Nrarp is a novel intracellular component of the Notch signaling pathway

Elise Lamar,[1] Gisèle Deblandre,[1] Daniel Wettstein,[1,3] Volker Gawantka,[2] Nicolas Pollet,[2] Christof Niehrs,[2] and Chris Kintner[1,4]

[1]The Salk Institute for Biological Studies, San Diego, California 92186, USA; [2]Division of Molecular Embryology, Deutsches Krebsforschungzentrum, D-69120 Heidelberg, Germany

The Lin12/Notch receptors regulate cell fate during embryogenesis by activating the expression of downstream target genes. These receptors signal via their intracellular domain (ICD), which is released from the plasma membrane by proteolytic processing and associates in the nucleus with the CSL family of DNA-binding proteins to form a transcriptional activator. How the CSL/ICD complex activates transcription and how this complex is regulated during development remains poorly understood. Here we describe Nrarp as a new intracellular component of the Notch signaling pathway in *Xenopus* embryos. *Nrarp* is a member of the Delta–Notch synexpression group and encodes a small protein containing two ankyrin repeats. *Nrarp* expression is activated in *Xenopus* embryos by the CSL-dependent Notch pathway. Conversely, overexpression of *Nrarp* in embryos blocks Notch signaling and inhibits the activation of Notch target genes by ICD. We show that Nrarp forms a ternary complex with the ICD of XNotch1 and the CSL protein XSu(H) and that in embryos Nrarp promotes the loss of ICD. By down-regulating ICD levels, Nrarp could function as a negative feedback regulator of Notch signaling that attenuates ICD-mediated transcription.

[*Key Words*: Nrarp; Notch target; transcription; feedback regulation; ternary complex]

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Cell differentiation during animal development is often regulated by local cell–cell interactions, many of which involve signaling by the Lin12/Notch family of receptors. In vertebrate embryos, for instance, Notch signaling is known to control neuronal and glial differentiation, lymphocyte differentiation, differentiation of epidermoblasts, hair follicle and ciliated cells in the skin, formation of the developing vasculature, and segmentation in the paraxial mesoderm (Beatus and Lendahl 1998; Pourquie 1999; Deftos and Bevan 2000; Krebs et al. 2000; Lin et al. 2000; Lowell et al. 2000; Wang and Barres 2000). Many human diseases may also be caused by mutations in the genes required for Notch signaling, as is expected for a pathway that plays such a significant role in development (Gridley 1997; Joutel and Tournier-Lasserve 1998).

Despite their diverse ranges of action, the Lin12/Notch receptors use a conserved mechanism of signal transduction in which proteolytic processing figures prominently (for reviews, see Mumm and Kopan 2000; Weinmaster 2000). The receptors are first cleaved by a furin-like convertase during transit through the secretory pathway, resulting in a heterodimeric molecule at the cell surface (Logeat et al. 1998). Activation of the Notch receptors by ligand binding leads to signaling by further proteolytic processing, beginning with cleavage of the ectodomain by a TACE-related metalloprotease (Brou et al. 2000; Mumm et al. 2000). Once the ectodomain is liberated, the carboxyl portion of the receptor becomes a substrate for processing by a presenilin-dependent, γ secretase activity that cleaves within the transmembrane domain (De Strooper et al. 1999; Struhl and Greenwald 1999; Ye et al. 1999). This cleavage releases the intracellular domain (ICD) from the plasma membrane; the ICD then translocates to the nucleus and associates with the CSL family of DNA-binding proteins (CBF1 in mammals, Su(H) in *Drosophila*, and Lag1 in *Caenorhabditis elegans*). Formation of the CSL/ICD complex activates the transcription of Notch target genes whose products act as downstream effectors of Notch signaling (Jarriault et al. 1995). Activation of target genes by Notch signaling is likely to be CSL-dependent in most cases, although there are examples in which a CSL-independent mechanism may be used (Weinmaster 1998).

Association with ICD changes the properties of CSL proteins as transcription factors, by displacing one group of interacting proteins that represses transcription while recruiting others that activate (for review, see Kadesch 2000). For example, the mammalian CSL protein, CBF1, can repress transcription of target genes by associating

[3]Present address: Myriad Genetics, 320 Wakara Way, Salt Lake City, UT 84108, USA.
[4]Corresponding author.
E-MAIL Kintner@salk.edu; FAX (858) 450-2172.
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with the HDAC-containing corepressor complexes, SMRT/NCoR and CIR/SAP30 (Kao et al. 1998; Hsieh et al. 1999). Because ICD displaces corepressor complexes from CBF1 (Hsieh et al. 1996), Notch signaling may induce the expression of Notch target genes by blocking the repressor activity of CBF1 (Morel and Schweisguth 2000). In most cases, however, loss of CSL function is phenotypically the same as loss of Notch signaling, indicating that derepression is not sufficient to activate the expression of Notch target genes. Instead, ICD binding to the CSL proteins also recruits additional proteins that promote transcription as coactivators. For example, proteins with histone acetyl transferase (HAT) activity such as PCAF and GCN5 are recruited to the CSL proteins by binding to ICD (Kurooka and Honjo 2000). These coactivators are used by a variety of DNA-binding proteins, suggesting that their recruitment to sites where the CSL proteins bind is also necessary to activate Notch target genes. CBF1 and ICD also bind to a protein called SKIP that can have a positive role in promoting Notch transcription (Zhou et al. 2000). SKIP potentiates transcriptional activation of reporter plasmids in transient transfection assays by ICD, and ablation of SKIP function in tissue culture cells using antisense oligonucleotides inhibits ICD-mediated repression of myogenesis (Zhou et al. 2000). Finally, the *lag3* gene in *C. elegans* and related genes called *mastermind* in *Drosophila* and mammals encode proteins that bind to the ankyrin repeats of ICD and the CSL proteins in a ternary complex (Petcherski and Kimble 2000a,b). Lag3 and the mastermind proteins have structural features typical of transcriptional coactivators (Doyle et al. 2000; Petcherski and Kimble 2000a) and are required for Notch signaling in *C. elegans* and *Drosophila*, respectively (Helms et al. 1999; Doyle et al. 2000; Petcherski and Kimble 2000a). Moreover, human mastermind promotes Notch-mediated transcription on reporter plasmids in transient transfection assays, indicating that these proteins are likely part of the CSL/ICD transcriptional complex that activates Notch target genes (Wu et al. 2000a).

Here we describe a new target of Notch signaling in *Xenopus* embryos that can act as a feedback regulator of the Notch pathway. This gene, originally called *5D9*, was first identified in a large-scale expression screen in *Xenopus* embryos as a member of the Delta–Notch synexpression group (Gawantka et al. 1998). The gene, now termed *Nrarp* (for Notch regulated ankyrin repeat protein) encodes a small protein with two ankyrin repeats. We show here that the CSL-dependent Notch pathway activates the expression of *Nrarp* in early embryos. Overexpression of *Nrarp* in embryos produces phenotypes consistent with a loss of Notch signaling and in animal cap assays inhibits the activation of other Notch target genes by ICD or XDelta1. We show that Nrarp binds to ICD and XSu(H), but only in a ternary complex. On one hand, formation of this ternary complex increases the levels of ICD associated with XSu(H). On the other, the overall levels of ICD decrease in total cell extracts when the complex forms. We provide several lines of evidence that Nrarp can modulate Notch-mediated transcription both in embryos and in transfected cultured cells. We conclude that *Nrarp* encodes a component of the Notch signaling pathway, its expression is activated by Notch signaling, and it binds to ICD and XSu(H) to modulate their activity.

Results

Expression pattern of Nrarp

A 1.2-kb cDNA encoding Nrarp was first isolated during a screen for developmentally expressed genes in *Xenopus* embryos (Gawantka et al. 1998). Rescreening of a *Xenopus* neurulae-stage library led to the isolation of three additional independent cDNAs whose sequences matched that of the original clone. The nucleotide sequences of these cDNAs predict a 1.6-kb transcript with rather large 5′ and 3′ untranslated regions and an open reading frame of 342 nucleotides encoding a small protein of 114 amino acids (Fig. 1A). The most distinguishing feature of the protein is two tandem copies of the ankyrin repeat, a structural motif that mediates protein–protein interactions and is present in a variety of proteins. Homology searches of the databases identified ESTs for both zebrafish and rat proteins that are remarkably similar to *Xenopus* Nrarp. For example, *Xenopus* and rat Nrarp differ at only 8 amino acids (Fig. 1B).

