miRNA-based buffering of the cobblestone-lissencephaly-associated extracellular matrix receptor dystroglycan via its alternative 3′-UTR

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Many proteins are expressed dynamically during different stages of cellular life and the accuracy of protein amounts is critical for cell endurance. Therefore, cells should have a perceptive system that notifies about fluctuations in the amounts of certain components and an executive system that efficiently restores their precise levels. At least one mechanism that evolution has employed for this task is regulation of 3′-UTR length for microRNA targeting. Here we show that in Drosophila the microRNA complex miR-310s acts as an executive mechanism to buffer levels of the muscular dystrophy-associated extracellular matrix receptor dystroglycan via its alternative 3′-UTR. miR-310s gene expression fluctuates depending on dystroglycan amounts and nitric oxide signalling, which perceives dystroglycan levels and regulates microRNA gene expression. Aberrant levels of dystroglycan or deficiencies in miR-310s and nitric oxide signalling result in cobblestone brain appearance, resembling human lissencephaly type II phenotype.
Developmental biologists typically are interested in phenotypes caused by gene loss-of-function; however, multiple stresses, unfavourable conditions, or developmental events can affect the precision of gene expression at the transcriptional level, resulting in altered protein levels. Elevated amounts of a critical protein can potentially be as deadly as its deficiency. In addition, the levels of numerous proteins constantly fluctuate due to different phases of cellular life, such as the cell cycle stage, cell shape rearrangements and motility. Many of the essential cellular functions are dependent on cell–extracellular matrix (ECM) contacts and are associated with cell shape reorganization1-3. The ECM provides signalling cues that coordinate multiple cell behaviour aspects to guarantee proper tissue formation and homeostasis4. The extracellular signals from the ECM to the cytoplasm are transmitted via ECM receptors (that is, integrins and dystroglycan) to activate specific signalling pathways inside the cell, modifying the gene expression programme. Conversely, changes inside the cell are transduced via these transmembrane proteins to adjust the surrounding ECM composition that in turn changes cell biochemistry5,6. The ECM constituents also establish a three-dimensional microenvironment that controls cell division and growth, survival and differentiation. As these processes are very dynamic, it suggests a need for a mechanism that restores proper levels of cell adhesion proteins. This mechanism should account for at least two major tasks: to sense abnormal levels of the protein and to send this information to induce a cellular response that will re-establish equilibrium.

Dystroglycan (Dg) is a conserved critical ECM receptor that is responsible for processes relevant to total organism health. It is a key component of the dystrophin (Dys) glycoprotein complex (DGC), connecting the ECM to the actin cytoskeleton via the cytoplasmic protein Dys5-7. The DGC is best known for its association with a large group of inherited neuromuscular diseases, muscular dystrophies. Owing to the high occurrence of these sometimes fatal diseases, the majority of research has focused on the role of the DGC in providing mechanical stability to the muscle sarcolemma to resist the vigour of contraction8-10. However, the DGC not only offers strength to the muscle sarcolemma, but also it has crucial roles in central nervous system development, as many muscular dystrophy patients experience cognitive impairment and learning disability, and develop some behavioural and neuropsychiatric disorders11. In particular, abnormal interaction of Dg and the ECM results in the cobblestone brain, which is an odd malformation, resulting from neuron overmigration and formation of an extracortical layer that resembles a bumpy cobblestone surface10,11. There are several vertebrate dystroglycanopathy models12, however, a Drosophila model has not been developed.

Previously, we have reported that Drosophila can serve as a great system to study muscular dystrophies and Dg deficiencies lead to muscle and nervous system disorders13,14. Drosophila Dg has distinct functions from Dys, and apart from its role in muscle maintenance it is involved in energy homeostasis, photoreceptor differentiation and neuromuscular junction establishment14-18. In addition, Dg deficiency has been shown to alter the microRNA (miRNA) profile, providing a link between the DGC and regulation of messenger RNA and protein levels19. Dg can be detected in various tissues in mammals and in Drosophila13,20,21. In both vertebrates and invertebrates, there are numerous Dg gene transcripts listed in GenBank, but it is not known why there is so much variation in the protein isoforms and what are regulatory events, including post-transcriptional control, which give rise to the mature, sugar-decorated protein. The broad expression pattern and the multiple cellular processes that Dg is being discovered to play a role in indicate that it can be considered as an ECM housekeeping gene (reviewed in ref. 3). Therefore, it is logical that there are various forms of regulation available to manage its levels, especially in the brain, where the exactitude of ECM–cell adhesion is particularly important for brain compartmentalization and greatly contributes to the brain complexity.

Now we report that precise levels of Dg per se are critical, as lower levels slow down neuronal stem cell division, while higher levels accelerate proliferation and perturb neuron differentiation. Interestingly, altered Dg levels lead to formation of cobblestone-like structures that outgrow the normal contour of the ECM-defined brain space. This phenotype is similar to the brain cortex abnormalities associated with dystroglycanopathies in humans, implying that Drosophila Dg mutants can serve as a model for cobblestone lissencephaly. We have found that misregulation of Dg affects the distribution of major cell adhesion proteins, which presumably alters cell–ECM interaction, consequently resulting in abnormal tissue assembly. The intensity of the cobblestone-like phenotype is proportional to the amount and the duration of Dg protein misregulation. Dg is subjected to alternative 3’-UTR processing, and our evidence suggests that this is a mechanism allowing for its partial visibility to the mir-310s complex of miRNAs. This regulation is critical in response to cell shape rearrangements that take place during neuronal stem cell division, neuron differentiation, and brain morphogenesis. As a consequence of the miR-310s regulation, only a portion of Dg mRNAs with an extended 3’-UTR can be targeted, preventing Dg levels from being reduced below a certain threshold that would affect nervous system development. In addition, miR-310s levels are sensitive to those of Dg and nitric oxide (NO) signalling alike, indicating a Dg-NO-miR-310s regulatory loop. As NO signalling regulates miRNA gene expression and the cellular localization of NO signalling components per se depends on Dg, it implies that Dg is involved indirectly in transcriptional activation of its own repressors, the miR-310s. Thus, this study is an example of the proposed perceptive-executive mechanism that manages the precision of protein amounts: the information about the higher ECM receptor Dg levels is relayed via signalling that induces transcription of miRNAs. miRNAs can posttranscriptionally regulate only a fraction of Dg mRNA with an alternative 3’-UTR, reducing the variance of the target and readjusting its levels via a negative feedback loop. We believe that this phenomenon might be indicative of a broader regulatory mechanism that could be in play to regulate ECM receptor levels in response to cell shape rearrangements in general.

