Snail-dependent repression of the RhoGEF pebble is required for gastrulation consistency in Drosophila melanogaster

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Abstract The Rho GTP exchange factor, Pebble (Pbl), long recognised as an essential activator of Rho during cytokinesis, also regulates mesoderm migration at gastrulation. Like other cell cycle components, pbl expression patterns broadly correlate with proliferative tissue. Surprisingly, in spite of its role in the early mesoderm, pbl is downregulated in the presumptive mesoderm before ventral furrow formation. Here, we show that this mesoderm-specific repression of pbl is dependent on the transcriptional repressor Snail (Sna). pbl repression was lost in sna mutants but was unaffected when Sna was ectopically expressed, showing that Sna is necessary, but not sufficient, for pbl repression. Using DamID, the first intron of pbl was identified as a Sna-binding region. Nine sites with the Sna-binding consensus motif CAGGT[GA] were identified in this intron. Mutating these to TAGGC[GA] abolished the ventral repression of pbl. Surprisingly, Sna-dependent repression of pbl was not essential for viability or fertility. Loss of repression did, however, increase the frequency of low-penetrance gastrulation defects. Consistent with this, expression of a pbl-GFP transgene in the presumptive mesoderm generated similar gastrulation defects. Finally, we show that a cluster of Snail-binding sites in the middle of the first intron of pbl orthologues is a conserved feature in the other 11 sequenced Drosophila species. We conclude that pbl levels are precisely regulated to ensure that there is enough protein available for its role in early mesoderm development but not so much as to inhibit the orderly progression of gastrulation.

Keywords Snail · Pebble · Gastrulation · Rho GTP exchange factor

Introduction

During embryonic development, genes can act at multiple times and locations and can perform different molecular and cellular functions in different contexts. The complex functional requirements for a gene can be reflected by intricate and dynamic patterns of expression, which are under tight genetic regulation. The need for precise spatiotemporal control of gene expression is particularly acute in the developing embryo of the fly, where gene regulatory events must be precisely controlled over short time frames as the developmental events unfold in rapid succession. In this paper, we investigate one aspect of the regulation of a gene with pleiotropic functions: the Rho GTP exchange factor, Pebble (Pbl).

Pbl has long been known as an essential activator of the Rho signalling pathway that controls the formation and constriction of the cytokinetic actomyosin contractile ring (Prokopenko et al. 1999). Consistent with its role in cell cycle progression, pbl is upregulated in tissues that are proliferating or have proliferative potential (Prokopenko et al. 2000). Given its role in such a fundamental aspect of cell biology, it was surprising, therefore, when it was discovered that Pbl is also required for mesoderm migration (Smallhorn
et al. 2004; Schumacher et al. 2004). The Drosophila mesoderm is formed by the invagination of a ventral band of epithelial cells during gastrulation. Following internalisation, the epithelial tube flattens down onto the basal surface of the ectoderm, cells become more rounded and dissociated as the epithelial tube undergoes an epithelial to mesenchymal transition (EMT) and, finally, cells extend cellular protrusions as they migrate out over the ectoderm. Each of these processes is dependent on Pbl function (Smallhorn et al. 2004).

One puzzling aspect of the expression pattern of \textit{pbl} has been that, in spite of its role in early mesodermal development, it is actually repressed in the presumptive mesodermal cells prior to furrowing (Smallhorn et al. 2004). Gastrulation is extremely rapid in \textit{Drosophila}: the progression from initial furrowing to the onset of migration occurs in less than an hour. It is counter-intuitive, therefore, that a critical mediator of mesodermal behaviour should be repressed just before it is required. In this study, we investigate \textit{pbl} repression in the presumptive mesoderm.

