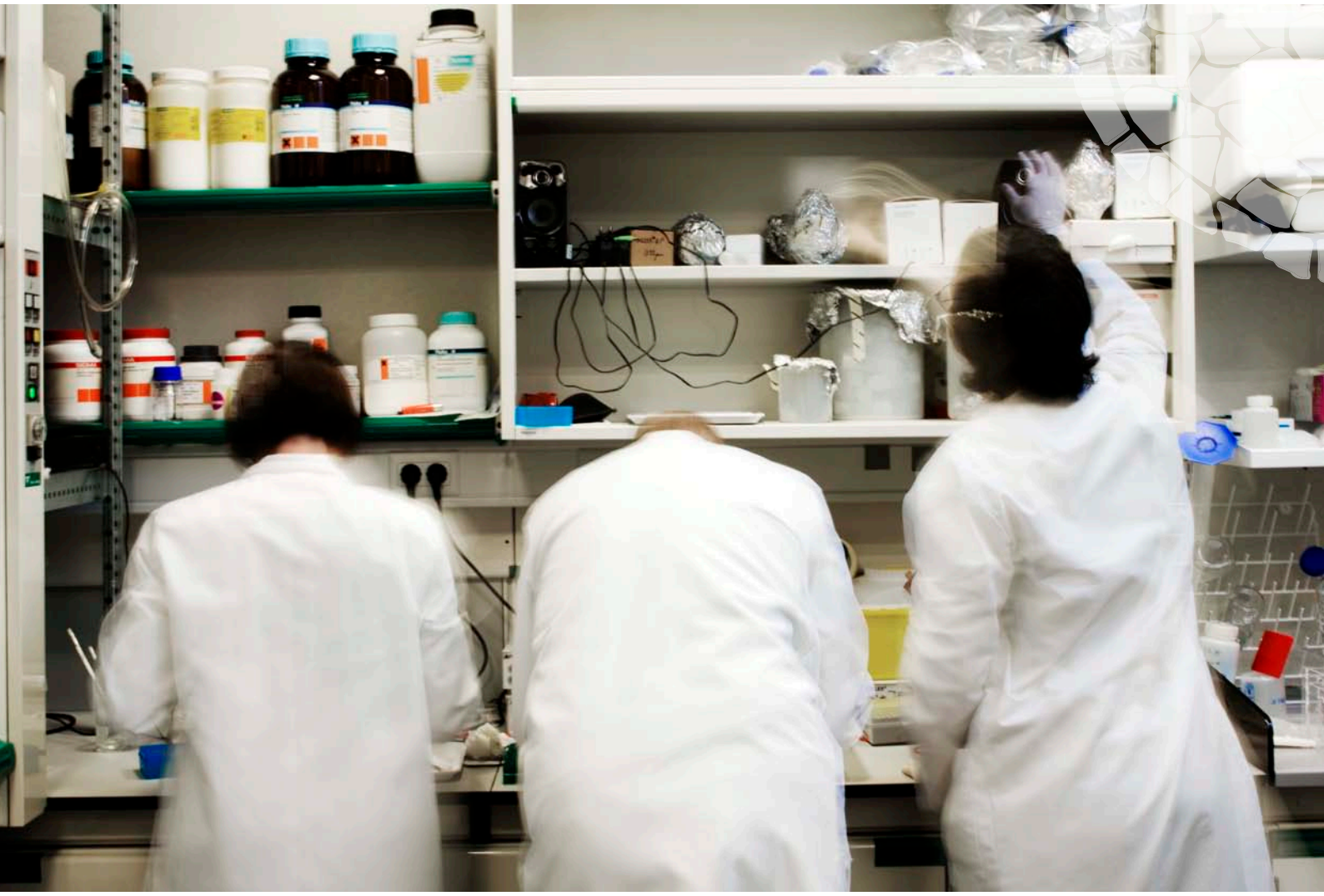
During the year we have been working on standardised protocols for the production of gene-targeting vectors. This has been made possible by the recruitment of a molecular biologist, whose tasks are to develop technologies and to assist research groups in the production of targeting and transgenic vectors. In 2008, we hosted two students, one who came to spend the summer with us, and the other who worked with us for eight months as part of an undergraduate degree.

Services for IRB Barcelona researchers

Experimental design. The Facility provides consultation on all aspects of the design of gene-targeting and transgenic DNA vectors. These designs usually begin by an examination of the gene structure, derived from data generated by the relevant research group, or from databases (such as Ensemble or Vega) or a combination of both. We then propose an appropriate experimental strategy and a suitable cloning protocol. We also provide advice on screening strategies for transgenic and gene-targeted mice and cells.

