An association between NUAK2 and MRIP reveals a novel mechanism for regulation of actin stress fibers

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Summary

Actin stress fiber assembly and contractility in nonmuscle motile cells requires phosphorylation of myosin regulatory light chain (MLC). Dephosphorylation and disassembly are mediated by MLC phosphatase, which is targeted to actin fibers by the association of its regulatory subunit MYPT1 with myosin phosphatase Rho-interacting protein (MRIP). In the present study, we identify the kinase NUAK2 as a second protein targeted by MRIP to actin fibers. Association of NUAK2 with MRIP increases MLC phosphorylation and promotes formation of stress fibers. This activity does not require the kinase activity of NUAK2 but is dependent on both MRIP and MYPT1, indicating that the NUAK2–MRIP association inhibits fiber disassembly and MYPT1-mediated MLC dephosphorylation. NUAK2 levels are strongly induced by stimuli increasing actomyosin fiber formation, and NUAK2 is required for fiber maintenance in exponentially growing cells, implicating NUAK2 in a positive-feedback loop regulating actin stress fibers independently of the MLC kinase Rho-associated protein kinase (ROCK). The identified MRIP–NUAK2 association reveals a novel mechanism for the maintenance of actin stress fibers through counteracting MYPT1 and, together with recent results, implicates the NUAK proteins as important regulators of the MLC phosphatase acting in both a kinase-dependent and kinase-independent manner.

Key words: Actin stress fibers, Kinase-independent, MRIP, MYPT, NUAK2

Introduction

The actin cytoskeleton in nonmuscle cells is a continuously remodeling organelle consisting of a wide array of actin filament assemblies. Contractile actin stress fibers represent one of these and are involved in cell adhesion, cell shape, cytokinesis and motility (Pellegrin and Mellor, 2007). In contrast with well-characterized lamellipodial or filopodial actin networks forming sheet- or finger-like protrusions at the plasma membrane (Chhabra and Higgs, 2007), actin stress fibers in nonmuscle cells are less extensively characterized in terms of the molecules required for assembly and disassembly (Hotulainen and Lappalainen, 2006; Small et al., 1998), as well as in their regulatory pathways, which typically involve kinases and phosphatases that enable rapid reorganization of actin stress fibers (Ito et al., 2004; Katoh et al., 2001a; Murata-Hori et al., 2001; Wilkinson et al., 2005; Yamashiro et al., 2003).

Myosin regulatory light chain (MLC or MRLC) of myosin II is a key molecule that monitors the assembly–disassembly balance and contractility of actin stress fibers (Bresnick, 1999). Phosphorylation of MLC on Thr18 and/or Ser19 is Rho-associated protein kinase (ROCK) (Riento and Ridley, 2003). The attenuation of ROCK using the specific inhibitor Y27632 leads to disassembly of actin stress fibers in exponentially growing cells, implicating NUAK2 in a positive-feedback loop regulating actin stress fibers independently of the MLC kinase ROCK. The identified MRIP–NUAK2 association reveals a novel mechanism for the maintenance of actin stress fibers through counteracting MYPT1 and, together with recent results, implicates the NUAK proteins as important regulators of the MLC phosphatase acting in both a kinase-dependent and kinase-independent manner.

Efficiently in various cell types, as well as a reduction of cell motility and invasiveness in in vitro and in vivo tumor models (Genda et al., 1999; Itoh et al., 1999; Somlyo et al., 2000; Takamura et al., 2001).

Dephosphorylation of MLC Thr18 and/or Ser19 is mediated by the myosin light chain phosphatase (MLCP), consisting of the 37 kDa protein phosphatase 1 (PP1) catalytic subunit, a 130 kDa myosin-targeting subunit (MYPT or MBS) and a small 20 kDa subunit (Alessi et al., 1992; Shimizu et al., 1994; Shirazi et al., 1994). MLCP is targeted to myosin by direct associations between both the catalytic subunit and MYPT with myosin (Hartshorne et al., 1998; Koga and Ikebe, 2008; Sellers and Pato, 1984; Tan et al., 2001). This interaction can be inhibited through phosphorylation of MYPT on Thr853 by ROCK (Muranyi et al., 2005; Velasco et al., 2002), whereas phosphorylation by ROCK or some other MLC kinases on Thr694 leads to decreased catalytic activity of the phosphatase (Feng et al., 1999; Muranyi et al., 2005; Wilkinson et al., 2005). In addition, MYPT can be dissociated from myosin through association with 14-3-3 proteins following phosphorylation of Ser472 by ROCK during re-stimulation of HeLa cells following serum withdrawal (Koga and Ikebe, 2008). Recently, the kinase NUAK1 was also reported to phosphorylate MYPT Ser472 during cell detachment (Zagorska et al., 2010), and this also required the activity of the NUAK1-activating kinase LKB1 (STK11) (Lizcano et al., 2004).

