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mRNA cycles through hypoxia-induced stress granules in live *Drosophila* embryonic muscles

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ABSTRACT In some myopathies, hypoxia can be the result of pathologic effects like muscle necrosis and abnormal blood flow. At the molecular level, the consequence of hypoxic conditions is not yet fully understood. Under stress conditions, many housekeeping gene mRNAs are translationally silenced, while translation of other mRNAs increases. Alterations to the pool of mRNAs available for translation lead to the formation of so-called stress granules containing both mRNAs and proteins. Stress granule formation and dynamics have been investigated using cells in culture, but have not yet been examined *in vivo*. In *Drosophila* embryonic muscles, we found that hypoxia induces the formation of sarcoplasmic granules containing the established stress granule markers RIN and dFMR1. Upon restoration of normoxia, the observed granules were decreased in size, indicating that their formation might be reversible. Employing photobleaching approaches, we found that a cytoplasmic reporter mRNA rapidly shuttles in and out of the granules. Hence, stress granules are highly dynamic complexes and not simple temporary storage sites. Although mRNA rapidly cycles through the granules, its movement throughout the muscle is, remarkably, spatially restricted by the presence of yet undefined myofiber domains. Our results suggest that in hypoxic muscles mRNA remains highly mobile; however, its movement throughout the muscle is restricted by certain boundaries. The development of this *Drosophila* hypoxia model makes it possible to study the formation and dynamics of stress granules and their associated mRNAs and proteins in a living organism.

KEY WORDS: *Drosophila, FRAP, hypoxia, stress granules, mRNA*

Introduction

The synthesis, transport and translation of mRNAs are regulated to maintain normal cell function and to allow cells to respond to external signals and changes in environmental conditions. A possible way of regulating translation is by compartmentalization of mRNAs. For example, mRNAs are guided along microtubules towards dendritic synapses to be locally translated in response to synaptic stimulation (Kindler *et al*., 2005). Recently, we observed in *Drosophila* embryonic muscle cells a reporter mRNA to move within domains bounded by structures of yet undefined physical composition (van Gemert *et al*., 2009). At least three distinct domains were determined in which the mRNA can move freely but exchange between the domains proved relatively slow. This finding was not unexpected because in adult myofibers nuclei are evenly spaced along the entire length of the fiber and each nucleus is surrounded by a volume of cytoplasm that is nurtured by the gene products exclusively derived from this nucleus (Allen *et al*., 1999). Understanding the movement of mRNAs within myofibers is important because it may have consequences for regenerative therapies that are currently being developed to cure myopathies (Cossu and Sampaolesi, 2007). These therapies aim to change an entirely diseased myofiber into a hybrid myofiber that consists of both healthy and diseased nuclei. Cell-based therapies, as described above, might have a limited effect if the mRNAs transcribed by

Abbreviations used in this paper: FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; PB, processing body; SG, stress granule.

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the healthy nuclei and the proteins they encode are restricted to their nuclear domain and cannot redistribute throughout the myofiber. Therefore, studying the dynamics and translation of mRNAs in an *in vivo* model could shed light on the chance for these therapies to succeed.

Environmental stress conditions, including restrictive growth conditions, UV irradiation, heat shock and hypoxia, have an impact on the distribution of mRNAs in the cell and consequently on protein synthesis. Here we have used the *Drosophila* embryonic muscle as a model to study the effects of hypoxia, a condition found in several myopathies including Duchenne muscular dystrophy (DMD), on the dynamic behavior of a reporter mRNA and that of stress granules (SGs) and processing bodies (PBs). Hypoxia results in the phosphorylation of the translation initiation factor eIF2α or the blockage of the eIF4A helicase, preventing the initiation of translation (Anderson and Kedersha, 2009). The stalled initiation complexes recruit other proteins which cause these ribonucleoprotein particles (RNPs) to aggregate and form SGs. The composition of SGs differs depending on the type of cellular stress applied. It is thought that the SGs form to temporarily repress the translation of "housekeeping mRNAs", allowing the preferential translation of proteins that are required for the cell to respond to the type of stress. The formation of SGs also likely depletes the cytoplasm of several factors, thus delaying apoptosis and allowing the translation machinery to produce proteins that promote cell survival (Arimoto *et al.*, 2008). In contrast to SGs, PBs are implicated in mRNA degradation. However, a subset of mRNAs present in PBs can also resume translation (Bhattacharyya *et al.*, 2006; Brengues *et al.*, 2005).

Recently, Fluorescence Recovery After Photobleaching (FRAP) experiments in cell culture have shown that both SGs and PBs are highly dynamic structures that contain proteins which rapidly move in and out (Aizer *et al.*, 2008; Barbee *et al.*, 2006; Kedersha *et al.*, 2000; Kedersha *et al.*, 2005; Mollet *et al.*, 2008). In addition to proteins, mRNAs present in SGs are also mobile. SGs induced in HeLa cells by arsenite treatment contain mRNAs that continuously cycle in and out of the SGs, with full repopulation achieved after a few minutes (Mollet *et al.*, 2008). Several observations suggest that mRNAs travel between PBs, SGs and polysomes. For example, trapping mRNAs within polysomes by using a polysome-stabilizing drug such as cycloheximide reduces the size and number of PBs and SGs, presumably because less mRNA molecules are available to be incorporated. Polysome-destabilizing drugs have, as would be expected, the opposite effect, PB and SG size and numbers increase (Kedersha and Anderson, 2007). The observation of PBs docked with SGs provides an indication of their close physical contact which may facilitate interchange of their components (Kedersha *et al.*, 2005).

