UNIVERSIDADE DE LISBOA
Faculdade de Medicina

Uncovering the Stressome: A Computational Approach to Define a Stress Granule Signature and its Implication in Cancer

Alexandre Miguel Kaizeler Guedes da Silva Afonso

Orientador: Prof. Doutor Nuno Luís Barbosa-Morais

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, Ramo de Biologia Computacional

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Instituição Financiadora: Fundação para a Ciência e Tecnologia
(UI/BD/153363/2022)
2026

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A impressão desta tese foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de DD de MMMM de YYYY.

Contents

Resumo

Os grânulos de stress (SGs) são condensados citoplasmáticos sem membrana que se formam de forma rápida e reversível em resposta a uma ampla variedade de estímulos celulares, incluindo hipóxia, privação de nutrientes, infeção viral, ativação de células T, choque térmico e stress oxidativo. Estes condensados estão conservados em todos os seres eucarióticos e resultam da agregação de ARN, complexos de pré-iniciação de tradução interrompidos e proteínas, aproximadamente metade das quais são proteínas de ligação a ARN (RBPs). A sua rápida formação (15 a 30 minutos, dependendo do estímulo) e dissipação, assim como a mobilidade contínua dos constituintes proteicos, revelam uma estrutura altamente dinâmica, sugerindo que os SGs desempenham papéis regulatórios adaptativos essenciais durante o stress celular. Inicialmente, os SGs foram descritos como elementos centrais na repressão global da tradução que caracteriza a resposta ao stress. Contudo, estudos subsequentes demonstraram que células incapazes de formar SGs ainda mantêm a inibição traducional global, o que levou à proposta atual de que os SGs atuam de forma seletiva, sequestrando proteínas e ARNs específicos, em vez de atuar unicamente na supressão global. Esta capacidade seletiva permite modular a resposta celular de maneira refinada, ajustando processos como apoptose, senescência e proteólise. Por exemplo, SGs podem reter caspases ativas ou DDX3X, prevenindo a pirocitose e favorecendo a sobrevivência celular; PAI-1, cuja acumulação atrasa a senescência; ou RB1, de forma dependente de RBFOX2. Apesar destes exemplos funcionais, o significado biológico completo destes mecanismos permanece parcialmente obscuro. A tradução contínua de alguns ARNs dentro dos SGs desafia a ideia de que o sequestro implica sempre inibição funcional, sobretudo no que diz respeito aos constituintes transcriptómicos. Além da função fisiológica, os SGs têm sido implicados em várias patologias. Mutações em proteínas nucleadoras de SGs, como TIA1, TDP43 ou ATXN2, estão associadas a doenças neurodegenerativas, como a esclerose lateral amiotrófica. Além disso, algumas variantes em DDX3X promovem agregados similares a SGs que comprometem a neurogénese. Contudo, estas associações devem ser interpretadas com cautela, uma vez que tanto os SGs como a fisiopatologia destas doenças dependem fortemente da agregação proteica. Em contexto antiviral, vírus como o da hepatite C e o SARS-CoV-2 interferem com a função de G3BP1, uma das proteínas nucleadoras chave, inibindo a formação de SGs e demons-

trando que a modulação destes condensados é explorada pelos vírus para contornar a resposta imunitária. No contexto oncológico, células tumorais enfrentam elevados níveis de stress intrínseco e extrínseco, resultantes de hipóxia, privação de nutrientes, inflamação e terapias como quimioterapia ou radioterapia, todos potenciais indutores de SGs. Observaram-se SGs em múltiplos tipos tumorais, sendo propostos como promotores de metastização e resistência terapêutica. Experiências de knockout de G3BP1 e COX-1/2 sugerem que a abolição dos SGs reduz a capacidade metastática e aumenta a sensibilidade aos tratamentos, embora não esteja claro se estes efeitos resultam exclusivamente da inibição dos SGs ou de outras funções independentes das proteínas-alvo. Apenas cerca de 20A caracterização dos SGs recorre principalmente a duas abordagens distintas: centrifugação diferencial (DC) e marcação de proximidade (PL). A DC isola frações celulares ricas em SGs por separação física, seguida de imunoprecipitação; a PL utiliza uma proteína-alvo para marcar moléculas próximas, permitindo identificar constituintes próximos da proteína de interesse. Ambas dependem de proteínas-chave como G3BP1 para a imunoprecipitação ou marcação. Em termos de constituintes proteicos, DC e PL apresentam forte concordância, isolando proteínas consistentes em diferentes células e condições de stress. No entanto, em termos de constituintes transcriptómicos, os métodos divergem: a DC frequentemente indica que ARNs mais longos são enriquecidos, enquanto a PL não reproduz esse padrão. Este fenómeno pode refletir um efeito biológico (ARNs longos possuem mais locais de ligação para RBPs e promovem nucleação) ou um viés metodológico, uma vez que a DC favorece a precipitação de moléculas maiores devido ao seu peso molecular elevado. Para resolver estas discrepâncias e caracterizar de forma precisa a composição transcriptómica dos SGs, realizámos uma análise não enviesada de múltiplos conjuntos de dados humanos, abrangendo diferentes linhas celulares, estímulos de stress e métodos de purificação. Os resultados confirmam que os perfis obtidos por DC são fortemente influenciados pelo método, com o comprimento do ARN a ser um preditor dominante. Após correção deste viés, persistem diferenças entre estudos, mas o enriquecimento em ARNs mitocondriais codificados pelo genoma mitocondrial (mas não pelo nuclear) surge como uma característica robusta, reproduzível também em PL e outros métodos. Estes achados permitiram-nos propor um modelo funcional: durante stress, a permeabilidade mitocondrial aumenta, permitindo a libertação de ARN de cadeia dupla (dsRNA) através de canais como VDAC e poros regulados por BAX/BAK. Este dsRNA, gerado naturalmente durante a transcrição do ADN mitocondrial circular, atua como sinal de dano celular, ativando sensores antivirais como MDA5 e RIG-I, posteriormente induzindo inflamação. Postulámos que os SGs sequestram este dsRNA, prevenindo a sua deteção pelos sensores e modulando a resposta inflamatória, funcionando como travões homeostáticos. Para testar esta hipótese, cultivámos células de osteossarcoma U2-OS em condições de stress hiperosmótico (sorbitol), com ou sem inibição da permeabilidade

