Insensitive is a corepressor for Suppressor of Hairless and regulates Notch signalling during neural development

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The Notch intracellular domain functions as a co-activator for the DNA-binding protein Suppressor of Hairless (Su(H)) to mediate myriad cell fate decisions. Notch pathway activity is balanced by transcriptional repression, mediated by Su(H) in concert with its *Drosophila* corepressor Hairless. We demonstrate that the *Drosophila* neural BEN-solo protein Insensitive (Insv) is a nuclear factor that inhibits Notch signalling during multiple peripheral nervous system cell fate decisions. Endogenous Insv was particularly critical when repressor activity of Su(H) was compromised. Reciprocally, ectopic Insv generated several *Notch* loss-of-function phenotypes, repressed most Notch targets in the *E(spl)-C*, and opposed Notch-mediated activation of an *E(spl)m3-luc* reporter. A direct role for Insv in transcriptional repression was indicated by binding of Insv to Su(H), and by strong chromatin immunoprecipitation of endogenous Insv to most *E(spl)-C* loci. Strikingly, ectopic Insv fully rescued sensory organ precursors in *Hairless* null clones, indicating that Insv can antagonize Notch independently of Hairless. These data shed first light on the *in vivo* function for a BEN-solo protein as an Su(H) corepressor in the Notch pathway regulating neural development.

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Introduction

The peripheral nervous system (PNS) of *Drosophila melanogaster* includes hundreds of mechanosensory organs arranged in characteristic patterns. Major aspects of the developmental progression of peripheral sensory organs are well understood (Lai and Orgogozo, 2004). Within an initially

undifferentiated ectodermal field, groups of cells termed proneural clusters (PNCs) selectively express basic helix-loop-helix (bHLH) activators, whose patterned activity defines territories of neural competence (Figure 1A). Cell interactions among PNC cells, mediated by the Notch receptor and its associated signalling cascade (Lai, 2004), restrict neural potential to singular cells known as sensory organ precursors (SOPs); the remaining PNC cells eventually adopt an ordinary epidermal fate. At this stage, a loss of Notch signalling results in multiple SOPs emerging from a PNC, while a gain of Notch signalling extinguishes the SOP fate.

Once stably selected, each SOP executes a stereotyped series of asymmetric cell divisions (Lai and Orgogozo, 2004). The first SOP division produces two cells termed pIIA and pIIB (Figure 1A). pIIA generates socket and shaft cells, which are visible on the fly exterior. pIIB undergoes two sets of divisions yielding several internal cells, a glial cell, a sheath cell, and the neuron; the glial cell is apoptotic in mechanosensory organ lineages. Notch signalling operates at each division to guarantee the distinct developmental choices of each pair of daughter cells (Posakony, 1994). The neuron escapes Notch activation throughout the sensory lineage, while the socket cell derives from cells that consistently activate the pathway. Consequently in *Notch* mutant clones, all cells of peripheral sensory lineages adopt the neural fate, while hyperactivation of Notch activity within the sensory lineage can yield mutant organs composed exclusively of sockets.

Upon activation by ligand, the Notch receptor undergoes a series of proteolytic cleavages, resulting in the release and nuclear translocation of its intracellular domain (NICD). This fragment binds directly to members of the CSL (for vertebrate CBF1, *Drosophila* Suppressor of Hairless (Su(H)), and nematode LAG-1) family of transcription factors, which mediate most if not all of the nuclear aspects of Notch signalling (Lai, 2004). Although originally recognized as a transcriptional repressor in cultured cells (Dou *et al*, 1994; Waltzer *et al*, 1995), CSL proteins were subsequently found to mediate activation of Notch target genes *in vivo* (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). These opposing activities have been reconciled by a 'switch' model in which CSL proteins repress target genes in the absence of signalling via associated corepressor molecules, but activate target genes via NICD and associated co-activator molecules (Lai, 2002).

The specific roles of CSL-mediated repression can be difficult to recognize owing to the massive and pleiotropic defects induced by loss of Notch signalling. Nevertheless, substantial mutant phenotypes have been observed in the appropriate genetic contexts. For example, *Drosophila* mutants of the dedicated Su(H) corepressor encoded by *Hairless* reveal many phenotypes in both inhibitory and inductive contexts of Notch signalling that reflect elevated

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Notch signalling (Bang *et al*, 1991; Maier *et al*, 1992; Morel *et al*, 2001; Barolo *et al*, 2002). The asymmetry of pIIa division is particularly sensitive to Su(H) repressor function, since *Hairless* heterozygotes exhibit a number of double-socket organs that reflect Notch pathway gain-of-function.

In this study, we characterized *Drosophila insensitive* (*insv*) that encodes a novel protein containing a BEN domain. Null mutants of *insv* were earlier reported to be lethal and to exhibit Notch gain-of-function phenotypes in notum clones (Reeves and Posakony, 2005). These phenotypes were confounded by simultaneous loss of the Notch antagonist *lethal giant larvae* from available alleles (Roegiers *et al*, 2009). Nevertheless, upon cleaning of these stocks, viable *insv* mutant animals maintained detectable Notch gain-of-function PNS phenotypes that were fully rescued by *insv* genomic DNA. Detailed genetic interaction analysis revealed the endogenous role of Insv to restrain Notch signalling during multiple cell fate decisions, including SOP specification, pIIA–pIIB decision, and socket–shaft decision. The nuclear localization of Insv suggested that it might regulate Notch target gene expression. Consistent with this hypothesis, ectopic Insv generated multiple Notch loss-of-function phenotypes, strongly repressed the expression of an array of Notch target genes across the *Enhancer of split-Complex*, and suppressed Notch-mediated activation of an *E(spl)m3-luc* reporter in cultured cells.

We determined that Insv is a direct corepressor for Su(H), as revealed by protein–protein interactions *in vitro* and strong binding of endogenous Insv to multiple Su(H) target genes by chromatin immunoprecipitation (ChIP). While both Insv and *Hairless* bind Su(H), ectopic Insv supported SOP specification in null clones of *Hairless*, and could in fact generate a lateral inhibition defect in *Hairless* clones, as in wild-type. Therefore, Insv is capable of inhibiting Notch signalling independently of *Hairless*. Altogether, these findings shed first light on a member of the BEN-solo protein family as an Su(H) corepressor that regulates multiple Notch-mediated cell fate decisions during neural development.

Results

Insv is a nuclear protein expressed in the PNS

Insensitive (*insv*) is an SOP-specific gene product of novel structure, containing only the domain of unknown function 1172 (DUF1172; Reeves and Posakony, 2005). An extended version of DUF1172 was recently recognized across a set of >100 animal and viral proteins, and renamed the BEN domain (Abhiman *et al*, 2008). BEN domains are often found in association with other domains with chromatin-relevant functions (e.g., POZ, SCML1, or MCAF N-terminal domains). However, Insv belongs to a family of invertebrate and vertebrate proteins containing only the BEN domain ('BEN-solo' proteins), which have been little studied to date.

