Chapter 5
Indirect visualization of mRNA

“Dynamic properties of Pumilio and dFMR1 and their contribution to the formation of RNA granules in Drosophila”

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Abstract

Regulation of mRNA translation, transport and degradation involve RNA binding proteins (RBPs). RBPs and mRNAs can be incorporated into different higher order structures as polysomes, Processing Bodies (PBs), Stress Granules (SGs) or transport granules depending on cellular requirements. We examined the dynamic properties of two RNA binding proteins that regulate translation, Pumilio (PUM) and the Fragile X Mental Retardation Protein (dFMR1), and show that the Green Fluorescent Protein (GFP) tagged Pumilio and dFMR1 in Drosophila accumulate in PB-like structures and can relocate to SGs. We demonstrate that these proteins become components of similar but distinct granules and exhibit different dynamics. PUM-GFP moves slowly while GFP-dFMR1 has a fast moving fraction allowing more rapid movement through the cell. Taken together, our results show the contribution of PUM and dFMR1 in the formation of RNA granules in Drosophila and suggest a role for the dynamic behavior of these mRNA binding proteins in their function.
Introduction

Gene expression is first regulated by the transcription of DNA but a significant step of regulation also occurs at the mRNA level. By translating, transporting, storing or degrading mRNA, each cell has a broad range of control mechanisms at its disposal to adapt protein levels to its specific needs. These processes are orchestrated by RNA Binding Proteins (RBP) which, in concert with a plethora of protein and RNA partners, collaborate to fine tune gene expression. It has been suggested that translation regulation through mRNAs and associated proteins, which together form RiboNucleoProtein particles (RNP), represents an alternative in higher eukaryotes to polycistronic regulation of mRNAs in prokaryotes [1]. Consistent with this proposition, several RBPs have been found to bind large sets of target mRNAs, making it possible to coordinate the collective fate of these transcripts. In addition, each mRNA can feature binding sites for different RBPs and then be regulated by their combinatorial effects of their respective protein partners.

Many RBPs have been found to play a role in the regulation of translation. Pumilio is a translational repressor binding more than a thousand mRNAs in adult Drosophila [2]. Its target mRNAs can be grouped in subsets coding for functionally or structurally related proteins. Fragile X mental retardation protein (FMRP) also down regulates translation of the fmr1 gene and is involved in the Fragile X mental retardation syndrome. FMRP binds 4% of all mouse brain mRNAs [3,4], including its own mRNA [5]. Recently Staufen 1 and Staufen 2, both involved in local translational control, were
found to be associated with large, distinct, but overlapping subsets of mRNAs [6,7].

Regulation of translation also entails mRNA transport, storage and decay. To accommodate these processes, RNPs and their respective mRNAs can move from polysomes, in which translation occurs, to other higher order structures such as transport particles, stress granules or processing bodies [8]. Stress granules (SGs) are RNP remodeling sites and processing bodies (PBs) correspond to mRNA degradation sites. Most of these structures have been shown to be dynamic. GFP-tagged proteins and reporter mRNA molecules have been reported to continuously move in and out of transport particles, PBs and SGs [9-13]. In addition, mRNAs present in polysomes, PBs and SGs can redistribute among these structures in response to changes in the translational status of the mRNAs brought about by environmental cues. Consistent with this finding, SGs or PBs can be characterized by artificially disturbing the balanced distribution of mRNAs with translation inhibitors and arsenite. In the presence of cycloheximide, mRNAs are sequestered by polysomes and are no longer able to relocate to other structures. As a consequence, SG and PB size diminishes and they eventually fall apart entirely. Conversely, inhibiting translation with puromycin liberates mRNAs from polysomes. These newly available mRNAs are incorporated into stress granules, and these structures subsequently become bigger [14]. Sodium arsenite also disturbs the equilibrium between polysomes and PBs or SGs by inducing oxidative stress. To avoid any damage caused by the reactive oxygen species (ROS), the cell has to reprogram translation and produce higher levels of ROS scavengers and antioxidants [15]. Consequently, a new set of messengers needs to be translated whereas previously translating mRNAs need to be stored or degraded, and therefore, SGs and PBs appear. Interestingly, the overexpression of several RNA binding proteins has been reported to induce the formation of granular structures containing SG components [16-18].

In this study, we show that overexpression of PUM and dFMR1 (Drosophila homologue of FMRP) in a Drosophila cell line of neuronal origin (ML-DmBG2-c2 cells) [19] results in the localization of these proteins to cytoplasmic foci that correspond to PB-like structures, as indicated by their susceptibility to translation inhibitors and sodium arsenite. Examination of the content of these granules subsequently revealed substantial heterogeneity and showed that the PB-like structures induced by the overexpression of PUM-GFP differ from the granules resulting from GFP-dFMR1 overexpression. In addition, these two fusion proteins can relocate to SG-like structures in the presence of puromycin or arsenite. To date, despite the fact that RNA granules play a key role in coordinately
adapting translation levels to environmental conditions and are obviously highly dynamic structures, the dynamic behavior of the proteins present in these granules has not been studied in great detail. Here, we compared the dynamics of PUM-GFP and GFP-dFMR1 using live cell imaging and photobleaching techniques. We show that most of the PUM-GFP and GFP-dFMR1 containing granules display a corralled movement and that both proteins constantly move in and out of the granules. GFP-dFMR1 moves faster through the cell than PUM-GFP. Upon release from its binding sites, GFP-dFMR1 does not immediately rebind but freely moves through the cytoplasm before attaching to a new binding site. By contrast, released PUM-GFP immediately binds the nearest unoccupied binding site, probably because the affinity of PUM-GFP for its substrates is higher than the affinity of GFP-dFMR1 for its own binding partners.

Results

PUM and dFMR1 in ML-DmBG2-c2 cells
To visualize PUM and dFMR1 in ML-DmBG2-c2 cells, we used immunocytochemistry as well as GFP-fusion proteins. This cell line was preferred above the more commonly used S2 cells because ML-DmBG2-c2 cells grow in a nicely spread layer, making them well suited for microscopic analysis. Figure 1A shows the endogenous localization of Pumilio. Small foci can be observed throughout the cytoplasm, their size varies from approximately 200 to 300 nanometers. A similar granular pattern can be observed for the endogenous localization of dFMR1 (Fig. 1B). This localization is consistent with the finding that many of the mRNA binding proteins studied so far are found in cytoplasmic granules. Double labeling revealed that PUM and dFMR1 are mostly present in separate granules (Fig. 1C). GFP-fusion proteins were used to study both PUM and dFMR1 granules. Cells transfected with PUM-GFP or GFP-dFMR1 (Fig. 1D and E, respectively) showed that the fluorescently tagged proteins have similar spatial expression patterns as their endogenous counterparts.