Nrarp is expressed in *Xenopus* embryos in a pattern

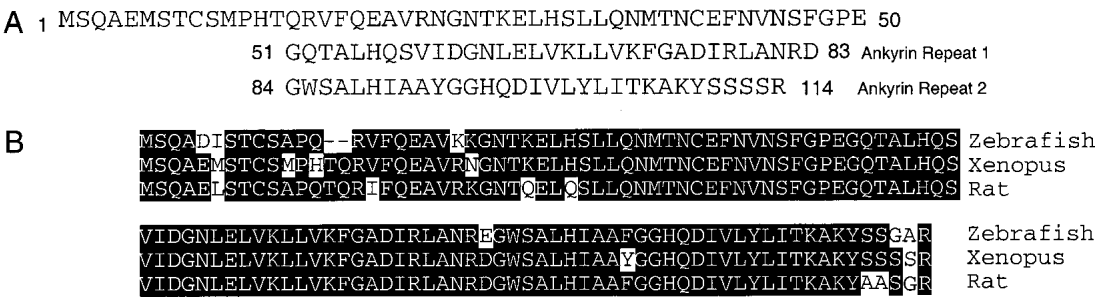

Figure 1. Predicted protein product of *Nrarp*. (*A*) *Nrarp* encodes a small novel protein of 114 amino acids. Analysis using the SMART program predicts two tandem ankyrin repeats (Ankyrin Repeats 1 and 2) that constitute the carboxyl half of the protein. Nrarp^{tr} is truncated after amino acid 51. (*B*) BLAST homology search of the EST databases identifies rat and zebrafish homologs that are nearly identical in amino acid sequence.

that resembles that of the Notch ligands, indicating that it may function in the Notch signaling pathway. This resemblance is first apparent at the earliest stages of embryonic development and in several germ layers where the Notch pathway is known to operate in cell fate decisions. For example, in *Xenopus*, as in other lower vertebrates, Notch signaling negatively regulates the number of primary neurons that form by a process called lateral inhibition. As part of this process, genes involved in Notch signaling are expressed in the neural plate in a characteristic pattern of three bilateral stripes. These genes include one encoding the Notch ligand XDelta1 (Fig. 2B; Chitnis et al. 1995) and one encoding a bHLH protein ESR1, whose expression is activated by Notch signaling (Fig. 2C; Wettstein et al. 1997). *Nrarp* expression in the neural plate closely mirrors this pattern: *Nrarp* RNA staining is already detected at late gastrulae stages within the prospective trigeminal placodes (data not shown), followed soon thereafter by stripes of staining in the posterior neural plate where the differentiation of primary neurons occurs (Fig. 2A). In addition, *Nrarp* expression is associated with neurogenesis throughout neural development as evidenced by *Nrarp* RNA staining in the ventricular zone of the neural tube (Fig. 2D,E). Finally, *Nrarp* is also expressed in the presomitic mesoderm in a pattern that resembles the expression of Notch pathway genes during the process of segmentation (Jen et al. 1999). Strong staining for *Nrarp* RNA is found in a ring of tissue that will form the early somites in gastrulating embryos and in a region of paraxial mesoderm called the tailbud domain, which grows out to give rise to somites in the tail in later-stage embryos (Fig. 2A,F). This expression of *Nrarp* in the presomitic, paraxial mesoderm closely mirrors *XDelta2*, which encodes a second *Xenopus* ligand for Notch, as well as two other Notch target genes, *ESR4* and *ESR5* (Jen et al. 1999). These data, therefore, suggest strongly that *Nrarp* is expressed in early embryos along with other genes involved in Notch signaling.

The CSL-dependent Notch pathway induces Nrarp *expression*

To determine whether Notch signaling regulates the expression of *Nrarp*, *Xenopus* embryos were injected with RNAs encoding proteins that activate or inhibit Notch signaling and then analyzed by whole-mount in situ hybridization for *Nrarp* RNA expression. The results show that *Nrarp* expression, like that of other Notch target genes, is strongly up-regulated in embryos that are injected with RNA encoding just the intracellular domain of XNotch1 (ICD) (Fig. 3A). To determine whether ICD

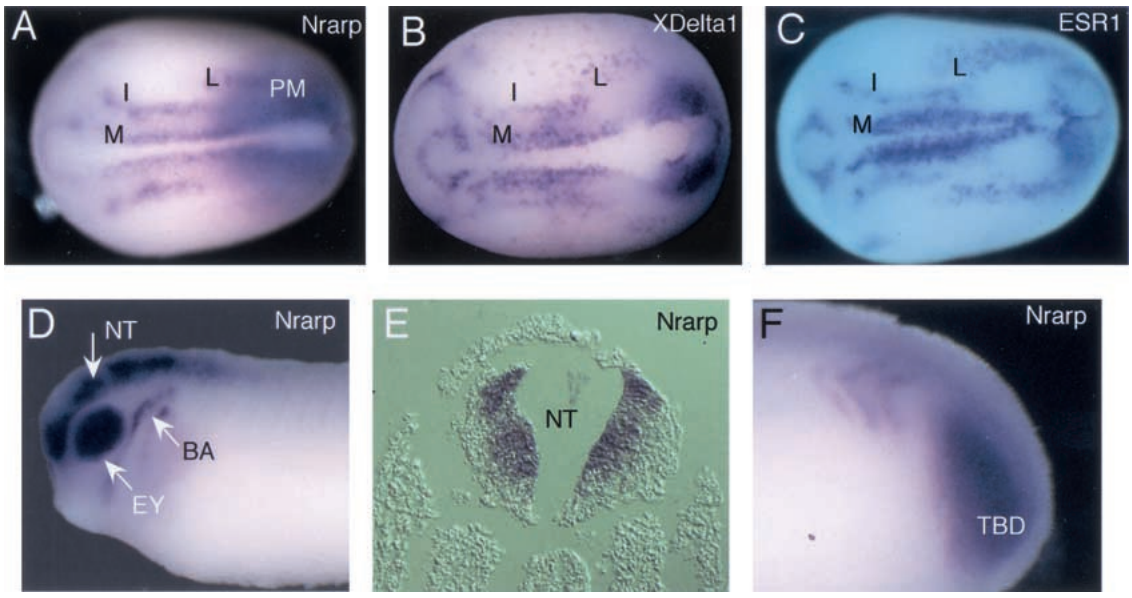

Figure 2. Expression of *Nrarp* mirrors that of other Notch pathway genes. *Xenopus* embryos were stained for the expression of *Nrarp* RNA using whole-mount in situ hybridization. (*A*) *Nrarp* expression at early neurulae stages. Shown is a dorsal view with anterior oriented to the left. Note that *Nrarp* RNA is expressed at high levels in the neural plate, localizing to the three bilateral domains where the Notch pathway is known to operate during the formation of the primary neurons (M, medial; I, intermediate; L, lateral). In addition, *Nrarp* RNA can be detected in the paraxial mesoderm (PM). (*B*,*C*) For comparison, note that the expression pattern of *Nrarp* is similar to that of *XDelta1* (*B*) and *ESR1* (*C*). (*D*) Expression of *Nrarp* RNA in a tadpole embryo stained in whole mount. Shown is a side view of the anterior end with anterior oriented to the left. Note expression in the eye (EY), neural tube (NT), and branchial arches (BA). (*E*) Tissue section of an embryo as in panel *D* shows that the expression of *Nrarp* RNA in the neural tube is confined to the ventricular zone where neurogenesis occurs. (*F*) Expression of *Nrarp* occurs at high levels in the outgrowing tailbud where Notch signaling is known to play a role in segmentation. High levels of *Nrarp* expression are apparent in the most posterior portion of the presomitic mesoderm called the tailbud domain (TBD) and in segments in a region containing prospective somites. This expression pattern in the presomitic mesoderm closely parallels that of *XDelta2* and two Notch target genes, *ESR4* and *ESR5*.

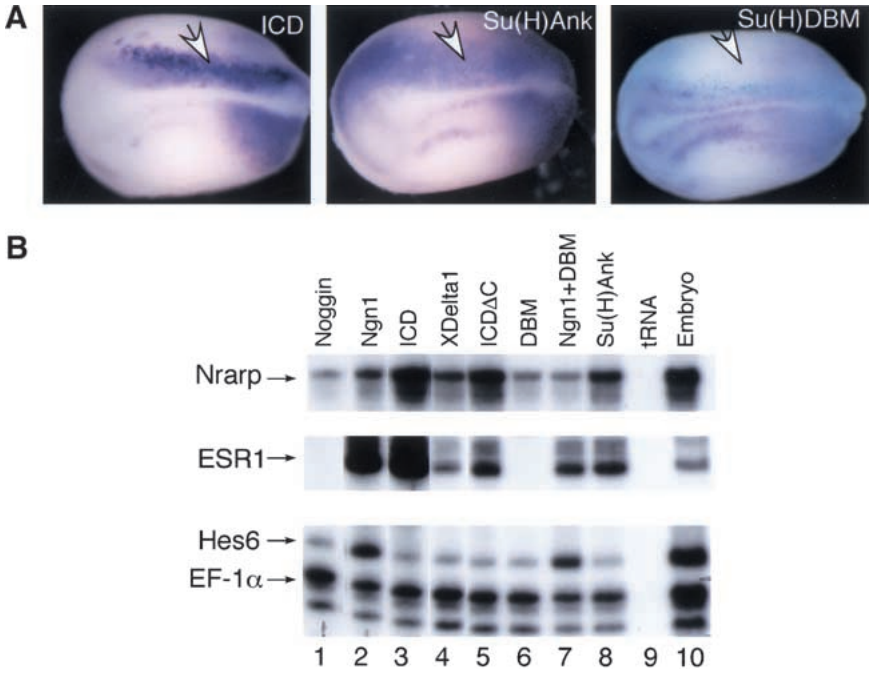

Figure 3. *Nrarp* expression is regulated by the CSL-dependent Notch pathway. (*A*) *Xenopus* embryos at the two-cell stage were injected with RNA encoding ICD, XSu(H)Ank, or XSu(H)DBM along with *LacZ* RNA as a tracer. At neural plate stages, embryos were fixed, stained for β-galactosidase activity, and analyzed for expression of *Nrarp* using whole-mount in situ hybridization. Shown is a dorsal view of the neural plate with anterior oriented to the left and the injected side of the embryos oriented up. Note that both ICD and XSu(H)Ank induce high levels of *Nrarp* expression, whereas XSu(H)DBM inhibits the expression of *Nrarp*. (*B*) *Xenopus* embryos at the two-cell stage were injected with RNA encoding *Xenopus* neurogenin (Ngn1) or various components of the Notch pathway, along with RNA encoding the neural inducer Noggin. (DBM) DNA-binding mutant of XSu(H); ICDΔC is depicted in Figure 6B. Neuralized ectoderm was removed from embryos at blastula stages and assayed at stage 16 (early neurulae) for the expression of *Nrarp*, *ESR1*, and *HES6* RNA using an RNase protection assay (RPA) (Wettstein et al. 1997). Each sample was also assayed for *EF-1*α RNA expression as a loading control. Note that *Nrarp* expression is activated by Notch signaling induced using either activated forms of the receptor, XSu(H), or with ectopic ligand expression.

induces *Nrarp* expression via the CSL protein, XSu(H), embryos were injected with RNA encoding forms of XSu(H) that activate or inhibit the expression of other Notch target genes (Wettstein et al. 1997). As predicted for a CSL-dependent gene, *Nrarp* RNA expression is induced by an activated form of *Xenopus* XSu(H), called XSu(H)Ank, and inhibited by a DNA-binding mutant of XSu(H), called XSu(H)DBM (Fig. 3A). Thus, activation of the CSL-dependent Notch pathway is one factor that regulates the expression of *Nrarp* in early embryos.

