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Suppressor of Hairless and *Presenilin* phenotypes imply involvement of canonical Notch-signalling in segmentation of the spider *Cupiennius salei*

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Abstract

Arthropods, vertebrates, and annelids all have a segmented body. Our recent discovery of involvement of Notch-signalling in spider segmentation revived the discussion on the origin of segmented body plans and suggests the sharing of a common genetic program in a common ancestor. Here, we analysed the spider homologues of the *Suppressor of Hairless* and *Presenilin* genes, which encode components of the canonical Notch-pathway, to further explore the role of Notch-signalling in spider segmentation. RNAi silencing of two spider *Suppressor of Hairless* homologues and the spider *Presenilin* homologue causes severe segmentation phenotypes. The most prominent defect is the consistent breakdown of segmentation after the formation of three (*Suppressor of Hairless*) or five (*Presenilin*) opisthosomal segments. These phenotypes indicate that Notch-signalling during spider segmentation likely involves the canonical pathway via *Presenilin* and *Suppressor of Hairless*. Furthermore, it implies that Notch-signalling influences both the formation and patterning of the spider segments: it is required for the specification of the posterior segments and for proper specification of the segment boundaries. We argue that alternative, partly redundant, pathways might act in the formation of the anterior segments that are not active in the posterior segments. This suggests that at least some differences exist in the specification of anterior and posterior segments of the spider, a finding that may be valid for most short germ arthropods. Our data provide additional evidence for the similarities of Notch-signalling in spider segmentation and vertebrate somitogenesis and strengthen our previous notion that the formation of the segments in arthropods and vertebrates might have shared a genetic program in a common ancestor.

Keywords: *Delta*; Somitogenesis; Arthropod evolution; *hairy*; *Drosophila*

Introduction

Segmentation is found in diverse animal phyla, including arthropods, annelids, and chordates. During the last two decades, genetic analyses in the fruit fly *Drosophila* (Pankratz and Jäckle, 1993; St. Johnston and Nüsslein-Volhard, 1992) and in various vertebrates (Holley and Takeda, 2002; Pourquié, 1999, 2001, 2003; Rida et al., 2004; Saga and Takeda, 2001) suggest fundamental differences in the underlying mechanisms of the segmentation processes in these different animal groups. However, this view has been challenged by our recent finding of the involvement of Notch-signalling in spider segmentation that shows similarities to Notch-signalling in vertebrate somitogenesis but that has not been described for *Drosophila* body segmentation (Stollewerk et al., 2003; see also Patel, 2003; Peel and Akam, 2003; Tautz, 2004).

Vertebrate somitogenesis involves a molecular oscillator–the so-called segmentation clock–that acts in the presomitic mesoderm (PSM) and is driven by Notch-signalling. This oscillator drives the cyclic expression of a number of genes in the PSM. Disruption of core components of Notch-signalling disturbs the cyclic gene expression and results in

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irregularly shaped somites and loss of rostro-caudal somite polarity (Holley and Takeda, 2002; Pourquié, 1999, 2001, 2003; Rida et al., 2004; Saga and Takeda, 2001).

The Notch-pathway is required for many different cell–cell signalling events during development and plays an important role in cell-type specification as well as in boundary formation events (Artavanis-Tsakonas et al., 1999; Lai, 2004). In the canonical mode of Notch-signalling, the Notch receptor is cleaved twice after binding of a ligand (Delta or Serrate/Jagged); the intracellular domain of Notch (NICD) subsequently locates to the nucleus. One of the cleavages of Notch is extracellular and is catalysed by members of the a disintegrin and metalloprotease (ADAM) domain family of proteases, whereas the other cleavage is intracellular and is mediated by a complex that contains members of the γ-secretase family and that depends on Presenillin (Mumm and Kopan, 2000). Loss of *Presenillin* function leads to *Notch*-like mutant phenotypes in *Drosophila*, mouse, and fish (Donoviel et al., 1999; Nornes et al., 2003; Struhl and Greenwald, 1999; Wong et al., 1997; Ye et al., 1999).

In the nucleus, NICD interacts with Suppressor of Hairless (Su(H)), a member of the CBF1, Su(H), Lag-1 (CSL) family of transcription factors that are highly conserved from human to *Drosophila* (Amakawa et al., 1993; Furukawa et al., 1991). This interaction converts the Su(H) protein from transcriptional repressor into transcriptional activator and results in the expression of *Notch* targets, like genes of the *h/E(spl)* family (Bray and Furriols, 2001; Furriols and Bray, 2000; Lai, 2002, 2004). The switch from repressor to activator involves distinct co-repressor and co-activator complexes. In absent of NICD, Su(H) associates with transcriptional co-repressors and actively keeps target gene expression switched off. Upon Notch activation, the Su(H) co-repressor complex is exchanged by a Su(H) co-activator complex coordinated by NICD.

The role of *Su(H)* during somitogenesis has been analysed in mouse and zebrafish (de la Pompa et al., 1997; Oka et al., 1995; Sieger et al., 2003). Somitogenesis is severely affected in *Su(H)* deficient embryos and the posterior somites do not form. The Morpholino knockdown approach in zebrafish furthermore demonstrated a disturbance of the cyclic expression of the *delta-C* gene and bHLH genes of the *h/E(spl)* family (Sieger et al., 2003). The defects in somite formation after *Su(H)* silencing are more severe than as seen in *Notch* and *Delta* mutants, nevertheless there is good evidence that the *Notch*-signal is mediated via *Su(H)* although additional *Notch*-independent pathways cannot be excluded (Sieger et al., 2003). Furthermore, the protease Presenilin is required for *Notch* activity in vertebrate somitogenesis (Donoviel et al., 1999; Nornes et al., 2003; Wong et al., 1997). Mice lacking *Presenilin* homologues are drastically shortened and fail to undergo proper segmentation; the somites are irregularly shaped and misaligned (Donoviel et al., 1999; Wong et al., 1997).

In the spider *Cupiennius salei*, Notch-signalling is involved in segmentation, showing a number of similarities to Notch-signalling in vertebrate somitogenesis (Stollewerk et al., 2003). The spider *Delta-1* gene is dynamically expressed in stripes in the growth zone of the embryo; this dynamic expression shows some similarities to the oscillating expression of the *delta-C* gene in the presomitic mesoderm of zebrafish. Furthermore, embryos depleted for *Notch* or *Delta* via RNAi show severe defects in segmental patterning. The phenotypes include malformations of the segments, fuzzy segmental boundaries, and an enlarged growth zone. In addition, the dynamic expression of the spider bHLH gene *hairy* (Damen et al., 2000) is disturbed and is no longer organised in stripes. But in contrast to vertebrates where Notch-pathway mutants cause also reduction in somite numbers, the number of segments is not altered in spider (Stollewerk et al., 2003).

To further analyse Notch-signalling in spider segmentation and to test whether Notch-signalling in the spider involves the canonical *Su(H)*-dependent pathway, we cloned and analysed the spider homologues of the *Suppressor of Hairless* (*Su(H)*) and *Presenilin* (*Psn*) genes. Knockdown analyses using RNAi for the spider *Su(H)* homologues *Cs-Su(H)-1* and *Cs-Su(H)-2* and the spider *Psn* homologue *Cs-Psn* show more severe developmental defects than observed by *Notch* or *Delta* RNAi. The most prominent defect is the consistent breakdown of the segmentation process after the formation of three (*Su(H)*) or five (*Psn*) opisthosomal segments. The *Su(H)* and *Psn* phenotypes indicate that Notch-signalling during spider segmentation likely involves the canonical pathway via *Psn* and *Su(H)* and further suggest that Notch-signalling influences both the formation of the segments and the proper specification of the segmental boundaries during spider segmentation. Furthermore, our data suggest that additional, partly redundant, pathways might act in the formation of the anterior segments that are not active in the posterior segments.

Material and methods

C. salei stocks

Fertilised females of the Central American wandering spider *C. salei* Keyserling (Chelicerata, Arachnida, Araneae, Ctenidae) were obtained from our colony bred in Cologne. Embryos were collected and treated as described before (Damen and Tautz, 1998).

