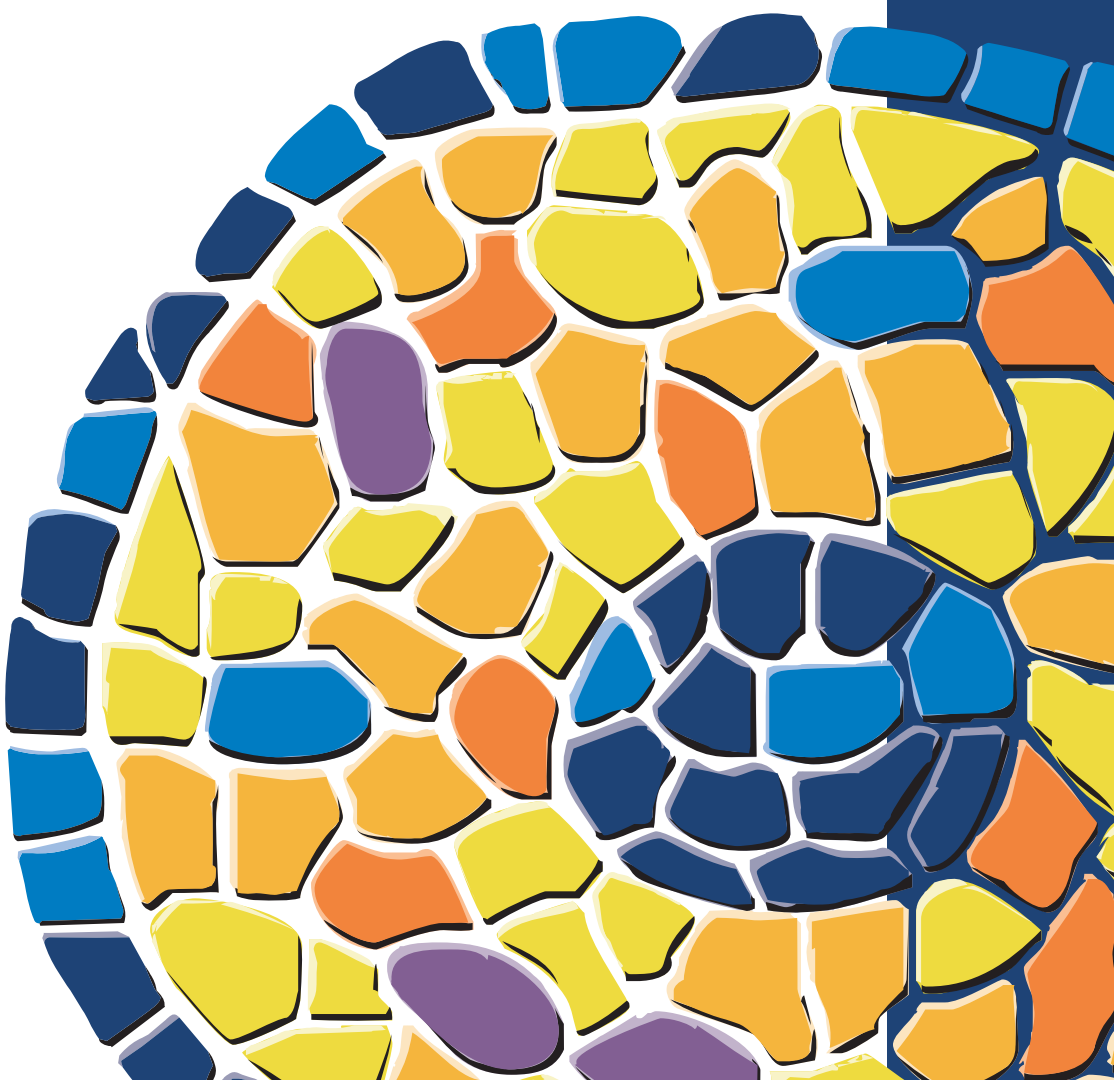
2007 Scientific Report

IRB
BARCELONA

INSTITUTE
FOR RESEARCH
IN BIOMEDICINE

Centres de recerca
de Catalunya

2007 Scientific Report



Institute for Research in Biomedicine

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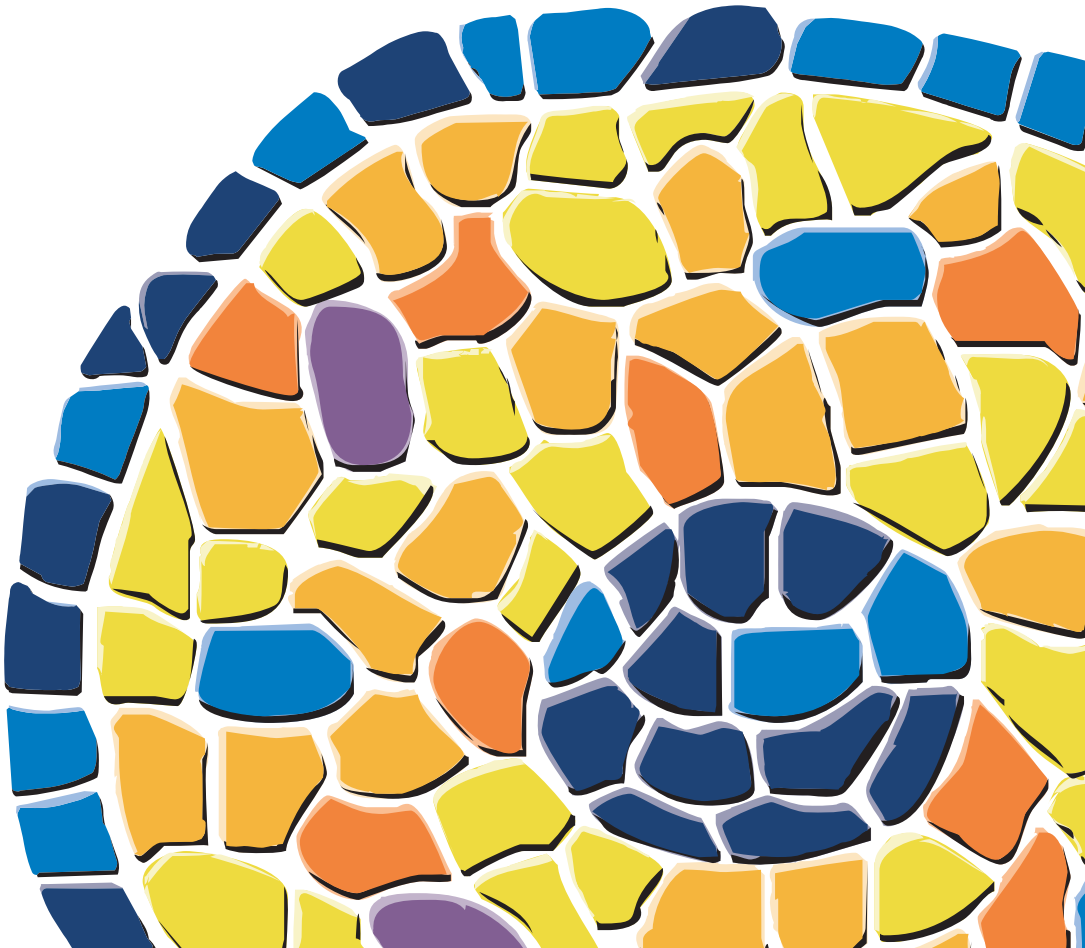
2007 Scientific Report



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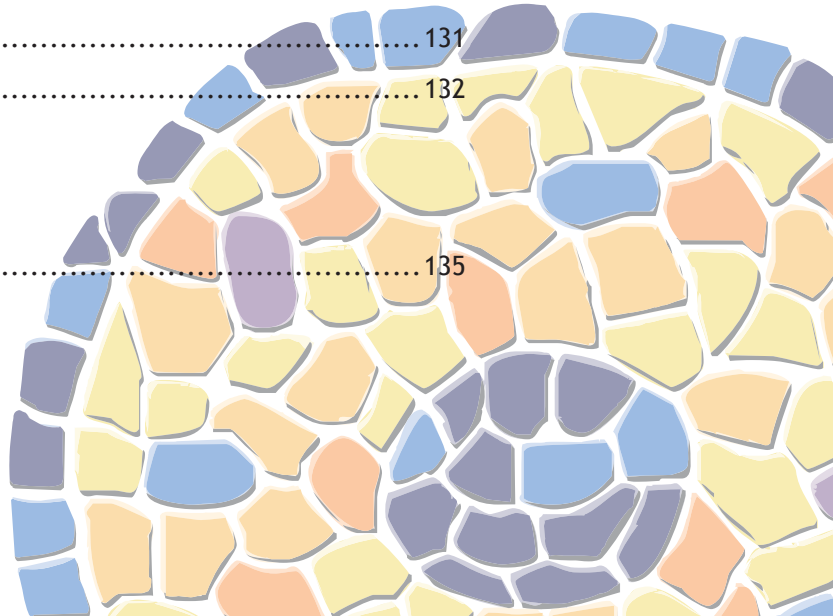


A year of consolidation and growth at IRB Barcelona

This is the second Annual Report of IRB Barcelona, a vibrant young research institute in the capital of Catalonia. It is composed of three volumes: this Scientific Report, which provides a detailed summary of the work carried out during the year by our research groups and core facilities, an Executive Summary, which includes facts and figures about the Institute, and Science Stories from IRB Barcelona, which highlights some of the research conducted over the past year and takes a look at the people behind the science at IRB Barcelona. The main goal of these documents is to provide a snapshot of the activities that have taken place at the Institute over the past year. Along the way, we hope readers will get a feeling for this unique new place and its importance in the landscape of biomedical research.

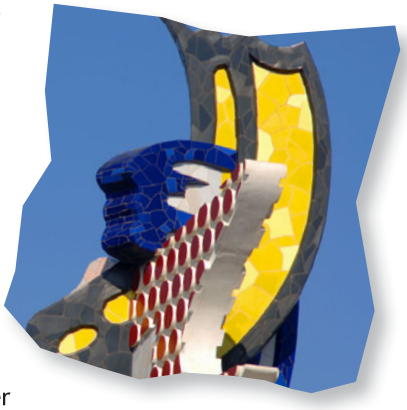
IRB Barcelona, formally founded just two years ago, has made a grand entrance onto the scientific scene, at the local, national and international levels. Over the past year we have recruited talented new researchers, established a number of core facilities and implemented administrative procedures in support of the scientific work done here. It has been a year of consolidation and growth and we are well on our way to establishing IRB Barcelona as one of the foremost biomedical research institutes worldwide.

A good measure of our progress can be seen in the list of publications authored by IRB Barcelona scientists, listed at the end of each group's entry in this Scientific Report, achieved in the short time that they have been working here. Specific scientific highlights include a collaboration between the groups of Joan Guinovart and Eduardo Soriano which demonstrated that an excess of glycogen induces neuronal death and causes Lafora disease, a fatal kind of epilepsy that affects adolescents (*Nat Neurosci*, 10(2), 1407-13). Researchers in Cayetano González's group made an important discovery about the mechanisms that regulate stem cell division. They showed that the process must occur asymmetrically along a pre-established axis in order to ensure the correct differential identity of the resulting cells (*Dev Cell*, 12(3), 467-74). 'Disoriented' division may give rise to uncontrolled proliferation and cancer. The Colorectal Cancer groups led by Eduard Batlle and Elena Sancho discovered a mechanism that prevents the spread of colon cancer (*Nat Genet*, 39(2), 1376-86). They found that benign tumour cells receive instructions to grow in confined compartments, and not to invade other areas of the tissue. In collaboration with the Barcelona Supercomputing Center and the Spanish National Institute for Bioinformatics, Modesto Orozco's group published a provisional 'atlas' of the dynamic behaviour of proteins, which provides important basic information on the flexibility of proteins and their ability to link with other molecules, an area of study which opens possibilities for the design of new drugs (*Proc Nat Acad Sci USA*, 104, 796-801). Antonio Zorzano, in collaboration with Manuel Palacín and Fernando Albericio, identified a new set of compounds that show potent anti-diabetic properties, thereby opening up possibilities for the development of a drug to treat type I diabetes (*Diabetes*, 56(2), 486-93).

Another measure is the success of IRB Barcelona scientists in increasing research resources obtained through competitive grants and private funding, both for individual researchers and for special programmes, such as Consolider and CIBER of the Spanish Ministries for Science and Education and Health and Consumer Affairs, respectively, and for EU-funded programmes. An overview of funding sources can be found on page 45 of the Executive Summary accompanying this volume, while details on each activity are included at the end of each group's report.

IRB Barcelona has quickly achieved respectable footing among the ranks of life sciences research institutes worldwide

IRB Barcelona has quickly achieved respectable footing among the ranks of life sciences research institutes worldwide. Our ability over the past year to recruit highly talented scientists to set up their research groups and support activities at IRB Barcelona attests to this. Jens Lüders (from Stanford University) was recruited to the Cell and Developmental Biology Programme and will focus his research efforts on the molecular mechanisms behind microtubule organisation. Xavier Salvatella (from the University of Cambridge) will join the Chemistry and Molecular Pharmacology Programme to work on the structure and dynamics of biomacromolecules and how they relate to disease. Both will take up their positions in 2008. In addition, managers were recruited for the Functional Genomics (Herbert Auer, from Ohio State University), Protein Expression (Nick Berrow, from Oxford University) and Mouse Mutant Core Facilities in 2007. Activities in the Mass Spectrometry Core Facility also began under the leadership of Marta Vilaseca (from the University of Barcelona).



No single research institute can effectively tackle the complexities of molecular life sciences on its own. In order to carry out research that will ultimately lead to benefits for human health, a multidisciplinary approach that draws on the knowledge and abilities of a variety of experts working in different fields is

IRB Barcelona scientists participate in numerous scientific collaborations and networks, at the local, national and international levels

essential. Transforming knowledge into new tools and therapies will require new types of partnerships with universities, clinical centres, and industry. To this end, IRB Barcelona scientists participate in numerous scientific collaborations and networks, at the local, national and international levels. Details are listed with each group's report.

At the institutional level, in 2007 a joint research programme was established between the Department of Life Sciences at the Barcelona Supercomputing Center (BSC) and the Structural and Computational Biology Programme at IRB Barcelona. The goal of this venture is to strengthen research in computational biology and favour collaborations between the two institutes. Through the initiative, IRB Barcelona researchers have direct access to the formidable computational resources of the BSC, while those at the BSC have access to the laboratory facilities at IRB Barcelona.

Research at IRB Barcelona

Our groups are organised into five programmes. Each has a core area of focus but includes themes and projects which overlap with the rest:

Cell and Developmental Biology studies how information in the genome is used to create structures within the cell, to guide the formation and regeneration of tissues, and to create a whole organism. High-throughput methods are used to watch the global activity of genes and proteins during these processes in healthy and diseased organisms.

Structural and Computational Biology begins at the molecular level, studying the structure of single molecules and their interactions. The chief methods that are used derive from physics and computational science: X-rays, NMR, electron microscopy, macromolecular biophysics, bioinformatics and molecular modelling.

Molecular Medicine probes the molecular bases of metabolic and genetic diseases, searches for diagnostic or therapeutic targets, and studies the behaviour of the entire genome and proteome during diseases.

Chemistry and Molecular Pharmacology specialises in the design and synthesis of small molecules and macromolecules that can be used to probe —and possibly repair— proteins and other biological molecules. The programme has a special emphasis on combinatorial chemistry. One focus involves building libraries of substances and optimising methods to produce them; a second involves examining how drugs affect molecules and how they can be modified in order to better control their effects.

Oncology studies diverse aspects of how tumours arise and develop, the relationship between stem cells and cancer, and the identification of cellular programmes that cause particular types of tumours to spread and metastasise in specific parts of the body.

Core facilities

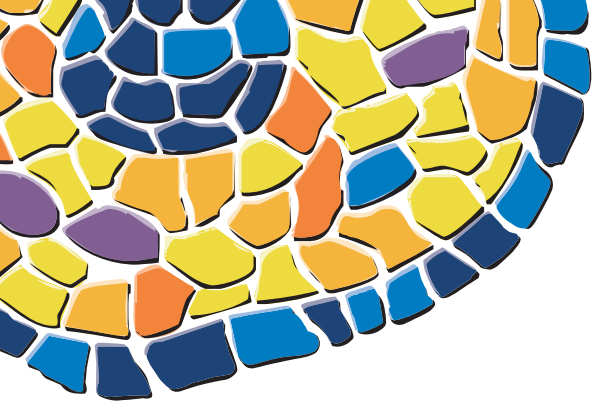
A few years ago laboratories often devoted themselves to the functions of just a few genes or molecules in a single model system; today's medically related projects typically involve monitoring the behaviour of the entire genome, in studies that shuttle from the computer, to the test tube, and to model organisms and human tissues. No single laboratory can master all the techniques needed to pursue these questions, so we have established several new service units to provide our researchers with state-of-the-art facilities. These widen an already considerable palette of platforms established and operated by the Barcelona Science Park and by the Scientific and Technical Services of the University of Barcelona.

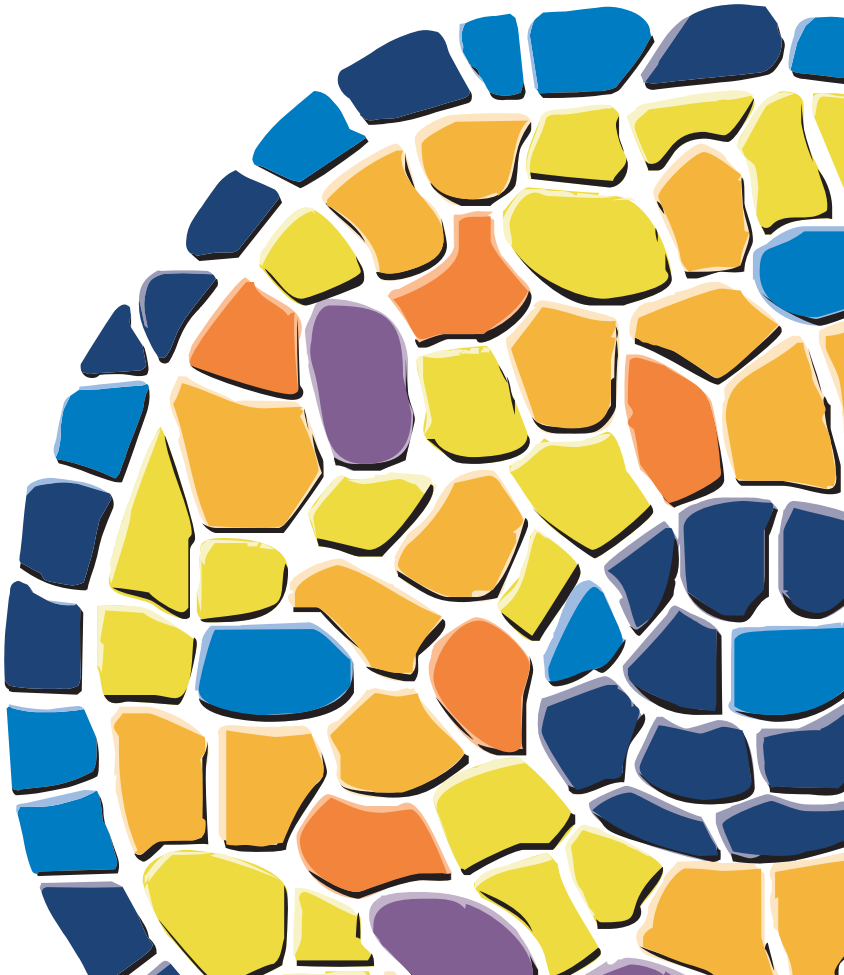
With 27 independent research groups, a large contingent of core facilities, and nearly 400 members, IRB Barcelona has rapidly coalesced into a significant presence in the landscape of biomedical research institutes. Yet, IRB Barcelona has much growth ahead and, most importantly, much potential to be realised. To this end, we plan in the upcoming year to fortify our efforts to recruit top international scientific talent to head new research groups and core facilities, to embrace initiatives of our PhD community to strengthen the activities and opportunities available to our students, and to expand on our outreach activities in order to bring the biomedical research carried out at IRB Barcelona closer to the public.

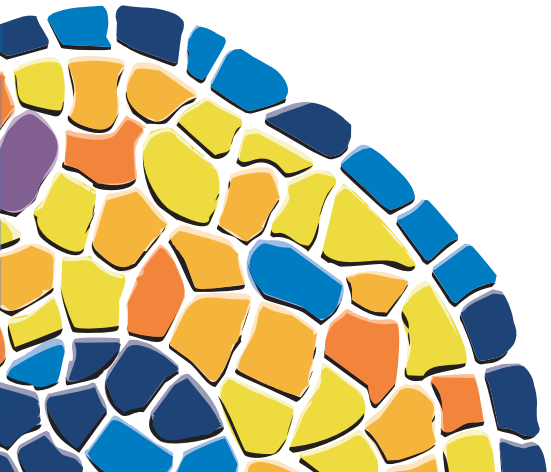
Joan J Guinovart
Director

Joan Massagué
Adjunct Director

Cell and Developmental Biology Programme







Chromatin structure and function

Ferran Azorín

Changes in chromatin structure are at the basis of many regulatory processes. Our understanding of the control of chromatin functions has improved through the identification of components and mechanisms that modify the structural and functional properties of this DNA-protein complex: chromatin assembly and remodeling complexes; histone modifications (ie, acetylation, methylation, phosphorylation, ubiquitination) and the corresponding enzymes; structural non-histone proteins (ie, HP1, Polycomb), which recognise specific histone modifications and contribute to the structural properties of distinct chromatin domains; histone variants, which localise to specific chromosomal locations (ie, CENP-A, H3.3, H2A.Z, macroH2A); etc. Our research focuses on the study of the molecular basis of the function and regulation of chromatin. In particular, we seek to elucidate the contribution of chromatin to the control of centromeric and telomeric function, and the regulation of gene expression.

RNA contributes to chromatin structure

Chromatin structure is influenced by multiple factors, such as pH, temperature, nature and concentration of counterions, post-translational modifications of histones, and binding of structural non-histone proteins. RNA is also known to contribute to the regulation of chromatin structure as chromatin-induced gene silencing depends on the RNAi machinery in *S. pombe*, plants and *Drosophila*. Moreover, both in *Drosophila* and mammals, dosage compensation requires the contribution of specific non-coding RNAs. However, whether RNA itself plays a direct structural role in chromatin remains unclear. We have shown (Rodríguez-Campos and Azorín, 2007) that RNA plays a general structural role in eukaryotic chromatin. RNA is associated with purified chromatin prepared from chicken liver, or cultured *Drosophila* S2 cells, and treatment with RNase A alters the structural properties of chromatin. Our results indicate that chromatin-associated RNAs, which account for 2%-5% of total chromatin-associated nucleic acids, are polyA$^-$ and show a size similar to that of the DNA contained in the corresponding chromatin fragments. Chromatin-associated RNAs are not likely to correspond to nascent transcripts as they are also found bound to chromatin when cells are treated with a-Amanitin. After treatment with RNase A, chromatin fragments of molecular weight >3,000bp of DNA show reduced sedimentation through sucrose gradients and increased sensitivity to micrococcal nuclease digestion. This structural transition, which is observed both in euchromatic and heterochromatic regions, proceeds without loss of histone H1 or any significant change in core-histone composition or integrity.

Structural and functional properties of *Drosophila* telomeric heterochromatin

Telomeres are essential to prevent cellular senescence and genome instability. Most eukaryotes use the telomerase enzyme to maintain their telomeres. In contrast, *Drosophila* uses mainly two retrotransposons, *HeT-A* and *TART*, with a few copies of *TAHRE*, which transposes exclusively to telomeres. These retrotransposons have unique features that are shared across all *Drosophila* species. In species as distant as *D. melanogaster* and *D. virilis*, the *HeT-A* element conserves most of its unusual characteristics while maintaining only 21% of amino acid identity. Most importantly, we have found (Casacuberta *et al*, 2007) that, both in *D. virilis* and *D. melanogaster*, *HeT-A* Gag localises to telomeres in interphase cells and in *D. melanogaster HeT-A* Gag is required for telomere targeting of the other telomeric retrotransposons. Moreover, the *HeT-A* Gag protein of *D. virilis* also localises to telomeres when expressed in *D. melanogaster* cells and, vice versa, the *HeT-A* Gag protein of *D. melanogaster* localises to telomeres in *D. virilis*. Telomeric heterochromatin comprises two domains; the most distal domain, which in the case of *Drosophila* is formed exclusively by head-to-tail arrays of the telomere retrotransposons (HeT-A, TART and TAHRE), and a proximal, or subtelomere domain, comprising complex satellite sequences, the telomere-associated sequences (TAS). Reports from

Research Group Members | **Principal Investigator:** Ferran Azorín | **Associate Researchers:** Jordi Bernués, Elena Casacuberta, Mª Lluisa Espinàs, Alejandro Vaquero | **Postdoctoral Fellows:** Clemènt Carrè, Francesc-Xavier Marsellach, Olga Moreno-Moreno, Mònica Torras | **PhD Students:** Lorena Aguilar, Xavier Aran, Marta Batlle, Marta Blanch, Elisabet Costa, Sergi Cuartero, Joan Font, Marta Lloret, Sonia Medina, David Piñeyro, Rute Sousa, Olivera Vujatovic | **Research Assistants:** Carles Bonet, Esther Fuentes, Gemma Mollà, Alicia Vera | **Visitors:** Tomasso Cabaza (Italy), Pablo Sirkin (Argentina)

PEV (position effect variegation) experiments as well as high resolution immunolocalisation have shown that the heterochromatin in these two domains is not identical. The two telomere subdomains are also affected differently by mutants of proteins known to be chromatin modifiers. We have studied several mutant lines of HP1, Jil-1 and Z4 and our results indicate that mutants of Jil-1 and Z4 affect gene expression in the distal (telomere) but not in the proximal (subtelomere) domain.

The contribution of chromatin to the regulation of homeotic gene expression: characterisation of new regulatory elements within the *Drosophila* bithorax complex

Chromatin makes an essential contribution to the regulation of homeotic gene expression. Expression of the homeotic gene *Abdominal-B (Abd-B)* depends on a modular cis-regulatory region divided into discrete functional domains *(iab)* that control the expression of the gene in a particular segment of the fly. These domains contain regulatory elements involved in both initiation and maintenance of homeotic gene expression, and elements that separate the distinct domains. We have performed an extensive analysis of the *iab-6* regulatory region (Pérez-Lluch *et al*, in preparation), which controls *Abd-B* expression at abdominal segment A6 (PS11), and we have characterised two new polycomb response elements (PREs) in this domain. Transgenic flies containing a P-element transgene white enhancer-mini-white carrying these sequences between the enhancer and promoter show repression of *white* and pairing-dependent silencing (silencing is enhanced in the presence of two copies of the transgene). Moreover, silencing and pairing-

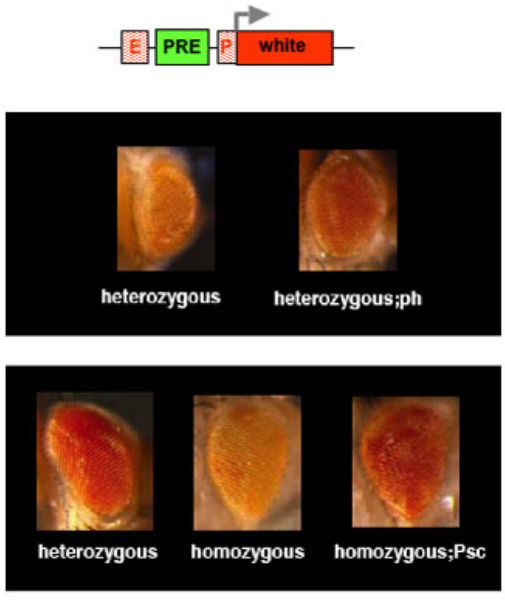


Figure 1. *Silencing and pairing-sensitive silencing of white expression in transgenic lines carrying the construct indicated on top depend on PcG proteins. Eye pigmentation of representative flies carrying the transgene indicated on top in PcG mutant backgrounds, polyhomeotic (ph) and Posterior sex combs (Psc).*

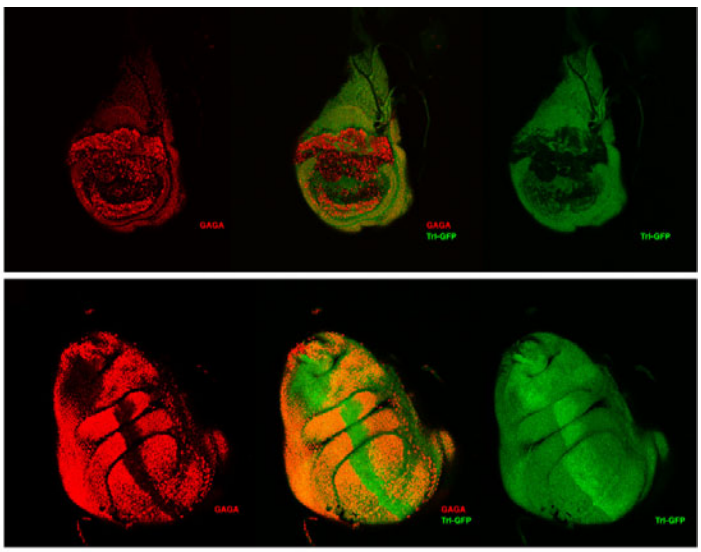


***Figure 2**. GAGA factor represses Trl expression. Using a GAL4-UAS system GAGA (in red) was overexpressed (a) or depleted (b) and the effect on a Trl promoter-GFP reporter construct was analysed in wing imaginal discs of transgenic flies. In the region of the disc where GAGA is overexpressed, GFP is reduced; in the region where GAGA is depleted, GFP expression is enhanced.*

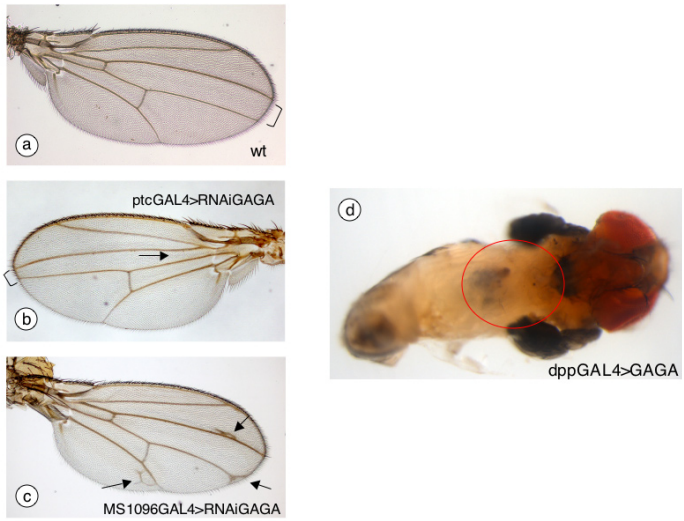


***Figure 3**. Alteration of GAGA factor levels results in abnormal phenotypes. (a) wt wing. (b) Depletion of GAGA factor directed by ptcGAL4 results in a reduced growth of the area affected (indicated by a bracket) and the loss of the anterior cross-vein (arrow) (compare wt a and b). (c) Depletion of GAGA factor directed by MS1096GAL4 leads to alteration of the vein pattern and the appearance of ectopic veins (arrows) (compare a and c). (d) Overexpression of GAGA factor with dppGAL4 results in strong defects in legs (red oval). A severe and abnormal phenotype is shown here (a late pupa missing its six legs).*

sensitive silencing depend on polycomb group (PcG) proteins (Figure 1). PcG silencing has been proposed to be associated with DNA compactation. However, we have found that PREs at *Abd-B* cis-regulatory domains present a particular chromatin structure which is nuclease-accessible throughout *Drosophila* development, and both in active and repressed states. This observation indicates that these cis-regulatory domains are depleted of nucleosomes in both ON and OFF transcriptional states.

A special class of cis-acting elements, known as boundary elements or insulators, has been proposed between each *iab* domain to allow their autonomy to properly specify segmental identity. However, until now only a few of these elements have been functionally identified and proteins responsible for the boundary function of these elements remain largely unknown. We have characterised a boundary element, *Fab-6*, capable of insulating the *iab-5* and *iab-6* cis-regulatory domains. We have found that insulator function of *Fab-6* and *Fab-7* elements depend on the CP190 protein. Our results allow us to conclude that boundary elements are present between each of the regulatory domains that control *Abd-B* expression and to propose that the CP190 insulator protein plays a general role in boundary function in the *Drosophila* bithorax complex.

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

In the fruit fly *D. melanogaster*, GAGA factor, which is encoded by the Trithorax-like gene (*Trl*), is essential in the regulation of chromatin structure and function. *Trl* is a crucial gene for the fly. It presents maternal effect and a homozygous null mutant is embryonic lethal. Little is known, however, about the requirement of GAGA factor at later developmental stages. Using the GAL4-UAS system, we have studied the effects of altering the levels of GAGA factor in transgenic flies both by overexpression and by RNAi-mediated depletion (Bernués *et al*, 2007). We have shown that GAGA factor continuously down-regulates its own expression (Figure 2). This negative feedback mechanism requires DNA-binding capacity but other features of GAGA factor like transactivation and oligomerisation are dispensable. *Trl* is the first gene on which GAGA action results in repression. On all other gene promoters tested GAGA factor always resulted in transcriptional activation. In addition, a statistically significant reduction in wing size has been observed when GAGA factor was depleted and there are indications that this is because cell proliferation is negatively affected in the wing. However, GAGA depletion can also alter wing morphogenesis, in particular venation. Moreover, early general embryonic depletion of GAGA factor results in larval lethality, thereby indicating that this factor is also essential at stages later than the embryonic one.

In general, GAGA factor overexpression is lethal (even when overexpression is only modest) and we have noticed a variety of abnormal phenotypes in response to increased GAGA levels depending on the GAL4 drivers used (Figure 3). Occasional escapers have been observed when overexpression experiments have been carried out at low doses (18°C using weak drivers). Our results indicate that the phenotypes observed depend on the particular stage and cell lineage affected, and are not produced because a general, ubiquitous mechanism is used. Thus, for example, dppGAL4-mediated overexpression of GAGA factor results in apoptosis with strong effects on leg formation that can be reverted by co-expression of DIAP 1 factor. In contrast, MS1096GAL4-mediated overexpression of GAGA factor in wing disk cannot be reverted and produces lethality. Remarkably, while both drivers are expressed in haltere disk, no abnormal phenotype is produced, thereby clearly indicating that GAGA can trigger a range of responses in distinct tissues.

These results suggest that GAGA exerts a complex regulatory programme of gene expression, which is pending further analysis.

Characterisation of *Drosophila* JmjC+N histone demethylases

Histone methylation at lysine residues is crucial in the regulation of a wide range of genomic functions, including heterochromatin formation, dosage compensation, gene expression and cell memory. The key for regulation is the possibility to revert the modification. Lysine-specific histone methyl-transferases (HMTs) have been known for some years. However, it was not until recently that enzymes capable of antagonising lysine-methylation were identified.

The first lysine-specific histone demethylase identified was LSD1, which is capable of demethylating mono- and di-, but not tri-, methylated H3K4 or H3K9. More recently, proteins containing the JumonjiC (JmjC) domain were found to act on trimethylated H3K9 and/or H3K36, H3K4 and H3K27. JmjC-containing proteins constitute an extensive phylogenetic family with multiple members in all eukaryotic species analysed to date, from yeasts to humans. In *D. melanogaster*, a homology search identified up to thirteen JmjC-containing proteins, whose enzymatic activity remains largely uncharacterised. In general, the JmjC-domain is found in combination with other protein domains. In particular, a subclass of JmjC-proteins contain, in addition to the catalytic JmjC-domain, a second highly conserved N-terminal domain (JmjN), which is also required for enzymatic activity. JmjC+N-proteins play crucial regulatory roles during development and cell cycle progression, and are frequently deregulated in cancer. *Drosophila* contains four JmjC+N-proteins: Lid (small imaginal discs), CG3654, CG15835 and CG33182. We have characterised the histone-demethylase activity of these proteins *in vivo* (Lloret-Llinares *et al*, 2008). Our results indicate that Lid demethylates H3K4me3. In addition, we also show that, in contrast to what would be expected from its enzymatic activity, *lid* antagonises gene silencing and it is required for acetylation of histone H3 (Figure 4). On the other hand, CG15835 and CG33182 demethylate H3K9me3 and H3K36me3. Moreover, overexpression of CG15835 results in the spread of HP1 into euchromatin (Figure 5). Finally, overexpression of CG3654 shows no significant effect on the levels of H3K4me3, H3K9me3, H3K27me3, H3K36me3 or H4K20me3.

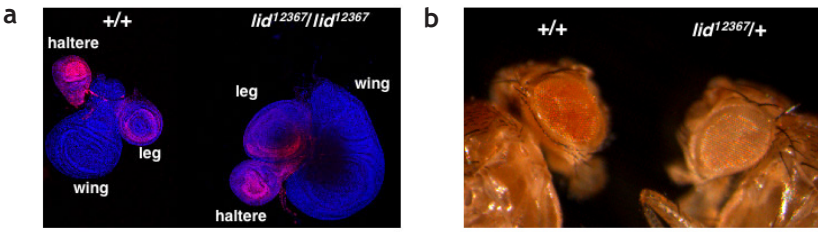


***Figure 4**. lid antagonises gene silencing. (a) Expression of the homeotic gene Ultrabithorax (Ubx) was determined by immunostaining with aUbx antibodies in imaginal discs from control wild-type larvae (+/+) and homozygous lid^{12367}/lid^{12367} mutant larvae. Haltere, wing and leg discs are indicated. (b) The effect of lid mutations on the PEV of $In(1)w^{m4}$ is shown. The eye phenotype of heterozygous $lid^{12367}/+$ flies is compared to that of control siblings wild-type for the locus (+/+).*

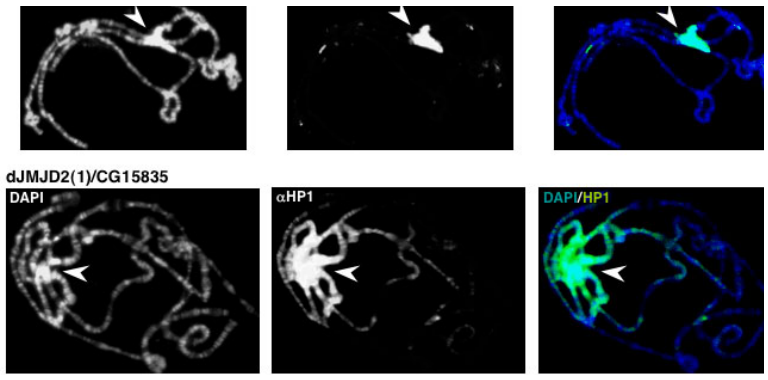


***Figure 5**. Overexpression of dJMJD2(1)/CG15835 induces the spread of HP1 into euchromatin. Polytene chromosomes from UASGAL4-CG15835-Flag; act5C-GAL4 larvae, where CG15835 is ubiquitously expressed (panels dJMJD2(1)/CG15835), and control wild-type larvae (panels +/+) were stained with specific aHP1 antibodies. DNA was stained with DAPI. Arrows indicate the position of the heterochromatic chromocenter.*

Publications

Casacuberta E, Azorín F and Pardue ML. Intracellular targeting of telomeric retrotransposon Gag proteins of distantly related *Drosophila* species. *Proc Natl Acad Sci USA*, **104**, 8391-96 (2007)

Lloret-Llinares M, Carré C, Vaquero A, de Olano N and Azorín F. Characterisation of *Drosophila* melanogaster JmjC+N histone demethylases. *Nucleic Acids Res*, **36**(9), 2852-63 (2008)

Rodríguez-Campos A and Azorín F. RNA is an integral component of chromatin that contributes to its structural organisation. *PLoS ONE*, **2**(11), e1182 (2007)

Vaquero A, Scher M, Erdjument-Bromage H, Tempst P, Serrano L and Reinberg D. SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. *Nature*, **450**(7168), 440-44 (2007)

Research Networks and Grants

Ànalisi estructural i funcional de la cromatina
CIRIT-Generalitat de Catalunya, 2005SGR678: 2005-2008
Research Director: Ferran Azorín

Cromatina silenciada: análisis de los factores y mecanismos implicados en su formación y mantenimiento
Ministerio de Educación y Ciencia, BFU2006-01627/BMC: 2007-2009
Research Director: Ferran Azorín

Characterisation of the role of histone H1 and its post-translational modifications in the functional regulation of chromatin
Marie Curie Action, International Reintegration Grant: 2007-2009
Research Director: Alex Vaquero

Drosophila telomere heterochromatin: Gene silencing and telomere targeting
Marie Curie Action, International Reintegration Grant: 2006-2008
Research Director: Elena Casacuberta

Epigenética: Mecanismos y enfermedad
Ministerio de Educación y Ciencia, Consolider Ingenio 2010, CSD2006-49: 2006-2011
Research Director: Miguel Beato

Episomal vectors as gene delivery systems for therapeutic application
European Commission, VI Framework Programme, FP6-2003-LSH-2: 2005-2008
Research Director: Ferran Azorín

Estudio de los telómeros de Drosophila; relación evolutiva y funcional con los telómeros de telomerasa. Los retrotransposones /HeT-A/ y /TART/ y su relación con otros componentes teloméricos
Ministerio de Educación y Ciencia, BFU2006-13934/BMC: 2007-2009
Research Director: Elena Casacuberta

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
Ministerio de Educación y Ciencia, BFU2007-64395/BMC: 2008-2010
Research Director: Jordi Bernués

Signalling in morphogenesis

Jordi Casanova

The development of multicellular organisms requires changes in cell populations in terms of their proliferation, differentiation, morphology and migration. These synchronized changes are controlled by the genes that specify cell fate and by the capacity of cells to respond to extracellular cues. They are achieved by means of signalling mechanisms that elicit cellular responses that ultimately will be responsible for morphogenetic events during development. Two key steps in these events are the mechanisms that regulate the appropriate spatial and temporal activation of the signalling pathways and the mechanisms that link these pathways to the cell effectors in order to elicit cell responses in terms of gene activity or cell morphology. Our research efforts focus on these phenomena in the context of the whole organism and for this purpose we use *Drosophila melanogaster* as a model. The basic similarity between developmental processes in different species justifies the choice of an organism as a model system. In particular, we analyse these mechanisms in two model systems in *Drosophila*, the Torso receptor signalling pathway and tracheal system morphogenesis.

Two distinct but convergent groups of cells trigger Torso receptor tyrosine kinase activation by independently expressing torso-like

Cell fate in development is often impinged by signalling interactions with neighbouring cells. In this regard, a key element is the spatial distribution of signalling molecules in a precise group of cells. This distribution can be achieved either by the regulated expression of the signalling molecule itself or by the restricted activity of a modifying enzyme that transforms an inactive precursor into a fully active signalling molecule. In addition, cells can exchange their neighbours and thus these molecules might be synthesised far from their final site of action. This is the case for the specification of the most anterior and posterior regions of the embryo by the Torso (Tor) RTK signalling pathway. The Tor receptor, which is present throughout the blastoderm membrane, is activated only at the poles by an unknown mechanism thought to involve the cleavage of Trunk (Trk), the putative Tor ligand. This mechanism of Tor activation is triggered by the expression of , which encodes a protein of unknown function thought to be required for the processing of Trk. Indeed, it is the restricted expression of that determines the localised domain of Tor activation.

is expressed in three cell populations in the egg chamber. One of these populations is a group of follicle cells at the posterior end of the chamber (PFCs), which are in close contact with the posterior end of the oocyte, one of the regions where the Tor pathway is activated upon oocyte fertilisation. At the anterior end, is expressed in two cell populations, border cells (BCs) and centripetal cells (CCs). BCs arise at the anterior of the egg chamber, far from the oocyte. CCs are specified later, at an anterior-lateral position close to the oocyte. In contrast to the situation at the posterior end, both BCs and CCs are originally distant from the anterior region of the oocyte where the Tor pathway will be activated after oocyte fertilisation, and it is only after the migration of these cells that they become juxtaposed to this region of the oocyte (Figure 1).

We have analysed the regulation of expression in order to determine how these distinct groups of follicle cells acquire the capacity to express a common

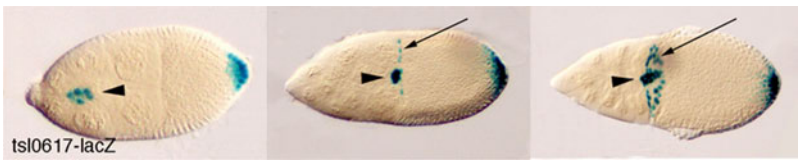


***Figure 1**. tsl is expressed in the border cells (arrowhead) and centripetal cells (arrow) in the anterior region of the oocyte and in some follicle cells at the posterior end of the eggchamber (see text).*

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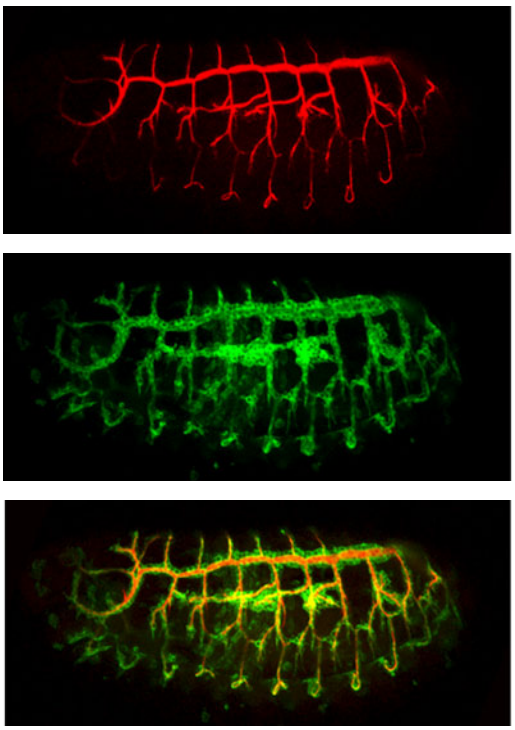

Figure 2. *The embryonic tracheal system of Drosophila is a complex tubular network that arises from sets of ectodermal cells that invaginate and migrate in stereotyped and distinct directions to form each of the primary tracheal branches. In red the tracheal lumen, in green the tracheal cells.*

signalling factor. We have found that JAK/STAT signalling, which is responsible for initially patterning the egg chamber epithelium, also regulates expression in PFCs and BCs. However, this pathway does not regulate expression in CCs, which, in these cells, is regulated by a different enhancer. We have also examined the functional significance of expression in BCs and CCs, and have found that both cell groups contribute to trigger Tor activation in the anterior embryonic region. Our results illustrate how independently acquired expression of a signalling molecule in distinct group of cells constitutes a mechanism by which these cells act together in the activation of a signalling pathway.

Signalling and cytoskeleton organisation in tracheal morphogenesis

We address the link between signalling and cytoskeleton by examining the mechanisms involved in tracheal morphogenesis. To this end, we are undertaking a complete description of tracheal morphogenesis at the cellular level to analyse the contribution of cytoskeleton elements and cell organelles to this process and to establish the changes in these elements that are under the control of signalling pathways and the mechanisms involved in this control.

The tracheal system of is a complex tubular network that conducts oxygen from outside the organism to internal tissues. This network arises from the tracheal placodes, clusters of ectodermal cells at each side of 10 embryonic segments from the 2nd thoracic segment to the 8th abdominal segment. Tracheal cells are specified by the activity of a set of genes whose expression in these cells is regulated by the genes

that specify positional cues along the embryonic body axes. Cells of each cluster invaginate and migrate in stereotyped and distinct directions to form each of the primary tracheal branches. The general conclusion from many studies is that the migratory direction of tracheal cells relies on a set of positional signals provided by nearby cells. In addition, the establishment of interactions between tracheal cells and their substrates is a crucial step in tracheal cell migration, a process ultimately determined by molecules expressed at their surface (Figure 2).

In this context, in collaboration with Marta Llimargas' group at the IBMB (CSIC), Sofia Araújo has been studying the role of the transcription factor Tramtrack (Ttk) in the development of the tracheal system. They have found that, in addition to a role in the specification of distinct tracheal cell identities, Ttk is directly involved and required for a range of cellular responses and morphogenetic events. In particular, Ttk appears to be a new positive regulator of tracheal cell intercalation. Analysis of this process in mutants has unveiled cell shape changes as a key requirement for intercalation and has identified Ttk as a novel regulator of its progression. In addition, Ttk is also a regulator of intracellular lumen formation and is autonomously involved in the control of tracheal tube size by regulating septate junction activity and cuticle formation. In summary, Ttk governs tracheal development by acting on the regulation of sequential steps of tube morphogenesis.

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Research Networks and Grants

Ajut per a grups de recerca singular
Generalitat de Catalunya, SGR2005-00508: 2005-2008
Research Director: Jordi Casanova

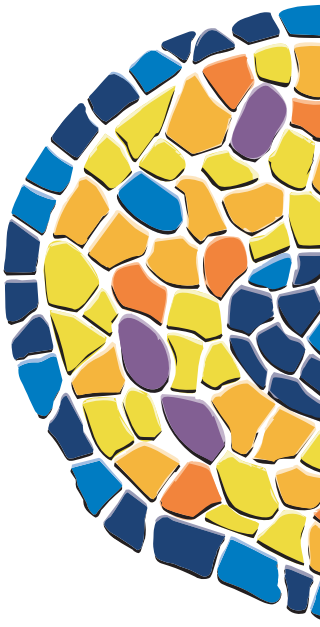
Mecanismos de señalización celular y morfogénesis en el desarrollo de Drosophila
Ministerio de Educación y Ciencia, BFU2006-01935/BMC: 2006-2009
Research Director: Jordi Casanova

Collaborations

Analysis of Drosophila tracheal development
Marta Llimargas, IBMB-CSIC (Barcelona, Spain)

Interaction between germ-cell determinants and Torso RTK signalling in Drosophila
Ruth Lehmann, Skirball Institute (New York, USA) and Rui Martinho, Instituto Gulbenkian de Ciência (Oeiras, Portugal)

On the origin of insect tracheal systems
Michalis Averof, IMBB (Crete, Greece)



Cell division

Cayetano González

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*.

