The Molecular Oncology Programme (MOP) is the largest research programme at the CNIO, hosting 9 Senior and 4 Junior Groups. Scientists at the MOP focus on trying to obtain a mechanistic understanding of how cells in our body work, as well as to identify the molecular alterations at the cellular level that drive carcinogenesis. To do so, MOP research groups use a wide range of technologies including molecular and cellular biology, mouse models, and genetic and chemical screens. The Programme encompasses expertise related to some of the most active areas of research in molecular oncology, including DNA and chromosome stability (Maria A. Blasco, Óscar Fernández-Capetillo, and Ana Losada), oncogenes and cell cycle kinases (Mariano Barbacid), DNA replication (Juan Méndez), mitosis (Marcos Malumbres), melanoma (María S. Soengas), metabolism and cell signalling (Alejo Efeyan), and metastasis (Manuel Valiente and Héctor Peinado). In addition, starting in 2019, the MOP will also host the Groups of Epithelial Carcinogenesis (Francisco X. Real), Growth Factors, Nutrients and Cancer (Nabil Djouder) and Brain Tumours (Massimo Squatrito), which will broaden our areas of interest and significantly strengthen the Programme.

In terms of scientific publications, this year once again, the Molecular Oncology Programme has continued its positioning on the frontline of oncology research. The top-level quality of the research conducted by each of these Groups is exemplified through 9 papers published in Nature journals (Nature Medicine, Nature Reviews Cancer, Nature Cell Biology, Nature Communications, Nature Structural and Molecular Biology), 4 papers in Cell Journals (Cancer Cell, Cancer Discovery) and 1 paper in a Science Journal (Science Translational Medicine), in addition to the excellent contributions in Circulation Research, Current Opinion in Cell Biology, Journal of Clinical Investigation, Journal of Experimental Medicine and Nucleic Acids Research.

Besides from publications, scientists at the MOP have continued to be leaders in their respective areas of research and have made important contributions in several areas such as generating patents, organising conferences or bridging the gap with the clinical world.

Maria A. Blasco, Director
Óscar Fernández-Capetillo, Vice Director
We study the mechanisms by which tumour cells are immortal and normal cells are mortal. Immortality is one of the most universal characteristics of cancer cells. 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 and are 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 research focuses on:

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

“We have demonstrated that telomerase activation in mouse models of pulmonary fibrosis can stop the progression of this fatal disease in mice.”
Telomerase gene therapy to cure pulmonary fibrosis in mice

Pulmonary fibrosis is a fatal lung disease that currently lacks effective treatment and is characterised by fibrotic foci and inflammatory infiltrates. Short telomeres can impair tissue regeneration and are found both in hereditary and sporadic cases. We have shown the therapeutic effects of AAV9-telomerase gene therapy in a mouse model of pulmonary fibrosis from a combination of bleomycin-induced lung damage short telomeres. AAV9 targets preferentially regenerative alveolar type II cells (ATII). Treated mice show improved lung function and lower inflammation and fibrosis at 1-3 weeks after treatment, and improvement or disappearance of the fibrosis at 8 weeks post treatment. Treatment results in longer telomeres and increased proliferation of ATII cells, as well as lower DNA damage, apoptosis, and senescence. We have provided a proof-of-principle that telomerase activation may represent an effective treatment for pulmonary fibrosis provoked by or associated to short telomeres.

Telomerase gene therapy does not increase risk of cancer-in-prone models

Telomeras are important epigenetic regulators

Telomeras are long non-coding RNAs that protect the telomeres. Our Group had already identified chromosome 20q as one of the main origins of human Telomeras and demonstrated that, by generating the first 20q-TERRA knockout models, they are

essential for telomere length maintenance and protection. Using 20q-TERRA knockout cells we have now addressed the direct role of Telomeras in telomeric heterochromatin formation. We discovered that Telomeras interact with components of the polycomb complex (PCRF), an important epigenetic regulator of gene expression, thus facilitating the assembly of telomeric heterochromatin. We analysed telomere heterochromatin marks in cells deficient for 20q-TERRA and observed that their telomeres had decreased heterochromatic marks; we discovered that they had lost a histone mark not previously recognised at telomeres (H3K27 methylation) that is catalysed by PCRF, a master regulator of gene silencing, which we found locates to the telomere in a TERRA-dependent manner. Our findings demonstrated an important role for TERRAs in telomeric heterochromatin assembly (FIGURE 2).

Telomerase gene therapy to cure pulmonary fibrosis

Telomerase gene therapy makes possible the deposition of heterochromatin marks. These events cannot take place when TERRAs are absent.

![Figure 1](image1.png)

**Figure 1** Representative images of lungs treated with gene therapy vectors (GT). Nuclei are in blue, alveolar type II cells in green and telomeres in red. Lung cells treated with telomerase present the most intense telomeres, indicating that they are the longest of all three scenarios.

**Figure 2** TERRAs regulate the status of telomeric chromatin. After binding to the PCRF complex TERRAs bring PCRF to the telomere. Binding of PCRF makes possible the deposition of heterochromatin marks. These events cannot take place when TERRAs are absent.

