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Loss of *Xenopus* cadherin-11 leads to increased Wnt/β-catenin signaling and up-regulation of target genes c-myc and cyclin D1 in neural crest

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ABSTRACT

Xenopus cadherin-11 (Xcadherin-11) is an exceptional cadherin family member, which is predominantly expressed in cranial neural crest cells (NCCs). Apart from mediating cell–cell adhesion it promotes cranial NCC migration by initiating filopodia and lamellipodia formation. Here, we demonstrate an unexpected function of Xcadherin-11 in NCC specification by interfering with canonical Wnt/β-catenin signaling. Loss-of-function experiments, using a specific antisense morpholino oligonucleotide against Xcadherin-11, display a nuclear β-catenin localization in cranial NCCs and a broader expression domain of the proto-oncogene cyclin D1 which proceeds c-myc up-regulation. Additionally, we observe an enhanced NCC proliferation and an expansion of specific NCC genes like AP2 and Sox10. Thereby, we could allocate NCC proliferation and specification to different gene functions. To clarify which domain in Xcadherin-11 is required for early NCC development we tested different deletion mutants for their rescue ability in Xcadherin-11 morphants. We identified the cytoplasmic tail, specifically the β-catenin binding domain, to be necessary for proper NCC development. We propose that Xcadherin-11 is necessary for controlled NCC proliferation and early NCC specification in tuning the expression of the canonical Wnt/β-catenin target genes cyclin D1 and c-myc by regulating the concentration of the nuclear pool of β-catenin.

Introduction

Neural crest cells (NCCs) are a multipotent stem cell population in vertebrates giving rise to a variety of cell types including glia cells and neurons of the peripheral nervous system, cartilage and bone of the craniofacial structures and pigment cells of the skin (Hall and Hörstadius, 1988; le Douarin and Kalcheim, 1999). The induction of NCCs starts at early gastrula stages at the lateral edges of the neural plate by formation of defined morphogen levels like BMP, Wnt, retinoic acid (RA), FGF and Notch signaling by which NC specification is activated (Basch et al., 2004; Steventon et al., 2005). In particular, there is strong evidence that the canonical Wnt/β-catenin pathway plays a direct role in the induction of NCCs. In *Xenopus*, depletion of the Wnt receptor Frizelled3, over-expression of the Wnt antagonist GSK3β or the over-expression of a dominant-negative Wnt ligand inhibits NCC formation (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Deardorff et al., 2001). Furthermore, De Calisto et al. (2005) showed that a Disheveled mutant disrupting the canonical Wnt/β-catenin pathway led to a strong inhibition of NCC induction. Additionally, the Lef/TCF transcription factors, which are the nuclear mediators of the canonical Wnt/β-catenin pathway, bind and regulate the promotor of Slug, which is an early marker of NCCs (Vallin et al., 2001).

The proto-oncogene c-myc, a basic helix-loop-helix zipper (bHLHZ) transcription factor, is a downstream target of Wnt-signaling in early embryonic carcinoma cells and colorectal tumors (He et al., 1998; Willert et al., 2002). It plays a role in different cellular events, such as proliferation, differentiation, cell growth and apoptosis. During NCC induction c-myc is expressed at the neural plate border and is one of the earliest NCC specifiers. Loss of function experiments revealed that c-myc is required for the initial induction of neural crest precursors independent upon changes in cell proliferation or cell death (Bellmeyer et al., 2003). Cyclin D1 is another target of the canonical Wnt/β-catenin pathway and is over-expressed in many colon carcinomas (Shtutman et al., 1999; Tetsu and McCormick, 1999). It is one of the main regulators of the GI to S-phase transition during the proliferative stage of the cell cycle (Baldin et al., 1993; Sherr, 1996). In the *Xenopus* embryo, cyclin D1 expression starts at the onset of gastrulation. During neurulation and tailbud stages transcripts are mainly localized in anterior domains like the developing eye, brain, neural tube and

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migrating NCCs (Vernon and Philpott, 2003). Promotor analysis revealed that its neural plate specific expression depends on a putative Lef/TCF binding site (Tanaka et al., 2003).

β-catenin has a dual role within the cell. Besides its function as an essential effector of the canonical Wnt-signaling pathway, β-catenin is localized at cell–cell adhesion complexes connecting cadherins to the actin cytoskeleton (Ozawa et al., 1989). Cadherins represent a multigene family of Ca^{2+}-dependent glycoproteins mediating cell–cell adhesion processes. Apart from the establishment of an epithelial cell polarization and the formation of robust cell–cell contacts, the *zonula adherens*, cadherins are responsible for cell proliferation as well as for the transfer of signals for cell differentiation (Takeichi, 1995; Geiger and Ayalon, 1992; Wollner et al., 1992). Classical cadherins consist of three domains, an extracellular domain mediating cell–cell adhesion *via* a zipper like mechanism, a hydrophobic transmembrane domain and a highly conserved cytoplasmic tail. This cytoplasmic domain is linked to the cytoskeleton by β-catenin and α-catenin to generate force within the cell (Gumbiner, 2000; Jamora and Fuchs, 2002). The role of E-cadherin is intensively investigated. Besides its function in an adhesive complex, E-cadherin can sequester β-catenin away from the nucleus acting as a tumor suppressor (Gottardi et al., 2001; Stockinger et al., 2001). Furthermore, the loss of E-cadherin is correlated with the invasion of epithelial tumor cells (Cavallaro and Christofori, 2004). Recently it was shown, that downregulation of E-cadherin activates β-catenin/TCF dependent transcription, which subsequently leads to the expression of invasion promoting genes like c-myc (Wang et al., 2010).

The mesenchymal cadherin-11 is highly expressed in prostate and breast cancer cells, where it leads to an accelerated invasion (Bussemakers et al., 2000; Tomita et al., 2000; Pishvaian et al., 1999). Additionally, cadherin-11 is involved in triggering inflammatory arthritis, where cells covering the joint capsule colonize the bone (Chang et al., 2010). During *Xenopus* embryogenesis cadherin-11 (Xcadherin-11) is mainly expressed in the migrating NCCs (Borchers et al., 2001). Gain of function analysis revealed that Xcadherin-11 restrains NCC migration and influences twist expression, which can be rescued by co-injection of β-catenin, indicating that Wnt/β-catenin signaling is required to maintain twist expression (Borchers et al., 2001). By loss of function analysis we could show that Xcadherin-11 initiates filopodia and lamellipodia formation in *Xenopus* NCCs, acting upstream of the GTP exchange factor (GEF) Trio and the small GTPases RhoA, Rac1 and cdc42 (Kashef et al., 2009).

In this study we report a novel function of the cell–cell adhesion protein Xcadherin-11 in NCC specification by regulating a precise level of β-catenin within NCCs. Loss of Xcadherin-11 leads to the accumulation of β-catenin within the nucleus of NCCs activating the expression of the Wnt/β-catenin target genes cyclin D1 and c-myc. This leads to an enhanced cell proliferation that can be rescued by HUA treatment. The proliferation is followed by an expansion of NCC marker gene expression and reduced expression of neural marker genes. Since broadening of cyclin D1 expression precedes c-myc upregulation we confirm c-myc as neural crest specifier and identified cyclin D1 as regulator of neural crest proliferation.

Materials and methods

Constructs

Xcadherin-11 and deletion constructs as well as Gap43-GFP (mbGFP) were described previously (Borchers et al., 2001; Kashef et al., 2009). Xcadherin-11 morpholino antisense oligonucleotide (Xcad-11-MO) and β-catenin-MO were designed as previously characterized (Heasman et al., 2000, Kashef et al., 2009) and purchased from Gene Tools, LLC.

Embryo micromanipulation, lineage tracing and in situ hybridization

Xenopus laevis embryos were obtained by *in vitro* fertilization and staged according to Nieuwkoop and Faber (1967). RNA for injection experiments was synthesized *in vitro* using mMessage mMachine Kit (Ambion). To identify the injected side dextran-FITC (4 pg; Molecular Probes) or membrane bound GFP (mbGFP, 200 pg mRNA) were used as lineage tracer.

In situ hybridization (ISH) was described earlier (Harland, 1991). Digoxigenin-labeled antisense RNA was generated according to manufacturer's (Roche) description using template cDNA encoding c-myc (Bellmeyer et al., 2003), cyclin D1 (Vernon and Philpott, 2003), sox2 (Mizuseki et al., 1998), N-CAM (Kintner and Melton, 1987), AP-2 (Luo et al., 2003), sox10 (Aoki et al., 2003), krox-20 (Bradley et al., 1993) and en2 (Landesman and Sokol, 1997). Results of at least three independent experiments were averaged, and statistical significance was calculated by using Student's *t*-test.

Hydroxyurea and aphidicolin treatment was performed as described elsewhere (Saint-Jeannet et al., 1997, Aybar et al., 2003). Embryos were incubated in HUA solution between stage 12 and 19.

Quantitative real-time-PCR (qRT-PCR)

For qRT-PCR embryos were injected in the animal dorsal blastomeres bilaterally in eight-cell-stage embryos. RNA was extracted from groups of five embryos of the recommended stage by using the High Pure RNA Isolation Kit (Roche). 150 ng of total RNA was reverse transcribed (M-MLV, Promega). cDNA was subjected to 40 cycles of PCR at an annealing temperature of 57 °C or 60 °C with primers as described (Suppl. Table 1). Expression levels were calculated relative to *ornithine decarboxylase* (*ODC*) and were normalized to uninjected controls. Results of at least three independent experiments were averaged, and statistical significance was calculated by using Student's *t*-test.

