MOLECULAR ONCOLOGY PROGRAMME

ÓSCAR FERNÁNDEZ-CAPETILLO Programme Director

Research at the Molecular Oncology Programme (MOP) aims to discover the genetic determinants that contribute to cancer onset and progression, as well as to provide new ideas and tools for the development of innovative therapies for cancer patients. To do so, we have Groups covering a wide range of topics in cancer research such as DNA and chromosome stability (Maria A. Blasco, Óscar Fernández-Capetillo, Felipe Cortés-Ledesma and Ana Losada), oncogenes and cell cycle kinases (Mariano Barbacid), DNA replication (Juan Méndez), mitosis (Marcos Malumbres), melanoma (María S. Soengas), epithelial carcinogenesis (Francisco X. Real), metabolism and cell signalling (Nabil Djouder and Alejo Efeyan), immunotherapy (María Casanova), and metastasis (Manuel Valiente, Eva González-Suárez and Héctor Peinado).

“During 2022, MOP scientists kept making significant discoveries that help us to understand the molecular bases of cancer and other age-related diseases, and provided new ideas for their treatment.”

During 2022, our scientists reported relevant contributions in many areas, and here I provide a few selected examples of their work. For instance, the Junior Group led by Manuel Valiente discovered biomarkers of resistance to radiotherapy in brain metastasis and developed an innovative platform to test new therapies for this disease *ex vivo*. Such a tool should facilitate the discovery of new treatments for brain tumours and has raised significant attention both by the scientific community and the pharmaceutical industry dedicated to oncology. On a related theme, the Group led by Nabil Djouder identified the cellular interactions that modulate the regeneration of the intestinal epithelia following radiation. In addition, Nabil´s team generated a novel mouse model of liver cirrhosis, which is a nice new tool for preclinical research on this lethal disease. Francisco Real´s team provided further insights into the role of GATA transcription factors that contribute to the changes in cell fate associated with pancreatic cancer, and made additional discoveries that help us to understand the still mysterious role of SA2 mutations in cancer onset. The Group led by Marcos Malumbres further developed our understanding about how mitotic kinases control asymmetric cell division in neural stem cells, which is at the basis of certain hereditary microcephaly disorders. Mariano Barbacid´s Group keeps making progress towards the development of inhibitors of the RAF1 kinase, which they previously identified as an actionable target in pancreatic cancer and, during 2022, they reported the atomic structure of RAF1. The Group led by Maria A. Blasco identified the relevant cell type that contributes to lung fibrosis, which is an important step towards developing targeted therapies against this age-related disease. Finally, in my own Group, we identified a novel mechanism that leads to multi-drug resistance in cancer cells, and some initial strategies for how this could be overcome. These examples provide a necessarily incomplete collage of our activities, yet they help to certify that our scientists keep an excellent level of scientific productivity.

I must end by saying that publications are just one of the outcomes of our activities. Our Group Leaders are often a reference in their fields of research and participate in many activities that contribute to raising awareness of the relevance of cancer research. Of note, this is not only done by principal investigators, as our technicians, students, postdocs and staff scientists are also very active in this regard. In addition, I am happy to see that scientists at the MOP are progressively increasing their interactions with clinicians and pharmaceutical environments, with the goal of contributing with their knowledge and discoveries to the development of new therapies, or improving the efficacy of existing ones. Finally, I am also personally glad to observe a progressive trend among our scientists to make their discoveries open as soon as possible in public repositories. I myself am convinced that, while publishing our research in journals of high visibility helps us reach a wider audience, it is also important that our most exciting discoveries are shared with the broader scientific community without extensive delays. If we want a different future for how science is reported, we should contribute to it. ■

TELOMERES AND TELOMERASE GROUP – *FUNDACIÓN HUMANISMO Y CIENCIA*

Maria A. Blasco
Group Leader

Research Scientists
Isabel López de Silanes, Rosa M. Marión, Paula Martínez

Post-Doctoral Fellows
Giuseppe Bosso, Buyun Ma, Arpita Saha (since May), Sarita Saraswati

Gradutate Students
José Carlos González, Óscar Laguía, Jessica Louzame, Amparo Sánchez, Raúl Sánchez

Technicians
Ana Guío (until Oct.) (TS)*, David Hernández (since Nov.) (TS)*, Rosa M. Serrano

**Titulado Superior* (Advanced Degree)

Visiting Students
Sarah Adetchessi (May -July) (PhD Student, Univ. of Paris, France), Stavroula Boukoura (Oct.-Dec.) (PhD Student, Danish Cancer Society Research Center, Denmark), Robson Diego Calixto (Aug.-Dec.) (*FAPESP* Internship, Univ. of São Paulo, Brazil), Ana Carolina Cintra (since Nov.) (Erasmus Fellowship, Tras-os-Montes e Alto Douro Univ., Portugal), Mariana Deli (June-Dec.) (Erasmus + Fellowship, Univ. of Ioannina, Greece), Julie Klein (June-Aug.) (PhD Student, *École de Biologie Industrielle*, France), Neetij Krishnan (until June) (Fulbright Commission Fellowship, USA), Cristina Pastor (Sep.-Dec.) (Traineeship, *Univ. Autónoma de Madrid*, Spain)

OVERVIEW

Immortality is one of the most universal characteristics of cancer cells. We study the mechanisms by which tumour cells are immortal and normal cells are mortal. The enzyme telomerase is present in more than 95% of all types of human cancers and is absent in normal cells in the body. Telomeres are nucleoprotein complexes located at the ends of chromosomes, essential for chromosome protection and genomic stability. Progressive shortening of telomeres associated with organism ageing leads to ageing. When telomeres are altered, adult stem cells have a maimed regenerative capacity.

"Our potential preclinical mouse model *ki-Pot1a*^{R117C} for Li-Fraumeni-Like syndrome presenting with high angiosarcoma incidence could be a very useful tool in the therapeutics of these tumours."

Our research focuses on:

- → Generating mouse models to validate telomeres and telomerase as therapeutic targets for cancer and age-related diseases.
- → Interplay between telomeres and DNA repair pathways.
- → Role and regulation of non-coding telomeric RNAs or TERRA.
- → Testing telomerase gene therapy in *telomere syndromes* and age-related diseases.
- → Role of telomerase and telomeres in adult stem cell biology and in nuclear reprogramming of differentiated cells to iPS cells.

RESEARCH HIGHLIGHTS

BRAF^{V600E} in adult mouse models elicits early differential responses

The BRAF gene, which encodes a master kinase of the RAS-pathway, is frequently mutated in human cancers. The most common genetic mutation is a single nucleotide transition that gives rise to a constitutively active BRAF kinase (BRAF^{V600E}), which in turn sustains continuous cell proliferation. The study of BRAF^{V600E} murine models has so far focused mainly on the role played by BRAF^{V600E} in tumour development, so much so that little was known about the early molecular impact of the *in vivo* expression of BRAF^{V600E}. We have now provided the first *in vivo* evidence that acute BRAF^{V600E} expression elicits instant DNA damage in an organ-specific fashion. The senescence marker p21CIP1, which may be activated by p53 upon genotoxic insults and by oncogene activation via pRb/E2F, promotes cell cycle arrest and senescence by inhibiting CDKs. Despite BRAF^{V600E} inducing both DNA damage and p21CIP1 activation *in vitro*, as well as in senescent lung adenomas, we did not find any differences in p21CIP1 levels either in liver or spleen upon BRAF^{V600E} expression. BRAF^{V600E} in lungs provokes an acute inflammatory state with tissue-specific recruitment of neutrophils to alveolar parenchyma and of macrophages to bronchi/bronchioles, as well as bronchial/bronchiolar epithelium transdifferentiation and development of adenomas.

A mouse model for Li-Fraumeni-Like syndrome with cardiac angiosarcomas associated to POT1 mutations

Although the telomeric protein POT1 is mutated in many different familial and sporadic cancers, so far there have been no mouse models to understand the pathobiology of these mutations. We have generated a mouse model for the human *POT1*^{R117C} mutation found in Li-Fraumeni-Like (LFL) families with cases of cardiac angiosarcoma (CAS) by means of introducing this mutation in the *Pot1a* endogenous locus, *knock-in* for *Pot1a*^{R117C}, thus generating *Pot1a*^{ki} mice. While homozygous *Pot1a*$^{ki/ki}$ are embryonic lethal, heterozygous *Pot1a*$^{+/ki}$ mice are viable. We also found that both mouse embryonic fibroblasts (MEFs) and tissues from *Pot1a*$^{+/ki}$ mice harbour longer telomeres than wild-type controls. Like human LFL patients, heterozygous *Pot1a*$^{+/ki}$ mice spontaneously develop a high incidence of angiosarcomas (FIGURE 1), including CAS, and this is associated with the presence of abnormally long telomeres in endothelial cells as well as in the tumours. The *Pot1a*$^{+/R117C}$ mouse model therefore constitutes a useful tool to understand human cancers initiated by POT1 mutations.

Impact of telomere dysfunction in fibroblasts, Club and basal cells on the development of lung fibrosis

Telomeric protein TRF1 is an essential component of the telomeric protective complex that prevents telomeric DNA damage, chromosome end-to-end fusions and telomere fragility. We previously showed that induction of telomere dysfunction in alveolar type II (ATII) cells is sufficient to induce progressive and lethal pulmonary fibrosis in mice. The pathological consequences of telomere dysfunction in lung fibroblasts, Club and basal cells remained to be investigated. We have now conditionally deleted *Trf1* in the former mouse tissues. We found that while TRF1 deficiency in fibroblasts does not lead to significant lung pathologies, *Trf1* deletion in Club and basal cells from male mice led to lung inflammation and airway remodelling. While dysfunctional telomeres in ATII cells led to alveolar DNA damage, senescence and apoptosis, as well as to interstitial lung fibrosis, their presence in Club and basal cells increased the presence of neutrophils, lymphocytes and macrophages in the lung, as well as airway collagen deposition and fibroblast abundance, features not observed in female mice upon telomere dysfunction. Depletion of TRF1 in fibroblasts, Club and basal cells did not lead to interstitial fibrosis, underscoring ATII cells as the relevant cell type for the origin of interstitial fibrosis (FIGURE 2). ■

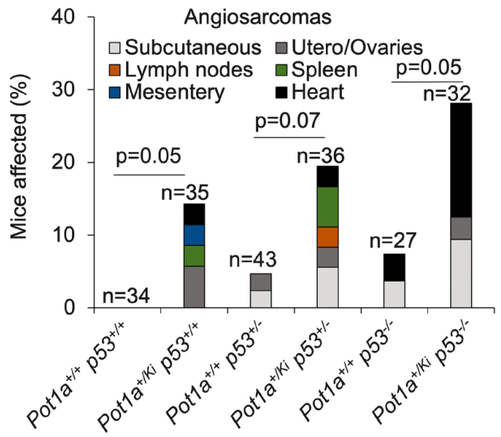

FIGURE 1 Higher incidence of angiosarcomas in *Pot1a*$^{+/ki}$ mice.

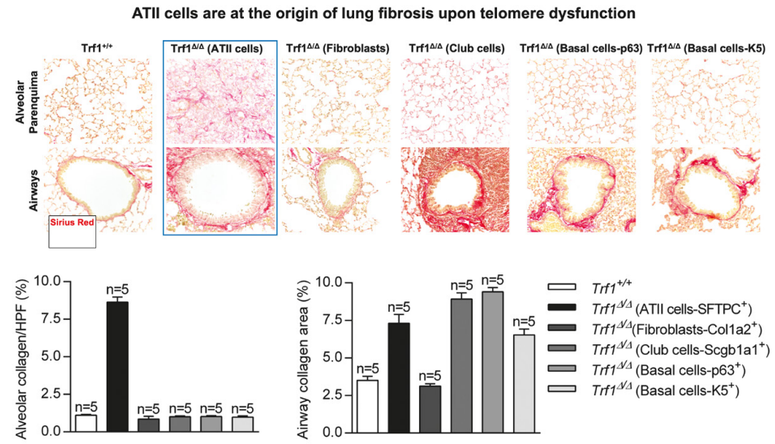

FIGURE 2 Pathological consequences of telomere dysfunction in fibroblasts, Club and basal cells in the lung. Dysfunctional telomeres in ATII cells led to alveolar DNA damage, senescence and apoptosis, and to interstitial lung fibrosis. TRF1 deficiency in Club and basal cells induced telomeric damage and cell cycle arrest, and reduced proliferation. TRF1 deletion in fibroblasts increased telomeric damage, cell cycle arrest, apoptosis, and proliferation. Depletion of TRF1 in fibroblasts, Club and basal cells did not lead to interstitial lung fibrosis.

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AWARDS AND RECOGNITION

- *Fundación Eugenio Rodriguez Pascual* 2022 Award, Madrid, Spain.
- *Averroes de Oro Ciudad de Córdoba* 2022 Award in Scientific Research, Córdoba, Spain.
- Full Member (*Académica de número*) of the Royal Spanish Academy of Pharmacy.
- Doctorate *Honoris Causa*, *Universidad Internacional de Valencia*, Valencia, Spain.
- Honorary Academician of *Academia Malagueña de Ciencias*, Málaga, Spain.
- Member of the Board of Trustees of the International Centre for Ageing Research (ICAR) Foundation, Valencia, Spain.
- President of the Scientific Advisory Board of the International Centre for Ageing Research (ICAR), Valencia, Spain.
- Member of the Advisory Board of the Spanish Foundation of Science and Technology (FECYT), Madrid, Spain.

