

MECHANISTIC INVESTIGATION AND PROTEIN ENGINEERING OF A
BIFUNCTIONAL PROTEIN REGULATING MITOSIS

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by

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MECHANISTIC INVESTIGATION AND PROTEIN ENGINEERING OF A BIFUNCTIONAL PROTEIN REGULATING MITOSIS

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Phosphoinositides (PIPs) are low-abundant yet essential phospholipids localized in the cytosolic leaflets of cellular membranes. A key function of PIPs is to serve as membrane identity markers, where they engage numerous types of PIP effector domains. Pleckstrin homology (PH) domain-containing proteins are the largest family of such “readers” of the PIP-based code of membrane identity, wherein PIP binding to PH domains controls subcellular location and protein activity toward downstream signaling events. PH domains often appear in multidomain proteins, linked to enzymatic or protein-protein interaction domains that can propagate signal transduction. Yet, there is a limited understanding of how all but a handful of the >250 PH domain-containing proteins transduce membrane recognition to downstream signaling.

This dissertation describes the biomolecular characterization and protein engineering of an understudied, microtubule- and plasma membrane-localized, PH domain-containing protein, PLEKHA5 (Pleckstrin homology domain-containing family A, member 5). At the microtubule network, PLEKHA5 functions as an adaptor of an E3 ubiquitin ligase, the Anaphase Promoting Complex/Cyclosome (APC/C), to

regulate the spatial organization of APC/C activity. Microtubule-localized proximity biotinylation tools reveal that PLEKHA5 promotes APC/C's subcellular localization to microtubules and facilitates its activation, thus ensuring the timely turnover of key mitotic APC/C substrates and proper progression through mitosis. Although PLEKHA5 predominately localizes to the microtubule cytoskeleton in interphase, a portion of the protein translocate to the plasma membrane (PM) via interaction with PI(4,5)P₂ specifically during mitosis, and the membrane association of PLEKHA5 is regulated by the phosphorylation status of a single serine near its PH domain. Harnessing this dynamic PM binding capability of PLEKHA5, we utilize protein engineering to develop a reversible, mitosis-specific protein recruitment platform at the PM we termed MARS (Mitosis-enabled Anchor-away/Recruiter System). We successfully apply MARS to recruit functional enzymes to generate signaling molecules at the PM in mitosis. In summary, the work presented in this dissertation highlights a link between the spatial organization of APC/C and the cellular functions of PLEKHA5 as a microtubule adaptor protein and develops a perturbation-free platform for conditional protein translocation capitalizing on the mitosis-specific plasma membrane localization of PLEKHA5.

BIOGRAPHICAL SKETCH

Xiaofu Cao was born and raised in Guangzhou, China. Xiaofu's curiosity towards science was sparked by bed-time stories about wonderful scientists and their inventions, and her interest in life sciences was further cultivated when she attended middle and high school at the Affiliated High School of South China Normal University. After high school, Xiaofu came to the United States for undergraduate education at Boston College as a biochemistry major. During sophomore year, Xiaofu joined the laboratory of Prof. Abhishek Chatterjee as an undergraduate research student, where she was first introduced to chemical biology research. In the Chatterjee lab, Xiaofu was mentored by Dr. Rachel Kelemen and learned how to functionalize Adeno-associated viruses with unnatural amino acids for the development of improved gene therapy vectors. Xiaofu graduated from Boston College in May 2017 with a BS degree and received the McCarthy Prize for natural science for her senior thesis, titled "Precise chemical modification of individual capsid proteins of Adeno-associated virus to understand and engineer cell-entry". After that, Xiaofu moved to Cornell University to obtain her graduate training under the guidance of Prof. Jeremy M. Baskin in the Department of Chemistry and Chemical Biology and Weill Institute for Cell and Molecular Biology, where she continued her research interest in Chemical Biology, but also learned new knowledge and skills in Cell Biology. In the Baskin lab, Xiaofu focused on elucidating the cellular functions and molecular mechanisms of a poorly characterized lipid-binding protein called PLEKHA5 and using protein engineering to develop a mitosis-specific plasma membrane protein recruiter based on PLEKHA5.

This dissertation is dedicated to my father, Mr. Huichao Cao, who was my first
mentor in science.

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I thank my committee members, Professor Fenghua Hu and Professor Richard A. Cerione, for their mentorship and support during my graduate training and for my future endeavors. I want to thank Professor Marcus B. Smolka, Ethan and Mateusz for their collaboration and great help in proteomic experiments. I also want to acknowledge my undergraduate advisor Professor Abhishek Chatterjee at Boston College for inspiring me to pursue a graduate degree to advance my research career and continuing to provide support during my time at Cornell.

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generations of Baskin lab members – Dr. Jessica Daughtry, Dr. Timothy Bumpus, Dr. Alex Batrouni, Dr. Dongjun Liang, and Dr. Reika Tei for creating and shaping an amazing lab culture before and when I started, and for giving me advises both in and outside of lab. I also want to thank Dr. Hongyan Sun and Shiyang Huang for being my sounding boards in lab. I want to acknowledge Dr. Brittany White, Dr. Masaaki Uematsu, and all the Baskin lab members for providing valuable intellectual inputs on the progress of my research. Additionally, I want to thank all the Weill Institute for Cell and Molecular Biology members and my cohorts at the Department of Chemistry and Chemical Biology who I have gotten to know over the years. I will cherish the great friendships forged over the years.

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For a complete list of hits from the SILAC MS proteomics experiment to determine the PLEKHA5 interactome, please refer to Table S1 in **Cao, X. et al. *ACS Chem Bio* (2022)**..... 113

LIST OF ABBREVIATIONS

ANAPC3	Anaphse Promoting Complex subunit 3
ANAPC4	Anaphse Promoting Complex subunit 4
ANAPC5	Anaphse Promoting Complex subunit 5
ANAPC6	Anaphse Promoting Complex subunit 6
ANAPC8	Anaphse Promoting Complex subunit 8
APC/C	Anaphse Promoting Complex/Cyclosome
APEX	Ascorbate peroxidase
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid Assay
BP	Basic Peptide
BSA	Bovine Serum Albumin
BTB	Broad-Complex, Tramtrack and Bric a brac domain
CC	Coiled coil domain
CD44	Cell Surface Glycoprotein CD44
CDC20	Cell Division Cycle protein 20
CDH1	CDC20 Homolog 1
CDK1	Cyclin depedent kinase 1
CK1	Casein Kinase 1
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats

CUL3	Cullin-3
DAPI	4',6-diamidino-2-phenylindole
DCX	doublecortin
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DTB	Double thymidine block
DTT	Dithiothreitol
DVL	Dishevelled
E. coli	Escherichia coli
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EDTA	Ethylenediaminetetraacetic Acid
EEA1	Early Endosome Antigen 1
EF1 α	Human elongation factor-1 alpha
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EMTB	Microtubule-binding domain of Ensin/Map7
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum

FIPI	5-gluoro-2-indolyl des-chlorohalopemide
FL	Full Length
FUCCI	Fluorescent Ubiquitination-based Cell Cycle Indicator
FYVE	Fab-1, YGL023, Vps27, and EEA1
G1	Gap 1 phase/Growth 1 phase
G2	Gap 2 phase/Growth 2 phase
GaAsP PMT	Gallium Arsenide Phosphide Photo Multiplier Tube
GAPDH	Glyceraldehyde phosphate dehydrogenase
GFP	Green Fluorescent Protein
H	Amphipathic Helix
H2B	Histone H2B
H3	Histone H3
HA tag	Human influenza hemagglutinin tag
HEK	Human Embryonic Kidney cells
HRP	Horseradish peroxidase
IDR	Intrinsically disordered region
IDT	Integrated DNA Technologies
IF	Immunofluorescence
IMPACT	Imaging Phospholipase D Activity with Clickable Alcohols via Transphosphatidylation
IP	Immunoprecipitation
IPTG	Isopropyl β -D-1-thiogalactopyranoside

iSH2	inter-Src homology 2
KLHL12	Kelch-like protein 12
Lyn10	First 10 residues in the Lyn kinase
M1R	Muscarinic Receptor
MAP4	Microtubule Associated Protein 4
MARS	Mitosis-enabled Anchor-away/Recruiter System
mCh	mCherry protein
MCS	Multiple cloning site
miRFP	monomeric Infrared Fluorescent Protein
MS ²	Tandem mass spectrometry
mSi	mCarlet-i protein
MT	Microtubule
n.s.	not significant
NEBD	Nuclear Envelope Breakdown
NES	Nuclear export signal
nGFP	GFP nanobody
OD600	Optical Density at 600 nm
ORF	Open Reading Frame
Oxo-M	Oxotremorine M
P/S	Penicillin/Streptomycin
PA	Phosphatidic acid
PAX2	Paired box gene 2

PBS	Phosphate buffered saline
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDZD11	PDZ domain-containing protein 11
PH	Pleckstrin Homology
PI	Phosphatidylinositol
PI(3,4,5)P ₃ /PIP ₃	Phosphatidylinositol 3,4,5-trisphosphate
PI(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphoinositide 3-kinase
PIPs	Phosphoinositides
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PLEKHA4	Pleckstrin Homology domain containing family A member 4
PLEKHA5	Pleckstrin Homology domain containing family A member 5
PLEKHA7	Pleckstrin Homology domain containing family A member 7
PLK1	Polo-like kinase 1

PM	Plasma membrane
PMA	Phorbol 12-Myristate 13-Acetate
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PP4	Protein Phosphatase 4
PPP	Phosphoprotein Phosphatase
PRD	Proline-Rich Domain
PX	Phox Homology
RING	Really Interesting New Gene
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
sh/ siRNA	short hairpin/ small interfering RNA
SILAC	Stable Isotope Labeling by Amino acids in Cell culture
STLC	S-trityl-L-cysteine
T/H buffer	Tyrode's-HEPES buffer
TB	Terrific Broth
Ub	Ubiquitin
UBE1	Ubiquitin activating enzyme E1
UBE2C	Ubiquitin conjugating enzyme E2 C
UBE2S	Ubiquitin conjugating enzyme E2 S
V5	V proteins of the paramyxovirus of simian virus 5
VSVg	Vesicular Stomatitis Virus Glycoprotein

WB

Western Blot

WT

Wild-type

LIST OF SYMBOLS

α	Alpha
β	Beta
δ	Delta
μ	Micro
Δ	Delta (deletion)
$>$	More than

CHAPTER 1

INTRODUCTION

Lipids are commonly considered as excellent energy sources and critical components for compartmentalization like different organelle membranes in cells. Beyond these well-known cellular functions, lipids are also versatile signaling moieties, existing as essential mediators in almost all branches of cellular signaling cascades¹. Lipids can be classified based on their structural diversities. Phospholipids harbor a characteristic hydrophilic, phosphate-containing head group and two hydrophobic acyl tails connected by the glycerol backbone². Abundant phospholipids in mammalian cell membranes include phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, which are generated by modifying the phosphate headgroup with metabolites choline, ethanolamine, and serine³.

1.1 Phosphoinositides

Phosphoinositides (PIPs) are low-abundant yet essential phospholipids in eukaryotic cells, encompassing about only 0.2-1% of total phospholipids⁴. There are seven naturally occurring PIP species generated via reversible phosphorylation of the *myo*-inositol headgroup of phosphatidylinositol (PI) at the 3, 4, and 5 hydroxy positions (Figure 1.1A)⁵. Different PIP species can be interconverted to one another through selective PIP-kinases and PIP-phosphatases, and they localize in the cytoplasmic leaflets of specific organelle membranes and the plasma membrane (PM) to serve as define membrane markers (Figure 1.1B)^{6,7}.

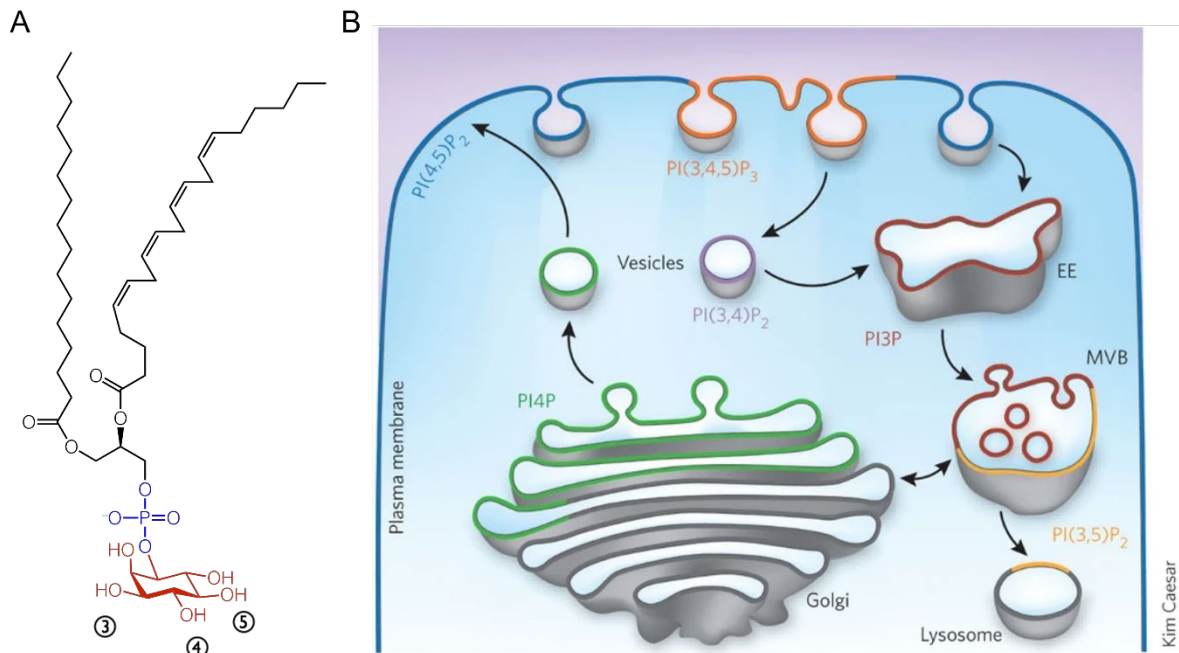


Figure 1.1. Structure of phosphatidylinositol (PI) and subcellular localizations of different phosphoinositides. (A) Phosphatidylinositol contains a myo-inositol headgroup (red) and two fatty acid tails (black) connected by the phospho-glycerol backbone. (B) Distribution of different phosphoinositides inside the cell. PI3P: phosphatidylinositol 3-phosphate; PI4P: phosphatidylinositol 4-phosphate; PI(3,4)P₂: phosphatidylinositol 3, 4-bisphosphate; PI(3,5)P₂: phosphatidylinositol 3, 5-bisphosphate; PI(4,5)P₂: phosphatidylinositol 4, 5-bisphosphate; PI(3,4,5)P₃: phosphatidylinositol 3, 4, 5-triphosphate. (Figure 1.1B re-printed with permission from reference 6).