Immunohistochemistry

Embryos were injected with 16 ng Xcad-11-MO into the animal hemisphere of one dorsal blastomere at eight-cell-stage unless described differently. Proliferation was estimated by phosphohistone H3 detection, as described earlier (Kunz et al., 2004). For β-catenin staining embryos were fixed in MEMFA. Cryosections of embryos were described earlier (Fagotto and Brown, 2008). Transverse sections of 12 µm were cut with a cryotome (CM 1900, Leica). The sections were washed three times with 1 × APBS and pre-incubated in blocking solution (1 × APBS, 10% FCS, 1% blocking reagent (BMB, Roche)) for 1 h (room temperature). The undiluted primary antibody was applied to the sections over night at 4 °C. Following three washing steps in 1 × APBS, sections were treated with secondary antibody (goat-anti-rat)-Cy3 (Dianova), 1:200 diluted in blocking solution for 1 h at 37 °C. Sections were washed twice and treated for 5 min with DAPI (0.4 µg/ml in 1 × APBS) to visualize the nuclei. Sections were mounted in mowiol. Images were taken with an Axio Observer.Z1 Spinning Disc confocal microscope (Carl Zeiss AG) using 63 × LCI-Neofluar-Imm-Corr-DIC objective (na 1.3) and 5 × Plan-Apochromat (na 0.16) and analyzed with the AxioVision software (4.7.2).

Statistical analysis of pH3 staining

ISH staining of c-myc or AP2 was performed to identify NCC tissue. Afterwards, embryos were cryosectioned and immunostained for phospho-histone H3. The area of NCC tissue was identified by the blue color from ISH staining. This area was selected as region of interest (ROI) and its volume was calculated by ImageJ software (version 1.46a). The number of pH3 positive nuclei was counted within ROI. To countervail biased measurements due to skewed section planes, the results of all sections of one embryo were accumulated that it resembled the whole mount measurements. Results of at least three independent experiments were averaged, and statistical significance was calculated by using Student's *t*-test.

Keller open-face explants assay

Embryos were injected with Xcad-11-MO (16 ng) alone or together with Xcad11Δc or Xcad11Δe in both dorsal blastomeres at four-cell stage. For analysis of convergent extension movements Keller open-face explants were prepared at stage 10.5 and cultured, imaged and scored as described previously (Keller, 1991; Unterseher et al., 2004). The results of at least three independent experiments were averaged, and statistical significance was calculated using Student's *t*-test.

Luciferase assay

Embryos were injected in one dorsal animal blastomere of eight-cell-stage with mbGFP for tracing and luciferase reporter constructs (cyclin D1 promoter (Tetsu and McCormick, 1999), siamois promoter (Brannon et al., 1997)) with or without Xcad-11-MO. As positive control constitutive active β-catenin (Aberle et al., 1997) was injected. At neurula stages NCCs were explanted. 20 NCC explants were collected and lysed in 25 μl 50 mM Tris–HCl (pH 7.5). The lysat was centrifuged and the supernatant was supplemented with additional 25 μl Tris–HCl and 210 μl reporter lysis puffer (luciferase assay kit, Promega). Samples were freeze-thawed in liquid nitrogen, centrifuged and resulting supernatant was analyzed for luciferase activity (20 μl sample with 100 μl luciferin reagent, 30 s incubation, 10 s measurement). Samples were normalized against protein concentration as determined by standard BCA assay. Results of at least three independent experiments were averaged and statistical significance was calculated by using Student's *t*-test.

Results

Depletion of Xcadherin-11 induces c-myc and cyclin D1 expression

In a previous study we observed an increase in cell proliferation and an increase of neural crest cells (NCCs) after depletion of Xcadherin-11 by injection of an antisense morpholino nucleotide (MO) (Kashef et al., 2009). In order to investigate this observation more closely we asked whether cyclin D1 and c-myc, two regulators of cell proliferation known to be expressed in NCCs (Bellmeyer et al., 2003, Vernon and Philpott, 2003), are increased in Xcadherin-11 morphants. Indeed, whole-mount ISH of embryos injected with 8 ng Xcad-11-MO in one blastomere of eight-cell-stage revealed an expansion of cyclin D1 expression (Fig. 1A,B). 58% (±8.9, $n=38$) of the embryos at stage 16 and 61% (±1.6, $n=33$) at stage 19 showed a broader and partially posteriorly shifted cyclin D1 expression domain (Fig. 1A,B). The expression of c-myc was slightly altered in a different manner. At stage 16 we observed in 35% (±1.9, $n=46$) of the embryos a more diffuse and broader expression of c-myc while 41% (±5.1) of the embryos displayed no change in the expression area (Fig. 1A,B). Interestingly, at stage 19 the expression of c-myc was dramatically increased on the injected side in the majority of the embryos (61%, ±4.7, $n=63$). To get more quantitative data about the expression levels of cyclin D1 and c-myc after Xcadherin-11 depletion we performed qRT-PCR with whole embryos that have been injected with Xcad-11-MO in two animal dorsal blastomeres of an eight-cell-stage embryo. Consistent with the ISH data (Fig. 1A), expression of cyclin D1 was clearly up-regulated at developmental stage 19 and also at stage 25 (128%; $p=0.0001$ and 195%, $p=0.06$; Fig. 1C) while at stage 16 the expression was unchanged compared to uninjected embryos indicating that the broadening of the expression observed in the ISH does not represent an increased expression in this neurula stage. This might be attributed to the differences in the methods; in qRT-PCR experiments the spatial resolution is ignored. For c-myc the qPCR data resembled the ISH data (Fig. 1A,B). Again, an increase in expression could be observed in stage 19 and 25 embryos (142%, $p=0.03$ and 177%, $p=0.1$) while at stage 16 the expression of the majority of the embryos was unaltered. However, both, ISH and qRT-PCR revealed that the expression of the NCC specific regulators of cell proliferation, c-myc and cyclin D1, is increased from stage 19 in NCCs lacking Xcadherin-11.

Loss of Xcadherin-11 increases cell proliferation

In principle, the observed expansion in cyclin D1 and c-myc expression might be resulting from changes in cell fate decisions leading to more NC specification. Alternatively, since cyclin D1 and c-myc are well characterized regulators of cell proliferation this mechanism might result in increased proliferation in NC area as indicated earlier by increased pH3 staining in Xcad-11 depleted embryos (Kashef et al., 2009). As seen by staining of mitotic nuclei in NC positive tissue the size of neural crest area and number of pH3 positive cell nuclei increased simultaneously (Suppl. Fig. 1), meaning the relative number of mitotic cell nuclei is unaltered. This indicates that both possibilities, involvement of cell fate changes or proliferation, can lead to an expansion of NC tissue.

To further analyze the role of proliferation on neural crest development by Xcad-11 depletion we repeated the Xcadherin-11 knockdown and incubated the embryos in the presence of the inhibitors of proliferation hydroxyurea and aphidicolin (HUA) (Saint-Jeannet et al., 1997; Aybar et al., 2003). In case of proliferation being responsible for the observed phenotype, treatment of Xcadherin-11 depleted embryos with HUA should rescue the expansion of c-myc and cyclin D1 staining in stage 19. Indeed, we observed a significant reduction in the number of embryos showing a broader expression domain of c-myc from 62% (±5.2, $n=49$, Fig. 2A,B) when Xcadherin-11 depleted embryos were treated only with DMSO to 31% (±3.7, $p=0.0008$, $n=84$; Fig. 2A,B) when treated with HUA. The number of embryos with broader c-myc expression in DMSO treated embryos is in a similar range than in not-DMSO treated injected embryos (61%, see Fig. 1). Similarly, the number of embryos with an expanded expression domain of cyclin D1 was reduced from 49% (±3.3; $n=63$) to 27% (±5.3; $p=0.02$; $n=75$). To confirm the functionality of HUA treatment in Cad-11 Mo injected embryos we analyzed pH3 staining after DMSO or HUA incubation. While treatment with DMSO did not interfere with proliferation and resulted in a high number of pH3 positive nuclei in the entire embryo treatment with HUA led to a nearly complete loss of cell proliferation (Suppl. Fig. 1D). This indicates that Xcadherin-11 depletion results in an increase of cell proliferation in NCC tissue.