Modelling cancer in *Drosophila*

We are starting to exploit *Drosophila* to study some basic principles of cell proliferation and malignant growth (Caussinus and González, 2005; Wodarz and González, 2006). This research focuses on the role of neural stem cells (larval neuroblasts) as the cell-of-origin of tumours.

Loss of cell polarity and cancer are tightly correlated; however, evidence of a causative relationship remains elusive. In stem cells, loss of polarity and impairment of asymmetric cell division could alter cell fates and thereby render daughter cells unable to respond to the mechanisms that control proliferation. To test this hypothesis, we generated larval neuroblasts from *Drosophila melanogaster* with mutations in various genes that control asymmetric cell division and then assayed the proliferative potential of these cells after transplantation into adult hosts. Larval brain tissue carrying neuroblasts with mutations in raps (also called pins), mira, numb or pros grew to more than 100 times its initial size, thereby invading other tissues and killing the host in two weeks. These tumours became immortal and could be retransplanted into new hosts for years. Six weeks after the first implantation, genome instability and centrosome alterations, two traits of malignant carcinomas, were detected in these tumours. Increasing evidence suggests that several tumours may be of stem cell origin. Our results show that loss-of-function of any of several genes that control the fate of stem cell daughters cause hyperproliferation, thus triggering a chain of events that subverts cell homeostasis in a general sense and leads to cancer.

Our recent findings include the observation that in stem cells, centrosomes are asymmetric in function and fate (Rebollo *et al*, 2007). Like the stem cells in other higher eukaryotes, *Drosophila* neural stem cells (NBs) undergo asymmetric division whereby one of the two daughters retains stem cell identity, while the other enters a differentiation programme. The molecular mechanisms that drive asymmetric division in these cells have been the subject of intensive research effort in the last decade. Briefly, the localisation of the Par complex, which includes Bazooka (Baz), Par-6 and atypical protein kinase C (aPKC) at the apical cortex, drives the basal localisation of the adaptor proteins Miranda and Partner of Numb. These, in turn, mediate the accumulation of cell fate determinants such as Numb Prospero at the basal cortex of the NB. Consequently, upon cell division, the determinants end up mostly within the small ganglion mother cell that buds off the basal side of the NB, thereby resulting in the unequal developmental fate of the two daughters (González, 2007).

A key step in the asymmetric segregation of the determinants is spindle orientation, which must be in line with the polarity axis of the cell. This process is governed by Inscuteable, another protein of the apical complex that binds to Baz and aPKC. Inscuteable mediates the organisation of a complex that includes Pins Gal and Mushroom body defective (Mud), the protein thought to ultimately mediate spindle orientation through direct interaction with one of the two mitotic asters, which is thereby singled out as the apical aster. It is unclear, however, how the system discriminates between the two asters of the NB so that only one engages in this interaction. Unlike embryonic NBs where, after assembly, the spindle rotates 90 degrees to align along the polarity axis, larval NBs assemble the spindle already aligned and no rotation occurs. The contribution of aPKC to spindle orientation also differs between larval and embryonic NBs. Directed assembly without rotation has also been observed in grasshopper embryonic NBs and in *Drosophila* germline stem cells (González, 2007).

To study this key process, we have recorded *Drosophila* larval NBs engineered to express fluorescent reporters for microtubules, pericentriolar material

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(PCM), and centrioles. We have found that early in the cell cycle, the two centrosomes become unequal: one organises an aster that remains near the apical cortex for most of the cell cycle, while the other loses PCM and microtubule-organising activity, and moves extensively throughout the cell until shortly before mitosis when, located near the basal cortex, it recruits PCM and organises the second mitotic aster. Upon division, the apical centrosome remains in the stem cell, while the other goes into the differentiating daughter cell. Apical aster maintenance requires the function of Pins. These results reveal that spindle orientation in *Drosophila* larval NBs is determined very early in the cell cycle and is mediated by asymmetric centrosome function (Rebollo *et al*, 2007).

Molecular analysis of centrosomes

We have recently cloned the gene that encodes a centriolar protein. We have mutant alleles, functional GFP fusions and antibodies that will be instrumental in the molecular dissection of this organelle (Lange *et al*, 2002; Tavosanis and González, 2003). Centrosomes are the major organisers of the microtubule network in most animal cells, and comprise centrioles embedded in a web of PCM. Recruitment and stabilization of PCM on the centrosome is a centriole-dependent function. Compared to the considerable number of PCM proteins known, the molecular characterisation of centrioles is still very limited. Only a few centriolar proteins have been identified to date in *Drosophila*, most of these related to centriole duplication.

We have cloned asterless (*asl*) and found that it encodes a 120 kD highly coiled-coil protein that is a constitutive pancentriolar and basal body component. Loss of *asl* function impedes the stabilization/maintenance of PCM at the centrosome. In embryos deficient for Asl, development is arrested immediately after fertilisation. Asl shares significant homology with Cep152, a protein described as a component of the human centrosome for which no functional data is yet available. In conclusion, the cloning of *asl* offers new insight into the molecular composition of *Drosophila* centrioles and a model for the role of its human homologue.

In terms of the roles that centrioles play during development, our results show that the first zygotic division never occurs in a cytoplasm deficient for Asl. This observation strongly suggests that functional centrosomes are required for embryogenesis in *Drosophila*. The finding that the first mitotic division does not take place in eggs derived from females lacking PCM components, like gTUB37C or D-TACC, leads to the same conclusion. However, the possibility remained that this early developmental arrest in gTUB37C or D-TACC-deficient embryos could be a downstream consequence of the meiotic defects caused by mutations in these genes. This caveat is now largely circumvented by the phenotype of embryos derived from *asl* mutant females in which both meiotic divisions proceed normally. Thus, although we cannot rule out a possible non-centrosomal function of Asl, the pheno-

type of embryos derived from *asl* mutant females is consistent with the hypothesis that centrosomes are required for *Drosophila* embryo development. Previous reports have shown that zygotic loss of key centrosomal proteins such as D-plp, Sas4, Sak/Plk4, or Cnn does not block progression of development into adult flies. However, the centrosome-less females that hatch are sterile, which strongly suggests that eggs defective for these centrosomal components do not support embryogenesis. How development can proceed in zygotic loss-of-function conditions for these genes is not clear. However, the initial stages of development of individuals homozygous for mutations in these centrosomal proteins are likely to be sustained by the wild-type RNA/protein contributed to the egg by the heterozygous females from which they derive. Thus, until a certain stage, which is hard to specify, development in these mutant individuals occurs when cells still have centrosomes.

The hatching of adults that have undergone the last stages of development without centrosomes proves a certain level of centrosome dispensability in *Drosophila* development, even though these adults are uncoordinated and sterile and die only hours after eclosion. Loss of centrosome function has been reported to impair a number of developmental stages in vertebrates. In humans, for instance, abnormal centrosomes have been linked to impaired neuronal migration, hereditary spastic paraplegia, Bardet-Biedl syndrome, the development of cystic kidneys, perturbed left-right asymmetry, microcephaly, and cancer. In mice, loss-of-function for Sak/Plk4 is a lethal condition, and haploinsufficiency for this gene results in a high incidence of tumours. The molecular dissection of centrioles in *Drosophila* may help to model the cellular basis of some of these processes.

Spindle assembly and cell division *in vivo*

Direct visualisation is becoming mandatory to unravel the complex processes that occur within the living cell. Using protocols developed in our laboratory, we are obtaining new information on the behaviour of specific proteins labelled with florescent tags (Rebollo and González, 2000; Lange *et al*, 2002; Sampaio *et al*, 2001; Rebollo *et al*, 2004).

Through this technology, we have unveiled a new role for *arf6* during cytokinesis. The dramatic morphological changes during cell cytokinesis requires the interplay between microtubules and the actomyosin contractile ring, and addition of membrane to the plasma membrane. Numerous membrane-trafficking components localise to the central spindle during cytokinesis, but it is still unclear how this machinery is targeted there and how membrane trafficking is coordinated with cleavage furrow ingression. Here we use an *arf6* null mutant to show that the endosomal GTPase ARF6 is required for cytokinesis in *Drosophila* spermatocytes. ARF6 is enriched on recycling endosomes at the central spindle, but it is required neither for central spindle nor actomyosin contractile ring assembly, nor for targeting of recycling endosomes to the central spindle. However, in *arf6* mutants the cleavage furrow regresses because of a failure in rapid membrane addition to the plasma membrane. We propose that ARF6 promotes rapid recycling of endosomal membrane stores during cytokinesis, which is critical for rapid cleavage furrow ingression (Dyer *et al*, 2007).

Publications

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Research Networks and Grants

Alteraciones en la localización subcelular y transformación cancerosa: Determinación de su utilidad diagnóstica
Fundación Mutua Madrileña Automovilística: 2004-2007
Research Director: Cayetano González

CENTROSOMA 3D; Hacia la comprensión estructural y funcional del centrosoma
Ministerio de Educación y Ciencia, Consolider Ingenio 2010, CSD2006-23: 2006-2011
Research Director: Cayetano González

Desarrollo de modelos tumorales en Drosophila melanogaster
Generalitat de Catalunya, 2005SGR-00821: 2006-2010
Research Director: Cayetano González

Identification of pathways that are relevant for the malignant transformation of stem cells in Drosophila
Ministerio de Educación y Ciencia, BFU2006-05813: 2007-2009
Research Director: Cayetano González

Integrative approach to cellular signalling and control processes: bringing computational biology to the bench, COMBIO.
European Commission, VI Framework Programme, STREP LSH-2004-503568: 2004-2007
Research Director: Cayetano González

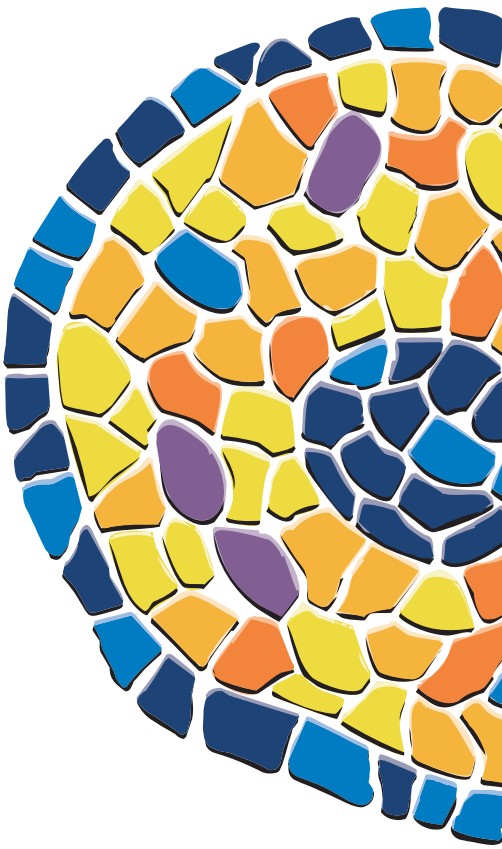
ONCASYM — Cancer stem cells and asymmetric division
European Commission,VI Framework Programme, STREP LSH-2005-2205: 2006-2009
Research Director: Cayetano González

Other Funding Sources

Oncostem. Collaboration agreement, University of Geneva

Collaborations

The role of arf6 in spermatocyte cytokinesis
Marcos González-Gaitán, Département de Biochimie, Sciences II (Geneva, Switzerland)



Drosophila limb development

Marco Milán

A central question in modern developmental biology is how the growth and patterning of tissues are controlled at a molecular and genetic level. *Drosophila melanogaster*, commonly known as the fruit fly, has become an indispensable model system to approach this question because it can be easily manipulated genetically and molecularly and there is abundant information available on its developmental biology. Systematic genetic screens for loss-of-function mutations, gain-of-function phenotypes and the detection of enhancers have revealed many of the genes involved in a number of developmentally critical processes. In addition, the completion of the genomic sequencing project for *Drosophila* makes it possible to use reverse genetic approaches, such as RNA-mediated interference and targeted gene disruption, as well as genome-wide expression analyses, to address a wide variety of issues concerning the developmental biology of the fruit fly.

During the development of multi-cellular organisms, groups of cells assemble to form tissues that are initially homogenous. The elaboration of spatial pattern often begins with the subdivision of a field of cells into smaller territories. The imaginal discs of *Drosophila* provide well-characterised experimental systems in which the subdivision of tissue depends on mechanisms that limit cell mixing, thereby producing stable boundaries. These stable subdivisions are called compartments. In the imaginal discs compartment boundaries serve as signalling centres. Short-range interactions between cells in adjacent compartments induce the expression of the signalling proteins Wingless (Wg) and Decapentaplegic (Dpp) in cells adjacent to the compartment boundaries. Wg and Dpp form long-range extracellular protein gradients centred on these boundaries. Stable boundaries between compartments result in tightly localised sources of these signalling proteins. Intermingling of cells at the compartment boundary causes disorganisation of the signalling centre with disastrous consequences for patterning and growth control.

The *Drosophila* wing imaginal disc is a monolayered epidermal sac. The wing primordium arises from the embryonic ectoderm as a group of around 30 cells, which proliferate extensively during larval development to achieve a final population of about 50,000 cells. The wing primordium is subdivided into an anterior (A) and a posterior (P) compartment by the restricted expression and activity of the homeodomain protein Engrailed in P cells. This subdivision is inherited from the embryonic ectoderm. When the wing primordium consists of around 100 cells, it becomes subdivided again into a dorsal (D) and a ventral (V) compartment by the restricted expression and activity of the LIM-homeodomain protein Apterous in D cells. Our research efforts focus on elucidating the generation of compartment boundaries, the induction of expression of the organizing molecules Wg and Dpp at the compartment boundaries, and the organisation of growth and patterning by the activity of Wg and Dpp at these boundaries. The following topics were addressed during 2007:

A novel molecular mechanism to restrict hedgehog expression in the *Drosophila* wing

Stable subdivision of *Drosophila* limbs into an A and a P compartment is the result of asymmetric signalling by Hedgehog (Hh) from P to A cells. The activity of the homeodomain protein Engrailed in P cells contributes to generating this asymmetry by inducing Hh expression in the P compartment and at the same time repressing the expression of the essential downstream component of the Hh pathway Cubitus interruptus (Ci). Ci is a transcription factor, which, in the absence of Hh signalling, is converted to a repressor form (Ci^{rep}). Ci^{rep} represses *hh* in A cells. The transcriptional co-repressor Groucho (Gro) also represses *hh* expression in A cells, thus helping to maintain the aforementioned asymmetry. Gro is ubiquitously expressed but it is required only in A cells that receive the Hh signal.

Over the last two years, Fernando Bejarano and Lidia Pérez, in collaboration with Christos Delidakis' group in Heraklion (Crete), have been analysing the role of Gro in this process (Bejarano *et al*, 2007). They have shown that it exerts its action by binding to the product of master of *thickveins (mtv)*, a target of Hh

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activity encoding a nuclear Zinc Finger protein. Two distinct mechanisms are then used to repress *hh* expression in A cells (Figure 1). The first is based on Ci^{rep} and acts mainly in those cells not receiving the Hh signal. The second is based on Hh restricting its own expression domain through the activity of its target gene *mtv*. We have shown that these two mechanisms are independent.

Many signalling molecules restrict their own expression domains (ie, Wg or Notch). To our knowledge, these results show, for the first time, that Hh does the same (Figure 1).

Robustness and stability of the gene regulatory network involved in DV boundary formation in the *Drosophila* wing

Gene regulatory networks in developing organisms have been conserved during evolution. The *Drosophila* wing and the vertebrate hindbrain share the gene network involved in the establishment of the boundary between D and V compartments in the wing and adjacent rhombomeres in the hindbrain (Figure 2). The activation of the receptor Notch at the compartment boundaries, as a result of the activity of Notch ligands in nearby cells, induces the expression of the signalling molecules Wg and Wnt-1 in boundary cells of the fly wing and the vertebrate hindbrain, respectively (Figure 2). Wg or Wnt-1 maintain the expression of Notch ligands in neighbouring cells, thus establishing positive feedback and ensuring high activity of Notch at the compartment boundaries. Notch activity then regulates the growth of the surrounding non-boundary cells and is required for maintaining the lineage restriction boundary.

By means of a Systems Biology approach that combines *in silico* and *in vivo* experiments, in collaboration with Javier Buceta's group in Barcelona, Héctor Herranz has generated a regulatory network for the establishment and maintenance of the DV boundary in the *Drosophila* wing (Buceta *et al*, 2007). This network shows how short-range cell interactions, medi-

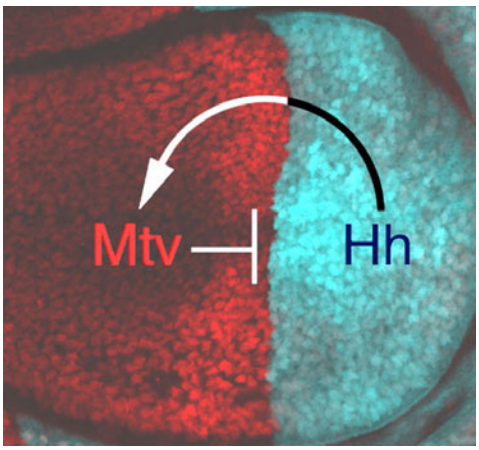

***Figure 1**. Wing imaginal disc labelled to visualize the expression of the mtv-lacZ reporter gene (antibody to β-gal, red) in a hh-GAL4;UAS-GFP background (GFP, blue).*

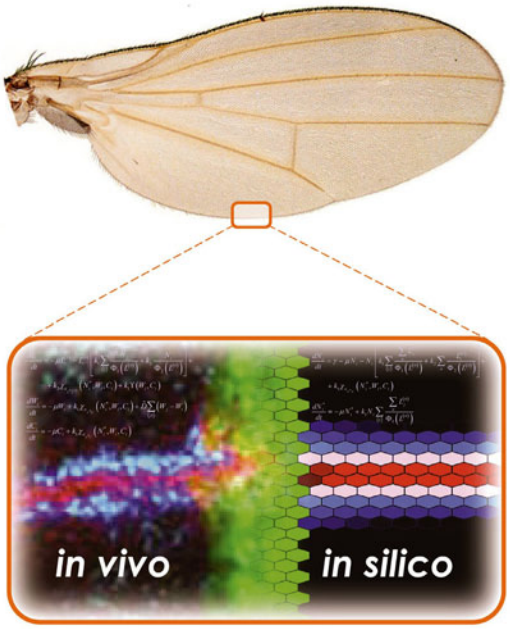

Figure 2. *DV Boundary formation in the fly wing relies on cell interactions between boundary (red) and non-boundary (white/blue) cells and is mediated by the activities of Notch and Wg.*

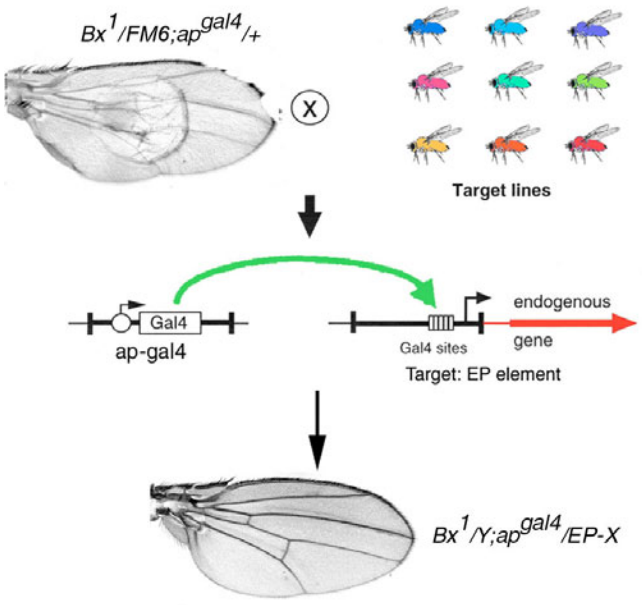

Figure 3. *Beadex^{1}/FM6; apterous^{Gal4}/CyO flies, which have a strong loss of wing margin phenotype, were crossed with a large number of independent Enhancer-Promoter (EP)-containing lines. Gal4 expressed in D cells should bind to Gal4 binding sites within the target element enhancer and activate an adjacent endogenous gene X. Those lines that rescued the wing margin phenotype were selected.*

ated by the receptor Notch and its ligands, together with long-range cell interactions, mediated by the Wg signalling molecule, shape the boundary and produce the gene expression pattern that is observed *in vivo*. The data from this research also provide *in vivo* and *in silico* evidence that a novel property, conferred by the activity of Notch in boundary cells and mediated by its target gene Cut, is required for the formation of a stable DV boundary: namely refractoriness to the Wg signalling molecule. We have addressed and explained concepts like spatially refined and polarized Notch signalling by invoking such a property. A robustness analysis of the regulatory network by means of *in silico* experiments complements these results and ensures the biological plausibility of the developmental mechanism proposed.

We wish to place our conclusions into a broader context. Boundary formation between adjacent rhombomeres in vertebrates relies on the same Wnt/Notch-dependent regulatory network. Therefore, we speculate that boundary cells also need to be refractory to the Wnt signal to generate stable boundaries. We conclude that the robustness and stability of this network, in which the interconnectivity of the elements is crucial and even more important than the value of the parameters used, might explain its use in boundary formation in other multicellular organisms (Figure 2).

A gain-of-function suppressor screen for genes involved in DV boundary formation in the *Drosophila* wing

The *Drosophila* wing primordium is subdivided into a D and a V compartment by the activity of the LIM-Homeodomain protein Apterous in D cells. Cell interactions between D and V cells induce the activation of Notch at the DV boundary. Notch is required for the maintenance of the compartment boundary and the growth of the wing primordium. *Beadex*, a gain-of-function allele of *dLMO*, results in increased levels of dLMO protein, which interferes with the activity of Apterous and results in defects in DV axis formation.

We have performed a gain-of-function screen to search for suppressors of *Beadex* when overexpressed in D cells. We have identified 53 lines corresponding to 35 genes. Loci encoding for micro-RNAs and proteins involved in chromatin organisation, transcriptional control and vesicle trafficking have been characterised in the context of *dLMO* activity and DV boundary formation.

Our results indicate that a gain-of-function screen in a *Beadex*-sensitized background to search for suppressors of the wing margin phenotype is efficient in identifying known and new genes involved in DV boundary formation as well as in the regulation of

Beadex/dLMO activity. We have shown that many of the *Beadex* suppressors involved in DV boundary formation are not essential during wing development. This observation suggests that these suppressors share redundant activities with other gene products. The gain-of-function approach has also been shown to be extremely efficient in unravelling new roles for the recently identified micro-RNAs.

Loss-of-function-based forward genetic screenings have not been as productive in this regard, probably because of the reduced size of these miRs or their redundant activities. Taken together, a suppressor gain-of-function screen in a sensitized background provides a suitable combination to identify new genes, including miRs and redundant genes, involved in a given process.

Redundancy and regulatory feedback loops contribute to the robustness of gene regulatory networks. Classical loss-of-function-based forward genetic screenings have been highly productive in identifying genes that behave as hubs in these networks. Essential genes in yeast are among those most highly connected. However, forward genetic screenings are not as effective in identifying redundant genes or regulators of these feedback loops, whose loss-of-function might not show an overt phenotype. More quantitative *in vivo* genetic screenings have been more efficient in this regard. Our results indicate that a gain-of-function *in vivo* genetic screen in a sensitized background is a strong alternative for the identification of redundant genes or regulators of feedback loops involved in developmental gene regulatory networks (Figure 3).

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Research Networks and Grants

Ayudas a grupos emergentes
Generalitat de Catalunya, 2005 SGR 00118: 2006-2009
Research Director: Marco Milán

Cell affinities in the development of multicellular organisms: the dorsal-ventral affinity boundary in the Drosophila wing
Ministerio de Educación y Ciencia, BFU2004-00167/BMC: 2004-2007
Research Director: Marco Milán

Compartments, organizing molecules and growth control in Drosophila
EMBO Young Investigator Programme: 2007-2010
Research Director: Marco Milán

Establishment and maintenance of compartment boundaries in the Drosophila wing imaginal disc
Ministerio de Educación y Ciencia, BFU2007-64127/BMC: 2007-2010
Research Director: Marco Milán

Collaborations

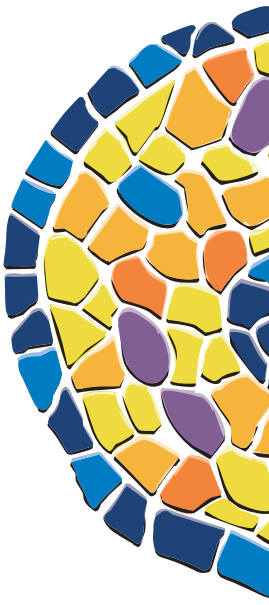
Growth control in the Drosophila wing
Francisco A Martín, Centro de Biología Molecular, Universidad Autónoma de Madrid/CSIC (Madrid, Spain)

In silico modeling of DV boundary formation
Javier Buceta, Centre especial de Recerca en Química Teòrica (CeRQT), Barcelona Science Park (Barcelona, Spain), Ramon Reiguada and Frances Sagues, Departament de Química-Física, University of Barcelona (Barcelona, Spain)

Regulation of Hh expression
Christos Delidakis, Institute of Molecular Biology and Biotechnology, FoRTH, and Department of Biology, Crete University (Crete, Greece)

Awards

EMBO Young Investigator Programme Award, European Molecular Biology Organization (2007)



Cellular networks linked to protein synthesis and human disease

Lluís Ribas de Pouplana

Our laboratory studies the connections between the protein synthesis machinery and the rest of the cellular environment. Gene expression and protein synthesis constitute the core of molecular biology and are therefore intimately linked to all the networks that regulate cells and tissues. The sophisticated machinery required to translate genes is well characterised, but the integration of genetic code components with the rest of the cellular metabolism is poorly understood. Alterations in the delicate balance that links protein synthesis to cell biology cause a vast array of illnesses. Elucidation of this balance is essential for the development of new therapeutic agents. Our research efforts focus on studying the associations between gene expression, protein synthesis and human disease. The projects undertaken in our laboratory can be divided into three general fields: gene expression and parasite pathogenicity, organelle protein expression and mitochondrial disease, and protein synthesis disorders and drug discovery.

Gene expression and parasite pathogenicity

(Manuel Castro, Alfred Cortes, Valerie Crowley and Thomas Jones)

Our research addresses the relationship between infectious processes in humans and the protein synthesis apparatus of the pathogens involved. Thomas Jones is currently characterising an unusual aminoacyl-tRNA synthetase in the human intracellular parasite *Mycoplasma penetrans*. This parasite presents remarkable genome reduction and yet some of its protein synthesis components display new domains of unknown function. The study of this protein has revealed a new mechanism for the discrimination of methionine and isoleucine during protein synthesis (Jones *et al*, 2008; Figure 1).

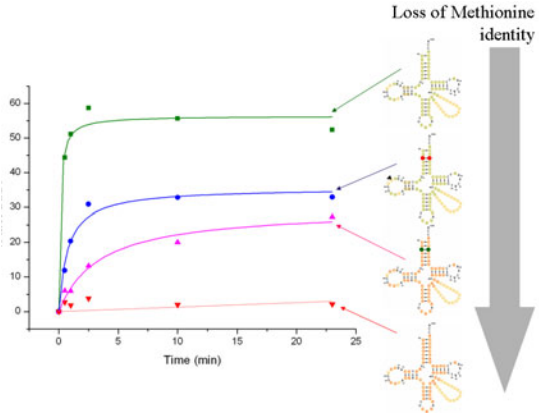
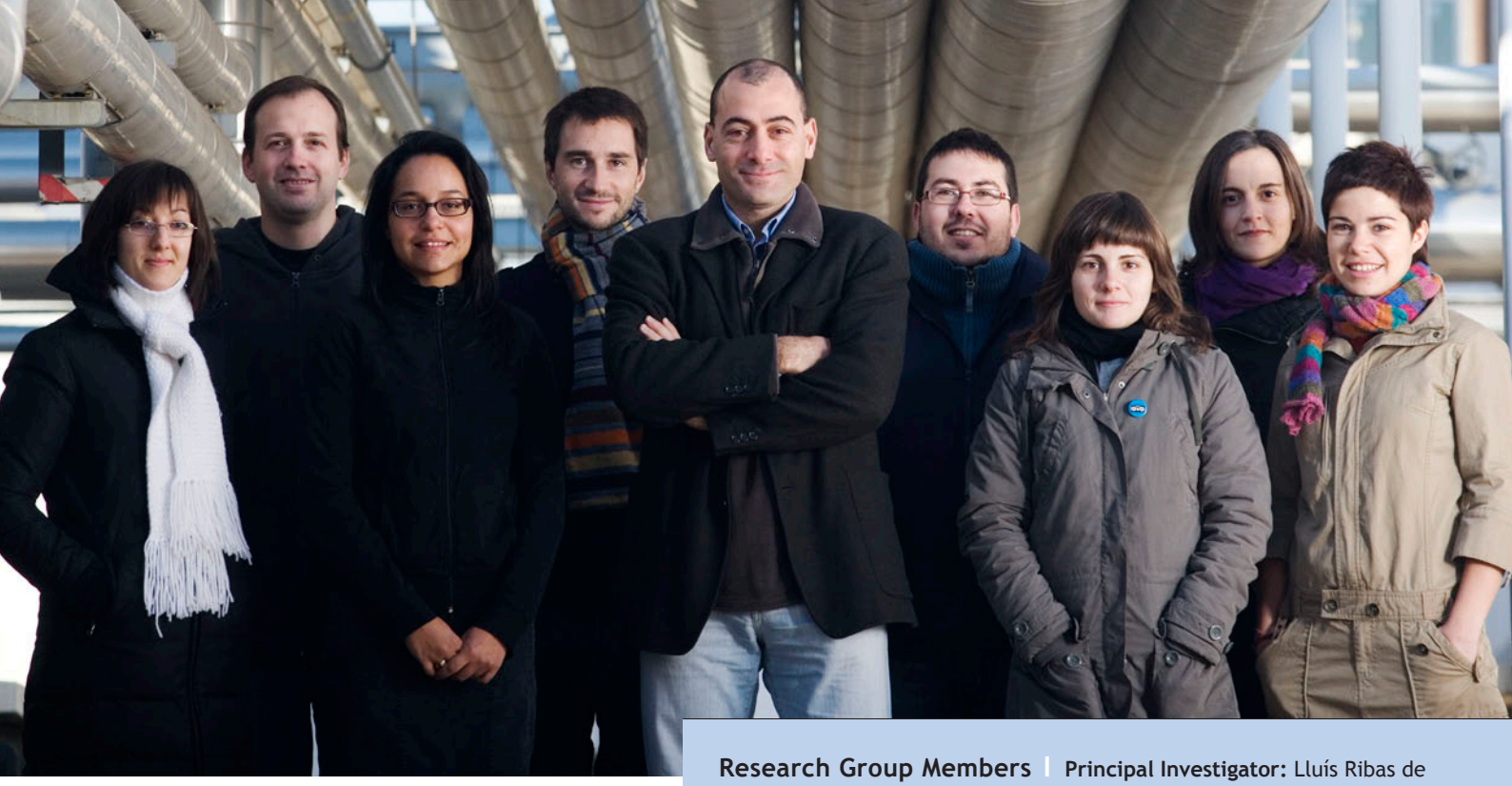


***Figure 1**. Manipulation of the methionine identity of Mycoplasma penetrans tRNAMet following new rules for methionine and isoleucine discrimination.*

Our studies also deal with genome dynamics and the functional role of a family of inflammation-activator domains that are transferred between enzymes involved in protein synthesis through a ubiquitous process of domain shuffling, which remains to be elucidated. In the human pathogen *Entamoeba histolytica*, one of these domains doubles its genomic dose through its selective integration into two unrelated enzymes. We have shown that *Entamoeba* generates a functional mimic of a human pro-inflammatory cytokine, and we are now examining its role in infection.

Our research effort also focuses on the contribution of gene regulation to the infection of red blood cells by the parasite *Plasmodium falciparum*, which is the main causal agent of malaria and responsible for over a million deaths a year. Using a multigenic family of proteins that display differential expression in clonal lines of *P. falciparum*, we study the role of chromatin structure and modification in the control of gene expression in *Plasmodium*. This research is directed by Alfred Cortes (Cortés *et al*, 2007).



Research Group Members | **Principal Investigator:** Lluís Ribas de Pouplana | **Associate Researcher:** Alfred Cortés Closas | **Postdoctoral Fellow:** Renaud Geslain | **PhD Students:** Manuel Castro de Moura, Valerie Crowley, Yaiza Español Fernández, Tanit Guitart Rodés, Thomas Jones | **Lab Technician:** Noelia Camacho Hinojosa | **Master Student:** Ana Bernardó | **Visitors:** Ana Catarina Gomes (Portugal), Assitan Sidibe (France)

Organelle protein expression and mitochondrial disease

(Yaiza Español and Tanit Guitart)

In this line of research, we examine the cellular mechanisms that control and coordinate protein synthesis in the lumen of cellular organelles, such as mitochondria. In humans, several forms of muscle-wasting diseases are caused by defects in mitochondrial protein synthesis machinery. The multi-clonal nature of mitochondria makes the study of these conditions extremely difficult. A long-term goal of our laboratory is to generate an animal model for mitochondrial disease in order to facilitate research into muscle-wasting disorders.

To this end, we have started characterising the mitochondrial protein synthesis machinery in *Drosophila*. Tanit Guitart is studying the seryl-tRNA synthetases of this organism and developing strains of *Drosophila* in which the expression of mitochondrial synthetases is experimentally repressible, in order to generate flies that reproduce the defects in mitochondrial protein synthesis that cause muscle-wasting in humans (Figure 2).

Most of the proteins required for protein synthesis in the mitochondria are nuclear-encoded, and are imported into this organelle. In contrast, most species have maintained their mitochondrial tRNA genes in the mitochondrial genome. Interestingly, several mitochondrial tRNA aminoacylation enzymes are organelle-specific. *Trypanosoma* represents an extreme case because their mitochondrial genomes code for only 4 tRNAs but the mitochondria maintains several specific nuclear-encoded aminoacyl-tRNA synthetases. What prevents the eukaryotic cell from reducing the complexity of its protein synthesis apparatus and using the same aminoacyl-tRNA synthetases (ARS) in the nucleus and the mitochondria? To answer this question, Yaiza Español is currently characterising the lysyl-tRNA synthetase system in *Trypanosoma brucei*.

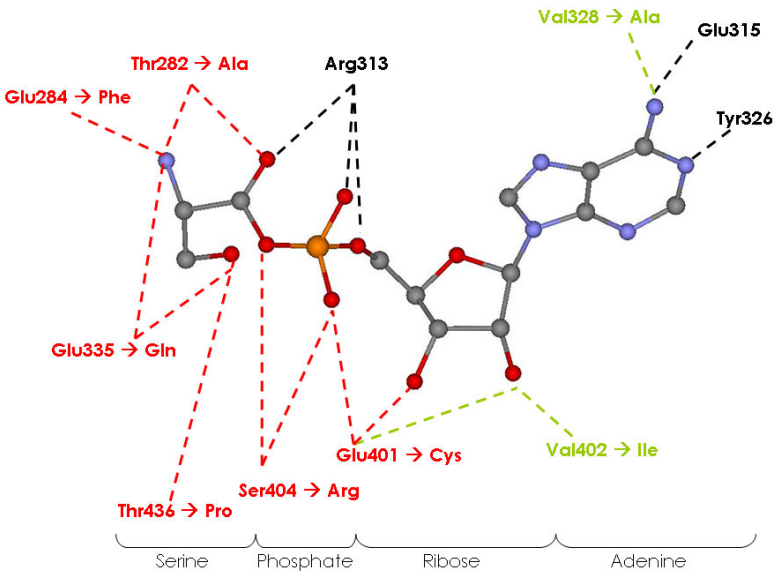


Figure 2. *Structural analysis of the catalytic centre of a Drosophila melanogaster mitochondrial seryl-tRNA synthetase in relation to the binding of the enzyme's reaction intermediate.*

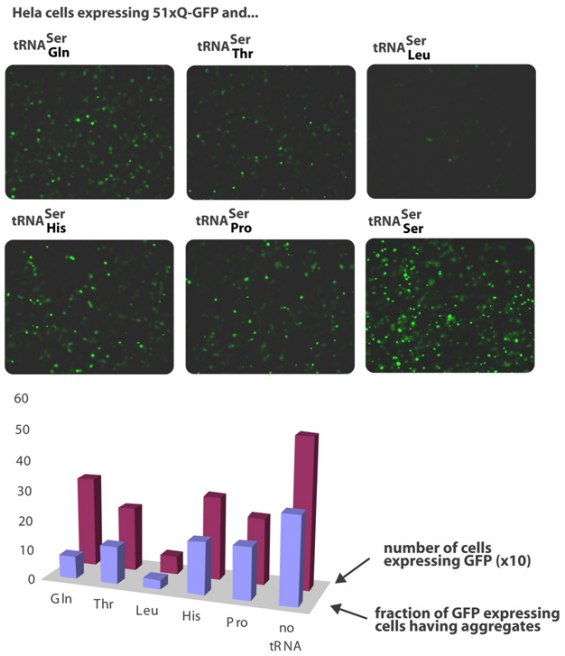


***Figure 3**. Analysis of the effect of generalised protein translation errors caused by mutant tRNAs and monitored through the detection of GFP fluorescence.*

Protein synthesis disorders and drug discovery

(Noelia Camacho and Renaud Geslain)

Laboratory efforts to develop a new screening procedure for molecular inhibitors of aminoacyl-tRNA synthetases have resulted in the creation of a spin-off company, Omnia Molecular, which will continue to develop this project commercially. This company is hosted by the bioincubator of the Barcelona Science Park, and has six employees.

We continue to study the effects of protein synthesis errors in human cells. Renaud Geslain is addressing the stress mechanisms that respond to gross misacylation of tRNAs to minimize the deleterious effect of generalised errors in protein synthesis (Figure 3). In parallel, we continue our collaboration with the Combinatorial Chemistry Laboratory of the Barcelona Science Park, which provides us with *ad hoc* chemical libraries designed to bind and inhibit the active sites of aminoacyl-tRNA synthetases. Noelia Camacho, in collaboration with Dr. Cortes, is testing these compounds in *Plasmodium*, and characterising the effect that protein synthesis inhibitors have on the infection of red blood cells by the malaria parasite (Farrera *et al*, in preparation).

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Research Networks and Grants

Desarrollo de un nuevo método para la selección de antibióticos
Spanish Ministry of Science and Education, BIO2006-01558: 2007-2009
Research Director: Lluís Ribas de Pouplana

Mecanismos de silenciamiento de genes de Plasmodium falciparum que codifican ligandos para la invasión de eritrocitos y fenotipos asociados al silenciamiento o activación de los mismos
Spanish Ministry of Health, PI070891: 2008-2010
Research Director: Alfred Cortés i Closas

Support to exceptional research groups
Generalitat de Catalunya, SGR00350: 2006-2008
Research Director: Lluís Ribas de Pouplana

Collaborations

Combinatorial libraries of aminoacyl-adenylate analogs
Miriam Royo, Combinatorial Chemistry Unit, Barcelona Science Park (Barcelona, Spain)

Development of positive selection screens for antibiotic discovery
Omnia Molecular SL (Barcelona, Spain)

Expression strategies for Plasmodium proteins
ERA Plantech SL (Barcelona, Spain)

Functional evolution of the glycogen metabolism
Daniel Cifuentes and Joan Guinovart, IRB Barcelona (Barcelona, Spain)

Inflammatory effect of an Entamoeba histolytica MetRS domain
Annabel Valledor and Antonio Celada, IRB Barcelona (Barcelona, Spain)

Methionine metabolism and pathogenicity in the human pathogen Mycoplasma penetrans
Rebecca Alexander, Wake Forest University (NC, USA)

Mitochondrial protein synthesis in Drosophila melanogaster development
Thomas Stratmann, University of Barcelona (Barcelona, Spain)

Developmental neurobiology and regeneration

Eduardo Soriano

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 studies have demonstrated that these steps are exquisitely controlled by a variety of molecular and cellular mechanisms, including expression of specific transcription factors, activity of morphogens and growth factors, 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.

Further roles of netrins and semaphorins in neuronal guidance

We have further studied the roles of several guidance molecules in the formation of complex brain structures, such as the cerebral cortex and the cerebellum. For instance, the way in which GABAergic interneurons in the cerebellar cortex migrate or the guidance cues that steer them are still unknown. 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 examined 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 through depletion of the inactive pool, is induced by a diffusible Sema6C1 form, thereby suggesting that this protein is involved 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, but not the contralateral, hippocampal hemisphere 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 kinaseAck1/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/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

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are detected in 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 expressions 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 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 could 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 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 that controls 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,* in preparation).

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 show 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 also demonstrate that Reelin induces the transcription of the early growth response 1 transcription factor (Simó *et al,* 2006). In addition, we show a novel role of Reelin in the migration of cerebellar granule cells, which is highly dependent upon Erk activation (Simó *et al,* in preparation). 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 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 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 overexpression of chondroitin sulfate (CS) proteoglycans (CSPG), 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 and chondroitinase ABC (ChABC), which degrades CS, thereby also 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 regeneration (Mingorance *et al,* 2005). We have also examined whether the combination of complementary strategies facilitates axonal regeneration in slice co-cultures. Both CS cleavage with ChABC and NEP1-40 strongly enhance the regrowth of entorhinal axons after axotomy, thereby 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 these 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 this gene in Purkinje cell specification, we are now exploring whether the induced expression of this gene in neuronal stem cells of distinct origins induces their phenotypic differentiation in a Purkinje cell-like phenotype. If this were confirmed, 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 dentate gyrus (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 or 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. These observations support the view that neuroprogenitor proliferation and/or 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 entorhinal cortex (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 in the control of 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.

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Research Networks and Grants

Identificació i caracterització d'un nou sistema de senyalització associat a exocitosis i neurotrofines: paper en la generació del dolor
Fundació MTV3, 071410: 2008-2010
Project Coordinator: Eduardo Soriano García

Implicación de las semaforinas transmembranales y sus receptores en plasticidad sináptica y en enfermedades neurales: Estudio celular y análisis de la transducción de señal
Ministerio de Sanidad y Consumo — ISC III, PI070500: 2008-2010
Project Coordinator: Ferran Burgaya Márquez

Papel de la reelina en la formación de conexiones sinápticas in vitro e in vivo y en el desarrollo de enfermedades neurodegenerativas
Ministerio de Sanidad y Consumo — ISC III, PI070715: 2008-2010
Project Coordinator: Albert Martínez García

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 Alzheimer y los procesos de potenciación a largo plazo
Ministerio de Sanidad y Consumo — ISC III, PI070942: 2008-2010
Project Coordinator: Jesús Mariano Ureña Bares

Collaborations

Functions of the novel tyrosin kinase Pyk1 in brain development
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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
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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, Zurich University (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, Zurich University (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 epilepsy
Javier de Felipe, Cajal Institute (Madrid, Spain)

Ultrashort lasers, axonal guidance, and brain repair
Pablo Loza, ICFO (Barcelona, Spain)

Structural and Computational Biology Programme

Structural bioinformatics and network biology

Patrick Aloy

Proteins are the main perpetrators of most cellular tasks; however, they seldom act alone. 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 centred mainly on unravelling these relationships. However, none of these interaction maps provide 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

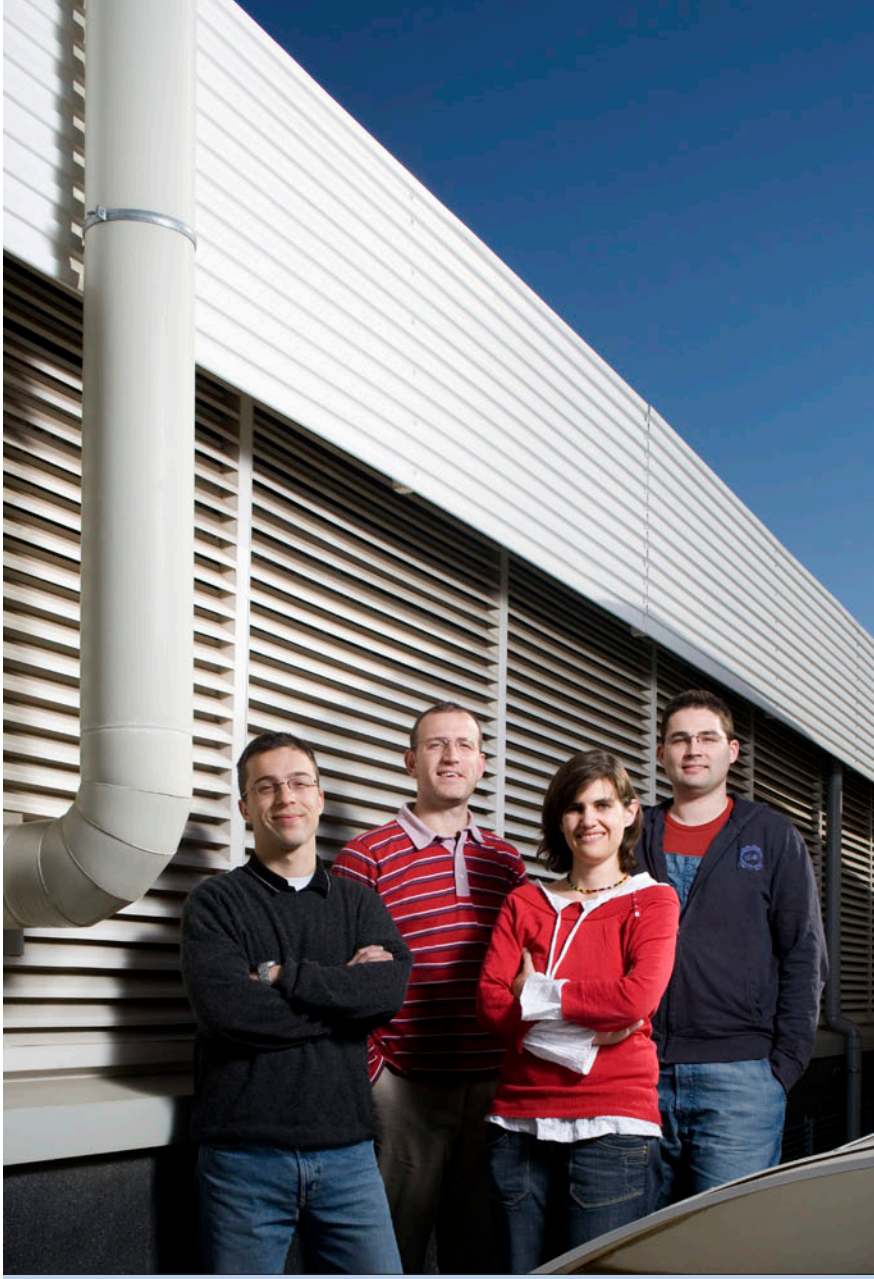
Recent years have seen the emergence of many large-scale proteomics initiatives that have identified thousands of new protein interactions and macromolecular assemblies. However, unfortunately, only a few of the discovered complexes meet the high-quality standards required to be used promptly in structural studies. Consequently, there is an increasing gap between the number of known protein interactions and complexes and those for which a high-resolution three-dimensional structure is available. We have developed and validated a computational strategy to distinguish complexes found in high-throughput affinity purification experiments that will stand the best chances to be successfully expressed, purified and crystallized 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 draw up a list of 20 complex candidates whose structural determinations will be attempted by groups within 3D Repertoire, a large European Integrated Project that seeks 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, thereby 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. Although 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 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 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 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 also have 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 mechanisms responsible for the dynamic nature of peptide-mediated interactions, and suggested a global evolutionary mechanism to maximize the binding specificity. Finally, we have investigated the viability of non-native interactions and highlight cases of potential cross-reaction that might compensate for individual protein failures and establish backup circuits to increase the robustness of cell networks.



Research Group Members | **Principal Investigator:** Patrick Aloy | **Postdoctoral Fellow:** Andreas Zanzoni | **PhD Students:** Roland Pache, Alejandro Panjkovich, Amelie Stein | **Research Assistant:** Verónica Martínez | **Visitor:** Judith Wodke (Germany)

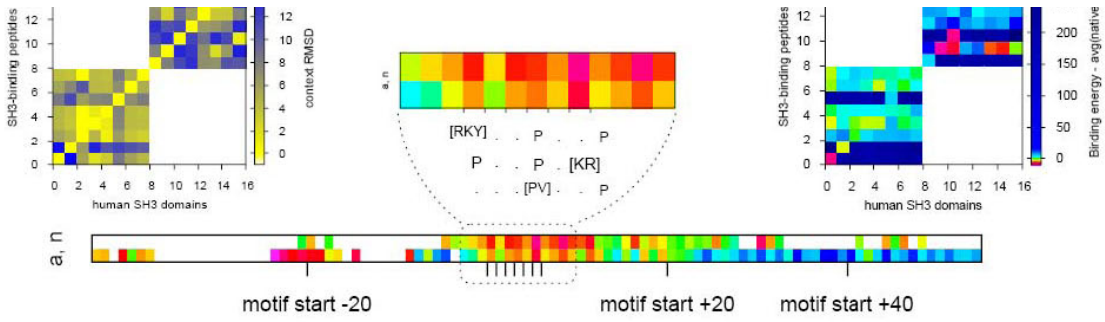


Figure 1. *Peptide exchange results for human SH3 domains. Upper heat maps show the topological distortion (left) and the energy variations (right) of all artificial (ie, non-native) interaction pairs constructed between human SH3 domains and their ligand proteins, with respect to the native topologies and the average native binding energy. SH3-binding peptides 1-9 correspond to class I and 10-16 to Class II. The lower figure shows the energy variation of motif and context, compared to the native binding energies, for each individual residue in the native (n) and artificial (a) interactions.*

Exploiting gene deletion fitness effects to understand 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 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 observed 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 several interesting cases of potential functional compensation between protein paralogs and, perhaps, a new piece to fit in the histone-code puzzle.

