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p120RasGAP mediates ephrin/Eph-dependent attenuation of FGF/ERK signals during cell fate specification in ascidian embryos

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SUMMARY

ERK1/2 MAP kinase exhibits a highly dynamic activation pattern in developing embryos, which largely depends on fibroblast growth factor (FGF) signals. In ascidian embryos, FGF-dependent activation of ERK1/2 occurs differentially between sister cells during marginal zone and neural lineage patterning. Selective attenuation of FGF signals by localised ephrin/Eph signals accounts for this differential ERK activation, which controls the binary fate choice of each sibling cell pair. Here, we show that p120 Ras GTPase-activating protein (p120RasGAP) is a crucial mediator of these ephrin/Eph signals. First, inhibition of p120RasGAP has a similar effect to inhibition of ephrin/Eph function during marginal zone and neural patterning. Second, p120RasGAP acts epistatically to ephrin/Eph signals. Third, p120RasGAP physically associates with Eph3 in an ephrin-dependent manner. This study provides the first *in vivo* evidence that the functional association between Eph and RasGAP controls the spatial extent of FGF-activated ERK.

KEY WORDS: p120RasGAP, Ephrin, Ascidian, *Ciona intestinalis*

INTRODUCTION

FGFs are secreted ligands that selectively bind to a class of receptor tyrosine kinases (RTKs), the FGF receptors (FGFRs). The prominent pathway downstream of FGFR is the Ras/MAPK cascade, whereby a GTP-bound form of Ras activates a cascade of kinases leading to activation of ERK1/2 MAP kinase (Turner and Grose, 2010). FGF/ERK signalling is implicated in many cell fate specification and patterning events during the development of a wide range of deuterostomes (Bertrand et al., 2011; Green et al., 2013; Dorey and Amaya, 2010; Lemaire, 2009). In mouse embryos, ERK1/2 (also known as MAPK3/1) activation is observed in a highly dynamic manner and is largely FGFR dependent (Corson et al., 2003). Consistently, the spatial distributions of ERK1/2 activation correspond well with the discrete expression domains of FGF genes (Corson et al., 2003). Several mechanisms contribute to the control of the spatial extent of ERK1/2 activation. Diffusion and receptor-mediated endocytosis of FGF ligands has been shown to set up a gradient of Fgf8 in zebrafish embryos (Scholpp and Brand, 2004; Yu et al., 2009). Another layer of regulation includes crosstalk with other signalling pathways. An antagonistic relationship between retinoic acid (RA) and FGF signals during body axis elongation in vertebrate embryos controls the spatial extent of graded ERK activation. This occurs via RA-mediated repression of FGF gene expression (Diez del Corral et al., 2003). Similarly, BMP signalling also results in repression of FGF gene expression during heart field, hindbrain and limb bud patterning in vertebrate embryos (Tirosh-Finkel et al., 2010; Weisinger et al., 2008; Zúñiga et al., 1999).

Ascidian embryos develop with a fixed cleavage pattern and small number of cells, enabling the spatial pattern of ERK1/2 activation to be visualised at the level of individual cells. In *Ciona intestinalis*, several cases of differential ERK activation between sister cells have been described during marginal zone and neural lineage patterning. This differential ERK activation between sister cell pairs dictates their differential fate specification (Hudson et al., 2007; Stolfi et al., 2011; Wagner and Levine, 2012; Yasuo and Hudson, 2007) (reviewed by Lemaire, 2009). Importantly, in several of these cases, differential ERK activation is achieved via ephrin/Eph-dependent attenuation of FGF/ERK signalling (Picco et al., 2007; Shi and Levine, 2008; Stolfi et al., 2011). However, it remains to be addressed how ephrin/Eph signals attenuate FGF signals in this embryonic context.