On actin stress fibers, MYPT1 (PPP1R12A) and its close family member MBS85 (PPP1R12C) also interact with the actin-filament-binding protein MRIP (also known as MPRIP and p116Rip)
(Mulder et al., 2004; Surks et al., 2003). Several observations suggest that this interaction participates in the recruitment of MLCP to dephosphorylate MLC on actomyosin fibers: (1) MRIP directly associates with actin filaments through a pleckstrin homology (PH) domain and an acidic stretch (Mulder et al., 2003) and with MYPT through its C-terminal coil-coiled regions (Mulder et al., 2004; Surks et al., 2003) (shown schematically in Fig. 1A), (2) enhanced MRIP expression in cells leads to disruption of actin stress fibers (Mulder et al., 2003), (3) MRIP downregulation increases actin stress bundles and MLC phosphorylation, and shifts MYPT1 from a particulate to a soluble fraction (Koga and Ikebe, 2005; Surks et al., 2005), and (4) actin stress fibers in cells where MRIP (Surks et al., 2005) or MYPT (Xia et al., 2005) is downregulated are not disassembled following inhibition of ROCK by Y27632, implying that MLCP requires the association of MYPT and MRIP for efficient dephosphorylation of MLC. In the present study, we find that MLCP activation, which is crucial for actin stress fiber dynamics, is counteracted by a previously unrecognized association between MRIP and the inducible kinase NUAK2 (also known as SNARK).

**Results**

**Disassembly of actin stress fibers by MRIP is inhibited by NUAK2**

We aimed to identify proteins interacting with the kinase NUAK2 (Fig. 1A) (Lefebvre et al., 2001) in nonmuscle cells. To this end, NUAK2 was used to screen cDNAs from fetal brain and liver libraries using yeast two-hybrid protein interaction screening. All of the 21 identified cDNA clones from a human fetal brain library and five out of seven clones from a human liver library (Fig. 1A) (Lefebvre et al., 2001) in nonmuscle cells. To this end, NUAK2 was used to screen cDNAs from fetal brain and liver libraries using yeast two-hybrid protein interaction screening. All of the 21 identified cDNA clones from a human fetal brain library and five out of seven clones from a human liver library encoded C-terminal fragments of MRIP (encoded by MRIP) varying from amino acids 684–1024 to 782–1024 (Fig. 1A). The results suggest that the association of NUAK2 and MRIP is through the MRIP coiled-coiled region, including the part crucial for MYPT1 interaction (Fig. 1A) (Mulder et al., 2004; Surks et al., 2003). To validate the NUAK2–MRIP association in mammalian cells, human osteosarcoma (U2OS) cell extracts expressing glutathione S-transferase (GST)-tagged NUAK2 together with green fluorescent protein (GFP)-tagged MRIP clones were incubated with glutathione–Sepharose beads. Subsequent purification of the GST-tagged proteins and western blotting of associated molecules indicated that NUAK2 specifically associated with the tested MRIP C-terminal clones (Fig. 1B). The association was also observed following immunoprecipitation of the full-length GFP-tagged MRIP from cells coexpressing MRIP and NUAK2 (Fig. 1C). The decrease of full-length MRIP and NUAK2 occurs preferentially on actin fibers. Alternatively, the affinity of the full-length proteins might be lower than with the truncated proteins.

Subsequent localization analyses of NUAK2 were performed in HeLa cells, which have been previously used to study the role of MRIP in actin stress fibers (Koga and Ikebe, 2005; Xia et al., 2005). Cells expressing GST–NUAK2 showed dotted cytoplasmic and nuclear staining, with partial colocalization with actin fibers demonstrated by phalloidin staining (Fig. 1D, insets in the right-hand panels). NUAK2 localization to actin fibers was more pronounced following a mild detergent (Triton X-100) treatment before fixation (supplementary material Fig. S1). Interestingly, the NUAK2-expressing cells also exhibited increased phalloidin staining, frequently located in central actin fibers (Fig. 1D and E).

