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REVIEW

miRNA targeting and alternative splicing in the stress response – events hosted by membrane-less compartments

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ABSTRACT

Stress can be temporary or chronic, and mild or acute. Depending on its extent and severity, cells either alter their metabolism, and adopt a new state, or die. Fluctuations in environmental conditions occur frequently, and such stress disturbs cellular homeostasis, but in general, stresses are reversible and last only a short time. There is increasing evidence that regulation of gene expression in response to temporal stress happens post-transcriptionally in specialized subcellular membrane-less compartments called ribonucleoprotein (RNP) granules. RNP granules assemble through a concentration-dependent liquid–liquid phase separation of RNA-binding proteins that contain low-complexity sequence domains (LCDs). Interestingly, many factors that regulate microRNA (miRNA) biogenesis and alternative splicing are RNA-binding proteins that contain LCDs and localize to stress-induced liquid-like compartments. Consequently, gene silencing through miRNAs and alternative splicing of pre-mRNAs are emerging as crucial post-transcriptional mechanisms that function on a genome-wide scale to regulate the cellular stress response. In this Review, we describe the interplay between these two post-transcriptional processes that occur in liquid-like compartments as an adaptive cellular response to stress.

KEY WORDS: Stress response, miRNA, Alternative splicing, RNP granule, Low-complexity sequence domain, Phase separation, Dicer, Drosha, Argonaute

Introduction

The stress response requires regulation of gene expression

Cells react to stress in several ways; this ranges from the initiation of survival programs that include cell repair mechanisms, a temporary adaptation to stress, to the execution of autophagy, and the activation of cell death that removes damaged cells (de Nadal et al., 2011; Poljšak and Milisav, 2012). The cell type and the nature and duration of stress determine whether a survival or destructive stress response pathway is chosen (Zhao et al., 2017). Minor and acute short-term stresses (de Nadal et al., 2011; Gehart et al., 2010; Richter et al., 2010) – such as fluctuations in food availability, temperature or sleep duration – are reversible and may occur frequently; therefore, cellular stress responses must be robust and immediate, but equally, they must be reversible. In contrast, genotoxic stress, aging and disease-related stress are of a more permanent nature (Chinta et al., 2013; Reinhardt and Schumacher, 2012; Suh et al., 2002); in the long term, they globally alter multiple intracellular signaling pathways that control almost every aspect of cellular physiology. Such persistent stresses cause cells to adjust their metabolism and even cellular architecture to survive.

The speed and scale of gene expression readjustment are crucial parameters for optimal cell survival upon stress, and this adjustment can happen at either the transcriptional or the post-transcriptional level (Fig. 1). During persistent stress, coordinated transcription factor networks are prominent regulators of the adaptive responses that result in global changes in gene expression, which is important for slow but long-lasting adaptation and recovery (de Nadal et al., 2011; Gray et al., 2014; Novoa et al., 2003). Conversely, it is becoming increasingly clear that, in conjunction with transcription factors and signaling pathways, microRNAs (miRNAs) play important roles in the maintenance of cellular homeostasis. In particular, they orchestrate an immediate and reversible stress response (Cicek et al., 2016; Edeleva and Shcherbata, 2013; Fan et al., 2013; Leung and Sharp, 2010; Mendell and Olson, 2012; Wu et al., 2011).

Moreover, such a stress response happens at the level of post-transcriptional regulation and involves mRNA metabolism; for example, alternative splicing of pre-mRNA, and mRNA transport, storage, translation and degradation (Courchaine et al., 2016; Nott et al., 2015; Stoecklin and Kedersha, 2013; Zhu and Brangwynne, 2015). This regulation occurs in specialized subcellular membrane-less compartments or ribonucleoprotein (RNP) granules, which are also called liquid droplets (Fig. 1). These compartments are formed through phase-separation of RNA-binding proteins that contain low-complexity sequence domains (LCDs) (Brangwynne et al., 2015; Hyman et al., 2014; Mateju et al., 2017). These proteins are intrinsically disordered, which makes them impossible to crystalize (Tompa et al., 2015; Uversky, 2016). They can promiscuously interact with multiple proteins (Kato et al., 2012; Kroschwald et al., 2015), which complicates defining their biological functions, and how they are regulated such that they are included in different RNPs upon reversible stress is poorly understood *in vivo*. Interestingly, many splicing factors and key enzymes that regulate miRNA biogenesis contain LCDs or bind to other LCD-containing proteins, and localize to stress-induced nuclear and cytoplasmic compartments during stress (Agranat-Tamir et al., 2014; Damianov et al., 2016; Kato et al., 2012; Kawahara and Mieda-Sato, 2012; Shen et al., 2013; Twyffels et al., 2011). In this Review, we discuss two critical mRNA regulation events that govern the adaptive stress response in cells: alternative pre-mRNA splicing and miRNA-based mRNA targeting. We define how these two processes are intercalated and how – upon stress – their key enzymes are redirected to various liquid-like subcellular compartments to assure a robust cellular stress adaptation and maximize survival upon unfavorable environmental changes.

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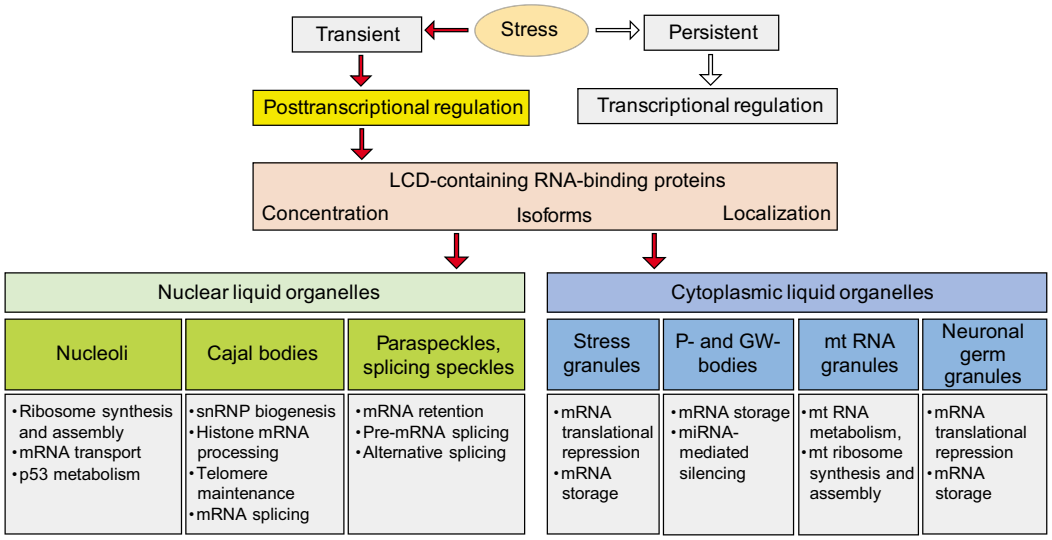