Eph receptors form the largest subfamily of RTKs and interact with membrane-bound ligands – the ephrins (Arvanitis and Davy, 2008). Although ephrin/Eph signals are involved in a wide range of developmental processes, their underlying biological function is attributed principally to modulation of cytoskeletal organisation and cell adhesion. Consistently, Eph receptors associate with guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) for small GTPases such as Rac and Rho (Klein, 2012). In certain cell lines, however, ephrin/Eph signalling affects cell behaviours by inhibiting ERK1/2 activation (Elowe et al., 2001; Miao et al., 2001; Kim et al., 2002; Minami et al., 2011). In some of these cases, this Eph-mediated ERK attenuation is mediated by p120RasGAP (also known as Rasa1) (Elowe et al., 2001; Kim et al., 2002; Minami et al., 2011). p120RasGAP is a multi-domain protein containing SH2-SH3-SH2, PH and C2 domains and a GAP catalytic domain (Boguski and McCormick, 1993). It inhibits Ras by increasing the intrinsic rate of GTP hydrolysis and has been

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shown to associate directly with several activated RTKs, including Eph receptors (Anderson et al., 1990; Margolis et al., 1990; Holland et al., 1997). However, the physiological and developmental roles of p120RasGAP remain to be fully addressed and, in particular, its *in vivo* role in association with ephrin/Eph signals remains unknown. In this study, we reveal that p120RasGAP mediates all cases of ephrin/Eph-dependent attenuation of FGF signals that have been described so far during *Ciona* embryogenesis.

MATERIALS AND METHODS

Embryo culture, electroporation and manipulations of *Ciona intestinalis*

Adult *Ciona intestinalis* were purchased from the Roscoff Marine Biological Station (Roscoff, France) and M-REP (San Diego, CA, USA). Electroporation and microinjection were carried out as described previously (Christiaen et al., 2009; Sardet et al., 2011). All the data presented in this study were collected from at least two independent experiments.

Reagents

RGΔGAP was generated by PCR amplifying cDNA fragments corresponding to amino acids 1 to 585 using cDNA clone cibd054f08 (Satou et al., 2002) and subcloning into pRN3 (Lemaire et al., 1995). RG(R818E) was generated by PCR-based introduction of a point mutation resulting in an arginine-to-glutamate substitution at amino acid residue 818 (Miao et al., 1996). The RG(R818E) ORF was subcloned into pRN3 for RNA injection or placed under the *fgf8* promoter for electroporation (Imai et al., 2009). *fgf8>RGΔGAP* was generated by digesting *fgf8>RasGAP* with *Eco*RV, followed by religation, which results in removal of a large part of the GAP domain. RNA concentrations used for microinjections were 0.5 μg/μl for *ephrin-Ad*, 1.0 μg/μl for *Eph3ΔC* and 1.5 μg/μl for *RGΔGAP* and *RG(R818E)*. Ectopic expression of *Bra* was also sometimes seen in neural lineages with a RasGAP morpholino (5′-CCATTTACACCAAACATC-TAAACAC-3′; Gene Tools). However, this morpholino is toxic and results were variable so we chose to pursue our analysis using dominant-negative forms of RasGAP.

For *FOG>ephrin-Ad* and *FOG>venus*, the ORF of each cDNA was subcloned in place of the RfA cassette of pSP1.72BSSPE-pFOGc::RfA (Roure et al., 2007). For *FOG>Eph3-3xHA*, the *Eph3* ORF was subcloned into pSP1.72BSSPE-pFOGc::RfA in place of the RfA cassette to generate *FOG>Eph3*. Annealed oligonucleotide pairs encoding three tandem HA tags were then subcloned in-frame into *FOG>Eph3*. To construct *FOG>RasGAP-6xMyc*, the *RasGAP* ORF was first subcloned into pCS2+-6xMyc, then RasGAP-6xMyc replaced the RfA cassette of pSP1.72BSSPE-pFOGc::RfA.

In situ hybridisation and immunohistochemistry

Chromogenic and fluorescent *in situ* hybridisation and β-galactosidase detection in *Ciona* embryos were carried out as described previously (Hudson et al., 2013; Beh et al., 2007). Dig-labelled probes were synthesised from the following cDNA clones: *Bra* (Corbo et al., 1997), *ETR* (Hudson et al., 2003), *Mnx* (Stolfi et al., 2011), *NoTrlc* (citb018l16) and *Titf* (ciad042d09). Images in Fig. 1 and Fig. 2L were taken on an Olympus BX51 and those in Fig. 3 on a Leica DM2500.

