Transcriptional repression by Suppressor of Hairless involves the binding of a Hairless-dCtBP complex in *Drosophila*

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Notch is the receptor for a conserved signaling pathway that regulates numerous cell fate decisions during development [1]. Signal transduction involves the presenilin-dependent intracellular processing of Notch and the nuclear translocation of the intracellular domain of Notch, NICD [2–6]. NICD associates with Suppressor of Hairless [Su(H)], a DNA binding protein, and Mastermind (Mam), a transcriptional coactivator [7–9]. In the absence of Notch signaling, Su(H) acts as a transcriptional repressor [10, 11]. Repression by Su(H) is relieved by the activation of Notch [12–16]. In the *Drosophila* embryo, this transcriptional switch from repression to activation is important for patterning the expression of the *single-minded* (*sim*) gene along the dorsoventral axis [12]. Here, we investigate the mechanisms by which Su(H) inhibits the expression of Notch target genes in *Drosophila*. We show that Hairless, an antagonist of Notch signaling [17–19], is required to repress the transcription of the *sim* gene. Hairless forms a DNA-bound complex with Su(H). Furthermore, it directly binds the *Drosophila* C-terminal Binding Protein (dCtBP), which acts as a transcriptional corepressor. The dCtBP binding motif of Hairless is essential for the function of Hairless in vivo. We propose that Hairless mediates transcriptional repression by Su(H) via the recruitment of dCtBP.

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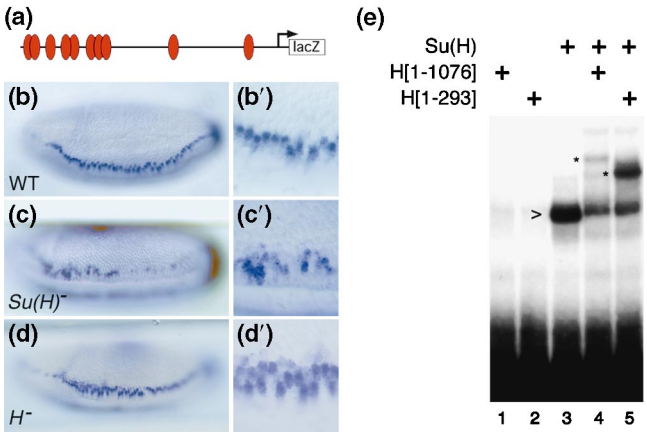
Results and discussion

The *sim* gene is expressed in a single row of cells abutting the mesoderm in the *Drosophila* embryo at the cellular blastoderm stage (Figure 1a–b′). Sim confers to these cells a mesectodermal identity [20]. We have previously shown that Su(H) has a dual function in the regulation of *sim* expression. First, Su(H) directly inhibits the expression of the *sim* gene in the neuroectoderm. In *Su(H)* mutant embryos derived from germ-line clones (GLC), both endogenous *sim* and a sim-lacZ transgene that mimics the expression of *sim* are ectopically expressed in 2–3 rows of neuroectodermal cells (Figure 1c,c′; see [12]). Second, Su(H) upregulates the expression of *sim* in the mesectoderm. Transcriptional activation by Su(H) depends on Notch signaling, but repression by Su(H) is independent of Notch activity [12].

Hairless is a nuclear protein that binds Su(H) and antagonizes Notch activity in numerous cell fate decisions [17, 18, 21, 19]. It is, however, unclear how Hairless inhibits transcriptional activation by Su(H). One hypothesis is that Hairless promotes repression by Su(H). Our previous analysis of the *sim* promoter allowed us to test for the first time whether Hairless is required for Su(H)-dependent repression. The expression of the *sim* gene was analyzed in embryos that lack both the maternal and the zygotic contributions of *Hairless*. In these *Hairless* GLC embryos, sim-lacZ is ectopically expressed in cells located dorsally to the mesectoderm (Figure 1d,d′). This ectopic expression in the neuroectoderm is similar to the one observed in *Su(H)* mutant embryos (Figure 1c,c′; see [12]). Thus, Hairless is required for the repression of *sim* in the same cells that also require Su(H) for repression.