**Results**

High levels of Dg result in cobblestone-like brain. Multiple studies imply that Dg is not only the transmembrane component of the DGC, but also is a well-designed platform that allows communication with many interacting proteins to modulate multiple cellular processes. Thus, we postulate that Dg levels need to be tightly controlled. To test this hypothesis, we used a previously established Drosophila muscular dystrophy model, where it was shown that Dg deficit leads to age-dependent muscle defects13,15,16. Now we tested Dg developmental requirements and found that Dg loss retards pre-adult development (Fig. 1a) and affects mutant animals’ viability (Fig. 1b), while its overexpression with a ubiquitous Gal4 driver (tub-Gal4) appears to be deadly (Fig. 1c). As Dg has been shown to play important roles in muscle and nervous tissues, we wanted to dissect whether this lethality was due to the requirement for exact Dg levels in muscles or in the nervous system using different tissue-specific drivers (expression exemplified in Supplementary Fig. 1). Increasing Dg levels in the muscle did not affect viability,
Loss of Dg leads to slower development.

Embryonic lethality induced by Dg overexpression.

Survival is reduced due to Dg deficiency.

Control    Control
DgO55/DgO55 DgO55/CyO
DgO55/DgO55 DgO55/DgO38
DgO55/DgO55 DgO55/DgO43
DgO55/DgO55 DgO55/DgO86

hsFlp; FRT42B GFP/FRT42B parental

Insc>Dg, GFP

DAPI DAPI DAPI

Eclosed flies
0% 20% 40% 60% 80% 100%
1 2 3 4 5 Days

Survival is reduced due to Dg deficiency.

Control
DgO55/DgO55
DgO55/DgO38
DgO55/DgO43
DgO55/DgO86

Insc>Dg, GFP

DAPI DAPI

Eclosed flies
0% 20% 40% 60% 80% 100%
1 2 3 4 5 Days

Embryonic lethality induced by Dg overexpression.

Control
DgO55/DgO55
DgO55/DgO38
DgO55/DgO43
DgO55/DgO86

Insc>Dg, GFP

DAPI DAPI

Eclosed flies
0% 20% 40% 60% 80% 100%
1 2 3 4 5 Days

Embryonic lethality induced by Dg overexpression.

Control
DgO55/DgO55
DgO55/DgO38
DgO55/DgO43
DgO55/DgO86

Insc>Dg, GFP

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Eclosed flies
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DAPI DAPI

Eclosed flies
0% 20% 40% 60% 80% 100%
1 2 3 4 5 Days
but in the nervous system caused high levels of embryonic lethality (Fig. 1c), suggesting that there might be a need to control Dg levels, specifically in the nervous system.

Next, we wanted to understand what is the cause for the lethality. Dg overexpression in the nervous system resulted in a peculiar phenotype: dense cellular masses that overgrow the normal brain contour (Fig. 1d,e; arrowheads). The majority of Dg-overexpressing mutants die during larval stages; however, a few escapers make it to adulthood. Their brains also contained lumps (Supplementary Fig. 2a–c), but in a lesser quantity than observed on average in larval brains, suggesting that the penetrance of the brain overgrowth phenotype is variable. We also noted that lump-like structures found within one brain could be irregular in size (Fig. 1e). As neuronal Gal4 drivers may have somewhat variable expression patterns, the size of the overgrowths should correlate with the localized strength of driver expression. To test this, we co-expressed green fluorescent protein (GFP) as a marker for the efficiency of Gal4 expression and full-length Dg (insc-Gal4 > UAS GFP, UAS Dg). We observed that clusters with the highest GFP levels (the strongest Gal4 activity, the highest Dg expression) produced qualitatively larger lumps (Fig. 1f), substantiating a direct correlation between the Dg amount and brain malformation. Similar effects were observed on Dg overexpression by pan-neuronal (elav-Gal4) and neuronal stem-cell-specific (wor-Gal4) drivers: some of the neuronal stem cells (neuroblasts (NBs)) expressed high, while others moderate levels of Dg (Supplementary Fig. 2d–l). Interestingly, NBs and their progeny that had similar amounts of Dg protein clustered together and grouped into separate lumps, indicating that lump size and assembly correlate with Dg levels.

To specify the time course of the abnormal lump formation and provide evidence that this effect is cell-autonomous, we took advantage of the temporally inducible FRT–FLP system to obtain Dg overexpression clonal cells. Early Dg-overexpressing clones (analysed 1 day after clone induction) did not show any obvious abnormalities; however, after 3 days, clonal cells that over-expressed Dg started to aggregate and formed clearly outlined three-dimensional masses, beginning to separate from the rest of the brain tissue (Supplementary Fig. 2g,h). Notably, induced Dg-deficient cellular clones or Dg downregulation (tub > DgRNAi) also resulted in similar brain malformations; however, the observed phenotype was milder in comparison with its overexpression (Fig. 1h and Supplementary Table 2). Thus, regardless of whether Dg levels are increased or decreased, the cobblestone-like brain phenotype appears.

These data demonstrate the following: (1) the precision of Dg expression levels affects brain morphology; (2) the severity of the observed brain defect depends on the amount and duration of abnormal Dg levels; and (3) the effect of Dg misregulation on the brain lump formation is cell autonomous, which together implies that Dg levels must be stabilized for normal brain development.

**Dg non-tumorous outgrowths caused by cell adhesion defects.** Next, we wanted to understand the nature of these brain defects. First, we suspected that the observed Dg-linked brain lumps were tumours that occurred due to neuronal stem cell division and/or neuron differentiation defects. However, staining of mutant brains with the antibody against the neuronal stem cell and neuron-specific markers proved that asymmetry of neuronal stem cell division and the major differentiation processes are not affected (Fig. 2a). Thus, we concluded that these lumps were not tumours.

Second, we hypothesized that Dg’s interaction with the ECM could be responsible for the cobblestone-like brain phenotype and the strength of the ECM–cell interaction could contribute to abnormal lump formation. Similar to that in vertebrates, *Drosophila* Dg contains two functional domains: the extracellular domain that connects to the ECM and the cytoplasmic domain that interacts with Dys and other DGC proteins. To establish which functional part of Dg is responsible for the observed brain overgrowth phenotype, we overexpressed a truncated Dg version that lacks the extracellular domain (Dg4AE, Supplementary Fig. 3a). Importantly, misregulation of this version of Dg had no effect on the brain shape (Supplementary Fig. 3a–c), suggesting that the altered brain morphology due to abnormal Dg levels results from impaired Dg-ECM communication.

Therefore, we analysed the expression pattern of cell adhesion proteins in tandem with Dg overexpression. In contrast to neuronal cell differentiation markers, we found the distribution of major cell adhesion receptors and ECM molecules to be highly abnormal. According to the genotype, Dg levels were extremely upregulated in the brain outgrowths (Fig. 2b). In vertebrates, Dg is a key receptor for the major class of ECM proteins, laminins, and defects in α-Dg posttranslational modifications cause aberrant interactions with different laminins, causing muscular dystrophy development and exacerbation.

