

RGM and its receptor neogenin regulate neuronal survival

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Repulsive guidance molecule (RGM) is an axon guidance protein that repels retinal axons upon activation of the neogenin receptor. To understand the functions of RGM–neogenin complexes *in vivo*, we used gene transfer technology to perturb their expression in the developing neural tube of chick embryos. Surprisingly, neogenin over-expression or RGM down-expression in the neural tube induces apoptosis. Neogenin pro-apoptotic activity in immortalized neuronal cells and in the neural tube is associated with the cleavage of its cytoplasmic domain by caspases. Thus neogenin is a dependence receptor inducing cell death in the absence of RGM, whereas the presence of RGM inhibits this effect.

RGM is a glycosylphosphatidylinositol (GPI)-anchored protein recently isolated from chick optic tectum. RGM induces the collapse of growth cones from temporal retinal axons and repels them in stripe assays, suggesting that it controls the development of the retinotectal projection¹. However the whole spectrum of biological functions modulated by RGM in the developing brain is still unknown.

Using *in situ* hybridization, we found that RGM expression starts very early in the developing central nervous system (CNS) in almost the entire neural plate at the 3-somite stage (data not shown). At stage HH17 (ref. 2; embryonic (E)2.5), RGM expression was observed in the neuroepithelium of the telencephalon, rostral mesencephalon and metencephalon, but not in the caudal mesencephalon (Fig. 1a, d). By HH21 (E3.5), RGM was still expressed in the mesencephalon in a high-rostral to low-caudal manner (Fig. 1b). It was by E6–E7 that the low rostral to high-caudal gradient of expression of RGM was established in the tectum¹. This widespread expression of RGM in the neural tube at very early stages of development suggested that it has other functions along with the control of retinal axon pathfinding.

Interestingly, a biochemical screen for an RGM ligand led to the observation that neogenin (that was initially identified as a homologue of DCC (deleted in colorectal cancer) and a netrin-1 receptor)^{3,4} was a high affinity receptor for RGM, mediating its repulsive activity for retinal axons⁵. Neogenin is also expressed in the CNS early in development. Thus at stage HH17–18 many neurons at the surface of

the mesencephalon expressed neogenin (Fig. 1c). At this stage, horizontal sections of the mesencephalon showed that neogenin and RGM were both expressed in the ventricular zone in a high-rostral to low caudal gradient (Fig. 1d, e). However, RGM mRNA was homogeneously distributed throughout the ventricular zone whereas neogenin expression was stronger on the lumen side (Fig. 1f, g).

We therefore examined whether neogenin also mediates the biological activities of RGM in the neural tube. We electroporated either RGM or neogenin together with a green fluorescent protein (GFP) expression construct in HH10–11 embryos. The electroporated area covers the dorsal metencephalon, mesencephalon and caudal diencephalon (see Supplementary Information, Fig. S1a). RGM over-expression promoted the expression of β -tubulin, an early pan-neuronal marker, thus suggesting that RGM either directly or indirectly is involved in differentiation events (E.M. and A.C., unpublished observations). Surprisingly, when a GFP expression construct was co-electroporated with neogenin, there was a net decrease of the number of GFP-expressing cells that could be detected in the neural tube at HH17, 24 h after electroporation (Fig. 2c; and see Supplementary Information, Fig. S1a, b). This suggested that neogenin expression could induce cell death. We obtained similar results in the mesencephalon and metencephalon (data not shown). Accordingly, we observed a strong increase of the number of TUNEL (TdT-mediated dUTP nick end labelling) positive cells on the experimental side 24 h after neogenin electroporation (Figs 2a, b, 5i; $n = 10/10$). Many cells stained with anti-activated caspase-3 antibody were also detected on the experimental side (Fig. 5e). Moreover, electroporation of the baculovirus p35 (a general and widespread inhibitor of caspase activity)^{6,7} completely inhibited neogenin-induced cell death ($n = 6/7$; $P < 0.0001$; Fig. 5h). Thus, neogenin triggers apoptotic cell death in the neural tube.

This pro-apoptotic activity of neogenin recalls the behaviour of the closely related receptor DCC. Indeed, DCC is a receptor for netrin-1 mediating the chemo-attractivity of this molecule⁴, but DCC was also shown to display a pro-apoptotic activity when unbound to netrin-1 (ref. 8). As such, DCC belongs to the so-called functional family of dependence receptors⁹ that also includes Patched (Ptc), Rearranged

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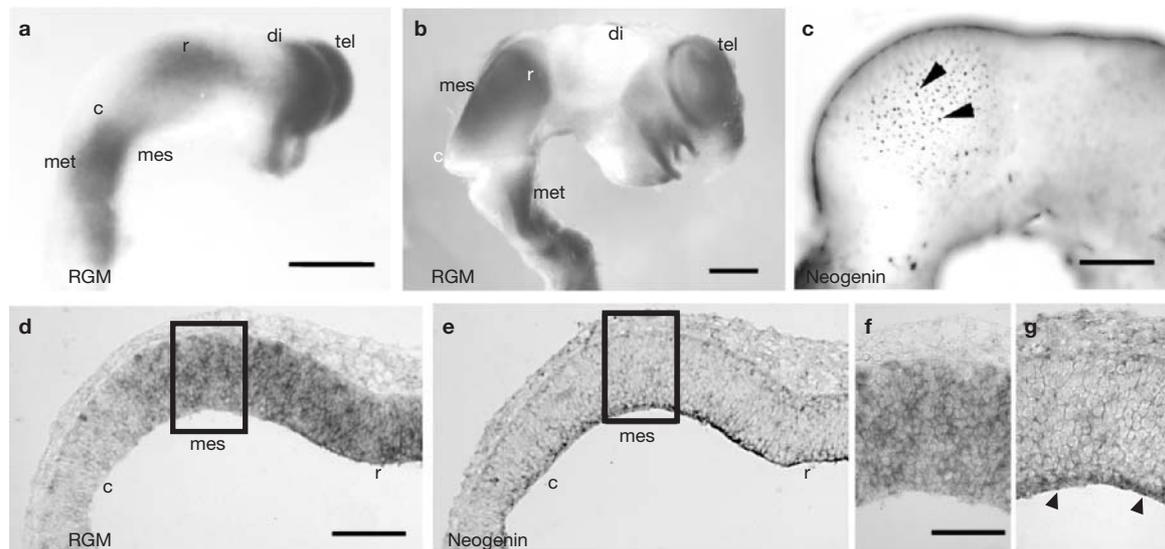