The regulation of *Nrarp* expression was also examined in animal cap assays. RNAs were injected into embryos at the two-cell stage. Animal caps were removed at blastula stage and cultured until stage 16, when they were analyzed in RNase protection assays (RPA) for the expression of *Nrarp* or *EF-1*α as a loading control (Fig. 3B). For comparison, these samples were also assayed for the expression of two genes that encode bHLH repressors but differ in that one is up-regulated by Notch signaling (*ESR1*; Wettstein et al. 1997) whereas the other is not (*HES6*; Koyano-Nakagawa et al. 2000). The results show that *Nrarp* expression occurs at low levels in both isolated ectoderm and ectoderm neuralized by injecting embryos with *noggin* RNA (Fig. 3B, lane 1; data not shown). Increased levels of *Nrarp* expression resulted when animal caps expressed proteins that activate the Notch pathway, including ICD (Fig. 3B, lane 3), XDelta1 (Fig. 3B, lane 4), a truncated form of ICD (ICDΔC, Fig. 3B, lane 5; see Fig. 6B, below), and the activated form of XSu(H), XSu(H)Ank, (Fig. 3B, lane 8). In contrast, *Nrarp* expression was unchanged when neuralized animal caps express the DNA-binding mutant of XSu(H), XSu(H)DBM (Fig. 3B, lane 6). The neural bHLH protein, XNGN1, also induced higher levels of *Nrarp* expression (Fig. 3B, lane 2), but this may be indirect, via the ability of XNGN1 to activate the expression of *XDelta1* and thus Notch signaling (Koyano-Nakagawa et al. 1999). Indeed, coinjection of XSu(H)DBM along with XNGN1 markedly reduced the induction of *Nrarp* expression, as expected if this regulation were indirect (Fig. 3B, lane 7). By comparison, the response of *Nrarp* to both Notch signaling and XNGN1 follows closely that for another Notch target gene *ESR1* (Wettstein et al. 1997) but differs from that of *HES6*, which has been shown to be regulated by XNGN1 but not by Notch (Fig. 3B; Koyano-Nakagawa 2000). Thus, these data provide further evidence that *Nrarp* expression is regulated by Notch signaling in early embryos.

Overexpression of Nrarp *in* Xenopus *embryos inhibits the Notch signaling pathway*

To gain insight into the potential role of Nrarp as a Notch target gene in embryonic development, we overexpressed the protein by injecting embryos with RNA encoding a myc-tagged form of Nrarp. Injected embryos stained with an α-myc epitope antibody revealed expression in nuclei and in the cytoplasm (data not shown). We first examined *Nrarp*-injected embryos by scoring the number of primary neurons in the neural plate or the number of ciliated cells in the skin. In both cases, increased Notch signaling markedly reduces the number of

differentiated cells that form, whereas disabling Notch signaling markedly increases their number (Chitnis et al. 1995; Deblandre et al. 1999). In a manner consistent with a reduction of Notch signaling, injection of *Nrarp* RNA markedly increases the number of both primary neurons and ciliated cells (Fig. 4A–C). In the neural plate, this increase in cell number is restricted to those domains where primary neurons normally form, as observed previously when Notch signaling is inhibited by other means (Fig. 4A; Wettstein et al. 1997). Identical results were obtained with a nontagged form of Nrarp (data not shown).

Nrarp overexpression could conceivably inhibit the Notch signaling pathway by targeting the expression and/or activity of the ligands, the receptors, or the downstream Notch target genes that act as effectors of the pathway. As a first step toward pinpointing where Nrarp acts, we asked whether *Nrarp* overexpression changes the expression pattern of *XDelta1* and *ESR1*, because these genes appear to play a key role in Notch signaling during primary neurogenesis (Chitnis and Kintner 1995; Wettstein et al. 1997). This analysis shows that the levels of *XDelta1* RNA expression in the neural plate increase in response to *Nrarp* overexpression (Fig. 4D). This result indicates that Nrarp is not likely to act by inhibiting *XDelta1* expression, but is consistent with a loss of Notch signaling in these embryos because Notch signaling is known to negatively regulate *XDelta1* expression (Wettstein et al. 1997). In contrast, the expression of the Notch target gene, *ESR1*, decreased in the neural plate in response to *Nrarp* overexpression (Fig. 4E,F), suggesting that *Nrarp* interferes with the activation of Notch target genes. This inhibition may account for the ability of Nrarp to disable the lateral inhibitory process, thereby producing an increase in the number of primary neurons and ciliated cell precursors.

Nrarp inhibits ICD-mediated transcription in animal cap assays

We next asked whether Nrarp inhibits Notch signaling by interfering directly with ICD-mediated transcription. Toward this end, we used the animal cap assay described above to determine whether Nrarp also inhibits the activation of Notch target genes by ICD. Different amounts of *ICD* RNA were injected into embryos at the two-cell stage, and the animal caps were removed at blastula stage and cultured until stage 16 when they were analyzed by RPA for the expression of *ESR1* and *ESR7*, as well as *EF-1*α as a loading control. RNA injections in all cases included the neural inducer *Noggin* because both of these Notch target genes are normally expressed in neural tissue (Wettstein et al. 1997; Deblandre et al. 1999). The results show that ICD induces the expression of both *ESR1* and *ESR7* in a dose-dependent fashion in neuralized animal caps, and that the coexpression of *Nrarp* consistently reduces the activity of *ICD* in this assay (Fig. 5A). As a negative control, a truncated form of Nrarp lacking the ankyrin repeats (Nrarp^{tr}, truncated after amino acid 51; Fig. 1A) either had no effect or slightly increased the activity of ICD. Similar results were obtained when more physiological levels of Notch signaling were activated in this assay using XDelta1 (Fig. 5A). Again, *Nrarp* overexpression decreased the levels of

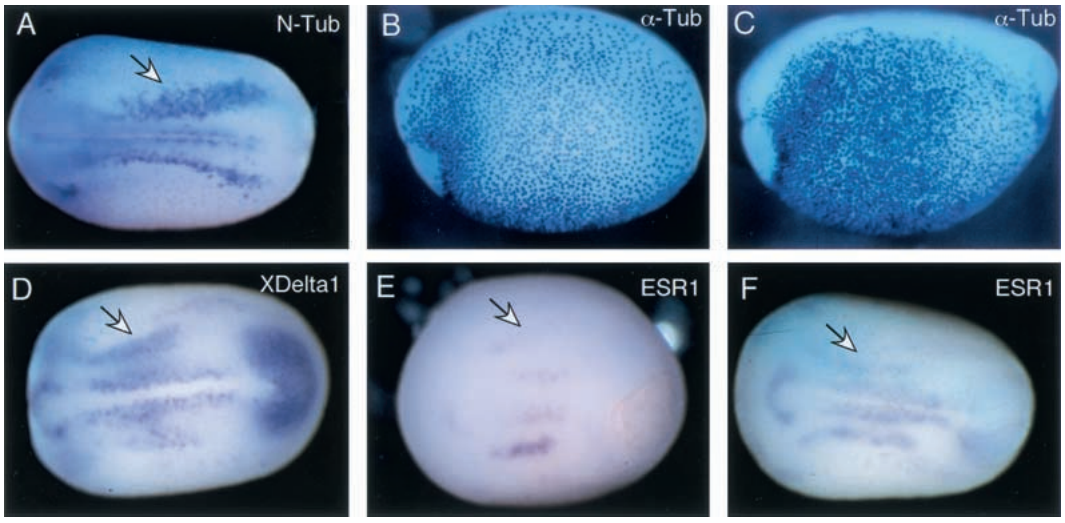

Figure 4. *Nrarp* overexpression blocks Notch signaling in embryos. *Xenopus* embryos at the two-cell stage were injected with RNA encoding Nrarp along with *LacZ* RNA as a tracer. At neural plate stages, embryos were fixed, stained for β-galactosidase expression, and double-labeled by whole-mount in situ hybridization for markers of neurons or ciliated cell precursors. (*A*) *Nrarp* overexpression induces the formation of additional primary neurons (arrow) as shown by the expression of a neural isoform of β-tubulin (N-Tub). Shown is dorsal view of the neural plate with the injected side oriented to the top of the panel. (*B*,*C*) *Nrarp* overexpression induces additional ciliated cell precursors that form in the skin as shown by the expression of an isoform of α-tubulin (α-Tub) (Deblandre et al. 1999). The uninjected and injected sides of the same embryo are shown in panels *B* and *C*, respectively. (*D–F*) Dorsal view of the neural plate with the injected side oriented up. Note that *Nrarp* overexpression induces more *XDelta1* expression (*D*) but reduces the expression of *ESR1* when scored at stage 12 or stage 14 (*E* and *F*, respectively).

