Maintenance of Cell Fate by the Polycomb Group
Gene Sex Combs Extra Enables a Partial Epithelial Mesenchymal Transition in Drosophila

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ABSTRACT Epigenetic silencing by Polycomb group (PcG) complexes can promote epithelial-mesenchymal transition (EMT) and stemness and is associated with malignancy of solid cancers. Here we report a role for Drosophila PcG repression in a partial EMT event that occurs during wing disc eversion, an early event during metamorphosis. In a screen for genes required for eversion we identified the PcG genes Sex combs extra (Sce) and Sex combs midleg (Scm). Depletion of Sce or Scm resulted in internalized wings and thoracic clefts, and loss of Sce inhibited the EMT of the peripodial epithelium and basement membrane breakdown, ex vivo. Targeted DamID (TaDa) using Dam-Pol II showed that Sce knockdown caused a genomic transcriptional response consistent with a shift toward a more stable epithelial fate. Surprisingly only 17 genes were significantly upregulated in Sce-depleted cells, including Abd-B, Abd-A, caudal, and nubbin. Each of these loci were enriched for Dam-Pc binding. Of the four genes, only Abd-B was robustly upregulated in cells lacking Sce expression. RNAi knockdown of all four genes could partly suppress the Sce RNAi eversion phenotype, though Abd-B had the strongest effect. Our results suggest that in the absence of continued PcG repression peripodial cells express genes such as Abd-B, which promote epithelial state and thereby disrupt eversion. Our results emphasize the important role that PcG suppression can play in maintaining cell states required for morphogenetic events throughout development and suggest that PcG repression of Hox genes may affect epithelial traits that could contribute to metastasis.

KEYWORDS epithelial mesenchymal transition Drosophila Polycomb Group Abdominal B Sex combs extra epigenetics Targeted DamID wing eversion

Epithelial mesenchymal transitions (EMT) are a fundamental mechanism in development, homeostasis and pathologies such as cancer metastasis (Thiery et al. 2009). Since the genes that regulate EMT are highly conserved, studies in model organisms like the vinegar fly, Drosophila melanogaster, can play an important role in identifying and analyzing EMT factors important to human health. To find new EMT regulators in the fly we made use of a partial EMT event that occurs during imaginal wing disc eversion (Pastor-Pareja et al. 2004; Manhire-Heath et al. 2013; Murray 2015).

Eversion is an early event during metamorphosis whereby wing imaginal discs, and other imaginal discs, break through the larval epidermis and join up to create the new epidermis of the adult body. During eversion, peripodial epithelial (PE) cells exhibit classic hallmarks of EMT: they lose epithelial features, such as apico-basal polarity and adherens junctions, they express matrix metalloproteases that breakdown the basement membrane, and they become migratory, extending F-Actin rich protrusions. These cellular changes allow...
them to invade the overlying larval epidermis, creating perforations that coalesce and allow the wing discs to be externalized, and subsequently lead the epithelial migration that results in thorax closure. Failure of any these events can disrupt eversion leading to loss of thoracic tissue and midline clefts, and disruptions to the wings, including internalization, mis-positioning and reduction in size (Martin-Blanco et al. 2000; Pastor-Pareja et al. 2004; Ishimaru et al. 2004; Srivastava et al. 2007; Manhire-Heath et al. 2013).

To find EMT factors, we conducted an RNAi screen in which the Ubx-GALA4 driver, which expresses strongly in peripodial cells, was used to knockdown genes during third-instar larval development, and adult flies (both eclosed and pharate), were scored for eversion defects (Golenkina et al. 2021). This screen identified Netrin-A (NetA) as a key regulator of the peripodial EMT (Manhire-Heath et al. 2013). NetA facilitates the breakdown of the adherens junctions of the peripodial epithelium (PE) via downregulation of its receptor Frizzled.

Here we present our analysis of another gene identified in this screen, the Polycomb Group (PcG) gene: Sex combs extra (Sce). Sce is a Drosophila ortholog of vertebrate RING1, an E3 ubiquitin-ligase that monoubiquitinates H2A at K118 leading to chromatin compaction (Fritsch et al. 2003; Gorfinkel et al. 2004). In Drosophila, PcG genes are well-known for their role in maintaining the patterns of Hox gene expression that are established during embryogenesis (Beuchle et al. 2001) but have not previously been associated with regulation of epithelial plasticity. In humans the PcG components EZH2 and Bmi1 have been linked with increased EMT and metastasis in cancer (Kleer et al. 2003; Wu and Yang 2011; Tong et al. 2012) as well as EMT during endometriosis (Zhang, Dong, et al. 2013). EZH2 forms a complex with Snail and HDAC1/HDAC2 to repress E-Cadherin expression (Cao et al. 2008; Tong et al. 2012), while Bmi1 cooperates with Twist to again silence E-Cadherin expression as well as the tumor suppressor p16INK4A (Yang et al. 2010; Wu and Yang 2011).