For immunodetection of diphosphorylated (dp) ERK1/2, the protocol described previously (Stolfi et al., 2011) was used with slight modifications. Embryos were fixed in 1 ml PIPES-sucrose-FA buffer for 30 minutes at room temperature with constant rotation. Fixed embryos were washed in PBS/0.1% Triton X-100 and then treated with PBS/0.1% Triton/3% H_2O_2 for 10 minutes. After washing in PBS/0.1% Triton, embryos were blocked in PBS/0.1% Triton/0.5% Blocking Reagent (Roche Applied Science) for 1 hour and then incubated overnight with monoclonal mouse anti-dpERK1/2 antibody (1:500; Sigma M9692) at 4°C. After washing in PBS/0.1% Tween 20, immunofluorescence signals were detected as described previously (Hudson et al., 2013). Images were acquired on a Leica SP5 confocal microscope and processed with ImageJ (NIH).

Western blot and co-immunoprecipitation

Western blot analyses of dpERK1/2 were carried out following standard protocols with mouse anti-dpERK1/2 (Sigma M9692), rabbit anti-ERK1/2 (Cell Signaling Technology, 9102) and HRP-linked goat anti-mouse and goat anti-rabbit (Jackson ImmunoResearch, 115-035-166 and 111-035-144) at a dilution of 1:1000.

For the co-immunoprecipitation assay, plasmids (25 μg each) were electroporated into *Ciona* fertilised eggs. At late gastrula stage, embryos were lysed on ice in PLC lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA) with 0.25% Triton X-100 supplemented with a mixture of protease inhibitors (Sigma). Lysates were treated with 10 μl agarose resin coupled with anti-HA antibodies (HA Tag IP/Co-IP Kit, Pierce) for 2 hours and the resin was then washed with lysis buffer. Bound proteins were denatured in 35 μl Laemmli sample buffer at 100°C and then analysed by western blot using rabbit anti-Myc (1:1000; Cell Signaling Technology, 2278) and mouse anti-HA (1:1000; Covance, HA.11). ECL signals (Pierce, 34077) were detected using Kodak Image Station 4000MM. Protein samples were normalised for Eph3-HA signal and then probed for RasGAP-myc.

RESULTS AND DISCUSSION

p120RasGAP is required for correct fate specification during marginal zone and motor ganglion patterning

A requirement for ephrin/Eph-mediated attenuation of FGF/ERK signals has been shown for several binary fate decisions during embryonic patterning of ascidian embryos (Picco et al., 2007; Shi and Levine, 2008; Stolfi et al., 2011). Two such examples are found during patterning of the marginal zone. At the 32-cell stage, notochord-neural and trunk lateral cell (TLC)-endoderm mother cells divide along the animal-vegetal (AV) axis (Fig. 1A). In both cases, the daughter cells on the vegetal pole side (notochord and endoderm precursors, respectively) exhibit FGF-dependent ERK1/2 activation, whereas those on the animal pole side (neural and TLC precursors, respectively) receive an ephrin-Ad signal derived from the animal hemisphere that attenuates ERK1/2 activation. This differential ERK1/2 activation between these sister cell pairs dictates their differential fates (Picco et al., 2007; Shi and Levine, 2008).