**PUBLICATIONS**

**OVERVIEW**

**KRAS** oncogenes have been identified in at least one fifth of all human cancers. In spite of recent successes with checkpoint inhibitors, most KRAS mutant tumours, including lung adenocarcinomas, are still treated with cytotoxic compounds approved over 2 decades ago. Moreover, attempts to block KRAS oncogenic activity with selective inhibitors of the MEK kinase, a downstream effector, have turned out to be major failures. Two MEK inhibitors, Trametinib and Selumetinib, have failed to show significant anti-tumour activity in large phase III clinical trials due to unacceptable toxicities. In our laboratory, we have continued our quest to validate therapeutic targets using a new generation of genetically engineered mouse tumour models that allow us to evaluate their anti-tumour properties as well as their potential toxic effects in tumour-bearing mice. These studies have allowed the identification of c-RAF as a target capable of inducing significant tumour regressions in advanced KRAS/TRP53 mutant lung tumours without inducing major toxicities. These observations suggest that forthcoming c-RAF inhibitors may provide significant therapeutic benefits in the clinic.
Regression of advanced K-Ras/Tp53 mutant lung adenocarcinomas upon systemic ablation of e-Raf expression

Almost a quarter of all solid tumours harbour K-Ras oncogenes. Yet, more than 30 years after their identification in human cancer, there are no selective therapies to treat these tumours. Inhibitors of K-Ras oncogenes activated by G12C mutations, a mutation frequently identified in lung adenocarcinomas, have already entered clinical trials. Yet, direct targeting of other mutations has proven to be challenging. Genetic interrogation of the MAPK pathway revealed that systemic ablation of Mek or Erk kinases in adult mice prevent tumour development but are unacceptable toxic. This year, we demonstrated that ablation of e-Raf expression in advanced mouse lung tumours driven by K-Ras/G12D/Tp53 mutations led to significant tumour regression with no detectable appearance of resistance mechanisms (Figure 1). Tumour regression results from massive apoptosis. Importantly, systemic abrogation of e-Raf expression does not inhibit canonical MAPK signalling, hence, resulting in limited toxicities. These observations suggest that therapeutic strategies aimed at inhibiting c-Raf kinase activity may not be suitable since they may only block MAPK Kinase activation. Indeed, three independent c-Raf kinase inhibitors have shown to be rather toxic even at non-therapeutic doses. Therefore, we need to explore other therapeutic strategies. Drugs capable of degrading the c-Raf protein could be most effective in the clinic, yet, drugs that promote protein degradation are still at very early stages of development. Inhibition of e-Raf by small interfering RNA is also possible, but still faces significant technical challenges. Finally, selective targeting c-Raf effectors regulated through non-kinase mechanisms such as ROKalpha, ASK1 and MST2 will also be challenging since they will require toxicities. These observations suggest that therapeutic strategies. In an attempt to clarify the role of the desmosomal stroma in PDAC development and progression, we decided to reprogram the CAPS tumours that make up the stromal tissue by identifying, and subsequently targeting, genes responsible for their pro-tumorigenic properties. First, we compared the transcriptional profile of PDGFβR+ CAPS isolated from genetically engineered mouse PDAC tumours with that of normal pancreatic fibroblasts (NFs) in order to identify genes potentially implicated in their pro-tumorigenic properties. We have observed that the most differentially expressed gene, Saa3, a member of the acute-phase protein family, is a key mediator of the pro-tumorigenic activity of PDGFβR+ CAPS. Whereas Saa3 competent CAPS stimulates the growth of PDAC tumour cells in an orthotopic model, Saa3 null CAPS inhibit tumour growth. Saa3 plays a role in the cross-talk between CAPS and tumour cells (Figure 2). Ablation of Saa3 in pancreatic tumour cells makes them insensitive to the inhibitory effect of Saa3 null tumour cells. As a consequence, germline ablation of Saa3 does not prevent PDAC tumour development in mice (Figure 2). The pro-tumorigenic activity of Saa3 in CAPS is mediated by Mpp6, a member of the palmitoylated membrane protein subfamily of the peripheral membrane-associated guanylate kinases. Finally, we interrogated whether these observations could be translated to a human scenario. Indeed, SAA1, the orthologue of murine Saa3, is overexpressed in human CAPS. Moreover, high levels of SAA1 in the stromal component correlate with worse survival. These findings support the concept that selective inhibition of SAA1 in CAPS may provide potential therapeutic benefit to PDAC patients.

**SAA1** is a key mediator of the pro-tumorigenic properties of cancer-associated fibroblasts in pancreatic ductal adenocarcinomas

Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant human tumours for which there are no efficacious therapeutic strategies. This tumour type is characterised by an abundant desmosomal stroma which promotes tumour progression. It is generally accepted that cancer-associated fibroblasts (CAFs) stimulate tumour progression and might be implicated in drug resistance and immunosuppression. Yet, recent studies have shown that physical or genetic elimination of the stroma leads to more aggressive tumour development. In an attempt to clarify the role of the desmosomal stroma in PDAC development and progression, we decided to reprogram the CAPS tumours that make up the stromal tissue by identifying, and subsequently targeting, genes responsible for their pro-tumorigenic properties. First, we compared the transcriptional profile of PDGFβR+ CAPS isolated from genetically engineered mouse PDAC tumours with that of normal pancreatic fibroblasts (NFs) in order to identify genes potentially implicated in their pro-tumorigenic properties. We have observed that the most differentially expressed gene, Saa3, a member of the acute-phase protein family, is a key mediator of the pro-tumorigenic activity of PDGFβR+ CAPS. Whereas Saa3 competent CAPS stimulates the growth of PDAC tumour cells in an orthotopic model, Saa3 null CAPS inhibit tumour growth. Saa3 plays a role in the cross-talk between CAPS and tumour cells (Figure 2). Ablation of Saa3 in pancreatic tumour cells makes them insensitive to the inhibitory effect of Saa3 null tumour cells. As a consequence, germline ablation of Saa3 does not prevent PDAC tumour development in mice (Figure 2). The pro-tumorigenic activity of Saa3 in CAPS is mediated by Mpp6, a member of the palmitoylated membrane protein subfamily of the peripheral membrane-associated guanylate kinases. Finally, we interrogated whether these observations could be translated to a human scenario. Indeed, SAA1, the orthologue of murine Saa3, is overexpressed in human CAPS. Moreover, high levels of SAA1 in the stromal component correlate with worse survival. These findings support the concept that selective inhibition of SAA1 in CAPS may provide potential therapeutic benefit to PDAC patients.
The Cell Division and Cancer Group is interested in deciphering the mechanisms by which cell division and cell proliferation are regulated in mammalian cells. During the last years, we have used different mouse models to understand the relevance of cell cycle regulators, including cell cycle kinases and phosphatases, as well as proteins involved in ubiquitin-dependent degradation, in the control of cell division and tissue physiology. Our interests are: i) to understand the basic control mechanisms that regulate the cell division cycle; ii) to characterise the physiological and therapeutic consequences of cell cycle deregulation; iii) understanding self-renewal and pluripotency in stem cell biology and tumour development; and iv) to find and validate new targets for cancer therapy. As a final goal, we aim to generate information that may be useful to improve therapeutic strategies against cancer cell proliferation.

“During 2018, we investigated the relevance of PLK1 and MASTL as oncogenes and as therapeutic targets in breast cancer, as well as the effects of MASTL mutations in patients with thrombocytopenia.”
Cell cycle progression is typically triggered by phosphorylation of a large number of proteins involved in different cellular pathways. Several families of protein kinases involved in cell cycle progression, such as Cyclin-dependent kinases (CDKs) or Polo-like kinases (PLK), have been thoroughly studied over the last few decades. However, the identity and relevance of phosphatases is less well-established. Recently, the cell cycle kinase Mastl (also known as Greatwall) emerged as a key player in cell cycle control by inhibiting the PP2A phosphatase during mitosis. Mastl phosphorylates 2 small proteins, endosulfine (ENSA) and ARPP19, which in their phosphorylated form bind and inhibit PP2A-B55 complexes, thus contributing to the phosphorylation of mitotic phosphoproteins (FIGURE 1).

However, its physiological relevance in normal tissue homeostasis or disease is less known. After an initial screening in several tumour types, we found that Mastl was upregulated in a significant fraction of breast tumours and correlated with poor prognosis in breast cancer patients (a collaboration with M.A. Quintela, CNIO, and C. Caldas, Cancer Research UK).

Importantly, genetic downregulation or ablation of Mastl results in defective proliferation of a subset of breast cancer cells, both in vitro and in vivo, suggesting the therapeutic potential of inhibiting this kinase in breast cancer (a collaboration with the Oncology R&D group at Pfizer, Álvarez-Fernández et al., Cell Death Differ 2018).

Before the function of Mastl in mitosis was proposed, the corresponding human gene was found mutated in patients with thrombocytopenia. To understand the effects of this mutation we recently generated a knockin mouse model carrying that mutation (Mastl E166D in the mouse). Mastl E166D mice developed thrombocytopenia but, unexpectedly, this defect was not due to abnormal cell cycle in megakaryocytes but to defective activation of mutant platelets. In the presence of this mutation, PP2A is constitutively inhibited resulting in hyperphosphorylation of proteins involved in actin cytoskeleton signalling during platelet activation. Mastl E166D mutant platelets were prematurely activated and displayed defective morphology and function, as well as decreased survival, thus contributing to thrombocytopenia (Hurtado et al., J Clin Invest, in press).

These data uncovered a new function of MASTL in the actin cytoskeleton in postmitotic cells, entailing important implications in human disease.

### MASTL: oncogene or tumour suppressor

Among the multiple kinases involved in cell cycle progression, PLK1 is considered an attractive cancer target and a few small-molecule inhibitors are currently under evaluation in clinical trials. However, our knowledge about the relevance of this protein in adult mammalian tissues is still limited. In 2017, we described a critical role for the mouse Plk1 in controlling the contraction of smooth muscle cells and blood pressure (de Cárcer et al., Nat Med 2017), suggesting possible toxic effects linked to the inhibiting of this kinase that need to be controlled in patients. More recently, we evaluated to what extent the expression levels of Plk1 contribute to tumour development in mouse models. Although Plk1 is frequently considered as an oncogene, we observed that Plk1 overexpression prevented proper cell proliferation by generating genomic aberrations in polyploid and aneuploid cells (FIGURE 2). Overexpression of Plk1 impaired breast cancer development induced by Kras or Her2 oncogenes, thereby suggesting a tumour suppressor function for this protein in these models (a collaboration with R. Sotillo, German Cancer Research Center, de Cárcer et al., Nat Commun 2018). Specifically, in human breast cancer, PLK1 overexpression correlated with better prognosis. Although these data do not argue against the use of PLK1 inhibitors in the clinic, they add new levels of knowledge that will be critical when optimising the use of mitotic inhibitors in cancer therapy.

