

# Vertebrate development requires ARVCF and p120 catenins and their interplay with RhoA and Rac

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Using an animal model system and depletion-rescue strategies, we have addressed the requirement and functions of armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) and p120 catenins in early vertebrate embryogenesis. We find that xARVCF and Xp120 are essential to development given that depletion of either results in disrupted gastrulation and axial elongation, which are specific phenotypes based on self-rescue analysis and further criteria. Exogenous xARVCF or Xp120 cross-rescued depletion of the other, and each depletion was additionally rescued with (carefully titrated) dominant-

negative RhoA or dominant-active Rac. Although xARVCF or Xp120 depletion did not appear to reduce the adhesive function of C-cadherin in standard cell reaggregation and additional assays, C-cadherin levels were somewhat reduced after xARVCF or Xp120 depletion, and rescue analysis using partial or full-length C-cadherin constructs suggested contributory effects on altered adhesion and signaling functions. This work indicates the required functions of both p120 and ARVCF in vertebrate embryogenesis and their shared functional interplay with RhoA, Rac, and cadherin in a developmental context.

## Introduction

The roles of armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) and p120 catenins in vertebrate development have remained open to question even as p120's modulation of cadherin-based adhesion and Rho family (small) GTPases (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001; Noren et al., 2000), associations with kinesin and microtubules (Chen et al., 2003; Franz and Ridley, 2004; Yanagisawa et al., 2003), and stabilization of cadherin (Ireton et al., 2002; Davis et al., 2003; Peifer and Yap, 2003; Xiao et al., 2003) have received attention in recent cell line studies. In the work presented here, we have sought to address the requirement of *Xenopus laevis* xARVCF and Xp120 in vertebrate embryogenesis, and the functional interaction of each protein with cadherin and small GTPases. We find that xARVCF exhibits functional interactions with

small GTPases that are similar to Xp120 and, most notably, that xARVCF and Xp120 are developmentally required.

The catenin proteins are distinguished by the various cellular compartments in which they function and the diversity of their protein associations, e.g., those occurring with the cytoplasmic domain of cadherins (plasma membrane; Gumbiner, 2000; Peifer and Polakis, 2000), Rho family GTPases (plasma membrane/cytoplasm; Anastasiadis and Reynolds, 2001), kinesin and microtubules (Chen et al., 2003; Yanagisawa et al., 2003; Franz and Ridley, 2004), and transcription factors (nucleus; Daniel and Reynolds, 1999; Kim et al., 2002; Bienz and Clevers, 2003). Certainly, the best characterized catenin family member is  $\beta$ -catenin (McCrea et al., 1991), which transduces canonical Wnt signals in multiple developmental and pathological (cancer) contexts (Behrens, 2000; Sharpe et al., 2001).

The p120 catenin subfamily is lesser understood and exhibits functions distinct from  $\beta$ -catenin (Sirotkin et al.,

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Abbreviations used in this paper: ARVCF, armadillo repeat gene deleted in velo-cardio-facial syndrome; DA, dominant-active; DN, dominant-negative; EC, ectodomain; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; JMR, juxtamembrane; MO, morpholino oligonucleotide.

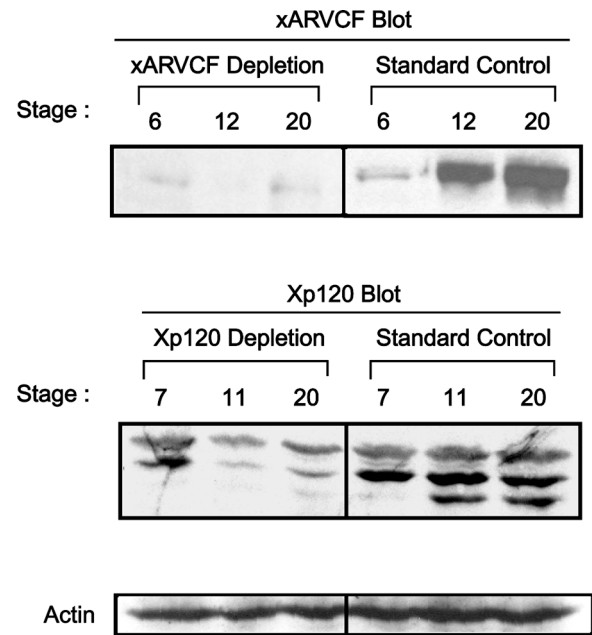
1997; Paulson et al., 1999; Anastasiadis and Reynolds, 2000). For example, at the plasma membrane, p120 and ARVCF competitively bind the membrane-proximal (“juxtamembrane”) region of cadherin cytoplasmic domains (Mariner et al., 2000; Paulson et al., 2000), whereas  $\beta$ -catenin and plakoglobin ( $\gamma$ -catenin) competitively bind the membrane-distal (“catenin-binding”) domain and indirectly associate with the cortical actin cytoskeleton (Knudsen et al., 1995; Rimm et al., 1995). Recent papers further demonstrate the association of p120 with kinesin and microtubules (Chen et al., 2003; Franz and Ridley, 2004) and with the POZ/zinc finger transcription factor Kaiso (Daniel and Reynolds, 1999; Kim et al., 2002; Yanagisawa et al., 2003), whereas nuclear partners of ARVCF and  $\delta$ -catenin remain to be identified.

Arising from effects on cadherin stability and clustering (Ireton et al., 2002; Davis et al., 2003; Peifer and Yap, 2003; Xiao et al., 2003) or on small GTPases and the cell cytoskeleton, p120 is thought to positively or negatively regulate cell adhesion and motility (Briehner et al., 1996; Yap et al., 1998; Aono et al., 1999; Ohkubo and Ozawa, 1999; Paulson et al., 2000; Thoreson et al., 2000). For example, depending on p120's intracellular localization to cell junctions or the cytoplasm and free cell edges, p120 may promote cell adhesion versus motility via activation of Rac and Cdc42 and inhibition of RhoA (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2000, 2001; Noren et al., 2000; Grosheva et al., 2001; Magie et al., 2002). In this manner, p120 has been likened to a molecular switch contributing to both cell adhesion and motility.

The junctional organization and adhesive activity of cell-cell contacts containing cadherins is further known to be responsive to Rho family GTPases (Braga, 2000; Fukata and Kaibuchi, 2001), which in turn are responsive to the levels and adhesive activity of cadherins (Kim et al., 2000; Nakagawa et al., 2001; Noren et al., 2001; Goodwin et al., 2003). Such functional interdependencies are believed to promote vertebrate gastrulation (Gumbiner, 2000; Tepass et al., 2000), wound healing, or pathologies, including a variety of human carcinomas (Behrens, 1999; Nollet et al., 1999; Anastasiadis and Reynolds, 2000; Van Aken et al., 2001; Thoreson and Reynolds, 2002).

At the biochemical level, the activation of Rac and Cdc42 by p120 appears to be mediated via its direct association with the guanine nucleotide exchange factor (GEF) Vav2 (Noren et al., 2000), which promotes the residence of GTP (as opposed to GDP) within the nucleotide binding pocket of Rho family GTPases such as Rac. In contrast, inhibition of RhoA is likely to occur as a consequence of direct p120–RhoA complex formation in which p120 functions as a guanine nucleotide dissociation inhibitor (GDI; Anastasiadis et al., 2000; Magie et al., 2002), favoring the residence of GDP within RhoA's nucleotide binding pocket.

