From Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

IDENTIFICATION OF NOVEL MODULATORS OF PROTEIN SYNTHESIS AND NUCLEOLAR BIOLOGY USING HIGH THROUGHPUT PHENOTYPIC SCREENS

Alba Corman

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Identification of novel modulators of protein synthesis and nucleolar biology using high throughput phenotypic screens

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Alba Corman

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Principal Supervisor:
Associate professor Jordi Carreras-Puigvert
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Genome Biology
Currently:
Uppsala University
Department of Pharmaceutical Biosciences and Science for Life Laboratory

Co-supervisor(s):
Professor Óscar Fernández-Capetillo
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Genome Biology

Opponent:
Associate professor Ola Larsson
Karolinska Institutet
Department of Department of Oncology and Pathology

Examination Board:
Associate professor Olov Andersson
Karolinska Institutet
Department of Cell and Molecular Biology

Associate professor Marianne Farnebo
Karolinska Institutet
Department of Cell and Molecular Biology, and Department of Biosciences and Nutrition

Dr. Stefan Kubicek
Head of Molecular Discovery Platform
CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences

To my family, my husband, my friends, and everyone that has been a part of this journey.

Para mi familia, mi marido, mis amigos, y todos los que habéis sido parte de este viaje.

*"**It takes time… Learning to fly."***

Keane (Tear Up This Town)

POPULAR SCIENTIFIC SUMMARY OF THE THESIS

How are medicines discovered? There are many ways, but in this work, we used a strategy known as **phenotypic screening** to discover molecules and genes that could benefit the treatment of diverse diseases. Phenotypic screens start with a **phenotype** or observable characteristic in a cell or organism, which we want to modify, either by exposure to medicines or doing genetic modifications. Most of the screens in this thesis are **chemical screens**, in which we had exposed cells to thousands of drugs to find new compounds that affect the production of proteins in the cell and potential new therapies for diseases like **Amyotrophic Lateral Sclerosis (ALS)** and **cancer**. We conduct these screens in small plates, of the size of a Nintendo Game Boy Color, which are divided into 384 small wells or compartments. In each well, we deposit human cells, and we add a different treatment. This means that we can rapidly screen 384 drugs in a single plate at the same time! To help us, we use robots and automated systems, allowing us to screen thousands of compounds in one go. To understand the effects of the drugs in cells, we just *color* the parts of them that we are interested into looking at, this is known as labelling and/or staining. Then, we take pictures using a microscope, and we extract information from these images using automated image analysis. Next, we evaluate the changes induced cells by the drugs comparing them to untreated cells. Imagine that we can color or label a *bad* protein produced in the cell in green, so that when this protein is produced (when we detect green signal), the cell is sick. Now, aiming to find potential cures of this disease, so we ran a phenotypic screen. Our hypothetical screen would try to find drugs that reduce the toxic green signal. In such a screen we would also other drugs that potentiate green signal, which is sometimes interesting to better understand the disease. Nevertheless, if our goal is to develop a medicine for this disease, we would take as a starting point the drugs that reduced green signal, and study them further.

In our first work (**paper I**), we aimed to understand the effects of known medicines in protein production. **Protein production** is a fundamental process in cells because proteins are the molecular machines that do most cellular functions, such as controlling how cells grow, move, or even become one cell type or another. For instance, neurons produce some proteins, sperm, others. The manual for making proteins is the DNA (genome) that is stored in the nucleus of the cell, for it to be protected, since it is the most valuable treasure of the cell. This manual or DNA is written in a language that needs to be translated into an amino acid code, in which proteins are written. This process is known as **translation**, and it is assisted by complex molecular factories called **ribosomes**. These ribosomes are produced in a cellular compartment known as **nucleolus**, inside the nucleus of the cell, where the DNA is. Translation is complex process that is tightly regulated and responds to the energy balance of the cell. If the cell is growing in optimal conditions, gets nutrients, and is *happy*, more proteins are produced, the cell grows, and it divides to generate new cells. If the cell is *under stress* and the conditions are suboptimal for growth, translation is shut down to cope with these challenging situations, until the stress is resolved; in the worst case, if the stress persists, the cell dies. Translation is so fundamental that when it is not well regulated it leads to disease. For example, **cancer** cells are addicted to translation, they over-produce proteins, which allow them to grow uncontrollably.

In other cases, there are diseases where cells translate less by default and cannot perform their normal functions. To identify drugs able to modulate translation, first we had to be able to *observe* translation in cells. To visualize changes in translation we labelled newly produced proteins with a molecule called **OPP**. OPP can be detected with microscopy, and its signal can be quantified using image analysis to monitor changes in translation. When cells translate more, more OPP signal can be detected, and *vice versa*. Thus, with the intensity of the OPP signal we get a snapshot of the amount of proteins that were produced in the cell at a certain point. OPP labelling allowed us to screen for compounds increasing or decreasing protein synthesis in cancer cells. We could not find drugs that consistently stimulated translation (increased OPP signal), probably because translation levels in cancer cells are so over the roof that cannot get any higher. However, we found compounds reducing translation (decreasing OPP signal). Most of them were already known to inhibit protein synthesis - telling us that we were on the right track – but we found a new drug reducing translation. This drug's name is **SKI-II**, and it was developed as an inhibitor of sphingosine kinases (SPHKs), which have been exploited as targets for cancer. However, our results show that SKI-II, and a similar compound used in the clinic, kill cancer cells by inhibiting translation in cells, independently of SPHKs (their expected target). In particular, **SKI-II reduces translation**, stressing the cell by destroying a cellular compartment essential for protein production. Our work is the first to report the effects of known drugs in translation and shows a new mechanism of toxicity for SKI-II, which could be explored to give better treatments to cancer patients.

In our second work (**paper II)**, we conducted a screen to find potential medicines for **ALS**. ALS is a terrible **neurodegenerative disease** characterized by the death of neurons that innervate muscles, resulting in paralysis. The physicist Stephen Hawking suffered from this disease, which kept him for over 50 years in a wheelchair and using assisted devices that he could control with the only muscles left that he could move, the ones in his eyes. While it is a tragedy, this was an exceptional case, since most patients die 2-5 years after diagnosis, when their diaphragm stops working, so they stop breathing. The "ALS ice bucket challenge" in 2014 brought us some awareness of this disease which today still has no cure, and the medicines approved for these patients only extend their life marginally. One of the reasons why ALS had been understudied is because its causes were not known. Nowadays, we know a bit more, mutations in some genes have been associated to ALS, being the presence of DNA repeats within the C9ORF72 gene the most frequent mutation in hereditary and sporadic cases of ALS. These repeats of DNA are abnormally translated and produce aberrant protein chains, some of which are toxic and kill cells. One of them is known as **poly-PR**, because it is a repeated chain of two amino acids Proline-aRginine), which goes to the cell nucleus, predominantly to the nucleolus, disrupts the production of ribosomes, proteins, and ultimately kills cells, of any kind, including neurons. We used synthetic poly-PR peptides to mimic the cell death that occurs in ALS. We conducted a screen where we added poly-PR to cells in culture looking for drugs able to **limit cell death induced by poly-PR**. To find novel therapies for ALS we screened medically approved drugs, since they have proven to be safe in humans, and our results could be fast-tracked into the clinic, since these patients need urgent help. Our screen identified a big

class of drugs, **epigenetic drugs**, that reduced cell death caused by poly-PR. We explored if more of these epigenetic drugs could achieve the same or better results and found three that were very efficient. They even protected neurons and tiny zebrafish embryos exposed to poly-PR! We sought to understand how these compounds fought ALS, and found that two of them, **protected the nucleolus from poly-PR.** The function of the nucleolus in producing ribosomes depends greatly on its structure. Upon stress conditions, the nucleolus dramatically changes its morphology, affecting ribosome production and protein synthesis. To explore if our compounds were protecting the nucleolus from any source of stress, we exposed cells to a drug known to cause nucleolar stress, Actinomycin D (**ActD**). Strikingly, our compounds ALSO protected the nucleolus from ActD. This was particularly interesting because the nucleolar stress produced by poly-PR is very different from the one produced by ActD. Poly-PR causes nucleolar aggregation and enlargement, BUT ActD triggers the segregation of the nucleolus as if it had exploded. Our work is the first to report the existence of **drugs maintaining nucleolar integrity**, which is relevant not only for ALS, but also for other diseases where the nucleolus is altered, such as Huntington's or Alzheimer's diseases.

Our results inspired us to conduct three additional screens to find new compounds modulating translation and nucleolar biology, including more protectors of nucleolar stress. To end this thesis, we conducted a genetic screen aiming to understand which mutations will sensitize cancer patients to drugs targeting the nucleolus.

In conclusion, here we used phenotypic screens to find potential new therapies for human diseases associated to dysfunctional protein production and nucleolar biology.

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RESUMEN DIVULGATIVO DE LA TESIS

¿Cómo se descubren los fármacos? Hay diferentes maneras, pero en este trabajo hemos usado una estrategia conocida como **cribados fenotípicos** para descubrir moléculas y genes beneficiosos para el tratamiento de varias enfermedades. Los cribados fenotípicos comienzan con un **fenotipo** o una característica que podemos observar en células o en organismos, y que podemos modificar, ya sea usando medicinas o haciendo modificaciones genéticas. La mayoría de los cribados que hemos hecho en esta tesis son **cribados químicos**, en los que hemos expuesto células a miles de fármacos para encontrar nuevos compuestos que afecten a la producción de proteínas en la célula y potencialmente nuevas terapias para enfermedades como **la Esclerosis Lateral Amiotrófica (ELA)** y el **cáncer**. Estos cribados los hacemos en pequeñas placas, que caben en la palma de una mano, del tamaño de una Nintendo Game Boy Color, que están subdivididas en 384 pocillos o compartimentos muy pequeños. En cada pocillo, depositamos células humanas y añadimos un tratamiento distinto. ¡Esto significa que podemos testar 384 fármacos a la vez en tan sólo una placa! Para ayudarnos utilizamos robots y sistemas automatizados, y así cribar miles de compuestos en una tirada. Para entender cuál es el efecto de cada fármaco en la célula, lo que hacemos es *colorear* las partes de la célula que nos interesa observar, este proceso se conoce como etiquetado, marcaje o tinción. Luego, tomamos imágenes usando el microscopio, de las que extraemos información usando sistemas automáticos de análisis de imagen. A continuación, podemos evaluar los cambios que las drogas han producido en las células comparándolas con células no tratadas. Ahora, imagina que podemos colorear o marcar una proteína *mala* producida en la célula en verde, y que cuando esta proteína se produce (cuando detectamos señal verde), la célula está enferma. Ahora queremos encontrar una potencial cura para esta enfermedad y hacemos un cribado fenotípico. Nuestro cribado hipotético tratará de encontrar fármacos que reduzcan la señal verde, de modo que las células estarán más sanas. Pero, en este cribado, también podemos encontrar otros compuestos que incrementen esta señal, lo que a veces es también interesante para comprender mejor la enfermedad. En todo caso, si nuestro objetivo es desarrollar un medicamento para esta enfermedad, utilizaremos compuestos que reduzcan la señal verde como punto de partida para estudiarlos más en profundidad.

En nuestro primer trabajo (**publicación I**), nuestro objetivo era entender el efecto de medicamentos conocidos en la síntesis de proteínas. La **producción de proteínas** en la célula es un proceso fundamental porque las proteínas son las máquinas moleculares que hacen la mayoría de funciones celulares, como regular cómo la célula crece, se mueve o incluso si se convierte en un tipo celular u otro. Por ejemplo, las neuronas producen ciertas proteínas y los espermatozoides, otras. Las instrucciones para hacer proteínas están en el ADN (genoma) que está guardado celosamente en el núcleo de la célula para estar bien protegido, ya que es el tesoro más valioso de la célula. Este manual de instrucciones, o ADN, está escrito en un lenguaje que se tiene que traducir a un código de aminoácidos en el que están escritas las proteínas. Este proceso se conoce como **traducción** y está asistido por complejas máquinas moleculares llamadas **ribosomas**. Los ribosomas se producen en un compartimento dentro del núcleo de la célula (donde el ADN está) llamado **nucléolo**. La traducción es un proceso

complejo y altamente regulado que responde a las necesidades energéticas de la célula. Si la célula está creciendo en condiciones óptimas, recibe nutrientes, y es *feliz,* se producen más proteínas, la célula crece y se divide para dar lugar a nuevas células. Por el contrario, si la célula está bajo condiciones de estrés, subóptimas para su crecimiento, se apaga la traducción hasta que se supere este bache; en el peor de los casos, si el estrés persiste, la célula se muere. El proceso de traducción es tan fundamental que si no está bien regulado da lugar a enfermedades. Por ejemplo, las **células cancerosas** son adictas a la traducción y producen proteínas en exceso, lo que les hace crecer fuera de control. En otros casos, hay enfermedades donde las células traducen muy poco y eso hace que no puedan realizar sus funciones normales. Para identificar fármacos que regulan traducción, lo primero que tenemos que hacer es *observarla* en células. Para visualizar cambios en traducción, marcamos proteínas de nueva síntesis con una molécula que se llama **OPP**. OPP se puede detectar por microscopía, y su señal se puede cuantificar usando análisis de imagen para monitorizar cambios en traducción. Cuando las células traducen más, más señal de OPP se puede detectar y viceversa. De modo que al final, con la señal de OPP podemos tener una instantánea de la cantidad de proteínas que se han producido en una célula en un determinado momento. La incorporación de OPP nos ayudó a hacer un cribado para encontrar compuestos incrementando o reduciendo síntesis de proteínas en células de cáncer. En nuestro estudio, no pudimos encontrar fármacos que estimulasen la síntesis de proteínas (aumento de señal de OPP) en células cancerosas, probablemente porque éstas tienen los niveles de traducción por las nubes, y no se pueden elevar más allá. Sin embargo, sí que encontramos compuestos que reducían traducción (decremento de señal de OPP). Muchos de ellos ya eran conocidos inhibidores de síntesis de proteínas – lo que nos indicaba que íbamos en buen camino – pero, también encontramos una nueva droga capaz de reducir traducción. Esta droga se llama **SKI-II**, y se había desarrollado como un inhibidor de unas enzimas llamadas esfingosín quinasas (SPHKs), que se han explotado como dianas para acabar con el cáncer. Sin embargo, nuestros resultados muestran que SKI-II y un compuesto similar (análogo) que se usa en la clínica, matan a células cancerosas inhibiendo traducción y que este efecto es independiente de su diana esperada (SPHKs). En particular, **SKI-II y su análogo reducen la traducción** porque destruyen una estructura esencial para la producción de proteínas en la célula. Nuestro trabajo es el primero en reportar los efectos de fármacos conocidos en traducción y muestra un nuevo mecanismo de toxicidad para SKI-II y su análogo de uso médico, que puede ser explorado para proveer de mejores tratamientos a los pacientes de cáncer.

En nuestro segundo trabajo (**publicación II**), hicimos un cribado para encontrar medicinas para tratar la **ELA**. La ELA es una terrible **enfermedad neurodegenerativa** caracterizada por la muerte de neuronas que inervan los músculos de nuestro cuerpo, lo que resulta en parálisis. El físico Stephen Hawking padeció esta enfermedad que lo dejó 50 años en una silla de ruedas, asistido de aparatos que podía controlar con los únicos músculos que podía mover, los de sus ojos. Por trágica que es su historia, se trata de un caso excepcional, ya que la mayoría de los pacientes de ELA muere 2-5 años después de su diagnóstico, cuando su diafragma deja de funcionar, y consecuentemente, dejan de respirar. El "ALS bucket challenge", el reto viral en 2014 en el que gente se tiraba un cubo de hielo en la cabeza nos concienció a todos sobre esta

enfermedad que a día de hoy no tiene cura, y para la que las dos medicinas disponibles para su uso, sólo alargan unos pocos meses la vida de los pacientes, si se da el caso. Una de las razones por las que la ELA no tiene aún una cura es, probablemente, porque ha sido una enfermedad poco estudiada, ya que sus causas no se conocían. Ahora sabemos un poco más de ella, se han identificado mutaciones en genes que se han asociado con ELA, y la presencia de fragmentos repetitivos de ADN en el gen C9ORF72 es la mutación más frecuente en casos hereditarios y esporádicos de la enfermedad. Estos fragmentos repetitivos de ADN dan lugar a proteínas aberrantes, algunas de las cuales son tóxicas y matan células. Una de estas proteínas se llama **poli-PR**, al ser una cadena repetitiva de dos aminoácidos Prolina-aRginina, que va al núcleo de las células, predominantemente al nucléolo, interfiriendo con la producción de ribosomas, proteínas y por último matando células de cualquier tipo, incluyendo neuronas. En nuestro estudio utilizamos muerte celular causada por poli-PR como modelo de ELA. En el cribado buscamos **compuestos que limitasen la toxicidad de poli-PR en células**. Para encontrar nuevas terapias para la ELA, usamos compuestos que están aprobados para uso médico, dado que se ha demostrado que su uso es seguro en humanos, y esto beneficiaría que estos fármacos llegasen más rápido a los pacientes de ELA, que necesitan ayuda urgente. En nuestro cribado una clase de compuestos estaba sobrerrepresentada (**compuestos epigenéticos**), siendo capaces de reducir muerte celular causada por poli-PR. Por ello, decidimos explorar si más compuestos epigenéticos podían tener el mismo o mejores resultados y encontramos que tres eran muy efectivos. Tanto, ¡que incluso protegían a neuronas y a minúsculos embriones de pez cebra de los efectos de poli-PR! A continuación, nos dirigimos a investigar cómo estos fármacos combatían la ELA, y encontramos que dos de ellos **protegían el nucléolo de los efectos de poli-PR.** La función del nucléolo en la producción de ribosomas depende en gran parte de su estructura. En condiciones de estrés, el nucléolo cambia dramáticamente su morfología, lo que afecta a la síntesis de ribosomas, proteínas y a la viabilidad celular. Para explorar si nuestros compuestos rescataban el nucléolo de cualquier estrés, expusimos células a una droga que causa estrés nucleolar, Actinomicina D (**ActD**). Sorprendentemente, nuestros compuestos TAMBIÉN protegían al nucléolo de ActD. Esto es particularmente interesante porque el estrés nucleolar generado por poli-PR es muy diferente del producido por ActD. Poli-PR causa la agregación y aumento en tamaño del nucléolo, mientras que ActD gatilla su segregación, tal como si el nucléolo hubiese explotado. Nuestro trabajo es el primero en reportar la existencia de compuestos que mantienen la integridad nucleolar, lo que no es sólo importante para la ELA, sino para otras enfermedades relacionadas con alteraciones nucleolares, como la enfermedad de Huntington o el Alzheimer.

Nuestros resultados nos inspiraron a hacer tres nuevos cribados para encontrar fármacos capaces de modular traducción y el nucléolo, incluyendo más protectores de estrés nucleolar. Para acabar esta tesis, hicimos un cribado genético con el fin de comprender qué mutaciones hacen más sensibles a los pacientes de cáncer a fármacos que tienen el nucléolo como diana.

En conclusión, en este trabajo hemos usado cribados fenotípicos para encontrar potenciales nuevas terapias para enfermedades humanas relacionadas con alteraciones en la síntesis de proteínas y en la biología del nucléolo.

ABSTRACT

Protein synthesis and **ribosome biogenesis** are fundamental steps in gene expression and constitute the most energy demanding processes in living cells. Dysregulation of these processes is associated to a variety of human disorders including cancer, metabolic diseases, immunodeficiency, neurological and developmental disorders, and physiological aging. Therapeutic strategies modulating protein synthesis and ribosome biogenesis or **nucleolar biology**, have proven to be efficient for several of these disorders, and some of them are already used in the clinic, predominantly in the context of cancer. However, the success of these drugs has been limited due to activation of mechanisms of resistance or lack of general effects among different cancer types. Additionally, the application of modulators of protein and ribosome production in other disease contexts is just starting to be explored. This is particularly important for disorders where altered translation control is a hallmark, such as in the case of some **neurodegenerative diseases**. Moreover, different disorders may require different therapeutic approaches, hence, research in less known disease areas opens possibilities of finding new ways of regulating protein synthesis and ribosome biogenesis, and perhaps new biology.

In this thesis we have used **high throughput phenotypic screens** to discover new modulators of protein synthesis and nucleolar biology. Phenotypic screening allows for the systematic identification of regulators of an organismal feature (phenotype) without having any prior knowledge.

In **paper I** we benefited from novel technologies allowing visualization of changes in protein synthesis to evaluate the effects of medically approved and well-characterized drugs in mRNA translation. Our screen failed to identify small molecules stimulating translation in cancer cells growing in complete media. Yet, it seems that translation can only be boosted when the translation machinery of cells is challenged, such as when cells are grown under starvation conditions. Nevertheless, our screen identified known down-regulators of translation, supporting the validity of our approach, and a new translation inhibitor, SKI-II. SKI-II was developed as a sphingosine kinase inhibitor (SPHK), and this group of compounds has been explored extensively as anticancer drugs. However, in our hands, SKI-II inhibited translation by inducing the integrated stress response (ISR), causing physical damage to the endoplasmic reticulum (ER), which resulted in cell death. The toxicity of SKI-II and its clinically relevant analog ABC294640 was not abrogated when knocking out sphingosine kinases, while it was partially rescued upon inhibition of the ISR. Our work is the first to systematically examine the effect of known drugs in translation in cells and to report cytotoxic properties of SPHK inhibitors that are independent of SPHKs.

In **paper II** we conducted a chemical screen to identify compounds limiting the toxicity of amyotrophic lateral sclerosis (ALS)-related dipeptide repeats (DPRs). ALS is a fatal neurodegenerative disease characterized by loss of upper and lower motor neurons, leading to muscular paralysis and death, within 3 to 5 years after diagnosis. The expansion of G_4C_2 repeats within the first intron of the C9ORF72 gene constitutes the most common cause of

ALS and frontotemporal dementia (FTD). Through repeat-associated non-ATG (RAN) translation, these expansions are translated into DPRs, some of which, poly-proline arginine (PR) and poly-glycine arginine (GR), bind to the nucleoli and lead to cell death. Here we conducted a screen to identify compounds reducing toxicity of twenty-repeats poly-PR peptides (PR_{20}) added exogenously to cells in culture. Our screen identified two BET bromodomain inhibitors (Bromosporine-1 and PFI-1) and sodium phenylbutyrate (Na-Phen), currently in clinical trials, as modifiers of PR_{20} toxicity in different cell lines and in developing zebrafish embryos. Our work shows that BET Bromodomain inhibitors rescue the nucleolar stress induced by PR_{20} and the known nucleolar stressor Actinomycin D (ActD). To our knowledge, this is the first time that compounds able to protect nucleolar integrity are reported in the literature, and therefore, they might have beneficial effects in diseases associated to nucleolar stress, such as ALS/FTD.

Inspired by our results, we conducted four additional screens that are collected in the section ***preliminary results***. Following **paper I**, we applied the same screening pipeline to identify novel modulators of translation among natural compounds (***preliminary results I***). Related to **paper II**, the literature points to two main issues with current modulators of ribosome biogenesis, promiscuity, even among the so-called selective modulators, and heterogeneity in the efficacy of compounds across different cancer types. Regarding the first, the discovery of regulators of ribosome biogenesis has advanced in parallel with the technology allowing their study. Current methods allow better characterization of the activities of these drugs and development of strategies to find more selective modulators, which we reviewed in **annex I.** Nevertheless, there is a growing need for novel modulators of nucleolar activity, and we benefited from publicly available image datasets to explore the effects of known drugs in the nucleolus (***preliminary results II***). Also, we conducted a genome-wide CRISPR/Cas9 screen to identify vulnerabilities to nucleolar stressors and systematically interrogate in which genetic backgrounds these drugs are suitable anticancer therapies (***preliminary results III***). Lastly, triggered by the discovery of "nucleolar protectors" in **paper II**, we conducted a chemical screen to explore novel nucleolar functions of known drugs using the Drug Repurposing Hub library [1] from the Broad Institute (***preliminary results IV***).

Altogether, here we have used high throughput phenotypic screens to discover new modulators of protein synthesis and nucleolar biology relevant for disease contexts, and to uncover new biology linked to these processes.

LIST OF SCIENTIFIC PAPERS

THESIS PUBLICATIONS

I. **Corman, A**., Kanellis, D. C., Michalska, P., Häggblad, M., Lafarga, V., Bartek, J., Carreras-Puigvert, J., Fernandez-Capetillo, O. (2021) A chemical screen for modulators of mRNA translation identifies a distinct mechanism of toxicity for sphingosine kinase inhibitors. *PLoS Biol.* 19, e3001263.

II. **Corman, A.*,** Jung, B.*, Häggblad, M., Bräutigam, L., Lafarga, V., Lidemalm, L., Hühn, D., Carreras-Puigvert, J., Fernandez-Capetillo, O. (2019) A Chemical Screen Identifies Compounds Limiting the Toxicity of C9ORF72 Dipeptide Repeats. *Cell Chem Biol.* 26, 235-243.

***** – Authors contributed equally to this work

RELATED MANUSCRITPS

I. **Corman, A**., Sirozh, O., Lafarga, V., Fernandez-Capetillo, O. Modulating nucleolar activity as a therapeutic strategy. Review Manuscript. *Trends Biochem Sci.*

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LIST OF ABBREVIATIONS

ActD	Actinomycin D
AD	Alzheimer's Disease
ADME-tox	Absorption, Distribution, Metabolism And Toxicity
ALS	Amyotrophic Lateral Sclerosis
AML	Acute Myeloid Leukemia
ASO	Antisense Oligonucleotide
ATCC	American Type Culture Collection
ATF4	Activating Transcription Factor 4
AVG	Average
BDNF	Brain-Derived Neurotrophic Factor
BET/BETi	Bromodomain And Extraterminal Domain Family / BET Inhibitors
BFP	Blue Fluorescent Protein
BIOGRID / BIOGRID ORCS	Biological General Repository For Interaction Datasets / Open Repository Of CRISPR Screens
BSP1	Bromosporine-1
Cas9	CRISPR Associated Protein 9
cHAUs	N,N0 -Diarylureas
CHX	Cycloheximide
cMAP	Connectivity Map
cmpds	Compounds
CPT2	Camptothecin-2
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CV%	Percentage of Coefficient Of Variation
DNA	Deoxyribonucleic acid
DBM	Dibenzoylmethane
DDR	DNA Damage Response
DEGS1	Desaturase 1
DFC	Dense Fibrillar Component
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

dsRNA	Double-Stranded RNA
E, P, A sites	(E)xit, (P)eptidyl, and (A)minoacyl Ribosomal Sites
EdU	5-Ethynyl-2′-Deoxyuridine
eEF-	Eukaryotic Elongation Factor
eIF-	Eukaryotic Initiation Factor
4E-BP	eIF4E-Binding Protein
ER	Endoplasmic Reticulum
FAM-PR20 / 5(6)-FAM-PR20-HA	PR_{20} Tagged With Fluorescein / PR_{20} Tagged with Fluorescein and HA
FC	Fibrillar Center
FDA	Food And Drug Administration
FIBL1	Fibrillarin
FKBP12	FK506-Binding Protein
5-FU	5-Fluorouracil
FTD	Frontotemporal Dementia
FUNCAT	Fluorescent Biorthogonal Noncanonical Amino Acid Tagging
GC	Granular Component
GCN2	General Control Non-de-repressible 2
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GTP/GDP	Guanosine Triphosphate / Guanosine Diphosphate
HA-tag	Hemagglutinin Tag
HD	Huntington’s Disease
HDAC/HDACi	Histone Deacetylase / HDAC Inhibitor
9HE	Hydroxyellipticine
Hpf	Hours Post Fertilization
HPG	Homopropargylglycine
HRE	Hexanucleotide Repeat Expansion
HRI	Heme-Regulated Inhibitor

HSP	Heat Shock Protein
HTM	High Throughput Microscopy
HU / HU2	Hydroxyurea / Hydroxyurea 2
IF	Immunofluorescence
IGF-1	Insulin-like Growth Factor
IP	Immunoprecipitation
IRBC	Impaired Ribosome Biogenesis Checkpoint
IRES	Internal Ribosome Entry Sites
ISR	Integrated Stress Response
ISRIB	Integrated Stress Response Inhibitor
Kb	Kilobase
KO	Knockout
LLPS	Liquid-Liguid Phase Separations
m7G	5' Methyl-7-guanosine Cap
MAGeCK	Model-Based Analysis Of Genome-Wide CRISPR/Cas9 Knockout
MAPK	Mitogen Activated Protein Kinases
MDM2/HDM2	Murine/Human Doble Minute 2
Met / Met-tRNAi	Methionine / Methionyl Transfer RNA
MOI	Multiplicity Of Infection
mRNA	Messenger Ribonucleic Acid
MRPL20	Mitochondrial Ribosomal Protein L20
mTOR	Mammalian Target Of Rapamycin
mTORC1/mTORC2	mTOR Complexes 1 And 2
Na-Phen	Sodium Phenylbutyrate
NCL	Nucleolin
NGS	Next Generation Sequencing
NLS	Nuclear Location Sequence
NORs	Nucleolar Organizing Regions
NPM1	Nucleophosmin 1
OPP	O-Propargyl-Puromycin
ORF	Open Reading Frame

p-eIF2α / eIF2α	Phospho and Total Eukaryotic Initiation Factor 2 Subunit 1 (α)
p70S6K1/pS6K1 and 70S6K1/S6K1	Phospho and Total Ribosomal Protein S6 Kinase
PABP	Poly(A)-Binding Protein
PD	Parkinson's Disease
PERK	PKR-Like Endoplasmic Reticulum Kinase
PERKi	PERK Inhibitor GSK2606414
PFA	Paraformaldehyde
PIC	Pre-Initiation Complex
PKR	Double-Stranded RNA-Dependent Protein Kinase
PP1/PP1c	Protein Phosphatase 1 / PP1 Catalytic Subunit
PR/GR	Poly-Proline-Arginine/Poly-Glycine-Arginine
PR20	Poly-Proline-Arginine, 20 Repeats
pre-rRNA (47S)	Precursor rRNA 47S
RAN	Repeat-Associated Non-ATG Dependent Translation
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RNA pol I/II/ III	RNA Polymerase I/II/III
RNase	Ribonuclease
RP	Ribosomal Protein
RPL-/RPS-	Ribosomal Protein (L)arge Or (S)mall Subunit
RRA	Robust Rank Aggregation
rRNA	Ribosomal RNA
S1P	Sphingosine-1-Phospate
sgRNA	Single Guide RNA
SL1	Selectivity Factor 1
snoRNAs	Small Regulatory RNAs
SPHK	Sphingosine Kinase
StDev	Standard Deviation
TC	Ternary Complex
TEM	Transmission Electron Microscopy

TIF-1A	Transcription Intermediary Factor 1- A
TL	Tupfel Long Fin Zebrafish
TOP1/TOP2	Topoisomerase I and II
tRNA	Aminoacyl Transference RNAs
TSA	Trichostatin A
UBF1	Upstream Binding Factor 1
UMI	Unique Molecular Identifier
uORF	Upstream ORF
UPR	Unfolded Protein Response
UTR	Untranslated Region
UV	Ultraviolet Light
VCP	Valosin-Containing Protein
VWM	Vanishing White Matter
WB	Western Blot
WGA	Wheat Germ Agglutinin
WT	Wild-Type
Z´	Z-Prime Factor

1 INTRODUCTION

Gene expression is the process by which the information encoded in genes (genotype) is used to synthesize proteins. The expression of the genotype contributes to the production of observable features, known as **phenotypes** [2]. Gene expression is a highly regulated multistep process that controls timing, location and abundance of a given gene product [3]. Regulation of gene expression drives cell proliferation, tissue differentiation, and ultimately, organismal development [2]. Proteins have direct phenotypic impact because they are the main effectors of cellular functions behind these processes [4]. Consequently, the catalogue and abundance of proteins in cells determines their fate and energetic balance [4,5]. The instructions for protein production are stored inside the nucleus in the DNA (genome) and are written in a nucleotide-based language that is transcribed into messenger RNAs (mRNAs) (transcriptome), which needs to be translated into amino acid-based polypeptide chains. **Translation** can be considered as the last step that dictates protein production (translatome), which is the input for further fine-tuning mechanisms of regulation of protein abundance (proteome), such as mRNA decay or post-translational modifications of proteins [2,3] (**fig. 1**).

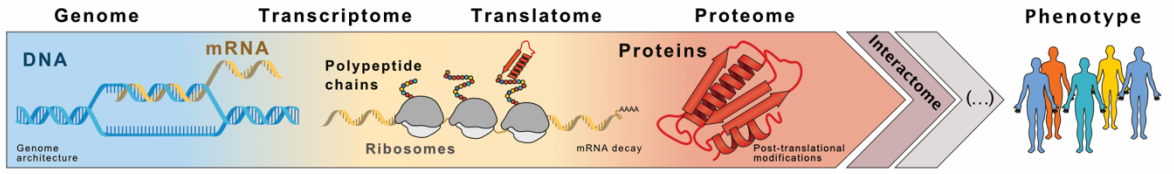


Figure 1. *Overview of the initial steps in gene expression connecting genotype to phenotype. Adapted from Buccitelli and Selbach, 2020* [2].

Translation is the most energy demanding process in the cell [6], and it is tightly regulated at different levels: initiation, elongation, termination, and ribosome composition. Importantly, deregulation of translation, including defects in **ribosome biogenesis**, has been associated to a variety of disorders, such as cancer, diabetes, neurodevelopmental and neurodegenerative disorders [7-10]. Altogether, the crucial role of translational control in regulation of cellular homeostasis, together with its effects on human health, have made translation an attractive target for therapeutic exploration [4,11].

Here, I present an overview of **mRNA translation** and **ribosome biogenesis**, their role in **disease**, as well as the **therapeutic strategies** targeting these processes to contextualize the research presented in this doctoral thesis.

1.1 mRNA TRANSLATION

Translation is a coordinated process by which a polypeptide chain is synthesized from mRNA. The machinery in charge of translation is constituted by ribosomes, aminoacyl transference RNAs (tRNAs) and translation factors, which intervene in different steps during protein synthesis. Eukaryotic ribosomes are comprised by a small subunit (40S) and a large subunit (60S) which together (80S) become the factory where the mRNA nucleotide-based code is read to amino-acid code (more about ribosome biogenesis in **section 1.2**). The transition from a nucleotide to an amino acid sequence occurs via base-pairing between triplets of nucleotides (codons) in the mRNA sequence with complementary bases (anticodons) in the tRNAs. Depending on its anticodon sequence, each tRNA is loaded with a particular amino acid, which constitutes the basis of the genetic code [12]. Translation is conventionally divided in three steps: initiation, elongation, and termination.