Fig. 1. RNA-containing membrane-less compartments involved in the stress response. Depending on the duration of the stress (transient or persistent), a cell adjusts gene expression through post-transcriptional (faster) or transcriptional (slower) regulation. The post-transcriptional regulation occurs in various nuclear (green) and cytoplasmic (blue) liquid-like organelles that have distinct functions in the regulation of RNA metabolism. These membrane-less organelles are formed through a process of biochemical phase separation, which is mediated by proteins that contain RNA-binding domains and LCDs. Potential mechanisms that affect phase separation are changes in protein concentration, isoform specificity or post-translational modifications that regulate the binding affinities of phase-separating proteins. mt, mitochondrial.

Stress and membrane-less subcellular compartments

Large-scale post-transcriptional regulation requires the cooperation of hundreds of components, many of which organize into complex macromolecular centers. These stress-responsive centers often involve the controlled reorganization of the cytoplasm and the formation of spatially restricted membrane-less compartments. Here, the majority of post-transcriptional gene regulation is achieved (Hyman et al., 2014; Lavut and Raveh, 2012; Müller-McNicoll and Neugebauer, 2013; Zhu and Brangwynne, 2015). In fact, such subcellular compartmentalization can minimize the delay time for individual steps of gene processing, which is especially crucial during stress, when large pools of RNAs have to be processed, stored, silenced or degraded (Pontius, 1993; Wright and Dyson, 2015).

There are many types of membrane-less compartments (Fig. 1). Among them are the cytoplasmic RNP assemblies – stress granules (SGs) (Kedersha et al., 1999), processing bodies (PBs) (Eulalio et al., 2007a; Jain and Parker, 2013), GW-bodies (Eulalio et al., 2009; Stoecklin and Kedersha, 2013), germline P-granules (also known as P-bodies) (Brangwynne et al., 2009; Voronina et al., 2011), neuronal granules (Buchan, 2014), mitochondrial RNA granules (MRGs) (Antonicka and Shoubridge, 2015), as well as the nuclear RNP assemblies – nucleoli (Brangwynne et al., 2011; Mahboubi and Stochaj, 2014), nuclear pores (Frey et al., 2006), Cajal bodies (CBs) (Machyna et al., 2013; Staněk and Neugebauer, 2006), promyelocytic leukemia (PML) bodies, histone locus body (Nizami et al., 2010), splicing speckles, other nuclear speckles and paraspeckles (Fox and Lamond, 2010; Knott et al., 2016; Shevtsov and Dundr, 2011).

Stress granules

Even in evolutionarily distant organisms, for example, plants, yeast, worms, insects and mammals, stress causes the transient formation of SGs, which are linked to the inhibition of translation (Anderson et al., 2015; Buchan and Parker, 2009; Gilks et al., 2004; Guil et al., 2006; Kedersha et al., 2005; Lavut and Raveh, 2012; Mateju et al., 2017; Molliex et al., 2015). SGs contain stalled translation pre-initiation complexes, which consist of mRNAs, translation initiation factors, ribosomal subunits and numerous other RNA-binding proteins that regulate mRNA functions (Buchan and Parker, 2009; Kayali et al., 2005). Induced by stress, SGs are very dynamic and continuously exchange RNAs and proteins with the cytoplasm, and their formation can be promoted or impaired by the overexpression and/or depletion of multiple factors (Anderson et al., 2015; Buchan and Parker, 2009; Kroschwald et al., 2015; Mahboubi and Stochaj, 2014; Mateju et al., 2017).

Processing bodies and GW-bodies

The other prominent cytoplasmic liquid-like granules are PBs, which contain mRNAs, a pool of mRNA-decay enzymes, proteins that are involved in translational repression and gene silencing, and factors for mRNA transport and modification (Eulalio et al., 2007a; Jain and Parker, 2013). Over the past decade, many studies have revealed the specific functions of PBs (Anderson et al., 2015; Eulalio et al., 2007a; Lavut and Raveh, 2012; Stoecklin and Kedersha, 2013). Previously, it was believed that PBs are centers for mRNA decay and turnover; however, it has been subsequently shown that mRNA decay does not occur in these granules, and the only confirmed function remains mRNA storage (Eulalio et al., 2007b,c). PBs also appear to be scaffolding centers for miRNA function (Liu et al., 2005a,b). Closely related to PBs are GW-bodies, which contain GW182 (also known as TNRC6) family proteins that are essential for miRNA-mediated gene silencing. GW182 proteins are recruited to miRNA targets through direct interactions with Argonaute (Ago) proteins, which promotes target silencing (Braun et al., 2013). Unlike SGs, PBs and GW-bodies are present under stress-free conditions, but their size and numbers also increase in response to stresses (Kedersha et al., 2005). Several studies have demonstrated an interaction between SGs and PBs; in particular, during stress, these two compartments dock onto each other, but do not mix together (Halstead et al., 2015; Stoecklin and Kedersha, 2013).

