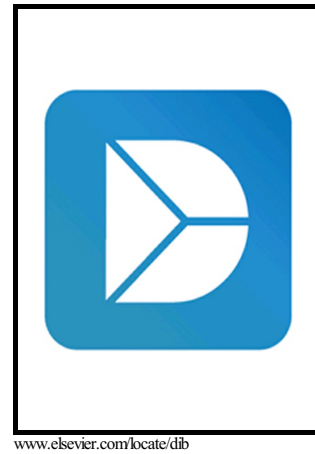


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# Data on the effects of N-cadherin perturbation on the expression of type II cadherin proteins and major signaling pathways

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## Abstract

This article contains data related to the research article entitled, "A catenin-dependent balance between N-cadherin and E-cadherin controls neuroectodermal cell fate choices" [1]. The data presented here include (1) proximity ligation assays using antibodies that recognize type I cadherins (N-cadherin and E-cadherin) co-incubated with antibodies against type II cadherins (Cadherin-6B and Cadherin-11) to test heterotypic interactions in vivo; (2) expression of Cadherin-6B and Cadherin-7 after electroporation with full length N-cadherin and N-cadherin translation-blocking morpholino; and (3) expression of WNT, Notch and TGF- $\beta$  signaling reporters and effectors upon loss of N-cadherin protein in chicken embryos.

## Specifications Table

|                            |   |
|----------------------------|---|
| Subject area               | <i>Biology</i>  |
| More specific subject area | <i>Developmental Biology</i>  |
| Type of data               | <i>Figures/Images</i>   |
| How data was acquired      | Fluorescence images were taken after gain and loss of function experiments using Zeiss ImagerM2 with Apotome.2 and Zen software or Axioskop 2 Plus with AxioVision software (Karl Zeiss).   |
| Data format                | <i>Data has been analyzed</i>   |
| Experimental factors       | Half embryos injected and electroporated with morpholino oligomer or DNA plasmid and were compared to either the uninjected or control side or embryos injected with GFP or control morpholino. Immunohistochemistry and proximity ligation we performed as described in methods. |
| Experimental features      | <i>Gain and loss of function, morpholino oligomers injected/electroporated into Gallus gallus embryos. Immunohistochemistry and proximity ligation assay (PLA)/Duolink.</i>   |
| Data source location       | <i>California State University Northridge, Northridge CA</i>  |
| Data accessibility         | <i>Data included only in this article.</i>  |
| Related research article   | Rogers, C.D.S., L.K.; Bronner, M.E., <i>A catenin-dependent balance between N-cadherin and E-cadherin controls neuroectodermal cell fate choices.</i> Mechanisms of Development, 2018. <b>In Press.</b> [1]   |

## Value of the data

- There is contrasting evidence in the field of cell biology to support heterotypic interactions between type I and II classical cadherins. These data test whether type I and type II cadherins heterotypically interact in vivo using the proximity ligation assay (PLA), described below.

- A related study [1] showed that type I cadherins compete for localization in the membrane *in vivo*. These data can be useful to test whether altering levels of N-cadherin effect the spatiotemporal expression of Cadherin-6B or Cadherin-7.
- Cadherins are not typically reported as major regulators of signaling molecules other than BMP [2]. These data can be useful to examine the effects of N-cadherin knockdown on the expression of reporters/effectors involved in the WNT, Notch and TGF- $\beta$  signaling pathways.

## 1. Data

Chicken embryos were cultured to various stages, fixed, and cryosectioned prior to performing the proximity ligation assays (PLA) using antibodies against type I cadherins, N-cadherin (Ncad) or E-cadherin (Ecad) together with antibodies against Cadherin-6B (Cad6B) or Cadherin-11 (Cad11) (Figure 1). Antibodies were used to detect the presence of either Ecad or Ncad with Cad6B or Cad11 in the PLA, which tested interactions between type I and type II cadherins. Red dots in Fig. 1A, D, G or J would indicate that the two proteins of interest are close enough to interact with each other. Immunohistochemistry using the same antibodies was performed side-by-side to show the endogenous expression of the proteins of interest and to verify that functional antibodies were used in the assay (Fig. 1C, F, I, L). An Ncad translation-blocking morpholino (NcadMO), non-specific control morpholino (ContMO), full-length N-cadherin-GFP, or GFP were injected into one side of a Hamburger-Hamilton stage 4 chicken embryo. Subsequent immunohistochemistry (IHC) was performed for Cad6B (Figure 2A-F", 2M-N) or Cad7 (Fig. 2G-2L', 2O-P). Embryos were injected with NcadMO and/or ContMO and IHC and *in situ* hybridization were used to detect the expression of the WNT reporter, Top-Flash-GFP (Fig. 3A-3F'), the Notch effectors, Hes1 and Hes5 (Figure 3G-H'), and the TGF- $\beta$  effector, Smad2/3 (Fig. 3I-J'). Confirmation of ectopic/exogenous Ncad expression after Ncad-GFP injection as well as confirmation of Ncad protein knockdown after morpholino injection can be found in Supplemental Fig. 2 from [1].

