Functional interactions between phosphatase POPX2 and mDia modulate RhoA pathways

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Summary

Rho GTPases and their downstream effectors regulate changes in the actin cytoskeleton that underlie cell motility and adhesion. They also participate, with RhoA, in the regulation of gene transcription by activating serum response factor (SRF)-mediated transcription from the serum response element (SRE). SRF-mediated transcription is also promoted by several proteins that regulate the polymerization or stability of actin. We have previously identified a family of PP2C phosphatases, POPXs, which can dephosphorylate the CDC42/RAC-activated kinase PAK and downregulate its enzymatic and actin cytoskeletal activity. We now report that POPX2 interacts with the formin protein mDia1 (DIAPH1). This interaction is enhanced when mDia1 is activated by RhoA.

The binding of POPX2 to mDia1 or to an mDia-containing complex greatly decreases the ability of mDia1 to activate transcription from the SRE. We propose that the interaction between mDia1 and POPX2 (PPM1F) serves to regulate both the actin cytoskeleton and SRF-mediated transcription, and to link the CDC42/RAC1 pathways with those of RhoA.

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Introduction

The Rho GTPases are a family of proteins that function as molecular switches to regulate diverse cellular signalling pathways. They are implicated in the control of cell cycle progression, transcription, differentiation, and of cell morphology, movement and motility. Many of these signalling pathways involve changes to and modulation of the actin cytoskeleton. Expression of active CDC42 and RAC1 leads to the formation of filopodia and lamellipodia, respectively, whereas expression of active RhoA increases the amount of stress fibres and focal adhesions (Etienne-Manneville and Hall, 2002; Lim et al., 1996; Van Aelst and D'Souza-Schorey, 1997).

Effectors downstream of CDC42 and RAC1 that are implicated in the remodelling of the actin cytoskeleton include PAK and the WASP-WAVE family of proteins. Active PAK was reported to cause the dissolution of stress fibres and focal adhesion complexes (Manser et al., 1997). WASP interacts directly with CDC42 and activates the ARP2-ARP3 (ARP2/3) complexes to catalyze actin nucleation and polymerization (Miki et al., 1996). Other proteins such as IRSp53 (BAIAP2) and the diaphanous-related-formin DRF3 (DIAPH3) have also been implicated in promoting filopodia formation downstream of CDC42 (Govind et al., 2001; Krugmann et al., 2001; Peng et al., 2003). The WAVE proteins act downstream of RAC1 to modulate the formation of lamellipodia, using IRSp53 as an intermediate (Miki et al., 1998; Miki et al., 2000). However, not all WAVE isoforms can interact with IRSp53. WAVE1 has been reported to bind to a protein complex that inhibits its function. Binding of RAC1 to this inhibitory complex results in its dissociation, setting WAVE1 free to activate the ARP2/3 complex (Eden et al., 2002). RhoA acts via Rho kinase (ROK, ROCK) and mDia (DIAPH) to modulate the stress fibres (Watanabe et al.,

1999). ROK phosphorylates and inactivates the myosin light chain (MLC) phosphatase (Kimura et al., 1996). MLC thus remains phosphorylated and enhances myosin-filament assembly, which leads to the bundling of actin filaments into stress fibres. ROK also phosphorylates LIM kinase, which in turn phosphorylates cofilin. The final outcome of this cascade of events is the inactivation of the actin-filament severing and depolymerization activities of cofilin. Interestingly, PAK also phosphorylates LIM kinase, leading to the inactivation of cofilin. mDia belongs to a group of proteins containing formin homology domains; these proteins exert their effect on the actin cytoskeleton and the microtubule network. There are three formin homology domains in mDia. FH1 binds to profilin and is reported to aid actin nucleation and polymerization, events that are catalyzed by the FH2 domain (reviewed by Wallar and Alberts, 2003). The function of the FH3 domain is not well understood.

In addition to their effect on the actin cytoskeleton, the Rho proteins also play a role in transcription control (Hill et al., 1995). In particular, transcription from the serum response element (SRE) by the serum response factor (SRF) is enhanced by active RhoA. The link between actin and SRF-mediated transcription has been reported recently (Posern et al., 2002; Sotiropoulos et al., 1999). Several proteins involved in the positive regulation of actin polymerization or stability also promote SRF transcription. The activation of SRF-driven transcription appears to be dependent on the ratio of the levels of filamentous actin and monomeric G-actin in the cells (Sotiropoulos et al., 1999). The transcription co-activator MAL (MKL1) is reported to be the 'sensor' for monitoring the levels of actin in the cytoplasm and for coordinated SRF transcription in some cell types (Miralles et al., 2003). MAL belongs to a family of myocardin-related transcription factors. It

associates with, and is sequestered by, G-actin in the cytoplasm. MAL is translocated into the nucleus upon depletion of G-actin and this translocation is promoted by Rho-induced actin polymerization. Once in the nucleus, MAL functions as a co-activator for SRF activity. A recent paper on the regulation of MAL has proposed a more complex regulation of MAL localization (Vartiainen et al., 2007). It is reported that MAL binds to nuclear actin upon translocation into the nucleus and SRF activity is not activated. In the presence of serum stimulation, MAL will dissociate from the nuclear actin and participate in SRF transcription. Therefore the nuclear import and export of MAL as well as actin are important in the regulation of SRF activities.

We have previously identified two PP2C-type phosphatases, POPX1 (PPM1E) and POPX2 (PPM1F), as binding partners of the CDC42/RAC1 exchange factor PIX (ARHGEF7) (Koh et al., 2002; Manser et al., 1998). POPX can dephosphorylate and inactivate PAK, a PIX partner. Hence, the effector, its activator and its negative regulator are all present in the same complex. PIX also binds GIT, which interacts with paxillin, a component of focal adhesion complexes (Turner et al., 1999; Zhao et al., 2000). The composition of PIX-containing complexes varies because of the temporal and spatial control of different overlapping signalling pathways that use some members in common. We now report that mDia1, involved in RhoA pathways, is also present in POPX-containing complexes that are normally associated with CDC42/RAC1 pathways. We find that the interaction between POPX2 and mDia1 affects the role of mDia1 in SRF-mediated transcription and nuclear localization of MAL. Thus, our results suggest a functional link between the CDC42/RAC1 and RhoA pathways.