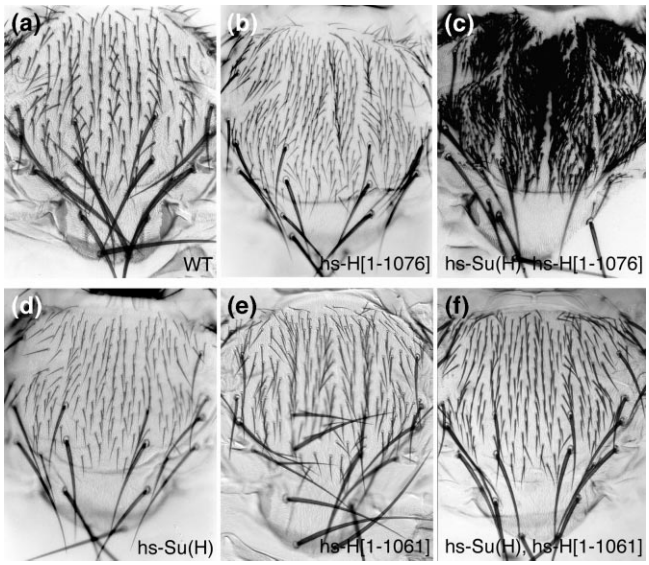
Hairless has previously been shown to bind Su(H) and inhibit transcriptional activation by Su(H) [22]. However, because Su(H)-Hairless complexes did not bind DNA in vitro, Hairless was proposed to inhibit the DNA binding activity of Su(H) [22]. In this model, Hairless inhibits Notch signaling by titrating Su(H) and not by repressing Notch target genes. This model does not explain, however, why the loss of *Hairless* activity leads to the same phenotype as the loss of *Su(H)* activity, i.e., derepression of *sim* in neuroectodermal cells. We therefore envisage that Hairless and Su(H) act together to repress transcription. This implies that Hairless must bind to a DNA bound form of Su(H). We have therefore reexamined whether Hairless and Su(H) can form a DNA bound complex in a gel retardation assay. Hairless and a truncated version of Hairless that binds Su(H) (H[1–293]) are shown to supershift a Su(H)-oligonucleotide complex (Figure 1e). We conclude that Hairless associates with DNA via Su(H).

Figure 1



Hairless represses the expression of the *sim* gene and binds DNA via Su(H). **(a)** Schematic diagram of the sim-lacZ construct. The 2.5 kb of *sim* 5′ regulatory sequences that drive lacZ expression contain ten Su(H) binding sites (in red) [12]. **(b–d′)** Lateral views of (b) a wild-type embryo and of (c) *Su(H)* and (d) *Hairless* (*H*) GLC mutant embryos. The expression of sim-lacZ was analyzed by in situ hybridization. $Su(H)^{del47}$ and H^{E31} mutant GLC embryos were obtained as described previously [12, 33]. Females were mated with sim-lacZ $Su(H)^{del47}$/CyO ftz-lacZ and sim-lacZ H^{2}/TM3 Sb ftz-lacZ males, respectively. Enlarged views are shown in (b′,c′,d′). Panels (b) and (c) are taken from [12]. **(e)** Gel retardation analysis of the Su(H)-Hairless complex. A radiolabeled 43mer oligonucleotide that contains two consensus binding sites for Su(H) (sequence available upon request) binds in vitro-translated Su(H) (lane 3, arrowhead) but neither in vitro-translated H[1–1076] nor H[1–293] (lanes 1 and 2; similar results are seen with oligonucleotides containing a single Su(H) binding site). H[1–1076] supershifts the Su(H)-oligonucleotide complex (lane 4, asterisk). H[1–293], which binds Su(H) [22], also supershifts the Su(H)-DNA complex (lane 5, asterisk). Gel retardation was carried out as previously described [22] except that salmon sperm DNA (0.2 mg/ml) was used as a nonspecific competitor.

