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# Antagonistic roles of full-length N-cadherin and its soluble BMP cleavage product in neural crest delamination

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During neural crest ontogeny, an epithelial to mesenchymal transition is necessary for cell emigration from the dorsal neural tube. This process is likely to involve a network of gene activities, which remain largely unexplored. We demonstrate that N-cadherin inhibits the onset of crest delamination both by a cell adhesion-dependent mechanism and by repressing canonical Wnt signaling previously found to be necessary for crest delamination by acting downstream of BMP4. Furthermore, N-cadherin protein, but not mRNA, is normally downregulated along the dorsal tube in association with the onset of crest delamination, and we find that this process is triggered by BMP4. BMP4 stimulates cleavage of N-cadherin into a soluble cytoplasmic fragment via an ADAM10-dependent mechanism. Intriguingly, when overexpressed, the cytoplasmic N-cadherin fragment translocates into the nucleus, stimulates cyclin D1 transcription and crest delamination, while enhancing transcription of β-catenin. CTF2 also rescues the mesenchymal phenotype of crest cells in ADAM10-inhibited neural primordia. Hence, by promoting its cleavage, BMP4 converts N-cadherin inhibition into an activity that is likely to participate, along with canonical Wnt signaling, in the stimulation of neural crest emigration.

**KEY WORDS: Adherens junctions, β-catenin, Cell cycle, Epithelial to mesenchymal transition, Wnt, Quail**

## INTRODUCTION

The neural crest (NC) is a transient population of embryonic progenitors that constitutes the dorsal midline of the early neural tube (NT) and generates a wide variety of derivatives (Le Douarin and Kalcheim, 1999). The molecular network underlying NC specification is being elucidated (Basch et al., 2004; Gammill and Bronner-Fraser, 2003). Following specification, NC cells successively emigrate from the NT and follow stereotypic migrations throughout the embryo to reach their homing sites and differentiate. To this end, premigratory cells undergo a process of epithelial-to-mesenchymal transition (EMT), an indispensable stage for generating cellular movement (Halloran and Bendt, 2003; Kalcheim and Burstyn-Cohen, 2005; Nieto, 2001).

Recent studies have demonstrated a role for dorsal NT-derived BMP and Wnt signals in initiating NC delamination, independent of their prior specification. We reported that a decreasing rostrocaudal gradient of BMP4 activity is established along the dorsal NT by a reciprocal gradient of its inhibitor, noggin. BMP then induces EMT of NC progenitors. Noggin downregulation is in turn triggered by the developing somites, which thus determine the timing of NC emigration (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Sela-Donenfeld and Kalcheim, 2002). NC emigration can also be stimulated by Cv-2, which promotes BMP activity in the avian NT (Coles et al., 2004), suggesting that BMP signaling is subject to both positive and negative regulation. The cell cycle was also found to play a pivotal role. NC cells synchronously emigrate in S phase, and the transition from G1 to S is necessary for the process: specific inhibition of G1-S transition blocked NC emigration, whereas arrest at S or G2 phases had no immediate effect (Burstyn-Cohen and Kalcheim, 2002). A subsequent study integrated the above findings, demonstrating that: (1) BMP regulates NC delamination in a cell cycle-dependent manner; and (2) canonical Wnt signaling acts downstream of BMP in the dorsal NT to mediate BMP activity in the context of both the G1-S transition and NC delamination (Burstyn-Cohen et al., 2004). Additional studies suggested that EMT of NC cells is modulated by transcription factors, cell adhesion molecules and other regulatory proteins (Barrallo-Gimeno and Nieto, 2005; Halloran and Bendt, 2003; Kalcheim, 2000; Nieto, 2001). However, very little is known about their precise mechanism of action or their possible relationship with the BMP/Wnt cascade described above; this knowledge would prove invaluable in establishing the molecular network underlying EMT of NC progenitors.

Here, we explored the mechanism by which N-cadherin acts on NC delamination and its regulation by BMP. N-cadherin belongs to a family of $Ca^{2+}$-dependent cell adhesion molecules (Hatta et al., 1988; Hatta et al., 1987; Hatta and Takeichi, 1986) important for various developmental processes (Gumbiner, 2000; Nelson and Nusse, 2004). N-cadherin is characterized by five extracellular cadherin-binding domains, a transmembrane and an intracellular β-catenin-binding domain (Tepass et al., 2000). The full-length 135 kDa protein is cleaved extracellularly by a rate-limiting metalloproteinase, ADAM 10, generating a 40 kDa C-terminal fragment termed CTF1, which is further processed by a γ-secretase-like activity into the soluble 35 kDa intracellular CTF2, which is involved in the regulation of gene expression (Fortini, 2002; Marambaud et al., 2003; Reiss et al., 2005). This places N-cadherin within a category of cell surface receptors, including Notch, amyloid precursor protein and Erb-B4, in which the intracellular domain is liberated by γ-secretase-mediated cleavage (Fortini, 2002). Whereas in the latter cases, the cleaved intracellular domain translocates to the nucleus to promote signaling events, there is no information as to whether a similar mechanism operates in the case of N-cadherin.

N-cadherin is initially expressed in the entire NT. Presumptive premigratory NC cells, however, lose the protein prior to departing from the NT (Akitaya and Bronner Fraser, 1992; Duband et al., 1988; Hatta et al., 1987), suggesting that loss of N-cadherin is a

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prerequisite for NC delamination. Functional studies using adenoviral-mediated overexpression of N-cadherin reported a failure of melanoblast emigration, the last NC cells to depart from the NT. Nevertheless, they showed a normal onset of cell emigration followed by successful formation of neural derivatives, except for a slight reduction in the number of dorsal root ganglion cells (Nakagawa and Takeichi, 1998). Reciprocally, loss of N-cadherin-mediated adhesion had no effect either on NC emigration or NT morphology (Nakagawa and Takeichi, 1998). Furthermore, injection of neutralizing antibodies into the cranial tube resulted mainly in NT deformities and some ectopic aggregates of NC cells, but the significance to EMT of NC was not studied (Bronner-Fraser et al., 1992). Additional loss-of-function approaches focused on its role in maintaining NT integrity without addressing the problem of NC development (Ganzler-Odenthal and Redies, 1998; Lele et al., 2002). Hence, the longstanding hypothesis proposing that N-cadherin prevents NC delamination has, at best, been only partially substantiated. Furthermore, the mode of N-cadherin activity on NC remains unexplored.

Using electroporation of the NT, we show that full-length N-cadherin is sufficient to completely block NC delamination while downregulating *cyclin D1* and the G1-S transition. Transfection of mutant forms of N-cadherin reveals that NC delamination requires the combined activity of both cell adhesion and β-catenin-binding domains. Hence, N-cadherin is required for maintaining all potential NC in a premigratory state and this function can be explained both by its adhesive properties and by inhibiting canonical Wnt-mediated signaling. Is N-cadherin part of the BMP-dependent cascade leading to NC delamination? We report that N-cadherin protein is expressed along the dorsal tube in a decreasing caudal-to-rostral gradient similar to that exhibited by noggin. Furthermore, we show that its normal downregulation is prevented by noggin and, reciprocally, is stimulated by BMP4. Thus, N-cadherin is part of the BMP-dependent pathway leading to NC delamination. We propose that BMP affects N-cadherin stability via ADAM10, as treatment of explanted neural primordia with GI254023X, a specific inhibitor of endogenous ADAM10, maintains membrane-bound cadherin and inhibits NC delamination when added either alone or in combination with BMP4. Most importantly, soluble CTF2, the end product of N-cadherin degradation, translocates into nuclei, stimulates transcription of *cyclin D1* and promotes delamination of NC cells. This is likely to result, at least partially, from enhanced β-catenin transcription. Taken together, we suggest that BMP-mediated downregulation of N-cadherin in the dorsal tube serves both to reduce intercellular adhesion and to facilitate transcriptional activity leading to the generation of NC cell movement.

## MATERIALS AND METHODS

### Embryos

Fertile quail (*Coturnix coturnix Japonica*) eggs from commercial sources were used as the source of embryos.

