Drosophila Ebi mediates Snail-dependent transcriptional repression through HDAC3-induced histone deacetylation

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The *Drosophila* Snail protein is a transcriptional repressor that is necessary for mesoderm formation. Here, we identify the Ebi protein as an essential Snail co-repressor. In *ebi* mutant embryos, Snail target genes are derepressed in the presumptive mesoderm. Ebi and Snail interact both genetically and physically. We identify a Snail domain that is sufficient for Ebi binding, and which functions independently of another Snail co-repressor, *Drosophila* CtBP. This Ebi interaction domain is conserved among all insect Snail-related proteins, is a potent repression domain and is required for Snail function in transgenic embryos. In mammalian cells, the Ebi homologue TBL1 is part of the NCoR/SMRT–HDAC3 (histone deacetylase 3) co-repressor complex. We found that Ebi interacts with *Drosophila* HDAC3, and that HDAC3 knockdown or addition of a HDAC inhibitor impairs Snail-mediated repression in cells. In the early embryo, Ebi is recruited to a Snail target gene in a Snail-dependent manner, which coincides with histone hypoacetylation. Our results demonstrate that Snail requires the combined activities of Ebi and CtBP, and indicate that histone deacetylation is a repression mechanism in early *Drosophila* development.

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Introduction

Transcriptional repressors have key functions in establishing localized and tissue-specific patterns of gene expression during embryo development (reviewed in Rivera-Pomar and Jackle, 1996; Mannervik *et al*, 1999). For example, the Snail family of repressors have central functions in morphogenesis by inducing mesoderm formation in several organisms, including flies and mammals (reviewed in Nieto, 2002). Vertebrate Snail proteins participate in the epithelial–

mesenchymal transition, a central process in embryonic development and metastasis of cancer cells, by repressing E-cadherin expression (reviewed in Huber *et al*, 2005). In *Drosophila*, Snail establishes a boundary between the presumptive mesoderm and the neurogenic ectoderm through downregulation of neuroectoderm-specific genes in the mesoderm (Alberga *et al*, 1991; Kosman *et al*, 1991). The Snail C terminus contains five zinc-fingers that are responsible for DNA binding, whereas the N terminus harbours repressor activity. In the early embryo, expression of Snail is turned on by high levels of Dorsal protein in ventral nuclei, which is critical for establishing a mesodermal cell fate as well as for mesoderm invagination (reviewed in Rusch and Levine, 1996). Snail target genes include transcriptional regulators, cell adhesion molecules and modifiers of signal transduction pathways (reviewed in Hemavathy *et al*, 2000; Barrallo-Gimeno and Nieto, 2005; De Craene *et al*, 2005; Peinado *et al*, 2007). Many of these genes are completely derepressed into ventral regions in *snail*^{−/−} mutants. However, it seems that the regulation of these genes requires different functional levels of Snail, as only some of them are affected in *snail* hypomorphs (Hemavathy *et al*, 1997, 2004).

Repressors often need co-repressors for their function. Previous studies have identified several co-repressors, including CtBP, Groucho, Sin3-Rpd3 (HDAC1) and the nuclear receptor co-repressor (NcoR)–silencing mediator for retinoic acid and thyroid hormone receptor (SMRT)–transducin β -like 1 (TBL1)–histone deacetylase 3 (HDAC3) co-repressor complex (reviewed in Mannervik, 2001; Jepsen and Rosenfeld, 2002). CtBP mediates transcriptional repression by Knirps, Krüppel and Snail in the *Drosophila* embryo (Nibu *et al*, 1998a,b). A short peptide motif, PXDLSXK/R, mediates binding of CtBP to repressor proteins (reviewed in Chinnadurai, 2002). In Snail, two such motifs are necessary for repressor function. The NCoR and/or the closely related SMRT are involved in repression by unliganded thyroid hormone and retinoic acid receptors, as well as several unrelated transcription factors in mammals (reviewed in Jones and Shi, 2003). It forms a stable complex with HDAC3 and TBL1 (Guenther *et al*, 2000; Li *et al*, 2000; Wen *et al*, 2000). In mammalian cells, the complex also contains TBLR1, which is closely related to TBL1. It was shown that TBL1 and TBLR1 can directly interact with histones H2B and H4, and that repression by the complex correlates with histone binding (Yoon *et al*, 2003, 2005). The *Drosophila* homologue of TBL1, Ebi, was first identified as a downstream regulator of epidermal growth factor receptor (EGFR) signalling during eye development (Dong *et al*, 1999). It contains six WD repeats and a divergent F-box domain. The Ebi F-box domain promotes association with the Seven in absentia (Sina) E3 ligase, which induces ubiquitination of the transcription factor Tramtrack (Boulton *et al*, 2000). SMRTER, the *Drosophila* orthologue of mammalian SMRT, was identified

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through its interaction with the ecdyson receptor (Tsai *et al*, 1999). SMRTER associates with Ebi, which mediates crosstalk between EGFR and Notch signalling by regulating Delta expression in the eye (Tsuda *et al*, 2002). Recently, it was reported that the Ebi-SMRTER complex directly regulates expression of the *charlatan* gene in *Drosophila* eye development together with the transcription factor Suppressor of Hairless (Tsuda *et al*, 2006). However, the presence and function of HDAC3 in the complex remain unexplored in *Drosophila*.

In this study, we discovered a new function of Ebi as a Snail co-repressor. Ebi directly interacts with the Snail repressor and regulates Snail repressor function in the early embryo and in tissue culture cells. We identified a minimal Ebi interaction motif within the Snail protein that is evolutionarily conserved among Snail-related proteins in insects. This motif constitutes a repression domain and is essential for Snail function *in vivo*. Furthermore, we provide evidence that Ebi-dependent repression relies on histone deacetylation mediated by HDAC3 rather than protein ubiquitination. Although histone deacetylation is known to participate in transcriptional repression in yeast and mammalian cells, these are the first results to imply histone deacetylation as a repression mechanism in early *Drosophila* development.

