Published in final edited form as:

Biochem Biophys Res Commun. 2015 April 17; 459(4): 686–691. doi:10.1016/j.bbrc.2015.03.005.

Phosphorylation of myosin II regulatory light chain by ZIP kinase is responsible for cleavage furrow ingression during cell division in mammalian cultured cells

Kosuke Hosoba¹, Satoshi Komatsu², Mitsuo Ikebe², Manato Kotani³, Xiao Wenqin³, Taro Tachibana³, Hiroshi Hosoya¹, and Kozue Hamao^{1,*}

¹Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan

²Department of Cellular and Molecular Biology, University of Texas Health Northeast, Tyler, TX 75708, USA

³Department of Bioengineering, Graduate School of Engineering Osaka City University, Osaka 558-8585, Japan

Abstract

Zipper-interacting protein kinase (ZIPK) is known to regulate several functions such as apoptosis, smooth muscle contraction, and cell migration. While exogenously expressed GFP-ZIPK localizes to the cleavage furrow, role of ZIPK in cytokinesis is obscure. Here, we show that ZIPK is a major MRLC kinase during mitosis. Moreover, ZIPK siRNA-mediated knockdown causes delay of cytokinesis. The delay in cytokinesis of ZIPK-knockdown cells was rescued by the exogenous diphosphorylation-mimicking MRLC mutant. Taken together, these findings suggest that ZIPK plays a role in the progression and completion of cytokinesis through MRLC phosphorylation.

Keywords

ZIP kinase; Myosin II regulatory light chain; Cytokinesis

Introduction

The motor activity of myosin II, composed of myosin heavy chains (MHCs), essential light chains and regulatory light chains (MRLCs), is regulated by MRLC phosphorylation. It has been demonstrated that monophosphorylation of MRLC at Ser19 (1P-MRLC) enhances myosin II activity [1,2], which is further promoted by diphosphorylation of MRLC (2P-MRLC) at Thr18/Ser19 [3]. The most well-known protein kinase responsible for MRLC

© 2015 Published by Elsevier Inc.

*Corresponding author: Kozue Hamao, kozue@hiroshima-u.ac.jp, Address: 1-3-1, Kagami-yama, Higashi-Hiroshima, 739-8526, Japan, Tel: +81-82-424-7444. Fax: +81-82-424-0734.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

phosphorylation is myosin light chain kinase (MLCK) [4,5], however, it has been realized that several other protein kinases including zipper-interacting protein kinase (ZIPK) / DAPK3 [6,7] can phosphorylate MRLC, thus controlling myosin activity in various aspects of cell contractility. ZIPK regulates myosin II activity through diphosphorylation of MRLC and inhibition of myosin light chain phosphatase (MLCP) to induce smooth muscle contraction [8]. We reported that overexpressed ZIPK induced diphosphorylation of MRLC and the rearrangement of actin stress fibers in interphase cells [9]. We also reported that phosphorylation of MRLC by ZIPK is responsible for cell motility [10,11]. However, while exogenously expressed ZIPK localizes at the contractile ring, the role of ZIPK during cytokinesis remains unclear.

During cytokinesis, a contractile ring, composed of actin filaments and myosin II [12], is formed at the cell equator allowing the cell to divide into two daughter cells. The motor activity of myosin II is a driving force of the cleavage furrow ingression through the actomyosin contraction [13]. Many studies report that phosphorylated MRLC localizes at the contractile ring [14,15,16]. It was reported that a nonphosphorylatable form of MRLC delays myosin II/actin turnover at the contractile ring [17], slows furrow ingression [18], and induces multinucleation [19,20], suggesting a major role of myosin II activation by MRLC phosphorylation in cytokinesis. In addition to MLCK and ZIPK, several kinases such as Rho-associated kinase-2 (ROCK2) and Citron kinase are reported to be able to phosphorylate MRLC [21,22]. However, the identity of the kinases responsible for MRLC phosphorylation during cytokinesis has been obscure.