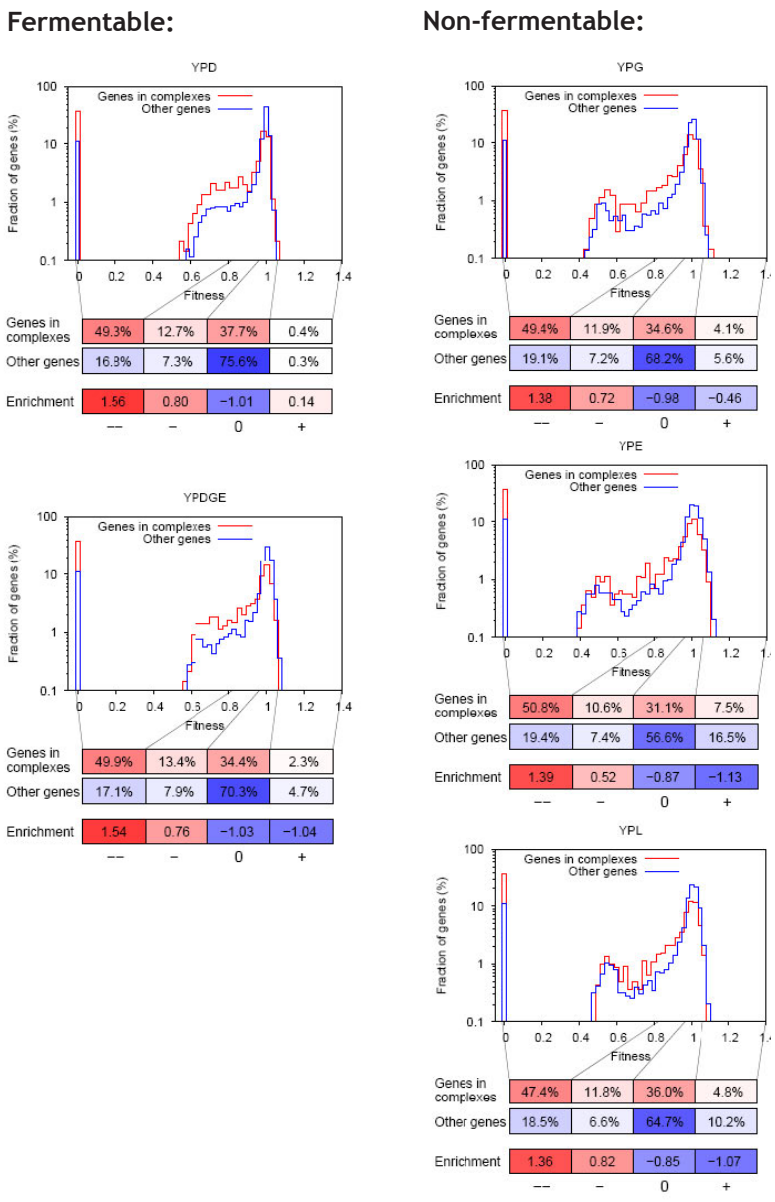


Figure 2. *Comparison of the fitness of yeast strains upon deletion of genes in complexes and other genes. Distributions of strain fitness upon deletion of genes in complexes (red) and genes not part of complexes (blue) in two fermentable and three non-fermentable media. Genes with a fitness of zero are essential. The fitness values of individual genes are partitioned into four categories: 'strong negative effect' (--), 'moderate negative effect' (-), 'weak or no effect' (0) and 'positive effect' (+).*

Towards a molecular characterisation of pathological pathways

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 interrelationships between macromolecules in an organism or monitoring how they coordinately 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 performed 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, clinical phases because of the poorly understood action mechanisms of the pathways they target or an inappropriate choice of the animal models, which proved ineffective at predicting off-target effects. It is therefore 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.

Probably, the main goal of our laboratory can be considered 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 required 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, and this process will reveal novel and unexpected connections between them. We will then choose to further study those of most relevance from an academic or clinical perspective. We have already started to implement our approach to study the molecular bases of aging-related diseases and metabolic syndrome in collaboration with academic and industrial partners. It is our belief that a deeper understanding of the molecular mechanisms underlying a disease phenotype will permit the discovery of new potential targets, lead to more effective combinations of already marketed products, and help to select the best model organisms to study a patho-physiological pathway or to identify specific biomarkers.

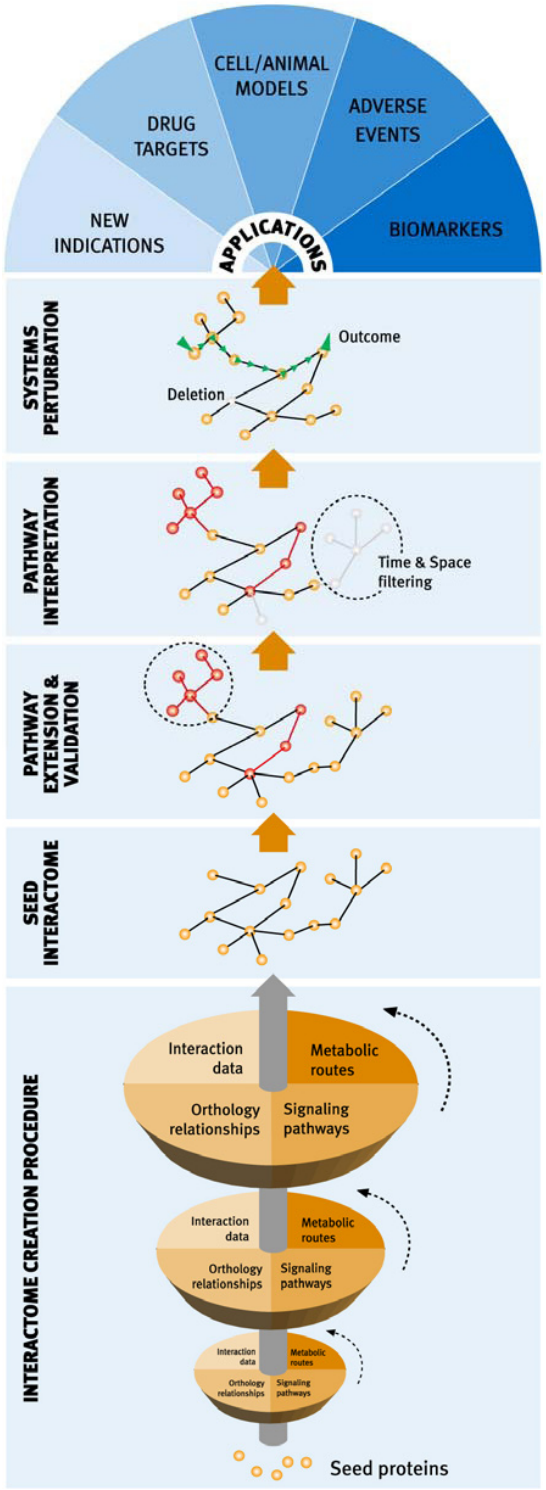


***Figure 3**. Global strategy for the molecular characterisation of pathways and potential clinical applications. Starting from a set of seed proteins, an initial interactome is built using known protein-protein interactions. The resulting seed interactome or pathway is then extended and validated before placing the pathway components into a spatiotemporal context based on gene expression data. Perturbation of the system finally allows us to unveil relationships between pathway topology and biological activity, with relevant implications for several kinds of clinical applications.*

Publications

Aloy P. Shaping the future of interactome networks. *Genome Biol*, **8**, 316 (2007)

Pache RA and Aloy P. Incorporating high-throughput proteomics experiments into structural biology pipelines: identification of the low-hanging fruits. *Proteomics*, **8**(10), 1959-64 (2008)

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Research Networks and Grants

Aproximación bioinformática al estudio de la especificidad contextual en redes de interacciones entre proteínas y sus posibles aplicaciones biomédicas y biotecnológicas
Ministerio de Educación y Ciencia, BIO2007-62426: 2007-2010
Research Director: 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
Ministerio de Educación y Ciencia, Plan Singular Estratégico, PSE-010000-2007-1: 2007-2008
Research Director: Patrick Aloy

Collaborations

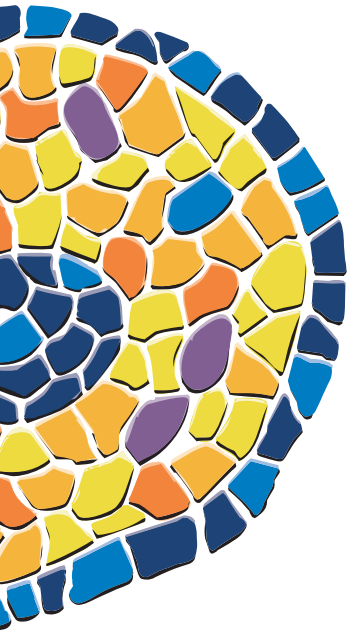
Identification of potential phosphorylation targets for AURORA A kinase in human
Isabelle Vernos, Centre for Genomic Regulation (Barcelona, Spain)

Modular architecture of protein complexes and gene deletion fitness in yeast
Madan Babu Mohan, MRC-LMB (Cambridge, UK)

Molecular characterisation of biological pathways related to aging
José Manuel Mas, InfoCiencia Clinical Research (Barcelona, Spain), Xavier Gomis-Rueth, IBMB-CSIC (Barcelona, Spain), Baldo Oliva, GRIB-UPF (Barcelona, Spain) and Xavier Daura, IBB-UAB (Barcelona, Spain)

Molecular characterisation of biological pathways related to metabolic syndrome, type II diabetes and obesity
José Manuel Mas, InfoCiencia Clinical Research (Barcelona, Spain), Andrés Fernández, Salvat Biotech (Barcelona, Spain), Albert Barberà and Ramón Gomis, IDIBAPS-Hospital Clínic de Barcelona (Barcelona, Spain)

Structural characterisation of macromolecular machines in yeast
Luís Serrano, Centre for Genomic Regulation (Barcelona, Spain) and Rob Russell (European Molecular Biology Laboratory, Heidelberg, Germany)



Structural biology of proteins, nucleic acids and their complexes

Miquel Coll

Our research focuses on the structural analysis of proteins, nucleic acids and their complexes with the aim to further our understanding of several essential mechanisms in the cell. For this purpose, we use a number of molecular and structural biology techniques, with a focus on X-ray crystallography. We study systems related to horizontal gene transfer that involve the translocation of DNA between cells. In addition, we address regulatory mechanisms of gene expression and the control mechanisms of DNA replication. We also study unique DNA structures, like DNA junctions, and novel drugs that target DNA.

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. This DNA translocation system can be divided in two modules: the relaxosome (Guasch *et al*, 2003; Boer *et al*, 2006), which triggers and participates in plasmid DNA processing and replication, and a type IV secretion system, which impels protein and single-stranded DNA through the membranes. In addition, a coupling protein (Gomis-Rüth *et al*, 2001) links both modules. We have studied the relaxosome and the coupling protein in Gram-negative bacteria (Gomis-Rüth and Coll, 2006; Russi *et al*, 2008) and are now starting to analyse the equivalent system in Gram-positive bacteria. Another mechanism for gene transfer is transduction, where DNA is transported from one bacterial cell to another by means of a bacteriophage. Packaging the DNA in the phage capsid is performed by a molecular machine that processes and pushes the DNA molecule into the preformed viral capsid through one vertex (Figure 1). We analyse the structures of several components and sub-complexes of the packaging machinery both in phages and herpes viruses.

Transcription regulation

To elucidate how transcription is regulated, we structurally analyse several transcription factors and their complexes with other proteins and DNA promoter regions. In one study we have 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 (Boer *et al*, 2006; Arribas-Bosacoma *et al*, 2007). A transcription initiation sub-complex, which includes the tandem-arranged dimer of PhoB effector domain, the *pho box* promoter DNA and the σ_4 domain of the σ70 subunit fused to the RNA polymerase β-flap tip-helix, has been solved, thereby showing how the RNA polymerase is recruited to the promoter region (Gomez-Blanco *et al*, in preparation; Figure 2).

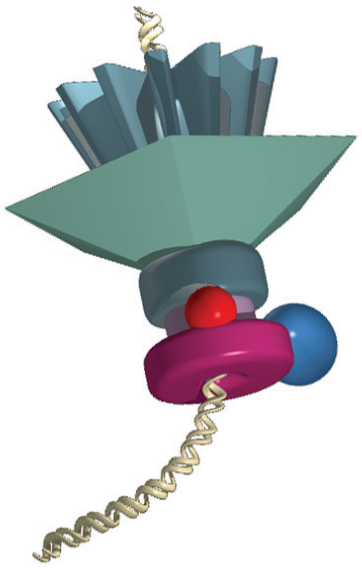


***Figure 1**. The herpes virus DNA packaging machinery formed by the terminase complex, the connector and other ancillary proteins.*

Research Group Members | **Principal Investigator:** Miquel Coll | **Associate Researchers:** Maria Solà, Maria Cristina Vega | **Postdoctoral Fellows:** Carme Arnan, Roeland Boer, Lionel Costenaro, Francisco José Fernández, Robert Janowski, Tomislav Kamenski, Sebastien Violot | **PhD Students:** Raquel Arribas, Sol Cima, Nereida Jiménez, Diana Martínez, Marta Nadal, Esther Peña, Silvia Russi | **Research Assistants:** Leonor Alloza, Maïlys Boutin, Rosa Pérez | **Lab Manager:** Albert Canals | **Lab Technician:** Esther Ferrando

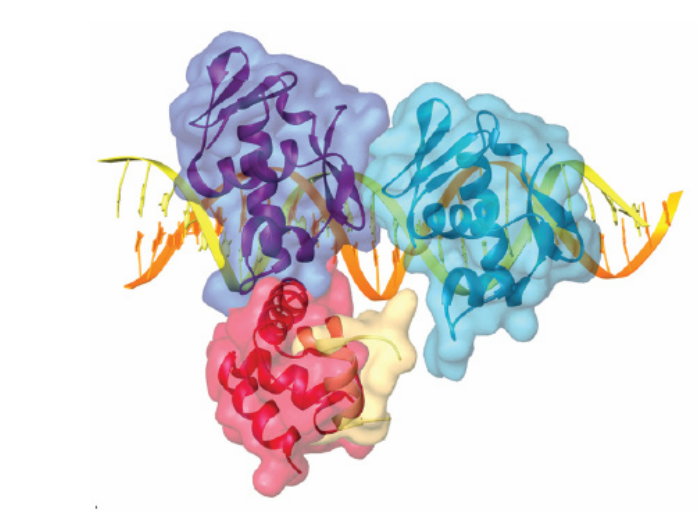


***Figure 2**. A transcription initiation quaternary subcomplex, including the pho box DNA, the PhoB activator and RNA polymerase subunit domains, shows how the polymerase is recruited to pho promoters.*

DNA replication control

DNA replication is a key cell event that is performed by diverse mechanisms in diverse organisms. Among these mechanisms, rolling circle replication (RCR) is a rapid one that leads to the generation of single or multiple copies of circular DNA or RNA 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 catalyzed by RCR initiator proteins, which thus provide a primer for the DNA or RNA polymerases to start synthesis. Initiators are also involved in termination of the replicative process. We have solved the three-dimensional structure of one of these initiator proteins and unveiled that it oligomerises as a hexamer with a central channel (Boer *et al*, in preparation; Figure 3).

DNA structure and drug-DNA interactions

Unique DNA structures, such as four-way and three-way junctions related to DNA recombination and other processes, have been structurally analysed. We previously showed that a novel cytotoxic drug consisting of a supramolecular helicate binds to a three-way junction DNA with a perfect fit in the central cavity of the junction (Oleksi *et al*, 2006). We have now determined other related structures with various helicates that show similar drug-nucleic acid interactions. Our findings thus indicate that the three-

way junction DNA is a target for this kind of prisma-shaped molecule.

Structural genomics

We implement medium/high throughput technologies for the expression and crystallisation of proteins and complexes. In relation to these activities, the group has participated in several National and European Structural Genomics consortia: VIZIER (www.vizier-europe.org), 3D-Repertoire (www.3drepertoire.org), GENES and SPINE2-Complexes (www.spine2.eu).

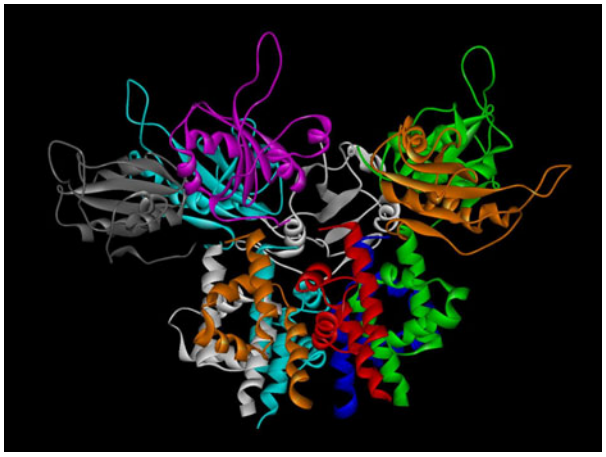


***Figure 3**. Three-dimensional structure of a plasmid DNA replication initiator.*

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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, VI PM LSHG-CT-2005-512028: 2005-2009
Research Director: Miquel Coll

Ayuda complementaria al proyecto: Genómica estructural comparativa para enzimas víricos
Ministerio de Educación y Ciencia, Acción complementaria de investigación, BFU2005-24122-E: 2006-2010
Research Director: Miquel Coll

Ayuda complementaria al proyecto: Una aproximación multidisciplinaria para determinar las estructuras de los complejos proteicos en un organismo modelo
Ministerio de Educación y Ciencia, Acción complementaria de investigación, BFU2005-24123-E: 2006-2010
Research Director: Miquel Coll

Centrosoma 3D: Hacia la comprensión estructural y funcional del centrosoma
Ministerio de Educación y Ciencia, Proyecto Consolider, CSD2006-23: 2006-2010
Research Director: Miquel Coll

Comparative structural genomics of viral enzymes involved in replication (VIZIER)
European Commission, VI PM LSHG-CT-2004-511960: 2004-2008
Research Director: Miquel Coll

Consorcio para el descubrimiento y desarrollo de nuevos fármacos (GENIUS PHARMA)
Ministerio de Industria, Turismo y Comercio, Proyecto CENIT, 050102060008: 2006-2009
Research Director: Miquel Coll

Epigenetic chromatin regulation proteins as targets in anticancer strategy

Fundació La Marató de TV3, 052810: 2005-2008
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Estructura de proteínas y complejos de unión al DNA
Ministerio de Educación y Ciencia, BFU2005-06758/BMC: 2005-2008
Research Director: Miquel Coll

From receptor to gene: structures of complexes from signalling pathways linking, immunology, neurobiology and cancer (Spine II Complexes)
European Commission, VI PM LSHG-6-031220: 2006-2010
Research Director: Miquel Coll

Genómica estructural: aplicación a proteínas y complejos proteicos relacionados con el cáncer
Ministerio de Ciencia y Tecnología, Acción estratégica de genómica y proteómica, GEN2003-20642: 2004-2007
Research Director: Miquel Coll

Grupo de cristalografía de proteínas
CIRIT, Generalitat de Catalunya, ayudas a grupos de investigación consolidados, 2005SGR-00280: 2005-2008
Research Director: Miquel Coll

Collaborations

Centrosomal proteins
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Chromatin-modifying proteins
Ferran Azorín, IRB Barcelona (Barcelona, Spain), Xavier Barril, University of Barcelona (Barcelona, Spain)

DNA-drugs
Mike Hannon, University of Birmingham (Birmingham, UK) and Cristina Vicent, Instituto de Química Orgánica General — CSIC (Madrid, Spain)

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

Genius Pharma
Crystax Pharmaceuticals, SL (Barcelona, Spain)

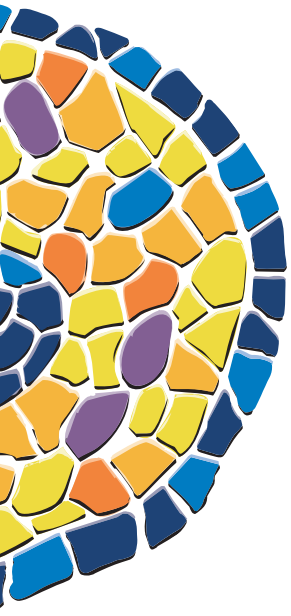
HTP protein expression
Darren J Hart, European Molecular Biology Laboratory (Grenoble, France)

Mobility of Rep protein hexamer
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

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)

Transcription regulation
Juan Aguilar, University of Barcelona (Barcelona, Spain), Ramón Díaz, Centro de Investigaciones Biológicas — CSIC (Barcelona, Spain), Antonia Herrero, Instituto de Bioquímica Vegetal y Fotosíntesis — CSIC (Sevilla, Spain), Margarita Salas, Centro de Biología Molecular — CSIC (Madrid, Spain)



Structural biology and oxidative stress: molecular aggregates

Ignasi Fita

During 2007 we have used experimental and theoretical (computational) approaches to perform structural and biochemical analyses of protein systems related to oxidative stress. In particular, we have done extensive work on the catalase-peroxidase system both for the intrinsic scientific interest of these moonlight enzymes and for their crucial role in the activation of isoniazide, one of the main anti-tubercular treatments. This work has been done in close collaboration with Prof PC Loewen at the University of Manitoba (Canada) and Dr C Rovira (an ICREA scientist at the Barcelona Science Park). Our group has also participated in the structural determination of a number of molecular aggregates. In particular, in collaboration with Prof V Rubio (CSIC-Valencia), we have determined the crystal structure of the complex of PII and acetylglutamate kinase. This study, which reveals how PII controls the storage of nitrogen as arginine, was recently published in PNAS. Furthermore, this study was the subject of a review in the Editor's choice section of Science (Vol. 318, p: 888).

Research Group Members | **Principal Investigator:** Ignasi Fita | **Postdoctoral Fellows:** Xavier Carpena, Antonio Rodríguez | **PhD Students:** David Aparicio, Barbara Calisto | **Lab Technician:** Maria Queralt

Publications

Alfonso-Prieto M, Borovik A, Carpena X, Murshudov G, Melik-Adamyan W, Fita I, Rovira C and Loewen PC. The structures and electronic configuration of compound I intermediates of *Helicobacter pylori* and *Penicillium vitale* catalases determined by X-ray crystallography and QM/MM density functional theory calculations. *J Amer Chem Soc*, **129**, 4193-205 (2007)

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Calisto BM, Perez-Gil J, Bergua M, Querol-Audi J, Fita I and Imperial S. Biosynthesis of isoprenoids in plants: structure of the 2C-methyl-D-erithrytol 2,4-cyclodiphosphate synthase from *Arabidopsis thaliana*. Comparison with the bacterial enzymes. *Protein Sci*, **16**(9), 2082-88 (2007)

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Gallego O, Ruiz FX, Ardevol A, Domínguez M, lvarez R, de Lera AR, Rovira C, Farres J, Fita I and Pares X. Structural basis for the high all-trans-retinaldehyde reductase activity of the tumour marker AKR1B10. *Proc Natl Acad Sci USA*, **104**, 20764-69 (2007)

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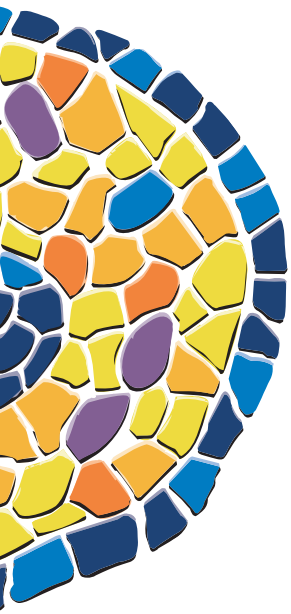
Llácer JL, Contreras A, Forchhammer K, Marco-Marín C, Gil-Ortiz F, Maldonado R, Fita I and Rubio V. The crystal structure of the complex of PII and acetylglutamate kinase reveals how PII controls the storage of nitrogen as arginine. *Proc Natl Acad Sci USA*, **104**, 17644-49 (2007)

Marco-Marín C, Gil-Ortiz F, Pérez-Arellano I, Cervera J, Fita I and Rubio V. A novel two-domain architecture within the amino acid kinase enzyme family revealed by the crystal structure of *Escherichia coli* glutamate 5-kinase. *J Mol Biol*, **367**(5), 1431-46 (2007)

Vidossich P, Alfonso-Prieto M, Carpena X, Loewen PC, Fita I and Rovira C. Versatility of the electronic structure of compound I in catalase-peroxidases. *J Am Chem Soc*, **129**, 13436-46 (2007)



Protein complexes determined by nuclear magnetic resonance

Maria J Macias

Our main focus is the study of protein-protein interactions at an atomic level, with the final aim of providing three-dimensional structures that can contribute to a better description of biological processes. For this purpose, we apply multidimensional nuclear magnetic resonance (NMR) spectroscopy in conjunction with other biophysical and biomolecular techniques, intended to characterise the properties of the proteins and ligands of interest. Thus, we not only seek to address the structural determination of proteins and complexes but also decipher the rules that contribute to defining and maintaining a given fold, or to controlling a given interaction.

Unraveling the protein-protein interactions scenario during transcription and splicing: characterisation of ligand recognition by WW domains

Group 2 WW domains are found mostly in proteins involved in splicing and transcription. They usually bind proline-rich ligands including the PPxyPP motif, where x/y can be hydrophobic residues that include prolines but not aromatic residues. In this study we have identified a new recognition motif, named PPLIPP, as one of the targets of the transcription factor CA150. We attempted to narrow down the main residues required for specificity by solving structures with three distinct ligands. We found that the motif can be accommodated using two distinct binding modes, a feature characterised for the first time in WW complexes. These two binding modes differ in the localisation of the isoleucine, which can be placed either in the XP or in the XP2 groove, as shown in Figure 1. The selection of the binding mode does not require electrostatic interactions, as observed for instance in SH3 domains, which also recognise proline-rich ligands. In the case of the FBP28/CA150 WW2 domain, the composition and size of the XP2 groove may help to establish whether to accommodate the non-proline residue in the XP2 or in the XP groove.

We have also analysed the binding of a WW domain tandem, that of the splicing factor FBP11WW1-2 to a long stretch of FH1 (a long proline-rich region of Formins), using fluorescence spectroscopy and NMR titrations. We found that the presence of two consecutive WW domains also influences the selection of the binding mode, particularly when the two domains interact with consecutive motifs in the ligand. Our results represent the first observation of protein-

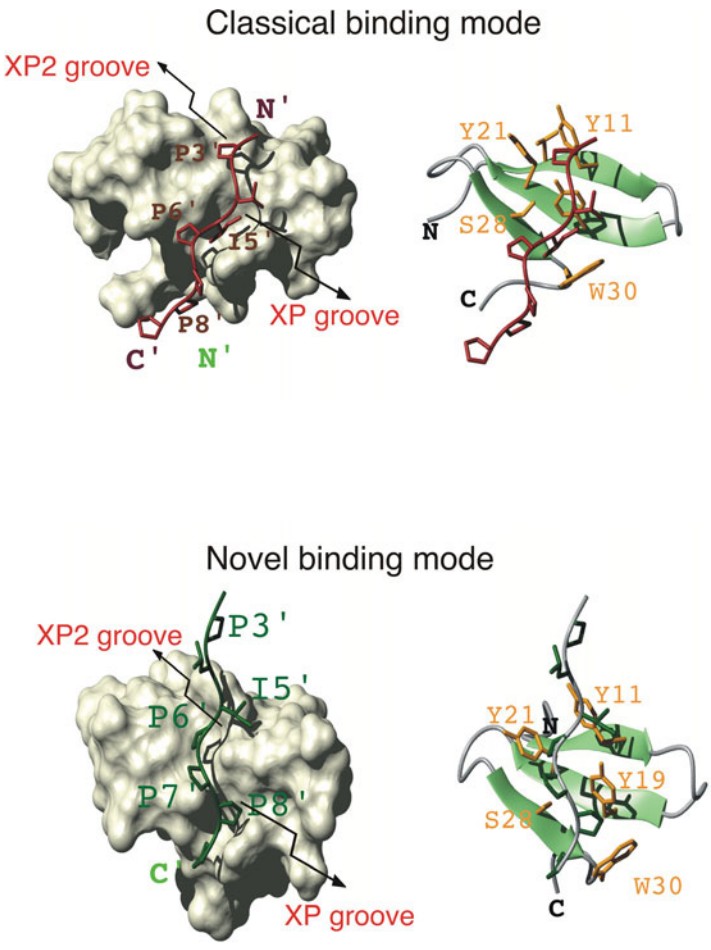
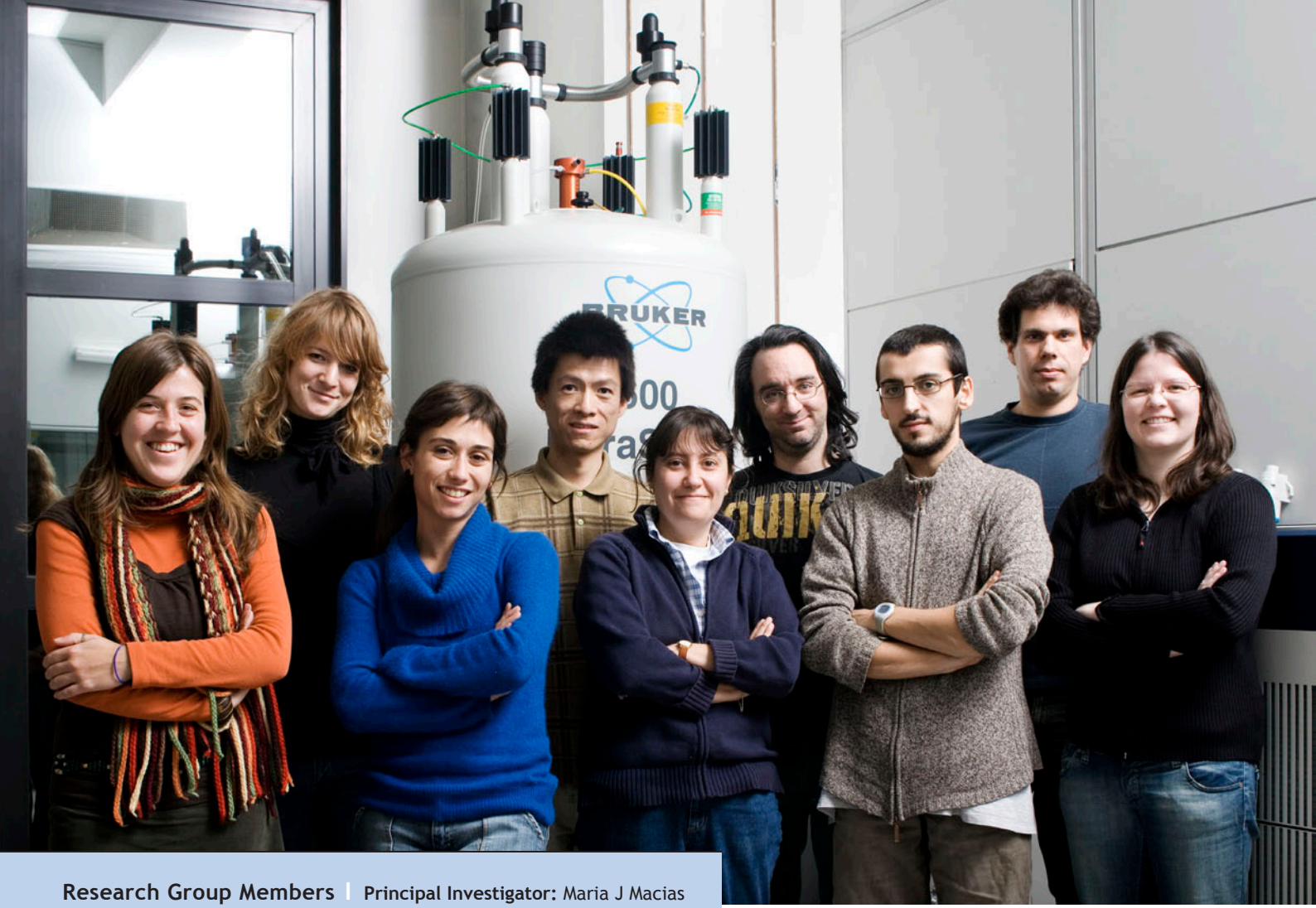


***Figure 1**. Solution structure of FBP28/CA150WW2 in complex with the PPPLIPPPP and PPLIPPPP peptides.*



Research Group Members | **Principal Investigator:** Maria J Macias | **PhD Students:** Román Bonet, Nina Goerner, Pau Martin, Begoña Morales, Lei Zhang | **Lab Technician:** Lidia Ruiz | **Master Student:** Claudia Flicker | **Visitors:** Eric Aragón (Spain)

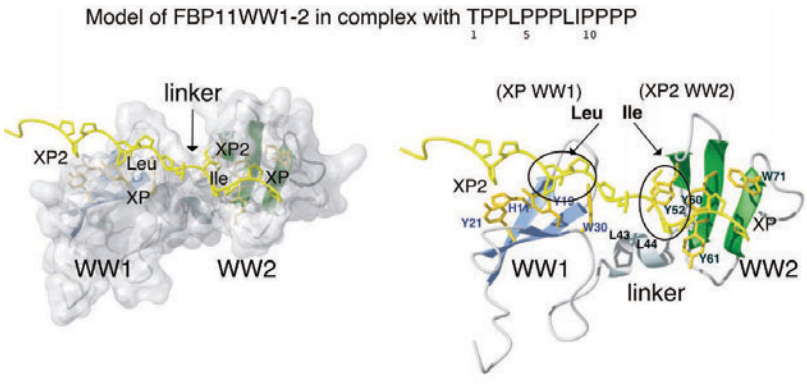


***Figure 2**. Diagram representation of a model interaction of a tandem of WW domains and two proline-rich motifs. Surface and diagram representation of the hypothetical binding of the tandem, assuming that the tandem will use both binding modes. Across the surface and on the side, elements of secondary structure and some side-chains are highlighted. The backbone of the peptide is shown in yellow.*

ligand recognition in which a WW tandem and two consecutive motifs participate simultaneously. The results presented here contribute to explaining why so many WW domains appear in two, three or four copies in the same protein. Certainly more structural information on other WW sequences belonging to this group, including tandem sequences, is required before we can start to outline the rules that govern the selection of binding modes. With respect to tandem complexes (so far ignored from both functional and structural points of view), the model of interaction that we describe may open a window towards explaining how and why WW domains operate either solo or as a tandem in regulating cellular mechanisms (Figure 2).

Regulation of the ubiquitin ligase Itch ligand recognition through WW domain phosphorylation

HECT-type E3 ubiquitin ligases participate in the direct recognition of substrates and catalyze the covalent attachment of ubiquitin to their target proteins. The Nedd4 family of ligases is conserved in evolution from yeast to mammals. These ligases comprise several WW domains, most of them belonging to Group 1 of WW domains, a C2 membrane localisation domain and the characteristic HECT (homologous to E6-AP COOH-terminus) catalytic domain.

The selection of proteins for poly-ubiquitination often occurs through specific interactions between the WW domains and motifs present in target proteins. Given the broad number of targets bound by the Nedd4 proteins as well as the observation that some targets are recognised by several ligases, it appears that each E3-ligase displays either specialized or redundant recognition, depending on functional requirements. Interestingly, mice homozygous for a loss-of-function in the itch gene develop immunological disorders, thereby suggesting that the Itch protein has at least one non-redundant function. It has been recently observed that Itch becomes phosphorylated in cells following T-cell activation. Tyrosine phosphorylation of the viral membrane LMP2A protein has also been described.

We have studied the potential role of phosphorylation directly at the interface of binding from both domain and ligand perspectives. We have found that phosphorylation of LMP2A peptides at the PPxY motif decreases the affinity of the interaction although does not abolish it. This observation indicates that the ItchWW3 binding site accommodates the tyrosine phosphate group but with more difficulty than the unmodified tyrosine. The reduction in affinity, however, can play a relevant biological role, since depending on the global affinity, a three-fold decrease may situate the binding threshold at the noise level. In this regard, it has been described that phosphorylation of the PPxY motif of cJun is enough to prevent its efficient degradation in T-lymphocytes (Figure 3).

In contrast to phosphorylation of the ligand, phosphorylation of residue T30 in the domain was found to fully inhibit binding to the PY ligand, although the structure of the phosphorylated domain is maintained as in the wild-type. This feature could be explained by the sterical hinderance introduced by the phosphate group (reducing the size of the XP groove) and the energetic cost that the loss of the hydrogen bond with the ligand implies (Figure 4).

Phosphorylation experiments with T-lymphocyte lysates and with purified kinases show that T30 can be phoshorylated *in vitro*, thereby supporting the hypothesis that domain phosphorylation also occurs in intact cells. Remarkably T30 is highly conserved in the WW domain family, with about 90% of all WW domains known to date displaying a hydroxyl-containing residue at this position in the sequence.

Itch phosphorylation by JNK1 in T-lymphocytes modulates its intramolecular interactions. Indeed, after JNK1 activation, Itch suffers a profound conformational rearrangement, shifting from the closed conformation, where the WW domains directly contact the catalytic HECT domain, to the open conforma-

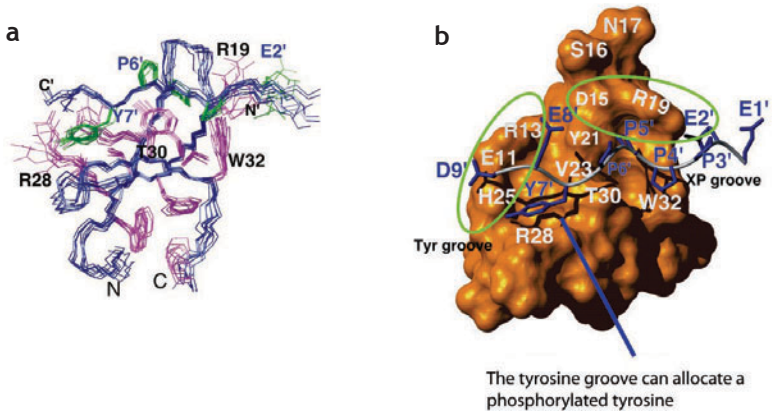


Figure 3. *(a) Superposition of the 10 lowest energy structures after water refinement. Backbone is shown in blue, with selected side-chains indicated in magenta (domain) and in green (peptide). Several selected residues are labelled. (b) Lowest energy structure of the complex with the domain shown as a solid surface representation (in gold) and with the same orientation as above. The peptide is shown in blue lines. Residues located in the binding site as well as both tyrosine- and proline-binding grooves are labelled. Green circles indicate additional contacts observed in the complex.*

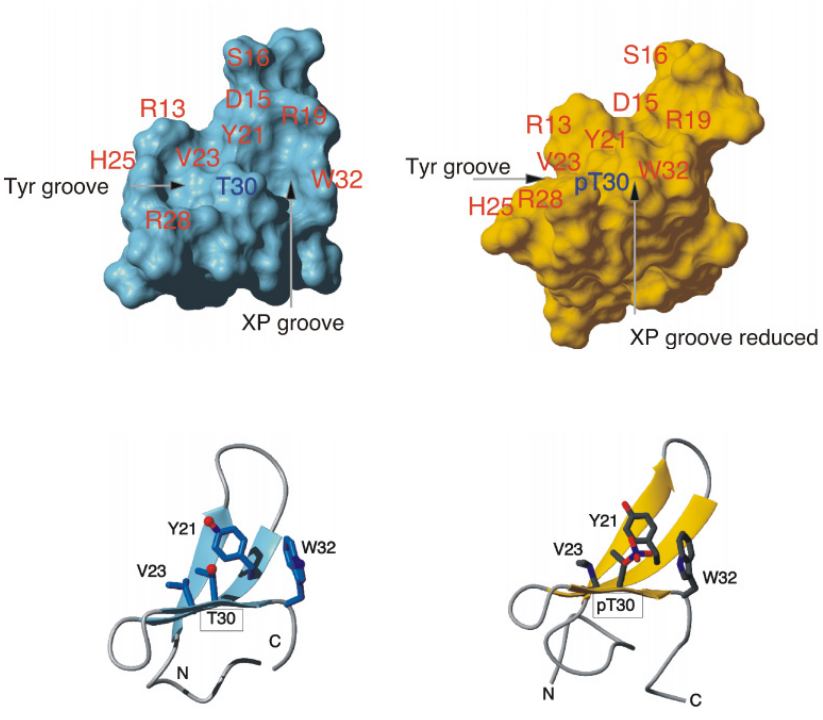


Figure 4. *Surface and diagram representation of the structures of ItchWW3 domain (left, in blue) and pT30 (right, in yellow) showing that the phosphorylation of T30 partially reduces the XP binding groove, used to interact with the ligand prolines.*

tion. In the latter, Itch WW domains interact with other proteins and can contribute to their ubiquitination and degradation. E3 ubiquitin ligases can also be phosphorylated at the WW sequence, thereby inhibiting their interactions. Our results suggest that while the WW domain is phosphorylated in a key residue for ligand recognition, such as T30, ligands are not efficiently recognised and targets can prevent their degradation. Thus, the initial change in conformation described in Itch after JNK1 activation could be the first step of a cascade of events that may include a WW domain phosphorylation/dephosphoylation equilibrium, thus providing a mechanism of target survival though inhibition of degradation. Further studies *in vivo* on whether phoshorylation can occur in these ancillary domains will make a crucial contribution to our understanding of activation and repression mechanisms of ubiquitin ligases. Structural work is underway to characterise the intramolecular interactions at an atomic level.

Set-up of the 600 MHz spectrometer

At the end of October, IRB Barcelona acquired a Bruker 600 MHz (14.1 Tesla) UltraShield magnet. Three weeks after its arrival, technical and scientific installations were finished. At present, we are running the standard set of triple resonance experiments to test the performance of this equipment. To date, the spectrometer is working as expected and giving high quality data sets.

Publications

Brucet M, Querol-Audí J, Serra M, Ramirez-Espain X, Bertlik K, Ruiz L, Lloberas J, Macias MJ, Fita I and Celada A. Structure of the dimeric exonuclease TREX1 in complex with DNA displays a proline-rich binding site for WW Domains. *J Biol Chem*, **282**(19), 14547-57 (2007)

Macias MJ, Teijido O, Zifarelli G, Martin-Malpartida P, Ramirez-Espain X, Zorzano A, Palacín M, Pusch M and Estévez R. Myotonia-related mutations in the distal C-terminus of ClC-1 and ClC-0 chloride channels affect the structure of a poly-proline helix. *Biochem J*, **403**(1), 79-87 (2007)

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Ramirez-Espain X, Ruiz L, Martin-Malpartida P, Oschkinat H and Macias MJ. Structural characterisation of a new binding motif and a novel binding mode in group 2 WW domains. *J Mol Biol*, **373**(5), 1255-68 (2007)

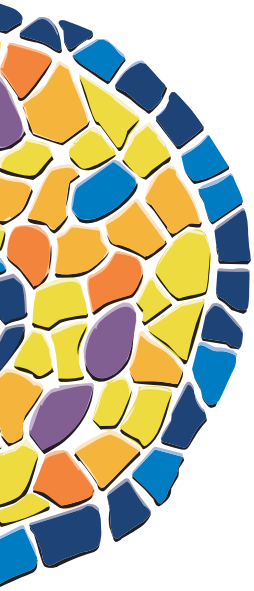
Research Networks and Grants

Aplicación de la RMN a la determinación y caracterización dinámica de estructuras de proteínas, a la identificación de ligandos y a la caracterización de los correspondientes complejos
Ministerio de Educación y Ciencia, GEN2003-20642-C09-04: 2004-2007
Research Director: Ernest Giralt

Determinación de estructuras de dominios FF de proteínas y de sus interacciones mediante la aplicación de la resonancia magnética nuclear multidimensional en solución
Ministerio de Educación y Ciencia, BFU2005-06276 IP: 2005-2008
Research Director: Maria J Macias

Collaborations

Synthesis of peptides and phosphorylated WW domains
Miriam Royo, Barcelona Science Park (Barcelona Spain)



Molecular modelling and bioinformatics

Modesto Orozco

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. To this end, we work with several methodologies, from the mining of biological databases to classical dynamics and quantum chemistry calculations. Such a broad spectrum of techniques allows us to explore a wide range of questions, from drug design to genome analysis. Special emphasis is placed on associating basic interactions with the global properties of biological systems. In general terms, our research focuses on four major fields: the study of small model systems, the analysis of stressed or unusual nucleic acids, genome mining studies, and protein dynamics.

Small model systems

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

During 2007 we have explored small models for ionic interactions, namely calyx-[4]-pyrroles, the focus of previous studies because of its biotechnological relevance (Blas *et al*, 2007), and modified nucleobases (Vázquez *et al*, 2007; Robles *et al*, 2007). From the methodological perspective, we have adapted our MST methodology within the framework of the RM1 semi-empirical Hamiltonian. This has allowed us to deal with solvation terms for biologically relevant molecules, and in particular pharmaceutical drugs, with accuracy and computational efficiency (Forti *et al*, 2007). During this year we have also presented a new method to derive better force-fields by explicit inclusion of polarisation effects. In our approach, atomic polarisabilities are fitted to second order perturbational approaches of the polarisation energy computed with our GMIPp method, which has allowed us to derive polarisability that is consistent with atomic charge distributions (Soteras *et al*, 2007). We are now exploring the possibility to develop a new force-field for nucleic acids based on these polarisation models.

Analysis of stressed or unusual nucleic acids

Major breakthroughs in the field of nucleic acid simulations have emerged from our work in 2007. Probably the two most influential are the development of a new force-field for nucleic acid simulations (parmbsc0) and the derivation of the first microsecond-long trajectory for duplex DNA. The development of parmbsc0 (Pérez *et al*, 2007) provides the community with one of the most accurate force-fields for the simulation of nucleic acids. The AMBER community now use parmbsc0 as the default force-field for nucleic acid simulations. Furthermore, the Ascona-B-DNA consortium is using it in massive simulations to describe the sequence-dependent properties of DNA. Pilot calculations by our group (Goñi *et al*, 2007) have revealed that parmbsc0 is accurate enough to decipher the secondary physical code that helps the cell to label particularly relevant regions of DNA (like promoters; see below).

The availability of parmbsc0 and the massive calculation capacity of the MareNostrum supercomputer has allowed us to run, for the first time in history, a microsecond-long simulation of DNA duplex (Perez *et al*, 2007b). Calculation that represents a 10-fold increase in the length of the longest published trajectory at that time (Orozco *et al*, 2007) was previously impossible because of the fragility of DNA in response to long simulations with older force-fields. Our mas-

Research Group Members | **Principal Investigator:** Modesto Orozco | **Associate Researchers:** Xavier De la Cruz, Agusti Emperador, Josep Lluis Gelpí | **Postdoctoral Fellows:** Oliver Carrillo, Marco d'Abramo, Rebeca García, Manuel Rueda | **PhD Students:** Annalisa Arcella, Damian Blanco, Ignacio Faustino, Carles Fenollosa, Josep Ramón Goñi, Adam Hospital, Sergio Lois, Tim Meyer, Agnés Noy, Laura Orellana, Alberto Pérez, David Piedra, David Talavera | **Research Assistants:** José Antonio Alcántara, Iván Párraga | **Administrative Assistant:** Margarita Pedro | **Visitors:** Jiali Gao (USA), Charles Anthony Laughton (UK)

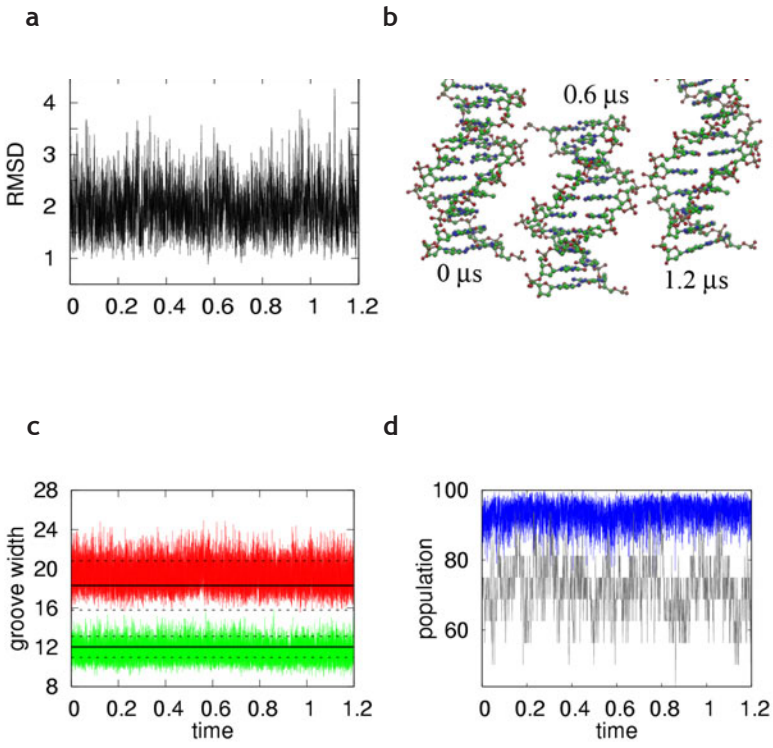


***Figure 1**. Examples of the use of parmbsc0 to obtain 1.2 microsecond trajectory of B-DNA. (a) RMSd with experimental structure, (b) detail of the structures at distinct time frames, (c) distribution of groove widths and (d) population of canonical □/□ and B_I/B_{II} conformers.*

sive analysis has provided accurate information on the rare events that control DNA flexibility (□/□, N/S, B_I/B_{II} transitions, base openings, local bending, ion insertions,…) and on the pattern of intrinsic DNA deformability that dominates transitions between DNA conformations and the binding of control proteins (Figure 1).

