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2009 Scientific Report

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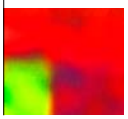

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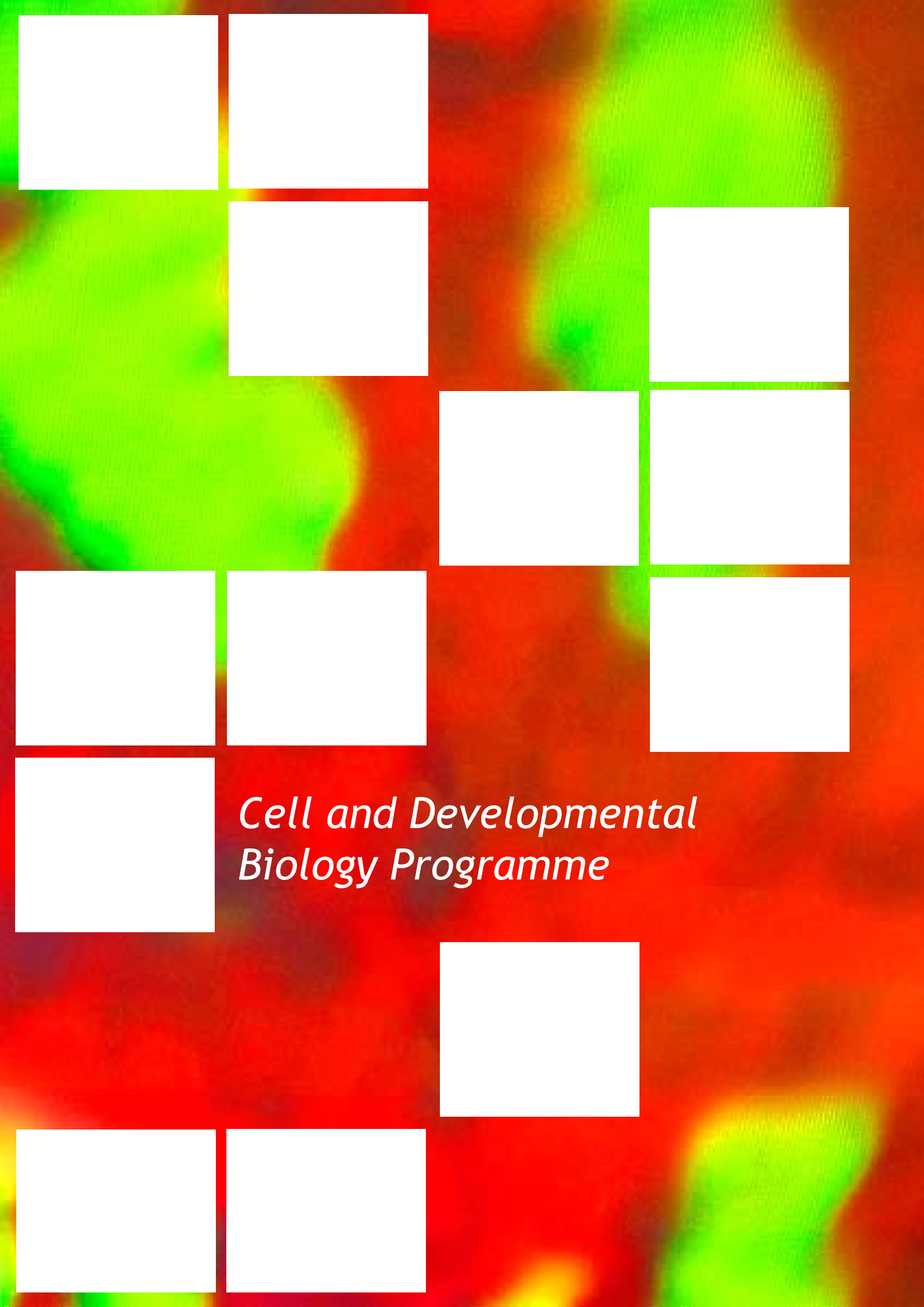
Cell and Developmental
Biology Programme

Ferran Azorín

Chromatin structure and function

Changes in chromatin structure play a fundamental role in the regulation of multiple genomic processes, from gene expression to chromosome segregation and the maintenance of genome integrity and stability. Increasing evidence indicates that alterations in chromatin structure and function are at the root of several human pathologies, including some types of cancer and neurological disorders. Our current knowledge about the regulation of chromatin functions has benefited from the identification of components and mechanisms that covalently and structurally modify chromatin. These include chromatin assembly and remodelling complexes, histone modifications (eg, acetylation, methylation, phosphorylation, ubiquitination, etc) and the corresponding enzymes (eg, HATs, HDACs, HMTs, HDMs, etc), non-histone proteins that recognise specific histone modifications and contribute to the establishment of distinct functional domains (eg, HP1, PC, etc), histone variants that localise to specific chromosomal locations (eg, CenH3, H3.3, H2A.Z, macroH2A, etc), and non-coding RNAs that modify chromatin structure and regulate gene expression. Our research focuses on the study of the molecular basis of chromatin function and its regulation. More precisely, we seek to elucidate the contribution of chromatin to the control of: (i) centromere identity and function; (ii) gene expression and (iii) long-distance genomic interactions.

Centromere identity and function

Centromere function ensures accurate chromosome segregation during mitosis and meiosis, as the centromere dictates the assembly of the kinetochore. This chromosomal protein structure, in turn, regulates the spindle attachment checkpoint (SAC), which delays anaphase onset until all chromosomes are correctly attached in a bipolar fashion to the mitotic spindle. In all eukaryotes, centromeres are characterised by the presence of a specific histone H3 variant (CenH3), which is essential for centromere function and cell viability. CenH3 dictates kinetochore assembly, being required for the localisation of all other inner as well as outer kinetochore proteins tested to date. However, the precise molecular interactions underlying the contribution of CenH3 to kinetochore assembly are not understood. Likewise, mechanism(s) that ensure centromere-only deposition of CenH3 are only partially known. We are currently addressing these questions.

Regulated proteolysis restricts centromeric localisation of CenH3

Centromere identity is determined by the deposition of CenH3, which replaces canonical H3.1 in all eukaryotic centromeres. CenH3 is exclusively found at centromeres. We are addressing how centromere-specific deposition of CenH3 is achieved in *Drosophila*. Contrary to canonical histones, which are deposited during DNA replication, CenH3 incorporates into chromatin independently of DNA replication (see Figure 1). In human cells, the deposition of newly synthesised CenH3^{CENP-A} occurs during mitosis, at late telophase, and early G1. In syncytial *Drosophila* embryos, where no G1/G2-phases are observed, CenH3^{CID} deposition also takes place during mitosis, at anaphase. As a result

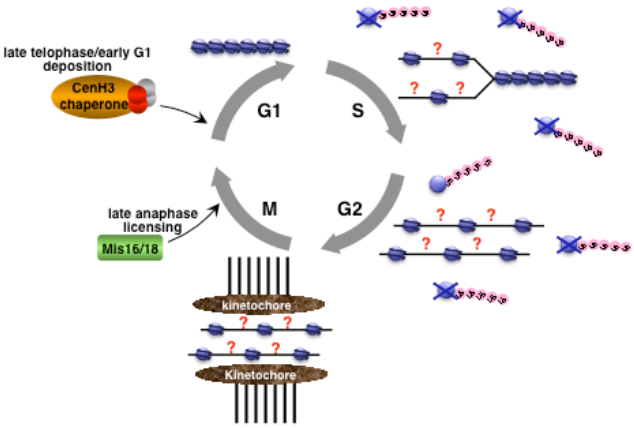

Figure 1. ***Assembly and dynamic behaviour of CenH3-chromatin during the cell cycle.*** *Like other histone variants, CenH3 incorporates into chromatin independently of DNA replication. Deposition of newly synthesised CenH3 takes place during mitosis, at late telophase, or early G1. Specific CenH3-chaperones localise to the centromere coincidentally with the deposition of new CenH3 and mediate the assembly of CenH3-nucleosomes. During assembly, CenH3 might become resistant to proteolysis, which otherwise degrades CenH3 and prevents deposition at non-centromeric sites. Prior to CenH3 deposition, at late anaphase, specific complexes (Mis16/Mis18) appear to modify centromeric chromatin to allow assembly of new CenH3 nucleosomes. During DNA-replication at S-phase, CenH3 concentration at centromeres is diluted and kinetochore assembly occurs before replenishment with new CenH3 nucleosomes. It is unclear whether 'gaps' generated during DNA replication remain nucleosome-free or are filled by replicative H3 nucleosomes. It may also be that CenH3 nucleosomes are disassembled into 'half-nucleosomes' to compensate for this deficit.*

of its loading outside S-phase, CenH3 concentration at centromeres is diluted during DNA replication, thereby generating 'gaps' that can remain nucleosome-free, ready for CenH3 deposition during mitosis, or that can be filled by replicative H3-nucleosomes, which are later replaced by CenH3-nucleosomes. During DNA replication, it is feasible that CenH3-nucleosomes are disassembled into 'half-nucleosomes'. The loading of CenH3 late in mitosis raises several intriguing questions. It implies that kinetochore assembly occurs before centromeres are fully replenished with CenH3-nucleosomes and also that loading occurs in close coincidence with chromosome segregation, thereby suggesting that signalling events occurring during segregation trigger CenH3 deposition. Finally, it raises the question as to how CenH3 assembly takes place during mitosis, when chromatin is believed to be more inaccessible and, in general, refractory to transactions. In this regard, the Mis16/Mis18 complex appears to be involved in licensing the centromeric chromatin for deposition of new CenH3.

The deposition of CenH3 nucleosomes is, however, a promiscuous process, as it may also occur during DNA replication, thereby leading to the mislocalisation of these nucleosomes throughout chromatin. Therefore, additional mechanisms must be present to either prevent the deposition of CenH3-containing nucleosomes at non-centromeric sites during DNA replication and/or to remove them afterwards. We have shown that, in cultured *Drosophila* S2 cells, proteasome-mediated degradation restricts the localisation of $CenH3^{CID}$ to centromeres by removing mislocalised CenH3CID as well as by regulating available $CenH3C^{CID}$ (Moreno-Moreno *et al*, 2006). Moreover, in the fly, proteasome mutants show increased expression and mislocalisation of $CenH3^{CID}$. Proteasome-mediated degradation appears to be an evolutionarily conserved mechanism that regulates available CenH3 to favour its preferential deposition at centromeres. This notion is supported by observation that the levels of $CenH3^{Cse4}$ in the yeast *Saccharomyces cerevisiae* are also regulated by the proteasome and proteolysis-resistant mutants mislocalise throughout chromatin. How is CenH3 proteolysis regulated? In this regard, using a yeast two-hybrid screen, we have identified the interaction of $CenH3^{CID}$ with *partner of paired* (*Ppa*), an F-box-containing protein that interacts with *Skp1*, an evolutionarily conserved component of SCF, an E3 ubiquitin ligase complex. The interaction of $CenH3^{CID}$ with SCF was confirmed *in vitro* by GST-pull down assays, as well as *in vivo*, where SCF mutant conditions result in the overexpression and mislocalisation of $CenH3^{CID}$.

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The contribution of CenH3 to kinetochore assembly

CenH3 is essential for centromere function. Cells deficient in CenH3 fail to localise kinetochore proteins and show strong chromosome segregation defects. These observations indicate that CenH3 is required for kinetochore formation. Kinetochore assembly at the centromere involves complex pathways of hierarchical, sometimes reciprocal, interactions. CenH3 is at the bottom of this network of interactions. However, at present, we are only just beginning to understand its true contribution to kinetochore assembly. Kinetochores are large macromolecular entities. Depending on the organism, kinetochores are composed by dozens to more than a hundred different protein components. Biochemical studies have identified a number of protein complexes/networks that act at several stages of assembly, some being constitutively associated with the centromere throughout the cell cycle while others localise to the kinetochore only transiently during mitosis (Figure 2). The complexes identified include the KNM-network (KNL1, NDC80 and MIND), which is involved in microtubule binding, and, in particular, the NAC/CAD-network, which directly associates with centromeric chromatin. In addition, a third protein-network that regulates chromosome movement, Dam1/DASH, has been identified both in *S. cerevisiae* and *S. pombe*. However, the precise molecular interactions that link CenH3 to kinetochore assembly have not been fully elucidated. We currently seek to identify the components that directly bind CenH3 nucleosomes and the features of CenH3 nucleosomes that these components recognise. In this regard, we have reported that deletion of the N-domain of $CenH3^{Cse4}$ in *S. cerevisiae* causes lethality, and mutation analyses have identified a 33 aa motif (END) within the N-domain of $CenH3^{Cse4}$. This motif is essential for viability and interacts with COMA, a kinetochore complex that is functionally related to NAC/CAD. We have also found that, in *Drosophila*, ectopic targeting of $CenH3^{CID}$ results in the recruitment of BubR1, an evolutionarily conserved outer kinetochore protein that is a core component of SAC, and that this interaction is mediated by the N-terminal domain of $CenH3^{CID}$. Despite its remarkable low degree of conservation, CenH3s from *S. cerevisiae*, *S. pombe* and humans show similar properties. Interestingly, in *S. cerevisiae*, END corresponds to an R-rich motif and the presence of R-rich domains at the N-terminal domain is conserved in CenH3s of distant species.

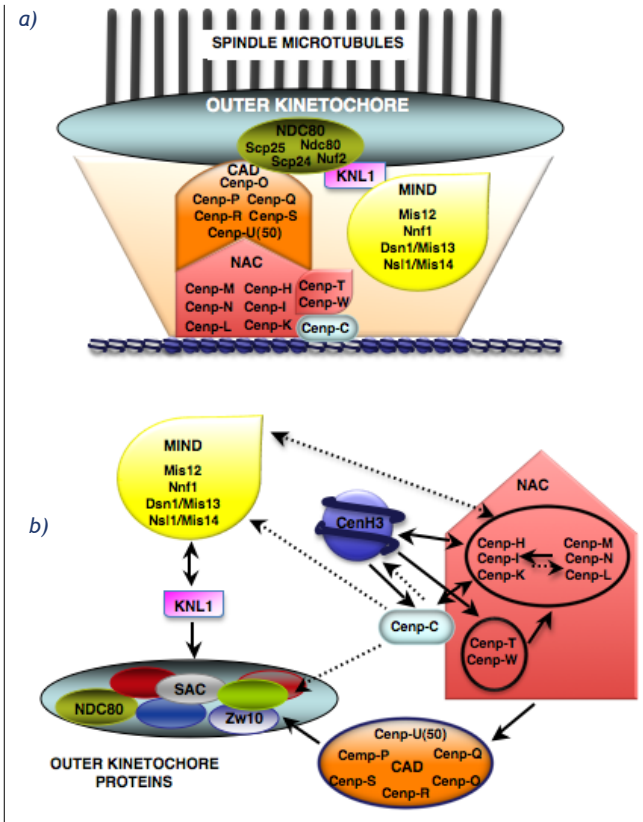


Figure 2. ***Kinetochores are large macromolecular entities.*** *(a) Various protein complexes/networks are known to act at different stages of kinetochore assembly. These include the KNM-network (KNL1, NDC80 and MIND), which is involved in microtubule binding, and the NAC/CAD-network, which directly associates with centromeric chromatin. (b) CenH3 is essential for kinetochore assembly. CenH3 is at the bottom of a complex network of interactions that ultimately leads to assembly of a fully functional kinetochore. Dependencies for centromeric/kinetochore localisation are indicated by solid arrows. Possible interactions, observed only in some species or not fully confirmed, are indicated by dotted arrows.*

Regulation of gene expression: The role of histone lysine (K)-methylation

Most frequently, regulation by chromatin involves the establishment of specific patterns of post-translational histone modifications, which result in the recruitment of regulatory non-histone proteins that bind chromatin and modify its functional state. In this context, methylation at K-residues plays a central role, as it is involved in regulating a wide range of genomic functions, including gene expression.

Characterisation of histone K-demethylases

K-specific histone methyl-transferases (KMTs) were first reported some years ago. However, enzymes capable of antagonising K-methylation (KDMs) have been identified only recently. In the last two years, proteins containing the JumonjiC (JmjC) domain were found to have the capacity to act as KDMs. In this context, we have characterised the demethylase activity of the four JmjC+N-proteins of *Drosophila*: dJARID1/Lid (small imaginal discs), dJMJD2(1), dJMJD2(2) and dJARID2 (Lloret-Llinares *et al*, 2008).

dJARID1/Lid demethylates H3K4me3 and, contrary to what would be inferred from its demethylase activity, contributes to the establishment of transcriptional-competent chromatin states. What are the molecular mechanism(s) underlying the contribution of dJARID1/Lid to transcription activation? The pattern of H3K4me3 depends strongly on the gene-expression status. Thus in active genes H3K4me3 is constrained to a relatively short region spanning the transcription start-site, while inactive genes show no significant enrichment in H3K4me3 throughout the *locus*. How does dJARID1/Lid contribute to the pattern of H3K4me3? Does it bind to coding regions to demethylate H3K4me3, or does the binding take place at promoter regions to regulate dynamic H3K4me3? How is dJARID1/Lid recruited

to chromatin? dJARID1/Lid contains three PHD-fingers (PHD1, PHD2, PHD3) that may mediate binding to methylated-K residues in chromatin. Are they required for binding to chromatin? What methylated-K residues in histones do they bind to? These are some of the questions we are currently addressing.

dJMJD2 demethylates both H3K9me3 and H3K36me3, and dJMJD2(1) influences heterochromatin organisation, as its overexpression in polytene chromosomes induces the spread of HP1 from heterochromatin to euchromatin. The contribution of dJMJD2(1) to heterochromatin organisation is, however, more complex than anticipated, as endogenous dJMJD2(1) is expressed only early in development. In fact, no significant dJMJD2(1) is detected in polytene chromosomes. Moreover, overexpressed dJMJD2(1) is excluded from heterochromatin, localising to multiple euchromatic sites, where it demethylates H3K36me3. In contrast, little is known about the expression, localisation or functional properties of dJMJD2(2). dJMJD2s demethylases may act early in development to establish heterochromatin/euchromatin boundaries. We are currently testing this hypothesis.

The contribution of HP1 proteins to regulation

HP1 is one of the best examples of a regulatory non-histone protein that is recruited to chromatin through the recognition of a specific histone modification, namely di- or tri-methylation of H3K9. HP1 is widely conserved in eukaryotes, with most species containing several isoforms. For instance, *Drosophila* has five HP1 isoforms, three of which are ubiquitously expressed (HP1a, HP1b and HP1c), while the other two (HP1d/Rhino and HP1e) are expressed in the germline. Ubiquitously expressed isoforms show differential chromosomal distributions, as HP1a associates with heterochromatin, while HP1c is excluded from heterochromatin and HP1b is found both at euchromatic and heterochromatic domains. All these observations indicate that current models suggesting that HP1s bind H3K9me2,3 and recruit factors involved in heterochromatin assembly are not likely to apply to all isoforms and functional contexts. In this context, we have shown that, although *Drosophila* HP1c efficiently binds H3K9me2,3 *in vitro*, its binding to chromatin strictly depends on two sequence-specific DNA binding proteins, WOC and ROW, which are putative transcription factors (Font-Burgada *et al*, 2008). HP1c regulates transcription since it extensively co-localises with active RNA polymerase II and H3K4me3, a modification that correlates with active chromatin domains. Moreover, expression-profiling analyses show that HP1c, WOC and ROW extensively co-operate to regulate gene expression during development. To a large extent, the binding of HP1b to euchromatin also depends both on WOC and ROW, which physically interact with HP1b. These findings suggest that HP1c and HP1b co-operate in gene expression regulation (Figure 3). The mechanism(s) behind the contribution of HP1b/c to transcription regulation are currently being studied.

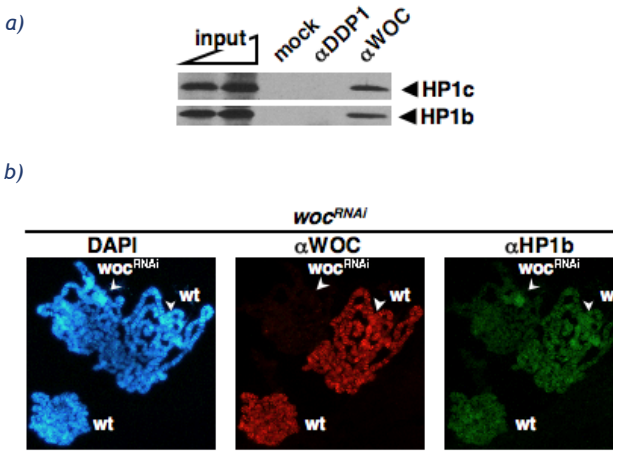


Figure 3. **Drosophila *HP1b interacts with the zinc-finger proteins WOC and ROW.*** *(a) HP1b co-immunoprecipitates with WOC. (b) Binding of HP1b to euchromatin is strongly impaired in the absence of WOC (wocRNAi chromosomes).*

Transcriptional regulation of genes under the control of GAGA factor

GAGA is a *Drosophila* transcription factor involved in many nuclear activities. Expression microarray analyses of GAGA knockdown and overexpression in cells and flies (carried out at the IRB Barcelona Functional Genomics Core Facility) have identified a list of genes that appear to be bona-fide GAGA targets as they are also positive in ChIP-on-Chip experiments. Although we are still analysing the results in flies, with the help of the IRB Barcelona Bioinformatics/Biostatistics Unit, several phenotypical defects have been noted. Among others, growth defects in GAGA-depleted flies that result in smaller wings have been observed in a variety of experimental conditions. We are still analysing other morphological parameters to evaluate whether these statistically significant differences in size (ranging from 10% up to 40%) are general or organ-dependent, and also to assign this and other defects to the genes differentially expressed in the microarray analysis.

Because of the multiplicity of processes in which GAGA factor is involved, we are also studying its molecular dynamics by means of Fluorescence Recovery After Photobleaching (FRAP) and related techniques (with the help of Julien Colombelli at the IRB-PCB Advanced Digital Microscopy Facility). Results obtained in stably transfected cell lines constitutively expressing a GAGA-PAGFP (photoactivatable GFP) fusion protein show that GAGA factor behaves much like a transcription factor. It displays high mobility, lower that free GFP but much higher than core histones, and similar to that shown by linker histone H1. GAGA mobility can be attributed to its binding to DNA because a GAGA mutant at the DBD that abolishes stable interaction with DNA ($GAGA_{DBDKO}$) behaves similarly to GFP (Figure 4). While wt GAGA factor shows a mobile fraction of ~60%, in the case of mutant $GAGA_{DBDKO}$ this fraction increases to ~95%. A ~10-fold difference in their exchange time has also been noted. These results indicate that GAGA factor mobility is restricted by its DNA binding capacity, protein-protein interactions representing a minor contribution, if any. Our results also indicate a half-life estimation of ~13h for wt GAGA factor in cells. This is remarkably long for a transcription factor, thereby suggesting the existence of additional regulatory mechanisms that modulate GAGA activities. A highly dynamic mechanism that may account for this modulation is acetylation. We have shown that, in addition to being phos-

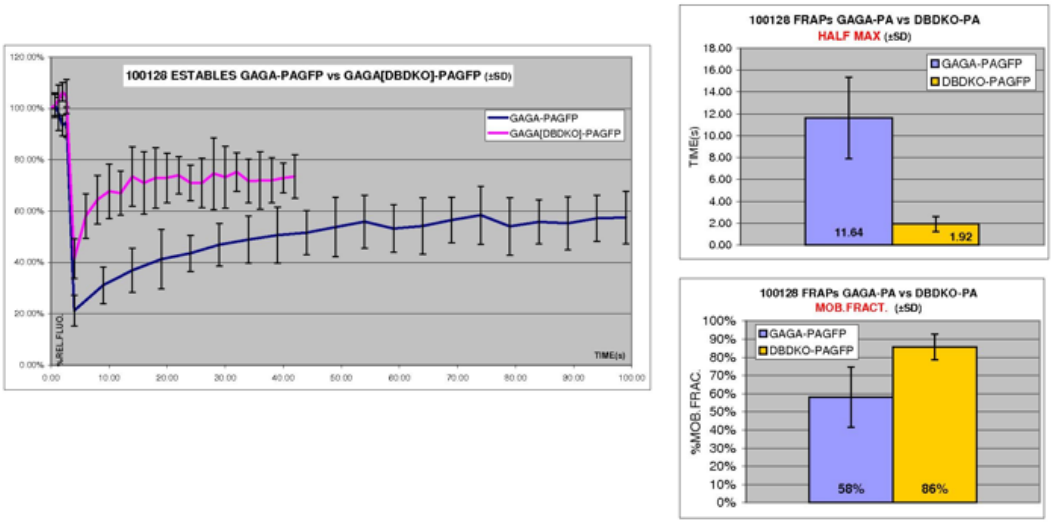


Figure 4. FRAP results of wtGAGA-PAGFP (blue line) and $GAGA_{DBDKO}$-PAGFP (pink line; left panel). Quantification of the exchange half-time and of the mobile fractions for wtGAGA-PAGFP (in blue) and $GAGA_{DBDKO}$-PAGFP (in yellow) are shown in the right upper and lower panels, respectively.

phorylated, GAGA factor is also acetylated *in vivo*. Two lysines at the DNA binding domain that are acetylated by PCAF have been mapped. This acetylation confers a notable reduction in affinity to DNA. Remarkably, while these two lysines are highly conserved in 11 *Drosophila* species, we found that their mutation to glutamines affected neither DNA affinity nor transcriptional activity, thereby suggesting that their conservation is for regulatory purposes.

Scientific output

Publications

Torras-Llort M, Moreno-Moreno O and Azorín F. Focus on the centre: the role of chromatin on the regulation of centromere identity and function. *EMBO J*, **28**(16), 2337-48 (2009)

Other references

Font-Burgada J, Rossell D, Auer H and Azorín F. *Drosophila* HP1c isoform interacts with the zinc-finger proteins WOC and Relative-of-WOC (ROW) to regulate gene expression. *Genes Dev*, **22**(21), 3007-23 (2008)

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)

Moreno-Moreno O, Torras-Llort M and Azorín F. Proteolysis restricts localization of CID, the centromere-specific histone H3 variant of *Drosophila*, to centromeres. *Nucleic Acids Res*, **34**(21), 6247-55 (2006)

Research networks and grants

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

Caracterización biológica de inhibidores de metil transferasas
Spanish Ministry of Science and Innovation, PET2007-0319-02 (2009-2011)
Principal investigator: Ferran Azorín

Cromatina silenciada: Análisis de los factores y mecanismos implicados en su formación y mantenimiento
Spanish Ministry of Science and Innovation, BFU2006-01627/BMC (2006-2009)
Principal investigator: Ferran Azorín

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

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

Collaborations

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

ChIP-seq analyses of histone modifications and chromatin binding proteins in Drosophila
Herbert Auer and David Rossell, IRB Barcelona (Barcelona, Spain)

Jordi Casanova

Signalling in morphogenesis

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

The major outcomes of our research in 2009 can be broken into the following sections:

A functional antagonism between the pgc *germline repressor and torso in the development of somatic cells*

Segregation of the germline is a fundamental event during early development. In *Drosophila*, germ cells are specified at the posterior pole of the embryo by the germplasm. As zygotic expression is activated, germ cells remain transcriptionally silent owing to the polar granule component (Pgc), a small peptide present in germ cells. Somatic cells at both embryonic ends are specified by the torso (Tor) receptor tyrosine kinase, and in tor mutants the somatic cells closest to germ cells fail to cellularise correctly. We have shown that extra wild-type gene copies of pgc cause a similar cellularisation phenotype, and that both excessive pgc and a lack of tor are associated with impaired transcription in somatic cells. Moreover, a lack of pgc partly ameliorates the cellularisation defect of tor mutants, thus revealing a functional antagonism between pgc and tor in the specification of germline and somatic properties. As transcriptional quiescence is a general feature of germ cells, similar mechanisms might operate in many organisms to 'protect' somatic cells that adjoin germ cells from inappropriately succumbing to such quiescence.

Closca, *a new gene required for both Torso RTK activation and vitelline membrane integrity. Germline proteins contribute to* Drosophila *eggshell composition*

The *Drosophila* eggshell is a specialised extracellular matrix (ECM) that surrounds and protects the oocyte and the embryo until its eclosion. In addition, the vitelline membrane, the innermost layer of the eggshell, holds the local determinant required to activate the Torso RTK pathway, which establishes the embryonic terminal regions. We have identified and characterised *closca*, a gene encoding a new member of a group of proteins which act non-redundantly in vitelline membrane biogenesis and in Torso signalling. We have also found that the Nasrat protein, another member of this group, is incorporated into the vitelline membrane, thereby indicating that the eggshell is a shared ECM that receives contributions from follicle cells and the germline. This observation also provides a new scenario that accounts for the long-known contribution of germline products to vitelline membrane biogenesis and to the follicle cell-dependent activation of the Torso receptor.

In vivo *coupling of cell elongation and lumen formation in a single cell*

Fine tubes form inside cells as they reach their target tissues in epithelial ducts and in angiogenesis. Although a very suggestive model of cell hollowing proposes that intracellular lumen arise

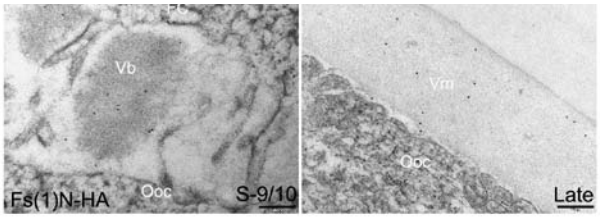


Figure 1. Immunoelectron microscopy of egg chambers bearing a fs(1)Nasrat-HA *construct fixed with high pressure freezing (HPF) and stained with an anti-HA antibody (3F10). In stage 9/10 egg chamber staining is detected mainly in the vitelline bodies. In late egg chambers, labelling is detected in the vitelline membrane. Control egg chambers show no significant staining. Ooc, oocyte; FC, follicle cells; Vb, vitelline bodies; Vm, vitelline membrane. Bars, 200 nm. (image obtained at the Electron Microscopy facility, SCT-UB).*

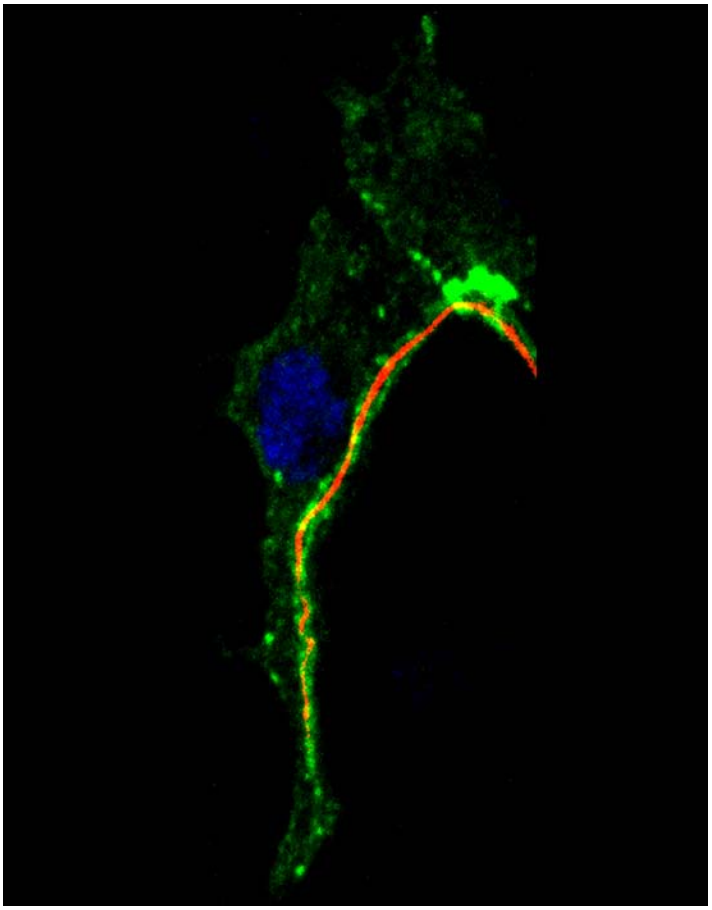
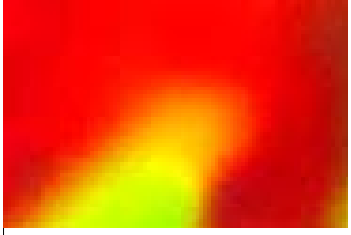


Figure 2. Confocal image of the terminal cell of a dorsal branch of the Drosophila *embryonic tracheal system (nucleus of the terminal cell is detected by DSRF expression in blue). Baz-YFP expression (green) labels the apical membrane and lower green staining allows observation of cell shape. The intracellular lumen is detected by CBP (in red; image from L Gervais, obtained at IRB Barcelona's Advanced Digital Microscopy Core Facility).*



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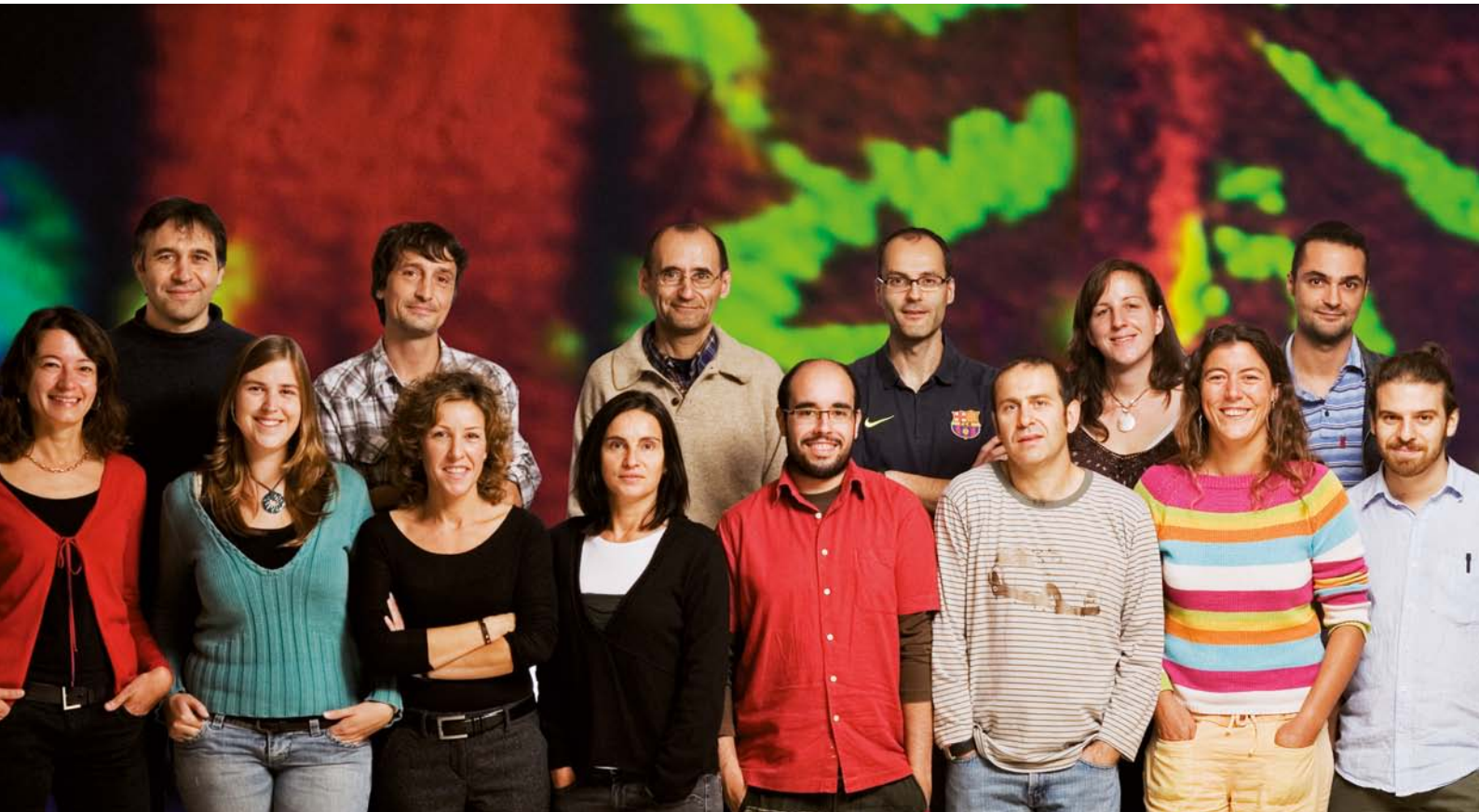

by coalescence of intracellular vacuoles, how these tubes form *in vivo* remains an open question. We have addressed this issue by examining intracellular lumen formation in the *Drosophila* trachea. The main branches of the *Drosophila* tracheal system have an extracellular lumen, as their cells fold to form a tube. However, terminal cells, specialised cells in some of the main branches, form unicellular branches by generating an intracellular lumen. In contrast to the above-mentioned model, we have found that the intracellular lumen arises when an apical membrane grows inwards the cell. In support of this observation, we have detected an appropriate subcellular compartmentalisation of distinct components of the intracellular trafficking machinery. We have also shown that both cellular elongation and lumen formation depend on a mechanism that is based on asymmetric actin accumulation and microtubule network organisation. Given the similarities in the formation of fine respiratory tubes and capillaries, we propose that an inward membrane growth model could account for lumen formation in these two processes.

Scientific output

Publications

De las Heras JM, Martinho RG, Lehmann R and Casanova J. A functional antagonism between the pgc germline repressor and torso in the development of somatic cells. *EMBO Rep*, **10**(9), 1059-65 (2009)

Research networks and grants

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Agency for Administration of University and Research Grants (AGAUR), SGR2008-00343 (2009-2013)
Principal investigator: Jordi Casanova

Cellular properties and morphogenesis. From genes to shape: analysis of morphogenesis in Drosophila *and vertebrates*
Spanish Ministry of Science and Innovation, Consolider CSD 2007-0008 (2007-2012)
Principal investigator: Jordi Casanova

Mecanismos de señalización celular y morfogénesis en el desarrollo de Drosophila
Spanish Ministry of Science and Innovation, Plan Nacional BFU2006-01935/BMC (2007-2009)
Principal investigator: Jordi Casanova

Regulación de los mecanismos celulares en la morfogénesis de Drosophila
Spanish Ministry of Science and Innovation, BFU2009-07629 (2009-2011)
Principal investigator: Jordi Casanova

Collaborations

A functional antagonism between the pgc and torso
Ruth Lehmann, New York University (New York, USA) and Rui Martinhol, Gulbenkian Institute (Lisbon, Portugal)

Identification and characterization of the gene closca
Vitor Barbosa, Gulbenkian Institute (Lisbon, Portugal)

Morphogenesis of the Drosophila *tracheal tube*
Veronique Brodu and Antoine Guichet, Institut Jacques Monod-CNRS (Paris, France)

New elements in the Drosophila *terminal system*
Stephan Luschnig, University of Zurich (Zurich, Switzerland)

Cayetano González

Cell division

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

Cancer stem cells and asymmetric division in Drosophila

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

Drosophila *as a model for cancer research*

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

The first *Drosophila* TSs were identified *in situ* by the growth of massive neoplasms in mutant third instar larvae (Gateff, 1978). The best established assay to discern between benign and malignant growth in *Drosophila* is to implant the affected tissue in a healthy host. This kind of allograft, or 'dauer' culture, is now a standard technique in our laboratory (González, 2007). Upon implantation, wild-type tissue never overgrows, and benign hyperplasias grow slowly, do not invade other tissues, and retain their capacity to differentiate. Malignant neoplasms, in contrast, display autonomous growth, the capacity to migrate

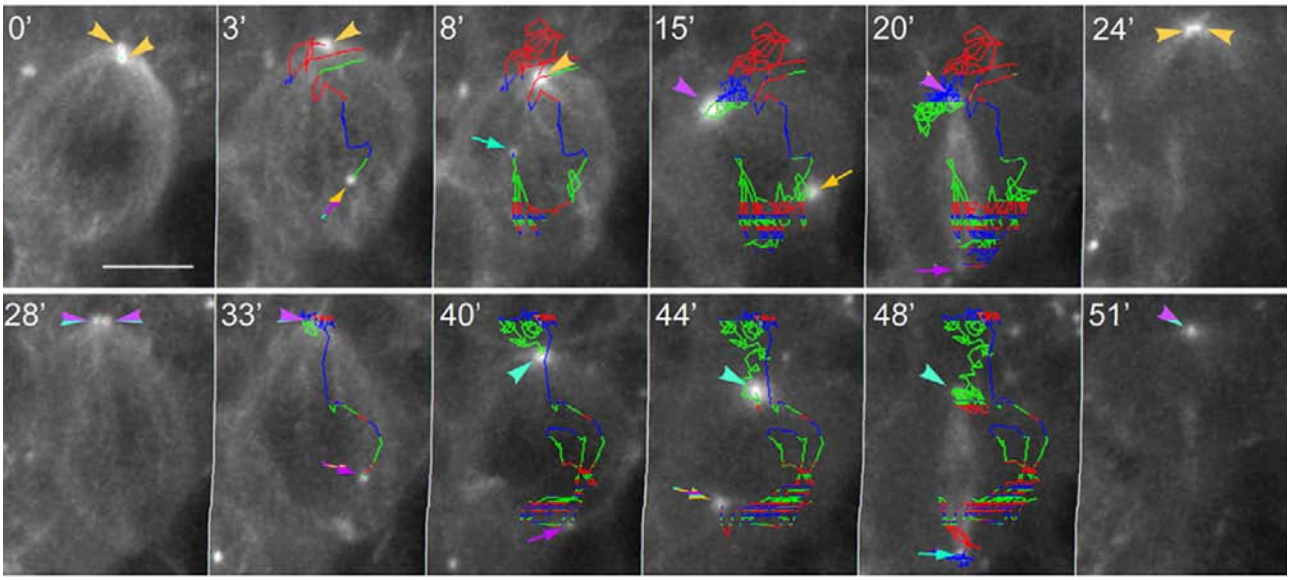

Figure 1. ***Asymmetry of centriole motility in an embryonic neuroblast (NB).*** *Centrosome movement was recorded during two consecutive cell cycles in a delaminated embryonic NB expressing YFP-Asl and GFP-α-TUB84B. Movements of the active apical centrosome (arrowhead) and the motile centrosome (arrow) are traced in red and green, respectively. Scale bar: 5 μm. Taken from: Rebollo E* et al, *2009.*

to and colonise distant organs, and lethality to the host. Moreover, malignant neoplasms frequently become immortal and can be expanded limitlessly through successive rounds of implantation into healthy hosts.

Drosophila *neural stem cells and controlled spindle orientation*

Neurons and glia in the developing central nervous system of *Drosophila* are generated by the self-renewing asymmetric division of neural stem cells (SCs), called neuroblasts (NBs). Acquisition of NB identity imposes a self-renewing asymmetric division mode whereby each of the two daughter cells acquires one of two possible developmental fates: NB or ganglion mother cell (GMC). GMCs can be considered as intermediate progenitors -to use the terminology that is common in vertebrates- that divide, normally just once, to generate cells that eventually differentiate into neurons or glia. Therefore, some of the key processes that characterise SCs occur in *Drosophila* NBs, which are probably the best understood models for animal SC asymmetric division.

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

Pioneering live microscopy studies carried out on embryos demonstrated the first reported mechanism of spindle alignment in *Drosophila* NBs: spindles assemble at an angle that is almost perpendicular to the apicobasal axis of the cell and later rotate to align with it (Kaltschmidt *et al*, 2000). More recent studies carried out on larval NBs revealed a different mechanism, by which spindles assemble already closely aligned along the cortical polarity axis of the NB and only minor rotations refine their alignment before division occurs (Rebollo *et al*, 2007; Rusan and Peifer, 2007). This second mechanism relies on the differential spatiotemporal control of the activity of the microtubule-organising center (MTOC) activity of the NB centrosomes (Rebollo *et al*, 2007; Rusan and Peifer, 2007). Because larval NBs originate from quiescent embryonic NBs, these observations raise the question of when the switch from the rotational to the predetermined spindle alignment mode occurs during development. We have

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recorded embryonic NBs that express centrosome and microtubule reporters, from delamination up to the fourth cell cycle, by two-photon confocal microscopy, and have found that the switch between these two modes occurs in the second cell cycle of the NB, the first one taking place after delamination. Therefore, predetermined spindle orientation is not restricted to larval NBs. On the contrary, this phenomenon occurs in all but the first cell cycle of embryonic NBs (Rebollo *et al*, 2009).

As we have discussed in a recent article published in collaboration with S. Tajbakhsh (Stem Cells and Development Department of Developmental Biology, Institut Pasteur, CNRS, Paris, France), given that old and newly synthesized centrosomes differ in their microtubule nucleating capacity, the asymmetric localisation of epigenetic marks and kinetochore proteins could lead to the differential recognition of sister chromatids and the biased segregation of DNA strands to daughter cells during cell division (Tajbakhsh and González, 2009). Such asymmetric localisation could be linked to biased chromatid segregation, which might also be associated with the acquisition of distinct cell fates after mitosis (Tajbakhsh and González, 2009).

Self-renewing asymmetric division in neural SCs and tumour suppression

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

Subsequent reports from several laboratories have confirmed our results and expanded the number of what is now a long list of genes known to play a role in neural SC polarity and tumour suppression in these cells, including cell fate determinants, some elements of the apical cortex complexes, and kinases that regulate SC polarity like AurA and Polo (Betschinger *et al*, 2006; Lee *et al*, 2006; Wang *et al*, 2006; Wang *et al*, 2007; Bowman *et al*, 2008; Knoblich, 2008; Castellanos *et al*, 2008). The main conclusion derived from these observations is that NBs can be transformed into malignant cells by disrupting their delicately balanced process of self-renewing asymmetric division. This provides additional support to the general hypothesis that malfunction of the asymmetric cell division machinery of SCs contribute tá their transformation. (For a review, see: Januschke and González, 2008.)

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

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

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

Other TSs in Drosophila

In collaboration with AM Martinez and G Cavalli (Institut de Génétique Humaine, France), we have studied the tumorigenic potential of loss-of-function conditions in the PcG gene polyhomeotic (ph). Polycomb Group (PcG) proteins silence critical developmental genes and modulate cell proliferation. Using the *Drosophila* eye as a model system, we show that cells mutant at the PcG polyhomeotic (ph) locus overproliferate, and lose the capacity to differentiate and also their normal polarity. They invade neighbouring tissues and when combined with an activated form of the Ras proto-oncogene they trigger metastasis formation. PcG proteins bind to multiple genes in the Notch pathway and control their transcription as well as Notch signalling. The massive cell-autonomous overproliferation of ph mutant cell clones can be rescued by ectopic expression of a dominant negative form of Notch or by RNAi-mediated Notch repression. Conversely, overexpression of ph induces a small eye phenotype that is rescued by activation of Notch signalling. These data show that ph is a TS locus that controls cellular proliferation by silencing multiple Notch signaling components (Martinez *et al*, 2009).

Scientific output

Publications

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Research networks and grants

Acción complementaria
Spanish Ministry of Science and Innovation, CGL2007-31170-E/ANT (2008-2009)
Principal investigator: Cayetano González

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

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

Centrosoma 3D: Hacia la comprensión estructural y funcional del centrosoma. Consolider Ingenio 2010
Spanish Ministry of Science and Innovation, CENTROSOME 3D, CSD2006-23 (2006-2011)
Principal investigator: Cayetano González

Grup reconegut de la Generalitat
Agency for Administration of University and Research Grants (AGAUR), SGR2005 (2009-2013)
Principal investigator: Cayetano González

Nuevas dianas para el tratamiento del cáncer
Spanish Ministry of Science and Innovation, *Oncológica* (2009-2012)
Principal investigator: Cayetano González

Collaborations

Cancer stem cell and asymmetric cell division (ONCASYM)
Marcos González Gaitán, University of Geneva (Geneva, Switzerland)

Centrosoma 3D
Luís Serrano Pubull, Center for Genomic Regulation (Barcelona, Spain)

Jens Lüders

Organisation of microtubules during cell division and differentiation

Microtubules are a component of the cytoskeleton and are required for many essential cellular processes. To carry out their functions, microtubules are assembled into highly ordered arrays. Defects in the structure and function of the microtubule network are frequently observed in cancer cells and are implicated in certain developmental disorders. Cells contain sites that nucleate the polymerisation of new microtubules and control where and when these structures are made. These nucleation sites provide a unique molecular environment, not only for the control of microtubule nucleation but also for regulating microtubule behaviour, and are thus central to our understanding of microtubule organisation. Microtubule nucleation sites are formed by interaction of the γ-tubulin ring complex, a microtubule nucleator, with microtubule organising centres such as the centrosome. However, the exact molecular composition of microtubule nucleation sites and the spatio-temporal regulation of their assembly are poorly understood. Our long-term goal is to achieve a molecular understanding of the function of centrosomal and non-centrosomal microtubule nucleation pathways in distinct cell types and how defects are linked to disease.

Centrosome maturation

The centrosome, the major microtubule organising centre (MTOC), contributes to the assembly of the mitotic spindle by organising the spindle poles and by nucleating microtubule (MT) polymerisation (Lüders and Stearns, 2007). Centrosomal defects, such as aberrant size, integrity and MT nucleation activity can impair proper spindle assembly and function, and result in genomic instability.

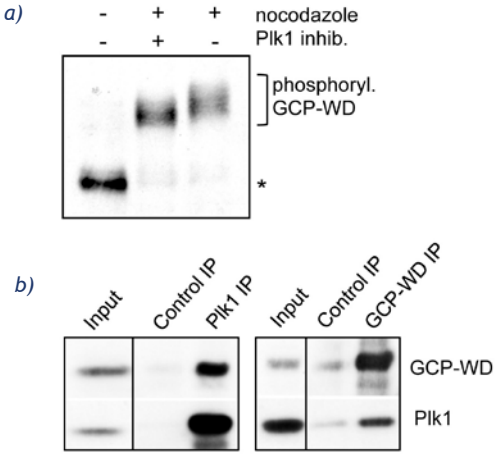

Figure 1. ***Plk1 interacts with GCP-WD in mitosis and contributes to its mitotic phosphorylation.*** *(a) Hela cell lysates immunoblotted for GCP-WD. Cells were synchronised in mitosis by nocodazole treatment and treated with the Plk1 inhibitor BI2536, as indicated. The asterisk shows the position of unphosphorylated GCP-WD. (b) Endogenous GCP-WD and Plk1 were immunoprecipitated from mitotic cell extracts and probed with antibodies against the indicated proteins after Western blotting.*

The centrosome is composed of a pair of barrel-shaped centrioles surrounded by a dense proteinaceous matrix, the pericentriolar material (PCM). The nucleation of MT polymerisation occurs within the PCM and requires recruitment of γ-tubulin ring complexes (γTuRCs) from the cytoplasm. These complexes contain γ-tubulin, a tubulin family member that is not incorporated into the MT polymer, and additional proteins. We have previously shown that the interaction of γTuRCs with centrosomes is mediated by the γTuRC subunit GCP-WD (also known as NEDD1) (Lüders *et al*, 2006).

In late G2 phase of the cell cycle, cells prepare for mitosis by increasing the size and MT nucleating activity of the centrosomes. This process, also termed centrosome maturation, depends on the activity of mitotic kinases such as Polo-like kinase 1 (Plk1), but the molecular details of this pathway are largely unknown.

We have discovered that Plk1 associates with GCP-WD, the γ-tubulin targeting factor, and that Plk1 activity contributes to mitotic phosphorylation of GCP-WD (Haren *et al*, 2009; Figure 1).

Plk1 depletion or inhibition revealed that accumulation of γ-tubulin at centrosomes is regulated by controlling the levels of centrosomal GCP-WD. Surprisingly, GCP-WD mutants that are defective in Plk1 binding and phosphorylation still accumulate at mitotic centrosomes and recruit γ-tubulin. Our studies further

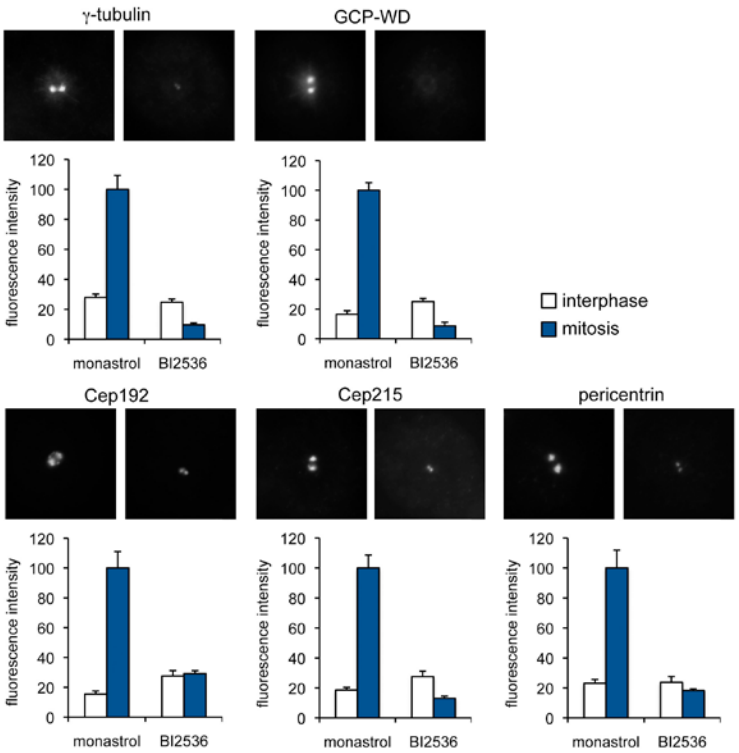

Figure 2. ***Plk1 inhibition interferes with the recruitment of Cep192, Cep215 and pericentrin to mitotic centrosomes****. HeLa cells were treated with monastrol or the Plk1 inhibitor BI2536. They were then fixed and immunostained for GCP-WD, γ-tubulin, Cep192, Cep215 or pericentrin. Fluorescence intensities at the centrosomes were quantified in interphase and in mitosis and plotted as a percentage of the intensities measured in mitotic cells treated with monastrol.*

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show that Plk1 also controls the recruitment of other PCM proteins implicated in centrosomal γ-tubulin attachment (Cep192/hSPD2, pericentrin, Cep215/Cdk5Rap2; Figure 2).

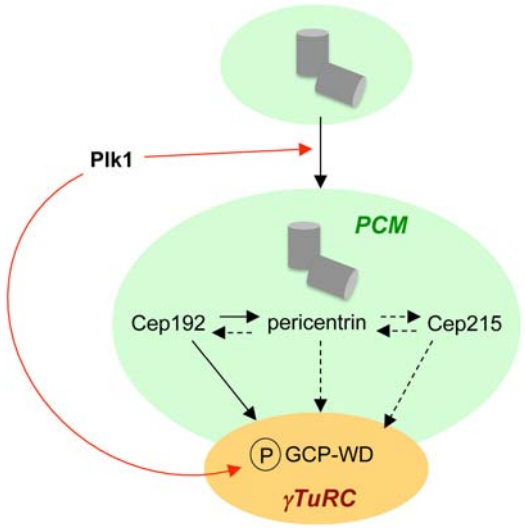

Figure 3. ***Interdependencies of components involved in the recruitment of γ-tubulin complexes to mitotic centrosomes.*** *During centrosome maturation, Plk1 phosphorylates PCM components, which results in the accumulation of PCM, including γTuRC recruitment factors, at mitotic centrosomes. Plk1-dependent phosphorylation of GCP-WD does not affect centrosome recruitment of γTuRCs, which is regulated upstream of GCP-WD. Upstream regulators and potential Plk1 substrates include Cep192, pericentrin, and Cep215. Pericentrin and Cep215 contribute to each other's centrosome localisation and to γTuRC binding. Pericentrin is also important for centrosome localisation of Cep192, which has the strongest impact on centrosome recruitment of GCP-WD/γTuRC (Gomez-Ferreria* et al*, 2007; Zhu* et al*, 2008). Solid black arrows indicate an essential role in the recruitment of the component they are pointing at, dashed black arrows indicate a partial role. Red arrows indicate Plk1-dependent regulation.*

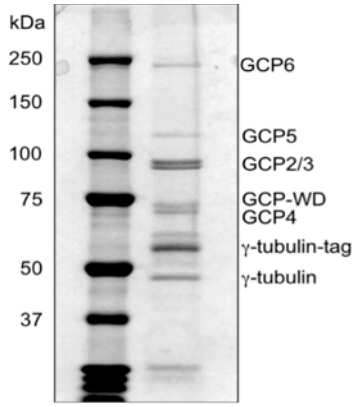

Figure 4. Purified human γTuRC analysed by PAGE and coomassie staining. The positions of the γTuRC subunits are indicated on the right.