Here, we report the role of ZIPK in cytokinesis. Biochemical analysis revealed that ZIPK is a major MRLC kinase during mitosis. ZIPK-knockdown decreases phosphorylated MRLC at the contractile ring and induces cytokinetic abnormalities. These abnormalities were rescued by the expression of diphosphorylation-mimicking MRLC mutant. These results suggest that ZIPK plays an important role in promoting cytokinesis ingression by phosphorylating MRLC.

Materials and Methods

Reagents

SM-1 peptide was synthesized as described [23]. Y27632 was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan) and ML-7 was purchased from Calbiochem Inc.

Antibodies

Rat monoclonal antibodies against 2P-MRLC (3H1012) and ZIPK (1D3D5) were produced as described previously [24]. The Pro-Gln-Arg-Ala-pThr-pSer-Asn-Val-Phe peptide for generating an anti-2P-MRLC antibody and GST-ZIPK for an anti-ZIPK antibody were used as antigens. The anti-1P-MRLC antibody was purchased from Cell Signaling Technology. A polyclonal anti-2P-MRLC antibody (pTS Ab), a monoclonal anti-1P-MRLC antibody (pSer19 Ab), a polyclonal anti-ZIPK antibody and phosphorylation-specific antibody against myosin binding large subunit (MBS) phosphorylated at Thr641 or at Ser799 were previously

described [25]. Antibodies against α -tubulin were purchased from Cedarlane Labs and Thermo Fisher Scientific, Inc.

Cell culture

HeLa cells and REF-2A cells were cultured in EMEM supplemented with 10% (V/V) fetal bovine serum and DMEM supplemented with 10% (V/V) new born calf serum, respectively.

Preparation of cell extracts and in vitro phosphorylation assay

REF-2A cells were treated for 3 h with 0.25 μ g/ml nocodazole. 40 min after release of mitotic arrest (M_{+40} , the majority of the cells are in metaphase and early anaphase), the cells were washed, and then lysed in buffer I (50 mM Tris-HCl (pH7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 5mM dithiothreitol, 5% glycerol, 0.2 mM Na-*p*-tosyl-L-lysine chloromethyl ketone, 0.2 mM N-tosyl-L-phenylalanine chloromethyl ketone, 2 mM phenylmethylsulfonyl fluoride). After added NP-40 (final 0.05%), cells were gently lysed and then added NaCl (final 0.4 M). The cell lysates were mixed and clarified by centrifugation at 10,000 g for 15 min.

The *in vitro* phosphorylation was carried out using two different buffers, A and B. Buffer A contains 30 mM NaCl, 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, 1 μ M microcystin-LR, 0.2 mM ATP and 30 mM Tris-HCl, pH7.5. Buffer B was the same as buffer A except for replacement 0.2 mM $CaCl_2$ by 5 mM EGTA. MRLC or isolated MBS (0.4 mg/ml) were phosphorylated in the presence of various concentrations of kinase inhibitors by M_{+40} extracts (0.1 mg/ml) in buffer A, and buffer B was used as the EGTA condition.

M_{+40} extracts were incubated with rabbit IgGs or anti- ZIPK Ab at 4°C for 3 h and then protein A-Support (Bio-Rad Laboratories) was added. After 1 h incubation, immunocomplex was removed. Immunodepleted extracts were incubated at 30°C for 15 min with MRLC in buffer B.

Immunostaining and microscopy

Immunostaining was performed as previously described [16] except for the use of Vectashield as a mounting reagent. Images were captured using a LSM 700 confocal microscope (Carl Zeiss).

RNAi, co-transfection and live-cell imaging

Targeting sequences of luciferase (5'-CGUACGCGGAAUACUUCGAdTdT-3'), ZIPK #1 (5'-CCAACAUCUCAGCCGUGAAAdTdT-3') and ZIPK #2 (5'-CCAGCUUGCCGCCAACAA-3') were designed. Transfection of siRNA and co-transfection of siRNA and each MRLC were performed according to manufacturer instructions using Oligofectamine, Lipofectamine RNAiMAX and Lipofectamine 3000 (Life technologies, USA). Live-cell imaging was performed using ECLIPSE Ti (Nikon, Japan) with a 40 $\times$ objective lens. Images were analyzed with the NIS Elements software (Nikon, Japan) to measure the width of cleavage furrow.

