広島大学学位請求論文

Functional analysis of mammalian ZIP kinase on phosphorylation of myosin II regulatory light chain during cytokinesis

（細胞質分裂時のミオシン II 調節軽鎖のリン酸化に関する ZIP キナーゼの機能解析）

細羽康介

2015 年

広島大学大学院理学研究科

生物科学専攻

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主論文

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2015

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Publication of the thesis

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

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Biochemical and Biophysical Research Communication, 459 (2015) 686-691

Chapter 1

General 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 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]. It was reported that overexpressed ZIPK induced diphosphorylation of MRLC and the rearrangement of actin stress fibers in interphase cells [9]. It was also 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.

In this study, I revealed that the role of ZIPK during cytokinesis. Cell biological and biochemical analysis revealed that ZIPK is a major MRLC kinase during mitosis. The siRNA mediated ZIPK depleation decreases phosphorylated MRLC at the contractile ring and induces cytokinetic abnormalities including delay of cleavage furrow ingression and multinucleation that is occurred by furrow regression. Moreover, these phenotypes were rescued by the induction of diphosphorylation-mimicking MRLC mutant. These results suggest that ZIPK phosphorylates MRLC and regulates cytokinesis ingression.

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Chapter 2

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, biochemical analyses show that ZIPK is a major MRLC kinase during mitosis. Moreover, ZIPK siRNA-mediated knockdown causes cytokinetic abnormalities including delay of cytokinesis and multinucleation. These phenotypes in 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.

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 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. HRP labeled anti-rabbit, mouse and rat antibodies were purchased from Jackson immuno labs. Alexa Fluor

568 or 488 labeled anti-rabbit, mouse and rat antibodies were purchased from Molecular Probes (USA) and TRITC or FITC labeled phalloidin were obtained from Sigma Aldrich (USA).

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.

SDS-PAGE and Western blot

The cells were lysed with 2× SDS sampling buffer (200 mM Tris-HCL (pH 6.8), 250 mM dithiothreitol, 5% SDS, 22.5% glycerol and bromophenol blue). Each samples were separated by SDS-PAGE and transferred to PVDF membrane (pore size 0.2 μM) (Millipore, USA) in transfer buffer (40 mM glycine, 48 mM Tris base). After that, membranes were blocked by 5% skim milk-PBS and incubated with each primary antibodies, anti-mouse α-tubulin (1/4000), anti-rat ZIPK (1/1000) and rabbit anti-GFP (1/1000) for 1 h at 37˚C. After primary antibody treatment, membranes were washed by PBS containing 0.02% Tween-20 for 4 times, membranes were

incubated with HRP labeled secondary antibodies. After chemiluminescence reagent treatment, signals were detected by ChemiDoc XRS Plus (Bio-Rad Laboratories)

Preparation of cell extracts and *in vitro* phosphorylation assay

REF-2A cells were treated for 3 h with 0.25 μg/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 Nα-*p*-tosyl-L-lysine chloromethl 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 μM microcystin-LR, 0.2 mM ATP and 30 mM This-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

Each cells on coverslips were fixed by 3.7% formaldehyde-PBS for 10 min and permeabilized by 0.2% Triton X-100-PBS for 3 min at room temperature. The cells were washed by PBS for 3 times, blocked by 1% BSA-PBS for 15 min. Then, the cells were incubated with each primary antibodies, rabbit anti-α-tubulin, rat anti-ZIPK (1D3D5), rat anti-2P-MRLC (3H1012) and rabbit anti-1P-MRLC for 1h at 37˚C. For staining of ZIPK, cells were re-fixed by 3.7% formaldehyde-PBS for 10 min and washed by PBS for 4 times. After that, cells were incubated with Alexa Fluor 568 or 488 labeled anti-rabbit, mouse, or rat IgG (H＋L) antibodies (1/200) for 30 min at 37˚C. To visualization of actin filaments, the cells were treated with

TRITC or FITC-phalloidin (1/200) for 30 min at 37˚C. After PBS washing, cells were mounted by Vectashield containing DAPI for staining DNA. Images were captured using a LSM 700 confocal microscope (Carl Zeiss) and FV1000D confocal microscope (Olympus).

si RNA and plasmids transfection

The HeLa cells were cultured on 3.5 cm glass-bottom dishes (IWAKI, Japan) for live cell imaging or 6 well plates. Targeting sequences of luciferase (5′-CGUACGCGGAAUACUUCGAdTdT-3′), ZIPK #1 (5′-CCAACAUCUCAGCCGUGAAdTdT-3′) and ZIPK #2 (5′-CCAGCUUGCCGCCAACAA-3′) were designed. RNAiMAX (Life technologies, USA) was used for siRNA transfection according to manufacturer instructions. Each siRNAs (final 20 nM), RNAiMAX reagent (4 μl) and 500μl OPTI-MEM were mixed at room temperature. After incubation, mixture were applied to glass-bottom dishes or 6 well plates and cells were cultured for 48 h.

The wild type MRLC cDNA and MRLC mutants that were mutated in phosphorylation sites (AA-, AD- and DD-MRLC) cDNAs were subcloned to

pEGFP-N1 vector (Clontech, USA). For co-transfection of siRNAs and each MRLC, Lipofectamine 3000 (Life technologies, USA) was used. The siRNAs (final 20 nM), each plasmids (300 ng) and 500μl OPTI-MEM were mixed and incubated at room temperature. After applying to glass-bottom dishes or 6 well plates, cells were cultured for 24 h.