As it has not been shown that in *Drosophila* brain Dg interacts with laminins, we performed co-immunoprecipitation analysis with the brain-specific GFP-tagged Dg (Supplementary Fig. 3d,e) and the ECM protein LamininB (LanB), demonstrating that Dg can act as a laminin receptor in the fly brain (Supplementary Fig. 3e). Moreover, LanB was notably upregulated in the forming cysts (Fig. 2b), Another ECM protein, Perlecan (Pcan) that binds Dg in *vitro* was also upregulated and differentially localized (Fig. 2b). LanB and Pcan are major macromolecular components of the neural lamella, the extracellular net-like.
ECM sheet that outlines the developing brain. On Dg misregulation, lumps protrude above this ECM-formed outermost brain layer (Fig. 2b). At the same time, levels of the major extracellular receptor required for cell–ECM interaction, integrin (βPS), is drastically reduced in these structures compared with Control (Fig. 2c). It is known that Dg competes with integrins for ECM binding, and levels of both Dg and integrins are co-dependently readjusted at the transcriptional level via the ECM27. Thus, Dg misregulation alters the βPS integrin expression pattern and ECM composition, such as LanB and Pcan levels and distribution. In addition, higher Dg levels cause reduction as well as mislocalization of cell–cell adhesion proteins at the cellular membrane (β-catenin = Arm and E-cadherin = Cad, Fig. 2c). Together, these data demonstrate that the Dg-malformed brain phenotype is associated with abnormal cell–ECM and cell–cell adhesion.

Thus, on Dg misregulation, neuronal cells express altered levels of ECM molecules and cell adhesion receptors, which cause abnormal interaction of these cells with the rest of the brain tissue. As a result of selective cell adhesion, these cells with aberrant Dg levels cluster together, forming lumps that are dissociated from other cells and are pushed out from the brain. Notably, similar cobblestone brain structure is a characteristic of lissencephaly type II in humans suffering from dystroglycanopathies10,28, which are also caused by abnormal α-Dg interaction with the ECM. Resemblance of the brain phenotypes proposes that Drosophila Dg mutants can be used as a model to study these neurodevelopmental diseases.
Dg can be accessible to miRNA regulation via a variable 3'-UTR. Owing to the evidence that Dg needs to be precisely regulated in the nervous system, we next studied the mechanism of how Dg can be regulated there. The Dg transcript has the possibility of having at least two different 3'-UTRs according to FlyBase annotation (Fig. 3a). Recently, it has been shown that 3'-UTRs of metazoan mRNAs contain various regulatory motifs that influence mRNA stability, localization and translational efficiency, affecting numerous cellular processes29,30. It is logical to hypothesize that longer 3'-UTRs may contain regulatory motifs that can be used to fine-tune spatiotemporal signaling. When we probed Dg complementary DNA with primers that recognize a region belonging to both the short and long 3'-UTR and a region of only the long 3'-UTR, we found that ~60% of Dg transcripts have the longer 3'-UTR version (Fig. 3b), mRNA in situ hybridization using a probe with primers that recognize the long 3'-UTR showed expression in the brain, imaginal disks, muscles and other tissues (Fig. 3c and Supplementary Fig. 4a) and no obvious difference in expression patterns was observed between the Dg protein13,14,16 and the Dg long 3'-UTR mRNA.

As Dg has an alternative 3'-UTR, it can be reasoned that there is a regulatory function for this diversity. Theoretically, by varying the 3'-UTR length, Dg becomes more or less visible to regulation by miRNAs. For example, the short 3'-UTR does not have, while the Dg-extended 3'-UTR has, a predicted binding site for a family of miRNAs, the mir-310s (miR-310, miR-311, miR-312, miR-313), miR-92a and miR-92b, which we found to be Dg-dependent in our miRNA microarray screen19. The long 3'-UTR is evolutionarily conserved and present among all Drosophila species, but the conservation in the sequence corresponding to the seed of the newly evolved miR-310s miRNA family (Fig. 3a) developed along with the emergence of the miR-310s, via miR-92a/b duplication, around 53 million years ago21. As the conservation of the corresponding sequence necessary for targeting by the miR-310s appeared at the same time as these miRNAs emerged, we thought that it might be physiologically relevant and, next, we examined whether the miR-310s can target Dg.

First, to determine whether the miR-310s target Dg’s extended 3'-UTR, we assayed a luciferase reporter gene with different 3'-UTR fragments in Drosophila S2R+ cells (Supplementary Table 3). The luciferase assay showed that the miR-310s can target Dg via the extended 3'-UTR in vitro and confirmed the miR-310 seed sequence specificity (Fig. 3d). Next, we also verified that Dg could be targeted by the miR-310s in Drosophila tissue, where we analysed ovarian follicle cell clones that ectopically express the miR-310s (Supplementary Fig. 4c). Together, these data demonstrate that the miR-310s can downregulate Dg in vitro and in vivo via its long 3'-UTR. Finally, we tested whether miR-310s-based

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Figure 3 | Dg’s variable 3'-UTR allows for alternative targeting by miR-310s. (a) Schematic drawing of the variable Dg 3'-UTRs, the short and the long that is extended by an additional 491 bp. Arrows mark the location of primers used to detect Dg mRNA with both the shorter and the longer 3'-UTR, and just the long 3'-UTR. (b) PCR analysis of cDNA from whole flies with primers that recognize the region of the long 3'-UTR, and the region common for both short and long 3'-UTR versions of Dg transcript show that ~60% of the Dg mRNA has the longer 3'-UTR region present (analysis was done taking into the account primer efficiencies). (c) In situ hybridization against the long 3'-UTR region shows that the long 3'-UTR containing Dg mRNA is present in the larval brain. Scale bar, 25 μm. (d) Consistent with the fact that only the extended version of the Dg 3'-UTR has the miR-310s binding sites, no reduction in luciferase activity is observed when fragments of the Dg 3'-UTR common for the short and long Dg 3'-UTRs (but without the miR-310s binding site) were assayed (P1, P2 and P3). When the reporter gene fused to an appropriate fragment of the Dg long 3'-UTR containing the miR-310s putative binding site is assayed (P4), a decrease in luciferase activity is observed even in the absence of miRNA transgenes (~1.5-fold), presumably due to endogenous levels of targeting miRNAs in the cells. When also transformed with plasmids expressing miR-310s (all), individual cluster miRNAs and miR-92a (but not mut-miR-92b that contains a single point mutation in the seed sequence, CACTGCA instead of CATTGCA), a further decrease in luciferase activity (~1.3- to 4.7-fold) is seen (Supplementary Table 3). (e) KT40 mutants (loss of miR-310s) have elevated levels of total Dg mRNA detected by primers that recognize both short and long 3'-UTRs. This upregulation is more pronounced when primers specifically against the long 3'-UTR region or RNA extracts from heads were used (Supplementary Table 4). Three to five independent biological experiments were analysed. The AVF ± s.d. is reported and two-tailed Student’s t-test was used to test for statistical analyses: *P≤0.05, **P≤0.01, ***P≤0.001, NS, not significant.
regulation of Dg has a functional role in living animals. We found that miR-310s loss-of-function mutants, KT40 (ref. 32) have higher levels of total Dg mRNA detected by primers that recognize the short and long 3' -UTRs and only the long 3' -UTR (Fig. 3e). This dramatic increase in Dg expression could be also seen at the protein level measured by western blot analysis (Supplementary Fig. 4b). Dg mRNA levels were increased even more in heads alone, indicating that Dg regulation is primarily occurring in the head. Moreover, Dg mRNA levels with the long 3' -UTR in the head increased ~8.5 times, presumably due to Dg targeting in the central brain and eyes (Fig. 3e). The determining mechanism for transcribed 3' -UTR length is ambiguous, but in the presence of mixed 3' -UTRs certain levels of Dg would be maintained via invisibility to miR-310s targeting, offering
miR-310s and Dg expression in the nervous system is dynamic.
We analysed the expression pattern of Dg and the miR-310s. Interestingly, we found that there are two possible modes of how miR-310s can regulate Dg levels: axonal bundling in the central brain neuropils express Dg and miR-310s in a mutually exclusive pattern (Fig. 4a–d), while cells located at the brain surface or in the developing visual system express both the miRNA and its target Dg (Fig. 4b–c). miRNA functions can be classified into two categories: expression tuning (when a miRNA is setting the mean of the expression level of the target gene resulting in the ‘on-off’ expression pattern) and expression buffering (when a miRNA reduces the variance of the target where the miRNA and its target are co-expressed). These two functions are independent and can co-exist (Fig. 4g, reviewed in ref. 33). Dg protein expression analysis in miR-310s deficient flies shows that miR-310s and Dg could co-exist in the expression tuning and buffering modes (Fig. 4d–f).