**Fig. 1. Association of NUAK2 with MRIP and the subsequent increase of actin stress fibers in NUAK2-expressing cells does not require the kinase activity of NUAK2.** (A) Schematic representations of NUAK2, with the kinase domain and the activating T-loop phosphorylation site (T208) indicated, and the 1024-amino-acid MRIP, with the PH and coiled-coiled (C) domains, as well as minimal regions for actin binding (F-actin, amino acids 1–212) (Mulder et al., 2003) and MYPT binding (amino acids 823–878) (Surks et al., 2003), indicated. Five representative MRIP clones (cl.) identified as interacting with NUAK2 in a yeast two-hybrid screen are shown schematically. (B) Western blotting analysis using anti-GFP (WB: α-GFP) or anti-GST (WB: α-GST) antibodies following GST pulldown of 200 μg of U2OS cell extracts coexpressing GST–NUAK2 or GST together with GFP–MRIP (cl. 684 or cl. 782). Input panels indicate 5% of the cell extracts used for pulldowns. (C) Western blotting analysis using anti-HA (WB: α-HA) and anti-GFP (WB: α-GFP) antibodies of anti-GFP-immunopurified U2OS cell extracts coexpressing full-length GFP–MRIP or GFP together with HA–NUAK2 or HA–QIK as a negative control. The asterisk marks an unspecific band. In B and C all images are from the same blot and exposure, with white lines indicating removal of intervening lanes. (D) Confocal microscope images following immunofluorescence analysis performed with HeLa cells expressing GST (control) or GST–NUAK2 using a rabbit polyclonal anti-GST (α-GST) antibody and Alexa-Fluor-488–phalloidin as indicated. The insets in the right-hand panels represent higher magnifications of merged images for the areas boxed in the phalloidin images to highlight NUAK2 localization along actin fibers. Asterisks mark untransfected or control cells with fewer actin fibers. Scale bar: 10 μm. (E) Box-plot analysis of integrated phalloidin intensity per cell area in HeLa cells expressing control, wild-type (wt), kinase-deficient (T208A) or constitutively active NUAK2 (T208E), as indicated. A total of 50 cells were analyzed from three independent experiments for each analysis. ***P<0.001 between the bracketed samples as analyzed using a Student’s t-test.
supplementary material Fig. S1; compare fibers in control cells outlined in yellow and in NUAK2-expressing cells outlined in red). Both localization to actin fibers and the increase in phalloidin staining were also clearly observed in cells expressing a kinase-deficient (GST–NUAK2-T208A) and a constitutively active NUAK2 (GST–NUAK2-T208E; supplementary material Fig. S1; Fig. 1E; ***P<0.001) indicating that kinase activity is not required for the NUAK2-mediated increase in stress fibers. Quantification of phalloidin intensities also demonstrated that there was a small but significant increase upon expression of the constitutively active mutant compared with that arising upon expression of wild-type or kinase-deficient NUAK2 (Fig. 1E), suggesting the existence of a minor kinase-dependent mechanism for increasing actin stress fibers by NUAK2.

Expression of GFP-tagged MRIP resulted in a decrease in total fibers, as expected (Mulder et al., 2003), but weak staining along actin fibers could still be detected following Triton X-100 pretreatment (Fig. 2A, GFP–MRIP, blue outline) as well as before Triton X-100 extraction (supplementary material Fig. S2). Interestingly, coexpression of NUAK2 together with MRIP rescued the decrease in phalloidin staining noted with MRIP alone (Fig. 2A, cells with red outlines) and demonstrated colocalization of NUAK2 and MRIP along actin stress fibers (Fig. 2A, ‘GST/GFP’ and ‘+phalloidin’). The ability of NUAK2 to rescue the MRIP-mediated decrease in fibers did not require the kinase activity of NUAK2 given the comparable rescue seen with wild-type and kinase-deficient NUAK2 (Fig. 2A,B, NUAK2-wt and NUAK2-T208A). As noted above, the constitutively active NUAK2 gave a further increase in actin fibers.

NUAK2 targeted to actin stress fibers by MRIP
MRIP binds to actin filaments through its N-terminal part (Mulder et al., 2003) and to NUAK2 through its C-terminal part (Fig. 1A), suggesting a direct mechanism for targeting of NUAK2 to actin stress fibers. An alternative indirect model is suggested by interactions between, on the one hand MRIP and MYPT1 (Mulder et al., 2004; Surks et al., 2003) and, on the other, between MYPT1 and NUAK2 (Yamamoto et al., 2008). To investigate this, the distribution of GST–NUAK2 was examined following small interfering RNA (siRNA)-mediated downregulation of either MRIP or MYPT1, where expression levels of ectopically expressed and downregulated proteins were analyzed by western blotting (Fig. 3B) or by mRNA analysis (MRIP; 85–95% decrease of mRNA levels in three separate experiments). As expected from previous studies (Koga and Ikebe, 2005; Surks et al., 2003; Xia et al., 2005), siMRIP or siMYPT1 transfections alone increased the number of actin fibers (data not shown; see also Fig. 6A–D). Subsequent comparison of actin-stress-fiber-associated NUAK2 showed that there was a striking loss of NUAK2 in cells transfected with siMRIP (Fig. 3A, arrowhead), but not with siMYPT1 (arrow) or control (siNT). This was not due to altered NUAK2 expression, as demonstrated by the comparable signals in total NUAK2 detected before Triton X-100 extraction (supplementary material Fig. S3A, arrowheads), indicating a requirement for MRIP but not MYPT1 for the association of NUAK2 with actin fibers. Coexpression of MRIP and NUAK2 in siMYPT1-treated cells enabled the association of NUAK2 with actin fibers, demonstrating that the NUAK2–MRIP association on actin fibers is independent of MYPT1 (Fig. 3A, double arrows). Similar results were obtained using two independent MRIP or MYPT1 siRNA oligonucleotides (supplementary material Fig. S3B).