![Cell cycle progression, maintenance of genomic stability, and support of tumour growth](image)

**Figure 2** A central role for Plk1 in controlling genomic stability. Plk1 controls several processes during the cell cycle, including centrosome maturation, spindle dynamics and the formation of the cytokinetic furrow. In normal conditions these functions support cell proliferation and tumour growth, and inhibiting Plk1 may prevent tumour cell proliferation.

*Note: All figures and images are placeholders and are not actual images.*
The Genomic Instability Group centres its research on understanding how cells respond to DNA damage, in particular to a specific type of harm known as replication stress (RS). Oncogene-induced RS has been confirmed as the main source of genomic rearrangements in cancer cells. In mammals, RS triggers a cellular response initiated by ATR and CHK1 kinases, known as the Replicative Stress Response (RSR). Throughout the years, our laboratory has developed a wide battery of cellular and animal tools for the study of the RSR. Among them, we have mice with enhanced or limited function of ATR and CHK1 kinases, cell lines in which the RSR can be activated at will and chemical inhibitors of ATR. Our studies have enhanced our understanding of the impact of RS on cancer and ageing, and have provided novel drugs with antitumoural potential that exploit the presence of RS in cancer cells. Overall, our goal is to understand the molecular mechanisms governing genome protection and repair – particularly during replication – and to exploit this knowledge as a way to fight against cancer.

“In 2018 we have, among other achievements, extended our line of research on the mechanisms of resistance to cancer therapies and revealed a novel mechanism by which cells couple DNA replication termination to mitotic entry.”
RESEARCH HIGHLIGHTS

Coupling DNA replication termination to mitotic entry

The cell cycle consists of a ordered sequence of events leading to the duplication of DNA and ultimately cell division. According to this model, once DNA replication is finished, there is a G2 transition phase that precedes mitosis. However, how and when mitosis is triggered is not yet fully understood. In fact, it has been long speculated that some yet-to-be-discovered checkpoint prevents mitotic entry until DNA replication is completed. We previously described that inhibition of the USP7 deubiquitinase leads to the ubiquitination of replisome components and replication termination. We have now seen that DNA replication triggered by USP7 inhibitors occurs concomitant to a generalised activation of the mitotic kinase CDK1 throughout the entire cell cycle, which impairs chromosome segregation and is toxic for mammalian cells. Accordingly, the toxicity of USP7 inhibitors is alleviated by CDK1 inhibition. Besides its interest in clarifying how USP7 inhibitors kill cells, this work provides direct evidence for the existence of a ubiquitin-based signalling code that couples DNA replication termination to mitotic entry.

Rescuing RAS deficiency in mammalian cells

Previous work in our laboratory revealed the mechanisms of resistance to genotoxic anticancer agents such as ATR inhibitors. We have now looked into potential mechanisms of resistance to targeted therapies. In the context of targeted therapies, RAS is assumed to be the ‘Holy Grail’. More than 30% of all human cancers are driven by mutations in the RAS family of genes and, if not RAS, another member of the pathway is frequently altered. Since RAS inhibitors have been technically very difficult to develop, most drugs that have reached the clinic actually target some other components of the signalling route such as EGRF, RAF or MEK. However, all of these targeted therapies invariably confront the emergence of resistance. To which extent resistance would also occur to RAS inhibitors remains unknown. In this regard, we have recently identified a mutation – the loss of the ETS-domain factor ERF – that enables the growth and differentiation of mouse embryonic stem cells (mESC) lacking all RAS genes (H-, N- and K-Ras). Strikingly, ERF deficiency supports the generation of RAS-less teratomas, this being the first example of a tumour that can develop in the absence of RAS proteins. We believe this work indicates that, even if potent and selective inhibitors of RAS are finally developed, they might likely confront resistance through mutations of other genes such as ERF. We are currently investigating the role of ERF and other ETS-domain factors in the context of resistance to targeted therapies in cancer.

\[ \text{Image: Rescuing RAS deficiency in mammalian cells} \]

\[ \text{Image: Coupling DNA replication termination to mitotic entry} \]

\[ \text{Image: Rescuing RAS deficiency in mammalian cells} \]

\[ \text{Image: Coupling DNA replication termination to mitotic entry} \]
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. Germ line 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 are dissecting the functional specificity of cohesin variant subunits to better understand how their mutation promotes carcinogenesis.”
Cohesin consists of four core subunits, SMC1, SMC3, RAD21 and SA. There are two versions of the SA subunit in vertebrate somatic cells, SA1 and SA2. Less of function mutations in the Stag2 gene encoding SA2 have been identified in bladder cancer, Ewing sarcoma and myeloid malignancies, among others. In cells lacking cohesin-SA2, cohesin-SA1 performs the essential functions of cohesin related to cohesion. We suspect, however, that cohesin-SA1 cannot accomplish other functions of cohesin-SA2 related with chromatin organisation and gene regulation. Importantly, lack of cohesin-SA2 may also generate vulnerabilities that could be exploited in cancer therapy. We aim to identify the specific functions of the two variant complexes in chromatin architecture and gene regulation.

