ECM stiffness regulates glial migration in Drosophila and mammalian glioma models

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ABSTRACT

Cell migration is an important feature of glial cells. Here, we used the Drosophila eye disc to decipher the molecular network controlling glial migration. We stimulated glial motility by pan-glial PDGF receptor (PVR) activation and identified several genes acting downstream of PVR. Drosophila lox is a non-essential gene encoding a secreted protein that stiffens the extracellular matrix (ECM). Glial-specific knockdown of Integrin results in ECM softening. Moreover, we show that lox expression is regulated by Integrin signaling and vice versa, suggesting that a positive-feedback loop ensures a rigid ECM in the vicinity of migrating cells. The general implication of this model of ECM regulation was tested in a mammalian glioma model, where a Lox-specific inhibitor unraveled a clear impact of ECM rigidity in glioma cell migration.

KEY WORDS: Drosophila, Human, Mouse, Glial cell migration, PVR, PDGF-receptor, Lysyl oxidase, Lox, Extracellular matrix

INTRODUCTION

The formation of the brain is one of the most fascinating processes in development. Once neurons are born, they settle and never divide again. By contrast, glial cells retain mitogenic and motile properties. Glial motility requires intensive interaction with the extracellular environment. On the one hand directional cues have to be perceived; on the other hand, a dynamic regulation of cell-cell or cell-matrix contact is needed to allow glial migration (Friedl and Gilmour, 2009; Klämbt, 2009).

In Drosophila, glial migration has been extensively studied in several parts of the nervous system (Klämbt, 2009). During embryogenesis, peripheral glia migration is in part controlled by Netrin signaling and the modulation of adhesive forces is provided by the expression of the NCAM homolog Fasciclin2 (von Hilchen et al., 2010; Sepp et al., 2000; Silies and Klämbt, 2010; Silies and Klämbt, 2011). During larval development, the forming visual system provides an easily accessible model system with which to study glial migration. Photoreceptor neurons emerge in the eye disc to decipher the molecular network controlling glial migration. We stimulated glial motility by pan-glial PDGF receptor (PVR) activation and identified several genes acting downstream of PVR. Drosophila lox is a non-essential gene encoding a secreted protein that stiffens the extracellular matrix (ECM). Glial-specific knockdown of Integrin results in ECM softening. Moreover, we show that lox expression is regulated by Integrin signaling and vice versa, suggesting that a positive-feedback loop ensures a rigid ECM in the vicinity of migrating cells. The general implication of this model of ECM regulation was tested in a mammalian glioma model, where a Lox-specific inhibitor unraveled a clear impact of ECM rigidity in glioma cell migration.

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Glia migration along the Bolwig nerve on the peripodial membrane of the eye imaginal disc, whereas in the remaining discs abnormal glial migration was noted on the basal side (Fig. 1E,F).

Pan-glial expression of activated FGF receptor results in dramatically increased glial number, but does not induce glial migration (Franzdóttir et al., 2009). Thus, signaling downstream of receptor tyrosine kinases diverts either to promote cell division or to stimulate cell migration. PVR-induced cell migration is manifested already very early in development and can be seen in second instar larvae (supplementary material Fig. S1A,B). In addition, concomitant misprojections of the photoreceptor axons are seen in ~40% of the eye discs dissected from third instar larvae (n=23, supplementary material Fig. S1C,D). Within the brain, glial cells expressing activated PVR are organized in an abnormal multilayered fashion (supplementary material Fig. S1E,F). Importantly, PVR signaling appears to primarily instruct cell migration. However, phenotypes were only apparent when animals were grown at 18°C, which has previously been associated with glial migration (Rangarajan et al., 2001) (Table 1). Hedgehog binding to Patched blocks its negative action on Smoothened (Briscoe and Thérond, 2013; Ingham et al., 2011) and thus it was surprising that knockdown of hedgehog or patched both shift the early lethality induced by glial PVR expression. In agreement with these findings, we were able to rescue the early lethal phenotype of PVR expression upon panglial co-expression of Patched. We also identified several members of the RTK and Wingless signaling pathways. The largest group of genes affecting PVR-induced lethality regulates transcription via chromatin regulation (Table 1). As Bap60 might simply affect UAS-mediated PVR expression, we focused on an analysis of the role of lox.

RNAi screen to identify cell-autonomously acting components required for glial migration

To further understand how PVR activity is transduced to increase cell motility, we followed a genetic approach and screened for genes that would alleviate the consequences of PVR over-activation by using a cell-type-specific knockdown of gene functions via Gal4-mediated expression of dsRNA (Dietzl et al., 2007). We generated a tester fly strain in which the Gal4-mediated expression of activated PVR is blocked by concomitant ubiquitous Gal80 expression (UAS-λPVR/UAS-APVR; repo-Gal4 UAS-CD8GFP/TM6 tub-Gal80). When these tester flies are crossed to wild-type flies, Gal80 function is removed genetically (supplementary material Fig. S2) and all non-TM6 flies, which can be recognized by the dominant marker Tubby, die due to the activation of PVR mediated by repo-Gal4 activity in glial cells.

