

Rock2 controls TGF β signaling and inhibits mesoderm induction in zebrafish embryos

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Summary

The Rho-associated serine/threonine kinases Rock1 and Rock2 play important roles in cell contraction, adhesion, migration, proliferation and apoptosis. Here we report that Rock2 acts as a negative regulator of the TGF β signaling pathway. Mechanistically, Rock2 binds to and accelerates the lysosomal degradation of TGF β type I receptors internalized from the cell surface in mammalian cells. The inhibitory effect of Rock2 on TGF β signaling requires its kinase activity. In zebrafish embryos, injection of *rock2a* mRNA attenuates the expression of mesodermal markers during late blastulation and blocks the

induction of mesoderm by ectopic Nodal signals. By contrast, overexpression of a dominant negative form of zebrafish *rock2a*, *dnrock2a*, has an opposite effect on mesoderm induction, suggesting that Rock2 proteins are endogenous inhibitors for mesoderm induction. Thus, our data have unraveled previously unidentified functions of Rock2, in controlling TGF β signaling as well as in regulating embryonic patterning.

Key words: Rho kinases, Rock2, TGF β , Zebrafish, Embryo

Introduction

The transforming growth factor β (TGF β) signal transduction pathway, which involves multiple ligands, including Activin, Nodal, Vg1 and GDFs, is a key player during early development of vertebrate embryos. Among the TGF β ligands, Nodal proteins are absolutely required for mesendoderm induction and the establishment of left-right asymmetry (Schier, 2003; Shen, 2007; Tian and Meng, 2006). These ligands initiate signal transduction by binding to type I and type II serine/threonine kinase receptors on the plasma membrane, which results in the activation of type I receptors (Shi and Massague, 2003). The activated receptors phosphorylate the downstream effectors Smad2 and/or Smad3, and the phosphorylated Smad2 or Smad3 form complexes with Smad4 and translocate into the nucleus to control the expression of target genes (Jia et al., 2008; Schmierer and Hill, 2007). The multiple steps that are involved in the TGF β signal transduction pathway allow for precise regulation by many modulators through diverse mechanisms (Chen and Meng, 2004).

Intracellular trafficking of internalized TGF β receptors has been found to play a role in regulating TGF β signaling. The membrane-anchored receptors are internalized by clathrin-dependent and clathrin-independent processes (Anders et al., 1997; Di Guglielmo et al., 2003; Lu et al., 2002; Mitchell et al., 2004; Zwaagstra et al., 2001). The internalized receptors are then recycled back to the membrane or degraded via proteasomal or lysosomal pathways (Di Guglielmo et al., 2003; Kavsak et al., 2000; Mitchell et al., 2004). Distinct targeting of the internalized receptors, a process regulated by multiple factors, determines the duration of the intracellular signaling, and thus has profound effects on vertebrate embryonic development (Jullien and Gurdon, 2005; Shim et al., 2006; Zhang et al., 2004).

Targeting and trafficking of intracellular proteins, including internalized receptors, rely on functions of actin-filaments and

microtubules (Salinas, 2007; Soldati and Schliwa, 2006). RhoA, a member of the Ras homology family of small GTPases, is a key regulator of the actin cytoskeleton during cell morphogenesis and motility (Etienne-Manneville, 2004; Ridley, 2006). The Rho-associated serine/threonine kinases Rock1 (also termed Rok1 and Rok β) and Rock2 (also termed Rok2, Rok α and Rho kinase) are immediate downstream targets of RhoA (Amano et al., 1997; Ishizaki et al., 1996; Leung et al., 1995; Matsui et al., 1996); they phosphorylate a variety of substrates, including myosin light chain (Amano et al., 1996; Totsukawa et al., 2000), the myosin-binding subunit (MYPT1) of the myosin-associated form of protein phosphatase 1 (Velasco et al., 2002), LIM kinases (Ohashi et al., 2000; Sumi et al., 2001), adducin (Fukata et al., 1999) and Zipper-interacting protein kinase (Hagerty et al., 2007). Rho kinases may intervene with intracellular transduction and propagation of various extracellular signals by assisting intracellular targeting of the signaling pathway components. Several reports have shown that RhoA and Rho kinases can be activated by TGF β signaling and are required for mediating the effects of TGF β signals in epithelial-to-mesenchymal transdifferentiation, actin filament rearrangement and growth inhibition (Bhowmick et al., 2001; Edlund et al., 2002; Kamaraju and Roberts, 2005). However, it is largely unknown whether RhoA and Rho kinases function to down-regulate TGF β signal transduction.

Rho kinases have been found to be important regulators of cell contraction, adhesion and migration as well as proliferation and apoptosis (Riento and Ridley, 2003; Shi and Wei, 2007). Many studies using Rho kinase inhibitors have indicated that Rho kinases are involved in various physiological and pathological processes (Shi and Wei, 2007; Wetschurck and Offermanns, 2002). Developmental roles of Rho kinases have been examined in mice by knockout approaches. *Rock1* null mice died soon after birth with failure of the

closure of the eyelids and the ventral body wall (Shimizu et al., 2005). Most *Rock2*-deficient mouse embryos died in uterus with placental dysfunction and intrauterine growth retardation; the few survivors were born runts and developed without gross anomalies (Thumkeo et al., 2003). In *Xenopus* and zebrafish, *Rock2* is required for cell convergence and extension movements during gastrulation (Marlow et al., 2002). These reports indicate that *Rock1* and *Rock2* play important roles in regulating cell movements in vertebrate embryos. However, it remains unclear as to whether they are involved in embryonic patterning during early development.

We have previously demonstrated that the late-endosomal protein Dpr2 binds to and facilitates lysosomal degradation of TGF β type I receptors, and that zebrafish Dpr2 functions to inhibit mesoderm induction by Nodal signals during early embryogenesis (Zhang et al., 2004). The function of Dpr2 is evolutionarily conserved in vertebrates (Su et al., 2007). In this study, we identified *Rock2* as binding partner of Dpr2. We disclose that, like Dpr2, *Rock2* attenuates TGF β signaling by promoting lysosomal degradation of TGF β type I receptors in mammalian cells. In zebrafish embryos, *Rock2* inhibits mesoderm induction by Nodal signals. Thus, our findings have revealed novel functions of *Rock2*, in signaling transduction and in embryonic development.

Results

Rock2 negatively regulates TGF β signaling in mammalian cells
Using a yeast two-hybrid screen to search for Dpr2-interacting factors in zebrafish, we identified *Rock2a* as a Dpr2-binding partner. We then looked at whether *Rock2a* also plays a role in regulating TGF β signal transduction. We first investigated the effects of zebrafish

Rock2a on TGF β signaling using the ARE-luciferase reporter that is driven by Activin responsive elements (Huang et al., 1995). The expression of ARE-luciferase in Hep3B cells was stimulated by the addition of TGF β 1 (Fig. 1A) or by transfection of the constitutively active (ca) form of type I receptors ALK5 (Fig. 1B) or ALK4 (data not shown), whereas the reporter activity was inhibited by co-transfection of zebrafish *Rock2a* in a dose-dependent manner. The inhibitory effect of zebrafish *Rock2a* on ARE-luciferase reporter expression was also observed in HepG2 cells (Fig. 1C). Furthermore, overexpression of human *ROCK2* also inhibited ARE-luciferase reporter expression (Fig. 1C). The inhibitory effect of human *ROCK2* overexpression on ARE-luciferase expression in response to TGF β 1 stimulation was blocked in the presence of the Rock kinase inhibitor Y-27632 in Hep3B cells (data not shown), indicating that the Rock catalytic activity is required. These results imply that *Rock2* functions to impede TGF β signal transduction, and that this function is evolutionarily conserved.