mitocondrial (DIDS). Imunofluorescência para mitocôndria, G3BP1 e dsRNA (anticorpo J2) revelou que o bloqueio da saída de dsRNA impede a formação de SGs e que, durante stress, existe forte colocalização entre dsRNA e SGs. Estes resultados suportam a necessidade de dsRNA para nucleação dos SGs e sugerem o papel destes condensados na modulação da inflamação celular, embora estudos adicionais sejam necessários para confirmação definitiva, visto o anticorpo de dsRNA utilizado marcar todo este tipo de moléculas, e não apenas as provenientes da mitocôndria. De forma complementar, definimos usando métodos de machine learning uma assinatura transcriptómica de SGs (SGScore) baseada em níveis de expressão, usando um estudo com células não stressadas, células stressadas sem SGs, e células stressadas com SGs. Esta assinatura permite inferir a presença de SGs em datasets de RNA-seq sem recurso a técnicas de imagem, facilitando o estudo destes em grandes estudos transcriptómicos de cancro, como o The Cancer Genome Atlas. Após validação num outro estudo independente, a SGScore revelou associação com pior prognóstico em quatro tipos de cancro: carcinoma adrenocortical, colangiocarcinoma, mesotelioma e adenocarcinoma gástrico. Comparações com tumores onde a SGScore não possui valor prognóstico mostraram que estes 4 cancros apresentam maior inflamação e características metastáticas, mas menor proliferação basal. Dentro destes 4 tumores, pontuações mais altas na SGS correlacionaram com maior expressão de HK2, que promove abertura de VDAC, e alterações em vias de sinalização como aumento de genes proliferativos e diminuição de genes associados à metastização, sugerindo uma modulação adaptativa da célula em stress. Em suma, este trabalho fornece avanços significativos na compreensão dos SGs: (i) demonstra e corrige um viés metodológico crítico na caracterização transcriptómica; (ii) identifica a presença consistente de RNA mitocondrial e propõe um modelo funcional integrando permeabilidade mitocondrial, dsRNA e modulação inflamatória por SGs; (iii) define uma assinatura transcriptómica capaz de prever impacto clínico em cancro, reforçando o potencial translacional desta análise. Assim, os SGs emergem não como simples subprodutos do stress, mas como condensados reguladores ativos, com relevância fisiológica, patológica e terapêutica, representando potenciais alvos para estratégias inovadoras de intervenção em doenças humanas.

Palavras-chave: Grânulos de stress; proteínas de ligação a ARN; transcriptómica; ARN mitocondrial; marcação de proximidade; centrifugação diferencial; viés de tamanho de ARN; modulação imune; resposta celular ao stress.

Summary

Stress granules (SGs) are dynamic, membraneless cytoplasmic condensates that form in response to diverse stress stimuli, including oxidative, osmotic, and heatshock stress. Composed of RNA, stalled translation initiation complexes, and RNA-binding proteins (RBPs), SGs have been proposed to modulate cellular stress responses by selectively sequestering specific molecules. However, despite extensive study, their precise molecular composition and biological function remain poorly defined. In particular, transcriptomic profiles of SGs differ markedly between studies, largely reflecting methodological discrepancies between differential centrifugation (DC) and proximity labeling (PL) approaches. To clarify these inconsistencies, we performed a comprehensive reanalysis of publicly available human SG transcriptomes encompassing multiple cell types, stresses, and purification strategies. We found that DC-based datasets are heavily influenced by RNA length, consistent with a physical bias inherent to sedimentation-based separation, which favors precipitation of longer, higher-molecular-weight RNAs. After correcting for this effect, inter-study concordance remained limited, but a reproducible enrichment in mitochondrially encoded RNAs was consistently observed across both DC and PL datasets. To explore the biological implications of this mitochondrial RNA enrichment, we hypothesized that SGs may function as buffers against inflammation by sequestering mitochondrial double-stranded RNA (dsRNA), which acts as a damage-associated molecular pattern that activates inflammatory antiviral pathways. Supporting this idea, immunofluorescence assays in U2-OS cells showed strong colocalisation of dsRNA with G3BP1-positive SGs under hyperosmotic stress, whereas SG formation was impaired when mitochondrial membrane permeability was blocked. These observations suggest that mitochondrial dsRNA may contribute to SG nucleation and that its sequestration within SGs could prevent aberrant activation of innate immune signaling. Finally, we derived an SG-associated gene signature from transcriptomic data of unstressed, and stressed cells with and without SG formation. This signature, validated in an independent dataset, revealed prognostic value across several human cancers, including adrenocortical carcinoma, cholangiocarcinoma, mesothelioma, and gastric adenocarcinoma, where higher SG activity correlated with poorer survival. Collectively, our results reveal that SG composition is strongly shaped by methodological bias, that mitochondrial RNA sequestration represents a conserved feature of SGs, and

that SG formation may serve as a regulatory "brake" on inflammation with potential implications in cancer biology, acting as putative new therapeutic targets.

Keywords: Stress granules; RNA-binding proteins; transcriptomics; mitochondrial RNA; proximity labelling; differential centrifugation; RNA length bias; immune modulation; cellular stress response.

Acknowledgements

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Abbreviations, acronyms and symbols

3' UTR 3' Untranslated Region.

5' UTR 5' Untranslated Region.

AA amino-acid.

ADAR adenosine deaminase acting on RNA.

ARE adenylate–uridylate-rich element.

ATP Adenosine Triphosphate.

ATXN ataxin.

CLIP Cross-linking and immunoprecipitation.

CPEB cytoplasmic polyadenylation element–binding protein.

CPSF30 cleavage and polyadenylation specificity factor 30.

DNA deoxyribonucleic acid.

dsDNA double-stranded DNA.

dsRNA double-stranded RNA.

eIF2α Eukaryotic Translation Initiation Factor 2 α.

eIF2β Eukaryotic Translation Initiation Factor 2 β.

eIF4A Eukaryotic Initiation Factor 4A.

ENCODE Encyclopedia of DNA Elements.

FDR false discovery rate.

G3PB1 Ras GTPase-Activating Protein-Binding Protein 1.

G3PB2 Ras GTPase-Activating Protein-Binding Protein 2.

GCN2 General Control Nonderepressible 2.

HIV-1 human immunodeficiency virus 1.

HRI Heme-Regulated Inhibitor.

HuR/ELAVL1 Hu antigen R/embryonic lethal, abnormal vision-like RNA binding protein 1.

IDD intrinsically disordered domains.

ISR integrated stress response.

KH K Homology domain.

LARP1 La ribonucleoprotein 1, translational regulator.

lncRNA long non-coding RNA.

MERS-CoV Middle East respiratory syndrome–related coronavirus.

mRNA messenger RNA.

MYC Myc proto-oncogene.

NELFE negative elongation factor complex member E.

nt nucleotide.

p15/NXT1 Nuclear transport factor 2 like export factor 1.

PABP Poly(A)-binding protein.

PAI-1 Plasminogen Activator Inhibitor-1.

PB Processing Bodies.