Results

ZIPK is a predominant protein kinase for MRLC phosphorylation during mitosis

To identify the protein kinases responsible for MRLC phosphorylation during cell division, mitotic cell extracts (M_{+40}) were prepared. As ROCK and MLCK are well-known MRLC kinases, we examined whether they are responsible for MRLC phosphorylation. Y27632 (ROCK inhibitor) had no effect on the MRLC phosphorylation activity (Fig. 1A and B). On the other hand, the activity of MBS phosphorylation at both Thr641 and Thr799 (ROCK phosphorylation sites) in the M_{+40} extracts was inhibited by Y27632 (Fig. S1A). The result indicates that there are ROCK activities in the M_{+40} extracts, but ROCK is not responsible for MRLC phosphorylation in the M_{+40} extracts. In contrast, ML-7 (MLCK inhibitor) inhibited MRLC phosphorylation (Fig. 1A and B). However, MRLC phosphorylation activity in the M_{+40} extracts was not affected at all by the elimination of Ca^{2+} (Fig. 1A). Moreover, SM-1 peptide, a MLCK specific peptide inhibitor for both native and constitutively active MLCK, showed no inhibition on the kinase activity in M_{+40} extracts (Fig. S1B). These results suggest that Ca^{2+} /CaM dependent MLCK is not primarily responsible for MRLC phosphorylation in the M_{+40} extracts, and that ML-7 inhibited other kinases. The present results are consistent with the previous work, and furthermore suggest that the kinases responsible for MRLC phosphorylation during mitosis phosphorylate Ser19 and Thr18 with same potency. We previously found that ZIPK phosphorylates MRLC at Ser19 and Thr18 with Ca^{2+} independent manner [4, 23] with the same rate constant [23] and that a high concentration of ML-7 inhibits ZIPK activity [10]. We next asked whether ZIPK contributes to MRLC phosphorylation during mitosis. To address this, the exogenous myosin II was incubated with M_{+40} extracts which were immunodepleted by ZIPK Ab. The immunodepletion of ZIPK significantly reduced MRLC kinase activity in the M_{+40} extracts as compared with the mock (Fig. 1C and D). These results suggest that ZIPK is a major kinase for MRLC phosphorylation during mitosis.

ZIPK localizes at the contractile ring and phosphorylates MRLC

To investigate the localization of ZIPK during mitosis, we generated a monoclonal anti-ZIPK antibody (1D3D5). This antibody recognized a single band of HeLa cell lysate at molecular mass of 53 kDa (Fig. 2A). ZIPKsi markedly decreased ZIPK band (Fig. 2B). We stained HeLa cells with this antibody and observed (Fig. 2C). When a cell entered mitosis, ZIPK co-localized with actin filaments on the cell surface. During cytokinesis, ZIPK localized at the equator of the cell, where contractile ring forms. The ZIPK signals were notably diminished by ZIPKsi. Moreover, ZIPK-knockdown was accompanied by a decrease in cortical actin bundles (Fig. 2C), suggesting that ZIPK regulates actin filaments through the myosin II stability by phosphorylating MRLC (as mentioned in Discussion).

We next asked whether ZIPK phosphorylates MRLC at the contractile ring. To address this, we examined the effect of ZIPK-depletion on MRLC mono- and diphosphorylation by immunostaining of 1P- and 2P-MRLC, respectively. The specificity of anti-2P-MRLC antibody (3H1012) was confirmed (Fig. S1C and D). Both 1P- and 2P-MRLC on the cell surface and at the contractile ring were notably diminished by ZIPK-knockdown using each siRNAs (Fig. 2D). As shown in Fig. 2E, the signal intensity of 1P- and 2P-MRLC at the

contractile ring was decreased by ZIPK-depletion. Taken together, these results suggest that ZIPK is responsible for MRLC phosphorylation during cytokinesis.