We next crossed the tester flies to flies carrying different UAS-dsRNA constructs. The non-TM6 progeny of these crosses were then scored for a shift in the lethal phase towards pupal stages. Following screening of 2000 lines, we identified several genes that, upon silencing, shifted the lethal phase to late third instar or even pupal stages (Table 1). Silencing of PVR function in this paradigm also shifts the lethal phase to the pupal stage and serves as a control in the screen. We noted a shift in lethal phase upon silencing several components of the hedgehog signaling pathway, which has previously been associated with glial migration (Rangarajan et al., 2001) (Table 1). Hedgehog binding to Patched blocks its negative action on Smoothened (Briscoe and Thérond, 2013; Ingham et al., 2011) and thus it was surprising that knockdown of hedgehog or patched both shift the early lethality induced by glial PVR expression. In agreement with these findings, we were able to rescue the early lethal phenotype of PVR expression upon panglial co-expression of Patched. We also identified several members of the RTK and Wingless signaling pathways. The largest group of genes affecting PVR-induced lethality regulates transcription via chromatin regulation (Table 1). The strongest suppression effects were noted upon silencing of Bap60 and lox (Table 1). As Bap60 might simply affect UAS-mediated PVR expression, we focused on an analysis of the role of lox.

**Fig. 1. The activation of PVR signaling causes increased glial cell migration.** Larval eye discs attached to the brain stained for the glial nuclei marker Repo (red), for glial-expressed membrane-bound GFP (repo-Gal4 UAS-CD8GFP, green) and for the neuronal marker HRP (blue). Projections of confocal stacks are shown. (A,D,E,F) Orthogonal sections taken at the positions indicated by a dashed line in A,D,E,F. In the orthogonal views, the positions of the eye disc are indicated by a dashed curved line. (A) In young third instar repo-Gal4, UAS-CD8GFP larvae the normal distribution of glial cells is seen. The arrow indicates the end point of glial migration in the eye disc. (B) A young third instar eye disc. Glial migration is initiated at the optic stalk (os) and follows the differentiating photoreceptor axons (red arrow) in the wake of the morphogenetic furrow (mf). The eye disc is shown in an orthogonal view. (C) Glial migration following pan-glial activation of PVR. Glial cells are now frequently found on both sides of the eye disc. The eye disc is shown in an orthogonal view. (D-F) Eye imaginal discs of young third instar larvae of the genotype repo-Gal4, UAS-CD8GFP UAS-λPVR. (D,F) In 22 out of 43 eye discs, we noted ectopic glial migration on both the apical and the basal side of the disc (the arrowhead indicates the apical migration stream; the arrow indicates the basal migration stream). (E,E') In four out of 43 cases, we noted ectopic migration on the basal side of the eye imaginal disc (arrow). (F,F') In 17 out of 43 cases, we noted ectopic migration along the Bolwig’s nerve (arrowhead), which resides in the peripodial membrane. Scale bar: 20 µm.
**Table 1. Suppressor screen**

<table>
<thead>
<tr>
<th>CG</th>
<th>Gene</th>
<th>ID</th>
<th>Result</th>
<th>Molecular function grouped</th>
<th>Off targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG8222</td>
<td>Pvr</td>
<td>13502</td>
<td>P</td>
<td>Signal transduction/receptor</td>
<td>–</td>
</tr>
<tr>
<td>CG15793</td>
<td>Dsor1</td>
<td>40025</td>
<td>L3</td>
<td>Signal transduction/receptor</td>
<td>1</td>
</tr>
<tr>
<td>CG3166</td>
<td>aop/Yan</td>
<td>BL26759</td>
<td>L3</td>
<td>Transcription</td>
<td>–</td>
</tr>
<tr>
<td>CG31163</td>
<td>SKIP</td>
<td>25702</td>
<td>L3</td>
<td>Signal transduction/receptor</td>
<td>–</td>
</tr>
<tr>
<td>CG11335</td>
<td>lox</td>
<td>1135R-3</td>
<td>P</td>
<td>Cell adhesion/ECM</td>
<td>–</td>
</tr>
<tr>
<td>CG11335</td>
<td>lox</td>
<td>Mutant</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CG11335</td>
<td>lox lox2</td>
<td>Double mutant</td>
<td>L3/P</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CG4402</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Following screening of 2000 UAS-dsRNA strains, we obtained the above list of constructs that are able to shift the lethal phase caused by expression of activated PVR in glial cells. The shift from early larval stages to either the third instar stage (L3) or pupal stage is indicated. The predicted molecular function is taken from FlyBase. For a few lines, off-targets are predicted. The ID numbers of Bloomington, VDRC or NIGFly are indicated. Genes are grouped according to their function.

*One additional line was tested with the identical results.

Two additional lines were tested with the identical results.

**loxl-like genes act downstream of PVR**

Lox encodes a Lysyl oxidase, which in mammals is known to exert ECM crosslinking functions to increase ECM stiffness (Lucero and Kagan, 2006). Moreover, lox expression promotes breast cell metastasis, as increasing ECM stiffness promotes cell migration (Levental et al., 2009; Moore et al., 2010; Ng and Brugge, 2009).

