Coronin-1C and RCC2 guide mesenchymal migration by trafficking Rac1 and controlling GEF exposure

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Abstract

Sustained forward migration through a fibrillar extracellular matrix requires localization of protrusive signals. Contact with fibronectin at a protruding tip activates Rac1, and for linear migration it is necessary to dampen Rac1 activity in off-axial positions and redistribute Rac1 from non-protrusive membrane to the leading edge. We identify interactions between Coro1C, RCC2 and Rac1 that focus active Rac1 to a single protrusion. Coro1C mediates release of inactive Rac1 from non-protrusive membrane and is necessary for Rac1 redistribution to a protrusive tip and fibronectin-dependent Rac1 activation. The second component, RCC2, attenuates Rac1 activation outside the protrusive tip by binding to the Rac1 switch regions and competitively inhibiting GEF action, thus preventing off-axial protrusion. RNAi of Coro1C or RCC2 causes loss of cell polarity that results in shunting migration in 1D or 3D culture systems. Furthermore, morpholinos against Coro1C or RCC2, or mutation of any of the binding sites in the Rac1/RCC2/Coro1C complex delays the arrival of neural crest derivatives at the correct location in developing zebrafish, demonstrating the critical role in migration guidance in vivo.

Keywords: Coronin-1C/Migration/Neural Crest/Rac1/RCC2
Introduction

The persistence and direction in vivo cell migration is essential during development and wound healing, meaning that localization and turnover of protrusive signals are crucial. As the regulator of membrane protrusion, Rac1 is a nexus in migration signaling and is necessary for fibroblast migration along ECM gradients, chiefly fibronectin, but not growth factor gradients (Wu et al., 2012). In vivo, fibroblast-specific Rac1 -/- mice suffer a wound healing defect (Liu et al., 2009) due to defects in fibroblast migration. Likewise, defective Rac1 signaling in Danio rerio (zebrafish) upon injection of morpholinos against the receptor responsible for Rac1 activation, syndecan-4 (Bass et al., 2007), causes developmental defects due to misregulation of Rac1 in migrating neural crest (Matthews et al., 2008).

One major obstacle to the use of matrix gradients as a guidance cue is that, in vivo, gradients along the length of a cell are very shallow (Neilson et al., 2011), and directional migration requires protrusion where the fibronectin signal is greatest, yet avoidance of additional stochastic protrusion. Classical Rac1 regulation has been found to comprise guanine nucleotide exchange factors (GEFs) to switch on Rac1, GTPase activating proteins (GAPs) to switch off Rac1 and guanine nucleotide dissociation inhibitors (GDIs) that sequester Rac1 in the cytoplasm (Burridge and Wennerberg, 2004). However, more recently, other putative Rac1 regulators have been identified that may provide the spatial and temporal resolution required in vivo. One such factor is RCC2 which, despite being initially characterized for its role in mitotic spindle assembly (Mollinari et al., 2003), was recently found as a component of fibronectin-associated adhesion complexes (Humphries et al., 2009). There is some controversy over the cytosolic role of RCC2 because it was previously predicted to act as a Rac1 GEF, based on homology to RCC1 (Mollinari et al., 2003). However, RNAi of RCC2 enhanced Rac1 activation (Humphries et al., 2009), suggesting an inhibitory role, and although buried residues that would result in formation of a 7-bladed propeller are conserved between RCC1 and RCC2, surface resides are poorly conserved, as is the flexible N-terminal extension (Mollinari et al., 2003). Other factors include the coronin family of actin-binding proteins that regulate actin branching by inhibition of the Arp2/3 complex and stimulation of actin depolymerization by cofilin (Chan et al., 2011). Coronin-1A (Coro1A), was recently
found to promote translocation of Rac1 from the cytosol to the plasma membrane (Castro-Castro et al., 2011), thereby potentially regulating Rac1 localization. By associating with the actin cytoskeleton or adhesion complexes, RCC2 and coronins are suitably localized to regulate GTPases and the key question is whether they act as non-canonical GEFs, GAPs or GDIs, second regulators of existing mechanisms or simply influence GTPases by localization.

In this manuscript we define the molecular mechanism by which active Rac1 is focused at the tip of a protrusion. We find that the putative Rac1 regulator, RCC2 protects Rac1 from GEF-mediated activation at the plasma membrane, thereby limiting the dynamics of activation and preventing off-axial protrusion. We find that RCC2 binds coronin-1C (Coro1C), which is itself essential for redistribution and reactivation of GDP-Rac1, and therefore formation of Rac1-dependent protrusions. We find that disrupting any of these interactions causes a loss of unidirectional migration. The defect results in inefficient developmental migration of neural crest cells, so that cartilage structures are misaligned during early zebrafish development, highlighting the importance of Rac1 localization by the RCC2/Coro1C complex during development.
Results

RCC2 as a non-canonical Rac1-sequestering molecule

Persistent migration requires polarization of signals such as Rac1 and, while concerted engagement of fibronectin receptors, $\alpha_5\beta_1$-integrin and syndecan-4, triggers Rac1 activation (Bass et al., 2007), it is less clear how the signal remains localized to a single protrusion as the composition of a 3D matrix changes. RCC2 has been identified as a negative regulator of Rac1 that associates with adhesion complexes (Humphries et al., 2009), leading us to investigate the role of a putative Rac1 inhibitor in a signaling complex traditionally linked to Rac1 activation. To synchronize receptor engagement and specifically interrogate receptor-signaling, we examined how RCC2 influences the kinetics of Rac1 activation in cells spread on the $\alpha_5\beta_1$-integrin-binding fragment of fibronectin, using the syndecan-4-binding fragment of fibronectin (H/0) as the trigger. RNAi of RCC2 consistently accelerated Rac1 activation compared to control, such that there was a 65% increase in active Rac1 at 10 minutes post stimulation, a 44% decrease at 30 minutes, and unstimulated activity was unaffected (Fig. 1A, S1A+B). Conversely, over expression of GFP-RCC2 suppressed activation of Rac1 but had no effect on basal activity (Fig. 1A+S1C). These experiments demonstrate a negative influence of RCC2 over Rac1 but suggest a block in activation, rather than inhibition per se.

Interaction between RCC2 and Rac1 was examined through binding assays. Both endogenous RCC2 and GFP-RCC2 could be isolated from 293T cells using GST-Rac1, but not GST as bait (Fig. 1B). Endogenous RCC2 and Rac1 coimmunoprecipitated (Fig. 1C), and direct interaction between bacterially-expressed proteins was also detected (Fig. 1D). RCC2 was originally characterized as a component of the centromeres of metaphase chromosomes (Mollinari et al., 2003), yet the effect on Rac1 kinetics indicates that RCC2 should be associated with the membrane. RCC2 was found in both membrane and nuclear fractions of fractionated cells and the distribution was not affected by syndecan-4-engagement (Fig. 1E). Rac1 was detected in membrane and soluble fractions with RCC2 and RhoGDI respectively (Fig. S1D). Membrane localization of RCC2 was confirmed by immunofluorescence.
and over expression of GFP-RCC2 clustered and colocalized with membrane-bound DsRed-Rac1, which is normally diffusely distributed (Fig. 1F). Together these experiments demonstrate that RCC2 and Rac1 localize to overlapping subcellular compartments and associate directly in fibroblasts.

To better understand the nature of the RCC2/Rac1 interaction, molecular docking was used to generate a model of the Rac1-GDP/RCC2 complex that would allow us to predict key interacting residues. A structure of RCC2 is yet to be determined, so a homology model was generated from the RCC1 structure (Renault et al., 2001) and docked with the available Rac1-GDP crystal structure (Tarricone et al., 2001), yielding a single high probability Rac1-GDP/RCC2 complex model (Fig. 1G). The “switch I” loop of Rac1, including Asp38 and Thr35 sits within with a positively charged groove on the surface of RCC2 including Lys439 (yellow outline) while “switch II” sits within a negatively charged cavity on the surface of RCC2 (green) and hydrogen bonds form at a third site (magenta)(Fig. 1H). Substitution of Lys439 of RCC2 or Thr35/Asp38 of Rac1 completely blocked the RCC2/Rac1 interaction (Fig. 1I) and RCC2-K439E failed to rescue normal Rac1 activation in knockdown cells (Fig. 1J), confirming that RCC2 binds to the switch region of Rac1 through complimentary electrostatics.

