**Xenopus** Kazrin interacts with ARVCF-catenin, spectrin and p190B RhoGAP, and modulates RhoA activity and epithelial integrity

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**Summary**

In common with other p120-catenin subfamily members, *Xenopus* ARVCF (xARVCF) binds cadherin cytoplasmic domains to enhance cadherin metabolic stability or, when dissociated, modulates Rho-family GTPases. We report here that xARVCF binds and is stabilized by *Xenopus* KazrinA (xKazrinA), a widely expressed conserved protein that bears little homology to established protein families, and which is known to influence keratinocyte proliferation and differentiation and cytoskeletal activity. Although we found that xKazrinA binds directly to xARVCF, we did not resolve xKazrinA within a larger ternary complex with cadherin, nor did it co-precipitate with core desmosomal components. Instead, screening revealed that xKazrinA binds spectrin, suggesting a potential means by which xKazrinA localizes to cell–cell borders. This was supported by the resolution of a ternary biochemical complex of xARVCF–xKazrinA–xβ2-spectrin and, in vivo, by the finding that ectodermal shedding followed deletion of xKazrin in *Xenopus* embryos, a phenotype partially rescued with exogenous xARVCF. Cell shedding appeared to be the consequence of RhoA activation, and thereby altered actin organization and cadherin function. Indeed, we also revealed that xKazrinA binds p190B RhoGAP, which was likewise capable of rescuing Kazrin depletion. Finally, xKazrinA was found to associate with δ-catenins and p0071-catenins but not with p120-catenin, suggesting that Kazrin interacts selectively with additional members of the p120-catenin subfamily. Taken together, our study supports the essential role of Kazrin in development, and reveals the biochemical and functional association of KazrinA with ARVCF-catenin, spectrin and p190B RhoGAP.

**Key words:** ARVCF, Cadherin, Kazrin

**Introduction**

Catenins were initially defined as molecules that bind cadherins (Ozawa et al., 1989) and, with the exception of α-catenin, that possess a central Armadillo (Arm) domain that mediates cadherin and other protein–protein interactions (Choi and Weis, 2005; Huber et al., 1997; Peifer et al., 1994). Catenins have numerous roles in varied cellular compartments. Members of the p120-catenin subfamily, such as p120-catenin, Armadillo-repeat protein deleted in velo-cardio-facial syndrome (ARVCF)-catenin and δ-catenin, modulate cadherin stability at cell–cell junctions (Davis et al., 2003; Fang et al., 2004; Gu et al., 2009; Freton et al., 2002; Xiao et al., 2003). They also directly or indirectly associate with and regulate small GTPases, enabling intracellular signaling and cytoskeletal control (for a review, see Anastasiadis, 2007). Most catenins additionally enter the nucleus (for a review, see McCrea et al., 2009). β-catenin, for example, is known to relieve TCF/LEF-mediated transcriptional repression in response to canonical Wnt-pathway stimulation, thereby activating target genes important in development or in pathologies such as cancer (for a review, see Cadigan and Peifer, 2009). Although further study is required to address unresolved issues (Ruzov et al., 2009a; Ruzov et al., 2009b), we and others find that p120 likewise contributes to Wnt signaling (Hong et al., 2010; Iioka et al., 2009; Kim et al., 2004; Park et al., 2006; Park et al., 2005; Spring et al., 2005). In comparison to β-catenin and p120-catenin, relatively little is known concerning the interactions and functional roles of ARVCF-catenin or other catenins.

ARVCF is one of several gene products deleted in velo-cardio-facial syndrome (Sirotnik et al., 1997). In the context of the cadherin complex, p120 subfamily members including ARVCF bind in a mutually exclusive manner to the cadherin juxtamembrane domain (Mariner et al., 2000). In the nucleus, p120- but not ARVCF-catenin binds the transcriptional repressor Kaiso (Daniel and Reynolds, 1999; Kim et al., 2002) (for a review, see Daniel, 2007). The C-terminal PDZ binding motifs within ARVCF, δ-catenin and p0071 (absent in p120-catenin) bind the ERBIN scaffolding protein (Laura et al., 2002), the ZO-1 and ZO-2 tight junction proteins (Kausalya et al., 2004), and the less-characterized
FRMPD2 (Stenzel et al., 2009). In brief, the p120-subclass proteins exhibit both shared and non-overlapping interactions.

To reveal novel ARVCF functions, we screened a *Xenopus* neurula (stage 18) cDNA library for proteins that interact with *Xenopus laevis* ARVCF (xARVCF) and identified *Xenopus laevis* Kazrin (xKazrinA). Biochemically, human KazrinA was previously shown to associate with the peripheral desmosomal proteins periplakin and envoplakin in human keratinocytes (Groot et al., 2004), with microtubules (isoform E) (Nachat et al., 2009), and to modulate RhoA (Sevilla et al., 2008a). We found that xKazrinA interacts directly with xARVCF but not with *Xenopus laevis* p120 (Xp120) or β-catenin, and as reported earlier is present at cell–cell junctions (Groot et al., 2004). Surprisingly, we found that the xARVCF–xKazrinA complex associates and colocalizes with the spectrin cytoskeleton, rather than with cadherins at adherens junctions (Kaufmann et al., 2000; Mariner et al., 2000; Paulson et al., 2000), or with desmosomal core proteins (Groot et al., 2004). Our depletion of xKazrinA resulted in lessened embryonic tissue integrity (Sevilla et al., 2008b). In parallel, xARVCF protein levels were reduced and, supporting their functional interaction, exogenous xARVCF significantly rescued xKazrinA depletion phenotypes. xKazrinA depletion additionally led to RhoA activation, microfilament alterations, and lowered cadherin and cell adhesion levels, which are probably relevant to ectodermal fragility. An additional screen for novel xKazrinA partners resolved Xp190B RhoGAP. In common with xARVCF, p190B partially rescued xKazrinA depletion effects, consistent with functional links existing between components of the xARVCF–xKazrinA–Xp190B complex. Finally, two additional *Xenopus* p120 subclass catenins, xδ-catenin and Xp0071 directly bound xKazrinA. Taken together, we propose that xKazrinA enables xARVCF association with the spectrin–actin network, and that the xARVCF–xKazrinA–Xp190B complex modulates RhoA activity and thereby cytoskeletal organization, cell adhesion and ectodermal integrity.

**Results**

**Yeast two-hybrid analysis identifies a novel ARVCF-associated protein**

Yeast two-hybrid analysis, using xARVCF as ‘bait’, was employed to screen a *X. laevis* stage18 neurula library for interacting proteins. Three independent clones corresponded to the *X. laevis* homolog of human KIAA1026 (GenBank accession #AB028949) (Kikuno et al., 1999). The fidelity of the screen was indicated by retrieving cadherin juxtamembrane domains known to bind p120-subclass catenins (data not shown) (Aono et al., 1999; Kaufmann et al., 2000; Mariner et al., 2000; Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998; Paulson et al., 2000; Thoreson et al., 2000; Yap et al., 1998). KIAA1026 became termed Kazrin (Groot et al., 2004). BLAST analysis demonstrated that xKazrin is highly homologous to human and mouse KazrinA (81% and 80.5% amino acid identity, respectively) (Table 1), and with *X. tropicalis* Kazrin (GenBank #EU404187; 92.6% identity) (Table 1) (Fig. 1A). Relative to xKazrin, *X. tropicalis* Kazrin contained 28 additional residues following the putative coiled-coil domain of Kazrin. RT-PCR and subsequent DNA sequencing of *X. laevis* stage18 neurula cDNA showed that this region (encoding the same 28 residues/exon 6) is alternatively spliced (Fig. 1B,D; data not shown). In keeping with another report (Groot et al., 2004), we named the short xKazrin isoform xKazrinA (structurally similar to human KazrinA), and the longer isoform xKazrinB.