Results

POPX2 interacts with mDia

mDia and ROK are crucial effectors for stress-fibre formation. Our initial observation indicated a possible role of POPX2 in the maintenance of stress fibres. To determine whether POPX2 has any functional link with the RhoA pathway, COS7 cells were transfected with Flag-tagged mDia1 and GFP-POPX2 with or without dominant-active RhoA (RhoAV14). Immunoprecipitates obtained using anti-Flag antibodies contained a substantial amount of POPX2 only in the presence of RhoAV14 (Fig. 1A, right panel, lanes 1 and 3). This suggests that POPX2 interacts with 'activated' mDia1. mDia is thought to be in a closed conformation via intra-molecular interaction of its N-terminal with its C-terminal diaphanous auto-regulatory domain (DAD) (Alberts, 2001). When RhoA-GTP binds to the GTPase binding domain (GBD), the auto-inhibition is relieved (Otomo et al., 2005) and 'activated' mDia1 is thus free to interact with other partners, as seen with POPX2. Because mDia is known to be activated by SRC binding (Tominaga et al., 2000), we tested the effect of SRC kinase on mDia1-POPX2 interaction. The presence of SRC did not substantially increase the amount of POPX2 in mDia1 immunoprecipitates, with or without RhoAV14 (Fig. 1A, right panel, lanes 2 and 4). An additional control experiment was also performed to show that the interaction between POPX2 and mDia1 is dependent on the presence of active RhoA. No co-immunoprecipitation of mDia1 and POPX2 was observed in the presence of dominant-negative RhoN19 (supplementary material Fig. S1A). Similarly, RhoV14 did not enhance the interaction between mDia-ΔC (residues 1-571) and POPX2 (supplementary material Fig. S1B), most probably because of the open conformation of mDia-ΔC.

The region of mDia1 responsible for the POPX2 interaction was determined using truncated constructs shown in Fig. 1B. The activated form of mDia1, resulting from truncation of the DAD (ΔDAD), interacted with POPX2 more efficiently than full-length mDia1 (Fig. 1C, compare lanes 1 and 4), possibly as a result of its 'open' conformation. Notable interaction with POPX2 was shown by mDia1 residues 261-571 (Fig. 1C, lane 2); this corresponds to a dominant inhibitory form of mDia1 (mDia-DN) previously described by Nakano et al. (Nakano et al., 1999). Neither the N-terminal truncated mutant (Dia-ΔN1; Fig. 1C, lane 3) nor the mDia-GBD construct (Fig. 1C, lane 5) was co-precipitated with GST-POPX2. These results suggest that the mDia1 domain responsible for the interaction with POPX2 is likely to be the FH3 domain lying within the mDia-DN fragment.

To determine whether POPX2 and mDia1 were in the same complex in vivo, we conducted immunoprecipitation (IP) using affinity-purified anti-POPX2 antibodies. Anti-POPX2 antibodies and random IgG were covalently coupled to Protein G Sepharose beads. The immunoprecipitated products were separated by SDS-PAGE and subjected to western blot analysis using anti-mDia1 antibodies. It was found that mDia1 was present in the POPX2 IP
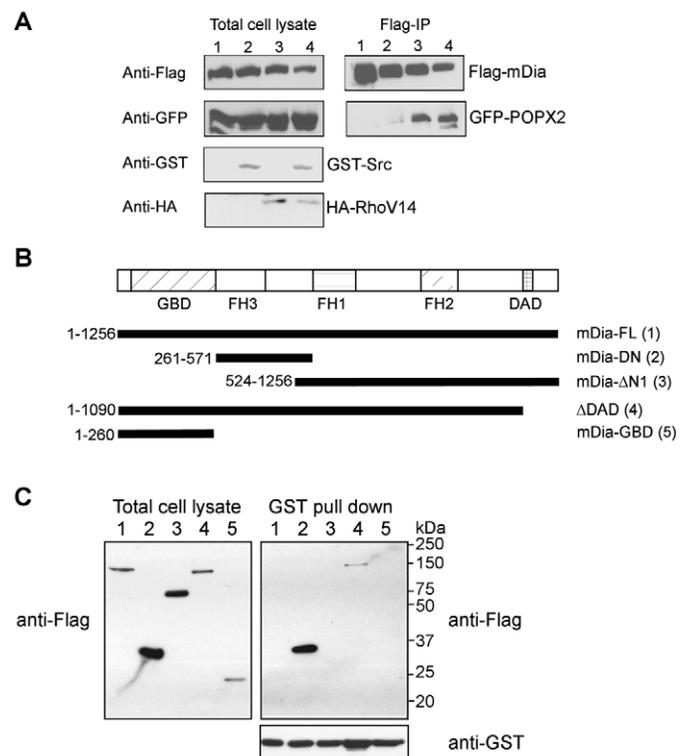

Fig. 1. POPX interacts with mDia1 in the presence of RhoAV14. (A) A construct of flag-tagged full-length mDia1 was co-transfected with GFP-*POPX2* and other cDNA constructs (HA-RhoAV14 or GST-SRC) into COS7 cells. Flag-immunoprecipitated proteins from cell lysates were analyzed by SDS-PAGE and western blotting using anti-GFP antibodies to detect co-precipitated POPX2. Lane 1, mDia-FL + POPX2; 2, mDia-FL + POPX2 + GST-SRC; 3, mDia-FL + POPX2 + HA-RhoAV14; 4, mDia-FL + POPX2 + HA-RhoAV14 + GST-SRC. (B) Schematic of the various mDia constructs. (C) GST-POPX2 was transfected with different Flag-tagged mDia1 constructs into COS7 cells. Cell lysates were incubated with glutathione beads. The bound protein was eluted and analyzed by SDS-PAGE and western blotting using anti-Flag antibodies to detect co-purification of mDia molecules. Lane 1, POPX2 + mDia-FL; 2, POPX2 + mDia-DN; 3, POPX2 + mDia-ΔN1; 4, POPX2 + mDia-ΔDAD; 5, POPX2 + mDia-GBD.

but not in the control IgG IP (Fig. 2A). These data demonstrate that POPX2 and mDia1 form a complex in vivo. We went on to confirm direct interaction by overlaying bacterial-expressed POPX2 onto mDia-DN protein in a dot-blot format (Fig. 2B). POPX2 clearly bound to mDia-DN but not to maltose binding protein (MBP), which was used as a negative control.