NUAK2 expression renders central actin stress fibers resistant to ROCK inhibition
Although treatment with the ROCK inhibitor Y27632 leads to rapid disassembly of actin stress fibers in normal cells, in MRIP-downregulated cells Y27632-mediated fiber loss is significantly impaired (Surks et al., 2005), which was suggested to reflect the inability of MYPT1 and MLCP to target to fibers. To investigate disassembly of actin stress fibers upon ROCK inhibition in NUAK2-expressing cells they were treated with 10 μM Y27632 for 30 minutes and analyzed for actin fibers. In control cells, actin fiber disassembly was already noted at 15 minutes (data not shown) and fibers were undetectable at 30 minutes (Fig. 4A, control). NUAK2-expressing cells were partially resistant to Y27632 treatment, exhibiting long centrally localized actin fibers (Fig. 4A, NUAK2-wt). The ability of NUAK2 to maintain fibers in the absence of ROCK activity does not require the kinase activity of NUAK2, as determined by the comparable activity of the wild-type and kinase-deficient NUAK2 in this assay (Fig. 4A,B), with both displaying significant increases in central actin stress fibers (Fig. 4B; P<0.001). NUAK2 expression by contrast did not affect loss of actin stress fibers following treatment with cytochalasin D, which blocks monomer addition at the barbed ends (data not shown). These results suggest that NUAK2 inhibits MRIP–MLCP-mediated actin stress fiber disassembly.

NUAK2 mRNA and protein levels are regulated by actin stress fibers
The increase in actin stress fibers upon ectopic expression of the wild-type or the kinase-deficient NUAK2 suggested an association between NUAK2 expression levels and actin stress fibers. To investigate whether NUAK2 levels correlate with actin fibers, NUAK2 mRNA levels in HeLa cells were assessed in circumstances where actin stress fibers are altered: serum withdrawal and re-stimulation, and on treatment with the ROCK inhibitor Y27632, the myosin II inhibitor blebbistatin or cytochalasin D (Fig. 5A). As expected, actin stress fibers and phosphorylated MLC (pMLC) decreased in serum-starved, Y27632- or blebbistatin-treated HeLa cells and increased in serum-stimulated cells [10% fetal calf serum (FCS)] compared with exponentially growing cells. Cytochalasin-D-treated cells displayed typical actin aggregates (Fig. 5A, cytochalasin D). The subsequent quantification of phalloidin and pMLC intensities confirmed the observed changes, which were statistically highly significant (P<0.001) except with cytochalasin D due to the bright actin aggregates (Fig. 5B,C). Interestingly, NUAK2 mRNA levels were downregulated during a 24-hour serum starvation to 16% of the level in exponentially growing cells (P=0.017), and thereafter strongly induced during 60-minute serum re-stimulation reaching 11-fold the level in comparison with 24-hour serum-starved cells (from 0.16 to 1.86, P=0.033) (Fig. 5D, compare ‘Starved’ and ‘10% FCS’). A 60 minute treatment with Y27632, blebbistatin or cytochalasin D in turn decreased NUAK2 mRNA levels to 32% (P=0.0098), 50% (P=0.066) and 48% (P=0.0040), respectively (Fig. 5D). NUAK2 protein levels were regulated similarly during serum starvation–re-stimulation (Fig. 5E). These results demonstrate that NUAK2 mRNA and protein are rapidly regulated in response to growth signals and directly by alterations in actin stress fibers, and identify exponentially growing HeLa cells as a suitable system to investigate the contribution of endogenous NUAK2 to the maintenance and regulation of actin stress fibers.
NUAK2 regulates MLC phosphorylation and actin stress fibers

To investigate the role of endogenous NUAK2 in actin stress fiber maintenance and MLC phosphorylation, as well as possible interactions with MRIP and MYPT1, the levels of MLC phosphorylated at Ser19 [pMLC (Ser19)] and phalloidin were analyzed from exponentially growing HeLa cells following siRNA-mediated downregulation of control MRIP, MYPT1 and NUAK2.