In *Drosophila*, perineurial glial cells migrate below the neural lamella, a dense ECM surrounding the entire nervous system (Stork et al., 2008; Xie and Auld, 2011). We therefore set out to test whether modulation of ECM rigidity suppresses glial cell migration evoked by expression of activated PVR. A well-established specific inhibitor of lysyl oxidase activity is BAPN (β-aminopropionitrile). This small compound irreversibly blocks the catalytic properties of Lox family members (Smith-Mungo and Kagan, 1998; Tang et al., 1983). When we reared the flies on food containing 1.25 mM BAPN, we also noted a shift from early larval stages to late larval stages, with few animals reaching pupal stages (1-4 pupae/small vial with four females and two males; for crossbreeding, see supplementary material Fig. S2). This is never observed when PVR-overexpressing flies are fed untreated food, when lethality in early larval stages is found. To verify that loxl plays a decisive role in regulating PVR-induced migration, we generated a lox mutant by imprecise excision of a P-element. In the allele *loxΔA38*, the entire *lox*-coding sequence is removed without affecting the neighboring loci (supplementary material Fig. S3A). Flies homozygous for this mutation are viable and show no obvious external phenotypes. Likewise, the larval visual system forms with no discernible abnormalities (Fig. 3B). Moreover, pan-glial overexpression of loxl does not interfere with normal glial migration (Fig. 2E,F).

In *Drosophila* S2 cells, N- or C-terminally-tagged Lox protein of the predicted size is quantitatively found in the supernatant, suggesting that no proteolytic processing occurs in S2 cells (Fig. 2B,C). When we treated the supernatants with Endo H or PNGase F, which removes N-linked glycosylation, we noted only a slight change, indicating a moderate glycosylation of the protein (Fig. 2C). When we followed expression of a Myc-tagged Lox protein during *Drosophila* development in a ubiquitous manner using the da-Gal4 driver we found unprocessed secreted Lox protein in the larval hemolymph (Fig. 2D). Interestingly, we could also detect secreted Lox protein in the hemolymph when we expressed Lox specifically only in glial cells using the repo-Gal4 driver (Fig. 2D).

**loxl-like genes suppress PVR-induced glial migration**

To verify that loxl plays a decisive role in regulating PVR-induced migration, we generated a lox mutant by imprecise excision of a P-element. In the allele *loxΔA38*, the entire *lox*-coding sequence is removed without affecting the neighboring loci (supplementary material Fig. S3A). Flies homozygous for this mutation are viable and show no obvious external phenotypes. Likewise, the larval visual system forms with no discernible abnormalities (Fig. 3B). Moreover, pan-glial overexpression of loxl does not interfere with normal glial migration (Fig. 2E,F).

Surprisingly, the *loxΔA38* mutation does not shift the lethal phase associated with glial expression of activated PVR. Given that the Lox inhibitor BAPN suppresses the PVR induced lethality, this may suggest that redundantly acting genes prevent a phenotypic rescue. The deduced Lox2 protein is highly related to the Lox protein in...
sequence (Fig. 2A). It carries two SRCR domains similar to the human Lox12 protein (Molnar et al., 2005). A transposon induced Drosophila lox mutation is available (supplementary material Fig. S3B). The transposon insertion disrupts the open reading frame and mostly likely removes the catalytic function of Lox2. lox2 mutants are fertile and no abnormal phenotypes were detected in the eye disc. By contrast, lox2 double mutant animals are male sterile but no defects in glial migration onto the eye imaginal disc were noted \((n=13, \text{Fig. } 3C)\). However, we noted that the optic stalk is shortened and thicker in 50% of the cases \((7/13)\). To demonstrate this phenotype, we analyzed intact third instar brains with the eye imaginal disc attached. In a single confocal section, this tubby-like optic stalk phenotype can be identified (Fig. 3C′, arrows). These phenotypes suggest at least partially redundant functions of \(lox\) and \(lox2\).

When activated PVR is expressed in a \(lox\ lox2\) double mutant background, the lethal phase is shifted to late larval stages with rare animals reaching pupal stages, as observed following BAPN treatment (see above). Importantly, the glial overmigration caused by expression of activated PVR onto the eye disc is suppressed (Fig. 3D,E, \(n=7\)). In addition, the optic stalk appears thicker than normal, resembling that of \(lox\ lox2\) double mutants (Fig. 3C′,E). In agreement with these observations, we found that \(lox\) and \(lox2\) mRNA expression is regulated by PVR activity. Interestingly, activated PVR is able to induce \(lox\) expression during embryogenesis, whereas \(lox2\) expression is activated only in larval tissues (Table 2).

**Lox is required for stiffness of the extracellular matrix**

Lox oxidizes peptidyl lysine residues to \(\alpha\)-aminoadipic-\(\delta\)-semialdehyde (e.g. of collagen), which permits covalent cross-linking and thus \(lox\) genes are crucial for ECM maturation (Lucero and Kagan, 2006). In humans, cutis laxa, a rare congenital disorder, is associated with \(lox\) deficiency and is characterized by a lack of collagen monomer cross-linking, which affects the elasticity and thus the rigidity of the ECM. In breast cancer, \(lox\) dysfunction has been implicated in metastasis (Levental et al., 2009).

During *Drosophila* eye development, glial cells migrate along a thick ECM called neural lamella (Silies et al., 2007b). We therefore assayed the elastic properties of the ECM in an area of the eye disc that is contacted by migrating glia using atomic force microscopy (AFM; Fig. 4A). The force required to indent the AFM tip about 200 nm into the neural lamella was measured and Young’s modulus, which describes the elasticity of the matrix, was calculated.