Results

Ebi specifically affects Snail repressor function in early Drosophila embryos

Dong *et al* (1999) previously reported that Ebi is involved in the EGFR pathway, and that *ebi* mutant embryos have a 'tail-up' phenotype. In our studies, we analysed phenotypes of the *ebi* mutant allele, *ebi*^{k16213} (also named *ebi*^P), which has a P-element insertion in the 5' untranslated region. By using FRT/FLP-induced mitotic recombination, embryos lacking the maternal contribution of *ebi* were generated (compare Figure 1E with D). The cuticle phenotype is similar to that previously described (Figure 1B; Dong *et al*, 1999). The tightly curled morphology indicates a failure in germ-band retraction, implying a defect in dorsal-ventral (D/V) patterning. This prompted us to examine expression of genes involved in D/V axis formation. The *rhomboid* (*rho*) gene is expressed in two lateral stripes that help to specify the neurogenic ectoderm, and is restricted from the ventral mesoderm region by the Snail repressor in wild-type (wt) pre-cellular embryos (Figure 2A). However, in *ebi* germline clone mutant embryos, *rho* is derepressed in ventral cells (Figure 2B), indicating a disruption in Snail function. To test if expression of other Snail targets is also affected, we analysed the expression pattern of the following genes: *short-gastrulation* (*sog*), *single-minded* (*sim*) and *brinker* (*brk*). As shown in Figure 2, both *sog* and *sim* exhibit an expanded expression pattern (compare Figure 2D and F with C and E). However, *brk* is normally expressed (data not shown). Importantly, the Snail protein is present at normal levels in *ebi* mutants (compare Figure 2G and H). The U-shaped phenotype of *ebi* mutants resembles that of *snail*^{-/-} mutant embryos (Figure 1B and C), indicating that these two factors may function together during dorsoventral patterning. Taken together, these results suggest that the function of Snail is specifically but partially affected in *ebi* germline clone embryos.

Figure 1 Similar developmental defect in embryos derived from *ebi*^{k16213} germline clones and *sna*¹⁸ embryos. Cuticle preparations of newly hatched wild type (A), *ebi*^{k16213} germline clone mutant (B) and *sna*¹⁸ zygotic mutant (C) embryos. The 'U-shape' phenotype is shared between *ebi* and *sna* mutants, suggesting a common biological function. *ebi* mRNA is maternally distributed in the whole embryo in wt (D), but is undetectable in *ebi*^{k16213} germline clone embryos (E) hybridized with a digoxigenin-labelled antisense *ebi* probe. All embryos shown in this study are oriented with anterior to the left.

To determine whether the effect on Snail target genes is at the transcriptional level, we introduced a *lacZ* reporter transgene driven by a modified *rho* neuroectoderm enhancer (NEE). This enhancer lacks the natural Snail repressor sites, but contains two synthetic Snail-binding sites (Gray and Levine 1996). Reporter gene expression is repressed by endogenous Snail protein in the presumptive mesoderm of wt embryos (Figure 2I). When crossed into *ebi* germline clone embryos, *lacZ* expression expands into the ventral mesoderm region, mimicking the derepression phenotype of endogenous *rho* (Figure 2J). However, a *lacZ* reporter driven by a similar NEE that replaces the Snail sites with synthetic Krüppel (Kr)-binding sites shows an identical expression pattern in wt and *ebi* mutant embryos (Figure 2K and L). This indicates that the effect of Ebi is specific to Snail function, and does not affect the activity of other proteins binding the *rho* NEE enhancer. Furthermore, we found no or very minor effects on expression of genes that pattern the anterior-posterior axis in *ebi* mutant embryos (not shown). Hence, we conclude that Ebi specifically regulates Snail-mediated repression on both endogenous targets and transgenic reporter genes.

ebi genetically interacts with snail

The single cell-wide pattern of *sim* expression defines the mesectoderm and is regulated by Snail and Notch signalling at the blastoderm stage (Morel *et al*, 2003). In cellularizing *ebi* mutant embryos, *sim* expression is normal, whereas in stage 8–9 embryos, when the mesoderm has invaginated and *sim* should be expressed only in the ventral midline (Figure 2E), *sim* is expanded into the ventral mesoderm territory in *ebi* mutants with 14% penetrance (Figure 2F and Table I). To investigate if this phenotype is related to

Figure 2 Snail target genes are expanded into the presumptive mesoderm in *ebi* mutant embryos. Ventral or ventro-lateral views of the embryos are shown. Wild-type (wt) (A, C, E, G) and *ebi* germline clone embryos (B, D, F, H) were hybridized with digoxigenin-labelled RNA probes or a Snail antibody. *Rhomboid* (*rho*) is expressed in two lateral stripes in pre-cellular stage wt embryos (A), but is derepressed into the ventral region in *ebi* mutants (B). *Short gastrulation* (*sog*) expression is similarly altered in the *ebi* mutant (D) compared to wt (C). Embryos at stage 9 have invaginated the mesoderm, and *single-minded* (*sim*) is only expressed along the ventral midline in wt (E), but is expanded to the mesoderm in *ebi* mutant embryos (F). Snail protein expression in *ebi* mutant cellularized embryos (H) is comparable to wt embryos (G). (I–L) Snail but not *Krüppel* (*Kr*) is unable to repress a modified *rho* neuroectoderm enhancer (NEE)-*lacZ* reporter gene in *ebi* mutant embryos. Two different *lacZ* reporter genes were introduced into wt embryos (I, K) or embryos derived from *ebi* germline clones (J, L) and stained with a *lacZ* RNA antisense probe. Ventro-lateral views of cellularized embryos are shown. (I, J) A *lacZ* reporter driven by a *rho* NEE containing synthetic Snail-binding sites. *lacZ* expression is repressed by endogenous Snail in the mesoderm in wt (I) but is derepressed in *ebi* mutant embryos (J). A *lacZ* reporter driven by a similar NEE that contains synthetic *Kr*-binding sites can be repressed by *Kr* in the central domain of the embryo in both wt (K) and *ebi* mutants (L). Schematic drawings of the reporter genes are shown underneath the embryo images.

impaired Snail function, we reduced the *sna* dosage by crossing females with *ebi* germline clones to *sna*¹⁸ heterozygous males. This way, 50% of the resulting *ebi* germline

clone embryos will contain half the amount of Snail. This increased the penetrance of *sim* derepression from 14 to 34% (Table I). By contrast, when *sna*¹⁸ heterozygous males were crossed with wt females *sim* derepression was observed in less than 1% of the embryos. As expected, approximately one-quarter (25.9%) of the embryos collected from the *sna*¹⁸ stock showed this phenotype (Table I). This genetic interaction provides further evidence that Ebi and Snail participate in the same biological pathway.