To investigate the effects of endogenous *ROCK2* on TGF β signaling, we made two siRNA constructs, pSilencer-*ROCK2*-siRNA1 and pSUPER-*ROCK2*-siRNA2 that were designed to target different regions of human *ROCK2* mRNA. Transfection of either of the constructs resulted in a decrease in HA-*ROCK2* expression levels (Fig. 1D). Quantitative RT-PCR analysis revealed that transfection in HEK293T cells with these siRNAs caused significant reduction of endogenous *ROCK2* mRNA levels but had little effect on *ROCK1* mRNA levels (data not shown). These results indicate that both *ROCK2*-siRNAs were able to effectively and specifically target *ROCK2*. When endogenous *ROCK2* in Hep3B cells was knocked down with pSilencer-*ROCK2*-siRNA1 or pSuper-*ROCK2*-siRNA2,

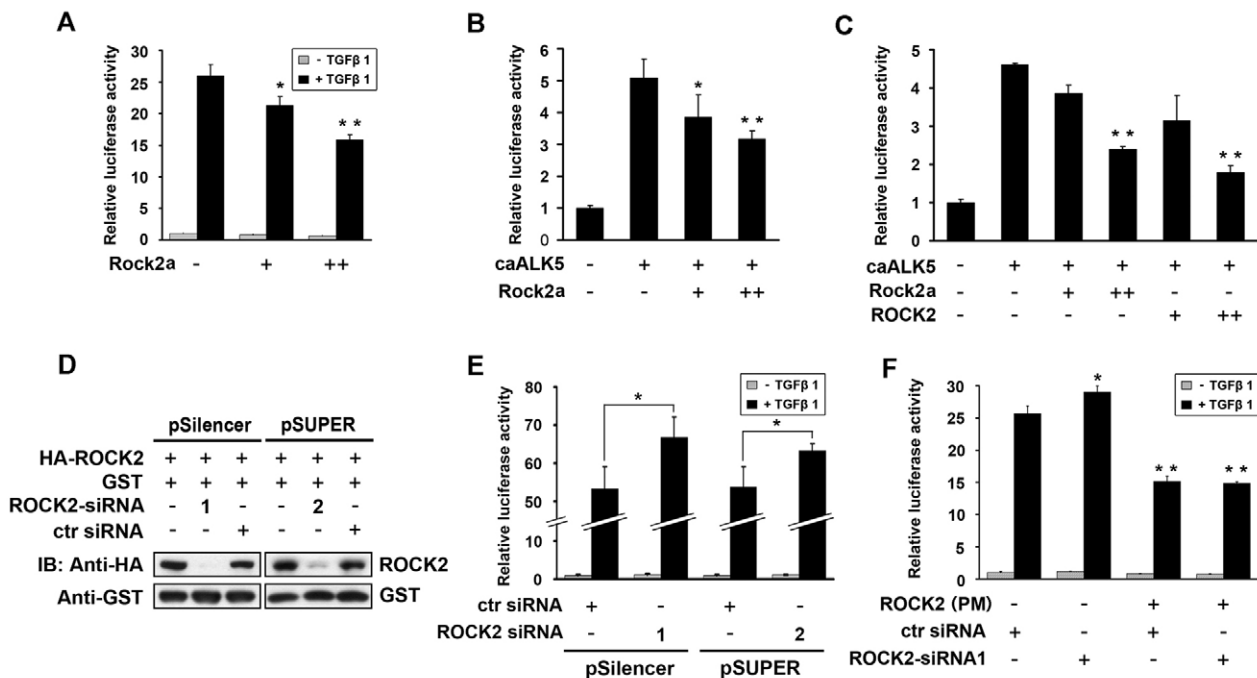


Fig. 1. *Rock2* inhibits TGF β signaling in mammalian cells. (A-C) Co-expression of fish *Rock2a* (A-C) or human *ROCK2* (C) attenuated TGF β 1-stimulated (A) or caALK5-induced (B,C) expression of the ARE-luciferase reporter in a dose-dependent manner in Hep3B (A,B) or HepG2 (C) cells. Doses: ARE-luciferase, 0.4 μ g; caALK5, 0.1 μ g; fish *Rock2a*, 0.2 μ g (+) or 0.4 μ g (++); human *ROCK2*, 1 μ g (+) and 2 μ g (++); and TGF β 1, 50 pM. (D) Efficiency of siRNAs targeting human *ROCK2*. HA-*ROCK2* and GST plasmids were transiently transfected into HEK293T cells with *ROCK2*-specific siRNAs, control siRNA or empty vector as indicated. The expression of HA-*ROCK2* and GST in cell lysates were detected by immunoblotting with anti-HA and anti-GST antibodies, respectively. (E) Knockdown of human *ROCK2* significantly enhanced the response of the ARE-luciferase reporter to TGF β 1 stimulation (100 pM) in Hep3B cells. (F) Effect of *ROCK2*-siRNA1 on TGF β 1-stimulated ARE-luciferase reporter expression was blocked by co-expression of the *ROCK2* mutant *ROCK2* (PM) in Hep3B cells. The relative luciferase activity from ctr siRNA transfection was used as the control for statistical comparisons. For all reporter assays, data represent the mean \pm s.d. of the triplicates after being normalized to Renilla activity. * P <0.05; ** P <0.01.

TGFβ1 induction of the ARE-luciferase reporter expression was further enhanced (Fig. 1E). Furthermore, the effect of ROCK2-siRNA1 on TGFβ1-stimulated ARE-luciferase reporter expression was blocked by co-expression of the ROCK2 mutant ROCK2 (PM) (Fig. 1F), which contains six mutations within the ROCK2-siRNA1 recognition site and is thus resistant to ROCK2-siRNA1. These results confirm that ROCK2 is able to inhibit TGFβ signaling in human cells.

Rock2 promotes degradation of TGFβ type I receptors via lysosomal pathway

Dpr2 has been found to inhibit TGFβ signaling by accelerating the degradation of TGFβ type I receptors (Su et al., 2007; Zhang et al., 2004). We speculated that Rock2 might regulate TGFβ signaling through a similar mechanism. To test this, we first examined the physical interaction between Rock2 and TGFβ type I receptors by co-immunoprecipitation. We found that overexpressed fish Rock2a could associate with co-expressed wild-type (ALK5-FLAG) and constitutively active (caALK5- FLAG) forms of the receptor ALK5

in human HEK293T cells (data not shown). Overexpressed HA-ROCK2 bound to a small amount of endogenous ALK5 without the stimulation of exogenous TGFβ1, but their association was intensified dramatically by the addition of TGFβ1 (Fig. 2A), suggesting that Rock2 preferentially associates with the activated receptor.