### Electroporation and expression vectors

DNA (3-5 μg/μl) was microinjected into the lumen of the NT of 15- to 18-somite-stage embryos at the level of the segmental plate and two recently formed somites. A four parameter Pulse*Agile* square wave electroporator (PA-4000, Cyto Pulse Sciences) was used to deliver three groups of sequential pulses as follows: 3×18V of 20 mseconds each; 3×26V of 15 mseconds each; 3×18V of 20 mseconds each. Embryos were then incubated for various times ranging from 8-24 hours, some followed by a 1-hour pulse of Brdu (10 mM) or by processing for in situ hybridization. Another series of embryos were incubated for 2 hours followed by explantation of isolated tubes on fibronectin (see below).

The expression vector pCAGGS-AFP (Momose et al., 1999) was used as a control (referred to as control-GFP). DNAs encoding full-length chicken N-cadherin, N-cadherin lacking part of the extracellular domain (cN390Δ) and N-cadherin lacking either the intracellular β-catenin-binding domain (CBR-) or the juxtamembrane domain (JMD-) (Fujimori and Takeichi, 1993; Horikawa and Takeichi, 2001; Nakagawa and Takeichi, 1998); N-cadherin tail (CTF2) (Sadot et al., 1998); *Xenopus* noggin and mouse BMP4 (Endo et al., 2003) were subcloned into the pCAGGS vector and fused in-frame to a GFP-encoding sequence. Experimental details are available upon request.

### Grafting of BMP4-coated beads

Heparin-acrylic beads (Sigma) were immersed in BMP4 (R&D, 50 ng/ml in 1% fetal calf serum in PBS) or BSA for 1.5 hours followed by repeated washings in PBS. To graft the beads, a slit was made along the dorsal aspect of the NT at the caudal segmental plate level of the axis. A single BMP-coated bead per embryo was then inserted, being held between the neural folds.

### Explants of neural primordia

Neural tubes containing premigratory NC were excised from segmental plate levels of 16- to 20-somite-stage embryos and then explanted onto 8-well chamber slides (Lab-Tek) pre-coated with fibronectin (Sigma, 50 μg/ml), as described (Burstyn-Cohen and Kalcheim, 2002). Culture medium consisted of CHO-S-SFM II (Gibco-BRL) to which BMP4 (100 ng/ml), or the ADAM10-inhibitor GI254023X (12 μM), or both were added.

### Tissue processing, immunocytochemistry and in situ hybridization

Embryos were fixed with 4% formaldehyde, embedded in paraffin wax and sectioned at 5 or 10 μm. Rabbit anti-GFP (Molecular Probes) was used at 1:500 in combination with HNK-1, N-cadherin, or Brdu immunolabelings or with in situ hybridization for *cyclin D1*. Additional in situ hybridizations were performed with *noggin*, *β-catenin* or with a chicken *N-cadherin* probe encompassing nucleotides 816 to 1523 of the coding domain. Monoclonal antibodies against the intracellular domain of N-cadherin (Zymed) were applied following antigen retrieval by boiling the slides in 0.1 M Tris buffer (pH 9.5) for 5 minutes. The GC4 antibody against the extracellular region of N-cadherin was obtained from Sigma. Nuclei were visualized with Hoechst. Whole-mount embryo preparations and sections were photographed using a DP70 cooled CCD digital camera (Olympus) mounted on a BX51 microscope (Olympus).

### Data analysis

Cell proliferation and NC delamination were monitored in at least five embryos per treatment out of 8-21 embryos showing a similar phenotype, as described (Burstyn-Cohen et al., 2004). Briefly, the proportion of Brdu+/GFP+ cells or the number of Hoechst+ nuclei located up to the migration staging area was measured in 25 sections of control versus experimental hemi-tubes, and expressed as the mean±s.d. of total cases monitored. The number of NC cells with mesenchymal morphology that exited from explanted neural tubes was counted in 33-43 microscopic fields per explant, each comprising an area of 2500 $\mu m^2$. Results represent the average number of cells per explant (±s.d. of 4-5 cultures counted out of at least 12 cultures per treatment showing a similar phenotype) normalized to the length of the NT fragment. Significance was examined using one-way analysis of variance (ANOVA). When significant differences were indicated in the F ratio test ($P<0.005$), the significance of differences between means of any two of these groups was determined using the modified Tukey method for multiple comparisons with an $\alpha$ of 0.05.

## RESULTS

### Loss of N-cadherin protein in the dorsal NT is associated with the onset of NC delamination and is directly correlated with the caudal-to-rostral decrease in *noggin* transcription

N-cadherin immunoreactivity in the dorsal NT was apparent at caudal regions of the axis opposite the segmental plate mesoderm, but was absent from the region that was already facing epithelial somites and further rostralward (Fig. 1A-C, arrowheads). Loss of N-

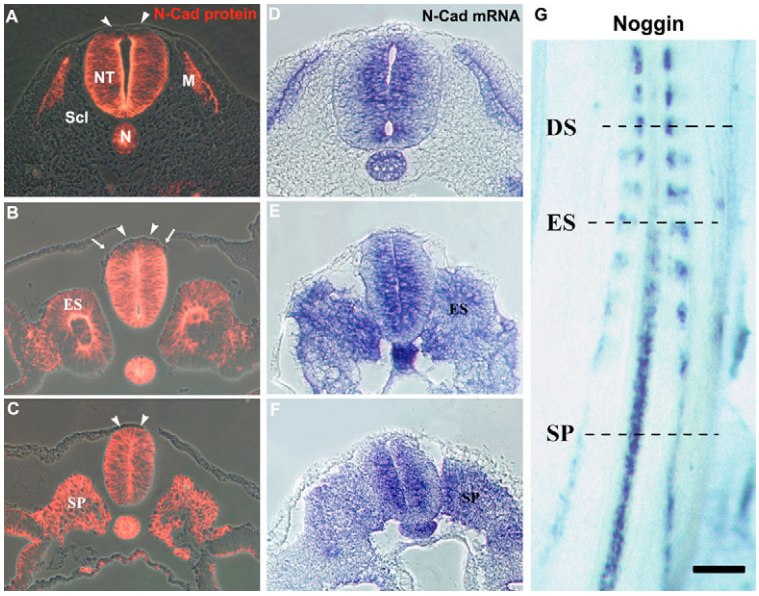


**Fig. 1. Axial-level-dependent downregulation of N-cadherin protein, but not mRNA, in the dorsal NT is correlated with loss of *noggin* transcripts.** (**A-C**) N-cadherin immunoreactivity. Arrowheads point to the dorsal NT of a 25-somite-stage embryo that expresses N-cadherin immunoreactivity opposite the segmental plate (C), but lacks the protein in the region facing epithelial somites (B) and dissociated somites (A). Arrows in B depict the onset of NC emigration from a N-cadherin-deficient dorsal NT. (**D-F**) N-cadherin mRNA showing no change in dorsal NT along similar axial levels. (**G**) Axial-dependent downregulation of *noggin* mRNA coincides with a similar pattern of N-cadherin disappearance. Abbreviations: DS, dissociated somites; ES, epithelial somites; M, myotome; N, notochord; NT, neural tube; Scl, sclerotome; SP, segmental plate. Scale bar: 80 μm.

cadherin positively correlated with the onset of NC delamination (Fig. 1B) and coincided with the axial-dependent downregulation of *noggin* mRNA, whose expression is intense in the dorsal NT opposite segmental plate levels and decreases rostralward (Fig. 1G) (Sela-Donenfeld and Kalcheim, 1999). By contrast, no change in *N-cadherin* mRNA expression was detected along the axis (Fig. 1D-F), suggesting that loss of N-cadherin is regulated post-transcriptionally.