ZIPK contributes to cytokinesis by regulating the furrow ingression

To address the role of ZIPK on cytokinesis, we measured the fraction of multinuclear cells. When the cells were treated by ZIPKsi, multinuclear cells appeared (Fig. 3A, arrows). The fraction of multinuclear cells was higher than control by ZIPK-knockdown (Fig. 3B), suggesting that ZIPK-knockdown interfered with proper cytokinesis. On the other hand, about 90% of ZIPK-knockdown cells were still able to complete cytokinesis. We next asked whether ZIPKsi influences the progression of cytokinesis (Fig. 3C). We used ZIPKsi #1 because of its higher knockdown efficiency than ZIPKsi#2. Ten minutes after the start of cytokinesis, the control cell, but not the ZIPK-knockdown cell, finished cytokinesis. For some ZIPK-knockdown cells, furrow regression was observed (data not shown). Fig. 3D shows the progression of furrow ingression of Fig. 3C. The furrow ingression of ZIPK-knockdown cell was slower than that of control cell (Fig. 3D). The width of the equator region of ZIPK-knockdown cells was significantly wider than that of control cells at each time points (Fig. S2A). Moreover, times required for achieving 50% furrow constriction and the end of cytokinesis were delayed by ZIPK-depletion (Fig. 3E and Fig. 3F). Transfection of ZIPK siRNA#2 also induced cytokinetic delay (data not shown). These results suggest that ZIPK plays an important role in progression and completion of cytokinesis.

ZIPK contributes to cytokinesis progression through MRLC phosphorylation

To test whether ZIPK regulates cytokinesis through phosphorylation of MRLC, we co-transfected ZIPKsi #1 and EGFP-MRLCs in HeLa cells (Fig. S3A). We used four kinds of EGFP-MRLCs, wild type (WT-MRLC), nonphosphorylatable form (AA-MRLC), and mono- and di-phosphorylation-mimicking forms (AD-MRLC and DD-MRLC, respectively). The co-transfected cells were examined for production of multinuclear cells (Fig. S3B, arrows). The expression of DD-MRLC, but not AD-MRLC, into ZIPK-knockdown cells decreased the fraction of multinuclear cells compared to that of WT-MRLC (Fig. 4A). AA-MRLC increased the fraction of multinuclear cells than WT-MRLC. These results suggest that multinucleation in ZIPK-knockdown cells can be attributed to furrow regression induced by the decrease in diphosphorylated MRLC.

We next examined whether the delay of furrow ingression in ZIPK-knockdown cells is rescued by the expression of phosphorylation-mimicking MRLCs. We carried out live-cell imaging (Fig. S3C). Cytokinesis of the ZIPK-knockdown cell expressing DD-MRLC was faster than the cells expressing other MRLC variants (Fig. S3C and Fig. 4B). Diameter of furrows was plotted as a measure of the furrow ingression (Fig. 4B and Fig. S2B). The expression of DD-MRLC made furrow ingression of ZIPK-knockdown cells faster than that of WT-MRLC. DD-MRLC, but not AD-MRLC, decreased the duration to reach 50% cytokinesis progression (Fig. 4C) and the end of cytokinesis (Fig. 4D) than WT-MRLC. AA-MRLC slows the furrow ingression compared to WT-MRLC. These experiments suggest that ZIPK plays a role in regulation of cytokinesis through MRLC diphosphorylation.