Western blot analysis of endogenous PUM expression revealed the three previously identified isoforms (98 kDa, 130 kDa and 156 kDa) of PUM [20] in ML-DmBG2-c2 cells (Fig. 1F, lane 1). The 130 kDa PUM isoform is GFP labeled. GFP adds 27 kDa to the endogenous protein and the 157 kDa fusion protein then overlaps with the endogenous 156 kDa isoform (Fig. 1F, compare lanes 1 and 2). In ML-DmBG2-c2 cells, the mouse antibody directed against dFMR1 outlined the expected two dFMR1 isoforms (Fig.
1F, lane 3) of 85 and 92 kDa [21] and also recognized GFP-tagged dFMR1 (Fig. 1F, lane 4). Attempts to generate PUM-GFP stable cell lines both using S2 and ML-DmBG2-c2 cells proved unsuccessful, suggesting that sustained overexpression of Pumilio is detrimental to the cell. All experiments were performed in cells with comparable distributions and low expression levels as shown in figures 1D and 1E.

**Figure 1.** Endogenous PUM, endogenous dFMR1, PUM-GFP and GFP-dFMR1 in ML-DmBG2-c2 cells. (A, B) Endogenous PUM and dFMR1, respectively, localize in small cytoplasmic foci. (C) Endogenous PUM and dFMR1 do not significantly overlap; nucleus stained with DAPI. (D, E) The corresponding GFP-fusion proteins adopt a similar localization. Scale bars 5 µm. (F) Western blots. Lanes 1 and 3 were loaded with lysates from untransfected cells; lanes 2 and 4 were loaded with lysates from cells transfected with PUM-GFP and GFP-dFMR1, respectively, after 24 hours of induction. Blot corresponding to lanes 1 and 2 was incubated with an anti-PUM antibody, whereas lanes 3 and 4 were incubated with an anti-dFMR1 antibody. Lane 1: the three previously described PUM isoforms (left black arrowheads) are present in ML-DmBG2-c2 cells. Lane 2 shows that the 130 kDa isoform is GFP-tagged, yielding a new strong band that overlaps with 156 kDa isoform (left green arrowheads). Lane 3 reveals the presence of the 85 and 92 kDa isoforms (right black arrowheads). Lane 4 shows that the antibody also recognizes the corresponding GFP-fusion protein (right green arrowheads). Marker sizes are indicated in kDa on each side of the blots.

**Characterizing PUM-GFP and GFP-dFMR1 granules**

To better characterize PUM-GFP and GFP-dFMR1 granules, we investigated their further protein content. We found that the majority of the PUM-GFP granules were dFMR1 negative, and that most of the GFP-dFMR1 granules were PUM negative (Fig. 2A-C and 3A-C, respectively). Because PUM and dFMR1 play a role in translation regulation, we examined if the observed PUM-GFP and GFP-dFMR1 granules in ML-DmBG2-c2 cells contained eIF4E and Cup, eIF4E being a cap-binding translation initiation factor [22] and Cup an eIF4E binding protein that inhibits translation [23]. Most of the PUM-GFP granules were eIF4E and Cup negative (Fig. 2D-I). Although no obvious colocalization with eIF4E was observed (Fig. 3D-F), a few GFP-dFMR1 granules were Cup positive.
(Fig. 3G-I), suggesting that some of the GFP-dFMR1 granules are translationally inactive and may contain a blocked initiation complex. dFMR1 has been shown to play a role in transport of silenced mRNAs [10,24]. Therefore we determined the localization of Stau and Yps, two other proteins also implicated in the regulation of transported mRNAs [9,25]. We found that some but not all PUM-GFP and GFP-dFMR1 granules contained Yps (Fig. 2M-O, 3M-O) and that only a few GFP-dFMR1 granules contained Stau (Fig. 3J-L). Interestingly, endogenous dFMR1 did not colocalize with eIF4E, Cup, Stau, or Yps (Figure 4), clearly showing that endogenous dFMR1 and GFP-dFMR1 granules differ from each other. Colocalization of endogenous PUM with the different markers could not be investigated because of conflicting antibody species (no antibody raised in other species commercially available to our knowledge). When dFMR1-GFP and PUM-mCherry are coexpressed, the two constructs localize to the same granules (data not shown). This further emphasizes the fact that the GFP- or mCherry-tagged proteins localize to different granules as their endogenous counterparts. As expected, the granules containing PUM and dFMR1 fusion proteins also contain mRNAs (Supplemental figure 1).

Figure 2. PUM-GFP partially colocalizes with dFMR1 and Yps. PUM-GFP transfected cells (A-O) stained with the antibody indicated on the left of each image (A, D, G, J, M). PUM-GFP localization (B, E, H, K, N). Overlays (C, F, I, L, O). Scale bars 5µm. C and O show that the bigger PUM-GFP foci colocalize with dFMR1 and Yps, respectively, whereas virtually no colocalization is observed with the other proteins.
Figure 3. GFP-dFMR1 partially colocalizes with Cup, Stau and Yps. GFP-dFMR1 transfected cells (A–O) stained with the antibody indicated on the left of each image (A, D, G, J, M). GFP-dFMR1 localization (B, E, H, K, N). Overlays (C, F, I, L, O). Scale bars 5 µm. I, L and O show that some GFP-dFMR1 granules colocalize with Cup, Stau and Yps, respectively.

Figure 4. Endogenous dFMR1 does not colocalize with any of the other proteins tested. Untransfected cells stained with MoAbs to dFMR1 (B, E, H, K) and with the antibody indicated on the left of each image (A, D, G, J). Overlays (C, F, I, L). Nuclei are stained with DAPI. No clear colocalization can be observed with any of the combinations used. Scale bars 5 µm.

Together these immunocytochemistry experiments show that, while the foci containing PUM-GFP and GFP-dFMR1 exhibit a similar distribution in the cell, their protein content slightly varies and they are present in different granules than their endogenous equivalents.
PUM and dFMR1 overexpression induces the formation of PB-like structures

Expression of exogenous PUM2 in rat hippocampal neurons was shown to lead to the formation of SG-like structures [16]. This is also true for the expression of human FMRP in STEK cells [18]. Hence, we postulated that PUM-GFP and GFP-dFMR1 foci in Drosophila corresponded to SG-like structures as well.

