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CELLULAR AND DEVELOPMENTAL FUNCTIONS OF THE XENOPUS ARVCF-CATENIN:KAZRIN COMPLEX

Kyucheol Cho

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CELLULAR AND DEVELOPMENTAL FUNCTIONS
OF THE XENOPUS ARVCF-CATENIN:KAZRIN COMPLEX

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CELLULAR AND DEVELOPMENTAL FUNCTIONS
OF THE XENOPUS ARVCF-CATENIN:KAZRIN COMPLEX

A

DISSERTATION

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In Partial Fulfillment

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for the Degree of

DOCTOR OF PHILOSOPHY

by

Kyucheol Cho, M.S.

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DEDICATION

I would first like to dedicate this work to God who created our world.

He guided me as my Lord and he was my strength when I was weak.

I would also like to dedicate this work to my loving wife Hye-won and my adorable daughter Lucy. Without their love and support this work would never have been accomplished.
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Finally, I would like to thank my parents, sister and brother for the love and support I needed to finish my studies.
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Xenopus ARVCF (xARVCF), a member of p120-catenin subfamily, binds cadherin cytoplasmic domains to enhance cadherin metabolic stability, or when dissociated, modulates Rho-family GTPases. We previously found that xARVCF binds directly to Xenopus KazrinA (xKazrinA), a widely expressed, conserved protein that bears little homology to established protein families. xKazrinA is also known to influence keratinocyte proliferation-differentiation and cytoskeletal activity.

In my study, I first evaluated the expression pattern of endogenous Kazrin RNA and protein in Xenopus embryogenesis as well as in adult tissues. We then collaboratively predicted the helical structure of Kazrin’s coiled-coil domain, and I obtained evidence of Kazrin’s dimerization/oligomerization. In considering the intracellular localization of the xARVCF-catenin:xKazrin complex, I did not resolve xKazrinA in a larger ternary complex with cadherin, nor did I detect its co-precipitation with core desmosomal components. Instead, screening revealed that xKazrinA binds spectrin. This suggested a potential means by which xKazrinA localizes to cell-cell junctions, and indeed, biochemical assays confirmed a ternary xARVCF:xKazrinA:xβ2-spectrin complex. Functionally, I demonstrated that xKazrin stabilizes cadherins by negatively modulating the RhoA small-GTPase. I further
revealed that xKazrinA binds to p190B RhoGAP (an inhibitor of RhoA), and enhances p190B’s association with xARVCF. Supporting their functional interaction in vivo, Xenopus embryos depleted of xKazrin exhibited ectodermal shedding, a phenotype that could be rescued with exogenous xARVCF. Cell shedding appeared to be caused by RhoA activation, which consequently altered actin organization and cadherin function. Indeed, I was capable of rescuing Kazrin depletion with ectopic expression of p190B RhoGAP. In addition, I obtained evidence that xARVCF and xKazrin participate in craniofacial development, with effects observed upon the neural crest. Finally, I found that xKazrinA associates further with delta-catenin and p0071-catenin, but not with p120-catenin, suggesting that Kazrin interacts selectively with additional members of the p120-catenin sub-family. Taken together, my study supports Kazrin’s essential role in development, and reveals KazrinA's biochemical and functional association with ARVCF-catenin, spectrin and p190B RhoGAP.
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CHAPTER I

INTRODUCTION
p120-catenin family

Catenins were initially defined as binding cadherins (1), and with the exception of alpha-catenin, as possessing a central Armadillo domain mediating cadherin and other protein:protein interactions (2-4). Catenins consist of three sub-families: the beta-catenin subfamily, the p120-catenin subfamily and the plakophilin subfamily (5). In vertebrates, the beta-catenin subfamily includes beta-catenin itself (homologous to Drosophila Armadillo), and plakoglobin. The p120-catenin subfamily includes p120 itself, ARVCF (Armadillo Repeated gene in Velo-Cardio Facial syndrome), delta-catenin and p0071 (Fig. 1). The plakophilin-catenin subfamily includes the plakophilins 1 to 3. These catenins were first found to localize to the plasma membrane as components of adherens junctions and desmosomes (1, 6-11). Cytoplasmic tails of classical cadherins bind to p120 subfamily proteins within the juxtamembrane region, and to beta-catenin subfamily proteins in the distal region (12-15). The cytoplasmic tails of desmosomal cadherins, desmoglein and desmocolin, interact with the plakophilin subfamily proteins, with p0071 (p120 subfamily), and with plakoglobin (beta-catenin subfamily) (7, 10, 11, 16, 17). Thus, p0071 and plakoglobin are generally considered to be localized both at desmosomes and at adherens junctions (16-19), although there is still some controversy about the localization of p0071 (20).

Catenins have numerous roles in varied cellular compartments (Fig. 2). Members of the p120-catenin subfamily, such as p120-, ARVCF- and delta-catenin, modulate classical cadherin stability at cell:cell junctions (21-25). p120 subfamily
Figure 1. Schematic diagram of p120-catenin subfamily proteins

Armadillo repeats and PDZ binding motifs are depicted as shaded boxes and dark boxes, respectively.
Figure 2. The roles of p120-catenin sub-family proteins (p120-catenin is depicted).

At cell-cell contacts, cadherins are stabilized upon their association with p120-catenin sub-family members (p120-, ARVCF-, delta- and p0071-catenins). p120 sub-family members also modulate Rho GTPase in the vicinity of cadherin mediated cell-cell contacts. Cytosolic (cadherin-free) p120 sub-family members modulate Rho GTPases. p120-catenin binds to the Kaiso transcriptional repressor, and de-represses transcription of Kaiso target genes. Other p120 sub-family members are expected to have distinct nuclear functions.
members further directly or indirectly associate with and regulate small GTPases, enabling intracellular signaling and cytoskeletal control (26). Further, most and perhaps all catenins enter the nucleus (27). Beta-catenin, for example, is well known to relieve TCF/LEF-mediated transcriptional repression in response to canonical Wnt-pathway stimulation, thereby activating target genes important in development or pathology/cancer (28). In the nucleus, p120-catenin binds the transcriptional repressor Kaiso (29-31). While further study is required to address unresolved issues (32, 33), we and others find that p120 likewise contributes to Wnt signaling through its binding and displacement of Kaiso from target gene promoters, and thereby the de-repression of Kaiso gene targets, some being shared targets of beta-catenin/ TCF (34-39). In comparison to beta- and p120-catenins, less is known concerning ARVCF and other catenin’s interactions and functional roles.

**ARVCF-catenin**

ARVCF was cloned as one of the gene products deleted in Velo-Cardio Facial syndrome (40). In mammals, ARVCF mRNA is expressed ubiquitously across tissues (40). However, the protein levels of ARVCF are at least 10 fold less than that of p120-catenin (41). Thus, it is believed that p120 is the major p120 subfamily protein binding to the juxtamembrane region (JMR) of classical cadherin proteins. Of note, ARVCF can bind to the JMR and compete with p120 for binding to the cadherin tail (41). This is supported by the lack of developmental defects in ARVCF knock-out (KO) mice (Raju Kucherlapati, Brigham and Women’s Hospital,
unpublished data) (see below for differences in *Xenopus*), whereas p120 KO mice are embryonic lethal (Albert B. Reynolds, Vanderbilt Univ., unpublished data). In contrast to p120-catenin, over-expression of mammalian ARVCF did not initially induce a dendritic phenotype in the NIH3T3 mouse fibroblast cell line, suggesting that ARVCF may be deficient in Rho modulation (41). However, our group’s research then indicated this to be incorrect. We found that *Xenopus* ARVCF depletion could induce gastrulation defects similar to that seen upon p120-catenin depletion, (and resulted in lowered cadherin protein levels), while over-expression of ARVCF in NIH3T3 cell induced dendritic phenotypes, each suggesting that ARVCF functionally interacts with Rho GTPases (22). In contrast to mice, our results in *Xenopus* indicated that ARVCF-catenin may be as central as p120 in amphibian development. Furthermore, the fact that ARVCF but not p120 interacts with protein partners such as the ERBIN scaffolding protein (42), the ZO-1 and ZO-2 tight junction proteins (43), and the less characterized FRMPD2 protein (44), suggests that ARVCF-catenin has additional functions not overlapping with p120-catenin functions.

**Cadherins and p120 catenin**

Cadherin proteins are calcium-dependent cell adhesion molecules and they serve as molecular linkers between neighboring cells (45). Most cadherin proteins are characterized by an extracellular (EC) domain, a single transmembrane domain and a cytoplasmic domain (46). More than a hundred cadherins have been
identified to date. This cadherin super-family is divided into the classical cadherin family, the desmosomal cadherin family, the protocadherin subfamily and additional EC-domain containing proteins according to the nature of their EC and cytoplasmic domains (47). While classical and desmosomal cadherins each have 5 EC domains, their cytoplasmic sequences are largely dissimilar. This suggests that different proteins bind to their cytoplasmic tails. Indeed, as introduced earlier, the p120-catenin subfamily proteins associate with classical cadherins, whereas the plakophilin-catenin subfamily proteins associate with desmosomal cadherins. The relevance of p120-catenins to cadherin function was first indicated by pioneering work of Ireton et al (21). In this report, the authors found that SW48 human carcinoma cells expressed low levels of p120 and E-cadherin. Ectopic expression of p120 increased E-cadherin expression, implying that p120 influences cellular levels of E-cadherin. Two subsequent papers support this by showing that siRNA mediated p120-catenin depletion in various cell lines reduced classical cadherin levels (E-, P-, VE-, N- cadherin) (23, 24). Furthermore cadherins were found to be internalized and degraded upon p120-catenin depletion (23). Thus, these early reports uncovered p120-catenin’s essential role as a rheostat of cadherin stability. However, the mechanism by which p120-catenin stabilizes cadherins is not yet well understood.

Generally, cadherin levels are believed to be regulated by four different mechanisms. First, E-cadherin transcription is down-regulated by transcriptional repressors such as slug, snail and twist during epithelial-mesenchymal-transition (EMT)(48). Second, E-cadherin proteins can be ubiquitilated by Hakai or MDM2 E3
ubiquitin ligases and subsequently degraded (49, 50). Third, the gamma secretase complex (including presenilin-1 protein as a component), ADAM10 metalloprotease or caspase-3 bind to cadherins and cleave their extracellular domains (51-54).

Fourth, cell surface cadherins are internalized by endocytosis (55-57). Internalized cadherins are recycled to another membrane region to form new adherens junctions or are lysosomally degraded (58). Thus, increased endocytosis and lysosomal degradation reduces cellular cadherin levels. Regarding the mechanism by which p120-catenin stabilizes classical cadherins, two models have been proposed. The first is that p120-catenin blocks the interaction of Hakai E3 ubiquitin ligase and presenilin-1 to the cytoplasmic tails of cadherins (49, 59). Thus, dissociation of p120 from cadherin tails exposes the binding sites of Hakai and presenilin, and subsequently, cadherin proteins are degraded or cleaved. However, since a presenilin inhibitor could not restore decreased cadherin levels after p120-catenin depletion (24), and Hakai binds only to E-cadherin (49), this model is unlikely to explain p120’s general mechanism in cadherin stabilization. As an alternative model, Xiao et al. showed that the vascular endothelial/ VE-cadherin tail fused to interleukin-2 receptor (IL-2R) is internalized by clathrin-mediated endocytosis and that p120-catenin inhibits internalization of this chimeric protein (60). This suggests that p120-catenin plays a role in inhibiting cadherin endocytosis. Although it is not presently clear how p120-catenin protects cadherins from endocytosis, one view is that p120 modulates cortical actin at adherens junctions via effects upon Rho GTPases, thereby inhibiting the endocytosis machinery. After an initial report in 2000, subsequent studies demonstrated the modulation of Rho GTPases including
RhoA, Rac1 and Cdc42, and this has thereafter been considered one of p120’s major molecular functions (26, 61-63). The cellular roles of p120-catenin as a modulator of Rho GTPases has been extensively studied in cell activities including actin cytoskeletal rearrangement, inflammation response via the NF-kB pathway, dendrite formation in neurons, malignant tumor cell migration and cell proliferation (64-68). There is much evidence that Rho GTPases play a role in endocytosis (69). For example, over-expression of active RhoA in MDCK cells increases endocytosis of cell surface molecules (70). Thus, it is plausible that p120-catenin stabilizes cadherins via modulating Rho GTPase’s activity, and thereby cortical actin (etc.), in the vicinity of cadherin tails (71).

**Kazrin**

*Kazrin* was initially cloned as an uncharacterized gene product expressed in human brain (72), and subsequently was shown to be expressed ubiquitously at the mRNA level in other human tissues (73). The Kazrin gene is located on human chromosome 1p36.21, and thus far has exhibited seven alternative splice isoforms (A-F and K) (Fig. 3). The KazrinA isoform has an amino-terminal leucine zipper motif, a central coiled-coil domain and Nuclear Localization Signal (NLS) sequence in its carboxyl-terminus. Our previous study found that ectopically expressed KazrinA protein localizes to cell:cell contacts, the cytoplasm and the nucleus in various cell lines. We found that mutation of the carboxyl-localized putative NLS sequence abolished KazrinA nuclear localization, suggesting the NLS is functionally
Figure 3. Schematic comparison of *Xenopus* and human Kazrin isoforms

The coiled-coil, poly-lysine tract (NLS) and sterile a motif (SAM) are respectively depicted as graded grey, black or dark grey shaded areas. LZ indicates the putative leucine zipper region. Alternative splicing at the carboxyl-terminus of the human KazrinA isoform results in an additional isoform, KazrinE, with 3 SAM domains, and KazrinK, lacking the poly-lysine tract. KazrinF contains an additional 97 amino acids at the amino terminus of Kazrin A. Each protein’s total number of amino acids is indicated. Protein structure was predicted with ScanProsite proteomics server.
active. The coiled-coil domain is likely a protein interaction domain because in human Kazrin it was earlier shown to bind the peripheral desmosomal proteins envoplakin and periplakin (73). In *Xenopus*, we found that this region of Kazrin binds ARVCF-catenin. Kazrin’s coiled-coil bears limited sequence similarity with known coiled-coil domains such as FERM, SMC, HOOK, Myosin tail and SbcC. The role of the leucine zipper motif within the coiled-coil of KazrinA is not clear at this point. However, it is notable that the KazrinB-D isoforms lack a leucine zipper motif. Thus, the study of the functional difference between KazrinA and KazrinB-D may uncover the role of the leucine zipper. The KazrinE isoform is similar to KazrinA but includes additional amino acids in the carboxyl-terminus (74). This additional sequence contains three Sterile Alpha Motifs (SAM) which is a well-described protein interaction domain. Indeed, KazrinE interacts with the leukocyte common antigen-related (LAR) protein tyrosine phosphatase via its three SAM domains (74). KazrinF was cloned from the cDNA of a human glioma cell line, U373MG (75). Relative to Kazrin A, it has an additional 97 amino acids at its amino-terminus, and it binds to the apoptotic protein ARC (Apoptosis Repressor with Caspase recruitment domain), and to Bax (Bcl2-associated X protein). At this point, is not clear if KazrinF’s additional amino acids are responsible for these interactions. It will be interesting to test whether other Kazrin isoforms also interact with ARC and Bax. During our prior study, we found that one Kazrin isoform from human brain lacks the NLS, that we termed KazrinK. We compared KazrinK’s intracellular localization with that of KazrinA in MCF7 cells. While KazrinA resided in the nuclei of multiple cells, human KazrinK was absent from nuclei. This pattern was likewise observed in
HEK293 and *Xenopus* A6 cells. In all cases, the existence of variable splicing, and localization of the protein products, is suggestive of functionally distinct roles for Kazrin isoforms.

In *Xenopus*, we and another lab (that of Dr. Fiona Watt, Cambridge U.) independently isolated the KazrinA isoform. BLAST analysis demonstrated *Xenopus laevis* Kazrin (xKazrin) is highly homologous to human and mouse KazrinA (81% and 80.5% amino acid identity, respectively), and with *Xenopus tropicalis* Kazrin (Xt-Kazrin; GenBank # EU404187; 92.6% identity). Interestingly, Xt-Kazrin contained 28 additional residues following Kazrin’s putative coiled-coil domain.

