

# Nicalin and its binding partner Nomo are novel Nodal signaling antagonists

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**Nodals are signaling factors of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily with a key role in vertebrate development. They control a variety of cell fate decisions required for the establishment of the embryonic body plan. We have identified two highly conserved transmembrane proteins, Nicalin and Nomo (Nodal modulator, previously known as pM5), as novel antagonists of Nodal signaling. Nicalin is distantly related to Nicastrin, a component of the Alzheimer's disease-associated  $\gamma$ -secretase, and forms a complex with Nomo. Ectopic expression of both proteins in zebrafish embryos causes cyclopia, a phenotype that can arise from a defect in mesendoderm patterning mediated by the Nodal signaling pathway. Accordingly, downregulation of Nomo resulted in an increase in anterior axial mesendoderm and the development of an enlarged hatching gland. Inhibition of Nodal signaling by ectopic expression of Lefty was rescued by reducing Nomo levels. Furthermore, Nodal- as well as Activin-induced signaling was inhibited by Nicalin and Nomo in a cell-based reporter assay. Our data demonstrate that the Nicalin/Nomo complex antagonizes Nodal signaling during mesendodermal patterning in zebrafish.**

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## Introduction

The transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily consists of a large number of structurally related, secreted proteins, which regulate a wide range of biological functions in developing and adult tissues including cell proliferation, differentiation, apoptosis and cell fate specification (Massague, 1998). In addition to their structural similarity, TGF $\beta$  ligands share a common signaling pathway, which includes the binding and activation of a serine/threonine kinase receptor complex, the phosphorylation and activation of intracellular signal transducers of the Smad family and the translocation of the Smad complex to the nucleus, where it associates with other transcriptional regulators to activate target gene transcription (Shi and Massague, 2003). Based on sequence similarity and the specific signaling pathway that they activate, the TGF $\beta$  proteins are divided into several subgroups including the Nodal subfamily, which plays a key role in the embryonic development of vertebrates (Schier and Shen, 2000; Schier, 2003). Nodals are required for processes as different as the formation of mesoderm and endoderm, positioning of the anterior–posterior body axis, specification of left–right symmetry and patterning of the nervous system. In contrast to other TGF $\beta$  ligands, Nodals require the presence of coreceptors of the EGF-CFC family in addition to TGF $\beta$ -type receptors to initiate signaling (Shen and Schier, 2000). The Nodal signal is then relayed to the nucleus, where specific transcription factors, most notably FoxH1 (FAST2), are activated.

In the zebrafish, mesendoderm formation and patterning are primarily controlled by the two Nodal-related genes *cyclops* and *squint* (Schier, 2001; Whitman, 2001). *cyclops*; *squint* double mutants lack all endoderm and head and trunk mesoderm including notochord, heart, kidney, blood, liver, pancreas and gut (Feldman *et al*, 1998). Gene expression and fate map analyses have revealed that Nodals act before gastrulation to specify the mes- and endodermal progenitors. A lack of Nodal signaling causes the abnormal specification of these cells followed by their abnormal movement (Feldman *et al*, 2000; Carmany-Rampey and Schier, 2001). Instead of internalizing to give rise to mesoderm and endoderm, cells stay on the outside and contribute to neuroectoderm. The induction and patterning of mes- and endoderm by Nodals largely depend on graded signaling: different signal strengths are achieved by the generation of an activity gradient within a field of cells through the interaction of Nodals and Nodal inhibitors (Chen and Schier, 2002; Feldman *et al*, 2002; Solnica-Krezel, 2003). This provides positional information to each cell resulting in the establishment of different cell fates, with endoderm requiring the highest Nodal levels, followed by the anterior mesendoderm (prechordal plate) and mesoderm. Several extracellular and intracellular Nodal inhibitors have been identified including the Lefty proteins, divergent members of the TGF $\beta$  family (Schier, 2003). Leftys are considered classical feedback inhibitors,

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since their expression depends on Nodal signaling. They represent long-range inhibitors contributing to the generation of Nodal activity gradients (Chen and Schier, 2002; Feldman *et al*, 2002). Leftys can directly bind and inhibit Nodals or compete with them for binding to EGF-CFC coreceptors (Chen and Shen, 2004; Cheng *et al*, 2004). Other extracellular Nodal inhibitors like Cerberus, Coco and Charon belong to the DAN/Cerberus family (Balemans and Van Hul, 2002; Hashimoto *et al*, 2004). These proteins act by binding directly to Nodals inhibiting their interaction with the receptor. Intracellular Nodal inhibitors include tomoregulin-1, which might also inhibit EGF-CFC coreceptors (Harms and Chang, 2003), and DRAP, which binds FoxH1 and prevents its DNA binding (Iratni *et al*, 2002).

The Notch pathway is another signaling pathway regulating cell fate decisions during embryonic development (Selkoe and Kopan, 2003). Activation of Notch leads to its proteolytic processing by  $\gamma$ -secretase, a high-molecular-weight membrane protein complex that cleaves its substrates within their transmembrane domain (Haass, 2004). This complex also mediates the final step in the proteolytic processing of the amyloid precursor protein (APP) leading to the generation of the amyloid  $\beta$ -peptide (A $\beta$ ), which is deposited in the brains of Alzheimer's disease (AD) patients (Hardy and Selkoe, 2002; Sisodia and St George-Hyslop, 2002). Genetic and biochemical approaches led to the identification of four components of this complex, the Presenilins, Nicastrin, APH-1 and PEN-2 (Francis *et al*, 2002; Haass, 2004). Presenilin 1 and 2 are polytopic membrane proteins mutated in the majority of patients with early-onset familial Alzheimer's disease (FAD) (Selkoe, 2001). They are believed to represent aspartyl proteases and provide the active site of the  $\gamma$ -secretase complex (Wolfe *et al*, 1999). Nicastrin, APH-1 and PEN-2 are required for complex assembly (Edbauer *et al*, 2002; Hu *et al*, 2002; Kimberly *et al*, 2002; Leem *et al*, 2002; Edbauer *et al*, 2003), but their precise role is unknown.  $\gamma$ -Secretase activity has been reconstituted in yeast by expressing all four components demonstrating that they represent the core proteins of the complex (Edbauer *et al*, 2003).

Nicastrin is a type 1 transmembrane protein (Yu *et al*, 2000) with a large extracytosolic domain containing an  $\sim$ 200-amino-acid region, which is predicted to adopt a fold similar to the aminopeptidase (AP) domain found in several active aminopeptidases and the transferrin receptor (TfR) (Fagan *et al*, 2001). We have now identified Nicalin, a novel Nicastrin-related AP domain protein, and its binding partner Nomo (Nodal modulator, pM5) as components of a novel protein complex involved in Nodal signaling. In zebrafish embryos, Nicalin and Nomo modulate mesendodermal patterning and interfere with Lefty function. In a cell-based assay, they inhibit Nodal- as well as Activin-dependent signaling. We, therefore, suggest a role of the Nicalin/Nomo complex in antagonizing Nodal signaling during mesendodermal patterning in zebrafish.