PERK Protein Kinase R-Like Endoplasmic Reticulum Kinase.

PKR Protein Kinase R.

PRPF8 pre-mRNA processing factor 8.

PTBP1 polypyrimidine tract binding protein 1.

QKI Quaking.

RB1 Retinoblastoma Transcriptional Corepressor 1.

RBFOX2 RNA-Binding Protein Fox-1 Homolog 2.

RBP RNA-binding protein.

RBP ribonucleoprotein.

RNA ribonucleic acid.

RNA-Seq RNA sequencing.

RNGTT RNA guanylyltransferase and 5'-phosphatase.

RNMT RNA Guanine-7 methyltransferase.

RRM RNA Recognition domain.

rRNA ribosomal RNA.

SARS-CoV-2 severe acute respiratory syndrome coronavirus 2.

SASP senescence-associated secretory phenotype.

SCA spinocerebellar ataxia.

SG stress granule.

shRNA short hairpin RNA.

SP1 specificity protein 1.

ssRNA single-stranded RNA.

TAP/NXF1 transport associated protein/nuclear RNA export factor 1.

TIA1 T-Cell-Restricted Intracellular Antigen-1.

TIAR T-Cell-Restricted Intracellular Antigen-1 Related.

tRNA transfer RNA.

TTP tristetraprolin.

WDR33 WD repeat-containing protein 33.

ZF Zinc Fingers domain.

°C Degree Celsius.

Preface

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Chapter 1

Introduction

1.1 Stress Granules

1.1.1 What are Stress Granules?

Stress granules (SGs) are membraneless ribonucleoprotein (RBP) condensates, formed in response to a wide variety of stimuli [1], including hypoxia, nutrient deprivation, viral infection, T-cell activation, heat-shock, and oxidative stress [2–7]. SGs comprise aggregations of ribonucleic acid (RNA), stalled pre-initiation complexes, and proteins [3], of which around half are RNA-binding proteins (RBPs) [8]. Characterised by rapid formation and swift disassembly upon cessation of the triggering stimuli (15 and 30 minutes with oxidative and heat-shock stress, respectively) [9], SGs are highly dynamic structures, with their protein constituents continuously transitioning into and out of them [10].

1.1.2 A brief history of Stress Granules

Cytoplasmic aggregations induced by stress were first described in 1983 in *Lycopersicon peruvianum* (Peruvian tomato plant), where exposure to heat-shock conditions (around 37–40 Degree Celsius (°C)) led to the aggregation of several types of heat-shock proteins [11]. These structures were termed heat-shock granules, named after the inducing stressor, and are often, albeit erroneously, cited as the first observation of SGs. Subsequent studies demonstrated, however, that these plant granules do not contain RNA and therefore represent a different class of cytoplasmic assemblies [12]. This distinction does not imply that plants lack SG; on the contrary, bona fide SGs do form in plants [12], concomitantly with heat-shock granules [12]. The first genuine SGs were in fact observed in chicken only a few years later, in 1986 [13].
Since then, SGs have been observed to form across virtually all eukaryotic lineages, ranging from unicellular organisms such as *Saccharomyces cerevisiae* [14] and *Schizosac-*

charomyces pombe [15], to classical model organisms including *Drosophila melanogaster* [16] and *Caenorhabditis elegans* [17], in plants such as the aforementioned tomato species, *Arabidopsis thaliana* [18], and *Oryza sativa* [19], and in mammals including *Mus musculus* [20] and *Homo sapiens* [21].

Such widespread evolutionary conservation is often taken as evidence of fundamental biological importance. However, this inference warrants caution. Numerous biological features persist across evolutionary time despite having little or no apparent functional relevance [**?**]. For instance, most extant whales retain rudimentary hind limb bones, vestigial structures that no longer contribute meaningfully to locomotion or survival [22]. Similarly, the vomeronasal organ is present in primates, including human fetuses, yet is functionally inactive (or missing) in adults [23]. Although these examples are taxon-specific and far less ubiquitous than SGs across eukaryotes, they illustrate a broader principle: persistence alone does not imply functional necessity.

Additional examples include the widespread presence of pseudogenes and large fractions of so-called "junk deoxyribonucleic acid (DNA)" in vertebrate genomes [24]. There is little evidence that evolution actively selected for these elements [24]. Rather, evolutionary dynamics are frequently dominated by negative selection, whereby traits are eliminated only when they confer a sufficiently deleterious effect. Classical examples include the rapid elimination of maladaptive phenotypes, such as the light-colored moths during the Industrial Revolution in London [25]. Traits that are selectively neutral, or only mildly disadvantageous, may persist indefinitely simply because they are not strongly selected against [24, 26].

This principle is particularly evident in tumour evolution. Beyond the initial stages of oncogenic transformation, much of tumour growth proceeds under near-neutral evolutionary dynamics, with limited clonal selection except against cells harboring severely deleterious mutations [26]. This neutrality helps explain the extensive intratumoural heterogeneity observed in many cancers [26]. Analogous to mass extinction events in macroevolution [27], strong selective pressures are often episodic, becoming prominent only under extreme conditions,such as the introduction of therapeutic interventions [26].

Consequently, a biological feature may be conserved throughout evolution not because it confers a selective advantage, but because it does not impose a sufficient fitness cost to be eliminated. Conservation, therefore, does not necessarily imply positive selection. This argument is not intended to suggest that SGs lack biological function; indeed, their potential relevance in oncology is what motivated this work. Rather, it serves to emphasize that evolutionary conservation can at best support the hypothesis of functional importance, but cannot, on its own, constitute definitive proof.

1.1.3 Stress Granule Assembly and Disassembly

SG assembly is most commonly initiated by phosphorylation of Eukaryotic Translation Initiation Factor 2 α (eIF2α) [28], a central regulator of translation initiation [28]. Phosphorylation of this factor is sufficient to block translation initiation and consequently trigger SG formation [29], and is mediated by four stress-responsive kinases that constitute the core of the integrated stress response (ISR) [30–32]: General Control Nonderepressible 2 (GCN2), Protein Kinase R-Like Endoplasmic Reticulum Kinase (PERK), Protein Kinase R (PKR), and Heme-Regulated Inhibitor (HRI). Each of these kinases is activated by distinct cellular insults and is required for an appropriate translational and granule-forming response to its corresponding stress [31, 32]. For example, HRI is essential for SG formation under oxidative stress [33], but is dispensable in response to heat-shock [31], whereas GCN2, PERK, and PKR are required, respectively, during amino-acid (AA) deprivation [34], perturbation of endoplasmic reticulum proteostasis [35], and cellular stress [36] induced by viral infection, heat-shock, or ultraviolet irradiation. Consistent with this model, genetic defects in eIF2α itself [29], or pharmacological inhibition of the ISR [37], markedly impairs SG assembly.