The nucleolus and Cajal bodies

In the nucleus, the nucleolus is the largest membrane-less RNP-containing organelle, and also functions as a stress sensor (Brangwynne et al., 2011; Feric et al., 2016). The nucleolus serves primarily as a site of ribosome synthesis and assembly; however, many additional functions have been revealed (Boisvert et al., 2007; Olson, 2004; Pederson, 1998; Raška et al., 2006; Rubbi and Milner, 2003), for example, telomerase activity, and regulation of the cell cycle, stress response and aging (Boulon et al., 2010; James et al., 2014; Zink et al., 2004). Stress influences nucleolar activity by affecting its morphology and three-dimensional structure, which is accompanied by alterations in nucleolar protein distribution and composition (Boulon et al., 2010). Under unfavorable environmental conditions, the nucleolar p53-linked stress-sensing mechanism allows the cell to halt the energy-consuming process of ribosome biogenesis, thereby slowing cell growth and proliferation (Holmberg Olausson et al., 2012; Zhang and Lu, 2009). Importantly, the nucleolus often acts together with CBs, which function as hubs for proteins that are involved in RNA processing and mRNA splicing (Staněk and Neugebauer, 2006). In addition, CBs have been implicated in histone mRNA processing and telomere maintenance (Gall, 2003; Machyna et al., 2013). Thus, the dynamic sequestration and release of specific proteins and RNA complexes in the nucleolus and CBs is an important mechanism by which cellular stress responses are regulated.

Paraspeckles

Paraspeckles are subnuclear RNP bodies that are located within the interchromatin space of mammalian cell nuclei (Fox et al., 2002). They are predominantly defined by colocalization of dynamic and multifunctional RNA-binding proteins, such as splicing factor, proline- and glutamine-rich (SFPQ), non-POU domain-containing octamer-binding protein (NONO) or paraspeckle component 1 (PSPC1). These factors belong to the *Drosophila* behavior/human splicing (DBHS) protein family that forms a dynamic scaffold with the long noncoding RNA *NEAT1* (Bond and Fox, 2009; Clemson et al., 2009; Fox et al., 2005; Knott et al., 2016). The major function of paraspeckles is nuclear retention of mRNA that contains double-stranded (ds)RNA structures at the 3′ untranslated region (UTR), which are generally formed by adenosine-to-inosine edited inverted repeats (Fox and Lamond, 2010). The nuclear retention of mRNA is involved in many cellular processes that include the stress response; for example, stress signals mediate 3′UTR cleavage and cause RNA release from the nucleus (Prasanth et al., 2005). Besides being essential paraspeckle components, DBHS proteins act as molecular scaffolds that synergistically associate with a broad spectrum of transcription factors, DNA and RNA. This allows the scaffold to promote transcription initiation, elongation and termination, to facilitate co-transcriptional processing – in particular splicing – and to regulate mRNA transport and cytosolic trafficking (Knott et al., 2016). In addition, paraspeckles may mediate regulatory crosstalk with the nucleolus: paraspeckle proteins localize to perinucleolar caps when RNA Pol II transcription is inhibited, and it has been observed that PSPC1 shuttles between paraspeckles and nucleoli (Fox and Lamond, 2010).

Liquid-like organelle formation through phase separation

Interestingly, electron microscopy studies have confirmed that there is no stable structural organization of liquid-like organelles, whose shape and size fluctuate considerably over time and under different conditions (Gilks et al., 2004). At the same time, multiple processes that globally regulate gene expression upon stress take place in these organelles, whose dynamic composition and physical properties in turn depend on stress and the cellular type, growth and cell cycle stage.

Recent work on the physical properties of liquid-like organelles, their protein content and protein domain structures has led to paradigm-shifting insights into their formation. It was revealed that membrane-less subcellular compartments are viscous liquid droplets of multiple components that are formed through a biochemical phase separation of proteins that contain LCD domains (Elbaum-Garfinkle et al., 2015; Kato et al., 2012; Molliex et al., 2015; Nott et al., 2015; Patel et al., 2015). In principal, these structures are condensed liquids that are separated from the surrounding liquid (e.g. cytoplasm or nucleoplasm) by means of molecular supersaturation. The potential mechanisms that cause such a liquid supersaturation, and consequently phase separation, include an increased concentration of LCD-containing proteins and post-translational modifications that affect the charge of proteins (Brangwynne et al., 2015; Kwon et al., 2013; Nott et al., 2015; Schüller and Eick, 2016). As a physical chemical phenomenon, the formation of phase-separated compartments is exquisitely sensitive to changes in environmental conditions. This could be, in part, due to changes in cellular salt and/or proton concentration, as a result of stress through a change in temperature, pH, or osmosis, or due to certain intrinsic molecular changes (Hyman et al., 2014; Jain and Parker, 2013; Kroschwald et al., 2015; Mateju et al., 2017; Wu et al., 2011). Intrinsically disordered sequences, such as LCDs, have been linked to protein–protein, protein–DNA and protein–RNA interactions; moreover, multiple post-translational modifications of disordered sequences can modulate their RNA-binding activity, subcellular localization, folding and self-association (Calabretta and Richard, 2015). Consequently, liquid-like organelle formation depends on many promiscuous interactions, which makes the material state of the RNP granule flexible (Kroschwald et al., 2015). However, such protein promiscuity, coupled with genetic mutations and stressful conditions, results in undesirable interactions that are observed in numerous human diseases. For example, LCD proteins can aggregate irreversibly, which causes subcellular accumulation of solid-like amyloid fibers that can result in pathologies (Fiumara et al., 2010). In fact, protein aggregates have been implicated in a variety of neurodegenerative diseases, known collectively as amyloidosis. They include amyotrophic lateral sclerosis, spinocerebellar ataxia, Huntington's, Alzheimer's and Parkinson's disease, and prion diseases (Wolozin, 2012; Wright and Dyson, 2015). Moreover, several studies suggest that age-related protein aggregation might be a widespread event, even in healthy organisms (Lindner and Demarez, 2009). The transition of physiological liquid droplets to potentially pathological solid-state aggregates may thereby rely on changes in protein concentrations (Brangwynne et al., 2015; Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). This hypothesis was confirmed experimentally for the LCD-containing RNA-binding proteins fused in sarcoma (FUS) and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), both of which are linked to human diseases. Moreover, misfolded proteins, such as ALS-linked variants of superoxide dismutase 1 (SOD1), can specifically accumulate and aggregate with SGs, which influences their composition and dynamics and triggers an aberrant liquid-to-solid transition (Mateju et al., 2017). Importantly, pathogenic ALS-causing mutations in FUS are sufficient to inhibit miRNA biogenesis (Eitan and Hornstein, 2016; Emde et al., 2015).

