

# 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

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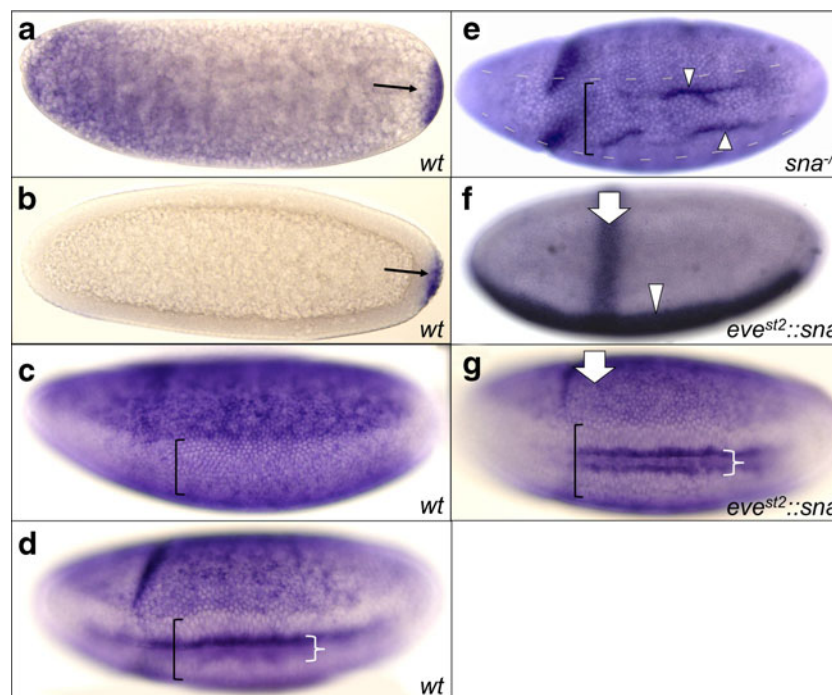
et al. 2004; Schumacher et al. 2004). The *Drosophila* mesoderm is formed by the invagination of a ventral band of epithelial cells during gastrulation. Following internalisation, the epithelial tube flattens down onto the basal surface of the ectoderm, cells become more rounded and dissociated as the 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 *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 *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 *pbl* repression in the presumptive mesoderm.

When considering how *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 *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, *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 *string* and *cycE* (Southall and Brand 2009) and can repress *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 *pbl* and identify Sna-binding sites in the first intron of *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 *pbl* expression in gastrulating embryos, therefore, appears



**Fig. 1** Sna is necessary but not sufficient for repression of *pbl*. Wild-type (**a–d**), *sna*<sup>18</sup> (**e**) and *eve*<sup>st2</sup>::*sna* (**f, g**) embryos stained for *pbl* (**a–e, g**) and *sna* mRNA (**f**). **a–d**. *pbl* mRNA expression in early wild-type embryos. In preblastoderm embryos, *pbl* is widely expressed but enriched at the posterior pole (**a**, arrow). By early stage 5, expression is confined to the pole cells (**b**, arrow). During stage 5, *pbl* is turned on throughout the ectoderm, but expression is significantly lower in a ventral band (**c–d**, black square brackets). Shortly before furrow formation and continuing as furrowing progresses, *pbl* is de-repressed in a central region near the midline (**d**, curly brackets). A *sna*<sup>18</sup> mutant

embryo at a similar stage to **d** (**e**). The main ventral furrow has failed to form, but ectopic “mini-furrows” (arrowheads) are apparent. There is no repression of *pbl* in the normal ventral furrow domain (region between dotted lines, black square bracket). An *eve*<sup>st2</sup>::*sna* embryo stained for *sna* mRNA, showing the normal band of ventral expression (arrowhead) and an ectopic band of expression corresponding to the *eve* stripe 2 region (arrow) (**f**). An *eve*<sup>st2</sup>::*sna* embryo showing normal patterns of *pbl* expression and no obvious ectopic band of *pbl* repression in the *eve* stripe2 domain (arrow) (**g**)