To test this proposition, we first investigated if the PUM and dFMR1 granules contained RIN. RIN is a Drosophila homologue of G3BP which is used as a stress granule marker in mammalian cell lines [26,27].

We found that RIN was present in very small granules throughout the cytoplasm (Fig. 5Aa’, 5Ac’ and 5Ac’) but was absent from the dFMR1, PUM-GFP and GFP-dFMR1 granules (Fig. 5Ab’’, 5Ac’’ and 5Ac’’). This suggests that dFMR1, PUM-GFP and GFP-dFMR1 containing structures do not correspond to bona fide SGs.

We next investigated if the granules contained DCP1. DCP1 is a cofactor enhancing the activity of the 5’→3’ decapping enzyme DCP2 [9,28-30], and thus plays a role in mRNA degradation. It is considered a core PB component [31].

In untransfected cells, two distinct types of DCP1 granules coexisted (Fig. 5Ab’): a large population of small granules and a few bigger foci were found within one cell. DCP1 does not colocalize with endogenous dFMR1 (Fig. 5Ab’’). The larger DCP1 granules disappeared with PUM-GFP or GFP-dFMR1 expression (Fig. 5Ad’ and 5Af’), suggesting that DCP1 was recruited to GFP-dFMR1 and PUM-GFP granules. We found that a subpopulation of PUM-GFP granules contained DCP1 (Fig. 5Ad) and that almost all the bigger GFP-dFMR1 granules colocalized with DCP1 (Fig. 5Af). Together, these results (summarized in supplemental table 1) suggest that the majority of PUM-GFP and GFP-dFMR1 granules indeed correspond to DCP1-containing PBs.

We next asked if PUM-GFP and GFP-dFMR1 foci, like PBs, are reactive to translation inhibitors and arsenite [14].
A. CONTROL  
B. + CYCLOHEXIMIDE  
C. + PUROMYCIN  
D. + SODIUM ARSENITE
**Figure 5.** PUM-GFP and GFP-dFMR1 overexpression induces the formation of PB-like structures that are responsive to regulators of translation. In A, B, C and D: (a, a’, b, b’) Untransfected cells. (c, c’, d, d’) PUM-GFP transfected cells. (e, e’, f, f’) GFP-dFMR1 transfected cells. (a, a’, b, b’, c’, d’, e’, f’) Cells are stained with the antibody indicated in each image. (a’’, b’’, c’’, d’’, e’’, f’’) Overlays, nuclei are stained with DAPI.

[A. control] The bigger PUM-GFP and GFP-dFMR1 foci colocalize with DCP1 but not with RIN. RIN does not colocalize with PUM-GFP and GFP-dFMR1, as seen in c’’ and e’’. Conversely, the bigger DCP1 granules are positive for PUM-GFP or GFP-dFMR1, as observed in d’’ and f’’, respectively.

[B. cycloheximide] Cells were incubated for 4 hours with 50µg.mL⁻¹ of cycloheximide. In untransfected cells, only the DCP1 pattern is modified (b’), the bigger granules have disappeared. c, d, e and f show that both PUM-GFP and GFP-dFMR1 adopt a less granular and more diffuse pattern upon cycloheximide incubation. This prevents us from drawing any relevant conclusions about their colocalization with DCP1 and RIN, as seen in c’’, d’’, e’’ and f’’.

[C. puromycin] Cells were incubated for 4 hours with 20µg.mL⁻¹ of puromycin. In untransfected cells, bigger RIN granules appear upon puromycin incubation (a’). All of the big PUM-GFP and GFP-dFMR1 granules that appeared upon puromycin incubation are DCP1 and RIN positive, as seen in c’’, d’’, e’’ and f’’.

[D. sodium arsenite] Cells were incubated for 45 minutes with 0.75mM of sodium arsenite. Endogenous dFMR1, RIN and DCP1 are affected by sodium arsenite, and dFMR1 colocalizes with RIN and DCP1 (a-b’’). The modified PUM-GFP and GFP-dFMR1 granules are both DCP1 and RIN positive. (c’’, d’’, e’’, f’’).

In the presence of cycloheximide ribosomes are stalled on mRNA and mRNAs that cycle between polyribosomes and any other structures will be trapped in polyribosomes, at the expense of the other structures involved. As a result cycloheximide induces SG and PB disassembly. Whereas cycloheximide had no effect on dFMR1 and RIN (Fig. 5Ba, a’’), the DCP1 pattern was changed (Fig. 5Bb’). The bigger DCP1 granules disappeared, leading to a distribution of DCP1 granules of more or less equal size throughout the cytoplasm. This suggest that excess of mRNAs trapped in polyribosomes has an effect on the larger DCP1 granules. These larger DCP1 granules may therefore correspond to effective PBs, whereas the smaller DCP1 foci are unreactive to cycloheximide. Upon cycloheximide incubation, both PUM-GFP and GFP-dFMR1 granules fell apart, resulting in a more homogeneous distribution of the GFP-tagged proteins throughout the cell (Fig. 5Bc-f). RIN localization was not affected by cycloheximide incubation (Fig. 5Ba’).

Puromycin destabilizes polyribosomes by promoting premature termination. Thus, polyribosomes fall apart, translation is stopped and mRNAs are sequestered into non-translating structures. Consequently puromycin is an agent used to induce large SGs. Accordingly, endogenous DCP1 distribution is not affected by puromycin treatment (Fig. 5Cb’). After puromycin treatment, PUM-GFP and GFP-dFMR1 formed large foci which were positive for both DCP1 and RIN (Fig. 5Cc-f’’). A few smaller PUM-GFP or GFP-dFMR1 granules remained that did not contain DCP1 (Fig. 5Cd’’, f’’).
In untransfected cells endogenous RIN localized to larger granules in 26% of the cells upon puromycin treatment suggesting that RIN localizes to SGs induced by puromycin.

Finally, SGs and PBs can be induced by sodium arsenite. Both PUM-GFP and GFP-dFMR1 patterns are modified after arsenite treatment. Bigger and irregular granules were formed (Fig. 5Dc-f’’). They were more abundant than foci generated by puromycin treatment, present throughout the cytoplasm and both contained DCP1 and RIN. In untransfected cells dFMR1, RIN and DCP1 also appear in large irregularly shaped granules after arsenite treatment (Fig. 5Da-b’’).

Surprisingly, endogenous PUM was not reactive to any of the drugs used (Fig. 6) clearly showing that endogenous PUM granules differ from PUM-GFP granules.