## Results

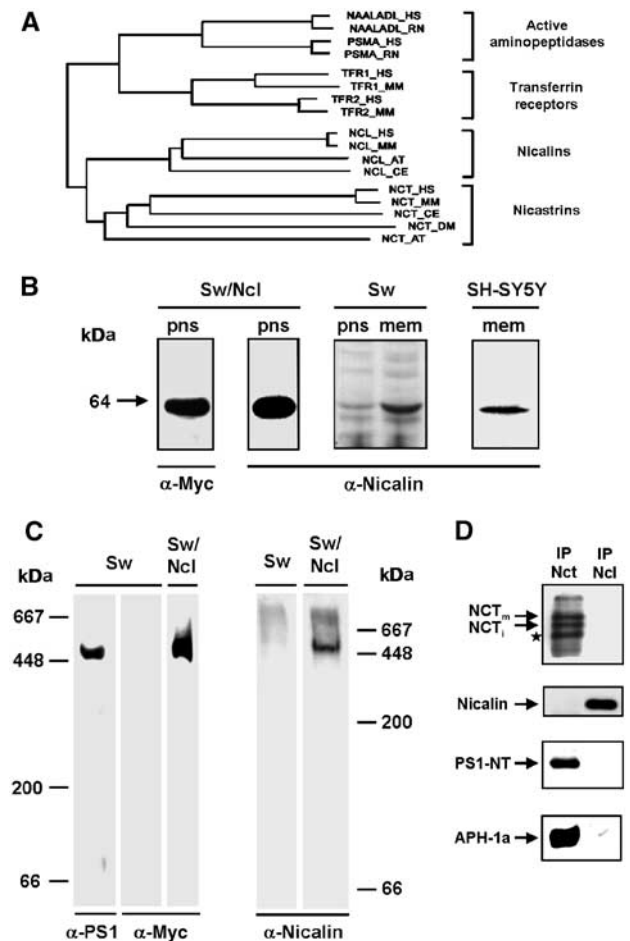
### **Nicalin is distantly related to Nicastrin and present in a high-molecular-weight complex**

We searched current protein and genome databases for proteins homologous to the  $\gamma$ -secretase complex component Nicastrin. By using standard database comparison methods

like BLAST, we found significant similarity only to Nicastrin orthologs from various organisms. In order to find more distantly related sequences, we searched the databases with generalized profiles (Bucher *et al*, 1996) constructed from the ectodomain of the Nicastrin family. This search yielded two highly significant matches ( $P < 1E-5$ ), the open reading frame (ORF) T05F1.1 from *Caenorhabditis elegans* and an unnamed human ORF with the SwissProt accession number Q96H48. These two sequences encode orthologs of a novel protein with the same phyletic distribution as Nicastrin (Figure 1A). Since this protein is predicted to represent a type I transmembrane protein and shares the same overall architecture with Nicastrin, we refer to it as Nicalin (Nicastrin-like protein). Significant sequence similarity is confined to a region of 180 residues (see Supplementary Figure), which roughly corresponds to the previously described aminopeptidase domain (Fagan *et al*, 2001). The dendrogram analysis shown in Figure 1A confirms that Nicastrin is the closest relative of Nicalin. Similar to Nicastrin, Nicalin lacks the amino-acid conservation required for catalytically active aminopeptidases.

To analyze a putative role of Nicalin in the processing of APP and the generation of amyloid  $\beta$ -peptide (A $\beta$ ), we used human embryonic kidney 293 (HEK293)/APP<sub>Sw</sub> (Sw) cells expressing the Swedish APP mutant (Citron *et al*, 1992) to generate a stable Nicalin-expressing cell line (Sw/Ncl). Immunoblotting of total protein extracts using an anti-myc or anti-Nicalin antibody resulted in the detection of a single band of about 60 kDa (Figure 1B), consistent with the calculated molecular weight of human Nicalin (63.7 kDa). Endogenous Nicalin levels were barely detectable in these lysates, but enriched in membrane preparations of HEK293 cells and SH-SY5Y human neuroblastoma cells (Figure 1B), suggesting that Nicalin is indeed a transmembrane protein expressed in neuronal and non-neuronal cells. Analysis of the levels of APP C-terminal fragments and of A $\beta$  in Sw and Sw/Ncl cells did not reveal significant differences (data not shown), indicating that Nicalin is not involved in  $\gamma$ -secretase-mediated processing of APP (see below).

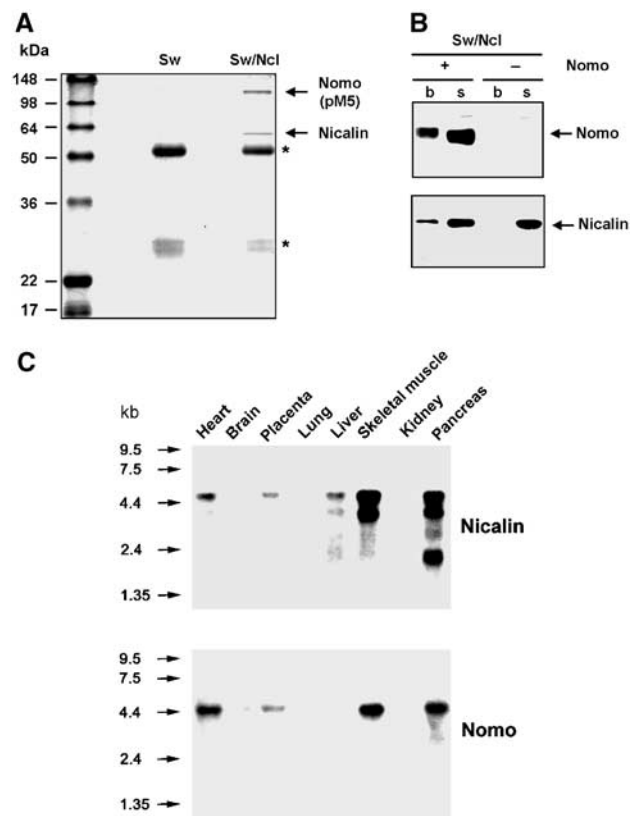
The  $\gamma$ -secretase complex can be isolated in its native form in DDM-solubilized membranes and detected as a high-molecular-weight band in Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) by immunostaining with anti-bodies against complex components (Steiner *et al*, 2002). We used this method to analyze if Nicalin is present in a similar complex. Whereas in Sw cells endogenous Nicalin levels were too low to be detected in BN-PAGE, in Sw/Ncl cells a band of 500–550 kDa was visible, indicating the presence of Nicalin in a high-molecular-weight complex (Figure 1C). Similar to  $\gamma$ -secretase, the Nicalin complex is disrupted upon treatment with Triton X-100 (data not shown). We next examined if Nicalin interacts with known  $\gamma$ -secretase complex components by co-immunoprecipitation experiments using detergents that allow the isolation of the intact complex (Steiner *et al*, 2002). Whereas the well-established  $\gamma$ -secretase complex components Presenilin 1 and APH1a co-precipitated with Nicastrin as expected, they could not be detected in Nicalin immunoprecipitates (Figure 1D). Likewise, Nicalin did not co-precipitate with Nicastrin. In addition, overexpression of Nicalin failed to rescue the assembly of a functional  $\gamma$ -secretase complex in Nicastrin-RNAi cells (Edbauer *et al*, 2002) (data not shown). Taken together, these data show that Nicalin forms a complex distinct from  $\gamma$ -secretase.