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

*sna*<sup>18</sup> and  $\beta$ 2-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[*pbl*<sup>WT</sup>], att[*pbl* <sup>$\Delta$ SNA</sup>], att[*pbl*-GFP], and att[*pbl* <sup>$\Delta$ SNA</sup>-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 CAGGT[AG] Sna sites to TAGGC[AG], multiple mutagenesis primers were used in a multiple-mutation reaction, with Ampligase<sup>®</sup> 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<sup>®</sup> 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-*pbl* <sup>$\Delta$ SNA</sup>] and P[acman-*pbl* <sup>$\Delta$ SNA</sup>-GFP]. Both constructs were then injected into embryos expressing  $\Phi$ C31 to obtain insertions at the landing site M{3xP3-RFP.attP}ZH-86Fb which were subsequently recombined onto *pbl*<sup>2</sup> and *pbl*<sup>3</sup> 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- $\beta$ -galactosidase MAbs 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.

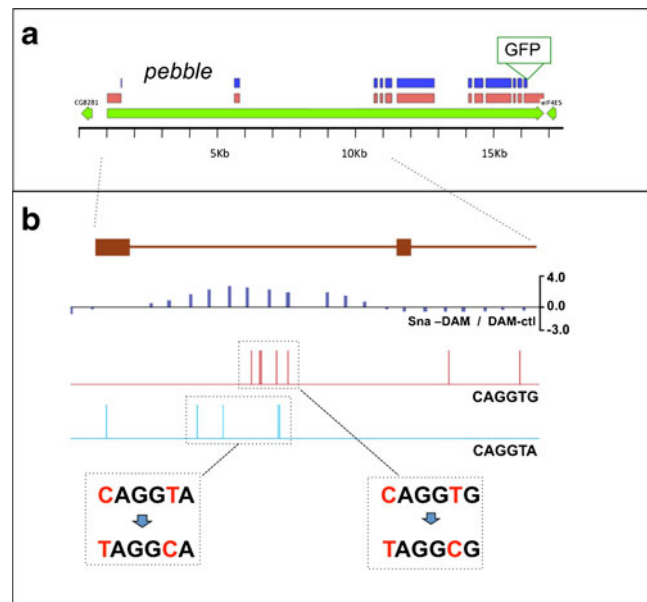
### Embryonic lethality estimates

Lethality estimates of embryos were based on hatching rates of embryos laid from crosses attP(*pbl*), *pbl*<sup>3</sup>/TM3fzlacZ  $\times$  attP(*pbl*), *pbl*<sup>2</sup>/TM3fzlacZ and attP(*pbl* <sup>$\Delta$ SNA</sup>), *pbl*<sup>3</sup>/TM3fzlacZ  $\times$  attP(*pbl* <sup>$\Delta$ SNA</sup>), *pbl*<sup>2</sup>/TM3fzlacZ. 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 TM3fzlacZ/TM3fzlacZ homozygotes). N values given in text are estimates of the number of embryos of the given genotype (i.e. 25 % of total number).