Because effectors of Rho family GTPases are numerous (Bishop and Hall, 2000), various outcomes likely follow p120 (or ARVCF) activation of Rac or Cdc42 or inhibition of RhoA. In addition to effects on the actin cell cytoskeleton, Rho family GTPases have roles in other essential biological processes, e.g., in transducing developmental signals within noncanonical Wnt pathways (Myers et al., 2002). Thus, although modulation of the actin cytoskeleton comprises a sig-



**Figure 1. Morpholino-directed depletion of xARVCF or Xp120.** xARVCF and Xp120 protein from total embryo extracts isolated at the indicated developmental stages were detected via SDS-PAGE/Western blotting using anti-xARVCF or anti-Xp120 polyclonal antibodies. Embryos at the 1-cell stage were injected with a combination of either xARVCF morpholinos (5 ng each of xARVCF MO I and MO II, total delivery) or of Xp120 morpholinos (40 ng each of Xp120 MO I and MO II, total delivery), while the standard control morpholino was injected at matched doses. Protein loads were assessed by Western blotting samples for actin. The anti-Xp120 antibody recognizes three major Xp120 isoforms. Developmental stages presented include 6 or 7 (blastula, before zygotic transcription/mid-blastula transition), 11 or 12 (late gastrula), and 20 (early tailbud). When comparing experimental samples with their stage-matched standard control, depletion efficiencies ranged from 20–90% depending on factors including embryo batch variation, the morpholinos used, and morpholino dose. xARVCF displayed an SDS-PAGE mobility of  $\sim$ 100 kD, whereas the Xp120 isoforms migrated between 92–98 kD; p120's lowermost band/isoform may arise as a consequence of protein degradation given its appearance in some but not all experiments.

nificant aspect of Rho family functions, each GTPase acts in additional cellular and developmental capacities.

Given cell line studies indicating that p120 modulates cadherin function and Rho family GTPases, we tested if p120 and/or ARVCF are required in early vertebrate development. Using early *X. laevis* embryos, we further assessed if ARVCF regulates Rac, Cdc42, and RhoA in a manner similar to that proposed for p120. Our work, conducted in *X. laevis* and using a depletion-rescue strategy in conjunction with biochemical and cell biological analyses, indicates that Xp120 and xARVCF are each developmentally essential, and that these two catenins share related functions in early embryogenesis via the stabilization of cadherin, activation of Rac, and inhibition of RhoA.

## Results

### xARVCF or Xp120 proteins are specifically depleted upon treatment with morpholino oligonucleotides (MOs)

To assess the requirement of xARVCF and Xp120 in early vertebrate development, we used an established MO ap-

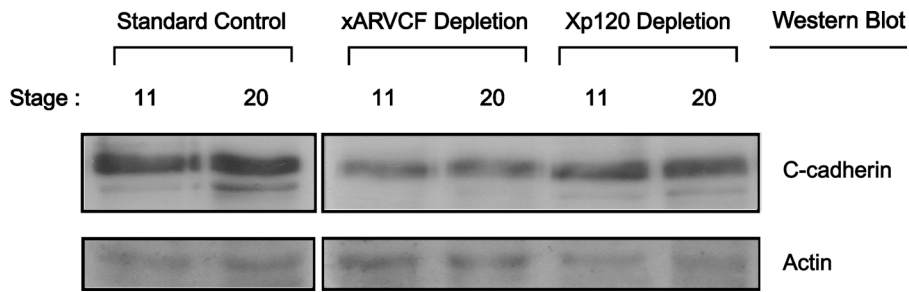


Figure 2. xARVCF or Xp120 depletion results in reduced C-cadherin levels. *X. laevis* C-cadherin protein in total embryo extracts was detected using anti-C-cadherin polyclonal antibody. Embryos were injected at the 1-cell stage with 20 ng each of xARVCF morpholino, Xp120 morpholino, or standard control morpholino and harvested at developmental stages 11 (gastrula) or 20 (early tailbud). Protein loads were assessed by Western blotting samples for actin. C-Cadherin reduction after embryonic depletion of xARVCF or Xp120 ranged from 10–50% depending on factors including embryo batch variation, the morpholinos used, and morpholino dose.

proach to deplete (“knockdown”) endogenous xARVCF or Xp120 in *X. laevis* (Heasman et al., 2000). MOs were designed to block the protein translation of xARVCF (xARVCF-MOI and -MOII) or Xp120 (Xp120-MOI and -MOII; see Materials and methods). In brief, oligonucleotide sequences were selected to complement the start ATG and adjoining proximal 5' UTR of the mRNA, thereby disrupting ribosome binding and progress along the transcript. For initial morpholino characterization, injections of 10–80 ng were made into the animal region of single-cell embryos, which were permitted to develop through gastrula, neurula, and early tailbud stages. To test MO effectiveness, extracts were isolated and xARVCF or Xp120 protein levels were resolved via SDS-PAGE/Western blotting using specific rabbit polyclonal sera or affinity-purified antibodies directed against xARVCF (Paulson et al., 2000) or Xp120 (see Materials and methods). In accordance with embryo batch variance, the morpholino selected, its dose, and whether used singly or in combination, depletions ranged from 20% to >90% (Fig. 1). Injection of an established standard control morpholino at comparable doses did not result in xARVCF or Xp120 depletion, suggesting specificity of our xARVCF or Xp120 MOs. xARVCF-MOII and Xp120-MOI exhibited the most favorable respective depletions of xARVCF or Xp120 and were used in subsequent experimentation. In accordance with the morpholino approach in general, xARVCF or Xp120 depletions were effective but not absolute (not equivalent to a genetic null), a point that will pertain to the later interpretation of our results.

### C-Cadherin levels are reduced after xARVCF or Xp120 depletion

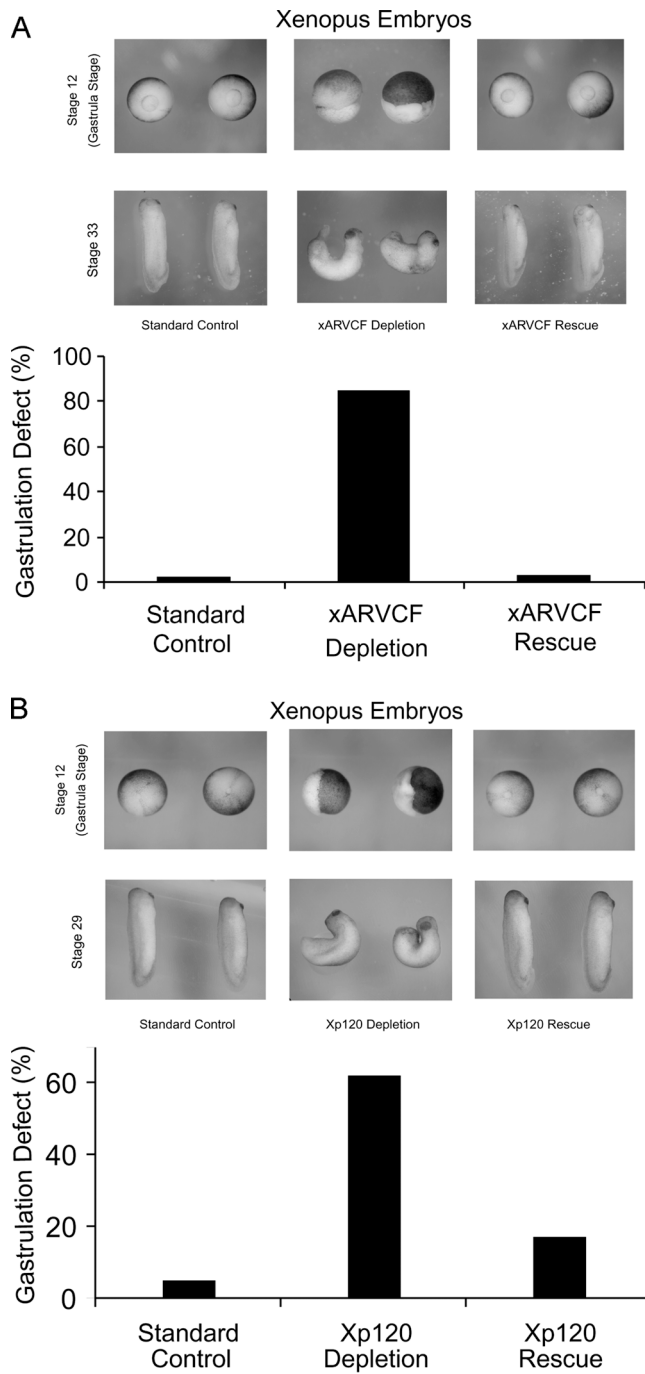
Given that p120 was recently shown to enhance cadherin stability in mammalian cell lines (Ireton et al., 2002; Davis et al., 2003; Peifer and Yap, 2003; Xiao et al., 2003), we wished to test in vertebrate embryos if xARVCF or Xp120 depletion results in reduced levels of C-cadherin, the predominant early embryonic cadherin of *X. laevis* (Choi et al., 1990; Angres et al., 1991). Indeed, total levels of C-cadherin were slightly to moderately reduced (10–50%) after MO injection at the 1-cell stage (Fig. 2). Injection at the 1-cell stage facilitated visualization of resulting decreases in protein levels, whereas injections at the 4-cell stage facilitated phenotypic depletion-rescue analyses (see Results sections using