Vector construction. DNA vector design and construction is important for the success of the transgenic or gene-targeting experiments. We have recently employed a full-time molecular biologist whose function is two-fold. The first aim is to design and generate a set of molecular and cellular tools and protocols in order to make the creation of recombinant DNA molecules and the screening for mutations both easier and faster. The second goal is to act as a consultant for those groups and researchers generating transgenic or targeting vectors.

Generation of gene-targeted and transgenic mice. In recent years, publically funded initiatives aimed at creating ready-made ES cell mutants, such as the European Conditional Mouse Mutagenesis Programme (EUCOMM) and the International Gene Trap Consortium (IGTC), have generated mutations in thousands of genes. We use these resources wherever possible, and in the last year we initiated the first of our gene-targeting projects using ready-made clones. The list of projects using these clones or vectors is rapidly increasing, and we expect to do many more in the coming year.



However, many types of genes and genetic modifications are not covered by the aforementioned consortiums, and these types of projects require the design and building of a gene/mutation-specific vector in collaboration with the research groups.

Mouse ES cell culture. The Facility has a dedicated tissue culture lab for the culture and manipulation of mouse ES cells and mouse embryonic fibroblast cells. We offer a complete gene-targeting service, from transfection of ES cells with gene-targeting vectors, drug selection of transfected cells, and the picking and expansion of drug-resistant clones, to the archiving of duplicate clones. After correctly targeted cell clones have been identified, potential positives are expanded and further analysed before being microinjected into pre-implantation mouse blastocysts.

Microinjection. The Facility has two dedicated microinjection stations equipped with state-of-the-art micromanipulators. Two specialist technicians carry out microinjection and associated microsurgery techniques in addition to overseeing breeding strategies for lines generated or maintained by the Facility.

NICK BERROW

Protein Expression Core Facility

The Protein Expression Core Facility Unit was founded to carry out High Through-Put (HTP) activities in which many variations of an experiment (*eg*, cloning and expression screening of truncations or mutants of a protein) can be performed in parallel. The capacity to simultaneously perform many experimental variations on a single theme can significantly decrease the time taken to solve a particular cloning- or protein-related problem, thereby bringing experiments to more rapid conclusions and, more importantly, leading to rapid publication of data. In addition to the time savings offered by HTP methods, they are also generally considered cost-effective and can significantly reduce project and laboratory costs. Many of the protocols are automated, with the Facility making full use of liquid handling robotics for small-scale HTP plate handling and automated purification systems for larger scale protein purifications. The Facility also offers many high quality reagents for cloning and expression, competent bacterio-phage-resistant *E. coli* strains, specialised expression media and recombinant enzymes at prices substantially lower than commercial ones. We also offer custom cloning and vector modification services.

Figure 1. *A selection of instruments purchased for the Facility. Caliper LabChipGX for HTP protein, DNA and RNA analysis and documentation, Äkta Xpress systems for automated large scale protein purifications and Theonyx liquid handling robot for HTP expression screening, plasmid purification, etc.*