Cloning of genes

Fragments for spider genes were obtained by RT-PCR (Damen et al., 2000). The oligonucleotide primers used in the initial PCR for *Su(H)-1* were *Su(H)-fw-1* (5′-CAY GCN AAR GTN GCN CAR-3′) and *Su(H)-bw-1*(5′-TC NGT NSW DAT DAT NGT CCA-3′). In a subsequent semi-

nested PCR, the primers *Su(H)-fw-1* and *Su(H)-bw-2* (5′-TG NSW NAC NGG RTC RTC NGC-3′) were used. In addition, the degenerated primers published by Sieger et al. (2003) were used to recover a second fragment (*Su(H)-2*). A larger fragment of *Su(H)-2* was obtained by RACE-PCR (Marathon cDNA amplification kit, Clontech).

For *Psn*, the primers *Psn-fw-1705* (5′-TAY GGN GCN MAR CAY GTN AT-3′) and *Psn-1715* (5′-GG NAR RTA YTT DAT RAA NAC-3′) were used in the initial PCR and the primers *Psn-1705* and *Psn-1714* (5′-AR NGG NCC YTK CCA RTG DAT-3′) were used in the subsequent semi-nested PCR.

Sequences were determined from both strands on an ABI-3100 automated sequencer (Applied Biosystems), using Big Dye dye-determinators (Perkin-Elmer). The sequences are available under accession numbers AJ717513 (*Cs-Su(H)-1*), AJ717514 (*Cs-Su(H)-2*), and AJ717515 (*Cs-Psn*).

Sequence alignments

Sequences were aligned using ClustalX (Thompson et al., 1994) and BLOSUM matrix with a gap penalty of 20 and a gap extension of 0.2.

In situ hybridisation and DAPI staining

Whole-mount in situ hybridisations were essentially performed as described for *Drosophila* (Klingler and Gergen, 1993; Tautz and Pfeifle, 1989) with modifications for spider embryos (Damen and Tautz, 1998, 1999). The 4,6-diamidino-2-phenylindole (DAPI) staining for the spider has been described previously (Damen and Tautz, 1999).

Double-stranded RNA interference (RNAi)

Preparations of double-stranded RNA (dsRNA), injections, and further treatment of embryos were performed as described before (Schoppmeier and Damen, 2001). As template for the dsRNA, we used the 663 bp fragment of *Su(H)-1*, the 1161 bp 3′RACE fragment of *Su(H)-2*, and the 405 bp fragment of *Psn*. As control, we injected 1000 bp dsRNA of the jellyfish *GFP* gene. The embryos were analysed for morphology of the germ band and segmental boundaries by staining for *engrailed* (*Cs-en*) and DNA (DAPI).

Results

Spider Suppressor of Hairless and Presenilin homologues

cDNA fragments of two different *Su(H)* genes, *Cs-Su(H)-1* and *Cs-Su(H)-2*, have been recovered from the spider *C. salei*. The two fragments were isolated by RT-PCR using different sets of primers. The 663 bp *Cs-Su(H)-1* cDNA fragment encodes an incomplete deduced protein fragment of 221 amino acids. The 1161 bp of *Cs-Su(H)-2* sequence is incomplete at its 5′ end and contains an open reading frame from nucleotide 1–1029 bp, encoding a deduced protein of 343 amino acids, and 100 bp of 3′UTR followed by short poly-A stretch. Sequence analysis using BLAST (Altschul et al., 1997) and ClustalX (Thompson et al., 1994) shows that proteins encoded by both spider genes exhibit high similarities to other CBF1, Suppressor of Hairless, Lag-1 (CSL) proteins indicating that *Cs-Su(H)-1* and *Cs-Su(H)-2* are the homologous genes of the spider.

The sequence alignment of Su(H)/CSL proteins shows that the proteins are highly conserved among arthropods and vertebrates. The available sequences of the *Cs*-Su(H)-1 and *Cs*-Su(H)-2 protein fragments show 95.7% identity to each other (Fig. 1A). *Cs*-Su(H)-1 and *Cs*-Su(H)-2 display 92.4% and 93.6% identity, respectively, to the corresponding regions of the *Drosophila* Su(H) protein. In comparison to the corresponding region of the mouse CSL protein RBP-jκ, *Cs*-Su(H)-1 is 90.1% identical and *Cs*-Su(H)-2 is 92.9% identical.

A 405 bp fragment of the spider orthologue of the *Drosophila Presenilin* gene has been recovered by RT-PCR. The 135 amino acid *Cs*-Psn protein fragment deduced from this sequence corresponds to amino acids 99–234 of the *Drosophila* Psn protein. Sequence analyses show that the *Cs*-Psn fragment is 69% identical to the corresponding region of the *Drosophila* Psn protein and also shares high similarities to chordate and mollusc Psn sequences (Fig. 1B).

Expression of spider Su(H) and Psn genes

The spider body consists of two tagmata: a prosoma (cephalothorax), that bears six pairs of appendages (chelicers, pedipalps, and four pairs of walking legs), and an opisthosoma (abdomen), that consists of twelve segments. The opisthosomal segments are sequentially added from a posterior growth zone (Seitz, 1966). As a first step to analyse whether spider *Su(H)* and *Psn* homologues are involved in spider segmentation, we have studied their expression by whole mount in situ hybridisation.

In situ hybridisation shows that there is a weak ubiquitous expression of *Cs-Su(H)-1*, *Cs-Su(H)-2*, and *Cs-Psn* at the earliest available stages (Figs. 2A–C). The transcripts are present in both the already formed segments and the growth zone. In these early stages, there is, in addition, some accumulation of *Su(H)-2* transcripts in the posterior regions of the last two formed segments and within the anterior region of the growth zone (Fig. 2B, arrowheads). Embryos of earlier stages, at which the prosomal segments form, are not accessible for in situ hybridisation. It therefore remains unsolved when the expression of these genes starts in the prosomal segments.

During later stages of development, the *Su(H)* genes and the *Psn* gene are still ubiquitously expressed, but there are

A Su(H)

```
Cs-1  AAKTLFISDSDKRKHFMLSVKMFYANGEDLGMFQSKRIKVISKPSKKKQSLKNADLCIASGTKVALFNRLRSQTVSTRYLHVENG
Cs-2                       -----G----I-V-H----------------I-----------------------------------
Dm    ------------------------G--H-I-V-N--------------------------N----------------------
Ag    ------------------------GS-H-I-V-------------------------------------------------N-
Mm    T----Y-----------------G-SD-I-V-L-----------------------------------------------G-

Cs-1  NFHASSTQWGAFTIHLLDDNESESEEFTVRDGYIHYGSTVKLVCSVTGMALPRLIIRKVDKQTALLDADDPVWQ  (aa73-231)
Cs-2  -------------------T-------------------------------------------------S-   (aa1-140)
Dm    H--------------------------Q---------A----------------------M---E-----S- (aa198-356)
Ag    H--------------------------Q-----V---A----------------V-------M---E-----S- (aa112-270)
Mm    ------Q-----Y------D---G-------------Q-----------------------------------S- (aa150-308)
```

B Psn

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Cs    YGAQHVIKLFIPVSLCMLVVVATTNSITFYTQTNTYLVYTPFTDQTVDTPTKVWQSFANAFILMGVIVCMTILLILLYKFRWYKV
Dm    ----------V------------I---S--NS-DV--L----HE-SPEPSV-F-SAL--SL---S-V-V--F---V---K-C-RI
Bf    ---K---M--A------------IS------EKNG--I----HEEGAS-ASK-GD-L-NGA-MV---LV--VF-V----Y-C-F-
Mm    ---K---M--V--T---I-----IK-VR---EKNGQ-I-----ED-PSVGQRLLN-VL-TL-MIS---V---F-VV---Y-C--F
Hl    ---K---M-----T---V-----IS---Y--SGGV--I----H-V-E--G--L---M---V--LC---V--VV-L----YKC-R-

