EXPERIMENTAL THERAPEUTICS PROGRAMME

JOAQUÍN PASTOR Programme Director

The following highlights some of the main achievements of the Experimental Therapeutics Programme during 2022:

Mastl inhibitors (MASTL-is). (In collaboration with Marcos Malumbres´ Group). In 2022, we further optimised our MASTL-is and PROTACs, putting special emphasis on their *in vivo* bioavailability. We studied the *in vivo* pharmacokinetic properties of ETP-715, our frontrunner MASTL-i. Unfortunately, the *in vivo* bioavailability (PO route) is still suboptimal. We will continue optimising it in the next stage of our work. By contrast, ETP-184 achieved plasma and tissue levels well above its cellular activity (Nanobret EC50). Furthermore, PROTAC ETP-823 displayed good bioavailability in plasma (IP route), ensuring compound levels above its DC50 in cells. Both compounds are now ready for more advanced *in vivo* PK-PD and efficacy studies to pharmacologically validate MASTL as a therapeutic target.

TRF1. (In collaboration with Maria A. Blasco's Group). In 2022, we focused our activities on the validation of previously identified hits as potential direct-TRF1 inhibitors. In addition to ETP-631 emerging from a virtual screening campaign, 3 other hits arose from screening a subset of our ETP library. All 4 compounds were active in the TRF1-dsTelDNA proximity assay and inactive in the corresponding counter-screens. Importantly, one of them showed the disruption of the interaction of TRF1 with dsTelDNA in ChIP experiments in cells (M. A. Blasco's laboratory). We are currently validating these compounds using an orthogonal EMSA assay to ensure that they disrupt the TRF1-dsTelDNA complex, and are testing their direct interaction with TRF1 using the Thermofluor assay. Notably, an international pharmaceutical company has shown interest in TRF1 as a therapeutic target and is now testing these compounds under an MTA agreement.

SETD8 inhibitors. (In collaboration with Óscar Fernández-Capetillo's Group). After several screening campaigns, we identified covalent and non-covalent high micromolar SETD8 biochemical inhibitors. During 2022, we characterised representative covalent hits by intact protein mass spectrometry. Regarding the non-covalent hits, we carried out an initial chemical exploration with the aim of improving the potency, although with limited success so far. In cells, we tested selected hits and analogues. We also measured the inhibition of the methylation of H4K20, a direct substrate of SETD8, and are currently analysing these results. In the next stage, we will continue optimising current hits and perform additional hit finding/generation activities to obtain better starting points to develop SETD8 inhibitors.

"The Experimental Therapeutics Programme (ETP) continues giving support to Drug Discovery and Chemical Biology projects at the CNIO and collaborates with external partners to discover new therapeutic agents."

FOXO activators. (In collaboration with Refoxy Pharmaceuticals GmbH). In 2020, the CNIO established a collaboration with Refoxy Pharma (Berlin, Germany) to discover FOXO activators for potential development in multiple diseases. We identified several FOXO activators after several cell-based screening campaigns, analoging of initial hits, characterisation in mechanistic studies, and preliminary off-target selectivity. Refoxy has expressed its interest in licensing some of these hits (under negotiation).

Others. ETP has worked in the early phases of other internal projects: RANK (Eva González-Suárez, CNIO) / NUDIX5 (*CRG-UIC*). ETP also provided support to several CNIO researchers in exploratory projects or contributed with internally synthesised tool compounds: Felipe Cortés-Ledesma, Juan Méndez, Héctor Peinado, Manuel Valiente, Joaquín Martinez-López, Mariano Barbacid, María S. Soengas, Óscar Fernández-Capetillo, Nabil Djouder, Francisco X. Real. ■

MEDICINAL CHEMISTRY SECTION

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Technicians
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** *Titulado Superior* (Advanced Degree)