Figure 1 RGM and Neogenin expression in early chick embryos. (a, b) Whole mount *in situ* hybridization for RGM at HH17 (a) and E3.5 (HH20) (b). (c) Whole mount *in situ* hybridization for neogenin. At HH18 neogenin is expressed in scattered neurons (arrowheads) at the surface of the mesencephalon. (d–g) Serial sections of the mesencephalon of HH17 embryos hybridized with RGM (d, f) or neogenin (e, g) riboprobes. f and g are

higher magnification of the boxed area in d and e. At this stage, both genes are expressed in a high rostral (r) to low-caudal (c) gradient throughout the ventricular zone (VZ). In addition, neogenin expression is higher on the lumen side of the ventricular zone (arrowheads in g). di, diencephalon; mes, mesencephalon; met, metencephalon; tel, telencephalon. Scale bars are 500 μ m (a, b), 200 μ m (c), 115 μ m (d) and 65 μ m (f).

during transfection (RET), uncoordinated gene-5 homologue (UNC5H1-3), androgen receptor (AR), p75 neurotrophin receptor (p75^{ntr}) or integrins such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Such receptors induce apoptosis when their ligand is unavailable and are consequently thought to induce a cellular state of dependence toward ligand availability.

To determine whether neogenin is also a dependence receptor, we evaluated the ability of RGM to rescue the pro-apoptotic activity of neogenin. When neogenin, GFP and RGM were simultaneously co-electroporated no difference in TUNEL staining was detected between the control and experimental side ($n = 5/7$; $P < 0.0001$; Figs 2d–f, 5i). When RGM was co-electroporated with GFP (at HH17) there was no difference in the number of TUNEL positive cells in the control and experimental side ($n = 4/4$; Fig. 5i and see Supplementary Information, Fig. S1c–f). A similar inhibition of neogenin-induced cell death was obtained using an expression construct encoding a diffusible form of RGM ($n = 5/5$; $P < 0.0001$; Fig. 2h, i) lacking its GPI anchor and fused to placental alkaline phosphatase (RGM–AP¹).

To examine further the function of the RGM–neogenin interaction in the control of apoptosis, RGM expression was repressed using small interference RNA constructs^{10,11} for RGM (RGM siRNA) electroporated in the neural tube. RGM siRNA expression specifically repressed RGM expression ($n = 6/6$, Fig. 3a, b). Similarly RGM siRNA was able to repress the expression of a RGM–GFP construct ($n = 8/8$; see Supplementary Information, Fig. S2a, b). As shown with TUNEL staining, the electroporation of RGM siRNA ($n = 10/13$) and not of control siRNA constructs ($n = 6/6$, see Methods) also induced apoptosis on the experimental side (Figs 3c–f, 5i). RGM siRNA also induced caspase-3 activation on the electroporated side (Fig. 5f). No evidence for increased cell death was detected in older embryos, probably due to the rapid death of cells expressing high levels of RGM siRNA. These results, together with the fact that neogenin expression induces cell death and that this activity is repressed by co-expression of RGM, suggests that RGM functions as a cell survival factor to counteract neogenin pro-apoptotic activity. This conclusion was supported by the results of co-electroporation of RGM siRNA and neogenin siRNA. In

this condition, the proportion of TUNEL positive cells was at control level ($n = 7/7$; $P < 0.0001$; Figs 3h, 5i). Electroporation of neogenin siRNA alone also did not modify the level of TUNEL positive cells compared to controls ($n = 8/8$; Figs 3g, 5i). Overall, these results show that neogenin is a *bona fide* dependence receptor *in vivo*.

We next investigated whether full-length neogenin also displays a pro-apoptotic activity in immortalized neuroblast 13.S.24 cells (Fig. 4a). As shown in Fig. 4a, expression of neogenin induces caspase activity, whereas addition of the general caspase inhibitor zVAD-fmk (ref. 7) or of the neogenin ligand RGM inhibited neogenin-induced caspase activation. Thus, both *in vivo* and in cell culture, unbound neogenin displays pro-apoptotic activity. Interestingly, although purified netrin-1 was able to block DCC-mediated apoptosis⁸ (data not shown), the addition of netrin-1 instead of RGM had no effect on neogenin-induced caspase activation. Similarly, electroporation of netrin-1 together with neogenin failed to inhibit neogenin-induced cell death in chick embryo ($n = 7/7$; Fig. 2g). This suggests that netrin-1 is not a dependence ligand for neogenin.