When considering how \textit{pbl} might be repressed, an obvious candidate is the transcription factor Snail (Sna). Snail family transcription factors are well known for their roles in promoting mesenchymal cell fates through repression of epithelial genes such as DEcadherin. In \textit{Drosophila}, Sna is specifically upregulated in the presumptive mesoderm by the master mesodermal factor Twist and is required for repression of a number of genes, including the DECad orthologue, \textit{shotgun} (Leptin 1999). In addition to inducing EMTs, Snail family members have also been linked with cell cycle control. In flies, Sna binds cell cycle regulators \textit{string} and \textit{cycE} (Southall and Brand 2009) and can repress \textit{string} expression in neuroblasts (Ashraf and Ip 2001). Similarly, in vertebrates, Snail can block cell cycle progression by repressing cyclin D2 (Vega et al. 2004) and when EMT is induced in human breast cancer cells by expression of SNA1, genes associated with the cell cycle, including the Pbl orthologue, Ect2, are generally downregulated as the EMT proceeds (Vetter et al. 2009).

In this paper, we show that Sna directly represses \textit{pbl} and identify Sna-binding sites in the first intron of \textit{pbl} that are required for this repression. We subsequently show that while this repression is not essential for viability, it is required for optimal fidelity of gastrulation. The transcriptional regulation of \textit{pbl} expression in gastrulating embryos, therefore, appears

![Fig. 1](image_url)
to be a finely tuned compromise between a requirement for Pbl in mesodermal development on the one hand and impairment of gastrulation due to lack of repression on the other.

Materials and methods

Drosophila genetics

sna18 and β2-tubulin-FLP fly stocks were obtained from the Bloomington stock centre. The eve-stripe2-FRT-STOP-FRT-sna P-element construct (a gift from S. Small) (Nibu et al. 1998) was injected, and transformants are made by standard methods.

pbl intron mutagenesis

To create att[pblWT], att[pblASNA], att[pbl-GFP], and att[pblASNA-GFP] transgenics, briefly, a genomic fragment containing pbl and the two flanking genes was rescued by gap repair from the cosmid cos34 (G. Hime, unpublished) into P[acman] to create P[acman-pbl]. The GFP sequence was then incorporated just prior to the STOP codon into P[acman-pbl] using galK recombineering to create P[acman-pbl-GFP]. To mutate CAGGTT[AG] Sna sites to TAGGCG, multiple mutagenesis primers were used in a multiple-mutation reaction, with Ampligase® Thermostable DNA Ligase (EPICENTRE Biotechnologies) and Pfu (Stratagene) at an annealing temperature of 45 °C. The initial reaction successfully incorporated mutations to six of the motifs. We then used QuikChange® Site-directed Mutagenesis Kit (Stratagene) to introduce the mutations to the other three sites. Further details on primers and methodology are available on request. An unexpected A to G mutation was found in the final fragment, but this was located well away from the sna motifs. The mutated sequence was then incorporated into P[acman-pbl] and P[acman-pbl-GFP] using galK recombineering to create P[acman-pblASNA] and P[acman-pblASNA-GFP]. Both constructs were then injected into embryos expressing Flp to obtain insertions at the landing site M{3xP3-RFP.attP}ZH-86Fb which were subsequently recombined onto pbl2 and pbl3 chromosomes.

In situ hybridisation, immunostaining and imaging

Immunohistochemistry and in situ hybridisation were performed using standard methods. Rabbit anti-Twist was used at 1:500, and Goat anti-Rabbit-Alkaline Phosphatase at 1:500 (Jackson ImmunoResearch, West Grove, PA), Mouse anti-β-galactosidase MAb 40-1a at 1:100 (Developmental Studies Hybridoma Bank). Confocal images of twist::pblGFP embryos were captured on a Leica SP2 microscope. Image processing and 3-D rendering utilised ImageJ. In situ images were captured on an Olympus BX60 microscope and DP71 camera.

