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Wnt-5A/Ror2 Regulate Expression of XPAPC through an Alternative Noncanonical Signaling Pathway

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SUMMARY

XWnt-5A, a member of the nontransforming Wnt-5A class of Wnt ligands, is required for convergent extension movements in *Xenopus* embryos. XWnt-5A knockdown phenocopies paraxial protocadherin (XPAPC) loss of function: involuted mesodermal cells fail to align mediolaterally, which results in aberrant movements and a selective inhibition of constriction. XWnt-5A depletion was rescued by coinjection of *XPAPC* RNA, indicating that XWnt-5A acts upstream of XPAPC. XWnt-5A, but not XWnt-11, stimulates *XPAPC* expression independent of the canonical Wnt/β-catenin pathway. We show that transcriptional regulation of *XPAPC* by XWnt-5A requires the receptor tyrosine kinase Ror2. XWnt-5A/Xror2 signal through PI3 kinase and cdc42 to activate the JNK signaling cascade with the transcription factors ATF2 and c-*jun*. The Wnt-5A/Ror2 pathway represents an alternative, distinct branch of noncanonical Wnt signaling that controls gene expression and is required in the regulation of convergent extension movements in *Xenopus* gastrulation.

INTRODUCTION

During gastrulation, the mesoderm undergoes highly coordinated mass cell movements. Four types of cell movements are observed in *Xenopus* gastrulation: vegetal rotation, substrate-dependent migration of the head mesoderm, convergent extension (CE) of the trunk mesoderm, and epiboly of the ectoderm. CE movements of the notochord and paraxial mesoderm make major contributions to shaping the dorsal body axis. After involution, the originally multipolar mesodermal cells polarize and acquire a typical bipolar shape, align with their long axes in parallel to the embryo's mediolateral axis, and start to intercalate mediolaterally (reviewed in Keller et al., 2003). These intercalation movements result in narrowing and simultaneous elongation of the tissue and are tightly regulated by a balanced activity of different signaling pathways, which include canonical and noncanonical Wnt pathways (Kuehl et al., 2001; Tada and Smith, 2000).

Wnt ligands are commonly subdivided into two classes according to their ability to induce secondary body axes in *Xenopus* embryos and to morphologically transform mouse C57MG mammary epithelial cells (Du et al., 1995; Wong et al., 1994). The transforming Wnt-1 class ligands (Wnt-1, Wnt-3a, Wnt-8) act through the canonical Wnt/β-catenin pathway (Miller, 2001; Shimizu et al., 1997), while the nontransforming members of the Wnt-5A class (Wnt-5A, Wnt-4, Wnt-11) stimulate β-catenin-independent, noncanonical pathways (Slusarski et al., 1997; Tada and Smith, 2000). In vertebrates, noncanonical Wnt pathways are currently further subdivided into the Wnt/PCP pathway, which is closely related to planar polarity signaling in *Drosophila* (Fanto and McNeill, 2004), and the Wnt/Ca^{2+} pathway (Kuehl et al., 2000b; Slusarski et al., 1997). Recent work showed that the Frizzled receptors and the presence of LRP 5/6 coreceptors play a crucial role in the selectivity between canonical and noncanonical pathways (Cadigan and Liu, 2006; Hsieh, 2004), and that Wnt ligands of the nontransforming Wnt-5A class can also activate canonical Wnt signaling (Mikels and Nusse, 2006; Tao et al., 2005). The recent discovery of non-Frizzled receptors for Wnt ligands, including Ryk and Ror2, adds further complexity to the current view of Wnt signaling (Hikasa et al., 2002; Keeble and Cooper, 2006; Oishi et al., 2003).

In our previous work, we have shown that coordination of cell polarity and movements during CE in *Xenopus* requires the activity of paraxial protocadherin (PAPC) in addition to noncanonical Wnt signaling (Unterseher et al., 2004).

Here, we report that XWnt-5A knockdown, but not XWnt-11 knockdown, phenocopies XPAPC loss of function, and that XWnt-5A regulates *XPAPC* gene expression in early *Xenopus* development. This function of XWnt-5A is mediated by the Ror2/JNK signaling cascade. We have further characterized this signaling pathway and show that Phosphoinositide 3 kinase (PI3K), cdc42, and MKK7 activate JNK downstream of Ror2, which, in turn, leads to upregulation of *XPAPC* transcription mediated by c-*jun* and ATF2. With this work we identify Wnt-5A/Ror2 signaling as a distinct branch of noncanonical Wnt signaling pathways and demonstrate a physiological function of this pathway in early embryonic development.

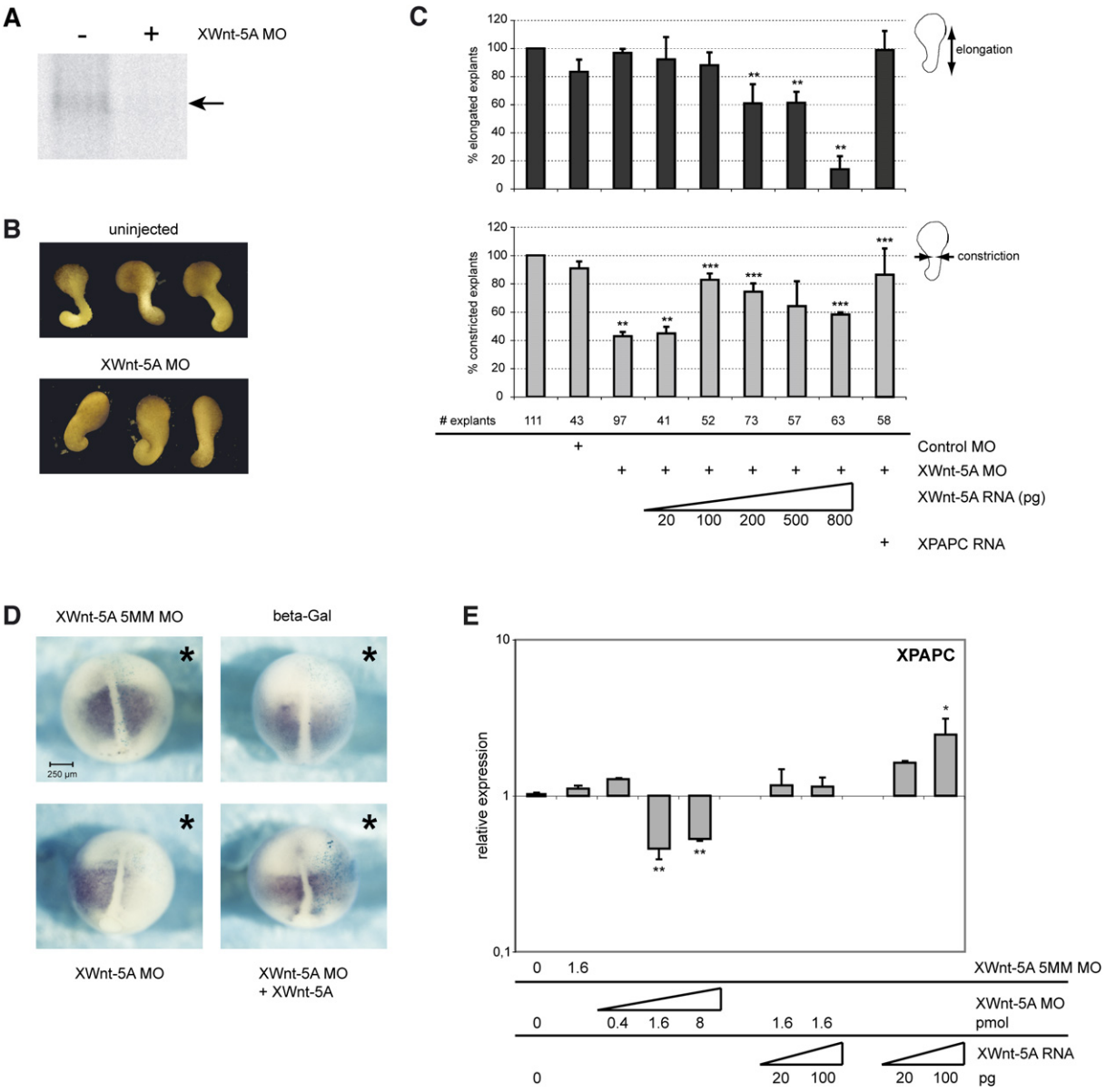


Figure 1. XWnt-5A Acts Upstream of XPAPC

(A) Autoradiograph of ^{35}S-methionine-labeled in vitro translation of a morpholino-sensitive XWnt-5A construct in the absence and presence of XWnt-5A MO shows blocking of XWnt-5A translation by the MO.

(B) XWnt-5A knockdown phenocopied the selective inhibition of constriction of XPAPC loss of function in Keller open-face explants.