Prior findings demonstrated that seven human Kazrin splice isoforms (KazrinA–KazrinF and KIAA1026) contain variable N- and C-termini (Groot et al., 2004; Kikuno et al., 1999; Nachat et al., 2009; Wang et al., 2009). As discussed below, KazrinA–KazrinF each encode a putative nuclear localization sequence (NLS), as do the *Xenopus* isoforms when assessed using PredictNLS server (http://cubic.bioc.columbia.edu/predictNLS) (Cokol et al., 2000) (Fig. 1A,C). KIAA1026, on the other hand, lacks this cluster of multiple lysine residues; we have designated KIAA1026 as KazrinK. As a preliminary test of the putative NLS functionality, we compared xKazrinA intracellular localization with that of human KazrinK (KIAA1026) in MCF7 cells. Human KazrinK appeared fully excluded, although xKazrinA resided in the nuclei of multiple cells (supplementary material Fig. S1A). This pattern was likewise observed in HEK293 and *Xenopus* A6 cells (data not shown). The putative NLS lysine residues of xKazrin (365–368; supplementary material Fig. S1B), were then mutated to glutamines to alter their charge but maintain the steric properties of the region (Cokol et al., 2000). Whereas full-length xKazrinA exhibited an even pattern within the cytoplasm and nucleus, or showed predominantly nuclear staining (supplementary material Fig. S1C, left panel), the NLS mutant was excluded from the nucleus. Collectively, these data suggest that the C-terminal polylsine tract of xKazrin functions as an NLS. Although KazrinK and KazrinE isoforms are represented in other mammals, we have not yet identified their homologs in *Xenopus*. In all cases, the existence of variably spliced and localized Kazrin isoforms is suggestive of functional distinctions.

Using predictive software, the central region of xKazrin and a limited N-terminal region (amino acids 82–255 and 16–41, respectively) were strongly predicted to be coiled coils (both scoring >90% using COILS) (http://www.ch.embnet.org/software/COILS_form.html) (Lupas et al., 1991). This region bears limited sequence similarity with known coiled-coil domains (Table 2). Using the structural property of 3.5 amino acid residues per helical turn, we modeled five coiled coils within the central region of xKazrin (leucine zipper), and an additional coil in the N-terminal region (supplementary material Fig. S1B and Fig. S2).

**Table 1. Amino-acid comparison of xKazrinA with KazrinA proteins from human, mouse, rat, *X. tropicalis* and puffer fish (**Tetraodon nigroviridis**)**

| Organism          | GenBank protein ID | Amino acid identity (%) |
|-------------------|--------------------|-------------------------|
| *X. tropicalis*   | ABZ01809.1         | 92.6                    |
| Human             | AAS86434.1         | 81.0                    |
| Rat               | AAH89223.1         | 80.5                    |
| Mouse             | NP_001103154.1     | 80.5                    |
| Puffer fish       | CAG07017.1         | 52.9                    |

**Table 2. Comparison of xKazrin coiled-coil domain with related domains in other protein families**

| Protein domain | Identity/similarity (%) |
|----------------|-------------------------|
| FERM           | 23/48                   |
| SMC            | 25/48                   |
| HOOK           | 23/45                   |
| Myosin tail    | 20/40                   |
| SbcC           | 23/45                   |

xKazrinA interacts with xARVCF-catenin but not with Xp120-catenin

To determine whether the xARVCF–xKazrinA complex could be isolated from in vivo extracts, Myc-tagged xKazrinA was
coexpressed in *Xenopus* embryos with HA-tagged xARVCF or HA-tagged Xp120. In agreement with our yeast two-hybrid results, coimmunoprecipitation assays showed that xKazrinA associates with xARVCF but not with Xp120-catenin (Fig. 2A).

To test whether xKazrin and xARVCF interact directly, each protein was purified as glutathione-S-transferase (GST)- or maltose-binding protein (MBP)-fusion constructs. Then, GST and MBP pull-down assays were performed (Fig. 2B, left versus right panels). GST–xKazrinA coimmunoprecipitated with MBP–xARVCF but not (or considerably more weakly) with MBP–Xp120, indicating specificity and a direct xARVCF–xKazrinA interaction. Using the same approach, *Xenopus* β-catenin (xβ-catenin) did not interact with xKazrinA (data not shown).

We further assayed for direct xARVCF–xKazrinA association using blot overlays (Fig. 2C) (Hall, 2004). Purified and immobilized GST–xKazrinA or GST were probed with purified MBP–xARVCF, MBP–Xp120 or MBP. A clear interaction was observed between membrane-bound GST–xKazrinA and MBP–xARVCF, although none was observed using MBP–Xp120 or MBP probes. Although cofactors might modulate their interaction in vivo, this data indicates that xARVCF and xKazrinA can selectively bind each other in vitro.

To outline the region interacting with xARVCF, we used GST–xKazrinA polypeptides representing the N-terminal, C-terminal, and coiled-coil regions of xKazrinA (GST–xKazrin NT, GST–xKazrin CT, and GST–xKazrin CC, respectively). Using a blot-overlay approach, these fusion proteins were assayed along with full-length GST–xKazrinA for association with MBP–xARVCF (Fig. 2D). xARVCF interacted with both full-length and coiled-coil GST–xKazrinA, but not with N-terminal or C-terminal fusions, suggesting that the central coiled-coil domain of xKazrin is required for xARVCF–xKazrin association.
xKazrinA associates with xARVCF within cells
To investigate the xARVCF–xKazrinA interaction within the physiological conditions provided by vertebrate cells, xARVCF-catenin or Xp120-catenin constructs were targeted to the mitochondrial outer membrane (MOM) via C-terminal fusion to the MOM localization domain of Bcl-XL (Kaufmann et al., 2003; Waibler et al., 2001). Such xARVCF or Xp120 fusion proteins were localized to the MOM when expressed in *Xenopus* A6 kidney epithelial cells, resulting in a characteristic punctuate pattern (Fig. 3A). xKazrinA relocalized to the MOM when co-transfected with xARVCF–MOM but not with Xp120–MOM (Fig. 3B, top versus bottom panels). Similar patterns appeared when using human embryonic kidney (HEK-293) or human breast cancer cells (MCF-7) (data not shown). These experiments support the specificity and authenticity of the xARVCF–xKazrinA interaction within a cellular context.