## Overexpression of N-cadherin inhibits NC delamination

To directly examine whether the temporal coincidence between loss of N-cadherin protein and onset of NC emigration are functionally related, we prevented the normal downregulation of N-cadherin by electroporating N-cadherin-GFP into hemi-tubes at segmental plate levels. By the time the transfected protein was expressed, endogenous N-cadherin had disappeared from the dorsal midline region but persisted elsewhere in the NT where it was enriched in apical adherens junctions (Fig. 2A-C). First, we noticed that neuroepithelial cells rounded up, losing their elongated pseudostratified morphology (Fig. 2B,D), which was likely to have resulted from homogeneous membrane distribution of the transfected protein. Full-length N-cadherin reduced the overall emigration of NC cells by 68±3.3%, with transfected cells almost completely failing to delaminate as compared with control-GFP (Fig. 2A-D and Fig. 3I). N-cadherin-GFP also prevented NC emigration from neural primordia explanted following electroporation; note that untransfected cells in these explants, or cells that received control-GFP, emigrated normally (Fig. 2E,F). The observed phenotype reflects a true failure of NC emigration rather than simply a delay because embryos grown for 35 hours revealed the presence of N-cadherin-GFP+ cells in the dorsal NT that expressed ectopic HNK-1 (Fig. 2H). These embryos had virtually no fluorescent cells in their peripheral ganglia, and this was associated with severely reduced size of sensory and sympathetic ganglia (Fig. 2G,H). Hence, N-cadherin present in the dorsal NT at segmental plate levels may contribute to preventing premature NC delamination and, conversely, its downregulation may be required for the onset of NC emigration.

## Membrane-anchored N-cadherin mutants have no effect on NC delamination but disrupt neuroepithelial morphology

To examine whether the inhibitory effect of N-cadherin on emigration of NC cells can be mimicked by overexpressing selected domains of the protein, three mutants were transfected at segmental plate levels. N-cadherin bearing a deletion in the extracellular domain, N-cadherin lacking the β-catenin-binding domain, and N-cadherin lacking the juxtamembrane domain, were fused to a GFP reporter to create cN390Δ-GFP, CBR-GFP and JMD-GFP, respectively (Fig. 3A). These mutants have been shown to act in a dominant-negative fashion on cell adhesion and morphology in tissues expressing endogenous N-cadherin (Fujimori and Takeichi, 1993; Horikawa and Takeichi, 2001; Nakagawa and Takeichi, 1998). Since most of the NT continuously expresses the protein, we expected the mutant DNAs to similarly act in this region in a dominant-negative fashion. By contrast, the dorsal-most region of the NT that contains the premigratory NC downregulates N-cadherin by the time the transgenes first become expressed (epithelial to dissociating somite levels, not shown). Hence, regarding the dorsal NT, we asked whether the mutant proteins were sufficient to compensate for the physiological loss of endogenous N-cadherin. None of the mutants had any measurable effect on delamination of NC cells as compared with control-GFP, with a similar number of labeled cells emigrating from the NT in all cases (Fig. 3B-I and data not shown). Furthermore, we noticed that in cN390Δ-GFP and CBR-GFP-treated embryos, the delaminating cells were round and spread apart (Fig. 3B,C,H and Fig. 2E; and data not shown). Hence, N-cadherin-mediated inhibition of NC delamination requires the integrated activity of all its domains as this effect cannot be reproduced by overexpressing individually any combination of two out of the three tested domains. Nevertheless, both cN390Δ-GFP and CBR-GFP provoked a loss of the pseudostratified conformation in NT cells that continuously express N-cadherin with a rounding up similar to that observed upon transfection of wild-type N-cadherin (Fig. 2B,D,F and Fig. 3C,E,F,H; see also Fig. 4B,C). Surprisingly,

transfection of JMD- had no effect on NT morphology (Fig. 3G), suggesting that maintenance of the epithelial conformation in this tissue is not likely to result from events linked to the JMD, such as binding to p120 and the resulting changes in cytoskeletal assembly (Gumbiner, 2000).

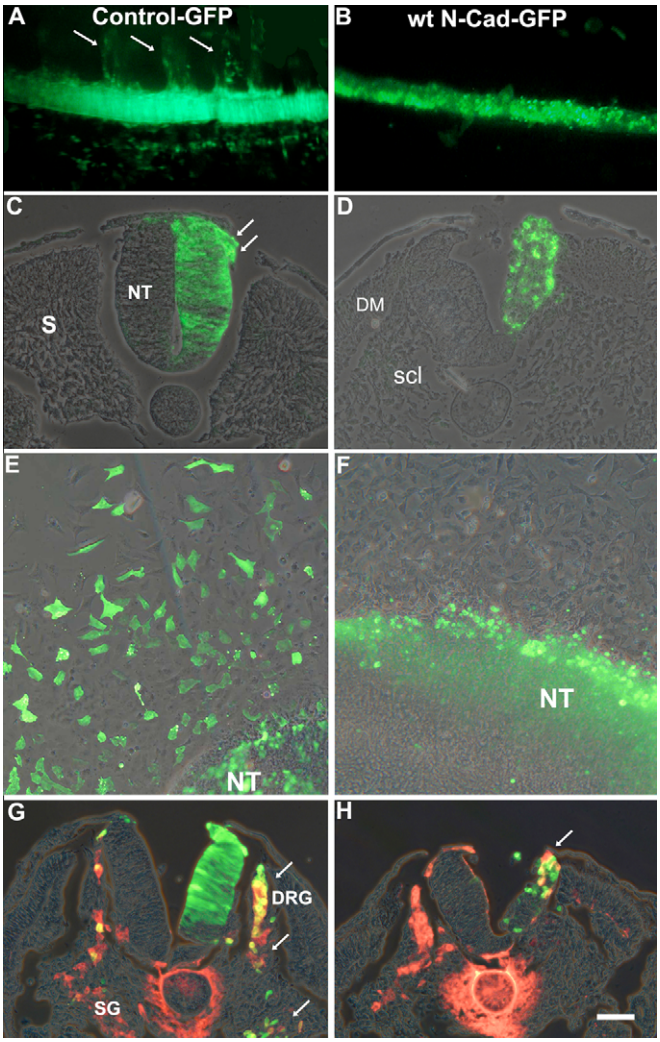


**Fig. 2. Overexpression of N-cadherin inhibits NC delamination.** (**A**,**C**,**E**) Electroporation of control-GFP. (A) Segmentally migrating labeled NC (arrows) in whole-mounts. (C) Early delaminating NC (arrows) opposite a dissociating somite (S) in transverse section. (E) GFP-labeled mesenchymal NC cells emigrate from NT explants along with unlabeled progenitors evident with phase contrast optics. (**B**,**D**,**F**) Electroporation of full-length N-cadherin. No NC delamination occurs in whole-mounts (B), in sections opposite an already dissociated somite (D), or from NT explants (F). Note that unlabeled cells emigrate normally, yet transfected cells round up as in vivo, with no evidence of cell death (F and data not shown). (**G**,**H**) Control and N-cadherin-treated embryos, respectively, fixed 35 hours after transfection when peripheral ganglia are already coalesced. (G) Note the presence of GFP+ cells (arrows, green) that colonize the HNK-1+ DRG and SG (red). (H) By contrast, N-cadherin+ cells remain in the dorsal NT where they express ectopic HNK-1 (arrow). *n*=6 for each treatment. Abbreviations: DM, dermomyotome; DRG, dorsal root ganglion; NT, neural tube; S, somite; scl, sclerotome; SG, sympathetic ganglion. Scale bar: 28 μm for C,D; 55 μm for E,F; 25 μm for G,H.