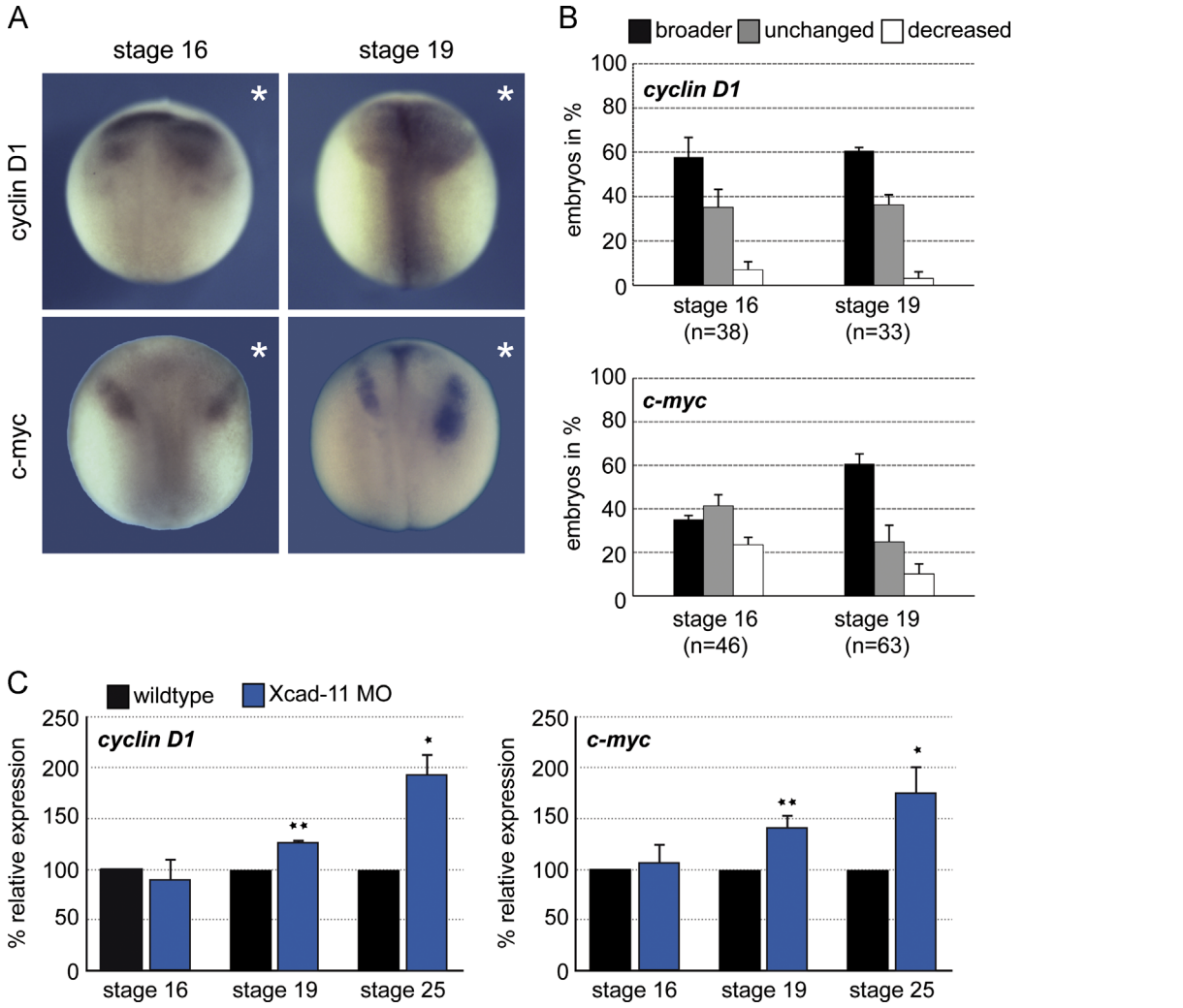

Fig. 1. Depletion of Xcadherin-11 increases c-myc and cyclin D1. (A) Whole mount ISH results of Xcad-11-MO (8 ng) injected (*) in one dorsal animal blastomere of eight-cell-stage embryos analyzed at stage 16 and 19 for cyclin D1 and c-myc expression (dorsal view, anterior to top). Expression area of cyclin D1 is increased in both stages, whereas c-myc expression seems to be first unaffected at stage 16 and subsequently broadend at stage 19. (B) Statistical analyses of whole mount ISH results for cyclin D1 and c-myc. (C) qRT-PCR comparing cyclin D1 and c-myc expression between uninjected and Xcad-11-MO injected embryos at stage 16, 19 and 25. The expression of cyclin D1 and c-myc is increased in stage 19 and even more in stage 25. n=number of embryos ($^{*}p \leq 0.1$; $^{**}p \leq 0.05$).

Loss of Xcadherin-11 induces NCC specification

C-myc is one of the earliest NCC specifiers and is required for the initial induction of NCC precursors (Bellmeyer et al., 2003). Thus, we asked whether the increase of c-myc expression after Xcadherin-11 depletion lead to an altered expression of other NCC marker genes which have been described to be regulated by c-myc. Using qRT-PCR we compared the expression of AP2 and sox10 between wildtype and Xcadherin-11 morphants through different developmental stages (stage 16, 19 and 25) (Fig. 3C). At stage 16 the expression levels of AP2 were significantly increased (160%, p=0.02). At stage 19 we monitored still a slight increase in AP2 expression (122%, p=0.068), while at stage 25 the expression was not altered in morphants in comparison to wildtype embryos. In contrast, the expression of sox10, a temporally later expressed NCC marker than AP2, was reduced in stage 16 and significantly decreased in Xcadherin-11 depleted embryos at stage 19 (42%, p=0.03). At tail bud stage we detected a distinct increase of sox10 transcripts in Xcad-11-MO injected embryos (139%, p=0.16). We further confirmed these findings by ISH (Fig. 3A,B). Xcadherin-11 morphants showed a broadening of AP2 expression in stage 16 (52%, ±7.8, n=53) and 19 (46%, ±3.7, n=79) while at stage 25 the expression domain was unaltered or slightly increased (44%, ±9.1, n=53). In comparison, sox10 expression was first decreased at stage 16 and 19 (60%, ±5.0, n=32 and 70%, ±5.7, n=34) and later increased (42%, ±4.0, n=30) at the Xcad-11-MO injected side (Fig. 3B). Taken together, these results indicate a subsequent activation of NCC marker genes following c-myc activation.

Xcadherin-11 is additionally expressed during gastrulation (Hadeball et al., 1998). Therefore, the depletion of Xcadherin-11 could have an influence on early NCC induction steps due to disturbed gastrulation movements that cause a shift of inductive fields, which are important for proper NCC induction. To examine this possibility we injected Xcad-11-MO into both dorsal blastomeres at four-cell-stage and analyzed the blastopore closure during gastrulation from stage 11 to stage 12 (Suppl. Fig. S2A). Here, we observed a delay of blastopore closure in Xcadherin-11 morphants. To analyze more intensively gastrulation movements we performed Keller open-face explants (Keller, 1991). Interestingly, the depletion of Xcadherin-11 had no influence on constriction but on the elongation of Keller open-face explants. 19% (±1, n=75) of Xcad-11-MO injected explants exhibited a reduced elongation (Suppl. Fig. S2B,C). To determine whether the adhesive or the signaling function of Xcadherin-11 is necessary for proper gastrulation movements we performed rescue experiments by co-injections of Xcadherin-11 lacking the extracellular (Xcad-11Δe, also see Fig. 4A) and

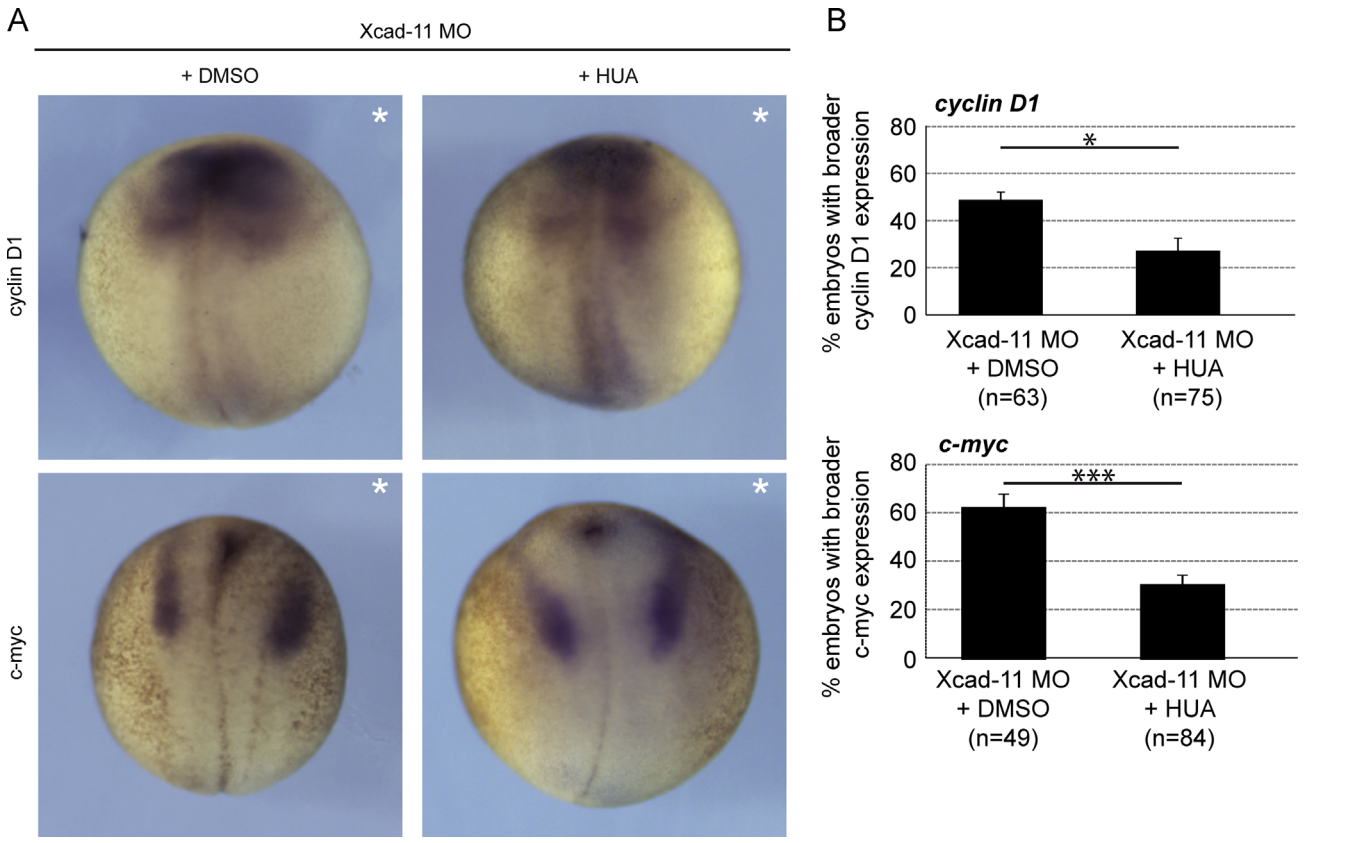

Fig. 2. Expanded c-myc and cyclin D1 expression domains result from increased proliferation. *Xenopus* embryos were injected with Xcad-11-MO in one dorsal animal blastomere of eight-cell-stage embryo and incubated in DMSO only or in HUA/DMSO from stage 12–19. (A) Depletion of Xcadherin-11 results in expanded expression domains of cyclin D1 and c-myc. (B) Statistical analysis of cyclin D1 and c-myc expression in Xcad-11 depleted embros tretated with DMSO or DMSO/HUA. Treatment with HUA was able to rescue the expansion significantly. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$) Injected side is marked with asterisk (*).