Dissecting the role of cohesin-SA1 and cohesin-SA2 in human cells

We analysed the genome-wide distribution of the two variant cohesin complexes in several human cell lines and applied functional genomics to assess their enrichment in different regulatory elements as well as their co-localisation with other factors involved in genome organisation such as CTCF. We then addressed how this distribution changes when one or the other variant is missing and the subsequent alterations in the transcriptome and in chromatin organisation, analysed by Hi-C in collaboration with M. A. Marti-Renom (CNAG-CRG). Our results show that the two complexes fulfil different functions (FIGURE 1). Cohesin-SA1 is important for the organisation of the topological domains or TADs, which make up the global structure of the genome, and works always alongside the CTCF protein. In contrast, cohesin-SA2 is more versatile and is capable of interacting with diverse transcription factors to form local chromatin loops that bring together enhancers and promoters. Cohesin-SA2 is also more dynamic in its chromatin association, and a large fraction of cohesin-releasing factor Wapl is found associated with SA2 than with SA1.

In the absence of cohesin-SA1, cohesin-SA2 can still cooperate with CTCF to demarcate contact domains although border strength is decreased. In the absence of SA2, however, cohesin-SA1 cannot occupy the non-CTCF sites present at many enhancers and SA. There are two versions of the SA subunit in vertebrate somatic cells, SA1 and SA2. Less of function mutations in the Stag2 gene encoding SA2 have been identified in bladder cancer, Ewing sarcoma and myeloid malignancies, among others. In cells lacking cohesin-SA2, cohesin-SA1 performs the essential functions of cohesin related to cohesion. We suspect, however, that cohesin-SA1 cannot accomplish other functions of cohesin-SA2 related with chromatin organisation and gene regulation. Importantly, lack of cohesin-SA2 may also generate vulnerabilities that could be exploited in cancer therapy. We aim to identify the specific functions of the two variant complexes in chromatin architecture and gene regulation.

Dissecting the role of cohesin-SA1 and cohesin-SA2 in mice

We have generated a conditional Stag2 knockout allele in collaboration with Francisco X. Real (CNIO). Embryos lacking cohesin-SA2 die by mid-gestation and we are currently addressing the cause of this lethality. We are also using mouse embryo fibroblasts (MEFs) to further understand the specific contribution of cohesin-SA2 to cohesion and genome organisation. We observed loosened centromere cohesion and slower proliferation in the Stag2-deficient MEFs, consistent with reports in other cell lines (FIGURE 2). However, the defects are milder than expected and are unlikely to be the sole cause of the embryonic lethality. Complementary to previous observations in Stag2-deficient MEFs, in which the distribution of cohesin changed to include new non-CTCF positions, the number of cohesin binding sites detected in Stag2-deficient MEFs is restricted to those overlapping with CTCF. This result is in line with the idea that cohesin-SA1 cannot replace cohesin-SA2 at many non-CTCF sites, as described in human cells. Experiments aimed to identify the molecular determinants of the distinct behaviour of the two cohesin variants are underway.

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Molecular Oncology Programme | Chromosome Dynamics Group

ANNUAL REPORT 2018

SPANISH NATIONAL CANCER RESEARCH CENTRE, CNIO
Recent epidemiology studies indicate that up to two thirds of the mutations found in tumours are the consequence of inaccurate DNA replication; the rest are inherited or caused by environmental factors. We study the process of DNA replication and its regulatory pathways, with a particular interest in the phenomenon of replicative stress (RS) caused by the temporal stalling or inhibition of the protein machinery responsible for DNA synthesis. In 2018, we focused on the following areas: (1) the activation of ‘dormant’ replication origins in response to RS; (2) the molecular connection between the speed of replication forks and the frequency of origin activation, the two main parameters affected by RS; and (3) the function of PrimPol primase in ‘replicative tolerance’, i.e. the duplication of chemically damaged DNA molecules in order to facilitate their subsequent repair. We have also applied single-molecule methods to analyse the impact of RS in several biological processes.

“We have developed a method to determine the primary cause of replicative stress as a necessary step towards the design of methods to restrict it in primary cells and/or enhance it in tumour cells.”
RESEARCH HIGHLIGHTS

Differential activation of replication origins upon replicative stress

Ten years ago, our laboratory reported that stalled replication forks induce the activation of extra origins as a backup mechanism to complete DNA replication. The genomic characteristics of these ‘dormant’ origins and their mode of activation remained largely unknown. We have now identified, in collaboration with Dr M. Gómez (Centro de Biología Molecular “Severo Ochoa”, CSIC-UMA, Madrid) and Dr V. Pancaldi (formerly at CNIO; currently at the Cancer Research Centre of Toulouse, CRCT), the genomic positions and efficiency of activation of thousands of replication origins in mouse embryonic stem cells, in normal growth conditions or under stress to trigger extra origin activation. This comparative analysis has revealed that the vast majority of ‘stress-responsive’ origins are active in a fraction of the control cell population, but their efficiency is significantly increased when stalled forks accumulate. The efficiency of activation of each individual origin correlates with its physical proximity to active or bivalent promoters, CpG islands, and the presence of ‘open chromatin’ epigenetic marks. The integration of linear origin maps into 3D chromatin interaction networks reveals a hierarchical arrangement in which local clusters of origins are brought together by long-range chromatin interactions.