Phosphorylation of eIF2α inhibits the guanine nucleotide exchange factor Eukaryotic Translation Initiation Factor 2 β (eIF2β), resulting in global translational repression [32]. This inhibition leads to ribosome run-off, polysome disassembly, and the accumulation of stalled pre-initiation complexes [38]. These translationally arrested messenger RNAs (mRNAs), together with associated translation initiation factors and RBPs, form the core molecular scaffold of SGs [38]. This nascent assembly subsequently recruits additional proteins through multivalent interactions, promoting further condensation and maturation of the granule [29, 38]. Of central importance in this process are SG-nucleating proteins such as Ras GTPase-Activating Protein-Binding Protein 1 (G3PB1), Ras GTPase-Activating Protein-Binding Protein 2 (G3PB2), T-Cell-Restricted Intracellular Antigen-1 (TIA1) and T-Cell-Restricted Intracellular Antigen-1 Related (TIAR) [31], which are also commonly used as SG markers [31].

SGs can also assemble through eIF2α-phosphorylation independent mechanisms, arising from inhibition of alternative steps in translation initiation. An example of one such mechanism involves Eukaryotic Initiation Factor 4A (eIF4A), an Adenosine Triphosphate (ATP)-dependent RNA helicase that is required for ribosome recruitment and for unwinding secondary structures within the 5' Untranslated Region (5' UTR) of mRNA [39, 40]. Pharmacological inhibition of eIF4A activity, for example by hippuristanol or other compounds, impairs translation initiation and has been shown to robustly induce SG formation in the absence of eIF2α phosphorylation [39, 40].

In addition to signaling-dependent mechanisms, physical changes in the intracellular environment can also promote SG assembly [41]. Hypertonic stress leads to cellular

shrinkage, resulting in an increased intracellular concentration of macromolecules and enhanced molecular crowding [41]. This elevated crowding strengthens weak, multivalent interactions among mRNA–protein complexes, thereby favouring their condensation and promoting SG formation independently of canonical translation initiation signaling pathways [41].

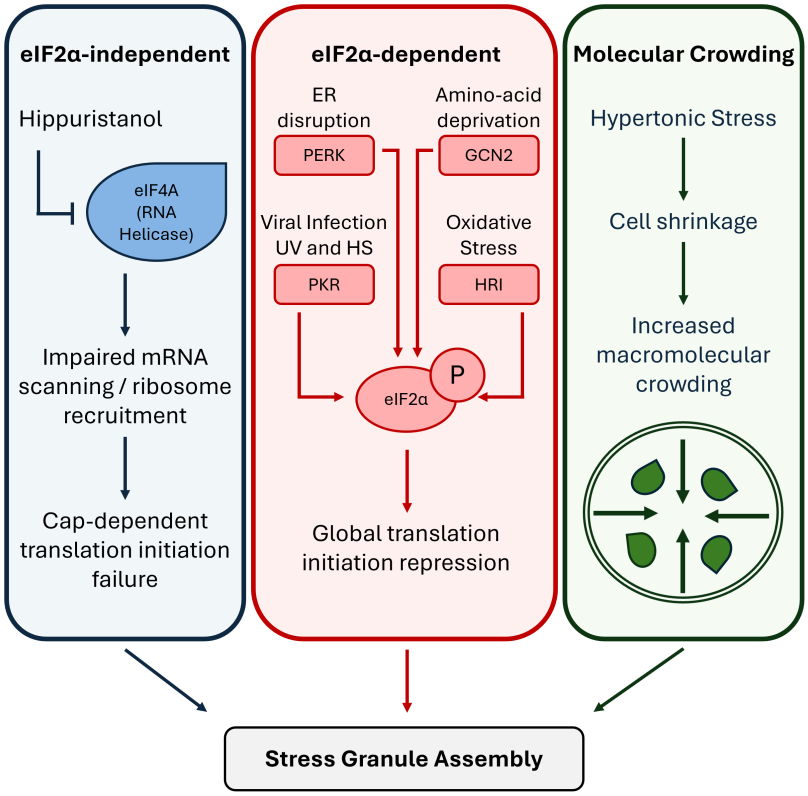

Figure 1.1: Different Mechanisms Leading to SG Assembly The schematic illustrates three mechanistically distinct pathways through which SGs can assemble. eIF2α phosphorylation–independent SG assembly (blue): In this pathway, SGs form independently of eIF2α phosphorylation. One representative example is shown, in which inhibition of the RNA helicase eIF4A by hippuristanol impairs mRNA scanning and ribosome recruitment [39, 40]. This results in failure of cap-dependent translation initiation, leading to the accumulation of non-translating messenger ribonucleoprotein complexes and subsequent SG assembly [39, 40]. eIF2α phosphorylation–dependent SG assembly (red): In response to diverse cellular stress stimuli, one or more eIF2α kinases (PERK, PKR, GCN2, or HRI) are activated, resulting in phosphorylation of eIF2α [30–32]. This modification reduces ternary complex availability and causes global repression of translation initiation, constituting activation of the ISR) and promoting SG assembly [30–32]. Molecular crowding–driven SG assembly (green): SGs can also assemble as a consequence of increased molecular proximity and macromolecular crowding. For example, hypertonic stress induces cell shrinkage, leading to elevated intracellular crowding that strengthens weak, multivalent interactions among mRNA–protein complexes [41]. This favors their condensation and promotes SG formation independently of canonical translation initiation signaling pathways [41]

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Because SG formation is intrinsically linked to polysome dynamics, pharmacological

agents that disrupt polysomes tend to trigger or enhance SG assembly, whereas compounds that stabilise polysomes inhibit their formation [42]. For example, edeine interferes with proper ribosome assembly and translation initiation, leading to polysome disassembly and robust induction of SGs [43]. Similarly, puromycin induces premature termination of translation and promotes ribosome release from mRNAs, thereby destabilising polysomes and enhancing SG formation [38, 42]. In contrast, cycloheximide stabilises polysomes by freezing elongating ribosomes on mRNAs, effectively preventing ribosome run-off and increasing the threshold of stress required to induce SG assembly [38, 42].

SG disassembly largely follows the reverse sequence of events that govern their assembly. Upon stress relief, eIF2α is dephosphorylated in the canonical pathway, leading to the restoration of translation initiation [44, 45]. Translation restart occurs in both canonical and non-canonical contexts and promotes the re-engagement of mRNAs with ribosomes, resulting in their reincorporation into polysomes [44, 45]. As translationally active mRNAs are withdrawn from SGs, the number of stabilising intermolecular interactions within the granules decreases, ultimately driving their disassembly [44, 45]. During this process, SGs progressively fragment into smaller assemblies, which are thought to be cleared through autophagy-dependent pathways [45], although the extent to which autophagy contributes to SG turnover remains an active area of investigation [46, 47].