EXPERIMENTAL ONCOLOGY GROUP

Mariano Barbacid
Group Leader

Research Scientists
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Post-Doctoral Fellows
Federico Virga (January-October)

Gradutate Students
Gonzalo M. Aizpurua, Sara Barrambana, Oksana Brehey, Laura de La Puente, Ana María Fernández, Fernando Fernández, Jing Li (until August), Vasiliki Liaki, Lucía Morales, Marina Salmón (until November), Pian Sun, Elena Zamorano (since December; CIBERONC, Madrid)

Technicians
Rebeca Barrero (since October), M. Carmen González (TS)*, Silvia Jiménez, Marta San Román, Raquel Villar

Titulado Superior (Advanced Degree)

Bioinformatician
Ruth Álvarez (until November)

Administrative Assistant
Patricia T. Guerra

Undergraduate Students
Lucía Baselga (since Oct.) (Undergraduate trainee, *Univ. Politécnica de Madrid*, Spain), Irene Herruzo (until June) (BS Thesis, *UAM,* Madrid, Spain), Mirto Kostopoulou (since Nov.) (Erasmus + Fellowship, National and Kapodistrian University of Athens, Greece), Lucía Lomba (since Oct.) (Master's Thesis, *UCM,* Madrid, Spain), Sonsoles Liria (Jan.-Apr.) (Master's Thesis, *Univ. Francisco de Vitoria*, Madrid, Spain), Laura Martínez (Mar.-June) (BS Thesis, *UCM,* Madrid, Spain), Melissa Minic (since Sept.) (Erasmus + Fellowship, *FH Campus Wien*, Austria), Blanca Rosas (since Sept.) (BS Thesis, *UCM,* Madrid, Spain)

External Associates
Alfredo Carrato (*Hosp. Ramón y Cajal*, Madrid, Spain), Matthias Drosten (*Centro de Investigación del Cáncer*, Salamanca, Spain), Monica Musteanu (*UCM,* Madrid, Spain), Bruno Sainz (*UAM,* Madrid, Spain), Juan Velasco (*Eli Lilly*, Alcobendas, Spain)

OVERVIEW

The main area of interest of our Group is to identify therapeutic strategies against KRAS mutant lung and pancreatic tumours. For almost 4 decades, KRAS oncoproteins were thought to be undruggable targets. However, selective KRAS inhibitors, at least against one of the KRAS oncogenic isoforms, $KRAS^{G12C}$, have been recently approved by the FDA. Yet patients develop drug resistance rather quickly indicating that successful treatment of KRAS mutant tumours will require combination with inhibitors of KRAS signalling pathways, such as the MAP kinase and the PI3 kinase pathways. Unfortunately, all inhibitors tested thus far in the clinic have failed due to excessive toxicities. A potential exception is RAF1. Ablation of this kinase induced significant levels of tumour regression with limited toxicities in experimental models. Ironically, the tumour-inducing effect of RAF1 is not mediated by its kinase activity. Hence, pharmacological targeting of RAF1 will require the use of strategies capable of degrading the protein. To identify such compounds, we have determined the tertiary structure of the full RAF1 protein using Cryo-Electron Microscopy (Cryo-EM) technologies. These results have identified structural vulnerabilities that will make it possible to design selective RAF1 degraders.

"The tertiary structure of RAF1, bound to the Hsp90 and Cdc37 chaperones, has revealed structural vulnerabilities that will make it possible to generate pharmacologically active RAF1 degraders capable of inhibiting KRAS mutant lung tumours."

RESEARCH HIGHLIGHTS

KSR induces RAS-independent MAPK pathway activation and modulates the efficacy of KRAS inhibitors

KSR1/2 have long been considered scaffolding proteins required for optimal MAPK pathway signalling. However, recent evidence suggests that they play a more complex role within this pathway. We have demonstrated that ectopic expression of KSR1 or KSR2 is sufficient to activate the MAPK pathway and to induce cell proliferation in the absence of RAS proteins. In contrast, ectopic expression of KSR proteins is not sufficient to induce cell proliferation in the absence of either RAF or MEK proteins, indicating that they act upstream of RAF. Indeed, KSR1 requires dimerization with at least 1 member of the RAF family to stimulate proliferation, an event that results in the translocation of the heterodimerized RAF protein to the cell membrane. Mutations in the conserved DFG motif of KSR1 that affect ATP binding impair induction of cell proliferation. We have also shown that increased expression levels of KSR1 decrease the responsiveness to the $KRAS^{G12C}$ inhibitor sotorasib in human cancer cell lines. These results suggest that high KSR1 or KSR2 expression levels in tumours could render strategies aimed at inhibiting RAS largely ineffective. Indeed, we further show that $KRAS^{G12C}$ inhibitors are less effective when KSR1 expression levels are elevated. In conclusion, our data should raise awareness that KSR1 or KSR2 expression levels are direct modulators of the effectiveness of RAS inhibition.

Structure of the RAF1 kinase bound to the HSP90 and CDC37 chaperones: identification of selective RAF1 degrons

We have described the structure of the full-length RAF1 protein in complex with HSP90 and CDC37 obtained by Cryo-Electron Microscopy (FIGURE 1A and B). The reconstruction reveals a RAF1 kinase with an unfolded N-lobe separated from its C-lobe. The hydrophobic core of the N-lobe is trapped in the HSP90 dimer, while CDC37 wraps around the chaperone and interacts with the N- and C-lobes of the kinase. The structure indicates how CDC37 can discriminate between the different members of the RAF family. Our structural analysis also reveals that the folded RAF1 assembles with 14-3-3 dimers, suggesting that after folding follows a similar activation as B-RAF. Finally, disruption of the interaction between CDC37 and the DFG segment of RAF1 unveils potential vulnerabilities to attempt the pharmacological degradation of RAF1 for therapeutic purposes (FIGURE 1C).

Despite the well-conserved sequence amongst members of the RAF family, they contain substantial functional differences. Whereas RAF1 and A-RAF are client proteins of the HSP90-CDC37 chaperone system, B-RAF is not. Therefore, the HSP90-CDC37 chaperone system adds an extra regulatory layer to this kinase family. The structure of the complex highlights the key interactions of the HSP90 chaperone and its cochaperone CDC37 with RAF1. Moreover, our combined biochemical and functional analysis of the interacting regions indicates that CDC37 can recognise segments of RAF1 that are different from their counterparts in B-RAF.

We propose a model in which RAF1 would be unstable until it becomes associated with CDC37, followed by binding to HSP90. The HSP90-CDC37 chaperone system couples the folding of the client protein with ATP hydrolysis cycles (FIGURE 1B). RAF1 is phosphorylated in residues S259 and S621, thereby, once the HSP90-CDC37 renders this protein folded, the complex is disrupted and RAF1 associates with 14-3-3 in a manner similar to B-RAF. We speculate that the interaction of RAF1 with the HSP90-CDC37 system could control the dynamics of RAF1 heterodimers formed with the 14-3-3 proteins, thus influencing the levels of homo or heterodimers of this signalling module, and thereby controlling cellular proliferation.

Our mutagenesis analysis of the interface between CDC37 and RAF1 highlights the importance of this association for RAF1 stability. Indeed, we observed a reduction in the levels of RAF1 when the mutant isoforms were co-expressed with HSP90 and CDC37 (FIGURE 1D and E). These observations raise the possibility that the interface between RAF1 and CDC37 may represent a vulnerable spot, which could be targeted to induce the degradation of RAF1, reproducing the therapeutic results obtained in experimental models of *Kras/Trp53*-induced lung tumours upon ablation of RAF1 expression. ■

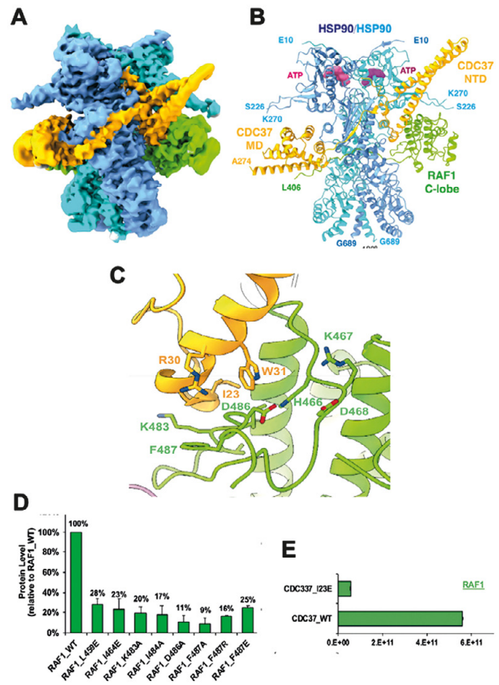

FIGURE 1 Cryo-EM structure of the RAF1-HSP90-CDC37 (RHC) complex. (**A**) Cryo-EM maps of the RAF1-HSP90-CDC37 complex at 3.67 Å global resolution. Blue and cyan for each monomer of the HSP90 dimer, yellow for CDC37 and green for RAF1. (**B**) Overview of the RAF1-HSP90-CDC37 assembly in complex with ATP. (**C**) Detailed view of the main interacting region between CDC37 and RAF1. (**D**) Levels of wild type and mutant RAF1 proteins co-expressed with HSP90 and CDC37. The levels of each protein were quantified by mass spectrometry, using RAF1 common peptides for all the mutants. (**E**) Mean levels of RAF1 bound to strep-tagged wild type and mutant CDC37 I23E proteins.

PUBLICATIONS

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- García-Alonso S, Mesa P, Ovejero LP, Aizpurua G, Lechuga CG, Zarzuela E, Santiveri CM, Sanclemente M, Muñoz J, Musteanu M, Campos-Olivas R, Martínez-Torrecuadrada J, Barbacid M*, Montoya G* (2022). Structure of the RAF1-HSP90-CDC37 complex reveals the basis of RAF1 regulation. *Mol Cell* 82, 3438-3452. *Co-senior authors.
- Paniagua G, Jacob HKC, Brehey O, García-Alonso S, Lechuga CG, Pons T, Musteanu M, Guerra C, Drosten M, Barbacid M (2022). KSR induces RAS-independent MAPK pathway activation and modulates the efficacy of KRAS inhibitors. *Mol Oncol* 16, 3066-3081.
- Drosten M, Barbacid M (2022). Targeting KRAS mutant lung cancer: light at the end of the tunnel. *Mol Oncol* 16, 1057-1071.
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- Diego-González L, Fernández-Carrera A, Igea A, Martínez-Pérez A, Real Oliveira MECD, Gomes AC, Guerra C, Barbacid M, González-Fernández Á, Simón-Vázquez R (2022). Combined inhibition of FOSL-1 and YAP using siRNA-lipoplexes reduces the growth of pancreatic tumors. *Cancers (Basel)* 14, 3102-3123.
- Casadevall D, Hernández-Prat A, García-Alonso S, Arpí-Llucià O, Menéndez S, Qin M, Guardia C, Morancho B, Sánchez-Martín FJ, Zazo S, Gavilán E, Sabbaghi MA, Eroles P, Cejalvo JM, Lluch A, Rojo F, Pandiella A, Rovira A, Albanell J (2022). mTOR inhibition and T-DM1 in HER2-positive breast cancer. *Mol Cancer Res* 20, 1108-1121.

Publications at other institutions

- París-Muñoz A, Aizpurua G, Barber DF (2022). Helios expression is downregulated on CD8+ Treg in two mouse models of lupus during disease progression (2022). *Front Immunol* 13, 922958.

AWARDS AND RECOGNITION

M. Barbacid:

- *"Santiago Ramón y Cajal"* National Research Prize 2022, Spain.
- Honorary Doctorate (*"Doctor Honoris Causa")* from the *Universidad Nacional de Educación a Distancia*, Madrid, Spain.
- *"Premio a la Excelencia en la Trayectoria Científica"* AstraZeneca Foundation Lifetime Achievement in Science Award, Spain.
- Member of the *Universidad Internacional Menéndez Pelayo´s* Advisory Board, Madrid, Spain.

CELL DIVISION AND CANCER GROUP

Marcos Malumbres
Group Leader

Clinical Investigator
Rodrigo Sánchez

Post-Doctoral Fellows
Begoña Hurtado, Miguel Ruiz, Diana Vara, Carolina Villarroya

Gradutate Students
Gloria Cristina Bonel, Mariona Cubells (PEJ, CAM)*, Alejandro García, José González, Fátima Guerra, Luis Rodrigo López, Enrique Nogueira (since October), Borja Pitarch, Agustín Sánchez

**Plan de Empleo Joven de la Comunidad de Madrid* (Youth Employment Plan, Community of Madrid)

Technicians
Cristina Aguirre (TS)**, Irene Díaz, Sandra Díez (since November), Aicha El Bakkali

***Titulado Superior* (Advanced Degree)

Students in Practice
Iciar Luna (until June) (*UCM*, Madrid, Spain), Sara Mozas (Oct.-Dec.) (*Univ. Francisco de Vitoria*, Madrid, Spain), María Fernanda Pérez (until June) (*UAM*, Madrid, Spain), Martina Svedin Figo (July-Sep.) (University of Manchester, UK)

Visiting Scientists
Mónica Álvarez (*Instituto de Investigación Sanitaria del Principado de Asturias*, Ovideo, Spain), Senn Wakahashi (Kobe University, Kobe, Japan)

OVERVIEW

The Cell Division and Cancer Group is interested in deciphering the mechanisms by which cell division and cell proliferation are regulated in mammalian cells. Our scientific interests are to: i) understand the basic control mechanisms that regulate the cell division cycle; ii) characterise the physiological and therapeutic consequences of cell cycle deregulation; iii) understand self-renewal and pluripotency in stem cell biology and tumour development; and iv) improve the use of old and new targets for cancer therapy. As a final goal, we aim to generate information that will be useful for understanding basic mechanisms of cell function and to improve therapeutic strategies against cancer cell proliferation.

"We have defined the role of mitotic kinases in neural and metabolic disease, and the immune response to chromosomal instability in patients with high tumour susceptibility."

RESEARCH HIGHLIGHTS

Mitotic kinases in developmental diseases

The cell cycle machinery regulates multiple aspects of cell biology, including the balance between proliferation and differentiation in multiple tissues. Several cell cycle kinases, such as polo-like kinase 1 (PLK1), modulate not only centrosome and chromosome biology but also other cellular processes such as the dynamics of the cytoskeleton, cell movements, etc. Our previous work showed critical implications of PLK1 in vascular biology and tumour development. By using gain- and loss-of-function mouse models of PLK1 function, we recently identified a new role for PLK1 in the control of cell fate in neural progenitors during development. Interestingly, centrosomal alterations are thought to be one of the aetiological reasons for primary microcephaly, a defect in which decreased cortex size is accompanied by mental retardation and other symptoms. By combining *Plk1*-mutant alleles with specific mutations in *Cep135* or *Cdk5rap2*, two genes mutated in microcephaly, we described new genetic interactions that lead to defective asymmetry of centrosomal components during the division of neural progenitors, microcephaly, and defective brain development (González-Martínez et al., 2022). Importantly, these phenotypes are also observed after inhibiting PLK1 with small-molecule inhibitors that are currently under evaluation in clinical trials for cancer therapy, raising a note of caution on the possible secondary effects of inhibiting PLK1 in neural progenitor cells.

Links between cell cycle regulation and metabolism

The serine/threonine kinase MASTL (also known as Greatwall) is a critical regulator of mitosis by inhibiting the PP2A phosphatase (2012). We previously reported that loss of MASTL results in mitotic defects such as defective chromosome condensation and segregation errors in mammalian cells. By using a variety of genetic and biochemical models, we have recently reported a mitotic-independent function of the MASTL-PP2A axis in modulating the response to glucose. In conditions of nutrient excess and high mTOR signalling, a negative feedback loop inhibits AKT activity, thus limiting the continuous activation of the AKT-mTOR pathway. In these conditions, MASTL is activated by phosphorylation and inhibits PP2A activity, thereby preventing the function of this phosphatase in allowing the continuous activation of AKT. These observations identify a new layer of control that interconnects a cell cycle module with the negative feedbacks regulating the AKT-mTOR pathway, and suggest the possible use of MASTL inhibition to specifically improve glucose metabolism in specific pathological conditions such as obesity or diabetes.

