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Essential role of MARCKS in cortical actin dynamics during gastrulation movements

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Myristoylated alanine-rich C kinase substrate (MARCKS) is an actin-binding, membrane-associated protein expressed during *Xenopus* embryogenesis. We analyzed its function in cytoskeletal regulation during gastrulation. Here, we show that blockade of its function impaired morphogenetic movements, including convergent extension. MARCKS was required for control of cell morphology, motility, adhesion, protrusive activity, and cortical actin formation in embryonic cells. We also demonstrate that the noncanonical Wnt pathway promotes the formation of lamellipodia- and filopodia-like protrusions and that MARCKS is necessary for this activity. These findings show that MARCKS regulates the cortical actin formation that is requisite for dynamic morphogenetic movements.

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Introduction

During *Xenopus* gastrulation, mesoderm migrates to the inside of the embryo and moves along the blastocoel roof to establish the three germ layer structure. This process involves several morphogenetic cell movements including mesendoderm extension and convergent extension. During mesendoderm extension, cells migrate along the blastocoel roof in contact with fibronectin (FN) fibrils (Winklbauer, 1990; Davidson et al., 2002). In convergent extension, cells are polarized and elongated mediolaterally, then the cells are intercalated. This movement forms the dorsal mesodermal structure and extends the anteroposterior body axis (Shih and Keller, 1992; Wallingford et al., 2002). The noncanonical Wnt pathway has been implicated in the regulation of convergent extension (Kuhl, 2002; Tada et al., 2002). One of the intracellular signaling components, *Xenopus* Dishevelled (Xdsh), plays a pivotal role in this process. When the function of Xdsh is inhibited, the polarity of the mesodermal cells is not established normally (Wallingford et al., 2000).

Because these cell movements are accompanied by dynamic changes in cell polarity, morphology, and motility, it is very likely that cytoskeletal dynamics are carefully regulated. Thus, we sought to analyze the regulatory mechanism of cytoskeletal dynamics during gastrulation. We decided to focus on myristoylated alanine-rich C kinase substrate (MARCKS). Mammalian MARCKS has been shown to interact with actin (Arbuzova et al., 2002). It has been reported that *Xenopus MARCKS* is expressed maternally and throughout embryogenesis (Ali et al., 1997; Shi et al., 1997), but its role in development was not well understood.

Here, we report that the loss of MARCKS function severely impaired gastrulation movements. MARCKS regulates the cortical actin formation, cell adhesion, protrusive activity, and cell polarity control during gastrulation. We further show that MARCKS is necessary for the protrusive activity regulated by the noncanonical Wnt pathway. These findings show that MARCKS regulates the cortical actin formation that is requisite for dynamic morphogenetic movements.

Results and discussion

To investigate the function of MARCKS in *Xenopus* development, we conducted loss of function experiments using antisense Morpholino oligonucleotides (Mo). First, we examined the specificity of *MARCKS* Mo (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200310027/DC1). The Mo specifically and effectively inhibited epitope-tagged MARCKS protein synthesis, leading us to expect that it could inhibit the endogenous MARCKS protein synthesis.

The online version of this paper includes supplemental material.

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Key words: cell adhesion; *Xenopus*; cell movement; convergent extension; Wnt pathway

Abbreviations used in this paper: DMZ, dorsal marginal zone; FN, fibronectin; MARCKS, myristoylated alanine-rich C kinase substrate, mb-Venus, membrane-binding Venus; Mo, Morpholino oligonucleotide; RFP, red fluorescent protein; RMA, RFP-moesin actin; Xdsh, *Xenopus* Dishevelled; XMLP, MARCKS-like protein.

