Identification of two binding regions for the Suppressor of Hairless protein within the intracellular domain of Drosophila Notch

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Running Title
Two Su(H)-binding regions in the Notch intracellular domain
Summary

Notch is a phylogenetically conserved transmembrane receptor that is required for many aspects of animal development. Upon ligand stimulation, a fragment of Notch is released proteolytically, and enters the nucleus to form a complex with the DNA-binding protein CSL and activate transcription of Notch/CSL target genes. The physical structure of the Notch/CSL complex remains unclear, however, clouding the interpretation of previous efforts to correlate Notch structure and function. We have therefore characterized the binding of Drosophila CSL (called Suppressor of Hairless, or Su(H)) to the intracellular domain of Drosophila Notch, both in vitro and in vivo. We report identification of two Su(H) binding regions in Notch. The first is in the juxtamembrane region (the “RAM” domain). The second is just C-terminal to the Notch ankyrin repeats, overlapping or identical to two previously proposed nuclear localization sequences (NLS). The ankyrin repeats themselves do not bind to Su(H), however, they substantially enhance binding of Su(H) to the more C-terminal region. Consistent with this picture, removal of either the Ram or PPD binding sites, separately, modestly reduces Notch activity in vivo, while removal of both renders Notch severely defective. These results clarify the relationship between Notch and CSL, help to explain the importance of the ankyrin repeats in Notch signaling and reconcile many apparently contradictory results from previous Notch structure/function studies. Moreover, they suggest a second function for the Notch “NLS” elements.
Introduction

The Notch pathway is a phylogenetically conserved signaling mechanism that mediates cell-cell communication events in the development of nearly all metazoan organisms that have been examined. Homologues of Notch have been found in echinoderms, ascidians, nematodes, insects and vertebrates. In all those organisms, the Notch pathway is essential for a variety of developmental processes involving intercellular communication, including asymmetric cell-fate decisions, formation of boundaries in developmental fields, cell proliferation, and apoptosis [1]. It is thought that the Notch pathway exerts these pleiotropic effects through a common signaling pathway involving the interaction of a few core elements: a signal, DSL (Delta, Serrate, LAG-2), a receptor, Notch (sometimes called LNG for LIN-12, Notch, GLP-1), and a DNA binding protein, CSL (CBF-1, Suppressor of Hairless (Su(H)), LAG-1) with its co-activator, Mam (Mastermind, MAML). Interaction of Notch with its ligand DSL initiates a series of proteolytic events at the membrane, which ultimately result in the release of the intracellular domain of Notch (N\textsuperscript{icd}) (for review see [2]). The free Notch intracellular domain then translocates into the nucleus where it forms a transcription control complex in association with CSL and Mam, binding to target genes that bear CSL binding sites and activating their transcription.

The intracellular domain of Drosophila Notch is large, 937 amino acids, and contains a variety of signaling domains and binding sites for regulators and effectors. (for a schematic representation see Figure 1A). It starts with the juxtamembrane RAM domain, which contains a conserved nuclear localization signal (NLS) sequence. RAM also contains a high affinity binding site for the PTB domain of Numb [3], involved in the trafficking of the receptor, particularly for endocytosis [4] and degradation [5]. C-terminal to RAM, seven ankyrin repeats [6-9] constitute a domain (ANK) that is essential to all Notch functions described so far, and which is highly conserved throughout the entire metazoan Notch family [1]. The molecular function of ANK remains elusive, but it includes a binding site for Deltex, a protein that is required for full Notch activity in vivo [10-12]. A domain that we have called in this study PPD for Potential Phosphorylated Domain follows ANK. It contains a large number of serines and threonines in consensus phosphorylation motifs for various kinases [13, 14], two stretches of basic residues with NLS homologies, and a transcriptional activation capability [15, 16]. The more C-terminal
part of the Notch intracellular domain contains a Glutamine rich region (OPA) and a PEST region involved in regulating the half-life of the protein [17]. In mouse and nematode the PEST region is phosphorylated \textit{in vivo} and becomes a binding site for the E3 ubiquitin ligase Sel-10 (for review [18]).

The association of Notch with CSL is thought to be the key to Notch signaling, since it is CSL that determines what genes are bound and activated by the Notch/Mam/CSL complex [1], but the physical basis for this association remains unclear. The RAM domain was first identified as the primary binding domain for CSL in mammalian Notch [19]. Mutation of a span of conserved residues in this region prevents \textit{in vitro} binding of CBF-1 with mNotch [19]. However, in mammalian cultured cells, RAM-deleted Notch derivatives retain Notch activity, suggesting either the presence of other CBF-1 sites or some CBF-1-independent activity [15, 20, 21]. More definitively, in \textit{Drosophila}, RAM-deleted Notch derivatives still provide significant Su(H)-dependent activity [22, 23]. It has been suggested that Su(H) may interact \textit{in vitro} with the ankyrin repeats of \textit{Drosophila} Notch [24], and indeed, in contrast to the RAM domain, the ANK repeats are essential for all CSL-dependent Notch functions in all studied organisms. However, the binding of CSL proteins to the ANK region seems to be very much weaker than the binding to the RAM domain [25], and has not even been detectable in all studies. Moreover, no CSL binding site has been specifically mapped to this part of the protein. Very recently, it has been shown in mammalian cells that the presence of the protein Mastermind (Mam) interacting with the ANK domain can reinforce the interaction between CBF-1 and RAM-deleted Notch derivatives [26].