Ebi physically associates with Snail

It was shown previously that CtBP is required for Snail-mediated repression and that the proteins directly associate through two PXDLSXXK/R motifs, which are both located in the N-terminal part of the Snail protein (Nibu *et al*, 1998a). To investigate whether Ebi is also able to directly interact with Snail, we performed glutathione S-transferase (GST) pull-down assays. As shown in Figures 3 and 5, full-length GST-Snail protein purified from bacteria pulls down *in vitro*-translated Ebi protein, demonstrating that these two proteins physically associate. We identified a minimal interaction domain in Snail that is required for Ebi binding by constructing a series of GST-Snail truncations (Figure 3A and B). As shown in Figure 3B, the truncated proteins, Sna 1–245, Sna 1–178, Sna 1–102 and Sna 1–50, can all interact with both Ebi and CtBP. By contrast, Sna 245–390 cannot interact with either protein. More interestingly, Sna 1–40, which lacks both CtBP interaction motifs, still binds to Ebi but not to CtBP. We further narrowed down the Ebi interaction domain to amino acids 4–21. Moreover, residues 8–14 (CPLKKRP) are essential for the association as deletion of this motif strongly reduced the interaction. As shown previously, this motif is identical in the Snail-related proteins Escargot and Worniu (Hemavathy *et al*, 2004). Alignment of insect Snail homologues demonstrated that the minimal Ebi interaction domain (aa 4–21) is highly conserved, and that an extended YXXCPLKKRP motif is identical among insect Snail proteins (Figure 3C). On the basis of these results, we propose that Sna 4–21 is a conserved Ebi interaction domain. As Ebi and CtBP interact with different parts of Snail, we tested whether they can bind to Snail simultaneously. We found that *in vitro*-translated Snail binds to GST-CtBP, whereas *in vitro*-translated Ebi fails to interact with GST-CtBP (Figure 3D). However, in the presence of *in vitro*-translated Snail, GST-CtBP is able to precipitate Ebi (Figure 3D, arrow), demonstrating that Ebi and CtBP can interact with Snail at the same time.

To determine which part of Ebi that binds to Snail, we made GST-Ebi fusion proteins. As shown in Figure 3E, the C-terminal WD repeat domain of Ebi is able to interact with Snail, whereas the N terminus cannot.

The Ebi interaction domain is a potent transcriptional repressor when tethered to DNA

We have shown that Snail requires Ebi function *in vivo*, and that Snail 1–40 directly associates with Ebi *in vitro*. Then we tested whether Sna 1–40 is able to repress transcription when tethered to a promoter. We fused different parts of Snail to the DNA-binding domain of the tetracycline repressor (TetR-DBD) and transiently expressed the fusion constructs in *Drosophila* S2 cells. We co-transfected a luciferase reporter construct driven by the *actin* 5C enhancer containing Tet operators (Ryu and Arnosti, 2003). We compared luciferase activity of

Table 1 Genetic interaction between *ebi* and *snail* mutants

Crosses	Normal <i>sim</i> pattern (n)	Derepressed <i>sim</i> pattern (n)	Total number of embryos (n)	Percent derepression (%)
<i>ebi</i> GLC × wt	103	17	120	14.1
<i>ebi</i> GLC × <i>snail</i> /CyO	144	76	220	34.5
wt × <i>snail</i> /CyO	517	5	522	1
<i>snail</i> /CyO × <i>snail</i> /CyO	286	100	386	25.9

cells that expressed TetR–Snail fusion proteins with those that expressed the TetR–DBD protein alone. We found that Sna 1–40 is able to repress transcription as efficiently as Sna 1–245, and that Sna 4–21 has repression activity as well, although less potent than Sna 1–40 (Figure 4A). Removing the Ebi interaction motif (amino acids 5–25) prevents repression by Sna 1–40, but not Sna 1–245 (Figure 4A). These results indicate that either one of the Ebi (1–40) or CtBP (1–245Δ5–25) interaction motifs in Snail are sufficient for repression in tissue culture cells.

We then extended this finding to *Drosophila* embryos with a transgenic assay. In this case, Sna 1–245 and Sna 1–40 were fused to the Gal4 DBD and placed under the control of the *Kr* CD enhancer, which directs expression in the central domain of the early embryo. A *lacZ* reporter containing a modified *rho* NEE lacking endogenous Snail repressor sites and containing three upstream activating sequence (UAS) sites was used to monitor Gal4–Snail repressor activity (Gray and Levine, 1996). In a wt background, the reporter gene is expressed in the ventral region of the embryo (Figure 4B). However, when crossed into transgenic embryos expressing either the Gal4–Snail 1–40 or the Gal4–Snail 1–245 fusion proteins, expression of the reporter is reduced in the central domain (Figure 4C and D). Repression by Gal4–Sna 1–40 is weaker than that by Sna 1–245, perhaps because Sna 1–245 is expected to recruit both CtBP and Ebi, whereas Sna 1–40 can only recruit Ebi.

In summary, when tethered to DNA the Ebi interaction domain, Snail 1–40, is sufficient for repression of transcription both in cells and in embryos.