Xcadherin-11 lacking the cytoplasmic domain (Xcad-11Δc) together with Xcad-11-MO. Interestingly, only Xcad-11Δc could rescue the elongation phenotype demonstrating the prominent adhesive function of the extracellular domain of Xcadherin-11 during convergent extension movements. Since we observed only a weak gastrulation phenotype and additionally injected in all experiments at eight-cell stage to target specifically NCCs we can propose that the observed effects of Xcadherin-11 depletion on NCC formation were not due to changes in early NCC induction processes by cell signaling events from the dorsolateral mesoderm during gastrulation. Instead, knockdown of Xcadherin-11 led to changes in cell specification during NCC induction by affecting c-myc expression. Furthermore, the restricted gastrulation movements *per se* were not affecting NCC induction.

The expansion of NCC tissue in response to Xcadherin-11 knockdown raised the question whether other cell fates are altered as well. Therefore, we analyzed the expression of neural markers that are closely related to NCC development. The expression of the panneurally expressed sox2 resulted in a diffuse broadening of the expression domain in stage 16, 19 and 25 (Suppl. Fig. S3A,B). This effect became most obvious in stage 16 (87%, ±6.7, $n=50$). The intensity seemed to be decreased and we could confirm this by qRT-PCR analysis (Suppl. Fig. S3C,D). The expression of sox2 and another neural marker, N-CAM, is reduced significantly (67%, $p=0.02$ and 63%, $p=0.02$) in Xcadherin-11 depleted embryos at stage 19. To see whether brain patterning is disturbed resulting in altered NCC induction areas we also analyzed krox-20 (rhomomere 3 and 5) and en2 (marking the mid-hindbrain boundary) expression by *in situ* hybridization. Both markers showed a strong decrease in expression in stage 16 and krox-20 remained diminished until stage 25 underlying the further observed decrease of neural tissue (Suppl. Fig. S3A,B).

The cytoplasmic domain of Xcadherin-11 mediates proper NCC specification

The surprising increase of cyclin D1 and c-myc in NCC tissue prompted us to investigate the underlying molecular mechanism. We asked whether the cell-cell adhesion function or the signaling function of Xcadherin-11 is responsible to trigger proliferation. Therefore we took advantage of different deletion mutants (Fig. 4A) and co-injected them together with the Xcad-11-MO. These reconstitution experiments clearly demonstrated that the cytoplasmic tail but not the extracellular domain could substitute for the loss of full-length cadherin (Fig. 4). C-myc expansion by Xcad-11-MO (61% of the embryos, ±4.7, $n=63$ was restored by full length Xcadherin-11 (26%, ±6.5; $n=66$, $p=0.004$) and Xcad-11Δe (31%, ±7.3; $n=60$, $p=0.017$) but not by Xcad-11Δc (47%, ±5.7, $n=56$; Fig. 4A–C). Consistently, proliferation in the NCC area as measured by area of AP2 or c-myc stained tissue sections and by pH3 staining in Xcadherin-11 morphants was reduced by co-injected Xcad-11Δe from 2fold (±0.2, $n=22$) and 1.8fold (±0.27), respectively to 1.2fold (±0.45, $n=13$; $p=0.0059$) and 1fold (±0.41, $p=0.045$), respectively but not by Xcad-11Δc (2.1fold, ±0.37, $n=24$; and 1.5fold, ±0.21; Fig. 4D,E). Thus, signaling by the cytoplasmic tail, but not adhesion via the extracellular cadherin domains is essential for regulation of proliferation in the NCC area. A candidate region within the cytoplasmic tail, which could mediate such a regulation, is the binding domain of the Wnt effector protein β-catenin. If this region, indeed, is essential, depletion of the β-catenin binding site in the Xcad-11Δe construct should result in a mutant unable to restore normal NCC marker expression in Xcadherin-11 morphants. This is exactly what we observed (Fig. 5). 68% (±13.5, $n=63$) of the Xcadherin-11 morphants showed a broader c-myc expression domain, even after

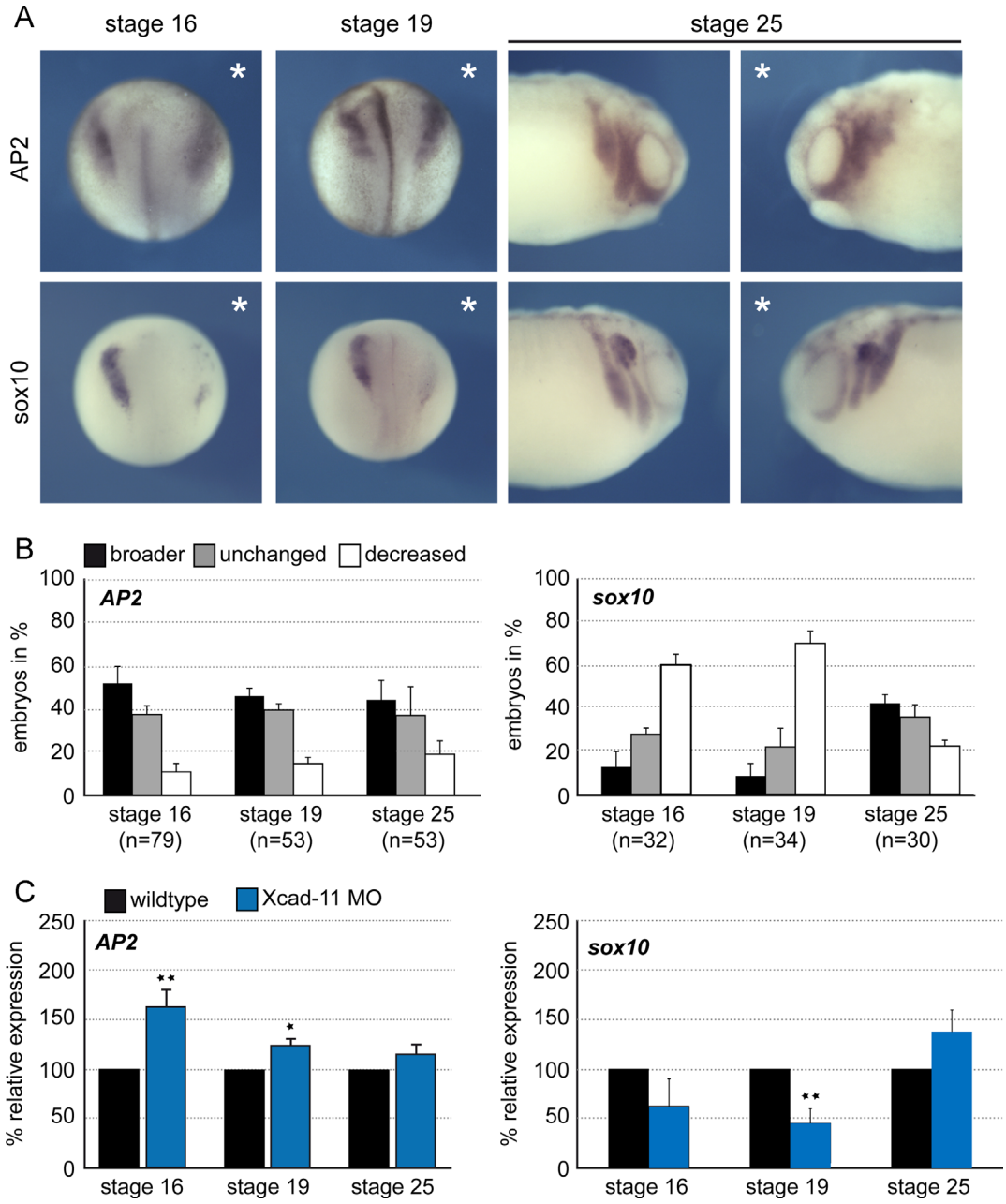

Fig. 3. Xcadherin-11 knockdown increases cranial NCC marker gene expression. (A) Whole mount ISH. Embryos were injected (*) with Xcad-11-MO (8 ng) in one dorsal animal blastomere of eight-cell-stage and analyzed at stage 16 and 19 (dorsal view, anterior to top) as well as stage 25 (lateral view) for AP2 and sox10 expression. At all stages investigated Xcadherin-11 morphants showed an increased AP2 expression. In comparison, almost all embryos revealed a reduced sox10 expression at stage 16 and 19 whereas at stage 25 most of the Xcad-11-MO injected embryos exhibited a broader expression domain of sox10. (B) Statistical analyses of whole mount ISH results for AP2 and sox10. (C) qRT-PCR analysis of AP2 and sox10 expression in stage 16, 19 and 25. We observed an increased expression of AP2 in stage 16 and 19 while sox10 was reduced in stage 19 and became increased in the later stage. n=number of embryos ($^{*}p \leq 0.1$; $^{**}p \leq 0.05$).

co-injection of the β-catenin binding mutant (ΔeΔβcat, Figs. 4A,5A,B). The results could be confirmed by a broader staining of AP2 in 53% (±5.6, n=63) and of c-myc in 67% (±6.8, n=63) of Xcad-11-MO injected embryos co-injected with Xcad-11ΔeΔβcat (Fig. 5A,B). Therefore, we hypothesized that β-catenin is released from the membrane in Xcadherin-11 morphants and may signal as transcriptional co-activator.

