The Ric-8A/Gα13/FAK signalling cascade controls focal adhesion formation during neural crest cell migration in *Xenopus*

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**ABSTRACT**

Ric-8A is a pleiotropic guanine nucleotide exchange factor involved in the activation of various heterotrimeric G-protein pathways during adulthood and early development. Here, we sought to determine the downstream effectors of Ric-8A during the migration of the vertebrate cranial neural crest (NC) cells. We show that the Gα13 knockdown phenocopies the Ric-8A morphant condition, causing actin cytoskeleton alteration, protrusion instability, and a strong reduction in the number and dynamics of focal adhesions. In addition, the overexpression of Gα13 is sufficient to rescue Ric-8A-depleted cells. Ric-8A and Gα13 physically interact and colocalize in protrusions of the cells leading edge. The focal adhesion kinase FAK colocalizes and interacts with the endogenous Gα13, and a constitutively active form of Src efficiently rescues the Gα13 morphant phenotype in NC cells. We propose that Ric-8A-mediated Gα13 signalling is required for proper cranial NC cell migration by regulating focal adhesion dynamics and protrusion formation.

**KEY WORDS:** Ric-8A, Cell migration, Heterotrimeric G-Protein, Focal adhesion, GEF, Protrusion formation, Neural crest

**INTRODUCTION**

Cell migration is the driving force of a plethora of biological processes and is crucial for morphogenetic movements of early developing embryos, wound healing, immune response and cancer metastasis (Chang et al., 2013; Mayor and Etienne-Manneville, 2016; Ridley et al., 2003; Vedula et al., 2013). The *Xenopus* cranial neural crest (NC) cells represent an excellent system with which to investigate the complex molecular mechanisms underlying epithelial-to-mesenchymal transition (EMT) and migration (Barriga et al., 2013; Kelleher et al., 2006; Mayor and Theveneau, 2013). The NC is a transient cell population that populates the vertebrate embryo and eventually differentiates into endocrine and pigment cells, glia, neurons of the peripheral neural system and the craniofacial skeleton, among other tissues (Bronner-Fraser, 1995; Duband et al., 2015; Dupin et al., 2006; Hall, 2008; Mayor and Theveneau, 2013). During the course of gastrulation and neurulation, NC induction occurs by a combination of bone morphogenetic protein (BMP), Wnt and fibroblast growth factor (FGF) signals produced by the mesoderm, neural ectoderm and non-neural ectoderm (LaBonne and Bronner-Fraser, 1998; Milet and Monsoro-Burq, 2012; Steventon et al., 2009; Steventon and Mayor, 2012). As NC cells undergo EMT, they alter their morphology and molecular features, acquire motility, lose their epithelial polarity and experience a switch in cadherin expression that leads to decreased adhesive properties (Nieto, 2013; Theveneau and Mayor, 2012).

During migration, cells produce transient attachment sites to the substrate, called ‘focal contacts’ (Lauffenburger and Horwitz, 1996; Parsons et al., 2010; Roycroft et al., 2018). Some focal contacts mature into larger structures called ‘focal adhesions’, which are formed by integrin molecules connected to the cytoskeleton by adaptor proteins, such as paxillin. Focal adhesions associate with stress fibres composed of myosin and actin microfilaments, and generate the traction and contraction forces required for cell migration. Finally, focal adhesions are disassembled in order to produce the contraction of the posterior cell region (Lauffenburger and Horwitz, 1996; Ridley et al., 2003).

At a molecular level, heterotrimeric G proteins control the migration of several cell types by promoting actin cytoskeleton reorganization through small GTPase family proteins, including Cdc42, Rac and Rho (Cotton and Claing, 2009; Nobes and Hall, 1995; Kjoller and Hall, 1999; Sah et al., 2000; Rohde and Heisenberg, 2007). Heterotrimeric G proteins are classified into four families according to the structural and functional similarities of their Gα subunits. These include the Gas, Gαi/o, Gαq/11 and Gα12/13 members, all of which are expressed in NC cells (Fuentesalba et al., 2016) and are involved in different embryonic processes (Wilkie et al., 1992; Malbon, 2005). We recently reported that the Ric-8A guanine nucleotide exchange factor (GEF) protein, for Gα13, Gαi and Gαq (Tall et al., 2003; Klattenhoff et al., 2003; Von Dannecker et al., 2005; Nishimura et al., 2006; Chan et al., 2011a,b; Malnic and Gonzalez-Kristeller, 2009), regulates cranial NC cell migration in *Xenopus* (Fuentesalba et al., 2013). Ric-8A loss of function results in a significantly decreased number of focal adhesions and induces craniofacial cartilage defects, suggesting that Ric-8A controls cell migration by regulating cell adhesion properties (Maldonado-Agurto et al., 2011; Fuentesalba et al., 2013; Toro-Tapia et al., 2017). However, the molecular mechanisms by which Ric-8A controls the migration of NC cells still remain to be elucidated. Here, we have explored the role of Ric-8A during embryonic *Xenopus* development by identifying its downstream effectors. Our findings reveal that Ric-8A acts upstream of Gα13 to control cranial NC cell migration. By combining explant and transplant assays with functional experiments, we provide evidence that Ric-8A and Gα13 activities are crucial for the migration of cranial NC cells in vivo and in vitro. We propose that a Ric-8A-dependent activation of Gα13 signalling is required for proper cell migration by controlling focal adhesion dynamic and protrusion formation.
**RESULTS**