Here we show that loss of Sce results in a general failure of the wing discs to undergo the partial EMT of the PE, with effects on both the breakdown of zonula adherens (ZA) and basement membrane (BM). DamID transcripational profiling revealed that Sce knockdown resulted in de-repression of the well-established PcG target genes abd-A and Abd-B along with a small group of other genes, which together comprise a strong epithelial signature. We found that Abd-B was upregulated in cells lacking Sce and RNAi knockdown of Abd-B was able to substantially repress the Sce RNAi phenotypes. Misregulation of Abd-B is clearly only partly responsible for the Sce phenotypes, however, as knockdown of other genes was also able to rescue to some extent, and ectopic expression of Abd-B, while having potent effects on epithelial morphology, did not, itself, recapitulate the Sce,IR phenotypes. Our results suggest that PcG activity in peripodial cells is required to keep them in a cell state that is competent to undergo the pEMT required for successful eversion. Loss of PcG repression causes a general shift in gene expression toward a more epithelial state, which inhibits eversion.

MATERIALS AND METHODS

Drosophila stocks and husbandry

The following fly stocks were used in this study: Ubx-GALA4 (Pallavi and Shashidhara 2003), puc-GALA4 (Pastor-Pareja et al. 2004), odd-GALA4 (Larsen et al. 2006). The following strains were obtained from the Bloomington Drosophila Stock Center at Indiana University: Tre-GFP (#59010), UAS-Abd-B (#913), UAS-Abd-A (#912).

All UAS-RNAi stocks were obtained either from the Vienna Drosophila RNAi Centre or the Bloomington Stock Centre. SceK0 (Gutiérrez et al. 2012) was a kind gift from J. Müller. Targeted DamID was carried out by crossing Ubx-GALA4,GAL80* or UAS-Sce.IR106328;Ubx-GALA4,GAL80* flies to UAS-mCherry-Dam-Pol II (attP2) and UAS-LT3-Dam(attP2), or TaDaG-Dam (attP2) and TaDaG-Polycomb (attP2) flies (Delandre et al. 2020). MARCM clones were created by crossing hsFLP;UAS GFP; tub-GALA4, FRT82B tubP-GAL80 males to w;FRT82B SceK0 virgins and heat-shocking larvae at approximately early second instar for 30 min.

Targeted DamID

The Targeted DamID protocol was as described (Marshall and Brand 2017), with minor alterations. For each replicate of each genotype, 30 wing discs were dissected from wandering third instar larvae in 1xPBS, pooled, excess PBS removed, and then frozen at -80°C until required. Tissue was processed using a Qiagen DNase Kit. For the Dam-Pol II experiments, tissue from the freezer was thawed, 40μl of 500mM EDTA, 180μl of ATL buffer, and 20μl Proteinase K added, mixed gently and incubated for 56°C overnight, cooled to RT and 20μl of RNAase (12.5μl/ul) added and incubated for 2 min 400μl of a 1:1 mix of Buffer AL and 100% ethanol was added and mixed gently, before processing the solution through the DNase kit spin columns. The genomic DNA was then digested overnight with DpnI, cleaned up with a Qiagen PCR Purification kit, and DamID Adapters blunted ligated with T4 ligase, digested again with DpnI, and then adapter-ligated fragments PCR amplified using DamID primers and Advantage PCR kit DNA polymerase (Clontech). Adapters were then removed with Alw digestion, and final DNA fragments processed by the Melbourne Australian Genome Research Facility with a shotgun library prep protocol and 100bp single end reads generated on an Illumina HiSeq machine. For the Dam-Polycomb experiment, wing discs were prepared in the same way, though MyTaq polymerase (Bioline) was used for amplification, a TrueQ Nano Low throughput kit (Illumina) was used for library preparation and 86 base single-end reads were obtained on an Illumina MiSeq.