In contrast, considerably less is known about the disassembly mechanisms of SGs formed through increased molecular crowding. Notably, while SGs assembled via both eIF2α-dependent and eIF2α-independent translational control pathways typically disassemble within approximately one hour following stress removal [41], crowding-induced SGs display markedly slower dynamics and can persist for several hours, with reported disassembly times extending up to nine hours [41].

1.1.4 Functions of Stress Granules

A process conserved across all eukaryotes inevitably implies functional relevance. Even prior to the discovery of SGs, it was well established that heat-shock and other forms of cellular stress induce a rapid arrest of translation [11, 48, 49]. The striking correlation between translational repression and SG appearance naturally led to the assumption of a causal relationship between the two phenomena [29]. Indeed, such causality does exist; however, it is not SGs that impose translational arrest. Rather, translational arrest is the upstream event that drives SG formation [28].

How did this confusion arise? Early observations showed that the RNA-binding proteins TIA1 and TIAR bind untranslated mRNAs and localise to SGs [29]. Mutations in these proteins strongly impaired mRNA recruitment into SGs, yet had no effect

on the global sequestration of stress-induced untranslated transcripts [29]. In parallel, although eIF2α phosphorylation was recognised as a key driver of translational arrest, cases were described in which translation inhibition occurred even in the absence of eIF2α phosphorylation [50], still accompanied by SG formation [29]. Because SGs contained untranslated mRNAs and were present under conditions of translational blockade, even when canonical eIF2α signaling was bypassed, it was postulated that SGs might play a central role in enforcing translation inhibition [29].

However, subsequent work has demonstrated that SGs are neither necessary nor sufficient for translational repression [40] and, in many contexts, have only modest effects on overall protein output [51]. Moreover, translation has been shown to occur within or in close association with SGs, challenging the long-standing notion that SGs function primarily as sites of mRNA storage to prevent translation [52]. Together, these findings indicate that SGs are a consequence, rather than a cause, of stress-induced translational arrest [53], and that their functional role, if any, must extend beyond simple translational repression.

As of the writing of this thesis, a growing hypothesis proposes that SGs function as fine-tuners of the cellular stress response through selective sequestration [47]. SGs have been reported to enhance cell survival by transiently sequestering specific pro-apoptotic [54–56] or senescence-associated factors, such as Plasminogen Activator Inhibitor-1 (PAI-1) [57], thereby limiting stress-induced senescence, and *Retinoblastoma Transcriptional Corepressor 1 (RB1)*, protecting it from degradation in an RNA-Binding Protein Fox-1 Homolog 2 (RBFOX2)-dependent manner [58], among other examples.

Nevertheless, although SG assembly generally correlates with improved cellular fitness under stress [7], SGs do not appear to represent a strict life-or-death determinant, as cells deficient in SG formation can still survive [7, 59, 60]. The picture becomes even more ambiguous when selective transcript sequestration is considered. In most cases, SGs recruit transcripts broadly, with the number of mRNA copies within SGs largely reflecting their abundance in the total cellular transcriptome [59–62]. One notable exception appears to be stress-induced transcripts [21, 63]. Indeed, even in the earliest descriptions of "stress granules", it was hypothesised that SGs might function to prioritise stress-responsive gene expression, as translation under stress conditions is largely restricted to these transcripts [11].

From this perspective, transcript selectivity may not arise from the preferential recruitment of specific mRNAs into SGs, but rather from the sequestration of the bulk of non-stress transcripts [63], effectively excluding stress-induced mRNAs from SGs and allowing their continued translation [63].

This model of selective sequestration is, however, directly challenged, at least at the transcript level as previously mentioned, by observations showing that mRNAs recruited to SGs can undergo translation at rates comparable to those of freely diffusing

transcripts under non-stressed conditions [52]. Such findings undermine the notion that transcript sequestration by SGs constitutes a dominant or exclusive mechanism for translational regulation [64].

In light of this apparent functional ambiguity, a fundamental question emerges: why are SGs still attributed such a wide range of cellular functions, and why are they so frequently implicated in diverse pathological contexts? Part of the answer may lie not in definitive mechanistic evidence, but in their conspicuous and reproducible presence. SGs are readily observable, form robustly across cell types and stresses, and appear whenever translation is perturbed. Their persistence and ubiquity make them natural focal points for hypothesis generation.

This phenomenon reflects a broader tendency in biological research: highly visible and evolutionarily conserved structures are rarely assumed to be functionless. SGs are consistently "there" (as George Mallory would put it), forming reliably under conditions of translational stress, and their very existence invites functional attribution. As a result, they have become central players in numerous models of stress adaptation and disease, even as definitive evidence for many proposed roles remains incomplete [64]. In this sense, the prominence of SGs in both physiological and pathological narratives may reflect not only their biological relevance, but also the interpretive weight we assign to persistent, conspicuous cellular structures whose functions are still being actively defined.

1.1.5 Stress Granules in Disease

As anticipated in the previous section, SGs, like most fundamental cellular processes, have been extensively implicated in disease. SGs are cytoplasmic assemblies characterised by the aggregation of mRNAs and proteins, approximately half of which are RBPs [8]. Many RBPs contain intrinsically disordered domainss (IDDs) [47, 65], which confer structural flexibility but also predispose these proteins to aberrant self-association and aggregation [47,65]. Notably, this same class of proteins is prominently implicated in neurodegenerative disorders [47]. For example, members of the ataxin (ATXN) protein family, whose dysfunction underlies several forms of spinocerebellar ataxia (SCA), exhibit aggregation-prone properties linked to disease pathology [66,67]. Given that both SG biology and many neurodegenerative diseases centrally involve protein aggregation, it was perhaps inevitable that mechanistic connections between the two would be proposed. Indeed, numerous studies have suggested that alterations in SG dynamics, particularly defects in assembly, maturation, or disassembly, may promote pathological aggregation [68]. One prevailing hypothesis posits that the insoluble aggregates characteristic of several neurodegenerative disorders may originate from SGs that fail to properly disassemble, thereby transitioning from transient, re-

versible assemblies into persistent, cytotoxic inclusions [47, 69–71].

SGs have also been extensively implicated in antiviral responses, a topic that has been reviewed in detail elsewhere [72]. Many viruses induce SG formation, most commonly through activation of PKR [72–74], leading to phosphorylation of eIF2α, although PERK-mediated eIF2α phosphorylation has also been reported in certain contexts [72, 75]. As discussed in the sections on SG assembly and disassembly, these signaling events position SG formation downstream of translational inhibition.