Chromosomal instability and cancer

Most human tumours display an abnormal number of chromosomes. A few mutations affecting mitotic regulators

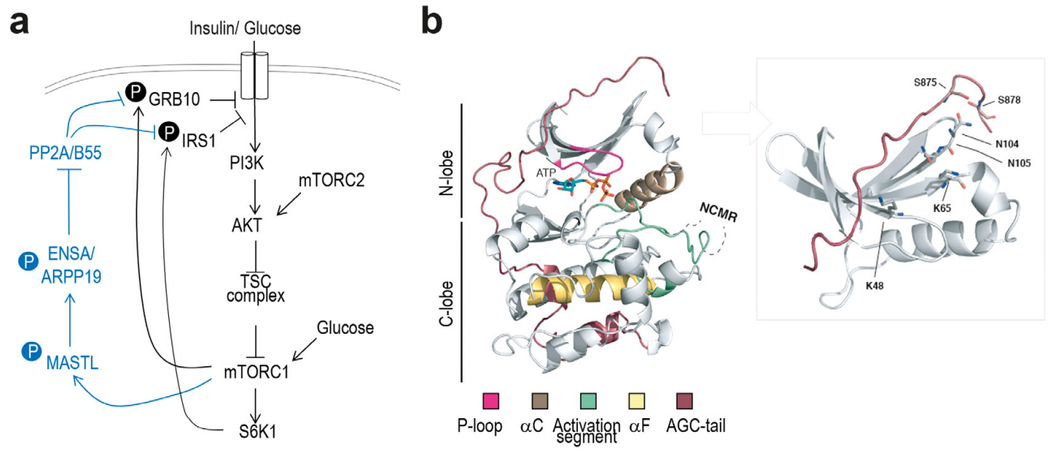

FIGURE 1 A new role for MASTL in the mTOR-AKT feedback loop. (**a**) A model for the new role (in blue) of MASTL in the negative feedback loop that controls AKT activity. (**b**) A homology structural model showing the position of the MASTL S875 residue phosphorylated by mTOR in response to high glucose signalling.

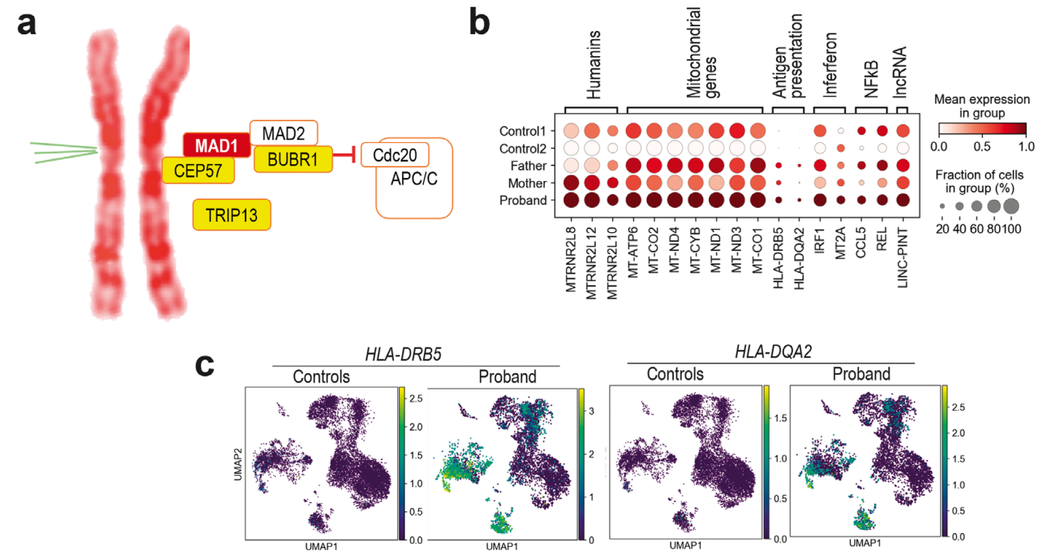

FIGURE 2 MAD1 mutations in a patient with high tumour susceptibility. (**a**) Critical components of the mitotic checkpoint, with genes previously identified in MVA in yellow. (**b**) Enrichment in mitochondrial and immune responses in peripheral blood cells from the proband. (**c**) Single-cell analysis showing upregulation of genes involved in the major histocompatibility complex.

are also detected in familial cancer (Villarroya-Beltri & Malumbres, 2022). In collaboration with the laboratories of Miguel Urioste and Sandra Rodríguez-Perales, we studied the effect of novel mutations in the mitotic checkpoint component MAD1 in a patient with unprecedented levels of tumour susceptibility. Our single-cell data in peripheral blood of the patient suggest that chromosomal instability induced by MAD1 mutations results in an immune response characterised by chronic activation of inflammatory signals (Villarroya-Beltri *et al.*, 2022). These data suggest a new variant of the Mosaic Variegated Aneuploidy (MVA) syndrome with high tumour susceptibility, and an immune response whose detailed analysis may lead to novel strategies for immunotherapy. ■

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OVERVIEW

The Genomic Instability Group focuses its research on understanding the molecular mechanisms leading to cancer and other age-related diseases, with the ultimate goal of translating this knowledge into novel therapeutic strategies. Our initial investigations centred on Replicative Stress (RS), a type of DNA damage sensed by the ATR kinase, and that is particularly abundant in some cancer cells. Our work in this area led to the discovery of selective ATR inhibitors that were further improved to enable their clinical development as anticancer agents. Next, we became increasingly interested in understanding the mechanisms of drug resistance to specific agents, such as inhibitors of ATR or USP7. More recently, our group has revealed that one of the most frequent mutations in human cancer, inactivation of the tumour suppressor FBXW7, leads to multidrug resistance (MDR). Importantly, we have also discovered strategies to overcome MDR, which is an important area of our current research.

"We have discovered a new mechanism that drives multidrug resistance. In addition, we have shown that the depletion of PD-L1 expressing cells might be a fruitful approach for cancer therapy."

RESEARCH HIGHLIGHTS

The Integrated Stress Response as a vulnerability of cancer cells

Last year, we reported that *FBXW7* mutations lead to multidrug resistance (MDR), limiting the efficacy of most available antitumor agents. Importantly, FBXW7 is one of the 10 most frequently mutated genes in cancer due to either inactivating mutations and/or allelic loss. Furthermore, mutations in this gene are among the most significantly associated with poor survival across all human cancers. Interestingly, we discovered that, despite their MDR phenotype, FBXW7 deficient cells were preferentially sensitive to therapies targeting mitochondria, such as the antibiotic tigecycline. Subsequently, we identified that that the toxicity of tigecycline for cancer cells is mediated by the Integrated Stress Response (ISR). In support of this, nuclear accumulation of ATF4, one of the hallmarks of ISR activity, was induced by tigecycline and reverted by the ISR inhibitor ISRIB. Moreover, and by searching for additional compounds that could target FBXW7 deficient cells, we found another set of seemingly unrelated compounds that did so, all of which activated the ISR (FIGURE 1). Surprisingly, these compounds were already known to have antitumor effects through very different mechanisms of action, such as inhibition of B-RAF or EGFR. This raises the important question as to what extent the anticancer effects of several clinically used drugs might be partly mediated by a previously unknown effect of these compounds in activating the ISR. We are currently investigating the basis of these observations, as they suggest the exciting possibility that a targeted activation of the ISR might be able to trigger cell death in cancer cells that are otherwise resistant to other chemotherapies.

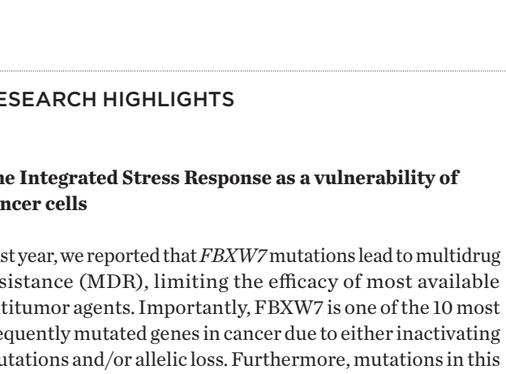

FIGURE 1 Multidrug resistance of FBXW7-deficient cells can be overcome by activation of the ISR. (**A**) Sensitivity of FBXW7-deficient DLD-1 to the ISR-activating drug tunicamycin. (**B**) Drugs that are similar to the antibiotic tigecycline, based on comparison of transcriptional signatures available at the connectivity map (CMAP). Note the presence of several seemingly unrelated tyrosine kinases in this set, all of which trigger a similar transcriptional signature to well established ISR-inducers such as tunicamycin or salubrinal. (**C**) Nuclear levels of ATF4 as evaluated by High-Content Microscopy in DLD-1 cells. Note that compounds to which FBXW7-deficient cells are resistant do not activate the ISR.

Targeting PD-L1 expressing cells in cancer therapy

The latest advances in immunotherapy for the treatment of cancer have incredibly improved the prognosis of a wide range of malignancies. Not surprisingly, the discovery of the immune checkpoint mediated by PD-1 and CTLA-4 receptors and of how targeting these pathways can be exploited for cancer therapy was awarded the Nobel Prize in Medicine in 2018. Antibodies targeting the PD-1/PD-L1 interaction are among the most widely used immunotherapy strategies, but, despite the indisputable success of these treatments, only 20-40% of the patients respond, and even fewer show durable responses. We hypothesised that the elimination of PD-L1 expressing cells, which may display additional checkpoint mediators on their membranes, could have broader antitumoral effects than targeting only the PD-1/PD-L1 interaction. To address this, we generated mice carrying an inducible suicidal reporter allele of PD-L1, which allows the isolation and identification of PD-L1-expressing cells, as well as their selective elimination upon treatment with an otherwise inert compound. Our work with these mice has revealed that the depletion of PD-L1 positive cells potentiates immune responses against different stimuli, such as a septic cytokine storm. In the context of cancer, we found that depletion of PD-L1-expressing cells favoured the clearance of tumour cells in a mouse model of peritoneal metastasis and, consequently, prolonged the survival of the animals (FIGURE 2). This work supports the usefulness of targeting PD-L1^{+} cells in cancer therapy, and provides the immunotherapy research community with a useful genetic tool for further investigations of the PD-1/PD-L1 checkpoint. ■

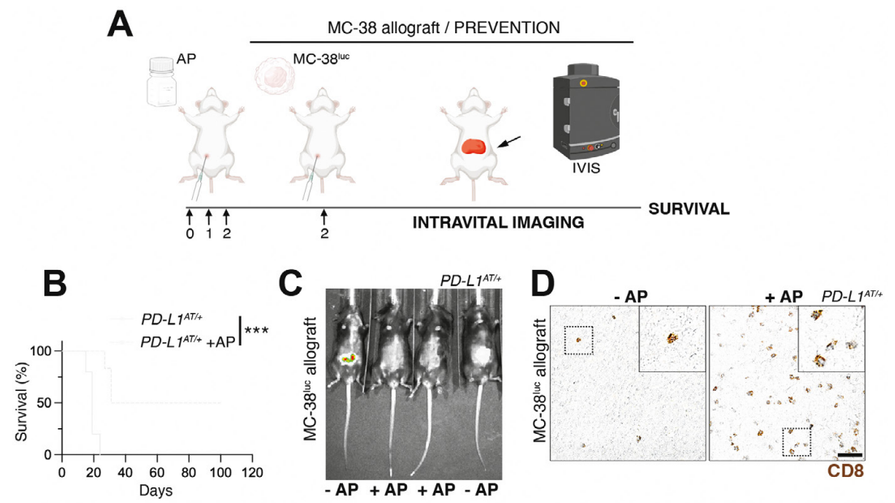

FIGURE 2 Depleting PD-L1^{+} cells prolongs survival in an immunocompetent model of peritoneal cancer metastasis. (**A**) MC-38 cells were intraperitoneally injected into mice previously treated with AP20187 to deplete PD-L1^{+} cells. (**B**) Survival curve of control and AP20187-treated *PD-L1*$^{AT/+}$ mice after i.p. inoculation of MC-38 allografts. (**C**) Representative IVIS from the experiment defined in (**B**) at day 4 post-tumour injection. (**D**) IHC of CD8 in intraperitoneal MC-38 allografts isolated from control and AP20187-treated *PD-L1*$^{AT/+}$ mice. Insets illustrate the presence of tumour-infiltrating CD8^{+} cells.

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OVERVIEW

DNA topoisomerases have a dual relationship with the genome. They are essential to solve supercoiling and other topological problems inherent to all DNA transactions, but their intrinsic mechanism of action can result in the formation of DNA breaks, either accidentally during normal cellular metabolism or upon chemotherapy treatment with the so-called topoisomerase poisons. Imbalances in DNA topoisomerase activity can therefore compromise cell survival and genome integrity, entailing serious consequences for human health, such as developmental and degenerative problems and, very importantly, neoplastic transformation processes and their subsequent response to treatment.

We are interested in understanding how DNA topoisomerase activity is regulated to integrate different aspects of genome dynamics, how an imbalance in these processes can lead to the appearance of pathological DNA breaks, and how cells specifically respond to these lesions to maintain genome stability.

"We have defined a complete map of the genetic pathways operating in the repair of topoisomerase II-induced DNA breaks, their relationships, and how this affects genome stability and tumorigenesis."

RESEARCH HIGHLIGHTS

During 2022, we had 2 main areas of interest. The first one is in line with the main research line of the laboratory on the repair of topoisomerase II (TOP2)-induced DNA double-strand breaks (DSBs), while the other one is completely different, and stems from the efforts initiated during the COVID-19 pandemic to develop novel genetic diagnosis methods that could be implemented in a point-of-care setting.

Repair of topoisomerase II-induced DNA breaks

TOP2-induced DSBs are particular DNA lesions in which the ends of the break are blocked by a protein adduct that needs to be removed to allow further repair to take place, and can arise spontaneously or as a consequence of chemotherapeutic regimes including TOP2 poisons. We have used unbiased genetic screening approaches to obtain a comprehensive view of the different factors specifically involved in the repair of these lesions. Our results outline 2 main pathways that operate hierarchically to remove the protein adduct (FIGURE 1). First, cells strongly rely on repair mediated by TDP2, an enzyme that directly removes the adduct without affecting the DNA molecule, thus promoting accurate repair and the maintenance of genome stability. Alternatively, but only if this pathway is overwhelmed or disturbed, cells use nucleolytic activities, such as Artemis or the MRN complex, which eliminate the adduct by trimming off DNA ends, allowing repair, but at the cost of compromising genome integrity. As expected from this model, removal of TDP2 in mouse models leads to increased cancer predisposition. Finally, we found that ATM, a common tumour suppressor and the most relevant kinase controlling the response to DSBs, is important for enforcing the hierarchical preference for the TDP2-mediated pathway, and that ATM deficient tumours specifically accumulate mutations that are compatible with the misrepair of TOP2 breaks. Altogether, these results open new avenues to improve the therapeutic use of TOP2 poisons, demonstrate the spontaneous occurrence of TOP2 lesions *in vivo*, and highlight their oncogenic potential when not appropriately repaired.