**Gu13 acts downstream of Ric-8A and controls cranial NC cell migration**

Considering that migrating cranial NC cells express Ric-8A and Ga13 transcripts (see Fig. S1A; Maldonado-Agurto et al., 2011; Fuentealba et al., 2016), and that Ga12/13 is known to regulate migration events of a wide variety of cell types (Bian et al., 2006; Kelly et al., 2006a,b; Lin et al., 2005, 2009; Offermanns et al., 1997; Parks and Wieschaus, 1991; Xu et al., 2003), we decided to further investigate the epistatic relationship between both genes. We first designed a Gu13 morpholino (Gu13MO) and a synthetic rescue mRNA (not recognized by the Gu13 morpholino) that lead to a reduction and increase in Gu13 levels, respectively (Fig. S1B). In agreement with our previous findings (Fuentealba et al., 2013), injection of 10 ng of Ric-8A morpholino (Ric-8AMO) in two collateral animal blastomeres at the eight-cell stage inhibits NC migration, as analysed by in situ hybridization against Snail2 (Fig. 1A,B). Such defects were rescued by the co-injection of Ric-8A mRNA (Fig. S1C) or Gu13 mRNA (Fig. 1A,B), suggesting that Gu13 acts downstream of Ric-8A. Furthermore, the Gu13 loss of function mimicked the Ric-8A morphant phenotype, and the Gu13MO effect was rescued by the Gu13 mRNA showing the specificity of the MO (Fig. 1A,B). By contrast, the Ric-8A mRNA was unable to rescue Gu13 morphant embryos, arguing in favour of an epistatic relationship that places Ric-8A upstream of Gu13. No effect on NC induction was observed in stage 16 Gu13 morphant embryos, as analysed by the expression of Snail2 (Fig. S1C). In order to evaluate whether Gu13 is required in NCs or in their surrounding tissues, we grafted control or Gu13MO-treated NCs into wild-type embryos, showing a clear inhibition of migratory behaviour for only the morphant NC cells (Fig. 1C). Taken together, these results show that Gu13 function is required specifically within NCs, and acts downstream of Ric-8A during cranial NC cell migration in vivo.

To further examine whether the defects observed in Ric-8A and Gu13 morpant cells are due to a general effect on cell motility, we performed a series of in vitro assays. To evaluate radial cell migration, we labelled cell membranes and nuclei by co-injecting Xenopus embryos with mRNAs coding for mGFP and nRFP, respectively, with either the ControlMO, Ric-8AMO or Gu13MO. Cranial NC explants from stage 17 embryos were cultured onto fibronectin-coated dishes, and their migratory behaviour was subsequently monitored by time-lapse imaging (Fig. 2, see also Movie 1). For such experiments, we exclusively selected and analysed the explants in which all the cells were GFP and RFP positive, thereby avoiding any interference due to mosaicism and non-cell-autonomous effects. Radial migration of cranial NC cells was severely impaired in explants from Ric-8A and Gu13 morphants, whereas migratory behaviour was unaffected in ControlMO-treated explants (Fig. 2, see also Movie 1). As expected, the area covered by cranial NC cells after 10 h was significantly reduced upon injection of Ric-8AMO and Gu13MO (Fig. 2B,C). Importantly, this inhibitory effect in Ric-8A and Gu13 morphant cells was rescued by the co-injection of Gu13 mRNA (Fig. 2). Therefore, in NC explants, the Ga13 mRNA is able to rescue the migration defects caused by the Ric-8A and Gu13 knockdown.

In order to analyse whether the impaired migration in the Ric-8A and Gu13 morphant cells could be due to an effect on EMT, we performed an immunostaining assay against E-cadherin and N-cadherin, two well-known EMT markers in Xenopus NC cells (Barriga et al., 2018). Our analysis shows that the E-cadherin and N-cadherin expression patterns were similar between the control conditions and the Ric-8A and Gu13 morphants (Fig. S2A,B). Taken together, these data suggest that Ric-8A does not affect EMT, whereas it controls cranial NC migration by regulating the Gu13 pathway both in vivo and in vitro.

**Gu13 and Ric-8A colocalize at the cell membrane in explanted migratory cranial NC cells**

In addition to its main role as a GEF, Ric-8A functions as a chaperone localizing the Go subunits at the cell cortex (Klattenhoff et al., 2003; Colombo et al., 2003; Gotta and Ahringer, 2001; Wang et al., 2011; Gabay et al., 2011). To further explore the Ric-8A/Gu13 molecular relationship, we analysed whether the subcellular localization of both proteins correlates with the migratory behaviour of cranial NC cells. For this purpose, embryos were co-injected with a mix of mRNAs coding for mGFP and for a Myc-tagged form of Ric-8A, and cranial NC cells were explanted from stage 17 embryos.
and cultured onto fibronectin-coated dishes (Fig. 3). We find that the endogenous Gα13 and the Myc-Ric-8A proteins mainly colocalize at the cell cortex leading edge, including in the cell protrusions of cranial NC cells, but are depleted from cell-cell contact regions (Fig. 3A). In addition, the overexpression of Ric-8A results in an increased recruitment of the endogenous Gα13 protein at the leading edge, leading to a homogenous Gα13 distribution and a less pronounced dot-like pattern observed for the endogenous Gα13 (Fig. 3A). Interestingly, under Ric-8A knockdown conditions, the Gα13 protein fails to localize at the cell cortex and becomes distributed at low levels throughout the cell (Fig. 3B). By contrast, the Gα13MO leads to a dramatic decrease in Gα13 levels, both by immunofluorescence and western blot (compare Fig. 3C with D). These observations suggest that Ric-8A regulates the localization of the Gα13 protein without overtly affecting its expression levels (Fig. 3B,D). In agreement with this view, RT-PCR (Fig. S3A) and western-blotting (Fig. 3D) analyses confirmed that the Gα13 transcript and protein levels are not decreased in Ric-8AMO morphant cells. Furthermore, co-immunoprecipitation assays showed that Ric-8A and Gα13 interact in HEK293 cells and in *Xenopus* embryos (Fig. 3E, see also Fig. S3B,C). In agreement with published results, our work supports the idea that the activation of the G-protein pathway is accompanied by a Ric-8-dependent translocation of Gα subunits to the cell cortex (Klattenhoff et al., 2003; Gabay et al., 2011). Here, we demonstrate for the first time a functional association between Gα13 and Ric-8A during cranial NC cell migration.