1.2 Pleckstrin Homology Domain-containing Proteins as Important Effectors for PIPs Cellular Functions

In addition to forming a PIP-based code for membrane identities, PIPs are also universal signaling molecules that participate in the regulation of a diverse array of cellular processes, including modulating membrane dynamics⁸, controlling vesicular trafficking⁹, remodeling cytoskeletal structures¹⁰, adjusting cellular metabolic programs¹¹, and affecting cell migration and division¹². PIPs achieve their regulative roles through direct interaction with integral membrane proteins, or through

membrane recruitment of cellular “reader” proteins (effectors) that contain PIP-binding domains^{13,14}. Multiple families of protein domains have been characterized to bind PIPs, including Pleckstrin Homology (PH), Phox Homology (PX), FYVE, and C2 domains (Figure 1.2).

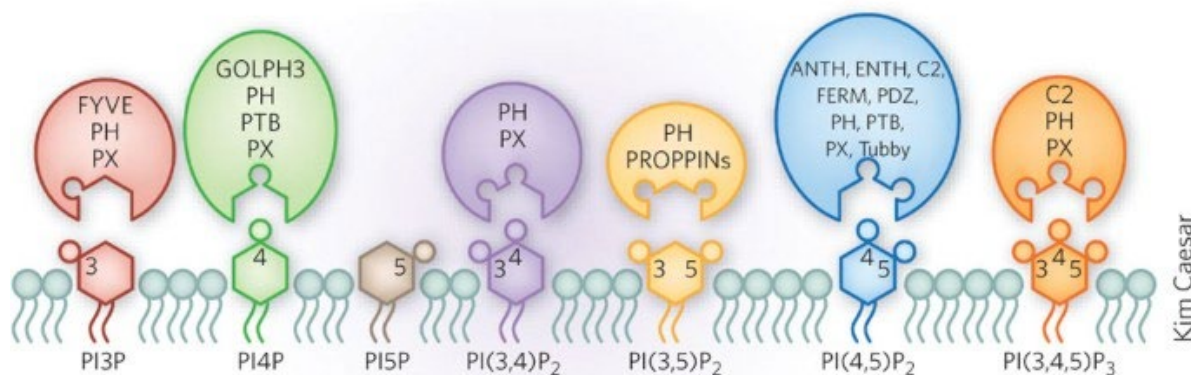


Figure 1.2. Protein domains interacting with PIPs. Different signaling domains and their target PIPs. Some of these domains can bind to more than one type of PIPs due to structural diversity, such the PH domains.

Among these PIP-interacting domains, the PH domains comprise the 11th most abundant protein domains in the human proteome¹⁵. PH domains often appear in multidomain proteins, linked to enzymatic or protein-protein interaction domains that can propagate signal transduction from lipid binding. However, despite detailed characterization of a handful of well-studied PH domain-containing proteins, many biological discoveries remain to be made for the rest of the members within this family of over 250 proteins¹⁶.

The Baskin lab has been interested in exploring the roles of how putative phosphoinositide-binding proteins might link lipid binding to the regulation of cell

signaling. Toward this effort, we have centered on poorly characterized members of the PLEKHA subfamily of PH domain-containing proteins. Several members of the PLEKHA subfamily function as adaptor proteins that can regulate the subcellular localizations and thus biological functions of other proteins or protein complexes. For example, PLEKHA7 localizes to adherent junctions and recruits PDZD11 (PDZ domain-containing protein 11) to these junctions¹⁷. The PLEKHA7–PDZD11 complex can stabilize the plasma membrane localization and modulate the functions of multiple transmembrane proteins, including nectins for junctional integrity and the Menkes copper ATPase and the plasma membrane calcium ATPase to maintain intracellular ion homeostasis^{17–19}. Furthermore, we recently discovered a new nexus between PIPs and the control of ubiquitination pathway and Wnt signaling through PLEKHA4²⁰. We showed that PLEKHA4 binds to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and functions as a plasma membrane adaptor to sequester the Cullin-3 E3 ubiquitin ligase adaptor KLHL12, thus elevating Dishevelled levels and enhancing the strength of Wnt signaling pathways in Wnt-receiving cells. In the context of melanoma cells, the enhanced Wnt signaling activity by PLEKHA4 promotes tumor proliferation²¹. The PLEKHA4 paralog PLEKHA5 shares a similar multidomain architecture (Figure 1.3) and has been linked to proliferation and metastasis in several disease models^{22–24}, although underlying mechanisms remained unknown, prompting us to investigate its molecular properties and cellular functions.

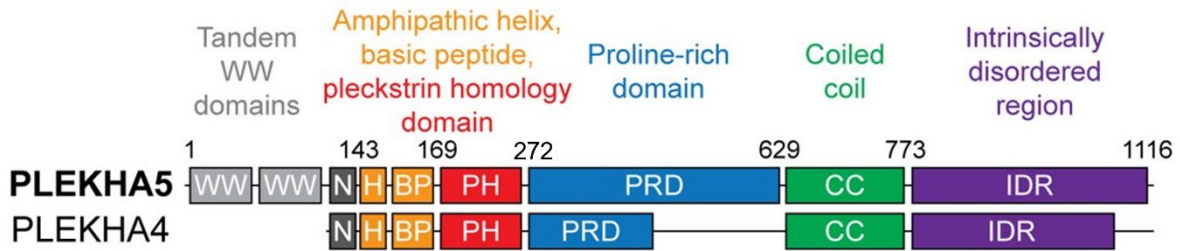


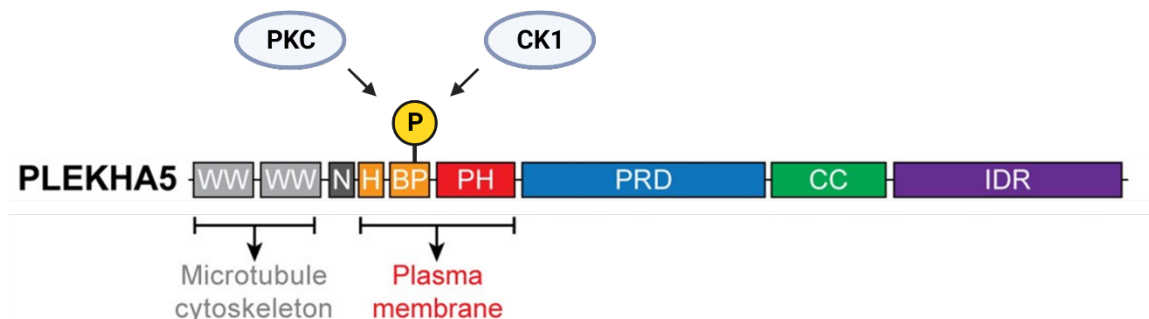
Figure 1.3. Domain architecture of PLEKHA4 and PLEKHA5.

In this dissertation, I will summarize the phenotypic characterizations and mechanistic investigations of PLEKHA5 facilitated by the development of microtubule-targeting proximity labeling tools and describe the design and engineering of a new mitosis-specific plasma membrane protein recruiter harnessing the cellular behavior of PLEKHA5.