damidseq_pipeline, genome visualization and statistical analysis

Sequencing data for Targeted DamID were mapped to release 6.03 of the Drosophila genome using damidseq_pipeline (Marshall and Brand 2015). Transcribed genes (defined by Pol II occupancy) were identified using a Perl script described in (Mundorf et al. 2019) based on one developed by (Southall et al. 2013) (available at https://github.com/tonysouthall/Dam-RNA_POLII_analysis). Drosophila genome annotation release 6.03 was used, with a 1% threshold. To compare data sets, log2 ratios were subtracted, in this case, producing 2 replicate comparison files (as 2 biological replicates were performed). These data were then analyzed as described above to identify genes with significantly different Pol II occupancy. Due to the presence of negative log2 ratios in DamID experiments, these genes were filtered to check that any significantly enriched genes were also bound by Pol II in the experiment of interest (numerator data set). A gene list was generated from the transcript data using the values from the associated transcript with the most significant FDR. Replicate bedgraph files for each genotype were scaled by dividing each dataset by its standard deviation and averaged to create the profiles shown in Figures 2 and Fig. S3 which were visualized using pyGenomeTracks (Ramirez et al. 2018). Gene Ontology enrichment analysis was carried out using Flymine (Lyne et al. 2007).
Figure 1  Sce expression in peripodial cells is required for wing eversion. (A-F) Ubx > Sce.IRV10638 pupae showing increasingly severe categories of adult eversion failure. (G) A SceKO MARCM pupa showing a thoracic cleft. (H) Quantification of adult eversion phenotypes. Knockdown of Sce using three different UAS RNAi lines, and three different peripodial GAL4 drivers (Ubx-GAL4, puc-GAL4, odd-GAL4) induces eversion failure phenotypes.
Table 1 Knockdown of the Polycomb Group genes, Sce and Scm, inhibits wing disc eversion

| Genotype                                      | Normal % | Weak % | Strong % | Early lethal % | n-val | p-val  |
|-----------------------------------------------|----------|--------|----------|----------------|-------|--------|
| +/+; Ubx-GAL4, GAL80+/-                       | 100.0    | 0.0    | 0.0      | 0.0            | 79    |        |
| Sce.IR^{274E9+}; Ubx-GAL4, GAL80+/-         | 49.1     | 2.7    | 32.1     | 16.1           | 112   | <0.0001|
| Sce.IR^{31E12+}; Ubx-GAL4, GAL80+/-         | 61.9     | 16.8   | 18.6     | 2.7            | 113   | <0.0001|
| Sce.IR^{106328+}; Ubx-GAL4, GAL80+/-       | 8.9      | 0.0    | 5.1      | 86.1           | 79    | <0.0001|
| Sce.IR^{106328+}; odd-GAL4; +/-             | 51.0     | 7.8    | 9.8      | 31.4           | 51    | <0.0001|
| Sce.IR^{106328+}; puc-GAL4 /+               | 88.1     | 2.4    | 2.4      | 7.1            | 42    | 0.0043 |
| Scm.IR^{31E12+}; Ubx-GAL4, GAL80+/-        | 19.5     | 0.0    | 80.5     | 0.0            | 41    | <0.0001|

p-values use two-tailed Fisher’s exact method on the proportion of normal adults.

RESULTS

Polycomb group gene expression in the peripodial epithelium is required for wing disc eversion

To find genes required for the peripodial EMT the Ubx-GAL4 driver was crossed to UAS-RNAi lines and pharate or eclosed adult flies screened for eversion defects. Phenotypes were categorized in increasing level of severity (Figure 1) as:

i. malformed wing; the thorax is normal but one or both wings are affected in some way such as being smaller, mispositioned, or crumpled (Figure 1B).

ii. thoracic cleft: both wings everted but a gap remaining in the middle of the thorax (Figure 1C).

iii. single-eversion failure: one wing failed to evert, resulting in an adult lacking half a thorax (Figure 1D).

iv. double-eversion failure: neither wing everted and thoracic tissue missing (Figure 1E).

v. early pupal lethal: adult structures such as wings, legs and head not discernible (Figure 1F).

As expected, knockdown of genes known to play a role in eversion such as components of the JNK (fos, slp) and TGFβ pathways (dpp, punt, Mad) generated eversion phenotypes (data not shown) as did NetA and NetB as previously described (Manhire-Heath et al. 2013). Two other genes with highly penetrant, and phenotypically severe, eversion defects were the PcG genes, Sex combs extra (Sce) and Sex combs midleg (Scm). Knockdown of these genes had similarly strong effects. RNAi to Sce using UAS-Sce.IR^{81664} resulted in a high proportion of single and double eversion failure (18.6%, n = 113) and crumpled wings (16.8%) (Table 1; Figure 1H). Similarly, knockdown of Scm with UAS-Scm.IR^{81664} produced high levels of single and double eversion failure (80.5%, n = 41; Table 1; Figure 1). For further analysis we focused our attention on Sce.