To start elucidating the molecular mechanisms of neogenin-induced cell death, we further analysed the involvement of caspases. The dependence receptors DCC, UNC5H and Ptc were shown to require a preliminary caspase cleavage to induce cell death^{6,7,9} because: (1) these receptors are cleaved *in vitro*, and (2) mutation of the cleavage site abrogated the receptor's pro-apoptotic activity. We therefore analysed the ability of the intracellular domain of neogenin to be cleaved by caspases. *In vitro* translation of the intracellular domain (IC) was performed and incubated in the presence of purified active caspase-3 or -8. Whereas caspase-8 had no effect on neogenin-IC, a single cleavage was seen with caspase-3 (Fig. 4b). Hence, neogenin-IC is cleaved *in vitro* by caspases, most effectively by caspase-3. The caspase cleavage site was mapped by constructing mutants based on: (1) the known requirement of an aspartic acid residue for cleavage, and (2) the consistency with the fragment size shown in Fig. 4b. Whereas the mutation of numerous aspartic acid residues had no effect on neogenin cleavage by caspase-3, the mutation D1323N (Fig. 4c and

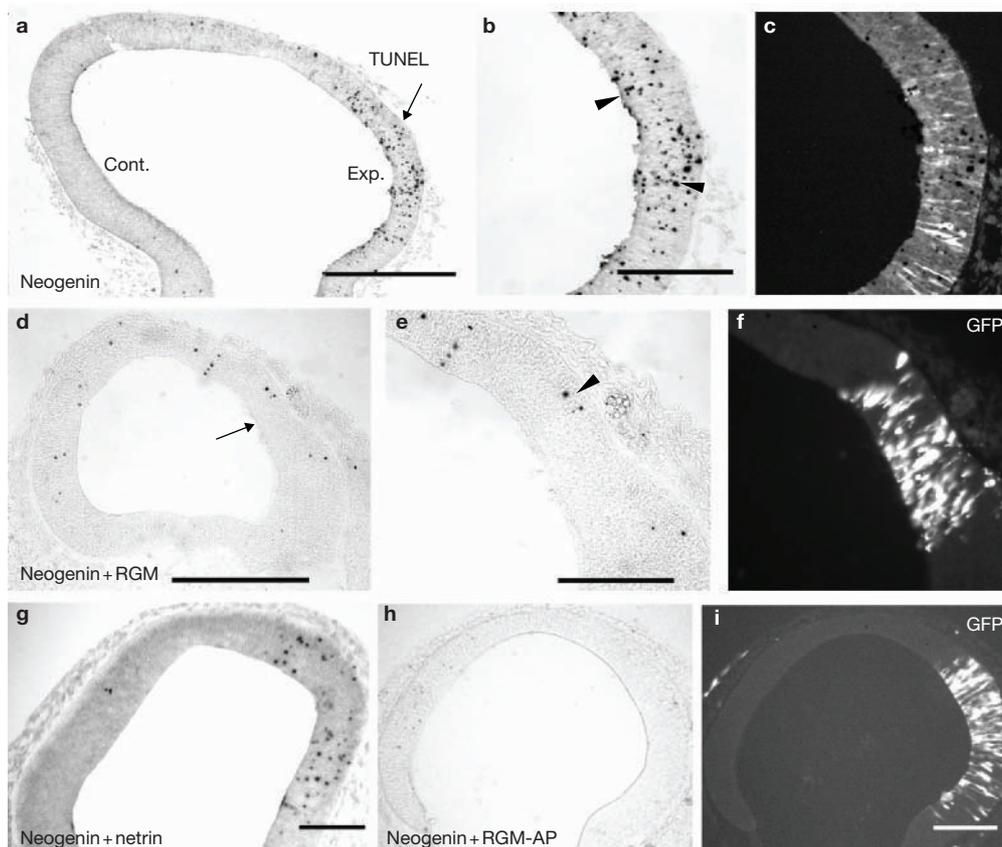


Figure 2 Neogenin over-expression induces cell death in the neural tube. All bright field images are TUNEL staining, others show GFP expression. (a–c) TUNEL staining 24 h after neogenin/GFP co-electroporation. **b** and **c** are higher magnifications of the area indicated by an arrow in **a**. The number of TUNEL positive cells is increased on the electroporated side (arrowheads in **b**) but only a few GFP positive cells are detected (**c**). (d–f) RGM represses the pro-apoptotic activity of neogenin. (**d**, **e**) TUNEL staining 24 h after neogenin/RGM/GFP co-electroporation. **e** and **f** are higher magnifications of the area indicated by an arrow in **d**. The

number of TUNEL positive cells is similar on both control and experimental side and the number of GFP expressing cells is strongly increased (compare with **b** and **c**). (**g**) Netrin-1 does not repress the pro-apoptotic activity of neogenin. TUNEL staining 24 h after netrin-1 and neogenin/GFP co-electroporation. (**h**) A soluble form of RGM (RGM-AP) also represses the pro-apoptotic activity of neogenin. TUNEL staining 24 h after co-electroporation with RGM-AP/neogenin/GFP. cont., control side; exp., experimental side. Scale bars are 300 μ m (**a**), 200 μ m (**d**), 175 μ m (**g**, **i**), 125 μ m (**b**) and 100 μ m (**e**).

data not shown) suppressed the main caspase-3 cleavage of neogenin-IC, demonstrating that neogenin is cleaved just after Asp 1323. Interestingly, the caspase cleavage consensus (usually composed of the four amino acids preceding the caspase cleavage) is CCTD, a non-classic caspase consensus site corresponding however to a highly conserved amino-acid sequence in neogenin from chicken to mammals.