Taken together, PUM-GFP and GFP-dFMR1 granules were in dynamic equilibrium with polysomes and were reactive to arsenite. These experiments also allowed us to outline the redistribution of proteins between two types of granules. First PUM-GFP and GFP-dFMR1 are present in (DCP1-containing) PBs in the absence of any treatment and they disappear upon cycloheximide incubation. Second, PUM-GFP and GFP-dFMR1 are relocated to granules that contain both DCP1 and RIN upon puromycin or arsenite treatment.

**Figure 6.** Endogenous PUM does not react to polysome destabilizing drugs or arsenite. Untransfected cells are stained against endogenous PUM. Neither cycloheximide, puromycin, nor sodium arsenite treatment changed the granular pattern observed in the control.

**PUM-GFP and GFP-dFMR1 granules movement and dynamics**

We first documented the movements of PUM-GFP and GFP-dFMR1 granules in the cell using time lapse imaging. Although a few granules showed a directed movement over a short period of time (Supplemental movies 2 and 4), it was clear that most of the granules oscillated around the
same position and are not actively transported (Supplemental movies 1 and 3). This is in agreement with previous experiments in mammals showing that FMRP granules exhibit both directed and oscillatory movements [18,32].

RNA granules have been reported to be highly dynamic. GFP-dFMR1 and PUM-GFP are located in different populations of PB-like structures. To establish if they also move in and out of the granules, we next investigated the mobility of the GFP-tagged proteins by performing Fluorescence Recovery After Photobleaching (FRAP) experiments. A region of the cytoplasm of a cell transfected either with PUM-GFP or GFP-dFMR1 was irreversibly bleached and fluorescence recovery was monitored in the same area over time. Values were collected from 14 cells for PUM-GFP and 20 cells for GFP-dFMR1, and the resulting average recovery curves are plotted in figure 7A. The recovery curves clearly showed that PUM-GFP and GFP-dFMR1 slowly repopulated the bleach area after the bleach event, suggesting that both of these proteins are mobile. Interestingly, GFP-dFMR1 recovered faster than PUM-GFP (50% recovery after 42.9 seconds and 97.3 seconds, respectively). The difference in molecular weight between GFP-dFMR1 and PUM-GFP is not sufficient to explain the difference in mobility [33]; therefore we assume that GFP-dFMR1 undergoes fewer or weaker interactions than PUM-GFP, allowing it to repopulate the bleach area faster than PUM-GFP.

Figure 7. PUM-GFP and GFP-dFMR1 dynamics: recovery and immobile fraction. (A) FRAP curves for PUM-GFP and GFP-dFMR1. A circular area of 3 µm in diameter was bleached with one frame at full laser power. 45 post-bleach images with a time interval of 5 seconds were collected. GFP-dFMR1 fluorescence recovers faster than that of PUM-GFP and reaches a plateau around 170 seconds. PUM-GFP fluorescence is still recovering after 200 seconds. (B, C) PUM-GFP and GFP-dFMR1 double FRAP. A circular area of 3 µm in diameter was bleached and recovery was monitored with 30 frames having a time interval of 10 seconds. The same region was then bleached again to obtain the second FRAP curve. Only the second FRAP curve for GFP-dFMR1 rises to 100% and is significantly different from the first recovery curve (P-value: 0.001). Asterisks indicate graphs in which the two represented curves are significantly different from each other. Error bars represent standard deviations.

When analyzing the recovery curves, we observed more differences between the dynamic behavior of GFP-dFMR1 and PUM-GFP. GFP-
dFMR1 did not fully recover and reached a plateau after 170 seconds, whereas PUM-GFP recovery continued well beyond that time point (Fig. 7A). These data suggested that an immobile population of GFP-dFMR1 is present inside the bleach area. We further investigated the presence of this immobile fraction by performing two consecutive FRAP experiments on the same region in the cytoplasm [34]. An immobile fraction that is irreversibly bleached during the first experiment cannot take part in the second FRAP experiment, resulting in two different recovery curves. The first recovery curve of GFP-dFMR1 reached approximately 90% during the time frame of the experiment while the second GFP-dFMR1 recovery curve achieved almost 100% over the same period (Fig. 7C). The difference between the first and second recovery curves proved highly significant (P-value = 0.001), clearly indicating the presence of an immobile fraction. The second recovery curve for PUM-GFP was not significantly different from the first recovery curve (P-value = 0.285) (Fig. 7B). These data clearly showed that GFP-dFMR1 has an immobile fraction of approximately 10% and that PUM-GFP does not contain an immobile fraction, but slowly recovers through the entire duration of the experiment which is typical of a so-called transiently immobile protein [35].

The presence of an immobile fraction for GFP-dFMR1 raised two questions. Is the immobile fraction located inside or outside the granules? If found inside the granules, is it equally distributed among the several subtypes of granules? To investigate if the immobile fraction is located within the granules, we bleached individual granules and measured fluorescence recovery within each granule. To ensure that the granules investigated did not move out of focus during the experiment, we cotransfected cells either with PUM-GFP and PUM-mCherry (Fig. 8A) or with GFP-dFMR1 and mCherry-dFMR1 (Fig. 8B) [36]. Green and red-tagged proteins are mixed and the granules appeared yellow. mCherry-tagged proteins were used to perform the FRAP experiment while GFP-tagged proteins served to monitor the granule throughout the experiment. A single granule was bleached using the 561 and 594 nm laser lines, ensuring the bleaching of mCherry-tagged proteins. In both cases only the GFP-tagged proteins were visible in the granules immediately after bleaching and appeared green (Fig. 8A, B). We found that exchange of mCherry-tagged proteins took place after photobleaching in all granules examined, resulting in yellow granules over time. In agreement with this observation, the average recovery curves over nine PUM granules and eleven dFMR1 granules showed that red fluorescence dropped with bleaching and slowly recovered (Fig. 8C, D).
Figure 8. PUM-mCherry and mCherry-dFMR1 respectively repopulate the same granules. (A) Cell cotransfected with PUM-GFP and PUM-mCherry. (B) Cell cotransfected with GFP-dFMR1 and mCherry-dFMR1. Images shown are the overlays of the red and green channels taken at different time points. Granules indicated by arrows in the pre-bleach images were bleached with the 561 and 594 nm laser lines. After bleaching, the respective granules are still visible because the GFP-tagged proteins have not been bleached. mCherry fluorescence recovery can then be measured without ambiguity in the same granule. Quantification of the 2-color FRAP experiments are shown in C and D for PUM and dFMR1, respectively. Error bars represent standard deviations.