In concordance with the existence of diverse isoforms, various functions have been proposed for Kazrins. An earlier study demonstrated that Kazrin over-expression inhibits clathrin-mediated endocytosis of transferrin receptor, suggesting a role for Kazrin in endocytosis (76). Research in human keratinocytes revealed multiple functions for Kazrin as a structural component of the cornified envelope in human skin, which protects the body from the outside environment; as a modulator of RhoA activity affecting actin cytoskeletal rearrangements; and as a regulator of keratinocyte differentiation (73, 77). During *Xenopus tropicalis* development, Kazrin is involved in axis formation, eye development and ectoderm integrity (78). Furthermore, Kazrin knock-down in U373MG cells induced caspase activation and increased apoptosis, implying a function for Kazrin in cell death signaling (75).

Although surprisingly diverse cellular effects of Kazrin have been reported, the mechanism(s) by which Kazrin is actually connected to such cellular activities is not well worked out. Our previous finding that ARVCF-catenin interacts with Kazrin
suggests that Kazrin may cooperate with p120-catenin family proteins in p120-mediated endocytosis, Rho GTPase modulation and cadherin stabilization. Thus, it is hoped that my study of the role of the ARVCF:Kazrin complex will shed light on our understanding of the multifunctional Kazrin protein and of catenins.

Resolving the cellular localization of Kazrin is also of interest to researchers. Cell line, mouse embryo and human tissue studies demonstrated its localization to cell-cell contacts as well as the cytoplasm and nucleus (73, 77, 79). KazrinA-F isoforms have an NLS sequence, and they each are detected in the nucleus when over-expressed. However, Kazrin’s nuclear function is not yet understood. In the cytoplasm, Nachat et al. observed KazrinE co-localizing with stable, acetylated microtubule structures although the role of Kazrin binding to microtubules remains unclear (74). At cell-cell contacts, Kazrin was reported to be localized to desmosomes in keratinocytes and human skin tissue by binding to plakin proteins, periplakin and envoplakin (73). However, we found *Xenopus* Kazrin directly binds to ARVCF-catenin, which is best known as a component of adherens junctions in association with cadherins. It is possible that Kazrin localizes to different junctions depending on the cell type. Likewise, ARVCF-catenin may localize to more than one junction type, or to adherens junctions by more than one means. In my studies, I will address the cellular localization of the ARVCF-catenin:Kazrin complex, to assist in broadening our knowledge of both the Kazrin and catenin proteins.
CHAPTER II

RESULTS
**Part One: Characterization of Xenopus Kazrin**

*Identification of a new Xenopus Kazrin isoform*

xKazrin is highly homologous to the human and mouse KazrinA isoforms, having 81% and 80% amino acid identity, respectively, whereas *Xenopus tropicalis* Kazrin (Xt-Kazrin; GenBank #EU404187), is 98.8% identical at the protein level with *Xenopus laevis* Kazrin (xKazrin) (Fig. 4). It is notable that Xt-Kazrin contains 28 additional amino acids in the middle of Kazrin’s putative coiled-coil domain. Analysis of the *X. tropicalis* genomic sequence (Ensembl ID #ENSTETG00000013469 and scaffold_257) revealed that Xt-Kazrin has 9 exons, with RT-PCR analysis using *X. laevis* stage 18 neurula cDNA showing that exon 6 is alternatively spliced (Fig. 5). DNA sequencing confirmed that the longer isoform coded for the expected 28 additional amino acid residues in the putative distal coiled-coil region. Being similar in structure and length to human Kazrin isoform A, we named the short xKazrin isoform xKazrinA. The long isoform was named xKazrinB.

*Expression of Kazrin during X. laevis development*

To assess xKazrin mRNA expression during *X. laevis* development, I conducted RT-PCR using primers designed to detect the two xKazrin splice isoforms. While xKazrinB transcripts were detected from the one-cell embryo through tadpole stages, xKazrinA transcripts were only found during or after gastrulation (stage 12) (Fig. 6A). It is notable that xKazrinA expression increased as xKazrinB transcripts decreased between embryonic stages 18 and 40.
Figure 4. Sequence alignment of *Xenopus laevis* Kazrin

Sequence alignment of *Xenopus tropicalis* Kazrin and two isoforms of human Kazrin (hKazrinA and hKazrinK). Identical and similar residues are highlighted in black or grey, respectively. The central coiled coil region (brackets), conserved potential leucine zipper and the predicted nuclear localization sequence (NLS) are indicated.
Figure 5. Genomic structure of *Xenopus tropicalis* Kazrin and identification of two isoforms in *X. laevis*

A) Genomic information was obtained from Ensembl database (Ensembl ID #ENSXETG00000013469 and scaffold 257). Asterisk (*) indicates an alternatively spliced exon in *X. laevis*. B) Identification of *Xenopus laevis* Kazrin isoforms using primers (F1 and R1) binding to the 5'- and 3'-flanking region of exon 6. cDNA was synthesized from embryo stage 18 genomic RNA. Open arrowhead indicates PCR product of xKazrinB cDNA and closed arrowhead indicates that of xKazrinA.
Figure 6. xKazrin expression during *Xenopus* development

A) RT-PCR of xKazrin transcripts during development. Primer set (F1+R1) was used to detect both xKazrin isoforms (top panel) and Histone H4 was used as a loading control (bottom panel). B) Equal amounts of *Xenopus* embryo lysates (0.5 embryo/lane) were resolved by SDS-PAGE and Western blotted for xKazrin (using IgG-purified anti-xKazrinA polyclonal antibody detecting the carboxyl terminal 200 amino acids), or GAPDH as a loading control (bottom panel). Non-specific signals were indicated as asterisks (*).
To evaluate the temporal expression of xKazrin at the protein level, embryonic lysates were prepared from various developmental stages and subjected to Western blotting using an IgG-purified polyclonal antibody, which I raised against the carboxy terminal 147 aminos acids of xKazrinA. The predicted molecular weights of xKazrinA and B are 46.8 kDa and 50 kDa, respectively. However, a 50 kDa band was not detected. Instead, 40 kDa, 47 kDa and 100 kDa appeared to be constitutively expressed from the oocyte to tadpole stage (Fig. 6B). Interestingly, the 40 kDa and 100 kDa bands correspond to the size of human KazrinC/D isoform and Kazrin E isoform, respectively, implying existence of other Kazrin isoform in Xenopus. Since xKazrinA RNA was not detected until the blastula stage, the detected 47 kDa band was expected to be the xKazrinB isoform. However, it cannot be excluded that the KazrinA isoform might migrate to the same position in SDS-PAGE and that these two proteins cannot be separated because ectopically expressed xKazrinA migrates at 47 kDa (data not shown).

**Expression of Kazrin in Xenopus adult tissues**

When the same primer pairs were used to examine mRNA from adult tissues, xKazrinA expression was ubiquitous, whereas xKazrinB was expressed in some but not all tissues (Fig. 7A). Interestingly, these two isoforms were often inversely expressed; xKazrinB appeared to a lesser extent in adult tissues having high levels of xKazrinA.

xKazrin proteins are variably expressed across tissues (Fig. 7B). Brain, heart, stomach, skin and lung contain a 47 kDa protein (maybe xKazrinA or B).
Figure 7. xKazrin expression in adult tissues

A) RT-PCR of xKazrin transcripts from adult tissues. B) Western blot of SDS-soluble extracts from Xenopus adult tissues as indicated at the top. xKazrin was detected as a 47 kDa isoform (closed arrowhead, KazrinA or B), a 40 kDa isoform (open arrowhead, KazrinC/D) and a 97 kDa isoform (asterisk, KazrinE). Equal amounts of total protein were loaded in each lane, and actin was detected as a relative loading control. Molecular weight is indicated in kilodaltons (kDa).
Brain expresses an additional 40 kDa xKazrin (xKazrinC/D) and even lesser amounts of a 40 kDa xKazrin appear in other tested tissues. 100 kDa xKazrin (xKazrinE) is abundantly expressed in the stomach. Heart and muscle also express detectable levels of 100 kDa xKazrin. It is notable that brain, stomach and liver contain an xKazrin protein higher than 100 kDa. As a comparison, the same lysates were used to blot for ARVCF-catenin. ARVCF-catenin is expressed in all tissues except muscle. Although more study is required, this result may suggest that an interaction between ARVCF-catenin and Kazrin occurs in various adult tissues. Brain expresses multiple ARVCF proteins, which due to our previous findings about isoforms in *Xenopus* brain are expected to be different, alternatively spliced isoforms.

**Spatiotemporal expression pattern of xKazrin**

The spatiotemporal expression patterns of xKazrin mRNA during development were studied using whole mount *in situ* hybridization (Fig. 8). xKazrin transcript is enriched in the animal pole region at the one-cell stage (Fig. 8A); in the dorsal neural fold and anterior future head region at neurula stages (Fig. 8B); and in the head region at tadpole stages.

**Prediction of xKazrinA coil structure**

Previously we used the COILS program to predict that the central domain of xKazrin would form a coiled-coil region.
**Figure 8.** Spatiotemporal expression pattern of xKazrin in *X. laevis*

A) Kazrin whole mount *in situ* hybridization at one-cell stage embryos. B) Neurulation stage embryo *in situ* hybridization. C) Tadpole stage embryo *in situ* hybridization. Anti-sense Dig-labeled RNA probe for full-length xKazrinA was used for whole mount *in situ* hybridization. As a negative control, the same amount of sense probe were used.
Experimental results demonstrated that Kazrin binds to other proteins such as envoplakin, periplakin and ARVCF-catenin through a coiled-coil domain (73). Thus, the predicted structural information for xKazrin will assist us in testing models addressing how Kazrin’s putative coiled-coil domain engages in protein-protein interactions. To obtain high resolution structural information for Kazrin, we have been collaborating with Dr. Richard Brennan (UT MDACC) to do X-ray crystallography of xKazrinA. Prior to crystallography, using the given structural property of 3.5 amino acid residues per helical turn, I modeled the coil structures for xKazrinA. I found five coiled-coils within xKazrin’s central region, and an additional coil (leucine zipper) in the amino terminal region (Fig. 9).

**Interaction between Kazrin isoforms**

In order to check whether Kazrin isoforms bind each other, myc-tagged *X. tropicalis* Kazrin (similar to *X. laevis* KazrinB form) and HA-tagged *X. laevis* KazrinA were co-expressed in *Xenopus* embryos and their interaction was tested using co-immunoprecipitation (co-IP). As shown in Fig. 10, two Kazrin proteins bind to each other. Because xKazrinA and xKazrinB are quite similar, Kazrin’s homophilic interaction is expected. For the binding site mapping, Kazrin deletion mutants were used in a co-IP experiment. I found that Kazrin proteins interact with each other via their coiled-coil region as expected (Fig. 11).
**Figure 9.** Predicted xKazrin coiled-coil structures

A) Based upon direct study of the sequence regions in combination with the use of available software, xKazrinA was predicted to form as many as six amphipathic coiled coil structures with the indicated amino acid sequences (coiled coils by definition exhibit 3.5 amino acid residues per helical turn). To aid in visualization, polar amino acid residues (asparagine (N), glutamine (Q), serine (S) and threonine (T)); nonpolar or hydrophobic amino acid residues (leucine (L), isoleucine (I), valine (V), alanine (A), methionine (M), phenylalanine (F)); acidic amino acid residues (aspartate (D), glutamate (E)) and basic amino residues (lysine (K), arginine (R)) are labeled and shaded in white, green, red and blue, respectively. Amino acid residues assigned to the “a” and “d” positions within the helix are most often nonpolar or hydrophobic and form a hydrophobic interface that favors interaction with a similarly nonpolar helical interface found within the same polypeptide (intramolecular interaction), or within another protein (intermolecular interaction: either homotypic or heterotypic). B) The alignment of the amino acid sequences encompassing the six predicted coiled-coil regions reveals the periodic appearance of nonpolar/hydrophobic amino acid residues at the “a” and “d” positions.
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**Figure 10.** Interaction between different xKazrin isoforms

Xt-KazrinA (myc tagged) and XI-KazrinA (HA tagged) were co-expressed in *Xenopus laevis* embryos. Lysates were immunoprecipitated for Myc-Xt KazrinA (A panel) or HA-XI-KazrinA (B panel), and immunoblotted as indicated.
Figure 11. Binding domain mapping of Kazrin homo-dimerization

A) Schematic diagram of xKazrin deletion mutants used in this study. The LZ indicates putative leucine zipper region. B) Indicated in vitro transcribed Kazrin deletion mutant RNAs were microinjected into one-cell stage embryos. Then early gastrula embryos were lysed and mutants were immuno-precipitated using Myc antibody, followed by SDS-PAGE/western blotting. Asterisks (*) indicate non-specific signals from HA antibody western blotting.
Part Two : Cellular Localization of the ARVCF-catenin: Kazrin complex

Cellular localization of xKazrinA

In mammalian cell lines and human skin tissues, Kazrin protein localizes at cell-cell contacts, in the cytoplasm and in the nucleus (74, 77). To determine xKazrin’s cellular localization in *Xenopus* tissues, I performed immunofluorescence using *Xenopus* blastula ectoderm (animal caps) (Fig. 12A). As endogenous xKazrin proved difficult to visualize by immunofluorescence using the *Xenopus* Kazrin antibody, I ectopically expressed Myc-tagged xKazrinA. *In vitro* transcribed Myc-xKazrinA RNA was injected into one-cell stage embryos. At the blastula stage, Myc-xKazrinA’s localization was examined in different cell layers within the ectoderm. *Xenopus* ectodermal (animal cap) tissue at this developmental time point consists of three to four cell layers; an outer layer that differentiates to epidermis and two to three inner cell layers. While most xKazrinA protein seems to be localized to cell-cell contacts within the outer cell layer and nuclear xKazrinA is also detected in some cells, inner cell layers displayed strong nuclear localization as well as cell-cell border localizations (Fig. 12B). Kazrin’s nuclear presence in *Xenopus* tissue is in accordance with its nuclear localization in cell lines and human skin. The weak nuclear signal in outer cell layers is thought to be due to difficulty in the penetration of the antibody through the outer cell membrane, because nuclear localization in outer cell layers was enhanced when a strong detergent was used during immunofluorescence to increase membrane permeability.
A. Blastula ectoderm (animal cap)
   - outer cell layer
   - inner cell layer

B. Images showing different cell layers:
   - outer cell layer
   - inner (deep) cell layer
   - Images labeled as 'Myc-xKazrin A' and 'Topro-3 nuclear staining'.
Figure 12. Cellular localization of xKazrin A in ectodermal explants of *Xenopus* blastula (stage 9-10) embryos

A) Cross-sectional view of *Xenopus* blastula. B) a) Myc-xKazrinA localization in the outer layer of the blastula ectoderm, detected using anti-Myc antibodies. b) Co-visualization of Myc-xKazrinA and nuclei (topro-3 dye) in the outer layer of blastula ectoderm. c) Myc-xKazrinA localization in the inner layer(s) of blastula ectoderm.
**xKazrinA and xARVCF co-localize at cell-cell contacts in Xenopus blastula ectoderm.**

To assess HA-xARVCF and Myc-xKazrinA co-localization following co-expression *in vivo*, immunofluorescent images were acquired from the outer ectodermal cell layer at blastula stages. Co-localization occurred along cell-cell borders in the X-Y plane (Fig. 13A-C), and to a significant extent, if not entirely along the Z-axis (Fig. 13D). 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.**

Since xARVCF directly binds cadherin juxta-membrane regions (41, 81, 82), I tested whether xKazrinA and xARVCF would co-localize with C-cadherin, a major cadherin essential for *Xenopus* early development (83, 84). Xenopus C (xC)-cadherin co-localized at cell-cell borders with xKazrinA as well as with xARVCF (Fig. 13E-L), consistent with a possible association of xKazrinA with adherens junction components.

