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Stress induced subcellular distribution of ALG-2, RBM22 and hSlu7☆

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ABSTRACT

ALG-2 is a highly conserved calcium binding protein in the cytoplasm which belongs to the family of penta-EF hand proteins. Recently, we showed that ALG-2 is interacting with RBM22, a highly conserved spliceosomal nuclear protein (Montaville et al. Biochim. Biophys. Acta 1763, 1335, 2006; Krebs, Biochim. Biophys. Acta 1793, 979, 2009). In NIH 3T3 cells expressing both proteins a significant amount of ALG-2mRFP is translocated to the nucleus due to the interaction with RBM22-EGFP. hSlu7, another spliceosomal nuclear protein, known to interact with RBM22 in yeast, has been shown to translocate to the cytoplasm under cellular stress conditions. Here we provide evidence that the 2 spliceosomal proteins differ significantly in their subcellular distributions under stress conditions, and that RBM22 enhances the cytoplasmic translocation of hSlu7 under stress, especially a stress induced by thapsigargin. On the other hand, in NIH 3T3 cells expressing RBM22-EGFP and ALG-2-mRFP, ALG-2 remains translocated into the nucleus under both stress conditions, i.e. heat shock or treatment with thapsigargin. We could further demonstrate that these stress conditions had a different influence on the splicing pattern of XBP-1, a marker for the unfolded protein response indicating that ER stress may play a role in stress-induced translocation of spliceosomal proteins. The article is part of a Special Issue entitled: 11th European Symposium on Calcium.

1. Introduction

Activation of cells due to an external signal often results in up to a 100-fold rise in the intracellular free Ca^{2+} concentration. This can be due to the uptake of extracellular Ca^{2+} and/or the release of Ca^{2+} from intracellular stores. Important for the Ca^{2+} homeostasis is the endoplasmic reticulum (ER) which is involved in many specialized functions of the cell including regulation of protein folding and cellular stress response.

ALG-2 (apoptosis linked gene-2) is a 22-kDa highly conserved Ca^{2+}-binding protein, which is ubiquitously expressed. It contains 5 EF-hand motifs and forms dimers through the fifth EF-hand as shown by the crystal structure analysis [1]. This could provide a new interface for the interaction with possible targets [2,3] which interact with ALG-2 in a Ca^{2+}-dependent manner indicating that ALG-2–like CaM–may have Ca^{2+}-sensing properties. Therefore, the involvement in apoptosis, which gave the protein its name, would be one, but not the exclusive signal transduction pathway. In addition, recent studies with ALG-2-deficient mice indicate that ALG-2 is not essential for apoptotic responses in T-cells from ALG-2-deficient mice [4] induced by either stimulating T-cell receptors (TCR) and Fas/CD95 or by glucocorticoids.

Using a yeast two-hybrid screen analysis we identified the conserved RNA-binding protein RBM22, a spliceosomal protein [5,6], as an interacting partner of ALG-2. Surprisingly, confocal analysis of ALG-2 and RBM22 localization in NIH-3T3 cells revealed that more than 95% of RBM22 was localized in the nucleus whereas ALG-2 was localized in the cytosol. However, if we co-injected the two fluorescent constructs into NIH 3T3 cells, it was of interest to note that on average more than 95% of the two proteins co-localized within the nucleus suggesting a functional connection between the two proteins [6]. By screening for essential proteins involved in the regulation of alternative splicing, cell division and development homologues of RBM22 have been identified [7–11] as discussed in Ref. [6]. ALG-2 may thus be an important modulator to link Ca^{2+} signaling to essential cellular processes such as alternative splicing, cell division and proliferation, and cell death.

During activation of the spliceosome for pre-mRNA splicing the formation of the U2/U6 helix is an important step which is conserved from yeast to mammals (see Wahl et al. [12] for a recent review; see also Sun & Manley [13]). In yeast the homolog of RBM22, i.e. slt11p, is involved in the base-pairing interaction of U2/U6 helix II [14]. Slu7, a factor that is required for the selection of the splice site at the 3′ end during the second step of splicing [15], is interacting with slt11p [14]. Recently, Shomron et al. [16] reported that in HeLa cells exposed to heat shock treatments up to 50% of hSlu7, the human homolog of yeast slu7, was localized in the cytoplasm with subsequent changes in the alternative splicing pattern of cellular genes. Here we report the influence of various stress stimuli on the subcellular distribution of

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the spliceosomal proteins RBM22 and hSlu7 in NIH 3T3 cells and its influence on splicing of XBP-1, a stress indicator of the endoplasmic reticulum. Importantly, we show that the distribution pattern of the 2 proteins under the same stress conditions differed significantly, and that these stress conditions had no measurable effect on the RBM22-dependent translocation of ALG-2 into the nucleus.