Interaction domain mapping of the xARVCF–xKazrinA complex
To resolve xARVCF–xKazrinA interaction domains, deletion constructs were employed. Seven xKazrinA constructs were tested for interaction with xARVCF–MOM in A6 cells (Fig. 3C; supplementary material Fig. S3A). Deletion of the 70 N-terminal amino-residues of xKazrinA (Fig. 3C, MT xKazrin ΔN1) resulted in a construct similar to the C and D isoforms of human KazrinA (Groot et al., 2004), which retained competence to relocalize with xARVCF–MOM. Deletion of the N-terminal 175 amino acid residues (Fig. 3C, MT xKazrin ΔN2), or C-terminal 235 amino acids (Fig. 3C, MT xKazrin ΔC2) abolished xARVCF–xKazrinA association. A smaller C-terminal deletion of 127 amino acid residues (Fig. 3C, MT xKazrin ΔC1), as well as a more modest deletion of the C-terminal 47 amino acid residues of xKazrinA, inclusive of the NLS (Fig. 3C, MT xKazrin ΔNLSc), or mutation of the NLS (Fig. 3C, MT xKazrin NLScm, see below), did not disrupt xARVCF–xKazrinA interaction. These data indicate that the region of xKazrinA necessary for interaction with xARVCF resides between amino acid residues 70–354, containing the coiled-coil domain of xKazrin up to the potential A/K isoform splice site at amino acid residue 354. This region is present in all identified isoforms of Kazrin (frog, human, mouse, rat and *Xenopus*), suggesting that ARVCF–Kazrin interactions might be shared. Indeed, as shown in supplementary material Fig. S9B, human KazrinE and KazrinK isoforms coimmunoprecipitate with xARVCF.

To map the interaction region(s) of xARVCF, we used MOM-targeted deletion constructs comprised of one or two of the three xARVCF domains (N-terminus, Arm domain and C-terminus; Fig. 3C and supplementary material Fig. S3B). When coexpressed with xKazrinA, only the full-length (Fig. 3C, HA xARVCFmom) and Arm–C-terminus (Fig. 3C, HA xARVCF-ACmom) constructs exhibited the capacity to relocalize xKazrinA to the MOM (supplementary material Fig. S3). xKazrin relocalization failed when using the isolated N- or C-terminal domains of xARVCF (Fig. 3C, HA xARVCF-Nmom or HA xARVCF-Cmom, respectively) [the latter binds ERBIN, ZO-1, ZO-2 and FRMPD2]...
Relocalization also failed when using the xARVCF Arm domain (Fig. 3C, HA xARVCF-Amom), or upon removal of the xARVCF C-terminus (Fig. 3C, HA xARVCF-NAmom). These data collectively indicate that a region(s) comprised of the Arm and C-terminal domains of xARVCF is required for interaction with the coiled-coil domain of xKazrin (Fig. 3D).

xKazrinA and xARVCF colocalize at cell–cell contacts in Xenopus blastula ectoderm

To assess xKazrinA and xARVCF colocalization in Xenopus tissue, immunofluorescence was performed of Xenopus blastula ectoderm (animal caps) expressing Myc–xKazrinA. Most xKazrinA protein was present at cell–cell borders, with some staining evident in the nucleus (data not shown). The nuclear presence of KazrinA in vivo is in accordance with its nuclear localization in cell lines and human skin (supplementary material Fig. S1) (Nachat et al., 2009; Sevilla et al., 2008a).

To assess HA–xARVCF and Myc–xKazrinA colocalization following coexpression in vivo, immunofluorescence images were acquired from the outer ectodermal cell layer at blastula stages. Colocalization occurred along cell–cell borders in the X–Y plane (Fig. 4A–C), and to a significant if not complete extent along the Z-axis (Fig. 4D). In general, these findings are consistent with the biochemical association of xARVCF and xKazrinA.

The xARVCF–xKazrinA complex is not a core component of adherens junctions or desmosomes

Because xARVCF directly binds cadherin juxtamembrane regions (Kaufmann et al., 2000; Mariner et al., 2000; Paulson et al., 2000), we tested whether xKazrinA and xARVCF colocalize with C-cadherin, a major cadherin essential for Xenopus early development (Heasman et al., 1994; Lee and Gumbiner, 1995). Xenopus C-cadherin (xC-cadherin) colocalized at cell–cell borders with xKazrinA as well as with xARVCF (Fig. 4E–L), consistent with a possible association of xKazrinA with adherens junction components.
To test whether xKazrinA and C-cadherin form a larger complex, presumably bridged by xARVCF, we overexpressed Myc–xKazrinA followed by C-cadherin immunoprecipitation and western blotting (Fig. 4M). As anticipated, endogenous C-cadherin co-precipitated endogenous xARVCF, as well as *Xenopus* β-catenin and plakoglobin. Importantly, however, it did not co-precipitate Myc–xKazrinA. In further tests, we simultaneously overexpressed three components: HA–xKazrinA, HA–xARVCF and the cytoplasmic tail of C-cadherin (Myc-tagged). Whereas xARVCF communoprecipitated with the cytoplasmic domain of C-cadherin as expected, HA–xKazrinA was not present in conjunction with cadherin (Fig. 4N). Furthermore, we could not detect an interaction between xKazrinA and xC-cadherin following chemical crosslinking [using DTSSP; 3,3'-(dithiobis(sulfosuccinimidyl) propionato)] (data not shown). These results indicate xC-cadherin–xARVCF association as anticipated, but not our communoprecipitation results, not cadherin association with xKazrinA.

Because Kazrin associates with envoplakin and periplakin at desmosomal or interdesmosomal regions in human keratinocytes (Groot et al., 2004), and ARVCF- and p120-catenins have been reported at desmosomes (Bormann et al., 2006; Johnson and Boekelheide, 2002; Kanno et al., 2008a; Kanno et al., 2008b), we wanted to know whether ARVCF is resident at desmosomes in human keratinocytes. As shown in Fig. 5, both Myc–xARVCF and human Kazrin partially colocalized with the desmosomal marker desmoplakin (Fig. 5A–C and Fig. 5D–F, respectively), and with desmoglein3 in human keratinocytes (Fig. 5G–I). We then employed transmission EM, but in our hands this did not further clarify ARVCF or Kazrin presence at desmosomes and/or adherens junctions (data not shown).

To further probe desmosomal associations, we turned to biochemical precipitations from *Xenopus* embryo (Fig. 5J) and human epithelial carcinoma cell extracts (A431) (Fig. 5K). The cytoplasmic tail of human desmoglein1 (Fig. 5J) or full-length desmoglein1 (Fig. 5K) failed to co-precipitate *Xenopus* or human ARVCF from either system. Plakoglobin and C-cadherin were positive controls for the respective binding functionality of desmoglein and ARVCF in *Xenopus* embryos (Fig. 5J). As evaluated by these criteria, our data do not support xARVCF as being a core component of the desmosome.