*** *Plan de Empleo Joven* (Youth Employment Plan)

OVERVIEW

The Medicinal Chemistry Section is part of the multidisciplinary Experimental Therapeutics Programme (ETP) focused on early drug discovery activities. ETP is integrated into the CNIO's structure, and acts as a bridge between basic research groups in cancer biology and the pharmaceutical industry, with the aim of transferring the results obtained in basic research laboratories to products, potential drugs that help to understand the biology of cancer, or the development of new therapies. The Section deals with the design, synthesis, and optimisation of compounds that are then characterised in the Biology Section of ETP, in order to evaluate their potency in biological targets *in vitro* and *in vivo* and ultimately to demonstrate their efficacy and mechanism of action in animal models (*in vivo* proof-of-concept). As a complementary strategy to the classic inhibitors, we also contemplate the degradation of particular targets using different chemical approaches such as the use of PROTACs. Additionally, we have entered the field of Chemical Biology in order to discover and identify novel drugs and targets from phenotypic screenings. In this regard, we contribute by synthesising high quality chemical tools needed for interrogating the observed phenotype.

"In our MASTL project, we generated the first MASTL PROTAC (ETP-823) that potently degrades MASTL protein via E3 ligase and proteasome recruitment."

RESEARCH HIGHLIGHTS

Our Section's activities focus mainly on the following projects:

Telomeric repeat binding factor 1 (TRF1) inhibitors

This project is led by Maria A. Blasco (Telomeres and Telomerase Group). In previous years, the ETP Biology Section developed an assay to measure the binding of TRF1 to telomeric DNA. After virtual and wet screening campaigns, we identified some disruptors of such binding that do not interfere with the assay system nor with DNA. During 2022, we analytically characterised the hits, resynthesised fresh batches, and synthesised some analogues to establish Structure Activity Relationships (SAR). The compounds are currently being evaluated in orthogonal assays by ETP's Biology Section and by a pharmaceutical company that is interested in the target, under an MTA agreement.

Microtubule-associated serine/threonine protein kinase-like (MASTL) inhibitors

This project is being undertaken in collaboration with Marcos Malumbres (Cell Division and Cancer Group). We have been involved in the fine optimisation of ETP-715, a potent cell active, selective compound without cardiotoxic alerts but with low exposure levels after oral administration in pharmacokinetic (PK) studies performed in BALBC mice. Seventy six new analogues have been synthesised so far, and the most promising ones, in terms of potency and *in vitro* ADMET, are being evaluated in PK studies. Additionally, we have started to work on back-up series, to reinforce the intellectual property and to determine the impact of different scaffolds on drug-like properties. Fifty seven new compounds from different chemical series have been synthesised, and we have identified new series with potent compounds that will be characterised in terms of *in vivo* PK. In addition, we continue with our activities developing PROTACs (Proteolysis Targeting Chimeras) to degrade MASTL protein. Previously, we identified ETP-823 as our first MASTL PROTAC that potently degrades MASTL protein via E3 ligase and proteasome recruitment. PK studies showed *in vivo* levels above its DC50 in cells in plasma after IP administration. We performed a fine optimisation of ETP-823, and 62 new PROTACs were synthesised by exploring different linkers, different functional groups in the growing vectors, and different E3 ligase ligands (FIGURE 1). So far, we have identified several new PROTACS with good degradation profiles in different cell lines.

HistoneH4-lysine20 N-methyltransferase (SETD8) inhibitors

In collaboration with Óscar Fernández Capetillo (Genomic Instability Group), we explored one of the initial hits identified in a cellular assay in Capetillo's laboratory, but the chemical exploration of this series was put on hold due to lack of activity in the biochemical assay. After 2 screening campaigns, we identified new hits (covalent and non-covalent), and we are currently working on their validation by re-synthesising the hits and synthesising some analogues to establish SARs.

Foxo activators (collaboration with Refoxy Pharmaceuticals GmbH)

We have been involved in the selection of new analogues of the hits identified in screening campaigns, as well as in the quality control analyses. Several Foxo activators have been identified, and negotiations for licensing the compounds to Refoxy are underway.

NUDT5 inhibition

We are collaborating with GRG-IUC to optimise a hit that inhibits the ATP generating activity of NUDT5 in a biochemical assay. Several analogues have been obtained, and we are currently characterising the compounds.

