Supporting Information

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SI Results
In addition to TrkA and p75NTR, sortilin, a member of the Vps10p-domain receptor family, was identified as a specific receptor for proNGF (1). No data are available regarding its expression in the chicken embryo. We were unable to detect sortilin in chicken embryos of various stages, by Western blot or immunohistochemistry. However, we cannot rule out the possibility that it is expressed at later stages or that the available anti-sortilin antibodies do not detect chicken sortilin.

SI Materials and Methods

Materials. Recombinant mouse NGF was expressed and refolded from E. coli inclusion bodies as described in (2). Rat monoclonal αD11 anti-NGF antibody (3) was prepared from hybridoma supernatant. Mouse mAb 9E10 (4) control antibody was purified from ascites.

ELISA. Chicken embryos of different developmental stages [η between 5 and 10 for each stage, from 3 d (HH 19–20) to 7 d (HH 30–31) of embryonic development] were collected and homogenized in ice-cold sample buffer (SB) [Tris HCl 0.1 M (pH 7.4), BSA 2%, NaCl 0.4 M, Protease Inhibitor Mixture; Roche] at the ratio of 0.5 mL SB/g of embryos. The homogenates were placed in ice for 20 min and then centrifuged for 30 min at 15,000 × g at 4 °C. The supernatant was used for the ELISA immunoassay.

The anti-NGF polyclonal antibody (Sigma) was immobilized on a microtiter 96-well plate (Falcon) in 100 mM NaHCO₃ (pH 9.6) at 3 μg/mL. After the coating step, the plate was blocked with 5% (wt/vol) BSA (Sigma) in PBS with Tween 0.05% (PBST) for 1 h at room temperature.

The calibration curve was obtained by preparing 10 serial dilutions of recombinant mouse NGF from 80 to 0.2 ng/mL in SB. The samples were first supplemented with four different concentrations of recombinant mouse NGF (40, 4, 0.4, and 0 ng/mL) and diluted 1:2 in PBST with BSA 5% (wt/vol). The samples were run in triplicate, incubated for 120 min at room temperature, and washed with PBST.

αD11 was used as primary antibody, incubated for 90 min at room temperature, and washed with PBST. Secondary HRP-conjugated anti-rat Ig antibody (Jackson ImmunoResearch) was incubated at a 1:7,000 dilution in PBST with 2.5% (wt/vol) BSA for 1 h at room temperature.

The developing step was carried out with TMB (Sigma). The reaction was stopped with a 1 M H₂SO₄ solution, and binding signal was detected at 450 nm with a Bio-Rad ELISA Reader. Triplicate readings for standard curve and sample points were averaged, and the average optical density of the zero standard point was subtracted. A standard curve was created by plotting the mean absorbance for each standard point (y axis) as a function of the natural logarithm of the NGF concentration (x axis). The best fit regression line was used to calculate sample values.

The assay was validated by supplementation with known amounts of recombinant NGF in the chicken embryo samples as an internal control. This allowed us to estimate that the assay, which measures the sum of mature NGF and proNGF (i.e., total NGF), underestimates the amount of detected NGF by ∼20–30%.

Immunohistochemistry. Fresh fertilized chicken (G. gallus) eggs were incubated at 37.5 °C until they reached the required stage of development according to HH. Whole embryos from stage HH 3–8 were collected and fixed in 4% (wt/vol) paraformaldehyde dissolved in PBS [2.7 mM KCl, 137 mM NaCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.4)] at 4 °C.

Embryos to be stained with anti-NGF, anti-proNGF, and anti-p75NTR were washed in PBS plus 0.3% Triton X-100 to remove the fixative and incubated with 40% (vol/vol) NGS (Vector Labtech) in PBS plus 0.3% Triton X-100 overnight at 4 °C. Embryos to be stained with anti-TrkA were incubated in PBS plus 0.3% Triton X-100 NGS 40% (vol/vol) for 8 h at room temperature. After removing NGS, embryos were incubated overnight at 4 °C with rabbit anti-NGF (1:1,000; Sigma-Aldrich), rabbit anti-proNGF (1:500, Chemicon), rabbit anti-TrkA (1:100; AbCam), or rabbit anti-p75NTR (1:500; Chemicon) polyclonal antibodies. All antibodies were diluted in PBS plus 0.3% Triton X-100 NGS 40% (vol/vol). Then, embryos were washed in PBS and incubated for 4 h at room temperature with goat anti-rabbit secondary antibody conjugated with alkaline-phosphatase (1:250; Sigma-Aldrich) diluted in PBS/NGS 40% (vol/vol).