Dg, detected by antibody staining, is broadly expressed in the nervous system and, as expected, its levels are variable. For example, some neuronal stem cells had higher, while others had lower or undetectable, Dg amounts (Fig. 4i). The fluctuating Dg levels were also characteristic for other cells in the brain, suggesting that its levels vary, possibly during different stages of the neuronal lifecycle, such as cell division or neuron maturation. We analysed in detail neuronal cells that co-express Dg and the miR-310s. If Dg levels fluctuate, they must be re-adjusted; therefore, we analysed in more detail neuronal cells that co-express Dg and the miR-310s. miR-310s expression (marked by nuclear lacZ, miR-310s > lacZ, driven by the endogenous miR-310s promoter) was detected in a subset of each Dg-expressing cell type on the brain surface and their expression levels were also fluctuating (Fig. 4h and Supplementary Fig. 5a). However, on miR-310s loss, Dg levels were visibly increased in all brain cells (Fig. 4i,j). Comparison of the expression patterns shows that Dg and the miR-310s are expressed in some of the same cell types and their levels are dynamic, confirming the expression-buffering mode of miR-310s-Dg interaction (Fig. 4g).

Considering that Dg levels are critical for proper formation of the nervous tissue and that the miR-310s can regulate only isoforms of the Dg mRNA with the extended 3′-UTR (~60% of total Dg mRNA), we propose that the miR-310s buffer the expression of its target Dg against fluctuation, perhaps dynamically.

Dg and miR-310s fine-tune the mitotic index of neuronal stem cells. As miRNA-based regulation could offer additional safeguarding, we next investigated the biological significance of Dg buffering by these miRNAs in the nervous system. We tested whether similar phenotypes are associated with Dg deregulation and the miR-310s loss in the developing brain. Importantly, we found that the miR-310s loss-of-function mutant, similar to Dg loss- and gain-of-function mutants, exhibits cobblestone brain (Supplementary Table 2), suggesting that Dg buffering by the miR-310s via Dg’s extended 3′-UTR is relevant for brain development.

Dg misregulation affects the amounts of multiple proteins involved in cell adhesion (Fig. 2c), intensity of which modifies multiple aspects of the cellular lifecycle, including cell proliferation. In particular, we found that the loss of Dg results in a decreased number of mitotically active neuronal stem cells, NBs (Fig. 5a–d). Moreover, Dg overexpression in miR-310s-expressing cells and miR-310s deficiency caused a significant overproliferation rate, while Dg deficit substantially decreased the NB mitotic index (Fig. 5d). These data show that managing the levels of miR-310s and the ECM receptor Dg is important to fine-tune the mitotic index of neuronal stem cells. We hypothesize that this is because NB cell divisions are normally accompanied by cell–ECM and cell–cell contact rearrangements requiring a proper ECM composition that depends on Dg amounts.

miR-310s regulation of Dg is necessary in the visual system. Other processes, accuracy of which highly depends on ECM composition, are neuron differentiation and axon pathfinding. Therefore, next we analysed whether the miRNA-based regulation of Dg levels takes place in the visual system, where Dg previously has been shown to play an essential role in terminal differentiation of Drosophila photoreceptor neurons. We found that only some of the photoreceptor neurons within ommatidium express the miR-310s and that Dg levels are miR-310s dependent; in miR-310s mutants, Dg was accumulated and its cellular localization was affected in these cells (Fig. 6a–c and Supplementary Fig. 5).

Photoreceptor neurons undergo complex reorganization during development: migration, axon growth and projection, and synapse formation, and all of these events are associated with dynamic cell shape rearrangements that are highly dependent on cell adhesion (reviewed in ref. 1). Ectopic Dg expression in photoreceptor neurons and specifically in miR-310s-expressing cells recapitulates the premature axon stops noted in miR-310s mutants (Fig. 6d–h). Importantly, reduction of Dg specifically in photoreceptor cells and miR-310s overexpression can rescue the premature axon termination phenotype in the miR-310s mutant background (Fig. 6h), showing that regulation of exact Dg levels via the miR-310s is important for proper photoreceptor neuron differentiation.

In summary, these data show that in the nervous system maintaining precise levels of the ECM receptor, Dg, is particularly important, as Dg abnormal levels affect distribution of cell adhesion proteins, subsequently influencing neuronal stem cell division speed, neuron differentiation and nervous tissue morphology.

Dg regulates miR-310s expression via a negative feedback loop. If the miR-310s are assigned to buffer Dg only when required, it is possible that miRNA expression itself could be sensitive to higher levels of the target. Thus, we hypothesize that the miR-310s promoter can be responsive to Dg amounts, which would reassure homeostatic Dg levels. To confer the responsiveness of miR-310s expression to abnormal Dg levels, we overexpressed Dg with the dynamic miR-310s promoter that is shared by all miRNAs in the miR-310s family and measured the levels of a representative miRNA, miR-312. We observed an ~5-fold increase in miR-312 levels due to Dg overexpression, reciprocally, Dg loss results in lower levels of miR-312 (Fig. 7a). This effect can also be visualized on the cellular level; for example, when in Control, the majority of ommatidia in the eye imaginal disk contain two cells that have the transcriptionally active miR-310s promoter, and Dg overexpression results in more cells actively expressing the miR-310s (Fig. 7b,c). These data imply that the miR-310s and Dg are in a negative feedback loop, where expression of miRNAs is induced in response to higher levels of their target (Fig. 7d).