The use of *Drosophila* embryos and confocal microscopy allows the direct visualization of hypoxia-induced granules in living muscle cells without prior dissection. SG formation was induced by exposing the embryos to low oxygen tension at a relatively high temperature to enhance the response. Here, we show that the granules arising under these conditions contain RIN and dFMR1. RIN is the homologue of G3BP which was shown to be present in SGs (Irvine *et al.*, 2004; Pazman *et al.*, 2000) and is involved in the aggregation of RNPs (Tourriere *et al.*, 2003). dFMR1 is a mRNA-binding protein which negatively regulates translation (Laggerbauer *et al.*, 2001) and is another known component of SGs (Kim *et al.*, 2006; Mazroui *et al.*, 2002). We also investigated the presence of DCP1 in these hypoxia-induced granules. DCP1 has been implicated in mRNA degradation and is a cofactor of the mRNA decapping enzyme DCP2 (Sakuno *et al.*, 2004; She *et al.*, 2008). Furthermore, DCP1 is also a core PB component (Ingelfinger *et al.*, 2002). We find that DCP1 localizes into small granules under normoxic conditions while hypoxia results in the formation of larger DCP1-containing granules.

Results obtained by performing photobleaching experiments demonstrate that a reporter mRNA expressed in *Drosophila* embryos shuttles in and out of SGs induced by hypoxia, suggesting that the proposed role of SGs to refresh the protein cover of arrested mRNA reflects their function in live animals (Mollet *et al.*, 2008). By reloading a protein or by adding a posttranslational mark on the protein cover it is thought that the mRNA is kept intact until translation resumes. This hypothesis is in accordance with the finding that SGs contain many RNA-binding proteins that are known to regulate mRNA structure and function (Anderson and Kedersha, 2009). After return to normoxia, we show that the mRNA is released from large granules but is still integrated into small granules, indicating that the formation of SGs might be reversible. In summary, we have established an *in vivo* model to study SG induction and the dynamics of a SG-associated mRNA.

Results

Localization of RIN, dFMR1 and DCP1 proteins in Drosophila *embryonic muscles*

In *Drosophila* embryonic muscle, RIN protein, involved in the aggregation of RNPs, is located rather diffusely throughout the cytoplasm (Fig. 1A), indicating that RIN-containing granules do not form when the embryo develops under normoxic conditions. dFMR1, a mRNA-binding protein, localizes in a somewhat granular pattern throughout the muscle, while an accumulation of dFMR1 is observed at the muscle tips (Fig. 1B, arrows) (Schenck *et al.*, 2002) where the muscle attaches to the tendon cells. DCP1 protein, a core PB component, was seen to localize into small granules distributed throughout the cytoplasm (Fig. 1C), likely corresponding to the PBs.

Reporter mRNA localizes to hypoxia-induced granules

To investigate the presence of mRNA in granules induced by hypoxia, we employed a reporter mRNA expressed in *Drosophila*

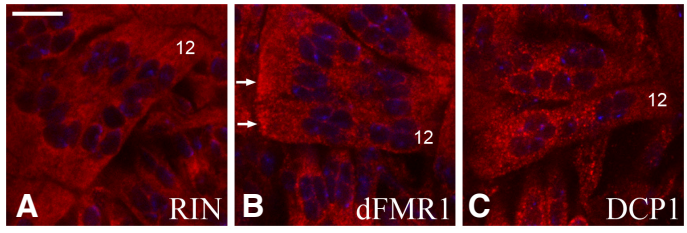

Fig. 1. RIN, dFMR1 and DCP1 protein localization in *Drosophila* embryonic muscles. (A) *RIN is localized rather diffuse throughout the cytoplasm,* **(B)** *dFMR1 localization is slightly granular, white arrows indicate an accumulation of granules at the muscle tip and* **(C)** *DCP1 localizes into granules (muscle ends are slightly out of focus due to muscle curvature). Nuclei are stained with Hoechst (blue). Muscle 12 is indicated on each image. Scale bar, 8 μm.*

embryos which can be visualized with the MS2-tagging system (Bertrand *et al.*, 1998; van Gemert *et al.*, 2009) by fluorescent labeling *in vivo*. The reporter mRNA contained the SV40 3'UTR (Untranslated Region) that is commonly present in *Drosophila* transgenes to increase mRNA stability. This 3'UTR, however, does not contain any known localization sequence.

When embryos were subjected to low oxygen tension, RIN protein-containing accumulations were observed (compare Fig. 2A with 2A'). The reporter mRNA localization, as visualized by anti-GFP antibody labeling, was also affected by hypoxia (compare Fig. 2B with 2B'); a part of it also shows to accumulate (Fig. 2B', arrow). Staining the GFP-tagged mRNA with an anti-GFP primary antibody was necessary because most of the GFP fluorescence is lost during the fixation procedure (Patel, 1994). Some of the GFP-fusion protein accumulates in the nucleus at the presumptive integration sites of the reporter transgene (Fig. 2 B-C, arrowheads). Larger granules located close to the nuclei appeared to contain both reporter mRNA and RIN protein and are possibly the result of GFP-tagged mRNA assembly in the perinuclear compartment (Fig. 2 A'-C', arrows). The same embryo was also stained for dFMR1, which showed to accumulate into the same structures as RIN (Fig. 2 A"-C", arrows), supporting the assumption that these are SGs.

Thus, hypoxia also affects dFMR1 protein localization in *Drosophila* embryonic muscles. After two hours of hypoxia, dFMR1-containing granules appeared predominantly around the nuclei (compare Fig. 2D with 2D'). Some of these dFMR1- positive granules also contain the GFP-tagged reporter mRNA (Fig. 2 D'-F', arrows), while others are GFP-negative (Fig. 2 D',F', hollow arrows). The arrowheads indicate again the presumptive integration sites of the reporter transgene inside a nucleus (Fig. 2E'-2F', arrowheads).