Xcadherin-11 reduction results in activation of canonical Wnt-pathway

To prove this hypothesis, we analyzed the subcellular localization of β-catenin after Xcadherin-11 depletion (Fig. 6). To target the NCCs *Xenopus* embryos were injected with Xcad-11-MO and mbGFP at eight-cell-stage into one animal dorsal blastomere (Fig. 6A,C). At stage 19 embryos were fixed, transversally sectioned and analyzed for β-catenin staining (Fig. 6D–F). On the uninjected side β-catenin is prominently localized at cell-cell contacts (Fig. 6E). Besides its localization at cell–cell contacts, we could find β-catenin on the Xcad-11-MO injected side additionally accumulated within several nuclei (Fig. 6F, arrows). A statistical analysis (Suppl. Fig. 4) of several sections for the ratio between β-catenin positive nuclei and total cell nuclei revealed a significant increase in nuclear localization of β-catenin on the injected side of Xcadherin-11 depleted embryos (35%, ±3.9, n=142 DAPI-positve nuclei) compared to the uninjected side (15%, ±0.6, n=131 DAPI-positve nuclei, p=0.007). These results displayed that after Xcadherin-11 depletion β-catenin gets translocated within the

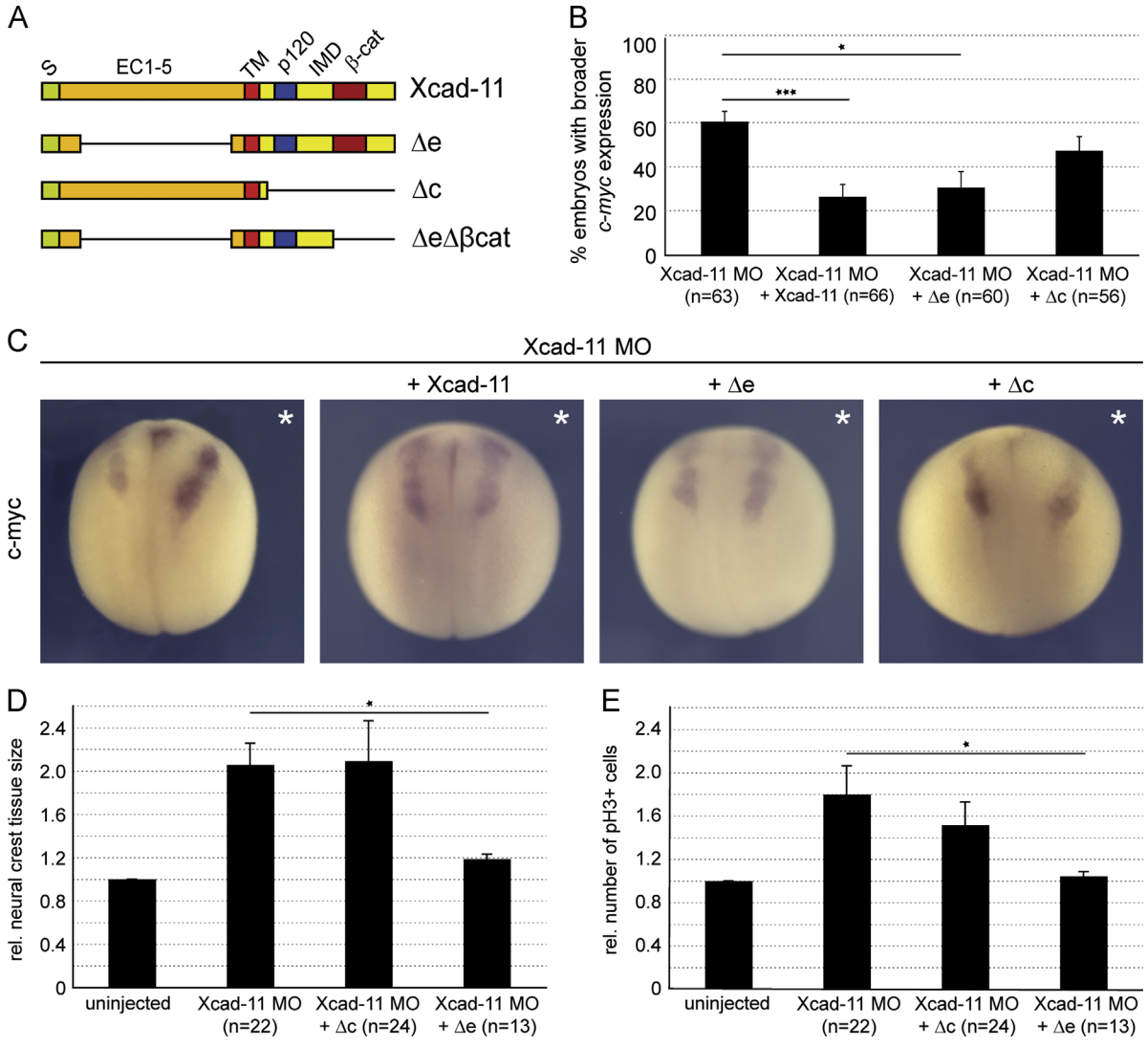

Fig. 4. The cytoplasmic domain of Xcadherin-11 mediates proper c-myc expression and proliferation. (A) Scheme of the Xcadherin-11 mutants used for rescue experiments. (B) Statistical analysis of whole mount ISH results for c-myc. Full length Xcad-11 and Xcad-11Δe, but not Xcad-11Δc co-injection was able to rescue c-myc expression. (C) Whole mount ISH. Embryos were injected with Xcad-11-MO (8 ng) alone or together with 75 pg full length Xcad-11, 75 pg Xcad-11Δc or 50 pg Xcad-11Δe, respectively, and analyzed for c-myc expression at stage 19. Dorsal view, anterior to top, asterisk marks the injected side. (D) Statistical analyses of whole mount ISH results. The area of c-myc stained tissue was compared in horizontally sliced embryos. The area of NCC positive tissue is increased by Xcad-11-MO, and this effect cannot be reverted by co-injection of Xcad-11 Δc. On the other hand co-injection of Xcad-11 Δe resulted in an area of a comparable size (1.2fold increase) as in uninjected tissue (set as 1). (E) Statistical analysis of pH3 positive nuclei at stage 19 in NCC positive tissue. PH3 positive nuclei on the uninjected side were set as 1. The increase of pH3 positive nuclei after Xcadherin-11 depletion could be significantly rescued after Xcad-11Δe co-injection. Only 1.05fold of pH3 positive nuclei could be detected by immunohistochemistry. Co-injection of Xcad-11Δc was not able to restore the increase of pH3 positive nuclei and resulted in an increase in mitotic nuclei by 1.52fold. n=number of sections ($^*p \leq 0.05$).

nucleus and is possibly activating target gene expression as transcriptional co-activator.

If the observed translocation of β-catenin to the nucleus is responsible for the observed effects on cell proliferation and marker gene expression the activation of the canonical Wnt-signaling pathway should be one result of Xcad-11-MO injection. This influence of increased nuclear β-catenin on canonical Wnt-signaling was tested by activation of Wnt dependent reporter genes. We injected Xcad-11-MO or constitutively active β-catenin together with mbGFP as lineage tracer and a siamois or a cyclin D1 promoter fused to luciferase as reporter into one animal dorsal blastomere of an eight-cell-stage embryo (Fig. 7A). At neurula stage the NCCs were explanted and analyzed by luciferase assay. The siamois promoter was activated 6.8fold ($\pm$1.51; p=0.0006, n=4) by Xcad-11-MO in a similar manner as by constitutively active β-catenin (6.4fold, $\pm$1.12; p=0.0032, n=3; Fig. 7B). The cyclin D1 promoter could be activated even 20.5 fold by Xcad-11-MO ($\pm$11.3, p=0.0466, n=5) while β-catenin activated 4.6fold ($\pm$1.5; p=0.0146, n=6; Fig. 7C).

To prove the conclusion drawn by the earlier experiments that the activation of canonical Wnt-signaling pathway is responsible for the observed phenotype we performed the experiments in β-catenin-MO background. We injected Xcad-11-MO alone or together with β-catenin-MO and compared the expression of NCC markers c-myc and AP2. As expected, the blocking of the Wnt-pathway prevented the increased expression domain of AP2 and c-myc by injection of Xcad-11-MO (Fig. 7D F). Compared with Xcadherin-11 morphants without β-catenin-MO the number of embryos with broader expression of the NCC markers was significantly reduced. As expected and previously shown (Hong et al., 2008), the injection of β-catenin-