## N-cadherin inhibits NC delamination by reducing *cyclin D1* transcription and G1-S transition

Given that a cell adhesion-mediated process cannot solely account for the activity of the full-length molecule, and that even membrane-anchored mutants bearing the β-catenin-binding region were ineffective, we examined the hypothesis that wild-type N-cadherin, but not its mutants, acts by inhibiting the G1-S transition, a prerequisite for NC delamination (Burstyn-Cohen and Kalcheim, 2002; Burstyn-Cohen et al., 2004). As previously documented, control-GFP had no effect on cell proliferation (Fig. 4A,F) as 45±6.65% of GFP+ nuclei in the NT were Brdu+. By contrast, only 7.94±5.47% of GFP+ cells were Brdu+ in the wild-type N-cadherin-

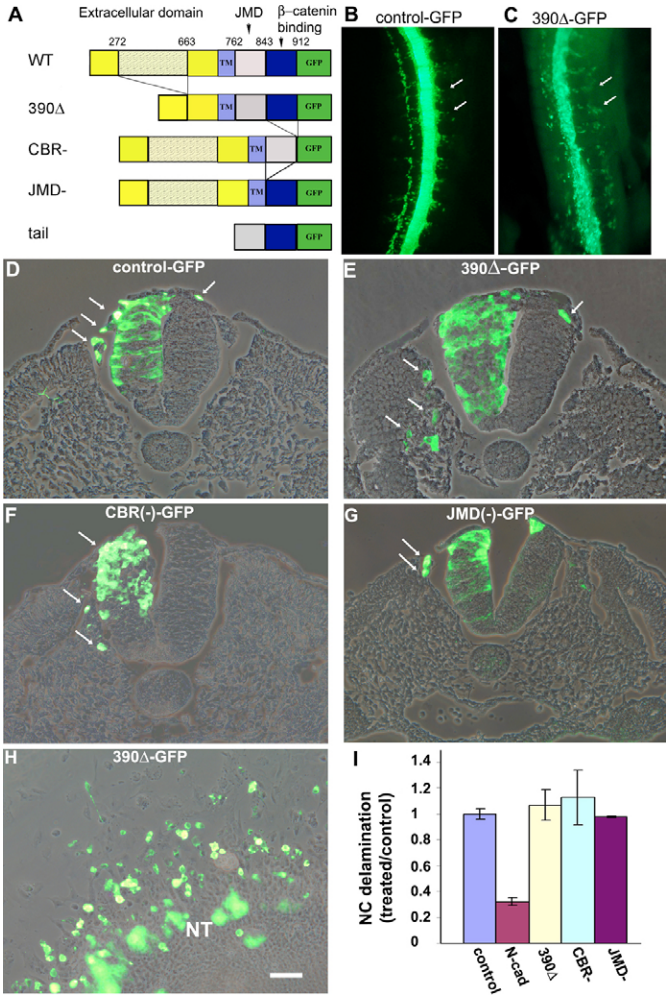


**Fig. 3. N-cadherin mutants do not affect NC delamination.** (**A**) Schematic of N-cadherin mutants used. (**B-G**) Transverse sections through embryos electroporated with: (B,D) control-GFP; (C,E) cN390Δ-GFP; (F) CBR-GFP; (G) JMD-GFP. (**H**) NT explanted on fibronectin following transfection with cN390Δ-GFP. (**I**) Quantification of NC delamination. Efficiency of electroporation was greater than 70%. Results represent the mean±s.d. of five embryos per treatment normalized to control values. The extent of NC emigration was not affected by the mutant DNAs (arrows in D-G, and I), but labeled emigrating NC cells appeared more dispersed than controls (arrows in B,C). Transfected NT cells lost epithelial morphology in cN390Δ-GFP and CBR-GFP-treated cases, and NC cells emigrated as round cells from NT explants (H). Scale bar: 38 μm for D-G; 45 μm for H.

treated tubes (Fig. 4A,B,D). Thus, full-length N-cadherin causes an 83% reduction in DNA synthesis in neuroepithelial cells. Next, we examined the effect of N-cadherin on transcription of *cyclin D1*, a target of β-catenin-dependent Wnt signaling in the NT (Burstyn-Cohen et al., 2004; Megason and McMahon, 2002). As expected from its effect on the G1-S transition, *cyclin D1* mRNA was downregulated in cells that received N-cadherin-GFP as compared with control-GFP and to untransfected regions of the NT (Fig. 4E-H). As a control, we examined the expression of *cyclin B2* mRNA, a G2-M cyclin, and observed that in contrast to *cyclin D1*, levels of *cyclin B2* were increased (not shown); hence, N-cadherin caused a selective inhibition of G1-specific gene transcription without producing a general inhibition of transcriptional activity. These data suggest that N-cadherin inhibits NC delamination by inhibiting the G1-S transition, a consequence of canonical Wnt signaling in this system. This is consistent with studies showing that cadherins antagonize β-catenin signaling by binding and sequestering it in catenin-cadherin complexes at the plasma membrane (see Discussion).

By contrast, neither cN390Δ-GFP, CBR-GFP nor JMD-GFP had any effect on the G1-S transition, with 40-50% of the Brdu+/GFP+ progenitors being found in the NT, and with delaminating NC cells mostly in the S-phase of the cell cycle (Fig. 4C (arrows),D and data not shown). These data substantiate previous results showing that NC delamination depends upon successful G1-S transition. In addition, taken together with the observed delamination phenotypes, our data indicate that maintenance of N-cadherin protein integrity is required in the NT for effectively antagonizing transcriptional signaling; retention of the β-catenin-binding domain in membrane-anchored mutants is not sufficient, however, contrary to what has been observed in several in vitro systems (see Discussion).

Notably, both cN390Δ-GFP and CBR-GFP, which caused the loss of the pseudostratified morphology of NT cells (Figs 3, 4), also revealed an abnormal pattern of interkinetic nuclear migration, defined as a cell cycle-dependent radial migration of cell nuclei across the epithelium. In these embryos, Brdu+/GFP+ nuclei were scattered throughout the apico-basal extent of the NT (Fig. 4C and data not shown), instead of being confined to its basal half where DNA replication normally occurs (Fig. 4A). The finding that cell proliferation occurs to a normal extent even in the disorganized hemi-NT suggests that cell proliferation and interkinetic nuclear migration are separable processes.

## N-cadherin is a component of the BMP-noggin cascade underlying NC delamination

In previous studies, we demonstrated that the downregulation of *noggin* in the dorsal NT at the level of epithelial somites (see also Fig. 1G) relieves BMP activity from the inhibition to which it is subjected along the caudal NT. BMP4 then triggers NC delamination via the canonical Wnt pathway. Since N-cadherin protein expression, but not mRNA, also disappears from the dorsal NT at comparable axial levels (Fig. 1), we examined the hypothesis that BMP4 downregulates N-cadherin immunoreactivity. The presence of BMP4-coated beads or the overexpression of a BMP4-encoding DNA in the dorsal NT opposite the caudal segmental plate, resulted in loss of N-cadherin 8 hours later, opposite both the caudal and rostral segmental plate; at these levels, N-cadherin was still present in control-treated and intact dorsal NTs (Fig. 5A-D, Fig. 1C and data not shown). We next examined the physiological relevance of this effect by inhibiting endogenous BMP. Transfection of control-GFP into the NT at segmental plate levels revealed a normal loss of N-cadherin 15 hours later, adjacent to dissociating levels of