Embryonic lethality estimates

Lethality estimates of embryos were based on hatching rates of embryos laid from crosses attP[pbl], pbl2/TM3ftzlacZ × attP[pbl], pbl2/TM3ftzlacZ and attP[pblASNA], pbl2/TM3ftzlacZ × attP[pblASNA], pbl2/TM3ftzlacZ. Lethality was calculated as proportion of unhatched, fertilised embryos minus 25 % (i.e. we assumed normal ratios of the four possible embryonic genotypes and full lethality of TM3ftzlacZ/ TM3ftzlacZ homozygotes). N values given in text are estimates of the number of embryos of the given genotype (i.e. 25 % of total number).

Results and discussion

Sna is necessary but not sufficient for pbl repression

To test whether Sna regulates pbl expression, we compared pbl mRNA in wild type and sna18 mutant embryos. In wild-type embryos, pbl is initially expressed throughout the preblastoderm embryo but becomes enriched at the posterior end of the embryo (Fig. 1a) and confined to pole cells by stage 5 (Fig. 1b). During stage 5, pbl is turned on throughout the ectoderm, but its
expression is partly repressed in a ventral band of the embryo (Fig. 1c, d; Smallhorn et al. 2004). pbl is also de-repressed in a narrow band near the midline just prior to, and during, furrow formation (Fig. 1d; Smallhorn et al. 2004).

In sna<sup>18</sup> mutants, pbl repression was abolished, with expression remaining constant throughout the ectoderm (Fig. 1e). Thus, Sna is necessary for pbl repression.

To test whether Sna was sufficient for repression, we ectopically expressed Sna in the lateral ectoderm at a time when Sna is usually active in the presumptive mesoderm. We used the male specific β<sub>2</sub>-tubulin-FLP to flipout the intervening STOP from the eve<sub>stripe2</sub> > STOP > sna construct and thereby induce sna expression in the even-skipped stripe 2 domain of stage 5 embryos.

As expected, the construct generated an ectopic stripe of sna expression (Fig. 1f), but pbl expression was unaffected (Fig. 1g), even when the copy number of the eve<sub>stripe2</sub> > STOP > sna construct was increased to four. Thus, Sna is

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Fig. 3 Evolutionary conservation of Sna-binding sites in the first intron of pbl in other Drosophila species. The positions of the Sna motif CAGGT[A/G] in the first introns of 11 Drosophila species (D. melanogaster (D. mel), D. simulans (D. sim), D. sechellia (D. sec), D. yakuba (D. yak), D. erecta (D. ere), D. ananassae (D. ana), D. pseudoobscura (D. pse), D. persimilis (D. per), D. willistoni (D. wil), D. mojavensis (D. moj), D. virilis (D. vir) and D. grimshawi (D. gri)) are shown. Seven of the nine motifs in D. melanogaster are conserved in the closely related species, while a core cluster of four to five sites is conserved in even more distantly related species such as D. mojavensis and D. grimshawi. Red CAGGTG, blue CAGGTA. Ticks above and below the line indicate forward and reverse strands, respectively.

Fig. 4 Sna-binding sites are required for pbl repression. a In situ stains for GFP in embryos expressing pbl-GFP (a–c) and pbl<sub>SNA</sub>-GFP (d–f). a–c Embryos carrying the wild-type attP[pbl-GFP] construct showing the normal pattern of pbl expression at early stage 5 in pole cells (a), throughout the ectoderm but with a ventral band of repression (brackets) in stage 5 (b) and stage 6 (c) embryos and upregulation nearer the midline (c, white braces). d–f Embryos carrying the same construct in which the nine Sna consensus binding motifs have been mutated. As in control embryos, expression is seen in pole cells (d) and throughout the ectoderm in stages 5 (e) and 6 (f) embryos, but there is no repression in the ventral domain (e, f, black square brackets), though upregulation of pbl near the midline still occurs (f, white braces).
necessary, but not sufficient, for repression of \( pbl \). This was unexpected since the putative Sna co-repressor dCtBP is maternally expressed and would therefore be expected to be present in the lateral ectoderm and suggests that there exists some other factor necessary for repression, which may not be present outside the mesoderm region.