To check for off-target effects, two other RNAi lines for Sce were tested: UAS-Sce.IR^{106328} and UAS-Sce.IR^{274E9}. At 29° these also produced eversion defects, though in one case (UAS-Sce.IR^{106328}) the primary phenotype was early lethality (86.1%, n = 79; Table 1; Figure 1). However, subsequent tests using a temperature shift regime to restrict knockdown to a tighter developmental window, also produced a high proportion of double-eversion failures for this RNAi line (see below), suggesting that the early lethality was due to a stronger
| Symbol | Name            | Ratio\textsuperscript{a} | FDR         | Molecular function                                      | Biological roles                                                                 | GO-terms (biological function)                      |
|--------|-----------------|---------------------------|-------------|---------------------------------------------------------|---------------------------------------------------------------------------------|------------------------------------------------------|
| Abd-B  | Abdominal-B     | 0.43916667 1.67E-19       | Hox          | Hox transcription factor                                 | Bithorax complex Hox gene controlling posterior abdominal segments; external genitalia and gonads, and post-mating-response | epithelium development, anatomical structure morphogenesis |
| oc     | ocelliless      | 0.17026667 2.66E-07       | Paired-like  | Paired-like homeobox transcription factor                | regulator of rhodopsin expression and axonal targeting in the retina             | epithelium development, anatomical structure morphogenesis |
| abd-A  | abdominal-A     | 0.11543333 3.91E-07       | Hox          | Hox transcription factor                                 | bithorax complex Hox gene controlling identity of embryonic segments            | epithelium development, anatomical structure morphogenesis |
| mirr   | mirror          | 0.1755 8.49E-06           | iroquois     | iroquois homeobox transcription factor                  | dorso-ventral axis; eye formation; embryonic segmentation; PNS development      | epithelium development, anatomical structure morphogenesis |
| nub    | nubbin          | 0.08573333 1.61E-05       | POU/homeodomain | POU/homeodomain transcription factor                 | wing formation; midgut stem cell proliferation and enteroctye differentiation      | anatomical structure morphogenesis                     |
| cad    | caudal          | 0.17613333 4.41E-05       | Hox-like     | Hox-like homeobox transcription factor                  | anterior/posterior patterning, organ morphogenesis                              | epithelium development, anatomical structure morphogenesis |
| Inx2   | Innexin 2       | 0.21316667 0.00027954     | gap junction | gap junction protein                                     | epithelial organization and polarity of epidermis, regulation of organ size and stem cell behavior | epithelium development, anatomical structure morphogenesis |
| CAH2   | Carbonic anhydrase 2 | 0.35613333 0.00038658 | Carbonic anhydrase | Carbonic anhydrase | Catalyze the CO2 hydration reaction |                                                                 |
| tup    | tailup          | 0.06526667 0.0015028      | LIM homeobox | LIM homeobox transcription factor                        | neuronal sub-type identity, including motor, serotonegenic and dopaminergic neuron identity. It regulates germ band retraction, dorsal closure, muscle and heart development | epithelium development, anatomical structure morphogenesis |
| Sp1    | Sp1             | 0.04683333 0.00190136     | Sp-family of Cys2His2-type zinc finger transcription factors | Sp-family of Cys2His2-type zinc finger transcription factors | ventral thoracic appendage specification; leg growth; type-II neuroblast development | epithelium development, anatomical structure morphogenesis |
| Pdk    | Pdk             | 0.13876667 0.00347056     | Pyruvate dehydrogenase kinase | Pyruvate dehydrogenase kinase | Pyruvate dehydrogenase kinase | Axon guidance receptor | epithelium development, anatomical structure morphogenesis |
| CG3777 | CG3777          | 0.08913333 0.00529072     | unknown      | unknown                                              | unknown                          | epithelium development, anatomical structure morphogenesis |
| pim    | pimples         | 0.5068 0.00719975         | Securin      | Inhibits Separase                                       | unknown                          | epithelium development, anatomical structure morphogenesis |
| CG3262 | CG3262          | 0.39193333 0.00728925     | unknown; interpro domain [Flagellum site-determining protein YkH/Fe-S cluster assembling factor NBP35] | unknown; interpro domain [Flagellum site-determining protein YkH/Fe-S cluster assembling factor NBP35] | unknown |                                                                 |
| Psc    | Posterior sex combs | 0.09333333 0.00911746     | Component of PRC1 complex | Component of PRC1 complex | PcG epigenetic repression | Anatomical structure morphogenesis |
| CG34293 | CG34293-RA      | 0.61326667 0.00994297     | unknown; Interpro domain [Small subunit of serine palmitoyltransferase-like] | unknown | CG34293-RA                                      |                                                                 |

\textsuperscript{a}Values are for transcript isoform with highest ratio - see Supplementary Data File 1.
Figure 2  Sce.IR derepression loci are also Dam-Pc binding sites. (A-E) Genome browser views of major de-repression loci. Traces show fold-change of Dam-Pol II fusion over Dam alone, and Dam-Pc fusion over Dam alone for control discs and Ubx > Sce.IR discs. The bithorax region encompassing abd-A and Abd-B (A) is markedly derepressed in Sce.IR discs. These regions also show clear binding of Dam-Pc indicating that they are regions of PcG repression. Note that Ubx expression is unaffected by loss of Sce. Similarly, loci for caudal (B), nubbin (C), mirror (D) and ocelliless (E) show varying degrees of increased Dam-Pol II binding but are all clearly regions of Dam-Pc binding.
RNAi effect. Occasional eversion defects could also be generated by creating random SceKO mutant clones using the MARCM technique (Lee and Luo 2001) (Figure 1G).