Analysis of live cell imaging

Live-cell imaging was performed using ECLIPSE Ti (Nikon, Japan) with a 40× 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 in 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 enters 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. In interphase, ZIPK co-localized with F-actin and localized at midbody (Fig. S2A). The ZIPK signals were notably diminished by ZIPKsi. Moreover, ZIPK-knockdown was accompanied by a decrease in cortical actin bundles and MHC IIB (Fig. 2C and Fig. S3A and B), 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 immunostaing of 1P- and 2P-MRLC, respectively. The specificity of anti-2P-MRLC antibody (3H1012) was confirmed (Fig. 3A and B). Both 1P- and 2P-MRLC on the cell surface and at the contractile ring were notably diminished by ZIPK-knockdown using each siRNAs (Fig. 3C and D). As shown in Fig. 3D, 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. 4A, arrows). The fraction of multinuclear cells was higher than control by ZIPK-knockdown (Fig. 4B), 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. 4C). 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. 4D shows the progression of furrow ingression of Fig. 4C. The furrow ingression of ZIPK-knockdown cell was slower than that of control cell (Fig. 4D). The width of the equator region of ZIPK-knockdown cells was significantly wider than that of

control cells at each time points (Fig. S4A). Moreover, times required for achieving 50% furrow constriction and the end of cytokinesis were delayed by ZIPK-depletion (Fig. 4E and Fig. 4F). 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. 5A). We used four kinds of EGFP-MRLCs, wild type (WT)-, nonphosphorylatable form (AA-), 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. 5B, 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. 5C). 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. 6A). Cytokinesis of the ZIPK-knockdown cell expressing DD-MRLC was faster than the cells expressing other MRLC variants (Fig. 6A and Fig. 6B). Diameter of furrows was plotted as a measure of the furrow ingression (Fig. 6B and Fig. S4B). 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. 6C) and the end of cytokinesis (Fig. 6D) 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 and MHC (Fig. 2C and Fig. S3A and B) 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. 4B). 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.

Acknowledgement

I gratefully thank my supervisor Associate professor Kozue Hamao (Hiroshima University) for her advise, encouragement and preparation of this thesis. I also thank Drs. T. Miyamoto and S. Matsuura (Research Institute for Radiation Biology and Medicine, Hiroshima University, Japan) for kindly giving Lipofectamine3000; and member of our laboratory for supporting the experiments.

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Figures and supplemental figures

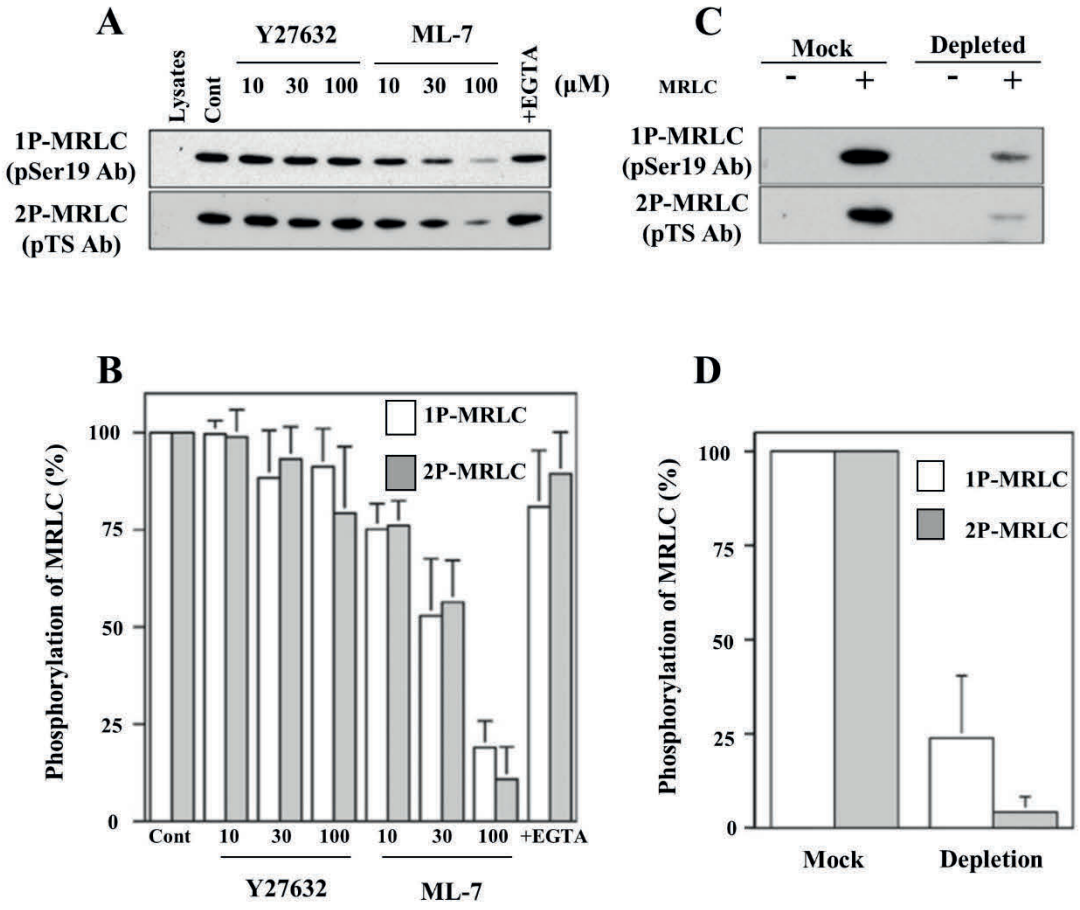
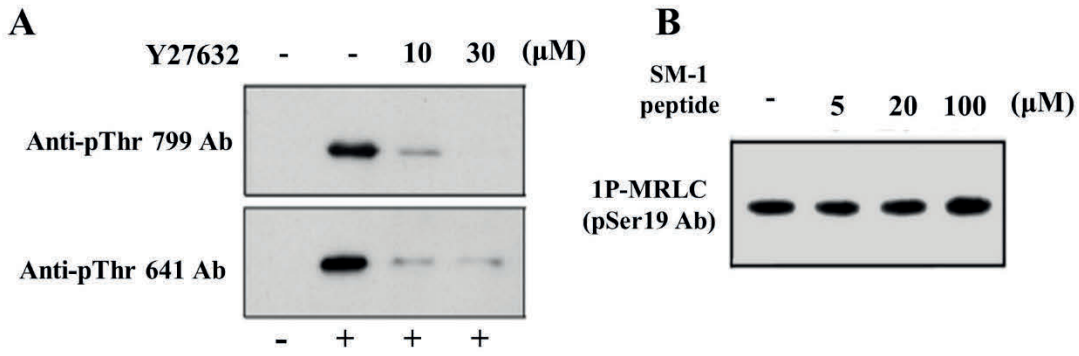