Systematic testing of interdependencies between Cep215, pericentrin and GCP-WD for their localisation to mitotic centrosomes using RNAi demonstrated that pericentrin and Cep215 strongly depend on each other for their localisation to mitotic centrosomes and that each protein only partially contributes to γ-tubulin recruitment. In contrast, GCP-WD is an absolute requirement for γ-tubulin recruitment and seems to function more proximal to the γTuRC in the recruitment pathway, most likely downstream of Cep215/pericentrin.

On the basis of our results, we propose that recruitment of γ-tubulin to mitotic centrosomes is regulated upstream of GCP-WD and involves multiple PCM proteins and potentially multiple Plk1 substrates (Haren *et al*, 2009; Figure 3).

Composition and regulation of the γTuRC, a nucleator of MT polymerisation

The γTuRC is a key component required for MT nucleation at MTOCs. γTuRCs are composed of γ-tubulin and additional subunits named gamma-tubulin complex proteins (GCPs) in humans. Assembly of the γTuRC involves subcomplexes composed of two molecules of γ-tubulin and one of GCP2 and GCP3, respectively (gamma-tubulin small complex, γTuSC). According to a current model, the additional GCPs 4-6 promote assembly of ~13 γTuSCs into the higher order ring-shaped γTuRC by forming a stabilising cap on one side of the ring. However, in flies only γ-tubulin and the homologues of GCP2 and 3 are essential and in the absence of the homologues of GCPs 4-6 γ-tubulin is still recruited to centrosomes and supports MT nucleation. Similar results were recently obtained in *Aspergillus*. It is likely that in these cases, as well as in budding yeast, which naturally lacks homologues of GCP4-6, assembly of the γTuSC into a higher order ring structure occurs only upon interaction with MTOCs. In addition to promoting the assembly of cytoplasmic γTuRCs, GCPs 4-6 might have other regulatory functions, for example in controlling MT length and dynamics, either directly or by interaction with other proteins.

It is currently unclear how γTuRC-dependent MT nucleation is regulated in space and time and how cooperation with other components involved in MT assembly and organisation occurs.

To address this issue, we are collaborating with Carme Caelles and Joan Roig (Molecular Medicine Programme, IRB Barcelona). We have developed a new method to purify γTuRC from human cells, which greatly improves yield and purity (Figure 4). By combining the purification of γTuRC with mass spectrometry, we will gain insight into cell cycle-dependent interactions as well as post-translational modifications such as phosphorylation. Our studies are the first to systematically analyse the cell cycle-dependent regulation of the γTuRC and will provide new insight into the spatio-temporal control of MT assembly during spindle formation.

Identification and characterisation of novel centrosome and spindle-associated proteins

The proteomes of specific structures of the MT cytoskeleton have been described, but many proteins still await functional characterisation. Assigning functions to these proteins and analysing

their interplay is crucial for our understanding of the architecture and regulation of complex MT arrays such as the mitotic spindle.

We screened uncharacterised centrosome proteins by expression as EGFP fusions in human cells for their localisation to specific cytoskeletal structures. Using this approach, we identified several proteins that localised to centrioles throughout the cell cycle and to mitotic spindle MTs in mitosis. This unique localisation pattern prompted us to study these proteins in more detail. Three of the proteins identified were recently described by other labs as subunits of the augmin complex (Lawo *et al*, 2009; Uehara *et al*, 2009). This complex associates with a subset of spindle MTs in mitosis and recruits γTuRC to promote proper spindle assembly and mitotic progression. According to a current model, augmin recruits γTuRC via its targeting factor GCP-WD, to induce non-centrosomal MT nucleation from the sides of existing MTs within the spindle. Our results indicate that spindle targeting of the γTuRC is regulated by mitotic phosphorylation of GCP-WD (Lüders *et al*, 2006). We are currently studying the molecular details of this novel pathway. In addition to augmin subunits, our expression screen also identified another uncharacterised protein. Experiments are underway to characterise this novel protein in human cells and to test whether it also plays a role in the augmin pathway.

Scientific output

Publications

Haren L, Stearns T and Lüders J. Plk1-dependent recruitment of gamma-tubulin complexes to mitotic centrosomes involves multiple PCM components. *PLoS One*, **4**(6), e5976 (2009)

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Zhu F, Lawo S, Bird A, Pinchev D, Ralph A, Richter C, Müller-Reichert T, Kittler R, Hyman AA and Pelletier L. The mammalian SPD-2 ortholog Cep192 regulates centrosome biogenesis. *Curr Biol*, **18**(2),136-41 (2008)

Research networks and grants

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

Organización molecular de los sitios de nucleación de microtubulos centrosómicos y no centrosómicos
Spanish Ministry of Science and Innovation, BFU2009-08522 (2009-2012)
Principal investigator: Jens Lüders

Collaborations

Composition and regulation of the human γTuRC
Carme Caelles and Joan Roig, Molecular Medicine Programme, IRB Barcelona (Barcelona, Spain)

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

Marco Milán

Development and growth control

During the development of a given tissue or organ, growth and fate specification are controlled in a coordinated manner by the activity of a discrete number of signalling molecules and their corresponding pathways to give rise to a well-formed structure with a particular size, shape and pattern. The activity or expression of these signalling molecules has to be tightly regulated since deregulation of their activity might cause uncontrolled growth and cancer. The Drosophila *wing primordium, a highly proliferative epithelium that arises as a group of 30-40 cells in the embryonic ectoderm and proliferates during five days to reach a final size of around 50,000 cells, is a highly suitable model system to analyse the mechanisms used to regulate the expression and activity of signalling pathways at a genetic, cellular and molecular level.*

Genetic and epigenetic mechanisms regulating hedgehog expression in the Drosophila *wing*

Drosophila limb primordia are subdivided into adjacent territories called compartments, cell populations that do not mix during development. Short-range cell interactions between adjacent compartments lead to the restricted expression or activity of organising molecules to the compartment boundaries. These organise the growth and pattern of the developing limb primordia.

Two orthogonal compartment boundaries behave as signalling centres and organise the growth and pattern of the developing wing primordium. Activation of the receptor Notch along the dorsal-ventral (DV) compartment boundary and the long-range activity of the signalling molecule Dpp, a member of the BMP/TGF-β family, expressed along the anterior-posterior (AP) compartment boundary, execute the organising activities of these signalling centres. The formation of the AP compartment boundary in *Drosophila* limbs and the restricted expression of Dpp along this boundary rely on asymmetric signalling of the secreted molecule Hedgehog (Hh) from P to A cells. This asymmetry is generated by the complementary activities of Engrailed/Invected and Cubitus interruptus (Ci) transcription factors in P and A cells, respectively. In P cells, the homeodomain proteins Engrailed (En) and Invected induce hh and repress ci expression, the essential downstream component of the Hh signalling pathway. Ci represses hh expression in A cells and, at the same time, confers the capacity to respond to Hh coming from P cells, thereby inducing Hh target gene expression. Thus, P cells express Hh while A cells respond to it.

The Polycomb (PcG) and the Trithorax (TrxG) group of proteins form the basis of a cellular memory system that maintains the transcriptional state of their target genes heritable during development. The genes controlled by the PcG/TrxG system have PcG response elements (PREs), to which these proteins bind and either keep the gene permanently repressed (PcG) or active (TrxG). Some PREs have been shown to maintain the initial transcriptional state of a nearby reporter gene through several rounds of mitosis during development, and as such they have been termed Cellular Memory Modules (CMMs). Interestingly, a 3.4-kb fragment situated upstream of the hh transcription start site behaves as a PRE and exhibits CMM activity. In A cells, PcG

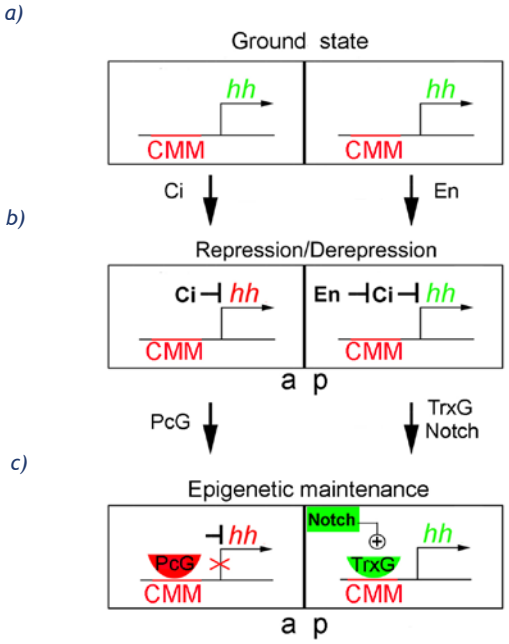

Figure 1. ***Regulation of hh expression in the*** **Drosophila** ***wing: an integrative model****. (a) The ground state in all wing cells is hh transcriptional activation. (b) Ci represses hh expression in anterior cells, while an En/Ci double-repression mechanism maintains hh expression in the posterior compartment. (c) The PcG proteins, presumably through the hh CMM, help to maintain the inactive transcriptional state of hh in anterior cells. In posterior cells, Notch, together with the TrxG proteins and through the hh CMM, contributes to the maintenance of the active transcriptional state of hh.*

genes are involved in maintaining the repressive transcriptional state of hh while TrxG genes maintain the active transcriptional state of hh once it is ectopically induced. However, the role of PcG and TrxG genes in maintaining hh expression in its endogenous expression domain, namely the P compartment, has not been studied to date.

Fernando Bejarano in the lab has analysed the contributions of En, Ci and the PcG/TrxG system in the regulation of hh expression in P cells of the *Drosophila* wing (Figure 1). First, his data indicate that the initial transcriptional state of hh is a direct consequence of the presence or absence of Ci. It is well known that Ci represses hh expression in A cells, while En activity represses Ci expression in P cells. He has demonstrated that in the absence of Ci and En activities, wing disc cells express hh. Thus, En is required in P cells

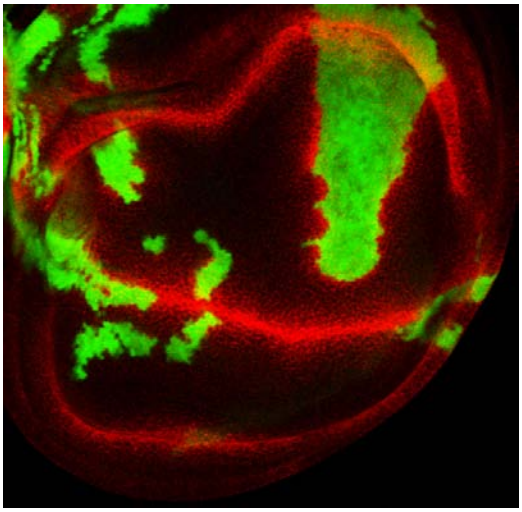

Figure 2. ***A non-autonomous role of Notch.*** *In* Drosophila *wing discs, removal of Notch in a group of cells (in green) induces the activation of the Notch pathway (in red) in nearby wild-type cells.*

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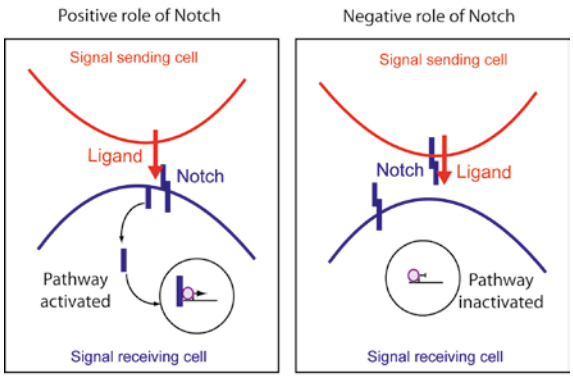

Figure 3. ***Two opposite roles of Notch receptor.*** *While binding of the ligand to the Notch receptor in the neighbouring cell induces the activation of the pathway, binding of this receptor to the ligand in signal-sending cells represses the activation of the pathway in the signal-receiving cells.*

to relieve Ci-mediated repression of hh. He has also presented evidence that *TrxG* genes are involved in the maintenance of hh expression in P cells. Interestingly, epigenetic maintenance of hh expression is positively regulated by the activity of Notch, whose activation is spatially restricted in the wing primordium. He has demonstrated that Notch is required, together with TrxG genes, to maintain hh expression and this is achieved through the previously defined hh CMM. To our knowledge, this is the first study in which Notch has been implicated in regulating the activity of a particular PRE (Bejarano and Milán, 2009).

A role of Notch receptor in ligand cis-inhibition in Drosophila

Notch and its ligands mediate short range cell interactions that play a conserved role in inducing cell fate specification. Several regulatory mechanisms have been described to ensure robust polarised signalling from signal-sending to signal-receiving cells. High levels of ligand expression activate Notch in nearby cells and exert a cell-autonomous dominant negative effect on Notch activity. This regulatory process is called cis-inhibition and it helps to restrict Notch activation to signal-receiving cells. By combining genetic mosaics in the *Drosophila* wing primordium (Figure 2) by means of cell culture assays, Isabelle Becam, in collaboration with Alfonso Martínez-Arias' lab in Cambridge (UK), has presented evidence that Notch promotes the clearance of Serrate ligand from the cell surface and exerts an inhibitory effect on the activity of Serrate expressed in the same cell (Figure 3). These regulatory mechanisms are independent of Notch- mediated transcription and are executed by the extracellular domain of Notch. Isabelle has shown that this process is required to block Serrate-mediated activation of Notch in the signal-sending cell population and helps to restrict Notch activation to signal-receiving cells. All together, her results, in concert with the previous results on ligand-mediated Notch cis-inhibition, indicate that mutual inhibition between ligand and receptor in signal-sending cells helps to block Notch activity in these cells and to restrict receptor activation in signal-receiving cells.

Scientific output

Publications

Bejarano F and Milán M. Genetic and epigenetic mechanisms regulating hedgehog expression in the *Drosophila* wing. *Dev Biol*, **327**(2), 508-15 (2009)

Research networks and grants

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

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

From genes to shape: analysis of morphogenesis in Drosophila *and vertebrates*
Spanish Ministry of Science and Innovation, CSD2007-00008 (2008-2010)
Principal investigator: Marco Milán

Collaborations

A role of receptor Notch in ligand cis-inhibition in Drosophila
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Lafora disease in Drosophila
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miRNAs and growth control
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Lluís Ribas de Pouplana

Understanding the connections between protein synthesis and disease

The machinery of protein synthesis is a central part of the cell and is in many ways linked to a number of human diseases. Our goal is to contribute to the understanding of genetic code biology and its integration within cellular metabolism. In recent years it has become evident that aminoacyl-tRNA synthetases (ARSs) play key roles in cell cycle regulation, the control of gene expression, cell-cell communication and tissue development. This array of new functions has directly linked ARSs and protein synthesis with regulatory cellular pathways and an increasing number of human diseases.

Study of the response mechanisms to proteomic errors in human cells

Misfolded proteins are caused by genomic mutations, aberrant splicing events, translation errors, and environmental factors. The accumulation of misfolded proteins is a phenomenon connected to several human disorders and is managed by stress responses specific to the cellular compartments affected. In wild-type cells mechanisms of stress response can be experimentally induced by expressing recombinant misfolded proteins or by incubating cells with large concentrations of amino acid analogues. We have developed a novel approach for the induction of stress responses to protein aggregation. Our method is based on engineered transfer RNAs (tRNAs) that can be expressed in cells or tissues, where they actively integrate in the translational machinery and cause general proteome substitutions.

We have engineered a battery of mutagenic tRNAs that introduce a range of ten distinct mutations in a human cell type and in a vertebrate embryonic model (in collaboration with Elisa Martí, IBMC-CSIC, and David Rossell, IRB Barcelona). In order to rapidly follow the effect of each tRNA, we have constructed a GFP protein that is not affected by the mutagenic tRNAs and that can be used as a marker of the overall physiological state of the cells. This method allows for the controlled induction of generalised proteome defects in a direct manner, without the potential for other secondary effects. This strategy also permits the uniform introduction of different types of mutations throughout the proteome, and can be applied to the analysis of the timing and grade of stress responses, as well as to the identification of new links between these responses (Figure 1).

This strategy allows the random introduction of mutations of increasing severity in the proteome, without exposing cells to unnatural compounds. We have shown that this approach can be used for the differential activation of the stress response in the endoplasmic reticulum. As an example of the applications of this method, we have applied it to the identification of human microRNAs activated or repressed during unfolded protein stress (Geslain *et al*, 2009).

Generation of an animal model for mitochondrial disease linked to translation defects

The mitochondrial tRNA serylation system is relevant in biomedical terms because mutations in human tRNASer cause mitochondrial encephalomyopathy and lactic acidosis (MELAS). MELAS constitutes a loose family of mitochondrial diseases often produced

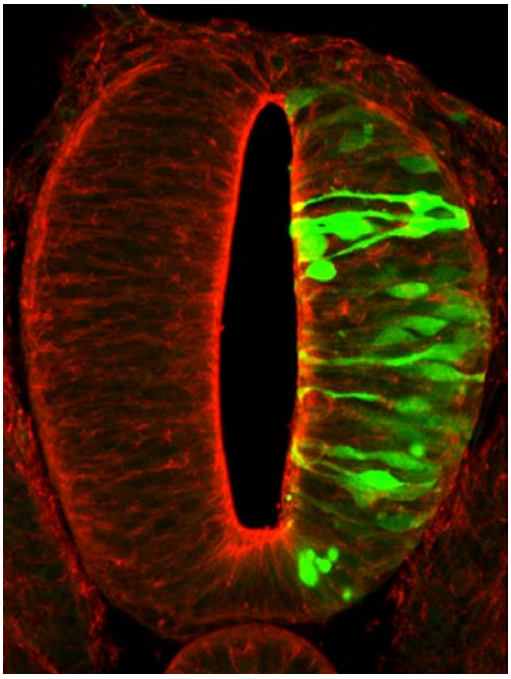

Figure 1. Expression of heterologous tRNAs in a chicken embryo model.

by mutation in mitochondrial tRNA genes and, occasionally, by mutations in nuclear-encoded mitochondrial ARS. The nature of mitochondria makes these disorders extremely difficult to study and this justifies the search for animal models that facilitate the characterisation of these illnesses and the search for palliative measures for their symptoms.

Seryl-tRNA synthetases (SRSs) are the enzymes responsible for the serylation of $tRNA^{Ser}$. SRSs are dimeric enzymes that belong to the subclass IIa of aminoacyl-tRNA synthetases (ARSs). In metazoans, SRSs are among the few enzymes that remain duplicated in the cell, one isoform acts in the cytosol and the second functions in the mitochondria, where it recognises the highly diverged structures of mitochondrial $tRNA^{Ser}$. During the process of building a model for human deficiency in mitochondrial $tRNA^{Ser}$ aminoacylation in *Drosophila melanogaster*, we realised that the *D. melanogaster* genome contains three genes coding for SRS. All three proteins coded by these genes contain the canonical class II ARS motifs, and share a significant level of sequence identity among them. In order to identify the mitochondrial SRS in *Drosophila*, we are characterising the products of these three genes.

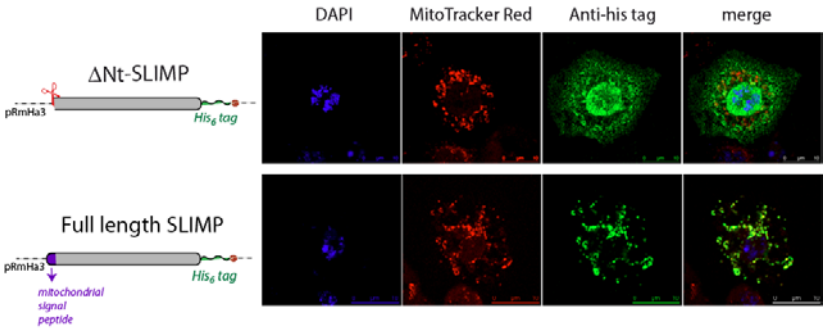

Figure 2. Cellular distribution of SLIMP.

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During 2009 we have functionally characterised one of these proteins, provisionally named SLIMP (SRS-Like Insect Mitochondrial Protein). This polypeptide is not a functional SRS. However, the gene coding for SLIMP is universally present in the insect genomes available, and the protein is expressed in species of diptera and coleoptera. SLIMP expression is developmentally regulated in *D. melanogaster*, and the protein is localised to the mitochondria through a signal peptide that is processed upon translocation.

The function of the protein is essential to *D. melanogaster* as repression of its expression by RNAi treatment is lethal to the animals. However, this lethal effect can be strongly suppressed by supplementing the flies' diet with known anti-oxidant molecules. This observation suggests that depletion of the protein causes oxidative stress in the affected animals (Guitart *et al*, in preparation; Figure 2).

Studying the biology of ARSs in human parasites

ARSs are multi-domain proteins responsible for the aminoacylation of tRNAs. Throughout the phylogenetic tree, ARSs and their related proteins carry out additional cellular functions and may be implicated in other metabolic pathways, cell signalling mechanisms, and developmental processes. The protein synthesis machinery represents one of the most useful targets for the development of new anti-infectives. Several widely used antibiotics exert their function by blocking the protein synthesis machinery.

However, very little is known about the specifics of the protein synthesis machinery in parasites. This lack of information about this key metabolic pathway in parasites clearly hinders the possibility of transferring knowledge in protein synthesis to the development of new drugs directed against the translational machinery of these organisms. We aim to contribute to closing this knowledge gap through the analysis of ARSs in human parasites of the genera *Plasmodium* (the causal agents of malaria), *Entamoeba* (causal agent of amoebic dysentery) and *Trypanosoma* (causal agents of Chaga's disease; Español *et al*, 2009; Novoa *et al*, in press; Castro *et al*, in preparation; Krog *et al*, in preparation).

In eukaryotes, it is well established that cytoplasmic ARSs form multi-enzyme complexes composed of up to eleven individual ARSs. These complexes are structurally stable and assemble around three additional proteins (AIMP1, AIMP2, and AIMP3), which are essential for the formation of the complex and also act as cytokines. Little is known about additional functions of ARS in protozoan parasites, but we are studying a potential example of such functions in the protozoan *Entamoeba histolytica*.

E. histolytica, an amitochondriate unicellular protozoa, is the leading cause of human death by dysentery worldwide. The main clinical complications caused by *E. histolytica* infections are due to the capacity of the parasite to traverse the intestinal wall and infect the internal organs of its host. Protozoan human parasites have evolved a variety of strategies to respond and modulate the immune responses of their host. We have discovered that several *Entamoeba* ARS genes contain a C-terminal domain homologous to the pro-inflammatory human AIMP1 cytokine. In collaboration with Antonio Celada's group (IRB Barcelona), we are characterising the biological role of the AIMP1-like domains in *Entamoeba*.

We have shown that the purified AIMP1-like domain that is coded by *Entamoeba* ARS genes displays significant dose-dependent cytokine activity in mammalian cell signalling assays, at concentrations comparable to those required by human AIMP1 to obtain similar effects. The protein is located in cell periphery and is readily cleaved by *Entamoeba* proteases to release the AIMP1-like domain. Our current hypothesis is that this protein is involved in the formation of liver granulomas during the systemic infection sometimes caused by *E. histolytica* in humans (Figure 3).

Identification of new anti-malarial molecules that target Plasmodium falciparum *ARS*

ARSs are essential enzymes that constitute well-known targets for antibacterial compounds (including the commercial antibiotic BACTRABAM); however, they have not been explored in the search for anti-malarial drugs. The goal of this project is to study the effect of known ARS inhibitors on the *Plasmodium* life cycle, to determine their effectiveness and specificity, and to use these data to identify target enzymes and chemical scaffolds of interest for further chemical design. This project forms part of *Mephitis*, a European consortium funded under FP7 and coordinated by our laboratory.

We have assembled an initial battery of known ARS inhibitors and tested them for their capacity to reduce the growth rate of *P. falciparum* in human blood. In initial tests, we have observed three inhibitory effects. After addition of drugs, borrelidin and the amino acid adenylate analogues showed an immediate effect on *P. falciparum* at pharmacologically relevant concentrations. Pseudomonic acid, as reported for other inhibitors of the protein synthesis machinery, showed a delayed effect that is evident at 96 hours of culture. This observation suggests that

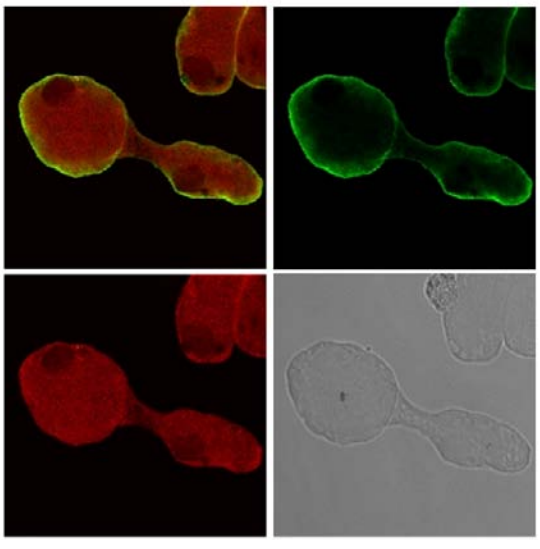

Figure 3. Distribution of AIMP1 (green) in Entamoeba histolytica.

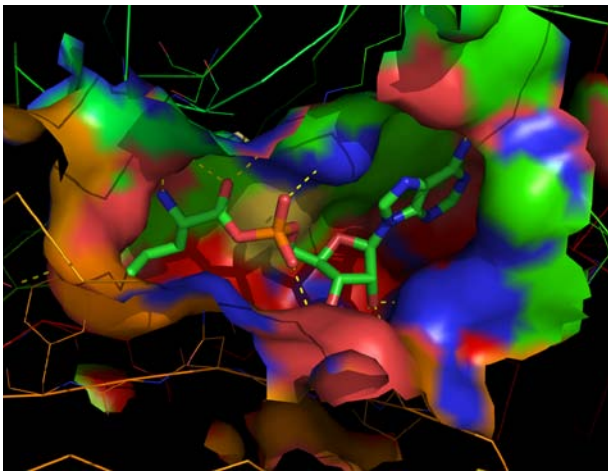

Figure 4. Three-dimensional model of a Plasmodium *ARS active site.*

the drug specifically inhibits the apicoplastic ARS, but not its cytosolic counterpart.

We have analysed the effect of all the components of our chemical battery on human cells and bacteria to determine their species specificity. The tests performed indicate that most of the compounds tested are highly toxic to HeLa cells. However, pseudomonic acid and borrelidin do not affect the growth of human cells despite being effective anti-bacterials. These two compounds have been tested in an animal model of *Plasmodium* infection (in collaboration with José Manuel Bautista, UCM, Madrid). Remarkably, borrelidin is as active as the anti-malarial drug chloroquine in protecting mice from malaria (Camacho *et al*, in preparation). We are characterising the behaviour of this molecule and of chemical analogues. In parallel, we have started a computational drug design project to obtain dual-inhibitors of *Plasmodium* ARS. This is a collaboration project with the Combinatorial Chemistry Laboratory, managed by Miriam Royo at the Barcelona Science Park (Figure 4).

Scientific output

Publications

Español Y, Thut D, Schneider A and Ribas de Pouplana L. A mechanism for functional segregation of mitohondrial and cytosolic genetic codes. *Proc Natl Acad Sci USA,* **106**(46), 19240-45 (2009)

Geslain R, Cubells R, Bori-Sanz T, Álvarez-Medina R, Rossell D, Martí E and Ribas de Pouplana L. Chimeric tRNAs as tools to induce proteome damage and identify components of stress responses. *Nucleic Acids Res*, Epub Dec 8 (2009)

Research networks and grants

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

Implicación de componentes del código genético en patologías humanas
Spanish Ministry of Science and Innovation, BIO2009-09776 (2009-2012)
Principal investigator: 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*
Carlos III Health Institute (ISCIII), BIO2006-01558 (2007-2010)
Researcher: Alfred Cortés

Targeting protein synthesis in the apicoplast and cytoplasm of Plasmodium (Mephitis)
European Commission, FP7-HEALTH-223024 (2009-2011)
Principal investigator and coordinator: Lluís Ribas de Pouplana

Collaborations

Analysis of protein mistranslation in a vertebrate model
Elisa Martí, IBMC-CSIC (Barcelona, Spain)

Characterisation of antimalarial activities of ARS inhibitors
José Manuel Bautista, UCM Madrid (Madrid, Spain)

Characterisation of Mycoplasma penetrans MetRS
Rebecca Alexander, Wake Forest University (Winston-Salem, USA)

Construction of a Plasmodium *KO for PfTRBP111*
Mafgali Frugier, IBMC-CNRS (Strasbourg, France)

Design of new dual ARS inhibitors
Miriam Royo, Barcelona Science Park (Barcelona, Spain)

Role of AIMP1 in Entamoeba *infections*
Antonio Celada, IRB Barcelona (Barcelona, Spain)

Role of AIMP1 in Entamoeba *infections*
Sunghoon Kim, Seoul University (Seoul, Korea)

Statistical analysis of protein mistranslation in eukaryotic cells
David Rossell, IRB Barcelona (Barcelona, Spain)

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Developmental neurobiology and regeneration

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

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

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

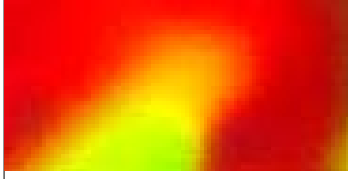
The tyrosine kinase ACK1/PYK1 in brain development and plasticity

Cytosolic tyrosine kinases play a critical role 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 are detected in the brain, particularly in the hippocampus, neocortex, and cerebellum. Electron microscopy studies show that Ack1/Pyk1 protein is expressed both at dendritic spines and presynaptic axon terminals, thereby indicating that this protein is involved in synaptic function. Furthermore, Ack1/Pyk1 mRNA levels are strongly up-regulated by increased neural activity, which points to a role of this protein 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 develop-

ing brain. In neuronal cultures, Ack1/Pyk1 is detected in developing dendrites and axons, including dendritic tips and growth cones. Moreover, Ack1/Pyk1 colocalises with Cdc42 GTPase in neuronal cultures and co-immunoprecipitates with Cdc42s (Ureña *et al*, 2006; De la Torre *et al*, 2006). Activation of integrins by cell adhesion on fibronectin leads to strong tyrosine phosphorylation and activation of Ack. Upon cell stimulation with EGF or PDGF, Ack is tyrosine-phosphorylated and recruited to activated EGF or PDGF receptors, respectively. Moreover, tyrosine-phosphorylated Ack forms a stable complex with the adapter protein Nck via its SH2 domain (Galisteo *et al*, 2006). Taken together, our findings indicate that Ack1/Pyk1 tyrosine kinase has a functional role as an early transducer of multiple extracellular stimuli, and that it may be involved in adult synaptic function and plasticity and in brain development.

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

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



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

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

Dissecting novel Reelin functions in development and neurodegenerative diseases

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

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

Stem cells, neuronal precursor specification, and brain repair

The nervous system is formed by hundreds of types of neurons. The mechanisms by which the different types of neurons are generated and specified remain unclear. We have shown that in the cerebellum the pancreatic transcription factor *Ptf1a* is required for the specific generation of Purkinje cells and interneurons. Moreover, we have reported that granule cell progenitors in the external granule cell layer appear to be unaffected by deletion of *Ptf1a*. Cell lineage analysis in Ptf1a$^{Cre/Cre}$ mice was used to establish that, in the absence of *Ptf1a* expression, E12/E13-proliferating progenitors —normally fated to produce Purkinje cells and interneurons— shift to a granule cell phenotype and aberrantly migrate to the external granule layer. These findings indicate that *Ptf1a* is necessary for the specification and normal production of Purkinje cells and cerebellar interneurons, two essential GABAergic cell types of the cerebellar cortex. We have also established that *Ptf1a* is required for the suppression of the granule cell specification programme in cerebellar ventricular zone precursors (Pascual *et al*, 2007). Given the key role of *Ptf1a* in Purkinje cell specification, we are now exploring whether the induced expression of this gene in neuronal stem cells of distinct origin induces their phenotypic differentiation into a Purkinje cell-like phenotype. If this is the case, we will have devised a method to produce Purkinje cells *in vitro*, thereby facilitating cell therapy approaches in murine models of cerebellar ataxia.

The production of neurons is a temporally restricted process that occurs during embryonic life, except in a few brain areas (the hippocampus, cerebellum, and the subventricular zone). In fact, new granule neurons are produced in the DG of rodents and humans throughout adult life. Understanding the mechanisms that control cell proliferation and neuron production in these areas is crucial to devise therapeutic strategies aimed at producing neurons from the natural 'niches' that contain neural stem cells. Recent studies have also reported adult neurogenesis in the cerebral cortex of healthy animals and after brain injury. We have analysed whether the absence of the synaptic input from the main hippocampal afferents induces neuronal generation in the hippocampus outside the DG and/or regulates the proliferation of DG neuroprogenitors. We have shown that the denervation of the hippocampus does not induce neurogenesis in hippocampal regions other than the DG. However, neuroprogenitor proliferation in the DG is reduced after fimbria-fornix lesions but not after entorhinal deafferentation. This observation sup-

ports the view that neuroprogenitor proliferation and differentiation in the DG are controlled from basal forebrain/septal neurons. We have also studied cell proliferation in the hippocampus of rodents and the intrinsic putative neurogenic potential of EC progenitors. We show that only the DG generates new neurons in the hippocampus. In addition, neurospheres from the EC have the capacity to differentiate into neurons and glia *in vitro* and after transplantation in the adult DG (Fontana *et al*, 2006). In a more recent study, we have identified Netrin1 as a key factor controlling neurogenesis and differentiation of neural stem cells, specifically in the DG (Barallobre *et al*, in preparation) and we are currently focusing our research efforts on elucidating the cellular mechanisms that control symmetrical versus asymmetrical neural cell division.

Scientific output

Publications

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Mathew M, Amat-Roldan I, Andrés R, Santos SI, Artigas D and Soriano E. Signalling effect of NIR pulsed lasers on axonal growth. *J Neurosci Methods*, **186**(2), 196-201, Epub Nov 27 (2009)

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Research networks and grants

CIBERNED (Enfermedades Neurodegenerativas)
Carlos III Health Institute (ISCIII), RCIBERNED (2007-2010)
Principal investigator: Eduardo Soriano

Desarrollo y maduración de la conexión septohipocámpica, implicaciones en la enfermedad de Alzheimer
Carlos III Health Institute (ISCIII), PI081891 (2009-2011)
Researcher: Marta Pascual

Identificació i caracterització d'un nou sistema de senyalització associat a exocitosis i neurotrofines: paper en la generació del dolor
'La Marató TV3' Foundation, MTV3-071410 (2008-2010)
Principal investigator: Eduardo Soriano

Identificación y caracterización de nuevos genes y vías de señalización implicados en el desarrollo cortical
Spanish Ministry of Science and Innovation, SAF2005-00171 (2009-2013)
Principal investigator: Eduardo Soriano

Implicación de las semaforinas transmembranales y sus receptores en plasticidad sináptica y en enfermedades neurales: Estudio celular y análisis de la transducción de señal
Carlos III Health Institute (ISCIII), PI070500 (2009-2010)
Researcher: Ferran Burgaya

Papel de la reelina en la formación de conexiones sinápticas in vitro *e* in vivo *y en el desarrollo de enfermedades neurodegenerativas*
Carlos III Health Institute (ISCIII), PI070715 (2009-2010)
Researcher: Albert Martínez

Papel de la tirosina quinasa Ack1 en la formación de dendritas y axones en neuronas de neocorteza y de cerebelo. Relación con la enfermedad de Alzheimer y los procesos de 'long-term potentiation'
Carlos III Health Institute (ISCIII), PI070942 (2009-2010)
Researcher: Jesús Ureña

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

Potencial del gen Ptf1a/p48 en la regeneración del cerebelo
'La Caixa' Foundation, BM06-335-0 (2006-2009)
Principal investigator: Eduardo Soriano

Collaborations

Functions of the novel tyrosine kinase Pyk1 in brain development
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Interactions between Ephrin and Trk signalling pathways in axonal navigation
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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 Netrin1 and NogoR in neural development and regeneration
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Role of Syntaxin1 and Podocalyxins in axonal guidance and brain development
Thomas Südhoff and José Rizo-Rey, Southwestern University (Dallas, USA) and Esther Stoeckli, University of Zurich (Zurich, Switzerland)

Role of the glycogen synthase enzyme in neuronal function and degeneration
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Role of the pdf1 gene in cerebellar development and repair
Paco X Real, Pompeu Fabra University/IMIM (Barcelona, Spain)

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

Ultrashort lasers, axonal guidance and brain repair
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Structural and Computational Biology Programme

Patrick Aloy

Structural bioinformatics and network biology group

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

Novel peptide-mediated interactions derived from high-resolution 3D structures

Many biological responses to intra- and extra-cellular stimuli are regulated through complex networks of transient protein interactions where a globular domain in one protein recognises a linear peptide from another, creating a relatively small contact interface. These peptide stretches are often found in unstructured regions of proteins and they contain a consensus motif complementary to the interaction surface displayed by their binding partners. While most current methods for the *de novo* discovery of such motifs exploit their tendency to occur in disordered regions, our work focuses on another observa-

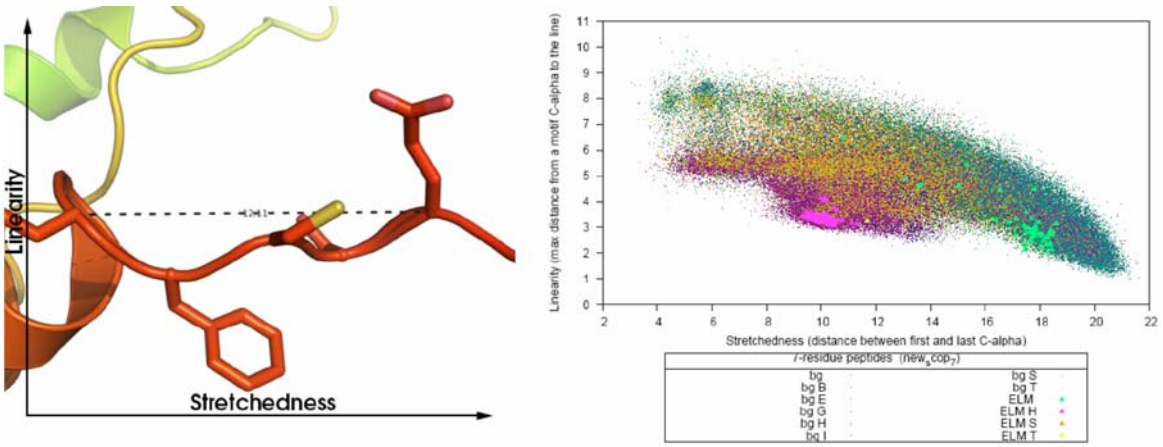

Figure 1. ***Linearity and stretchedness of linear motifs.*** *Linearity is defined as the maximum deviation of any C_α in the motif from the line through the first and last C_α. Comparison of the linearity of known peptides [11] with that of random peptides shows that the linear motifs tend to be more linear, but there is no clear distinction between the two distributions. Stretchedness values for known Eukaryotic Linear Motifs (ELM) peptides tend to be higher than those for random peptides, but again there is no clear distinction. Linearity and stretchedness divide into groups on the basis of the most frequently assigned secondary structure class (DSSP [42]) shown for 7 residue peptides. Combining linearity, stretchedness and secondary structure class with data from the SCOP background (bg), shown as dots, we can observe that known linear motifs fall into distinct regions of the parameter space.*

tion: upon binding to their partner domain, motifs adopt a well-defined structure. Indeed, through the analysis of all peptide-mediated interactions of known high-resolution 3D structure, we found that the structure of the peptide may be as characteristic as the consensus motif and may help identify target peptides even though they do not match the established patterns. Our analyses of the structural features of known motifs reveal that they tend to have a particular stretched and elongated structure, unlike most other peptides of the same length. Accordingly, we have implemented a strategy based on a support vector machine that uses these features, along with other structure-encoded information about interaction interfaces, to propose novel peptide-mediated interactions. Whenever enough information has been available, we have also derived consensus patterns for these interactions -and compared our results with established linear motif sequences and their binding domains. Finally, we have cross-validated our newly derived patterns on interactome network data from several model organisms, and presented a list of 64 peptide-mediated interactions, 47 of which have not been described before, involving 46 distinct domains, along with their respective high-resolution 3D structures and consensus motifs.

Pushing structural information into the yeast interactome by high-throughput protein docking experiments

Recent years have seen the consolidation of high-throughput proteomics initiatives to identify and characterise protein interactions and macromolecular complexes in model organisms. In particular, more that 10,000 high-confidence protein-protein interactions have been described in the roughly 6,000 proteins encoded in the budding yeast genome (Saccharomyces cerevisiae). However, unfortunately, high-resolution 3D structures are available for fewer than one hundred of these interacting pairs. In this project, we expand this structural information on yeast protein interactions by running the first-ever high-throughput docking experiment with some of the best state-of-the-art methodologies. To increase the coverage of the interaction space, we also explore the possibility of using homology models of varying quality in the docking experiments, instead of experimental structures, and assess how they affect the

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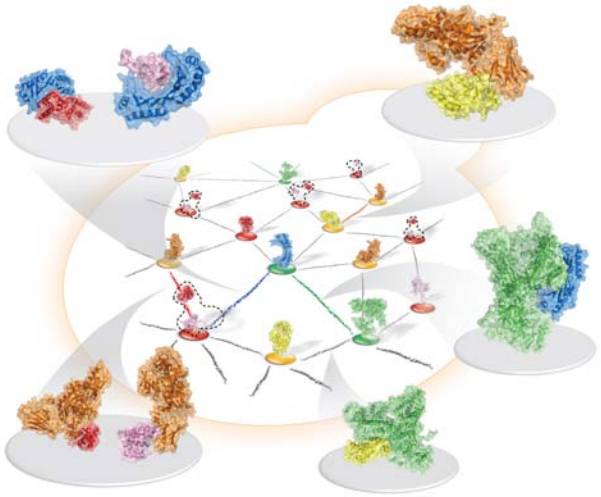

Figure 2. Artistic representation of the structured yeast interactome.

global performance of the methods. In total, we have applied the docking procedure to 217 experimental structures and 1,023 homology models, providing putative structural models for over 3,000 protein-protein interactions in the yeast interactome. Finally, we analyse in detail the structural models obtained for the interaction between SAM1-anthranilate synthase complex and the MET30-RNA polymerase III, to illustrate how our predictions can be used straightforwardly by the scientific community. The results of our experiment will be integrated into the general 3D-Repertoire pipeline, a European initiative to solve the structures of protein complexes in yeast at the best possible resolution. All docking results are available at http://gatealoy.pcb.ub.es/HT_docking/.

Unveiling the role of network and systems biology in drug discovery

Network and systems biology offer a novel way to approach drug discovery by developing models that consider the global physiological environment of protein targets and the effects derived from tinkering with them, without losing the key molecular de-

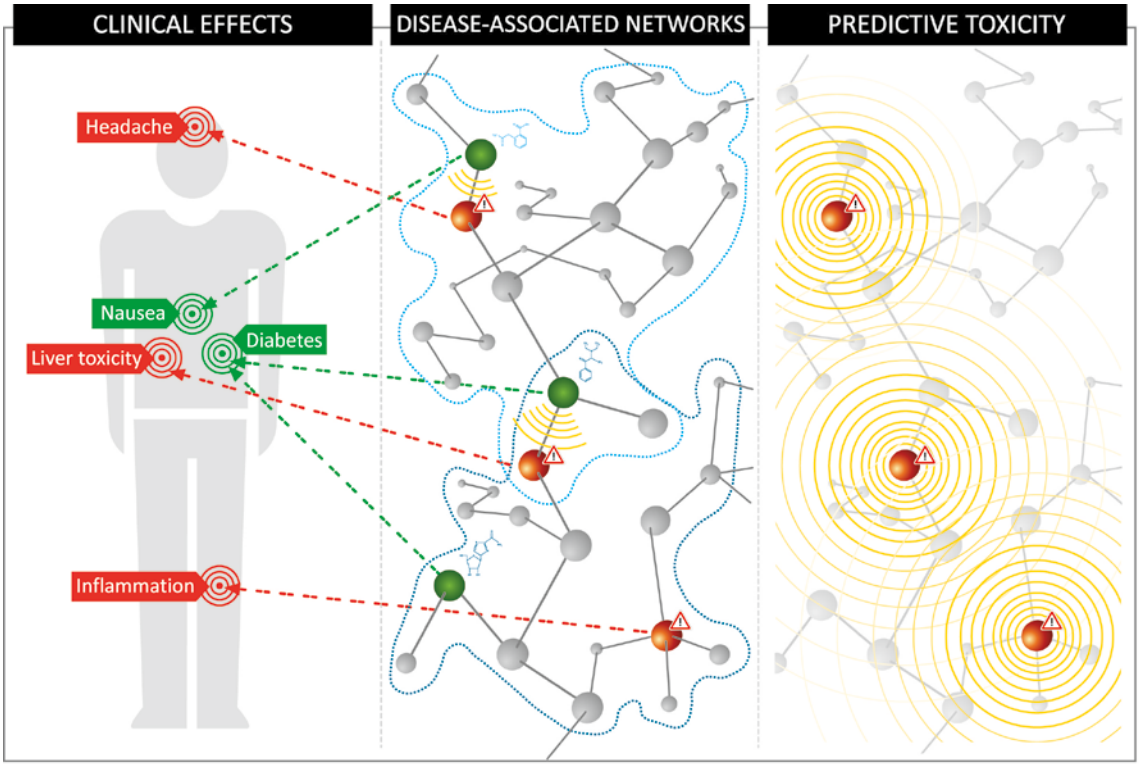

Figure 3. ***Network biology applied to predictive toxicology and drug repurposing****. The disease-associated networks for diabetes (dark blue dashed lines) and nausea (light blue dashed lines) contain several proteins that have been reported to be possible causes of some frequent adverse effects when their normal functioning is affected (red nodes). In addition, the networks contain drug targets annotated to their specific diseases (green nodes). Intense research is carried out to develop models with the capacity to identify the areas of influence of proteins leading to undesired effects and to explore how they are related to network connectivity. If successful, these models could help to discard potential drug targets that are likely to trigger severe adverse reactions at early stages of the discovery process, and to rationally design the toxicity tests required to check the safety of other under the area of influence of a certain red node. In addition, a detailed description of the molecular networks associated with certain diseases can unveil the existence of validated drug targets for a given therapeutic indication in key enclaves of the network that describe a distinct disease, thereby suggesting candidates for drug repurposing (ie, finding new indications for a target).*

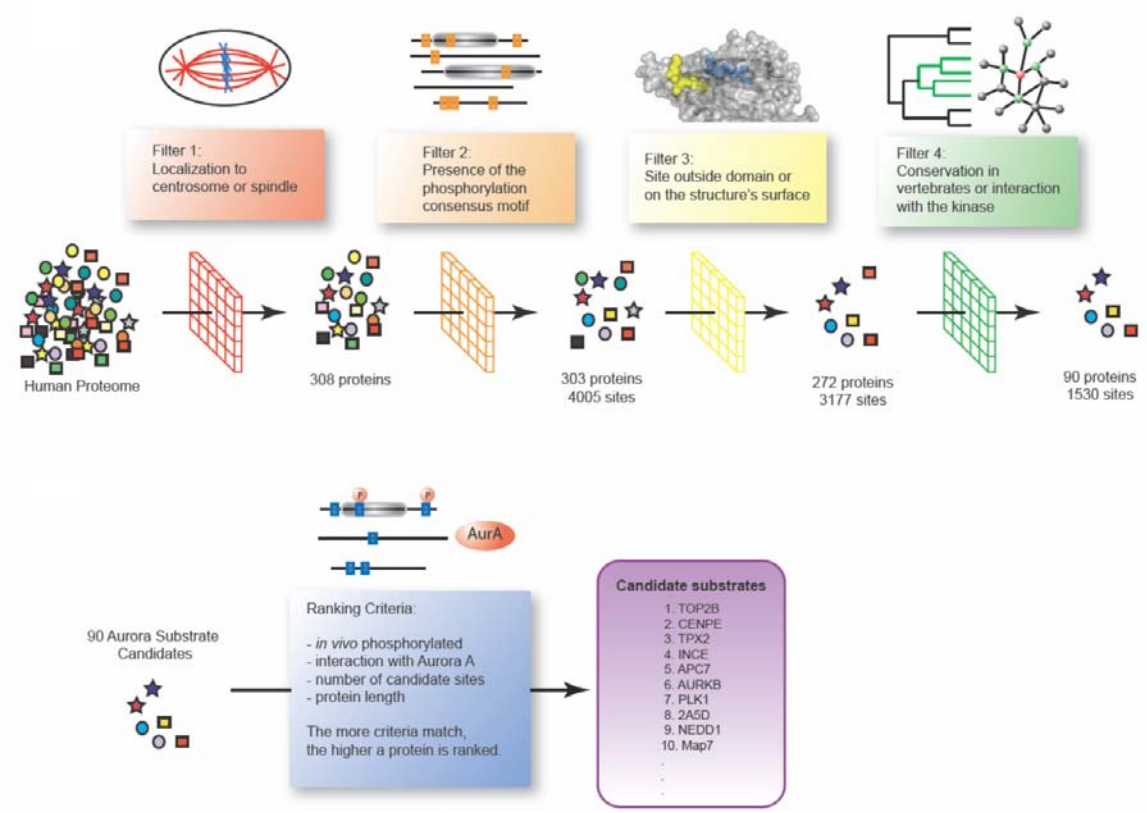

Figure 4. ***Schematic representation of the bioinformatics approach developed to uncover new Aurora kinase substrates.*** *Candidate substrate selection. Aurora substrate candidates were selected based on a series of filters applied to the whole human proteome: presence of an Aurora phosphorylation motif in the sequence, localisation to the centrosome or the spindle, accessibility of the consensus motif and conservation of the potential phosphorylation site among vertebrates. The 90 proposed Aurora substrates were ranked following several criteria.*

tails. In this paper, we reviewed some recent advances in the fields of network and systems biology applied to human health, and discussed their impact on some of the hottest areas of drug discovery. In particular, we claim that network biology will play a central role in the development of novel polypharmacological strategies to fight complex multi-factorial diseases, where efficacious therapies will need to centre on bringing down entire pathways rather than single proteins. In this area of research, we focus mainly on developing novel strategies in the two fields in which we consider network and system biology strategies are most likely to make an immediate contribution: predictive toxicology and drug repurposing.

Uncovering novel substrates for Aurora A kinase

Aurora A is a serine/threonine kinase that is essential for cell cycle progression, centrosome maturation and spindle assembly. Although the participation of Aurora A in these events is well established, its mechanism of action is poorly understood in most cases. Moreover, the relatively small number of known substrates for this kinase does not account for its many roles.

In this study, we present and validate a novel strategy to identify Aurora A substrates, along with their specific phosphorylation sites. We have developed a computational approach that integrates distinct types of biological information to generate a ranked list of 90 potential Aurora substrates, of which 76 are novel. Experimental validation on a randomly selected group of candidates, using *in vitro* kinase assays and mass spectrometry analyses, indicates a prediction accuracy of about 80%. Our results open the way to a better understanding of Aurora A function during cell division and point to novel unexpected roles for the Aurora kinase family. We estimate that our approach can be readily applied to more that 30 human kinases.

Scientific output

Publications

Aloy P and Oliva B. Splitting statistical potentials into meaningful scoring functions: testing the prediction of near-native structures from decoy conformations. *BMC Struct Biol*, **16**, 9-71 (2009)

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Stein A, Panjkovich A and Aloy P. 3did Update: domain-domain and peptide-mediated interactions of known 3D structure. *Nucleic Acids Res*, **37**, D300-4 (2009)

Zanzoni A, Soler-López M and Aloy P. A network medicine approach to human disease. *FEBS Lett*, **583**(11), 1759-65 (2009)

Research networks and grants

A bioinformatics approach to the study of contextual-specificity in protein interaction networks and potential applications to biomedicine and biotechnology
Spanish Ministry of Science and Innovation, BIO2007-62426 (2007-2010)
Principal investigator: Patrick Aloy

A multidisciplinary approach to determine the structures of protein complexes in a model organism
European Commission, LSHG-CT-2005-512028 (2006-2010)
Principal investigator: Patrick Aloy

Grup de recerca emergent
Generalitat de Catalunya, 2009 SGR 1519 (2009-2013)
Principal investigator: Patrick Aloy

Identification of secondary targets and drug design through the structural and functional analyses of biological networks
Spanish Ministry of Science and Innovation, PSE-010000-2009-1 (2009-2010)
Principal investigator: Patrick Aloy

Identification and validation of novel drug targets in Gram-negative bacteria by global search: a trans-system approach
European Commission, 223101 (2009-2011)
Principal investigator: Patrick Aloy

4th CAPRI evaluation meeting
Genoma España (2009)
Principal investigator: Patrick Aloy

Collaborations

Novel strategy for network-based therapeutics
José Manuel Mas, Infociencia & Anaxomics Biotech (Barcelona, Spain)

Novel ways of assessing protein-DNA interactions
Anastassis Perrakis, Nederlands Kanker Instituut (Amsterdam, The Netherlands)

Structural systems biology
Juan Fernández-Recio, Barcelona Supercomputing Center (Barcelona, Spain); M Madan Babu, LMB-MRC (Cambridge, UK); Baldomero Oliva, Pompeu Fabra University (Barcelona, Spain); Miquel Pons, IRB Barcelona (Barcelona, Spain)

Miquel Coll

Structural biology of protein-nucleic acid complexes and molecular machines

Our research effort focuses on the structural characterisation of proteins and nucleic acids, and their complexes in order to elucidate their mechanism of action. Using X-ray crystallography and other structural and molecular biology methods, we address the following: i) the initiation of DNA replication in plasmids by analysing the initiator protein RepB; ii) the termination of transcription in prokaryotes, which is mediated by the Rho factor; and iii) DNA packaging in herpesvirus, in which a molecular machine called terminase plays a key role. In addition, we analyse a number of unique DNA structures formed by DNA three-way junctions and supramolecular drugs.

Replication initiation

DNA replication is a key biological event performed by diverse mechanisms that deal with the incapacity of DNA polymerases to start *de novo* DNA synthesis. Among these mechanisms, rolling circle replication (RCR) is used by a variety of genetic entities (transposons, plasmids, bacteriophages and viruses) that replicate autonomously. RCR is initiated by a triggering reaction that consists of the site-specific cleavage of one of the strands of the DNA duplex at the origin of replication. This endonuclease reaction is catalysed by initiator proteins, which provide the primer for the polymerase to start DNA synthesis. Initiators also participate in the termination of DNA replication in a strand-transfer event.

The structure of the full-length RepB, the replication initiator of a streptococcal plasmid, has been solved by X-ray crystallography and electron microscopy (Boer *et al*, 2009), revealing a hexameric ring molecule where each protomer has two domains (Figure 1). The origin-binding and catalytic domains show an α/β plate fold and are highly mobile, which would account for recognition of two distinct DNA sites. The oligomerisation domains are all-helical, and form a compact ring with a central channel, a feature found in ring helicases and, in particular, in the initiator proteins of oncogenic viruses, such as papillomavirus and SV40. This observation suggests that, in a similar way, RepB encircles one of the DNA strands during replication to confer high processivity to the replisome complex.

Transcription termination

The Rho factor is a ring-shaped ATP-dependent helicase that mediates transcription termination in most prokaryotic cells by disengaging the transcription elongation complex formed by the RNA polymerase, DNA and the nascent RNA transcript. We have solved the structure of the early RNA-free state of Rho from *Thermotoga maritima* to 2.3 Å resolution (Canals *et al*, 2009;

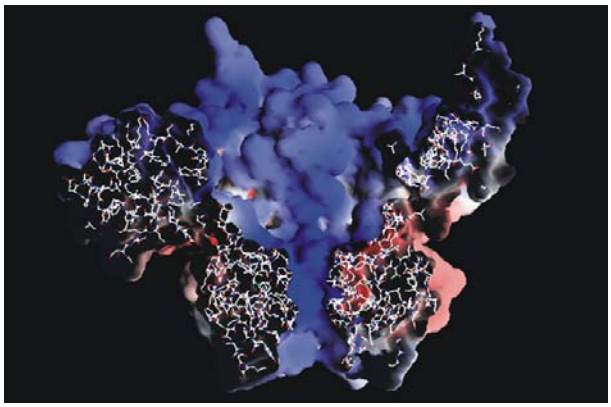

Figure 1. The replication initiator protein RepB.