The Ebi interaction motif is essential for repression by Snail

We have demonstrated that Ebi is an important component of Snail-mediated repression. Next we wanted to assess the significance of the Ebi interaction motif in the Snail repressor *in vivo*. The *Kr* CD enhancer was used to mis-express full-length Snail in transgenic embryos (Figure 5A). In addition to endogenous expression in the ventral region, *snail* is ectopically expressed in the central domain of these embryos. The presence of Snail in the neuroectoderm results in repression of the two lateral *rho* stripes (Figure 5C, arrow). As Snail is a short-range repressor, binding to the *rho* NEE does not interfere with expression of the dorsal, Dpp-dependent *rho* pattern (Figure 5C). By contrast, when we mis-express Snail with a deletion of amino acids 5–25, no repression of *rho* expression is observed although it is expressed at levels comparable to the wt transgene (Figure 5B and D, and Supplementary Figure S1). The failure of Snail Δ5–25 to repress transcription is not due to a defect in nuclear localization, as the mutant protein is localized to the nucleus in S2 cells and embryos (Supplementary Figure S1). Rather, as deletion of amino acids 5–25 disrupts the association with

Ebi but not CtBP in a GST pull-down assay (Figure 5E), we suggest that Ebi binding is required for full Snail repressor activity. In summary, deletion of Snail amino acids 5–25 mimics the *ebi* mutant phenotype where Snail function is impaired, and demonstrates the importance of this motif *in vivo*.

Ebi-dependent Snail repressor function requires HDAC activity of HDAC3

Tsuda *et al* (2002) previously showed that Ebi associates with the SMRT orthologue SMRTER in S2 cells. We wanted to determine whether Ebi also associates with *Drosophila* HDAC3. Protein extracts prepared from *Drosophila* embryos were immunoprecipitated with HDAC3 or control antibodies, followed by a western blot with anti-Ebi antibody. We could detect Ebi in HDAC3 as well as in Ebi immunoprecipitates, but not in those of HDAC1 or the negative controls (Figure 6A). Reprobing the membrane with an HDAC3 antibody showed that HDAC3 can be co-precipitated with Ebi as well (Figure 6A, second panel). As a further control, we performed immunoprecipitations from *Drosophila* S2 cells and knocked down HDAC3 or Ebi levels by addition of double-stranded RNA (dsRNA; Figure 6A). Immunoprecipitation from dsRNA-treated cells reduced the intensity of the Ebi and HDAC3 bands, demonstrating that co-immunoprecipitation depends on the presence of HDAC3 and Ebi. Hence, we conclude that endogenous Ebi and HDAC3 associate in S2 cells and early embryos.

Next we used cell culture assays to assess the requirement of HDAC3 in Snail-mediated repression. S2 cells were stably transfected with either TetR–Sna 1–245 or TetR–Sna 1–40 together with the TetO-luciferase reporter construct. A third stable cell line carrying the luciferase reporter alone was used as a control. These cells were treated with dsRNA synthesized from either *ebi*, *HDAC3*, *HDAC1* or *CtBP* sequences. Knockdown of the corresponding gene product was assayed by western blot (Figure 6B). As shown in Figure 6C, both Ebi and HDAC3 knockdown partially relieve Sna 1–40-mediated repression, whereas HDAC1 or CtBP knockdown has no effect. However, in the Sna 1–245 cell line, Ebi, HDAC3, as well as CtBP knockdown weakly derepressed reporter gene expression. Treatment with Ebi + CtBP dsRNA led to further derepression. Control cells expressing the luciferase reporter alone were not significantly affected by any dsRNA treatment. Our results suggest that repression by Sna 1–40 depends on both Ebi and HDAC3.

We also treated the cell lines with the HDAC inhibitor trichostatin A (TSA) to assess the requirement of histone deacetylation for Snail-mediated repression. The addition of TSA greatly attenuates the repression activity of Sna 1–40, but has no effect on the control cell line, on Sna 1–245, nor on Sna 1–245Δ5–25 (Figure 6D). This observation is consistent with previous data that CtBP-dependent repression is

Figure 3 Dissection of an Ebi interaction domain in the Snail protein. (A) GST pull-down assay. A series of GST-Snail fusion proteins showed different binding abilities to *in vitro*-translated Ebi or CtBP. GST alone served as a negative control. Notably, Sna 1-40 and Sna 4-21 interact only with Ebi. (B) A schematic drawing summarizing the binding activities of all tested Sna fusions to Ebi or CtBP. In the right panel, '+' or '-' symbol indicates strong binding or weak binding/no binding, respectively. (C) Alignment of the N termini of insect Snail proteins with Clustal W (DNASTAR Lasergene). Amino acids 5 and 8-14 in *Drosophila melanogaster* Snail are identical among all insect Snail-related sequences. Flies (*D.m.*, *Drosophila melanogaster*, *D.p.*, *Drosophila pseudoobscura*, *D.v.*, *Drosophila virilis*, *D.g.*, *Drosophila grimshawi*), mosquito (*A.g.*, *Anopheles gambiae*), silkworm (*B.m.*, *Bombyx mori*), honeybee (*A.m.*, *Apis mellifera*) and flour beetle (*T.c.*, *Tribolium castaneum*) were compared. (D) Snail is able to interact with Ebi and CtBP simultaneously. Ebi and Snail were *in vitro*-translated and incubated with GST fusion proteins separately or together. GST-CtBP interacts strongly with Snail (lane 6), but not with Ebi alone (lane 7). However, in the presence of Snail, GST-CtBP can also bind to Ebi (lane 5, arrow), suggesting that Snail can bridge the interaction between CtBP and Ebi by binding both proteins at the same time. (E) GST-Ebi fusion proteins binding to *in vitro*-translated Snail. Full-length Ebi and the Ebi C terminus bind to Snail, but the Ebi N terminus does not interact.