We generated a rabbit antiserum that recognizes Insv in SOPs of third instar wing imaginal discs (Figure 1B), consistent with prior *in situ* hybridization data (Reeves and Posakony, 2005). This reactivity was not observed in discs from *insv* deletion animals (Figure 1C), demonstrating the specificity of this reagent. Insv similarly accumulated in pupal SOP/pI cells at 14 h after puparium formation (APF), colocalizing with the nuclear transcription factor Senseless

(Sens). Notably, Insv appeared exclusively nuclear (Figure 1D), potentially reflecting a chromatin-associated role.

We traced Insv expression through the bristle lineage. Insv was detected in both pIIA and pIIB (Figure 1E), and was later seen in their daughters at the 4-cell stage (Figure 1F). However, Insv was strongly downregulated in all but one of the lineage cells (Figure 1F and G). Insv was extinguished in this cell before expression of typical markers of terminal PNS cell fates, such as Prospero (marking the sheath cell) or Elav (marking the neuron). However, we could visualize weak co-expression of Insv and Elav at a number of positions (Figure 1G'), while Insv was never colocalized with Pros (Figure 1G''). This identified the last Insv+ cell in the microchaete lineage as the neuron. The accumulation of Insv in SOPs and nascent neurons was analogous in the sense that neither of these cells activates Notch signalling during PNS development (Figure 1A).

Insv and *Notch[ts]* mutants exhibit mutual phenotypic suppression

Homozygous *insv* deletion animals were viable and exhibited grossly normal patterning of notum mechanosensory organs (Figure 2A and B). However, careful quantitation revealed about 10% fewer notum microchaetes in *insv* null animals compared with wild-type. Although the effect was mild, it was rescued by either of two independent transgene insertions bearing 3.7 kb *insv* genomic DNA (Figure 2C and D). This indicated that it was genuinely due to *insv* loss-of-function, and ruled out any contribution of other second-site mutations that might potentially have remained on *insv* chromosomes. A decrease in notum sensory organs is potentially consistent with increased Notch signalling. A mild reduction of Notch function, achieved in viable *N[ts]* animals raised at 22°C, results in increased microchaete density (Figure 2E). Ectopic sensory organs induced by *N[ts]* were suppressed in the *insv* mutant (Figure 2F), providing evidence that the decrease of sensory organs in *insv* was due to excess Notch pathway activity.

The abdominal mechanosensory organs are developmentally and genetically similar to their counterparts in the notum (Long *et al*, 1996; Norga *et al*, 2003). Further inspection revealed that *insv* homozygotes consistently exhibited ~10.4 double-socketed sensory organs per abdomen ($n = 50$ animals, 25°C; Figure 2G), and scanning electron micrography clearly revealed that shaft cells were replaced by socket cells in the mutant organs (Figure 2H). This phenotype indicates a gain in Notch pathway activity during pIIA division, during which the shaft cell is the sender and the socket cell is the recipient of the Notch signal (Figure 1A). Although this phenotype was mild, it was observed in both homozygotes and trans-heterozygotes of the deletion alleles *insv[23B]* and *insv[23L]*. As with the sensory organ number defect, this double socketing was fully rescued by a single copy of an *insv* genomic transgene ($n = 50$ animals, no double sockets observed; Figure 2J). Moreover, it was largely suppressed by introduction of *N[ts]* alleles at 25°C (Figure 2I), again reflecting its status as a Notch gain-of-function phenotype.

Finally, we took note of the viability of *N[ts]* alleles in the *insv* background at 25°C, since *N[ts]* is otherwise nearly completely pupal lethal at this temperature. We examined the dose sensitivity of this interaction, and found that

Figure 1 Insensitive (Insv) is nuclear protein expressed in the *Drosophila* peripheral nervous system. **(A)** Model of the external mechanosensory organ lineage. A proneural cluster (PNC, blue) is differentiated from other epidermal cells (grey) by spatially patterned activity of bHLH activator proteins Achaete (Ac) and Scute (Sc). A presumptive sensory organ precursor (SOP) or *pl* cell activates Notch signalling in non-SOP cells of the PNC, which then adopt an ordinary epidermal fate. The SOP undergoes a series of asymmetric divisions in which the sister cells are net senders (in red) or receivers (in blue) of Notch signalling. A glial cell is apoptotic leaving four cells in the mature sensory organ. Timepoints for dissection are indicated in hours after puparium formation (APF), and cell-specific markers used in this study are indicated. **(B)** Late third instar wing disc stained for Insv, revealing expression in all SOPs. **(C)** *insv*[23B] disc stained for Insv (in green, **C**) and Senseless (Sens, in red, **C'**), lack of Insv demonstrates antibody specificity. **(D)** 14 h APF notum stained for Insv (in green, **D**) and Sens (in red, **D'**); merge shows nuclear colocalization of these proteins (**D''**). **(E)** Sensory development at the 2-cell stage reveals colocalization of Insv and Cut in both cells. **(F)** Sensory development at the 4-cell stage reveals that Insv is maintained at a higher level in one cell (asterisks) in each cluster (dotted ovals). **(G)** Insv is extinguished before full expression of terminal cell markers, but we could observe colocalization of Insv with nascent Elav, but never with Prospero (Pros). This identified the last sensory cell to express Insv as the neuron.

Figure 2 Mutual suppression of *insv* and *N[ts1]* phenotypes indicates that endogenous *Insv* opposes Notch function. (A) Normal pattern of microchaetes in wild-type. Dashed line indicates the midline; there are five rows of microchaetes from the midline to the dorsocentral macrochaete row. (B) *insv[23B]* female seemingly shows normal notum development, although ‘holes’ in the microchaete field (arrows) hint at a lower sensory organ density. (C) A normal notum pattern is seen in *insv[23B]* female carrying an *insv* genomic rescue transgene. (D) Quantification reveals decreased numbers of microchaetes in *insv* females, and its rescue by either of two *insv* rescue transgene insertions. Student’s two-tailed *t*-tests were performed; $n = 59$ wt, 65 *insv*, 18 rescue#1, 13 rescue#2 animals. (E) *N[ts1]* females cultured at 22°C exhibit an increase in the density of microchaetes, with ~6–7 disorganized rows of microchaetes from the midline to the dorsocentral macrochaete row. (F) *N[ts1]; insv[23B]* females cultured at 22°C show a relatively normal pattern of microchaetes. (G) Abdomen of *insv[23B]* female cultured at 25°C contains a number of double-socketed organs (circles). (H) Scanning electron micrograph illustrating the conversion of the shaft (sh) cell into a socket (so) cell in *insv* double-socketed organs. (I) *N[ts1]; insv[23B]* female abdomen cultured at 25°C shows a strong reduction in double sockets. (J) Quantitation of double socketing in *insv* ($n = 50$), *insv; P[insv]* ($n = 40$), and *N[ts1]; insv* ($n = 48$) mutants. (K) *insv* loss-of-function suppressed the pupal lethality of *N[ts1]* animals raised at 25°C. Fifteen females and males were used in each condition.