(C) Explants were scored for elongation (upper panel) and constriction (lower panel). XWnt-5A MO was rescued by coinjection of MO-insensitive *XWnt-5A* RNA and by *XPAPC* RNA (***p > 0.99 to XWnt-5a MO).

(D) In situ hybridization with an XPAPC probe showed downregulation of *XPAPC* by XWnt-5A MO, but not by XWnt-5A 5MM MO, and was rescued by coinjection of a MO-insensitive *XWnt-5A* RNA. The injected side is labeled with X-Gal and is indicated with an asterisk.

(E) Transcriptional regulation of *XPAPC* by XWnt-5A and the specificity of the effect were confirmed by real-time RT-PCR.

All charts show mean ± SEM (*p > 0.95; **p > 0.99 to controls).

RESULTS

XWnt-5A Regulates *XPAPC* Transcription

We analyzed the effects of Wnt-5A loss of function on CE movements by using antisense morpholino oligonucleotides (MOs). Suppression of XWnt-5A protein translation was confirmed in an in vitro transcription assay (Figure 1A). In Keller open-face explants we observed that XWnt-5A MO injection phenocopied the XPAPC-depletion phenotype (Unterseher et al., 2004), a selective inhibition of constriction, but not elongation of explants (Figures 1B and 1C). The phenotype was rescued by coinjection

of a morpholino-insensitive *XWnt-5A* RNA in a dose-dependent manner. At higher doses, we again observed a decrease in constricted explants as well as a strong inhibition of explant elongation (Figure 1C), as reported previously for XWnt-5A overexpression (Kuehl et al., 2001; Torres et al., 1996). Coinjection of *XPAPC* RNA was sufficient to restore normal constriction and did not affect elongation in Keller open-face explants, resulting in a full rescue of the XWnt-5A MO phenotype (Figure 1C) and confirming that the observed XWnt-5A MO phenotype in Keller open-face explants correlated with XPAPC loss of function. In situ hybridizations revealed that XWnt-5A knockdown resulted in downregulation of *XPAPC*, while control injections had no effect, and coinjection of morpholino-insensitive *XWnt-5A* RNA restored the normal *XPAPC* pattern (Figure 1D).

These observations indicate that XWnt-5A acts upstream of XPAPC in the regulation of CE movements and point toward a transcriptional regulation. Therefore, we analyzed the effects of XWnt-5A on *XPAPC* mRNA levels by quantitative real-time RT-PCR. XWnt-5A MO, but not XWnt-5A 5-mismatch (5MM) MO, injections resulted in a significant downregulation of *XPAPC* mRNA (Figure 1E). Coinjection of morpholino-insensitive *XWnt-5A* RNA restored *XPAPC* transcription to the levels of uninjected control embryos, and XWnt-5A overexpression upregulated *XPAPC* (Figure 1E).

These results indicate that XWnt-5A is required for CE movements in the regulation of *XPAPC* expression. During gastrula stages, XWnt-5A was expressed in the deep layer of the ectoderm, a cell layer that lies directly adjacent to the XPAPC-expressing involuting mesoderm (Figure S1; see the Supplemental Data available with this article online). Thus, XWnt-5A could be involved in the regulation of *XPAPC* expression in the underlying involuting mesoderm.

To investigate whether this regulation is specific for *XPAPC*, we analyzed the transcriptional levels of a number of other genes expressed in Spemann's organizer or the dorsal mesoderm. Transcription of *chordin*, *goosecoid* (*gsc*), *Xbra*, and *XB-cadherin* was not significantly altered by XWnt-5A depletion or by XWnt-5A overexpression; however, *Xlim-1* was regulated in a manner similar to *XPAPC* (Figure S2A).

To investigate the mechanism of XPAPC regulation by XWnt-5A, we tested XWnt-5A's ability to induce *XPAPC* transcription in Animal Cap explants. The Animal Cap of blastula-stage embryos is a pluripotent tissue of ectodermal origin that is able to differentiate into a number of tissues depending on the combination of growth factors. In naive Animal Caps, only very few copies of *XPAPC* RNA were detectable (data not shown); however, injection of *XWnt-5A* RNA was sufficient to induce a robust *XPAPC* expression (Figure 2A). The upregulation of *XPAPC* was not due to general mesoderm induction, as XWnt-5A failed to induce the pan-mesodermal marker *Xbra*. To exclude early effects of the injected RNA, we also used conditioned medium (CM) of a Wnt-5A-producing cell line (Kispert et al., 1998). *XPAPC* was induced to the same extent by both Wnt-5A CM and *XWnt-5A* RNA injection, while a control medium had no effect (Figure 2A). We next investigated whether protein translation was required for Wnt-5A-induced *XPAPC* expression. The addition of Cycloheximide (CHX) did not abolish *XPAPC* induction by Wnt-5A CM, and *XPAPC* was not induced significantly by the addition of CHX to the control medium (Figure 2B). In a control experiment, we observed the induction of *gsc* in a CHX-dependent manner (Figure S2B).

These results show that protein translation is not required and indicate direct regulation of *XPAPC* gene expression. It has been reported that *XPAPC* becomes upregulated in response to mesoderm induction (Hukriede et al., 2003; Medina et al., 2004), by canonical Wnt signaling (Wessely et al., 2004), and by Xlim-1 (Hukriede et al., 2003). We therefore investigated whether XWnt-5A interacted with any of these known activators of *XPAPC* transcription. These experiments did not yield hints on the potential mechanism of *XPAPC* regulation by XWnt-5A and excluded the Wnt/β-catenin pathway and Xlim-1 as mediators of XWnt-5A signaling (Figures S2C, S2D, and S3). In addition, the insensitivity of XWnt-5A-stimulated *XPAPC* transcription to CHX indicated a translation-independent, direct regulation. This prompted us to carry out a more thorough evaluation of the pathway downstream of XWnt-5A.

JNK Mediates Regulation of *XPAPC* by XWnt-5A

In the literature, PKC and CamK II (Kuehl et al., 2000a; Moon et al., 1993; Slusarski et al., 1997; Torres et al., 1996), JNK (Yamanaka et al., 2002), and NF-AT (Saneyoshi et al., 2002), effectors of Wnt/Ca^{2+} signaling, have been reported to act downstream of XWnt-5A. Therefore, we investigated the potential of these factors to modulate *XPAPC* expression.

The activation or inhibition of the Wnt/Ca^{2+} signal transducer PKC or injection of constitutively active mutants of CamK II and NF-AT had no effect (Figure 2C). However, activation of the JNK signaling cascade by injection of *ca MKK7* RNA induced upregulation of *XPAPC* to the same level as injection of *XWnt-5A* RNA. Consistently, JNK Inhibitor reduced *XPAPC* mRNA levels comparable to injection of XWnt-5A MO (Figure 2C). Western blot analysis confirmed that XWnt-5A overexpression increased, and XWnt-5A depletion significantly decreased the amount of the active phosphorylated forms of MKK7 and JNK, which was restored by coinjection of MO-insensitive *XWnt-5A* RNA (Figure 2D). Total levels of MKK7 and JNK were not affected by XWnt-5A overexpression or depletion (Figure 2D).

JNK is involved in multiple cellular signaling processes and activates the transcription factors jun, fos, and ATF2. To investigate whether transcriptional regulation of XPAPC was indeed mediated by the JNK signaling cascade, we injected dominant-negative mutants of c-*jun* and ATF2. Both, dn jun and dn ATF2 downregulate *XPAPC* (Figure 2E), confirming that *XPAPC* gene expression depends on c-*jun* and ATF2.