The direct binding to the Rac1 switch region suggests that RCC2 inhibits Rac1 by either acting as a GAP, or protecting Rac1 from activation by a GEF. GFP-RCC2, purified by GFP-trap, did not show GAP activity toward Rac1, RhoA, Cdc42 or Ras (Fig. 2A). To investigate whether RCC2 might protect Rac1 from GEF action, binding of RCC2 to nucleotide-free, GDP and GTPγS-loaded Rac1 were compared. GFP-RCC2 bound to all three forms of bacterially-expressed Rac1 with preference for the GDP-bound form, suggesting a sequestering role (Fig 2B). The result was consistent with further modeling experiments that predicted that GTP-Rac1 would dock more weakly with RCC2, because the interface is confined to the Switch I loop (Fig. S1E-F). To test directly whether RCC2 prevents GEF-mediated activation of Rac1, we conducted a fluorescent guanine nucleotide exchange assay, measuring the increase in fluorescence that occurs when mant-GTP binds to Rac1. The Rac1 GEF, TrioD1 (van Rijssel et al., 2012), caused rapid loading of mant-GTP onto Rac1, compared to Rac1 alone. Addition of GFP-RCC2 to the TrioD1 reaction inhibited
loading of mant-GTP, whereas addition of GFP-RCC2-K439E did not, demonstrating
that RCC2 does indeed protect Rac1 from GEF-catalyzed GTP-loading (Fig. 2C). RCC2
had no effect on the slow, spontaneous loading of mant-GTP (Fig. 2D). RCC2
failed to inhibit p50 RhoGAP-catalyzed hydrolysis of GTP (Fig. 2E), which is
consistent with the preference of RCC2 for the GDP-bound form of Rac1 and the
accelerated Rac1 activation observed in RCC2-knockdown cells. Canonical
mechanisms of Rac1 sequestration involve RhoGDI, but RCC2-knockdown had no
effect on RhoGDI binding to GFP-Rac1, and RhoGDI did not co-precipitate with
GFP-RCC2 (Fig. 2F-G), demonstrating that actions of RCC2 and RhoGDI are not
directly linked. Together these experiments demonstrate that RCC2 retards Rac1
activation by protecting Rac1 from GEF-mediated activation.

Identification of novel RCC2-binding Rac1-regulators

To better understand how RCC2 functions within the cell, we used Stable Isotope
Labeling with Amino acids (SILAC) mass spectrometry to identify proteins that co-
precipitate with GFP-RCC2, but not GFP alone. The majority of RCC2-binding
partners were transcription/translation regulators, consistent with the nuclear
localization of overexpressed RCC2. The list of proteins potentially linked to Rho-
family GTPase regulation was short (Fig. 3A+B) and included Rac1 and Cdc42 as
well as known regulators and effectors. Coro1C was an interesting hit as the
hematopoietic homologue, Coro1A (but not Coro1B), was found to form a complex
with RhoGDI and Rac1 in the cytosol, causing release of Rac1 from the GDI and
translocation to the plasma membrane (Castro-Castro et al., 2011), while Coro1C
affects Rac1 in tumor cells by an unknown mechanism (Wang et al., 2013). We
hypothesized that Coro1C might affect Rac1 localization by interaction with the
RCC2/Rac1 complex. Co-precipitation of Coro1C with GFP-RCC2 or endogenous
RCC2 was confirmed by western blotting (Fig. 3C-D). Coronins comprise a
conserved β-propeller that includes an actin-binding site and acts as a protein
scaffold, a linker region that is unique to each coronin and a coiled-coil domain that
binds Arp2/3 (Fig. S1G-H (Chan et al., 2011)). Endogenous or in vitro transcribed
RCC2 were found to bind to the linker/coiled-coil tail domain of Coro1C using GFP-
Coro1C-tail or GST-Coro1C-tail as respective baits (Fig. 3E-F), and GST-Coro1C-tail
still bound to GFP-K439E-RCC2, demonstrating that the interaction is not dependent on the Rac1-binding motif of RCC2 (Fig. 3G). The RCC2-binding site was mapped to the linker region of Coro1C, and further subdivision of the linker into two sections caused binding to be lost (Fig. S1I). Together these experiments demonstrate that RCC2 binds directly to the linker region of Coro1C, which is poorly conserved between coronins and suggests a specific relationship between RCC2 and the 1C isoform (Fig. 3H).

When the role of Coro1C in Rac1 regulation was tested, RNAi of Coro1C had no effect on unstimulated Rac1 activity but blocked activation of Rac1 in response to syndecan-4 engagement (Fig. 3I, S1J-K). Rac1 activation could be rescued by re-expression of Coro1C, but not Coro1A (Fig. 3I) demonstrating that the Rac1-regulating functions of Coro1C and 1A are not redundant.

The contrasting effects of Coro1C and RCC2 on Rac1, the former allowing and the latter retarding activation, were reflected by the morphology and Rac1 localization of knockdown cells. Compared to the control fibroblasts, which formed a dominant protrusion on fibronectin, RCC2-knockdown cells formed very large or multiple membrane protrusions and ruffles (Fig. 4A-B, S2A, Movie 1 (red markers)). In control cells, GFP-Rac1 or endogenous Rac1 were diffusely distributed but, in RCC2-knockdown cells, Rac1 accumulated in protruding membrane ruffles, consistent with accelerated activation (Fig. 4B, S2A-B red outlines and arrowheads, Movie 1). By contrast, knockdown of Coro1C slightly decreased, but did not abolish the formation of protrusions and caused Rac1 to accumulate in non-protruding membrane (from here on termed lateral membrane) (Fig. 4A-B, S2A-B, arrows). The alterations in morphology are indicative of positive and negative effects of Coro1C and RCC2 on localized Rac1 activation, which we examined using the Raichu-Rac1 FRET probe in response to syndecan-4 engagement. In control cells, Rac1 activation was polarized into a single protrusion, peaking at 30 minutes (Fig. 4C-D, Movie 2). By contrast, Rac1 activity peaked at 10 minutes in multiple protrusions in RCC2-knockdown cells, and was not activated above basal in Coro1C-knockdown cells in agreement with the biochemical and confocal data.

**Coro1C facilitates redistribution of Rac1**
The lateral mislocalization of Rac1 in Coro1C-depleted cells caused us to examine the distribution of Rac1 and Coro1C between membrane microdomains. Fractionation of cells into total membrane, nuclear and cytoskeletal fractions revealed that, as well as localizing to the cytoskeletal fraction, Coro1C could be detected in the membrane fraction, allowing proximity to RCC2 and Rac1 (Fig. 5A). Knockdown of Coro1C did not prevent association of Rac1 or RCC2 with total membrane.

Cell membranes were segregated further by separating cells into detergent soluble (including cytosol (tubulin), early endosomes (EEA1) and soluble plasma membrane (\(\beta_1\)-integrin)) and detergent-insoluble (including flotillin microdomains) fractions (Fig. 5B). Coro1C and RCC2 were found predominantly in insoluble and soluble fractions respectively, but were also detected in the alternate fractions, demonstrating that the locations do overlap. Rac1, normally found in the cytoplasm and soluble membrane of control cells, became trapped in the detergent-resistant pellet upon knockdown of Coro1C (Fig. 5C). This was the opposite of the reported effect of Coro1A-knockdown, which caused increased association with RhoGDI (Castro-Castro et al., 2011). Rac1 was restored to the soluble fraction by reexpression of Coro1C, but not Coro1A (Fig. S3A). The amount of Rac1 detected in lysates prepared with 0.1% SDS was only slightly reduced, demonstrating that Coro1C regulates distribution, rather than absolute protein level of Rac1 (Fig. S3B).

Accumulation of Rac1 in the detergent-resistant pellet was due to membrane-association, as a cysteine-to-serine substitution in the CAAX box caused GFP-Rac1 to remain in the soluble fraction of Coro1C-depleted cells, although the endogenous Rac1 was still detected in the detergent-resistant pellet (Fig. 5D). The shift of Rac1 to the insoluble membrane upon Coro1C-knockdown, suggested that Coro1C might be responsible for release of Rac1 from detergent-resistant membrane, particularly as Coro1C localized predominantly to that fraction in control cells. The hypothesis was supported by imaging, which revealed that both GFP-tagged and endogenous Coro1C localized to the lateral membrane, in proximity to actin stress fibers, as well as ruffling membrane, and was not affected by RNAi of RCC2 (Fig. 5E, S3C-E).
Release of Rac1 from lateral membrane was measured by following dispersion of photoactivatable-GFP-tagged Rac1. Following photoactivation of a 1.5μm square of lateral membrane, fluorescence quickly dispersed in control cells (t½=2.30s), but was retarded by Coro1C-knockdown (t½=3.96s) (Fig. 5F-G, S3F-G, Movie 3). Cysteine-to-serine substitution of the Rac1 CAAX box, to prevent membrane-binding, caused dispersion of activated GFP that was as rapid as dispersion of a cytoplasmic spot (t½=1.86 and 2.03s). Coro1C-knockdown did not slow movement of CAAX-mutant or cytosolic Rac1 (t½=1.76 and 1.80s), demonstrating that loss of Rac1 mobility in Coro1C-knockdown cells is due to membrane association. In fixed cells, where Rac1 is crosslinked to the membrane, the fluorescent signal persisted beyond the duration of the experiment. Finally, photoactivation of a 4.5μm strip of membrane yielded similar rate constants to activation of the 1.5μm square, demonstrating that the loss of fluorescent signal was predominantly due to inward movement of Rac1, rather than sideways diffusion within the membrane.