removal of both alleles of *insv* was necessary for rescue (Figure 2K). Note that at 25°C, *N[ts1]* exhibits stronger effects on microchaete patterning that were partially, although not fully, rescued by loss of *insv* (data not shown). The changes in PNS patterning may not be sufficient to account for the rescue of lethality of *N[ts1]* animals, since other mutants with stronger increases in microchaete density are viable (Levitin and Posakony, 1996; Lai *et al*, 2000a); therefore, it may reflect another location of *Insv* function. Nevertheless, this viability interaction provides further evidence that endogenous *Insv* opposes Notch activity. As well, the interactions of these loci in sensory organ number and socket–shaft decisions implied that endogenous *Insv* functions at multiple steps during peripheral neurogenesis to oppose Notch activity.

Endogenous *Insv* regulates multiple cell fate decisions during PNS development

We used additional genetic interaction tests to survey the endogenous requirements of *Insv* during PNS development. Activation of *UAS-Su(H)* using *Eq-gal4*, which is broadly active in the developing thorax, resulted in a substantial balding phenotype (Figure 3A). This was caused by failure

of notum SOP commitment as marked by loss of *Sens* at 14 h APF (Figure 3C and D). We observed similar loss of SOPs and adult macrochaetes in third instar wing imaginal discs of the *dpp-gal4 > UAS-Su(H)* genotype (Supplementary Figure S1A–D). These phenotypes are indicative of a gain of Notch pathway activity, which extinguishes the SOP fate (Figure 1A). Misexpression of *Su(H)* in a homozygous *insv* mutant yielded a stronger loss of adult bristles (Figure 3B) and microchaete SOPs (Figure 3E); the bristle phenotypes were quantified in Supplementary Figure S1E–G. These data are consistent with the notion that the presence of *Insv* in SOPs protects against Notch-mediated activation of the epidermal fate (Figure 3F), as suggested by the decrease in microchaete numbers in straight *insv* mutants (Figure 2A–D).

We next assessed the requirement for *Insv* for asymmetric division of the SOP to generate pIIA and pIIB cells (Figure 1A). We sensitized this assay by delivering a brief pulse of ectopic *Numb*, a well-characterized inhibitor of Notch signalling, just before SOP division. In *hs-numb* animals, a 1-h heat-shock at 37°C at 14 h APF induced a strong balding phenotype (Figure 3G). Unlike balding caused by elevation of Notch signalling during SOP selection, the

Figure 3 *Insv* exhibits genetic interactions with *Su(H)* and *numb* that reflects its antagonism of Notch-directed cell fate decisions. (A) Misexpression of *Su(H)* using *Eq-gal4* causes sensory organ loss due to failure to specify SOPs (see also Supplementary Figure S1). (B) Misexpression of *Su(H)* in an *insv* mutant background causes enhanced SOP loss. (C–E) 14 h APF left heminota stained for the SOP marker Sens. (C) Wild-type pattern of SOP cells. (D) *Eq>myc-Su(H)* shows reduced SOPs. (E) *Eq>myc-Su(H)* in an *insv[23B]* mutant background yields enhanced SOP loss. (F) Cellular interpretation of bristle loss phenotype. (G) Activation of *hs-numb* during division of microchaete SOP cells (14 h APF) results in extensive bristle loss due to pIIA → pIIB transformation. (H) Ectopic Numb is unable to drive cell fate transformations effectively in an *insv* mutant. (I–K) Notum sensory lineages at 24 h APF stained for the sheath cell marker Prospero and the neural marker Elav. (I) Wild-type sensory organs contain single Pros+ and Elav+ cells. (J) *hs-numb* pulsed at 37°C for 1 h at 14 h APF yields organs with pairs of Pros+ and Elav+ cells, due to pIIA → pIIB conversion. (K) Similar elevation of numb in an *insv* mutant fails to yield efficient cell fate conversions. (L) Cellular interpretation of bristle loss phenotype induced by ectopic Numb, and its rescue by absence of *Insv*.

balding phenotype caused by loss of Notch signalling results from transformation of the pIIA cell into a pIIB cell. This fate conversion is evidenced by pairs of sheath cells and neurons, labelled by Prospero and Elav staining, at 24 h APF (Figure 3I and J). However, when the *hs-numb* regimen was performed in an *insv* mutant background, we observed a strong suppression of balding (Figure 3H), concomitant with a restoration of sensory organs with single Prospero/Elav-positive nuclei (Figure 3K). Therefore, efficient inhibition of Notch signalling by ectopic Numb during pl division requires *Insv* (Figure 3L).

Finally, we studied the asymmetric division of pIIA, which generates the socket and shaft cells, in further depth. The major known direct corepressor of *Su(H)* is Hairless (H) (Morel *et al*, 2001; Barolo *et al*, 2002). The importance of *Su(H)* autorepression during shaft cell specification is manifested by the haploinsufficient double socketing of *H* mutants (Bang *et al*, 1991), seen at some macrochaete and microchaete positions (Figure 4A and A'). Strikingly, *insv* mutant animals heterozygous for *H* exhibited nearly complete failure of shaft cell differentiation, resulting in a field of double-socketed sensory organs (Figure 4B and B'). We

Figure 4 *Ins* exhibits potent genetic interactions with *Hairless*, an Su(H) corepressor. (A) *H[31]/+* heterozygote exhibits dominant double socketing of a few macrochaetes (arrow); (A') scanning electron micrograph (SEM) illustrates a double socket in this genotype more clearly. (B) *insv; H[31]/+* shows double socketing of nearly all external sensory organs; (B') SEM illustrates a double socket in this genotype more clearly. See also Supplementary Figure S2 for additional allelic combinations that yielded identical results. (C) The complete double socketing in *insv; H[31]/+* is fully rescued back to the *H[31]/+* phenotype upon introduction of one copy of an *insv* genomic transgene. (D, E) Triple label of sensory organs at 32 h APF to mark socket cells [Su(H) + nuclei], shaft cells (large D-Pax2 + nuclei), sheath cells (small D-Pax2 + nuclei), and neurons (Elav + nuclei). (D) Most sensory organs contain the normal complement of differentiated cells in *nota* doubly heterozygous for *insv* and *H*, including single socket (arrow), shaft (large arrowhead) and sheath (small arrowhead) cells. (E) *insv; H[31]/+* notum exhibits complete double socketing at the expense of shaft specification. (F, G) Cellular interpretation of double-socketing phenotype.

verified this exquisite genetic interaction with several *H* alleles, including *H[1]*, *H[2]*, and *H[31]* (Supplementary Figure S2). Abdominal double socketing was also enhanced, but was less profound owing to the more substantial *H/+* phenotype extant in the abdomen (Supplementary Figure S2). We were wary to rule out that second-site aberrations might contribute to this potent genetic interaction. Importantly, introduction of one copy of *insv* genomic transgene to *insv/insv; H/+* animals restored them to *H/+* phenotypic status

(Figure 4C), demonstrating that this massive sensory organ defect was specifically attributable to loss of *insv*.