1.3 Chapter Overviews

Chapter 2. Investigation of the subcellular localizations of PLEKHA5: Chapter 2 characterizes the dynamic subcellular localizations of PLEKHA5 and investigates the molecular mechanism regulating such behavior. We report that PLEKHA5 localizes to the microtubule network throughout the cell cycle via its N-terminal tandem WW-domain, and the protein interacts with the plasma membrane (PM) specifically during mitosis by binding to PI(4,5)P₂ via the H-BP-PH motif. The membrane association of PLEKHA5 is regulated by the phosphorylation status of a single serine residue located adjacent to the PLEKHA5 PH domain. Further, modulation of protein kinase

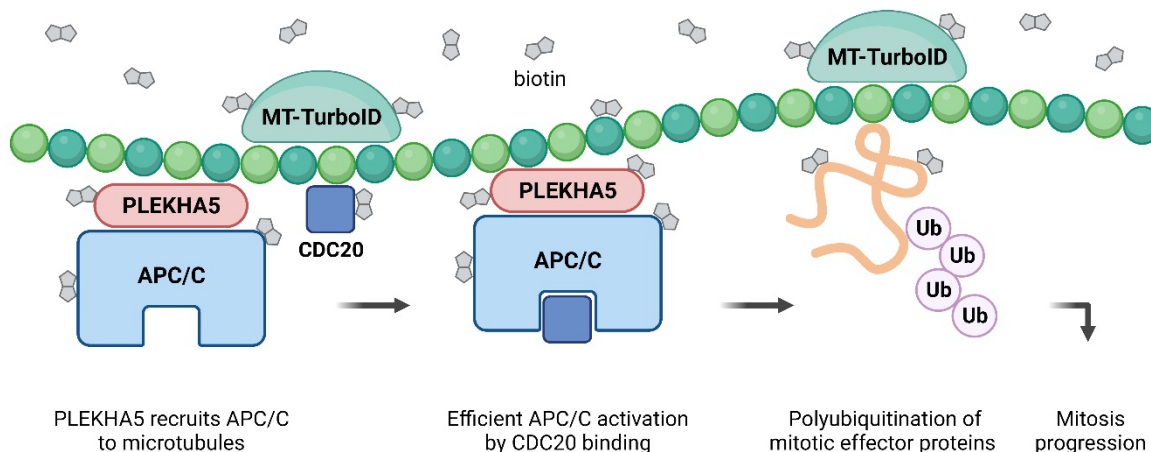
C and casein kinase 1 activity suggests roles for both of these kinases in this phosphorylation.



Chapter 2 author contributions: Xiaofu Cao carried out experiments, analyzed data, made the figures, and wrote the manuscript. Mateusz M. Wagner performed the phosphoproteomics experiment and data analysis, made Figure 2.3C (MS² spectrum of the phosphopeptide), and edited the manuscript. Marcus B. Smolka revised the manuscript, acquired funding, and provided valuable intellectual input. Jeremy M. Baskin supervised the project, wrote and edited the manuscript and figures, and provided valuable intellectual input.

Chapter 3. PLEKHA5 functions as a microtubule adaptor for the spatial regulation of APC/C activity: Chapter 3 investigates the cellular functions of PLEKHA5 at the microtubules in modulating the E3 ligase activity of the Anaphase Promoting Complex/Cyclosome (APC/C) and regulating mitosis progression. We identified APC/C as a protein interacting partner of PLEKHA5 by immunoprecipitation followed by mass spectrometry. Utilizing microtubule-targeted TurboID-based proximity biotinylation, we demonstrated that PLEKHA5 recruits a pool of APC/C to the microtubule network to facilitate efficient APC/C activation by its co-activator

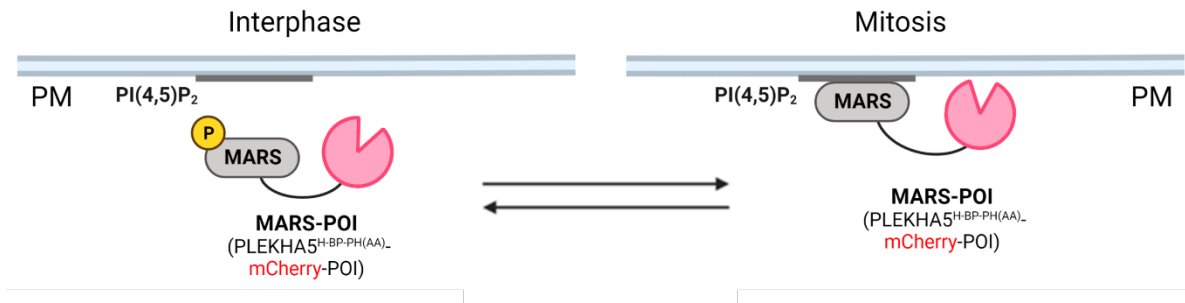
CDC20 and promote timely polyubiquitination and proteasomal degradation of APC/C substrates, therefore ensuring proper mitosis progression.



Chapter 3 author contributions: Xiaofu Cao carried out experiments, analyzed data, made the figures, and wrote the manuscript. Adnan Shami Shah tested conditions and purified proteins for the in vitro ubiquitination assay, edited the manuscript, and offered valuable intellectual input. Ethan J. Sanford performed the SILAC proteomics experiment and data analysis, generated Figure 3.1B (volcano plot for all the hits from the MS experiment), and edited the manuscript. Marcus B. Smolka revised the manuscript and provided valuable intellectual input. Jeremy M. Baskin supervised the project, wrote and edited the manuscript and figures, and provided valuable intellectual input.

Chapter 4. Development of a reversible, mitosis-specific plasma membrane protein recruiter: Chapter 4 outlines the design and engineering of the Mitosis-enabled Anchor-away/Recruiter System (MARS) at the plasma membrane. We harnessed the dynamic plasma membrane binding capability of PLEKHA5 to develop

a reversible, perturbation-free protein recruitment platform at the plasma membrane to regulate signal transduction using mitosis as an endogenous switch.



Chapter 4 author contributions: Xiaofu Cao carried out experiments, analyzed data, made the figures, and wrote the manuscript. Jeremy M. Baskin supervised the project, wrote and revised the manuscript and figures, and provided valuable intellectual input.

Chapter 5. Conclusions and Outlook: Chapter 5 summarizes the results presented in this dissertation and discusses future studies of PLEKHA5. We have started to explore additional functions of PLEKHA5 in more broadly regulating ubiquitination pathways through its interaction with two Cullin-RING E3 ligase substrate adaptors, KCTD3 and SHKBP1.

Chapter 5 author contributions: Xiaofu Cao, Lin Luan, Jingyi Hu, and Shun Enomoto performed experiments and analyzed data. Xiaofu Cao made the figures and wrote the chapter. Hongyan Sun helped design experiments and offered valuable intellectual input. Jeremy M. Baskin supervised the project, revised the chapter, and provided valuable intellectual input.