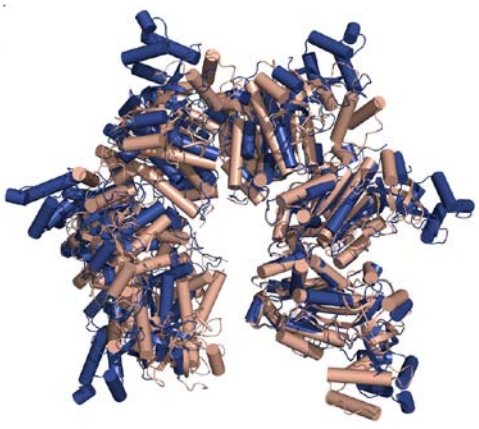

Figure 2. Superposition of the generated T. maritima Rho ring (blue) and the crystallographic ring from E. coli *(green).*

Canals *et al*, in press). This RNA-free structure had eluded crystallisation for many years but now completes previous studies. The structure allows the characterisation of the apo-form of Rho and reveals an RNA-recruiting site that becomes hidden after occupancy of the adjacent specific primary RNA-binding site. These findings suggest an enriched model for mRNA capture that is consistent with previous data (Figure 2).

Herpesvirus DNA packaging

During viral replication herpesviruses package their DNA into the procapsid by means of the terminase molecular machine. In human cytomegalovirus (herpesvirus 5), subunit UL89 of the terminase cleaves the long DNA concatemers into unit-length genomes for encapsidation. We used an ultra-high throughput screening method to identify a soluble and purifiable fragment (UL69-C) from 18,432 randomly truncated constructs (Figure 3). We crystallised this fragment and solved its 3D structure to 2.15 Å resolution (Nadal *et al*, in preparation). The structure reveals that UL89-C belongs to the RNase H/integrase superfamily, a vast group of nucleases and polynucleotidyl transferases that includes resolvases, integrases, transposases, RNA slicers, spliceosomal proteins and Okazaki-fragment cleaving RNases. On the basis of the structural similarities, we tested the inhibitory effect of HIV integrase inhibitors on the nuclease activity of the terminase and found that one of them is a potent inhibitor of UL89, a result that could facilitate the development of novel antiherpes molecules.

Complexes of DNA three-way junctions and supramolecular nano-cylinders

Metallo-supramolecular cylinders are a class of helicate coordination compounds displaying three-fold symmetry and exhibiting high affinity for DNA. We previously showed (Oleksy *et al*, 2006) that one of these cylinders has the capacity to induce three-way junctions in palindromic DNA by occupying the central junction cavity with extraordinary shape complementarity. We have solved a number of additional DNA-cylinder complex structures (Figure 4), all revealing a similar drug-DNA binding mode (Boer *et al*, in press). These structures also indicate that non-covalent cylinder-DNA interactions may be used for the directed assembly of DNA-based nanomaterials.

Research Group Members

Group Leader:
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Lab Technician:
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Scientific output

Publications

Boer DR, Ruíz-Masó JA, López-Blanco JR, Blanco AG, Vives-Llàcer M, Chacón P, Usón I, Gomis-Rüth FX, Espinosa M, Llorca O, del Solar G and Coll M. Plasmid replication initiator RepB forms a hexamer reminiscent of ring helicases and has mobile nuclease domains. *EMBO J*, **28**(11), 1666-78 (2009)

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Other references

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Oleksy A, Blanco AG, Boer R, Usón I, Aymamí J, Rodger A, Hannon MJ and Coll M. Molecular recognition of a three-way DNA junction by a metallo-supramolecular helicate, *Angew Chem Int Ed Engl*, **45**, 1227-31 (2006)

Research networks and grants

A multidisciplinary approach to determine the structures of protein complexes in a model organism (3D-Repertoire)
European Commission, LSHG-CT-2005-512028 (2005-2010)
Principal investigator: Miquel Coll

Ajuts a grups de recerca reconeguts
Agency for Administration of University and Research Grants (AGAUR), 2009 SGR 1309 (2009-2012)
Principal investigator: Miquel Coll

Ayuda complementaria al proyecto europeo 'Spine2-complexes'
Spanish Ministry of Science and Innovation, BFU2007-29798-E (2008-2009)
Principal investigator: Miquel Coll

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

Ayuda complementaria al proyecto 'Una aproximación multidisciplinaria para determinar las estructuras de los complejos proteicos en un organismo modelo'
Spanish Ministry of Science and Innovation, BFU2005-24123-E (2006-2009)
Principal investigator: Miquel Coll

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

Centrosoma 3D: Hacia la comprensión estructural y funcional del centrosoma
Spanish Ministry of Science and Innovation, Consolider CSD2006-23 (2006-2010)
Principal investigator: Miquel Coll

Comparative structural genomics of viral enzymes involved in replication (VIZIER)
European Commission, LSHG-CT-2004-511960 (2004-2009)
Principal investigator: Miquel Coll

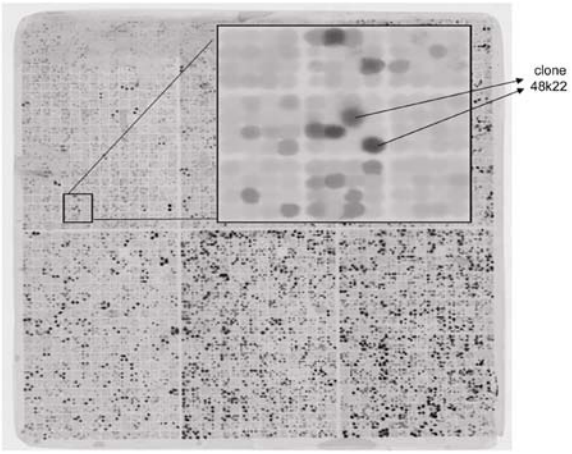

Figure 3. Blot of protein expression screen of 18,432 random deletion constructs of ul89.

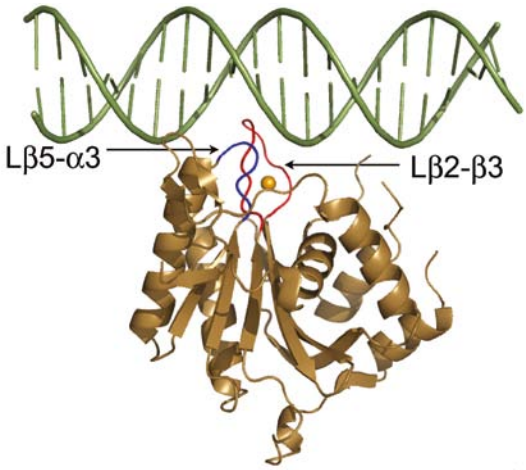

Figure 4. Herpesvirus terminase UL89-C structure with modelled DNA bound.

Estructura de proteínas y complejos de unión al ADN
Spanish Ministry of Science and Innovation, BFU2008-02372/BMC (2009-2011)
Principal investigator: Miquel Coll

Spine2-complexes
European Commission, LSHG-2006-031220 (2006-2010)
Principal investigator: Miquel Coll

TEACH-SG: Advanced strategies for expression of protein complexes in yeast
Agency for Administration of University and Research Grants (AGAUR), ARCS1 00152-TEACH-SG (2009)
Principal investigator: Miquel Coll

Training and education in high volume and high value structural genomics
European Commission, LSSG-CT-2007-037198 (2008-2009)
Principal investigator: Miquel Coll

Collaborations

Centrosomal proteins
Cayetano González, IRB Barcelona (Barcelona, Spain); Isabelle Vernos, Center for Genomic Regulation (Barcelona, Spain); Juan Carlos Zabala, University of Cantabria (Cantabria, Spain)

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

DNA-drug complexes
Joan Aymamí, Universitat Politècnica de Catalunya (Barcelona, Spain); Michael Hannon, University of Birmingham (Birmingham, UK); Per Lincoln, Chalmers University of Technology (Göteborg, Sweden); Cristina Vicent, Instituto de Química Orgánica General-CSIC (Madrid, Spain)

DNA packaging
José Carrascosa, Centro Nacional de Biotecnología-CSIC (Madrid, Spain); Ignasi Fita, IRB Barcelona (Barcelona, Spain)

EM of protein complexes
Montserrat Samsó, Brigham and Women's Hospital, Harvard Medical School (Boston, USA)

Fragment screening
Ernest Giralt, IRB Barcelona (Barcelona, Spain)

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

PETRI project METHISTO
CrystaX Pharmaceuticals (Barcelona, Spain)

Plasmid replication and transfer
Fernando de la Cruz, University of Cantabria (Cantabria, Spain); Gloria del Solar, Centro de Investigaciones Biológicas-CSIC (Madrid, Spain); Manuel Espinosa, Centro de Investigaciones Biológicas-CSIC (Madrid, Spain)

Transcription regulation
Josefa Badia, University of Barcelona (Barcelona, Spain); Ramón Díaz, Centro de Investigaciones Biológicas-CSIC (Madrid, Spain); Margarita Salas, Centro de Biología Molecular-CSIC (Madrid, Spain)

Ignasi Fita

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

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

Structure determination of oxidative stress systems and other large molecular aggregates

Our group works on the structural determination of a number of large molecular aggregates. In particular, in a collaboration project led by Nuria Verdaguer (Institute of Molecular Biology of Barcelona, IBMB-CSIC), we have determined the structures of the seven N-terminal domains of the major protein from the ribonucleoproteic vault particles at almost atomic resolution, and of the intact vault particles at 8 Å resolution (Querol-Audí, Casañas *et al*, 2009). With a mass of 13 Megadaltons and overall dimensions of 400*400*700 Å, the vault complex is the largest ribonucleoprotein particle found in eukaryotes. In mammals, vaults contain three proteins: the 100-kDa major vault protein (MVP), the 193-kDa vault poly(ADP-ribosyl)ating polymerase and the 240-kDa telomerase-associated protein. In addition, at least one small and untranslated RNA is found as a constitutive component. Approximately 75% of the vault particle mass is due to MVP. When rat MVP is expressed in insect cells it has the capacity to produce vault-like particles similar to endogenous vaults. Despite their diverse origin, vaults are uniform in size and morphology, presenting a barrel-like structure with an invaginated waist and two protruding caps, as observed by electron microscopy. The finding that the murine MVP is orthologous to the earlier described human lung resistance-related protein, known to be overexpressed in multiple chemotherapy resistance models, prompted the association of vaults with intrinsic drug resistance. Vaults have also been implicated in the regulation of several cellular processes, including transport mechanisms, signal transduction and immune responses. A large and increasing number of proteins have been found to interact with vaults, in particular, it is now well established that the tumour-suppressor phosphatase PTEN binds to N-terminal repeats R3-R4 of MVP in a Ca2+ dependent manner.

In continuing and highly productive collaboration with Vicente Rubio (Instituto de Biomedicina de Valencia, IBV-CSIC) for more than ten years, we have participated in the structural determination and analysis of a number of kinase and large kinase complexes. Two new papers have derived from this research, one is now in press (Ramon-Maiques *et al*) and the other under revision (Gil-Ortiz *et al*).

We have also continued our research into systems related to oxidative stress, including mammalian peroxidases (Carpena *et al*, 2009) and plant peroxidases (Vidossich *et al*, in press). In particular, we have done extensive work on the catalase-peroxidase system in an attempt to clarify the biochemical mechanisms that allow the function of these moonlight enzymes and also because of its crucial role in the activation of isoniazide, one of the main anti-tubercular treatments. This work has been done in close collaboration with Peter C Loewen at the University of Manitoba (Canada) and Carme Rovira (ICREA scientist at the Barcelona Science Park).

Also in the field of oxidative stress, we have continued our collaboration with Xavier Parés and Jaume Ferrés (Autonomous University of Barcelona-UAB) on the human enzymes P53-inducible quinine oxidoreductase (Porte *et al*, 2009) and aldo-keto reductase AKR1B10 (Xavier-Ruiz *et al*, 2009). The critical tumour-suppressor P53 regulates the expression of P53-induced genes (PIGs), which trigger apoptosis. PIG3 is the only known member of the medium-chain dehydrogenase/reductase super-

family induced by P53 and it is used as a pro-apoptotic marker as the participation of PIG3 in the apoptotic pathway is extensively documented. In the study by Porte *et al*, we found that *in vitro* activity and *in vivo* overexpression of PIG3 leads to the accumulation of reactive oxygen species (ROS). Accordingly, an enzymatically inactive PIG3 variant (Ser151Val) did not produce ROS in cells. This observation supports the notion that PIG3 action is exerted through oxidative stress produced by its enzymatic activity, thus providing essential information for the potential control of apoptosis. Aldo-keto reductases (AKRs) are monomeric NAD(P)H-dependent enzymes. These molecules exert detoxifying activity and therefore contribute to phase I drug metabolism. AKR1B10 (aldose reductase-like or human small intestine reductase) is the most active AKR with all-trans-retinaldehyde, a crucial molecule in the retinoic acid synthesis pathway. AKR1B10 is induced in several types of cancer and is proposed to be involved in the onset of carcinogenesis, thus stressing its potential use as a diagnostic marker for smoking-related lung cancer and as a potential therapeutic target.

Deciphering membrane proteins through X-ray crystallography

For the last few years, our laboratory has devoted considerable effort to membrane proteins, a major challenge in protein crystallography. Some very promising results have now been obtained thanks to close collaboration with Manuel Palacín (Molecular Medicine Programme, IRB Barcelona), who leads research on the structure-function relationship of amino acid transporters.

The new structural and mechanistic insights into the PKC α-C2 domain association (Guerrero-Valero *et al*, 2009) and on the prokaryotic secreted lipoxygenases (Carpena *et al*, in preparation) have also contributed to our experience on X-ray crystal studies of membrane and membrane-related proteins.

Research Group Members

Group Leader:
Ignasi Fita

Postdoctoral Fellows:
Xavi Carpena, Antonio Rodríguez

PhD Students:
David Aparicio, Bárbara Machado, Luca Martinelli

Lab Technician:
Mª Queralt Garcia

Visiting Student:
Maria Adell (Spain)

Scientific output

Publications

Carpena X, Vidossich P, Schroettner K, Calisto BM, Banerjee S, Stampler J, Soudi M, Furtmüller PG, Rovira C, Fita I and Obinger C. Essential role of proximal histidine-asparagine interaction in mammalian peroxidases. *J Biol Chem*, **284**(38), 25929-37 (2009)

Guerrero-Valero M, Ferrer-Orta C, Querol-Audí J, Marin-Vicente C, Fita I, Gómez-Fernández JC, Verdaguer N and Corbalán-García S. Structural and mechanistic insights into the association of PKCalpha-C2 domain to PtdIns(4,5)P2. *Proc Natl Acad Sci USA*, **106**(16), 6603-07 (2009)

Porté S, Valencia E, Yakovtseva EA, Borràs E, Shafqat N, Debreczeny JE, Pike AC, Oppermann U, Farrés J, Fita I and Parés X. Three-dimensional structure and enzymatic function of proapoptotic human p53-inducible quinone oxidoreductase PIG3. *J Biol Chem*, **284**(25), 17194-205 (2009)

Querol-Audí J, Casañas A, Usón I, Luque D, Castón JR, Fita I and Verdaguer N. The mechanism of vault opening from the high resolution structure of the N-terminal repeats of MVP. *EMBO J*, **28**(21), 3450-57 (2009)

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Ruiz FX, Gallego O, Ardèvol A, Moro A, Domínguez M, Álvarez S, Álvarez R, de Lera AR, Rovira C, Fita I, Parés X and Farrés J. Aldo-keto reductases from the AKR1B subfamily: retinoid specificity and control of cellular retinoic acid levels. *Chem Biol Interact*, **178**(1-3), 171-77 (2009)

Research networks and grants

Ajuts a grups de recerca reconeguts
Agency for Administration of University and Research Grants (AGAUR), 2009-SGR-1309 (2009-2012)
Principal investigator: Ignasi Fita

Análisis estructural de las proteínas peroxisomales: Enzimas metabólicos y peroxinas
Spanish Ministry of Science and Innovation, BFU2009-09268 (2009-2012)
Principal investigator: Ignasi Fita

Biología estructural del peroxisoma
Spanish Ministry of Science and Innovation, BFU2008-01539 (2009)
Principal investigator: Ignasi Fita

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

Collaborations

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

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

Oxidative stress-related systems
Peter C Loewen, University of Manitoba (Winnipeg, Canada); Carme Rovira, ICREA-PCB (Barcelona, Spain)

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

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

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

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

Maria Macias

Protein NMR spectroscopy laboratory

Inter- and intra-cellular communication is fundamental for the existence and survival of multi-cellular organisms, and defects in the process often lead to disease. At the molecular level, the basis for transferring information is the organisation of complex networks, many of which are based on protein-ligand interactions performed by domains (independently structured fragments of proteins) specialised in the recognition of specific sequence targets. Our research is devoted to the study of protein complexes involved in degradation processes, splicing, and transcription, and also the regulatory mechanisms behind these interactions. For these purposes, we apply solution nuclear magnetic resonance spectroscopy in conjunction with other biophysical and biomolecular techniques.

Inter-molecular interactions in E3 Ubiquitin ligases

Smad proteins function as intracellular signalling effectors for the TGF-β superfamily of secreted polypeptides. As a result of their capacity to bind DNA and to induce transcriptional responses through interactions with other transcription factors, Smads act as transcription factors. Smads are modular proteins containing two conserved MH1 and MH2 domains, but they differ in the linker sequence connecting these domains. Accumulating evidence suggests that E3 ubiquitin ligases are critical regulators of transcription factors and growth factor receptors. Ubiquitination occurs through a three-step process involving

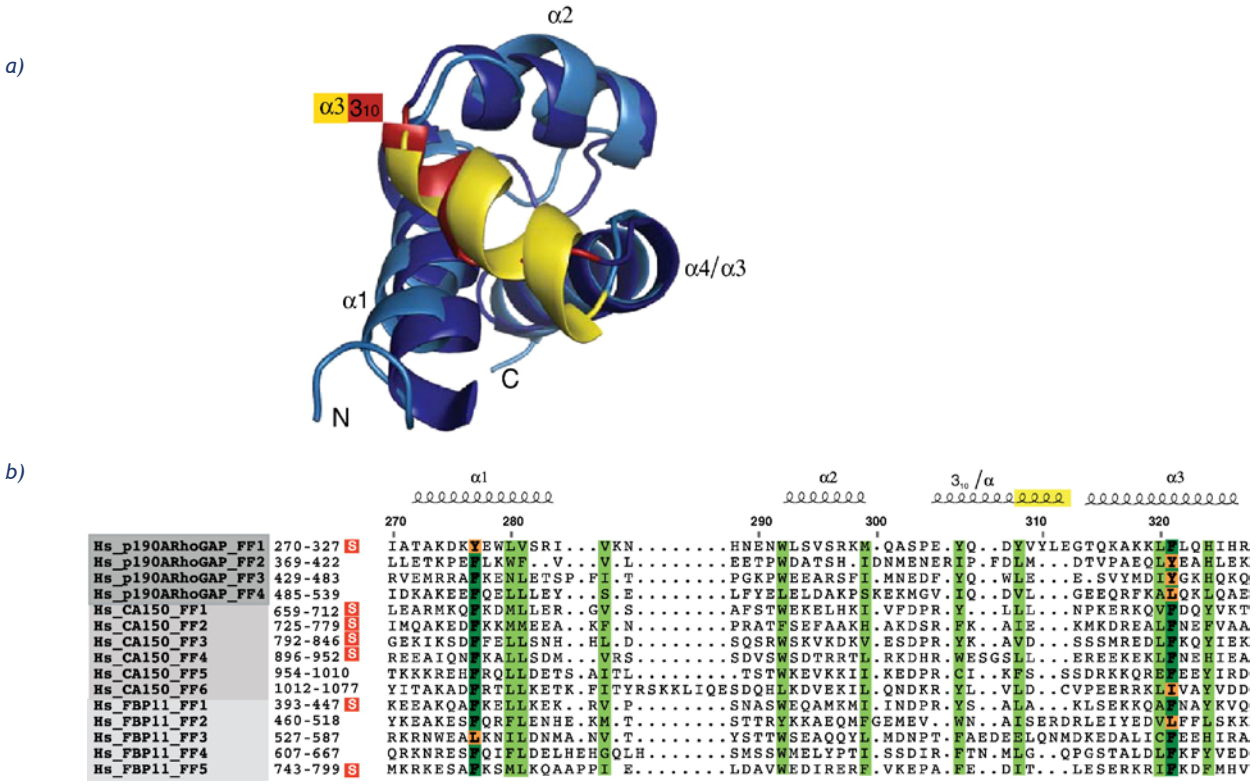


Figure 1. ***Comparison of RhoGAPFF1 with other FF domains.*** *(a) Superimposition of the typical 3_{10} helix (in red) of the FBP11FF1 domain (in dark blue) and the extended a helix (in yellow) that replaces the 3_{10} helix in the RhoGAPFF1 domain (in sky blue). The residues that form part of this extended a helix are marked in a yellow box in the alignment in (b). (b) Sequence alignment of FF domains from human p190-A RhoGAP, CA150, and FBP11. The alignment was generated with ClustalX and edited manually.*

ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. Only the HECT domain-containing E3 ligases recognise and also directly catalyse the transfer of ubiquitin to the substrate. Thus, much research effort has focused on the identification of motifs in the targets that are recognised by the ligases or on studying whether a given ligase recognises one or more targets. In the case of ubiquitin ligases belonging to the Nedd4 family and the Smads, the interaction between the ligase and the target is driven by contacts from the WW domain of the ligase towards the PY motif of the Smad proteins. However, additional contacts from other regions in the proteins or from auxiliary proteins forming transient complexes may control the specificity of the interactions. We seek to elucidate the factors involved in controlling this specificity. However, in order to fully understand how the interactions occur at a molecular level, the actors needed and their order, a combined approach of *in vivo* experiments with detailed structural work is required.

In this context, Joan Massagué and co-workers identified Nedd4L as the ubiquitin ligase responsible for the polyubiquitination of Smad2/3. They also mapped the interaction domains of Nedd4L and Smad3 using a series of expression vectors encoding several fragments of Nedd4L and Smad3. When expressed in HEK293T cells, the second WW domain (WW2) of Nedd4L bound to the Smad3 linker region, whereas the other three WW domains, the C2 domain, and the HECT domain did not. Mutation of the PY motif (PPGY to AAGY) abolished this interaction, as did mutation of the four-linker phosphorylation sites in Smad3. Furthermore, using Smad3 constructs with individual mutations in these phosphorylation sites or with mutation of all these sites but one, they determined that T179 is the only phosphorylation site required for the Smad3-Nedd4L interaction. T179 (T220 in Smad2) lies directly upstream of the PY motif, suggesting that the WW2 domain of Nedd4L specifically recognises a phosphothreonine-PY (pT-PY) motif in Smad2/3.

On the basis of this information, and in order to quantify the interactions shown to occur *in vivo*, we measured the affinity of the four individual Nedd4L WW domains for synthetic peptides comprising 13 amino acids, containing the T-PY motif of Smad2 or Smad3 with either a threonine or a phosphothreonine residue. Isothermal titration calorimetry analysis revealed high affinity of the WW2 domain for the pT-PY motif

Research Group Members

Group Leader:
Maria Macias

PhD Students:
Eric Aragón, Albert Escobedo, Nina Görner, Jordi Mas

Masters Student:
Tiago López

Research Assistant:
Pau Martín

Lab Technician:
Lidia Ruiz

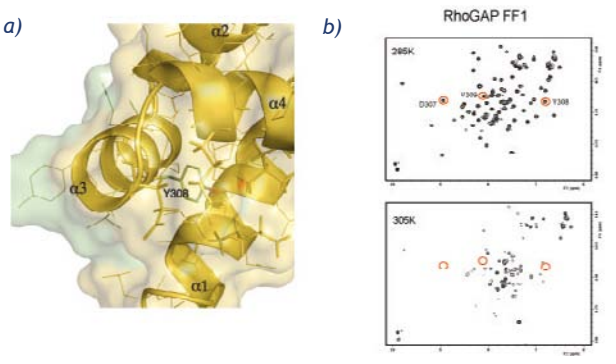


Figure 2. ***Tyr phosphorylation on RhoGAPFF1 domain.*** *(a) Solid surface representation of the RhoGAPFF1 domain. The region corresponding to the consensus site (QDYVYL) for the kinase recognition is shown in green, and residues are labelled. The position of the hydroxyl group of Tyr308 is indicated in red. (b) The effects of temperature on the unfolding process.*

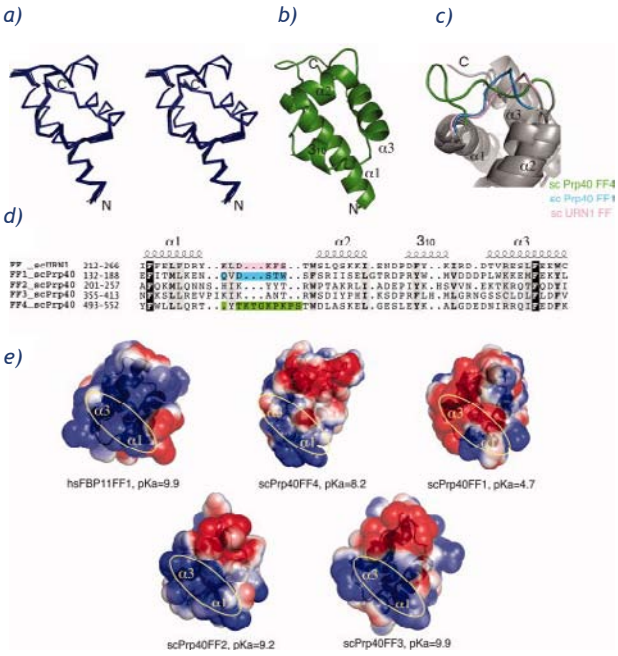


Figure 3. ***Structure of Prp40 F4 domain.*** *(a) Overlay of the backbone 15 lowest-energy conformers of the Prp40FF4 domain after water refinement. (b) Ribbon representation of the lowest-energy structure of Prp40FF4. (c) An expanded view of the superimposition of Prp40FF4 to Prp40FF1 and URN1FF structures in the region of the loop connecting helices 1 and 2. The loop is colored in green for Prp40FF4, in light blue for Prp40FF1, and in pink for URN1FF. (d) Sequence alignment of Saccharomyces cerevisiae FF domains. The alignment was generated with ClustalX and edited manually. Conserved residues are shaded in grey. The region corresponding to the first loop is shaded in colours following the superimposed FF domains of the previous figure. (e) Electrostatic surface plots of the Prp40 FF1, FF4, and FBP11FF1 structures and the Prp40 FF2 and FF3 models.*

peptides (K_d = 7.8 μM and 4.1 μM, respectively). This affinity is among the highest reported to date for a WW-PY domain interaction. The affinity of WW2 for the unphosphorylated T-PY motif was 7-15-fold lower. The Nedd4L WW3 domain also preferentially bound to the phosphorylated T-PY motifs, but with lower affinity than the WW2 domain. The WW1 and WW4 domains bound even more weakly and with no preference for the phosphorylated T-PY motifs. Interestingly, Smad1 (and Smad5) also contains a conserved T-PY motif. However, this threonine residue was not phosphorylated *in vivo* under any of the agonist or antagonist stimuli tested and it was poorly phosphorylated by CDK8/9 *in vitro*. The Smurf1 WW2 domain binds a synthetic peptide of 13 residues including the T-PY motif of Smad1 with a Kd of 32 μM and the phosphorylated Smad3 pT-PY motif with a K_d of 36 μM. These values are in agreement with the observation that Smurf1 plays a minor role in Smad3 turnover and it requires contacts with the phosphorylated SerPro cluster in order to target Smad1. A detailed analysis of these interactions, including the determination of the different complexes between Nedd4L WW domains and PY motifs of Smads, is also underway.

Unravelling the protein-protein interaction scenario during splicing: implication of FF domains

FF domains are protein-protein interaction modules of about 70 amino acids and are often found in several copies. They are present in three protein families: the splicing factors FBP11, Prp40 and URN1, the transcription factors CA150, and the p190RhoGTPase-related proteins. However, the simplicity of their distribution contrasts with the difficulty to define their biological roles. Indeed, for each FF domain studied, there appears to be a ligand that does not contain conserved features when compared to others previously characterised.

p190RhoGAP FF1 domain shields its phosphorylation site in the domain core

p190-A and -B Rho GAPs (guanosine triphosphatase activating proteins) are the only cytoplasmatic proteins containing FF domains. In p190-A Rho GAP, the region containing the FF domains has been implicated in binding to the transcription factor TFII-I. Moreover, phosphorylation of Tyr308 within the first FF domain inhibits this interaction. Because the structural determinants governing this mechanism were unknown, we sought to solve the structure of the first FF domain of p190-A Rho GAP (RhoGAPFF1) and to study the potential impact of phosphorylation on the structure. We found that RhoGAPFF1 does not fold with the typical (α1-α2-3(10)-α3) arrangement of other FF domains. Instead, the NMR data obtained at 285 K show an α1-α2-α3-α4 topology. In addition, we observed that specific contacts between residues in the first loop and the fourth helix are indispensable for the correct folding and stability of this domain.

The structure also revealed that Tyr308 contributes to the domain hydrophobic core. Furthermore, the residues that compose the target motif of the platelet-derived growth factor receptor alpha kinase form part of the alpha 3 helix. We observed that the phosphorylation reaction requires a previous step including domain unfolding, a process that occurs at 310 K. The unfolding capacity of this FF domain was not observed in other domains used as controls. Furthermore, in the absence of phosphoryla-

tion, the temperature-dependent RhoGAPFF1 folding/unfolding process was reversible. However, phosphorylation caused an irreversible destabilisation of the RhoGAPFF1 structure, which probably accounts for the inhibitory effect that it exerts on the TFII-I interaction. Our results link the capacity of a protein domain to be phosphorylated with conformational changes in its three-dimensional structure.

Phosphorylation sites in proteins are often found in the loops or in linkers connecting domains. Instead, one of the phosphorylation sites of RhoGAP forms part of the protein core, and it is inaccessible to kinases in the folded state. Some proteins may have developed a double-check system, shielding phosphorylation sites in the protein core. Thus, the inhibitory role that phosphorylation plays in this regulatory process appears to cover the energetic cost of shifting the folding-unfolding equilibrium towards the unfolded state in a non-reversible manner. The removal of the phosphate group allows the system to recover the basal structure.

Solution structure of the fourth FF domain of yeast Prp40 splicing factor

Prp40 protein was originally identified as a suppressor of 5' end U1 RNA point mutations. [1] Prp40 is a U1 snRNP-associated protein that participates in the early steps of yeast pre-messenger RNA splicing. Prp40 associates with the branch-binding point protein to bring the 5' end splicing site and the intron branch point into spatial proximity. In addition, Prp40 has been implicated in the binding to the phosphorylated C-terminal domain (herein referred as phospho-CTD) of RNA polymerase II through regions involving the WW and FF domains. However, a subsequent study on the structure of the Prp40 WW domain pair also showed that, in the absence of additional FF domains, the WW domains do not interact with the phospho-CTD repeats.

We determined the solution structure of the first FF domain of Prp40 in 2006. That study also examined the binding of Prp40FF1 to the splicing factor Clf1 and to a pair of bisphospho-CTD repeats. The binding site for the association with the first TPR motif of Clf1 involves helices 2 and 3_{10}, and the N-terminal half of helix 3. In contrast, no interaction was detected for the Prp40FF1 domain with the phospho-CTD repeats and for the Prp40FF4 domain with the TPR motif of Clf1.

In this study we report the solution structure of the Prp40FF4 domain. Furthermore, prompted by the observation that the charge distribution of the FBP11FF1 region involved in the interaction with the bisphospho-CTD repeats is partially conserved in Prp40FF4, we also examined whether this domain interacted with the phospho-CTD repeats; however, no binding was detected under our experimental conditions.

Scientific output

Publications

Alarcón C, Zaromytidou AI, Xi Q, Gao S, Yu J, Fujisawa S, Barlas A, Miller AN, Manova-Todorova K, Macias MJ, Sapkota G, Pan D and Massagué J. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell*, **139**(4), 757-69 (2009)

Bonet R, Ruiz L, Aragón E, Martín-Malpartida P and Macias MJ. NMR structural studies on human p190-A RhoGAPFF1 revealed that domain phosphorylation by the PDGF-receptor alpha requires its previous unfolding. *J Mol Biol*, **389**(2), 230-37 (2009)

Bonet R, Ruiz L, Morales B and Macias MJ. Solution structure of the fourth FF domain of yeast Prp40 splicing factor. *Proteins*, **77**(4), 1000-03 (2009)

Gao S, Alarcón C, Sapkota G, Rahman S, Chen PY, Goerner N, Macias MJ, Erdjument-Bromage H, Tempst P and Massagué J. Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling. *Mol Cell*, **36**(3), 457-68 (2009)

Research networks and grants

Determinación de estructuras de dominios FF de proteínas y de sus interacciones mediante la aplicación de la resonancia magnética nuclear multidimensional en solución

Spanish Ministry of Science and Innovation, BFU2008-02795 (2009-2011)
Principal investigator: Maria Macias

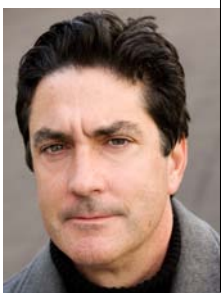
Collaborations

Inter- and intra-molecular interactions in E3 ubiquitin ligases: Recognition of new proline-rich motifs by WW domains and its implications in protein degradation and transcription
Joan Massagué, Memorial Sloan-Kettering Cancer Center (New York, USA)

Structural studies of cell-penetrating peptides (γ-peptides) based on proline derivatives and of somatostatin analogues
Antoni Riera, IRB Barcelona (Barcelona, Spain); Miriam Royo, Barcelona Science Park (Barcelona, Spain)

Unravelling the protein-protein and protein-RNA scenario during splicing: Exon skipping
Juan Valcárcel, Center for Genomic Regulation (Barcelona, Spain)

Modesto Orozco



Molecular modelling and bioinformatics group

Our long term objective is to decipher the behaviour of living organisms by means of theoretical models, whose roots are anchored in the basic principles of physics and chemistry. For this purpose, we work with a range of methodologies, from the mining of biological databases to classical dynamics and quantum chemistry calculations. The use of such diverse approaches allows us to explore problems as diverse as drug design and genome analysis. Special emphasis is given to connecting basic interactions with the global properties of biological systems. During this period, our work has focused on three major areas: i) the study of small model systems; ii) the analysis of stressed or unusual nucleic acids; and iii) the dynamics of proteins.

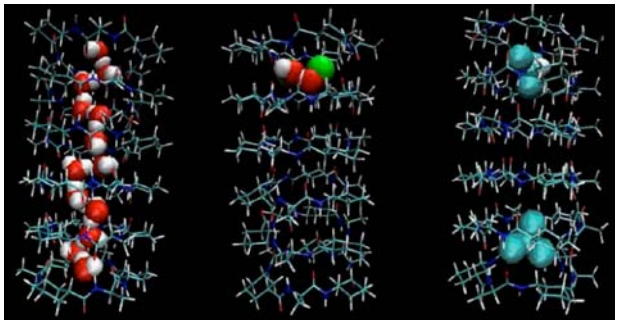


Figure 1. Detail of solvent migration in a α,γ-peptide nanotube.

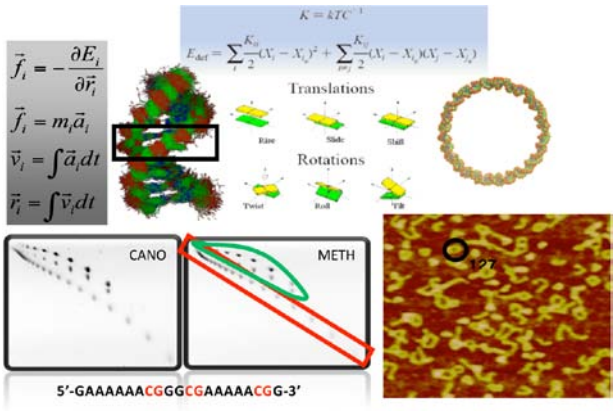


Figure 2. Derivation of mesoscopic descriptors of DNA flexibility and validation by cyclation experiments at EBL.

Small model systems

The study of simple systems can provide clues to enhance our understanding of the behaviour of much more complex biological molecules. In this area of work our effort has been traditionally focused on the development of methods for the treatment of solvation and the analysis of supramolecular systems of biological impact. During 2009, we have advanced in the refinement of our MST method for the representation of solvent (Klamtz *et al*, 2009), which in recent blind tests (Soteras *et al*, 2009) was found to be superior to most self-consistent reaction field approaches, providing not only good estimates of solvation free energies, but also enthalpies (Bidon-Chanal *et al*, 2009). Applying this and other methods, we have studied several supramolecular systems of biological/technological impact. Special mention is given to our work on α,γ-peptide nanotubes (Garcia-Fandiño *et al*, 2009; Figure 1), in which we characterise the structure, dynamics and transport properties of these molecules, which have been proposed as potential carriers of small molecules across membranes.

Analysis of stressed or unusual nucleic acids

Major breakthroughs in the field of nucleic acid simulations emerged from work performed by our group in 2007 related to the development of the PARMBSC0 force-field. This formalism has become the default force-field for nucleic acid simulations and was selected by the Ascona B-DNA consortium to simulate the sequence-dependent geometrical and dynamic properties of DNA (Lavery *et al*, 2009; reviewed in Laughton & Orozco, 2009). Using this force-field, we have advanced considerably in the characterisation of the elastic properties of DNA and their dependence on environmental conditions, external stress or chemical modifications. In summary, during 2009 we have analysed the impact of ionic atmosphere on DNA (Noy *et al*, 2009), the structure of unusual loop-structures in DNA quadruplexes (Fadrna *et al*, 2009), the elastic properties of all DNA tetraplexes in physiological conditions (Lavery *et al*, 2009), and the changes induced by epigenetic modifications on DNA (Perez *et al*, in preparation). Our models are now being validated through

cyclation experiments performed at the Experimental Bioinformatics Laboratory (EBL). Following this line of work, we are in the process of completing the development of a new nucleosome predictor, which is currently being validated by next- generation sequencing and tilling arrays.

Following a well-established research line in the laboratory, we have explored the structure of nucleic acids containing modified nucleotides. In particular, alone or in collaboration with other research groups, we have analysed the impact of introducing restricted nucleotides in the G-DNA structure of the thrombin aptamer (Saneysoshi *et al*, 2009), the impact of thio-thymines (Faustino *et al*, 2009), seleno derivatives of thymine (Vázquez-Mayagoitia *et al*, 2009) and fluoro-arabino compounds (Watts *et al*, 2009) in duplexes, and the 8-amino purine derivatives in hairpins and triplexes (Aviño *et al*, 2009). Finally, of note are our recent efforts in the characterisation of nucleic acid folding and unfolding pathways (Pérez *et al*, in press; Portella *et al*, in preparation), studies that will crystallise during 2010.

Dynamics of proteins

The creation and data-mining of the MODEL (Molecular Dynamics Extended Library) database has involved a huge amount of work. We have not only completed the database, but used it to characterise the connection between evolutionary and physical deformation patterns in proteins (Velázquez-Muriel *et al*, 2009). This general analysis has been centred on the RAS family (Raimondi *et al*, 2009), where flexibility was found to be crucial to explain the relation between flexibility and function. We have also used the information in MODEL to parametrise a wide variety of coarse-grained methods, which have greatly facilitated the study of deformability in systems containing thousands of proteins (Emperador *et al*, 2009). Recently these different approaches are being implemented in automatic tools for the description of protein flexibility, such as FlexServ (http://mmb.pcb.ub.es/FlexServ; Figure 4).

The latest technological breakthrough in the group in this field is related to the development of applications for automatic trajectory generation (MdWeb), an application for which a beta-version is already available (Figure 5), and the COCO utility for combing NMR and physical description of flexibility (Laughton *et al*, 2009).

Research Group Members

Principal Investigator:
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Research Associates:
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Postdoctoral Fellows:
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PhD Students:
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Research Assistants:
José Alcántara, Carlos Fenollosa, Chiara Lara, Margarita Pedro

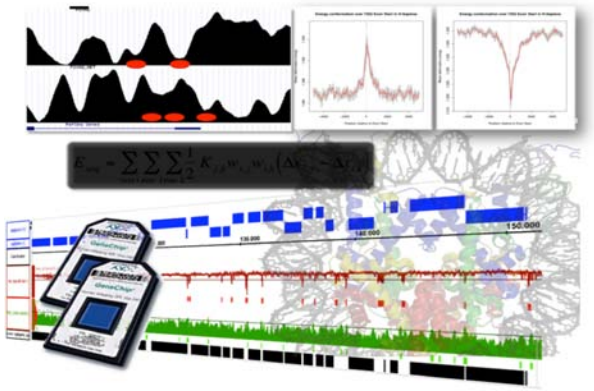

Figure 3. Prediction and experimental validation by tilling array of nucleosome positioning in S. cerevisae genome.

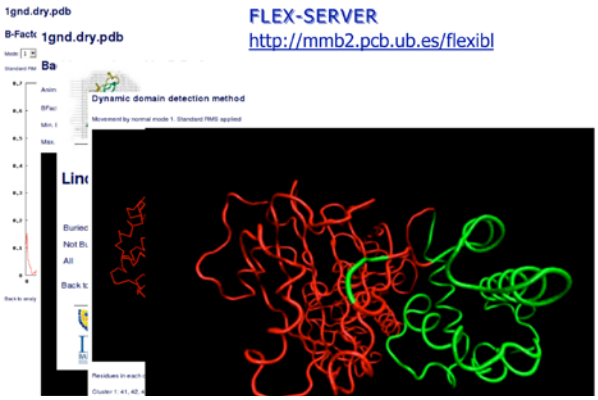

Figure 4. Examples of output of our FlexServ application.

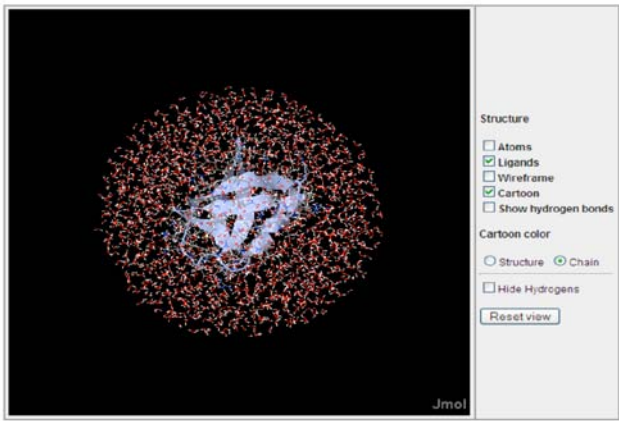

Figure 5. Graphical interface in the MDWeb application.

Significant work has been done during 2009 on the description of protein structure and flexibility under extreme conditions, particularly on gas phase (Meyer *et al*, 2009). The MD simulations demonstrated that the gas-phase ensemble of conformations is not far from the solution conformation, and, in fact, we demonstrated that collision cross-sections determined for gas-phase ensembles contribute to refining structural models derived from threading, *ab initio* or homology modelling (D'Abramo *et al*, 2009). The study opens up the possibility to use gas-phase structural information derived from X-free electron laser microscopy to obtain the protein structure in solution.

Scientific output

Publications

Bidon-Chanal A, Huertas O, Orozco M and Luque FJ. Solvation enthalpies of neutral solutes in water and octanol. *Theor Chem Acc*, **123**, 11-20 (2009)

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Research networks and grants

Acción complementaria
Spanish Ministry of Science and Innovation, ExpandingBio (2009)
Principal investigator: Modesto Orozco

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

IFLPD
European Commission, Marie Curie-FP7-PEOPLE-2007-4-1-IOF (2009-2013)
Principal investigator: Manuel Rueda

Molecular recognition
'Marcelino Botin' Foundation, IO FMBotin-M Orozco (2007-2010)
Principal investigator: Modesto Orozco

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

Simulaciones de formas inusuales o tensionadas de los ácidos nucleicos de potencial interés biotecnológico o biomédico
Spanish Ministry of Science and Innovation, BIO2009-10964 (2009-2012)
Principal investigator: Modesto Orozco

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

Collaborations

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

Drug design
Lluís Ribas de Pouplana, IRB Barcelona (Barcelona, Spain)

Dynamics of proteins
Francesca Fanelli, University of Modena (Modena, Italy); José Maria Carazo, Centro Nacional de Biotecnología-CSIC (Madrid, Spain)

Mixed QM-MM methods for protein simulations
Donald Truhlar, University of Minnessotta (Minnessotta, USA); Jiali Gao, University of Minnessotta (Minnessotta, USA)

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

Study of modified nucleobases
Ramon Eritja, IRB Barcelona (Barcelona, Spain); Miguel Fuentes-Cabrera, Oak Ridge National Laboratory (Oak Ridge, USA); Carlos González, Rocasolano Institute—CSIC (Madrid, Spain); Francisco Javier Luque, University of Barcelona (Barcelona, Spain)

Study of physical properties of DNA
ABC Consortium; Charlie Laughton, Centre for Biomolecular Sciences, Nottingham University (Nottingham, UK); Richard Lavery, Institut de Biologie et Chimie des Protéines, University of Lyon (Lyon, France)

Awards and honours

Distinguished fellow, 'Marcelino Botin' Foundation
Awardee: Modesto Orozco

Miquel Pons

Proteins involved in cell signalling and regulation

Living organisms are adaptive machines that respond dynamically to their environment. Higher organisms have evolved specific features to cope with their increasing regulatory needs. Intrinsically Disordered Proteins (IDPs) represent 30% of eukaryotic proteins. The association of IDPs with regulation is highlighted by the fact that 80% of cancer-associated proteins contain large disordered portions. Our group focuses on the disordered Unique domain of c-Src family kinases and its role in the integration of a variety of signalling inputs. Kinases and competing phosphatases, which are also studied in our lab, are key elements of many signalling cascades. Unicellular organisms, like bacteria, are exposed to drastic changes in the environment and these trigger the coordinated regulation of a large number of genes. Changes that allow the colonisation of higher organisms are particularly relevant from the perspective of human health. Most virulence genes belong to a gene pool that can be acquired horizontally. Enterobacteria have increased their success as pathogens by evolving selective regulatory mechanisms that minimise the burden of maintaining horizontally transferred genes. Our group studies the proteins involved in these mechanisms as possible targets for sustainable anti-bacterial treatments. For our research purposes, extensive use is made of NMR, Small Angle X-ray Scattering, and molecular biology and computational methods.

The Unique domain of c-Src and other intrinsically disordered proteins

The Src family of non-receptor protein tyrosine kinases (SKFs) is formed by at least ten members (Src, Frk, Lck, Lyn, Blk, Hck, Fyn, Yrk, Fgr and Yes) and it plays a key role in the initiation of various signal transduction pathways that regulate cell growth, differentiation, proliferation and survival. All SKFs comprise six functional domains: SH4, Unique domain, SH3, SH2, kinase (catalytic), and the C-terminal regulatory region. The sequence homology within the family is relatively high with the exception of the N-terminal region comprising the SH4 and Unique domains. The reported X-ray structures of c-Src do not contain the N-terminal region, a section that hinders crystallisation and is easily degraded by proteases. The Unique domain is intrinsically disordered and, in spite of the relevance of SFKs, and in particular c-Src, this domain has attracted very little attention to date.

Yolanda Pérez has completed the NMR assignment and the structural study of the Unique domain of c-Src in solution (Perez *et al*, 2009). A partially structured region was detected using residual dipolar couplings and paramagnetic relaxation enhancement experiments.

Further studies have shown that this region is involved in a variety of interactions that include specific lipid binding as well as intra- and inter-molecular protein-protein contacts. Taken together, the new results uncover an unexpected role of the Unique domain of c-Src in the integration of various signalling inputs (Pérez *et al*, in preparation).

In collaboration with Martin Blackledge's group (Institut de Biologie Structurale), Pau Bernadó has developed specific methods to study intrinsically disordered proteins (Jensen *et al*, 2009; Estrada *et al*, 2009; Bernadó *et al*, 2009) and has collaborated in the study of disordered synuclein in an aggregation-prone state (Cho *et al*, 2009).

Bernadó and Pons, together with Peter Wright at the Scripps Research Institute, are preparing a Barcelona Biomed meeting on intrinsically disordered proteins, which will take place in October 2010.

Tyrosine phosphatases and weak oligomerisation processes

Although phosphatases have received far less attention than kinases, they revert the action of the latter and are essential components of signalling cascades. The regulation of kinases is still poorly understood. We have used a low molecular weight phosphatase as a model system in a number of studies that address weak protein-protein interactions. Weak interactions result in mixtures of the free and associated species, which hinder study of the individual species. During 2009 we published a seminal methodological paper in which we showed that it is possible to obtain Small Angle X-ray Scattering (SAXS) curves of the monomer and dimer of the low molecular weight phosphatase from

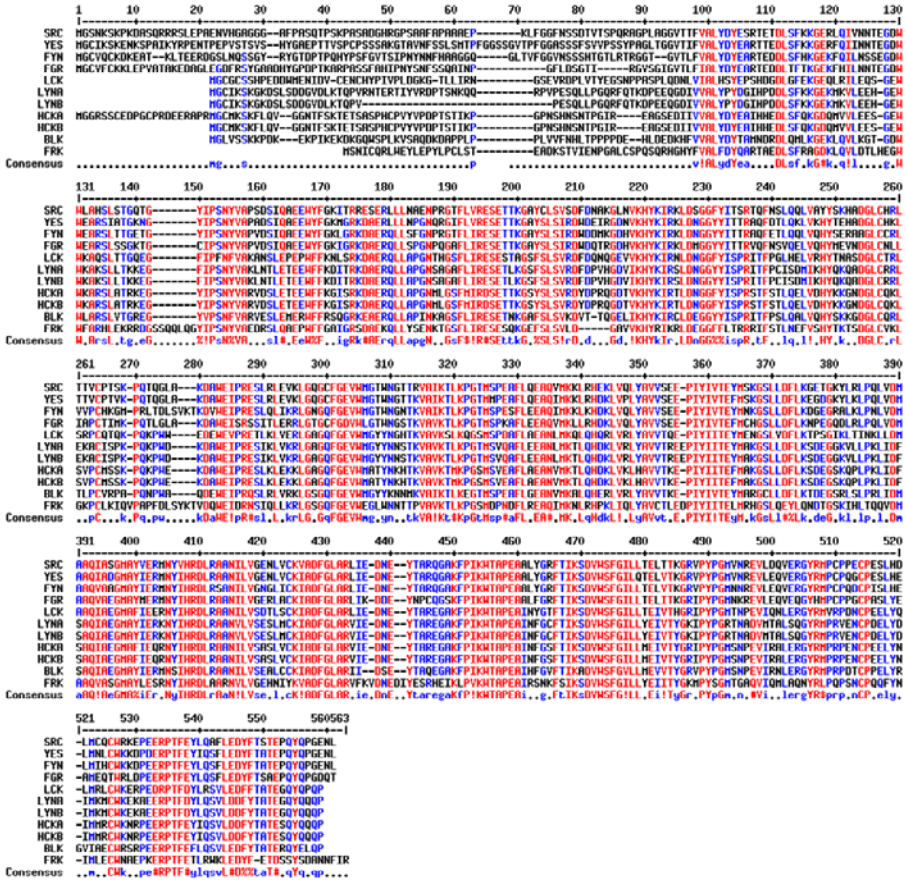

Figure 1. Sequence alignment of several SFKs showing the large sequence diversity in the Unique region.

Research Group Members

Group Leader
Miquel Pons

Research Associates:
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Postdoctoral Fellows:
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Lab Technician:
Isabel Latorre

Visiting Student:
Lucas Gelain (Brazil)

a)

b)

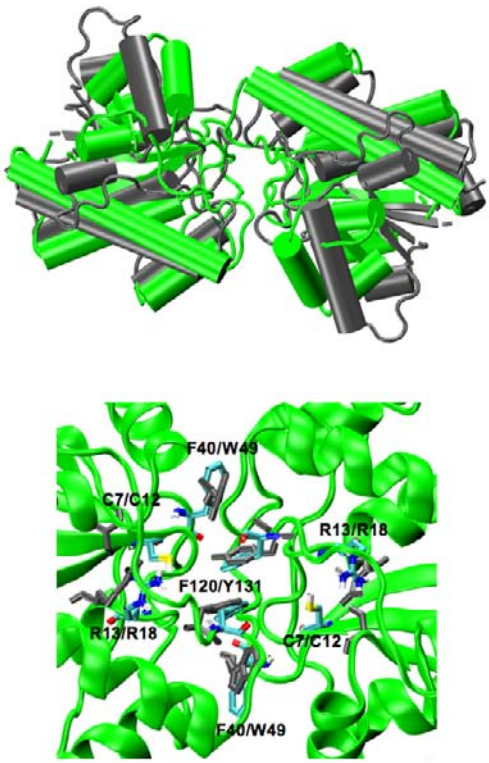

Figure 2. Comparison of dimers and the dimerisation interface of the low molecular weight phosphatases of B. subtilis and B. taurus.

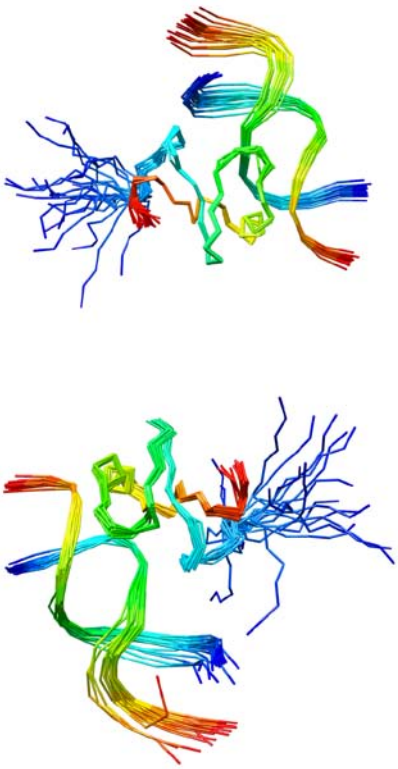

Figure 3. Structures of two complexes between the DNA-binding domain of Ler and the same native DNA fragment.

the combined analysis of scattering data obtained in a range of protein concentrations.

The extracted curves were fully consistent with those expected from the pure components known from crystal structures obtained under conditions in which only one of the two species crystallised (Blobel *et al*, 2009). In a less demanding example, the identification of the correct dimer of STAT5a from a range of possible structures was achieved using only three protein concentrations (Bernadó *et al*, 2009).

Phosphatase dimerisation involves the interaction of active sites and therefore the dimer is intrinsically inactive. We had previously proposed that this dimerisation represents a regulatory mechanism that allows the accumulation of inactive phosphatase dimers —which do not interfere with the action of kinases— but that releases active monomers when increasing concentrations of its substrate compete for its active site. Support for this hypothesis has been obtained by the observation that a low molecular weight phosphatase from a bacteria (*Bacillus subtillis*) shows a very similar dimerisation process (Blobel *et al*, 2009).

Selective regulation of horizontally acquired genes in bacteria

Horizontally transferred genes (HTGs) can be shared between individuals of the same or even different bacterial species. Genes encoding virulence factors and antibiotic resistance determinants belong to the HTG pool. By gene transfer from resistant strains, a given bacterial population not previously exposed to antibiotics may acquire resistance to these drugs. The acquisition and maintenance of HTGs may provide a competitive advantage under selective conditions (*eg*, in the presence of antibiotics). However, when the selective pressure is released (*eg*, when no antibiotics are present), the presence of additional genes decreases the fitness of the bacteria. In a stable collaboration project with Antonio Juarez's group (University of Barcelona and IBEC), we have found that *enterobacteria* have developed the capacity to selectively regulate HTGs, without affecting the 'resident' gene pool. The discovery came from the analysis of plasmidic forms of the general regulator H-NS. The plasmid R27 contains a H-NS variant that is selective for HTGs. This discovery, published in *PLoS Genetics*, is the first demonstration of the presence of two gene pools in bacteria and helps to explain the virulence of well-known pathogens of the genus *Yersinia* (*eg*, the cause of the Plague), *Salmonella* (a common cause of food poisoning) and the pathogenic forms of *E. coli* (Baños *et al*, 2009).

