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Autoregulation of the glial gene *reversed polarity* in *Drosophila*

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During development, cells of the nervous system begin as unspecified precursors and proceed along one of two developmental paths to become either neurons or glia. Work in the fruit fly *Drosophila melanogaster* has established the role of the transcription factor Glial cells missing (Gcm) in directing neuronal precursor cells to assume a glial cell fate. Gcm acts on many target genes, one of which is *reversed polarity (repo)*. *repo* encodes a homeodomain transcription factor and is necessary for the terminal differentiation of glial cells. Transient Gcm expression is followed by maintained expression of *repo*. Evidence supports autoregulation to be one of the mechanisms that maintains *repo* expression, as ectopic *repo* expression in embryos can activate *repo-lacZ* reporter constructs. In this paper we further explore the ability of *repo* to activate reporter constructs in transgenic embryos and in cultured S2 cells. We provide further evidence that Repo protein acts as a transcription factor on its own regulatory DNA sequence. We report that three canonical Repo binding sites (RBSs) are located within the upstream 4.3 kilobase *repo cis*-regulatory DNA (CRD). The upstream 2 kb within the *repo* CRD has remarkable *repo*-dependent gene expression activity, and mutagenesis of RBS1 in this 2 kb region results in a significant decrease in *repo*-induced reporter gene expression in both systems. Our results in cell culture experiments also show that RBS2 and/or RBS3 can affect *repo*-dependent gene expression in the context of the whole upstream *repo* CRD. Mutagenesis of both RBS2 and RBS3 in the *repo* CRD, leaving RBS1 intact, significantly reduces *repo*-induced reporter gene expression. These results suggest that all three canonical RBSs may be cooperatively involved in autoregulation of *repo* expression.

Two cell types compose a functional nervous system in most animals: neurons and glia. Neurons are the most well-studied of the two types as these cells transmit information throughout the system using electrical and chemical signals in response to both internal and external stimuli. Glial cells are also required for the proper function of the nervous system, both during development and in the mature nervous system, serving as the support network for the neurons. Glial cells provide nourishment to neurons, insulate neurons, maintain synapses, and assist in the conduction of action potentials[1–3].

Drosophila melanogaster is an excellent model system to study how glial cells differentiate from their neuronal cousins during early development. Specifically, *Drosophila* provides an avenue in which to study, and manipulate, the genetic mechanisms that underlie this developmental process. Glial cells in *Drosophila* are collectively termed "lateral glia" (with the exception of the midline glia) and differentiate from progenitor cells derived from the neurogenic ectoderm. These progenitor cells can give rise to either neurons or glia, but the expression of a single gene, *glial cells missing (gcm)*, determines if this progenitor cell will become a glia. If *gcm* is expressed, the cell becomes a glia; if *gcm* is not expressed, the cell becomes a neuron[4–6]. In this way, *gcm* expression acts as a binary switch to control cell fate. Gcm protein, acting as a transcription factor, activates the expression of other genes; some act to complete differentiation of glial cells, while others act to repress the neuronal program. We are interested in how Gcm acts on these target genes, specifically *reversed polarity (repo)*, and how *repo* expression is maintained after transient Gcm expression.

The *repo* gene encodes a homeodomain transcription factor, and expression of *repo* is necessary for the terminal differentiation of glial cells[7–9]. Repo protein is expressed in all lateral glia, and embryos mutant for *repo* will show normal initial formation of glial cells, but defects in the final stages of development. The region of DNA located directly 5' of the *repo* coding sequence was presumed to be a target of Gcm due to the presence of Gcm binding sites (GBSs)[10,11]. This 4.3 kb sequence was confirmed as *cis*-regulatory DNA (CRD) of *repo* through reporter expression analysis, and it was shown that the initial activation of *repo* is dependent upon Gcm[12]. The *repo* CRD contains eleven GBSs, and *repo-lacZ* reporter constructs recapitulated the native Repo expression pattern. When GBSs were mutated in these *repo-lacZ* reporter constructs, reporter expression was greatly reduced, indicating *repo* is a direct target of Gcm and Gcm is necessary for *repo* expression[12].

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However, activation by Gcm does not fully explain the observed expression pattern of Repo. Gcm has a relatively short expression time in the newly forming glial cells, disappearing by stage 15[5]. *repo*, once expressed, is maintained throughout the life of *Drosophila*, throughout all larval stages and adult stages. As Gcm is not continuously acting on the CRD of *repo*, another mechanism must be in place to sustain the expression of *repo*.

Lee and Jones[12] showed that ubiquitous expression of Repo in *repo -4.3-lacZ* embryos produces ectopic expression of β-galactosidase (β-gal) in epidermal patches[12]. This observation suggested that Repo can interact with its own CRD. We hypothesize the mechanism of sustained *repo* expression is that of autoregulation, whereby Repo protein interacts with its own CRD in a manner of positive feedback to maintain its own expression. Once Gcm is produced in the newly developing glial cell, it will activate the expression of *repo*. Repo, also a transcription factor, will act on other genes necessary for the completion of glial cell development, but will also act on its own CRD to produce more of itself. This model requires the presence of Repo binding sites (RBSs) within the *repo* CRD.

In this paper we further explore the ability of *repo* to activate reporter constructs in transgenic embryos and in cultured S2 cells. We report that three canonical RBSs are located within the upstream 4.3 kilobase *repo* CRD which we designate RBS1-3. We show that the upstream 2 kb within the repo CRD has remarkable *repo*-dependent gene expression activity, and mutagenesis of RBS1 in this 2 kb region results in a significant decrease in *repo*-induced reporter gene expression in both systems. Our results in cell culture experiments also show that RBS2 and/or RBS3 can affect *repo*-dependent gene expression in the context of the whole upstream *repo* CRD. Mutagenesis of both RBS2 and RBS3 in the *repo* CRD, leaving RBS1 intact, significantly reduces *repo*-induced reporter gene expression. These results suggest that all three canonical RBSs may be cooperatively involved in autoregulation of *repo* expression.