Figure 4 The Ebi interaction domain in Snail is a potent repression domain. (A) Snail amino acids 4–21, 1–40, 1–40Δ5–25, 1–245 or 1–245Δ5–25 were fused to the tetracycline repressor (TetR) DNA-binding domain. These plasmids were co-transfected into S2 cells with a *luciferase* reporter gene driven by the *actin* 5C enhancer containing Tet operators, as well as an *actin* 5C-driven *lacZ* gene to control for transfection efficiency. Luciferase activity (normalized for β-galactosidase activity) of unfused TetR is set to 1, and activity of TetR–Snail fusion proteins was plotted relative to the TetR. The value in the plot is the average from at least three independent experiments and error bars indicate the standard deviation. A schematic drawing of the reporter plasmid is depicted below the histogram. (B–D) Lateral views of transgenic embryos expressing *lacZ* under the control of a modified *rho* NEE into which Gal4 upstream activating sequences (UASs) have been inserted. *LacZ* is expressed in ventral cells in embryos containing only the reporter gene (B). Embryos that additionally express a Gal4 DNA-binding domain–Snail 1–40 (C) or Gal4 DNA-binding domain–Snail 1–245 (D) transgene under control of the *Kr* CD enhancer are shown.

resistant to TSA treatment in cell culture (Ryu and Arnosti, 2003). We conclude that Ebi cooperates with HDAC3 to regulate Snail 1–40 repressor activity in S2 cells, and that histone deacetylation is part of this repression mechanism.

Histone hypoacetylation and Ebi binding to a Snail target enhancer in early embryos

To investigate if Ebi is recruited to a Snail-regulated gene in the early embryo, and if this results in histone deacetylation, we performed chromatin immunoprecipitation (ChIP) experiments. Extracts were prepared from wt embryos, as well as from embryos lacking nuclear Dorsal activator and Snail repressor (*gastrulation defective*, *gd*), embryos containing intermediate levels of nuclear Dorsal but lacking Snail in all cells (*Toll^{rm9/rm10}*), and embryos with high levels of nuclear Dorsal and consequently also containing the Snail repressor along the entire D/V axis (*Toll^{10b}*). In these embryos, the *rho* gene is either expressed in two lateral stripes (wt), is absent because the lack of the Dorsal activator (*gd*), is expressed throughout the D/V axis (*Toll^{rm9/rm10}*), or is absent due to the presence of Snail along the D/V axis (*Toll^{10b}*) (Figure 7A). Antibodies recognizing Ebi, histone 3 (H3), acetylated H3 (Ac-H3) or control IgG were used in ChIP and precipitation of the endogenous *rho* NEE enhancer was assayed by PCR (Figure 7B). The results show that Ebi binds to the *rho* NEE in wt embryos, but not in embryos that lack Snail (*gd* and *Toll^{rm9/rm10}*). In *Toll^{10b}* embryos, which contain

Snail in almost all cells, strong binding of Ebi to the *rho* NEE is observed (Figure 7B). However, Ebi does not bind to DNA upstream of the *rho* NEE, nor to a modified *rho* NEE that lacks Snail-binding sites in *Toll^{10b}* embryos (data not shown). Taken together, these results demonstrate that binding of Ebi to the *rho* NEE *in vivo* is Snail dependent.

We then determined the histone acetylation status of the *rho* NEE enhancer in these embryos. In *gd* embryos that lack the Dorsal activator, low levels of H3 acetylation were observed (Figure 7B). In wt embryos, more Ac-H3 is found on the *rho* NEE, and high levels of H3 acetylation is found in *Toll^{rm9/rm10}* embryos that contain the Dorsal activator in all cells and express *rho* along the entire D/V axis (Figure 7B). Although *Toll^{10b}* embryos contain high levels of Dorsal in all cells, binding of Snail and Ebi to the *rho* NEE results in H3 hypoacetylation (Figure 7B). Importantly, the amount of total H3 on the *rho* NEE was not changed in the different genotypes (Figure 7B). We conclude that the *rho* NEE enhancer is hyperacetylated in cells that contain the Dorsal activator, but hypoacetylated in cells containing both Dorsal and Snail. This indicates that Snail recruits a HDAC to the *rho* NEE in early embryos.

Discussion

The *Drosophila* embryo is a particularly useful system to study transcriptional co-regulators in development.

Figure 5 The Ebi interaction motif is essential for Snail-mediated repression. Cellularizing embryos were hybridized with digoxigenin-labelled *snail* (*sna*) (A, B) or *rho* (C, D) antisense RNA probes and oriented with anterior to the left and dorsal up (A, B) or shown from a ventro-lateral view (C, D). Snail is mis-expressed with the *Kr* CD enhancer in transgenic embryos that contain either full-length Snail coding region (A, C) or Snail with a deletion of amino acids 5–25 (B, D). *snail* expression is observed both in the ventral region (the endogenous pattern) and in the *Kr* domain. The expression level of Snail is similar in these transgenic strains. (C) *rho* expression pattern in the transgenic strain shown in (A). The ectopic *snail* stripe creates a gap (see arrow) in the *rho* lateral stripes, but not in the pattern present in the dorsal-most region of the embryo that is regulated independently of Snail. (D) *rho* expression pattern in the transgenic strain shown in (B). Mutant Snail does not alter the *rho* pattern, suggesting that amino acids 5–25 are essential for Snail-mediated repression. (E) GST pull-down assay showing that deletion of amino acids 5–25 abolishes the binding of Ebi to full-length Snail, whereas CtBP binding is unaffected.

Transcriptional hierarchies and gene regulatory networks have been extensively characterized in early embryos, which makes it possible to assign highly specific functions to ubiquitous co-regulators. We have presented strong evidence that Ebi functions as a co-repressor for the *Drosophila* Snail protein. We identified an evolutionarily conserved interaction motif in Snail that is necessary for Ebi binding and for the repression activity of Snail. We found that HDAC3 is associated with Ebi and is involved in Snail-mediated repression.

Previous studies have suggested that CtBP mediates transcriptional repression by Snail in the early embryo (Nibu *et al*, 1998a). However, disruption of Snail repressor activity in *ebi* mutant embryos cannot be due to an indirect effect on CtBP, based on several observations. We detected comparable CtBP protein levels in *ebi* mutant and wt embryos using a CtBP-specific antibody (data not shown). In addition, the *ebi* mutation did not affect the function of the *Kr* repressor, which also requires CtBP, on the NEE-lacZ reporter gene (Figure 2). Furthermore, the segment polarity gene *engrailed* that is indirectly regulated by CtBP-dependent repressors such as *Kr* and *Knirps* is normally expressed in *ebi* mutant embryos (data not shown), indicating that CtBP activity is not disrupted by the *ebi* mutation.