Novel nucleic-acid detection method

The capacity of CRISPR-Cas systems being programmed to recognise specific nucleic acid sequences has boosted their biotechnological applications. One of them is the detection of the genetic material of pathogens or genetic markers in diagnosis. Systems to detect specific nucleic acid sequences based on CRISPR-Cas technology have been recently developed and promise to revolutionise point-of-care diagnostics in the near future. These systems rely on the fact that, upon recognition and cleavage of the desired target, which is highly specific and easily programmable, the Cas protein becomes activated with a sequence-independent, unscheduled nucleolytic activity that can be easily detected with nuclease reporter substrates, and whose signal can therefore be used as a readout for the presence of the given nucleic acid of interest.

These CRISPR-Cas diagnostics, however, despite their great specificity and versatility, are currently limited by the levels of sensitivity, which are outside the range of the concentrations required for diagnostic purposes, and currently rely on pre-amplification of the target sequences by methods such as PCR or LAMP. This introduces a complication to the reactions, limiting their current use in point-of-care applications. We have developed and patented a conceptually novel solution that, instead of amplifying the target nucleic acid, focuses on boosting Cas activation, so the reaction is carried out in a single step at room temperature, providing an ideal setting for point-of-care diagnostics. Due to its versatility in the detection of any nucleic acid of interest, this invention should constitute the platform for the development of a wide range of specific genetic testing kits and devices, including pathogen and genetic marker detection. ■

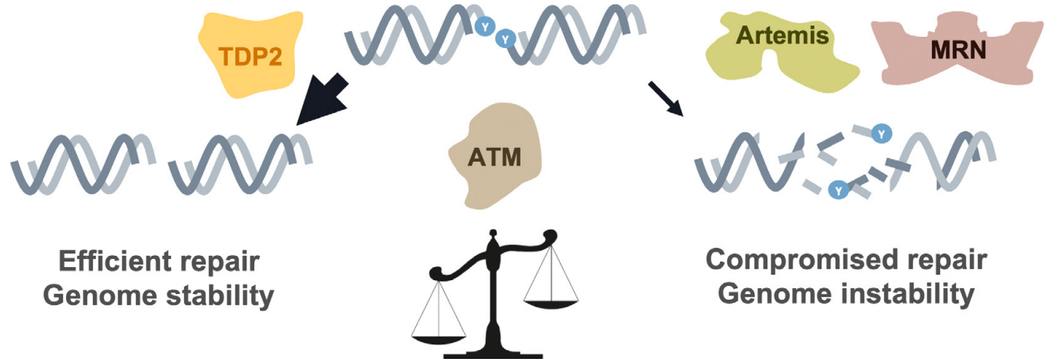

FIGURE 1 Scheme of the different cellular pathways involved in the repair of TOP2-induced DSBs. Protein adducts are removed by either TDP2, which results in accurate repair, or nucleolytic pathways, which compromise genome integrity. ATM establishes a hierarchical preference for the TDP2-dependent pathway.

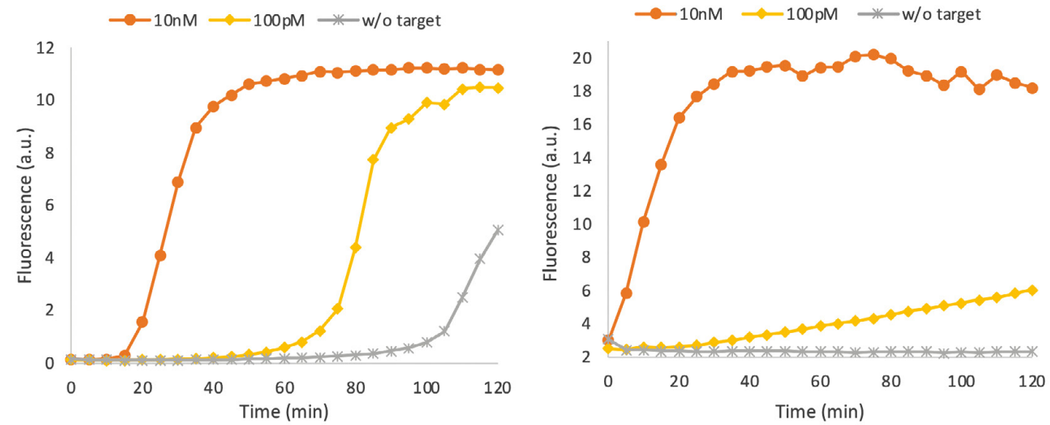

FIGURE 2 CRISPR-Cas12a DNA detection. Our improved method with amplification of Cas activation (left) is compared to direct detection (right).

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OVERVIEW

Our research focuses on a protein complex named cohesin that embraces DNA to mediate sister chromatid cohesion, a process essential for chromosome segregation and faithful DNA repair by homologous recombination. Cohesin also plays a major role in the spatial organisation of the genome by promoting long-range DNA looping, which in turn contributes to transcriptional regulation. Mutations in cohesin have been found in several tumour types, most prominently in bladder cancer, Ewing sarcoma and acute myeloid leukaemia. Germline mutations in cohesin and its regulatory factors are also at the origin of human developmental syndromes collectively known as cohesinopathies.

Our goal is to understand how cohesin works, how it is regulated, and how its dysfunction contributes to cancer and other human diseases. In particular, we are intrigued by the existence of different versions of the cohesin complex. We use human cells and mouse models carrying *knock out* alleles of genes encoding variant cohesin subunits to investigate their functional specificity.

"We have identified a differential requirement of cohesin-STAG1 and cohesin-STAG2 for NIPBL, a key regulator of cohesin activity and the gene most commonly mutated in cohesinopathy patients."

RESEARCH HIGHLIGHTS

NIPBL is not required for loading cohesin on chromatin

The spatial organisation of the genome inside the nucleus is critical for transcription, DNA replication and repair. Cohesin mediates 3D genome organisation by binding to chromatin and extruding DNA loops that become stabilised at several locations along the genome, most notably at sites bound by CTCF. In this way, the complex facilitates contacts between promoters and distal enhancers while restricting such interactions within topological associated domains (TADs). Loop extrusion by cohesin also promotes intermixing of active/inactive chromatin compartments.

There are two versions of the cohesin complex in all somatic vertebrate cells that carry SMC1A, SMC3, RAD21, and either STAG1 or STAG2. Results from our group and others indicate that the two complexes make specific contributions to 3D genome architecture, and further suggest that their different chromatin association dynamics are responsible for these specific functions. In turn, chromatin association is modulated by the interactions of cohesin with its regulators. STAG2 is more often found associated with the unloading factor WAPL, while cohesin acetyltransferase ESCO1 preferentially acts on cohesin-STAG1 at CTCF-bound sites. What it is not known is how the two complexes respond to limited availability of NIPBL.

NIPBL is currently considered the cohesin loader. It activates the cohesin ATPase and is essential for loop extrusion by cohesin *in vitro*. *NIPBL* is an essential gene, and heterozygous mutations have been identified in over 70% of patients with Cornelia de Lange Syndrome (CdLS), the most common developmental syndrome due to cohesin dysfunction. To assess the consequences of NIPBL knock down (KD), we combined a flow cytometry assay that measures chromatin-bound proteins with analyses of genome-wide distribution of cohesin-STAG1 and cohesin-STAG2 by ChIP-seq and of genome contacts by *in situ* Hi-C. Strikingly, we found that cohesin-STAG1 increases on chromatin and further accumulates at CTCF positions after NIPBL knock down, while cohesin-STAG2 diminishes genome-wide. These effects are independent of the presence of the other complex and are epistatic to downregulation of CTCF, ESCO1, or WAPL. Despite the presence of cohesin-STAG1 on chromatin, loop formation is severely impaired. These and additional data support a model in which, contrary to current thinking, NIPBL is not required for association of cohesin with chromatin. However, it is required for loop extrusion, which in turn facilitates stabilisation of cohesin-STAG2 at CTCF positions after being loaded elsewhere (FIGURE 1, right). In contrast, cohesin-STAG1 is loaded and stabilised at CTCF sites even under low NIPBL levels, although in that condition it is unable to form long loops (FIGURE 1, left). These results add to our understanding of the different behaviour of cohesin-STAG1 and cohesin-STAG2. More importantly, they provide a new perspective on the role of NIPBL on cohesin dynamics that needs to be considered when thinking of potential therapies for CdLS.

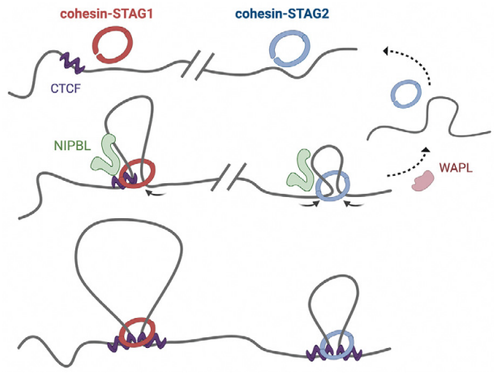

FIGURE 1 Model for the different NIPBL requirement of cohesin-STAG1 and cohesin-STAG2 in the process of DNA loop formation that drives 3D genome organisation. Created with Biorender.com.

Contribution of STAG2 mutations to aggressive Ewing sarcoma

Ewing sarcoma (EWS) is the second most frequent type of bone cancer in children and young adults. It is driven by a fusion protein, most often EWS-FLI1, which alters the gene expression programme of the cell initiating the tumour. It is a highly aggressive cancer with a 5-year survival below 30% in patients that present metastasis. Among the few recurrent mutations identified in EWS, in addition to the oncogenic fusion, are those that inactivate *STAG2*. Importantly, *STAG2* mutations are often present in the most aggressive EWS tumours, suggesting that the loss of cohesin STAG2 may facilitate the acquisition of the aggressive form of EWS.

From the bioinformatic analysis of transcriptomic data from EWS patients and cell lines, we have identified a gene signature dependent on STAG2 loss that correlates with poor survival. We are currently exploring the contribution of these genes to the metastatic phenotype by analysing, both *in vitro* and *in vivo*, the migration and invasion capabilities of EWS cell clones knocked out for these genes (FIGURE 2). In collaboration with E. de Álava (*Hospital Virgen del Rocío-IBIS*, Sevilla), we are also assessing whether they can serve as biomarkers to predict the presence of metastases before their actual detection using immuno-histochemistry (IHC) in tissue microarrays (TMAs) from patient samples. Finally, with the help of Carmen Blanco (Experimental Therapeutics Programme, CNIO), we are carrying out drug screens to identify vulnerabilities in EWS cells lacking STAG2. ■

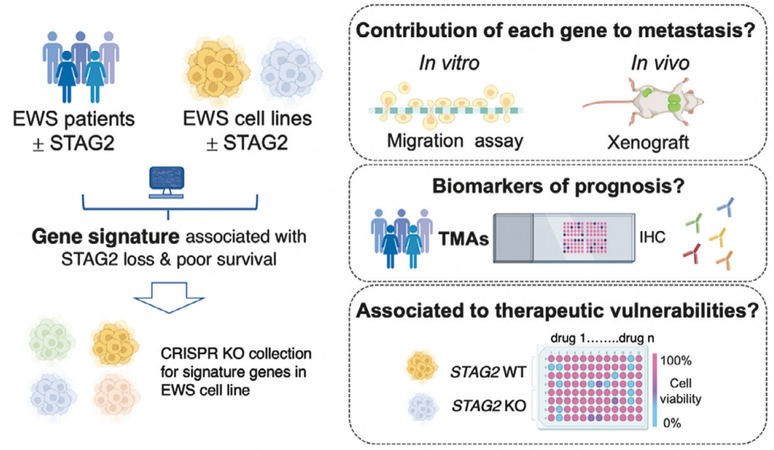

FIGURE 2 Strategy to understand and exploit the contribution of *STAG2* mutations to aggressive Ewing sarcoma.

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DNA REPLICATION GROUP

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OVERVIEW

Despite the biochemical complexity of the DNA replication process, the molecular machinery that duplicates our genome displays a remarkable capacity to adapt to different cell types, each one with its own transcriptional programme and specific patterns of chromatin organisation. In addition, the replisome proteins react to endogenous and exogenous factors that induce replicative stress (RS) and may cause DNA breaks, recombination events, and genomic instability. Our Group studies the mechanisms that confer operational flexibility to the replicative process, combining molecular and cellular approaches in human and mouse cells. In 2022, we completed two studies describing the cellular responses to specific situations of stress, which involve the regulation of origin activity and the control over replication fork progression. We also continued to study the dynamics of DNA replication and the impact of RS in other cellular contexts, including the acquisition of metastatic capacity by tumour cells.

"We have described how PRIMPOL facilitates DNA synthesis during stress-induced proliferation of haematopoietic stem cells, allowing the haematopoietic system to reconstitute itself after a bone marrow transplantation."

RESEARCH HIGHLIGHTS

Three-dimensional chromatin organisation underlies the efficiency of replication origins

In earlier work, we had reported that a fraction of mammalian replication origins remains inactive ("dormant") in S phase but can be activated as a backup mechanism in response to RS. To investigate the regulation of active *vs* dormant origins, we mapped origin activity in mouse embryonic stem cells (mESCs) undergoing mild RS triggered by aphidicolin, a DNA polymerase inhibitor, or by the ectopic expression of CDC6, an origin licensing factor. The main stress-induced response was an increase in the frequency of activation of existing initiation sites that were used with lower efficiency in unchallenged conditions. This phenotype reflects, at the cell population level, the combined effect of the activation of dormant origins in millions of individual cells. By intersecting origin mapping and Hi-C chromosomal conformation data, we found that origin efficiency is directly proportional to the number of three-dimensional (3D) contacts established between origin-containing chromatin fragments. Origins that cluster in 3D tend to fire with similar efficiencies and share their timing of replication, supporting the organisation of origins in higher-level replication factories (Jodkowska *et al.*, 2022; see FIGURE 1).