Dg regulates miR-310s expression via NO signalling. Dg is a transmembrane receptor and the question of how it can regulate miR-310s expression at the transcriptional level needs to be answered. Previously, it has been shown in vertebrates that the DGC acts as a scaffold responsible for the membrane localization
**Figure 5 | The mir-310s and Dg regulate proliferation of neuronal stem cells.** (a,b) On Dg deficiency, the frequency of actively dividing cells (S phase, marked by EdU) is significantly decreased in the larval brain in comparison with Control. Fourteen to 16 lobes were counted per genotype. (c,d) On Dg deficiency (Dg086, Dg055 and FRT42B Dg086 clones) or Dg downregulation using RNAi driven by the ubiquitous and the dynamic mir-310s Gal4 drivers (tub > DgRNAi and mir-310s > DgRNAi), the frequencies of NBs in M phase (marked by PH3 and Mira, yellow) are significantly decreased. Dg overexpression in mir-310s-expressing cells results in overproliferation defects comparable to the defects occurring due to the loss of the mir-310s (KT40 and KT40/Df(Exel)6070), the mitotic index of larval brain NBs significantly increased compared with Control. Six to 17 lobes were counted per genotype. To prove that this decrease in the number of PH3- and EdU-positive NBs is not due to increased apoptosis induced by the absence of the principal ECM receptor, activated Caspase3 staining was performed in Dg loss-of-function (Dg055) mutants and no elevated numbers of apoptotic cells per brain were observed; four out of nine brains had one apoptotic cell where the maximum number of positive cells seen was four in one brain, which was similar to Control. Dg knockdown cannot rescue the mir-310s deletion phenotype (compare KT40; tub > DgRNAi and tub > DgRNAi), plausibly because RNAi-based downregulation of Dg is more efficient than Dg upregulation resulting from mir-310s loss. Overexpression of another confirmed mir-310s target, Khc-73 (ref. 32) (mir-310s-Gal4/UAS-Khc-73), decreases the mitotic index, which is opposite to the increase seen in KT40 and mir-310s-Gal4/UAS-Dg mutants. These data confirm previous findings about the role for Khc-73 in NB division; however, this shows that the increased proliferation phenotype observed in mir-310s loss-of-function mutants is not due to an mir-310s-Khc-73 interaction in NBs. See also Supplementary Table 5. The AVE ± s.d. are reported and significance was tested using the two-tailed Student’s t-test: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, NS, not significant. Scale bar, 25 μm.
of signalling proteins (Fig. 8a). In particular, Dg, via Dys or Dystrobrevin, anchors Syntrins (Syn) that bind neuronal NO synthase and, as a result, regulate generation of NO35,36. NO nitrosylates the histone deacetylases that regulate gene transcription and in vertebrate muscles the Dys-Syn-neuronal NO synthase signalling pathway regulates histone modifications that control transcription of miRNA genes37. Recently, we showed that in Drosophila, Dg-Dys-Syn signalling also controls the miRNA expression profile19. Therefore, we decided to test whether Dg-mediated regulation of miR-310s expression occurs via NO signalling.

First, we addressed whether the outgrowth phenotype induced by Dg overexpression depends on the DGC being a platform for NO signalling. To test this, we analysed Dys expression in the mutant brains and found that on Dg overexpression Dys amounts at the protein (but not mRNA) levels are significantly increased.
Supplementary Fig. 6). Plausibly, Dys is stabilized when bound to Dg and it is anchored to the membrane. The increase of both major components of the complex, specifically in brain lumps, allows the assumption that the outgrowth phenotype induced by overexpression of Dg does occur through the DGC. Next, we tested whether the levels of miR-310s expression depend on DGC-NO signalling. To provide the mechanistic evidence for NO regulation of miR-310s-Dg expression, we fed Drosophila

Figure 7 | miR-310s expression depends on Dg levels. (a) Dg mRNA levels are upregulated in flies where Dg is expressed under control of the miR-310s endogenous promoter. High levels of Dg coincide with increased, while lack of Dg with decreased amounts of miR-312, one of the miRNAs from the miR-310s complex. Three independent biological replicates were analysed. The AVE ± s.d. is reported and the two-tailed Student’s t-test was used to test for statistical significance: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. See Supplementary Table 4. (b) Dg overexpression (miR-310s-Gal4/+; UAS-Dg/UAS-mCD8-GFP, UAS-nLacZ) in miR-310s-expressing cells marked by anti-GFP and β-Gal staining leads to an increased number of cells positive for GFP and β-Gal in comparison with animals that do not overexpress Dg (miR-310s-Gal4/+; UAS-mCD8-GFP, UAS-nLacZ). Scale bar, 2 μm. (c) Histogram showing the frequency of miR-310s-positive cells/ommatidium (miR-310s-Gal4/+; UAS-mCD8-GFP, UAS-nLacZ, n = 29 ommatidia, miR-310s-Gal4/+; UAS-Dg/UAS-mCD8-GFP, UAS-nLacZ, n = 24). The two-tailed Mann–Whitney U-test predicts statistical significance with P = 0.0027. (d) Schematic model showing the miR-310s and Dg regulation in a negative feedback loop.
Model for perceptive-executive mechanism of miRNA-based Dystroglycan buffering
nitroglycerin (NG) as a NO donor and measured the levels of miR-310 and miR-312. Importantly, NG treatment significantly increased, while NO synthase deficiency \((\text{ANOS})\) or SynRNAsi significantly decreased these miRNAs levels, conferring the miR-310s promoter responsiveness to NO signalling (Fig. 8b). Even more, similar to miR-310s mutants, Dg levels were upregulated on NO synthase deficiency (Fig. 8c).

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**Table 1 | 3'-UTRs of known Drosophila ECM receptors.**

| Gene name | Binding partners | Extended 3'-UTR variants | miRNA binding sites in extended regions of 3'-UTR (TargetScan) | Nervous system phenotypes |
|-----------|------------------|--------------------------|---------------------------------------------------------------|--------------------------|
| Dystroglycan (Dg) | Laminin, Perlecán | 1,609–2,100 nt | miR-315, miR-33, miR-985, miR-927, miR-310s/92a,b | NMJ defects, reduced number of synaptic boutons |
| division abnormally delayed (daily) | Hedgehog, Decapenta-plegic, Magu | 834–1,873 nt | n/a | Cell cycle defects of lamina precursors |
| Syndecan (Sdc) | Slit, Robo, Nrx IV, Laminin A, DLAR | 335–945 nt | miR-989, miR-281-2-5p, miR-967 | Pathfinding errors in CNS midline, retinal and motoraxons |
| dally-like protein (dlp) | Wingless, Hedgehog, Branchless | none | — | Misrouted CNS midline and retinal axons |
| multiple edematous wings (mew, αPS1 integrin) | Laminin A, Slit | 160–460 nt | miR-932, miR-961 | Misrouted motor nerves |
| inflated (if, αPS2 integrin) | Laminin W, Slit, Tenectin, Thrombospondin | None | — | Misrouted motor nerves, errors in CNS pathfinding |
| scab (αPS3 integrin) | Laminin W, Slit | None | — | Errors in CNS pathfinding, learning defects |
| αPS4 | ND | None | — | ND |
| αPS5 | ND | None | — | ND |
| myospheroid (myo, βPS integrin) | αPS1-5 | None | — | Misrouted motor nerves, errors in CNS pathfinding, NMJ defects |
| βint-ν (βν integrin) | ND | None | — | ND |

CNS, central nervous system; ECM, extracellular matrix; NMJ, neuromuscular junction; n/a, extended 3'-UTR sequence is not present; ND, not determined; UTR, untranslated region.