2. Materials and methods

2.1. Cell culture and transfection

NIH 3T3 cells were plated in 6-well dishes and cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), containing 4.5 g/L D-glucose and 110 mg/L pyruvate, supplemented with 10% fetal calf serum, 2 mM glutamine and streptomycin/penicillin as described in detail in Ref. [6]. After 24 hours the cells were transfected with specific expression vectors encoding either RBM22-EGFP or ALG-2-mRFP as described in detail in [6]. Transfection was carried out at 80–90% confluency using the effectene kit from Qiagen according to the manufacturer's instructions. Transfections were performed with the single construct (0.5 μg plasmid DNA) or as co-transfections of the ALG-2-mRFP and the RBM22-EGFP expression vectors encoding the fusion proteins (each 0.5 μg plasmid DNA) [6]. The efficiency of transfection was more than 80%. Forty-eight hours after transfection cells were exposed to heat shock at 42 °C for a given time (for details see legends to the figures), and afterwards for 3 hours back to normal temperature at 37 °C before cells were analysed. Cells were also treated with 1 μM thapsigargin (TG) for 16 hours at 37 °C.

For confocal microscopy cells were fixed with 3.7% formaldehyde 24 to 48 hours after transfection [6]. Images were captured using the confocal laser-scanning microscope LSM 5 from Zeiss (Carl Zeiss Canada Ltd.). The subcellular distribution of the different proteins was analysed and quantified by using the software from ImageJ (NIH).

HSlu7 was detected using a specific polyclonal antibody raised in goat (Santa Cruz Biotechnology; see also [16]) and a secondary anti-goat antibody (Jackson Immunoresearch) coupled to Cy3.

2.2. Reverse transcriptase polymerase chain reaction

After stress exposure of the cells either by heat shock or exposure to TG total RNA was isolated from cells expressing recombinant proteins followed by cDNA synthesis as described by Coe et al. [17]. Briefly, RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies), cDNA synthesized by reverse transcriptase (Invitrogen) and amplified with Taq polymerase (Sigma) using primers specific for the unspliced or spliced isoform of Xbp1. The PCR products were separated on a 7.5% acrylamide gel.

2.3. Luciferase assay

NIH 3T3 cells were transfected with pRL–XFL vector encoding Renilla luciferase, mXBP1 and firefly luciferase reporter genes as described previously [17,18]. Forty-eight hours after transfection cells were treated either by heat shock for different times as indicated in Fig. 7, or by incubating with 1 μM TG for 16 hours, lysed with 150 μl passive lysis buffer (Promega), frozen in −80 °C and thawed for 3 times and incubated for 15 min at RT with gentle shaking. After centrifugation the supernatant was used for analysis. The assay was performed with the dual-luciferase assay kit (Promega) using a luminometer (Berthold-Lumat LB 9501). Relative light units (RLUs) were normalized to internal control (pRL–XF) or to Renilla luciferase reporter gene under control of the CMV promoter (pRL–CMVor pGL3–Xbp1) [18]. Average values of three independent experiments ($n = 3$) were reported.

3. Results and discussion

The subcellular localization of RNAs and proteins can be influenced in many ways [19,20]. Here we report how different cellular stress conditions can influence the subcellular distribution of 2 nuclear proteins, the spliceosomal proteins RBM22 and hSlu7, and the cytoplasmic Ca^{2+}-binding protein ALG-2. hSlu7 was shown by Shomron et al. [16] to decrease its nuclear concentration by exposing HeLa cells to various stress treatments, either heat shock or irradiation by UV light. The authors provided evidence that with increasing length of stress treatments up to 50% of hSlu7 localized within the cytoplasm with a subsequent functional consequence in alternative splicing [16].