To test if xKazrinA and xC-cadherin form a larger complex, presumably bridged by xARVCF, I over-expressed Myc-xKazrinA followed by xC-cadherin immunoprecipitation and Western blotting (Fig. 14). As anticipated, endogenous xC-cadherin co-immunoprecipitated endogenous xARVCF, as well as xβ-catenin and Xplakoglobin. Importantly, however, it did not co-precipitate Myc-xKazrinA. In further tests, I simultaneously over-expressed three components: HA-xKazrinA, HA-
Figure 13. Co-localization of xKazrinA with xARVCF and xC-cadherin in ectodermal explants of Xenopus blastula (stage 10) embryos

A-D) Localization and co-localization of co-expressed Myc-xKazrinA and HA-xARVCF in the outer ectodermal layer, detected using anti-Myc and anti-HA antibodies respectively. The area outlined by a white rectangle in (C), was viewed using Z-stack section images shown in (D). E-H) Localization and co-localization of HA-xARVCF and C-cadherin, detected respectively using anti-Myc and anti-C-cadherin antibodies. I-L) Localization and co-localization of Myc-xKazrinA and xC-cadherin. Bars = 50 mm.
Figure 14. Co-immunoprecipitation of Myc-xKazrinA with adherens junction components

Myc-xKazrinA was expressed in early embryos, lysed at stage 9-10 and immunoprecipitated for endogenous xC-cadherin. Co-precipitated proteins were detected by immuno-blotting using the indicated antibodies.
xARVCF and the cytoplasmic tail of xC-cadherin (Myc-tagged). Whereas xARVCF co-immunoprecipitated with C-cadherin's cytoplasmic domain as expected, HA-xKazrinA was not present in conjunction with cadherin (Fig. 15). These results indicate that xC-cadherin can form a complex with xARVCF but not with xKazrinA.

Since human Kazrin associates with envoplakin and periplakin at desmosomal or inter-desmosomal regions in human keratinocytes (73), and ARVCF- and p120-catenins have been reported at desmosomes (85-88), I asked if ARVCF is present at desmosomes using biochemical precipitations from A431 human epithelial carcinoma cell extracts and *Xenopus* embryos. In A431 cell extracts, endogenous Desmoglein1 (Dsg1) did not co-precipitate with human ARVCF while E-cadherin did (Fig. 16). Similarly, the cytoplasmic tail of human Dsg1 (Fig. 17) or full-length mouse Dsg3 (Fig. 18) failed to co-precipitate with *Xenopus* ARVCF. Plakoglobin and xC-cadherin were positive controls for the respective binding functionality of Dsg1 and ARVCF in *Xenopus* embryos (Fig. 17). Our data indicate that xARVCF is not a core component of the desmosome.

Furthermore, we could detect neither an interaction between xKazrinA and xC-cadherin, nor an interaction between xARVCF and xDsg1 under chemical cross-linking conditions (DTSSP; 3,3’-Dithiobis Sulfosuccinimydilpropionate), while the interaction between xARVCF and xC-cadherin was increased as expected (positive control) (Fig. 19). This result supports the idea that the xARVCF:xKazrin complex is not a core component of the adherens junction.
Figure 15. Co-immunoprecipitation assay following the co-expression of Myc-xC-cadherin tail, HA-xARVCF and HA-xKazrinA

Myc-xC-cadherin tail was precipitated using anti-Myc antibodies. Co-precipitated HA-xARVCF and HA-xKazrinA were detected (versus not) using anti-HA antibody. Asterisk (*) indicates migration of IgG heavy chains (50 kDa).
Endogenous Desmoglein 1 or E-cadherin was immuno-precipitated from confluent A431 human epithelial carcinoma cells. Bound endogenous ARVCF was detected with human ARVCF specific antibody. As a negative control, mouse IgG was used for immunoprecipitation at the same time.
**Figure 17.** The interaction between desmosomal cadherin Desmoglein 1 and xARVCF in *Xenopus* embryo lysates

Indicated amounts of *in vitro* transcribed RNAs were microinjected into one cell stage embryos. Then early gastrula embryos were lysed and human Desmoglein tail or xARVCF were immuno-precipitated, followed by SDS-PAGE/western blotting.
Figure 18. The interaction between mouse desmosomal cadherin Desmoglein 3 and xARVCF in Xenopus embryo lysates

Indicated amounts of in vitro transcribed RNAs were microinjected into one-cell stage embryos. Then early gastrula embryos were lysed and mDesmoglein 3 or xARVCF were immuno-precipitated, followed by SDS-PAGE/western blotting.
**Figure 19.** Chemical cross-linking using the DTSSP (3,3′-Dithiobis Sulfosuccinimidylpropionate) chemical cross linker, followed by immuno-precipitation

A) After cross-linking of embryo lysates expressing Myc-xKazrinA, lysates were subjected to SDS-PAGE/western blotting to check cross-linking status. Reversible DTSSP-induced chemical cross-links were cleaved using the reducing reagent, β-mercaptoethanol. B) Cross-linked lysates were precipitated using antibodies directed against ARVCF or C-cadherin and co-precipitated proteins were detected with the indicated antibodies.
**xKazrinA and xC-cadherin do not compete for xARVCF**

Interestingly, the interaction between xKazrinA and xARVCF decreased as more xC-cadherin bound to xARVCF under cross-linking conditions, suggesting that xC-cadherin binding to xARVCF may be mutually exclusive with xKazrinA. In accordance with this, our previous binding domain mapping study demonstrated that both xC-cadherin and xKazrinA bind to the armadillo repeats of xARVCF-catenin.

To test this hypothesis, I monitored the effects of xC-cadherin over-expression on the xARVCF:xKazrinA interaction (Fig. 20), and the effect of xKazrinA over-expression on the xARVCF:xC-cadherin interaction (Fig. 21). Contrary to my expectation, I found that xC-cadherin and xKazrinA did not compete for binding to xARVCF.

Based on the cross-linking co-IP and competition IP experiments, I hypothesized the existence of two ARVCF complexes at the plasma membrane. First, the known presence of ARVCF at the adherens junction via cadherin association, and second, an ARVCF:Kazrin complex anchored to the plasma membrane by an unknown mechanism. In this model, ARVCF-catenin shuttles between these complexes, but the cadherin:ARVCF complex at adherens junctions is predominant. Thus, under chemical cross-linking conditions, ARVCF was reduced in its capacity to shuttle to Kazrin, lowering its detection in complex with Kazrin.
A) 1 ng Myc-xKazrinA RNA was micro-injected at the one-cell stage together with different amounts of HA-xC-cadherin RNA. The expression of each protein was detected from blastula embryo lysates using SDS-PAGE/immuno-blotting. B) Endogenous ARVCF was precipitated from lysates described in (A), and co-precipitated xKazrinA and xC-cadherin were detected using Myc- and HA-antibodies, respectively.

**Figure 20.** Competition assay I
**Figure 21.** Competition assay II

After mixture of the indicated level of components, Myc-xKazrinA RNA and β-gal RNA were micro-injected at the one cell stage, endogenous ARVCF was precipitated from late blastula embryo lysates (stage 10), and co-precipitated xKazrinA and xC-cadherin were detected using Myc- and C-cadherin specific antibodies, respectively. Expression of each protein was detected using SDS-PAGE/immuno-blotting.
**xKazrin depletion decreases membrane localized xARVCF**

It has been generally thought that cadherin proteins are the only significant membrane-binding partners of p120-catenin proteins, and thus that they determine the membrane localization of p120-catenin proteins. However, my results suggest that Kazrin may comprise another partner localizing ARVCF to cell-cell contacts. The effect of xKazrin on membrane localization of ARVCF was tested using a membrane fractionation assay (Fig. 22). Surprisingly, I found Kazrin depletion reduced membranous ARVCF-catenin protein and increased cytosolic ARVCF-catenin slightly, suggesting that xKazrin may play a role in ARVCF-catenin retention at the plasma membrane. However, it cannot be precluded that the decrease of cadherin levels after Kazrin depletion (see Part III), indirectly freed ARVCF-catenin from the plasma membrane.

**xKazrinA binds to the xβ2-spectrin cytoskeletal protein at cell-cell contacts**

To further address how the xARVCF:xKazrinA complex localizes to cell contacts, I collaborated with Hybrigenics® to perform a yeast two-hybrid screening of xKazrinA. From an adult mouse brain cDNA library, we obtained 64 positive clones (partial list in Table 1). Notably, 9 clones encoded plasma membrane-associated proteins. These included two p120-catenin sub-family members (delta-catenin and p0071), the tight junction protein symplekin (89), the spectrin binding protein Camsap1 (90, 91), and three spectrin family members (α1-, α2- and β2-spectrins) (92). This screen was not sensitive as we did not isolate ARVCF-catenin, envoplakin or periplakin, perhaps because ARVCF expression is lower in mammals.
Figure 22. Membrane fractionation assay after xKazrin depletion

xKazrin depleted embryo lysates were prepared at late blastula stage (stage 10) and membrane fractionated. To control for complete fractionation, C-cadherin was detected for membrane fraction and actin for cytosolic fraction. Closed arrowhead indicates intact C-cadherin and open arrowhead indicates cytosolic cleaved C-cadherin.
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**Table 1.** Selected list of xKazrin interactors from yeast-two-hybrid screen
than amphibians (41), and because there is little envoplakin/ periplakin expression in neural tissues (93).

Spectrin as a potential Kazrin binding partner was intriguing given that our earlier structural analysis of xKazrinA with help of Dr. Richard Brennan predicted that its coiled-coil domain would be similar to the spectrin-repeat region of α-spectrin (GenThreader program in PSIPRED Protein Structure Prediction Server; http://bioinf.cs.ucl.ac.uk/psipred/; p-value = 4e-04) (94). In mammals, there are two α-spectrin genes and five β-spectrin genes. Their gene products form αβ heterodimers that orient end-to-end to form α2β2 tetramers. Tetramers in turn interact with microfilaments, neighboring spectrin tetramers and other proteins such as band 4.1, ankyrin, adducin and calmodulin, to form a multifunctional, cytoskeletal matrix (92). Anchoring points, such as provided by band 4.1 and ankyrin, facilitate the spectrin cytoskeleton’s plasma membrane association, as do junctional cadherins (92). In *Xenopus*, two spectrins are known, α-spectrin (also known as α-fodrin) and β-spectrin (95, 96). Xα-spectrin expression, while high in oocytes, is reduced following fertilization (95). Xβ-spectrin mRNA on the other hand, is expressed throughout development as assessed via semi-quantitative RT-PCR (Fig. 23).

I wondered if the spectrin network might be involved in the localization of xARVCF:xKazrinA 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 *Xenopus* β-spectrin, and is homologous to human β2-spectrin isolated from our yeast two-hybrid screen (74.6%
Figure 23. *Xenopus* β2-spectrin mRNA expression during development

Total RNA was isolated from embryos at the indicated stages, and RT-PCR was performed using a primer pair for *Xenopus* β2-spectrin. *Histone H4* served as a loading control.
identical). It encodes β2-spectrin’s N-terminal actin-binding domain (ABD; which binds band 4.1 and adducin), and five spectrin repeats (Fig. 24). Isolation of shorter yeast clones from our two-hybrid screen suggested that the β1- or β2-spectrin regions extending from the end of the second spectrin repeat to the middle of the third repeat are sufficient to bind Kazrin (not shown). The xKazrinA:β2-spectrin interaction was supported using co-precipitation tests of *Xenopus* embryo lysates in which full length xKazrinA and partial xβ2-spectrin proteins were co-expressed (Fig. 25). Furthermore, using confocal microscopy, I showed that xKazrinA and xβ2-spectrin co-localized to cell-cell borders in blastula ectoderm, as expected (Fig. 26).

**xARVCF, xKazrinA and xβ2-spectrin form a ternary complex**

To test if 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 endogenous xARVCF complexes isolated and assayed (Fig. 27). As shown in Fig. 27, xβ2-spectrin as well as xKazrinA were co-precipitated with xARVCF. As xβ2-spectrin expression did not alter xARVCF:xKazrinA co-precipitation, we expect that a ternary xARVCF:xKazrinA:xβ2-spectrin complex was formed. However, since a prior study reported β2-spectrin’s association with E-cadherin (through ankyrin) (97), it was conceivable that xARVCF association with xβ2-spectrin occurred via their mutual association with cadherin.
Figure 24. Comparison of Human and *Xenopus* β2-spectrin cDNA

A) Sequence alignment of human and *Xenopus* β2-spectrin. Identical and similar residues are highlighted in grey. The actin binding domain (brackets), and the putative Kazrin binding domain (black box) suggested from our yeast two-hybrid screen are indicated. B) Schematic comparison of human β2 spectrin (gene bank ID # NM_003128) and *Xenopus* partial β2 spectrin (BC046267) Actin binding domain (ABD) and putative Kazrin binding region are depicted.
Enzymatically synthesized Myc-xKazrinA and HA-xβ2-spectrin mRNA were co-injected into one-cell stage embryos and blastula embryo lysates were HA immuno-precipitated followed by Myc immuno-blotted.

**Figure 25.** *In vivo* binding of xKazrinA and xβ2-spectrin
Figure 26. Co-localization of xKazrinA and xβ2-spectrin in the blastula ectoderm

Animal caps were isolated from blastula embryos (stage 9-10) expressing Myc-xKazrinA and HA-xβ2-spectrin, followed by their respective (Myc and HA) immuno-fluorescent detection.
**Figure 27.** Formation of xARVCF, xβ2-spectrin and xKazrinA ternary complex

Blastula lysates from embryos injected with the indicated amounts of each mRNA were immuno-precipitated with xARVCF antibody, followed by immuno-blotting for Myc-xKazrinA and HA-xβ2-spectrin.
We tested this possibility using co-precipitation assays. As shown in Fig. 28, xARVCF but not xC-cadherin associated with xβ2-spectrin and xKazrinA. Thus, the ARVCF:Kazrin complex likely associates with spectrin through Kazrin binding to spectrin.
Figure 28. ARVCF-specific ternary complex

Lysates from blastula embryos expressing xβ2-spectrin and xKazrinA were immuno-precipitated using the indicated antibodies. Co-precipitating xβ2-spectrin or xKazrinA were detected by immuno-blotting.
Assessment of xKazrin morpholinos

In an effort to address the function of Kazrin in the *Xenopus* system, I used two independent morpholinos; one that blocks translation initiation of mature xKazrin mRNA (Kmo-T: Kazrin Morpholino targeting RNA Translation), and one that blocks nuclear processing of xKazrin pre-mRNA (Kmo-S: Kazrin Morpholino targeting RNA Splicing).

Kazrin depletion using the translation blocking morpholino in early *Xenopus tropicalis* embryos was previously reported to result in ectodermal blistering, a shortened body axis, head reductions, and somatic defects (78). Because the mRNA sequence targeted by the morpholino used in *X. tropicalis* is completely conserved with that in *X. laevis*, I used it in my study (Kmo-T), and demonstrated its efficacy in blocking *in vitro* translation of Kazrin mRNA, and in knocking-down *X. laevis* Kazrin to 60% of endogenous levels by the neurulation stage (significant maternal protein loading precluded obtaining earlier effects) (Figs. 29A and B, respectively).

Kmo-S was designed to bind the 3’ end of exon 4 (Fig. 30A). RT-PCR analysis following morpholino injection revealed that Kazrin RNA expression was decreased in a dose-dependent manner (Fig. 30B). DNA sequencing of the product generated upon morpholino treatment (sequencing of the K1/ K2 PCR product) confirmed that exon 4 was skipped during splicing, producing a frame shift in exon 5 followed by a non-sense mutation.
A. 

\[ 0.5 \mu g \text{ xKazrinA} \quad 0.5 \mu g \text{ Myc-xKazrinA} \]

\[
\begin{array}{c|c|c|c|c|c}
& \text{Cmo} 20 \mu g & \text{Kmo-T} 20 \mu g & \text{Cmo} 20 \mu g & \text{Kmo-T} 20 \mu g \\
\hline
\text{anti-xKazrin Blot} & & & & & \\
\end{array}
\]

B. 