Since p120RasGAP has been implicated in Eph-dependent attenuation of ERK1/2 activation in mammalian cell lines (see Introduction), we first tested whether p120RasGAP is required for the specification of cell fates previously shown to be under the control of ephrin/Eph-mediated attenuation of ERK1/2 in *Ciona*. RNAs encoding two dominant-negative forms of *Ciona* p120RasGAP were injected into *Ciona* eggs. RGΔGAP lacks the entire GAP domain (Elowe et al., 2001), whereas RG(R818E) contains a point mutation that compromises GAP-mediated Ras-GTP hydrolysis (Miao et al., 1996). Effects were compared with embryos in which ephrin/Eph signals were inhibited by injecting a truncated form of Eph3 that lacks the intracellular domain (Eph3ΔC) (Picco et al., 2007). The marker genes monitored were *ETR* for neural, *Brachyury* (*Bra*) for notochord, *no trunk lateral cells* (*NoTrlc*) for trunk lateral cells and *Titf* for endoderm (Fig. 1B) (Hudson et al., 2003; Corbo et al., 1997; Tokuoka et al., 2004; Ristoratore et al., 1999). Injection of dominant-negative forms of RasGAP phenocopied the effect of Eph3ΔC albeit with lesser efficiency, with RGΔGAP having a stronger phenotype than RG(R818E) (Fig. 1C-H). Notably, when RasGAP was compromised, the neural and TLC lineages expressed markers of the alternative sister cell fates of notochord and endoderm, respectively.

