Title: Revisiting the Stress concept in the context of solid tumors prognostic: a role for the Stress Granules?

Running title: Stress granules as novel actors in cancer er progression

Anaïs Aulas[1], Pascal Finetti[1], Shawn Lyons[2,3], François Bertucci[1], Daniel Birnbaum[1], Claire Acquaviva[1] & Emilie Mamessier[1]

[1]Predictive Oncology Laboratory, Cancer Research Center of Marseille (CRCM), Inserm U1068, CNRS UMR7258, Institut Paoli-Calmettes, Aix Marseille Université, Marseille, France

[2] Department of Biochemistry, Boston University School of Medicine, Boston, MA, USA;

[3]The Genome Science Institute, Boston University School of Medicine, Boston, MA, USA

Corresponding Author:

Anais.aulas@inserm.fr

ABSTRACT

Stress Granules formation is a pro-survival mechanism helping cells to cope with environmental challenges. Stress Granules have been studied for two decades in fundamental research, and are now being examined in the context of human pathogenesis. Here, we review studies highlighting stress granules' involvement in cancer development through translational pattern modification.

Keywords: Stress granules, G3BP1, G3BP2, Caprin-1, USP10, TIA1, TIAR, cancer prognosis, biomarker, metastasis, resistance, cell death, pro-survival properties

Introduction

The *stress response* is an ancestral evolutionary mechanism acquired by the first cellular organisms to protect them from sudden environmental or intracellular changes [1]. In response to stress, the cell first responds by activating pathways that promote its survival in the prospect of recovering from the insult. If the noxious stimulus persists, the cell then elicits a programmed cell death to eliminate the damaged/non-viable cell. As cell survival critically depends on the ability to elaborate an appropriate response towards environmental or intracellular stress stimuli, we can easily understand that this mechanism was highly conserved in evolution [2]. For example, heat shock proteins, which are among the most studied actors of the *stress response*, are activated by environmental stressors, such as heat, UV, tissue remodeling… and can be found in the lower organisms and the mammals. There are many different types of stress and even more possible stress responses that can be designed in return. Indeed, the effective stress response depends on the type and level of the insult. For example, protective responses such as the heat shock response or the unfolded protein response, mediate an increase in chaperone protein activity which enhances the protein folding capacity of the cell, thus counteracting the stress and promoting cell survival. The cell fate is ultimately determined by the cell's adaptive capacity and ability to recover from the stress.

At the level of the cell, any change in the environment that diverges from its optimal growth condition is considered as a stress and induces a *stress response*. All the processes involved are part of what is now known as the *stress response*, and include the activation of stress response genes, such as those coding for heat shock proteins, known for decades, but also of another mechanism recently discovered: the formation of *Stress Granules* (SGs) [1].

In this review, we will focus on stress granules (SGs), as actors of the stress response specifically induced by sudden noxious changes. In a second section, we will discuss the pro-survival effect of the SG and how they could participate in various pathologies. Then we will analyze their prognostic value in cancer. Finally, we will discuss the involvement or contribution of cellular stress responses to disease states.

The SGs are composed of proteins involved in the regulation of mRNA translation

Stress granules are membrane less cytoplasmic condensates, visible by conventional and electron microscopy, that were first discovered in 1999 by the laboratory of Dr. P. Anderson [3-6]. They have been reported in plants, yeast, worms, insects and mammalian cells [3, 7-11]. This high degree of conservation throughout multiples species highlights their importance for cell survival and cell integrity maintenance [12]. These cytoplasmic foci are composed of mRNA, RNA binding proteins and 40S ribosomes. The absence of membrane surrounding SGs and the extreme lability of the components has hindered the purification of these structures and the precise identification of SGs components by global analysis. Currently, even if methods are reported to purify SG markers [13, 14], the candidate approaches was for a long time the only way for identifying specific components. Most of the studies use immunofluorescence and FISH to robustly identify proteins and mRNAs included in these structures. This was not an easy task because the SGs are composed of proteins that generally switch their localization and functions between basal and stress conditions. In 2015, an inventory of the literature mentioned more than a hundred of proteins known to be recruited to SGs. These proteins form an eclectic mix belonging to various signaling pathways. Even if there is still no consensus to predict the recruitment of specific proteins to SGs, most of them are RNAs interacting proteins or are involved in the metabolism of RNA. There are also components involved in translation initiation such as eIF3 and eIF4 (Eukaryotic Initiation Factor) complexes proteins or PABP (PolyA Binding Protein) [15]. The presence of those components is not surprising, as SG form in response to a general translation inhibition. In homeostatic conditions, active translation is facilitated by the formation of a closed-loop mRNA (**Figure 1A**). This is a situation where the 5' and 3' ends of an mRNA are brought in close proximity. The 5' mRNA cap is bound by eIF4E and the 3' poly(A) tail is bound by PABP. These two proteins are bridged by the large scaffolding protein eIF4G. To initiate translation, the ternary complex, composed of eIF2:$tRNA_i^{Met}$:GTP facilitates decoding of the start codon which results in GTP to GDP hydrolysis. In response to stress, translation is rapidly inhibited, which, in most cases, results in SG formation. Two translation inhibition pathways can induce the formation of SGs