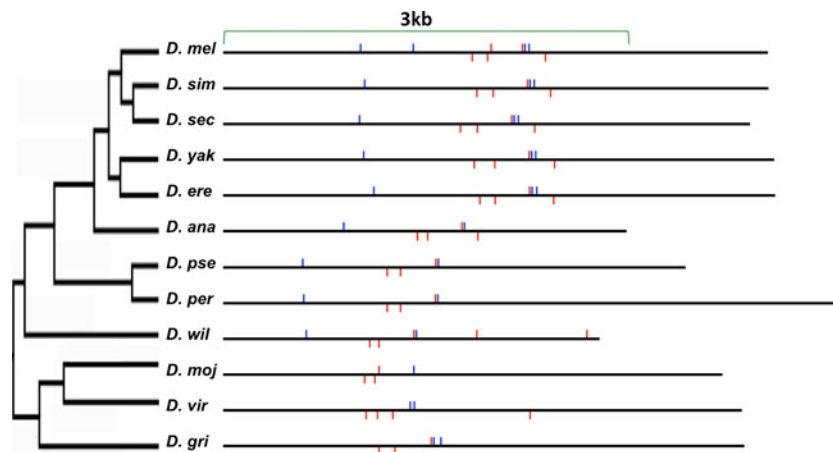
## Results and discussion

### Sna is necessary but not sufficient for *pbl* repression

To test whether Sna regulates *pbl* expression, we compared *pbl* mRNA in wild type and *sna*<sup>18</sup> 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



**Fig. 2** Identification of Sna-binding sites at the *pbl* locus. **a** Diagrammatic representation of the *pbl* genomic region that was cloned into p[acman] and modified by the insertion of a C-terminal GFP tag. Green represents the *pbl* transcript; pink, the exon structure; and blue, the open reading frame. **b** Binding of Dam-Sna in the first intron of *pbl*. Bar heights are proportional to the average of two normalised log<sub>2</sub>-transformed ratio of intensities from DamID in vivo binding site experiments. The intron contains five CAGGTG (red) and four CAGGTA (blue) Sna consensus binding motifs. In the *pbl* <sup>$\Delta$ SNA</sup> constructs, the CAGGTG and CAGGTA sites are mutated to TAGGCA and TAGGCG, respectively



**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 or 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

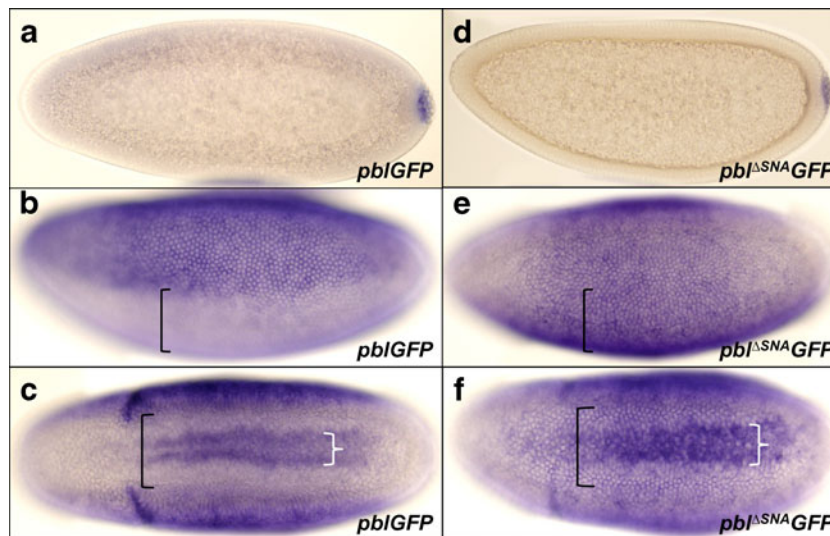
expression is partly repressed in a ventral band of the embryo (Fig. 1c, d; Smallhorn et al. 2004). *pbl* is also de-repressed in a narrow band near the midline just prior to, and during, furrow formation (Fig. 1d; Smallhorn et al. 2004).