**Fig. 2. NUAK2–MRIP association prevents MRIP-mediated actin stress fiber disassembly in a kinase-independent manner.** (A) Confocal microscope images following immunofluorescence analysis of Triton X-100-pretreated HeLa cells expressing GFP–MRIP (blue outlines) or GFP–MRIP together with wild-type (wt) NUAK2 or kinase-deficient NUAK2 (T208E) mutants (red outlines), as indicated. Examples of control and untransfected cells are marked with yellow outlines. NUAK2 was detected using a rabbit polyclonal anti-GST antibody (αGST), and actin fibers were detected using Alexa-Fluor-647–phalloidin. GST or GFP expression alone was not detected owing to their solubility upon Triton extraction. Scale bar: 10 μm. The smaller images on the right-hand side represent merged images of MRIP and NUAK2 fluorescence (GST/GFP), and an image also including actin fibers (+phalloidin) to display possible colocalization. (B) Box-plot analysis showing phalloidin intensity per cell area for the experiments shown in A. ***P<0.001; **P<0.002. A minimum of 50 cells were analyzed for each data set.
As expected from previous results (Koga and Ikebe, 2005; Surks et al., 2005; Xia et al., 2005), siMYPT1 and siMRIP transfections increased actin stress fibers and pMLC signals, the latter of which were particularly notable as dot-like patterns along long thick actin cables (Fig. 6A, arrows). Interestingly, in NUAK2-downregulated cells, both actin stress fibers and pMLC levels were significantly reduced (Fig. 6A, arrowheads). The specificity of the siRNA effects was demonstrated by a correlation between knockdown efficiency and biological effect (Fig. 6B–D). The observed pMLC changes were also detectable by western blotting (Fig. 6E). These results indicate that NUAK2 promotes MLC phosphorylation and thereby maintenance of actin stress fibers in exponentially growing HeLa cells.

NUAK2 requires MRIP and MYPT1 to regulate actin stress fibers

If NUAK2 was required for actin stress fiber maintenance, owing to its association with MRIP, it would be expected that downregulation of MRIP together with NUAK2 would overcome the effects of NUAK2. To investigate this, siRNAs targeting MRIP and NUAK2 were sequentially transfected into HeLa cells and analyzed 2 days after the second transfection. Knockdown efficiencies were 88–93% (supplementary material Fig. S4). Analysis of phalloidin-stained samples revealed that NUAK2-siRNA-transfected cells contained decreased levels of actin stress fibers (Fig. 7A, ‘siNT->siNUAK2’), whereas cells with both MRIP and NUAK2 downregulation did not display decreased levels of fibers (Fig. 7A–C, ‘siMRIP->siNUAK2’). Similarly, MYPT1 knockdown rescued actin stress fiber decrease following NUAK2 loss (Fig. 7A–C, ‘siMYPT1->siNUAK2’). Similar results were obtained using two independent siRNAs targeting MRIP or MYPT1 (supplementary material Fig. S5). The requirement for both MRIP and MYPT1 for NUAK2 regulation of actin fibers is interesting given that NUAK2 localization only requires MRIP.

Colocalization and inhibition of MYPT-mediated fiber disassembly by NUAK2

To further investigate the molecular mechanisms by which NUAK2 inhibits MLC dephosphorylation on actin stress fibers, MYPT localization was investigated in NUAK2- or MRIP-expressing cells. MYPT1 was not efficiently expressed in our hands possibly owing to toxicity, whereas the functionally closely related (Mulder et al., 2004; Zagorska et al., 2010) MBS85 cDNA (PPP1R12C) was readily detectable. GFP–MBS85 alone localized partially along actin fibers, the levels of which were significantly decreased (Fig. 7E,F; P<0.002 compared with control). NUAK2 expression by contrast increased actin fibers (Fig. 7E,F; P<0.001 compared with control). Interestingly, in cells expressing both MBS85 and NUAK2, staining patterns on actin fibers were very similar, demonstrating colocalization on actin stress fibers (Fig. 7E, merged images in the right-hand panels). NUAK2 coexpression in MBS85-expressing cells also led to stronger central actin fibers, and this did not require the kinase activity of NUAK2. These results with MBS85 provide indirect evidence that the mechanism by which NUAK2 inhibits MLC dephosphorylation, although clearly involving both MRIP and MYPT1, is not dependent upon dissociation of MYPT1.
Discussion