Results

Ectopic expression of Repo induces ectopic β-galactosidase expression with specific *repo-lacZ* constructs

After identifying the 4.3 kb *cis*-regulatory DNA of *repo*, Lee and Jones[12] created reporter constructs containing different segments of the *repo* CRD coupled to the *lacZ* gene; different restriction enzymes were used to create the various constructs (Fig. 1). Many of these reporter constructs expressed in embryos largely recapitulated the glial cell specific expression of the *repo* gene[12]. However, one reporter (*repo -4.3/2.3*), in addition to glial expression, showed epidermal expression of *lacZ* in patches of clusters of lateral epidermal cells (Fig. 2G). To determine if Repo could interact with any part of its CRD, we used *Act5C-Gal4* to drive ubiquitous expression of *UAS-repo* during embryogenesis in individual embryos that carried each of these reporter constructs (*repo-lacZ* lines), as well as the full length 4.3 kb CRD, creating what we call *Act-repo* embryos. *Act-repo* embryos were generated by crossing *UAS-repo* flies with each of the *repo-lacZ* lines. The resulting F1 males of this cross were then crossed with *Act5C-Gal4/CyO* females, from which embryos were collected and stained for β-galactosidase (β-gal) expression. 1/8 of the embryos should be heterozygous for all three transgenes (*Act5C-Gal4*, *UAS-repo*, and *repo-lacZ*). We compared the β-gal staining pattern in each of these *Act-repo* embryos to those of the *repo-lacZ* CRD lines alone (which we called "wild type"). Our results are summarized in Fig. 1. In 4 out of 10 lines, a portion of embryos collected showed ectopic β-gal expression in the epidermis, while in the 6 other lines, embryos showed only wild type patterns of β-gal. We inferred that in those 4 lines, the embryos with ectopic β-gal carried all three transgenes, while in the other 6 lines, embryos carrying all three transgenes showed the wild type pattern.

Figure 2 shows β-gal staining of the full length construct (*repo -4.3*) and the three additional constructs where ectopic expression was observed compared with wild type embryos. All constructs show a significant increase in cells expressing β-gal in the epidermis ($p < 0.0001$; Fig. 2I). No ectopic β-gal expression was seen in the epidermis of embryos if the Sal/Xho fragment of the CRD was not present; in addition, removal of Sca/Xho fragment of the CRD resulted in the loss of the ability of the constructs to be ectopically expressed. However, the reporter construct *repo -2.8/-2.3* containing the Sca/Xho fragment alone did not express ectopically in an *Act-repo* background; these *Act-repo* embryos were identical to wild type in the staining pattern as shown in Johnson, et al.[13] (Fig. S3).

Repo CRD contains three canonical Repo binding sites

The canonical DNA binding site for Repo protein has been known for years; in 2003 Yuasa, et al.[14] extended observations by Halter, et al.[9] that Repo directly interacts with the specific 6 bp sequence 5'CAATTA3', and both groups show that the AAT region is the most critical for Repo binding. Repo binding sites (RBSs) have been found and required for the expression of several downstream genes regulated by Repo[15–17]. We looked for RBSs in the *repo* 4.3 kb CRD and found three CAATTA motifs, one located in the Sal/Xho fragment, and two located in the Xho/Spe fragment (Fig. 3). We compared the sequence of these RBSs to eleven other *Drosophila* species using the UCSC Genome Browser (http://genome.ucsc.edu)[18] and found a high level of conservation amongst all three (Fig. 3). We have designated these RBS1-3. Complete conservation for RBS1 and RBS2 is seen for all 12 *Drosophila* species, and RBS3 is lacking in only 4 of the 12 species. Interestingly, all 3 RBSs are located close to a Gcm binding site (GBS), with RBS2 overlapping a GBS by a single base pair (Fig. 3).

Luciferase assays in S2 cells show similar results with in vivo transgenic *repo* reporter constructs

Our transgenic embryo data showing ectopic β-gal expression suggested that Repo was capable of acting on its own regulatory sequence. We wanted to know if the same results could be obtained in a cell culture system using *Drosophila* S2 cells as a more direct measure of this interaction. We used the pAc-Gal4 plasmid (with *Actin 5C* promoter) to constitutively make Gal4, which acted on the pUAST-repo plasmid[12] to make Repo protein,