depletion-rescue approaches). In summary, we find that in addition to Xp120 catenin, xARVCF contributes to cadherin stability during vertebrate embryogenesis.

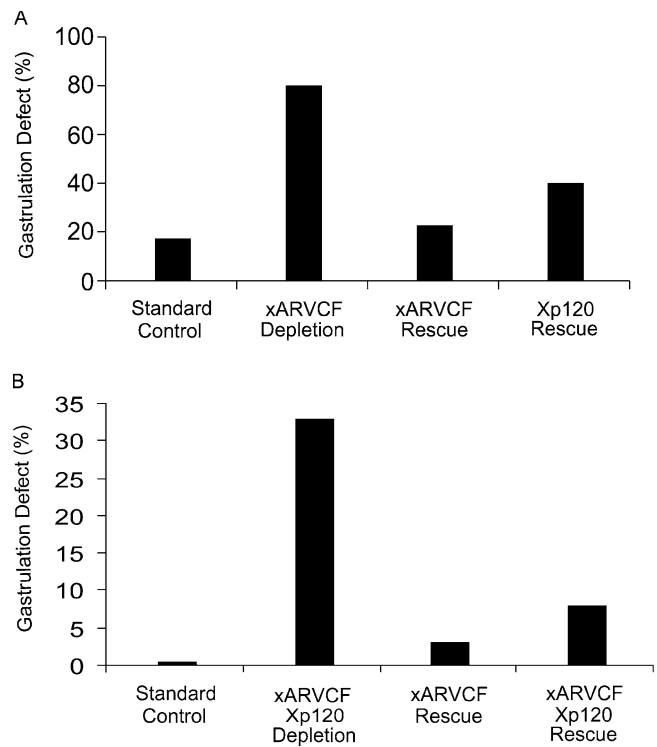
### xARVCF and Xp120 are each required in early *X. laevis* development

To assess the developmental outcome of reduced xARVCF or Xp120 levels, MOs were injected into the animal pole of one or two dorsal cells within 4-cell embryos (Fig. 3 and Tables I and II). Although introduced at early (cleavage) stages of embryogenesis, when maternal mRNAs predominate, morpholinos continue to function after the mid-blastula transition (stage 8) when zygotic transcription/translation is initiated in *X. laevis*. At doses ranging from 10–40 ng, we observed gastrulation defects whose penetrance varied from 20–90% of embryos, as a function of the morpholino, its dose, and the batch of embryos used. To control for the effect of MO delivery (injection) and its presence during embryogenesis, similar or greater doses of an established “standard control” morpholino were introduced. As anticipated, injection of the standard control morpholino resulted in considerably lesser effects relative to experimental morpholinos, and in many cases were in keeping with background levels observed in water-injected or uninjected embryos (Figs. 3 and 4; and Tables I and II).

In contrast, injection of xARVCF-MOII or Xp120-MOI at doses of  $\geq 10$  ng produced significantly larger fractions of embryos displaying aberrant gastrulation. Gross morphological examination of injected embryos indicated normal development at early cleavage and blastula stages, whereas gastrula stage embryos exhibited blastopore closure defects in association with subtle extents of extruded endoderm, phenotypes characteristic of aberrant convergent extension, and mesoderm involution (Fig. 3 and Tables I and II). Most depleted embryos retained the capacity to proceed through gastrulation, early neurogenesis, and neural fold closure, whereupon effects included reduced extension of the dorsal axis made apparent in a bowed appearance at late neurula and tailbud stages, effects again typical of aberrant convergent-extension (Fig. 3 and Tables I and II). When compared within the same experiment, embryos depleted for both xARVCF and Xp120 displayed more severe gastrulation phenotypes than single depletions and did not progress into neurulation stages (unpublished data). Likewise, greater outward effects fol-



**Figure 3. xARVCF and Xp120 are essential to embryonic development.** Embryos were depleted for xARVCF or Xp120 (20 ng morpholino injected into one dorsal cell at the 4-cell stage) or injected with 20 ng of standard control morpholino. Substantial rescues were effected via coinjection of morpholino with xARVCF or Xp120 in vitro transcribed mRNA (0.01 ng), indicating specificity of the depletion phenotypes. Embryos were scored at stage 12 (gastrula) for defects (histogram), which included partial/improper closure of the blastopore. As depicted, depleted embryos at later stages (tailbud and tadpole) displayed a bowed appearance characteristic of reduced dorso-axial mesoderm elongation, which was likewise rescuable to the standard control (normal embryo) phenotype. (A) xARVCF depletions and rescue upon coinjection of xARVCF mRNA. (B) Xp120 depletions and rescue upon coinjection of Xp120 mRNA. See also combined depletion/rescue data presented in Tables I and II.



**Figure 4. Xp120 partially rescues depletion of xARVCF, and xARVCF largely rescues codepletion of xARVCF and Xp120.** (A) Gastrulation defects in embryos depleted for xARVCF (20 ng morpholino injected into one dorsal blastomere at the 4-cell stage) were largely rescued upon coinjection of xARVCF in vitro transcribed mRNA (0.01 ng, positive control for rescue) as well as substantially cross-rescued upon coinjection of Xp120 mRNA (0.01 ng). (B) Gastrulation defects in embryos codepleted for xARVCF and Xp120 (20 ng of respective morpholino coinjected into one dorsal blastomere at the 4-cell stage) were largely rescued upon coinjection of xARVCF and Xp120 in vitro transcribed mRNAs (0.02 ng of respective mRNA, positive control for codepletion rescue) or upon coinjection of xARVCF mRNA alone (0.02 ng).

lowed dorsal versus ventral depletions, which is consistent with the more graphic morphogenic requirements of dorsal tissues. These findings indicate that xARVCF and Xp120 are each required in early vertebrate development.