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CHAPTER 2

INVESTIGATION OF THE SUBCELLULAR LOCALIZATIONS OF PLEKHA5

Adapted in part with permission from:

“Proximity Labeling Reveals Spatial Regulation of the Anaphase-Promoting Complex/Cyclosome by a Microtubule Adaptor”

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2.1 INTRODUCTION

Phosphoinositide (PIP) lipids are anionic phospholipids localized in cytosolic leaflets of organelle membranes and the plasma membrane (PM)^{1,2}. The seven naturally occurring PIP species reside in the cytosolic leaflets of different membranes and form a PIP-based code of membrane identity, which can be deciphered by numerous types of PIP effector domains^{2,3}. Pleckstrin Homology (PH) domain-containing proteins are the largest family of “readers” for such PIP-based code, wherein PIP binding to PH domains controls subcellular location and protein activity toward downstream signaling events^{4–6}. Yet, there is a limited understanding of how all but a handful of the >250 PH domain-containing proteins transduce membrane recognition to downstream signaling⁷. PLEKHA5 (Figure 2.1A) belongs to the

PLEKHA4–7 protein family, members of which serve as intracellular adaptor proteins to promote specific subcellular localization of their interacting proteins to carry out various functions^{8,9}. PLEKHA5 is critical for proliferation and invasiveness of melanoma cells with a high propensity for brain metastasis¹⁰. Other studies have implicated PLEKHA5 in the regulation of malignant progression in diffuse-type gastric carcinoma and breast cancer metastasis^{11,12}. These reports underscore the importance of elucidating the fundamental molecular and cellular functions of PLEKHA5.

2.2 RESULTS

2.2.1 PLEKHA5 Localizes to Microtubules via the Tandem WW Domains and to the Plasma Membrane via the H-BP-PH Motif

We first examined the cellular localization of PLEKHA5. Unlike PLEKHA4, which localizes to the plasma membrane⁸, endogenous PLEKHA5 was detected predominantly along the microtubule network in HeLa cells by immunofluorescence (Figures 2.1B and Appendix Figure A1a) and imaging of green fluorescent protein (GFP)-tagged full-length PLEKHA5 (EGFP-PLEKHA5^{FL}, Figure 2.1C and Appendix Figure A1b). As a member of the WW-PLEKHA subfamily of proteins¹³, PLEKHA5 contains two N-terminal WW domains (residues 1-105) absent from PLEKHA4 (Figure 1.3). We hypothesized that the microtubule localization of PLEKHA5 might be mediated by its tandem WW domains. Indeed, we found that an EGFP-tagged PLEKHA5 construct lacking the two WW domains failed to localize to microtubules and an EGFP fusion to the tandem WW domains localized to microtubules similar to

the full-length protein Figure 2.1C). These data indicate that the tandem WW domain is necessary and sufficient to recruit PLEKHA5 to the microtubule network. These observations are consistent with recent studies demonstrating that the tandem WW domain of PLEKHA5 associates with PDZD11 and that this interaction was crucial for PLEKHA5 to localize to the microtubule network¹⁴.

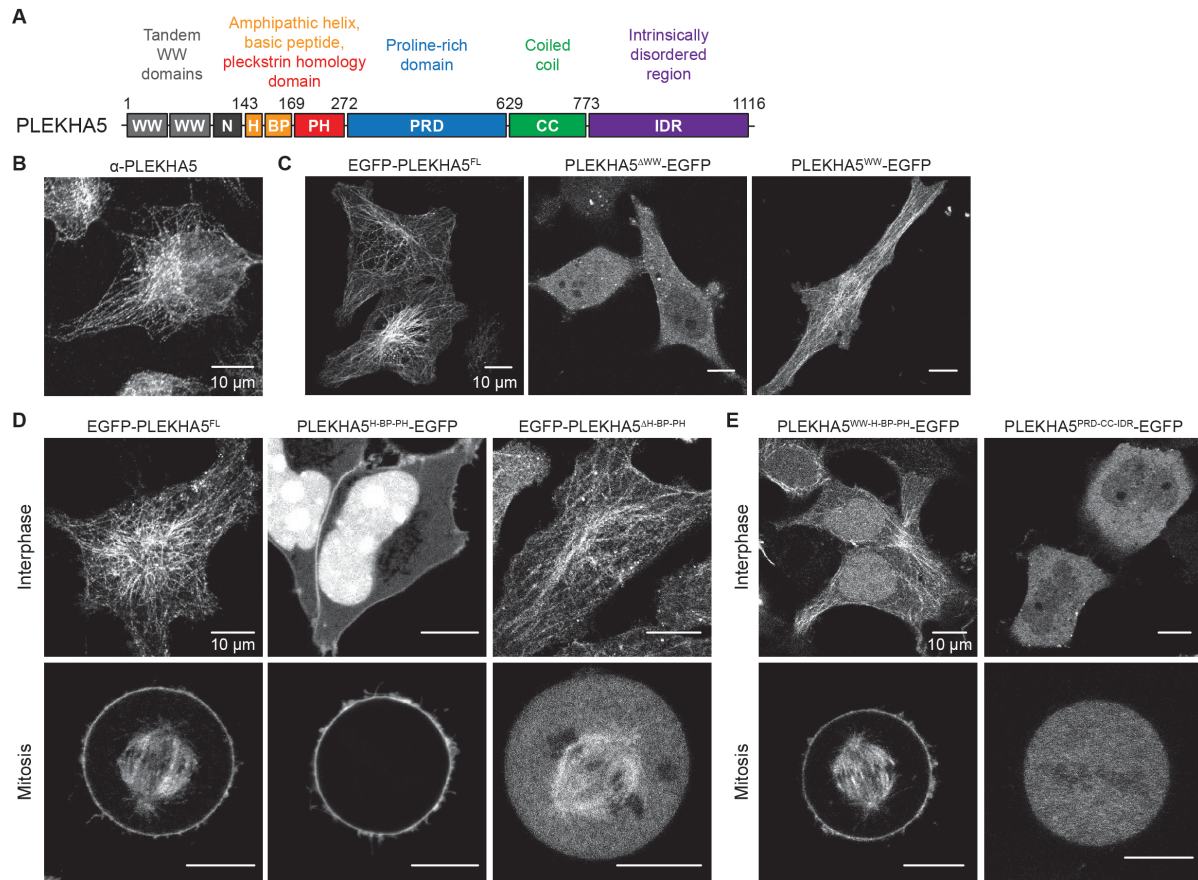


Figure 2.1. PLEKHA5 exhibits microtubule and plasma membrane localizations. (A) Domain map of PLEKHA5. (B) Immunofluorescence (IF) of endogenous PLEKHA5 in HeLa cells by confocal microscopy. (C-E) Confocal microscopy of HeLa cells transfected with different GFP-tagged PLEKHA5 constructs during interphase and mitosis. The name of each construct imaged is displayed on top of the image panel.

Upon further analysis, we found that although the protein predominantly localizes to the microtubules during interphase, a substantial pool of EGFP-tagged PLEKHA5 translocate to the PM during mitosis (Figure 2.1D). Downstream of the tandem WW domains, PLEKHA5 contains a putative membrane binding motif (residues 143-271) consisting of an amphipathic helix (H), a basic peptide (BP) enriched with positively charged lysine and arginine residues, and a pleckstrin homology (PH) domain (Figure 2.1A). This motif (H-BP-PH) shares homology with a motif in the PLEKHA5 paralog PLEKHA4 (Figure 1.3), which localizes to the PM via binding to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)⁸.

Indeed, GFP-tagged PLEKHA5^{H-BP-PH} exhibited constitutive PM localization during interphase and mitosis, whereas a PLEKHA5 construct lacking this motif, GFP-PLEKHA5^{ΔH-BP-PH}, showed no PM interaction (Figure 2.1D), demonstrating that the H-BP-PH motif is sufficient and necessary to recruit PLEKHA5 to the PM. Further, the longer N-terminal fragment WW-H-BP-PH exhibited similar localizations as the full-length protein, whereas the PRD-CC-IDR fragment remained in the cytosol, establishing the minimal motifs required for the dual localizations of PLEKHA5 during the cell cycle (Figure 2.1E). Extended time-lapse imaging of asynchronous HeLa cells expressing PLEKHA5^{WW-H-BP-PH}-GFP and H2B-mCherry (Appendix Figure A1c) revealed the first appearance of PM localization at the stage of nuclear envelope breakdown (NEBD), with PM fluorescence peaking in metaphase and then decreasing starting in anaphase and terminating upon completion of cytokinesis, accompanying chromosome decondensation.