Cause and effect in replicative stress phenotypes

Replicative stress (RS) phenotypes are normally identified by specific nuclear patterns of markers γH2AX and RPA, but their detailed characterisation requires single-molecule analyses of fork speed and frequency of origin activation using DNA fibres. The interpretation of these assays is complicated because primary alterations in fork speed trigger the secondary activation of extra origins, and conversely, primary changes in the number of active origins also affect fork speed. We have designed interventions in which primary effects of RS on fork speed can be distinguished from primary effects on origin firing, and have applied them to our current research on PrimPol protein (FIGURE). Identifying the primary cause of RS may inform us about new methods to enhance it in cancer cells, increasing their susceptibility to chemotherapeutic agents that target DNA repair.

Primpol protein and its potential applications in cancer therapy

Besides Polα/primase, PrimPol is the only other primase in mammalian cells and it facilitates replication through damaged DNA templates. In 2018, we used Crispr/Cas9 technology to eliminate PrimPol expression in cancer cells, making them hypersensitive to DNA crosslinking agents. These results open the possibility of inhibiting PrimPol as a coadjuvant in chemotherapy. In collaboration with Dr L. Blanco (Centro de Biología Molecular “Severo Ochoa”, CSIC-UMA, Madrid), we have characterised a variant of PrimPol in which amino acid Tyr100 is changed to His, a mutation identified in certain types of lung cancer. Tyr100 mediates the enzyme selection of dNTPs over rNTPs, and Y100H is unusually proficient at using the latter, which may provide a cellular advantage during oncogenic transformation when the dNTP/rNTPs balance is disrupted.

Single-molecule analysis of DNA replication: shedding light on relevant biological processes

RS potentially impinges on all biological processes that involve cell proliferation. Over the past year, we participated in two collaborative projects to analyse RS in specific contexts. First, a study led by Dr J. Moreno de Albornoz (Centro Nacional de Biotecnología, CSIC-UMA, Madrid), has uncovered the replicative defects linked to the loss of transcription factors c-Myc and Max during the differentiation of B lymphocytes. The second study, in collaboration with Dr G. Stoecklin (Heidelberg University) and Dr O. Fernández-Capetillo (CNIO), has led to the functional characterisation of TIAR, an RNA-binding protein that controls mitotic entry and is required for genomic stability.

Figure New methods to determine the primary cause of replicative stress. (A) Test based on a CDC7 kinase inhibitor (CDCTi) to separate cause and effect when fork speed is reduced and origin density is increased. A complementary test can be applied in the opposite situation (fork rate increased, origin density reduced; not shown). (B) CDC7i test applied to U2OS cells undergoing RS after PrimPol downregulation. In this case, RS is due to a primary defect in fork speed. Representative images of DNA fibres used to measure fork speed and origin usage are shown. Bar, 10 μm. Adapted from Rodríguez-Acebes et al. (2018).
Melanomas are a prime example of how basic and translational research has been translated into improved prognosis for affected patients. Nevertheless, clinical responses are still incomplete. The long-term goals of our Group are to identify new progression biomarkers and therapeutic agents. Focusing on stress response programmes involving apoptosis, autophagy and endosome mobilisation, we have discovered lineage-specific oncogenes that define the melanoma ‘fingerprint’. Transcriptomic and proteomic analyses of the melanoma secretome have enabled us to define how tumour cells remodel the (lymph)angiogenic vasculature and avoid immune recognition. Moreover, we have generated a unique set of animal models for non-invasive imaging of melanoma progression in vivo. These systems have led to the validation of nanoparticle-based treatments that are currently being tested in clinical trials. Our ultimate objective is to improve the management of patients with otherwise refractory metastatic melanomas.

“Combining a series of -omic studies with in vivo imaging in mouse models, we have identified a melanoma-associated signature of prometastatic genes that make this tumour uniquely aggressive.”
ANNUAL REPORT 2018

Alonso-Curbelo identified a cluster of endolysosomal-associated genes that we have previously is to discover new melanoma drivers and mechanisms that enable melanoma cells to disseminate already from lesions of barely 1 mm in depth. Last year, we reported a series of mouse models of melanoma that have the unique feature of revealing how these cells act ‘a distance’ from very early stages of tumour development, activating the lymphatic vasculature and preparing metastatic niches before their colonisation (Olmeda et al., Nature 2017). These ‘MetAlert’ animals, together with histological validation in patient biopsies, revealed the growth factor MIDKINE as a new driver of lymphangiogenesis and melanoma metastasis. We have now exploited the MetAlert mice for pharmacological analyses of anti-cancer agents. These studies revealed the doxorubicin-loaded micelles (CNIO) is now being tested in Phase I clinical trials.

NCIO Melanoma Group: objectives and model systems

Melanomas are aggressive solid tumours and provide a prime example of how integrated basic and clinical research has significantly improved patient prognosis. Nevertheless, despite great successes achieved with targeted and immune-based therapies, sustained clinical responses are still limited. Moreover, the field lacks molecular markers of diagnosis, and the knowledge on how melanomas progress and metastasise is largely incomplete. In addition, one of the main hurdles to advance in this disease is the lack of animal models to monitor melanoma initiation and progression in vivo. To this end, our Group focuses on 3 main objectives (FIGURE 1):

 Aim 1: Oncogenic pathways selectively deregulated in melanoma that may represent new diagnostic indicators.
 Aim 2: Risk factors and prognostic markers.
 Aim 3: Animal models that allow for non-invasive monitoring of pre-metastatic niches.