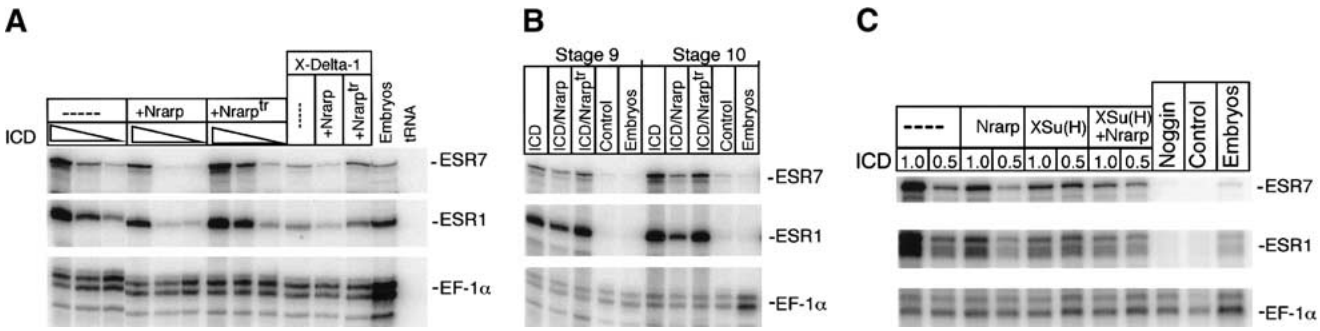

Figure 5. *Nrarp* overexpression alters ICD-mediated transcription. (*A*) *Xenopus* embryos at the two-cell stage were injected with different concentrations of *ICD* RNA in either the presence or absence of RNA encoding Nrarp or Nrarp^{tr}. At blastula stage, animal caps were removed and cultured to the equivalent of stage 16, when they were assayed for expression of *ESR1*, *ESR7*, and *EF-1*α as described in Materials and Methods. Note that *Nrarp* overexpression reduced the levels of *ESR1* and *ESR7* expression induced by ICD. As a negative control, Nrarp^{tr} either had no effect or even slightly increased the levels of *ESR* gene expression, suggesting that it might have weak dominant-negative effects. Similarly, when *ESR1* and *ESR7* expression is induced by XDelta1, *Nrarp* overexpression reduces *ESR* expression, whereas *Nrarp^{tr}* slightly increases it. All assays included RNA encoding noggin to neuralize the ectoderm. (*B*) Time course experiments in which neuralized animal caps injected with the designated RNAs were extracted and assayed at the denoted developmental stages.

ESR1 and *ESR7* induced by XDelta1, whereas *Nrarp^{tr}* slightly increased their levels. The effects of *Nrarp* on ICD activity were examined further by assaying the isolated animal caps as early as an hour after the beginning of zygotic transcription (Fig. 5B). Even at these early stages, overexpression of *Nrarp* decreases the levels of *ESR1* and *ESR7* induced by ICD. Thus, Nrarp inhibits the activation of target genes by ICD, suggesting that it might interfere with ICD-mediated transcription.

Nrarp forms a ternary complex with XSu(H) and the ankyrin repeats of ICD

To determine whether Nrarp alters ICD-mediated transcription by interacting physically with either ICD or XSu(H), we injected embryos at the two-cell stage with RNA encoding Flag-tagged Nrarp along with RNA encoding myc-tagged ICD and XSu(H). Extracts were prepared from isolated animal pole tissue at blastula stages and subjected to immunoprecipitation with an α-Flag antibody, followed by Western analysis with an α-myc antibody. The results show that ICD or XSu(H) are not detectably coimmunoprecipitated (co-IPed) in a complex with Nrarp when expressed alone (Fig. 6A, lanes 6,7). Strikingly, however, when ICD and XSu(H) are expressed in combination, they are co-IPed with Nrarp, indicating that Nrarp forms a ternary complex with XSu(H) and ICD (Fig. 6A, lane 8).

Different deleted forms of ICD and XSu(H) were tested in this co-IP assay to determine roughly what regions were required for binding to Nrarp (Fig. 6B). The results show that a ternary complex can form with ICDΔC, which lacks sequences downstream of the ankyrin repeats (Fig. 6C, lane 6). A complex does not form with the juxtamembrane region of ICD, called RAM23 (ICDram23; Fig. 6C, lane 7), which has the strongest interaction with XSu(H) in vitro (Tamura et al. 1995). However, the ankyrin repeat region of ICD (ICDAnk; Fig. 6C, lane 8) is sufficient to form a ternary complex with XSu(H) and Nrarp (see also Fig. 7, lane 12). Finally, we tested a form of XSu(H) lacking 117 amino acids from the carboxyl terminus [XSu(H)^{tr}; Fig. 6B], which fails to activate the expression of Notch target genes in embryos (Wettstein et al. 1997). XSu(H)^{tr} also fails to form a ternary complex with ICD and Nrarp (Fig. 6C, lane 2). Thus, Nrarp interacts physically with XSu(H) and ICD in a ternary complex, in which the ankyrin repeats of ICD are sufficient for complex formation.

The formation of a ternary complex between Nrarp, XSu(H), and the ankyrin repeats of ICD was tested further by co-IP with a Flag-tagged XSu(H). In line with the results described above, myc-tagged Nrarp does not bind detectably to Flag-tagged XSu(H) unless ICD is also present (Fig. 6D, cf. lanes 6 and 5 in lower panel). Again, the ankyrin repeats of ICD are sufficient to promote a strong interaction between Nrarp and XSu(H) (Fig. 6D, lane 12), whereas the RAM23 region shows a much weaker interaction (Fig. 6D, lane 10). Significantly, the results also show that Nrarp can increase the amount of ICD that associates with XSu(H) in a co-IP complex (Fig. 6D, cf. lanes 6 and 4). Again, this effect of Nrarp occurs with just the ankyrin repeat region of ICD (Fig. 6D, lane 12 vs. 11), but does not occur with the RAM23 region (Fig. 6D, lane 10 vs. 9). These results support further the conclusion that a ternary complex forms between Nrarp, XSu(H), and the ankyrin repeats of ICD and moreover show that Nrarp markedly increases the amounts of ICD that can associate with XSu(H).

Nrarp can form a complex with XSu(H) and ICD that includes Mastermind

Both Nrarp and Mastermind form ternary complexes with the CSL proteins and ICD. We asked, therefore, whether the binding of Nrarp and Mastermind to Su(H) and ICD is mutually exclusive or whether these proteins