Cs    IHGWLIISSLMLLFLFAYIYLGEVLRAYNIPMDYITVSIIMWNFGVVGMM      (aa1-135)
Dm    ------L--F----I-T-L--E-L----------P--ALL----------     (aa99-234)
Bf    ------L------------------Q----------LA-V-----A---V     (aa89-224)
Mm    ------M---------T-I------KT--V----P-LFLAV----A---V     (aa83-216)
Hl    -N---VL--I----F-SFM--EQI-----A----V--A--------G-LF    (aa114-245)
```

Fig. 1. Alignment of Suppressor of Hairless (Su(H)) protein sequences (A) and Presenilin (Psn) protein family sequences (B). Dashes indicate identical amino acids (aa). Accession Numbers: *Cs*-Su(H)-1 AJ717513, *Cs*-Su(H)-2: AJ717514, *Dm*-Su(H): NP_788069, *Ag*-Su(H): XP_319690, *Mm*-RBP-jκ: NP_033061, *Cs*-Psn: AJ717515l *Dm*-Psn: NP_524184, *Bf*-Psn: AAL4014, *Mm*-Psen2: AAH10403, *Hl*-Psn: AAG28518. *Cs*: *C. salei*, *Dm*: *Drosophila melanogaster*, *Ag*: *Anopheles gambiae*, *Bf*: *Brachiostoma floridae*, *Mm*: *Mus musculus*, *Hl*: *Helix lucorum*.

additional places with stronger expression of *Su(H)* genes, such as in the head lobe, in spots in neuro-ectodermal tissue, in the developing heart precursors, in particular cells of the leg that presumably represent peripheral nerve cells, and in rings in the forming appendages (not shown). These patterns differ for *Su(H)-1* and *Su(H)-2* transcripts. The observed expression patterns resemble the situation in vertebrate and *Drosophila* development where *Su(H)* homologues are up-regulated in some tissues of higher *Notch* activity (Oka et al., 1995; Schweisguth and Posakony, 1992; Sieger et al., 2003).

Su(H) RNAi severely interferes with segment formation

To interfere with the function of *Su(H)* genes in the spider, we injected double-stranded RNA (dsRNA) corresponding to *Su(H)-1* and *Su(H)-2* into embryos. RNAi for either *Su(H)-1* or *Su(H)-2* resulted in severe but identical developmental defects (Figs. 3D–I, Table 1). The segmentation process breaks down after the formation of the third opisthosomal segment (Figs. 3F,I). The embryos essentially stop growing and no additional segments are added from the growth zone. The remaining

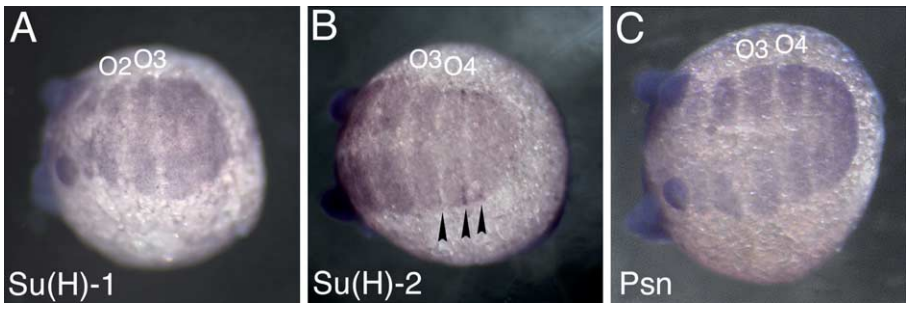

Fig. 2. Expression of *Su(H)* and *Psn* in the spider *C. salei*. Expression of *Cs-Su(H)-1* (A), *Cs-Su(H)-2*, (B) and *Cs-Psn* (C) in young spider embryo (stage with three to four opisthosomal segments). Only the posterior segments and the growth zone are shown. All three genes are expressed uniformly in prosomal and opisthosomal segments and in the growth zone. There is an accumulation of *Cs-Su(H)-2* in the posterior region of the last two formed segments and within the anterior region of growth zone (arrowheads in panel B). All panels: anterior to the left. O: opisthosomal segment.

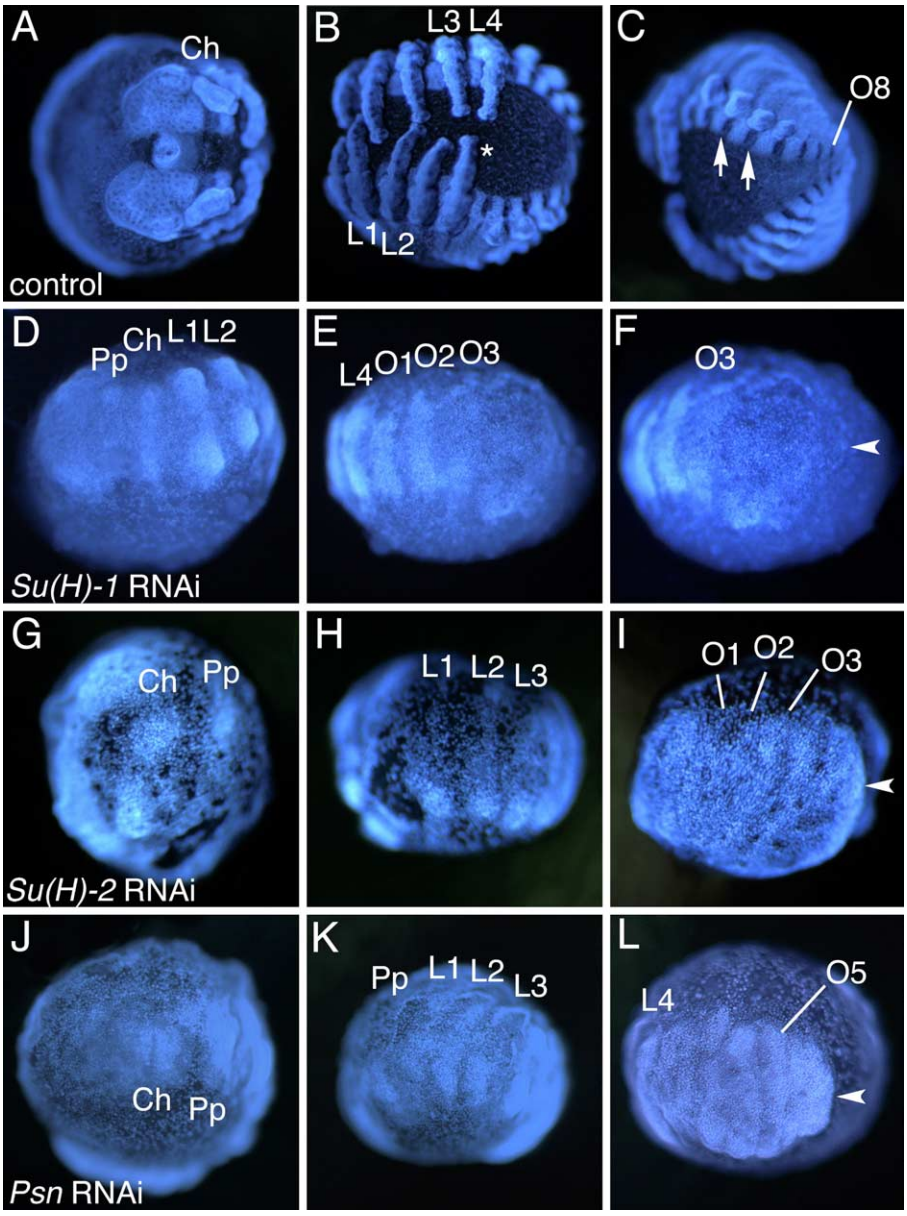

Fig. 3. *Su(H)* and *Psn* RNAi result in segmentation phenotypes. Embryos were injected with dsRNA for *GFP* (control) (A–C), *Cs-Su(H)-1* (D–F), *Cs-Su(H)-2* (G–I), or *Cs-Psn* (J–L). All panels show epi-fluorescence images. Embryos are stained with DAPI (bright blue staining) and for the segmental marker *engrailed* (dark blue staining). (A–C) Head, prosomal, and opithosomal view respectively of the same embryo after control injection with *GFP* dsRNA. The embryo displays a wild-type phenotype, *engrailed* expression is in the posterior portion of the segments (arrows in panel C) and the appendages (asterisk in panel B); *engrailed* staining is obvious as dark blue staining that quenches the bright fluorescent DAPI staining. (D–F) Embryo injected with dsRNA for *Cs-Su(H)-1*. The head region is malformed (D) and the appendages are dramatically shortened (D, E). Only the first three opisthosomal segments (O1–O3) form (E). The segments are reduced in size and width, the growth zone is enlarged. The posterior end of the embryos is marked by an arrowhead (F). The *engrailed* staining is completely absent. (G–I) *Cs-Su(H)-2* RNAi embryo with segmentation phenotypes similar to those of *Cs-Su(H)-1*. (G) Head region, (H) prosomal segments, (I) opisthosomal segments and the growth zone. Again, the *engrailed* staining is absent. (J–L) Embryo injected with dsRNA corresponding to *Cs-Psn*. The head lobe is deformed (J), appendages are reduced (J, K), and the segments are malformed and vary in size and width (K). Segmentation stops after the formation of the fifth opisthosomal segment (L). No *engrailed* transcripts are detectable. Arrowheads in panels F, I, and L point to the end of the growth zone. All panels: anterior to the left. Ch: chelicere, Pp: pedipalp; L: walking leg; O: opisthosomal segment.

growth zone is enlarged (Figs. 3F,I). From the DAPI staining, it becomes obvious that the growth zone of the *Su(H)* RNAi embryos is less compact and seems to consists of loosely aggregated cells (Figs. 3F,I). The segments that have formed are irregularly shaped and reduced in size. Their segmental borders are not properly arranged and are less defined compared to control embryos (Figs. 3B,D,H). In addition, the cephalic lobe is reduced and the appendages are reduced or even absent. We do not observe intermediate or mosaic phenotypes; the embryos either stop segmentation after the third opisthosomal segment (34% and 37% after

Table 1
Effects of *Su(H)-1*, *Su(H)-2*, and *Psn* RNAi in spider embryos

	Total (n)	Segmentation phenotype	No effects	Unspecific effects
No injection	71	0 (0%)	65 (91.5%)	6 (8.5%)
GFP dsRNA	98	0 (0%)	88 (90%)	10 (10%)
Su(H)-1 dsRNA	180	61 (34%)	102 (57%)	17 (9%)
Su(H)-2 dsRNA	114	42 (37%)	58 (51%)	14 (12%)
Su(H)-1 + Su(H)-2 dsRNA	86	35 (41%)	45 (52%)	6 (7%)
Psn dsRNA	152	75 (49%)	65 (43%)	12 (8%)

The table shows the number and percentage of embryos that display segmentation defects after the injection of double-stranded RNA (dsRNA). "Segmentation phenotype" is defined as block of segmentation after the formation of the third opisthosomal segment in case of Su(H) RNA injection or block of segmentation after the formation of the fifth opisthosomal segment in case of Psn dsRNA injection. "No effect" embryos develop same number of segments as in control embryos. Control embryos were either not injected ("No injection") or injected with dsRNA corresponding to the jellyfish *GFP* gene.

Su(H)-1 or *Su(H)-2* RNAi respectively) or do not show a segmentation phenotype and form the same number of segments as the control embryos (57% and 51% respectively for *Su(H)-1* and *Su(H)-2)* (Table 1). These uniform defects demonstrate that the observed segmentation blockade after *Su(H)* RNAi is consistent and specific.