**xKazrinA and xARVCF form a complex with the xβ2-spectrin cytoskeletal protein**

To further address how the xARVCF–xKazrinA complex localizes to junctional areas, we performed yeast two-hybrid screening of xKazrinA to identify novel binding partners (Hybrigenics). From an adult mouse brain cDNA library, we obtained 64 positive clones (partial list in supplementary material Table S1). Notably, nine clones encoded plasma-membrane-associated proteins. These included two p120-catenin subfamily members (δ-catenin and p0071), the tight junction protein symplekin (Keon et al., 1996), the spectrin binding protein Camsap1 (Baines et al., 2009; Yamamoto et al., 2009), and three spectrin family members (α1-, α2- and β2-spectrins) (for a review, see Bennett and Healy, 2009). This screen was not saturating because we did not isolate ARVCF-
heterodimers that orient end-to-end to form $\beta$-spectrin genes and five $\alpha$-spectrin genes. Their gene products form $\alpha\beta$-tetramers, and other proteins such as band 4.1, ankyrin, adducin and calmodulin, forming a cytoskeletal web that functions in many contexts (for a review, see Bennett and Healy, 2009). Anchoring points, such as provided by band 4.1 and ankyrin, facilitate the plasma membrane association of the spectrin cytoskeleton, as do junctional cadherins (see later) (Bennett and Healy, 2009). In Xenopus, two spectrins are known, $\alpha$-spectrin (also known as $\alpha$-fodrin) and $\beta$-spectrin (Giebelhaus et al., 1987; Klein et al., 2002). Xenopus $\alpha$-spectrin (xα-spectrin), although highly expressed in oocytes, becomes reduced following fertilization (Giebelhaus et al., 1987), whereas xβ-spectrin-encoding mRNA is expressed throughout development, as assessed via semiquantitative RT-PCR (supplementary material Fig. S4B).

We wondered whether the spectrin network might be relevant to xARVCF-xKazrinA localization to cell–cell borders. Confocal microscopy and biochemical approaches were employed using a partial xβ2-spectrin cDNA (GenBank #BC046267.1). This construct is the longest available xβ-spectrin, and is homologous to human $\beta$-spectrin isolated from our yeast two-hybrid screen (74.6% identical). It encodes the N-terminal actin-binding domain of $\beta$-spectrin (ABD; which binds band 4.1 and adducin), and five spectrin repeats (Fig. 6B). Furthermore, using confocal microscopy, xKazrinA and xβ2-spectrin colocalized as expected to cell–cell borders in blastula ectoderm (Fig. 6C). To test whether xβ2-spectrin binds to the xARVCF–xKazrinA complex in vivo, varying amounts of spectrin were expressed in Xenopus embryos with a constant amount of xKazrinA, and the endogenous xARVCF complexes isolated and assayed (Fig. 6D). xβ2-spectrin as well as xKazrinA co-precipitated with xARVCF. Because xβ2-spectrin expression did not alter xARVCF–xKazrinA co-precipitation, we expect that a ternary xARVCF–xKazrinA–xβ2-spectrin complex was formed. However, because a prior study reported association of $\beta$-spectrin with E-cadherin (through ankyrin) (Kizhatil et al., 2007), it was conceivable that xARVCF association with xβ2-spectrin occurred via their mutual association with cadherin. We tested this possibility using co-precipitation assays. As shown in Fig. 6E, xARVCF but not xC-cadherin associated with xβ2-spectrin and xKazrinA. Thus, the ARVCF–Kazrin complex probably associates with spectrin through Kazrin binding to spectrin.

xARVCF partially rescues embryonic phenotypes resulting from xKazrin depletion

Morpholino-directed Kazrin depletion in early X. tropicalis embryos was previously reported to result in ectodermal blistering, as well as a shortened body axis, head reductions and somitic defects (Sevilla et al., 2008b). Because the mRNA sequence targeted by the X. tropicalis morpholino is completely conserved with that in X. laevis, we used X. tropicalis morpholino (KMO1) in our study, and demonstrated its efficacy in blocking in vitro translation of Kazrin-encoding mRNA, and in knocking-down X. laevis Kazrin to 60% of endogenous levels by neurluration stages (significant maternal protein loading precluded obtaining earlier effects) (supplementary material Fig. S6A and Fig. S6B, respectively). The phenotypes appearing in X. laevis were similar to those previously indicated in X. tropicalis (Sevilla et al., 2008b). However, Kazrin depletion in X. laevis appeared to have a more severe impact upon the ectoderm, as evident in abnormal
neural tube closure during neurulation (supplementary material Fig. S6C, upper panels), and in regional ectoderm shedding in addition to blistering (noted previously) at early tailbud stages (supplementary material Fig. S6C, bottom panels and Fig. 7A, middle panel). The fraction of embryos exhibiting such phenotypes increased in a dose-dependent manner (supplementary material Fig. S6D). Because we obtained the same results using an independent non-overlapping Kazrin morpholino in *X. laevis*, the specificity of our phenotype was supported (data not shown).

To test for an in vivo functional interaction between xARVCF and xKazrinA, we attempted to rescue ectodermal shedding through ectopic expression of xARVCF (Fig. 7, Table 3). As shown in Fig. 7B, Kazrin depletion was partially rescued by an appropriately titrated dose of ectopic xARVCF (dose subphenotypic in isolation), consistent with xKazrin–xARVCF functional interactions in maintaining ectodermal integrity.

**Kazrin depletion decreases cadherin protein levels and cell–cell attachment**

Our data suggested that xKazrinA and xARVCF form a complex that is capable of associating with spectrin, and furthermore that the xKazrinA–xARVCF complex participates in maintaining ectodermal integrity. However, it remained unclear at the mechanistic level how xKazrinA depletion alters ectodermal integrity. Given their established contributions to cell adhesion, we first considered the effects upon C-cadherin and E-cadherin. Interestingly, the levels of both cadherins were decreased after xKazrin depletion (Fig. 8A), as was that of desmoglein1, a desmosomal cadherin (supplementary material Fig. S5). A previous report employing cell lines used immunofluorescence to indicate that Kazrin overexpression lowers desmoplakin and perhaps E-cadherin levels (Sevilla et al., 2008a). Intriguingly, both in our and others’ hands (Sevilla et al., 2008b) (data not shown), Kazrin overexpression in *Xenopus* does not generate an obvious phenotype(s). Although speculative, it is possible that effects occur but are more effectively compensated for in vivo than in vitro. To evaluate xKazrin depletion effects on cell–cell adhesion in a more controlled setting, we performed cell dissociation and cell re-aggregation assays using blastula ectoderm tissue (animal caps). xKazrin-depleted tissue exhibited accelerated dissociation compared to controls (Fig. 8B) and, correspondingly, they experienced delayed reaggregation (data not shown). Considering the key roles of cadherins in determining the outcome of such dissociation–reaggregation assays (Turner et al., 1992), together with our biochemical evidence, the observed reduction of ectoderm integrity is probably due to reduced cadherin levels following xKazrin depletion. In support of this proposition, xKazrin depletion phenotypes were partially rescued upon ectopic expression of an appropriately titrated dose of C-cadherin (subphenotypic in isolation).
stabilization and/or endocytosis (Akhtar and Hotchin, 2001; Charrasse et al., 2006; Izumi et al., 2004; Lamaze et al., 1996; Leung et al., 1999) (for a review, see Anastasiadis, 2007; Xiao et al., 2007). Interestingly, a non-biased screen earlier correlated Kazrin overexpression with inhibition of clathrin-mediated endocytosis of transferrin receptor (Schmelzl and Geli, 2002). Thus, Kazrin together with p120 subfamily catenins might be relevant to cadherin endocytosis. Indeed, cadherin levels after Kazrin depletion were restored when embryos were incubated with a specific dynamin inhibitor (Dynasore; Fig. 8D) (Macia et al., 2006), and ectodermal defects were partially rescued in parallel (data not shown). This suggests a potential role of Kazrin in cadherin endocytosis, although we never resolved Kazrin in complex with cadherin using standard immunoprecipitation (Fig. 4M,N) or chemical crosslinking (using DTSSP, data not shown). Furthermore, although less compelling, we never observed that association of xARVCF with cadherin was altered by xKazrinA expression (data not shown).