\text{Stage 18 embryos}

\text{Set I} \quad \text{Set II}

\[
\begin{array}{c|c|c|c|c|c}
40 \text{ ng Cmo} & 40 \text{ ng Kmo-T} & \text{uninjected} & 40\text{ng Cmo} & 40\text{ng Kmo-T} & \text{uninjected} \\
\hline
\end{array}
\]

\text{Kazrin} \quad \text{GAPDH}
**Figure 29. Assessment of xKazrin morpholino, Kmo-T**

A) *In vitro* translation blocking activity of xKazrin morpholino. *In vitro* transcribed xKazrinA and Myc-xKazrinA mRNAs were *in vitro* translated in the presence of Kmo-T or Cmo as a control. As expected, Kmo-T blocked the translation of xKazrinA mRNA and not that of Myc-xKazrinA, since the latter is an amino-terminal fusion product (w/ Myc) (thus offering incomplete sequence complementary with the morpholino targeting the endogenous xKazrinA translation initiation site). B) xKazrin morpholino knock-down of endogenous xKazrin protein. Morpholinos were injected into one-cell stage embryos and lysates were prepared at neurulation (stage 18). Endogenous xKazrin (47 kDa) was detected by SDS-PAGE/ immuno-blotting using polyclonal antibody raised against the carboxy-terminal 147 amino acids of xKazrinA. Standard control morpholino (Cmo) was used as a negative control.
Figure 30. Assessment of xKazrin morpholino, Kmo-S

A) Schematic diagram of morpholino binding region and primers used for testing morpholino activity (left panel). B) Effect of xKazrin morpholino S (Kmo-S) on expression of endogenous xKazrin RNA, assayed using RT-PCR. Embryos were injected with two different doses of Kmo-S (40 ng and 80 ng), total RNA was isolated from stage 18-19 embryos, and RT-PCR was conducted using the indicated primer pairs.
Kazrin depletion decreases cadherin protein levels and cell:cell attachment

My data showed that xKazrin depletion during X. laevis development induced loss of ectodermal integrity as reported in X. tropicalis (this phenotype will be detailed in Part four). To address the mechanism by which xKazrin depletion alters ectodermal integrity, I first considered effects upon C-cadherin and E-cadherin. Interestingly, both cadherins' levels were decreased after xKazrin depletion using either Kmo-T (Fig. 31) or Kmo-S (Fig. 32), as was that of Desmoglein 1, a desmosomal cadherin (Fig. 33). As a control, endogenous Kazrin protein was detected using a specific antibody, with Fig. 31 showing that Kazrin protein was decreased about 30% following Kazrin depletion, which is similar to an about 40% decrease in the previous experiment (Fig. 29). A previous report employing cell lines in combination with immunofluorescence methods indicated that Kazrin over-expression lowers desmoplakin and perhaps E-cadherin levels (77). This suggests that Kazrin plays a role in the stability of junctional proteins.

To evaluate xKazrin depletion effects upon cell:cell adhesion in a more controlled setting, I performed cell dissociation and cell re-aggregation assays using blastula ectoderm tissue (animal caps). xKazrin-depleted tissue exhibited accelerated dissociation compared to controls even without EGTA which weakens cadherin-mediated cell contacts (Fig. 34), and correspondingly, they experienced delayed re-aggregation (Fig. 35). Together, our data suggest that Kazrin positively modulates cell:cell contacts and affects cadherin levels.
Figure 31. The effects of xKazrin depletion using Kmo-T upon cadherin and catenin protein levels

Decrease of cadherin and catenin protein levels after xKazrin depletion. Embryos injected at the one-cell stage with different amounts of the indicated morpholinos were lysed at the early gastrula (stage 10) and neurula (stage 18) stages. Indicated protein levels were visualized by immuno-blotting.
Figure 32. The effects of xKazrin depletion using Kmo-S on cadherin and ARVCF-catenin

Decrease of cadherin and ARVCF-catenin protein levels after xKazrin depletion using Kmo-S morpholino. Embryos injected at the one-cell stage with different amounts of the indicated morpholinos were lysed at the early gastrula (stage 10) stage. Indicated protein levels were visualized by immuno-blotting.
Figure 33. The effects of xKazrin depletion on desmosomal cadherin, desmoglein 1

Embryos injected at the one cell stage with two different amounts of the indicated morpholinos were lysed at the neurula (stage 18) stage. Desmoglein 1 protein levels were visualized by immuno-blotting using a specific antibody. As a positive control, classical cadherins levels were visualized, too.
Figure 34. Cell dissociation assay using animal cap explants

Blastula ectoderm (animal caps; stage 9-10) from embryos injected with control (Cmo), Kazrin morpholino T (Kmo-T) or ARVCF morpholino 1 (ARVmo-1) were dissociated in the absence (upper panel) or presence of 2 mM EGTA (lower panel). Photos were taken at 0hr, 1 hr and 2 hr following EGTA addition.
**Figure 35.** Cell re-aggregation assay using animal cap explants

Inner cells of blastula ectoderm (animal caps; stage 9-10) from embryos injected with control (Cmo) or Kazrin morpholino T (Kmo-T) were isolated and dissociated in Ca, Mg-free medium. Then, cells were re-aggregated in the presence of 2 mM CaCl₂. Photos were taken at 2 hr following CaCl₂ addition.
**Kazrin depletion decreases catenin proteins levels**

As for cadherin, the level of xARVCF, Xp120 and xβ-catenin decreased in response to xKazrin depletion (Figs. 31 and 33). One possible explanation for Kazrin’s modulation of cadherin protein levels is its above-shown relationship with ARVCF. Based upon published reports, lowered ARVCF levels, as for lowered p120 levels, are expected to result in lowered cadherin levels (21-24). To test whether xKazrin affects cadherin levels through regulation of xARVCF, the levels of cadherins and catenins were examined after xARVCF depletion (Fig. 36). Whereas classical cadherin levels were down-regulated by xARVCF depletion as expected, desmoglein 1 and p120-catenin levels were not changed. This result suggested that xKazrin-mediated regulation of ARVCF-catenin stability may not be a main cause of decreased levels of various cadherins and catenins after xKazrin depletion. One alternative model is that cadherin levels are reduced after xKazrin depletion by an unknown mechanism and then catenins are destabilized because lowered cadherin presence can result in reduced catenin levels (e.g. beta-catenin)(13, 98).

It is notable that one xARVCF morpholino did not reduce cadherin levels although endogenous xARVCF-catenin levels were reduced (Fig. 36). The reason for this discrepancy will require further investigation.

**Kazrin depletion leads to increased RhoA activity and cortical actin disorganization.**

One unanswered question revolves around how xKazrinA, or the xARVCF:xKazrinA complex, modulates cadherin stability. p120 sub-family catenins
Figure 36. The effects of xARVCF depletion on cadherin and catenin expression

Embryos were injected at the one cell stage with two independent morpholinos and lysates were prepared at the neurula (stage 18) stage. Indicated protein levels were visualized by immuno-blotting.
including ARVCF are thought to stabilize cadherins by binding cadherin membrane-proximal domains to reduce protein associations (such as Hakai or presenilin-1) (49, 51, 59) or modifications promoting cadherin degradation/endocytosis (21, 23, 24). Rho GTPases that are modulated by p120 sub-family catenins are further relevant to cadherin stabilization/endocytosis (26, 57, 70, 71, 99-101). Interestingly, a previous non-biased screen correlated Kazrin over-expression with inhibition of clathrin-mediated endocytosis of the transferrin receptor (76). Thus, Kazrin, together with p120 sub-family catenins, may be relevant to cadherin endocytosis. Indeed, cadherin levels after Kazrin depletion were restored when embryos were incubated with a specific dynamin inhibitor (Dynasore; Fig. 37) (102), and ectodermal defects induced by Kazrin depletion were partially rescued (data not shown). This suggests a potential role for Kazrin in cadherin endocytosis.

None the less, given known ARVCF- (and p120-) catenin effects upon small GTPases (22, 61-63), I turned my attention to xKazrinA effects on RhoA and Rac, and on cortical actin organization in plasma membrane regions. I initially evaluated RhoA activity in embryos following the depletion of xKazrin and/or xARVCF (Fig. 38). Interestingly, xKazrin depletion significantly increased RhoA activity, an effect likewise observed (and expected) following xARVCF knock-down (22). Coordinate xKazrin and xARVCF depletion had an additive effect upon RhoA activity. As Rac1 was not affected by xKazrin depletion (data not shown), Kazrin may be a RhoA specific modulator. My results are in line with a report indicating that Kazrin over-expression inhibits RhoA in human keratinocytes, with no apparent effect on Rac1 (77). Thus, while Kazrin and xARVCF appear to have shared inhibitory effects upon
Figure 37. Effect of inhibiting clathrin-mediated endocytosis on the reduced xE-Cadherin levels observed following xKazrin depletion

One cell stage embryos were injected with the indicated morpholinos. Embryos were subsequently (32-64 cell stages) incubated with 50mM Dynasore (versus DMSO solvent). Lysates from neurula (stage 18) embryos were immuno-blotted for xE-cadherin and GAPDH.
Figure 38. Rho activity in response to xARVCF and/ or xKazrin depletion

The indicated morpholinos were injected into one cell stage embryos, and lysates from late blastula (stage 9-10) embryos were tested for RhoA activity in vitro.
RhoA, one distinction (relative to ARVCF and related p120 sub-family members) is that Kazrin is not likely to modulate Rac1 in this context (22, 61-63).

Since ectopic RhoA activation modulates actin and cell structure/morphology (103), I employed phalloidin staining to grossly resolve the effects of Kazrin depletion on actin organization in embryos (Fig. 39). 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. The cytoskeletal disorganization resolved in vivo appears to be in keeping with a previous report employing keratinocytes (77). To test whether increased RhoA activity after Kazrin depletion contributes to cadherin destabilization, different forms of RhoA and Rac1 were ectopically expressed and E-cadherin levels were monitored. As expected, active RhoA specifically decreased E-cadherin levels (Fig. 40). In summary, Kazrin appears to be a negative regulator of RhoA in vivo, suggesting that increased RhoA activity on Kazrin depletion contributes to microfilament reorganization, and possibly to lowered cadherin levels as previously published (70, 99).

**xKazrinA interacts with Xp190B RhoGAP.**

I wondered if Kazrin might modulate RhoA via another protein(s), since predictive analysis suggested it was unlikely to directly bind RhoA. Common modulators of RhoA include RhoGDIs (Rho GDP Dissociation Inhibitors), RhoGAPs (Rho GTPase Activation Proteins) and RhoGEFs (Rho Guanine nucleotide Exchange Factors). Intriguingly, from the xKazrinA yeast two-hybrid screen, I
Figure 39. Actin staining of blastula ectoderm after Kazrin depletion

Animal caps were isolated and filamentous actin in deep (inner) cells were visualized using phalloidin-Alexa 488 fluorescence.
Figure 40. The effects of Rho GTPases on E-cadherin levels

The indicated mutant RhoA and Rac1 RNAs were micro-injected into one cell stage embryos and embryos were later lysed at the blastula stage (stage 10). E-Cadherin levels were visualized by SDS-PAGE/Western blotting. GAPDH was detected as a loading control.
identified multiple independent clones of mouse p190B RhoGAP (Table 1). 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 (104). Recently, p120-catenin was shown to mediate RhoA inhibition via the recruitment of p190A RhoGAP (105, 106). 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), I deduced that xKazrinA binds to a region within or spanning the 3rd-4th FF domains of p190B, an α-helical structure including two conserved phenylalanine residues at the end of the N- and C-termini (Figs. 41 and 42) (107). 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 may be competitive with that of Rac1 and Rnd3 (108, 109).

To further test the validity of Kazrin’s interaction with p190B, I performed co-IP experiments from embryo extracts and used pull-down experiments with GST-purified-components, using human p190B as well as the longest available (partial) Xenopus p190B construct (79% sequence identity with human) (Figs. 41 and 42). Following expression in vivo, both full-length human p190B and partial Xp190B construct robustly co-immunoprecipitated with xKazrinA (Fig. 43), and they co-localized to cell:cell borders (Fig. 44). Finally, in a reductionist context, GST-xKazrin
Rho GAP domain

GTPase domain

Kazrin binding region
**Figure 41.** Schematic comparison of Human and *Xenopus* p190B

Identical and similar residues are highlighted in grey. The GTPase domain (brackets), RhoGAP domain (black box), and Kazrin binding domain (shaded box), were suggested based upon published literature for p190A or p190B RhoGAP, or our yeast two-hybrid results.
Figure 42. Schematic comparison of Human p190B (GeneBank ID # NM_001030055) and Xenopus p190B (BC084299.1)

Protein domains were predicted using the SMART website (http://smart.embl-heidelberg.de/). The predicted binding region of Rnd proteins and Kazrin is shown.
A.

\[
\begin{array}{c}
\text{Myc-xKazrinA} \\
\text{HA-x}\beta 2\text{-spectrin} \\
\hline
\text{Lysates} & \text{IgG} & \text{HA} \\
\text{Lysates} & \text{IgG} & \text{HA} \\
\text{Myc-xKazrin} \\
\text{\textasciitilde HA-xp190B} \\
\text{\textasciitilde HA-x}\beta 2\text{-spectrin} \\
\text{IgG}
\end{array}
\]

B.

\[
\begin{array}{c}
\text{Myc-xKazrinA} \\
\text{HA-xp190B} \\
\hline
\text{Lysates} & \text{IgG} & \text{Myc} \\
\text{Lysates} & \text{IgG} & \text{Myc} \\
\text{HA-Hp190B} \\
\text{HA-xp190B} \\
\text{Myc-xKazrin} \\
\text{IgG}
\end{array}
\]
Figure 43. Interaction of xKazrinA with Xp190B

A) *In vivo* binding of xKazrinA to *Xenopus* p190B. The indicated mRNAs were injected into one-cell embryos. Immuno-precipitates of HA-tagged proteins were obtained from early gastrula (stage 10-11) extracts, followed by immuno-blotting for Kazrin (Myc antibody). B) *In vivo* binding of xKazrinA to human p190B. Myc-Kazrin was precipitated from embryo lysates expressing indicated proteins, followed by immuno-blotting for p190Bs.
Figure 44. Co-localization of xKazrinA and Xp190B in blastula ectoderm

Blastula ectoderm (stage 9-10) co-expressing Myc-xKazrinA and HA-Xp190B was isolated, fixed and visualized by confocal fluorescent imaging for Myc and HA.
was co-precipitated with \textit{in vitro} translated p190B suggesting their direct interaction (Fig. 45).

Given their physical association, I asked if the xKazrinA:p190B interaction might be functionally evident in cadherin stability. E-cadherin levels decreased upon Kazrin depletion and were restored by ectopic expression of human p190B RhoGAP (Fig. 46), consistent with the possibility that lowered cadherin levels in Kazrin knock-down embryos might result from abnormal activation of RhoA.

To investigate if xKazrin might facilitate p190B association with xARVCF, I asked if Kazrin expression had an impact upon p190B’s association with xARVCF. As indicated in Fig. 47 (lane 3), a trace but reproducible amount of p190B was detected in a complex with ARVCF only upon Kazrin co-expression. I next turned to \textit{in vitro} pull-down assays to evaluate how p190B might associate with ARVCF (Fig. 48). As expected, based upon the precipitations from \textit{Xenopus} extracts, \textit{in vitro} translated Xp190B was pulled down with MBP-xARVCF in the presence of GST-xKazrinA (lane 3). This suggests that these three proteins form a ternary complex. Even in the absence of xKazrinA, Xp190B appeared to weakly bind xARVCF (lane 1). Thus, while requiring later study, it appears that Kazrin may recruit and/or enhance p190B association with ARVCF. In all cases, our data supports xKazrin’s direct association and functional interaction with Xp190B RhoGAP.
**Figure 45.** *In vitro* binding assay involving xKazrinA and Xp190B

*In vitro* transcribed and translated *Xenopus* p190B RhoGAP protein was subjected to GST pull down using bacterially purified GST-xKazrinA protein. Bacterially purified GST served as a negative control.
Figure 46. Ectopic p190B rescues xE-Cadherin levels, reduced following xKazrin depletion

The indicated morpholinos and mRNAs were injected into one cell embryos. Neurulation (stage 18) extracts were immuno-blotted as indicated.
**Figure 47.** *In vivo* ternary complex formation of xARVCF:xKazrinA:Xp190B

Extracts from gastrula stage embryos expressing the indicated proteins and endogenous xARVCF were precipitated for xARVCF. Co-precipitated xKazrinA and Xp190B were detected using antibodies respectively directed against Myc and HA.
**Figure 48.** *In vitro* ternary protein complex of xARVCF:xKazrinA:Xp190B

*In vitro* transcribed and translated Xp190B was incubated with bacterially purified GST-xKazrinA and MBP-xARVCF. Pull-down of xARVCF occurred using amylose-agarose beads, followed by immuno-blotting as indicated.
Part Four : Developmental Roles of the ARVCF-catenin:Kazrin complex

Function in Ectoderm Integrity

Morpholino depletion of xKazrin results in disruption of tissue integrity

Morpholino-directed Kazrin depletion in early *Xenopus tropicalis* embryos was previously reported to result in ectodermal blistering, shortened body axis, head reductions and somatic defects (78). The phenotypes appearing in *X. laevis* were similar to those previously indicated in *X. tropicalis*. Like embryos injected with standard-control morpholino (Cmo), those injected with xKazrin morpholinos (Kmo-T or Kmo-S) did not produce gross morphological changes through gastrulation (data not shown). However, xKazrin depletion with Kmo-T morpholino (which has the same sequence as the morpholino used in *X. tropicalis*) in *X. laevis* appeared to have a severe impact on the ectoderm, as evident in abnormal neural tube closure during neurulation, and in regional ectoderm shedding in addition to blistering at early tailbud stages (Fig. 49A). The fraction of embryos exhibiting such phenotypes increased in a dose dependent manner (Fig. 49B and Table 2). As I obtained the same results using an independent Kmo-S morpholino in *X. laevis*, the specificity of our phenotype was supported (Fig. 50). During neurulation, embryos micro-injected with Kmo-S began to exhibit varying extents of defects in neural fold closure (Fig. 50A a-a’), neural fold formation (Fig. 50A b-b’), and ectodermal integrity (Fig. 50A c-c’). Overall, greater than 80% of Kmo-S injected embryos (40 or 80 ng doses) displayed these overt phenotypes (Fig. 50B and Table 2).
A. Embryos at the one-cell stage were microinjected with the indicated morpholinos, and embryos were evaluated at neurulation (stage 18) and early tailbud stages (stage 20).