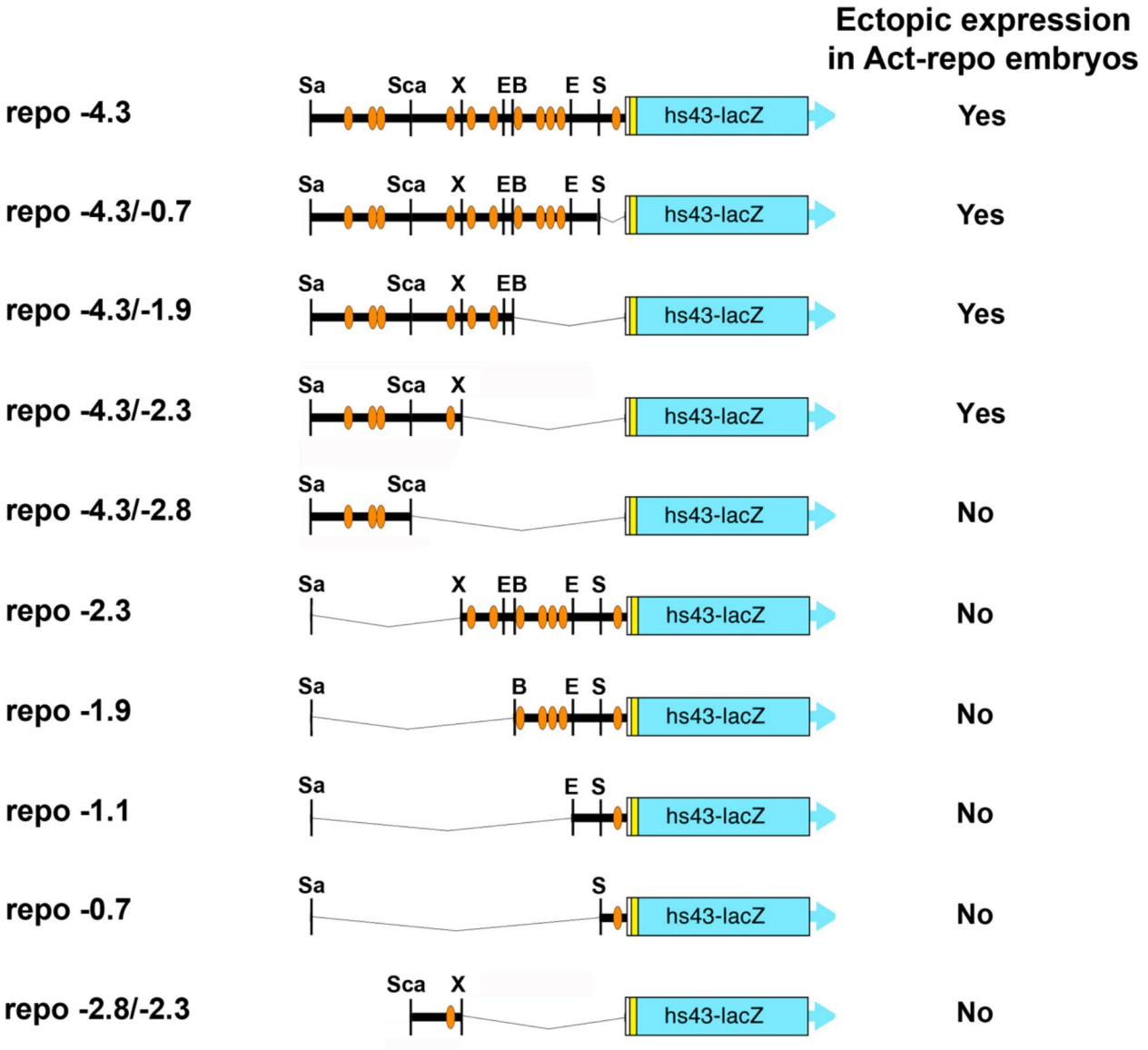

Fig. 1. Four *repo-lacZ* reporter constructs containing *repo 5' cis*-regulatory DNA (CRD) are ectopically expressed by ubiquitous Repo expression. Ten transgenic fly lines carrying the *repo-lacZ* reporter constructs shown here were crossed into an *Act5C-Gal4/UAS-repo* (Act-repo) background. Ectopic expression was determined by the appearance of β-gal staining in the epidermis of the trans-heterozygotes. Only four constructs resulted in ectopic expression, and all contained the SalI/XhoI fragment of the *repo* CRD. All other constructs (those that lack the Sca/Xho fragment) resulted in staining patterns indistinguishable from wild type. The *repo -2.8/-2.3* reporter construct which contains the Sca/Xho fragment alone also resulted in staining patterns indistinguishable in staining patterns indistinguishable from wild type (see Fig. S3). Orange ovals represent locations of Gcm binding sites as reported in Lee and Jones (2005)[12]. Restriction sites indicated: Sa, SalI; Sca, ScaI; X, XhoI; E, EcoRI; B, BamHI; S, SpeI.

combined with firefly luciferase reporter plasmids containing *repo* CRD fragments in transfections of S2 cells. To ensure the changes we observed in firefly luciferase expression were due to the induced expression of Repo, we first stained the S2 cells for Repo protein. The S2 cells that were not induced to produce Repo protein do not show any staining using our anti-Repo antibody, but cells do show staining with DAPI. Only transfected cells that were induced by Gal4 to produce Repo protein stain with the Repo antibody (Fig. S1). When reading the assays, firefly luciferase expression was normalized against the renilla luciferase as part of the dual-luciferase assay protocol (Promega). The replacement of pAc-Gal4 plasmid with the empty pAc vector lacking Gal4 co-transfected with pUAST-repo served as a negative control.

Our results indicate that the induced expression of Repo protein in the S2 cells results in an increase in luciferase activity, and this increase is dependent on which reporter construct is present in the cells (Fig. 4). The