**Figure 1** Nicalin is distantly related to Nicastrin and part of a high-molecular-weight complex unrelated to  $\gamma$ -secretase. (A) Phylogenetic tree showing that the Nicalins are more closely related to Nicastrins than to transferrin receptors and active aminopeptidases. NAALADL: N-acetyl-alpha-linked acidic dipeptidase-like protein; PSMA: prostate-specific membrane antigen; TFR: transferrin receptor; NCL: Nicalin; NCT: Nicastrin; HS: *Homo sapiens*; RN: *Rattus norvegicus*; MM: *Mus musculus*; DM: *Drosophila melanogaster*; CE: *C. elegans*; AT: *Arabidopsis thaliana*. (B) Western blot of postnuclear (pns) and membrane (mem) protein fractions of cells expressing endogenous Nicalin (Sw and SH-SY5Y) or myc-tagged Nicalin (Sw/Ncl). Immunodetection with  $\alpha$ -myc or  $\alpha$ -Nicalin antibodies reveals a 60 kDa protein enriched in membrane fractions. (C) Immunoblot of Blue-Native gels of Sw and Sw/Ncl cell membrane protein extracts. Nicalin immunoreactivity is seen in a high-molecular-weight complex with a size similar to  $\gamma$ -secretase, which is visualized using an  $\alpha$ -Presenilin 1 antibody ( $\alpha$ -PS1). (D) Nicastrin and Nicalin were immunoprecipitated from Sw/Ncl cells and the bound material was analyzed by Western blotting. In Nicastrin immunoprecipitates (IP Nct), Presenilin N-terminal fragments (PS1-NT) and APH-1a were present, but Nicalin could not be detected. In Nicalin immunoprecipitates (IP Ncl), neither Nicastrin nor PS1-NT or APH-1a could be detected (band denoted with \* is unspecific).

### Nicalin interacts with the 130 kDa protein Nomo (pM5)

To identify binding partners of Nicalin that may contribute to its native molecular weight, we immunoprecipitated Nicalin from DDM-solubilized membranes and separated the bound material by SDS-PAGE. Coomassie blue staining identified two bands of 60 and 130 kDa that were specifically enriched in material from Sw/Ncl cells (Figure 2A). The two bands were isolated, digested with trypsin and peptide mass finger-



**Figure 2** Nomo (pM5) is a major binding partner of Nicalin. (A) Myc-tagged Nicalin was immunoprecipitated from Sw/Ncl cell membrane preparations and the bound proteins were visualized by Coomassie staining. Bands of molecular weight 60 and 130 kDa were specifically enriched in precipitates from Sw/Ncl cells. Peptide mass fingerprints generated by MALDI analysis identified these bands as Nicalin and pM5, which we refer to as Nomo (bands denoted with \* represent immunoglobulin heavy and light chains). (B) FLAG-tagged Nomo was transfected into Sw/Ncl cells and immunoprecipitated. Western blot analysis of bound (b) and unbound (s) material revealed co-precipitation of Nicalin in Nomo-overexpressing cells (+), but not in control cells (-). (C) Northern blot analysis using full-length Nicalin and Nomo probes and 2  $\mu$ g each of mRNA from various human tissues. Molecular weight markers in kilobases are shown on the left.

prints were generated by MALDI analysis. Whereas the 60 kDa band, as expected, corresponded to Nicalin, the 130 kDa band was identified as pM5, an uncharacterized protein that had emerged in a PCR-based screen for new members of the collagenase family, a subfamily of metalloproteases (Templeton *et al*, 1992). However, pM5 does not contain the HEXXH motif conserved in all metalloproteases and does not show any homology to collagenases. Instead, it appears to represent a type I transmembrane protein with no recognizable functional motif. Based on our data on pM5 function (see below), we refer to it as Nomo (Nodal modulator). To verify the interaction between Nicalin and Nomo, FLAG-tagged Nomo expressed in HEK293 cells was immunoprecipitated, and analysis of the bound material revealed co-precipitation of Nicalin (Figure 2B). These data suggest that Nomo is a major binding partner of Nicalin.

To examine the tissue distribution of Nicalin and Nomo, we performed Northern blot analysis with mRNA from various human tissues. Both proteins show an almost identical expression pattern, with highest mRNA levels in pan-

creas, skeletal muscle and, at somewhat lower levels, in heart (Figure 2C). After longer exposure, transcripts of both proteins were found in all tissues examined (data not shown). Whereas only one Nomo transcript of about 4.5 kb was detected, three different Nicalin mRNAs of 4.6, 4.0 and 2.3 kb exist, the sizes of which correspond roughly to cDNA sequences present in the databases. Since these sequences differ only in the length of their 3' untranslated regions, the various Nicalin transcripts are most likely generated through alternative polyadenylation. In addition to the overlapping tissue distribution of Nicalin and Nomo, we also found colocalization of both proteins on the subcellular level. In iodixanol density gradients, endogenous Nicalin as well as Nomo cofractionized with endoplasmic reticulum (ER) membranes (data not shown). Taken together, these data supported a possible functional relationship between Nicalin and Nomo.

### Ectopic expression of Nicalin and Nomo in zebrafish embryos causes cyclopia

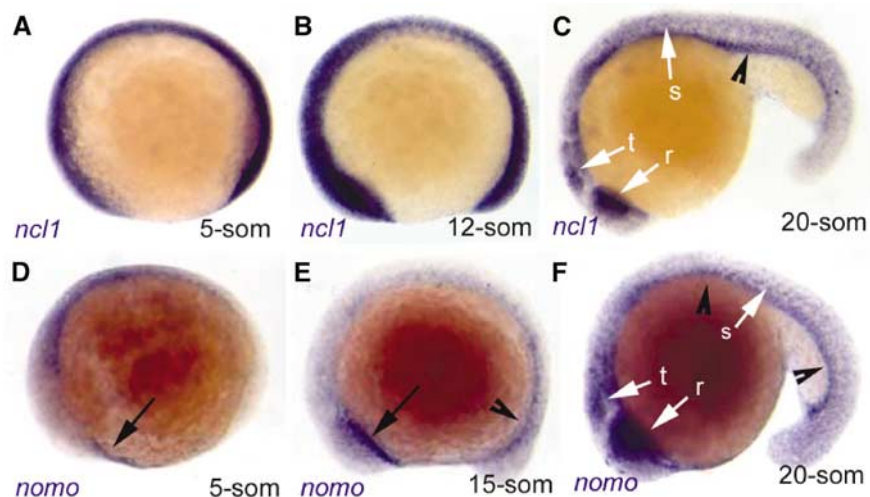
The  $\gamma$ -secretase-mediated cleavage of Notch is a highly conserved process required for normal embryonic development (Selkoe and Kopan, 2003). The evolutionary conservation of Nicalin and Nomo in metazoans and plants indicated that the Nomo/Nicalin complex could have a similarly important role. To analyze its function in development, we chose the zebrafish system, which has been successfully used to recapitulate the defects in somite formation associated with the inhibition of Notch signaling (Geling *et al*, 2002). Database analysis revealed that the zebrafish genome contains two Nicalin homologs with 72 and 47% identity to human Nicalin and one Nomo homolog with 70% identity to human Nomo (named *ncl1*, *ncl2* and *nomo*, respectively, according to the Zebrafish Nomenclature Committee). *In situ* hybridization demonstrated maternal expression as well as ubiquitous early zygotic expression of *ncl1* and *nomo*, while *ncl2* was not transcribed at appreciable levels during early embryogenesis (not shown). *ncl1* is strongly and ubiquitously expressed at