Some HTGs are incorporated in the main bacterial chromosome. Selective regulation of HTGs by chromosomic H-NS is also possible with the help of a co-regulator Hha, which is also exclusively found in *enterobacteria*, in contrast to H-NS, which is found in all Gram-negative bacteria. In order to gain insight into the H-NS forms of *enterobacteria* and other Gram-negative species, we compared the *E. coli* and *Vibrio cholerae* forms of H-NS. We discovered that a single mutation can toggle the capacity to bind Hha between H-NS from both species (García *et al*, 2009). Intestinal lesions caused by enteropathogenic (EPEC) and en-

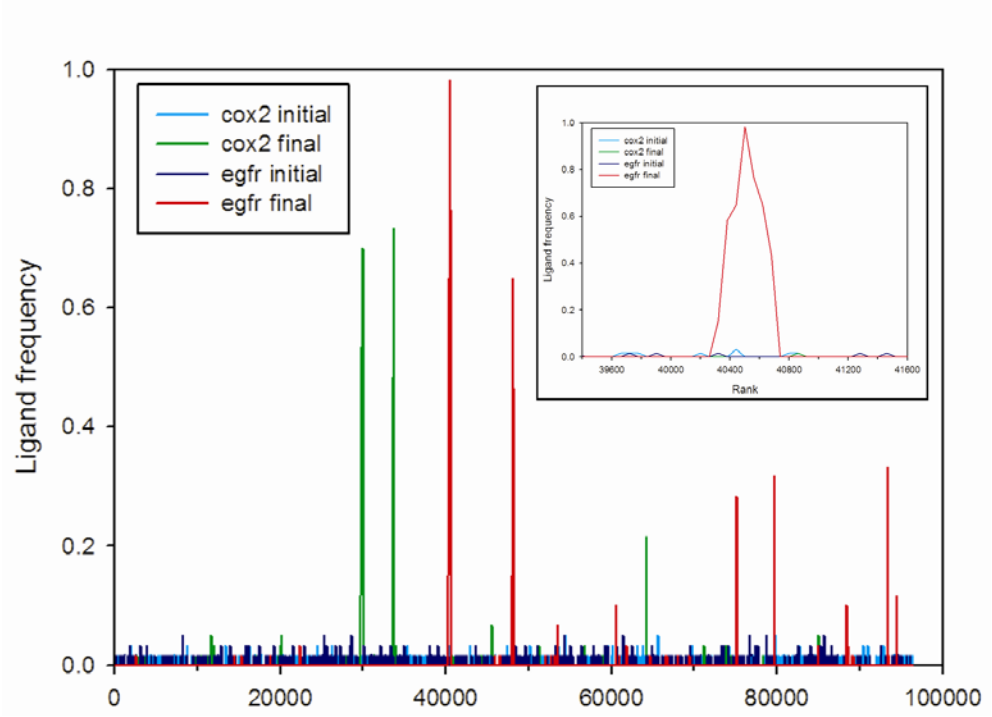

Figure 4. Virtual LINGO chromatography showing the distribution of known ligands of cox2 and the epithelial growth factor receptor in the original (initial) and ordered (final) list of all the compounds present in the DUD database.

terohemorragic (EHEC) forms of *E. coli* are mediated by proteins encoded in a pathogenicity island called 'locus of enterocyte effacement' (LEE). The protein Ler is the master regulator of this portion of horizontally acquired DNA. We have solved the structure of two complexes of the DNA binding domain of Ler with different regions of a native DNA sequence. The structures show binding in the DNA minor groove with the insertion of a protein α-helix that widens the groove considerably. The two complexes have similar stabilities; however, in the context of the DNA sequence studied, binding to one of the sites prevented the interaction with the second site, probably as a result of the distortions that complex formation introduces into the DNA itself. DNA distortion, or its capacity to be easily distorted, seems to be the target recognised by the protein. In our study, we show that the protein binds preferentially to sites that show departures from the canonical B DNA form, even in the absence of the protein.

The DNA binding domain of Ler shows considerable sequence similarity with the C-terminal domains of other H-NS-like proteins. Interestingly, it is most similar to the plasmidic form of H-NS, with which it shares the capacity to bind selectively to HTGs. This is the first high resolution structure of a protein-DNA complex of a domain of the H-NS family of proteins to be reported.

Structuring chemical space

The number of small molecules in the NIH-sponsored PubChem database exceeds 26 million. Of these, several million are commercially accessible for screening. Experimental screening of such large collections is unfeasible. Even virtual screening using structural information about the target can be extremely slow.

In recent years we have developed a collection of computational tools for the virtual screening of large databases. These include the LINGO application, which is based on similarity and property prediction methods, and the MP-algorithm, which combines similarity and docking information in a genetic algorithm. We have also modified the Autodock scoring function to improve the performance of the virtual search for compounds that have low affinity for the target (Cincilla *et al*, 2009). This is a key point as the MP-algorithm is a 'learning' one that uses the information from weak complexes to explore the most promising regions of chemical space.

During 2009 we have completed the development of a hierarchical clustering method derived from the affinity propagation algorithm introduced by Fred and Dueck. With this method, large databases can be structured so that compounds with predicted similar properties are placed together. The result of the method corresponds to the 'yellow pages' of the parent database, in which one does not search for a particular compound but for unknown compounds with similar properties.

In a linear representation of the structured database, compounds are listed so that similar compounds appear closer in the list. When collections of compounds binding to the same biological target are mapped in the list, 'activity peaks' are observed. This is reminiscent of the location of biological activity in certain fractions of a chromatographic separation of a complex mixture of compounds. We have coined the term 'Virtual LINGO Chromatography' to describe the process that allows the prediction of possible biological activity from the location of compounds in the ordered list of similarities (Cincilla *et al*, in preparation).

Scientific output

Publications

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Jensen MR, Markwick PR, Meier S, Griesinger C, Zweckstetter M, Grzesiek S, Bernadó P and Blackledge M. Quantitative determination of the conformational properties of partially folded and intrinsically disordered proteins using NMR dipolar couplings. *Structure*, **17**(9), 1169-85 (2009)

Pérez Y, Gairí M, Pons M and Bernadó P. Structural characterization of the natively unfolded N-terminal domain of human c-Src kinase: insights into the role of phosphorylation of the unique domain. *J Mol Biol*, **391**(1), 136-48 (2009)

Stein A, Pache RA, Bernadó P, Pons M and Aloy P. Dynamic interactions of proteins in complex networks: a more structured view. *FEBS J*, **276**(19), 5390-405 (2009)

Research networks and grants

Dominis únics de quinases de la família Src implicats en malalties cardiovasculars
'La Marató TV3' Foundation, IO-MaratóTV3 (2009-2012)
Principal investigator: Miquel Pons

EMAR-Multidisciplinary frontiers of magnetic resonance support
Spanish Ministry of Science and Innovation, PCI2006-A9-0690 (2007-2012)
Principal investigator: Miquel Pons

Investigación de sistemas supramoleculares por RMN
Spanish Ministry of Science and Innovation, PHB2008-0089-PC (2009)
Principal investigator: Miquel Pons

Nuevos métodos de RMN aplicados al estudio de proteínas relacionadas con la patogenicidad bacteriana
Spanish Ministry of Science and Innovation, BIO2007-63458 (2007-2010)
Principal investigator: Miquel Pons

Programa de cooperación interuniversitaria
Spanish Ministry of Foreign Affairs and Cooperation (MAEC)/Spanish Agency for International Cooperation (AECI), A/019105/08 (2009)
Principal investigator: Miquel Pons

Renewal of 500 MHz NMR instrument
Spanish Ministry of Science and Innovation, ICTS-2007-08

Renewal of 600 NMR instrument
Spanish Ministry of Science and Innovation, ICTS-2009-43

Research networking programme
European Science Foundation, 05-PGM-022 (2007-2012)
Principal investigator: Miquel Pons

3rd Iberoamerican NMR meeting
Spanish Ministry of Science and Innovation, PHB2008-0082-TA (2009)
Principal investigator: Miquel Pons

Collaborations

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

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

Computational studies in nucleoid-associated proteins
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Ler structure
Christian Griesinger, Max-Planck Institute (Göttingen, Germany)

New radicals for Dynamic Nuclear Polarization (DNP)
Anita Marsaioli, UNICAMP (Campinas, Brazil); Antoni Riera, IRB Barcelona (Barcelona, Spain); Jaume Veciana, Institute of Material Sciences of Barcelona (Barcelona, Spain)

NMR- and SAXS-based protein-protein docking
Juan Fernández Recio, Barcelona Supercomputing Center (Barcelona, Spain); Javier Sancho, University of Zaragoza & Institute for Biocomputation and Physics of Complex Systems-BIFI (Zaragoza, Spain)

Relaxation dispersion NMR and uroporphyrinogen
Oscar Millet, CIC bioGUNE (Bilbao, Spain)

Ribosomal proteins and combinations of SAXS and NMR
Mikael Akke, Lund University (Lund, Sweden)

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

Solid-state NMR studies of H-NS
Marc Baldus, Bijvoet Center for Biomolecular Research, Utrecht University (Utrecht, The Netherlands)

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

Awards and honours

Steering Committee member, ISMAR

Member of the Board of Trustees, EUROMAR

Chair of the Steering Committee, EMAR

Director of the Spanish National NMR large-scale facility (ICTS)

Experimental Bioinformatics Laboratory

Recent progress in genomics and high-throughput techniques has brought an explosion of biological data, which in turn has provided a great opportunity to computationally predict complex biological networks with high accuracy in living organisms. However, the implementation of computational methods in research may raise questions about the predictive capacity of computer simulations. The Experimental Bioinformatics Laboratory (EBL) is part of a collaborative research programme between IRB Barcelona and the Barcelona Supercomputing Center (BSC). The EBL is devoted to experimentally verifying in silico *models performed by computational scientists in the fields of systems biology (protein-protein interaction networks) and genome regulation. For this purpose, experimental functional genomics techniques (eg high-throughput yeast two-hybrid screening or genome-wide nucleosome position mapping), in combination with biochemical and cell biology methods, are implemented.*

Genome-wide approaches to studying chromatin modifications

Chromatin modifications have been shown to have a profound impact on the regulation of gene expression. By addressing several approaches, we ultimately plan to derive a full integrative model to predict nucleosome positioning and repositioning as a function of gene activity and methylation pattern.

Genomic profile of nucleosome positioning

Knowing the precise positions of nucleosomes in a genome is crucial to understand how genes are regulated, since their organisation along the DNA sequence controls the accessibility of DNA to the regulatory factors. While the underlying DNA sequence may be involved in the rotational placement of nucleosomes around the histone core, most nucleosomes depend on exclusion signals, which may play a wider role in regulating the translational positioning of nucleosomes along the genome. With the implementation of high-throughput genomic scale techniques, such as microarray hybridisation and next generation sequencing, nucleosome maps of a similar resolution in yeast, worms, flies and humans have been reported.

Following Modesto Orozco interests (IRB Barcelona), and in collaboration with Ferran Azorin's group at IRB Barcelona, the EBL (Özgen Deniz's work) is pursuing the mapping of nucleosome positions in a variety of yeast species in order to study the factors that influence the positioning of these repeating units. For these purposes, our first goal was to reproduce the already reported genome-wide nucleosome positioning map of *Saccharomyces cerevisiae* (Lee *et al*, 2007). We isolated mono-

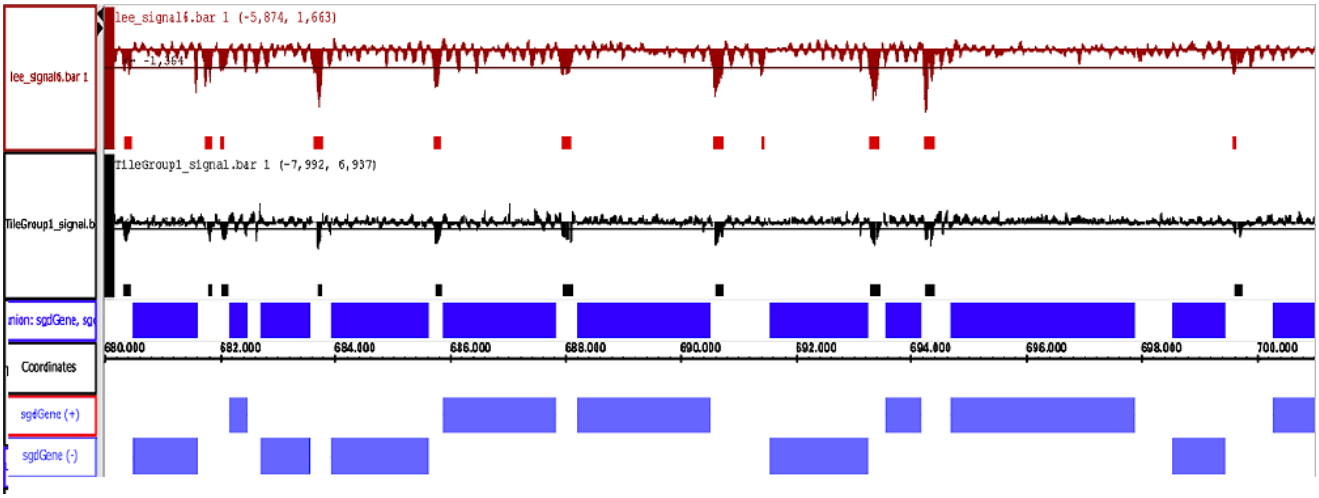

Figure 1. Genome-wide analysis of nucleosome positioning of S. cerevisiae *from Lee (red spectrum) and from our study (black spectrum), with the genes indicated as blue boxes.*

nucleosomal DNA from *S. cerevisiae* strain BY4741 by partial nuclease digestion. To define and align the locations of the mononucleosomes in the genome, we used genomic DNA as a reference, which was partially MNase-digested to 100-400 bp fragments. Both samples were hybridised to *GeneChip S. cerevisiae Tiling 1.0R* arrays (Affymetrix), in collaboration with the Functional Genomics Core Facility at IRB Barcelona. To validate and compare the genome-wide nucleosome positioning map of *S. cerevisiae*, samples were also analysed by massive parallel sequencing using Solexa technology. The data are being analysed by computational scientists in Orozco's group, where comparison of our nucleosome positioning map and Lee's map has been completed in the genome-wide manner (Figure 1). However, a more detailed analysis is required.

In order to gain insight into the possible factors affecting nucleosome positioning, we will map the nucleosome positions of a variety of yeast species like *S. pombe*. Furthermore, nucleosomal DNA of synchronised yeast cultures will be analysed at different stages of the cell cycle in order to examine the effect of the chromatin structure on the positioning pattern.

DNA methylation and its effect on DNA flexibility

Bioinformatic simulations by Orozco's group predict that DNA methylation produces both DNA structural alterations and changes in DNA flexibility. To confirm these results, the EBL (Chiara Castellazzi's work) performed *in vitro* experiments through a cyclation assay, a robust method to study the conformational features of DNA. Starting from linear fragments of dsDNA, we carried out a ligation reaction to form concatamers and eventually to form circles, thereby generating a situation whereby there is competition between cyclation and oligomerisation. We subsequently analysed the affectation of circle formation depending on their degree of methylation by converting cytosines to methylcytosines.

Data were analysed by Atomic Force Microscopy (AFM) to directly distinguish linear from circular DNA (Figure 2a); *Exonuclease III* digestion (enzyme that digests only linear DNA); and 2D-polyacrylamide gel electrophoresis, which allowed us to separate and compare the resulting two species of circular DNA (Figure 2b). The first analyses allowed us to identify 10 different DNA sequences with the capacity to oligomerise and circularise, with an obvious affectation of circle formation depending on their degree of methylation. Further analyses involved the quantification of circle sizes and the de-

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Research Assistant:
Chiara Castellazi

Lab Technician:
Ricart Lluís

Visiting Student:
Elisa Duran (Spain)

termination of the formation rate of circular vs. linear species.

Our research efforts also include the study of the DNA properties (torsional flexibility or bending) that are most affected by methylation. Depending on the results, we will analyse the behaviour of methylated RNA in flexibility.

Genome-wide DNA methylation and its effect on nucleosome positioning

We also aim to determine how DNA methylation affects genome-wide nucleosome positioning in lower eukaryotes and mammalian cells with differing capacities for DNA methylation. This work involves the research performed by Kathryn Collinet in collaboration with Orozco's group.

One of the few model organisms known to lack naturally occurring DNA methylation is the yeast *S. cerevisiae*. Its small genome size and existing nucleosome positioning maps make this organism a favorable model to initiate such a study. We transformed yeast strains to express a prokaryotic methyltransferase (MT) along with two murine MTs to catalyse the transfer of methyl groups to CpG dinucleotides along the genome. To analyse global DNA methylation, in collaboration with the Scientific and Technical Services of the University of Barcelona, we performed high performance liquid chromatography (HPLC) to quantify the amount of genome-wide methylcytosine with respect to cytosine. In addition, we selected genome areas that may be more accessible to modification by DNA MTs, such as those with a higher probability of being located in a nucleosome-free region. Using bisulfite sequencing, we identified several regions on chromosome 11 that appear to harbour some of the DNA methylation observed in the HPLC analysis (Figure 4c).

We will continue with the bisulfite sequencing of various genomic regions in order to localise DNA methylation. Furthermore, we aim to detect changes in gene expression caused by DNA methylation by performing an expression array comparing wild-type and mutant strains. Finally, we will focus on mapping nucleosomes in the yeast strains expressing DNA MTs and we will compare their positions with those in the wild-type yeast genome using massive parallel sequencing.

Genome-wide analysis of gene regulation

Although information regarding protein function has increased dramatically, there are limited experimental data on the underlying mechanisms that control the expression of the encoding genes. Assuming that promoter regions display unusual physical properties, computational biologists in Orozco's group developed a promoter predictive algorithm to identify novel human promoters (ProStar). In order to validate the method, the EBL is currently measuring the potential of these putative promoter sequences *in vivo* by means of a luciferase-based expression reporter system in mammalian cells. This task involves the work of Elisa Durán, in close collaboration with Orozco's and David Torrents' (Barcelona Supercomputing Center) computational groups. The identification and validation of novel potential genes is the first step towards a broader study that aims to characterise and determine the relationship between physical chromatin characteristics and gene expression regulation.

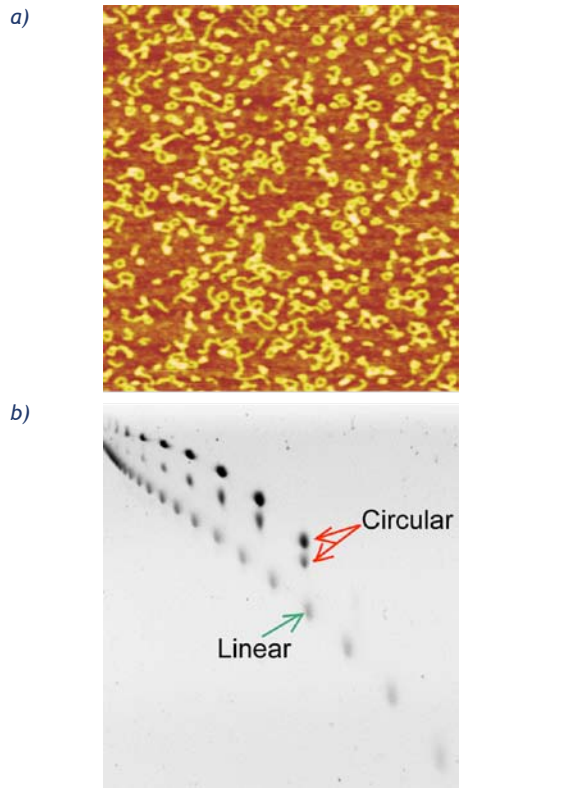


Figure 2. ***DNA cyclation assay.*** *(a) AFM image of a cyclated DNA sample displaying the different topologies. (b) 2D-gel showing the migration of linear and circular DNA species.*

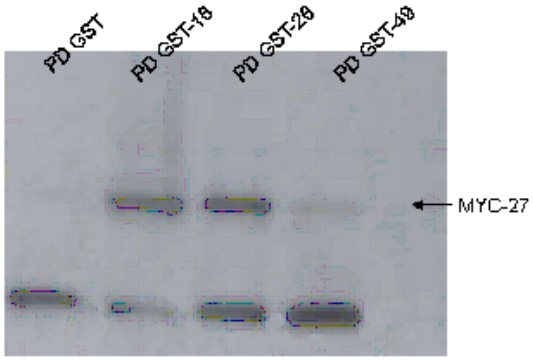


Figure 3. ***Validation of Y2H interactions by pull-down assays in mammalian cells.*** *Western blot showing positive interactions of GST-fused proteins 16, 26 and 49 with Myc-tagged protein 27, respectively. PD: pull-down.*

Molecular characterisation of pathological pathways

A deeper knowledge of the molecular bases of human disease will have a significant impact on the discovery of new drug targets and biomarkers, the optimisation of preclinical models, and our understanding of how biological networks change from

a healthy state to a diseased one. Following Patrick Aloy's (IRB Barcelona) interests, a real dry-wet cycle approach is applied to describe pathological pathways at the molecular level, using a combination of computational biology and interaction discovery techniques.

The EBL has implemented a number of experimental approaches to analyse protein-protein interaction networks. These approaches have allowed the identification of protein-protein interactions at physiological concentrations, and thus provide an efficient screening tool. Three projects related to molecular mechanisms associated with Alzheimer's disease, colon cancer and breast cancer are currently underway at the EBL.

Alzheimer's disease interaction network

Alzheimer's disease (AD) is the most common form of dementia. This incurable, degenerative, and terminal disease is diagnosed in people over 65 years of age, although the less prevalent early onset AD can occur much sooner. Research studies indicate that the disease is associated with plaques and tangles in the brain. However, the cause and progression of AD are not well understood. Although the treatments currently used offer some symptomatic relief, no treatments to delay or halt the progression of the disease are available.

The EBL (work by Montse Soler and Ricart Lluís, in collaboration with Aloy's computational group, has analysed the protein-protein interaction network of an initial list of 55 putative AD-causing genes with 9 AD-causing genes. Of the 2200 pair-wise protein interactions examined by the yeast-two-hybrid system (Y2H), we have identified 322 interactions among 53 proteins (44 putative genes). The Y2H interactions were further validated by *in vitro* co-affinity purification (pull-down) in mammalian cell cultures. Because most of the genes encode for membrane proteins, only 16 genes yielded detectable protein overexpression in COS-7 cells. This allowed us to test 42 protein-protein interactions by pull-down assay, 11 of which resulted positive (Figure 3).

Similarly, Y2H interactions were also validated by co-immunoprecipitation binding experiments in SH-SY5Y neuronal cells. The first interaction network was further expanded by a Y2H screen of an adult brain cDNA library against the 9 AD-causing genes. After 45 library assays, we isolated 155 distinct genes involved in 186 interactions, with 26 genes interacting with more than one AD-causing gene. Fifty-eight of the interactions identified were further observed by pair-wise Y2H. Aloy's group is currently expanding the experimental interaction data with data reported in the literature in order to gain a deeper insight into the pathological pathway of AD.

Colon cancer interaction network

Colorectal cancer (CRC) is the third leading cause of cancer-related death in the Western world. At the cellular and molecular level, CRC genes along the *Wnt* signalling pathway are damaged, in particular the most common mutated gene is *APC*. Some of the mutations are inherited while others are acquired. However, other mutations must occur for the cell to become cancerous. The EBL (Clara Berenguer), in collaboration with Patrick Aloy's group and Elena Sancho from the Oncology Programme (IRB Barcelona), is studying the protein-protein interaction network of 48 CRC-causing genes with 53 putative CRC-causing genes (which are located in a CRC susceptibility locus or present altered expression in CRC tissues). Experiments are performed following a similar protocol to that described above. We are currently examining 2544 pair-wise protein interactions by Y2H screens.

Breast cancer interaction network

Breast cancer (BC) is the most frequent malignancy in the female population. BC is the second most common type of cancer worldwide after lung cancer and the fifth most common cause of death from cancer. The EBL (Guillermo Suñé and Rodrigo Arroyo), in collaboration with Aloy's group, Roger Gomis, and Travis Stracker (Oncology Programme, IRB Barcelona), is examining the protein-protein interaction network of 59 BC-causing genes with 58 putative BC-causing genes. Of the 2400 pair-wise protein interactions studied by Y2H screens, 805 interactions resulted positive. A Y2H library approach against 13 BC-causing genes and *in vitro* validating binding assays are currently underway, respectively.

Scientific output

Publications

Zanzoni A, Soler-López M and Aloy P. A network medicine approach to human disease. *FEBS Lett*, **583**(11), 1759-65 (2009)

Collaborations

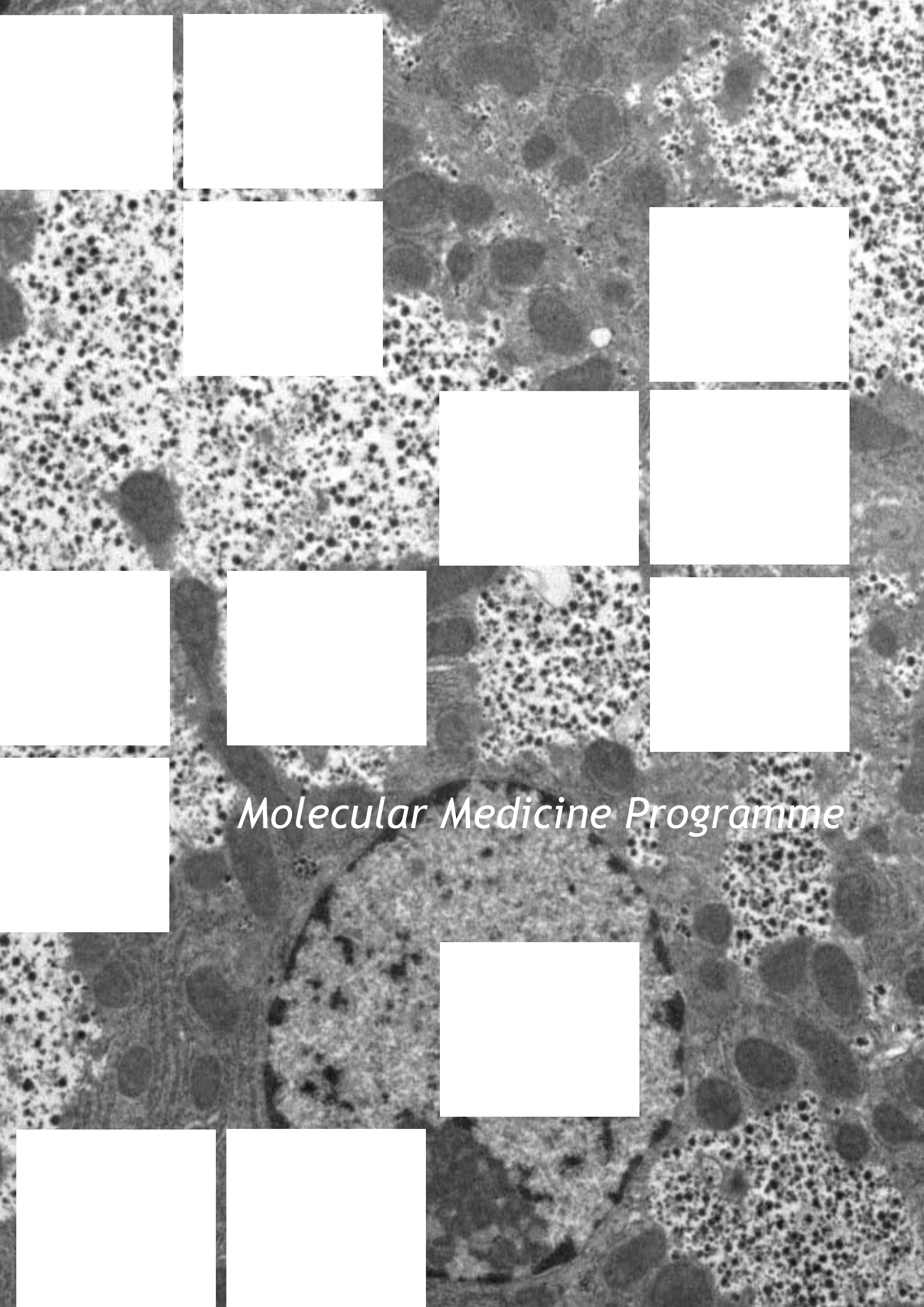
Alzheimer's disease interaction network
Ulrich Stelzl, Max-Planck-Institut für Molekulare Genetik (Berlin, Germany)

Breast cancer interaction network
Roger Gomis, IRB Barcelona (Barcelona, Spain); Travis Stracker, IRB Barcelona (Barcelona, Spain)

Colon cancer interaction network
Elena Sancho, IRB Barcelona (Barcelona, Spain)

Genomic profile of nucleosome positioning
Ferran Azorín, IRB Barcelona (Barcelona, Spain)

Genome-wide analysis of gene regulation
David Torrents, Barcelona Supercomputing Center (Barcelona, Spain)



Molecular Medicine Programme

Carme Caelles

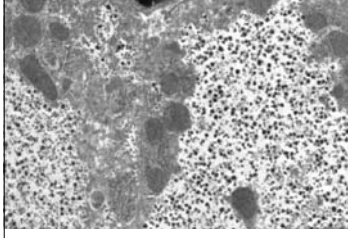
Cell signalling: Regulation and function

We study the mechanisms that regulate signal transduction and their role in physiology and pathology, with the aim to improve and/or develop new therapeutic tools. We focus on two major research lines, the negative cross-talk between the nuclear receptor (NR)-stress activated protein kinase (SAPK) pathway, and the Nek9/Nek6/7 NIMA-family signalling cassette. In relation to the former, we center on a subset of NRs, namely the glucocorticoid receptor (GR) and the members of the peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) subfamilies, which share the capacity to down-regulate inflammatory responses. We have shown that, upon ligand binding, all these NRs inhibit the activation of the SAPK c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, all crucial mediators of pro-inflammatory signals. Therefore, inhibition of SAPK pathways by these NRs may be a mechanism by which to exert anti-inflammatory action, but also other relevant pharmacological activities, such as anti-diabetic and anti-atherosclerotic activity. In relation to the second research line, we address how phosphorylation regulates the execution of mitosis. In this regard, we study the signalling module composed of the NIMA-family kinases Nercc1/Nek9, Nek6 and Nek7, with the aim to elucidate its regulation, its relationship with other mitotic signalling components and its function, and to determine whether these kinases could be used as targets of pharmacological drugs.

NR-SAPK pathway cross talk: mechanisms and actions

Inflammation is a body response triggered by pathogens and noxious stimuli, such as chemicals or physical injury, which damage tissues and cells. Inflammatory responses underlie a wide variety of physiological and pathological processes. The classical events that trigger short-term or acute inflammation are infection and tissue injury, and considerable progress has been made to unravel this response at the molecular and cellular level. However, stress and dysfunction in tissues similarly induce a process characterised by being a systemic-chronic-low grade inflammatory response. This process is probably responsible for the chronic inflammatory conditions associated with many diseases that are highly prevalent in modern societies and that include classical chronic inflammatory diseases, such as rheumatoid and psoriatic arthritis, inflammatory bowel disease, chronic obstructive pulmonary disease, but also metabolic diseases, for instance, obesity, type 2 diabetes (T2D), atherosclerosis, and cancer (Medzhitov, 2008). In the last decade it has become evident that inflammation is a crucial factor at the origin and/or for the progression of all these chronic diseases, a circumstance that may explain the beneficial effects of anti-inflammatory therapy in these diseases. Therefore efforts and resources are being dedicated to the search, development, and improvement of anti-inflammatory drugs (Karin, 2005). Furthermore, supporting this strong link between inflammation and metabolism, NRs initially identified for their role in the regulation of glucose and lipid metabolism, such as PPARs and LXRs, were found to have anti-inflammatory properties and are expressed in cells of the immune system, such as macrophages (Bensinger and Tontonoz, 2008). In addition, the protein kinases of the same pathways that orquestrate the inflammatory response, JNK and IKK (IkB kinase), were found to contribute to promoting insulin resistance (in addition to their involvement in the production of pro-inflammatory and diabetogenic cytokines) by an inhibitory phosphorylation of the insulin receptor substrate (IRS)-1 (Hotamisligil, 2005). In this context, our studies have shown that the inhibition of the JNK pathway by the glucocorticoid receptor (GR) is responsible for the interference of these hormones with the AP-1 complex (Caelles *et al*, 1997; Caelles *et al*, 2002).

More recently, we have shown that thiazolidinediones (TZDs), which are synthetic ligands for PPARγ with insulin-sensitising activity, also have the capacity to inhibit the JNK pathway. Moreover, our results indicate that the inhibition of this pathway by TZDs mediates the anti-diabetic action of these drugs, and consistently, genetic ablation of *jnk*1 abrogates the hypoglycemic action of TZD in mice (Díaz-Delfín *et al*, 2007). This study expanded the scope of pharmacological actions (that is to say anti-inflammatory and anti-diabetic activities) mediated by the negative interference of NRs on SAPK pathways. Other members of the PPAR family, as well as LXRs inhibit LPS-induced activation of the JNK and p38MAPK pathways upon ligand activation in primary macrophages (peritoneal- and bone marrow-derived).



Given that the kinetics of this inhibitory action is compatible with the requirement of gene transcription, we are performing transcriptomic analyses to identify potential candidates to mediate the interference of these NRs on SAPK pathways. Potential candidate genes were identified as those encoding known inhibitors of SAPKs, such as the cell cycle regulator p21*waf*-1, which in addition is a transcriptional target of all these NRs, and the dual specificity phosphatase MKP-1, which mediates MAPK inhibition by the GR. Although we have observed that waf-1 expression is increased specifically in white and brown adipose tissue in various mouse models of obesity, this potential candidate has been discarded, as waf-1-deficient mice are resistant to the development of diet-induced obesity and insulin resistance. MKP-1 involvement in the inhibition of SAPK pathways by PPARs and LXRs has also been discarded, as we have found no evidence that this gene is a transcriptional target of any of these receptors. In summary, the inhibition of SAPK pathways by NRs is exerted at different levels along the signal transduction cascade and is mediated by distinct mechanisms depending on the cell type and the NR involved.

The observation that the NR-SAPK pathway cross-talk is negative and mutual implies that activation of SAPK pathways may alter the functionality of these NRs. This notion was previously proposed as a mechanism to account for the resistance to GCs found in the clinic (Adcock and Lane, 2003). In addition, JNK is activated in diseases that are treated with ligands of the NRs we are currently studying. In this context, we generated a transgenic mouse model (GFP-MKK7D) in which JNK activation depends on the Cre recombinase-dependent expression of a constitutively-activated mutant of MKK7 (MKK7D). We have already floxed/activated the transgene in pancreatic β-islets (by crossing these mice with the strain B6.Cg-Tg(Ins2-cre)25Mgn/J), and mice became glucose-intolerant as a result of increased insulinemia in response to hyperglycemia. In this particular model, we are characterising at molecular level the mechanism by which activation of the JNK pathway leads to pancreatic dysfunction and that, unexpectedly, is not caused by the induction of pancreatic cell death or by major structural abnormalities of the islets, as determined by morphometric analysis (Figure 1).

Research Group Members

Group Leader:
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Postdoctoral Fellow:
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Lab Technician:
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The signalling module formed by the NIMA-family kinases Nek9/Nek6/7: regulation and function

Nek9 (also known as Nercc1) is a member of the NIMA-family of protein kinases. Nek9 in collaboration with the related Nek6 and Nek7 has a crucial although not well understood role in the control of mitotic progression. Nek9 is activated on the centrosomes and spindle poles during mitotic entry. Once active, Nek9 interacts with Nek6 and Nek7, two highly similar kinases of the NIMA family that can be directly phosphorylated and activated by Nek9. A number of studies have shown that Nek9, Nek6 and Nek7 are required for normal spindle formation, chromosome segregation, mitotic progression and cytokinesis, although the molecular basis for these observations is currently unknown.

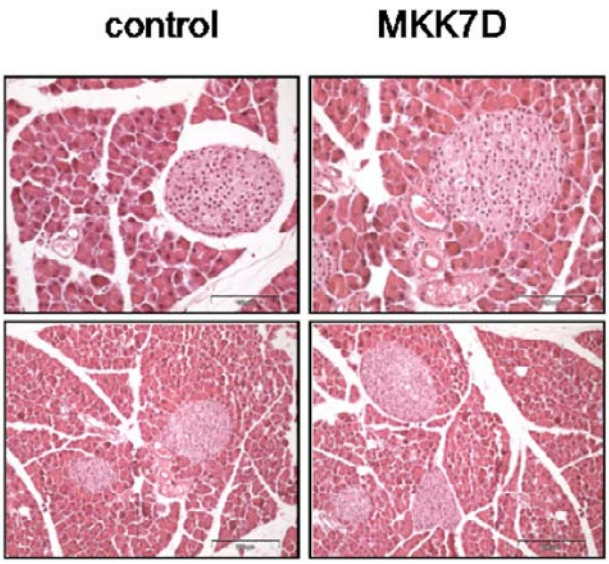

Figure 1. In vivo ***JNK activation in insulin-producing cells does not produce significant changes in the number or morphology of pancreatic β-islets.*** *Pancreata of unfloxed (control) and floxed (MKK7D) GFP-MKK7D transgenic mice stained by eosine-hematoxilin.*

Our group studies the mitotic function of the Nek9/6/7 cassette as well as the regulation of its upstream kinase, Nek9. For this purpose, we have performed various experiments aimed to identify proteins that interact with the protein kinases. One of the proteins identified as a Nek9 interactor, LC8 (also known as DYNLL), was previously found to be a component of several macromolecular complexes, such as the motor dynein. We have characterised LC8-Nek9 binding and produced different point mutants of these proteins that are not able to interact. Using these and other tools, we have shown that LC8 does not act as an adaptor between Nek9 and dynein, and that although LC8 is not necessary for Nek9 dimerization it is involved in Nek9 multimerisation and collaborates in its activation mechanism, most probably by amplifying Nek9 activity during *in vivo* activation. More importantly, we have shown that LC8 binding to Nek9 is regulated by phosphorylation, as we have identified a Nek9 residue which, once phosphorylated, impedes LC8 binding to the kinase. This residue is autophosphorylated in response to Nek9 activation, thus inducing LC8 release from active Nek9. Using a range of approaches, such as RNAi, we have demonstrated that LC8 allows Nek6/7 interaction with Nek9, and thus their subsequent activation. We propose that LC8 is a key controller of signal transduction through the Nek9/6/7 module and show for the first time that LC8 binding to other proteins can be regulated through partner phosphorylation.

Following our interest in unravelling how phosphorylation controls the execution of mitosis, we have started a project in collaboration with Jens Lüders' group (Cell and Developmental Biology Programme) to study the regulation through phosphorylation of the γ-tubulin ring complex (γ-TURC), a multiprotein complex formed by γ-tubulin and a number of associated proteins that is indispensable for microtubule nucleation (and thus spindle formation), and that we have previously shown to interact with Nek9 in various systems. To this end, we have developed a method based on the expression of tagged γ-tubulin that allows the purification of significant amounts of γ-TURC from both exponentially growing and mitotic cells; we expect that this approach will allow us to analyse both the protein content and phosphorylation of the complex and to study its spatiotemporal regulation during the cell cycle.

Regarding the study of the function of the Nek9/6/7 module, we are currently addressing its relationship with Eg5, a mitotic kinesin that we previously demonstrated to be an *in vivo* substrate of Nek6. We are also in the process of producing Nek9 -/- knock out mice, and have generated heterozygous Nek6 +/- mice. This has allowed us to determine that Nek6 is required for normal development as Nek6 -/- knock out mice die at early stages of embryogenesis. At present we are characterising this mice model, and we anticipate that the culture of Nek6 -/- MEF cells will be a valuable tool to better understand the molecular role of this kinase.

Finally, we have also made significant advances in the study of the activation mechanism of Nek9 and identified the proteins responsible for the regulation of this kinase. Other ongoing projects in our group address the following: the determination of the structure of an inactive form of Nercc1; the binding of Nek6/7 and LC8 to this kinase (in collaboration with David Reverter, Institute of Biotechnology and Biomedicine, Autonomous University of Barcelona, Spain); and the Nek9/6/7 signalling module in *Xenopus* mitotic egg extract (in collaboration with Isabelle Vernos, Center for Genomic Regulation, Spain).

Scientific output

Publications

Casals-Casas C, Álvarez E, Serra M, de la Torre C, Farrera C, Sánchez-Tilló E, Caelles C, Lloberas J and Celada A. CREB and AP-1 activation regulates MKP-1 induction by LPS or M-CSF and their kinetics correlate with macrophage activation versus proliferation. *Eur J Immunol*, **39**(7), 1902-13 (2009)

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Research networks and grants

Ajuts a grups de recerca reconeguts
Generalitat de Catalunya, 2009-SGR-163 (2009-2013)
Principal investigator/Researcher: Carme Caelles, Joan Roig

El módulo de señalización Nercc1/Nek6/7; regulación y funciones
Spanish Ministry of Science and Innovation, BFU2008-03441/BMC (2009-2011)
Researcher: Joan Roig

Papel de la c-Jun N-terminal kinase en las acciones fisiológicas y farmacológicas de los glucocorticoides y los ligandos de PPARs y LXRs
Spanish Ministry of Science and Innovation, BFU2007-62087 (2007-2009)
Principal investigator: Carme Caelles

Relación de la expression del receptor de insulina y la activación de la vía PI3K/AKT con la expresión de enzimas glicogénicas y gluconeogénicas en células tubulares de riñón de ratas normales y diabéticas
'Marcelino Botin' Foundation, IO-FMBotin (2008-2010)
Principal investigator: Carme Caelles

Collaborations

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

LXR-MAPK pathways cross talk
Annabel F Valledor, University of Barcelona (Barcelona, Spain)

Regulation of MAPK pathways in macrophage
Antonio Celada, IRB Barcelona (Barcelona, Spain)

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

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

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

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

Antonio Celada

Macrophage biology: The regulation of gene expression

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

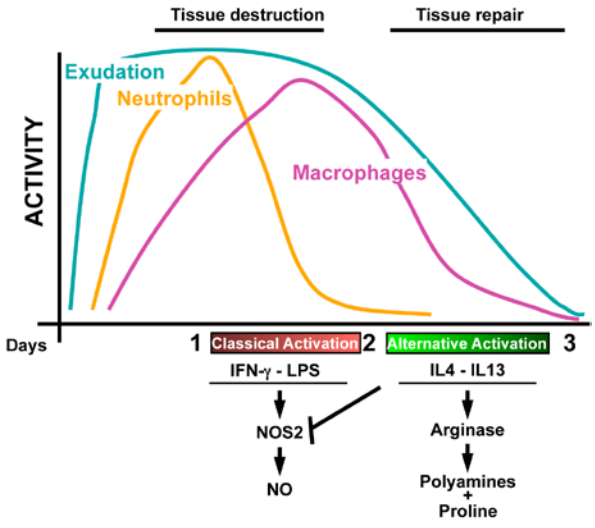


Figure 1. Dual activity of macrophages at the inflammatory loci.

Signal transduction and gene regulation mediating the proliferation, activation and apoptosis of macrophages

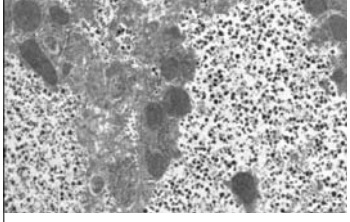
The mitogen-activated protein kinase (MAPK) cascade is one of the best characterised signal transduction pathways involved in the sequential activation of Ras, Raf-1, mitogen/extracellular signal-regulated kinase (MEK) and the extracellularly regulated kinase (ERK). Activated Raf-1 phosphorylates MEK1 and MEK2, which in turn activate ERK1 and ERK2. In unstimulated cells, ERK1 and ERK2 are found in the cytoplasm and they relocate to the nucleus after being phosphorylated. Once in the nucleus, they phosphorylate a series of transcription factors. These kinases also participate in the synthesis of nucleotides and in protein translation processes, both required for 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 the latter also blocks proliferation. The duration and time of initiation of ERK phosphorylation determines whether the cell proliferates

(short phosphorylation) or whether it 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 (Casals *et al*, 2009). In both cases, MKP-1 induction is mediated by PKCε and is independent of ERK phosphorylation. Furthermore, IFN-γ, which also inhibits proliferation, blocks MKP-1 induction by M-CSF by elongating ERK phosphorylation. Inhibition of MKP-1 induction by RNA interference (RNAi) blocks proliferation and elongates ERK activation.

We have also defined all the intracellular pathways required for the regulation of the MKP-1 gene that is mediated by phosphorylated c-jun and CREB. We have cloned the MKP-1 promoter and, by means of luciferase mutations and activity assays, we have localised an AP-1/CRE box that is critical for MKP-1 induction by M-CSF and by LPS. By electrophoretic mobility shift assays and chromatin immunoprecipitation, we have determined that this box is bound by Jun and CREB factors. c-Jun and the phosphorylation of CREB are induced by LPS and M-CSF with the same kinetics as MKP-1 (Casals *et al*, 2009; Figure 2).

Our group has devoted many years to the study of the regulation of MHC class II molecules. Peptides derived from processed proteins bind to a cleft in the MHC class II molecule surface and are presented to T-lymphocytes. Thus, the expression of MHC class II molecules regulates not only the generation of the T-lymphocyte repertoire, but also the induction and maintenance of immune response. MHC class gene transcription depends on the interaction and co-operation of several transcription factors that bind to the regulatory elements found in the promoter. However, all the transcription factors described to date show ubiquitous expression, which does not correlate with the differential tissue expression of MHC class II molecules. CIITA (class II transactivator), which does not bind directly to DNA, has been described and shown to be required for the expression of these genes. We have determined that an AP-1 box acting as an enhancer is responsible for the induction of expression in B-lymphocytes and dendritic cells treated with LPS. Also, the upstream regulatory elements interact with the proximal promoter, thereby blocking the transcription. This loop is open when CIITA is present (Serrat *et al*, in press).

In collaboration with Víctor Puntes (Catalan Institute of Nanotechnology—ICN) and Er-



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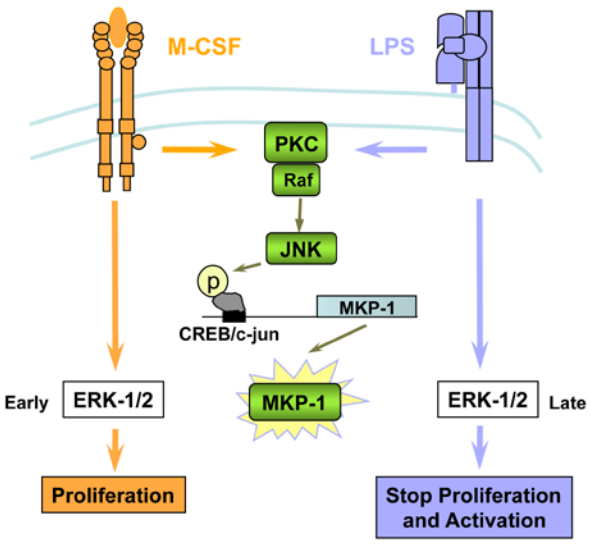


Figure 2. Signal transduction of M-CSF and LPS inducing the expression of MPK-1.

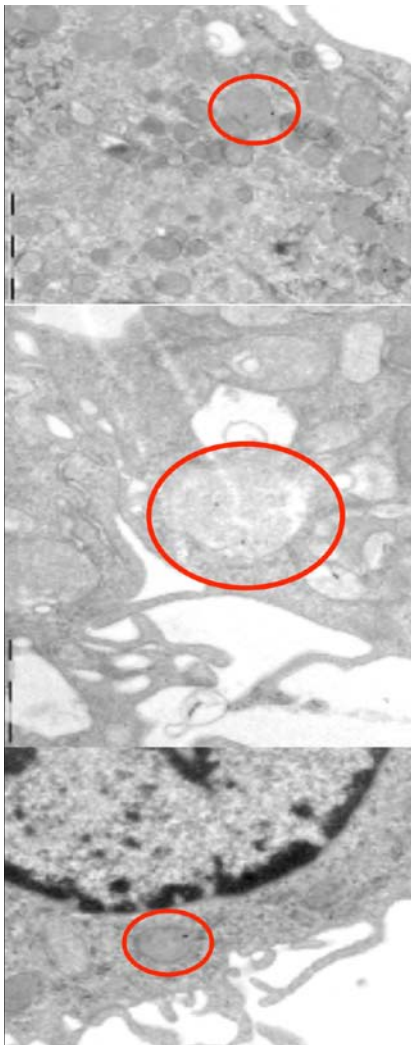


Figure 3. Nanoparticles ingested by macrophages.

nest Giralt (IRB Barcelona), we have found that nanoparticles have the capacity to activate macrophages by interacting with Toll-like receptor 4 (TLR4) (Bastús *et al*, 2009a and 2009b; Figure 3).

Finally, we have reviewed the role of the CDK inhibitor $p21^{waf1}$ as a regulator of macrophage pro-inflammatory activity that prevents the development of septic shock (Lloberas and Celada, 2009; Figure 4).

Molecular mechanisms involved in classical and alternative activation of macrophages

Classical or pro-inflammatory activation of macrophages is induced by IFN-γ or LPS while that triggered by IL-4, IL-10 or IL-13 is known as alternative or anti-inflammatory activation. Apart from a series of structural and functional modifications, the main difference between these phenotypes is the biochemical pathway used for processing the amino acid arginine. IFN-γ and LPS induce Nitric Oxide Synthase 2 (NOS2) enzyme, thereby producing nitric oxide (NO), which has great destructive power and in the first phases of inflammation kills microorganisms. In anti-inflammatory macrophages, arginase is induced and produces proline and polyamines, which catalyse the reconstitution of the damaged extracellular matrix, an event that occurs during the final phases of inflammation. We have found that activation with IL-4 or IFN-γ blocks proliferation in G1/S. However, while the mechanism behind IL-4 inhibition of the proliferation is $p21^{waf1}$- and Stat6-dependent, the mechanism used by IFN-γ differs (Arpa *et al*, 2009). Also, we have reviewed the methods to determine the classical and alternative activation of macrophages (Classen *et al*, 2009).

Role of TREX1 exonuclease in transcription

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

In collaboration with IRB Barcelona experts in crystallography (Ignasi Fita) and in NMR (Maria Macias), we have determined the structure of TREX1 alone and its binding to DNA. TREX1 binds preferentially to certain DNA sequences that correlate with the exonuclease activity. TREX1 has a proline-rich domain that is not found in TREX2. This domain allows interaction with SH3 or WW domains, which we have demonstrated by NMR and co-immunoprecipitation. These data, together with the nuclear localisation of the protein, have led us to study whether TREX1 is involved in transcription. In addition, we have identified a new active histidine conserved in DEDDh exonucleases that is required for functional activity (Figure 5).

Deregulated gene expression in aging

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

The effect of aging was determined on bone marrow-derived macrophages produced *in vitro* from aged mice. In these cells, compared to those of young mice, the telomeres were shorter and GM-CSF- but not macrophage (M)-CSF-dependent proliferation was impaired as a result of decreased phosphorylation of STAT5a. The same defects were found in macrophages from knock-out (KO) mice for telomerase (*terc*$^{-/-}$). Macrophages from aged and *terc*$^{-/-}$ mice showed increased susceptibility to oxidants and an accumulation of intracellular reactive oxygen species. In these macrophages, STAT5a oxidation was reduced, which led to the decreased phosphorylation observed. These results suggest that telomere loss produces enhanced oxidative stress, reduced STAT5a oxidation and phosphorylation and, ultimately, impaired GM-CSF-dependent macrophage proliferation (Sebastian *et al*, 2009a). Finally the molecular and cellular aspects of macrophage aging have been reviewed (Sebastian *et al*, 2009b).

Ly-6C+CD11b+ cells and monocyte homing for inflammatory tissues

In inflammation, blood monocytes migrate into tissues and give rise to dendritic cells and macrophages. In the mouse, circulating monocytes that migrate to non-lymphoid tissues in inflammation express the cell surface marker Ly-6C. Myeloid Ly-6C+ CD11b+ cells are recruited to early atherosclerotic and relapses of neuroinflammatory lesions. Thus these cells constitute a highly relevant subset of myeloid cells with which to study the mechanisms involved in proliferation, differentiation and apoptosis in macrophages.

To date, myeloid Ly-6C+CD11b+ cells have been studied only *in vivo* through complex and time-consuming procedures. We have developed an *in vitro* method to generate Ly-6C+CD11b+ cells from mouse bone marrow cells. We are characterising this cell population. We are developing an animal model of cutaneous inflammation both *in vitro* and *in vivo* by *in vivo* imaging (IVIS). This project will contribute to translate several molecular mechanisms involved in proliferation, differentiation, activation and apoptosis of macrophages in inflammation identified by our group into an *in vivo* context.

The Ly-6C+CD11b+ monocyte subset has a human equivalent in the CD16-CLA+ monocyte subset. The CLA (cutaneous lymphocyte-associated antigen) constitutes the skin-homing receptor, an adhesion molecule that identifies immune cells and tumour cells with cutaneous tropism. We have a close collaboration with the Dermatology Service of the Hospital del Mar in Barcelona on the immunopathology of CLA+ cells in psoriasis, eczema and cutaneous T-cell lymphoma. This collaboration allows us access to pathological material and the technology to study these cells, and thus the opportunity to be one step ahead in the translation of this project by characterising the monocyte equivalents of mouse Ly6-C+CD11b+ in an *ex vivo* model of psoriatic lesion (Antúnez *et al*, 2009; Ferran and Santamaría-Babi; Santamaría-Babi, in press).

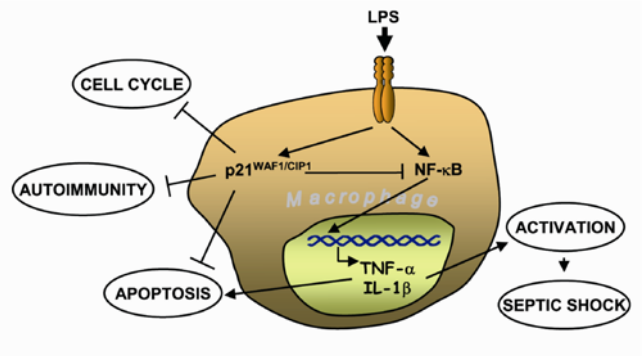
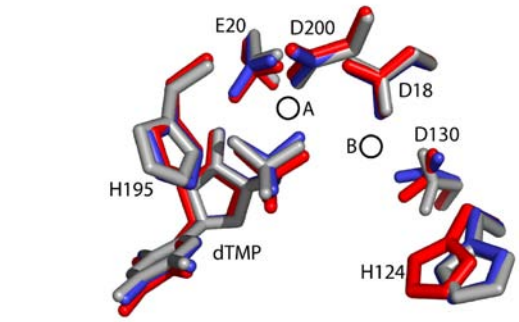


Figure 4. p21^{waf1} plays a crucial role in the biology of macrophages.

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                Exo I                Exo II                Exo III
                                     H124                  H195
hTREX1   (15)IFFDMEATGLPFSQ  (121)LVAHNGDRYDFPLLQ  (192)PDSHTAEGDVLALLS
mTREX1   (15)IFLDLEATGLPSSR  (121)LVAHNGDRYDFPLLQ  (192)TDSHTAEGDVLTLLS
hTREX2   (11)VFLDLEATGLPSVE  (114)LVAHNGFDYDFPLLC  (185)SAAHSAEGDVHTLLL
mTREX2   (11)VFLDLEATGLPNMD  (114)LVAHNGFDYDFPLLC  (185)SAAHSAEGDVHTLLL

Eco-DP3E  (9)IVLDTETTGMNQIG   (95)LVIHN-AAFDIGFMD  (159)RTLHGALLDAQILAE
Eco-RNT  (20)VVIDVETAGFNAKT  (117)MVAHN-ANFDHSFMM  (178)TQAHSALYDTERTAV
Bsu-DP3 (422)VVFDVETTGLSAVY  (502)LVAHN-ASFDMGFLN  (564)--HHRAIYDTEATQY

Eco-POL1(352)FAFDTETDSLDNIS  (416)KVGQN-LKYDRGILA  (493)EAGRYAAEDADVTLQ
BPT4-DPO(109)ANCDIEVTGDKPPD  (210)FTGWNIEGFDVPYIM  (316)RYISYNIIDVESVQA
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*Figure 5. **Histidine 124 participates in TREX1 activity.** Superimposition of the active site of the TREX1-dTMP-ion complexes. The conserved residues DEDDh, the His124, and the dTMP are represented as sticks and are red (magnesium complex), blue (lithium complex), or gray (sodium complex).*

Scientific output

Publications

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Valledor AF, Lloberas J and Celada A. Macrophage foam cells. In Encyclopedia of Life Sciences, John Wiley & Sons, in press (2009)

Research networks and grants

Mecanismos moleculares y celulares en enfermedades inflamatorias crónicas y autoinmunes
Genoma España, Research Project PE MACIA (2009-2011)
Principal investigator: Antonio Celada

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

Collaborations

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

Estudio piloto de inmunización
EraBiotech (Barcelona, Spain)

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

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

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

Inflammation and polymerases
Antonio Bernard, Spanish National Centre for Cardiovascular Research (Madrid, Spain)

Inflammation in dermatology
Ignacio Umbert, Clínica Corachan (Barcelona)

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

Telomerase and macrophaging
María Blasco, Spanish National Cancer Research Centre (Madrid, Spain)

Awards and honours

Augusto Stiefel Prize
Spanish Academy of Dermatology and Venereology (2009)
Awardee: Luís F Santamaría Babi

Joan Guinovart

Glycogen metabolism in health and disease

Our group is devoted to the study of the regulatory mechanisms of glycogen metabolism. We focus on the physiological regulation of glycogen deposition and the pathological implications of its alteration. We have a long tradition in the study of glycogen synthase (GS), the key enzyme in the regulation of glycogen synthesis. In order to address relevant biological issues, 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. Against the general belief that everything was known about the regulation of glucose metabolism, our discoveries have open new areas of investigation and brought renewed attention to this field. Since altered glycogen deposition may have a causal relation to Lafora disease and other pathologies, we aim to gain a deeper understanding of its regulatory mechanisms and to characterise new therapeutic targets.