Next, we tested whether overexpression of Rock2 promoted degradation of ALK4 or ALK5. When fish Rock2a was co-overexpressed, protein levels of caALK4 or caALK5 were markedly reduced in a dose-dependent fashion (Fig. 2B). Even in the presence of cycloheximide (CHX), a protein synthesis inhibitor, transfection of fish Rock2a resulted in apparent reduction of the caALK5 level (Fig. 2C) and led to a shortened half-life for caALK5 (Fig. 2C'). This implied that the Rock2a-induced caALK5 reduction was not due to impaired transcription or translation. Similarly, transfection of human ROCK2 caused reduction of the caALK5 level in the presence of CHX (Fig. 2D), suggesting a conserved function of Rock2 from fish to human. Consistent with these observations, knockdown of *ROCK2* in human HEK293T cells by ROCK-siRNAs

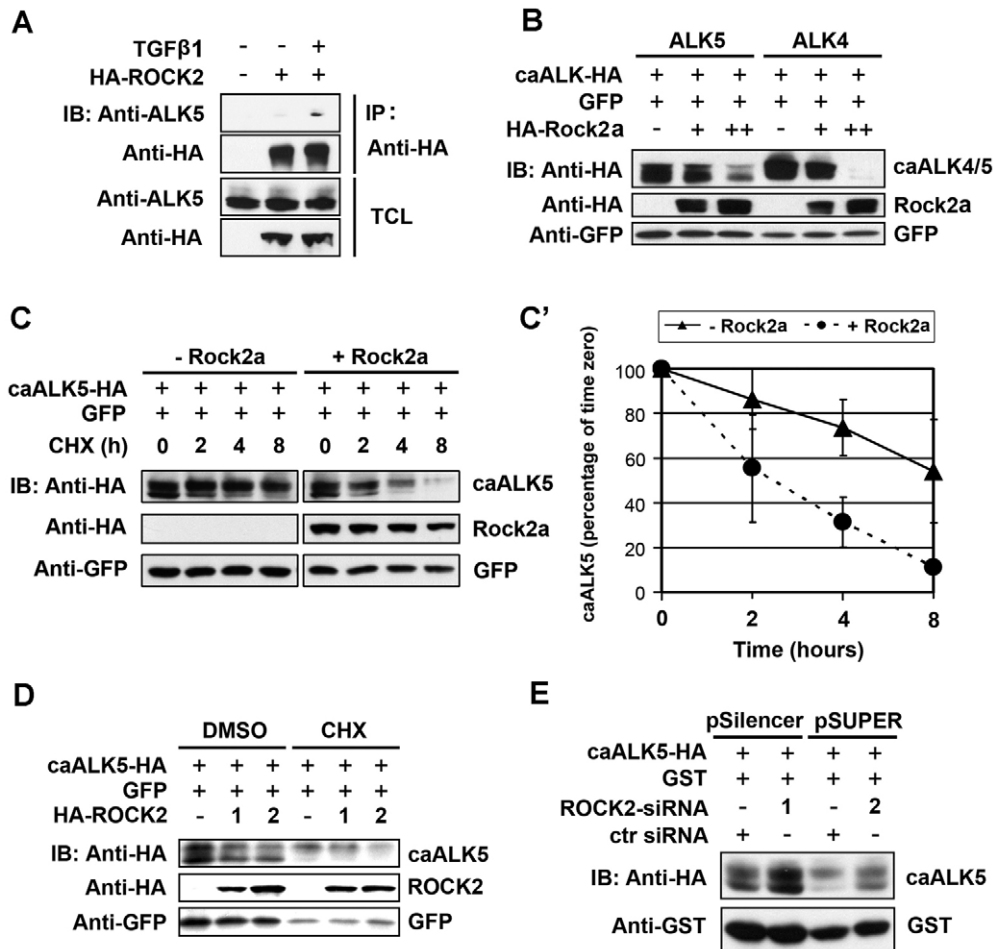


Fig. 2. Rock2 binds to and accelerates degradation of ALK5. (A) Co-immunoprecipitation detected association of endogenous ALK5 with overexpressed HA-ROCK2. Note that the addition of 100 pM TGFβ1 promoted the ALK5-ROCK2 association. (B) The amount of caALK4 or caALK5 decreased by co-transfection of fish Rock2a in a dose-dependent manner. HA-Rock2a dose: +, 1 μg; ++, 2 μg. Lysates of transfected cells were immunoblotted with anti-HA to detect caALK4 or caALK5 and Rock2a, and with anti-GFP to detect the internal standard GFP. (C) Rock2a transfection accelerated the degradation of caALK5 protein in the presence of 100 μg/ml cycloheximide (CHX). The duration of CHX treatment is indicated in hours. GFP levels were used as controls. Control assays using DMSO and the short-lived protein mouse Tob1 were performed, but the data are not shown here. (C') Time-course of caALK5 turnover. Three independent experiments were performed as described in C. The intensity of caALK5 and GFP bands were scanned and quantified using ImageJ. After normalization with the internal control (GFP) the relative quantity of caALK5 (% of time zero) was indicated as the mean ± s.d. of three independent experiments at different time points. (D) Overexpression of human ROCK2 induced caALK5 degradation in a dose-dependent manner in the presence of CHX. HA-ROCK2 dose: +, 1 μg; ++, 2 μg. (E) Knockdown of endogenous ROCK2 resulted in higher levels of caALK5 protein. GST was used as a control. All the experiments were performed in HEK293T cells. TCL, total cell lysates; IP, immunoprecipitation.

gave rise to an increase in the amount of overexpressed caALK5 (Fig. 2E). Thus, Rock2 controls TGF β signaling by accelerating the degradation of TGF β type I receptors.

We then made efforts to identify the pathway(s) through which Rock2 promotes degradation of ALK4 and ALK5. We found that the presence of the lysosomal inhibitors NH₄Cl, bafilomycin A1 or chloroquine, but not of the proteasome inhibitor MG132, prevented Rock2a-induced caALK5 degradation (Fig. 3A). We obtained similar results for caALK4 (data not shown). Thus, Rock2 promotes the receptor degradation through the lysosomal pathway but not the proteasomal pathway. We then adopted a biotinylation method (Lu et al., 2002) to investigate the effect of Rock2 on the degradation of TGF β receptors anchored on the cell surface. In the precipitates pulled-down with streptavidin 3 hours after activation of internalization, the amount of endogenous cell surface ALK5, detected with anti-ALK5 antibody, was markedly reduced by overexpression of HA-ROCK2 (Fig. 3B). Taking these results together, we hypothesize that Rock2 binds to and promotes the degradation of endogenous ALK5 internalized from the cell surface.

Effect of Rock2 on TGF β signaling depends on its kinase activity
As a serine/threonine kinase, Rock2 exerts its biological effects by phosphorylating substrates (Riento and Ridley, 2003). We then looked at whether the kinase activity of Rock2 was required for its ability to inhibit TGF β signaling. We first used the ROCK kinase inhibitors Y27632 (Ishizaki et al., 2000) and HA1077 (Davies et al., 2000) to address this issue. Treatment with either of the inhibitors in mammalian Hep3B cells resulted in elevated levels of TGF β 1-induced ARE-luciferase reporter expression (Fig. 3C). The presence of Y27632 or HA1077 also caused an increase in caALK5-HA levels and attenuated the HA-ROCK2-induced caALK5-HA degradation (Fig. 3D; data not shown for HA1077 treatment). These results suggest that the kinase activity of ROCKs is required for the inhibitory effect on TGF β signal transduction. We further tested the role of Rock2 kinase activity in the inhibition of TGF β signaling by using several mutant forms of human ROCK2 (Amano et al., 1997; Amano et al., 1999; Tan et al., 2002), including the constitutively active mutant ROCK2-CAT, which retains the N-terminal region (6-553 aa) only, ROCK2-CAT KD (identical to