RANK antagonists as a novel therapeutic approach for the treatment of TNBC patients

We are collaborating with Eva González-Suárez (CNIO) to develop small molecules that specifically target the RANK receptor. The activities in 2022 focused on acquiring the virtual hits and assessing the quality control to validate them in wet assays (SPR, cells, etc.) and to generate robust data.

Apart from the drug discovery activities, we give support to several Groups by synthesising reference or tool compounds. During 2022, we carried out such work for the following Groups: Brain Metastasis, Genomic Instability, and Telomeres and Telomerase. ■

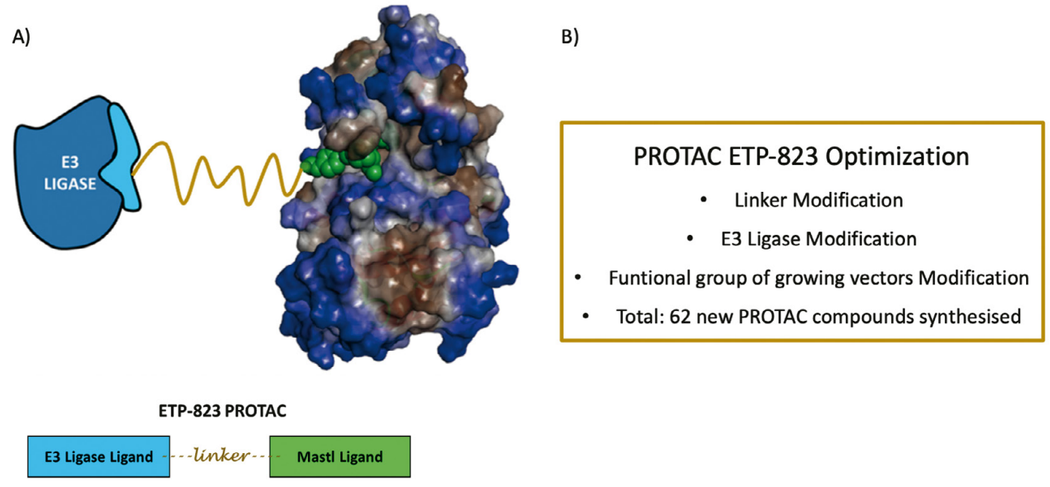

FIGURE 1 (**A**) Representation of PROTAC ETP-823 binding to the kinase domain of human MASTL protein (PDB 5LOH) through MASTL Ligand, and to E3 Ligase through the corresponding Ligand. (**B**) Strategies for the optimisation of ETP-823.

PUBLICATION

- Zhu L, Retana D, García-Gómez P, Álvaro-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 Á, Hernández-Laín A, Toldos O, Ruano Y, Alcázar L, Blasco G, Fernández-Alén J, Caleiras E, Lafarga M, Megías D, Graña-Castro O, Nör C, Taylor MD, Young LS, Vareślija D, Cosgrove N, Couch FJ, Cussó L, Desco M, Mouron S, Quintela-Fandino M, Weller M, Pastor J, Valiente M (2022). A clinically compatible drug-screening platform based on organotypic cultures identifies vulnerabilities to prevent and treat brain metastasis. *EMBO Mol Med* 14, e14552.

PATENTS

- Pastor Fernández J, Martínez González S, Blanco-Aparicio C, García Campos FJ, Cebriá Gómez A. Thiazolopyrimidones as inhibitors of DDR1/2 and therapeutic uses thereof. PCT application (2022). *PCT/EP2022/051064. WO2215716 6A1.*
- Pastor Fernández J, Martínez González S, Blanco-Aparicio C, González Cantalapiedra E, García García AB, Pastor Fernández M, Hernández Higueras AI, Albarrán Santiño MI, Cebriá Gómez A. Imidazo[1,2-a]pyrazines as inhibitors of HASPIN and therapeutic uses thereof. PCT application (2022). *PCT/EP2022/054626. WO2218015 0A1.*
- Pastor Fernández J, Martínez González S, Blanco-Aparicio C, García García AB, Rodríguez Aristegui S, Gómez de la Oliva CA, Albarrán Santiño MI, Cebriá Gómez A, Malumbres Martínez M. Imidazo[1,2-b] pyridazine based tricyclic compounds as inhibitors of HASPIN and therapeutic uses thereof. PCT application (2022). *PCT/2022/057636. WO2022004 33A1.*