- **Initiation.** Canonically, eukaryotic translation starts in the cytoplasm with the recognition of the start codon (AUG) in the 5' untranslated region (UTR) of mRNAs. First, the eukaryotic initiator factor (eIF) eIF2, the initiator methionyl transfer RNA (Met-tRNAi) and GTP form the **ternary complex (TC),** eIF2-GTP- Met-tRNAi. Then, the TC associates with the 40S ribosomal subunit together with other eIFs (eIF1, eIF1A, eIF3, and eIF5) to form the 43S ribosome **pre-initiation complex (PIC)**. Next, the PIC is recruited to the 5' methyl-7-guanosine (m^7G) cap of the mRNA. This structure is present in the majority of transcripts in the cell, and together with the poly(a)-binding protein (PABP), at the 3' terminal end of the mRNA, drives circularization of mRNAs, which facilitates translation [4]. In the 5'cap, the PIC interacts with the **cap-binding protein complex eIF4F**, constituted by cap-binding protein eIF4E, scaffolding protein eIF4G, and helicase eIF4A. Association of eIF4G with the PIC triggers the formation of the 48S ribosome complex, and initiates scanning of the 5'UTR until encountering with the start codon (**cap-dependent scanning**) [4,13]. Additional interaction between eIF4G and PABP improves mRNA stability and enhances translation [14]. Commitment to the start codon leads to hydrolisis of eIF2-GTP, ejection of the eIFs from the PIC, assembly of the 60S ribosomal subunit, and formation of the 80S ribosome, initiating elongation phase. Then, inactive eIF2-GDP is converted to eIF2-GTP by the guanine nucleotide exchange factor (GEF) eIF2B to start another round of translation [4] (**fig. 2**). However, translation can also be initiated from non-AUG start codons, such as near-cognate AUG codons in the 5'UTRs, or, as described for non-ATG (RAN) translation, from repeated stretches of RNA. Furthermore, some mRNAs do not require of a cap structure to be translated. The presence of internal ribosome entry sites (IRESs) in mRNAs allow direct recruiment of ribosomes and protein synthesis, bypassing scanning of the 5'UTR [4,15,16]. These alternative mechanisms of translation play important roles in modulating the translatome under physiological conditions and in response to stress, but they have also been associated to disorders, such as in the case of many IRESs in cancer, promoting tumorigenesis [15]; or RAN translation in the

production of toxic proteins in amyotrophic lateral sclerosis (ALS) and Hungtington's disease [17].

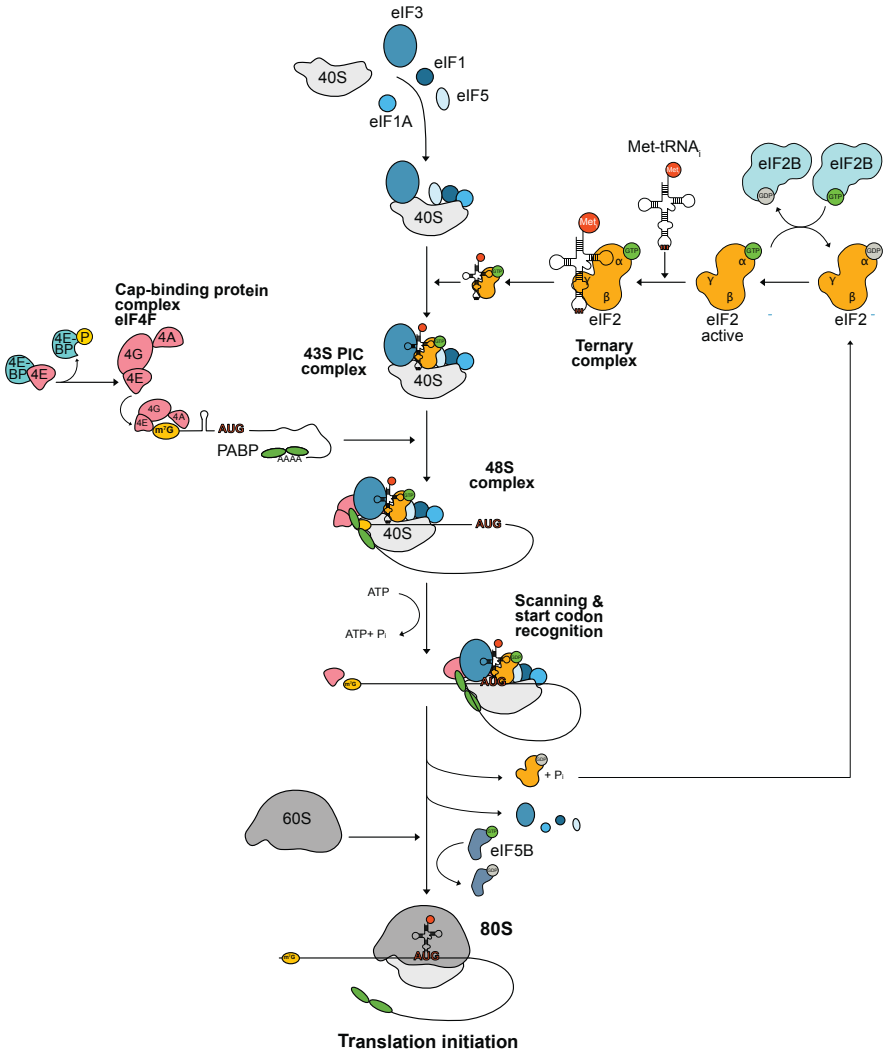


Figure 2. *Cap-dependent translation initiation in eukaryotes. Adapted from Komar and Merrick, 2020* [18].

- **Elongation.** Translation elongation starts with the formation of the 80S ribosome at the start codon, which begins protein synthesis from an open reading frame (ORF). The addition of the initial amino acid methionine is achieved by base-pairing the anticodon of Met-tRNAi with the start codon of the mRNA at the peptidyl (P) site of the 80S ribosome [19,20]. Then, eukaryotic elongation factor eEF1A bound to an aminoacyl-tRNA and GTP, form the eEF1A-GTP-aminoacyl-tRNA complex, which is delivered to the aminoacyl (A) site, already accomodating the next three nucleotides of the transcript. Complementary base-pairing of the codon in the A site with the anticodon of aminoacyl-tRNA triggers GTP hydrolisis of eEF1A [21]. The aminoacyl-tRNA remains in the A site, while the eEF1A-GDP complex is released, to be reactivated by the GEF eEF1B. Next, the peptidyl transferase centre at the 60S catalyses the formation of the first peptide bond of the polypeptide chain between the aminoacyl residues of the tRNAs at the P and A sites. This results in ribosome translocation, shifting the tRNAs

at the P and A sites to the next position, the exit (E) and P sites, respectively. This leaves the P site occuppied by the peptidyl-tRNA, while the A site is vacant for the next aminoacyl-tRNA. This transition is assisted by eEF2-GTP upon GTP hydrolisis [22] (**fig. 3**).

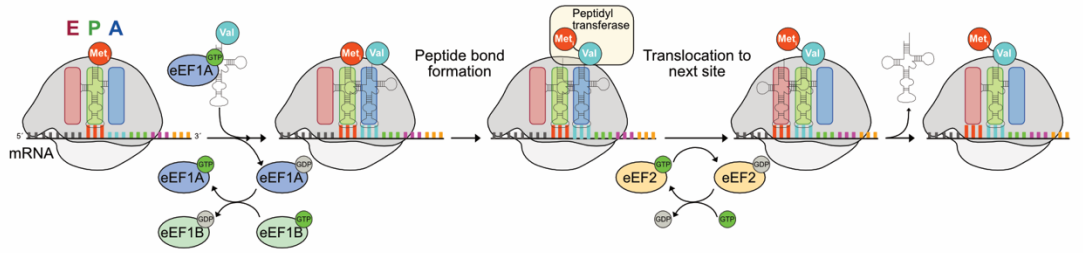


Figure 3. *Overview of translation elongation. After the formation of every new peptide bond and displacement of tRNAs from the (E)xit site, the cycle re-starts. P, peptidyl site; A, aminoacyl site.*

- **Termination.** Translation termination occurs when ribosomes encounter a stop codon (UAA, UGA or UAG) in their A site, releasing the polypeptide chain and ribosome from the transcript template. This process is mainly assisted by the eRF1 and eRF3, which form a complex with GTP. In this complex, eRF1 recognizes the stop codon, while eRF3 hydrolyzes GTP, enhancing peptide release. Then, 80S ribosomes are disassembled, by releasing 60S subunits, deacylated tRNAs and mRNAs from 40S subunits. Ribosomes, mRNAs and tRNAs are recycled for multiple rounds of translation [23,24].

Since protein synthesis is energetically the most expensive process for cells, several mechanisms modulate translation rates and accuracy, depending on the metabolic status of the cell. In response to nutrients and external cues, several pathways modulate translation, including mammalian target of Rapamycin (mTOR), mitogen activated protein kinases (MAPK) and integrated stress response (ISR), all of which particularly target translation initiation [4,25]. For this thesis, the most relevant of these pathways are mTOR and the ISR. In response to nutrients, mTOR regulates the formation of the eIF4F complex, and different stress inputs lead to activation of the integrated stress response (ISR), which controls the ternary complex [4,9]. Furthermore, translation initiation has been the most exploited mechanism to modulate protein synthesis using chemical and genetic approaches [11]. An additional level of translation control is achieved by regulating ribosome biogenesis, which is highly sensitive to stress sources and its therapeutic potential has been extensively explored [26].

1.1.1 Regulation of mRNA translation by mTOR signaling

The mTOR pathway is one of the main regulators of translational control. mTOR is a serine/threonine protein kinase that forms two multi-subunit complexes in the cell, mTORC1 and mTORC2 [27]. mTORC1 plays a pivotal role in translation, phosphorylating components of the translation machinery and protein kinases that modulate translation factors (**fig. 4**). The best well-studied substrates of mTORC1 involved in translation control are eIF4E-binding proteins (4E-BPs) and ribosomal protein S6 kinase (p70S6K1/pS6K1) [27]. In the presence of growth factors and nutrients, mTORC1 phosphorylates 4E-BPs, which sequester eIF4E, triggering their dissociation from the translation initiation factor, allowing formation of the cap-binding protein complex, and initiation of translation [4]. Simultaneously, mTORC1 phosphorylates ribosomal protein S6K1, which phosphorylates translation initiation and elongation factors, and ribosomal protein S6 (RPS6), which is a component of the 40S small ribosomal subunit [28,29]. Additionally, activation of mTORC1 promotes ribosome biogenesis in the nucleolus, stimulating the transcription of ribosomal DNA (rDNA) [30]. Altogether, activation of mTORC1 promotes cellular growth and proliferation. Under non-favorable growth conditions, such as starvation, mTORC1 is not active, leading to hypo-phosphorylation of its substrates, resulting in global attenuation of translation. Furthermore, additional substrates of mTORC1 have been identified regulating different steps in translation and influencing in translational output in response to different stimuli [25]. Also, the mTOR pathway is further regulated by upstream signaling cascades, such as MAPK [25]. Collectively, mTORC1 is a master integrator of energetic cues and it has a key role in the regulating cellular homeostasis, and consequently in human health. For instance, hyperactivation of mTORC1 is common in most cancers, and it has been characterized as a driver of tumorigenesis, of which inhibition sufficiently impaired neoplastic growth [27,31,32].

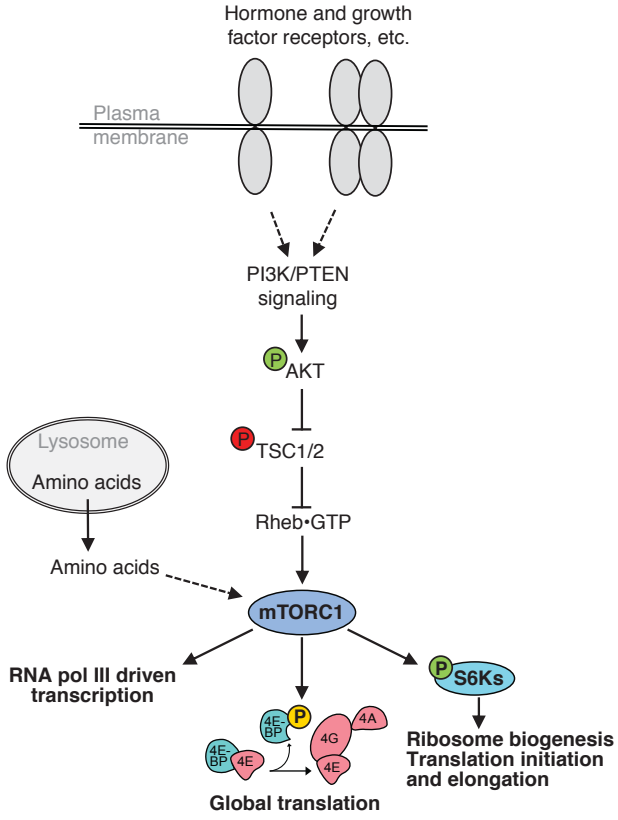


Figure 4. *Simplified overview of modulation of translation by mTORC1 in response to nutrients. Activating phosphorylation is shown in green; in red, is inhibitory phosphorylation; in yellow, phosphorylation of 4E-BPs triggers its dissociation from 4E and formation of the eIF4E. Adapted from Proud, 2020* [25].

1.1.2 Regulation of mRNA translation by the ISR

The integrated stress response (ISR) is a central signaling network conserved among eukaryotes, which is activated by a variety of stress sources that converge in the phosphorylation of eukaryotic initiation factor 2 subunit 1 (eIF2α) at serine 51 [4,33] (**fig. 5**). Phosphorylation of eIF2α leads to inhibition of global protein synthesis and activation of selective transcriptional and translational programs to cope with cellular stress, maintaining protein homeostasis (proteostasis), and ultimately promoting cell recovery and survival [34,35]. Nevertheless, if the stress cannot be mitigated, due to its severity or persistence, the ISR triggers apoptosis to eliminate the damaged cells.

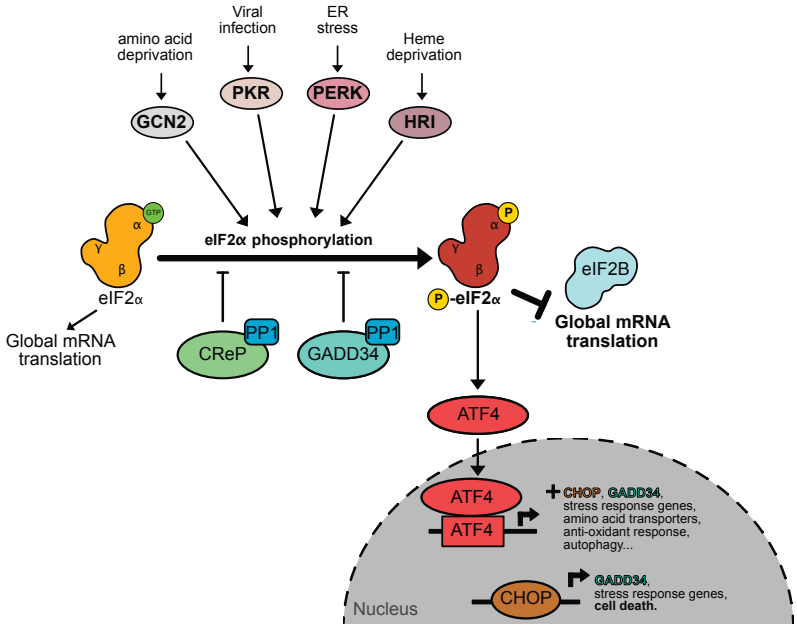


Figure 5. *Overview of the ISR. Four kinases (GCN2, PKR, PERK and HRI) trigger phosphorylation of eIF2α, which inhibits general translation, leads to translocation of ATF4 to the nucleus and activation of stress-response transcriptional and translational programs. Termination of the ISR is achieved by dephosphorylation of eIF2α. By CReP-PP1 or GADD34-PP1. Adapted from Derisbourg, 2021* [36].

1.1.2.1 Four kinases activate the ISR

In metazoans, four serine/threonine kinases phosphorylate eIF2α: HRI (heme-regulated inhibitor), PKR (double-stranded RNA-dependent protein kinase), GCN2 (general control non-de-repressible 2), and PERK (PKR-like endoplasmic reticulum (ER) kinase) [37]. These kinases are structurally very similar, except for their distinct regulatory domains allowing them to respond to different environmental and physiological stresses [26,35]. Stress signals detected by regulatory domains trigger the dimerization and activation of these kinases.

- **HRI** is activated upon heme-deprivation [38]. HRI is mostly expressed in erythroid cells, and its involved in erythropoiesis, where translation of globin needs to be coupled with the availability of heme to produce hemoglobin. Hence, activation of the ISR by HRI protects erythroid cells from accumulation of toxic globin aggregates and iron deficiency. Interaction of heme with the regulatory domains of HRI inhibits the kinase.

Interestingly, HRI is also activated by arsenite-induced oxidative stress, heat shock, proteasome inhibition, and nitric oxide [35].

- **PKR** is mainly activated by double-stranded RNA (dsRNA) after viral infection [39]. Activation of PKR results in translational shutdown of viral and host mRNAs. Additionally, PKR activation is triggered by other sources of stress, including oxidative and ER stress, growth factor deprivation, cytokines, bacterial infection, ribotoxic stress, stress granules, heparin, and by caspase activity in early stages of apoptosis [40].
- **GCN2** is activated by amino acid deprivation, which is sensed by binding of deacylated tRNAs to the kinase [41]. Activation of GCN2 helps in attenuating translation when there are no amino acids available. Additionally, GCN2 has been reported to be stimulated by ultraviolet light (UV), serum starvation, oxidative stress, and viral infection [35].
- **PERK** is activated in response to ER stress [42], which could be consequence of an accumulation of unfolded proteins in the lumen of the ER or due to changes in the lipidic composition of the membranes of the ER (**fig. 6**). Of note, PERK is one of the arms that regulate the unfolded protein response (UPR), together with kinases IRE1 and ATF6 [43]. These kinases are activated upon imbalance in the amount of unfolded proteins and chaperones in the lumen of the ER, and activate gene expression programs modulating synthesis, processing, maturation and secretion of proteins. Both PERK and IRE1 respond to changes in the lipid composition of the ER, which are essential for mainting calcium levels in the ER, necessary for protein folding, trafficking and secretion [44]. Additional roles of PERK in metabolite sensing related to insulin resistance, mitochondrial development and thermogenesis have been reported [26].

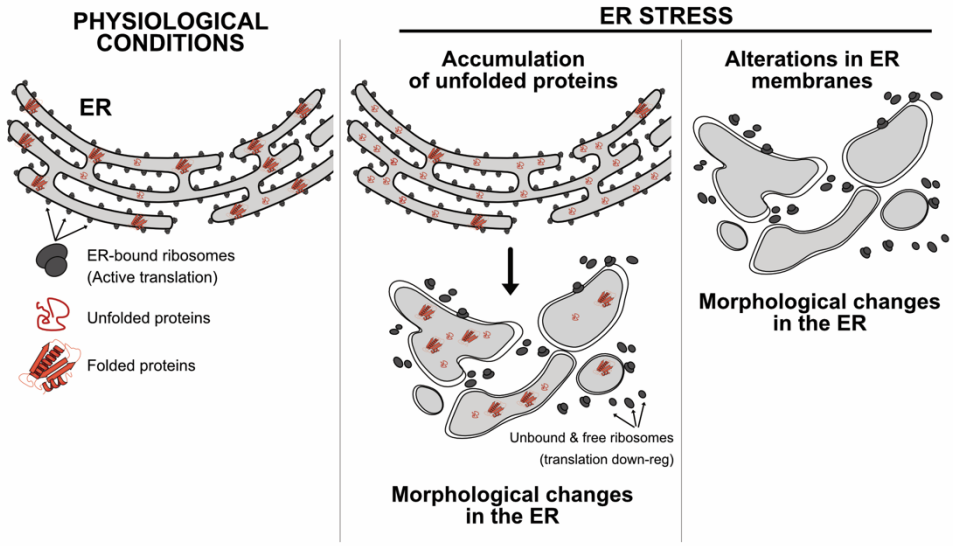


***Figure 6**. Changes in the ER induced by different sources of ER stress. In physiological conditions proteins are folded, even co-translationally, in the lumen of the ER. The rough ER is characterized to be decorated by ribosomes in its cytoplasmic face. ER stress can occur because of accumulation of unfolded proteins in the ER, leading to morphological changes of the organelle. However, ER stress can be produced also but alterations in the biophysical properties of ER membranes.*

Nevertheless, these kinases have overlapping functions, as shown in knockout cells for specific kinases, which still activate the ISR after exposure to their canonical stressors [37].

1.1.2.2 Phosphorylation of eIF2α as the core of the ISR

The ISR attenuates translation by modulation of the ternary complex (TC), specifically of eIF2. eIF2 is constituted by three subunits, α, β and γ. For the ternary complex to be active, eIF2 needs to be loaded with GTP. Upon commitment to translation, GTP is hydrolyzed to GDP, and the exchange of GDP for GTP needs to be assisted by GEF eIF2B. However, in response to stress, eIF2α phosphorylated at serine 51 inhibits eIF2B-mediated exchange of eIF2-GDP to eIF2-GTP, preventing the formation of the 43S PIC and translation of cap-dependent mRNAs [33,34]. However, attenuation of global translation allows translation of cap-independent mRNAs, which encode for proteins involved in stress responses, such as ATF4 (activating transcription factor 4), the best characterized effector of phosphorylated eIF2α [35].

1.1.2.3 Cellular effects of the ISR

ATF4 is a crucial regulator of the ISR. It is a leucine zipper transcription factor belonging to the family of AMP response element binding protein (ATF/CREB family) [45]. Phosphorylation of eIF2α triggers translocation of ATF4 to the nucleus, promoting transcription of genes involved in cellular stress adaptation, such as CHOP, with which forms a heterodimer that stimulates the expression of other stress-responsive genes, such as ATF3, GADD34, TRIB3, WARS, and RPL7 [35]. Additionally, most of these stress response genes present short inhibitory upstream ORFs (uORFs) in their 5'UTR, which prevent their expression under normal physiological conditions. However, due to ISR-driven down-regulation of cap-dependent translation, these stress-response genes can be expressed, amplifying the overall ISR response. Activation of the ISR reduces protein load overwhelming the ER with unfolded proteins and promotes the synthesis of chaperones that assist protein folding. The ISR can stimulate macroautophagy, thus removal of unfolded proteins or damaged organelles resulting in replenishment of the amino acid pool, providing of energy to starved cells. Finally, activation of the ISR up-regulates the expression of negative modulators of apoptosis and pro-survival signaling, to restore physiological conditions once the stress is resolved [35].

1.1.2.4 Termination of the ISR

Termination of the ISR is essential to restore cellular homeostasis, and it occurs via dephosphorylation of eIF2α by the protein phosphatase 1 (PP1) [46]. The PP1 complex is constituted by the PP1 catalytic subunit (PP1c) associated to either GADD34, which expression is induced by the ISR, or to CReP, a constitutively expressed repressor of eIF2α phosphorylation. Under physiological conditions, PP1-CReP maintains low levels of eIF2α phosphorylation [47]. But, under stress, ATF4, and downstream CHOP and ATF3, stimulate the synthesis of GADD34, to substantially achieve eIF2α dephosphorylation [48]. Thus, formation of the GADD34–PP1 complex acts as a negative feedback loop to restore protein synthesis. Additionally, ATF4 has a relatively short half-life, which facilitates termination of the ISR [49].

However, if the stress persists, ATF4, ATF3, and principally CHOP, can promote cell death [35]. CHOP induces pro-apoptotic members of the BCL2 family, expression of death receptors, and

oxidases which destabilize the ER environment, causing additional damage. CHOP in association with ATF4 and ATF3, also up-regulates the expression of other genes promoting cell death. Of note, CHOP is not a sufficient driver of ISR-toxicity [50]. Additionally, activation of the ISR regulates NRF2, which is involved in the oxidative stress response [51], expression of the caspase inhibitor XIAP [52], and crosstalk with mTORC1 [53], which could become active under non-optimal conditions, contributing to cellular catastrophe and death [35].

As expected, control of cellular translation by the ISR plays a central role in various diseases, such as diabetes, cancer, and viral infection [34,35].

Until recently, it was considered that regulation of translation by mTORC1 and the ISR were independent from each other. So that, in response to stimuli, mTORC1 controlled translation of a set of genes, and the ISR of another [54], even though the two pathways converge in regulating translation initiation [4]. However, a recent study using mass spectrometry by Klann and colleagues [55] revealed that mTORC1 and the ISR regulate translation of the same set of proteins. So, translational repression of specific genes is determined by the strength of changes in global translation, rather than on specific activation of mTORC1 or the ISR. Thus, attenuation of translation can be considered as a dose-dependent event, where some transcripts are more sensitive to others in response to stress. These findings suggest that strategies directed to study changes general translation, as done in this thesis, might be more impactful than focusing on targeting specific pathways.

1.2 CONTROL OF RIBOSOME PRODUCTION

1.2.1 Ribosomal DNA

Ribosomes are molecular machines formed by ribosomal proteins (RPs) and ribosomal RNA (rRNA). In eukaryotes, three RNA polymerases (RNA pol I, II and III) participate in ribosome production. RNA pol I exclusively transcribes ribosomal DNA (rDNA) to ribosomal RNA (rRNA); RNA pol II transcribes most mRNAs in the cell, including the ones coding for RPs; and, RNA pol III, synthesizes small regulatory RNAs (snoRNAs), tRNAs, and the 5S rRNA, an essential component of the 60S large ribosomal subunit [56]. Human cells contain hundreds of copies of rDNA, most of which are arranged head-to-tail in tandem arrays of 50-300 repeating units that are included in nucleolar organizer regions (NORs) located in the short arms of acrocentric chromosomes 13, 14, 15, 21 and 22 [57]. Each rDNA repeat is approximately 43 kb, of which 30 kb correspond to the intergenic spacer region containing regulatory elements and 13 kb to the precursor rRNA (47S, pre-rRNA) [58] (**fig. 7**). 47S pre-rRNA contains rRNAs constituting both ribosomal subunits, 18S for the 40S small ribosomal subunit, and 5.8S and 28S for the 60S large subunit. The polycistronic nature of 47S pre-rRNA ensures equimolar production of ribosomal subunits. In fact, altered stoichiometry in ribosomal subunits triggers inhibition of rRNA synthesis, cellular stress, and it is the cause of diseases such as ribosomopathies [59]. The 5S rRNA is encoded separately in the long arm of chromosome 1 (1q42.13) [60]. Interestingly, the number of rDNA repeats is variable among human individuals, although whether this has a physiological effect is unknown [60,61]. In most cells, about half of

these genes are epigenetically silenced [58]. Interestingly, changes in growth conditions predominantly affect transcriptional efficiency of already active genes, rather than activate silent genes. Hence, inactive rDNA plays a structural role in chromatin organization and maintenance of genome integrity [62,63]. Due to its repetitive nature and high transcriptional rates, rDNA is one of the most unstable genomic regions and a recombination hotspot, which requires precise surveillance by the DNA damage response (DDR) machinery [59].

1.2.2 Ribosome biogenesis

Ribosome biogenesis initiates in **the nucleolus** with the transcription of rDNA by the RNA pol I, which accounts for up to a 60% of total transcriptional activity in eukaryotic cells [64] (**fig. 7A**). RNA pol I transcription is often referred to as **nucleolar activity**. Several factors specifically interact with RNA pol I, constituting the **Pre-Initiation Complex (PIC)** at the rDNA promoter. These specific interactions provide a framework for specific and early modulation of nucleolar activity. Briefly, the Selectivity factor 1 (SL1) complex binds at the rDNA promoter and confers specificity for RNA pol I and promotes its recruitment to the transcription start site [56,65]. RNA pol I-specific transcription initiation factor RRN3 interacts with the polymerase and assists conformational transition into its transcriptionally active form [66]. RRN3-SL1 complex-RNA pol I form the PIC at the rDNA promoter. Another level of regulation at chromatin is necessary for rRNA transcription [56,66]. Here, Upstream binding factor 1 (UBF1) plays a key role binding to rDNA repeats and promoting substantial topological changes opening chromatin for rRNA transcription [56,58]. After commitment to the rDNA promoter, RNA pol I must dissociate from PIC factors to start rRNA synthesis in a process known as promoter escape [67]. Co-transcriptionally, 47S pre-rRNA is covalently modified by 2′-O-ribose methylation and pseudouridylation by snoRNAs [68]. These changes fine-tune rRNA structure and function and are physiologically relevant [69]. Next, 47S is cleaved into intermediates that will give rise to the 18S, 5.8S and 28S rRNAs, which are associated to RPs progressively restraining rRNA flexibility into pre-ribosomal scaffolds [70]. Of note, every single rRNA continues to be chemically modified and further cleaved during this process [70]. Many factors interact transiently with pre-ribosomal particles to shape them, initially in the outskirts of the nucleolus, and then in the nucleoplasm. It is here where the 5S rRNA transcribed by RNA pol III gets incorporated into the pre-60S subunit. In the nucleoplasm, pre-ribosomal particles are further remodeled and coupled to other proteins to become competent for export to the cytoplasm [70,71]. In the cytoplasm, pre-ribosomal subunits undergo the last modification steps, including release from some RPs and interaction partners, final cleavage of 18S rRNA, and functional activation of now mature 40S and 60S subunits [71].

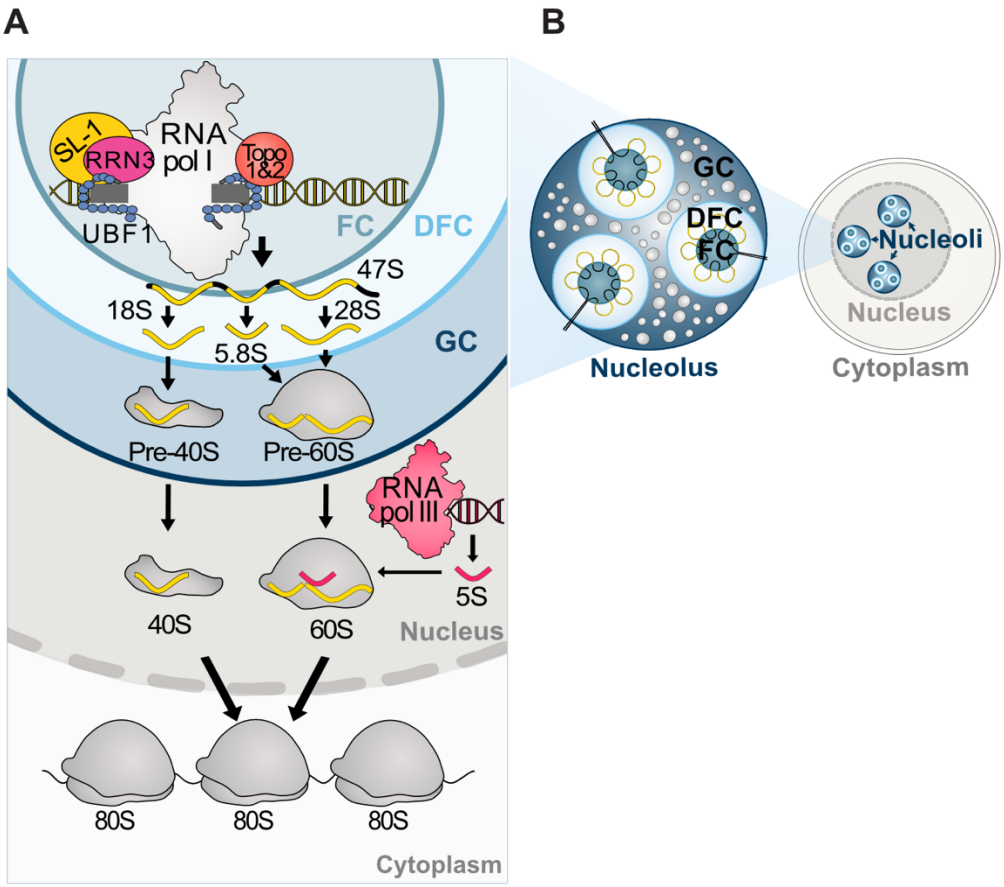


Figure 7. *Scheme illustrating ribosome biogenesis (A) and the tripartite nucleolar structure (B). Transcription of rDNA to pre-rRNA (47S) by RNA pol I, occurs in the FC or at the FC-DFC border, processing of the pre-RNA into the 18S, 5.8S and 28S takes place in the DFC, and it is followed by pre-ribosome subunit assembly at the GC. In the nucleoplasm, the 5S rRNA is transcribed by RNA pol III, and it is incorporated into the 60S. Both 40S and 60S subunits are exported to the cytoplasm and assembly into mature 80S ribosomes. FC, fibrillar center; DFC, dense fibrillar component; GC, granular component.*

1.2.3 The nucleolus

The nucleolus is a nuclear membrane-less organelle highly structured around tandem repeats of rDNA distributed in different chromosomal regions, known as nucleolar organizing regions (NORs) together with hundreds of proteins [58]. Besides ribosome biogenesis, previously described, the nucleolus regulates other fundamental biological processes such as cell cycle progression, DNA replication and repair, hypoxia, osmotic stress, nutrient deprivation, oncogene activation, and viral infection [72]. In fact, only a 30% of the nucleolar proteome constituted by 1,318 proteins is dedicated to ribosome biosynthesis [73,74]. Furthermore, recent studies have identified a new nucleolar compartment dedicated to chromosome segregation during mitosis [74], and a role in protein folding quality control [75]. Nevertheless, in the next sections we will focus on the role of the nucleolus in ribosome synthesis.

1.2.3.1 Nucleolar structure

The characteristic structure of the nucleolus is divided in three main sub-compartments (**tripartite model**) facilitates progression of ribosome synthesis. These three layers are: the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC)

[76,77]. The FC is enriched in components of the RNA pol I machinery, such as UBF1 and Transcription intermediary factor 1- α (TIF-1A), and it is in the border between the FC and the DFC where transcription of rDNA to rRNA takes place. The DFC includes rRNA processing factors, such as Fibrillarin (FBL1) and Nucleolin (NCL). At the GC, rRNAs assemble with RPs, such as the histone chaperone Nucleophosmin (NPM1) [76,78] (**fig. 7B**).

Nucleolar organization is governed by a biophysical phenomenon known as liquid-liguid phase separations (LLPS). Basically, the three nucleolar layers behave as oil droplets, which organize themselves always in the same manner, and which keep the nucleolus physically separated from the rest of the nucleoplasm [79]. The different biophysical properties of the proteins constituting each nuclear sub-compartment drive their organization as immiscible centers [80]. In fact, experiments using purified FBL1 and NPM1 show spontaneous formation of nucleolar-like structures out of the cell [81]. Similarly, chemical disruption of the nucleolar structure in *Xenopus* cells is followed by restoration of the initial three layers [81].

Phase separations have revolutionized the field and helped explaining the highly dynamic structure of the nucleolus, which had been reported for the last two decades [77]. The nucleolus responds rapidly to external and internal stimuli [77]. In fact, nucleolar morphology and number are variable across different species and cell lines, even within the same cell type. These parameters are subjected to changes during cell cycle and due to energetic cues [78]. For instance, when mammalian cells start dividing, nucleoli disassemble and are reconstituted in the end of mitosis around NORs [82] (**fig. 8**). Furthermore, already 200 years ago, scientists observed that cancer cells, addicted to translate, had bigger and more numerous nucleoli [83].

1.2.4 Nucleolar stress

Nucleolar stress can be defined as the impairment of nucleolar function and integrity, which when persistent can result in cell death, for yet poorly understood reasons. RNA pol I inhibition or hyperactivation, DNA damage, nutrient starvation, heat-shock, hypoxia and viral infection induce nucleolar stress [77]. Upon these insults, changes in rRNA output and in nucleolar morphology can be observed, such as translocation of proteins from the nucleolus to the nucleoplasm and cytoplasm, disintegration of the nucleolar area into numerous small nucleolar foci, segregation of the GC from the FC, formation of nucleolar caps, or formation of large nucleoli [77]. These changes were studied using image-based studies and spatial proteomics and built the first collection of data of the mammalian nucleolar proteome under stress [72,84] (**fig. 8**).