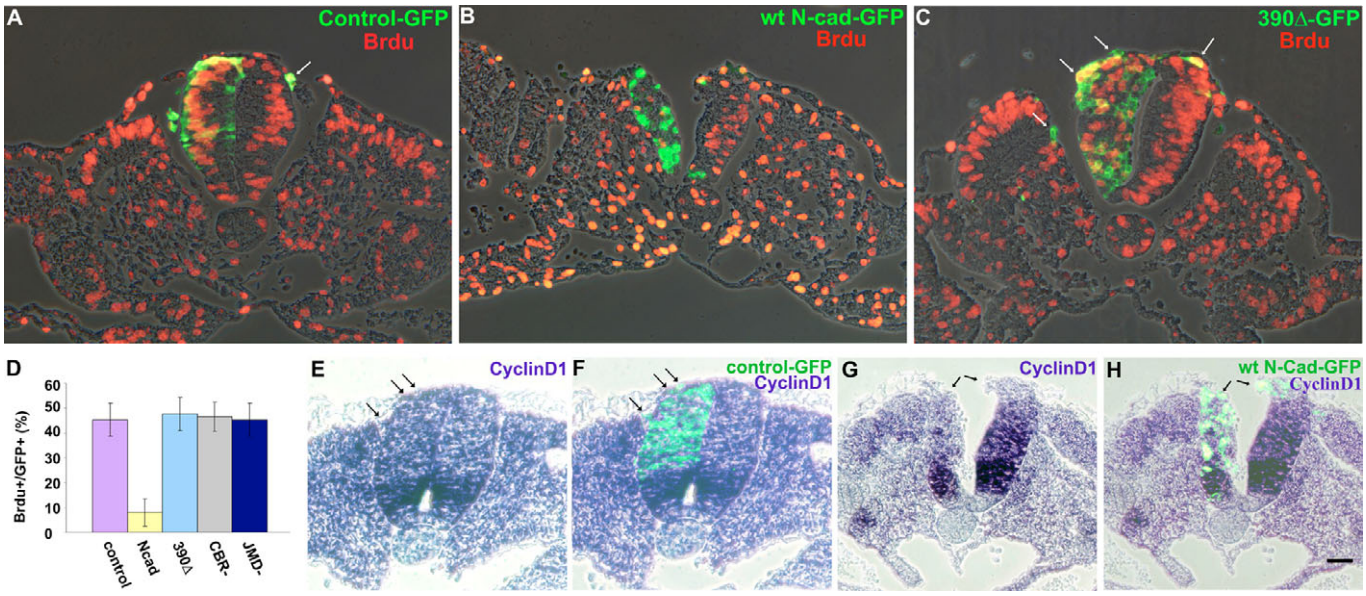


**Fig. 4. N-cadherin-mediated inhibition of NC delamination is associated with a downregulation of *cyclin D1* transcription and G1-S transition.** (**A-C**) Brdu incorporation (red nuclei) following electroporation (green) with control-GFP (A), wild-type N-cadherin (B) and cN390Δ-GFP (C). (**D**) Quantification of the percentage of Brdu+/GFP+ nuclei in the various treatments (data are the mean±s.d. of at least five embryos per treatment). Cells that received wild-type N-cadherin failed to incorporate Brdu+ and no GFP+ NC cells exited the treated side of the tube. By contrast, Brdu+ NC cells emigrate normally from control-GFP and cN390Δ-GFP-treated hemi-tubes (arrows in A,C). Note that Brdu+ nuclei are spread across the apico-basal thickness of the NT (C) contrary to their normal localization to the basal half of the epithelium (A). (**E-H**) *cyclin D1* mRNA (blue) is reduced in cells that received wild-type N-cadherin-GFP (G,H, green) but not in control-GFP-treated hemi-tubes (E,F) where delaminating NC cells express *cyclin D1* (arrows). Scale bar: 25 μm for A-C,E,F; 30 μm for G,H.

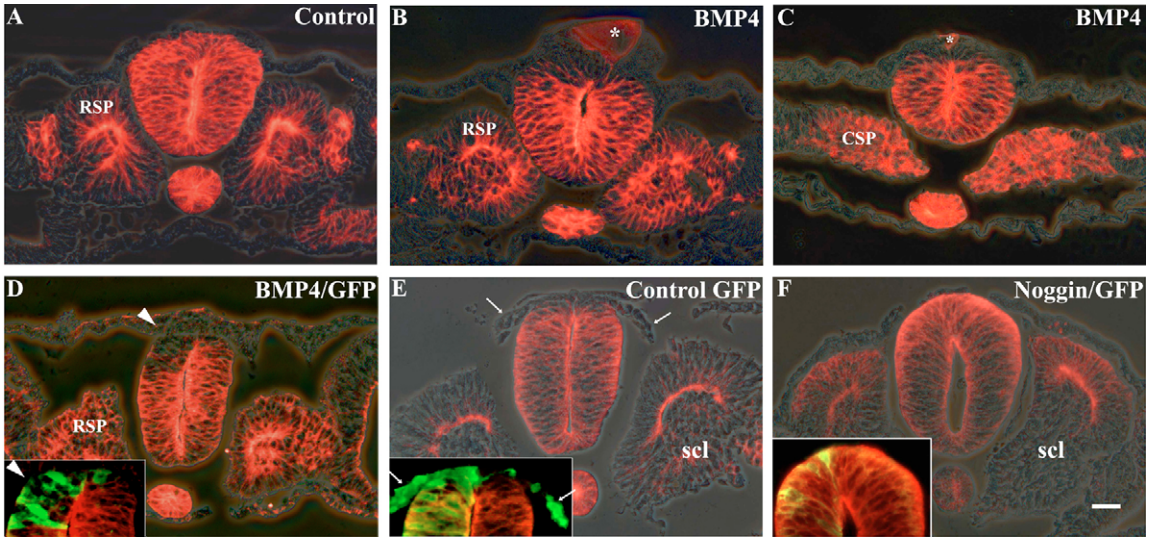


**Fig. 5. The normal downregulation of N-cadherin protein along the axis is regulated by BMP4.** (**A-D**) Treatment with BMP4-coated beads (* in B,C; *n*=7) or electroporation with BMP4-DNA/GFP (arrowhead in D; insert depicts localization of transfected BMP/GFP; *n*=8) for 8 hours downregulates N-cadherin immunoreactivity at levels of rostral and caudal segmental plates. At these levels, N-cadherin is still expressed in the dorsal NT under normal conditions (A) and in matching controls (not shown). (**E**,**F**) Sections through a dissociating somite level 15 hours after electroporation of control-GFP (E; *n*=8) and noggin-GFP (F; *n*=9). (E) Note the absence of N-cadherin in the dorsal NT from which N-cadherin-negative NC cells emigrate (arrows depict phase-bright NC in main image and GFP+ NC in insert). (F) By contrast, noggin overexpression (green cells in insert and surrounding area) maintains N-cadherin and no NC emigration is apparent. Similar results were obtained by staining with anti-N-cadherin antibodies directed to either the extracellular or intracellular domains of the protein; the data presented stem from the latter. Abbreviations: CSP, caudal segmental plate; RSP, rostral segmental plate; scl, sclerotome. Scale bar: 25 μm.

the somite (Fig. 5E), a phenotype accompanied by delamination of labeled NC cells (Fig. 5E, arrows in insert). By contrast, a similar overexpression of noggin-encoding DNA prevented the normal downregulation of N-cadherin and inhibited NC emigration (Fig. 5F). No change in N-cadherin mRNA levels could be detected under these conditions (not shown), consistent with the uniform pattern of N-cadherin mRNA apparent along the neuraxis under normal conditions. Thus, the normal loss of N-cadherin protein in the dorsal NT is initiated by BMP4, suggesting that BMP4 triggers NC delamination in a N-cadherin-dependent manner.

## BMP4 stimulates N-cadherin proteolytic cleavage and NC delamination via ADAM10

To further investigate the relationship between BMP and N-cadherin, we examined the hypothesis that BMP stimulates its proteolytic degradation. N-cadherin is cleaved extracellularly by ADAM10, generating a membrane-bound fragment termed CTF1; this metalloproteinase is the rate-limiting step of the reaction. CTF1 is further processed by a γ-secretase-like activity to yield a soluble N-cadherin cytoplasmic tail, CTF2 (Fortini, 2002; Marambaud et al., 2003; Reiss et al., 2005). Furthermore, ADAM10 has been shown to be expressed in the dorsal NT and in emigrating avian NC in vitro (Hall and Erickson, 2003), and also in the E9.5 mouse NT colocalized with N-cadherin (Reiss et al., 2005). We prevented ADAM10-dependent N-cadherin degradation by treating neural primordia with the selective inhibitor GI254023X (Hundhausen et al., 2003; Ludwig et al., 2005). Neural primordia containing premigratory NC were explanted onto fibronectin. Fifteen hours later, NC cells were present on the substratum of control cultures; the densely packed cells close to the NT still expressed N-cadherin, but cells with a mesenchymal morphology had already lost membrane-bound immunoreactivity as visualized with antibodies to extracellular or intracellular domains, suggesting a complete cleavage of the protein (Fig. 6A-C). Treatment with BMP4 caused an 11-fold increase in the number of emigrating mesenchymal cells with a complete loss of membrane-bound N-cadherin (Fig. 6D-F,M; $P<0.002$). Treatment with GI254023X alone inhibited emigration of NC cells by 67±12% compared with controls (Fig. 6G,M; $P<0.001$). The combination of GI254023X and BMP4 allowed an initial flattening of the dense epithelial sheet, but inhibited EMT of NC cells by 83±10% ($P<0.002$) and 98.0±1.3% ($P<0.001$) of control and BMP-treated values, respectively (Fig. 6J,M). We then verified that GI254023X prevented the cleavage of N-cadherin. Indeed, staining with antibodies to the extracellular and intracellular protein domains revealed retention of membrane-associated immunostaining in GI254023X-treated explants (Fig. 6H,I), and also in the combined GI254023X+BMP4-treated explants (Fig. 6K,L). Altogether, these results suggest that BMP downregulates N-cadherin (Fig. 5) by promoting its degradation via ADAM10, and that cleavage of N-cadherin by ADAM10 is necessary for NC EMT.