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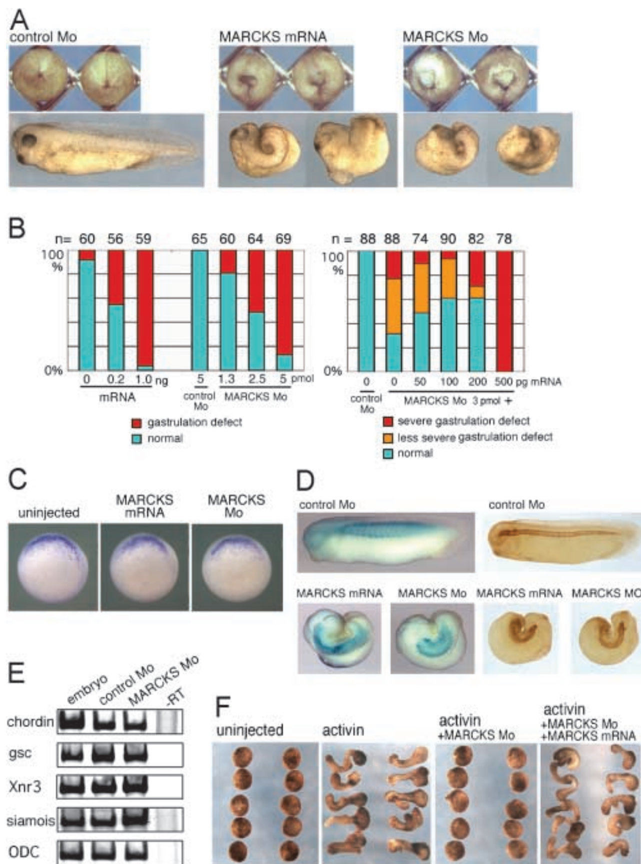

Figure 1. **MARCKS is essential for gastrulation movements.** (A) Both 500 pg of *MARCKS* mRNA and 5 pmol of *MARCKS* Mo impaired gastrulation movements, when either was injected into the dorsal marginal region. (B) Statistical data of the gastrulation-defective phenotype caused by *MARCKS* mRNA and Mo. (C) Expression of *chordin* at the gastrula stage, detected by in situ hybridization. (D) Somites (left) and notochord (right) were immunostained with 12/101 and MZ15 antibodies, respectively. (E) 5 pmol of *MARCKS* Mo was injected into the two dorsal blastomeres at the four-cell stage; the DMZ explants were isolated, and the expression of mesodermal markers was detected by RT-PCR. gsc, *goosecoid*. (F) 2 pmol of *MARCKS* Mo inhibited the activin mRNA-induced elongation of animal caps. This inhibition was rescued by coinjection of 200 pg of *MARCKS* mRNA.

Using *MARCKS* Mo, we analyzed MARCKS function in development. When it was injected into the dorsal marginal zone (DMZ) of four-cell embryos, the embryos showed a gastrulation-defective phenotype (Fig. 1 A). The involution of the mesoderm was impaired and the blastopore remained open. A similar phenotype was observed when *MARCKS* mRNA was injected. The phenotype of *MARCKS* Mo was partially rescued by coinjection of *MARCKS* mRNA (Fig. 1 B). The rescue was imperfect probably because *MARCKS* overexpression also inhibited gastrulation movements. As discussed below, however, cell biological effects of *MARCKS* Mo were efficiently rescued by *MARCKS* mRNA. Over- and under-expression of *MARCKS* may have opposite effects at a cellular level, but both of these effects may negatively influence gastrulation movements. MARCKS is essential for gastrulation and its level must be tightly regulated.

It has been reported that *MARCKS-like protein* (*XMLP*) is also expressed in *Xenopus* embryo (Zhao et al., 2001). Although XMLP is similar to MARCKS (23% amino acid identity), *XMLP*-Mo injected embryos showed malformations of the anterior axis and eye defect, but the gastrulation defect was not reported (Zhao et al., 2001). They seem to play distinct roles in *Xenopus* development.

To determine whether this gastrulation defect was caused by a defect in mesodermal differentiation, we examined the expression of the dorsal mesodermal markers. At the gastrula stage, *MARCKS* Mo-injected embryos expressed *chordin* at the same level as control embryos (Fig. 1 C). In tadpoles, the notochord and somites were formed in the *MARCKS* Mo-injected embryos, but the extension of these tissues was severely inhibited (Fig. 1 D). We also tested the expression of the mesodermal markers in DMZ explants by RT-PCR (Fig. 1 E). The expression of these markers was not inhibited by *MARCKS* Mo. These results indicated that the phenotype was caused, not by a defect in mesoderm differentiation, but by a defect in morphogenetic movements.