Thus, based on these findings, it is becoming clear that aspects of RNA metabolism depend on the type and concentration of phase

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separation proteins. Therefore, it is critical to understand how their expression is controlled – especially under stress conditions. miRNAs are prime candidates to control gene expression, as the miRNA expression profile is substantially changed in response to different stresses or diseases (Emde et al., 2015; Freiesleben et al., 2016; Lu et al., 2005; Marrone et al., 2012; Piwecka et al., 2015). This change in miRNA expression has a profound impact on the ability of the cell, or even the whole organism, to manage stress responses (Cicek et al., 2016; Edeleva and Shcherbata, 2013; Leung and Sharp, 2010; Mori et al., 2012). Stress also affects the subcellular localization of miRNAs. Numerous miRNAs, their mRNA targets and RNA-induced silencing complex (RISC) components have been detected in stress-induced and/or stress-sensitive RNP granules (SGs and PBs) (Stoecklin and Kedersha, 2013). This implies that miRNA-biogenesis and/or miRNA-based targeting is linked to subcellular membrane-less compartments.

miRNA biogenesis and liquid-like organelles

The microprocessor complex

miRNAs comprise a class of non-coding RNAs that are transcribed as large primary transcripts (pri-miRNAs). The pri-mRNAs form hairpin structures that are recognized and cleaved by the microprocessor complex [Drosha and diGeorge syndrome critical region 8 (DGCR8) proteins] into intermediate ∼70-nucleotide precursor miRNAs (pre-miRNA) in the nucleus. In addition, many miRNA genes are located within host genes and processed by Drosha at the same time as pre-mRNA splicing (Kim and Kim, 2007), which suggests that miRNA biogenesis and splicing are interconnected (Fig. 2). It has been observed that flanking exons increase pri-miRNA retention at active transcription sites, which contributes to increased levels of intronic pri-miRNAs (Pawlicki and Steitz, 2008). Interestingly, cleaved and polyadenylated pri-miRNAs that escape processing at transcription sites appear immune to cleavage by Drosha and accumulate in nuclear foci that normally contain unspliced pre-mRNAs, microprocessor components and serine/arginine-rich splicing factor (SC35; also known as SRSF2) (Pawlicki and Steitz, 2008). SC35 promotes stress-induced alternative splicing of neuronal mRNA and promotes transcriptional elongation (Lin et al., 2008; Meshorer et al., 2005).

Drosha and DGCR8, as well as pre-miRNAs, are also detected in spliceosomes and splicing speckles, where nuclear post-transcriptional processing is executed (Kataoka et al., 2009) (Fig. 2). Moreover, inhibition of splicing increases the expression of miRNAs, whereas knockdown of Drosha increases splicing (Agranat-Tamir et al., 2014). Drosha per se acts as an alternative splicing factor that enhances inclusion of exons that are capable of