In wild type, Young’s modulus is around 400 Pa (median 384; mean 426, both values are indicated; \(n=488\) independent measurements in ten imaginal discs; Fig. 4B,C, all measurements are summarized in supplementary material Table S1). A pronounced reduction in rigidity was noted in flies homozygous for the excision allele \(lox^{Δ438}\) (222; 221; \(n=165\), four imaginal discs; Fig. 4C). By contrast, pan-glial overexpression of \(lox\) resulted in increased rigidity in the overlying ECM (431; 522; \(n=170\), four imaginal discs; Fig. 4C) when compared with wild-type or repo−Gal4 control imaginal discs.

Given the decrease in ECM stiffness noted in \(lox\) mutants, we analyzed the ultrastructure of the neural lamella in wild type and \(lox\) mutants by transmission electron microscopy. The ECM generated in \(lox\) mutants appeared structurally similar to the wild-type ECM. When we analyzed the ECM of \(lox\) and \(lox\ lox2\) mutant larvae, we noted no significant increase in its thickness. Areas with reduced and increased ECM thickness are found in about equal numbers (Fig. 4D-G, arrows). For quantification, we determined the actual size of the ECM at 24 positions per optic stalk, defined by randomly placing a star on the TEM image and measuring the thickness of the ECM at the respective intersections (Fig. 4H,I). The ECM thickness varies from 150 nm to 700 nm \((n=5\) optic stalks per genotype). In summary, this shows that ECM rigidity, but not its apparent structure, is crucially regulated by \(lox\).

**Integrin signaling establishes a positive-feedback loop to increase ECM stiffness**

Most cell-matrix interactions are mediated by Integrin receptors and we therefore asked whether the rigidity of the ECM is...
Fig. 3. Loss of lox activity suppresses the glial migration induced by PVR activation. Eye discs are stained as described in Fig. 1. (A) In mature eye discs, glial migration stops at the position where photoreceptor axons are formed (arrow). (B) Eye disc of a homozygous Df(3R)loxA334 larva. (C) Fifty percent of the lox<sup>Δ34</sup>lox2<sup>Δ60317</sup> double mutant eye discs exhibit a shorter and broader optic stalk. The shape of the tissue was labeled using anti-Nidogen (Ndg) antibodies. (D,E) Single confocal section. (D) Pan-glial activation of PVR (repo-Gal4; UAS-ΔPvr) leads to ectopic migration of glial cells (arrowhead). (E) In UAS-ΔPvrlox<sup>Δ60317</sup>/lox<sup>Δ2xrepo</sup>lox<sup>Δ334</sup>repo-Gal4 UAS-CD8GFP lox<sup>Δ318</sup>/lox<sup>ΔA38</sup> double mutant eye discs, migration appears normal (double arrowhead). (B,C,E) The arrows indicate the optic stalk. Scale bar: 20 µm.

also influenced by integrins. Glial-specific knockdown of myospheroid encoding a β-Integrin subunit also resulted in a softer more-flexible matrix, demonstrating that Integrin signaling not only mediates the adhesion to the ECM but also signals towards the regulation of matrix rigidity (184; 211; Ozawa et al., 2010), we analyzed the expression of human LOX genes in different glioblastoma cell lines (human glioma cell lines). As glioma cells are able to secrete extracellular matrix, the relevant genotypes, the tissues analyzed and the expression level about 12% in U343 and 5% in A172 cells after the knockdown of PVR expression, this experiment could not be performed in late third instar larvae. Upon overexpression of integrin subunits, we detected an increase of lox expression in the whole embryo and in the CNS of late third instar larvae. lox2 expression is normally not detected in the CNS of late larval third instar (ND). Upon expression of integrin or lox, however, we detected lox2 expression. We also noted an increase in mys expression when we overexpressed lox, and a slight decrease in mys expression in the CNS of lox mutants.

### Table 2. Relative expression of lox, lox2 and mys

<table>
<thead>
<tr>
<th>Age/tissue</th>
<th>Genotype</th>
<th>lox</th>
<th>lox2</th>
<th>mys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 14-16 whole embryo</td>
<td>repo&gt; CD8GFP</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>repo&gt; ΔPvr, CD8GFP</td>
<td>1.751***</td>
<td>0.656**</td>
<td>2.395**</td>
</tr>
<tr>
<td></td>
<td>2x repo&gt; Integrin, CD8GFP</td>
<td>1.741***</td>
<td>0.618***</td>
<td>1.651***</td>
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<tr>
<td>Early third instar larval CNS</td>
<td>repo&gt; CD8GFP</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>repo&gt; ΔPvr, CD8GFP</td>
<td>0.272**</td>
<td>7.464**</td>
<td>14.689**</td>
</tr>
<tr>
<td>Third instar larval CNS</td>
<td>lox&lt;sup&gt;Δ338&lt;/sup&gt;</td>
<td>1.000</td>
<td>ND</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Fak&lt;sup&gt;CG1&lt;/sup&gt;</td>
<td>0.000***</td>
<td>ND</td>
<td>0.714***</td>
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<tr>
<td></td>
<td>2x repo&gt; CD8GFP</td>
<td>0.400**</td>
<td>4.9***</td>
<td>0.839***</td>
</tr>
<tr>
<td></td>
<td>2x repo&gt; Integrin, CD8GFP</td>
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<td>25.723***</td>
<td>7.621*</td>
</tr>
<tr>
<td></td>
<td>2x repo&gt;ΔPvrlox&lt;sup&gt;Δ318&lt;/sup&gt;/lox&lt;sup&gt;ΔA38&lt;/sup&gt;</td>
<td>85.430***</td>
<td>5.809***</td>
<td>18.722*</td>
</tr>
</tbody>
</table>