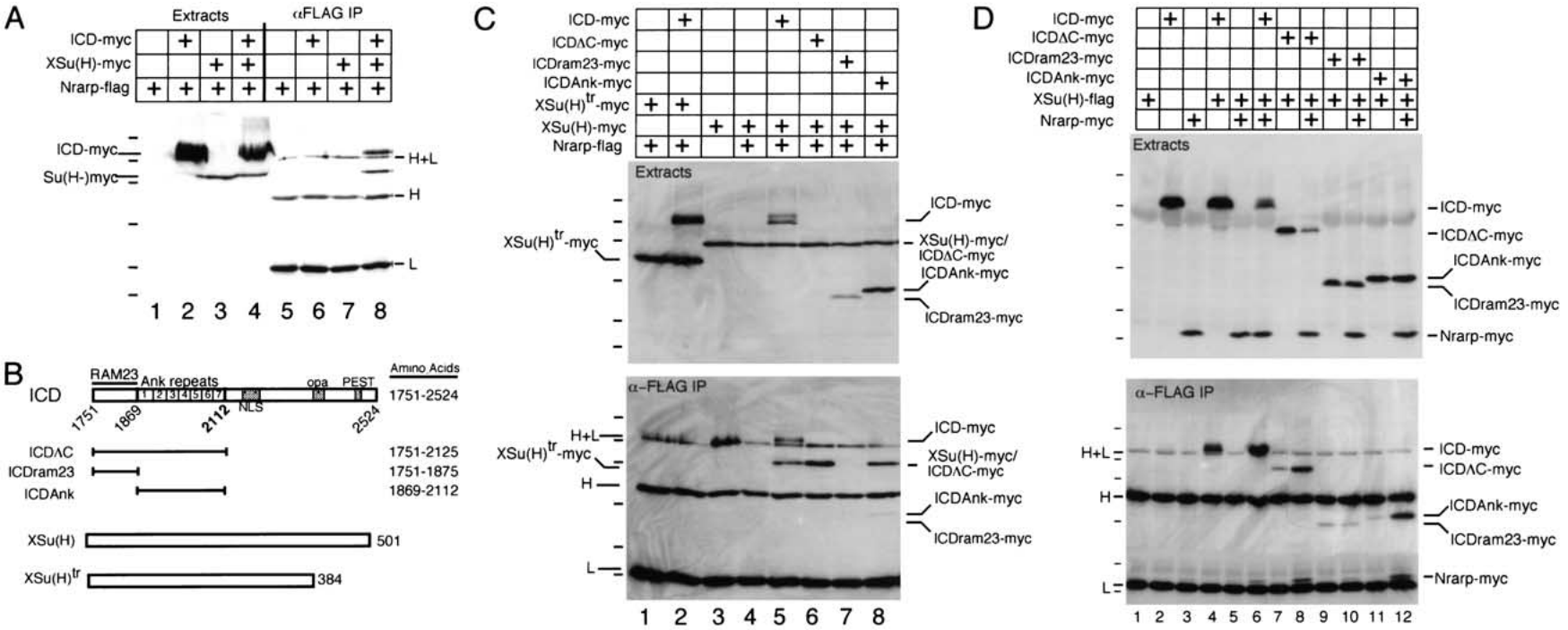

Figure 6. Nrarp forms a complex with XSu(H) and ICD. (*A*) RNA encoding myc-tagged forms of ICD and XSu(H) was injected along with Flag-tagged Nrarp into two-cell stage embryos. Extracts were prepared from animal tissue at blastula stages and subjected to Western analysis either directly (*left*; Extracts) or after coimmunoprecipitation (co-IP) with an α-Flag antibody (*right*; αFLAG IP) as described in the Materials and Methods. The myc-tagged proteins on Western blots were detected using the 9E10 α-myc monoclonal antibody, a secondary α-mouse IgG conjugated with horseradish peroxidase, and the ECL reagent. Note that neither ICD (lane *6*) nor XSu(H) (lane *7*) co-IP with Flag-tagged Nrarp unless both are present (lane *8*). (H and L) Position of heavy and light chains, respectively, of the α-Flag antibody in this and subsequent figures. Similarly, the position of molecular weight markers is denoted here and elsewhere by a series of hatches left of the panel. (*B*) Diagram of the various forms of ICD and XSu(H) used for deletion analysis. Note that these deletions of ICD include a nuclear localization signal (NLS) that was added by cloning into the CS2-NLS vector (Turner and Weintraub 1994). (*C*) Co-IP of various myc-tagged ICD and XSu(H) with Nrarp-Flag. Shown is the Western analysis using the α-myc antibody of total extracts (*top*) or a co-IP with the α-Flag antibody (*bottom*). Note that both XSu(H)-myc and ICD-myc co-IP with Nrarp-Flag when all three are present (lane *5*), but that this complex does not form with a truncated form of XSu(H) (lane *2*). Moreover, both ICDΔC-myc (lane *6*) and ICDAnk-myc (lane *8*) form a complex with XSu(H)-myc and ICD-myc, but ICDram23-myc (lane *7*) does not. Note that XSu(H)-myc and ICDΔC-myc comigrate at the same position on the gel. (*D*) Co-IP and Western analysis of embryo extracts expressing XSu(H)-Flag, as well as ICD-myc and Nrarp-myc. (*Top*) Western analysis of total extracts with the α-myc antibody; (*bottom*) same analysis of the proteins co-IPed with XSu(H)-Flag. Note that the amount of ICD associated with XSu(H) increases in the presence of Nrarp (cf. lanes *4* and *6*). Similar results are obtained with ICDΔC (cf. lanes *7* and *8*) and ICDAnk (cf. lanes *11* and *12*) but not with ICDram23 (cf. lanes *9* and *10*). Note also that Nrarp-myc co-IPs with XSu(H)-Flag in the presence of ICD-myc (lane *6*), ICDΔC-myc (lane *8*), and ICDAnk-myc (lane *12*), but not ICDram23-myc (lane *10*).

can exist in a complex together. Binding of human Mastermind (hMM) to XSu(H) and ICD from XNotch1 was examined first by co-IP analysis in which extracts were prepared from embryos injected with RNA encoding Flag-tagged XSu(H), myc-tagged hMM, and myc-tagged ICDΔC. Flag-tagged XSu(H) was recovered from total extracts, and associated proteins were analyzed by Western analysis. The results show that hMM is co-IPed detectably with XSu(H), but only in the presence of ICDΔC (Fig. 7A, lanes 5,8). Moreover, the amounts of ICDΔC associated with XSu(H) in a co-IP complex increase markedly in the presence of hMM (Fig. 7A, cf. lanes 8 and 6). Both of these results are consistent with the idea that hMM binds to XSu(H) and ICD in a ternary complex, as reported by others (Petcherski and Kimble 2000b; Wu et al. 2000a). Myc-tagged Nrarp is also co-IPed with XSu(H) in the presence of hMM and ICDΔC, consistent with the formation of multimeric complexes (Fig. 7A, lane 9). However, this finding is inconclusive as to whether these proteins can form a quaternary complex. To address this issue, embryos were injected with RNA encoding Flag-tagged Nrarp along with myc-tagged hMM, XSu(H), and ICD. Flag-tagged Nrarp was recovered from extracts by immunoprecipitation, and associated proteins were analyzed by Western analysis using an α-myc antibody. The results show that the immunoprecipitation of Nrarp recovers not only XSu(H) and ICD, but hMM as well (Fig. 7B, lane 5), indicating that Nrarp and hMM can bind in tandem to XSu(H)/ICD to form a quaternary complex.

Nrarp decreases the levels of ICD

In the Western analyses described above, the levels of ICD in total embryonic extracts were consistently less in the presence of Nrarp than in its absence (e.g., in Fig. 6D, cf. lanes 6 and 4, upper panel), indicating that Nrarp promotes the loss of ICD. To examine this possibility further, embryos were injected at the two-cell stage with different amounts of RNA encoding a myc-tagged ICD, either alone or with RNA encoding myc-tagged Nrarp or myc-tagged XSu(H). Animal caps isolated from these embryos were extracted, and the amounts of myc-tagged ICD were measured by Western analysis using an α-myc antibody. The results confirm that the levels of ICD present in these extracts were reduced markedly in the presence of Nrarp (Fig. 8A, cf. lanes 6,8 and lane 2). ICD loss was evident in embryos injected with only *Nrarp* and ICD RNAs, suggesting that Nrarp may act without XSu(H). However, because XSu(H) is expressed maternally and at relatively high levels in early embryos, Nrarp likely requires endogenous XSu(H) to promote ICD loss. Consistent with this interpretation, exogenous myc-tagged XSu(H) potentiates a decrease in ICD levels when coexpressed with Nrarp (Fig. 8A, lane 8 vs. lane 6) but has no effect on ICD levels when expressed alone (Fig. 8A, lane 4). Coupled with our findings that ICD, XSu(H), and Nrarp form a complex (Fig. 6), these results indicate that ICD levels are reduced when that complex includes Nrarp, perhaps accounting for the lower levels of Notch signaling in these embryos.

We next asked whether ICD loss also occurs when ICD and XSu(H) form a ternary complex with hMM. Such loss might be predicted if any factor that promotes a complex between XSu(H) and ICD also promotes a loss of ICD. Different levels of RNA encoding myc-tagged ICD, along with constant amounts of RNA encoding myc-tagged XSu(H), were injected into embryos, either alone or with RNA encoding Flag-tagged Nrarp or myc-tagged hMM. Western analysis of total extracts made

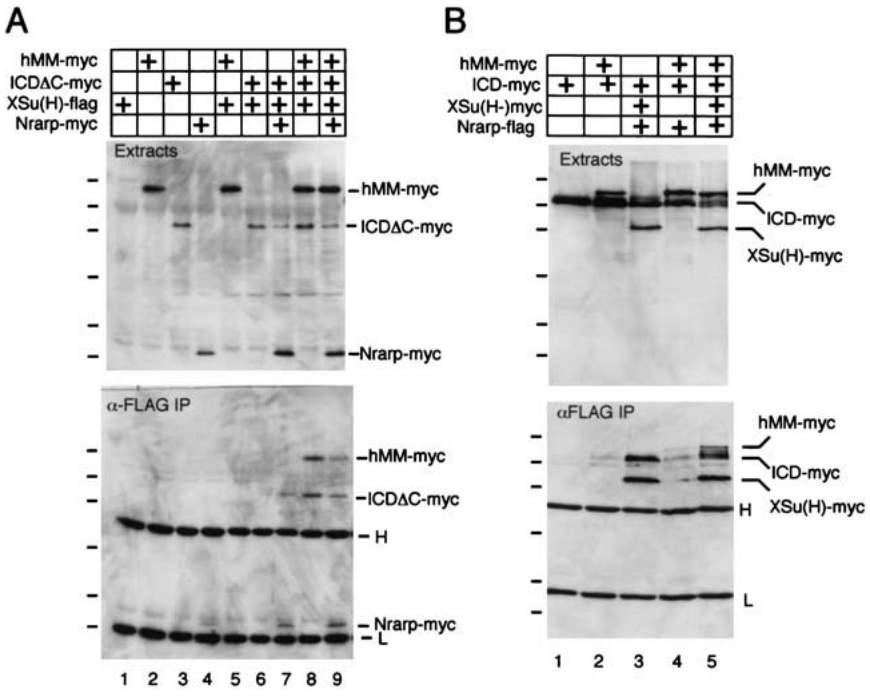

Figure 7. The Nrarp complex can include Mastermind. (*A*) Human Mastermind (hMM) forms a complex with XSu(H) and ICD. Embryo extracts expressing Flag-tagged XSu(H), as well as myc-tagged ICDΔC, Nrarp, and human Mastermind were subjected to co-IP and Western analysis using an α-myc antibody. (*Top*) Total extracts; (*bottom*) products that co-IP with an α-Flag antibody. Note that hMM binds to Su(H), but only in the presence of ICDΔC, and increases the levels of ICDΔC associated with XSu(H) (lane *8* vs. lane *6*). Inclusion of Nrarp forms a complex that is co-IPed with XSu(H) (lane *9*). (*B*) Co-IP and Western analysis of embryo extracts expressing Nrarp-Flag, XSu(H)-myc, ICD-myc, and hMM-myc. Note that hMM is included in a co-IP complex with ICD and XSu(H) that is recovered using Nrarp-Flag (lane *5*, *bottom*). The low levels of ICD and hMM recovered in lane *4* presumably reflect the fact that XSu(H) is expressed ubiquitously and thus already present in extracts.