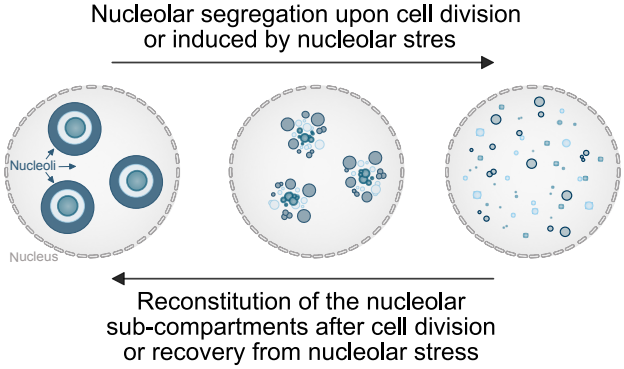


Figure 8. *Changes in nucleolar structure. Upon cell division, nucleolar components segregate, and after division, re-nucleate among themselves, preserving the tripartite structure. Under stress conditions, the nucleolus disintegrates, and it is reconstituted when stress is resolved.*

Follow-up studies focused on the mechanistic association between nucleolar stress and cell cycle arrest, also referred to as **nucleolar surveillance pathways**. Nucleolar stressors trigger the release of RPs and nucleolar factors to the cytoplasm, such as NPM1, which binds and interferes with the activity of murine/human doble minute 2 (MDM2/HDM2), the E3 ubiquitin ligase responsible for p53 degradation, resulting in p53 stabilization, cell cycle arrest and/or apoptosis [85]. Additionally, stress conditions can affect ribosomal stoichiometry and lead to cell cycle arrest. One of the best characterized mechanisms is the Impaired Ribosome Biogenesis Checkpoint (IRBC) response [59], where an excess in production of ribosomal proteins triggers the interaction of proteins of the large ribosomal subunit (RPL11 and RPL5) coupled to 5S rRNA with MDM2/HDM2, stabilizing p53. Nevertheless, cell cycle arrest due to alterations in ribosome biogenesis does not depend exclusively on RPL5 and RPL11 [59]. Additionally, rDNA structures are very sensitive to DNA breaks, which in turn can activate p53 signaling [62,85]. However, there are other p53-independent mechanisms that are activated in response to nucleolar stress, which have been identified in p53-null backgrounds, but that are not as in-depth characterized [86]. Some examples of p53-independent nucleolar stress response are linked to E2F1, PIM kinase, RPL13, and PeBoW nucleolar complex [86]. The transcription factor E2F-1 is stabilized by MDM2/HDM2. Upon nucleolar stress, these factors dissociate due to RPL11 displacement of MDM2/HDM2, and proteasomal degradation of E2F-1 triggers cell cycle arrest and down-regulation of rRNA synthesis [87]. PIM1 kinase interacts with the small ribosomal protein RPS19. If RPS19 levels are reduced, this interaction is broken, PIM1is degraded, leading to p27 stabilization, cell cycle arrest and apoptosis [88]. An increase in RPL3, promotes the formation of a complex with NPM1 at the p21 promoter, resulting in cell cycle arrest [89]. PeBoW nucleolar complex is involved in processing pre-rRNA during 60S assembly [90]. This complex appears upregulated in cancer cells independently of their p53 status, and when knocked down, it triggers upregulation of p27, and results in cell cycle arrest [86,90]. Nevertheless, the independency from p53 for inducing cell cycle arrest or apoptosis is of pharmacological interest since approximately half of the cancer types are p53 null [77,86]. Overall, the nucleolus is a potent biomarker of cellular health.

1.3 PROTEIN SYNTHESIS AND RIBOSOME BIOGENESIS IN CANCER AND NEURODEGENERATIVE DISORDERS

Alterations in protein synthesis and nucleolar function have been associated to several diseases, including cancer, ribosomopathies, metabolic disorders, immunity, neurodegeneration and other aging disorders, plus physiological aging, which is covered in excellent reviews [9,10,34,91]. However, here the focus will be on cancer and neurodegenerative disorders, especially amyotrophic lateral sclerosis (ALS), since these have been explored the most during this doctoral thesis.

In the context of **cancer**, overexpression of oncogenes and loss of function of tumor suppressors stimulate ribosome biogenesis and protein synthesis, contributing to tumor growth. In fact, for more than a hundred years, pathologists had observed a correlation between increased nucleolar size and number with poor prognosis for many cancer types [92]. Several oncogenic pathways, including mTOR and MAPK, promote ribosome biogenesis, protein synthesis, and proliferation. Additionally, pharmacologically targeting of factors involved in mRNA translation and ribosome production, improves therapeutic outcome. However, while these processes have been assumed to stimulate cancer pathogenesis, it has not been until recently that two studies have demonstrated that excessive ribosome biogenesis can drive malignant transformation [93-95]. Ebright and colleagues conducted a genome wide clustered regularly interspaced short palindromic repeats (CRISPR) activation screen in circulating tumor cells and identified overexpression of genes coding for RPs and translation factors as drivers of metastasis in mice [94]. These results were supported by an enrichment in ribosome and protein synthesis signatures from freshly isolated human circulating tumor cells correlating with poor clinical outcome. Furthermore, there is evidence showing tumoral transformation upon overexpression of translation initiation factors (eIF4E [96]), oncogenic-driven transcription of components of the translation machinery, and differential translation of oncogenes, due to distinct structural elements on their coding mRNAs [11]. Moreover, reduction in the levels of eIF4E or eIF4A1 have shown to delay tumor onset in CRISPR-engineered heterozygous mice [97]. Due to proteotoxic stress, eIF2α is phosphorylated in most cancer types, which outcome is difficult to predict since it can have pro-survival consequences or lead to cell death. For instance, expression of a non-phosphorylatable eIF2α mutant (S51A) and inhibition of the PKR branch of the IRS, hence that cannot inhibit translation, were shown to transform mouse cells and promoted tumor formation in immune-deficient mice [98-100].

Paradoxically, mutations in factors involved in ribosome biogenesis and translation are associated with cancer and **ribosomopathies** [32]. Briefly, ribosomopathies are a group of genetic disorders caused by mutations in ribosomal proteins, rRNA genes, or other proteins involved in ribosome biogenesis [101]. Most ribosomopathies are characterized by severe anemia, skeletal abnormalities, growth retardation, and predisposition to cancer. Intriguingly, haploinsufficiency of ribosomal genes exhibit a variety of phenotypes, often tissue-specific [9]. Approximately, 43% of human sporadic cancers bear hemizygous deletions of chromosomal regions containing RP genes [102], most of which are present in TP53 mutant cancers [59]. So far it has been proposed that mutations in RPs contribute metabolic changes, including secondary

transcriptional programs, that generate a hostile environment selecting for able to proliferate under those conditions, and therefore becoming malignant [95]. Nevertheless, the association between defects in RPs and cancer remains to be explained.

Alterations in proteostasis and ribosome biogenesis have been related to **aging** and aging-associated diseases, such as neurodegenerative disorders. Aging can be described as progressive loss of physiological integrity and systemic performance, increased disease vulnerability and decreased lifespan [36]. The cellular hallmarks of aging include genomic instability, deregulated nutrient sensing and loss of protein homeostasis [103]. Therefore, it is not surprising that protein synthesis and nucleolar activity are dysregulated with aging. An increase in nucleoli area, rRNA content and nucleolar proteins have been reported in senescent cells in culture, in aging primary human fibroblasts, in models of premature aging, and in oocytes from old mice [104]. This stimulation of nucleolar activity has been proposed as a compensation mechanism due to faulty proteostasis in aged organisms [104]. However, overall translation is down-regulated in aged cells [105]. Yet, mTORC1 appears to be hyperactivated in aging, promoting protein synthesis in a context prone to error, where the cellular capabilities for protein folding are saturated. Additionally, when mTORC1 is activated, autophagy is inhibited. Autophagy is fundamental in refreshing the cellular machinery by degrading damaged proteins and organelles [27]. During aging, stress signaling pathways become dysfunctional having effects in protein homeostasis, and, consequently, the ISR appears activated in aged animals and in human tissues [36]. Nevertheless, genetic and pharmacological inhibition of mTORC1 and ribosome biogenesis have been successful in extending lifespan and improving health status in several model organisms [104].

Aging is the greatest risk factor for several **neurodegenerative disorders**, which are characterized by progressive loss of specific neuronal populations in the central or peripheral nervous system [106]. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS) are well-known examples. The above-mentioned disorders have different etiology, but share phenotypes based on production of toxic aggregating proteins and/or RNA meta-stable structures in the nucleus and cytoplasm of cells [107]. Defects in nucleolar biology and protein synthesis have emerged as a common denominator among this group of disorders. Regarding ribosome biogenesis, some common features are epigenetic silencing of rDNA promoters and aberrant distribution of nucleolar proteins, often due to sequestration by protein aggregates and RNA stable structures, these latter being prominent in repeat expansion disorders, such as ALS and HD [108-110]. Anecdotally, nucleoli in AD and PD patients are smaller compared to healthy patients, while for HD and ALS, they appear enlarged [111]. Therefore, therapeutic interventions have been directed to both stimulate and reduce nucleolar activity, being the latest the more successful [107,112]. Additionally, stimulation of autophagy has proven to be relevant in degrading protein oligomers in AD, PD, HD and several forms of ALS, and overall inhibition of mTORC1 have shown more beneficial than detrimental effects [113]. Therefore, ways of inducing autophagy without severely inhibiting mTORC1 are being explored as potential candidates for treating these disorders [27,114]. Defective proteostasis linked to these disorders activates the ISR chronically [34,35]. Because of this, the

ISR has been targeted in these neurodegenerative disorders. While there is a fine line to draw for whether activation of the ISR could be protective or not, chemical or genetic interference with ISR activation have shown to be protective in models of AD, PD, HD and ALS [34,115]. Interestingly, eIF2α phosphorylation has been shown to promote repeat-associated non-ATG dependent (RAN) translation, an unconventional mechanism of protein synthesis, relevant for HD and ALS pathologies [116,117].

Focusing on **ALS,** this a fatal disease that affects one very 350 people [118]. ALS is characterized by progressive degeneration of upper and lower motor neurons, leading to muscular paralysis and death within 3 to 5 years after diagnosis. Mutations in ALS are associated to an ever-growing number of genes, the most classically studied being SOD1, TDP-43, FUS and C9ORF72 [118]. Mutations in these genes have shown to form inclusions and interfere with RNA metabolism, and these pathologies are not mutually exclusive within the same individual [119]. However, the presence of G_4C_2 hexanucleotide repeat expansions (HREs) within the first intron of C9ORF72 is the most frequently inherited mutation in both in ALS and Frontotemporal Dementia (FTD) [120,121]. These repeats are present in less than a dozen copies in unaffected individuals and are up to over a thousand copies in patients [122]. C9ORF72 HREs are translated into five different poly-dipeptide repeats (DPRs) through RAN translation. Poly-PR and poly-GR are the most toxic species of C9ORF72 DPRs, showing toxicity in cells and in a variety of model organisms [123,124], even when added exogenously [125]. Both arginine-containing DPRs (poly-PR and poly-GR) are positively charged and localize in the nucleolus given to its high content of negatively charged nucleic acids [125], and based on this property, they interfere with all the cellular processes involving RNA and DNA (negatively charged) [126]. This property explains the reported effects of DPRs in nucleolar stress, nucleocytoplasmic transport, limiting protein synthesis, disturbing phase separations, among others involving RNA and DNA binding activities [108]. A proteomics approach identified in ribosomal and nucleolar proteins as the main interactors of poly-PR and poly-GR in cells [109]; probably also based on electrostatic protein-nucleic acid interactions. Moreover, poly-PR and poly-GR affect the distribution of FBL1, NPM1, NCL and many other nucleolar proteins, as also our results show [110,127,128]. Additionally, C9ORF72-HRE transcripts localize to the nucleolus, interact with NCL and sequester other key ribosomal binding proteins. However, RNA gain of function seems to contribute to the neuronal toxicity in ALS, but it is insufficient to cause cell death [129], as well as haploinsufficiency of C9ORF72 [117,123,130]. While in the case of ALS, as well as for HD, many of the current therapeutical interventions are focused in stopping expression of repeats, other efforts have been put in modulation of nucleolar activity and translation.

1.4 THERAPEUTIC INTERVENTIONS

Due to their relevance in human disease, many chemical and genetic approaches have been developed to modulate eukaryotic mRNA translation and ribosome biogenesis. As presented, these are multi-step processes, with different factors and complexes involved, which can be source of regulation. Below, Dmitriev and colleagues [131] illustrated the described the mechanism of action for some of the small molecules modulating protein and ribosome production (**fig. 9**). This section will focus on compounds regulating some of the steps of these processes that have been further described before.

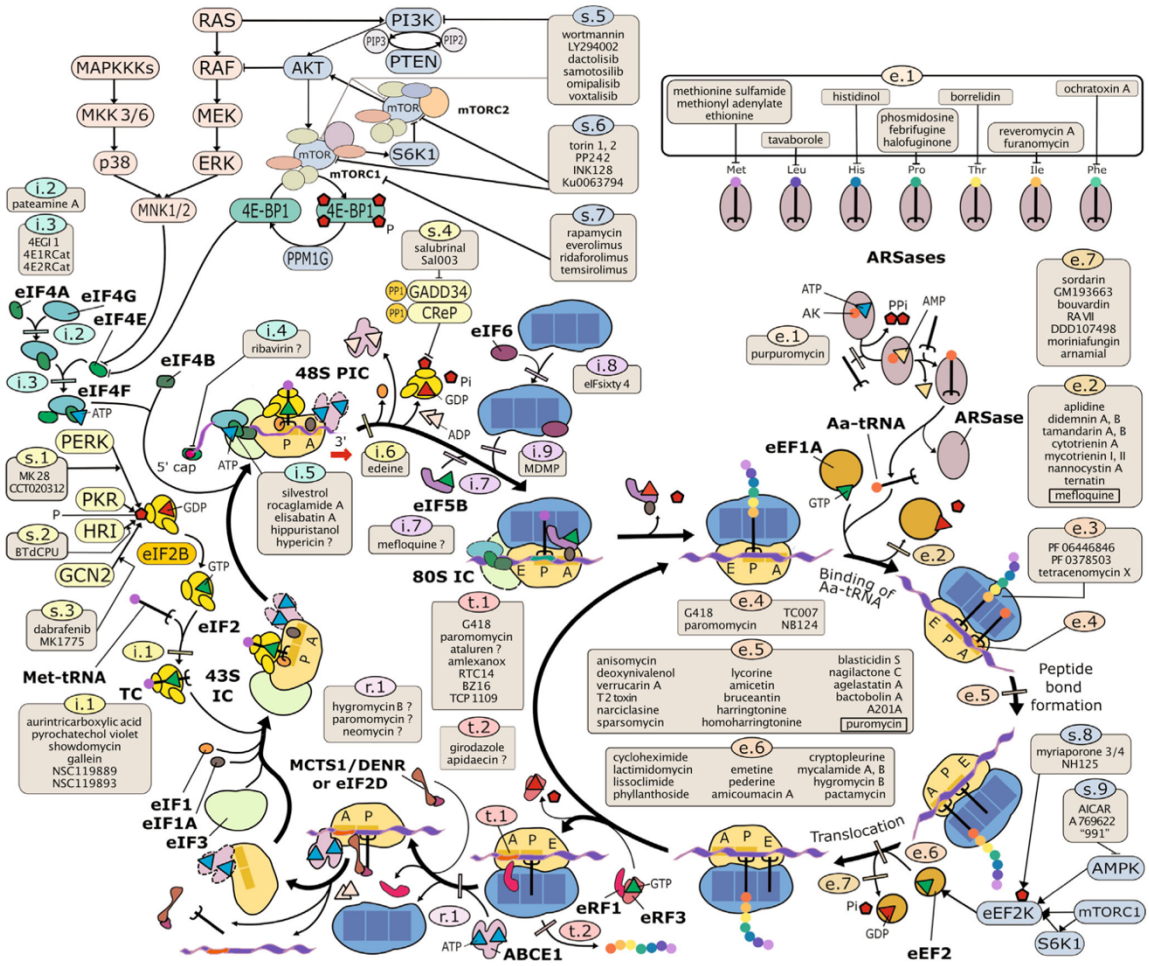


Figure 9. *Overview of small molecules regulating eukaryotic translation* [131].

1.4.1 Modulators of the cap-binding complex eIF4F

Components of the cap-binding complex eIF4F (eIF4E, eIF4G and eIF4A) are upregulated in many cancers, therefore have been target for therapeutical modulation [11].

- **Cap-binding protein eIF4E**. Antisense oligonucleotides (ASOs) against eIF4E (***LY2275796***) have suppressed eIF4E expression, shown anti-tumor potential and have been well tolerated in mice [132]. Additionally, cap analogs interfering with eIF4E-cap interaction have been identified and designed thanks to structural studies. In this category, the most successful has been ***4Ei-1***, which inhibits translation in cells and in zebrafish embryos [133]. Compounds able to interrupt eIF4E:eIF4G interaction (***4EGI-1***,

4E1RCat, and ***4E2RCat***) were discovered in two high throughput screens [134,135]. Interestingly, 4EGI-1 increases 4E-BP1 binding, besides triggering disassociation of eIF4G from eIF4E (Moerke et al. 2007). These compounds have inhibited translation *in vitro* and *in vivo* [11].

- **Scaffolding protein eIF4G.** Compound ***BI-69A11*** and analog ***SBI-756*** inhibit eIF4G [136,137]. However, BI-69A11 was designed to inhibit AKT, and even though it was found to interact with eIF4G using chemoproteomics, these compounds also inhibit AKT and NF-κB activities [137].
- **Helicase eIF4A**. Three natural products, ***pateamine A (PatA)***, ***hippuristanol***, and ***rocaglates*** have been identified by biochemical assays as selective and potent eIF4A inhibitors. In fact, rocaglates have proven selective for the eIF4A1 homolog, as it is not toxic when the allels is edited by CRISPR/Cas9 [138]. The compounds have shown preclinical efficacy in cells and animal models inhibiting tumorigenesis [11].

1.4.2 Modulators of eIF2 and the ISR

The ternary complex formed by eIF2–GTP–Met-tRNAi is tightly regulated at different levels which have been targeted with small molecules (**fig. 10**).

- **Targeting eIF2B.** A high throughput chemical screen identified ***ISRIB*** as an inhibitor of the ISR based on ATF4 expression using a luciferase reporter system [139]. ISRIB enhances GEF activity of eIF2B, and therefore reverses the effects caused by eIF2α without affecting its phosphorylation. Similarly, ***2BAct*** was developed to enhance pharmacokinetic properties of ISRIB [140]. Another screen using a CHOP luciferase reporter identified ***Trazodone*** and ***Dibenzoylmethane (DBM)*** acting downstream of eIF2α phosphorylation [36,141]. However, these compounds do not affect eIF2B dimerization, contrary to ISRIB [141]. Interestingly, trazodone and DBM cross the blood-brain barrier, which is interesting for treating neurological disorders [142]. Compounds targeting eFI2B have shown to be neuroprotective *in vivo,* amelliorating neurodegenerative phenotypes, including different forms of ALS [143,144]. Additionally, these drugs improve congnitive functions and help in other neurodevelopmental disorders such as vanishing white matter (VWM) disease, characterized by myelin loss [140]. Paradoxically, activators of the ISR also seem beneficial for VWM models [36,115]. In the context of cancer, ISRIB induced tumor regression and extended survival in xenograph models of MYC-driven prostate cancer, characterized by PERK activation [145].
- **Blocking eIF2α Function.** In this case, the fluorescein derivatives ***NSC119889*** and ***NSC119893*** prevented binding of Met-tRNAi with eIF2, stimulating IRES-driven translation [146].
- **Inhibiting dephosphorylation of eIF2α.** The phosphatase PP1 inhibitor ***Salubrinal*** was identified in a chemical screen for compounds limiting ER stress induced cytotoxicity in rat neuronal PC12 cells [147]. Salubrinal and a more potent and soluble analog (***Sal003***) likely blocked the conserved PP1-binding domain of GADD34 and

CReP [147,148]. These drugs have shown protective effects against amyloid β- protein and Huntingtin aggregation, aberrant expression of α-synuclein in Parkinson's disease, and for accumulation of unfolded proteins in ALS [11]. In cancer, Salubrinal has been able to resensitize cells to the proteasome and UPR activator Bortezomib [149]. ***Guanabenz*** maintains eIF2α phosphorylation, but it specifically inhibits GADD34 and PP1 interaction [150]. Interestingly, Guanabenz is an α2-adrenergic agonist that was used for treating hypertension, and that has been repurposed as a modulator of the ISR, which is encouraging in terms of safety for future clinical uses. Guanabenz amelliorates protein folding stress, and inhibits proliferation and migration of cancer cells [151]. Guanabenz derivative ***Sephin1*** is another selective inhibitor of GADD34 [150]. Guanabenz have shown to delay onset in ALS mouse models, yet there is more evidence supporting that ISR inhibition helps in ALS [34,36]. Additionally, Guanabenz improved WVM phenotypes [36]. ***Raphin1*** is another derivate of Guanabenz that specifically binds to CReP, examined in a CReP null background [152]. Exposure to Raphin 1 results in transient increase of eIF2α phosphorylation, which are reverted due to an increase in GADD34 levels. Raphin1 reduces neurological decline in HD mouse models [152].

- **Modulating eIF2α activity.** Many compounds have been identified to increase phospho-eIF2α levels, yet their mechanism of action or specific regulation of eIF2 remains to resolved. Some obvious regulators of eIF2α have an effect in the eIF2α kinases.
    - **HRI**. **Aminopyrazolindane** was found as an inhibitor of HRI in a chemical screen [153], however it is not bioavailable. Another screen idenfied ***N,N0 - diarylureas (cHAUs)*** as a direct activator of HRI and the ISR [154,155]. Lead cHAUs inhibit proliferation in cancer cell lines and in melanoma xenografts [156].
    - **PKR**. There are several PKR inhibitors in the literature, the first one reported was ***2-amino purine***, but was not potent nor selective [11,157]. ***Cmpd #16*** was discovered using docking to the ATP-binding site of PKR and testing if it was able to prevent PKR translational inhibition in cell extracts of rabbit reticulocytes [158]. Using the same strategy, other inhibitors were identified by the same laboratory that need further characterization in terms of selectivity and bioavailability [11].
    - **GCN2.** Three compounds (***Indirubine-30-monoxime***, ***SP600124***, and ***staurosporine***) have been identified to inhibit GCN2 using a GCN2-autophosphorylation assay [159]. Investigation of indirubine-30-monoxime derivatives led to the discovery of ***Syk1 inhibitor*** (spleen tyrosine kinase inhibitor), which prevented phosphorylation of eIF2α in cells exposed to ultraviolet (UV) light [160]. Syk1 inhibitor has been used in mouse experiments to reduce inflammation. ***GCN2iA*** and ***GCN2iB*** were generated as competitors of the ATP-binding site of GCN2, blocking activation of ISR via GCN2.

GCN2iB is more specific and works in mice, and has shown to contribute in reducing tumor growth [161].

- **PERK**. A chemical screen using a CHOP luciferase reporter identified ***TGD31*** and ***TG45BZ*** inducing ISR activation via PERK [162]. However, these compounds induce the ISR by additional mechanisms since they are active in PERK-null cells [162]. A collection of PERK inhibitors was designed using *in silico* docking followed by eIF2α phosphorylation assay [163,164]. ***GSK2606414***, also referred to as PERK inhibitor (PERKi) in **paper I** included in this thesis, and derivative ***GSK2656157*** showed high selectivity for PERK and reduced tumor growth in mouse models [165]. GSK2606414 have shown to reduce neuronal loss in different *in vivo* models of neurodegeneration, including ALS [115]. However, GSK2606414 induces pancreatic toxicity [166], hence ISRIB has been used instead as a safer alternative. However, both PERK inhibitors have an off-target effect inducing RIPK1-kinase associated toxicity that should be considered [167]. Also in **paper I**, we found that the sphingosine kinase (SPHK) inhibitor ***SKI-II*** activates PERK by inducing physical damage to the membranes of the ER.

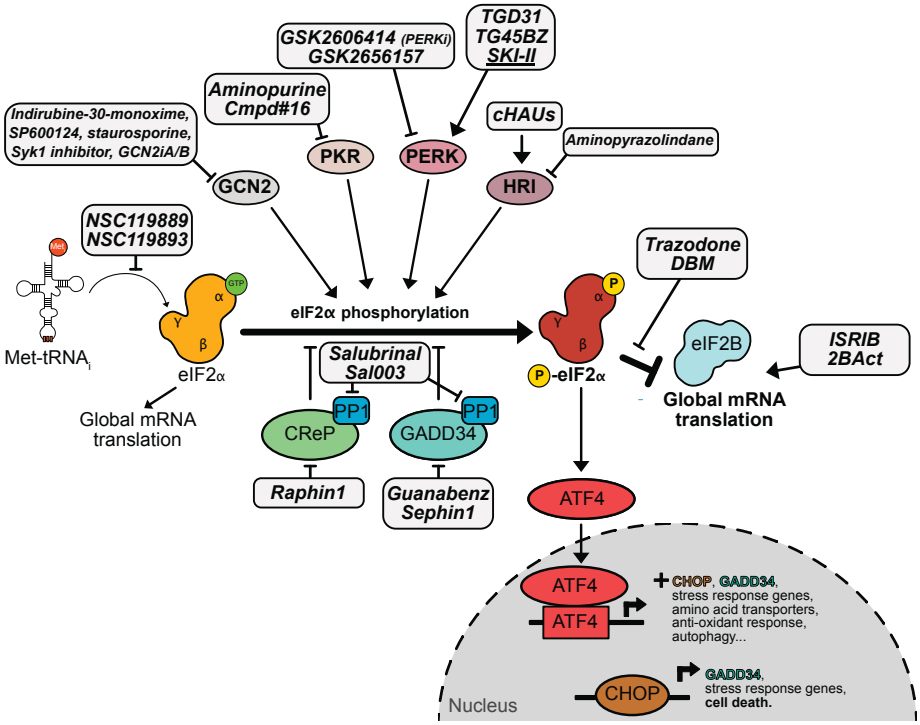


Figure 10. *Overview of chemical modulators for eIF2 and the ISR at different levels. Underlined is SKI-II, the hit we identified in* ***paper I*** *as a modulator of the ISR via induction of ER stress.*

1.4.3 Modulators of mTORC1

The mTOR pathway has been extensively targeted for modulation due to its relevance in regulating metabolism and physiology. Some mTORC1 inhibitors are Food and Drug Administration (FDA) drugs currently used in the clinic as anticancer agents and immunosuppressives for transplants [168]. According to their mechanism of action, mTORC1 inhibitors can be classified as:

- **Rapalogs.** Rapamycin and analogs (rapalogs) are the first generation of mTORC inhibitors. These compounds selectively bind to mTORC1 in complex with FK506-binding protein (FKBP12). ***Rapamycin***, also known as Sirolimus, was isolated from soil bacteria from the island of Rapa Nui and was characterized as an antifungal, immunossupressive and antiproliferative compound [169,170]. Rapamycin analogs with better pharmacokinetics are used as anticancer agents [171]. For instance, ***Everolimus*** and ***Temsirolimus*** are used in advanced renal carcinoma [172]. Also, Everolimus is prescribed for advanced breast cancer and pancreatic neuroendocrine tumors. Incomplete inhibition of mTORC1 susbtrates by rapalogs results in modest effects in solid tumors when used as a monotherapy [173,174]. Additionally, mantained inhibition of S6K leads to AKT activation, promoting pro-oncogenic signalling [175]. Mutations in KRAS, BRAF, and TSC1/2 generally make cells resistant to mTOR inhibitors, while mutations in PIK3CA sensitize them [168]. However, these associations cannot be generalized, as metastatic renal cell carcinoma patients with mutations in mTOR and TSC1/2 (mTOR suppressors) are more sensitive to rapalogs [176]. Of note, rapalogs usually arrest cell cycle but do not induce apoptosis, which helps in activating secondary mechanisms of drug resistance [168]. Interestingly, for ribosomopathies it has been debated whether if activation of inhibition of translation would be beneficial, while amino acid L-Leucine supplements stimulating mTORC1 have been part of the standard of care for these patients [91]. However, recent experiments have shown that inhibition of translation with Rapamycin helps to cope with protein synthesis when the translational machinery is not fully functional [177]. Rapalogs have shown great potential in neurodegenerative disorders and aging, since they induce autophagy and lysosomal flow, which degrades damaged proteins and organelles, but also also cytoplasmic inclusions characteristic of these disorders [112]. Additionally, Rapamycin-driven inhibition of translation can reduce production of toxic proteins linked to these disorders, such as α-synuclein in PD [112]. The beneficial effects of rapalogs for neurodegenerative disorders have been confirmed in animal models for AD, PD, HD and ALS [112]. Overall, rapalogs have shown to delay disease onset, decrease neuronal loss, reduce cognitive deterioration, and extend lifespan [112]. In the same line, rapalogs have shown geroprotective effects in several animal models [27,104]. Supporting this data, genetic ablation of proteins driving activation of mTORC1 have extended lifespan in aging models and improved phenotypes associated to neurodegeneration [27,104]. Furthermore, rapalogs and mTOR inhibitors in general, reduce ribosome biogenesis, slowing down general translation in cells, supporting accuracy in protein synthesis and maintenance of energetic homeostasis [104].

About possible side-effects derived from chronic use of rapalogs, we can learn from data of patients undergoing mTOR inhibitors therapies for cancer or organ transplantation, which generally involve high-dose regimens, compared to antiaging interventions [27]. Intermittent and low dosing of Rapamycin extended the lifespan of mice without affecting glucose metabolism [178]. Nevertheless, more studies are needed to explore the potential of these compounds and appropriate dosing in humans.

- **ATP-competitive mTOR inhibitors.** To this group belong compounds binding to the ATP-binding site of mTOR, completely inhibiting mTORC1 and mTORC2 [171]. In preclinical models, ATP-competitive TOR inhibitors are generally superior to rapalogs due to the complete inhibition of mTOR and sustained inhibition of 4E-BP1 phosphorylation. Moreover, ATP-competitive TOR inhibitors induce apoptosis [168,171]. Among these compounds are: ***Torin 1***, ***Torin 2***, ***MLN0128 (INK128, TAK-228)***, ***PP242 (Tokinib)***, ***AZD2014*** and its analog ***AZD8055*** [168]. These compounds reduced tumor growth *in vitro* and *in vivo*, in different cancer models including leukemia, gastric cancer, sarcoma, and breast cancer [171]. Interestingly, all these drugs resensitized tumors that were resistant to rapalogs, chemotherapy and hormone therapy [168]. The downside of these drugs is related to side effects, proposed to result from inhibition of other kinases similar to mTOR, such as PI3K. Nevertheless, some of these drugs are enrolling in clinical trials and seem to be tolerated at effective doses [179]. However, as for rapalogs, prolonged exposure to this compounds could activate feedback loops reactivating AKT, and promoting uncontrolled proliferation [175]. While some of these compounds have been tested for aging and neurodegeneration, their blunt effect on inhibiting mTORC1 seems to be the reason limiting their success in these contexts [112].
- **Dual PI3K/mTOR inhibitors.** Inhibition of mTORC1 and mTORC2 paradoxically can enhance the PI3K/PDK1 axis, stimulating proliferation. Therefore this third generation of mTOR inhibitors also targeted PI3K, aiming for better anti-cancer effects and to overcome mTOR resistance [168]. While several compounds have been classified into this category due to inhibiting mTOR and PI3K, many inhibit other kinases [168]. Nevetheless, they have shown promissing results and mono- and combinational therapies for different cancer types [168]. However, the flagship of this compounds is ***RapaLink1*** [180]. RapaLink1 links Rapamycin to the ATP-competitive mTOR inhibitor, MLN0128, enabling it to overcome resistance to existing first- and second-generation inhibitors. Additionally, RapaLink1 is selective for mTORC1 at low doses, which could be used as a tool for discerning the effects linked to specific mTORC1/2 [181]. This compound is showing promising results in different cancer types, such as glioblastoma, a type of brain cancer, since RapaLink1 can cross the blood-brain barrier [180,182].

There are several compounds which indirectly inhibit mTOR, which now are being reexamined to see whether they specifically target mTORC1, since most of the above commented pitfalls in mTOR inhibition are related to inhibition of mTORC2 activity. Some of these small molecules are already medically approved, and can be developed as pharmacologically safe mTORC1 inhibitors in the future [183].

Besides mTORC1 inhibitors, two activators have been reported in the literature: MHY1485 [184] and NV-5138 [185]. ***MHY1485*** was reported to inhibit autophagy by increasing mTORC1 activity [184]. However, examination of the current literature where this drug has been used shows (1) no evident effects in mTORC1 activation markers, (2) the effects of the drug are more related to counteracting autophagy when it is induced chemically or under specific genetic backgrounds. In our hands, MHY1485 failed in increasing translation under control and starvation conditions (data not shown). Regarding ***NV-5138***, it is a brain orally bioavailable mTORC1 activator [185]. NV-5138 stimulates mTORC1 binding to Sestrin2, communicating sufficiency of amino acids in the cell; in fact, the structure of the compound was designed based on L-Leucine. NV-5138 is progressing in clinical phase II as an antidepressant [186]. mTORC1 modulation is relevant in neurodevelopment and cognitive processes [4], which are beyond the scope of this thesis.

1.4.4 Modulators of the nucleolus and ribosome biogenesis

Modulation of the nucleolus and ribosome biogenesis has been extensively exploited in cancer for two reasons, sufficiency, and efficiency. Cancer cells are more sensitive to nucleolar stress than somatic cells, and for this reason many compounds have been developed to inhibit rRNA synthesis [187]. Usually, inhibitors of ribosomal biosynthesis are classified by their selectivity, however, better characterization of these drugs keeps revealing that they are not only committed to this process [187]. For this reason, and inspired by Burger and colleagues [188], we classified these drugs by the step of ribosome biogenesis that they modulate. This classification integrates nucleolar modulation in other disease and physiological contexts, such as in neurodegenerative diseases and aging. Below is a summary of this classification, which is extended in our review manuscript (**annex I**) focused on compounds modulating the nucleolus and ribosome biogenesis and strategies used for their identification and development.

A) Modulators of RNA pol I transcription

A.1. Negative modulators of RNA pol I PIC formation

- **Compounds interferring with SL1-RNA pol I.** ***CX-5461*** is considered a selective inhibitor of RNA pol I. CX-5461 is a DNA intercalator that stabilizes G-quadruplex secondary RNA structures, very common on rDNA, and inhibits rRNA pol I [189]. It displaces SL1 from rDNA promoter *in vitro* [189], yet this mechanism is now under debate. Recently, it has been characterized as a topoisomerase II inhibitor [190]. CX-5461 is in clinical trials for hematological and breast cancers [187,191]. ***Hydroxyellipticine (9HE) and other ellipticines*** are structurally similar to CX-5461 and affect SL1 occupancy at rDNA promoter [192]. CX-5461 is on clinical trials phase II for breast cancer [191] [187].
- **Compounds interferring with RRN3-RNA pol I interaction.** ***Cerivastatin sodium*** [193] and ***Small molecule peptide*** [194] reduce rDNA transcription and viability in cellular models.
- **Compounds affecting UBF1 binding.** Platinum-based drugs ***Oxaliplatin*** and ***Cisplatin*** are rDNA crosslinking agents that affect RNA pol I progression and additionally bind to nucleolar factos, such as UBF1, affecting its localization [187,195]. These drugs are used for treating sarcoma, lymphoma, and carcinoma [188].