After washing in PBS, the reaction was detected by incubating embryos in avidin-alkaline phosphatase system (1:100 in PBS; Vector Labtech) and then washed in PBS before incubating for 30 min with 1 mM levamisole to deactivate endogenous alkaline phosphatase activity. The reaction was developed in nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (BCIP) for 10 min, and then embryos were allowed to dry, were dehydrated in methanol, cleared in xylene, and mounted in DPX for microscopical analysis (Eclipse 90i; Nikon Instruments).

To verify the specificity of labeling, chicken embryos were incubated in a PBS plus 0.3% Triton X-100 NGS 40% (vol/vol) solution in which the primary antibody or the secondary antibody or both were omitted.

Measure of Axial Rotation. Morphological analysis was performed using a Nikon 90i microscope connected to a Nikon DMX 2000 videocamera. Across the different treatments, embryos were first checked for the absence of general gross abnormalities (such as spina bifida), the presence of which would make them excluded. Then, using a 2× objective and the image analysis program NIS (Nikon) the angle formed by the intersection between the mid-sagittal plane of the body (blue line in Fig. 3 A and J) and the line symmetrically dividing the posterior portion of the neural tube (red or green line in Fig. 3 A and J) was measured. The criterion was chosen to consider as negative those angles directed to the left of the body axis of the embryo.

Measurements were performed at two levels. The first one corresponded to the section at the level A of plate 76 of the “Atlas of Chick Development” (5) (Fig. S1, red line), referred to as the proximal angle; the second one in more dorsal sections corresponding to a plane passing through the centre of the pharynx (Fig. S1, green line), referred to as the proximal angle. Performing two series of measurements allows minimizing variability due to possible artifacts in the histological preparation. Indeed, at the level of the red line, the caudal part of the body (which includes the tail and the posterior limbs) is more mobile with respect to the trunk and the head, and this could determine angle variability during the procedures used to embed chick embryos in paraffin. For this reason we also measured axial rotation at the level of the green line, which is formed essentially by the head and a caudal part corresponding to the neck and shoulder and therefore tightly connected by a straight portion of the neural tube.
RNA Isolation. Embryos were incubated as described above, until HH 11-12, injected with 1 μg of αD11 or 9E10, stored in RNAlater (QIAGEN) at HH 11-12 and 30 min and 48 h after injection, total RNA was isolated using Trizol (Invitrogen) and DNase, by Qiagen columns (Invitrogen protocol). RNA quantity was determined on a NanoDrop UV-VIS. Only samples with an absorbance ratio in the range 1.8 < OD\textsubscript{260}/OD\textsubscript{280} < 2.0 were selected. Quality of RNA samples was checked for integrity with the Agilent BioAnalyzer 2100 (Agilent RNA 6000 nano kit); samples with a RIN index lower than 8.0 were discarded.

Real-Time PCR. Real-time PCR was carried out to study the expression and modulation of three candidate mRNAs: CSK, RTTN, and FURIN.

The first-strand cDNA template was synthesized from 500 ng of total RNA using random primers and SuperScript III reverse transcriptase (Invitrogen) in a final volume of 100 μL. RNA was subjected to real-time PCR using the two-step iCycler iQ Real-Time Detection system from Bio-Rad in the following cybergreen PCR program: 95 °C for 3 min and 35 cycles at 95 °C for 15 s, followed by 30 s at 60 °C. For quantification of gene expression changes, the ΔΔCt method was used to calculate relative fold changes normalized against the housekeeping gene TATA box binding protein (Tbp). Each data point was obtained from four biological replicates each of them in duplicate.

Primers for quantitative real-time PCR analysis were designed with the assistance of Universal Probe Library Software (Roche Applied Science). The following primers were selected to amplify 70-bp G. gallus CSK: 5′-AAGTGCTGGACGCTGGAC-3′ (forward) and 5′-TGATATGCACTAGCTGTTCACG-3′ (reverse); 60-bp G. gallus Rotatin (RTTN): 5′-TGAGGCTGTGAAGATGGTC-3′ (forward) and 5′-CCTTTGCTGGAACCGTACTT-3′ (reverse); and 63-bp G. gallus FURIN: 5′-CCTTTGCTGGAACCGTACTT-3′ (forward) and 5′-ATAACCGGGAGGACTGCT-3′ (reverse).

Microarray. A whole genome microarray analysis (Agilent platform) was performed on RNA extracted from embryos injected with 1 μg of αD11 or 9E10 and from noninjected reference embryos, following the two-color protocol (Agilent): two injected embryos for both antibodies and the reference were labelled with Cy3 and Cy5 respectively, and hybridized to Agilent 4x44k whole chicken genome oligonucleotide microarrays (G4122F). Post-hybridization image acquisition was accomplished using the Agilent scanner G2564B and analyzed by Agilent Feature Extraction. Data filtering was performed in Microsoft Excel by discarding spots close to the background level. Data analysis was performed with Agilent GeneSpring GX and Microsoft Excel.