*Non-conserved miRNA binding site (D. melanogaster specific)
As Dg is an anchor protein required for localization of NO signalling components, its amounts potentially can regulate NO signalling strength and play an indirect role in control of at least miR-310s expression. Therefore, we tested NO levels in flies that overexpress Dg in the nervous system and observed that relative NO levels were increased, which was comparable to the amounts measured on NG treatment, while in NOS-deficient flies these were significantly reduced (Fig. 8d). In addition, miR-310s and NOS deficiencies phenotypically copied, to a lesser extent, the cobblestone brain phenotype caused by Dg overexpression (Fig. 8e,f). Consistently, the milder brain phenotype in KT40 and ΔNOS mutants, relative to nervous system overexpression of Dg (insc > Dg), correlated with the measured Dg protein amounts (Supplementary Fig. 4b), again correlating that the severity of the brain dysplasia directly depends on the extent of Dg deregulation.

Similar to Dg mutant brains, cobblestone-like structures observed on miR-310s deficiency were also associated with aberrant cell adhesion protein expression (Supplementary Fig. 7). Importantly, the miR-310s mutant brain phenotype could be significantly rescued by reducing Dg levels (Fig. 8g). In addition, NO treatment had some positive effect on the brain outgrowth phenotype in Dg-overexpressing and NOS-deficient flies (Fig. 8g); however, it could not fully rescue it. This implies that some early NO-dependent developmental processes are irreversible and could not be repaired by transient NG administration. Taken together, these data show that the miR-310s promoter is responsive to the presence of higher amounts of Dg and NO signalling. This proposes that Dg is involved in transcriptional activation of the miR-310s via NO signalling, while NO signalling and the miR-310s in turn attenuate Dg abundance via a negative feedback loop.

Discussion

In summary, our data propose a novel perceptive-executive model for protein levels adjustment: it is perceptive, because it senses high levels of a critical protein, and it is executive, because it results in transcriptional activation of miRNAs that target the protein to restore its normal levels (Fig. 8h). As we showed, this regulation is potentially of great importance for stabilization of Dg expression levels, which modulates multiple cellular processes in the developing nervous system (Fig. 8h). As a consequence of miR-310s regulation, only a portion of Dg mRNAs with an extended 3′-UTR can be targeted, preventing Dg levels from being reduced below a certain threshold, which also is damaging for brain formation. Potentially, having a form of the target that is insensitive to miRNAs (alternatively processed 3′-UTR) allows adjustment of the target amount only to a critical level. Thus, the variability of 3′-UTR sequences has great significance in regulation of gene expression, where miRNAs act as managers of this extra layer of precision. Currently, the mechanisms coordinating alternative 3′-UTR processing for specific mRNA isoforms remain elusive; therefore, it would be important to understand what signalling controls the specifics of Dg 3′-UTR processing. Intriguingly, we found that the majority of the non-integrin ECM receptors have alternative 3′-UTRs that can be subjected to differential miRNA targeting (Table 1). This implies that the presented perceptive-executive model might be a more general buffering mechanism to readjust levels of essential molecules. Further, more detailed molecular evidence is required for the proposed here mechanism of protein expression regulation.

Alternative 3′-UTRs are common, sometimes tissue-specific, and often correlate with developmental stages, becoming shorter as the animal ages.

Recent bioinformatic work has shown that a high number of vertebrate miRNAs are alternatively polyadenylated and cleaved, leading to transcripts with different 3′-UTR lengths. Interestingly, the longest 3′-UTRs among adult tissues are found in the brain and 3′-UTR length also correlates with cell proliferation: tumour cells contain shorter 3′-UTRs. Moreover, proliferating cells can have conserved miRNA transcripts with shorter 3′-UTRs in diverse cell types and tissues, and interestingly expression of the full-length 3′-UTRs results in reduced protein levels that can be reversed by mutating predicted miRNA target sites. Therefore, the variability of 3′-UTR sequences offers an additional step, safeguarding the precision of gene expression where miRNAs play a key role. In this study, we report that Dg is subject to variable 3′-UTR processing and such processing determines its visibility to at least one family of miRNAs—the miR-310s. Interestingly, the 3′-UTR of the human Dg (DAG1) gene contains multiple miRNA binding sites and most of them are evolutionary conserved between vertebrates, suggesting that miRNA-based regulation of this important housekeeping gene might also take place in vertebrate organisms.

Prior studies in vertebrate muscle demonstrated that a signalling pathway involving DGC-regulated histone modification manages the proper transcription of miRNA genes. In addition, regulation of mRNA levels is changed in human muscular dystrophies, correlating with mis-expressed miRNA–mRNA modules. Interestingly, restoring of NO signalling or using histone deacetylase inhibitors return mis-expressed miRNAs to normal levels in mdx mice and affectively ameliorate muscular dystrophy. This proposes that the DGC via regulation of NO signalling maintains equilibrium between acetylation and deacetylation of histones and has an impact on muscle maintenance and/or the regenerative potential of muscle stem cells. In a miRNA microarray screen conducted on dystrophic flies, we found that on DGC deficit the expression profile of multiple miRNAs is altered. Moreover, some of these miRNAs are predicted to be involved in multiple processes in the nervous system. In addition, our study is the first report showing that Dg at least in Drosophila can be regulated posttranscriptionally via miRNAs in the nervous system. Here we propose that in the nervous system, Dg is able to regulate miRNA gene expression presumably via a NO-dependent mechanism. This implies that Dg and NO signalling can actively adjust epigenetic characteristics of neuronal cells. As Dg is also a signal-transducing module involved in crosstalk between the internal and external environments, this study suggests that Dg is involved in highly managed regulation of gene expression required to adapt cellular homeostasis that is compromised under dystrophic conditions. It would be important in the future to study in more detail how Dg via NO signalling manages gene expression in the nervous system.

We propose that Drosophila can be used as a model organism to study cobblestone lissencephaly, because Dg misregulation mutants present all typical syndromes associated with dystroglycanopathy: muscle degeneration, cobblestone brain and eye defects. Dystroglycanopathies are very pleiotropic, their associated pathologies range from very severe to few or no structural defects. This wide symptomatic range can be explained by the nature of the mutations causing these neuromuscular disorders, which occur in multiple proteins that decorate the Dg glycoprotein with different sugars in a tissue-specific manner; other Dg modifications, such as phosphorylation and proteolysis, also have a profound effect on its physiological function. Thus, the common mechanism underlying the assortment of the defects observed in secondary dystroglycanopathies is abnormal cellular interaction with the ECM, composition of which is also altered due to abnormal z-Dg posttranslational modifications. Dg interacts with various laminins in a tissue- and cell type-specific manner, and
abnormal α-Dg posttranslational modifications generally alter the strength of neuron adhesion to the ECM. As our findings show that either decreased or increased Dg levels result in the cobblestone brain, we propose that proper brain architecture formation requires the intensity of Dg-coordinated cell–ECM interactions to be accurate. As cells produce their own ECM, there is bidirectional signalling between the extra- and intracellular machinery. For example, we showed that in Drosophila, Dg modulates the levels of the ECM protein laminin non-cell and cell autonomously, thus adjusting the ECM constitution to be in accordance with the amounts of Dg expressed by the cell.27