A.2. Stimulators of RNA pol I PIC formation

Molecules promoting formation of the PIC have been studied in neurodegenerative diseases, where rDNA synthesis appears to be down-regulated [107]. Neurotrophic factors, such as ***Brain-derived neurotrophic factor (BDNF)*** and ***Insulin-like growth factor (IGF-1)***, and Histone deacetylase (HDAC) inhibitors, such as ***Sodium phenylbutyrate (Na-Phen)*** and ***Trichostatin A (TSA)***, enhance UBF1 acetylation and promote opening of rDNA chromatin [196]. Most of these approaches have failed in different phases of clinical trials, only Na-Phen is currently on trials for ALS [110,197].

A.3. RNA pol I destabilizers

The following compounds trigger the degradation of RNA pol I main catalytic subunit (RPA194), reducing rRNA synthesis. This activity was first discovered for ***BMH-21*** [198,199], and derivates, and then for other compounds, including ***Amodiaquine*** [200], ***Hernandonine*** [201], acridin derivatives ***CID-765471***, ***Aminacrine*** and ***Ethacridine*** [202], ***Metarrestin*** [203] and ***Sempervirine*** [204]. Most of these drugs are additionally drug intercalators with high affinity for rDNA secondary structures known as G-quadruplexes, interfering with RNA pol I progression. These drugs are being evaluated on cancer preclinical models [187], except for Amodiaquine, Aminacrine and Ethacridine which are already FDA-approved drugs that could be repurposed for treating cancer.

A.4. Inhibitors of RNA pol I progression

- **Antibiotics.** Some antibiotics act as DNA intercalating agents and are able to generate crosslinks that affect the rDNA structure inhibiting rRNA transcription. In this group are found: ***Actinomycin D (ActD)*** (used in kidney cancer known as Wilms' tumor, and sarcoma [205]), ***Mitomycin C*** (it has been tested in a variety of cancer types, and recently has got FDA approval for low-grade upper tract urothelial cancer (https://clinicaltrials.gov/ct2/show/NCT02793128), and the anthracyclines, ***Doxorubicin*** and ***Mitoxantrone*** (used in the clinic for haematological cancers, bladder, breast, stomach, lung, ovarian and thyroid cancer, sarcoma [206]).
- **Topoisomerase I and II inhibitors.** Camptothecins such as ***Irinotecan*** and ***Topotecan*** (ovarian, lung, cervical cancer [191]), ***Etoposide*** (sarcoma, glioblastoma, lung, testicular, haematological cancers [191]).
- ***CX-3543*** or ***Quarfloxacin*** is often classified as a selective inhibitor of RNA pol I. Its mechanism of action relies on disrupting rDNA G-quadruplex and NCL complexes. It failed clinical trials phase II for neuroendocrine tumors [187].

RBI1 and ***RBI2*** have been recently identified as early inhibitors of rRNA synthesis and to reduce viability of cancer cells lines [207]. Additionally, the ***small molecule Y-320*** have shown to stimulate ribosome production and rRNA synthesis after long exposure (24 - 48h) by undetermined mechanisms [208].

B) Modulators of rRNA processing

- **Antimetabolites**, such as nucleotides analogs, inhibit specific enzymes causing DNA synthesis inhibition and have shown to affect rRNA processing, for yet not well defined reasons. Some examples are ***5-Fluorouracil (5-FU)*** (used in the clinic to treat colon, rectum, head, neck cancers [191]) and ***Methotrexate*** (used in chemotherapy for breast cancer, lung cancer, certain head and neck cancer, some types of lymphoma, and leukemia [209]).
- **Cdk inhibitors, translation inhibitors and proteasome inhibitors** have shown to interfere in rRNA processing by undertermined mechanisms [188].

C) Modulators of RNA pol III

There are no currently avaiable modulators of RNA pol III, however, genetic interventions in RNA pol III and associated protein regulators have shown to reduce ribosome biogenesis and extend lifespan in yeast, worms and flies [210].

D) Modulators of ribosome assembly

Ribosome assembly has been overlooked when targeting ribosome biogenesis. Two compounds, ***Diazaborine*** [211] and ***Rbin-1*** [212] inhibit AAA-ATPases essential for the formation of the 60S ribosomal surbunit. These compunds activate nucleolar stress pathways by disrupting ribosomal stoichiometry.

E) Modulators of nucleolar structure

Many of the mentioned compounds affect nucleolar structure [188] by inhibiting RNA pol I activity. However, BET Bromodomain inhibitors (BETi) such as ***PFI-1*** and ***JQ-1***, have shown maintain the structure of the nuleolus when insulted by nucleolar stressors [128].

F) Signalling modulating ribosome biogenesis and nucleolar stress response pathways

As already stated, inhibition of mTOR signalling results in inhibition of ribosome biogenesis, and similarly happens for other pro-proliferative pathways. Rapamycin has shown to reduce nucleolar area and ribosome biogenesis, showing potential in extending and improving lifespan in aging and models of neurodegeneration [104]. In cancer several compounds have been discovered targeting the HDM2-p53 interaction, promoting stabilization of p53 and triggering cell cycle arrest or apoptosis, such as in the case of ***nutlins*** and derivatives that have entered clinical trials for haematological and solid tumors, osteosarcoma, head and neck cancer [191,213]).

1.5 PHENOTYPIC SCREENS

Drug discovery is the process through which chemical compounds with desirable properties to potentially treat a disease are found [214]. Two main strategies have been used for identifying new drugs: phenotypic screening and target-based approaches [215,216].

Phenotypic screens evaluate the effects of different chemicals or genes against a phenotype, defined as an organism's observable characteristic, which can be linked to a disease or a cell state [217]. Many of the drugs we use today that are first-in-class small molecules, such as ***Rapamycin***, were identified through phenotypic screening [218]. However, from 2003 to 2011, **target-based approaches** reigned drug discovery pipelines [219]. Target-based drug discovery is centered in interfering with specifically one gene or protein that is implicated in a disease [220]. This strategy has also achieved important milestones, such as ***Imatinib***, the first-to-market BCR-ABL inhibitor, which has been successful treating KIT-driven gastrointestinal stromal tumors [218]. However, a target-based approach is not synonym of success; only 10% of the leads from target-ID discovery are approved in terms of safety and efficacy by health agencies [219]. New techniques such as Next Generation Sequencing (NGS) revealed heterogeneity in disease and among patients, supporting that "one disease, one target, one drug" is an oversimplification [217]. Consequently, in the last decades phenotypic screening has re-emerged as a drug discovery approach [214,221,222].

Phenotypic screens are designed to select **hits** or lead compounds according to quantifiable phenotypic endpoints without previous knowledge of the drug target [217]. This agnostic approach is extremely relevant for human disorders of which biology is poorly understood [214,217,223]. These screens benefit from working in a complex biological system, which can be an intact cell or a whole organism that can be designed to model distinct disease states [214,224]. Often, these disease models are based on molecular target hypotheses and allow phenotypic discovery of novel and therapeutically relevant mechanisms of action, this practice is called **molecularly informed phenotypic discovery** [223].

The typical workflow of a phenotypic screen is characterized by the use of a **model** where to conduct the screen, a defined **assay set up**, a selection of **perturbagen**s, which can be a set of chemicals or they can be based on genetic interventions, and ultimately a **readout or endpoint**, according to which hits will be selected and validated [215,225] (**fig. 11**). Some hits could be further developed to be tested in preclinical models, and hopefully become a candidate drug.

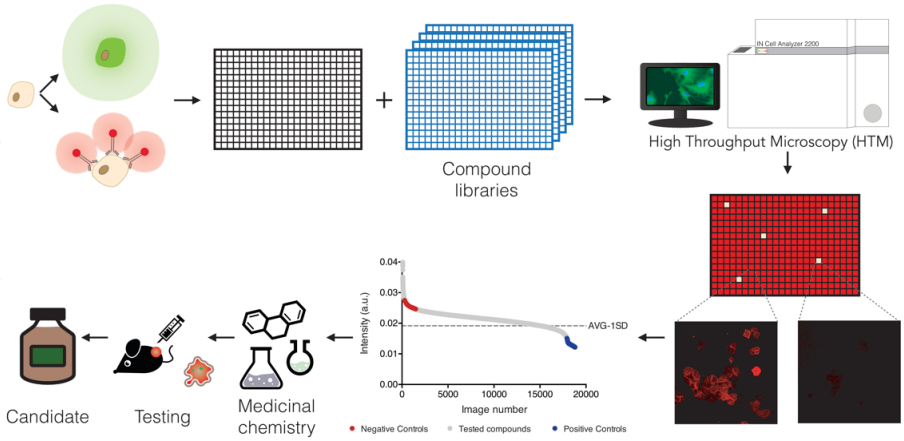


***Figure 11**. Overview of workflow image-based phenotypic screens. Cells showing a phenotype that can be detected by expression of reporters, antibody-based staining, among others, are seeded into 384-well plates and exposed to compound libraries. HTM images are analyzed to identify hits, which could be further developed as candidate drugs.*

Before conducting a phenotypic screen, it is especially important to establish the **assay window**, which is the distinction between negative and positive controls, and will help with **hit calling**. Additionally, it is relevant to optimize the assay for high throughput, including the automatization process and assessment of variability of the experimental procedure by using miniaturized set ups and/or by performing mock screens, with only controls, for instance. After conducting the screen, the next step is to validate the hits, which can be done using the same assay as for the primary screen and with **orthogonal assays** [226,227]. Orthogonal assays allow to distinguish drugs that generate false positives in the primary screen from those genuinely inducing phenotypic changes. Additionally, these secondary screens can be directed to provide more information about a reduced list of compounds, compared to the starting point of the primary screen. Secondary screens can help in gaining insights of the mechanism of action of the hits and to extend an initial concrete question to a broader research frame [217]. For instance, in our studies where compounds able to reduce nucleolar defects caused by ALS-dipeptide repeats (DPRs) were able to prevent from difference sources of nucleolar stress. Another example would be running a validation screen in neuronal-like cells, which is a more relevant model for ALS. In these secondary screens, new sets of compounds, including analogs, could be screened to inspect whether modulation of specific pathways or specific targets are enriched [217,223], such as the ad-hoc screen that we conducted using epigenetic libraries to assess the relevance of these compounds in limiting toxicity of ALS-DPRs in **paper II**. Another possibility would be the use of *in vivo* model organisms to run a small validation screen, as when we used zebrafish embryos. Zebrafish has become a popular model organism for preclinical studies due to their high similarity to humans in genetic and physiology and the capacity to scale experiments to high throughput technologies. More information about zebrafish as a preclinical model in **section 3.4**.

Image-based high-content screens facilitate in many aspects extracting more information from the same set of cells that have been exposed to perturbation agents [217,224,228]. This is the approach that we have mostly explored during this thesis. Imaged-based screens can be based

on single or multiple readouts, building **low** or **high complexity** datasets respectively [224,225] (**fig. 12**). The approach depends on the question to be assessed, the catalogue of features that can be extracted from the images, and the laboratory's capabilities and infrastructure. A phenotype can be monitored based on changes in number, morphology or functionality of cellular structures detected with specific dyes or due to the expression of proteins defining their morphology; or it can be followed due to changes in the levels or localization of proteins detected using antibody staining or by genetic modification of target proteins with tags; or a phenotype can reflect changes in the kinetics of cellular processes measured thanks in the incorporation of modified metabolites or substrates [225]. In our studies, we focused on a discrete number of phenotypes, in **paper I** and in ***preliminary results***, we considered changes in protein synthesis levels measured by the incorporation of an analog of Puromycin or changes in the area occupied by Fibrillarn together with changes in the number of nuclei stained with Hoechst. Even though we used very few features, images hold more information that can be used and explored for different questions. This idea of extracting multiple features from images and classifying compounds according to their phenotypic fingerprint has grown in the last years with the development of more sophisticated methods for image analysis and automatization, including **machine learning** tools [217,224,228]. The extended use of **Cell painting** [229,230], a technique that allows staining of several organelles and cellular structures simultaneously in cells, have revolutionized the field in this regard. Hence, without prior knowledge, compounds exerting similar effects, as similar features in different image channels (changes in the organelles), are predicted to have similar mechanistic effects [229,231].

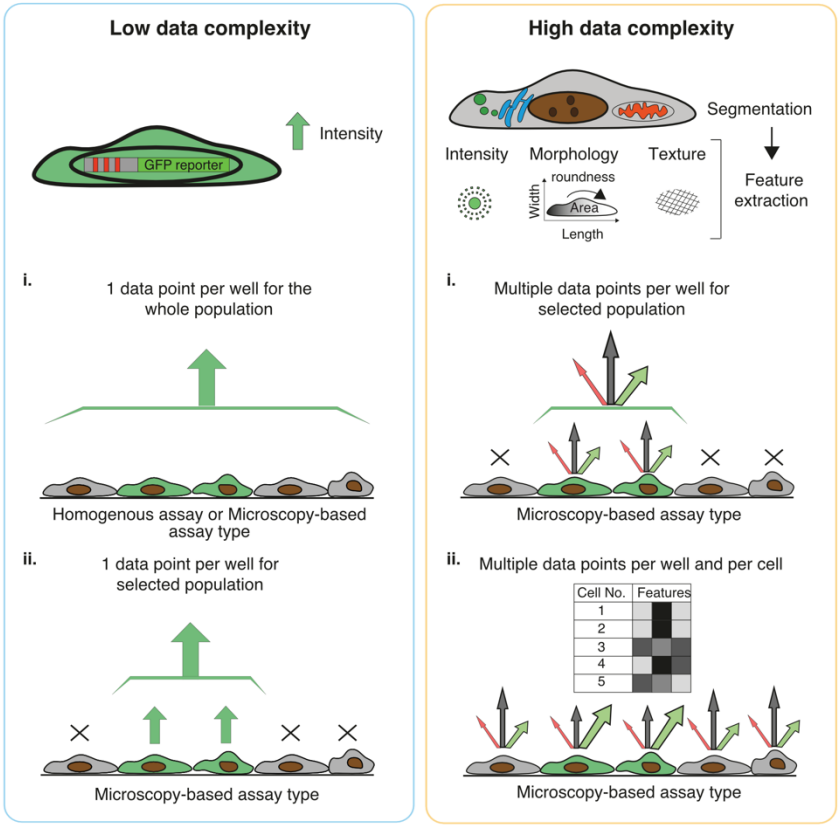


***Figure 12**. Schematic example of low and high data complexity set ups adapted from Lin, 2020 [224].*

The capacity of new assays to help in target discovery is another game changer regarding the starting point when choosing between target and phenotypic approaches. Nevertheless, target-based discovery approaches can assist phenotypic screening efforts and *vice versa*. For instance, phenotypic screens provide new drug target spaces to feed target-based discoveries and can help in exploring **off-target effects** of drugs designed for a specific target [217,232]. Whereas the use of technologies developed for target-based approaches can assist phenotypic screening with its biggest challenge: **target deconvolution** [215]. Target deconvolution is the association of the phenotype resulting from pharmacological perturbation with a specific cellular or extracellular molecular target [223]. While target deconvolution is not strictly necessary for a candidate drug to progress into clinical development, it is often preferable for drug discovery groups, investors, funders, and health agencies to further develop drugs into the market [223,233].

In this sense, using reference drugs for conducting phenotypic screens can help in the identification of targets and offer a frame for classification of compounds into same categories. For instance, after comparing phenotypic signatures of compounds, if several of them clustering together are annotated under the same terms, such as mTORC1 inhibitors, we can establish that (1) this group of drugs exert their effect through mTORC1 inhibition and (2) preliminary re-annotate drugs clustering together as modulators of this pathway, following the principle of **guilt by association** [218,234]. **Drug repurposing** is a strategy for identifying new applications to medically approved or tool compounds in new medical indications [218,235]. Besides helping as a starting point to examine the mechanism of action of compounds, drug repurposing offers other advantages compared to developing a drug from scratch for a medical indication [218,235]. The risk of failure of these drugs is lowered, since they have been tested in preclinical and clinical set ups, also, in many cases their formulation has been already optimized for clinical use, which saves time and money [223]. Additionally, in terms of investment, it is less risky and there is more rapid return in developing repurposed drugs [223]. Importantly, drug repurposing has been historically characterized by serendipity [214].

During this thesis, we had mostly used characterized libraries and medically approved drugs and worked in the characterization of the mechanism of action of various hits. While we could not identify "the" single target exerting the phenotypic outcome observed, we found the pathway through which modulation was achieved [217]. As highlighted by others [236,237], often small molecules interact with multiple targets or non-protein targets, so the concept target deconvolution is slowly evolving into **pathway deconvolution** or **network pharmacology** [217]. Network pharmacology contemplates the complexity of a disease and helps in the rational development of strategies, such as different drug combinations, to achieve a desired modulation [226]. This new way of approaching drug development holds big promises thanks to the recent advances in phenotypic, transcriptomic, and proteomic technologies which data can be integrated in chemical and biological databases, helping in elucidating the mechanism of action of phenotypic screening hits and in the rational design for these screens [218,223]. For instance, understanding these molecular phenotypes can reduce the number of compounds to be screened, which helps in costs, efficacy and during the difficult process of hit definition and

validation. Using integrative databases can help in predicting whether drugs can work in other contexts besides for the one they were discovered; hence a more systematic drug repurposing can be achieved [223] [218]. Additionally, this information can be used to filter out targets and compounds that repetitively fail to progress in clinical trials due to associated toxicity, which is common when by targeting pan-essential genes [238]. Databases such as The Connectivity Map (cMap) [239], which consists of gene expression profiles generated by exposing a variety of cell lines to more than 5,000 compounds, are key to network pharmacology and better patient stratification [217,218,239]. This database has a user-friendly interface allowing matching drug-disease and drug-drug similarities, and it has been proficiently used to understand new mechanisms of action of old drugs, the targets of novel uncharacterized compounds, and for drug repurposing [217,226]. Besides cMap there are other complementary databases and resource tools used for the study of the role of the genome in drug response, which is known as **pharmacogenomics** [217]. The use of CRISPR-based perturbation screens has been pivotal in unveiling novel drug targets, inferring off-target effects and polypharmacologies, exploring drug vulnerabilities, and providing of tailor-made therapeutic frames for heterogeneous disorders [217,218]. Additionally, CRISPR-based methods have helped in the development of disease models and reporting systems for high throughput phenotypic screens [217,218,226].

High throughput phenotypic screens have been efficient in identifying compounds able to regulate signaling targeting translation, such as inhibitors of the ISR and mTOR, or compounds inducing nucleolar stress. Modulation of general processes such as protein synthesis and nucleolar biology in contexts where these are a targetable vulnerability can be referred as **impersonalized precision medicine** [187]. During this thesis we have used high throughput techniques to identify and investigate new modulators of mRNA translation and the nucleolus, and by regulating these fundamental processes we found potential therapeutic agents for diverse disorders.

2 RESEARCH AIMS

The aim of this thesis was to exploit cell-based phenotypic screens to systematically identify modulators of mRNA translation and nucleolar biology in mammalian cells, as tools for understanding new biology and as potential therapies for diseases characterized by dysregulation of these molecular mechanisms.

In **paper I**, our objective was to evaluate the potential of known drugs to modulate global protein synthesis benefiting from techniques allowing visualization of newly synthesized proteins in cells.

In **paper II**, we conducted a chemical screen using medically approved and characterized libraries to identify compounds able to limit toxicity of ALS-C9ORF72 dipeptide repeats. These toxic dipeptides predominantly localize in nucleoli and interfere with ribosome biogenesis and protein synthesis. Two out of three hits protected the nucleolus from different sources of nucleolar stress besides ALS-C9ORF72 dipeptide repeats. This is the first time that nucleolar protectors have been reported, and these findings could lead to the discovery of new biology.

In preliminary results we report initial data from four additional screens conducted to answer the following questions inspired by our results from **paper I** and **paper II**:

- *Can we identify novel translation regulators among uncharacterized compounds?* Here we explored natural compounds libraries using the same approach as in **paper I** (***preliminary results I***).
- *Can we identify new modulators of nucleolar stress among known drugs*? There is a need for better inhibitors of ribosome biogenesis. Here, we conducted an image-based *in silico* screen to identify new modulators of nucleolar stress (***preliminary results II***).
- *Which are the genetic contexts that could benefit from the use of nucleolar stressors?* Drugs inducing nucleolar stress have been effective in the clinic, yet they are not equally efficient for all cancer types. Here we conducted a genome-wide CRISPR/Cas9 loss of function screen in cells exposed to nucleolar stressors to help us understand why (***preliminary results III***).
- *Could we identify more compounds protecting from nucleolar stress?* Here, we screened the Drug Repurposing Hub library to identify drugs preventing nucleolar stress (***preliminary results IV***).

3 METHODOLOGY

This section will provide an overview of the strategies used for conducting phenotypic screens and key methodologies used for hit characterization, which in are mostly common within the projects included in this thesis. Detailed descriptions of the techniques used can be found in the individual study publications (**papers I** and **II)** and in the preliminary results section.

3.1 CELL-BASED HIGH THROUGHPUT CHEMICAL SCREENS

3.1.1 Overview cell-based high throughput chemical screens

First, we start with **a question,** a **cellular model**, and **a methodology** to examine phenotypic changes of interest. In this thesis we screened for compounds modulating mRNA translation (**paper I**, and ***preliminary results I***), limiting toxicity of ALS-related poly-proline arginine (PR_{20}) toxic peptides (**paper II**), and modulating nucleolar biology (***preliminary results II*** and ***IV***) (**fig. 1, table 1**). All these screens were done using osteosarcoma U2OS cells, since they are technically easy to work with, as they remain attached to microwell plates after many steps of processing and washing, they grow relatively fast, and their morphology helps for imaging and image analysis. With U2OS cells, we could study changes in translational rates and nucleolar biology, as these parameters changed upon addition of control compounds modulating these processes. Despite being cancer cells, exogenous addition of ALS-PR_{20} to U2OS in culture, led to cell death, as it had been demonstrated by others [125]. For these screens we measured changes in protein synthesis rates based on incorporation of metabolites into newly synthesized proteins which can be detected by immunofluorescence (**OPP and HPG labelling)**; changes in cell viability based on **nuclei count** by staining cells with the nuclear dye Hoechst; and **changes in nucleolar area** using nucleolar markers, such as Fibrillarin (FBL1).

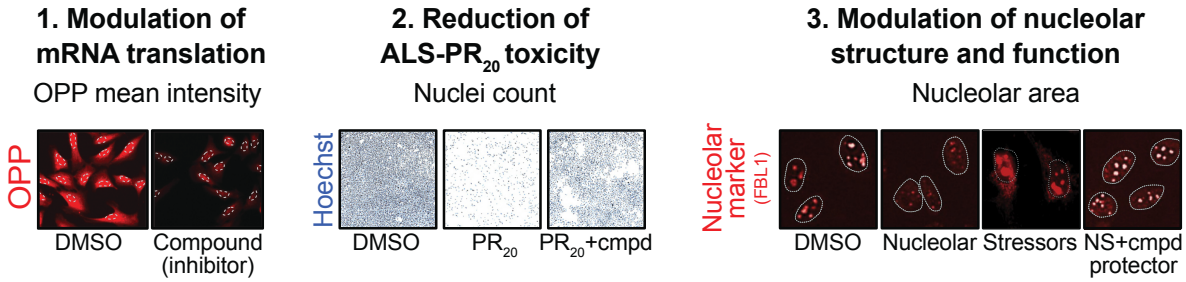


Figure 1*. Summary of phenotypes and screening readouts included in this thesis.*

Next, we select a perturbagen, in this case **compound libraries**. The compound libraries are prepared in DMSO and are provided by the Chemical Biology Consortium Sweden at SciLifeLab by the Laboratories for Chemical Biology at Karolinska Institutet (LCBKI). Most of our screens start with using medically approved and characterized tool compounds from different vendors that are collected in the CBCS in the so-called FDA-approved library. Working with **characterized libraries** can facilitate the characterization of the mechanism of action of hits and in drug repurposing, which is particularly relevant when finding potential

therapeutic agents for unmet diseases such as ALS. Moreover, the size of this library is relatively small and easy to handle when screening using novel assays and models. In terms of repurposing, we have also explored the Drug Repurposing Hub library[1] defined by the Broad Institute, which includes the vast majority of approved drugs as well as many in advanced preclinical development. In addition, we have also performed screens using a library of natural compounds available at CBCS. An important part of conducting chemical screens is **selecting libraries**, but also **concentration ranges** and a **dosing schedule**. These choices will affect the screen outcome since it is nearly impossible for academic labs to test all possible scenarios. However, establishing these conditions based on known controls can help in the rational selection of these parameters. In this thesis, the primary screens were done at a single concentration of compound in triplicates and the exposure time varied depending on the screen. Nevertheless, in following **validation** or **secondary screens**, a wider range of concentrations had been used and different extension of treatments have been considered. For instance, direct modulators of translation are expected to act rapidly, therefore after running a primary screen and validation exposing cells for 24h, the lead compounds followed were tested at 3h, based on the dynamics for **control compounds**.

Before running the screen, we **set up the assay** that would be automated and scaled up for high throughput. Usually, we start setting up these assays in 96-well plates to test different cell densities, fixation protocols, staining or labelling methods, image acquisition and analysis pipelines. Having established the assay, then we adapt it for high throughput. This means that we optimize the number of cells that will be seeded in 384-well plates using liquid dispensing devices. Also, different strategies for addition of compounds, which can be either resuspended in media and added to cells, or that cells can be seeded onto plates pre-spotted with compounds. Additionally, the staining and labelling protocols that often involve multiple and timed steps, including addition of reagents and washings, need to be automatized and adapted to liquid handling devices, including their limitations and functionalities, to achieve the most optimal results. In this phase, definition of controls, and consequently of an **assay window** is very relevant, since it will determine the power and feasibility of the screen on finding potential hits, or the need for more optimization to improve robustness of the experiment. At this stage, setting efficient pipelines for imaging and statistical methods of analysis is fundamental to gain the same depth of knowledge while using fewer images, faster image acquisition protocols, less computational power, and storage space, as well as to automate ways of compiling and representing data.

Work	Objective	Readout	Model	Treatment	Controls	Features	Imaging
Paper I	Modulators of translation	Changes in translation rates	U2OS 750 cells/well	CBCS FDA-approved library v2 (4,166) 10µM, 24h In triplicate	Negative control DMSO Positive controls Torin 2 and CHX	OPP signal in the cytoplasm Nuclei count based on Hoechst	10X 4 fields
Paper II	Compounds limiting toxicity of ALS-PR_{20} dipeptides	Changes in viability	U2OS + PR_{20} (5µM, 48h) 500 cells/well	CBCS FDA-approved library v1 (4,126) 10µM, 24h (24h after PR_{20}) In triplicate	Negative control DMSO Positive control DMSO + PR_{20}	Nuclei count stained with Hoechst	4X 1 field
PR I	Modulators of translation	Changes in translation rates	U2OS 750 cells/well	Natural compounds library (4,038) 10µM, 24h In triplicate	Negative control DMSO Positive controls Torin 2 and CHX	OPP signal in the cytoplasm Nuclei count stained with Hoechst	10X 4 fields
PR II	Modulators of nucleolar stress	Changes in nucleolar area	Image set from U2OS 1500 cells/well	Medically approved and tool compounds (1,600) 10µM, 48h	Negative control DMSO	Nucleolar area defined by nucleic acid dye SYTO14 Nuclei count stained with Hoechst	20X 9 fields
PR IV	Modulators of nucleolar stress	Changes in nucleolar area	U2OS + ActD (5nM, 4h) 750 cells/well in pre-spotted plates	Drug Repurposing Hub library (5,280) Pre-exposure 48h, 1µM. Then addition of ActD for 4h. In triplicate	Negative control DMSO Positive controls DMSO+ActD PFI-1+ActD JQ-1+ActD	Nucleolar area defined by Fibrillarin staining Nuclei count based on Hoechst	20X 4 fields

Table 1. *Overview of the screening set ups from the work included in this thesis. Here, PR I-IV stands for preliminary results I-IV; v1, v2 stand for versions of the CBCS library of medically approved and tool compounds. The number of fields refers to the number of images taken per well.*

Once the assay is set up, then the screen is conducted and analyzed, which will be explained later. Then, selected hits are validated. For **validation screens** we have consistently used the same methodology as for the primary screen, and, in some cases, an orthogonal assay, as summarized in **table 2**. In all these screens, we had exposed cells to three concentrations of compounds. After validation, the hits selected for characterization were purchased from vendors and assayed using the same methods, to verify effectivity and dosing of these drugs, since there could be slight differences with the compounds kept on the libraries. With that, the process of characterization of compounds using different readouts and techniques starts, and so their functional testing in other models of interest.

Work	Objective	Validation
Paper I & PR I	Modulators of translation	Validation of compounds at 1, 3 and 10 μM Exposure 24h. Validation using OPP labelling and orthogonal assay HPG labelling.
Paper II	Compounds limiting toxicity of ALS-PR_{20} dipeptides	Ad-hoc secondary screen with CBCS Epigenetic library (94). Validation of compounds at 1, 3 and 10 μM. U2OS cells (125 cells/well) pre-exposed to compound for 72h + 48h after re-addition compound and PR_{20}.
PR II	Modulators of nucleolar stress	Validation of the results of the *in silico* screen (45) at 1, 5, 10 μM. Two time points, 4h and 48h exposure to compounds, in triplicate. Validation using SYTO14 staining for nucleic acids and additional Fibrillarin staining to mark the nucleolus. Positive controls ActD, BMH-21 and rapamycin were added.
PR IV	Modulators of nucleolar stress	Validation to be conducted. Proposal: dose response 0.5, 1, 3 μM, following the same parameters as before and in the absence of ActD.

Table 2*. Overview of the validation screens included in this thesis. Here are technical aspects and strategies included in the secondary screen, anything else not specified in here was set as in the primary screen. Here, PR I-IV stands for preliminary results I-IV.*

3.1.2 Image analysis techniques

For these screens, the images were acquired using an IN Cell Analyzer 2200 (GE Healthcare) scanning microscope and analyzed using self-made pipelines built in CellProfiler (v.2.0) [240]. During this thesis we predominantly used three pipelines that were adapted to different purposes described below (**fig. 2**).

1. **Identification of nuclei using Hoechst staining**. This pipeline used nuclear shape and intensity of Hoechst signal in contrast to the background to identify nuclei. Definition of nuclei allowed assessing cell viability based on nuclei count, which was the endpoint for **paper II**, where we screened for drugs able to reduce toxicity induced by ALS-related PR_{20}. We additionally integrated changes in nuclei number as an aspect to consider when intrinsic drug cytotoxicity can influence our readout of interest. For example, in the case of **paper I** and ***preliminary results I***, compounds affecting cell number, would reduce protein synthesis without necessarily regulating this process. Furthermore, segmentation of the nucleus allows to calculate the intensity of nuclear signals different to Hoechst. For instance, when expososing cells to activators of the ISR, as done in **paper I**, the transcription factor ATF4 translocates from the cytoplasm to the nucleus. This translocation is measured as an increase of ATF4 intensity, which signal is in a different channel to Hoechst, in the nucleus, defined by Hoechst. Similarly, in **paper II**, we used this strategy to follow changes in cell cycle based on Hoechst and 5-Ethynyl-2′-deoxyuridine (EdU) intensity. EdU is a thymidine analog that is incorporated into the DNA of dividing cells [241].
2. **Definition of cytoplasmic objects using the nucleus as a seed**. This pipeline uses the cell nucleus, defined by Hoechst, as a starting point from which it expands to the cytoplasm based on the intensity of the cytoplasmic staining of interest. The resulting region of interest includes both cytoplasmic and nuclear signals. Next, the nuclear signal can be subtracted, so only the cytoplasmic region remains. We used this pipeline in **paper I** to measure changes in protein synthesis rates derived from OPP and HPG labelling, which stain the nucleus and the area surrounding the nucleus that corresponds to the endoplasmic reticulum (ER), where translation takes place. In the same work, we used this pipeline to detect phenotypic changes in different cellular organelles, such as the ER, mitochondria, and Golgi appparatus, which were stained using Cell painting dyes.
3. **Segmentation of nucleoli.** This pipeline defines objects (nucleoli) due to their size, shape and intensity inside the nucleus as foci. We used it to measure intensities of nucleolar proteins, and also number and area occupied by these foci. The identification of nucleoli has been done using different nucleolar markers, such as by detection of FBL1 and UBF1 (**paper II** and ***preliminary results II*** and ***IV***), and the fluorescent nuclei acid dye SYTO14, which accumulates in the nucleoli since rRNA is the most abundant nucleic acid in the cell. In **paper II**, for profiling changes in a subset of nucleolar proteins in U2OS, nuclei and nucleoli were defined by Hoechst and FBL1 staining, respectively. Then, the rest of proteins were detected with primary antibodies

in different wells, and for all of them the same fluorescent secondary antibody was used. This allowed the detection of changes from the channel where proteins of interest were imaged in the nucleus and nucleolus.

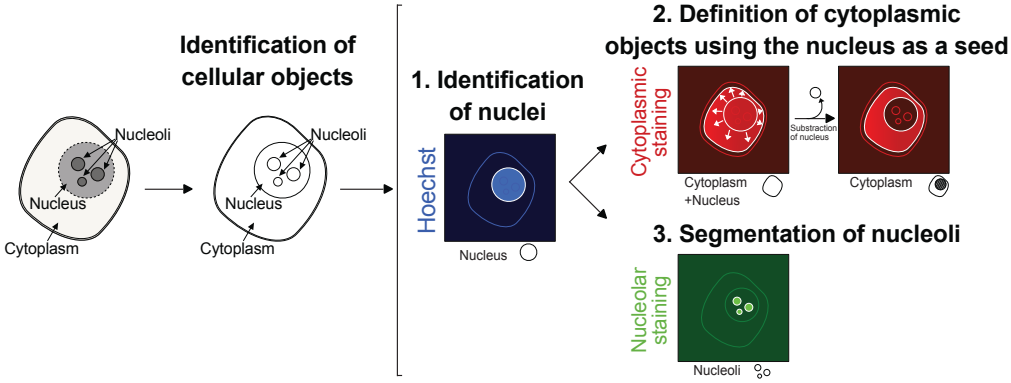


***Figure 2**. Summary of image analysis pipelines to define cellular objects such as the nucleus, cytoplasm, and nucleoli to measure different features in these compartments.*

3.1.3 Statistical analysis pipelines for high throughput screening analysis

Statistical analyses of high content imagining data from the chemical screens were conducted using TIBCO Spotfire (**paper I** and **II,** and ***preliminary results I***) and open source modular KNIME Analytics Platform [242], with own pipelines based on the HTS-workflow [243]. While different criteria have been applied for the different screens, in general, the data has been **normalized to the negative control DMSO**, and hits have been selected to modulate the phenotype over or below several standard deviations from the average of DMSO samples; or, alternatively, according to a percentage of modulation based on this variation in control samples. Additionally, for OPP and nucleolar modulators screens we filtered compounds based on nuclei count reducing viability over a 70%, which usually coincided with the standard deviation of nuclei counts in cells exposed to DMSO. For different screens we chose using either the mean or the median values depending on the robustness of the data and based on pre-set criteria tested when optimizing the assay in high throughput. The screens were done in replicates and the variation amongst them was accounted by calculating the **coefficient of variation (CV%)**, a statistical measure of the relative dispersion of data points around the mean, when comparing the data values for the different compounds among the replicates. Additionally, we contemplated that the hits were identified in several replicates. Definition of a window of assay is fundamental to run a screen, and for that we used control compounds to understand the potential magnitude of changes modifying the phenotypes of study. When analyzing our screens we considered the **Z-prime factor (Z´)** statistic to measure assay quality, showing the separation between positive and negative controls, and indicating the likelihood of false positives or negatives. All these aspects were included in our screen analyses workflows. Additional statistical analyses were carried out with Microsoft Excel and Graphpad Prism software.