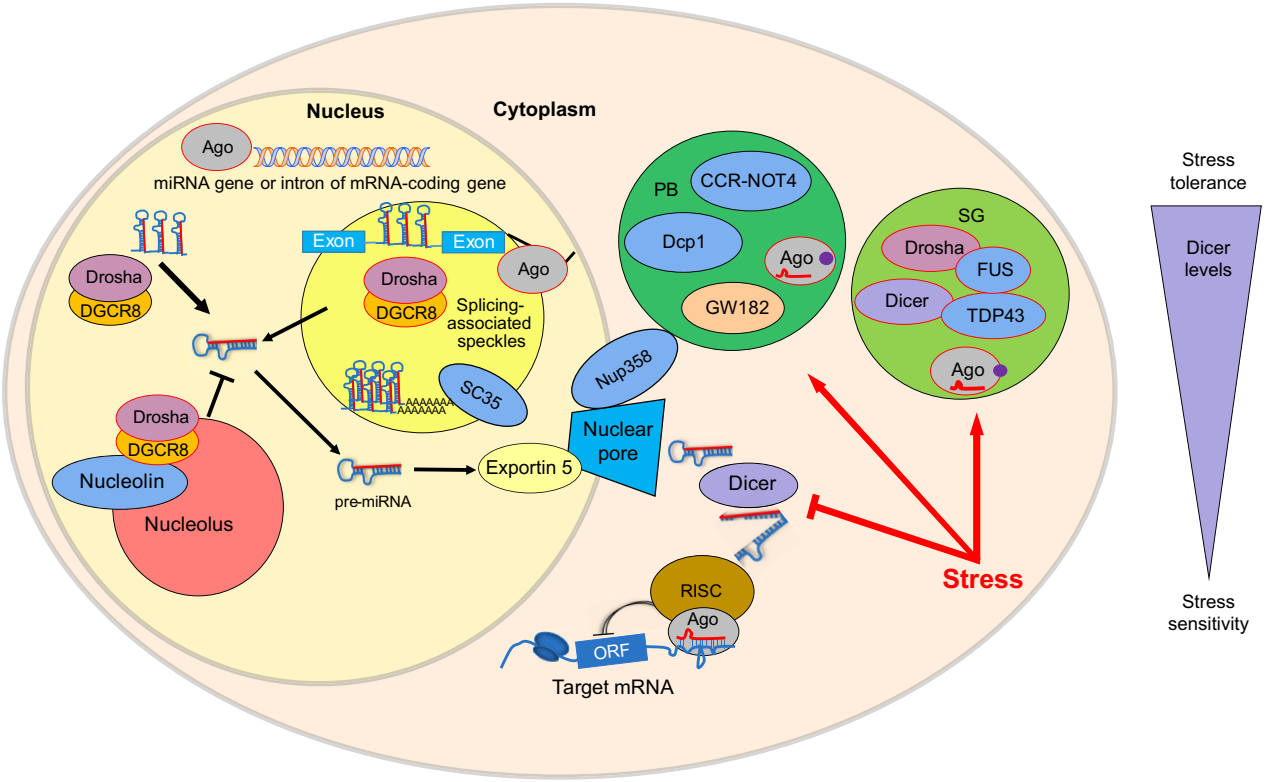

Fig. 2. Influence of stress on function and localization of key enzymes of miRNA biogenesis. The nuclear microprocessor complex (Drosha–DGCR8) processes pri-miRNAs into intermediate ∼70-nucleotide pre-miRNA. Cleaved and polyadenylated pri-mRNAs that have escaped processing by Drosha accumulate in SC35-containing nuclear splicing-associated speckles, in which the microprocessor complex produces pre-miRNAs from exons of pre-mRNAs concurrently with pre-mRNA splicing. Upon stress, Drosha interacts with nucleolin and localizes to the nucleolus, or accumulates in cytoplasmic stress granules (SGs), which may also contain Dicer, Ago proteins, TDP43 and FUS. Pre-miRNA is transported by exportin 5 via the hydrogel-like nuclear pore to the cytoplasm, where it is cleaved by Dicer into a mature miRNA and loaded into the Ago-containing RISC for mRNA targeting. Nup358 associates with P-bodies (PBs) that contain proteins involved in mRNA degradation (the CCR-NOT4 complex, Dcp1, and the miRNA effector complex, which includes mature miRNA, GW182, Ago proteins and other RISC proteins). Upon stress, Ago is modified, which affects its subcellular localization and function. In the nucleus, Ago either binds near gene promoters and regulates gene expression or localizes to splicing-associated speckles and controls the alternative splicing of pre-mRNAs. In the cytoplasm, Ago phosphorylation or hydroxylation enhances its association with SGs and PBs, which decreases efficiency of miRNA-based targeting. The localization of proteins under normal conditions is shown by black outlines, and under stress by red outlines. ORF, open reading frame.

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forming a pre-miRNA-like hairpin structure that resembles a canonical Drosha substrate (Havens et al., 2014). These data demonstrate that an RNA sequence has the potential to function as an exon or a miRNA, and that Drosha can mediate this decision; this implies that an intimate association between miRNA biogenesis and pre-mRNA splicing exists. Taking into account that the microprocessor complex is associated with the spliceosome and that RNA hairpins are the most common RNA structure, cleavage by microprocessor could significantly influence mRNA processing.

Moreover, Drosha itself is subjected to alternative splicing (Fig. 3) that results in the appearance of isoforms that are differentially localized to the nucleus or cytoplasm (Link et al., 2016). In particular, the subcellular localization of Drosha, its stabilization, and its response to stress depends on the presence of alternatively spliced arginine/serine-rich (RS-rich) domains. It has been found that upon stress, Drosha is highly enriched in the cytoplasm (Yang et al., 2016). Thus, the function of Drosha is stress-dependent and at the same time, Drosha influences cell survival; at lower levels, it sensitizes cells to stress, promoting cell death, whereas at higher levels, stress-induced death is reduced (Fan et al., 2013; Link et al., 2016; Yang et al., 2015).

In addition, in the nucleus, microprocessor complex components are associated with nucleolin, a nucleolar protein that is critical for rRNA processing (Fig. 2). Its deregulation affects miRNA biogenesis specifically at the primary to precursor stage of processing (Pickering et al., 2011). This suggests that the stress-sensing nucleolus may also play a role in the modulation the initial steps of miRNA biogenesis.

The Dicer complex

After being cleaved in the nucleus by the microprocessor complex, pre-miRNAs are transported through the nuclear pore by Exportin 5 (Fig. 2). The nuclear pore, which also exhibits features of a hydrogel (Frey et al., 2006), dynamically associates with PBs and SGs; in particular, the nucleoporin Nup358 (also known as RANBP2) interacts with Argonaute (Ago) and GW182 proteins, and its depletion disrupts PBs and impairs the miRNA pathway (Sahoo et al., 2017). In the cytoplasm, the pre-miRNAs are further processed by Dicer to form mature, ∼22-nucleotide miRNAs. Interestingly, levels of Dicer have been shown to directly correlate with cellular stress resistance (Ho et al., 2012; Mori et al., 2012; Wiesen and Tomasi, 2009). For example, Dicer depletion in murine fat tissue causes hypersensitivity to stress, whereas, conversely, Dicer overexpression results in stress tolerance (Mori et al., 2012). Cellular stresses, such as reactive oxygen species (ROS), phorbol ester and Ras oncogene activation also inhibit Dicer protein expression in several cell types (Wiesen and Tomasi, 2009), and Dicer expression and activity is important for adaptive cellular responses to chronic hypoxic stress (Ho et al., 2012). In addition, upon stress, Dicer and Drosha can directly bind to the LCD proteins TAR DNA-binding protein 43 (TDP43; also known as TARDBP) and FUS in SGs, which affects the efficiency of their function in miRNA processing (Eitan and Hornstein, 2016; Emde et al., 2015; Kawahara and Mieda-Sato, 2012). These data demonstrate that Drosha and Dicer, key components that regulate the first steps of miRNA biogenesis, are controlled by stress, which in turn affects the cellular ability to cope with stress.

The effector complex

After being cleaved by Dicer, mature miRNAs are incorporated into RISC to form the so-called miRISC (Fig. 2), in which an Ago protein acts as the major catalytic component that is involved in post-transcriptional repression of targeted mRNAs (Hutvagner and Simard, 2008; Hutvagner and Zamore, 2002; Mourelatos et al., 2002). Several studies have shown that stress causes post-translational modifications of Ago proteins (Qi et al., 2008; Shen et al., 2013; Wu et al., 2011; Zeng et al., 2008). This is often coupled with its subcellular translocation to RNP granules, thereby ultimately influencing miRNA biogenesis and/or function. For example, in human cells, the EGFR-controlled tyrosine phosphorylation of Ago2 takes place in response to hypoxic stress, which affects miRNA maturation (Shen et al., 2013), whereas the p38 MAPK family-mediated serine phosphorylation of Ago2 in response to sodium arsenite or anisomycin, which induce oxidative stress and inhibit