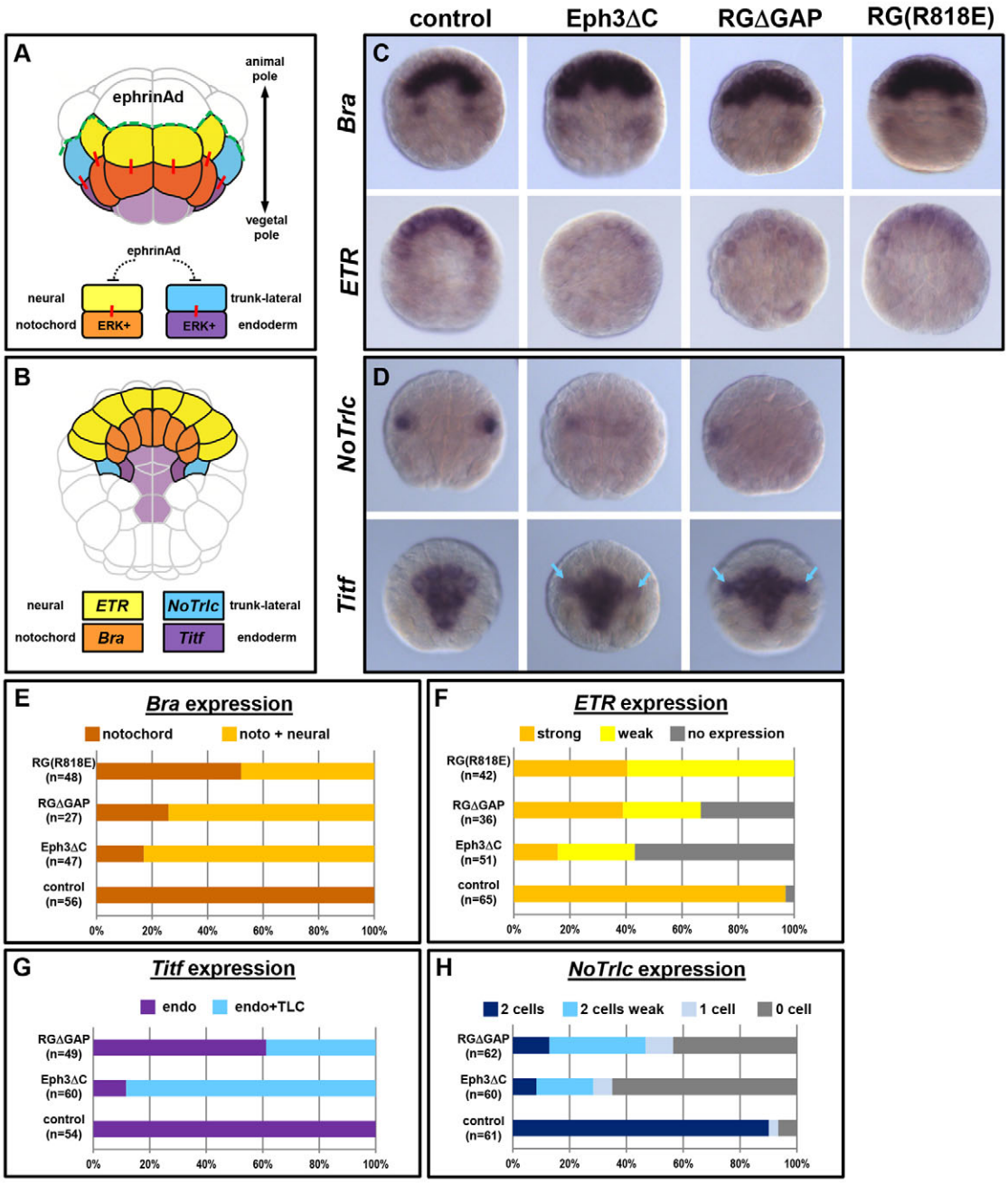

Fig. 1. RasGAP plays a role in marginal zone patterning in *Ciona* embryos. (**A**) Schematic representation of the notochord side view of a 44-cell stage embryo illustrating the cell lineages (colour coded). Red bars indicate sister cell relationships. Dashed green line indicates the frontier between the animal and vegetal hemisphere. (**B**) Schematic representation of the early gastrula embryo (vegetal pole view). Expression domains of marker genes assessed in C and D are shown following the colour code. (**C**,**D**) *In situ* hybridisation analysis to assess the effect of dominant-negative forms of RasGAP and a truncated form of Eph3 on the indicated lineage marker genes, at the early gastrula stage (C) or the 76-cell stage (D). Blue arrows point to ectopic expression of *Titf* in trunk lateral cell precursors. (**E-H**) Quantification of marker gene expression.

Consistent with the above observations, inhibition of p120RasGAP results in ectopic activation of ERK1/2 in neural and TLC precursors (Fig. 2). RNA encoding RGΔGAP or Eph3ΔC was injected into one cell of the 2-cell stage embryo and ERK1/2 activation was analysed at the 44-cell stage. The ectopic activation of ERK1/2 in neural and TLC precursors in RGΔGAP-injected embryos strongly implicates p120RasGAP in the ephrin/Eph-driven attenuation of FGF/ERK signals (Fig. 2A-J) (Picco et al., 2007; Shi and Levine, 2008). To confirm this, we showed that p120RasGAP is required for ephrin-induced attenuation of ERK activation. Overexpression of *ephrin-Ad* in *Ciona* embryos results in a dramatic decrease of activated ERK1/2 at the 44-cell stage (Fig. 2K) and a concomitant loss of *Bra* expression at the 64-cell stage (Fig. 2L) (Picco et al., 2007). However, co-injection of *RGΔGAP* RNA with *ephrin-Ad* RNA results in the recovery of ERK1/2 activation (Fig. 2K) and in *Bra* expression that resembles the pattern obtained following *RGΔGAP* injection alone (Fig. 2L, Fig. 1C).