**Ric-8A and Gα13 loss of function induce actin cytoskeleton and cell protrusion defects**

In an effort to identify the molecular mechanism by which Ric-8A and Gα13 regulate cranial NC cell migration, we analysed the effect of Ric-8A and Gα13 knockdown on cytoskeletal organization and cell morphology. For this purpose, we analysed the actin cytoskeleton at the leading edge of explanted cranial NC morphant cells (Fig. 4). In ControlMO-injected cranial NC explant cells, the cortical actin cytoskeleton forms normal lamellipodia and filopodia (Fig. 4A). In contrast, Ric-8A and Gα13 morphant cells present an abnormal cytoskeleton characterized by disorganized cortical actin cables and a decreased number and size of cellular protrusions (Fig. 4A, see also Fig. S4). Furthermore, these Ric-8A-induced defects were rescued by Gα13 (Fig. 4A, see also Fig. S4).

The cortical actin cable phenotype observed in morphant conditions prompted us to examine the stability, number and morphology of cell protrusions *in vitro*. Hence, cranial NCs were explanted at stage 17 from embryos expressing lifeActCherry, cultured onto fibronectin, and examined by time-lapse recording. Control cranial NC cells formed a low number of new protrusions during the examined time frame, and such protrusions appeared to be very stable (Fig. 4B). By contrast, cranial NC cells from Ric-8A or Gα13 morphant explants exhibited a dramatically reduced ability to form stable filopodia and lamellipodia, thereby producing a high number of novel, transient, membrane blebbings (Fig. 4B and see also Movie 2). Importantly, co-injection of Gα13 mRNA rescued the Ric-8A morphant phenotype, increasing protrusion stability and generating structures similar to those of the control (Fig. 4B and see also Movie 2).

**Ric-8A acts upstream of Gα13 to control cell attachment**

As we had previously shown that Ric-8A loss of function significantly decreases focal adhesions, we sought to analyse whether the abnormal protrusion formation observed in Ric-8A and Gα13 morphant cells is associated with changes in their adhesion properties (Fuentea et al., 2013). We first performed phospho-paxillin and integrin immunostaining, and found a strong reduction in the number and area of the focal adhesion complexes in the Gα13 and Ric-8A morphant cells (Fig. 5A,B, see also Fig. S5A). Three-dimensional reconstructions confirmed that Gα13 morphant explants exhibit abnormal actin cytoskeleton organization and a decreased number and size of their focal adhesion complexes (Fig. S5B; see also Movie 3). The Ric-8A loss-of-function phenotype was rescued by overexpressing Gα13 (Fig. 5A,B). We next performed adhesion assays on fibronectin-coated surfaces and found that the Gα13MO-treated explants exhibit a marked dose-dependent reduction in their adhesive properties (Fig. 5C, left panel), similar to the phenotype previously described for Ric-8A morphant cells (Fuentea et al., 2013). Such adhesion defects were rescued by the co-injection of Gα13 mRNA (Fig. 5C, left panel). Interestingly, the extracellular matrix adhesion defects of Gα13 morphants were rescued in a dose-dependent fashion by
the addition of Mn$^{2+}$ to the culture media (Fig. 5C, right panel; see also Fig. S5C), an ion known to improve the affinity of integrins to fibronectin, and, therefore, extracellular matrix binding (Mould et al., 1998, 2002). Mn$^{2+}$ treatment increased the number of phospho-paxillin-positive focal adhesions and rescued the G$\alpha_{13}$ morphant cell phenotype (Fig. 5C, right panel; see also Fig. S5C). Taken together, these results strongly suggest that Ric-8A regulates G$\alpha_{13}$ to control integrin-mediated adhesion properties.

G$\alpha_{13}$ acts upstream of FAK and Src

FAK is a cytoplasmic kinase able to form complexes with integrins, paxillin and Src, playing a key role in integrin-mediated signalling, Rho GTPase-dependent focal adhesion assembly and disassembly, and cell migration (Chen et al., 2001; Golubovskaya et al., 2002; Parsons et al., 2010; Zhao and Guan, 2011). To further assess the molecular mechanism by which Ric-8A-regulated G$\alpha_{13}$ is controlling adhesive properties and their possible association with the integrin pathway, we evaluated focal adhesion dynamics by performing time-lapse imaging of a GFP-tagged form of FAK expressed in explanted NC cells. For this purpose, Xenopus embryos were co-injected with mRNAs coding for a FAK-GFP fusion and for lifeact-RFP together with a ControlMO, a G$\alpha_{13}$MO or a Ric-8AMO. When compared with controls, G$\alpha_{13}$ and Ric-8A morphant explants exhibit small and unstable focal adhesions and cellular protrusions (Fig. 6A,B, and also see Movie 4 and Fig. S6A,B). We further show that FAK and G$\alpha_{13}$ partially colocalize in the protrusions of the cell leading edge (Fig. 6D).

In addition, co-immunoprecipitation assays showed that FAK, G$\alpha_{13}$ and Ric-8A are part of the same protein complex (Fig. 6C and see also Fig. S6C). Collectively, these data suggest that, at the leading edge of cranial NC cells, Ric-8A regulates G$\alpha_{13}$, which, in turn, controls focal adhesion dynamics by forming a protein complex with focal adhesion scaffold proteins, thereby promoting migration.