RNAi for *Su(H)-1* and *Su(H)-2* showed identical defects. We co-injected dsRNA corresponding to *Su(H)-1* and *Su(H)-2* to analyse whether we could detect cumulative defects. These double RNAi embryos displayed the same developmental defects as described for the single injections of either *Su(H)-1* or *Su(H)-2* dsRNA (Table 1). The co-injection of dsRNA for both *Su(H)* genes thus does not result in additional defects. The most likely explanation is that both genes are targeted and down-regulated by injection of dsRNA complementary to either one of the *Su(H)* transcripts as particular regions in the *Su(H)-1* and *Su(H)-2* sequences are almost identical at the DNA level (not shown). Injection of dsRNA directed against just one of the spider *Su(H)* transcripts thus likely results in targeting of both transcripts by the RNAi machinery and leads to the silencing of both *Su(H)* genes. Similarly, a silencing mechanism has been proposed for related genes in *Caenorhabiditis elegans* (Parrish et al., 2000).

Su(H)-1 and *Su(H)-2* RNAi embryos have also been analysed for expression of the segmental marker *engrailed* (Fig. 3). In normal development, the *engrailed* gene is expressed in segmental stripes that define the parasegmental boundary (Figs. 3A–C) (Damen, 2002). However, in *Su(H)-1* and *Su(H)-2* RNAi embryos, we could not detect traceable amounts of *engrailed* transcripts (Figs. 3D–I). The lack of *engrailed* expression after *Su(H)* RNAi is a more severe defect compared to the effect after *Notch* or *Delta* RNAi; in *Notch* and *Delta* RNAi embryos, the segments do still express *engrailed*, although the *engrailed* stripes are not as well-defined as in control embryos (Stollewerk et al., 2003).

Down-regulation of dynamic expression of Cs-hairy after Su(H) RNAi

Silencing of *Notch* or *Delta* results in a disorganisation of the expression of the bHLH gene *hairy* in the growth zone of the spider embryo. In normal development, *hairy* is expressed in a dynamic way in stripes in the growth zone (Damen et al., 2000), but, in *Notch* or *Delta* RNAi embryos, the *hairy* expressing cells are no longer organised in stripes and the *hairy* gene is expressed in a salt-and-pepper pattern in the growth zone (Stollewerk et al., 2003). In contrast to *Notch* or *Delta* RNAi, *Su(H)* RNAi results in a dramatic down-regulation or even complete absence of *hairy* expression (Figs. 4E–L), the growth zone is almost free of staining (Figs. 4G, L). Thus, although these embryos still have a growth zone, the *hairy* gene is no longer expressed in this growth zone or its expression is strongly reduced and unorganised. The already formed segments neither show any sign of organised *hairy* expression (Figs. 4E,I); these segments normally show a secondary phase of expression of *hairy* predominantly in cells of the forming central nervous system (Fig. 4C).

The spider hairy promoter region contains a putative Su(H) binding site

The *Su(H)* RNAi strongly suggests a regulation of the spider *hairy* gene via *Su(H)*. To obtain additional evidence for the involvement of *Su(H)* in the regulation of *hairy* in the spider, we searched the 5′ regulatory region of the spider *hairy* gene for presumptive Su(H) binding sites. One putative *paired-Su(H)-binding-site* (SPS) is present within the available almost 8 kb of the 5′ regulatory sequence (our unpublished data) of the spider *hairy* gene (Fig. 5). SPS sites are specific enhancer elements in target genes with which Su(H) interacts (Bailey and Posakony, 1995; Gajewski and Voolstra, 2002). SPS sites consist of two Su(H) binding sites with an additional invariant element, the SPS Hexamer. The motif is highly conserved and has been identified in the regulatory region of various *Su(H)*-dependent genes like the *Drosophila E(spl)* genes or some vertebrate bHLH genes (Gajewski and Voolstra, 2002). The presumptive SPS in the spider bHLH gene *hairy* shows high similarity to SPS sites in *Drosophila* and vertebrate bHLH genes (Fig. 5). The functionality of the SPS site in the spider *hairy* gene remains to be tested. Nonetheless, the presence of a SPS site in the *hairy* gene provides additional evidence that the spider *hairy* gene likely is regulated via *Su(H)*.

Disruption of dynamic gene expression of Delta-1 after Su(H) RNAi

We also analysed the expression of the *Delta-1* gene in *Su(H)* RNAi embryos. The spider *Delta-1* gene, similar as the spider *hairy* gene, is expressed in a dynamic way in

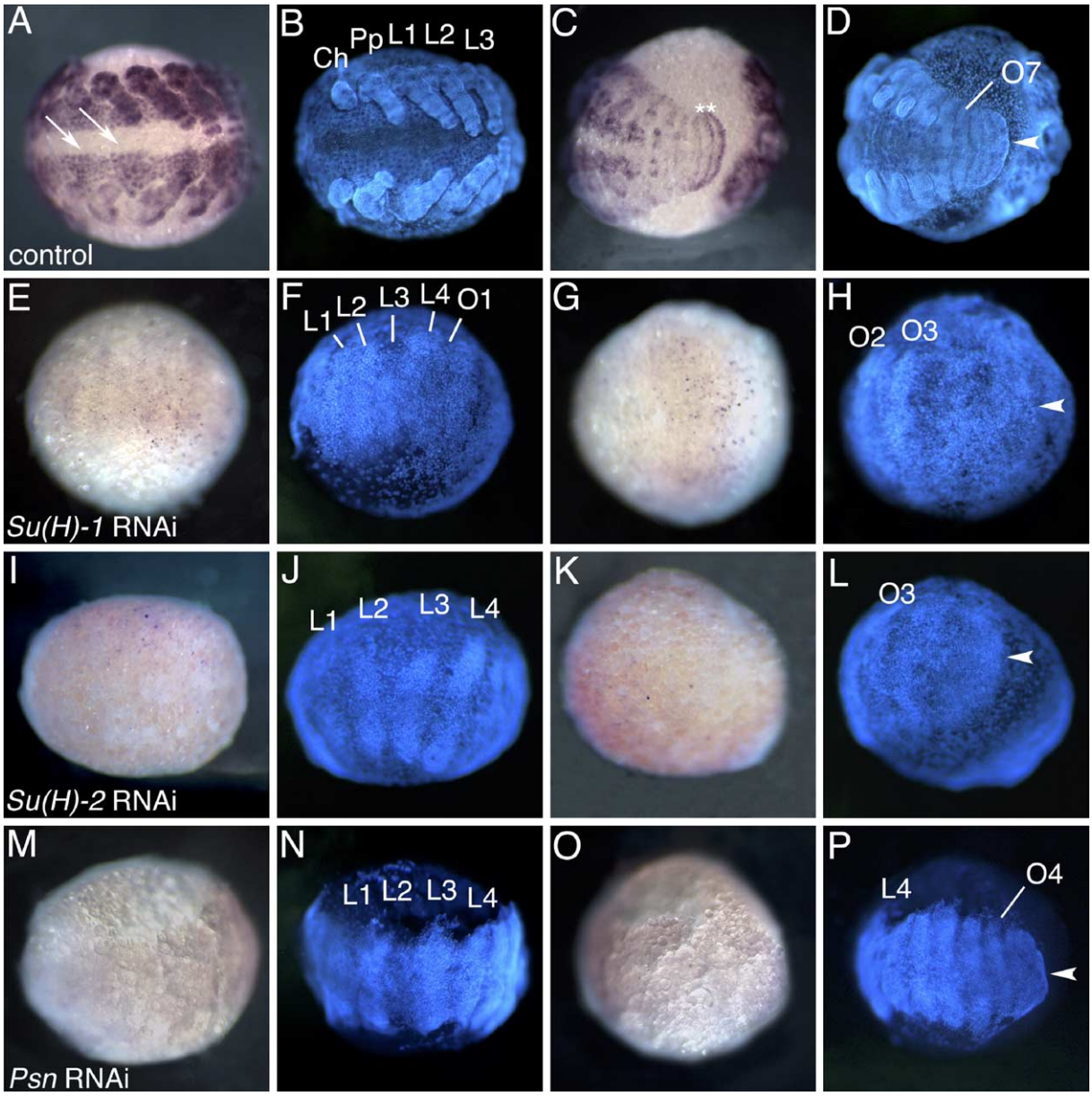

Fig. 4. The expression of *hairy* in *Su(H)* and *Psn* RNAi embryos is lacking. Embryos injected with dsRNA for *GFP* (control) (A–D), *Cs-Su(H)-1* (E–H), *Cs-Su(H)-2* (I–L), and *Cs-Psn* (M–P). All embryos were stained for the spider *hairy* gene by in situ hybridisation. The panels show bright field (A, C, E, G, I, K, M, O) and epi-fluorescence images of the same embryos (B, D, F, H, J, L, N, P) respectively. (A–D) *GFP* dsRNA, prosomal (A, B) and opisthosomal (C, D) view of the same embryo. (A,B) *hairy* transcripts are present in the developing nervous system (arrows) and in the appendages. (C, D) Expression of *hairy* is stripes in the growth zone (C, asterisks). (E–H) *Cs-Su(H)-1* RNAi embryo, prosomal (E, F) and opisthosomal view (G, H). The *hairy* gene is no longer expressed, neither in the nervous system nor in the growth zone (E, G). (I–L) *Cs-Su(H)-2* RNAi embryo, prosomal (I, J) and opisthosomal (K, L) view. Embryos no longer express *hairy* (I, K). (M–P) *Cs-Psn* RNAi embryo, prosomal (M, N) and opisthosomal (O, P) view. Similar as in the Su(H) RNAi embryos, there is neither in the nervous system nor in the growth zone *hairy* expression after *Psn* RNAi. Arrowheads in panels D, H, L, and P point to the posterior end of the growth zone. All panels: anterior to the left. Ch: chelicere; Pp: pedipalp; L: walking leg segment; O: opisthosomal segment.

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Cs-hairy        TGTGGGAGCCTGAGAGCGTGTGTAGCCTCACACTTTCCCACG
Dm-E(spl)m8     TGTGGAGAAACTTACTTTCAGT---------GGGTTCCCACG
Xl-hairy-2      CGTGGGAAAGAATGCA-GTAAGGT----------TTCACACG
Dr-her1/7       GGTGAGAGAGTGTGTGCTAAAGTTTCAA-----ATTCCACC-
Fr-her1/5       TGTGGGAAACTTTGCCGCATGAGA-------ACTTTCACACA