To determine the functional role of the caspase cleavage, full-length neogenin mutated in its caspase cleavage site (neogenin^{D1323N}) was expressed both in 13.S.24 cells or *in ovo*. Mutation of the caspase cleavage site completely inhibited neogenin pro-apoptotic activity *in vitro* (Fig. 4d) and failed to induce cell death in the neural tube ($n = 5/6$; Fig. 5a, b, i). Thus, similarly to DCC, UNC5H or Ptc, neogenin needs to be cleaved by caspase to be a pro-apoptotic molecule. However, even though the cleavage seems important *in vivo* (because the mutation of the cleavage site inhibits neogenin pro-apoptotic activity) it should be noted, that as for DCC, UNC5H or Ptc, we failed to detect the cleavage of neogenin in cell culture or *in vivo*, probably because of the short half-life of these cleaved receptors⁹. Similarly, because caspases are usually thought to be inactive unless activated upon pro-apoptotic stimuli, it is yet unclear, how the

process of receptor cleavage is initiated. One possibility is that the process may be initiated by a non-caspase protease, then propagated through caspase cleavage. Only a few cleavage events by a non-caspase protease would be sufficient to initiate the cell death pathway by activating local caspase to generate a caspase amplification loop through these receptors. An alternative view is that the dogma suggesting that caspases are completely inactive in non-apoptotic cells and are only activated massively upon pro-apoptotic stimuli, is wrong⁹. Thus, cell death induction could be the result of caspase activation amplification rather than caspase initiation *per se*.

Because the caspase cleavage of neogenin was consequently hypothesized to release/expose a pro-apoptotic domain, we then expressed the two resulting fragments of neogenin issued from the caspase cleavage, both in 13.S.24 cells or *in ovo*. Whereas the carboxy-terminal fragment after Asp 1323 displays no pro-apoptotic activity, neogenin truncated after amino acid 1323 (neogenin^{1–1323}) induced caspase activation in 13.S.24 cells (Fig. 4d). Similarly, electroporation of neogenin^{1–1323} induced cell death in the neural tube (Fig. 5c–d, i), suggesting that the caspase cleavage authorized the exposure of a pro-apoptotic domain located in the amino-terminal part of the

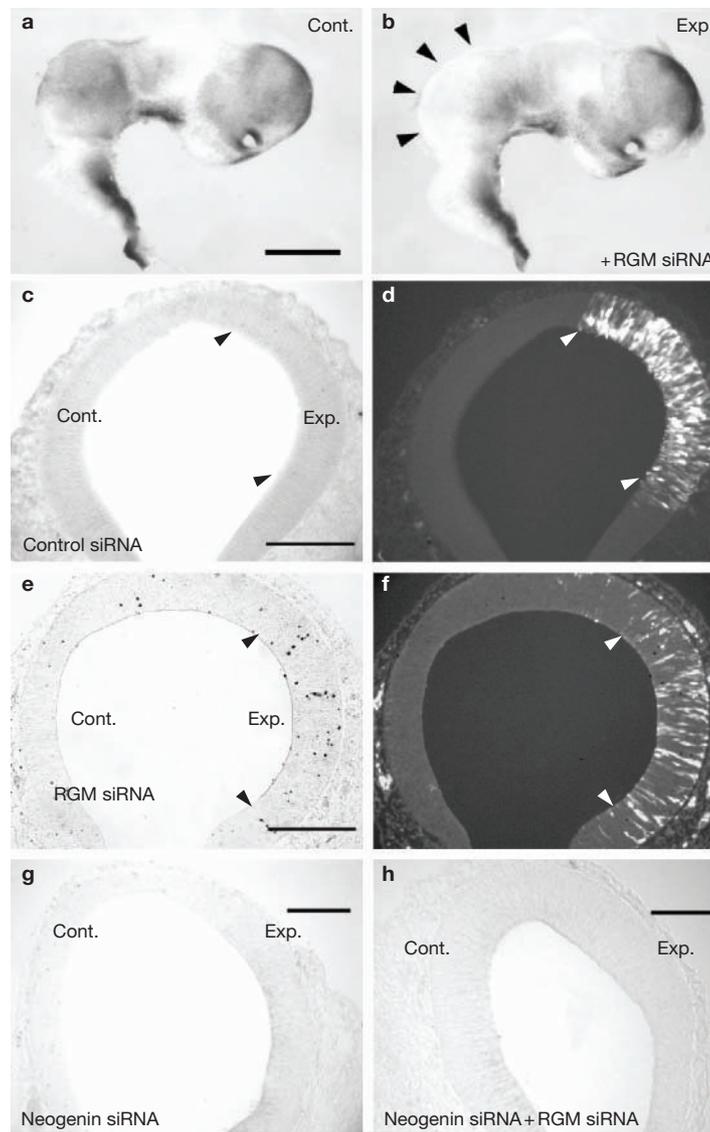


Figure 3 RGM downregulation induces cell death *in vivo*.

(a, b) RGM expression is downregulated after electroporation with RGM siRNA. Whole mount *in situ* hybridization for RGM of HH17 embryos 24 h after electroporation. RGM expression in the mesencephalon (arrowheads in b) is strongly decreased on the experimental side (exp., b) compared with the control side (cont., a). (c, d) TUNEL staining 24 h after electroporation of control siRNA. No increased cell death is detected in the electroporated area (arrowheads in c and d). (e, f) TUNEL staining 24 h after electroporation of RGM siRNA. The

number of TUNEL positive cells is increased on the experimental side (exp., arrowheads in f and d) compared with the control side (cont.). GFP expression is also lower compared with embryos electroporated with control SiRNA (compare d and f). (g) TUNEL staining 24 h after electroporation of neogenin siRNA. No increased cell death is detected in the experimental side (exp.). (h) TUNEL staining 24 h after co-electroporation of neogenin siRNA and RGM siRNA. No TUNEL positive cells are detected on the experimental side (exp.). Scale bars are 500 μm (a), 150 μm (c, e) and 120 μm (g, h).

intracellular domain of neogenin before the caspase cleavage site. Moreover, whereas wild-type neogenin triggers cell death only in the absence of RGM, the pro-apoptotic activity of neogenin¹⁻¹³²³ failed to be inhibited by co-electroporation of its ligand RGM ($n = 5/5$; Fig. 5g, i). Thus, RGM binding to neogenin antagonized the caspase cleavage of neogenin, an event that otherwise allows the exposure of a pro-apoptotic domain located upstream of the caspase cleavage site and consequently triggers cell death.