BIOLOGY SECTION

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**Titulado Superior* (Advanced Degree)
#*Plan de Empleo Joven* (Youth Employment Plan, until January)

Students in Practice
Andrea Álvarez (Feb.-July) (Bachelor's Degree Final Project, *Univ. Autónoma de Madrid*, Spain), Jada Li (June - July) (MISTI Internship, USA), Noelia Martin (Feb.-July) (Bachelor's Degree Final Project) and María Cuerda (since Oct.) (Master's Thesis)(*Univ. Complutense de Madrid*, Spain)

Visiting Scientists
Lucía Jiménez and Wolfgang Link (until November) (*IIBm (CSIC-UAM),* Madrid, Spain)

OVERVIEW

A high-quality small-molecule probe for target validation has to be cell permeable and demonstrate target engagement and selectivity, as well as pharmacological and phenotypic response. PROTACs (PROteolysis TArgeting Chimeras) have emerged as new promising pharmacological modalities. Moreover, PROTACs represent the chemical equivalent of small interfering RNA (siRNA), albeit allowing removal of a protein at a post-translational level. Parameters such as the maximum level of target degradation (Dmax), confirmation of a proteasome dependent degradation mechanism, and kinetic parameters of POI degradation and selective degradation have to be taken into account to use PROTACs for target validation. In collaboration with Marcos Malumbres, we started an early drug discovery project to develop MASTL inhibitors and PROTACs, as non-advanced inhibitors have already been described. We have been able to develop both types of molecules, generating a set of PROTACS that meet the requirements to be used as chemical tools for target validation and to define their clinical niche.

"We identified selective and potent MASTL PROTACs with *in vivo* levels needed to perform PK/PD and proof of concept studies."

RESEARCH HIGHLIGHTS

Microtubule-associated serine/threonine protein kinase-like (MASTL)

This project is undertaken in collaboration with the CNIO Cell Division and Cancer Group. In 2022, we tested in our biochemical assay using active human full-length MASTL protein around 190 new compounds, both MASTL-i and MASTL PROTAC-like molecules. We measured MASTL engagement in cells (BRET assay). In the case of PROTACs, we also evaluated their MASTL degradation capacity in cells, both in a dose response and time dependent manner. We identified a set of nanomolar MASTL degraders with different linker and E3 ligase ligand that have been used to study their broad degradation capacity with proteomics. In addition, we performed pharmacokinetics studies of several MASTL inhibitors and PROTACs, identifying a MASTL inhibitor and a PROTAC that have achieved enough plasma levels to allow PK/PD studies to be performed (FIGURE 1).

Telomeric repeat binding factor 1 (TRF1)

This project is carried out in collaboration with the CNIO Telomeres and Telomerase Group. We are working to identify disruptors of TRF1 binding to ds telomeric DNA, and so far we have identified several hits from different chemical series after virtual screening and wet assays, and screening of a collection of 1500 molecules selected from our ETP-library and analogue searching. We confirmed the specific disruption of the binding of TRF1 to dsTelDNA with screen and counter screen alpha assays, and a fluorescent displacement assay to discard the binding of the compounds to dsTelDNA. Now we are validating these hits by applying orthogonal assays against TRF1 and the dsTelDNA probe, such as EMSA and thermofluor assay with freshly prepared and/or resynthesised samples. Compounds that disrupt the binding of TRF1 to ds telomeric DNA by binding to TRF1 will be tested in a TRF1 phenotypic assay.