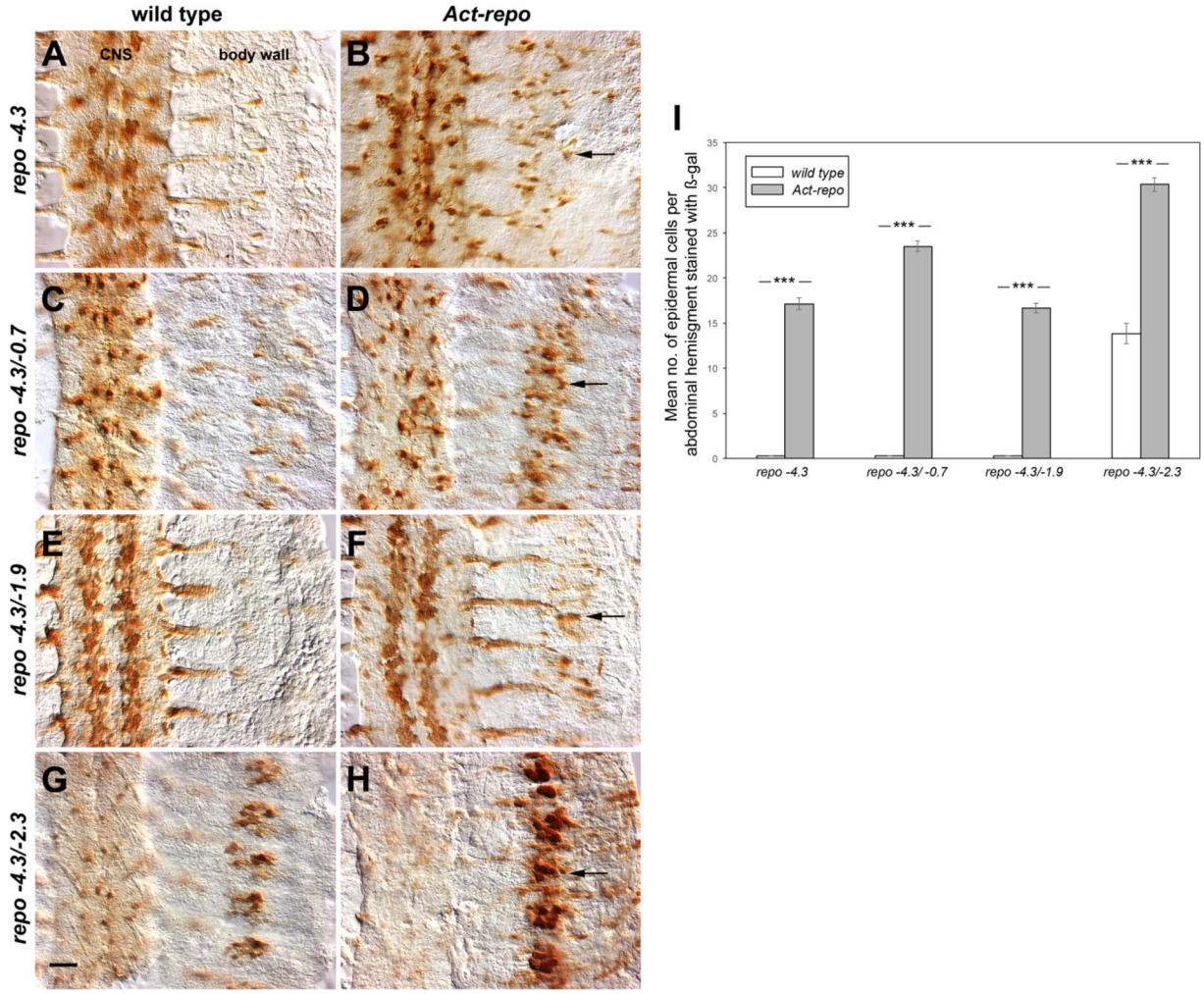

Fig. 2. Ectopic expression of *repo-lacZ* reporters with specific fragments of *repo* CRD. (A-H) Filleted late stage 16 embryos are pictured showing 5 adjacent abdominal segments of the ventral nerve chord (CNS) and the right lateral body wall, as stained with anti-β-gal antibody using HRP immunohistochemistry. Anterior is up. Scale bar, 20 µm. In all embryos the objective is focused on the epidermal cell layer of the body wall that lies below the musculature; glial cells in the CNS and PNS are in or out of focus depending on the flatness of the preparation. (A, B) *repo-4.3-lacZ* recapitulates the native Repo staining pattern in wild type embryos, but ectopic patches appear in the lateral epidermis of *Act-repo* embryos (arrow). (C, D) *repo-4.3/-0.7* and (E, F) *repo -4.3/1.9* also show epidermal ectopic patches in *Act-repo* embryos where none appear in wildtype (arrows). (G, H) *repo -4.3/-2.3* shows patches of epidermal staining in wild type embryos, but these patches are increased in the *Act-repo* embryos, extending over to the dorsal side of the embryo (arrow). (I) The mean number of epidermal cells per abdominal hemisegment expressing β-gal are represented for each construct expressed in either a wild type or *Act-repo* background. For *repo -4.3*, $n = 3$ embryos of each genotype, cells in 15 hemisegments were counted. For *repo – 4.3/-0.7*, $n = 4$ embryos of each genotype, cells in 19 hemisegments were counted. For *repo -4.3/-1.9*, $n = 4$ embryos of each genotype, cells in 20 hemisegments were counted. For *repo -4.3/-2.3 wild type*, $n = 4$ embryos, cells in 19 hemisegments were counted. For *repo -4.3/-2.3 Act-repo*, $n = 3$ embryos, cells in 15 hemisegments were counted. Data are presented as mean ± SEM. $^{***}p < 0.0001$.

full CRD sequence, repo -4.3, results in ~ 27 fold increase in luciferase activity. However, when repo -4.3/-2.3 is tested in culture, the fragment that contains only RBS1, we see a ~ 50 fold increase over no Repo protein present, which is a significant increase when compare to the full-length repo -4.3 ($p = 0.0026$) The repo -2.3 construct provides an interesting result; this fragment of the CRD contains RBS2 and RBS3, but results in only ~ 12 fold increase in luciferase expression compared to when no Repo is present, a decrease from both repo -4.3 ($p = 0.073$) and repo -4.3/-2.3 ($p < 0.0001$).

Mutation of canonical RBSs affects reporter expression both in culture and in vivo

We wanted to test if the canonical RBSs in the *repo* CRD were the sites of interaction with the induced Repo protein. We started with RBS1 as *repo -4.3/-2.3* gave a significant increase in reporter expression both culture and in vivo. We mutated RBS1 from the canonical sequence of 5'CAATTA3' to 5'CCCGTA3' through site directed