Figure.1 ZIPK is a major MRLC kinase during cell division.

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 ± SD from three independent experiments. (C) MRLC was phosphorylated by M_{+40} extracts immunodepled 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 ± SD from three independent experiments.



Supplementary Figure. 1 Confirmation of the kinase activity in mitotic cell extracts.

Supplementary Figure. 1 Confirmation of the kinase activity in mitotic cell extracts and the generated anti-2P-MRLC antibody specificity. (A) MBS was incubated with M_{+40} extracts containing Y27632. Phosphorylated MBS was detected by immunoblotting with anti-pThr 641 Ab or anti-pThr 799 Ab. (B) MRLC was incubated with M_{+40} extracts containing SM-1 peptides. Phosphorylated MRLC was detected by immunoblotting with pSer19 Ab.

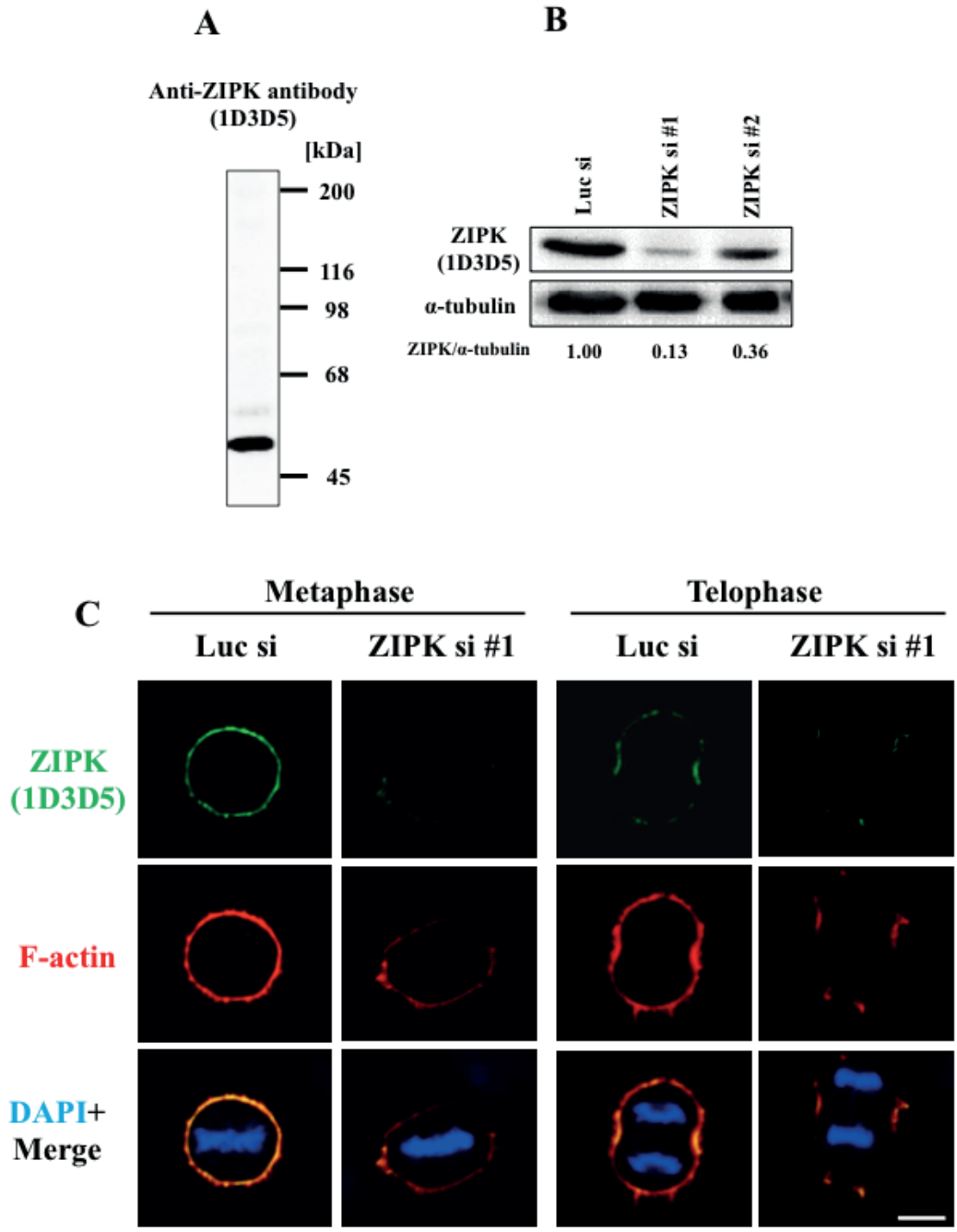
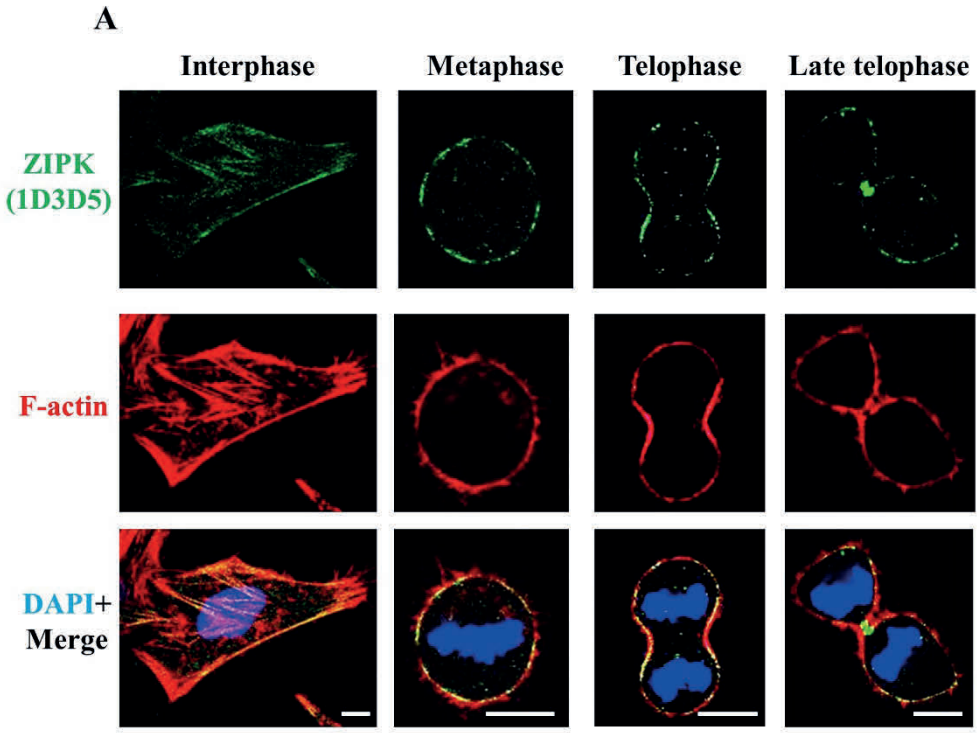


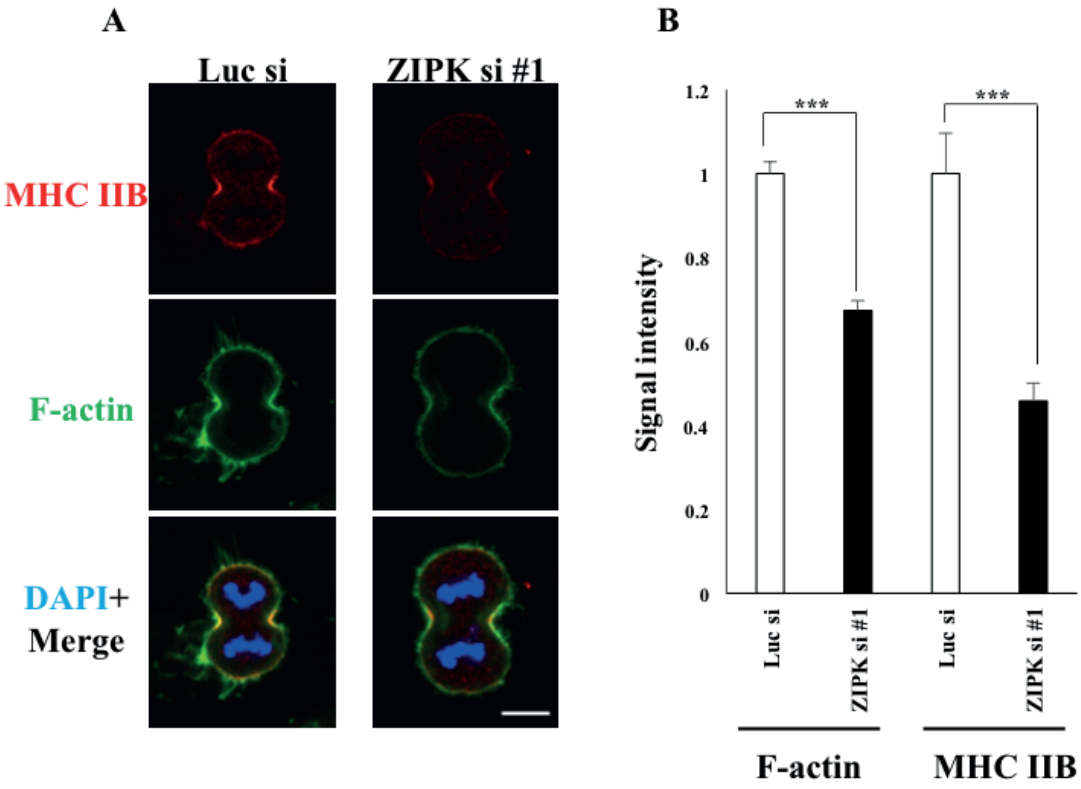
Figure.2 ZIPK localizes at the contractile ring.