The efficiency of POPX2 binding to mDia-ΔC (residues 1-571) and to mDia-DN was compared with that of RhoA, a well-established physiological partner of mDia1 (Watanabe et al., 1997). As expected (Fig. 2C), mDia-ΔC, but not the smaller mDia-DN, co-precipitated with RhoAV14. POPX2 and RhoAV14 exhibited comparable efficiency of co-immunoprecipitation with mDia-ΔC (Fig. 2C, lanes 2 and 4). The mDia-interacting domain of POPX2 was found to be located within an N-terminal region (data not shown). The larger POPX1 also interacted with mDia1 (supplementary material Fig. S2B), probably via conserved residues in the N-terminal region (Koh et al., 2002). Other mDia

proteins [mDia2 and DRF3] also interact with POPX1 and POPX2 (supplementary material Fig. S2).

POPX2 participates in the regulation of transcription from the SRE

Because RhoA acts via mDia to induce SRF-mediated transcription (Hill et al., 1995; Tominaga et al., 2000), we next investigated whether the association of POPX2 with mDia1 affected this nuclear event. As expected, mDia-DN behaved like a dominant-negative mutant and inhibited SRF activity induced by either RhoAV14 (Fig. 3A, lane 3) or the active form of mDia1, mDia-ΔGBD (residues 261-1256; Fig. 3B, lane 3). In this assay, POPX2 was able to inhibit the SRF activity induced by RhoAV14 or active mDia-ΔGBD mutant (Fig. 3A,B, lane 4). Such RhoA- and mDia-induced SRF transcription was reduced to about 15% by the presence of POPX2. A similar inhibitory effect was also observed with the phosphatase-deficient mutant POPX2m (Fig. 3A,B, lane 5). However, PAK-KID, the inhibitory domain of PAK, was a far less-effective inhibitor compared with POPX2 or POPX2m (Fig. 3A,B,
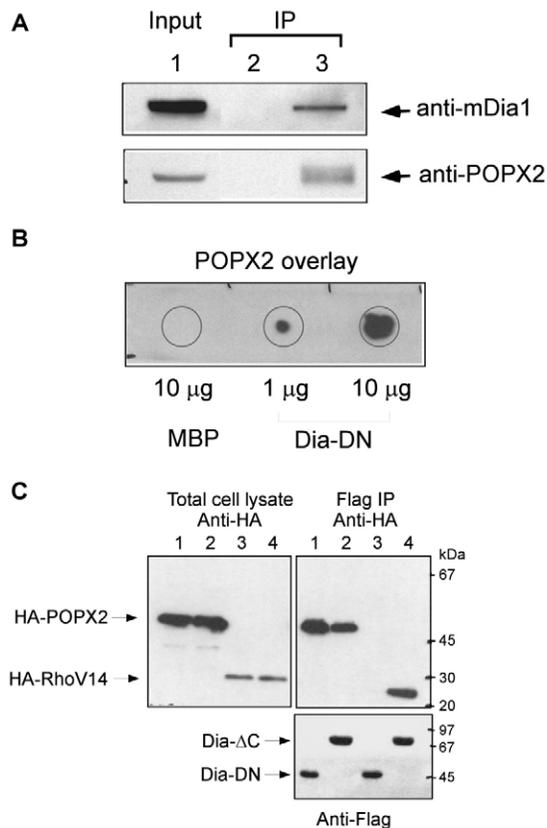

Fig. 2. mDia1 co-immunoprecipitates with POPX2. (A) Protein lysates from COS7 cells were incubated with Protein G Sepharose beads covalently coupled with random IgG or affinity-purified anti-POPX2 antibodies. The eluates were analyzed by SDS-PAGE and western blotting using anti-mDia1 antibodies. Lane 1, COS7 total-protein lysate; 2, eluate from co-IP with random IgG; 3, eluate from co-IP with anti-POPX2 antibodies. (B) Dot-blot using 10 μg of MBP as control protein, 1 μg 6×His-tagged mDia-DN and 10 μg 6×His-tagged mDia-DN. The nitrocellulose membrane was dotted with MBP and 6×His-tagged mDia-DN and incubated with bacterial-produced GST-POPX2. Far-western analysis was done using anti-GST antibodies to detect direct binding. (C) COS7 cells transfected with mDia-DN or mDia-ΔC were co-transfected with POPX2 or RhoV14 to determine the binding by co-IP and western blotting. Lane 1, HA-POPX2 + Flag-mDia-DN; 2, HA-POPX2 + Flag-mDia-ΔC; 3, HA-RhoV14 + Flag-mDia-DN; 4, HA-RhoV14 + Flag-mDia-ΔC.
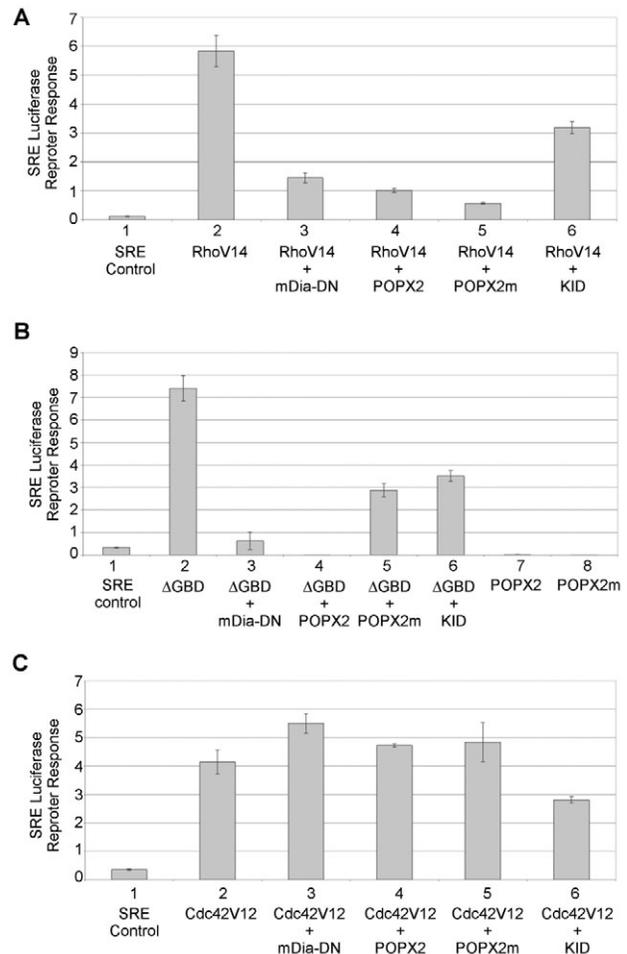