The parmbsc0 force-field also provides a key tool to analyse conformational changes in nucleic acids, such as the A<-->B transition of DNA, which we found to follow the intrinsic deformation pattern of B-DNA (Noy *et al*, 2007), and the transition in ONA·RNA hybrids, which are required for RNase H recognition and cleavage (Noy *et al*, 2007).

We are now exploring the limits of parmbsc0 and using this simulation technique to describe highly stressed DNA, which might behave out of the harmonic limit, and to explore unusual structures like triplexes and tetraplexes, both in mild and aggressive environments.

Genome mining

Like many bioinformatics groups, we seek to obtain information on key biological processes by analysing biological databases. In recent years our research effort has focussed on the analysis of the structural and genetic consequences of alternative splicing and the analysis of genomes to determine a pattern of DNAs with unusual structures or properties.

Our analysis of alternative splicing has yielded considerable scientific output during 2007, such as the characterisation of the degree of genetic variability induced by alternative splicing in mammals and the similarity of this variability with that induced by gene duplication (Talavera *et al*, 2007). Furthermore, a remarkable achievement in this field has been the development of tools for the correct annotation of

alternative splicing events, which helps in the correct annotation of protein isoforms in distinct tissues or development stages (Talavera *et al*, 2007). The resulting web server (SPLASH) has been implemented as the default procedure for the annotation of splicing events at the National Institute of Bioinformatics (Figure 2).

During 2007, the development of tools for the description of the physical properties of DNA based on atomistic simulations has opened the door to genome-wide analysis of these properties (Goñi *et al*, 2007). While performing this analysis we observed that promoter regions display unusual physical properties (Figure 4). On the basis of this finding, we used a simple method (named ProStar) which, despite its simplicity, showed excellent performance to find human promoters, even in cases where the promoters are located in unusual positions such as exons, introns or 3'UTRs. The method is especially powerful for the location of unusual promoters, where it shows greater a capacity than methods based on orthology and gene structure conservation. ProStar is now available as a web-service of the National Institute of Bioinformatics.

Along the same lines, we are now completing the physical atlas of human DNA and providing the community with DNalive, a very powerful tool that plots all the known 3D structural information on long DNA fibers, including protein complexes. The algorithms implemented in DNalive will allow, for example, the determination of putative protein-protein complexes mediated by the chromatin fiber (Figure 3; Figure 4).

Dynamics of proteins

The creation of the MODEL (Molecular Dynamics Extended Library) database has been the focus of intense effort by the group. During 2007, we have completed 90% of the library, and reported the first meta-analysis of a small subset of proteins covering all protein metafolds (Rueda *et al*, 2007), which were analysed with the four most popular force-fields (AMBER, CHARMM, OPLS and GROMOS). Our studies on this subset of MODEL (named mMODEL) contributed to validating our force-field calculations by extensive comparison with all available experimental data and to determining a number of interesting properties of protein flexibility, such as the mixed liquid-solid character of proteins or hydrogen bonds as the major determinants of protein flexibility (Rueda *et al*, 2007). We are now in the process of finishing Cluster-90 calculations for MODEL. We are also completing two parallel studies using mMODEL, in order to determine how environmental changes affect proteins. We expect to finish these two massive studies during 2008 (Figure 5).

The availability of several terabytes of trajectories has opened many interesting possibilities for the anal-

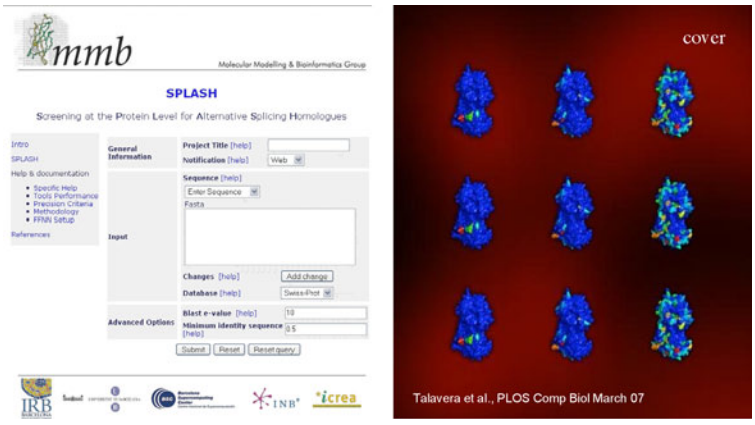


***Figure 2**. Detail of the web server Splash for annotation of alternative splicing events and examples of alternative splicing mapped on protein structure.*

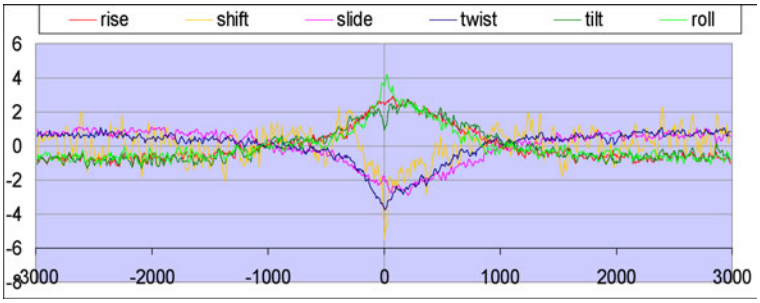


***Figure 3**. Representation of average helical stiffness parameters in human DNA as a function of the distance to transcription start site (TTS).*

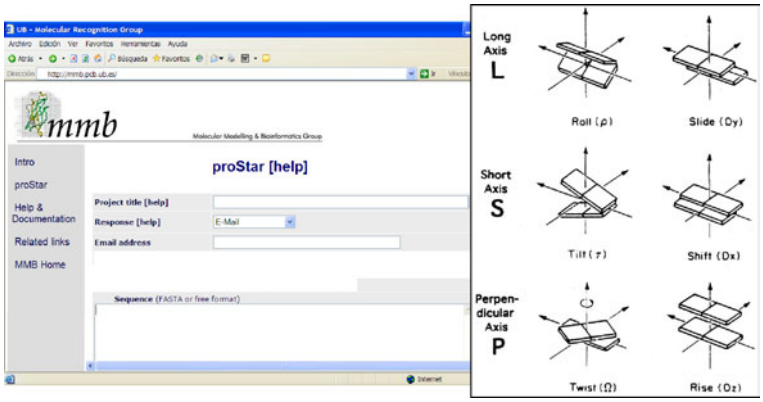


***Figure 4**. Main page of the ProStar web application with a graphical indication of the six stiffness parameters used to predict promoter location.*

ysis of protein properties at the genome-wide scale. For example, we are now applying a new technique developed by the group and based on MD (molecular dynamics) simulations of force-grids, to analyse and characterise all protein channels (Carrillo *et al*, 2007).

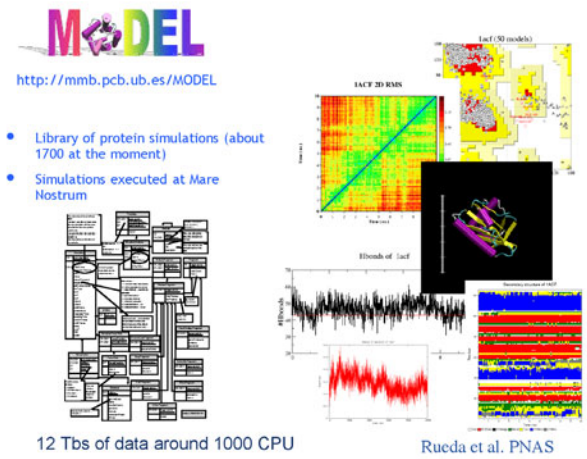


***Figure 5**. MODEL server and database, with some details about the amount of data collected.*

Similarly, MODEL infrastructure is now used to improve the resolution of NMR-derived protein structures within the COCO approach developed in collaboration with groups at the European Bioinformatics Institute and Nottingham University (Laughton *et al*, 2008).

During 2007 we have explored the possibility to use MODEL as a benchmark for the development of coarse-grained models of protein flexibility. Particularly, we have analysed the suitability of NMA methods based on quasi-GO models and found that after a careful calibration they can provide a reasonable representation of protein dynamics (Rueda *et al*, 2007). More recently, we have demonstrated that these simple potentials can be incorporated into Brownian dynamics protocols to analyse flexibility in protein clusters (Emperador *et al*, 2007) and that ultra-simplified discrete MD simulations can also satisfactorily reproduce complete MD trajectories (Emperador *et al*, 2007). These results open up the possibility to perform massive analysis (at the organelle or cellular level) of multi-protein dynamics.

Finally, within this subarea of research, we have made contributions to computer-assisted therapy design, both in the determination of possible targets (Fort *et al*, 2007) and in the development of new drugs (Soliva *et al*, 2007) for the treatment of inflammation.

Publications

BioMoby Consortium (including Orozco M, Gelpí JL and others). Interoperability with Moby 1.0--it's better than sharing your toothbrush! *Brief Bioinform*, **9**(3), 220-31 (2008)

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Talavera D, Vogel C, Orozco M, Teichmann SA and De la Cruz X. The (in)dependence of alternative splicing and gene duplication. *PLoS Comput Biol*, **3**, 375-88; front cover article (2007)

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Research Networks and Grants

Bioinformática y modelización en genómica estructural
Ministerio de Ciencia y Tecnología, GEN2003-20642-C09-07: 2004-2007
Research Director: Modesto Orozco

ELIXIR — European Life-science Infrastructure for Biological Information
European Commission, PE00030012: 2007- 2010

Estudio de formas inusuales o tensionadas del DNA. Implicaciones biotecnológicas y biomédicas
Ministerio de Educación y Ciencia, BIO2006-01602: 2006-2009

Reconeixement molecular
Generalitat de Catalunya, 2005SGR0286: 2006-2009
Research Director: Modesto Orozco

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

Structural bioinformatics
Genoma España, GN4: 2007-2010
Research Director: Modesto Orozco

Other Funding Sources

Fundación Marcelino Botín. Extended 5-year support

Genmedica Therapeutics. Collaboration agreement

Laboratorios Salvat. Collaboration agreement

Neuropharma. Collaboration agreement

Palau Pharma, Grupo Uriach. Collaboration agreement

Collaborations

Design of P39a-MAP kinase inhibitors
Robert Soliva, Palau Pharma, Grupo Uriach (Barcelona, Spain)

Development of coarse-grained models for protein flexibility
Pablo Chacon, Centro de Investigaciones Biológicas, CSIC (Madrid, Spain)

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

Development of nucleobase analogues
Enrique Pedroso, University of Barcelona (Barcelona, Spain), Francisco Javier Luque, University of Barcelona (Barcelona, Spain)

Development of strategies for improvement of NMR-samplings
Charles Anthony Laughton, University of Nottingham (Nottingham, UK), William Vranken, European Bioinformatics Institute, European Molecular Biology Laboratory (Cambridge, UK)

Force-field refinement for nucleic acid simulations
Jiri Sponer, Brno University of Technology (Brno, Czech Republic), Thomas E Cheatham, University of Utah (Utah, United States), Charles Anthony Laugton, University of Nottingham (Nottingham, UK)

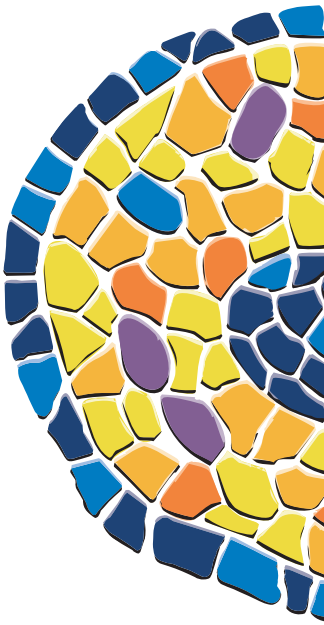
Introduction of polarisation effects in force-field calculations
Christoph Chipot, Nancy University (Nancy, France), Francisco Javier Luque, University of Barcelona (Barcelona, Spain)

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

Study of genetic variability induced by alternative splicing
Sarah A Teichman, MRC (Cambridge, UK)

Awards

Distinguished fellowship, Fundación Marcelino Botín (2007)



Structure and dynamics of protein interactions

Miquel Pons

Weak interactions provide the rich dynamic background that sustains the regulatory processes responsible for the stability of living organisms. The transient nature of these interactions and competition between alternative interactions present a research challenge that is best addressed using NMR. The co-existence of rapidly exchanging alternative structures also requires a conceptual approach that differs from the static single geometric picture. Instead, it relies on the statistical characterisation of the properties of structural ensembles, including all accessible structures within a given time scale in a particular environment. The time and/or ensemble average associated with most experimental measurements causes a loss of information. This loss can be partially recovered by combining several experimental techniques with distinct averaging properties and by modelling. Currently, we are combining NMR with other solution techniques, especially Small Angle X-ray Scattering (SAXS) and fluorescence spectroscopy.

Small molecules can non-specifically modulate the environment of a protein by binding strongly to it, specifically interfering with its activity, or by interacting weakly, but specifically, as molecular spies reporting on the interactions of the protein with other macromolecules. Our group focuses on three main fields of direct biomedical interest: i) nucleoid-associated proteins of potentially pathogenic bacteria. These proteins modulate gene expression in response to environmental changes and are essential for infection; ii) the regulation of low molecular weight phosphatases and Src kinase, two key proteins in tyrosine phosphorylation signalling; and iii) drug design and the characterisation of the space of potential low molecular weight protein ligands.

Bacterial nucleoid-associated proteins

H-NS is an abundant DNA-binding protein known to be a general regulator of gene expression in response to environmental challenges, such as changes in osmolarity or temperature. The structure of its N-terminal domain, involved in protein-protein interactions, has been reported, although two dimer topologies have been described for very similar constructs. We have used paramagnetic tagging and NMR to confirm the anti-parallel topology of the 1-46 H-NS construct (manuscript in preparation).

Hha is a nucleoid-associated protein that does not bind directly to DNA but co-regulates gene expression by interaction with H-NS (Madrid *et al*, 2007a). Previous extensive mutational studies of H-NS clearly defined the Hha binding site as having an essential arginine residue. These results prompted an analysis of known bacterial genomes. This study has highlighted the presence of the Hha gene in all species of the enteric group and even in endosymbionts that have undergone dramatic genomic reduction (Madrid *et al*, 2007b). This observation points to H-NS as having a central role in the biology of the enteric group, which includes many life-threatening pathogens, such as Yersinia (causing the plague), Salmonella (causing a large number of cases of food poisoning), and the urophathogenic or enteropathogenic strains of *E. coli*. Previous studies by our group showed the plasticity of Hha, as demonstrated by it changing conformation to bind H-NS. Until now, dynamic effects have prevented the direct determination of the structure of the Hha-H-NS complex. We have carried out an exhaustive mutational study of Hha, which has allowed the identification of negatively charged residues essential for H-NS binding (manuscript in preparation). In addition, paramagnetic tagging is being used to define the relative topology of Hha and H-NS in the complex. In collaboration with the group led by Juan Recio (Barcelona Supercomputing Center), we are combining these experimental results with macromolecular docking calculations.

Preliminary NMR relaxation dispersion experiments have identified one of the regions most affected by conformational exchange in the absence of H-NS, namely the loop between helices 1 and 2 that contains a single conserved cysteine residue. Replace-

Research Group Members | **Principal Investigator:** Miquel Pons | **Associate Researchers:** Pau Bernadó, Jesús García, Juan Carlos Paniagua | **Postdoctoral Fellow:** Yolanda Pérez | **PhD Students:** Eric Aragón, Jascha Blobel, Giovanni Cincilla, Tiago Cordeiro, Carles Fernandez de Alba, Arola Fortian, Oriol Marimón | **Master Students:** Lidia Ballester, Xiodi Sun | **Visitor:** Catalina Granados (Colombia)

ment of this cysteine residue by isoleucine results in an increased affinity for H-NS. However, expression of the mutant *in vivo* leads to a sharp decrease in viability. This observation points to a direct role of H-NS-bound Hha in the activity of the Hha-H-NS complex (Cordeiro, in preparation).

We are currently extending our studies to other proteins that are equally essential or structurally related. Jesus Garcia, in our group, has already identified new relevant interactions involving the N-terminal domain of the ε-subunit of the multi-protein DNA polymerase III complex, with YdgT, a member of the Hha/YmoA/YdgT family. Also, we are determining the structure of the DNA-binding domain of Ler, a general regulator of virulence, with homology to H-NS in this domain. We have also established new collaborations with the aim to characterise, by means of complementary techniques, the interaction of full-length H-NS with DNA. This research line is a long-term collaboration with Antonio Juarez (University of Barcelona and IBEC).

Regulation of proteins involved in tyrosine phosphorylation signalling

Our group has a long standing interest in mammalian low molecular weight tyrosine protein phosphatases (lmwPTPs) and their oligomerisation. Bovine lmwPTP forms enzymatically inactive homodimers at high protein concentration. We hypothesise that phosphatase oligomerisation is a regulatory mechanism of the recovery stage of kinase-mediated signalling events under crowding conditions. In our search for a suitable mimetic of crowding conditions, we have explored the use of arginine-glutamic mixtures and we have characterised bovine lmwPTP oligomerisation using ^{129}Xe-NMR chemical shifts and ^{15}N-NMR relaxation (Blobel, Schmidl *et al*, 2007). We have demonstrated that arginine-glutamic mixtures have intriguing emerging properties: enhancement of specific macromolecular interactions and suppression of non-specific contacts. In the presence of arginine-glutamic acid, ^{129}Xe NMR becomes a selective probe of the presence of one of the oligomeric species.

We have recently focused our attention on the highly relevant human Src tyrosine kinase, the paradigm of the Src kinase family that includes, in addition to Src, the kinases Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrc. Src kinase is inactive in the metabolic basal state. The inactive form involves the interaction of a phosphorylated tyrosine with an SH2 domain. By the action of a phosphatase, Src is activated. SAXS data of a mimic of the activated form show that, in contrast to previously reported X-ray data, the active form is not structurally open but is involved in an equilibrium in which 85% of the species are closed (Bernadó *et al*, 2007b). This result sheds light on the relative stability of the open and closed forms and explains the

weak affinity of the natural phosphorylated sequence in the closed form: the need to find the right compromise between low basal activity in the resting state, which requires a 'closed' form, and accessibility to the activating phosphatase, which needs at least a small quantity of an 'open' form. This study has also demonstrated the power of an analytical algorithm developed by Pau Bernadó and also applied to other systems (Bernadó, Mylonas *et al*, 2007; Cho *et al*, 2007; Mukrasch *et al*, 2007; Grela *et al*, 2007).

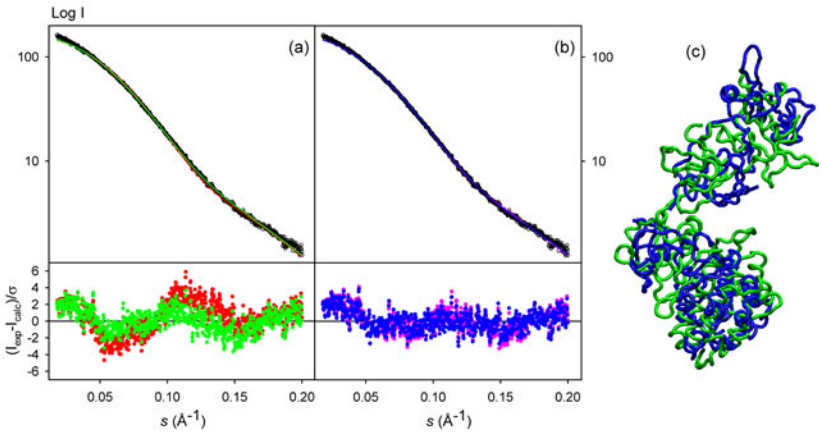


***Figure 1**. Small Angle X-ray Scattering (SAXS) profile of active human Src kinase fitted with models including an equilibrium between the inactive closed form and several representations of open forms.*

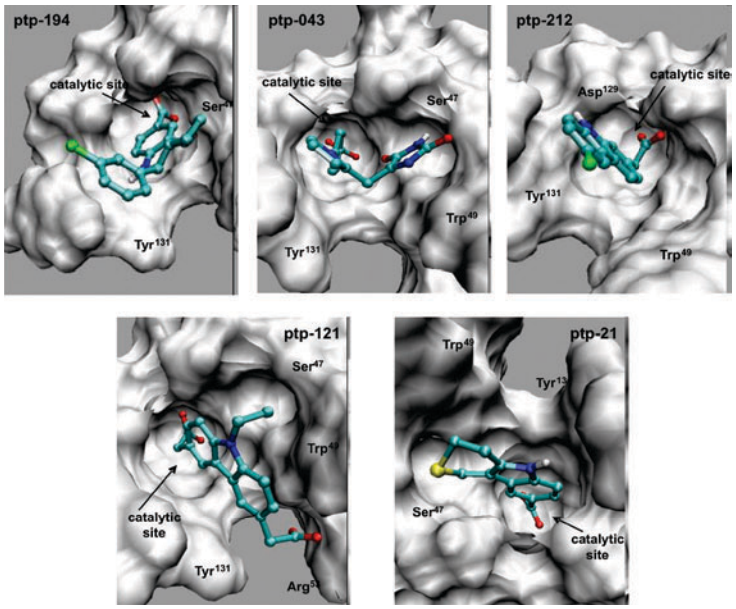


***Figure 2**. Calculated structures of complexes of inhibitors of low molecular weight tyrosine protein phosphatase.*

In addition to the well folded multi-domain core, which is well conserved among several family members, the Src family of kinases has a native unfolded domain that is unique for each protein species. This unique domain is essential for substrate specificity and is currently being studied by our group by a combination of NMR and SAXS. We have performed complete NMR assignment and have measured the residual dipolar couplings (Figure 1).

Interaction between small molecules and proteins

We have recently developed a new highly efficient strategy for virtual ligand screening based on the LINGO concept. The performance of this strategy has been demonstrated experimentally by the successful development of inhibitors for monomeric lmwPTP (Vidal *et al*, 2007). NMR techniques used to detect transient ligand binding make use of the distinct relaxation properties of small molecules and large complexes. We have shown that saturation transfer difference (STD) experiments can be used to detect protein-protein interactions indirectly through the change of intensity in the signal of NMR signals of small molecules acting as reporters of the change of correlation time of the target protein when it interacts with another macromolecule. The use of small molecule reporters allows the study of protein-protein interactions at very low concentrations and does not require isotopically labelled proteins (Blobel *et al*, 2008a).

We have extended the LINGO concept, initially developed for SMILES representations, to IUPAC names (Thormann *et al*, 2007). This new development allows the prediction of quantitative molecular properties simply from the IUPAC name and converts the complete chemical literature, where IUPAC names are routinely used, into a searchable database of molecules that have been studied, and therefore are likely to be accessible and have a property of interest within a given range. We are currently developing strategies to extract the inherent structure present in large databases by combining new clustering techniques with LINGO similarity approaches (Figure 2).

Publications

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Madrid C, García J, Pons M and Juarez A. Molecular evolution of the H-NS protein: interaction with Hha-like proteins is restricted to enterobacteriaceae. *J Bacteriol*, **189**, 265-68 (2007b)

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Research Networks and Grants

Acción de mejora de la infraestructura científico técnica singular de RMN de Barcelona
Ministerio de Educación y Ciencia, ICTS-2006-05: 2007
Research Director: Miquel Pons

Acción de mejora de la infraestructura científico técnica singular de RMN de Barcelona
Ministerio de Educación y Ciencia, ICTS-2007-08: 2007-2008
Research Director: Miquel Pons

EMAR—European Science Foundation
Ministerio de Educación y Ciencia, PCI2006-A9-0690: 2007-2012
Research Director: Miquel Pons

EMAR-Multidisciplinary frontiers of magnetic resonance
European Science Foundation, Research Networking Programme, 05-PGM-022: 2007-2012
Research Director: Miquel Pons

EUROMAR 2007
Generalitat de Catalunya, 2006/ARCS1/0036: 2007
Research Director: Miquel Pons

EUROMAR
Ministerio de Educación y Ciencia, BIO2006-27045-E: 2007
Research Director: Miquel Pons

Evaluación de Hha y H-NS como dianas antibacterianas
Agencia Española de Cooperación Internacional, B/9539/07: 2007
Research Director: Miquel Pons

Genómica estructural: aplicación a proteínas y complejos proteicos relacionados con el cáncer
Ministerio de Educación y Ciencia, GEN2003-20642-C09-04: 2004-2007
Research Director: Miquel Coll

Inhibidores de la adaptación: una nueva estrategia antibacteriana
Fundación Caja Navarra, 10055: 2007
Research Director: Miquel Pons

Nuevos métodos de RMN para el estudio de interacciones proteína-proteína
Ministerio de Educación y Ciencia, BIO2004-5436: 2005-2007
Research Director: Miquel Pons

Nuevos métodos de RMN para el estudio de proteínas relacionadas con la patogenicidad bacteriana
Ministerio de Educación y Ciencia, BIO2007-63458: 2007-2010
Research Director: Miquel Pons

Collaborations

Bacterial nucleoid-associated proteins
Antonio Juarez, 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 BIFI (Zaragoza, Spain)

Origins of the Hoffmeister effect
Oscar Millet, CIC BIOGUNE (Bilbao, Spain)

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

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

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

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)

129Xe- NMR spectroscopy
Eike Brunner, Technical University Dresden (Dresden, Germany)

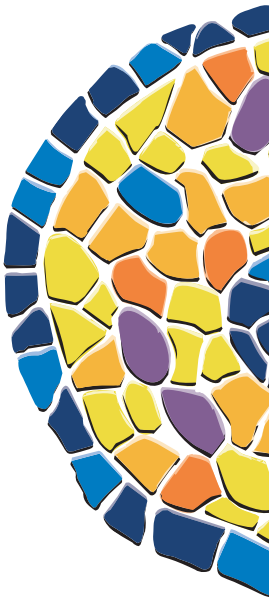
Honours

Chairman, European NMR conference EUROMAR 2007

Selection committee member, 2007 Russel Varian Prize

Editorial board member, The Open Magnetic Resonance Journal

Council member, International Society for Magnetic Resonance (ISMAR)



Molecular Medicine Programme

Cell signalling: regulation and function

Carme Caelles

With the aim to improve and develop new therapeutic tools, we study regulatory and cross-talk mechanisms that underlie protein kinase-mediated signal transduction. We focus on two research lines, the nuclear receptor-MAPK pathway negative cross-talk and the Nercc1/Nek6/7 NIMA-family signalling cassette. With regards to the former, our research addresses the cross-talk between the glucocorticoid receptor (GR) and the peroxisome proliferator-activated receptor (PPAR) γ, and the C-Jun N-terminal kinase (JNK) pathway, since each of these negative interactions appears to mediate some of the pharmacological actions of the ligands for these nuclear receptors. We explore the molecular mechanisms that mediate the inhibition of the JNK pathway by these two nuclear receptors, and analyse the pharmacological actions of these nuclear receptor ligands. In relation to the second research line, we study the regulation and function of the signalling module formed by NIMA-family kinases Nercc1/Nek9, Nek6 and Nek7. Previous data from our group and others show that Nercc1 is activated during mitosis, when it binds and activates Nek6/7, and that these kinases have a central role during spindle formation and mitotic progression. Our present objectives are to unravel the mechanism of Nercc1 activation, to identify Nercc1 and Nek6/7 substrates, and to validate these as drug targets and/or prognosis markers in diseases related to cell cycle malfunction.

Nuclear receptor-MAPK pathway crosstalk: mechanisms and actions

JNK is a group of serine/threonine protein kinases that belongs to the MAPK family, and together with the p38MAPK group are known as stress-activated kinases (SAPKs). The JNK pathway is activated mainly by pro-inflammatory cytokines and stress stimuli, such as stress in the endoplasmic reticulum (ER stress) triggered by the activation of the unfolded protein response or oxidative stress caused by the accumulation of reactive oxygen species. JNK activation results in the phosphorylation and functional regulation of its substrates, which are nuclear proteins like the component of the AP-1 complex and c-Jun, or cytoplasmic proteins, such as the insulin receptor substrate (IRS)-1. JNK activity is negatively regulated by protein phosphatases such as the dual specificity MAP kinase phosphatase (MKP)-1. JNK is involved in a number of pathological conditions in which chronic inflammation is a key component, such as cancer, arthritis, asthma, neurodegeneration, obesity and diabetes. This observation has prompted intensive search for JNK inhibitors because of their potential therapeutic use.

Ligands of several members of the nuclear hormone receptor superfamily, such as glucocorticoids (GCs), retinoids or ligands of PPARs and LXRs, have anti-inflammatory properties. All these molecules, in a receptor-dependent manner, share the capacity to down-regulate the activation of signalling pathways that are crucial for the inflammatory response, such as the JNK/AP-1 and IKK/NFκB signal transduction pathways. We analyse this/these cross-talk mechanism(s) and the role of JNK pathway inhibition in conducting the anti-inflammatory action of these ligands. Our analysis focuses on GCs, since they are first choice anti-inflammatory drugs for the treatment of arthritis and asthma, and the synthetic PPAR¤ ligands, thiazolidinediones (TZDs), because their insulin-sensitising action makes them therapeutic tools for the treatment of type 2 diabetes.

The GC/GR-JNK pathway negative cross-talk

In relation to cross-talk of the GR-JNK pathway, we have shown that in several cell types, such as primary bone marrow and thioglycollate-elicited macrophages, GCs up-regulate the expression of the MKP-1 gene, a negative regulator of the JNK and p38MAPK pathway. GCs trigger accumulation of MKP-1 mRNA in a transcription- and GR-dependent manner. Consistently, we have characterised three GC response elements (GREs) in the 5' regulatory region. All of these GREs are functional since each mediates tran-

Research Group Members | **Principal Investigator:** Carme Caelles | **Associate Researcher:** Joan Roig | **Postdoctoral Fellow:** Marta Nicolàs | **PhD Students:** Maria Isabel Arévalo, Maria Teresa Bertrán, Laura Regué, Mariana Teixeira | **Lab Technician:** Cristina Vila

scriptional activation in response to GCs, as shown by transient transfection reporter assays, and binds hormone-activated GR *in vitro* and *in vivo*, as shown by EMSA and ChIP assays, respectively.

We also have analysed the anti-inflammatory action of GCs in a genetic background deficient in the MKP-1 gene, taking advantage of previously generated MKP-1 knock-out mice. As a first approach, we examined the capacity of GCs to inhibit lipopolysaccharide (LPS)-induced activation of MAPK pathways and pro-inflammatory gene expression in bone marrow and thioglycollate-elicited macrophages. In both primary cell types, in the absence of MKP-1, GCs lost their capacity to inhibit LPS-induced activation of the JNK and p38MAPK pathways, while no effect was observed on activation of the ERK pathway either in wild-type (WT) or in MKP-1-deficient macrophages. In relation to the action of GC on pro-inflammatory cytokine gene expression, in general in most of the genes analysed the inhibitory property of GCs was decreased by MKP-1 deficiency although to a different extent depending on each particular gene, IL-1β being the most greatly affected.

Despite the partial loss of GC inhibitory action on the pro-inflammatory pathway and gene expression in both types of macrophages derived from MKP-1 knock-out mice compared to WT, at high doses GCs were fully competent in rescuing mice from lethal endotoxic shock and in decreasing the amount of pro-inflammatory cytokines in blood to a level similar to that found in WT mice. These results indicate that GC anti-inflammatory action in sepsis is independent of MKP-1 gene expression. In MKP-1 knock-out mice, we also analysed the capacity of GCs to inhibit local inflammation caused by the topical application of the irritant TPA. In this test, GC anti-inflammatory action was achieved in the MKP-1-deficient mice at higher GC doses than in the WT animals, thereby indicating a certain degree of resistance to GCs.

The TZD/PPAR□-JNK pathway negative cross-talk

Insulin signalling depends on IRS-1 interaction with and phosphorylation on tyrosine residues by the hormone-activated IR. Finally, insulin signalling activates JNK, which, in turn down-regulates insulin sig-

nalling by IRS-1 phosphorylation on serine residues, a mechanism that constitutes a physiological negative feedback loop. Nonetheless, in obesity, abnormally elevated JNK activity, caused by increased levels of pro-inflammatory cytokines, such as TNF-α or ER stress, inhibits insulin signalling, thereby promoting insulin resistance.

Thiazolidinediones (TZDs), a class of drugs used clinically as insulin-sensitising agents, reduce blood glucose, insulin, triglyceride and free fatty acid (FFA) levels in animal models of insulin resistance and type 2 diabetes, and in humans with these conditions. Despite the pharmacological significance of TZDs, little is known about the molecular mechanism(s) behind the insulin-sensitising action of these anti-diabetic agents. These drugs ameliorate insulin resistance during early steps of the insulin signal transduction cascade by restoring IR and IRS-1 tyrosine phosphorylation levels in response to insulin.

In this context, we have analysed the effect of agonist ligands of PPARγ, including the anti-diabetic drugs of the TZD family, on the activation of the JNK pathway. We showed that rosiglitazone down-regulates JNK activity in cultured cells and *in vivo*. We demonstrated that this drug inhibits the JNK activity triggered by TNF-α in 3T3-L1 pre-adipocytes and adipocytes and by IL-1β in the insulin-secreting INS-1E and βTC-3 cell lines. To study the inhibitory action of rosiglitazone *in vivo*, we took advantage of the abnormally elevated JNK activity in the obese diabetic *ob/ob* mouse and in the diet-induced model of insulin-resistant obese mouse. Treatment with rosiglitazone produced a significant decrease in JNK activity in peripheral tissues in both murine models of obesity. In addition, our data indicate that PPAR□ mediates the action of this drug on the JNK pathway. Given the central role of JNK in insulin resistance and the abrogation of the hypoglycemic action of rosiglitazone in JNK1 knock-out mice, we propose that the inhibitory action of TZD/PPARγ on the JNK pathway is involved in conducting the insulin-sensitising properties of these drugs and their receptor *in vivo* (Figure 1). In addition, given the role of the IL-1β/JNK cascade in pancreatic β-cell death, the inhibitory action of rosiglitazone on the JNK pathway may also be a relevant mechanism that mediates the protective properties of TZD/PPARγ on pancreatic β-cells *in vivo*.

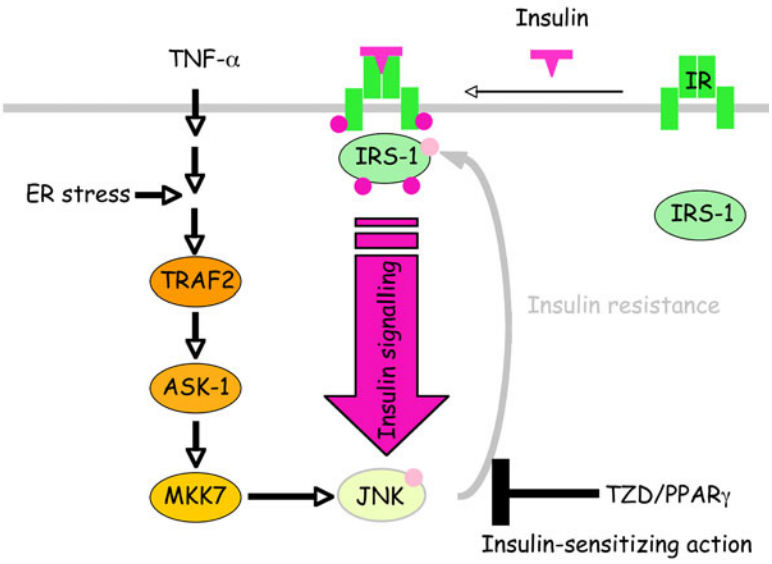


Figure 1. *Inhibition of JNK pathway activity by TZDs mediates insulin-sensitising action. Insulin signalling depends on IRS-1 interaction with and phosphorylation on tyrosine residues by the hormone-activated IR. Finally, insulin signalling activates JNK, which, in turn down-regulates insulin signalling by IRS-1 phosphorylation on serine residues, a mechanism that constitutes a physiological negative feedback loop. Nonetheless, in obesity, abnormally elevated JNK activity, caused by increased levels of pro-inflammatory cytokines such as TNF-□ or ER stress, inhibits insulin signalling, thereby promoting insulin resistance. Agonist ligands of PPARγ, including the anti-diabetic drugs of the TZD family, inhibit the activation of the JNK pathway in a PPARγ-dependent manner. This TZD/PPARγ negative interaction with the JNK pathway participates in conducting the insulin-sensitising action of these drugs.*

The Nercc1/Nek6/7 NIMA-family signalling cassette: regulation, function and implication in cellular transformation

To further our understanding of the regulation and function of Nercc1, Nek6 and Nek7, we have performed several two-hybrid screenings, which have led to the identification of several proteins that interact with these kinases. Complementing these experiments, we have identified additional interacting proteins by a copurification/mass spectrometry approach. We are currently assigning the interactors identified to one of two groups: regulators of Nercc1/Nek6/7 activity or subcellular localisation, or substrates. We have started an in-depth study of some of these proteins. This approach has lead to the identification of a novel Nek6 substrate that has a central role during spindle formation and we can explain at least part of the phenotype that results from Nercc1/Nek6/7 interference.

Our data suggest that the regulation of Nercc1 activity depends on a mechanism that involves both the mitotic phosphorylation of the kinase as well as the effects of one or more inhibitory proteins. Our research efforts currently address the identification of these inhibitors through the study of proteins that interact with Nercc1, and in parallel we have determined Nercc1 mitotic phosphorylation sites. We are now advancing in the identification of the kinase(s) responsible for this modification and its/their relationship with Nercc1 activation.

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Research Networks and Grants

A novel mitotic signalling pathway composed by the NIMA-family protein kinases Nercc1, Nek6 and Nek7. Regulation, functions and possible implication in cellular transformation
Marie Curie International Reintegration Grants, European Commission, (MIRG-CT-2005-031088): 2006-2007
Research Director: Joan Roig Amorós

Estudio de una nueva via de señalización mitotica compuesta por las NIMA quinasas Nercc1, Nek6 y Nek7. Regulación y funciones
Ministerio de Educación y Ciencia, Programa Ramón y Cajal, RYC-2004-001074: 2005-2007
Research Director: Joan Roig Amorós

Estudio de una nueva via de señalización mitotica compuesta por las NIMA quinasas Nercc1, Nek6 y Nek7. Regulación y funciones
Ministerio de Educación y Ciencia, (BFU2005-05812): 2006-2008
Research Director: Joan Roig Amorós

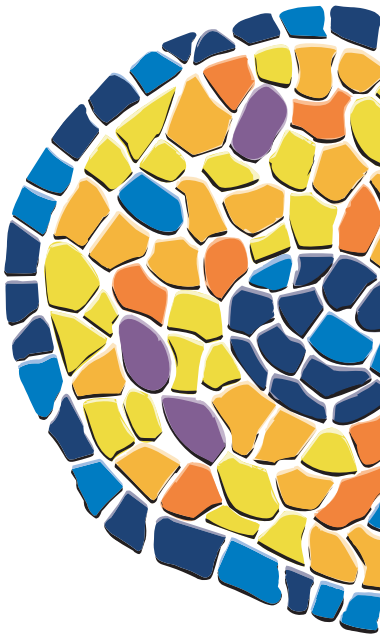
Estudio in vitro e in vivo de la interacción de los glucocorticoides y la ruta de la Jun N-terminal kinase (JNK): mecanismos y acciones fisiopatológicas y farmacológicas mediadas por esta interacción
Ministerio de Educación y Ciencia, BFU2004-02096/BMC: 2005-2007
Research Director: Carme Caelles

Papel de la c-Jun N-terminal kinase (JNK) en las acciones fisiológicas y farmacológicas de los glucoccorticoides y los ligandos de PPARs y LXR
Ministerio de Educación y Ciencia, BFU2007-62087: 2007-2009
Research Director: Carme Caelles

The Cell Signalling Research Group is member of the Consolidated Research Group 2005SGR00857 recognised by the Generalitat de Catalunya.

Collaborations

Methods for identifying inhibitors of mitosis
Mercury Therapeutics, Inc (Woburn, USA)



Macrophage biology: regulation of gene expression

Antonio Celada

Inflammation occurs when the body suffers aggression either by microbes, traumatisms or a variety of physical agents such as heat, radiation, etc. Inflammation is also involved in the pathogenesis of chronic diseases of auto-immune origin (ie, rheumatoid arthritis) and cancer. In the early stages of this process, 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. In addition, these phagocytic cells 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 changes from being pro-inflammatory to being anti-inflammatory, when these cells remove all the tissue debris that arises during healing (Figure 1). Our project is the continued work of many years devoted to the biology of macrophages and dendritic cells. These two cell types are crucial 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 inflammation 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. 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

One of the best characterised signal transduction pathways is 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 kinases, which in turn activate ERK1 and ERK2. In non-stimulated cells, ERK1 and ERK2 are found in the cytoplasm and 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, both required for proliferation and cellular activation. In macrophages, we have observed that ERK activation is required not only for proliferation, but also for lipopolysaccharide (LPS)-mediated activation, although this activation also blocks proliferation. The duration and time of initiation of ERK phosphorylation determines whether the macrophage proliferates (short phosphorylation) or becomes activated (long phosphorylation). This is explained by the fact that MKP-1, the phosphatase responsible for ERK dephosphorylation, is induced rapidly in response to macrophage colony-stimulating factor (M-CSF) or slowly in response to LPS. In both cases, MKP-1 induction is mediated by protein-kinase C (PKC)ε and is independent of ERK phosphorylation.

We have also reported that Jun N terminal kinase (JNK) activation is required for this induction (Sánchez-Tilló *et al*, 2007). Furthermore, IFN-γ, which also inhibits proliferation, blocks MKP-1 induction by

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M-CSF by elongating ERK phosphorylation (Valledor *et al*, 2007; Figure 2). Inhibition of MKP-1 induction by RNA interference (RNAi) blocks proliferation and elongates ERK activation. IFN-γ also cross-talks with the MAP kinases (Valledor *et al*, 2007).

We have cloned the MKP-1 promoter, and by means of luciferase mutations and activity assays, we have localised an AP-1/CRE box which 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 is induced by LPS and M-CSF with the same kinetics as MKP-1 (Figure 3).

Macrophage proliferation is independent of calcineurin but requires immunophilin, without which ERK is inactivated. M-CSF induces the opening of K^+ channels, which are required for proliferation (Villalonga *et al*, 2007).

Our group has devoted many years of research 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 de-

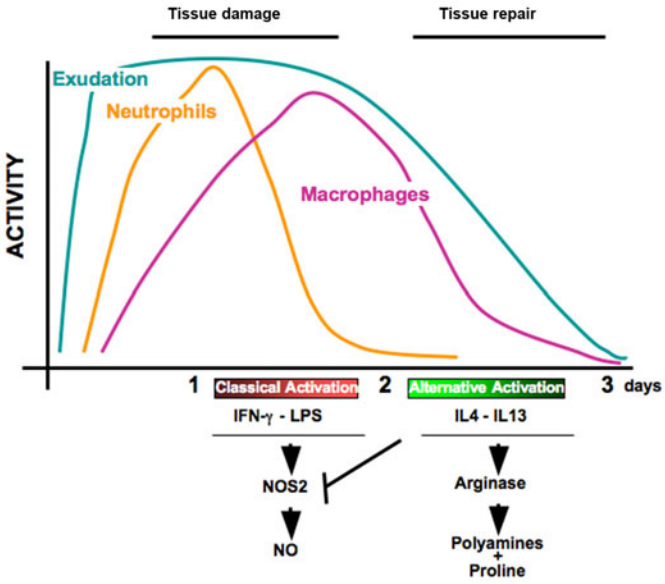

***Figure 1**. Dual activity of macrophages at the inflammatory loci. Initially there is a proinflammatory phase where microbes are destroyed followed by an anti-inflammatory phase where particular macrophages eliminate apoptotic bodies and reconstruct the tissues.*

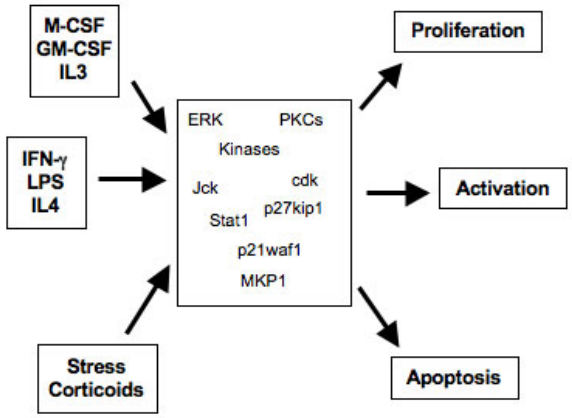

Figure 2. *Group factors, activators or inducers of apoptosis use several signal transduction molecules to achieve proliferation, activation or apoptosis.*

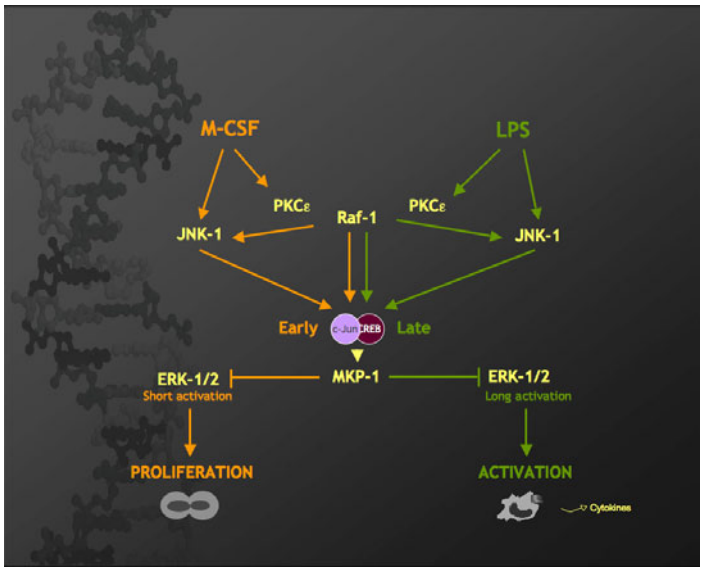

Figure 3. *Signal transduction of M-CSF and LPS inducing the expression of MPK-1.*

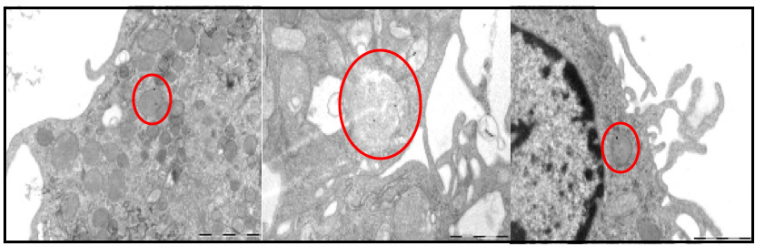

Figure 4. *Nanoparticles ingested by macrophages.*

scribed to date show ubiquitous expression, a finding that does not correlate with the differential tissue expression of MHC class II molecules. A transactivator that does not bind directly to DNA, CIITA (class II transactivator), has been 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 expression in B lymphocytes and dendritic cells treated with LPS (Casals *et al*, 2007). Also the upstream regulatory elements interact with the proximal, thereby blocking the transcription. This loop is open when CIITA is present.

In collaboration with Victor Puntes (Institut Català de Nanotecnologia) and Ernest Giralt (IRB Barcelona), we have found that nanoparticles activate macrophages by interaction with the Toll-like receptor4 (Figure 4).

Molecular mechanisms involved in classical and alternative activation of macrophages

Classical or pro-inflammatory activation of macrophages (phenotype M1) is induced by IFN-γ or LPS while activation triggered by IL-4, IL-10 or IL-13 is known as alternative or anti-inflammatory activation (phenotype M2). 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 nitric oxyde synthase 2 (NOS2), which produces nitric oxide (NO), a molecule that 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 by which IL-4 inhibits proliferation is $p21^{waf1}$- and Stat6-dependent, the mechanism used by IFN-γ differs.

Role of TREX1 exonuclease in transcription

We have cloned a protein that corresponds to TREX1 exonuclease. 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, develop inflammatory myocarditis and have 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 auto-immune 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 experts in crystallography (Ignasi Fita) and in NMR (Maria Macias) at IRB Barcelo-

na, we have determined the structure of TREX1 alone and its binding to DNA (Brucet *et al*, 2007; Figure 5). TREX1 binds preferentially to certain DNA sequences that correlate with the 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 and the nuclear localisation of the protein have led us to study whether TREX1 is involved in transcription. Also, we have identified a new active histidine that is conserved in DEDDh exonucleases and is required for functional activity (Brucet *et al*, in preparation).

Deregulated gene expression in aging

We are currently testing the molecular changes that occur in the genome of macrophages during aging. By growing macrophages alone *in vitro*, we eliminate the effects that other cells could exert on them. In addition, we have recently reported that deacetylase activity is required for GM-CSF-dependent functional response of macrophages and dendritic cell differentiation (Sebastian *et al*, 2007). Since deacetylase activity plays a relevant role in aging in lower organisms, these results have prompted us to study whether it is involved in macrophage aging.