Moreover, the Ebi interaction motif (Sna 1–40) that does not bind to CtBP *in vitro* still has repression activity in S2 cells

and in transgenic embryos, suggesting that Ebi functions directly as a cofactor for Snail through a physical association. Removing this motif from the Snail protein abolishes its repression activity (Figure 5). This result is consistent with data from Hemavathy *et al* (2004) that used *snail* transgenes to rescue *snail*^{−/−} embryos. They found that Snail lacking amino acids 6–25 fails to repress *sim* expression in *snail*^{−/−} embryos. It was proposed that this part of Snail might be involved in nuclear localization of the protein. However, we demonstrate that the mutant protein is normally localized to nuclei in S2 cells and embryos (Supplementary Figure S1). This suggests that mutant Snail loses the ability to repress because it is unable to interact with Ebi. Taken together, we conclude that Ebi specifically regulates Snail-mediated repression through a new, CtBP-independent pathway.

Our study suggests that Snail mediates repression through two pathways, a CtBP-dependent and an Ebi-dependent pathway. Several repression activities in one protein could contribute qualitatively or quantitatively to repression. In some cases, different target genes are repressed through distinct co-repressors (Hasson *et al*, 2001; Winter and Campbell, 2004). By contrast, the CtBP-dependent and -independent repression activities in *Knirps* and *Hairless* exerts an effect quantitatively (Struffi *et al*, 2004; Nagel *et al*, 2005). Our experiments show that in the presumptive mesoderm, repression of several Snail target genes requires both CtBP and Ebi (Figure 2; Nibu *et al*, 1998a), that Snail recruits both CtBP and Ebi to the same *rho* enhancer (Figure 7; manuscript in preparation) and that CtBP and Ebi can interact simultaneously with Snail (Figure 3). Deletion of either the Ebi or CtBP interaction motifs impairs Snail function in transgenic mis-expression and rescue assays (Figure 5; Nibu *et al*, 1998a; Hemavathy *et al*, 2004). Furthermore, derepression of Snail target genes is not complete in either *ebi* or *CtBP* mutant embryos (Figure 2; Nibu *et al*, 1998a). In *ebi* mutant embryos, and *snail* mutant embryos rescued with Snail lacking amino acids 6–25, gene repression is impaired but ventral furrow formation and mesoderm invagination normal, which is similar to the situation in the *snail* hypomorphic allele V2 (data not shown; Hemavathy *et al*, 1997, 2004). Taken together, these results strongly suggest that Snail requires both Ebi and CtBP for full repressor activity.

By what mechanism does Ebi contribute to repression? Previous studies have shown that Ebi and its mammalian homologue TBL1 can function through two different complexes, the NCoR-SMRT-HDAC3 complex and a Sina E3 ubiquitin ligase complex. We tested if ubiquitin-dependent protein degradation is involved in Snail-mediated repression by adding a proteasome inhibitor to Tet-Sna-expressing cells. No change in luciferase activity was observed in response to this drug (Supplementary Figure S2). This indicates that proteasomal degradation is not necessary for Snail repressor activity, which is also supported by the lack of *rho* derepression in *sina* germline clone mutant embryos (data not shown).

It is well established that histone deacetylation correlates with transcription repression. Local deacetylation of histones by HDAC3 results in repression of gene transcription (Ishizuka and Lazar, 2003). HDAC3 was purified as a core subunit of the NCoR-TBL1 (Ebi) complex in mammalian cells, suggesting that histone deacetylation is functionally linked to the activity of this complex. Although the

Figure 6 Ebi associates with HDAC3 and both proteins are required for Snail-mediated repression in S2 cells. **(A)** Ebi can be co-immunoprecipitated with endogenous HDAC3 from *Drosophila* embryos and S2 cells. Panels 1 and 2: normal rabbit serum (NRS), guinea-pig HDAC3, rabbit HDAC1, mouse Ebi or mouse GFP antibody was used in immunoprecipitation, and a mouse Ebi antibody used in the western blot to detect the presence of Ebi. Lane 1 is 1% of the input embryo extract. The same membrane was reprobed with a rat HDAC3 antibody. Panels 3 and 4: lanes 1–3 are the input extract (2%) from normal S2 cells and cells treated with *HDAC3* or *ebi* dsRNA, respectively. The guinea-pig HDAC3 antibody was used to immunoprecipitate HDAC3 and its associated proteins, and Ebi was detected by western blot. The blot was reprobed with a rat HDAC3 antiserum. Panels 5 and 6: the Ebi monoclonal was used in immunoprecipitation, and rat HDAC3 antiserum was used in western blot. The membrane was reprobed with the Ebi antibody. The input panels are shown at a longer exposure than the IP panels. **(B)** Efficiency of RNAi in the Sna 1–40/luc cell line was determined by western blot. Panel 1: Ebi protein levels are reduced by *ebi* RNAi treatment. Panel 2: HDAC3 protein levels are reduced by *HDAC3* RNAi treatment, and to some extent by *ebi* or *HDAC1* RNAi treatment. Panel 3: HDAC1 protein levels are reduced by *HDAC1* RNAi, and to a lesser extent by *HDAC3* RNAi. Panel 4: *CtBP* RNAi knocked down both CtBP protein isoforms as detected by a CtBP antibody. Panel 5: tubulin was used as a loading control. **(C)** RNAi assay performed in stable cell lines containing luciferase reporter alone (luc), luciferase + TetR–Sna 1–40 (Sna40/luc) or luciferase + TetR–Sna 1–245 (Sna245/luc). The repression activity of Snail 1–40 is attenuated by knock down of either Ebi or HDAC3, but not affected by knock down of CtBP or HDAC1. The activity of Sna 1–245 is affected by knockdown of Ebi, HDAC3 or CtBP. Ebi and CtBP RNAi treatment together gives a stronger effect. The luciferase activity in the control cell line is largely unaffected by the RNAi treatments. Error bars indicate the standard deviation. **(D)** The HDAC inhibitor TSA relieves Sna 1–40-mediated repression, suggesting that histone deacetylation is involved in Ebi-dependent repression. The cell lines expressing Sna 1–245 or Sna 1–245Δ5–25 are insensitive to TSA treatment, indicating that repression occurs by a CtBP-dependent mechanism.