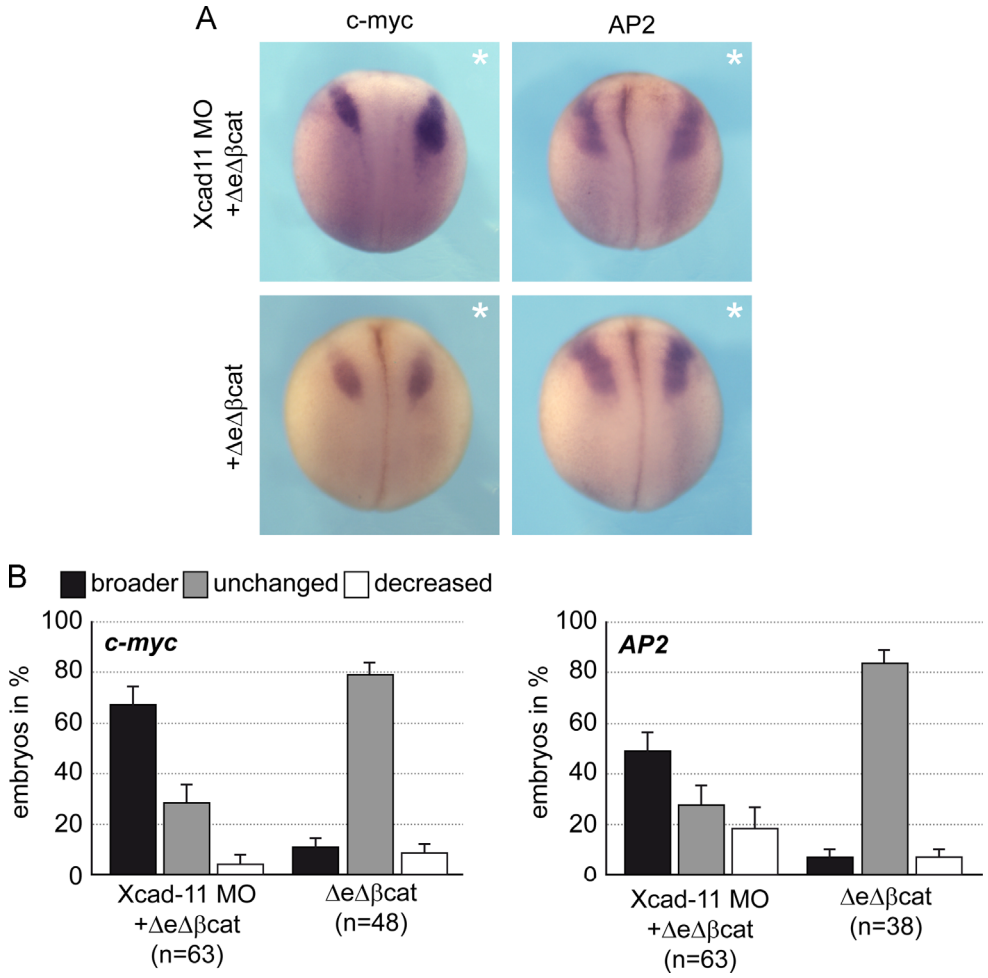

Fig. 5. The β-catenin binding site is essential for Xcadherin-11 function. (A) Whole mount ISH. Embryos were injected with Xcad-11ΔeΔβcat (50 pg) either with or without Xcad-11-MO in one dorsal animal blastomere of eight-cell-stage and analyzed at stage 19 for c-myc and AP2 expression. While overexpression of Xcad-11ΔeΔβcat did not result in a distinct phenotype the expansion of c-myc and AP2 staining by Xcad-11-MO could not be rescued by Xcad-11ΔeΔβcat. Dorsal view, anterior to top, asterisk marks the injected side. (B) Statistical analysis of whole mount ISH results. 67% of the analyzed embryos with Xcad-11-MO and Xcad-11ΔeΔβcat showed an expanded c-myc expression domain. The overexpression of Xcad-11ΔeΔβcat did not show any significant phenotype (80% normal expression). Similar results were obtained for AP2 (53% increased expression in embryos co-injected with Xcad-11-MO and Xcad-11 ΔeΔβ-cat). *n*=number of embryos.

MO alone resulted in a decrease or complete loss of NCC tissue (Fig. 7D–F).

Discussion

Cadherins are not just mediating cell-cell adhesion to organize a proper tissue organization. Additionally, they have a prominent role in cell migration and cell differentiation (Geiger and Ayalon, 1992; Wollner et al., 1992, Takeichi, 1995; Becker et al., 2012). In this study we show an unexpected function of the classical type II Xcadherin-11 in NCC specification. Loss of Xcadherin-11 protein function led to an accumulation of β-catenin within the nucleus and to an increased expression domain of c-myc and cyclin D1, which are both direct target genes of the canonical Wnt-signaling pathway and expressed in *Xenopus* NCCs (He et al., 1998; Shtutman et al., 1999; Bellmeyer et al., 2003; Vernon and Philpott, 2003). Additionally, depletion of Xcadherin-11 induces the expression of early NCC specifiers with a simultaneous decrease of neural marker genes. Furthermore, we identified the C-terminal β-catenin binding site to be essential for regulation of NCC specification and proliferation by modulating a balanced nuclear β-catenin level and therefore regulating Wnt/β-catenin signaling. Importantly, the critical time-point for a balanced nuclear β-catenin pool coincides with neural crest proliferation and specification (stage 16) but not with induction during gastrulation (stage 11) as demonstrated by the different rescue constructs.

The development of NCC compromises the processes of induction, maintenance, migration and differentiation (Steventon et al., 2005). These studies proposed a novel, two-step model for NCC development where increasing levels of Wnt and inhibition of BMP are required at gastrula stages for NCC induction, whereas at neurula stages constant high levels of Wnt and BMP are essential for NCC specification and maintenance (Steventon et al., 2009). Xcadherin-11 is expressed in a two-phase manner (Fig. 8A). A moderate expression can be detected at the time of gastrulation (stage 10), which increases during neurulation and at the onset of NCC migration (stage 18) (Hadeball et al., 1998). Therefore, early expression of Xcadherin-11 could correlate with a function in gastrulation, due to (1) the adhesive function of Xcadherin-11 during convergent extension movements or (2) the signaling function by interference with the canonical Wnt/β-catenin pathway, which could lead to disturbed NCC induction. The adhesive function of Xcadherin-11 is needed for proper gastrulation movements (this work). However, from our data we can exclude a role of Xcadherin-11 in cell signaling phase during NCC induction, because we observed an expanded expression area of the early NCC marker gene c-myc in stage 19 and stage 25 embryos depleted for Xcadherin-11. This increased c-myc expression seems not to depend on disturbed gastrulation movements which could

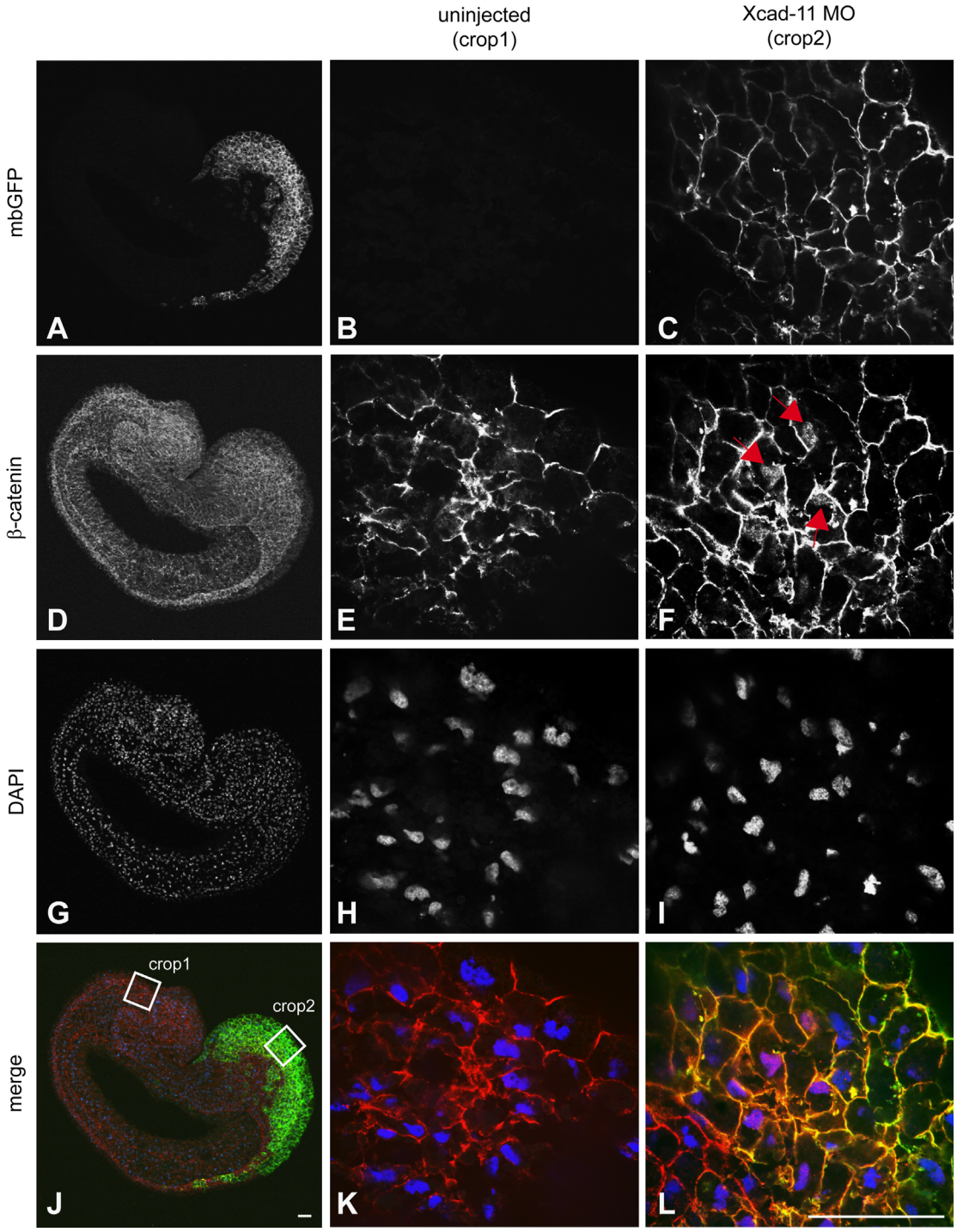

Fig. 6. β-catenin is translocated in the nucleus after Xcadherin-11 depletion. Transversal sections of Xcad-11-MO injected (one dorsal animal blastomere at eight-cell-stage) embryos analyzed at stage 19. (A–C) Co-injection with mbGFP (200 pg) was used as lineage tracer and for membrane staining. (D–F) Immunostaining for β-catenin. Note nuclear β-catenin staining at the injected side (red arrows in F). (G–I) Nuclei were stained with DAPI. (J–L) Merged images, squares indicate areas of higher magnification. Crop1: uninjected side, crop2: injected side. Scale bar 50 μm.

cause a shift of inductive fields, that are important for proper NCC induction. A recent study by Steventon and Mayor (2012) highlights a new three-step model for NCC induction where at early gastrulation Wnt signals need to be repressed for neural plate border induction. At mid-gastrula activation of Wnt together with intermediate BMP signals is required for NCC specification and at early neurula both BMP and Wnt are required for NCC maintenance. Therefore, activation of the Wnt-pathway by Xcadherin-11 depletion activates the expression of NCC specifiers like c-myc and AP-2 (Figs. 1 and 3). Overexpression of Xcadherin-11 is not affecting NCC induction but is important for Wnt/β-catenin signaling for late events in NCC development. In particular, Xcadherin-11 is needed for the maintenance of the NCC marker twist during the period of NCC specification (Borchers et al., 2001). Therefore, both loss of function and gain of function experiments allocate Xcadherin-11 a role in NCC specification.