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. At the physiological level, LXRs play a crucial role in the positive regulation of genes involved in lipid homeostasis.

We seek to explore whether the activation of LXRs exerts anti-inflammatory and neuroprotective actions in the central nervous system (CNS). 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 the progression of neurodegenerative disorders. Our preliminary studies show that both primary microglia and the microglial cell line BV-2 express the LXRα and β, and RXRα and β isoforms. In BV-2 microglial cells and in primary microglia from neonatal mice, stimulation with endogenous ligands of LXR resulted in the activation of known LXR target genes involved in lipid metabolism. 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 therefore studied the role of LXRs in the regulation of microglial activation *in vitro*. Our results indicate that a number of pro-inflammatory factors induced by endogenous cytokines are inhibited by LXR agonists (Figure 6). We are currently using microarray technology to establish a more extensive list of pro-inflammatory genes susceptible to down-modulation by LXR agonists and to determine the relative contribution of the macrophage phenotype (M1 vs. M2) in this context.

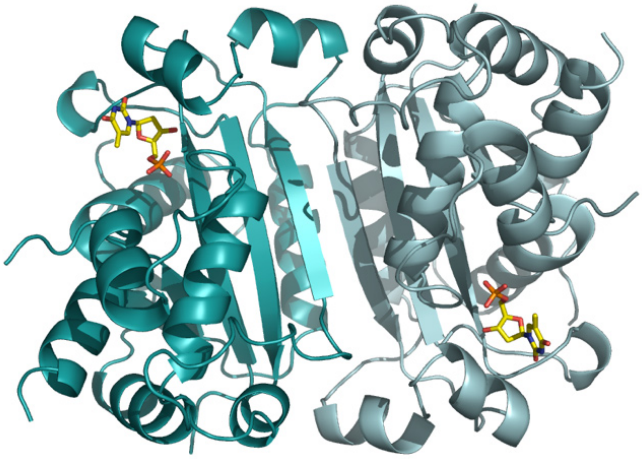

***Figure 5**. Three-dimensional structure of TREX1.*

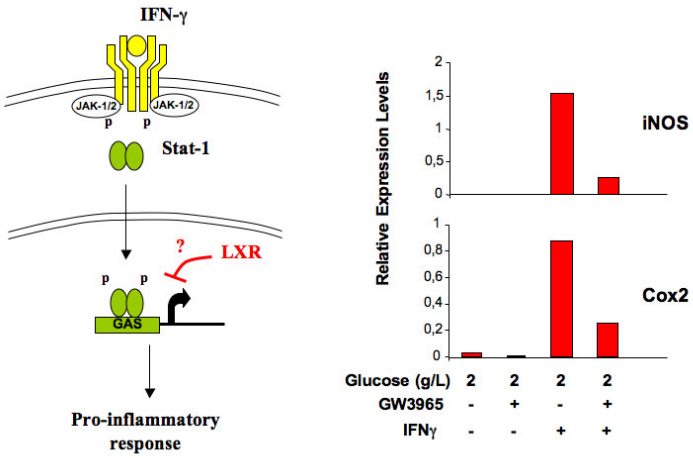

***Figure 6**. LXR agonists inhibit specific pro-inflammatory responses in microglia activated by IFNg.*

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, such as the genes Bcl-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 led us to propose that this factor mediates paracrine anti-apoptotic actions on other neighbour cells in the CNS, such as 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 to determine direct effects of LXR agonists on these 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*.

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Research Networks and Grants

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

Ayuda para potencializar los grupos de investigación consolidados
Pla de Recerca de Catalunya, 2005SGR 00910: 2005-2008
Research Director: Antonio Celada

Nanoparticles as activators of phagocytic cells for the clearance of toxic aggregates of proteins in the brain
Ministerio de Educación y Ciencia, SAF2006-26676-E: 2007
Research Director: Jorge Lloberas

Programas transcripcionales regulados por LXR en microglia y neuronas: implicaciones en neuroinflamación y neuroprotección
Ministerio de Educación y Ciencia, SAF2007-63543: 2007-2009
Research Director: Annabel Fernández Valledor

Regulation of the expression of genes involved in the proliferation, differentiation, activation and apoptosis of macrophages and dendritic cells
Ministerio de Educación y Ciencia, BFU2004-05725/BMC: 2005-2007
Research Director: Antonio Celada

Regulation of the expression of genes involved in the proliferation, differentiation, activation and apoptosis of macrophages and dendritic cells
Ministerio de Educación y Ciencia, BFU2007-63712/BMC: 2007-2011
Research Director: Antonio Celada

Collaborations

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

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

LXR and brain
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Signal transduction
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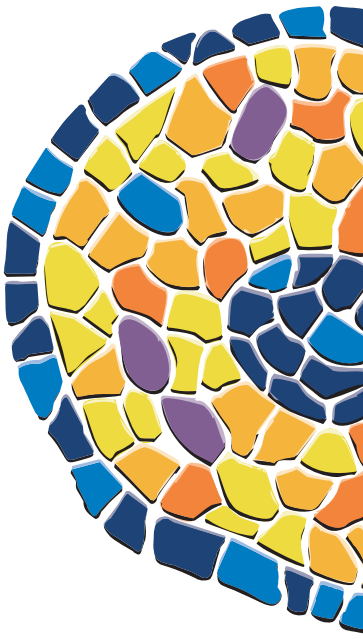
Telomerase and macrophaging
María Blasco, Centro Nacional de Investigaciones Oncológicas (Madrid, Spain)



Study of the regulatory mechanisms of glycogen metabolism, its alterations in pathologies and characterisation of new therapeutic targets

Joan J Guinovart

Our group is devoted to the study of the regulatory mechanisms of glycogen metabolism. We have a long tradition in the study of glycogen synthase (GS), the key enzyme in the regulation of glycogen synthesis. To this end we combine our knowledge of biochemistry and metabolism with a wide variety of techniques from molecular biology, cell biology, proteomics, RNA silencing, gene transfer, mutant mouse generation and structural biology, thereby allowing us to address biological issues using a multidisciplinary approach. We have made a series of discoveries that have contributed to revitalizing the field of glycogen metabolism. Our main achievements during 2007 are summarized below.

Neurons have the enzymatic machinery to accumulate glycogen, which is pro-apoptotic for this cell type

Although glycogen is present in most cells, its metabolism has been studied mainly in liver and muscle. Nevertheless, there are some cell types that do not accumulate this polysaccharide, like neurons. The central nervous system is an interesting case regarding glycogen metabolism. In embryonic stages, glycogen appears both in glial and neuronal cells but in adults this polysaccharide is present exclusively in astrocytes. Although the total concentration of glycogen in brain is lower than in muscle or liver, glycogen is a crucial source of energy for neurons. It is accepted that neurons, through neurotransmitters and neuromodulators, stimulate the mobilization of astrocyte glycogen reserves, which are converted into lactate to be taken up and utilized by neurons.

GS is the only enzyme able to synthesise glucose polymers in mammals. We have recently demonstrated that neurons express GS, specifically the muscle isoenzyme of GS (MGS). This is a remarkable finding since, as noted above, these cells do not normally accumulate glycogen. Our results also show that neurons have the capacity to synthesise glycogen when MGS becomes active. Since these cells do not express glycogen phosphorylase (the key enzyme for glycogen degradation) once glycogen is synthesised it cannot be degraded. Interestingly, the glycogen accumulated in neurons in these conditions is poorly branched (Vilchez *et al*, 2007). Furthermore, the deposition of this glycogen is pro-apoptotic. Therefore, when synthesised inside the neurons, glycogen acts as a Trojan horse, triggering mechanisms that lead to neuronal death. The concept that glycogen is harmful for neurons completely changes our vision of the field. A review of the literature indicates that the presence of glycogen in neurons in certain neurological diseases was reported long ago. Moreover, the presence of intracellular bodies composed mainly of glucose polymers has been recognised in many pathologies. The nomenclature used to describe these structures is varied: polyglucosan bodies, corpora amylacea and Lafora bodies among others, although all these structures have in common that they are essentially formed by poorly branched glycogen (Vilchez *et al*, 2007).

New regulatory mechanism of glycogen metabolism: laforin-malin complex blocks glycogen synthesis in neurons by inducing the proteasome-dependent degradation of MGS and PTG

We have shown that although neurons have the enzymatic machinery for synthesising glycogen, it is kept silent by a series of well-coordinated intracellular mechanisms: a) GS is confined in the nucleus; b) GS is fully inactivated by phosphorylation; and c) GS and PTG, a regulatory subunit of protein phosphatase 1 that activates GS by stimulating its dephosphorylation, are degraded by a novel regulatory mechanism involving the proteasome. Failure to keep GS under control,

Research Group Members | **Principal Investigator:** Joan J Guinovart | **Associate Researchers:** Jorge Domínguez, Mar García Rocha | **Postdoctoral Fellows:** Daniel Cifuentes, Adelaida Díaz-Vilchis, Carlos J Rodríguez, Delia Zafra | **PhD Students:** Oscar Blanco, Carles Martínez-Pons, Laura Nocito, Susana Ros, Isabel Saez, Jordi Vallès, David Vilchez | **Research Assistant:** Anna Adrover | **Lab Manager:** Carme Carrion | **Lab Technician:** Mari Carmen Romero, Emma Veza | **Visitors:** Juan Carlos Slebe (Chile), Alejandro Yañez (Chile)

which results in glycogen synthesis, damages neurons by triggering apoptotic signalling. In addition, the glycogen that neurons synthesise is abnormal and causes the accumulation of granular deposits that cannot be mobilized. Such deposits, referred to as Lafora bodies, are characteristic of a form of progressive myoclonus epilepsy, Lafora disease (Vilchez *et al*, 2007).

The laforin-malin degradation system regulates PTG stability in hepatic cells

In collaboration with Pascual Sanz (IBV—CSIC) and Santiago Rodríguez de Córdoba (CIB—CSIC), we have also reported that the laforin-malin complex down-regulates PTG-induced glycogen synthesis in hepatic cells. Furthermore, the interaction between laforin and malin is a regulated process that is modulated by the AMP-activated protein kinase (AMPK). These data unravel a novel link between the energy sensor AMPK and glycogen metabolism (Solaz-Fuster *et al*, 2007).

Study of the mechanisms which drive glycogen accumulation in embryonic liver in the absence of glucokinase

Glucokinase (GK, hexokinase type IV) is required for the accumulation of glycogen in adult liver. During development, rat liver expresses hexokinase type I (HKI) and hexokinase type II (HKII) but not GK. It is when pups are weaned that the first solid carbohydrate-rich ingestion triggers an insulin peak, which stimulates the insulin-dependent promoter of GK in liver. Surprisingly, mammalian embryonic livers accumulate glycogen despite the absence of GK expression. We have addressed how mammalian embryonic livers, but not adult livers, manage to accumulate glycogen in the absence of this enzyme. Although HKI or HKII would not normally have the capacity to build up high enough levels of glucose-6-phosphate to activate the liver isoform of GS (LGS), embryonic livers choose to express massive levels of HKI and HKII. In these conditions HKI and HKII can synthesise sufficient amounts of glucose-6-phosphate to activate LGS and consequently hepatic glycogen synthesis. Our results in fasted pregnant mice provide a teleological explanation for the hexokinase reorganisation in embryonic liver. Glycogen plays a key role during embryonic development as it ensures pup survival in the period of time between birth and first receiving their 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. In this scenario, by greatly increasing the expression of the high affinity hexokinases HKI and HKII, embryos not

only ensure their capacity to use glucose in all circumstances, but at the same time they may produce enough glucose-6-phosphate to ensure hepatic glycogen accumulation even if the mother is fasting and blood glucose levels decrease. By this mechanism, embryos safeguard their liver glycogen stores and thereby provide a crucial advantage at the moment of birth (Cifuentes *et al*, 2008).

Study of the effects of sodium tungstate and its possible application in the treatment of diabetes, obesity and neurodegenerative diseases

Tungstate is an oral glucose-lowering and anti-obesity agent discovered by our group. This compound has a low toxicity profile in animals and humans, and has successfully completed Phase I clinical trials and is currently undergoing Phase II clinical trials. Tungstate normalises carbohydrate metabolism in liver, stimulates insulin secretion, and regenerates pancreatic beta-cells in neonatally streptozotocin-treated diabetic rats. Tungstate is also efficient in ZDF rats, a genetic model of type 2 diabetes. Much effort has been devoted in the study of the physiological and metabolic actions of tungstate but little information is available on its molecular targets. To this end, we have focussed on the study on the actions of this compound at the molecular level. Our previous results show that tungstate treatment stimulates glycogen synthesis, which is correlated with a transient activation of ERK1 and ERK2. We now have consistent data showing that this compound activates glycogen synthesis through a non-canonical mechanism involving G-proteins (Gα and G$\beta\gamma$ subunits) and ERK phosphorylation (Zafra *et al*, in preparation).

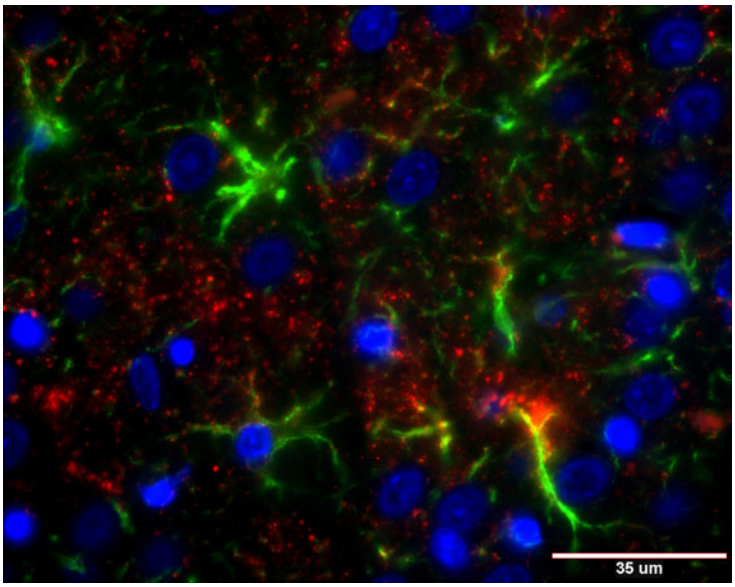


***Figure 1**. Analysis of the distribution of glycogen in rat brain cortex by immunofluorescence. Glycogen (red), astrocytes (green) and nuclei (blue).*

In addition, in collaboration with the group directed by Ramon Gomis (IDIBAPS, Barcelona), we have shown that tungstate also induces phosphorylation and subsequent activation of p38 and PI3K in insulin-secreting cells (Piquer *et al*, 2007). In collaboration with Rafael Salto (University of Granada), we have shown that tungstate increases glucose transport in muscle through a MEF-dependent mechanism (Salto *et al*, in press).

In collaboration with the group directed by Joan Enric Rodríguez Gil from the Veterinary Science Faculty at the Universitat Autònoma de Barcelona, we have shown that tungstate administration improves the sexual and reproductive function in male and female diabetic rats (Ballester *et al*, 2007).

In collaboration with Jesus Avila (CBM-CSIC) and Ramon Gomis, we have performed a study of the effects of tungstate treatment on tau protein, which is involved in Alzheimer's disease. We have demonstrated that tungstate reduces tau phosphorylation in sites that are involved in the aggregation of this protein, which occurs in this disease.

This research line has produced three patents which protect the use of sodium tungstate for the treatment of diabetes, obesity and neurodegenerative diseases. The rights of the first two have been transferred to Bayer.

Publications

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Research Networks and Grants

Ayudas para potenciar y dar soporte a los grupos de investigación
Generalitat Catalunya, 2005-SGR0570: 2005-2009
Project Coordinator: Joan J Guinovart

Efectos del tungstato sobre el síndrome metabólico: Análisis de acciones a nivel plasmático, hepático y muscular. Determinación de dianas terapéuticas
Instituto de Salud Carlos III, ISCIII-PI042402: 2005-2008
Project Coordinator: 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
Ministerio de Educación y Ciencia, SAF 2007-64722: 2007-2008
Project Coordinator: Joan J Guinovart

Investigación de las dianas terapéuticas del agente anti-diabético oral de tungstato de sodio
Ministerio de Educación y Ciencia, SAF2004-06962: 2004-2007
Project Coordinator: Joan J Guinovart

Red de Diabetes y Enfermedades Metabólicas Asociadas
Instituto de Salud Carlos III, Ministerio de Sanidad, RD06/0015/0030: 2007
Project Coordinator: Ramon Gomis de Barbará (network coordinator)

Regulación del metabolismo del glucógeno hepático, muscular y neuronal. Alteraciones en situaciones patológicas
Ministerio de Educación y Ciencia, BFU2005-2253/BMC: 2005-2008
Project Coordinator: 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
Secretaría de Estado de Cooperación Internacional, Ministerio de Asuntos Exteriores, A/6647/06: 2007-2008
Project Coordinator: Joan J Guinovart

Other Funding Sources

Enfermedad de Lafora: papel de laforina y malina
Fundación La Caixa, BM06-340-1: 2007-2009
Project Coordinator: Joan J Guinovart, Santiago Rodríguez de Córdoba

Mejora de la predicción traslacional de los ensayos de seguridad no clínica al hombre
Neuropharma, Consorcio Melius (CENIT project): 2007-2011
Project Coordinator: Joan J Guinovart

Molecular basis of progressive myoclonus epilepsy of the Lafora type
Fundación La Marató de TV3, 061930: 2007-2009
Project Coordinator: Joan J Guinovart, Santiago Rodríguez de Córdoba, Pascual Sanz Bigorra

Nuevos fármacos y dianas para el tratamiento de la diabetes mellitus
Fundación Marcelino Botín: 2006-2010
Project Coordinator: Joan J Guinovart

Collaborations

Analysis of the toxicity and anti-diabetic potential of GSK3 inhibitors
Neuropharma (Madrid, Spain)

Characterisation of glycogen metabolism in reproductive tissue: analysis of alterations in pathological situations
Joan E Rodríguez-Gil, Universitat Autònoma de 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
Joan Carles Ferrer, University of Barcelona (Barcelona, Spain) and Ignasi Fita, IRB Barcelona (Barcelona, Spain)

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

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

Molecular dissection of the mechanisms of action of the anti-diabetic agent sodium tungstate in skeletal muscle
Rafael Salto, Universidad de Granada (Granada, Spain)

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

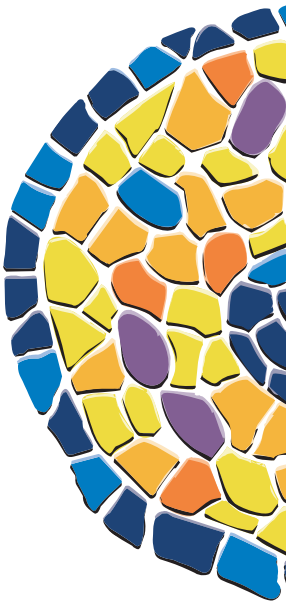
Study of the anti-diabetic actions of tungstate on diabetes induced by immunosupressant treatment
Armando Torres, Hospital Universitario de Canarias (Canary Islands, Spain)

Patents

Método de identificación de compuestos para terapia de enfermedades relacionadas con la acumulación de glucógeno y uso de compuestos para preparar medicamentos contra dichas enfermedades
Patent application number: P200702755
IRB Barcelona (2007)

Awards

Diplôme d'Honneur, Federation of European Biochemical Societies (2007)
Awardee: Joan J Guinovart



Molecular pathology and therapy in heterogenic and multigenic diseases

Manuel Palacín

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 goal is to develop animal models to study the impact of several renal amino acid transporters on cystinuria; to identify mechanisms of pathology in this inherited disorder; to search for new drugs for the treatment of lithiasis in cystinuria; and to generate and characterise a mouse model for LPI. Finally, our group works towards developing the 3D structure of HATs, using both human transporters and prokaryotic homologues.

The molecular bases of renal reabsorption of amino acids

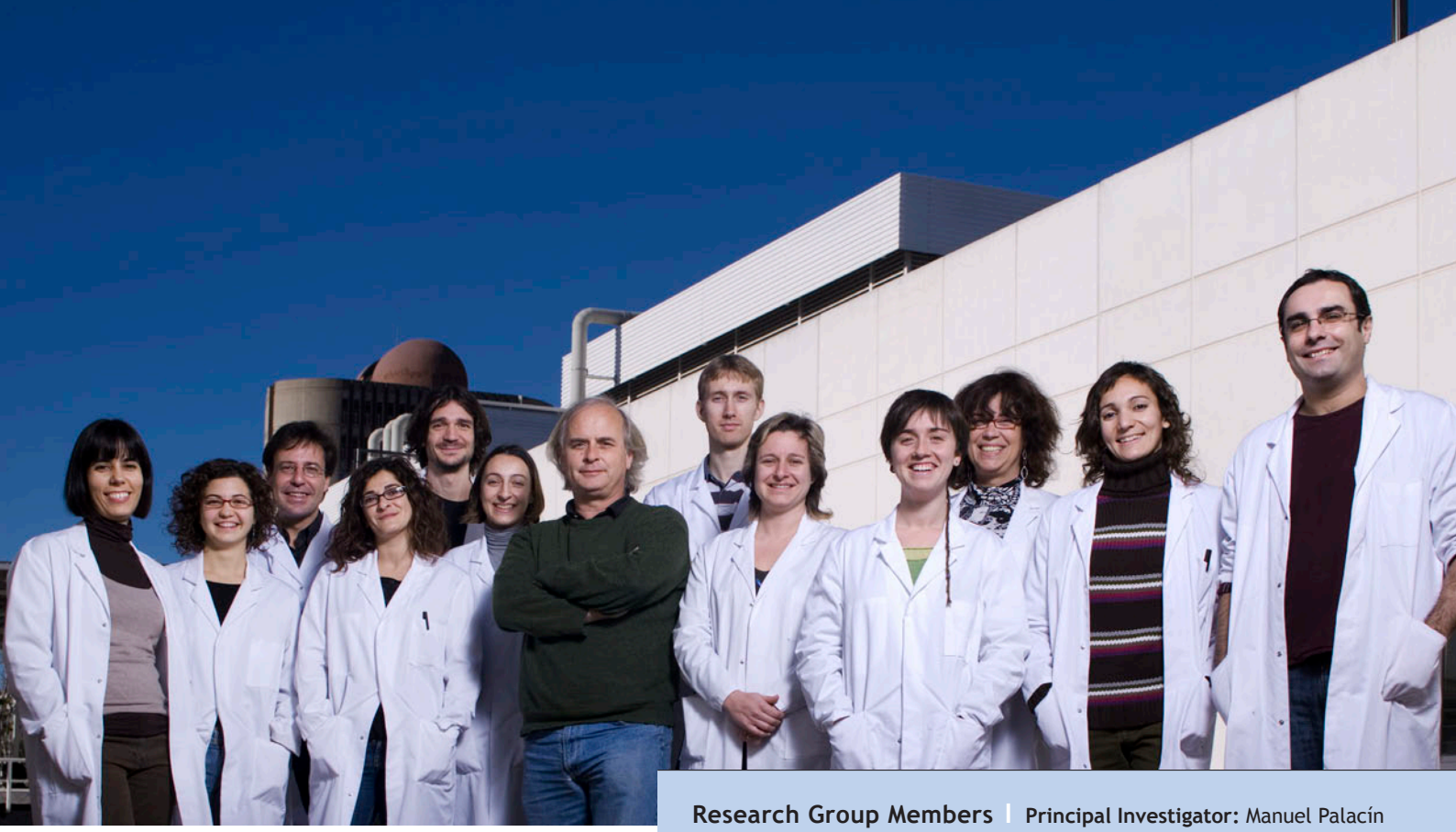
Our laboratory has identified three amino acid transporters involved in the renal reabsorption of amino acids: systems $b^{0,+}$ (heterodimer rBAT-b0,+AT), y+L (heterodimer 4F2hc-y+LAT1) and exchanger L (heterodimer 4F2hc-LAT2). We also demonstrated the role of systems b0,+ and y+L in cystinuria and 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. This activity was initiated within the European Union project EUGINDAT.

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 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: a) the identification of molecular mechanisms to explain the distinct phenotypes in cystinuria, using animal and cell models; b) the identification of modulator genes of lithiasis in cystinuria, using animal models; c) the search for new drugs to treat lithaisis in cystinuria, using our murine cystinuria model *Stones*; and d) the identification of the mechanisms that lead to alveolar proteinosis in LPI, using a newly generated LPI mouse model.

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

Our laboratory has identified most of the members of the heteromeric amino acid transporters (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), the light subunit as the catalytic component, the membrane topology of the light subunits and the key residues for transport. At present we are developing the 3D structure of prokaryotic homologues of the light subunits with Dimitrios Fotiadis (University of Bern) and within the



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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 allows 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.

Publications

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Research Networks and Grants

Determinantes moleculares del metabolismo y la nutrición Biocomunicación hormonal. Nuevas estrategias terapeúticas
Red de centros, enfermedades metabólicas de la nutrición, ISCIII C03/08: 2003-2005
Research Director: Manuel Palacín

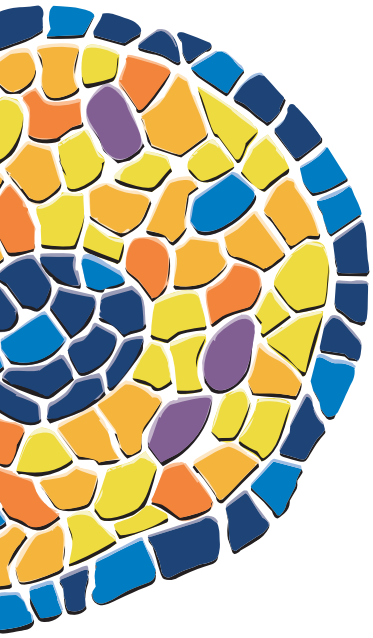
European drug initiative on channels and transporters (EDICT)
European Commission, 7th Framework Programme, FP7-HEALTH-2007-A: 2008-2011
Research Director: Peter Henderson
Group Leader: Manuel Palacín

European genomic initiative on disorders of plasma membrane amino acid transporters (EUGINDAT)
European Commission, LSH2002-211-7-502852: 2004-2007
Research Director: Manuel Palacín

Red de enfermedades metabólicas humanas
Red de grupos, REDEMETH, ISCIII G03/54: 2003-2005
Research Director: Manuel Palacín

Role of 4F2hc in tumorogenesis
La Marató-TV3: 2006-2009
Research Director: Manuel Palacín

Transportadores heteroméricos de aminoácidos: estructura, genómica funcional y fisiopatología (cistinuria y lisinuria con intolerancia a proteínas)
Ministerio de Educación y Ciencia, BFU2006-14600-C02-01: 2006-2009
Principal Investigator: Manuel Palacín



Identification of novel mechanisms involved in the development of insulin resistance and search for new therapeutic strategies for diabetes

Antonio Zorzano

It has been estimated that between 200 million 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 over 40% of people over 60 years of age. Recent epidemiological and biological data indicate that the etiology of these diseases may share unexpected and common genetic and biochemical mechanisms.

A major step towards understanding the metabolic syndrome is the identification of susceptibility genes; a breakthrough of this nature may lead to the acquisition of additional therapeutic targets for future drug design. In this regard, we aim to determine the molecular mechanisms involved in the development of insulin resistance and, in particular, to identify novel susceptibility genes for obesity and type 2 diabetes. Our research interests are the following: the identification of genes responsible for the development of insulin resistance associated with obesity or type 2 diabetes, with special emphasis on genes encoding proteins involved in nuclear gene expression and in signalling; the role of mitochondrial dynamics and function in metabolic regulation and in insulin sensitivity; and the identification of novel targets and development of new compounds for the treatment of the metabolic syndrome.

Identification of a novel protein required for thyroid hormone action

We have recently identified a novel component of thyroid hormone action, named *DOR*, which is abundantly expressed in muscles and brain and is repressed in skeletal muscle from obese and diabetic rats. To identify genes responsible for the alterations associated with diabetes, we screened genes differentially expressed in Zucker diabetic fatty (ZDF) rats and non-diabetic lean rats by PCR-select cDNA subtraction. After obtaining the subtracted cDNA library, we isolated several clones using differential screening by PCR-selection. One of these clones contained an insert of 260 base-pair (bp) and allowed the detection of a 4.5 kb mRNA species (we named it DOR for Diabetes and Obesity-Regulated gene) in various tissues.

Human *DOR* encodes a protein of 220 residues. Human, rat and mouse DOR polypeptides are well conserved; human and mouse show 84% identity, human and rat 83%, and rat and mouse 85%. The only homologous protein is TEAP/SIP/p53DINP1, reported to regulate p53-dependent apoptosis (Okamura *et al*, 2001). The overall identity of human DOR and TEAP/SIP/p53DINP1 proteins is moderate (36%). DOR contains a strong positive charge in its C-terminal region, which is predicted to form an alpha-helix structure while the rest of the protein is predicted to be largely unstructured.

We examined the distribution of *DOR* mRNA in RNA samples obtained from human and rat tissues by Northern blot. In humans, transcripts were predomi-

Research Group Members | **Principal Investigator:** Antonio Zorzano | **Associate Researcher:** Manuela Sanchez Feutrie | **Postdoctoral Fellows:** Vicent Ribas Serra, David Sebastián Muñoz, Eleonora Sorianello | **PhD Students:** Victor Francis, Maribel Hernandez, Marc Liesa, Caroline Mauvezin, Deborah Naon, José Carlos Paz, Sonia Pereira da Veiga, David Sala, Ana Sancho Medina, Jessica Segales | **Lab Technician:** Juan Carlos Monasterio | **Project Manager:** Olga Bausà

nant in skeletal muscle, heart and brain and lower expression was detected in adipose tissue and other tissues. The expression of *DOR* mRNA was maximal in rat skeletal muscle and heart and substantial in white adipose tissue, kidney, brain and liver. *DOR* expression in skeletal muscle from ZDF rats was reduced by 77% compared with healthy rats, thereby corroborating the original subtraction hybridization assay.

Using differential screening, we thus identified a novel protein which is strongly repressed in obese diabetic rats, and highly expressed in tissues involved in metabolic homeostasis. Next, we analysed DOR cellular function in order to establish whether alterations in DOR expression contributed to the pathophysiology of diabetes.

DOR is a nuclear protein that enhances the activity of thyroid hormone receptors

DOR is localised mainly in nuclei (Figure 1). Subcellular fractionation assays confirmed this observation and DOR was detected in nuclear extracts and was distributed both in soluble and non-soluble nuclear fractions. DOR migrated as a 40-kDa protein in SDS-PAGE. During the course of the immunofluorescence assays, we observed that DOR concentrated in nuclear bodies, so we next performed double-labelling to determine their nature. DOR colocalised with promyelocytic leukemia protein (PML, a marker of PML nuclear bodies); this localisation was specific and no colocalisation was detected between DOR and SC-35 or RNA polymerase II (Figure 1). The localisation of DOR in PML nuclear bodies was not due to its overexpression in HeLa cells since endogenously expressed DOR was also detected in these bodies in murine 1C9 muscle cells derived from the immortomouse.

As theoretically predicted, DOR was localised within the nucleus. On the basis of this observation, and given that DOR is homologous to a nuclear protein involved in transcriptional regulation, we proposed that DOR also regulates transcription. Furthermore, the high DOR expression in tissues characterised by high metabolic requirements led us to speculate on a regulatory role of this protein on thyroid hormone action. To this end, HeLa cells were transfected with DNA encoding thyroid hormone receptors (TRα1) and CAT or luciferase reporter gene fused to thyroid hormone receptor elements, in the presence or absence of DOR. TRα1 transactivated the reporter gene, whereas DOR alone showed a small stimulatory effect on reporter activity. The cotransfection of DOR and TRα1 enhanced the transcriptional activity of the reporter gene in a dose-dependent manner. DOR did not cause any effect on the reporter activity induced by transcription factors p53 or c-Myc. In addition, DOR did not stimulate the activity of the chimeric protein GAL4-VP16, generated by fusion of the GAL4 DNA-binding domain and the VP16 activation domain. In all, these observations indicate that DOR specifi-

cally potentiates the activity of thyroid hormone receptors. The effect of DOR is not a consequence of a generalised stimulation of transcription since basal reporter activity, activity driven by c-Myc or p53, and GAL4-VP16 activity remained unaltered.

To analyse whether DOR acts as an activator when tethered to DNA, full-length DOR or distinct cDNA fragments were fused to a GAL4 DNA-binding domain (Gal4-DBD) and its transcriptional activity was assayed by cotransfection with a Gal4 reporter plasmid in HeLa cells. Gal4-DBD fused to full-length DOR caused a moderate increase (3-fold) in reporter activity and deletion of the C-terminal half of the protein (fragment 1-120) markedly increased this activity (8.5-fold). The fragment encompassing amino acid residues 31-111 showed the maximal stimulatory activity (47-fold). In contrast, the C-terminal half of DOR showed no transcriptional activity. These data suggest that the N-terminal half of DOR shows transcriptional activity, and this activity is increased when the C-terminal half of the protein is deleted.

DOR loss-of-function reduces the action of thyroid hormones in muscle cells

To determine whether DOR is required for thyroid hormone action, we generated lentiviral vectors encoding for siRNA to knock-down (KD) DOR expression in mouse cells. The siRNA lentiviral infection in C2C12 muscle cells markedly reduced DOR protein expression (80% reduction) compared to levels found in cells infected with scrambled RNA (control group). Once the KD system had been validated, control and KD cells were transiently transfected with a reporter gene driven by a TRE, in the presence or absence of TRα1 or T3. In control muscle cells, while thyroid hormone caused a 5-fold stimulation of reporter activity as a result of the activation of endogenous TRα1, the addition of exogenous TR increased the stimulation of transcriptional activity up to 10-fold. DOR loss-of-function markedly reduced the effect of T3, TRα1 and T3.

On the basis of these data, next we tested whether the reduced DOR expression altered the effect of thyroid hormones on endogenous target genes. In control C2C12 muscle cells, stimulation with T3 markedly enhanced the expression of genes such as *myogenin*, *IGF-II*, *actin* α*1*, *caveolin-3*, *creatine kinase* or *UCP2*. Stimulation of α-*actin* and *myogenin* in response to thyroid hormones has been previously reported. Under these same conditions, DOR-KD cells markedly reduced the stimulatory effect of thyroid hormones on the expression of the same subset of genes.

On the basis of these data and the previous observation that DOR enhances the transcriptional activation of TRα1, we propose that DOR is a regulator of TR-mediated cellular responses.

Functional role of DOR in myogenic differentiation

Given that DOR expression is markedly repressed in muscle from ZDF rats and that diabetes is linked to skeletal muscle atrophy, we next studied whether DOR participates in myogenesis. To this end, the expression of several genes and proteins in scrambled or DOR siRNA C2C12 cells was studied during myogenic differentiation (from myoblasts to myotubes). Muscle differentiation in C2C12 cells caused a 3-fold stimulation of DOR expression, which was blocked in DOR KD cells. During C2C12 myoblast differentiation, several muscle-specific genes, such as *myogenin*, *creatine kinase*, *caveolin 3*, *actin* α*1* and *IGF-II*, were markedly induced in control cells (from 10- to 20-fold). Under these conditions, DOR-KD cells showed an altered induction in the expression of these genes. However, the pattern of each particular gene was not identical. Myogenin, a transcription factor which plays a

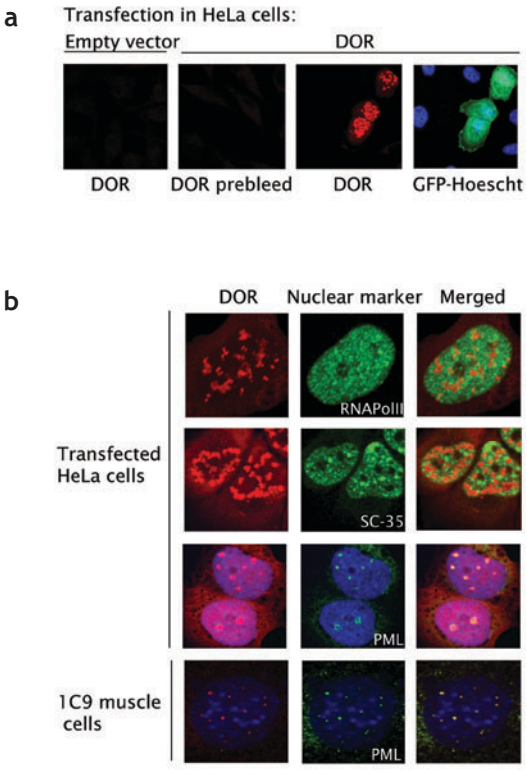


***Figure 1**. DOR protein is localised in nuclear bodies. (a) HeLa cells were transfected with a DOR expression vector or with the empty vector and with GFP. After 48 hours, cells were fixed and stained to view DOR, GFP or the DOR pre-immune serum (negative control). Cells were also counterstained with Hoescht. The arrow indicates a GFP-positive cell, also DOR-positive. DOR is shown in red; GFP in green; nuclei counterstained in blue. (b) DOR-transfected HeLa cells or wild-type mouse 1C9 myoblasts were fixed and stained to view DOR and markers of subnuclear domains, such as the splicing speckles (SC-35), PML bodies (PML) or transcriptionally active sites (RNA Pol. II). DOR is shown in red in the images on the left, and SC-35, PML and RNA Polymerase II are shown in green in the middle. Merging is shown on the right.*

unique function in the transition from a determined myoblast to a fully differentiated myotube, was rapidly induced at early stages of differentiation. While control cells normally induced myogenin mRNA levels (5-fold stimulation at day 1 of differentiation), DOR-KD cells showed a delay in stimulation. However, at day 3 of differentiation no differences were detected between control and KD cells. In the case of the pattern shown by *actin α1*, *creatine kinase* or *IGF-II*, the inhibition of expression was greater at the onset of differentiation (days 1 and 2). Finally, the expression of muscle-specific genes at the protein level was also analysed and this further confirmed that DOR siRNA reduced the abundance of myogenin, glycogen synthase or caveolin-3 compared to control cells. In all, our results indicate that DOR plays a regulatory role in the myogenic programme, and more specifically, during early stages of muscle differentiation.

In this study we have identified a novel protein, named DOR, as a result of a substractive hybridization screening aimed to detect genes down-regulated in skeletal muscle from ZDF rats. DOR is abundantly expressed in tissues with high metabolic rates, such as skeletal muscle and heart. The experimentally induced DOR repression in muscle cells markedly reduces the action of thyroid hormones and alters muscle differentiation. In this regard, it has been reported that type 2 diabetes is characterised by reduced thyroid function. In addition, skeletal muscle atrophy is a well-documented complication of diabetes and is characterised by a reduction in the diameter of myofibers and a decreased number of myonuclei. All these data, together with the marked reduction of DOR expression in skeletal muscle from ZDF rats, allow us to hypothesize that DOR is involved in the pathophysiology of type 2 diabetes.

We have also demonstrated that DOR participates in thyroid hormone action. The evidence we have obtained is as follows: a) DOR overexpression enhances 4-fold the transcriptional activity of thyroid hormone receptor TRα1; b) DOR loss-of-function represses the stimulatory effect of thyroid hormones on the expression of genes such as *actin α1*, *caveolin-3*, *creatine kinase*, *IGF-II*, *UCP2* and *myogenin* in muscle cells; c) DOR binds to TRα1 *in vitro* and *in vivo* in the context of a T3-responsive promoter (human dio1 promoter); and d) DOR undergoes a rapid and transient intranuclear movement from PML nuclear bodies in response to T3. The rapid changes in the nuclear localisation in response to T3 may be relevant in the ligand-dependent DOR-mediated potentiation of TRα1 thyroid hormone receptor activity.

Thyroid hormones stimulate the development and differentiation of muscle. In addition, they stimulate myogenin, myotube formation in muscle cells and also induce the expression of muscle-specific genes such as *α-actin*, or *GLUT4*. In our study, we have demonstrated that, in C2C12 muscle cells, thyroid hormones also potently stimulate the expression of other genes such as *caveolin-3*, *creatine kinase*, *IGF-II* or *UCP2*. The induction of *IGF-II* may be particularly relevant since this growth factor modulates the biology of muscle cells. In addition, and more central to our study, we have found that DOR loss-of-function markedly reduced the myogenic effect of thyroid hormones in muscle cells, as assessed by the expression of *myogenin*, *α-actin*, *caveolin-3*, *creatine kinase*, *IGF-II* and *UCP-2*. Thus, our data implicate DOR in the specific stimulatory effects of thyroid hormones on muscle differentiation. In fact, DOR loss-of-function also had an impact on the capacity of myoblasts to undergo myogenesis. DOR-KD C2C12 muscle cells showed a lower induction of *myogenin* expression, and a reduced expression of *creatine kinase*, *α-actin* and *caveolin-3*. These results indicate that DOR regulates muscle differentiation, at least in part, by controlling *myogenin* expression.

On the basis of the data presented in this study, we propose that DOR repression participates in a deficient response of muscle to thyroid hormones and in the alterations in muscle biology associated with the diabetic condition.

Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells

Neuregulins are essential growth factors for myogenesis and also regulate muscle metabolism. The addition of a recombinant neuregulin-1 isoform, heregulin-β1 177-244 (Hrg), containing the bioactive EGF-like domain, to developing L6E9 myocytes has acute and chronic effects on glucose uptake and enhances myogenesis. We have recently demonstrated that chronic treatments with Hrg causes a marked stimulation of mitochondrial activity, which is parallel to enhanced insulin sensitivity.

Neuregulins increase oxidative metabolism and mitochondrial activity

To test the metabolic effects of neuregulins without affecting myogenesis, a process which occurs above 30-100 pM (recombinant neuregulin-β1) in distinct muscle cells, we treated L6E9 with a low concentration (3 pM) of Hrg for 48 hours. At this concentration, Hrg-treated cells did not display any change in myotube formation, in the protein levels of myogenic markers, such as myosin heavy chain (MHC) and caveolin-3, or in the expression of neuregulin receptors, ErbB2 and ErbB3. Chronic treatment with 3 pM Hrg increased GLUT4 levels, while GLUT1 levels were not significantly different to those of control cells. The increase in total GLUT4 levels was reflected in intracellular membranes, where this transporter is located in basal conditions, while at the cell surface

the levels remained unaffected. Consequently, we observed no changes in basal glucose uptake between controls and cells treated with 3 pM Hrg. However, Hrg treatment increased glucose (85%) and palmitate (89%) oxidation, while lactate release decreased in comparison with control levels.

To test whether the increase in substrate oxidation was due to changes in cellular mitochondrial content in L6E9, we measured the abundance of mitochondrial markers in total cell lysates. Cells treated with Hrg showed increases in total protein levels of porin, an outer-mitochondrial membrane protein, CPT-1, a mitochondrial acyl group transporter and a limiting-step in fatty acid oxidation, and COX-I, a subunit of OXPHOS complex IV that is expressed by mitochondrial DNA. Furthermore, the mitochondrial membrane lipid dye NAO confirmed the increase in mitochondrial content induced by Hrg. Immunofluorescence confocal studies using COX-I antibody showed that Hrg treatment did not alter the mitochondrial network architecture of L6E9 cells.

The increase in COX-I induced by Hrg was proportionally higher than that of porin or CPT-1. To test whether mitochondria were more enriched in OXPHOS complexes in neuregulin-treated than in control cells, we analysed the abundance of distinct OXPHOS complex subunits in mitochondrial fractions from L6E9 cells incubated with Hrg for 48 hours. Hrg increased the abundance of all OXPHOS complexes in mitochondria, while the porin and cytochrome C content per mg of mitochondrial protein was similar. Furthermore, mitochondrial membrane potential, measured with JC-1 staining, was increased in Hrg-treated cells. These data suggest that Hrg increased not only mitochondrial cell content but also the oxidative capacity of these cells.

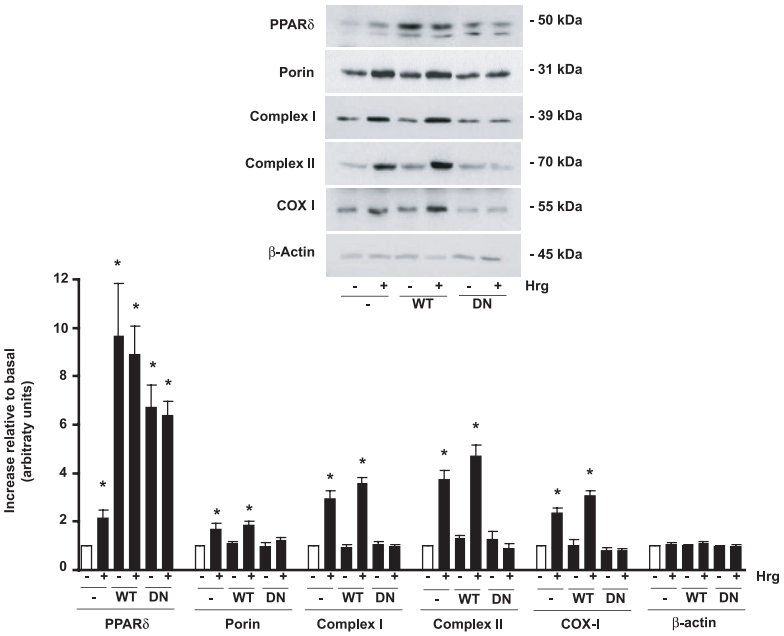


***Figure 2**. PPARδ mediates neuregulin effects on mitochondrial protein levels. C2C12 control cells (-), cells over-expressing wild type PPARδ (WT) or dominant negative PPARδ (DN) were treated with 3 pM Heregulin for 48 hours. Total cell lysates were used for Western blot analysis. * Significant difference vs untreated cell group (absence of Heregulin) at P<0.05.*

Neuregulins stimulate PGC-1□ and PPAR□ expression

To explore how Hrg increased mitochondrial content, we analysed whether it affected PGC-1α expression, since this protein is a master regulator of mitochondrial biogenesis in skeletal muscle. Chronic treatment with Hrg promoted an 82% increase in PGC-1 α protein levels and a 3-fold increase in PGC-1α RNA expression. Next, we determined whether Hrg affected the expression of PPARδ or PPARγ. These two members of the PPAR family have been described to regulate lipid metabolism and oxidative metabolism in skeletal muscle and are co-activated by PGC-1α. As previously reported, no protein expression of PPARγ was detected in control or in Hrg-treated L6E9 cells. In contrast, there was a clear increase in PPARδ protein levels in response to Hrg in L6E9 cells.

PPARδ mediates neuregulin-induced increases in mitochondrial proteins

To test the role of PPARδ in mediating neuregulin effects on mitochondrial genes, we used stable retrovirally infected wild-type or dominant-negative C2C12 cells. Tranfected cells did not show any difference in the levels of late myogenic markers, caveolin 3 and MHC, in comparison to non-transfected cells. As in L6E9 cells, Hrg treatment of control C2C12 cells led to an increase in the protein levels of mitochondrial markers, OXPHOS complexes and PPARδ (Figure 2). While wild-type PPARδ showed a tendency to potentiate Hrg effects on the protein levels of the mitochondrial markers tested, dominant-negative PPARδ completely abolished the effects of Hrg on OXPHOS complexes and porin protein levels (Figure 2). Moreover, Hrg was unable to modulate mitochondrial activity, measured with the JC-1 probe, in the dominant-negative PPARδ cells. These results clearly suggest that PPARδ is involved in neuregulin action on mitochondrial activity. In contrast, Hrg did not affect total PPARδ levels in non-transfected, wild-type or dominant-negative C2C12 cells.

Neuregulins increase insulin sensitivity

Given that the oxidative capacity of skeletal muscle correlates with insulin sensitivity, we tested whether chronic treatment with Hrg also increased insulin sensitivity. We examined glucose uptake at a range of insulin concentrations in cells pre-treated with 3

pM of Hrg for 48 hours or left untreated. Hrg greatly increased insulin sensitivity by almost one order of magnitude. However, although higher total GLUT4 levels were attained in Hrg-treated cells, maximal insulin response was not altered. The increase in insulin sensitivity was also observed in GLUT4 recruitment at the plasma membrane. To test whether the increase in insulin sensitivity was due to augmented activation of insulin signalling effectors, we measured the abundance and response of several insulin mediators of glucose uptake, such as the insulin receptor, IRS-1, p85 subunit of PI3K, PKB and PKCζ, at submaximal insulin concentration (100 nM) in L6E9 cells chronically pre-treated or not with Hrg. Hrg-treated cells increased expression of insulin receptors, IRS-1, p85 regulatory subunit of PI3K, PKB and PKCζ and also enhanced insulin-stimulated activation of these proteins. In conclusion, pre-treatment with Hrg increased insulin sensitivity regulating GLUT4 translocation and glucose uptake, probably because of an increase in the abundance of insulin-signalling mediators in response to submaximal concentrations of insulin. Next, using dominant-negative C2C12 cells, we examined whether the effect of Hrg on insulin sensitivity was dependent on PPARδ. Hrg-treated control cells increased insulin sensitivity on IRS-1 tyrosine-phosphorylation, IRS-1 binding to the p85 subunit of the PI3K and Ser473-PKB phosphorylation, at a submaximal insulin concentration (10 nM). These Hrg effects were abrogated in PPARδ dominant-negative C2C12 cells, thereby indicating that Hrg required PPARδ to induce insulin sensitivity.

Our study demonstrates that the sustained action of low Hrg concentration increases oxidative metabolism, mitochondrial cell content and expression of OXPHOS subunits by a PPARδ-dependent mechanism. Moreover, Hrg markedly enhanced insulin sensitivity in muscle cells. On the basis of this global pattern and on the observation that neuregulins are released from muscle during contraction, we propose that neuregulins are crucial mediators of the adaptive metabolic responses of skeletal muscle to contraction. Hrg effects on the stimulation of mitochondrial activity confirm recent data on isolated cardiomyocytes showing that neuregulins promote a reprogramming that leads to increased expression of many OXPHOS- and β-oxidation-related genes, and that inhibition of ErbB2 causes mitochondrial dysfunction, which involves decreased oxidative capacity.