Study of glycogen metabolism in diabetes and the search for therapeutic tools

Glycogen synthase (GS) is the only enzyme that catalyses glycogen synthesis. In vertebrates there are two GS isoforms, one expressed exclusively in liver (LGS), and the other expressed in muscle and in most other tissues (MGS). GS expression is elevated in tissues involved in glucose homeostasis and glycogen storage, namely liver, skeletal muscle, heart and even adipose tissue. Liver glycogen metabolism plays a central role in glucose homeostasis and the control of glycemia. In fact, the reduced capacity to accumulate glycogen in the liver of diabetic patients dramatically contributes to hyperglycemia. Thus, LGS activation and stimulation of liver glycogen deposition are potential targets for the treatment of diabetes mellitus.

Analysis of liver glycogen synthase

GS activity is regulated by reversible phosphorylation, in addition to other regulatory mechanisms including alosterism, subcellular localisation and protein stability. While much research effort has focused on the functional consequences of MGS phosphorylation, little has been devoted to the liver isoform. Nine phosphorylation sites have been described in MGS, and by sequence alignment 7 of these are conserved in LGS. By systematically mutating these 7 serine residues to alanine in the LGS sequence, individually or in pairs, we have identified site 2 (Ser7) as the most potent regulatory site of the activity of the enzyme (Ros *et al*, 2009). We generated and characterised a constitutively active mutant LGS form. Next we tested the efficacy of increasing LGS activity to improve blood glucose homeostasis in rats in fed and fasted states. The adenovirus-mediated transfer of wild-type LGS to the liver of rats had no effect on blood glucose homeostasis in either state. In contrast, the expression of the active LGS form caused a significant lowering of blood glucose in fed rats but not in fasted ones. Moreover, it markedly

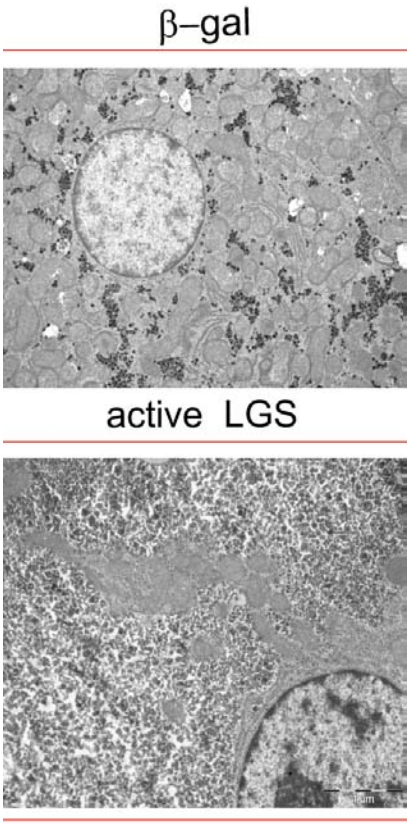


Figure 1. ***Effects of active LGS overexpression on ultracellular structure as shown by electron microscopy analysis of liver sections.*** *Cellular ultrastructure analysis by electron microscopy of liver biopsies from the rats overexpressing β-gal or the active LGS, fed ad libitum. Note the electrodense glycogen particles distributed throughout the cytoplasm. The ruler represents 5 nm (taken from Ros* et al*, in press).*

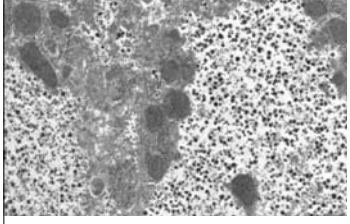
enhanced the clearance of blood glucose when fasted rats were challenged with a glucose load. Hepatic glycogen stores in rats overexpressing active LGS were enhanced in the fed state and in response to an oral glucose load, but showed a net decline during fasting. We conclude that LGS activation improves glucose tolerance in the fed state without compromising glycogenolysis in the post-absorptive state (Ros *et al*, in press). On the basis of these findings, we propose that LGS activation may provide a potential strategy for improving glucose tolerance and normalising glycemia in diabetic states.

In a complementary study, mass spectrometry techniques were used to identify LGS phosphorylation states characteristic of several metabolic conditions. New phosphorylation sites, not conserved in the MGS sequence, were identified in these studies. Next, rat LGS was engineered to mimic phosphorylated species by mutating phosphorylatable serine residues to glutamic acid. Various mutant LGS forms, including single and multiple Ser-to-Glu and Ser-to-Ala substitutions, were generated, subcloned into adenoviral vectors and transferred to primary hepatocytes. We are currently characterising the effects of each mutation on LGS activity, sensitivity to regulation, and subcelullar localisation.

Identification of the molecular targets of the anti-diabetic and anti-obesity agent sodium tungstate

Sodium tungstate is an oral glucose-lowering and anti-obesity agent discovered and patented by our group. After demonstration of the activity of this compound in an animal model of type 2 diabetes (ZDF rats), it has completed Phases I and II clinical trials. In collaboration with the groups led by Ramon Gomis (IDIBAPS-Hospital Clínic de Barcelona), Rafael Salto (University of Granada) and Joan Enric Rodríguez Gil (Autonomous University of Barcelona), we have devoted much research effort to unravelling the molecular targets and physiological effects of tungstate. Previous work indicated that this compound induces the activation of the MAP kinase pathway. Our recent experiments show that G-protein activation is involved in the mechanism of tungstate, since Pertussis Toxin, a G-protein inhibitor specific for G_i and G_o, blocks both tungstate-induced ERK phosphorylation and glycogen deposition in primary hepatocytes. These results are being validated by combining tungstate treatment with G protein knock-down (shRNA) or signalling disruption in human and rat cells.

In addition to ERK phosphorylation, we have observed a tungstate-induced normalisa-



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tion of PEPCK expression in treated diabetic rats. This result indicates that tungstate reduces the activation of the gluconeogenic pathway associated with diabetes mellitus, thereby contributing to the lowering of circulating glucose. We extended this research by studying the effects of tungstate on several gluconeogenesis-related genes. We have detected significant changes in the expression of regulatory proteins affecting the control of this pathway.

Study of glycogen metabolism in neurons and the consequences of its deregulation

Although glycogen is present in most cells, its metabolism has been studied mainly in liver and muscle. Nevertheless, there are some cell types, like neurons, that do not accumulate this polysaccharide. We have demonstrated that neurons express GS, specifically MGS. This is a remarkable finding because these cells do not normally accumulate glycogen. However, GS activity in neurons is tightly blocked through previously described regulatory mechanisms (phosphorylation, subcellular localisation) and through a new mechanism that involves the coordinated action of malin and laforin proteins. Mutation or inactivation of these two genes, together representing more than 90% of the genetic defects found in Lafora disease patients, results in the suppression of one control level of glycogen accumulation and in the formation of non-degradable glycogen aggregates. Our results also show that excessive glycogen accumulation in neurons induces apoptosis (Vilchez *et al*, 2007).

The concept that glycogen is harmful for neurons has completely changed our vision of the field. During 2009 we have oriented our research to gaining a better understanding of the following: the physiological role of GS in neurons; the (pathological) conditions that induce glycogen accumulation in neurons; and the apoptotic pathway activated by glycogen accumulation. In addition, we have generated a number of transgenic mouse models of (conditional) gain- and loss-of-function of GS and associated regulatory proteins. Using Cre-recombinase technology, we are able to direct the overexpression or deletion of our gene of interest to specific cell types. These animal models will provide further insight into the physio-pathological implications of abnormal glycogen accumulation *in vivo*.

Study of physiological role of GS expression in neurons

As stated above, neurons express GS but do not normally accumulate glycogen. Furthermore, glycogen accumulation is harmful for neurons. The obvious question is why neurons use energy to express GS and keep it strictly blocked. We are addressing this issue through a range of approaches. First, using immunhistochemistry, we are studying the expression of MGS in the mouse brain at various developmental stages, in order to identify the neurons and stages in which higher levels of MGS are expressed, and whether this increased expression is related to transient periods of tolerated glycogen deposition. Second, we hypothesised that MGS has a moonlighting activity in addition to its function in glycogen synthesis. Since MGS translocates to the nucleus in cells lacking glycogen deposits, we have studied the putative nuclear function of this enzyme. By co-immunoprecipitation techniques, we have identified MGS-binding proteins that participate in RNA processing. We are currently testing the capacity

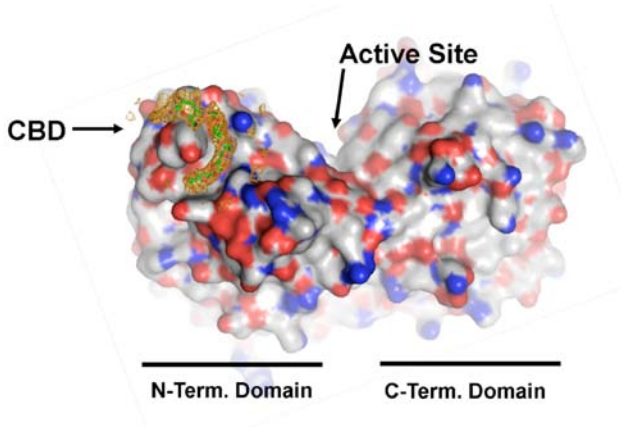

*Figure 2. **Crystallographic structure of monomeric PaGS.** The enzyme is represented by coloured surfaces (carbon atoms in grey, nitrogen in blue and oxygen in red) while the bound maltotetraose molecule is shown by sticks, including its electron density. CBD, Carbohydrate Binding Domain.*

of MGS to directly bind RNA. Furthermore, in order to dissect the regulatory events that determine MGS subcellular localization and proteasome-mediated degradation, we have generated a collection of mutant MGS forms.

Study of the (pathological) conditions that induce glycogen accumulation in neurons

A review of the literature indicates that the presence of glycogen in neurons in certain neurological diseases was reported many years ago. Moreover, the presence of intracellular bodies composed mainly of glucose polymers has been recognised in many pathological conditions. The nomenclature used to describe these structures is varied: polyglucosan bodies, *corpora amylacea* and Lafora bodies, among others. However, all these structures share a common feature, namely they are essentially formed by poorly branched glycogen. Indeed, abnormal glycogen deposits are found not only in Lafora disease patients, but also in those with other syndromes commonly designated as Glycogen Storage Diseases (GSDs) or glycogenoses. Several of these rare diseases have a recessive monogenic origin, involving a number of proteins related to glycogen synthesis and disposal. In addition, polyglucosan bodies can be found as a consequence of prolonged hyperglycemia in diabetic patients.

In collaboration with Ramon Gomis (IDIBAPS-Hospital Clínic de Barcelona) and Rafael Simó (Hospital Vall d'Hebron), and in the context of a CIBERDEM coordinated project, we have studied the presence of abnormal glycogen deposits in pancreatic β-cells, retinal neurons, and nephrons, and the relation of these deposits with the diabetic complications affecting these organs (*ie* retinopathy, nephropathy). We have demonstrated the expression of MGS in these cell types and the accumulation of glycogen in the retina of diabetic patients. In addition, we are generating tissue-specific transgenic mouse models in order to assess whether glycogen accumulation by itself is causative of said pathologies. Finally, we are interested in the potential relation

between hypoxia, aging, glycogen accumulation and neurodegeneration. In this regard, we have used an *in vitro* model to study the effects of hypoxia in neurons. We have observed that under these conditions MGS is strongly activated. We are currently extending these studies, as well as testing human samples obtained from elderly subjects and patients with neurodegenerative diseases.

Study of the apoptotic pathway activated by glycogen accumulation in neurons

In an effort to characterise the apoptotic pathway activated by abnormal glycogen deposition in neurons, we have studied the level of activation of several proteases that drive the cell death programme. First, by expressing a constitutively active GS form, we confirmed that enforced glycogen accumulation induces neuronal apoptosis. Second, we observed that glycogen accumulation induces the activation of Bid, Caspase 8 and Caspase 3, thereby suggesting the activation of the extrinsic apoptosis signalling pathway. Finally, a major effort has been made in the generation of *in vivo* models for the study of glycogen-induced neurodegeneration. In addition to the above-mentioned transgenic mouse models, we have optimised intracranial stereotactic injection for the delivery of viral vectors to specific brain areas in mice. This technique will allow us to force the expression or silencing of genes of interest and thus to study their role in the regulation of neuronal glycogen metabolism and glycogen-induced apoptosis.

Structural approach to the elucidation of the catalytic mechanism and the regulation of GS

The expression and purification of mammalian GS has proven extremely difficult. However, we have been able to purify GS from *Pyrococcus abyssi* (*Pa*GS) and crystallise it as a trimeric complex. In order to reduce the flexibility of the trimeric structure and improve resolution, we have recently generated a monomeric *Pa*GS mutant form, which can be purified and crystallised in the presence of substrates. Crystals obtained in the presence of UDPG and maltohexaose were subjected to X-Ray diffraction to generate a new 2.6 Å resolution structure. Surprisingly, this structure shows the enzyme in an open conformation with no substrate present in the active site. Instead, a glucose polymer is found tightly bound to the surface of the N-terminal domain of the protein (Figure 2). This result offers structural evidence of the existence of a carbohydrate binding domain in the N-terminal part of GS, which most likely facilitates the binding and retention of the enzyme to the growing glycogen particles *in vivo*. Importantly, this carbohydrate binding site is structurally conserved in *Escherichia coli* GS and in glycogen phosphorylase, and a similar domain can be deducted by sequence alignment in mammalian GSs. We are currently studying the functional role of this glycogen binding site in human MGS by generating single amino acid mutant forms and testing their binding affinity to glycogen and their specific activity. FRAP results demonstrate a marked reduction of glycogen binding capacity when conserved tyrosine residues in the carbohydrate binding domain are mutated to alanine.

Scientific output

Publications

Guinovart JJ. Mind the gap: bringing scientists and society together. *Cell*, **137**(5), 793-95 (2009)

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Research networks and grants

Actividades Comité IUBMB
Spanish Ministry of Science and Innovation, MEC-IUBMB (2007-2009)
Principal investigator: Joan J Guinovart

Diabetes and obesity treatment by tungstate: metabolic and molecular targets
Carlos III Health Institute, CIBERDEM—DOTUM (2009-2010)

Diabetes y enfermedades metabólicas asociadas (CIBERDEM)
Carlos III Health Institute, CB07-08-0045 (since 2008)
Principal investigator: Joan J Guinovart

Enfermedad de Lafora: papel de laforina y malina
'La Caixa' Foundation (2006-2009)
Principal investigator: Joan J Guinovart

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

Glycogen-induced dysfunctions in pancreas and retina and their involvement in the ethiogenesis of diabetes mellitus (GIDIPRED)
Carlos III Health Institute, CIBERDEM—GIDIPRED (2009-2010)

Mejora de la predicción traslacional de los ensayos de seguridad no clínica al hombre
NOSCIRA SA (2007-2010)
Principal investigator: Joan J Guinovart

Molecular basis of progressive myoclonus epilepsy of the Lafora type
'La Marató TV3' Foundation (2007-2009)
Principal investigator: Joan J Guinovart

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

Collaborations

Analysis of the 3D structure of glycogen synthase
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Characterization of glycogen metabolism in reproductive tissue: analysis of alterations in pathological situations
Joan E Rodríguez-Gil, Autonomous University of Barcelona (Barcelona, Spain)

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

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

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

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

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

Laser induced forward transfer: a direct writing technique for biosensors preparation
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Mechanism of action of anti-hyperglycaemic compounds and development of in vitro *methods for screening mode of action*
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Molecular basis of Lafora disease
Santiago Rodríguez de Córdoba, Centro de Investigaciones Biológicas, CSIC (Madrid, Spain); 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 and Mª Dolores Girón, University of Granada (Granada, Spain)

Relation between the diabetic syndrome and the key glucose homeostasis enzymes, fructose-1,6-Biphosphatase and glycogen synthase
Juan Carlos Slebe, Instituto de Bioquímica, Universidad Austral de Chile (Valdivia, Chile)

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

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

Study of the alterations in glycogen metabolism associated with colon cancer
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Study of the alterations of glycogen metabolism in animal models with neurological diseases
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Study of the molecular targets and biological actions of sodium tungstate
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Study of the proteomic alterations induced by tungstate treatment of diabetic animals
Carmen Cámara, Universidad Complutense de Madrid (Madrid, Spain)

The use of Drosophila melanogaster *as model system for the study of Lafora disease*
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Awards and honours

Prat de la Riba award
Institut d'Estudis Catalans (2009)
Awardee: Joan J Guinovart

Manuel Palacín

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

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

The molecular bases of renal reabsorption of amino acids

Our laboratory has identified and characterised three amino acid transporters involved in the renal reabsorption of amino acids: systems $b^{0,+}$ (heterodimer rBAT-$b^{0,+}$AT), y^+L (heterodimer 4F2hc-y^+LAT1) and exchanger L (heterodimer 4F2hc-LAT2; Figure 1). We have also demonstrated the role of systems $b^{0,+}$ and y^+L in cystinuria and lysinuric protein intolerance (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. The characterisation of mutated mouse models of LAT2 and EEG1 might shed light on this issue. Moreover, in collaboration with Paolo Gasparini, we are studying whether there is an association between amino acid transporter polymorphisms and renal reabsorption of amino acids in genetically isolated human populations. In this regard, we have identified groups of amino acids with co-variation in urinary excretion (D'Adamo *et al*, in press). This activity was initiated within the European Union project EUGINDAT (European Union Genomic Initiative on Disorders of Amino acid Transporters).

Physiopathology of inherited aminoacidurias cystinuria and 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 as follows: i) identification of molecular mechanisms to explain the distinct phenotypes in cystinuria, using animal and cell models; ii) identification of modulator genes of lithiasis in cystinuria, using animal models; iii) search for new drugs to treat lithiasis in cystinuria, using our murine cystinuria model Stones; and iv) identification of the mechanisms that lead to immunological disorders associated with LPI, using a newly generated floxed y^+LAT1 mouse line. Expression of CRE recombinase under the control of tamoxiphen in these animals has resulted in the first mouse model for LPI.

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

Our laboratory has identified most of the members of the HATs. Moreover, we have approached the structure-function relationships of HATs by defining the transport mechanisms as obligatory exchangers, the oligomeric state, 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 key residues for transport. Recently, in collaboration with Dimitrios Fotiadis (EUGINDAT project), we obtained the first projection map of a prokaryotic homologue of the light subunits of HATs (LAT family of transporters) at a subnanometer scale (6.5 Å). This map revealed striking similarities with unrelated transporters with the so called '5 + 5 transmembrane repeat' fold (Casagrande *et al*, 2008). Recently, we offered evidence

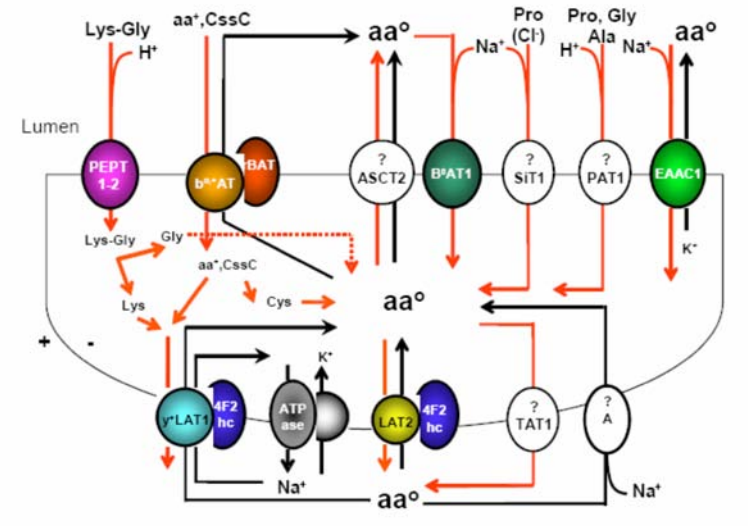
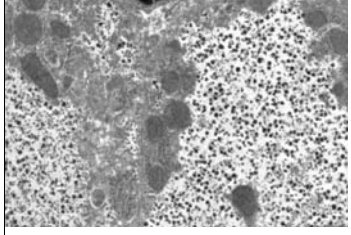


Figure 1. ***Proximal tubule model for amino acid transporters involved in renal and intestinal reabsorption of amino acids.*** *Transporters with a proved role in renal reabsorption or intestinal absorption of amino acids are coloured, whereas those expressed in the plasma membrane of epithelial cells of the proximal convoluted tubule (or of the small intestine) but with no direct experimental evidence supporting their role in reabsorption are shown in white. Amino acid fluxes in the reabsorption direction are in red. PEPT1 and PEPT2 are expressed in the small intestine and kidney respectively. Adapted from Moe, Wright and Palacín; Brenner & Rector's The Kidney, chapter 6, 214-47 (2008)*

by force spectroscopy that substrate binding increases the conformational flexibility of a LAT transporter (Bippes *et al*, 2009). At present, we are working on the atomic resolution of a prokaryotic homologue of the light subunits of HATs (Figure 2). Functional studies in parallel seek to identify key residues for amino acid transport function within HATs.



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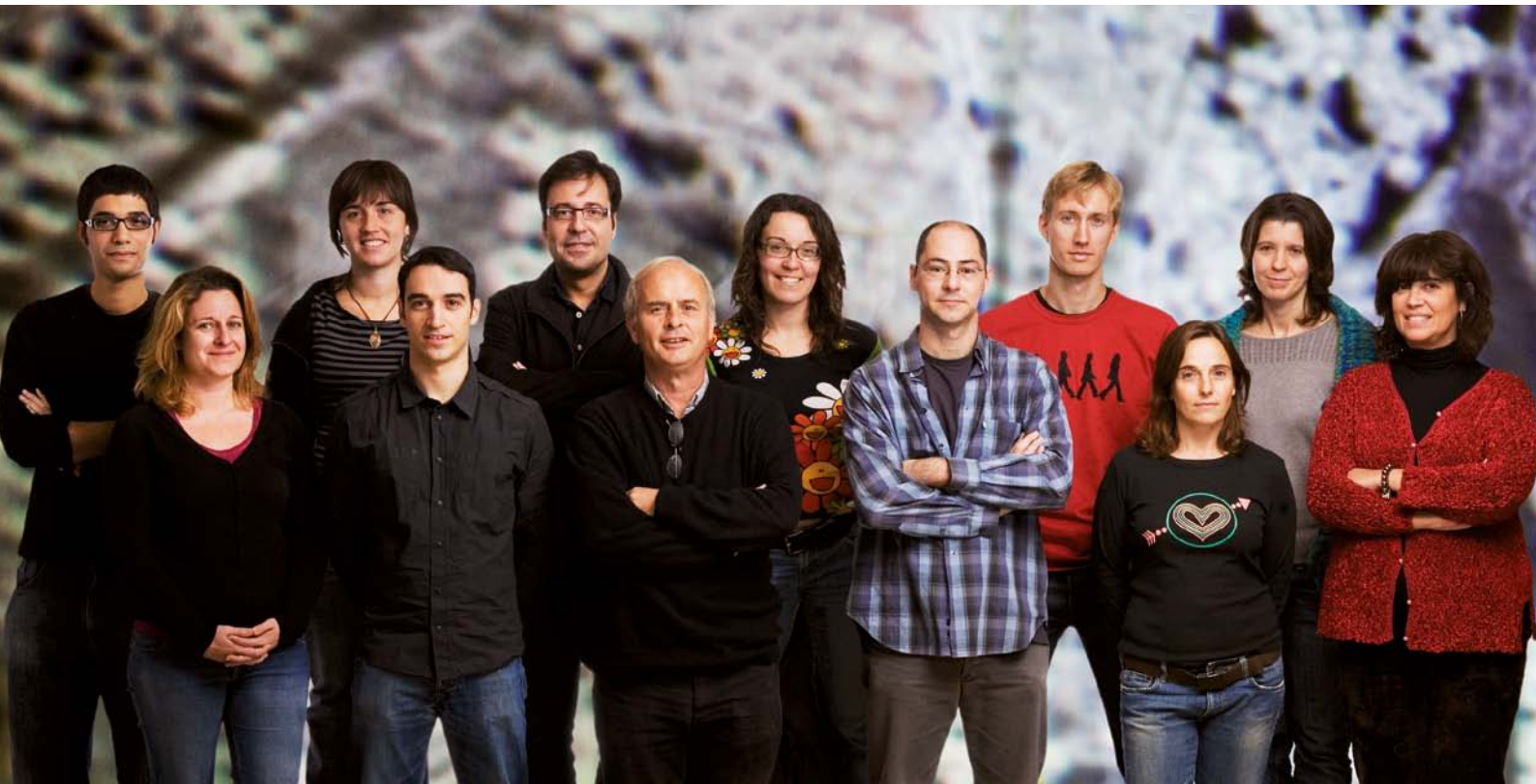

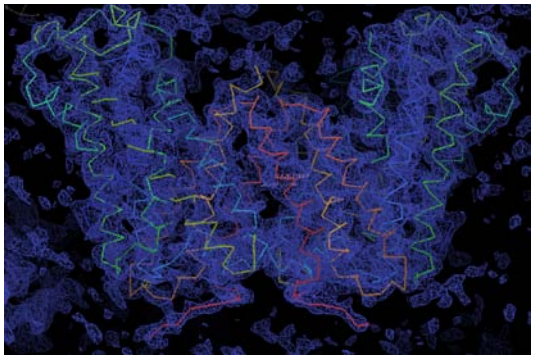

Figure 2. ***Electronic density of a prokaryotic homologue of the light subunits of HATs.*** *The atomic structure (Ca backbone; rainbow colour) has been solved at a resolution of 3.5 Å. Electronic density (1 σ) (violet).*

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 ß1 integrins. Moreover, the recombinant extracellular domain of 4F2hc is a powerful tool for the identification of potential ligands of 4F2hc.

Scientific output

Publications

Bippes CA, Zeltina A, Casagrande F, Ratera M, Palacin M, Muller DJ and Fotiadis D. Substrate binding tunes conformational flexibility and kinetic stability of an amino acid antiporter. *J Biol Chem*, **284**(28), 18651-63 (2009)

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Research networks and grants

CIBER de enfermedades raras (CIBERER)
Carlos III Health Institute (since 2007)
Principal investigator: Manuel Palacín

European Drug Initiative on Channels and Transporters (EDICT)
European Commission, 201924 (2008-2012)
Principal Investigator: Manuel Palacín

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

Role of 4F2hc in tumorogenesis
'La Marató TV3' Foundation (2006-2009)
Principal investigator and coordinator: Manuel Palacín

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

Collaborations

Physiopathology of inherited aminoacidurias cystinuria and lysinuric protein intolerance (LPI)
Josep Chillarón, University of Barcelona (Barcelona, Spain); Virginia Nunes, IDIBELL (Barcelona, Spain); Gianfranco Sebastio, Università Federico II (Naples, Italy)

Structure-function relationship in heteromeric amino acid transporters (HATs)
Steve Baldwin, University of Leeds (Leeds, UK); Ignacio Fita, IRB Barcelona (Barcelona, Spain); Dimitrios Fotiadis, University of Bern (Bern, Switzerland); Eric Gouaux, Vollum Institute (Portland, USA); Modesto Orozco, IRB Barcelona (Barcelona, Spain); Matthias Quick, Cornell University (New York, USA)

Study of the multiple functions of heavy chains of HATs
Joaquín Abian, Autonomous University of Barcelona (Barcelona, Spain); Chloe Feral, Nice Sophia Antipolis University (Nice, France); Mark Ginsberg, University of California San Diego (La Jolla, USA); María Antonia Lizarbe, Universidad Complutense de Madrid (Madrid, Spain)

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

Antonio Zorzano

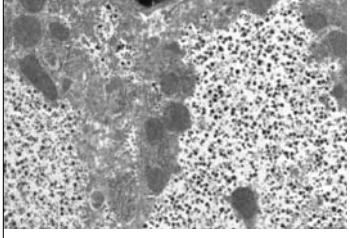
Identification of defective mechanisms in specific forms of type 2 diabetes

It has been estimated that between 200 million and 300 million people worldwide currently meet World Health Organization diagnostic criteria for diabetes mellitus. This epidemic of predominantly type 2 diabetes is largely mediated 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. The major factors proposed to participate in the development of insulin resistance are inflammation, excessive lipid availability, oxidative stress, endoplasmic reticulum stress and mitochondrial dysfunction. A key step towards a complete understanding of type 2 diabetes is the identification of insulin resistance susceptibility genes, which will lead to the acquisition of therapeutic targets for future drug design. Our global aim is to determine the molecular mechanisms involved in the development of insulin resistance, and to identify novel susceptibility genes for insulin resistance and type 2 diabetes. The specific research projects are as follows: i) Analysis of the relationship between mitochondrial activity and insulin signalling. Role of mitochondrial dynamics proteins in metabolic homeostasis and in the control of insulin resistance; ii) Autophagic machinery, and metabolism; iii) Role of regulators of nuclear gene expression in adiposity and in insulin resistance; iv) Identification of novel targets and development of new compounds for the treatment of diabetes.

Muscle mitochondrial metabolism is reduced in type 2 diabetes. This type of diabetes is characterised by insulin resistance, which affects skeletal muscle and other insulin-sensitive tissues, and by defective insulin secretion. Muscle insulin resistance occurs as a result of alterations in intracellular signalling and is manifested by a reduced capacity of insulin to stimulate glucose uptake. In addition to these alterations, insulin-resistant subjects show a reduced muscle capacity to properly oxidise substrates –glucose and lipids– during fasting conditions and after a meal. The switch between glucose and lipid oxidation, depending on energy requirements, is referred to as 'metabolic flexibility'. In this regard, type 2 diabetic subjects show metabolic inflexibility since they present a higher capacity to oxidise lipids in insulin-stimulated conditions, instead of switching to glucose oxidation.

Insulin-resistant conditions are characterised by alterations in mitochondrial activity in skeletal muscle. Elderly insulin-resistant subjects show a reduction in mitochondrial oxidative and phosphorylation activity, as assessed by *in vivo* by 13C/31P NMR spectroscopy, and also increased fat accumulation in muscle and liver. The skeletal muscle of type 2 diabetic patients shows a decrease in the activity of the Krebs cycle and of the respiratory chain. In keeping with these observations, plasma levels of lactate are enhanced and the rate of whole-body lactate production is also increased in these patients. In addition, it has been reported that oral administration of dichloroacetate to diabetic patients reduces fasting hyperglycemia, and plasma lactate, cholesterol and triglycerides without affecting circulating insulin. Several lines of evidence suggest that the alterations in mitochondrial metabolism in skeletal muscle occur before the development of type 2 diabetes. Thus, offspring of type 2 diabetic parents show reduced ATP synthesis, which was the first indication that this may be an inherited defect.

Several mechanisms contribute to the reduction of mitochondrial activity in insulin-resistant conditions, namely changes in mitochondrial density or intrinsic alterations in mitochondrial metabolism. In this regard, there is evidence that insulin-resistant obese individuals with type 2 diabetes have approximately 30% fewer mitochondria in their skeletal muscles than age-matched healthy controls. Thus, the skeletal muscle of type 2 diabetic



patients shows a lower mitochondrial DNA content, and these patients also present reduced citrate synthase activity. It is likely that the decreased mitochondrial mass is a defect in both obesity and type 2 diabetes. Consequently, no differences in muscle mitochondrial DNA copy number or in citrate synthase are found in muscle of obese type 2 diabetic patients compared to non-diabetic obese subjects. A reduced mitochondrial density has been demonstrated in muscle of insulin-resistant offspring of type 2 diabetic parents.

Several studies have reported that the mitochondrial alterations found in muscle of type 2 diabetic patients reflect a functional impairment of mitochondria since these alterations are present even after correction by mitochondrial mass. However, other studies have not detected differences in electron transport chain or in oxygen consumption after correction by surrogates of mitochondrial mass. In all, current data support the view that mitochondrial mass is decreased in skeletal muscle in obesity and in type 2 diabetes, and some evidence supports the notion of a functional impairment of mitochondria in these conditions.

Alterations in the density and function of mitochondria have been demonstrated in muscle of insulin resistant-offspring of type 2 diabetic subjects. This observation points to the presence of inherited defects that lead to mitochondrial dysfunction; however, solid demonstration of this hypothesis is still pending.

A reduction in the expression of genes encoding for oxidative phosphorylation has been proposed to explain the alterations in mitochondrial metabolism in type 2 diabetes. In addition, the expression of the nuclear co-activators PGC-1α and PGC-1β is reduced in muscle of type 2 diabetics and in offspring of insulin-resistant subjects with this disease. In this regard, hypermethylation of PGC-1α within non-CpG nucleotides has been detected in skeletal muscle of type 2 diabetic patients. The reduced activity of PGC-1α and PGC-1β may explain, at least in part, the defective expression of genes encoding respiratory chain subunits and the lower mitochondrial biogenesis that occurs in muscle in type 2 diabetes. In contrast to these findings, no changes in PGC-1α or PGC-1β expression have been reported in diabetic Asian Indians.

Type 2 diabetes is also associated with reduced expression of genes involved in oxidative metabolism as well as with the repression of Mfn2. Mfn2 loss-of-function decreases glucose oxidation and mitochondrial membrane potential in muscle and non-muscle

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cells. This observation suggests that Mfn2 dysregulation plays a relevant role in the pathophysiology of type 2 diabetes. It is unlikely that the dysregulation of Mfn2 expression is a consequence of reduced insulin action. Thus, Mfn2 expression in healthy, obese or type 2 diabetic subjects is not altered in response to 3 hours of hyperinsulinemia during euglycemic-hyperinsulinemic clamps nor is the expression of this protein affected when cultured muscle cells are incubated for up to 48 hours with supramaximal insulin concentrations.

Furthermore, it has been shown that Mfn2 is induced by PGC-1α or by PGC-1β through interaction with the transcription factor ERRα. This may be particularly relevant since it has been reported that the nuclear co-regulators PGC-1α and PGC-1β are repressed in type 2 diabetes.

Mitochondrial dysfunction and insulin resistance

The association found between insulin-resistant states and mitochondrial dysfunction has led to the proposal that a reduced mitochondrial metabolism causes insulin resistance. Several intervention studies have tested this hypothesis and have generated discordant findings. Obese/overweight subjects underwent a 4-month intervention in which they performed physical exercise and had further weight loss induced by dietary restriction. This intervention led to increased mitochondrial size and stimulated ETC, citrate synthase and succinate dehydrogenase in muscle, in parallel to ameliorated insulin sensitivity. Similar findings were reported in type 2 diabetic subjects in response to weight loss/physical activity intervention. These studies illustrate that the amelioration of mitochondrial metabolism and improved insulin sensitivity run in parallel (Zorzano *et al*, 2009a).

In contrast, several other studies indicate that the amelioration of insulin resistance can occur in the absence of changes in mitochondrial metabolism. Thus, dietary restriction for 16 weeks caused improved insulin sensitivity in obese/overweight subjects in the absence of changes in mitochondrial metabolism or cardiolipin content. In addition, treatment for 8 weeks with rosiglitazone decreased insulin resistance in type 2 diabetic patients, without any improvement in mitochondrial function. Comparison of mitochondrial content and insulin sensitivity in a range of ethnic groups also casts doubts on a strict relationship between mitochondrial dysfunction and insulin resistance. Thus, Asian Indians displaying higher mtDNA content and increased oxidative enzyme activity are more insulin-resistant than age-, sex- and BMI-matched North American counterparts.

In all, the data available on humans indicate that not all interventions that improve insulin sensitivity are a consequence of parallel changes in muscle mitochondrial activity.

Specific alterations in morbidly obese type 2 diabetic subjects

Weight reduction and physical exercise are the best approaches to ameliorate insulin sensitivity; however, compliance with lifestyle changes has proven to have little beneficial effect. In recent decades, bariatric surgery has emerged as a potential

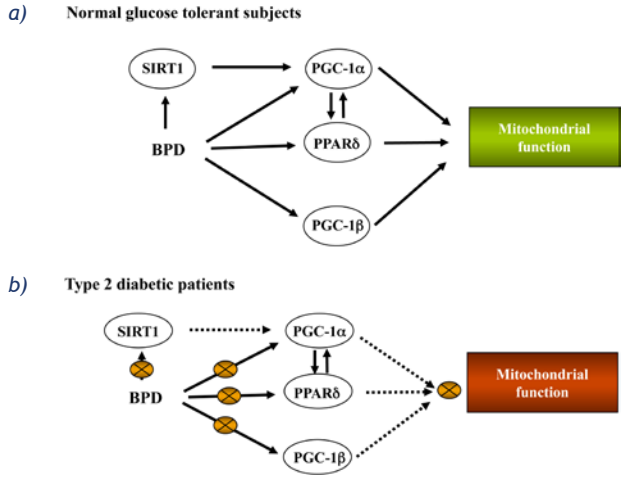

Figure 1. Scheme of the changes in gene expression that occur in non-diabetic (panel a) and type 2 diabetic subjects (panel b) in response to bilio-pancreatic diversion (BPD).

therapy for diabetes. Bilio-pancreatic diversion (BPD) is a bariatric surgical technique characterised by a massive weight loss mainly as a result of lipid malabsorption. BPD causes a net improvement in insulin sensitivity, long before the normalisation of body weight. In addition, BPD regulates substrate oxidation and modulates the expression of genes involved in lipid synthesis and oxidation in both muscle and adipose tissue.

BPD surgery improves insulin sensitivity both in type 2 diabetic and in nondiabetic subjects. We had previously described increased Mfn2 mRNA expression in skeletal muscle of morbidly obese subjects with normal glucose tolerance (NGT) after BPD. In the studies reviewed here, we aimed to determine whether the expression of genes involved in mitochondrial biogenesis/function was induced in response to BPD. To this end, we selected nuclear genes that regulate mitochondrial biogenesis such as PGC-1α, PGC-1β, and PPARδ; genes that regulate mitochondrial metabolism and fusion such as Mfn2; genes that regulate PGC-1α such as SIRT1; and genes that encode for constitutive proteins such as Porin or Citrate synthase. In addition, to focus on the mechanisms leading to reversal of diabetes after BPD, we analysed the effect of comparable weight loss caused by BPD on the expression of the aforementioned genes and whether there were differences in the response between morbidly obese NGT subjects and morbidly obese type 2 diabetic subjects. All patients were characterised before and after weight loss with respect to insulin sensitivity by the euglycemic-hyperinsulinemic clamp, and glucose and lipid oxidation, as assessed by the respiratory chamber.

NGT and type 2 diabetic morbidly obese subjects responded to BPD by losing weight to a similar extent, improving insulin sensitivity, and lowering glycemia, insulinemia, and the plasma concentration of cholesterol and triglycerides. Under these conditions, in which both groups showed a similar clinical biochemistry, NGT subjects showed a higher rate of glucose oxidation

and lower lipid oxidation than diabetic patients. Furthermore, in response to BPD, non-diabetic subjects showed an induced expression of genes encoding for mitochondrial proteins (Mfn2, citrate synthase or porin), and regulatory proteins (PGC-1α, PGC-1β, PPARδ, or SIRT1). However, under similar conditions, diabetic patients did not show any increase in the expression of these genes (Figure 1).

We propose that the pattern of changes in gene expression detected in skeletal muscle of non-diabetic subjects in response to BPD serves to trigger mitochondrial biogenesis in skeletal muscle, thereby favouring increased glucose oxidation under conditions of reduced lipid availability. This notion requires confirmation by direct analysis in muscle but is consistent with observations that whole body glucose oxidation is increased in subjects after BPD. Subjects undergoing BPD show reduced lipid availability provided that BBD causes lipid malabsorption. This observation may account for the detection of lower whole-body lipid oxidation after BPD in non-diabetic subjects.

In addition, our findings suggest that weight loss induced by BPD exerts a beneficial effect on insulin sensitivity via mechanisms that are independent of the skeletal muscle expression of genes involved in mitochondrial biogenesis/function. Furthermore, the observation that gene expression is not altered with weight loss in type 2 diabetic patients while it is induced in subjects with NGT points to the contribution of a heritable component.

Scientific output

Publications

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Research networks and grants

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

Ajuts a grups de recerca reconeguts
Agency for Administration of University and Research Grants (AGAUR), 2009-SGR215 (2009-2013)
Principal investigator: Antonio Zorzano

CIBERDEM (Diabetes y Enfermedades Metabólicas Asociadas)
Carlos III Health Institute (2007-2011)
Principal investigator: Antonio Zorzano

Determinantes genéticos de las alteraciones metabólicas de la obesidad y diabetes de tipo 2
Spanish Ministry of Science and Innovation, SAF2008-03803 (2008-2013)
Principal investigator: Antonio Zorzano

Integration of the system models of mitochondrial function and insulin signalling and its application in the study of complex diseases (MITIN)
European Commission, HEALTH-F4-2008-223450 (2008-2011)
Principal investigator and coordinator: Antonio Zorzano

Transnational cooperation for technological innovation in the development of molecules for the treatment of diabetes and obesity
Interreg—IVB, DIOMED, SOE1/P1/E178 (2009-2011)
Principal investigator and coordinator: Antonio Zorzano

Collaborations

Aquaporins in adipose tissue
Graça Soveral, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (Lisbon, Portugal)

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

Effect of salicylate conjugate compounds in obese and diabetic mice
Alec Mian and Luc Martí, Genmedica Therapeutics (Barcelona, Spain)

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

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

Functional role of DOR homologue genes
Aurelio Teleman, German Cancer Research Center—DFKZ (Heidelberg, Germany)

Generation of a screening platform
Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Generation of a screening platform
Mabel Loza, Universidad de Santiago de Compostela (Santiago de Compostela, Spain)

In vivo *role of neuregulins*
Anna Gumà, University of Barcelona (Barcelona, Spain)

Mitochondrial dynamics in cardiac cells
Sergio Lavandero, Universidad de Chile (Santiago, Chile)

Regulation of gene transcription in skeletal muscle
Ubiratan F Machado, Institute of Biomedical Sciences, University of São Paulo (São Paulo, Brazil)

Role of mitofusin 2 in endoplasmic reticulum
Luca Scorrano, Venetian Institute of Molecular Medicine (Padova, Italy)

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


Chemistry and Molecular
Pharmacology Programme

Fernando Albericio

Medicinal chemistry

Our project is organised around the following three cornerstones: (i) the training of researchers; (ii) the creation of knowledge; and (iii) the transfer of knowledge to society. In this regard, this is an integrated project, because although building on a robust chemical platform, the main goal is to identify compounds, mainly natural products or analogues, with biological/therapeutic activity (drug discovery), and to facilitate their reaching the target (drug delivery systems). Very often containing complex structural features that cover a more diverse chemical space than combinatorial chemistry libraries, natural compounds offer great opportunity to discover novel biological activities. Furthermore, a combination of natural products and combinatorial chemistry provides a potent approach for the discovery of new and safer therapeutic compounds.

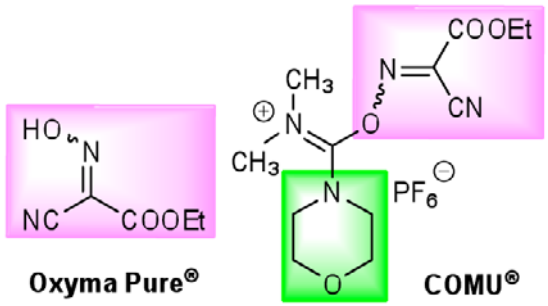


Figure 1. Structures of Oxyma Pure® and COMU®, a new family of uronium-type coupling reagents.

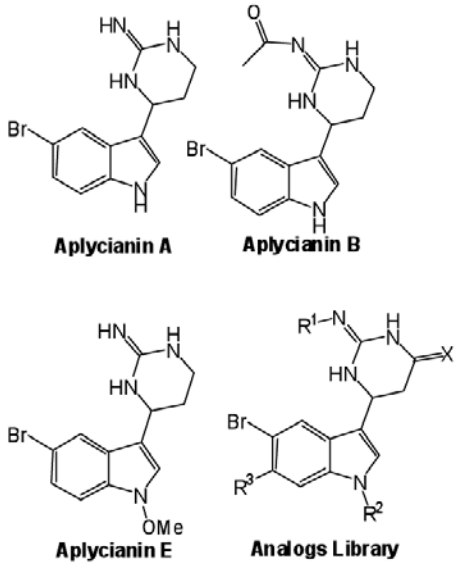


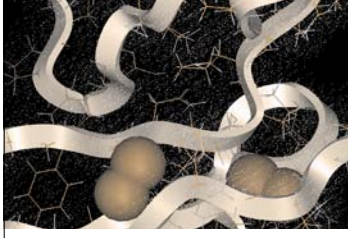
Figure 2. Aplycianin structures.

Peptide synthesis is based on an appropriate combination of protecting groups and a suitable choice of coupling method. In this regard, a new protecting group for Arginine (Arg) and a novel family of coupling reagents have been developed.

We have developed 1,2-Dimethylindole-3-sulfonyl (MIS) for the side-chain of Arg. The protection of Arg side chains is a crucial issue in peptide chemistry because of the propensity of the basic guanidinium group to produce side reactions. Currently, sulfonyl-type protecting groups, such as 2,2,5,7,8-pentamethylchroman (Pmc) and 2,2,4,6,7-penta methyldihydrobenzofurane(Pbf), are the most widely used for this purpose. Nevertheless, Arg side chain protection remains problematic as a result of the acid stability of these two compounds. This issue is even more relevant in Arg-rich sequences, acid-sensitive peptides and large-scale syntheses. The MIS group is more acid-labile than Pmc and Pbf and is therefore a better option for Arg side chain protection. In addition, MIS is compatible with tryptophan-containing peptides (Isidro-Llobet *et al*, 2009).

At present, almost all peptide bonds are formed in the presence of 1-hydroxybenzotriazole (HOBt) or its derivatives. After September 11, HOBt has been declared to have a high risk of explosion. Our group has proposed Oxyma Pure® as a substitute for HOBt. Oxyma Pure® displays a remarkable capacity to inhibit racemisation, together with impressive coupling efficiency in both automated and manual synthesis, superior to that shown by HOBt. Calorimetry assays showed decomposition profiles for HOBt-based additives that were consistent with their reported explosiveness and suggested a lower risk of explosion in the case of Oxyma Pure® (Subirós-Funosa *et al*, 2009).

Furthermore, we have introduced (El-Faham *et al*, 2009) a new family of uronium-type coupling reagents based on the presence of Oxyma Pure®. COMU® contains a morpholino group in conjunction with Oxyma Pure®. COMU® shows less tendency to racemise and higher coupling efficiency than HOBt derivatives.



Both Oxyma Pure® and COMU® are currently on the worldwide market (El-Faham *et al*, 2009; Subirós-Funosa *et al*, 2009).

Synthesis of natural products. Medicinal chemistry programmes

Aplicyanins are a new family of indole alkaloids recently isolated from the ascidian *Aplidium cyaneum*. These compounds are cytotoxic to the human tumour cell lines MDA-MB-231 (breast adenocarcinoma), A549 (lung carcinoma), and HT-29 (colorectal carcinoma) and also exhibit antimitotic activity. We have reported the first total synthesis of the indole alkaloids (±)-aplicyanins A, B, and E, plus 17 analogues, all in racemic form. Modifications to the parent compound included changing the number of bromine substituents on the indole, the nature of the substituents on the indole nitrogen (H, Me, or OMe), and/or the oxidation level of the heterocyclic core tetrahydropyrimidine. Each compound was screened against three human tumour cell lines, and 14 of the newly synthesised compounds showed considerable cytotoxicity. The assay results were used to establish structure-activity relationships (SARs). These results suggest that the presence of the bromine at position 5 of the indole is critical for activity, in the same way as the acetyl group on the imine nitrogen is in some compounds (Sisa *et al*, 2009).

Lamellarins form a family of more than forty members of marine alkaloids that show relevant bioactivity. Lamellarin D (Lam-D) is a cytotoxic agent against various tumour cells, an inhibitor of topoisomerase I, and a potent pro-apoptotic agent. We recently described the total synthesis of Lam-D, the preparation of a library of more than forty analogues, and SAR studies. We have reported the preparation of PEG-conjugates to improve the solubility of this compound, studies of cell penetration, and the apoptotic mechanism of cell death. A second generation of bio-conjugates with a nuclear localisation signal peptide and a poly(ethylene glycol)-based dendrimer has also been studied (Pla *et al*, 2009).

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

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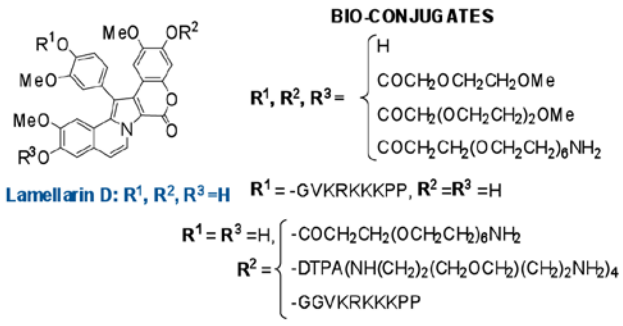

Figure 3. Structures of Lamellarin D bioconjugates.

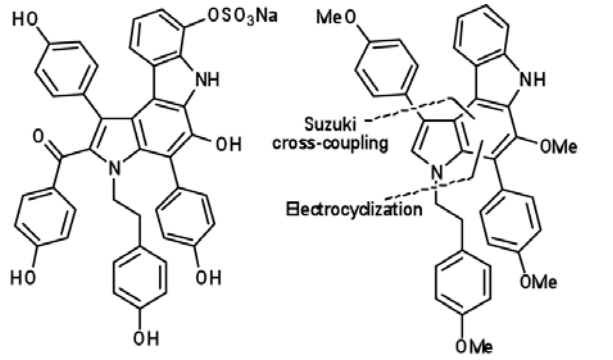

Figure 4. Studies toward the synthesis of Dictyodendrin B.

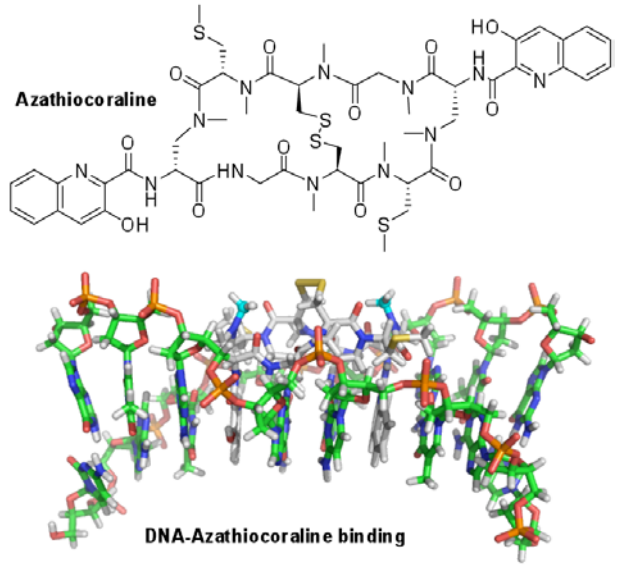

Figure 5. Structure of Thiocoraline and analogues, and binding of Azathiocoraline to DNA.

between a pyrrole fragment and an indole fragment, followed by tandem photochemical 6π-electro-cyclisation/aromatisation (Ayats *et al*, 2009).

Taking Thiocoraline, a potent marine anti-tumoral cyclic thiodepsipeptide, as a model, we have demonstrated that bridged *N*-methyl amides are isosteres for thiodepsi bonds. The introduction of *N*Me-amides in bridges mimicked the thioester bonds without imposing steric hindrance and allowed conservation of the hydrogen bonding map of the natural product. *N*Me-azathiocoraline displayed nanomolar activity in the same order as the natural product, and the same mode of action. In fact, modelling of *N*Me-azathiocoraline bonded to a TCGA sequence showed how the methyl groups remained further away from the DNA strand, without changing the recognition pattern of thiocoraline. This synthetic approach could be used in other depsipeptides and side-chain to side-chain cyclic peptides. Taking advantage of the molecule symmetry, we have synthesised NMe-azathiocoraline by a total solid-phase convergent approach, using a complete arsenal of coupling methods and protecting groups (Tulla-Puche *et al*, 2009).

Siamese depsipeptides as new constrained bicyclic architectures

Taking as a model sansalvamide A (SA), which is produced by a marine fungus and shows cancer cell cytotoxicity, we constructed cyclic depsipeptide dimers connected by a *CC* single bond. Remarkably, these analogues showed greater activity than the natural product, thereby providing additional information on the SAR (Ruiz-Rodriguez *et al*, 2009).

We have reported the leishmanicidal activity of Kahalalide F (KF), which is a tumoricidal cyclic depsipeptide currently under phase II clinical trials for several types of cancer and psoriasis, and its synthetic analogues at a micromolar range of concentrations (Cruz *et al*, 2009). The lethality of this compound is strongly linked to the alteration of the plasma membrane (PM) of the parasite and is based on: (i) rapid depolarisation of the PM and uptake of the vital dye SYTOX Green upon addition of the dye; (ii) evidence of severe morphological damage to the membrane of the parasite, as shown by transmission electron microscopy; and (iii) a rapid drop in the intracellular ATP levels, which correlates significantly with the leishmanicidal activity for active analogues. In addition to the basic knowledge obtained, this class of lethal mechanism is considerably less prone to the induction of resistance than classical drugs. All together, these observations foster further studies for the optimisation of KF and its analogues as novel anti-Leishmania leads with a new mode of action (Cruz *et al*, 2009).

In addition, two other small molecule-type compounds designed and synthesised in our group are entering the pre-clinical phase at the Bellvitge and Vall d'Hebron hospitals, respectively.

Nanotechnologies. Drug delivery systems

Kahalalide F (KF) conjugated to gold nanoparticles (GNPs). Two Cys-containing analogues of the anticancer drug KF were synthesised and conjugated to 20- and 40-nm GNPs. The self-

assembly capacity of a peptide dramatically influences the final ratio number of molecules per nanoparticle, saturating the nanoparticle surface and prompting multilayered capping on the surface. In such way, the nanoparticle could act as a concentrator for the delivery of drugs, thereby increasing bioactivity. GNP size and conjugation have an influence on biological activities. KF analogues conjugated with GNPs are located subcellularly at lysosome-like bodies. This distribution may be related to the action mechanism of KF. The results suggest that the selective delivery and activity of KF analogues are improved by conjugating the peptides to GNPs (Hosta *et al*, 2009).

Using optical tweezers (OT) and atomic force microscopy (AFM), we have studied the topoisomerase activity of Lam-D. OT exhibited a large increase in hysteresis of the force cycles as a result of the initial nicking activity of Topo I. The presence of Lam-D prevents the religation step and blocks enzyme turnover, as evidenced by the absence of a higher force plateau and by large, non-vanishing hysteresis between the stretching and relaxing paths of the force cycle. AFM showed that Lam-D blocks Topo I cleaving activity, as indicated by the prevalence of supercoiled topoisomers. Taken together, the data obtained indicate that, upon Lam-D inhibition, Topo I keeps a non-covalent interaction with the 50 end of the cleaved DNA strand. This interaction is strong enough to prevent supercoil relaxation in solution (Pla *et al*, 2009).

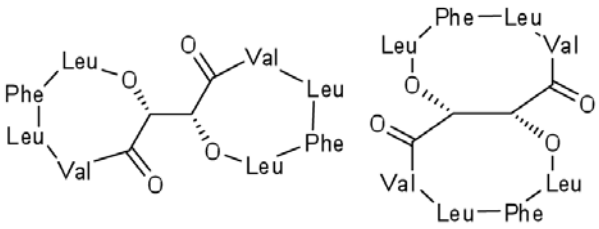

Figure 6. Structure of Siamese Depsipeptide analogues of SA.