## The soluble β-catenin-binding cytoplasmic tail (CTF2) stimulates transcription of β-catenin and cyclin D1 and enhances NC delamination

The end product of N-cadherin cleavage is a soluble cytoplasmic tail termed CTF2 (Fig. 3A). Since this fragment binds to β-catenin, a key mediator of canonical Wnt signaling that is necessary for NC delamination (Burstyn-Cohen et al., 2004), its effect on the above process was examined. We electroporated CTF2-GFP into hemi-NTs and embryos were incubated for a further 16 hours. CTF2-GFP stimulated the delamination of NC cells by 1.9±0.2-fold as compared with controls; the emigrating NC cells were HNK-1+ and mostly Brdu+ (Fig. 7A,B), as previously documented. Notably, this effect was preceded and accompanied by a prominent upregulation

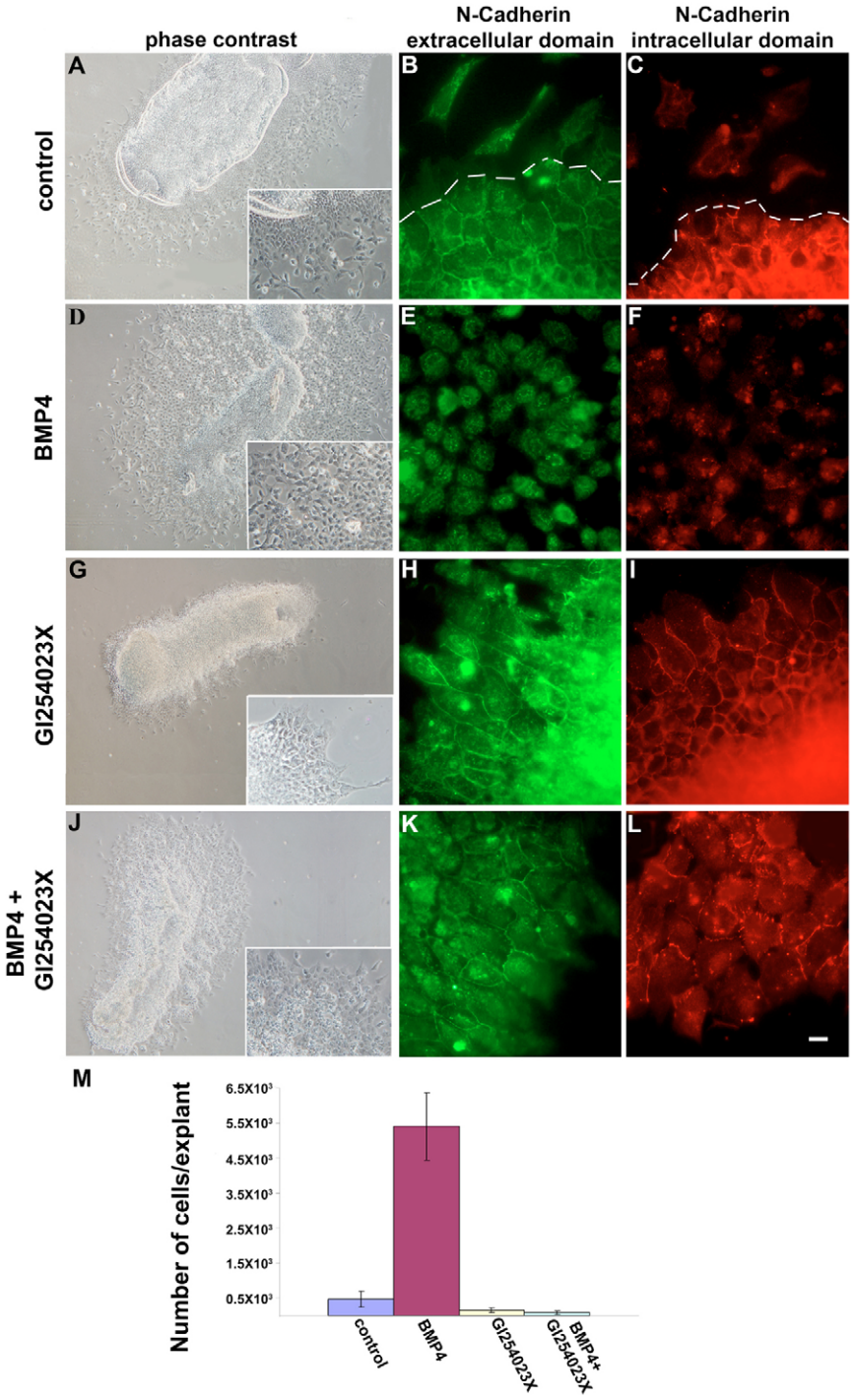


**Fig. 6. BMP4 stimulates N-cadherin cleavage and NC delamination via ADAM10.** Explants of neural primordia that received control medium, BMP4, GI254023X or BMP4+GI254023X. (**A**,**D**,**G**,**J**) Phase contrast. (**B**,**E**,**H**,**K**) GC4 anti-N-cadherin antibody that recognizes the extracellular domain. (**C**,**F**,**I**,**L**) N-cadherin antibody against intracellular region. (**M**) Quantification of the number of mesenchymal NC cells that exited from explanted NTs (mean±s.d. of 4-5 similar cultures per treatment). Note that whereas BMP4 dramatically enhanced NC EMT, GI254023X inhibited both basal and BMP-induced NC delamination and maintained full-length membrane-bound N-cadherin under both conditions ($n \geqslant 12$ explants per treatment stained with each antibody). The broken white line (B,C) delineates the border between the explant and emigrated cells. E and F exhibit only emigrated cells, whereas H,I,K and L exhibit only densely packed but non-mesenchymal cells. Scale bar: 100 μm in A,D,G,J; 10 μm in B,C,E,F,H,I,K,L.

of both *β-catenin* and *cyclin D1* transcription in the transfected hemi-tubes and in the emigrating NC cells (Fig. 7C,D). In addition, CTF2 was highly enriched in the nuclei of transfected progenitors, a phenotype clearly apparent in NC cells emigrating from explanted primordia (Fig. 7E,F and data not shown). These results indicate that CTF2 is likely to act through β-catenin at various levels, by enhancing β-catenin synthesis and perhaps also by translocating along with β-catenin into the nucleus to promote transcription of *cyclin D1* and the G1-S transition.

Next, we examined whether CTF2 is sufficient to rescue the inhibition of NC EMT caused by treatment with GI254023X. CTF2 was electroporated into hemi-NTs, which were then explanted onto fibronectin in the presence of GI254023X. Explants were fixed following overnight incubation and then stained with antibodies directed to the intracellular domain of N-cadherin; these also recognize transfected CTF2 that translocates into cell nuclei (Fig. 7E,F,I,J). In explants treated with GI254023X alone, NC cells failed to undergo EMT and exhibited membrane-associated N-cadherin immunoreactivity (Fig. 7G,H and Fig. 6G-I). Treatment with CTF2 alone or in combination with GI254023X revealed that in all explants examined, the CTF2-positive cells (arrows in Fig. 7I,J and data not shown) exited the neural tubes and were found on the substrate. Most importantly, CTF2-positive NC cells lost membrane-associated N-cadherin staining and appeared mesenchymal, whereas