hSlu7 is interacting with RBM22 during early steps of the activation process of the spliceosome [14]. Therefore we became interested whether stress conditions had a similar effect on the subcellular distribution of RBM22 and whether the presence of RBM22 could influence the stress-dependent distribution of hSlu7 within the cell. As shown in Fig. 1 in NIH 3T3 cells expressing a fluorescent RBM22 and exposed to a heat shock at 42 °C for up to 3 hours less than 5% of RBM22 became visible in the cytoplasm as shown in Fig. 2, for which several cells have been investigated at the different times exposed to heat shock. By comparing a control cell with one exposed to heat shock for 3 hours an exclusive nuclear localization of RBM22 could be observed in most cases (Fig. 1A and B). Similarly, nuclear localization of RBM22 in cells was observed if the cells were exposed to 1 μM TG for 16 hours (Fig. 1C), an inhibitor of SERCA pump of the ER thereby preventing Ca^{2+} reuptake into the lumen of the ER. These observations were in contrast to those findings reported by Shomron et al. [16] for hSlu7. However, by transfecting NIH 3T3 cells with expression vector encoding RBM22 and determining the subcellular distribution of hSlu7 by immunofluorescence we noticed an enhanced cytoplasmic translocation of hSlu7 during a

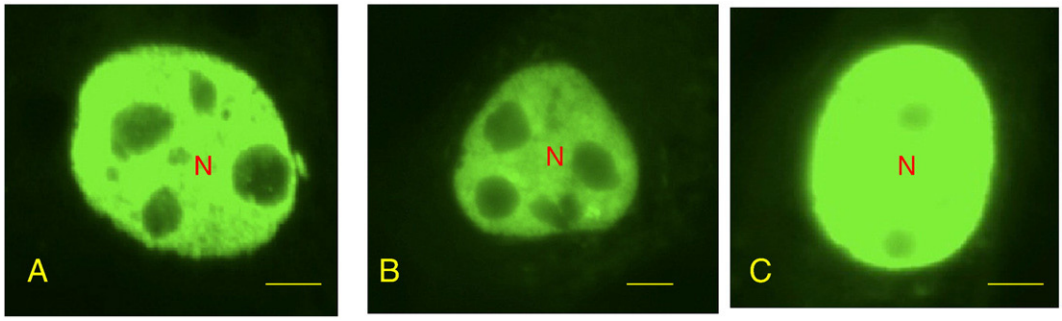

Fig. 1. Subcellular distribution of RBM22 in NIH 3T3 cells under different stress conditions. Cells were transfected with RBM22-EGFP and exposed to (A) no stress, (B) 180 min heatshock at 42 °C, (C) exposed to 1 μM TG for 16 hours. Images were taken with the filter for green fluorescence (excitation at 488 nm). N = Nucleus. Scale bars represent 5 μm.

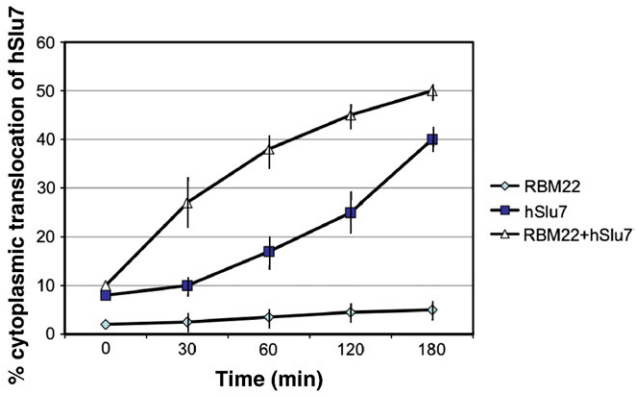

Fig. 2. Cytoplasmic localization of RBM22 and hSlu7 in NIH 3T3 cells. Cells have been either untransfected or transfected with expression vector encoding RBM22-EGFP and the localization of the proteins has been determined after exposing cells to heat shock for different length of times. Localization of hSlu7 was determined either in the absence or presence of RBM22-EGFP and quantified using software of ImageJ (NIH). The results represent an average of 3 different experiments.