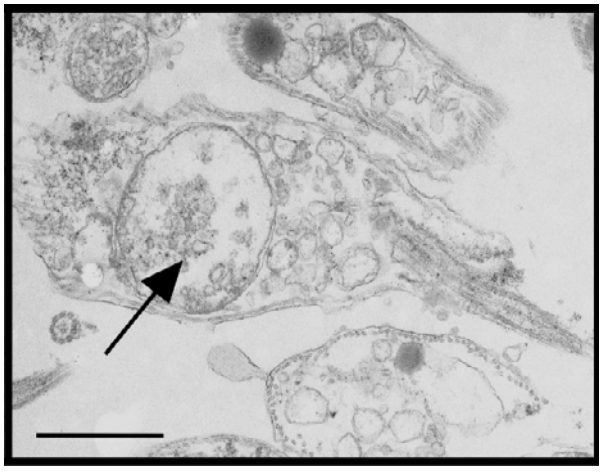

***Figure 7. Electron microscopy of L. donovani promastigotes treated with 5 µM of KF analogue [Nal 3]-KF.** Arrows show the formation of large vacuoles inside the parasites treated with this compound.*

Scientific output

Publications

Acosta GA, del Fresno M, Paradis-Bas M, Rigau-DeLlobet M, Côté S, Royo M and Albericio F. Solid-phase peptide synthesis using acetonitrile as a solvent in combination with PEG-based resins. *J Pept Sci*, **15**(10), 629-33 (2009)

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Research projects and networks

Advancing the field of drug delivery - Combined targeted treatments against human breast cancer
Spanish Ministry of Science and Innovation, EUI2008-00174 (2009-2011)
Principal investigator: Fernando Albericio

Cooperación transnacional para la innovación tecnológica en el desarrollo de moléculas para el tratamiento de la obesidad y de la diabetes
SUDOE – Interreg IV, DIOMED - SOE1/P1/E178 (2009-2012)
Principal investigator: Fernando Albericio

Creación de un laboratorio de nanobiotecnología para el desarrollo de nuevas herramientas para el diagnóstico y terapia de enfermedades de interés regional con la Universidad de Santiago de Chile
Spanish Agency for International Cooperation (AECI), D/021016/08 (2009)
Principal investigator: Fernando Albericio

Identificación de moléculas antimicrobianas miméticas obtenidas a partir de mapeo de péptidos activos con la Universidad Católica de Valparaíso
Spanish Agency for International Cooperation (AECI), A/016856/08 (2009)
Principal investigator: Fernando Albericio

Plataforma combiquímica basada en productos naturales: descubrimiento y administración de fármacos
Spanish Ministry of Science and Innovation, CTQ2006-03794 (2006-2009)
Principal investigator: Fernando Albericio

Química combinatòria per al desenvolupament de nous compostos
Generalitat de Catalunya, 2009SGR1024 (2009-2013)
Principal investigator: Fernando Albericio

Synthesis and evaluation of antimycobacterial peptides targeting MDR and XDR strains
Spanish Ministry of Science and Innovation, HS2008-0009 (2009-2010)
Principal investigator: Fernando Albericio

Collaborations

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

Anti-leishmania compounds
Luis Rivas, CSIC (Madrid, Spain)

Antimicrobial peptides
Sergio Marshall and Fanny Guzmán, Catholic University of Valparaiso (Valparaíso, Chile)

Antimycobacterial peptides targeting MDR and XDR strains
Thavi Govender, University of KwaZulu Natal (KwaZulu Natal, South Africa)

Antitumoral compounds
Rosa Aligué, Faculty of Medicine, University of Barcelona (Barcelona, Spain)

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

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

Delivery systems for SiRNA
Ramon Eritja, IRB Barcelona (Barcelona, Spain)

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

Development of nanoparticles as vehicles for the treatment of metastatic colorectal cancer
Ramon Mangues, Institut de Recerca Hospital de Sant Pau (Barcelona, Spain)

Multiple agonists for D1 and D2 dopamine receptors
Rafael Franco, University of Barcelona (Barcelona, Spain)

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

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

Synthesis and conformational analysis of cyclodepsipeptides from marine origin
Ernest Giralt, IRB Barcelona (Barcelona, Spain)

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

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

Therapeutic compounds
Antonio Zorzano, IRB Barcelona (Barcelona, Spain)

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

Other punctual collaborations: Norbert de Kimpe, University of Ghent (Ghent, Belgium); Javier de Mendoza, Institute of Chemical Research of Catalonia (Tarragona, Spain); José Antonio del Rio, Institute for Bioengineering of Catalonia (Barcelona, Spain); Ramon Estevez, University of Santiago de Compostela (Santiago de Compostela, Spain); Roser González, University of Barcelona (Barcelona, Spain); John Joule, University of Manchester (Manchester, UK); José Luís Mascareñas, University of Santiago de Compostela (Santiago de Compostela, Spain); Andrés Parra, University of Granada (Granada, Spain)

Ramon Eritja

Synthesis and properties of modified oligonucleotides

Synthetic oligonucleotides are convenient tools for a large number of studies. With the aim to obtain novel compounds with new and/or improved properties, we focus on the methodology used for the synthesis of DNA and RNA derivatives. The projects undertaken during 2009 have addressed: i) the conjugation of small molecules to DNA and RNA for potential use in DNA/RNA therapeutics; ii) the effect of modified bases in the structural and biological properties of oligonucleotides; and iii) the use of modified oligonucleotides in the assembly of nanomaterials and biosensors.

Chemical modification to control the inhibitory properties of nucleic acids over gene expression

The use of synthetic oligonucleotides to regulate gene expression has triggered the search for new oligonucleotide derivatives with improved therapeutic potential. In these cases, nucleic acids are used to inhibit a specific gene by blocking gene translation or gene transcription or by stimulating the degradation of a particular messenger RNA (mRNA). Several strategies are possible. In the antisense strategy, synthetic oligonucleotides complementary to the mRNA of a given gene are used to inhibit the translation of mRNA to protein. In the siRNA strategy, small RNA duplexes complementary to mRNA bind to a protein complex named RISC. siRNA duplexes contain two strands: the antisense or guide strand, which binds to RISC and the sense or passenger strand, which is released as a result of the interaction of the siRNA duplex with RISC. The complex formed by the antisense or guide RNA strand and the protein complex RISC catalyses the efficient degradation of a specific mRNA, thereby lowering the amount of target protein.

This year has witnessed the completion of a 3-year study on siRNA aimed to answer whether it is possible: i) to increase siRNA stability without affecting RISC recognition; ii) to increase cellular uptake/biodistribution without affecting RISC recognition; iii) to modulate RISC recognition by chemical modification of the guide strand; iv) to prevent off-target effects by chemical modification of siRNA duplexes; and v) to fabricate a simple pharmaceutical formulation based on siRNA to cure a disease. We used luciferase and TNF-α as target genes. The latter was selected because it is a major mediator of apoptosis as well as inflammation and immunity and it has been implicated in the pathogenesis of a wide spectrum of human diseases. Luciferase was selected because it can be measured by chemiluminescence. In the dual luciferase assay, cells were transfected with two plasmids, one with the firefly luciferase gene and the other carrying the Renilla luciferase gene. One of the genes was inhibited by specific siRNA duplexes while the other was used as a control. Using this assay, it is possible to measure the inhibition of gene expression of one luciferase gene by means of chemiluminescence.

i) The question of stability versus inhibitory properties has been addressed by modifying siRNA duplexes with bicyclohexane pseudo-sugars. The pucker of the furanose ring is a crucial structural parameter in DNA/RNA. In standard B-DNA, the pucker is 2'-endo or 'South' (S) whereas A-DNA and RNA are characterised by 3'-endo or 'North' (N) pucker of N-type conformation. Several authors have focused on the synthesis of novel nucleosides as potential therapeutic agents that are biased toward one specific ring puckering. We have studied the effect of the N-type derivatives in RNA interference experiments. A few pseudo-nucleoside modifications either at guide or passenger strands have a strong stabilising effect towards degradation without decreasing the inhibitory properties of siRNA duplexes. These compounds have been prepared by Víctor Márquez (National Institutes of Health, USA).

ii) To address cellular uptake, we designed several siRNA conjugates carrying peptides, lipids, steroids, intercalating agents, carbohydrates and so on. More than 30 new siRNA duplexes have been produced. Jose Carlos Perales' group (University of Barcelona) assist us in evaluating the inhibitory properties of the conjugates. The first manuscript derived from this work has been published. In that study, we describe the synthesis of RNA carrying nucleoplasmine and the efficient delivery of these siRNA duplexes to HeLa cells. These conjugates entered the RNAi pathway to silence gene expression as efficiently as unmodified and 3'-cholesterol modified siRNA duplexes (Aviñó *et al*, 2009).

iii) The modulation of the affinity of siRNA to RISC has been addressed by synthesising siRNA duplexes with guide strands carrying several groups designed to fit on a hydrophobic pocket of RISC. We have observed that the inhibitory properties of siRNA duplexes carrying modified guide strands are affected by the size of the group at the 3'-end while the same modification on the passenger strand does not yield any change in activity. This observation is relevant for the design of new siRNA derivatives with higher potency.

iv) We have tested the effect of small chemical modifications on the passenger strand on the innate inmunostimulation described

as a source of undesired off-target effect. Some modifications were shown to produce reduced inmunostimulation and a strong and prolonged specific inhibitory action.

v) We have started a preclinical study on a mouse model. Modified siRNA duplexes have been tested in a mouse model of inflammatory bowel disease. Biomedical markers of this disease were clearly improved with one of the modified siRNA duplexes. This research has been performed in collaboration with José Carlos Perales (University of Barcelona) and Esther Fernández (Autonomous University of Barcelona).

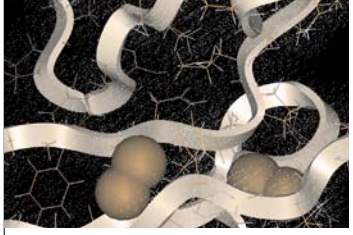
Synthesis of oligonucleotides carrying base analogues

Aberrant DNA methylation is common in cancer. Several drugs that inhibit this process 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 that lacks the 4-amino group of the other cytosine analogues, has been shown to inhibit DNA methylation and may have activity against cancer. To carry out a detailed comparison of the interaction between purified DNA methyltransferases (bacterial M.HhaI and mammalian Dnmt1) and oligonucleotides, we synthesised oligonucleotides containing either 5-azacytosine or 2-(1H)-pyrimidinone in place of the cytosine targeted for methylation. This study, performed by Judith Christman (University of Omaha, USA), supports the hypothesis that the efficacy of zebularine as an inhibitor of DNA methylation *in vivo* is dependent on its capacity to be incorporated into DNA (van Bemmel *et al*, 2009).

In addition, we have examined the base-pairing properties of 2-thio- and 4-thiothymidine derivatives. Previous results cited in the literature suggested that the replacement of carbonyl oxygen atoms by sulphur atoms leads to dramatic changes in the tautomeric properties of these pyrimidine derivatives. We have shown that the presence of thiothymines induces only mild changes in DNA structure, stability and fidelity. Thus thiothymines are excellent molecules to introduce thiolated nucleosides into DNA (Faustino *et al*, 2009).

Oligonucleotides and nanotechnology

A remarkable development in the field of DNA nanotechnology was the use of stable DNA Holliday junctions with addressable sticky ends to form two-dimensional DNA crystals.



Research Group Members

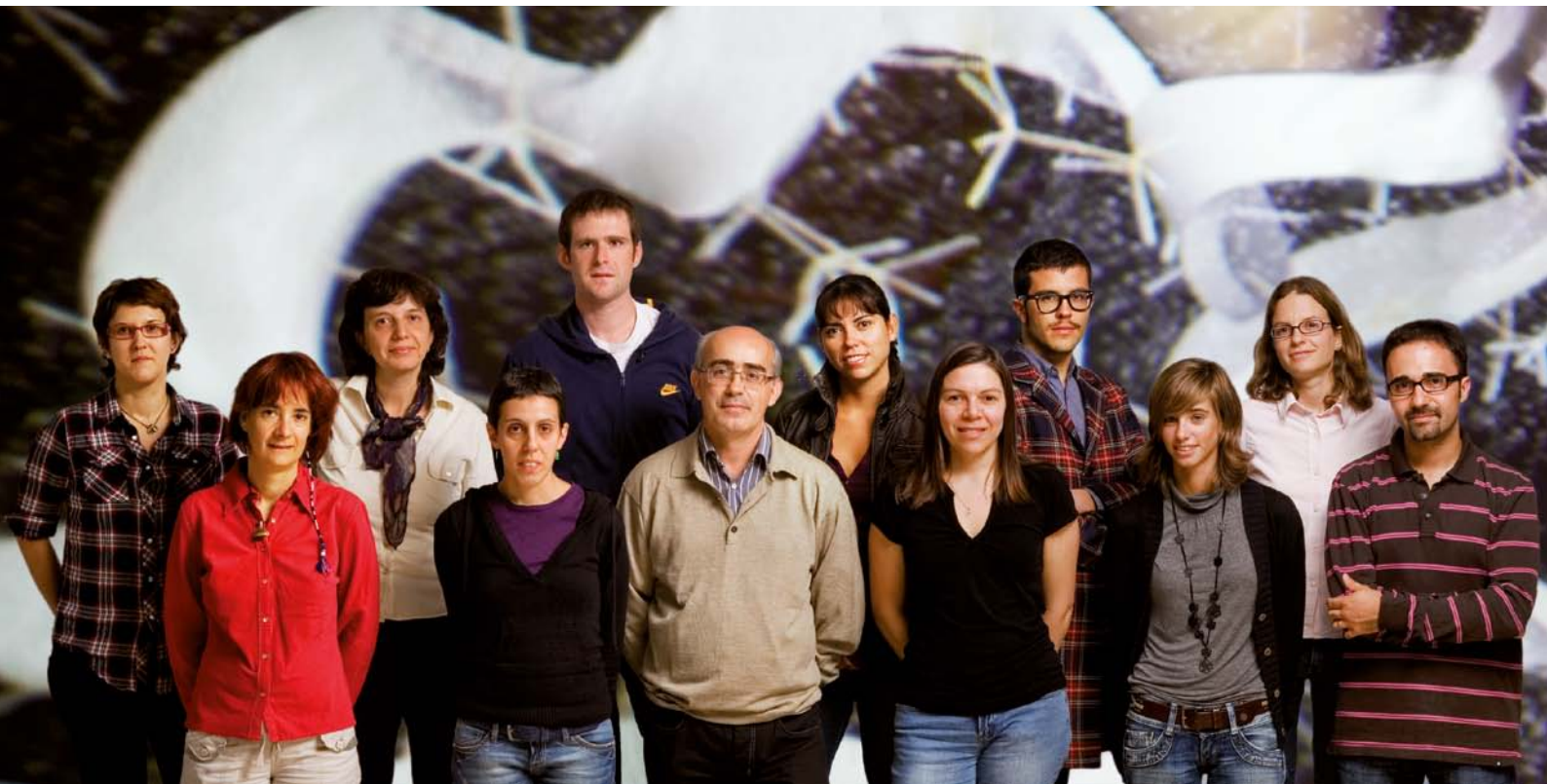
Group Leader:
Ramon Eritja

Research Associates:
Anna Aviñó, Carme Fàbrega

Postdoctoral Fellows:
Alejandra Garibotti, Santiago Grijalvo, Sonia Pérez, Montserrat Terrazas

PhD Students:
Margarita Alvira, Rubén Ferreira, Brendan Manning, Sandra Ocampo

Masters Student:
Maria Tintoré



We used the principles of construction described by Seeman and adapted then to generate systems with fine control of shape and function. For example, we transformed large DNA lattices into highly regular two-dimensional (2D) DNA networks on surfaces that provide templates for the deposition of gold nanoparticles. Our interest has focused on the preparation of thiolated 2D DNA arrays because the special reactivity of the thiol group will allow the functionalisation of 2D DNA arrays. Thiol groups have a strong affinity for gold surfaces and can also be used to introduce peptides and proteins as well as large number of molecules that have been functionalised with maleimido groups or bromo- and iodo-acetyl groups. We inserted reactive thiol groups at the nucleobase of specific sites of a well-characterised bidimensional DNA lattice to study the formation of the DNA lattices on gold, a surface that allows electrical contacts. We have demonstrated that DNA lattices carrying a single thiol derivative in each topological hairpin marker can be prepared and deposited on mica substrates. However, and most importantly, we have also shown that, in contrast to unmodified 2D DNA arrays, these thiolated 2D DNA arrays are readily deposited on gold surfaces (Garibotti *et al*, 2009; Figure 1).

In addition, we have developed a new photolithographic method that uses photolabile DNA hairpins to make patterns on silicon oxide wafers. The method described offers an attractive option for the fabrication of patterned surfaces of potential interest in the electronics and biosensor sectors (Ramos *et al*, 2009 and Manning *et al*, 2009).

In the framework of the strategic action on nanotechnology, we have provided modified oligonucleotides to Mª Teresa Martínez (CSIC, Zaragoza) to perform a study of DNA hybridisation on carbon nanotube field-effect-transistors (CNTFETs) at the Molecular Foundry of Lawrence Berkeley National Laboratory (Berkeley, USA). Using oligonucleotides and a special polymer developed by Iraida Loinaz at CIDETEC and the facilities at the Molecular Foundry for the fabrication of CNTFETs, Martínez has achieved high precision measurements of DNA hybridisation using electrical means (Martínez *et al*, 2009).

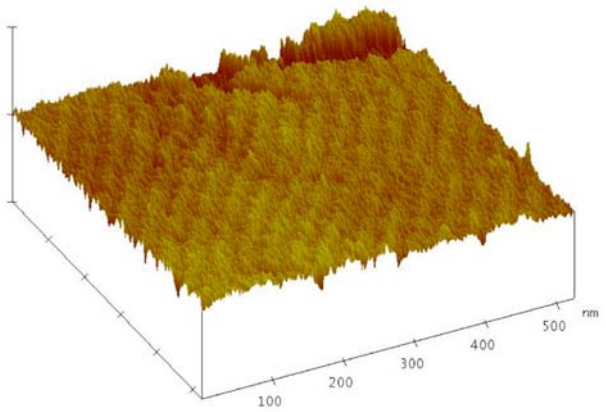


Figure 1. ***Topological AFM image of the thiolated A-B* DNA lattice assembled on mica****. This DNA array was described by Winfree* et al *(Winfree E, Liu F, Wenzel LA and Seeman NC (1998),* Nature, ***394****, 539-44). The lattice is formed by annealing of 10 oligonucleotides and subsequent deposition of DNA crystal on mica. Note the regular alignment of DNA hairpins that are used as topological markers. The functionalisation of these hairpins is the main goal pursued by our group.*

Furthermore, in this same strategic action and in collaboration with the groups headed by Pilar Marco (CSIC, Barcelona) and Josep Samitier (Institute for Bioengineering of Catalonia, Barcelona), we have prepared oligonucleotide conjugates carrying steroids. These conjugates are being used for the development of analytic devices for anti-doping and food control of illegal steroidal anabolic hormones (Tort *et al*, 2009).

Finally, using atomic force microscopy (AFM), we have characterised peptide nanotubes formed by cyclic peptides, which were prepared by Juan Granja's group (University of Santiago de Compostela) (Reiriz *et al*, 2009).

G-quadruplex

Aptamers are oligonucleotides that were originally derived from an *in vitro* evolution process known as SELEX (systematic evolution of ligands by exponential enrichment). This process selects aptamers on the basis of their specific and tight binding affinity to a ligand of choice from a library of sequences. Through this approach, aptamers with very high affinity have been developed for diagnostic, therapeutic and other technical applications. One of the most studied aptamers is the 15-base long thrombin binding aptamer (TBA). This oligonucleotide binds specifically to thrombin at nanomolar concentrations and thus shows anti-coagulant properties of interest. TBA is characterised by a chair-like, anti-parallel quadruplex structure consisting of two G-tetrads connected by two TT loops and a single TGT loop (Figure 2). We have studied the effect of 2'-deoxyguanine (dG) residues with locked North- or South-bicyclo[3.1.0] hexane pseudo-sugars when inserted in TBA. Individual 2'-deoxyguanosines at four positions of the aptamer were replaced by these analogues where the North/anti and South/syn conformational states were confined. We conclude that locked bicyclo[3.1.0]hexane nucleosides appear to be excellent tools in the study of the role of critical conformational parameters for the formation of a stable, antiparallel G-tetrad DNA structures (Saneyoshi *et al*, 2009). This work was performed in close collaboration with the groups of Modesto Orozco (IRB Barcelona), Víctor Márquez (National Institutes of Health, USA), Stefania Mazzini (University of Milan, Italy) and Carlos González (CSIC, Madrid).

Moreover, guanine-rich sequences capable of forming G-quadruplex structures have been found in telomeres and in transcriptional regulatory regions of critical oncogenes such as c-myc, and c-kit. Ligands that selectively bind and stabilise these structures have become anticancer drugs. We have initiated the study of the G-quadruplex structures present at the initiation sites of oncogenes as well as their interaction with small drugs and with the complementary C-rich strand, which may also form a quadruplex structure known as the i-motif. This research is done in collaboration with Raimundo Gargallo's group (University of Barcelona). A detailed analysis of the equilibrium

formed by the G-quadruplex of bcl-2 and c-kit oncogenes and the corresponding complementary C-rich sequences has been made in order to determine the relative amount of duplex or separate quadruplexes that forms at a range of pH (del Toro *et al*, 2009; Bucek *et al*, 2009).

Design of DNA repair inhibitors in cancer chemotherapy

Chemotherapy is the main pharmacological approach used against cancer. Anti-proliferative drugs are highly cytotoxic and aggressive agents. Under attack, the biochemical repair systems of the cancer cell machinery responds, attempting to mitigate the cellular damage induced by these agents. As a result, the clinical efficacy of these drugs is often limited. High doses are required and consequently serious secondary effects are commonplace. Recent advances in the molecular biology of cancer have identified key pathways involved in the DNA repair pathways induced by chemotherapeutic agents. Regarding methylating agents, two main mechanisms have been envisaged. One involves the O6-methylguanine-DNA-methyltransferase (hAGT), which removes the methyl/alkyl group from the O6 position of guanine. A second mechanism is the base excision repair (BER) pathway, which is involved in the repair of adducts resulting from methylation of the N7 position of guanine (N7-mG)s. This project seeks to develop potent inhibitors of hAGT and APE1, the latter a key endonuclease in the BER pathway. To this end, a combination of X-ray crystallography and *in silico* virtual screening of chemical libraries is being performed. This new research line is supervised by Carme Fàbrega.

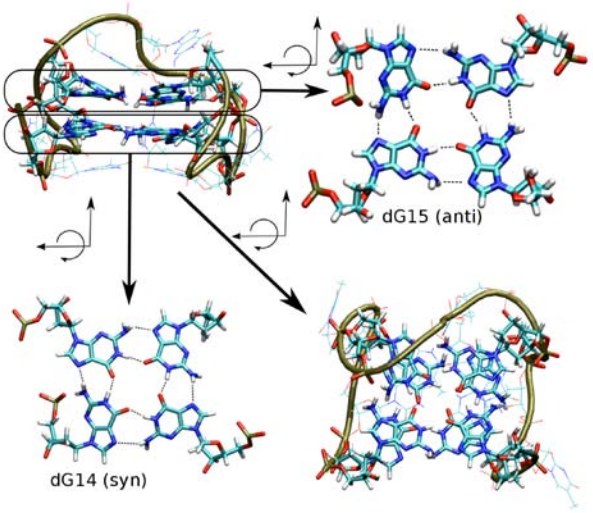


Figure 2. Structure of the thrombin binding aptamer showing the positions that were modified with locked North- or South-bicyclo[3.1.0] hexane pseudo-sugars (figure from Guillem Portella and Modesto Orozco).

Scientific output

Publications

Aviñó A, Ocampo SM, Caminal C, Perales JC and Eritja R. Stepwise synthesis of RNA conjugates carrying peptide sequences for RNA interference studies. *Mol Divers*, **13**(3), 287-93 (2009)

Aviñó A, Pérez-Rentero S, Garibotti AV, Siddiqui MA, Márquez VE and Eritja R. Synthesis and hybridization properties of modified oligodeoxynucleotides carrying non-natural bases. *Chem Biodivers*, **6**(2), 117-26 (2009)

Bucek P, Jaumot J, Aviñó A, Eritja R and Gargallo R. pH-Modulated Watson-Crick duplex-quadruplex equilibria of guanine-rich and cytosine-rich DNA sequences 140 base pairs upstream of the c-kit transcription initiation site. *Chemistry*, **15**(46), 12663-71 (2009)

Del Toro M, Bucek P, Aviñó A, Jaumot J, González C, Eritja R and Gargallo R. Targeting the G-quadruplex-forming region near the P1 promoter in the human BCL-2 gene with the cationic porphyrin TMPyP4 and with the complementary C-rich strand. *Biochimie*, **91**(7), 894-902 (2009)

Faustino I, Aviño A, Marchán I, Luque FJ, Eritja R and Orozco M. Unique tautomeric and recognition properties of thioketothymines? *J Am Chem Soc*, **131**(35), 12845-53 (2009)

Garibotti AV, Sisquella X, Martínez E and Eritja R. Assembly of two-dimensional DNA crystals carrying N4-[2-(tert-Butyldisulfanyl) ethyl]cytosine residues. *Helv Chim Acta*, **92**(8), 1466-72 (2009)

Jaumot J, Eritja R, Navea S and Gargallo R. Classification of nucleic acids structures by means of the chemometric analysis of circular dichroism spectra. *Anal Chim Acta*, **642**(1-2), 117-26 (2009)

Manning B, Pérez-Rentero S, Garibotti AV, Ramos R and Eritja R. Modified oligonucleotides for biosensing applications. *Sensor Lett*, **7**(5), 774-81 (2009)

Martínez MT, Tseng YC, Ormategui N, Loinaz I, Eritja R and Bokor J. Label-free DNA biosensors based on functionalized carbon nanotube field effect transistors. *Nano Lett*, **9**(2), 530-36 (2009)

Ramos R, Manning B, Aviñó A, Gargallo R and Eritja R. Photocleavage of peptides and oligodeoxynucleotides carrying 2-nitrobenzyl groups. *Helv Chim Acta*, **92**(4), 613-22 (2009)

Reiriz C, Brea RJ, Arranz R, Carrascosa JL, Garibotti A, Manning B, Valpuesta JM, Eritja R, Castedo L and Granja JR. Alpha,gamma-peptide nanotube templating of one-dimensional parallel fullerene arrangements. *J Am Chem Soc*, **131**(32), 11335-37 (2009)

Saneyoshi H, Mazzini S, Aviñó A, Portella G, González C, Orozco M, Márquez VE and Eritja R. Conformationally rigid nucleoside probes help understand the role of sugar pucker and nucleobase orientation in the thrombin-binding aptamer. *Nucleic Acids Res*, **37**(17), 5589-601 (2009)

Tort N, Salvador JP, Eritja R, Poch M, Martínez E, Samitier J and Marco MP. Fluorescence site-encoded DNA addressable hapten-microarray for anabolic androgenic steroids. *Trac Trends Anal Chem*, **28**(6), 718-28 (2009)

Van Bemmel DM, Brank AS, Eritja R, Marquez VE and Christman JK. DNA (Cytosine-C5) methyltransferase inhibition by oligodeoxyribonucleotides containing 2-(1H)-pyrimidinone (zebularine aglycon) at the enzymatic target site. *Biochem Pharmacol*, **78**(6), 633-41 (2009)

Research networks and grants

Ajuts a grups de recerca reconeguts
Agency for Administration of University and Research Grants (AGAUR), 2009SGR-208 (2009-2012)
Principal investigator: Ramon Eritja

CIBER Bioingeniería, Biomateriales y Nanomedicina
Carlos III Health Institute, CIBERBBN (2006-2013)
Principal investigator: Ramon Eritja

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

Diseño combinado de inhibidores de los mecanismos de reparación del ADN hAGT y BER como terapia contra el cáncer
Carlos III Health Institute, PI061250 (2006-2010)
Principal investigator: Carme Fàbrega

Multi-scale formation of functional nanocrystal-molecule assemblies and architectures (FUNMOL)
European Commission, STREP-NMP-2007-213382 (2008-2011)
Principal investigator: Ramon Eritja

Self-assembly guanosine structures for molecular electronic devices
European Commission, COST action MP0802 (2008-2012)
Principal investigator: Ramon Eritja

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

Synthesis of RNA interference linked to lipids
Research contract with Sylentis SAU
Principal investigator: Ramon Eritja

Collaborations

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

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

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

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

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

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

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

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

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

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

Awards and honours

25 years at CSIC
Consejo Superior de Investigaciones Científicas (2009)
Awardee: Ramon Eritja

Ernest Giralt

Design, synthesis and structure of peptides and proteins

Proteins constitute the working machinery and structural support of all organisms. Understanding the molecular basis of a disease, namely where and how the protein network fails, is crucial for developing a therapeutic strategy. The breakthrough concept that proteins function as a contact network rather than as independent agents is not only one of the most important advances in our comprehension of living systems, but it also translates into a new era in drug discovery. The few reported examples of diseases caused by 'impolite' social behaviour of proteins are merely the tip of the iceberg. Therapeutic intervention through molecules designed to selectively modulate the strength and specificity of protein-protein interactions is becoming a reality. Mass spectrometry (MS), nuclear magnetic resonance (NMR) and atomic force microscopy (AFM) are emerging as privileged tools to study the structure of proteins and the complex molecular recognition events that take place in protein-protein interactions, and the interaction of designed ligands - potential new drugs - with protein surfaces. However, the efficient use of these spectroscopic tools is still hampered by numerous difficulties. Regarding MS, the high dynamic range of protein concentrations in living organisms is one of the major challenges in contemporary proteomics. In structure-based drug design, a previous knowledge of the 3D structure of the therapeutic target is mandatory. When this target is a high molecular weight protein, the use of NMR to determine the structure is not possible due to the following two problems associated with this high molecular weight: i) crowding of spectral signals; and ii) fast magnetic relaxation with the subsequent loss of sensitivity. Finally, in amyloid diseases, the study of molecular interactions by AFM or other techniques is strongly hampered by the strong tendency of amyloid proteins to self-assemble.

Using peptidyl aldehydes in activity-based proteomics

Activity-based proteomics (ABP) is a chemical strategy that uses probes that covalently bind the active site of an enzyme. This approach is applied to address protein activity profiling and to discover new therapeutic targets and enzyme inhibitors. Natural substrates and covalent inhibitors of diverse enzymatic classes (*eg*, proteases, glycosidases, and phosphatases) have been used to design several activity-based probes. In most cases, the reactive moiety of these probes consists of electrophilic functional groups such as fluorophosphonates, epoxides, and acyloxymethylketones. Aldehydes are electrophilic functional groups that show inhibitory properties towards several kinds of proteolytic enzymes. Many compounds with an aldehyde moiety have recently been described as covalent reversible inhibitors of serine and cysteine proteases, such as trypsin, thrombin, and cathepsins, among others.

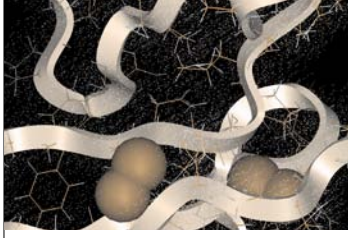
The broad inhibitory spectrum of aldehydes and the possibility that amino acid residues modulate their specificity point to the potential of using peptidyl aldehydes as activity-based probes. We have explored for the first time the potential of peptidyl aldehydes in ABP. For this purpose, we have synthesised various probes and, as a proof of principle, we have used them to specifically label a well-known serine protease in an activity-dependent manner.

Prolyl oligopeptidase (POP; EC 3.4.21.26) is a post-proline serine protease that hydrolyses small proline-containing peptides. POP is involved in the regulation of many bioactive peptides *in vivo*, such as substance P and thyrotropin-releasing hormone, among others, and has been associated with several neuropsychiatric disorders, including schizophrenia and bipolar affective disorder. Although the mechanism of action of this protease remains unknown, several studies have proposed that POP pro-

duces its effect through the metabolism of inositol-1,4,5-triphosphate, a key molecule in the transduction cascade of neuropeptide signalling. In our laboratory, POP was recently cloned from human brain RNA, expressed in *Escherichia coli*, and a homologous model based on the X-ray structure of porcine POP was obtained. To evaluate the use of peptidyl aldehydes in ABP, we synthesised and used three peptidyl aldehyde activity-based probes, Aha-Bpa-Pro-Pro-H (1), Aha-Bpa-Ahx-Pro-Pro-H (2), and Aha-Bpa-Peg-Pro-Pro-H (3) (Aha: hexynoicacid; Ahx: e-aminohexanoic acid; Bpa: benzoylphenylalanine; Peg: 15-amino-4,7,10,13-tetraoxapentadecanoic acid) to label active POP (Figure 1). It is worth mentioning that unlike the phosphonates, sulfonates and other reactive groups traditionally used in activity-based probes, the use of aldehydes facilitates probe synthesis through straightforward and rapid solid-phase peptide synthesis (SPPS) strategies.

Having synthesised the peptidyl aldehyde probes and confirmed their inhibitory properties, we focused on verifying whether these compounds were proper activity-based probes, that is, whether they label POP in an activity-dependent manner and distinguish its active form from heat-denatured samples. To confirm this point, active and heat-denatured POP were incubated with probes 1–3 at 1 and 25 μM for 15 min at rt and crosslinked by UV light irradiation for an additional 60 min at 4 °C. Afterwards, samples were labelled using a trifunctional tag that incorporates both a biotin and a rhodamine moiety49 (TriN3), and were then analysed by SDS-PAGE. These assays showed that peptidyl aldehydes are satisfactory activity-based probes when used at low concentrations. These results were further confirmed with pull-down experiments where the crosslinked probe–protein complex was labelled with the TriN3 tag, extracted from the sample using avidin beads and detected by Western Blot using either an in-house a-POP antibody or streptavidin. This assay was performed with probe 3 (1 μM), and in both cases only the active form of POP was taken from the sample.

Probe 3 was also used for direct mass spectrometry protein identification using an nLC–MS/MS approach. In this case, probe–protein complexes were also labelled with the TriN3 tag, extracted from the sample with avidin, but analysed by reverse-phase chromatography followed by MS/MS identification. MS/MS data were analysed with the SEQUEST software using the IPI database. The maximum false positive rate was set at 1% with PeptideProphet and ProteinProphet. This data analysis led to the identification of POP in active samples but not in heat-denatured controls. These results show that peptidyl aldehydes are adequate activity-based probes not only for specific in-gel



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activity profiling of proteases but also for other ABP applications like protease identification using MS. Unlike other probes that target only one specific type of enzyme at a time (*eg*, serine proteases), peptidyl aldehydes can simultaneously target a variety of proteolytic enzymes.

Therefore, peptidyl aldehydes could provide a valuable tool for identifying unknown proteases by substrate recognition (*eg*, those involved in the activation of neuropeptide precursors), which could eventually lead to the establishment of new therapeutic targets. Moreover, the specificity resulting from the peptide sequence is a crucial feature of peptidyl aldehyde probes and could also be used to successfully monitor the activity of a particular subset of proteins.

A cost-effective labelling strategy for the NMR study of large proteins

NMR spectroscopy is a useful tool for the study of protein structure, protein dynamics and molecular recognition processes, including both protein-protein and protein-ligand interactions. However, the application of NMR experiments to large proteins remains a challenge. Transverse relaxation processes are accelerated as the macromolecule grows and the perdeuteration of proteins with low tumbling rates may be required. Thus, cells must be grown in D_2O, which, in general, reduces protein expression levels and significantly raises the cost of the NMR sample. Moreover, the assignment of spectra is limited by signal overlap. Consequently, simplification of spectra by an appropriate selective labelling scheme is often required. Selective labelling of specific amino acids can be achieved by using auxotrophic cell strains and adding the amino acid with a suitable isotope label to the medium. However, given that the biosynthetic pathways of amino acids are complex, cell growth may be limited when one of these pathways is disrupted, and this leads to lower expression levels.

A less intrusive approach consists of exploiting the cell's metabolic machinery to produce selectively labelled proteins and the particular choice of precursor determines whether a subset or a specific amino acid ends up labelled. Various authors have described such labelling approaches. As an example, when ^{13}C-labelled α-ketobutyrate and α-ketoisovalerate are added to the growth medium, the cell incorporates the ^{13}C-label into valine, leucine and isoleucine side chains. Similarly, addition of [2-^{13}C]- or [4-^{13}C]-labelled indole to the medium allows the labelling of tryptophan residues. Tryptophan, tyrosine and arginine are the most common amino acids in the hot spots of a given protein and are therefore involved in most of the binding energies in protein-protein and protein-ligand interactions. An inexpensive and reliable labelling procedure for the above-mentioned residues that is applicable to large proteins would be extremely useful.

We have set up a cost-effective labelling strategy for the NMR study of large proteins. In this approach, the ^{15}N-label is selectively incorporated into the tryptophan side chains of the protein and the spectrum can be acquired without the need for deuteration. We applied this labelling strategy to POP, a serine protease of 80 kDa (Figure 2).

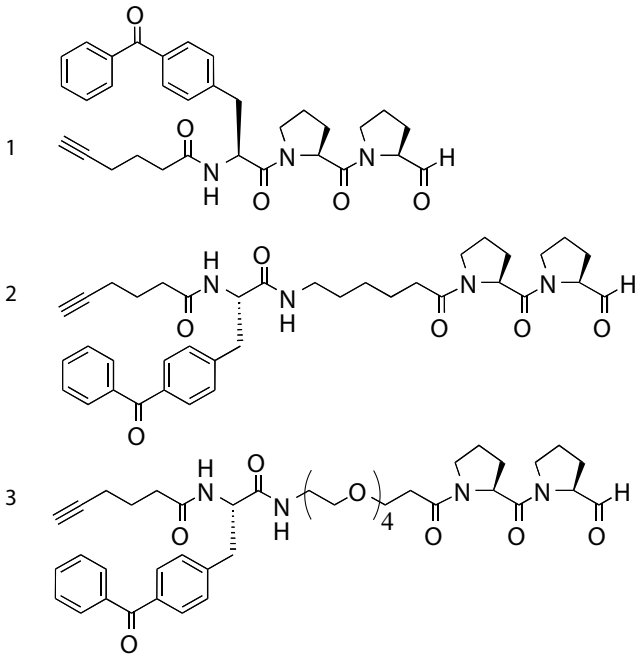

Figure 1. Structure of peptidyl aldehyde probes Aha-Bpa-Pro-Pro-H (1), Aha-Bpa-Ahx-Pro-Pro-H (2), and Aha-Bpa-Peg-Pro-Pro-H (3).

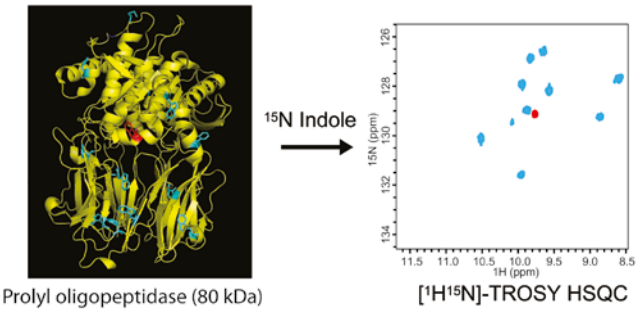

Figure 2. An ^{15}N-label is selectively incorporated into the tryptophan side chains of a large protein and a simplified [^{1}H, ^{15}N]-TROSY HSQC spectrum is obtained without the need for deuteration.

In recent years POP has gained relevance as a target for the treatment of cognitive disturbances. An array of strategies is currently being used to identify POP inhibitors, as these compounds show neuroprotective and cognition-enhancing effects in experimental animals. The X-ray structure of POP from porcine muscle revealed a distinctive two-domain structure: a catalytic domain with an α/β hydrolase fold and an unusual β-propeller domain. The co-structure of POP in the presence of Z-prolyl-prolinal (ZPP), a canonical POP covalent inhibitor, shows that the specificity of the binding between the enzyme and the proline-containing inhibitor is provided by the hydrophobic interaction between POP tryptophan 595 (Trp595) and the ZPP proline ring.

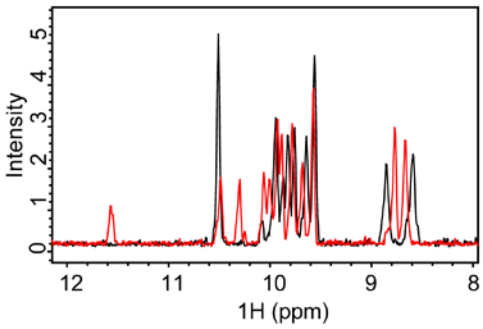

Figure 3. ***1H-projection of the [1H,15N]-TROSY HSQC spectra of a selectively Trp[15N-indole]-labelled POP sample.*** *Wild-type POP (black) and POP wild-type with ZPP (red).*

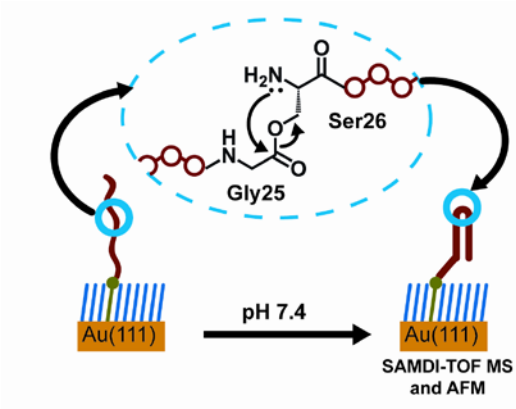

Figure 4. Schematic representation of the intramolecular O-N-acyl migration of Aβ40 on the functionalised gold surface.

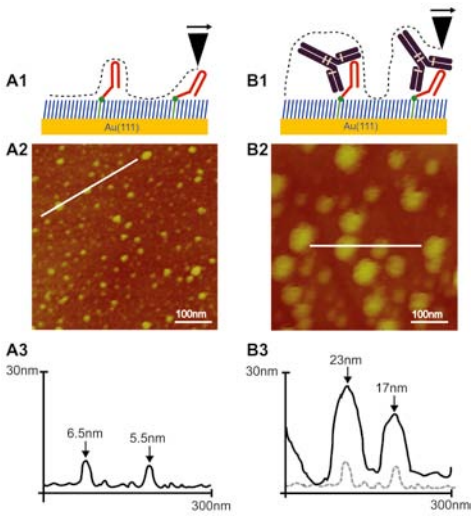

Figure 5. Schematic representation of AFM study of the Aβ40-functionalised gold surface before (A1) and after (B1) incubation; AFM images of the Aβ40-functionalised gold surface before (A2) and after (B2) incubation; surface morphology measurements (AFM) of the Aβ40-functionalised gold surface before (A3) and after (B3) incubation.

The acquisition of a spectrum of a perdeuterated and uniformly ^{15}N-labelled POP sample (U-[^{2}H,^{15}N]-POP) showed that even with perdeuteration and transverse relaxation-optimised spectroscopy (TROSY), signal overlap was still a considerable handicap when dealing with a large protein of 80 kDa. Therefore, a POP sample that was selectively labelled at tryptophan side chains (Trp[^{15}N-indole]-POP) was produced by supplementing the minimal growth medium with ^{15}N-Indole.

The incorporation of the label was checked by MS. The resulting [1H,^{15}N]-TROSY HSQC spectrum recorded with the Trp [^{15}N-Indole]-POP sample showed a satisfactory signal-to-noise ratio (S/N) and eleven of the twelve expected tryptophan signals were present and well-dispersed (Figure 3). It is noteworthy that, despite of the high molecular weight of POP, this result was obtained with a non-perdeuterated protein sample.

Toward the preparation of well-defined amyloid surfaces

Amyloids are a family of self-aggregating proteins related to various central nervous system disorders, including Alzheimer's disease (AD). The two most distinctive brain pathologies of AD are neuritic plaques, which contain mainly insoluble aggregates of the β-amyloid peptide (Aβ), and neurofibrillary tangles of abnormally phosphorylated Tau protein. Cleavage of the β-amyloid precursor protein (APP) by proteases such as β-secretase and γ-secretase produces a predominant product of 40 amino acid residues: β-amyloid peptide (Aβ40). Other Aβ species of various lengths are also produced, including a fibrillogenic peptide of 42 amino acids (Aβ42). The deposition of Aβ40 and Aβ42 in cerebral plaques begins with nucleation of soluble Aβ42 into fibrils, followed by accumulation of soluble Aβ40. In fact, soluble prefibrillar forms of amyloid peptides, including monomers, are suspected to be the main pathogenic factor in AD. Although the mechanism of Aβ toxicity remains unclear, we believe that the study of how Aβ peptides interact with proteins may lead to a better understanding of AD pathogenesis.

We have recently fabricated well-defined, functionalised, monomeric β-amyloid peptide surfaces for the study of protein-protein interactions. We first prepared a non-aggregating analogue of the β-amyloid peptide and then attached it to a gold surface covered with a self-assembled monolayer (SAM) of alkanethiols. After attachment, the native form of the β-amyloid peptide (Aβ40) was obtained by surface-level intramolecular O-N migration (Figure 4). The surface was characterised by atomic force microscopy (AFM) and self-assembled monolayer for matrix-assisted laser desorption/ionization time of-flight mass spectrometry (SAMDI-TOF MS). The interaction between the surface-bound Aβ40 and monoclonal anti-Aβ40 antibody was tracked by AFM and chemiluminescence, which revealed that Aβ40 was attached mainly in its monomeric form and that the protein-protein complex was assembled on the surface (Figure 5). Finally, we used a proteomics approach to demonstrate the specificity of the Aβ40-functionalised surface in surface-binding experiments using serum amyloid P (SAP) and bovine serum albumin (BSA).

Scientific output

Publications

Bastús NG, Sánchez-Tilló E, Pujals S, Farrera C, Kogan MJ, Giralt E, Celada A, Lloberas J and Puntes V. Peptides conjugated to gold nanoparticles induce macrophage activation. *Mol Immunol*, **46**(4), 743-48 (2009)

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Research networks and grants

Ajuts per potenciar i donar suport als grups de recerca
Generalitat de Catalunya, 2009SGR001005 (2009-2013)
Principal investigator: Ernest Giralt

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

Funcionalización de nanopartículas metálicas para favorecer su paso a través de la barrera hematoencefálica. Aplicaciones en la enfermedad de Alzheimer
Spanish Agency for International Cooperation (AECI), A/018203/08 (2009)
Principal investigator: Ernest Giralt

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

Reconocimiento molecular de superficies proteicas
Spanish Ministry of Science and Innovation, BIO2008-00799 (2009-2013)
Principal investigator: Ernest Giralt

Structural and dynamic characterization of ab aggregation. Examination of aβ aggregation peptide inhibitors
'La Marató TV3' Foundation, 092 (2006-2009)
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Studies of neurosecretion by remote control of exocytosis and endocytosis (OpticalBullet)
European Commission, FP7-ERC-StG08-210355 (2008-2013)
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Activity-based proteomics
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Amyloid recycling
Christopher M Dobson, Cambridge University (Cambridge, UK)

Anàlisi conformacional de tres mostres problema d'hormona de creixement (hGH) mitjançant la utilització de tècniques de DC, RMN i LS
Ipsen Pharma SA (Barcelona, Spain)

Applications of the Suzuki reaction to the synthesis of conformationally contrained peptides
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Cell-penetrating peptides
Shiroh Futaki, Kyoto University (Kyoto, Japan)

Design, synthesis and study of P53 ligands
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Ion-mobility mass spectrometry
Carol Robinson, Oxford University (Oxford, UK)

Nanoparticles for drug delivery
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Remote manipulation of protein aggregation
Marcelo Kogan, University of Chile (Santiago, Chile)

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

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

Antoni Riera

Syntheses of biologically active molecules and development of new synthetic methodologies

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 a 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 new compounds for biological screening.

Catalytic and asymmetric reactions. Basic synthetic methodologies

New ligands for metal-catalysed reactions

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 insertion of a carbon monoxide molecule to give a cyclopentenone. We use these cyclopentenones as starting materials for the synthesis of biologically active substances. To date, the best approach to attain an asymmetric version of the PKr is to use chiral ligands. Some years ago, 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 1). We successfully synthesised these chiral non-racemic *N*-diphenylphosphino *tert*-butylsulfinamides and found that they bound to dicobalthexacarbonyl complexes with high selectively. The resulting complexes gave excellent yields and high enantiomeric excesses (up to 99% ee) in the intermolecular PKr (Solà *et al*, 2007).

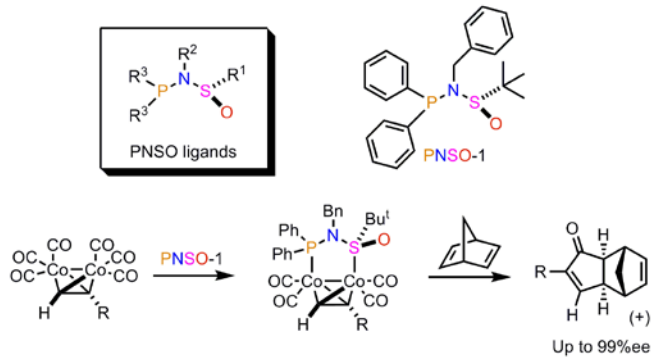


Figure 1. Asymmetric Pauson-Khand reaction using PNSO ligands.

In 2009 we have explored the scope of these highly modular P,S ligands. Firstly, we have tested the effect of the substituent at sulfur (R^1). A family of PNSO ligands with electron-deficient sulfinyl groups has been synthesised. Reaction with Co_2-alkyne complexes yields P,S-bridged complexes. These complexes have been used to study the metal-bonding of the distinct sulfinyl groups. Infrared spectroscopy (IR), X-ray and Pauson-Khand reactivity studies indicated that electron-deficient sulfinyl groups provide enhanced sulfur-metal bonding. Among the sulfinyl groups studied the trifluoromethyl PNSO ligand afforded the strongest S-Co bond. We have therefore established that the electron-deficient substituents linked to the sulphur atom of the ligand favour backbonding with the metal. Consequently, the resulting cobalt complexes are less hemilabile (Revés *et al*, 2009).

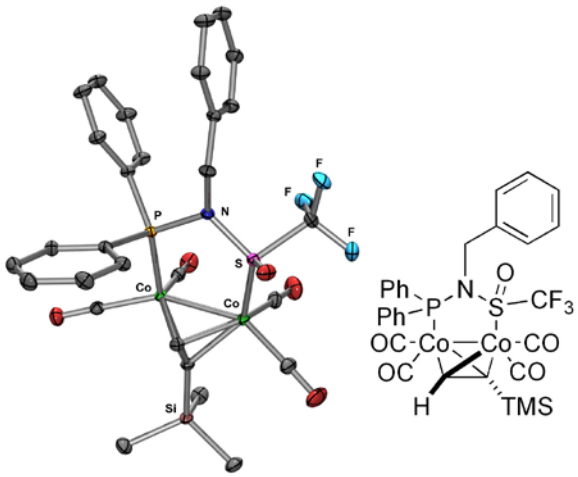


Figure 2. X-ray structure of a cobalt carbonyl complex coordinated to a trifluoromethyl PNSO ligand.

Furthermore, we considered that the replacement of the nitrogen atom by a carbon atom would increase the stability of both

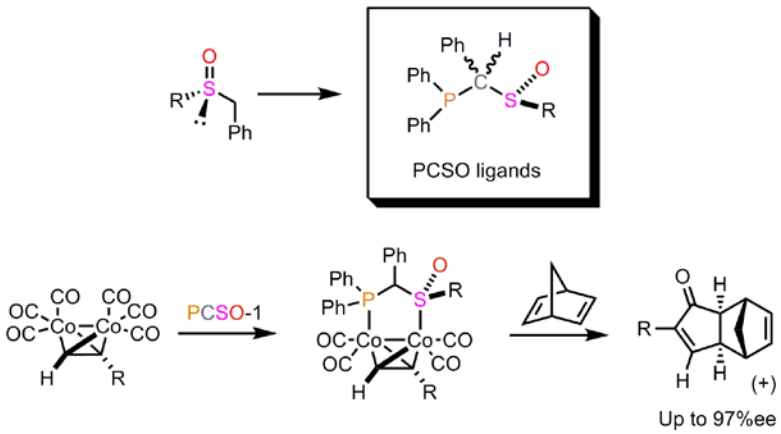
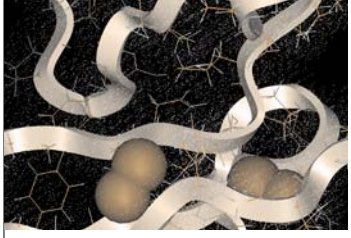


Figure 3. Asymmetric Pauson-Khand reactions with PCSO ligands.

ligands and the corresponding metal complexes. Ligands with a central carbon atom (PCSO) have been prepared by phosphinylation of benzyl and homobenzyl sulfoxides. Therefore, a new family of enantiomerically pure p-tolyl and *tert*-butyl sulfinylmethylphosphine ligands (PCSO ligands) with an extra chiral centre at the carbon atom have been described. Ligand exchange reaction of these compounds with Co_2-alkyne complexes afforded up to 6:1 dr. The resulting bridged complexes were tested in the intermolecular PKr to provide up to 97% ee (Ferrer *et al*, 2009).



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Although initially designed for PKr, PNSO ligands have the capacity to bind to other metals and, a priori, can be useful in other types of metal organometallic asymmetric reactions. We have studied this possibility thoroughly and have pursued the formation of new organometallic complexes. We have prepared and characterised the rhodium (I) complexes of *N*-phosphino-*tert*-butylsulfinamide ligands that are potentially useful in asymmetric hydrogenation. In this case PNSO ligands work either as P,O or P,S chelating ligands when attached to the square planar rhodium centre. Complexes bearing diene ligands, such as [Rh(PNSO)(NBD)][TfO] and [Rh(PNSO)(COD)][TfO], provided P,O coordination, while [Rh(PNSO)$_2$][TfO]-type complexes provided P,S coordination. Ligand exchange experiments with mono and bidentate phosphines afforded evidence of the hemilabile behaviour of the PNSO ligands (Achard *et al*, 2009).

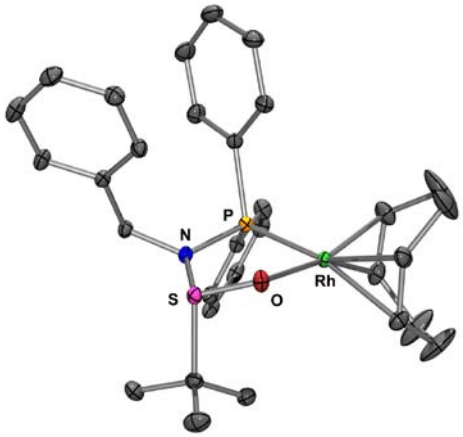

Figure 4. X-ray structure of a rhodium complex with a P,O coordination to a PNSO ligand.

Figure 5. Asymmetric PKr of symmetrically substituted alkynes.

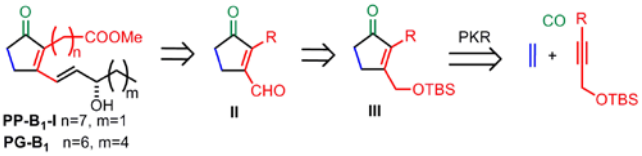

Figure 6. Retrosynthetic scheme of our synthesis of prostaglandin and phytoprostane B_1.

New developments in the PKr

Prior to our work, no asymmetric PKr with symmetrically substituted alkynes had been developed. These acetylenes have the advantage that when coordinated to a bidentate ligand they can give rise to only one diastereomer, thereby preventing the diastereomeric separation of the complexes. However, the asymmetric version is especially difficult because the olefin insertion must select between the two almost identical C-Co bonds. We discovered that PNSO ligands can be efficiently used in this process. The chirality of the cobalt S-bonded sulfinyl moiety was found to direct olefin insertion into one of the two possible cobalt–carbon bonds in the alkyne complex. Therefore, we developed the first asymmetric PKr with symmetrically substituted alkynes (Ji *et al*, 2009).

Synthesis of biologically active compounds

Synthesis of five-membered-ring compounds

As mentioned above, one of the best ways to prepare five-membered-ring compounds is through the PKr. Prostaglandins are among the most relevant cyclopentanic compounds because they exhibit a wide variety of functions and biological activities. We have described a new approach to synthesise prostaglandin and phytoprostanes B_1. The key step in this strategy is an intermolecular PKr between a silyl-protected propargyl acetylene and ethylene. This reaction, promoted by NMO in the presence of 4Å molecular sieves, afforded the 3-*tert*-butyldimethylsilyloxymethyl-2-substituted-cyclopent-2-en-1-ones (III) in good yield and with complete regioselectivity. Deprotection of the silyl ether, followed by Swern oxidation, gave 3-formyl-2-substituted-cyclopent-2-en-1-ones (II). Julia olefination of the aldehydes II with the suitable chiral sulfone allowed preparation of PPB1 type I and PG-B_1 (Vázquez-Romero *et al*, 2009).

Enantioselective synthesis of amino acids and peptides

We have prepared several new non-natural amino acids and used them in the preparation of Somatostatin analogues. The biological activity of these new compounds revealed that they are as active as the natural hormone in some receptors but much more selective. We are now studying their structure by NMR, in collaboration with Maria Macias (IRB Barcelona). We aim to correlate the biological activity with the structure in order to design analogues with an improved selectivity profile and higher stability in plasma.