Cellular analysis of terminal PNS cell fate markers in the notum confirmed that this phenotype was due to complete transformation of shaft cells (as marked by large D-Pax2 + nuclei) into socket cells (as marked by Su(H) + nuclei), with little change in the differentiation of sheath cells (marked by small D-Pax2 + nuclei) or neurons (marked by Elav) (Figure 4D and E). These data demonstrate that endogenous *Ins* is

critical for normal sensory organ development when Su(H) corepressor function is mildly compromised (Figure 4F and G). Altogether, the strong genetic interactions observed with *Notch*, *Su(H)*, *Numb*, and *Hairless* (Figures 2–4) report on the involvement of endogenous *Insv* to inhibit Notch pathway activity for robust execution of three different steps in PNS development: SOP selection, pLIB specification, and shaft cell specification.

Ectopic *Insv* generates Notch loss-of-function phenotypes

We next used gain-of-function approaches to assess *Insv* activity with respect to Notch signalling. Misexpression of *Insv* in PNCs, using *sca-Gal4*, resulted in ectopic macrochaetes (Figure 5A and B). Activation of *UAS-insv* using *Eq-Gal4* strongly increased the density of notum microchaetes (Figure 5C). These defects were due to ectopic commitment to the SOP fate, as reported by aberrant expression of *Sens* (Figure 5D and E). Therefore, *Insv* gain-of-function generated a lateral inhibition phenotype that was opposite to the decrease in sensory organ number seen in *insv* mutants (Figure 2A–D), and similar to the reduction of Notch activity during SOP specification (Figure 2E).

Specification of the wing margin also requires Notch signalling but is presumably not under endogenous *insv* control, since *Insv* is restricted to SOPs in the wing disc (Figure 1B). Nevertheless, misexpression of *Insv* with multiple wing drivers, including *ptc-Gal4*, *dpp-Gal4*, and *vg-Gal4*, yielded wing notching (Figure 5F and G and data not shown). Immunostaining of third instar wing discs revealed loss of Cut and *Sens* expression in the developing wing margin (Figure 5H), indicating defective Notch signalling. Because ectopic *Insv* could phenocopy defective Notch signalling in both endogenous (PNS) and ectopic (wing margin) contexts, these data implied that the neural-specific *Insv* factor likely acts via a core component of the Notch pathway.

***Insv* represses an array of N/Su(H) target genes**

By both loss-of-function and gain-of-function tests, then, *Insv* functions as an inhibitor of Notch signalling. Moreover, reduction of Su(H) corepressor function (in *Hairless* heterozygotes) renders *Insv* essential for sensory organ development. We, therefore, assayed the influence of *Insv* on the expression of Notch/Su(H) target genes. Critical effectors of Notch signalling during PNS development are encoded by the *Enhancer of split-Complex* [*E(spl)-C*], which contains seven bHLH repressors and four Bearded family genes that are directly activated during Notch signalling via high affinity Su(H) binding sites (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Nellesen *et al*, 1999; Cooper *et al*, 2000; Lai *et al*, 2000a,b). We analysed the accumulation of endogenous *E(spl)*bHLH and Bearded family transcripts in embryos that overexpressed *insv* under heat-shock control, and observed that nearly all of them were downregulated in response to this treatment (Figure 6A). By contrast, *E(spl)m1*, a Kazal protease-encoding gene embedded in the *E(spl)-C* (Lai *et al*, 2000b), was not affected by *insv* gain-of-function (Figure 6A). Therefore, *Insv* specifically regulates Notch target genes in the *E(spl)-C*.

To provide more direct evidence for inhibition of Notch target gene transcription by *Insv*, we performed reporter assays. The *E(spl)-C* is mostly transcriptionally silent in S2

cells, even in the presence of dominantly active NICD (Krejci and Bray, 2007). However, *E(spl)m3* can respond to NICD in S2 cells (Mukherjee *et al*, 2005; Krejci and Bray, 2007). We constructed an *E(spl)m3-luc* reporter that was responsive to co-transfection with *pMT-NICD* and *ub-Gal4* in S2 cells. In this assay, *UAS-insv* compromised the ability of NICD to activate *E(spl)m3-luc* (Figure 6B), confirming the ability of *Insv* to antagonize Notch-mediated transcriptional activation.

***Insv* binds Su(H) directly via its BEN domain**

We wished to probe the mechanism of *Insv* function further. We used western blotting of transfected S2 cells to check for possible effects of *Insv* on the stability of various nuclear components of the Notch pathway. However, co-expression of *Insv* did not substantially affect the accumulation of NICD, Su(H), or *Hairless* proteins (Supplementary Figure S3). These negative data do not rule out, but certainly do not support, models in which *Insv* antagonizes Notch pathway transcriptional outputs by degrading NICD or Su(H), or by stabilizing *Hairless*. This left a possibility that *Insv* might participate as a component of the Su(H) corepressor complex.

We sought evidence for *Insv*–Su(H) complexes, first by performing co-immunoprecipitation (co-IP) experiments from S2 cells. We observed modest, although demonstrably specific signal for endogenous Su(H) in *Insv*-V5 IPs (Supplementary Figure S4A). We attempted to enhance such signals by co-expressing *Insv* and myc-Su(H), and could also detect Su(H) in *Insv* IPs in these conditions (Supplementary Figure S4B). The relatively modest interactions in these co-IP studies might potentially reflect transient complexes *in vivo*, but did not address how direct such interactions might be. Therefore, we turned to GST-pulldown assays. Indeed, we found that full-length GST-*Insv*, but not GST alone, could effectively pulldown *in vitro* translated Su(H) (Figure 7A).