Next, we tested whether the loss of MARCKS function affects the animal cap elongation, which mimics convergent extension movements during gastrulation (Fig. 1 F). *MARCKS* Mo blocked the elongation by activin, and it was rescued by coinjecting *MARCKS* mRNA without the UTR, suggesting that MARCKS is required for convergent extension.

During mesodermal convergent extension, the cells become polarized, align mediolaterally, and are then intercalated. To test how MARCKS is involved in this process, the convergent extension in DMZ explants was observed microscopically. *MARCKS* Mo, Rhodamine dextran, and mRNA encoding membrane-binding Venus (mb-Venus) were coinjected into one of the two dorsal blastomeres (Fig. 2 A). As a control, mb-Venus mRNA alone was injected into the other dorsal blastomere. At the gastrula stage, the DMZ explants were isolated and cultured on a cover glass coated with FN. These explants adhered to the FN, and convergent extension movements occurred subsequently in the mesoderm (Kinoshita et al., 2003). In the absence of *MARCKS* Mo, red and nonred cells were polarized and intercalated. In the *MARCKS* Mo-injected explants, the nonred cells, which were assumed to lack the Mo, were polarized and showed convergent extension. In contrast, the red cells (Mo-injected cells) were not polarized and did not participate in the intercalation. Thus, MARCKS is essential for the cell polarization and movement during convergent extension.

In addition to convergent extension, an important mechanism regulating gastrulation movements is mesendoderm extension (Davidson et al., 2002). To test whether MARCKS is required for this process, DMZ explants were cultured on FN-coated dishes according to the method developed by Davidson et al. (2002). Mesendodermal cells migrated on the FN substrate as an intact mantle (Fig. 2 B). When Venus mRNA and the control Mo were coinjected, the Venus-expressing cells dispersed broadly, and some cells migrated to the front. In contrast, *MARCKS* Mo-injected cells rarely migrated on the FN substrate. We examined 15 explants and confirmed that none of the *MARCKS* Mo-injected cells reached the leading edge of the migrating mesendoderm.