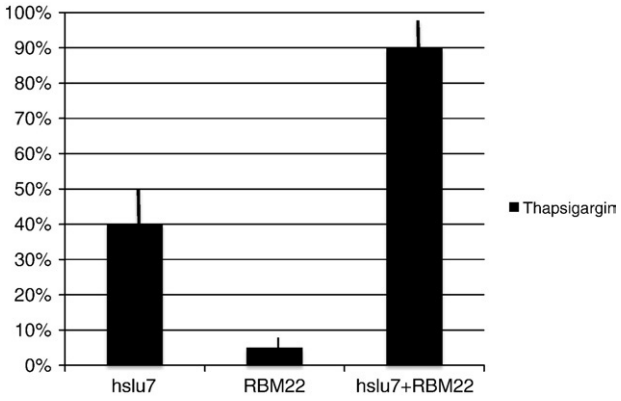

Fig. 4. Thapsigargin-dependent cytoplasmic translocation of hSlu7 and RBM22. Cytoplasmic localization of RBM22 and hSlu7 in NIH 3T3 cells has been determined after exposing cells to 1 μM TG for 16 hours. Localization of hSlu7 was determined either in the absence or presence of RBM22-EGFP and quantified using a software of ImageJ (NIH). The results represent an average of 3 different experiments.

time-dependent exposure of the cells to heat shock (see Fig. 2). In addition, these observations were underlined by comparing Fig. 3B and D, and Fig. 3C and E, respectively, representing the subcellular distribution of hSlu7 in the presence and absence of RBM22 in response to heat stress and to TG, respectively. Especially the comparison of Fig. 3C and E made it evident that the presence of RBM22 significantly enhanced the cytoplasmic translocation of hSlu7 if TG was used as a stress signal resulting in about 90% translocation of hSlu7 into the cytoplasm (Fig. 4). As an example, Fig. 3C shows cellular nuclei devoid of hSlu7. Fig. 4 shows quantitative analysis of the cytoplasmic translocation of RBM22 alone, and of hSlu7 in the absence and presence of RBM22 for a number of cells from 3 different experiments under the influence of TG as a stress signal. The calculation was made possible by using the software of ImageJ.

Of interest was the apparent punctuate accumulation of hSlu7 in the cytoplasm after heat stress (especially visible in Fig. 3B, note the arrows). Even if we do not have direct evidence for it, but this cytoplasmic concentration of hSlu7 in vesicle-like organelles is reminiscent of stress granules [21]. These organelles have been reported to concentrate due to stress spliceosomal proteins like RBM4 or hnRNP A1, among others [22]. In this context the observation is of interest that the stress-dependent accumulation of RBM4 in stress granules is dependent on phosphorylation [23]. Since Shomron et al. [16] reported that by inhibiting especially the JNK phosphorylation pathway which is known to be influenced by the ER stress response [24] reduced the cytoplasmic localization of hSlu7, it is conceivable that the cytoplasmic concentration of hSlu7 we observe indeed represent stress granules. Interestingly, RBM22 does not contain consensus sequences responsible for phosphorylation as reported for RBM4 or hSlu7 which may be the reason that RBM22 remained nuclear even during stress conditions, but further experiments have to confirm this hypothesis.

Recently we reported that in NIH 3T3 cells the cytoplasmic protein ALG-2 was translocated to the nucleus due to its interaction with RBM22 [6]. This observation could be made not only in tissue culture, but also *in vivo* during the development of zebrafish [6]. Next we were interested whether RBM22 is still able to translocate ALG-2 into the nucleus under ER stress conditions. Fig. 5 shows that cells exposed to heat stress at 42 °C for 1 hour show no significant effect on ALG-2