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Fig. 5. The spider *hairy* gene contains a putative Su(H) binding site. Alignment of the presumptive *Su(H) paired binding site* (SPS) of the spider *hairy* gene with SPS sequence of other bHLH genes. The SPS consist of two Su(H) binding sites (**in bold**) with an additional invariant element, the SPS Hexamere (underlined). The sequence between the two Su(H) binding sides is variable. Dashes indicate gaps introduced for alignment. The information for *Dm*, *Xl*, *Dr*, and *Fu* is adapted from Gajewski and Voolstra (2002). *Cs*: *C. salei*, *Dm*: *Drosophila melanogaster*, *Xl*: *Xenopus laevis*, *Dr*: *Danio rerio*, *Fr*: *Fugu rubripes*.

stripes in the growth zone (Stollewerk et al., 2003). After knocking down either *Su(H)-1* or *Su(H)-2*, the *Delta-1* expression is disturbed (Figs. 6G,K). The dynamic pattern in stripes in the growth zone breaks down and *Delta-1* is no longer expressed in stripes (Figs. 6E–L), indicating that the dynamic expression of *Delta-1* in stripes depend on *Su(H)*. Furthermore, there is no longer specific expression of *Delta-1* in the developing central nervous system (Figs. 6E,I). Instead, *Delta-1* transcripts are present throughout the whole germ band in an unorganised fashion (Figs. 6E–L). However, in contrast to *hairy* expression, *Delta-1* expression appears not be down-regulated in *Su(H)* RNAi embryos.

Presenilin RNAi results in segmentation defects

To further explore the role of Notch-signalling in spider segmentation, we analysed the spider *Presenilin* gene.

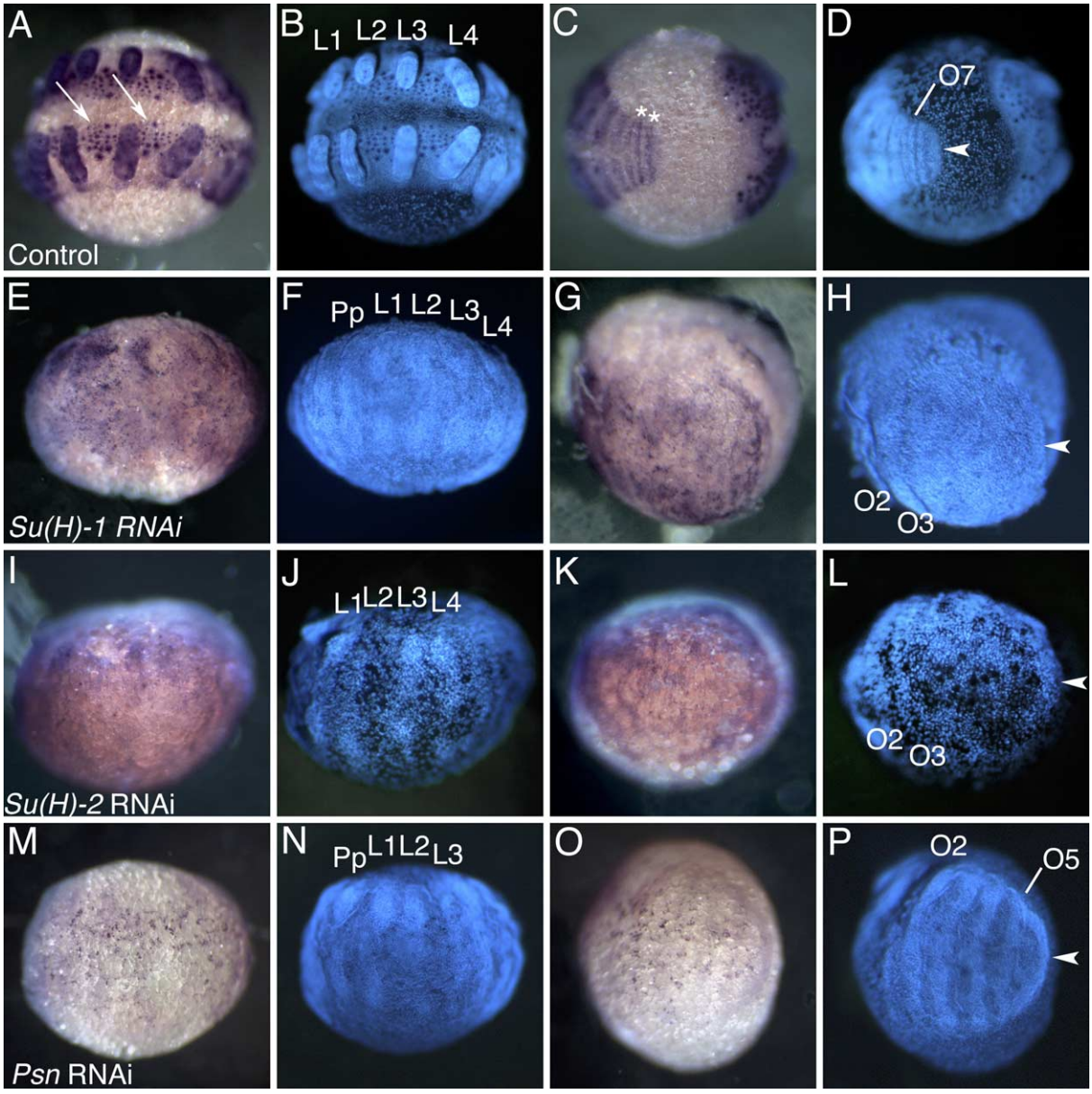

Fig. 6. The expression of *Delta-1* is disturbed after *Su(H)* and *Psn* RNAi. Embryos were injected with dsRNA for *GFP* (control) (A–D), *Cs-Su(H)-1* (E–H), *Cs-Su(H)-2* (I–L), and *Cs-Psn* (M–P). All embryos were stained for in situ hybridisation of the spider *Delta-1* gene. The panels show bright field (A, C, E, G, I, K, M, O) and epi-fluorescence image of the same embryos (B, D, F, H, J, L, N, P) respectively. (A–D) Prosomal (A, B) and opisthosomal (C, D) view of the same control embryo. (A, B) *Delta-1* is expressed in the developing nervous system (arrows) and in the appendages. In the growth zone (C, D), *Delta-1* is expressed in stripes (asterisks). (E–H) *Delta-1* expression in *Cs-Su(H)-1* RNAi embryo, prosomal (E, F) and opisthosomal view (G, H). The *Delta-1* staining is disturbed and no longer organised. *Delta-1* transcripts accumulate in an unspecific way in both the segments and the growth zone. (I–L) *Delta-1* expression in *CsSu(H)2* RNAi embryo, prosomal (I, J) and opisthosomal (K, L) view. (M–P) *Delta-1* expression in *Cs-Psn* RNAi embryo. Hardly any expression of *Delta-1* is visible. Arrowheads in panels D, H, L, and P point to the posterior end of the growth zone. All panels: anterior to the left. Pp: pedipalp; L: walking leg segment; O: opisthosomal segment.

Presenillin is another component of the Notch-signalling pathway and encodes a protease that is involved in the intracellular cleavage of the Notch receptor and causes the release the Notch intracellular domain NICD (Mumm and Kopan, 2000).

Injection of dsRNA corresponding to *Cs-Psn* (Figs. 3J–L) results in severe segmentation phenotypes. However, there are some differences compared to the RNAi phenotype observed for *Su(H)*. Like in *Su(H)* RNAi embryos, the segmentation process breaks down, but after *Psn* RNAi, this breakdown takes place after the formation of the fifth opisthosomal segment (Fig. 3L), while in *Su(H)*, RNAi segmentation stops after the formation of the third opisthosomal segment (Figs. 3F,I). The defects detected in the segments that form are similar in *Psn* and *Su(H)* RNAi embryos. The segments are irregularly shaped and reduced in size and width (Figs. 3J–L). The appendages are shortened or even absent and the cephalic lobe is malformed. Furthermore, the segmental borders are not sharp and the *engrailed* gene is no longer expressed after *Psn* RNAi (Figs. 4M–P). Similar as for *Su(H),* we do not observe mosaic or intermediate phenotypes. The formation of segments stops either after the fifth opisthosomal segment (49%) or the embryos develop the normal number of segments (43%) (Table 1). We did not observe embryos with an in-between number of segments. Thus, also the observed segmentation blockade after *Psn* RNAi is consistent and specific.

Apart from differences in the moment of segmentation breakdown, there is another apparent difference between *Su(H)* and *Psn* RNAi embryos. The growth zone of *Psn* RNAi embryos is not enlarged and still is compact (Fig. 3L), in contrast to the enlarged growth zone with loosely arranged cells in *Su(H)* RNAi embryos (Figs. 3F,I).

Presenilin RNAi interferes with the dynamic gene expression of hairy and Delta

Similar as after *Su(H)* RNAi, we analysed the expression of the *hairy* and *Delta-1* gene after *Psn* RNAi. Silencing of *Psn* completely blocks the *hairy* expression (Figs. 4M–P), similar as in *Su(H)* RNAi. Both the dynamic *hairy* expression in the growth zone (Fig. 4O) as well as the expression in the anterior segments is abolished (Fig. 4M). As for *hairy* expression, there is hardly any *Delta-1* expression detectable in the growth zone after *Psn* RNAi (Fig. 6O), however, some *Delta-1* transcripts can still be detected in the anterior segments of *Psn* RNAi embryos, but the level of expression is very low and in a dot-like random scattered pattern (Figs. 6M–P). This remaining *Delta-1* expression presumably forms remnants of the nervous system expression (Fig. 6A). In conclusion, both *Psn* and *Su(H)* are essential for the dynamic expression of *hairy* and *Delta-1* in the growth zone of the spider embryo.

Discussion

Su(H) and Presenillin genes are essential for spider segmentation and suggest a role of Notch-signalling in spider segment formation and segment boundary specification

The present analysis of the *C. salei Su(H)* and *Psn* orthologues clearly demonstrates that these genes are necessary for spider segmentation. Both *Su(H)* and *Psn* are components of the Notch-signalling pathway and are highly conserved among arthropods and vertebrates. The sequence conservation of *Su(H)* and *Psn* genes in combination with their phenotypes suggests that the canonical or "standard" Notch-pathway is used in spider segmentation.