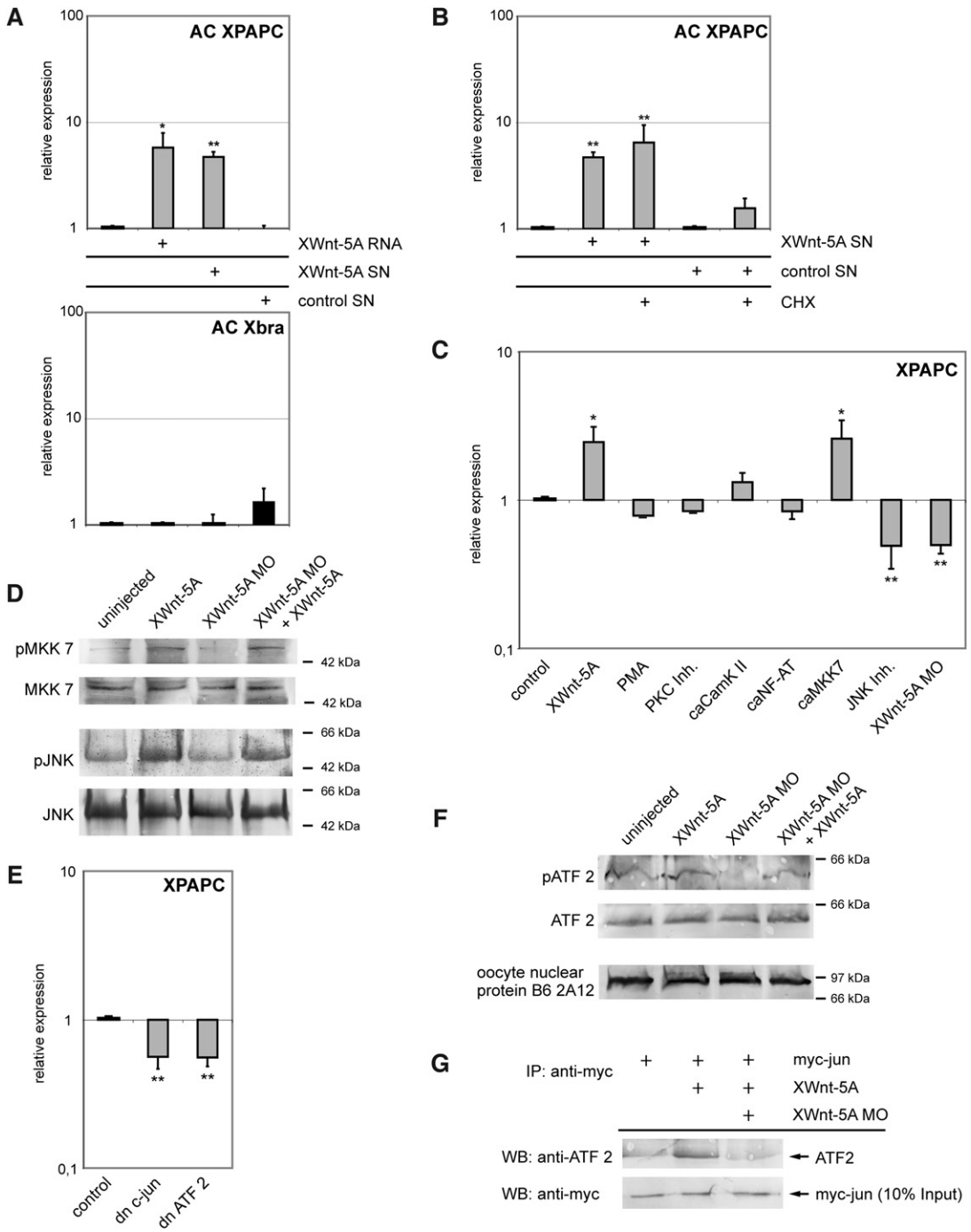


Figure 2. XWnt-5A Regulates *XPAPC* Transcription through a JNK Signaling Pathway

(A) XWnt-5A was sufficient to induce *XPAPC* in Animal Caps. Similar levels of *XPAPC* transcription were obtained in Animal Caps injected with *XWnt-5A* RNA and in Animal Caps treated with Wnt-5A CM (XWnt-5a SN), but not with control medium (control SN). *Xbra* was not induced by either of these treatments.

(B) XWnt-5A induced *XPAPC* independent of protein translation. Animal Caps were treated with Wnt-5A CM or control medium in the absence and presence of Cycloheximide (CHX).

(C) *XPAPC* was upregulated by injection of *XWnt-5A* or *ca MKK* RNA and was downregulated by XWnt-5A knockdown or inhibition of JNK. Neither treatment with PMA or PKC Inhibitor nor injection of *ca CamK II* or *ca NF-AT* RNA had any effect.

(D) Western blot of embryo extracts showed that XWnt-5A depletion reduced the amounts of phospho-MKK7 and phospho-JNK, which were restored by coinjection of the MO-insensitive RNA. Full-length JNK and full-length MKK7 amounts were unchanged.

(E) Injection of dn jun or dn ATF2 significantly downregulated *XPAPC*.

The XWnt-5A-dependent regulation of c-*jun* and ATF2 activity was shown by western blot analysis of phospho-ATF2 and coimmunoprecipitation of c-*jun* and ATF2 in XWnt-5A-overexpressing and XWnt-5A-depleted embryos. *XWnt-5A* RNA injection caused a moderate increase in phospho-ATF2, but XWnt-5A knockdown led to significantly reduced levels of endogenous phospho-ATF2 in gastrula-stage *Xenopus* embryos and was rescued by coinjection of MO-insensitive *XWnt-5A* RNA (Figure 2F). The total protein level of ATF2 remained unchanged. Consistently, the amount of ATF2 that coimmunoprecipitated with a myc-c-*jun* fusion construct was significantly increased by XWnt-5A overexpression and decreased by XWnt-5A MO injections (Figure 2G). These data clearly indicate that XWnt-5A regulates *XPAPC* expression by a JNK signaling pathway and requires c-*jun* and ATF2 as transcriptional activators.

XWnt-5A Signals through Xror2

It has recently been reported that Wnt-5A-induced JNK signaling can be mediated by the Ror2 receptor tyrosine kinase (Mikels and Nusse, 2006; Oishi et al., 2003). The *Xenopus* homolog of Ror2 is expressed in the dorsal mesoderm and ectoderm of gastrula-stage embryos and functionally interacts with noncanonical Wnt signaling pathways in the regulation of CE movements (Hikasa et al., 2002). Based on our data, we speculated whether Ror2 might be the receptor of XWnt-5A in this signaling cascade and investigated the role of Xror2. Xror2 MO efficiently depleted the embryos of Xror2 protein, as shown by western blotting against the endogenous protein (Figure 3A). Injection of Xror2 MO, but not Xror2 5MM MO, caused a significant downregulation of *XPAPC* transcription that was rescued by coinjection of a morpholino-insensitive *Xror2* RNA in a dose-dependent manner (Figure 3B). In Animal Caps, coinjection of *XWnt-5A RNA* with Xror2 MO completely abolished the ability of XWnt-5A to induce *XPAPC*, while coinjection of *Xror2* RNA or Xror2 5MM MO did not significantly alter the induction of *XPAPC* compared to *XWnt-5A* RNA alone (Figure 3C).

In the embryo, coinjection of XWnt-5A MO with *Xror2* RNA restored *XPAPC* transcription to control levels (Figure 3D). We also coinjected Xror2 3I (Hikasa et al., 2002), an Xror2 construct carrying three point mutations in the kinase domain. Xror2 3I was not able to restore *XPAPC* transcription in XWnt-5a- or Xror2-depleted embryos (Figure 3D), indicating that kinase activity was required for Xror2 function downstream of XWnt-5A. Functional studies with Keller open-face explants further demonstrated that Xror2 MO injections phenocopied XPAPC depletion and caused a strong inhibition of constriction with little effect on explant elongation (Figure 3E). Constriction was rescued by coinjection of the morpholino-insensitive RNA, and also by *ca MKK7* RNA and by *XPAPC* RNA (Figure 3F), which clearly confirmed that Xror2 acted upstream of MKK7 and XPAPC.

PI3K and cdc42 Mediate XWnt5A/Xror2 Signals

In *Xenopus*, overexpression phenotypes of Xror2 could be rescued by coinjection of dominant-negative cdc42 (Hikasa et al., 2002). cdc42 belongs to the Rho family of small GTPases, which can be activated downstream of receptor tyrosine kinases through PI3K, as in the case of PDGF receptor (Heldin et al., 1998), and they activate the JNK signaling cascade through mixed-lineage kinases (reviewed in Gallo and Johnson, 2002). A recent study also showed that Ror2-mediated Wnt-5A signaling was insensitive to Pertussis toxin (PTX) (Mikels and Nusse, 2006).