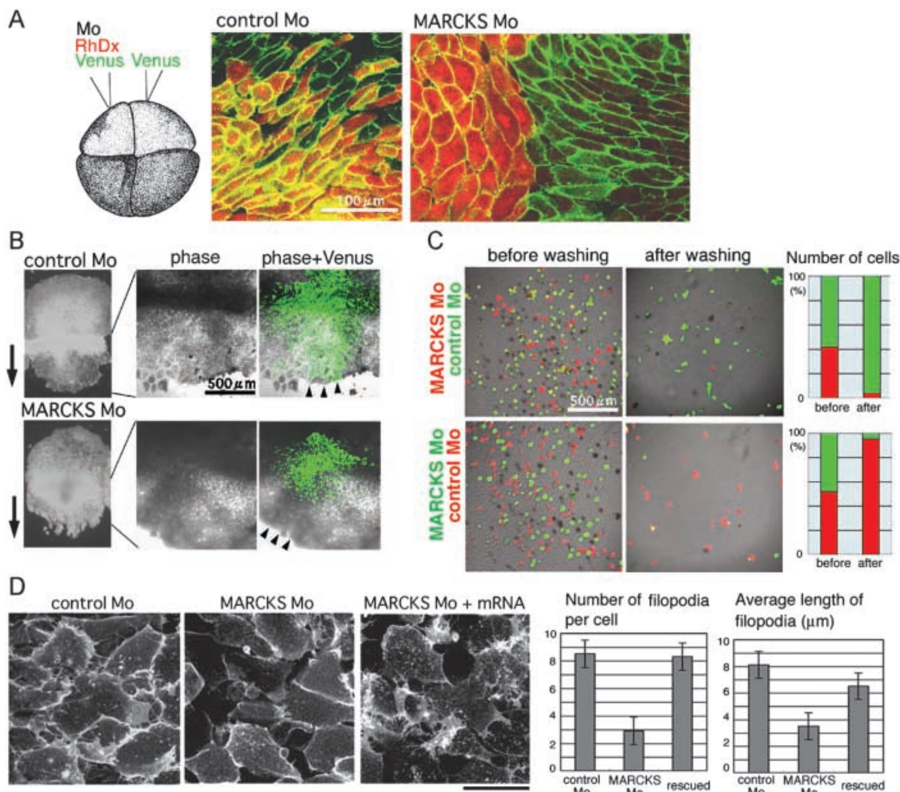

Figure 2. **MARCKS is essential for controlling cell polarity, motility, and adhesion.** (A) 5 pmol of *MARCKS* Mo, Rhodamine dextran, and the mRNA for 100 pg of mb-Venus were coinjected into one of the two dorsal blastomeres at the four-cell stage. mb-Venus mRNA alone was injected into the other dorsal blastomere. DMZ explants were cultured on a cover glass coated with FN, and convergent extension movements were observed. (B) Control or 5 pmol of *MARCKS* Mo was coinjected with 100 pg of Venus mRNA into two blastomeres of four-cell embryos. DMZ explants were cultured on an FN-coated dish until sibling embryos reached the late neurula stage. Arrows indicate the direction of mesendoderm migration. Arrowheads indicate the leading edge. (C) *MARCKS* Mo inhibited the adhesion on FN. *MARCKS* Mo, control Mo, Venus mRNAs (green), and Rhodamine dextran (red) were coinjected dorsally as indicated. Cells were dissociated from the DMZ explants. Cells from the control- and MARCKS-Mo–injected explants were mixed, plated on FN-coated dishes, incubated for 6 h, and fixed in formaldehyde. Cells that did not adhere to the dish were removed by washing five times with PBS. (D) 3 pmol of *MARCKS* Mo inhibited the protrusive activity of cells in DMZ explants. *MARCKS* Mo or control Mo was coinjected dorsally with mb-Venus mRNA. DMZ explants were cultured on an FN-coated dish until sibling embryos reached the early neurula stage. The effect of *MARCKS* Mo was rescued by 200 pg of *MARCKS* mRNA. Bar, 50 µm. The graph shows statistical data obtained by analyzing 15 cells for each sample. The error bars represent statistical significance ($p < 0.05$).

This indicated that MARCKS was required for mesendoderm extension as well as convergent extension.

We examined whether *MARCKS* Mo affects the adhesion to FN of cells dissociated from the DMZ explants. *MARCKS* Mo and Venus mRNA (green) or Rhodamine dextran (red) was injected dorsally (Fig. 2 C). DMZ explants were isolated and dissociated in Ca^{2+}-Mg^{2+}–free medium. Dissociated cells were cultured on FN-coated dishes, and cells that adhered to the dish were counted (Fig. 2 C). When *MARCKS* Mo was coinjected with Venus, the adherence of Venus-expressing cells was extremely reduced. In contrast, when *MARCKS* Mo was coinjected with Rhodamine dextran, these red cells rarely adhered to the dish. A few cells containing *MARCKS* Mo were found on the dish, but these cells were rounded up and did not spread out on the dish. This indicates that MARCKS is essential for cell adhesion and spreading on FN. The effect of *MARCKS* Mo on the cell adhesion was rescued by coinjection with *MARCKS* mRNA (unpublished data).

Next, we tested whether *MARCKS* Mo affected the protrusive activity in mesodermal cells. Mesodermal cells had many filopodia-like protrusions when the DMZ explants adhered to a FN-coated dish (Fig. 2 D). *MARCKS* Mo severely reduced the number and the length of these protrusions. The effect of *MARCKS* Mo on the protrusive activity was rescued by coinjection with *MARCKS* mRNA. Thus, MARCKS is required for the protrusive activity, which may directly correlate with the control of cell adhesion and motility. The inhibition of cell adhesion and migration on the FN fibrils that cover the blastocoel roof may contribute to the gastrulation defect caused by *MARCKS* Mo.