Immunostaining confirmed that Sce was expressed ubiquitously throughout the wing disc, including the peripodial epithelium, was predominantly nuclear, and appeared relatively constant between third instar and white prepupal stages (Fig. S1A, E). As expected there was a marked reduction of Sce levels in \texttt{Ubx.Sce.IRV106328} peripodial cells (Fig. S1C).

We next wished to see if \texttt{Sce} RNAi knockdown using other peripodial GAL4 drivers could also disrupt eversion. The PE has genetically distinct subdomains and different drivers express in different regions. The \texttt{Ubx-GAL4} driver has a broad expression domain throughout the central area of the PE but posterior to the anterior/posterior border, while the \texttt{odd-GAL4} driver expresses in the medial anterior cells, and the \texttt{puc-GAL4} driver, a reporter for JNK-activation, expresses strongly in peripodial cells nearest the stalk region (Pastor-Pareja et al. 2004; Tripura et al. 2011; Aldaz et al. 2013) (Fig. S2). Knockdown of \texttt{Sce} with both \texttt{odd-GAL4} and \texttt{puc-GAL4} produced eversion failures though the penetrance was less than for \texttt{Ubx-GAL4} (Table 1; Figure 1H).

Note that although \texttt{Ubx} is part of the bithorax complex along with \texttt{abd-A} and \texttt{Abd-B}, and that region is known to be regulated by PcG repression, our TaDa expression profiling showed that the \texttt{Ubx} locus was not affected by loss of \texttt{Sce} (see below) making it unlikely the \texttt{Ubx-GAL4} driver was itself being affected by loss of PcG repression.

Taken together these results show that \texttt{Sce} is required for eversion and suggest that target genes of PcG repression must remain repressed for successful eversion to occur.

\textbf{Sce RNAi affects the partial EMT of the wing discs}

Since eversion is a complex multi-step process it can be affected at several stages: the initial apposition of the wing disc to the body wall, the degradation of the BM, the pEMT of the PE, the invasion of the epidermis, or the subsequent epithelial migration (Pastor-Pareja et al. 2004). Previously, we and others have found that the first steps of
eversion, the pEMT and BM breakdown, can occur when discs are cultured in the presence of ecdysone (Milner 1977; Aldaz et al. 2010; Manhire-Heath et al. 2013). This provides an opportunity to determine if eversion failures are due to those early events, or later stages of the process. At 29°C, eversion typically begins after 6-7 hr of culturing and is complete by 9-10 hr. To obtain an overall readout of eversion success we cultured discs for 16 hr, a period long enough to ensure complete eversion. Under these conditions we have found discs fall into three categories (Golenkina et al. 2021):

i. successfully everted. discs that have flattened, wing-like morphologies and the PE forms a disorganised clump;

ii. partially everted. discs show evidence of breakdown of the PE but have not flattened out,

iii. uneverted. discs show no evidence of PE and BM breakdown although the DP may have undergone some bending.

When Ubx > Sce.IR discs (hereafter Sce.IR discs) were cultured overnight there was a significant change in eversion outcomes. The proportion of discs that were uneverted increased from 28.8% (n = 80) to 45.45% (n = 77) (P = 0.0007), while successful eversion fell by half, from 57.5 to 29.87% (Figure 1I).

Next, we looked at discs after 7 hr of culturing, which, at 29°C, is a time when most discs are initiating epithelial dissociation by dismantling their AJs and are breaking down their BM. Discs were fixed and immunostained for E-Cadherin, Rhodamine-Phalloidin, and anti-Laminin to label AJs, F-Actin and BMs, respectively (Figure 1J-M). The 7hr results were consistent with overnight eversion. In control discs only 9.1% (n = 66) of discs showed an intact AJs compared to 52% (n = 50) in Sce.IR discs (P = 0.0001) – the remaining discs showing either a loss of AJs or small to large perforations in the PE (Figure 1N). Similarly, the proportion of discs with an intact BM was doubled from 32.8% of control discs to 58% of Sce.IR discs (P = 0.0005) (Figure 1O). Thus, there was overall inhibition of these processes in Sce.IR discs but no other obvious qualitative differences were detected.

Next, we tested whether two other key events in wing eversion were affected by loss of Sce: activation of the JNK pathway and expression of abd-A. abd-A is partially repressed by Sce. (A-F) Third instar wing discs stained for abd-B and E-Cadh. In control discs (A-B) there is no nuclear expression of abd-B though some cytoplasmic staining in PE cells was apparent. (C-D) Ubx > Sce.IR discs, appeared the same, though the cytoplasmic staining appeared somewhat stronger. (E-F) In Sce MARCM discs there was clearly some nuclear expression of abd-A in some clones (E, E’, F, F’, arrows) though this was of varying strength within a clone (F’, arrow), and some clones showed no expression (F’, arrowhead).