In this study, we show that RGM, which was originally isolated as a repulsive axon guidance molecule¹⁻⁵, also controls cell survival. We also

provide direct *in vitro* and *in vivo* evidence showing that neogenin is a dependence receptor. Interestingly, DCC and UNC5H, which were shown to bind the secreted factor netrin-1 similarly to neogenin, are also dependence receptors and axon guidance molecules^{6,8,12}. Thus, a conserved feature of many receptors for axon guidance molecules may be to trigger cell death of neurons either because these neurons have failed to make stable contacts with their target cells or because they have migrated or extended axons in inappropriate regions where the ligand is not or only poorly expressed. At later stages of chick development when neuronal differentiation is more complete, perturbation of

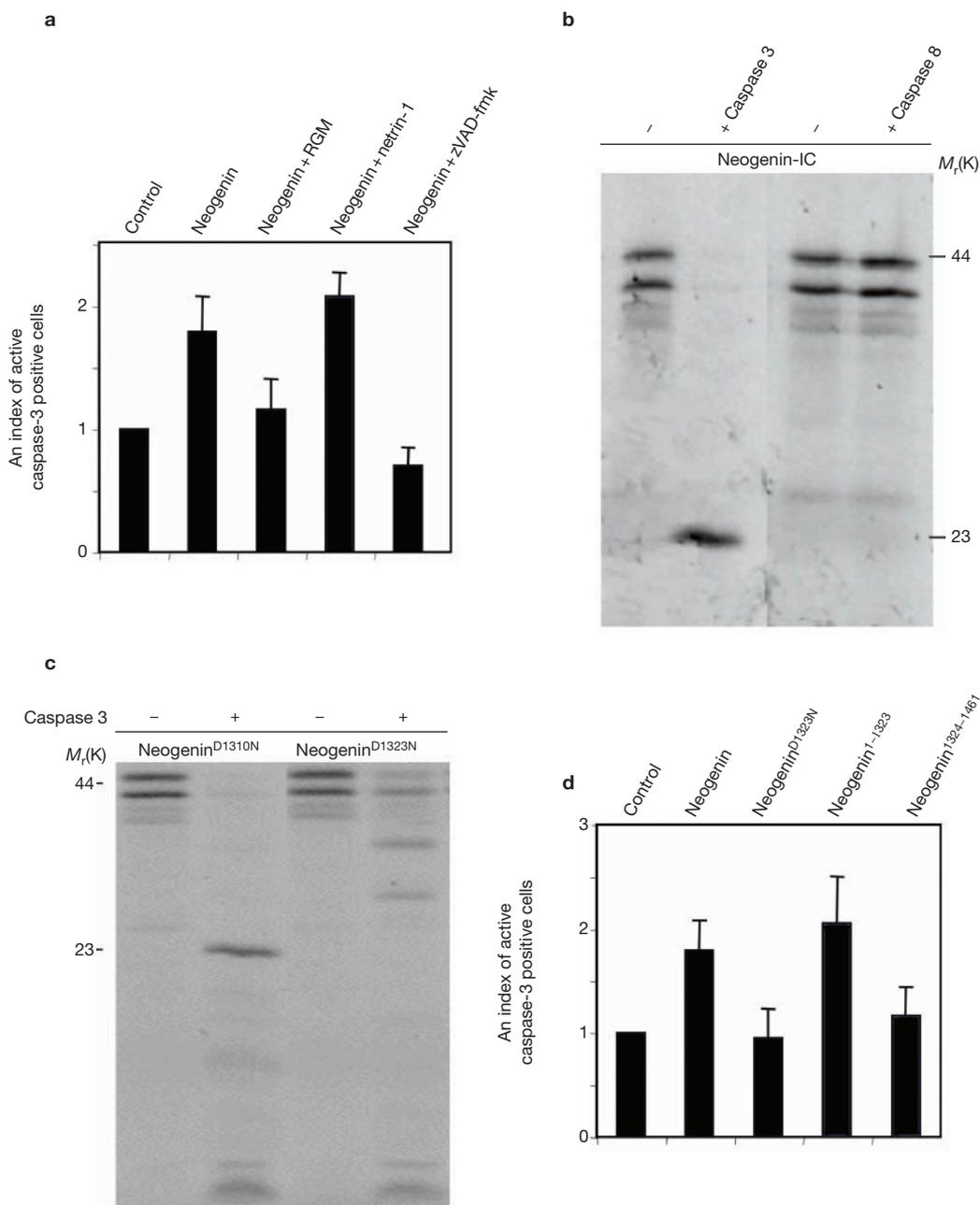


Figure 4 Neogenin is a dependence receptor. **(a)** Neogenin-induced caspase activation measured by numeration of cells stained with anti-active caspase-3 antibody. Note the increase of 13.S.24 cells that display caspase-3 activity when transfected with neogenin. An index of cells with active caspase-3 staining is indicated with standard deviations ($n = 3$). Neogenin-induced cell death is inhibited in the presence of RGM but not in the presence of netrin-1. **(b)** Neogenin is a caspase substrate. *In vitro*-translated neogenin-IC was incubated in the absence of caspase (-) or with purified caspase-3 (0.3 μ M) or caspase-8 (0.3 μ M). An

autoradiograph is shown. **(c)** The *in vitro* cleavage was performed as in **b** with neogenin-IC^{D1310N} or neogenin-IC^{D1323N}. Note that only the mutation neogenin-IC^{D1323N} prevents the presence of the 23 kDa cleavage fragment. Note also, in this case, the apparition of a few aberrant cleavage fragments that probably result from cryptic caspase sites. **(d)** Neogenin caspase cleavage is required for Neogenin proapoptotic activity. An index of 13.S.24 cells stained with anti-active caspase-3 antibody is presented after transfection with either wild-type neogenin, neogenin^{D1323N}, neogenin¹⁻¹³²³ or neogenin¹³²⁴⁻¹⁴⁶¹.