The actin-binding domain of mammalian MARCKS binds to actin filaments and cross-links them in vitro (Hartwig et al., 1992). The corresponding domain of *Xenopus* MARCKS is 100% identical (Shi et al., 1997), suggesting that it may also interact with F-actin. At first, we examined the colocalization of MARCKS with F-actin. Cells expressing MARCKS-Venus were dissociated from DMZ explants and cultured on an FN-coated dish. The cells were then fixed and F-actin was stained with phalloidin. As shown in Fig. 3 A, MARCKS and cortical actin were colocalized.

We then constructed two mutants, GA and SD (Fig. 3 B). GA is an unmyristoylated mutant in which the second glycine residue is replaced with alanine. SD is a pseudophosphorylation mutant whose potential phosphorylation sites were replaced with aspartic acid, which is expected not to bind to actin filaments (Hartwig et al., 1992). To detect F-actin, we used the F-actin–binding domain of moesin fused to red fluorescent protein (RFP; Campbell et al., 2002), designated RMA (RFP-moesin actin–binding domain). It has been shown biochemically that this domain binds to F-actin (Turunen et al., 1994; Pestonjamasp et al., 1995). In *Drosophila* embryos, the corresponding domain of moesin fused with GFP was successfully used to analyze actin dynamics (Dutta et al., 2002). We confirmed that our construct (Venus-moesin actin-binding domain) colocalized with stress fi-

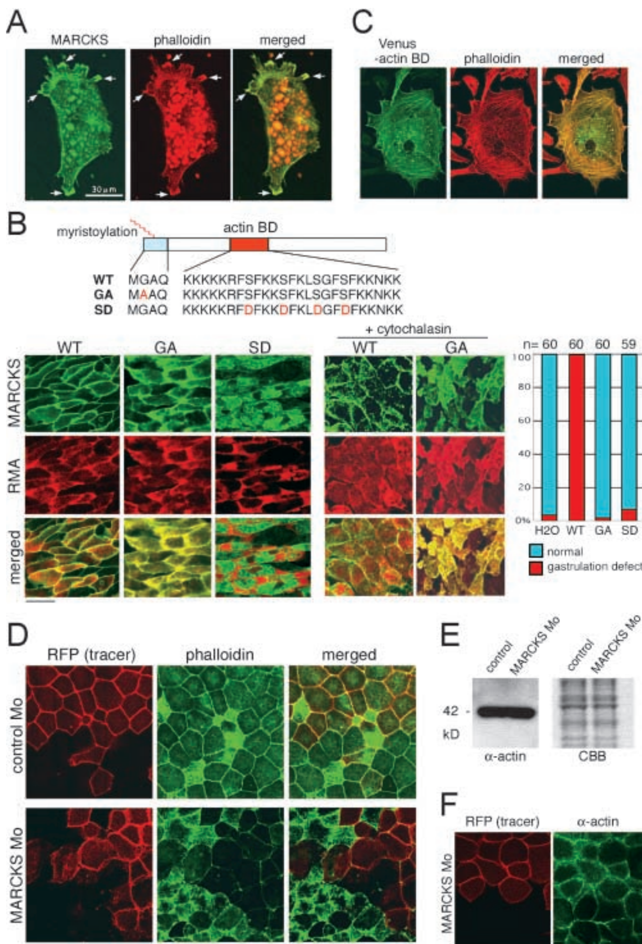

Figure 3. **MARCKS regulates cortical actin formation.** (A) Cells expressing 200 pg of *MARCKS-Venus* mRNA were dissociated from DMZ explants and plated on a FN-coated cover glass. Arrows indicate protrusions where both MARCKS and F-actin were enriched. (B) Wild-type, GA, and SD mutants were expressed in the DMZ explants and observed. RMA, RFP fused with the actin-binding domain of moesin. The explants were treated with 200 μM of cytochalasin B for 30 min. Bar, 50 μm. The graph shows percentages of the gastrulation-defective phenotype. (C) The actin-binding domain of *Xenopus* moesin was fused with Venus (Venus-actin BD) and expressed in CHO cells. (D) Mo was injected into one blastomere of two-cell embryo with mb-RFP mRNA as a tracer. Animal cap explants were fixed and stained with phalloidin. Bar, 50 μm. (E) Western blot with an antiactin antibody (left) and Coomassie Blue (CBB) staining (right). Mo was injected into both of the blastomeres of two-cell embryos. Lysates were prepared from the animal caps. (F) *MARCKS* Mo and mb-RFP were coinjected and animal cap cells were immunostained with antiactin antibody.

bers and cortical actin stained with phalloidin in CHO cells (Fig. 3 C).