The induction of hypoxia also resulted in alterations in DCP1 localization. Under normoxic conditions DCP1 is localized into small granules, while during hypoxia DCP1 was concentrated in larger granules (compare Fig. 2G with 2G'). Interestingly, a subset of these granules was closely juxtaposed or colocalizing with the GFP-tagged mRNA granules (Fig. 2I', white circle). Due to the limited resolution of light microscopy, we could not determine

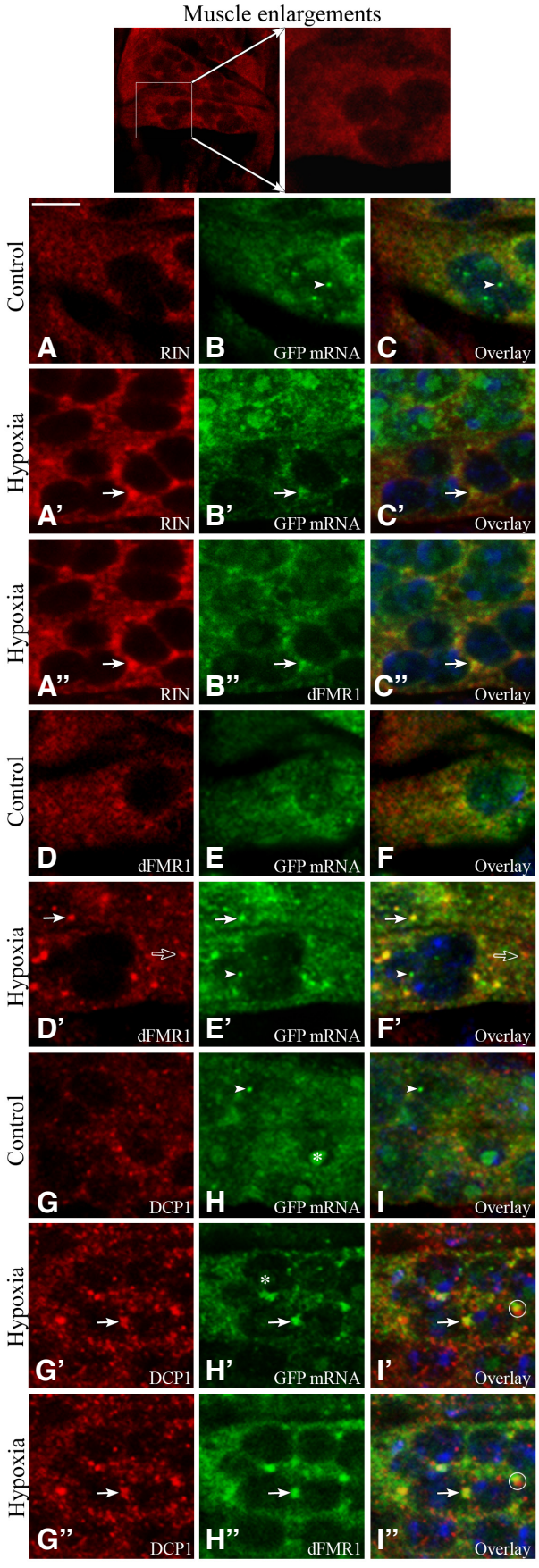

Fig. 2. RIN, dFMR1, DCP1 proteins and reporter mRNA localization under normoxic conditions (control) and hypoxic conditions. *Enlargements representative of the entire muscle as depicted in the first row are shown for each staining.* **(A,A',A",D,D',G,G',G")** *show antibody stainings directed against RIN, dFMR1 or DCP1, as indicated in the bottom right corner of each image.* **(B,B',E,E',H,H')** *show an anti-GFP staining and reveals the localization of the GFP-tagged reporter mRNA.* **(B",H")** *show an anti-dFMR1 staining.* **(C,C',C",F,F',I,I',I")** *are overlays including Hoechst nuclear staining (in blue). After two hours of hypoxia the number of granules containing the reporter mRNA increases* **(B', E', H')**. *Hypoxia and a high temperature also induce the formation of RIN- and dFMR1-positive granules from which a few dFMR1 granules are GFP-negative* **(D',F')** *(hollow arrows) and a few are positively stained for the GFP-tagged mRNA (indicated by arrows)* **(A'-C', D'-F')**. *Colocalization was mainly observed in the larger granules which are often located around the nuclei. Larger DCP1 granules induced by hypoxia are often in close proximity to or colocalizing with the GFP-tagged mRNA and dFMR1 granules* **(I', I")** *(white circles). Arrowheads indicate the DNA integration site of the mRNA; asterisks indicate nucleoli that occasionally contain excess of GFP-fusion protein. All incubations were done at 29°C. Scale bar, 4 μm.*