We proceeded to narrow down the interaction. The first half of the *Insv* protein is predicted to have coiled-coil structure, but otherwise its only recognizable motif is the C-terminal BEN domain (Figure 7B). GST alone or GST fused to the predicted coiled-coil region did not pulldown Su(H) (Figure 7B). However, the second half of *Insv* effectively bound Su(H), and this interaction could be further refined to just the BEN domain. *Insv* did not pulldown the nuclear repressor Groucho that also regulates Notch signalling (Supplementary Figure S5), indicating the specificity of *Insv* interaction with Su(H). The protein–protein interaction of the *Insv*–BEN domain with Su(H), together with our abundant genetic data (Figures 2–6), support a model in which *Insv* functions as a component of the Su(H) corepressor complex to oppose Notch pathway activity during *Drosophila* peripheral neurogenesis.

Endogenous *Insv* binds Su(H)-regulated enhancers in an array of *E(spl)-C* genes

A prediction from our observation of direct binding of *Insv* to Su(H) is that *Insv* should be recruited to Su(H) target genes *in vivo*. We assessed this using ChIP from embryos. We observed strong (10–20-fold) enrichment of *Insv* to the N/Su(H)-regulated enhancers of two Bearded family genes in the *E(spl)-C* [*E(spl)malpha* and *E(spl)m4*] that are known to regulate nervous system development (Figure 7C). Perhaps

Figure 5 Ectopic Insv inhibits Notch pathway activity. (A) Wild-type adult notum showing normal pattern of mechanosensory bristles; anterior and posterior dorsocentral positions (aDC and pDC) are labelled, as is the microchaete field (μ C). (B) *sca-Gal4>UAS-insv* notum exhibits macrochaete tufting, especially at aDC and pDC. (C) *Eq-Gal4>UAS-insv* notum exhibits a strong increase in microchaete density. (D) In 14 h APF wild-type notum, Insv (green) colocalizes with Senseless (Sens) (red) in all pl cells. The regular columns of microchaete pl cells are easily discerned; the midline is indicated with a dashed line. (E) Following misexpression of *insv* using *Eq-gal4*, the regular arrangement of pl columns is disturbed, and there are many intervening pl cells as marked by Sens. (F) Wild-type adult wing. (G) *vg-Gal4>UAS-insv* wing exhibits extensive notching. (H) *ptc-Gal4>UAS-insv* disc, in wing margin cells that overlap the domain of ectopic Insv (H) there is a gap in Sens (H') and Cut (H'').

more impressively, we also observed strong ChIP signals to the N/Su(H)-regulated enhancers of all seven bHLH repressor genes in the E(spl)-C, which are the major conserved effectors of metazoan Notch signalling (Figure 7C).

We checked the promoters of several randomly selected genes not known to be regulated by Notch signalling (CG12290, CG34245, and CG18559). As we did not observe any ChIP enrichment of Insv to these genes (Figure 7C), Insv is, therefore, not generally bound to gene promoters. Finally, to firmly confirm that observed signals were due to endogenous Insv, we repeated ChIP assays from *insv* homozygous mutant embryos. These samples exhibited no ChIP signals for any of

the tested targets, including the Bearded family gene *E(spl)m4* and the bHLH repressor gene *E(spl)m8*, which exhibited 20–30-fold enrichment of Insv in wild-type (Figure 7C). We conclude from these data that Insv is recruited to Su(H) target genes *in vivo*, consistent with our observation of protein–protein interaction between Insv and Su(H) *in vitro*, and the ability of Insv to repress the expression of these N/Su(H) target genes.

Insv can antagonize Notch pathway activity independently of Hairless

Endogenous Insv contributes to Su(H) corepressor function during multiple stages of peripheral neurogenesis, but

Figure 6 *Insv* inhibits the expression of Notch target genes. (A) qrt-PCR was used to demonstrate downregulation of N/Su(H) target genes in the *E(spl)-C*, in embryos overexpressing *Insv* under *hs-Gal4* control. Both bHLH-R and Bearded family genes were repressed by *Insv*; in contrast, the Kazal protease encoded by *E(spl)m1* is not regulated by Notch signalling and was not affected by ectopic *insv*. Values are the mean from two independent RNA extractions, each quantified in quadruplicate. (B) Cell culture assays demonstrate that *Insv* antagonizes transcriptional activation of *E(spl)m3-luc* by NICD; total amounts of transfected plasmids were equalized with empty expression vectors. Value is the mean from three independent experiments, each of which included four independent transfections. Student's two-tailed *t*-tests were performed.

Hairless is apparently required more critically for Notch inhibition during neural development. Since *Insv* does not substantially alter *Hairless* accumulation (Supplementary Figure S3), one might envision that *Insv* supplements or potentiates *Hairless* function. We wished to know whether *Insv* requires *Hairless* to antagonize Notch pathway activity, or whether it might be able to function independently of *Hairless*. We performed a strict genetic test of this by using the MARCM technique to generate *Hairless* null clones *in vivo* that concomitantly expressed GFP or *Insv*. We then analysed their respective phenotypes in adult morphology and pupal cell fate commitment.

H null clones fail to develop adult mechanosensory bristles, resulting in bald notum patches that exhibit only epithelial hairs (Figure 8A and B). This reflects the essential role of *H* to antagonize Notch signalling during lateral inhibition, since all SOPs succumb to Notch-mediated suppression. In contrast, *H* null clones that expressed *Insv* could differentiate adult sensory organs (Figure 8C and D), although these typically exhibited multiple (2–4) sockets, a Notch gain-of-function phenotype. Occasionally, we observed shaft differentiation from multisocket structures (Figure 8D, inset shows an organ with two sockets and one shaft). Nevertheless, the major conclusion from this experiment was the very fact that *Insv*-expressing *H* clones could develop any PNS sensory cells at all. Thus, *Insv* could protect SOPs against ectopic Notch signalling in *H* mutant cells.

Adult morphologies do not report fully on the cellular bases, including the potential ability of internal cells (pIIIB daughters) to be specified. We, therefore, examined SOP specification at 14 h APF by staining for Hindsight (Hnt). As expected, *H* null clones were completely unable to specify SOPs (8/8 clones examined, Figure 8E and F). Reciprocally, there was a clear non-autonomous effect induced by *H*

clones, in that wild-type cells bordering the clonal territory preferentially adopted the SOP fate (Figure 8F'). Notably, expression of *Insv* in *H* clones completely rescued their ability to specify SOPs (10/10 clones examined). These clones also suppressed the non-autonomous capacity of bordering wild-type cells to adopt the SOP fate (Figure 8G and G'). Moreover, SOP density within these clones was actually increased above neighbouring control tissue, paralleling the effect of elevated *Insv* expression in *H* +/+ conditions (Figure 5). Therefore, *Insv* not only fully substituted for the complete absence of *H* during SOP selection, but in fact could induce a Notch loss-of-function phenotype without *H*.