Fig. 3. POPX2 inhibits RhoAV14-induced and active-mDia-induced SRF-mediated transcription. The different plasmid constructs were transfected into COS7 cells as described in the Materials and Methods section. (A) POPX2 inhibits RhoA-induced SRF activity. The transfections were as labelled. (B) POPX2 inhibits active-mDia-induced SRF activity. The transfections were as labelled. (C) POPX2 does not inhibit CDC42-induced SRF activity. The transfections were as labelled.

lane 6), implying that POPX2 does not act primarily by inhibition of PAK. Because POPX2m could also reduce RhoA- or mDia1-induced transcription, it implies that the phosphatase activity of POPX2 is not crucial for the transcriptional inhibition. It is the interaction of POPX2 with mDia1 or with an mDia-containing complex that affects the transcription from the SRE. The POPX2 and POPX2m proteins by themselves have no effect on the SRF-mediated transcription (Fig. 3B, lanes 7 and 8). Knocking down of endogenous POPX2 using siRNA resulted in enhanced SRF-mediated transcription compared with control (supplementary material Fig. S3).

Active CDC42 (CDC42V12) is known to induce transcription from SRE (Hill et al., 1995). Although PAK-KID partially blocked CDC42V12-induced SRF-mediated transcription (Fig. 3C, lane 6), neither mDia-DN nor POPX2 affected the CDC42-induced transcription from the luciferase reporter construct (Fig. 3C, lanes 2, 3 and 4). This suggests that POPX2 specifically affects RhoA-induced transcription from SRE.

POPX2 can affect nuclear translocation of MAL in the cells

Our data indicated that the interaction between POPX2 and mDia1 could influence the SRE response downstream of active mDia1 and RhoA. MAL is a co-activator in SRF-mediated transcription and is reported to function downstream of RhoA (Miralles et al., 2003). Activators of F-actin accumulation – such as RhoAV14, mDia, LIM kinase and profilin – were found to promote nuclear translocation of MAL (Miralles et al., 2003). We wished to determine whether the localization of MAL is influenced by POPX2. A construct of haemagglutinin (HA)-tagged *MAL* and various *POPX2* cDNA constructs were transfected into NIH3T3 cells. The cells were grown in media with 0.5% serum for 18 hours before they were fixed and immunostained for the visualization of MAL localization. This low level of serum was sufficient to trigger MAL nuclear translocation (Fig. 4A, lower panels). POPX2 was able to prevent this translocation (Fig. 4A, upper panels). Consistent with previous reports, the majority of MAL was translocated into the nucleus in the vector control. Whereas only 17% of the cells contained MAL in the cytoplasm (Fig. 4B, lane 1) in the control sample, the presence of POPX2 increased this proportion to 44% (Fig. 4B, lane 3). A similar effect was observed with co-transfection of *POPX1* (Fig. 4B, lane 2). The negative effect of POPX proteins on MAL nuclear translocation appeared to be independent of its phosphatase activities, because the phosphatase-dead mutant, POPX2m, was equally efficient in inhibiting nuclear translocation of MAL (Fig. 4B, lane 4). A truncated version of POPX2 containing the mDia-interacting domain, POPX2N, inhibited MAL translocation to the same extent as full-length POPX2 (Fig. 4B, lane 5). However, expression of the catalytic domain of POPX2 alone (PPc), without the mDia-interacting domain, failed to prevent MAL translocation (Fig. 4B, lane 6); the same result occurred using a phosphatase-dead catalytic domain, PPm (Fig. 4B, lane 7). The behaviour of these mutants was identical to that of the vector control. These data suggest that POPX2 impairs nuclear translocation of MAL, possibly via interaction with mDia or an mDia1-containing complex.