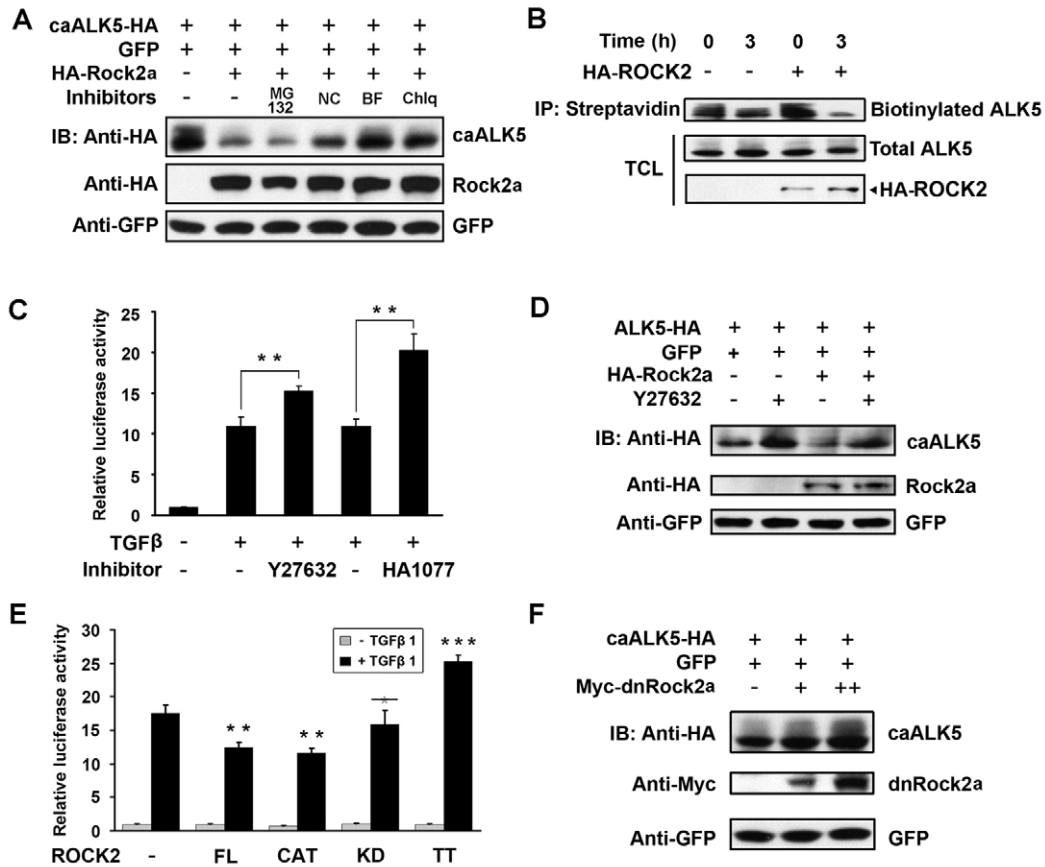


Fig. 3. Rock2 promotes the lysosomal degradation of ALK5 depending on its own kinase activity. (A) Lysosomal inhibitors, but not proteasomal inhibitor, attenuated caALK5 degradation induced by fish Rock2a. HEK293T cells were co-transfected with 2 μ g of HA-Rock2a or with empty vector as a control. At 24 hours post-transfection, cells were treated with the proteasomal inhibitor MG132 (25 μ M), with the lysosomal inhibitors NH₄Cl (NC, 25 mM), bafilomycin A1 (BF, 500 nM) or chloroquine (Chlq, 100 μ M), or not treated for 6 hours and then harvested for anti-HA and anti-GFP immunoblotting to detect expression levels of caALK5-HA, HA-Rock2a or GFP, respectively. (B) ROCK2 promotes the turnover of biotinylated endogenous cell surface ALK5 in HeLa cells. The incubation times at 37°C in the presence of 100 pM TGF β 1 following biotin labeling at 4°C, which allowed internalization before harvest, are indicated. Note that the cell surface ALK5 amount was remarkably lower in the cells transfected with Rock2 than in the control cells following 3-hour internalization. TCL, total cell lysates; IP, immunoprecipitation. (C) TGF β 1 (50 pM)-stimulated ARE-luciferase reporter expression in Hep3B cells was intensified by treatment with Y27632 (10 μ M) or HA1077 (20 μ M). (D) Inhibition of Rock kinase activity with 10 μ M Y27632 counteracted the effect of ROCK2 on the degradation of overexpressed ALK5 protein in HEK293T cells. Cells were stimulated by 100 pM TGF β 1 in the presence of 100 μ g/ml CHX, and were collected after 6 hours for protein detection by western blotting. (E) Effect of different ROCK2 deletion mutants on caALK5-induced ARE-luciferase reporter expression in Hep3B cells. The mutants are described in the text. ** P <0.01; *** P <0.001; asterisk with a line through, P >0.1. (F) Co-transfection of dnRock2a, which is a dominant negative form of fish Rock2a, elevated caALK5 levels in HEK293T cells in a dose-dependent manner. DnRock2a doses: +, 1 μ g; ++, 2 μ g. Protein levels were examined by western blotting.

ROCK2-CAT except for loss of kinase activity) and the dominant negative mutant ROCK2-RB/PH(TT), which carries the C-terminal region (1125-1388 aa) but lacks RhoA-binding activity. Like the full-length ROCK2, ROCK2-CAT overexpression inhibited the caALK5-stimulated ARE-luciferase reporter expression in Hep3B cells (Fig. 3E). By contrast, overexpression of ROCK2-CAT KD had no effect, but ROCK2-RB/PH(TT) overexpression enhanced the reporter expression (Fig. 3E). Furthermore, overexpression of dnRock2a, a dominant negative mutant of zebrafish Rock2a that is similar to ROCK2-RB/PH(TT) (Marlow et al., 2002), elevated caALK5-HA protein levels in HEK293T cells (Fig. 3F). Taken together, these data support the idea that the kinase activity of Rock2 is required for regulating TGFβ type I receptor degradation.

rock2a overexpression inhibits mesoderm induction in zebrafish embryos

As shown above, fish Rock2a is able to downregulate TGFβ signaling by facilitating the degradation of ALK4 and ALK5 receptors in mammalian cells. We wondered whether Rock2a could also regulate TGFβ signaling in zebrafish embryos. Because Nodal signaling is essential for mesoderm induction (Tian and Meng, 2006), we looked at whether *rock2a* had an effect on mesoderm induction in zebrafish embryos. A previous study by Marlow et al.

demonstrated in zebrafish that *rock2a* plays a role in cell convergence and extension movements during gastrulation by mediating non-canonical Wnt11 signaling (Marlow et al., 2002). To avoid interpretation ambiguity due to abnormal convergence and extension movements, we examined the expression of the Nodal targets *gooseoid* (*gsc*), *chordin* (*chd*) and *no tail* (*ntl*) at the sphere stage, well before the onset of gastrulation, following injection of zebrafish *rock2a* mRNA into one-cell embryos. As shown in Fig. 4A,A', overexpression of *rock2a* led to reduction or elimination of *gsc*, *chd* and *ntl* expression in the dorsal margin where dorsal mesoderm precursors are located, whereas injection of *lacZ* mRNA had little effect on *gsc* and *ntl* expression. Unexpectedly, injection of *lacZ* mRNA also caused reduction of *chd* expression, which might have occurred through unknown mechanisms. To confirm the in situ hybridization results, we performed quantitative RT-PCR analysis. As shown in Fig. 4B, *rock2a* overexpression caused significant reduction in *gsc*, *ntl* and *chd* transcript levels, which excludes the possibility that the change of the marker expression patterns is owing to abnormal convergent extension movements. By contrast, neither quantitative RT-PCR nor whole-mount in situ hybridization detected any decrease following *rock2a* overexpression, even if no increase, in the expression of *bozozok* (*boz*) (Fig. 4B,C), which is a direct target of canonical Wnt/β-catenin