B. Dose-dependent effect of Kmo-T morpholino on ectoderm integrity. Two doses of xKazrin morpholino (Kmo-T) were injected into one-cell stage embryos, and embryos having defective ectoderm were counted at tailbud stages (stages 20-22). Bar graph shows the average value of three independent experiments.

Figure 49. Reduced ectoderm integrity results following xKazrin morpholino-T injection
Figure 50. Reduced ectoderm integrity results following xKazrin morpholino-S injection

A) Stage 19-20 embryos are shown following Kmo-S (a’, b’ and c’) versus standard-control morpholino (a, b, and c) injections into the animal region of one cell stage embryos, or into both cells of two-cell stage embryos. B) Bar graph indicates the percentage of defective embryos following xKazrin depletion.
Table 2. Summary of xKazrin depletion

Xenopus embryos were injected with xKazrin morpholinos at the one cell stage, and scored at stage 18-20 for defects in proper neural tube formation and ectoderm shedding. This table shows the combined results from multiple experiments.
Intriguingly, both in our and others’ hands (78) (data not shown), Kazrin over-expression in *Xenopus* does not generate an obvious phenotype(s). While speculative, it is possible that effects occur but are more effectively compensated for *in vivo* than *in vitro*.

**xARVCF partially rescues embryonic phenotypes resulting from xKazrin depletion.**

To test for an *in vivo* functional interaction between xARVCF and xKazrinA, I attempted to rescue ectodermal shedding through ectopic expression of xARVCF (Fig. 51 and Table 3). As shown in Fig. 51B, Kazrin depletion was partially rescued by an appropriately titrated dose of ectopic xARVCF (dose sub-phenotypic in isolation), consistent with xKazrin:xARVCF functional interactions in maintaining ectodermal integrity.

**xC-cadherin and p190B Rho GAP rescues embryonic phenotypes resulting from xKazrin depletion**

From the study of cellular function in part three, I concluded that the ARVCF-catenin:Kazrin complex modulates RhoA via p190B RhoGAP and subsequent RhoA activity regulates cadherin endocytosis and stability. To evaluate this, I used phenotypic rescue of ectodermal shedding in embryos depleted of xKazrin as a read-out.
Figure 51. Rescue of ectoderm integrity, reduced upon xKazrin depletion, through ectopic expression of xARVCF

A) The indicated morpholinos and mRNAs were co-injected into one cell stage embryos. After vitelline membrane removal, representative tailbud stage embryos were imaged (stages 20-22). B) Graphical depiction of xARVCF rescue of xKazrin depletion effects upon ectoderm integrity. Two concentrations of xARVCF mRNA were used in the rescues, with similar results being observed from four independent experiments.
### Table 3. xARVCF rescue of xKazrin depletion

The indicated mixture of morpholino and RNA was micro-injected into one-cell stage embryos. Then, embryo phenotypes were observed and counted at tailbud stages (stage 20-22). The table represents a summary of multiple independent experiments. Dead embryos without a loss of ectoderm integrity were also included in the total number (N).

<table>
<thead>
<tr>
<th>Injection</th>
<th>Number of embryos</th>
<th># expts.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total (N)</td>
<td>normal</td>
</tr>
<tr>
<td>morpholino RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ng CMO 100 pg β-gal</td>
<td>127</td>
<td>123</td>
</tr>
<tr>
<td>20 ng KMO1 100 pg β-gal</td>
<td>132</td>
<td>26</td>
</tr>
<tr>
<td>20 ng KMO1 20 pg xARVCF + 80 pg β-gal</td>
<td>122</td>
<td>67</td>
</tr>
<tr>
<td>20 ng KMO1 100 pg xARVCF</td>
<td>138</td>
<td>75</td>
</tr>
<tr>
<td>20 ng CMO 100 pg xARVCF</td>
<td>72</td>
<td>69</td>
</tr>
<tr>
<td>uninjected uninjected</td>
<td>94</td>
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</tr>
</tbody>
</table>
Considering the outcome of dissociation/re-aggregation assays (Fig. 34 and 35), together with biochemical evidence (Fig. 31, 32 and 33), the observed reduction of ectoderm integrity (Fig. 49 and 50) is likely due to reduced cadherin levels following xKazrin depletion. Thus, I first tested whether ectopic expression of xC-cadherin rescued this loss of ectoderm integrity after xKazrin depletion. Defective phenotypes were partially rescued upon ectopic expression of an appropriately titrated dose of C-cadherin (sub-phenotypic in isolation) as shown in Fig. 52 and Table 4, supporting the function of ARVCF:Kazrin complex in cadherin stability.

Since RhoA activation might have contributed to the ectoderm fragility we observed following Kazrin depletion, I tested the rescuing capacity of carefully titrated dominant-negative RhoA (versus dominant-active RhoA), upon co-injection with Kazrin morpholino (Kmo-T). In line with RhoA being functionally relevant and possibly downstream of Kazrin, co-injection of DN-RhoA mildly rescued observed phenotypes, while titrated DA-RhoA doses only worsened developmental defects (data not shown).

Finally, 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. 53 and Table 5). These results support the concept that p190B and Kazrin are functionally coupled.
Figure 52. Rescue of ectodermal integrity, reduced upon xKazrin depletion, through expression of xC-Cadherin

Embryos injected with the indicated morpholino and mRNA were assessed for ectoderm shedding at tailbud stages (stage 20-22).
**Table 4.** xC-cadherin rescue of xKazrin depletion

Experiment was performed as described in Fig. 52. Table shows combined results of three independent experiments.
**Figure 53.** Partial rescue of ectoderm integrity, reduced following xKazrin depletion, upon expression of human p190B (Hp190B)

Embryos were injected with the indicated amounts of morpholino and mRNA at the one cell stage. Ectoderm integrity was evaluated at tailbud stage (stage 20-22) in three independent experiments.
### Table 5. Hp190B rescue of xKazrin depletion

Experiment was performed as described in Fig. 53. Table shows the combined results of three independent experiments. Dead embryos without loss of ectoderm integrity were included in the total number (N).

<table>
<thead>
<tr>
<th>Injection</th>
<th>Number of embryos</th>
<th># expts.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total (N)</td>
<td>normal</td>
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<tr>
<td>morpholino RNA</td>
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<td></td>
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<tr>
<td>20 ng Cmo 500 pg β-gal</td>
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<td>99</td>
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<tr>
<td>20 ng Kmo-T 500 pg β-gal</td>
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<td>10</td>
</tr>
<tr>
<td>20 ng Kmo-T 100 pg Hp190B + 400 pg β-gal</td>
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</tr>
<tr>
<td>20 ng Kmo-T 500 pg Hp190B</td>
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</tr>
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<td>20 ng Cmo 500 pg Hp190B</td>
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<td>70</td>
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</table>
**Function in Craniofacial Development**

**xKazrin depletion in the head region induces eye and craniofacial developmental defects**

Whole mount in situ hybridization revealed that Kazrin mRNA is expressed in the head region of tailbud embryos (Fig. 8) and comparable amounts of various Kazrin isoform proteins are detected in adult brain tissue (Fig. 7). Together with earlier cloning of Kazrin from a mouse brain cDNA library, these results suggested Kazrin’s specific role during head or brain development (72). To address this possibility, xKazrin was specifically depleted in the future head region by injecting xKazrin morpholinos (Kmo-T and Kmo-S) into the animal region of one dorsal blastomere at the four cell stage. As shown in the bottom panels of Fig. 54, the eye was smaller in the xKazrin-depleted side (right side) and in addition, the head axis was distorted to the right side. Careful observation suggested that the axis kink was due to a smaller head size in the xKazrin morpholino-injected side. This effect was specific to dorsal animal depletion compared to ventral vegetal depletion as a control (Fig. 55). The specificity of xKazrin depletion was demonstrated by the rescue of defects using ectopic expression of xKazrinA (Fig. 56 and Table 6).

**xARVCF depletion in the head region induces eye and craniofacial developmental defects**

Since xARVCF is a binding partner of xKazrin and since specific Xp120-catenin depletion in the head region was reported to result in eye and craniofacial
**Figure 54.** Eye and craniofacial defects after xKazrin or xARVCF depletion in the dorsal animal region

xKazrin or xARVCF morpholinos were micro-injected into the animal region of one dorsal blastomere at the four cell stage. Embryos were fixed with MEMFA buffer and pictures were taken at the tadpole stage (stage 43). The white dotted line indicates the middle of the embryo head. Injected sides were stained pink due to co-injection of β-gal RNA, which was detected by Red-gal staining.
Figure 55. Injection region specific effects of xKazrin depletion

Kmo-S morpholino was micro-injected into either the animal region of one dorsal blastomere or the vegetal region of one ventral blastomere. Pictures of the embryos were captured at the tadpole stage.
**Figure 56.** xKazrinA rescues craniofacial defects after xKazrin depletion

*In vitro* transcribed xKazrinA mRNA was co-injected with Kmo-S morpholino into the dorsal animal region of four cell stage embryos. Embryo phenotypes were investigated and counted at the tadpole stage (stage 43).
Table 6. Rescue of craniofacial defects from xKazrin depletion with ectopic expression of xKazrinA

Experiments were performed as described in Fig. 56.

<table>
<thead>
<tr>
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<th>Number of embryos</th>
<th></th>
<th></th>
<th># expts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>morpholino</td>
<td>RNA</td>
<td>total (N)</td>
<td>normal</td>
<td>craniofacial defects</td>
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<td>500 pg myc-xKazrinA</td>
<td>46</td>
<td>21</td>
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</tr>
</tbody>
</table>
defects similar to xKazrin depletion (110), the effects of xARVCF depletion in head region was tested in parallel (Fig. 54). As expected, xARVCF depletion also induced eye and craniofacial defects, suggesting shared role of xARVCF and xKazrin during Xenopus head development.

**xARVCF partially rescues the eye and craniofacial defects from xKazrin depletion**

To test the functional interplay between xARVCF and xKazrin during Xenopus eye and craniofacial development, I performed rescue experiments with sub-phenotypic levels of xARVCF (Fig. 57). Notably, xARVCF can rescue eye and craniofacial defects as much as similar amounts of xKazrinA (Fig. 57B and Table 7), implying that the xARVCF-catenin:Kazrin complex plays a role in Xenopus head development.

**xKazrin and xARVCF depletion induces malformation of the craniofacial cartilage**

To check craniofacial defects of abnormally developed Xenopus skulls after xARVCF-catenin or xKazrin depletion, cartilage from embryos that were injected with each morpholino were investigated after alcian blue staining. As shown in Fig. 58, depletion of either xARVCF or xKazrin by morpholino induced underdevelopment of both ceratohyal cartilage and ceratobranchial cartilage as compared to the normal cartilage seen in the un-injected side.
**Figure 57.** xARVCF as well as xKazrinA partially rescue craniofacial defects from xKazrin depletion

Mixtures of the indicated morpholinos and *in vitro* transcribed RNA were micro-injected into the animal region of a one dorsal blastomere at the four cell stage. Embryos were allowed to develop at 18°C until the tadpole stage after which the phenotypes were observed and counted. Pictures were taken of representative embryos (A) and results are graphically presented in B.
Table 7. Rescue of the craniofacial defects from xKazrin depletion with ectopic expression of xARVCF and xKazrinA

Experiment was performed as described in Fig 57.
Figure 58. Developmental defects of *Xenopus* skull cartilage after xKazrin or xARVCF depletion

After embryos were injected with the indicated morpholinos at the four cell stage, tadpoles (stage 43) were fixed and cartilage was stained with Alcian blue dye. Asterisks (*) indicate the morpholino-injected side. CH: ceratohyal cartilage; CB: ceratobranchial cartilage.
xKazrin or xARVCF depletion decreased detection of migrating neural crest cells

Since the craniofacial skeleton originates from neural crest cells (NCC) (111), it is possible that xARVCF-catenin:xKazrin complex play a role in NCC development. As expected, whole mount in situ hybridization with twist neural crest marker revealed that depletion of xKazrin or xARVCF decreased all three streams of NCC; branchial, hyoid and mandibular NCCs (Fig. 59). This result is consistent with the developmental defects of ceratohyal cartilage and ceratobranchial cartilage, which originate from hyoid NCCs and brachial NCCs, respectively (Fig. 58). A defect of Meckel’s cartilage from mandibular NCCs was also observed in the xKazrin or xARVCF depleted side. Interestingly, it was previously reported that abnormal activation of RhoA inhibits NCC establishment in *Xenopus* (112). Therefore, ARVCF-catenin:Kazrin complex may affect NCC establishment by modulating RhoGTPase activity.
Figure 59. *Twist* in situ hybridization after xKazrin- or xARVCF-depletion

The indicated morpholinos were injected into the animal region of one dorsal blastomere at the four cell stage. At the neurula stage (stage 19) embryos were fixed and neural crest cells expressing *twist* mRNA were visualized using whole mount in situ hybridization. Asterisks (*) indicate the injected side. Br: Brachial neural crest cells; H: Hyoid neural crest cells; M: mandibular neural crest cells.
CHAPTER III

DISCUSSION
In my study, I have evaluated the expression of xKazrinA, the subcellular localization and developmental roles of the xARVCF-catenin:xKazrinA complex. Distinct from reported protein interactions of ARVCF or KazrinA at the adherens junction or desmosome, I found that KazrinA associates with spectrin. Spectrin forms a cytoskeletal network that includes interactions with junctional constituents (schematic Fig. 60) (92, 97). While requiring further study, Kazrin appears to enhance p190B RhoGAP association with ARVCF-catenin. In any case, Kazrin’s modulation of RhoA, likely occurring in part via p190 RhoGAP, is relevant (based upon rescue analysis) to xKazrin-depletion effects in *Xenopus* embryos. In early amphibian development, I also observe that Kazrin depletion results in actin reorganization and reductions in cadherin levels and ectoderm integrity (modeled in Fig. 60). Finally, I find that xKazrin as well as xKazrinA play important roles in neural crest cell development and subsequent craniofacial development. My results form an initial outline of Kazrin’s role in the context of catenins, RhoA and p190B RhoGAP.

**Kazrin isoforms, expression and structure**

*Xenopus* Kazrin and its well-conserved vertebrate homologs are likely to represent a new protein group. Kazrin was originally identified as a cDNA (KIAA1026) expressed in human brain (72). In human keratinocytes, five Kazrin splice isoforms (A-E) were then reported, along with biochemical interactions and functional effects upon keratinocyte differentiation, small GTPases and actin or microtubule networks (73, 74, 77). Adding to Kazrin’s functional complexity is KazrinF, recently reported to
Figure 60. Working model of xARVCF:xKazrinA complex localization and function at the plasma membrane.