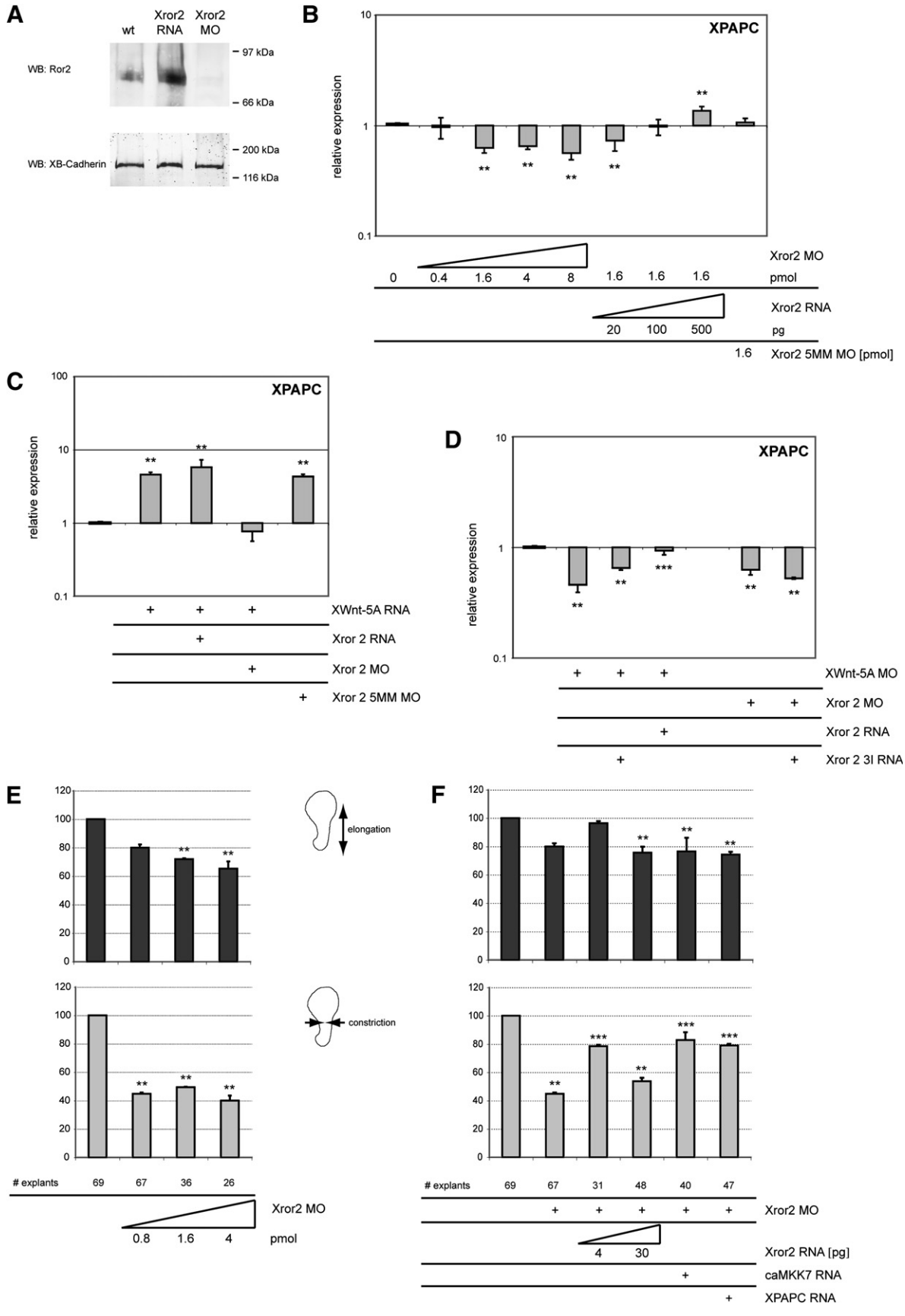
To investigate the role of small GTPases, we injected dominant-negative mutants of Rac 1, Rho A, and cdc42 and found that only dn cdc42 considerably downregulated *XPAPC* mRNA levels (Figure 4A). The effect in the case of dn cdc42 was not as strong as for XWnt-5A MO or Xror2 MO; however, increasing the dose of injected *dn cdc42* RNA was lethal before the embryos reached gastrula stage. These results indicate that cdc42 might be part of the Wnt-5A/Ror2 pathway. To allow stimulation with Wnt-5A CM and application of inhibitors, we again used Animal Caps. We observed that the PI3K inhibitor Wortmannin, dn cdc42, and JNK Inhibitor significantly blocked XWnt-5A stimulated induction of *XPAPC*, while PTX had no effect (Figure 4B).

For further confirmation of these findings, we analyzed the physiological role of cdc42, JNK, c-*jun*, and ATF2 in Keller open-face explants. We observed that Wortmannin treatment, injection of dn cdc42, treatment with JNK Inhibitor, and injection of dn jun or dn ATF2 phenocopied XWnt-5A depletion (Figure 4C). Coinjection of ca cdc42 or ca MKK7 fully rescued the XWnt-5A knockdown in Keller open-face explants (Figure 4C), which demonstrated that cdc42 and MKK7 acted downstream of XWnt-5A. Consistently, ca cdc42 and ca MKK7 rescued *XPAPC* transcriptional levels in XWnt-5A-depleted embryos (Figure 4D). We could further confirm that Wortmannin downregulated *XPAPC* mRNA levels, downstream of XWnt-5A and upstream of ca cdc42 and ca MKK7, and that cdc42 acted upstream of MKK7 and c-*jun* in this signaling cascade (Figure S4).

XWnt-5A Function in CE Is Not Redundant to XWnt-11/PCP Signaling

The role of Wnt-11/PCP signaling in the control of CE movements in *Xenopus* and zebrafish has been studied intensively in the past. In zebrafish, Wnt-11 and Wnt-5A show partially redundant functions (Westfall et al., 2003). Therefore, we investigated the relationship between the Wnt-5a/Ror2 pathway described here and XWnt-11 signaling in gastrulating *Xenopus* embryos. We coinjected

(F) Western blotting of embryo extracts as in (D) confirmed the specific reduction of phospho-ATF2, but not the total amount of ATF2 protein, as a result of XWnt-5A MO injection; oocyte nuclear protein (B62A12 mAb) was used as the loading control. (G) *XWnt-5A* RNA increased and XWnt-5A MO decreased the amount of ATF2 that coimmunoprecipitated with a myc-tagged c-*jun* construct.
All bars represent means ± SEM (**p > 0.99; *p > 0.95 to controls).


A
wt
Xror2 RNA
Xror2 MO
WB: Ror2
97 kDa
66 kDa
WB: XB-Cadherin
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XPAPC RNA

XWnt-5A MO with increasing doses of *XWnt-11* RNA and analyzed Keller open-face explant phenotypes and *XPAPC* mRNA levels. In Keller open-face explants coinjection of XWnt-5A MO with XWnt-11 did not restore constriction, but caused a significant inhibition of explant elongation (Figure 5A). The inhibition of explant elongation corresponds to the XWnt-11 overexpression effect (data not shown). Real-time RT-PCR experiments showed that coinjection of *XWnt-11* RNA did not alter *XPAPC* mRNA levels in XWnt-5A-depleted embryos, and that neither XWnt-11 overexpression nor XWnt-11 depletion affected *XPAPC* transcription (Figure 5B).

We also investigated the ability of DshΔDIX, ca Rho A, and ca Rac 1 as effectors of Wnt/PCP signaling to rescue XWnt-5A knockdown phenotypes in Keller open-face explants. DshΔDIX and ca Rac 1 were not able to restore constriction in XWnt-5A-depleted explants (Figure 5C). With ca Rho A, we observed a partial rescue (Figure 5C), which is likely due to the role of Rho A downstream of XPAPC (Unterseher et al., 2004). In contrast to XWnt-5A or XPAPC depletion, XWnt-11 knockdown affected only explant elongation, not constriction. The inhibition of elongation was dose dependent (data not shown) and was rescued by coinjection of MO-insensitive *XWnt-11* RNA, but not by *XWnt-5A* RNA (Figure 5D). Furthermore, XWnt-11 MO was rescued by coinjection of DshΔDIX, but not by ca MKK7 or XPAPC (Figure 5D). These results further confirm that XWnt-5A and XWnt-11 act in a nonredundant manner and through distinct pathways to regulate CE movements.

A detailed analysis of cell shape and behavior further supports the distinct roles of XWnt-5a and XWnt-11 in gastrulation.

The change from multipolar to elongated bipolar cell shape was not affected in XWnt-5A-depleted cells, as reflected by an average length-to-width ratio (LWR) of 2.28 in XWnt-5A MO-injected cells compared to 2.5 in control cells (Table 1). However, XWnt-5A-depleted cells failed to align to the mediolateral axis of the embryo. Only 53% of XWnt-5a-depleted cells, but 93% of control cells, showed mediolateral orientation (Table 1). The trajectories of cells in XWnt-5A MO-injected explants showed random movements and frequent changes of orientation, which resulted in a strong reduction of net dorsal movement and net dorsal speed, while the overall speed was only slightly reduced compared to control cells (Figures 6A and 6B; Movies S1 and S2). This behavior was very similar to that found in Xror2- (Table 1) or XPAPC-depleted cells (Unterseher et al., 2004). In contrast, XWnt-11 MO injection resulted in a strong inhibition of cell motility and in a decrease of both total and net dorsal speeds (Figures 6A and 6B; Movie S3). In addition, XWnt-11 loss of function prevented cell polarization; cells showed an average LWR of 1.25, making an evaluation of mediolateral orientation not applicable (Table 1).

When we took a closer look at the cellular behavior, we observed that XWnt-5A-depleted cells showed a 7-fold higher number of filopodia compared to control cells, while the number of lamellipodia was slightly reduced (Figures 6C and 6D). Additionally, we observed shorter lifetimes of both lamellipodia and filopodia (Table 1). XWnt-11 MO-injected cells did not form lamellipodia at all, while the number of filopodia was similar to that of control cells. Additionally, we observed short-lived blebbing in XWnt-11-depleted cells, which was not seen in control or XWnt-5A MO-injected cells (Figures 6C and 6D). Xror2-depleted or XPAPC-depleted cells behaved very similarly to XWnt-5A MO-injected cells (Table 1). These results further stress the different effects of XWnt-5A and XWnt-11 on mesodermal cells during CE movements and confirm the functional coupling of XWnt-5A, Xror2, and XPAPC.