FAK, Src and paxillin are known integrin downstream effectors; and the adhesion-dependent autophosphorylation of FAK leads to the recruitment and activation of Src, which eventually regulates cytoskeletal activity and adhesion dynamics (Hynes, 2002; Lietha et al., 2007; Mitra et al., 2005; Parsons et al., 2010). Hence, we employed a constitutively active form of Src (SrcY527F) to perform epistasis assays and evaluate whether Ric-8A and G$\alpha_{13}$ act through integrin signalling to control cell adhesion dynamics. Our results show that SrcY527F is able to rescue the G$\alpha_{13}$ morphant phenotype over cranial NC migration (Fig. 7A). Finally, by immunostaining cranial NC explants using phospho-paxillin antibody, we showed that active Src is able to rescue the focal adhesion formation defects induced by the loss of function of G$\alpha_{13}$ (Fig. 7B,C). As a control,
the injection of the SrcY527F mRNA alone affected neither cranial NC cell migration (Fig. 7A) nor focal adhesion formation (Fig. 7B, C). Taken together, these results show that Src is a downstream effector of Gα13 signalling that controls focal adhesion formation during cranial NC cell migration.

**DISCUSSION**

We show here that the loss of function of Ric-8A and Gα13 does not disrupt NC induction and the switch in E- to N-cadherin expression. We therefore favour a model where the major function played by Ric-8A and Gα13 occurs during active NC migration. Indeed, we and others have shown that the loss of Ric-8A function impairs the migration of cranial NC cells (Fuentealba et al., 2013), of mouse embryonic fibroblasts and B lymphocytes (Boularan et al., 2015; Wang et al., 2011), and of zebrafish gastrulating cells (Kanesaki et al., 2013; Peters and Rogers, 2013). In addition, several studies have shown that heterotrimeric G-protein signalling regulates this process during embryogenesis (Offermanns and Wettichaureck, 2005; Saint-Jeannet, 2006; Rohde and Heisenberg, 2007; Lin et al., 2009). In this study, we connect these two fields and propose a novel molecular mechanism to explain how Ric-8A potentiates Gα13 signalling to control cell focal adhesion formation and cranial NC cell migration.

Our experiments performed in vivo and in explanted NC cells show that whereas Gα13 efficiently rescues the Ric-8A morphant phenotype, the overexpression of Ric-8A is not sufficient to rescue the loss of function of Gα13. By contrast, the overexpression of wild-type Gα13 does rescue the Ric-8A morphant phenotype. We argue that at least three factors might explain the biological activity of the exogenous Gα13 under Ric-8A morphant conditions. Indeed, Gα13 activation might occur as a result of an interaction with its cognate GPCR or with residual Ric-8A proteins (the Ric-8AMO decreases the protein levels of Ric-8A without completely abolishing them, see Fuentealba et al., 2013), or because the
Fig. 5. Ric-8A and Gα13 morphant cells have defects in focal adhesions. Embryos were injected with nuclear RFP and Gα13MO or Ric-8AMO or a Ric-8AMO and Gα13 mRNA mixture, and the cranial NC cells were extracted and cultured onto fibronectin. (A) Immunostaining against phospho-paxillin (green) and phalloidin (red) was performed in X. laevis cranial NC. Ric-8AMO and Gα13MO cells showed a decrease in the number and area of focal adhesions compared with control cells. Gα13 mRNA was able to rescue the Ric-8A morphant phenotype. (B) The graphs show the number of focal adhesions per condition normalized to the control, and the total area of focal adhesion per cell in each condition normalized to control. (C) Adhesion assay performed on Gα13 morphant explants to evaluate the attachment of the explants over fibronectin. Gα13 morphant explants do not attach properly to the extracellular matrix in comparison with the control explants, suggesting a severe defect in focal adhesions. MnCl2 rescues the Gα13MO phenotype, increasing the attachment in a dose-dependent fashion. Data are mean±s.e.m., *P<0.1, **P<0.01, ***P<0.001, n=40. The images are representative of four independent experiments analysing 10 explants per each condition. Scale bars: 25 μm.

amount of the exogenous Gα13 largely exceeds endogenous levels, forcing the binding and stabilization of a fraction of the overexpressed Gα13 to GTP. We therefore resolve the epistatic relationship of both proteins, establishing Gα13 as a likely downstream effector of its GEF Ric-8A. In fact, both proteins are probably involved in a positive-feedback loop, because Gα13 is required for the Ric-8A phosphorylation by Src family kinases (SFKs) and its subsequent translocation to the membrane (Yan et al., 2015). Consistently with this regulatory model, the Ric-8A and Gα13 transcripts are co-expressed during embryogenesis (Fig. S1A; see Maldonado-Agurto et al., 2011; Fuentealba et al., 2016), and, as we show here, the Ric-8A and Gα13 proteins interact in co-immunoprecipitation assays. As Ric-8A and Gα13 homologues are known to be involved in the migration of Drosophila embryonic cells and mouse embryonic fibroblasts (Peters and Rogers, 2013; Wang et al., 2011), our results might be applicable to a wide variety of cellular contexts. Importantly, in whole embryos, the Gα13 loss of function phenocopies Ric-8A morphants in terms of cranial NC cell migration, inducing defects in the distribution of snail2-positive cells, which remain dorsally localized at stage 22 (Fuentealba et al., 2013). The fact that homotypically transplanted Gα13 morphant cranial NC cells fail to properly migrate through the cranial routes suggests that, under normal conditions, Gα13 function is required directly within the NC cell population and not in its surrounding tissues. We observed similar results in vitro, showing that Gα13 deficiency impairs cell dispersion over a fibronectin substrate, and that the Gα13 overexpression rescues the Ric-8A morphant phenotype. Although both Gα13 and Ric-8A knockout mouse die during early development (Gabay et al., 2011; Gu et al., 2002; Tönissoo et al., 2010), their phenotypes display interesting differences. Indeed, the Gα13 knockout embryos die at E10 stage, owing to angiogenesis defects (Gu et al., 2002); and the Ric-8A knockout embryos display gastulation defects and die at the E6.5-E8.5 stages (Tönissoo et al., 2010). Thus, Ric-8A may regulate several signalling pathways involving different heterotrimeric G proteins in addition to Gα13. A possible redundancy between different heterotrimeric G proteins may explain the milder phenotype of Gα13 mutants, which is consistent with the fact that Ric-8A is not an exclusive Gα13 regulator (Offermanns, 2001) and that Ric-8A is co-expressed with four distinct Gα subunits family members in Xenopus cranial NC routes (Fuentealba et al., 2016).