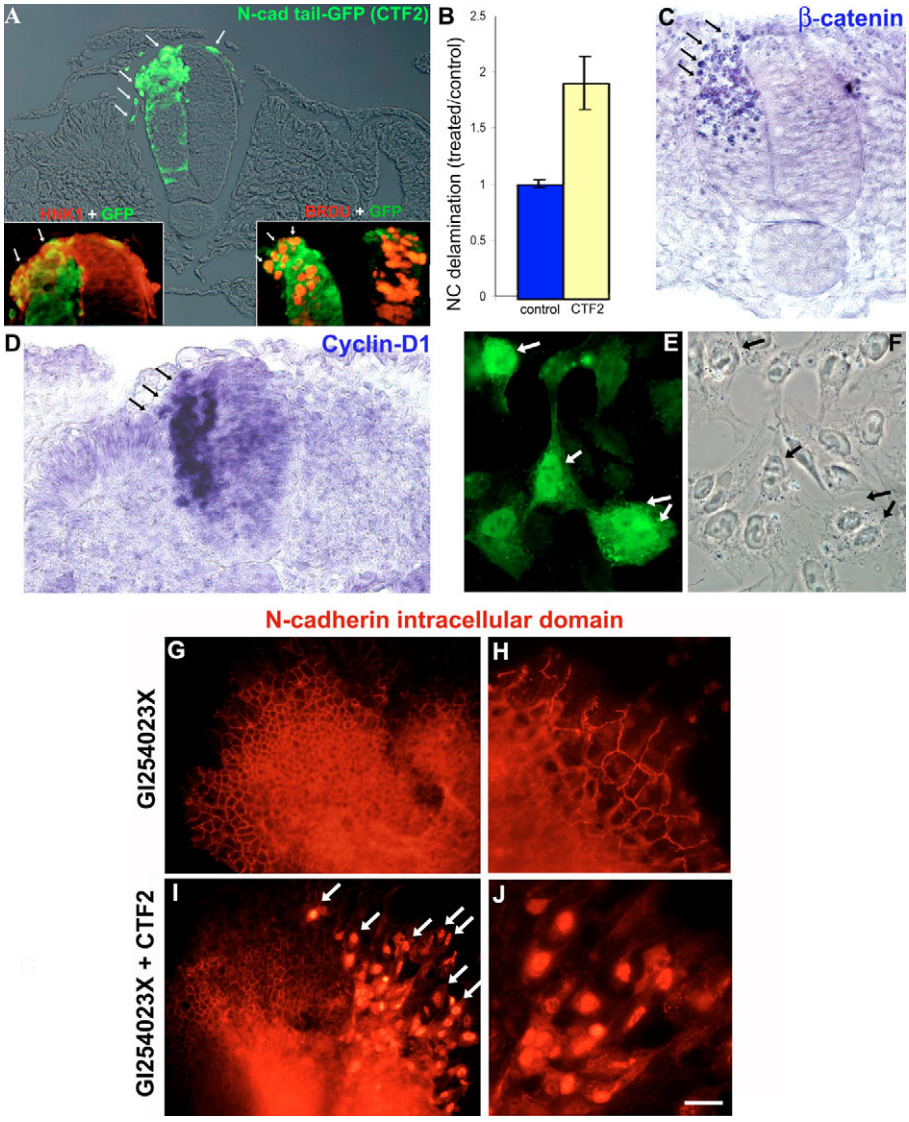


**Fig. 7. N-cadherin cytoplasmic tail (CTF2) stimulates *cyclin D1* transcription and NC emigration.** (**A**) Delamination of NC cells that received CTF2-GFP (arrows); CTF2-GFP+ delaminating cells co-express HNK-1 (arrows, left insert) and Brdu (arrows, right insert). (**B**) Quantification of NC delamination. Results represent the mean±s.d. of 5 embryos per treatment normalized to control values. (**C**,**D**) The transfected hemi-tubes and delaminating NC (arrows) reveal enhanced *β-catenin* and *cyclin D1* transcription (*n*=4 and 7, respectively); note the round appearance of the overexpressing cells. (**E**,**F**) Anti-GFP and phase-contrast images, respectively, showing that CTF2-GFP is enriched in nuclei of emigrated NC cells (arrows; *n*=8). (**G-J**) Electroporation of neural tubes with CTF2 followed by explantation in the presence of GI254023X. All explants were stained with antibodies directed to the intracellular domain of N-cadherin that also react with transfected CTF2 evident in cell nuclei. (G,H) Explants that received control-GFP and GI254023X (*n*=13). Note that cells retain membrane-bound N-cadherin immunoreactivity and are adhered to each other. (I,J) Explants treated with CTF2 and GI254023X (*n*=14). Note that cells expressing CTF2 in their nucleus (arrows in I and higher magnification in J) have emigrated from the tube, lack membrane immunostaining and are detached from each other. By contrast, untransfected cells in the same field still express membrane N-cadherin and are epithelial (I, compare left and right sides). Similar results were obtained upon transfection of CTF2 alone (data not shown; *n*=12). Scale bar: 35 μm in A (25 μm in left insert; 33 μm in right insert); 25 μm in C,D; 15 μm in E,F; 40 μm in G,I; 17 μm in H,J.

cells lacking nuclear CTF2 in the same cultures retained membrane-bound N-cadherin (Fig. 7I, compare left and right sides of explant). Hence, CTF2 overcomes the inhibition exerted by GI254023X, suggesting that it plays a physiological role in NC EMT. Altogether, our data suggest that whereas full-length N-cadherin prevents NC EMT by inhibiting *cyclin D1* transcription and G1-S transition (Fig. 4), BMP-dependent N-cadherin cleavage generates a β-catenin-binding fragment that stimulates these processes.

## DISCUSSION

We have shown that N-cadherin maintains premigratory NC progenitors in an epithelial state by inhibiting Wnt-dependent β-catenin signaling rather than by solely maintaining intercellular adhesiveness. In addition, we show that N-cadherin is cleaved by BMP4 in an ADAM10-dependent manner, and that BMP-mediated delamination depends on the proteolytic degradation of N-cadherin, hence implicating N-cadherin as a crucial component of the BMP-dependent molecular network previously characterized (Fig. 8). Finally, we demonstrate that CTF2, the soluble end product of N-cadherin cleavage, translocates into cell nuclei and promotes *cyclin D1* transcription followed by the generation of NC cell movement, events previously demonstrated to result from β-catenin-dependent signaling (Burstyn-Cohen et al., 2004). We therefore propose that BMP-dependent downregulation of N-cadherin in the dorsal NT opposite the level of developing somites serves not only to relieve intercellular adhesion and signaling inhibition, but also to enhance transcriptional activity, leading to the onset of NC emigration (Fig. 7G). Consequently, the process of NC emigration illustrates the complexity of N-cadherin regulation, as demonstrated here for the first time, involving opposite roles elicited by the full-length protein and by its soluble degradation product.

We report that in the dorsal NT, full-length N-cadherin inhibits transcription of β-catenin-dependent genes such as *cyclin D1* and subsequent G1-S transition and NC delamination. Similar effects were reported upon overexpression of β-catenin-engrailed, dominant-negative Lef1 or mutant dishevelled, which interfere with canonical Wnt activity (Burstyn-Cohen et al., 2004). Likewise, *cyclin D1* transcription in the dorsal NT is low at caudal axial levels where noggin and N-cadherin are active, and increases in association with the downregulation of the these two proteins (Burstyn-Cohen et al., 2004) (see also this study). Hence, our data suggest that N-

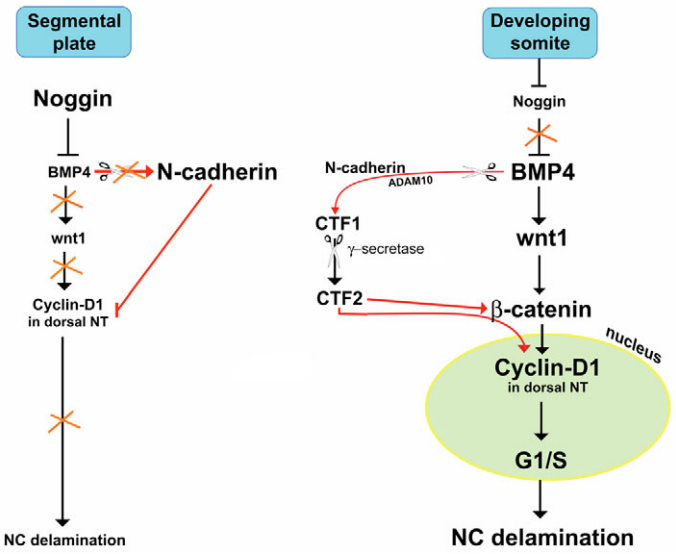


**Fig. 8. An updated model for NC delamination.** Opposite the segmental plate mesoderm, high levels of noggin result in low BMP activity, no Wnt1 transcription, low *cyclin D1* transcription in dorsal NT and no NC cells emigrating from the caudal NT. N-cadherin at this stage is expressed in the dorsal NT where it contributes to maintaining low *cyclin D1* transcription and lack of NC emigration. With ongoing development, opposite mature epithelial and dissociating somites, a factor emitted by the dorsomedial portion of the paraxial mesoderm inhibits *noggin* transcription in the dorsal NT, thereby releaving BMP activity. BMP4 in turn positively regulates *Wnt1* transcription. Wnt signaling, via the canonical pathway, positively modulates transcription of *cyclin D1*, G1-S transition and NC cell delamination. In parallel, BMP4, via ADAM10, promotes N-cadherin protein cleavage into CTF1. CTF1 is in turn cleaved by γ-secretase to generate soluble CTF2. CTF2 may act in at least two ways: by upregulating levels of *β-catenin* transcription and by binding β-catenin protein. We propose that the CTF2–β-catenin complex translocates into the cell nucleus where transcription of target genes such as *cyclin D1*, followed by G1-S transition and EMT of NC, are stimulated. Hence, BMP activity transforms N-cadherin from an inhibitory cue into a stimulatory signal. Altogether, these data suggest that NC emigration from the NT is the result of at least two separate yet converging pathways, both stimulated by BMP signaling in coordination with somite development.