DISCUSSION

In this work, we have characterized the role of the Wnt-5A/Ror2 pathway as a transcriptional regulator of *XPAPC* during gastrulation in *Xenopus*. The Wnt-5A/Ror2 pathway has to be considered a noncanonical Wnt pathway, because it does not involve β-catenin and LEF/TCF transcription factors. Like other noncanonical Wnt pathways, both gain of function and loss of function inhibited CE movements. However, while XWnt-5A depletion selectively affected constriction in Keller open-face explants, coinjection of high amounts of an MO-insensitive *XWnt-5A* RNA caused a strong inhibition of Keller open-face explant elongation. This phenotype had been reported earlier for XWnt-5A overexpression (Moon et al., 1993), and similarly for Xror2 overexpression (Hikasa et al., 2002), and is most likely due to the inhibition of canonical Wnt signaling (Kuehl et al., 2001; Mikels and Nusse, 2006). Our results further exclude a PTX-sensitive G protein, PKC, CamK II, and NF-AT as downstream effectors of Wnt-5a/Ror2 signaling, which is in agreement with the observation that Wnt-5a/Ror2 antagonized canonical Wnt

Figure 3. XWnt-5A Acts through Xror2 to Induce *XPAPC*

(A) Amounts of detected Xror2 protein were increased in extracts from *Xror2* RNA-injected embryos compared to uninjected controls. Injection of Xror2 MO efficiently depleted the embryos of endogenous Xror2; XB-Cadherin was used as the loading control.

(B) Xror2 depletion resulted in downregulation of *XPAPC* and was rescued by coinjection of an MO-insensitive *Xror2* RNA.

(C) Coinjection of 4 pmol Xror-2 MO, but not Xror2 5MM MO, abolished the ability of *XWnt-5A* RNA to induce *XPAPC* in Animal Cap explants, while coinjection of *Xror2* RNA slightly enhanced *XPAPC* upregulation.

(D) Kinase-dead Xror2 3I could not rescue *XPAPC* transcription in XWnt-5A- or Xror2-depleted embryos, but wild-type *Xror2* RNA did (***$p > 0.99$ to XWnt-5A MO).

(E) Xror2 loss of function had little effect on Keller open-face explant elongation (upper panel), but it significantly blocked constriction (lower panel) and thus phenocopied XWnt-5A and XPAPC depletion.

(F) Constriction was rescued in Xror2-depleted explants by coinjection of MO-insensitive *Xror2* RNA, ca MKK7, and XPAPC (***$p > 0.99$ to Xror2 MO). All graphs show means ± SEM (**$p > 0.99$; *$p > 0.95$ to uninjected controls).

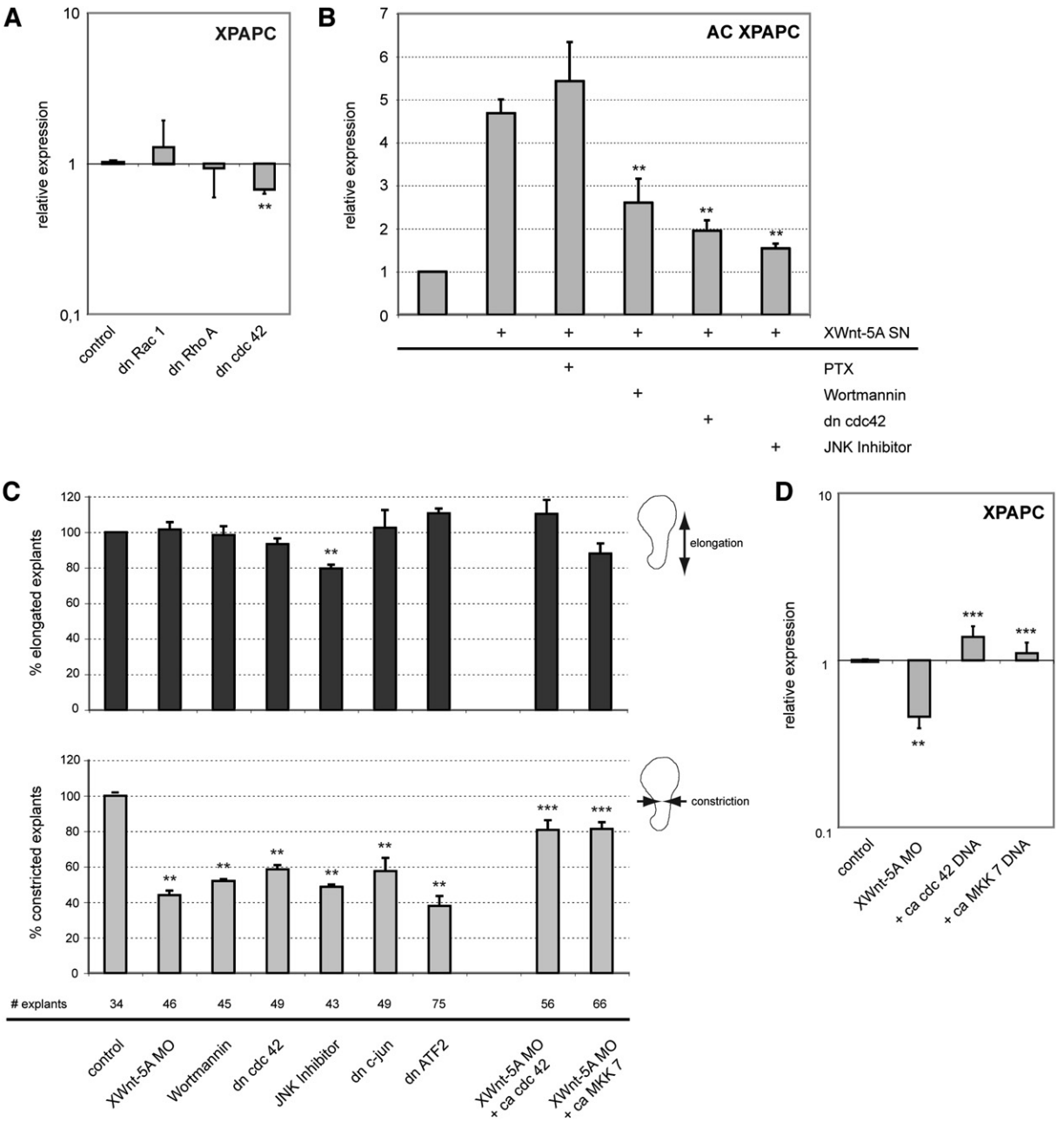

Figure 4. PI3K and cdc42 Are Involved in the Regulation of *XPAPC* Expression

(A) Injection of *dn cdc42* RNA, but not of *dn Rho A* or *dn Rac 1* RNA, downregulated *XPAPC* expression.

(B) Induction of *XPAPC* expression by Wnt-5A-conditioned medium (XWnt-5a SN) in Animal Caps was not affected by Pertussis toxin (PTX), but it was significantly blocked by Wortmannin, injection of *dn cdc42* RNA, or treatment with JNK Inhibitor (**p > 0.99 to Wnt-5A-conditioned medium).

(C) In Keller open-face explants, injection of dn cdc42, dn jun, or dn ATF2 as well as JNK Inhibitor phenocopied XWnt-5A depletion. Coinjection of XWnt-5A MO with ca cdc42 or ca MKK7 restored constriction (***p > 0.99 to XWnt-5A MO).

(D) Real-time RT-PCR confirmed that coinjection of XWnt-5A MO with ca cdc42 or ca MKK7 raised *XPAPC* mRNA back to or higher than control levels (***p > 0.99 to XWnt-5A MO). All results are shown as means ± SEM (**p > 0.99 to controls).

signaling in cell culture independent of PTX and without modulation of intracellular Ca^{2+} levels (Mikels and Nusse, 2006). Thus, Ror2-mediated Wnt-5A signaling is clearly not related to the Wnt/Ca^{2+} pathway that has so far been associated with XWnt-5A in early *Xenopus* development (Kuehl et al., 2000a; Moon et al., 1993; Saneyoshi et al., 2002; Slusarski et al., 1997; Torres et al., 1996).