To clarify the structure of the Notch/Su(H) signaling complex and its relationship to the sequence of the receptor, we have mapped Su(H) binding sites on \textit{Drosophila} Notch \textit{in vitro}, in \textit{Drosophila} cells and in extract of \textit{Drosophila} embryos. We identified two Su(H) binding regions within the intracellular domain of \textit{Drosophila} Notch. One is in the RAM domain, and confirms the conservation of a strong CSL binding site in the juxtamembrane region of the intracellular domain. A second Su(H) binding region is more complex and was found just C-terminal to the ankyrin repeats, within the domain we called PPD. The ankyrin repeats themselves do not bind directly to Su(H) but they substantially enhance binding of Su(H) to the PPD region, and may be
linked to Su(H) indirectly in vivo, via a bridging adaptor protein [27, 28]. The Ram and PPD sites each contribute to Notch function in vivo, and deletion of both regions severely reduces Notch activity. Together, these data allow us to reconcile a great deal of seemingly conflicting data on the function of Notch domains and the effects of Notch mutations.
Experimental Procedures

Plasmid constructions

Briefly, constructs were obtained as followed:

**GST-Su(H):** To create a baculovirus construct for the expression of GST fusion proteins, the
GST coding sequence, polylinker region, and stop codons of pGEX-2T (Pharmacia Biotech Inc.)
were amplified by PCR and subcloned into the baculovirus transfer vector pVL1393 transfer
vector (pharmingen) to create the vector pVL-GST. pGEX-KG-Su(H) (provided by Posakony,
[29]) was cut EcoRI and HindIII (blunt) and subcloned into pVL-GST cut EcoRI and XbaI
(blunt).

**Notch fragments:** His tagged Notch fragments RAM and OPA were PCR amplified and
subcloned between the Nhel and BgII sites of pRSET A and have been previously described
[30]. The others similar constructs of this study: RAMD, RAMD, RAMD, ANK,
ANK-PPD, the different PPD-OPA, N1766-2262 and N1779-2262 were made the same way. In
each case, a stop codon was introduced after the final amino acid and the amplified fragment was
fully sequenced.

**pT7/C1 EGFP and pT7/C1 EGFP-PPD:** pT7/C1 EGFP was derived from pEGFP-C1 (Clontech)
with addition of a T7 promoter. pT7/C1 EGFP-PPD was obtained by subcloning pRSET-ANK-
PPD cut BamHI and PstI into pT7/C1 EGFP cut BglII and PstI.

**pUAS Flag-N1766 and pUAS Flag-N1779:** The constructs were obtained after amplification by
PCR of a fragment from pUAS N1-2262 (provided by C. S. Wesley [31]) with addition in 5’ of
a BgII site, a *Drosophila* initiation of translation sequence and 3 Flag tags in frame with amino
acid 1766 or 1779 and the sequence ending in 1962 at the XhoI site. The amplified fragments
were sequenced and then subcloned into pUAS N1790 (provided by S. Kidd [16]) cut by BgIII
and XhoI.

**pUAS NFL WT:** To get the pUAS NFL WT we cleaned the stop mutation of pUAS N1-2262
[31] by replacing its Xhol/XbaI fragment by the one from pUAS N1790 [16].

**pUAS Su(H)-HA:** The triple HA tag was derived from IRES hrGFP2a (Stratagene), cut XhoI and
BstEII (blunt) and subcloned into PBS-SK cut XhoI and KpnI (blunt). Su(H) was PCR amplified
from PBS-KS-Su(H) (provided by F. Schweisguth, [32]) with a KpnI site in front of the ATG
and a XhoI site in place of the stop codon putting it in frame with the three HA tags. The KpnI-
XbaI insert was fully sequenced before being subcloned into pUAS.
**Mutagenesis**

All the deletions or mutations in the Notch derivatives were done by directed mutagenesis according to the protocol of the Quick Change Directed mutagenesis kit (Stratagene). Briefly, for all the mutations in the RAM domain, the NheI-Ncol fragment of pUAS N1-2262 was subcloned into pRSET and then mutagenised. For all the mutations in the PPD domain, the Xho-NarI fragment of pUAS Nintra1790 was subcloned into BS-SK and then mutagenised. All the mutagenised fragments were fully sequenced before being re-subcloned in their appropriate backbones (pUAS NFL WT, pUAS N1766 or N1779, pRSET N1766 or N1779, etc.).

**Recombinant protein**

**Baculovirus:** Baculovirus stocks were produced by cotransfecting into SF9-insect cells pVL-GST or pVL-GST-Su(H) plasmids along with a mixture of AcNPV DNA according to the manufacturer procedures (BaculoGold™ Pharmingen).

**Purification:** GST and GST-Su(H) fusion protein expressed in SF9 were purified on glutathione-sepharose beads (Pharmacia). Briefly, 36 hours post-infection with baculovirus expressing pVL-GST or pVL-GST-Su(H), SF9 cells were harvested, washed in phosphate-buffered saline and lysed in 25mM Hepes (pH 7.5), 300mM NaCl, 0.5% NP40, 15mM [-mercaptoethanol, 5mM NaF, 10mM Na pyrophosphate and 25mM [-glycerophosphate. After 30min of gentle agitation at 4°C and 30min of centrifugation at 14,000rpm, the supernatant was diluted with lysis buffer lacking NaCl to reduce NaCl to 125mM. Preblocked glutathione-sepharose beads were added to the lysate and incubate for 1 hour with gentle rocking. Beads were washed 4 times in lysis buffer 50mM NaCl and stored in presence of azide 2mM. GST and GST-PTB purified from bacteria have been previously described [30].

**In vitro binding assay**

Notch fusion proteins were *in vitro* transcribed and translated (IVT) in the presence of \(^{35}\)S methionine using rabbit reticulocyte lysates (TNT, Promega) and following the manufacturer protocol. As indicated, some of the constructs (ANK-PPD derivatives) were digested and purified before being IVT. Binding of *in vitro* translated polypeptides or fly extracts to GST fusion proteins was performed as previously described [30].