Scientific output

Publications

Achard T, Benet-Buchholz J, Riera A and Verdaguer X. Cationic rhodium (I) complexes of N-phosphino-tert-butylsulfinamide ligands: Synthesis, structure and coordination modes. *Organometallics*, **28**, 480-87 (2009)

Ferrer C, Riera A and Verdaguer X. Sulfinylmethyl phosphines as chiral ligands in the intermolecular Pauson-Khand reaction. *Organometallics*, **28**, 4571-76 (2009)

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Research networks and grants

Nous catalizadors quirals amb lligands de tipus fosfinosulfinamida per a síntesi asimètrica
Enantia SL (2009)
Principal investigator: Antoni Riera

Síntesis enantioselectiva de compuestos biológicamente activos y desarrollo de nueva metodología sintética
Spanish Ministry of Science and Innovation, CTQ2008-00763 (2009-2011)
Principal investigator: Antoni Riera

Collaborations

Molecular orbital calculations
Santiago Olivella, CSIC (Barcelona, Spain)

Structural NMR studies of peptides
Maria Macias, IRB Barcelona (Barcelona, Spain)

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

Synthesis of cucurbituril derivatives
Miquel Pons, IRB Barcelona (Barcelona, Spain)

Synthesis of PPAR-γ inhibitors
Annabel Fernández Valledor, University of Barcelona (Barcelona, Spain)

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

Màrius Rubiralta

Peptidomimetics and Bioactive Heterocycles Group (Biosyner)

Scientific output

Publications

Arévalo MJ, Kielland N, Masdeu C, Miguel M, Isambert N and Lavilla R. Multicomponent access to functionalized mesoionic structures based on TFAA activation of isocyanides: Novel domino reactions. *Eur J Org Chem,* **5**, 617-25 (2009)

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Chas M, Riobé F, Sancho R, Minguillón C and Avarvari N. Selective monosulfoxidation of tetrathiafulvalenes into chiral TTF-sulfoxides. *Chirality*, **21**(9), 818-25 (2009)

Pérez AM and Minguillón C. Retention of fluorinated chiral selectors in biphasic fluorinated solvent systems and its application to the separation of enantiomers by countercurrent chromatography. *J Chromatogr A*, Epub Oct 2 (2009)

Rubio N and Minguillón C. Preparative enantioseparation of (+/-)-N-(3,4-cis-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide by centrifugal partition chromatography. *J Chromatogr A*, Epub Dec 18 (2009)

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Research projects and networks

Building blocks for lead finding
Almirall Prodesfarma, APF-004
Researcher: Rodolfo Lavilla

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

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

Plataforma combiquímica basada en productos naturales: descubrimiento y administración de fármacos
Spanish Ministry of Science and Innovation, BQU2006-03794 (2007-2009)
Researcher: Rodolfo Lavilla

Synthesis of bioactive molecules
Ferrer Laboratories, CNV-FERRER

Collaborations

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

Derivatització de polisulfona
Ivo Vankelecom, Bioengineering Department, Catholic University of Leuven (Leuven, Belgium)

New inhibitors of acetylcholinesterase with anti-Alzheimer activity
Pelayo Camps, Pharmacology and Chemistry Department, University of Barcelona (Barcelona, Spain); Javier Luque, Faculty of Pharmacy, University of Barcelona (Barcelona, Spain)

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

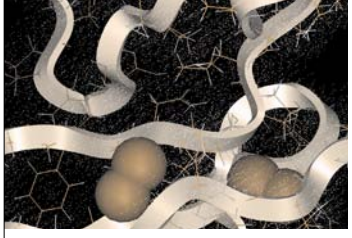
Preparative separation of chiral sulfoxides
Narcis Avarvari, Laboratoire Chimie, Ingénierie Moléculaire et Matériaux, Université d'Angers (Angers, France)

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

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

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

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



Other collaborations

Amirall Laboratories (Barcelona, Spain)

Ferrer Laboratories, Ferrer Group (Barcelona, Spain)

LEITAT (Barcelona, Spain)

Sigma-Aldrich (Madrid, Spain)

Awards and honours

Best poster award: Multiple dual-mode countercurrent chromatography applied to chiral separations (Rubio N, Ignatova S, Sutherland I and Minguillón C)
15th International Conference on Biopartitioning and Purification (London, UK)
Awardee: Nuria Rubio

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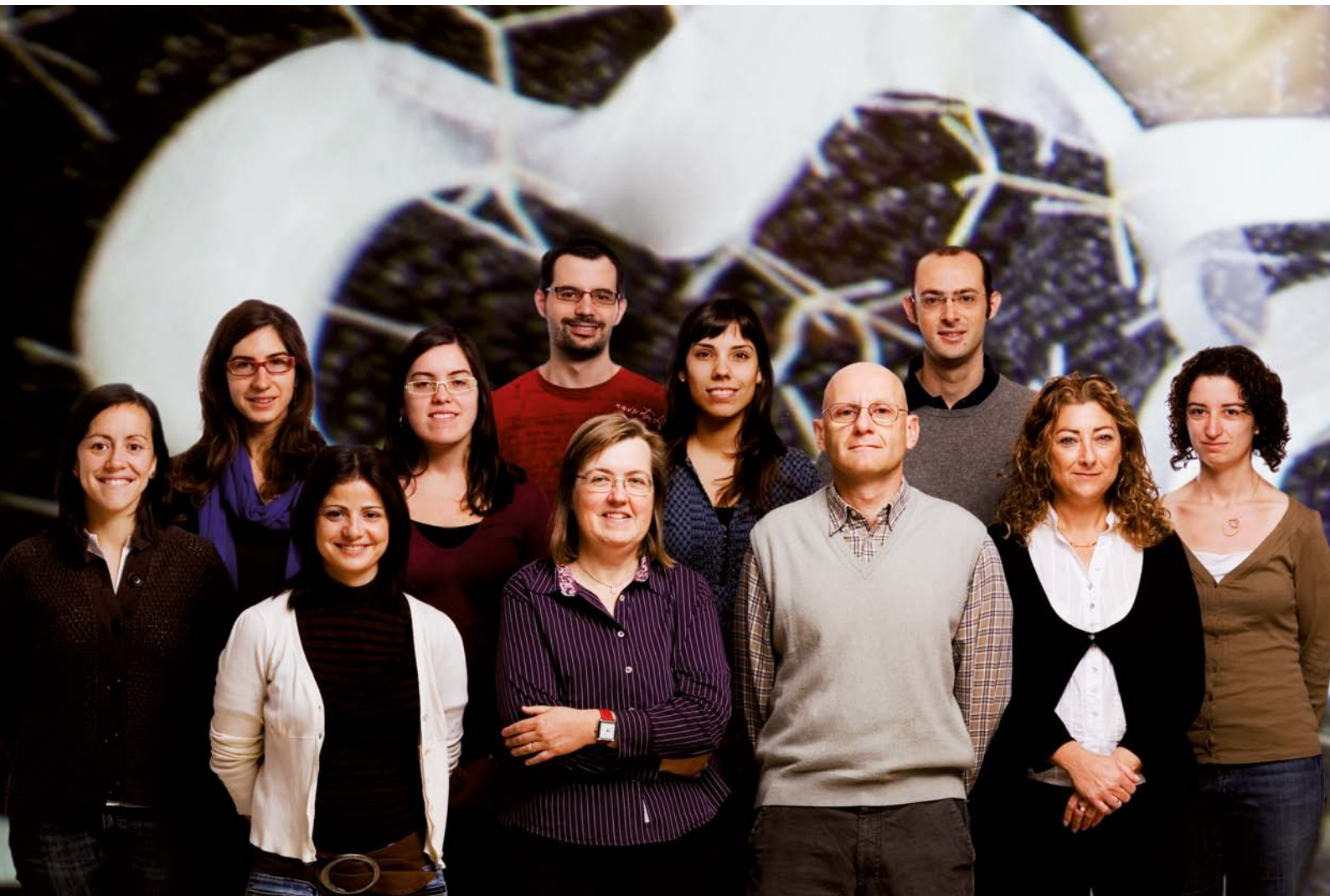
This group left IRB Barcelona in December 2009.



Xavier Salvatella

Laboratory of Molecular Biophysics

The Laboratory of Molecular Biophysics addresses how the structure and motions of proteins relate to disease, with a strong emphasis on diseases that involve the formation of protein aggregates. Members of the laboratory develop new methods to experimentally characterise the flexibility of proteins using Nuclear Magnetic Resonance (NMR) and molecular simulations and then apply these tools to study the molecular bases of specific diseases. The methodological aspect of the work performed by the lab is essential because there is a lack of tools to describe in detail the conformational properties of intrinsically disordered proteins - devoid of persistent secondary and tertiary structure - that are often involved in the formation of aggregates in protein aggregation diseases. The availability of these newly developed tools allows the lab to test the effect of mutation and environmental changes on the properties of these proteins, a procedure that is crucial for unravelling the molecular bases of this class of diseases.

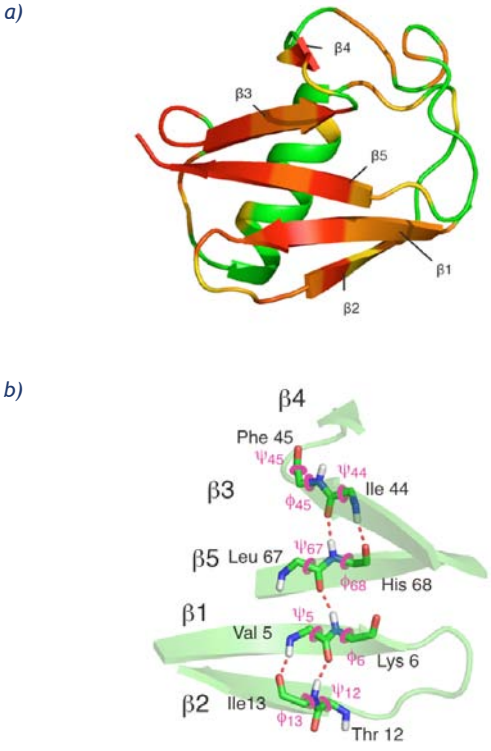
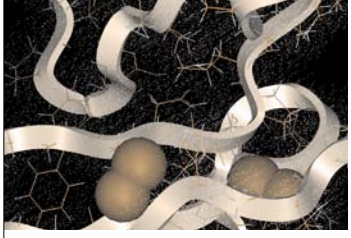


Figure 1. (a) Structure of ubiquitin coloured on the basis of the extent to which the backbone motions of the residues of the protein are correlated to those of residues distant in sequence, where red indicates strong correlation and green absence of correlation. (b) Network of hydrogen bonding interactions that dynamically link the backbone torsion angles of residues Ile 12 (strand β2) to Phe45 (strand β3), which are separated by a distance of ca. 15 Å.

During 2009 the lab focused its efforts on developing methods to determine the structure and dynamics of proteins from Residual Dipolar Couplings (RDCs) measured using NMR as well as on establishing structure-activity relationships in the aggregates formed by the protein lysozyme in non-neuropathic systemic amyloidoses. These efforts have led to the following: i) the identification of concerted conformational changes that transfer information across the structure of proteins through correlated backbone motions; ii) the development of a method to accurately characterise the structure and dynamics of disordered proteins; and iii) the identification of the structural and dynamical properties of the fibrils formed by lysozyme that render them toxic to cells.

Identification of long-range correlations in a surface patch of folded ubiquitin

Motions in folded proteins play key roles in biological functions ranging from enzyme catalysis to molecular recognition. Correlated motions, which occur when distinct sites in the structure fluctuate in concert, are candidate mechanisms for processes that require information transfer across large distances, such as allostery and signal transduction. In addition, they are thought to underlie folding co-operativity, which prevents proteins from undergoing local conformational changes that expose hydrophobic side chains and can trigger aggregation (for an example see last section). Correlated motions are challenging to observe because they involve concerted local conformational changes of low amplitude that are difficult to detect in the laboratory. RDCs are sensitive to the orientation of bond vectors and have recently revealed correlations in the motions of residues that are close in space.



To study the presence of long-range correlated motions in the backbone of ubiquitin, the laboratory has determined, in collaboration with Christian Griesinger's group at the Max Planck Institute for Biophysical Chemistry in Göttingen (Germany), an ensemble of conformations, named ERNST (ensemble refinement for native proteins using a single alignment tensor), which represents the structure and the sub-ms dynamics of the protein in solution. ERNST was obtained by using ensemble simulations restrained by 36 sets of RDCs and its quality is illustrated by its agreement with independently measured NMR parameters that are sensitive to backbone motions. The average structure of ERNST is also consistent with the static X-ray and the average NMR structures of ubiquitin. Moreover, a structural comparison of the ensemble members with a variety of bound conformations of ubiquitin confirmed the recent finding that conformational selection is the most likely mechanism by which this protein recognises its binding partners. Most importantly, ERNST cross-validates well against cross-correlated relaxation rates and trans-hydrogen bond scalar couplings. These NMR parameters are optimal for the analysis of correlated motions because they average on the same timescale as RDCs; however, unlike RDCs, they are sensitive to the relative motions of two distinct sites of the structure. The back-calculated values of cross-correlated relaxation and trans-hydrogen bond scalar couplings from ERNST are in better agreement with experimental data than those computed from the static X-ray structure, the average NMR structure and from other dynamic ensembles.

Using ERNST, the lab has identified and characterised, for the first time, correlations in the motions of residues separated by up to 15 Å (Figure 1) and has shown that they result from concerted conformational changes mediated by the hydrogen bonding network of the protein ubiquitin as well as by steric clashes. This novel collective motion spans four β-strands of ubiquitin and dynamically links residues that form a surface patch recognised by ubiquitin binding domains (UBDs) in ubiquitination, a post-translational modification that controls endocytosis, DNA repair and protein degradation. These results provide a detailed description of the mechanism by which conformational selection operates in molecular recognition and illustrate that the analysis of NMR parameters sensitive to correlated motions is a powerful approach to characterise information transfer in biology. In addition, our findings reveal the mechanism re-

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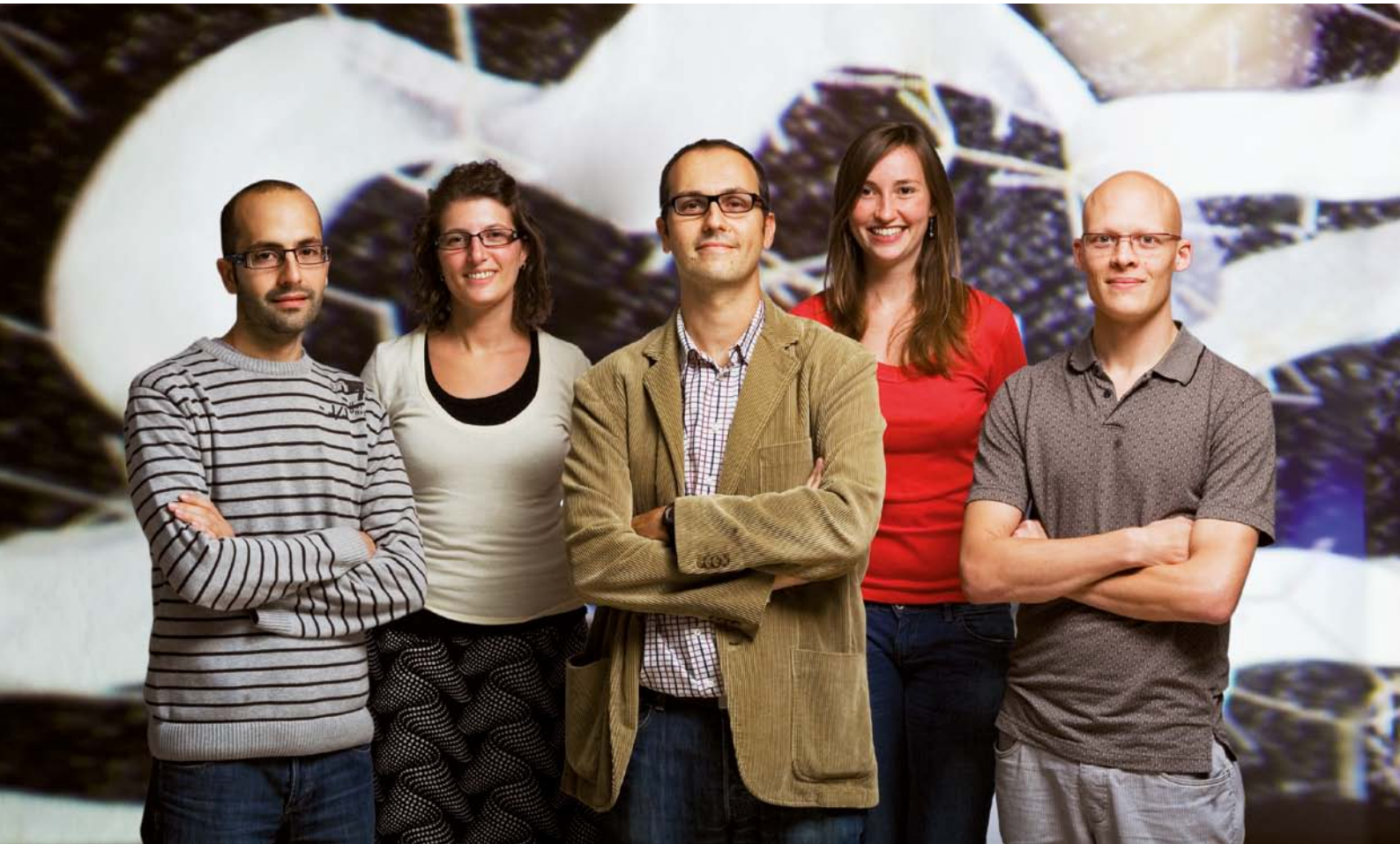


sponsible for folding co-operativity and are therefore relevant for our understanding of the origin of amyloidosis, as this disease is often triggered by mutations or environmental changes that lead to local unfolding and to the formation of potentially cytotoxic protein aggregates (for an example see last section).

Refinement of ensembles describing unstructured proteins

As shown in the characterisation of the dynamics of folded ubiquitin presented in previous section, RDCs are unique probes of the structural and dynamical properties of biomolecules in the sub-ms time scale and can be used as restraints in ensemble molecular dynamics simulations to study the relationship between macromolecular motion and biological function. Until now, however, this powerful strategy was applicable only to molecules that do not undergo shape changes in the timescale sampled by RDCs, such as folded ubiquitin, thus preventing the study of key biological macromolecules such as intrinsically disordered proteins involved in protein aggregation diseases. To circumvent this limitation, researchers in the laboratory have developed the ERIDU algorithm - ensemble refinement of intrinsically disordered and unstructured molecules - that explicitly computes the individual alignment tensors of the ensemble members from their coordinates at each step in the simulation. As a first application, the laboratory has determined an ensemble of conformations that accurately describes the structure and dynamics of chemically denatured ubiquitin, a model system often used to develop algorithms to characterise the structural properties of disordered proteins.

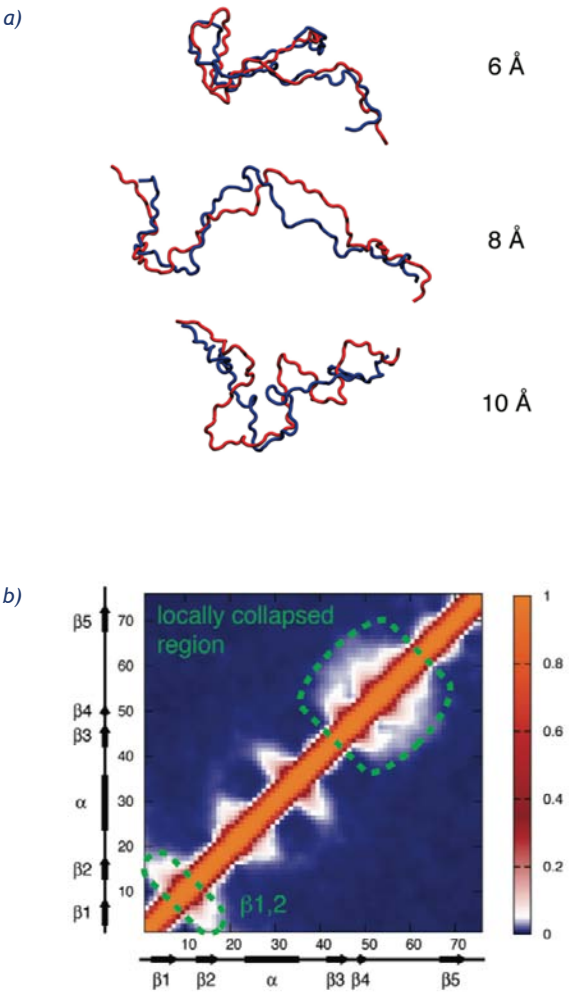


Figure 2. (a) Illustration of how the ERIDU algorithm corrects the conformation of three representative members of the SCM. (b) Contact map of the refined ensemble, where pairs of residues are coloured on the basis of the fraction of ensemble members where the Ca atoms are closer than 10 Å and where the mid-range inter-residue interactions detected using alternative techniques have been highlighted in green.

In analogy to dynamic refinement of folded globular proteins, where simulations are initiated from average structures determined using X-ray crystallography or NMR spectroscopy, the researchers used statistical coil models (SCMs) as starting configuration because these represent the best structural descriptions available for unstructured proteins. They found that refinement causes significant structural corrections (Figure 2a) and produced an ensemble that was in complete agreement with experiments and presented transient mid-range inter-residue interactions between strands β1 and β2 of the native protein (Figure 2b), also observed in other studies based on trans-hydrogen bond scalar couplings and paramagnetic relaxation enhancements. The availability of ERIDU increases the range of systems that can be studied using ensemble simulations restrained by RDCs and will be particularly useful to characterise the conformational properties of intrinsically disordered proteins involved in protein aggregation diseases.

Structure-activity relationships in amyloid fibrils

Amyloid fibrils are non-covalent assemblies of proteins that form in the tissues of patients suffering from protein aggregation diseases, including sporadic and transmissible neurodegenerative disorders as well as various non-neuropathic amyloidoses. The observation that non-fibrillar species transiently populated in the early phase of *in vitro* aggregation are more cytotoxic than the corresponding mature amyloid fibrils suggests that they have generic and currently poorly understood structural properties and add to a significant body of evidence linking the onset of Alzheimer's and Parkinson's diseases with the formation of similar species in the brains of patients.

However, other neurodegenerative disorders, such as prion diseases, are caused by the propagation of infectious particles that carry all the information required to exhibit distinct phenotypic traits in identical hosts and, contrary to what is the case for non-fibrillar oligomers, are clearly fibrillar. These observations raise the possibility that the cytotoxicity of protein aggregates in the biological milieu is not directly related to their oligomeric nature but rather to structural properties common to non-fibrillar and certain fibrillar aggregates. Given that, in contrast to highly evolved native structures, the structures of protein aggregates are influenced by pH, buffer components, protein concentration and temperature, these prospects have led to intense research efforts aimed at establishing structure-activity relationships in protein aggregates.

A particularly useful system to study these structure-activity relationships is lysozyme, a protein that is well characterized and forms amyloid deposits in patients suffering from familial lysozyme systemic amyloidosis, a disease that occurs when amyloidogenic mutations in the protein lead to the formation of partially unfolded amyloidogenic intermediates. By incubating lysozyme under various destabilising conditions, researchers in the lab produced fibrils differing in morphology, molecular structure and stability, thereby generating a range of cytotoxic effects (Figure 3). These results, obtained in collaboration with Christopher Dobson's lab at the University of Cambridge (UK), illustrate that the energy landscape of aggregation is significantly more rugged than the normal folding landscape and that the pathogenic properties of certain protein aggregates are related to the size of their cross-β core.

During 2009, the lab has made relevant contributions to the study of the molecular basis of protein aggregation diseases by developing methods to examine the conformational changes that occur in the earliest stages of disease as well as by identifying the properties of protein aggregates that lead to cytotoxicity. Our goals for 2010 include the application of these newly developed tools and principles to key protein aggregation diseases, such as Alzheimer's and Parkinson's disease, as well as to the rare neurodegenerative disease Spinal Bulbar Muscular Atrophy (SBMA), in which nuclear inclusions of Androgen Receptor (AR) molecules with an expanded polyglutamine tract cause motor neuron death.

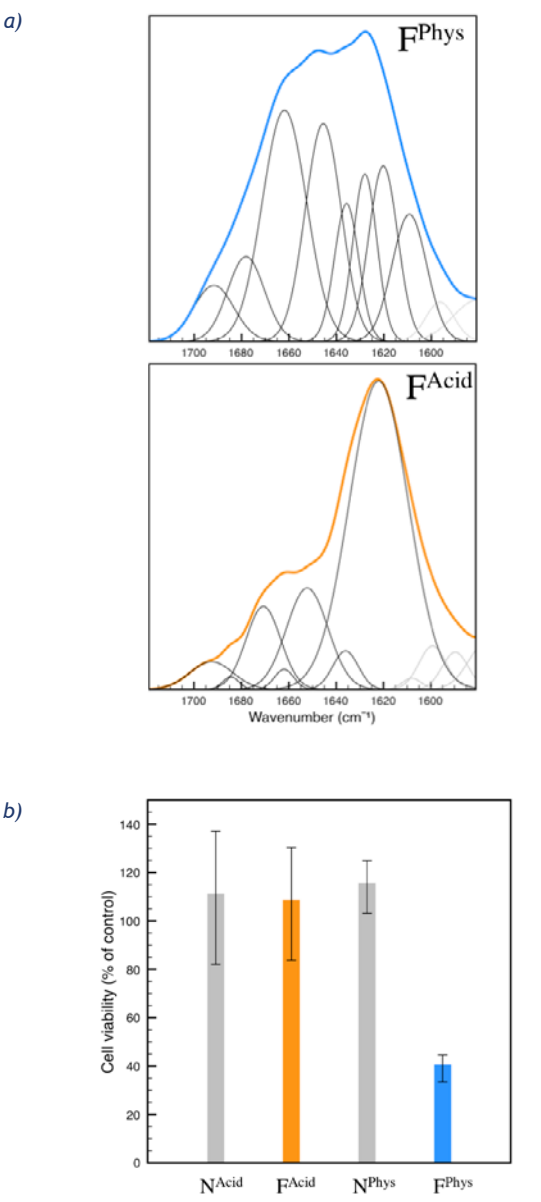


Figure 3. (a) Deconvolution of the FTIR spectrum of fibrils formed under physiological conditions (in blue) and those formed under acidic conditions (in orange) that present, respectively, a low and high degree of cross-β structure that gives rise to the band at 1620 cm⁻¹ right). (b) Viability of SH-SY5Y neuroblastoma exposed to native lysozyme under acidic pH (N^{Acid}), to fibrils formed under acidic conditions (F^{Acid}), to native lysozyme under physiological conditions (N^{Phys}) and to fibrils formed under physiological conditions (F^{Phys}).

Scientific output

Publications

De Simone A, Richter B, Salvatella X and Vendruscolo M. Toward an accurate determination of free energy landscapes in solution states of proteins. *J Am Chem Soc*, **131**(11), 3810-11 (2009)

Robustelli P, Cavalli A, Dobson CM, Vendruscolo M and Salvatella X. Folding of small proteins by Monte Carlo simulations with chemical shift restraints without the use of molecular fragment replacement or structural homology. *J Phys Chem B*, **113**(22), 7890-96 (2009)

Research networks and grants

Structural characterization of key conformational transitions in protein deposition diseases
Spanish Ministry of Science and Innovation, CTQ2009-08850-BQU (2009-2012)
Principal investigator: Xavier Salvatella

Suport a grups de recerca
Agency for Administration of University and Research Grants, 2009-SGR-1514 (2009-2013)
Principal investigator: Xavier Salvatella

Collaborations

Analysis of protein dynamics
Christian Griesinger, Max Planck Institute for Biophysical Chemistry (Goettingen, Germany)

Analysis of RNA dynamics
Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Structure-activity relationships in protein aggregates
Christopher Dobson, University of Cambridge (Cambridge, UK)

Use of protein native ensembles in drug discovery
Crystax Pharmaceuticals (Barcelona, Spain)

Use of protein native ensembles in protein-protein docking
Juan Fernández-Recio, Barcelona Supercomputing Centre (Barcelona, Spain)


Oncology Programme

Eduard Batlle

Signalling in intestinal stem cells and colorectal cancer

Colorectal cancer (CRC) is the third most common type of cancer and the second cause of death by cancer in the western world, causing around 650,000 deaths worldwide per year. The development of a full-blown malignant colorectal tumour is a slow process that often takes more than a decade. Most CRCs originate from pre-neoplastic lesions called adenomas that initially are benign and occur frequently. The transformation of an intestinal adenoma into an aggressive cancer requires the accumulation of multiple genetic alterations. The most common alteration in CRC is the inactivation of the Adenomatous Polyposis Coli (APC) tumour suppressor gene, which is a central component of the Wnt signalling pathway. Loss of APC function results in activation of Wnt signalling via constitutive transcription mediated by the β-catenin/Tcf complex. Alterations in APC (and less frequently in other Wnt pathway components) affect up to 80% of all types of neoplastic lesions in the intestine. Even the earliest precursors of intestinal tumours, the so-called dysplastic crypts, show mutational activation of the Wnt pathway. Remarkably, most β-catenin/Tcf target genes induced after APC mutations in intestinal cells were also found to be physiologically expressed in crypt intestinal stem cells (ISCs) and/or in transient amplifying progenitor cells (van de Wetering et al*, 2002). Mice engineered to lack physiological Wnt signalling in the intestinal epithelium lose the crypt progenitor/stem cell compartment (Korinek* et al*, 1998). Conversely, constitutive activation of the Wnt pathway results in massive expansion of crypt progenitor/stem cell numbers* in vivo *and the onset of CRC (Sansom* et al*, 2004).*

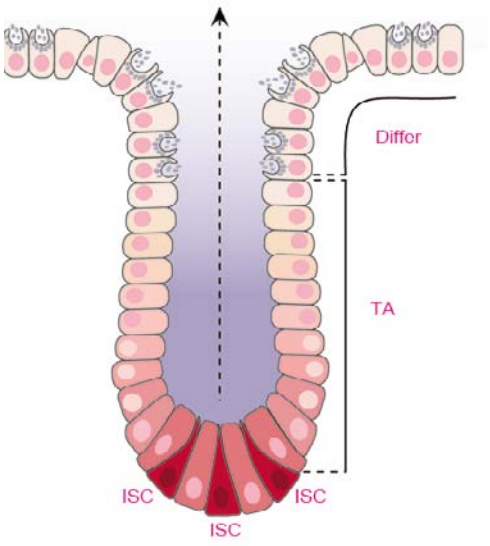
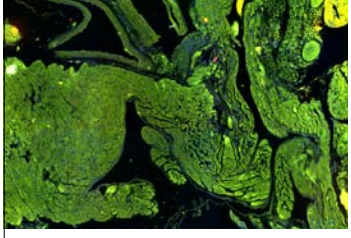


Figure 1. ***The mammalian intestinal epithelium.*** *Organisation of a mammalian colon crypt. ISC; Intestinal stem cells, TA; Transient amplifying cells, and Differ; differentiated cells. Arrow indicates the direction on migration.*

ISCs and CRC development

The columnar epithelium that lines the adult small intestine is shaped into glandular invaginations, called crypts, and finger-like protrusions known as villi. Hundreds of thousands of epithelial intestinal cells, generated daily at the base of the crypts, enter a migratory flow towards the tips of villi. Along their migratory path, intestinal cells differentiate into one of the four cell lineages characteristic of the intestine (enterocytes, enteroendocrine, goblet or Paneth cells). Differentiated cells exert their functions only for a few days as they are then shed into the lumen when they reach the villus tip. Self-renewing stem cells and Paneth cells reside at the crypt bottom and escape the upward migratory flow. The progenitors of differentiated cells, which arise from stem cell divisions, have limited self-renewing capacity and after a few rounds of mitosis, undergo lineage commitment and differentiation. While single crypts are fuelled by one stem cell clone and are monoclonal, each villus receives cells originating from different crypts, thereby explaining their polyclonal nature. The colonic epithelium has an overall structure similar to the small intestine but lacks villi. Differentiated cells are shed into the luminal space when they reach the surface epithelium. Colonic proliferative progenitor cells are lo-



cated within the bottom two-thirds of the crypts, with stem cells residing at the bottom-most positions (Figure 1).

The location and the precise identity of mammalian ISCs are controversial issues because of the lack of specific marker genes and assays to study their properties (Batlle, 2008). To assess the gene programmes that operate in ISCs, we have recently developed a method to purify crypt cell populations. To this end, we used EphB2 as surface marker, a Wnt target gene that is expressed in gradient from the crypt base to the surface epithelium (Batlle *et al*, 2002). FACS sorting of cells showing varying degrees of EphB2 expression has allowed us to obtain the expression profiles of ISCs, transient amplifying cells (TA) and differentiated cells. These profiles are instrumental tools to study the biology of crypt ISCs and also their participation in the initiation and progression of CRC. We are currently studying the role of several ISC-specific genes discovered in this screen (Figure 2). We have also performed extensive bioinformatic analysis of the expression profiles of our purified crypt cell populations versus several publicly available databases containing data from CRC patients. This has led to the discovery of an ISC gene signature that predicts poor outcome in CRC.

In addition to the work on mammalian ISCs, we are collaborating with Jordi Casanova and Andreu Casali (Developmental Biology Programme, IRB Barcelona) in the analysis of adult *Drosophila* ISCs. Recent studies have shown that in a similar fashion to the mammalian intestinal epithelium, Wnt and Notch signalling play essential roles in the specification and maintenance of midgut ISCs (reviewed in Casali and Batlle, 2009). We are currently investigating the extent to which the *Drosophila* midgut intestine represents a good model to study the role of ISCs in intestinal cancer.

EphB receptors as suppressors of CRC progression

As target genes of the beta-catenin/TCF complex, EphB2 and EphB3 receptors are highly expressed in all early CRC lesions, such as dysplactic crypts and small adenomas. However, we observed that these receptors become down-regulated at the adenoma-carcinoma transition in virtually all patients analysed (>100). The level of EphB down-regulation strongly correlated with higher histological tumour grade, a parameter associated with malignancy. More importantly, by using transgenic and

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Lab Technicians:
Javier Hernando, Marta Sevillano

Elena Sancho's research group merged with Eduard Batlle's group in 2009.

knock-out animals, we engineered mice where APC mutations were placed in a background of low EphB activity in the intestine. These mice developed dozens of malignant tumours in the colon and the rectum, including invasive carcinomas. These findings thus demonstrate a causal role for EphB down-regulation in CRC progression. Thus, while progressing tumours maintain the beta-catenin/Tcf-imposed progenitor features as an essential component of their transformed phenotype, they are apparently selected to silence a subset of betacatenin/TCF target genes (*ie* EphB genes) to progress beyond the initial stages (Batlle *et al*, 2005).

Our research efforts address the mechanism of EphB-mediated tumour suppression in the intestine. We demonstrated that EphB signalling compartmentalises the growth of CRC cells *in vivo*. In $Apc^{Min/+}$ mice, $EphB^{+}$ tumour cells that form incipient adenomas are in continuous contact with normal intestinal epithelial cells expressing ephrinB ligands. Through the use of mice models deficient in EphB or ephrinB ligands, we demonstrated that Apc mutant tumour founder cells cannot colonise the regions of the normal epithelium that express high levels of ephrinB1, owing to EphB repulsive signals. We have proposed that tumour cell compartmentalisation may be a general mechanism of tumour suppression in tissues whose architecture is defined by Eph-ephrin interactions. Overall, our observations imply that fully malignant CRC cells bearing multiple mutations in oncogenes and tumour suppressors respect the boundaries imposed by EphB-ephrinB interactions (Cortina *et al*, 2007).

In this work we also generated *in vitro* models that mimic EphB/ephrinB interactions in CRC cell lines. We took advantage of CRC cell lines that do not express EphB receptors or ephrinB ligands to generate two populations of the same cell line expressing 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 (Cortina *et al*, 2007). We are currently exploring the mechanisms and cell dynamics downstream of EphB signalling by extensive usage of *in vitro* models and sophisticated microscopy methods. During 2009, we have identified the critical involvement of a metalloproteinase downstream of EphB-mediated cell sorting (manuscript in preparation).

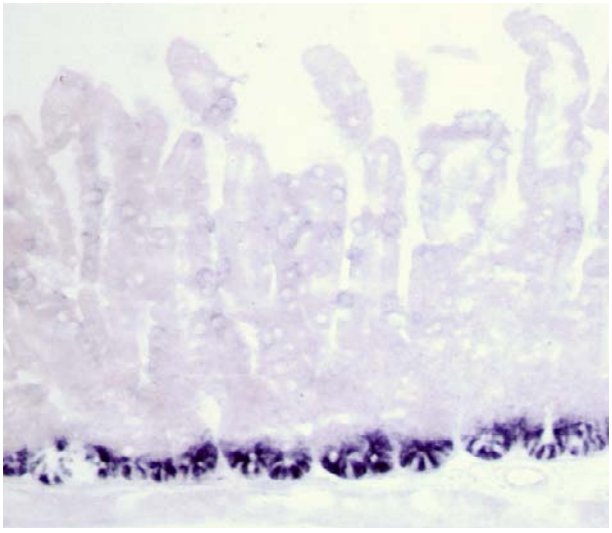

Figure 2. **In situ *hybridisation of a new ISC-specific gene, OLFM4.*** *Cells detected by OLFM4 probe at the crypt base (in blue) correspond to ISCs.*

TGF-beta signalling during CRC progression

One of the most prevalent mutations found during CRC progression are those that inactivate the TGF-beta signalling pathway (reviewed in Xu and Pasche, 2007). The TGF-beta 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 homo dimers. Ligand-bound receptor II recruits TGF-beta receptor I homodimers, which are subsequently transphoshorylated and thus activated by receptor type II. Phosphorylation of the intracellular mediators smads by activated receptor I allows dimer formation with smad-4 and translocation to the nucleus where the specific outcome of the signalling will depend on the cell type and the context of the cell itself (reviewed in Massagué, 2008).

Around 80% of all microsatellite instable CRCs contain mutations in type II TGF-beta receptor (TGFBR2) that impair signalling. In addition, inactivation of downstream TGF-beta pathway effectors, in particular SMAD4 and SMAD2, have also been found in a significant fraction of microsatellite stable CRCs. Overall, the incidence of TGF-beta resistance in CRCs appears to be around 30%. In addition, virtually all CRC cell lines have lost their TGF-beta response. Modelling CRC progression in mice has revealed that disruption of TGF-beta signalling in the intestinal epithelium does not initiate intestinal tumorogenesis *per se*. However, when the onset of CRC is triggered by deficiency of the tumour suppressor APC, compound Tgfbr2 or Smad4 null alleles accelerate adenoma to carcinoma progression in the intestinal tract.

Collectively, the data described above strongly support the notion that TGF-beta signalling suppresses CRC. This is in accordance with data obtained for solid tumours such as breast cancer, prostate cancer and skin tumours, among others, which have led to the general believe that TGF-beta acts as a tumour suppressor in the initial stages of carcinogenesis. However, several studies have proposed additional roles for TGF-beta in CRC progression (reviewed in Xu and Pasche, 2007). Our lab currently focuses on the role of TGF-beta signalling in CRC progression. For many years, tumorogenesis was studied from the perspective of tumour cells alone. Recently, much attention has been given to the contribution of the non-epithelial component of solid tumours during disease progression. The tumour microenvironment is a complex mixture of cell types that includes fibroblasts, immune cells, blood ves-

sels and a multitude of factors. The knowledge and control of stromal changes within a developing tumour has become a major topic of research in oncology that has drawn the attention of some of the leading groups in cancer worldwide. We are studying the transcriptional events controlled by TGF-beta in CRC epithelial cells and also in stromal cells. We have identified changes in approximately 500 genes in response to TGF-beta in CRC both in epithelial and stromal cells and have studied their modulation during CRC progression. Remarkably, the TGF-beta responding signature robustly classified benign and malignant colorectal tumours with 100% accuracy in unsupervised analysis. This finding implies that these genes may contain the information that drives the adenoma/carcinoma transition (Figure 3).

Our laboratory is dedicated to dissecting this information in order to identify TGF-beta genes that play an executive role in CRC progression. We are approaching this research from a multidisciplinary perspective that includes screening to identify TGF-beta regulated genes that are crucial for CRC progression, and the development of orthotopic models of colorectal tumours in nude mice that would be instrumental to investigate the role of the TGF-beta controlled gene signature.

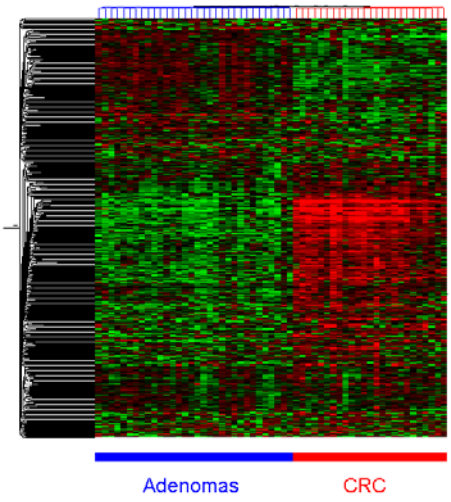

Figure 3. ***The TGF-beta signature discriminates between adenomas and carcinomas.*** *Unsupervised clustering analysis of a collection of tumours of known transcriptomes, on the basis of target genes controlled by TGF-beta signalling, clearly classifies adenomas and carcinomas in two separate branches.*

Scientific output

Publications

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Research networks and grants

Biología del cáncer (ONCOBIO)
Spanish Ministry of Science and Innovation, CSD-2007-00017 (2007-2012)
Principal investigator: Eduard Batlle

Convenio de colaboración entre la Fundación Científica de la Asociación Española contra el Cáncer y la Fundación Privada Institut de Recerca Biomèdica en materia de ayudas para investigadores en el ámbito de la oncología
Spanish Association Against Cancer (2009-2010)
Principal investigator: Eduard Batlle

Dissecting the roles of the beta-catenin and Tcf genetic programmes during colorectal cancer progression
European Commission, ERC Starting Grant 208488 (2008-2013)
Principal investigator: Eduard Batlle

Estudio de la modulación del programa genético controlado por beta-catenina Tcf durante la progresión del cáncer colorrectal
'La Caixa' Foundation (2006-2009)
Principal investigator: Eduard Batlle

Señalización por Wnt, receptores Eph y cáncer de colon: un análisis funcional del inicio de la tumorigénesis intestinal
Spanish Ministry of Science and Innovation, SAF2008-01512 (2009-2011)
Principal investigator: Eduard Batlle

Collaborations

Common genes in pancreas cancer and CRC
Francisco X Real, Spanish National Cancer Research Center (Madrid, Spain)

Drosophila *gut as a model for CRC development*
Andreu Casali, IRB Barcelona (Barcelona, Spain)

Intestinal stem cells in CRC
Hans Clevers, Hubrecht Laboratory (Utrecht, The Netherlands)

Mediators of EMT in Drosophila *and CRC*
Jordi Casanova, IRB Barcelona (Barcelona, Spain)

Regulation of Wnt signalling pathway in CRC
Antonio García de Herreros, IMIM (Barcelona, Spain) and Mireia Duñach, Autonomous University of Barcelona (Barcelona, Spain)

Role of cdk6 in intestinal development
Mariano Barbacid and Marcos Malumbres, Spanish National Cancer Research Center (Madrid, Spain)

TGF-beta target genes in CRC
Joan Massagué, Memorial Sloan-Kettering Cancer Center (New York, USA)

Awards and honours

Debiopharm Life Science award
École Polytechnique Fédérale de Lausanne (since 2006)
Awardee: Eduard Batlle

Roger Gomis

Tumoral Metastasis Laboratory (MetLab)

Intricate signalling networks control cell division, differentiation, movement, organisation and death. Cancer cells disobey these signals during tumour progression and metastasis, which is the final step in 90% of all fatal solid tumours. Metastasis is therefore a grave public health problem and consequently a field of considerable pharmaceutical interest. A major research focus of our laboratory is to identify and study the genes and functions that allow tumour cells to achieve metastatic colonisation of vital organs.

Growth control and cancer metastasis

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

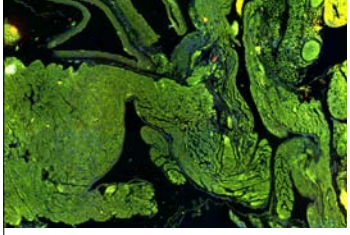
The Tumoral Metastasis Laboratory (Metlab) focuses on the study of the molecular mechanisms involved in metastasis. Our research focuses on aberrant gene responses that enable invasion and metastasis in tumour cells. We seek to elucidate the mechanisms that mediate tissue-specific metastasis, in particular breast cancer metastasis. Metastasis, a complex process caused by elaborate interactions between tumour cells and the surrounding healthy tissues in several vital organs, accounts for 90% of all deaths from cancer in patients with solid tumors. The molecular and cellular mechanisms that lead primary tumours to form metastases must be understood in order to better address this major life-threatening disease. The identification of metastatic genes and mechanisms is essential for understanding the basic biology of this lethal condition and its implications for clinical practice. Previous research has revealed the complexity of the metastatic process. However, it has largely failed to explain how and why metastasis from Estrogen Receptor-positive (ER+) breast cancer subtype occurs. Neither has it identified the mechanisms that make metastasis a tissue-specific process, the events that allow dormant metastases to become active and lethal many years after removal of a primary tumour, or the metastasis-mediating genes with potential as therapeutic targets.

Our contribution to the field builds on an experimental approach based on the use of moderately metastatic cells that are injected into a mouse model for the selection of highly metastatic ER+ breast cancer subpopulations. Live-animal imaging techniques are used to track the spread, homing, and outgrowth of the metastatic cells in different organs. After harvesting metastatic lesions and verifying that highly metastatic cells have been selected, we plan to use genome-wide transcriptomic profiling to identify and clinically validate metastasis-linked genes. Gene transfer techniques are then used to assess the contribution of individual genes to various steps (invasion, homing, outgrowth, angiogenesis, and stroma adaptation) of metastasis. Using this approach, the laboratory has recently identified a preliminary set of genes that cooperatively mediate ER+ breast cancer metastasis to the bone. We also aim to define the role of bone metastasis genes in ER+ breast cancer metastasis, and how ER status influences the choice of metastatic mechanisms. Finally, we will evaluate the potential use of metastatic mediators as targets of therapy.

Two years ago, we initiated a project in the laboratory that focuses on breast cancer metastatic suppressor genes and their functions in metastasis. Our initial efforts are devoted to study the group of metastatic suppressor genes necessary for breast to lung metastasis, first identified at Joan Massagué's laboratory (Minn *et al*, 2005). With 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 and lung cancer patients in order to identify new metastatic gene signatures.

For this purpose, on the basis of collaborations with clinical and basic researchers at the Hospital Clínic and Hospital de Sant Pau, in Barcelona, and the Memorial Sloan-Kettering Cancer Center, in New York, the MetLab team continues to work on the isolation of metastatic cells from pleural effusions derived from lung and breast cancer patients. Once in-

jected into mice, these metastatic cells are labeled with the GFP-Luciferase-TK protein fusion and visualised by bioluminescent techniques. On the basis of these metastatic cell populations, we intend to isolate highly aggressive subpopulations with tropism to specific tissues. 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.



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Esther Fernández

Lab Manager:
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Visiting Scientists:
Xabier Adrian García, Cristina Nadal (members of the Institut d'Investigacions Sanitàries IDIBAPS-Hospital Clínic/IRB Barcelona)

Scientific output

References

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Research networks and grants

Estudio de los mecanismos moleculares de la metástasis del cáncer de mama a pulmón: función y potencial terapéutico de genes supresores de metástasis
Spanish Association Against Cancer (2008-2011)
Principal investigator: Roger Gomis

Papel de C/EBPb en los mecanismos moleculares de regulación de la respuesta citostática al TGFb; implicaciones fisiológicas y sus alteraciones en el cáncer de mama
Spanish Ministry of Science and Innovation, SAF2007-62691 (2007-2009)
Principal investigator: Roger Gomis

Other funding sources

Mechanisms of metastasis
BBVA Foundation

Collaborations

Cristina Nadal, Oncology Service, Hospital Clínic Barcelona (Barcelona, Spain)

Eduard Batlle, IRB Barcelona (Barcelona, Spain)

Awards and honours

Josep Sala Trepat award
Institut d'Estudis Catalans (2009)

Travis Stracker

The DNA damage response, genome instability and cancer

The research in our laboratory focuses on understanding the cellular DNA damage response (DDR) and its role in safeguarding human health. This response to diverse DNA lesions is crucial for maintaining the stability of the genome. Genome instability and a defective DDR are a hallmark of cancer cells and can predispose to cancers and other debilitating pathologies. Detection of DNA damage leads to changes in cell behaviour, including cell cycle checkpoint arrest and in some cases the activation of cell death pathways. We investigate the molecular signalling pathways that control these responses in order to understand their role in tumour suppression and to identify ways to manipulate them for clinical applications.

The DNA damage response in human disease

The integrity of the genome is threatened by diverse types of DNA damage. This damage can originate from cellular processes, such as DNA replication, or result from exposure to exogenous environmental insults, such as ionizing radiation or chemical mutagens. If not dealt with appropriately, unrepaired DNA damage can be amplified or exacerbated during cell division, cause defects in cellular function, and result in the generation of potentially oncogenic mutations or rearrangements.

The detection of DNA lesions by damage-sensing proteins activates the DNA damage response (DDR), triggering signal transduction cascades that alter cell behaviour to promote genome stability (Figure 1). These alterations include the activation of cell cycle checkpoints, the recruitment and/or activation of appropriate repair factors, and, in some cases, the initiation of specialised responses such as programmed cell death (apoptosis) or permanent exit from the cell cycle (senescence). These mechanisms prevent DNA damage from being passed on to daughter cells and are known to play crucial roles in tumour suppression.

Different types of DNA lesions require different repair pathways and affect cell behaviour in distinct ways. DNA double strand breaks (DSBs) are considered one of the most dangerous types of

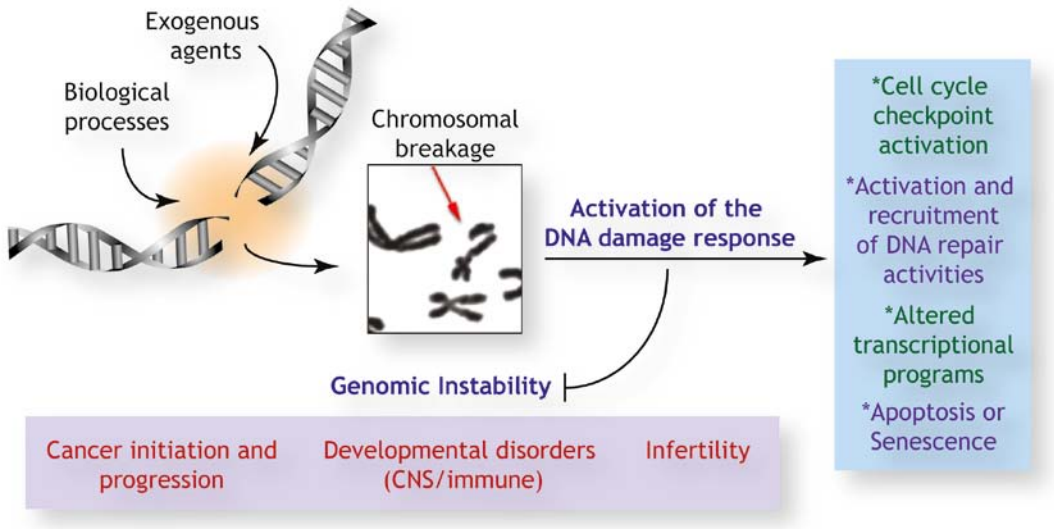
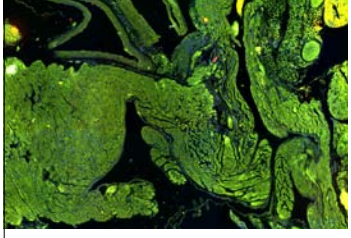


Figure 1. ***The activation of the DNA damage response.*** *Chromosomal breakage arising from endogenous or exogenous sources activates the DNA damage response. This leads to changes in cell behaviour (blue box) that prevent the accumulation of genomic instability that can promote various human pathologies (violet box), including cancer.*



DNA damage because they can be cytotoxic or cytostatic and can lead to the generation of oncogenic translocations if not repaired correctly. Defects in the DDR to DSBs underlies many severe human hereditary disorders that predispose patients to cancer and other serious pathologies. For example, the human disorders Ataxia-telangiectasia (A-T) and Nijmegen Breakage Syndrome (NBS) are characterised by DSB sensitivity, predisposition to cancer, immunodeficiency, and severe neuronal pathology. In addition to hereditary cancer predisposition, genes encoding proteins that respond to DSBs are often found mutated in spontaneous human cancers.

DNA DSBs and the G2/M cell cycle checkpoint

DSBs are rapidly detected by the highly conserved Mre11-Rad50-Nbs1 (MRN) sensor complex. Mutations in the genes that encode all 3 members of the complex have been found to underlie human genetic instability syndromes (reviewed by Lavin, 2007). Recognition of DSBs by MRN activates signal transduction kinases, such as Ataxia-telangiectasia mutated (ATM). Both the MRN complex and ATM play important roles in the regulation of cell cycle checkpoints, particularly during S-phase DNA replication and in G2.

Previously, we have employed genetic approaches to identify the role of the MRN complex in tumour suppression (Stracker *et al*, 2007; Stracker *et al*, 2008). These studies implicated the G2/M cell cycle checkpoint activities of MRN as playing a crucial role. This checkpoint is activated following DNA damage and prevents G2 cells from initiating mitosis with broken chromosomes. This checkpoint has also received considerable interest in clinical research as the ability to inactivate it during chemotherapy can enhance the toxicity of DNA-damaging drugs on dividing tumour cells.

Activation of the G2/M checkpoint following DSB detection requires the processing of double-stranded DNA (dsDNA) breaks 5' to 3' to form single-stranded DNA (ssDNA) tails at the break sites, a process called resection. The formation of ssDNA tails is a crucial event for both cell cycle checkpoint activation as well as homologous recombination-mediated repair of DNA breaks. Resection of dsDNA to ssDNA requires

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This group joined IRB Barcelona in May 2009.

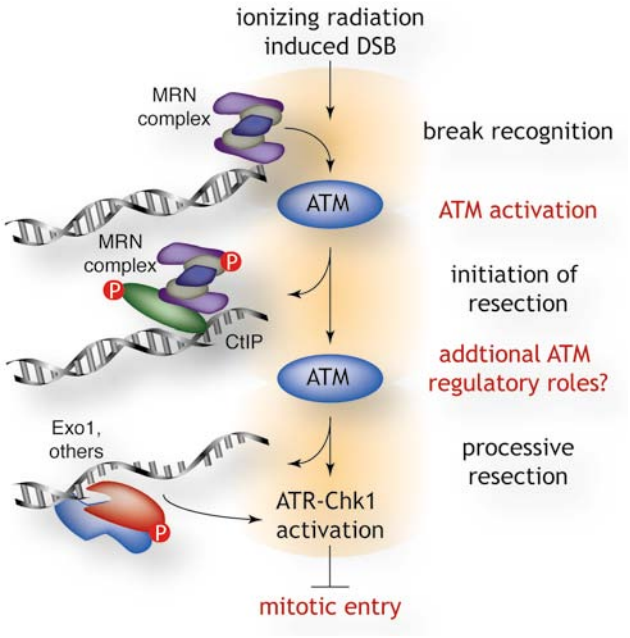

Figure 2. ***The activation of the G2/M checkpoint****. Detection of breaks by the MRN complex leads to the activation of ATM kinase activity. The MRN complex and CtIP are substrates of ATM and initiate resection at DSB ends. Following short resection of the ends, additional factors such as Exo1 continue the resection process. Many of these proteins are substrates of ATM, thereby suggesting a regulatory role. Resection is required for the efficient activation of the ATR and Chk1 kinases that inhibit mitotic entry through modulation of cyclin-dependent kinase activity.*

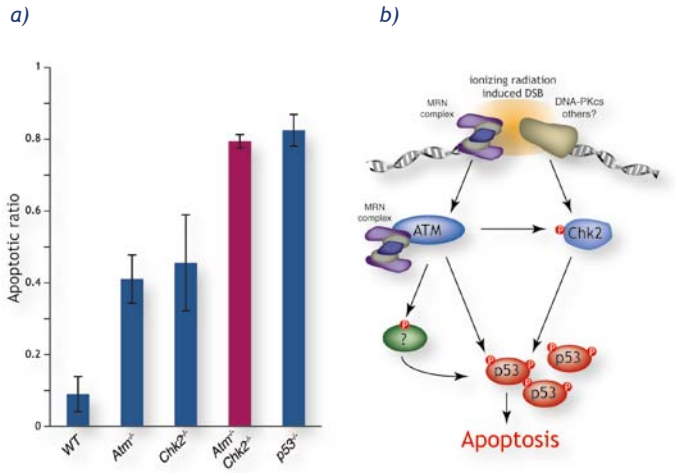

Figure 3. ***Apoptotic signalling in thymocytes****. (a) Analysis of apoptosis in primary thymocytes 20 hours following exposure to 5 Gy of ionizing radiation. Genetic analysis revealed that Chk2 is required for ATM independent apoptosis in response to ionizing radiation (from Stracker* et al*, 2007). (b) Working model of apoptosis in thymocytes. The MRN complex activates ATM and plays a secondary role in the phosphorylation of some ATM substrates required for apoptosis. Chk2 is phosphorylated by ATM but additional kinases can activate Chk2 in the absence of ATM. DNA-PKcs is a prime candidate for this activity and promotes ATM independent apoptosis (Callén* et al*, 2009).*

the concerted action of multiple nucleases, helicases and regulatory proteins. It remains unclear as to precisely which enzymatic activities are required for resection but recent advances in yeast and biochemical systems have provided much insight and resulted in a 2-step model of resection, summarized in Figure 2 (Mimitou and Symington, 2009). The MRN complex binds DSBs and recruits Sae2 (CtIP in mammalian cells) to promote the first step, removing a short stretch of nucleotides from the break ends. This is then followed by processive resection, which can be carried out by the Exo1 nuclease and a helicase or the DNA2 protein that has both nuclease and helicase activities. Biochemical data in mammalian cells implicate many of the same activities in the resection process but their roles in checkpoint activation remain unclear.