Figure.2 ZIPK localizes 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 μm.



Supplementary Figure. 2 Localization pattern of ZIPK during cell cycle.

Supplementary Figure.2 Localization pattern of ZIPK during cell cycle. (A) Localization of ZIPK during cell cycle in HeLa cells was observed by a confocal microscopy. Scale bar, 10 μm.



Supplementary Figure. 3 Localization of F-actin and MHC IIB in ZIPK knockdown cells

Supplementary Figure.3 Localization of F-actin and MHC IIB in ZIPK knockdown cells. (A) Localization of F-actin and MHC IIB in ZIPK depleated HeLa cells was observed by a confocal microscopy. Scale bar, 10 μm. (B) The signal intensity of F-actin and MHC IIB at the contractile ring was measured by the ZEN software (Carl Zeiss). The values represent means ± SEM (n = 20). ***$P < 0.001$ (one-way-ANOVA, Ryan's method).

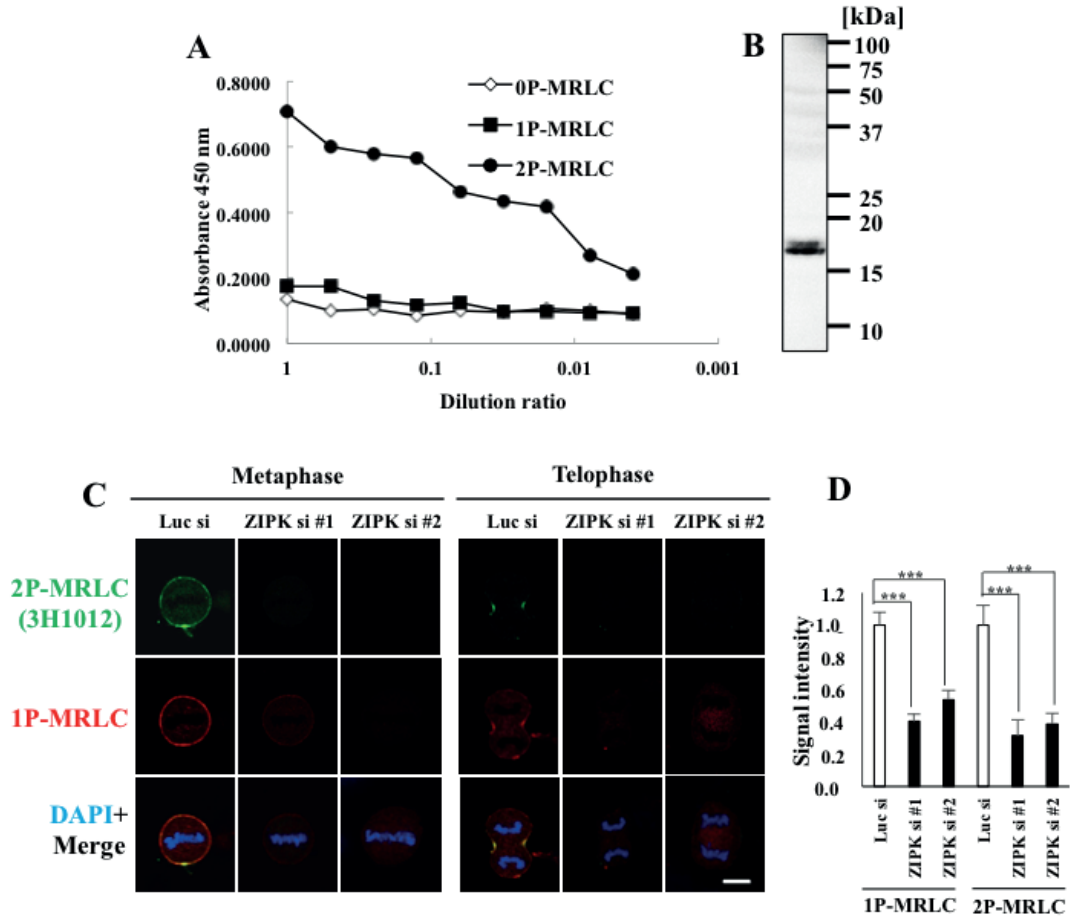


Figure.3 ZIPK phosphorylates MRLC at the contractile ring.

Figure.3 ZIPK phosphorylates MRLC at the contractile ring. (A) The Line chart shows the ELISA data for specificity confirmation of the generated anti-2P-MRLC antibody (3H1012). (B) Immunoblotting of generated anti-2P-MRLC antibody (3H1012) using whole HeLa cell lysate. (C) The siRNA-transfected cells were stained with anti-1P-, 2P-MRLC antibody and DAPI, and observed by a confocal microscopy. Scale bar, 10 μm. (D) The signal intensity of 1P- and 2P-MRLC on the contractile ring was measured by the ZEN software (Carl Zeiss). The values represent means ± SEM (n = 20). ***$P < 0.001$ (one-way-ANOVA, Ryan's method).