early stages of somitogenesis (Figures 3A and B). Later, prominent sites of expression include the developing retina, optic tectum and somites (Figure 3C) as well as endoderm (Figure 3C, arrowhead). *nomo* expression follows a very similar pattern at the 20-somite stage (Figure 3F). At earlier stages, *nomo* is most abundantly transcribed in the endoderm (Figures 3E and F, arrowheads) and anterior mesendoderm (hatching gland) (Figures 3D and E, arrows).

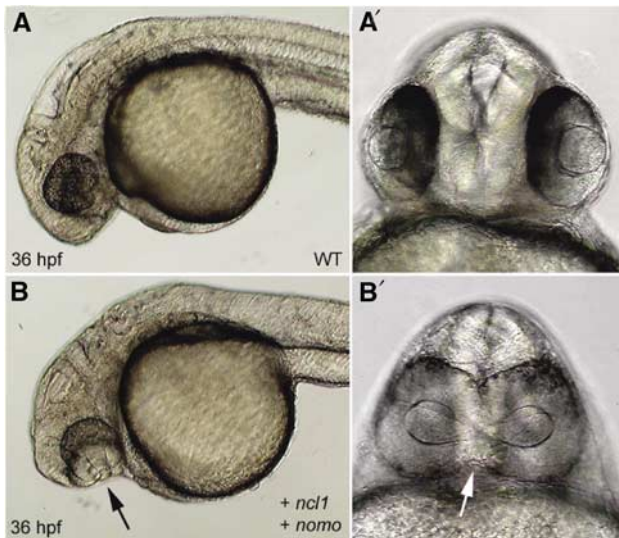
The expression patterns of *ncl1* and *nomo* are compatible with a function in embryonic development and we, therefore, analyzed the effects of ectopic expression of both proteins. Whereas injection of *ncl1* or *nomo* capped RNA alone produced no discernible phenotype (not shown), simultaneous injection of both RNAs led to cyclopic embryos (27% of cases,  $n=26$ ) (Figures 4B and B'), a phenotype known to arise through developmental failure of the basal forebrain (Blader and Strahle, 1998; Varga *et al*, 1999; Chow and Lang, 2001). Thus, when overexpressed, Nicalin and Nomo can collaborate in a process that impairs forebrain patterning, providing evidence for the existence of a functional Nomo/Nicalin complex.

### Nomo is required to restrict the formation of anterior axial mesendoderm

Basal forebrain identity itself is induced by the underlying anterior axial mesendoderm (Beddington and Robertson, 1998; Rubenstein *et al*, 1998; Knoetgen *et al*, 1999; Kiecker and Niehrs, 2001). Thus, we next investigated whether endogenous Ncl1 and/or Nomo are required for mesendoderm patterning. Blocking *ncl1* activity by the injection of antisense oligonucleotides (*ncl1*-grip) into wild-type zebrafish oocytes did not trigger a specific phenotype (data not shown). Blocking the production of Nomo protein using a *nomo*-grip, however, resulted in the development of massively enlarged hatching glands (Figures 5A–B', arrows) (68% of cases,  $n=33$ ). At the molecular level, *nomo* knockdown embryos display an increased number of *gsc*-, *hgg1*- and *hlx1*-positive cells, which mark the anterior



**Figure 3** Zebrafish *ncl1* and *nomo* are expressed in largely overlapping domains at somitogenesis stages. Expression of *ncl1* (A–C) and *nomo* (D–F) revealed by whole-mount *in situ* hybridization (blue staining) at the stages indicated (anterior left, dorsal up). *ncl1* expression is ubiquitous, with higher levels in the presumptive tectum (t), retina (r) and somites (s) (white arrows) and in the endoderm (black arrowhead) at 20 somites. *nomo* is transcribed at lower levels during earlier stages, with the most prominent expression in the anterior mesendoderm (black arrows) and endoderm (black arrowhead). At the 20-somite stage, *nomo* is expressed in a pattern very similar to *ncl1*.



**Figure 4** Coexpression of *ncl1* and *nomo* impairs forebrain patterning leading to cyclopia. 36 hpf morphology of wild-type embryos (**A, A'**) and embryos coinjected with *ncl1* and *nomo* capped RNAs (150 ng/ $\mu$ l each) at the one-cell stage (**B, B'**). Note the prominent cyclopia of the injected embryo (arrows).

axial mesoderm (Figures 5C–H, arrows). Correlatively, the amount of immediately posterior axial mesoderm (notochordal tissue), as revealed by *znot* expression, is reduced (81% of cases,  $n = 26$ ) (Figures 5I and J). The effect of blocking Nomo was dose dependent, as the amount of *hgg1*-positive cells was in inverse proportion to the concentration of the injected *nomo*-grip (data not shown). Importantly, the coinjection of a *nomo\** capped RNA that carries point mutations preventing the *nomo*-grip binding but encodes a wild-type Nomo protein rescues this phenotype (66% of cases,  $n = 31$ ) (Figures 5K and L), arguing for its specificity. Staining with the endoderm marker *sox17* did not reveal differences between wild-type and *nomo*-grip-injected embryos, indicating that endoderm formation is not affected by Nomo (Figures 5M and N). The effect of *nomo*-grip injection on mesendoderm patterning was quantified by counting *hgg1*-positive cells of flat-mounted tail-bud-stage embryos ( $n = 5$ ): whereas in *nomo*-grip-injected embryos, the cell number ( $714 \pm 90$ ) was threefold higher compared to the wild-type embryos ( $240 \pm 48$ ), in embryos coinjected with *nomo*-grip and *nomo\** capped RNA, the number of *hgg1*-positive cells ( $279 \pm 60$ ) was not increased. Thus, Nomo is required to limit the amount of anterior axial mesoderm and allow proper axial mesoderm patterning. Together with the above gain-of-function data, this suggests that Nomo and Ncl1 cooperate to regulate mesoderm patterning *in vivo*. The failure to get a phenotype in *ncl1* knockdown embryos might result from an incomplete downregulation of *ncl1* expression or the presence of an *ncl1*-redundant factor.