Summary of the relative expression data of lox, lox2 and myospheroid (mys) as determined by qRT-PCR normalized to expression of rp49. The relevant genotypes, the tissues analyzed and the P-values are indicated (*P<0.1, **P<0.01, ***P<0.001). repo, repo-Gal4; 2x repo, two copies of repo-Gal4. All experiments were repeated at least three times. In embryos, expression of activated PVR causes an elevation of lox and mys expression. lox2 expression is slightly reduced. By contrast, in young third instar larvae, lox expression is reduced, whereas lox2 and mys expression is increased. Owing to the lethal effect of PVR expression, this experiment could not be performed in late third instar larvae. Upon overexpression of integrin subunits, we detected an increase of lox expression in the whole embryo and in the CNS of late third instar larvae. lox2 expression is normally not detected in the CNS of late larval third instar (ND). Upon expression of integrin or lox, however, we detected lox2 expression. We also noted an increase in mys expression when we overexpressed lox, and a slight decrease in mys expression in the CNS of lox mutants.

### Lox inhibition restricts glioma cell migration

The above data demonstrate the crucial role of ECM modulation during glial migration in response to PVR activation in *Drosophila*. Because, in humans, PDGF receptor activation is often linked to glioma progression and to a worse prognosis (Brennan et al., 2009; Ozawa et al., 2010), we analyzed the expression of human LOX genes. The human genome encodes LOX and four members of the LOX-like family (Lucero and Kagan, 2006; Molnar et al., 2003). As glioma cells are able to secrete extracellular matrix components (Gladson, 1999), Lox expression by glioma cells in cell culture may be linked to their migration. To prove this hypothesis, we performed a scratch assay (see Materials and Methods). Following knockdown of expression of LOX4, which is most similar to *Drosophila* lox, cell migration is slightly reduced (expression level about 12% in U343 and 5% in A172 cells after 24 h; and 15% after to 96 h of culture; Fig. 5K). However, when we...
stimulated U343 cell migration with 40 ng/ml PDGF-BB, relative migration was significantly decreased in the LOXL4-depleted cells (Fig. 5H). In conclusion, lox-like genes appear to have a function during cell migration in the mammalian system.

**Lox promotes tumor invasion in vivo**

To directly test whether inhibition of the activity of the Lox-like enzymes also suppresses glioblastoma invasion, we then turned to a mouse xenograft model. The glioma cell line C6 is derived from a rat glioma and is widely used (Benda et al., 1968). To follow the C6 mouse xenograft model. The glioma cell line C6 is derived from a rat enzymes also suppresses glioblastoma invasion, we then turned to a To directly test whether inhibition of the activity of the Lox-like Lox promotes tumor invasion in vivo

**DISCUSSION**

Cell migration is of prime relevance during the development of the nervous system and is often triggered by the activation of the PDGF receptor. The *Drosophila* PDGF-receptor homolog PVR is required in a number of cell types for their normal migratory responses. In the fly ovary, *pvr* instructs the migration of border cells in part by influencing actin dynamics (Duchek et al., 2001; Fulga and Rørth, 2002; Rosin et al., 2004; Wang et al., 2006). In addition, migration of hemocytes and some embryonic glial cells depends on PVR activity (Cho et al., 2002; Janssens et al., 2010; Sears et al., 2003; Wood et al., 2006). In line with these findings, expression of activated PVR efficiently induces glial migration and generates phenotypes resembling a metastatic tumor cell phenotype (Vidal and Cagan, 2006; Witte et al., 2009). Likewise, PDGF-receptor activity instructs cell migration during normal mammalian development, but also during tumor metastasis (Hoch and Soriano, 2003; Jones and Cross, 2004; Shih and Holland, 2006).

Interestingly, the effects of activated PVR on glial motility appear to be a specific feature of this receptor tyrosine kinase, as activation of the related FGF-receptor results in extensive proliferation but does not trigger cell migration. Here, we used a genetic suppressor screen to better understand PVR-induced motility. We identified components of hedgehog and wingless signaling pathways. As both loss and gain of *patched* function is able to suppress the early lethality induced by PVR activation in glia, we suspect that the
Integrin might act in a positive-feedback loop enforcing a local extracellular environment favoring migration (Fig. 7).

In the mammalian system, integrins may suppress tumor metastasis (Ramirez et al., 2011). Generally, however, integrins are thought to be important mediators of cell adhesion and constitute attractive therapeutic targets to treat tumor progression (Weaver et al., 1997). In line with these findings, Integrin signaling is also key in regulating glioma cell migration (D’Abaco and Kaye, 2007; Fukushima et al., 1998). As BAPN can suppress glioma cell migration in vivo, this may indicate that the Lox/Integrin feedback loop identified in Drosophila is evolutionarily conserved to couple ECM stiffness to glioma cell migration.

The regulation of ECM stiffness via Lox is not required for viability. In humans, loss of lox leads to recessive connective tissue disorders (Smith-Mungo and Kagan, 1998), whereas no abnormal phenotypes were so far detected in Drosophila mutants (Molnar et al., 2005). In humans and Drosophila, lox activity is upregulated in wound repair (Smith-Mungo and Kagan, 1998; Stramer et al., 2008), reflecting the need for increased migration of cells into the wound area. Thus, Lox might be primarily involved in the regulation of plastic responses encountered during injury and disease.