Kazrin facilitates formation of the trimeric ARVCF:p190B RhoGAP complex, which localizes to the plasma membrane via association of Kazrin with spectrin. The p190B (and p190A) RhoGAP complex reduces RhoA activity locally in cortical/junctional regions, thereby stabilizing the actin cytoskeletal structure supporting cell-cell contacts. An interaction of p120 with p190A RhoGAP at adheres junctions was previously reported (105).
inhibit apoptosis through binding BAX (Bcl-2-associated X protein) and ARC (apoptosis repressor with caspase recruitment domain) in a human glioma cell line (75). We recently found that the original Kazrin cDNA isolated, KIAA1026, that we named it KazrinK, lacks the nuclear localization signal (NLS) sequence present in other isoforms. In Xenopus, our lab cloned the xKazrinA isoform from X. laevis, finding it highly homologous to human KazrinA, with 81% amino acid identity. Interestingly, the X. tropicalis KazrinA isoform is almost identical to that of X. laevis, except for 28 additional amino acids in the middle of coiled-coil region (Fig. 4). In my study, I found that X. laevis expresses both forms of xKazrinA (similar to human KazrinA), and named the longer form (similar to X. tropicalis KazrinA) as xKazrinB (Fig. 5). It is notable that the xKazrinB mRNA is maternally loaded (present in oocytes), and is thus more abundant in very early development, while xKazrinA shows a slightly later zygotic expression pattern (initiation coinciding with the mid-blastula transition) (Fig. 6A). In adult tissues, xKazrinA is expressed ubiquitously whereas xKazrinB is present in more restricted contexts (Fig. 7A). Even as the functional differences of Kazrin isoforms are not yet known, these results suggest that xKazrinB may predominantly function during early development and in limited adult contexts, while xKazrinA operates slightly later in development and rather widely in the adult. So far, other isoforms of Kazrin have not been identified or cloned in Xenopus. However, my immuno-blotting using an antibody raised against the carboxy terminal 147 amino acids of xKazrinA, indicated the potential existence of KazrinC/D, and the SAM domain containing KazrinE, in Xenopus embryo and adult tissues (Fig. 6B and 7B). To further analyze the presence of such isoforms at
the mRNA and protein levels, RT-PCR primers and antibodies specific for each isoform need to be generated and applied. At present, we don’t understand why the xKazrinA and xKazrinB proteins are not resolved from one another in my immuno-blots, or why xKazrinB appears to migrate on SDS-PAGE gels at the size of xKazrinA. To address these questions, we need to ectopically express both xKazrinA and xKazrinB and compare their migration. Also, it is possible that an unknown alternative splice variant of xKazrin reduces what we have assumed is xKazrinB to a size approaching that of xKazrinA.

Temperally, *Xenopus* Kazrin RNA and protein are expressed from the oocyte through adult-tissue stages, leaving open the possibility that Kazrin may continuously be needed for vertebrate life (Figs. 6 and 7). During embryonic development, Kazrin RNA is most prominently localized in the animal region of one-cell stage embryos, in the anterior and dorsal parts of neurula embryos, and in the head region of tadpoles (Fig. 8). This expression pattern coincides with the developmental defects we observe upon xKazrin depletion in vivo, which includes abnormal neural fold and craniofacial development (Figs. 49, 50 and 54). As noted, an unsolved question is why xKazrinA and xKazrinB are not resolved from one another following immuno-blotting (Fig. 6B).

Structural prediction of the Kazrin protein using the “ScanProsite” proteomics server (http://expasy.org/tools/scanprosite/) indicated the presence of an amino-terminal leucine zipper motif and central coiled-coil region (Fig. 3). Five helices were predicted in the coiled-coil sequence (Fig. 9). Functionally, the coiled-coil domain appears to be a binding module for other proteins such as envoplakin.
and periplakin (73), with our previous work additionally indicating the binding of ARVCF-catenin. In the future, it will be interesting to determine which helix (or helices) out of the five predicted engages in each of Kazrin’s protein-protein interactions. Additionally, I demonstrated that the coiled-coil region is required for the homo-dimerization of xKazrinA (Fig. 11). At present, it is not clear whether Kazrin forms a dimer and/or higher oligomers. By running non-denaturing native gels of bacterially-purified Kazrin protein, one may predict how many Kazrin molecule form a complex. Notably, Kazrin’s coiled-coil structure seems to be highly labile to protein degradation based on the results shown in Fig. 11, in which deletion of either Kazrin’s N- or C-terminal region decreased protein stability dramatically, while deletion of its coiled-coil region has no effect on stability. This result also suggests that both the N-terminal and C-terminal regions may work together to protect the coiled-coil domain. On the contrary, intact Kazrin protein is expected to be stable, because whereas Kazrin morpholino efficiently blocked Kazrin protein translation in vitro (Fig. 29A), a detectable decrease of endogenous Kazrin protein levels following Kazrin morpholino injection was not observed until neurula stages (Figs. 29B and 31). This indicates that the maternally loaded Kazrin protein is stable enough to survive until neurula stages. Thus, it will be interesting to study how Kazrin protein stability is regulated. One speculation is the post-translational modification of Kazrin. Conceivably, for example, phosphorylation may lead to the opening/ increased access of the coiled-coil domain, and Kazrin degradation.
Subcellular localization of the xARVCF: xKazrinA complex

In common with the related p120- and delta-catenins, ARVCF is an established component of adherens junctions associating with cadherin membrane-proximal regions (41, 81, 82). My tests indicate, however, that KazrinA is not associated with cadherin complexes (Figs. 14, 15 and 19).

Kazrin is reported to directly bind periplakin and envoplakin, proteins present at peripheral desmosomal or inter-desmosomal regions (73). In cornified epithelia and other primary and cultured cell types, Kazrin was visualized at plasma-membrane areas containing desmosomal and intermediate filament proteins, extra-desmosomal areas enriched in cortical actin, and partially within cytoplasmic and nuclear compartments (73). In considering if ARVCF might localize to desmosomes in addition to adherens junctions, we considered the case of p0071-catenin, which likewise belongs to the p120 sub-family, and has been reported at both types of junctions (17, 19, 113). However, my experiments using mammalian cell or Xenopus embryo extracts, do not support ARVCF’s endogenous association with desmoglein 1 (Figs. 16 and 17). Furthermore, mouse desmoglein 3 and Xenopus ARVCF, over-expressed in Xenopus embryos, did not appear to interact (Fig. 18). This suggests that the ARVCF: KazrinA complex is not likely to be a core component of desmosomal junctions.

Yeast two-hybrid 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 sub-domains, transduction of nerve action potentials, and
contraction of heart cells (92, 114). In this study, I found that the xARVCF:xKazrinA
complex associates with xβ2-spectrin (Figs. 25 and 27), suggesting a novel
interaction and localization of Kazrin and, by association, ARVCF-catenin.
Previously, it was reported that the spectrin network associates with E-cadherin via
ankyrin, contributing to lateral cell membrane integrity (97). I, however, found
neither C-cadherin nor p120 co-precipitating with β2-spectrin, despite significant
homology within the cyto-domains of Xenopus E- and C-cadherin (80.5% identity)
(Fig. 28). Possibly, cadherins such as C-cadherin form lesser interactions with the
spectrin cytoskeleton in developmentally transitioning embryonic tissues.

It has been thought that the membrane localization of p120-catenin depends
upon its association with cadherins in junctional areas. However, my study suggests
that the ARVCF-catenin may additionally locate to the plasma membrane by binding
to the spectrin cytoskeleton via Kazrin. Our yeast two hybrid screening, and
preliminary in vitro binding data, showed that other p120-catenin subfamily proteins,
namely delta-catenin and p0071, also bind to Kazrin. This implies that Kazrin
mediated membrane localization may be a shared mechanism among p120-catenin
subfamily members (Table 1 and Fig. 61). The decrease of ARVCF at the
membrane, with increased ARVCF in the cytosol following Kazrin depletion
supports this idea (Fig. 22), although I cannot exclude the possibility that decreased
cadherin levels after Kazrin depletion contributed or was responsible for the
cytoplasmic localization of ARVCF.
Figure 61. In vitro binding of *Xenopus* p120 sub-family catenins to xKazrinA.

In vitro transcribed and translated *Xenopus* p120 sub-family catenins (HA-tagged) were tested for association with GST-xKazrinA following GST pull-down and immuno-blotting as indicated. Positive and negative controls respectively included xARVCF- and Xp120-catenins. In agreement with our yeast two-hybrid assays, xARVCF, xdelta (δ)-catenin and Xp0071-catenin bound xKazrinA, whereas Xp120 did not. This suggests that Kazrin’s biochemical and presumably functional interactions involve some but not all p120 sub-family catenins.
Another interesting question is the interplay that might occur between the two ARVCF distinct complexes suggested from my findings, namely the ARVCF:cadherin and ARVCF:Kazrin complexes. Surprisingly, two competition assays (Figs. 20 and 21) demonstrated that cadherins and Kazrin do not compete for binding to ARVCF, even though the two complexes are separable. This implies that a “free pool” of cytosolic ARVCF is able to move to either to cadherins at adherens junctions and/or to the spectrin cytoskeleton where Kazrin is present, and that other unknown factors must allow the two ARVCF complexes to remain biochemically distinct, even if they reside in proximity at cell-cell contact regions.

Under chemical cross-linking conditions, I observed increased cadherin:ARVCF binding as expected, but with reduced Kazrin:ARVCF association. Although further study is needed, I speculate this is due to ARVCF becoming covalently attached to more prevalent interacting proteins, or to those having higher affinity interactions (possibly including cadherin, PAPIN, ZO-1 and 2, FRMPD2, Vav2 and Erbin). Proteins binding weakly to ARVCF, such as Kazrin, might thus exhibit reduced interaction following cross-linking. Other possibilities are that the chemical modifications involved in cross-linking block ARVCF:Kazrin interactions from occurring, such that the loss of their association is more evident than any gains obtained from covalently linking existing complexes of the two proteins together.

Cellular roles of the xARVCF:xKazrin complex

My study in Xenopus, and previous reports in human keratinocytes resolved Kazrin localization to cell:cell borders (73, 77). Earlier work predicted Kazrin’s
involvement in clathrin mediated endocytosis (76), which is known to contribute to
cadherin internalization and thereby the modulation of junction function (55, 58, 60,
115-118). Perhaps related are reports that p120 sub-family members, such as
p120, ARVCF and delta-catenin, protect cadherins from endocytosis and lysosomal
degradation, and may also chaperone cadherins to the plasma membrane (21-25,
60, 71, 119-121). I speculate that Kazrin’s role in endocytosis, or the role of the
ARVCF:Kazrin complex, may 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 modulate cadherin endocytosis at the molecular level.
Endocytosis requires cortical actin to be locally reorganized, with such changes
involving the actions of small GTPases (69, 122). Multiple groups have reported
Rho and Rac (and Cdc42) modulation by p120 sub-family catenins including
ARVCF (22, 26, 61-63). Kazrin is similarly implicated in RhoA modulation (77). My
results point to negative xARVCF and xKazrin effects upon RhoA activity (Fig. 38),
with the actin network becoming reorganized upon Kazrin depletion in vivo (Fig. 39).
Requiring future study, I speculate that the ARVCF:Kazrin complex modulates
RhoA activity at cell contacts, having an impact upon actin organization and thereby
cadherin stability/endocytosis. Interestingly, Charrasse et al. found that
constitutively active RhoA induces M-cadherin endocytosis and degradation
(following p120-catenin dissociation from M-cadherin) (99). Although there is a
discrepancy between my results and theirs regarding p120 (Kazrin knock-down in
Xenopus embryos decreased catenin protein levels, whereas RhoA activation in
mouse myoblasts does not change endogenous p120 protein levels), both groups find that altered RhoA activity affects cadherin levels.

We identified p190B RhoGAP, ubiquitously expressed in mammals, as a novel binding partner of xKazrinA (Figs. 43 and 44). Since p190A RhoGAP interacts with p120-catenin (105, 106), and Xp190B with xARVCF (our results), p190 RhoGAP members may be core RhoA effectors of the p120 subfamily proteins (or vice versa). This view may also relate to an earlier report implicating p190 RhoGAP in RhoA inhibition upon cadherin engagement (123). While I detected only weak endogenous association of Xp190B RhoGAP with xARVCF in vivo (Fig. 47), their interaction might be regulated, conceivably in response to Rac1, Rnd proteins or tyrosine phosphorylation of p190B by Src or Arg (Abl related gene) kinase (108, 109, 124, 125).

**Developmental roles of the xARVCF:xKazrin complex**

I resolved two distinct developmental phenotypes relating to the xARVCF:xKazrin complex following Kazrin depletion. General Kazrin knock-down by injecting the morpholino at the one-cell stage induced abnormal dorsal structures and ectoderm shedding at neurula stages, whereas dorso-anterior (e.g. future head/brain region) depletion induced eye and craniofacial defects at tadpole stages. My results appear partially consistent with a previous report of Kazrin depletion in X. tropicalis. Sevilla et al. showed that Kazrin depletion (one-cell stage injections) in X. tropicalis embryos resulted in defects involving axis elongation, the eye and head, epidermal integrity and cardiac edema (78). Axis defects and cardiac edema were
not obvious in my study using *X. laevis*. Instead, I observed defects in dorsal epithelial closure after neural tube formation and the subsequent leakage of inner cells. Also, the loss of epidermal integrity following Kazrin morpholino injection at the one-cell stage was so severe in *X. laevis* that the embryos did not progress to tadpole stages when eye and head defects could be observed. At this time, I cannot readily explain the observed differences in Kazrin’s developmental phenotypes in these two closely related species. It should be pointed out that I used twice the dose of morpholino (20 ng) in my study, because lower doses (10 ng) did not induce a phenotype. Of note, axial elongation defects and cardiac edema are developmental defects generally originating in the mesoderm. Relative to the *X. tropicalis* study, it is possible that I did not deplete *X. laevis* Kazrin as effectively in the mesoderm due to differences in my injection methods (e.g. injection location or depth). To test this possibility, I could deplete Kazrin in a more directed fashion in mesodermal regions. Also, the more ectoderm-directed Kazrin depletion may help explain why there was not a greater than a 30-40% decrease in overall Kazrin protein level (Figs. 29 and 31), the large rearrangement of ectodermal filamentous actin at blastular stages (Fig. 39), and graphic defects in dorsal neural fold and epidermal integrity (Figs. 49 and 50), which originate from ectoderm.

During neural tube formation, ectoderm tissue on each longitudinal side of the dorsal midline first form a neural fold that moves towards the midline, makes contact and attaches. In my study, Kazrin depletion (morpholino injecting into one-cell stage embryos), resulted in defects in dorsal midline sealing of the neural folds, leading to cell dissociation/escape from the unsealed region (Figs. 49 and 50). This
phenotype appears is in keeping with my in situ hybridization showing that Kazrin mRNA is normally locally enriched within the neural fold region at these developmental stages (Fig. 8B). I found that ectopic expression of cadherin partially rescued such abnormalities following Kazrin depletion (Fig. 52). Taken together, this suggests that Kazrin plays a role in neural fold closure (dorsal epithelial closure after neural tube formation), perhaps via effects upon cadherin levels.

As mentioned earlier, Kazrin depletion in X. tropicalis gave rise to defects in eye and head development (78). Similarly, specific depletion of Kazrin (or ARVCF) in the future head region produced small head and eye phenotypes in X. laevis (about 90% after 20ng Kmo-S injection) (Figs. 54 and 56). Looking at later embryonic stages, my findings indicated that xKazrin and xARVCF are needed for craniofacial development, apparently as a consequence of their roles in neural crest cells (NCC). Neural crest is often described in three stages, neural crest cell establishment, migration and differentiation. Previous reports have demonstrated that RhoA participates in neural crest cell establishment (112), while my findings support Kazrin and ARVCF modulation of RhoA. Thus, the ARVCF:Kazrin complex may be involved in neural crest cell establishment. It is also conceivable that the xARVCF:xKazrin complex regulates migration of neural crest cells. For example, Xenopus cadherin-11 is required for neural crest cell migration (126), and in human cancer cells (MDA231 and UMRC3), cadherin-11 mediated cell motility depends upon association of p120-catenin and p120-mediated RhoA inhibition (65). To clarify which stage(s) of neural crest formation require Kazrin, further studies are
needed, such as more extensive in situ analysis of neural crest markers (slug, snail, sox9 and etc.), as well as neural crest cell migration assays.

Technically, Kazrin or ARVCF morpholino was injected into the dorsal animal region of four cell stage embryos. However, in addition to later neural contributions, this region contributes to other tissues such as the epidermis, somites and notochord. To improve upon the specificity of targeting of Kazrin depletion to the future head region and to neural crest cells, the appropriate morpholinos need to be injected into head-fated blastomeres at the 16-cell or 32-cell stages.