The functional consequences of SG assembly during viral infection, however, remain ambiguous. In some cases, SG formation correlates with impaired viral replication, consistent with a role in host defense [76]. In other systems, SG induction appears to have little or no measurable impact on viral propagation [73]. On the other hand, they have also been found to be hijacked by viruses, having their assembly linked to increased viral replication [77].

This variability suggests that the relationship between SGs and viral replication is at least partly context-dependent, influenced by viral species, host cell type, and the specific mechanisms by which viruses interact with the host translational machinery.

Perhaps more striking than SG induction is the remarkable diversity of viral strategies that actively impair SG formation [72]. Viruses across essentially all major classes—including single-stranded RNA (ssRNA) positive- (Middle East respiratory syndrome–related coronavirus (MERS-CoV) [78], severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [79] and Zika virus [80]) and negative-sense (Influenza A virus [81]) viruses, double-stranded RNA (dsRNA) (Pseudorabies virus [82]), double-stranded DNA (dsDNA) viruses (Kaposi's Sarcoma-associated herpesvirus [83]), and retroviruses (human immunodeficiency virus 1 (HIV-1) [84]), have evolved mechanisms to interfere with SG assembly. Some viruses achieve this by promoting eIF2α dephosphorylation [82] or by inhibiting upstream kinases such as PKR or PERK [78, 81, 83], thereby preventing translational arrest and the downstream formation of SGs. Others employ more direct strategies, including the cleavage, sequestration, or functional inhibition of core SG-nucleating proteins such as G3PB1 [79, 80] or TIA1 [85].

In several cases, viral inhibition of SG formation is associated with enhanced viral replication [78–80], supporting the view that SGs predominantly exert antiviral functions. This observation has led to the proposal that certain viruses actively antagonize SGs to evade host defenses [72, 78], whereas others may tolerate or even exploit SG assembly [72, 73, 77]. Nonetheless, such interpretations warrant caution. Many viral strategies that inhibit SG formation act at very upstream levels of cellular regulation, for example, by preventing eIF2α phosphorylation [82], an event central to the ISR and to global translational control [30–32]. It is therefore plausible that these viruses evolved primarily to suppress the ISR as a whole, with SG inhibition arising as a downstream consequence rather than as a direct selective target.

More compelling evidence for a direct antiviral role of SGs comes from viruses that specifically target SG-nucleating RBPs such as G3PB1 or TIA1 [79,80,85]. These proteins are less universally essential than the ISR itself, lending support to the idea that SGs, or at least SG-associated functions, pose a direct obstacle to viral replication. Interestingly, viruses that directly target these SG components appear to be enriched among positive-sense ssRNA viruses [72]. A possible explanation is that this viral class also infects the broadest range of eukaryotic hosts [86]. From an evolutionary perspective, it would be informative to determine when, phylogenetically, viral strategies shifted from targeting the ISR globally to directly antagonising SG components, an adaptation that likely emerged after the evolution of eukaryotes. However, given that most viral lineages are thought to have evolved independently from prokaryotic viruses (brilliant reviewed elsewhere [86]), the repeated emergence of SG-targeting mechanisms instead points toward convergent evolution. This convergence, in turn, supports the notion that SGs represent a meaningful barrier to viral replication. Nevertheless, disentangling SG-specific antiviral effects from the broader RNA-regulatory functions of these RBPs remains a significant challenge in defining the precise contribution of SGs to antiviral defense [64].

SGs have also been implicated in cellular senescence and, by extension, in ageing. They have been reported to counteract senescence by selectively sequestering PAI-1 [57], a well-established promoter of cell-cycle arrest and a key component of the senescence-associated secretory phenotype (SASP) [57]. While cellular senescence is generally considered beneficial in the context of tumour suppression, persistent senescence is detrimental and contributes to tissue dysfunction [87]. Moreover, the SASP itself can paradoxically promote tumour progression [87], a duality that mirrors the context-dependent roles attributed to SGs in cancer biology [2].

Given that both SGs and senescence are induced by cellular stress [88], it is tempting to conceptualise them as alternative, and potentially antithetic, stress responses that are balanced according to cellular context and stress severity. Supporting this notion, senescent cells have been shown to suppress SG formation through depletion of key SG-nucleating RBPs, G3PB1, TIA1 and TIAR, as well as their transcription factor specificity protein 1 (SP1) [89]. As a result, SG assembly is impaired even under conditions of robust eIF2α phosphorylation, which is commonly observed during senescence [89]. This finding suggests that senescence actively enforces a cellular state refractory to SG formation [57,89].

However, this relationship appears to be more complex and context-dependent than a simple antagonism. Notably, G3PB1 has been reported to be required for the establishment of the SASP [88], placing it functionally upstream of a hallmark senescence program, countering its reported depletion in senescent cells [89]. Importantly, depletion of G3PB1 does not abolish senescence itself but selectively impairs SASP

expression [88], indicating that senescence maintenance and SASP execution can be uncoupled [88]. One speculative interpretation is that SGs may represent an early or transient attempt by the cell to buffer stress and avoid irreversible senescence. Failure of this adaptive response could then favor commitment to senescence and the emergence of the SASP, ultimately promoting chronic inflammation. While this model remains speculative, it highlights a potentially dynamic interplay between SG biology and senescence programs that warrants further investigation.
Beyond the roles discussed above, and some others not addressed at length (inflammation [7]) SGs have also been extensively implicated in cancer biology [2]. Given the breadth and complexity of their functions in tumourigenesis and cancer progression, this topic is addressed separately and in greater detail in Chapter 2.

1.2 RNA-Binding Proteins

1.2.1 What are RNA-Binding Proteins?

RBPs are proteins that interact with RNA molecules in both single-stranded and double-stranded conformations [90]. Far from representing a specialised or recently evolved protein class, RBPs are thought to be among the most ancient functional proteins [91–93]. Current models of early molecular evolution suggest that the first biological systems were not purely protein-based, but instead relied on intimate cooperation between RNA and peptides [92]. While the classical RNA world hypothesis posits RNA as the sole primordial biopolymer, more recent views favor an RNA–peptide world, in which short peptides interacted with structured RNAs to stabilise them and enhance catalytic efficiency [92]. In this context, primitive RNA-binding peptides associated with proto-ribosomal structures, are thought to have facilitated the emergence of early metabolism by conferring structural stability and functional versatility to RNA [92,94]. As biological complexity increased, the repertoire of proteins expanded and diversified [92]. Many newly evolved proteins gradually lost RNA-binding capacity as they specialised toward enzymatic, structural, or signaling roles [92, 93]. Nonetheless, a substantial fraction of modern proteins retained RNA-binding functions, reflecting the central role of RNA in gene expression and cellular regulation [92–94]. Today, RBPs constitute a large and functionally diverse class of proteins that expanded broadly in eukaryotes [90, 95] and that govern virtually every aspect of RNA metabolism, and as such, cellular function and homeostasis [90, 93–97].
At the molecular level, RBPs recognise RNA through a variety of conserved RNA-binding domains, such as RNA Recognition domains (RRMs), K Homology domains (KHs), Zinc Fingers domains (ZFs), and DEAD-box helicase domains, as well as through IDDs that enable dynamic and multivalent interactions [90, 93]. The pre-