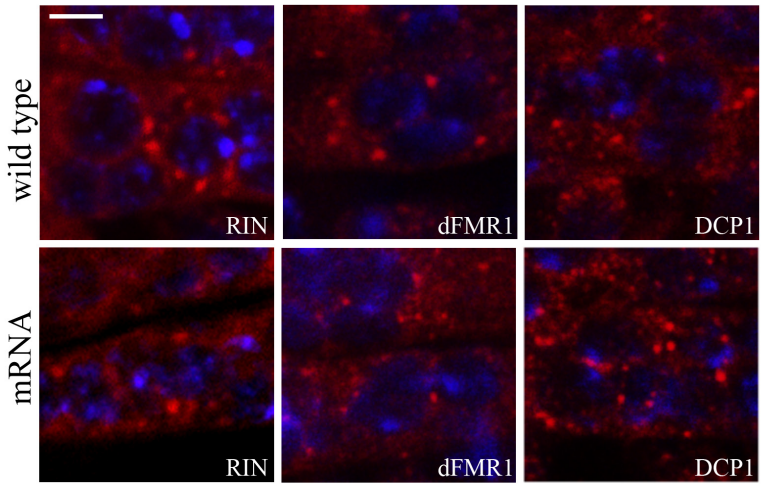

Fig. 3. The localization of RIN, dFMR1 and DCP1 proteins is not modified by the presence of mRNA. *Enlargements representative of the entire muscle are shown. RIN, dFMR1 and DCP1 protein localizations, as indicated in the right lower corner of each image, are shown under hypoxic conditions in either wild type embryos (upper row) or embryos expressing the reporter mRNA construct (lower row). Nuclei are stained with Hoechst (blue). Scale bar, 2.7 μm.*

whether these granules colocalize, make physical contact or even exchange certain components. Labeling of dFMR1 in the same embryo showed that the same subset of large granules were stained (Fig. 2 G''-I'', arrows). Colocalization of RIN and DCP1 could not be tested since both antibodies are raised in the same species of animal. To conclude, RIN-, dFMR1- and DCP1-containing granules form under hypoxic conditions and mainly the large RIN- and dFMR1 granules also include GFP-tagged reporter mRNA. Due to the limited resolution of our microscope it is unclear whether DCP1 is located inside these larger granules or in their close proximity.

In our experimental setup *Drosophila* embryos were exposed to normal oxygen tension and low oxygen tension at 29°C. To investigate whether this temperature could evoke a heat shock response that facilitates the formation of SGs under hypoxic conditions we investigated the localization of dFMR1 and GFPmRNA in embryos incubated at 25°C (Supplementary Fig. 1). Large granules staining positive for dFMR1 and/or GFP mRNA were no longer observed in muscle cells. Instead, small granules were observed that in general did not show a colocalization of dFMR1 and GFPmRNA (Supplementary Fig. 1 D-F). Embryos incubated at normal oxygen tension at 29°C showed a more diffuse staining (Supplementary Fig. S1 A-C). This indicates that a high temperature alone does not result in the formation of large stress-induced granules. It is the combined effect of hypoxia and a relatively high temperature that leads to the formation of the large SGs.

The expression of the reporter mRNA does not influence RIN, dFMR1 and DCP1 protein localizations

To investigate whether the granules induced by hypoxia at 29°C were not solely observed in the fly line expressing the reporter mRNA, we also subjected wild type embryos to low oxygen tension and used antibody labeling for the detection of the granules. RIN-, dFMR1- and DCP1-containing granules were observed in wild type embryos under hypoxic conditions (Fig. 3, upper row) as was observed in the embryos expressing the reporter mRNA (Fig. 3, lower row). This confirms that the formation of granules is not related to the expression of the reporter mRNA but is the result of hypoxia and a relatively high temperature.

mRNA shuttles in and out of hypoxia-induced stress granules and is released after return to normoxia

mRNA has been reported to shuttle in and out of SGs in cultured cells (Mollet *et al.*, 2008). To determine whether this is the case in hypoxic *Drosophila* muscle fibers *in vivo*, we performed FRAP experiments in live embryos. FRAP includes the irreversible bleaching of a specific region in a cell expressing a fluorescently tagged protein with a high power laser beam and subsequently monitoring the recovery of fluorescence intensity in the same region over time. If the fluorescently tagged proteins are fully mobile, fluorescence intensity recovers in the bleached area to its initial level (100% recovery) upon redistribution of fluorescent proteins. Conversely, if the fluorescently tagged proteins are partially immobile, fluorescence intensity does not recover to its initial level. A single SG inside the muscle cytoplasm, containing the GFP-tagged mRNA, was bleached and recovery under continued low oxygen tension at room temperature was monitored over time. After bleaching, fluorescence intensity dropped in the SG and rapidly recovered to a level of 70% within eleven seconds (Fig. 4, blue curve). This demonstrates that the reporter mRNA moves in and out of the SGs induced by hypoxia at 29°C. The observed flattening of the curve towards a plateau lower than 100% suggests the presence of an immobile fraction (Reits and Neefjes, 2001) in addition to the presence of the relatively fast recovering fraction. Unfortunately, recovery of the GFP-mRNA

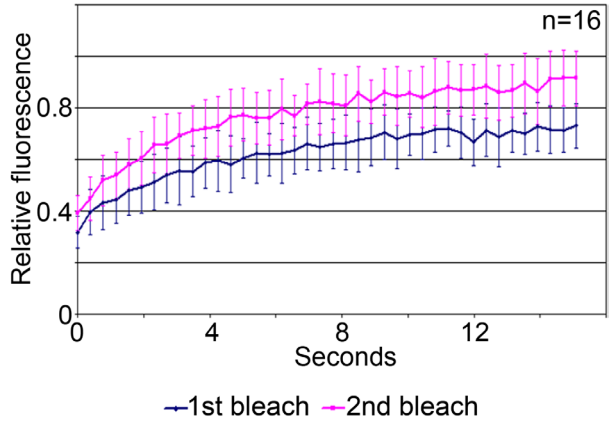

Fig. 4. Double FRAP (fluorescence recovery after photobleaching) measurement on hypoxia-induced stress granules in *Drosophila* embryonic muscle. *The average first recovery curve, showing a fast but not complete recovery of fluorescence signal, indicates that the mRNA is highly mobile inside the granule. The average second recovery curve reaches higher relative fluorescence intensities than the first one, suggesting the presence of an immobile fraction. Error bars represent standard deviation for each time point. Recovery curves were found to be significantly different from each other (n=16) (P-value = 0).*