Identification of Sna-binding sites in the first intron of \( pbl \)

In a genome-wide DamID analysis of Sna-binding sites (Southall and Brand 2009), the first intron of \( pbl \) was identified as a Sna-binding region (Fig. 2b). In vitro analyses of Sna-binding consensus sequences, and ChIP-chip de novo motif discovery, have previously identified CAGGTG as a highly conserved core binding motif (Mauhin et al. 1993); (Zeitlinger et al. 2007). Consistent with this, five CAGGTG sites were identified in close proximity to the DamID peak (Fig. 2b).

To identify further binding motifs, we have previously developed the MICRA software tool (Southall and Brand 2009) which identifies sequences that are overrepresented in genomic regions with DamID peaks. This analysis revealed that although the CAGGTG binding motif was indeed enriched (43 % relative to background levels) in Dam-Sna-binding regions, the enrichment of the motif CAGGTA was higher (71 % relative to background levels). Interestingly, the CAGGTA sequence was also found to efficiently bind Sna in vitro (Mauhin et al. 1993). Four CAGGTA motifs were located near the DamID peak (Fig. 2b).

Thus, Sna binds to the first intron of \( pbl \), and this intron has nine potential Sna-binding sites.

Evolutionary conservation of Sna-binding sites

We next tested whether the enrichment of Sna motifs was conserved in related species. We examined the location of CAGG[T/G]A motifs in the first intron of \( pbl \) orthologues in 11 other species of \( Drosophila \). In each case, the arrangement, size and spacing of exons of the \( pbl \) locus was preserved, allowing us to unambiguously identify the first intron. In all \( Drosophila \) species, a cluster of four to five motifs towards the centre of the intron was conserved, including the more distantly related species such as \( Drosophila psuedoobscura \) and \( Drosophila grimshawi \) (Fig. 3). Analysis of five other arbitrarily chosen motifs showed conservation out to \( Drosophila erecta \) but not for the more distantly related species (Fig. S1).
Thus, the cluster of Sna-binding sites in pbl intron 1 has been conserved throughout the *Drosophila* genus.

Next, we checked whether Sna repression of *pbl* was conserved in a much more distantly related insect, the mosquito. Although this might seem unlikely, genetic regulation of dorsoventral patterning in *Anopheles gambiae* shows surprising levels of conservation with flies, with key genes such as *sna*, having comparable patterns of expression to *Drosophila* (Goltsev et al. 2007). The *pbl* orthologues in the mosquitoes *A. gambiae* (AGAP005796) and *Aedes aegypti* (AAEL000984) had a similar genomic structure to *Drosophila*, though the first annotated exon in both cases clearly matched exon 2 of *Drosophila* (Fig. S2). Analysis of CAGGT[GA] motifs over the entire loci of AGAP005796 and AAEL000984 (including 20 kb upstream sequence) did not reveal a cluster of Sna motifs similar to that found in *Drosophila*. Interestingly, in the mosquito, internalisation of presumptive mesodermal cells does not proceed by a whole-scale furrowing event but, rather, by the ingestion of individual mesodermal cells (Goltsev et al. 2007). The lack of Sna motifs, therefore, may be indicative of a different mode of gastrulation.

The Sna-binding sites in the first intron of *pbl* are required for Sna-mediated repression

To test whether these nine sites were functionally important, we generated genomic rescue constructs in which they were mutated. We cloned a 17.5-kb genomic region encompassing the *pbl* gene and the two flanking genes (Fig. 2a). This construct, P[acman-*pbl*], was inserted onto the third chromosome and introduced into a *pbl*−/− null mutant background. The construct was able to completely rescue the known cyto-kinetic and mesoderm migration phenotypes of *pbl* mutants (data not shown), and attP[ *pblWT* ], *pblΔ* /attP[ *pblWT* ], and *pbl*Δ (hereafter *pblΔ*WT) adults were viable and fertile.