composition of a similar complex in *Drosophila* has not been determined, a physical association and functional connection between Ebi and SMRTER have been reported (Tsuda *et al*, 2002). In this study, we found that HDAC3 and Ebi associate and that both are required for Snail repression domain

function in S2 cells, as determined by RNAi and inhibition of HDAC activity. The observation that Sna 1–245 and 1–245Δ5–25 are resistant to TSA treatment implies that these proteins can repress by an Ebi-independent mechanism. Taken together, our results suggest that Ebi-dependent

Figure 7 Binding of Ebi to the *rho* NEE enhancer in the embryo is Snail-dependent and coincides with histone hypoacetylation. (A) *In situ* hybridization showing expression of *snail* and *rho* in wt embryos, in *gd⁷* embryos where no Dorsal activator protein translocates to nuclei, in *Toll^{rm9/rm10}* embryos that contain intermediate levels of nuclear Dorsal in all cells and in *Toll^{10b}* embryos where high levels of nuclear Dorsal activator are present in all cells. (B) Chromatin immunoprecipitation of the *rho* NEE enhancer using embryo extracts derived from different genetic backgrounds. Whole cell extract (WCE) represents the input DNA in each extract. In the upper panel, an Ebi-specific antibody was used to show that Ebi binding to the *rho* NEE occurs in wt embryos and in *Toll⁷* embryos that contain Snail, but not in embryos that lack Snail. The bottom panel uses an antibody specific to acetylated lysine 14 in histone 3 (H3K14ac). H3 acetylation of the *rho* NEE enhancer is observed in wt embryos and in *Toll^{rm9/rm10}* embryos that contain the Dorsal activator but lack the Snail repressor. In embryos with both Dorsal and Snail present throughout the dorsal-ventral axis (*Toll^{10b}*), H3K14 is hypoacetylated, indicating that Snail recruits a histone deacetylase. The total amount of histone H3 on the *rho* NEE does not change in the different genotypes (middle panel). A quantification of the DNA present in the immunoprecipitates is shown at the bottom.

repression requires histone deacetylation, whereas CtBP-dependent repression does not in this assay.

In contrast to the situation in embryos where the Ebi interaction domain and the CtBP interaction domain in Snail cooperate, in the cell culture assay these domains (1–40 and 1–245Δ5–25) can repress transcription independently of one another (Figures 4A and 6D). It is surprising, therefore, that Ebi or HDAC3 RNAi weakly relieved repression by Sna 1–245 containing both repression domains, and produce stronger effects together with CtBP RNAi (Figure 6C). This indicates that repression in the cell culture assay may involve further components.

The role of SMRTER in this process remains to be determined. Unfortunately, SMRTER knock down by RNAi results in cell cycle arrest and is cell lethal (Pile *et al*, 2002; data not shown), precluding an investigation of its function in Snail-mediated repression. However, it has been shown that mammalian NCoR and SMRT contain a deacetylase-activating domain (DAD) that is essential for catalytic activity of HDAC3 (Guenther *et al*, 2001). The DAD is evolutionarily conserved and present also in SMRTER (Codina *et al*, 2005). Presumably, *Drosophila* HDAC3 also requires SMRTER binding for activation of its enzymatic activity. Moreover, the association between TBL1 and HDAC3 in mammalian

cells is bridged by SMRT (Yoon *et al*, 2003). For these reasons, it is likely that the Ebi-HDAC3 complex also includes SMRTER. Reciprocal BLAST searches also reveal a homologue of the SMRT-NCoR complex core component GPS2 in *Drosophila* (data not shown). It appears that the composition and dependence on histone deacetylation by HDAC3 for SMRT-NCoR complex function has been evolutionarily conserved.

Ever since the discovery that Rpd3 is a HDAC over 10 years ago (Taunton *et al*, 1996), a strong link between histone hypoacetylation and transcriptional repression has been established (reviewed in Yang and Seto, 2003). In *Drosophila*, five HDACs of the class I and II type, and five Sir2-like HDACs are present (Cho *et al*, 2005; Foglietti *et al*, 2006; Kusama *et al*, 2006). However, it is not known whether regulation of histone acetylation contributes to transcriptional control during the rapid nuclear divisions in early *Drosophila* embryogenesis. Although Rpd3 or the Mi-2-Rpd3 complex has been implicated in repression by the Even-skipped, Runt, Knirps, Tramtrack and Hunchback repressor proteins, and as part of Groucho and Atrophin co-repressor complexes (Kehle *et al*, 1998; Chen *et al*, 1999; Mannervik and Levine, 1999; Murawsky *et al*, 2001; Wheeler *et al*, 2002; Struffi and Arnosti, 2005; Wang *et al*, 2006; Yamasaki and Nishida, 2006), a direct role of histone deacetylation in repression has not been established in these instances. A recent report has invoked regulation of transcription elongation in repression by the pair-rule proteins Runt and Fushi tarazu in early embryos (Wang *et al*, 2007). In this case, no change in histone acetylation was observed on the target gene in transcriptionally active cells compared with inactive cells (Wang *et al*, 2007). By contrast, we demonstrate that H3 becomes hypoacetylated at a Snail-regulated enhancer in the presence of Snail (Figure 7), and suggest that histone deacetylation participates in Snail-mediated repression based on our cell culture assay (Figure 6). This is the first evidence that histone deacetylation may be involved in cell-fate specification during *Drosophila* embryo development.