**Cell culture and cell extracts**
Drosophila Schneider 2 (S2) cells were obtained from Invitrogen and maintained in Schneider’s Drosophila Medium supplemented in 10% heat-inactivated Fetal Calf Serum, 50 units/ml penicillin and 50 µg/ml streptomycin according to the manufacturer’s instructions. The cells were transfected with calcium phosphate as recommended by the manufacturer. Briefly, 3 × 10^6 S2 cells were plated in a 35mm plate in 3ml, grow for 20 hours before being transfected with 1 µg of each plasmid, as indicated, and 1 µg of pRK241 (Actin-Gal4, from R. Kostriken and P. O’Farrell) to allow the expression of the UAS constructs. Twenty four hours after transfection, the cells were harvested, washed with phosphate-buffered saline and suspended in 300 µl of ice-cold 25mM Hepes (pH 7.5), 100mM NaCl, 0.5%NP40 (v:v), 10% glycerol (v:v), 15mM β-mercaptoethanol and 1mM Phenylmethylsulfonyl fluoride. After 30min of gentle agitation at 4°C and 30min of centrifugation at 14000rpm, the supernatants were collected as whole cell extracts.

Transgenic fly strains and fly extracts

Transgenic strain: Germ line transformation was performed using standard procedures. w^1118 flies were injected with UAS constructs at 1mg/ml and Delta2-3 helper plasmid at 0.5mg/ml. Other fly strains: The strong mutant allele Notch[55e11] (from E.H. Grell and Y.N. Jan) was used as the background for transgene rescue experiment. GAL4 drivers for various experiments were 69B (A. Brand): expression throughout embryonic ectoderm; scabrous-GAL4 (C.S. Goodman): expression in neuroectoderm and its derivatives; hairy-GAL4 (S. Parkhurst): expression in every other segment of the early embryo.

Whole embryo lysates: UAS expressing flies were crossed to 69B to obtain expression in embryos. F1 embryos were collected on grape juice plates O/N (~18 hours at 25°C), harvested with 0.7% NaCl, 0.3% Triton (NaCl/Tx), dechorionated with 50% bleach, washed with NaCl/Tx and transferred to an ice-cold Dounce homogenizer. Embryos were then washed twice with cold H₂O and once with cold lysis buffer. Embryos were suspended with 3 volumes of 25mM Hepes (pH 7.5), 100mM NaCl, 0.5mM DTT, 0.5% NP-40 (v:v), 10% glycerol (v:v), 1mM Phenylmethylsulfonyl fluoride and lysed with 25 strokes of an ice-cold A pestle, followed by 25 strokes with a cold B pestle. Embryo lysates were transferred to microfuge tubes and centrifuged at 14,000rpm for 10min at 4°C. The supernatants were collected as whole embryo extracts.

Immunoprecipitation and western blot
**Antibodies:** antibodies, their sources, and the concentrations used for western blotting (WB) and immunoprecipitation (IP) were as follows: anti-Notch (C17.9C6, Developmental Studies Hybridoma Bank (Iowa)), 1:50 (WB), 30μl/IP, Anti-HA.11 (16B12, Covance) 1/2,000(WB) 0.5μl/IP, anti-Flag (M2, Sigma) 1/1,000 (WB), 0.5μl/IP. Peroxidase-conjugated anti-mouse secondary (Jackson) was used at 1:10,000 (WB), Rb anti-mouse IgG (Jackson) was used at 1μg/5μl packed Protein A sepharose beads.

**Immunoprecipitation:** Protein A sepharose beads (Amersham) were blocked with lysis buffer containing 0.5% BSA, prebound with Rb anti-mouse IgG and washed 2x. Extract was thawed on ice (if previously frozen), and 5μl of protein A/Rb anti-mouse beads was added per IP sample (typically 200 μl of extract, diluted to 350 μl final volume) for pre-clearing. Sample was rocked at 4°C for 1hr, then cleared by centrifugation at 14,000rpm 10min. Supernatant was removed, added to appropriate primary antibody and rocked at 4°C for 1h, 5μl of protein A/Rb anti-mouse beads were added per IP sample and rocked for 2 hours. Beads were spun down for 5sec in a microfuge, unbound material was removed, and the beads were washed 3x with lysis buffer. Beads were then boiled in 20μl Laemmli buffer.

**Western blot:** Proteins separated by SDS-PAGE (6.5% 29:1 acrylamide:bis) were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 90min in PBST (PBS with 0.05% tween 20) containing 3% non fat milk and were incubated with primary antibodies diluted in blocking solution for 3 hours. After washing with PBST, secondaries diluted in PBST were applied for 30min. The membranes were finally washed with PBST, developed with Supersignal West Pico chemoluminescent substrate (Pierce) and exposed to Kodak Biomax MR Films.

For densitometry quantification, non-saturated chemoluminescence exposed film were scanned and densitometry analysis was performed with NIH Image Software.

**Immunohistochemistry and immunofluorescence**

Embryos were collected at 25°C, fixed with 4% formaldehyde and stained with antibodies by standard methods. Whole mount embryos visualized by HRP histochemistry were examined with a Nikon Optiphot microscope using Nomarski optics and photographed with a digital camera (Coolscan). Fluorescently-labelled embryos were examined by confocal microscopy (Leica TCS). Antibodies used were: anti-Notch C17.9C6 (1:50), rat anti-Elav (1: 20) and anti-Sex Lethal (1:50): Developmental Studies Hybridoma Bank; anti-FLAG M2 (Kodak; 1:300);
anti-β-galactosidase (Cappel; 1:10,000). For in situ detection of nuclear Notch protein, we used TSA amplification (PerkinElmer) of the Notch signal, detected with DTAF-streptavidin.
Results