Figure 5 Knockdown of de-repressed loci substantially represses Sce.IR eversion phenotypes. Effects on adult eversion failure when Ubx-GAL4 knockdown of Sce is accompanied by expression of the indicated UAS RNAi lines, or UAS-GFP control. Co-expression of GFP does not significantly decrease the rates of eversion failure in Ubx > Sce.IR discs, but co-expression of UAS RNAi lines for Abd-B, Abd-A, cad, and nub all repress eversion failure. Ubx-GAL4 expression of Abd-Bm produces a high proportion of weak phenotypes in which the thorax is normal, but wings are deformed or mispositioned (56%, n = 222). Expression of Abd-A has no effect (n = 84). Error bars = 95% confidence interval (Wilson score method).

4466 | G. Jefferies et al.
We performed Gene Ontology enrichment analysis on the lists of significantly changed genes, and on significantly expressed genes in the two genotypes (see Materials and Methods). For the 17 de-repressed genes, the most significant terms for biological function are “epithelium development” (10/17 genes; $P = 0.001178$; Holm-Bonferroni correction used for all enrichment analysis; Table 2) and “anatomical structure morphogenesis” (12/17 genes; $P = 4.3e-4$).

There is also significant enrichment of genes with molecular function of transcription factors (8/17 genes; $P = 4.3e-4$), seven of which contain homeodomains. In contrast, for genes whose expression significantly decreased in Sce.IR discs there is no GO Term enrichment in any category.

Similar results were obtained when analysis was expanded to the entire set of significantly expressed genes in the two genotypes. The most strongly enriched biological function in Sce.IR discs is “epithelium development” (333/2045 genes; $P = 1.84e-45$), whereas for control discs it is “cellular-metabolic-process” (917/1898 genes; $P = 2.8e-11$). In Sce.IR discs there is also an enrichment of “cellular component” for cell junction proteins (62 genes; $P = 2.0e-11$) and of “molecular function” for actin binding (51 genes; $P = 3.1e-7$) consistent with cellular changes impacting upon pEMT processes.

Since direct targets of the PcG complexes would be expected to have increased expression we focused our attention on the 17 de-repressed genes. Changes in expression levels for these genes, averaged across the whole gene locus were relatively modest, ranging from 0.61 to 0.047 log2 (i.e., fold-change of 1.5 to 1.03) averaged over the gene locus.

To confirm that these genes corresponded to regions of PcG repression we again used TaDa to examine the binding profile of the PcG component, Polycomb using UAS-myr-GFP-Dam-Polycomb and a UAS-myr-GFP-Dam control (Materials and Methods). The Dam-Pc ratio profile exhibited the expected genomic patterns of Polycomb binding for known PcG target areas, such as the engrailed/invected and the bithorax regions (Tolhuis et al. 2006) indicating that the method had worked. For each of the 17 genes we then calculated the average level of Pc-binding in control discs (Fig. S4B). The genes with the most significant fold-change in Sce.IR discs vs. controls (FDR < 1e-4) (Figure 2) also tended to have higher levels of Pc-binding (Fig. S4). We also examined the Dam-Pc profile in Sce.IR discs but found the pattern of binding largely unchanged from control discs though the average ratio levels across the genome were reduced (Figure 2; Fig. S4; and data not shown).

Thus, the loss of Sce has resulted in increased expression of a small number of genes in PcG-repression regions, and this is accompanied by a genome-wide change in genes from those associated with cellular metabolism to those involved in epithelial development, consistent with an inhibition of the PE pEMT.

**Abd-B is upregulated in the peripodial epithelium of Sce.IR discs and required for eversion failure**

Based on the expression profiles of the de-repressed genes, we conducted further tests on four of the genes that had a distinct change in expression profile and higher levels of Pc-binding: *abd-A*, *Abd-B*, *cad* and *nub*.