In *Xenopus* embryonic cells, the RMA was localized to the cell cortices, and cytochalasin B treatment, which disrupts actin filaments, dispersed the RMA to the cytoplasm (Fig. 3 B). This indicates that the RMA should be useful for monitoring F-actin dynamics. When these MARCKS-Venus genes were expressed, the wild-type and GA forms were associated with the plasma membrane and colocalized with RMA, but SD was in the cytoplasm. When the cells were treated with cytochalasin B, GA dispersed to the cytosol with RMA, whereas wild-type remained on the membrane. This result suggested that the association of MARCKS with the membrane was regulated by two mechanisms, myristoylation and binding to the cortical actin. We also found that GA and SD mutants did not inhibit gastrulation movement when they were overexpressed (Fig. 3 B). These mutants also did not rescue the embryo phenotype caused by *MARCKS* Mo. These results suggest that both myristoylation and actin biding are required for its function.

To test whether MARCKS regulates cortical actin formation, *MARCKS* Mo was injected into one blastomere near the animal pole of two-cell embryos. Animal caps were isolated at the late blastula stage, fixed, and stained with phalloidin. Membrane-binding RFP was coinjected with Mo for tracing the injected cells. As shown in Fig. 3 D, *MARCKS* Mo significantly reduced the amount of cortical actin stained by phalloidin. The amount of actin protein was not affected, however, judging from Western blotting and immunocytochemistry using an antipan actin antibody (Fig. 3, E and F). This result suggests that MARCKS plays an important role in cortical actin formation.

The noncanonical Wnt pathway has been implicated in convergent extension. It has been demonstrated that Xdsh, an essential cytoplasmic component in this pathway, regulates cell polarity and protrusive activity in DMZ cells (Wallingford et al., 2000). The Wnt pathway activates RhoA and Rac (Habas et al., 2001, 2003), which have been shown to regulate the protrusive activity (Tahinci and Symes, 2003). Thus, the pathway may directly regulate actin cytoskeletal dynamics. To investigate the relationship between the Wnt pathway and cortical actin, we examined the localization of Xdsh. Cells were dissociated from the DMZ explants expressing *Xdsh-Venus*, cultured on an FN-coated dish, and stained with phalloidin. As shown in Fig. 4 A, Xdsh was colocalized with cortical actin, even in the lamellipodial and filopodial protrusions.

When RMA was expressed during convergent extension, it was located at the tips of elongated mesodermal cells (Fig. 4 B). This indicates that F-actin is enriched in this region. We showed previously that Xdsh-Venus was also accumulated in the same region (Kinoshita et al., 2003). Mammalian Dishevelled interacts with actin filament through the NH_2-terminal DIX domain (Capelluto et al., 2002). To test whether the tip localization of Xdsh was due to the interaction between the DIX domain and F-actin, we tested the localization of Xdsh lacking the DIX-domain (XdshΔDIX). As shown in Fig. 4 B, the XdshΔDIX was located at the tip, indicating that this localization is not due to interaction between F-actin and the DIX domain. This result is consistent with the finding that DishevelledΔDIX can mediate the noncanonical Wnt signaling in *Xenopus* and zebrafish (Heisenberg et al., 2000; Tada and Smith, 2000). The actin depolymerizing reagent, Latrunculin A, dispersed both RMA and XdshΔDIX to the cytosol. Essentially, the same result was also obtained using cytochalasin B (unpublished data). These results strongly suggest that Xdsh interacts with F-actin either directly or indirectly and mediates the Wnt signaling to the actin cytoskeleton.