Su(H) interacts with two separate regions of N^{icd} in vitro

To map the Su(H) interaction region(s) within the *Drosophila* Notch intracellular domain (N^{icd}) in vitro, we purified GST-Su(H) fusion protein produced in SF9 cells infected by recombinant baculovirus and we in vitro translated and ^{35}S-labeled different functional regions of the Notch intracellular domain (Figure 1A). We tested those various regions for their ability to bind to GST-Su(H) on glutathione beads (Figure 1B). The RAM domain is sufficient to bind efficiently to GST-Su(H) (Figure 1B, lane 1) whereas it does not bind to GST alone (data not shown). This indicates that the RAM domain of *Drosophila* Notch, just like the RAM domain of vertebrate Notch [19], contains an interaction surface for CSL. We also found that a fragment containing both ANK and PPD bound to Su(H) (Figure 1B, lane 3) with similar efficiency as RAM. Furthermore, a fragment containing both PPD and OPA bound to GST-Su(H), though very weakly (Figure 1B, lane 4). Since neither the ANK domain alone (containing the full seven ankyrin repeats [8, 9]) nor the OPA domain alone bound to Su(H) (lane 2 and 5), we suspected that the PPD domain was the region of interaction and tested that hypothesis by creating a fusion protein between PPD and EGFP (Figure 1C). Whereas EGFP did not bind at all to GST-Su(H), EGFP-PPD bound GST-Su(H) about as well as did PPD-OPA (Figure 1C, compare lanes 1 and 2). These data suggest that the PPD domain can interact with Su(H) but somehow this interaction is strongly improved by the presence of the ANK domain. Together, these data suggest the existence of two separate Su(H) binding sites within *Drosophila* N^{icd}: one in the PPD domain that is sensitive to the presence of the ankyrin repeats, and one in the RAM domain.

Molecular characterization of the Su(H) binding site within the RAM domain of Notch

To narrow down the location of the Su(H) binding site within the RAM domain of Notch, we divided that domain into four subregions (A, B, C, and D, see Figure 2A) and tested the ability of deleted forms of RAM to bind to GST-Su(H) (Figure 2B). All subdomains except A were dispensable for Su(H) binding (Figure 2B). The A subdomain is highly conserved between species and includes a strong match to the previously described CBF-1 binding region in the RAM domain of mammalian Notch [19]. Therefore, we introduced a triple point mutant WFP to LLA at position 1776-1778 in the *Drosophila* RAM domain. As in mammals, this mutation in the RAM domain of *Drosophila* Notch prevented binding to Su(H) (Figure 2B, lane 8, middle
panel). In contrast, it did not prevent the binding of a different Notch-interacting protein, a fusion protein between GST and the PTB domain of Disabled (Dab) that we have shown previously to interact with RAM [30] and that also binds to the RAM A subregion (Figure 2B, lanes 2 and 7 and 8, lower panel).

**Molecular characterization of the Su(H) binding site within the PPD domain of Notch**

We set out to characterize binding of Su(H) to the PPD domain, using the same strategy that we had used for RAM. We started with the ANK-PPD fragment, digested its coding DNA with 5 restriction enzymes deleting progressively the C-terminus part of that encoded peptide and produced the encoded protein by *in vitro* transcription and translation (Figure 3A). Whereas Su(H) can bind to the peptide encoded by the constructs cut at the EcoRI or PvuII site (Figure 3B, lanes 1-2), it cannot bind to any shorter peptide (Figure 3B, lanes 3-5). Conversely, we generated different versions of the PPD-OPA fragment starting at those different restriction sites (Figure 3A) and showed that whereas a construct starting at the SfiI site bound as well as full length PPD-OPA (Figure 3B, lanes 8-9), another construct starting at the PvuII site interacted little, if at all, with Su(H) (Figure 3B, lane 7). Those results suggest that amino acids 2201-2247, coded by the DNA sequence between the SfiI and PvuII sites, are essential for the interaction of Su(H) with the PPD domain of Notch.

We next mutated all the codons between SfiI and PvuII in groups of four adjacent codons and to our surprise, none of these mutations prevented binding to Su(H) (data not shown). This suggested that the binding region might be complex or redundant. Looking at the primary amino acid sequence of the PPD region, we noticed two stretches of basic residues (lysines and arginines) that are strongly conserved within the Notch family (Figure 3C and Table 1). These peptides were previously suggested in human Notch to be Nuclear Localization Signals (NLS) [20]. The Su(H) interaction site in the RAM domain is also in a region with a block of positively charged residues [19]. We therefore wondered whether those blocks of basic residues might be important for the binding of Su(H) to the PPD region. We made deletions in the PPD region of *Drosophila* Notch that removed each of those stretches of charged residues separately (1 or 2) or both together (1+2 and 3) (Figure 3C) and we tested their effect on the binding to GST-Su(H). We made those deletions in a construct starting at codon 1779 (N1779-2262)
lacking the Ram site, as well as in a construct starting at codon 1766 (N1766-2262) that retains the Ram site and acts as a positive control for the activity of the construct. As shown in Figure 3D, deletion of one (D1, lane 7) or the other (D2, lane 8) block of basic residues does not affect the binding to GST-Su(H). However, deletion of both blocks (D1+D2 or D3, lanes 9-10) strongly reduces the interaction of Su(H) with N1779-2262. All proteins bearing the RAM site (N1766-2262 derivatives) still bound Su(H) (lanes 1-5), suggesting that the deletions did not grossly inactivate the proteins. These results suggest that the PPD region contains two small peptides either of which is sufficient to allow binding of Su(H) to N1779-2262. The deletion D3 was used in the experiments below as a mutant form of the PPD region that prevents binding to GST-Su(H).