SET domain containing lysine methyltransferase 8 (SETD8)

This project is conducted in collaboration with the CNIO Genomic Instability Group. Our main objective is to generate and optimise novel SET8 inhibitors as new therapeutic agents. After 2 different screening campaigns, we identified both reversible and irreversible possible hits with micromolar activity. The covalent mechanism of action of the hits was validated by time dependent biochemical assays and the formation of adducts by proteomics with purified SETD8. In order to identify the reactive amino acid in SETD8, we are going to perform biochemical assays with a mutant protein and proteomics studies in cells to evaluate their selectivity. In addition, all possible hits have been tested in a cellular assay that measures monomethylation of H4K20 in order to prioritise chemical serials to improve their biochemical activity.

Collaborations with other CNIO Groups

The ETP-Biology Section performed *in vivo* studies of selected compounds and drugs such as pharmacokinetics and distribution studies in collaboration with the Microenvironment and Metastasis Group, the Brain Metastasis Group, and the Genomic Instability Group. Furthermore, we performed screening campaigns with the Topology and DNA Breaks Group and the H12O - CNIO Haematological Malignancies Clinical Research Unit, identifying several hits that are under validation. Finally, we collaborated with the Experimental Oncology Group, the Melanoma Group, the DNA Replication Group, and the Chromosome Dynamics Group giving support to perform cellular screenings.

Collaborations with other institutions

Refoxy collaboration: We gave logistics and data analysis support.

Collaboration with CRG/UIC: This project is conducted in collaboration with Dr R. Wright. We characterised, in terms of ADME-T and pharmacokinetics, a NUDIX5 inhibitor previously identified by the researcher. ■

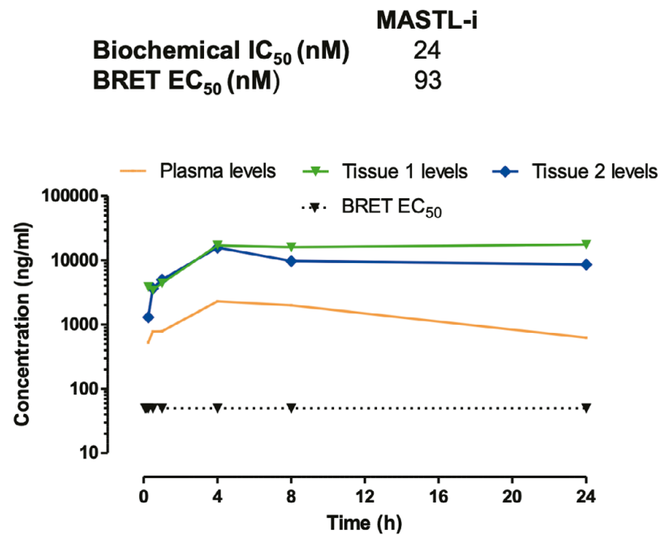

FIGURE 1 Plasma and tissue levels of MASTL inhibitor after oral administration. Nanomolar biochemical and cellular MASTL-I achieves levels in mice clearly above the EC_{50} to modulate MASTL in cells, which guarantees *in vivo* target modulation.

PUBLICATIONS

- Zhu L, Retana D, García-Gómez P, Álvaro-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 Á, Hernández-Laín A, Toldos O, Ruano Y, Alcázar L, Blasco G, Fernández-Alén J, Caleiras E, Lafarga M, Megías D, Graña-Castro O, Nör C, Taylor MD, Young LS, Vareslija D, Cosgrove N, Couch FJ, Cussó L, Desco M, Mouron S, Quintela-Fandino M, Weller M, Pastor J, Valiente M (2022). A clinically compatible drug-screening platform based on organotypic cultures identifies vulnerabilities to prevent and treat brain metastasis. *EMBO Mol Med* 14, e14552.
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- Jimenez L, Amenabar C, Mayoral-Varo V, Mackenzie TA, Ramos MC, Silva A, Calissi G, Grenho I, Blanco-Aparicio C, Pastor J, Megías D, Ferreira BI, Link W (2022). mTORC2 is the major second layer kinase negatively regulating FOXO3 activity. *Molecules* 27, 5414.