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

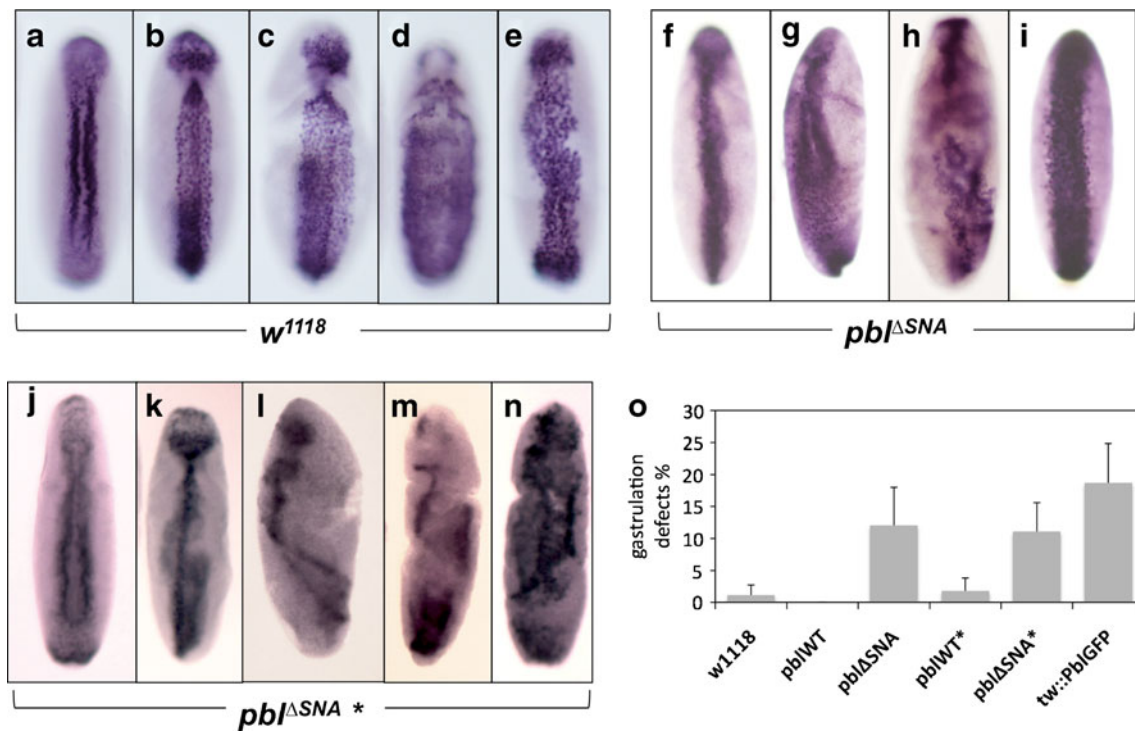
when Sna is usually active in the presumptive mesoderm. We used the male specific  $\beta 2$ -tubulin-FLP to flipout the intervening STOP from the *eve*<sup>STRIPE2</sup> > STOP > *sna* construct and thereby induce *sna* expression in the *even-skipped* stripe 2 domain of stage 5 embryos.

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*<sup>STRIPE2</sup> > STOP > *sna* construct was increased to four. Thus, Sna is



**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*<sup>ΔSNA</sup>-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)



**Fig. 5** Gastrulation defects in  $pbl^{ASNA}$  mutants. Embryos were immunostained for Twist to reveal mesoderm development. **a–e**  $w1118$  control embryos showing normal mesodermal invagination (**a**), dissociation (**b**) and spreading out into a monolayer (**c–d**). **e** An example of a small percentage (2 %) of  $w1118$  embryos in which the invaginated mesoderm shows unevenness. **f–n**  $pbl^{ASNA}$  embryos derived from parents with one  $pbl$  mutant chromosome (**f–i**) and  $pbl^{ASNA*}$  embryos

derived from  $pbl^{ASNA}$  parents (**j–n**), showing examples of the approximately 10 % of embryos exhibiting gastrulation defects. These included an irregular/wavy internalised mesoderm (**f, k**), variations in the progression of gastrulation along the anterior–posterior axis (**g, h, j, l**), twisting (**g, h, l, m**), and severe failures of internalisation (**i, n**). **o** Chart showing total proportions of gastrulation defects for different genotypes. Error bars show 95 % confidence interval

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 CAGGT[GA] 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 pseudoobscura* 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 mosquitos *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 ingress 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<sup>3</sup>/pbl<sup>2</sup>* 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[*pbl<sup>WT</sup>*], *pbl<sup>2</sup>/attP[*pbl<sup>WT</sup>*]*, and *pbl<sup>3</sup>* (hereafter *pbl<sup>WT</sup>*) 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-*pbl<sup>ΔSNA</sup>*] (Fig. 2b).