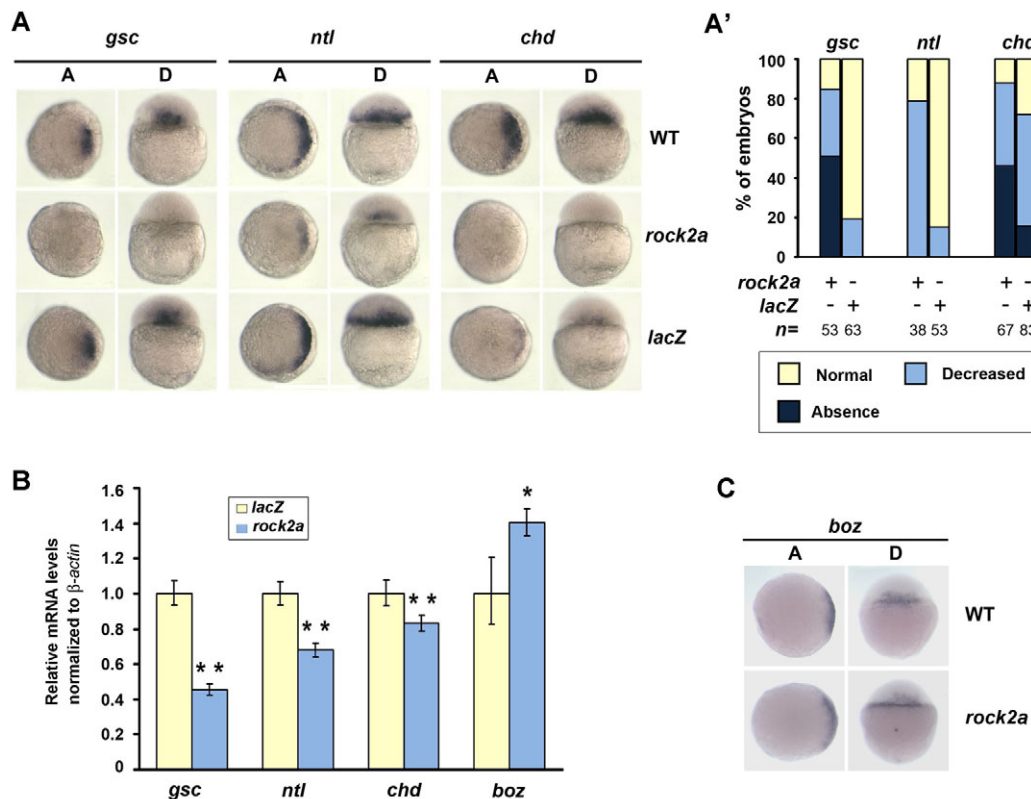


Fig. 4. Overexpression of *rock2a* inhibits mesodermal induction in zebrafish embryos. (A) Overexpression of *rock2a* downregulated expression of *gsc*, *ntl* and *chd*. Zebrafish embryos were injected at the one-cell stage with 400 pg of *rock2a* or *lacZ* mRNA. The expression of marker genes (named on the top of each panel) in injected or wild-type (WT) embryos was detected by whole-mount in situ hybridization at the sphere stage. For each marker, embryos were shown in animal-pole view ('A', left) and dorsal view ('D', right). Note that *lacZ* mRNA injection had little effect on *gsc* and *ntl* expression but interfered with *chd* expression through unknown mechanisms (three independent experiments produced similar results). (A') Statistical data for A. Percentages of embryos with variable expression levels (in different colors) of indicated genes were calculated. The number of observed embryos (*n*) is indicated below each bar. (B) *rock2a* overexpression resulted in decrease of *gsc*, *ntl* and *chd* transcript amounts. Embryos were injected with 400 pg of *rock2a* or *lacZ* mRNA at the one-cell stage, and were analyzed at the sphere stage by real-time PCR for *gsc*, *ntl*, *chd* and *boz* expression. *actin* was also examined as an internal control. The fold change in transcript levels (*y*-axis) is given relative to control embryos and the data represent the mean ± s.d. from triplicates. Note that *boz* transcript level was not decreased but increased by *rock2a* overexpression. Statistical significance levels: **P*<0.05; ***P*<0.01. (C) The expression pattern of *boz*, a canonical Wnt target, was unaltered in embryos injected with *rock2a*.

signaling (Leung et al., 2003; Ryu et al., 2001). This implies that *rock2a* does not downregulate canonical Wnt/ β -catenin signaling in vivo. Thus, *rock2a* overexpression negatively regulates the expression of the Nodal targets during mesoderm induction, irrespective of the canonical Wnt signaling.

rock2a overexpression impedes mesoderm induction activity of Nodal signals

The next issue to investigate was whether *rock2a* inhibited mesoderm induction by antagonizing Nodal signaling in zebrafish embryos. As reported before (Rebagliati et al., 1998), overexpression of *squint* (*sqt*), a zebrafish Nodal gene, caused dramatic expansion of *gsc*, *chd* and *ntl* expression domains at the

sphere stage (Fig. 5A). The inductive effect of *sqt* was obviously compromised by co-injection of *rock2a* mRNA. Zebrafish *tarama* encodes a TGF β -related type I receptor required for Nodal signaling, and overexpression of its constitutively active form *tar** induces mesendodermal marker expression (Aoki et al., 2002; Renucci et al., 1996). We found that induction of *gsc*, *chd* and *ntl* expression by injection of *tar** mRNA was also inhibited by co-injection of *rock2a* mRNA (Fig. 5B). Lefty1 (also known as Antivin) is a potent antagonist of Nodal ligands, and its overexpression in zebrafish embryos eliminates the formation of most mesendodermal tissues (Cheng et al., 2000; Thisse and Thisse, 1999). When 10 pg of *lefty1* mRNA was injected, the expression levels of *gsc*, *chd* and *ntl* were decreased at the sphere stage (Fig. 5C). When the same