We first used immunostaining to determine if any of the four genes showed significant upregulation in the PE of Sce.IR discs. Of the four genes, only Abd-B showed a clear change in expression in PE cells with nuclear staining apparent in the Sce.IR discs but not in control discs (Figure 3A-F). We further confirmed that loss of Sce was responsible for Abd-B upregulation by examining MARCM clones for the null allele SceKO. Clones in both the PE and DP showed clear upregulation of Abd-B (Figure 3G-I). In addition, there was a morphological change in both PE and DP clones in that they showed a “segmentation-phenotype” whereby they became more rounded and developed furrowing/invagination at the borders with wild type cells as previously reported for several PcG genes (Beuchle et al. 2001; Fritsch et al. 2003; Gandille et al. 2010; Curt et al. 2013).
Figure 6 Abd-B affects epithelial cells and wing morphology. (A-C) Pharate adults showing eversion phenotypes. The primary phenotype was for one or more malformed and mispositioned wings (A’, A”). (B) An adult with both wings affected, and legs malformed. (C) Adult with a more severe phenotype in which the thorax is disrupted and only one wing has evverted, but is malformed. (D) Expression of Abd-Bm with the Ubx-GAL4 driver.
Although no obvious change in abd-A, Nub or Cad expression/localization was seen in Ubx > Sc.eIR PE cells, a subset of Sc.eKO MARCM clones also showed clear upregulation of abd-A, though the levels were variable (Figure 4). We speculate that while Abd-B is directly controlled by PcG complexes, Abd-A faces more complex regulation and may be being suppressed by Abd-B and/or the non-coding RNA mir-iab-8 which is also located in the de-repressed region between abd-A and Abd-B. In the case of Nub and Cad there was no nuclear expression though we cannot discount the possibility of a mild increase in cytoplasmic signal.

Next, we tested whether RNAi knockdown of any of the four genes could suppress the Sc.eIR phenotypes. We utilized the Sc.eIR\textsuperscript{Y106528} RNAi line but used a temperature shift regime to restrict the period of GAL4 expression to third instar stages, thereby avoiding the excessive early pupal lethality. Two independent RNAi lines were used for each gene (Figure 5). Knockdown of any of the four genes was able to partly rescue the defects while co-expression of an arbitrary UAS construct, UAS-GFP, had no effect (normal progeny = 4.8%, n = 165, P = 0.65). Of the four genes loss of Abd-B had the strongest effect increasing the proportion of normal eversions from 4% in Sc.eIR discs (n = 379) to 47.15% in Sc.eIR;Abd-BIR discs (n = 397, Figure 5; Table 3; P < 0.0001). The results suggest that the inhibition of eversion may not be due to any one of these genes, but rather to a genome-wide change in transcriptional profile toward an epithelial state. The other implication is that the maintenance of epithelial/BM integrity in Sc.eIR discs is relatively unstable, since knockdown of any of the four PcG targets was enough to substantially restore successful eversions.

Finally, we tested whether over-expression of either of the two genes with strongest rescue, Abd-B and abd-A, could phenocopy loss of Sc.eIR. Ubx-GAL4-driven expression of Abd-B in the PE did not block eversion, though a high proportion of adults had reduced/misplaced wings (Figures 5, 6A-C). Clonal expression of Abd-B did, however, recapitulate the epithelial invagination/segregation phenotype, as has previously been described (Gandille et al. 2010) (Figure 6F-I). Ubx-GAL4-driven expression of abd-A had no effect, however clonal expression of abd-A also created invaginations suggesting that this phenotype is a conserved ability of Hox genes to regulate epithelial morphology (data not shown). Since sole expression of Abd-B was not able to recapitulate the Sc.eIR phenotypes we conclude that while the epithelial morphology changes induced by Abd-B, and to a lesser extent, abd-A, may contribute to eversion failure, they are not sufficient.

Overall, our results imply that the maintenance of epithelial/BM integrity in Sc.eIR discs is due to a genome-wide change in gene expression toward an epithelial state, and that Abd-B likely plays the major role in this change.

**DISCUSSION**

We have uncovered a new role for PcG repression during *Drosophila* development: maintenance of the state of peripodial cells such that they are able to undergo the partial EMT that allows eversion to proceed. Loss of *Sce* leads to de-repression of a small number of target genes and an overall shift in gene expression toward a cell-state associated with “epithelial development”, and hence eversion is impeded. Thus, PcG repression is not only crucial for maintaining segmental identity but also for maintaining cells in a state of readiness for the epithelial plasticity events that occur later during development and which are necessary for successful eversion.

Our TaDa analysis of Dam-Pol II binding identified a surprisingly small number of genes that were upregulated in *Sce*IR discs. Only 17 genes had an FDR < 0.01 and two of these were the known PcG targets, *abd-A* and *Abd-B*. Using Dam-Pc we confirmed that, for most of these genes, their loci corresponded to Polycomb binding regions of the genome.

In contrast there were 110 genes that were significantly down-regulated in *Sce*IR discs but these showed no GO-term enrichments and did not include well-known *Drosophila* EMT regulators, such as Snail and Serpent. However, one gene that is linked to EMT in mammals, and was among the most significantly reduced genes, was the lipid raft protein Flotillin-1 (Flol). In *Drosophila* Flol has been shown to regulate collagen turnover (Lee et al. 2014) which could well promote the eversion process. In mammals Flotillins are more strongly linked to EMT, where they promote endocytosis and turn-over of both cell adhesion molecules and ECM proteins and promote cancer metastasis (Gauthier-Rouvière et al. 2020). Interestingly, the *Drosophila* paralog Flolo2, is also upregulated during wound healing (Juarez et al. 2011), a cellular event with many parallels to thorax closure, including the involvement of Src42A and the JNK pathway. It will be of great interest, therefore, to explore the role of the two Flotillins in the eversion process.