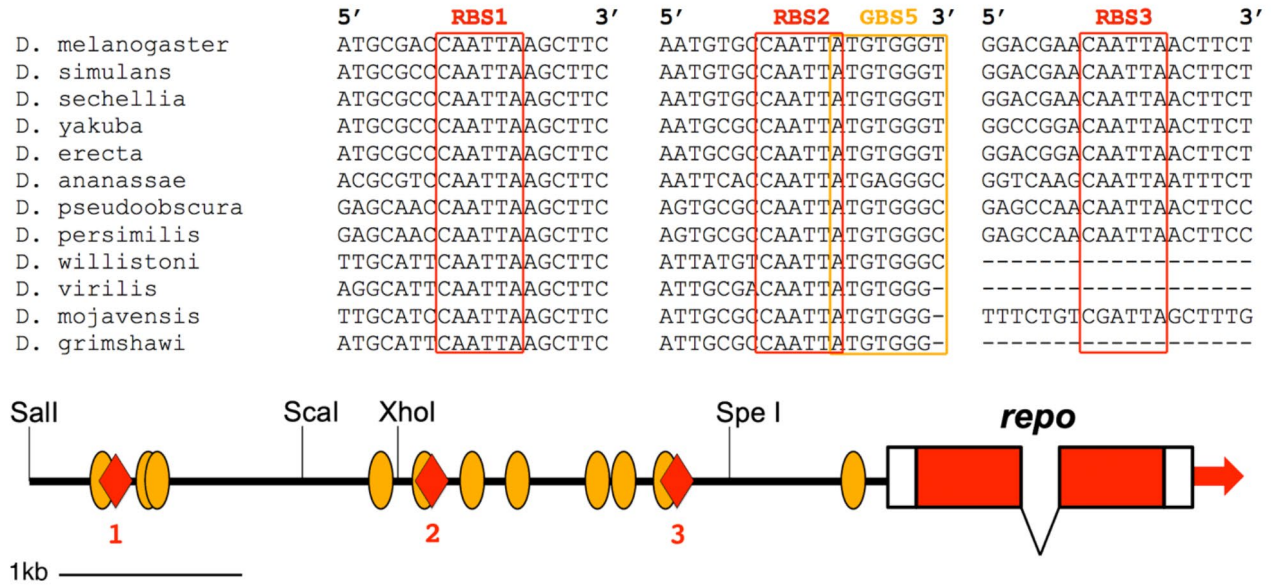

Fig. 3. The *repo* CRD contains three conserved canonical Repo binding sites (RBSs). (A) Alignment of 12 *Drosophila* species at RBS locations in the *repo* CRD with conserved RBS sequences (RBS1, 2, and 3) in red boxed areas. A Gcm binding site (GBS5) is directly adjacent to RBS2 (orange box). (B) Map of the *repo* CRD showing location of RBSs (red diamonds) and GBSs (orange ovals). RBS1 is located within the SalI/ScaI fragment and is completely conserved among the *Drosophila* species examined. RBSs 2 and 3 are located within the larger XhoI/SpeI fragment. RBS2 shows complete conservation, while RBS3 is only conserved among eight species of *Drosophila*. All three RBS sequences have the reverse complement sequence 5' to 3' with respect to the *repo* promoter .

mutagenis using the same Sal/Xho fragment of the *repo* CRD, and we used this new construct containing this single RBS1 mutation, called repo -4.3/-2.3ΔRBS1, in both our S2 cell culture and in vivo systems. Figure 5 shows our cell culture results. The amount of luciferase expression in the S2 cells is decreased significantly ($p < 0.001$), with only ~7 fold increase in expression as compared to no Repo protein present in the cells. We also constructed fly lines that contained the RBS1 mutant Sal/Xho fragment of the *repo* CRD in a *lacZ* reporter construct; examination of the *repo -4.3/-2.3ΔRBS1* embryos in *Act-repo* background found no ectopic expression (Fig. S2).

To determine the effect of the RBS1 mutation in the full 4.3 kb CRD, we made the same mutation in the plasmid containing the -4.3 kb sequence in the firefly luciferase reporter plasmid. We made two additional constructs creating mutations in RBS2 and RBS3 in the -4.3 kb sequence (Fig. 5A). We also made a third construct that contained the wild type RBS1 sequence with mutations in RBS1 and RBS2 (Fig. 5A). The results of these cell culture experiments are also shown in Fig. 5B. Mutagenesis of RBS1 results in a significant decrease in luciferase activity ($p < 0.01$); mutation of RBS2 and RBS3 in addition to RBS1 showed no significant decrease in reporter gene expression, and are not significantly different from mutation of all three RBSs in one construct. However, restoring wild type RBS1 while maintaining mutations in RBS2 and RBS3 in the repo -4.3ΔRBS23 construct does not restore activity back to that of wild type repo -4.3, indicating that RBS1 is not sufficient for this activity in repo -4.3, and that mutuation of RBS2 and RBS3 has a comparable affect to mutation of RBS1 in reduced reporter gene expression.

Discussion

We present evidence here that demonstrates that Repo is capable of interacting with its own *cis*-regulatory DNA (CRD) and can control its own expression as measured by two different methods with reporter constructs. We show that RBS1 is necessary for strong activation of *repo* reporter expression in our cell culture and in vivo systems. We also show that mutations in RBS2 and RBS3 reduced *repo*-dependent reporter gene expression our cell cultrure system.

Ectopic reporter expression is correlated between *in vivo* and culture systems

We observe excellent correlation in the amount of reporter protein produced in our expression systems when corresponding fragments of the *repo* CRD are fused to the different reporter genes. *repo -4.3-lacZ* produces patches of epidermal staining in *Act-repo* embryos, and results in ~27-fold increase in luciferase activity in S2 cells compared to no Repo controls. *repo -4.3/-2.3-lacZ* greatly increases β-gal in *Act-repo* embryos, and a correlating increase is observed using this fragment in the luciferase assay, with an additional ~25-fold increase in expression over the *repo -4.3*-luciferase construct. No ectopic reporter expression is observed in vivo with the