#### Nomo and Nicalin attenuate Nodal signaling

Patterning of the mes- and endoderm during vertebrate gastrulation is primarily controlled by Nodal signaling (Schier, 2001; Whitman, 2001). Specifically, increasing levels of Nodal account for the formation of mes- and endodermal derivatives of progressively more anterior character. Thus,

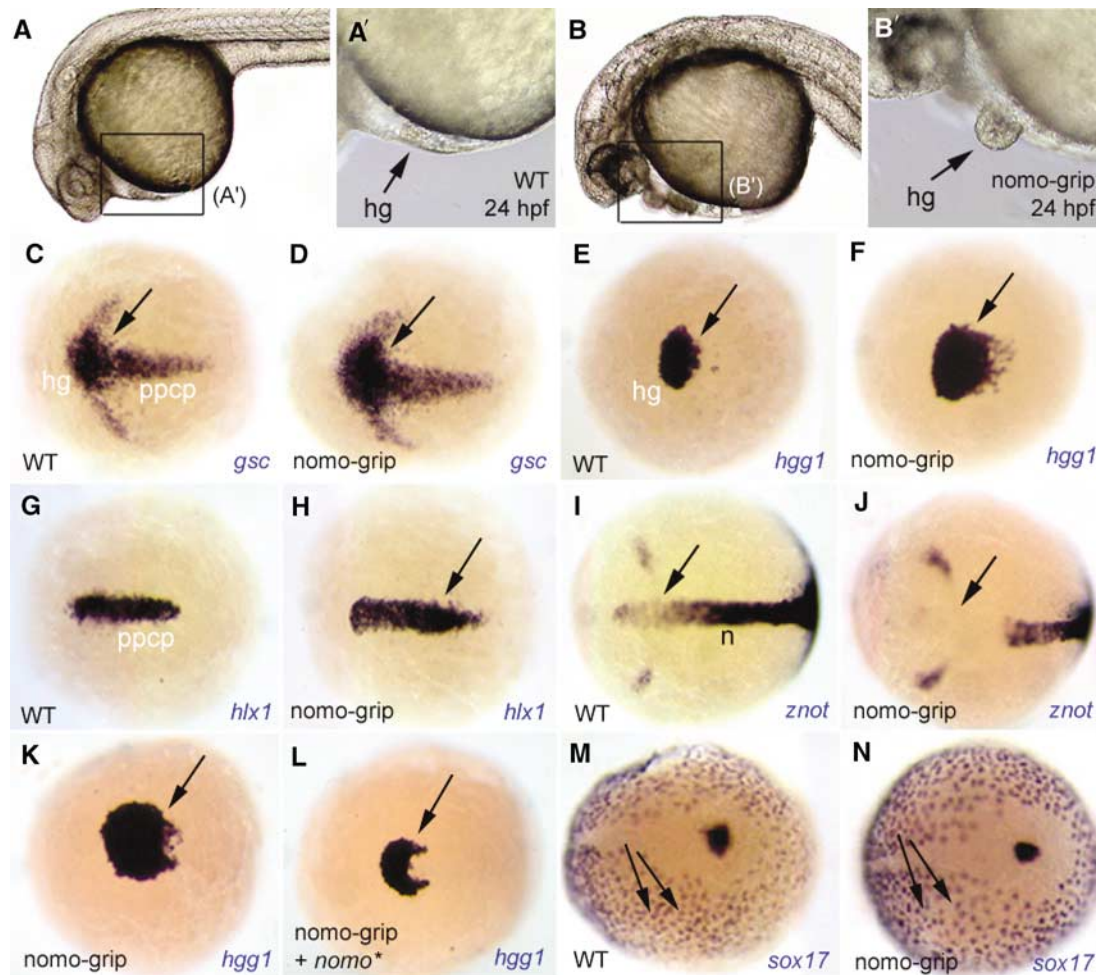
ectopic expression of the Nodal inhibitor Lefty leads to the sequential disappearance of the hatching gland, posterior prechordal plate derivatives and notochord in a dose-dependent manner (Cheng *et al*, 2000; Thisse *et al*, 2000; Branford and Yost, 2002; Chen and Schier, 2002; Feldman *et al*, 2002). Therefore, we hypothesized that Nomo, like Lefty, might inhibit Nodal signaling. To test this hypothesis, we activated *lefty* and blocked *nomo* by the coinjection of *lefty* capped RNA and the *nomo*-grip. While expression of *lefty* alone severely reduced the number of hatching gland precursor cells (Figure 6A) (70% of cases,  $n = 28$ ), the concomitant inhibition of *nomo* function restored the cell number to a level similar to or higher than in wild-type controls (Figure 6B) (100% of cases,  $n = 41$ ). We, therefore, conclude that *nomo* controls mesendoderm patterning *in vivo* by interfering with Nodal activity (Figures 6C–F).

To demonstrate that the effect of Nomo (and possibly Nicalin) on Nodal signaling is direct, we used a reporter assay based on the A3-lux reporter construct that contains three tandem copies of the Nodal- and activin-responsive element from the *Xenopus Mix2* promoter (Chen *et al*, 1996). This construct has been successfully used as a reporter of Nodal signaling in HEK293T cells (Yan *et al*, 2002). To reconstitute the Nodal pathway in these cells, we transfected Nodal, the EGF-CFC coreceptor Cripto and the transcription factor FAST2, all of which are not expressed endogenously. As shown before (Yan *et al*, 2002), this led to a strong increase in reporter activity (Figure 7A). Addition of increasing amounts of Nicalin or Nomo expression plasmids to the transfection mix resulted in a dose-dependent inhibition of Nodal signaling, whereas expression of Nicastrin had no effect. The reduction in reporter activity corresponded with Nicalin and Nomo expression levels as determined by immunoblotting (Figure 7A). Nicalin was about 10-fold more efficient than Nomo in this assay. This may reflect interaction of ectopic Nicalin with excess amounts of endogenous Nomo (see below). To exclude a possible effect of Nicalin and Nomo on the expression, processing or secretion of Nodal, we directly activated the pathway using a purified ligand. Activin is known to use the same receptors and intracellular signal transducers as Nodals except that they do not require a coreceptor of the EGF-CFC family. Accordingly, we observed reporter activation in 293T cells transfected with FAST2 only and treated with purified Activin (Figure 7B). Similar to Nodal signaling, Nicalin and Nomo were able to inhibit Activin signaling in a dose-dependent manner. The dose-response curves were almost identical in both assays, strongly suggesting that the mechanism of inhibition is the same. In cells transfected with both Nomo and Nicalin, we observed a more potent inhibition compared to cells expressing only one of the proteins (Figure 7C), similar to the situation in zebrafish embryos (see Figure 4). In summary, these data demonstrate a direct involvement of the Nicalin/Nomo complex in Nodal and Activin signaling.