**Specificity of the xARVCF or Xp120 depletion phenotype indicated via self-rescue**

To assess if the xARVCF or Xp120 depletion phenotype is specific, we tested the respective capacity of exogenous xARVCF or Xp120 to rescue their depletion in vivo. Indeed, morpholino-directed depletion of xARVCF was rescued upon expression of exogenous xARVCF (injection of in vitro transcribed mRNA), and likewise the depletion of Xp120 rescued upon expression of exogenous Xp120 (Fig. 3 and Tables I and II). Importantly, we used minimal rescuing doses of xARVCF or Xp120 mRNAs that had been titrated to exhibit no or very minimal effects when injected alone (Tables I and II). This process was required given that p120 perturbs development when expressed at higher doses (Geis et al., 1998; Paulson et al., 1999). Because nearly complete rescues followed minimal-dose injections of xARVCF or Xp120 mRNA, our depletion phenotypes

Table I. Rescue of gastrulation defects after xARVCF depletion

Morpholino oligo ± mRNA injected	Normal	Gast. defect	Embryos	Expt.
	%	%		
xARVCF morpholino	31	69	233	7
xARVCF morpholino + xARVCF mRNA	84	16	128	3
xARVCF morpholino + DN-RhoA mRNA	85	15	115	3
xARVCF morpholino + DA-Rac mRNA	84	16	167	2
xARVCF morpholino + C-cadherin mRNA	82	18	60	2
xARVCF mRNA	86	14	66	3
DN-RhoA mRNA	84	16	89	3
DA-Rac mRNA	85	15	60	2
C-Cadherin mRNA	86	14	59	2
Standard control morpholino	94	6	195	7
Uninjected	97	3	272	7

*Xenopus* embryos were injected with xARVCF morpholino (20 ng, one cell at the 4-cell stage), and rescues were undertaken with xARVCF in vitro transcribed mRNA (0.02 ng) or RhoA or Rac mRNA (0.5 pg). To avoid overexpression phenotypes, the injection doses of all rescuing constructs were carefully titrated to produce minimal effects when injected alone. Embryos (*n*) were scored at gastrula stages for defects. This table shows the combined data of multiple experiments.

likely resulted from the targeted reduction of xARVCF or Xp120. Furthermore, because our rescuing constructs lack any 5' UTR sequence, they did not complement (xARVCF-MOI and Xp120-MOI) or only partially complemented the morpholinos used (xARVCF-MOII and Xp120-MOII). Thus, the rescues are unlikely to have been successful as a consequence of morpholino sequestration, again suggesting the specificity of our observed phenotypes (see the following paragraph).

### Depletion of xARVCF is partially rescued by exogenous Xp120 and vice versa

Because xARVCF and Xp120 are structurally similar members of the p120 sub-class of catenins, we assessed if depletion of one could be functionally compensated by increasing the level of the other. As in the aforementioned experiments, we assayed a range of Xp120 or xARVCF mRNA concentrations to optimize their potential effectiveness. We found that Xp120 largely cross-rescues depletion of xARVCF, and conversely, that xARVCF largely rescues codepletion of

xARVCF and Xp120 (Fig. 4). Because the Xp120 and xARVCF mRNAs lack sequence complementarity in the regions targeted by their respective morpholinos, observed phenotypes likely resulted from selective xARVCF or Xp120 depletion (Figs. 1 and 2). However, complicating matters somewhat is that depletion of one catenin produced slight but measurable decreases of the other (not depicted), perhaps as an indirect consequence of lowered cadherin stability (Fig. 2) or an unknown indirect effect. In any case, given the cross-rescues, we propose that xARVCF and Xp120 share significant functional overlap in early vertebrate development (see following Results sections).

### C-Cadherin partially rescues depletion of xARVCF or Xp120

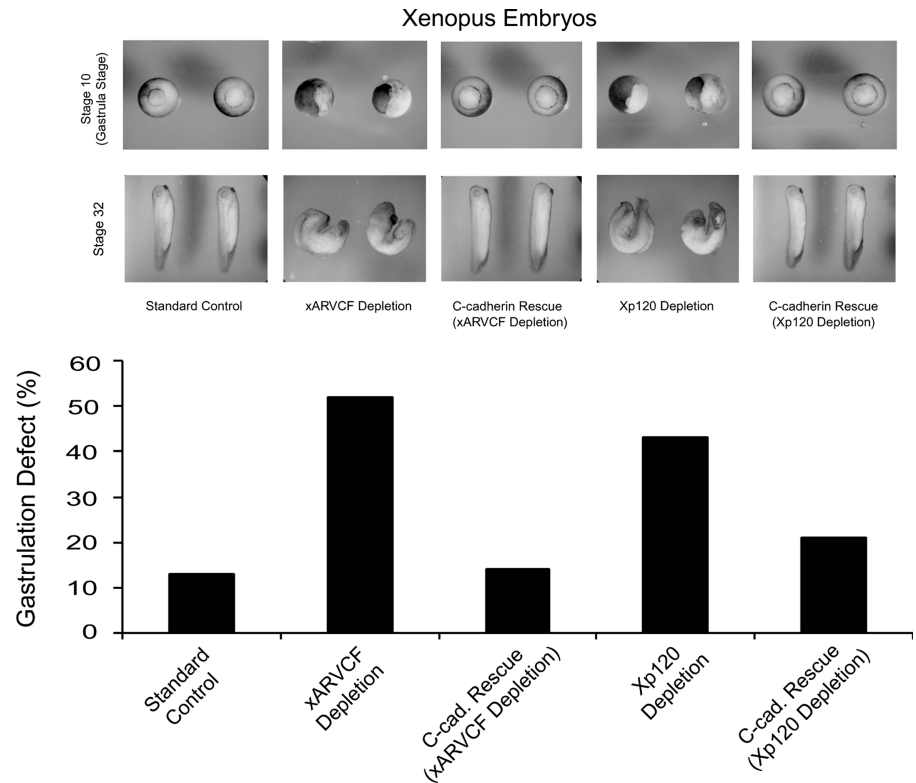
Because the depletion of xARVCF or Xp120 resulted in slightly reduced C-cadherin levels (Figs. 1 and 2), we tested if exogenous C-cadherin would rescue embryogenesis in depleted embryos. We found that a carefully titrated level of C-cadherin mRNA rescued to a significant extent pheno-

Table II. Rescue of gastrulation defects after Xp120 depletion

Morpholino oligo ± mRNA injected	Normal	Gast. defect	Embryos	Expt.
	%	%		
Xp120 morpholino	39	61	240	8
Xp120 morpholino + Xp120 mRNA	83	17	127	2
Xp120 morpholino + DN-RhoA mRNA	82	18	176	4
Xp120 morpholino + DA-Rac mRNA	84	16	89	2
Xp120 morpholino + C-cadherin mRNA	81	19	155	3
Xp120 mRNA	86	16	56	2
DN-RhoA mRNA	90	10	126	4
DA-Rac mRNA	83	17	63	2
C-Cadherin mRNA	86	14	109	3
Standard control morpholino	91	9	207	8
Uninjected	98	2	166	8

*Xenopus* embryos were injected with Xp120 morpholino (20 ng, one cell at the 4-cell stage), and rescues were undertaken with Xp120 in vitro transcribed mRNA (0.02 ng) or RhoA or Rac mRNA (0.5 pg). To avoid overexpression phenotypes, the injection doses of all rescuing constructs were carefully titrated to produce minimal effects when injected alone. Embryos (*n*) were scored at gastrula stages for defects. This table shows the combined data of multiple experiments.

**Figure 5. C-Cadherin rescues xARVCF or Xp120 depletion.** Embryos were injected with 20 ng of standard control morpholino or depleted for xARVCF or Xp120 (20 ng of respective morpholino injected into one dorsal cell at the 4-cell stage). Rescues were effected by coinjection of xARVCF or Xp120 morpholino with C-cadherin *in vitro* transcribed mRNA (2 ng) and scored at stage 12 (gastrula) for defects including partial/improper closure of the blastopore. Resulting gastrulation phenotypes are shown in A with percentages presented in B. Depletion phenotypes and C-cadherin rescues were apparent at both gastrula and later developmental stages (tailbud and tadpole).



types arising from xARVCF or Xp120 depletion (Fig. 5), suggesting that reduced C-cadherin levels are contributory to our observed developmental phenotypes.

### C-Cadherin adhesive function is not graphically altered after xARVCF or Xp120 depletion

Given that C-cadherin levels were reduced in embryos depleted for xARVCF or Xp120 (Fig. 2), and given the capacity of exogenous C-cadherin to rescue such embryos (Fig. 5), we tested if xARVCF or Xp120 depletion altered C-cadherin-mediated adhesion using a well established blastomere re-aggregation assay (see Materials and methods). Contrary to our initial expectations, no observable effects on cell reaggregation followed xARVCF or Xp120 depletion (Fig. S1, available at <http://www/jcb.org/cgi/content/full/jcb.200307109/DC1>). Using three additional assays, we likewise did not observe altered tissue integrity or aberrant cell-cell contacts in xARVCF, Xp120, or doubly-depleted embryos or animal caps (Figs. S2–S4, available at <http://www/jcb.org/cgi/content/full/jcb.200307109/DC1>).