To examine whether the Wnt pathway regulates the protrusive activity, we coexpressed *Xwnt11* and *Xfz7 (Xenopus*

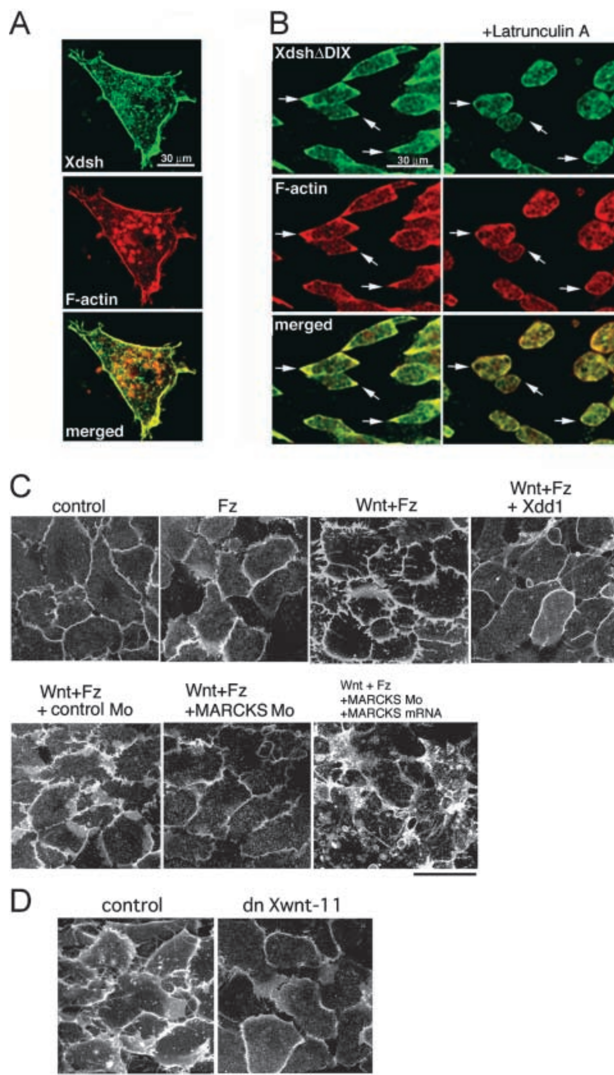

Figure 4. **MARCKS is required for the cortical actin dynamics regulated by the noncanonical Wnt signaling pathway.** (A) 250 pg of *Xdsh-Venus* mRNA was expressed in DMZ explants. Cells were dissociated and plated on an FN-coated dish. Xdsh-Venus was colocalized with the cortical actin. (B) 100 pg of *XdshΔDIX-Venus* mRNA was expressed in DMZ explants. The explants were cultured on an FN-coated dish. F-actin was probed with RMA. XdshΔDIX and RMA were colocalized (arrows). (Right) Treatment with 30 μM Latrunculin A for 30 min. (C) *Xwnt11* and *Xfz7* mRNAs (200 pg each) were coexpressed in animal cap explants with mb-RFP. The coexpression of *Xwnt11* and *Xfz7* promoted the protrusive activity. 5 pmol of *MARCKS* Mo and Xdd1 inhibited it. The effect of *MARCKS* Mo was rescued by coinjection of 200 pg of *MARCKS* mRNA. Bar, 50 μm. (D) mb-RFP was injected with or without mRNA encoding dominant-negative Xwnt-11 (2 ng). Bar, 50 μm.

frizzled-7) in animal cap explants with the membrane-binding RFP. As shown in Fig. 4 C, the coexpression of Xwnt11 and Xfz7 dramatically promoted lamellipodia- and filopodia-like protrusions and it was inhibited by Xdd1, a dominant negative Xdsh mutant (Sokol, 1996; Wallingford et al., 2000). When *MARCKS* Mo was coinjected, this activity was severely inhibited (Fig. 4 C). It was rescued by coinjection with *MARCKS* mRNA. In addition, dorsal mesodermal cells expressing dominant-negative Xwnt-11 (Tada and Smith, 2000) significantly reduced the number of protrusions (Fig. 4 D), which is consistent with the observation by Wallingford et al. (2000) that cells expressing Xdd1 maintain significantly fewer stable protrusions. These results strongly suggest that the Wnt signaling pathway regulates cortical actin dynamics and that MARCKS is required for this process.