Interestingly, release of Rac1 from protrusive membrane was unaffected by Coro1C-knockdown (Fig. S3H), despite Coro1C localizing to both lateral and protrusive membrane. This suggested that Coro1C-mediated release of Rac1 is directional, moving Rac1 from lateral to protrusive membrane, where it is activated. Lateral PAGFP-Rac1 redistributed to protruding membrane, in control cells, but was blocked by Coro1C-knockdown (Fig. 5H, Movie 4), demonstrating that Coro1C facilitates the redeployment of Rac1 from the edges of the cell. Furthermore, PAGFP-Coro1C itself redistributed from lateral to protrusive membrane and Rac1 and Coro1C colocalized in small vesicles, demonstrating that they do indeed co-traffic (Fig. 5I-J, Movie 5). Collectively, these experiments demonstrate that Coro1C redistributes Rac1 from lateral to protruding membrane and is necessary for fibronectin-induced activation, while the GEF inhibitor, RCC2, fine-tunes the kinetics and localization of activation.

**Transfer of Rac1 between Coro1C and RCC2**

The role of Coro1C in Rac1 trafficking caused us to examine possible interaction between Coro1C and Rac1. Endogenous Rac1 coimmunoprecipitated with Coro1C (Fig. 6A) and direct binding between bacterially expressed GST-Coro1C and Rac1 was also detected (Fig. 6B). A docking simulation between Coro1C and GDP-Rac1 suggested that GDP-Rac1 docks with the propeller domain of Coro1C in an
arrangement similar to the RCC2/Rac1 complex. No viable solution for interaction of Coro1C with GTP-Rac1 could be found, and GFP-Coro1C bound more strongly to nucleotide-free and GDP-Rac1 than GTP-Rac1 in pull-down experiments, demonstrating that Coro1C binds poorly to active Rac1 (Fig. 6C). The docking experiment predicted that Coro1C binds to the switch regions of GDP-Rac1, including an electrostatic interaction of Arg31 of the Coro1C propeller with Thr35 and Asp38 of Rac1, and substitution of these residues perturbed the Coro1C/Rac1 interaction (Fig. 6D+S3I). Substitution R31E had no effect on the Coro1C/RCC2 interaction or binding of Coro1C to actin (Fig. S3J-K) and, like RCC2-knockdown, Coro1C-knockdown had no effect on RhoGDI binding to Rac1 (Fig. 2F). Therefore the binding experiments collectively demonstrate that RCC2, Coro1C and Rac1 each bind to the other components and association is not reliant on formation of a ternary complex or linked to RhoGDI.

We have demonstrated that Coro1C and RCC2 bind overlapping sites (Thr35/Asp38) on Rac1, the former allowing release from lateral membrane, the latter retarding GEF-mediated activation. This caused us to look for binding competition between RCC2 and Coro1C (Fig. 6E-G). Coro1C could be titrated off GST-GDP-Rac1 beads by increasing concentrations of RCC2. Conversely, an equivalent concentration of Coro1C had no effect on RCC2 binding but did bind to the Rac1/RCC2 complex. We reasoned that this interaction must be due to Coro1C binding to RCC2, rather than competing for the overlapping site on Rac1 and indeed the Coro1C propeller, which includes the Rac1 but not the RCC2-binding site, did not bind except at very high concentration. Similarly, the amount of Coro1C that could be precipitated from cell lysates with GDP-Rac1 was increased by RCC2-knockdown, but Coro1C-knockdown had no effect on precipitated RCC2 (Fig. S3L-M). Together these experiments demonstrate competition between RCC2 and Coro1C, where RCC2 is the preferred partner. The dominant influence of RCC2 was also demonstrated by effects on Rac1 regulation. Unlike Coro1C-knockdown alone, cells with double RCC2/Coro1C-knockdown did not shift Rac1 to the detergent insoluble pellet, and exhibited rapid Rac1 activation and multiple Rac1-rich membrane protrusions, similar to RCC2-knockdown (Fig. 6H-I, S3N-O).
Finally we examined Rac1 release from RCC2 for activation. As RCC2, Coro1C and GEFs bind to the switch I loop, we reasoned that RCC2 and GEF would also compete for binding. At higher concentrations, Trio-D1 did indeed compete RCC2 off GDP-Rac beads (Fig. 6J), which in the presence of GTP would allow nucleotide exchange (Fig. 2C), reducing affinity of Rac1 for RCC2 still further (Fig. 2B)

Collectively, these data demonstrate that Coro1C allows release of GDP-Rac1 from lateral membrane, causing redistribution to protruding membrane where Coro1C is out-competed by RCC2 to form a sequestered pool of Rac1. Only under conditions of high GEF concentration is Rac1 activated, with the result that formation of multiple, unstable protrusions is unfavourable (Fig. 6K).

Rac1 localization is necessary for sustained forward migration in a fibrous environment

In vivo, the extracellular matrix is arranged into fibers along which cells migrate by forming narrow protrusions. To examine the influence of RCC2 and Coro1C on localization of active Rac1 in a fibrous matrix, we seeded Raichu-Rac-transfected MEFs into cell-derived matrix (CDM) (Bass et al., 2007). In control MEFs, active Rac1 was focused into one or two dominant protrusions (Fig. 7A+B, Fig. S4A).

RCC2-knockdown resulted in multiple narrow protrusions that resembled the formation of multiple active-Rac1 lamellae in 2D, and reflected the Rac1-sequestration defect of RCC2-depleted cells. Like the 2D FRET and biochemical analysis, Coro1C-knockdown prevented activation of Rac1 in protrusions and retarded release from lateral membrane (Fig. S4B). Therefore depletion of either RCC2 or Coro1C ablates the formation of a dominant protrusion.

We compared the migration on CDM of knockdown MEFs with wild-type, and also Sdc4-/--MEFs that we previously reported to have a directional migration defect, due to constitutively high, mislocalized Rac1 activity (Bass et al., 2007). RCC2 or Coro1C-knockdown or Sdc4-knockout had negligible effect on migration speed (Fig. 7C). However control MEFs migrated persistently along matrix strands, whereas compromised expression of RCC2, Coro1C or Sdc4 reduced directional persistence, suggesting random migration (Fig. 7D).
Inspection of the migration paths revealed that the modes of migration were very different. Sdc4-/- MEFs moved randomly, whereas RCC2 and Coro1C-knockdown MEFs recognized the topography of the matrix but failed to commit to a dominant protrusion and shunted backwards and forwards on the fibers (Fig. 7E+ Movie 6-7). The two types of behavior were quantified by calculating the curvature of each track (Fig. S4C). The random migration of Sdc4-/- resulted in a curvature value that was almost double that of control MEFs. By contrast, the curvature of RCC2 and Coro1C-depleted MEFs was similar to control MEFs, demonstrating that they followed a linear trajectory, albeit in a non-persistent fashion (Fig. 7F). The shunting migration of RCC2-depleted MEFs was consistent with the presence of active Rac1 protrusions at either end of the cell. Knockdown of RCC2 in MEFs stably expressing β1-integrin-GFP that allowed the cell boundaries to be clearly visualized, revealed that direction changes of RCC2-depleted cells occurred when a different protrusion achieved dominance, rather than due to a tail-retraction defect (Fig. S4D, Movie 8-9). Notably, RNAi of Coro1C resulted in an intermediate persistence (Fig. 7D) as without a dominant protrusion Coro1C MEFs would show less commitment to a particular direction than control but less cause to change direction than RCC2-knockdown. In both RCC2- and Coro1C-knockdown MEFs, expression of wild-type protein restored persistence, but expression of Rac1-binding mutants did not (Fig. 7D, S4E). To ensure that the shunting behavior was not due to differences in matrix organization, we analyzed migration on fibronectin stripes. Control MEFs migrated forward throughout the movie, but Coro1C-knockdown cells shunted, and RCC2-knockdown even more so, in agreement with the CDM data (Fig. 7G).