To assess the PM localization of endogenous PLEKHA5 during mitosis, we expressed a PM-targeted Lyn₁₀-TurboID-V5¹⁵ fusion to use proximity biotinylation to tag all proteins at or near the PM, followed by streptavidin agarose enrichment and western blot analysis to probe for endogenous PLEKHA5 in the enriched PM-proximal proteome (Appendix Figure A2a). Immunofluorescence microscopy and western blotting demonstrated the correct subcellular localization and PM-specific biotinylation activity of Lyn₁₀-TurboID-V5 (Appendix Figure A2b, c). Indeed, levels of PLEKHA5 biotinylation were higher in prometaphase-synchronized cells relative to asynchronous cells, indicating that endogenous PLEKHA5 also exhibits cell cycle-dependent changes to its PM localization (Figure 2.2A).

2.2.2 PLEKHA5 Localizes to the Plasma Membrane via PI(4,5)P₂ Binding

Previous work from our lab reported that the H-BP-PH domain of PLEKHA4 directs its PM localization through interactions with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)⁸. The PI(4,5)P₂ binding and PM association of PLEKHA4 was abolished by alanine mutation of either one of two arginine residues, R75 and R129, in the PH domain predicted to be critical for contacting the PIP headgroup^{8,16}. According to amino acid sequence alignment of the PLEKHA4 and PLEKHA5 PH domains, these two key arginine residues correspond to R190 and R244 in PLEKHA5 (Appendix Figure A2d).

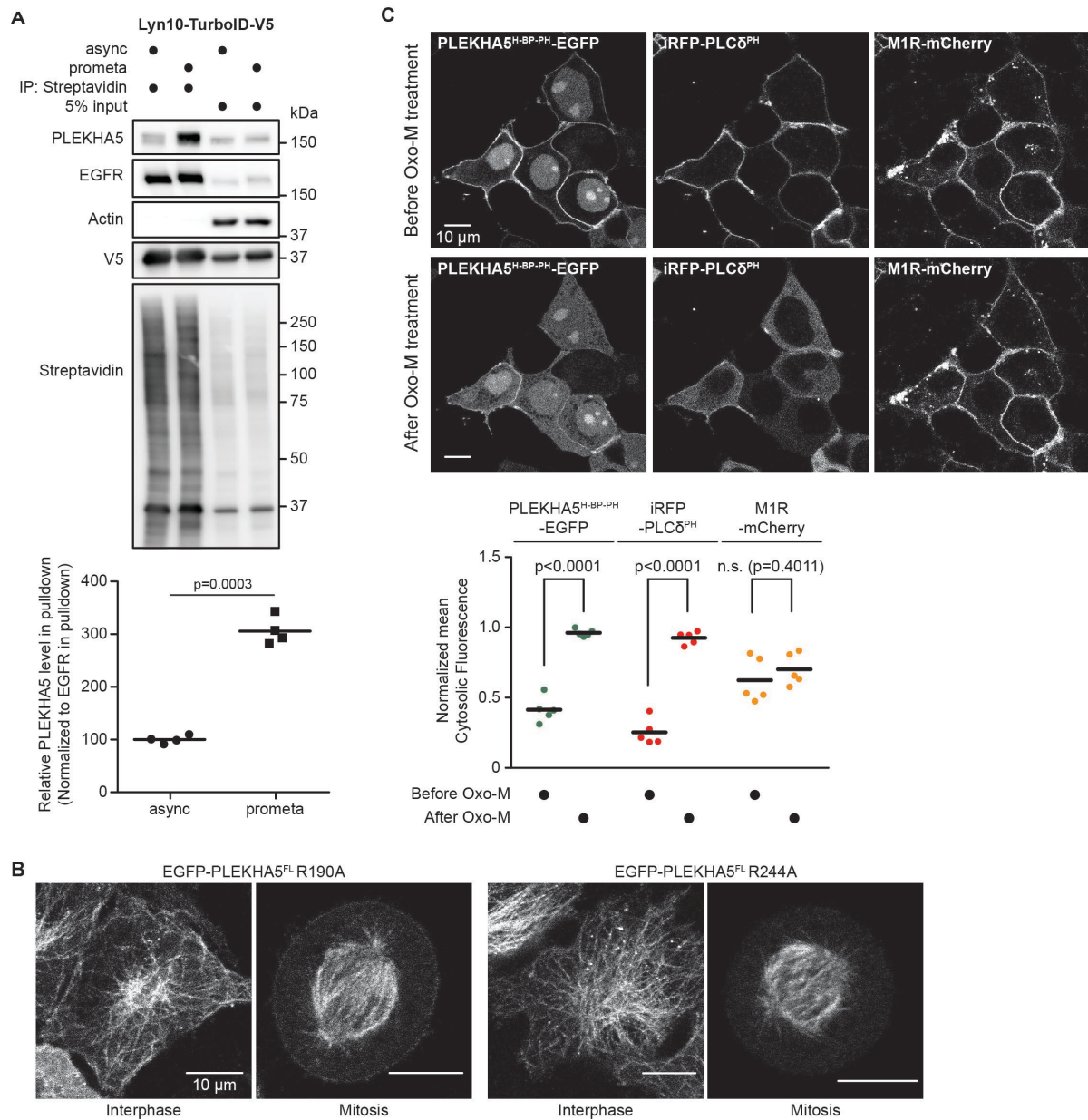


Figure 2.2. PLEKHA5 binds to PI(4,5)P₂ at the plasma membrane. (A) A larger portion of endogenous PLEKHA5 was biotinylated by the PM-targeted TurboID construct in isolated prometaphase cells than in the asynchronous populations. Representative western blots are shown at top with quantifications at bottom ($n=4$ biological replicates, Student's t-test). (B) Confocal microscopy of PI(4,5)P₂ binding mutants (R190A and R244A) of EGFP-PLEKHA5^{FL}. (C) Acute PI(4,5)P₂ depletion assay in cells expressing M1R-mCherry, the PI(4,5)P₂ reporter iRFP-PLC δ^{PH} , and PLEKHA5^{H-BP-PH}-GFP reveals the PI(4,5)P₂-binding capacity of PLEKHA5^{H-BP-PH}-GFP. Shown are representative micrographs and quantification of normalized mean fluorescence intensity in the cytosol before and after treatment with the M1R agonist oxotremorine-M (Oxo-M) ($n=5$ cells, Student's t-test).

Upon R190A and R244A mutation, the PM localization of GFP fusion to the full-length protein, the WW-H-BP-PH fragment, and the H-BP-PH motif were largely abolished (Figure 2.2B and Appendix Figure A2e). For the H-BP-PH motif, the fluorescence signal from the mitotic spindle, the chromosomes, and to a partial extent, the plasma membrane was potentially due to non-specific electrostatic interactions of the enriched positively charged residues and these negatively charged cellular structures. We further tested the requirement of PI(4,5)P₂ in PM interaction of PLEKHA5 using a PI(4,5)P₂ depletion assay (Figure 2.2C). Depletion of PI(4,5)P₂ from the PM resulted in the loss of PM-localized PLEKHA5^{H-BP-PH}-GFP, indicating that the H-BP-PH motif associates with the PM by binding to PI(4,5)P₂.