Ric-8A has been described as a receptor-independent GEF, interacting directly with the Gβγ-free GαGDP subunit, promoting its guanine nucleotide exchange and its translocation to the plasma membrane, thereby extending the lifetime of active Gα subunits (Chan et al., 2011a,b; Klattenhoff et al., 2003; Malnic and Gonzalez-Kristeller, 2009; Nishimura et al., 2006; Von Dannecker et al., 2005; Tall et al., 2003; Hinrichs et al., 2012). Upon palmitoylation, Gα13 localizes to the membrane region where it interacts with specific protein partners and exerts its signalling activity (Suzuki et al., 2009). In agreement with these observations, our results show that Ric-8A and Gα13 are excluded from cell-cell contact regions and colocalize below the leading edge membrane during cranial NC cell migration, including in filopodia and lamellipodia. In addition, our results reveal a subcellular expression pattern for the endogenous Gα13 protein in the form of small and discrete foci (dots like), which is comparable with the punctate localization of radixin actin-binding proteins from the ERM (ezrin, radixin and moesin) family that are known to directly activate Gα13 (Vaiskunaite et al., 2000). According to previous studies, Ric-8A is
a molecular chaperone for different Gα subunits and is required for the initial association of nascent Gα subunits with cellular membranes, thus increasing their stability (Gabay et al., 2011; Wang et al., 2005, 2011; Saare et al., 2015). Consistent with such a chaperone function, the Ric-8AMO causes the endogenous Gα13 protein to lose its cell membrane localization. Interestingly, our western blot analyses showed that the Gα13 protein levels remain largely unaffected in the absence of Ric-8A (Fig. 3D), supporting previous findings Peters and Rogers (2013). These data suggests that, in contrast to other Gα subunits, Ric-8A regulates the translocation of Gα13 to the cell membrane without increasing its stability. However, this result seems at odds with the apparent Gα13 reduction observed by immunofluorescence in Ric-8A morphant conditions (Fig. 3B). This discrepancy might be explained by loss of subcellular localization and subsequent cytoplasmic dilution experienced by the Gα13 protein in the absence of Ric-8A, or, alternatively, by the fact that some of the Gα13 pool may be masked to the primary antibody because of conformational changes.

In this work, we demonstrate that the loss of Ric-8A or Gα13 function strongly affects the cortical actin cytoskeleton and alters cranial NC cell morphology, producing a sharp increase in the amount of novel, unstable, membrane blebbing. This observation is consistent with a recent study from our laboratory, showing that Ric-8A morphant cells present a more rounded phenotype, together with a decreased number and size of protrusions, implying a cell polarity role for Ric-8A (Leal et al., 2018, in press). In addition, Ric-8A and Gα13 influence the migration of other cell types by regulating the turnover of membrane ruffles or the cortical actomyosin pool required for cellular contraction (Peters and Rogers, 2013; Wang et al., 2011). Furthermore, the G-protein signalling pathway reinforces the cell cortex by regulating cortical actin organization, allowing cells to cope with hydrostatic stress during tissue folding (Kanesaki et al., 2013). Several studies have also associated Gα13 with actin cytoskeleton reorganization, showing that it activates the small G-protein RhoA through a direct interaction with RhoGEF proteins, such as p115RhoGEF and LARG (Suzuki et al., 2009; Lin et al., 2005; Malbon, 2005; Moers et al., 2008; Cotton and Claing, 2009). As it has recently been reported that radixin promotes cell migration, by regulating Rac-mediated epithelial polarity and adherens junctions formation through VavGEF (Valderrama et al., 2012), it will be interesting to investigate whether radixin functionally interacts with the Ric-8A/Gα13 axis. Interestingly, Gα13 it is not able to entirely rescue the Ric-8A morphant phenotype. It is tempting to propose that, in addition to Gα13, Ric-8A is probably also acting as a GEF for Gαi and Gαq (Tall et al., 2003). In the future, it will be interesting to examine how Ric-8A controls different aspects of cell migration by fine-tuning the biological activity of a diverse array of Gα subunits.

The observed decrease in protrusion stability prompted us to also analyse cell adhesion properties. Similar to the previously reported...
morphant Ric-8A phenotype (Fuentealba et al., 2013), Gα13 morphant cells present with a strong reduction in the number of cell adhesions. These defects are specific because they can be rescued by the overexpression of Gα13 itself. The fact that the addition of Mn2+ into the culture media also rescues the adhesion of cranial NC cell explants suggests the participation of αβ integrin heterodimers known to activate a variety of signalling pathways (Mould et al., 1998, 2002; Takada et al., 2007). Such a possibility would be consistent with previous studies reporting that the recruitment of scaffold proteins and stress fibres either promote new focal adhesions or potentiate the binding of integrins to the extracellular matrix in the presence of cations (Dormond et al., 2004; Lydolph et al., 2009). We therefore examined paxillin, a key integrin downstream effector whose phosphorylation at focal adhesion sites activates Rac-1 and inhibits RhoA GTPases, thereby generating protrusions at the leading edge during cell spreading and migration (Turner et al., 1990; Brown and Turner, 2004; Price et al., 1998). FAK, Src and Gα-protein subunits are among the proteins known to regulate the phosphorylation of paxillin tyrosine residues in response to cell adhesion cues (Rozengurt, 2007). Several studies have shown that Gα13, Gα12, Goq and thrombin regulate FAK by Rho-dependent or -independent mechanisms (Chikumi et al., 2002). In addition, LARGE and RhoGEF are phosphorylated by FAK in response to thrombin, suggesting a positive feedback between FAK and Rho (Chikumi et al., 2002). In ovarian cancer cells, both the LPA and Gα12/13-RhoA-ROCK pathways control FAK autophosphorylation, resulting in Src-mediated cell migration (Bian et al., 2006). In addition, Rgnef (p190RhoGEF) has been reported to be a new effector for Gα13 downstream of gastrin and the type 2 cholecystokinin receptor, mediating FAK activation to promote colon carcinoma cell motility and tumour progression (Masià-Balagué et al., 2015). Moreover, MEF cells from Rgnef knockout mice displayed suppressed haptotaxis migration and wound closure motility, as well as a reduced number of focal adhesions and RhoA GTPase activation, following fibronectin-integrin stimulation (Miller et al., 2012). This evidence is consistent with the phenotype observed in the present study, revealing an important interaction between Go13 and FAK in the control of cell migration and where Gα13 and Ric-8A downregulation strongly decreases the number and size of the focal adhesions. Therefore, it...
will be interesting to investigate the possible association with Rgnef and this signalling pathway.