PRIMPOL-mediated repriming of DNA synthesis during stress-induced proliferation of haematopoietic stem cells

Since its discovery in 2013, our laboratory has been involved in the characterisation of the PRIMPOL enzyme, a DNA primase specialised in damage tolerance. In a recent study, we described how PRIMPOL mediates the replicative tolerance of DNA inter-strand crosslinks (ICLs; González-Acosta *et al.*, *EMBO J* 2021). Inefficient ICL repair causes Fanconi Anaemia (FA), a rare but severe disease characterised by frequent congenital defects, bone marrow failure, aplastic anaemia and cancer predisposition. In 2022, we completed a study in collaboration with M. Lopes (Institute of Molecular Cancer Research, University of Zurich), showing that mouse haematopoietic stem cells (HSCs) that are forced to proliferate by a simulated viral infection display accelerated fork progression and accumulate extensive DNA damage. In this critical situation, HSCs rewire their DNA damage response and engage PRIMPOL primase, favouring re-priming of DNA synthesis over fork reversal. Competitive bone marrow transplantations confirmed that PRIMPOL activity is required for HSC amplification and efficient reconstitution of the haematopoietic system (Jacobs *et al.*, 2022; FIGURE 2). This study opens the possibility that in some cases, PRIMPOL-mediated bypass of damaged DNA could also contribute to the onset of leukaemia. In this regard, we are pursuing the identification of small inhibitors of PRIMPOL, in collaboration with the CNIO Experimental Therapeutics Programme.

DNA replication and RS in other cellular contexts

We have participated in two collaborative studies related to the main research topics described above: (a) the characterisation of a protective function of human p38 SAP kinase to maintain genome integrity in response to osmo-stress, mediated by claspin/Mrc1 phosphorylation (Ulsamer *et al.*, 2022); and (b) the analysis of DNA replication in cells harbouring a truncated variant of RAD51B associated with primary ovarian insufficiency (Franca *et al.*, 2022).

Other ongoing projects in the DNA Replication Group include: (i) a genome-wide analysis of the formation of pre-replicative complexes in human and mouse cells, using CUT&RUN with initiator proteins; (ii) a comparative analysis of replisome composition in *naive* and *primed* mESCs, which could explain the changes in fork speed observed during cell reprogramming; (iii) an investigation of the influence of RS during epithelial-to-mesenchymal transition, a process that underlies the acquisition of resistance to chemotherapy in some tumour cells. ■

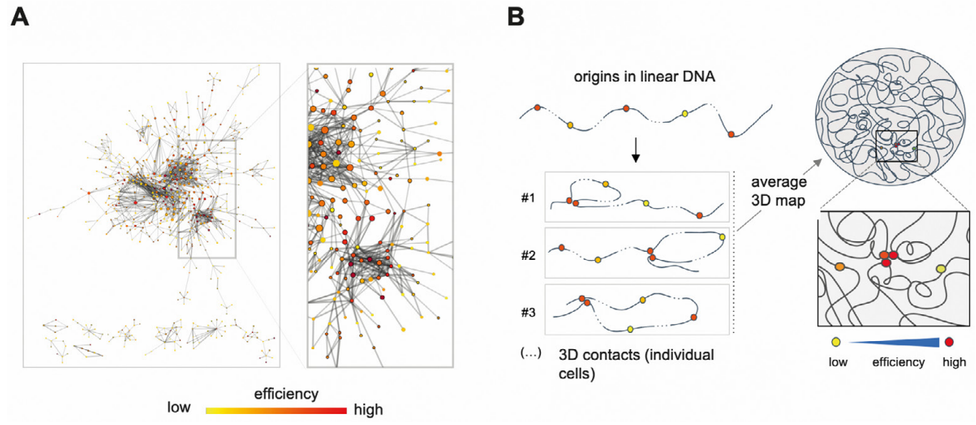

FIGURE 1 Integration of origin maps into 3D chromatin interaction networks. (**A**) Network of chromatin contacts derived from Hi-C data in mESCs (chrom 1). Origins (coloured circles) located at more connected hubs are activated with higher frequency. (**B**) Model of a replication factory formed by clustered origins. Adapted from Jodkowska *et al.* (2022).

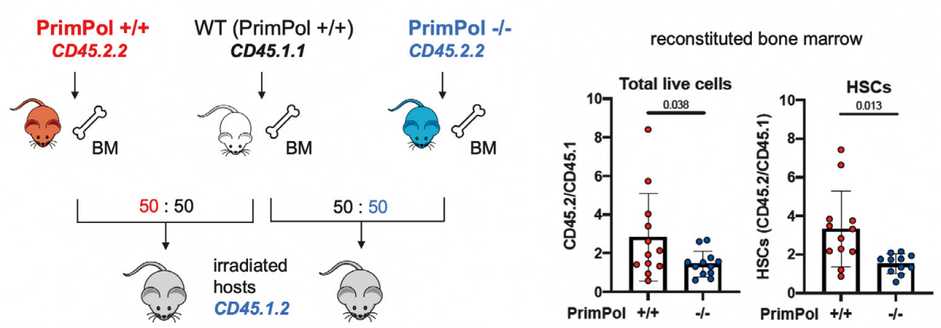

FIGURE 2 Efficient reconstitution of the haematopoietic system requires PRIMPOL activity. (**A**) Experimental design of a competitive bone marrow (BM) transplantation. (**B**) Donor chimerism in the reconstituted BM (total live cells and HSCs). Red, PRIMPOL-proficient; blue, PRIMPOL-deficient cells. Adapted from Jacobs *et al.* (2022).

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MELANOMA GROUP

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OVERVIEW

Melanomas are prime examples of aggressive diseases where basic and translational research have significantly improved patient prognosis. Nevertheless, clinical responses are still incomplete. The long-term goals of our Group are to identify new progression biomarkers and therapeutic agents. We are particularly interested in mechanisms of cellular stress that, being selectively deregulated in melanoma, define lineage-specific vulnerabilities (publications in *Nature, Cancer Cell, Nature Cell Biology, Nature Communications*, among others). Our laboratory has also reported first-in-class lymphoreporter (*MetAlert*) mice for non-invasive imaging of pre-metastatic niches in melanoma (*Nature*). These systems have led to the identification of new mechanisms of immune resistance (*Nature Medicine*) and the generation of nanoparticle-based treatments (*Cancer Cell, EMBO Mol Med*), with derivatives now being tested in clinical trials. Our ultimate objective is to improve the management of patients with otherwise refractory metastatic melanomas.

"We have visualised and targeted (pre)metastatic niches in melanoma and defined mechanisms of immune suppression with clinical implications for cancer patients."

RESEARCH HIGHLIGHTS

The long-term goals of our Group are to (see FIGURE 1):

1. Define the "fingerprint" that distinguishes melanomas from other cancer types.
2. Visualise and target melanoma progression at the whole body level *in vivo*.
3. Determine and target signalling cascades that turn immunologically "hot" melanomas into "cold" and refractory tumours.
4. Develop new therapeutic strategies to overcome immune suppression and immune tolerance in melanoma.

New tumour drivers that favour melanoma progression

One of the long-term objectives of our Group is to discover novel melanoma drivers. We have previously identified endolysosomal-associated genes (RAB7) and RNA binding proteins (CEFL1, CUGBP1 and IGF2BP1) with lineage-specific protumorigenic functions that are not shared by over 25 cancer types (Alonso-Curbelo *et al.*, *Cancer Cell* 2014; García-Fernández *et al.*, *Autophagy* 2016; Perez-Guijarro *et al.*, *Nat Commun* 2016; Cifdaloz *et al.*, *Nat Commun* 2017; Karras *et al.*, *Cancer Cell*, 2019). In addition, we have pursued melanoma-secreted factors that exert long-range activities, particularly in the generation of premetastatic niches. A prime interest of our Group has been modulators of neolymphangiogenesis, as this is an early stage in melanoma dissemination. Exploiting "lymphoreporter" models generated by Sagrario Ortega's Group at CNIO, we developed the first *"Melanoma-MetAlert"* mice. These animals have the unique feature of allowing for spatio-temporal analyses of tumour-activated lymphangiogenesis *in vivo* as a way to define premetastatic niches (Olmeda *et al.*, *Nature* 2017). *'MetAlert'* animals, in combination with human tissue specimens, revealed the growth factor MIDKINE (MDK) as a new melanoma driver with a potent ability to act in a systemic manner to promote neolymphangiogenesis and melanoma metastasis (Olmeda *et al.*, *Nature* 2017). Our expertise in lymphangiogenesis also contributed to collaborative studies to define an unexpected crosstalk of lymphatic genes with lipid metabolism and autophagy (Mece *et al.*, *Nat Commun* 2022). In the course of these studies, we generated computational tools and experimental models that have served to characterise novel dsRNA binding proteins and various immune modulators. In particular, our ability to mine large tumour datasets has helped us to describe immune suppressive roles of IL22 favouring lung metastasis (Briukhovetska *et al.*, *Immunity* 2023).

Impact of the melanoma secretome in the rewiring of the immune system towards tumour-promoting phenotypes

Melanomas are a prime example of tumours quite efficient at bypassing antigen presentation and promoting immunologically "cold" or tolerogenic phenotypes, but the underlying mechanisms are not well understood. Analysing downstream effectors of MDK, we found new immune suppressive roles of this protein, whereby macrophages are recruited to tumours, but instead of attacking the cancer cells, promote dysfunctional CD8+ T cells (Cerezo-Wallis *et al.*, *Nat Medicine* 2020). More recently, we discovered that MDK acts as a multifaceted suppressor of antigen presentation. Mechanistically, MDK was found to repress all main aspects of the differentiation, activation, and function of dendritic cells (DCs), particularly of conventional type 1 (cDC1). Moreover, we uncovered an MDK-associated signature in DCs that defines bad prognosis and resistance to immune checkpoint blockers actively used in human patients (FIGURE 2). MDK-associated downregulation of cDC1-dependent immune scores were also identified in a variety of other tumour types, further emphasising the translational relevance of MDK as a target to boost antigen presentation in otherwise immune refractory cancers (Catena *et al.*, *BioRxiv* 2022; Catena *et al.*, submitted). In light of the tumour-promoting and immune-suppressive roles of MDK, we are actively pursuing this protein as a therapeutic target. We have previously reported dsRNA mimetics that repress MDK mRNA expression (Olmeda *et al.*, *EMBO Mol Med* 2021) and are now developing small molecule inhibitors and blocking antibodies. ■

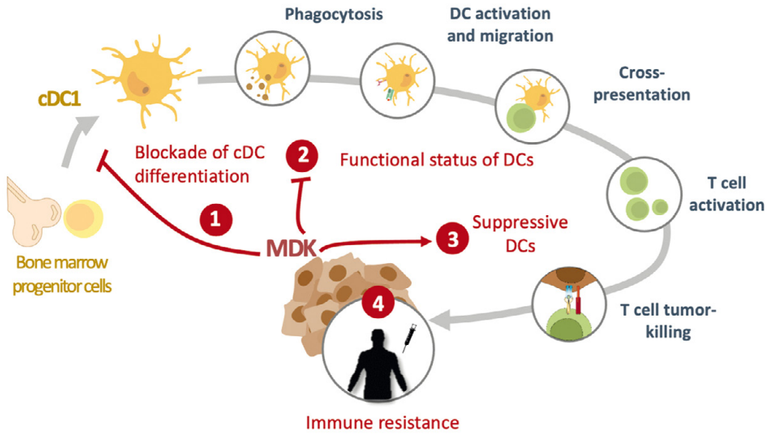

FIGURE 2 Multi-stage rewiring of dendritic cell (DC) differentiation and function by tumour-secreted Midkine (MDK). MDK was found to block DC differentiation and impair all main DC associated functions (phagocytosis, activation, cross-presentation, and T cell activation), shifting DCs into suppressive features. Ultimately, these DC-driven effects reduce the efficacy of immune-based therapies.

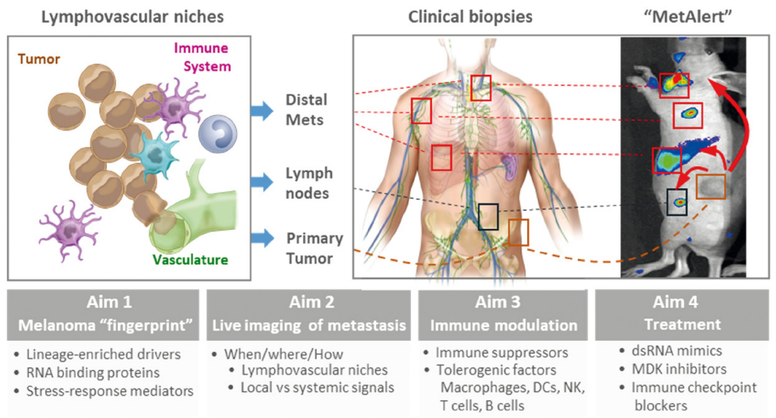

FIGURE 1 Melanoma Group at glance: main aims and experimental models to identify new tumour drivers and therapeutic targets, with a particular emphasis on the crosstalk between lymphatic vasculature and the immune system.

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AWARDS AND RECOGNITION

- Excellence in Science Award by the *Colegio de Biólogos y Biólogas de Galicia*, Spain.
- International Award in Oncology, *Ramiro Carregal* Foundation, Spain.
- Leadership in Science Award ("*Premio Liderando en Ciencia*"), *Comunidad Autónoma de Madrid*, Spain.
- President, Spanish Association for Cancer Research (*Asociación Española de Investigación Contra el Cáncer, ASEICA*).
- Elected EMBO Member.
- Top 100 Women Leaders in Spain, *Mujeres & Cia*.

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OVERVIEW

We focus on the molecular pathophysiology of pancreatic ductal adenocarcinoma (PDAC) and urothelial bladder carcinoma (UBC) taking a disease-oriented approach. These tumours present very distinct clinical challenges. We learn from patient samples, cultured cells/organoids, and genetically modified mice. To translate the findings, we bring this knowledge to a "population" level leveraging on information and samples from large patient cohorts together with Núria Malats (CNIO).

"We have found that antibiotic administration and gut flora depletion rescues a genetic defect present in *Nr5a2* heterozygous mice that sensitises them to acute pancreatic damage and to PDAC."

PDAC has a dismal prognosis even when diagnosed early. We aim to dissect the molecular mechanisms involved in very early steps of tumour development, harnessing the excellent genetic mouse models available. A main hypothesis is that cell differentiation is an early and potent tumour suppressor mechanism. Understanding the contribution of early molecular events is crucial to design better strategies for prevention and early tumour detection.

UBC presents with very wide clinical and pathological heterogeneity. We aim to acquire knowledge about the underlying molecular pathways and to apply it for improved prediction of outcome and therapy.