However, one functional strategy did suggest effects on C-cadherin adhesive function resulting from Xp120 depletion. A carefully titrated level of C-cadherin ectodomain (EC) construct partially rescued Xp120 depletion phenotypes (construct included the C-cadherin transmembrane domain but not cytoplasmic sequence; Fig. S5, available at <http://www/jcb.org/cgi/content/full/jcb.200307109/DC1>). C-Cadherin's juxtamembrane (JMR) domain also displayed partial rescuing activity in isolation (tethered to the inner plasma membrane via an engineered myristylation sequence), but to a lesser extent than the EC construct or native (full-length) C-cadherin (Fig. S5 and Fig. 5). Given that C-cad-

herin's EC reproducibly exhibited rescuing activity, we conjecture that the adhesive function of C-cadherin is reduced after Xp120 (or xARVCF) depletion, but to a subtle extent that is not readily detectable using accepted direct or indirect assays of cadherin function (Figs. S2–S4).

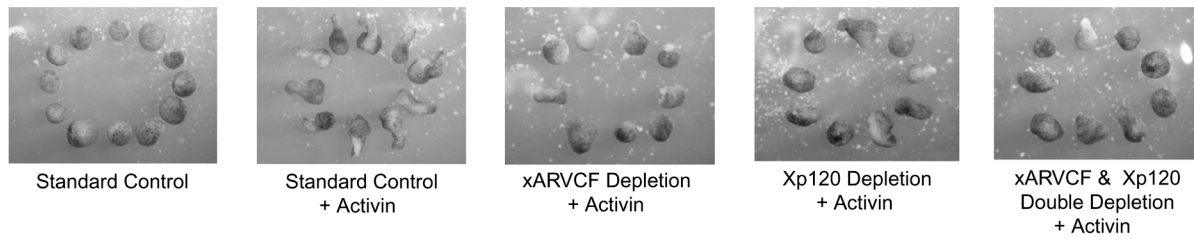
### xARVCF or Xp120 depletion inhibits morphogenic movements

To test depletion effects on cadherin-dependent morphogenic movements (convergent-extension), we used an *in vivo* explant assay in which activin-induced naive ectoderm (animal caps) reflect aspects of the more elaborate *in vivo* process, wherein dorsal mesoderm cells polarize and converge toward the dorsal midline during gastrulation to drive mesoderm involution as well as subsequent embryo elongation along the anterior–posterior axis.

Naive ectoderm (animal caps) were excised from late blastula embryos (stage 9), and convergent-extension responses were scored after the addition (versus not) of activin, a member of the TGF- $\beta$  family of morphogenic factors. Consistent with our embryonic phenotypes (Fig. 3 and Tables I and II), animal caps depleted for xARVCF or Xp120 exhibited a significantly reduced ability to converge and extend (Fig. 6), suggesting that xARVCF and Xp120 contribute to the directed interdigitation of cells *in vivo*.

### Depletion of xARVCF or Xp120 is rescued by dominant-negative (DN)–RhoA or dominant-active (DA)–Rac

Given mammalian cell line studies indicating that p120 inhibits RhoA and activates Rac and Cdc42 (Introduction), we asked if this functionality is evident in vertebrate embryogenesis. Upon the coinjection of either DN-RhoA or



Condition	Score 0 Caps	Score 1 Caps	Score 2 Caps	Score 3 Caps	Total Animal Caps	Total Score
Standard Control	22	0	0	0	22	0
Standard Control + Activin	0	0	2	19	21	61
xARVCF Depletion + Activin	18	2	2	0	22	6
Xp120 Depletion + Activin	17	3	2	0	22	7
xARVCF & Xp120 Double Depletion + Activin	8	3	2	0	13	*

Score 0: not extended. Score 1: slightly extended. Score 2: moderately extended. Score 3: fully extended.

**Figure 6. xARVCF and/or Xp120 depletion inhibits convergent-extension of naive ectoderm explants.** Embryos were injected with standard control morpholino or depleted for xARVCF and/or Xp120 (40 ng of respective morpholino injected into animal hemisphere of each cell of 2-cell embryos). Animal caps were subsequently isolated from stage 8 (blastula) embryos and incubated in the absence or presence of 20 ng/ml human activin A. Elongations were scored on a scale of 0–3, respectively reflecting no, slight, moderate, or full extension relative to activin-treated caps injected with the standard control morpholino. Total scores were calculated by multiplying the number of caps receiving a particular score by that score, and then summing such products across the four score categories. Depletion of xARVCF and/or Xp120 significantly reduced the fraction of caps displaying strong elongations. The chart displays aggregate data from two experiments. The asterisk notes that the score distribution of the xARVCF + Xp120 double depletion resembles that of xARVCF or Xp120 single depletions, although fewer total caps were examined.

DA-Rac (whose levels had been titrated to generate no or little effect when injected in isolation; Tables I and II), we observed the nearly complete rescue of embryos depleted of xARVCF or Xp120 (Figs. 7 and 8). In contrast, neither DA-Cdc42, DN-Cdc42, DA-Rho, or DN-Rac effected rescue of xARVCF- or Xp120-depleted embryos (Fig. S6, available at <http://www.jcb.org/cgi/content/full/jcb.200307109/DC1>). Thus, we demonstrate that xARVCF and Xp120 are functionally linked with RhoA and Rac in a vertebrate developmental system; in contrast to cell line work (Noren et al., 2000), a linkage with Cdc42 was not made evident.

### xARVCF produces a dendritic phenotype when expressed in NIH-3T3 cells and associates with Vav2 and RhoA

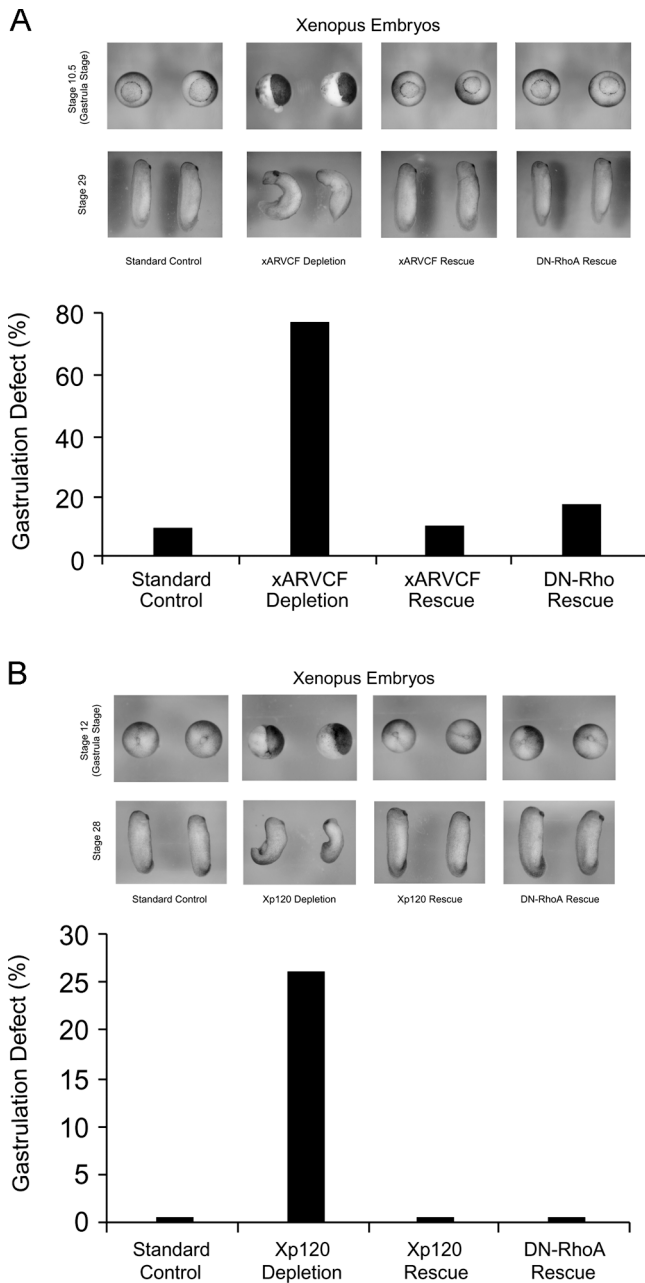
To further test the capacity of xARVCF to functionally interact with RhoA and Rac, we turned to phenotypic assays using mammalian cell lines and coimmunoprecipitation tests from *X. laevis* extracts. When transiently overexpressed in NIH-3T3 cells, xARVCF produced the same dendritic phenotype known to arise after overexpression of p120, a phenotype that further bore similarity in being rescuable upon the coexpression of DA-RhoA (Fig. 9; Anastasiadis et al., 2000).