The precision of cell adhesion protein expression is extremely important in the nervous system. Brain development, compartmentalization and even synapse formation rely on the quantity and quality of cell adhesion molecules expressed by different types of neurons.50 Based on expression patterns, we propose that there are different requirements for Dg as a cell adhesion receptor during different stages of neuron differentiation: first, to properly cluster, axons of fully differentiated neurons express certain levels of cell adhesion molecules; therefore, they have low affinity for α-Dg; second, to properly divide and differentiate, NBs, ganglion mother cells and not yet fully differentiated neurons readjust Dg levels according to their life cycle. In addition, we found that Dg is an important neuronal stem cell cycle regulator, as its misregulation leads to loss of cell cycle control in the brain. Together, our data show that the accuracy of neuronal proliferation, differentiation and axon pathfinding greatly depend on Dg levels and that miRNAs are candidates to buffer the levels of Dg expression against fluctuation in these processes. Overall, we believe that our Drosophila cobblestone lissencephaly model will allow for the better understanding of the molecular basis of dysgrycocanapathies.

Methods

Fly strains and genetics. Fly stocks were maintained at 25°C on a standard cornmeal-agar diet unless otherwise stated. The following Drosophila fly stocks were used: wild-type strain Oregon-R-C and w1118 (which were used as Controls; GMR-Gal4, elav-Gal4, tub-Gal4, act-Gal4, D42-Gal4, 24B-Gal4, MHC-Gal4, wor-Gal4, insc-Gal4, the deficiency spanning the mir-310s locus D exce6707 (Bloomington Drosophila Stock Center); and UAS-Gal4, w1118 (ref. 51). We used a miR insertion inserted to Dg52 (ref. 32) domain52; and DgO43/CyO, DgO43/CyO (kindly provided by Frank Hirth, and Wu-Min Deng. To analyse the specificity of the phenotypes and to control of the similar genetic background.

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Immunohistochemistry. Tissues were dissected and fixed in 4% formaldehyde. For western blotting and staining we performed hybridization as described before18. The following primary antibodies were used: rabbit anti-DgC-term52 (1:2,000), rabbit anti-DgEx8 (ref. 53) (1:500), mouse anti-22C10, anti-β-Gal, anti-Elav, anti-IPS Integrin, anti-Pcan, anti-Arm, anti-Pros and rat anti-DE-Cad (1:50; Developmental Studies Hybridoma Bank), chicken anti-GFP (1:2,000; Abcam), rabbit anti-β-Gal (1:1,000; Invitrogen), rabbit anti-Phospho-Histone-3 (anti-PH3, 1:2,000; Upstate Biotechnologies), rabbit anti-Miranda (gift from Andreas Wodarz, 1:1,000), rabbit anti-Caspase3 (1:200; Abcam), rabbit anti-LanB (1:1,000, Abcam ab47651), rabbit anti-Dxy186 (gift from Lee Fradkin), Alexa 488/568 goat anti-mouse, Alexa 488/568/633 goat anti-rabbit, Alexa 488 goat anti-mouse, Alexa 568 goat anti-guinea pig, Alexa 488 goat anti-mouse (1:200; Invitrogen). Samples were mounted on slides in 70% glycerol, 2% n-propyl gallate, 1 × PBS and analysed using a confocal microscope (Zeiss LSM 700). Quantification of staining intensities was done using the Zeiss software.

Histology. To prepare Drosophila tissue sections, flies wereimmobilized incollars in the required orientation and fixed in Carnoy fixative solution (6:3:1, ethanol:chloroform:methacrylic acid) at 4°C overnight. Tissue dehydration and embedding inparaffin was performed as described previously54. Ten-micrometre histological sections were prepared using a Hyrax M25 (Zeiss) microtome and stained with haematoxylin and eosin. All chemicals for these procedures were obtained from Sigma Aldrich. Analysis was done using a light microscope (Zeiss).

Induction of loss- and gain-of-function clones. Females of genotype hsFlp: act>FRT-C2-DFRT>FRT<Gal, UAS-GFP were crossed to males of genotype UAS-mir-310s/TM3. Two- to three-day-old females of the correct genotype were subjected to heat-shock treatment at 37°C for 2–3 h to permit for 2 days of development to fresh food with wet yeast for 3 days at which point ovaries were dissected and subjected to immunohistochemistry. To obtain Dg gain-of-function clones, hsFlp: act>FRT-C2-DFRT>FRT<Gal, UAS-GFP were crossed either toOregonR (Control) or UAS-Dg flies and to obtain Dg loss-of-function clones, females of genotype hsFlp; FRT42B Ubi-GFP/Cyo were crossed to males of genotype w1118, FRT42B Dg086/Cyo or to parental FRT 42B as a control. Fl larvae from the cross were heat shocked for 2 h at 37°C for 2 consecutive days and dissected as L3 to analyse the brains.

EdU labelling. To label cells that are in S phase, brains from L3 larvae where dissected in Schneider’s Drosophila Medium (Life Technologies) and incubated in medium containing 0.1 μM EdU (5-ethyl-2′-deoxyuridine) for 4 h by following a protocol in 4% formaldehyde in 1 × PBS for 20 min. Next, brains were washed two times with PBT (0.2% Triton X in 1 × PBS) (20 min each) and blocked with PBTB (1 × PBT, 0.2% bovine serum albumin, 5% normal goat serum, 0.05% sodium azide) for 30 min. Labelling of EdU-positive cells was performed using Click-IT Edu Alexa Fluor 555 Imaging Kit (Invitrogen/Molecular Probes) according to the manufacturer’s protocol, followed by standard antibody staining protocol.

Nitroglycerin feeding. Thirsty 3- to 7-day-old adult flies were put overnight in vials containing filter paper soaked in 5% sucrose with 0.2 ng/ml−1 NG (VWR EHERLA15586000AL) or just 5% sucrose (Control). 0.2 ng/ml−1 was the concentration of NG on which the lethality was <5%. Larvae were kept in vials with standard fly food supplemented with 500 μl of 5% sucrose with or without 0.2 ng/ml−1 NG. L3 larvae were collected ∼12 h after NG treatment.

Measurement of NO levels. To measure NO levels OxiSelect in Vitro Nitric Oxide Assay Kit was used (Cell Biologabs STA-802). Measurements of total nitrite/ nitrate amounts were done according to the manufacturer’s protocol. Samples were prepared from three whole larvae in the standard western blotting lysis buffer.

In situ hybridization. Probes were designed to target the longer region of the 3′-UTR annotated in Flybase release FB2012.02 to belong to the Dg-RD transcript. Primers used to generate an ~350 bp sense fragment were: forward→−5′ TAATA CAGCAGTGTTAGTGATTATCTC3′ and reverse→5′ ATAGAAGACAGTATACAGC3′, where the underlined region corresponds with the T7 RNA polymerase promoter region. Primers used to generate an ~350 bp anti-sense fragment were: forward→−5′ TACCAGCGATTTATGATTATT CTC3′ and reverse→5′ TTTAATCGACTC ACATACGCTGCGTGGACAGAAGA TTGACAGC3′.