RESEARCH HIGHLIGHTS

Pancreatic cancer molecular pathophysiology

In recent years, GWAS have identified a variety of common genetic variants associated with PDAC risk. Several of them are associated with genes involved in acinar cell biology, including *NR5A2* and *HNF1A*, coding for transcription factors required for full acinar differentiation that we have extensively studied. A few other GWAS hits associate with genes involved in acinar function, such as *XBP1* and *CTRB1/2*. These observations have strengthened the notion, pioneered by our lab, that cell differentiation is the first tumour suppressor mechanism in the pancreas. Among the processes participating therein are inflammation and the ER stress response. *Nr5a2* heterozygous mice display more damage and are not able to recover properly upon induction of a mild acute pancreatitis. In addition, they are more susceptible to mutant *KRas*-driven PDAC. Among the modifiable factors that may cooperate with this genetic defect to drive PDAC, we tested diet and the gut microbiome. A high fat diet does not add to the pancreatitis phenotype of *Nr5a2* heterozygous mice. In contrast, antibiotic administration and depletion of the gut microbiota rescues the genetic defect observed upon pancreatitis induction. 16S rDNA analysis does not reveal major differences in the faecal microbiome of wild type and *Nr5a2* heterozygous mice. A variety of experiments fail to support the contribution of heterozygosity at the intestinal level. Transcriptomic changes analysis of the pancreas reveals significant changes both in basal conditions and during pancreatitis. Most notably, in mice that received antibiotics we find an up-regulation of the acinar programme and of mitochondrial pathways and a down-regulation of cell cycle and inflammatory pathways. *Nr5a2* heterozygous mice have higher levels of CD4+ cells in blood, and antibiotic administration reduces their CD4+ cell levels. Gut microbiome reconstitution results in increased CD4+ cell counts (FIGURE 1). Our observations suggest that the gut microbiome induces a basal inflammatory state that contributes to disease, the modulation of which could be exploited therapeutically.

Urothelial bladder carcinoma (UBC) genetics, biology, and clinical translation

We focus on understanding 2 new UBC tumour suppressor genes that we identified through exome sequencing: *STAG2* and *RBM10*. *STAG2* codes for a cohesin subunit, and *RBM10* codes for a splicing regulator. We have generated conditional mouse models for these 2 genes and are exploring their role in development and urothelial biology, as well as their cooperation with other cancer genes.

Increasing evidence shows that STAG2 acts as a tumour suppressor through rather unique mechanisms, largely unrelated to the canonical role of cohesin in chromosome segregation. *STAG2* alterations occur early during tumourigenesis. Therefore, we are using both normal urothelial cells and tumour cell lines to identify the impact of STAG2 at the genomic and cellular levels. Using RT112 cells, we have integrated ChIP-Seq, HiC chromatin interaction data, and RNA-Seq to assess the impact of STAG2 knockdown. The cohesin-STAG2 complex mediates short- and mid-range interactions that engage genes at higher frequency than cohesin-STAG1. STAG2 knockdown results in the down-regulation of luminal differentiation programmes and up-regulation of basal programmes. These findings are at odds with the fact that *STAG2* mutations are associated with luminal-type bladder cancers, suggesting an intermediate luminal differentiation phenotype. STAG2 knockdown does not affect compartment and domain boundaries, but it rewires intra-TAD DNA interactions and leads to the de-repression of lineage specifying genes (in collaboration with M. Martí-Renom, *CRG*, Barcelona).

Our translational studies expand several clinical trials with a strong translational component carried out in collaboration with Núria Malats and Spanish uro-oncologists. ■

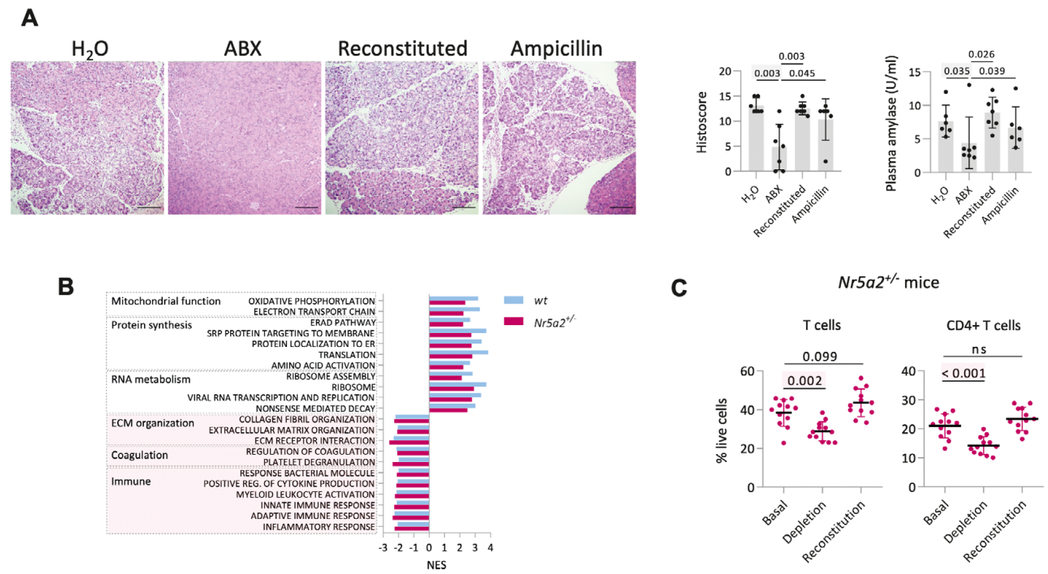

FIGURE 1 Antibiotic-mediated gut flora depletion rescues the genetic defect of *Nr5a2*$^{+/-}$ mice, as shown at the histological level upon induction of acute pancreatitis (**A**). In basal conditions, antibiotic administration has anti-inflammatory effects in the pancreas as shown by RNA-Seq pathway analysis (**B**). Antibiotic administration induces a significant decrease of CD4+ cells in the blood that is reversed upon gut flora reconstitution by co-housing (**C**).

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OVERVIEW

Research over the last 20 years has focused mainly on understanding the functions and roles of newly discovered mutated genes in the development of cancer and associated diseases. However, it remains largely unknown how environmental factors can alter the host's eukaryotic epithelial cells to cause various pathologies that can progress to cancer. Identifying likely causal links between environmental stresses and diseases that progress to cancer will help to elucidate mechanisms of disease and to identify targets with preventive and therapeutic value for treating frequent lethal human disorders with increased worldwide incidence and unmet medical needs.

"We continuously strive to generate new and unique preclinical mouse models to elucidate the mechanisms of diseases and capture the complexity of human disorders, with a particular focus on diseases associated with obesity and the digestive tract."

In our laboratory, we focus on understanding the mechanisms of diseases associated with the ingestion of toxic diets or nutrient overload that can lead to obesity and associated disorders, including diseases of the digestive system. We have a particular interest in liver disease, including non-alcoholic steatohepatitis and cirrhosis, and their progression to hepatocellular carcinoma (HCC), one of the most aggressive and lethal liver cancers. We also study intestinal disorders that can lead to colorectal cancer. Our ultimate goal is to guide the design of new medicines.

RESEARCH HIGHLIGHTS

Our research interest is mainly driven by the discovery of two components initially identified in our laboratory to be downstream targets of the growth factor and nutrient signalling cascades: the **URI (Unconventional prefoldin RPB5 Interactor)** and **MCRS1 (Microspherule protein 1)** proteins. URI and MCRS1 are respectively part of 2 independent protein complexes: the **URI prefoldin-like** and the **non-specific lethal (NSL) complexes**. Importantly, URI and MCRS1 expression turned out to be also regulated by environmental factors (nutrients, radiations, bacteria, viruses, etc.), which may compromise their functions and activate pleiotropic circuits supporting complex cell signalling networks, thereby provoking severe outcomes.

Using genetically engineered mouse models generated in our lab for URI and MCRS1 gain- and loss-of-functions, combined with other model systems and cutting-edge technologies and human data, our laboratory has devoted substantial efforts over the last years to determine the molecular, cellular, and pathophysiological mechanisms that link environmental stresses to obesity and disease pathogenesis of the digestive system, with the aim of developing more effective therapeutic strategies. In particular, we have focused on diseases associated to the liver, intestine, and pancreas, as these organs are primarily impacted by environmental stressors, including nutrient overload, but are also physiologically interconnected through their exocrine and/or endocrine functions. In this regard, the following highlights summarise our major achievements during 2022:

- → The liver has an exceptional ability to regenerate itself to maintain tissue homeostasis, but this process can be impacted by stress signals, potentially leading to liver cancer. We have reviewed the mechanisms of hepatic regeneration under homeostasis or upon injury (Rigual *et al.*, *Trends Cancer*, 2022).
- → Additionally, we have developed a novel murine model that mimics the pathological features of cirrhosis, and uncovered a new function of MCRS1 in regulating histone acetylation, maintaining gene expression and liver homeostasis. The loss of MCRS1 in hepatocytes activates the bile acid/FXR axis in liver fibroblasts, a significant event in cirrhosis development, with important implications for treatment (Garrido *et al.*, *J Hepatol*, 2022).
- → We have also determined the mechanisms of regeneration of the intestinal epithelium and demonstrated that URI+ cells play a crucial role in maintaining intestinal homeostasis by controlling R-spondin 1 levels, supporting $Lgr5^{high}$ intestinal stem cell proliferation. These findings highlight the unexpected role of transit-amplifying cells in controlling $Lgr5^{high}$ intestinal stem cell proliferation (Chaves-Peréz *et al.*, *J Exp Med*, 2022).

Future work

Obesity is becoming one of the most increasingly growing risk factors for liver and intestinal disorders, including cancer. By employing multi- and inter-disciplinary approaches, including the use of preclinical mouse models generated in our laboratory combined with human data, we will continue to determine the mechanisms of diseases associated with obesity. In particular, with a special focus on diseases of the digestive system, we aim to: find out what goes wrong in diseased and cancerous tissues; understand how organs can regenerate; potentially engineer new tissues; and, if regeneration goes awry, determine how it contributes to cancer. Our ultimate goal is to help guide the design of new medicines against obesity and its associated disorders (FIGURE 1). ■

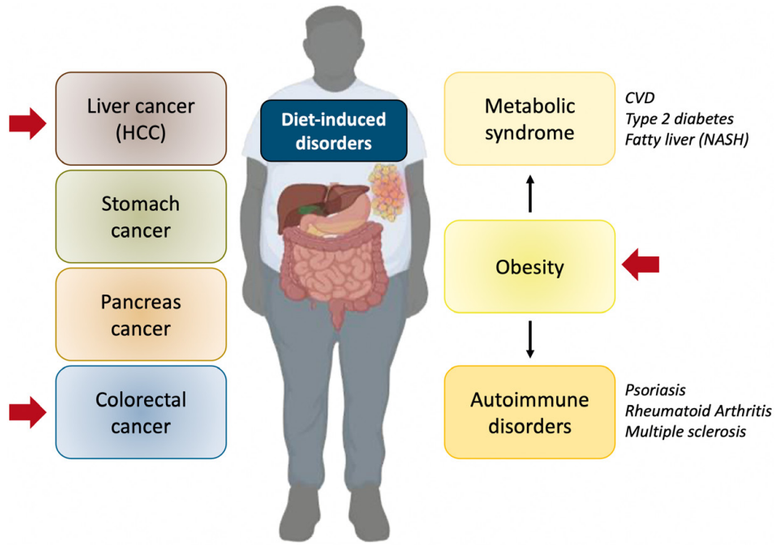

FIGURE 1 Representation of some of our research directions. Obesity is one of the most increasingly growing risk factors for liver and intestinal disorders, including cancer. By employing multi- and inter-disciplinary approaches, including the use of preclinical mouse models generated in our lab combined with human data, we aim to: find out what goes wrong in diseased tissues; understand how organs can regenerate; potentially engineer new tissues; and, if regeneration goes awry, determine how it contributes to disorders. Our final goal is to guide the design of new medicines against obesity and its associated disorders, including metabolic, liver and intestinal diseases. CVD: cardiovascular diseases. NASH: Non-alcoholic steatohepatitis.

PUBLICATIONS

- Chaves-Pérez A, Santos-de-Frutos K, De la Rosa S, Herranz-Montoya I, Perna C, Djouder N (2022). Transit-amplifying cells control R-spondins in the mouse crypt to modulate intestinal stem cell proliferation. *J Exp Med* 219, e20212405.
- Garrido A, Kim E, Teijeiro A, Sánchez Sánchez P, Gallo R, Nair A, Matamala Montoya M, Perna C, Vicent GP, Muñoz J, Campos-Olivas R, Melms JC, Izar B, Schwabe RF, Djouder N (2022). Histone acetylation of bile acid transporter genes plays a critical role in liver cirrhosis. *J Hepatol* 76, 850-861.
- Rigual MDM, Sánchez Sánchez P, Djouder N (2022). Is liver regeneration key in hepatocellular carcinoma development? *Trends Cancer*. PMID: 36347768.

AWARDS AND RECOGNITION

- BBVA grant Award in Biomedicine 2022, Spain.
- Spanish national grant from the Ministry of Science and Innovation (*Retos* 2022).
- Member of the European Association for the Study of Diabetes (EASD).

TRANSFORMATION AND METASTASIS GROUP

Eva González Suárez
Group Leader

Research Scientists
Patricia González, María Jiménez, Gema Pérez

Post-Doctoral Fellows
Sara Lázaro (April-August), Angélica Santiago (since April)

Gradutate Students
Alexandra Barranco, Marina Císcar (until September), Alejandro Collado, Jaime Redondo, Andrea Vethencourt (Clinical Oncologist at *ICO/IDIBELL*, Barcelona, Spain)

Undergraduate Student
Pedro Luis Echevarria (until February)

Technicians
Lucía de Andrés (since April), Víctor López, Sergi Velasco (until September) (PEJ, CAM)*

'Plan de Empleo Joven de la Comunidad de Madrid (Youth Employment Plan, Community of Madrid)

Students in Practice
Pablo Blanco (February-July) (Master's Thesis, *UAM,* Madrid, Spain), Isabel Jiménez (Jan.-July) (Master's Thesis, *UCM,* Madrid, Spain), Teresa Martí (Jan.-July) (*AECC* Traineeship and BS Thesis, *Univ. Francisco de Vitoria*, Spain)

Visiting Scientists
Emilia Brizzi (June-July) (Pathology resident, *Hospital La Paz*, Madrid, Spain), Marta Matas (Sept.-Nov.) (Althaia, *Xarxa Assistencial Universitària de Manresa*, Spain)

OVERVIEW

Tumours exploit and manipulate for their benefit the same mechanisms that regulate homeostasis in healthy tissue. In the Transformation and Metastasis Group, we aim to understand normal mammary gland development and the key events that lead to tumour initiation, progression, and metastasis in order to identify novel therapeutic targets to combat breast cancer. We use complementary tools, including primary cell cultures and organoids, lineage tracing mouse models, and clinical samples with the goal of translating basic knowledge into clinically relevant findings.

"Analyses of clinical samples and functional experiments in patient-derived xenografts demonstrate that RANK protein expression in tumour cells is associated with poor survival in ER negative breast cancer, and its inhibition improves chemotherapy response."