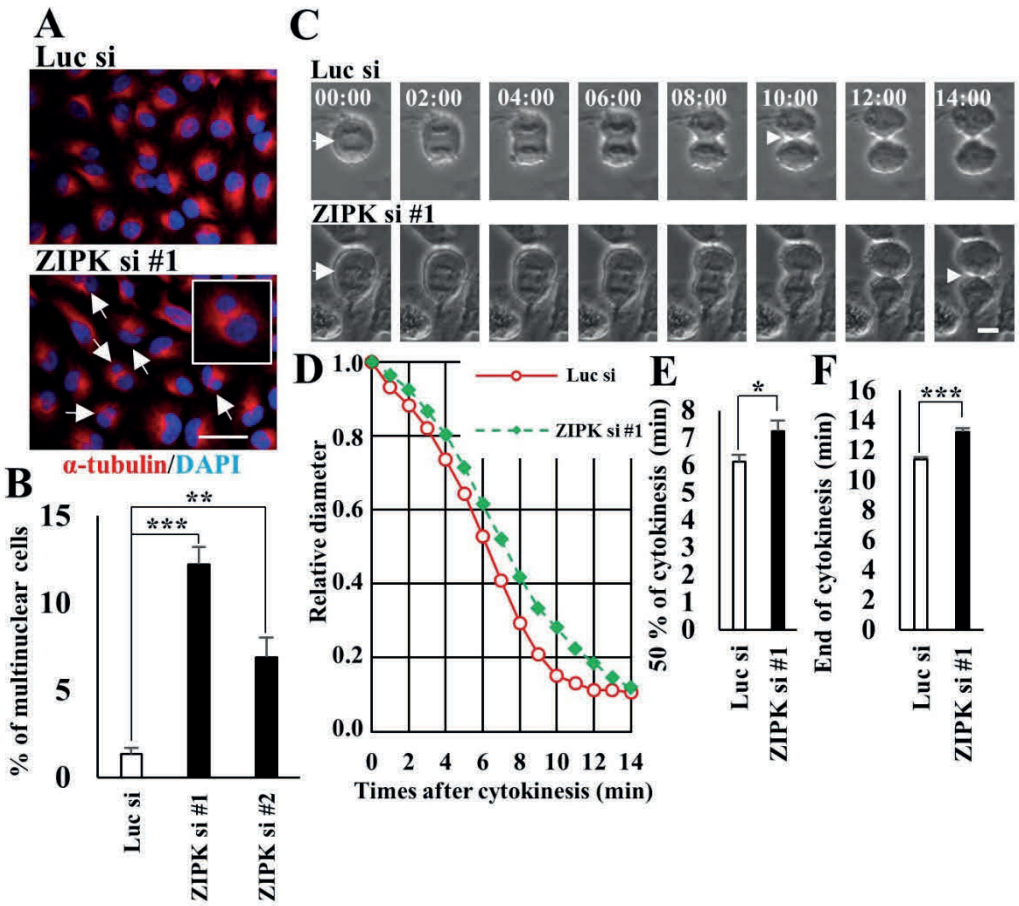


Figure.4 ZIPK contributes to cytokinesis by regulating the furrow ingression.

Figure.4 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 μm. (B) The percentage of multinuclear cells in control or ZIPK-knockdown was shown. The values represent means ± 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 μm. (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 ± SEM (n = 15). $*P < 0.05$, $**P<0.01$, $***P < 0.001$ (one-way ANOVA, Ryan's method and t-test).