3.2 MONITORING CHANGES IN PROTEIN SYNTHESIS

3.2.1 OPP labelling

O-propargyl-puromycin (OPP, also seen in the literature as OP-Puro), is an analog of the translation inhibitor Puromycin, which structure is similar to an aminoacyl tRNA, allowing its positioning into the ribosome A site and incorporation to the C-terminus of nascent polypeptide chains during translation elongation, leading to premature termination and drop-off the ribosome from the mRNA [244]. OPP bears an alkyne moiety that allows detection of OPP-labelled peptides via Cu(I)-catalyzed click chemistry. This signal can be quantified using high throughput microscopy (**fig. 3).** Use of cycloheximide (CHX) is recommended as a technical control, since it abrogates the activity of translating ribosomes, hence OPP cannot get incorporated into new peptides [244].

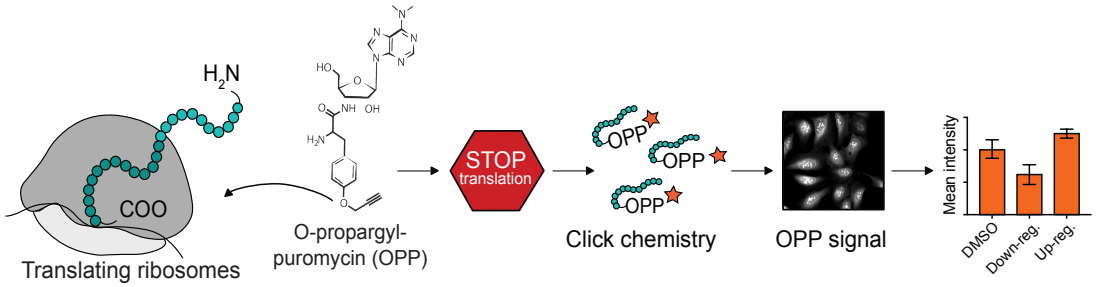


Figure 3*. Scheme of OPP labelling.*

OPP labelling is commercially available (Thermo Fisher Scientific, C10458), however, for the screens we purchased the components separately and prepared our own homemade reagents. OPP is efficiently integrated in cells growing in complete media. Briefly, the protocol for OPP labelling starts by diluting OPP in media to a final concentration of 1:1000 (which can be diluted up to 1:2000). Then, OPP solution is added to plated cells for 30 min up to 1h, the plates are kept at 37°C, labelling newly synthesized peptides during this time. Next, cells are fixed in either 80% or 100% cold methanol (20 or 5 min, respectively), after which cells were washed with 1xPBS. Alternatively, cells can be also fixed with 4%PFA, however, in our hands, methanol fixation worked best for imaging. Then cells are permeabilized with 0.1% or 0.5% Triton X-100 (for 20 or 10 min, respectively). After which, plates are washed and incubated with click reaction cocktail (88 mM Na-phosphate (pH 7), 20 mM CuSO4, 10 mM Na-Ascorbate, 2 µM Alexa Azide 647) for 30 min in the dark. Then, nuclei were stained with 2 µM Hoechst 33342 for 15 min in the dark. Lastly, plates were washed and imaged.

3.2.2 HPG labelling

Another method for studying changes in translation rates is the incorporation of fluorescent biorthogonal noncanonical amino acid tagging (FUNCAT) [245,246]. The most used are analogs of methionine, that can be incorporated during translation initiation and elongation in various positions of the polypeptide chain, since contrary to OPP, they do not inhibit translation. We also used alkyne-bearing homopropargylglycine (HPG) labelling as an orthogonal assay for validation of hits from OPP screens (**paper I** and ***preliminary results I***). Importantly, for incorporation of these analogs, first cells need to be in media deprived from the amino acid

methionine. For that, prior to HPG pulse, cells were washed with PBS and methionine free media was added for 30 min; HPG solution was prepared in the same media. Labelling, processing of plates and detection using click chemistry followed the same steps as for OPP. For these experiments we used commercial HPG labelling kit (Thermo Fisher Scientific, C10186).

3.2.3 Polysome profiling

Polyribosome (polysome) profiling is a technique in molecular biology to study the association of mRNAs with ribosomes [247]. This technique can be used to study general translation rates in the cells and of specific mRNAs in a genome-wide fashion. Additionally, this method can be used to identify ribosome and polysome associated factors. This technique consists in the fractionation of polysomes by sucrose density gradient centrifugation, which separates polysome from monosomes, ribosomal subunits and ribonucleotide particles. Ribosomes are immobilized on mRNA by using translation inhibitors, such as CHX, in different buffers while preparing the cytosolic lysates. Hence, the mRNAs that are heavily translated are associated with more ribosomes, while the poorly translated, with less. It is possible to extract mRNA across the different gradient fractions and study their distribution and translational profile. This technique allows for in depth analysis of the translatome, yet its throughput is quite limited, it is technically challenging, and it requires a bigger amount of starting material, compared to OPP labeling, for instance [245]. We used this technique to visualize the effects of one of our hits (SKI-II) in translation based on the abundance of polysomes and the commitment of the ribosomal subunits into actively translating mRNA. For getting polysome gradients, lysates were subjected to ultracentrifugation using a SW41Ti rotor, gradients were analyzed in a piston gradient fractionator (Biocomp), and profiles were acquired with Gradient profiler v.2.0 (Biocomp, Spain) and represented using Graphpad Prism.

3.3 MONITORING CHANGES IN NUCLEOLAR BIOLOGY

Changes in the nucleolus were mostly studied by immunofluorescence (IF) following alterations in features of specific nucleolar proteins upon diverse treatments in different cell lines. Briefly, cells were seeded into microwell plates, fixed with 4% PFA for 15 min, permeabilized with 0.1% Triton X-100 for 10min at room temperature, blocked with 3% BSA and 0.1% Tween-20 in PBS) for 30min, and incubated with primary antibodies in blocking buffer either overnight at 4°C or 1h at room temperature. Next, plates were washed and incubated with fluorescently tagged secondary antibodies. This general protocol for immunostaining was used for to characterize markers for other projects, for instance to visualize ATF4 translocation into the nucleus in **paper I**. Additionally, Fibrillarin staining also worked when fixing cells with 100% cold methanol for 5 min, which allowed simultaneous detection of PR_{20} tagged with HA in **paper II**. Also, Fibrillarin staining can be achieved skipping permeabilization with TritonX100, as done for ***preliminary results IV***. In **paper II**, we did a general microscopy-based profiling of changes in a subset of nucleolar proteins together with the Cell profiling facility at SciLifeLab, where we exposed U2OS cells to our compounds and PR_{20}, and they conducted fixation, permeabilization and immunostaining using

their protocols (Stadler et al., 2010). For all these experiments, changes in the levels, localization, area and number of nucleolar proteins and nucleoli were assessed using image analysis pipelines explained in above (**3.1.2**). Among the nucleolar proteins characterized, the levels of UBF1 were lowered when cells were exposed to PR_{20}, suggesting a decrease in rRNA synthesis induced by DPRs. We conducted immunoprecipitation (IP) experiments expressing different versions of UBF1 using standard protocols for IP and DNA transfection. We found that PR_{20} was preferentially interacting with the C-terminal domain of UBF1, which is rich in acidic amino acids that have high affinity for positively charged arginines that are present in poly-PR and poly-GR. These acidic domains are common among nucleolar proteins.

3.4 ZEBRAFISH AS A PRECLINICAL MODEL FOR DRUG DISCOVERY

Zebrafish (*Danio rerio*) are small (2–5 cm) tropical fish that live in rivers in Southern Asia. Zebrafish were introduced in the lab as a model organism more than 40 years ago to study development, and they have become a prominent vertebrate model for disease [248]. Zebrafish share 70% of their genome with humans, including more than 80% proteins associated to diverse diseases, as well as many drug targets [249]. In many aspects, such as physiology, drug metabolism and pharmacology, zebrafish are comparable to humans, particularly when they are embryos [248]. In fact, for some drugs zebrafish recapitulates better the effects of drugs observed in humans than mice. For instance, thalidomide, a drug prescribed for helping with nausea during pregnancy, and known to cause morphological limb defects in human fetuses, did not show these defects in mice while it did in zebrafish [248,249]. Another interesting feature is the small size and minimal needs of zebrafish embryos, since they get nutrients from their yolk sacs, which makes them suitable to be housed in multi-well plates and screened for multiple phenotypes in the context of a living whole vertebrate organism [248]. Furthermore, drugs can be actively absorbed from the water by the embryos. Hence, the effects of drugs on different tissues can be scored, as well as compound absorption, distribution, metabolism and toxicity (ADME-tox) [249]. Additionally, due to their fast development (90 days from egg to adult), zebrafish provides relatively rapid insights on how drugs affect cell and tissue over time [248,250]. Furthermore, in terms of screening, they show obvious signs of toxicity that can be seen by-eye, and traced using image-based analysis, such as measuring changes in the curvature of their tail fin and spine or based on their heartbeat [249,251]. Also, zebrafish embryos are practically transparent, which helps when using fluorescent reporter lines or fluorescently tagged molecules, and which facilitates using stainings [248,251]. Other technical advantages of using zebrafish are the possibility of dispensing them into microwell plates using fish sorter systems, and monitoring changes in their behavior based on their movement using automated detection and tracing systems [251,252]. This last, has been particularly interesting when studying neurodevelopmental, neurodegenerative and locomotor disorders. In **paper II**, we used zebrafish as a preclinical model for testing leading compounds able to ameliorate toxicity of ALS-PR_{20}.

3.4.1 Zebrafish to validate models of toxicity

In **paper II**, we used zebrafish embryos to study toxicity of ALS-PR_{20} and understand the potential of leading compounds in limiting these effects *in vivo*. For that, we used wild-type Tupfel long fin (TL) zebrafish that were maintained at 28.5°C on a 14h light/dark cycle. The zebrafish facility at Karolinska Institute set up pairwise breeding and provided us from the embryos used for experiments. After 28 - 30h post fertilization (hpf), the chorion from the embryos was remove using pronase, a non-specific protease, to improve diffusion of compounds. Compounds were added to the water saline E3 medium, in which the embryos are kept for 24h. Then, embryos were co-treated with compounds and PR_{20}, when they were at the stage of 54-58 hpf. After 16h, embryos were scored as dead or alive based on their heartbeat. For these experiments we used a version of PR_{20} tagged with fluorescein (FAM-PR_{20}) which allowed us to follow distribution of the PR_{20} in the whole fish embryo and at cellular level. The experimental set up was done based on the treatment schedule used in cells and considering the distribution of PR_{20} during development. FAM-PR_{20} was more equally and extensively distributed in zebrafish embryos exposed for 24h to compounds than a day later, as it was done cells, due to more complex compartmentalization of organs.

3.4.2 Nucleolar staining in zebrafish tissue sections

Since PR_{20} localizes predominantly in the nucleoli of cells, we used IF to mark nucleoli. Embryos from the toxicity experiments were used to do IF in zebrafish sections. For that, zebrafish embryos were embedded in Richard-Allan Scientific™ NEG50TM frozen section medium. Then, sagittal tissue sections were cut using a cryostat. The sections were fixed in 4% PFA for 20 min and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Next, sections were blocked with 3% BSA in 0.1% Tween-20 in PBS for 1h, sections were incubated with anti-Fibrillarin antibody (1:500, Abcam, ab4566) at 4°C overnight, and then with secondary antibody 1h at room temperature, and nuclei were stained with Hoechst for 15 min. Finally, sections were mounted in ProLong™ Gold Antifade Mountant (Thermo Fisher scientific, P36934) for image acquisition using Nikon Ti2 inverted microscope.

3.4.3 OPP labelling in zebrafish

Alive embryos that had been exposed to another version of PR_{20} tagged with fluorescein and HA (5(6)-FAM-PR_{20}-HA) for 16 - 18h (72 hpf) were pulse labelled for 1h with 0.5 mM OPP in E3 medium. The protein synthesis inhibitor CHX was added for 2h prior to OPP pulsing to some embryos as a negative control. Then embryos were washed, prepared for cryosectioning, and slides were fixed and permeabilized as before. Tissue slides were incubated with click reaction cocktail overnight at room temperature, as adapted from a protocol for metabolic labelling in worms [253]. Next day, nuclei were stained, and sections were mounted and imaged as before. The sections were analyzed considering the profiles for OPP staining and PR_{20} signal, in their corresponding channel, in a defined region of interest.

3.5 USING CELL PAINTING FOR FUNCTIONAL STUDIES

Cell painting is a high-content image-based assay for morphological profiling of multiple cellular organelles simultaneously using fluorescent dyes, which are: Hoechst 33342 (nucleus), SYTO14 (predominantly nucleoli), MitoTracker (mitochondria), Wheat Germ Agglutinin (WGA, Golgi apparatus), Phalloidin (cytoskeleton), and Concanavalin A (ER) [230]. Changes in different features associated to these stainings - such as intensity of signal, texture, distribution of the signal, among others – can be clustered together and provide information of the mechanism of action of cells exposed to different perturbagens or provide insights about the cell's state, when, for instance, studying disease models. We took a different approach in **paper I** and in ***preliminary results II***, and we used Cell painting to functionally to characterize the effects of compounds exploring the intrinsic properties of the staining dyes.

In **paper I**, we explored whether the sphingosine kinase (SPHK) inhibitor SKI-II, identified as a down-regulator of translation, was altering different cell membrane-bound organelles which composition is rich in sphingolipids using Cell painting. Here, U2OS cells that had been exposed to SKI-II were incubated with MitoTracker Deep Red Alexa-647 (1:1000, Thermo Fisher Scientific, M22426) for 20 min at 37°C. Then, cells were fixed in 4% PFA for 15 min and permeabilized with 0.5% TritonX100 for 10 min and stained with a cocktail of Hoechst (1:1000), Concanavalin A Alexa-488 (1:200, Thermo Fisher Scientific, C11252), and WGA Alexa-555 (1:500, Thermo Fisher Scientific, W32464) for 20 min in the dark. After identifying that the ER was specifically affected, next we sought to understand whether the compound was affecting protein folding or the physical structure of the organelle. The dye Concanavalin A is a lectin that binds to α-mannopyranosyl and α-glucopyranosyl residues in glycoproteins found in the ER during protein folding. Hence, if the effect of the compound depended on accumulation of unfolded proteins, leading to a reduction in Concanavalin A-associated intensity, inhibitors of the unfolded protein response (UPR) should limit the effects in Concanavalin A staining. Whereas, if the damage done is on the physical structure of the ER, inhibiting the UPR would not prevent the decrease of Concanavalin A signal, as it was the case for SKI-II. In ***preliminary results II,*** incorporation of the nucleic acid dye SYTO14, which we used as a readout for nucleolar changes, it is additionally an indicator of RNA pol I activity. SYTO14 is preferentially accumulated in the nucleoli, since rRNA is the more abundant nucleic acid species in the cell. Hence, changes in SYTO14 area and intensity are indicative of rRNA synthesis in cells. Other functional applications can be based on using specific versions of these dyes. For example, mitochondrial trackers that differently labelled either mitochondria or functional mitochondria. Hence, Cell painting dyes can be used for functional studies without the need of multiplexing and using high-complexity analysis.

3.6 TRANSMISSION ELECTRON MICROSCOPY (TEM)

In **paper I**, we used transmission electron microscopy (TEM) to visualize changes in the ER upon exposure to compounds inducing ER stress, including our hit compound SKI-II. This level of resolution is achieved when an electron beam goes through the specimen, an ultra-thin section mounted on a suspension grid, to form an image. For that, we prepared samples

exposing U2OS cells to compounds, and then samples were resuspended in glutaraldehyde-based fixative. Samples were subsequently processed and ultra-thin sections were prepared by the Electron Microscopy (EMil) Unit at Karolinska Institutet. Next, together with them, we acquired EMT images using a Veleta camera (Olympus Soft Imaging Solutions, Germany).

3.7 CRISPR/Cas9 EDITING

3.7.1 Validation of drug targets

In **paper I**, we found the sphingosine kinase (SPHK) inhibitor SKI-II to down-regulate protein synthesis by activating the ISR. To assess if these effects were dependent on the modulation of targets SPHK1 and SPHK2 we generated single and double knockout (KO) U2OS cell lines using CRISPR/Cas9 editing. We used SYNTHEGO knockout pools v2, in which a pool of three single guide RNAs (sgRNAs) targeting a gene of interest are provided and then they are coupled with a Cas9 nuclease from *Streptococcus pyogenes* that is fused to two nuclear location sequences (NLSs). These ribonucleoprotein (RNP) complexes are then transfected into cells, in this case U2OS, using Lipofectamine CRISPRMAX Cas9 Transfection Reagent. We first generated single-clone knockouts for SPHK1 and SPHK2, and then, we repeated the transfection process to generate double knockout clones.

3.7.2 CRISPR screen

In ***preliminary results III***, we conducted a genome-wide CRISPR/Cas9 loss of function screen to study cancer vulnerabilities in cells exposed to nucleolar stressors ActD and BMH-21. For this screen, we used A375 melanoma cell lines stably expressing a construct coding for Cas9 nuclease from *S. pyogenes,* blue fluorescent protein (BFP), and a selection marker of resistance to Blasticidin. Then, these cells were sorted, selected, and transduced with the CRISPR guide library in two replicates at an approximate multiplicity of infection (MOI) of 1000 cells/guide in the presence of 2 μg/ml polybrene. For this screen, we used the genome-wide Brunello sgRNA library [254] including Unique Molecular Identifiers (UMIs) [255]. Guides were cloned in pool and packaged into lentivirus. Transduced cells were selected with Puromycin (2 μg/ml) for five days. Library and cell line preparation was done by the High throughput Genome Engineering (HTGE) Facility at Karolinska Institutet. Next, 80 million (M) cells were seeded for each treatment (DMSO/ActD/BMH-21) in T175 flasks, each of them containing 6 million cells. After 24h, media was removed and cells were exposed to DMSO, ActD 0.75nM and BMH-21 0.2 μM. Cells were passaged, counted, seeded, and exposed to compounds every three days until day 12 of treatment. Every time that cells were split, 80M cells were harvested for each condition for following sequencing, having a coverage of 1000 cells/guide. Only before starting with compound treatments (T_0) 100M cells were collected from each replicate as a reference. Samples from time 0 (T_0, before treatment), and at days 3 (DMSO/ActD), 6 (DMSO/BMH-21) and 12 (DMSO/ActD/BMH-21) were used for sequencing. Next-Generation Sequencing (NGS) data was analyzed using Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout (MAGeCK) [256] by the HTGE facility, and then we conducted network analysis and selected hits for validation, as it is explained below (**3.8**).

3.8 DATABASES AND DATA MINING

In **papers I** and **II** we used Gene Ontology (GO) [257] and Gene Set Enrichment Analysis (GSEA) [258] to analyze the data from mass spectrometry of cells exposed to SKI-II and RNA sequencing data for cells exposed to PR_{20} and hit compounds, respectively; without discovering any significant enrichments for **paper II**.

In **paper I**, we used the Connectivity map (cMAP) [239] to identify potential pathways triggered by SKI-II. Using the CMap Touchstone tool we compared signatures from different perturbagens – such as compounds, knockdown or overexpression of genes – with the expression signature of SKI-II. For this, we used as a queries (1) SKI-II, which compound signature was already included in cMap, and (2) the top upregulated genes in U2OS cells exposed to SKI-II from our mass spectrometry data. Additionally, we used the PRISM Repurposing dataset from the Broad Institute [259,260], to identify compounds sharing the same mechanism of toxicity with SKI-II.

In ***preliminary results II,*** we conducted a virtual screen for modulators of nucleolar biology using publicly available images of a Cell Painting experiment on U2OS cells exposed to compounds from the Broad Bioimage Benchmark Collection [261].

In ***preliminary results III,*** we used web-based GSEA tool WebGesalt [262] and STRING functional protein association networks [263] tools for network analysis of the hits from the CRISPR screen. For plotting and identifying the intersecting genes among conditions we generated an UpSet plot and retrieved the gene list using web-tools Intervene [264] and Venn diagram tool (https://bioinformatics.psb.ugent.be/webtools/Venn/). Additionally, we searched for some of the hits from our screen in the BioGRID Open Respository of CRISPR Screens (ORCS) [265], and against the repository from Durocher's lab for CRISPR screens done with genotoxic compounds [266], which includes multiple nucleolar stressors, using their webtool (https://durocher.shinyapps.io/GenotoxicScreens/). We used cBioportal to explore the alteration frequency amongst different cancer types of genes identified in our screen against the dataset Pan-cancer analysis of whole genomes from 2,658 donors across 38 cancer types [267].

3.9 ETHICAL CONSIDERATIONS

This work has been done using commercially available cell lines from ATCC donated to science, and that are spared from ethical clearance. The experiments in zebrafish embryos were carried out until they were five days of age, which are exempt from ethical requirements. This work was done following the guidelines defined by the Stor Stockholm djuretiska ethics committee and of the EU directive 2010/63/EU for animal testing. Zebrafish were housed in the central facility at Karolinska Institutet (Solna, Sweden) in accordance with the Swedish animal welfare legislation and acknowledged guidelines of the Society of Laboratory Animals (GV-SOLAS) and of the Federation of Laboratory Animal Science Associations (FELASA).

4 RESULTS

4.1 PAPER I: A chemical screen for modulators of mRNA translation identifies a distinct mechanism of toxicity for sphingosine kinase inhibitors

4.1.1 Summary

Regulation of mRNA translation has become an attractive therapeutic target due its association to an ever-growing number of disorders together with the success achieved by interventions in this process [4,9]. However, most of the regulators of translation have been discovered either by serendipity or by to targeting specific components of the translation machinery and pathways controlling protein synthesis [7,11]. Yet, a systematic identification of modulators of global mRNA translation had not been conducted in cells, most probably due to the limited throughput of classically used methods to study protein synthesis. In this sense, recent development of techniques allowing monitoring translation in cells by incorporation of noncanonical amino acids or Puromycin derivatives, which can be detected using fluorophores or tags, has revolutionized the field [245]. In this project, we capitalized on the use of these tools to conduct a high throughput image-based screen to evaluate how all medicines and drugs under development affect translation rates in human cells.

To monitor translation, we used OPP labelling [244]. OPP is an analog of Puromycin that gets incorporated into the C-terminus of newly produced peptides, stopping translation, and which can be visualized using click chemistry.

First, we set up the conditions to conduct a chemical screen in human osteosarcoma (U2OS) cells in 384-well plates using changes in intensity of OPP signal quantified by high throughput microscopy as a readout.

Next, we screened 4,166 characterized compounds (a third of which are medically approved drugs) by exposing U2OS cells to 10 µM compound for 24h. The primary screen identified 54 compounds increasing OPP signal (up-regulators of translation) and 48 down-regulators, the majority of which were mTOR/PI3K/MAPK inhibitors, as it would be anticipated (**fig, 1**). As expected, translation inhibitors included as controls, cycloheximide (CHX) and the mTOR inhibitor, Torin 2, were distributed among the down-regulators. Of note, selected hits did not have effects in cell viability to avoid interference with translational readout. Then, hits were validated using two orthogonal assays to measure changes in protein synthesis, OPP and HPG labelling.

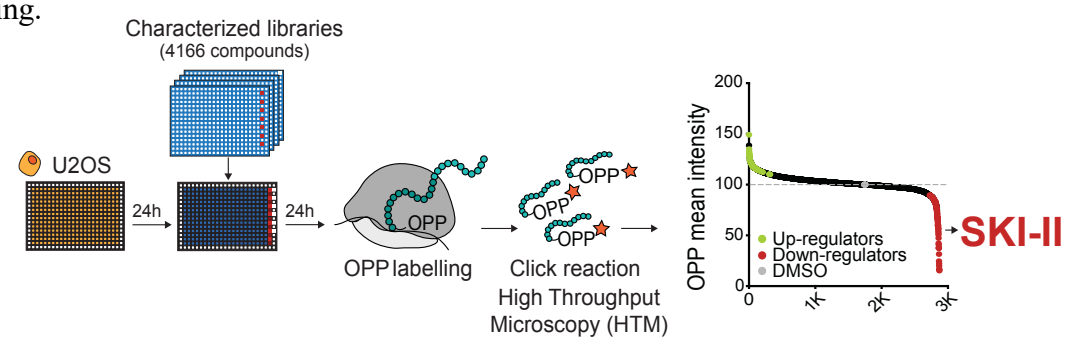


Figure 1. *Schematic overview of screen for modulators of protein synthesis using OPP labelling.*

Interestingly, among the compounds screened, we were unable to identify a single drug that substantially up-regulated translation. The ones initially identified as up-regulators showed inconsistent results in following experiments and failed to stimulate translation after short exposure, suggesting that, if any, the effects observed in OPP incorporation were indirect. To evaluate the capability of our assay to find up-regulators, we exposed a panel of cell lines to insulin, a known stimulator of mTORC1, and consequently, translation. As previously reported, insulin only improved translation in cells where translation rates are reduced, as in this case, serum starved [247]. Altogether, seems doubtful that protein synthesis can be boosted in cancer cell lines grown in complete media, and our screen was limited to discovering drugs decreasing translation. That said, identification of novel chemicals able to rescue translation in compromised cells characterized by intrinsically low translation rates (stressed, diseased, etc.), could be a valuable therapeutic approach for a variety of disorders.

The sphingosine kinase (SPHK) inhibitor SKI-II stood out as the only down-regulator of translation which was not annotated as an mTOR/PI3K/MAPK inhibitor. SKI-II inhibited translation independently of mTOR by activating the Integrated Stress Response (ISR) pathway, causing endoplasmic reticulum (ER) stress. Supporting this, inhibition of ER stress and ISR signaling with known inhibitors, PERKi and ISRIB, prevented down-regulation of translation by SKI-II. Interestingly, SKI-II physically damaged the membranes of the ER, contrary to what happens with other known ER-stressors, which promote accumulation of unfolded proteins (**fig. 2**).

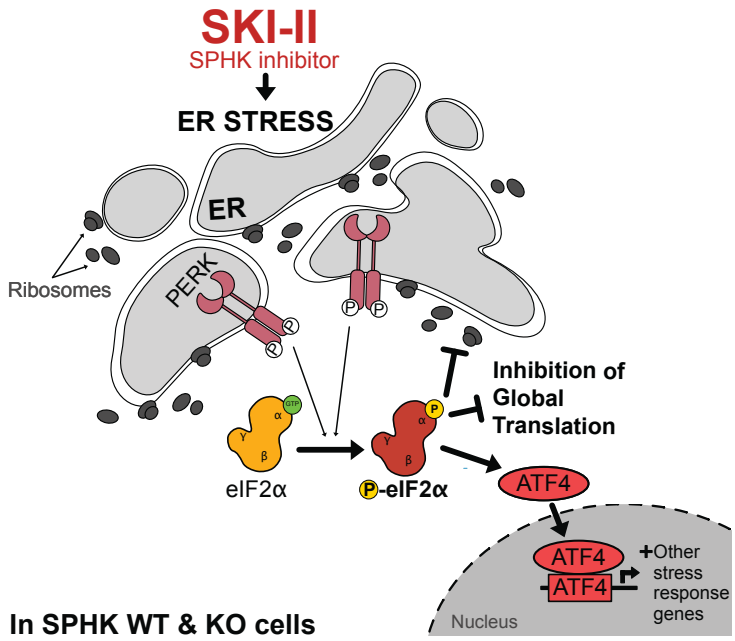


Figure 2. *Overview of SKI-II mechanism of toxicity by induction of physical damage to the ER, resulting in ER stress, activation of the ISR and inhibition of global translation in SPHK WT and KO cell lines.*

SKI-II, and other SPHK inhibitors, have been investigated as anticancer drugs, and their proposed mechanism of toxicity is based on accumulation of ceramides, which triggers apoptosis [268]. We assessed the contribution of the ISR to the mechanism of toxicity of SKI-II in U2OS and in mouse acute myeloid leukemia (AML) cells, since SKI-II have been efficient

in vivo for this malignancy [269]. Treatment with ISR inhibitors rescued toxicity caused by SKI-II. Importantly, genetic ablation of SKI-II annotated targets – SPHK and SPHK2 – did not prevent activation of the ISR, downregulation of translation and toxicity exerted by the compound. Of relevance, the same phenotypes, including independence from SPHKs, were observed for the structural analog of SKI-II, ABC294640. ABC294640 was developed as a more selective SPHK2 inhibitor [270] and is in several clinical trials for cancer and tested for treatment of COVID-19-associated pneumonia [271].

Our work raises a word of caution as to what is the real target of these drugs that mediates their anticancer activity. Additionally, our results provide a rationale for drug combinations of SPHK inhibitors with other drugs able to induce ER stress by activation of the UPR, exacerbating ER stress. Lastly, while ISR activators might be toxic for cancer cells, these compounds might be beneficial for other conditions, as it has been proposed for demyelinating disorders, such as Charcot-Marie-Tooth Disease [115].

4.1.2 Follow-up studies

4.1.2.1 Which is the target of SKI-II and ABC294640 that activates the ISR?

In this work we identified that activation of the ISR by SKI-II and ABC294640 was independent of SPHKs, yet we did not discover which is the target of SKI-II responsible for inducing ER damage, and here are summarized our efforts on **target deconvolution**.

Initially, we aimed to isolate interactors of SKI-II and identify potential candidates by pull-down experiments followed by mass spectrometry (**fig. 3A**). For these experiments, we got synthesized a clickable version SKI-II, bearing an alkyl group, courtesy of Martin Haraldsson (CBCS). The compound was able to diffuse into cells, the drug accumulated principally in the nucleus, and co-localized with the ER (**fig. 3B**). We followed different protocols for pull-down, based on coupling the alkylated drug to biotin-azide followed by isolation via biotin-streptavidin interaction [272]. These experiments are technically challenging and we were not able to immunoprecipitate SPHK1, with which SKI-II has been crystalized [273], hence we discarded following with mass spectrometry experiments.

Inspired by [274], we tried to generate SKI-II resistant cell lines, to identify genes differentially regulated compared to SKI-II responsive cells. While the cells continuously exposed to SKI-II were able to divide, they seemed chronically stressed, similar to what has been reported in [275], and were not substantially more resistant to high concentrations of SKI-II than control cells.

Lastly, we used the Connectivity Map (CMap) developed by the Broad Insitute and MIT to compare the perturbational signature SKI-II to the 1.5M signatures contained in this database, obtained from exposing human cancer cell lines to approximately 5,000 drugs and genetic perturbations (https://www.broadinstitute.org/connectivity-map-cmap) [239]. We used used as input to CMap the top 20 significantly up-regulated proteins from proteomic analyses of cells exposed to SKI-II for 6 h, included in **paper I** (**fig. 3C**). Satisfactorily, SKI-II (SA-792728), which is included in the CMap perturbagen collection, appeared among the most significantly

enriched compounds matching the expression signature used as a query. Comparison of the expression signature of both top 20 up-regulated proteins and SKI-II (SA-792728), revealed inhibition or down-regulation of Heat Shock Proteins (HSPs) and Valosin-Containing Proteins (VCPs) as the most prominent classes enriched in similarity to SKI-II. Of note, bonafide ER-stress inducers, such as Tunicamycin or Cyclosporin-A, and inhibitors of protein synthesis, such as Rapamycin, were also enriched. We cross-validated our list of potential candidates with the PRISM Repurposing dataset from the Broad Institute [259,260], in which compounds sharing the same mechanism of toxicity appear clustered together, after searching for SKI-II. We settled for a sensible list of drugs to test, which where HSP and VCP inhibitors or shared both transcriptional and cytotoxic signatures with SKI-II. We assessed whether these compounds could recapitulate SKI-II-phenotypes, and if SKI-II behaved as a HSP or VCP inhibitor using commonly use readouts for these activities. Our results revealed that neither SKI-II or ABC294640, were inhibitors of HSP90, as they did not promote stabilization of HSP70 and degradation of c-Raf [276], as Geldanamycin and Radicicol did (**fig. 3D**). Similarly, exposure to SKI-II, failed to result in accumulation of Lysine 48-linked polyubiquitin chains (K-Ub48), which is used as a readout for VCP inhibition [277] (**fig. 3E**).

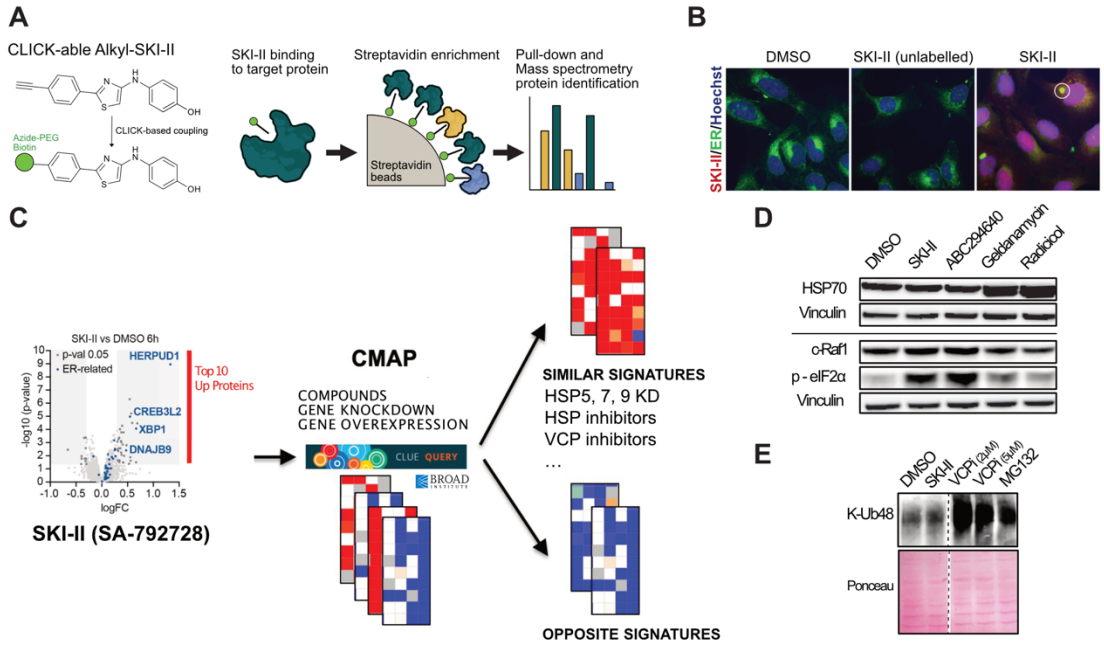


Figure 3. *Overview of target deconvolution experiments for SKI-II. (A) Scheme for pull-down experiments using alkylated version of SKI-II followed by mass spectrometry. (B) Intracellular localization of alkyl-SKI-II (red), primarily in the nucleus (blue) and co-localizing with ER (green). (C) Overview of datamining strategies in CMap and PRISM. (D) Immunoblot of U2OS cells exposed for 6h to SKI-II, ABC294640, and HSP90 inhibitors Geldanamycin and Radicicol. Exposure to HSP90 inhibitors led to accumulation of HSP70 and decreased levels of c-Raf1, with almost no effect on eIF2α, hence not activating the ISR. While SPHK inhibitors did not affected HSP70 and c-Raf1 levels. Vinculin was included as a loading control. (E) Immunoblot of U2OS cells exposed to SKI-II 10 μM, VCP inhibitor (VCPi) at 2 and 5 μM, and proteasome inhibitor MG-132 10 μM. VCPi and MG-132 led to accumulation of K-Ub48, while SKI-II did not show any effect compared to cells exposed to DMSO. Ponceau staining is shown as loading control.*

Regardless of our lack of success, these strategies have proven valuable for target identification [278]. Indeed, there are more approaches available for target identification, including loss of function genetic screens and Cellular Thermal Shift Assay [232], and some other perturbagens identified in perturbation datasets could have been explored. However, it would be possible that despite of investing more time and resources we could not find this target still, and pondered the contribution of our work in relation to characterizing SKI-II and ABC294640 as inhibitors of translation.