The second, well-characterized branch of noncanonical Wnt signaling, the Wnt/PCP pathway, is mediated by Frizzled, Dishevelled, Daam 1, and the small GTPases Rho A

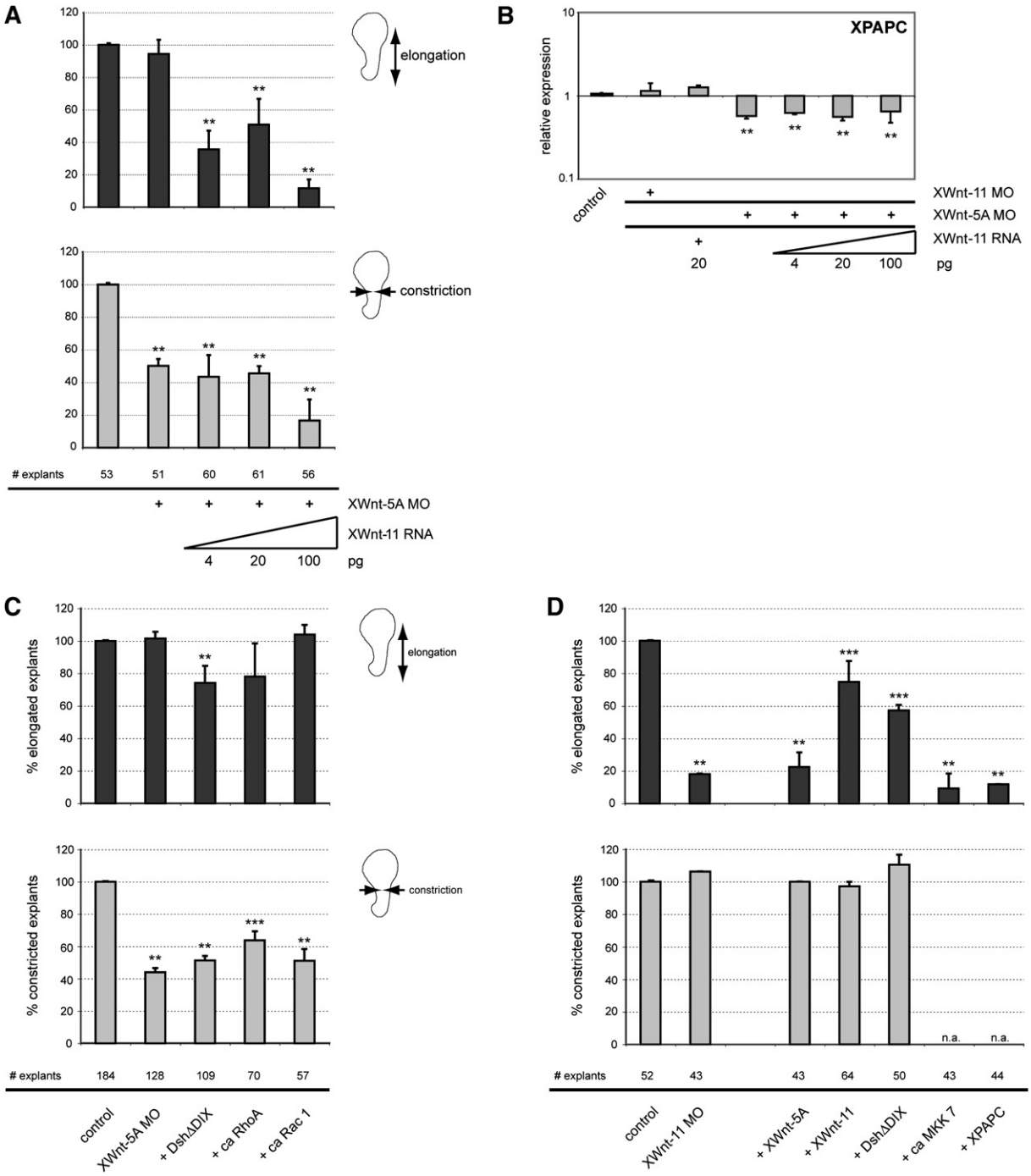

Figure 5. XWnt-11 Is Not Able to Rescue XWnt-5A Knockdown

(A) Coinjection of the indicated amounts of *XWnt-11* RNA did not restore constriction in XWnt-5A MO-injected Keller open-face explants, but led to a significant inhibition of explant elongation.

(B) XWnt-11 overexpression or XWnt-11 knockdown had no effect on *XPAPC* mRNA levels, and coinjection of XWnr-5A MO with the indicated amounts of *XWnt-11* RNA could not restore *XPAPC* transcription.

(C) Coinjection of ca Rho A partially rescued constriction in XWnt-5A-depleted Keller open-face explants, while DshΔDIX and ca Rac 1 did not (***p > 0.99 to XWnt-5A MO).

(D) XWnt-11 MO injection blocked elongation, but not constriction, in Keller open-face explants. The XWnt-11 MO phenotype was not rescued by coinjection of XWnt-5A, ca MKK7, or XPAPC, while coinjection of *XWnt-11* and *DshΔDIX* RNA (partially) restored elongation. In coinjections of ca MKK7 and XPAPC, explants were not scored for constriction because of the low proportion of elongated explants (***p > 0.99 to XWnt-11 MO). Results are shown as means ± SEM (**p > 0.99 to controls).

Table 1. Cell Shapes, Orientation, and Behavior

		Control	Xwnt-5A MO (1.6 fmol)	Xwnt-11 MO (1 fmol)	XPAPC MO (1.6 fmol)	Xror2 MO (0.8 fmol)
Average total speed (μm/hr)		50.6 ± 2.6	41.6 ± 3.0	13.4 ± 0.6	37.2 ± 1.2[a]	53.5 ± 2.8
Average net dorsal speed (μm/hr)		46.4 ± 2.7	17.5 ± 2.5	7.4 ± 0.3	19.9 ± 0.6[a]	23.6 ± 1.3
Length-to-width ratio (LWR)		2.50 ± 0.07	2.28 ± 0.10	1.25 ± 0.06	2.22 ± 0.10[a]	2.08 ± 0.12
Mediolaterally oriented cells (%)		93 ± 2	53 ± 1	n.a.	39 ± 3[a]	45 ± 4
Lamellipodia/cell/hr	Stable[b]	3.25 ± 0.25	0.18 ± 0.10	0	0.27 ± 0.12	0.18 ± 0.12
	Transient	1.5 ± 0.34	1.46 ± 0.22	0.13 ± 0.09	1.32 ± 0.14	3.23 ± 0.36
Filopodia/cell/hr	Stable[b]	1.08 ± 0.26	2.61 ± 0.82	0	2.00 ± 0.76	1.64 ± 0.41
	Transient	0.92 ± 0.31	11.04 ± 1.21	1.54 ± 0.25	13.27 ± 1.73	15.68 ± 1.68
Blebbing/cell/hr	Stable[b]	0	0	0	0	0
	Transient	0	0	1.33 ± 0.25	0	0

[a] Unterseher et al., 2004.
[b] Lifetime > 15 min.

and Rac 1 (Axelrod et al., 1998; Habas et al., 2001, 2003). Dsh is a multidomain protein involved in canonical and PCP signaling. It has been shown that canonical signaling requires the DIX and PDZ domains, while PCP signaling utilizes the PDZ and DEP domains of Dsh (Axelrod et al., 1998; Wallingford and Habas, 2005). Deletion mutants that lack specific domains are used to discriminate between canonical and noncanonical activity of Dsh (Axelrod et al., 1998). A mutant lacking the DIX domain has been shown to activate PCP signaling and to rescue the phenotypes induced by a dn Wnt-11 mutant in *Xenopus* (Tada and Smith, 2000). Consistent with the assumption that XWnt-11 activates the Wnt/PCP pathway, XWnt-11 depletion was rescued by coinjection of DshΔDIX, but XWnt-5A MO was not. Additionally, XWnt-5A loss of function was not rescued by ca Rac 1 and was only partially rescued by ca Rho A. The latter is probably due to the role of Rho A downstream of XPAPC (Unterseher et al., 2004). Together with the observation that XWnt-11 was not able to replace XWnt-5A and had no influence on *XPAPC* transcription, our results demonstrate that XWnt-5A and XWnt-11 are not redundant, and that XWnt-5A/Xror2 signaling is not related to the Wnt/PCP pathway. We conclude that Wnt-5A/Ror2 signaling should be considered as an additional, distinct branch of noncanonical Wnt signaling.

An earlier study in *Xenopus* embryos showed that the overexpression phenotypes of Xror2 were less frequent and less severe if kinase-domain deletion or point mutants were injected, which suggested partially kinase-independent functions of Xror2 (Hikasa et al., 2002). Similar observations have been reported for the *C. elegans* homolog Cam-1, which regulates cell migration independent of kinase activity (Forrester et al., 1999; Kim and Forrester, 2003). The kinase-domain point mutant Xror2 3I was not able to rescue *XPAPC* transcription in XWnt-5a- or Xror2-depleted embryos, indicating that Xror2 kinase activity is required for Wnt-5A/Ror2-mediated transcriptional regulation.