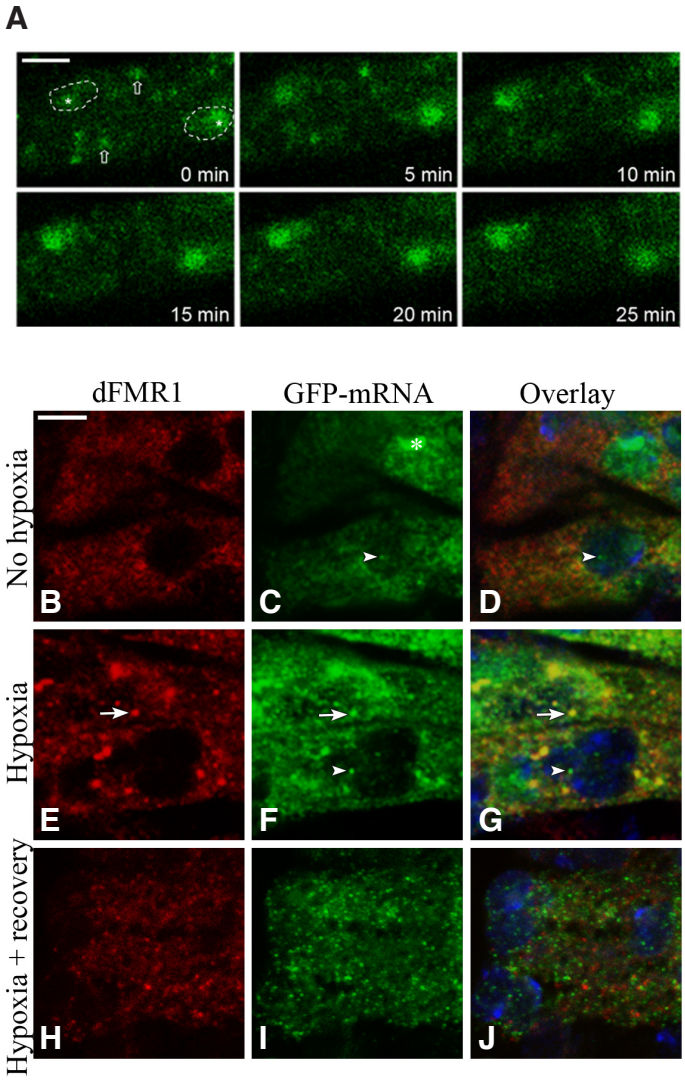

Fig. 5. The formation of large stress granules (SGs) is reversible in *Drosophila* embryonic muscles. (A) *After two hours of hypoxia at 29°C, live embryos were subsequently imaged every five minutes at room temperature after restoration of normoxia. Large SGs (indicated by a hollow arrow) gradually disappear after stress release. Nuclei and nucleoli, marked by excess of GFP-fusion protein, are indicated by white dotted circles and asterisks, respectively. Scale bar 3,1 μm.* **(B-J)** *dFMR1 protein and reporter mRNA localization under normoxic conditions, hypoxic conditions and after recovery from hypoxia. As shown in Figure 2, in contrast to normoxia* **(B-D)**, *hypoxia gives rise to the formation of large dFMR1-positive SGs* **(E-G)** *which in general colocalize with the GFPmRNA (arrows). Recovery of 30 minutes at normal oxygen tension after two hours of hypoxia results in the loss of the large colocalizing SGs but smaller granules remain showing no colocalization between dFMR1 and GFPmRNA* **(H-J)**. *Notice that there is more GFP present in the nuclei during recovery* **(I)**. *Arrowheads indicate the DNA integration site of the mRNA. Nuclei are stained with Hoechst (blue). Scale bar, 4 μm.*

inside the living embryo could not be monitored until a possible completion due to movements inside the embryo, such as the first contractions of the somatic musculature or the gut. These movements also result in a relatively high variance between experiments.

A second bleach event was performed in the context of a double FRAP experiment to further investigate the existence of an immobile fraction (Krouwels *et al.*, 2005; Stavreva and McNally, 2004). This includes a second bleach of the same region; only the molecules recovered from the first bleach will be bleached and can recover after this second bleach event which theoretically would result in a 100% recovery curve. The experiment yielded a recovery curve (Fig. 4, pink curve) that was significantly different from the first one (P-value = 0). The average recovery curve (n=16) did not reach a 100% recovery level within the time frame of the experiment. However, the curve has not reached a plateau yet, suggesting that full recovery is possible. These FRAP results show that, besides a mobile population of reporter mRNAs moving in and out of the SGs, a fraction of mRNA is apparently immobilized inside the granules presumably by association with yet undefined molecules.

Monitoring the SGs in live embryos at room temperature after stress release by reoxygenation showed that the granules progressively disassemble (Fig. 5A, hollow arrows and Supplementary Movie 1), which is in accordance with data obtained from SGs induced by arsenite in COS cells (Kedersha *et al.*, 2000). Already after ten minutes of normoxia hardly any SGs could be observed. Embryos that were allowed to recover for 30 minutes at normal oxygen tension showed by immunolabeling very small granules in their muscles only containing either dFMR1 or GFPmRNA (Fig. 5 H-J). Notably, the large granules observed in hypoxic muscles containing both dFMR1 and GFPmRNA (Fig. 5 E-G, arrows) were no longer observed. Whether the small granules will disassemble completely after a longer recovery period is not investigated because at this stage the embryos develop into larvae.