2.2.3 The PM Localization of PLEKHA5 is Regulated by the Phosphorylation Status of S161

Intrigued by the cell cycle-dependent PM interaction of PLEKHA5, we set out to investigate the underlying molecular mechanism governing the dynamic PM localization of this protein. Because PI(4,5)P₂ levels do not increase during mitosis¹⁷, we hypothesized that posttranslational modifications to PLEKHA5 might regulate its localization, in particular phosphorylation^{18,19}. Therefore, we generated phosphodeficient and phosphomimetic mutations of nine residues that were adjacent to the PH domain and reported to be phosphorylated in PhosphoSitePlus²⁰ (Figure 2.3A), within both GFP-PLEKHA5^{FL} and PLEKHA5^{WW-H-BP-PH}-GFP. Mutations at eight positions caused no change in localization by confocal microscopy (Appendix Figure A3). Strikingly, however, mutation of S161 strongly

affected the subcellular localization. The S161A phosphodeficient mutants of both GFP- $\text{PLEKHA5}^{\text{FL}}$ and $\text{PLEKHA5}^{\text{WW-H-BP-PH}}$ -GFP displayed increased PM association during interphase, whereas the S161D phosphomimetic mutants exhibited no PM localization during mitosis (Appendix Figure 4a-d). We also used TurboID-based proximity labeling of the PM-associated proteome to confirm the changes in localization observed by microscopy (Appendix Figure A4e, f). The S161 mutations were also incorporated into the $\text{PLEKHA5}^{\text{H-BP-PH}}$ -GFP, and imaging analyses demonstrated the expected changes in PM localization (Figure 2.3B). Because these data indicate that the S161A mutation enhances PM binding and the S161D mutation eliminates such localization, these results suggest that phosphorylation at S161, mimicked by S161D, might prevent the PM localization of PLEKHA5 during interphase and that dephosphorylation during mitosis, mimicked by S161A, might enable PM binding.

First, to confirm the phosphorylation of PLEKHA5 at S161, we performed phosphoproteomics analysis of enriched $\text{PLEKHA5}^{\text{H-BP-PH}}$ -GFP and detected a miscleaved tryptic phosphopeptide with MS² analysis confirming phosphorylation at S161 (Figure 2.3C). We also exploited Phos-tag SDS-PAGE²¹, wherein phosphorylated forms of a given protein migrate more slowly than the non-phosphorylated forms, to interrogate the mechanisms underlying S161 phosphorylation. Analysis of lysates from asynchronous or prometaphase-synchronized cells expressing either wild-type (WT) or S161A forms of $\text{PLEKHA5}^{\text{H-BP-PH}}$ -GFP revealed a loss of a slower-migrating band in the S161A samples that likely correspond to $\text{PLEKHA5}^{\text{H-BP-PH}}$ -GFP phosphorylated at S161 (Figure 2.3D).

The intensity of this band in WT PLEKHA5^{H-BP-PH}-GFP was significantly lower in prometaphase-synchronized compared to asynchronous cells, consistent with the phosphomutant imaging results suggesting that a larger portion of PLEKHA5^{H-BP-PH}-GFP was dephosphorylated during mitosis.

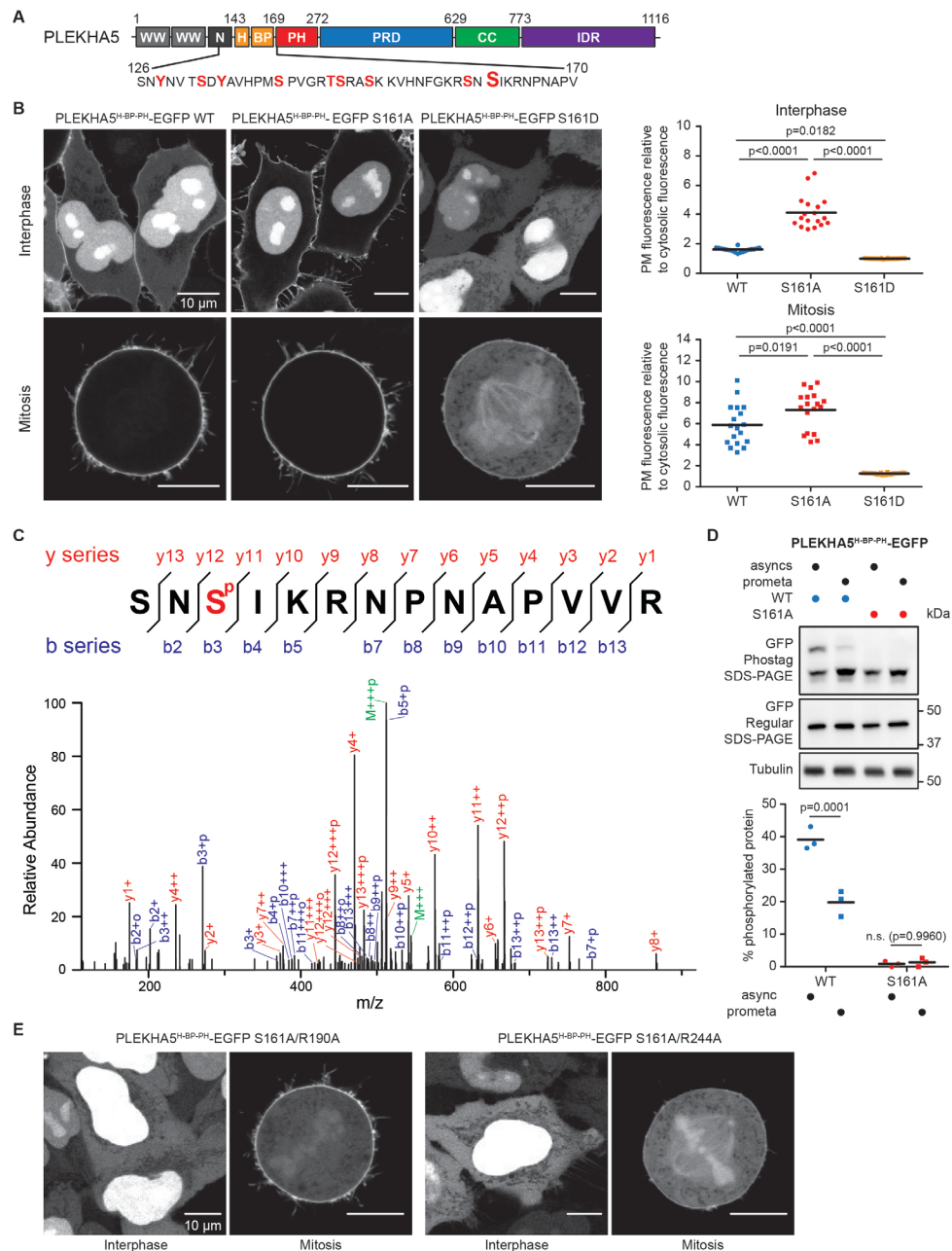


Figure 2.3. PM localization of PLEKHA5 is regulated by S161 phosphorylation. (A) Primary amino acid sequence of the region of PLEKHA5 immediately upstream of the PH domain. Residues highlighted in red were selected for phospho-