Lineage-specific oncoenic dependencies in melanoma

One of the long-term objectives of the Melanoma Group is to discover new melanoma drivers. We have previously identified an endolysosomal-associated genes that distinguish melanoma from over 35 additional malignancies (Alonso-Curbelo et al., Cancer Cell 2014). Further analyses of lysosomal-dependent pathways also revealed unique features of autophagy genes (ATG5) in melanoma (Garcia-Fernandez et al., Autophagy 2016). Other melanoma-enriched regulatory mechanisms were identified by focusing on RNA binding proteins (RBPs). We selected RBPs because they are largely dispensable for autolysosome maturation in melanoma (RBPs). We selected RBPs because they are largely dispensable for autolysosome maturation in melanoma and interactomic analyses showed that p62 was largely protein to autophagy. However, proteomic, transcriptomic based on previous reports in the literature linking this protein to autophagy described in a broad spectrum of types, this protein was found in melanoma to bind a selected set of RNA binding proteins (RBPs), here exemplified by CUGBP1. -Omic studies in cell lines combined with histopathological studies in genetically modified mouse models (GEMM) and histopathological validation in clinical biopsies identified the scaffolding factor FERM2 as a downstream target of the p62/ CUGBP1 axis. Both, p62 and FERM2 were found overexpressed in advanced melanomas, representing new indicators of poor prognosis.

Figure 2  New functions for pro-metastatic drivers in melanoma. Schematic representation of a set of pro-tumourigenic factors with no previous links to melanoma identified by addressing the expression and functional requirement of p62/CUGBP1 in this disease. Different from roles of p62 in autophagy described in a broad spectrum of types, this protein was found in melanoma to bind a selected set of RNA binding proteins (RBPs), here exemplified by CUGBP1. -Omic studies in cell lines combined with histopathological studies in genetically modified mouse models (GEMM) and histopathological validation in clinical biopsies identified the scaffolding factor FERM2 as a downstream target of the p62/CUGBP1 axis. Both, p62 and FERM2 were found overexpressed in advanced melanomas, representing new indicators of poor prognosis.

Publications

- Figures 1 and 2: schematic representation of the main experimental systems and representative publications. Indicated are main experimental systems and representative publications.

- Figure 1: main objectives of the CNIO Melanoma Group and achieved new progression biomarkers and validate more efficient anti-cancer agents. Indicated are main experimental systems and representative publications.

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and prepare pre-metastatic niche formation, aiding metastatic progression. Exosomes secreted from the tumour can reach metastatic organs that contain specific signatures of molecules (e.g. proteins, DNA). Our data support that tumour-secreted exosomes can communicate with the tumour and surrounding cells. This data support that combination of therapies targeting both the tumour and the microenvironment decreases metastatic spread.

OVERTVIEW

Cancer treatment is no longer only focused on tumour cell analysis. The Microenvironment and Metastasis Group studies the communication between tumour and stromal cells along tumour progression. Cancer treatment requires the analysis of the tumour microenvironment to define specific therapies targeting both the tumour and surrounding cells. Data support that combination of therapies against the tumour and its microenvironment are the future of cancer treatment. In our laboratory, we have focused on understanding the message of a novel ‘language’ between tumour cells and the environment, these small extracellular vesicles called exosomes. Our data support that tumour-secreted exosomes contain specific signatures of molecules (e.g. proteins, DNA). Exosomes secreted from the tumour can reach metastatic organs and prepare pre-metastatic niche formation, aiding metastatic homing and further metastatic progression.

“Tumour-secreted exosomes are the forefront edge of tumour metastasis, they reach local and distant microenvironments facilitating metastatic spread.”

RESEARCH HIGHLIGHTS

Novel factors involved in melanoma progression, the future of liquid biopsies

In this project, we study the function of tumour-secreted exosomes in the establishment of pre-metastatic niches within the lymph node (FIGURE). We are analysing the role of the matrix-anchored proteins and neurotrophic receptors in lymph node metastasis and melanoma progression. We are also using novel biofluids (e.g. lymph node exudative seroma obtained post-lymphadenectomy) as a source of biomarkers, analysing protein cargo and BRAF mutations. Our laboratory is also interested in the study of microenvironmental factors influencing melanoma progression such as obesity.

Obesity modulates breast cancer behaviour

Obesity has drastically increased to become one of the most serious health problems worldwide and is now recognised as a risk factor for breast cancer incidence, progression, and prognosis. In this project, we aim to understand cellular and molecular mechanisms that underlie inflammation, obesity, and breast cancer metastasis. Furthermore, we are analysing the interaction of cancer cells with immune cells and platelets in metastasis and evasion of immune supervision. Our goal is to understand how obesity modulates breast metastatic behaviour defining novel factors involved and to define new therapies.