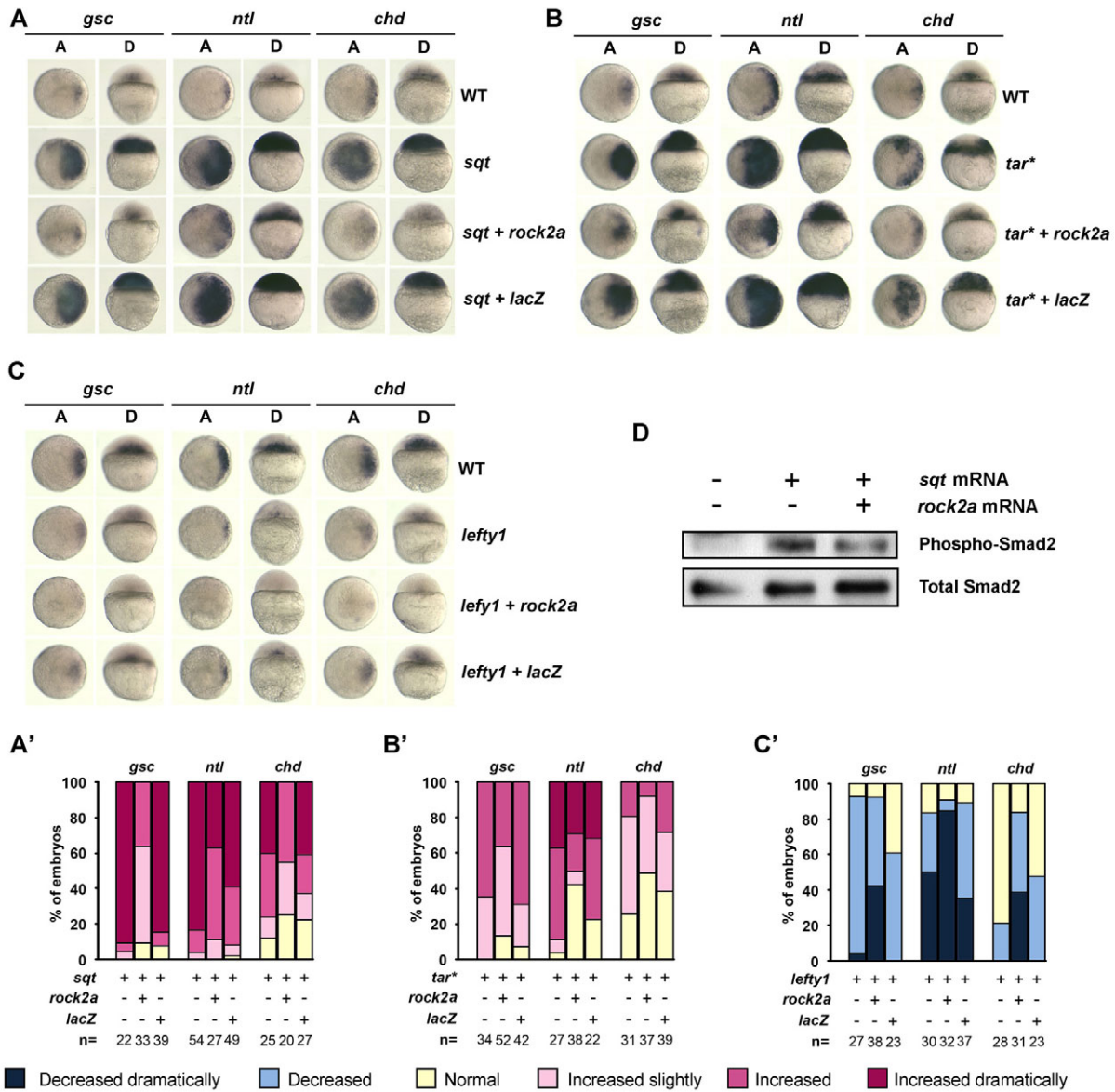


Fig. 5. Overexpression of *rock2a* impedes mesoderm induction of Nodal signals. Expression of marker genes in embryos injected with the indicated mRNAs or in wild-type (WT) embryos was detected by whole-mount in situ hybridization at the sphere stage. For each marker, embryos are shown in animal-pole view ('A', left) and dorsal view ('D', right). (A,B) *rock2a* overexpression inhibited *sqt*-induced (A) or *tar**-induced (B) ectopic expression of the marker genes *gsc*, *ntl* and *chd*. Doses: *sqt* mRNA, 0.25 pg; *tar** mRNA, 0.6 pg; *rock2a* mRNA, 200 pg. (C) *rock2a* overexpression intensified the effect of *lefty1*. Embryos were injected with 10 pg *lefty1* mRNA alone or together with 200 pg *rock2a* mRNA. Note that *lacZ* mRNA injections at a comparable dose served as controls. (A'-C') Statistical data for A-C, respectively. (D) Induction of endogenous phospho-Smad2 by *sqt* was suppressed by *rock2a* co-injection. Embryos were injected at the one-cell stage with 0.5 pg of *sqt* mRNA alone or in combination with 400 pg of *rock2a* mRNA and lysed at the sphere stage for immunoblotting. Phospho-Smad2 and total Smad2 were detected with anti-phospho-Smad2 and anti-Smad2 antibodies, respectively.

amount of *lefty1* mRNA was co-injected with 200 pg of *rock2a* mRNA, most of the injected embryos showed further decrease in the expression levels of these markers. Taken together, these data indicate that *rock2a* overexpression is able to block mesoderm induction by ectopic Nodal signaling and to enhance the inhibitory effect of *lefty1* on mesoderm induction in vivo.

To obtain direct evidence of the effects of *rock2a* on Nodal signaling in fish embryos, we examined phosphorylated Smad2 levels after overexpression of *sqt* and *rock2a*. As shown in Fig. 5D, injection of *sqt* mRNA led to an increase in the phospho-Smad2 levels, but co-injection with *rock2a* mRNA compromised the stimulatory effect of *sqt*, while the total amount of Smad2 remained comparable. These results demonstrate that *rock2a* downregulates the Nodal signaling upstream of Smad2 phosphorylation in fish embryos, which is consistent with the fact that Rock2 accelerates the turnover of TGF β receptors in mammalian cells.

Endogenous Rock2 is required for mesendoderm induction in zebrafish embryos

A previous study carried out by Marlow and coworkers (Marlow et al., 2002) indicated that knockdown of *rock2a* with antisense morpholino oligonucleotides in zebrafish embryos fails to cause any detectable phenotypes, probably due to the presence of the abundant maternal deposit of Rock2 proteins (Marlow et al., 2002). Because the dominant negative mutant *dnrock2a* can attenuate the degradation of TGF β receptor by interfering with endogenous ROCK2 in mammalian cells (Fig. 3F), we evaluated the biological function of endogenous Rock2 proteins by injecting *dnrock2a* mRNA into one-cell embryos and examining *gsc*, *chd* and *ntl* expression using whole-mount in situ hybridization and quantitative RT-PCR analysis at the sphere stage. In situ patterns of these markers were not altered obviously by *dnrock2a* overexpression (data not shown). However, quantitative RT-PCR analysis revealed that overexpression of *dnrock2a* resulted in significant increases in *ntl* and *chd* transcript levels, although the *gsc* transcript level was only marginally increased (Fig. 6A). These results suggest that endogenous Rock2 is required for mesoderm induction during the early development of zebrafish embryos.

We also took another approach, *dnrock2a* and *lefty1* co-injection, to investigate the requirement of endogenous Rock2 for mesendoderm induction. As shown in Fig. 6B,B', *lefty1*-induced reduction of *gsc*, *ntl* and *chd* expression at the sphere stage was compromised by co-injection of *dnrock2a* mRNA. Overexpression of *lefty1* led to reduction of the expression of the endodermal marker *sox32* (also a Nodal target) at the 75% epiboly stage and of *ntl* expression in the axial mesoderm at the bud stage, but caused expansion of the expression domains of the ventral ectodermal marker *gata2* at the 80% epiboly stage (Fig. 6C,C'). These effects of *lefty1* overexpression were also alleviated by co-expression of *dnrock2a*. Our observations support the idea that endogenous Rock2 proteins play an inhibitory role in mesendoderm induction during zebrafish embryogenesis.

Rock2 inhibits TGF β signaling independent of Dpr2

To confirm the functional relationship between Rock2 and Dpr2, we first tested their physical interaction by co-immunoprecipitation assay. As shown in Fig. 7A, overexpressed HA-Rock2a in HEK293T cells was co-immunoprecipitated with Myc-Dpr2, and vice versa. Moreover, endogenous human ROCK2 was found to associate with overexpressed Myc-tagged mouse Dpr2 in HEK293T cells (Fig. 7B). We next investigated whether the functions of Dpr2

and Rock2a in regulating TGF β signaling were interdependent. Like the full-length Rock2a, transfection of HA-Rock2a Δ Dpr2, a Rock2a mutant that was depleted of 1068-1110 aa and failed to interact with Dpr2 (data not shown), attenuated the caALK5-stimulated expression of the ARE-luciferase reporter in Hep3B cells (Fig. 7C), suggesting that the effect of Rock2a is independent of its Dpr2-binding ability. When Dpr2 in HEK293T cells was knocked down by Dpr2-siRNA1 (Su et al., 2007), co-expression of HA-Rock2a was still able to effectively accelerate caALK5-HA degradation (Fig. 7D). Thus, it appears that Rock2a inhibits TGF β signaling in a Dpr2-independent manner. By contrast, transfection of Dpr2 had little effect on the TGF β 1-stimulated ARE-luciferase reporter expression in Hep3B cells upon knockdown of endogenous ROCK2 by pSilencer-ROCK2-siRNA1 (Fig. 7E). Consistent with this observation, overexpression of Dpr2 failed to promote caALK5-HA degradation when ROCK2 was knocked down in HEK293T cells (Fig. 7F). These data imply that the inhibitory effect of Dpr2 on TGF β signaling relies on ROCK2 function.