Genomic DNA was PCR amplified using the above primers. One microgram of DNA in a 20-μl reaction was converted into RNA using a DIG DNA labelling kit (2 μl 10 × transcription buffer, 2 μl 10 × NTP labelling mixture (Roche), 1 μl RNase inhibitor (Applied Biosystems) and 2 μl of T7 RNA polymerase (New England Biolabs). The reaction was carried out for 2 h at 37°C. The solution was then treated with 2 μl of DNase1 (Invitrogen) for 15 min at 37°C, and the reaction was stopped by the addition of 2 μl of 0.2 M EDTA. One microlitre of the solution...
run on a agarose gel to ensure that the RNA product was of the expected size. The probes were then stored at −20°C in 10 μl of 1 M Trs pH 7.5 and 100 μl of hybridization buffer (50% formamide, 25% 20 x SSC, 5 mg ml−1 Torula yeast RNA (Sigma), 0.1% Tween 20) until their use.

Tissues were dissected, fixed for 30 min in 4% formaldehyde in PBS, dehydrated and stored overnight or longer. Tissues were then rehydrated, treated for 30 min to 1 h with Proteinase K solution (50 μg ml−1) and subjected to standard PCR on a T100 Thermocycler (Bio-Rad) using the appropriate primers as described above to target the short and long 3′-UTR. The PCR products were separated via electrophoresis on a 2% agarose gel. Gel densitometry analysis was used to determine the relative amounts of PCR products. Amplification efficiency was determined via densitometric analysis of the relative levels of unused primers for each reaction visualized at the bottom of the gel. PCR product levels were adjusted for the respective primer efficiencies and compared against one another to determine relative levels. The PCR product generated from primers that target the short and long 3′-UTR were used as the normalizing quantity.

Reverse transcription quantitative PCR. Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s protocol and treated with DNaseI (Invitrogen). To detect miRNA levels TaqMan microRNA assays (Applied Biosystems) were used to reverse transcribe from RNA and amplify cDNA specific to miR-310, mir-311, mir-312, mir-92a, mir-92b and 25S rRNA as an endogenous control on a StepOne Plus thermocycler (Applied Biosystems). Quantitative PCR (qPCR) was done using the Taqman qPCR master mix (Applied Biosystems). For the determination of transcript levels, mRNA was converted into cDNA using the High-Capacity reverse transcription kit (Applied Biosystems) following the manufacturer’s directions. cDNA for Dg mRNA was amplified using the SYBR green master mix (Applied Biosystems). To detect Dg cDNA corresponding to the 3′-UTR long isoform, the forward and reverse primers were used respectively: 5′-TGGCTCTAATGGACGAGCTCT-3′ and 5′-TCAGGAGCTTAATGTTCTCC-3′ (Fig. 3a). To detect Dg cDNA corresponding to the 3′-UTR long and short isoforms, following forward and reverse primers were used respectively: 5′-TTCCAACAAGCTCCTGGTTG-3′ and 5′-TACATTTTCCATCTCTGATTG-3′. As an endogenous control for all qPCR reactions, a product was also amplified corresponding to Ribosomal Protein L32 (RpL32) with the following forward and reverse primers respectively: 5′-GAAAGTGCACCCCTGGGACGACG-3′ and 5′-TAAGAGAAAACGCTCGTCTCTCTG-3′. The reactions were run at least in triplicate with appropriate blank controls. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. The ΔCt value is determined by subtracting the average RpL32 or 25S rRNA Ct value from the average tested Ct value. The ΔΔCt value is calculated by subtracting the ΔCt of the control sample from the ΔCt of the suspect sample. The relative amount of miRNA or mRNA is then determined using the expression 2−ΔΔCt.

Luciferase reporter assay. Drosophila S2R+ cells (DGRC Indiana) were seeded in a 96-well cell culture plate 1 day after splitting 1:5. Cells were transfected using the Effectene transfection reagent (Qiagen) with the following amounts: 50 ng of empty psiCHECK-2 (Promega) or 50 ng of psiCHECK-2 with the Dg-long-3′-UTR sensor cloned in, 25 ng of act-Gal4 and/or 50 ng of the pUAST-miR test plasmid. After 72 h of transfection, the cells were subjected to the cell lysis buffer (Promega) and luciferase assay (Promega). Both firefly (control reporter) and Renilla luciferase) counts were divided by control luciferase activity to determine the downregulation ratio (control). The ratio of Renilla luciferase activity in the presence of the psiCHECK-2-Dg-3′-UTR plasmid with an appropriate Dg 3′-UTR fragment (P1–P4) was divided by the fold decrease in the presence of the empty psiCHECK-2 plasmid without the presence of transfections or plasmids to determine the downregulation ratio (control). Next, the Renilla luciferase activity was determined in the same way with the empty psiCHECK-2 and the psiCHECK-2-Dg-3′-UTR plasmid in the presence of the test pUAST-miR and the downregulation ratio between these values was calculated. The downregulation ratio was then normalized to the plasmid psiCHECK-2-Dg-3′-UTR plasmid in the presence of the test pUAST-miR which was then normalized to the downregulation ratio between the psiCHECK-2 plasmid and the psiCHECK-2-Dg-3′-UTR plasmid without the test pUAST-miR to determine the relative reduction in Renilla luciferase expression caused by the presence of the test miRNA. The two-tailed Fisher’s test was used to determine the effects statistically. All transfections were done three independent times to determine an average and s.e.m. The DNA plasmids pUAST-miR-310s, pUAST-miR-312, pUAST-miR-313, pUAST-miR-92a and pUAST-miR-92b were generated in the Lai lab25 and obten from the Drosophila Genomics Resource Center. To generate sensors for Dg 3′-UTR regions, fragments of interest were amplified from genomic Drosophila melanogaster DNA by PCR with primers that included enzymatic digestion sites for NotI and XhoI as follows: P1-Dg-3′-UTR: Fwd—5′-TACCTTGCGGCCGCTTGGCAATTATGGCG-3′, Rev—5′-CCACATTGCGGCTGTCCTGATTACTT-3′. P2-Dg-3′-UTR: Fwd—5′-TACCTTGCGGCCGCTTGTCCTGATTACTT-3′, Rev—5′-CCACATTGCGGCTGTCCTGATTACTT-3′. P3-Dg-3′-UTR: Fwd—5′-TCAGTGGCGGGCGGCTTGACAGGATCATGTGGTCGATC-3′, Rev—5′-CCACATTGCGGCTGTCCTGATTACTT-3′. P4-Dg-long-3′-UTR: Fwd—5′-TACCTTGCGGCCGCTTGACAGGATCATGTGGTCGATC-3′, Rev—5′-CCACATTGCGGCTGTCCTGATTACTT-3′. Western blotting. Sample preparation and SDS-PAGE have been described previously.26 Bio-Rad ready-made 4–20% polyacrylamide gels were used for protein separation. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) using a semi-dry transfer apparatus. Non-fat-tailed Student’s t-test was used to determine the effects.

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