Oral insulin-mimetic compounds that act independently of insulin

In collaboration with Luc Marti (Genmedica Therapeutics), Fernando Albericio (IRB Barcelona) and Miriam Royo (Barcelona Science Park), we have synthesised and characterised a novel family of compounds which show anti-diabetic properties of interest. These compounds are arylalkylamine vanadium salts that show insulin-mimicking effects in adipose cells, muscle and *in vivo*. These compounds represent a novel therapeutic approach for diabetic patients with severe insulin resistance.

Effects of arylalkylamine vanadium salts on isolated adipocytes and in muscle

We have previously reported that the combination of SSAO/VAP-1 (Semicarbazide Sensitive Amine Oxidase/Vascular Adhesion Protein-1) substrates and low doses of vanadate show anti-diabetic effects in streptozotocin (STZ)-induced diabetic and Goto-Kakizaki diabetic rats. During these studies we noted the possibility of generating vanadium salts containing arylalkylamines that are also substrates of SSAO/VAP-1, which may offer some pharmacological advantages compared to the administration of vanadate and SSAO/VAP-1 substrates separately. On the basis of this observation, we optimised the synthesis of salts composed by a series of arylalkylamines combined with vanadate. Three salts were prepared starting from benzylamine and vanadate: hexaquis(benzylammonium) decavanadate ($(C_7H_{10}N)_6V_{10}O_{28}.2H_2O$; B6V10), pentaquis (benzylammonium) decavanadate ($(C_7H_{10}N)_5HV_{10}O_{28}$; B5V10) and tetraquis(benzylammonium) decavanadate ($(C_7H_{10}N)_4H_2V_{10}O_{28}$; B4V10). B6V10 stimulated glucose transport in rat adipocytes in a concentration-dependent manner with maximal effect at 85% relative to maximal stimulation caused by insulin. The stimulatory effect of B6V10 was completely blocked by semicarbazide, which indicates that SSAO activity is required. In contrast, sodium decavanadate salt (V10) alone at concentrations ranging from 5 to 50 μM did not stimulate glucose transport. Similar stimulatory effects of B6V10 were detected in isolated mouse adipocytes.

In silico modelling studies of SSAO binding allowed us to determine the basic structural requirements of potential substrates. Commercial compounds that met these basic structural requirements as well as novel compounds obtained through combinatorial chemistry were included in a library that was subsequently screened for attractive SSAO substrates. Screening yielded four novel and high affinity SSAO substrates: 2-(4-fluorophenyl)ethylamine, 3-phenylpropylamine, 4-fluorobenzylamine and 4-phenylbutylamine. Some of these compounds, in fact, showed high Vmax values and high affinity for rat SSAO compared to benzylamine. Salts of these compounds and vanadium were prepared and characterised and their insulin-mimicking activity was assayed in isolated rat adipocytes. All four compounds markedly stimulated glucose transport of these cells. These studies indicate that arylalkylamine vanadium salts are a novel class of insulin-mimetic agents that can be generated from several amine compounds and vanadate.

We have also examined the mechanism of action of B6V10 in isolated rat adipocytes. B6V10 initially stim-

ulated insulin signalling downstream of the insulin receptor in isolated rat adipocytes and this was followed by a transient stimulation of insulin receptors. The activation of IRS-1, protein kinase B or GSK-3 induced by B6V10 was blocked by semicarbazide and it was not observed with decavanadate alone.

Next, we analysed the effects of B6V10 in skeletal muscle glucose transport and insulin signalling in preparations of incubated rat soleus muscles. To this end, soleus muscles were incubated with or without insulin (100 nM), decavanadate or B6V10 in the absence or in the presence of membranes from rat adipocytes as a source of SSAO. Our results indicated that B6V10 but not decavanadate stimulated glucose transport by muscle only in the presence of adipocyte membranes. The stimulation was blocked by the inhibitor semicarbazide. Under these conditions, B6V10 caused a limited stimulation of insulin receptor (less than 25% of the effect of insulin), and more substantial stimulation of IRS-1 phosphorylation (40% of the effect of insulin) or Akt phosphorylation (80% of the effect of insulin). These stimulatory effects depended upon the presence of adipocyte membranes, were blocked by semicarbazide and were not shown by decavanadate.

Anti-diabetic effects of B6V10

To determine whether B6V10 shows metabolic effects *in vivo*, we examined whether its acute administration improved glucose tolerance in conscious mice. An intraperitoneal injection of B6V10 15 minutes prior to a glucose load reduced the hyperglycemic response in normoglycemic C57BL/6J mice. Similar glucose tolerance tests were performed 15 minutes after B6V10 injection in mice made glucose intolerant by a very high-fat diet. Again, the acute B6V10 administration enhanced glucose tolerance in these mildly obese and diabetic mice. Prior administration of B6V10 ameliorated the profile of plasma insulin during the hyperglycemic response, which was characterised by reduced basal values and increased insulin after a glucose load.

We have also examined the chronic *in vivo* efficacy of B6V10 in STZ-induced diabetic rats and in db/db mice. Chronic subcutaneous administration of B6V10 for 12 days resulted in significant correction of hyperglycemia in STZ-induced diabetic rats (Figure 3). Daily oral administration of B6V10 for 17 days also resulted in a significant correction of hyperglycemia in diabetic rats. Treatment with identical doses of decavanadate did not alter glycemia in STZ-induced diabetic rats. Intraperitoneal treatment with B6V10 also reduced glycemia in *db/db* mice, thereby indicating that this compound was also effective in animal models of type 2 diabetes.

In all, we have identified a novel family of anti-diabetic compounds. We propose that the development of these compounds is appropriate for not only type 1 and type 2 diabetes but particularly for the treatment of conditions characterised by the lack of insulin receptor activity, such as type A or type B insulin resistance syndromes, or in other types of severe insulin resistance that are refractory to treatment with insulin sensitizers and in which the only effective therapy is the use of large doses of insulin.

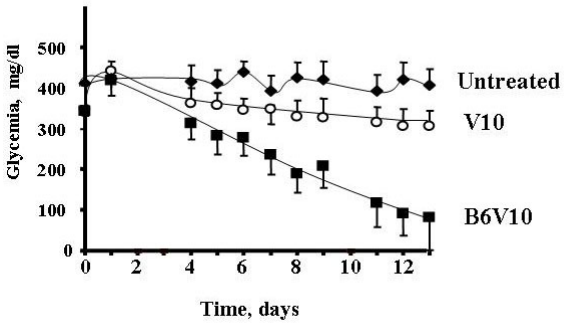


***Figure 3**. Anti-diabetic efficacy of administered hexaquis(benzylammonium) decavanadate in streptozotocin-induced diabetic rats. Streptozotocin-induced (45 mg/kg) diabetic rats were subcutaneously treated with hexaquis(benzylammonium) decavanadate (2.5 μmol/kg) (B6V10, solid squares) or with decavanadate (2.5 μmol/kg) (V10, open circles) delivered subcutaneously by osmotic minipumps. Diabetic rats were also sham-operated (untreated, solid diamonds). Two way ANOVA indicated significant differences between the B6V10 and the untreated or V10 groups, at P<0.01. Post-hoc tests indicated significant differences in the B6V10 group compared to the untreated group after day 8 of treatment, at P<0.01.*

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Research Networks and Grants

CIBER de Diabetes
Instituto de Salud Carlos III, DR06/0015/0035: 2007
Research Director: Antonio Zorzano

Functional analysis of novel candidate genes of insulin resistance
Plan Nacional de Biomedicina, SAF2005-0445: 2005-2008
Research Director: Antonio Zorzano

Mitofusin-2 gene and risk of obesity and of type 2 diabetes mellitus
Instituto de Salud Carlos III, PI041123: 2005-2007
Research Director: Antonio Zorzano

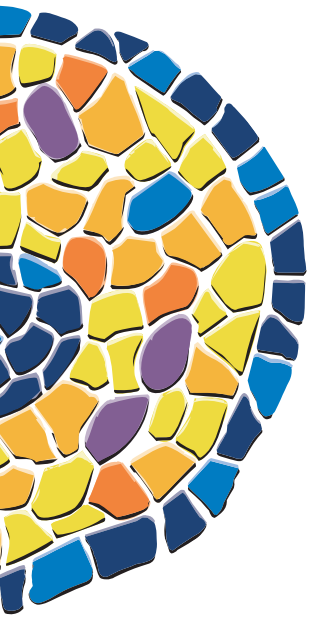
Molecular bases of pathologies associated to membrane transport processes
Generalitat de Catalunya, 2005SGR00947: 2005-2007
Research Director: Antonio Zorzano

REDIMET (RETICS Programme)
Instituto de Salud Carlos III, DR06/0015/0035: 2007
Research Director: Antonio Zorzano

Other Funding Sources

Molecules purification of biotechnological interest
MELLITUS, Collaboration agreement: 2004-2007
Research Director: Antonio Zorzano

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



Chemistry and Molecular Pharmacology Programme

Medicinal chemistry

Fernando Albericio

Our research is devoted to the discovery and synthesis of bioactive compounds principally for use in the treatment of cancer and inflammation. We adopt an integrated approach based on a strong methodological platform and solid-phase and combinatorial science techniques to synthesise peptides and small molecules. Collaborations with biologists and researchers from industrial sectors allow us to hold a unique position in the field.

Synthesis and structural assignation of peptides with bioactivity

Marine peptides

In this sub-project we focus on structurally different compounds: kahalalide F (currently in Phase III clinical trials), thiocoraline (at the preclinical stage), and peptides with concatenated azoles. Our main objectives are to develop synthetic methodologies for these compounds and to synthesise analogues to increase their potency, such as in the kahalalide F project, or to improve absorption, distribution, metabolism and elimination (ADME) properties, such as in the thiocoraline project. Finally, to improve pharmacokinetics, derivatization of the natural products by PEGylation, encapsulation, or anchoring onto nanoparticles is also examined. (See Figures 1 and 2.)

Figure 1. Structure of oxathiocoraline as example of a natural product with biological activity.

Synthesis of polyheterocyclic nitrogen-containing systems

Marine invertebrates, such as sponges, tunicates, ascidians and corals, provide a rich arsenal of new bioactive compounds. These are characterised by unprecedented structures and are highly active against various tumour cell lines. We have synthesised the following marine alkaloids in solution or on solid-phase, and are currently preparing libraries of related compounds and developing the total syntheses of new marine alkaloids (Figure 3).

Solid-phase synthesis of bioactive compound libraries

Peptidomimetic libraries

Azabicyclo-δ,γ-bilactam as a new scaffold for caspase-3 inhibitors: We have used hydantoins as a new scaffold to synthesise a novel peptidomimetic caspase-3 inhibitor by introducing a basic group into the S3 subsite. This introduction will reduce the overall negative charge of caspase-3 inhibitors and improve their cell permeability. Figure 4 shows the interactions of the most potent analogue with caspase-3, as shown by molecular dynamics. New libraries have been prepared.

New resins and linkers

Solid-phase synthesis requires properly functionalised resins and linkers. The resins can be used not only as supporting material for anchoring the scaffold and growing the compound but also as protecting group of a functionality of the reactive starting material. Our research in this field includes the modification of commercially available resins and the synthesis of new linkers (Figure 5).

Solid-phase protecting groups and linkers

The synthesis of multifunctionalised complex molecules often requires the use of orthogonal protecting groups that can be removed independently under mild conditions. In the particular case of solid-phase syn-

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thesis, the resin itself should also provide orthogonal protection, which is achieved by modification of the resin with a suitable linker. In this line of research we address the development of new orthogonal protecting groups and linkers which are removed under mild conditions. Application of the developed tools to novel synthetic strategies for peptides and heterocyles of biological relevance is also performed (Figure 5).

Focused Ion Beam (FIB)

FIB uses Ga^+ ions to scan an object's surface, similar to the electrons in a scanning electron microscope (SEM). The resulting secondary electrons form the image of the sample. With FIB, the sample surface may be modified and holes may be made in order to obtain cross sections, thus allowing the internal structure to be studied (Figure 6).

Hexafluorine chemistry

We are developing new applications of the bidentate protecting/activating reagent hexafluoroacetone (HFA) in emerging fields of peptide synthesis:

- solid-phase coupling protocols with high atomic economy
- native chemical ligation strategy at C-terminal Ser and Thr residues
- preparation of bioactive compounds (depsipeptides)
- generation of libraries for combinatorial chemistry

(See Figure 7.)

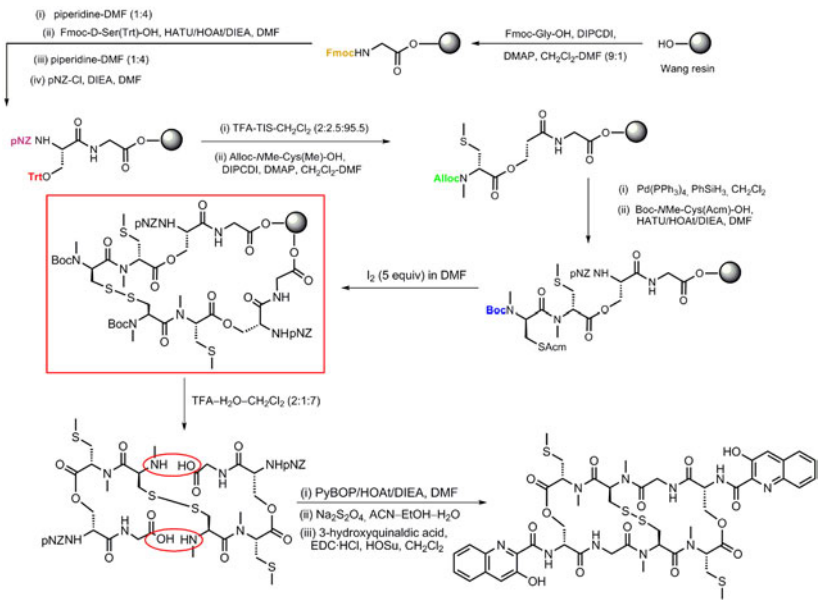

***Figure 2**. Solid-phase synthesis of oxathiocoraline used a restricted conformation.*

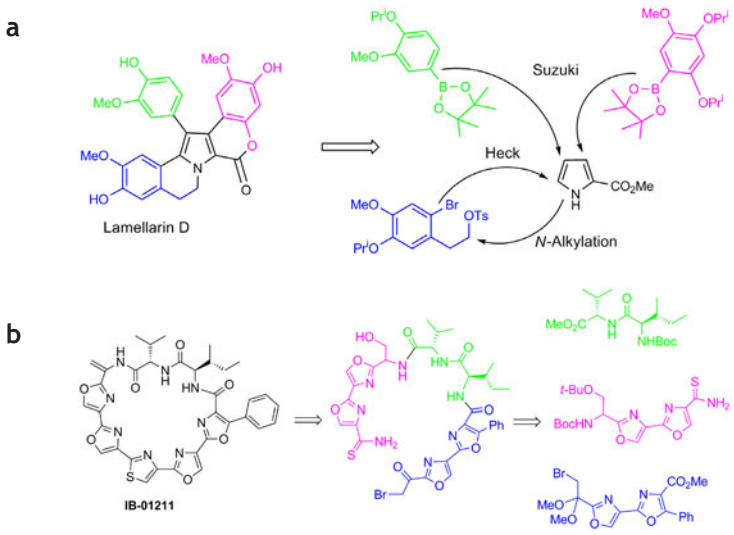

***Figure 3**. Synthetic strategies. (a) Pd(O) cross-coupling reactions. (b) Macrocyclization reactions.*

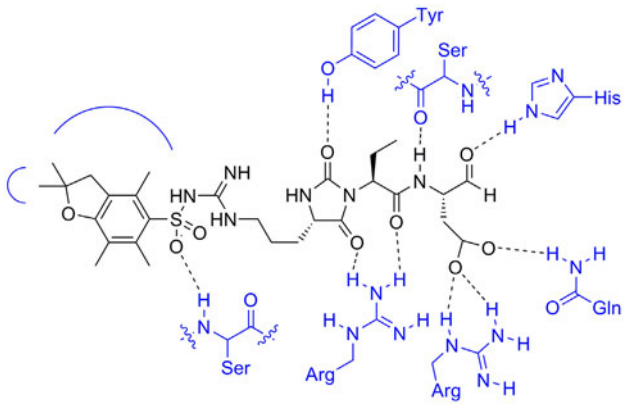

***Figure 4**. Interactions by molecular dynamics.*

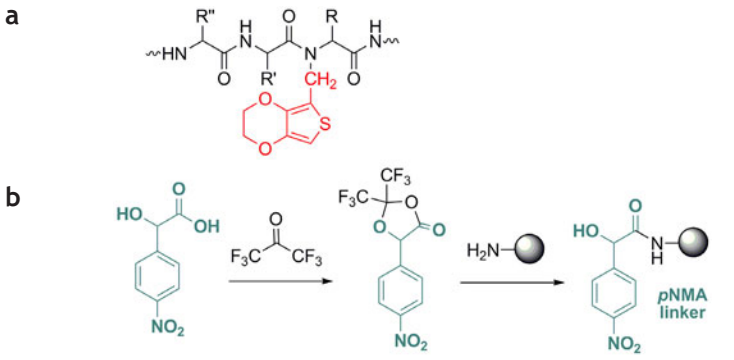

***Figure 5**. (a) EDOTn: an acid labile and less sterically hindered alternative to the widely used Dmb group for amide backbone protection. (b) p-Nitromandelic (pNMA), a novel reduction labile safety-catch linker.*

Synergy between nanobiotechnology and solid-phase synthesis

We are using all our solid-phase strategy expertise for the development of molecules that are increasingly required for nanobiotechnology applications. Thus, we have recently described a highly efficient synthesis of BATs compared with solution-phase syntheses reported to date (Figure 8).

Drug delivery

We have shown that the activity of a bioactive molecule such as the anti-tumour peptide Kahalalide F can be heightened by conjugation to nanoparticles. Furthermore, we have demonstrated that nanoparticles of a certain size can be directed to targets within cells. Additive or more than additive cytotoxic effect was observed when conjugating the peptides and nanoparticles. The nanoparticle acts as a presenter of the anti-tumour agent, concentrating numerous peptide molecules on its surface, which then guide the nanoparticle to the lysosomal compartment (Figure 9).

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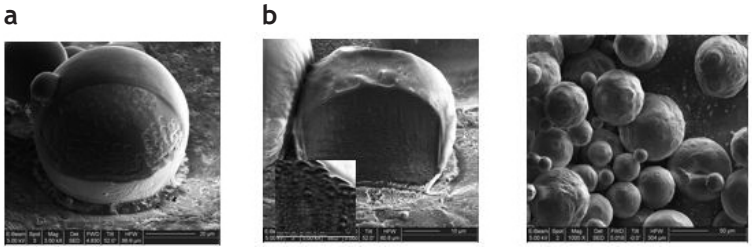

***Figure 6**. (a) Elimination of the external surface of a PEG resin. (b) Cross-section of a PEG resin, created with FIB.*

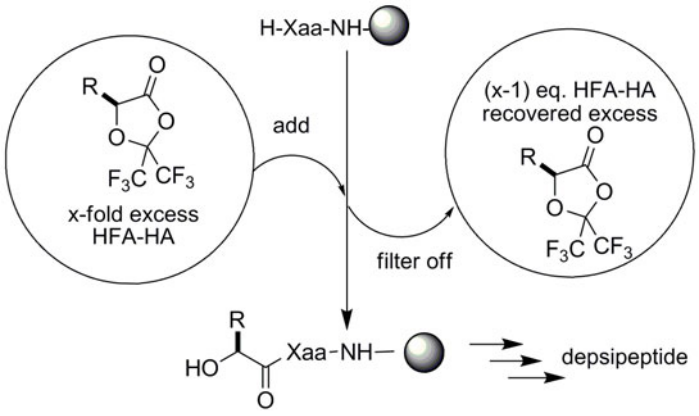

***Figure 7**. Hexafluoroacetone as protecting and activating group of □-hydroxy acids.*

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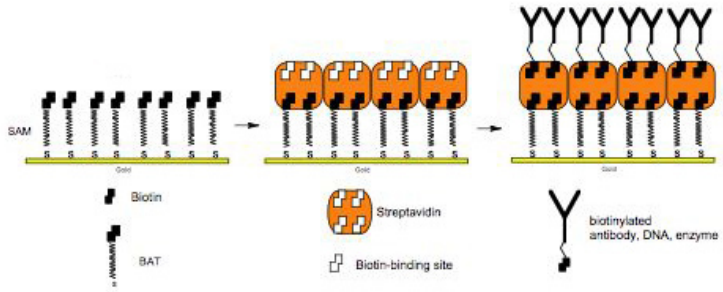

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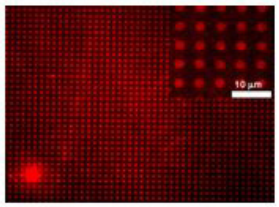
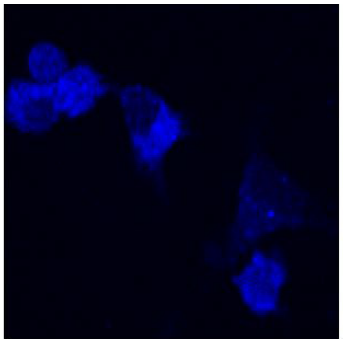


***Figure 8**. (a) Biotinylated alkyl thiols (BAT) with the capacity to graft avidin proteins are in increasing demand for the development of self-assembled monolayers on gold. (b) Fluorescent micropatterned gold surface with BAT showing the interaction between streptavidin labelled with a fluorescent dye and biotin.*

a



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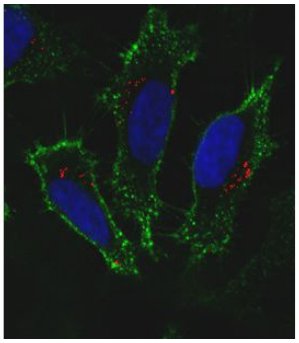

***Figure 9**. Confocal microscopy to detect cell penetration. (a) Localisation of gold nanoparticles in HeLa cells: the red dots represent reflections of the nanoparticles. Membranes (green) were stained with a fluorescence marker (WGA), and nuclei (blue), by a DNA marker (Hoechst). (b) Penetration studies of marine alkaloid bioconjugates in tumoral cells.*

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Research Networks and Grants

Combinatorial chemistry for drug discovery
Ministerio de Educación y Ciencia, CTQ2006-03794/BQU: 2006-2008
Research Director: Fernando Albericio

Nanobiotechnology for diagnosis and therapy of solid tumours
Secretaría de Estado de Cooperación Internacional (AECI), Ayudas para proyectos conjuntos de investigación, colaboración con la Universidad de Santiago, Chile, A/6654/06: 2007
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Networking centre on bioengineering, biomaterials and nanomedicine
Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, CIBER-BBN 0074: 2006-2009
Research Director: Fernando Albericio

Purification of protein with potential pharmaceutical interest by affinity chromatography with peptidic ligands
Secretaría de Estado de Cooperación Internacional (AECI), Ayudas para proyectos conjuntos de investigación, colaboración con la Universidad de Buenos Aires, A/6524/06: 2007
Research Director: Fernando Albericio

Other Funding Sources

New resins for drug delivery
Matrix Innovation (Montreal, Canada)

Synthesis of new therapeutic agents
PharmaMar SA (Madrid, Spain)

Collaborations

New antitumoral agents development
Ramón Mangues, Institut de Recerca, Hospital de Sant Pau (Barcelona, Spain)

Synthesis of new therapeutic agents
Carmen Cuevas, PharmaMar SA (Madrid, Spain)

Synthesis and properties of modified oligonucleotides


Ramon Eritja

Solid-phase synthesis protocols have become highly optimised and consequently synthetic oligonucleotides are ubiquitously found in most laboratories. These protocols allow the synthesis of a large variety of modified oligonucleotides. Our group studies the methodology used for the synthesis of DNA and RNA derivatives, in order to obtain new compounds with new and/or improved properties. The projects undertaken during 2007 address: the conjugation of small molecules (peptides, lipids, carbohydrates) to DNA and RNA for potential use in DNA/RNA therapeutics; the effect of modified bases on the structural and biological properties of oligonucleotides; the use of modified oligonucleotides in the assembly of nanomaterials and biosensors; and the preparation of new DNA-binding drugs. A review of our results in the field of oligonucleotide synthesis has been published this year (Eritja, 2007).

Synthesis of oligonucleotide-peptide conjugates

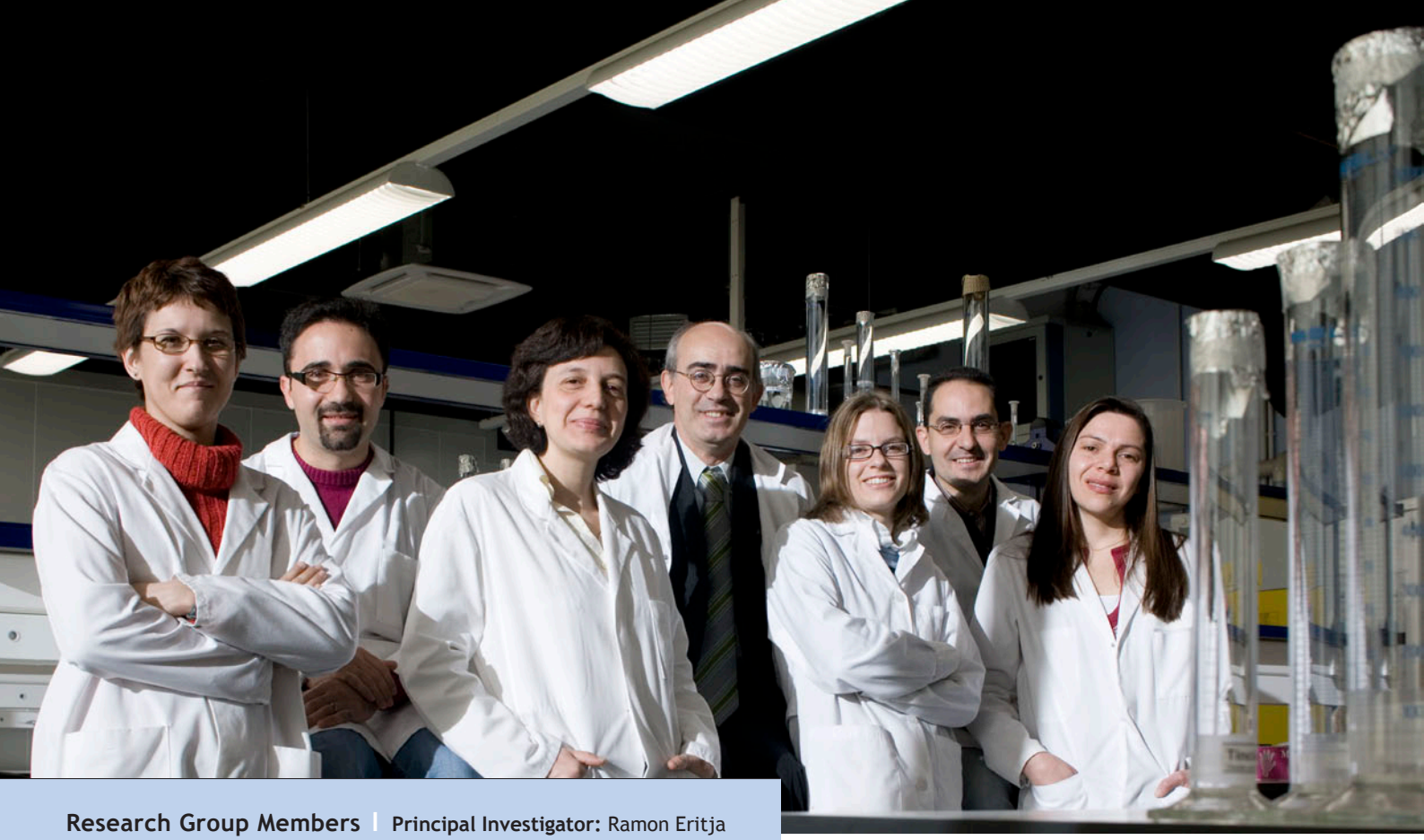
The use of synthetic oligonucleotides to control gene expression has triggered the search for new oligonucleotide derivatives with improved therapeutic potential. Oligonucleotide-peptide conjugates are chimeric molecules consisting of oligonucleotides covalently linked to peptides. As a result, synthetic oligonucleotides acquire some of the biological and/or biophysical properties of peptides.

In collaboration with the groups directed by Fernando Albericio (IRB Barcelona) and José Luis Mascareñas (University of Santiago de Compostela), we have undertaken the preparation of conjugates comprising double-stranded DNA (dsDNA) and selected fragments of biologically relevant transcription factors. Specifically, we have focused on the synthesis of conjugates between DNA and peptide regions from the leucine zipper domain of FOS. These constructs, which display a dual recognition surface provided by the presence of both the peptide and the DNA, were devised as potential specific receptors for selected FOS partners, such as the well known oncogenic transcription factor JUN. Hypothetically, recognition involves a specific interaction between the leucine-rich region of JUN and the hybrid, and an additional interaction between the oligonucleotide portion of the constructs and the basic region of JUN. The synthesis of these conjugates was complex due to the presence of long peptide sequences with a large number of trifunctional amino acids. In spite of these difficulties, the desired oligonucleotide-peptide conjugates were prepared in good yields and showed JUN-binding properties of interest (Portela *et al*, 2007).

Synthesis of oligonucleotide conjugates carrying lipids, steroids and carbohydates

Modified oligonucleotides are used to inhibit gene expression. 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, linked to oligonucleotides enhance the antisense activity of these compounds as well as the silencing properties of small interfering RNA (siRNA).

During the year we have prepared several oligonucleotide (DNA and RNA) conjugates carrying lipids, steroids and carbohydrates. The properties of these new compounds are now being studied. We are collaborating with several groups in this field. The group led by José Carlos Perales (University of Barcelona) is working on the evaluation of the inhibitory properties of conjugates *in vivo*. Juan Carlos Morales' group (CSIC, Seville) is preparing carbohydrate derivatives required for solid-phase synthesis. There is a collaborative project with Sylentis-PharmaMar. Oligonucleotide-steroid conjugates are also being used for the development of bioanalytical devices for anti-doping and food control of the illegal use of steroidal anabolic hormones, in collaboration with the groups direct-



Research Group Members | **Principal Investigator:** Ramon Eritja | **Associate Researcher:** Anna Avinyó | **Postdoctoral Fellows:** Clara Caminal, Alejandra Garibotti, Santiago Grijalvo, Sónia Pérez | **PhD Students:** Margarita Alvira, Francesc X Blasco, Sandra Ocampo | **Lab Manager:** Roger Ramos | **Visitors:** Stefania Mazzini (Italy)

ed by Pilar Marco (CSIC, Barcelona), Josep Samitier (IBEC, Barcelona), Iraida Loinaz (CIDETEC, San Sebastian) and Maria Teresa Martínez (CSIC, Zaragoza).

Synthesis of oligonucleotide clamps for triplex formation

Oligonucleotides can interact in a sequence-specific manner with homopurine-homopyrimidine sequences of duplex and single-stranded DNA and RNA to form triplexes. Nucleic acid triplexes have potential applications in diagnostics, gene analysis, and therapy. Depending on the composition and orientation of the third strand vis-à-vis the central homopurine Watson-Crick strand, triplexes are classified into two main categories: (i) parallel and (ii) antiparallel. The most-well characterised parallel triplex is the one formed between a double-stranded homopurine-homopyrimidine helix (duplex DNA) and a single-stranded homopyrimidine track (triplex-forming oligonucleotide). In this type of triple helix, the triplex-forming oligonucleotide binds to the major groove (parallel to the homopurine strand of Watson-Crick double-helical DNA) via Hoogsteen hydrogen bonding, and is stabilized under acidic conditions. In the antiparallel triplexes, the third strand comprising purine bases binds in a pH-independent and antiparallel fashion to the homopurine duplex strand via reverse-Hoogsteen hydrogen bonds.

Our group, in collaboration with Modesto Orozco (IRB Barcelona) found that the introduction of an amino group at position 8 of the Watson-Crick purine produces a high stabilization of parallel triplexes. The triplex-stabilization properties of the amino group at this position results from a combined effect of the gain of one Hoogsteen purine-pyrimidine H-bond and the propensity of the amino group to be integrated into the 'spine of hydration' located in the minor-major groove of the triplex.

Sequence-specific triple-helix structures can also be formed by DNA clamps. Parallel-stranded DNA clamps consist of purine residues linked to a homopyrimidine chain of inverted polarity by 3'-3' or 5'-5' internucleotide junctions, which interact with single-stranded homopyrimidine nucleic acid targets. In this triplex, the homopurine strand of the clamp binds the homopyrimidine target through Watson-Crick bonds; and the homopyrimidine strand of the clamp forms the triplex via Hoogsteen bonding. Our group has found that the stability of triple helices is enhanced by replacing natural bases with some modified bases, such as 8-aminopurine residues (Aviñó *et al*, 2007).

The classical method for the preparation of parallel-stranded clamps requires the use of reversed phosphoramidites, which are less efficient and more expensive than standard phosphoramidites. Recently, our group has reported that copper-catalysed 1,3-dipolar cycloaddition reactions between oligonucleotides carrying azido and alkyne groups can be used for the

non-templated chemical ligation of two oligonucleotides (Alvira *et al*, 2007). The cycloaddition reaction can also be performed when one of the oligonucleotides is still linked on the solid support, thereby facilitating the removal of the excess of the reagents. We have also found that solid-phase ligation of oligonucleotides using Cu^{+}-catalysed cycloadition reactions is clearly enhanced using ChemMatrix supports because of the good swelling of the support in polar (acetonitrile/water) solvents. In addition ChemMatrix supports allow both the synthesis of oligonucleotide sequences and the analysis of the binding properties of these sequences in spite of the range (anhydrous/salty aqueous) of solvent conditions used in each experiment. We believe that these properties will lead to the development of a broad number of applications in the field of drug- and protein-nucleic acid interactions (Mazzini *et al*, 2008).

Oligonucleotides and nanotechnology

There is a 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 hybridization as well as to detect a particular nucleic acid sequence of interest. Our group collaborates actively with several teams working in this field. This line of research has been summarized in a review (Eritja, 2007) and a progress report (Eritja *et al*, 2007).

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 the active sites of proteins and directly block interactions with natural substrates. In addition to this direct mode of enzymatic regulation, it is possible to use drugs that interact with nucleic acids. These compounds have become relevant antibiotic, antiviral and anticancer agents. In collaboration with the group led by Fernando Albericio (IRB Barcelona) and Crystax Pharmaceuticals, we are designing and synthesising new drugs that interact with DNA. In this project, we are applying the knowledge acquired from peptide and oligonucleotide synthesis to obtain new and larger molecules in order to increase affinity for a particular DNA site.

In collaboration with the group directed by Raimundo Gargallo (University Barcelona), we have studied the interaction of actinomycin D (ACTD) with its target DNA sequences: 5'-CAAAGCTTTG-3', 5'-CATGGCCATG-3' and 5'-TATGGCCATA-3'. We have observed the formation of an interaction complex with a stoichiometry 1:1 (ACTD:duplex) and log of formation constants 5.1 ± 0.3, 6.4 ± 0.2, and 5.6 ± 0.2, respectively. An additional interaction complex at higher temperatures has been detected in the case of AGCT and GGCC_G sequences, which has been related to the formation of a hairpin structure stabilized by the terminal G-3' base (Vives *et al*, 2007).

Synthesis of oligonucleotides carrying DNA methyltransferase inhibitors

Aberrant DNA methylation is common 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 highly stable, and it can be administered orally. However, the disadvantage of this drug is that it is a less potent inhibitor of DNA methylation compared to azacytosine derivatives. It is believed that zebularine is not metabolised as efficiently to

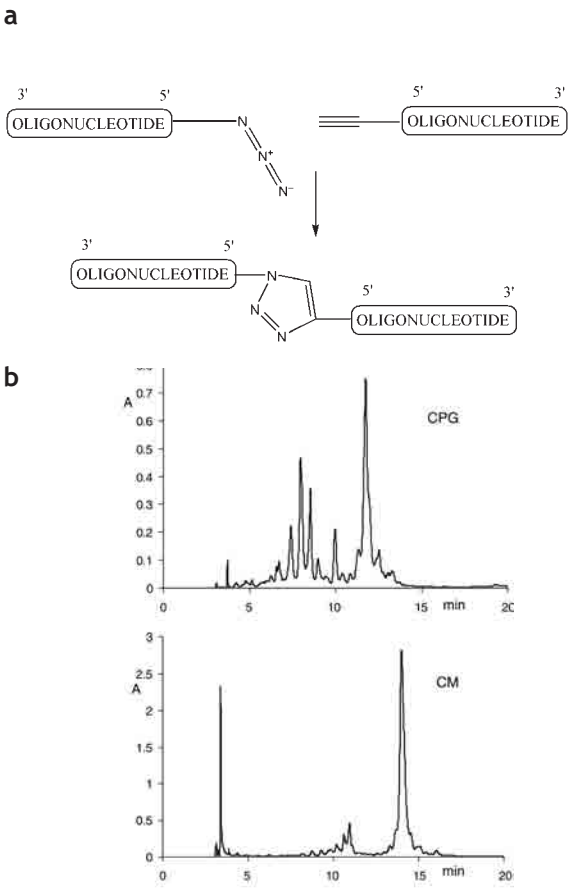


Figure 1. *(a) Copper-catalysed [3+2] cycloaddition or 'Click Chemistry' between oligonucleotides carrying azido and alkynyl groups. (b) HPLC profile of Cu-catalysed cycloaddition between T8-5'propargyl and 5'azido-oligonucleotides attached to controlled pore glass (CPG, above) and ChemMatrix (CM, below) supports to yield the corresponding 5'-5' linked oligonucleotides (Mazzini et al, 2008).*

its triphosphate form as 5-azacytidine and 5-aza-2'-deoxyctyidine, and therefore not efficiently incorporated into DNA. In collaboration with Victor Márquez and Allen Yang, we have prepared oligonucleotides carrying 2'-deoxyzebularine in order to measure the efficiency of incorporation of the drug to DNA. When zebularine was included in the template strand, dGTP was preferentially incorporated by the Klenow fragment opposite zebularine, but dATP and dTTP were incorporated with 8.1% and 5.2% the efficiency of dGTP. In addition zebularine in the template strand inhibited 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).

G-quadruplex and i-motif

G-rich oligonucleotides may form intra- or inter-molecular structures involving the formation of G tetrads. These structures are naturally present at the end of chromosomes or telomeres. Recently, G-quadruplex structures have been reported in promoter regions of oncogenes and several authors have suggested that G-quadruplex structures regulate the expression of these proteins. Given the potential biological relevance of the G-quadruplex, we have initiated the study of its structure and its complementary C-rich strand, which may also form a quadruplex structure known as the i-motif.

In collaboration with Raimundo Gargallo, we have studied the G-quadruplex/i-motif present in the B-cell lymphoma-2 (bcl-2) promoter. Bcl-2 is a potent oncoprotein that plays an essential role in cell survival and functions as an inhibitor of cell apoptosis. The human bcl-2 gene contains a guanine-cytosine-rich region upstream of the P1 promoter that is implicated in the regulation of gene expression. We have examined the structural transitions of the guanine-rich and cytosine-rich sequence by means of acid-base, mole-ratio and melting experiments monitored by molecular absorption, circular dichroism, and NMR spectroscopies. One intramolecular i-motif structure has been detected in the pH range 3-7, with maximal formation at pH 6. At pH 7.1 most species have been associated with a hairpin involving Watson-Crick base pairs. Upon addition of the quadruplex-interacting ligand TmPyP4, both bcl-2 structures yield two identical interaction species with stoichiometries 1:1 and 1:2 (DNA : ligand) (Khan *et al*, 2007).

Delivery of oligonucleotide derivatives

One of the problems encountered when oligonucleotides are used to inhibit gene expression is cellular uptake. The group led by Rafael Gómez (University of Alcalá de Henares) has developed carbosilane dendrimers with positive charges that can be used to improve oligonucleotide delivery. Thus, we have initiated a collaboration project with the groups led by Rafael Gómez and Angeles Muñoz (Hospital General Universitario Gregorio Marañón) and Maria Bryszewska (University of Lódz). In this research, we have produced several oligonucleotides to be used to study complexation and cellular uptake. This study has shown a higher affinity of oligonucleotides to dendrimers (Bermejo *et al*, 2007). Moreover, oligonucleotides bound to dendrimers do not interact with serum albumin, thereby increasing the inhibitory properties of oligonucleotides (Shcharbin *et al*, 2007; Chonco *et al*, 2007).

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Research Networks and Grants

Design and functionality of non-linear electrochemical nanoscale devices (Dynamo)
European Commission, STREP, NEST-2004-ADV-028669-1: 2006-2008
Research Director: Kyösti Kuntturi

Development of nanobio-analytical platforms based on biomolecular recognition using optical and/or electrical detection (Nanobiomol)
Ministerio de Educación y Ciencia, Strategic action on nanotechnology, NAN2004-09415-C05-03: 2005-2008
Research Director: Josep Samitier

Development of new nanosensors functionalised with DNA
Agencia Española de Cooperación Internacional, Ministerio de Educación y Ciencia, Tunez-Spain collaborative project, A/2673/05: 2006-2007
Research Directors: Zouhair M Baccar, Abdelhamid Errachid

Group of synthesis and structure of biomolecules
Generalitat de Catalunya, Consolidated groups, 2005SGR00693: 2006-2008
Project Coordinator: Enrique Pedroso

Inhibitory carbohydrate-ARN conjugates as new antiviral agents by several mechanisms: RNA interference (siARN), antisense, aptamers and ribozymes
Consejo Superior de Investigaciones Científicas, Frontier projects, PIF06-045: 2007-2008
Project Coordinator: Juan Carlos Morales

Modified oligonucleotides for the study of triplex formation and for obtaining other structures with potential technological and structural interest
Ministerio de Educación y Ciencia, BFU-2004-02048/BMC: 2004-2007
Research Director: Ramon Eritja

Nucleic acids chemistry group
Instituto de Salud Carlos III, CIBER Network on Bioengineering, Biomaterials and Nanomedicine, CB06/01/0019: 2006-2009
Research Director: Ramon Eritja

Precision chemical nanoengineering: integrating top-down and bottom-up methodologies for the fabrication of 3-D adaptive nanostructures architectures (Nano-3D)
European Commission, STREP, NMP4-CT2005-014006: 2004-2008
Research Director: Jon Preece

Rational synthesis of molecules with affinity to double-stranded DNA constituted by several units with DNA-intercalating properties
Office for the Transfer of Research (OTRI), PTR1995-0976-OP: 2006-2008
Research Director: Fernando Albericio

Synthesis and properties of modified oligonucletides of biomedical and structural interest (OMIBE)
Ministerio de Educación y Ciencia, BFU2007-63287: 2007-2010
Research Director: Ramon Eritja

In addition the group is active in the following publicly funded networks:

European Platform on Nanomedicine, a network of European scientists working in nanomedicine

NanoSpain, a network of Spanish scientists working in nanosciences

Oncostem. Collaboration agreement, University of Geneva

RIBORED, a network of Spanish scientists working in the field of RNA field

Plataforma Española de Nanomedicina, a network of Spanish scientists working in nanomedicine

RANN, a network of Spanish scientists working in nucleosides, nucleotides and nucleic acids

Other Funding Sources

RNA-lipid conjugates
Research contract with Sylentis, Pharmamar: 2006-2007
Research Director: Fernando Albericio

Synthesis and properties of modified oligonucleotides with potential anticancer activity
Fundació La Caixa, BM04-52-0: 2004-2007
Research Director: Ramon Eritja

Collaborations

Research on nanosensors
Arben Merkoçi, National Center on Nanotechnology, Bellaterra (Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps
Carlos Cuidad, University of Barcelona (Barcelona, Spain), Anna Nadal, University of Girona (Girona, Spain), 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 binds DNA
Joan Aymamí, Crystax Pharmaceuticals, Barcelona Science Park (Barcelona, Spain), Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Synthesis of new RNA derivatives
Ana Isabel Jiménez, Sylentis-PharmaMar (Madrid, Spain), Fernando Albericio, IRB Barcelona (Barcelona, Spain)

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

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

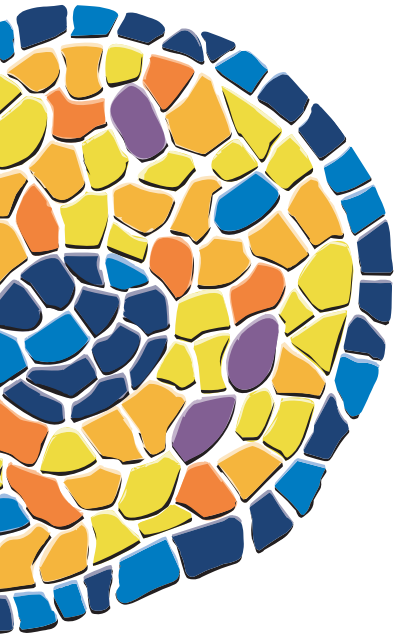
Synthesis of oligonucleotide-esteroid conjugates
Mª Pilar Marco, IIQAB-CSIC (Barcelona, Spain)

Synthesis of oligonucleotide-peptide conjugates
José Luis Mascareñas, University of Santiago de Compostela (Santiago de Compostela, Spain)

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

Research on nanosensors functionalised with oligonucleotides
Josep Samitier, IBEC, Barcelona Science Park (Barcelona, Spain)

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



Design, synthesis and structure of peptides and proteins

Ernest Giralt

Improving our knowledge of the rules that govern molecular recognition of how molecular recognition is regulated is behind all our endeavours in this field. The study of protein-protein interactions in general and protein self-assembly in particular affords many opportunities to improve our understanding of molecular recognition. Of greater importance still, from a more applied perspective, these studies provide numerous possibilities for drug discovery. Many additional unknowns have yet to be addressed. To make good progression in this field, several of these unknowns are also studied by our group. How can we design a peptide to ensure efficient cellular uptake? Is it possible to achieve remote control of the disruption of amyloid fibrils? Can we use peptides to shuttle drugs across the blood-brain barrier? Finally, methodological improvements are constantly required in all scientific activities. This is the focus of our more recent work in using NMR to study protein-ligand and protein-protein interactions, improving solid-phase methods for peptide and protein synthesis and developing computational evolutionary algorithms for structure-based drug design.

Intracellular delivery by cell-penetrating peptides

Attaining satisfactory intracellular delivery is crucial for many drugs. A successful delivery strategy is the use of peptide sequences that have the capacity to translocate through the cytoplasmic membrane, the so-called cell-penetrating peptides (CPPs). The most common feature of CPPs is the presence of positive charges, from arginines or lysines.

A CPP class of particular interest comprises amphipathic proline-rich peptides (Pujals, 2008), derived from the N-terminal domain of □-zein, a maize storage protein. On the basis of the observation that a Pro content of 50% is enough to maintain PPII structure, we transformed a PPII helix into an amphipathic sequence by placing hydrophobic residues at positions i/i+2, i+6/i+8, etc, and hydrophylic amino acids at i+3, i+7, etc. Several peptides with the structure $(VXLPPP)_n$ were synthesised, where X=His, Arg or Lys and n=1-3. The hydrophobic residues Val and Leu were chosen in an analogy with the aforementioned N-terminal domain of □-zein. Evaluation of the internalisation properties of the distinct compounds by plate fluorimetry showed CF-$(VRLPPP)_3$ (where CF=5(6)-carboxyfluorescein) to be the peptide most efficient at crossing the cellular membrane.

This new family of intracellular vectors holds several advantages: non-viral origin, high solubility in aqueous media, and absence of cytotoxicity at very high concentrations. The best candidate, (VRLPPP)3, was named SAP (Sweet Arrow Peptide) for the aforementioned reasons.

Efforts have been made to improve the cell penetration capacity of SAP by increasing its hydrophobicity and amphipathicity, and developing fatty acyl derivatives (Fernández-Carneado *et al*, 2005) and a silaproline derivative (Pujals *et al*, 2006).

The mechanism by which SAP is internalised in the cell has been examined (Pujals *et al*, 2008) through colocalisation studies and experiments with ATP depletion. This study concluded that the internalisation mechanism is lipid raft- or caveolae-mediated endocytosis. The most relevant property of lipid rafts and caveolae is that during transport, unlike in internalisation via clathrin-mediated endocytosis, the pH remains neutral and no degradation of cargo occurs, ie, late endosomes do not fuse with lysosomes. This is crucial for carrying cargo susceptible to proteases or nucleases inside the cell. Furthermore, a totally human-serum-stable version of SAP built with non-natural D-amino acids (D-SAP) has been developed (Pujals *et al*, 2008). The enantiomer retained the non-cytotoxic and translocation properties of SAP and was stable to high concentrations of trypsin and in 90% human serum.

The toxicity of D-SAP was also evaluated in Balb/c mice over one week (Pujals *et al*, 2007). The com-

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pound was administered on alternate days, and the most relevant blood biochemical parameters, as well as weight, were measured. No significant differences were found between treated and untreated animals. We then performed *in vivo* internalisation studies using the carboxyfluoresceinated version of D-SAP. The uptake of D-SAP in white blood cells was monitored by flow cytometry, which showed that internalisation was time-dependent, with great fluorescence 1 and 4 h post-injection and low fluorescence at 24 h. Confocal laser scanning microscopy showed distribution of fluorescence signal in the kidney, heart, spleen and liver.