From the experiments performed on living organisms we conclude that the mRNAs cycle through the SGs although some are immobilized inside these granules and that the accumulation of GFP-tagged reporter mRNA into large SGs is reversible upon release of stress. It remains unclear if the small granules present after 30 minutes of stress release will eventually disassemble. If the immobile part of the mRNA undergoes prolonged binding inside the SGs for the duration of stress and to what proteins it binds is yet unknown.

Muscle domain boundaries are resistant to hypoxia

In a previous study, we showed that the movement of the GFP-tagged reporter mRNA under normoxic conditions was restricted by the presence of at least three different domain boundaries in *Drosophila* embryonic muscles (van Gemert *et al.*, 2009). Such a restricted movement could limit the distribution of mRNAs and proteins needed to increase muscle viability. If the boundaries are abolished when the cell undergoes hypoxia, as in some myopathies, therapies to treat myopathies might have more chance to succeed. Here, we address the question whether the domains delineated by the movement of the reporter mRNA are still present under hypoxic conditions. Embryos, exposed to low oxygen tension at 29°C were used for Fluorescence Loss In Photobleaching (FLIP) experiments. FLIP is performed by repeatedly bleaching a specific region in the cell expressing a fluorescently tagged protein with a high power laser beam and by monitoring the loss of fluorescence intensity in the rest of the cell. Any fluorescent molecule able to move through the bleach

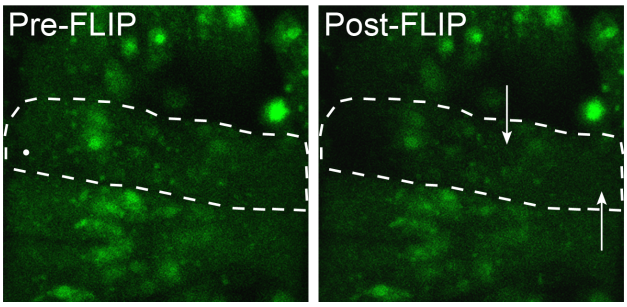

Fig. 6. FLIP (fluorescence loss in photobleaching) reveals the presence of muscle subdomains under hypoxic conditions. *Embryos under continuous low oxygen tension were submitted to 21 cycles of bleaching and imaging. The resulting pre- and post-FLIP images show that only the domain containing the bleach spot (indicated with a white spot) is bleached during the FLIP experiment (here the left muscle end domain). Fluorescence intensity does not drop in the remaining muscle indicated by arrows (muscle center and right muscle end). However, fluorescence intensity decreases uniformly throughout the entire field due to photobleaching by recurrent scanning of the sample. The white dotted line indicates the muscle outline.*

spot is eventually bleached and areas in which molecules can freely move can be identified. The bleach spot (Fig. 6, white spot) was positioned in the left muscle end domain in the cytoplasm of a *Drosophila* embryonic muscle in which hypoxia was maintained. The result of the FLIP experiment, showing a muscle expressing the GFP-tagged reporter mRNA under hypoxic conditions before and after bleaching, is illustrated in Fig. 6. In the post-FLIP image, the fluorescence signal has disappeared in the left muscle end domain but not in the rest of the muscle (Fig. 6, arrows). This result illustrates, that the domains previously delineated by the movement of the reporter mRNA under normoxic conditions at this stage of development (van Gemert *et al.*, 2009) remain during hypoxia.

Discussion

Here, we have shown that hypoxia not only affects the localization of several proteins implicated in mRNA processing but also affects the localization of a reporter mRNA in *Drosophila* embryonic muscles. This study using live embryos provides more insight into the dynamics and localization of mRNA after exposure to a hypoxic environment and its consequences for therapeutic approaches.

Hypoxia induces the formation of granules containing, among others, the RIN and dFMR1 proteins. The accumulation of RIN, the *Drosophila* homologue of G3BP which is an effector for assembly (Tourriere *et al.*, 2003) and a known component of SGs (Irvine *et al.*, 2004; Pazman *et al.*, 2000), under hypoxic conditions supports our identification of these granules as SGs. Furthermore, the presence of dFMR1 in these hypoxia-induced granules is in agreement with the presence of FMRP in rat hippocampal SGs induced by electrode injury (Kim *et al.*, 2006; Kim *et al.*, 2007). A combination of hypoxia and a relatively high temperature gave rise to large SGs, where RIN, dFMR1 and a GFP-tagged reporter mRNA were found to colocalize. Hypoxia alone led to the formation of much smaller granules, showing no longer colocalization of dFMR1 and GFP-mRNA. The formation of large SGs during hypoxia and thermal stress could possibly be mediated by heat shock proteins (HSPs). Recent findings suggest that HSPs provide tolerance to hyperthermia as well as to hypoxia, ischemia, inflammation, among other stress conditions. It has been shown that sustained hypoxia (1.0% for 2 h) increases the expression of the Hsp70 family proteins, Hsp68 and Hsp23. This increase was associated with a higher survival rate of *Drosophila* exposed to prolonged hypoxia (1.5% for 7 days). Mutants that have no copies or a few copies of Hsp70 mRNA were unable to survive, while overexpression of Hsp70 in specific parts of the brain or heart significantly increased *Drosophila* survival under chronic hypoxic conditions (Azad *et al.*, 2009).

The observation of DCP1 redistribution into larger granules suggests that low oxygen concentrations and hyperthermia also result in the formation of large PBs. Due to the limited resolution of light microscopy it remains unclear whether granules containing the reporter mRNA, dFMR1 and the typical SG-component RIN colocalize with DCP1-positive granules or are only in close proximity to each other. Colocalization would suggest that they are PBs storing their content for possible degradation. Current developments in super-resolution microscopy could possibly help to answer this question. Nevertheless, the possibility that PBs and SGs do not intermingle but are located close to each other is supported by a previous ultrastructural study where in stressed human cells SGs and PBs were found to be closely associated but distinct from each other (Souquere *et al.*, 2009).