Each array was normalized by the Lowess algorithm. Differentially expressed mRNAs were identified by ANOVA and by SAM (6). Only genes who were either up- (fold change >1.25) or downregulated (fold change in the interval 0.00 < fc < 1/1.25) for each group (αD11 and 9E10) and that were differentially expressed between the two groups were considered.


Fig. S1. Axial rotation in normal embryos. Images modified by permission of Judith Cebra-Thomas (Swarthmore College) showing the stage at which embryos were injected (blue arrow) and observed: green and red lines show the section where proximal (green) and distal (red) angle have been calculated.
Fig. S2. NGF influence on developing somites. Different extent of cell death in the myomer, in horizontal sections from embryos fixed 48 h after the injection of 1 μg of 9E10 (B and E) or αD11 (C and F) and noninjected embryos (A and D), at the level of the distal (A–C) and proximal (D–F) angle. Arrows point to dead cells.

Fig. S3. Multiple sequence alignment of the amino acid sequences of NGF from different species. The UniProt entry for the different proteins are indicated in front of the alignment. The regions of the epitope for the αD11 antibody are highlighted in yellow (loop I of NGF as in the tertiary structure) and green (loop II of NGF as in the tertiary structure). The multiple sequence alignment was performed with ClustalW2.
Table S1. Functions of genes regulated by anti NGF

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Function</th>
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| CDCA1       | Forms a complex essential for mitotic progression  
              | Regulatory role in chromosome segregation  
              | Stimulation of cell growth and division  
              | Essential for normal formation of enteric nerves and melanocytes  
              | Mediates neural proliferation and anti-apoptotic effect in postnatal rat cerebellum  
| EDNRB       | Important for organizing the molecular machinery that mediates adrenergic control of calcium reabsorption into sarcoplasmonic reticulum  
              | Stimulation of cell growth and division  
              | Essential for normal formation of enteric nerves and melanocytes  
              | Mediates neural proliferation and anti-apoptotic effect in postnatal rat cerebellum  
| AKAP7       | The earliest known marker of prospective feather tracts  
              | Blocks myogenic differentiation  
              | Negative regulator for gene transcription and apoptosis  
              | Role in notochord and somite differentiation  
| CDERMO-1    | Important for organizing the molecular machinery that mediates adrenergic control of calcium reabsorption into sarcoplasmonic reticulum  
              | The earliest known marker of prospective feather tracts  
              | Blocks myogenic differentiation  
              | Negative regulator for gene transcription and apoptosis  
              | Role in notochord and somite differentiation  
| CCND2       | Supports cortical intermediate progenitor cells divisions in embryonic cortex  
              | Developmental regulator  
              | Regulator of the G1/S transition of the cell cycle  
| CUBN        | Required for embryonic development and somite formation  
              | Suggested important role in the development of the peri-implantation embryo  
| WNT11       | Important in cardiac specification and morphogenesis  
              | Essential role during chick myogenesis in the oriented elongation of the miocytes  
              | Role in regulation of gastrulation cell movements in avian embryo; essential for normal CE movements in vertebrate gastrulation  
              | Regulator in skeletogenesis  
              | May be involved in formation of somites  
| S100A11     | Can play dual role in growth regulation of epithelial cells  
| GSTK1       | Antioxidant enzyme that function in detoxification  
| AQP5        | Regulate water homeostasis in several organs as nephron, salivary and lacrimal glands, the inner ear, the reproductive system, the gastrointestinal tract  
              | Involved in inducing cell invasion in tumors  
| MALL        | Role during implantation  
| RGS2        | In yest has a role in cell cycle control and is essential for mitochondrial function and maintenance of mitochondrial genome  
| PPA2        | One of its isoforms was associated with myogenesis  
| KCNAB2      | Role in gastrulation and cell movements  
| CSK         | Antiproliferative activities  
              | Regulate apoptosis and cell cycle arrest concurrently  
              | Essential role in cell number control during retinal development  
              | Involved in the control of embryonic growth  
| PLAGL1      | Suggested role in the control of cell fate during neurogenesis, chondrogenesis and myogenesis  
| MRPL39      | Mitochondrial ribosomal protein  
| SNF8        | Mediate sorting of ubiquitinated membrane proteins into intraluminal endosomal vesicles destined for degradation in lysosomes  

Functions of genes either up or down-regulated 48 h after the injection of 1 μg of αD11 or 9E10 as described in Table 1.