RGM or neogenin functions cause a degree of axonal mis-targeting in the retinectal pathway (A.C. and E.M., unpublished observations) supporting previous *in vitro* work.

It seems that RGM controls a variety of biological functions in the developing CNS that are mediated to a large extent by neogenin. However, as netrin-1 was also shown to bind to neogenin it is possible

that neogenin exerts additional function upon netrin-1 binding. Alternatively, netrin-1 could modulate RGM function in some neurons.

The neogenin related receptors UNC5H and DCC are all putative tumor suppressors that are submitted to loss of expression or mutation in a wide number of cancers^{13,14}. Interestingly, sustained RGM over-expression using the RCAS (replication-competent ASLU long

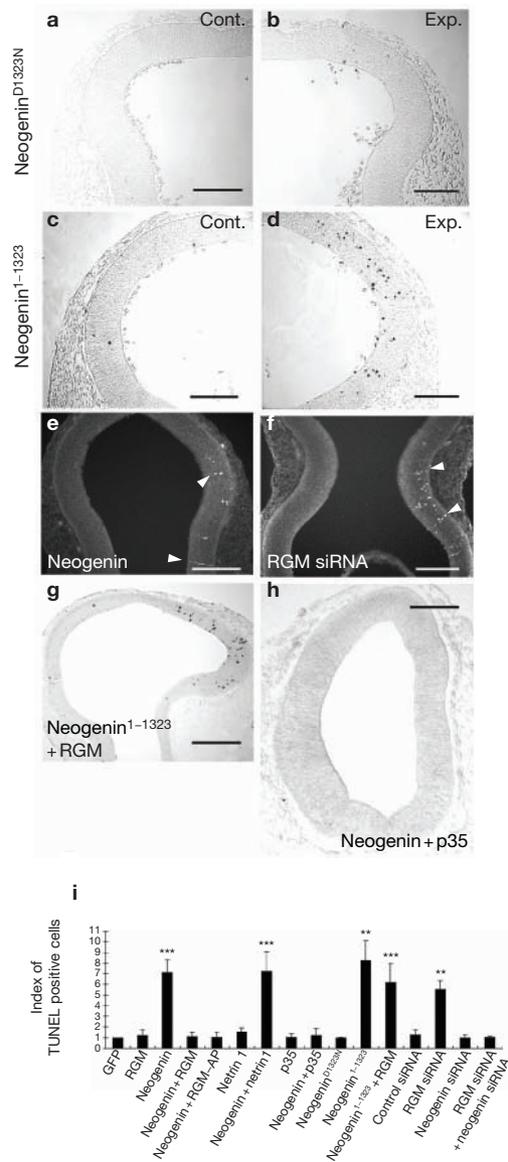


Figure 5 Pro-apoptotic potential of neogenin mutants *in vivo*. (a, b) TUNEL staining 24 h after electroporation of a full-length neogenin mutated in its caspase cleavage site (neogenin^{D1323N}). The number of TUNEL positive cells is similar on the experimental side (exp.) and the control side (cont.). (c, d) TUNEL staining 24 h after electroporation of Neogenin¹⁻¹³²³. A massive cell death is induced on the experimental side (d, exp.) compared the control side (c, cont.). (e, f) Immunostaining for activated caspase-3 after neogenin (e) or RGM siRNA (f) electroporation. Many cells expressing activated caspase-3 (arrowheads) are observed on the electroporated side. (g) TUNEL staining 24 h after co-electroporation of RGM together with Neogenin¹⁻¹³²³. (h) TUNEL staining 24 h after co-electroporation of neogenin together with the caspase inhibitor p35. (i) Quantification of cell death in neural tube after electroporation of the different constructs shown on all previous figures (see Methods). The index, with standard deviations ($n = 4-6$), is the ratio of the number of TUNEL positive cells on the electroporated side : control side in each experimental conditions. Three asterisks $P < 0.0001$; two asterisks $P < 0.001$, others are non significant. Scale bars are 225 μm (g), 150 μm (e, f), 125 μm (a, b, c, d) and 80 μm (h).

terminal repeat (LTR) with a splice acceptor) virus induces the disruption of the ventricular zone neuroepithelium (A.C. and E.M., unpublished observations) similar to phenotypes seen in tumorigenesis¹⁵, and neogenin may be involved in breast cancer¹⁶. Thus, as RGM and neogenin (that have been shown here to control cell survival) are expressed in many non neural tissues (ref. 17; and E.M. and A.C., unpublished observations), they may turn out to be crucial regulators of tumorigenesis. The recent phenotypic analysis of mouse knockouts for RGM-A¹⁸ has shown that mice deficient for this molecule exhibit neural tube closure defects, supporting the idea that RGM is not only an axon guidance molecule. Cell death was examined at E8.5 but no increase or decrease in apoptotic cell death was observed in cephalic neural folds. In the light of our results a detailed quantification should be performed in RGM-A knockout mice at later developmental stages. However, it will be important to study knockout mice of RGM-B, RGM-C and neogenin to get a full picture of the diversity of cell functions mediated by RGM and its receptor. □