[4] (**Figure 1**). The phosphorylation of a subunit of eIF2, EIF2α (or EIF2S1), by one (or more) kinases, notably HRI (or EIF2AK1), PKR (or EIF2AK2), PERK (or EIF2AK3) and/or GCN2 (or EIF2AK4) [16], prevents the hydrolyzed GDP from leaving the ternary complex EIF2α-tRNA^{met}-GTP by block the formation of an active complex with ATP necessary for translation initiation (**Figure1 A-B**). Another stress response pathway centers on mTOR (**Figure 1C**). Under basal conditions, mTOR is active and constitutively phosphorylates eIF4E-BP protein (4EBP). Hyperphosphorylated 4EBP cannot interact with eIF4E, the mRNA cap binding protein. However, induction of a stress response inactivates mTOR leading to a rapid dephosphorylation of 4EBP thereby allowing it to interact with eIF4E. The eIF4E:4EBP interaction prevents eIF4E:eIF4G complex formation. It is worth pointing out that these pathways are not mutually exclusive. Depending on the type of stress, either or both pathways could be activated [16, 17]. While it is intuitive to understand that cell shut down translation to preserve energy, we could wonder what would be the survival advantage of being able to form SGs in response to environmental stress.

Stress Granules are pro-survival entities at the cellular level that can be involved in pathological conditions

Stressors triggering the formation of SGs can be as diverse as extreme temperatures (hot or cold), oxidative stress, osmotic stress, endoplasmic reticulum (ER) stress, mitochondrial stress, or UV irradiation (Previously reviewed [15]). Several lines of evidence point toward pro-survival benefits of SG formation, explaining the evolutionary conservation of this process. Upon mutations or knock-out of specific proteins involved in SG formation, or treatments decreasing the ability to form SG, cells die more easily and rapidly after stress exposition [18-23]. This pro-survival effect of SG formation could be explained by several independent mechanisms.

First, many pro-apoptotic signaling molecules are sequestered in SGs and it has been proposed that it prevents them from activating the pro-apoptotic cascade. It is the case for RACK1 (Receptor of

activated protein C kinase 1), TRAF2 (TNF receptor-associated factor 2) and RSK2 (Ribosomal S6 kinase 2) [24-26].

Second, while not fully characterized, SGs seem to protect cells from oxidative insults by reducing the level of cellular ROS [21, 27]. Indeed, the expression level of a major SG regulator G3BP1 **(**Ras GTPase-activating protein-binding protein 1), inversely correlates with the generation of reactive oxygen species (ROS) after exposition to oxidative insult. Moreover, overexpression of G3BP1 reduces the level of ROS compared to wild type cells. Cells expressing a truncated form of the protein that abrogates SG formation have an increased production of ROS. Similar results were obtained with USP10 (Ubiquitin carboxyl-terminal hydrolase 10), another SG regulating protein.

Lastly, the translation repression upstream SGs formation reduces the cellular energetic needs during stress by restricting the process of translation, which is consuming much ATP. By protecting mRNAs from stress-induced degradation, this allows cells to restart translation as soon as the stress is resolved without having to re-synthetize fresh RNAs [28]. Also, SGs sequester the untranslated mRNAs consecutively to the global inhibition of translation [29]. Some mRNAs, such as chaperone mRNAs, are excluded from the SG structures so that they can be preferentially translated during the time of the stress and participate to proper protein folding and avoid functional defects [29, 30]. By those actions, SGs are described as a triage center for translation of mRNAs during stress exposure. One growing hypotheses is that SGs are able to reshape translation pattern under stress exposure [31]. Some questions remain open: are the mRNA stored in SGs pre-selected according to the stress condition (mRNA important for cell survival in case the noxious condition is still present) or in the prospect of restarting a “normal” activity (most important mRNA insuring a proper the cell metabolism)?