We reasoned that if the migration phenotype of Coro1C-depleted MEFs is indeed due to constitutively low Rac1 activity, it should be possible to recapitulate the behavior by other means. To test this hypothesis, we took Sdc4-/- MEFs that are unable to activate Rac1 in response to fibronectin but move randomly due to constitutively high Rac1 activity (Bass et al., 2007) and reduced Rac1 expression by RNAi, to a create a second cell type with constitutively low Rac1 activity. This cell type phenocopied the Coro1C-knockdown cells exactly, with low curvature and intermediate persistence values (Fig. 7H-J). Therefore we find that failure to localize Rac1 activity (RCC2-
depleted), limit Rac1 activity (Sdc4-/-), or activate Rac1 in response to a fibronectin stimulus (Coro1C-depleted or Sdc4-/- Rac1-depleted) each compromise efficient migration through a fibrillar environment.

**RCC2 and Coro1C are necessary for proper localization of neural crest derivatives in zebrafish**

Finally we investigated the effect of RCC2 and Coro1C on developmental migration. RCC2 and Coro1C are widely expressed, in mammals and zebrafish (Chan et al., 2011; Thisse and Thisse, 2004). Injection of morpholinos against Coro1C or RCC2 into single-cell embryos reduced protein expression to 52% and 17% respectively at 3 days post fertilization (dpf) (Fig. 8A). Morphants survived to 5 dpf with no gross anatomical defects (Fig. S4F), demonstrating that RCC2 and Coro1C are not essential for developmental migration *per se*. Given that RCC2- or Coro1C-knockdown affected fibroblast migration, we examined the effect on mesenchymal lineages during development. We injected morpholinos into embryos carrying Fli1:eGFP or Sox10:eGFP transgenes, which are expressed in the migratory neural crest that populates the pharyngeal arches that will later form the cartilage elements of the ventral jaw (Lawson and Weinstein, 2002; Wada et al., 2005). Larvae were imaged at 32 hours post fertilization (hpf), at which stage the crest populations of the first and second pharyngeal arches in control fish are separated by the, GFP-negative, pharyngeal pouch 1. Coro1C and RCC2 morphants demonstrated mixing of the 1st and 2nd arch cells suggesting altered migratory behavior (Fig. 8B). At 5 dpf defects were also detected in the chondrocytes that are derived from the migratory crest cells. Col2a1::BAC:mCherry-expressing chondrocytes mislocalized between the Meckel’s cartilage and ceratohyal (derived from the first and second pharyngeal arches respectively), such that in extreme cases the two regions merged together (Fig 8C). The RCC2-knockdown phenotype was subtler, resulting in misalignment of 1st and 2nd arch cartilage elements such as the ceratohyal (Fig. 8C). Staining with Alcian blue revealed cartilage maturation to be normal in morphant fish, demonstrating that reduction of RCC2 or Coro1C expression had no effect on full chondrocyte differentiation or proteoglycan synthesis (Fig. 8D). To test whether the neural crest migration defect was indeed due to the interaction between RCC2 and Coro1C, human mRNAs were coinjected with the Coro1C morpholino. Full-length Coro1C
rescued separation of pharyngeal arches in 53% of fish, but a Coro1C propeller mRNA, lacking the RCC2-binding motif, failed to do so (Fig. 8E+S4G). Furthermore, although coinjection of RCC2 or Coro1C mRNAs rescued the respective morpholino, Rac1-binding mutant mRNAs failed to do so (Fig. 8F-G). Thus, loss of any of the interactions between RCC2, Coro1C and Rac1 cause subtle but significant alterations in mesenchymal migration in vivo.
Discussion

In this manuscript we discover a novel Rac1 regulation complex comprising RCC2 and Coro1C. We find that: 1) Coro1C redistributes Rac1 from lateral to protrusive membrane; 2) RCC2 limits GEF activation of Rac1 by obscuring the switch regions and thereby prevents formation of multiple protrusions; 3) perturbation of RCC2 or Coro1C mislocalizes Rac1 and results in shunting migration; 4) suppression of RCC2 or Coro1C results in mislocalization of neural crest derivatives in developing zebrafish.

The competitive nature of the interactions of Rac1 with RCC2, Coro1C and GEFs means that local concentrations of the individual binding proteins will be important factors in determining activation rates. The bound nucleotide will also play a role. The high affinity of RCC2 for GDP-bound Rac1 will retard the initial binding of GEF, but the equilibrium will shift toward GEF-binding as nucleotide exchange proceeds. This means that, in regions of low GEF activity, RCC2 would sequester Rac1, preventing stochastic activation, but in areas of high GEF activity, Rac1 will be activated efficiently, causing a cell to form a single dominant protrusion. Equally, the poor affinity of Coro1C for GTP-Rac1 means that Coro1C will effectively redistribute inactive from lateral membrane, but not active Rac1 from protrusions (Fig. S3H), pushing the equilibrium towards active Rac1 in a protrusion. Although the relationship of RCC2 and Coro1C could be explained in terms of relative affinity, it is probable that there will be additional regulation events. Indeed, phosphorylation of the coiled-coil domain of trimeric Coro1C prevents Arp2/3 binding and therefore organization of the actin cytoskeleton (Xavier et al., 2012). Although RCC2 binds to the linker region, rather than the coiled-coil, phosphorylation of the coiled-coil may influence RCC2 binding, either directly or by altering the localization of Coro1C. The possibility of other regulatory events affecting RCC2 or Coro1C is certainly a topic for future investigation.

It was notable that Cdc42 was also found in the RCC2 interactome and that the switch regions of all Rho family GTPases share considerable homology. This could mean that RCC2 will have broader applications than those we identify here, and focus the activation of other Rho family members, with the exact sites of activity depending on
local GEF concentrations. It has also been predicted that *D. discoideum* coronin might bind both Rac1 and Cdc42 through a CRIB-like domain (Swaminathan et al., 2014). However, the CRIB-like domain is poorly conserved in Coro1C and the critical histidine residue buried. The tail domains are also poorly conserved between *D. discoideum* coronin and Coro1C, and the functions of Coro1C and Coro1A are not redundant, making it less likely that Coro1C has the broad specificity attributed to coronin in *D. discoideum*.

During mesenchymal cell migration, Rac1 must be delivered to the leading edge for activation by adhesion receptor-associated GEFs. The two known mechanisms of Rac1 redistribution exert a negative influence on Rac1 signaling: caveolin-mediated endocytosis of polyubiquitinated Rac1 results in Rac1 degradation (Del Pozo et al., 2005; Nethe et al., 2010), while RhoGDI extracts Rho-family GTPases for sequestration in the cytosol (Nomander et al., 1999). In this manuscript we describe a third possibility. Coro1C releases Rac1 from lateral membrane and trafficks it to the leading edge. Because the retrafficking and the sequestering properties of the RCC2/Coro1C complex are distributed between two molecules, the sequestering phase of Rac1 redistribution can be avoided at the protruding tip, unlike the RhoGDI pathway, which sequesters Rac1 as part of the extraction process. The benefit of the multi-component system is that Rac1 could pass through the Coro1C redistribution loop faster than it could pass through the RhoGDI sequestration loop, allowing better temporal resolution of Rac1 signaling.

In the Rac1 FRET experiments, a soluble syndecan-4 ligand caused localized Rac1 activation (Movie 2), despite the diffuse nature of the stimulus, demonstrating the predisposition of certain regions of membrane to Rac1 activation. A role of Coro1C in maintaining high, local Rac1 activity fits well with the other known functions of type I coronins. The archetypal role of coronins is prevention of Arp2/3-mediated actin branching that focuses lamellapodial protrusion. One would expect Rac1 to be retrieved from membrane where branching is inhibited and time-lapse, single-molecule analysis of this process is now necessary to test the hypothesis. It was notable that the SILAC analysis that identified Coro1C as an RCC2-binding protein did not identify components of the Arp2/3 complex. The role of RCC2 in sequestering Rac1 is consistent with this finding. One would expect to find Arp2/3 at
points where Rac1 is delivered for immediate reactivation, but not at points where Rac1 is sequestered. Indeed the benefit of separating the retrafficking/sequestering properties of Coro1C/RCC2 would be lost if the proteins constitutively associated.