cadherin-dependent inhibition of NC delamination results from the inhibition of β-catenin-dependent Wnt signaling (Fagotto et al., 1996; Funayama et al., 1995; Gottardi and Gumbiner, 2001; Heasman et al., 1994; Sanson et al., 1996; Wong and Gumbiner, 2003). This is consistent with increasing evidence that β-catenin is involved in signaling through cadherins, given that β-catenin has dual and mutually exclusive functions in cadherin-mediated adhesion at the plasma membrane and in transcriptional regulation as part of the canonical Wnt pathway (Nelson and Nusse, 2004). Increased cell proliferation has been observed upon loss of N-cadherin in several in vivo systems (Ganzler-Odenthal and Redies, 1998; Lele et al., 2002), confirming that endogenous N-cadherin negatively modulates the Wnt pathway.

Mutant analysis in vitro has suggested that the growth-inhibitory and signaling activities of cadherins are retained by constructs that have the β-catenin-binding domain (Gottardi et al., 2001; Sadot et al., 1998; Stockinger et al., 2001). By contrast, in the present in vivo context, we observed that only full-length N-cadherin inhibited the G1-S transition and *cyclin D1* mRNA transcription, whereas membrane-anchored mutants retaining only the ectodomain or the β-catenin-binding domain were ineffective. This suggests the need for functional coupling between adhesion and signaling motifs, a situation likely to reflect the complexity of cell-to-cell interactions that characterize the embryonic environment. Conversely, soluble CTF2 has a prominent positive effect on signaling. Hence, our functional assays suggest that the β-catenin-binding domain of N-cadherin has either a negative, positive or no effect on signaling followed by NC delamination, depending on the structural context of the molecule (as full-length N-cadherin, CTF2 or cN390Δ, respectively).

The precise mechanism by which CTF2 stimulates *cyclin D1* transcription and NC delamination remains to be investigated, yet it is likely to involve several levels of regulation through β-catenin. First, in our system, CTF2 activates β-catenin transcription, which is consistent with recent in vitro data that in addition documented reduced β-catenin degradation (Uemura et al., 2006). Increased cytoplasmic β-catenin could facilitate nuclear accumulation of the protein and activation of target genes. Indeed, elevated cytoplasmic and nuclear β-catenin were detected in vitro upon overexpression of CTF2 (Uemura et al., 2006). Second, because we detected CTF2 in the nucleus of emigrating NC, it is possible that in addition to stimulating β-catenin transcription, CTF2 complexes with β-catenin and the two undergo nuclear translocation followed by *cyclin D1* activation. FRET analysis of SH-SY5Y cells expressing an inducible form of CTF2 revealed an interaction between the two proteins (Uemura et al., 2006). Notably, cadherins and TCF/Lef bind to the same sequence of β-catenin, hence predicting that CTF2 would abrogate rather than stimulate signaling events. Yet, in vivo, this complex might have a more rapid turnover than that of the isolated component proteins (Huber et al., 2001), and the released β-catenin could then bind to TCF. Alternatively, nuclear CTF2/β-catenin could signal independently of TCF (Olson et al., 2006; Stadeli et al., 2006; Wong and Gumbiner, 2003; Xu et al., 2000). The situation is further complicated in our system because BMP activates, in parallel, both N-cadherin degradation to CTF2 and canonical Wnt signaling (Fig. 7G), and possible interactions between these pathways cannot be isolated from each other.

The cN390Δ-GFP and CBR-GFP mutants distorted the normal neuroepithelium, with misplaced S-phase nuclei observed across the apico-basal thickness of the tube. This was followed by a failure of normal peripheral gangliogenesis and disordered central neurogenesis (I.S. and C.K., unpublished). Consistent with these results, both cN390Δ-GFP and CBR-GFP have been shown to cause an initial loss of epithelial morphology in cells of the avian dermomyotome, yet they had opposite effects in driving cell segregation into dermal or myotomal domains, respectively (Cinnamon et al., 2006). These constructs also altered the morphology of epithelial cell lines (Fujimori and Takeichi, 1993), but, surprisingly, when misexpressed using an adenoviral approach, they had no effect on NT morphology (Nakagawa and Takeichi, 1998). These results suggest that adenovirus-mediated gene delivery was less effective at the time points analyzed as compared with electroporation. This assumption is further substantiated when considering the effect of adenoviral-mediated overexpression of wild-type N-cadherin, which mainly inhibited emigration of melanocyte progenitors (Nakagawa and Takeichi, 1998), which are the last NC cells to leave the NT, in contrast to the present study in which electroporation of wild-type N-cadherin prevented emigration of all potential NC cells and further gangliogenesis.

It is of interest to note that the dorsal NT continues expressing cadherin 6B, a type II cadherin (Liu and Jessell, 1998; Nakagawa and Takeichi, 1998; Sela-Donenfeld and Kalcheim, 1999) after N-cadherin is downregulated. However, these two cadherins share no structural or functional similarity (Nakagawa and Takeichi, 1995) and, moreover, they respond differently to BMP signaling. Whereas N-cadherin protein is downregulated by BMP-mediated degradation (Figs 5, 6), maintenance of cadherin 6B transcription is under positive regulation by BMP4 (Liu and Jessell, 1998; Sela-Donenfeld and Kalcheim, 1999). Since BMP triggers NC delamination it is possible that, in contrast to N-cadherin, cadherin 6B has a pro-delamination effect and/or serves to segregate the premigratory NC from the adjacent CNS progenitors, which continue expressing N-cadherin (Nakagawa and Takeichi, 1995). Together with the finding that cadherin 7 is expressed in migrating NC, these results suggest that cadherins are not a generic property of epithelial cells and that type II cadherins in particular may regulate morphogenetic movements.

An intriguing finding of our study is that BMP4 activity triggers NC delamination through an ADAM10-dependent-mechanism, as both the basal level of cell emigration and that enhanced by exogenous BMP4 were inhibited by GI254023X. This is expected because failure of ADAM10 activity, the rate-limiting enzyme in cadherin cleavage, preserves N-cadherin in its full-length conformation (Fig. 6); as such, N-cadherin actively antagonizes β-catenin-dependent signaling induced by BMP/Wnt and consequently prevents NC emigration (Figs 2, 4). This inhibition can be overcome upon treatment with CTF2, which on the one hand reduces cell adhesion and on the other hand promotes signaling, further stressing the antagonistic relationship between the two processes. Consistent with our data, ADAM10-deficient cells exhibited reduced transcriptional activity and lower expression levels of β-catenin-dependent genes such as those encoding cyclin D1, c-myc and c-jun (Reiss et al., 2005). Hence, we propose that BMP4 stimulates at least two distinct molecular pathways: (1) it triggers transcription of Wnt1 in the dorsal NT, which in turn acts via its canonical pathway; and (2) it promotes N-cadherin degradation, which releases the system from a pre-existing inhibition and at the same time produces CTF2, a β-catenin-binding product with a pro-delamination activity. As a result, BMP-dependent Wnt and CTF2 activities might converge to drive the onset of NC movement via a common β-catenin-dependent mechanism.

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