The *Su(H)* and *Psn* phenotypes infer that Notch-signalling influences both segment formation and segmental boundary specification during spider segmentation. First, Notch-signalling has a crucial role in the specification of the posterior segments since these segments do not form after *Su(H)* or *Psn* silencing; this goes together with a severe disturbance of the dynamic expression of *hairy* and *Delta-1* in the growth zone. Second, Notch-signalling plays an important role in the specification of the segmental borders. The anterior segments that still form after blocking Notch-signalling require Notch-signalling for the proper formation of the segmental borders as becomes obvious from the fuzzy segment borders and the missing *engrailed* expression. An alternative explanation for the misformed segments and the lack of *engrailed* expression would be that the RNAi effects are not fully penetrant and that a partial silencing leads to the formation of improperly patterned segmental primordia. However, in the case of such a partial disruption of Notch-signalling, one would expect that the effects display mosaic phenotypes and variation in the number of segments that form. The latter is clearly not the case, as no variance in the number of formed segments is observed after *Su(H)* or *Psn* RNAi. The affected embryos consistently form three (*Su(H)*) or five (*Psn*) segments. The most likely explanation therefore is that Notch-signalling influences (1) the prepatterning of the posterior segments in the unsegmented growth zone and (2) proper specification of segment borders. A comparison with vertebrate somitogenesis shows that two crucial phases of Notch-signalling can be distinguished in vertebrate somitogenesis: first, in prepatterning of the unsegmented presomitic mesoderm (PSM) and, second, in formation of the somite borders (reviewed in Pourquié, 2003; Rida et al., 2004; Saga and Takeda, 2001). The two phases of involvement of Notch-signalling in vertebrate somitogenesis show remarkable similarities to what we find in spider segmentation. In the first phase, it is required for the prepatterning of unsegmented tissue: the growth zone (spider) or the PSM (vertebrates). Interference with Notch-signalling leads to a disturbance of this patterning and is also obvious from the disturbed expression patterns of *hairy/E(spl)* and *Delta* genes in both systems. In

the second phase, Notch-signalling is required for proper formation of the segment or somite borders. Notch-signalling thus seems to play comparable roles in vertebrate somitogenesis and spider segmentation.

Loss of anterior–posterior polarity of the segments

Comparisons reveal a number of similarities between *Su(H)* phenotypes in the spider and those seen in mouse mutants or zebrafish Morpholino knockdown embryos (de la Pompa et al., 1997; Oka et al., 1995; Sieger et al., 2003). Morpholino knockdown of *Su(H)* results in developmental defects in zebrafish; somitogenesis stops after the formation of five to seven somites, while the somites that form are irregularly shaped (Sieger et al., 2003). These defects are comparable to those seen in the spider. Interestingly, there is, in addition, a disturbance of the anterior–posterior polarity of the zebrafish somitic tissue. Similar phenotypes are observed in mice mutant for the *Su(H)* homologue *RBP-jκ* where the identity of the posterior somite halves is lost (de la Pompa et al., 1997; del Barco Barrantes et al., 1999; Oka et al., 1995).

The anterior–posterior polarity of the spider segments might also be lost after *Su(H)* and *Psn* silencing as these segments lack expression of the segment-polarity orthologue *engrailed.* The *engrailed* gene not only defines the parasegmental boundaries, but also the anterior–posterior polarity of the segments (Damen, 2002; Martinez-Arias and Lawrence, 1985). Thus, the absence of *engrailed* expression suggests that specification of segment-polarity may form an additional role of Notch-signalling in spider segmentation.

Differences in specification of anterior and posterior segments?

The specific and consistent breakdown of segmentation in *Psn* and *Su(H)* RNAi points to a difference in the specification of anterior and posterior segments. *Su(H)* RNAi embryos still form nine segments: the six appendage-bearing prosomal segments as well as the first three opisthosomal segments. *Cupiennius* normally forms twelve opisthosomal segments (Damen, 2002) implying that the nine most posterior segments do not form in *Su(H)* RNAi embryos. The number of segments missing in RNAi embryos is very consistent and there is no variation or grading in the number of affected segments. There seems to be a well-defined distinction between the anterior segments that still can form and the posterior segments that do not form at all after *Su(H)* RNAi. It is unlikely that these differences between anterior and posterior segments are caused by incomplete silencing as in this case one would expect more variation in the number of segments that forms after RNAi. Currently, however, no antibodies are available to test the remaining protein level and to finally exclude this possibility. The most likely explanation in our opinion therefore is that these nine posterior segments have a different dependency on *Su(H)* than the more anterior segments. This would suggest that the specification of the anterior segments is either independent of *Su(H)* and *Psn* implying that Notch-signalling is only required for proper segment borders in these segments, or alternatively, additional redundant and *Su(H)*-independent pathways act in these anterior segments that are not active in the formation of the posterior segments. Additional experiments are required for final prove and to distinguish between the two possibilities.

The comparison with vertebrate somitogenesis again shows similarities as similar differences have been described for the formation of the anterior and posterior somites. One of the effects of mutations in Notch-pathway components is the loss of somites (Conlon et al., 1995; de la Pompa et al., 1997; Oka et al., 1995; Sieger et al., 2003; van Eeden et al., 1996). The most prominent example is observed in zebrafish embryos after *Su(H)* Morpholino knockdown, when only the first five to seven somites form. The authors concluded that additional pathways might be active in the anterior somites (Sieger et al., 2003). This situation is very similar to what we observe in *Su(H)* RNAi the spider. The knockdown of *Su(H)* thus leads to similar phenotypes in both the spider and vertebrates suggesting the existence of additional *Su(H)*-independent pathways for patterning anterior segments in both systems.