Figure 2

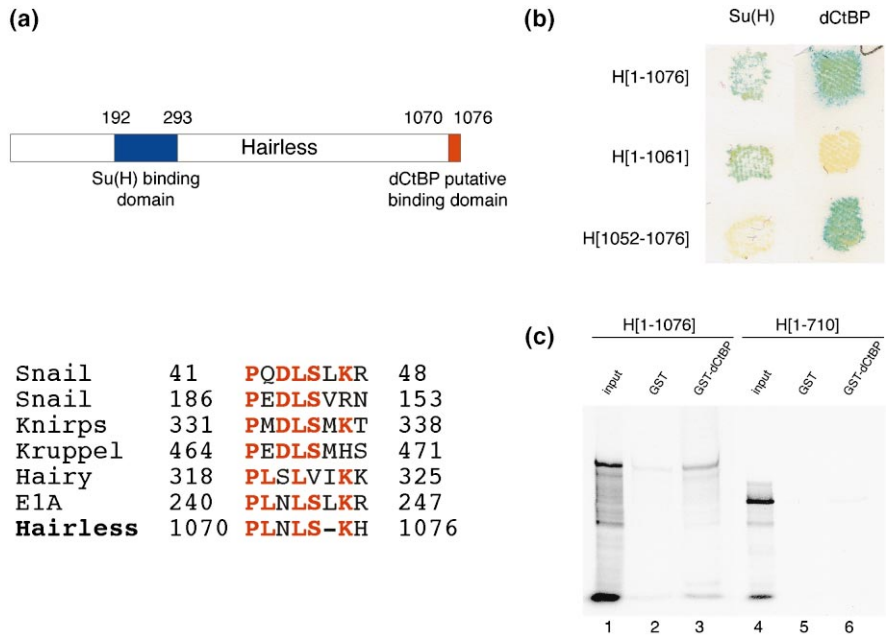


Hairless cooperates with Su(H) to inhibit Notch signaling. Cuticular preparations from **(a)** wild-type, **(b)** hs-H[1–1076]/+, **(c)** hs-Su(H)/hs-H[1–1076], **(d)** hs-Su(H)/+, **(e)** hs-H[1–1061]/+, and **(f)** hs-Su(H)/hs-H[1–1061]. The hs-Su(H)/+, hs-H[1-1076]/+, and hs-Su(H)/hs-H[1–1076] flies were heat-shocked for 30 min at 37°C 6 hr after pupae formation (APF). The hs-H[1–1061]/+ and hs-Su(H)/hs-H[1–1061] flies were heat-shocked 3 times for 30 min at 37°C between 3 and 7 hr APF. Under these heat shock conditions, hs-Su(H)/hs-H[1–1076] pupae do not develop. Su(H) strongly enhances the inhibition of Notch signaling by H[1–1076]. In contrast, Su(H) does not enhance inhibition by H[1–1061]. The hs-Su(H), hs-H[1–1076] and hs-H[1–1061] lines are described in [17, 30, 34].

To test whether Hairless is able to cooperate with Su(H) in vivo, we have used an assay based on the expression of Hairless and Su(H) during pupal development. Lateral inhibition mediated by Notch signaling controls the spacing of bristle sensory organs on the dorsal thorax of the fly (Figure 2a; [23]). Increasing the level of Notch signaling results in the determination of a reduced number of sense organs. Conversely, decreasing the level of Notch signaling leads to an increased density of sense organs. Similarly, overexpression of Su(H) under heat-shock control decreases sense organ density, while overexpression of Hairless has the opposite effect [17, 24]. Control flies in which the expression of either Su(H) or Hairless was induced under mild heat shock conditions (30 min at 37°C) display only a weakly decreased or increased bristle density, respectively (Figure 2b,d). The titration model proposed earlier [22] predicts that when Su(H) and Hairless are simultaneously overexpressed, they should counteract each other's activity to produce an intermediate phenotype. In contrast to this prediction, low-level expression of Hairless and Su(H), under the same mild heat shock conditions, leads to a dramatic increase in sense organ density on the dorsal thorax (Figure 2c). This shows that Su(H) and Hairless strongly synergize to inhibit Notch signaling in this experimental situation. This synergy between Hairless and Su(H) was also seen for the regulation of *sim* expression in the mesectoderm (data not shown), as well as during wing development [25]. These findings suggest that this synergy represents a general feature of the function of these two genes. These observations are not consistent with the titration model described above, but rather they support the hypothesis that Hairless acts in a Su(H)-dependent manner to antagonize Notch signaling activity.

We next investigated the mechanism by which Hairless might regulate transcription. Sequence analysis of Hairless identifies a putative binding site for the *Drosophila* C-terminal Binding Protein (dCtBP; Figure 3a). This site is located at the very C terminus of the Hairless protein. In *Drosophila* and mammals, CtBP is a transcriptional corepressor [26–29]. We therefore tested whether Hairless binds to dCtBP. The full-length Hairless protein, H[1–1076], interacts with dCtBP in a yeast two-hybrid assay (Figure 3b). In contrast, a truncated version of Hairless in which the last 15 amino acids had been deleted, H[1–1061], did not bind to dCtBP. This shows that the Hairless-dCtBP