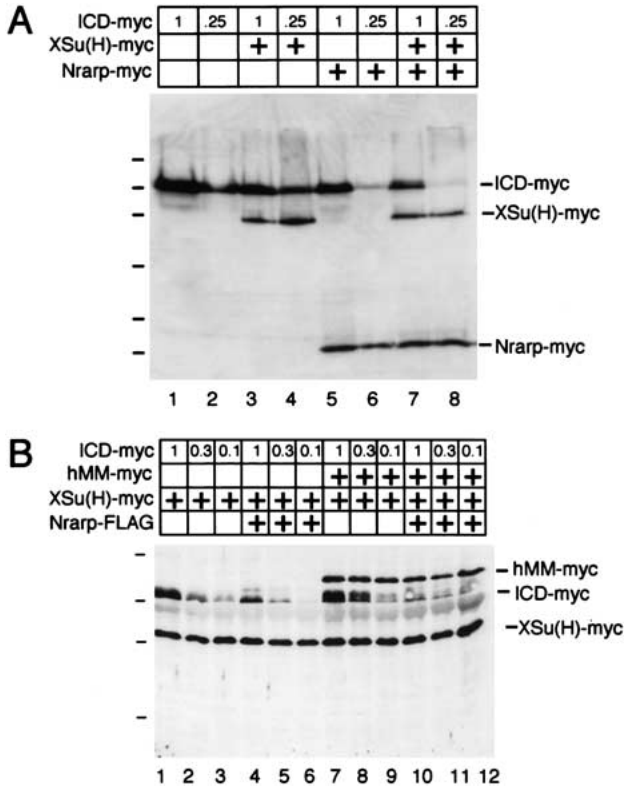

Figure 8. Nrarp reduces the levels of ICD in total cell extracts. (*A*) Two different concentrations of RNA encoding ICD-myc were injected into embryos alone or in the presence of XSu(H)-myc and Nrarp-myc. Shown is a Western analysis of the extracts prepared from these embryos using an α-myc antibody. Note that the presence of Nrarp decreases the levels of ICD-myc in the extracts (cf. lanes *6* and *2*), and this decrease is greater in the presence of XSu(H) (cf. lanes *8* and *6*). (*B*) Three different levels of ICD RNA were injected into embryos along with XSu(H)-myc in the presence and absence of Nrarp-Flag or hMM-myc. Shown is a Western blot with total extracts probed with a anti-myc antibody. Note that hMM-myc does not detectably change the levels of ICD-myc (lanes *7–9*; see also Fig. 7A), nor does hMM-myc block the degradation promoted by Nrarp-Flag (lanes *10–12*).

from these embryos shows that hMM did not noticeably alter the levels of myc-tagged ICD (Fig. 8B, lanes 7–9; see also Fig. 7B). In the presence of hMM, the reduction of ICD promoted by Nrarp still occurs or is slightly enhanced (Fig. 8B, lanes 10–12). Together, these results indicate that hMM on its own does not cause a significant loss of ICD in embryos, although it may potentiate the loss that occurs in response to Nrarp.

Nrarp forms a complex with Su(H) and ICD in cultured cells and promotes ICD-mediated transcription

One model based on the results above is that Nrarp forms a complex with XSu(H) and ICD that leads to a loss of ICD, thus attenuating the levels of ICD-mediated transcription and resulting in loss of Notch signaling. To verify these results, we tested whether Nrarp inhibits ICD-mediated transcription using reporter assays in cultured cells. For this assay, HeLa cells were transfected with a reporter gene consisting of multimerized CBF1/Su(H) binding sites cloned upstream of a basal promoter driving luciferase expression. This reporter is relatively silent in the absence of ICD but has been shown by others to be stimulated following misexpression of activated forms of human and mouse Notch (Hsieh et al. 1996; Kato et al. 1997; Kao et al. 1998). Surprisingly, when *Xenopus* ICD was transfected at levels eliciting a submaximal ICD response, cotransfection of Nrarp produced a marked stimulation over the activity seen with ICD alone (Fig. 9A, left panel). Transfection of Nrarp with ICD and a reporter with mutant Su(H) binding sites had no effect on basal level transcription (data not shown), indicating that ICD activity in this assay with or without Nrarp requires Su(H). The stimulation of ICD transcription mediated by Nrarp was comparable in magnitude to that seen when hMM was cotransfected with ICD (Fig. 9A, left panel). When ICD was transfected in this assay at even higher levels, Nrarp did not promote further reporter activity, as has also been observed for Mastermind (Fig. 9A, right panel; Wu et al. 2000a). However, coexpression of Nrarp and Mastermind along with ICD resulted in a further increase in reporter activity. Thus, in cultured cells, Nrarp promotes transcriptional activation by the Su(H)/ICD complex both in the presence and absence of the coactivator protein Mastermind.

Nrarp has opposite effects on ICD activity in embryos and cultured cells: In the former it promotes ICD degradation and blocks Notch signaling, whereas in the latter it potentiates the ability of ICD to activate a Su(H)-dependent reporter. We reasoned that based on its ability to potentiate transcription, Nrarp was likely able to form a complex with Su(H) and ICD in cultured cells, but that Nrarp might not be able to efficiently promote ICD loss in this context if cultured cells were deficient in components required for degradation to occur. To determine whether Nrarp forms a complex with XSu(H) and ICD and whether complex formation promotes loss of ICD in cultured cells, CS2 constructs encoding myc-tagged ICD, XSu(H), and Flag-tagged Nrarp were transfected in 293T cells, and extracts were prepared and analyzed by Western analysis directly or after co-IP with the α-Flag antibody. The results show that Nrarp brings down both ICD and XSu(H) in a complex but only when both are present, as was seen in embryos (Fig. 9B, lane 3). However, in contrast to what we observed in embryos, ICD levels were not altered detectably in the presence of Nrarp (Fig. 9B, cf. lanes 2,3 and lane 1). We next correlated levels of exogenous ICD protein with transcriptional activity in HeLa cells in reporter assays comparable to those described above. Under conditions of high ICD expression, Nrarp potentiated ICD activity modestly (Fig. 9C), and Western analysis of the same transfected cells showed that Nrarp did not appreciably change the steady-state levels of ICD-myc protein (Fig. 9D). Similarly, Nrarp also

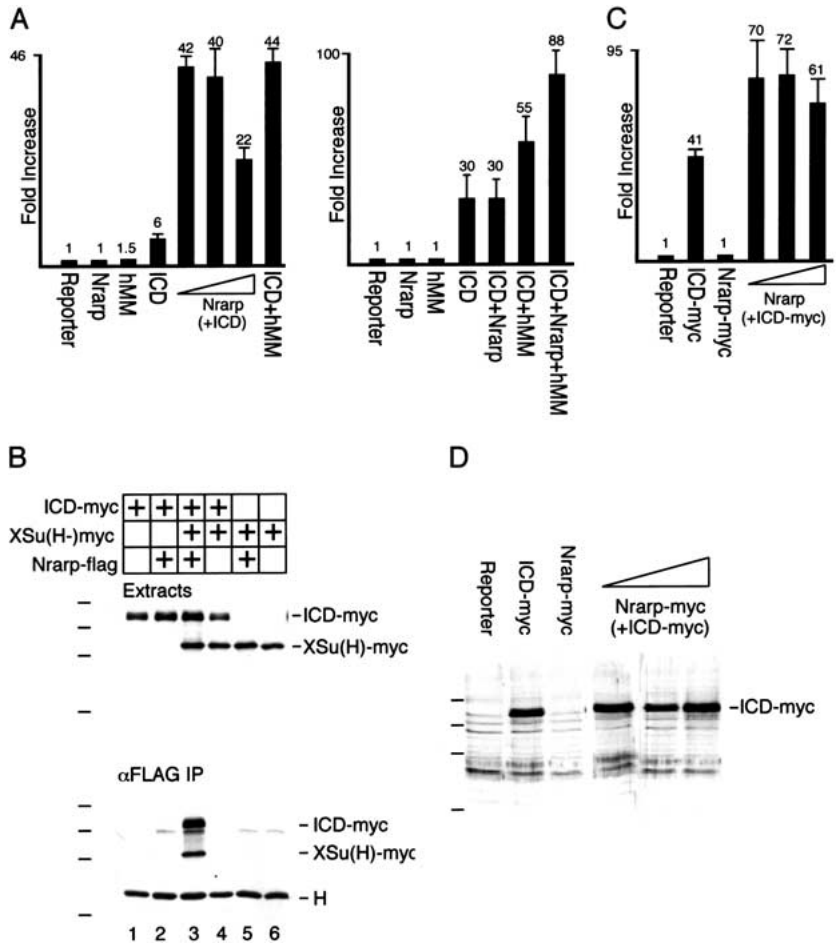

Figure 9. Nrarp potentiates Su(H)-dependent ICD activity in vitro. (*A*) HeLa cells were transfected transiently with a luciferase reporter containing multimerized Su(H) binding sites plus effectors. (*Left*) Activation of the reporter by ICD, ICD plus increasing levels of Nrarp, and ICD plus hMM. Cells were transfected with an ICD expression vector at a level resulting in submaximal (sixfold) activation. For details of transfection conditions see Materials and Methods. Cotransfection of Nrarp with ICD produced an approximately sevenfold increase in activity over ICD alone, an increase comparable to that seen with cotransfection of hMM with ICD. (*Right*) When ICD was transfected at levels producing higher activation of the reporter (30-fold), addition of Nrarp or hMM with ICD had little effect on activity. A combination of both Nrarp and hMM with ICD, however, produced an approximately threefold increase in activity over ICD alone. Bars represent means of triplicate measurements, and the standard error of the mean is shown. The activity of the reporter alone is defined arbitrarily as 1. Luciferase values are normalized to the activity of a cotransfected β-galactosidase vector. (*B*) Co-IP and Western analysis of 293T cells transfected with a tagged form of Nrarp (Nrarp-flag), as well as with myc-tagged forms of ICD and XSu(H). (*Top*) Western analysis of cell extracts with the α-myc antibody; (*bottom*) parallel analysis of proteins co-IPed with Nrarp-Flag. Note that ICD-myc or XSu(H)-myc coprecipitate with Nrarp only in a ternary complex (cf. lane *3* and lanes *2*,*5*). (*C*) Reporter assay and (*D*) corresponding Western blot of Nrarp-myc cotransfected with ICD-myc in HeLa cells. In this experiment a modest potentiation of luciferase activity is observed when Nrarp-myc is cotransfected with high levels of ICD-myc, as is the case in the *right* panel of *A*. However, in HeLa cells no degradation of ICD-myc is observed in the presence of even the highest levels of Nrarp-myc.

did not appreciably change the levels of ICD detected by Western blot under conditions in which it promotes ICD transcription (data not shown). Together, these results indicate that in HeLa and 293T cells, in which Nrarp-mediated loss of ICD is not favored, Nrarp forms a complex with Su(H) and ICD that can promote ICD-mediated transcription.

Discussion

The Notch signaling pathway plays a prominent role in determining cell fate in embryos by activating the expression of downstream target genes. Here we report the analysis of *Nrarp*, whose expression pattern in early embryos suggested that it could be part of the Notch pathway as predicted by its synexpression group (Niehrs and Pollet 1999). The results of this analysis provide strong evidence that *Nrarp* is indeed activated by Notch signaling and encodes a product that feeds back on the activity of the Notch pathway by forming a complex with XSu(H), ICD, and other proteins associated with Notch-mediated transcription.