It has recently been reported that Gz13 is required to promote the integrin ‘outside in’ signalling by Src activation and transient RhoA inhibition (Gong et al., 2010; Shen et al., 2015). ‘Outside-in’ signalling mediates cellular responses induced by ligand binding to integrins, leading to cell spreading, retraction, migration, proliferation and survival (Shen et al., 2012). These studies indicate that Gz13 exerts a dual function by (1) activating RhoA by GPCR signalling and (2) inhibiting RhoA function by integrin signalling via the Src-dependent activation of p190RhoGAP (Gong et al., 2010; Shen et al., 2015). Consistent with these reports, our results showed that a constitutively active form of Src rescues the Gz13 morphant phenotype, recovering cranial NC cell migration in vivo as well as focal adhesion formation. Previous studies have also shown that a crosstalk between Src and Gz13 regulates the stability of endothelial cell adherens junctions through the direct activation of Src (Gong et al., 2014). In addition, Gz13 signalling can promote Ric-8A phosphorylation by SFKs, which are required for translocating Ric-8A to the plasma membrane (Yan et al., 2015). Although we did not observe an interaction between Gz13 and Src by co-immunoprecipitation (data not shown), we found that Gz13 morphant cranial NC cells display a strong reduction in the size and stability of focal adhesions. In addition, the interaction and colocalization between Gz13 and FAK indicates that these proteins may be part of the same protein complex, possibly with the integrin intracellular domains. However, a co-immunoprecipitation assay showed a weak interaction between FAK and Gz13, raising the possibility that this interaction may be highly dynamic in order to allow migration. Thus, these proteins may be mediating the ‘outside in’ signalling pathway in cranial NC cells.

In addition to Ric-8A, another non-receptor GEF has been identified for the heterotrimeric G proteins, metastasis-associated G protein, GIV/Girdin, which mediates integrin signalling by activating Goi (Leyme et al., 2015). As several GPCRs are known to control cranial NC cell behaviour, our current work sheds light on G protein, GIV/Girdin, which mediates integrin signalling by

X. tropicalis

embryo manipulation and microinjection of RNAs/antisense morpholinos

**X. tropicalis** and **X. laevis** (3 years old) were obtained by in vitro fertilization after gonadotropin stimulation (human chorionic gonadotropin) as previously described (Maldonado-Agurto et al., 2011). All embryos were dejellied in 3% cysteine (pH 7.9), washed and cultured in 1/9×MR. The embryos were collected at different stages according to Nieuwkoop and Faber (1967). Eight-cell-stage embryos were microinjected in one dorsal and one ventral animal blastomere (collateral). Morpholinos oligonucleotides were designed for Ric-8A (Ric-8AMO: 5′-GAG GGT ACC CGG ATC CAT GGC TGG C-3′) and Gz13 (Gz13MO: 5′-GGA AGT CCC GAG CTA TAC ACA C-3′) to target the translation initiation site of **X. tropicalis** and **X. laevis** transcripts. The **X. tropicalis** and **X. laevis** embryos were injected with 10 ng and 15 ng of Ric-8AMO or Gz13MO, respectively. A random MO (ControlMO: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′) was used as a control sequence (Gene Tools). Rhodamine dextran or fluorescein was used as a lineage tracer. The following mRNAs were in vitro transcribed and then co-injected as previously described above: nuclear-RFP (100 pg and 200 pg), membrane-GFP (100 pg), FAK-GFP (400 pg), constitutively active Src (Y527F) (100 pg and 200 pg), Gz13 (100 pg and 300 pg), Ric-8A (300 pg) and Lifeact-Cherry (300 pg).

**NC cell dissection, culture and grafts**

The NC cells were dissected as described by Alfandari et al. (2003). In brief, NC cells were removed from injected embryos at stage 17 by micro-dissection on 3/4 normal amphibian medium (NAM) [1× NAM: 110 mM NaCl, 2 mM KCl, 1 mM Ca(NO3)2, 1 mM MgSO4, 0.1 mM EDTA, 1 mM NaHCO3, 2 mM PBS (pH 7.4)]. The explants were collected in Danilchik’s for Amy (DFA) medium [53 mM NaCl, 5 mM Na2CO3, 4.5 mM K-Gluconate, 32 mM Na-Gluconate, 1 mM MgSO4, 1 mM CaCl2, 0.1% BSA (pH 8.3 adjusted with 1 M bicine) and 50 µg/ml streptomycin] and cultured on fibronectin-coated petri dishes or coated coverslips (10 or 50 µg/ml, respectively), in DFA medium for several hours (at 18°C **X. laevis** and 26°C **X. tropicalis**). The fibronectin was prepared for incubating 1 h at 37°C at the corresponding concentration. After that, the fibronectin matrix was washed with PBS and blocked with PBS/BSA (0.1%) for 30 min at 37°C. For NC transplantation experiments, the cells were microdissected from the donor embryo (injected embryo) and grafted into a wild-type host embryo (immobilized in modelling clay) in which the NC had been removed. Embryos were photographed at stage 26 with a stereoscope Olympus SZ61 coupled to a Leica DFC450 camera.