The interactions between Rac1, RCC2 and Coro1C had pronounced effects on neural crest localization in the developing zebrafish. Likewise, syndecan-4 morphant fish exhibit a defect in neural crest migration, due to aberrant Rac1 regulation, that blocks cartilage development altogether (Matthews et al., 2008). The severity of the syndecan-4 phenotype is consistent with the migration defect of fibroblasts, because while Sdc4-knockout MEFs exhibit entirely random migration, Coro1C or RCC2-depleted MEFs can still recognize matrix fibers but lack the polarity to move processively along them, so that developmental migration is retarded, rather than completely blocked. Given the similarities in vitro and in zebrafish, it will be interesting to see whether Coro1C or RCC2 defects in mammals result in the defective wound healing that is the hallmark of syndecan-4 knockout mice.
Materials and Methods

Cell Culture
Immortalized wild-type and Scd4 -/- MEFs and human fibroblasts has been described previously (Bass et al., 2007). For RNAi, siRNA duplexes with ON TARGET™ modification were transfected with Dharmafect2 reagent (Thermo Fisher Scientific). Sequences targeted the sense strand of mouse RCC2 (CCAACGUGGUGGUUCGAGA or UCCAAGCGAUUCAACGUUA), Coro1C (CCGUUUGAAAUAAUACGUA or GUAUAAACACUCACGAGAA), Rac1 (AGACGGAGCUGUUGGUAAAUU), human RCC2 (AGACGGAGCUGUUGGUAAAUU), and Coro1C (GCACAAGACUGGUCGAAUU), using siGLO as control.

Cell spreading for biochemical assays
Tissue culture-treated plastic dishes (Corning BV) were coated with 20 μg/ml recombinant fibronectin polypeptide encompassing type III repeats 6-10 that comprises the α5β1-integrin ligand (50K) (Danen et al., 1995). To prevent de novo synthesis of ECM and other syndecan-4 ligands, MEFs were pretreated with 25 μg/ml cycloheximide (Sigma) for 2 hours and spread for 2 hours in DME, 4.5 g/l glucose, 25 mM HEPES, 25 μg/ml cycloheximide. Spread cells were stimulated with 10 μg/ml recombinant fibronectin polypeptide encompassing type III repeats 12-14 that comprises the syndecan-4 ligand (H/0) (Sharma et al., 1999).

GTPase assays
Active Rac1 was precipitated from cell lysates using GST-PAK-CRIB as bait. Fluorescent RhoGEF exchange assays using 1.8 μM recombinant GTPase, 0.75 μM MantGTP, 3 μM GFP-TrioD1 and 2 μM GFP-RCC2, and RhoGAP assays using 6.2 μM recombinant GTPase and 200 μM GTP incubated with 3.7 μM GFP-RCC2, 3.7 μM GFP or 1 μM p50RhoGAP for 20 minutes, before adding CytoPhos dye were performed according to manufacturer’s instructions (Cytoskeleton).

Cell fractionation
Spread cells were fractionated into total membrane, nuclear and cytoskeletal using a QProteome, subcellular fractionation kit (QIAGEN) (Bunger et al., 2009). Detergent-resistant membrane and cytoskeletal components were isolated using 20 mM HEPES (pH 7.4), 10% (v/v) glycerol, 140 mM NaCl, 1% (v/v) Nonidet P-40, 4 mM EGTA, 4 mM EDTA.

GFP-Trap
HEK-293T cells transfected with GFP-RCC2 (M. Humphries, University of Manchester), GFP-K439E-RCC2, GFP-TrioD1 (J. van Buul, Sanquin, Amsterdam), GFP-Coro1C (J. Bear, Howard Hughes Institute, North Carolina), GFP-R31E-Coro1C, GFP-Coro1C propeller, GFP-Coro1C tail or GFP-C1 (Clontech) were purified by GFP-Trap (ChromoTek). Where required, proteins were eluted in glycine, pH 2.5 and neutralized with Tris-HCl, pH 10.4. For mass spectrometry, cells were cultured in R0K0 (GFP) or R6K4 (GFP-RCC2) SILAC media, before GFP-trap, and analyzed using a LTQ-Orbitrap Velos mass spectrometer, filtering data to satisfy false discovery rate of less than 1%.

Binding assays
GST-Rac1 dynabeads were loaded with 6 mM GDP, 0.6 mM GTPγS or no nucleotide and used to pull down target proteins from non-clarified cell lysates or in titrations with purified proteins, where 0.25 µM GFP-Coro1C or GFP-RCC2 were bound with increasing concentrations of competitor.

GST, GST-RCC2, GST-Coro1C or GST-Coro1C tail domain, immobilized on agarose beads, were incubated with soluble recombinant GDP-loaded Rac1 or in vitro translated RCC2, prepared using TnT quick transcription/translation kit (Promega). For cosedimentation, 21 µM F-actin and 2 µM GFP-Coro1C proteins were cosedimented at 150000xg.

Homology modeling and docking studies
The RCC2 homology model was generated using the ESyPred3D server (Lambert et al., 2002) (http://www.fundp.ac.be/urdm/bioinf/esypred/), followed by model building in MODELLER and macromolecular docking with the Rac1-GDP crystal structure (Tarricone et al., 2001) (PDB 1I4D) using the ClusPro sever (Comeau et al., 2004)
ImmunoFluorescence

Fibroblasts transfected with pDsRed-Rac1, GFP-RCC2 or GFP-Coro1C under the control of a pMSCV promoter were spread on glass coverslips coated with 10 μg/ml fibronectin (Sigma) or CDM for 4 hours in DME, 10% FBS, 4.5 g/l glucose, 25 mM HEPES. Where appropriate, fixed cells were stained for Rac1, Coro1C, cortactin or with phalloidin and photographed on a Leica SP5-II confocal laser scanning microscope using a 100x, NA 1.4 PlanApo objective. Maximum projection images were compiled, bandpass filtered, and analyzed using ImageJ software.

Migration Analysis

CDMs were generated as described previously by culturing confluent fibroblasts for 10 days before removing the fibroblasts by NH4OH lysis (Bass et al., 2007). Cells were spread on CDM or 2D fibronectin in media with 10% serum for 4 hours before capturing time-lapse images at 10-minute intervals for 10 hours on a Leica AS MDW microscope using a 5x NA 0.15 Fluotar or 40x NA 0.55 N PLAN objective and Roper CCD camera. The migration paths of all non-dividing, non-clustered cells were tracked using ImageJ software.

Persistence was calculated by dividing linear displacement of a cell over 10 hours by the total distance migrated.

The total absolute curvature of a track curvature, C, is defined by

\[ C = \int_0^l |\kappa| ds = \sum_{i=1}^{N} k_i |\Delta s_i| \]

where \( k_i \) is the curvature at the mid-point of a straight track segment \( i \), \( \Delta s_i \) is the length of that segment and the curvature \( \kappa \) for a parametric curve \( \dot{x} = x(s), \dot{y} = y(s) \) is given by

\[ \kappa = \frac{\dot{x} \dot{y}' - \dot{y} \dot{x}'}{\left( \dot{x}^2 + \dot{y}^2 \right)^{3/2}} = \frac{1}{R} \]

with
\[ x'' = \frac{dx}{ds}; x' = \frac{d^2x}{ds^2}; y'' = \frac{dy}{ds}; y' = \frac{d^2y}{ds^2} \]

where \( s \) is the arc length along the track (Cui et al., 2009). Interpretation of \( C \), illustrated in figure S4C, is straightforward. Regions of high curvature correspond to regions with a small radius of curvature, where the track is tightly curved and changing direction rapidly.

For migration on 5-μm fibronectin stripes (Cytoo), cells were seeded in media with 2% serum and allowed to spread for 2 hours before capturing time-lapse images at 10-minute intervals for 6 hours on a Leica AS MDW microscope using a 10x NA 0.3 objective and Roper CCD camera. Cells were scored for number of direction changes during the movie.

**FRET analysis of Rac activity**

Fibroblasts transfected with the Raichu Rac probe (Itoh et al., 2002) were filmed on 50K-coated MATTEK dishes for 10 min, before stimulation with H/0 for 40 min. Images were acquired on a Leica DM IRBE inverted microscope using a 63x NA 1.32 objective, Sutter DG5 light source and Photometrics CoolSnap HQ2 camera, capturing images through CFP and YFP filters upon excitation through the CFP channel every 2 minutes. FRET ratios were calculated as described previously (Machacek et al., 2009) using ImageJ software. Briefly, aligned CFP and YFP images were corrected for uneven illumination, photobleaching and background subtracted. A binary mask was used to define the borders of the cell, and the YFP image divided by the CFP image to yield a ratio image reflecting the distribution of Rac1 activity across the cell. The same result was obtained when the order of acquisition of CFP and YFP images was reversed or when fixed cells were analyzed, eliminating motion artifacts.