Design and synthesis of □-amyloid aggregation inhibitors

One hundred years after the discovery of Alzheimer's disease (AD), current treatments offer only symptomatic benefits to patients. Although this is discouraging, during these one hundred years, basic research has led to the identification of many of the pathways that contribute to this devastating disease, thereby providing an excellent opportunity to develop new treatments that target the root causes (Carulla *et al*, 2006).

AD is associated with aggregation of the β-amyloid (Aβ) peptide, a 39- to 42-residue peptide. Soluble Aβ converts into insoluble amyloid fibrils or plaques that accumulate in the brain of patients suffering from AD (Figure 1). Thus, one strategy to defeat AD involves the design of compounds with the capacity to interfere with and/or prevent Aβ aggregation. Within this context, our laboratory addresses the design of peptide-based Aβ aggregation inhibitors. We have used the self-recognition property of Aβ, that is to say its capacity to bind to itself, and employed a central Aβ fragment, Aβ(16-20) as the recognition element.

The end products of Aβ aggregation are amyloid fibrils, which are stabilised by intermolecular β-sheets with hydrogen bonds expanding in the direction of the fibril growth. Our inhibitor peptides contain an N-methyl amino acid, which allows the inhibitor peptides to present two unique hydrogen-bonding faces when arrayed in an extended, β-strand conformation. One face of the inhibitor peptide has normal hydrogen bonding capacity, which allows interaction of the inhibitor with the Aβ molecules. The other face has an N-methyl group in place of a backbone amide proton, which prevents hydrogen bond formation precluding further Aβ molecules from binding to the inhibitor and thus capping fibril growth.

Peptide drugs offer high activity, high specificity and low toxicity, but are susceptible to proteolytic deg-

radation. A strategy to overcome this problem is to produce peptides using non-natural D-amino acids. Although all-D peptides are not recognised by proteases, they may be less active than their corresponding all-L peptides because of differences in the orientation of amino acid side-chains. To preserve the orientation of the side-chains of the parent L-peptide in a D-analogue, the D-amino acid residues can be linked in the reverse order to that of the parent peptide in order to produce a retro-enantio peptide. This approach emphasises the maintenance of the topological native orientation of the side-chains but not of the backbone.

After considering the pros and cons of an all-D peptide and a retro-enantio peptide versus an all-L peptide, we chose to study the use of the retro-enantio approach in the development of peptide-based Aβ aggregation inhibitors for the treatment of AD. We have worked with three inhibitors, inL (an all-L peptide), inD (an all-D peptide), and inrD (a retro-enantio peptide). inD and inrD were designed to improve proteolytic stability since they are made of D amino acids. The work with inrD allowed us to test the utility of the retro-enantio approach to obtain peptides able to interact with β-sheet proteins. Using biophysical methods, we evaluated the capacity of the three inhibitors to interfere with Aβ aggregation.

Furthermore, we have screened their capacity to inhibit Aβ cytotoxicity in neuroblastoma cell cultures. The inhibitor peptides have been subjected to trypsin and cerebrospinal fluid (CSF) to evaluate their stability to proteases.

Finally, we have modelled the interaction of the inhibitor peptides with an Aβ fibril model to examine their possible mode(s) of action. From these studies, we have learned that inrD, the one designed using the retro-enantio approach, is the peptide with the highest capacity to interfere with Aβ aggregation and decrease Aβ cytotoxicity, while being stable to proteases (Figure 1; Grillo-Bosch *et al*, submitted for publication). Given that during Aβ aggregation β-sheet formation is one of the predominant conformations sampled and inrD interferes with Aβ aggregation, we consider the use of retro-enantio peptides a valuable approach for obtaining peptides able to interact with β-sheet proteins.

This observation may have major implications for the design of bioactive peptides for myriad therapeutic indications, including those targeted at disrupting protein-protein interactions. As a result of the success of this study and with the idea to go one step further in our goal towards developing drugs to treat AD, we have initiated a collaboration with Isidre Ferrer (Institut de Neuropatologia de Bellvitge) to study the effect of inrD in transgenic AD mouse models.

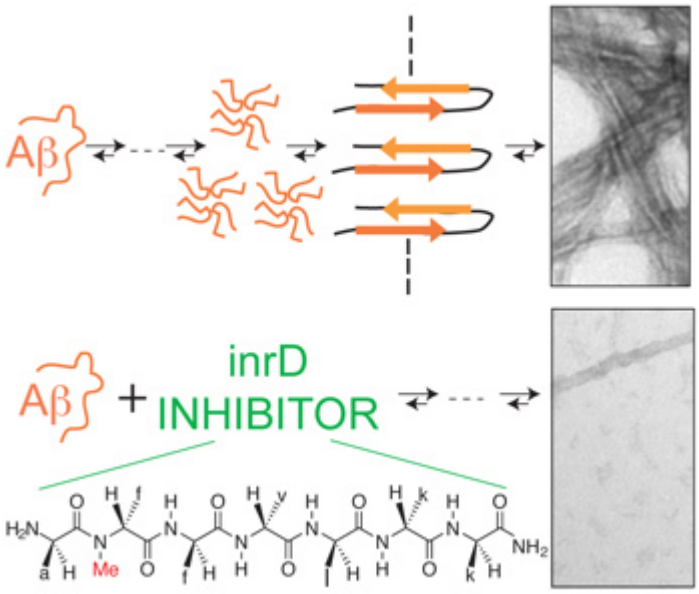

***Figure 1**. Schematic representation of the effect of inrD on Aβ aggregation. (Top) Schematics of a possible mechanism for Aβ aggregation and amyloid fibril formation. Monomeric Aβ is converted into amyloid fibrils through a series of steps. At the end of Aβ aggregation, electron micrographs show the presence of abundant Aβ amyloid fibrils. (Bottom) Schematics of Aβ aggregation in the presence of inrD. The electron micrograph reveals the presence of very few protofilaments as well as spherical oligomers in the βA samples incubated with inrD. The amino acid sequence of inrD is depicted in the figure, all the amino acids are in the D configuration and the N-Me group is shown in red.*

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Research Networks and Grants

Ajuts per potenciar i donar suport als grups de recerca
Generalitat de Catalunya, 2005SGR00663: 2005-2008
Research Director: Ernest Giralt

Design, synthesis and structural studies of new VIH protease dimerisation inhibitors
FIPSE – Fundación para la investigación y la prevención del SIDA en España, 36606/06: 2006-2009
Research Director: Ernest Giralt

Design of peptide ligands for protein-surface recognition
Ministerio de Educación y Ciencia, BIO2005-00295: 2005-2008
Research Director: Ernest Giralt

Design of peptidic 'mirror-molecules' as novel ligands using evolutive algorithms
Acciones Complementarias – Programa Explora Ingenio, Ministerio de Educación y Ciencia, BIO2006-26119-E: 2007
Research Director: Ernest Giralt

NANOFAR-Use of peptides for intracellular nanoparticle delivery
Ministerio de Educación y Ciencia, Acción estratégica de nanociencia y nanotecnología, NAN2004-09159-C04-02: 2006-2008
Research Director: Ernest Giralt

Nanotechnologies in biomedicine (Nanobiomed)
Ministerio de Educación y Ciencia, CONSOLIDER-INGENIO 2010: 2006-2008
Research Director: Ricardo Ibarra

Novel nanobiomaterial development: Modification of auto-aggregation and protein conformation to reduce toxicity
Secretaría de Estado de Cooperación Internacional, Ayudas para proyectos conjuntos de investigación, collaboration with the University of Santiago, A/5987/06: 2007
Research Director: Ernest Giralt

Structure and dynamics of □-amyloid oligomeric and fibrilllar species. Hydrogen/deuterium exchange experiments analysed by nuclear magnetic resonance spectroscopy (nmr) and mass spectrometry (ms)
Fundació La Caixa, BM05-60-0: 2006-2008
Research Director: Ernest Giralt

Structural and dynamic characterisation of □□ aggregation. Examination of □□ aggregation peptide inhibitors
Fundació La Marató TV3, 092: 2006-2009
Project Coordinator: Ernest Giralt

Synthesis of proline-rich prolyl oligopeptidase (POP) inhibitors
Ministerio de Educación y Ciencia, Programa Hispano-Brasileño de Cooperación Interuniversitaria, estancias de movilidad, PHB2005-0068-PC: 2006-2007
Research Director: Ernest Giralt

Collaborations

Applications of the Suzuki reaction to the synthesis of conformationally constrained peptides
Paul-Lloyd Williams, 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 -ICIQ (Tarragona, Spain)

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

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

Synthesis and structural studies of □-peptides
Rosa Mª Ortuño, Universitat Autònoma de Barcelona (Barcelona, Spain)

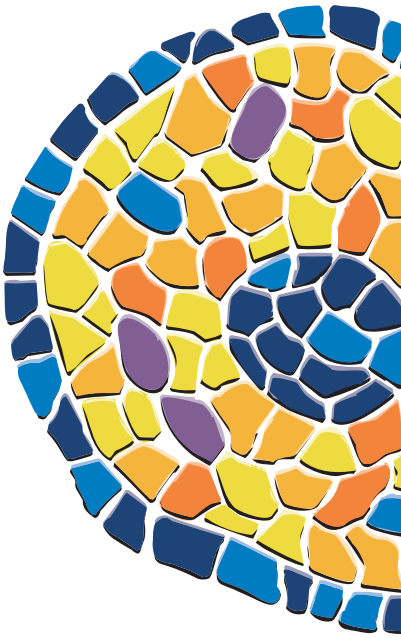
Awards

Peptide Idol Award for young investigators, American Peptide Society, 20th American Peptide Symposium (Canada, 2007).
Awardee: Meritxell Teixidó

Patents

Compounds as blood brain barrier shuttles and shuttle-cargo constructs
Giralt E and Teixidó M
Patent application number: PCT/ES2007/0401
University of Barcelona (2007)

Inhibition of alpha-synuclein aggregation
Zurdo J, Fowler S, Stallwood Y, Giralt E, Teixidó M and Carulla N
Patent application number: PCT/GB2007/002469
Zyentia, Ltd (2007)



Synthesis of biologically active molecules and development of new synthetic methodologies

Antoni Riera

Our group focuses on synthesising biologically active compounds for the various stages of drug development. Several of our projects are devoted to the development of basic synthetic methodology, with special emphasis 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.

Catalytic and asymmetric reactions. 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. We use these cyclopentenones as starting materials for the synthesis of biologically active substances such as phytoprostanes or carbanucleosides. In 2007, we have published two papers on basic aspects of the catalytic PKR. Using a pressure reactor with an FT-IR probe, we have monitored catalytic PKRs for the first time (Figure 1). Using this technique, we have studied the kinetics of the intermolecular PKR between trimethylsilylacetylene and norbornadiene (Cabot *et al*, 2007). We have also reported the first asymmetric intermolecular cobalt-catalysed PKR. Although the selectivities were modest, our hemilabile ligands CamPHOS provided good conversion, thereby showing that we are on the right track to develop an efficient catalytic asymmetric reaction (Lledó *et al*, 2007; Figure 2).

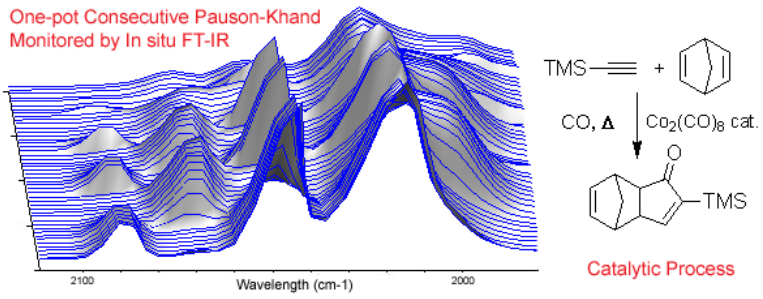


***Figure 1**. Plot of a catalytic Pauson-Khand reaction (PKR) monitored by in situ FT-IR.*

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. We designed a second generation of hemi-labile P,S-ligands with an unprecedented structure showing a backbone constituted by four linked heteroatoms (P-N-S-O; Figure 3). We successfully synthesised these chiral non-racemic N-phosphinosulfinamides and found that they bound to dicobalthexacarbonyl complexes with high selectivity. The resulting complexes gave excellent yields and high enantiomeric excesses (up to 99% ee) in the intermolecular PKR (Solà *et al*, 2007). Applications of the new N-phosphinosulfinamide ligands to other metal-catalysed processes are now being studied in our laboratory.

Synthesis of other chiral ligands

Triphenylethylene oxide is a useful precursor of several chiral ligands in asymmetric syntheses. During our work on an efficient synthesis of this compound from triphenylethylenediol, an unexpected phosphine-dependent stereoselectivity was found for the Mitsunobu cyclodehydration (Garcia-Delgado *et al*, 2007).

Research Group Members | **Principal Investigator:** Antoni Riera | **Associate Researcher:** Xavier Verdaguer Espaulella | **Postdoctoral Fellows:** Thierry Achard, Noemí García Delgado, Agustí Lledó, Jordi Solà | **PhD Students:** Carles Alegret Molina, Yining Ji, Thierry Leon, Maria Moreno, Rosario Ramón, Marc Revés, Ana Vázquez | **Lab Technician:** Ferran Santacana

Synthesis of biologically active compounds

Synthesis of five-membered ring compounds

One of the best ways to prepare five-membered ring compounds is via the PKR. Prostaglandins are among the most important cyclopentanic compounds since they exhibit a wide variety of functions and biological activities. On route to a synthesis of prostaglandins, we addressed the introduction of a hydroxymethyl fragment into a PK adduct and uncovered a novel photochemical rearrangement (Figure 4). We have studied the scope of this new reaction (Lledó *et al*, 2007) and are currently examining the synthetic potential of the rearranged products as well as the mechanism of the reaction.

Enantioselective synthesis of amino acids

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. In 2007, we have described enantioselective syntheses of two types of compounds: mesityl amino acids and methylpipecolic acids. The synthesis of mesityl glycine and mesityl alanine was performed starting from ethyl 3-mesityl-2-propenoate. The source of chirality was Sharpless asymmetric dihydroxylation. By means of this reaction, both enantiomers of the subsequent chiral diol were obtained in excellent yield and complete enantioselectivity (>99% ee). Each diol was converted into the corresponding amino acid through

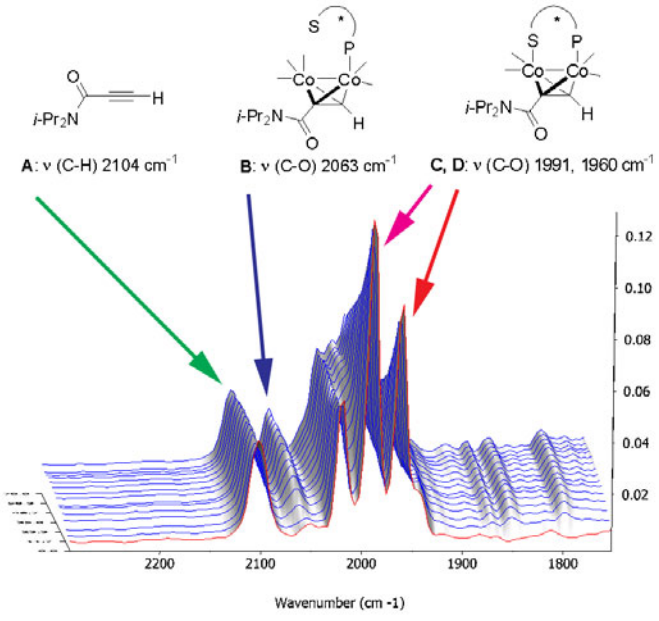


***Figure 2**. Asymmetric Pauson-Khand reaction using a bidentate P,S ligand monitored by FT-IR. The starting material and some intermediates are shown.*

high yielding stereospecific syntheses. Furthermore, methyl pipecolic acids were prepared from 5-methyl-2,5-hexadien-1-ol using Sharpless epoxidation as a source of chirality. The key steps of this synthesis were the ring-closing-metathesis to construct the six-membered ring and a diastereoselective hydrogenation to fix the chiral centre at C-4 (Figure 5).

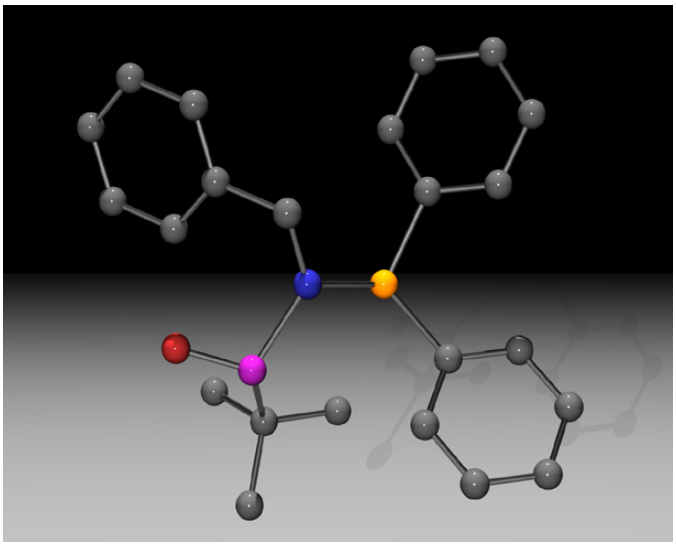


***Figure 3**. Structure of a chiral P,S ligand developed in our group.*

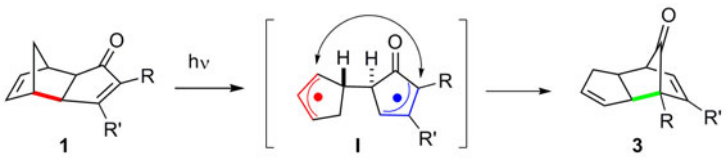


***Figure 4**. New photochemical rearrangement of Pauson-Khand adducts.*

***Figure 5**. Protected unnatural amino acids prepared by asymmetric synthesis in our group.*

Enantioselective synthesis of aza-sugars

Glycosidase inhibitors are among the most important enzymatic inhibitors. These compounds have been studied for the treatment of metabolic disorders such as diabetes, as well as for anti-viral and anti-cancer indications. Many glycosidase inhibitors have similar structures to those of monosaccharides in which the oxygen atom of a functional group is replaced by a nitrogen atom (*eg*, aza-sugars, amino sugars and amino cyclitols). The combination of the regioselective opening of unsaturated epoxides by nitrogenated nucleophiles with RCM reactions has enabled us to prepare aza-sugars from (E)-2,4-pentadien-1-ol. This year we have completed the synthesis of several polyhydroxylated pyrrolidines such as 1,4,-dideoxy-1,4-imino-D-ribitiol from the same starting material (Murruzzu *et al*, 2007).

Furthermore, we have described the preparation of this intermediate labelled with three ^{13}C atoms. Therefore, many ^{13}C-labelled aza-sugars are now available for protein-binding experiments using NMR methods (Murruzzu *et al*, 2007; Figure 6). In collaboration with Jesus Jiménez-Barbero, we have used the binding between (4,5,6-^{13}C)-deoxymannojirimycin and jack bean α-mannosidase as a test model. We have reported information on binding features that can be extracted when labelled compounds are used in HSQC and HMQC-trNOESY experiments.

Synthesis of new chemical libraries for drug discovery

Synthesis of specific inhibitors of β-catenin

This project is being developed in collaboration with Mireia Duñach (Universitat Autònoma de Barcelona) and Antonio Garcia de Herreros (Universitat Pompeu Fabra). The progressive accumulation of nuclear β-catenin, which deregulates cellular proliferation, differentiation, and migration, has been described as an initial event for the development of colon tumorigenesis. In addition to its structural role in epithelial junctions, β-catenin activates the TCF-4-mediated transcription of genes required for cell proliferation. Our hypothesis is that tumoral progression can be aborted by blocking the aberrant transcription mediated by β-catenin. Our main goal consists of identifying small molecules that specifically block β-catenin-mediated transcription, which is essential for tumour development. These molecules could therefore be of potential use in colon tumour treatment.

We are looking for small molecules that do not alter the interaction of β-catenin with: a) factors involved in the establishment of adherent junctions (E-cadherin, β-catenin) or b) factors involved in the degradation complex (APC, axin). To this end, we have prepared hundreds of compounds, which have been screened in Duñach's and Garcia de Herreros' laboratories and have shown promising results.

Synthesis of peptide analogues

This year has witnessed increased collaboration with BCN-Peptides, a leading company in API (Active Pharmaceutical Ingredient) peptides. We have prepared several adequately protected unnatural amino acids, which have been given to this company to be used in the synthesis of several peptidic analogues. The biological activity of these new compounds is now being tested.

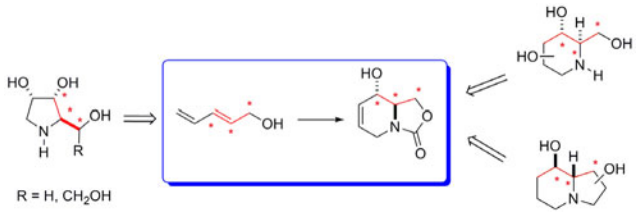


Figure 6. *Synthetic scheme for the preparation of ^{13}C-labelled glycosidase inhibitors.*

Publications

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Cabot R, Lledó A, Reves M, Riera A and Verdaguer X. Kinetic studies on the cobalt-catalysed norbornadiene intermolecular Pauson-Khand reaction. *Organometallics,* **26**, 1134-42 (2007)

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Lledó A, Solà J, Verdaguer X, Riera A and Maestro MA. PuPHOS and CamPHOS ligands in the intermolecular catalytic Pauson-Khand reaction. *Adv Synth Catal,* **349**, 2121-28 (2007)

Murruzzu C, Alonso M, Canales A, Jimenez-Barbero J and Riera A. Synthesis and NMR experiments of (4,5,6-13C)-deoxymannojirimycin. A new entry to 13C-labeled glycosidase inhibitors. *Carbohydr Res,* **342**, 1805-12 (2007)

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Ramon R, Alonso M and Riera A. A unified approach to mesityl amino acids based on Ssharpless dihydroxylation. *Tetrahedron: Asymmetry,* **18**, 2797-02 (2007)

Solà J, Reves M, Riera A and Verdaguer X. N-phosphino sulfinamide ligands: An efficient manner to combine sulfur chirality and phosphorus coordination behavior. *Angew Chem Int Ed,* **46**, 5020-23 (2007)

Research Networks and Grants

Identificació d'inhibidors específics de l'activitat transcripcional de la beta-catenina en la progressió tumoral
Fundació La Marató de TV3, Ajuts econòmics a projectes de recerca sobre càncer, 050630/31/32: 2006-2008
Project Coordinator: Mireia Duñach Masjuan
Research Director of the subproject: Antoni Riera

Identificación de inhibidores específicos de la actividad transcripcional de la beta-catenina en cáncer de colon
Fundació La Caixa, Investigación Biomédica, BM 05-68-0: 2006-2008
Project Coordinator: Mireia Duñach Masjuan
Research Director of the subproject: Antoni Riera

Síntesis enantioselectiva de moléculas bioactivas mediante catálisis asimétrica: Reacciones de Pauson-Khand, organocatálisis y oxidaciones de Sharpless
DIGI — Dirección General de Investigación, NCTQ — Programa Nacional de Ciencias y Tecnologías Químicas, CTQ2005-00623/BQU: 2006-2008
Research Director: Antoni Riera Escale

Other Funding Sources

Three contract research projects with Enantia SL through the Fundació Bosch i Gimpera.

Collaborations

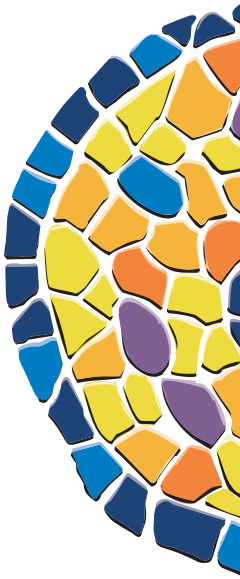
Asymmetric catalysis
Miquel A Pericàs, Institute of Chemical Research of Catalonia-ICIQ (Tarragona, Spain)

Synthesis and biological activity of phytoprostanes
Paul Evans, Trinity College, University of Dublin (Dublin, Ireland), Martin Mueller, Julius-von-Sachs-Institut of Biosciences, Universität Würzburg (Würzburg, Germany)

Synthesis of peptide analogues
Berta Ponsati, Jimena Fernández-Carneado and Marc Gomez, 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 Biomédica and Universitat Pompeu Fabra (Barcelona, Spain), Mireia Duñach, Universitat Autònoma de Barcelona (Barcelona, Spain)



Peptidomimetics, bioactive heterocycles and enantiomeric recognition

Màrius Rubiralta

Drug discovery relies on the preparation of new chemical entities (NCEs) to be used in preliminary pharmacological and biochemical bioactivity assays. The purity of NCEs is a crucial issue in this context in order to properly attribute activity to these compounds. Therefore, synthetic and purification technologies are essential for the rapid and successful development of this kind of project. Our research interests focus on exploring new reactivity pathways that lead to novel peptide-like molecules and heterocycles, substructures frequently occurring in drugs, and in the efficient separation of enantiomers. This research contributes to the development of highly relevant methodological tools in the early stages of drug discovery. Our main objective is to synthesise new useful molecules for use in the field of biomedicine. To this end, our approach is based on heterocyclic and peptide synthesis. We focus on the development of new synthetic pathways for rapid and efficient synthesis of heterocyclic systems. In this regard, we explore new multi-component reactions (MCRs) using nitrogen- and oxygen-containing heterocycles. We also study chiral molecules as a tool in the separation of enantiomers, and other products of high added value by several chromatographic and related technologies.

Conformationally constrained peptidomimetics

Conformationally restricted surrogates of model peptides of known activity are widely used in medicinal chemistry to determine the sites of action and the active conformations of peptides. These rigid surrogates can also lead to compounds acting specifically on a target receptor, and therefore to potential drugs with enhanced activity, devoid of undesired effects. On this basis, we design small libraries of peptidomimetics in which dipeptide sections are replaced by conveniently functionalised 3-amino-2-piperidone moieties. The synthesis of the suitable lactams and of the libraries is completed by biological screening. We are working on four peptide models of relevance in several pharmacological fields: i) antibiotics, derived from the cycloheptapeptide stylostatin 1, that act on the prokaryote cell membrane; ii) inhibitors of the dimerisation of HIV1-PR, whose structure is based on that of the natural protein; iii) peptides with a miraziridine A template; and iv) peptides modelling melanotan II. Our ultimate aim is to obtain anticancer agents.

In each case, a first library has been produced and the activity of the compounds has been screened by expert partners working both in the private and public sectors. During 2007, a PhD thesis devoted to the study of HIV1-PR inhibitors has been completed and successfully defended (Eulàlia Pinyol). The experimental section of a second PhD thesis, dedicated to pseudo-miraziridines as anticancer agents, has been concluded and the results will be presented shortly by Patricia López-Roldan. The results of this work will soon be submitted for publication in international journals. In the meantime, we have presented our preliminary results in several international and national meetings.

Synthesis and reactivity of bioactive heterocycles

We study new reactivity pathways for relevant heterocycles (pyridines, indoles, oxygen-heterocycles, etc). Our main goal is to develop efficient synthetic methodologies for the preparation of bioactive compounds. Recently, we have started a project for the incorporation of N- and O-heterocycles in MCRs. In these processes, several molecules react to afford a single product, thereby increasing molecular diversity and exploratory power. These features make this approach very powerful in the development of Structure Activity Relationship (SAR) studies of drug-like

Research Group Members | **Principal Investigator:** Màrius Rubiralta | **Directors of Research:** Anna Diez Pascual, Rodolfo Lavilla Grifols, Cristina Minguillón Llombart | **Postdoctoral Fellows:** Mª José Arévalo, Ana Belén, Federica Catti, Montse Cruz Gatell, Nicolas Isambert, Sánchez Maya | **PhD Students:** Biotza Gutierrez Arechederra, Nicola Kielland, Carlos López Martínez, Patricia López Roldán, Jordi Mas Pons, Carme Masdeu Margalef, Miriam Miguel Sala, Anna María Pérez Montero, Eva Pérez Palomar, Eulàlia Pinyol i Ollé, Núria Rubio Esplugas, Raquel Sancho Ponce | **Master Student:** Anna Alcaide López | **Administrative Assistant:** Montse Moreno Olivas

compounds. We have established new multi-component procedures with pyridine derivatives, the most frequently found subunit in drugs (substituted pyridines, quinolines and other azines, dihydroderivatives, etc) and oxa-heterocycles (5- and 6-membered rings, like those found in carbohydrates and other bioactive compounds). The MCRs we have developed fall into two main categories: Mannich-type processes (Povarov-like reactions) and isonitrile-derived transformations (Ugi and Passerini reactions). Both types of reactions share a number of features: the search for structural diversity, selectivity, and practical protocols (Figure 1).

We have recently described the participation of enol esters in MCRs of the first category. Interestingly, the final adduct displays a lactam core, which makes the process attractive for the preparation of drug-like structures (Isambert *et al*, 2007; Figure 2). Regarding isonitrile reactions, we have reported an extraordinary transformation involving dihydropyridines, iodine and isocyanides to yield benzimidazolium salts in just one step. The process, involving a cascade reaction, forms 3 carbon-carbon bonds and 1 carbon-nitrogen bond (Masdeu *et al*, 2007; Figure 2). Furthermore, the compounds display potent and selective inhibition of prolyl oligopeptidase.

Thanks to technology transfer, the scientific knowledge generated in this research field is being applied by pharmaceutical companies via two research contracts now being developed in our laboratories (collaborations with laboratories at Almirall and Grupo Ferrer). Furthermore, we are currently exploring the bioactivity of the scaffolds prepared. In collaboration with Antoni Riera (IRB Barcelona), our research

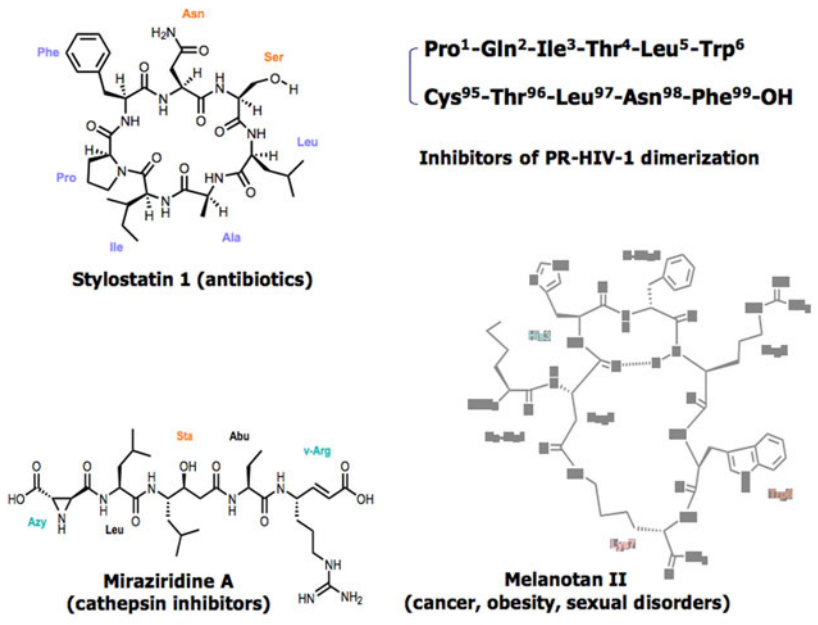


***Figure 1**. The four peptide models used for the synthesis of new peptidomimetic libraries.*

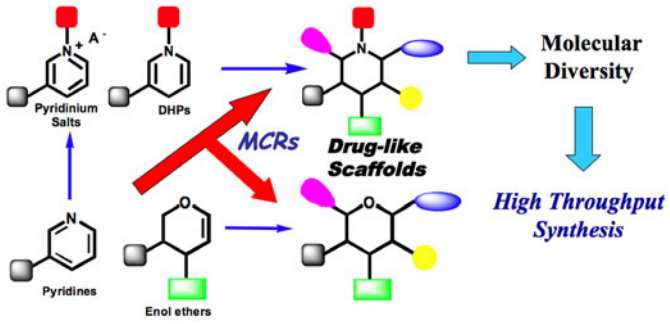


Figure 2. *Multi-component reactions based on O- and N-heterocycles.*

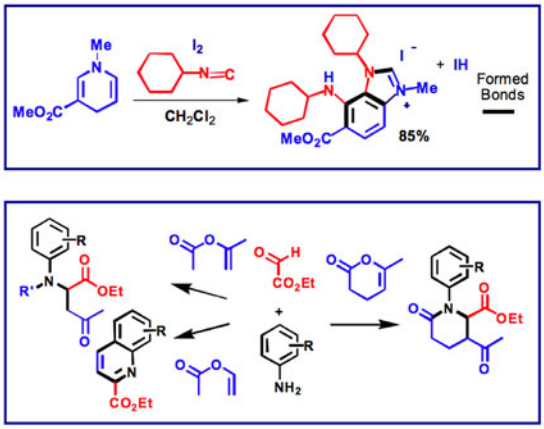
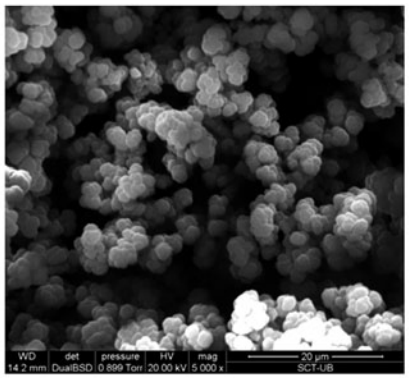


Figure 3. *Access to functionalised benzimidazolium salts, lactams, amides and quinolines through new multi-component reactions.*



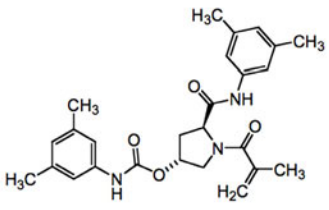


Figure 4. *ESEM image (Environmental Scanning Electron Microscopy) of the material obtained in the polymerization of the chiral monomer used in a monolithic chromatographic column.*

has targeted β-catenin antagonists; with Ernest Giralt (IRB Barcelona), the identification of prolyl oligopeptidase inhibitors; and with Pelayo Camps (University of Barcelona), the rapid synthesis of designed acetylcholinesterase inhibitors with anti-Alzheimer properties. Several compounds displayed potent and selective activities against these targets, and we are now developing SAR studies to optimise their biological performance.

Enantioselective molecular recognition

Enantioselective recognition leading to the separation of enantiomers by distinct techniques is the result of the selective association between a chiral molecule in its enantiomeric pure form, the so-called chiral selector (CS), and each of the two enantiomers of a given chiral compound. Following our interest in elucidating how this process occurs, we have applied STD NMR (Saturation Transfer Difference NMR) experiments to the study of the association between a number of polysaccharide derivatives and the enantiomers of chiral drugs, such as pindolol, propranolol and warfarin. Results allow correlation of enantioseparation by liquid chromatographic techniques (HPLC or centrifugal partition chromatography – CPC) (Pérez *et al*, 2006) with the differences observed by STD NMR experiments (Pérez *et al*, in preparation). This is the first time that STD NMR experiments have been applied to chromatographic enantioselective association.

One of the research interests of our group is to demonstrate the applicability of countercurrent chromatography (CCC), as a preparative tool, to the separation of enantiomers. In this regard, we have synthesised several CSs with L-proline or (4R)-hydroxy-L-proline as a fundamental scaffold, among others. At present, CCC enantioselectivity studies using the newly prepared CSs are in course (PhD theses of AM Pérez and N Rubio). Particular effort also focuses on the preparation of materials for membrane separations. Thanks to a pre-doctoral stay of B Gutierrez in the Max-Planck-Institut für Dynamik Komplexer Technischer Systeme (Magdeburg, Germany), working under the supervision of A Seidel-Morgenstern, we have incorporated Molecular Imprinting Technology (MIPs, Molecularly Imprinted Polymers) to the preparation of chiral chromatographic columns and enantioselective membranes. The partial separation of several racemates has been achieved in HPLC using this kind of material and application to membranes is in course.

We recently described outstanding chromatographic results obtained using a polyproline derivative as a CS in HPLC (Sancho *et al*, 2006) when bonded to silica gel beads as chromatographic support. Several of the properties of this new chiral chromatographic material (the broader application domain and also the higher loadability than simple amino acid-derived chiral stationary phases) have been attributed to the

helical nature of the CS. Moreover, significant changes in chromatographic behaviour have been observed when the nature of the solvent constituting the mobile phase is changed. During 2007, we have been examining the significance that the bonding site on the CS, amino-term or acidic-term of the peptide, has on the final enantioselectivity of the resulting material (Sancho *et al*, in preparation). In parallel, and thanks to the pre-doctoral stay of R Sancho in the Department of Chemistry at the University of California, (Berkeley, California, USA), working under the supervision of Frantisek Svec, we have studied the effect of using a monolithic silica column as a matrix instead of the classical silica gel bead matrix. The increase in specific surface that this modification implies has resulted in an augmented application domain for our CS. Moreover, a number of structural studies on this CS have recently been undertaken. The behaviour observed when using several solvents in the mobile phase could be the result of the change in the CS conformation. Thus, the CS has been submitted to several conditions in solution to confirm these changes using various techniques. Circular dichroism as well as FRET (Fluorescence Resonance Energy Transfer) have been applied, after moderate success using 13C NMR (cis/trans disposition of peptide bonds), to observe differences in helicity and, therefore, in the length of the peptoid CS. TEM (Transition Electron Microscopy) has also been considered with the aim to assess the possible supramolecular self-association of the CS.

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Research Networks and Grants

Diseño, síntesis y evaluación biológica de peptidomiméticos de conformación restringida
Dirección General de Ciencia y Tecnología, CTQ2004-01757/BQU: 2005-2007
Research Director: Anna Diez Pascual

Diseño, síntesis y evaluación biológica de peptidomiméticos de conformación restringida con interés farmacológico
Dirección General de Ciencia y Tecnología, CTQ2007-60764: 2007-2009
Research Director: Anna Diez Pascual

Diseño, síntesis y estudio estructural de nuevos inhibidores de la dimerización de la proteasa del VIH
VII FIPSE, IRB-FIPSE: 2007-2009
Project Coordinator: Ernest Giralt
Research Director: Anna Diez Pascual

Nuevas tecnologias para la separación preparativa de enantiómeros: cromatografía en contracorriente y membranas enantioselectivas
Dirección General de Ciencia y Tecnología, CTQ2006-03378/PPQ: 2007-2009
Research Director: Cristina Minguillón

Plataforma combiquímica basada en productos naturales: descubrimiento y administración de fármacos
DGICYT, BQU2006-03794: 2007-2009
Project Coordinator: Fernando Albericio
Research Director: Rodolfo Lavilla

Other Funding Sources

Assessorament i investigació aplicada en el camp de la Química Farmacéutica i Orgánica
Fundació Bosch Gimpera, FBG 100027
Coordinator: Cristina Minguillón

Building blocks de interés para el proyecto de quimiocinas
Fundació Bosch Gimpera, FBG 302256
Project Coordinator: Rodolfo Lavilla

Building blocks for lead finding
Fundació Parc Científic de Barcelona (Research project funded by Almirall Prodesfarma), APF 004: 2006-2007
Project Coordinator: Rodolfo Lavilla

Síntesis de building blocks
Fundació Bosch i Gimpera. Lab contract, Esteve-University of Barcelona
Research Director: Anna Diez

Síntesis de moléculas bioactivas
Fundació Parc Científic de Barcelona (Research project funded by Laboratorios Ferrer), CNV.FERRER 01: 2007-2008
Project Coordinator: Rodolfo Lavilla

Collaborations

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

Development of new inhibitors for acetylcholinesterase
Pelayo Camps, University of Barcelona (Barcelona, Spain)
Coordinator: Rodolfo Lavilla

Diseño, síntesis y estudio estructural de nuevos inhibidores de la dimerización de la proteasa del VIH
Ernest Giralt, IRB Barcelona (Barcelona, Spain)
Coordinator: Anna Diez

Molecular imprinting technology applied to the preparation of polymers to by used in enantioselective chromatographic columns and membranes
Andreas Seidel-Morgenstern, Max-Planck-Institut für Dynamik Komplexer Technischer Systeme (Magdeburg, Germany)
Coordinator: Cristina Minguillón

Polyproline-derived chiral selectors bonded to monolithic silica gel chromatographic columns
Frantisek Svec, University of California (California, USA)
Coordinator: Cristina Minguillón

Preparative enantioseparation of chiral sulfoxides
Narcis Avarvari, Laboratoire Chim Ingénierie Moléculaire et Matériaux, CNRS-Université d'Angers (Angers, France)
Coordinator: Cristina Minguillón

Proline derived chiral selectors as monomers in the preparation of monolithic capillary columns and its use in enantioselective electrochromatography
Guillermo Ramis and JM Herrero, University of Valencia, Burjassot (Valencia, Spain)
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Synthèse de synthons pipéridiniques bicycliques à application dans le domaine des peptidomimétiques
Laurent Micouin, Laboratoire de Chimie Thérapeutique, UMR CNRS, Université Paris (Paris, France)
Coordinator: Anna Diez

Screening of libraries for catenine antagonists
Antoni Riera, IRB Barcelona (Barcelona, Spain)
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Synthesis and structure-activity relationships of proline oligopeptidase inhibitors
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Synthetic methodology. Synthesis of bioactive compounds
Fernando Albericio, IRB Barcelona (Barcelona, Spain)
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Laboratorios Amirall (Barcelona, Spain)
Coordinator: Rodolfo Lavilla

Laboratorios Ferrer – Grupo Ferrer (Barcelona, Spain)
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Enantia (Barcelona, Spain)
Coordinator: Cristina Minguillón

Panreac Quimica (Barcelona, Spain)
Coordinator: Cristina Minguillón

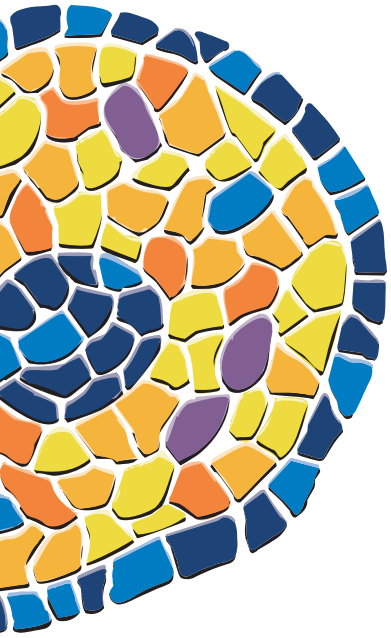
Laboratorios Esteve (Barcelona, Spain)
Coordinator: Anna Diez

Enantia (Barcelona, Spain)
Coordinator: Anna Diez

Procter & Gamble (Brussels, Belgium)
Coordinator: Anna Diez

Awards

Poster Award, 19th International Symposium on Chirality, San Diego (2007)
Awardee: Raquel Sancho
(4R)-Derivatized-L-proline oligomers as HPLC chiral selectors. Dependence of enantioselectivity on fixation to the chromatographic matrix. Authors: Raquel Sancho and Cristina Minguillón



Oncology Programme

Wnt signalling and EphB-ephrin interactions in intestinal stem cells and CRC progression

Eduard Batlle

Eph receptor tyrosine kinases and their ligands, ephrins, play key roles in the regulation of migration and cell adhesion during development, thereby influencing cell fate, morphogenesis and organogenesis. Recent findings suggest that Eph signalling also controls the architecture and physiology of several tissues in the adult body under normal and pathological conditions, such as cancer. Major research efforts in our laboratory in recent years have focused on elucidating the role of Eph-ephrin signalling in the intestinal epithelium and colorectal cancer (CRC).

Eph receptors in intestinal cell positioning

Eph receptors constitute the largest subfamily of transmembrane tyrosine kinase receptors described to date, with 14 members identified in mammals. Their ligands, the ephrins, are membrane-anchored proteins which are grouped into two subclasses: type-A ephrins (ephrinA1-ephrinA6), which are attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor, and type-B ephrins (ephrinB1-ephrinB3), which contain transmembrane and intracellular domains (Figure 1). Depending on their sequence similarity and on their affinity for ephrins, Eph receptors are also classified into two groups. In general, EphA receptors (EphA1-EphA10) bind ephrinAs and EphB receptors (EphB1-EphB6) bind ephrinBs, yet promiscuity in their binding specificities has been described for some members. Upon cell-to-cell contact and ligand-receptor engagement, intracellular signalling is induced in a bidirectional fashion: 'forward signalling' starts in receptor-expressing cells, while 'reverse signalling' initiates in cells expressing the corresponding ligand (Figure 1). Eph-ephrin signalling regulates a number of cellular events during embryonic development such as cell migration, repulsion vs adhesion, and cell-to-cell communication. Most of these responses are achieved through the capacity of Eph-ephrin signalling to regulate actin cytoskeleton dynamics (reviewed in Pasquale, 2005).

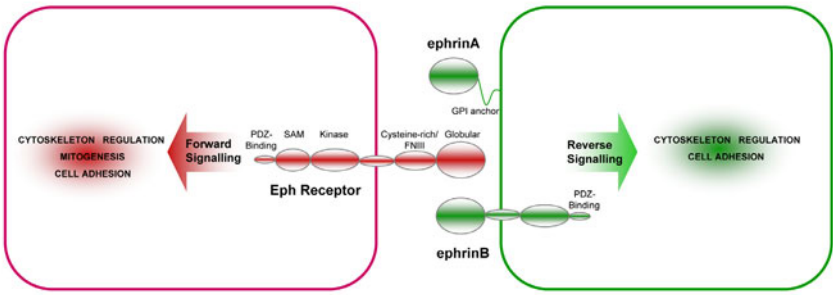


***Figure 1**. Eph receptors and ephrins. Eph receptors are tyrosine kinase transmembrane molecules. Their extracellular domain consists of a ligand-binding globular region, a cysteine-rich domain and two fibronectin type III (FNIII) repeats. The tyrosine kinase domain, the sterile-alpha motif (SAM) and the PDZ-binding motif are located in the intracellular region and are responsible for the interaction with downstream effector molecules, which regulate events such as cytoskeleton dynamics, cell proliferation or cell adhesion. Ephrins are also plasma membrane-anchored molecules with an extracellular Eph-binding domain. EphrinAs are tethered to the cell surface through a glycosylphosphatidylinositol (GPI) anchor, while ephrinBs have an intracellular domain, containing a PDZ-binding motif which allows interaction with proteins affecting cytoskeleton organisation and cell adhesion.*

Our laboratory pioneers the study of Eph signalling in the intestinal epithelium. The innermost layer of the intestinal tube is a mono-stratified epithelium which is folded into millions of bag-shaped invaginations called crypts. At the base of each crypt reside a handful of exceptionally active stem cells that constantly regenerate the epithelium (Barker *et al*, 2007). Cell renewal is accomplished in a bottom-up fashion. Intestinal Stem Cells (ISCs) continuously regenerate three cell lineages that coexist in the small intestine and colon (ie, mucosecreting, absorptive and enteroendocrine cells). These cell types migrate towards the lumen as they undergo terminal differentiation. The small intestine contains a fourth epithelial cell type, Paneth cells (PCs), that escape from this migratory flow and remain at the base of the crypts as mature cells. The main driving force behind intestinal cell renewal is Wnt signalling. A still uncharacterised source of Wnt factors present at the base of each

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crypt promotes beta catenin/Tcf-driven transcription in cells localised within this niche, ie, in ISCs and PCs. Blockage of Wnt signalling in the gut results in loss of the progenitor compartment and defects in PC maturation whereas constitutive activation of the Wnt pathway causes the expansion of the crypt progenitor compartment and the onset of tumorigenesis (reviewed in Clevers and Batlle, 2006). We originally showed that the expression of EphB2 and EphB3 in crypts is driven by Wnt signalling (Batlle *et al*, 2002). EphB2 is present at highest levels in ISCs and its expression gradually decreases in progenitor cells as they migrate towards the lumen (Figure 2). EphB3 displays a restricted localisation in cells present at the bottom-most positions of the crypts (ie, PCs, ISCs and probably early progenitors). Conversely, the expression of ephrinB1 and ephrinB2 is negatively controlled by beta-catenin/Tcf activity and these isoforms show the highest expression levels in differentiated cells (Batlle *et al*, 2002). In the intestine of EphB3 null mice, localisation of PCs is no longer restricted to the crypt base, instead this cell type migrates upwards and is found dispersed throughout the epithelium (Batlle *et al*, 2002). We have recently demonstrated that this phenotype is also observed in intestine specific conditional ephrinB1 knockout mice (Cortina *et al*, 2007). In double EphB2-/-; EphB3-/- mice, the boundary between the proliferative and differentiated cell compartments is lost and crypt cells intermingle instead of undergoing unidirectional upward migration (Batlle *et al*, 2002). Thus, EphB/ephrinB signalling is required to establish cell compartments and to organise ordered migration of

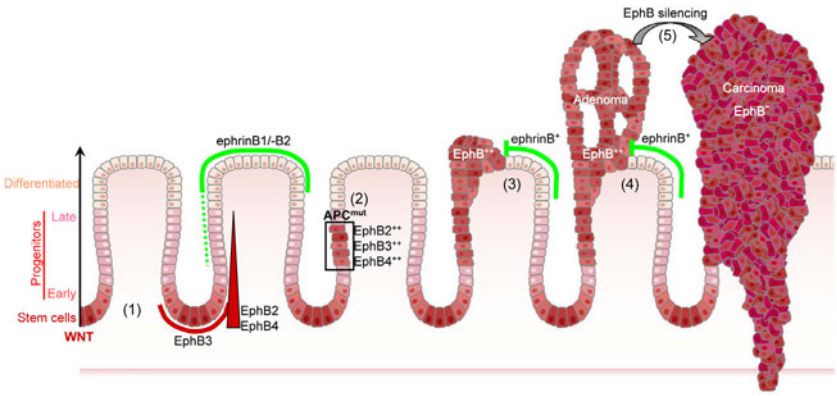

Figure 2. *EphB-ephrinB interactions during CRC progression. (1) Expression domains of EphB and ephrinB genes in the colon. Wnt signalling occurs at the bottom-most positions of the crypts. (2) Mutations in the tumour suppressor gene APC activates the Wnt pathway and transforms intestinal epithelial cells into tumour-initiating cells (cells within the square). As a result of constitutive beta-catenin/Tcf activity, Apc mutant cells express high levels of EphB2, EphB3 and EphB4 receptors. (3) Tumour-initiating cells acquire stem cell properties and repopulate the crypts with their mutant descendants until they reach the surface epithelium. There, tumour cells accumulate and form benign polyp-like outgrowths known as adenomas. Contact of tumour cells with normal differentiated cells that express high levels of ephrinB ligands results in the activation of EphB signalling. (4) Expansion of adenomas is blocked by EphB repulsive signals that limit the spread of tumour.*

epithelial cells along the crypt axis. Furthermore, EphB2 and EphB3 receptors are also expressed in ISCs. As a follow up of this work, our laboratory is currently attempting to provide evidence that EphB signals may also be required for stem cell retention within the crypt niche.