4.1.3 Discussion and future perspectives

Here we have conducted a chemical screen to explore the effects on translation of medically approved drugs and characterized compounds, which could serve as a resource for other studies. This is the first chemical screen based on changes in translation levels ever performed in living cells. In fact, only two chemical screening campaigns had been directed to find modifiers of global translation, the first used changes in levels of reporter proteins in cells as a readout [279], with the caveat associated to exogenous expression systems, and the second, used *in vitro* translation systems in rabbit reticulocytes lysates [280]. This was particularly interesting, since new technologies with improved throughput for measuring protein synthesis rates have been used for different purposes [245,281], but not exploited in drug screening. Our work using OPP/HPG labelling illustrates this. However, probably one of the next milestones in this area of research would be based on in depth interrogation of the translatome in response to multiple perturbations, in this sense, technologies like riboPLATE-seq show promise [282].

Our screen failed to identify compounds stimulating translation, which seems not to achievable in proliferating cells growing in the presence of nutrients. Only in the case of starved cells, exposure to insulin increased translation, but to the same extent as for cells growing in complete media. This happened for cancer cell lines and for the immortalized epithelial cell line, RPE. Yet, while our screen might have been biased towards finding inhibitors of translation due to using a cancer cell line, there are not many stimulators reported, and the few of them had only an effect in compromised cells. For instance, in starved MCF7 cells, addition of insulin promotes engagement of polysomes to translating mRNAs [247]. Whereas expression of a constitutively active version of S6K together with deletion of 4E-BP, both of which activate mTORC1, did not result in accumulation of polysomes in experiments in *Drosophila* [283]. Similarly, we and others, have reported that inhibition of the ISR does not increase translation levels when cells are not stressed [139]. Given than protein synthesis is energetically expensive, the possibility of having mechanisms controlling that translation is not supraphysiolocally stimulated could be interesting to examine. These studies could benefit from techniques that allow more in depth interrogation of changes in the translatome, such as ribosome or polysome profiling followed by RNA sequencing or mass spectrometry [245]. Nevertheless, discovery of drugs able to increase translation could be of benefit for many disease models, where protein synthesis is impaired. For instance, in ALS overexpression of the translation factor eIF1A rescued protein synthesis and viability, without having any effects in healthy cells [284]. Additionally, discovering stimulators of translation that can solely improve translational rates

in compromised cells could circumvent safety concerns regarding risks of neoplastic transformation.

Our screen identified the sphingosine kinase inhibitor SKI-II as a novel inhibitor of mRNA translation. Our data is consistent with previous observations of SKI-II inducing ER stress [285,286]. Nevertheless, our work is the first reporting that SKI-II and its clinically relevant analog ABC294640 produce ER stress by physically damaging the ER. SPHK inhibitors have brought interest for treating cancer since they are overexpressed in tumors [268]. The mechanism of toxicity of these compounds was assumed to result from accumulation of ceramide, which induces apoptosis, with simultaneous decrease in pro-survival factor sphingosine-1-phospate (S1P) [287]. However, discrepancies among the effects of chemical and genetic modulation of SPHKs raised questions regarding toxicity induced by ceramide/S1P balance. For instance, some studies reported that knockdown of SPHK1 or SPHK2 led to a decrease in S1P with no increase in pro-apoptotic ceramides [286,288-290]. Additionally, specific inhibition of SPHK1 with PF-543 does not induce cell death in cancer cell lines [273], while SPHK1 knockdown does [291]. Furthermore, reported accumulation of ceramides by SKI-II and ABC294640 is not recapitulated in SPHK2 knockdowns [292-294]. Additionally, our results show that inhibition of DEGS1, another target of SKI-II and ABC294640, does not generate ER stress. Our results bring some light to the mechanism of action of these compounds and might be beneficial to improve their potential use. For example, combination of ISR inhibitors with these SPHKs, might be counterproductive aiming for cytotoxicity. Meanwhile, the effects of SKI-II and ABC294640 might be enhanced by other chemicals activating the UPR, in this sense combination of SKI-II with proteasome inhibitor Bortezomib has been successful in preclinical models (Wallington-Beddoe et al., 2017). Our work also highlights the importance of using genetic models to validate target specificity, or to de-associate the mechanism of toxicity of a drug to its initial target, as it has been systematically explored in [236]. Additionally, activation of the ISR could be of interest for other diseases, such as Charcot-Marie-Tooth disease [295].

4.2 PAPER II: A chemical screen identifies compounds limiting the toxicity of C9ORF72 dipeptide repeats

4.2.1 Summary

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that kills patients 2-5 years after diagnosis. ALS is characterized by the loss of upper and lower motor neurons resulting in muscular paralysis. Today, there is no actual treatment for ALS. The only FDA-approved drugs for ALS are Riluzole and Edaravone, which modestly extend lifespan and have no effect in maintaining muscle function [296,297]. Thus, there is a need to identify drugs that substantially help these patients.

Mutations in several genes have been associated to ALS, most of them related to RNA metabolism and nuclear biology [298-300]. However, the discovery of G_4C_2 hexanucleotide repeat expansions (HRE) within the first intron of C9ORF72 was a big breakthrough in the field, since this is the most frequent inherited mutation in both in ALS and Frontotemporal Dementia (FTD) [120,121,301]. These HRE are present in 2-8 copies in unaffected individuals and are up to 1,600 copies in patients [122]. Furthermore, C9ORF72 HRE account from 5-15% of sporadic cases of ALS.

Through repeat-associated non-ATG (RAN) translation, these expansions are translated from the six possible open reading frames into dipeptide repeats (DPRs), some of which are toxic, such as poly-proline-arginine (poly-PR) and poly-glycine-arginine (poly-GR) [125,302,303]. Both poly-PR and poly-PR bind to nucleoli, disrupt nucleolar activity and mRNA translation, and lead to cell death. The work from Kwon and colleagues [125] recapitulated these phenotypes using synthetic versions of 20 repeats of poly-PR (PR_{20}) and poly-GR (GR_{20}), which were added exogenously to cells in culture, and the effects were not limited to cells from neuronal lineage, such as the human osteosarcoma cell line U2OS.

We benefited from this model to conduct a chemical screen to identify small molecules able to limit toxicity of C9ORF72 DPRs. We specifically used PR_{20}, since it is more toxic and has a longer half-life than GR_{20} [125]. We screened the library of medically approved and characterized compounds, bearing in mind that our findings could be translatable to the clinic faster. In our search, we did not only identify three compounds preventing PR_{20} toxicity in cell lines and in zebrafish embryos, but we discovered new nucleolar functions for some of these compounds.

The primary chemical screen was conducted exposing U2OS cells to PR_{20} (5 μM) for 24h, and the next day, compounds (4,126) were added at a single concentration of 10 μM for other 24h, after which cells were fixed and nuclei were stained with Hoechst (**fig. 1**). Changes in nuclei count quantified by high throughput microscopy were used as a readout. Two classes of compounds where mostly enriched: small molecules targeting redox enzymes and epigenetic modifiers, and we decided to further explore the therapeutic potential of the latest, given that redox-related drugs have been widely studied for ALS [297,304].

Next, an ad-hoc secondary screen was done using the epigenetic compound library (94 compounds) to examine the potential of epigenetic modifiers in PR_{20}-mediated toxicity. In this case, U2OS cells were exposed to three concentrations of the compounds (1, 3, and 10 μM) for 48h, and then to PR_{20} (5 μM) for 48h. Three compounds improved viability in a dose-dependent manner: two BET (Bromodomain and extra-terminal domain family) inhibitors (BETi), PFI-1 and Bromosporine (BSP.); and the histone deacetylase inhibitor (HDACi), Sodium phenylbutyrate (Na-Phen).

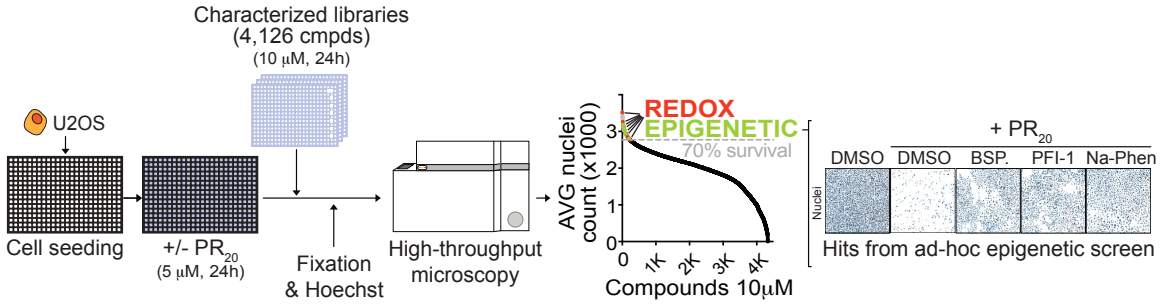


Figure 1. *Overview of chemical screens conducted to identify compounds limiting toxicity of ALS-PR_{20} peptides in U2OS cell using characterized compounds libraries. Exposure to BSP., PFI-1, and Na-Phen reduced cell toxicity induced by PR_{20}, as shown in changes in nuclei count stained with Hoechst.*

The three compounds ameliorated PR_{20} toxicity in U2OS cells and in NSC-34 cells, a mouse motor-neuron-like cell line. Additionally, the hits rescued neurite length in differentiated NSC-34 cells which was critically reduced upon exposure to PR_{20}, supporting an improvement in neuronal health. Addition of PR_{20} induced alterations in nucleolar morphology, which had been already reported [125], such an increase in area occupied by the nucleolar protein Fibrillarin, which had effects in nucleolar activity. Exposure to the compounds, and predominantly to BETi, improved these phenotypes (**fig. 2**). Moreover, other phenotypic changes induced by PR_{20} reported by Kwon and colleagues, were ameliorated in the presence of the compounds.

We then conducted some *in vivo* experiments in zebrafish embryos. First, we explored the effects of exogenously adding PR_{20} to the water of developing zebrafish embryos. These experiments recapitulated some of the phenotypes seen for cells in culture. In zebrafish tissue sections, we found PR_{20} in the nuclei of cells, predominantly in nucleoli, leading to nucleolar disfunction and a decrease in protein synthesis, marked by lower OPP levels. Addition of PR_{20} killed zebrafish embryos, and treatment with PFI-1 and Na-Phen improved viability in this model.

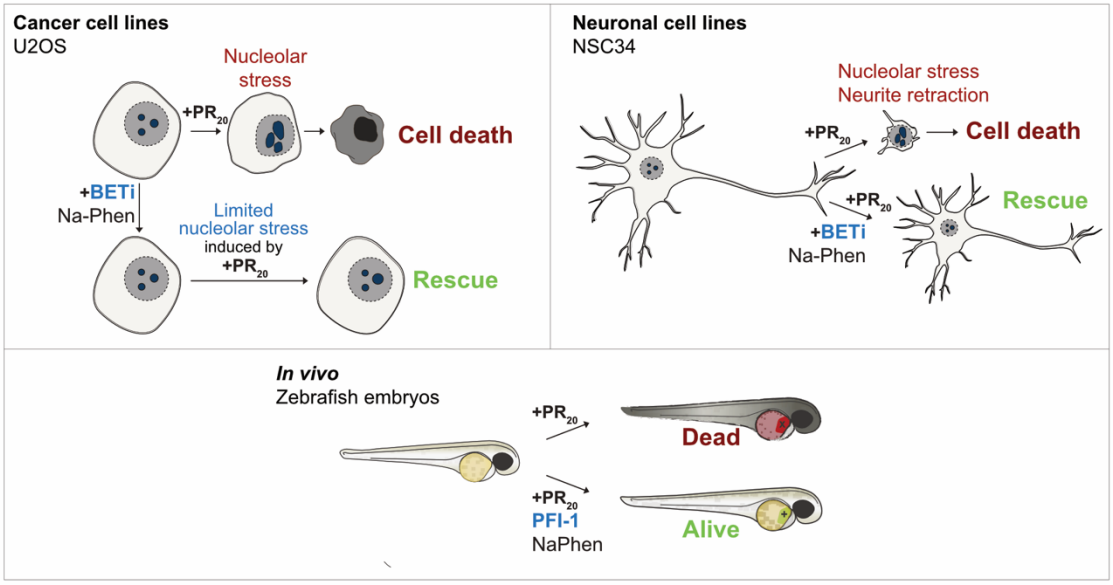


Figure 2. *Schematic overview of PR_{20} induced phenotypes rescued by hit compounds in cancer (U2OS) and neuronal cell lines (NSC-34) and in vivo in zebrafish embryos. Phenotypes more associated to BETi appear in blue.*

Our results suggested that the hits contributed to phenotypes downstream the nucleolus, such as protein synthesis, and, ultimately, in viability. Next, we evaluated if the mechanism of action of these compounds in improving ALS-related phenotypes was due to preserving nucleolar integrity. For that, we challenged cells with the known nucleolar stressor Actinomycin D (ActD). Nucleolar alterations induced by PR_{20} and ActD are quite different, while PR_{20} increases the area occupied by Fibrillarin, ActD triggers nucleolar segregation, reducing this area (**fig. 3A**). Nevertheless, treatment with BETi, and not with Na-Phen, alleviated nucleolar stress induced by both PR_{20} and ActD (**fig. 3B**). Furthermore, we found that PFI-I prevented alterations in the distribution and levels in other 9 nucleolar proteins induced by PR_{20}. Additionally, JQ-1, a clinically approved BETi, also ameliorated PR_{20} -related phenotypes and protected from nucleolar stress induced by ActD. Altogether, our results support that inhibition of BET Bromodomain proteins provides a strategy to alleviate the consequences of nucleolar stress, including those triggered by ALS-related DPRs.

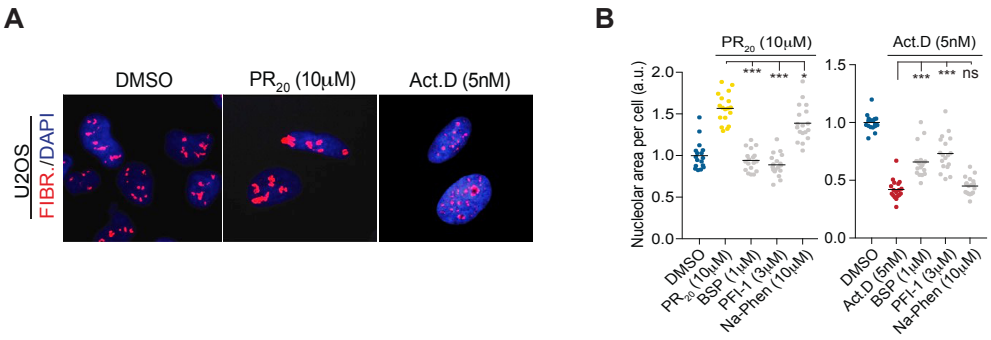


Figure 3. *Figure from **paper II**, showing different induction of nucleolar stress induced by PR_{20} and ActD in U2OS cells (A), and how predominantly BETi, BSP and PFI-1, rescued these phenotypes (B).*

4.2.2 **Discussion and future perspectives**

The presence of G_4C_2 hexanucleotide repeat expansions (HRE) in the first intron of the C9ORF72 gene is the most frequent cause of ALS/FTD, hence a better understanding of their mechanism of toxicity could help in developing new therapies for these diseases. Translation of these HRE in toxic dipeptide-repeat polypeptides (DPRs), poly-PR and poly-GR, plays an important role in pathology of *C9ORF72-HRE* patients. These peptides accumulate in the nucleoli and kill cells. Different therapeutic strategies could be used to stop the cellular effects triggered by these peptides.

Some approaches focused on preventing the expression of DPRs. For instance, via identification of factors involved in the transcription of mutant C9ORF72 genes, such as SUPT4H1 [305]. In this line, anti-sense oligonucleotides (ASOs) targeting G_4C_2 repeats have been effective [306], and some leads are progressing on clinical trials phases I/II [307]. Anti-sense oligonucleotides ASOs are single-stranded sequences of synthetic oligonucleotides that are designed to complement target mRNAs for RNase H enzyme-mediated target degradation or against primary transcripts inducing alternative splicing. ASOs have proven to be safe and stable in humans, and some of them are already FDA-approved therapies [308]. This strategy has been extended to targeting other ALS-associated genes, and it has been explored for other neurodegenerative diseases, particularly related to repeat-extensions [307]. Unfortunately, ALS-SOD1 ASOs recently failed on clinical trial phase III, yet the rest are still on the roll [307]. Similarly, adenovirus-mediated gene silencing using RNA interference and CRISPR technologies have been advancing in clinical trials for preventing C9ORF72 expression [309]. Recently, a new small molecule has been developed to bind to the G_4C_2 stretches and recruit a nuclease to the repeats, eliminating these repeat-bearing stretches from C9ORF72 mRNAs. This strategy has worked *in vitro* and in mouse models, ameliorating ALS-pathology [310]. Other methods have been based on small molecules able to affect the stability of mRNA secondary structure emerging from C9ORF72-HRE repeats. Such as a molecule binding to RNA G-quadruplex structures on HRE C9ORF72 repeats, which reduced the expression of DPRs in motor neurons derived from patients and improved viability in fly models [311]. Furthermore, some efforts have identified compounds that inhibit or regulate RAN translation [312-314].

Other strategies aimed to reduce the toxicity of already expressed DPRs. Genetic screens in different model systems have identified interesting candidates affecting or involved in the cellular effects of these peptides. These studies helped in understanding that the toxicity of DPRs is associated to processes involving nucleocytoplasmic transport, RNA splicing, or RNA non-mediated decay, among others [315-320]. Nevertheless, while promising, translation of these findings into actual treatments is not trivial [321].

Our work here capitalizes on the use of non-targeted phenotypic screens to identify compounds with potential usefulness in the context of C9ORF72-ALS/FTD pathology. Our screen identified BET bromodomain inhibitors and the HDAC inhibitor Na-Phen to reduce toxicity of PR_{20}. Interestingly, Na-Phen is currently investigated in clinical trials for ALS (https://www.clinicaltrials.gov/ct2/show/NCT03127514). However, in our hands, the effects

of Na-Phen seemed the result of general improvement of cell fitness, yet it did not substantially rescue nucleolar alterations triggered by PR_{20}. Whereas BETi improved viability and nucleolar integrity not only in cells exposed to PR_{20}, but to ActD. While we do not understand yet how BETi protect the nucleolus from stress, nucleolar activity is regulated by the interaction between bromodomains and acetylated histones [322]. Furthermore, the potential of BETi has not been explored for ALS/FTD. Interestingly, a CRISPR screen identified that deletion of bromodomain containing proteins BRD1 and BRD2 conferred resistance to PR_{20} in human leukemia K562 cells [315].

Our results support a role of nucleolar stress in neurodegeneration, which has started to be considered as a potential therapeutic target for ALS, Huntington's, Alzheimer's and Parkinson's diseases [107,111]. Additional interventions improving nucleolar stress have shown to be beneficial for these disorders, such as the small molecule BIND, which interferes with sequestering of nucleolar proteins by mRNA repeat stretches, rescuing motor function and viability in ALS and Huntington's disease in flies. While other strategies have been focused on stimulation of nucleolar activity, discussed in ***Annex I.*** Additionally, genetic interventions on elements of the nucleolar stress response, such as partial inhibition of p53 [323] and overexpression of NPM1 [324], have been reported to ameliorate cell toxicity in ALS models. Moreover, genetic targeting of processes downstream ribosome biogenesis, such as mRNA translation, have shown beneficial effects in *in vivo* models of ALS [284,325].

Nevertheless, now we know more about the functions of DPRs in cells and their mechanism of toxicity [326]. Poly-PR and poly-GR affect RNA metabolism [327] because they interfere with any process in the cell involving DNA or RNA [126]. While the authors strengthen that this is a model on how DPRs work, these results could open new therapeutic venues for modulators of toxicity of ALS.

Additionally, given the need of finding a cure for ALS, during this thesis we conducted a high throughput screen of 100K uncharacterized drugs exposing cells to PR_{20}. Due to variability related to synthetic peptides, we could not validate our results. Nevertheless, this task has been resumed by colleagues thanks to more robust genetic models recently available.

To summarize, in this work we identified compounds able to limit toxicity by ALS-PR_{20} added exogenously to cells and zebrafish embryos. If our findings can be validated in other models of ALS or neurodegeneration remains to be explored. Remarkably, our study is the first to identify compounds able to prevent nucleolar stress, which could benefit conditions where the nucleolus is compromised, setting new possibilities in discovering new drugs and understanding novel nucleolar functions. These results have led us to screen for compounds able to protect from nucleolar stress described in ***preliminary results IV***.

"When you're curious, you find lots of interesting things to do."

- *Walt Disney*

5 PRELIMINARY RESULTS

5.1 PRELIMINARY RESULTS I: Identification of novel modulators of mRNA translation using non-characterized compound libraries

5.1.1 Introduction

After our first screen for modulators of translation [328], we sought to discover new potentially interesting translation regulators among compounds from non-characterized libraries. As a proof of concept, and due to the throughput of the assay, we screened the natural compounds library (4,038 compounds) available from CBCS, which is of similar size as the medically approved library used in **paper I**. Natural products are chemicals or substances produced by living organisms or found in nature with some biological or pharmaceutical activity. They have been used in medicine from the early days, and, in fact, around a 40% of all medicines are either natural products or derivatives, including Aspirin, Digotoxin or Penicillin [214,329]. Natural compounds are often good chemical leads for further drug development because their biology, target engagement and mechanism of action have been optimized by evolution [214]. A variety of natural compounds have been described in the literature to modulate eukaryotic translation and interact with components of the translation machinery, including ribosomes [330]. In fact, Rapamycin, the namesake of mTORC1, is a natural compound that was purified from soil bacteria from the island of Rapa Nui in 1972 [169]. In this work, we have conducted a chemical screen to evaluate the potential of natural products in regulating global mRNA translation.

5.1.2 A chemical screen for natural compounds regulating mRNA translation

The screen was done in triplicate exposing U2OS cells with a single concentration of compound (10 μM) for 24h, followed by OPP labelling and staining (**fig. 1A**). The libraries used were Timtec (3,040 compounds) and Analyticon (998). Since we did not have any information regarding the target or mechanism of action of these compounds, we applied very stringent selection criteria for hit calling: (a) for up-regulators, an increase in OPP intensity greater than 3 standard deviations over the average signal of DMSO controls (115%); (b) for down-regulators, which are easier to identify, a decrease in OPP intensity greater than a 25% (approximately 5 standard deviations) below the average signal for DMSO control (below 75% OPP signal); (c) an effect in cellular viability not greater than a 30%; (d) and, last, that the coefficient of variation (%CV) between triplicate plates was lower than a 20% for both OPP intensity and nuclear counts. Regarding quality control, for plate 7, Torin 2 and CHX failed lowering OPP signal (**fig. 1B**), which is apparent when looking at the Z' (Z-prime) statistic, used to measure assay quality, which indicated that the positive and negative controls were not well separated this plate for neither of the controls (**fig. 1C**). Hence, we discarded any potential up-regulators from this plate. Nevertheless, in plate 7, three compounds consistently decreased OPP intensity, when the controls did not. So, to not prematurely discard some potent down-regulators of protein synthesis, these three compounds were included in the validation. As well, we re-introduced to our list of hits a compound in the top 10 of up-regulators of OPP signal, which was initially discarded due to a variation coefficient, but that was borderline to the

established 20% (21.2%). In total, the screen yielded potential 74 up-regulators and 28 down-regulators of translation (**fig. 1D**). Controls Torin 2 and CHX were amongst the compounds lowering OPP signal, with no compound equally effective as CHX.

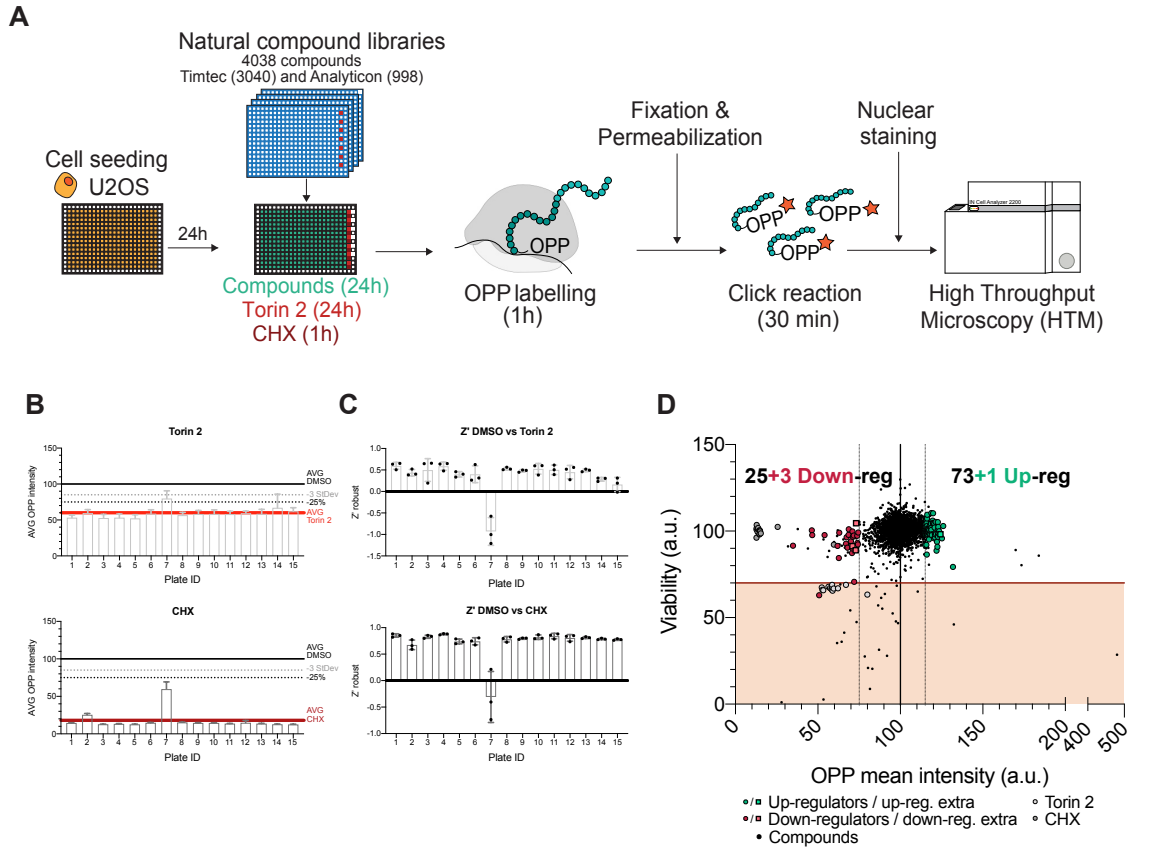


Figure 1. A chemical screen for natural compounds modulating protein synthesis. *(A) Schematic overview of the phenotypic screen workflow. U2OS cells were plated in 384-well plates. Next day, cells were exposed to compounds at 10 µM or 0.5 µM of Torin 2 as a control in specific wells. After 23 h, CHX (100 µg/mL) was added for an hour as an additional control. Then, cells were pulsed with OPP for an hour, after which they were fixed and processed for HTM-dependent quantification of the OPP signal and nuclei counts. (B) Average OPP mean intensity and (C) CV% of controls Torin 2 and CHX across the plates in the screen. (D) Compound distribution from the screen, based on OPP mean intensity and nuclei count. Compounds increasing (up-regulators, GREEN) or decreasing (down-regulators, ORANGE) OPP incorporation over 3 standard deviations compared to the DMSO control (DMSO) are shown. Extra modulators (extra RED and GREEN) included for validation due to their potency. Compounds exceeding 30% toxicity are shown under the ORANGE shade.*

5.1.3 Validation of the screen using OPP and HPG labelling

Next, we validated the hits exposing U2OS cells for 24h to compounds at three concentrations (1, 3, 10 µM), using OPP and HPG labelling. The same criteria as in the primary screen was used for hit-calling compounds modulating OPP signal, only that for nuclei count a CV under 25% was considered. Three down-regulators and one up-regulator were classified as hits based on changes in OPP intensity. HPG labelling showed more variation than OPP (**fig. 2A**), hence hits for HPG were defined by an increase or decrease of intensity greater than a 40%,

approximately over one standard deviation, compared to the average of DMSO controls (**fig. 2B**). For HPG, eight up-regulators and nine down-regulators were identified. The greater number of hits for HPG was probably due to the high variability of the assay. Both OPP and HPG labelling identified three down-regulators of translation, which seemed interesting for follow-up characterization (**fig. 2C**).

However, the number for potential HPG up-regulators showing a dose-response behavior, made us look at the OPP validation, and define a new category of compounds mildly increasing OPP signal (over 110%). Considering the results for both OPP and HPG validation screens, four compounds were annotated as mild up-regulators of protein synthesis (**fig, 2D, E**). While is unlikely to increase translation using cancer cells grown in complete media, as discussed before, the role of these compounds could be explored exposing cells for shorter times, and additionally, on cells that have been grown under starvation conditions.

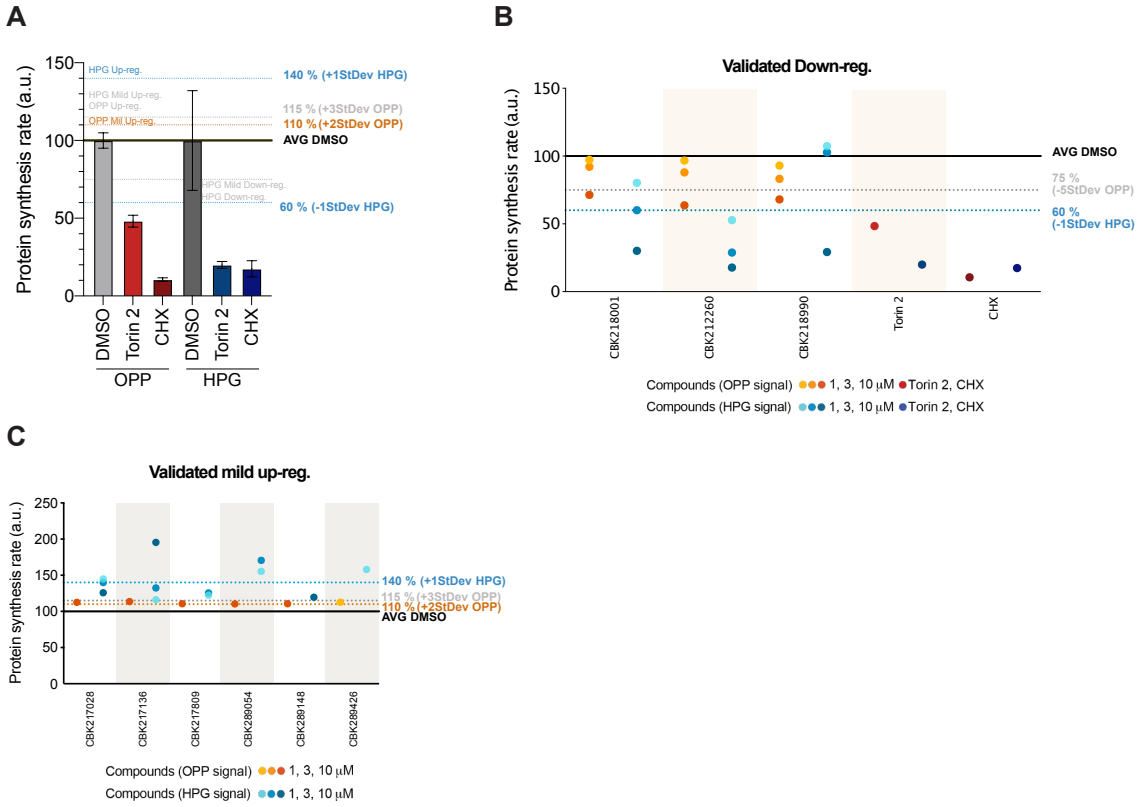


Figure 2. Validation of the natural compounds screen for mRNA translation using OPP and HPG labelling. *(A) Average (AVG) measurements for OPP and HPG mean intensities for controls DMSO, Torin 2 and CHX in the validation screen. Threshold strategies to define up- and down-regulators of translation are defined by modulating intensity of OPP and HPG over shown standard deviations (StDev) (B) Compounds that were validated as down-regulators of protein synthesis in a dose-dependent manner (1, 3, 10 µM). (C) Compounds classified as mild up-regulators.*

5.1.4 Next steps

Our next experiments will focus on validating if these compounds downregulate translation after short exposure, and on assessing whether this regulation occurs via mTORC1, ISR, ribosome biogenesis, or other pathways.

5.2 PRELIMINARY RESULTS II: A virtual screen for modulators of nucleolar activity using publicly available images

5.2.1 Introduction

The nucleolus has emerged as an interesting target for treating or improving diverse disorders, including metabolic and neurodegenerative diseases, and this strategy has been widely exploited in cancer [95]. However, while there is a considerable number of drugs available affecting nucleolar integrity, it is often the case that these compounds have pleiotropic activities besides modulation of RNA pol I, including genotoxicity [191]. Remarkably, most of the current knowledge about nucleolar biology and the techniques to interrogate it have progressed hand-in-hand with the development and characterization of drugs altering the nucleolus. However, it currently resonates in the field a need for more specific inhibitors of RNA pol I activity, or, at least, drugs showing better toxicity profiles and less off-target effects [191]. In our review under preparation, ***annex I***, we have summarized some already in use and new technologies for the identification of modulators of nucleolar biology. In the next section is described our attempt of discovering drugs regulating the nucleolus benefiting from public image repositories using our own image-analysis pipelines. We used images from a Cell painting experiment in U2OS cells exposed to compounds mostly from characterized libraries. Cell painting has revolutionized drug discovery allowing to investigate and group drugs based on their mechanism of action reflected in the morphological changes (features) induced by these compounds in different cellular compartments [228]. Here, instead of interrogating a wide range of phenotypes, we just searched for compounds able to modulate nucleolar area, which is a common readout for assessing nucleolar fitness.