**Photoactivatable-GFP-Rac1**

Control or Coro1C-knockdown MEFs were cotransfected with pmCherry and PAGFP-tagged Rac1, Rac1-CAAX (cysteine to serine substitution) or Coro1C, and spread on fibronectin or CDM. A 1.5x1.5μm or 1.5x4.5μm box on the lateral membrane was photoactivated at 405 nm and then GFP fluorescence at 488 nm was recorded on an Ultraview Spinning Disk confocal microscope (Perkin Elmer) using a 63x, NA 1.3 PlanApo objective. For diffusion experiments, images were captured at
2 images per second for 5 seconds prior to, and 15 seconds after photoactivation. For re-trafficking experiments, PAGFP was photoactivated at 3-4 separate 1.5x4.5μm boxes and then images captured at 1 image per minute for 10 minutes. For diffusion experiments, pixel intensity within a 1.5x1.5μm box at the centre of the activated area was measured and one-phase decay curves fitted using GraphPad Prism. Significant difference between decay curves was tested using F-tests.

Zebrafish husbandry and analysis

All experiments were carried out according to UK Home Office regulations. Lines used were Tg(col2a1BAC:mCherry) (Hammond and Schulte-Merker, 2009), Tg(Fli1:eGFP)y1 (Lawson and Weinstein, 2002) and Sox10:eGFP (Wada et al., 2005). Morpholinos (Gene Tools) against coronin-1ca (ENSEMBL gene ID: ENSDARG00000035598) (translation blocking: TCGTACAACCCGTTTGAACATATCT, 3.5 nM) and RCC2 (ENSEMBL gene ID: ENSDARG00000011510) (splice blocking: ATACAAGAAGCATCCTTACAATCTT, 1 ng) were injected at the 1 cell stage. For rescue experiments, 200 pg human mRNA, prepared using a mMessage mMACHINE kit (Ambion), were coinjected with the morpholino. Images were captured on a Leica SP5-II confocal laser-scanning microscope. Alcian blue and Alizarin red staining is as previously described (Walker and Kimmel, 2007).
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Author Contributions
RCW, CAMC, CLH and JR conducted experiments and contributed to the design of the project. DB and YF conducted experiments. TCSR wrote software for migration tracking. PR performed in silico modeling. MDB conducted experiments, designed the project and wrote the manuscript.


Figure Legends

Fig. 1. RCC2 retards Rac1 activation at the membrane

(A) H/0-stimulated Rac1 activation in control, RCC2-knockdown MEFs (oligo #1, n=7), and MEFs overexpressing GFP-RCC2 (n=4). RNAi of RCC2 with an alternative oligo, pairwise comparison of time points and RCC2 expression from the same experiments shown in Fig. S1A-C.

(B) Pull down of endogenous RCC2 (60 kDa) and exogenous GFP-RCC2 (87 kDa) from 293T cells using GST or GDP-loaded GST-Rac1 as bait, n=6.

(C) Endogenous RCC2 and Rac1 coimmunoprecipitate from MEFs, n=6.

(D) Pull down of recombinant GDP-loaded Rac1 using GST-RCC2, n=8.

(E) Protein distribution between total membrane, nuclear and cytoskeletal fractions using a Qproteome kit. Comparison of control and RCC2-knockdown fibroblasts, with and without syndecan-4 engagement. n=7.

(F) Images of fibroblasts expressing GFP-RCC2, dsRed-Rac1 or both spread on fibronectin with serum and fixed. Colocalization tested by ImageJ. Bar=10 μm.

(G) Ribbon diagram of the modeled GDP-Rac1/RCC2 complex.

(H) Open-book representation of the binding interface between RCC2 and GDP-Rac1 outlining the first (yellow), second (green) and third (magenta) interaction sites.

(I) Interaction between GFP-RCC2 and recombinant GDP-loaded Rac1 was blocked by mutation of an interaction site of either molecule. n=4.

(J) H/0-stimulated Rac1 activation in RCC2-knockdown MEFs rescued with GFP-RCC2-K439E or GFP-RCC2. n=4.

Error bars indicate s.e.m. Significance tested by ANOVA. *=p<0.05, **=p<0.005.

Fig. 2. RCC2 inhibits GEF-mediated activation of Rac1

(A) Comparison of GTP hydrolysis by GTPases in the presence of GFP (negative control), p50 RhoGAP (positive control) and GFP-RCC2. n=4.

(B) GFP-RCC2 from 293T lysates bound preferentially to GDP-loaded, rather than nucleotide-free or GTPγS-loaded GST-Rac1. n=7. Significance tested by ANOVA, *=p<0.05.
(C-D) GFP-RCC2, but not the K439E mutant inhibited TrioD1-mediated loading of mant-GTP on to Rac1 (C), but had no effect on spontaneous GTP loading (D). n=12.

(E) GFP-RCC2 failed to inhibit p50-catalyzed GTP hydrolysis by Rac1. n=12.

(F) GFP-Rac1 was precipitated by GFP-Trap from control, RCC2-knockdown and Coro1C-knockdown MEF lysates. Coprecipitated RhoGDI, RCC2 and Coro1C were detected by Western blot. n=4.

(G) GFP-RCC2 was precipitated by GFP-Trap and blotted for RhoGDI and RCC2. n=4.

For (A+C-E), tagged RCC2 and TrioD1 proteins were purified from 293T cells by GFP-Trap and relative protein loading was demonstrated by coomassie-stained gel or Western blot. Error bars indicate s.e.m.

**Fig. 3. The RCC2-binding protein, Coro1C, is necessary for syndecan-4-stimulated Rac1 activation**

(A+B) Plot of the 1636 proteins identified by SILAC mass spectrometry that associate with GFP-RCC2 better than GFP. Proteins linked to GTPase signalling in red and (B), nuclear and ribosomal proteins in grey, and other proteins in black.

(C) Coro1C coprecipitated with GFP-RCC2 but not GFP from 293T cells in a GFP-Trap. n=6

(D) Endogenous RCC2 and Coro1C coimmunoprecipitated from fibroblasts. n=7.

(E) RCC2 coprecipitated from 293T lysates with GFP-Coro1C full length and tail domain, but not propeller domain. n=5.

(F) *In vitro* translated RCC2 bound to GST-Coro1C tail in a pull down assay. n=5.

(G) GFP, GFP-RCC2 or GFP-RCC2-K439E beads were incubated with GST-Coro1C tail and blotted for bound tail protein.

(H) Schematic of the domain structures of RCC2 and Coro1C.

(I) Syndecan-4-stimulated Rac1 activation in Control (n=6) Coro1C-knockdown (n=8), Coro1C-rescue (n=4) and Coro1C-knockdown/Coro1A rescue (n=6) and RCC2/Coro1C-double-knockdown MEFs (n=5). Significance tested by ANOVA, *=p<0.05, error bars indicate s.e.m. Coro1C-knockdown with an alternative oligo shown in Fig. S1K.

**Fig. 4. RCC2 and Coro1C regulate Rac1 activity in protrusions**
Control, RCC2 or Coro1C-knockdown MEFs expressing GFP-Rac1 were filmed on fibronectin with serum. (A) Frames were scored for number of protrusions, representative frames indicating protrusion between adjacent frames (red outline+arrowhead) and non-protrusive lateral membrane (arrow). Representative of 21 movies. (C-D) Engagement of syndecan-4 causes activation of a Raichu-Rac1 activity reporter (arrows) in a single protrusion in control, multiple protrusions in RCC2-knockdown, and no protrusions in Coro1C-knockdown MEFs. (C) Images are frames from Movie 2. (D) FRET intensity across protruding membrane of individual cells. n=12. Error bars indicate s.e.m. Bar=10μm.