We mutated the nine sites to see if they were required for the normal ventral repression of *pbl* expression. To achieve strong disruption to Sna binding with minimum DNA changes, we mutated just two highly conserved bases within each binding site. Based on published Sna-binding motifs (Ip et al. 1992; Mauhin et al. 1993; Zinzen et al. 2006) and in vitro mutation tests in which individual bases were mutated (Mauhin et al. 1993), we chose the C in position 1 and the T in position 5 as the bases to be mutated to T and C, respectively, to create the construct P[acman-*pblΔSNA*] (Fig. 2b).

To examine the transcriptional expression pattern of this construct, without the complication of endogenous *pbl* mRNA, we tagged both *pblWT* and *pblΔSNA* with GFP to create P[acman-*pblWTGFP*] and P[acman-*pblΔSNA_GFP*]. These constructs were introduced into the same attP site on the third chromosome as that used for the wild-type constructs, and expression of the GFP fusion transgenes was assessed with in situ hybridisation to GFP.

*pblWTGFP* was expressed in the same pattern as endogenous *pbl* with pole-cell expression (Fig. 4a), general ectodermal expression with downregulation in a ventral band (Fig. 4b, c) and upregulation near the midline (Fig. 4c). In contrast, *pblΔSNA-GFP* was expressed throughout the ectoderm with no ventral repression either before (Fig. 4e) or during gastrulation (Fig. 4f). Other endogenous patterns of *pbl* such as the expression in pole cells (Fig. 4d), upregulation near the midline (Fig. 4f) and in the longitudinal visceral muscle precursors (data not shown) were normal, indicating that the mutations did not wholly disrupt *pbl* regulation. Thus, one or more of the nine Sna consensus sites are required for repression of *pbl* in the presumptive mesoderm.

Loss of Sna repression of *pbl* increases the frequency of gastrulation defects

Next, we tested the functional consequences of loss of *pbl* repression. The *pblΔSNA* rescue construct described above was recombined with *pbl*Δ and *pbl*Δ mutant alleles. Surprisingly attP[ *pblΔSNA* ], *pblΔ* /attP[ *pblΔSNA* ], *pblΔ* (hereafter *pblΔSNA*) flies were also viable and fertile, showing that Sna repression of *pbl* is not essential.

To test whether loss of Sna repression of *pbl* might nevertheless affect gastrulation, we immunostained embryos for the mesodermal marker Twist. In *w*1188 control embryos, the great majority of embryos (98.9 %, *n* = 174) had normal gastrulation (Fig. 5a–d), though two embryos showed an irregular invaginated tube (Fig. 5e), *pblΔSNA* embryos were more frequently disrupted with defects in 12.1 % (n = 116) of embryos (Fig. 5f–i). In contrast, *pblWT* embryos were all normal (n = 112). Gastrulation defects seen in *pblΔSNA* embryos were (a) an irregular “wavy” invaginated mesodermal tube (n = 5; Fig. 5f), (b) regions of delayed invagination along the anterior-posterior axis and twisting (n = 4; Fig. 5g, h), and (c), a severe failure to internalise mesodermal cells (n = 5; Fig. 5i). Embryonic lethality was also increased from ~5 % (n = 116) in *pblWT* embryos to ~9 % (n = 268) in *pblΔSNA*.

Since *pblΔSNA* and *pblWT* flies were viable and fertile, we were also able to examine embryos derived from *pblWT* or *pblΔSNA* parents (hereafter *pblWT* and *pblΔSNA* embryos). Again, gastrulation defects were more common in *pblΔSNA* (11.1 % n = 189) embryos than *pblWT* (1.8 % n = 166) embryos. These frequencies were not significantly different than those from embryos derived from heterozygous parents with one wild-type copy of *pbl* (two-tailed Fisher’s exact test), and the range of defects was comparable (Fig. 5j–n). This result implies that gastrulation defects are associated only with zygotic, not maternal, loss of *pbl* Sna motifs.