5.2.2 A virtual screen for modulators of RNA pol I activity

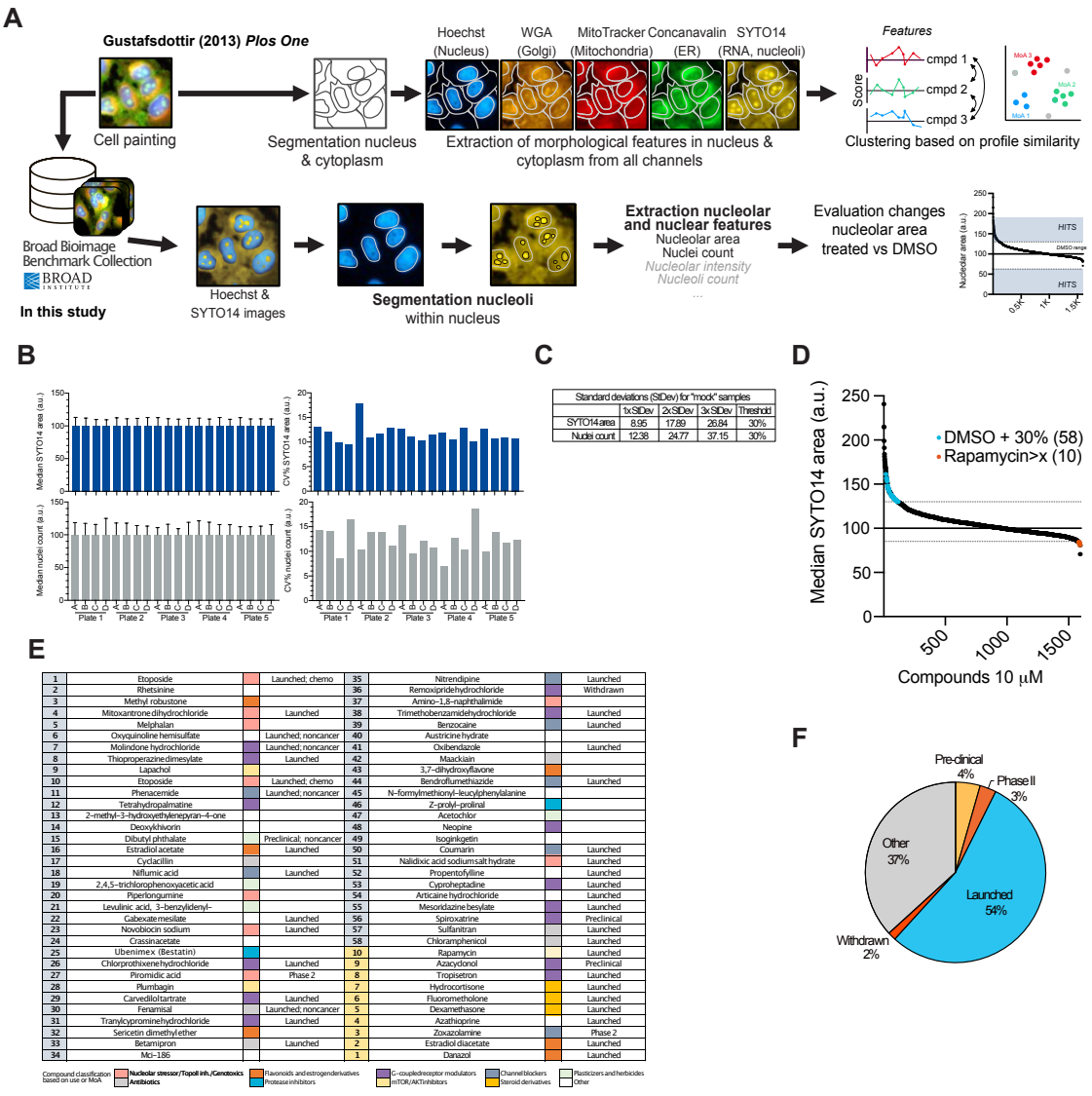
Here we used the image-based dataset "Human U2OS cells – compound-profiling Cell Painting experiment" (BBBC022) from the Broad Bioimage Benchmark Collection [261](https://bbbc.broadinstitute.org/BBBC022/). In this screen, U2OS cells were exposed to 1,600 compounds at a single concentration of 10 μM for 48h [231]. Most of the drugs used for this screen are included in the current Drug Repurposing Hub library from the Broad Institute [1] or are commercially available. The library range was covered with five 384-well plates, due to the presence of controls, and the screen was done with four technical replicates for each plate (A-D). After treatment, cells were processed using the Cell painting protocol [230]. Briefly, mitochondria and Golgi apparatus were live stained with MitoTracker and Wheat Germ Agglutinin (WGA), respectively. Then cells were fixed in 4% PFA, and permeabilized, and nuclei, nucleoli, cytoskeleton and ER were stained with Hoechst, SYTO14, Phalloidin and Concanavalin A, in this order.

We used the set of images showing nucleoli (SYTO14) and nuclei (Hoechst) to segment nucleoli using a self-made Cellprofiler pipeline. For each well there were nine images taken at 20X magnification. Of note, SYTO14 in a fluorescent nucleic acid stain, however, since rRNA is the most abundant nucleic acid in the cell, SYTO14 signal is concentrated in the nucleolus.

Importantly, changes in SYTO14 signal can be read as changes in RNA pol I activity. In the original study, they considered changes in all the channels corresponding to the organelles labelled within the delimited areas established by segmenting nuclei and cytoplasm. They extracted 824 features (signal distribution, granularity, intensity, etc.) that allowed them to cluster compounds according to similarity, integrating data from all the organelles. Here, we just examined changes in area of a specific cellular structure, to concretely interrogate modulation of the nucleolus (**fig. 1A**). For instance, in the previous work, compounds with fused nucleoli were separated into two clusters, while our analysis could identify them as a single class of compounds. Different questions can benefit from different readouts starting from the same group of images.

First, we tested the segmentation of our pipeline measuring nuclei count and nucleolar area in cells exposed to DMSO control, here annotated as "mock". Statistical analysis was done using KNIME. Below are the median values of nucleolar area and nuclei count, and their associated coefficient of variation for each plate (**fig. 1B**). For hit calling, first we considered as hits compounds able to produce a change in nucleolar area greater than three standard deviations compared to the mock control, this was approximately a 30% modulation **(fig. 1C**). Additionally, we discarded drugs reducing viability up to a 70%. We were not stringent in this aspect since cells were exposed for over 48h, and among our best hits, such as Etoposide, a known inducer of nucleolar stress, we found that this level of toxicity was not masking nucleolar effects caused by the compounds. Since for validation we would test lower concentrations and exposure times, we kept these drugs and just flagged them. Also, we set as a criterion that each compound should have come as a hit in at least three out of four replicates. Our first round of analysis identified 58 compounds increasing nucleolar area over a 30% (**fig. 1D**). Interestingly, among these drugs there were known nucleolar stressors, such as topoisomerase I and II (TOPO I/II) inhibitors and other genotoxic drugs (**fig. 1E**). Inspecting the data, we found that Rapamycin was mildly decreasing the area occupied by SYTO14, and that there were other drugs that were reducing it beyond it. Due to the success of Rapamycin in extending lifespan in diverse cellular and animal models of aging and ribosomopathies [27], and the growing interest in drugs decreasing rRNA synthesis, we included these nine compounds and rapamycin in our validation screen. Of note, most compounds identified in the primary screen are already used in the clinic for different purposes (**fig. 1F**).

Interestingly, among most of our hits we identified compounds known to induce nucleolar stress, such as TOPO II inhibitors [187]. Additionally, as described in the original paper using this dataset [231], our approach identified channel blockers and G-protein receptor modulators inducing nucleolar changes. Moreover, among the compounds classified as mTOR/AKT inhibitors are two naphtoquinones, which are chemically related to Chloroquine and Amodiaquine, and which are known to induce nucleolar stress [200]. Altogether, these results support that our approach can identify compounds altering nucleoli.



1	Etoposide		Launched; chemo	35	Nitrendipine		Launched
2	Rhetsinine			36	Remoxipridehydrochloride		Withdrawn
3	Methyl robustone			37	Amino–1,8–naphthalimide		
4	Mitoxantrone dihydrochloride		Launched	38	Trimethobenzamidehydrochloride		Launched
5	Melphalan			39	Benzocaine		Launched
6	Oxyquinoline hemisulfate		Launched; noncancer	40	Austricine hydrate		
7	Molindone hydrochloride		Launched; noncancer	41	Oxibendazole		Launched
8	Thioproperazine dimesylate		Launched	42	Maackiain		
9	Lapachol			43	3,7–dihydroxyflavone		
10	Etoposide		Launched; chemo	44	Bendroflumethiazide		Launched
11	Phenacemide		Launched; noncancer	45	N–formylmethionyl–leucylphenylalanine		
12	Tetrahydropalmatine			46	Z–prolyl–prolinal		
13	2–methyl–3–hydroxyethylenepyran–4–one			47	Acetochlor		
14	Deoxykhivorin			48	Neopine		
15	Dibutyl phthalate		Preclinical; noncancer	49	Isoginkgetin		
16	Estradiol acetate		Launched	50	Coumarin		Launched
17	Cyclacillin			51	Nalidixic acid sodium salt hydrate		Launched
18	Niflumic acid		Launched	52	Propentofylline		Launched
19	2,4,5–trichlorophenoxyacetic acid			53	Cyproheptadine		Launched
20	Piperlongumine			54	Anticaine hydrochloride		Launched
21	Levulinic acid, 3–benzylidenyl–			55	Mesoridazine besylate		Launched
22	Gabexate mesilate		Launched	56	Spiroxatrine		Preclinical
23	Novobiocin sodium		Launched	57	Sulfanitran		Launched
24	Crassinacetate			58	Chloramphenicol		Launched
25	Ubenimex (Bestatin)			10	Rapamycin		Launched
26	Chlorprothixene hydrochloride		Launched	9	Azacyclonol		Preclinical
27	Piromidic acid		Phase 2	8	Tropisetron		Launched
28	Plumbagin			7	Hydrocortisone		Launched
29	Carvedilol tartrate		Launched	6	Fluorometholone		Launched
30	Fenamisal		Launched; noncancer	5	Dexamethasone		Launched
31	Tranylcypromine hydrochloride		Launched	4	Azathioprine		Launched
32	Sericetin dimethylether			3	Zoxazolamine		Phase 2
33	Betamipron		Launched	2	Estradiol diacetate		Launched
34	Mci–186			1	Danazol		Launched

Compound classification based on use or MoA: Nucleolar stressor/Topoll inh./Genotoxics; Antibiotics; Flavonoids and estrogen derivatives; Protease inhibitors; G–coupled receptor modulators; mTOR/AKT inhibitors; Channel blockers; Steroid derivatives; Plasticizers and herbicides; Other

Figure 1. A virtual screen to identify modulators of ribosome biogenesis*. (A) Scheme showing the approach used originally to cluster compounds based on multiple features measured in nucleus and cytoplasm from a Cell painting experiment [231] compared to the approach used in this work, where only the channel for the nucleolus (SYTO14) and the nucleus (Hoechst) were considered, and the analysis focused on changes in area of nucleoli segmented with a self-made pipeline. (B) Median and coefficient of variation (%CV) of SYTO14 area and nuclei count among cells treated with DMSO control ("mock") across the screen. (C) Average of the median and CV% values of the mock samples in the screen and establishment of thresholding parameters considering the standard deviation (StDev) of these measurements within mock samples. (D) Distribution of the hits of the screen. 58 compounds (BLUE) increased the area occupied by SYTO14 over a 30% compared to the values for mock samples without having an effect in cell viability, considered by a reduction of nuclei count greater than a 30%. 10 compounds reduced SYTO14 area more than Rapamycin (ORANGE). (E, F) Classification of the hits based on their mechanism of action and their development or clinical status.*

5.2.3 Validation of the hits based on SYTO14 staining and fibrillarin area

For the validation screen, we selected 45 compounds, since some of the hits were not available in the CBCS library, and we also reduced overrepresentation of known inducers of nucleolar

stress. For this secondary screen, we decided to expose cells for 48h and 4h to compounds, to replicate the original screen conditions and to identify direct modulators of nucleolar stress exposing cells for a shorter time to compounds, since changes in nucleolar dynamics occur rapidly [77] (**fig. 2A**). Additionally, besides monitoring the abundance of rRNA with SYTO14, here we marked the nucleolus with Fibrillarin. Our idea was to test whether we could distinguish compounds: (a) affecting both rRNA synthesis (SYTO14) and nucleolar structure (Fibrillarin), (b) altering rRNA synthesis without significantly modifying nucleolar structure, (c) with no significant effect in rRNA synthesis yet altering the area occupied by Fibrillarin. The validation screen was done seeding U2OS cells in 384-well plates, the next day exposing them to three compound concentrations (1, 5, 10 μM) for the 48h set; and, treating the 4h plates, four hours before fixing both 4h and 48h sets of plates. Additionally, we included several controls, including known nucleolar stressors ActD and BMH-21, at doses known to trigger nucleolar segregation after 4h treatment, and Rapamycin, which was identified in the screen, worked as an additional internal control (**fig. 2B**). After fixation with 4% PFA, permeabilization with 0.1% TritonX100, and subsequent blocking with 3% BSA in 0.01% PBS-Tween20, plates were incubated with anti-fibrillarin antibody (1:2000, ab5821 Abcam) overnight. The next day, plates were incubated for one hour with secondary antibody and Hoechst, which was followed with addition of SYTO14 (1:2000, S7576, Thermofisher) for 15 minutes. Next, plates were imaged using InCell Analyzer. The validation screen was done in triplicates for each time point.

After validation, we identified several compounds that changed the area occupied by SYTO14 and Fibrillarin, or solely Fibrillarin (**fig. 2C, D**), and which were not highly toxic. For both, parameters hits were compounds changing nucleolar above three standard deviations from the DMSO control. Hits for SYTO14 had an increase in area greater or lower than 110% and 90%; while for Fibrillarin it was 130% and 70%, respectively, except for at 48h where the lower threshold was set to 80%. Interestingly, it seems that Fibrillarin is a more sensitive or faster marker of nucleolar disruption than changes in rRNA. Seven compounds, including Rapamycin, were validated; five of them with an effect in the area occupied by both SYTO14 and Fibrillarin, and two of them, Trimethobenzamide hydrochloride and Fluorometholone, exclusively affected Fibrillarin. Surprisingly, Fluorometholone did not reduce SYTO14 area as it happened when analyzing images of the primary screen. Similarly, here none of the compounds increased the area occupied by SYTO14, instead reduced it. These discrepancies could be due to the quality of the images themselves, for instance, for the validation we used 2D-deconvolution in the Fibrillarin channel to improve the definition of the nucleolar structures, and the images from the Broad Benchmark were not treated the same way. Also, for the validation we modified the parameters used to segment nucleoli using controls ActD, BMH-21, and Rapamycin as an additional reference to DMSO. Furthermore, differences in purity of the compounds from the library used in the primary and the validation screens can play a part in these results. Nevertheless, our virtual screen has identified potential modulators of the nucleolus.

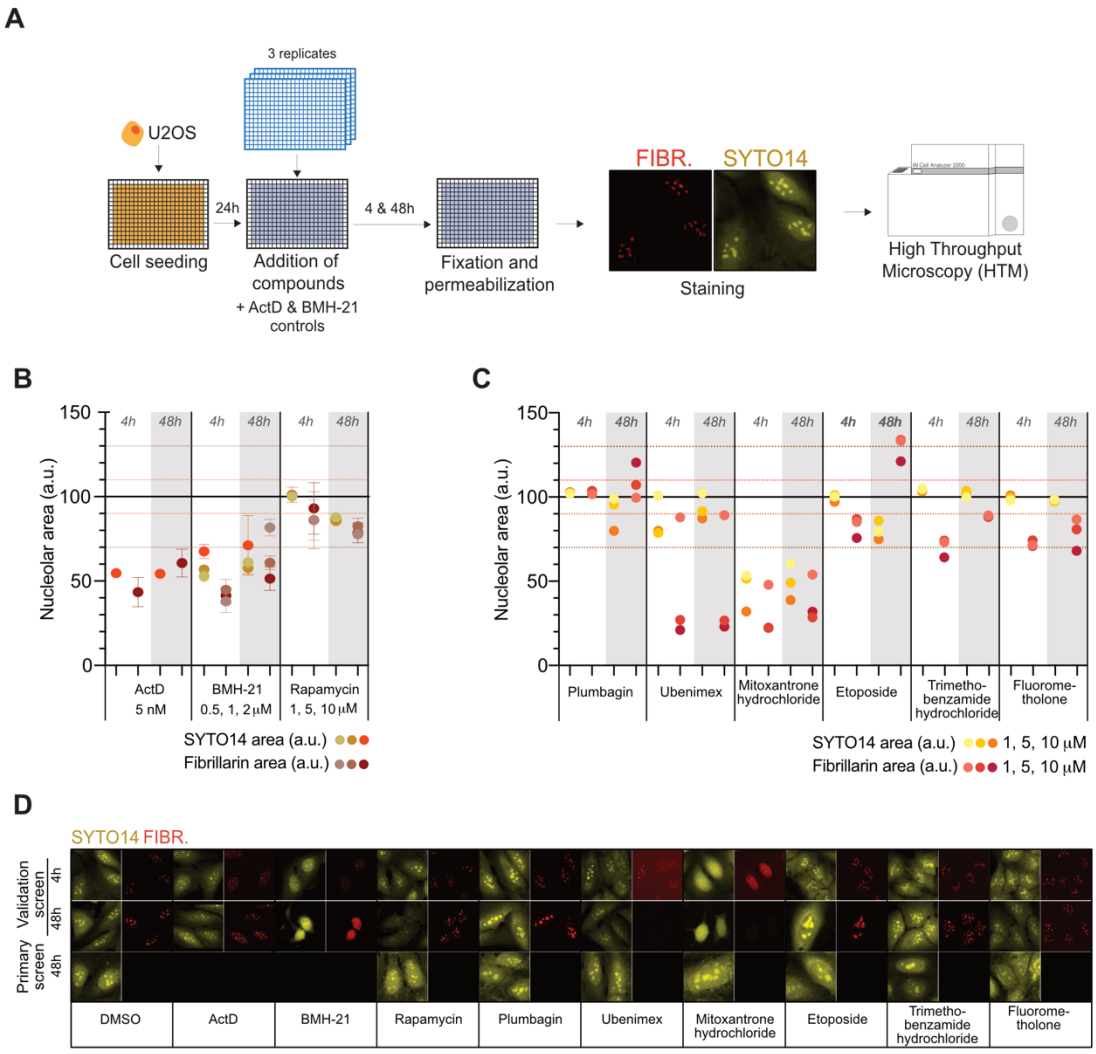


Figure 2. Validation of the virtual screen. *(A) Assay set up for the validation screen in U2OS exposed to three concentrations of compounds (1, 5, 10 µM) for 4 and 48h. Controls ActD (5nM) and BMH-21 (0.5, 1, 2 µM) were added to the screen. Validation readout was based on SYTO14 (yellow) and Fibrillarin (FIBR., red) staining. Changes in nucleolar area marked by SYTO14 and Fibrillarin staining in cells exposed to controls (B) and validated hits (C) at indicated concentrations. (D) Representative images of controls DMSO, ActD (5 nM), BMH-21 (2 µM), Rapamycin and validated hits (10 µM) from the validation and the primary screens. In the primary screen section, there are black squares for conditions for which we do not have images for, such as Fibrillarin staining and the controls ActD and BMH-21.*

5.2.4 Next steps

Next, we will focus on the characterization of Plumbagin, Ubenimex (Bestatin), Trimethobenzamide hydrochloride and Fluorometholone, given that none of them have been described as modifiers of nucleolar structure or function. For this characterization we will follow some of the guidelines included in **annex I**, such as assessing the effect of the compounds on different nucleolar markers, modulation of ribosome biogenesis, activation of the nucleolar stress response, and further functional characterization.

5.3 PRELIMINARY RESULTS III: Identification of cancer vulnerabilities to nucleolar stress using a genome-wide CRISPR/Cas9 screen

5.3.1 Introduction

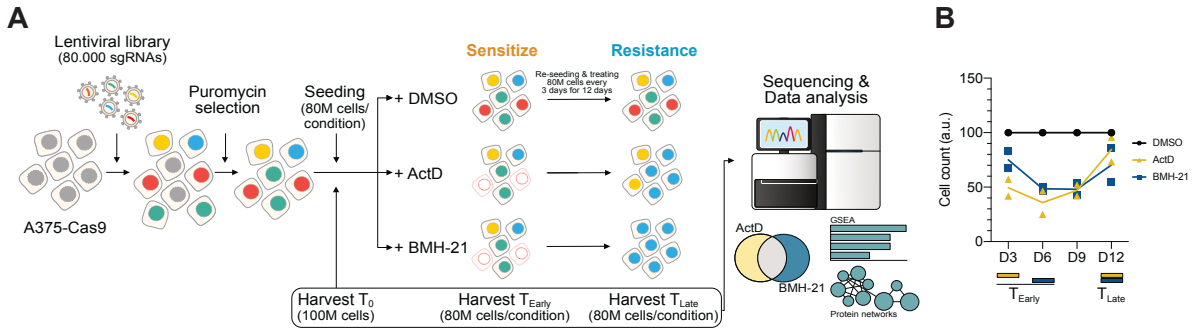
Nucleolar stressors have shown to be beneficial for treating different malignancies in the clinic as shown in **section 1.4.4**. Targeting the nucleolus offers better alternatives to more aggressive and non-selective traditional strategies to induce apoptosis by activating p53, such as with genotoxic stressors [77]. Additionally, cancer cells have shown to be selectively vulnerable to nucleolar stressors, both *in vitro* and *in vivo* [331]. Despite these interesting preclinical observations, nucleolar stress inducers used in the clinic are DNA intercalating agents, such as the antibiotic actinomycin D (ActD), so that it is unlikely that their effects rely solely on causing nucleolar stress [187]. A new generation of compounds classified as selective RNA Pol I inhibitors, CX-5461, CX-3543 or BMH-21 are progressing into the clinic [189,199,332]. However, recent studies using DNA repair-deficient cells revealed that some of these drugs are also genotoxic and that their activities are not RNA pol I-related [190,333], leaving BMH-21 as the most specific RNA pol I inhibitor available [334]. Despite of the emerging interest in this field, the range of toxicity exerted by these compounds is variable across cell lines, independently from the tumor type or p53 status, indicating that sensitivity is associated to specific molecular footprints that remain to be determined [335]. To gain a better understanding of the most suitable genetic contexts that would benefit from using drugs targeting the nucleolus, here we conducted a genome-wide CRISPR/Cas9 loss of function screen in A375 melanoma cells exposed to nucleolar stressors ActD and BMH-21. These two stressors have different activities associated. ActD inhibits RNA pol I and II, with higher specificity for the first at lower concentrations, and can trigger the DNA damage response. Whereas, BMH-21 does not activate the DNA damage response [334] and triggers degradation of RNA pol I [199]. Also, we considered these drugs due to their clinical status, being ActD used in chemotherapy and BMH-21 in preclinical studies.

5.3.2 A genome-wide screen to explore vulnerabilities to nucleolar stressors

Melanoma A375 cells stably expressing Cas9 were transduced in duplicate with the Brunello sgRNA library [254], which includes four sgRNAs per gen, covering 20,000 human genes (**fig. 1A**). Cells were selected for five days with Puromycin. Cell line and library preparation were done by the High throughput Genome Engineering (HTGE) Facility at Karolinska Institute. Next, 100M cells per replicate were harvested for measuring library coverage before exposing cells to compounds (T_0), and 80M cells were seeded per condition into T175 flasks (6M cells/flask). The next day, cells were exposed to either DMSO, ActD (0.75nM) or BMH-21 (0.2 μM) for three days. Then, cells were split, resuspended in new media containing compounds and 80M cells were seeded into new flasks, while 80M cells were harvested for sequencing. By harvesting 80M cells per condition a coverage of 1000 cells/sgRNA is expected. The same protocol was repeated every three days for 12 days in total. To enrich for genes sensitizing and giving resistance to compounds, samples at early (T_{Early}) and late (T_{Late}) time points were sequenced. According to cell count data during the screen, ActD killed cells

more rapidly than BMH-21, reaching to a decrease of about 50% of the population after 3 days treatment (**fig. 1B**). Hence, the early time point for ActD was 3 days and for BMH-21 6 days, while for both the latest time point used was 12 days.

Figure 1. *A genome-wide CRISPR/Cas9 loss of function screen to explore vulnerabilities to nucleolar stressors. (A) Scheme of the screen. Cells were exposed to ActD (0.75 nM) and BMH-21 (0.2 μM) for 12 days. (B) Changes in cell count induced by compound treatment during the screen (A).*



5.3.3 Analysis of potential genetic modulators of ActD and BMH-21 toxicity

RNA sequencing data was analyzed using MAGeCK algorithm, which considers statistical significance of each sgRNA, ranking genes according to consistent higher significance using robust rank aggregation (RRA). Additionally, RRA score applies pathway enrichment among the genes listed [256]. For each treatment effects were compared to the DMSO control samples, the genes were ranked according to their negative or positive contribution to the phenotype (cell viability), respectively acting as so-called sensitizers or providing resistance to the drugs. Hence, for each gene there is a negative and positive RRA rank and p-value associated. Since the screen was done in duplicate, paired analysis was applied to find top hits consistent between the two samples. Paired analysis considers the guides for each sample as independent, hence 8 sgRNAs per gene. Negative and positive p-values were collapsed into a single value per gene by calculating the -log10 of each p-value, subtracting one from each other, and calculating its absolute value.

Volcano plots representing the fold change and significance for each gene depict common and differential effects of gene-KOs in the different conditions just by looking at the top ranked genes (**fig. 2A**). Depletion of TP53 (encoding for p53 protein) consistently improved survival in cells exposed to ActD and BMH-21, as p53 cannot stop cells from cycling by inducing autophagy or apoptosis. In fact, resistance acquired due to TP53 loss is often used a quality control for viability screens [256]. Loss of ABCB1 multidrug resistant pump sensitized cells to ActD, which would be expected since ActD is a substrate of ABCB1, which lowers the intracellular concentration of the drug [336]. For all conditions, negative regulators of the mTORC1 pathway, such as PTEN, TSC1, and TSC2, made cells more vulnerable to both nucleolar stressors. To have a clearer view of the effects of these genes we compared the top 100 negatively and positively selected genes for each condition using an UpSet plot (**fig. 2B**). This analysis found common genes for ActD and BMH-21, as well as specific genes for each treatment, and others showing differential effects for these drugs. To narrow down the list, from

the top 100, we just considered the genes for which more than 5 sgRNAs out of 8 were detected. This criterion would increase the likeability of the hit to be highly ranked for both replicates. To this list, we added some of the common and differential hits from the UpSet plot, independently of the number of their sgRNAs, due to their general interest. Next, to investigate the hits we conducted protein network analysis using STRING, based on evidence from text-mining, experimental work, and databases; and GSEA analysis using the web-based tool webgestalt, against GO-cellular components and Reactome pathways (data not included). The information from these analyses was used to classify genes according to their molecular function or cellular components (**table 3**). Additionally, we added to this classification the phenotypic effects cause by depletion of these genes, such as sensitizing, conferring resistance or differential between ActD and BMH-21 (**tables 1** and **2**).

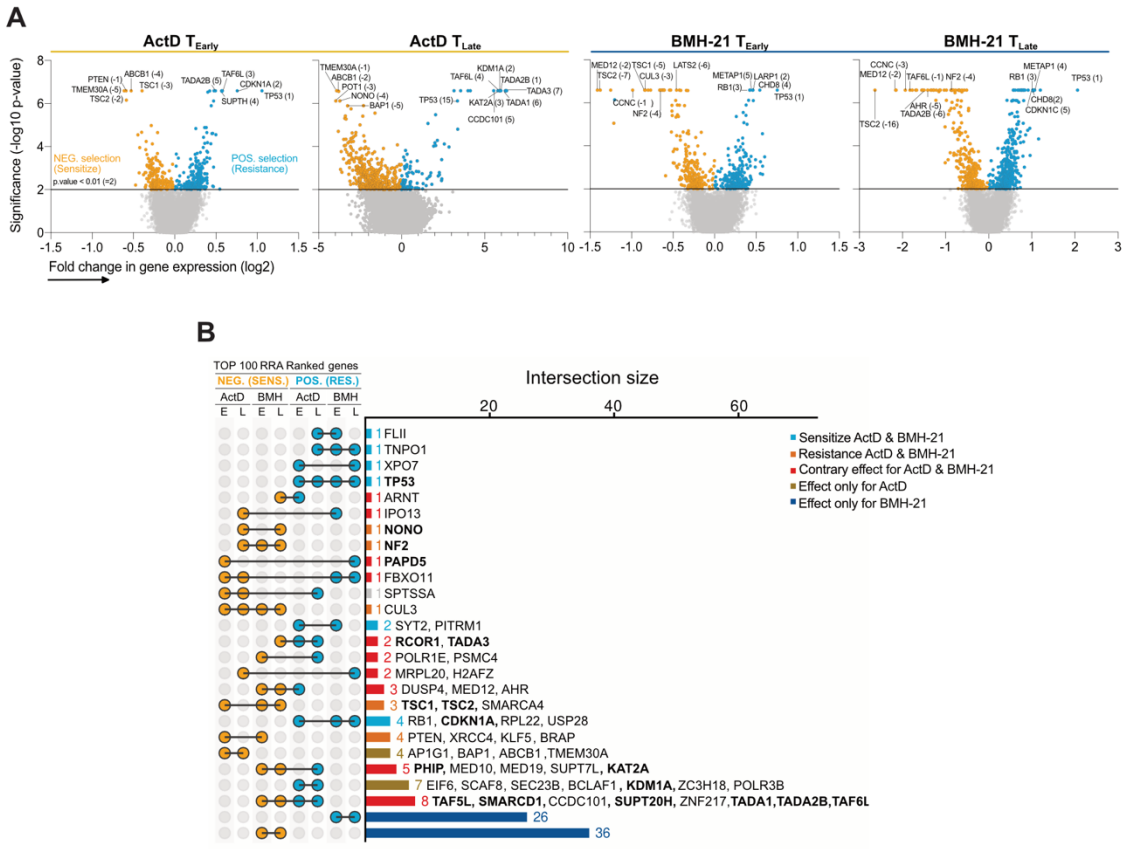


Figure 2. *Overview of RRA top ranked genes. (A) Volcano plots showing top 100 genes having a negative (orange) and positive (blue) effect in cells exposed to compounds and their significance. Black bar in -log10 p-value 2 (p-value < 0.01). (B) UpSet plot showing common and different genes in different conditions from top 100 list. In bold genes represented with more than 5 sgRNAs in the conditions depicted.*

TABLE 1

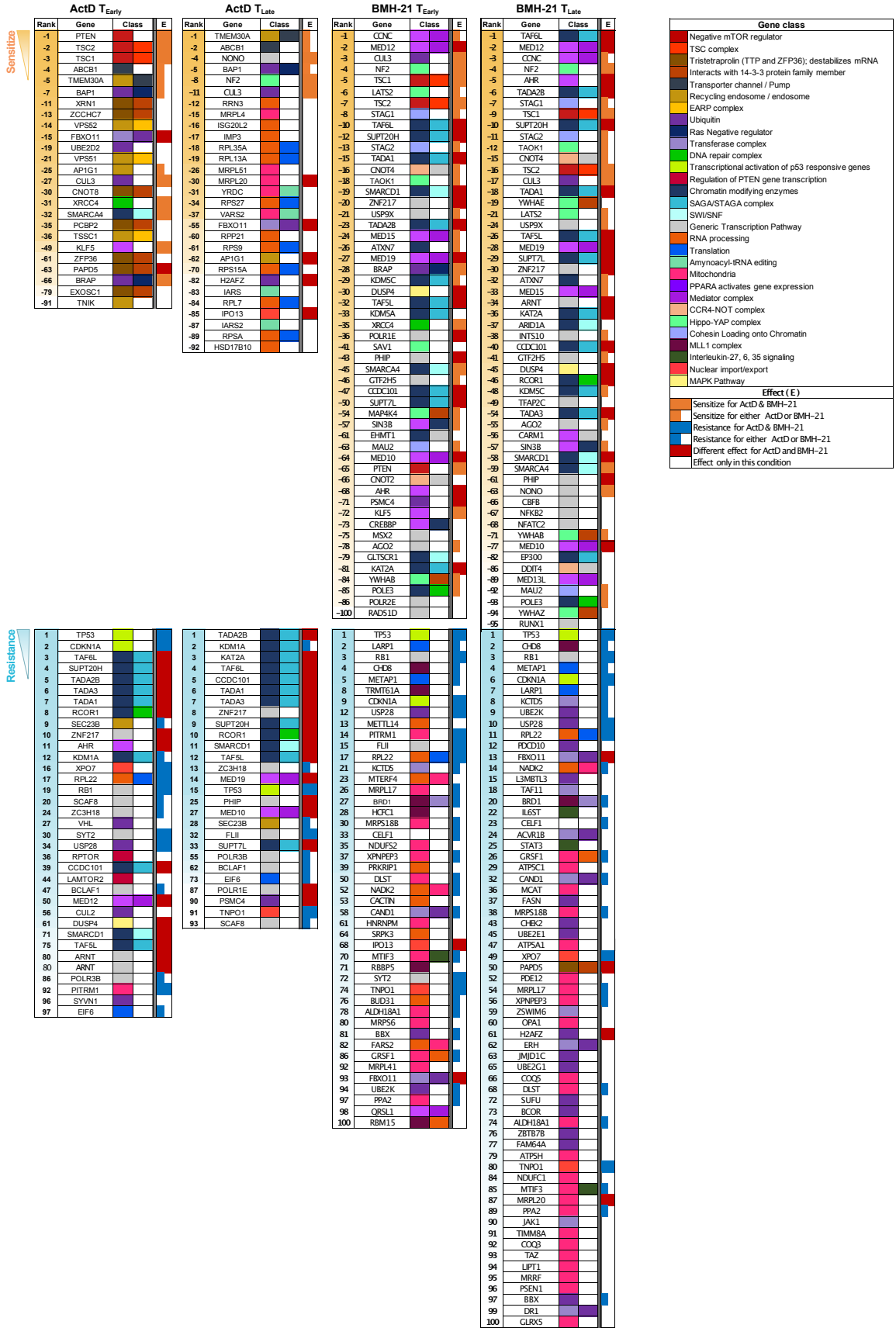


Table 1. *Table of hits from CRISPR/Cas9 screen, annotated gene class, and phenotypic effects in response to ActD and BMH-21.*

TABLE 2

Sensitize
Resistance

Gene	Effect		RRA Rank ActD T_{Early}	ActD T_{Late}	BMH-21 T_{Early}	BMH-21 T_{Late}	Gene class
	ActD	BMH					
FBXO11			-15	-55	93	13	
H2AFZ				-82		61	
IPO13				-85	68		
MRPL20				-30		87	
PAPD5			-63			50	
AHR			11		-68	-5	
ARNT			80			-34	
CCDC101			39	5	-47	-40	
DUSP4			61		-30	-45	
KAT2A				3	-81	-36	
MED10				27	-64	-77	
MED12			50		-2	-2	
MED19				14	-27	-28	
PHIP				25	-43	-61	
POLR1E				87	-36		
PSMC4				90	-71		
RCOR1			8	10		-46	
SMARCD1			71	11	-19	-58	
SUPT20H			4	9	-12	-10	
SUPT7L				33	-50	-29	
TADA1			7	6	-15	-18	
TADA2B			5	1	-23	-6	
TADA3			6	7		-54	
TAF5L			75	12	-32	-26	
TAF6L			3	4	-10	-1	
ZNF217			10	8	-20	-30	
CDKN1A			2		9	6	
FLII				32	15		
PITRM1			92		14		
RB1			19		3	3	
RPL22			17		17	11	
SYT2			30		72		
TNPO1				91	74	80	
TP53			1	15	1	1	
USP28			34		12	10	
XPO7			16			49	
BCLAF1			47	62			
EIF6			97	73			
KDM1A			12	2			
POLR3B			86	55			
SCAF8			20	93			
SEC23B			9	28			
ZC3H18			24	13			
ALDH18A1					78	74	
BBX					81	97	
BRD1					27	20	
BUD31					76		
CAND1					58	32	
CELF1					33	23	
CHD8					4	2	
DLST					50	68	
GRSF1					86	26	
KCTD5					21	8	
LARP1					2	7	
MRPL17					26	54	
MRPS18B					30	38	
MTIF3					70	85	
NADK2					52	14	
PPA2					97	89	
UBE2K					94	9	
XPNPEP3					37	56	
METAP1					5	4	
BRAP			-66		-28		
CUL3			-27	-11	-3	-17	
KLF5			-49		-72		
NF2				-8	-4	-4	
NONO				-4		-63	
PTEN			-1		-65		
SMARCA4			-32		-45	-59	
TSC1			-3		-5	-9	
TSC2			-2		-7	-16	
XRCC4			-31		-35		
ABCB1			-4	-2			
AP1G1			-25	-62			
BAP1			-7	-5			
TMEM30A			-5	-1			
AGO2					-78	-55	
ATXN7					-26	-32	
CCNC					-1	-3	
CNOT4					-16	-15	
GTF2H5					-46	-41	
INTS10						-38	
KDM5C					-29	-48	
LATS2					-6	-21	
MAP4K4					-54		
MAU2					-63	-92	
MED15					-24	-33	
POLE3					-85	-93	
SIN3B					-57	-57	
STAG1					-8	-7	
STAG2					-13	-11	
USP9X					-21	-24	
YWHAB					-84	-71	
TAOK1					-18	-12	

Gene class
Negative mTOR regulator
TSC complex
Tristetraprolin (TTP and ZFP36); destabilizes mRNA
Interacts with 14-3-3 protein family member
Transporter channel / Pump
Recycling endosome / endosome
Ubiquitin
Ras Negative regulator
Transferase complex
DNA repair complex
Transcriptional activation of p53 responsive genes
Chromatin modifying enzymes
SAGA/STAGA complex
SWI/SNF
Generic Transcription Pathway
RNA processing
Translation
Mitochondria
PPARA activates gene expression
Mediator complex
CCR4-NOT complex
Hippo-YAP complex
Cohesin Loading onto Chromatin
MLL1 complex
Interleukin-27, 6, 35 signaling
Nuclear import/export

Table 2. *Table of hits from CRISPR/Cas9 screen having an effect in viability for both ActD and BMH-21 or for each treatment.*

As indicated, negative regulators of mTORC1 - PTEN, TSC1, and TSC2 - made cells more vulnerable to ActD and BMH-21. Hence, cells with higher translation activity would respond better to these treatments. In fact, the opposite link has been reported, since mTOR inhibition reduces nucleolar stress induced by ActD [337]. We investigated if the same happened for other genotoxic drugs, including nucleolar stressors, comparing our results to the Genetic Map of the response to DNA damage [266]. This resource collects the data from 31 CRISPR/Cas9 loss of function screens in retinal pigment epithelium-1 (RPE1) cells depleted from TP53 (TP53-KO) exposed to 27 genotoxic agents, which does not include ActD nor BMH-21. In our search we compared all the common genes negatively and positively selecting for ActD and BMH-21 (**fig. 3A**). Most of the sensitizers from our screen were common with at least other eight compounds in the genetic map, which had different mechanism of action. For instance, TSC1/TSC2-KOs sensitized to ICRF-187 (TOP2 inhibitor), HU2 (DNA replication stress induced), Doxorubicin (caused DNA strand breaks), Duocarmycin (alkylating agent), UV (causes helix distorting lesions). Hence it is possible that the sensitivity observed among these sensitizers is due to DNA damage, which still can be consequence of nucleolar stress. In the case of CUL3, a key regulator in polyubiquitination and subsequent degradation of specific protein substrates, it seems a common sensitizer among genotoxic stressors, and similar profile is observed for PTEN. Interestingly, depletion of the top sensitizers of our screen confer resistance to Pyridostatin (G-quadruplex stabilizer), when both ActD also stabilizes these structures [187]. However, not many compounds shared the same resistance signature as ActD and BMH-21. Additionally, depletion of importin protein TNPO1 was identified as a common element of resistance for both treatments, hence it is possible that some substrates of this protein are required to induce ActD and BMH-21 toxicity.