## Discussion

### Nicalin is a component of a high-molecular-weight complex unrelated to $\gamma$ -secretase

We have identified Nicalin, a novel transmembrane protein of the aminopeptidase/transferrin receptor (TfR) superfamily, distantly related to the  $\gamma$ -secretase component Nicastrin. Both



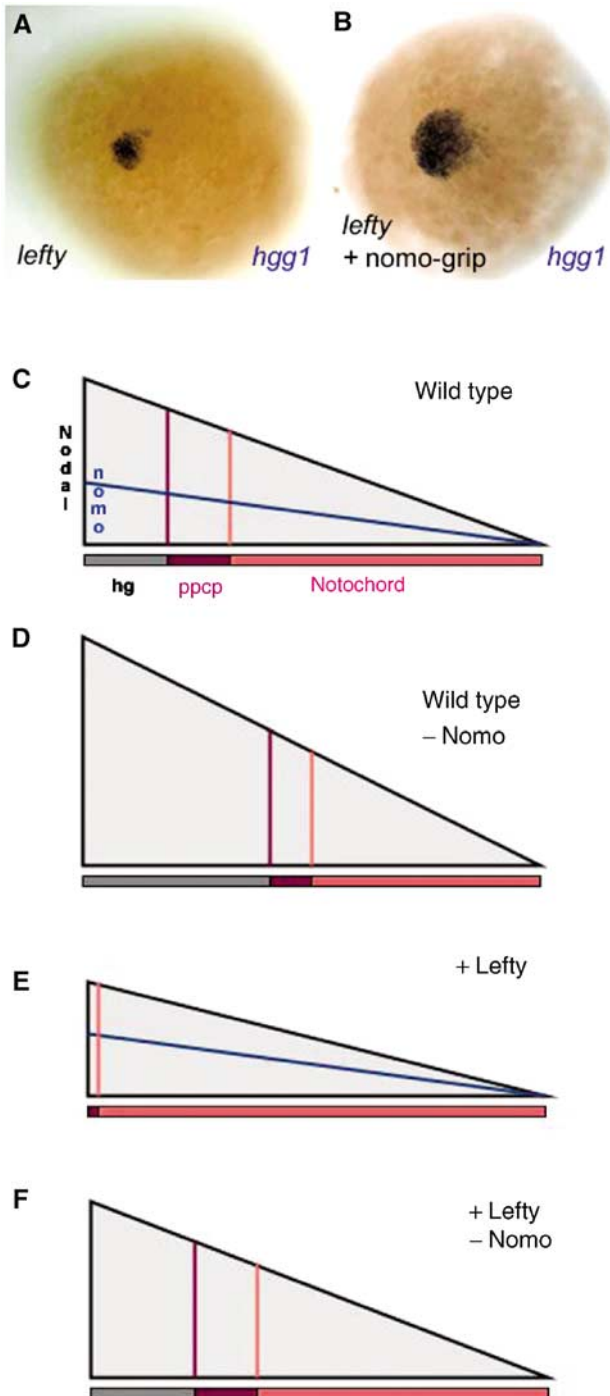
**Figure 5** Nomo activity controls the extent of anterior mesendoderm. (A–B') Morphology at 24 hpf of wild-type embryos (A, A') and embryos injected with an antisense gripNA oligonucleotide blocking translation of endogenous *nomo* (*nomo-grip*) (B, B'). All embryos are anterior left, dorsal up; (A') and (B') are higher magnifications of the territories boxed in (A) and (B), respectively. Blocking Nomo activity leads to the formation of enlarged hatching glands, the most anterior mesendodermal derivative (arrows). (C–J) Expression of the markers indicated (bottom right of each panel) at the end of gastrulation (tail-bud stage) in wild-type (C, E, G, I) or *nomo-grip*-injected embryos (D, F, H, J). All panels are dorsal views, anterior left. The expression of anterior mesendodermal markers (*gsc*: presumptive hatching gland + prechordal plate; *hgg1*: presumptive hatching gland; *hlx1*: prechordal plate) is enlarged in injected embryos (C–H, arrows to the enlarged domains), while *znol* expression in the anterior notochordal domain is absent (I, J, arrows). (K, L) The number of hatching gland precursors, labeled with *hgg1*, is restored when *nomo-grip* is coinjected with a capped RNA encoding Nomo (*nomo\**) (L, compare with K and E, F). (M, N) Expression of the endodermal marker *sox17* is similar in wild-type (M) and *nomo-grip*-injected embryos (N).

proteins have a similar topology, are conserved in higher eukaryotes and can be found in high-molecular-weight membrane protein complexes. However, we did not detect interactions between Nicalin and the  $\gamma$ -secretase complex components Nicastrin, Presenilin 1 and APh-1a by co-immunoprecipitation. Moreover, we were unable to rescue the assembly of a functional  $\gamma$ -secretase complex in Nicastrin knockdown cells (Edbauer *et al*, 2002) by overexpressing Nicalin (data not shown). Finally, Nomo (pM5), a protein unrelated to  $\gamma$ -secretase, was identified as major Nicalin-binding partner and the Nicalin/Nomo complex was found to have a function different from  $\gamma$ -secretase (see below). Taken together, these data demonstrate that Nicalin is part of a novel membrane protein complex.

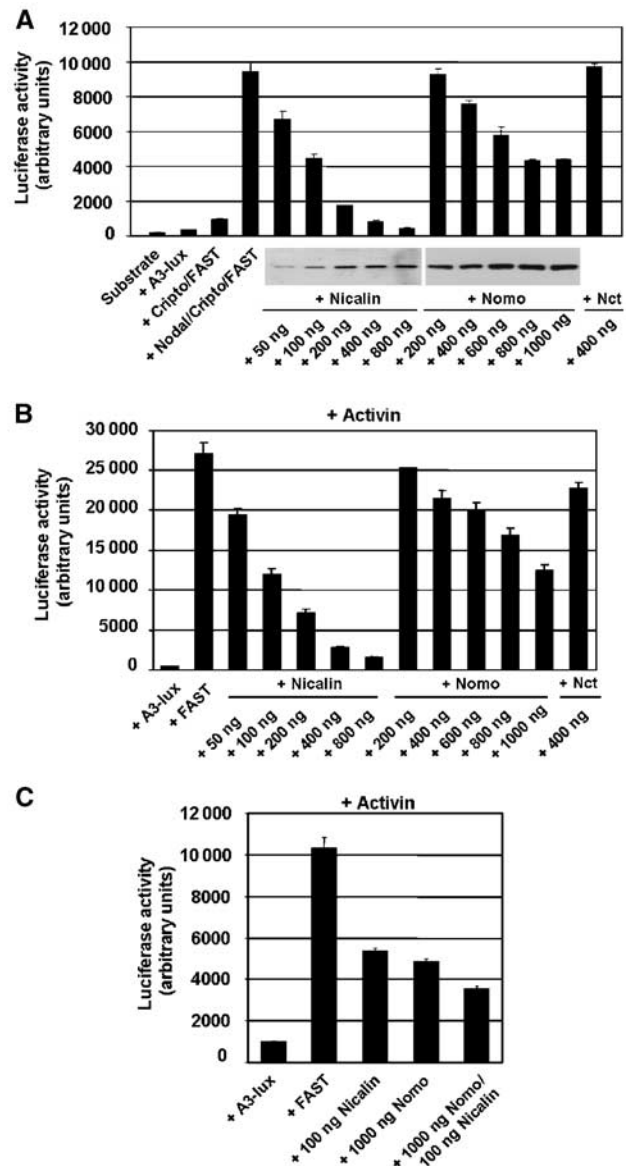
#### **Nomo is a major Nicalin-binding protein**