We then investigated whether ROCK kinase activity is required for binding to and facilitating the function of Dpr2. We generated a ROCK2 mutant, ROCK2K121G, by mutating K121 to G121 at the ATP-binding site. Like wild-type ROCK2, ROCK2K121G was able to bind to mouse Dpr2 (mDpr2) (Fig. 7G), suggesting that the kinase activity of ROCK2 is not required for associating with Dpr2. However, the presence of the ROCK kinase inhibitor Y-27632 reduced the inhibitory effect of fish Dpr2 overexpression on the TGF β 1-stimulated ARE-luciferase expression (Fig. 7H). Thus, the kinase activity of ROCK2 is dispensable for binding to Dpr2 but is necessary for assisting the function of Dpr2 in the control of TGF β signaling.

Discussion

In this study, we have demonstrated that Rock2, which was identified as a binding partner of Dpr2, acts to inhibit TGF β signal transduction in mammalian cells. Mechanistically, Rock2 binds to and promotes the degradation of endocytosed TGF β type I receptors via the lysosomal pathway. In zebrafish embryos, *rock2a* overexpression inhibits mesoderm induction by Nodal signals. Interference with endogenous Rock2 proteins by injecting a dominant negative mutant (*dnrock2a*) of *rock2a* compromises the inhibitory effect of *lefty1* on mesendoderm marker expression, suggesting an involvement of endogenous Rock2 proteins in mesendoderm induction during early embryogenesis. Thus, our study discovered a novel role of Rock2 in modulating TGF β /Nodal signaling and in controlling mesendoderm induction.

Rock1 and Rock2 have been found to play pivotal roles in cell motility. They regulate actin-cytoskeleton assembly and cell contractility by phosphorylating specific substrates, which ultimately exert their effects on the cytoskeleton (Riento and Ridley, 2003). We found that Rock2 could promote lysosomal degradation of endocytosed TGF β type I receptors. Hypothetically, this effect of Rock2 may be associated with cytoskeleton-dependent intracellular trafficking of the endosomes containing TGF β receptor towards lysosomes. Several studies have shown that RhoA and Rho kinases regulate endocytosis of transferrin and epidermal growth factor receptors (Lamaze et al., 1996), low-density lipoprotein receptors (Hrboticky et al., 2002), and equine herpesvirus 1 (Frampton et al., 2007). Kaneko et al. demonstrated that Rho kinases might inhibit endocytosis of epidermal growth factor receptors by phosphorylating endophilin A1, a key regulator of clathrin-mediated endocytosis (Kaneko et al., 2005). Accelerated degradation of the

endocytosed TGF β receptors by Rock2 might be ascribed to its ability to phosphorylate substrates that facilitate transportation of the TGF β -receptor-bound endosomes. Further studies are needed to test this hypothesis.

On the one hand, we found that Rock2 was physically associated with Dpr2 and TGF β type I receptors; on the other hand, Dpr2 is primarily located in the late endosomes, and it also binds to endocytosed TGF β type I receptors (Zhang et al., 2004). What then is the relationship between Rock2 and Dpr2 in controlling TGF β signaling? We note that Dpr2-induced degradation of TGF β receptors is largely Rock2-dependent in mammalian cells, whereas the effects of Rock2 on TGF β -receptor degradation are independent of Dpr2 function. We hypothesize that Dpr2 binds to TGF β

receptor(s) located on endosomes and presents such receptors to Rock2, which promotes movement of such 'labeled' endosomes towards lysosomes along the cytoskeleton. Most probably, other unidentified adaptor proteins may have a function similar to Dpr2, which recognizes and presents TGF β receptors to Rock2.

In zebrafish, *rock2a* is maternally expressed and its transcripts are distributed in all blastomeres, implying that it may be involved in specification of cell fate during early embryogenesis. A previous study found that knockdown of *rock2a* with antisense morpholinos failed to cause any detectable defects in early zebrafish embryos, even in convergence and extension movements during gastrulation (Marlow et al., 2002). One reason for this could be the presence of the abundant maternally derived Rock2a protein that is not targetable

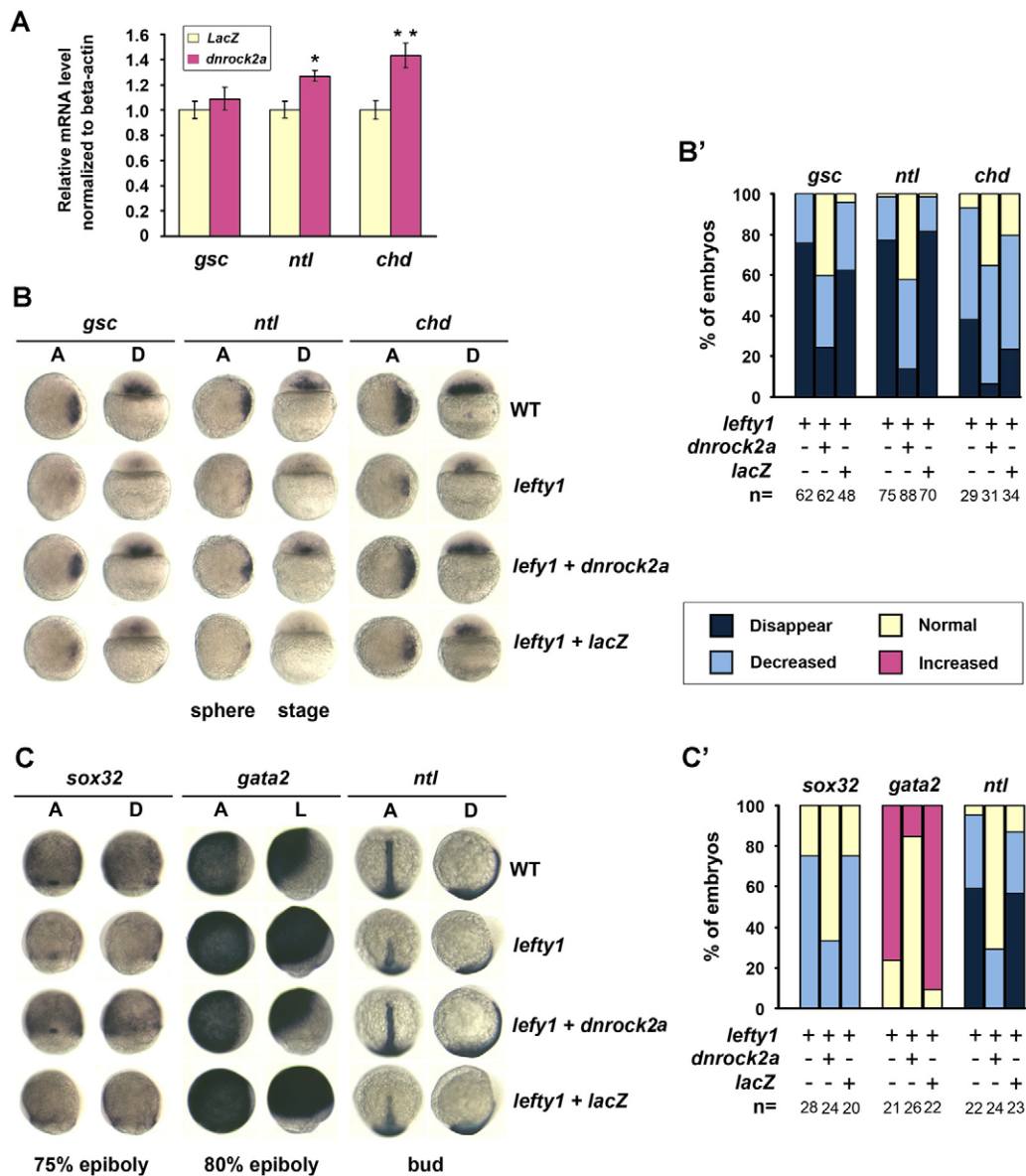


Fig. 6. Interference with endogenous Rock2 proteins promotes mesodermal induction in zebrafish embryos. (A) Real-time RT-PCR analysis of mesodermal makers in embryos injected with *dnrock2a*. Embryos were injected at the one-cell stage with 400 pg of *dnrock2a* or *lacZ* mRNA, and total RNAs were extracted at the sphere stage for real-time RT-PCR analysis. (B,C) The expression changes of marker genes caused by ectopic expression of *lefty1* (20 pg) were neutralized by 50 pg of *dnrock2a* mRNA co-injection. The expression of the marker genes (indicated on the top) was detected by whole-mount in situ hybridization at the indicated stages. Embryos are shown in animal-pole views with dorsal to the right ('A'), in dorsal views with animal pole to the top ('D'), or in lateral views with dorsal to the right ('L'). Corresponding statistical data are presented in B' and C'. Percentages of embryos with variable expression levels of indicated genes are shown in different colors. The number of observed embryos (*n*) is indicated below each bar. **P*<0.05; ***P*<0.01.