**Binding of Su(H) to N\text{ned} in fly extracts**

We further tested the significance of the two apparent Su(H) binding regions by performing pull downs of Notch derivatives from *Drosophila* embryo extract. We used the GAL4/UAS system to express in fly embryos [33, 34] different intracellular domains of Notch containing deletions or mutations in the two binding regions described above. Those constructs were tagged at the N-terminus with three FLAG epitopes to distinguish them from cleavage products of endogenous Notch (Figure 4A). Flag-N1766 has the entire intracellular portion of Notch, starting at the amino acid 1766 up to the last amino acid of Notch (2703) and contains the two Su(H) binding regions described above, whereas Flag-N1779 is missing the Su(H) binding site within the RAM domain. Incorporating the D3 deletion in those two constructs allows us to eliminate the second Su(H) binding region within the PPD domain. We tested the ability of these N\text{ned} derivatives to interact with GST or GST-Su(H) \textit{in vitro}. As shown on Figure 4B middle panel, none of the constructs bound to GST alone. Deletion of either the RAM site or the PPD region reduced the binding to GST-Su(H) (lanes 2-3). Deletion of both sites prevented binding completely (lane 4).

We always noticed a strong difference in expression levels of the constructs having or lacking the RAM Su(H) binding site (compare Figure 4B lanes 1-2 with 3-4, upper panel): proteins bearing the Ram site were present at much lower levels in vivo. This difference was reproducible with independent insertion lines and with expression through different GAL4 drivers (data not
Su(H) interacts with two separate domains of N\text{\textsuperscript{\textmd{ledd}}} in vivo

To test the binding of Notch to Su(H) in vivo, we co-transfected Drosophila S2 cells with C-terminus HA-tagged Su(H) and non-tagged full-length derivatives of Notch bearing or lacking the various Su(H) binding regions (Figure 5A). Drosophila S2 cells do not express endogenous Notch. Upon immunoprecipitation of Notch derivatives with an anti-Notch antibody, we detected both full-length as well as cleaved products of Notch (Figure 5B, top panels), and we tested whether we could detect co-immunoprecipitated Su(H)-HA (Figure 5B, bottom panels). Because of the different levels of expression of Notch derivatives (see above), the level of co-immunoprecipitation of Su(H) was normalized to the levels of full-length Notch and/or cleaved Notch in each experiment (Figure 5C). We found that removing either the Su(H) binding site in the RAM domain (with the triple point mutations or the \text{\textsuperscript{\textmd{\textmd{D}}}}\text{\textsuperscript{\textmd{A}}} deletion, respectively) or the Su(H) binding region in the PPD (with the \text{\textsuperscript{\textmd{\textmd{D}}}}\text{\textsuperscript{\textmd{3}}} deletion) domain reduces the binding to Su(H), suggesting that both binding regions contribute to the association of these proteins in vivo. We noted that some residual binding of Su(H) to Notch was detected even in the double mutant. Based just on the biochemical data, we cannot distinguish unambiguously whether this reflects non-specific binding or authentic association of Su(H) with Notch in some other way (see below).

Both Su(H) binding regions contribute to Notch activity in vivo

We employed a functional assay of Notch activity in vivo to assess the physiological significance of the Su(H) binding sites identified biochemically, above. Notch mutant embryos display severe hyperplasia of the central nervous system (CNS) and peripheral nervous system (PNS) (Fig 6 A,B), and this phenotype can be rescued almost completely by expression of wild type Notch throughout the neuroectoderm under control of an appropriate \text{\textmd{GAL4}} driver (\text{\textmd{scabrous-GAL4}}; Fig 6 C). We therefore used this driver to express Notch derivatives lacking the Ram binding site for Su(H), the PPD sites, or both (Fig 6 D-H). Embryos that expressed Notch
proteins lacking either Su(H) binding region alone showed residual neural hyperplasia in the rescued embryos (Fig 6 E-H). Since the mutant proteins are expressed at levels equivalent to or greater than that of wild type Notch (Fig 5 and data not shown), this demonstrates that the modified proteins had reduced activity relative to wild type Notch. A Notch derivative lacking both Su(H) binding regions provided only very weak rescue of the mutant phenotype (Fig 6D), demonstrating that it is severely reduced in Notch activity. The phenotype of embryos rescued with the double mutant was clearly less severe than that of the original Notch allele, however, indicating that the multiply mutant gene still retains some activity. These phenotypic data therefore are consistent with the co-IP experiment of Fig 5, in which we observed residual association of Su(H) with the multiply mutant Notch protein.

While the data above show that the basic motifs in the PPD region contribute to Notch activity in vivo, they do not distinguish whether these sequences are required for Su(H) binding or for nuclear targeting. We therefore prepared embryos expressing mutated Notch derivatives in alternate segments (driven by hairy-GAL4) and used immunofluorescence to investigate the subcellular localization of Notch. Embryos expressing a FLAG-tagged form of the Notch intracellular domain lacking the PPD Su(H) sites (N[intra$^{1779}$ D3]) showed clear nuclear concentration of FLAG immunofluorescence (Fig 7A). We further verified this result by analysis of untagged, full-length Notch bearing the D3 deletion (Notch[full-length, Ram* D3]; Fig 7 B, C). In cells lacking expression of the transgene, endogenous Notch can be clearly detected around the cell periphery, but consistent with previous studies, the level of protein in the nucleus is below the limit of detection by immunofluorescence. In cells in which the transgene is expressed, in contrast, a modest increase is evident in labeling of the cell periphery, and labeling
is now also detectable in the center of the cell, in the position of the nucleus (Fig 7B).

Examination of a cross section of this sample (Fig 7C) further supports the interpretation that this represents staining of the cell nucleus. In some experiments, this was verified by double-labelling with an authentic nucelar marker (anti-Lola; data not shown). We infer, therefore, that the expressed Notch protein lacking the two PPD basic motifs is present in the nucleus in these embryos.
Discussion

We have shown in this study that Su(H) binds to Drosophila N\textsuperscript{incd} through two separate binding regions. The first is in the RAM domain and was previously suspected by analogy with mammalian Notch. The second is bipartite, located downstream of the ankyrin repeats and overlapping if not identical to the second Notch “nuclear localization signal”. The efficiency of Su(H) binding to this second region is strongly enhanced by the presence of the ankyrin repeats. The two binding regions were identified \textit{in vitro}, suggesting a direct interaction between Su(H) and Notch, and were confirmed \textit{in vivo} both biochemically, by co-immunoprecipitation from Drosophila cell extracts, and functionally, by demonstrating that each contributes to Notch activity \textit{in vivo}.