METHODS

Site-directed mutagenesis and plasmid construction. Chick RGM cDNA was inserted into MiwiIII vector containing the Rous sarcoma virus (RSV) enhancer¹⁹ (a gift from H. Nakamura) or RCASBP(B) (RCAS Bryan Polymerase (B)) retroviral vector^{20,21} (kindly provided by S. H. Hughes). The expression vector encoding RGM-GFP fusion protein was made by inserting GFP cDNA C-terminal to RGM cDNA. The RGM-AP construct was previously described¹. The plasmid pcDNA3-neogenin-IC encoding the intracellular domain of neogenin was constructed by inserting the intracellular domain of neogenin into pcDNA3.1 (Invitrogen, Carlsbad, CA) obtained by polymerase chain reaction (PCR) with 5'-TGACCATGTGTACCCGTCGTACCACCT-3' and 5'-TTTCATGCTGTTGTGATAGCGTTTAGG-3' primers with pcDNA-neogenin as template. The site mutation in neogenin^{D1323N} was obtained using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) with pcDNA3-neogenin-IC as a template and the following primers: 5'-CAAACATGCTGCACTAATCACCAGGACCCTGAAG-3'; 5'-CTTCAGGGTCTCGGTGATTAGTGCAGCATGTTT-3'.

Neogenin¹⁻¹³²³ was obtained by Quikchange using pcDNA3-neogenin-IC as template and the primers 5'-CAAACATGCACTTAACACGACCCTGAAG-3' and 5'-CTTCAGGGTCTCGGTGTTAAGTGCATGTTT-3'. The construct for expression of the full-length neogenin, neogenin^{D1323N} or neogenin¹⁻¹³²³ was obtained by inserting the *Bgl*II fragment from pcDNA3-neogenin-IC^{D1323N} or pcDNA3-neogenin-IC¹⁻¹³²³, respectively into *Bgl*II cleaved pcDNA-neogenin vector. The construct pcDNA3-neogenin¹³²⁴⁻¹⁴⁶¹ was generated by inserting into pcDNA3.1 Topo (Invitrogen) the PCR fragment issued from using pcDNA-Neogenin as template and the primers 5'-CACCATGGAT-CACCAGGACCCTGAAG-3' and 5'-TTTCATGCTGTTGTGATAGCGTT-TAGG-3'. For siRNA, 5'-CTTCATGCTGGCTGCTTAT-3' was chosen as a target sequence of RGM. For Neogenin, 5'-CATTACCTCCCCTTACT-3' was chosen as the target sequence. For the control, the siRNA construct encoding the sequence 5'-GACACCCATATCTCTTCT-3' was used. To drive siRNA expression in the neural tube, all cDNAs were inserted in the pSilencer1.0-U6 vector (Ambion, Huntingdon, Cambridgeshire, UK).

In ovo electroporation. Fertilized chicken eggs were obtained from a local farm and were incubated at 38 °C. The various constructs described above were introduced into stage 10-11 chick embryos by *in ovo* electroporation²²: pMiwIII-RGM, RCASBP(B)-RGM (1.5 or 2 mg ml⁻¹), pMiw-RGM-GFP (3 mg ml⁻¹) and pcDNA-human neogenin (kindly provided by E. R Fearon) (1.5 or 3 mg ml⁻¹), neogenin^{D1323N} (2.0 mg ml⁻¹), neogenin¹⁻¹³²³ (2 mg ml⁻¹), pBabe-p35 (ref. 11) (1.0 mg ml⁻¹), netrin-1 (ref. 6) (1.5 mg ml⁻¹) or siRNA constructs (3 mg ml⁻¹ each). Electroporation was done with CUY-21 electroporator and CUY-610 standard electrode (NEPA gene, Chiba, Japan). We routinely carried out electroporation with 4 pulses of 50 ms duration at 20 V. A GFP expression vector (pMiwIII-GFP, 0.5 mg ml⁻¹) was co-electroporated to check the electroporation efficiency.

TUNEL assay. For TUNEL assay, ApopTag *in situ* Apoptosis detection kit (Chemicon, Temecula, CA) was used according to the manufacturer's instructions. For the secondary antibody, alkaline phosphatase (PLAP)-conjugated anti-digoxigenin goat-polyclonal antibody (Roche Molecular Biochemicals, Mannheim, Germany) was used. Signal was detected with 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics). For RGM-AP electroporated embryos, AP activity was killed by pre-incubation in 5% H₂O₂ for 30 min. The GFP fluorescence and TUNEL assay were not affected by this treatment. The index factor is the ratio between the number of TUNEL cells on the electroporated side : control side. A minimum of four embryos were counted and three sections per embryo for each experimental condition. The statistical significance was determined by variance analysis (ANOVA) with a Fisher PLSD test, using Statview (Abacus Concepts, Channel Islands, UK).

In situ hybridization. Whole mount *in situ* hybridization was performed as described by Bally-Cuif *et al.*²³. For *in situ* hybridization on sections, 20 µm thick sections were cut on a cryostat (Leica, Bannockburn, IL) and processed according to Marillat *et al.*²⁴. DIG-labelled antisense RNA probes were generated using Riboprobe System (Promega, Madison, WI).

Immunohistochemistry. Whole-mount immunostaining was carried out as previously described¹⁹. Embryos were incubated with anti-GFP (1 : 200, Molecular Probes, Eugene, OR), and then with Cy3-conjugated anti-rabbit antibody (Jackson IR, West Grove, PA) overnight at 4 °C. Embryos were cut along the dorsal midline and ventral midline and then flat-mounted. Immunostaining for sections were carried out as previously described¹⁹. Anti-activated caspase 3 (Asp 175) (1 : 200; Cell Signaling Technology, Beverly, MA) primary antibody was used followed by Cy3-conjugated anti-rabbit secondary antibody (Jackson IR, West Grove, PA).

Cell cultures and transfection procedures. Immortalized rat olfactory neuroblast 13.S.24 cells, previously described⁶, were transfected with Lipofectamine as described⁶. The RGM-AP construct and purification was as described previously¹. The general caspase inhibitor zVAD-fmk⁷ was from Tebu-bio (Le Perray en Yvelines Cedex, France).