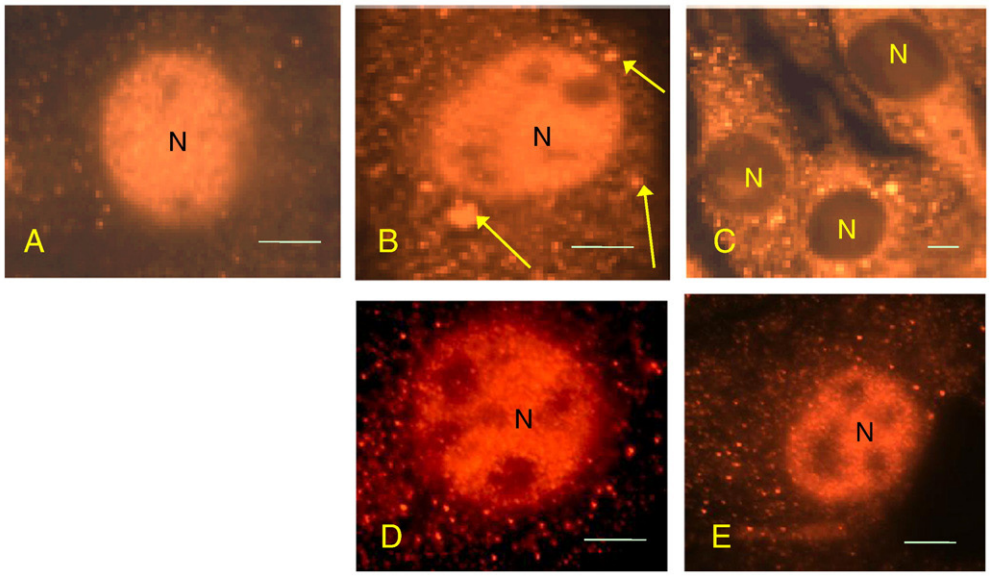

Fig. 3. Subcellular distribution of hSlu7 in NIH 3T3 cells under different stress conditions. NIH 3T3 cells, transfected with RBM22-EGFP, were incubated with antibodies against hSlu7. Distribution of hSlu7 was made visible by incubating the cells with a fluorescent secondary antibody (excitation at 543 nm). (A) control, (B) after heat stress for 180 min, (C) after stress with TG. (D) after heat stress for 180 min in the absence of RBM22-EGFP, (E) after stress with TG in the absence of RBM22-EGFP. Arrows in 2B indicate punctuate matters as discussed in the text. N = Nucleus; scale bars represent 5 μm.

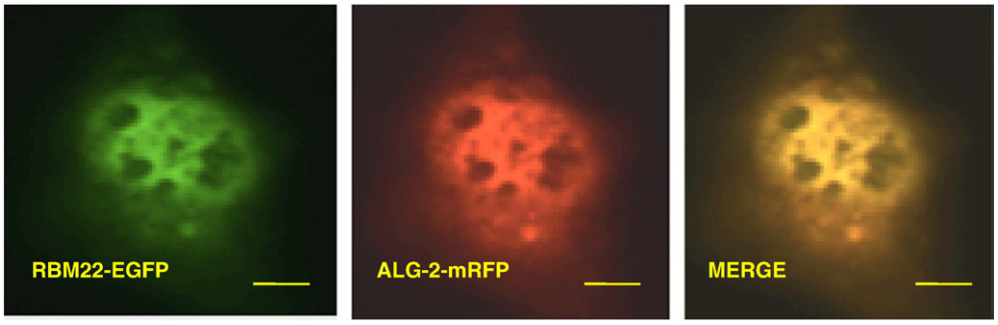

Fig. 5. Subcellular distribution of RBM22 and ALG-2 under heat stress. NIH 3T3 cells were double-transfected with RBM22-EGFP and ALG-2-mRFP and exposed to heatstress for 60 min. Images were taken either with the filter for green fluorescence (excitation at 488 nm; visualized RBM22-EGFP), or for red fluorescence (excitation at 543 nm; visualized ALG-2-mRFP), or merged. Scale bars represent 5 μm.

cellular localization, i.e. it is still translocated to the nucleus by RBM22 as indicated by merging Fig. 5A and B into C. Similar observations were made by exposing cells to 1 μM TG for 16 hours (data not shown).

Exposing cells to stress conditions results in the unfolded protein response (UPR) or ER stress response. The signaling is mediated by ER associated kinases such as PERK and IRE1. PERK is a kinase which phosphorylates the α subunit of the eukaryotic initiation factor with the consequence of reducing translation. IRE1 is also a kinase and, in addition, an endonuclease which, if activated, is responsible for the splicing of Xbp1, a transcription factor of genes encoding ER chaperones [25]. The splicing pattern of Xbp1 is a very sensitive signal of ER stress [25] in the sense that usually cellular stress favors the production of the spliced isoform of Xbp1 which as a specific transcription factor enhances the expression of ER chaperones [25]. Therefore, we analysed whether co-transfection of NIH 3T3 cells with the RBM22 and ALG-2 constructs had any influence on the splicing pattern of Xbp1. When cells were exposed to heat shock of increasing duration it appeared that less Xbp1 was spliced the longer the cells were exposed to heat shock (Fig. 6). This observation was supported if we quantified Xbp1 splicing using a luciferase assay as developed by Back et al. [18]. The firefly luciferase reporter gene is activated only when the 26-nt intron from Xbp1 is removed by splicing in an UPR-dependent manner (see Coe et al. [17] and Back et al. [18] for details of the assay). Less splicing of Xbp1 was observed with increasing duration of heat stress, especially if the cells were double transfected with the RBM22 and ALG-2 constructs (Fig. 7). Surprisingly, this effect was even stronger if cells were stressed by TG (Fig. 7) in which Xbp1 was completely spliced in untransfected cells [17], but splicing was significantly impaired if cells were transfected with either RBM22 or ALG-2, and especially if cells were expressing both proteins.