We focused our attention on four of the genes with a clear change in Dam-Pol II profile and tested whether RNAi knockdown could repress the eversion defects of *Ubx > Sc.eIR*. Surprisingly, we found that all had a significant effect on rescue, though the knockdown of *Abd-B* was the most significant. It is possible that co-expression of multiple UAS lines might result in a reduction in the strength of the UAS-Sc.eIR phenotype, simply due to competition for GAL4. However, we found no effect of combined expression of UAS-GFP. We speculate that PE breakdown and the eversion process as a whole, are “threshold events” that tend to proceed to completion once begun - like a membrane tearing. In a genotype such as Sc.eIR, where eversion is failing about half the time, the PE is presumably poised at that critical threshold - such that a small change in gene expression can have a large effect. Other dominant modifier tests we have conducted involving eversion have shown a similar sensitivity to genetic perturbation (data not shown). Although the expression of these genes was clearly important in blocking eversion, over-expression of *Abd-B* and *abd-A* on their own, was unable to recapitulate the eversion blockage, suggesting that it is the combined expression that produces a cell state necessary to inhibit the pEMT and BM breakdown.

Others have shown previously that loss of various PcG genes in wing discs results in ectopic expression of Ubx, Abd-B and Cad, and epithelial morphogenesis changes (Beuchle et al. 2001; Fritsch et al. 2003; Gandille et al. 2010; Curt et al. 2013). Interestingly, the results of this study for Sce and Scm clones (Beuchle et al. 2001) was that only Ubx and Abd-B were expressed in the time-window used. Our results agree with these in that we saw Abd-B upregulation, occasional abd-A upregulation but no Caudal. We did not look at Ubx protein expression in disc-proper cells. Abd-B expression was the clearest effect of loss of *Sce* and could induce clear morphological changes on
epithelial cells. Abd-B plays a well characterized role in the formation of posterior spiracles in the embryo, and this also involves invagination of epithelial tissue. In that case a small downstream regulatory network has been established involving the four immediate target genes, cut, split, upd1, and csm, as well as crumb, Gfe64C, and five cadherins (Lovegrove et al. 2006). None of these genes showed significant upregulation in Sce.IR discs, however, suggesting that there may exist other Abd-B targets that affect epithelial plasticity.

The importance of PcG repression of Abd-B has also been seen in the context of tests development and the closure of the tergites. PcG repression of Abd-B in cyst stem cells of the tergites is critical for normal cell fate identity and self-renewal of the stem cells (Zhang, Pan, et al. 2017). Mutation of regulatory elements the Boundary Elements and Polycomb Response Elements can also cause increased and ectopic expression of Abd-B that results in dorsal closure defects in the adult abdominal epithelium (Singh and Mishra 2015).

While Abd-B was always derepressed in cells lacking Sce (i.e., Sce.IR and SceKO mutant cells) abd-A was intermittently and variably expressed. We speculate that this may be a manifestation of the posterior dominance rule, whereby expression of Abd-B expression can repress abd-A (Karch et al. 1990; Macias et al. 1990; Sánchez-Herrero 1991). It is also possible that abd-A is being regulated by the non-coding RNA mir-iab-8 (Gummalla et al. 2012) since it is also located in the region of increased Dam-Pol II binding.

In conclusion, we have demonstrated a new role for PcG repression in maintaining cell competency for a developmental EMT event and shown that silencing of abd-A and Abd-B is crucial in this process. An important question now is what downstream targets of Abd-B and abd-A, and perhaps other TFs like Caudal and Nubbin, are inhibiting the pEMT and are these gene-regulatory interactions conserved in mammals. Based on the effects of EHZ2 and Bmi1 on E-Cadherin, we expected increased expression at the shg locus in the Sce.IR discs, but this was not seen. Mammalian Hox genes control many processes involving epithelial plasticity such as cancer metastasis, wound healing and angiogenesis, but they can have both positive and negative effects (Abate-Shen 2002; Kachgal et al. 2008).

For example, HOXB9 promotes differentiation and mesenchymal-epithelial transition, while inhibiting migration and invasion, in both colon adenocarcinoma (Zhan et al. 2014) and gastric carcinoma cells (Chang et al. 2014) Conversely, other studies have found the same gene is overexpressed in breast carcinoma cells and correlates with high tumor grade (Hayashida et al. 2010) and overexpression in colon cancer cells promotes metastasis and poor prognosis (Huang et al. 2014). Thus, understanding how epithelial plasticity is regulated by Hox genes is likely to be complex and context dependent, but remains an important future goal.

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