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- Pastor Fernández J, Martínez González S, Blanco-Aparicio C, García García AB, Rodríguez Aristegui S, Gómez de la Oliva CA, Albarrán Santiño MI, Cebriá Gómez A, Malumbres Martínez M. Imidazo[1,2-b] pyridazine based tricyclic compounds as inhibitors of HASPIN and therapeutic uses thereof. PCT application (2022). *PCT/2022/057636. WO2022200433A1.*

CNIO - LILLY CELL SIGNALLING AND IMMUNOMETABOLISM SECTION

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Technicians
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Titulado Superior (Advanced Degree)

OVERVIEW

Our laboratory, in collaboration with Loxo@Lilly Oncology, is working on the identification and validation of novel molecular targets engaged in the induction of chromosomal instability (CIN). Our goal is to find novel therapies that would make tumours bearing CIN more susceptible to destruction, either with the use of single agents, or acting synergistically with other anti-tumour therapies. Exploring how to better target these mechanisms would lead to better and more efficient therapeutic options, including more personalised therapies.

A combination of *in vitro* and *in vivo* approaches is being utilised to obtain a complete understanding of the role of CIN in tumour development and anti-tumour response. Each target goes through an *in vivo* validation process using xenografts, allografts, and mouse models developed at the CNIO that includes the use of non-invasive *in vivo* imaging technologies, and immune histochemical characterisation of tumours for different metabolic, immune, and tumour markers. The final step is the validation in human samples using tumour tissue arrays.

RESEARCH HIGHLIGHTS

Chromosomal instability (CIN) and whole genome duplication (WGD) are a hallmark of human cancer and are associated with poor prognosis, metastasis, and therapeutic resistance (FIGURE 1). CIN results from errors in chromosome segregation during mitosis, leading to structural and numerical chromosomal abnormalities, including loss or amplification of DNA segments, rearrangements, extrachromosomal DNA, and micronuclei formation. These abnormalities lead to the activation of oncogenes or the inactivation of tumour suppressor genes, as well as other genes aiding in the processes of metastasis, drug resistance, and immune scape. The causes of CIN are diverse, including mitotic errors, replication stress, homologous recombination deficiency (HRD), and telomere crisis. ■

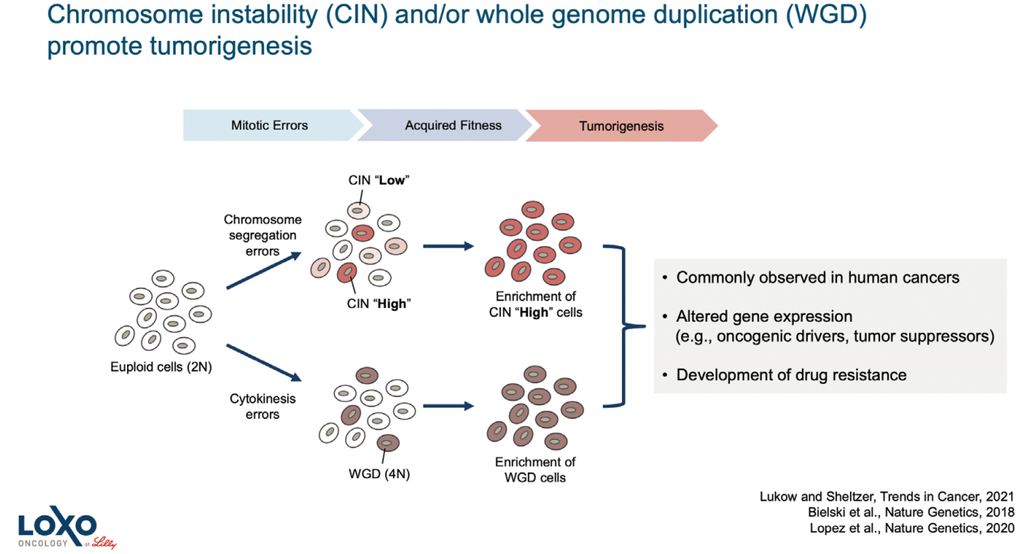

FIGURE 1 Chromosome instability (CIN) and/or whole genome duplication (WGD) promote tumorigenesis.