valence of IDDs among RBPs endows them with structural plasticity, allowing rapid assembly and disassembly of RBP complexes and facilitating the formation of higher-order RNA–protein assemblies, including membraneless organelles such as SGs and Processing Bodiess (PBs) [47, 65, 94, 98].
Given their central role in post-transcriptional regulation, RBPs are critical determinants of cellular identity and adaptability [95, 96, 99]. Perturbations in RBP function have been linked to a wide range of pathological conditions, including neurodegenerative diseases, cancer, viral infections, and ageing [47, 65, 96, 98]. Understanding the fundamental biology of RBPs is therefore essential for elucidating how cells integrate RNA regulation with stress responses and how dysregulation of these processes can contribute to disease.

1.2.2 Functions of RNA-Binding Proteins

As outlined above, RBPs act as master regulators of RNA biology, exerting control over virtually every stage of the RNA life cycle. Their functions span all major RNA classes, including ribosomal RNA (rRNA), transfer RNA (tRNA), mRNA, long non-coding RNA (lncRNA), and other regulatory RNA species [93,96]. Through direct and dynamic interactions with these RNAs, RBPs orchestrate RNA synthesis, processing, modification, transport, localisation, translation, and degradation [93, 96].
negative elongation factor complex member E (NELFE) binds to transcripts of the proto-oncogene *Myc proto-oncogene (MYC)*, stabilising the mRNA and promoting its translation, thereby contributing to tumour progression [96]. Hu antigen R/embryonic lethal, abnormal vision-like RNA binding protein 1 (HuR/ELAVL1) interacts with adenylate–uridylate-rich elements (AREs) within the 3' Untranslated Region (3' UTR) of target mRNA, leading to their stabilisation, particularly for transcripts involved in inflammatory responses [96]. In contrast, tristetraprolin (TTP) recognises similar ARE on overlapping sets of transcripts but promotes their deadenylation and degradation instead [96]. Beyond their direct effects on RNA stability, these examples illustrate how RBPs constitute an additional regulatory layer within signaling pathways, fine-tuning gene expression outcomes by selectively stabilising or destabilising specific RNA populations.
Another clear example of this functional dichotomy is found in the role of RNA-binding proteins in translational control. Poly(A)-binding proteins (PABPs) enhance cap-dependent translation by stabilising pre-initiation complexes [100]. In contrast, cytoplasmic polyadenylation element–binding proteins (CPEBs) generally function as translational repressors, although their activity can be modulated in response to specific cellular cues [100]. La ribonucleoprotein 1, translational regulator (LARP1) exemplifies the context-dependent nature of RBP function, as it has been shown to both repress and

promote translation depending on cellular conditions and signaling status [100–102].
RBPs also play central roles in RNA editing. The most prominent example is the adenosine deaminase acting on RNA (ADAR) family of enzymes, which bind dsRNA and catalyse the deamination of adenosine to inosine [103]. This form of RNA editing is particularly prevalent in the nervous system, where it is essential for proper neuronal development and function [103].
Beyond editing and stability control, RBPs are already critically involved at the earliest stages of RNA maturation. Proteins such as cleavage and polyadenylation specificity factor 30 (CPSF30) and WD repeat-containing protein 33 (WDR33) are required for 3'-end processing and polyadenylation [104], while RNA guanylyltransferase and 5'-phosphatase (RNGTT) and RNA Guanine-7 methyltransferase (RNMT) are essential for the formation of the 5' cap structure [105]. Both polyadenylation and capping are prerequisite steps for mRNA stability, translational competence, and subsequent nuclear export [97]. Export to the cytoplasm is mediated by RBPs such as transport associated protein/nuclear RNA export factor 1 (TAP/NXF1) and its cofactor Nuclear transport factor 2 like export factor 1 (p15/NXT1) [106].
In addition to their role in nuclear export, RBPs are also key determinants of RNA localisation within the cytoplasm, ensuring the spatial regulation of gene expression [93, 96, 97]. In the context of this thesis, RBPs are of particular relevance to SG biology, where they constitute more than half of the protein components [8]. SG-nucleating RBPs such as G3PB1/G3PB2 and TIA1/TIAR are essential for proper stress granule assembly [31], underscoring the central role of RBPs in the formation and regulation of these dynamic RBP assemblies.

1.2.3 RBPs and Splicing

One of the most fundamental functions of RBPs is their role in pre-mRNA splicing [97]. First discovered in 1977 because of adenovirus infection [107,108], splicing is a defining feature of eukaryotic gene expression and is particularly expanded in higher eukaryotes such as mammals, where it occurs in circa 95% of genes and greatly increases proteomic diversity [109]. Through alternative splicing, different combinations of exons can be joined from a single gene, allowing the production of multiple protein isoforms from a single genomic locus [109]. Together with the use of alternative promoters [87, 109], alternative splicing represents a principal mechanism by which the functional breadth of the genome is expanded beyond gene number alone.
RBPs are essential for the assembly, regulation, and function of the spliceosome, a large and highly dynamic RBP complex responsible for intron removal [109]. Beyond constitutive splicing, RBPs also regulate alternative splicing by promoting or inhibiting the inclusion of specific exons, thereby shaping transcript identity in a cell type–

and context-dependent manner [97,109,110]. By binding to cis-acting elements within pre-mRNAs, RBPs can either enhance or repress splice site recognition, influencing both exon inclusion and exclusion [97,109,110].

As a substantial fraction of splicing occurs co-transcriptionally [97,109], many RBPs function in close physical and temporal proximity to the transcription and splicing machiner [97,109]. Because splicing takes place predominantly within the nucleus, RBPs involved in this process must be efficiently localised to the nuclear compartment [97,109]. Consequently, following their synthesis in the cytoplasm, these proteins rely on regulated nuclear import mechanisms to reach the nucleus [111], where they execute their splicing-related functions.

How do RBPs exert control over alternative splicing decisions? In most cases, they do so by binding directly to specific sequence or structural elements within pre-mRNAs, although indirect regulation through interactions with spliceosomal components can also occur [97]. Depending on both the identity of the RBP and the position of its binding site relative to splice sites, this binding can either promote or inhibit intron removal or alternative exon inclusion [97,110].