The expression of Xcadherin-11 is induced by canonical Wnt-signaling (Hadeball et al., 1998). Posttranslationally, the expression of Xcadherin-11 can be modulated by proteases of the ADAM matrix-metalloprotease family, namely ADAM13, which is

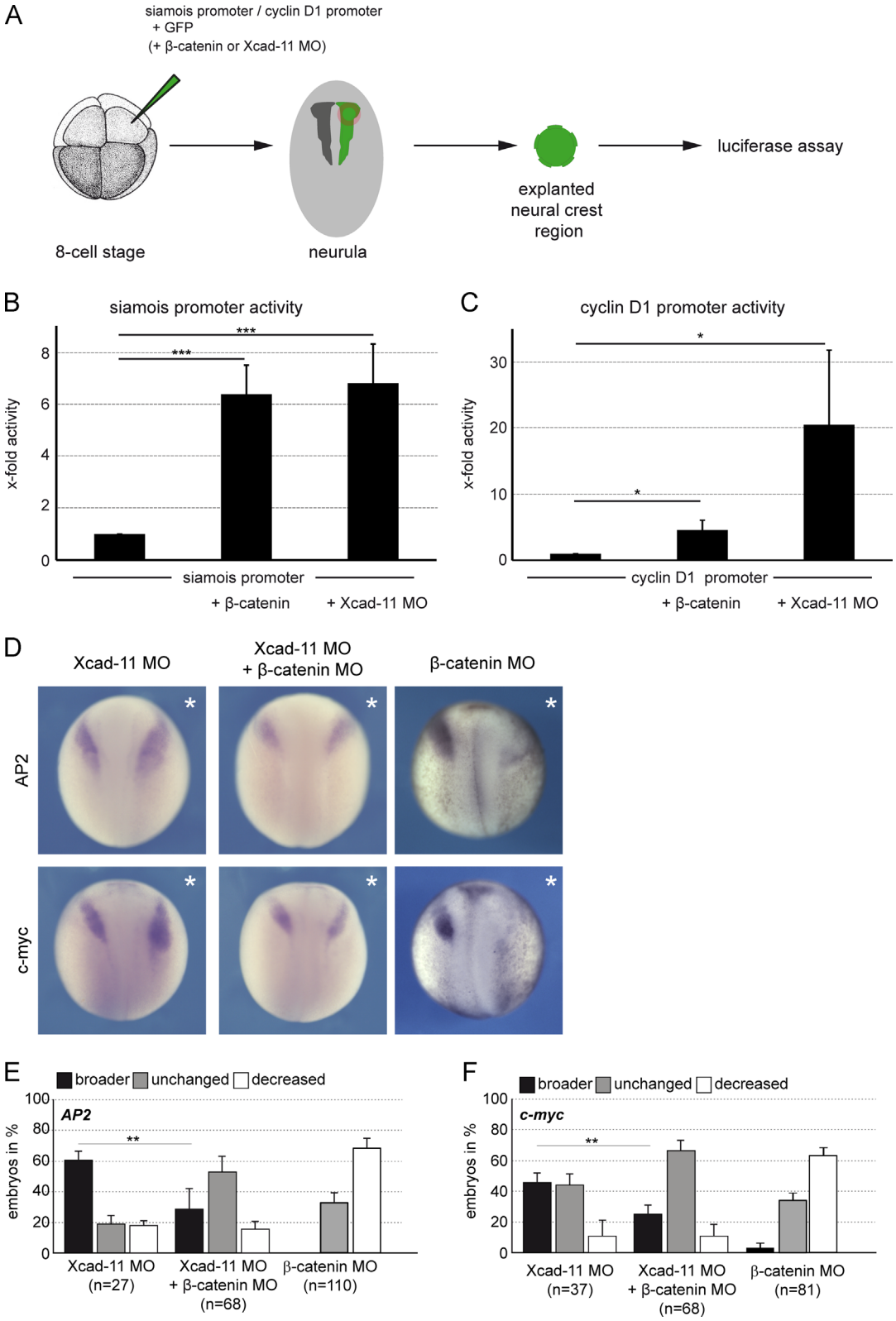

Fig. 7. Depletion of Xcad-11 results in an activation of the Wnt-signaling pathway. (A) Scheme of reporter gene experiment. Embryos were injected with the displayed constructs in one dorsal animal blastomere of eight-cell-stage embryo. At neurula stages the NCC area was explanted and underwent luciferase assay. (B,C) Injection of Xcad-11-MO resulted in strong activation of the siamois (B) and cyclin D1 (C) promoters. While the siamois promoter was activated in a similar manner by constitutively active β-catenin as positive control and Xcad-11-MO, the increase in cyclin D1 expression was even more prominent in Xcad-11-MO injected embryos ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.005$). (D) Enhanced expression of c-myc and AP2 by Xcad-11-MO can be prevented by co-injection of β-catenin-MO. (E,F) Statistical analysis of ISH. In Xcad-11-MO injected embryos 63% of the embryos show an increased expression domain of AP2 (E) and 44% of c-myc (F). When β-catenin-MO was co-injected, only 29% of the embryos resulted in an increased AP2 and only 24% in an increased c-myc expression area. ($^{**}p \leq 0.01$) β-catenin-MO injection alone led to decreased NCC formation.

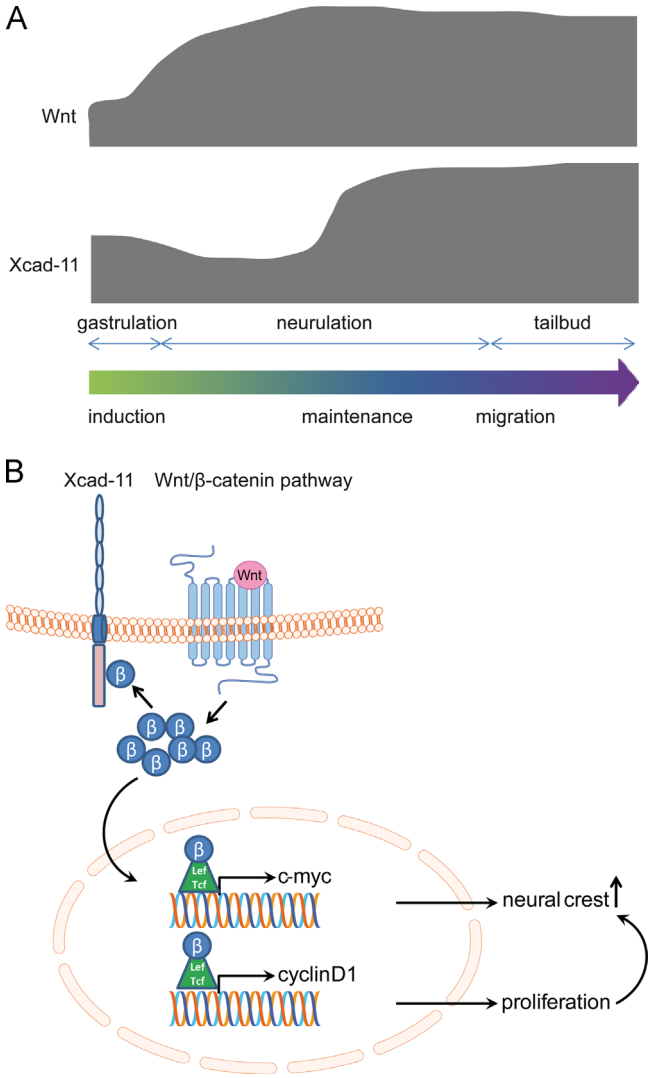

Fig. 8. Model of Xcadherin-11 function in NCC specification. (A) Temporal course of Wnt and Xcadherin-11 protein levels during embryo development stages and the corresponding phases of NCC formation (color code see Steventon et al., 2005). Increased expression of Xcadherin-11 at neurula stages is correlated with an increased Wnt level. (B) Competition of Xcadherin-11 and Wnt for the cytoplasmic pool of β-catenin. Knockdown of Xcadherin-11 leads to increased levels of β-catenin in the cytoplasm and an enhanced nuclear localization resulting in an up-regulation of NCC specifiers c-myc and cyclin D1.

expressed in NCCs from stage 13 onward (Alfandari et al., 2001). Although it has been shown that specific cleavage of Xcadherin-11 by ADAM13 starts with onset of NCC migration (McCusker et al., 2009), other metalloproteases like ADAM9 may overtake the role of early regulation of Xcadherin-11 localization to the membrane. On the other hand ADAM13 enhances canonical Wnt-signaling required for NCC induction (Wei et al., 2010, 2012). This indicates a regulatory loop of canonical Wnt-signaling inducing Xcadherin-11 expression on the one hand and regulation of Wnt-signaling by ADAM13 (Wei et al., 2010, 2012) and Xcadherin-11 (this paper). Whether the regulation of Wnt via ADAM13 is a direct regulation or mediated by Xcadherin-11 needs to be clarified.