Within arthropods, there is additional evidence for differences in the mechanisms for making anterior and posterior segments. Short germ segmentation, where anterior segments form more or less simultaneously and posterior segments are added sequentially, is the ancestral mode of arthropod segmentation and is found in most arthropods (Davis and Patel, 1999, 2002; Tautz et al., 1994). Segmentation in the spider *C. salei* is clearly short germ segmentation (Damen, 2002; Seitz, 1966). The appearance of transcripts of the segmental marker *engrailed* in anterior and posterior segments of short germ arthropods shows an important difference. The *engrailed* stripes of anterior segments appear rapidly in a particular species-specific order within a pre-existing field of cells, while posterior *engrailed* stripes form sequentially from a posterior growth zone in a strict anterior to posterior sequence, as has been described for varies arthropods like spider (chelicerate), grasshopper (insect), amphipod (crustacean), millipede (myriapod), and centipede (myriapod) (Chipman et al., 2004; Damen, 2002; Davis and Patel, 2003; Janssen et al., 2004; Patel et al., 1989; Scholtz et al., 1994). Other examples include the order of appearance of *wingless* and *pairberry* expression in the grasshopper *Schistocerca* (Davis et al., 2001; Dearden and Akam, 2001). These differences in the appearance of segmental gene expression suggest at least partial distinct segmentation mechanisms for the anterior and posterior segments (Peel and Akam, 2003).

Thus, both the *Su(H)* and *Psn* phenotypes and the appearance of *engrailed* stripes provide indications for differences in the specification of anterior and posterior

segments in the spider, although the anterior–posterior border of the effects in the spider slightly differs for *Su(H)*, *Psn*, and *engrailed*. The similarities in *engrailed* appearance in different arthropods even infers that such differences may be present in all short germ arthropods and are an ancestral feature of arthropod development. So far, however, it is not clear whether complete different patterning mechanisms are acting or whether additional and presumably partial redundant genetic pathways act in the anterior segments that are not active in the formation of the posterior segments.

Su(H) and Psn in canonical Notch-signalling during spider segmentation

Canonical Notch-signalling in *Drosophila* and vertebrates acts via *Su(H)* (Bray and Furriols, 2001; Lai, 2004). The presence of the highly conserved *Su(H)* and *Psn* genes in the spider and their segmentation phenotypes therefore strongly suggests that also spider segmentation depends on canonical Notch-signalling. At first glance, one would expect similar phenotypes in the RNAi for the different components of the Notch-signalling pathway. However, the phenotypes we detected in *Su(H)* and *Psn* RNAi (this paper) are more severe than the ones we observed for Notch or *Delta* RNAi (Stollewerk et al., 2003). Although other options cannot be excluded, these differences largely may be due to a functional redundancy of the Notch and *Delta* genes. Two *Delta* genes and one *Notch* gene have been described (Stollewerk, 2002) and we cannot exclude that additional *Notch* and/or *Delta* genes have not been identified from the spider yet. Similarly, most vertebrates contain multiple copies of *Notch* and *Delta* genes (Bierkamp and Campos-Ortega, 1993; del Barco Barrantes et al., 1999; Haddon et al., 1998; Westin and Lardelli, 1997) and the stronger phenotypes of *Su(H)* compared to *Notch* or *Delta* mutants in vertebrates are assumed to be caused by functional redundancy among the *Notch* and *Delta* genes (de la Pompa et al., 1997; Oka et al., 1995; Sieger et al., 2003). Another argument in favour of involvement of canonical Notch-signalling in spider segmentation are the similarities in the phenotypes for *Su(H)* and *Psn* while both genes have different functions in Notch-signalling. *Psn* encodes a protease that is required for the cleavage of the Notch receptor, while *Su(H)* encodes a transcriptional regulator that binds to the DNA in the nucleus (Lai, 2002, 2004; Mumm and Kopan, 2000; Struhl and Greenwald, 1999).

Although a functional redundancy may largely explain the weaker phenotypes in *Notch* or *Delta* RNAi, additional explanations should also be considered (Martinez-Arias et al., 2002). One of them is a *Notch*-independent activity of *Su(H)* as has been reported for the development of bristle sensory organ precursors (SOP) in *Drosophila* (Klein et al., 2000; Koelzer and Klein, 2003). Su(H) acts here independent of *Notch* as a repressor and suppresses the activity of negative regulator(s) of *senseless*. However, only some of the defects in spider segmentation, like the less compact growth zone, may be caused by *Notch*-independent activities of *Su(H)* as most of the other defects are also found for *Psn*.

Despite the similarities in phenotypes of *Notch*, *Delta*, *Psn*, and *Su(H)*, there are also some differences that may be caused by their different roles in the Notch-signalling pathway (Bray and Furriols, 2001; Furriols and Bray, 2000; Lai, 2002, 2004). In the switch model for the activity of Su(H), Su(H) binds to the promoter region of target genes and acts as a repressor; this repressor function turns into an activator function after interaction with NICD that forms upon Notch activation (Figs. 7A,B). Due to this dual function of Su(H), silencing of *Su(H)* may cause different effects than silencing *Psn*, *Notch,* and *Delta*. Interference of Notch-signalling by silencing *Psn*, *Notch,* or *Delta* prevents the formation of NICD and the Su(H) repressor complex cannot switch into an activator complex. The *Notch* target genes remain repressed (Fig. 7C). On the other hand, after silencing *Su(H)*, there is no Su(H) that

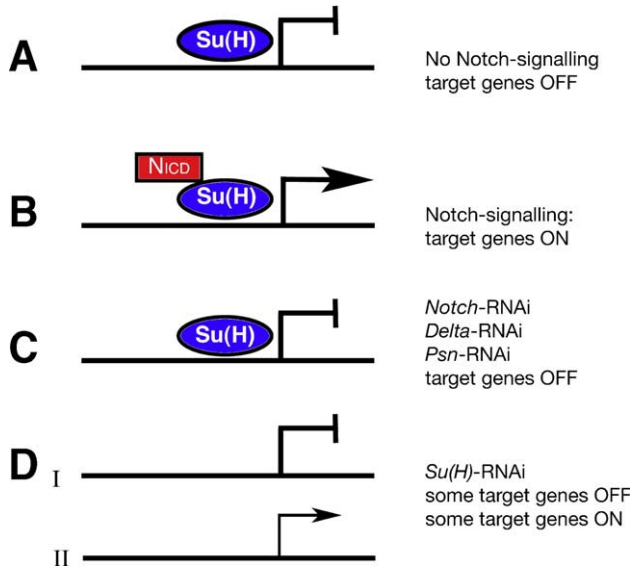

Fig. 7. Su(H) acts as a transcriptional switch in Notch-signalling. The figure is based on Bray and Furriols (2001). (A) In the absence of NICD, DNA bound Su(H) prevents target gene activation (OFF state). This likely is an effect mediated by co-repressors (not shown in drawing). (B) NICD is able to remove the repression as it forms a complex with Su(H) and additional co-activators and promotes transcription of the target genes (ON state). (C) Inhibition of ligand-receptor binding by *Notch*- or *Delta*-RNAi or inhibition of Notch-proteolysis by *Psn*-RNAi results in prevention of the formation of NICD and consequently Su(H) stays on as a repressor. The target genes remain in OFF state. (D) Removing Su(H) via *Su(H)*-RNAi abolishes this repression, and depending on the requirements of the target gene promoters, these either do not become activated as they need the NICD–Su(H) activator complex for activation (I) or become activated in a NICD/Su(H) independent way via other factors that normally are repressed by Su(H) (II). Thus, some target genes may be OFF, while other may be ON. The different effects on target genes may explain the differences in effects seen after RNAi.