A useful conceptual framework to describe this positional logic is provided by RNA splicing maps (Figure 2). Splicing maps are schematic representations that integrate RBP binding positions along a pre-mRNA with the corresponding splicing outcomes [97]. They depict where an RBP binds, such as within an exon, upstream or downstream introns, or near splice junctions—and indicate whether binding at each position enhances or represses exon inclusion [97]. Importantly, splicing maps reveal that the same RBP can exert opposite effects depending on binding location [97]. For example, binding downstream of an alternative exon may promote its inclusion, whereas binding within the exon or upstream intron may favor exon skipping [97].

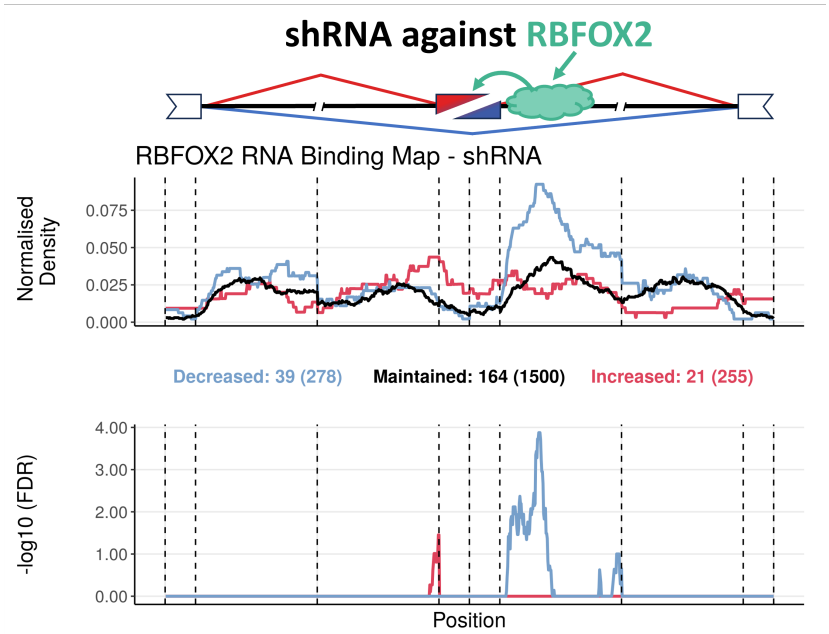

Figure 1.2: RNA binding map of HepG2 and K562 cells exposed to short hairpin RNA (shRNA) against RBFOX2 (Top) Schematic of an alternative exon skipping event, regulated by an RBP, RBFOX2. (Middle) Normalised binding of RBFOX2 to metagenomic regulatory sequences of alternative exons. The plot discriminates excluded (blue), included (red) or maintained (black) alternative exons upon RBFOX2 knockdown, obtained from Encyclopedia of DNA Elements (ENCODE) [112, 113]. The total number of events in each category can be found beneath the plot in brackets, preceded by the number of events for which there is at least one binding site for that RBP. (Bottom) false discovery rate (FDR) of excluded (blue) or included (red) normalised binding, in relation to maintained events. Vertical lines delimitate the following metagenomic splicing regulatory sequences, in order: last 50 nucleotides (nts) of upstream constitutive exon; first 200 nts of upstream intron; last 200 nts of upstream intron; first 50 nts of alternative exon; last 50 nts of alternative exon; first 200 nts of downstream intron; last 200 nts of downstream intron; first 50 nts of downstream constitutive exon. This example shows normalised RBFOX2 binding enriched downstream of alternative exon. Upon RBFOX2 knockdown, this binding is therefore lost, consistent with increased exon skipping. These results align with the known role of RBFOX2 in promoting exon inclusion [97], providing a proof of concept for this approach.

These maps are typically derived from the integration of transcriptome-wide binding data (e.g., Cross-linking and immunoprecipitation (CLIP)-based approaches) with splicing outcome measurements (e.g., RNA sequencing (RNA-Seq)) [97]. By providing a spatial and functional overview of RBP activity, RNA splicing maps offer a powerful way to predict and rationalise how RBPs regulate alternative splicing across different transcripts and cellular contexts [97].

Well-characterised examples illustrate how positional binding of RBPs determines alternative splicing outcomes. RBFOX2 and Quaking (QKI) generally promote inclusion of alternative exons when binding to intronic regions downstream of the exon [97]. In contrast, polypyrimidine tract binding protein 1 (PTBP1) typically promotes exon skipping by binding to upstream intronic elements flanking alternative exons [97, 114].

Other RBPs, such as pre-mRNA processing factor 8 (PRPF8), can directly lead to alternative exon inclusion by biding near to it, or to exclusion by binding to the 5' of the upstream intron [97]. These positional effects were defined through the integration of CLIP-based approaches with RNA sequencing analyses following experimental perturbation of RBP function, most commonly through knockdown strategies such as shRNA-mediated depletion [97, 114].

Importantly, RNA splicing maps are not limited to describing the effects of RBP depletion alone. Once established, they provide a predictive framework for interpreting splicing changes arising from any perturbation that alters RBP activity [114]. Such perturbations include chemical inhibition, altered expression levels, disruption of interacting partners within the same regulatory complex, post-translational modifications that affect binding or activity, or changes in subcellular localization [114]. For instance, sequestration of RBPs into stress granules effectively removes them from the nucleus [111], thereby impairing their ability to regulate splicing despite unchanged expression levels.

Altogether, understanding how a given RBP binds RNA and modulates splicing decisions opens new avenues for targeted manipulation of alternative splicing. This strategy has gained increasing interest in disease contexts such as cancer, where specific splice isoforms contribute to tumorigenesis and disease progression [115]. Modulating RBP activity to shift splicing patterns can therefore represent a therapeutic opportunity, either by suppressing oncogenic isoforms or by promoting intron retention to generate neoantigens that enhance anti-tumor immune responses [115].

1.3 Mitochondria

1.3.1 What is a Mitochondrion?

A	B	C
1	2	3
4	5	6

Table 1.1: Basic table

Group 1		Group 2
X	Y	Z
1	2	3

Table 1.2: Table with merged header

Figure 1.3: This is a basic figure.

Figure 1.4: Figure with custom dimensions.

(a) First image **(b)** Second image

Figure 1.5: Two images side by side

Column A	Column B
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Table 1.3: Table with fixed width columns

Chapter 2

Objectives

Some objectives here...

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Chapter 3

Materials and Methods

3.1 Section

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3.1.1 Subsection

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Chapter 4

Name Chapter I

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Chapter 5

Name Chapter II

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Chapter 6

Name Chapter III

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Chapter 7

Discussion

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