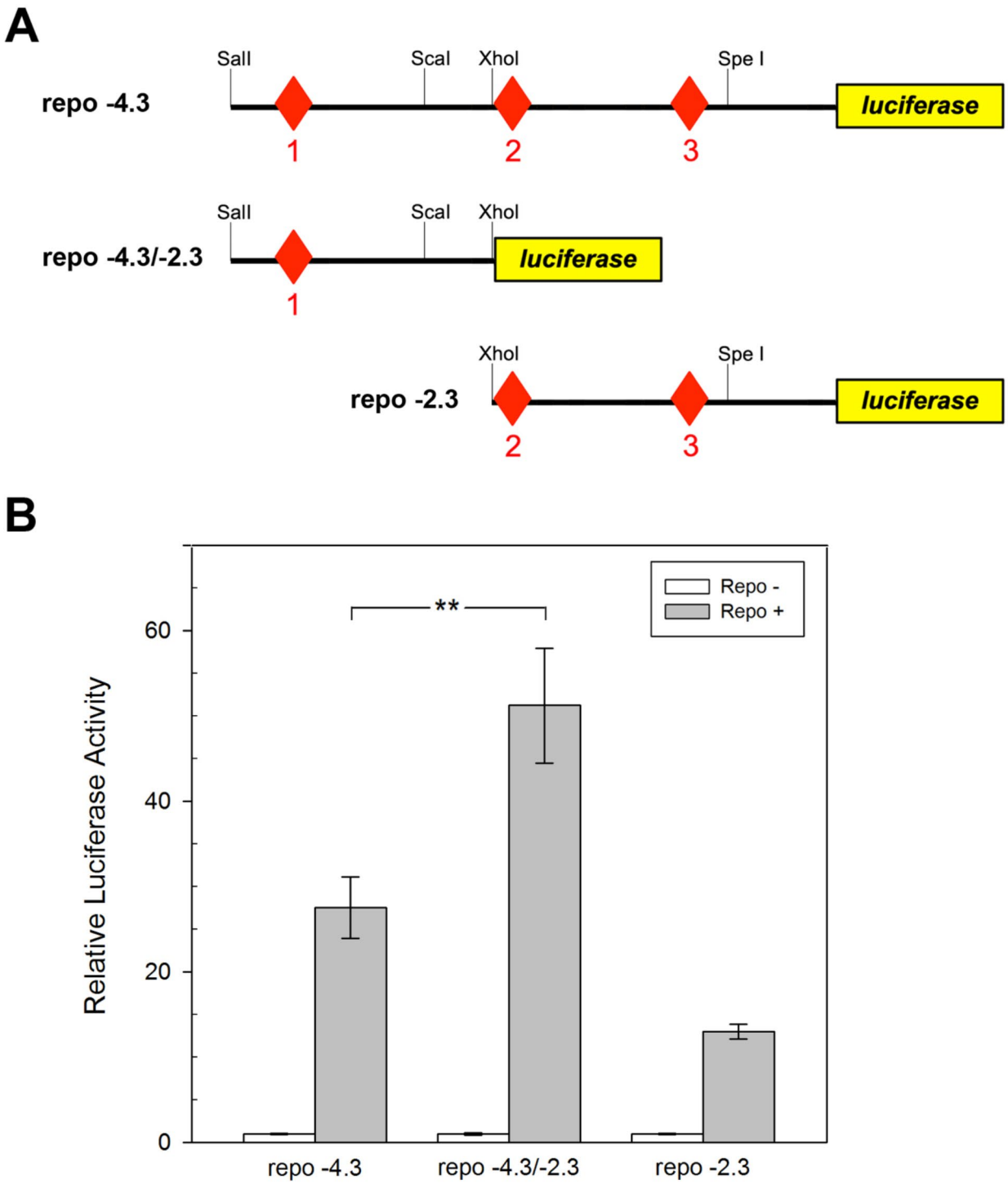

Fig. 4. Repo expressed in S2 cells can interact with *repo* CRD luciferase reporter constructs. (A) Construct diagrams showing selected fragments of the *repo* CRD that were fused to the luciferase reporter gene and used to transfect S2 cells. These *repo* fragments were chosen based on results from in vivo studies. (B) *repo -4.3/-2.3* gives the largest increase in relative luciferase activity and has only RBS1, compared to *repo-4.3*, which has all three RBSs, and *repo -2.3*, which has RBS2 and RBS3. Luciferase activity was normalized to renilla luciferase. Data are presented as mean ± SEM. $^{**}p < 0.01$.