The pro-survival role of SGs was demonstrated in various human pathological conditions, particularly in neurodegenerative diseases. In Amyotrophic Lateral Sclerosis (ALS), a plethora of proteins from many different pathways have been implicated [32]. For a long time, it was difficult to connect this diversity with the disease. But pioneer studies demonstrate that many ALS-relevant

proteins are recruited to SGs, and their mutation or mislocalisation cause aberrant regulation of SG and increased sensitivity to stress exposure [18, 33-38]. Now, the defect in stress response has become one of the leading hypothesis that explains neurons loss [15]. Another example for SGs pro-survival role was shown during antibiotic induced internal ear cells toxicity, which results in cilia loss [31]. The induction of SGs (using hydroxamate (-)-9) is able to rescue this defect [8].

However, in some cases, SGs pro-survival role might also not to be beneficial for the host. For example a pro-survival effect of SGs is not desired in cancer cells. Those cells are exposed to stresses such as hypoxia and nutrient deprivation, two stressors able to induce the formation of SGs [39]. Hypoxia has also been reported to promote resistance to therapies which could suggest a pro-survival role of SGs in this context [40]. Chemotherapies (CT) could be considered as stressors and induce the formation of SGs [41-46]. The cancer cell capacity to form SGs in response to CT is anti-correlated with cell survival *in vitro* [41-46]. Blocking the induction of these chemotherapy-induced granules by interfering with the phosphorylation of EIF2α increases the efficiency of the CT treatment [46]. Along the same line, some molecules can prevent SGs formation and restore the sensibility to the CT. This was shown in a study using hypoxia to induce chemo-resistance in HeLa cells. A screen of small molecules revealed that β-estradiol, progesterone and stanolone prevent SGs formation and restore the sensibility to the CT. Of note that the same molecules used in the MCF7 cancer cell line did not block the SG formation neither the chemoresistance induction [40, 46]. Blocking SG formation in cancer might look like an interesting option against cancer cells.

SGs major proteins and their prognosis role in cancer progression

During cancer development and propagation, cells acquired driver mutations that are responsible for cell transformation, then aggressiveness of the disease. Malignant transformation is a very complex and multifactorial mechanism, involving major changes in the initial genome, transcription and translation programs of the cell. For example, Epithelial-to-Mesenchymal Transition (EMT), acquisition of stemness or acquisition of drug resistance involve specific modifications of

these programs. Recently, some studies demonstrated that these changes of translational patterns occur after exposure to hypoxia stress [40, 47-49]. We know that a growing tumor is an extremely dynamic environment where stressors, such as mechanical constriction, hypoxia and/or starvation play a role at multiple levels. SGs could play a critical role in integrating those stressors with changes in translation that leads to cancer progression [31, 40].

SGs are composed of numerous proteins, but we will focus on some regulators: TIA-1 (T-cell-restricted intracellular antigen-1), TIAR (TIA-1-related protein), G3BP1 (Ras GTPase-activating protein-binding protein 1), G3BP2 (Ras GTPase-activating protein-binding protein 2), Caprin-1 (Cell Cycle associated protein 1) and USP10 (Ubiquitin carboxyl-terminal hydrolase 10) (**Figure 2**).

- TIA1 and TIAR have documented roles in immunity, RNA splicing and translation. Structurally, they bind RNA through RNA Recognition Motifs (RRM) (**Figure 2**). They are the historical markers for SG [3] [15]. Their overexpression induces spontaneous formation of SGs [3], but the knockout of each of them individually or simultaneously has never been reported to have an impact on SG formation. Unexpectedly, depletion of TIAR using a Dox inducible system triggers stress by activation of PKR and SGs induction in 50% of the cells [50].
- The G3BP1 protein also contains a RRM (**Figure 2**) and has been reported to have helicase and RNAse activity under normal conditions [6, 51]. G3BP1 is closely related to another protein, G3BP2, with which it shares 98% identity. Nowadays, G3BP1 and G3BP2 are considered as the master regulators of SGs. Their overexpression also induces spontaneous formation of SGs [6, 52]. Individual knockout partially inhibits or delays the formation of SGs [28], but the double knock out completely abolishes the formation of SGs [53].
- Finally, USP10 and CAPRIN1 are two interactors of G3BP1 (**Figure 2**), which compete with each other's to interact with their target. Both are binding G3BP1 on a short linear motif Phe-Gly-Asp-Phe, or FGDF-motif, and have opposing effects on SG formation: CAPRIN1/G3BP1/2 interaction favors SG formation, whereas USP10:G3BP interaction inhibits their formation

[53]. Those two proteins are not the sole regulators of G3BP1 aggregation, as the removal of the FGDF-motif does not influence the formation of SGs.