POPX2 prevents stress-fibre loss induced by dominant-negative mDia1

RhoA is known to recruit the downstream targets ROK and mDia to promote the formation of stress fibres; however, overexpression of dominant-negative mDia1 (mDia-DN) results in a dramatic loss
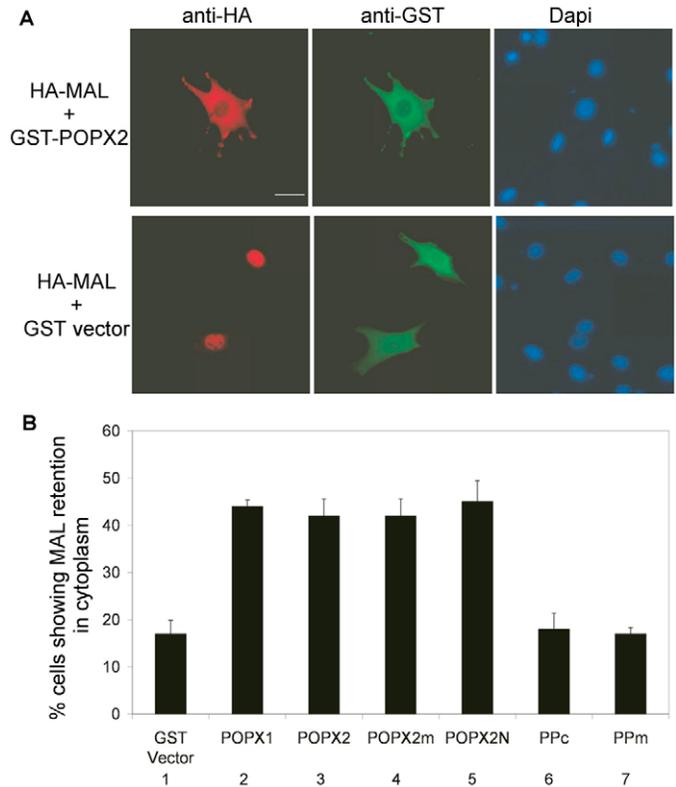

Fig. 4. POPX2 blocks the nuclear translocation of MAL. (A) HA-tagged MAL was co-transfected with GST-POPX2 or GST control vector into NIH3T3 cells. The cells were fixed and immunostained with antibodies to the tags to detect the location of MAL after growing in media containing 0.5% serum. The top panels show that HA-MAL was localized in the cytoplasm in the presence of GST-POPX2. The bottom panels show the nuclear location of HA-MAL in the absence of POPX2. DAPI stained the nuclei. (B) Various GST-tagged *POPX* cDNA constructs were co-transfected with HA-MAL and immunostained as in A. The transfected cells were scored for MAL nuclear localization. Lane 1: HA-MAL + GST control vector; 2, HA-MAL + GST-POPX1; 3, HA-MAL + GST-POPX2; 4, HA-MAL + GST-POPX2m; 5, HA-MAL + GST-POPX2N; 6, HA-MAL + GST-PPc; 7, HA-MAL + GST-PPm. Each transfection was repeated three times and approximately 100 transfected cells were scored per transfection. Scale bar: 25 μm.

of stress fibres (Nakano et al., 1999). Interestingly, we found that POPX2 expression can suppress this inhibitory effect of mDia-DN with many cells still displaying substantial amounts of stress fibres (Fig. 5A). Whereas approximately 50% of cells expressing mDia-DN showed an absence of actin stress fibres (Fig. 5B, lane 2), co-expression of POPX2 reduced this proportion to 17% (Fig. 5B, lane 5). POPX2 expression alone exerted a positive effect on stress-fibre formation in that only approximately 1% of the POPX2-expressing cells contained no detectable stress fibres compared with 25% in the GFP-expressing control cells (Fig. 5B, lanes 1 and 3). The phosphatase-deficient mutant POPX2m was not as potent a suppressor of mDia-DN activity, with ~35% of cells lacking stress fibres (Fig. 5B, lane 6). The introduction of the kinase inhibitory domain (KID) of PAK also suppressed mDia-DN (Fig. 5B, lane 7), suggesting that mDia inhibition and PAK kinase activity cooperate to block stress-fibre formation. The binding of POPX2 to mDia1 is indeed involved in stress-fibre regulation because the phosphatase domain alone (PPc) or the phosphate-dead version (PPm) could not block the effect of mDia-DN (supplementary
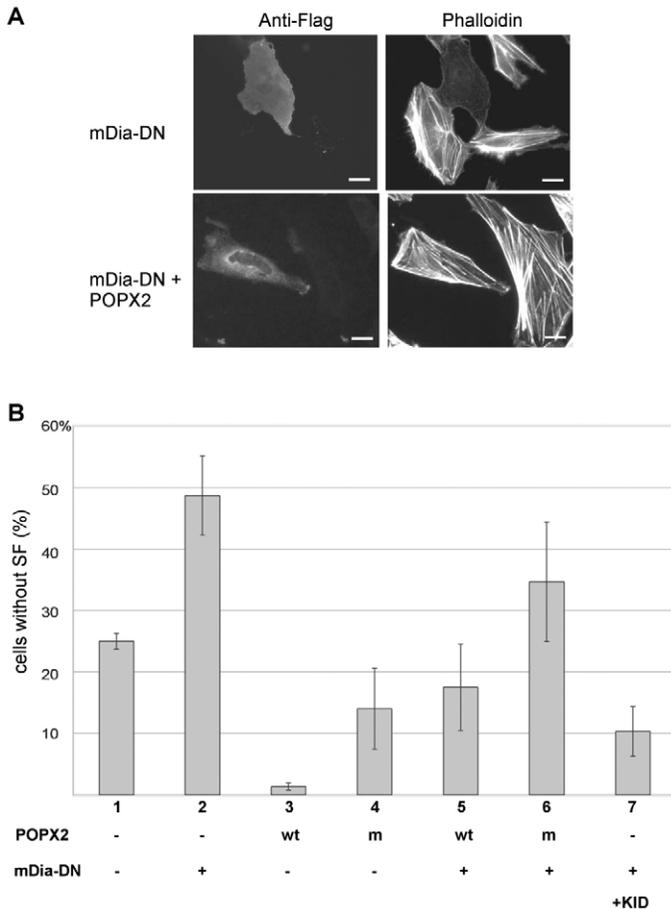
A

B

	1	2	3	4	5	6	7
POPX2	-	-	wt	m	wt	m	-
mDia-DN	-	+	-	-	+	+	+
							+KID

Fig. 5. POPX2 blocks the effect of dominant-negative mDia. (A) Flag-mDia-DN was transfected either with or without *POPX2* into HeLa cells. The cells were fixed and immunostained with anti-Flag antibodies and rhodamine-coupled phalloidin. Cells transfected with just mDia-DN alone showed loss of stress fibres. Cells co-transfected with mDia-DN and POPX2 still contained stress fibres. (B) Transfection with different cDNAs and detection of stress fibres were performed as in A. The phenotype was scored by analyzing at least 100 cells per transfection. Each transfection was repeated three times. Lane 1, GFP; 2, mDia-DN; 3, POPX2; 4, POPX2m; 5, mDia-DN + POPX2; 6, mDia-DN + POPX2m; 7, mDia-DN + KID. Scale bars: 10 μm.