RESEARCH HIGHLIGHTS

RANK is a poor prognosis marker and a therapeutic target in ER-negative postmenopausal breast cancer

Despite strong preclinical data, the therapeutic benefit of denosumab in breast cancer, beyond the bone, is unclear. Aiming to select patients who may benefit from denosumab, we analysed RANK and RANKL expression in more than 2000 breast tumours (777 oestrogen receptor-negative, ER^-) from 4 independent cohorts. RANK expression was more frequent in ER^- tumours, where it associated with poor outcome and poor response to chemotherapy. In patient-derived orthoxenografts (PDXs) of ER^- breast cancer, RANKL inhibition reduced tumour cell proliferation and stemness, regulated tumour immunity and metabolism, and improved response to chemotherapy.

Intriguingly, RANK expression was associated with poor prognosis in postmenopausal breast cancer patients, activation of NFKB signalling, and modulation of immune and metabolic pathways, suggesting that RANK signalling increases after menopause. Indeed, RANKL inhibition showed greater therapeutic benefit in ER^- breast cancer PDXs under postmenopausal conditions. Our results demonstrate that RANK expression is an independent biomarker of poor prognosis in postmenopausal patients with ER^- breast cancer and support the therapeutic benefit of RANK pathway inhibitors in breast cancer patients with $RANK^+$ ER^- tumours after menopause (FIGURE 1).

Luminal Rank loss decreases cell fitness leading to basal cell bipotency in parous mammary glands

Rank signalling is a known regulator of mammary gland homeostasis, being critical for stem cell maintenance and epithelial cell differentiation. Although the Rank receptor is highly expressed by basal cells and luminal progenitors, its role in each individual cell lineage remains unclear. By combining temporal/lineage specific *Rank* genetic deletion with lineage tracing techniques, we found that loss of luminal Rank leads to aberrant alveolar-like differentiation in virgin mammary glands, reminiscent of pregnancy, and an increase in hormone-sensing luminal population (PR/Rankl-positive cells). During a first pregnancy, Rank-deleted luminal cells are unable to produce milk and expand following successive pregnancies. This results in a "tissue-damage like" scenario in the developing alveoli leading to basal bipotency and the replacement of "unfit luminal cells" by Rank-proficient cells to restore lactation. Transcriptomic analysis and functional assays point to a dual role for luminal Rank signalling in the control of protein translation. In the virgin mammary gland, Rank-depleted luminal cells show aberrant expression of lactogenic genes and increased protein synthesis. This aberrant differentiation exhausts the protein synthesis capability of the parous Rank-depleted luminal cells, making them unable to cope with the high translational demands required for milk production upon pregnancy. Consequently, basal bipotency is awakened through the activation of Rank/NF-κB signalling in basal parous cells of the alveoli in successive pregnancies to restore lactation and tissue homeostasis (FIGURE 2). ■

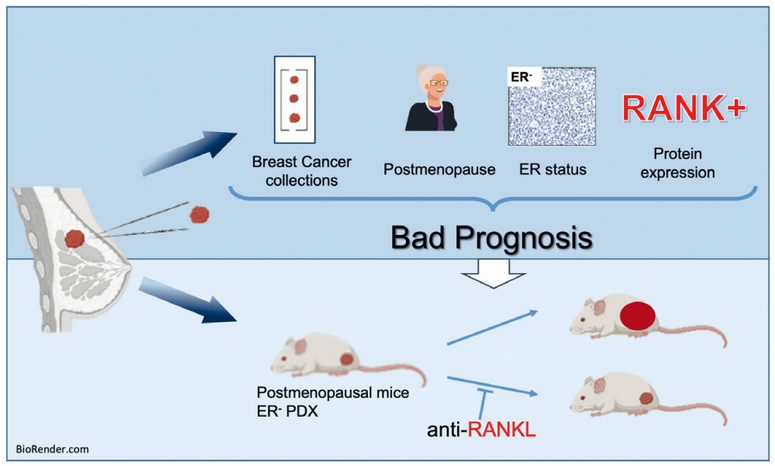

FIGURE 1

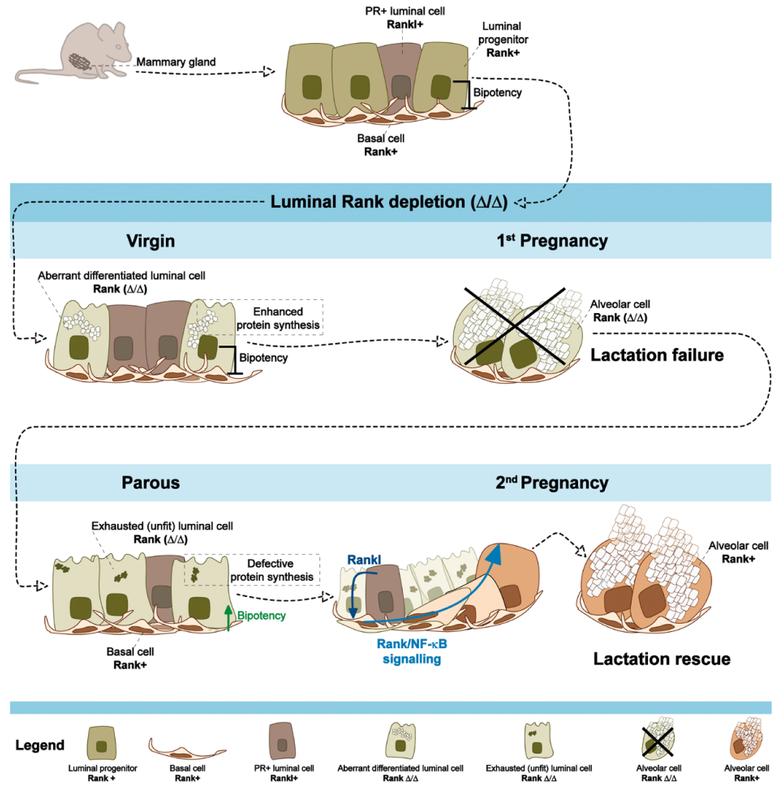

FIGURE 2

PUBLICATION

- Vethencourt A, Trinidad EM., Petit A., Soler Monso MT, Gomez Aleza C, Urruticochea A, Garcia-Tejedor A, Guma Martinez A, Obadia V, Vazquez S, Villanueva R, Fernanez A, Cejuela M, Penabad R, Stradella A, Gil-Gil M, Pernas S, Gonzalez-Suarez E, Falo C (2022). First results of the randomized window of opportunity clinical trial D-Biomark: immunomodulatory effect of denosumab in early breast cancer. *Cancer Res* 82 (4_Supplement), P2-10-P2-08-10.

AWARDS AND RECOGNITION

Eva González Suárez:
- ERC Proof of Concept Grant 2022, European Research Council.
- SenesceX-CM Consortium Coordinator, Community of Madrid (*CAM*).
- Invited Speaker, Basel Breast Consortium annual meeting on "Personalized breast cancer treatments", November 2022, Basel, Switzerland.
- Forum Participant, Forbeck Forum "Aneuploidy in cancer development, prognosis and treatment", April 2022, Lago Maggiore, Italy.

Alejandro Collado:
- Selected oral presentation, Gordon Conference in Mammary Gland Biology 2022, Barga, Italy.
- Selected oral presentation, Workshop "*Fat sensing and the brain control of puberty, 2022,* Baeza, Spain.

Alexandra Barranco:
- ECI Best Poster Award 2022, The European Congress of Immunology.

Jaime Redondo:
- Selected oral presentation, annual meeting of the Spanish Cell Senescence Network, September 2022, Valencia, Spain.

MICROENVIRONMENT & METASTASIS JUNIOR GROUP

Héctor Peinado
Junior Group Leader

Research Scientist
Susana García

Post-Doctoral Fellows
Marta Hergueta, Laura Nogués

Gradutate Students
Enrique Bastón, Elena Castellano (until December), Juan García-Agulló, Teresa González (until March), Alberto Hernández

Technicians
Sara Sánchez-Redondo, Vanesa Santos

Students in Practice
Eduardo Garvín (until Feb.) (*Univ. Francisco de Vitoria*, Madrid, Spain), Sandra López (March-July) (Bachelor's Degree Final Project Student, *Univ. Francisco de Vitoria*, Madrid, Spain)

Visiting Scientist
Marion Pascale (January-June) (*Instituto de Oncología Vall D´Hebron*, Barcelona, Spain)

OVERVIEW

In the Microenvironment and Metastasis laboratory, we are interested in understanding the crosstalk between tumour and stromal cells along metastatic progression. We are interested in how tumour cells can extrinsically influence the evolution of cancer during metastatic spread. For this purpose, we are analysing 1) the role of small extracellular vesicles (sEVs) in primary tumour evolution and pre-metastatic niche formation in melanoma, prostate and pancreatic cancer, and 2) the influence of obesity in breast cancer metastasis, as well as defining 3) the relevance of nerve growth factor receptor (NGFR) in melanoma, oral squamous cell carcinoma, and bladder cancer metastasis, aiming to develop new targeted therapies.

"We analyse the intrinsic and extrinsic mechanisms involved in metastatic dissemination, aiming to develop novel therapeutic targets."

RESEARCH HIGHLIGHTS

Relevance of extracellular vesicles in tumour cell evolution and metastasis. Extracellular vesicles (EVs) contain different biomolecules including DNA and RNA. However, the importance of nucleic acids in EVs and the consequences of its transfer to the tumour microenvironment are poorly understood. We are exploring the influence of tumour-shed EVs in surrounding tumour cells, stroma, and healthy tissue during tumour progression. In addition, we are analysing EV-associated nucleic acids as surrogate markers of tumour progression, developing highly-sensitive methods for detecting minimal residual disease and metastatic risk. Moreover, we are currently investigating the role of extracellular vesicles in prostate cancer premetastatic niche formation through the analysis of their molecular cargo and their influence in the lymph node microenvironment. We aim to define novel biomarkers of early dissemination by liquid biopsy and potentially new anti-metastatic therapies.

Understanding the link between obesity and breast cancer metastasis. Since obesity is linked to hypercoagulability and increased risk of breast cancer, we are evaluating if high-fat diet (HFD) influences breast cancer metastasis. We observed that HFD increases tumour-platelet-endothelial cell interaction favouring tumour cell homing and metastasis. Importantly, our data support that anti-platelet therapies reduce tumour cell homing and metastasis in HFD-fed mice, supporting the observation that anticoagulant agents or caloric intake reduction could modify premetastatic niche formation, decreasing metastasis in obesity models of triple-negative breast cancer (TNBC).

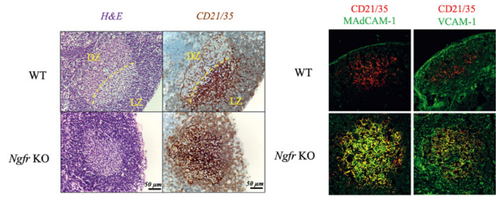

FIGURE 1 *Ngfr* loss leads to lymph node germinal center (GC) hyperproliferation, aberrant structure, and loss of polarisation. Representative H&E and CD21/35 IHC staining (left panels) and immunofluorescence (right panels) of the indicated markers. Immunised wt mice and *Ngfr* KO germinal centres are shown in left panels. Yellow dashed lines divide GC dark zone (DZ) and light zone (LZ). Wt and KO *Ngfr* KO GCs are shown in right panels analysing the expression of the follicular dendritic cell activation markers CD21/35 (red), MadCAM1 or VCAM1 (green); co-expression of markers is shown in yellow. Observe the lack of polarisation and structure in *Ngfr* KO GCs.

Defining the role of NGFR in tumour progression, lymphoproliferative diseases, and autoimmunity. NGFR is emerging as a key gene for metastatic spread and therapy resistance in several tumour types. We are analysing the role of NGFR in tumour metastasis and developing new therapies targeting NGFR to improve immunotherapy treatment in metastatic melanoma and other tumours such as oral squamous cell carcinomas (OSCC) and bladder carcinomas. Moreover, our data support a novel role for NGFR regulating immunity and cell proliferation in lymph nodes, suggesting an important role in follicular lymphoma or autoimmune disorders (FIGURE 1). ■

PUBLICATIONS

- Carretero-González A, Hergueta-Redondo M, Sánchez-Redondo S, Ximénez-Embún P, Manso Sánchez L, Gil EC, Castellano D, de Velasco G, Peinado H (2022). Characterization of plasma circulating small extracellular vesicles in patients with metastatic solid tumors and newly diagnosed brain metastasis. *Oncoimmunology* 11, 2067944.
- González-Muñoz T, Kim A, Ratner N, Peinado H (2022). The need for new treatments targeting MPNST: the potential of strategies combining MEK inhibitors with antiangiogenic agents. *Clin Cancer Res* 28, 3185-3195.
- Leary N, Walser S, He Y, Cousin N, Pereira P, Gallo A, *et al.* (incl. Peinado H) (2022). Melanoma-derived extracellular vesicles mediate lymphatic remodelling and impair tumour immunity in draining lymph nodes. *J Extracell Vesicles* 11, e12197.
- Almansa D, Peinado H, García-Rodríguez R, Casadomé-Perales Á, Dotti CG, Guix FX (2022). Extracellular vesicles derived from young neural cultures attenuate astrocytic reactivity in vitro. *Int J Mol Sci* 23, 1371.
- Cardeñes B, Clares I, Bezos T, Toribio V, López-Martín S, Rocha A, Peinado H, Yáñez-Mó M, Cabañas C (2022). ALCAM/CD166 is involved in the binding and uptake of cancer-derived extracellular vesicles. *Int J Mol Sci* 23, 5753.
- Santos-Coquillat A, González MI, Clemente-Moragón A, González-Arjona M, Albaladejo-García V, Peinado H *et al.* (2022). Goat milk exosomes as natural nanoparticles for detecting inflammatory processes by optical imaging. *Small* 18, e2105421.

PATENT

- Peinado Selgas H, Saragovi HU, García Silva S, Nogués Vera L, Hernández Barranco A. THX-B for treating and preventing cancer and metastasis. PCT Appliction (2022). *PCT/EP2022/070597. WO2023002008A1.*

AWARDS AND RECOGNITION

Héctor Peinado:

- XII National Cancer Research Award "*Doctores Diz Pintado*", Spain.
- Listed in the "World Ranking Top 2% Scientists", 2022 edition of the Stanford University list of World Top 2% scientists.