To examine the transcriptional expression pattern of this construct, without the complication of endogenous *pbl* mRNA, we tagged both *pbl<sup>WT</sup>* and *pbl<sup>ΔSNA</sup>* with GFP to create P[acman-*pbl<sup>WT</sup>*-GFP] and P[acman-*pbl<sup>ΔSNA</sup>*-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.

*pbl<sup>WT</sup>*-GFP 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, *pbl<sup>ΔSNA</sup>*-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.

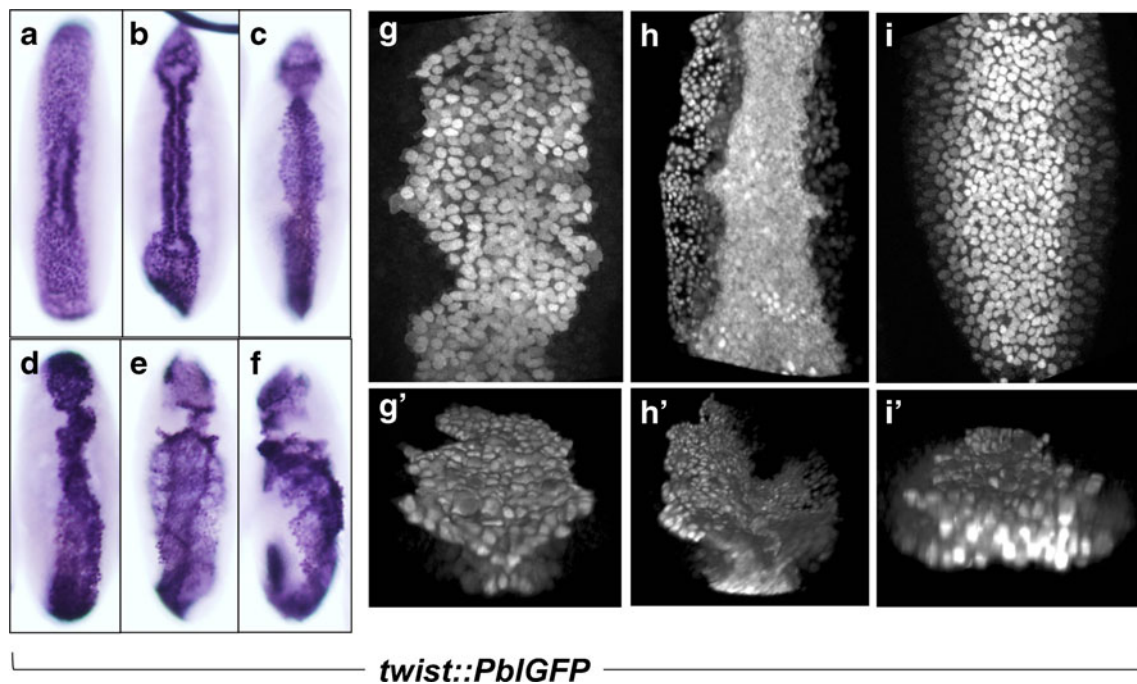
Loss of Sna repression of *pbl* increases the frequency of gastrulation defects

Next, we tested the functional consequences of loss of *pbl* repression. The *pbl<sup>ΔSNA</sup>* rescue construct described above was recombined with *pbl<sup>2</sup>* and *pbl<sup>3</sup>* mutant alleles. Surprisingly attP[*pbl<sup>ΔSNA</sup>*], *pbl<sup>2</sup>/attP[*pbl<sup>ΔSNA</sup>*]*, *pbl<sup>3</sup>* (hereafter *pbl<sup>ΔSNA</sup>*) 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<sup>1118</sup>* 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). *pbl<sup>ΔSNA</sup>* embryos were more frequently disrupted with defects in 12.1 % ( $n=116$ ) of embryos (Fig. 5f–i). In contrast, *pbl<sup>WT</sup>* embryos were all normal ( $n=112$ ). Gastrulation defects seen in *pbl<sup>ΔSNA</sup>* 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 % ( $n\sim 192^1$ ) in *pbl<sup>WT</sup>* embryos to ~9 % ( $n\sim 268$ ) in *pbl<sup>ΔSNA</sup>*.