Of note, our evaluation of neurula embryo sections (collaboration with Dr. Malgorzata Kloc, Methodist), demonstrated that the size of the neural tube is reduced after Kazrin depletion using Kmo-S (data not shown). This suggests another possible cause of eye and craniofacial defects. That is, a decrease in neural tissue upon Kazrin depletion may produce small eyes, a decreased number of neural crest cells and thereby subsequent cranial defects. It would be informative to score for defects in neural tube formation resulting from Kazrin depletion versus ARVCF depletion (Kmo-S or T, or ARVCF morpholinos), evaluating changes in neural tube size. Furthermore, Brdu (Bromodeoxyuridine) staining, or phospho Histone H3 staining of cross-sectioned neurula embryos, will assist in determining if there is a decrease in neural cell proliferation in response to Kazrin depletion, potentially contributing to reduced size of the neural tube.
**Concluding remarks**

My work indicates that Kazrin is a novel modulator of cadherin stability and Rho GTPase, likely occurring via its interaction with ARVCF-catenin and with p190B RhoGAP. My findings also suggest a new means by which ARVCF associates with junctional or cortical regions, and that is via Kazrin’s association with the spectrin cytoskeleton. In keeping with a prior study (78), I found that xKazrin is essential for ectoderm integrity and craniofacial development, with my rescue analysis extending Kazrin’s known functional interactions with xARVCF, cadherin and RhoA.

*Xenopus* Kazrin’s role in determining cadherin stability via RhoA modulation is in line with previous reports showing that Kazrin over-expression inhibits clathrin-mediated endocytosis (76), and that Kazrin inhibits RhoA in human keratinocytes (77). Generally, however, there has been limited knowledge regarding Kazrin’s roles. Recently, Kazrin was found to be involved in keratinocyte differentiation and the cornified envelope formation of skin (73, 77). Here, my work suggests that Kazrin plays a role in cadherin-mediated cell-cell adhesion through interactions with p120-catenin subfamily members. With respect to such p120 subfamily proteins, classical cadherins have been considered as the sole plasma membrane “anchors”. However, my results raise an alternative possibility of membrane-localization via association with the spectrin cytoskeletal network. This will be discussed more in the following Future Directions.

p120-catenin has been proposed to modulate RhoA in three manners: 1) p120 directly binds to RhoA and inhibits GDP dissociation as a RhoGDI (Rho GDP Dissociation Inhibitor) (61); 2) p120 activates Rac1 via Vav2 RhoGEF (Rho
Guanidine nucleotide Exchange Factor) and activated Rac1 indirectly inhibits RhoA activity (62); and 3) p120 binds to p190A RhoGAP (Rho GTPase Activating Protein), and membrane-localized p190A inhibits RhoA locally (105). My results indicate that members of the *Xenopus* p120-catenin subfamily (excluding p120 itself) inhibit RhoA via p190B RhoGAP, supporting the role of p190 RhoGAP family members in p120-catenin subfamily-mediated RhoA inhibition.

A remaining question is if/how Kazrin itself is regulated. Our study identified several novel binding partners of Kazrin including ARVCF-catenin, delta-catenin, p0071, spectrin and p190B RhoGAP. I propose that Kazrin is a scaffolding protein, enabling other proteins to interact. For example, ARVCF is complexed with spectrin via Kazrin, and thereby with the spectrin-actin cytoskeleton. However, it is unlikely that Kazrin binds to all of its binding proteins at the same time. Thus, a question is how the various possible interactions of Kazrin are regulated. It is conceivable that post-translational modifications may regulate Kazrin’s binding. It will be interesting to identify upstream signals that have an impact upon Kazrin and its interaction with other proteins.

Overall, my study has examined novel cellular and developmental roles of Kazrin, and broadens our understanding of p120-catenin subfamily proteins.
CHAPTER IV

FUTURE DIRECTIONS
Interaction between p120-catenin subfamily members and Kazrin in mammalian cells

While my studies focused upon *Xenopus* (amphibian) Kazrin, it would be expected that mammalian Kazrin binds to mammalian p120-catenin subfamily proteins such as ARVCF and delta-catenin (but not p120 itself) given that Kazrin is highly conserved between human and *Xenopus* (81% amino acid identity). Our preliminary immuno-precipitation results show that *Xenopus* ARVCF binds to human KazrinK and E isoforms (data not shown), and based upon our yeast two-hybrid screen, that *Xenopus* Kazrin may further bind to mouse delta-catenin and p0071. Interactions between the mammalian homologues of Kazrin and p120-catenin subfamily members need to be tested by using co-immunoprecipitation (etc.) approaches from mammalian cell lines or tissues. Using mammalian systems may also provide benefits in overcoming difficulties I confronted using *Xenopus*. These will be discussed further below.

Localization of endogenous Kazrin protein

Our currently available antibodies directed against Kazrin did not reliably detect endogenous *Xenopus* Kazrin protein in immuno-fluorescence experiments. For example, my attempts using two rabbit polyclonal antibodies directed against human Kazrin, or three rabbit polyclonal antibodies directed against *Xenopus* Kazrin, failed to detect endogenous Kazrin in blastula ectodermal tissues. Thus, my co-localization studies were performed with exogenously expressed Kazrin. For this reason, it is possible that the signals I obtained did not accurately reflect the
localization of endogenous Kazrin. If the interaction between endogenous mammalian Kazrin and mammalian ARVCF is verified, co-localization of endogenous Kazrin and ARVCF (or other p120-catenin subfamily members) can be tested via immuno-fluorescence in mammalian cell lines and tissues. Also, using immuno-gold electron microscopy, the localization of Kazrin to p120-catenin subfamily members such as ARVCF (to adherens or desmosomal junctions), can be studied further.

**P120-catenin subfamily localization in relation to the spectrin cytoskeleton**

It has generally been thought that p120 subfamily members localize to junctional areas via their association with cadherins. Interestingly, however, my study suggests that p120-catenin (and perhaps delta-catenin and p0071) indirectly interact with the spectrin cytoskeleton via Kazrin, thus becoming localized to plasma membrane areas independently of cadherins. To address this possibility, the effect of spectrin knock-down or knock-out on the membrane localization of p120-catenin subfamily members needs to be tested. It would be technically difficult to knock-down spectrin using a morpholino approach in *Xenopus*, because information regarding the spectrin isoforms, genomic sequence and expression pattern is presently not fully available. Alternatively, spectrin knock-out mice, or derived knock-out (or knock-down) cell lines could be used in studies of spectrin’s role in p120-subfamily membrane localization. For this approach, β2-spectrin would be a reasonable target for knock-out (or knock-down) because this spectrin is expressed
ubiquitously and also known to localize to the lateral membrane of epithelial cells similar to catenin proteins (97).

**Kazrin in human tissues**

Work by others has shown that Kazrin participates in human keratinocyte differentiation and cornified envelope formation via RhoA modulation and binding to envoplakin and periplakin (73, 77). However, an RNA expression study showed that Kazrin RNA is expressed in most human tissues, implying Kazrin’s general and/or diverse functions in various tissues. Since envoplakin and periplakin are thought to be skin specific proteins, they are not likely to represent a general interaction of Kazrin. On the other hand, p120-catenin subfamily members are expressed ubiquitously in human tissues, with the exception of the more neural specific delta-catenin (as an interesting aside, delta-catenin is much more widely expressed in *Xenopus*). Thus, it is plausible that Kazrin forms a more general complex with p120-catenin subfamily proteins (excluding p120 itself). To address this possibility, the association of Kazrin with other p120-catenin subfamily members needs to be performed using extracts from varied mammalian cell lines or tissues.

I propose here that one molecular function of the ARVCF (and perhaps delta-catenin and p0071):Kazrin complex is to inhibit RhoA activity. However, this must take into consideration findings that p120-catenin modulates Rho GTPase activity differently depending on cell context. For instance, p120 knock-down in MCF-7 cells increases Rac1 activity whereas it reduces it in MDA231 cells (64). Because I used extracts from whole *Xenopus* embryos for the RhoA activity assays, my results
reflect the net effects of Kazrin and the ARVCF:Kazrin complex on the modulation of RhoA activity. Thus, it is possible that this modulation occurs with opposing outcomes in differing tissues. To obtain a better over-view on the roles of p120-catenin subfamily members, and of Kazrin, in small GTPase modulation, RhoA and Rac1 activity assays could be performed across cell lines derived from different tissues, or from extracts from different tissues, after depletion of Kazrin or p120-catenin subfamily members. Perhaps also the use of in vivo reporters of RhoA and Rac1 activity would prove useful (127).

**Binding specificity of Kazrin**

A number of proteins have been shown to directly bind ARVCF-catenin. These include cadherins, RhoA, Vav2, ERBIN, FRMPD2, and ZO-1 and 2 (22, 41-44, 62, 81, 82, 128, 129). The latter four bind the PDZ motif present in the carboxy-terminus of ARVCF, delta-catenin and p0071. Our 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 likely other catenins), encompassing portions of the central Armadillo and carboxy-terminal domains. In turn, Kazrin’s extended coiled-coil domain associates with ARVCF, such that each protein’s coiled-coil regions presumably interact. Since the Armadillo domain of ARVCF exhibits some homology with p120-catenin (55% identity/ 74.4% similarity) (40), it may prove interesting to resolve the basis of specificity among different p120 subfamily proteins.
Thus far, it seems that the coiled-coil domain is a protein-protein interaction module of Kazrin since it binds enoplakin, periplakin as well as ARVCF with this domain. In addition, my study showed that Kazrin also dimerizes via the coiled-coil domain. Remaining questions include whether other novel binding proteins, spectrins and p190B RhoGAP, interact with the coiled-coil domain of Kazrin. Binding site mapping with Kazrin deletion mutants, and ultimately point mutants (possibly resolved via alanine scanning) will be useful to address this question.

As shown in Figure 9, Kazrin is predicted to have six helices, five of which are located in the putative coiled-coil domain. We would very much like to solve the crystal structure of Kazrin (e.g. in collaboration with Dr. Richard Brennan), to better test our prediction. From this structural information, especially if Kazrin were co-crystalized with ARVCF and other binding partners, we would obtain a deeper understanding of the binding specificity of Kazrin (and of ARVCF).

**Nuclear roles for the ARVCF-catenin:Kazrin complex**

We find that most Kazrin isoforms possess a defined functional NLS, and reside in both the nucleus and cytoplasm, as well as at cell-cell borders. However, Kazrin’s nuclear functions are uncertain. We previously determined that an NLS point mutant of Kazrin is excluded from the nucleus. One way to test the importance of the nuclear function of Kazrin will be compare the capacity of wild type Kazrin versus the NLS mutant in rescuing phenotypes arising from endogenous Kazrin depletion. For example, as shown in Fig. 56, wild type Kazrin can partilly rescue
Kazrin-depletion eye and craniofacial defects. Thus, one can test whether the NLS mutant Kazrin succeeds or fails to rescue this phenotype as well.

Kazrin has been observed by others at the meiotic apparatus in unfertilized mouse eggs, with its localization changing following egg activation (79). In human keratinocytes, the liprin-like KazrinE isoform was found to associate with stable acetylated microtubules (74). Kazrin further binds plakin proteins in complex with intermediate filaments (73), and thus may conceivably associate with nuclear lamins. Nuclear lamin is a major component of a proteinous mesh/network called the nuclear lamina. It is known that nuclear lamins and their associated proteins are involved in transcription through regulating the availability of transcription factors playing roles in signal transduction pathways (e.g. MAPK, Wnt/beta-catenin, TGF-beta and Notch) (130). Interestingly, several transcription factors were found as putative interacting partners of xKazrinA from our yeast two-hybrid assay (results not shown). Thus, it can be postulated that Kazrin may associate with transcription factors near or at the nuclear lamina. Post-translational modification (e.g. phosphorylation) of such transcription factors by upstream signals may decrease transcription factor association with Kazrin, release them from the nuclear lamina to function in the nucleoplasm and more directly modulate gene expression.

My and a prior study indicate that Kazrin affects the actin cytoskeleton via RhoA modulation (77), speculatively providing further means through which Kazrin might modulate gene activity. First, RhoA activates signal transduction pathways including JNK (Jun N-terminal Kinase), p38 MAP kinase and the NF-kB pathway, up-regulating gene targets (131). Thus, by regulating RhoA, Kazrin may affect gene
programs. Second, the spatial organization of chromosomes is known to be important for correct gene expression. For example, transcriptionally inactive heterochromatin is localized to perinuclear and perinucleolar regions. When activated, gene regions are looped-out and positioned near transcriptionally active nuclear compartments such as cajal bodies (132). It is known that nuclear actin and myosin play roles in chromosomal movements (133, 134). Interestingly, a previous report suggested that Kazrin binds to coilin, a nuclear protein enriched in cajal bodies where snRNPs are involved in RNA splicing, and are modified and assembled (135). Thus, it is conceivable that Kazrin may affect transcriptional regulation via RhoA GTPase, or other protein partners.

ARVCF further localizes to varied compartments including the nucleus (41, 82, 136). Given that beta-catenin directly binds transcriptional modulators (e.g. TCF/LEF to activate genes within the canonical Wnt pathway), as does p120 (27, 29, 31), it will be interesting to discern if Kazrin directly contributes to ARVCF (or delta- or p0071-catenin) effects upon gene activity.

Roles of Kazrin in human tumor metastasis

E-cadherin is a well-known suppressor of tumor metastasis. Thus, malignant metastatic tumor cells show reduced levels of E-cadherin expression and ectopic expression of E-cadherin in malignant tumor cell lines inhibits metastasis. Consistent with its role in cadherin stability, p120-catenin levels are down-regulated in many malignant tumor tissues. Thus, to better understand the process of tumor
metastasis, it is significant to study the mechanism by which p120-catenin subfamily members stabilize cadherins.

I propose that Kazrin is a novel factor regulating cadherin stability in X. laevis and that Kazrin stabilizes cadherins through interactions with p120 sub-family members (excluding p120 itself) and small GTPases. It should be noted that p120 sub-family members themselves are likely to have additional mechanisms of modulating cadherin levels, such as via their direct interactions with cadherin cytoplasmic tails, thereby reducing access of the endocytic machinery (exposure of endocytic signals) (137). In any case, to extend our knowledge of Kazrin in mammalian systems, cadherin protein level changes need to be monitored following Kazrin depletion in human cell lines. The effect of Kazrin on cell motility and invasion should also be tested, for example, using scratch-wound and cell-matrix invasion assays. In a more in vivo context, one could conduct tumor xenograft assays, using cell lines depleted for or over-expressing Kazrin. Alternative, more elegant transgenic or inducible knock-out approaches could be undertaken. Such experiments would assist in addressing the question of whether Kazrin plays a role as a metastasis suppressor, conceivably via the stabilization of cadherins. To determine the relevance of p120 sub-family members, cadherin and RhoA during this process, rescue experiments could be attempted via expression of p120 sub-family members or cadherin and inhibition of RhoA activity in xenograft assays.

At present, no chromosomal mutation of the Kazrin locus (1p36.21) has been reported in human disease including tumors. However, it is known that tumor suppressor genes can be down-regulated at the epigenetic level such as by
promoter methylation. Thus, as a translational approach, changes in Kazrin RNA and protein levels during tumor progression should be studied by comparing Kazrin expression in normal tissue samples versus those from differing cancer stages (methods might include real time PCR, and \textit{in situ} hybridization and immuno-histochemistry of tumor tissue microarrays). To address if epigenetic changes might contribute to Kazrin expression in disease, promoter DNA methylation could be monitored by MSP (methylation specific PCR) and COBRA (combined bisulfite restriction analysis) (138, 139). Posttranslational modification of promoter histones in tumor tissues could also be monitored by chromatin-IPs using specific antibodies.

\textbf{Roles for the p120-catenin subfamily: Kazrin complex in eye development}

Kazrin or ARVCF depletion in the head region induced eye and craniofacial developmental defects. Notably, in a previous study, p120 depletion in \textit{Xenopus} showed a similar phenotype, and we recently found that delta-catenin depletion also evidenced the same defects, suggesting a general function for p120-catenin subfamily members in eye and craniofacial development. Since the craniofacial cartilage is a derivative of neural crest cells, defective neural crest cell establishment after Kazrin or ARVCF depletion explains craniofacial defects. However, the means by which the p120-catenin subfamily or Kazrin is involved in eye development remains to be determined.

The eye develops through a delicate, multi-step processes including primary eye field specification in the forebrain, neuroectoderm evagination from the forebrain, optic vesicle formation, lens placode induction, optic cup formation, lens
placode invagination, lens vesicle formation after separation from the epithelium, lens differentiation, retina development and neurogenesis, retinal pigment epithelium (RPE) development, vasculogenesis and iris development (140). Thus, it is difficult to predict process(es) in which the p120-catenin family and Kazrin play roles.