Defining novel targets in rare diseases

Malignant peripheral nerve sheath tumours (MPNSTs) are rare tumours that are commonly related to neurofibromatosis type I (NF1) disease. In this project, we aim to find new biomarkers and novel therapeutic targets to prevent MPNST progression. The data obtained from a multidrug screening on MPNSTs cell lines and the mass spectrometry analysis of their exosomes identified several proteins as top candidates. Thus, we are currently testing the combination of MEK inhibitors, which are already used in the clinic, with novel drugs targeting these two proteins in order to define a new therapeutic window for MPNSTs.

• PUBLICATIONS


Colletti M et al. (incl. Peinado H). Expression profiles of exosomal miRNAs isolated from plasma of patients with desmoplastic small round cell tumor. Epigenomics. PMID: 30569756.


• PATENT

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 benefits for patients and the disease inevitably progresses. The rise in the number of systemic therapies that work extra-cranially but are unable to provide metastasis is partially due to the increasing number of systemic therapies that work extra-cranially but are unable to provide effective treatments. 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 in order to challenge the current status of this unmet clinical need.

**OVERVIEW**

**PUBLICATIONS**


**AWARDS AND RECOGNITION**

- Elected Member of the Scientific Committee of the European Association of Neuro Oncology.
- Keynote speaker at the Annual Congress of the European Society of Veterinary Oncology.
- Laura Alvarez Espinosa was recipient of a MinED Gillette-Ochser PhD Fellowship.
- Nealia Priego received the CNIO Award for Excellence in Research by Postdoctoral Staff Investigators, CNIO Lab Day.
- Lucia Zhu received the “Best Poster” Award at the CNIO Lab Day.
- STAT3 labels a subpopulation of reactive astrocytes required for brain metastasis, as per Priego et al., was selected as paper of the month by the Spanish Society for Biochemistry and Molecular Biology.

**RESEARCH HIGHLIGHTS**

We pioneered the first report proving the importance of glial heterogeneity associated with metastatic brain tumours. As previously shown in other diseases affecting the brain, understanding the contribution of specific glial subpopulations could provide novel therapeutic targets.

The use of genetic and pharmacological approaches has enabled us to disentangle the critical role of this disease-specific subpopulation of reactive astrocytes in brain metastasis, which is characterised by activation of the STAT3 pathway. Its presence, induced by metastatic cells, involves the establishment of an immunosuppressive local environment that favours tumour growth.

In collaboration with four different national and international clinical institutions we have proved the importance of this finding in patients with brain metastasis. Treatment of stage IV lung adenocarcinoma patients with the STAT3 inhibitor silibinin reduced brain metastasis in 75% of them, which led to an increased survival. This finding involves a proof-of-concept regarding the possibility of developing effective therapies against metastasis by targeting the microenvironment.

**Figure** A subpopulation of reactive astrocytes (GFAP), characterised by activated STAT3 (pSTAT3), is present in experimental brain metastasis models (a, b) and human samples (c, f). Targeting this glial subpopulation in mice (c) and humans (f) impaired the viability of intracranial metastases. pSTAT3 reactive astrocytes are required to maintain a pro-metastatic niche (d).
RESEARCH HIGHLIGHTS

Nutrient signalling in B cell lymphoma

One of the most rapid proliferation bursts in mammalian cells is that of B lymphocytes upon encountering certain pathogens or antigens. This proliferation suddenly multiplies the energetic and metabolic demands of the activated B cell and, accordingly, precise nutrient sensing and signalling are key to successfully accomplish the energetically onerous rounds of growth and division. Recently, components of the Rag GTPase pathway, a key nutrient signalling pathway that enables the anabolic capacity of the cell for rapid proliferation, were found mutated in follicular lymphoma (FL), an incurable B lymphocyte tumour. By means of novel strains of mice that express mutant variants of the RagC GTPase, we found that subtle increases in nutrient signalling unleash activation and proliferation of B cells, suppress cell death and drive the development of FL (FIGURE). These results pave our way towards a novel therapeutic strategy against B cell lymphoma, aimed at targeting its corrupted nutrient signalling. In addition, and surprisingly, this mild increase in the signalling of nutrient abundance in B lymphocytes also drives an autoimmune disease.

Chronic signalling of elevated nutrients and premature ageing

The study of genetically engineered mice expressing a mildly activating form of RagC revealed that, in the absence of lymphoma, these mice suffer from symptoms and pathologies consistent with premature ageing, including a shortened lifespan (FIGURE). While caloric restriction (CR) and other fasting-like regimes are well-known to delay ageing, as is also the case with the pharmacological inhibition of mTOR with rapamycin in mammalian model organisms, this is the first time that a moderate increase in nutrient signalling in mice shows compromised longevity. We are currently investigating the cellular and molecular alterations responsible for this shortening of the life span.

OVERVIEW

In the Metabolism and Cell Signalling Lab we study the interplay of nutrients, metabolism and cancer. Every cell in the organism integrates signals emanating from the abundance of intracellular nutrients and from the nutritional state of the organism as a whole. Integration of cellular and systemic nutrient abundance cues is key for adequate cellular and organismal functions, and importantly, the components of these signalling cascades are generally corrupted in disease states, such as cancer. Together with genetic mutations, environmental perturbations (such as those occurring in obesity) corrupt the cellular signalling cascades that control the responses to nutrients and hormones. In the lab, 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.

“Mouse models with a very mild genetic activation of nutrient signalling foster cancer, autoimmunity, and ageing; this has profound implications when thinking about the consequences of human nutrient overload.”