Since *pbl<sup>ΔSNA</sup>* and *pbl<sup>WT</sup>* flies were viable and fertile, we were also able to examine embryos derived from *pbl<sup>WT</sup>* or *pbl<sup>ΔSNA</sup>* parents (hereafter *pbl<sup>WT\*</sup>* and *pbl<sup>ΔSNA\*</sup>* embryos). Again, gastrulation defects were more common in *pbl<sup>ΔSNA\*</sup>* (11.1 %  $n=189$ ) embryos than *pbl<sup>WT\*</sup>* (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.

<sup>1</sup> See Materials and Methods.



**Fig. 6** Gastrulation defects in *twist::PblGFP* embryos. Embryos expressing *twist::PblGFP* stained for Twist (a–f) or GFP (g–i) showing gastrulation defects, which are similar to those observed in *pbl<sup>ΔSNA</sup>* embryos (Fig. 5) though more penetrant. These included variations in

progression of gastrulation along the anterior–posterior axis (a, b, d), twisting (b, f), irregular/wavy mesoderm (c, g) and severe internalisation defects (e, h, i). g', h' and i' show three-dimensional renderings of g, h and i, viewed from the anterior end of the embryo

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 *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 *pbl* mutants (data not shown), and the PblGFP genomic rescue construct described earlier can rescue *pbl* null mutants to viability.

In *twist::PblGFP* embryos PblGFP was expressed in a pattern that recapitulated endogenous *twist* patterns (e.g.

Fig. 6g and data not shown), and homozygous *twist::PblGFP* flies (in a wild-type background) were viable and fertile. *twist::PblGFP* embryos exhibited similar gastrulation defects to *pbl<sup>ΔSNA</sup>* embryos though they were more penetrant (18.7 %,  $n=155$ ) (Fig. 6 and Table 1). We conclude that loss of *Sna*-mediated *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 *pbl<sup>ΔSNA</sup>* embryos

Genotype	Number	Defects	Percent	<i>p</i> value
<i>w<sup>1118</sup></i>	174	2	1.1	–
<i>attP(Pbl), pbl<sup>3</sup>/attP(Pbl), pbl<sup>2</sup></i>	112	0	0	0.5218 <sup>a</sup>
<i>attP(Pbl<sup>ΔSNA</sup>), pbl<sup>3</sup>/attP(Pbl<sup>ΔSNA</sup>), pbl<sup>2</sup></i>	116	14	12.1	<0.0001 <sup>b</sup>
<i>attP(Pbl), pbl<sup>3</sup>/attP(Pbl), pbl<sup>2</sup>*</i>	166	3	1.8	0.2747 <sup>b</sup>
<i>attP(Pbl<sup>ΔSNA</sup>), pbl<sup>3</sup>/attP(Pbl<sup>ΔSNA</sup>), pbl<sup>2</sup>*</i>	189	21	11.1	0.0011 <sup>c</sup>
<i>twist::PblGFP</i>	155	29	18.7	<0.0001 <sup>a</sup>

\*The given genotype is that of the parents (see text)

<sup>a</sup> *p* value represents two-tailed Fisher's exact test with respect to *w<sup>1118</sup>*

<sup>b</sup> *p* value represents two-tailed Fisher's exact test with respect to *attP(Pbl), pbl<sup>3</sup>/attP(Pbl), pbl<sup>2</sup>*

<sup>c</sup> *p* value represents two-tailed Fisher's exact test with respect to *attP(Pbl), pbl<sup>3</sup>/attP(Pbl), pbl<sup>2</sup>\**

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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