Expression of Nrarp *is activated by Notch signaling*

Diverse genes have been shown to be direct targets for transcriptional regulation by the Notch pathway in *Drosophila* (Bray and Furriols 2001). In contrast, relatively few direct Notch target genes are known in vertebrate embryos, and those are related to genes in the *Drosophila* enhancer of split locus encoding transcriptional repressors. Evidence presented here shows that Nrarp expression is regulated by Notch signaling, indicating that it is a new, perhaps direct, transcriptional target of CSL/ICD. *Nrarp* expression is detected in regions where active Notch signaling is known to regulate cell fate, including the developing nervous system, during primary and secondary neurogenesis (Chitnis et al. 1995), and in the presomitic mesoderm during segmentation (Jen et al. 1999). Moreover, the expression of *Nrarp* in these tissues changes predictably as Notch signaling is perturbed experimentally. Thus, expression of *Nrarp* is up-regulated in response to an activated form of XNotch1, ICD, and to an activated form of XSu(H), XSu(H)Ank. Conversely, expression of *Nrarp* is reduced in response to a domi-

nant-negative form of XSu(H), XSu(H)DBM (Wettstein et al. 1997). The very strong up-regulation of *Nrarp* expression in response to ICD is indicative of a direct response, although definitive support for direct regulation requires analysis of the Nrarp promoter and the identification of functional Su(H) binding sites.

The expression of *Nrarp* in animal cap assays as well as in embryos can be induced by ectopic expression of XNGN1 (Fig. 3). This regulation may be indirect because XNGN1 activates Notch signaling by inducing expression of the Notch ligand, XDelta1. Nonetheless, many of the genes regulated directly by Notch in *Drosophila* are coregulated by E-box binding proteins (Nellesen et al. 1999), raising the possibility that *Nrarp* expression may also depend on regulation by the bHLH proteins.

Overexpression of Nrarp inhibits the activity of Notch in embryos

As a Notch target gene, Nrarp could act as a downstream mediator of Notch signaling, for example, by inhibiting cell differentiation. Overexpressing Nrarp in embryos, however, caused the opposite phenotype: a dramatic increase in the number of primary neurons and ciliated cells in the neural plate and skin, respectively. This increase in cell differentiation by Nrarp overexpression is identical to that observed when Notch signaling is blocked in embryos using dominant-negative reagents (Chitnis and Kintner 1995; Wettstein et al. 1997). Moreover, *Nrarp* overexpression induces the expression of *XDelta1*, a gene whose expression is normally inhibited by the Notch pathway in a negative feedback loop. Such phenotypes indicate that Nrarp overexpression disables the output of Notch signaling. Consistent with this interpretation, Nrarp overexpression inhibits expression of other Notch target genes, *ESR1* and *ES7*, one of which, *ESR1*, is a direct transcriptional target of ICD (Wettstein et al. 1997). Both of these genes are candidate downstream effectors of Notch signaling because they are activated strongly by Notch signaling and can repress primary neuron formation in ectopic expression experiments (Wettstein et al. 1997; Takke et al. 1999; Koyano-Nakagawa et al. 2000). Finally, in animal cap assays the activation of *ESR1* and *ESR7* by ICD is inhibited by *Nrarp* overexpression. The simplest model to explain these results is that *Nrarp* overexpression interferes with the ability of ICD to activate the expression of downstream Notch target genes, thereby blocking Notch signaling and promoting cell differentiation.

Nrarp forms a ternary complex with XSu(H) and ICD

Because Nrarp overexpression interferes with ICD activity in embryos, we tested whether Nrarp physically interacts directly with ICD. The results of these analyses provide several lines of compelling evidence that Nrarp acts by binding tightly to ICD in a ternary complex with XSu(H). First, Nrarp binding to ICD and XSu(H) can be detected by co-IP of tagged proteins both in embryos and in tissue culture cells. Binding can also be shown in vitro using bacterially expressed proteins in gel shift assays (data not shown). Efficient binding requires the presence of all three components and, moreover, results in a complex in which each component seems to be represented in equal proportions. Thus, these results indicate that a ternary complex forms between Nrarp, ICD, and XSu(H), involving protein–protein interactions that are tripartite in nature. Second, formation of a complex occurs with just the ankyrin repeats of ICD, a region that is critical for Notch activity and for ICD-mediated transcription (Kodoyianni et al. 1992; Roehl and Kimble 1993; Kopan et al. 1994; Hsieh et al. 1996; Roehl et al. 1996; Wettstein et al. 1997). Conversely, the complex does not form with a nonfunctional mutant of XSu(H) in which 117 amino acids are removed from the carboxyl terminus. The binding of Nrarp to these critical regions of XSu(H) and ICD is a strong indication that the activities of these proteins are modulated by Nrarp. Third, ICD is known to bind to XSu(H) via the juxtamembrane RAM23 region (Fig. 6B; Tamura et al. 1996) and to a lesser extent the ankyrin repeats (Fortini and Artavanis-Tsakonas 1994). However, our results suggest that a binary interaction between ICD and Su(H) is not nearly as tight as a ternary interaction among ICD, XSu(H), and Nrarp. Indeed, a tight association among these three components occurs even in the absence of RAM23. Together, these results indicate that Nrarp promotes an interaction between ICD and XSu(H), involving regions of these proteins that are critical for their function.

Nrarp promotes ICD loss in embryos

By forming a ternary complex with XSu(H) and ICD, Nrarp could inhibit Notch signaling in embryos by several mechanisms, none of which are mutually exclusive. Nrarp could interfere directly with the transcriptional activity of XSu(H)/ICD, change the subcellular location of XSu(H)/ICD in a way that reduces their transcriptional activity, or promote the degradation of either ICD or XSu(H). The last possibility is supported strongly by the results obtained with tagged proteins in embryos in which Nrarp in combination with XSu(H) causes a marked reduction in the levels of ICD. Indeed, this loss of ICD in response to Nrarp correlates well with the inhibitory effects of Nrarp on ICD-mediated transcription in animal cap assays. Nrarp-mediated loss occurs with both the full-length ICD as well as with ICDΔC, which lacks the carboxy-terminal PEST sequences, and it may occur even more efficiently in the presence of hMM. Because Nrarp does not have the structural features of a protease or an ubiquitin ligase, it is unlikely to promote ICD instability directly. Indeed, in both HeLa and 293T cells, the loss of ICD in response to Nrarp and Su(H) was not detectable, suggesting further that additional components are required, such as the appropriate ubiquitin ligase. Nrarp may also promote the loss of ICD via other posttranslational modification such as phosphorylation, because higher molecular weight forms of

ICD are often generated in the presence of Nrarp (e.g., Fig. 7B, lane 5).

Nrarp promotes ICD-mediated transcription in reporter assays

Because Nrarp is likely to promote a loss of ICD indirectly, we do not rule out the possibility that it modulates the activity of the Su(H)/ICD complex by other mechanisms. However, several lines of evidence suggest that inclusion of Nrarp in a Su(H)/ICD complex is compatible with an active transcriptional complex. First, as noted, ICD, Su(H), and Nrarp bind as a complex to Su(H) binding sites in gel shift assays, indicating that Nrarp does not inhibit DNA binding by Su(H) (data not shown). Second, the results of co-IP analysis indicate strongly that Su(H), ICD, Mastermind, and Nrarp can exist in a quaternary complex, indicating that Nrarp does not block the interaction of ICD with a coactivator thought to be key in ICD-mediated transcription. Third, Nrarp does not interfere with ICD-mediated transcription of Su(H)-dependent reporters in transient transfection. Not only does Nrarp fail to disable transcription in this assay, it potentiates transcription activity stimulated by subthreshold levels of ICD and can synergize with Mastermind to increase transcription further at saturating levels of ICD. These observations suggest that ICD-containing ternary or quaternary complexes are transcriptionally more potent than ICD and Su(H) dimers, either by virtue of their higher affinity for each other or for some other reason, that is, that they have higher affinity for DNA. Taken together, these results indicate that in the absence of ICD degradation, the ability of ICD to act as a transcriptional activator either alone on in combination with mastermind is not compromised by Nrarp.

Nrarp as a negative regulator of Notch signaling

Although Nrarp may contribute to the formation of an active Su(H)/ICD transcriptional complex, we propose that its primary role is to regulate Notch signaling by promoting the degradation of ICD (Fig. 10). Several lines of evidence indicate that only very small amounts of ICD need to be released from the membrane to produce maximal stimulation of Notch target genes. For example, ICD is very difficult to detect in the nucleus of cells undergoing Notch signaling even though these levels of ICD are apparently sufficient to produce significant changes in gene expression (Schroeter et al. 1998). One would predict, therefore, that under normal physiological conditions Nrarp is likely to have a dramatic effect on ICD-mediated transcription, in which a low level of ICD is already a rate-limiting factor. Moreover, Nrarp binds to ICD only when part of a complex with Su(H), suggesting that only after ICD forms an active transcriptional complex does it become a target of degradation via Nrarp (Fig. 10). This mechanism is consistent with recent findings showing that activation of some transcription factors is coupled to their degradation (Thomas and Tyers 2000). For example, the melanocyte factor Mi is phosphorylated in response to c-Kit signaling, resulting in both recruitment of coactivator proteins and ubiquitination and subsequent degradation of Mi (Wu et al. 2000b). Similarly, ligand binding converts the estrogen receptor into a transcriptional activator but also promotes its degradation (Nawaz et al. 1999).