NUAK2–MRIP association inhibits MLCP

The present study uncovers a novel molecular mechanism that promotes actin stress fibers in nonmuscle cells through NUAK2-mediated inhibition of MLC dephosphorylation. On the basis of biochemical and genetic evidence, we have shown that this occurs through an association between NUAK2 and MRIP, and is only noted in the presence of the MYPT1 targeting subunit of MLCP. Together with previous observations indicating that MLCP targeting to actin myofilaments is mediated at least in part through an interaction between MRIP–MYPT1 (Mulder et al., 2004; Surks et al., 2005), which has also been reported to enhance phosphatase activity (Koga and Ikebe, 2005), the results suggest that the association of NUAK2 with MRIP on actin fibers inhibits dephosphorylation of MLC by MLCP. The present studies using the MBS85 MYPT family member also indirectly argue that inhibition by NUAK2 through MRIP binding is not likely to involve competitive binding with MYPT1, despite the overlapping binding domain on MRIP (Mulder et al., 2004; Surks et al., 2003).

A novel kinase-independent function for NUAK2

The ability of NUAK2 to inhibit MLCP and promote actin stress fibers through association with MRIP represents a kinase-independent function of NUAK2 on the basis of comparison of a wild-type, a kinase-deficient and a constitutively active NUAK2. This is supported by the observation that NUAK2 downregulation significantly decreases actin stress fibers and MLC phosphorylation in HeLa cells lacking the NUAK2-activating kinase LKB1 (Lizcano et al., 2004). It is interesting to note the similarity between the mechanisms by which NUAK2, in a kinase-independent spatial manner, controls actin fibers through MRIP association and the mechanism by which the Raf-1 kinase regulates actin fibers and

Fig. 4. NUAK2 expression renders central actin stress fibers resistant to inhibition of ROCK. (A) Immunofluorescence analysis using confocal microscopy of GST (control), wild-type (GST–NUAK2-wt) or a kinase-deficient NUAK2 (GST–NUAK2-T208A) expressing HeLa cells treated with 10 μM Y27632 inhibitor for 30 minutes before fixation and staining with a rabbit polyclonal anti-GST antibody (αGST), Alexa-Fluor-488–phalloidin and Hoechst 33342 (phalloidin/hoechst). Quantification of the central actin fibers was performed by identifying nuclei and quantifying phalloidin overlapping this region (grey outlines). Scale bar: 10 μm. (B) Box-plot analysis of phalloidin staining per nuclear area following Y27632 treatment in the indicated transfectants. ***P<0.001. A minimum of 36 transfected cells were analyzed for each sample.

Fig. 5. NUAK2 levels are regulated by actin fibers. (A) Alexa-Fluor-488–phalloidin and anti-phosphorylated MLC (Ser19) staining of exponentially growing (Exp. gr.) HeLa cells and HeLa cells following serum withdrawal (for 24 hours; Starved), after serum re-stimulation (for 60 minutes; 10% FCS) or after 60 minute treatments with Y27632, blebbistatin or cytochalasin D, as indicated. Scale bar: 20 μm. Box-plot analysis of (B) phalloidin or (C) pMLC intensities per cell area following the treatments indicated in A. (D) Relative NUAK2 mRNA levels from HeLa cells shown in A. Quantitative PCR values were first compared with GAPDH levels and then normalized to mRNAs isolated from exponentially growing cells. Error bars indicate the s.d. between duplicate samples. (E) Western blotting analysis of serum re-stimulated lysates at 0, 2 and 9 hour timepoints with the indicated antibodies, where the CDK7 blot demonstrates comparable protein loading. Owing to the relatively low signal-to-noise ratio of the anti-NUAK2 antibody, biological duplicates were used to demonstrate changes in NUAK2 levels. The white line indicates removal of irrelevant lanes from a single gel.
cell migration through ROCK association and localization, also in a kinase-independent fashion (Ehrenreiter et al., 2005).

Recently, the closely related kinase NUAK1 was shown to inhibit MLCP and to promote cell detachment through binding to the catalytic subunit PP1β and phosphorylating MYPT on a 14-3-3 binding site (Ser472 on MYPT1) (Zagorska et al., 2010), and NUAK2 was reported to phosphorylate MYPT1 (Yamamoto et al., 2008). Reciprocally, NUAK1 also efficiently associates with MRIP (our unpublished data), and in our assays a small but significant kinase-dependent component of NUAK2 was evident (Fig. 1E, Fig. 2B and Fig. 7F). Thus the NUAK family regulates MYPT1 and MLCP in both a kinase-dependent and kinase-independent manner.