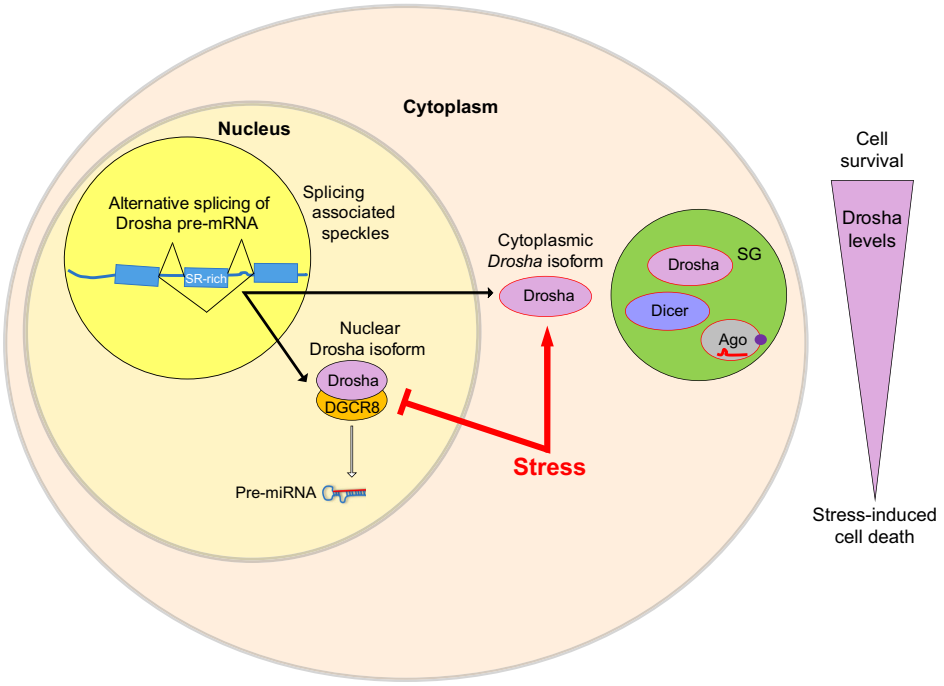

Fig. 3. Alternative splicing of Drosha affects its subcellular localization. *Drosha* pre-mRNA is subjected to stress-dependent alternative splicing, which affects Drosha protein function and localization. Drosha is predominantly localized in the nucleus, where it is involved in pre-miRNA production. Upon stress, increased levels of Drosha are found in the cytoplasm, particularly in the stress granules (SGs) that may also contain other proteins that are involved in miRNA biogenesis, such as Dicer and Ago. Low levels of Drosha accelerate stress-induced cell death, while its high levels promote cell survival. The localization of proteins under normal conditions is shown by black outlines, and under stress by red outlines.

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protein biosynthesis, respectively, facilitates its relocation to PBs (Zeng et al., 2008). Another study has shown that proline hydroxylation of Ago2 influences its stability (Qi et al., 2008), which, under hypoxia, leads to Ago2 association with SGs (Wu et al., 2011). Recently, it has been proposed that miRISC bound to target mRNA progressively matures from a form that scans and recognizes mRNA targets into an mRNA-bound effector mRNP particle that sequentially recruits and tethers the CCR4-NOT complex for mRNA decapping and decay (Wu et al., 2017). This demonstrates dynamic steps in miRNA-mediated silencing of effector mRNP assembly and the interaction with PB proteins. However, even though a significant fraction of RISC proteins localize to PBs, PB formation is not required for RNA-mediated gene silencing (Braun et al., 2013; Eulalio et al., 2007b, 2008). By contrast, blocking the miRNA silencing pathway at any step prevents PB formation and stability, indicating that PBs arise as a consequence of silencing (Eulalio et al., 2007b). Moreover, binding of mature miRNAs to the key RISC protein Ago2 is essential for Ago2 recruitment to PBs and SGs, which negatively regulates the efficiency of the RNAi pathways (Detzer et al., 2011; Pare et al., 2011). Most studies link the stress-dependent Ago translocation to PBs and SGs to attenuation of miRNA processing (Detzer et al., 2011; Shen et al., 2013), whereas others report increases in miRNA expression and, concomitantly, enhanced silencing of targeted mRNAs (Wu et al., 2011). Recently, it has been shown that the ability of a miRNA to regulate its target was enhanced by the granules that can be induced by overexpressing the PB components Dcp1a and GW182 (Wang et al., 2017). It is likely that PB- and SG-linked miRNA regulation has a cell type-, stress type- and stress duration-specific character. In addition, nuclear Ago2 regulates alternative splicing through its binding to G-rich sites of pre-mRNAs and also binds to chromatin sites near gene promoters to negatively regulate transcription; importantly, both of these activities are independent of the catalytic activity of Ago2 (Taliaferro et al., 2013).

Thus, similar to many other RNA-binding proteins that contain LCDs and assemble RNA granules, multiple proteins that are involved in miRNA biogenesis localize to nuclear and cytoplasmic subcellular RNP compartments in a stress-dependent manner. However, mechanistic studies are required for a more complete understanding of miRNA-based regulation in membrane-less subcellular compartments upon stress. Taking into account that the key players of miRNA biogenesis (Drosha, Dicer and Ago) are recruited to cytoplasmic RNA granules upon stress, which negatively regulates their ability to produce mature and functional miRNAs, it is logical to assume that, in general, the formation of stress-induced RNA compartments impairs the ability of miRNAs to silence genes under stress.

miRNAs control cellular stress responses by targeting alternative splicing proteins and vice versa

Even though the role of the miRNA pathway in mediating a stress reaction is well recognized, the functions of specific miRNAs in regulating particular aspects of cellular stress-responsive mechanisms are just beginning to emerge. In response to stress, miRNAs can regulate various targets and processes to readjust cellular homeostasis for survival. First, miRNA expression patterns can be temporally and spatially highly dynamic within the tissue and upon changes in environmental conditions (Cicek et al., 2016; König et al., 2011; Pasquinelli and Ruvkun, 2002; Rougvie, 2005; Yatsenko and Shcherbata, 2014). This suggests that miRNAs act strictly in the cells that require readjustment of gene expression. Second, miRNAs frequently operate in regulatory loops to control their own upstream regulators in order to increase miRNA levels and amplify target mRNA silencing (Konig and Shcherbata, 2015; Kucherenko et al., 2012). Third, a miRNA can be involved in simultaneous repression of several regulatory factors that control the same signaling cascade; for example, upon starvation, miRNAs from the *miR-310s* complex target several components of the conserved cholesterol-dependent hedgehog signaling pathway, thereby governing a quick and robust dietary stress response (Cicek et al., 2016). Thus, stress-dependent alterations in miRNA expression can affect multiple mRNAs simultaneously through direct targeting. However, recent data also support the idea that miRNAs can regulate multiple RNAs indirectly by targeting LCD-containing proteins that assemble various RNP granules that are involved in RNA metabolism regulation (Boguslawska et al., 2016; Boutz et al., 2007; Kucherenko and Shcherbata, 2018; Pare et al., 2011).