The ability of Nrarp to regulate the levels of ICD may

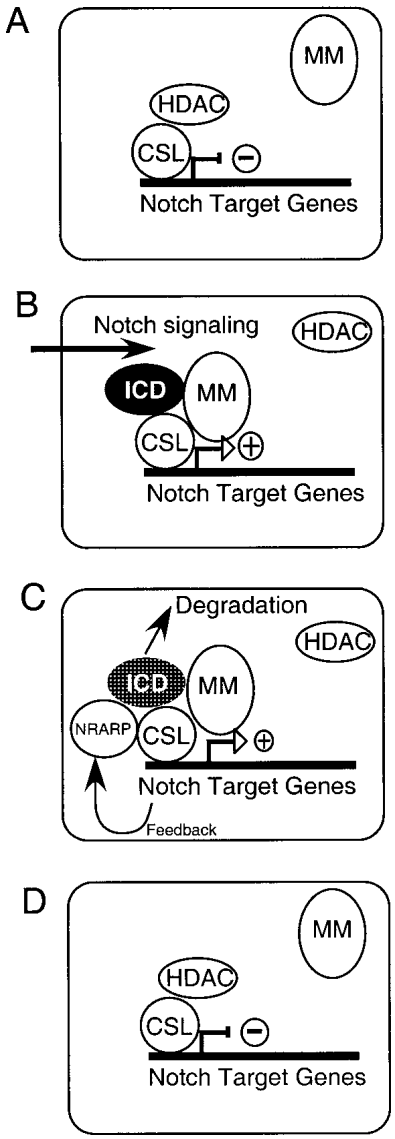

Figure 10. Model for Nrarp feedback regulation of Notch signaling. (*A*) In the absence of ICD, the CSL proteins associate with HDAC-containing complexes, thereby repressing transcription of Notch target genes. (*B*) On Notch signaling, ICD is released from the membrane and forms a complex with the CSL proteins and Mastermind (MM), thereby generating a transcriptional activator of Notch target genes, including *Nrarp*. (*C*) Nrarp feeds back and forms a complex with the CSL proteins, ICD, and Mastermind, which leads indirectly to a degradation of ICD. (*D*) Loss of ICD precludes the further association of Mastermind and Nrarp with the CSL proteins, thereby converting these proteins back into transcriptional repressors.

constitute a negative feedback loop that attenuates Notch signaling, assuring that activation of Notch target genes is transient. Notch is used ubiquitously to amplify differences between cells, thus allowing certain patterns of cell fate to be established (Artavanis-Tsakonas et al. 1999). As a result, Notch signaling is often used in rapid succession to control multiple cell fate decisions in the same lineage. A mechanism for down-regulating the levels of ICD produced after each decision by Nrarp would allow each decision to remain separate from the next. This rapid resetting of the Notch signaling pathway is particularly evident during segmentation of the paraxial mesoderm in vertebrate embryos, in which the transcription of a number of Notch pathway genes has been shown to oscillate in hourly intervals with a pattern suggestive of a segmental clock (Pourquie 1999). The role of Nrarp in the formation of this periodic pattern of gene expression is of particular interest based on its expression in the presomitic mesoderm and its potential role in down-regulating the levels of ICD required for Notch-mediated transcription.

Materials and methods

Embryos

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg-laying and in vitro fertilization using standard methods. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967).

Nrarp constructs

Templates for generating synthetic *Nrarp* RNA were produced from the open reading frame of Nrarp cloned into a myc- or Flag-tagged CS2 vector. The Nrarp open reading frame was amplified from a cDNA clone by PCR and primers corresponding to sequences immediately upstream and downstream of the initiation and termination codons, respectively.

In situ hybridization

Xenopus embryos were stained by whole-mount in situ hybridization according to Harland (1991) with modifications described by Knecht et al. (1995) using digoxigenin-labeled antisense riboprobes. Before in situ hybridization, RNA-injected embryos were stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) (Detrick et al. 1990). Plasmids used for generating in situ hybridization probes for *Xenopus* embryos are as described previously: *ESR1* (Wettstein et al. 1997); *XDelta1* (Chitnis et al. 1995); *ESR7* (Deblandre et al. 1999); *N-tubulin* (Oschwald et al. 1991); and α-*tubulin* (Deblandre et al. 1999).

RNA injection and RPA

Synthesis and injection of RNA was performed as described previously (Chitnis et al. 1995). For embryos assayed by whole-mount in situ hybridization, 0.2–1 ng of test RNAs was injected into single blastomeres of albino embryos at the two-cell stage, along with a synthetic *n-lacZ* RNA (500 pg) encoding a nuclear-localized form of β-galactosidase. For animal cap assays, both blastomeres of two-cell stage embryos were injected in the animal region with 0.3–2.0 ng of the indicated synthetic RNAs. Unless otherwise indicated, RNA injections for animal cap assays included *noggin* RNA to neuralize the injected animal cap tissue (Lamb et al. 1993).

Animal caps were dissected at stage 9 and cultured on agarose-coated Petri dishes in 0.5× MMR containing gentamycin until sibling controls reached the stage noted. RNA was isolated and analyzed by RPA using ^{32}P-labeled antisense RNA probes as described previously (Wettstein et al. 1997). Templates for generating antisense probes are described elsewhere: *ESR1*, *EF-1*α (Wettstein et al. 1997); *Hes6* (Koyano-Nakagawa et al. 2000). The probe for *Nrarp* RNA was generated by linearizing the cDNA with *Dde*I and transcribing with T3 polymerase. Quantification of RPA results was performed on a PhosphorImager (Molecular Dynamics), and, for each lane, specific band intensities were normalized to the amount of *EF-1*α RNA.

Expression constructs

Synthetic RNAs for injection into embryos were generated from templates based on the CS2$^+$ vectors (Turner and Weintraub 1994). Where appropriate these constructs included six copies of a myc tag, one copy of a Flag tag, or a nuclear localization signal sequence, which were placed at the amino end of the construct. Templates described previously are: *Notch-ICD*, *XSu(H)DBM*, *XSu(H)Ank*, *XSu(H)Ank^{tr}*, *ICDΔC*, *ICDAnk*, *ICDram23*, and *nlacZ* (Wettstein et al. 1997). A template for human Mastermind was constructed by inserting the open reading frame from the cDNA KIAA0200 into the CS2$^+$MT vector (Turner and Weintraub 1994).

Immunoprecipitation and Western analysis

RNAs were injected into *Xenopus* embryos at equal concentrations (0.2 ng/embryo) into the animal pole of each blastomere at the two-cell stage. At blastula stages, the animal tissue was dissected away and cultured until sibling embryos reached the start of gastrulation. Animal tissue from 20 embryos was homogenized in 200 µL of cold lysis buffer (50 mM Tris-HCl at pH 7.5, 300 mM NaCl, 1.0% NP40) with protease inhibitors (Wettstein et al. 1997). Homogenates were centrifuged at 4°C at 15,000 rpm in a microcentrifuge to remove insoluble material. One-tenth volume was brought to 1× in Laemmli loading buffer and boiled. The remaining supernatants were preadsorbed by adding 20 µL of a 50% slurry of protein A-coupled Sepharose (Pharmacia), which was blocked previously by incubation in 1.0 mg/mL BSA for 1 h and then washed. Homogenates were cleared again by centrifugation at low speed to remove the protein A-Sepharose beads. Immunoprecipitation was performed by adding 1 µL of an α-Flag mouse monoclonal antibody (M2, Sigma), rocking gently for 1 h, and then adding 20 µL of a 50% slurry of protein A-coupled Sepharose as prepared above. After an hour of incubation, the beads were pelleted, washed three times with an excess of lysis buffer, resuspended in Laemmli buffer, and boiled. One half of the extracts or the immunoprecipitates were loaded on 10% polyacrylamide gels and subjected to electrophoresis and Western blotting as described previously (Wettstein et al. 1997). Myc epitopes were detected on Western blots using the 9E10 α-myc mouse monoclonal antibody, followed by a secondary HRP-conjugated rabbit α-mouse IgG, followed by detection by chemiluminescence (ECL, Amersham). Each gel contained prestained molecular weight markers (Bio-Rad).

Tissue culture assays

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). For transfection,

50,000 cells were plated in 24-well dishes and transfected the next day in serum-free medium with 2 μg of DNA per well using the PEI (ethylene imine polymer) reagent (Fluka) according to published protocols. Generally, each transfection included 750 ng of a firefly luciferase reporter construct, 750 ng of a βgal expression vector, and 500 ng of effectors plus carrier DNA containing the CMV promoter. The reporters contained eight multimerized wild-type or mutant Su(H) binding sites cloned upstream of a basal promoter driving luciferase expression (Zhou et al. 2000). The effectors were CMV-based CS2 vectors driving expression of *Xenopus* ICD (Wettstein et al. 1997) and Nrarp. Cell lysates were prepared approximately 48 h posttransfection using Passive Lysis Buffer (Promega) according to the manufacturer's instructions. Transfection efficiency was determined by measuring lacZ activity using ONPG (Sigma) as substrate. Luciferase activity was measured on a Berthold Lumat luminometer using the Luciferase Assay Reagent (Promega). All transfections were performed in triplicate.

293T cells were grown in DMEM containing 10% FBS, *L*-glutamine, penicillin, and streptomycin. One day before transfection, 500,000 cells were plated per well in six-well dishes. Cells were transfected for 6 h using the calcium phosphate method, washed, and covered with 2 mL of fresh media. One microgram of each construct was used per well and the total amount of DNA was normalized to 3 μg with carrier CS2 vector. Forty-eight hours after transfection, cells were washed in PBS and lysed in 400 μL of ice-cold lysis buffer (25 mM Tris at pH 7.5, 300 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 μM pepstatin, 1 μM leupeptin). Cell lysates were cleared by centrifugation for 15 min at 15,000 rpm at 4°C. Tagged proteins in cell extracts were analyzed by Western blotting as described above. Two hundred microliters of cell lysates was brought to 1 mL with lysis buffer and subjected to immunoprecipitation using the monoclonal M2 antibody as described above.

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