The curves representing red and green fluorescence often demonstrated the same fluctuations suggesting that these fluctuations reflect cell and granule movements. It proved more difficult to measure the recovery of fluorescence in individual granules containing dFMR1 than granules containing PUM because the GFP-dFMR1 granules displayed a more pronounced oscillatory movement than the PUM-GFP granules and readily moved in and out of the focus plane. This is reflected by the larger error bars observed along the curves representing the recovery of GFP-dFMR1 in figure 8D. As a result, and because only a small part of the entire GFP-dFMR1 population is immobile, it proved impossible to determine the extent to which fluorescence recovered in the individual granules. Therefore, these experiments did not allow us to determine the localization of the immobile fraction of mCherry-dFMR1 and made it difficult to speculate on the function of the immobile fraction. However, these experiments did suggest that none of the granules consist exclusively of immobile proteins.
Figure 9. PUM-GFP and GFP-dFMR1 dynamics: fast fraction and diffusion. (A, B) Bleach depth in fixed or live cells was compared either for PUM-GFP or GFP-dFMR1. A few images were taken before and after bleaching a circular region of 3 µm in diameter in fixed or in live cells. The bleach depth achieved in fixed cells for GFP-dFMR1 is slightly lower than in live cells (P-value: 0.008) whereas the same bleach depth is reached in both conditions for PUM-GFP. (C) Confocal image of a GFP-dFMR1 transfected cell. White line indicates the half of the cell that was bleached with 2 frames at full laser power in the experimental setup for graphs D and E. Fluorescence recovery was monitored either in two segments that were located at different distances from the bleach boundary. Segment 1 bordered the bleach boundary (orange) while segment 3 was positioned at some distance from the bleach boundary (yellow). (D, E) PUM-GFP fluorescence recovery in segment 1 is faster than in segment 3 whereas GFP-dFMR1 fluorescence recovery is similar in both segments. (F) Ratios of the fluorescence intensities in segment 1 and 3 for PUM-GFP and GFP-dFMR1 through time. The ratio increases for PUM-GFP whereas it remains constant for GFP-dFMR1. Asterisks indicate graphs in which the two represented curves are significantly different from each other. Error bars represent standard deviations.

Besides an immobile fraction, a population of proteins can also include a very fast moving fraction. We next investigated the possible presence of such a fraction among the PUM-GFP and GFP-dFMR1 molecules. FRAP experiments showed that the fluorescence intensity of the bleached area did not reach background levels after bleaching with full laser power (bleach depth approximately 87%), neither for PUM-GFP nor GFP-dFMR1. Incomplete bleaching could be explained by the presence of a fast moving population that has already recovered when the first post-bleach image is taken [37]. As the laser needs some time to switch from bleaching intensity to imaging intensity, we compared the bleach depth in live cells and fixed cells expressing either PUM-GFP or GFP-dFMR1 (Fig. 9A, B). A small, yet significant difference in bleach depth was found for GFP-dFMR1 (Fig. 9B; P-value = 0.008) but not for PUM-GFP (Fig. 9A; P-value = 0.817). Our data therefore suggested that only GFP-dFMR1 contains a small fast moving fraction.
The presence of a fast moving fraction for GFP-dFMR1 and not for PUM-GFP led us to hypothesize that diffusion could play different roles in their respective dynamics [33]. If the proteins of interest diffuse faster than they bind to their attachment sites, two regions within the bleached area that are located at different distances from the bleach boundary will have the same recovery curves (diffusion-uncoupled recovery). By contrast, if the protein of interest attaches to its binding sites equally fast or faster than it diffuses, then the bleached area is only gradually repopulated. In that case, two regions within the bleached area with different distances from the bleach boundary will have different recovery curves, with the region closest to the bleach boundary recovering faster (diffusion-coupled recovery). The presence of a fast moving fraction suggested that GFP-dFMR1 could repopulate the entire bleached area before binding to its attachment sites, resulting in diffusion-uncoupled recovery. By contrast, we expected PUM-GFP to have a diffusion-coupled recovery, as it lacks a fast moving fraction. To test this, we bleached half of a cell transfected either with PUM-GFP or with GFP-dFMR1 and measured the fluorescence recovery within two segments: segment 1 is adjacent to the bleach boundary, and segment 3 is positioned 4 µm away from the bleach boundary (Fig. 9C). Segment 1 for PUM-GFP recovered to higher relative fluorescence intensities than segment 3 (Fig. 9D), this was also true for GFP-dFMR1 (Fig. 9E). Also, the immediate post-bleach fluorescence intensity value for GFP-dFMR1 was slightly higher in segment 1 than in segment 3 and the two curves were parallel (Fig. 9E), suggesting that the fluorescence recovery rates in segment 1 and 3 are similar for GFP-dFMR1. To further investigate the difference in recovery rates between these two segments for the two proteins of interest, we decided to calculate the ratios between the relative fluorescence intensities in segment 1 and segment 3, assuming that a varying ratio through time indicates a difference in recovery rate between the two segments, whereas a constant ratio argues that the segments recover at the same rate. This ratio significantly increased through time for PUM-GFP (Fig. 9F; P-value = 0.019). This indicates that fluorescence recovered faster in segment 1 than in segment 3, and thus, the binding of PUM-GFP to its binding sites is faster than or at least as fast as its diffusion. We conclude that PUM-GFP recovery is diffusion-coupled. Conversely, the GFP-dFMR1 ratios between the fluorescence intensities in segments 1 and 3 did not significantly increase over time yielding a horizontal line (Fig. 9F; P-value = 0.78). This indicates that fluorescence recovered with the same rate in the two segments: GFP-dFMR1, or at least a subpopulation of GFP-dFMR1, diffuses faster than it attaches to its binding sites. This suggests that GFP-dFMR1 recovery is diffusion-uncoupled, as expected for a protein that has a
small fast moving fraction. We found that the relative post-bleach fluorescence intensity immediately after photobleaching was higher in segment 1 than in segment 3 for GFP-dFMR1 but not for PUM-GFP (Fig. 9D, E). This could be explained by our finding that GFP-dFMR1 granules exhibit a more pronounced oscillatory movement than PUM-GFP granules. The granules that cross the bleach boundary and move onto the bleached area right after photobleaching might well contribute to slightly higher post-bleach fluorescence intensity in the segment closest to the bleach boundary.