miRNAs target alternative splicing factors

In particular, several miRNAs have been shown to regulate factors of alternative splicing (Fig. 4), which has a genome-wide effect on mRNA expression profiles (Boguslawska et al., 2016; Fu and Ares, 2014; Kalsotra et al., 2010; Kucherenko and Shcherbata, 2018). For example, miRNAs coordinate networks of alternative splicing events in postnatal heart development through targeting of CUG-binding protein, Elav-like family (CELF) proteins. Particularly,

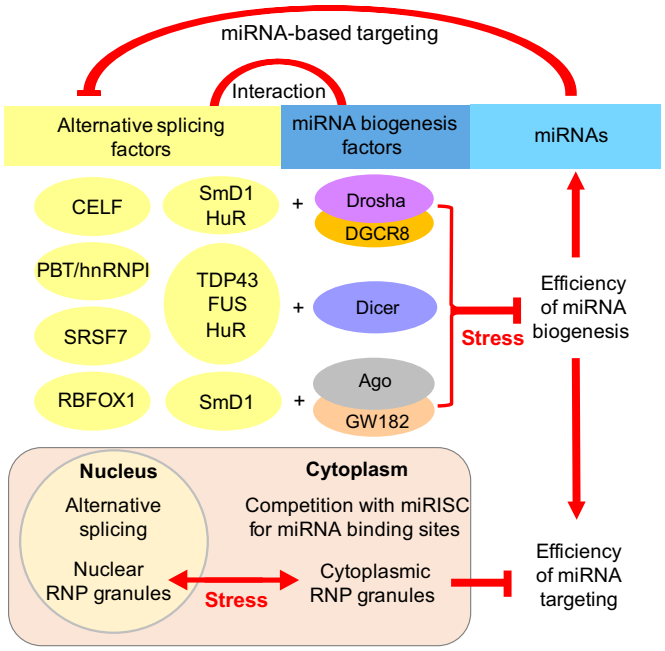

Fig. 4. The cellular interplay of alternative splicing and RNA-binding proteins. Alternative splicing factors travel between the nucleus and cytoplasm and associate with RNA-binding protein complexes, which allows them to perform different functions. In the nucleus, they control alternative splicing of pre-mRNAs; in the cytoplasm, they compete with miRNAs for binding to mRNAs, which negatively affects miRNA targeting efficiency. In addition, alternative splicing factors interact with miRNA biogenesis enzymes, which negatively affects miRNA production efficiency. Notably, these interactions are stress-dependent, suggesting their importance in the cellular stress response. On the other hand, miRNAs can directly target several alternative splicing factors, which globally influences the alternative splicing profile. Thus, the interplay between miRNA-based gene silencing of mRNAs and alternative splicing of pre-mRNAs is a crucial post-transcriptional mechanism that occurs in liquid-like organelles to regulate the cellular stress response.

miR-23a and *miR-23b* target CUGBP and ETR-3-like factor (CELF1 and CELF3), which control nearly half of the alternative splicing choices in the heart (Kalsotra et al., 2010). Not only do CELF proteins bind to introns in pre-mRNAs to mediate alternative splicing in the nucleus, they are also powerful modulators of mRNA decay, as they compete with other RNA-binding proteins for the binding to GU-rich elements (Liu et al., 2015; Vlasova-St Louis et al., 2013). In the cytoplasm, CELFs bind to 5′ and 3′UTRs in mature mRNAs to regulate deadenylation, mRNA stability and translation (Dasgupta and Ladd, 2012). Interestingly, several regions of CELF2 that control its subcellular localization have been identified, such as exclusion from the nucleolus, localization in the perinuclear compartment and translocation to cytoplasmic SGs (Fujimura et al., 2008; Ladd and Cooper, 2004). In addition, *miR-133* targets a key factor for alternative splicing, the polypyrimidine tract-binding protein (PTB, also known as hnRNPI) (Boutz et al., 2007). During viral infection, hnRNPI relocalizes from the nucleus to SGs, where it binds genomic and subgenomic RNAs that regulate posttranscriptional viral gene expression and viral stress response (Sola et al., 2011).

It has been also demonstrated that the subcellular localization of another group of alternative splicing factors, the serine/arginine-rich splicing factor (SRSF) proteins undergoes major alterations upon stress. Besides their nuclear functions in splicing, several SRSF proteins shuttle between the nucleus and the cytoplasm, participate in nonsense-mediated mRNA decay (NMD) and mRNA translation, and localize to SGs (Twyffels et al., 2011). Moreover, SRSF family members contribute to the integrity of the nuclear speckle (Morimoto and Boerkoel, 2013). It has been shown that *miR-30a-5p* and *miR-181a-5p* target one of the SRSFs, SRSF7, and this targeting is important for splicing and mRNA export of cancer-related genes, suggesting that miRNAs contribute to SRSF-dependent mechanisms of translational gene regulation (Boguslawska et al., 2016).