We further examined the specification of lineage cells in these mutant clones. As expected from the lack of SOPs, *H* notum clones completely lack differentiated PNS cells (9/9 clones, Figure 8H and I). In contrast, *H* clones expressing *Insv* maintained an increased density of sensory organs (17/17 clones, Figure 8J), consistent with the increased density of SOPs (Figure 8G). These clones usually exhibited multiple large Cut+ nuclei indicative of pIIA daughters (Figure 8J). Staining for the socket marker Su(H) showed that these clones differentiated multiple sockets (Supplementary Figure S6), as seen in the adult cuticle. We observed many 4-socket organs, which correspond to sensory lineages, in which Notch signalling was overactivated at each cell division following SOP specification. However, we also observed a high frequency of small Cut+ nuclei in these clones (Figure 8J), indicative of pIIIB daughters and partial restoration of asymmetric division of SOPs.

Staining for the neural marker Elav confirmed the capacity of these clones to generate neurons (Figure 8J), which is the cell type most protected from Notch signalling throughout the sensory lineage (Figure 1A). Quantification showed that 65% of *H* mutant sensory lineages expressing *Insv* could differ-

Figure 7 Insv is a direct partner of Su(H). **(A)** GST-pulldown assays showed that GST-Insv could specifically pulldown Su(H), relative to a large excess of GST alone. **(B)** Domain structure of Insv, with predicted coiled-coil domain in yellow and BEN domain in blue. GST-pulldown assays demonstrate that the Insv-BEN domain alone can mediate protein-protein interaction with Su(H). **(C)** ChIP assays. Chromatin from 2.5 to 6.5 h embryos was immunoprecipitated with antibodies to endogenous Insv, and the indicated target amplicons were checked by qPCR. Insv was bound to the Su(H)-regulated promoters of 10 genes in the *E(spl)*-C, including both Bearded family and bHLH-R genes. Insv did not associate with multiple control promoters, and the strong 20–30-fold ChIP enrichments to *E(spl)m4* and *E(spl)m8* were abolished in chromatin prepared from *insv* homozygous mutant embryos.

entiate neurons (6 clones and 86 sensory organs examined). We conclude that *Insv* is capable of inhibiting Notch signaling at multiple steps during the PNS lineage in the complete absence of *Hairless*, the main known nuclear Notch repressor in *Drosophila*. The low frequency of shaft specification in the rescued *H* clones is consistent with the notion that shaft specification requires a very high level of Notch inhibition, as evidenced by occasional double socketing in *H* heterozygotes and the full-blown double socketing seen in *insv* mutants heterozygous for *H*. On the other hand, *Insv* can antagonize

Notch quite effectively during SOP specification, during pIIA–pIIB specification, and during sheath–neuron specification in an H-independent manner.

Discussion

Default repression by members of the conserved CSL transcription factor family is critical for proper cell fate decisions mediated by Notch signalling. Curiously, while activation of Notch target genes involves a conserved

Figure 8 Insensitive can rescue sensory organ development in *Hairless* null clones. (A) SEM of adult notum bearing *H* null MARCM clones (*hs-flp*, *tub-Gal4*, *UAS-GFP*; *FRT82B H[E31]/FRT82B tub-Gal80*); $\times 205$. (B) Close-up of *H* clonal territory (boxed region in A) showing lack of external sensory organ structures; $\times 1150$. (C) SEM of adult notum bearing *H* null MARCM clones expressing *Insu* (*hs-flp*, *tub-Gal4*, *UAS-GFP*; *UAS-insu*; *FRT82B H[E31]/FRT82B tub-Gal80*); $\times 205$. (D) Closeup of *H* clonal territory expressing *Insu* (boxed region in C) showing lack of external sensory organ structures; $\times 1150$. Circles highlight multisocket organs bearing 2–4 sockets; inset shows a multisocket organ with a shaft. (E–G) Fourteen hours APF notum bearing MARCM clones marked by GFP (green) and stained for Hindsight to mark SOPs (red) and DAPI as a counterstain (blue); all of the (') panels show the clone border as a dashed line. (E, E') Control clone shows normal patterning of SOPs; some have just divided and are visible as stained pairs of cells. (F, F') *H* MARCM clone exhibits complete lack of SOP specification in the mutant territory. Reciprocally, there is a non-autonomous effect in that wild-type cells bordering the clones are more likely to adopt the SOP fate (asterisks). (G) *H* MARCM clone expressing *Insu* exhibits rescue of SOP specification and depletion of wild-type SOPs neighbouring the clone; in fact the density of SOPs is greater than in wt. (H–J) Thirty hours APF notum bearing MARCM clones marked by GFP (green) and stained for Cut to mark all sensory organ cells (red), Elav to mark neurons (white), and DAPI as a counterstain (blue). (H) Control clones show that each cluster of four sensory cells includes one neuron. (I) *H* null clones fail to differentiate any sensory lineage cells, consistent with the failure to specify SOPs; the non-autonomous effect persists (asterisks). (J) *H* MARCM clone expressing *Insu* exhibits sensory lineages with multiple large cells determined to be *Su(H)*-expressing sockets (see D and Supplementary Figure S6); however, about half of the organs could differentiate neurons (arrows).

N[ICD]–Mastermind–CSL complex, a diversity of corepressor complexes have been defined in invertebrate and vertebrate systems (Lai, 2002). The major corepressor for the *Drosophila* CSL protein Su(H) is Hairless, an adaptor protein that recruits both CtBP and Groucho repressor complexes (Morel *et al*, 2001; Barolo *et al*, 2002). Mammalian Hairless proteins have not been identified; however, it should be noted that Hairless is extremely rapidly evolving and not trivial to identify even in other insects (Maier, 2006; Maier *et al*, 2008). Therefore, the absence of mammalian proteins aligning to Hairless is not necessarily conclusive. On the other hand, mammalian SHARP and CIR were reported to bind the mammalian CSL protein CBF1, and recruit SMRT/N-CoR and HDAC repressor complexes (Hsieh *et al*, 1999; Oswald *et al*, 2005). Recently, the histone demethylase KDM5A/Lid was reported to be a direct partner of both CBF1 and Su(H) corepressor complexes (Moshkin *et al*, 2009; Liefke *et al*, 2010), although KDM5A/Lid is also documented to have pleiotropic functions involving diverse DNA binding partners such as Rb, Myc, and PRC2 (Fattaey *et al*, 1993; Secombe *et al*, 2007; Pasini *et al*, 2008).