In common with cadherin, the level of xARVCF, Xp120 and β-catenin became decreased in response to xKazrin depletion (data not shown). One possible explanation for the modulation of cadherin protein levels by Kazrin is its above-shown relationship with ARVCF. On the basis of published reports, lowered ARVCF (as for p120) levels are expected to result in lowered cadherin levels (Davis et al., 2003; Fang et al., 2004; Ireton et al., 2002; Xiao et al., 2003). Symmetrically, lowered cadherin presence can result in reduced cadherin levels (e.g. of β-catenin) (Papkoff, 1997; Thoreson et al., 2000). At this time, we do not know the temporal hierarchy of events following Kazrin perturbation that result in altered levels and/or functions of cadherin–catenin complex components. This will require further study.

Nonetheless, given the known effects of ARVCF-catenin (and p120-catenin) on small GTPases (Anastasiadis et al., 2000; Fang et al., 2004; Grosheva et al., 2001; Noren et al., 2000), we turned our attention to xKazrinA effects on RhoA and Rac, and on cortical actin organization in plasma-membrane regions. We initially evaluated RhoA activity in embryos following the depletion of xKazrin and/or xARVCF (Fig. 8E). Interestingly, xKazrin depletion significantly increased RhoA activity, an effect likewise observed (and expected) following xARVCF knockdown (Fang et al., 2004). Coordinate xKazrin and xARVCF depletions had an additive effect on RhoA activity, as seen by comparing 10 ng deliveries of each morpholino (Fig. 8E, lane 6 versus 2 and 4). Because Rac1 was not affected by xKazrin depletion (data not shown), Kazrin might be a RhoA-specific modulator. Our results are in line with a report indicating that Kazrin overexpression inhibits RhoA in human keratinocytes, with no apparent effect on Rac1 (Sevilla et al., 2008a). Thus, although Kazrin and xARVCF appear to have shared inhibitory effects on

Table 3. xARVCF rescue of xKazrin depletion

| Injection | Number of embryos | Number of experiments |
|-----------|------------------|----------------------|
| 20 ng KMO | 20 ng xARVCF + 80 pg β-gal* | 122 67 43 4  |
| 20 ng KMO | 100 pg ARVCF | 138 75 61 4 |
| 20 ng KMO | 100 pg xARVCF | 94 4 0 4 |
| Uninjected | Uninjected 127 123 2 4 |

CMO, control morpholino; KMO1, X. Laevis morpholino. *80 pg of β-galactosidase mRNA was co-injected with 20 pg xARVCF mRNA to equalize the total amount of injected mRNA.

(Fig. 8C). Together, our in vivo data suggests that Kazrin positively modulates cell–cell contacts, with the xARVCF–xKazrinA complex probably being pertinent to effects upon cadherin levels.

Kazrin depletion leads to increased RhoA activity and cortical actin disorganization

A remaining issue was how xKazrinA, or the xARVCF–xKazrinA complex, modulates cadherin stability. p120 subfamily catenins including ARVCF are thought to stabilize cadherins by binding cadherin membrane-proximal domains and blocking certain protein associations (such as with Hakai or presenilin-1) (Baki et al., 2001; Fujita et al., 2002; Marambaud et al., 2002), that would otherwise promote cadherin degradation and/or endocytosis (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003). Regulation of Rho-GTPases by p120 subfamily catenins is further relevant to cadherin associations (such as with Hakai or presenilin-1) (Baki et al., 2001; Fujita et al., 2002; Marambaud et al., 2002), that would otherwise promote cadherin degradation and/or endocytosis (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003). Symmetrically, lowered cadherin presence can result in reduced cadherin levels (e.g. of β-catenin) (Papkoff, 1997; Thoreson et al., 2000). At this time, we do not know the temporal hierarchy of events following Kazrin perturbation that result in altered levels and/or functions of cadherin–catenin complex components. This will require further study.

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RhoA, one distinction (relative to ARVCF and related p120 subfamily members) is that Kazrin is not likely to modulate Rac1 in this context (Anastasiadis et al., 2000; Fang et al., 2004; Grosheva et al., 2001; Noren et al., 2000). Because RhoA activation might have contributed to the ectoderm fragility we observed following Kazrin depletion, we tested the rescuing capacity of carefully titrated dominant-negative RhoA (versus dominant-active RhoA), upon co-injection with KMO1. In line with RhoA being functionally relevant and possibly downstream of Kazrin, co-injection of dominant-negative RhoA mildly rescued observed phenotypes, whereas titrated dominant-active RhoA doses only worsened developmental defects (data not shown).

Because ectopic RhoA activation modulates actin and cell structure (Paterson et al., 1990), we employed phalloidin staining to grossly resolve Kazrin-depletion effects on actin organization in embryos (Fig. 8F). Whereas control embryos exhibited a defined enrichment of cortical actin at cell–cell borders, Kazrin-depleted embryos displayed a disorganized pattern and an actin signal of reduced thickness along the Z-axis (Fig. 8F). The cytoskeletal disorganization we resolved in vivo appears to be in keeping with a previous report employing keratinocytes (Sevilla et al., 2008a). In summary, Kazrin appears to be a negative regulator of RhoA in vivo, suggesting that increased RhoA activity upon Kazrin depletion contributes to microfilament reorganization, and possibly to lowered cadherin levels and ectodermal integrity as previously noted (Charrasse et al., 2006; Leung et al., 1999).

**xKazrinA interacts with Xp190B RhoGAP**

We wondered whether Kazrin might modulate RhoA via another protein(s), because predictive analysis suggested that Kazrin was unlikely to directly bind RhoA. Common modulators of RhoA include Rho GDIs (Rho GDP dissociation inhibitors), RhoGAPs (Rho GTPase activation proteins) and Rho GEFs (Rho guanine nucleotide exchange factors). Intriguingly, from our xKazrinA yeast two-hybrid screen, we identified multiple independent clones of mouse p190B RhoGAP (supplementary material Table S1). This novel Kazrin interaction suggested one means by which it might influence RhoA activity and function.