**Discussion**

Regulation of mRNA storage and degradation are key mechanisms to control gene expression and take place in SGs and PBs. These granules are found to be highly dynamic with both the constituent mRNAs and proteins readily moving in and out of these granules. In the present study, we set out to analyze the mobility of two different GFP-tagged mRNA binding proteins that localize to granular structures.

When expressing PUM-GFP and GFP-dFMR1 these proteins accumulate in PB-like structures, as revealed by the sensitivity of the formed particles to translation inhibitors and the presence of a PB marker within the granules. Besides, these granules contain varying amounts of proteins involved in mRNA transport, translational arrest and degradation, demonstrating that these granules are highly heterogeneous. In the presence of puromycin and arsenite, PUM-GFP and GFP-dFMR1 relocate to granules that contain RIN and DCP-1. The presence of DCP1 in the granules induced by puromycin might be surprising as it is a PB component and puromycin is supposed to induce large stress granules [14]. However, the presence of DCP1 has previously been shown in SGs resulting from the overexpression of CPEB1 [13]. The presence of DCP1 in these granules induced by puromycin in cells expressing the GFP fusion proteins could be a “default localization” of the DCP1 [38]. Moreover, our finding that DCP1 has not been redistributed in untransfected cells is consistent with the suggestion that DCP1 is present in different types of granules depending on the relative concentrations of their respective components [13]. Our data clearly supports the idea that granule composition varies with the nature of the stress [16,39], and with the relative amounts of PB and SG components [13,38].

When examining the dynamics of PUM-GFP and GFP-dFMR1, we found that GFP-dFMR1 recovers faster than PUM-GFP. This suggested that dFMR1-GFP has less potential binding sites than PUM-GFP or binds them
with lower affinity. Both suggestions may be true as our data showed that transfected ML-DmBG2-c2 cells always bear slightly more PUM-GFP granules compared to GFP-dFMR1 granules, suggesting that more binding sites might be available. In addition, the assembly of stress granules has been shown to be dependent on the aggregation of glutamine rich areas in the proteins recruited to the stress granules [16,40]. Both dFMR1 and PUM contain regions enriched in glutamines; however PUM contains a substantial longer stretch of glutamines [41] than dFMR1 [42]. This might render PUM more prone to aggregation and might contribute to the higher affinity of PUM-GFP for its granules. However PUM might also undergo stronger interactions with its other partners in the cell (proteins or mRNA) than dFMR1.

The presence of fast moving GFP-dFMR1 proteins within the cell led us to examine the contribution of diffusion to the dynamic properties of the proteins under investigation. To this end, we bleached half the cell and monitored the fluorescence recovery in two regions within the bleached area that are located at different distances from the part of bleach boundary. In figure 10, fluorescence recovery is schematically represented. PUM-GFP first repopulates segment 1, directly flanking the bleach boundary, and then gradually moves further into bleached area and subsequently repopulates area 3 (Fig. 10, upper row). This finding clearly indicates that PUM-GFP recovery is diffusion-coupled: when PUM-GFP is released, it binds to the nearest available binding site without first diffusing throughout the cell: in this case diffusion is slower than binding.

By contrast, the fast moving fraction of GFP-dFMR1 moves rapidly throughout the bleached area after which exchange at the binding sites take place. As a result, segments 1 and 3 are repopulated simultaneously (Fig. 10, lower row). This shows that GFP-dFMR1 recovery is diffusion-uncoupled: GFP-dFMR1 partially diffuses faster than it binds. The fact that GFP-dFMR1 recovers faster than PUM-GFP could also mean that GFP-dFMR1 is actively transported more often or faster than PUM-GFP. This would be in agreement with the description of FMRP as a “processivity factor for mRNA transport” [10] and with our finding that GFP-dFMR1 recovery is diffusion-uncoupled. Although care was taken to perform experiments on cells expressing comparable low levels of GFP-fusion proteins, we cannot exclude that difference in expression levels contributes to the divergent dynamic characteristics as well.
Figure 10. Schematic representation of PUM-GFP and GFP-dFMR1 dynamics (adapted from Sprague and McNally, 2005). PUM-GFP and GFP-dFMR1 localization is comparable in ML-DmBG2-c2 cells, granules can be observed throughout the cytoplasm. The upper part of the cell is bleached. The fast moving fraction of GFP-dFMR1 (represented in light green throughout the cytosol) is also bleached during this bleach event. Immediately after bleaching, there has been no exchange of PUM-GFP between the two halves yet, whereas the GFP-dFMR1 fast moving fraction (both bleached and non-bleached released from the granules in the lower part of the cell) rapidly repopulates the whole cell. As seen in post-bleach 1 and 2, recovery is diffusion-coupled for PUM-GFP: segment 1 (orange), closer to the bleach boundary, recovers faster than segment 3 (yellow). GFP-dFMR1 recovery is diffusion-uncoupled: segments 1 and 3 recover equally fast. GFP-dFMR1, but not PUM-GFP, also contains an immobile fraction that does not recover during the time frame of the experiment. The localization of this immobile fraction is still unclear and is therefore not represented (UL: undefined localization). Also not depicted here is that PUM-GFP recovers slower than GFP-dFMR1.

In this paper, we have concentrated on the qualitative analysis of the FRAP curves. Recovery curves can be used to determine the on- and off-rates and residence times of the proteins of interest. However, we have shown that PUM-GFP and GFP-dFMR1 are part of a heterogeneous population of RNA granules. We therefore can not rule out that the binding characteristics of the proteins for these diverse sets of granules might vary substantially. In addition, to date, most models used to analyze FRAP curves require a homogeneous distribution of binding sites. As the distributions obtained here are very inhomogeneous, we refrained from quantitative analysis of our data.
We were not able to generate stable cell lines expressing GFP-dFMR1 or PUM-GFP and transfection efficiency was relatively low. This made it difficult to insure that both fusion proteins retained their biological functions. However the finding that both fusion proteins are reactive to cycloheximide, puromycin and sodium arsenite shows that these proteins do not form inert aggregates and exhibit behavior that is typical for RNA binding proteins.

RNA binding proteins and mRNAs constantly cycling in and out of either stress granules or processing bodies [9-13,43]. This would allow the cell to continuously reorganize the protein coat of mRNAs and thus, always achieve the optimum level of translation for each mRNA at each time point [13].