Figure 3



Hairless binds to dCtBP. **(a)** Schematic representation of the Hairless protein. The position of the Su(H) binding domain [22, 35] and of the putative binding site for dCtBP are indicated in blue and red, respectively. The putative dCtBP interaction motif of Hairless is compared with the dCtBP binding sites of various *Drosophila* proteins and with the CtBP binding site of the viral E1A protein [26–29]. **(b)** β-galactosidase filter assay showing that the H[1–1076] protein and the H[1052–1076] peptide binds to dCtBP. No interaction was detected between H[1–1061] and dCtBP. Su(H) was used as a positive control. The Su(H)-B42, dCtBP-VP16, GAL4-H[1–1076], and GAL4-H[1–1061] constructs are described in [29, 30, 36]. The GAL4-H[1051–1076] construct was obtained by PCR subcloning. **(c)** A GST-dCtBP fusion protein [29] binds the H[1–1076] protein (lanes 1–3) but not the H[1–710] protein (lanes 4–6). Input lanes show 10% of the radiolabeled proteins used for interaction. Interactions were performed as previously described [22] except that 0.75 M NaCl was used in the last wash.

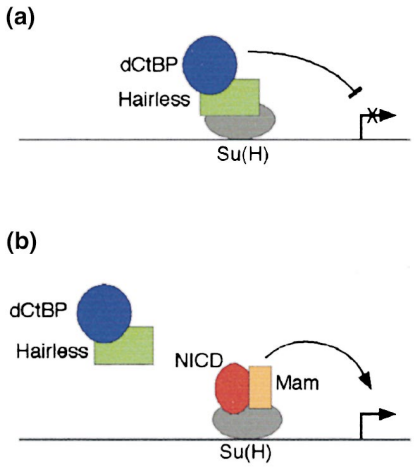
interaction strictly depends on the conserved C-terminal part of Hairless that contains the dCtBP binding site. Furthermore, a small C-terminal peptide, H[1052–1076], is sufficient to bind dCtBP (Figure 3b). Finally, a specific interaction between Hairless and dCtBP is also observed in vitro with a GST pull-down assay. H[1–1076], but not H[1–710] or H[1–1061], is efficiently retained by a GST-dCtBP fusion protein (Figure 3c; data not shown). This in vitro interaction indicates that the Hairless-dCtBP interaction is likely to be direct. We conclude that the conserved C-terminal part of Hairless contains a motif necessary and sufficient to bind dCtBP.

To test the functional significance of this binding site, we have used the in vivo assay described above (Figure 2). The expression of a truncated version of Hairless that does not bind dCtBP, H[1–1061], does not lead to an increased density of sense organs (Figure 2e; see also [30]) and does not rescue the loss of *Hairless* function [30]. Thus, the last 15 amino acids of Hairless are required for the activity of the protein. Interestingly, flies overexpressing both H[1–1061] and Su(H) display a wild-type phenotype (Figure 2f). This shows that H[1–1061] is unable to cooperate with Su(H) to block Notch signaling. Nevertheless, H[1–1061] expression suppresses the loss-of-bristle phenotype associated with increased levels of Su(H) (Figure 2f and data not shown). Since H[1–1061] binds Su(H) [22, 30], it is possible that H[1–1061] proteins form nonproductive complexes with Su(H). Accordingly, the residual activity of the mutant H^{RP1} protein, which carries a 68 amino acid C-terminal deletion [30], might result from its ability to sequester Su(H) without actively repressing transcription.

These results therefore suggest that Hairless requires the binding of dCtBP to repress the expression of Notch target genes.

The role of dCtBP in repressing the expression of *sim* cannot easily be tested genetically. Indeed, dCtBP is a corepressor of Snail, and in *dCtBP* mutant embryos derived from GLC, the repression activity of Snail is abolished [27]. This in turn leads to the ectopic expression of *sim*

Figure 4



Hairless links Su(H) to dCtBP. The repression of Notch target genes by Su(H) is mediated by Hairless and dCtBP. NICD would compete with Hairless to bind Su(H). NICD would then allow the recruitment of the coactivator Mam.

in a broad ventral domain [27]. This derepression effect, which is, at least in part, due to lack of repression of *sim* by Snail [31, 32], prevented us from analyzing the possible role of dCtBP in mediating repression by Su(H).

In summary, our findings indicate that Hairless links Su(H) to the dCtBP corepressor. We therefore propose that Hairless antagonizes Notch signaling activity by recruiting dCtBP to repress Notch target gene expression. The activation of the Notch receptor would then lead to a competition between NICD and Hairless to assemble DNA-bound regulatory complexes of opposite activities (Figure 4).

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