**Whole-mount in situ hybridization**

In situ hybridization was performed as described previously (Harland, 1991). Briefly, antisense RNA probes were synthesized by in vitro transcription using digoxigenin-11-UTP and T7 or Sp6 RNA polymerase, and detected with alkaline phosphatase-conjugated anti-DIG antibodies (Roche). Staining was performed with NBT/BCIP, used as the alkaline phosphatase substrate. Hybridization and washing were performed at 65°C. Finally, the embryos were post-fixed in Bouin solution and bleached in 0.5× SSC, 5% formamide and 1% H2O2, Twist (Hopwood et al., 1989) and Snail2 (LaBonne and Bronner-Fraser, 2000; Mayer et al., 2000) probes were used.

**Immunofluorescence and time-lapse microscopy**

NC cells were cultured on fibronectin-coated coverslips and fixed in 3.7% formaldehyde in PBS for 30 min. Permeabilization was performed with 0.2% Triton-X in PBS for 10 min followed by PBS washes. The following primary antibodies were used: anti-c-myc (9E10, Santa Cruz Biotechnology) at 1:100; anti-Gz13 (A-20, Santa Cruz) at 1:20; anti-phospho paxillin (pY 118, p118, Invitrogen) at 1:20, anti-β-integrin [8C8, Developmental Studies Hybridoma Bank (DSHB)] at 1:10, anti-N-cadherin (MNC22, DSHB) 1:10 and anti-E-cadherin (5D3, DSHB) at 1:10. The following secondary antibodies were used: anti-rabbit Alexa fluor 647 (Jackson Immunoresearch) at 1:200; anti-mouse Alexa fluor 546 (Life Technologies) at 1:200 and anti-rabbit Alexa fluor 488 (Life Technologies) at 1:200 in presence of phalloidin 546 and 633 (1:1000, Life Technologies) and Hoechst (1:1000, Thermo Fisher Scientific). The fixed cells were mounted in fluorescent mounting medium (Dako, Agilent) and the imaging was performed at room temperature using a confocal Zeiss LSM780 microscope with a Plan Apochromat 63×/1.4 NA oil immersion objective and Zen 2012 Zeiss acquisition software. Focal adhesions were quantified using the ‘threshold’ command and executing the ‘analyse particles’ command to measure structures >1 µm using ImageJ software. 3D reconstruction was undertaken using an Imaris Bitplane V7.5.2. The cell dispersion was recorded by time-lapse microscopy at room temperature using a DMX6000 Leica microscope equipped with a motorized stage and a camera (DFL420; Leica) with an N Plan Apochromat...
Fig. 8. Regulation of cranial NC cell migration through the regulation of adhesion properties by Ric-8A and Goα13 signalling. Ric-8A/Goα13 regulates focal adhesion proteins, stabilizing the protrusions at the leading edge during migration. The scheme shows a representation of the Ric-8A/Goα13 pathway during cranial NC migration. First, Goα13 is activated by a G-protein-coupled receptor bound to its ligand. Once the signalling is activated, the Ric-8A GEF activity maintains the monomeric Go protein in an active state and dissociated from the Gβγ dimer, thereby amplifying the signal. In addition, Ric-8A coordinates Goα13 translocation at the leading edge of the cell, where Goα13 interacts with the focal adhesion complex, and regulates focal adhesion formation and/or turnover through FAK and Src as part of a protein complex with integrin (see Discussion).

10×/0.25 NA objective controlled by LAS EZ software (Leica). Frames were obtained every 5 min for a total of 10 h. The dispersion was quantified by Delaunay triangulation (Carmona-Fontaine et al., 2011) using an ImageJ plug-in, comparing the final and initial average triangle area for each condition and was normalized against the control condition. The protrusion time-lapse sequence was obtained using a 40× (objective and frames were collected every 15 s for 30 min). The frames were edited using SimplePCI v 6.6.0.0 software, ImageJ and Photoshop CC. For TIRF microscopy, time-lapse sequences were acquired by Olympus TIRF System confocal microscope using a PLAPON 60× GTIRFM/1.45 NA objective at room temperature. The frames were capture every 30 s for 10 min and were processed using ImageJ.

**Cell substrate adhesion assay**

Cell adhesion was evaluated by turning the dishes upside down after 30 min of culture, as previously described (Carmona-Fontaine et al., 2011). The number of adhered explants was used as a measure of cell-substrate adhesion. When needed, DFA medium was supplemented with 0.5 mM, 1 mM or 2 mM MnCl2 to measure the attachment to extracellular matrix.

**Cell culture and transfection**

HEK293T (ATCC, CLR-11268, mycoplasma free) cells were grown in DMEM containing L-glutamine, 10% foetal bovine serum (FBS) and penicillin/streptomycin. Cells were maintained at 37°C in 5% CO2 and transfected with Lipofectamine 2000 and 3 μg of plasmid DNA (pCS2+6myc-XtRic-8A; pcDNA/His-XtGoα13; pcsp2+FAK-GFP).