## 2. Experimental Design, Materials and Methods

### 2.1 Embryos

Fertilized chicken eggs were obtained from local commercial sources (McIntyre Farms, San Diego, CA and AA Farms, CA) and incubated at 37°C to the desired stages according to the criteria of Hamburger and Hamilton (HH). All use of embryos was approved by the California State University Northridge IACUC protocol: 1516-012a.

### 2.2 Electroporation of antisense morpholinos and vectors

A translation blocking antisense fluorescein -labeled morpholino to Ncad (NcadMO) was designed (5'-GCGTTCCCGCTATCCGGCACATGGA-3') [3], as well as a non-specific control morpholino (ContMO) (5'-CCTCTTACCTCAGTTACAATTATA-3'). Injections of the fluorescein-tagged morpholinos (0.75–1 mM plus 0.5–1.5 mg/ml of PCI carrier plasmid DNA; as in [4] were performed by air pressure using a glass micropipette targeted to the presumptive neural plate region at HH stages 4–5. DNA plasmids pCS2-Ncad-GFP (Ncad-GFP)[5], or 12Tf-d2EGFP [6] were used (1 mg/ml) and were introduced in a similar manner to morpholinos described above. HH stage 4–5 electroporations were conducted on whole chick embryo explants placed ventral side up on filter paper rings. The Ncad morpholino and vectors were injected on the right side of the embryo and where indicated, controls were injected on the left side of the same embryo.

Platinum electrodes were placed vertically across the chick embryos and electroporated with five pulses of 6.3 V in 50 ms at 100-ms intervals.

### 2.3 Immunohistochemistry

Immunohistochemistry (IHC) for Ncad (DSHB, MNCD2)[7], Cad6B (DSHB, CCD6B-1) [8], Cad7 (DSHB, CCD&-1) [8], Cad11 (Invitrogen, 5B2H5) [9], and Ecad (BD Transduction Laboratories, 610181) was performed as follows: Embryos were fixed in 4% paraformaldehyde made in phosphate buffer for 15-40 minutes at room temperature. All washes were performed in TBST + Ca<sup>2++</sup> with 0.5% triton x-100. Blocking was performed with 10% donkey serum in the same buffer. The primary antibodies (1:5-1:10 for all hybridoma antibodies and 1:200-1:1000 for all others, see [1]) were incubated in the TBST buffer from overnight to two days at 4°C and secondary antibodies (Alexa Fluor, Thermo Fisher Scientific 1:500 to 1:1000) were applied in the same buffer for either three hours at room temperature or overnight at 4°C.

### 2.4 In situ hybridization

In situ hybridization was performed as follows: Embryos were fixed for 1h at room temp or O/N at 4°. Embryos were dehydrated in methanol and then rehydrated in PBS with 0.1% Tween-20. They were incubated in hybridization buffer [3, 10] at 70° for one hour, then incubated with the anti-sense mRNA Dig or Fluorescein labeled probe diluted in hybridization buffer at 70° O/N. Embryos were then washed with 2X SSC and 1X MAB with Tween-20. Next, embryos were incubated with blocking agent in 1X MAB and 1:1000 anti-Dig antibody, then washed with 1X MAB multiple times. Embryos were then incubated with NBT/BCIP until the chromogenic reaction was complete. Embryos were imaged in whole mount and imaged as described below.

### 2.5 Imaging and Fluorescence Quantification

Fluorescence images were taken using Zeiss ImagerM2 with Apotome.2 and Zen software or Axioskop 2 Plus with AxioVision software (Carl Zeiss). Fluorescence was quantified using NIH ImageJ64 by averaging the relative intensity of 1-6 images per embryo. Background was subtracted uniformly across the images using the background subtraction function in NIH ImageJ64 with a rolling-ball radius of 50.00 pixels before quantitation [11]. Half embryos injected with morpholino or DNA plasmid were compared to the uninjected side of the embryos.

### 2.6 Proximity Ligation Assay (Duolink)

The proximity ligation assay (PLA) was performed on previously cryosectioned embryos on glass slides. All sections are transverse cryosections created on a Microm HM 550. PLA was performed directly on sections of fixed tissue. The methods were performed as described in the instructions in the Duolink Assay (#DUO92101) by Sigma Aldrich (St. Louis, MO)[12]. Embryos were fixed, sectioned and incubated with primary antibodies (see IHC). After primary antibody incubation, sections were washed with TBST+ Ca<sup>++</sup> and subsequently incubated with the PLA probe set at 37°C for 1 hour. They were then washed in buffer A, placed in ligation mix for 30 min-1 hour at 37°C. Next, sections were washed in buffer A then incubated with polymerase mix for 110-200 min at 37°C. Finally, sections were washed in 1X and 0.1X buffer B, and subsequently mounted with Duolink mounting media with DAPI.