Downstream of Ror2 we identified PI3K, cdc42, and JNK as effectors in the Wnt-5A/Ror2 pathway. Inhibition of PI3K or JNK as well as injection of dn cdc42 blocked XWnt-5A-mediated upregulation of *XPAPC* and indicated that all three components are required as signal transducers in this pathway. In addition, we observed dephosphorylation of MKK7, JNK, and ATF2 in XWnt-5A-depleted embryos and could show that c-*jun* and ATF2 are required to upregulate *XPAPC* transcription.

PI3K signals to small GTPases of the Rho family, which mediate the activation of MEKK4 and mixed-lineage kinases (MLK) and their downstream targets MKK4 and MKK7 (Gallo and Johnson, 2002). Our rescue experiments confirmed that cdc42, not Rho A or Rac 1, was the effector downstream of XWnt-5A and Xror2, which is in agreement with the report that dn cdc42 was able to rescue the Xror2 overexpression phenotype in *Xenopus* embryos (Hikasa et al., 2002). Similar rescue experiments confirmed the requirement of MKK7/JNK signaling. The ability of ca cdc42 and ca MKK7 to restore constriction in XWnt-5A-depleted embryos could also be explained if they acted downstream of XPAPC. However, in our previous work, we have shown that the activity of cdc42 was not affected by XPAPC gain- or loss of function (Unterseher et al., 2004). Although the same work also identified JNK as a mediator of XPAPC signaling, the RT-PCR results presented in this work strongly argue that cdc42 and the JNK cascade are part of the XWnt-5A/Xror2 pathway and are required to regulate *XPAPC* transcription.

With this work, we have shown that the Wnt-5A/Ror2 pathway plays a role in the control of gene expression and identified *XPAPC* as a target of this pathway. We could show that the regulation seems to be direct, as no

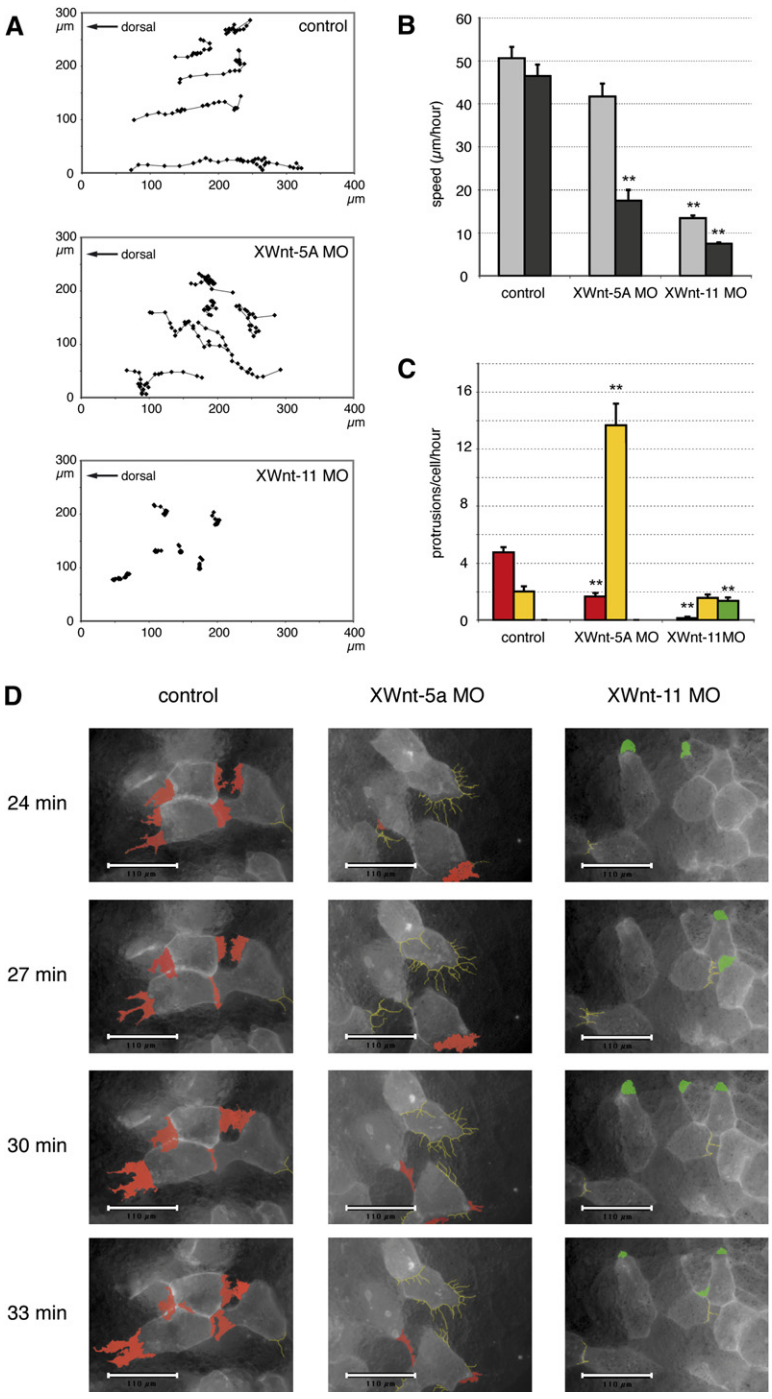

Figure 6. Time-Lapse Movies from Control, XWnt-5A MO-, or XWnt-11 MO-Injected Explants Show Distinct Cell Behavior

(A) Control cells moved predominantly straight toward the dorsal midline, while XWnt-5A-depleted cells showed random movement and frequent turns. XWnt-11 MO-injected cells scarcely moved at all and did not cover significant distances.

(B) The total speed (direction independent, gray bars) and net dorsal speed (black bars) calculated from time-lapse movies.

(C) The number of lamellipodia (red), filopodia (yellow), and bleb-like protrusions (green) was calculated per cell and per hour.

(D) Images show representative cells from control, XWnt-5A MO-injected, and XWnt-11 MO-injected explants (the scale bar is 110 μm). Lamellipodia are colored in red, filopodia in yellow, and bleb-like protrusions in green for better visualization.

Results are shown as means ± SEM (**p > 0.99 to controls).

protein translation was required to induce *XPAPC* in Animal Caps. This indicated that all effectors, including Xror2, were present in the Animal Cap. The published spatial expression pattern of Xror2 showed a clear enrichment in the dorsal marginal zone (Hikasa et al., 2002), but we detected *Xror2* transcripts in Animal Cap explants by real-time RT-PCR (A.S., unpublished data). *XWnt-5A* transcripts are also found in the Animal Cap (Moon et al., 1993); however, only negligible amounts of *XPAPC* RNA are detectable in naive Animal Cap tissue. This could be due to the presence of inhibitory signals that block *XPAPC* expression and antagonize Wnt-5A/Ror2 signaling. *XPAPC* expression is limited to the dorsal blastopore lip at the onset of gastrulation and is upregulated selectively in the paraxial mesoderm in late gastrula-stage embryos with a clear anterior border of the expression area (Kim et al., 1998; Unterseher et al., 2004). This restricted expression pattern and the number of factors involved,

including Xlim-1, Activin/BVg 1, and canonical Wnt signaling (Hukriede et al., 2003; Medina et al., 2004; Wessely et al., 2004), implies a complex network and tight regulation of *XPAPC* transcription. Xlim-1 depletion did not prevent *XPAPC* induction in Animal Cap explants in response to XWnt-5A. As injection of Xlim-1 MO substantially decreased Xlim-1 protein levels in the Animal Cap, it is unlikely that Xlim-1 mediates XWnt-5A function. If this was the case, reduced amounts of Xlim-1 protein should result in a correspondingly weaker upregulation of *XPAPC*, which was not true. Moreover, in late gastrula stages, when the effects of XWnt-5A knockdown on *XPAPC* are still clearly detectable, Xlim-1 becomes restricted to the notochord and head mesoderm (Taira et al., 1997). However, at the same time, *XPAPC* is downregulated in these tissues; thus, Xlim-1 is unlikely to mediate this later expression of *XPAPC*. Expression of Xror2 (Hikasa et al., 2002) and XWnt-5A persists in the paraxial mesoderm and the adjacent deep layer of the ectoderm, respectively, and thus could still participate in *XPAPC* transcriptional regulation. Similarly, we could exclude that XWnt-5A acted through the canonical Wnt/β-catenin pathway to regulate *XPAPC* transcription; however, β-catenin injections were sufficient to induce a moderate *XPAPC* expression in Animal Caps in this study, and *XPAPC* was upregulated by overactivation of the canonical Wnt pathway by LiCl treatment (Wessely et al., 2004). Overall, these observations indicate that *XPAPC* gene expression is regulated by multiple signals, including canonical Wnt signaling, Xlim-1, and the XWnt-5A/Xror2 pathway.