**Cell lysates, co-immunoprecipitation and western blot analyses**

Cells were harvested in lysis buffer [0.5% IGEPAL CA030, 10 mM HEPES (pH 7.9), 1 mM DTT, 100 mM NaCl, 0.5 mM PMSF and protease inhibitors (Roche)] and were centrifuged at 2000 g at 4°C for 15 min. Embryos were collected in lysis buffer [0.5% IGEPAL CA030, 10 mM HEPES (pH 7.9), 1 mM DTT, 100 mM NaCl, 0.5 mM PMSF and protease inhibitors (Roche)] and were resuspended by pipetting to obtain a grey homogenous solution. The solution was centrifuged for 10 min at 400 g four times. Proteins were quantified by the Bradford method. Lysates were precleared with protein A/G beads (Thermo Fisher Scientific) and incubated with antibody or control IgG associated with A/G agarose. Beads were collected and rinsed in lysis buffer. Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes, and proteins were detected with an anti-Myc antibody (1:5000, 9E10, Santa Cruz), an anti-Goα13 antibody (1:500, A-20, Santa Cruz) or an anti-6His antibody (1:500, #631212, Clontech), followed by HRP secondary antibodies. Blots were developed using a Western Lightening Plus-ECL (PerkinElmer) and Ultracruz Autoradiography Film Blue (Santa Cruz Biotechnology). Re-bLOTS were performed in low pH stripping solution (25 mM glycine-HCl, 1% p/v SDS).

**Statistical analysis**

Data sets were tested for normality using D’Agostino-Pearson or Kolmogorov–Smirnov’s tests. For normal dataset distribution, Student’s t-test (two-tailed) or one-way ANOVA with a Dunnett’s multiple comparisons was performed. Datasets with non-normal distribution were compared using a Kruskal–Wallis non-parametric test with Dunn’s multiple comparisons. Prism6 (GraphPad) was used for all statistical analyses.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.164269.supplemental

**References**


Figure S1: (a) *In situ* hybridization confirms the presence of Ga13 and Ric8A transcripts in migrating cranial NC cells at stage 26, as previously reported in Maldonado-Agurto et al. (2011) and Fuentealba et al. (2016). (b) Ga13 gain and loss of function controls. Left panel: Anti-Myc Western blot in embryos injected with a myc-Ga13 mRNA. Right panel: Anti-Ga13 Western blot showing that morphant embryos display diminished levels of endogenous Ga13 protein. GAPDH and α-tubulin were used as loading controls. (c) The rescue assay of the Ric-8A morpholino was performed using a myc-Ric-8A mRNA, as previously reported in Fuentealba et al.(2013). Snail2 was used as a migratory cranial NC marker at stage 23 (left panel). Snail2 expression is unaffected in stage 16 Ga13 morphant embryos (right panel). Legend: Is, injected side; Nis, non-injected side. All experiments shown here were performed in *X. tropicalis*. 
Figure S2: Cranial NC morphant cells for Ric-8A and Gα13 undergo EMT. (a) The panels show N-cadherin and E-cadherin expression in green and actin labeled with phalloidin in red at migratory stages of control, Ric-8A and Gα13 cranial NC morphant explants. E-cadherin expression at pre-migratory stages in control explant is also shown. (b) Intensity of N- and E-cadherin in green at cell-cell contact per each condition in migratory stages. Actin in red was labeled with phalloidin. A.u. arbitrary units.
Figure S3: (a) RT-PCR from Ric-8A morphant embryos shows no change at the Gα13 transcript. (b) Repetitions of co-immunoprecipitation assay from Figure 3e, was performed using anti-his (Gα13 tag) and anti-myc (Ric-8A tag) antibodies, confirming that both proteins interact. (c) Co-immunoprecipitation controls from Figure 3e showing that when each protein is expressed alone (without its partner) no pull-down occurs.
**Figure S4:** Intensity of cortical actin labeled with phalloidin (red) at the cell edge in control, Gα13 and Ric-8A morphant and rescue (Ric-8AMO + Gα13 mRNA) conditions. A.u.: arbitrary units.
**Figure S5:** (a) Visualization of β-integrin (immunofluorescence, green) and actin (fluorescent phalloidin, red) were performed in *X. tropicalis* cranial NC. Ric-8AMO and Ga13MO cells showed a decreased number of focal adhesions compared with control cells. Nuclear GFP was used as a lineage tracer. (b) 3D cell reconstruction was performed from an immunostaining of control and Ga13MO-treated cranial NC cells using phospho-paxillin (green) and phalloidin (red). Morphant cells exhibit an altered actin cytoskeleton and display abnormal focal adhesion. (c) Representative images from an adhesion assay performed on control and Ga13 morphant cells to evaluate the attachment of the explants over fibronectin. Phospho-paxillin (green) shows that treatment with MnCl₂ rescues the Ga13MO phenotype, increasing the number of focal adhesion in a dose-dependent fashion.
Figure S6: (a) Control cells and Ric-8AMO-treated cells were analyzed by time-lapse microscopy using FAK-GFP as a marker. The inset in each panel shows a zoom of the red dotted boxes, which indicate stable protrusion and focal adhesions in controls cells and collapsed protrusions and focal adhesions in Ric-8AMO cells (60× magnification). (b) Graphs showing that Ric-8AMO cells exhibit a mildly decreased stability of focal adhesions, which are significantly shorter and smaller than controls. Error bar: Standard deviation, *** p<0.001. (c) Repetitions of co-immunoprecipitation from Figure 6c, was performed using FAK-GFP, Myc-Ric-8A and His-Gα13 in HEK293T cells, illustrating that FAK, Gα13 and Ric-8A interact. Hence Ric-8A, Gα13 and FAK belong to the same protein complex.
Movie 1: Gα13 and Ric-8A are required for cranial NC cell migration *in vitro* and Gα13 subunit rescues the Ric-8A morphant effect.

Movie 2: Gα13 and Ric-8A down-regulation affect actin cytoskeleton reorganization and protrusions formation.
Movie 3: 3D cell reconstruction. Ga13 morphant cells have an altered actin cytoskeleton and displayed abnormal focal adhesion.

Movie 4: Ga13 morphant explants exhibit small and unstable focal adhesions and cellular protrusions.