by morpholinos. Another reason could be the presence of other *rock* genes in the zebrafish genome, e.g. *rock2b*, *rock2bl* and probably multiple *rock1* genes, which play redundant functions, in addition to *rock2a*. Further genetic studies are needed to disclose the requirement of endogenous Rho kinases in embryonic patterning.

Rho kinases act on many different substrates, implying that they may regulate multiple signaling pathways. Although Rock2 proved to be a negative regulator of TGFβ signaling in this study, several

other studies have found that RhoA and Rho kinases positively regulate convergence and extension movements by mediating non-canonical Wnt signaling in zebrafish and *Xenopus* gastrulas (Jopling and den Hertog, 2005; Kim and Han, 2005; Marlow et al., 2002; Zhu et al., 2006). Our unpublished data indicate that Rock2a may also be a positive regulator of the canonical Wnt/β-catenin signaling pathway. Since these signaling pathways are all involved in embryonic patterning, weak phenotypes caused by genetic

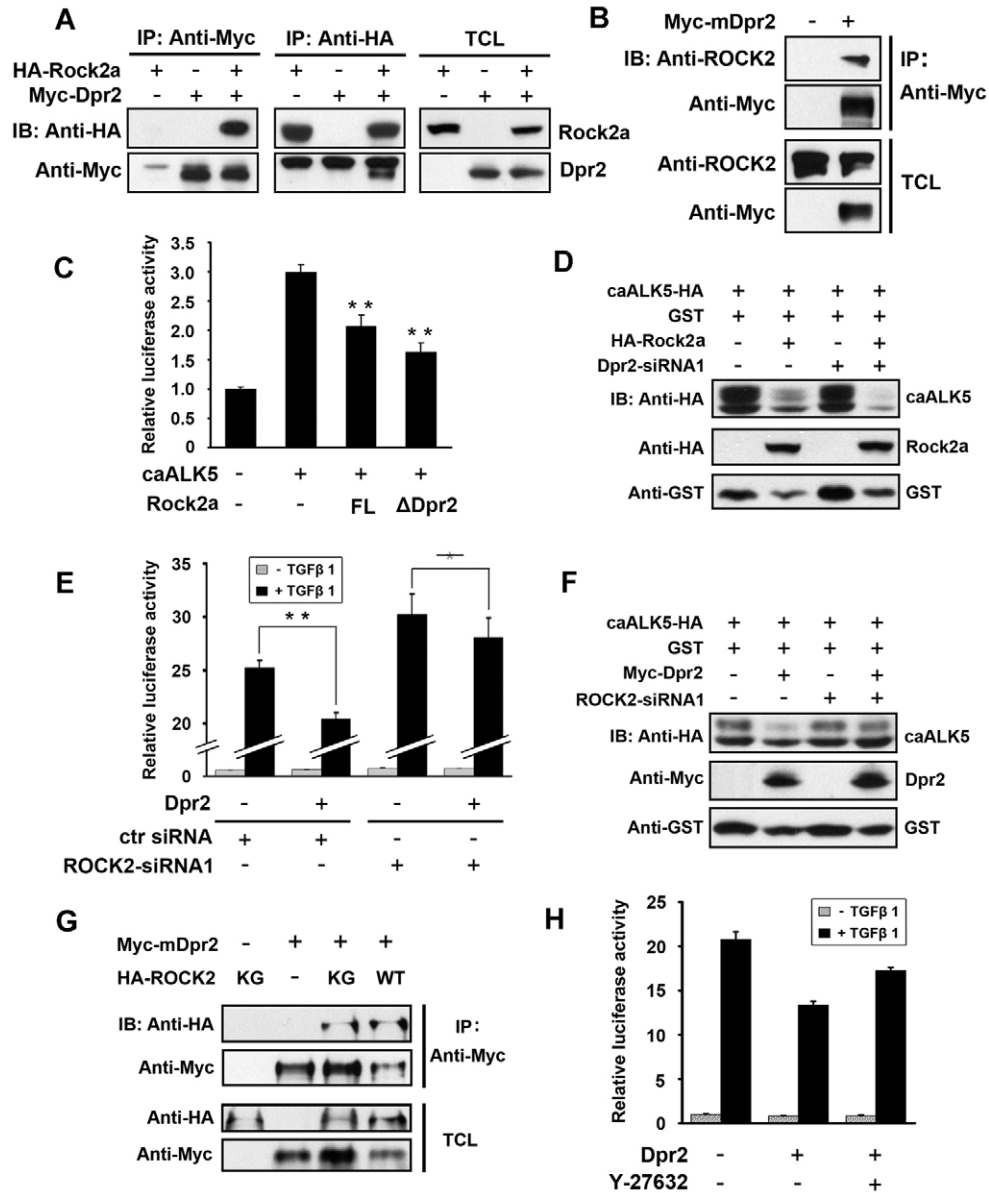


Fig. 7. Interdependence of Rock2 and Dpr2 functions on TGFβ signaling. (A) Physical interaction between zebrafish Rock2a and Dpr2 was verified by reciprocal co-immunoprecipitation in HEK293T cells. (B) Interaction between human endogenous ROCK2 and overexpressed mouse Dpr2 (Myc-mDpr2) was verified by co-immunoprecipitation assay in HEK293T cells. Cells lysates were subjected to immunoprecipitation with anti-Myc antibody, and then the immunoprecipitates together with total cell lysates were immunoblotted with anti-ROCK2 and anti-Myc antibodies, respectively. (C) Rock2aΔDpr2 defective in Dpr2 binding retained the ability to inhibit caALK5-induced ARE-luciferase expression in Hep3B cells. (D) Knockdown of endogenous Dpr2 by siRNA had no impact on Rock2a-induced caALK5 degradation. (E) Dpr2-induced suppression of the ARE-luciferase activity was compromised by ROCK2 knockdown. Hep3B cells were transfected with 0.3 μg of Dpr2 and 0.6 μg of ROCK2-siRNA1, and were stimulated 36 hours later with 50 pM TGFβ1 for an additional 12 hours before analysis. (F) Dpr2-induced caALK5 degradation was diminished by knockdown of endogenous ROCK2. Immunoblotting in D and F was performed essentially as described in the Materials and Methods. (G) Interaction between mouse Dpr2 (Myc-mDpr2) and ROCK2K121G (KG) or ROCK2 (WT) was verified by co-immunoprecipitation assay in HEK293T cells. ROCK2K121G was the kinase-dead full-length ROCK2 mutant. (H) Fish Dpr2-inhibited ARE-luciferase reporter expression was restored by Y-27632 treatment. At 30 hours post transfection, Hep3B cells were stimulated by 100 pM TGFβ1 with Y-27632 (10 μM) pretreatment or not, and then incubated for 16 more hours before harvest. TCL, total cell lysates; IP, immunoprecipitation. ***P*<0.01; asterisk with a line through, *P*>0.1