Flies have a relatively simply structured nervous system; however, relatively few glial cells share pronounced motility with their mammalian counterparts (Klambt, 2009). The Drosophila glioma model used here has disclosed a crucial role for Lox-related genes in maintaining the migratory state of mammalian glioma cells. Indeed, the cancer clinical genomics database ‘Repository of Molecular Brain Neoplasia Data’ (Rembrandt), which comprises a large number of glioma patient data (http://rembrandt.nci.nih.gov; Madhavan et al., 2009), demonstrates a significant correlation between the upregulation of LOX and LOXL1 and overall survival. As BAPN treatment can block the diffuse infiltration of glioblastoma cells in a mouse model, new therapeutic strategies for a currently incurable disease might be in reach.

**MATERIALS AND METHODS**

**Fly strains and genetics**
All crosses were performed on standard food at 25°C unless indicated otherwise. The lox-excision mutant was generated from P[acman]sor952. The following strains were used: repo-Gal4, repo-Gal4, UAS-δat, UAS-δat, UAS-ΔPvr, UAS-inflated-UAS-nemo [provided by V. Auld (University of British Columbia, Vancouver, Canada); B. Jones (University of Mississippi, Oxford, USA); M. Leptin (University of Cologne, Cologne, Germany); B. Shilo (Weizmann Institute, Rehovot, Israel); P. Rorth (University of Copenhagen, Copenhagen, Denmark); N. Brown (University of Cambridge, Cambridge, UK)]; lox (Exelixis collection, Harvard University, Cambridge, USA); and lox (Exelixis collection, Harvard University, Cambridge, USA); and lox (Bloomington Stock Center, Bloomington, USA). UAS-dsRNA strains were from Bloomington, VDRC or NIGFly. For details see Table 1. The different tagged UAS-based lox transgenic fly strains were generated according to standard procedures.

**Molecular genetics**
DNA cloning was conducted using Gateway-based vectors (Invitrogen). All constructs were verified by DNA sequencing. Proteins were extracted from cells and analyzed by western blot as described previously (Bogdan et al., 2005). For the glycosidase assay, proteins were extracted by TCA precipitation and deglycosylated by Endo H (Roche) or PNGase F (New England Biolabs) according to the manufacturer’s instructions. We used RNase B as a positive control.

**Quantitative RT-PCR (qRT-PCR)**
Total RNA was isolated from cultured cells or tissue with GenElute (Sigma-Aldrich) or TRIzol (Invitrogen). cDNA templates were synthesized using SUPERscript II polymerase with Oligo(dT)12-18 primers and RNaseOUT.
(Invitrogen) following the manufacturer’s instructions. qRT-PCR was performed using SYBR Green for monoplex reaction (Bioline), TaqMan Gene Expression Assays for duplex reaction (Applied Biosystems) and Realplex Mastercycler (Eppendorf) following the manufacturer’s instructions. The analysis of the data was conducted using the REST 2009 software (Qiagen; Pfaffl et al., 2002). For primers see methods in the supplementary material.

Elasticity measurements
Elasticity measurements were performed as described in a HEPES-buffered solution using a Nanoscope III Multimode-AFM (Veeco Instruments).

Deflection of colloidal probe cantilevers with a sphere radius of 5 µm was determined and Young’s modulus was calculated as follows:

\[ E = \frac{3}{4} \left( \frac{\Delta f}{\Delta \delta} \right)^{\frac{3}{2}} \frac{1 - \nu^2}{R} = \frac{3}{4} \text{slope}^{\frac{3}{2}} \frac{1 - \nu^2}{\sqrt{R}} \]

where \( E \) is Young’s modulus, \( f \) is force ({{f}}), \( \nu \) is Poisson ratio of the sample, \( \delta \) is deformation or indentation depth, and \( R \) is radius of the sphere. The initial part of the curves corresponding to indentation of 200 nm was analyzed using the linear implementation of equation 4 of the Hertz-Model. All force-indentation data were analyzed with PUNIAS (Protein Unfolding and Nano-Indentation Analysis Software, http://punias.voila.net/klmenu/punias0.htm), a custom-built semi-automatic processing and analysis software.

Immunohistochemistry
Fixation and treatment of tissues for immunohistochemistry on third instar larval brains and eye imaginal discs were performed as described previously (Silies et al., 2007a; Stork et al., 2008). For a list of antibodies used, see methods in the supplementary material. LOX, LOXL1 and LOXL4 staining was evaluated semi-quantitatively by scoring the percentage of stained cells \([0 \text{ (absent)}, 1 (<10\%), 2 (10-50\%), 3 (51-80\%), 4 (81-100\%)]\), as well as staining intensity \([0 \text{ (absent)}, 1 \text{ (weak)}, 2 \text{ (strong)}]\). Both scores were multiplied resulting in a maximum staining score of eight.

Scratch assay
U343 or A172 glioblastoma cells were transfected 1 day after plating using two siRNAi sequences (LOXL4_1 and LOXL4_4 siRNA, Qiagen) or control siRNA not able to bind known RNA sequences (AllStar negative siRNA, Qiagen). After 24 h the confluent cell layer was scratched with a sterile 100 µl pipette tip. The same area was documented after 0 h, 24 h, 48 h...
and 72 h, and analyzed using ‘analySIS FIVE’ (Olympus) and TScratch software (ETH Zürich, Switzerland).