There are two p190 RhoGAPs, p190A and p190B, and both are widely functioning negative regulators of Rho GTPases (Vincent and Settleman, 1999). Recently, p120-catenin was shown to mediate RhoA inhibition via the recruitment of the p190B RhoGAP (Niessen and Yap, 2006; Wildenberg et al., 2006). This suggested that xARVCF-catenin might analogously associate with p190B, bridged or facilitated by Kazrin. Upon analysis of our yeast two-hybrid findings (alignment of limiting sequences from 22 independent
clones), we deduce that xKazrinA binds to a region within or spanning the third to fourth FF domains of p190B (Fig. 9A; supplementary material Fig. S7) (Bedford and Leder, 1999). Interestingly, this portion of p190 RhoGAP also associates with activated Rac1 and Rnd3 (Rnd3 being a constitutively active RhoA antagonist), leaving open the possibility that Kazrin association with p190 RhoGAP might be competitive with that of Rac1 and Rnd3 (Fig. 9A) (Bustos et al., 2008; Wennerberg et al., 2003).
To further test the validity of the interaction of Kazrin with p190B, we undertook precipitations from embryo extracts and GST purified-component systems, using cDNA encoding human p190B, as well as the longest available (partial) cDNA encoding Xenopus p190B (79% sequence identity with human) (Fig. 9A; supplementary material Fig. S7). Following expression in vivo, both full-length human p190B and our partial Xp190B construct robustly co-immunoprecipitated with xKazrinA (Fig. 9B), and they colocalized to cell–cell borders (Fig. 9C). Finally, in a reductionist context suggesting their direct interaction, GST–xKazrin was co-precipitated with in vitro translated p190B (supplementary material Fig. S8).

Given their physical association, we asked whether the xKazrinA–p190B interaction might be functionally evident in vivo. For a read-out, we evaluated phenotypic rescue of ectodermal shedding, as described above for embryos depleted of xKazrin. Indeed, shedding phenotypes were partially but significantly rescued by titrated amounts of ectopic p190B (human full-length), and this occurred in a dose-dependent manner (Fig. 9D and supplementary material Table S3). These results support the concept that p190B and Kazrin are functionally coupled. Furthermore, the decrease in E-cadherin levels upon Kazrin depletion was restored by ectopic expression of human p190B RhoGAP (Fig. 9E), consistent with the possibility that lowered cadherin levels in Kazrin knockdown embryos might result from abnormal activation of RhoA.

To investigate whether xKazrin might facilitate p190B association with xARVCF, we asked whether Kazrin expression (versus not) had an impact on p190B association with xARVCF. As indicated in Fig. 9F (lane 3), a trace but reproducible amount of p190B was detected in complex with ARVCF only upon Kazrin coexpression. We next turned to in vitro pull-down assays to evaluate how p190B might associate with ARVCF (Fig. 9G). As expected on the basis of the precipitations from Xenopus extracts, in vitro translated Xp190B was pulled down with MBP–xARVCF in the presence of GST–xKazrinA (Fig. 9G, lane 3). This suggests that these three proteins form a ternary complex. Even in the absence of xKazrinA, Xp190B appeared to weakly bind xARVCF (Fig. 9G, lane 1). Thus, although requiring further study, it appears that Kazrin might recruit and/or enhance p190B association with ARVCF. In all cases, our data support the direct association and functional interaction of xKazrin with Xp190B RhoGAP.

In addition to xARVCF-catenin, xKazrinA binds xδ-catenin and Xp0071-catenin

As mentioned, our xKazrin yeast two-hybrid screen suggested that, in addition to xARVCF-catenin, two related members of the p120 subfamily interact with xKazrinA, namely xδ-catenin and Xp0071-catenin (supplementary material Table S1). Pull-down validation tests were performed using purified GST–xKazrinA protein and in vitro translated xδ-catenin or Xp0071-catenin. Positive and negative controls, respectively, included xARVCF- and Xp120-catenin (Fig. 10). In agreement with our yeast two-hybrid assays, xARVCF-catenin, xδ-catenin and Xp0071-catenin bound xKazrinA, whereas Xp120-catenin did not. This suggests that the biochemical and presumably functional interactions of Kazrin involve some but not all p120 subfamily catenins.

Discussion

In this study we describe the identification of xKazrinA as a direct binding partner of xARVCF-catenin, a member of the p120-catenin subfamily. We additionally resolved the association of of xKazrinA with spectrin and p190B RhoGAP. Distinct from reported protein interactions of ARVCF or KazrinA at the adherens junction or desmosome, we find that KazrinA associates with spectrin. Specrin forms a cytoskeletal network that includes interactions with junctional constituents (Bennett and Healy, 2009; Kizhatil et al., 2007). Although requiring further study, Kazrin appears to enhance the association of ARVCF with p190B RhoGAP, with the reduction of RhoA activity by Kazrin being likely to occur via these same interactions (at least in part). Borrowing from independent studies (Gliem et al., 2010; Lamaze et al., 1996; Schmalzing et al., 1995; Sullivan et al., 1999), we postulate that such RhoA modulation is relevant to the organization of cortical actin and cadherin stability. On the basis of our rescue analyses, these biochemical and functional relationships appear to be relevant to effects observed following Kazrin depletion in Xenopus embryos, which included reduced ectodermal integrity (see Fig. 11). Finally, we find that some, but not all, p120 subfamily members (not p120-catenin) bind xKazrinA. Our results thus constitute an initial outline of the role of Kazrin in the context of catenins, RhoA and p190B RhoGAP.

Kazrin isoforms and structure

Kazrin was originally identified as a cDNA (KIAA1026) expressed in human brain (Kikuno et al., 1999). In human keratinocytes, five Kazrin splice isoforms (KazrinA–KazrinE) were then reported, along with their biochemical interactions and functional effects on keratinocyte differentiation, small GTPases and actin or microtubule networks (Groot et al., 2004; Nachat et al., 2009; Sevilla et al., 2008a). Adding to the functional complexity of Kazrin is KazrinF, recently reported to inhibit apoptosis through binding BAX (Bel-2-associated X protein) and ARC (apoptosis repressor with caspase recruitment domain) in a human glioma cell line (Wang et al., 2009).

Our evaluation of KIAA1026 in particular showed that it lacks the NLS we characterized in other Kazrin isoforms, resulting in its exclusion from the nucleus. We propose that KIAA1026 be renamed KazrinK. Although the nuclear roles of all NLS-containing Kazrin isoforms are uncertain, both human KazrinE and human KazrinK associate with xARVCF (supplementary material Fig. S9B), possibly suggesting they have shared cytoplasmic and cytoskeletal functions.
Structurally, xKazrin and its well-conserved vertebrate homologs are likely to represent a new protein group. All Kazrins examined share limited homology with the known coiled-coil regions as represented in Table 2. Secondary structural prediction of the coiled-coil region of Kazrin further indicated resemblances to the folding of spectrin repeats within α-spectrin (Pascual et al., 1997). Each of these proteins possesses extensive α-helical or coiled-coil structure engaging in homotypic or heterotypic protein–protein interactions (Burkhard et al., 2001). Indeed, we find that the heterotypic interactions between Kazrin and ARVCF requires the predicted coiled-coil domain of Kazrin (Fig. 3C,D). Although possible homotypic coiled-coil associations will require further study, we observed that xKazrinA constructs bearing different epitope tags co-precipitate (supplementary material Fig. S9A), and that human Kazrin isoforms co-associate in a human cell line (Nachat et al., 2009).