Here, we have shown that MARCKS plays an essential role in regulating cortical actin dynamics in *Xenopus* development. *MARCKS* Mo inhibited cell movements, cell shape change, cell adhesion, and interaction with FN probably through the defect it caused in the cortical actin dynamics. MARCKS is required not only for gastrulation but also for the neural tube formation. When *MARCKS* Mo was injected into the dorso-anterior blastomeres of eight-cell embryos to target the neuroectoderm, neural tube closure was severely impaired (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200310027/DC1). It has been shown that *MARCKS*-deficient mouse shows neural tube closure defect (Stumpo et al., 1995), suggesting the conserved function between frogs and mice. MARCKS may be required for highly organized actin dynamics to effect dynamic tissue reorganization.

The regulation of the cortical actin cytoskeleton by MARCKS may be important for a proper cellular response to signals such as Wnt and the FN/integrin pathways. It is also possible that these signaling pathways regulate the activity of MARCKS. It has been shown that MARCKS is a PKC substrate. PKC has been involved in the noncanonical Wnt pathway (Sheldahl et al., 1999) and the integrin pathway (Vuori and Ruoslahti, 1993). It would be interesting to determine how the activity of MARCKS is regulated during development.

Materials and methods

Plasmids, RNA synthesis, and Morpholino oligos

Procedures for the plasmid construction, RNA synthesis and sequences of Morpholino oligos were described in the online supplemental material. The RFP plasmid is a gift from R. Tsien (University of California, San Diego, CA).

In situ hybridization and RT-PCR analysis

In situ hybridization in *Xenopus* was performed as described by Harland (1991). For RT-PCR analyses, RNA from the explants was prepared with TRIzol (Life Technologies). cDNA was synthesized with reverse transcriptase (TRT-101; Toyobo). Sequences of the primers were described in the online supplemental material.

Whole-mount immunostaining and Western blotting

The procedure for whole-mount immunostaining was performed as described in Kurata et al. (2001). The antibodies were MZ15 for notochord (a gift from F. Watt, Imperial Cancer Research Fund, London, UK) and 12/101 for somites (Development Studies Hybridoma Bank). Western blotting was performed using a mouse monoclonal antipan-actin antibody was purchased from NeoMarkers (MS-1295-P0).

Dissecting explants and cytological observations

For the animal cap explants, *MARCKS* mRNA or Mo was coinjected with 0.5 pg activin mRNA into the animal pole of two-cell embryos. The animal cap was dissected from stage-9 embryos. For DMZ explants, mRNA or a Mo was injected into the two dorsal blastomeres of four-cell embryos. Explants were isolated at stage 10+. These explants were cultured in 1× Steinberg's solution until sibling embryos reached stage 17. To dissociate cells from the explants, the explants were incubated in the Ca^{2+}-Mg^{2+}–free medium for 2 h. For the cytological observation, explants and dissociated cells were cultured in 1× Steinberg's solution on an FN-coated dish

(4000–030; Iwakil), or on a cover glass coated with FN (~1 μg/cm^2, F1141; Sigma-Aldrich). To stain F-actin, cells were fixed in 4% PFA and stained with PBS 0.5% Triton X-100 containing a 40-fold dilution of BODIPY 581/589 phalloidin (B-3416; Molecular Probes) or Alexa Fluor 488 phalloidin (A-12379; Molecular Probes). For confocal microscopy, images were captured using 510 software (Carl Zeiss MicroImaging, Inc.). All images were prepared for publication using Adobe Photoshop software.

Online supplemental material

Fig. S1 shows that *MARCKS* Mo specifically inhibits MARCKS protein synthesis. Fig. S2 shows that *MARCKS* Mo inhibits neural tube closure. Supplemental material is available online at http://www.jcb.org/cgi/content/full/jcb.200310027/DC1.

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