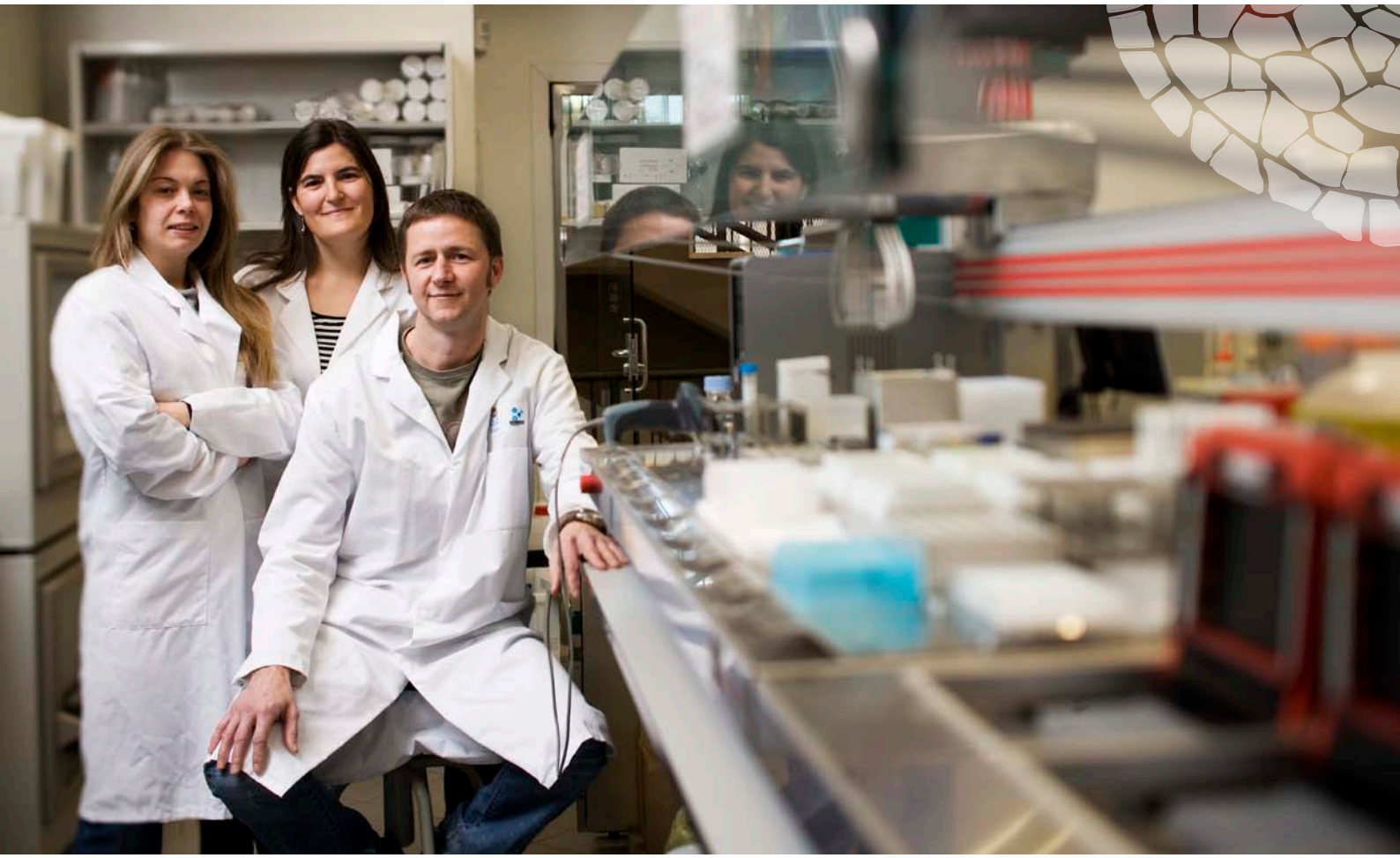
The Facility (founded in the autumn of 2007) moved into its current laboratory (Laboratory PBB12) when refurbishment was completed in spring 2008. Since then, the instruments required for its full operation have been purchased and staff have been recruited to ensure that the Facility can offer a full portfolio of services (Mª Carmen Romero and Raquel Garcia joined in October and December of 2008, respectively). Throughout 2008 the Facility has been implementing the first phase (mainly HTP cloning and expression screening in *E. coli*) of the planned services, which are now available as standard services (documented below).

The Steering Committee for the Facility approved an initial group of seven project applications of various sizes and complexity arising from our first call for projects, and many of these have been completed, or are nearing completion.

The Facility has already begun to deliver purified proteins to IRB Barcelona researchers and we hope to be able to deliver many more in the coming year. In addition, it has also completed many smaller scale cloning projects to help IRB Barcelona researchers and others from the local academic community.

The end of 2008 also saw the completion of equipment purchasing and installation for the tissue culture room. The Facility is now fully equipped for expression screening in mammalian

Facility Manager Nick Berrow **Senior Research Officer** Raquel García
Research Technician Mª Carmen Romero



(HEK293) cells, the rapid production of recombinant baculoviruses and follow-up expression screening in insect (*Sf*9) cells.

Services offered to IRB Barcelona researchers

The services currently available include:

- **Custom HTP cloning to generate expression vectors.** The Facility has introduced some of the latest cloning technologies to simplify often complex cloning (DNA manipulation) procedures, thereby allowing them to be performed more easily, reliably and efficiently. These methods allow the Facility to generate a microtitre plate of 96 expression-ready clones within 1-2 weeks of receiving the template and primers. Vectors currently available include popular fusion proteins such as MBP, GST, SUMO, His-Tags (cleavable N- or C-terminal), GFP and Strep II tags to facilitate the solubility or yield of the proteins of interest and provide simple purification or detection strategies.

- **In-Fusion™**, a ligation and restriction enzyme-independent cloning technique, allows the precise production of user-defined constructs, including the production of mutant and chimaeric constructs.