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**Figure 1. PLA examining putative interactions between type I and type II cadherins.** PLA performed on transverse sections of various stages of chicken embryos. (A, B) PLA in a 7 somite stage (SS) embryo using rb  $\alpha$  Ncad IgG with ms  $\alpha$  Cad6B IgG1. (A) Red channel showing PLA results, (B) DAPI identifying nuclei, and (C) IHC using same primary antibodies in adjacent section, but incubated with AlexaFluor secondary antibodies identifying HNK1 (blue), Cad6B (green), and Ncad (red). (D, E) PLA in a 7 SS embryo using rb  $\alpha$  Ecad IgG with ms  $\alpha$  Cad6B IgG1. (D) Red channel showing PLA results, (E) DAPI identifying nuclei, and (F) IHC using same primary antibodies in adjacent section, but incubated with AlexaFluor secondary antibodies identifying DAPI (blue), Cad6B (green), and Ecad (red). (G, H) PLA in a 12 SS embryo using rb  $\alpha$  Ncad IgG with ms  $\alpha$  Cad11 IgG1. (G) Red channel showing PLA results, (H) DAPI identifying nuclei, and (I) IHC using same primary antibodies in adjacent section but incubated with AlexaFluor secondary antibodies identifying HNK1 (blue), Ncad (green), and Cad11 (red). (J, K) PLA in a 10 SS embryo using rb  $\alpha$  Ecad IgG with ms  $\alpha$  Cad11 IgG1. (J) Red channel showing PLA results, (K) DAPI identifying nuclei, and (L) IHC using same primary antibodies in adjacent section but incubated with AlexaFluor secondary antibodies identifying DAPI (blue), Cad11 (green), Ecad (red). Dashed lines indicated location of neural tubes. Each experiment was performed at least three times.

**Figure 2. Expression of Cad6B and Cad7 after electroporation with Ncad translation-blocking morpholino or Ncad-GFP.** Gastrula stage embryos (Hamburger Hamilton stage 4) were unilaterally injected with either Ncad translation-blocking morpholino oligomer or non-specific control morpholino, or full length Ncad-GFP or GFP DNA and subsequently electroporated. IHC was performed using antibody against Cad6B (A-F'') or Cad7 (G-L'). (A, B') IHC for Cad6B after injection with NcadMO (N= 16), Ncad-GFP (D-E', N= 9), ContMO (C-C'', N= 10), or GFP (F- F'', N= 7). (A, D, C, and F) are images of whole mount chicken embryos with anterior to the top and posterior to the bottom. (B, E) are transverse sections at the cranial level. (G-H') IHC was performed using antibody against Cad7 after electroporation of NcadMO (G-H', N= 9), Ncad-GFP (J-K', N= 13), ContMO (I, I', N= 13), or GFP (L, L', N= 11). (G, J) are images of whole mount chicken embryos with anterior to the top and posterior to the bottom and (H, I, K, L) are transverse sections at the cranial level. (M-P) Fluorescence intensity was measured using ImageJ and uninjected sides were compared to the side injected with either NcadMO or Ncad-GFP. Relative fluorescence intensity was calculated by dividing the intensity of the injected side by the uninjected side. Bar graphs represent average fluorescence intensity. Error bars are standard deviation of raw data and p values were calculated using a student's T-test. The average intensity of Cad6B after injection with NcadMO was 1.07 (Fig. 2M, N= 16, std. dev. = 0.14, p= 0.5) and after Ncad-GFP injection it was 1.04 (Fig. 2N, N= 9, std. dev. = 0.05, p= 0.09). The average intensity of Cad7 after injection with NcadMO was 0.99 (Fig. 2O, N= 13, std. dev. = 0.09, p= 0.8) and after Ncad-GFP injection it was 0.87 (Fig. 2P, N= 13, std. dev. = 0.17, p= 0.8). Uninjected side is normalized to 1 for all treatments.

**Figure 3. Expression of WNT, Notch or TGF- $\beta$  reporters and effectors after Ncad knockdown.** (A-F) Gastrula stage chicken embryos were electroporated with WNT reporter construct 12xTCF-GFP (Top-flash) bilaterally alone (A-C') and collected at (A-A', C-C') 4 SS and (B-B') 8 SS. Gastrula stage chicken embryos were injected with NcadMO (unilaterally) +

Top-flash (bilaterally, N= 17). Active WNT signaling is demonstrated by expression of the GFP construct (blue). IHC was simultaneously performed for Ecad (red). Whole mount in situ hybridization for Notch targets, (G) hairy and enhancer of split 5 (Hes5) (N= 8) and (H) hairy and enhancer of split 1 (Hes1) (N= 7) after ContMO/NcadMO electroporation (G', H'). IHC for (I-J') phosphorylated Smad2/3 (red) after NcadMO electroporation (N= 12). Embryos are injected with NcadMO on right side (asterisk) and ContMO where indicated. Dashed lines indicated axial region of transverse section. IHC was performed in whole mount and embryos were cryosectioned and imaged in transverse section as well.

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