Considering that phosphorylation of MYPT1 on Ser472 by NUAK1 leads to 14-3-3 binding (Zagorska et al., 2010), that phosphorylation of Ser472 was reported, in an independent study, to cause dissociation of MYPT1 from actin stress fibers (Koga and Ikebe, 2008) and also that complexes between NUAK, PP1β and MYPT have been noted in lysates (Zagorska et al., 2010), whereas NUAK–MRIP complexes are prominent on actin fibers, the kinase-dependent and kinase-independent functions of NUAK proteins could provide two integrated levels of regulation of MLCP activity. In this model, induction of NUAK would lead to its association with MRIP, resulting in a local inhibition of MLCP activity even in absence of the kinase activity of NUAK. Activation of the kinase activity of NUAK would in turn lead to MYPT1 phosphorylation, 14-3-3 binding and dissociation of the phosphatase from actin fibers. Clearly, alternative models such as that the two regulatory mechanisms are used in specific spatial or temporal settings of MLCP regulation (Matsumura and Hartshorne, 2008), such as in central compared with peripheral actomyosin fibers (Katoh et al., 2001b; Peterson et al., 2004; Tan et al., 2008), should also be considered.

The identification of kinase-dependent and kinase-independent roles for NUAK in regulating MYPT1 and MLCP phosphorylation indicates that it is important to consider their relative contribution in the variety of cellular (Kim et al., 2008; Legembre et al., 2004; Ng et al., 2007; Suzuki et al., 2003) and organismal (Hoppe et al., 2009; Tsuchihara et al., 2008) conditions where NUAK has been implicated. Of special interest is the requirement of NUAK for myosin filament organization and proper cytoskeletal reorganization during Caenorhabditis elegans growth (Matsumura and Hartshorne, 2008), such as in central compared with peripheral actomyosin fibers (Kato et al., 2001b; Peterson et al., 2004; Tan et al., 2008), should also be considered.

NUAK2 as part of a positive-feedback loop contributing to actin stress fiber maintenance

The existence of kinase-independent regulation of actin stress fibers by NUAK2 emphasizes the relevance of regulation of
NUAK2 levels. Results from the present and previous studies indicate NUAK2 mRNA and protein levels are highly inducible following stimuli such as serum stimulation (Fig. 5D,E) and application of tumor necrosis factor (TNF)-α (Legembre et al., 2004; Yamamoto et al., 2008). These inductions are very likely to be at least partly due to NUAK2 levels responding to the increased actin fiber levels occurring in both stimulations (Hanna et al., 2001; Ridley and Hall, 1992), as NUAK2 was highly responsive to relaxation and disassembly of actin fibers by Y27632 and blebbistatin as well as to cytochalasin D. On the basis of the detailed mechanisms of these drugs (Sotiropoulos et al., 1999; Vartiainen et al., 2007), NUAK2 is probably not regulated by the serum response factor (SRF)–megakaryocytic acute leukemia (MAL) complex (Miralles et al., 2003) but rather by another G-actin regulatable transcriptional activator and/or repressor such as YY1 (Favot et al., 2005). Importantly, the strong regulation of NUAK2 levels by actin fibers, together with the positive regulation of actin fiber formation by NUAK2, identifies a positive feedback loop for actin stress fiber maintenance, cooperating with e.g. RhoA–ROCK-mediated fiber formation.

Assessing NUAK2 levels in pathological conditions characterized by increased stiffness, reflecting MLC phosphorylation and actomyosin formation (Butcher et al., 2009; Clark et al., 2007), might provide further information on the relevance of NUAK2 in these processes. In this regard, it is interesting to note that a meta-analysis of NUAK2 mRNA expression analysis from 43 normal and 63 tumor tissues (www.genesapiens.org) (Kilpinen et al., 2008) indicates a significant increase in two ovarian cancer samples and a peritoneal adenocarcinoma sample that represent highly invasive cancers.

**Materials and Methods**

**Yeast two-hybrid screenings**

Yeast two-hybrid screenings were conducted at the Biomedicum yeast two-hybrid core facility using the Gateway ProQuest two-hybrid System (Invitrogen) essentially as described in the manufacturer’s protocol. To generate a bait plasmid, a full-length NUAK2 in pEBG2T vector (Lizcano et al., 2004) was used as a template for a PCR
To obtain NUAK2 cDNA containing attB1 and attB2 sites, enabling cloning into the Gateway cloning system (Invitrogen), forward primers used for the PCR were 5’-GGGGACCAAGATCTTACAAAAGACGACGTTCCACACATTGAGTTCTGGGTGAGCG-3’ and the reverse primer was 5’-GGGG- ACCAATCTTTGACAAAGACTGCTGAGGTGCTTGGAGCCACC-3’. The created NUAK2 entry clone was sequenced, transferred to the bait pDEST32 plasmid, fused with the GAL DNA-binding domain and transformed into the MAV203 yeast strain. A ProQuest pre-made human fetal brain cDNA library fused with the GAL activating domain in EXP-AD2, or a human liver cDNA library fused with the GAL activating domain in EXP-AD502 (Invitrogen) was transformed into the pDEST32-NUAK2 yeast strain to perform screenings. A total of 5.2 million and 1.8 million colonies were screened from the human fetal brain and human liver cDNA libraries, respectively, and positive clones were subjected to sequencing or analyzed by digestion.