The shared ARVCF, p120, and rescue phenotypes exhibited in both in vitro and in vivo contexts suggest that xARVCF and Xp120 have underlying protein–protein interactions in common. As previously demonstrated for p120 in mammalian cell line and purified in vitro systems (Introduction), we therefore tested if xARVCF binds to Vav2 (an activator of Rac and other Rho family GTPases) and to RhoA (p120 is believed to act as a Rho GDI). Indeed, after their coexpression in NIH-3T3 cells, we resolved xARVCF's specific coimmunoprecipitation with Vav2 (Fig. S7, available at <http://www.jcb.org/cgi/content/full/jcb.200307109/DC1>). From *X. laevis* embryo extracts, we likewise resolved xARVCF's

specific coimmunoprecipitation with DN-RhoA (6× Myc epitope tagged) as well as Xp120's expected coassociation with DN-RhoA (Fig. S8, available at <http://www.jcb.org/cgi/content/full/jcb.200307109/DC1>). DN-RhoA was used for these coimmunoprecipitations tests because it largely exists in the GDP-bound state to which Rho GDIs preferentially associate. Thus, in vertebrate embryogenesis, xARVCF and Xp120 are each essential and likely to be direct GDIs of RhoA as well as activators of Rac in complex with the activator/GEF protein Vav2.

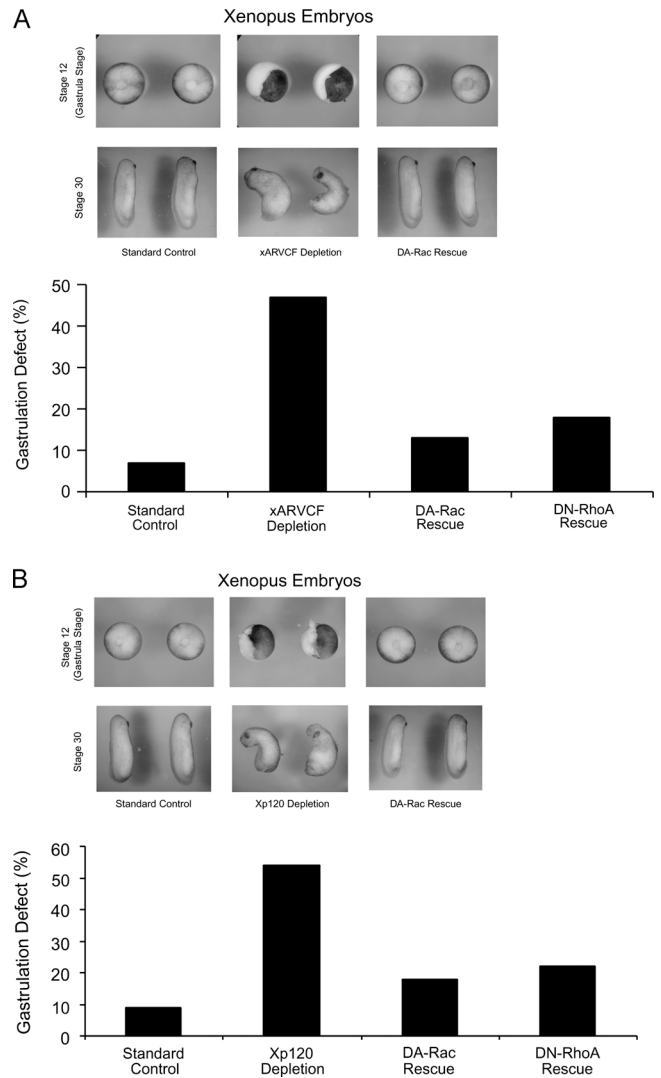
## Discussion

In this work, we find that the structurally homologous xARVCF and Xp120 catenins share down-stream effector pathways based on multiple criteria. First, the targeted (morpholino-directed) depletion of either protein in early *X. laevis* embryos generates similar gross morphological phenotypes, which indicate the requirement of both xARVCF and Xp120 in vertebrate development. Second, the depletion of xARVCF or Xp120 is rescuable via the (properly titrated) exogenous expression of the other catenin, suggesting overlapping embryonic functions. Third, the depletion of xARVCF or Xp120 is rescued to significant degrees upon (properly titrated) exogenous expression of DN-RhoA, DA-Rac, or C-cadherin, indicating that Rho family GTPases and cadherin are mediators of both catenins' functions in vertebrate embryogenesis. Fourth, the overexpression of xARVCF in NIH-3T3 cells produced a phenotype similar to that observed after p120 overexpression, which was further alike in being rescuable via DA-RhoA coexpression. Finally, on the biochemical level, we determined that both xARVCF and Xp120 coprecipitate with RhoA and that xARVCF coprecipitates with Vav2, which is a Rho family (Rac) GEF known to bind p120.



**Figure 7. DN-RhoA rescues xARVCF or Xp120 depletion.** Embryos were injected with standard control morpholino or depleted for xARVCF or Xp120 (20 ng respective morpholino injected into one dorsal cell of 4-cell embryos). Rescues were effected by coinjection of xARVCF or Xp120 morpholino with xARVCF or Xp120 in vitro transcribed mRNA (0.02 ng, self-rescue positive control) or with DN-RhoA mRNA (0.5 pg) and scored at gastrula stages 10–12. (A) DN-RhoA rescues xARVCF depletion to almost self-rescue and standard control injection levels. (B) DN-RhoA rescues Xp120 depletion to self-rescue and standard control injection levels. As depicted, depletion phenotypes and DN-RhoA rescues were apparent at both gastrula and later developmental stages (tailbud and tadpole). See also aggregate depletion/rescue data presented in Tables I and II.