As was previously found for SGs induced in tissue culture cell models, the hypoxia-induced granules we observed in our preparations also contain mRNA (Nover *et al.*, 1989). We showed that the reporter mRNA remains mobile with respect to the SGs as we observed a significant fluorescence recovery, although not complete, by FRAP (70% recovery was obtained in 15 seconds). It was shown in a previous study that a reporter mRNA present in arsenite-induced SGs in HeLa cells recovered to approximately 77% in 15 seconds and to 90% after 140 seconds (Mollet *et al.*, 2008). Unfortunately, a recovery of the mRNA inside the living embryo could not be monitored for a longer period of time because of movements within the embryo that are associated with its development. Such movements preclude accurate fluorescence intensity measurements. Since the recovery curve had not yet reached a plateau we believe that the mobility of mRNA inside SGs of living *Drosophila* embryos is comparable to that of mRNA inside SGs of cultured mammalian cells. Nevertheless, it should be taken into account that the experimental conditions are different. For example, kinetics inside cultured cells are measured at 37°C and in *Drosophila* muscles at room temperature.

We also demonstrated the presence of a relatively small immobile fraction inside the population of reporter mRNA localized in hypoxia-induced SGs. Fluorescence intensity after photobleaching recovered to a higher level after the second bleach event than after the first one, and yet, recovery was not complete (91%). As SGs were thought to act as storage sites, we expected to find an immobile fraction, but in addition we detected a bigger part of the mRNA cycling constant and rapidly in and out of the granules.

We observed that the large SGs disassemble upon release of stress, which is in accordance with observations that have been made on SGs induced by arsenite in COS tissue culture cells (Kedersha *et al.*, 2000). Some of the effects of hypoxia are reversible, for example, hypoxia induces cell cycle arrest in *Drosophila* embryos but cell cycle activity resumes about 20 min after re-establishing normoxia (DiGregorio *et al.*, 2001; Douglas *et al.*, 2001). However, antibody labeling revealed that after 30 minutes of reoxygenation

there are still small granules present showing a distinct localization of dFMR1 and GFPmRNA while colocalization was found under hypoxic conditions. It is possible that the mRNA is directed to other pathways for longer storage or degradation. Further investigation is needed to determine the function and composition of the small granules and to investigate if they will completely disassemble upon restoration of normoxia for a longer period of time.

The *Drosophila* muscle provides a powerful tool to further investigate the formation and dynamics of SGs *in vivo* as well as their involvement in several diseases. We previously found that the mobility of the reporter mRNAs is restricted by the presence of as yet undefined myofiber domain borders (van Gemert *et al*., 2009). Here, we report that also under hypoxic conditions mRNA continues to be limited in their movement, indicating that the domain boundaries are resistant to hypoxia at this stage of development. These findings may have consequences for gene and cell fusion-based approaches being developed to treat myopathies (Cossu and Sampaolesi, 2007). If therapeutic mRNAs cannot redistribute throughout the muscle fiber syncitium, therapeutic approaches that do not achieve expression in a high percentage of the nuclei in a treated myofiber will have a limited effect, unless the therapeutic protein itself is capable of moving throughout the muscle.

Hypoxia and respiratory failure are important features of DMD disease and a number of questions exist regarding the pathophysiological responses to hypoxia in muscle cells. *Drosophila* is an excellent genetically tractable model to study muscular dystrophies and neuronal abnormalities. A *Drosophila* model for DMD has been used extensively to study the effects of Dystrophin gene mutations (Pilgram *et al*., 2010; Shcherbata *et al*., 2007; van der Plas *et al*., 2007). From DMD muscles it is known they are affected by hypoxia (Mosqueira *et al*., 2010). It would be interesting to further explore the function of SGs and their involvement in animal models for myopathies. The comparison of mRNA dynamics under hypoxic conditions in Dystrophin knock out animals versus the wild type musculature will be an additional step towards understanding the effects of hypoxia on the disease progression of muscular dystrophies.

Materials and Methods

Transformation plasmids

Plasmids and constructs used were described previously (van Gemert *et al*., 2009)

Drosophila *stocks*

The wild type control embryos were derived from the w^{1118} stock. The 24B-Gal4 driver line (Brand and Perrimon, 1993) was used for transgene expression in the somatic embryonic musculature.

Transgenic **Drosophila** *stocks expressing GFP-tagged mRNA*

Transformation constructs referred to above were used to generate transgenic flies (van Gemert *et al*., 2009). The highest expressing independent homozygous fly founder line obtained from the UAS-lacZ-MS2bs was selected for the experiments in combination with the lowest expressing line obtained from the UAS-GFP-MS2-nls. To analyze GFP-tagged reporter mRNA in somatic muscles of stage 16 embryos the following crosses were made: 24B-Gal4 and UAS-GFP-MS2-nls were crossed, off spring was crossed with UAS-lacZ-MS2bs.