Fortini and Artavanis-Tsakonas were the first to report a direct physical interaction between Su(H) and Drosophila Notch [24]. Using the yeast 2-hybrid system, they detected an interaction between Su(H) and a portion of Notch centered on the ANK repeats; the deletion of the ANK repeats strongly reduced that interaction. Co-localization of transfected Notch and Su(H) in the nucleus of S2 cells was also dependent on the integrity of the ANK repeats. Surprisingly, however, Tamura \textit{et al.} reported compelling evidence based on yeast 2-hybrid experiments and GST pull-downs showing that CSL bound strongly within the RAM domain of Notch [19], but only weakly, if at all, with an extended region including the ANK repeats [25]. These data of Tamura \textit{et al.} were also difficult to reconcile with earlier genetic data in flies showing a strong gain of function phenotype of Drosophila Notch intracellular domains missing the RAM Su(H) binding domain [22, 23]. Subsequent studies in nematodes and vertebrates recapitulate the same contradictions, indicating a strong binding site within the Ram domain that could not always be linked with functional activity and variable and inconsistent binding to more C-terminal sequences, which failed to correlate simply with the activity of truncated derivatives and were not precisely localized. [15, 21, 27, 35, 36].
Our data now help to reconcile these earlier studies. We confirm in *Drosophila* the interaction domain identified by Tamura *et al.* within the RAM region, both *in vitro* and *in vivo* in fly cells. Moreover, we describe the functional conservation of the three amino acids shown to be essential to the interaction of mNotch with CBF-1[19]. We also identify a second binding region in the PPD domain immediately downstream of the 7th ANK repeat. The PPD binding site is bipartite, with its two elements being redundant in most experimental contexts, explaining why it has previously been difficult to identify by mutation or deletion. The previously described requirement for the ANK repeats in CSL-dependent Notch signaling [15, 25, 36] is consistent with our data showing that the presence of the ANK repeats greatly increases the effectiveness of Su(H) binding to PPD. Perhaps ANK holds PPD in a conformation that is favorable for Su(H) binding. We also find that the expression levels of *Drosophila* Notch derivatives *in vivo* are systematically dependent on the ability of those derivatives to interact with Su(H), complicating the functional mapping of Notch domains unless such experiments are normalized for Notch expression levels.

Previous studies offer independent support for the interpretation we propose. Notch derivatives lacking the RAM binding site that were shown to bind CSL *in vitro* and *in vivo* and to activate the CSL pathway retained one or both of the PPD sites (for example [37]; summarized in [9]). Moreover, Jeffries and Capobianco used both a neoplastic transformation assay and a luciferase reporter assay to show for human Notch1 the importance of what we are calling the PPD domain: the minimal transformation domain (TFD) they identified in human Notch1 corresponds to the ANK repeats together with the PPD domain we define in *Drosophila* Notch, and a deletion that removed only a portion of the PPD domain gave an intermediate level of activity in their transformation assay [38]. Finally, Oswald, et al [39] characterized transcriptional activation by a series of mouse Notch1 intracellular domain derivatives with C-terminal truncations that ended in and around the PPD domain. In that study, the shortest derivative that provided full Notch activity deleted one of the basic peptides in the PPD, but retained the other. A derivative that was just 22 codons shorter and lacked both of the basic peptides was reduced in activity by ~50% compared with wild type Notch, even though both expression level and nuclear localization of the derivatives tested appeared to be unimpaired in that assay system.
It is striking that the Su(H) binding region that we have identified within the PPD domain is the previously described “double NLS region” of Notch [20, 38]. This region contains two small basic peptides that resemble nuclear localization signals and are believed to contribute to nuclear accumulation of N\textsuperscript{icd}. We find that these same peptides are essential for \textit{in vitro} binding of Su(H). While our data do not exclude the possibility that those residues may also provide a classical NLS signal, we find that deletion of these sequences does not prevent nuclear entry of Notch, in agreement with previous data from mouse Notch [39, 40]. We note that it has not been demonstrated whether these basic peptides promote nuclear localization by direct interaction with the nuclear targeting machinery or by some other mechanism. For example, they could in principle promote nuclear retention by allowing interaction with a nuclear protein like Su(H). Our data, however, provide a plausible rationale for the strong conservation of the “double NLS” motif even though it is not essential for nuclear targeting of N\textsuperscript{icd}.

We note that we still observed residual co-immunoprecipitation of Su(H) with Notch derivatives deleted for both the Ram and PPD binding sites (Fig 5 B,C), even though those mutations abolished binding \textit{in vitro} (Fig 4 B). While we cannot fully exclude the possibility that the residual interaction reflects nonspecific background in the assay, an alternative possibility consistent with our functional data is that Su(H) is capable of associating with Notch indirectly, for example, through a “bridging” protein that itself provides the direct contact to Notch. Such a model has been proposed previously for Notch orthologs in nematodes and mammals. Roehl \textit{et al.} provided compelling data that just the ankyrin repeats of GLP-1 by themselves have substantial Notch pathway activity [27, 35]. Since they observed colocalisation of GLP-1 and the CSL homologue Lag-1 \textit{in vivo} but did not observe direct binding \textit{in vitro}, they postulated that some other protein(s) might provide a link between them, perhaps the Mastermind (Mam) homologue Lag-3 [41]. Similarly, in vertebrates, it has been proposed that the presence of Mam may stabilize or induce interaction of Notch with CBF-1 independently of the RAM domain [42], mediated perhaps by interaction of Mam with both CBF-1 and the Notch ankyrin repeats [26, 28]. Our evidence that the presence of the ANK repeats enhances binding of Notch to the PPD site \textit{in vitro} suggests that ANK may also play a more direct role promoting Notch(PPD) and Su(H) association, while the co-immunoprecipitation data supports the idea that the importance
of the ANK repeats in signaling is reinforced by their role in also mediating an indirect association through Mam, and perhaps other proteins.