Our genetic and biochemical studies show that Insv is a neural nuclear protein that functions as a direct Su(H) partner to antagonize Notch pathway activity during multiple steps of *Drosophila* peripheral neurogenesis. These data shed first light on the *in vivo* function for a BEN-solo protein as a neural corepressor in the Notch pathway. Although the phenotypes of *insv* mutants are mild, they were seen in multiple allelic combinations and were fully rescued by *insv* genomic DNA. More substantially, *insv* mutants exhibited strong genetic interactions with several Notch pathway alterations. This genetic situation is not unique to *insv*, as other critical components of the Notch pathway exhibit redundancy in the nervous system (e.g., multiple E(spl)bHLH-encoding genes must be removed to reveal strong neurogenic defect; Schrons *et al*, 1992) and both Notch ligands must be removed to reveal PNS lineage defects (Zeng *et al*, 1998). Perhaps most striking was the fact that shaft cell specification completely failed in *insv* mutants where Su(H) corepressor function was reduced by heterozygosity of *Hairless*, the major direct Su(H) corepressor identified to date. Reciprocally, elevation of Insv level could completely compensate for the null condition of *Hairless* during SOP specification, and could partially rescue the specification of internal cells including neurons. In fact, ectopic Insv could still generate a Notch loss-of-function lateral inhibition defect without *Hairless*. These data do not rule out the possibility of a trimeric Su(H)–Hairless–Insv repression complex, but they indicate that Insv does not require *Hairless* to mediate *in vivo* repression by Su(H). Our preliminary tests indicate that Insv may not bind directly to Groucho, as shown for *Hairless* (Barolo *et al*, 2002). However, now that a molecular function has been assigned to Insv, future studies can be aimed at understanding how it interfaces with other silencing proteins and perhaps eventually to chromatin modifying enzymes.

The *Drosophila* genome contains other loci encoding BEN domains (Abhiman *et al*, 2008), including other BEN-solo factors (*CG9883* and *CG12205*) and *mod(mdg4)*, a highly alternatively spliced locus that encodes proteins with BEN and POZ domains. It remains to be seen whether the *Drosophila* BEN proteins exhibit any functional overlap.

More generally, our data shed first light on the *in vivo* function for a BEN-solo protein as a corepressor in the Notch pathway. Other BEN domain proteins containing BTB/POZ domains have been linked to transcriptional repression (vertebrate NAC1; Korutla *et al*, 2005) and enhancer blocking (*Drosophila* *mod(mdg4)*; Cai and Levine, 1997) activities, and the mammalian BEN-solo protein SMAR1/BANP recruits the SIN3/HDAC1 repressor complex (Rampalli *et al*, 2005). Our data add to a growing theme for BEN factor involvement in transcriptional repression. While there are not clear mammalian orthologues of Insv, they do express several BEN-solo proteins (Abhiman *et al*, 2008). In light of the relatively specific effects of Insv in Notch-mediated cell fate decisions in both endogenous and ectopic contexts, our studies generate hypotheses to direct the study of mammalian BEN-solo proteins.

Finally, we note that BEN domains are also encoded by viral genomes, including the BEN-solo protein Chordopox E5R_VVC_137623 (Abhiman *et al*, 2008). Viral proteins such as Epstein Barr viral oncoprotein EBNA2 and the adenoviral oncoprotein 13S E1A bind CBF1 and function as NICD mimics (Grossman *et al*, 1994; Henkel *et al*, 1994; Ansieau *et al*, 2001). Our elucidation of a BEN-solo protein as a CSL corepressor raises the possibility that viruses may have co-opted cellular proteins to dominantly repress Notch signalling.

Materials and methods

Drosophila stocks

insv deletion alleles (23B and 23L) were a gift of James Posakony. As described recently, both alleles harboured deletions of *lethal giant larvae* (*lgl*) which accounted for their strong multisolet phenotype in mutant clones (Roegiers *et al*, 2009). We backcrossed these to *w[1118]* and screened 400–500 candidate recombinants of each allele for complementation of *lgl* and maintenance of *insv* deletions, yielding homozygous viable *insv[23B]* and *insv[23L]* stocks.

We used the following stocks for genetic interaction tests with the cleaned *insv* alleles: *Notch[ts1]* (from Bloomington Stock Center), the *Hairless* (*H*) alleles *H[1]*, *H[2]*, and *H[E31]* (from James Posakony), *UAS-myc-Su(H)* (from Mike Young), and *hs-numb* (from Yuh Nung Jan). For experiments with *Notch[ts1]*, we raised animals at 22 or 25°C; animals are viable and exhibit a mild increase in bristle density at the former temperature, and pupal lethal at the latter temperature. For experiments with *hs-numb*, we collected white pre-pupae and induced the transgene at 14 h APF, then moved them to 25°C to develop further.

To verify that *insv* mutant phenotypes were genuinely due to loss of *insv*, we amplified a 3.67-kb *insv* genomic region that extends into the terminal regions of both 5' and 3' flanking genes using PCR primers GGGGTACctgcacgcgatttggcaaca and ATAAGAATGCGGCCGCaccatcccatctgtgggtaa. We cloned this into the *KpnI/NotI* sites of pW8 and generated transgenic animals using standard injection with delta2–3 plasmid. This transgene rescued the nearly complete double-socketing phenotype of *insv/insv*; *H/+* animals. For conditional expression of *insv*, we amplified its coding region using the primers CGGAATTCatggaaacaaatcatcagcgc and GCTCTAGACtaataatgttctctttctgtg, and cloned it into the *EcoRI* and *XbaI* sites of pUAST. Multiple independent transgenic insertions were generated (BestGene). These were tested against a panel of Gal4 driver lines obtained from the Bloomington Stock Center: *sca-Gal4*, *ptc-Gal4*, *vg-Gal4*, and *bx-Gal4*; *Eq-Gal4* was a gift of Hugo Bellen (Baylor). The Gal4 crosses were all done at 25°C, and all *UAS-insv* lines generated qualitatively similar phenotypes.

For MARCM analysis, we recombined the null allele *H[E31]* onto FRT82B, and introduced this into background bearing *UAS-insv* on the second chromosome. MARCM clones were generated by crossing FRT82B, *H[E31]/TM6B* or *UAS-insv*; FRT82B, *H[E31]/*

TM6B stocks to *hsFLP*, *UAS-GFP*; *tub-Gal4*; *FRT82B tub-Gal80*/*TM6B*. We heat-shocked first instar larvae at 37°C for 1 h, let them recover at 25°C, and then dissected non-Tb animals bearing clones.

Immunostaining

We amplified a fragment of *insv* encoding amino acids 150–260 using the PCR primers *cgaattctgaacagcctgacacctctg* and *ccgctcgaggttcgggccaatggaccacatc*, and cloned it into the *EcoRI* and *XhoI* sites of pGEX-5x-2. This construct was suitable for isolation of soluble fusion protein and used to immunize rabbits (Covance). Following pre-adsorption with 0–1 h embryos, the raw Insv sera were effective for immunostaining at a dilution of 1:1000. Other primary antibodies used were guinea pig anti-Sens (1:5000, gift of Hugo Bellen), rabbit anti-D-Pax2 (affinity purified, 1:50, gift of Markus Noll), rat anti-Su(H) (1:2500, gift of Cedric Wesley), and the following monoclonal antibodies obtained from the Developmental Studies Hybridoma Bank: rat anti-Elav (7E8A10) and mouse anti-Elav (9F8A9), -Cut, -Hindsight, and -Prospero (MR1A), all used at 1:100. Secondary antibodies were Alexa-488-, 568-, and 647-conjugated goat secondary antibodies (1:1000, Molecular Probes). For some experiments with mouse Elav, primary antibodies were directly conjugated with Alexa Fluor 647 using a monoclonal antibody labeling kit (Invitrogen).