Discussion

The data shown in this paper suggest that ZIPK contributes for regulation of progression and completion of cytokinesis through MRLC diphosphorylation. We previously reported that a ROCK inhibitor induces cytokinetic delay and this delay was rescued by DD-MRLC [15], suggesting that ROCK is also involved in furrow ingression through MRLC phosphorylation. Since ROCK can diphosphorylate MRLC [21,22], it is plausible that ROCK also directly phosphorylate MRLC during cytokinesis. Another possibility is that ROCK may regulate ZIPK through phosphorylation. It was reported previously that ZIPK is phosphorylated at least three sites, Thr180, Thr225, and Thr265, is essential for the full activity of ZIPK [26]. It was also reported that ROCK1 phosphorylates ZIPK at Thr265 and Thr299 [27] and phosphorylation at Thr265, but not Thr299, promotes its kinase activity [28]. Another scenario is ROCK regulates MRLC phosphorylation via regulation of MLCP, and it was reported that ROCK phosphorylates MBS of MLCP and inhibits MLCP activity, thus increasing MRLC phosphorylation [29].

Citron kinase has been also reported to regulate MRLC phosphorylation during mitosis [30], while recent studies demonstrate that citron kinase contributes to cytokinesis through midbody formation [31] rather than MRLC phosphorylation. It is likely that MRLC phosphorylation during mitosis is concertedly regulated by these protein kinases, and relative contribution on MRLC phosphorylation is different among the cell types, presumably due to the expression levels and the presence of factors regulating these kinases.

It is assumed that the constriction of a contractile ring requires actomyosin-dependent force generation. Present result supports this view since the inactivation of myosin II, due to the decrease in phosphorylated MRLC, induced a delay of furrow ingression. Since actin filaments are important for actomyosin contractile activity, it is reasonable that the inhibition of actin polymerization by latrunculin A suppresses the contraction of contractile ring in fission yeast [32] and in cultured mammalian cells [33]. Interestingly, we found that ZIPK-knockdown induced a decrease in actin filamentous structure (Fig. 2C) and MHC (data not shown) at the contractile ring. We previously reported that diphosphorylated MRLC stabilizes filamentous structure of myosin [34]. Consistently, ZIPK-depletion disrupts myosin and actin filamentous structure in interphase cells [10]. We also reported previously that ZIPK-overexpression induced the rearrangement of actin filaments in interphase cells through MRLC diphosphorylation [9]. While the overexpression of AA-MRLC has no effects on actin filaments at the contractile ring [18], it enhances actin turnover at the contractile ring [17]. These findings suggest that phosphorylation of MRLC by ZIPK regulates the formation and dynamics of actin filaments and filament bundles at the contractile ring, presumably due to the stabilization of myosin II filaments. Supporting this view, it was reported recently that cortical actin dynamics is important for contractile ring constriction, which is controlled by actin cross-linker proteins and myosin II [35].

We showed that ZIPK-depletion induces multinucleation (Fig. 3B). What is the fate of these multinuclear cells? It has been reported that tetraploid cells produced due to cytokinesis failure induce aneuploidy and lead to oncogenesis [36]. In tetraploid cells, p53-dependent apoptosis is activated [37], suggesting that the cell failed in cytokinesis may induce

apoptosis. However, we didn't observe apoptotic cells by ZIPK-knockdown (data not shown). It is necessary to examine whether ZIPK-knockdown cells cause aneuploidy without apoptosis. Since ZIPK is reported to be a tumor suppressor [38], ZIPK may regulate apoptosis of the cells that failed in cytokinesis, thus preventing oncogenesis.

In summary, ZIPK controls cytokinesis through phosphorylation of MRLC. Phosphorylated MRLC may regulate the formation of actin filaments or filament bundles through stabilization of myosin II filaments in addition to the activation of myosin II motor activity, thus facilitating cytokinesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. T. Miyamoto and S. Matsuura (Research Institute for Radiation Biology and Medicine, Hiroshima University, Japan) for kindly giving Lipofectamine3000. We also thank Enago (www.enago.jp) for the English language review. This work was supported by JSPS KAKENHI (21770143) to KH. This work was in part supported by NHLBI grants to MI (HL070530, HL106461 and HL111696) and to SK (R21-HL-094983). This work was also partially supported by AHA research grant (0535419T) to SK.