In summary, our biochemical characterization of the binding of Su(H) to two regions within the Notch intracellular domain clarify the molecular role of the Notch ANK repeats, suggest an additional function for the two conserved nuclear localization sequences just downstream of those repeats and help to explain the phenotypes of a number of Notch mutations and Notch derivatives from a wide variety of experimental systems.

Acknowledgements
We are grateful to D. Crowner and L. Luke for technical assistance, and to L. Arnaud and all the members of our laboratory for useful discussions. We thank D. Barrick, S. Carlson, W. Carter, T. Reh and V. Vasioukhin for critically reading the manuscript. We also thank C.S. Wesley, F. Schweisguth, J. Posakony and R. Kostriken for providing DNA constructs. MLG was supported, in part, by a fellowship from the Association pour la Recherche Contre le Cancer. These experiments were supported by NIH grant GM 57830.
References


Footnotes

1: Abbreviations are as follows: CSL: CBF1/Suppressor of Hairless/Lag1; Su(H): Suppressor of Hairless protein. N\textsubscript{icd}: intracellular domain of Notch; NLS: nuclear localisation signal, PTB: phosphorylation binding domain EGF repeats: epidermal growth factor-like repeats; LNG: LIN-12, Notch, GLP-1 motif; TM: transmembrane domain; ANK: ankyrin repeats; PPD: domain bearing conserved potential phosphorylation motifs; OPA: poly-glutamine repeat-containing region: PEST: domain rich in proline, aspartate, serine and threonine residues; EGFP: enhanced green fluorescent protein.
Figure legends

**Fig 1: Su(H) interacts with two separate regions of N\textsuperscript{icd} in vitro.**

(A) schematic representation of the different regions of the Notch intracellular domain (with their corresponding amino acid numbers) *in vitro* translated in the following binding assays. (B and C) comparison of the binding of various regions of Notch to GST-Su(H). In each binding assay, \(^{35}\text{S}-\text{labeled} \textit{in vitro} \text{translated} \text{Notch proteins were mixed with GST-Su(H) glutathione sepharose beads, bound proteins were eluted in Laemml buffer and analyzed by SDS-PAGE followed by fluorography. The input lanes correspond to 10\% of the total proteins.}

**Fig 2: Molecular characterization of the Su(H) binding site in the RAM domain of Notch**

(A) schematic representation of the different constructs tested in B. The RAM domain was subdivided in 4 regions and derivatives deleted for those regions (”[]” with corresponding amino acid numbers) were tested for their binding to GST-Su(H) and to a fusion of GST to the PTB domain of the \textit{Drosophila} Disabled protein (GST-DAB(PTB)). Binding was performed as described for the experiments of Fig 1. The amino acid sequence of the triple point mutation RAM* is also shown, and its position indicated by a vertical, striped bar. (B) comparison of the binding of various deletions and mutations in the RAM domain of Notch to GST-Su(H) or GST-DAB(PTB).

**Fig 3: Molecular characterization of the Su(H) binding region within the PPD domain of Notch**

(A) schematic representation of the different PPD derivatives tested in B. All the ANK-PPD derivatives were obtained by digestion of the coding DNA of ANK-PPD with the indicated
restriction enzymes before \(^{35}\)S-labeled \textit{in vitro} translation, whereas all the PPD-OPA derivatives are independent constructs. B, comparison of the binding of the various deletions in ANK-PPD or PPD-OPA regions to GST-Su(H). Binding was assayed as for the experiments of Fig 1. The region between amino acid 2201 and 2247 is necessary and sufficient to allow binding of GST-Su(H) with ANK-PPD or PPD-OPA. Lanes 6-9 are reproduced from an experiment with a rather longer fluorographic exposure than lanes 1-5, due to the less efficient binding of PPD-OPA as compared with ANK-PPD. C, schematic representation of the two sets of constructs tested in D. N1779-2262 is deleted for the Su(H) binding site in the RAM domain (black rectangle), as compared with N1766-2262. The amino acid sequence encoded by the SfiI–PvuII region is also indicated, together with the deletions used in D. Note the presence of two blocks of basic residues in the Su(H)-binding interval. D, comparison of the binding of GST-Su(H) with Notch derivatives deleted for the putative Su(H) binding sites. Deletion of the Su(H) binding site in the RAM domain in combination with deletion of both stretches of basics residues strongly reduces the binding to Su(H).

\textbf{Fig 4: Su(H) interacts with two separate domains of \textit{N}icd in flies}

(A) schematic representation of the different constructs used in (B). The complete Notch intracellular domain and derivatives of it were labeled with three FLAG tags (indicated by open circles) and expressed in the ectoderm of \textit{Drosophila} embryos, using the GAL4 driver 69B. Vertical black bar represents the position of the N-terminal Su(H) binding site; thin, horizontal lines represent the location of deleted sequences. (B) Binding of Notch derivatives, made in \textit{Drosophila} embryos, to GST or GST-Su(H). Bound proteins were eluted in Laemmli buffer and
analyzed by SDS-PAGE followed by Western Blot analysis (WB) with anti-FLAG antibody. Input lanes represent 5% of total proteins.