- **Expression screening in *E. coli*.** A microtitre plate of 96 (Facility- or user-derived) expression clones can be screened in *E. coli* in approximately one week. The screen currently consists of the use of two expression strains, with expression in each strain being tested using both IPTG and auto-induction methods. Additional (DE3) *E. coli* strains can be incorporated into the screening process if required.

- **HTP plasmid mini-preparation-96 mini-preps** from *E. coli* pellets in less than 2 hours.

- **Custom protein expression and purification.** The introduction of common affinity 'tags' to proteins of interest during the cloning process enables the rapid purification of proteins at the milligram scale (dependent upon the particular protein being studied). Purity levels in excess of 95% are anticipated.

- **Expression screening** in mammalian, *eg*, HEK293, cells. A microtitre plate of 96 (Facility- or user-derived) expression clones can be screened in HEK293 cells in 1-2 weeks.

- **Other services** to be introduced early in 2009 include: recombinant baculo-virus generation and expression screening in insect (*Sf*9) cells and production of vectors for expression screening in *P. pastoris* or *K. lactis*.

The Facility also offers many high quality reagents for cloning, protein expression, protein labelling and also specialised *E. coli* competent cell strains (for expression or cloning) for purchase by individual researchers. In addition, we offer custom vector modification. Purchasing reagents through the Facility often leads to considerable cost savings for researchers.

The Facility has active projects with many IRB Barcelona teams, including the groups devoted to metabolic engineering and diabetes therapy, cell signalling, molecular pathology and therapy in heterogenic and multi-genic diseases, structural biology of proteins, nucleic acids and their complexes, experimental biology, and biomolecular NMR, in addition to local academic research groups. We hope to build many more collaborations within IRB Barcelona, the Barcelona Science Park, the University of Barcelona and the Autonomous University of Barcelona in the near future.

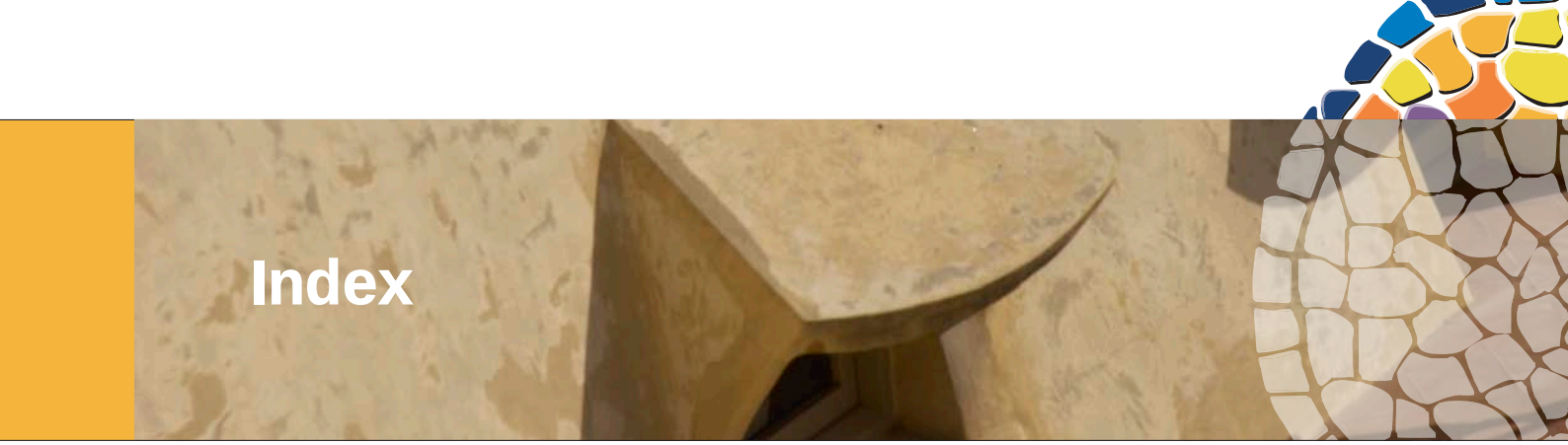
SCIENTIFIC OUTPUT

Collaborations

Adaptation of HTP cloning and screening pipeline for use with membrane proteins
Manuel Palacín, IRB Barcelona (Barcelona, Spain)

Adaptation of HTP cloning and screening pipeline for use with P. pastoris expression system
Francisco José Fernández, IRB Barcelona (Barcelona, Spain)

Continued development of pOPIN vector suite
Ray Owens, Oxford Protein Production Facility (Oxford, UK)



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