Transmission electron microscopy
Fixation and sectioning was performed as described previously (Stork et al., 2008). All measurements were made using Photoshop. Five different animals were analyzed for each genotype.

Human tissues
Glioblastomas (grade IV, WHO), obtained by biopsy (frozen, \( n=10 \); formalin-fixed and paraffin-embedded, \( n=17 \)), and non-pathologic frontal lobe brain tissue, obtained by autopsy (frozen, \( n=2 \), formalin-fixed and paraffin-embedded, \( n=2 \)), were examined. Ethical approval was obtained from the local Ethical Committee of the Ärztekammer Westfalen-Lippe, Germany (Az.2007-420-f-S).

Xenotransplantation and BAPN-administration
C6-SP-GFP cells (\( 4\times10^4 \) in 2 µl PBS) were transplanted into the right striatum anterior to the interaural line, 2.5 mm lateral to the midline, at a depth of 3.5 mm into the brain. One day before transplantation, intraperitoneal injection of BAPN (100 µg/g/day in 50 µl of PBS) was started in 10 animals, while 10 control mice were injected with 50 µl of PBS. Intraperitoneal injection of BAPN or PBS was continued daily. On day 10 and day 15, MRI was carried out. At day 15 after injection, brains were prepared. Five brains of each group were immediately frozen in liquid nitrogen, while five brains of each group were fixed in formalin and embedded in paraffin. Ethical approval was obtained from the local Ethical Committee of the Ärztekammer Westfalen-Lippe, Germany (Az.2007-420-f-S).

MRI
MRI measurements were performed on a 3T Philips Achieva System. A 3D gradient echo dataset was acquired in 8.50 min scan time using SPIR fat suppression and the following parameters: isotropic resolution, 200 µm; reconstructed to 90×90×200 µm³; field of view (FOV), 20×14×8 mm³; echo time/repetition time (TE/TR), 5.5/28 ms; excitation pulse angle, 35°; averages, 6. As contrast agent, Gd-DTPA (Magnevist) was applied at 0.5 mmol/kg body weight through a tail vein catheter 5 min prior to measurement start. Hyperintense-appearing tumor tissue was segmented semi-manually using Amira (Visage Imaging) and the volume was calculated.

Morphometry
Serial coronal sections (3 µm) were made from paraffin-embedded brain tissue of xenotransplanted mice (\( n=5 \) for BAPN and control mice). Whole sections were photographed using an automated Corvus high resolution stage control. Areas of tumor core and infiltration zone were quantified using Analysis software and a fixed threshold of light intensity. Fluorescent cell clusters detected outside the tumor core were investigated with respect to size (µm³) and number. We used ImageJ to determine the nuclear sizes. If the nuclei were located in the wild type, almost all glial nuclei appeared round. By contrast, glial nuclei appeared more oval upon expression of activated PVR. The longest diameter of a glial nucleus was measured in ImageJ and was taken as size value.

Statistics
Comparison of tumor volume (MRI investigation) was analyzed using two-way ANOVA [treatment as between-subject factor and timepoints (10 days and 15 days after implantation) as within-subject factor]. Number of GFP-positive tumor cell clusters in the infiltration zone was compared by Mann–Whitney U-test. Results of the migration assay were analyzed by Student’s t-test. \( P<0.05 \) was considered significant. Experiments were performed independently three times. All statistical analyses were performed using IBM SPSS Statistics 18 version 18.0.

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Competing interests
The authors declare no competing financial interests.

Author contributions
S.N.K. designed the work and performed the Drosophila experiments; A.J. designed the work and performed the mammalian immunohistochemistry and xenograft experiments; K.H. performed the scratch assay; H.T.W. established the Drosophila strains used in this work and performed the initial experiments; M.W. performed the AFM experiments; T.M. performed the TEM analysis; H.S. analyzed the AFM data; C.F. performed the MRI measurements; V.S. participated in the xenograft experiments; W.P. designed experiments; C.K. designed experiments and wrote the manuscript.

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Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.106039/-/DC1

References


Supplementary Methods

Quantitative RT-PCR (qRT-PCR)

For the quantitative RT-PCR we used the following primers:

reaction lox (5’ GTCTGCAGCAGCCACGAAA; 3’ TACCGGAAGGGGCCCCAGGG), lox2 (5’ CATTCCATGCGAGTCCG; 3’ GAATCCCCACTCGTACGCA), myospheroid (5’ TGTCGAGCGGATACGACATC; 3’ ACTGCCGATACGAGTACG), Actin5c (5’ GAGCGGCTTACTACTCTTAC; 3’ ACTTCTCCACGAGGAGCTG), RpL32 (5’ GTTCGATCCGTAACGGT; 3’ CCAGTCGGATCGATATG), Gapdh1 (5’ CCGACGGTAAGATCCACA; 3’ CCGCCAGAGACCATC), elav (5’ GGCACAAACCTCATCC; 3’ CGATGGAGAGATCAGG), EF1, Gapdh2, alphaTub84B, RpL13A, Ef1a48D and Hsp70 for SYBR green-monoplex reaction, and lox (Dm02151941_g1; FAM-labeled), Act5c (Dm02361909_s1; VIC-labeled) and RpL32 (Dm02151827_g1; VIC-labeled) for TaqMan Gene Expression Assays for duplex reaction (Applied Biosystems).