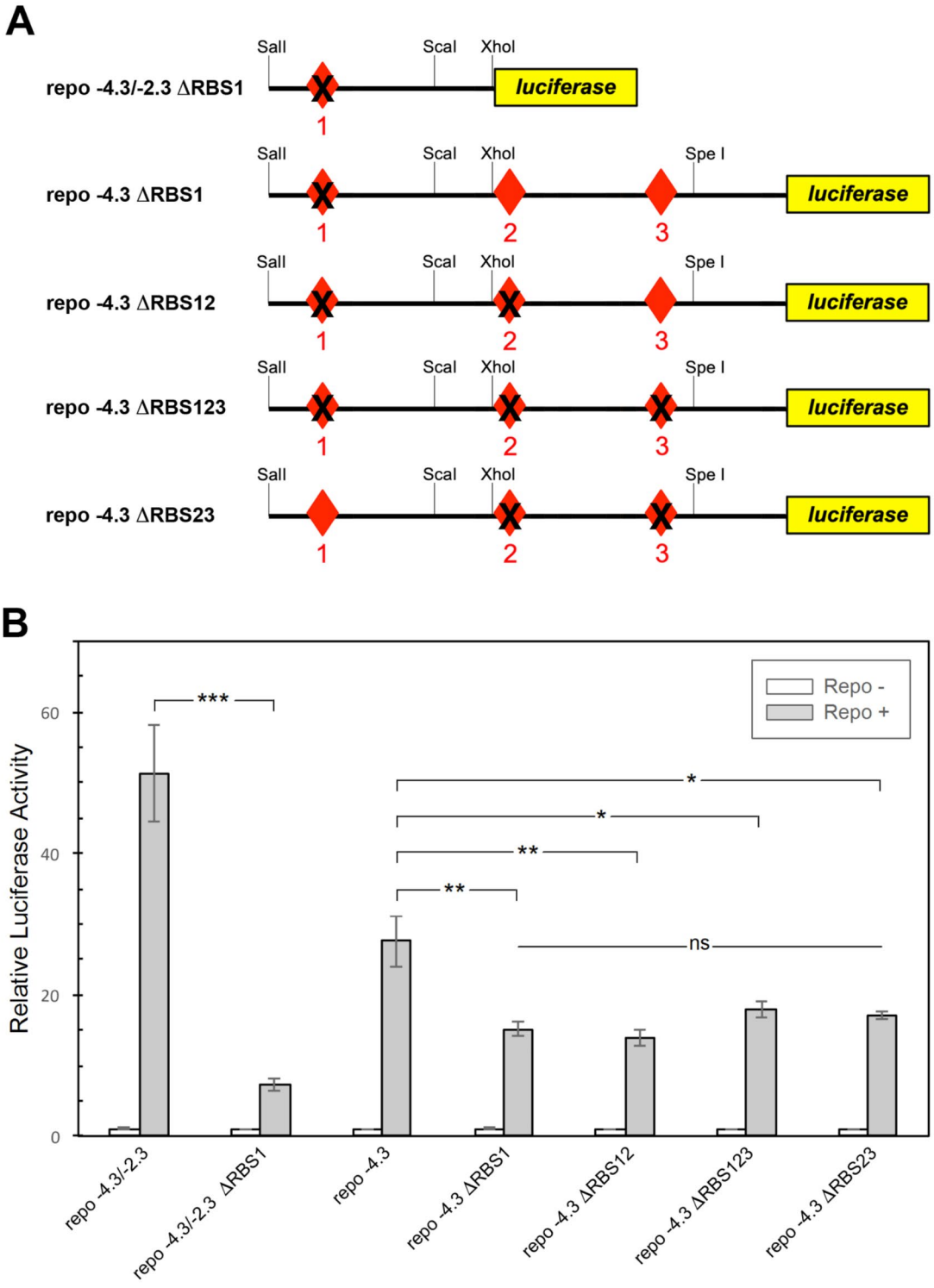

Fig. 5. Mutation of the canonical RBSs significantly decreases luciferase expression in culture. (A) Construct diagrams of *repo* CRD luciferase constructs showing mutations generated to canonical RBSs, indicated by the black "X". (B) Mutation of RBS1 causes a significant decrease in luciferase expression in repo -4.3/-2.3, from ~ 50 fold to ~ 7 fold, indicating this is the site of Repo interaction. Mutation of RBS1 also causes a significant decrease in repo -4.3 luciferase activity; mutation of RBS2 and RBS3 do not result in further decreases in activity. However, restoring RBS1, while maintaining RBS2 and RBS3 mutations in the repo-4.3ΔRBS23, does not restore activity back to that of repo -4.3. Luciferase activity was normalized to renilla luciferase. Data are presented as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, ns $p > 0.1$.

repo -2.3 fragment, despite the presence of RBSs 2 and 3, while mutation of RBS1 in the *repo -4.3/-2.3ΔRBS1* luciferase construct significantly reduced reporter expression in both systems to below that of this fragment alone and *repo -4.3*.

Repo autoregulation is dependent on RBS1

Our data presented here provides strong evidence that RBS1 is a necessary site of Repo interaction within its own CRD. Mutation of this site decreases the amount of reporter expression in both of our expression systems. While our results implicate a necessary role for RBS1 in autoregulation, our in vitro results indicate that it is not sufficient for autoregulation in the context of the whole repo -4.3 CRD. RBS1 alone was not able to restore luciferase activity when RBS2 and RBS3 are mutated, indicating that one or both of these sites are needed for activation, suggesting cooperation between RBSs. The high levels of conservation of these sites suggests a role for all RBSs. However, that role was not observed in our in vivo experiments as designed.

Our data also indicate that in addition to the RBS1 canonical site, an additional sequence of DNA, which we have previously described as the epidermal enhancer of *repo* located in the Sca/Xho fragment of the CRD, is required for ectopic *repo* reporter gene expression in epidermis. This Sca/Xho fragment is sufficient to drive β-gal expression in lateral epidermal patches (Johnson et al.[13]; Fig. S3, this study). RBS1 is located in the adjacent Sal/Sca fragment, within 23 bp of GBS1; however, when the Sal/Sca fragment was tested for its ability to drive ectopic β-gal expression in embryos, no staining was observed. In fact, not only was there no ectopic expression, there is no glial cell staining; these embryos are completely clear in both wild type[12] and *Act-repo* background. This indicates to us that while the interaction of Repo with the canonical RBS1 is necessary, RBS1 is not sufficient to drive expression of the reporter in vivo. We only observe glial cell staining and ectopic expression in embryos when the Sca/Xho fragment is present. The Sca/Xho fragment is also absent from reporters that have RBS2 and RBS3 alone. Taken together the Sca/Xho fragment seems to potentiate the ability of RBSs to mediate ectopic epidermal expression of reporters.

Repo regulation may need additional factors

Our cell culture data with *repo* -4.3-luciferase constructs with sequential RBS mutations indicate that this fragment is capable of giving some level of reporter activity even when Repo cannot interact with the canonical binding sites. Several explanations exist for the results we found. First, the ectopic Repo may be interacting with non-canonical binding sites. As Repo contains a homeodomain, it may be capable of interacting with other sequences of DNA that contain a core sequence of ATTA. Crocker et al.[19] show that many low-affinity binding sites are necessary in enhancers for proper gene expression in the correct developmental context. Second, the regulation of *repo* may require the presence of additional transcription factors.

The reason for the epidermis as the site of ectopic expression is not completely clear. When *Act5C-Gal4* flies are crossed with *UAS-repo* flies, Repo protein should be produced throughout the embryo. However, the strongest expression of ectopic Repo is, in fact, in the epidermis. The results we see in our *Act-repo* embryos may be a product of the particular *UAS-repo* lines we have; however, this does not explain why *repo -4.3/-2.3* creates patches of epidermal staining in a wild type background. A logical explanation is the presence of repressors acting in the epidermis to prevent the expression of *repo* under wildtype conditions. Chromatin factors would be a reasonable hypothesis as to the type of repressive factors operating in these conditions.