The important finding of this study is the different localization of RBM22, ALG-2 and hSlu7 upon cellular stress. The nuclear localization of hSlu7 was reduced as a result of the stress response which was dramatically enhanced due to the presence of RBM22, especially if cells were stressed by TG (Fig. 4). This was in sharp contrast to RBM22 which remained nuclear independent of the stress conditions, and promoted translocation of ALG-2 to the nucleus under stress.

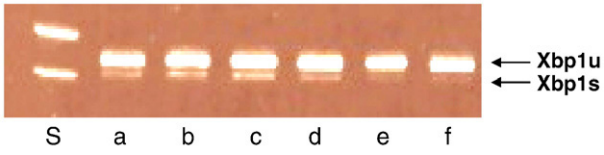

Fig. 6. Splicing of Xbp1 under stress induced by heatshock. NIH 3T3 cells were transfected with either RBM22-EGFP (a), or ALG-2-mRFP (b) or double-transfected with both constructs (c-f). Cells were exposed to no heat shock (a-c; controls) or to heatshock for 30 (d), 60 (e) or 120 min (f). S=DNA standards; Xbp1u=Xbp1 unspliced, Xbp1s=Xbp1 spliced.

Unexpectedly we observed that due to expression of the RBM22-EGFP and/or ALG-2-mRFP in NIH 3T3 cells Xbp1 splicing was reduced either during heat shock (Figs. 6, 7) or especially by exposure to TG in which case Xbp1 splicing was less than 25% as compared to controls (Fig. 7). These observations may indicate a reduced stress response of the cells under these conditions. In this context the report of Mao et al. [26] who were interested in the role of GRP94, a component of the GRP78/BiP chaperone system of the ER, should be considered. These authors demonstrated that in mice *GRP94*−/− embryonic stem cells a significant reduction of the level of ER stress could be observed, as demonstrated by a decrease of the spliced isoform of Xbp1, both on the mRNA as well as on the protein level [26]. Therefore, if the influence on the ER stress level is dependent on the interaction between those proteins described here which may be Ca^{2+}-dependent as it is known for the RBM22-ALG-2 interaction [27] it could be of interest that specific isoforms of the SERCA pump are located in the perinuclear membrane controlling nuclear Ca^{2+} stores [28,29] which would be inhibited by TG and thereby influencing the stress response. Future studies will address this hypothesis, especially whether the level of nuclear Ca^{2+} has an influence on the stress response under the conditions described here.

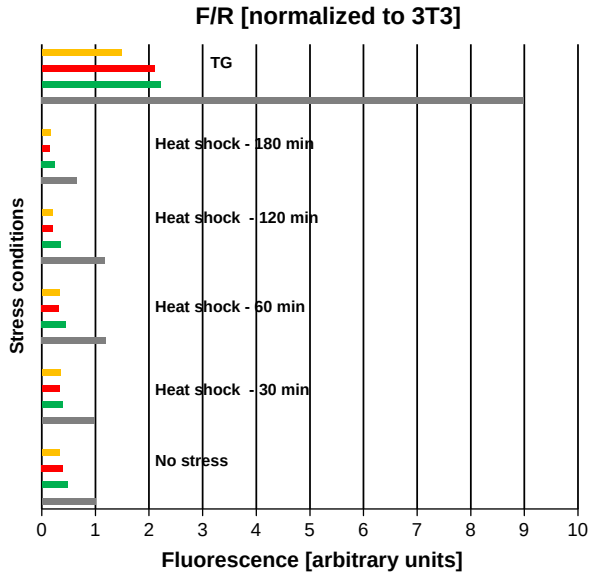

Fig. 7. Quantification of Xbp1 splicing using the luciferase assay. Cells were either untransfected (controls) or transfected with expression vector encoding either RBM22, or ALG-2, or both. The amount of spliced mRNA in control NIH 3T3 cells without stress was set to 1. TG=thapsigargin Grey=control; Green=RBM22; Red=ALG-2; Yellow=RBM22 + ALG-2 transfected cells.

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