Consistent with our interpretation that Wnt-5A/Ror2 signaling is distinct from XWnt-11-stimulated Wnt/PCP signaling, we observed different effects on cellular behavior in XWnt-5A and XWnt-11 knockdown. XWnt-11-depleted cells showed only little protrusive activity, polarization, and movement, which caused an almost complete inhibition of explant elongation. Similar defects have been observed in explants that overexpress dn Wnt-11 (Tada and Smith, 2000), DshΔDEP, or DshΔPDZ (Wallingford et al., 2000). Interestingly, the phenotypes shown by Pandur et al. (2002) for the same XWnt-11 MO are less severe, which is probably due to different targeting of the MO. Here, the injections targeted the dorsal marginal zone and not the presumptive cardiac region, which could be expected to have a stronger effect on CE movements.

The effects of XWnt-5A knockdown on cellular behavior were fundamentally different. The LWR and the overall speed were comparable to those of control cells; however, the cells failed to align mediolaterally. This lack of coordinated polarity resulted in randomized movements and thus ineffective intercalation, which is most likely the cause for the broadened, but still elongated, Keller open-face explants observed after XWnt-5A depletion. These results suggest that knockdown of XWnt-5A or XPAPC as shown earlier (Unterseher et al., 2004) partially uncouples convergence and extension, although the mechanism of elongation, despite disrupted mediolateral orientation, remains unresolved. For a detailed discussion of the explant phenotype and cellular behavior, see Unterseher et al. (2004). In the same work, we have shown that Frizzled 7 loss of function also inhibits constriction, as well as, partially, elongation, in Keller open-face explants. This phenotype was rescued by DshΔDIX, as was the case for the XWnt-11 MO phenotype reported in this work, and Frizzled 7 was required in parallel to XPAPC. These observations further support the hypothesis of distinct roles of XWnt-5A/Ror2 and Wnt/PCP signaling in CE movements.

A recent study reported that Ror2 was required for filopodia formation in mouse embryonic fibroblasts (Nishita et al., 2006). However, when we investigated the protrusive activity of the cells in Keller open-face explants, we found that knockdown of XWnt-5A, Xror2, or XPAPC induced the formation of filopodia and inhibited the stabilization of protrusions observed in control cells. An increase in the number of filopodia, although less dramatic, has been reported for cells that overexpress either dn Rho A or ca Rac 1 (Tahinci and Symes, 2003). The stronger effect in our experiments likely results from a combination of Rho A inhibition and Rac 1 activation, due to the downregulation of XPAPC, which activates Rho A and inhibits Rac 1 (Medina et al., 2004; Unterseher et al., 2004). Although the number of filopodia was increased about 7-fold, the lifetime of filopodia and lamellipodia in XWnt-5A, Xror2, or XPAPC knockdowns was decreased compared to control cells. Decreased stability of protrusions, but not the increased number of filopodia, has also been reported for PCP loss of function induced by Xdd1 overexpression, a mutant that lacks a large part of the PDZ domain (Wallingford et al., 2000). Interestingly, XWnt-11 MO-injected explants showed only very little protrusive activity, indicating that Xdd1 is not equivalent to depletion of XWnt-11. The observed changes in the type and stability of protrusions after XWnt-5A, Xror2, or XPAPC knockdown are most likely a cumulative effect that results from XPAPC depletion and thereby inhibition of downstream signaling by Rho and Rac 1. In the case of XWnt-5A MO and Xror2 MO, the observed phenotypes are clearly XPAPC loss-of-function phenotypes, which further stresses the role of Wnt-5A/Ror2 in early *Xenopus* development in the regulation of *XPAPC* gene expression, but does not exclude other functions in different cells or later in development.

The characterization of the Wnt-5A/Ror2 pathway in this work adds a new, to our knowledge, distinct branch to the known noncanonical Wnt signaling pathways that seems to be evolutionary conserved. Recent studies showed that murine Wnt-5A binds to mRor2 in vitro, cooperates with mRor2 in the JNK-dependent stimulation of c-*jun* phosphorylation (Oishi et al., 2003), and antagonizes canonical Wnt signaling (Mikels and Nusse, 2006). However, it remains to be shown that all effectors identified in this work are conserved in other species. Wnt-5a$^{-/-}$ mice and Ror2$^{-/-}$ mice exhibit dwarfism, short tails and limbs, and craniofacial defects (Oishi et al., 2003). These phenotypes, together with the expression of Xror2 in the branchial arches (Hikasa et al., 2002), the role of Ror2 in neurite extension (Paganoni and Ferreira, 2005), and the requirement of the Wnt-5A/Ror2 pathway for XPAPC expression in CE movements of *Xenopus* shown in this work, point

toward a general role of this signaling cascade in morphogenetic processes and open perspectives for further and more detailed functional studies.

EXPERIMENTAL PROCEDURES

Frog Handling, Plasmids, and Microinjections

Embryos were obtained by in vitro fertilization, cultured, and injected as described previously (Unterseher et al., 2004). Embryos were injected at the four-cell stage in both dorsal blastomeres. If not indicated otherwise, injection amounts were 100 pg XWnt-5AΔUTR, XPAPC, or β-catenin; 500 pg Xlim-1ΔUTR, ca NF-AT, LefΔHMG, conductin, DshΔDIX, ca MKK7, dn jun, myc-jun, or dn ATF2; 1 ng ca CamK II (T286D); 40 pg dn cdc42, dn Rho A, or dn Rac 1, or 20 pg *XWnt-11* RNA; 20 pg ca cdc42, 5 pg ca Rho A, or 10 pg ca Rac 1 DNA; 1.6 pmol XWnt-5A MO, XWnt-5A 5MM MO, Xror2 MO, Xror2 5MM MO, or standard control MO; 16 pmol Xlim-1 MO or Xlim-1 5MM MO; and 1 pmol XWnt-11 MO (all MOs: GeneTools, Philomath, OR, USA, sequences are given in Table S1). A total of 20 μM JNK Inhibitor, 100 μM PKC Inhibitor, 10 μM PMA (MerckBiosciences, Darmstadt, Germany), 10 nM Wortmannin, or 100 ng/μl PTX (SigmaAldrich, Munich, Germany) were added to the culture medium at stage 8 and to all subsequent media.

Keller Open-Face Explants

Keller open-face explants for analysis of convergent extension movements and time-lapse experiments were prepared at stage 10.5 and cultured, imaged, and scored as described previously (Unterseher et al., 2004). For time-lapse movies, images were captured in 3 min intervals for 2 hr.

In Situ Hybridization

Two-cell-stage embryos were injected in one blastomere with 40 pg β-galactosidase RNA and XWnt-5A MO or RNA. After fixation, the embryos were stained for β-galactosidase activity. Whole-mount in situ hybridizations were carried out by using the Digoxygenin/Alkaline Phosphatase detection system (Roche, Mannheim, Germany) as described (Hollemann et al., 1999).

Real-Time RT-PCR

At stage 10.5, total RNA was extracted (Nucleospin II, Macherey Nagel, Düren, Germany) and reverse transcribed (MMLV, Promega, Madison, WI, USA). Real-time PCR was carried out by using iQ-Sybr-Green Supermix on an iCycler instrument (BioRad, Hercules, CA, USA). Expression levels were calculated relative to Ornithin-Decarboxylase (ODC) and were normalized to uninjected controls.

Results of at least three independent experiments were averaged, and statistical significance was calculated by using a Student's t test. Primer sequences are given in Table S1.

Animal Cap Explants

Two-cell-stage embryos were injected into both blastomeres close to the animal pole. Animal Caps were prepared at stage 8 and were cultured in Barth's solution for 4 hr.

3T3 cells stably transfected with Wnt-5A or an empty vector (Kispert et al., 1998) were cultivated in serum-free medium (DMEM, Cambrex, NJ, USA) for 5 days; after that, the medium was collected, filter sterilized, and diluted 2:1 with sterile water.

Conditioned media were applied for 1 hr and then replaced by Barth's solution for an additional 3 hr. Cycloheximide was added to the embryos at 5 μM 1 hr prior to preparation of the Animal Caps and was added at 17.8 μM to all media.

SDS-PAGE and Western Blot

Western blots and immunoprecipitations were carried out as described by Unterseher et al. (2004). Primary antibodies were anti-FL JNK, anti-pJNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pMKK7, anti-Ror2, anti-pATF2, anti-ATF2 (Cell Signaling, Beverly, MA, USA), anti-FL MKK7 (Merck, Darmstadt, Germany), and monoclonal antibodies B62A12 (oocyte nuclear protein), 9E10 (myc), and 6D9 (XB-Cadherin). Secondary antibodies were goat-anti-mouse AP, goat-anti-rabbit AP, and donkey-anti-goat AP (all from Dianova, Hamburg, Germany).

Supplemental Data

Supplemental Data include oligonucleotide sequences; supporting experimental data to Figures 1, 2, and 4; and time-lape movies corresponding to Figure 6 and are available at http://www.developmentalcell.com/cgi/content/full/12/5/779/DC1/.

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