The observation that the activation of the ATM kinase is crucial for checkpoint activation in G2 suggests that ATM participates in regulating resection. Consistent with this, many of the enzymatic activities involved, including the MRN complex members, CtIP and Exo1, are substrates of ATM and related kinases. In ATM-deficient cells, the activation of the G2/M checkpoint is severely impaired and this correlates with reduced activity of the ATR and Chk1 kinases known to be required for G2 arrest. In cells expressing hypomorphic Nbs1 mutants that lack the N-terminal FHA and BRCT phospho-peptide binding domains, the G2/M checkpoint is impaired, but to a lesser degree than in ATM mutants. These Nbs1 mutants are expressed at very low levels and structural studies have shown that the N-terminal domains of Nbs1 are important for interactions with CtIP, another protein that is also crucial for initiating resection (Williams *et al*, 2009). Thus, the mild G2/M defect in Nbs1 mutant cells is surprising. In yeast, deletion of both Nbs1 and Exo1 leads to a synthetic growth defect and mutation of both genes produces more severe resection defects in the resection process (Mimitou and Symington, 2009). A potential explanation for the less severe G2/M checkpoint defect of Nbs1 mutants compared to ATM null mammalian cells is that partially redundant activities, such as Exo1, suppress the defects of Nbs1 mutants. Consistent with this possibility, Exo1 has been identified as a substrate of ATM/ATR activity and its overexpression can enhance ATR-Chk1 activation in response to synthetic oligonucleotide substrates (Shiotani and Zou, 2009).

In 2009 we have initiated projects to examine the mechanism of DNA resection and G2/M checkpoint activation. We are testing the hypothesis that Exo1 suppresses the severity of checkpoint defects in Nbs1 mutants. We are doing this using multiple cell culture-based approaches to determine the relevance of Exo1 in normal and Nbs1 mutant backgrounds during G2/M checkpoint induction. Complementary genetic and biochemical approaches have also been initiated to identify proteins involved in the resection process that may provide insight into its regulation following DSB detection.

The activation of radiation-induced apoptosis

Exposure to ionizing radiation results in severe toxicity characterized by cell death in both the hematopoietic system and the gastrointestinal tract. While occupational exposure to high doses of radiation is rare, ionizing radiation is frequently used as

a therapy for cancer. The Chk2 kinase that is activated following DSB recognition is important for preventing radiation toxicity (reviewed in Stracker *et al*, 2009). We, and others, have shown it is a tumour suppressor in the context of particular mutations that affect genome stability (Stracker *et al*, 2008). While Chk2 activation is well understood at the biochemical level, we have identified alternative pathways of Chk2 activation that are important for radiation-induced apoptosis and may play a role in tumour suppression.

Following DSB recognition by MRN, the Chk2 kinase is rapidly phosphorylated in trans by the ATM kinase that promotes its homodimerization and trans autophosphorylation. This MRN-ATM dependent activation of Chk2 is believed to be the primary mechanism of Chk2 activation following radiation exposure. In previous studies we found unexpectedly that Chk2 dependent apoptosis was still active in cells lacking ATM or in Mre11 hypomorphic mutants that fail to activate ATM properly (Stracker *et al*, 2007; Stracker *et al*, 2008). We have found that damage induced Chk2 phosphorylation occurs rapidly after ionizing radiation treatment in the absence of ATM, implicating additional kinases in the apoptotic response. We are currently investigating the kinase(s) responsible for ATM-independent activation of Chk2 in order to better understand how apoptosis is initiated after radiation exposure. DNA-PKcs is a prime candidate for alternative activation of Chk2 in apoptosis as it has been shown to be required for apoptosis in ATM deficient cells and modifies Chk2 *in vitro*. Our current working model is presented in Figure 3.

Concluding remarks

Our laboratory is taking a wide array of approaches to better understand aspects of the cellular response to DNA damage that have been linked to cancer prevention. Available data implicates both the G2/M checkpoint and apoptosis as playing central roles in tumor suppression. Understanding the molecular events that take place during these processes and how they influence cell behavior will potentially identify new prognostic markers for cancer screening and identification. Additionally, both checkpoint arrest and apoptosis influence the efficacy and toxicity of many chemotherapeutic approaches. Thus the identification of novel factors and regulatory mechanisms that influence these processes may open new inroads to enhance the potency and safety of current cancer treatment regimens.

Scientific output

Publications

Stracker TH, Usui T and Petrini JH. Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. *DNA Repair*, **8**(9), 1047-54 (2009)

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Williams RS, Dodson GE, Limbo O, Yamada Y, Williams JS, Guenther G, Classen S, Glover JN, Iwasaki H, Russell P and Tainer JA. Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. *Cell*, **139**(1), 87-99 (2009)

Research networks and grants

The regulation of apoptosis and chromosome integrity by the cellular DNA damage response
Spanish Ministry of Science and Innovation (2009-2012)
Principal investigator: Travis Stracker

Collaborations

Breast cancer interaction network
Roger Gomis and Montse Soler, IRB Barcelona (Barcelona, Spain)

Awards and honours

Leukemia and Lympoma Society Special Fellowship
Leukemia and Lymphoma Society (2006-2009)
Awardee: Travis Stracker


Core Facilities

Julien Colombelli

Advanced Digital Microscopy Core Facility

In the past two decades the advent of fluorescent proteins has revolutionised the role of light microscopy in the biological and medical sciences. Digital imaging now goes beyond morphological studies by offering scientists ways to study the functions of proteins, cells and tissues. The applications of fluorescence illumination and observation constantly expand, and together with the most recent developments in laser technology, modern optical microscopes have tremendously diversified to address research of increasing complexity and specificity. State-of-the-art fluorescence microscopy is rapidly growing on several fronts: high resolution multidimensional live imaging, full volume and deep penetration imaging, high-content imaging, time-resolved protein dynamics, laser manipulation of living systems, image processing and analysis, and super resolution.

The Advanced Digital Microscopy Core Facility offers access and support to state-of-the-art instruments, from automated conventional and spectral confocal microscopy to emerging techniques for cell manipulation and imaging. Inaugurated in January 2009 in a newly constructed laboratory optimised for microscopy, the facility offers scientists open access to the instruments necessary to perform all the steps of digital imaging, from sample preparation through image acquisition up to image analysis and interpretation. The facility puts emphasis on offering customised and adapted solutions for optical imaging and image analysis by combining instruments, developing emerging technologies and tailoring custom image analysis to each scientific project.

Since its inauguration, the facility has set up an automatic web-based platform for open access to instruments and for the management of user access and interventions. In one year, it has registered nearly 200 users accross IRB Barcelona and the Barcelona Science Park, for a total usage of nearly 14,000 hours on six advanced systems and six routine microscopes. Confocal microscopy reached an average use of 8 to 15 hours per day, depending on the system.

Since January 2009, the facility has set up new technologies at IRB Barcelona and the Barcelona Science Park. To diversify and improve the various possibilities of multidimensional confocal microscopy, two new technologies were implemented: spinning disk confocal microscopy, which offers fast 3D live imaging of multiple labels in cells and organisms; and multiphoton microscopy, tailored to our most advanced confocal microscope, where an ultrashort pulsed laser now allows excitation of fluorophores deeper into organisms and tissues to increase depth of observation and also to decrease phototoxicity through thick samples.

High content imaging is now possible at IRB Barcelona with a new fully automated widefield microscope that records whole plate and full slide preparations, with highly stable fluorescence excitation for a reliable statistical analysis of multispectral fluorescence images. High contrast surface imaging is now also possible with TIRF microscopy, which offers imaging of the thinnest optical layer (<100nm) at glass interfaces, for cellular dynamics (membrane, cytoskeleton, ...) or single molecule dynamics.

Laser manipulation made a step forward with the implementation of a custom laser-based platform to perform combined laser nanosurgery and FRAP. The system performs cell ablation, subcellular compartments surgery, single filaments nanodissection, organ manipulation, etc... With a tailored spinning disk unit, the system offers cell and developmental biologists access to critical biophysical experiments to study multicellular and intracellular dynamics.

Imaging processing and analysis is emphasized at ADM where access is given to image analysis workstations on which commercial and custom software solutions are available to IRB Barcelona and PCB scientists. In October 2009, ADM hosted an image processing workshop organised by Bitplane, the European Imaris User Group Meeting 2009, which gathered 30 scientists at IRB Barcelona to learn and improve their skills in 3D image processing.

Services for IRB Barcelona researchers

Visitor lab

Our installations offer access to equipment for on site experiments to scientists and external visitors. Live samples can be stored in cell culture incubators, access to hoods, centrifuge, fridge and freezers are also given to allow sample preparation and manipulation pre- and post- acquisition.

Spectral confocal microscopy

We offer three spectral confocal microscope systems, two inverted and one upright, for 3D to 5D imaging (x,y,z,t and up to 5 spectral channels), *in vivo* imaging (*ie* FRET) and photobleaching experiments (*ie* FRAP). All systems are equipped with

405nm excitation for photoactivation, and environmental control of temperature and CO_2 to allow live imaging.

Spinning disk confocal
Our Andor spinning disk unit offers fast and sensitive imaging (up to 10 frames per second) with optical sectioning for *in vivo* live imaging with two laser lines (488nm, 561nm) and EMCCD technology.

Multiphoton microscopy
A tunable (710-990nm) ultrashort pulsed Ti:Sapphire is tailored to our SP5 confocal microscope to perform multiphoton imaging and imaging is performed with non-descanned external detectors. Among the applications available: Live and fixed imaging deep in embryos and tissues, Second Harmonic Generation.

Total internal reflection fluorescence microscopy is implemented on our automated widefield CellR microscope, to perform high contrast fluorescence imaging at glass surfaces with an axial extent around 100nm, with two laser lines (488nm, 561nm).

High content 'screening' and long-term live imaging
A fully automated inverted microscope, ScanR CellR from Olympus, offers highly stable fluorescence imaging for quantification of cellular assays on live samples, fixed preparations, multi-well plates, and multi-slides. An advanced incubation chamber precisely controls CO_2 and temperature for long-term imaging (from 1 hour to 1 week imaging).

Laser-based manipulation of living cells and organisms
Several lasers are coupled and controlled with x,y scanners to perform laser manipulation of fluorescent cells. A pulsed UV laser can be precisely scanned to perform laser ablation of cells and subcellular components, DNA damage, microdissection. The laser can also be used to perform correlative microscopy by inscribing the cell location inside the glass sample holder. A second laser can be scanned to perform photobleaching in wide field fluorescence mode.

Research Group Members

Core Facility Manager:
Julien Colombelli

Research Officers:
Lídia Bardia, Anna Lladó

The combination of laser nanosurgery and photobleaching gives access to unique cellular dynamics experiments where the distribution of fluorescent proteins can be manipulated and further analysed. For instance, DNA damage is performed within nuclei, and the dynamics of the recruited proteins can be quantified with subsequent FRAP. The system is also equipped with a spinning disk module for optical sectioning in thick samples.

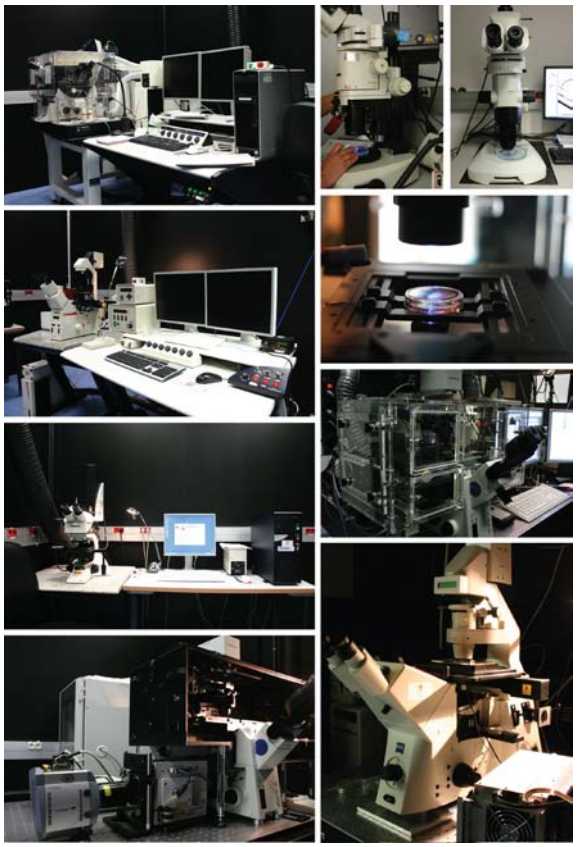


Figure 1. ***Overview of some advanced microscopy systems at ADM.*** *(From left to right and top to bottom): SP5 multiphoton spectral confocal, fluorescent and reflection stereoscope and macroscope, SP2 spectral confocal, live cell imaging, SPE confocal microscope, high-throughput automated widefield system with TIRF, spinning disk with EMCCD camera, laser nanosurgery and FRAP for live cells and organisms.*

Microdissector for fixed sample isolation

Implemented on an inverted microscope, a pulsed laser system from Olympus-MMI serves as a microscopic knife to dissect around subpopulations of cells within a flat sample preparation, *eg* a histology section. The isolated subpopulations can be transferred by laser expulsion into a recipient for further biochemical analysis (*ie*, PCR, etc...).

Fluorescence and reflection stereoscopy and macroscopy

A stereoscope and a macroscope offer low magnification and stereoscopic view from 0.6x to over 20x. Time sequences in fluorescence and in 3D can be performed, as well as automated reconstruction of in focus information through large volumes.

Conventional epifluorescence

Available on four microscopes, three upright and one inverted, widefield fluroescence microscopy is available with CCD cameras. Transmission and reflection microscopy contrast are also available with color camera to perform phase contrast, DIC (Differential Interference Contrast, or Normasky), color imaging of histological preparations.

Image processing

Two image processing workstations are freely available for data processing, visualization and interpretation, and are equipped with Imaris and other software packages. ADM also invests efforts in developing custom software applications for post acquisition image analysis, adapted to the needs of each users, typically giving the possibility to overcome limitations of comercial packages and focus on specific quantification tasks or automation of computing routines to process large datasets.

Scientific output

Publications

Solon J, Kaya-Copur A, Colombelli J and Brunner D. Pulsed forces timed by a ratchet-like mechanism drive directed tissue movement during dorsal closure. *Cell*, **137**(7), 1331-42 (2009)

Other references

Colombelli J, Besser A, Kress H, Reynaud EG, Girard P, Caussinus E, Haselmann U, Small JV, Schwarz US and Stelzer EHK. Mechanosensing in actin stress fibers revealed by a close correlation between force and protein localization. *J Cell Sci*, **122**, 1665-79 (2009)

Timinszky G, Till S, Hassa PO, Hothorn M, Kustacher G, Nijmeijer B, Colombelli J, Altmeyer M, Stelzer EHK, Scheffzek K, Hottiger MO and Ladurner AG. A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. *Nat Struct Mol Biol*, **16**, 923-29 (2009)

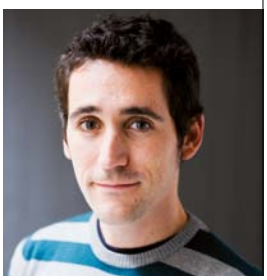
Collaborations

Advanced manufacturing technologies for microscopy
CIM Foundation-UPC (Barcelona, Spain)

Cellular mechanics during Drosophila *morphogenesis*
Jerome Solon, Centre for Genomic Regulation (Barcelona, Spain)

Microscopy development
European Molecular Laboratory & EMBLEM GmbH (Heidelberg, Germany); Olympus Soft Imaging Solution (Munich, Germany); Izasa SA (Barcelona, Spain)

David Rossell



Biostatistics/Bioinformatics Unit

Modern biomedical research has fostered the development of novel technologies capable of generating vast amounts of data. High-throughput genomic or proteomic experiments, for instance, produce data from as few as hundreds up to millions of genes or proteins. Nowadays researchers face not only the challenge of obtaining relevant scientific data, but also of extracting valuable information from it. Statistics is the science that transforms data into information. Founded on probability theory, it provides a disciplined and scientifically sound framework to test hypotheses and to learn about the systems and processes that generate biomedical data. The experimental design theory also guides researchers to conduct experiments in such a way that the subsequent data analysis can provide the information they need, that is, that the experimental goals are met.

Our collaboration with IRB Barcelona researchers focuses on the design and analysis of high-throughput experiments: next-generation sequencing, microarrays, tiling arrays and mass spectrometry. Regarding next-generation sequencing, we have participated in ChIP-Seq studies to characterise genome-wide transcription factor binding sites and histone methylation, and in RNA-Seq studies to characterise and quantify alternative splicing patterns. With respect to microarrays and tiling arrays, we have worked not only with datasets produced in-house but also with an increasing number of publicly available datasets, assessing the extent to which findings obtained in animal systems can be extrapolated to human patients. Regarding mass spectrometry, we have helped to design and analyse iTRAQ and to label free quantification experiments to find differentially expressed proteins that can serve as biomarkers. In addition to these study-specific collaborations, we placed emphasis on developing tools and methodology that benefits the IRB Barcelona community at large. We have developed statistical methods to infer alternative splicing based on RNA-Seq studies (Figure 1), and a novel Bayesian framework which offers important advantages in a wide range of hypothesis-testing problems.

In terms of informatics, we have structured a number of public gene expression datasets so that their contents are easily accessible. Furthermore, we have developed statistical data analysis software to screen them for patterns.

We have developed computer programmes to reduce the time needed to perform routine tasks and to implement novel data analysis methodology. We have made all our software available and have developed interfaces to facilitate its use.

Other activities during 2009 include providing assistance with statistical methodology and organising workshops to train IRB Barcelona researchers in the use of several data analysis software packages.

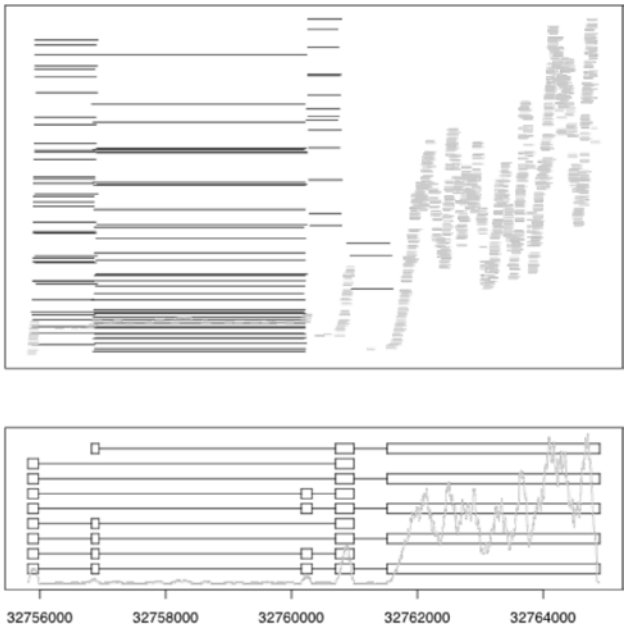


Figure 1. Inferring alternative splicing from paired end RNA-Seq data.

Services for IRB Barcelona researchers

Experimental design

Sample size, study design and planning of statistical methodology

Data analysis

Clinical or biomedical databases, and genomics data

Assistance with statistical methodology in own or others' research

Software
Help in using software, development of software to meet special data analysis or study design needs

Research Group Members

Unit Manager:
David Rossell

Research Officer:
Evarist Planet

Scientific output

Publications

Geslain R, Cubells L, Bori-Sanz T, Alvarez-Medina R, Rossell D, Martí E and de Pouplana LR. Chimeric tRNAs as tools to induce proteome damage and identify components of stress responses. *Nucleic Acids Res*, Epub Dec 8 (2009)

Rossell D. GaGa: a parsimonious and flexible model for differential expression analysis. *Ann Appl Statist*, **3**(3), 1035-51 (2009)

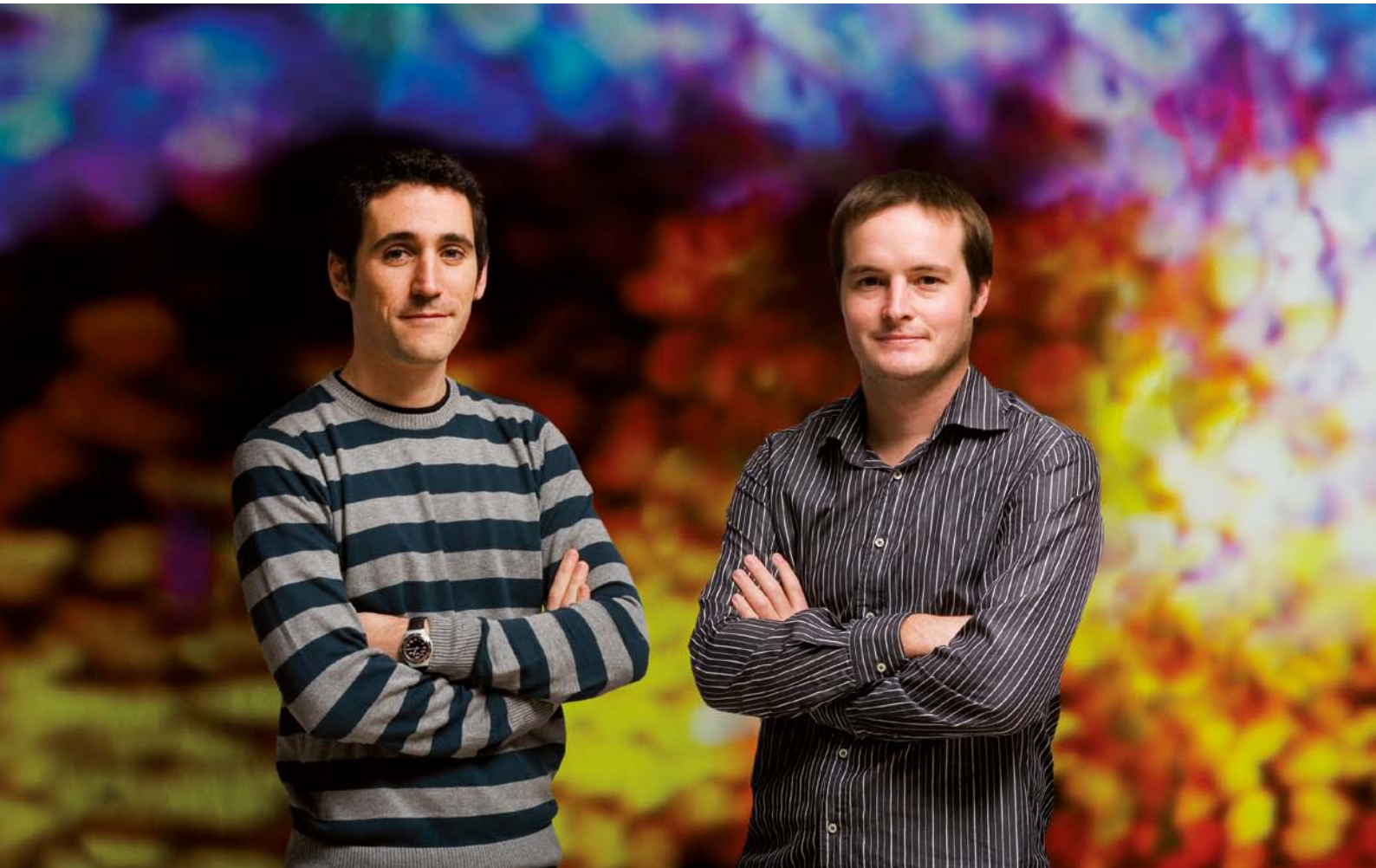
Collaborations

Evaluation of CSF immunodepletion and fractionation strategies for MS-based biomarker discovery
Jacques Borg and Joan Guinovart, IRB Barcelona (Barcelona, Spain)

Non-local priors for high-dimensional variable selection
Valen E Johnson, MD Anderson Cancer Center (Houston, USA); Donatello Telesca, University of California (Los Angeles, USA)

Prior densities for default Bayesian hypothesis tests
Valen E Johnson, MD Anderson Cancer Center (Houston, USA)

Sequential sample sizes for high-throughput experiments
Peter Müller, MD Anderson Cancer Center (Houston, USA)



Herbert Auer

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 need an increasing number of tools to either interrogate or alter genes at a genome-wide scale.

The Functional Genomics Core Facility provides state-of-the-art genomic tools for researchers at IRB Barcelona and other organisations. These tools fall into two categories:

i) Genome-wide analysis of transcription, DNA polymorphisms, and chromatin immunoprecipitation (ChIP). These analyses are performed using microarrays and Next Generation Sequencing. For all these analysis methods, the facility provides a complete service, including initial consultation during the design of the project, quality control of starting material, sample and array processing, data analysis in collaboration with statisticians, and data interpretation and validation by real-time PCR.

ii) Alteration of gene expression. For knock-down of gene expression, the facility provides genome-wide human and mouse shRNA libraries (Sigma), each containing approximately 100,000 clones, covering the majority of all known transcripts. For over-expression, the facility provides a human open-reading-frame library (Open Biosystems) containing 15,000 clones, covering three quarters of all human genes.

During 2009, the facility performed projects with over 20 research groups from all five programmes at IRB Barcelona and from other institutions throughout Barcelona, Catalonia and Spain.

Using products provided by Affymetrix, the facility performs genome-wide expression analysis at gene and exon level, as well as comparative genome hybridisation analysis (CGH analysis). These technologies are provided for over 20 organisms, including all standard model organisms and humans. For CGH analysis, resolution is further increased by using tiling arrays. These provide probes tiled across the entire genome without prediction

Figure 1. ***SNP density analysis in*** **Drosophila** ***strains.*** *A chromosomal region of 1.2 million nucleotides was analysed in four DNA samples for their density of single nucleotide polymorphisms relative to the reference genome. Two regions of high SNP density are highlighted. SNP analysis was performed using Next Generation Sequencing.*

of genes; therefore, this type of array provides the most comprehensive picture of genomic alterations currently available in microarray technology.

Services based on NimbleGen microarray products are also offered. NimbleGen technology provides longer probes than Affymetrix and therefore higher specificity. In addition, NimbleGen microarray production is extremely flexible and consequently facilitates the design of customized microarrays, even for small projects. This technology is currently used for expression analysis and CGH analysis.

Next Generation Sequencing on Illumina's Genome Analyzer II is offered for the qualitative and quantitative analysis of nucleic acids. Services include ChIP-Seq, miRNA-Seq, mRNA-Seq and genomic sequencing.

The tools for altering gene expression, namely the shRNA libraries and the open-reading-frame library, contain over 200,000 clones. These clones are centrally stored and a database has been developed for their administration. It also provides information about knock-down efficiency and the accuracy of clone annotation.

Services for IRB Barcelona researchers

DNA/RNA quantification and quality control

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

Microarray services

- Expression profiling on gene arrays and 3' biased arrays containing one probe set per gene, and on Exon arrays, tiling arrays and miRNA arrays.
- DNA polymorphism analysis for copy number variation (CNV) and single nucleotide polymorphisms (SNPs).

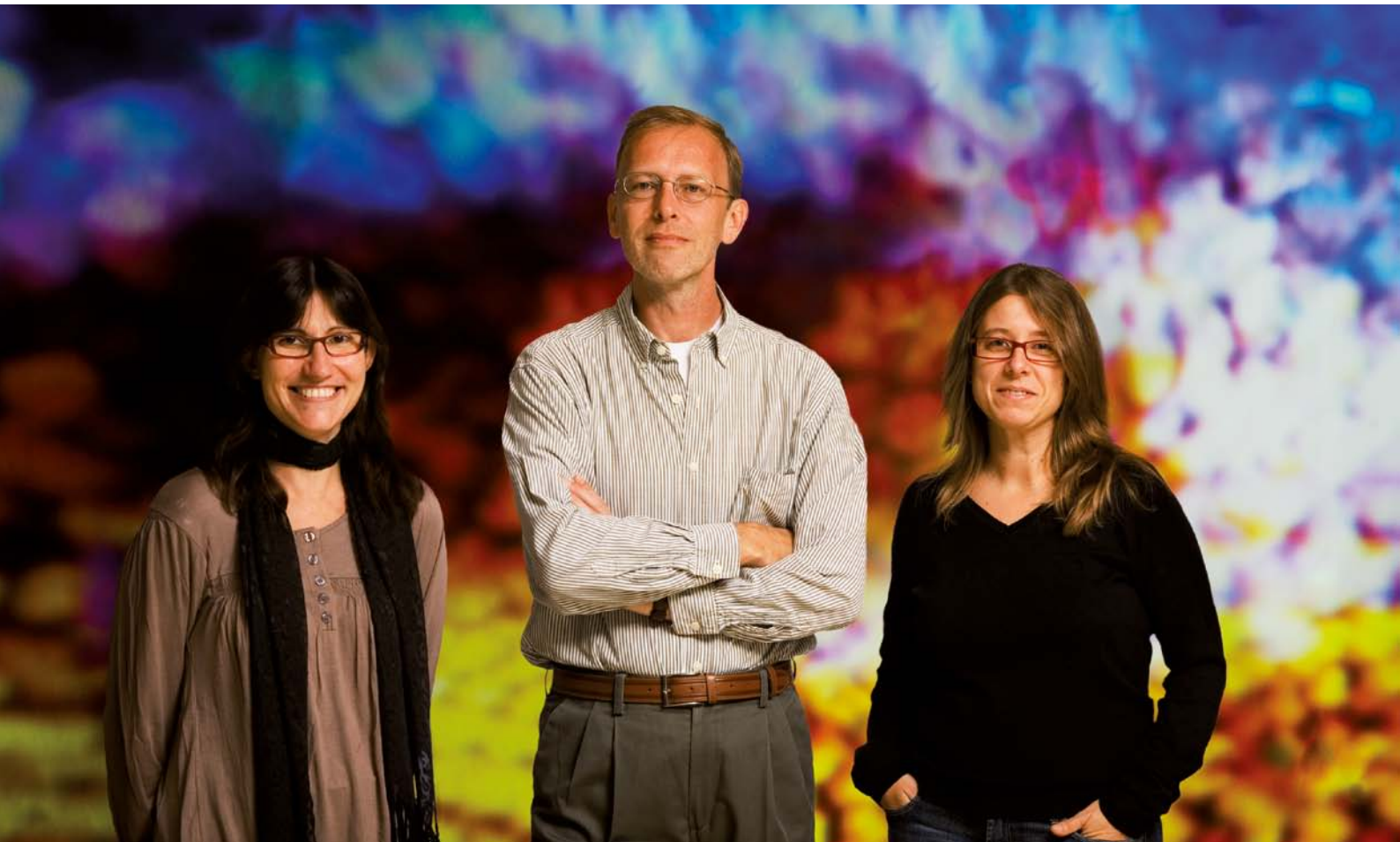
Next generation sequencing service

- ChIP-Seq for chromatin immune precipitation and input material is usually performed by single-end reads of 40 nucleotides per molecule.

Research Group Members

Core Facility Manager:
Herbert Auer

Senior Research Officers:
Eva González, Silvia Rodríguez



- mRNA-Seq for the discovery of unknown transcripts and splice variants is usually performed by paired-end reads of 40 or more nucleotides per molecule.
- miRNA-Seq for the quantification of known miRNAs and the discovery of unknown small RNAs are performed by single-end reads of 40 or fewer nucleotides.
- Genomic DNA sequencing is performed by single-end or paired-end sequencing, dependent on the underlying scientific question. Read lengths of up to 100 nucleotides are possible.

Validation of results by real-time PCR

For real-time PCR validation of microarray data, assays are designed, preformed and data are analysed for differential expression.

Alteration of gene expression

Bacterial clones are provided for the knock-down of almost all well characterised human and mouse transcripts. Multiple clones targeting the same transcript are available to assess off-target effects. For over-expression, one open-reading-frame clone is available per human gene. Clones are centrally administrated at the facility and are provided as bacterial stocks to IRB Barcelona researchers. The clone database provides information about knock-down efficiency and the accuracy of clone annotation.

Scientific output

Publications

Auer H. Expression divergence and copy number variation in the human genome. *Cytogenet Genome Res,* Epub Mar 11 (2009)

References

Auer H, Newsom DL and Kornacker K. Expression profiling using Affymetrix GeneChip microarrays. *Methods Mol Biol*, **509**, 35-46 (2009)

Cuscó I, Medrano A, Gener B, Vilardell M, Gallastegui F, Villa O, González E, Rodríguez-Santiago B, Vilella E, Del Campo M and Pérez-Jurado LA. Autism-specific copy number variants further implicate the phosphatidylinositol signaling pathway and the glutamatergic synapse in the etiology of the disorder. *Hum Mol Genet*, **18**(10), 1795-804 (2009)

Marta Vilaseca

Mass Spectrometry Core Facility

The high demand to discover new drugs and to identify new therapeutic agents has led researchers to focus on cellular proteins. This has been greatly facilitated by significant advances of MS in the field of proteomics. MS now forms an integral part of proteomics and drug discovery processes and can also provide relevant information about structural biology. The Mass Spectrometry Core Facility provides the research community at IRB Barcelona with modern chromatographic and mass spectrometric tools for the identification and characterisation of proteins and other biological species.

The facility implements intact protein analysis (Top-down approach), which aims to provide the complete characterisation of proteins. In this approach, protein ions are introduced into the gas phase and subsequently fragmented in the mass spectrometer, thereby yielding the molecular mass of both the protein and the fragment ions. Top-down is successful for the analysis of targeted proteins of less than 100 kDa; however, no platform is available for extending this approach to whole proteome analysis and the approach still requires improvements for high quality fractionation.

Figure 1. Advion Triversa Nanomate coupled to the LTQ-FT mass spectrometer. *Instrument configuration implemented for Top-down protein analysis.*

The facility also works with the classical proteomic approach (Bottom-up), which consists of the MS analysis of peptides resulting from previous digestion of proteins with an enzyme for their identification, the determination of post-translational modifications (PTMs), and quantitation. Moreover, the novel ion mobility-MS coupling methodology is being used to study the macromolecular structure and conformation of proteins and nucleic acids. Along the same line, non-covalent protein-protein and protein-ligand interactions can be directly detected and studied, thereby providing clues as to the mechanisms of action of these proteins in biological processes.

Set up in September 2007, the Mass Spectrometry Core Facility provides service to 17 research groups from the five IRB Barcelona programmes and has established collaborations within the Institute and with external institutions. For the complete characterisation of intact proteins, in 2009 the facility has implemented the dynamic Top-down methodology. By Top-down, proteins are introduced into the gas phase and fragmented inside of the mass spectrometer by several techniques. In doing this, the goals are to have the intact protein mass, plus a sufficient number of informative fragment ions that can provide a complete description of the primary structure of the protein and reveal all its modifications, as well as any correlations between these modifications. To undertake this approach, we use a chromatographic device coupled to an Advion Triversa Nanomate, which in turn is coupled to an LTQ-FT mass spectrometer (Figure 1). NanoESI is performed by chip technology and full MS data is acquired on the LTQ-FT at a chromatographic scale. Liquid chromatography (LC) fractions are simultaneously collected and reanalysed off-line by static Top-down, performing the fragmentation of selected intact ions by CID (collision-induced dissociation), ECD (electron capture dissociation) or IRMPD (infrared multiphoton dissociation). ProSight software was purchased in May 2009 to analyse Top-down data. We are applying this approach to examine moderately sized proteins in non-pure mixtures. In this regard, Histone 1 in *Droshophila* is being studied in order to provide an accurate map of its PTMs, thereby contributing to the understanding of its functions. Moreover, proteins up to 80 kDa are being analysed by Top-down or Middle-down

(cleaved with CNBr) techniques to determine their binding sites to covalent inhibitors or to determine catalytic mechanisms in the case of enzymes.

In 2009 we have been using the technology implemented last year for the detection of intact non-covalent complexes in our Synapt mass spectrometer. ß-amyloid protein oligomers (Figure 2), DNA complexes and protein bound to organometalic compounds, among others, have been analysed. These studies have been complemented with data from ion mobility (IM) experiments, performed in the same instrument. The capacity of the instrument to measure experimental cross sections implies that these experiments provide information about the structure of the macromolecule or the macromolecule-complex. The facility is working on the application of IM-MS methodology to study intact proteins in more complex samples, using a nanoLC device (purchased in April 2009) coupled to IM-MS. The purpose is to complement the Top-down analysis performed in the LTQ-FT in order to enhance the detection and structural characterisation of posttranslational modified proteins by using the potential offered by IM gas phase fractionation.

Throughout 2009, iTraq (Isobaric Tag for relative and absolute quantitation) and free-label quantitation methodologies have been used, in collaboration with the PCB Proteomics Platform, to search for biomarkers of amyotrophic lateral sclerosis (ALS) in cerebrospinal fluid originating from familiar and sporadic ALS patients. Statistical studies have been performed in conjunction with the IRB Barcelona Biostatistics/Bioinformatics Unit to identify significant changes in the level of expression of identified proteins between SALS, FALS and controls.

Services for IRB Barcelona researchers

The services offered include MS, MS/MS and MSn analysis using atmospheric pressure ionization techniques (electrospray and APcI) coupled to LC, nanoLC or infusion inlets. The facility also provides consultancy services and analytical method development for specific applications, as well as mass spectra data processing. Samples are analysed either directly by the service or by researchers (previously trained by facility members), who can use mass spectrometers through an open-access system.

Research Group Members

Acting Core Facility Manager:
Marta Vilaseca

Senior Research Officer:
Claudio Diema

Research Officer:
Nuria Omeñaca

Equipment and specialised applications

LTQ FT Ultra (Thermo Scientific)

Hybrid Mass Spectrometer consisting of a linear Ion Trap, combined with a Fourier Transform Ion Cyclotron Cell. It is also provided with ECD and IRMPD for complementary protein fragmentation in the mass spectrometer. This instrument is used to perform Top-down MS protein analysis and Bottom-up applications. In its normal configuration, this instrument works coupled to an LC device (Surveyor LC (Thermo) and a chip-based nanoESI interface (Advion Triversa Nanomate).

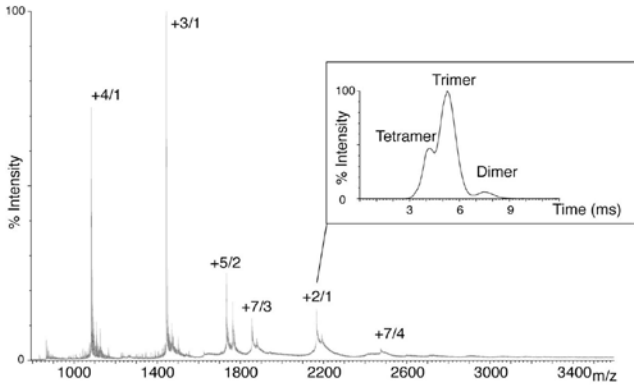


*Figure 2. **IM gas phase separation allows distinction between the dimer, trimer and tetramer of Aβ40.** Work performed in collaboration with researchers Rosa Pujol, Ernest Giralt and Natàlia Carulla.*

Synapt High Definition MS System (Waters-Micromass)

Hybrid QTOF instrument with an incorporated Triwave Cell. This instrument allows tandem MS to be combined with ion mobility, thus permitting the analysis of samples differentiated not only by their mass to charge ratio, but also by their shape and size. The instrument is used to analyse the macromolecular structure and conformation of intact proteins and to study non-covalent interactions. When working in its normal configuration, it is attached to a chip-based nanoESI interface (Advion Triversa Nanomate), thus combining the analysis of samples by infusion mode and by nanoLC coupling (NanoAcquity UPLC, Waters; purchased in 2009).

LCT-Premier XE (Waters-Micromass)

Orthogonal acceleration time-of-fight mass spectrometer ideal for the analysis of high molecular weight compounds. This instrument has been modified to achieve inert conditions inside the ionisation source, in order to allow amide H/D exchange experiments for the study of the dynamic and structural properties of proteins and their complexes. In another set-up configuration, it is used to analyse small molecules for their structural characterisation and provides accuracies of less than 3ppm. For LC-purifications, it is normally attached to an LC device (UPLC Acquity, Waters).

Scientific output

Collaborations

Amide H/D exchange determined by ESI. Method development to study molecular recycling in Aβ(1-42) amyloid fibrils
Natàlia Carulla, IRB Barcelona (Barcelona, Spain)

Evaluation of CSF immunodepletion and fractionation strategies for MS-based biomarker discovery
Jacques Borg, Jean Monet University (Saint-Étienne, France); Àlex Campos, Eliandre de Oliveira, PCB Proteomic Platform (Barcelona, Spain); Joan Guinovart, IRB Barcelona (Barcelona, Spain) and David Rossell, IRB Barcelona (Barcelona, Spain)

Evaluation of Top-down and Middle-down MS strategies for the location of specific inhibitors binding sites to prolyloligopeptidase
Ernest Giralt and Teresa Tarragó, IRB Barcelona (Barcelona, Spain); Michaela Scigelova and Vlad Zabouskov, Thermo Fisher Scientific (Bremen, Germany)

Experimental cross section determination by Ion Mobility Mass Spectrometry of DNA non-covalent complexes
Ramon Eritja and Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Study of the catalytic mechanism of glycosyltransferases: method development for the moniotorization of the transference of glycosidic groups by LC-MS
Joan Carles Ferrer, University of Barcelona (Barcelona, Spain)

Top-down analysis of glucose 6-phospate dehydrogenase: MS and MS/MS approaches to study its modification with methylglyoxal
Javier Luque, University of Barcelona (Barcelona, Spain); Eduardo Silva, Pontificia Universidad Católica de Chile (Santiago de Chile, Chile)

Top-down MS development for the analysis of PTMs in Drosophila melanogaster *Histone 1*
Ferran Azorín and Carles Bonet, IRB Barcelona (Barcelona, Spain)

Mouse Mutant Core Facility

Genetically modified (GM) mice play a vital role in both fundamental and applied biomedical research. Examples of their use include the modelling of disease processes, the study of individual genes, and the testing of novel drugs and treatments on disease models.
A wide range of modifications can now be made to the mouse genome, including the introduction of simple expression cassettes, targeted deletions and insertions, conditional sequences, point mutations and other more complex 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 purpose of the Mouse Mutant Core Facility is to generate genetically modified mouse models for use in the study of development and disease. The facility has been in existence since January 2007 and is staffed by scientists with extensive experience in cell culture, embryo manipulation and molecular biology.

The facility has two separate laboratories. Our main lab is a 60-sq m space containing a small self-contained tissue culture lab and a fully equipped lab for carrying out molecular biology work. In addition, we have a dedicated microinjection laboratory within the Animal Research Center (SEA) that houses two independent microinjection stations, as well as other equipment required for embryo manipulation and mouse surgery.

We have generated several lines of GM mice during the course of 2009, both transgenic and gene-targeted. Recently, various improvements and modifications have been made to the established protocols for the generation of GM mice. We have been assessing some of these modifications in an attempt to improve efficiencies.

We continue to develop and adapt existing molecular biology technologies used in both the generation of transgenic and gene-targeting vectors, and in screening for the resultant genetic modifications. The main aim of this is to assist research groups in the production of recombinant DNA molecules required for the production of GM mice. Much work has been carried out in the last 12 months to improve and simplify cloning protocols, particularly recombineering protocols.

Services for IRB Barcelona researchers

Experimental design

The facility provides consultation on all aspects of the design of gene-targeting and transgenic DNA vectors. These designs usually begin with an examination of the gene structure, derived from data generated by the relevant research group, or from databases (such as Ensemble or Vega) or a combination of both. We then propose an appropriate strategy and a suitable cloning protocol.

We can also give advice on screening strategies for transgenic and gene targeted mice and cells.

Vector construction

DNA vector design and construction is crucial for the success of the transgenic and gene-targeting process. We have a full-time molecular biologist who provides support for IRB Barcelona researchers. This member of staff designs targeting vectors and cloning plans, designs screening strategies, and develops and maintains a set of molecular and cellular tools and protocols. All of this work is designed to make the creation of recombinant DNA molecules and the screening for mutations both easier and faster.

Generation of gene-targeted and transgenic mice

In recent years, publically funded initiatives aimed at creating ready-made ES cell mutants, such as the European Conditional Mouse Mutagenesis Programme (EUCOMM) and the International Gene Trap Consortium (IGTC), have generated mutations in thousands of genes. We use these resources wherever possible, and in the last year we have been involved in several gene-targeting projects using ready-made ES clones or ready-made gene-targeting vectors.

However, many types of genes and genetic modifications are not covered by the aforementioned consortiums, thus these types of projects require the design and building of gene/mutation-specific vectors in collaboration with the research groups.

Mouse embryonic stem cell culture

The facility has a dedicated tissue culture laboratory devoted to the culture and manipulation of mouse ES cells and mouse embryonic fibroblasts. We offer a complete gene-targeting serv-

ice, from the transfection of ES cells with gene-targeting vectors, drug selection of transfected cells, picking, and expansion of drug resistant clones, to the archiving of duplicate clones. After correctly targeted cell clones have been identified, potential positives are expanded and further analysed before being microinjected into pre-implantation stage mouse embryos.

Microinjection

The facility has two dedicated microinjection stations equipped with state- of-the-art micromanipulators. Two specialist technicians carry out microinjection and associated microsurgery techniques, in addition to overseeing breeding strategies for lines generated or maintained by the facility.

During 2009, we have assessed some recent developments in the protocols used for the generation of GM mice. These include the use of improved media for embryo culture, microinjection techniques into early stage embryos, and the transfer of embryos into foster mice using non-surgical methods.

Scientific output

Publications

Samuel MS, Munro J, Bryson S, Forrow S, Stevenson D and Olson MF. Tissue selective expression of conditionally-regulated ROCK by gene targeting to a defined locus. *Genesis*, **47**(7), 440-46 (2009)

Nick Berrow

Protein Expression Core Facility

*The Protein Expression Core Facility was founded to deliver 'High Through-Put' (HTP) cloning and expression screening activities in which many variations of an experiment (*eg *cloning and expression screening of truncations or mutants of a protein) can be performed in parallel. In addition, the facility has the expertise and equipment necessary to produce and purify milligram amounts of protein from prokaryotic and eukaryotic expression hosts (currently* E. coli*, Sf9 insect and HEK293T mammalian cells). Many of the protocols are automated, with the facility making full use of liquid handling robotics for HTP plate handling for small-scale (µg) expression screening and automated purification systems (Äkta Xpress) for larger (mg) scale protein purification. The facility also offers many high quality reagents for cloning and protein expression such as competent bacterio-phage-resistant* E. coli *strains, specialised expression media, and recombinant enzymes. It also offers custom cloning and vector modification services.*

Since spring 2008, the facility has cloned almost 400 genes into expression constructs, most of which have been expression screened in at least one of our expression hosts. These expression constructs are also available for use either by the original investigator or for larger scale protein production and purification within the facility (in either *E. coli* or HEK293T cells). These include proteins from many different research projects with very different requirements; for example researchers may need seleno-methionine-labelled proteins for use in crystallisation and structure solution studies or secreted glycosylated proteins for use as tissue culture reagents.

The facility has already delivered purified proteins to IRB Barcelona researchers and this number is rapidly increasing. In addition, it has completed many smaller scale cloning projects to assist both IRB Barcelona researchers and others from the local academic community. During 2009 the baculoviral expression system was incorporated into the facility's services and we are in the process of optimising large-scale expression cultures of baculoviral-infected Sf9 cells, either in shaken flasks or in sterile 'wave' bags using an Appliflex Bioreactor.

The pPEU suite of In-Fusion-ready, multi-host, expression vectors was also developed during 2009, adding eleven new expression vectors to our vector list. Eight of these new vectors allow the production of fusion proteins with either enhanced Green Fluorescent Protein (eGFP) or Cherry fluorescent protein tags combined with either Hexa-Histidine or Strep-II tags. These vectors can be used in protein localisation studies (in conjunction with the Advanced Digital Microscopy Core Facility) or for the expression and purification of membrane proteins. The new pPEU1, pPEU10 and pPEU11 vectors can be used to produce GST C-His, N-His-thioredoxin or N-His Z-tag fusion proteins, respectively. All pPEU vectors have been produced in such a way that they are compatible with the pOPIN expression vector suite (University of Oxford, UK).

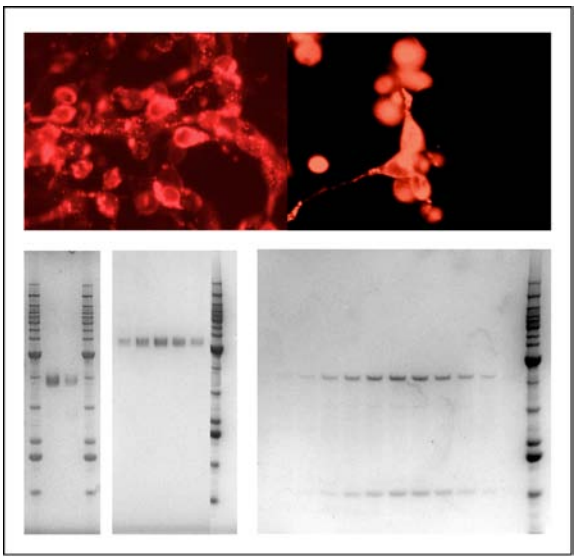


Figure 1. (Top panels) Using the pPEU vectors to study protein expression and localisation in HEK293T cells, two proteins fused to Cherry fluorescent protein are visualised by fluorescence microscopy. (Lower left panels) Purification of secreted and glycosylated proteins from HEK293T cells, SDS-PAGE gels of final products. (Lower right panel) Purifying protein dimers expressed in E. coli *from bi-cistronic vectors. SDS-PAGE of purified products. Note that only the larger protein is his-tagged.*

Services for IRB Barcelona researchers

Custom HTP Cloning to generate expression vectors

The In-Fusion™ ligation and restriction enzyme-independent cloning technique allows the precise production of user-defined constructs, including the production of mutant, chimaeric, and bi-cistronic (*E. coli* only) constructs. There are currently 18 pOPIN or pPEU vectors available.

***Expression screening in* E. coli**

A microtitre plate of 96 (facility- or user-derived) expression clones can be screened in *E. coli* in approximately one week. The screen currently consists of the use of two expression strains, with expression in each strain being tested using both IPTG and auto-induction methods. Additional (DE3) *E. coli* strains can be incorporated into the screening process if required.

HTP plasmid mini-preparation-96 mini-preps from* E. coli *pellets in less than two hours

Custom protein expression and purification at the milligram scale (depending on the particular protein being studied)

Purity in excess of 95% is anticipated for most proteins. The hosts currently available for large-scale expression cultures are *E. coli* and HEK293T cells; we are currently introducing large-scale insect cell culture to our list of services.

Production of seleno-methionine-labelled proteins for crystallographic structure determination in auxotrophic or prototrophic* E. coli *strains

Expression screening in mammalian (eg HEK293T) cells

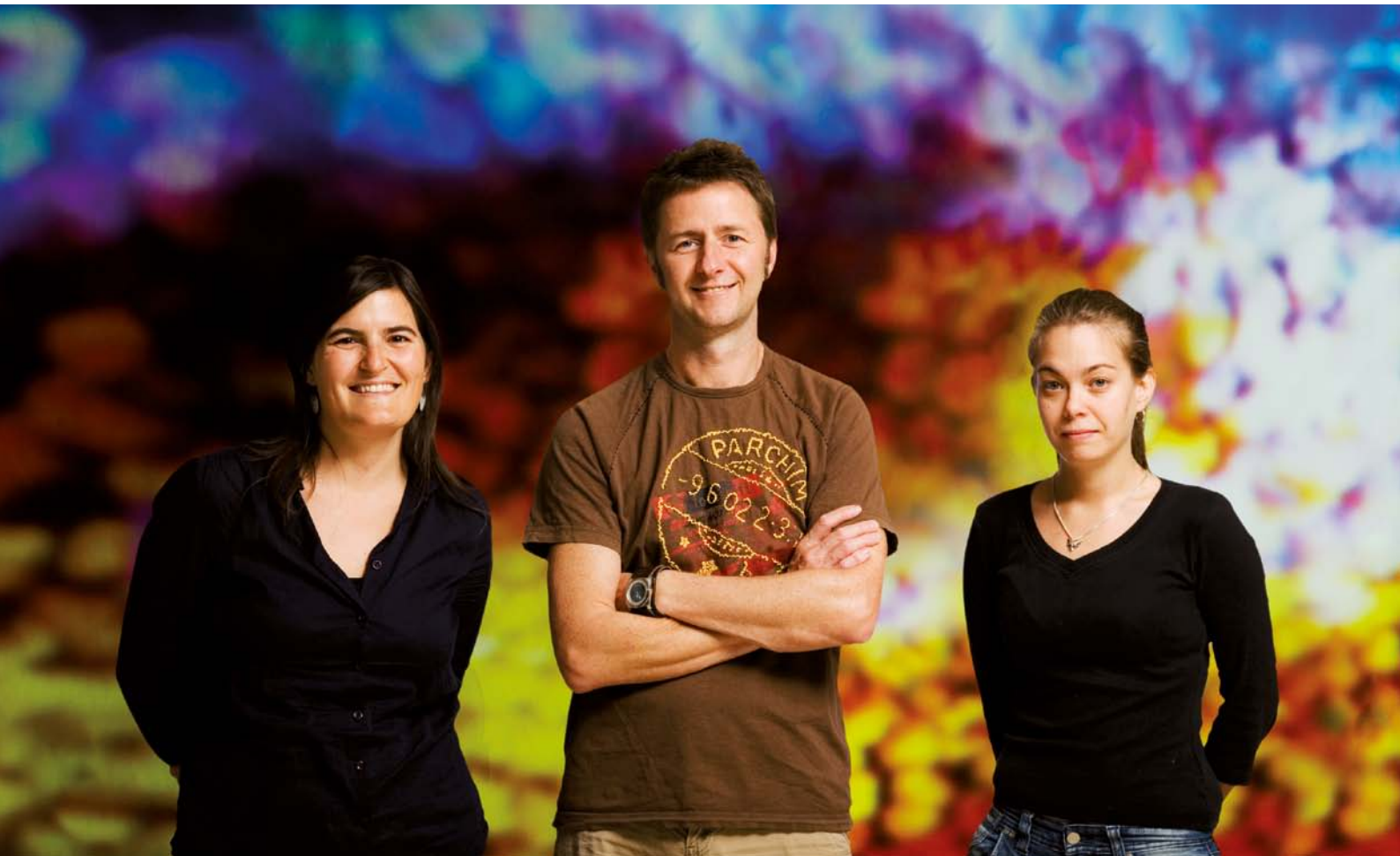
A microtitre plate of 96 (facility- or user-derived) expression clones can be screened in cells in 1-2 weeks.

Research Group Members

Core Facility Manager:
Nick Berrow

Senior Research Officer:
Raquel García

Technical Officer:
Mª Carmen Romero



Production of recombinant baculo-viruses either via the pOPIN or pPEU vector suites or from existing (*eg* pFastBac) constructs

Recombinant his-tagged 3C(PreScission) and SUMO proteases are available for the removal of fusion 'partners' from expressed proteins. We also hope to make the recombinant glycosidases PNGase and EndoF1 available for the removal of sugar moieties from recombinant proteins prior to crystallisation.

The facility also sources many high quality reagents, ranging from specialised *E. coli*-competent cell strains and reagents for protein expression to labelling and cloning reagents for use by individual researchers. Purchasing reagents through the facility generally produces considerable cost savings for researchers.

Scientific output

Collaborations

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

We collaborate with many different research groups to develop improved methods for the production, labelling and detection of proteins, and also improved cloning methods and expression vectors.

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