As it has been described for E-cadherin the binding of β-catenin to the corresponding binding site of the cadherin inhibits the simultaneous binding of β-catenin to transcription factors of the LEF/TCF family (Orsulic et al., 1999). Thus, there are different possible mechanisms of interacting with the canonical Wnt-signaling pathway. (1) One idea is the shedding of the cadherin leading to mobilization of the cytoplasmic domain which can translocate into the nucleus and co-transport β-catenin for a stronger induction of Wnt signal as it is described for N-cadherin (Shoval et al., 2007). (2) On the other hand the binding of β-catenin to the membrane bound or also mobilized cytoplasmic tail after ADAM cleavage might still keep β-catenin from binding to LEF/TCF transcription factors. Since we observe a strong activation of Wnt-signaling in luciferase reporter assays this seems to support the former idea. In a similar way E-cadherin shedding by ADAM10 also enhances Wnt-signaling and leads to increased expression of cyclin D1 (Maretzky et al., 2005). The switch from canonical to non-canonical Wnt-signaling by recruiting β-catenin to Xcadherin-11 and thus eliminating it from the signaling pathway may be one prerequisite for inducting NCC migration (De Calisto et al., 2005).

Wnt/β-catenin is required for NCC specification and maintenance during *Xenopus* neurulation (Steventon et al., 2009). One of the key mediators of the canonical Wnt-signaling pathway is β-catenin, which gets accumulated within the nucleus after Wnt response activating different target genes (Moon et al., 2002, Klaus and Birchmeier, 2008). Due to their β-catenin binding site within the cytoplasmic domain cadherins can compete with the canonical Wnt-signaling pathway for β-catenin. Studies in *Xenopus* demonstrated that overexpression of cadherins led to an inhibition of dorsal axis formation what is a clear function of canonical Wnt-signaling (Heasman et al., 1994; Fagotto et al., 1996). Additionally, E-cadherin$^{-/-}$ embryonic stem cells displayed an accumulation of β-catenin in the nucleus and the activation of a Wnt reporter (Orsulic et al., 1999). Moreover, the overexpression of Xcadherin-11 led to posteriorized phenotypes with strong head defects, which could be rescued by co-injection of β-catenin (Hadeball et al., 1998). In this study, we could detect a nuclear localization of β-catenin after depletion of Xcadherin-11, which is underlying the competition of cadherins and canonical Wnt-signaling pathway for β-catenin resulting in an increased expression domain of the Wnt/β-catenin target gene and NCC specifier c-myc as we could also demonstrate by siamois and cyclin D1 reporter gene activation. A similar mechanism was recently shown for E-cadherin, where its down-regulation activates β-catenin/TCF dependent transcription, which subsequently leads to the expression of c-myc (Wang et al., 2010). We were able to rescue the increase of c-myc expression by co-injection of the membrane anchored cytoplasmic tail of Xcadherin-11. Interestingly, we failed to rescue this phenotype by co-injecting this construct lacking the β-catenin binding site (Xcad-11ΔeΔβcat). More important, we were able to rescue the increase in c-myc expression in a β-catenin deficient background (Fig. 7D–F). Additionally, the loss of β-catenin alone led to loss of c-myc expression and NCC formation (Fig. 7D–F). These findings support again the Wnt/β-catenin dependent expression of c-myc and the competition for β-catenin between the cytoplasmic tail of Xcadherin-11 and the canonical Wnt-signaling pathway (He et al., 1998).

Simultaneously with an expanded c-myc expression we observed an increase in cell proliferation. C-myc plays an important role in cell cycle progression by regulating cyclin D2 expression and by influencing the stability and activity of cyclin/cdk-complexes (Steiner et al., 1995; Mateyak et al., 1999; Bouchard et al., 2004; Chiles, 2004). However, in *Xenopus* c-myc is a key regulator of NCC specification independent of NCC proliferation (Bellmeyer et al., 2003). This is in line with our data. When we overexpressed c-myc we could only detect a slight increase in cell proliferation (data not shown). Furthermore, the broadening of the expression domain of c-myc in Xcadherin-11 morphants is delayed compared to cyclin D1 which is expanded already in stage 16. Nevertheless, the development of the quantitative expression level of c-myc and cyclin D1 is similar. This indicates that the spatial induction of neural crest

inducers precedes the quantitative enhancement. Work on *Drosophila* reporting that mutations in c-myc fail to cause defects in cell proliferation also support alternative c-myc functions (Johnston et al., 1999). It is easily conceivable that the interference with cell cycle control in Xcadherin-11 morphants must depend on additional target genes, which can be activated through β-catenin. We identified cyclin D1, one of the main regulators in GI to S-phase transition during the cell cycle (Baldin et al., 1993; Sherr, 1996). In this study, knockdown of Xcadherin-11 resulted in a strong increase in cyclin D1 expression through mostly all stages analyzed correlating with a simultaneous increase in cell proliferation allocating cyclin D1 instead of c-myc a prominent role in NCC proliferation. We significantly rescued the increase in cell proliferation by co-injection of the cytoplasmic domain of Xcadherin-11. This suggests that the cytoplasmic domain could interfere with the canonical Wnt-signaling pathway by recruiting the cytoplasmic pool of β-catenin to the membrane and inhibiting cyclin D1 mediated cell proliferation. Our data support former studies, which showed that overexpression of β-catenin led to an increase of cyclin D1 expression whereas the overexpression of the cytoplasmic tail of N-cadherin decreased protein levels of cyclin D1 (Shtutman et al., 1999). However, comparing proliferation rate and neural crest tissue size we could not distinguish between cell fate changes and cell proliferation. Inhibition of proliferation is possible by hydroxyurea and aphidicolin (HUA) which induce a rapid replication arrest (Hammond et al., 2003). Thus, treatment of Xcadherin-11 depleted embryos with HUA prevented the broadening of the c-myc expression domain proving the role of proliferation as a causal initiator of the observed phenotype. Apart from increased cell proliferation upon Xcadherin-11 depletion (Kashef et al., 2009, this manuscript) recent studies in a murine retinoblastoma model revealed a decrease in apoptosis in absence of cadherin-11 due to up-regulation of β-catenin mRNA (Marchong et al., 2010). Thus, cadherin-11 might suppress tumor growth context dependent either by limiting cell proliferation or promoting apoptosis.

Besides c-myc we could observe an increased expression of the NCC specifier AP2. Recent studies highlighted the important function of AP2 in initiating the neural border, inducing NCCs and acting as a NCC specifier. The authors demonstrated that AP2 is sensitive to Wnt-signaling (Luo et al., 2003) and acts downstream of c-myc (Bellmeyer et al., 2003; de Croze et al., 2011). In our studies the increase in AP2 expression can be caused directly by activated Wnt/β-catenin signaling or indirectly through increased c-myc expression. In response AP2 is able to induce the expression of later NCC marker genes like sox10, sox9 and snail2 and to inhibit expression of neural markers like sox2 (de Croze et al., 2011). AP2 and c-myc are specifiers of neural plate border (NPB) formation. While AP2 restricts NPB towards the ectoderm, c-myc demarcates the neural tissue from NC. Both are expressed at similar time points around stage 10 (Winning et al., 1991; Honoré et al., 2003). Additionally, Schneider et al. (2010) revealed that expression levels of AP2 and snail1 are regulated by c-myc. The expression of sox10, which mediates specification, and survival of *Xenopus* NCCs (Honoré et al., 2003) is temporally subordinated to c-myc and AP2 (Light et al., 2005; de Croze et al., 2011; Milet et al., 2013) and it becomes prominent in the prospective NCC region in stage 14 (O'Donnell et al., 2006; Milet et al., 2013) indicating that sox10 is one of the late NC specifiers in the gene regulatory cascade. The observed delay in sox10 expression in our study (Fig. 3B) could be caused by the expanded c-myc expression or by the increased AP2 expression resulting in a delayed manifestation of the neural plate border. Thus, in absence of Xcadherin-11 NCCs still persist in the early specification phase and temporally subordinated genes like sox10 are not yet expressed. Nevertheless, sox10 has been reported to be a Wnt dependent target gene. Overexpression of Wnt or of GSK-3β inhibitors results in an increased expression of Sox10 (Aoki et al., 2003). Since depletion of Xcad-11 leads to activation of Wnt signaling sox10 expression should be increased. Due to its late position in the regulatory network of NC induction and by the delay in the NC specification process the prospective NC field remains in an early state thereby preventing the expression of later specifier genes. Dutton et al. (2008) showed that the expression of Sox10 does not only depend on Wnt signaling but that a combination with other transcription factors is necessary which are also embedded in the genes regulatory cascade. During progression of NCC specification the expression of Sox10 is then activated and enhanced. This results in increased pigmentation in Xcadherin-11 depleted embryos (Kashef et al., 2009) since later emigration from the neural tube results in increased formation of melanocytes (Thomas and Erickson, 2009).

Conclusion

Increasing levels of Wnt and inhibition of BMP are required at gastrula stages for NCC induction, whereas at neurula stages constant high levels of Wnt and BMP are essential for NCC specification and maintenance (Steventon et al., 2009). Therefore, a balanced Wnt/β-catenin level is essential for NCC development. In *Xenopus*, overexpression of dominant negative Wnt-8 leads to loss of the expression of snail2. In contrast, β-catenin overexpression results in an increased and expanded expression of snail2 at the expense of the neural plate marker sox2 (LaBonne and Bronner-Fraser, 1998). This is similar to what we show after Xcadherin-11 depletion. Interestingly, the increase of needed Wnt-signaling for NCC specification during neurulation is correlated with the increase of Xcadherin-11 expression (Hadeball et al., 1998). Apart from controlling cell migration Xcadherin-11 contributes to balancing the nuclear β-catenin level by sequestering cytoplasmic β-catenin to the membrane.

Taking our results together we propose following model for the role of Xcadherin-11 in NCC specification (Fig. 8): increased expression of Xcadherin-11 at neurula stages is correlated with an increased Wnt level (Fig. 8A). Xcadherin-11 normally recruits β-catenin to the plasma membrane. After depletion of Xcadherin-11 β-catenin can translocate into the nucleus activating Wnt target genes like cyclin D1 and c-myc. The increase of cyclin D1 is correlated with an increase in proliferation, which lead to more NCCs. In parallel, increase of the early NCC specifier c-myc activates the expression of other NCC genes like AP2 and sox10 (Fig. 8B).

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.08.007.

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