can bind and repress target genes. When there is no Su(H) to act as a repressor, some target genes may remain silent as their activation fully depends on the NICD–Su(H) activator complex, while other target genes may become activated independently of the NICD–Su(H) activator complex (Fig. 7D). Thus, *Notch*, *Delta*, and *Psn* silencing results in an inactivation of target genes as Su(H) is not switched into an activator but still can act as a repressor, while after *Su(H)* silencing, the target genes are no longer repressed by Su(H) repressor and some of them may become activated in a Su(H) independent way. These differences may explain some of the differences in phenotypes.

Su(H) and Psn are required for the dynamic gene expression of hairy and Delta-1

Our current and previous work shows that Notch-signalling is required for the activation of *hairy* as well as for the organisation of the *hairy* expressing cells in stripes. *Su(H)* and *Psn* silencing leads to an almost complete inhibition of the *hairy* expression, showing that Notch-signalling is required for the activation of *hairy* (this paper). Furthermore, our data suggest that the spider *hairy* gene may be controlled directly by Notch-signalling, as its promoter region contains a presumptive SPS binding site for Su(H). On the other hand, silencing of *Notch* or *Delta* does not lead to an inhibition of *hairy* expression but to a disturbance of its organisation in stripes (Stollewerk et al., 2003). These less severe defects presumably show a hypomorph effect caused by the assumed redundancy of *Notch* and *Delta* genes as discussed above. These less severe phenotypes however demonstrate that Notch-signalling controls the organisation in stripes.

The *Delta-1* gene also is controlled by Notch-signalling, but it is however not clear whether this control is direct or indirect. The dynamic *Delta-1* expression is strongly affected after *Psn* or *Su(H)* RNAi. Interestingly, these data infer that *Notch* activity is required for the regulation of its ligand *Delta*. *Drosophila* and vertebrate *Delta* genes also are controlled by Notch-signalling (de la Pompa et al., 1997; Heitzler et al., 1996; Holley et al., 2002; Oates and Ho, 2002). In *Drosophila*, a regulatory loop between *Notch* and *Delta* has been described for sensory organ precursors of the peripheral nervous system. This loop is under transcriptional control of the bHLH *E(spl)-C* gene (Heitzler et al., 1996). In vertebrate somitogenesis, a genetic circuit comprised of the Notch-pathway and bHLH target gene of *Notch* has been proposed as Notch-signalling is required to promote the oscillating gene expression of the *delta-C* gene as well as the bHLH gene *her-1* in zebrafish (Holley et al., 2002). Similarly, a regulatory loop consisting of Notch-signalling and the bHLH gene *hairy* may exist in the spider, although there is no direct evidence yet that *hairy* regulates Notch-signalling in the spider.

There is an interesting difference in the effects of *Psn* and *Su(H)* silencing on *Delta-1* expression. *Psn* silencing results in a strong reduction of *Delta-1* expression, while *Su(H)* silencing results in a disturbance of the organisation of the *Delta-1* expression but the expression is not abolished. This difference is also seen in vertebrates although the data come from two different vertebrates. In zebrafish *Su(H)*-morphants, *delta-C* expression is still present but no longer organised in dynamic stripes (Sieger et al., 2003), while in *Presenilin-1* mutant mouse, the expression of the *Delta-like-1* gene is strongly reduced in the PSM (Wong et al., 1997). Interestingly, the difference between the regulation of the spider *hairy* and *Delta-1* gene matches with the proposed different effects on target genes after *Su(H)* RNAi (Fig. 7D). The switch model for Notch-signalling predicts that *Su(H)* RNAi results in the OFF state of some target genes and in an ON state of other target genes. In the spider, one target gene (*hairy*) is OFF, while the other gene (*Delta-1*) is ON, although we cannot exclude that the latter is an indirect effect. As predicted by the switch model, *Psn* RNAi results in down-regulation of both *hairy* and *Delta-1* (Fig. 7C). Thus, Notch-signalling is required for the activation of the *hairy* and *Delta-1* expression as well as for their organisation in stripes but there are at least some other factors that in addition can activate the *Delta-1* gene in absence of *Su(H)*.

The evolution of segmentation

There is an ongoing discussion whether segmented animal body plans share a common origin (Davis and Patel, 1999). The impact of our previous discovery of the involvement of *Notch* and *Delta* in spider segmentation and the similarity to Notch-signalling in vertebrates has been discussed previously (Patel, 2003; Peel, 2004; Peel and Akam, 2003; Stollewerk et al., 2003; Tautz, 2004). The present data corroborate a firm involvement of the canonical Notch-pathway in spider segmentation as has been shown for vertebrate somitogenesis. Furthermore, the comparison between spider segmentation and vertebrate somitogenesis uncovered additional similarities like the supposed two phases of involvement of Notch-signalling in segmentation, presumptive differences in anterior versus posterior segment patterning, and the existence of a possible feedback loop between *Notch*/*Delta* and the bHLH gene *hairy.* These data therefore provide additional similarities of Notch-signalling in arthropod and vertebrate segmentation and underline our previous notion that the formation of the segments in arthropods and vertebrates might have shared a genetic program in a common ancestor (Stollewerk et al., 2003). However, additional support for this assumption should come from the analysis of bilaterian animals that are not obviously segmented, as well as from animals that possess a more basal phylogenetic position.

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