Our search for potential Nicalin complex components resulted in the detection of a 130 kDa protein in Nicalin

immunoprecipitates from kidney cell membranes. Using mass spectrometry, this protein was identified as pM5, the cDNA of which had originally been cloned by PCR and suggested to represent a new member of the collagenase family (Templeton *et al*, 1992). However, no data were provided to support this assumption. Instead, pM5 does not show homology to any class of protein nor does it contain a known functional domain. Based on our analysis of pM5 function *in vivo* (see below), we, therefore, refer to it as Nomo (Nodal modulator). The significance of the Nicalin/Nomo interaction is underscored by the following observations: both proteins are highly conserved from *C. elegans* to mammals and in plants, and their tissue distribution in humans is almost identical. In addition, Nicalin and Nomo show overlapping expression patterns during zebrafish embryogenesis. These data indicated that they interact functionally.



**Figure 6** Nomo antagonizes Nodal signaling. Expression of the anterior mesendodermal marker *hgg1* in embryos injected with 1 ng capped *lefty* RNA (A) or with *lefty* RNA and *nomo-grip* (B). Activation of Nodal signaling by Lefty reduces the number of *hgg1*-positive cells dramatically (compare to Figure 5E). Coinjection of the *nomo-grip* rescues this effect. (C–F) Model of the effects of *lefty* and *nomo* on Nodal signaling. In wild-type embryos, increasing levels of Nodal account for the generation of mesendodermal derivatives of progressively more anterior character (ppcp and hg) (C). Their formation is enhanced upon downregulation of *nomo*, indicating upregulated Nodal signaling (D). The endoderm, which requires highest Nodal activity, is not affected (not represented). Ectopic expression of *lefty* leads to inhibition of Nodal signaling and the formation of primarily posterior structures (n) (E). This effect can be reversed by simultaneously downregulating *nomo* (F). hg: presumptive hatching gland; n: notochord; ppcp: posterior part of the prechordal plate.



**Figure 7** Nicalin and Nomo inhibit Nodal- and Activin-induced signaling in 293T cells. Transient transfection assays were performed with the A3-lux luciferase reporter plasmid containing three tandem copies of a Nodal-responsive element. Luciferase activity is shown in arbitrary units with each bar representing four measurements from two independent transfections. (A) Strong induction of luciferase activity was measured in cells transfected with expression plasmids encoding Nodal, Cripto and FAST2. Adding increasing amounts of plasmids encoding Nicalin or Nomo led to a dose-dependent reduction of the luciferase signal, which corresponded to Nicalin and Nomo protein levels as demonstrated by immunoblotting. Transfection of Nicastrin (Nct) had no effect. (B, C) Luciferase activity was induced by transfection of FAST2 and treatment of the cells with 20 ng/ml recombinant, purified Activin for 18–22 h. Again, Nicalin and Nomo cause a dose-dependent reduction of luciferase activity (B). The reduction of Activin-induced luciferase activity is enhanced in cells transfected with both Nicalin and Nomo, suggesting a synergistic effect (C).

**Nomo/Nicalin complex regulates mesendodermal patterning in the zebrafish by attenuating Nodal signaling**

A possible functional relationship between Nicalin and Nomo was examined in the zebrafish model system, which

is highly suitable for transient functional tests at embryonic stages. Interfering with Nomo and/or Nicalin 1 (Ncl1) expression levels failed to produce phenotypes related to Notch signaling deficiencies, pointing to the distinct functions of the  $\gamma$ -secretase and the Nomo/Nicalin complex *in vivo*. In contrast, we found that Nomo and Ncl1 collaborate to modulate the activity of another crucial signaling pathway driven by the TGF $\beta$  factor Nodal. Our arguments are threefold: (i) ectopic expression of Nomo and Ncl1, but not of each factor alone, interferes with midline development, a known Nodal target, (ii) blocking Nomo function increases in a dose-dependent manner the amount of anterior mesendodermal derivatives at the expense of more posterior ones, thus mimicking enhanced Nodal activity, and (iii) blocking Nomo function counteracts the effect of the Nodal inhibitor Lefty. Thus, we propose that the Nomo/Ncl1 complex is a new modulator of the Nodal pathway that acts dose-dependently to attenuate Nodal signaling and refine patterning of the mesendoderm (Figures 6C–F). This hypothesis was confirmed in a well-defined reporter assay system. Using a reporter specific for the Nodal/Activin family of TGF $\beta$  ligands, we could show that (i) the antagonistic effect of Nicalin and Nomo on Nodal signaling is direct and is not based on crosstalk with another pathway, (ii) the Nicalin/Nomo complex acts in a cell-auto-nomous manner, that is, it interferes with the signal transduction in the signal-receiving cell, and (iii) Nicalin and Nomo also block Activin signaling, suggesting that they do not affect the function of EGF-CFC coreceptors.

To date, and in spite of the involvement of Nodal signaling in multiple developmental processes, only a few down-modulators of this pathway have been identified. These can act as long-range secreted antagonists such as Lefty (Chen and Schier, 2002; Sakuma *et al*, 2002; Cheng *et al*, 2004), as membrane-bound competitors for binding to the Nodal coreceptor Cripto/One-eye-pinhead (Oep) (Harms and Chang, 2003) or as intracellular transcriptional repressors of Nodal targets (Yamamoto *et al*, 2001; Iratni *et al*, 2002; Bell *et al*, 2003). The ER localization of Nomo and Nicalin may suggest that this new inhibitory complex could act by yet another mechanism, possibly by modifying and/or trapping Nodal pathway components that route through the ER. The trafficking of various plasma membrane proteins has been shown to be regulated at the level of the ER (Ma and Jan, 2002). Recently, the ER-resident membrane proteins BAP29 and BAP31 were shown to be involved in the ER retention of membrane-bound IgD (Schamel *et al*, 2003), and it is conceivable that the Nomo/Nicalin complex might have a comparable function. It remains, however, important to determine the exact biochemical action of Nomo and Nicalin, as well as whether its activity extends to other developmental processes involving Nodal. The necessity for additional local cofactors is suggested by the nonresponse of endodermal precursors to Nomo/Nicalin.

## Materials and methods

### Cloning of Nicalin and Nomo cDNAs

Database searches for Nicastrin homologs were performed with a nonredundant data set constructed from current releases of SwissProt, TrEMBL and GenPept. Generalized profiles from the

ectodomain of the Nicastrin family were constructed using the BLOSUM45 substitution matrix (Bucher *et al*, 1996) and default penalties of 2.1 for gap opening and 0.2 for gap extension. The searches were run locally using the pftools package, version 2.1 (program available from the URL <ftp://ftp.isrec.isb-sib.ch/sib-isrec/pftools/>). Human Nomo and zebrafish sequences were identified using BLAST searches against the GenBank database or the zebrafish EST database at the NCBI, respectively. All cDNA clones described were obtained from the German Resource Center for Genome Research (RZPD). For expression studies, full-length human Nicalin (acc. no. BG422523) and human Nomo (acc. no. AL832855) were subcloned into the pCDNA4/TO/myc-His vector (Invitrogen).