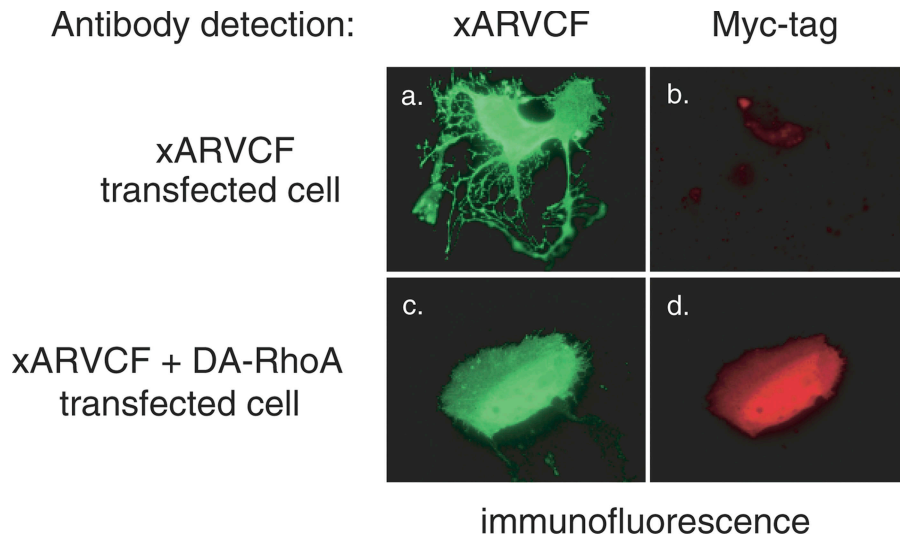
Prior studies conducted in mammalian cell lines are consistent with a number of these observations (Anastasiadis et al., 2000; Noren et al., 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001). Interestingly, the neural cell-specific  $\delta$ -catenin was also shown to functionally interact



**Figure 8. DA-Rac rescues xARVCF or Xp120 depletion.** Embryos were injected with standard control morpholino or depleted for xARVCF or Xp120 (20 ng respective morpholino injected into one dorsal cell of 4-cell embryos). Rescues were effected by coinjection of xARVCF or Xp120 morpholino with 0.5 pg DA-Rac and scored at gastrula stages 10–12. DA-Rac rescues xARVCF (A) or Xp120 (B) depleted embryos to substantial extents relative to standard control injections. As depicted, depletion phenotypes and DA-Rac rescues were apparent at both gastrula and later developmental stages (tailbud and tadpole). See also combined depletion/rescue data presented in Tables I and II.

with RhoA (and cortactin), suggesting that multiple members of the p120 subfamily may interact with small GTPases (Martinez et al., 2003). Thus, in both embryological and cell line contexts, it appears that ARVCF and p120 inhibit RhoA while activating Rac. A question that arises is if xARVCF and Xp120 are redundant with respect to functional interactions with Rho family GTPases or other molecular pathways. Certainly, each catenin's presence is required in the embryo given that xARVCF or Xp120 depletion results in perturbed gastrulation. Furthermore, because xARVCF or Xp120 depletion does not result in the complete absence of either protein product, the nondepleted (surviving) fraction of each catenin is likely to continue ex-





**Figure 9. Overexpression of xARVCF produces a dendritic phenotype in NIH-3T3 cells that is rescuable by DA-RhoA.** NIH-3T3 cells were transfected with xARVCF, together with pcDNA3 (control vector) or pcDNA3-RhoA-(V12)-Myc (DA-RhoA). After 24 h, cells were fixed in PFA and double labeled for immunofluorescent microscopy using anti-xARVCF (polyclonal) and anti-Myc (monoclonal) antibodies to detect xARVCF and RhoA, respectively. (a and c) Cells transfected with xARVCF and stained for xARVCF. (b and d) The same cells stained for the presence of RhoA (Myc epitope-tag). The dendritic phenotype is observed in xARVCF-transfected cells (a), while the reversal of this phenotype upon coexpression of V12-RhoA (DA-RhoA) is evident in panel c.

cutting a subset of xARVCF- versus Xp120-specific functions, which will escape phenotypic detection. Thus, we propose that xARVCF or Xp120 have required and overlapping relationships with Rho family GTPases and cadherins during early *X. laevis* embryogenesis, while additional functions unique to one or the other catenin exist and likely play important biological roles.

In the mouse, ARVCF or p120 knockouts have not yet been published, although work in progress indicates that the functional inactivation of p120 is embryonic lethal (Reynolds, A., L. Elia, and L. Reichardt, personal communications). However, in *Drosophila melanogaster* or *Caenorhabditis elegans*, deletion or depletion (siRNA) of the single p120/ARVCF-like gene product surprisingly results in animals of wild-type appearance and fertility (Myster et al., 2003; Pettitt et al., 2003). However, genetic backgrounds sensitized for cadherin/junctional function reveal significant genetic interactions when crossed with p120-deficient animals, indicating their coupled contribution to invertebrate development. Furthermore, flies deficient in RhoA (*D. melanogaster* Rho1) display functional interactions with p120 (siRNA depletion), while biochemical tests demonstrated RhoA's direct physical association with p120 and  $\alpha$ -catenin (Magie et al., 2002). Thus, although both invertebrate and vertebrate p120/ARVCF appear to interact with Rho family GTPases and with cadherins, the respective impact of reducing p120/ARVCF function differs with vertebrates exhibiting the greater functional dependence.

In our work, effects arising from the morpholino-directed depletion of xARVCF or Xp120 were largely rescued by exogenous introduction of titrated levels of DN-RhoA, DA-Rac, or C-cadherin. Each of those embryonic rescues can be accounted for if endogenous xARVCF/Xp120 inhibits RhoA, activates Rac, and contributes to the function/stabilization of cadherins. We are uncertain as to why human ARVCF overexpression was earlier found to lack activity in producing a dendritic phenotype in mammalian cells (Anastasiadis et al., 2000), but the distinction may conceivably be due to a difference in construct expression levels or an isoform or mutational difference (indeed, upon sequencing the human ARVCF construct used in the other work, we de-

tected a P→L transition at amino acid 222). In any case, consistent with xARVCF acting as an inhibitor/GDI of RhoA in vivo, we find that xARVCF (and Xp120) coprecipitates with DN-RhoA from *X. laevis* extracts and that DA-RhoA rescues the dendritic phenotype of NIH-3T3 cells overexpressing xARVCF. Thus, we expect that xARVCF is an inhibitor/GDI of RhoA as demonstrated for p120 (Anastasiadis et al., 2000; Magie et al., 2002). In further likeness to p120 (Noren et al., 2000), we find that xARVCF biochemically associates with Vav2, an activator/GEF of Rac, and more impressively that the embryonic depletion of xARVCF (or Xp120) is rescued by DA-Rac. Finally, xARVCF or Xp120 depletion results in reduced C-cadherin levels, which when exogenously increased, rescues normal embryogenesis. As indicated in recent papers (Fujita et al., 2002; Ireton et al., 2002; Davis et al., 2003; Xiao et al., 2003), we expect that the mechanism by which xARVCF and Xp120 protein levels are coupled to that of C-cadherin involves the catenins' modulation of the complex's metabolic stability.

Given that DN-Rho, DA-Rac, and C-cadherin rescue xARVCF or Xp120 depletion in vivo, all five proteins likely have coupled functions in embryogenesis. For example, exogenous DA-Rac or DN-Rho have the capacity to compensate for reduced C-cadherin protein levels after xARVCF or Xp120 depletion, perhaps by enhancing the remaining cadherin adhesive (extracellular) and/or signaling (intracellular) function. As alluded to previously, a further possibility is that rescues mediated by DN-Rho and DA-Rac occur via effects on cadherin metabolic stability by way of modulating cadherin endocytosis/destruction or delivery to the plasma membrane (Akhtar and Hotchin, 2001; Fujita et al., 2002; Le et al., 2002; Paterson et al., 2003; Yanagisawa et al., 2003).