**Protein partners of ARVCF-catenin**

A number of proteins have been shown to directly bind ARVCF. These include cadherins, RhoA, Vav2, ERBIN, FRMPD2, ZO-1 and ZO-2 (Fang et al., 2004; Izawa et al., 2002; Kaufmann et al., 2000; Kausalya et al., 2004; Laura et al., 2002; Magie et al., 2002; Mariner et al., 2000; Noren et al., 2000; Paulson et al., 2000; Stenzel et al., 2009). The latter four each contain PDZ domains, which are thought to associate with PDZ binding motifs within the C-termini of ARVCF, δ-catenin and p0071. Our present study adds Kazrin as the newest binding partner of p120 subfamily members, excluding p120 itself. Kazrin seems to bind a relatively broad region of ARVCF (and probably other catenins), encompassing portions of the central Arm and C-terminal domains. In turn, the extended coiled-coil domain of Kazrin associates with ARVCF, such that the coiled-coil regions of each protein presumably interact. Because the Arm domain of ARVCF exhibits some homology with p120-catenin (55% identity and 74.4% similarity) (Sirotkin et al., 1997), it may prove interesting to resolve the basis of specificity among different p120 subfamily proteins. It will furthermore be relevant to test whether the larger ARVCF–Kazrin complex includes proteins known to bind via the PDZ binding motif within ARVCF, such as ERBIN (Laura et al., 2002), as this would provide further insight to the functions of the complex.

**Subcellular localization of the xARVCF–xKazrinA complex**

In cornified epithelia and other primary and cultured cell types, Kazrin was visualized at plasma-membrane areas containing desmosomal and intermediate filament proteins, extradesmosomal areas enriched in cortical actin, and partially within cytoplasmic and nuclear compartments (Groot et al., 2004). In accordance with these results, and in common with ARVCF-, p120- and δ-catenin, we find that xKazrinA localizes to three subcellular regions: cell–cell borders, cytosol and the nucleus. Consistent with its nuclear localization, we resolved an NLS in the C-terminal region of xKazrinA (supplementary material Fig. S1). Interesting questions to address in future work include the nuclear function of Kazrin, possibly in a nuclear complex with ARVCF.

Initially, we addressed xARVCF and xKazrin localization to cell–cell borders, because although Kazrin is reported to bind periplakin and envoplakin at peripheral desmosomal or interdesmosomal regions in human keratinocytes (Groot et al., 2004), ARVCF is present at adherens junctions via binding cadherins (Kaufmann et al., 2000; Mariner et al., 2000; Paulson et al., 2000). Our present study indicates that the xARVCF–xKazrinA complex is not likely to be a core component of either adherens or desmosomal junctions. Instead, screening of xKazrinA resolved three (α2-, β1- and β2-) spectrin proteins as possible binding partners. Spectrins form a cytoskeletal network with actin filaments at the plasma membrane cortex, assisting in functions including plasma membrane and junctional strengthening, restriction of select proteins to plasma membrane subdomains, transduction of nerve action potentials, and contraction of heart cells (for a review, see Baines, 2009; Bennett and Healy, 2009). In this study, we found that the xARVCF–xKazrinA complex associates with ββ2-spectrin (Fig. 6), suggesting a novel interaction and localization of Kazrin and ARVCF-catenin (perhaps also δ-catenin and p0071). Previously, it was reported that the spectrin network associates with E-cadherin via ankyrin, contributing to lateral cell membrane integrity (Kizhatil et al., 2007). We, however, found neither C-cadherin nor p120 co-precipitating with ββ2-spectrin, despite significant homology within the cyto-domains of Xenopus E- and C-cadherin (80.5% identity). Possibly, cadherins such as C-cadherin form lesser interactions with the spectrin cytoskeleton in developmentally transitioning embryonic tissues.

**Roles of the ARVCF–Kazrin complex**

Earlier work predicted the involvement of Kazrin in clathrin-mediated endocytosis (Schmelzl and Geli, 2002), which is known to contribute to cadherin internalization and thereby to the modulation of junction function (Ivanov et al., 2004; Le et al., 1999; Xiao et al., 2005) (for reviews, see Bryant and Stow, 2004;
D’Souza-Schorey, 2005; Delva and Kowalczyk, 2009; Yap et al., 2007). Perhaps related are reports that p120 subfamily members protect cadherins from endocytosis and lysosomal degradation, and might also chaperone cadherins to the plasma membrane (Chen et al., 2003; Davis et al., 2003; Fang et al., 2004; Gu et al., 2009; Iretón et al., 2002; Xiao et al., 2003; Xiao et al., 2005) (for reviews, see Kowalczyk and Reynolds, 2004; Reynolds and Carnahan, 2004; Xiao et al., 2007). In desmosomal regions, Kazrin colocalizes with desmoglein3 (Fig. 5G–I), a desmosomal cadherin prone to accelerated endocytosis and degradation in the autoimmune disease pemphigus vulgaris (Calkins et al., 2006). We speculate that the role of Kazrin in endocytosis, or the role of the ARVCF–Kazrin complex, could be linked to reduced levels of cadherin and adhesion following Kazrin depletion.

If this were the case, one question would be how Kazrin, or the ARVCF–Kazrin complex, modulates cadherin endocytosis at the molecular level. Endocytosis requires cortical actin to be locally reorganized, with such changes involving the actions of small GTPases (for reviews, see Ellis and Mellor, 2000; Symons and Rusk, 2003). Multiple groups have reported Rho and Rac (and Cdc42) modulation by p120 subfamily catenins including ARVCF (Anastasiadis et al., 2000; Fang et al., 2004; Grosheva et al., 2001; Noren et al., 2000) (for a review, see Anastasiadis, 2007). Kazrin is similarly implicated in RhoA modulation (Sevilla et al., 2008a). Our results here point to negative xARVCF and xKazrin effects on RhoA activity (Fig. 8E), with the actin network becoming reorganized upon Kazrin depletion in vivo (Fig. 8F). Requiring future study, we speculate that the ARVCF–Kazrin complex modulates RhoA activity at cell contacts, having an impact on actin organization and thereby on cadherin stability and endocytosis. Interestingly, Charrasse and co-workers found that constitutively active RhoA modulates M-cadherin endocytosis and degradation (following p120-catenin dissociation from M-cadherin) (Charrasse et al., 2006). We speculate that the role of Kazrin in endocytosis, or the role of the ARVCF–Kazrin complex, could be linked to reduced levels of cadherin and adhesion following Kazrin depletion.