### Antibodies

The polyclonal anti-Nicalin antibody 2221 was generated by Eurogentech (Seraing, Belgium) by immunizing rabbits with a mixture of two peptides (LESHRDGQRSSIMDV, NQPRAAQLVDK DSTF) of the ectodomain of human Nicalin. The following other antibodies were used: monoclonal  $\alpha$ -myc (9E10, ATCC), monoclonal  $\alpha$ -FLAG (Kodak), polyclonal  $\alpha$ -Nicastrin (N1660, Sigma), monoclonal  $\alpha$ -Presenilin 1 (Capell *et al*, 1997), polyclonal  $\alpha$ -calnexin (Stressgen) and polyclonal  $\alpha$ -APH1a (K Shirotni and C Haass, unpublished).

### Cell lines and transfection

HEK293 cells and SH-SY5Y neuroblastoma were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 2 mM L-glutamine. HEK293 cells overexpressing the APP<sub>Sw</sub> mutant (Sw) (Citron *et al*, 1992) and HEK293/TR cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) and selected with the appropriate antibiotics.

### Blue-Native PAGE and immunoprecipitation

Membrane proteins for Blue-Native PAGE were extracted as described (Edbauer *et al*, 2002) and subjected to immunoprecipitation or analyzed on 4–12.5 or 4–16% Blue-Native gels as described (Schagger and von Jagow, 1991). Immunoprecipitation was performed as described (Edbauer *et al*, 2002).

### MALDI analysis

Coomassie-stained gel bands were excised from the gel, washed with water, shrunk twice with 50% acetonitrile and air dried for 5 min. Proteins were trypsinized for 3 min with 0.2  $\mu$ g/ $\mu$ l modified trypsin solution (Promega) and trypsin fragments were eluted overnight with 40 mM ammonium bicarbonate solution (pH 7.8), concentrated using ZipTips (Millipore), bound onto the reversed phase material, washed and eluted with a saturated matrix ( $\alpha$ -cinnamic acid) solution (0.3% TFA, 50% acetonitrile) directly onto the stainless steel target plate. Mass spectra were recorded on a Voyager DE STR (Applied Biosystems) according to the manufacturer's instructions. Mass spectra were calibrated internally with the autolysis products of porcine trypsin with a mass of 842.51 and 2211.1046 Da. Mass spectra were processed, trypsin peaks was removed and the peak list was submitted to Mascot (Matrixscience) for database searching and protein identification. Database searches were performed with a mass tolerance of 50 ppm or less after internal calibration.

### Northern blot analysis

Nicalin and Nomo probes were generated using [<sup>32</sup>P]dCTP (Amersham Biosciences), the Random Primers DNA Labeling System (Invitrogen) and human cDNAs as templates. A human multiple tissue Northern blot (Clontech) was hybridized to the probes according to the manufacturer's directions and exposed to Super RX film (Fuji) for 4–16 h.

### Fish strains

Wild-type embryos were obtained from natural spawning of AB adults, and raised and staged according to Kimmel *et al* (1995).

### Antisense experiments

The antisense oligonucleotides (gripNAs) for *ncl1* (5'-ACCAGCC TCCTCGAACAT-3') and *nomo* (5'-TTTAATCCACCCATCGT-3') were purchased from Active Motifs Europe (Brussels, Belgium), dissolved to a stock concentration of 1 mM in H<sub>2</sub>O and pressure-

injected into 1-cell-stage embryos at 0.5 or 1 mM using an Eppendorf microinjector.

### Capped RNA injections

For *in vitro* transcription experiments, a full-length zebrafish *ncl1* clone (*ncl1*) was generated by PCR (forward primer: 5'-GCGAATTCTCCACGATGTTGAAGAAGCGGGCGAGGTGTTGGAG-3'; reverse primer: 5'-GCGTCGACTCAGTGTCTTTGACC-3') using *ncl1* (acc. no. BI472741) as template and subcloned into the pCS2+ vector. A full-length *nomo* clone was generated by subcloning human *nomo* (*pM5*) (acc. no. AL832855) into the pCS2+ vector. For the rescue experiment, a gripNA-resistant version of zebrafish *nomo* (*nomo\**) was constructed in the pXT7 vector using an N-terminal fragment (acc. no. BM859276) modified by PCR (forward primer: 5'-GCGGTACCGCCACCATGGGAGGTATAAAGGAGCTAGCAATTCTT-3'; reverse primer: 5'-GCGAATTCGATATCCTCTCGTTAC-3'), a central fragment obtained by PCR (forward primer: 5'-AGTGTCTTCACGCATGCTGGAG-3'; reverse primer: 5'-GCCCAA TAGGAGAATTCATAGT-3') from tail-bud-stage cDNA and a C-terminal fragment (acc. no. BI887091). *lefty* encodes a soluble antagonist of Nodal signaling and was described previously (Thisse and Thisse, 1999). All capped RNAs were synthesized using the mMessage mMachine kit (Ambion) following the manufacturer's instructions, resuspended in H<sub>2</sub>O and injected at the following concentrations: *ncl1\**, *nomo* and *nomo\** RNA at 100–150 ng/μl and *lefty* RNA at 1 ng/μl.

### Whole-mount *in situ* hybridization

*In situ* hybridization was carried out on embryos according to standard protocols (Hammerschmidt *et al*, 1996). The following marker probes were used: *gsc*, *hgg1* (Thisse *et al*, 1994), *hlx1* (Seo *et al*, 1999), *znot* (Melby *et al*, 1996) and *sox17* (Alexander and Stainier, 1999).

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### Nodal reporter assay

The luciferase reporter assay in 293T cells was performed essentially as described (Yan *et al*, 2002). A total of  $2.5 \times 10^5$  cells were plated on polylysine-coated 24-well plates, grown overnight and transfected with 200 ng each of A3-lux (Chen *et al*, 1996), pcDNA3-Nodal, pcDNA3/FLAG-Cripto, pcDNA3/myc-FAST2 (Yan *et al*, 2002) and varying amounts of pcDNA4/myc-Nicalin or pcDNA4/myc-Nomo using Lipofectamine 2000 (Invitrogen). After 22–24 h, cells were washed once with PBS and lysed in 25 mM glycyl-glycine, pH 7.8, 10 mM MgSO<sub>4</sub>, 10 mM dithiothreitol and 0.2% N-P40. After removal of the insoluble material, 20 μl cell lysate was mixed with 100 μl assay buffer (25 mM glycyl-glycine, pH 7.8, 10 mM MgSO<sub>4</sub>, 10 mM dithiothreitol, 1 mM ATP, 35 μM D-luciferin) and luminescence was measured in a Wallac Victor 1420 luminometer.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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