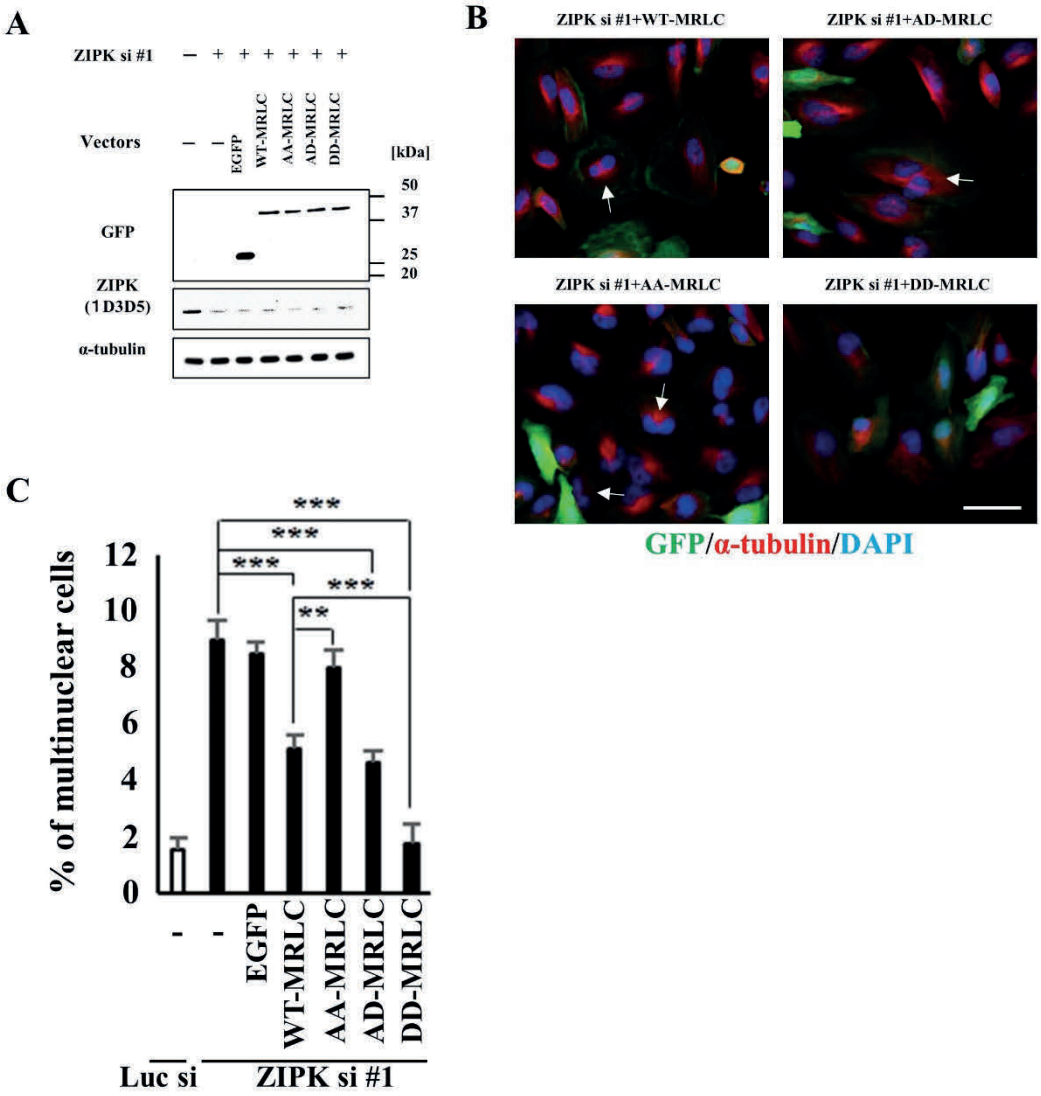


Figure. 5 The increase of multinuclearcells by ZIPK depleation are rescued by diphosphorylation-mimicking MRLC.

Figure. 5 The increase of multinuclearcells by ZIPK depleation are rescued by diphosphorylation-mimicking MRLC. (A) The co-transfection of siRNA and each MRLCs in HeLa cells was confirmed by immunoblotting. (B) HeLa cells co-transfected siRNA and each MRLC were stained with an anti-α-tubulin antibody and DAPI and observed by a confocal microscopy. Scale bar, 50 μm. (C) The ratio of multinuclear cells by co-transfection of siRNA and each MRLC was measured. The values represent means ± SEM (n = 3; at least 130 cells per experiment).