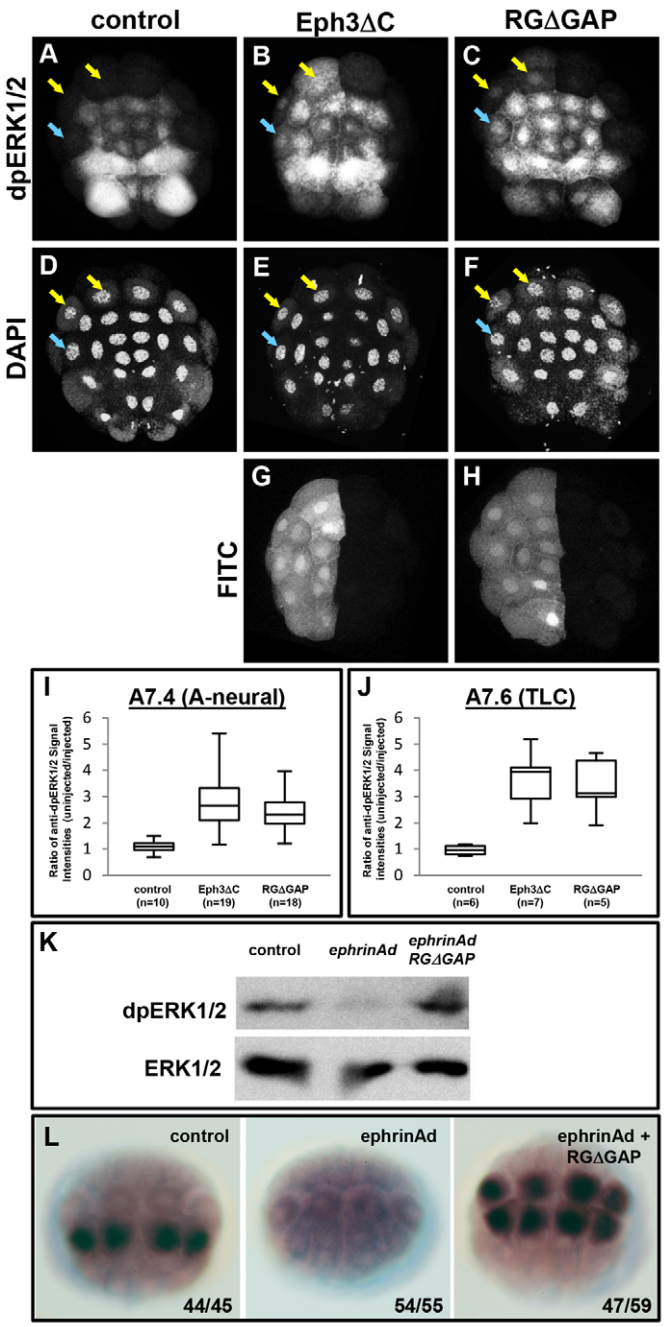

Fig. 2. RasGAP is required for the correct pattern of ERK1/2 activation in the developing marginal zone.
(**A-C**) Immunofluorescent detection of diphosphorylated (dp) ERK1/2 following expression of either Eph3ΔC or RGΔGAP on one side of the embryo. All embryos are at the 44-cell stage and in vegetal pole view. Arrows point to neural (yellow) and trunk-lateral cell (blue) precursors. (**D-F**) Counterstaining with DAPI. (**G**,**H**) FITC-dextran was co-injected with RNA to mark the injected side. All images are confocal stacks. (**I**,**J**) Boxplots of the ratios of nuclear dpEKR1/2 staining intensity between injected and uninjected sides in control, *Eph3ΔC*-injected and *RGΔGAP*-injected embryos. (**K**) Western blot showing the level of dpERK1/2 and total ERK1/2 in 44-cell stage embryos under the conditions indicated. (**L**) *In situ* hybridisation analysis of *Bra* expression at the 64-cell stage under the conditions indicated. Numbers (bottom right) indicate the proportion of embryos represented by the images. Embryos are tilted to show the notochord and neural precursors.

Inhibition of RasGAP had a similar effect on neuronal patterning in the motor ganglion (MG) (Fig. 3A). The MG contains five pairs of cholinergic neurons that project axons along the muscle band in the larval tail (Horie et al., 2010). Among these five pairs of neurons, the rostral four pairs originate from a pair of neural plate cells called A9.30. A9.30 divides along the rostral-caudal axis to generate A10.60 and A10.59, and FGF-dependent ERK activation is observed only in the rostral cell (A10.60), resulting in the transcriptional activation of *engrailed* (*en*) (Fig. 3A) (Stolfi et al., 2011). A10.59 then divides along the same axis to generate two postmitotic neurons termed A11.118 and A11.117. This cell division is also associated with ERK1/2 activation only in A11.118, where it induces expression of *Mnx* (Fig. 3A) (Stolfi et al., 2011). Functional studies indicated that these two rounds of differential ERK activation are controlled by ephrin-Ab ligand expressed in cells in contact with the caudally positioned sister cells (Fig. 3A) (Stolfi et al., 2011).