Fig. 5. Coro1C mediates relocalization of Rac1 from lateral to protrusive membrane

(A) Protein distribution between total membrane, nuclear and cytoskeletal fractions using a Qproteome kit. Comparison of control and Coro1C-knockdown fibroblasts, with and without syndecan-4-engagement. n=4. (B) Coro1C localized to detergent (1% Nonidet P-40) resistant membrane that includes flotillin-2. Rac1 and RCC2 localized to detergent soluble lysate that includes EEA1, β1-integrin and tubulin. n=8. (C) Rac1 was displaced from the detergent soluble to insoluble fraction upon knockdown of Coro1C. Significance tested by T-test, *=p<0.05, n=8. (D) Mutation of the CAAX box caused GFP-Rac1 to remain in the detergent soluble fraction of Coro1C-knockdown MEFs. n=8. (E) GFP-Coro1C localized to both lateral (arrows) and ruffling (arrowheads) membrane. Representative of 100 cells. (F-G) Coro1C slows release of photoactivated GFP-tagged Rac1 from the membrane. 1.5μm or 4.5μm sections (red boxes) of lateral membrane, identified by cotransfection with mCherry, were photoactivated at 405nm, and dispersion of activated-GFP-tagged Rac1 from the central 1.5μm (arrows) followed at 488/525nm. Panels show sample images 3 seconds post-activation, taken from Movie 3. (G) Rate constants of fluorescent decay, comparing Coro1C-knockdown with control MEFs, using full length or CAAX-mutant PAGFP-Rac1. Significance tested by F-test, **=p<0.005, n=18.
(H-I) In control, but not Coro1C knockdown MEFs, PAGFP-tagged Rac1 (H) and Coro1C (I) activated in lateral membrane was recruited to protruding membrane. Frames from Movies S4-5, n=15.

(J) GFP-Coro1C and DsRed-Rac1 colocalize in vesicles. Error bars indicate s.e.m, bar=10μm.

Fig. 6. RCC2 and Coro1C compete for an overlapping binding site on Rac1
(A) Endogenous Coro1C and Rac1 coimmunoprecipitated from fibroblasts. n=4.
(B) Recombinant GDP-loaded Rac1 bound directly to GST-Coro1C. n=7.
(C) GFP-Coro1C from 293T lysates bound poorly to GTPγS-loaded, compared to GDP-loaded or nucleotide-free GST-Rac1. n=6.
(D) Interaction between GFP-Coro1C and recombinant GDP-loaded Rac1 was blocked by mutation of an interaction site of either molecule. n=4.
(E-G, J) GST-GDP-Rac1 beads loaded with 0.25 uM GFP-Coro1C or GFP-RCC2 in the presence of increasing concentrations of GFP-RCC2, GFP-Coro1C, propeller or GFP-TrioD1 competitor. n=4
(H) Rac1 remained in the detergent soluble fraction upon knockdown of both Coro1C and RCC2. n=11.
(I) Syndecan-4-stimulated Rac1 activation in RCC2/Coro1C-double-knockdown MEFs (n=6).

Significance tested by ANOVA, *=p<0.05, error bars indicate s.e.m.

K) Schematic of the roles of Coro1C and RCC2 in Rac1 retrafficking and sequestration.

Fig. 7. Localization of Rac1 signals by RCC2 and Coro1C is necessary for processive migration
(A) Distribution of active Rac1 (arrows) in MEFs embedded into cell-derived matrix measured using a Raichu-Rac1 activity reporter. Images representative of 10 experiments, bar=10 μm.
(B) MEFs embedded into CDM were scored for cortactin-positive protrusions, images in Fig. S4A. n=56.
(C-F) 10-hour migration characteristics of cell types embedded into CDM. (C) Speed (distance/time). (D) Persistence (displacement/distance), grey bars indicate the
experimentally determined threshold for random migration on 2D. (E) Example
migration tracks, bar=100 μm. (F) Curvature (see methods and Fig. S4D).
(G) Frequency of migration turns on 5-μm fibronectin stripes.
(H) Knockdown of total Rac1 in sdc4 -/- MEFs caused a concomitant loss of GTP-
Rac1.
(I-J) 10-hour migration characteristics of cell types embedded into CDM.
Results represent analysis of >100 cells per condition. Error bars indicate s.e.m.
Significance was tested by Kruskal-Wallis test, *=p<0.05, **=p<0.005.

Fig. 8. Morpholino knockdown of Coro1C and RCC2 leads to migration defects
in the developing zebrafish
(A) Coro1C and RCC2 expression in morpholino-injected whole zebrafish lysate at 3
dpf relative to control morpholino.
(B) 32 hpf lateral views of Fli1:eGFP transgenic embryos exhibited altered migration
of neural crest in the 1st and 2nd pharyngeal arch elements (red dotted line), leading to
mixing of the two arches (red arrows), upon injection of Coro1C (23/32 fish) or
RCC2 (24/28 fish) morpholinos.
(C) col2a1BAC:mCherry transgenic embryos injected with control (n=49), Coro1C
(n=31) or RCC2 (n=32) morpholinos, ventral views at 5 dpf, white arrow shows
mislocalized chondrocytes between the meckel’s cartilage (Mc) and ceratohyal (ch).
yellow arrow shows misaligned elements.
(D) Alcian blue and Alizarin red stained larvae at 5 dpf, ventral views (n=28-32).
(E-G) Position of Sox10:eGFP-expressing neural crest cells at 32 hpf in fish injected
with control, Coro1C or RCC2 morpholinos plus mRNA as appropriate.
Williamson Fig. 4

A

B

Ctrl  RCC2 KD  Coro1C KD

GFP-Rac1

Zoom

C

Unstimulated

Syndecan-4 engagement

-11

29

-11

13

23

FRET ratio:

5

4

3

2

1

0

D

Ctrl

RCC2 KD

Coro1C KD

FRET Ratio

µm from leading edge

µm from leading edge

µm from leading edge
Williamson Fig. 8

A
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B

C

D

C'

E

F

G

E

F

G

Williamson Fig. 8

A

B

C

D

C'

E

F

G
Supplemental Information

A coronin-1C/RCC2 complex guides mesenchymal migration by trafficking Rac1 and controlling GEF exposure

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Fig. S1. RCC2 and Coro1C are Rac1 regulators

(A) Syndecan-4-stimulated Rac1 activation in RCC2 knockdown MEFs. Experiment uses an alternative oligo sequence to that shown in Fig. 1A. n=3.

(B) Pairwise comparison of syndecan-4-stimulated Rac1 activation at 0 and 10 minutes stimulation, in control and RCC2-knockdown MEFs (oligo #1), the complete timecourses are shown in Fig. 1A. n =7.

(C) Comparison of Rac1 activation in unstimulated control MEFs and MEFs overexpressing GFP-RCC2. n = 4.

(D) Distribution of Rac1, RhoGDI and RCC2 between soluble (CCT2) and total membrane (β1-integrin) fractions using a Qproteome kit. n=7.

(E) Scores for in silico docking experiments between RCC2/Coro1C and GDP/GTP-loaded Rac1.

(F) Binding interface between RCC2 and GTP-Rac1 from in silico docking experiments, demonstrating that interactions are confined to the Switch 1 loop of GTP-Rac1.

(G-H) Coro1C can be divided into functional subdomains. (E) Arp3 can be pulled-down from lysate with GST-Coro1C-tail. (F) GFP-Coro1C full length and propeller domain cosediment with filamentous actin. n=4.

(I) RCC2 coprecipitated from 293T lysates with GFP-Coro1C linker region (residues 351-435), but not the coiled-coil domain (residues 436-474). Further subdivision of the linker into N- and C-terminal parts (residues 351-397 and 393-435 respectively) also caused loss of binding. n=4.

(J) Knockdown of Coro1C had no effect on Rac1 activity in unstimulated MEFs. n=8.

(K) Syndecan-4-stimulated Rac1 activation in Coro1C knockdown MEFs. Experiment uses an alternative oligo sequence to that shown in Fig. 3I. n=4.
Fig. S2. Coro1C and RCC2 regulate Rac1 localization

(A) Control, RCC2-knockdown, Coro1C-knockdown, RCC2/Rac1-knockdown, or Coro1C/Rac1-knockdown MEFs were spread on fibronectin in the presence of serum. Cells were fixed and stained with phalloidin and immunostained for endogenous Rac1. Rac1 accumulated in actin-rich lamella (arrowheads) of RCC2-knockdown MEFs and lateral membrane (arrows) of Coro1C-knockdown MEFs. Images are representative of 100 cells on 4 separate occasions.

(B) GFP-Rac1 accumulation in protrusions of RCC2-knockdown MEFs and along the sides of Coro1C-knockdown MEFs spread on fibronectin in the presence of serum. Intensity profiles were measured across protrusions (red) and lateral membrane (green). 13 profiles, randomly selected from 100 cells from 4 separate experiments are displayed.

Fig. S3. Competition between Coro1C and RCC2 cause Rac1 redistribution

(A) Rac1 was restored to the detergent-soluble fraction of Coro1C-knockdown MEFs by exogenous expression of Coro1C, but not Coro1A. n=4.

(B) Knockdown of Coro1C has no affect on total Rac1 protein levels, lysates prepared with 0.1% SDS to ensure extraction of Rac1 from all membrane fractions. n=4.