What are the consequences of the differences in dynamic characteristics for the function of PUM and dFMR1? In ML-DmBG2-c2 cells, the rapid diffusion of GFP-dFMR1 throughout the cytoplasm might allow modifications of dFMR1, like methylation or phosphorylation [44-49], to rapidly disseminate throughout the cell. Interestingly, both methylation and phosphorylation play a vital role in dFMR1 performance and the rapid distribution of the modified proteins might be essential for the functioning of dFMR1 in the cell.

To date, the activity of PUM does not seem to rely on post translational modifications, although PUF proteins do contain putative phosphorylation sites [50]. By contrast, its activity rather depends on the interaction with other RNA binding proteins [51]. In addition, PUM is thought to regulate groups of mRNAs that encode proteins that are functionally or structurally related and therefore often present at distinct locations within the cell. The restricted movement of PUM-GFP through the cell might reflect the need to regulate the translation of distinct groups of mRNAs at a defined area in the cell.

We have studied the dynamic behavior of PUM-GFP and GFP-dFMR1 present in RNA granules. The dynamic characteristics of both proteins seem to accommodate the different properties described for these proteins so far. We can however not rule out that the presence of GFP within the fusion proteins and the redistribution of these fusion proteins to similar but distinct sets of heterogeneous structures influences the movement of the molecules within the cell. Indeed, one has to keep in mind that each cell type and each developmental stage requires different interactions of the translation regulators with their targets, or different levels of regulation, so the dynamics of each RNA binding protein might well vary with cell type and stage of development.
Taken together, we have demonstrated that the expression of PUM-GFP and GFP-dFMR1 in a Drosophila cell line induces the formation of PB-like structures. Interestingly, these tagged proteins feature different dynamic properties that reflect differences in the function of PUM and dFMR1. Our studies therefore suggest that proper regulation of transcription not only needs multiple RBPs binding different sets of mRNAs, but also requires that these proteins exhibit a variety of dynamic characteristics.

Experimental methods

Immunocytochemistry
Primary antibodies used are listed in supplemental table 2. Secondary antibodies used were Goat anti Rabbit Alexa 488 (Invitrogen), Goat anti Rabbit Alexa 594 (Invitrogen) and Goat anti Mouse Alexa 594 (Vector Laboratories). Cultured cells were fixed for 10 minutes with 3.7% formaldehyde in PBS, permeabilized for 15 minutes with 0.1% Triton X-100 in PBS and blocked for 30 minutes in TND (Tris buffered Saline with Dupont blocking reagent and 0.004% thimerosal). Primary and secondary antibodies were diluted in TND and incubated with the cells for 45 minutes at 37°C. After washing and dehydrating, cells were mounted in citifluor AF1 (Agar Scientific) containing 400 ng.mL$^{-1}$ DAPI. Images were taken with a Leica DMRXA microscope equipped with a Photometrix quantix camera, a 100 W mercury arc lamp, and a 100x NA1.3 plan Apo objective, using ColourProc software [52].

Constructs
pAc-EGFPdFMR1, kind gift from S.C. Ling [53], was digested with KpnI/BamHI. EGFPdFMR was subcloned into pBlueScript and then cloned into the KpnI and XbaI sites of pUAST [54]. mCherry was amplified from pBAD-mCherry with primers tataaatctagaggtatcgtacgctgcatgcc (forward) and tataatctagaggtatcgtacgctgcatgcc (reverse), cut with EcoRI and cloned into pUAST-dFMR1, resulting in pUAST-GFPdFMR1.

It proved impossible to clone EGFP at the 5'- end of Pumilio, and we therefore only generated pUAST-PUMGFP. EGFP was amplified from pEGFP-C1 (Clontech) with primers containing XbaI sites [atatattctagaggtatcgtacgctgcatgcc (forward) and tatattctagaggtatcgtacgctgcatgcc (reverse)] and cloned into the XbaI site of pUAST-PUM (kindly provided by B. Ye)[55]. mCherry was amplified from pBAD-mCherry (kind gift of R. Tsien) with primers...
atatatatagagagaatgtggagcaaggcaggggag (forward) and
tatattctagaaccgtttacttacagctgtgccatgcc (reverse), cut with XbaI and cloned
into pUAST-PUM, resulting in pUAST-PUMGFP.

Cell culture and transfections
ML-DmBG2-c2 cells were cultured in 3.5 cm glass-bottom Petri dishes
(MatTek) containing Schneider’s Drosophila medium supplemented with
8.77% fetal bovine serum, 1.75 mM L-glutamine, 87.7 U/mL penicillin,
87.7 µg/mL streptomycin (Gibco) at 25°C. Cells were transfected with
pUAST-PUMGFP or pUAST-GFPdFMR1 together with MTgal4 [56] using
calcium phosphate. Expression was induced one day after transfection by
adding 0.7 mM CuSO₄. Cells were imaged the day after induction.

Time lapse imaging
Cells were transfected either with pUAST-PUMGFP or with pUAST-
GFPdFMR1. Movies were recorded with a Leica TCS SP2 DMIRBE or SP5
DMI6000 confocal microscope with a 100x NA1.3 plan apo objective. Images
were taken every 2 seconds using 25 or 7% (SP2 and SP5,
respectively) of the Argon 488 nm laser line (operating at 3.2 and 3.8mW
respectively).

FRAP measurements
Photobleaching experiments were performed on a Leica SP5 DMI6000
confocal microscope with a 100x NA1.3 plan Apo objective. Pre- and post-
bleach images were taken with the 488 nm line of the argon laser, with a 10
seconds time interval and 7% laser power. A circular region, 3 µm in
diameter (containing 15 and 11 granules on average for PUM-GFP and
GFP-dFMR1, respectively), was bleached with a single frame at full laser
power. Recovery was monitored for approximately 5 minutes. Acquired
data were analyzed using Leica Application Suite-Advanced Fluorescence
and Excel. Raw data were processed as described in [34]. In brief,
background intensity was subtracted to each value, the whole cell was used
to correct for scanning and fluorescence intensities were normalized to pre-
bleach values.

For two-color FRAP experiments, pre and post-bleach images were taken
using 7% of the 488 nm laser line and 25% of the 561 nm laser line
(operating at 1.3mW), with a time interval of 12 seconds. A circular area (1
µm diameter) was bleached for one frame with 100% of the 561 and 594 nm
laser lines (operating at 1.3 and 0.4mW respectively).
For all the bleaching experiments, we took great care to keep scan bleaching
below 10%, to reach a bleach depth higher than 70% (except when
bleaching half of the cell where we compromised between bleach depth and short bleaching) and to reduce the bleach time as much as possible (one frame of 386 milliseconds for FRAP experiments involving a circular bleach ROI, and two frames of 386 milliseconds each when bleaching half of the cell).
To compare the bleach depth in live and fixed cells (Fig. 6 D and E), we first ensured that scanning fixed and live cells induced similar levels of fluorescence loss (not shown).