When looking at genes that influenced viability in ActD-treated samples (**tables 1** and **2**) we observe 4 interesting features. (1) Components of the endosome pathway, particularly of the EARP complex (VPS51, VPS52, and EARP interactor TSSC1), the Tristetraprolin (TTP, ZFP36) complex involved in destabilization of mRNA, and 14-3-3 interactors, involved in MAPK signaling, sensitize to ActD. (2) In late time points, RNA processing and mitochondrial related genes sensitize to ActD, while these group of genes, particularly the latest, confer resistance to BMH-21. (3) Depletion of components of the translation machinery seem to sensitize more cells to ActD than to BMH-21. (4) Loss of chromatin remodelers from SAGA-STAGA, SWI/SNF, and, to a smaller extent, transcription modulator Mediator complex, positively selected cells treated with ActD while negatively selecting for BMH-21. The SAGA-STAGA complexes acetylate chromatin [338,339] and SWI/SNF complex remodels the nucleosome landscape facilitating gene expression [340]. Similarly, Mediator complex promotes RNA transcription by assisting the RNA pol II PIC assembly at promoter regions [341], where it can be recruited with the help of SAGA-STAGA [342]. Interestingly, SUPT3H, a member of SAGA-STAGA, also sensitized HCCT116 cells to BMH-21 in a CRISPR/Cas9 screen to find vulnerabilities to CX-5461, Pyridostatin, and BMH-21 [333]. Acquired resistance due to loss of components of the SAGA-STAGA complex has been reported to be associated with up-regulation of ABCB1 [343], which could explain the differences between ActD1 and BMH-21,

assuming BMH-21 is not a substrate of ABCB1. Exploring the CRISPR screen repository BIOGRID ORCS, it seems that these genes may confer resistance to other drugs inhibiting protein synthesis pathways, as it is found for MAPK inhibitors Vemurafenib, Selumetinib and Trametinib. Henceforth, when studying the effect of deleting chromatin remodelers SAGA-STAGA and SWI/SNF, it would be necessary to control changes in ABCB1 levels and in the intracellular accumulation of ActD. Surprisingly, ActD signature seems to be quite different from other genotoxic compounds (**fig. 3A**). The only genotoxic compounds with distinct behavior from ActD are based on resistance acquired from loss of deubiquitinating enzyme BAP1.

Regarding BMH-21, (1) depletion of elements of the Hippo-Yap pathway, which controls cellular size [344], are enriched among sensitizers. In ActD samples, only the NF2 was found to sensitize, whilst for BMH-21 were NF2, LATS2, MAP4K4, SAV1, TAOK1, YWHAB, YWHAE, and YWHAZ. Additionally, TAOK1, appeared to negatively select in cells exposed to BMH-21 in another CRISPR/Cas9 screen [333]. Interestingly, and related to our previous results (**paper II**) where BETi prevented nucleolar stress, depletion of elements of the Hippo-YAP pathway confer resistance to the BETi JQ-1 [345]. Also, in our screen, depletion of BRD1 makes cells less vulnerable to BMH-21. Hence, changes in this pathway can be potentially related to differential phenotypes between nucleolar stressors and BETi. Furthermore, Hippo-YAP pathway collaborates with mTORC1 to orchestrate cell growth and protein biosynthesis [344]. Also, the genes enriched for Hippo-YAP, are growth repressors, as they were TSC1, TSC2, and PTEN. In the same line as before, among BMH-21 samples we find another link between translation activity and response to nucleolar stressors in LARP1 and METAP1 (2). LARP1 binds to PABP at mRNAs and promotes their translation [346]. For BMH-21, depletion of LARP1, expected to reduce translational rates, makes cells less sensitive to the RNA pol I inhibitor. METAP1 is an aminopeptidase that co-translationally removes N-terminal initiation methionine from nascent peptide chains ensuring optimal translation [347]. As well, cells depleted of METAP1 are more resistant to BMH-21. (3) Then, specifically for BMH-21, proteins involved in the loading of cohesins onto chromatin (STAG1, STAG2) are enriched as sensitizers in our screen. However, STAG2 appears to sensitize to CX-5461 as well [333], which could indicate that this complex plays a role in stability of GC-rich regions, as it is a common mechanism for BMH-21 and CX-5461. (4) Intriguingly, the contribution of mitochondrial proteins in cell viability by BMH-21 is not common among other genotoxic compounds (**fig. 3A**). Also, looking at MRPL20 (Mitochondrial Ribosomal Protein L20) in BIOGRID ORCS, it appears as a hit for multiple screens (312 hits/1082 screens), yet not for any specific drug treatment, and the same happens for other mitochondrial genes identified in BMH-21 treated cells. Hence, mitochondrial genes might be an interesting vulnerability to explore for BMH-21. (5) Additionally, among the genes positively selecting cells to BMH-21, there are many related to protein ubiquitylation and degradation by the proteasome. Then, it might be possible that among them are the factors in charge of RNA pol I degradation by BMH-21. To be more specific, BMH-21 triggers proteasomal degradation of the main catalytic RNA pol I subunit RPA194 [199]. Comparison of a selection of genes enriched in BMH-21 samples to other

genotoxic compounds showed that resistance upon CAND1 depletion was only common with Pyridostatin (**fig. XA**). CAND1 is a key factor that regulates the exchange of F-box proteins in the SCF complex, which is a E3 ubiquitin ligase complex that catalyzes the ubiquitination of proteins destined for degradation. F-box proteins confer specificity towards substrates. Hence, it would be interesting to test if exposure to Pyridostatin triggers degradation of RPA194 as BMH-21 does.

Along the same lines, it is particularly interesting the case of F-box protein FBXO11, since its depletion discriminates ActD and BMH-21 samples for all time-points. FBXO11-KO makes cells more sensitive to ActD and more resistant to BMH-21. Therefore, it could be an interesting marker to consider when deciding on treating cells with either ActD or BMH-21 and could be potentially involved in BMH-21 driven degradation of RPA194. Also, the zinc-finger transcription factor ZNF217 differentiates the response of ActD versus BMH-21. ZNF217 has been proposed as an interesting oncogenic biomarker, it appears to be frequently amplified in tumors, which have been related to poor prognosis [348]. ZNF217 seems to interfere with different cancer hallmarks, including genome instability and evasion of tumor suppressors.

Lastly, to explore the effect from identified gene candidates across different cancer types we used cBioportal, and queried TSC1 and TSC2 (top sensitizers) against the Pan-cancer analysis of whole genomes dataset (38 cancer types, 2,658 donors) [267] (**Fig. 3B**). Both TSC1 and TSC2 tend to be overexpressed and amplified amongst different cancers. For TSC1, head and neck cancer, breast cancer, renal cancer, ovarian cancer, and mature B-cell lymphoma show two clear cohorts where the gene is either overexpressed/amplified or underexpressed/deleted. Similarly, for TSC2, there are differentiated groups for breast cancer, renal cell carcinoma, hepatobiliary cancer, pancreatic cancer, esophagogastric cancer, mature B-cell lymphoma, glioma. Also, TSC1 appears to be deleted in approximately a 1% of embryonal tumors, malignant brain cancers that start in fetal cells. While TSC2 is downregulated in 8% non-small cell lung cancer, 1.5% melanoma, and, also deleted in 1% embryonal tumors, as well as TSC1. Interestingly, ActD is used as a therapy in renal cancers, such as Wilm's tumors, and this analysis could potentially spot responders based on TSC1 and TSC2, if our results hold true. Also, in B-cell lymphomas, such as Burkitt lymphoma, nucleolar stressors such as oxaliplatin have shown value [191]. Additionally, drugs altering ribosome biogenesis are being extensively studied for targeting medulloblastoma and glioma, and particularly for the more aggressive type glioblastomas [349,350]. Hence, within these cancer types, patients with lower levels of TSC1 and TSC2 could benefit from ActD treatment, since it is already in the clinic, if these markers are proven sufficient to stratify patients into responders and non-responders.

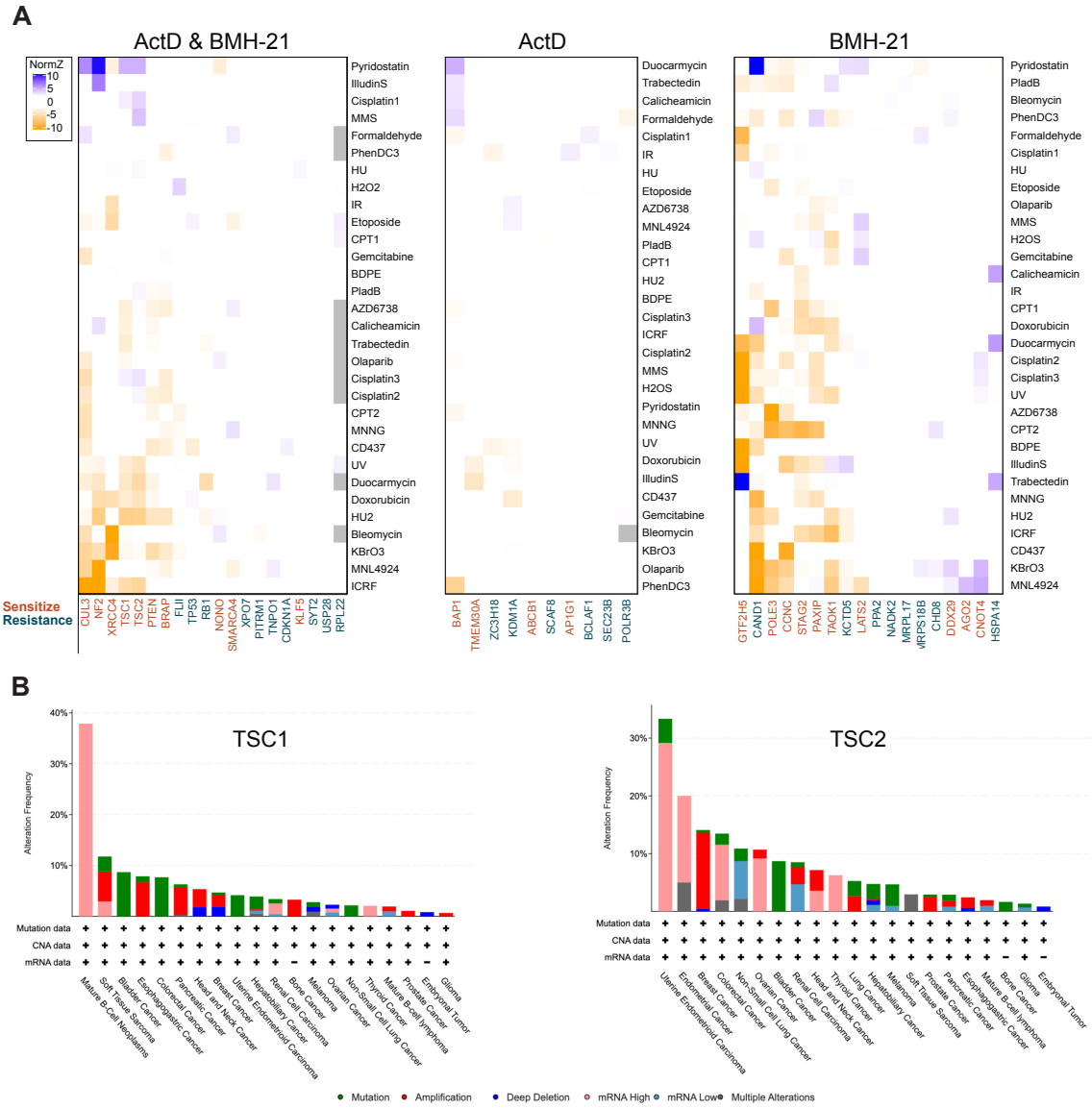


Figure 3. *Exploration of gene candidates using databases. Negative and positive selection of gene-KOs against the Genetic Map of the response to DNA damage of common hits for ActD and BMH-21 (A), specific genes for ActD (B) and for BMH-21 (C). Negatively selected gene-KOs appear in brown, and the ones conferring resistance in blue. (B) Alteration frequency of TSC1 and TSC2 across cancer types.*

5.3.4 Next steps

The next experiments consist in validation of some of the interesting gene candidates. We have started to generate knockout cells lines and conducting low-throughput validations for some of the hits using sgRNAs different from the ones in the Brunello library. Additionally, we will check if the hits hold in different cell lines, and if the response is dependent of p53, using isogenic p53 control and KO cells. This screen might be limited to changes in cell viability of ActD and BMH-21, which might not necessarily reflect nucleolar effects. Hence, we will test the response of depleting these candidate genes in the presence of other known nucleolar stressors, as well as using genetic models, such as by RNA pol I depletion using protein degraders (explained in **annex I**). Our ultimate goal with this project is to improve the use of nucleolar stressing chemotherapies in the clinic by understanding the genetic backgrounds that would particularly benefit from such treatments in cancer patients.

5.4 PRELIMINARY RESULTS IV: Exploration of novel nucleolar functions of known drugs using the Drug Repurposing Hub library

5.4.1 Introduction

The nucleolus has been exploited as a target for anticancer drugs but neglected for other disorders linked to nucleolar alterations. Most of the compounds having an effect in the nucleolus are inducers of nucleolar stress, except of perhaps Rapamycin and derivatives, which reduce nucleolar activity [27,104]. After our work on ALS, where we identified compounds able to protect from nucleolar stress, we understood there might be new ways to be discovered of regulating the nucleous. First, we decided to explore if more compounds were able to prevent nucleolar stress, as in the case of BET bromodomain inhibitors (BETi). To start with, we screened the Drug Repurposing Hub library [1] for compounds able to protect, or potentially enhance, from nucleolar stress induced by ActD. Nucleolar stress has emerged as a common element among different neurodegenerative disorders, and there is evidence that reduction of nucleolar stress could be beneficial in these contexts [351]. Thus, systematic identification of modulators of nucleolar stress could help to identify new therapies.

5.4.2 A high throughput chemical screen for protectors of nucleolar stress

For setting up the screen, we decided to use 384-well plates pre-spotted with compounds over which U2OS cells were seeded and exposed to drugs for 44h prior to adding 5nM of ActD for 4h. The treatment was set according to our previous work, and it was sufficient for BETi to prevent nucleolar stress induced by ActD, measured by changes in area occupied by fibrillarin (**fig. 1A**). The advantage of using pre-spotted plates it that all replicates for each plate can be independent. However, before running the actual screen, we had to test that our protocol could be coupled to using pre-spotted plates. For that, we ran a pre-screen where all wells were spotted with DMSO, except for the ones with BETi controls PFI-1 (3 μM) and JQ-1 (1 μM), expected to limit the nucleolar effects of ActD. The assay window of this test between cells solely exposed to DMSO compared to where ActD was added to was of about a 60% (**fig. 1B**). For cells exposed to JQ-1 and PFI-1 together with ActD, Fibrillarin area was around an 80-90% of the DMSO control, hence this set up allowed prevention of nucleolar stress by BETi.

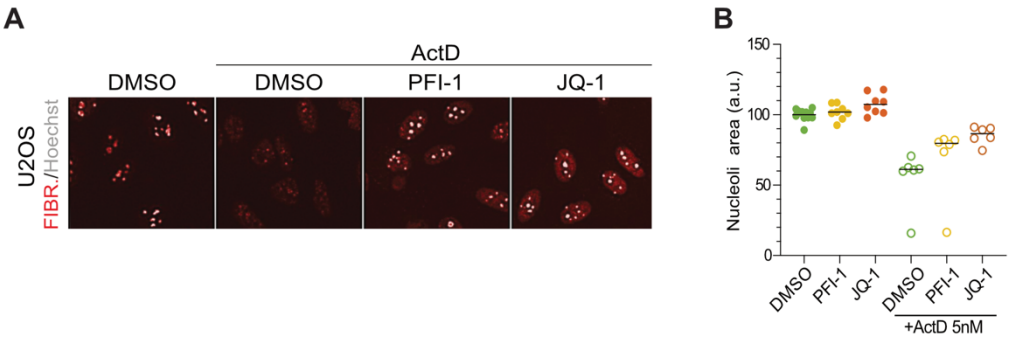


Figure 1. Definition of controls conditions with compounds preventing nucleolar stress *(A) HTM images of U2OS cells pre-exposed for 4h to DMSO, PFI-1 (3μM) and JQ-1 (1μM), and then to ActD 5nM for 4h. (B) Quantification of changes in nucleoli area defined by Fibrillarin of (A).*

Next, we screened the Drug Repurposing Hub library (5,280 compounds) in U2OS. This library is highly curated and annotations regarding mechanism of action, targets and clinical status of the drugs included are frequently revised [1]. Moreover, expression signatures of many of these compounds are included in cMap, which could potentially help in unraveling new mechanisms of action or similarities among small molecules. The extent of the library was covered in 19 plates. The screen was done in triplicates that were processed in three batches (1-19A, 1-19B, 1-19C) (**fig. 2A**). For every batch, U2OS cells were seeded on pre-spotted plates containing 1μM of compound per well for 44h, after which ActD (5nM) was added for other 4h, then cells were fixed with 4% PFA for 15 min. Next, plates were incubated in 3% BSA in 0.01% PBS-Tween20 for an hour, and anti-Fibrillarin antibody (ab5821 Abcam) solution was added to each well to a concentration of 1:2000, and plates were kept at 4°C overnight. Next day, the plates were further incubated with secondary antibody solution and Hoechst and imaged using InCell Analyzer. Image analysis was done using Cellprofiler, using a self-made pipeline to segment nucleoli within the cell nucleus based on Fibrillarin staining. Statistical analysis was done using KNIME. The window between the negative (DMSO) and positive (DMSO + ActD) controls was approximately of a 30% (**fig. 2B**). Hits were defined by the following criteria: (a) having an effect in Fibrillarin area greater to three standard deviations (3xStDev), meaning approximately a 15% increase or decrease of the area for DMSO; (b) compounds should not exhibit effects in cell viability, measured by nuclei count, beyond a 30%, which was about 3xStDev from the negative control. BETi controls did not prevent the effects of ActD as much as in the pre-screen, probably because the window of assay was narrower this time (**fig. 2C**). However, we identified 58 compounds limiting the changes in Fibrillarin area induced by ActD (**fig. 2D**). While it happened that for most triplicates one plate out of three appeared as an outlier, their contribution did not have a great effect on the overall results, as they did not affect the number of hits per plate (**fig. 2E**). When looking at the images it was apparent that these compounds limited the effect of ActD in nucleoli (**fig. 2F**). Examining the hits and classification based on mechanism of action, epigenetic compounds, including HDACi and JQ-1 were represented, supporting our previous results and premises for this screen (**fig. 2G**). Also, filtering hits from the plates that showed variation, going from 58 to 43 compounds, did not affect the representation of compound classes enriched (**fig. 2H**). Hence, we decided to take the 58 initial hits for validation. Importantly, a quarter of these compounds are available in the clinic or in the market and half of them are undergoing preclinical studies (**fig. 2I**).

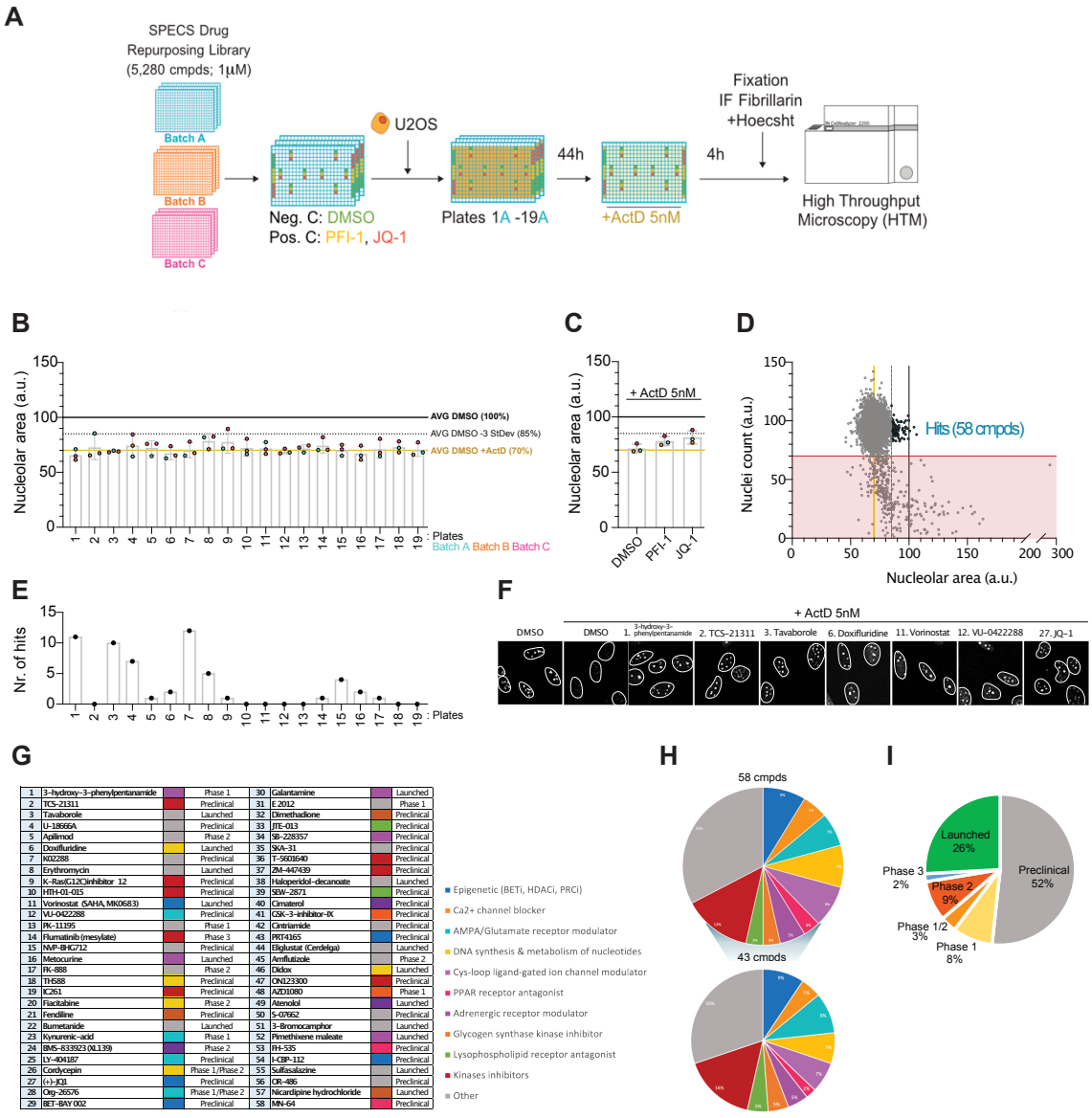


Figure 2. A chemical screen to identify modulators of nucleolar integrity. *(A) Schematic overview of the phenotypic screen workflow. Pre-spotted plates covering the Drug Repurposing Hub library (5,280 compounds) at 1 µM in triplicate (batches A, B, C, in BLUE, ORANGE and PINK respectively) and with the negative (DMSO) and positive controls (PFI-1 3 µM and JQ-1 1 µM) were prepared. One batch at a time, U2OS cells were seeded onto the 384-well plates, and after exposing cells to compounds for 44h, 5nM of ActD was added for 4h. Next, plates were fixed and processed for immunofluorescence (IF) for detection of Fibrillarin, and later nuclei were stained using Hoechst, and changes in nucleoli area and nuclei count were quantified by HTM. (B) Average of median nucleolar area defined by Fibrillarin across the plates in the screen from controls DMSO (BLACK line). The graph shows a threshold in 70% based on the average nucleolar area for DMSO + ActD samples (YELLOW line and on 85% according to three times the average of the standard deviation (StDev) of DMSO samples. (C) Average of median nucleolar area from cells exposed to DMSO or controls PFI-1 and JQ-1 with ActD in the screen, showing a rescue of about a 10%. (D) Compound distribution from the screen, based on Fibrillarin area and nuclei count. 58 compounds (BLUE) prevented nucleolar changes incduced by ActD. Compounds exceeding 30% toxicity are shown under the RED shade. (E) Distribution of hits per plate. (F) HTM images from some of the hits. (G) Rank of the hits, and classification based on their mechanism of action and clinical development. (H) Distribution of the hit classes before (58) and after (43) filtering compounds from plates showing variation. (I) Distribution of the 58 hits regarding their clinical status.*

5.4.3 **Next steps**

Next, hits will be validated in a secondary dose-response screen following the same experimental set up as before, and additionally by solely exposing cells to compounds to gain knowledge of their effects on nucleolar dynamics in the absence of ActD. If the hits hold, we will test if they limit toxicity induced by ActD and other nucleolar stressors. Ultimately, we would like to explore the potential of compounds maintaining nucleolar integrity as therapies for aging and neurodegenerative disorders.

6 CONCLUSIONS AND POINTS OF PERSPECTIVE

This thesis exploited the potential of high throughput screens to discover molecules and genes able to modulate molecular processes of interest, in this case protein synthesis and nucleolar activities, and potentially new compounds with potential therapeutic value, such as for BETi limiting ALS-PR_{20} toxicity.

In **paper I**, we screened the effects in mRNA translation of medically approved and characterized compounds. We could not identify drugs able to significantly increase translation in cancer cells grown in complete media, and our data suggests that supra-regulation of translation in cancer cells growing in optimal conditions might be not possible. The only case where up-regulation of translation has been reported in cells growing in normal conditions, has been after exposure to a compound that also increases ribosome biogenesis [208]. Hence, identifying compounds that stimulate both processes might be an interesting angle. Also, from our results we propose that conducting the same screen in cells that have been starved or under certain stress conditions, could help in identifying stimulators of translation. Nevertheless, the idea is always to translate our findings to disease models that can benefit from these compounds. Hence, perhaps screening in a model where translation is intrinsically reduced might be more interesting. For instance, using models for neurodegenerative disorders where translation is reduced or for ribosomopathies, and then understand the applicability of these compounds to other translational challenged models. This last strategy would be similar to **paper II**, where we went from an ALS model to a potential molecular mechanism (maintenance of nucleolar integrity) that could be broaden to other scenarios. Also, when selecting disease-related phenotypes where to conduct screens, genetic models might be preferred to draw conclusions, such as WT and mutant proteins related to any ribosomopathy. In any case, in our screen we identified sphingosine kinase inhibitor SKI-II as a down-regulator of translation, inducing the ISR by physically damaging the ER. We were not able to narrow down the target of SKI-II responsible for ER damage and it might be the case that it is not one specific protein, but some, or that the compound directly affects the membranes of the ER. Nevertheless, we showed that SPHKs are not responsible for toxicity associated to SKI-II induction of ER damage, which has been proposed in the literature. SPHK inhibitors have been extensively explored as anticancer therapies, and SKI-II analog ABC294640 is in clinical trials for cancer. Our work highlights the need for genetic validation of drug targets for (1) probing the relevance of the target in disease and (2) for assessing off-target effects of drugs. Additionally, understanding if SKI-II or ABC294640 might benefit other disease contexts where activation of the ISR can be helpful remains to be explored.

In **paper II**, we described that BETi and Na-Phen rescued viability in cells and zebrafish embryos exposed to ALS-PR_{20}. BETi rescue was due to maintenance of nucleolar integrity against stressors such as PR_{20} and ActD, and these results inspired us to conduct an additional screen for molecules able to achieve the same effects (***preliminary results IV***). However, the mechanism by which BETi and potential candidates from our last screen, protect from nucleolar stress remains unknown, and we hope to explore it further. Also, we had not assessed

directly rescue of cell viability in cells exposed to ActD, and if these protectors prevent the effects of specific sources of nucleolar stress, this could be interesting to compare with follow-ups from our *in silico* and CRISPR screens (***preliminary results II and III***). Critically, we have tested BETi in U2OS cells expressing inducible PR_{97} and viability was not rescued. But this could be a matter of fine tuning of experimental conditions. Yet, these compounds could be tested in genetically different models for ALS (TDP43, FUS, etc.) to see whether they might ameliorate toxicity and be of use, as well as models of neurodegeneration characterized by nucleolar defects. Additionally, validation of BETi in genetic models where bromodomains can be depleted or targeted for degradation, which is possible using already available proteolysis targeting chimeras (PROTAC) for BET proteins, would be of interest [352]. As well as using the same systems for selectively degrading RNA pol I [353], which would be a genetic approach to systematically induce nucleolar stress, and a way to explore additional hits from the projects related to nucleolar stress.

As experienced by us and others, selection of phenotypes where to conduct screens is challenging, since it can be limited to one aspect of a disease, which intellectually might be relevant but might not be necessarily translatable [223]. Screening pipelines using patient-derived cells and organelles are becoming more popular in this sense. Additionally, molecular phenotyping [223,226] could provide of better insights in understanding the drivers of disease, in-depth characterization of the phenotypes used and possibly predict response to compounds or even assist the selection of libraries to screen for, even though the beauty of phenotypic screens is not having any prior knowledge.

Withal, these resource tools could orient library selection, which is a difficult choice on its own, as well as it is dosing. For instance, traditionally high throughput phenotypic screens are conducted using a single dose of compound to gain more coverage of different drug libraries at the same time. Since most of these screens have been directed towards killing cancer cells, using a concentration of 10 μM, which is considered relatively high in terms of cytotoxicity, is common practice. However, is it the right dose when we try to limit toxicity caused by other challenge, such as PR_{20}? Perhaps this is something to be considered. Same happens with duration of the experiment, as conducting mRNA translation screens exposing cells for sorter times could help in finding specific modulators of protein synthesis. Back to the compounds the libraries, revised annotations, compound redundancies and comparisons to expression/phenotypic profiles, could reduce and refine compound selection without compromising unbiased interrogation of the phenotype of interested. Alternatively, there are groups of molecules that due to their intrinsic characteristics are interesting on their own, such as natural compounds libraries, since their targets have been optimized by nature [214], as we rationalized in ***preliminary results I***. In any case, what it is apparent is that the future of drug discovery for the next years is going to be driven by integrative data sources, including transcriptional, proteomic, chemical, and phenotypic profiles [217,226,354]. Furthermore, application of machine learning algorithms in this field will help in elaboration of prediction models for the response to drug treatments, but also, for compound selection. For instance, Stokes and colleagues trained machine learning-based neuronal networks to predict molecules

with antibiotic activity structurally different from antibiotics using the Drug-Repurposing Hub library of the Broad Institute to fight antibiotic-resistant bacteria [355].

Altogether, phenotypic screens are powerful tools for discovering new potential therapies for diseases, which will benefit from new integrative tools that have the potential to improve systematic evaluation of compounds and predicting their effects for diverse disorders. During this thesis, we have used phenotypic screens to identify compounds able to modulate protein synthesis, nucleolar activity, and drugs benefiting ALS models by regulation of these fundamental processes. Ours results have triggered us to formulate new questions to be answered in an unbiased manner by conducting new chemical and genetic screens to have a better understanding of the regulation of these processes and their potential in human health.

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Alba

"Mischief Managed."

- *J.K. Rowling (Harry Potter and the Prisoner of Azkaban)*

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