Knowing that these proteins are deeply involved in the composition and the regulation of SGs, their presence can be evaluated to estimate SGs formation capabilities of cells composing a tissue. Open access data on breast [54-88], colon [89-98] and pancreatic [99-112] cancer reveals that mRNA levels from *G3BP1, TIA1, TIAR* and *CAPRIN1* are mostly upregulated in primary tumors comparing to healthy tissues (**Figure Sup 1-3, Table 1 upper**). This suggests that tumor cells divert and exacerbate a pro-survival mechanism, here based on SGs related proteins, potentially to facilitate their survival in response to the numerous stresses encountered during tumor oncogenesis. This is true for most primary solid tumors. CAPRIN1 and G3PB1 show the most noticeable/prominent transcriptomic upregulation between healthy and primary tumor tissues. Of note that the overexpression between normal tissues and metastases was not as pronounced as in primary tumors, but still significant in most tumor types (**Figure 1-3, Table 1 lower**). Kaplan-Meier curves did not reveal major correlation with patient overall survival and disease/recurrence/metastasis free survival. The data analyses were pooled according to cancer type for representation, because no better correlations were found if the analysis were performed onto cancer subtypes.

Those data are surprising because correlations between SG proteins upregulation and poor prognosis have been reported on several occasions. G3BP proteins expression is of poor prognosis in various tumors, including sarcoma [113] colon [114], breast [114-117], thyroid [114], lung [118], head and neck [114], gastric [119, 120], hepatic [121] and prostate cancers [114, 122]. Higher TIA-1 protein levels correlate with poor prognosis in patients with colorectal cancer [123] and lymphoma [124]. High expression of CAPRIN1 protein correlates with poor prognosis for osteosarcoma [125], and hepatocellular carcinoma [126, 127]. Consistent with an inhibitory effect of USP10 on SG formation, high protein expression correlates with better prognostic in patients with gastric [128], ovarian [129], lung [130], small intestine [131], prostate [122] and gastric carcinoma [128].

All together, these contradictory results between mRNA and proteins levels suggest (**Figure 6-7**):

- *A transcriptional regulation early in the pathology;* to insure basal levels of the proteins involved in SGs function. This basal level is potentially the result of cancer cells subversion of the SGs mechanism to ensure their survival.
- *A post-transcriptional regulation;* impacting the final level of proteins. The translation rate of single mRNA encoding a SG protein is more efficient resulting in increased protein level without after mRNA level
- Or *a post translational modification*; enhancing the half-life of a protein. The degradation of the protein is delayed and enhances the overall level of protein.

Perspectives

Despite global survival improvement of cancer patients over the years, the cancer field is still facing the challenge of metastasis and chemoresistance in patients. Tumoral transformation is a multifactorial mechanism, involving accumulation of mutations and changes in the transcription and translation programs. Of course, the search for molecular alterations have been successful in the past, contributing to better tumors classification and to the development of efficient targeted therapies. But still, some patients are not answering or become refractory to a given therapy, meaning that treatments able to counter drug-induced mechanisms of resistance is the future challenge of the upcoming years. One reason that has limited progress in this field so far, is the fact that mechanisms of resistance might only be present during the course of the treatment, and may return to normal once the therapy is stopped. The reversibility of the phenomenon, also term “plasticity”, made it really difficult to apprehend, not being identifiable in the resected specimen [132]. It is highly possible that numerous biological processes required during metastasis occurrence are following the same pattern, for example when tumor cells are travelling in the blood flow. In all case, the mechanisms involved are most probably fast, “acute” and involved *ready-to-use* components, like those being part of the stress pathway. In this line, the cancer field is accumulating evidence for the SGs role in the adaptation and survival of cancer cells during tumor growth [114-116] and

chemoresistance [41-46]. SGs might thus turn out to be important actors of tumor cells plasticity in response to the various stress encountered, which will bring them as a target of choice in the fight against tumors development, progression and prevention of chemo-resistances.

SGs formation is the mechanism used by the cell to improve the translation of specific protein for a short period of time for survival. This overview of the literature, coupled to open data bases analyses, shows that tumor cells should have increased ability to form SG than normal ones based on their increased expression of SG regulator protein. In addition prognostic data correlates this SG expression with poor survival in patients. The exact mechanism for this increased protein level without transcriptional change is not known yet and could be the result of increased specific translation or decreased protein degradation. Further studies are warranted to understand the mechanism and to target this pathway for patient care because Inhibition of SG formation could be a promising therapy for refractory patients or in prevention of chemo-resistances.

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