A

B

C

Fig. 6. POPX2 knock-down causes rearrangement of the actin cytoskeleton and loss of stress fibres. (A) HeLa cells were microinjected with *POPX2* siRNA and immunostained with rhodamine-coupled phalloidin. (B) HeLa cells were transfected with combinations of pXJ constructs of GFP or KID and *POPX2* siRNAs. The cells were immunostained as in A. Three transfections were performed per construct. More than 100 cells were scored for each transfection. Lane 1, GFP; 2, GFP + control siRNA; 3, GFP + *POPX2*; 4, GFP + *POPX2*-siRNA1; 5, GFP + *POPX2*-siRNA2; 6, KID + *POPX2*-siRNA1. Scale bars: 10 μm.

material Fig. S4). It has been reported previously that active PAK promotes stress-fibre loss (Manser et al., 1997) and that POPX2 helps to stabilize stress-fibres through downregulation of PAK (Koh et al., 2002). Thus, our observations suggest that, in addition to its effect on PAK, POPX2 phosphatase is directly involved in the RhoA/mDia/stress-fibre pathway.

POPX2 is involved in the maintenance of actin stress fibres
To confirm that endogenous POPX2 is involved in stabilizing actin stress fibres, two small interfering RNAs (*POPX2* siRNA1 and 2) targeted to human *POPX2* were introduced into HeLa cells. Both siRNAs were effective in knocking down the protein levels of POPX2 in the cells as indicated by western blotting (Fig. 6B, lanes 3 and 4). Cells containing the *POPX2* siRNA showed a considerable loss of stress fibres with concomitant relocation of actin filaments to the cell periphery (Fig. 6A). Approximately 60-70% of cells transfected with *POPX2* siRNA contained no stress fibres, as opposed to 25% in control cells (Fig. 6C, lanes 1, 4 and 5). Because POPX2 acts as a negative regulator of PAK, one
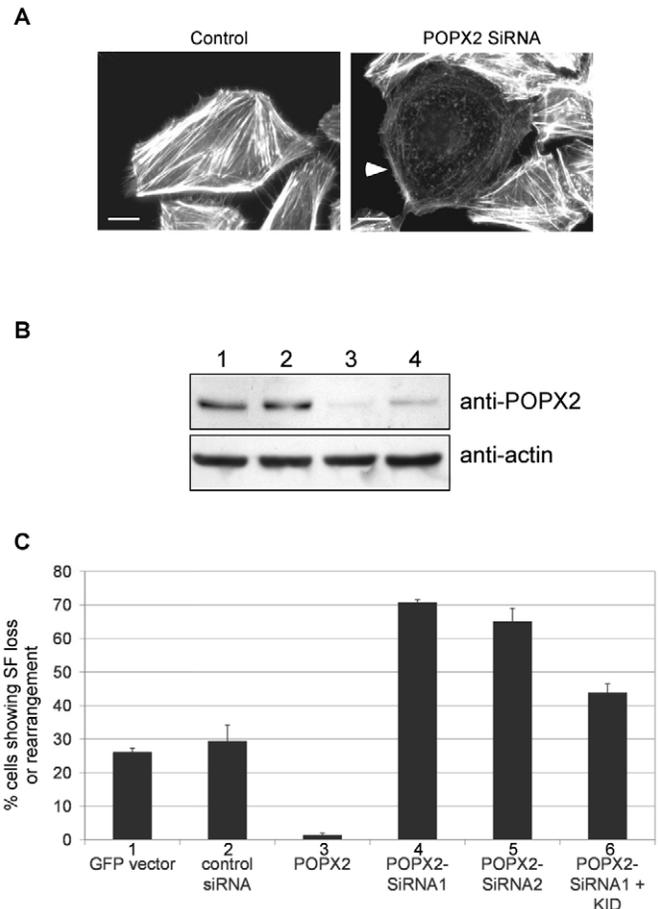
possible explanation is that PAK activity is elevated due to decreased levels of POPX2 protein. When *POPX2* siRNA was transfected with the *PAK*-KID cDNA, a significant proportion of cells (46%) still showed loss of stress fibres (Fig. 6C, lane 6). Although the proportion was less compared with cells transfected with *POPX2* siRNA alone (70%), it was still higher than in GFP controls (25%). Thus, effects on PAK alone cannot fully account for the stress-fibre loss caused by *POPX2* siRNA, suggesting that POPX2 might regulate other kinases or proteins involved in the control of the actin cytoskeleton. These observations clearly implicate POPX2 in the maintenance of actin stress fibres in both PAK-dependent and PAK-independent pathways.

Discussion
RhoA and CDC42/RAC elicit vastly different effects on the actin cytoskeleton (Etienne-Manneville and Hall, 2002). Many reports imply that their activities are antagonistic. Previous studies have also suggested some degree of cross-talk between these two major pathways. Here, we provide evidence for functional links between