References

1. Ikebe M, Hartshorne DJ. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem.* 1985; 260:10027–10031. [PubMed: 3839510]
2. Mizutani T, Haga H, Koyama Y, et al. Diphosphorylation of the myosin regulatory light chain enhances the tension acting on stress fibers in fibroblasts. *J Cell Physiol.* 2006; 209:726–731. [PubMed: 16924661]
3. Ikebe M, Hartshorne DJ, Elzinga M. Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J Biol Chem.* 1986; 261:36–39. [PubMed: 3079756]
4. Tan JL, Ravid S, Spudich JA. Control of nonmuscle myosins by phosphorylation. *Annu Rev Biochem.* 1992; 61:721–759. [PubMed: 1497323]
5. Stull JT, Tansey MG, Word RA, et al. Myosin light chain kinase phosphorylation: regulation of the Ca²⁺ sensitivity of contractile elements. *Adv Exp Med Biol.* 1991; 304:129–138. [PubMed: 1803895]
6. Murata-Hori M, Suizu F, Iwasaki T, et al. ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS Lett.* 1999; 451:81–84. [PubMed: 10356987]
7. Niino N, Ikebe M. Zipper-interacting protein kinase induces Ca(2+)-free smooth muscle contraction via myosin light chain phosphorylation. *J Biol Chem.* 2001; 276:29567–29574. [PubMed: 11384979]
8. Ihara E, MacDonald JA. The regulation of smooth muscle contractility by zipper-interacting protein kinase. *Can J Physiol Pharmacol.* 2007; 85:79–87. [PubMed: 17487247]
9. Murata-Hori M, Fukuta Y, Ueda K, et al. HeLa ZIP kinase induces diphosphorylation of myosin II regulatory light chain and reorganization of actin filaments in nonmuscle cells. *Oncogene.* 2001; 20:8175–8183. [PubMed: 11781833]
10. Komatsu S, Ikebe M. ZIP kinase is responsible for the phosphorylation of myosin II and necessary for cell motility in mammalian fibroblasts. *J Cell Biol.* 2004; 165:243–254. [PubMed: 15096528]
11. Komatsu S, Ikebe M. ZIPK is critical for the motility and contractility of VSMCs through the regulation of nonmuscle myosin II isoforms. *Am J Physiol Heart Circ Physiol.* 2014; 306:H1275–1286. [PubMed: 24633547]