For notum preparations, white pre-pupae were collected and aged for the designated period of time APF at 25°C in a humid chamber before dissection. For experiments with *hs-numb*, pupae were moved to a 37°C water bath for 1 h at 14 h APF, then returned to 25°C to develop further. Dissected tissues were fixed in 4% paraformaldehyde and stained as previously described (Lai and Rubin, 2001).

Reverse transcription-PCR

For the comparison between *w¹¹¹⁸* and overexpression of *insv* by *hs-Gal4*, embryos were incubated at 25°C for 1 h, treated in a 37°C water bath for another hour and then allowed to recover for 9 h before RNA extraction. Total RNA was isolated by use of TRIzol reagent (Invitrogen) and further cleaned with Turbo DNase (Ambion). Four micrograms of total RNA was reverse transcribed with Superscript III reverse transcriptase (RT) (Invitrogen) using random primer mix (New England Biolabs). To ensure absence of genomic DNA, qPCR was performed on a mock reverse transcribed RNA sample. Primer sequences are listed in Supplementary Table 1.

Cell culture and transient transfection

A tandem duplication of the *E(spl)m3* promoter was reported to be responsive to activated Notch in cultured cells (Mukherjee *et al*, 2005). To provide a more natural context, we replacing the SV40 enhancer/promoter between *NheI/BglII* sites in the psiCHECK2 vector (Promega) with the endogenous promoter/enhancer of *E(spl)m3* including 258 bp of 5' UTR and 1532 bp of upstream sequence. *Drosophila* S2-R+ cells were grown in Schneider *Drosophila* medium containing 10% fetal calf serum, 1 × Glutamax (Gibco), 0.05 mg/ml Gentamycin, 50 IU/ml Penicillin, and 50 µg/ml Streptomycin. Cells were transfected in 24-well plate using the Effectene Transfection kit (Invitrogen). In each well, 0.5×10^6 cells in 500 µl medium were transfected with 100 ng of *m3-psiCHECK2* reporter alone, co-transfected with 80 ng of pMT-NICD, or co-transfected with a mixture of 80 ng pMT-NICD, 100 ng pUAST-Insv, and 50 ng ub-Gal4 plasmids. Total plasmid delivered was normalized by adding pBluescript to the control transfection, and by including empty pUAST vector in the control NICD transfection. After 24 h incubation, 0.5 µl of 500 mM copper sulphate was added to each well to induce pMT-NICD. Cells were further cultured for overnight before harvest. Luciferase activity was measured in living cells using the Dual Luciferase Assay System (Promega), and promoter activity was defined as the ratio between the *Renilla* and firefly luciferase activities.

GST-pulldown assays

GST and GST-fusion proteins were expressed in *E. coli* BL21 cells by induction with IPTG for 3 h at room temperature, and purified on glutathione sepharose beads (Sigma). ³⁵S-labelled proteins were translated *in vitro* with TnT coupled reticulocyte lysate (Promega) and pre-cleared with GST-sepharose. Then, they were incubated with GST-fusion proteins on GSH sepharose in NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) for 1 h.

Sephadex beads were collected by a brief centrifugation at 1000 r.p.m. and washed four times in NETN before elution by boiling in loading buffer. Proteins were separated on a 10% polyacrylamide-SDS gel, stained with Coomassie blue, and vacuum dried at 80°C and exposed to a phosphorimager (FLA-3000, Fujifilm).

Constructs used in the GST-pulldown assay (Insv 6–178, Insv 179–361, and Insv 248–361) were amplified from *insv* cDNA with primers containing *EcoRI* and *XhoI* restriction sites, and cloned into the corresponding sites of pGEX5X-2. *In vitro* translation was performed using pET-His-Su(H) (Lai *et al*, 2000b) or pET-Groucho (Jimenez *et al*, 1997).

ChIP-quantitative PCR

ChIP assays were performed as described (Negre *et al*, 2006) with a few modifications. In brief, 2.5–6.5 h embryos from *w¹¹¹⁸* (wild-type) or *insv^{23B}* homozygous mutants were homogenized and fixed in 1.8% formaldehyde in buffer A1 (60 mM KCl, 15 mM NaCl, 4 mM MgCl₂, 15 mM HEPES (pH 7.6), 0.5% Triton X-100, 0.5 mM DTT, and protease inhibitor cocktail (Roche)) for 15 min at room temperature. After three washes with buffer A1 and once with lysis buffer (140 mM NaCl, 15 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 1% Triton, and 0.5 mM DTT), the chromatin in lysis buffer containing 0.1% SDS was sheared by sonication on a Branson Digital Sonifier 450 machine to an average length of 0.5 kb. For each immunoprecipitation, 0.5 ml supernatant containing sheared chromatin was incubated overnight with 40 µl Gammabind G agarose (GE Healthcare), coated with BSA to remove non-specific background. The pre-cleared chromatin was then treated overnight with pre-absorbed anti-Insv antibody or pre-immune serum. The precipitated complexes were washed and eluted, and crosslinks were reversed for overnight at 65°C. After treatment with proteinase K, DNA was purified using QIAprep Spin columns (Qiagen) and recovered in 50 µl of elution buffer containing RNase A.

Real-time PCR was performed on a BIO-RAD CFX96 machine using Power SYBR Green (Applied Biosystems). Primers were designed with online tools (Genescript) and their sequences are listed in Supplementary Table 1. PCR was performed on 1 µl template DNA in triplicate samples, and immunoprecipitated DNA was compared against standard curves from serial dilutions of input DNA. The values are plotted as folds of enrichment normalized to the mock reference sample, and the standard deviation within the triplicate samples indicated. Similar results were obtained in independent ChIP experiments.

Supplementary data

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

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Author contributions: HD generated the Insv antibody and *insv* transgenes, and performed genetic analysis of *insv* mutants. QD helped with genetic analysis and performed the qPCR, GST-pulldown, and ChIP analyses. JK analysed Insv expression and helped with immunostainings. FB performed some of the *insv* gain-of-function analysis. GM helped to re-isolate *insv* mutations onto clean backgrounds. NN helped with ChIP analysis. ECL helped design experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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