**Fig 5: Su(H) interacts with two separate domains of N\textsuperscript{icd} in vivo**

(A) Schematic representation of different Notch full-length derivatives expressed in *Drosophila* S2 cells. Vertical, striped bar represents the position of the N-terminal Su(H) binding site; thin horizontal lines depict the locations of sequences that are deleted in particular derivatives. (B) Co-immunoprecipitation of Su(H)-HA with Notch full-length derivatives in S2 cells. S2 cells, co-transfected with Su(H)-HA and Notch Full-length derivatives, were lysed and incubated with anti-Notch antibody (IP), then with rabbit anti-mouse-bound Protein A sepharose. Immunoprecipitated proteins were eluted in Laemmli Buffer and subjected to SDS-PAGE and Western Blot (WB) analysis with anti-Notch (top) or anti-HA (bottom). Input lanes represent 1% of total proteins. (C) Quantification of binding data. Su(H)-HA signal was quantified by densitometry and normalized for the total amount of immunoprecipitated Notch (light speckles) or for the amount of N\textsuperscript{icd} (dark speckles). Data shown are the average of 4 independent experiments. □A is a deletion of Notch amino acids 1765-1801; Ram* bears a triple point mutation of the Ram binding site for Su(H); □3 is a deletion of both basic peptides of the PPD; Ram*□3 bears both the Ram site point mutation and the □3 deletion.

**Fig. 6: Su(H) binding sites are required for Notch function in vivo**

Embryos of the indicated genotypes were allowed to develop to embryonic stage 16/17, fixed, stained with anti-Elav antibodies to label all neuronal nuclei and visualized by peroxidase histochemistry. (A) wild type embryo, (B) *Notch[55e11]*. Central nervous system (CNS) and
Peripheral nervous system (PNS) are indicated. Inset in (A) shows a higher magnification view of the dorsal sensory neuron cluster of segment A3 (indicated by a dashed box).

(C-H) *N[55e11]* embryos in which a *Notch* transgene has been expressed throughout the neuroectoderm under the control of *scabrous-GAL4*, as follows:

(C) UAS-wild type *Notch* transgene; (D) UAS-*Notch*[^ Ram*, [3]; (E) UAS-*Notch*[^ Ram*] (PNS view only); (F) UAS-*Notch*[^[ [3] (PNS only); (G) UAS-*Notch*[^ Ram*] (CNS only); (H) UAS-*Notch*[^ D3] (CNS only). (A-F) show lateral views of embryos (anterior to the left; dorsal up). Insets in (C-F) show the segment A3 dorsal sensory cluster from those embryos. (E, F) are focused on dorsal and lateral sensory clusters; v' and ventral clusters are largely out of view. The severity of the PNS phenotype is not fully evident in (B, D-F) since labelled cells are piled-up in multiple focal planes. (G, H) ventral views of embryos. Inset in (H) shows the CNS of a wild type embryo for comparison. Arrows in (G, H) point to bulges in the CNS indicative of neuronal hyperplasia.

**Fig. 7:** PPD “NLS” motifs are not essential for nuclear entry in vivo.

Embryos were prepared that express the specified transgenes in alternate segments, under control of *hairy-GAL4*. Stage 9-11 embryos were fixed and stained with the indicated antibodies, and visualized by confocal microscopy. (A) Embryo expressing *FLAG-Notch*[^[ intra][779] [3], visualized with anti-FLAG. (B) Embryo expressing *Notch*[^[ full length, RAM*][ [3], visualized with anti-Notch. Endogenous Notch does not accumulate in the nucleus to detectable levels, but Notch immunoreactivity is clearly observed in the position of the nucleus in cells expressing the transgene. Nuclear positions are indicated with magenta arrows. (C) Cross section of a z-series of the same embryo in (B), taken at the position of the white arrows.
Table 1 Conservation of the Su(H) binding sites in the Notch family

Identical or conserved basic residues are in bold. The three conserved amino acids of the 1st Su(H) binding site are highlighted in gray. The bipartite binding region, identified in the PPD domain in the fly sequence corresponds to the bipartite NLS identified in human Notch1; both of them are in boxes.

1st Su(H) binding region in the RAM domain of Notch

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2nd Su(H) binding bipartite binding region in the PPD domain of Notch

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Figure 1

A

EGF-repeats  LNG TM RAM ANK PPD OPA PEST

RAM (1766-1896)
ANK (1896-2155)
ANK-PPD (1896-2262)
PPD-OPA (2155-2606)
OPA (2262-2606)
EGFP
EGFP-PPD (2155-2262)

B

C

Input

GST-Su(H)

1 2 3 4 5

Input

GST-Su(H)

1 2
Figure 2

A

- RAM (1765-1896)
- RAMΔA (Δ1765-1801)
- RAMΔB (Δ1802-1825)
- RAMΔC (Δ1826-1860)
- RAMΔD (Δ1861-1896)
- RAM* (1776-1778 WFP-LLA)

B

Input

GST-Su(H)

GST-DAB(PTB)

1 2 3 4 5 6 7 8
Figure 3

### A

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- I896-2262 (ANK-PPD EcoRI)
- I896-2247 (ANK-PPD PvuII)
- I896-2211 (ANK-PPD BspEI)
- I896-2201 (ANK-PPD SfiI)
- I896-2155 (ANK BamHI)
- 2262-2606 (OPA EcoRI)
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Input

- 1
- 2
- 3
- 4
- 5
Figure 3

C

D
Figure 4

A

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B

Input
GST
GST-Su(H)

1 2 3 4

WB: anti-Flag
Figure 5

A

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B

![WB: anti-Notch](image1) ![Input](image2) ![IP: anti-Notch](image3)

C

![Graph of % Binding](image4)
Figure 6

A. Wild type

B. N[55e11]

C. N[55e11]; UAS-N[WT]

D. N[55e11]; UAS-N[Ram*Δ3]

E. N[55e11]; UAS-N[Ram*]

F. N[55e11]; UAS-N[A3]

G. N[55e11]; UAS-N[Ram*] (arrowed)

H. N[55e11]; UAS-N[A3] (arrowed)
Identification of two binding regions for the suppressor of hairless protein within the intracellular domain of drosophila notch
Maude Le Gall and Edward Giniger

J. Biol. Chem. published online April 29, 2004

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