Developmental events such as gastrulation require cadherins to be organized in a fashion that is responsive to developmental cues (Gumbiner, 1996; Keller et al., 2000; Teepass et al., 2000). Given the partial reductions in C-cadherin levels observed after xARVCF or xp120 depletion, we were surprised that few effects were evident using various cell–cell adhesion or tissue integrity assays. One possibility is that C-cadherin extracellular adhesive activity is indeed reduced but

occurs too subtly to be detected in the assays used. This view is supported by our observation that of the partial C-cadherin constructs tested, the EC produced the largest fractional rescue of embryos depleted for xARVCF or Xp120. Further, it is possible that C-cadherin's intracellular signaling (Rac activation?) capacity is reduced upon xARVCF or Xp120 depletion as a simple consequence of lessened C-cadherin levels, perhaps accounting for the fractional capacity of C-cadherin's JMR domain to rescue Xp120 depletion. Interestingly, because JMR mutants incapable of direct associations with xARVCF or Xp120 continued to display fractional rescuing activities, unknown JMR associations are likely to play a significant role in C-cadherin's cytoplasmic rescuing activity (Fedor-Chaiken et al., 2003; Ozawa, 2003). In any case, various laboratories will undoubtedly continue to direct attention to the role of cadherin in activating or inhibiting Rho family members (outside-in signaling; Kim et al., 2000; Nakagawa et al., 2001; Noren et al., 2001; Goodwin et al., 2003) as well as the activity of Rho family GTPases on cadherin function (inside-out signaling; Wunnenberg-Stapleton et al., 1999; Braga, 2000; Fukata and Kaibuchi, 2001; Ehrlich et al., 2002; Magie et al., 2002). Our principal contribution here is in demonstrating that ARVCF and p120 are required for vertebrate development and that each catenin is closely functionally linked to cadherin, RhoA, and Rac in vivo.

## Materials and methods

### MOs

MOs (25-mer) were obtained from Gene Tools, LLC and used in *X. laevis* embryos in accordance with published procedures (Heasman et al., 2000). The sequences of two *X. laevis* p120 morpholinos (Xp120-MO I and Xp120-MO II) and of two *X. laevis* xARVCF morpholinos (xARVCF-MO I and xARVCF-MO II) were as follows: Xp120-MO I, 5'-ACTCTGGCTCATC-CATATAGAAAGG-3'; Xp120-MO II, 5'-AGAGACCCAAAGTTCACACTC-CAGGC-3'; xARVCF-MO I, 5'-CTGTTGGAGTTCGGCAGGCATCTAT-3'; and xARVCF-MO II, 5'-ACACTGGCAGACCTGAGCCTATGGC-3'. A morpholino standard (negative) control oligo was also obtained from Gene Tools, LLC (5'-CCTCTTACCTCAGTTACAATTATA-3'). Morpholinos were targeted against the following mRNA sequences relative to the translational start site (ATG): Xp120-MO I (-9 to +13), xARVCF-MO I (-4 to +18), Xp120-MO II (-34 to -10), and xARVCF-MO II (-75 to -50). GenBank searches failed to detect significant complementarity of the Xp120 morpholinos to xARVCF mRNA or of the xARVCF morpholinos to Xp120 mRNA. The morpholinos were resuspended in Nuclease-Free Water (Ambion) to the experimental concentrations required for microinjection.

### *X. laevis* embryos and microinjection

*X. laevis* females were induced to lay eggs, which were fertilized and microinjected using published procedures (Montross et al., 2000). MOs and mRNA constructs were microinjected into the animal hemisphere of one or two blastomeres in 1-, 2-, or 4-cell cleavage stage embryos. The embryos were placed within a solution of 5% ficoll in 1× MMR for culture at 18°C. The total volume of MOs and/or mRNA constructs injected was 20 nl, with the total doses of 20 or 40 ng for morpholinos, 0.02 ng for Xp120 or xARVCF mRNA, and 0.5 µg for RhoA or Rac mRNA. Embryonic phenotypes were observed and evaluated using a standard binocular dissecting microscope (model SMZ-U; Nikon).

### RNA and in vitro transcription

The Xp120<sup>ctn</sup> isoform 1 (Xp120 iso1) construct was PCR generated using primers 5'-GAATTCGATGGATGAGCCAGAG-3' and 5'-GAATTCACCGCTGATCTTC-3' and subcloned into pCS2+MT (Myc epitope tag) at the EcoRI site (Kim et al., 2002). xARVCF isoform 1A (xARVCF-1A) construct was PCR generated using primers 5'-GCTCTAGAATGATGCAG-GAAC-3' and 5'-GCTCTAGACCCAAAAAGGGTCACTGC-3' and subcloned into pCS2+HA (HA epitope tag) at the XbaI site (Kim et al., 2002).

RhoA and Rac (rat) constructs were a gift from M. Symons (North Shore Long Island Jewish Research Institute, Bronx, NY). They were moved from the original EXV vector by EcoRI digestion and subcloned into the EcoRI site of pCS2+.

Capped mRNAs encoding these constructs were generated in vitro using the SP6 mMessage mMachine kit (Ambion) according to the manufacturer's protocol. Unincorporated nucleotides were removed by filtration through Sephadex G-50 Quick Spin Columns (Roche Applied Science). The quantity and quality of transcribed mRNA products were evaluated on the basis of migration within 1% agarose formaldehyde gels and by optical density (OD 280/260).

### Antibodies

Xp120 polyclonal antibodies were generated against the NH<sub>2</sub>- or COOH-terminal domains of Xp120 (corresponding to amino acids 43–176 and 789–860). Each fragment was subcloned into the pQE32 vector having an NH<sub>2</sub>-terminal 6× His tag (QIAexpress System; QIAGEN). The 6× His-Xp120 fusion proteins were expressed and purified using Ni-NTA matrices according to the manufacturer's instructions (QIAGEN) and raised at the institutional antibody core facility (University of Texas M.D. Anderson Cancer Center). The xARVCF polyclonal antibody was generated as described previously (Paulson et al., 2000). The C-cadherin polyclonal antibody was generated against the EC of *X. laevis* C-cadherin protein purified in the same manner as Xp120 but was raised by Sigma-Genosys. Actin polyclonal antibody was purchased from Sigma-Aldrich.

### Western blots

To evaluate endogenous or exogenous protein levels, embryos were harvested at the indicated developmental stages and lysates were prepared for SDS-PAGE/Western blotting according to published procedures (1:5,000 dilution of antibodies directed against xARVCF, Xp120 NH<sub>2</sub> terminus, Xp120 COOH terminus, or C-cadherin; Kim et al., 2002).

### Explant elongation assay

Morpholino oligos and/or mRNAs were injected at the 2-cell stage in the animal pole region of both blastomeres. Animal caps were dissected from injected embryos at stage 8 and incubated at 18°C overnight in 0.4× MMR with or without human activin A (R&D Systems) at a final concentration of 20 ng/ml. Elongations of the isolated animal caps were evaluated using a standard binocular dissecting microscope (model SMZ-U; Nikon).

### Immunofluorescence of NIH-3T3 cells

Immunofluorescence localization procedures have been described previously (Anastasiadis et al., 2000). The primary antibodies used were directed against xARVCF (polyclonal, 1:20,000) or Myc (9E10 monoclonal, 1 µg/ml). Anti-murine-p120 mAb 12F4 was used in positive control assays (1 µg/ml). The secondary antibodies used were cross-absorbed goat anti-mouse Alexa 488 (green; Molecular Probes) and goat anti-rabbit Alexa 596 (red; Molecular Probes; 1:600 dilutions). Coverslips were mounted on glass slides using PolyMount (Polysciences, Inc.) and visualized under a fluorescent microscope (model Optiphot 2; Nikon) with Plan Fluor (Nikon) 40× magnification (0.85 numerical aperture). Photos were acquired with the Metamorph program using a CCD camera (model CoolSnap Fx; Photometrics) and compiled in Photoshop and PowerPoint.

### Image acquisition and manipulation

Image acquisition and manipulation procedures are described elsewhere in Materials and methods in accordance with the indicated assays.

### Online supplemental material

Eight supplemental figures and legends indicate the following: (a) cell-cell interactions or tissue integrity is not graphically altered upon xARVCF or Xp120 depletion (Figs. S1–S4); (b) C-cadherin deletion or point mutants partially rescue Xp120 depletion (Fig. S5); (c) DA-Cdc42, DA-Rho, or DN-Rac do not rescue xARVCF or Xp120 depletion (Fig. S6); (d) xARVCF coimmunoprecipitates with Vav2 (Fig. S7); and (e) xARVCF and Xp120 coimmunoprecipitate with DN-RhoA from *X. laevis* embryo extracts (Fig. S8). Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200307109/DC1>.

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