12. Mabuchi I, Okuno M. The effect of myosin antibody on the division of starfish blastomeres. *J Cell Biol.* 1977; 74:251–263. [PubMed: 141455]
13. Sellers JR. Myosins: a diverse superfamily. *Biochim Biophys Acta.* 2000; 1496:3–22. [PubMed: 10722873]
14. Murata-Hori M, Murai N, Komatsu S, et al. Concentration of singly phosphorylated myosin II regulatory light chain along the cleavage furrow of dividing HeLa cells. *Biomedical Research.* 1998; 19:111–115.
15. Matsumura F, Ono S, Yamakita Y, et al. Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of cultured cells. *J Cell Biol.* 1998; 140:119–129. [PubMed: 9425160]
16. Kondo T, Isoda R, Uchimura T, et al. Diphosphorylated but not monophosphorylated myosin II regulatory light chain localizes to the midzone without its heavy chain during cytokinesis. *Biochem Biophys Res Commun.* 2012; 417:686–691. [PubMed: 22166199]
17. Kondo T, Hamao K, Kamijo K, et al. Enhancement of myosin II/actin turnover at the contractile ring induces slower furrowing in dividing HeLa cells. *Biochem J.* 2011; 435:569–576. [PubMed: 21231914]
18. Asano S, Hamao K, Hosoya H. Direct evidence for roles of phosphorylated regulatory light chain of myosin II in furrow ingression during cytokinesis in HeLa cells. *Genes Cells.* 2009; 14:555–568. [PubMed: 19371382]
19. Dean SO, Spudich JA. Rho kinase's role in myosin recruitment to the equatorial cortex of mitotic *Drosophila* S2 cells is for myosin regulatory light chain phosphorylation. *PLoS One.* 2006; 1:e131. [PubMed: 17205135]
20. Komatsu S, Yano T, Shibata M, et al. Effects of the regulatory light chain phosphorylation of myosin II on mitosis and cytokinesis of mammalian cells. *J Biol Chem.* 2000; 275:34512–34520. [PubMed: 10944522]
21. Ueda K, Murata-Hori M, Tatsuka M, et al. Rho-kinase contributes to diphosphorylation of myosin II regulatory light chain in nonmuscle cells. *Oncogene.* 2002; 21:5852–5860. [PubMed: 12185584]
22. Yamashiro S, Totsukawa G, Yamakita Y, et al. Citron kinase, a Rho-dependent kinase, induces diphosphorylation of regulatory light chain of myosin II. *Mol Biol Cell.* 2003; 14:1745–1756. [PubMed: 12802051]
23. Ikebe M, Stepinska M, Kemp BE, et al. Proteolysis of smooth muscle myosin light chain kinase. Formation of inactive and calmodulin-independent fragments. *J Biol Chem.* 1987; 262:13828–13834. [PubMed: 3654638]
24. Yoshimi T, Ohkawa Y, Azuma M, et al. A panel of specific monoclonal antibodies directed against various phosphorylated histones H3. *Monoclon Antib Immunodiagn Immunother.* 2013; 32:119–124. [PubMed: 23607347]
25. Takizawa N, Niiro N, Ikebe M. Dephosphorylation of the two regulatory components of myosin phosphatase, MBS and CPI17. *FEBS Lett.* 2002; 515:127–132. [PubMed: 11943207]
26. Graves PR, Winkfield KM, Haystead TA. Regulation of zipper-interacting protein kinase activity in vitro and in vivo by multisite phosphorylation. *J Biol Chem.* 2005; 280:9363–9374. [PubMed: 15611134]
27. Hagerty L, Weitzel DH, Chambers J, et al. ROCK1 phosphorylates and activates zipper-interacting protein kinase. *J Biol Chem.* 2007; 282:4884–4893. [PubMed: 17158456]
28. Weitzel DH, Chambers J, Haystead TA. Phosphorylation-dependent control of ZIPK nuclear import is species specific. *Cell Signal.* 2011; 23:297–303. [PubMed: 20854903]
29. Kimura K, Ito M, Amano M, et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science.* 1996; 273:245–248. [PubMed: 8662509]
30. Madaule P, Eda M, Watanabe N, et al. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature.* 1998; 394:491–494. [PubMed: 9697773]
31. Bassi ZI, Audusseau M, Riparbelli MG, et al. Citron kinase controls a molecular network required for midbody formation in cytokinesis. *Proc Natl Acad Sci U S A.* 2013; 110:9782–9787. [PubMed: 23716662]
32. Pelham RJ, Chang F. Actin dynamics in the contractile ring during cytokinesis in fission yeast. *Nature.* 2002; 419:82–86. [PubMed: 12214236]

33. Murthy K, Wadsworth P. Myosin-II-dependent localization and dynamics of F-actin during cytokinesis. *Curr Biol*. 2005; 15:724–731. [PubMed: 15854904]
34. Ikebe M, Koretz J, Hartshorne DJ. Effects of phosphorylation of light chain residues threonine 18 and serine 19 on the properties and conformation of smooth muscle myosin. *J Biol Chem*. 1988; 263:6432–6437. [PubMed: 2966156]
35. Mendes Pinto I, Rubinstein B, Kucharavy A, et al. Actin depolymerization drives actomyosin ring contraction during budding yeast cytokinesis. *Dev Cell*. 2012; 22:1247–1260. [PubMed: 22698284]
36. Lv L, Zhang T, Yi Q, et al. Tetraploid cells from cytokinesis failure induce aneuploidy and spontaneous transformation of mouse ovarian surface epithelial cells. *Cell Cycle*. 2012; 11:2864–2875. [PubMed: 22801546]
37. Castedo M, Coquelle A, Vivet S, et al. Apoptosis regulation in tetraploid cancer cells. *EMBO J*. 2006; 25:2584–2595. [PubMed: 16675948]
38. Brognard J, Zhang YW, Puto LA, et al. Cancer-associated loss-of-function mutations implicate DAPK3 as a tumor-suppressing kinase. *Cancer Res*. 2011; 71:3152–3161. [PubMed: 21487036]

Highlights

- ZIPK is responsible for phosphorylation of MRLC at the contractile ring.
- The cytokinetic abnormalities in ZIPK depleted cells were rescued by phosphorylation-mimic MRLC expression
- We revealed that ZIPK regulates cytokinesis through MRLC phosphorylation.