Cell culture, constructs, antibodies and reagents
Human U2OS osteosarcoma and HeLa cell lines were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine at 37°C under a 5% CO2 atmosphere.

To inhibit actin stress fibers (Fig. 5) HeLa cells were treated for 60 minutes with 10 µM ROCK inhibitor Y27632 (Tocris Bioscience), 80 µM myosin II inhibitor blebbistatin or 0.7 µM lipophilic binding cyclohexalin D (Sigma).

To generate a full-length MRIP clone, three fragments were used. Initially, a partial Invitrogen clone (GenBank accession number BE675880) was digested using NdeI and AgeI enzymes to clone it into the pSPORT-CMV6 vector. The second fragment was added to this construct by utilizing an EcoRV and AgeI digestion of the second Invitrogen clone (GenBank accession number BG171134). Finally, to obtain a full-length MRIP clone, the third partial Invitrogen clone (GenBank accession number BC009982) was digested using EcoRI and AvrII enzymes, resulting in MRIP in the pUTB7 plasmid, which was transferred into the pENTR2B entry vector, sequenced and further transferred into the pCDNA-DEST53 destination vector (Invitrogen). To generate GST–tagged MRIP and pDEST27 destination vector (Invitrogen), to generate GST-tagged MRIP.

The partial MRIP clones in yeast EXP-AD22 or EXP-AD502 plasmids were transferred into entry clones using the BP clonase reaction (Invitrogen), and from entry clones transferred into the pCDNA-2.N-EmGFP-DEST destination vector (Invitrogen) using the Gateway cloning instructions. For overexpression studies, Fugene 6 transfection reagent was used at a 3:1 ratio with a 1:200 for immunoprecipitations. Filamentous actin was stained with 1:5000 for western blotting and 1:200 for immunoprecipitations. Staining coverslips were fixed and imaged using a Zeiss Axioplan 2 upright epifluorescence microscope with a 63× objective (Zeiss, Plan-Neofluar, N.A. 1.25) and a Zeiss AxioCam HRc charge-coupled device (CCD) camera, or with a Zeiss LSM 510 Meta confocal inverted microscope, with a 63× objective (Zeiss, Plan-Neofluar, N.A. 1.4). Final images were generated using Adobe Photoshop CS4, version 11.0. Quantifications were performed from multichannel images obtained using a 63× or 20× objective using ImageJ, marking either the cell perimeter or the nucleus as the region of interest and calculating integrated densities or integrated densities per area from the appropriate channel (phalloidin or pMLC). A minimum of 36 (as indicated in the figure legends) cells were analyzed for each data set. In box-plot representations, whiskers indicate the lowest datum still within 1.5 interquartile range (IQR) of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile. Outliers are not indicated in figures. Statistical analyses for significance of results were performed using Student’s t-test, assuming a two-tailed distribution and unequal variance.

Quantitative PCR analysis
RNAs of duplicate or triplicate samples were isolated using the RNeasy kit (Qiagen) according to the manufacturer’s protocol and total RNA was reverse-transcribed using Taqman reverse transcription reagents (Applied Biosystems). The ABI Prism 7500 was used with the Power SYBR Green PCR master mix (Applied Biosystems) and relative mRNA amounts were assayed by using 7500 Fast Real-Time PCR System software. Values were first compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then normalized to mRNAs isolated from control cells. The primers used were: NUAK2, forward 5’-CACACCACCC-TCA CTATGGG-3’ and reverse 5’-TGAATTTCACTTCTCTCCAC-3’; MRIP, forward 5’-CTTCCCAAGACCAACGGTG-3’ and reverse 5’-TGCTTGCTGTTGTTGTTCT-3’; MYPT1, forward 5’-AAAGCAGACGGCAACAGAAGAG-3’ and reverse 5’-CCACCAGAAGATCATACCTG-3’; GAPDH, forward 5’-CGAACACTTGGTCAGCTCA-3’ and reverse 5’-AGGGGAGATTGCTTGTGG-3’. Statistical analyses for significance of results were performed using either Student’s t-tests assuming a two-tailed distribution or by Student’s t-squared test assuming a two-tailed distribution and unequal variance.

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