(C) Immunofluorescent staining of endogenous Coro1C demonstrating localization to both actin-rich ruffles (arrowheads) and lateral membrane (arrows). Staining is ablated by knockdown of Coro1C. Images are representative of 100 cells on 3 separate occasions.

(D) Fibroblast expressing GFP-Coro1C following RCC2 knockdown, spread on fibronectin with serum and fixed. Image representative of 50 cells on 2 separate occasions.

(E) Protein distribution between soluble, total membrane, nuclear and cytoskeletal fractions. Coro1C is still found in soluble, total membrane and cytoskeletal fractions upon RCC2-knockdown. n=7.

(F) Fluorescent decay curves compare redistribution of photoactivated GFP-tagged Rac1 from lateral membrane between control and Coro1C-knockdown MEFs.

(G) Rate constants of fluorescent decay, comparing Coro1C-knockdown with control MEFs, using alternative oligos to those presented in Fig. 5G. n=18.
(H) Rate constants of fluorescent decay, comparing dispersion of PAGFP-Rac1 from protrusive membrane Coro1C-knockdown with control MEFs, including dispersion from lateral membrane of control cells for comparison. n=18.

(I) Binding interface between Coro1C and GDP-Rac1 from *in silico* docking experiments, demonstrating that interactions include Thr35 and Asp38, similar to the RCC2/GDP-Rac1 complex.

(J) RCC2 coprecipitates with GFP-Coro1C and GFP-Coro1C-R31E with similar efficiency, using a GFP-Trap from 293T cells. n=3

(K) Wild type and R31E Coro1C cosediment with freshly polymerized filamentous actin at 150,000xg, whereas the previously charactized actin-binding mutant of Coro1C, R28D/2xKE, does not.

(L-M) Competition between RCC2 and Coro1C for binding to Rac1. Pull down assays from lysates of control, RCC2-knockdown or Coro1C-knockdown cells using GST or GDP-loaded GST Rac1 as bait. n=6

(N-O) Knockdown of both RCC2 and Coro1C results in a morphology that resembles RCC2 knockdown. Cells form multiple lamellae (arrowheads) on fibronectin, to which both immunostained endogenous Rac1 and GFP-Rac1 localize. Images are reproduced, in part, in Fig. S2B. n=70.

Error bars indicate s.e.m. Significance was tested by T-test, ** p<0.005. Bar = 10 µm.

**Fig. S4. RCC2 and Coro1C regulate migration**

(A) MEFs spread on CDM with serum, fixed and stained for cortactin and fibronectin. Images representative of 100 cells on 2 separate occasions. Bar = 10 µm.

(B) Fluorescent decay curves compare redistribution of photoactivated GFP-tagged Rac1 from lateral membrane between control and Coro1C-knockdown MEFs plated on CDM. n=18.

(C) Schematic of how the angle of each step of a migration path was assessed to calculate curvature of path.

(D) Cell outlines illustrating a migration sequence, red>yellow>green>cyan>blue to show that control cells are processive, while RCC2-depleted cells shunt. Individual frames derived from Movies S8-9.

(E) Lysates of control, RCC2, and Coro1C knockdown and rescued MEFs, used in Fig. 7C-F) were blotted for RCC2 to confirm knockdown and rescue.
Lateral views of control, Coro1C or RCC2 morphants, at 4 dpf.

Confocal stacks at 32 hours post fertilisation of the neural crest reporter line: Tg(-4.9sox10:EGFP)ba2 (Wada et al, 2005). Images are all of left-facing zebrafish heads, anterior to top. 1=neural crest stream from which 1st arch skeletal elements will be derived, 2= neural crest stream from which 2nd arch skeletal elements are derived, 1/2 indicates failed separation of these two streams. Fish were staged as 32 hpf by reference to migration of sox10:GFP labelled pigment precursors in the trunk. Ctrl are control morpholino injected (n=70), Mo only= Coro1C morpholino injected only (at 2ng per embryo)(n=41), Mo+mut RNA = Coro1C morpholino (2ng) in addition to 200 pg truncated Coro1C RNA (n=37), Mo+wt RNA= Coro1C morpholino (2ng) in addition to 200 pg full-length Coro1C RNA (n=30). All embryos were injected directly into the first cell at the 1-cell stage of development. Error bars indicate s.e.m. Significance was tested by T-test, ** p<0.005.

Movie 1. RCC2 and Coro1C regulate Rac1 localization and membrane protrusion. Control, RCC2 or Coro1C knockdown MEFs were transfected with GFP-Rac1 and filmed on a confocal microscope for 3.5 hours at 1 frame every 3 minutes. Red dots indicate protrusion between consecutive frames. Movie frames reproduced in Fig. 4B.

Movie 2. Localization of Rac1 activation is perturbed in RCC2 or Coro1C knockdown MEFs. Rac1 activity distribution was detected using a Raichu-Rac FRET probe in cells spread on 50K before addition of H/0 (white flash). A non-activatable mutant probe (Y40C) was used as a control to confirm that changes in FRET signal are caused by changes in Rac1 activity, not relocalization. Movie captured at 1 frame every 2 minutes, for 11 minutes prior to, and up to 49 minutes after stimulation. Images are false-colored for FRET intensity. Movie frames reproduced in Fig. 4C.

Movie 3. Release of photoactivated GFP-tagged Rac1 from the membrane is delayed in the absence of Coro1C expression. PAGFP was photoactivated in a 1.5x1.5 µm or 1.5x4.5 µm box at the lateral edge of cells spread on fibronectin and release of Rac1 followed by decay of GFP fluorescence. Fixed cells (no diffusion), control MEFs (large and small boxes), Coro1C-depleted MEFs (large and small
boxes) and Coro1C-depleted MEFs using a CAAX mutant Rac1 (no association of PAGFP-Rac1 with membrane) were analyzed. Images are false-colored for fluorescence intensity. Images were captured at 2 images per second for 5 seconds prior to, and 15 seconds after photoactivation, and displayed at 2 frames per second. Movie frames reproduced in Fig. 5F.

Movie 4. Retrafficking of Rac1 from lateral to protrusive membrane is reliant on Coro1C. PAGFP-Rac1 was photoactivated in boxes at the lateral edge of control or Coro1C KD cells spread on fibronectin, and arrival at protrusive membrane recorded. Images are false-colored for fluorescence intensity. Images were captured at 1 image per minute for 10 minutes following photoactivation and displayed a 1 frame per second. Movie frames reproduced in Fig. 5H.

Movie 5. Retrafficking of Coro1C from lateral to protrusive membrane. PAGFP-Cor1C was photoactivated by 3 pulses within 1 minute at boxes on the lateral membrane of cells spread on fibronectin, and arrival at protrusive membrane recorded over 10 minutes at 1 image per minute for 10 minutes following photoactivation. Movie frames reproduced in Fig. 5I.

Movie 6. RCC2 and Coro1C expression are necessary for processive migration. Control, RCC2 and Coro1C knockdown and Sdc4 -/- MEFs migrating through a cell-derived matrix. Movie captured with a 5x lens at 1 image every 10 minutes for 10 hours, displayed at 5 frames per second, bar = 100 µm.

Movie 7. RCC2 and Coro1C expression are necessary for processive migration. Control, RCC2 and Coro1C knockdown MEFs migrating through a cell-derived matrix. Movie captured with a 40x lens at 1 image every 10 minutes for 10 hours, displayed at 5 frames per second, bar = 10 µm.

Movie 8. Shunting migration of RCC2 knockdown MEFs is not due to a tail retraction defect. β1-integrin-GFP-expressing MEFs transfected with control oligos were filmed migrating through a cell-derived matrix to allow the rearmost attachment point to be seen. Movie captured at 1 frame every 10 minutes, displayed at 6 frames per second.
Movie 9. Shunting migration of RCC2 knockdown MEFs is not due to a tail retraction defect. β1-integrin-GFP-expressing MEFs transfected with RCC2-targeted antisense oligo were filmed migrating through a cell-derived matrix to allow the rearmost attachment point to be seen. Movie captured at 1 frame every 10 minutes, displayed at 6 frames per second.

Movie 10. RCC2 and Coro1C expression are necessary for processive migration. Control, RCC2 and Coro1C knockdown MEFs migrating along 5-µm fibronectin stripes. Movie captured at 1 image every 10 minutes for 6.5 hours, displayed at 3 frames per second, bar = 50 µm.
Figure S1.
Figure S2.
Figure S3.
Figure S4.
Movie 1.

Movie 2.

Movie 3.
Movie 4.

Movie 5.

Movie 6.