BRAIN METASTASIS JUNIOR GROUP

Manuel Valiente
Junior Group Leader

Post-doctoral Fellows
Mariam Al-Masmudi, Lluís Cordón, Neibla Priego

Gradutate Students
Laura Adriana Álvaro, Ana de Pablos Aragoneses, Pedro García, Carolina Hernández, Irene Salgado

Technicians
Patricia Baena, Virginia García-Calvo (since April), María Perea (until November), Diana Patricia Retana, Oliva Ana Sánchez

Lab Administrative Manager
Jorge Guillermo Ortiz (since March)

Visiting Student
Irene Cornejo (June-December) (Summer Traineeship, *Universidad Francisco de Vitoria*, Madrid, Spain)

OVERVIEW

Brain metastasis is the most common neurological complication of cancer. When metastatic cells reach the brain, prognosis is poor given that local therapies (i.e., surgery and radiation) have limited benefit for patients, and the disease inevitably progresses. The rise in the number of patients with brain metastasis is partially due to the increasing number of systemic therapies that work extra-cranially but are unable to provide the same therapeutic benefit in the brain. Consequently, cancer cells present at this secondary site have additional time to evolve and to grow into clinically detectable lesions. In the laboratory, we study why and how cells from different cancer types (breast cancer, lung cancer and melanoma) are able to access the brain, survive and colonise this vital organ. We dissect the biology of these processes *in vivo* using experimental models and patient-derived material in order to challenge the current status of this unmet clinical need.

"We reported the first strategy involving a liquid biopsy biomarker and a non-toxic radiosensitizer to personalise the use of radiotherapy in patients with brain metastasis."

RESEARCH HIGHLIGHTS

In 2022, we established a novel research line in Cancer Neuroscience, aiming to understand the biology underlying the neurocognitive impact of brain metastasis.

Among other activities, additional single cell approaches (i.e., spatial transcriptomics) were incorporated into our experimental pipeline.

We also consolidated research findings, with an impact on various aspects relevant for brain metastasis, such as novel strategies for immunotherapy, new cellular targets within the pro-metastatic microenvironment, and an unexpected avenue for preventing metastasis.

And, finally, we consolidated our scientific strategy as a productive source of findings to be translated from bench to bedside. The most recent examples are the clinical studies following from the discovery of a biomarker of radiosensitivity compatible with liquid biopsy (now part of a prospective observational multicentric clinical study) and the clinical trial combining a RAGE inhibitor and radiotherapy (now in phase I/II trial). ■

FIGURE (**a**) The microenvironment enhances the secretion of S100A9 from cancer cells that binds to RAGE, which could be targeted with a specific inhibitor. (**b**) Targeting of S100A9 blocks brain metastasis radioresistance. (**c**) S100A9 is a biomarker of radioresistance from liquid biopsy.

PUBLICATIONS

- Monteiro C**, Miarka L**, Perea-García M, Priego N, García-Gómez P, Álvaro-Espinosa L, de Pablos-Aragoneses A, Yebra N, Retana D, Baena P, Fustero-Torre C, Graña-Castro O, Troulé K, Caleiras E, Tezanos P, Muela P, Cintado E, Trejo JL, Sepúlveda JM, González-León P, Jiménez-Roldán L, Moreno LM, Esteban O, Pérez-Núñez Á, Hernández-Lain A, Mazarico Gallego J, Ferrer I, Suárez R, Garrido-Martín EM, Paz-Ares L, Dalmasso C, Cohen-Jonathan Moyal E, Siegfried A, Hegarty A, Keelan S, Varešlija D, Young LS, Mohme M, Goy Y, Wikman H, Fernández-Alén J, Blasco G, Alcázar L, Cabañuz C, Grivennikov SI, Ianus A, Shemesh N, Faria CC, Lee R, Lorigan P, Le Rhun E, Weller M, Soffietti R, Bertero L, Ricardi U, Bosch-Barrera J, Sais E, Teixidor E, Hernández-Martínez A, Calvo A, Aristu J, Martin SM, Gonzalez A, Adler O, Erez N; RENACER, Valiente M*. Stratification of radiosensitive brain metastases based on an actionable S100A9/RAGE resistance mechanism (2022). *Nat Med* 28, 752-756. (**) Shared authorship. (*) Corresponding author.
  - *A prospective multicentric observational study will be initiated based on these results.*
  - *A phase I/II clinical trial will be initiated based on these results combining a RAGE inhibitor with radiotherapy.*

  See also:
  - *Nature Medicine*. DOI: 10.1038/s41591-022-01776-5 (11 April 2022).
  - *Cancer Research*. DOI: 10.1158/0008-5472.CAN-82-11-BI (6 June 2022).
- Zhu L, Retana D, García-Gómez P, Alvaro-Espinosa L, Priego N, Masmudi-Martín M, Yebra N, Miarka L, Hernández-Encinas E, Blanco-Aparicio C, Martínez S, Sobrino C, Ajenjo N, Artiga MJ, Ortega-Paino E, Torres-Ruiz R, Rodríguez-Perales S; RENACER, Soffietti R, Bertero L, Cassoni P, Weiss T, Muñoz J, Sepúlveda JM, González-León P, Jiménez-Roldán L, Moreno LM, Esteban O, Pérez-Núñez A, Hernández-Laín A, Toldos O, Ruano Y, Alcázar L, Blasco G, Fernández-Alén F, Caleiras E, Lafarga M, Megías D, Graña-Castro O, Nör C, Taylor MD, Young LS, Varešlija D, Cosgrove N, Couch FJ, Cussó L, Desco M, Mouron S, Quintela-Fandino M, Weller M, Pastor J and Valiente M*. A clinically-compatible drug-screening platform based on organotypic cultures identifies vulnerabilities to prevent and treat brain metastasis (2022). *EMBO Mol Med* 14, E14552. (*) Corresponding author.

PATENT

- Zhu L, Graña-Castro O, Valiente M (2022). Signature for the prognosis of brain metastasis relapse after brain surgery. *EP 22382484.8*.

AWARDS AND RECOGNITION

Manuel Valiente:
- Chair of the EANO Scientific Committee, the European Association of Neuro-Oncology.
- Board Member-Elect of the Metastasis Research Society (MRS).
- EACR Reviewers' Panel (panel member), the European Association for Cancer Research.

METABOLISM AND CELL SIGNALLING JUNIOR GROUP

Alejo Efeyan
Junior Group Leader

Research Scientist
Bárbara Martínez

Post-Doctoral Fellow
Yurena Vivas

Gradutate Students
Lucía de Prado, Nerea Deleyto, Elena Fernández (since October), Ana Belén Plata, Elena Sánchez

Technicians
Alba Sanz

Titulado Superior (Advanced Degree)

Students in Practice
Elena Fernández (until June)

(Master's Thesis, *UCM*, Madrid, Spain), Paula Seghers (since Oct.) (Undergraduate Student, *Universidad Politécnica de Madrid*, Spain), Andrea Pinilla (since Nov.) (Undergraduate Student *Universidad Industrial de Santander*, Spain),

Camila Silva (until Aug.) (Master's Thesis, *UAM*, Madrid, Spain)

Visiting Scientists
Cristina Lebrero and Ana Ortega (*Centro de Biología Molecular Severo Ochoa*, *CBMSO*, Madrid, Spain),

Sebastian Thompson (*IMDEA Nanociencia* Institute, Madrid, Spain)

OVERVIEW

In the Metabolism & Cell Signalling Lab we study the links between nutrients, cancer and ageing. All our cells integrate signals emanating from the abundance of intracellular nutrients and from the nutritional state of the entire organism. Integration of these signals is key for adjusting metabolic functions, as well as for energy storage and expenditure, and importantly, the components of these signalling cascades are generally corrupted in cancer and are drivers of the metabolic complications of chronic nutrient overload. Conversely, dietary restriction regimes are extremely efficacious interventions against tumorigenesis and to delay the process of ageing, albeit we still ignore the fundamental molecular underpinnings of such protective effects. We combine mouse genetics and cell biological tools to gain insight into the genetic and environmental corruptions of nutrient signalling cascades, aiming to conceive therapeutic interventions in the context of cancer, obesity, and the process of ageing.

"We are beginning to understand how excessive nutrient levels deregulate cellular metabolism as well as cell-to-cell and interorgan communication, contributing to tumour development and the process of ageing."

RESEARCH HIGHLIGHTS

Cellular nutrients, such as amino acids and glucose, and systemic metabolic hormones such as insulin, are key mediators of cellular metabolism by control of the mTORC1 kinase, a master switch for most anabolic processes in the cell. We and others have previously dissected the impact of deregulated nutrient signalling (N-ON mice, mimicking a chronic increase in intracellular nutrient levels) and deregulated hormonal signalling (H-ON mice, mimicking chronically high levels of insulin signalling) in the mouse liver. While genetic activation of either input resulted important to unleash the metabolism of the fasted state, chronic nutrient surplus in humans typically causes synchronous, concomitant activation of nutrient and hormonal signalling. Thus, we generated a mouse strain harbouring deregulated nutrient and hormonal signalling to mTORC1 in hepatocytes (N+H-ON). Genetic activation of either nutrient or hormonal signalling on their own resulted in high mTORC1 activity, regardless of the fed/fasted state of the mice. To our surprise, simultaneous activation of both nutrient and hormonal signalling (N+H-ON) resulted in a minimal additional increase in mTORC1 signalling, as compared to either H-ON or N-ON livers (FIGURE 1A). In contrast to this mild increase, the livers of the N+H-ON mice showed multiple evidence of a synergic interaction between nutrient and hormonal signalling. These include a large increase in liver size, accumulation of several markers of liver damage, and aberrant bile acid and bilirubin metabolism (FIGURE 1B, C and D). In addition, N+H-ON mice experience rapid development of liver carcinomas, starting at 15 weeks of age (FIGURE 1E). Pharmacological suppression of mTORC1 by rapamycin (FIGURE 1F) dramatically delays tumorigenesis and corrects metabolic defects, while chronic restriction in caloric intake (diminishing circulating nutrients and circulating growth factors) fails to correct the phenotype (FIGURE 1G). These findings support a critical role for nutrient and hormonal signalling through mTORC1 in the beneficial effects of dietary restriction. ■

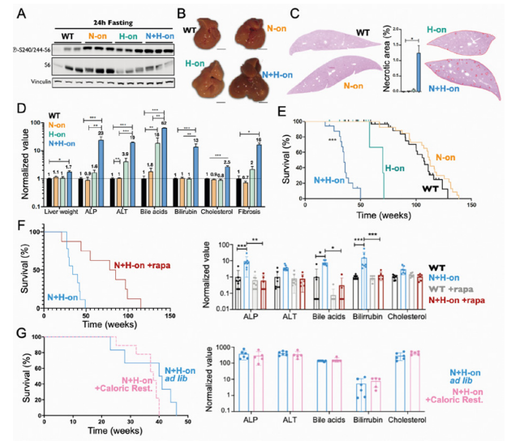

FIGURE 1 (**A**) Increase in mTORC1 activity (seen by phospho-S6) in livers from fasted N+H-ON mice. (**B**) Enlarged N+H-ON livers. (**C & D**) Readouts of liver damage in livers from N+H-ON mice. (**E**) Premature death of N+H-ON mice due to liver carcinomas. Rapamycin (**F**) corrects survival and readouts of liver damage in N+HON mice, but caloric restriction (**G**) does not.

PUBLICATIONS

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AWARDS AND RECOGNITION

- Yurena Vivas was recipient of a *Fundación Domingo Martinez* CNIO Friends Fellowship.
- Ana Belen Plata received an Award for best Selected Short Talk in the EMBO Meeting on Energy Balance in Metabolic Disorders, October 2022, Torremolinos, Spain.
- Nerea Deleyto, excellent *Cum Laude* thesis defence and 'Outstanding PhD Thesis' award in the field of molecular biosciences, Autonomous University of Madrid, Spain.

CANCER IMMUNITY JUNIOR GROUP

María Casanova-Acebes
Junior Group Leader

Gradutate Students
Eduardo Garvín (since December), Federico Lupo (until April), Mariola Munárriz (since November), Enrique Nogueira (until October)

OVERVIEW

The Cancer Immunity lab studies myeloid cells in the different tumour microenvironments. By focusing on the remarkable heterogeneity of these cells in a tissue-based manner, we aim to uncover their functional roles in shaping T cell responses.

First, we focus on how myeloid training can impact long-term anti-tumour responses. Next, we study how resident macrophages in the lung and in the ovary shape tumour-associated fibroblasts and metabolic responses, respectively. Lastly, we analyse how circadian biology impacts the initiation, progression and unresponsiveness to current therapies in lung cancer.

"Our laboratory is dissecting novel modulators of tumour immunity by analysing the crosstalk of myeloid cells with the stroma and other physiological cues, such as time-dependency of immune responses and diet-modulatory effects on suppressive and malignant haematopoiesis in solid tumours."

Technicians
Nines Sanguino Acosta (until October), Sheila Artesero (since April)

Bioinformatician
Gonzalo Soria (since April)

Students in Practice
Sheila Artesero (since May) (Master´s Thesis, *ENS-ISCIII*, Madrid, Spain),

Lucía Córdoba (until April) (Bachelor's Degree Final Project, *UCM,* Madrid, Spain), Ainhoa Muñoz (until May) (Bachelor's Degree Final Project, *UAM,* Madrid, Spain)

HIGHLIGHTS

During 2022, we consolidated our laboratory and achieved competitive national and international funding.

We also hosted and trained 2 bioinformaticians, 2 medical doctors and 3 undergraduate students.

In 2023, we aim to expand our team and to continue to fight for cancer cures using innovative myeloid targeting. ■

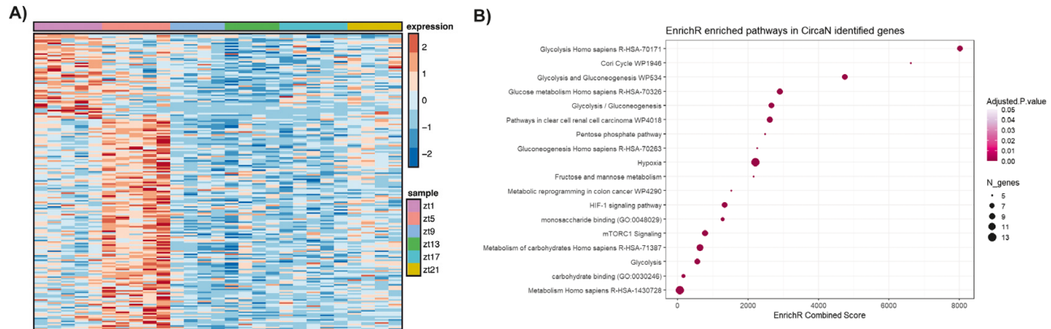

FIGURE 1 (**A**) Bulk transcriptomics on lung tumour cells (KP orthotopic model) over a diurnal cycle (24h) showing circadian genes. ZT, *zeitgeber time*. ZT1, indicates 1h after lights are turned on (i.e., 8am). (**B**) Gene ontology pathways enriched in the morning in lung cancer cells.

PUBLICATION

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AWARDS AND RECOGNITION

- *XXII Beca FERO* 2022 in Translational Oncology Research, FERO Foundation for Oncology Research, Spain.
- Education Committee Member, AACR Annual Meeting 2023.