Hypoxia experiments

Embryos were collected for five hours at 21°C on apple juice agar plates, aged for three hours at 21°C, then for 16 hours at 18°C and 2½ hours at 21°C. They were dechorionated for two minutes in 50% bleach. For antibody labeling (Patel, 1994) embryos were placed back onto the agar plates for two hours of incubation with 1% oxygen at 29°C in a water jacketed incubator controlling oxygen levels by nitrogen gas injection. They were subsequently fixed for immunohistochemistry (Patel, 1994). For live embryo experiments staged dechorionized embryos were lined up on slides using heptane glue and covered with Halocarbon oil (Halocarbon, New Jersey, U.S.). Embryos were incubated for two hours with 1% oxygen at 29°C in the incubator and immediately transferred to the microscope for analysis. To diminish the pressure on the embryos during the microscopic analysis three 18x18 mm cover slips were attached to the slide using heptane glue, surrounding the lined up embryos. Hypoxia was maintained throughout the measurement by positioning a cover slip (24x50 mm) on top of the embryos.

To study the recovery from hypoxia, embryos were transferred from the low oxygen tension incubator to an incubator at normal oxygen tension and 21°C and incubated for 30 minutes prior to fixation for immunohistochemistry. For live embryo imaging the embryos were lined up on cover slips that fit into the microscope stage insert (25x75mm, Electron Microscopy Sciences, Hatfield, USA). After two hours of incubation with 1% oxygen at 29°C the embryos were imaged every five minutes at room temperature and normal oxygen tension for a total time span of 50 minutes.

Immunohistochemistry

Antibody labelings were performed as described (Patel, 1994). Primary antibodies were rabbit-anti-GFP (Invitrogen, Breda, The Netherlands), mouse-anti-GFP 3E6 (Invitrogen), rat-anti-GFP (Nacalai Tesque, Kyoto, Japan), rabbit-anti-RIN (Pazman *et al*., 2000), rabbit-anti-DCPI (Barbee *et al*., 2006), mouse-anti-dFMR1 (developed by K.S. Broadie under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Secondary antibodies: AlexaFluor^{488}, AlexaFluor^{568}, AlexaFluor^{633} and Cy5-conjugated secondary antibodies (Invitrogen). Nuclei were stained by incubating the embryos for 20 minutes with 0,1 μg/ml Hoechst 33342 (Sigma-Aldrich, Zwijndrecht, The Netherlands) during the second last washing step. Embryos were dissected and mounted in Citifluor (Agar scientific Ltd., Essex, UK). Images were taken on a Leica TCS SP5 DMI6000 confocal microscope (Leica Microsystems, Wetzlar, Germany) (12 bit resolution, 1024x1024 pixels, 400 Hz speed, pinhole 1 Airy disc), (Fig. 1 and 3: HCX PL APO 63x/1,4 NA oil-immersion objective, zoom factor 6), (Fig. 2, 5 and Supplementary Fig. S1: HCX PL APO CS 100x/1,4 NA oil-immersion objective, zoom factor 3). The following laserlines were used for imaging in a sequential scanning mode: the 405 nm diode was used for exciting Hoechst with emission captured between 410 nm and 480 nm, the 488 nm line from an Argon laser was used for exciting AlexaFluor^{488} with emission captured between 505 and 555 nm, the 561 nm line was used for exciting AlexaFluor^{568} with emission captured between 585 and 620 nm and the 633nm line was used for exciting AlexaFluor^{633} and Cy5 with emission captured between 645 and 750nm.

Imaging living embryos using confocal laser scanning microscopy

All live embryo experiments were carried out on a Leica TCS SP5 DMI6000 confocal microscope (Leica Microsystems) (HCX PLAPO 63×/1.4 NA oil-immersion objective, 12 bit resolution, 1024×1024 pixels, 1400 Hz speed, pinhole 2.1 Airy discs, zoomfactor 6) at room temperature for a maximum of 1 hour. Imaging was performed using the 488 nm line from an Argon laser operating at 0,57 mW (measured through the 10x objective), collecting emission between 500 and 600 nm.

FRAP protocol

FRAP experiments were performed using the 488 nm line from an Argon laser collecting emission between 500 and 600 nm. Each FRAP experiment starts with taking two pre-bleach images (minimum scanning time: 386 ms) (0,38 mW, measured through the 10x objective) followed by bleaching of a 1μm circular region of interest (ROI) (3.8 mW, zoomfactor 64) with a single

scan and monitoring the recovery by taking 40 images (minimum scanning time: 386 ms) (0,38 mW). For the double FRAP experiment this session was repeated (after approximately 5.7 seconds) with the same bleach ROI (Stavreva and McNally, 2004). Loss of fluorescence due to the recurrent scanning that is part of the FRAP protocol was never more than 12 %.

Acquired data were analyzed using LAS AF software (Leica Microsystems). To create FRAP curves, the fluorescence intensities were background-subtracted (region chosen in an area devoid of any myofibers), scan-corrected by dividing through the whole muscle intensity, and normalized to pre-bleach values (Stavreva and McNally, 2004). Averages were plotted in Microsoft Office Excel 2003. To determine a significant difference between the recovery curves, the area under a curve (AUC) was analyzed by first restructuring data in SPSS 16.0 followed by computing the AUC in NCSS 2007. P-values were calculated with a paired-sample T-test with two-tailed distribution. Series were considered different when the resulting p-value was less than 0.05.

FLIP protocol

FLIP experiments were performed using the 488 nm line from an Argon laser collecting emission between 500 and 600 nm. FLIP experiments span a total of 185 seconds and start with two pre-bleach images (minimal frame scanning time 386 ms per image) (acquired with ~0.57 mW) followed by a loop of 21 cycles composed of ten bleach events (8 s which includes bleaching and switching of the microscope from image to bleach mode and back) of one circular (diameter 1 μm) ROI (~3.8 mW, zoomfactor 64) and two images (772 ms). Loss of fluorescence due to the recurrent scanning that is part of the FLIP protocol was never more than 5%. Acquired data were analyzed using LAS AF software (Leica Microsystems).

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