We expressed dominant-negative forms of RasGAP in the A9.30 lineage using the *fgf8* promoter (Imai et al., 2009). We first analysed the effect on the cell fate decision of the A9.30 daughters A10.60 and A10.59 by monitoring activation of an *en>mCherry* reporter that faithfully recapitulates endogenous *en* expression (Stolfi et al., 2011). Whereas RG(R818E) had little effect on this cell fate decision, RGΔGAP expression in the A9.30 lineage resulted in ectopic activation of *en>mCherry* in the A10.59 lineage in 21% of embryos (Fig. 3B). We then analysed the effect of RasGAP inhibition on the cell fate decision of the A10.59 daughters A11.118 and A11.117 by monitoring the expression of *Mnx*. In 60% of *fgf8>RG(R818E)* embryos, *Mnx* was expressed ectopically in A11.117 (Fig. 3C).

Furthermore, we demonstrated that the phenotype associated with ectopic Eph activation in the MG requires RasGAP function. Expression of a constitutively active form of Eph3 (caEph3) in the A9.30 lineage resulted in loss of *en* expression (Fig. 3D) (Stolfi et al., 2011). However, when caEph3 was co-expressed with RG(R818E) or RGΔGAP, *en>mCherry* expression was restored (Fig. 3D).

Altogether, these results show that p120RasGAP is required for all cell fate specification events currently known to be controlled by ephrin/Eph-dependent attenuation of FGF/ERK signals in *Ciona* embryos.

Eph3 and p120RasGAP physically associate in an ephrin-dependent manner in *Ciona* embryos

Co-immunoprecipitation assays were used to demonstrate that Eph3 and p120RasGAP physically associate in an ephrin-dependent manner in *Ciona* embryos. HA-tagged Eph3 and myc-tagged RasGAP were expressed using the *friend of GATA* promoter (*FOG*), which becomes active from the 16-cell stage in the animal hemisphere (Rothbächer et al., 2007). *FOG>RasGAP-6xMyc* and *FOG>Eph3-3xHA* were co-electroporated with either *FOG>venus* or *FOG>ephrin-Ad* and embryonic lysates were analysed at the late gastrula stage. In these experimental conditions, RasGAP co-immunoprecipitated with Eph3 only when ephrin-Ad was co-expressed (Fig. 4).

Conclusions

Altogether, our results provide strong evidence that p120RasGAP mediates the ephrin/Eph signal that attenuates FGF-dependent ERK activation in *Ciona* embryos. This mechanism is used repeatedly, in at least the four ERK-driven binary cell fate choices that we describe here. The membrane-tethered nature of ephrin ligands