the members of the RhoA and CDC42/RAC pathways in the context of stress-fibre maintenance and SRF-mediated transcription. We report that – besides RhoA, profilin and SRC (Tominaga et al., 2000; Watanabe et al., 1997) – POPX2 is a binding partner for mDia. Co-immunoprecipitation of mDia1 with POPX2 using anti-POPX2 antibodies (Fig. 2A) suggests an endogenous interaction between POPX2 and mDia1. The GST pull-down assays demonstrate similar affinities between mDia1 and POPX2, and between mDia1 and its physiological binding partner RhoA (Fig. 2C). There is also evidence of direct binding between the two proteins, as seen in the dot-blot data (Fig. 2B). mDia1 and POPX2 interaction is also enhanced in the presence of active RhoA (Fig. 1A). One possible explanation for this enhancement is that the binding of active RhoA causes conformational changes in mDia1 resulting in the dissociation of the C-terminal auto-inhibitory domain (Alberts, 2001; Otomo et al., 2005). The 'activated' mDia1 could then bind to its other partners, such as POPX2. A similar observation has been reported for mDia2 and DIP, whereby the binding of CDC42 to mDia2 relieves the auto-inhibition of mDia2 and allows its interaction with DIP (Eisenmann et al., 2007). Thus, the interaction of mDia1 with POPX2 might provide a mechanism for fine modulation of mDia1 activity. This interaction is likely to be mediated via the FH3 domain of mDia1 (Fig. 1C). Although the FH1 and FH2 domains of mDia1 are responsible for profilin binding and actin nucleation/polymerization, respectively, hitherto, no function has been attributed to the FH3 domain. Our observation suggests that the FH3 domain acts as a docking site for POPX2, which in turn influences the activity of mDia1.

Based on our observations, we propose that POPX2 might be involved in the negative regulation of RhoA and mDia1 signalling in SRF-mediated transcription. Knocking down of endogenous POPX2 using siRNA appears to enhance SRF-mediated transcription (supplementary material Fig. S3). POPX2 inhibits RhoA or activates mDia-induced SRF transcription to an extent similar to that of mDia-DN. The fact that inhibition occurred by expressing either mDia-DN or POPX2 (which binds to active mDia1) suggests that it occurs via interference with the interaction of mDia1 with its downstream effector (Fig. 3). Thus, the ability of POPX2 to associate with RhoA-activated mDia provides an additional regulatory mechanism in mDia-SRF transcriptional signalling.

Our data also suggest that POPX2 can prevent the nuclear translocation of MAL. The exact mechanism of MAL translocation is not known. It has been reported recently that the activities of MAL can be enhanced by the activation of mDia1/mDia2 signalling (Staus et al., 2007). MAL is highly phosphorylated upon serum stimulation (Miralles et al., 2003). However, the link between the phosphorylation status of MAL and its nuclear localization has not yet been established. Our results indicate that the phosphatase activity of POPX is not required to prevent MAL translocation because both POPX2 and the phosphatase-dead mutant of POPX2 inhibited MAL nuclear translocation (Fig. 4B). The N-terminus of POPX2, which contains the mDia-interacting domain, blocked nuclear translocation of MAL to the same extent as full-length POPX2. Moreover, the phosphatase domain alone had no detectable effect. We believe that it is the interaction between POPX2 and mDia1, but not its phosphorylation status, that influences MAL localization. A recent report has also proposed that the nuclear export of MAL could play an important role in controlling the amount of MAL in the nucleus (Vartiainen et al.,

2007). It has been suggested that MAL shuttles continuously between the cytoplasm and the nucleus, and that serum stimulation blocks the nuclear export of MAL.

Although the phosphatase activity of POPX2 is not required for its inhibition of SRF transcription and MAL nuclear translocation, it does exert a positive effect on actin stress-fibre maintenance. The dominant-negative mutant of mDia1 induces a loss of stress fibres [(Nakano et al., 1999) and this report]. Overexpression of wild-type POPX2 reverses the phenotype elicited by dominant-negative mDia1 more effectively than the phosphatase-dead mutant (Fig. 5B, lanes 5 and 6), suggesting that phospho-regulation does contribute to this phenotype. Cells overexpressing POPX2 exhibited robust stress fibres, such that only about 1% of cells were devoid of stress fibres (Fig. 5B, lane 3). By contrast, 14% and 25% of cells failed to form stress fibres when the phosphatase-dead mutant or GFP vector was overexpressed, respectively (Fig. 5B, lanes 4 and 1). The finding that siRNA-mediated knockdown of endogenous POPX2 resulted in 70% of cells losing stress fibres (Fig. 6C, lane 4) links POPX2 to stress-fibre maintenance. Because POPX2 can act as a negative regulator of PAK (Koh et al., 2002), knocking down endogenous levels of POPX2 might contribute to elevated activity of PAK, which in turn might cause stress-fibre dissolution (Manser et al., 1997). However, because the PAK inhibitory domain (KID) did not fully counteract the effect of *POPX2* siRNA (Fig. 6B, lane 6), this suggests that POPX2 might also act via other effectors, such as the non-conventional PAK4, in maintaining stress fibres.

It has been reported previously that stress fibres form from an existing actin (F-actin) pool after RhoA activation (Machesky and Hall, 1997). The stress fibres induced by activation of ROK appear as thick fibres (SF_R) because of the bundling of actin and myosin, whereas those induced by active mDia appear long and thin (SF_M) due to direct actin nucleation and assembly (Kimura et al., 1996; Leung et al., 1996; Li and Higgs, 2003). The fact that the balance of expression of ROK and mDia is important in the makeup of the stress fibres suggests that ROK and mDia cooperate to form both types of stress fibres observed when RhoA is activated (Watanabe et al., 1999). Indeed, our findings (supplementary material Fig. S5)
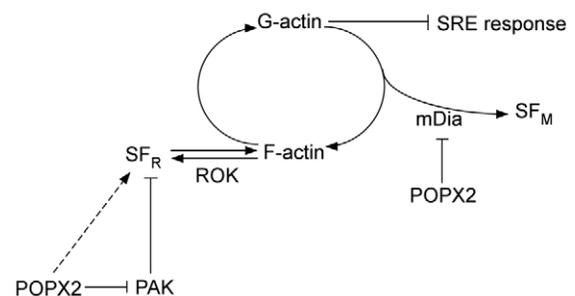