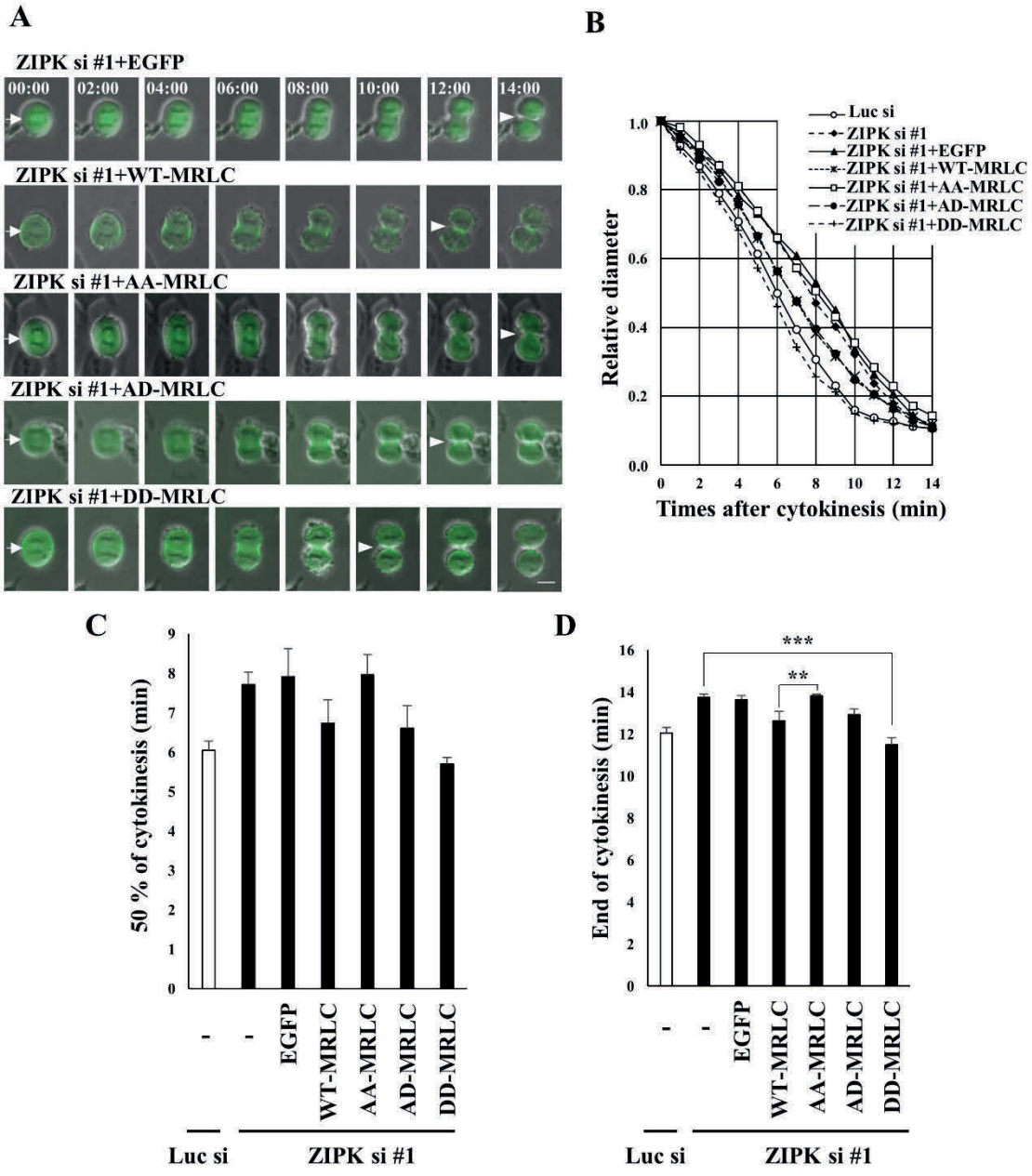
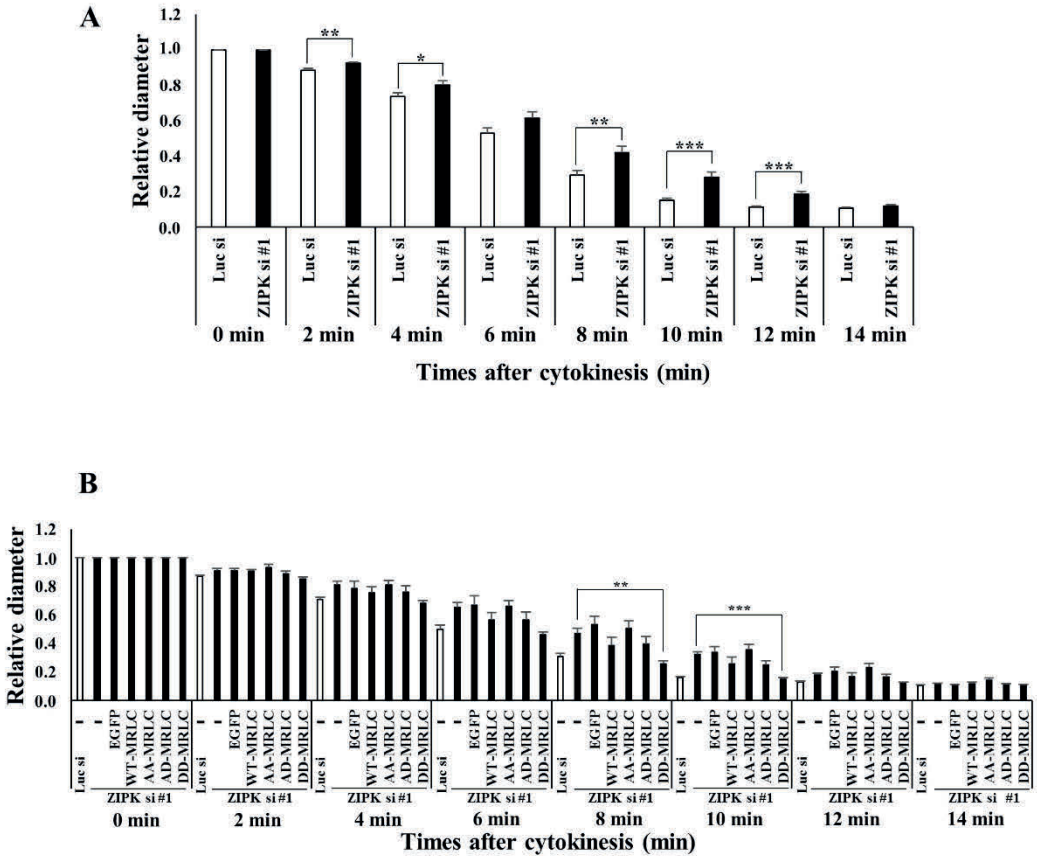


Figure. 6 The cytokinetic delay in ZIPK knockdown cells are rescued by diphosphorylation-mimicking MRLC.

Figure. 6 The cytokinetic delay in ZIPK knockdown cells are rescued by diphosphorylation-mimicking MRLC. (A) HeLa Cells co-transfected siRNA and each MRLC 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 μm. (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 ± SEM ($n \geq 7$). $^{**}P < 0.01$, $^{***}P<0.001$ (one-way ANOVA, Ryan's method).



Supplementary Figure. 4 The histogram of furrow ingression in cells transfected siRNA and co-transfected siRNA and each MRLC.

Supplementary Figure. 4 The histogram of furrow ingression in cells transfected siRNA and co-transfected siRNA and each MRLC. (A) The histogram represents the relative diameter of the width of the cleavage furrow at each time point divided by that at cytokinesis start based on the results of Fig. 4D. The values represent means ± SEM (n=15). (B) The bar shows the relative diameter of the width of the cleavage furrow at each time point divided by that at cytokinesis start based on the results of Fig. 6B. The values represent means ± SEM ($n \geq 7$). *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA, Ryan's method and t-test).