**Statistical analysis**
Areas under the curve (AUC) were calculated with NCSS2007 for each cell from fig. 7 and fig. 9A, B. AUCs from the two series of each graph were compared with a T-test performed in SPSS 14.0. The two tested series were considered different when the resulting p-value was inferior to 0.05.
For figure 9F, the hypothesis that the coefficient (a) corresponding to the slope of the regression curves from the different ratio/time representations was not equal to zero, was tested with a linear mixed model performed in SPSS 16.0. The coefficient was considered different from zero when the resulting p-value was inferior to 0.05.

**Acknowledgements**
We are grateful to R.P. Wharton, S.R. Haynes, J.E. Wilhelm, L.G. Fradkin, D. St Johnston, the Developmental studies Hybridoma bank, K. Kosik, V.I. Gelfand, S.C. Ling, Y.N. Jan, B. Ye and R.Y. Tsien who kindly provided us with antibodies and constructs. We thank Roeland Dirks, Adriaan Houtsmuller and Ron Wolterbeek for useful comments on this work. We also acknowledge Karien Wiesmeijer and Willem Sloos for technical support.

**Reference list**


Supplemental data
Supplemental figure 1. PUM-mCherry and mCherry-dFMR1 granules contain mRNA. (A) Localization of MS2-GFP-tagged hunchback reporter mRNA (Murata and Wharton, 1995; Wreden et al., 1997). (B) PUM-mCherry localization. (C) Overlay. Some PUM-mCherry granules contain the reporter mRNA (arrows). (D) Localization of the MS2-GFP-tagged dFMR1 reporter mRNA (Ashley, Jr. et al., 1993; Brown et al., 1998). (E) mCherry-dFMR1 localization. (F) Overlay. Some mCherry-dFMR1 granules contain the reporter mRNA (arrows). Scale bars 5 µm.

Supplemental table 1. Colocalization between PUM or dFMR and RIN or DCP1 in the absence or presence of translation inhibitors and arsenite. Colocalization is indicated by “+”, the absence of colocalization is indicated by “-”. “ND” indicates that the colocalization was not determined. Red indicates a condition in which the control localization is modified. Asterisk indicates that the diffuse pattern of the GFP constructs prevents any relevant conclusion on the colocalization with DCP1 or RIN.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PUM</th>
<th>PUM-GFP</th>
<th>dFMR1</th>
<th>dFMR1-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>Granular pattern</td>
<td>Granular pattern</td>
<td>Granular pattern</td>
<td>Granular pattern</td>
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<tr>
<td>DCP1</td>
<td>Numerous small foci and a few bigger ones</td>
<td>ND</td>
<td>*+ (bigger foci)</td>
<td>-</td>
</tr>
<tr>
<td>RIN</td>
<td>Granular pattern, 6% of the cells have a few bigger granules</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ cycloheximide</td>
<td>No effect</td>
<td>Smaller granules and more diffuse</td>
<td>No effect</td>
<td>Smaller granules and more diffuse</td>
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<tr>
<td>DCP1</td>
<td>Small foci remain, bigger foci disappear</td>
<td>ND</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>RIN</td>
<td>No effect</td>
<td>ND</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>+ puromycin</td>
<td>No effect</td>
<td>A few large foci appear</td>
<td>No effect</td>
<td>A few large foci appear</td>
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<td>No effect</td>
<td>ND</td>
<td>+ (bigger foci)</td>
<td>-</td>
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<tr>
<td>RIN</td>
<td>26% of the cells have a few bigger granules</td>
<td>ND</td>
<td>+ (bigger foci)</td>
<td>-</td>
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<td>+ arsenite</td>
<td>No effect</td>
<td>Bigger irregular granules appear, small foci still present</td>
<td>Less than half of the cells have bigger irregular granules</td>
<td>Bigger irregular granules appear</td>
</tr>
<tr>
<td>DCP1</td>
<td>Bigger irregular granules appear, small foci still present</td>
<td>ND</td>
<td>* (bigger foci)</td>
<td>* (bigger foci)</td>
</tr>
<tr>
<td>RIN</td>
<td>Bigger irregular granules appear</td>
<td>ND</td>
<td>* (bigger foci)</td>
<td>* (bigger foci)</td>
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Supplemental table 2. List of primary antibodies used.

<table>
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<tr>
<th>Antigen</th>
<th>Species</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>PUM</td>
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<td>Sonoda and Wharton, 1999</td>
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<tr>
<td>dFMR1</td>
<td>Mouse</td>
<td>Developed by K. S. Broadie under the auspices of the NICHD, maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242</td>
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<td>eIF4E</td>
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<td>L. Fradkin</td>
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<td>Yps</td>
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<td>Wilhelm et al., 2000</td>
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<td>DCP1</td>
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<td>Barbee et al., 2006</td>
</tr>
<tr>
<td>RIN</td>
<td>Rabbit</td>
<td>Pazman et al., 2000</td>
</tr>
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Supplemental movie 1. PUM-GFP granules oscillate around the same position. Images collected with a Leica SP2 confocal microscope, one image every 2 seconds.

Supplemental movie 2. Very few PUM-GFP granules have a short directed movement. Images collected with a Leica SP2 confocal microscope, one image every 2 seconds.

Supplemental movie 3. GFP-dFMR1 granules oscillate around the same position. Images collected with a Leica SP5 confocal microscope, one image every 2 seconds.

Supplemental movie 4. Very few GFP-dFMR1 granules have a short directed movement. Images collected with a Leica SP5 confocal microscope, one image every 2 seconds.

Supplemental material and methods

Constructs

dFMR1 3’UTR was generated by PCR on genomic DNA with primers caaagcggtaggaggagca (forward) and gcttgatggtttgttttg (reverse). Hunchback 3’UTR was generated by PCR on a plasmid (look up) with primers atataagatctgttccccatcaccatcacct (forward) and tatatgcggccgctattgcataaaaacatttcg (reverse). Both 3’UTR were digested with BglII and NotI and inserted into the reporter mRNA construct UAS-lacZ-MS2bs (Gemert et al., 2009). The MS2-GFP fusion protein was brought to expression with the UAS-GFP-MS2-nls construct (Gemert et al., 2009).