Human materials were examined with TaqMan Gene Expression Assays (Applied Biosystems) LOX (Hs00942480_m1), LOXL1 (Hs00173746_m1) and LOXL4 (Hs00260059_m1) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using primers GAPDH-fwd 5’-ACCCACCTCCTCCACTTACGAC-3’, GAPDH-rev 5’-CATCCAGGAATGACTTTGAC-3’, and GAPDH-probe 5’-CTGGCATGCCCTCAAGGACCA-3’. Rat materials were analyzed using LOX (Rn00566984_m1), LOXL1 (Rn01418038_m1) and LOXL4 (Rn01410863_m1) and normalized to GAPDH expression using primers GAPDH-fwd 5’-CAAAGGATGCCCTGGAGGATC-3’, GAPDH-rev 5’-CAAAGGATGCCCTGGAGGAT-3’ and GAPDH-probe 5’-ACTAACAGGCGATCTGGACAC-3’. All experiments were performed in triplicate.

Immunohistochemistry

Generally, fly specimens were mounted in Vectashield (Vector Laboratories). The following antibodies were used: Mouse anti-Reo antibodies (1:5) were obtained from the Developmental Studies Hybridoma bank (Iowa City, IA), anti-Nidogen (1:2,000, gift of S. Baumgartner). Rabbit anti-GFP (1:1,000, Invitrogen) and goat anti-HRP (DyLight™649 conjugated AffiniPure HRP) (1:500, Dianova GmbH) and Alexa 488, 568 or 647 (1:1,000, Molecular Probes) and biotinylated goat anti-rabbit secondary antibodies (1:500, Dako) were used as secondary antibodies. Anti-hLOX (1:1,500, Abnova), anti-hLOXL1 (1:400, Deciphergen Biotechnology), anti-hLOXL4 (1:400, Enzo Life Sciences), anti-Ki67 (1:100, Acris Antibodies). For mammalian tissue and cells, staining was done using the ABC method (Vector Laboratories) and sections were counterstained with hematoxylin.
Supplementary Figure 1. PVR causes early onset of glial migration. Staining of eye imaginal discs for expression of CD8GFP specifically expressed by all glial cells (green), Repo protein to highlight all glial nuclei (red), and the HRP antigen which is found on all neuronal membranes (blue or grey). Scale bars are 20 µm. A) Second instar larva. Glial migration onto the eye disc has not yet started. os, optic stalk. B) Second instar larva overexpressing activated PVR in all glial cells. The arrow denotes glial cells prematurely migrating onto the eye disc. A’,B’) Only glial nuclei and neuronal membranes are shown. C,C’) Third instar larva. Note that all photoreceptor cells born in the eye imaginal disc project through the optic stalk towards the brain. D,D’) Third instar larva overexpressing activated PVR in all glial cells. Ectopic glial migration can be observed. In addition aberrant axonal projections can be seen (arrow). E,F) PVR affects surface glia in the brain. E) In larvae of the genotype repo-Gal4, UAS-CD8GFP glial cells show a normal appearance on the brain surface. F) Following pan-glial expression of activated PVR a multilayered organization of glial cells is detected and larger glial nuclei are seen (asterisk). Same magnification as in (E).
Supplementary Figure 2. Crossing scheme. A fly strain with genotype \{UAS-\(\lambda\) Pvr / CyO tub-Gal80; repo-Gal4 UAS-CD8GFP/TM6 Tb\} was generated. Here no Gal4-mediated expression is observed due to the activity of the Gal80 protein. Upon crossing to flies carrying no Gal80 element all non-TM6 progeny express activated PVR and CD8GFP in all glial cells. Depending on the culture temperature, these flies die as late embryos/early L1 (25°C) or as young L3 larvae (18°C).

Supplementary Figure 3. Genomic organization of \textit{lox} and \textit{lox2}. A) Schematic organization of the Drosophila \textit{lox} gene. The P-element used to generate the small deficiency is indicated. The deletion \textit{Df(3R)\textit{lox}\text{\_A38}} removes the \textit{lox} gene but leaves the neighboring transcription units intact. B) Schematic organization of the Drosophila \textit{lox2} gene. The insertion site of the Minos element close to the LTQ domain is indicated. This insertion likely disrupts the catalytic function of the Lox2 protein.
**Supplementary Figure 4.** *myospheroid* affects glial migration onto the eye imaginal disc. Eye imaginal discs where we suppressed *myospheroid* expression through RNAi were stained for expression of CD8GFP specifically expressed by all glial cells (green), Repo protein to highlight all glial nuclei (red), and the HRP antigen which is found on all neuronal membranes (blue). Upon pan-glial silencing of *myospheroid* three different phenotypic classes were observed. 

A) In the most extreme case, no glial migration onto the eye disc was noted. 

B) In an intermediate class dramatically reduced migration of 10 or less glial cells on the eye disc was noted. 

C) In a third class the number of glial cells moving onto the eye disc was reduced but migration distances appeared relatively normal.
Table S1. Summary of all AFM measurements

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<th>Genotype</th>
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sd, standard deviation; sem, standard error of the mean. Pa, Pascal.