1 See Materials and Methods.
Finally, we directly tested whether increased levels of Pbl in the presumptive mesoderm could affect gastrulation. To obtain transgene expression prior to furrowing, we fused the twist promoter to a \textit{pbl} transgene tagged with a C-terminal GFP. We know that PblGFP is a functional transgene since it is able to rescue both cytokinesis and mesoderm migration defects of \textit{pbl} mutants (data not shown), and the PblGFP genomic rescue construct described earlier can rescue \textit{pbl} null mutants to viability.

In \textit{twist::pblGFP} embryos PblGFP was expressed in a pattern that recapitulated endogenous twist patterns (e.g. Fig. 6g and data not shown), and homozygous \textit{twist::pblGFP} flies (in a wild-type background) were viable and fertile. \textit{twist::pblGFP} embryos exhibited similar gastrulation defects to \textit{pbl}$^{\Delta SNA}$ embryos though they were more penetrant (18.7 \%, \textit{n}=155) (Fig. 6 and Table 1). We conclude that loss of Sna-mediated \textit{pbl} repression significantly increases the frequency of gastrulation defects, most likely due to increased levels of Pbl.

Although Pbl has not previously been linked with ventral furrowing, apical constriction of the invaginating mesoderm is a Rho-dependent process requiring correct apical

**Table 1** Gastrulation defects in \textit{pbl}$^{\Delta SNA}$ embryos

| Genotype | Number | Defects | Percent | \(p\) value |
|----------|--------|---------|---------|-------------|
| \(w^{1118}\) | 174 | 2 | 1.1 | – |
| \(att(P\beta),pbl^I/att(P\beta),pbl^I\) | 112 | 0 | 0 | 0.5218\(a\) |
| \(att(P\beta)\Delta^{SNA}, pbl^I/att(P\beta)\Delta^{SNA}, pbl^I\) | 116 | 14 | 12.1 | <0.0001\(b\) |
| \(att(P\beta), pbl^I/att(P\beta), pbl^I*\) | 166 | 3 | 1.8 | 0.2747\(b\) |
| \(att(P\beta)\Delta^{SNA}, pbl^I/att(P\beta)\Delta^{SNA}, pbl^I*\) | 189 | 21 | 11.1 | 0.0011\(c\) |
| \textit{twist::PblGFP} | 155 | 29 | 18.7 | <0.0001\(a\) |

\(a\) \(p\) value represents two-tailed Fisher's exact test with respect to \(w^{1118}\)

\(b\) \(p\) value represents two-tailed Fisher's exact test with respect to \(att(P\beta), pbl^I/att(P\beta), pbl^I\)

\(c\) \(p\) value represents two-tailed Fisher's exact test with respect to \(att(P\beta), pbl^I/att(P\beta), pbl^I*\)

*The given genotype is that of the parents (see text)
localisation of another RhoGEF, RhoGEF2 (Hacker and Perrimon 1998). When expressed in early mesodermal cells PblGFP localises evenly around the cell cortex. We speculate, therefore, that higher levels of Pbl might alter the balance of apical/basal Rho-dependent cortical contractile forces, which disrupts the apical contraction of cells leading to occasional failures in furrowing.

In conclusion, we have shown that Sna represses transcription of pbl in the mesoderm primordium of D. melanogaster via one or more Sna-binding sites, which are conserved among species of the Drosophila genus, but not in the mosquito, correlating with the different modes of gastrulation in the different genuses. Although Sna-mediated repression of pbl was found not to be essential for viability, the selective pressure to maintain the mechanism is likely to come from our observation that loss of repression of pbl in the mesoderm primordium disrupts furrowing and lowers viability. Following pbl repression in the mesoderm primordium, pbl is again upregulated near the centre of the presumptive mesoderm just prior to furrowing. This upregulation may be required to reinstate the levels of Pbl required for mesoderm migration and the cytokineses that occur during the migration phase. The genetic basis for this upregulation awaits further study. These results speak to the importance of tight spatiotemporal control in fine tuning developmental events so as to maximise the efficiency of the developmental process.

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