Another example of stress-dependent regulation of alternative splicing factors by miRNAs is the targeting of an LCD-containing RNA-binding protein Rbfox1 by *miR-980*, which promotes cell survival upon stress (Kucherenko and Shcherbata, 2018). *Drosophila* Rbfox1 is the single homolog of the human RBFOX family proteins, which have been linked to multiple diseases and pathological conditions; examples are spinocerebellar ataxia, mental retardation, epilepsy, attention-deficit hyperactivity disorder, autism, hand osteoarthritis, congenital heart defects, obesity, diabetes and glioblastoma (Bhalla et al., 2004; Davis et al., 2012; Gehman et al., 2011; Hamada et al., 2015; Joshita et al., 2010; Ma et al., 2010; Martin et al., 2007). Human RBFOX proteins associate with a multimeric complex of proteins called the large assembly of splicing regulators (LASR), which resides in specific nuclear fractions; namely, the insoluble high molecular mass fraction that contains chromatin, nuclear speckles and unspliced RNA (Damianov et al., 2016). Interestingly, several components of this complex contain LCDs and have been identified as liquid droplet components (Han et al., 2012). When *miR-980* is lost or repressed upon stress, Rbfox1 levels are increased. This affects a whole range of cellular processes that include cellular differentiation, adhesion, autophagy, apoptosis and cell survival (Kucherenko and Shcherbata, 2018). The protein promiscuously incorporates into various nuclear and cytoplasmic RNP granules, and, depending on the severity of cellular stress, these Rbfox1-containing RNP granules range in their chemical properties from liquid droplets to amyloid-like fibers (Kucherenko and Shcherbata, 2018). Importantly, our study also showed that a similar RBFOX–RNP-granule control of adaptive cellular stress responses also exists in humans, and might contribute to a wide range of RBFOX-associated pathologies. The stress-linked *miR-980–Rbfox1* regulation is an intriguing example of how a single miRNA can have a broad impact on cellular RNA metabolism by targeting an LCD-containing alternative splicing factor.

Alternative splicing factors modulate miRNA biogenesis and targeting efficiency

Conversely, not only do miRNAs regulate factors involved in alternative splicing, in different organisms, several splicing factors have been found to modulate the miRNA pathway (Bhattacharyya et al., 2006; Carreira-Rosario et al., 2016; Chen et al., 2016; Kawahara and Mieda-Sato, 2012; Lee et al., 2016; Mukherjee et al., 2016; Xiong et al., 2015). This indicates the evolutionarily widespread dual roles of alternative splicing factors in post-transcriptional gene regulation (Fig. 4). For example, in *Drosophila*, SmD1, a core component of the small nuclear ribonucleoprotein particle (snRNP) that is implicated in splicing, has been shown to be required for miRNA biogenesis and function (Xiong et al., 2015). In the nucleus, SmD1 interacts with both the microprocessor component Pasha (the DGCR8 homolog in *Drosophila*) and pri-miRNAs. In the cytoplasm, it colocalizes with components of the miRISC, including Ago1 and GW182, and this interaction is indispensable for optimal miRNA biogenesis (Xiong et al., 2015).

Another example of the regulation of miRNAs that is dependent on alternative splicing is the human ELAV protein HuR (also known as ELAVL1), which acts as a negative regulator of miRNA function. In human cells that are subjected to stress, HuR uncouples miRNAs from the target messages, thus relieving them of miRNA-mediated translational repression (Bhattacharyya et al., 2006). At the same time, HuR positively regulates extracellular vesicle-mediated export of *miR-122* and augments the stress response in human hepatic cells, in which in turn defective stress-induced *miR-122* export results in poor stress response and autophagy (Mukherjee et al., 2016). Furthermore, TDP43, a critical factor that drives SG dynamics, promotes miRNA biogenesis as a component of the Drosha and Dicer complexes (Kawahara and Mieda-Sato, 2012).

RBFOX proteins are alternative splicing factors that are also closely interconnected with the miRNA pathway. It has been demonstrated that human nuclear RBFOX2 regulates miRNA biogenesis by binding to miRNA precursors and repressing their processing (Chen et al., 2016). In the *Drosophila* germline, cytoplasmic Rbfox1 binds to specific sequences in 3′UTRs, which results in mRNA destabilization and translational silencing (Carreira-Rosario et al., 2016). In addition, in human neurons, cytoplasmic RBFOX1 competes with miRNAs for 3′UTR binding, which affects the stability of target mRNAs that are involved in cortical development and autism (Lee et al., 2016). Since Rbfox1 is not only involved in regulation of miRNA biogenesis and function, but is itself regulated by a miRNA (Kucherenko and Shcherbata, 2018), this suggests that Rbfox1 and miRNAs together are integrated in a stress-responsive signaling cascade. In this cascade, alterations in miRNA expression in response to stress result in the upregulation of a protein with broad pro-survival properties that affects alternative splicing, miRNA function and the formation of liquid-like organelles.

Conclusions and perspectives

Upon unfavorable conditions, a cell first adjusts its RNA metabolism in an attempt to adjust for a period of reversible

stress. This regulation is achieved globally through the formation of specialized subcellular compartments that contain RNAs and various RNA-binding proteins with LCDs. Interestingly, most eukaryotic proteins that regulate RNA biogenesis contain intrinsically disordered domains or LCDs. As they are characterized by little diversity in amino acid composition, this implies that they can interact with other LCD proteins and be included in various liquid-like organelles. In fact, RNA-binding proteins often shuttle between the nucleus and cytoplasm and associate with different partners and RNAs, which changes their functionality. Such subcellular dynamics are achieved by the presence of alternative splice forms, miRNA-dependent regulation of mRNA expression levels and protein modifications. This is true for key enzymes of miRNA biogenesis, as well as multiple alternative splicing factors that tend to associate with liquid-like organelles upon stress. This allows the increase of local activity of enzymes or the storage of a collection of functionally related molecules. Owing to high concentrations of RNA-binding proteins and RNAs in liquid-like organelles, competition for their binding partners occurs. This offers an enormous level of combinatorial possibilities and suggests a model of complex circuitry in order to harmonize gene regulation upon stress. Although there has been much progress in our understanding of how cellular compartmentalization is adapted upon stress, evidence is just emerging on how post-transcriptional gene regulation is employed to manage this process. Of particular interest is how miRNA expression and alternative splicing events allow cells to achieve elevated protein concentrations and isoform specificity to trigger formation of different liquid-like organelles in response to stress. The complexity of the interplay between the miRNA pathway and RNA regulatory proteins (Fig. 4) implies that multiple aspects should be considered while directing further efforts towards deciphering the role of miRNAs in the regulation of the cellular stress response. Nevertheless, such research will potentially allow development of miRNA-based therapeutics and uncover miRNAs that can act as biomarkers for age- and stress-related diseases, in particular neurodegeneration, muscle wasting and cancer.

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