Figure 1. ZIPK is a major MRLC kinase during cell division

(A) MRLC was incubated with M_{+40} extracts in the presence of ATP and kinase inhibitors or EGTA. Phosphorylated MRLC was detected by immunoblotting using pSer19 Ab or pTS Ab. (B) The amount of phosphorylated MRLC was quantitatively determined by scanning densitometry (NIH image program). The values shown are means $\pm$ SD from three independent experiments. (C) MRLC was phosphorylated by M_{+40} extracts immunodepleted with either rabbit IgGs (mock) or ZIPK Ab. Phosphorylated MRLC was detected by immunoblotting using pSer19 Ab or pTS Ab. (D) The signal intensity of phosphorylated MRLC was determined by scanning densitometry (NIH image program). The values shown are mean $\pm$ SD from three independent experiments.

Figure 2. ZIPK phosphorylates MRLC at the contractile ring

(A) Immunoblotting using the anti-ZIPK antibody (1D3D5) on HeLa cell lysate. (B) Immunoblotting of an anti-ZIPK antibody using ZIPK-depleted HeLa cell lysates. (C) The localization of ZIPK and actin filaments of HeLa cells transfected each siRNA was observed by a confocal microscopy. Scale bar, 10 μ m. (D) The siRNA-transfected cells were stained with anti-1P-, 2P-MRLC antibody and DAPI, and observed by a confocal microscopy. Scale bar, 10 μ m. (E) The signal intensity of 1P- and 2P-MRLC on the contractile ring was measured by the ZEN software (Carl Zeiss). The values represent means $\pm$ SEM (n = 20). *** P < 0.001 (one-way-ANOVA, Ryan's method).

Figure 3. ZIPK contributes to cytokinesis by regulating the furrow ingression

(A) HeLa cells transfected each siRNA were stained and observed by a confocal microscopy. Scale bar: 50 μ m. (B) The percentage of multinuclear cells in control or ZIPK-knockdown was shown. The values represent means $\pm$ SEM ($n = 3$; 150 cells per experiment). (C) Mitotic cells transfected each siRNA were observed by live-cell imaging from cytokinesis start to end. Time 0 indicates cytokinesis start (arrows). Arrowheads indicate the end of cytokinesis. Scale bar, 10 μ m. (D) The progress of furrow ingression was plotted from cytokinesis start to end. The results are shown as relative diameter of the width of the cleavage furrow at each time point divided by that at cytokinesis start ($n = 15$). (E) The bars show the times for 50 % cytokinesis progression from cytokinesis start. (F) The histogram shows the end of cytokinesis in each cell. The values represent means $\pm$ SEM ($n = 15$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA, Ryan's method and t-test).

Figure 4. ZIPK contributes to cytokinesis progression through phosphorylation of MRLC
 (A) The ratio of multinuclear cells by co-transfection of siRNA and each MRLC was measured. The values represent means $\pm$ SEM ($n = 3$; at least 130 cells per experiment). (B) The progress of furrow ingression in each cell from cytokinesis start to end was plotted. The result is expressed as the relative diameter of the width of the cleavage furrow at each time point divided by that at cytokinesis start. (C) The times for 50% cytokinesis progression from cytokinesis start in each co-transfected cell was measured. (D) The histogram shows the end of cytokinesis in each cell. The values represent means $\pm$ SEM ($n = 7$). $**P < 0.01$, $***P < 0.001$ (one-way ANOVA, Ryan's method).