Materials and Methods
DNA constructs
For yeast two-hybrid screening, pGBT7-ARVCF–1ABC was generated via PCR and cloned into pGBK7 (Clontech). pMAL/ARVCF–1ABC was generated by cloning pARVCF–1ABC from pGBT9 into pMALT2 (New England Biolabs). pCS2/HA–xARVCF–1ABC was generated from pCS2/Myc–xARVCF–1ABC. pCS2/MT–xKazrinA was produced via PCR from Xenopus cDNA. pGEX/KazrinA, pGEX/KazrinA NT (amino acid residues 1–268) and pGEX/KazrinA CT (amino acid residues 263–409) were purchased from BD Transduction Laboratories 610273; plakoglobin, BD Transduction Laboratories A2066; GAPDH, Santa Cruz Biotechnology SC-25778; RhoA, RhoB, RhoC, Millipore (Berkley, CA) (amino acids 1–361) was generated by PCR into pCS2/Myc. pCS2/MT–xKazrinA ANLS (amino acids 1–361) was generated by PCR into pCS2/Myc. pCS2/MT–xKazrinA NC2 (amino acids 1–281), pCS2/MT–xKazrinA DC1 (amino acids 1–281), pCS2/MT–xKazrinA DC2 (amino acids 1–173) were made by excising DNA encoding pCS2/MT–xKazrinA with restriction enzymes and re-ligating to pCS2/Myc. pCS2/MT–xKazrinA ANLS (amino acids 1–361) was generated by PCR into pCS2/Myc. pCS2/MT–xKazrinA ANLSm was generated with the QuickChange kit (Stratagene). pPMOM/HA was made by PCR of the Bcl-Xl MOM targeting sequence of Bcl-x [pEGFP-X-TMB (Bcl-x)] was a gift of Christof Borner (Albert-Ludwigs-University of Freiberg, Germany) [Kaufmann et al., 2003] and cloned into pCS2/HA. pMOM/HA–xARVCF–1ABC, pMOM/HA–Xp120–1, pMOM/HA–xARVCF–Nmom (amino acids 1–297) were generated by PCR of the respective cDNAs and placed into pPMOM/HA. cDNAs encoding Xenopus β2–spectrin and p90Rb (IMAGE 5557105 and 5663526, respectively) were purchased from Open Biosystems (Thermo Fisher Scientific), and placed into pCS2/HA. cDNA encoding human p190Rb RhoGAP was a gift of Jeffrey M. Rosen (Baylor College of Medicine, Houston, TX) (Vargo-Gogola et al., 2006), and subcloned into pCS2/HA. pCS2/MyC–GFP–desmoglein1-tail was obtained as a gift of Michael W. Klymkowsky (University of Colorado).

Antibodies and reagents
Anti-Myc (9E10) and anti Xenopus E-cadherin antibody (5D3), respectively developed by J. Michael Bishop and Barry M. Gumbiner, were obtained from the Developmental Studies Hybridoma Bank (NIH/CD and University of Iowa). Human Kazrin (L57), C-cadherin, xARVCF, Xp120 and xKazrin antibodies are previously described (Fang et al., 2004; Nachat et al., 2009). Other antibodies were purchased from commercial sources (HA, Sigma H9658 and Santa Cruz Biotechnology SC805; β-actin, Sigma A2066; GAPDH, Santa Cruz Biotechnology SC-25778; RhoA, RhoB, RhoC, Millipore 05-822; desmoglein1, BD Transduction Laboratories 610273; plakoglobin, BD Transduction Laboratories 610253; human ARVCF, Abnova H0000421-M01; desmoplakin I and 2, BD Transduction Laboratories; β-catenin, BD Transduction Laboratories; and Myc for keratinocyte immunofluorescence, Bethyl Labs. Phosphate Inhibitor Cocktail Set V was purchased from Calbiochem (EMD chemical), and included during the detection of in vivo p190B RhoGAP binding to xARVCF.
Yeast two-hybrid Screenin using full-length XARVC-1ABC employed the Saccharomyces cerevisiae strain AH109 (Clontech), which was transformed with pGBT9/XARVC-1ABC to generate the strain AH109/XARV. A. Y. lapsei stage 18 cDNA library (Sparrow et al., 1998) in pGAD10 was transformed into AH109/XARV and 1×10⁶ clones screened. Plasmids were isolated from colonies showing positive growth following replating and were sequenced. Screening using full-length xKazrinA employed an adult mouse brain cDNA library in collaboration with Hybrigenics ULMtame Y2H (Rain et al., 2009). We obtained 281 positive clones that were then sequenced and analyzed.

xKazrin RT-PCR primers and morpholino Primers used for RT-PCR analysis of xKazrin were: F1, 5’-AGTCTCCGGGC-CAGAACCAGG-3’ and R1, 5’-ACTCTTGACACTTCCTAATCCATCTTGAG-3’. We employed a previously characterized morpholino directed against xKazrin (KMO1) (xKazrin cDNA –4 to +24: 5’-GGCTTGTATCTTCCTCCTATCCATCTTGAG-G-3’) (Sevilla et al., 2008b). A standard control morpholino (CMO) from Gene Tools LLC was used as a negative control (5’-CTCTTCACTCTAGTTACAATTTATA-3’).

GST pull-downs and blot overlay For in vitro pull-down assays involving xARVC and xKazrinA, 1 mg of bacterially purified MBP–xARVC or MBP–xKazrinA, 4 µg of DNA encoding pc5/26 HA–Xp190b was in vitro transcribed and translated using the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega), following the manufacturer’s protocol. Synthesized Xp190b protein was then incubated with 1 mg of GST–xKazrinA and/or MBP–xARVC and pulled-down using amylase-resin.

For blot overlays, purified GST–xKazrinA or GST (250 ng each) was subject to SDS-PAGE and transferred to nitrocellulose. Resulting blots were incubated in PBS with 0.1% Tween-20 and 5% non-fat dry milk overnight at 4°C to facilitate refolding of the purified proteins, and then incubated for 1 hour at room temperature with 1 mg/ml purified MBP–xARVC, MBP–xKazrinA or MBP diluted in the same buffer. Associated MBP fusion proteins were detected using anti-MBP antibody.

Xenopus embryo manipulations, communoprecipitations and cell–cell dissociation assays Induction of female X. laevis, in vitro fertilization of eggs, and embryo injections were carried out using standard methods (Sive et al., 2000). Capped mRNAs for injection were generated using mMessage mMachine kits (Ambion). Embryos were injected at 18°C to stage10 (early gastrula), lysed in embryo IP buffer (50 mM Tris-HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml purified MBP–xARVC, MBP–xKazrinA or MBP diluted in the same buffer. Associated MBP fusion proteins were detected using anti-MBP antibody.

In vitro Rho activity assay In vitro Rho activity assays were performed as described (Gu et al., 2009). Briefly, Xenopus embryos injected with xARVC and/or xKazrinA morpholinos were subjected to lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 10 mg MgCl2, 1% NP-40, 1 mM EDTA and 2% glycerol), and lysates incubated with recombiant GST–Rhoetkin RBD (Rho binding domain) immobilized to glutathione-Sepharose 4B (GE Healthcare Life Science), followed by pull-down and subsequent SDS-PAGE and western blotting detection of active Rho.

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