Caspase activation analysis. Cell death was analysed using active caspase-3 staining procedures as described previously⁶. Briefly, the number of positive cells is determined by counting the number 'positive cells' (that is, active caspase-3 stained) in series of 10 fields of about 300 hundred cells. The index factor is the ratio between the number of dead in the tested condition and the number of dead cells in the control.

In vitro transcription/translation and caspase cleavage reactions. Purified caspases were a generous gift from G. Salvesen. *In vitro* transcription/translation and incubation with caspases 3 or 8 were performed as described previously⁸.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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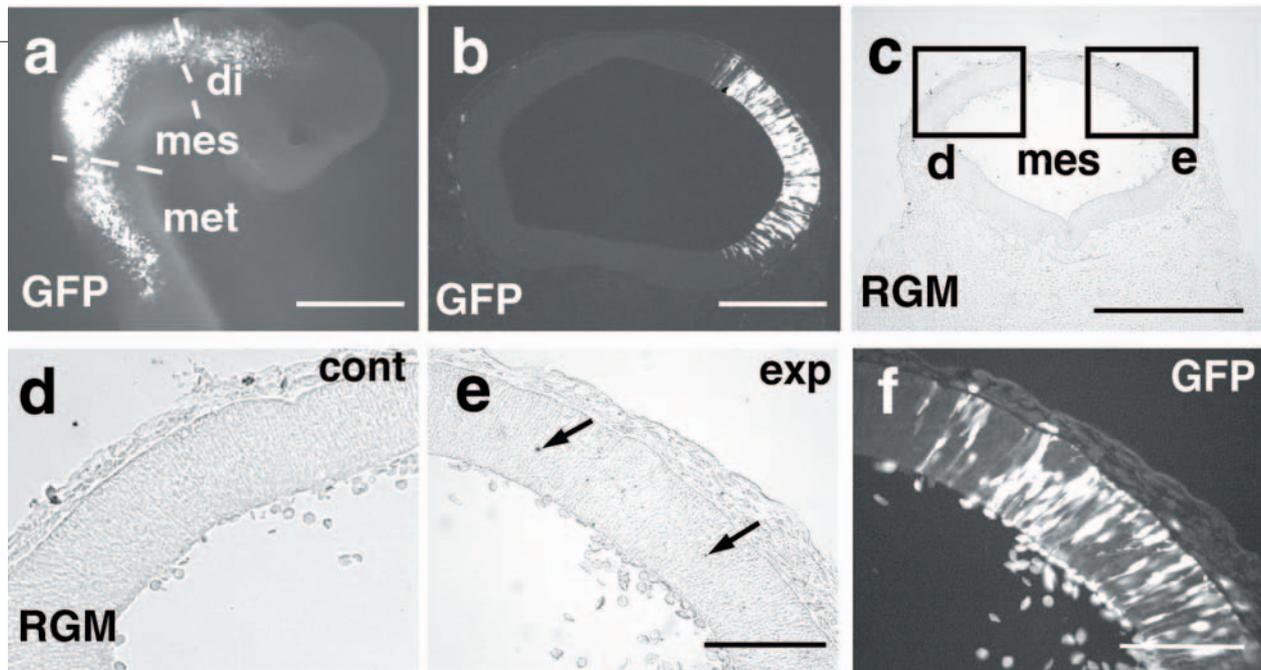


Figure S1 RGM overexpression does not induce cell death. **(a)** whole-mount GFP fluorescence of an embryo electroporated with a GFP expression construct. GFP is expressed in the metencephalon (met), mesencephalon (mes) and caudal diencephalon (di). **(b)** is a cross section at the level of the mesencephalon of a GFP electroporated embryo. **(c-f)** RGM overexpression does not induce apoptosis. **(d-f)** TUNEL staining 24 hours after RCASBP(B)-

RGM and pMiwIII-GFP electroporation. **(d)** and **(e)** are higher magnifications of corresponding areas in **(c)**. In the mesencephalon, only a few TUNEL positive cells are detected on the control **(d)** and experimental **(e)** sides. Many GFP positive cells are found on the experimental side **(f)**. Scale bars are 750 μm **(a)**, 500 μm **(c)** and 100 μm **(e, f)**.

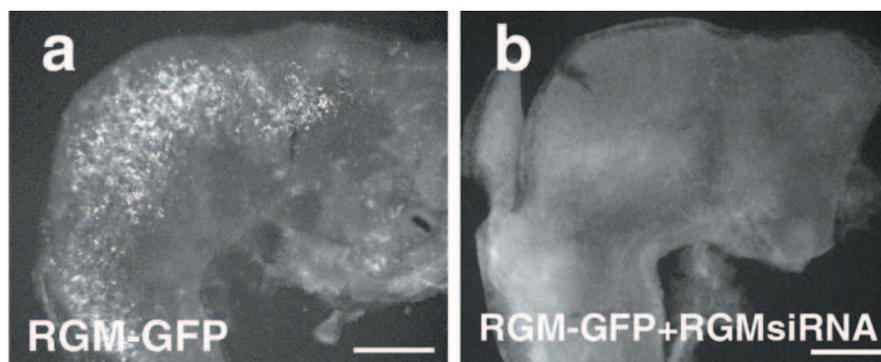


Figure S2 RGM siRNA downregulates RGM-GFP expression. **(a, b)** are whole mount HH17 embryos immunostained with anti-GFP, 24 hours after electroporation of a RGM-GFP construct **(a)** or co-electroporation of

RGM-GFP and RGM siRNA **(b)**. No GFP positive cells are detected after co-electroporation with RGM siRNA. Scale bars are 450 μm **(b)**, 350 μm **(a)**.