Eph signalling in CRC progression

Most colorectal tumours are initiated by mutations in the Wnt pathway which lead to the activation of the beta-catenin/Tcf complex in intestinal epithelial cells (reviewed in Clevers and Batlle, 2006; Sancho *et al*, 2004). We originally proposed that mutational activation of the Wnt pathway imposes a constitutive crypt progenitor phenotype on early tumour cells (van de Wetering *et al*, 2002). As a result, intestinal adenomas, the precursors of CRC, are characterised by high expression of crypt Wnt target genes, including EphB2 and EphB3 receptors (Batlle *et al*, 2005). However, as adenomas become aggressive, the expression of EphB receptors is silenced despite the persistence of Wnt pathway mutations (Batlle *et al*, 2005; Figure 2). We demonstrated through genetic analysis in mouse models that the combination of Apc mutations with blockage of EphB activity promotes the formation of aggressive colorectal tumours (Batlle *et al*, 2005; Cortina *et al*, 2007). Thus, EphB signalling blocks the acquisition of malignancy during CRC progression. The mechanism responsible for EphB silencing in malignant colorectal tumours remains unknown; however, promoter methylation as well as point mutations in EphB2 and EphB4 genes have been found in some cases. Our group performs intensive research into the regulation of the EphB3 gene. We have identified an enhancer region which directs EphB3 expression in CRC cells. We are currently screening for transcription factors and signalling pathways that regulate the activity of this promoter.

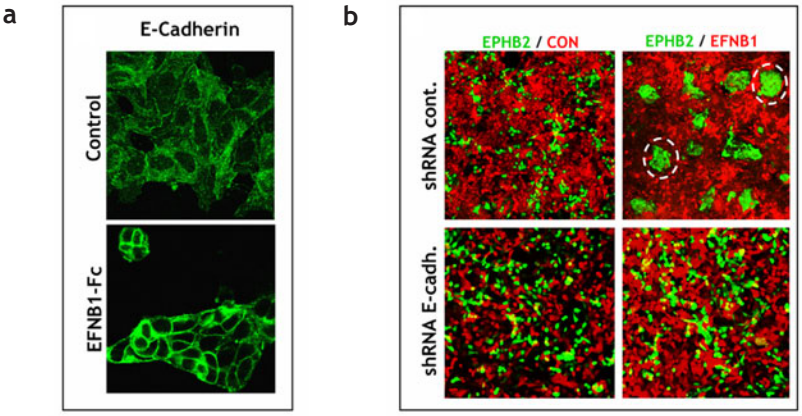

***Figure 3**. E-cadherin adhesion is essential for EphB-mediated tumour cell compartmentalisation. (a) EphB activation results in fast cell contraction, aggregation and re-localisation of E-cadherin to the membrane of DLD-1 EphB2-expressing cells. Activation of EphB signalling was achieved by treatment with ephrinB1/Fc fusion protein (EfnB1-Fc) for 1 h. (b) Co-culture of EphB2-GFP-expressing cells with control-RFP results in cell intermingling between both populations (top left). However, ephrinB1-RFP cells restrict the growth of EphB2-GFP cells to compact clusters (top-right panel; dashed circles). Down-regulation of E-cadherin levels by shRNA inhibits EPHB-mediated cell compartmentalisation (bottom-right).*

A major achievement in this period has been the identification of the mechanism by which EphB suppresses CRC progression (Cortina *et al*, 2007). We generated *in vitro* models that mimic EphB/ephrinB interactions in CRC. We took advantage of fully malignant 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 (Figure 3). More importantly, *in vivo*, EphB+ tumour-initiating cells become compartmentalised upon contact with normal intestinal cells that express high levels of ephrinB1, thus limiting the space available for adenoma expansion (Figure 2). Overall, our experiments also demonstrate that even fully malignant CRC cells bearing multiple mutations in oncogenes and tumour suppressors respect the boundaries imposed by EphB-ephrinB interactions. We proposed that tumour cell compartmentalisation is a general mechanism of cancer suppression in tissues whose architecture is defined by Eph-ephrin interactions.

Publications

Cortina C, Palomo S, Iglesias M, Fernandez-Masip JL, Vivancos A, Whissell G, Huma M, Peiro N, Gallego L, Jonkheer S, Davy A, Lloreta J, Sancho E and Batlle E. EphB ephrin-B interactions suppress colorectal cancer progression by compartmentalising tumour cells. *Nat Genet*, **39**, 1376-83 (2007)

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Research Networks and Grants

Cancer Biology (Consolider Network)
Ministerio de Educación y Ciencia, CSD2007-00017: 2007-2011
Research Director: 13 groups including Eduard Batlle, coordinated by CNIO

Grant for emerging groups
Generalitat de Catalunya, Agència de Gestió d'Ajuts Universitaris i de Recerca, 2005SGR 00775: 2006-2009
Research Directors: Eduard Batlle and Elena Sancho

Papel de los receptores EPHB en el posicionamiento de las células epiteliales y en el cáncer colorectal
Ministerio de Educación y Ciencia, SAF2005-04981: 2005-2007
Research Director: Eduard Batlle

The modulation of the beta-catenin/Tcf genetic programme during CRC progression
Fundació La Caixa, Biomedical Research Projects — Oncology: 2007-2009
Research Directors: Eduard Batlle and Elena Sancho

Collaborations

A role for TGF-beta in CRC progression
Elena Sancho, IRB Barcelona (Barcelona, Spain)

Control of intestinal stem cell positioning
Hans Clevers, Hubrecht Laboratorium (Utrecht, The Netherlands)

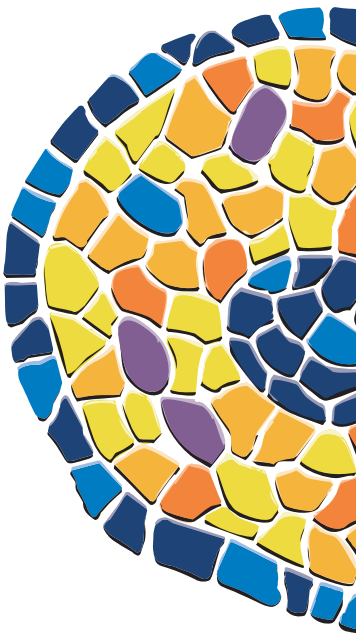
Eph signalling in pancreas development
Francisco X Real, Institut d'Investigació Mèdica (Barcelona, Spain)

Isolation of colorectal cancer stem cells using Wnt target genes
Gabriel Capellà, Institut Català d'Oncologia (L'Hospitalet de Llobregat, Spain)

Stem cell gene expression signatures in the prediction of CRC outcome
José Baselga, Vall d'Hebrón Hospital (Barcelona, Spain)

The genetic programmes linked to EphB down-regulation during CRC progression
Giancarlo Marra, Institute of Molecular Cancer Research, University of Zurich (Zurich, Switzerland)

The role of c/EBP isoforms in intestinal development and cancer
Claus Nerlov, European Molecular Biology Laboratory, Monterotondo (Rome, Italy)



Molecular mechanisms involved in colorectal cancer initiation and progression

Elena Sancho

Colorectal cancer (CRC) is the third most common type of cancer and the second cause of death by cancer in the Western world. CRC results in around 650,000 deaths worldwide per year. Most sporadic CRCs 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 performed 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.

WNT signalling and the initiation of CRC

Around 70% of sporadic colorectal tumours show biallelic 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 the Wnt-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 tumorogenesis in the intestine in a process characterised by the formation of dysplastic crypts 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 is responsible for the initiation of CRC (reviewed in Sancho *et al*, 2004; Figure 1).

***Figure 1**. Genetic alterations frequently associated with CRC progression.*

Mutations in Wnt signalling components that lead to CRC result in the stabilization 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 represents 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 normal non-transformed intestinal progenitors cells at the bottom of crypts (van de Wetering *et al*, 2002; see Figures 2 and 3).

Our results, together with those obtained from several animal models in which Wnt signalling had been 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 in CRC the first step towards malignancy 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 totally changed views on the initiation of this disease. We are currently developing

animal models that will formally confirm this notion and help to shed light on why Wnt signalling mutations are an important pre-requisite for the development of CRC.

Our studies are oriented towards the identification of the nature of the founding CRC cell and the mechanisms by which it escapes cell renewal. During 2007, we have generated several DNA constructs to enable 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 could be targeted to block the progression of this disease. Likewise, in collaboration with Eduard Batlle's laboratory (IRB Barcelona), and by systematic analysis of colorectal samples at different stages of the disease, we aim to identify the core set of instructions imposed by Wnt signalling mutations that remains unaltered throughout the carcinogenetic process. This research will provide crucial information on the molecular targets for CRC at all stages of the malignancy.

Having identified that the initial event triggering transformation is the blockage of founder tumour cells into a progenitor phenotype, our laboratory now seeks to identify differences between the true physiological progenitors and initial founder mutant cells. To this end, during 2007, we have developed a protocol which allows us to isolate epithelial cells from the bottom of colonic crypts (ie, stem cells and early progenitors) from fresh tissue. This protocol can also be used to isolate tumour cells from early adenomas or dysplastic 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 for targeting by the pharmaceutical industry. These targets will be particularly useful for patients suffering Familial Adenomatous Polyposis (FAP). These patients inherit a mutation in the APC gene, and due to 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

In recent years, some of the leading scientific teams, including ours, have reported that the onset and progression of CRC can be understood using several concepts taken from the Darwinian evolution model. Under this view, the sequence of mutations acquired during CRC progression (Figure 1) can be explained by colorectal tumours evolving through a series of bottlenecks or restriction points at which only those cell cells acquiring the correct mutational event expand and progress to the next stage of malignancy. Our research interest focuses on determining how the acquisition of mutations in other signalling pathways may modulate the initial progenitor phenotype imposed by Wnt signalling to overcome the bottlenecks associated with CRC progression.

One of the most prevalent types of mutation during CRC progression are those that inactivate the TGF-beta signalling pathway (reviewed in Grady and Markowitz, 2003; Figure 1). This pathway is involved in numerous processes in the 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

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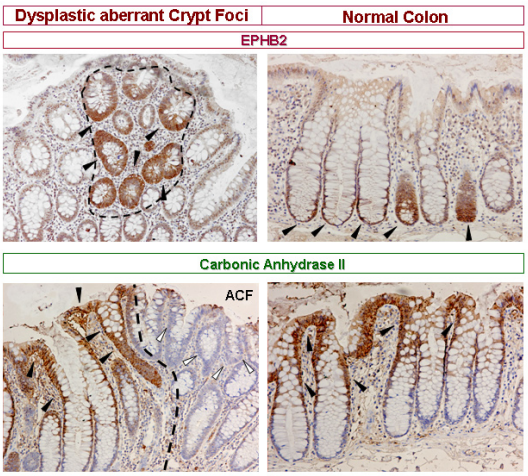

Figure 2. *Expression pattern of EphB2 (a,b) and Carbonic Anhydrase II (c,d) in early tumour lesions. EphB2, an example of a target gene under the control of beta-catenin/TCF, is expressed by tumour cells from dysplastic crypts (a, black arrows), but also by normal progenitor cells at the bottom of intestinal crypts (b, black arrows). In a complementary fashion, the differentiated marker carbonic anhydrase II is expressed only in the top half of the crypts and surface epithelium (c, d black arrows), and is absent from tumour lesions (c, white arrows). This expression pattern is observed for the beta catenin/Tcf target genetic programme identified so far.*

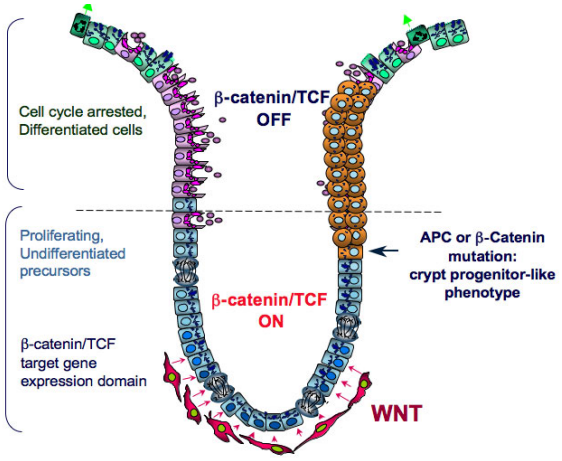

Figure 3. *Depiction of a colon crypt and proposed model for CRC initiation. The intestinal epithelium is organised in invaginations called crypts of Lieberkühn. Epithelial cells within these crypts are in constant renewal thanks to a small group of stem cells that reside at the bottom of the crypts. Stem cells divide asymmetrically to give rise to early progenitors, which rapidly divide whilst migrating towards the intestinal lumen. As they do so, they become predetermined towards differentiation into one of the functional cell types present in the intestine (adsorptive, mucosecreting or enteroendocrine cells). 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 proliferation and differentiate. Cells mutant in components of wnt signalling become independent of these signals, exhibit constitutive activation by beta-catenin/Tcf, and are blocked in a progenitor proliferative phenotype.*

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 Massagué, 2003). Mutations found in CRC affect mainly the TGF-beta receptor type II and the intracellular smads, smad-2 and smad-4, by abolishing the transcriptional effects mediated by TGF-beta.

Our lab currently focuses on the role of TGF-beta signalling in CRC progression. We are studying the transcriptional events controlled by TGF-beta in CRC cells. We have already identified changes in approximately 500 genes in response to TGF-beta in these cells. Unsupervised analysis of a collection of tumours of known transcriptomes on the basis of the TGF-beta signature obtained in our laboratory perfectly discriminates adenomas from carcinomas, thereby implying that these genes contain the information that drives the adenoma/carcinoma transition.

We are now dissecting this information in order to identify TGF-beta genes that play an executive role in the adenoma/carcinoma transition. Our studies in 2007 pinpoint a relevant role for TGF-beta signalling in the modification of several cellular responses required for tumour progression. Our results reveal a gain-of-function in TGF-beta signalling during CRC progression. This increase appears to translate into the expression of a series of TGF-beta target genes in several cell types within the tumour, including mesenchymal cells (see Figure 5). We have developed orthotropic models of colorectal tumours in nude mice to test the role of the TGF-beta-controlled gene signature. We are currently performing systematic shRNA-mediated down-regulation of genes contained in this signature in order to screen for TGF-beta-regulated genes that are crucial for CRC. In addition, we are studying whether the TGF-beta signature in CRC has a predictive value for clinical outcome.

Publications

Cortina C, Palomo S, Iglesias M, Fernandez-Masip JL, Vivancos A, Whissell G, Huma M, Peiro N, Gallego L, Jonkheer S, Davy A, Lloreta J, Sancho E and Batlle E. EphB ephrin-B interactions suppress colorectal cancer progression by compartmentalising tumour cells. *Nat Genet*, **39**, 1376-83 (2007)

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Research Networks and Grants

Molecular mechanisms involved in colorectal cancer initiation
Spanish Ministry for Education and Science, SAF2005-002170: 2006-2008
Research Director: Elena Sancho

Start-up grant for emergent research groups
Generalitat de Catalunya, Agència de Gestió d'Ajuts Universitaris i de Recerca, 2005SGR 00775: 2006-2009
Research Directors: Eduard Batlle and Elena Sancho

Variations in the genetic program under the control of beta-catenin/Tcf during colorectal cancer progression
Fundació La Caixa, BM06-241-0: 2007-2009
Research Directors: Eduard Batlle and Elena Sancho

Collaborations

TGF-beta target genes in CRC
Giancarlo Marra, Institute of Molecular Cancer Research (Zurich, Switzerland)

Wnt signalling in CRC
Hans Clevers, Hubrecht Laboratory (Utrecht, The Netherlands)

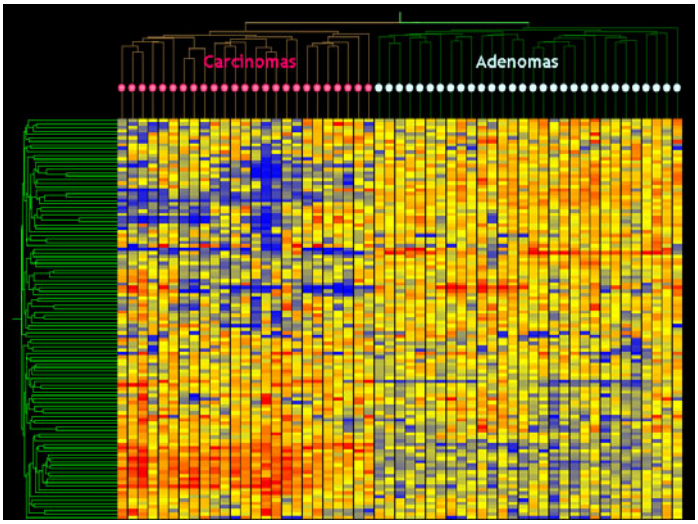


***Figure 4**. The TGF-beta signature discriminates between adenomas and carcinomas. Unsupervised clustering analysis of a collection of tumours of known transcriptomes on the basis of target genes controlled by TGF-beta signalling clearly classifies adenomas and carcinomas in two separate branches.*

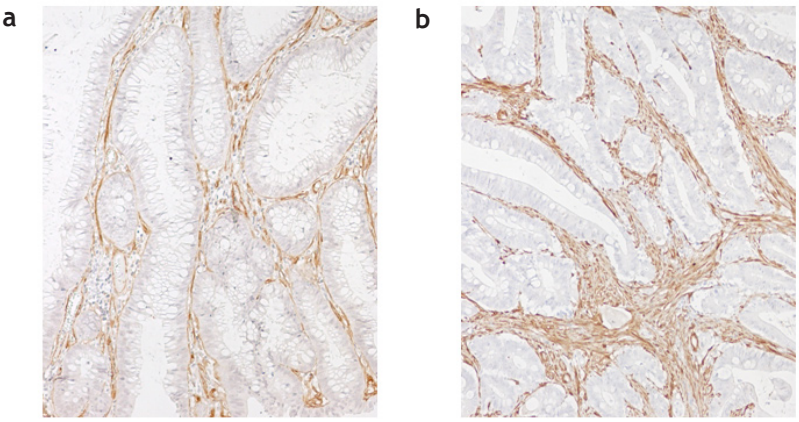


***Figure 5**. Representative example of the expression pattern of TGF-beta target genes in colorectal adenomas (a) and carcinomas (b).*

Tumoral Metastasis Laboratory (Metlab)

Roger Gomis

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 colonization of vital organs.

Growth control and cancer metastasis

Our research centres on the growth factors, signalling pathways, and gene expression programmes underlying cancer cell metastasis. We study the ways in which cancer cells evade tumour suppressor mechanisms and engage in metastatic behaviour. Focusing on a TGF-beta cytostatic programme involving the transcriptional regulation of cell cycle inhibitors and growth-promoting factors, we are examining how tumour cells evade these gene responses in order to pursue metastatic behaviour. By combining *in vivo* selection of human metastatic cells, transcriptomic profiling and functional testing, we seek to identify genes that selectively mediate breast metastasis to specific organs. Gene transfer techniques and RNAi-mediated gene silencing are used to functionally corroborate candidate genes. We are encouraged by the recent validation of these findings in clinical samples.

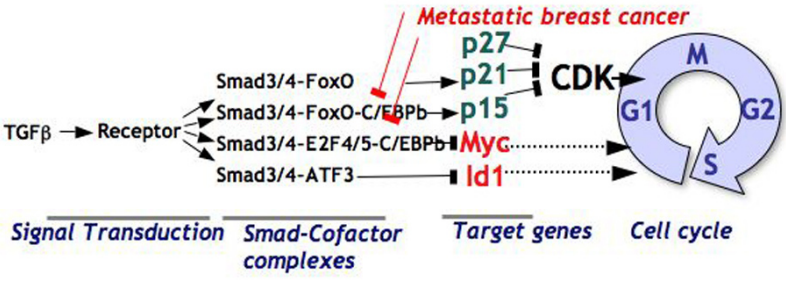
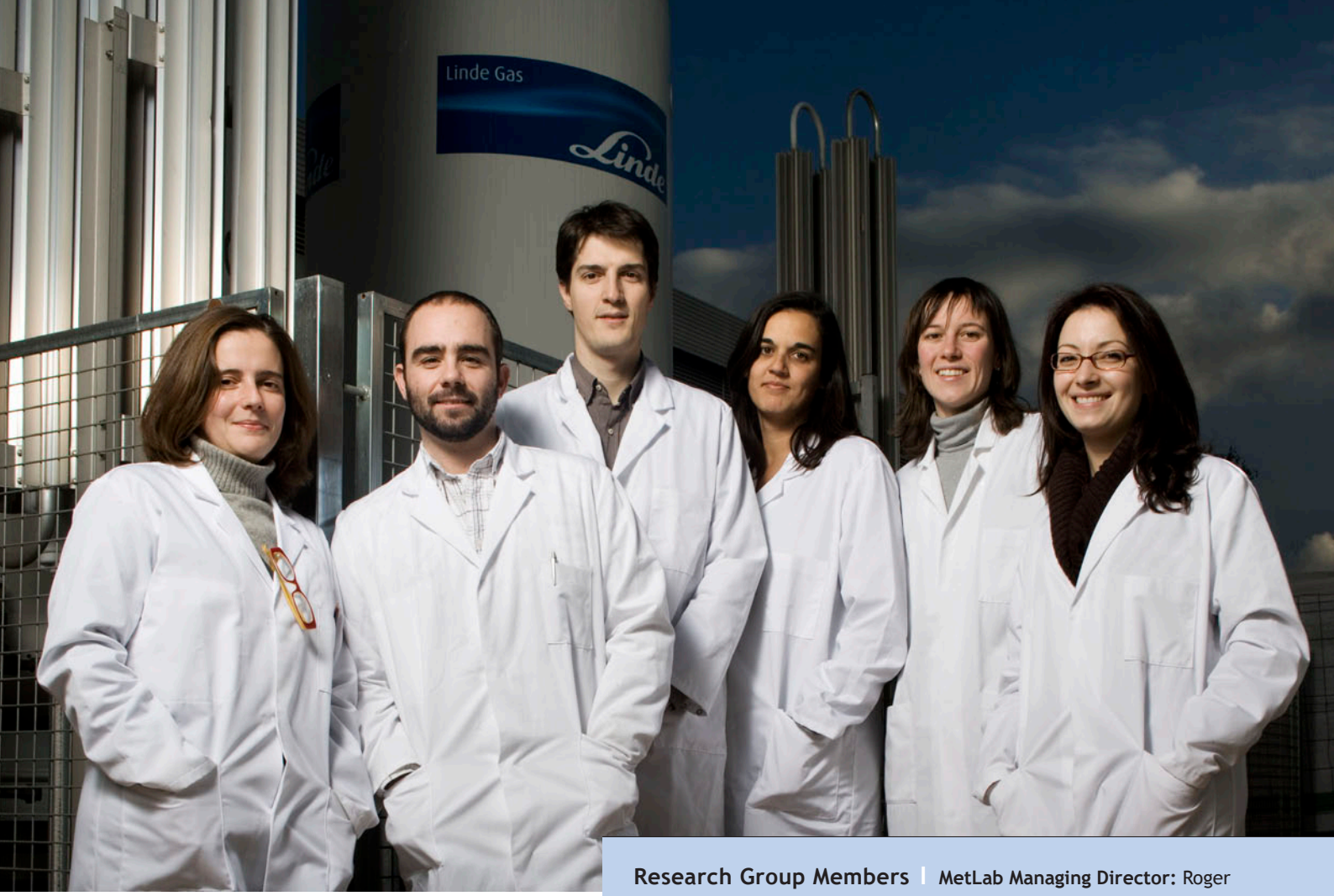


***Figure 1**. The TGF-beta cytostatic programme. Shown are the transcriptional components underlying the principal TGF-beta cytostatic responses in epithelial cells. Indicated in red are the targets of alterations present in certain human breast cancers either at the level of signal transduction events or Smad-Cofactor complexes converging on the target genes that mediate cell cycle arrest.*

Several of these genes encode products that are susceptible to therapeutic targeting.

Current research builds, 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/EBP transcription factor in the TGF-beta cytostatic programme in epithelial cells. Recent results have provided a new approach to explain the molecular mechanisms that control this programme. The TGF-beta signalling process is based on the formation of a TGF-beta-activated receptor complex that phosphorylates SMAD transcription factors, which in turn assemble molecular complexes that regulate the expression of target genes. Several of these gene responses act in concert to cause cell cycle arrest. This TGF-beta cytostatic programme includes repression of the proliferation-promoting genes *c-MYC* and *Id1*, as well as induction the cyclin-dependent kinase (CDK) inhibitors p15INK4b and p21CIP1 (Padua *et al*, 2008). Repression of *c-MYC* and *Id1* is mediated by a complex of SMAD with E2F4/5 and ATF3, respectively.

FoxO factors were identified as partners of TGF-beta-activated SMAD3 in the induction of the CDK inhibitor, p21CIP1, in epithelial cells. In recent work we have taken a genetic approach to identify other TGF-beta target genes that are regulated by a common SMAD3/FoxO transcription complex. By using siRNA techniques coupled with gene expression microarray data analysis, ten new genes whose TGF-beta expression is induced by the same complex were identified (Gupta *et al*, 2007). *p15INK4b* stands out among these genes. Surprisingly, a detailed analysis of the *p15INK4b* promoter has led to the finding of a role for C/EBP-beta in *p15INK4b* induction by TGF-beta (Gomis *et al*, 2006b).



Research Group Members | **MetLab Managing Director:** Roger Gomis | **IRB Barcelona Adjunct Director:** Joan Massagué | **Associate Researcher**: Mònica Morales | **PhD Students:** Anna Arnal Estapé, Maria Tarragona Sunyer | **Lab Manager:** Marc Guiu Comadevall | **Lab Technician:** Esther Fernández Rivas

Breast cancer cells are refractory to TGF-beta-mediated growth arrest, thus leading to further tumour progression and metastasis. The molecular characterisation of TGF-beta-mediated cytostasis in keratinocytes has positioned C/EBP-beta at the core of this response. Furthermore, deregulation of C/EBP-beta mediates evasion of the TGF-beta-induced cytostatic effects in metastatic breast cancer cells. We found that the transcription factor C/EBP-beta 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 TGF-beta 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/EBP-beta inhibitory isoform LIP, which has been implicated in tumour progression. By normalizing the LIP:LAP ratio, we restored these TGF-beta 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.

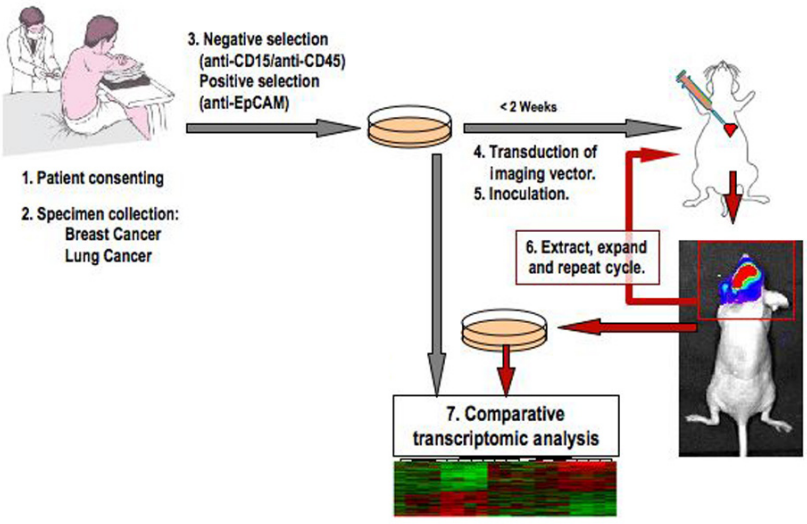


***Figure 2**. Scheme representing the experimental approach developed to discover new metastatic gene signatures.*

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/EBP-beta function and consequent loss of the TGF-beta cytostatic response in cancer cells. In addition, our laboratory focuses on extending these findings to other cell types in which the TGF-beta cytostatic response is absent either permanently or temporally.

The second research project started in our laboratory aims to identify gene groups that drive metastatic cells to one tissue or another. Particularly, we focus on metastatic suppressor genes and their functions in the metastatic process. Our initial studies are devoted to research into 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. Furthermore, we are also screening new metastatic cell populations from pleural effusions derived from breast or lung cancer patients in order to identify new metastatic gene signatures. For this purpose, on the basis of collaborations with clinical and basic investigators at the Hospital Clinic, 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.

Publications

Padua D, Zhang XH-F, Wang Q, Nadal C, Gerald W, Gomis RR and Massagué J. TGF-beta primes breast tumours for lung metastasis seeding through angiopoietin-like 4. *Cell*, **133**, 66-77 (2008)

Other references

Gomis RR, Alarcon C, He W, Wang Q, Seoane J, Lash A and Massagué J. A FoxO-Smad synexpression group in human keratinocytes. *Proc Natl Acad Sci USA*, **103**, 12747-52 (2006a)

Gomis RR, Alarcon C, Nadal C, Van Poznak C and Massagué J. C/EBPbeta at the core of the TGF-beta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell*, **10**, 203-14 (2006b)

Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Todorova-Manova K and Massagué J. Mediators of vascular remodeling co-opted for sequential steps in lung metastasis. *Nature*, **446**, 765-70 (2007)

Massagué J and Gomis RR. The logic of TGF-beta signalling. *FEBS Lett*, **580**, 2811-20 (2006)

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
Ministerio de Educación y Ciencia, SAF2007-62691: 2007-2009
Research Director: Roger Gomis

Study of the molecular mechanisms of metastasis of breast cancer to the lung: therapeutic function and potential of metastasis suppressor genes
Asociación Española Contra el Cáncer (Madrid, Spain)
Research Director: Roger Gomis

Other Funding Sources

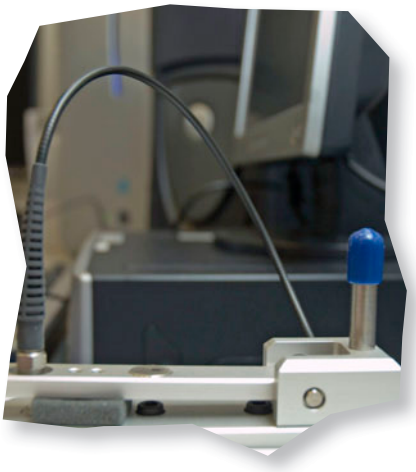
Mechanisms of metastasis
Fundación BBVA

Collaborations

Cristina Nadal, Servei D'Oncologia, Hospital Clínic Universitari de Barcelona (Barcelona, Spain)

Core Facilities

Functional Genomics Core Facility



During the last decade, molecular biology has 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 field, researchers require an increasing number of tools to either analyse 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:

- Genome-wide analysis of transcription, DNA polymorphisms, and chromatin immunoprecipitation (ChIP-chip). These analyses are performed using microarrays produced by Affymetrix. For all of these analytical methods, the Functional Genomics Core Facility provides a complete service, including initial consultation during the design of a project, quality control of starting material, sample and array processing, initial data analysis, and data interpretation and validation by real-time-PCR.
- Knock-down of gene expression by shRNAs. For knock-down of gene expression, the Functional Genomics Core Facility provides a genome-wide human shRNA library (Sigma), containing approximately 75,000 clones covering the majority of all known transcripts.

The Functional Genomics Core Facility was established in April 2007 and by end of the year it was performing projects for over 15 research groups from three programmes at IRB Barcelona and from external institutions. The first service offered was expression profiling on a gene-by-gene level. Soon after, expression profiling was also established at higher resolution by measuring the exons of genes individually. For the detection of unknown transcripts, tiling array analysis is also now available. Alternative chemistries are available to perform expression analysis of a few hundred cells. Arrays are available for over 20 organisms, including all of the frequently used model organisms and humans. Validation of microarray expression profiling by real-time PCR has been performed since October 2007.

For the analysis of DNA polymorphisms, genome-wide interrogation of DNA copy number variation (amplifications and deletions) has been performed since July 2007 on a gene-resolution level. Measurement of amplifications and deletions is especially useful to identify oncogenes and tumour suppressor genes.

In December 2007, the complete human shRNA library became available. It contains on average four distinct constructs targeting each transcript. Bacterial stocks of these clones are provided by the Facility to researchers at IRB Barcelona. A database is available to collect information about bacterial growth, sequences of inserts and knock-down efficiency.

Services for IRB Barcelona researchers

DNA/RNA quantification and quality control

Various analyses are provided for assessment of purity, integrity and concentration of nucleic acids.

Expression profiling

Genome-wide analysis of transcripts is provided at three levels of resolution:

- 3' biased arrays containing one probeset per gene; these arrays are available for more than 20 organisms.
- Exon arrays containing one probeset per exon; these arrays are currently available for human, mouse and rat.
- Tiling arrays interrogating the entire genome at a 35-bp resolution; these arrays are currently available for human, mouse, *Drosophila* and yeast.

DNA polymorphism analysis

Genome-wide analysis of DNA polymorphisms comes in two forms:

Core Facility Members | **Manager:** Herbert Auer | Senior **Research Officer:** Silvia Rodriguez

- For over twenty 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 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.

Knock-down of gene expression
Bacterial clones are provided for almost all well characterised human transcripts. Multiple clones targeting the same transcripts are available to assess off-target effects.

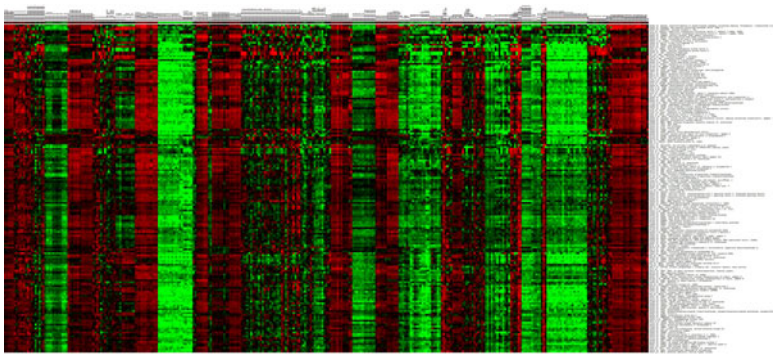


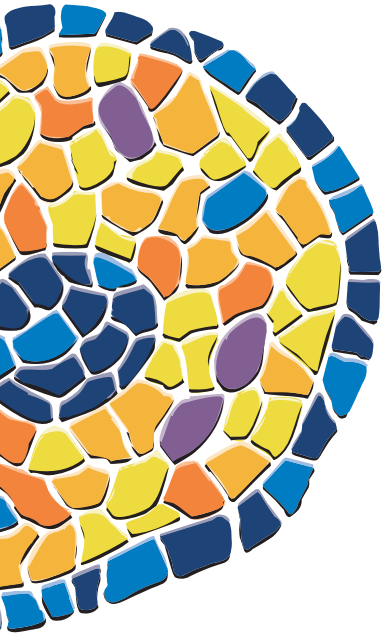
Figure 1. *A cluster of co-regulated genes in 336 samples including purified cord blood, B cell subpopulations, large B cell lymphomas, Burkitt-, follicular-, primary effusion-, and mantle cell-lymphoma, B cell chronic lymphocytic leukemia, hairy cell leukemia, Hodgkin disease, B cell lymphoma cell lines and lymphoblastic cell lines (Collaboration with K Kornacker, Nationwide Children's Research Institute).*

Collaborations

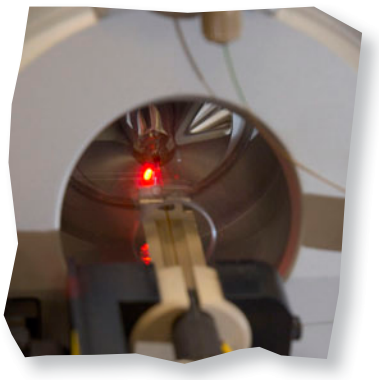
Comparison of comparative genomic hybridization technologies across microarray platforms
Susan Hester, Environmental Protection Agency (Durham, USA), Laura Reid, Expression Analysis Inc (Durham, USA), Agnes Viale, Memorial Sloan Kettering Cancer Center (New York, USA), Norma Nowak, Roswell Park Cancer Institute (Buffalo, USA), Kevin Knudtson, University of Iowa (Iowa, USA), William Ward, Environmental Protection Agency (Durham, USA), Jay Tiesman, Procter & Gamble (Cincinnati, USA), Caprice Rosato, Center for Genome Research and Biocomputing, Oregon State University (Corvallis, USA), Aldo Massimi, Albert Einstein College of Medicine (New York, USA), Greg Khitrov, Mount Sinai School of Medicine (New York, USA) and Nancy Denslow, University of Florida (Gainesville, USA)

Development of microarray quality control metrics
Karl Kornacker, Nationwide Children's Research Institute (Columbus, USA)

The ABRF MARG microarray survey 2008: Sensing the state of microarray technology
Chris Harrington, Oregon Health and Science University (Portland, USA), Susan Hester, US EPA (Durham, USA), Nadereh Jafari, Northwestern University (Chicago, USA), Steve Potter, Children's Hospital Medical Center (Cincinnati, USA), Jay Tiesman, Procter & Gamble (Cincinnati, USA), Richard Jensen, Virginia Bioinformatics Institute (Blacksburg, USA), Laura Reid, Expression Analysis (Durham, USA), Aldo Massimi, Albert Einstein College of Medicine (New York, USA), Agnes Viale, Memorial Sloan Kettering Cancer Center (New York, USA), Nancy Denslow, University of Florida (Gainesville, USA)



Mass Spectrometry Core Facility



The interdisciplinary nature of Mass Spectrometry (MS), which crosses the borders of physics, chemistry and biology, and the tremendous technological improvements that it has undergone have led to its increased use in biotechnology and biomedical research.
Classically, MS is considered a tool for the identification and characterisation of molecules. It has been defined as 'the art of measuring atoms and molecules to determine their molecular weight'.
Furthermore, MS has also been used to address questions about conformation and structural biology as mass analysis techniques have the capacity to detect changes in protein conformation under a wide range of conditions. Moreover, the development that MS has experienced in recent years allows intact protein complexes to be directly detected. Therefore, these analytical techniques now permit the study of non-covalent protein-protein and ligand-protein interactions, thereby improving our understanding of the mechanisms of action of these proteins in several biological processes. The capacity of MS to identify and, increasingly, to study protein-ligand interactions is expected to have a significant impact on biological and medical research. The Mass Spectrometry Core Facility provides scientific and technical support to the scientific community at IRB Barcelona, thereby combining the powerful capacity of the new MS techniques in life sciences with ongoing structural and biological research at the Institute.

The Mass Spectrometry Core Facility at IRB Barcelona is equipped with high accuracy and high resolution mass spectrometers, which are used for the identification and characterisation of a broad range of biological species, from small molecules to large biomolecules (eg intact proteins), as well as conformation, structural biology and non-covalent interactions of these biomolecules and complexes. The Facility also plans to include the study of protein biomarkers, which can indicate the presence and progression of a variety of diseases or response to drugs.

Services for IRB Barcelona researchers

The services offered include MS, MS/MS and MS^n analysis using atmospheric pressure ionization techniques (electrospray and APcI) coupled with LC 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 trained by facility members to use mass spectrometers through an open-access system.

The Facility includes three new generation mass spectrometers, equipped with high accuracy and high resolution analysers.

LTQ FT Ultra (Thermo Scientific)
The LTQ-FT Ultra system is a fully integrated hybrid mass spectrometer consisting of a linear Ion Trap Mass Spectrometer, LTQ XL™, combined with a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer. The instrument is provided with ESI, mESI, nanoESI and ApcI ionisation sources and CID, ECD, IRMPD fragmentation technologies.

The whole configuration system has a quaternary micro LC pump attached (Mod Surveyor MS), and a micro-autosampler (Micro AS). For its installation, this mass spectrometer required the handling of 500 l of liquid N_2 and 500 l of liquid He to cool down the magnet from room temperature.

With an accuracy of 0.5-1 ppm at m/z 400, the LTQ-FT has exceptional applications in chemistry for the identification and structural characterisation of compounds. For applications in biology, it permits both bottom-up (proteomics) and top-down approaches for the analysis of intact proteins, including post-transductional modifications (Instrument Mass Range: 50-4000 Da).

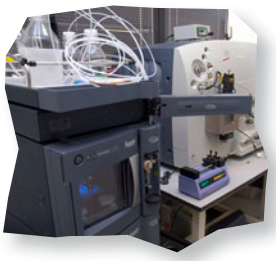
Core Facility Members | **Mass Spectrometry Research Specialist:** Marta Vilaseca | **Research Technician:** Núria Omeñaca

Synapt High Definition MS System (Waters-Micromass)

The Synapt is a hybrid QTOF instrument which has a Tri-wave Cell between the two analysers. This configuration allows high performance tandem MS to be combined with high efficiency 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 has ESI, nanoESI and ESCI™ (ESI, ApcI) capacity. Samples can be introduced by syringe pump or LC inlets. The instrument can also be attached to an Advion Triversa Nanomate, a robot which combines the strengths of LC, fraction collection and chip-based infusion into a single integrated system.

The instrument is used to analyse and study the macromolecular structure and conformation of intact proteins. It also has applications for the study of non-covalent complexes which can survive the transfer from solution to the gas phase thanks to the incorporation of 'ion cooling'. In addition, the Synapt has potential for top-down protein sequencing by increasing the sequence coverage obtained from fragmentation of multiple charged ions with respect to conventional QTOF instruments.



LCT-Premier XE (Waters-Micromass)

The LCT-Premier XE system is an orthogonal acceleration time-of-fight mass spectrometer. The system is provided with ESI, nanoESI (modified to perform H/D experiments) and ESCI™ (ESI, ApcI) capacity. Samples can be introduced by syringe pump or LC inlets. The instrument has a UPLC Acquity Chromatography system attached.

The instrument allows the detection of large single- or multiply-charge species with accurate mass measurements. Its extended mass range up to m/z 30000 allows the detection of large biomolecule complexes. Like the Synapt, it is provided with ion cooling, or intermediate vacuums, which allow the transmission of non-covalent complexes when ejected from solution to the gas phase. This system has also been modified to achieve inert conditions inside the ionization source, in order to allow amide hydrogen H/D exchange experiments and prevent the exchange during ionization and desolvation. These kinds of experiments will permit the study of the dynamic and structural properties of proteins and their complexes.

Mouse Mutant Core Facility

The purpose of the Mouse Mutant Core Facility is to generate murine models of disease and development for researchers at IRB Barcelona and their collaborators. The Facility was initiated in January 2007 and consists of three members of staff, with an additional member joining in March 2008.

The transgenic mouse plays a vital role in fundamental 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, the introduction of 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.

The period from January 2007 to December 2007 has been the first full operational year of the Mouse Mutant Core Facility. During this time, we have grown from 2 to 3 members of staff, with the recruitment of an additional microinjection specialist. This has enabled us to produce over 40 transgenic founder lines during the year. Part of the future service will include the design and cloning of gene targeting vectors. Therefore, we have begun to develop and implement the various molecular techniques required for this aspect of the service. Very recently we have moved into a new fully equipped laboratory space dedicated to the molecular and cellular facets of our work.

Services for IRB Barcelona researchers

The Mouse Mutant Core Facility aims to offer a full and comprehensive range of services for the production of genetically modified mice, including:

Experimental design

We provide consultation on all aspects of design for transgenic and gene targeting projects, such as the design and construction of recombinant vectors, screening for mutations and analysis of mutant mice.

Construction of gene targeting vectors

An eventual aim of the facility will be to offer the construction of various types of recombinant DNA vectors for genome modification. These will include gene knock-out vectors, conditional gene knock-out vectors, modified bacterial artificial chromosomes (BACs), point mutation vectors, knock-in vectors and other types of vectors. Most modifications will be carried out using the techniques of 'recombineering'. The recruitment of a full-time molecular biologist will enable us to fully implement this aspect of the service in the coming year.

Mouse embryonic stem cell culture

The Facility has a dedicated tissue culture laboratory used exclusively for the culture of mouse ES cells and related cell lines. We offer a complete gene targeting service, from transfection of stem cells with gene targeting vectors, selection and picking of drug resistant clones to archiving of clones and preparation of DNA from duplicate plates and expansion of targeted clones prior to microinjection. In addition, the facility offers karyotyping of clones and derivation of novel ES lines as required.

Microinjection

The Facility has two experienced microinjection specialists, who have the skills required to inject plasmid and BAC-based vectors into single-cell embryos, or modified ES cells into blastocysts. These modified pre-implantation embryos are then placed back into the reproductive tract of recipient mice and allowed to develop to term.

The technologies for creating mutant mice are continually being refined and advanced, and it is the aim of the Facility to keep abreast of these developments, and incorporate them into our methodologies where appropriate.

Protein Expression Core Facility

The Protein Expression Core Facility is currently completing its instrument purchasing and is expected to be able to begin operations shortly in order to provide a wide range of services to support IRB Barcelona researchers. Traditionally, researchers tackle a particular problem with a protein in an iterative process of trial and re-design that can potentially be time-consuming and costly. In contrast, the Facility will concentrate on delivering High Throughput (HTP) activities where many variations of an experiment (eg truncations or mutations of a protein) will be run in parallel. The capacity to perform up to ninety-six experimental variations on a theme, in parallel, can significantly decrease the time taken to solve a particular protein-related problem, thus bringing experiments to faster 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 economical and can significantly reduce project and laboratory costs. In the future, the Facility will offer many high quality reagents eg aliquots of competent bacterio-phage resistant *E. coli* strains for cloning and expression, specialized expression media and cloning reagents for use by individual researchers.

The Protein Expression Core Facility commenced purchasing specialised equipment and laboratory instruments in September 2007. All the equipment required for implementation of the first 'phase' (HTP cloning and expression screening) of the Facility was ordered and a number of instruments were operational by the end of the year. A variety of custom-prepared reagents were also purchased and delivered. In addition, an advisory board was created to help direct the diverse research activities in order to ensure maximum benefit to users of the Facility.

Services for IRB Barcelona researchers

The Protein Expression Core Facility plans to offer the following services to IRB Barcelona researchers:

Custom HTP cloning to generate expression vectors

The Facility will provide 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 will allow the Facility to generate a micro-titre plate of 96 expression-ready clones within 1-2 weeks of receiving template and primers. The plate may be comprised of:

- constructs (defined regions plus fusion 'tags') of many different 'target' proteins from a single organism under investigation (ie, a structural genomics approach)
- or, alternatively, multiple constructs of fewer, more complex proteins, where expression may have previously proven difficult and the researcher wishes to explore more expression options.

Constructs may contain defined N- or C-terminal deletions of the proteins in combination with a choice of 'fusion' proteins (eg, Maltose Binding Protein, MBP, Glutathione-S-Transferase, GST, or Small Ubiquitin-like Modifier, SUMO) to aid the solubility and yield of the proteins of interest. The In-Fusion™ cloning technique and the pOPIN suite of vectors (originally developed at the Oxford Protein Production Facility) will be used to generate expression constructs. A His_6 tag is included in all constructs to simplify parallel expression screening and protein purification.

Expression screening in *E. coli*

A micro-titre plate of 96 (Core Facility or user-derived) expression clones can be screened in *E. coli* in approximately one week. The screen generally 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.

Core Facility Members | **Manager:** Nick Berrow | **Research Technicians:** The Protein Expression Core Facility will be recruiting two technicians in 2008.

Expression screening in mammalian cells

A micro-titre plate of 96 (Core Facility or user-derived) expression clones can be screened in *HEK293* cells in 1-2 weeks. The introduction of this process is planned for the third quarter of 2008.

Other services to be introduced during 2008 and 2009 include: recombinant baculovirus generation, expression screening in *Sf9* cells, custom vector production and vectors for expression screening in *P. pastoris* or *K. lactis*.

The Facility also plans to offer many high quality reagents eg aliquots of competent bacterio-phage resistant *E. coli* strains for cloning and expression, specialized expression media (including auto-induction media and seleno-methionine) and cloning reagents for use by individual researchers. The purchase of these items through the Facility should also result in considerable cost savings for researchers compared to normal distributor prices.

Collaborations

Continued development of pOPIN vector suite
Ray Owens, Oxford Protein Production Facility (Oxford, UK)

Other future collaborations include:

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)

The Protein Expression Core Facility is currently advising many groups within IRB Barcelona on cloning and expression techniques and hopes to build many more collaborations within the Institute, the Barcelona Science Park, the University of Barcelona and the Universitat Autònoma de Barcelona in the near future.

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