Vertebrate Snail proteins contain a different conserved motif, the SNAG domain in their very N termini (reviewed in Nieto, 2002). The Snail SNAG domain is necessary to recruit a Sin3A-HDAC1/HDAC2 co-repressor complex to the E-cadherin promoter, which is sensitive to the HDAC inhibitor TSA (Peinado *et al*, 2004). This indicates that both vertebrate and insect Snail proteins rely on histone deacetylation for their repressor function, but that they recruit different co-repressor complexes. Whereas vertebrate Snail depends on Sin3A-HDAC1/HDAC2, insect Snail proteins require an Ebi-HDAC3 complex for maximal activity.

Materials and methods

Drosophila stocks, germline clones and genetics

We used a P-element insertion allele of *ebi*, *ebi*^{k16213} that we recombined to a FRT^{40A}-containing chromosome. Precise excision of this P-element has been shown to revert the lethality of the chromosome (Boulton *et al*, 2000). The *snail* loss of function allele *snail*¹⁸ was used in cuticle preparation and genetic interaction experiments. Embryos from *gd*⁷ or *Toll*^{10b} homozygous mothers, or from *Toll*^{rm9}/*Toll*^{rm10} trans-heterozygous females were used in Figure 7. *w*¹¹¹⁸ was used as a wt control. *yw* hs-FLP; *Sco*/CyO and *yw*; FRT^{40A} *ovo*^{D1} were for the FLP-FRT-dominant female sterile technique to create germline clones (Chou and Perrimon, 1996).

FRT *ebi* mutant females were crossed with males of the genotype hs-FLP/Y; FRT *ovo*^{D1}/CyO, and larvae were heatshocked for 3 h at 37°C on days 3, 4 and 5 after egg laying to induce expression of the FLP recombinase. *Cy*⁺ females were mated with *ebi* or *snail* heterozygous males, with wt males or with males expressing various transgenes, and embryos were collected and fixed for *in situ* hybridization.

To test the repressor activities of Snail and Kr proteins in an *ebi* germline clone background, we crossed wt females or females with *ebi* germline clones with males containing modified *rho* NEE enhancers. The NEE-*snail*-lacZ contains synthetic Snail-binding sites but lacks endogenous Snail sites. The NEE-Kr-lacZ contains synthetic Kr sites but lacks Snail sites and is described in Gray and Levine (1996).

To assess the activities of Snail 1-245 and Snail 1-40 fused to the Gal4 DBD, flies containing a modified *rho* NEE-lacZ reporter gene with three UAS sites (Gray and Levine, 1996) were crossed to wt, Kreggy-Snail 1-245 or Kreggy-Snail 1-40 transgenic flies (Supplementary data), embryos were collected and lacZ reporter gene expression was analysed by *in situ* hybridization.

To compare the activities of full-length Snail and SnailΔ5-25, males containing transgenes mis-expressing Snail proteins were crossed with wt females. Expression in a central domain of the embryo was achieved by use of the Kr CD enhancer (Lilja *et al*, 2003). The constructs contain transcriptional stop signals flanked by FRT sites downstream of the Kr enhancer to allow maintenance of transgenic lines. Expression was activated by crossing in a β2-tubulin-FLP transgene (Struhl *et al*, 1993). Male progeny containing both FLP and Snail transgenes was crossed with wt virgins, embryos were collected and processed for *in situ* hybridization.

Cuticle preparations and *in situ* hybridization

ebi germline clone embryos and *snail* mutant embryos were aged, dechorionated in bleach, devitellinized in methanol, transferred to microscopic slides, cleared in lactic acid at 65°C and cuticles were examined using dark-field microscopy (Wieschaus and Nüsslein-Volhard, 1998). RNA *in situ* hybridization using digoxigenin-labelled probes was performed as previously described (Tautz and Pfeifle, 1989; Jiang *et al*, 1991). Immunohistochemistry is described in Supplementary data.

Molecular cloning

Molecular cloning is described in Supplementary data.

GST pull-down assay

GST and GST fusion proteins were expressed in *Escherichia coli* BL21 cells by induction with IPTG for 3 h at room temperature. The proteins were purified on glutathione sepharose beads (GE Healthcare). Snail, Ebi and CtBP cDNAs were *in vitro*-translated with the TNT-coupled reticulocyte lysate system (Promega) using ³⁵S-methionine (GE Healthcare). *In vitro*-translated protein was pre-cleared with glutathione sepharose beads for 30 min; 30 µl was mixed with equivalent amount of GST or GST fusion protein on glutathione sepharose beads for 1 h at room temperature. The beads were washed three times in NTEN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Proteins were eluted from the beads by boiling in SDS-PAGE sample buffer and run on 10% SDS-PAGE, which was exposed to an FLA 3000 phosphorimager (Fujifilm).

Immunoprecipitation

Co-immunoprecipitation was performed by preparing protein extracts from 4 × 10⁶ mock treated, HDAC3 dsRNA-treated or *ebi* dsRNA-treated S2 cells, or from 0–5 h *w*¹¹¹⁸ embryos that were dechorionated and grinded in lysis buffer (10 mM Tris pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 1% NP-40 and protease inhibitor cocktail). Normal rabbit serum (5 µl), 5 µl of affinity-purified guinea-pig dHDAC3 antibody, 5 µl of rabbit dHDAC1 serum (gift from Lori Pile), 20 µl of mouse monoclonal Ebi antibody (KX11 hybridoma supernatant, a gift from Nick Dyson) or 2 µl mouse monoclonal GFP antibody (Sigma) was added to samples for immunoprecipitation. Protein samples were separated on an SDS–10% PAGE gel and transferred to PVDF membrane. The Ebi monoclonal (1:10) or affinity-purified rat dHDAC3 (1:2000) antibodies were detected with HRP-coupled secondary antibodies, and the signals were developed with ECL or ECL Plus reagent (GE Healthcare).

Cell culture and RNAi

Transfection of S2 cells and luciferase assays were performed according to Haecker *et al* (2007) and is described further together with drug and RNAi treatment in Supplementary data.

ChIP

ChIP assays were performed as previously described (Lilja *et al*, 2007) with a few modifications, and is further described in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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