According to my research and that of others, the p120-catenin subfamily and Kazrin share molecular functions with respect to cadherin stability and RhoA modulation. Interestingly, double knock-outs of E- and N-cadherin in the mouse lens placode induced an absent-lens phenotype and microphthalmia (abnormally small eye) because the lens cells failed to separate from the surface epithelium and form the lens vesicle (141). Furthermore, inhibition of RhoA activity in the lens resulted in abnormal lens development (cataract) and microphthalmia (142). These suggest that p120-catenin subfamily members and Kazrin may play crucial roles at least in lens formation. To test this, lens structures need to be carefully observed after Kazrin or p120-subfamily member depletion in *Xenopus*. To better test their potential downstream contributions to the observed eye defects, rescue experiments could be performed using ectopic expression of cadherins or Rho constructs.

In *Xenopus* and zebra fish, the non-canonical Wnt pathway is known to be important in early eye field specification. For example, depletion of either *Xenopus* Wnt4 or the *Xenopus* frizzled 3 (Xfz3) receptor reduced the expression of early eye markers such as Pax-6, Rx and Otx2, and induced eye defects (143, 144). Wnt4 depletion-mediated eye defects were rescued by xFz3 and ΔDix-Disheveled (a non-
canonical pathway activator). Because beta-catenin could not rescue this defect, it suggests that the non-canonical Wnt pathway (beta-catenin independent) plays a role in the specification of the *Xenopus* eye field. In fish, Wnt11 and Fz5 are important in eye field formation (145). Coincidentally, we previously reported that *Xenopus* p120-catenin activates the non-canonical Wnt pathway by de-repressing Kaiso-mediated repression of *Xenopus* Wnt11. Thus, there is a possibility that other p120 subfamily proteins such as ARVCF (and thus perhaps also Kazrin) may be involved in Wnt11 expression, and thereby in eye field formation. To test these possibilities, one could first examine changes in eye field specification after ARVCF or Kazrin depletion by performing whole-mount in situ hybridization for early eye markers such as Pax-6, Rx and Otx2. One could also monitor the changes in Wnt11 mRNA levels after Kazrin or ARVCF depletion using RT-PCR and whole mount in situ hybridization.

Alternatively, it is possible that the *Xenopus* Wnt4/Frizzled3 (xWnt4/xFz3) mediated non-canonical Wnt pathway is a target of p120-catenin subfamily members:Kazrin complexes. Depletion of xWnt4, xFz3, or two known Wnt4 gene targets (ELL-associated factor 2 or pescadillo) induced defects in craniofacial cartilage development and eye defects, which are similar to the phenotypic results following Kazrin or p120 sub-family members depletions. This may imply a functional interaction between xWnt4/xFz3 non-canonical pathway and p120-catenin subfamily:Kazrin complex during *Xenopus* eye and craniofacial cartilage development. Furthermore, xWnt4 also plays a role in *Xenopus* kidney development (146), and our preliminary result showed that depletion of delta-catenin induced
abdominal edema and abnormal fluid accumulation due to kidney failure. Even though evidences are circumstantial yet, it will be interesting to test whether the p120-catenin subfamily:Kazrin complex affects the Wnt4 pathway. First, one can check the change of $xWnt4$ or $xFz3$ RNA level after Kazrin or ARVCF depletion. As mentioned above, p120-catenin protein up-regulates $Wnt11$ RNA via inhibition of Kaiso repressor. Similarly, nuclear p120-catenin subfamily:Kazrin complex may regulate transcription of $xWnt4$ or $xFz3$ by an uncharacterized mechanism. The activity change of non-canonical Wnt4 pathway can be further monitored with a Jun reporter assay, which has been used as a (less than ideal) read-out of non-canonical Wnt pathway activity. Second, one can test physical association of the ARVCF:Kazrin complex with ELL-associated factor 2 (EAF2) or pescadillo using co-immunoprecipitation assays. EAF2 and pescadillo are gene targets of non-canonical Wnt4 pathway and depletion of either protein induces eye defects, loss of expression of eye markers and craniofacial defects (144, 147). At the molecular level, EAF2 is a component of the RNA Pol II elongation complex and pescadillo is a multi-functional nuclear protein involved in ribosome biogenesis, cell proliferation, RNA transcription, DNA replication and chromosome stability. Thus, it is expected that these proteins play a role in expression of genes required for eye and neural crest cell development. If interaction between p120-catenin subfamily:Kazrin complex and EAF2 (or pescadillo) is authenticated by binding assays, further experiments identifying gene targets will be performed. For example, microarray results from depletions of Kazrin, p120-catenin subfamily, EAF2 or pescadillo in *Xenopus* embryos will be compared and genes co-regulated by these depleted
proteins will be categorized as potential gene targets of interaction between p120-catenin:Kazrin and EAF2 (or pescadillo).

Summary

During my thesis work, I characterized the roles of *Xenopus* Kazrin at both cellular and developmental levels, examining for example, properties such as cadherin stabilization via RhoA modulation, ARVCF-membrane localization, ectoderm integrity and neural crest cell development. With respect to future directions, I am proposing to extend our knowledge obtained from use of *Xenopus* to study of mammalian systems and human cancer. In addition, the uncharacterized roles of Kazrin and p120-catenin sub-family members during eye development needs to be addressed.
CHAPTER V

MATERIALS AND METHODS
**DNA Constructs**

pCS2/myc-xKazrinA, pCS2/myc-xKazrinA ΔN, pCS2/myc-xKazrinA ΔM, pCS2/MT-xKazrinA ΔC, pCS2/HA-xARVCF-1ABC, pMAL/xARVCF-1ABC were generated by Travis Vaught. pCS2/xKazrinA was made by PCR amplification of xKazrinA cDNA from pCS2/MT-xKazrinA and cloning into pCS2 vector. pGEX/xKazrinA was produced by cloning PCR-amplified xKazrinA cDNA into pGEX4T-1 (GE healthcare). *Xenopus* β2-spectrin and p190B cDNAs (IMAGE #5571051 and # 6635626, respectively) were purchased from Open Biosystems (Thermo Fisher Scientific Inc.), and placed into pCS2/HA. Human p190B RhoGAP cDNA was a gift of Jeffrey M. Rosen (148), and subcloned into pCS2/HA. pCS2/myc-GFP-Dsg1-tail was obtained as a gift of Michael W. Klymkowsky (U. Colorado). pCS2/flag-mDsg3 was cloned by Hong Ji. pCS2/HA-Xdelta-catenin construct was cloned previously (25). pCS2/HA-Xp0071 was a gift of Kris Vleminckx (unpublished; U. Ghent).

**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 (NICHD and U. Iowa). xC-cadherin, xARVCF, Xp120 and xβ-catenin antibodies are previously described(22). To make Xenopus Kazrin specific antibody, carboxy-terminal 147 amino acids were cloned in pGEX 4T-1 vector and bacterially purified protein was injected into a rabbit for inoculation (UT MDACC Core Facility, Bastrop TX). IgG was purified from a rabbit
bleed using protein A sepharose and specificity of purified antibody was tested against ectopically expressed Xenopus Kazrin in embryo lysates. Other antibodies were purchased from commercial sources (HA, Sigma H9658 and Santa Cruz SC805; β-actin, Sigma A2066; GAPDH, Santa Cruz SC-25778; Rho (-A,-B,-C), Millipore 05-822, ; Desmoglein 1, BD Transduction Laboratories 610273; plakoglobin, BD Transduction Laboratories 610253; human ARVCF, Abnova H00000421-M01).

Dynasore and alcian blue dye were purchased from Sigma (D7693 and A3157, respectively). DTSSP was purchased from Thermo scientific (21578).

**Yeast Two-Hybrid**

Screening using full-length xKazrin employed an adult mouse brain cDNA library in collaboration with Hybrigenics’ ULTImate Y2H™ (149). 281 positive clones were obtained, then sequenced and analyzed.

**xKazrin RT-PCR Primers and Morpholinos.**

Primers used for RT-PCR analysis of xKazrin were: F1: 5’-AGCTTCGGCGCCAAGCCAAGG-3’, R1 : 5’-ACTCTGACAGCTTGCCGGTC-3’, K1: 5’-CGGACCTAGTAAGCCAAATGG-3’, K2: 5’-CTGTGGATGTCCGTGGTACAG-3’, K3: 5’-CCAGGCATTGCTAGAGAGTGG-3’ and K4: 5’-TCCGTTGCTTCCTTGGCTTG-3’.

We employed a previously characterized translation blocking morpholino directed against xKazrin (Kmo-T) (xKazrin cDNA –4 to +21: 5’-
GCTTGGTATCTTCCATCATCTTCAG-3') (78), and newly designed splicing blocking morpholino against xKazrin pre-mRNA (Kmo-S) (5'-AAACCATTTTTGCCCTCACCTTTCT-3'). A standard control morpholino (Cmo) from Gene Tools LLC was used as a negative control (5'-CCTCTACCTCAGTTACAATTATA-3').

**Pull-down Assays**

For in vitro MBP pull-downs involving Xp190B, xARVCF and xKazrinA, 4mg of pCS2/HA-Xp190B DNA 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 1mg of bacterially purified GST-xKazrinA and/or MBP-xARVCF in pull-down buffer (PBS, 1mM EDTA, 0.1% NP-40, 100ng/ml BSA) and pulled-down using amylose-resin.

A GST pull-down assay between GST-xKazrinA and Xp190B was performed following the same procedure. For pulling down GST-xKazrinA, Glutathione Sepharose 4B (GE healthcare) was used.

**Xenopus Embryo Manipulations and Co-Immunoprecipitations**

Induction of female *Xenopus laevis*, in vitro fertilization of eggs, and e-embryo injections were carried out using standard methods (150). Capped mRNAs for injection were generated using mMessage mMACHINE kits (Ambion). Embryos were incubated at 18°C to stage10 (early gastrula), lysed in embryo IP buffer (50mM Tris-HCl pH 7.4, 1% Triton X-100, 150mM NaCl, 1mM EDTA, 1 mM PMSF, 1mM
Na$_3$VO$_4$, 1mM NaF, and 1mg/ml each of Aprotinin, leupeptin and pepstatin), centrifuged, and the supernatants divided for immunoprecipitation. Immune complexes were precipitated using Protein-A/G agarose beads (Santa Cruz), and subjected to Western blotting.

**Cell-cell dissociation assay and re-association assay**

For assay of cell-cell dissociation, naive ectoderm tissue fragments (animal caps) were isolated from late blastula embryos (stages 9-10), placed in 0.6X MMR solution with or without 2mM EGTA and rotated at room temperature for 2-3 hours until control morpholino injected animal caps were completely dissociated.

For cell re-association assay, after isolation of animal caps outer layer was separated from inner cells and discarded. Remained inner cells were completely dissociated in Ca/Mg free MBS solution by pipetting, and then rotated at room temperature in the presence of 2mM CaCl$_2$.

**Immunofluorescence**

For *Xenopus* ectoderm confocal microscopy, in vitro transcribed Myc-xKazrinA RNA and/or HA-xARVCF RNA were injected into the animal pole of one-cell stage embryos. Vitelline membranes of late blastula embryos (stage 9) were manually removed, followed by 2 hour fixation in MEMFA and methanol dehydration. Embryos were then serially re-hydrated with PBS-diluted methanol, incubated 1 hour in blocking buffer (20% goat serum in PBST), incubated 3 hours with primary antibody, washed 1 hour with PBST, incubated 3 hours with secondary
antibodies and washed (3-4 times) with PBST. Blastula ectoderm tissue (animal caps) were isolated, and mounted on glass slides. Images obtained from an Olympus IX-70 microscope were captured using Olympus Fluoview FV500 software, and adjusted for brightness in select cases using Adobe Photoshop software.

For actin staining, naive ectoderm (animal caps) isolated at stage 10 was fixed with 3.7% Paraformaldehyde and 0.25% glutaraldehyde in PBST for 30 minutes at room temperature. After rinsing with PBST, samples were incubated overnight at 4°C with Alexa Fluor 488 – phalloidin (Molecular Probes/ Invitrogen). Stained ectodermal tissues were washed with PBST, mounted and viewed under a confocal microscope.

**In Vitro Rho Activity Assay**

In vitro Rho activity assays were performed as described, previously (25). Briefly, *Xenopus* embryos injected with xARVCF and/or xKazrinA morpholinos were subject to lysis buffer (25mM HEPES pH7.5, 150mM NaCl, 10mg MgCl₂, 1% NP-40, 1mM EDTA and 2% glycerol), and lysates incubated with recombinant GST-Rhotekin RBD (Rho Binding Domain) immobilized to glutathione-Sepharose™ 4B (GE Healthcare Life Science), followed by pull-down and subsequent SDS-PAGE/Western blotting detection of active Rho.

**Crosslinking co-IP**
For crosslinking co-IP, embryos were lysed in crosslinking buffer (50mM HEPES pH 7.5, 150mM NaCl, 1% Triton X-100, 2mM EDTA and protease inhibitors) and extract was crosslinked with DTSSP dissolved in 5mM sodium citrate buffer for 30 minutes at room temperature. Reaction was stopped by adding Tris pH 7.5 (50mM final concentration) and proteins were precipitated with specific antibodies. Precipitants were de-crosslinked by adding β-mercaptoethanol and DTT, and subjected to SDS-PAGE/western blotting.

**Membrane fractionation**

Membrane fractionation was performed as described previously (151). Embryo extract was prepared with homogenizing buffer (250mM sucrose, 10mM HEPES, 2mM MgCl, 1mM EGTA and 0.5mM EDTA) and then centrifuged at 750g for 5 minutes. For isolating cytoplasmic soluble fraction, supernatant was transferred to new tube and re-centrifuged at 21,000g for 5 minutes. Supernatant was taken as cytosolic fraction extract. For isolating membrane fraction, pellet from initial centrifugation was dissolved in Y buffer (0.5% NP40, 150mM NaCl, 10mM HEPES and 1.5mM EDTA), re-centrifuged at maximum speed for 10mins, and supernatant was kept as membrane fraction.

**Alcian blue staining**

For cartilage staining, embryos were fixed in 95% ethanol overnight and incubated in alcian blue solution (150mg alcian blue in the mixture of 800ml 95% ethanol and 200ml acetic acid) overnight. Stained embryos were washed with 95%
ethanol for 4-5 hours. Embryos were soaked in 2% KOH for 1-2 hour(s) until skin became soft. After removal of the skin, cartilage pictures were taken.

**Whole mount In situ hybridization**

Whole mount in situ hybridization was performed following the protocol reported previously (150). In brief, embryos were fixed in MEMFA fixative, dehydrated in methanol, re-hydrated with serially diluted methanol, re-fixed with 4% paraformaldehyde, pre-hybridized in hybridization buffer (50% formamide, 5X SSC, 1mg/ml yeast RNA, 100ug/ml heparin, 1X Denhart’s solution, 0.1% Tween 20, 0.1% CHAPS and 10mM EDTA) at 60°C for 6 hours and hybridized with digoxigenin labeled RNA probes at 60°C overnight. After washed with SSC solution, embryos were incubated in blocking solution (2% blocking reagent in maleic acid buffer) at room temperature for 1 hour and then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase overnight at 4°C. Then, target RNAs were visualized by color reaction with NBT/BCIP substrate for alkaline phosphatase and embryos were re-fixed in Bouin’s fixative overnight. After washing with 70% ethanol several times until yellow color from Bouin’s fixative disappeared, embryos were bleached in bleaching solution (0.5X SSC, 5% formamide and 1.2% peroxide) and observed under stereomicroscope.
REFERENCES


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Kyucheol Cho was born in Kang-neung, Republic of Korea, on June 16th, 1973 to Minhwan Cho and Okja Lee. After attending Kang-neung High School, he pursued a Bachelor of Science degree in Biology at Korea University in Seoul, Korea, and he earned a degree in 1997. He then went on to earn a Master of Science in Biochemistry from the same university in Department of Biotechnology, where he conducted research on yeast transcription factor Gcn4p. After earning a master degree in 2000, Kyucheol had more research experiences in neuroscience and cancer biology fields as a research assistant at Department of Biochemistry, College of Medicine, Korean Catholic University and Department of Pathology, College of Medicine, Kyung-Hee University, respectively. In 2003 Kyucheol left Korea to pursue the degree of Doctor of Philosophy in Biomedical Science at the University of Texas-Houston Graduate School of Biomedical Sciences. Travis currently lives with his wife, Hye-won Song, Ph.D., and daughter, Lucy Eujean Cho in San Diego, California.

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