Fig. 7. A scheme representing a possible mechanism by which POPX2 negatively regulates transcription from the SRE. An increase in G-actin level results in inhibition of transcription from the SRE. Two different types of stress fibres are induced; one by ROK (SF_R) and the other by mDia (SF_M). SF_R is formed from the bundling of existing actin filaments (F-actin) with myosin. SF_M formation is catalyzed by the actin-nucleation and -polymerization activities of mDia. Downregulation of PAK activity by POPX2 results in stabilization and accumulation of SF_R, which leads to less free F-actin in the cell. The increased G-actin:F-actin ratio will then inhibit SRE transcription. mDia has a positive effect on actin polymerization and enhances SRE transcription. Our current study shows that POPX2 blocks mDia enhancement of SRE response by its interaction with the mDia protein.

and those reported by others (Anderson et al., 2004; Takaishi et al., 2000) are consistent with the idea that there are at least two different types of actin filaments, which are regulated by ROK and mDia, respectively. The effectors of RhoA – ROK and mDia – will increase SF_R and SF_M formation, respectively. As G-actin is recruited for the polymerization of these actin filaments, the resultant decrease in G-actin will lead to an increase of SRE-mediated transcription (Posern et al., 2002; Sotiropoulos et al., 1999). We propose that the interaction between POPX2 and mDia1 provides a mechanism to fine-tune the stress-fibre content in the cell (Fig. 7). It is possible that binding to POPX2 prevents mDia1 from catalyzing actin assembly. This would in turn lead to an increase in the intracellular concentration of G-actin, resulting in the dampening of SRE-mediated transcription.

It might appear paradoxical that POPX2, although exerting a positive effect on stress-fibre maintenance (Fig. 6), inhibits RhoA- and mDia-induced SRF activities (Fig. 3). As shown in Fig. 7, POPX2 downregulates PAK activity and prevents it from causing the dissolution of SF_R (Koh et al., 2002; Manser et al., 1997). This will enhance the appearance of SF_R without any new actin polymerization. With the G-actin:F-actin ratio remaining high, the SRE response would not be stimulated.

The downregulation of PAK activity by POPX2 could also have an impact on LIM kinase. It has been shown that LIM kinase and mDia cooperate to regulate SRF activities (Geneste et al., 2002). Phosphorylation by PAK activates LIM kinase, which then leads to the phosphorylation and inactivation of cofilin, inhibiting its severing and F-actin-depolymerization activity. Thus, the negative effect of POPX2 on transcription from the SRE can be achieved by attenuation of both PAK and mDia1 activities.

In conclusion, POPX2 can be considered not only as a phosphatase in the context of targeting phospho-proteins, but also as a scaffolding protein via its N-terminal non-catalytic domain. Because of its link to both CDC42/RAC1 pathways [via PIX-PAK (Koh et al., 2002) and RhoA pathways (via mDia1, this report)], POPX2 represents an important player in cross-talk between the various Rho GTPase signalling pathways that underlie diverse activities; including morphological changes, transcription and the cell cycle.

Materials and Methods
Plasmid constructs
All the cDNA constructs were made in the pXJ40 vector with their respective Flag or GST tags. All mDia1 mutants were constructed by cloning the PCR fragment generated with primers targeting different regions. mDia-DN was made as described by Nakano et al. (Nakano et al., 1999), which corresponded to aa 261-571 of mDia1. The active mDia1 mutant used, mDia-ΔGBD, corresponded to aa 261-1256; it contained the FH3, FH1 and FH2 domains and the DAD. The mDia-ΔN1 (FH1→C) construct corresponded to aa 524-1256. The mDia-ΔC mutant corresponded to aa 1-571. The ΔDAD and GBD constructs corresponded to aa 1-1090 and aa 1-260, respectively. The POPX2N and PPc constructs corresponded to aa 1-269 and 269-454 of POPX2, respectively. The phosphatase-dead mutant of POPX2, POPX2m, was described in Koh et al. (Koh et al., 2002).

Immunoprecipitation
Affinity-purified antibodies against POPX2 and random IgG were covalently coupled to Protein G Sepharose beads. The beads were then incubated with total cell lysate harvested from COS7 cells for 2 hours at 4°C. The beads were washed thrice with $1\times$PBS to remove unbound proteins. The protein complex binding to Protein G beads was eluted and analyzed by SDS-PAGE and western blotting using mDia1 antibodies (BD Transduction Laboratory).

Luciferase assays
The SRE reporter plasmid contained three copies of SRE elements, as well as the thymidine kinase promoter upstream of the firefly luciferase gene. The pGLTK control containing the thymidine kinase promoter and Renilla luciferase gene was from Promega. The assays were performed using the Promega Dual Luciferase Reporter Assay System. The different constructs together with the SRE reporter and pGLTK control plasmid were transfected into COS7 cells using Lipofectamine 2000 (Invitrogen Life Technologies). For the SRE control, pXJ40 vector, the SRE reporter and pGLTK control plasmids were transfected into COS7 cells. Every transfection was done in triplicate. The cells were serum-starved in media containing 0.5% serum the day after transfection. After 24 hours of serum starvation, the cells were induced with 15% serum in media for 7 hours. The cells were then washed with PBS, lysed and assayed as described in the manufacturer's protocol.

siRNA, transfection and microinjection
The *POPX2* siRNA sequences used were 5′-CCUACUUUGCUGUGUUUGA-3′, which corresponded to nucleotide sequence from 572 to 590, and 5′-GGGAUGUCUUCCAGAAGCC-3′, which corresponded to nucleotide sequence from 983 to 1001 of *POPX2*. 4 nM of siRNA was co-injected with pXJ-GFP plasmid (50 µg/ml) into HeLa cells using Eppendorf InjectMan microinjection apparatus. In some experiments, *POPX2* siRNA was co-transfected with other plasmid constructs using Fugene 6 (Roche) or Lipofectamine 2000 according to the manufacturer's protocols. The cells were incubated for 24 hours after injection before fixing with 3% paraformaldehyde, which was then followed by immunostaining. For microscopy images, Carl Zeiss axiovert microscope and Zeiss objective plan neo-fluar 40×/1.3 oil were used. The MetaMorph software programme was used to capture images using a Roper Scientific CoolSNAP CCD camera.

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