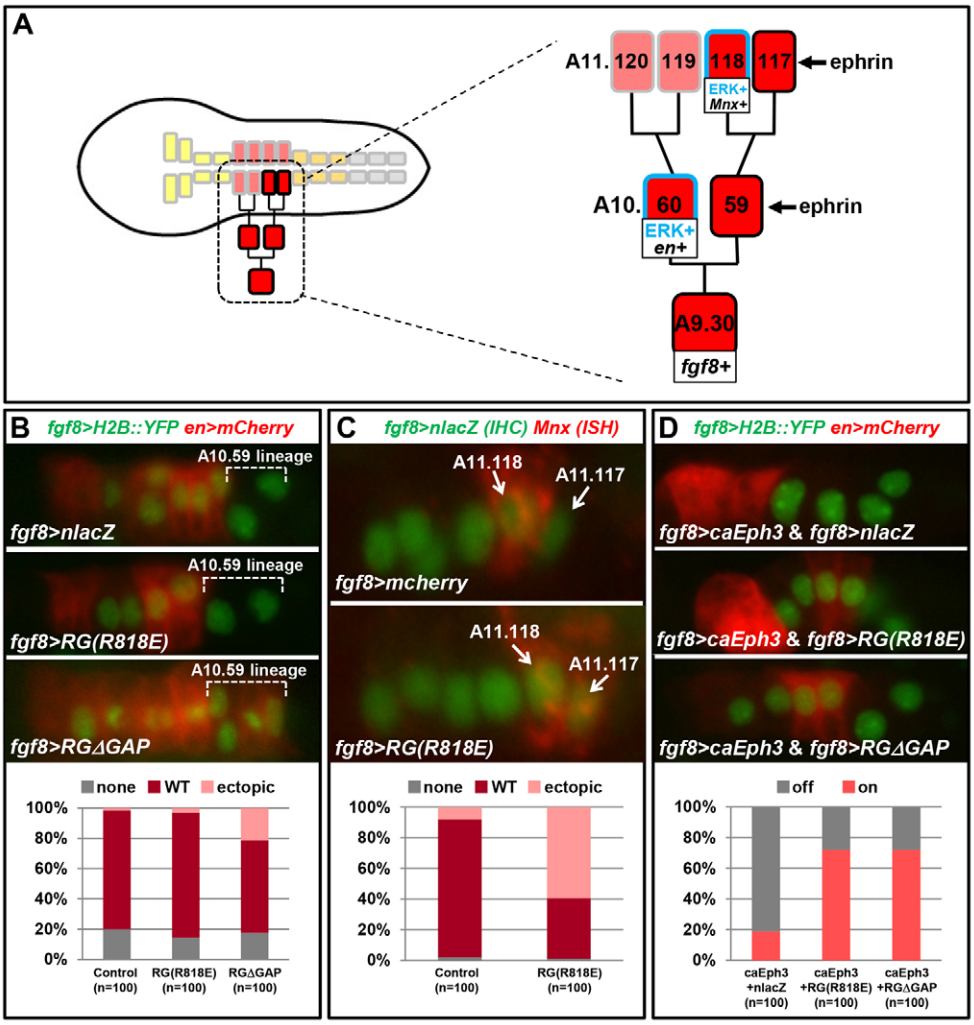

Fig. 3. RasGAP plays a role in motor ganglion patterning in *Ciona* embryos. (**A**) An early tailbud stage embryo showing the relative position of the A9.30 lineage, with gene expression and ERK activation illustrated to the right. (**B-D**) Electroporated constructs or *in situ* hybridisation probes used as lineage markers are indicated above the panels, whereas electroporated constructs used to modulate RasGAP or Eph3 activity are indicated in each panel. Quantification of each set of results is shown beneath. (B) Effect of RasGAP inhibition on the cell fate decision of A10.59 and A10.60. (C) Effect of RasGAP inhibition on the cell fate decision of A11.117 and A11.118. (D) RasGAP is required downstream of the Eph receptor during A10.59/A10.60 patterning. The large *en>mCherry*-positive domain seen in the top two panels corresponds to expression outside of the A9.30 lineage. WT, wild type.

might make this mechanism particularly suitable for embryos that develop with a small number of cells, such as those of *Ciona*, which are more likely to rely on direct cell-cell contact for patterning rather than diffusible signals over large fields of cells (reviewed by Lemaire, 2009). Thus, it is possible that this mechanism is an ascidian-specific adaptation to this particular mode of embryogenesis. Nonetheless, the *in vitro* observation that ephrin/Eph/RasGAP signalling can attenuate ERK activation in mammalian cell lines (Elowe et al., 2001; Kim et al., 2002; Minami et al., 2011) shows that this mechanism is operational beyond ascidians and thus might well be deployed *in vivo* in additional model animals.

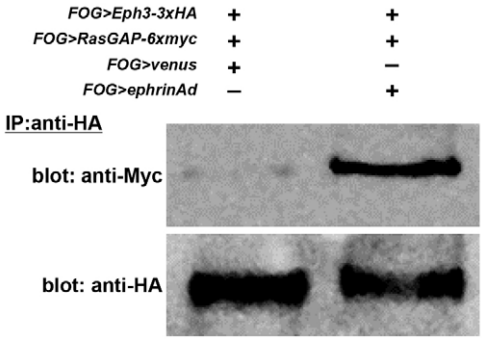

Fig. 4. RasGAP physically associates with Eph3 in an ephrin-dependent manner. Co-immunoprecipitation showing association of Eph3 and RasGAP in an ephrin-Ad-dependent manner. Electroporated constructs are indicated above the blot and the antibodies used on the left. The Eph3-3xHA level was used as a loading control.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions

N.H., A.S., C.S., V.P. and H.Y. designed and performed experiments. M.L., L.C. and H.Y. obtained funding for the research and experiments were performed in their laboratories. H.Y. wrote the manuscript and all authors participated in the editing process.

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