We also observe an interesting result with the *repo -4.3/-2.3* fragment upon mutation of different binding sites. Lee and Jones previously mutated the GBSs in this fragment and expressed this construct in embryos in a wild type background[12]. While very little staining in glial cells was present, the patches of epidermal staining were still present. However, when we use this same fragment and mutate only RBS1, leaving the GBSs intact, the epidermal staining observed when the GBSs were mutated disappears – RBS1 is necessary for epidermal patch expression, even though Repo protein is not not normally expressed there. One hypothesize is that another protein, possibly a transcription factor containing a homeodomain, can interact with the canonical RBS1 and produce reporter expression in the epidermis in a wild type background. Autoregulation as a means of controlling *repo* expression makes sense in that we see this mechanism employed in other glial genes, most notably in *gcm*[17,20,21].

Methods

Construction of luciferase reporters

We used the *patchΔ136-luciferase* plasmid[22], a kind gift from Dr. Stacey Odgen (St. Jude Children's Research Hospital) to clone selected fragments of the *repo* CRD that produced ectopic expression in vivo for expression in the S2 assays. Fragments were cloned into the KpnI/HindIII sites of the multiple cloning region. For consistency, we also cloned the Xho/Hind III fragment, containing wildtype RBSs 2 and 3 into this vector.

Mutation of repo binding sites

We used the Quikchange II Site Directed Mutagenesis kit (Agilent Technologies) to mutate all three RBSs from 5'CAATTA3' to 5'CCCGTA3'. The following oligonucleotides (reverse not shown) were used for each of the three mutations; underlined base pairs represent the mutations from the wildtype: RBS1 5'ATGCGG GATTTAAATTGATCTTAACGAAGCTTA*CGG*GGTCGCATCTGTATGTG3'; RBS2 5'CCTTGAAGCCA GACCCACATA*CGG*GGCACATTGGCTAATGCAAAATAC3'; RBS3 5'GGAATTCCTCGGCTAGAAGTT A*CGG*GTTCGTCCAACATGTGTGACGATG3'. Sequencing of clones confirmed successful mutagenesis.

Drosophila S2 cell culture luciferase assays

Our protocols were based on those used in the laboratory of Dr. Stacey Odgen (St. Jude Children's Research Hospital). Briefly, 350,000 cells were added to each well of a 24 well dish and grown overnight. Transfections

were carried out using 5 µl of Lipofectamine (Invitrogen) with the pAc-Gal4 plasmid (or empty pAc vector as a negative control), pUAST-repo[12], the designated *repo* CRD-luciferase construct, and PacRenilla (pAC-hRluc, Addgene) for normalization in serum free media. Four hours after transfection, S2 complete media was added to the cells and growth continued for 48 h. Cells were then assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. For each *repo*-CRD construct, assays were done in triplicate and each experiment was repeated three times for a total of nine replicates. Statistical tests include paired one-tailed T-test to compare Repo-/Repo+; comparison between constructs were analyzed by one-way ANOVA with Tukey multiple-comparison test and are presented as means ± SEM. pAc-Gal4 and empty pAc plasmids were obtained from Ogden lab.

Generation of *repo -4.3/-2.3ΔRBS1* reporter lines

The Sal/Xho fragment of the *repo* CRD with the RBS1 mutation was cloned into the P-element reporter vector pCasPeR-hs43-LacZ[23]. The reporter constructs were incorporated into flies via P-element mediated germ line transformation[24] to generate *repo -4.3/-2.3ΔRBS1* fly lines. Three independent lines were saved and compared. Fly line y^1w^{67c23} was used to generate transgenic lines.

Immunohistochemistry

Horseradish peroxidase (HRP) immunohistochemistry and embryo dissections were carried out as previously described (Patel, 1994)[25]. Rabbit anti-β-galactosidase (β-gal) antibodies (Cappel) were prepared at a 1:10,000 diluton. HRP-conjugated secondary antibodies (Jackson Immunoresearch) were prepared at a 1:300 dilution. Secondary antibodies were detected via the HRP/diaminobenzidine (DAB) reaction. For consistency, the DAB reactions were stopped after 15 min.

S2 cells were stained according to a protocol obtained from the Rebay Lab (University of Chicago) with minor changes. 100 µl of cells were applied to a well of a multi-well microscope slide coated with poly-L lysine. Cells that adhered to the slide were fixed with 3.7% formaldehyde. After antibody staining, cells were mounted using VectaShield with DAPI (Vector Laboratories, Inc.). Anti-Repo monoclonal antibody MAb 8D12[26] was used at a 1:5 dilution. Cy3 conjugated goat anti-mouse IgG secondary antibody was used at a 1:1000 dilution (Jackson, 115-165-146).

Counting cells

Cell counts were generated by counting epidermal cells expressing β-gal in 4 to 5 adjacent hemisegments in dissected embryos pictured in micrograph and dividing the total cell count by the number of hemisegments scored. ImageJ (National Institutes of Health) was used to assist in counting. Results were analyzed between wild type and *Act-repo* by paired t-tests.

D. Melanogaster stocks and genetics

UAS-repo and *repo-lacZ* reporter lines were generated in the lab previously[12,13]. The *Act5C-Gal/CyO* fly line was obtained from the Bloomington Drosophila Stock Center (BDSC #4414). *Act-repo* embryos were generated by crossing *repo-lacZ* flies with transgenes on the X or 3rd chromosomes, to flies with *UAS-repo* on the 2nd chromosome. F1 males were then crossed with *Act5C-Gal4/CyO*. In the resulting offspring 1/8 of the embryos will be heterozygous for all the genes necessary to test the effect of ectopic Repo expression on the *repo -4.3-lacZ* reporter. For each cross, a collection of over 300 stained embryos were screened for ectopic expression. Wild type controls were *repo-lacZ* lines crossed to y^1w^{67c23} flies.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

J.L.W. and B.W.J. designed the research and wrote the manuscript. J.L.W. and S.N. conducted the experiments and statistical analysis.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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