mutagenesis analysis. (B) The S161A mutation increased the PM localization of PLEKHA5^{H-BP-PH}-GFP, whereas the S161D mutation diminished the PM association of this construct. Shown at left are representative micrographs and at right the quantification of PM to cytosolic fluorescence ratios for each construct in interphase and mitosis (n=18 cells, one-way ANOVA with Tukey post hoc test). (C) MS² spectrum of phosphoproteomics analysis of GFP immunoprecipitates from asynchronous HeLa cells transfected with PLEKHA5^{H-BP-PH}-GFP. Labeled peaks show fragmented phosphate-containing ions. “o” following the charge descriptor refers to a neutral water loss; “p” refers to water and phosphate loss. “M” marks precursor ions. The sequence of the detected mis-cleaved peptide from tryptic digest with corresponding b and y series ions is shown at top. The S161 site is highlighted in red. (D) Phos-tag and regular SDS-PAGE analysis of lysates harvested from asynchronous or prometaphase-synchronized HeLa cells expressing WT or S161A forms of PLEKHA5^{H-BP-PH}-GFP. Shown are representative western blots (top) and quantification of phosphorylated proteins (bottom) (n=3 biological replicates, one-way ANOVA with Tukey post hoc test). (E) Live-cell imaging of HeLa cells expressing combinational mutant of S161A with either R190A or R244A for PLEKHA5^{H-BP-PH}-GFP illustrating the requirement for PI(4,5)P₂ binding for PM association independent of S161 phosphorylation status.

To further understand how S161 phosphorylation modulates the PM association of PLEKHA5 and MARS, we analyzed the localizations of the PI(4,5)P₂ binding deficient mutants, R190A and R244A, in the S161A background. The S161A mutation did not restore the PM localizations of GFP-PLEKHA5^{FL}, PLEKHA5^{WW-H-BP-PH}-GFP, or the PLEKHA5^{H-BP-PH}-GFP constructs harboring either R190A or R244A (Figure 2.3E, Appendix Figure B5a, b), implying that the S161 phosphorylation switch on the BP requires an intact PI(4,5)P₂-binding PH domain to regulate PM association.

Interestingly, the PLEKHA5 paralog PLEKHA4 exhibits plasma membrane localization in both interphase⁸ and mitosis (Figure 2.4B), and a sequence alignment of the BP motifs from these proteins revealed that human PLEKHA4 contains an alanine at position 46, which corresponds to S161 in human PLEKHA5

(Figure 2.4A). Examination of the localization of A46S and A46D mutants of GFP-
PLEKHA4 revealed that, though the A46S mutant exhibited a similar PM localization
to the WT protein, the A46D mutation abolished the PM localization during both
interphase and mitosis (Figure 2.4B). These results suggest the importance of this
residue in regulating the PM binding of protein members across the PLEKHA sub-
family.

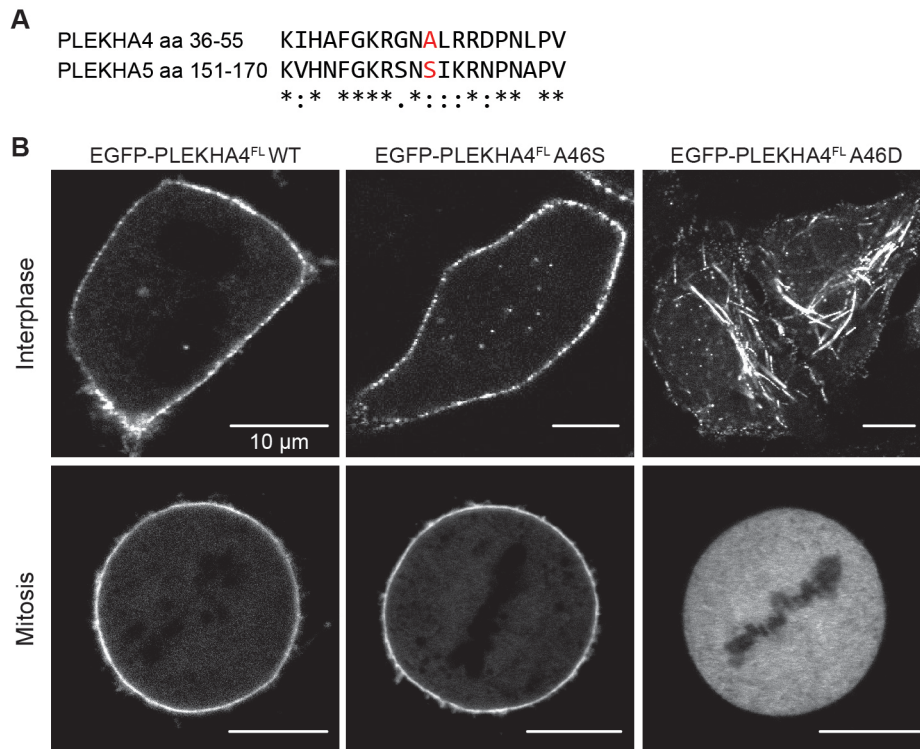


Figure 2.4. PLEKHA4 A46D mutation eliminates its PM localization. (A) Clustal Omega alignment of amino acid sequences around A46 in PLEKHA4 and S161 in PLEKHA5. Symbol “*” indicates a fully conserved residue, symbol “:” indicates conservation of strongly similar properties, and symbol “.” indicates conservation of weakly similar properties. (B) Confocal microscopy of HeLa cells transfected with either the WT, A46S, or A46D forms of full-length PLEKHA4.

2.2.4 PKC and CK1 Kinases Mediate PLEKHA5 S161 Phosphorylation

Next, we sought to determine the kinases responsible for PLEKHA5 S161 phosphorylation. According to a kinase/phosphosite prediction tool²², several protein kinase C (PKC) isoforms were among the top predicted kinases for recognizing the linear amino acid sequence around S161, prompting us to examine the PKC family (Figure 2.5A). We first treated cells expressing either the WT or S161A forms of PLEKHA5^{H-BP-PH}-GFP with one of two PKC agonists, phorbol 12-myristate 13-acetate (PMA) or Bryostatin 1, or a DMSO control, with or without pretreatment with a pan-PKC inhibitor cocktail (PKCi). Subsequent Phos-tag SDS-PAGE analysis revealed that treatment with either PKC agonist significantly increased the intensity of the slower-migrating, phospho-S161 band, and that this effect was eliminated in the presence of PKC inhibition (Figure 2.5B). Furthermore, live-cell, time-lapse imaging of both PLEKHA5^{H-BP-PH}-GFP and PLEKHA5^{WW-H-BP-PH}-GFP upon PKC activation revealed that addition of PMA or Bryostatin 1 induced a loss of PM localization within 25 minutes, an effect that was abolished upon pretreatment of PKC inhibitors or by using S161A mutants (Figure 2.5C, D and Appendix Figure A6a, b).

Though phosphodeficient/mimetic mutants of the nearby S159 did not affect PLEKHA5 PM localization, this residue is reported to be phosphorylated²⁰, and a different set of kinases were predicted to mediated S161 phosphorylation in the presence of phospho-S159, notably several casein kinase 1 (CK1) isoforms²² (Appendix Figure A6c). Indeed, Phostag SDS-PAGE analysis revealed that CK1 inhibition significantly decreased the level of S161 phosphorylation of PLEKHA5^{H-BP-}

^{PH}-GFP, and combinational treatment with both CK1 and PKC inhibitors eliminated the majority of S161 phosphorylation (Appendix Figure A6d). Collectively, these data point to the PKC and CK1 kinase families as important mediators of PLEKHA5 and MARS S161 phosphorylation during interphase and imply that such phosphorylation activity toward PLEKHA5 S161 is reduced during mitosis.

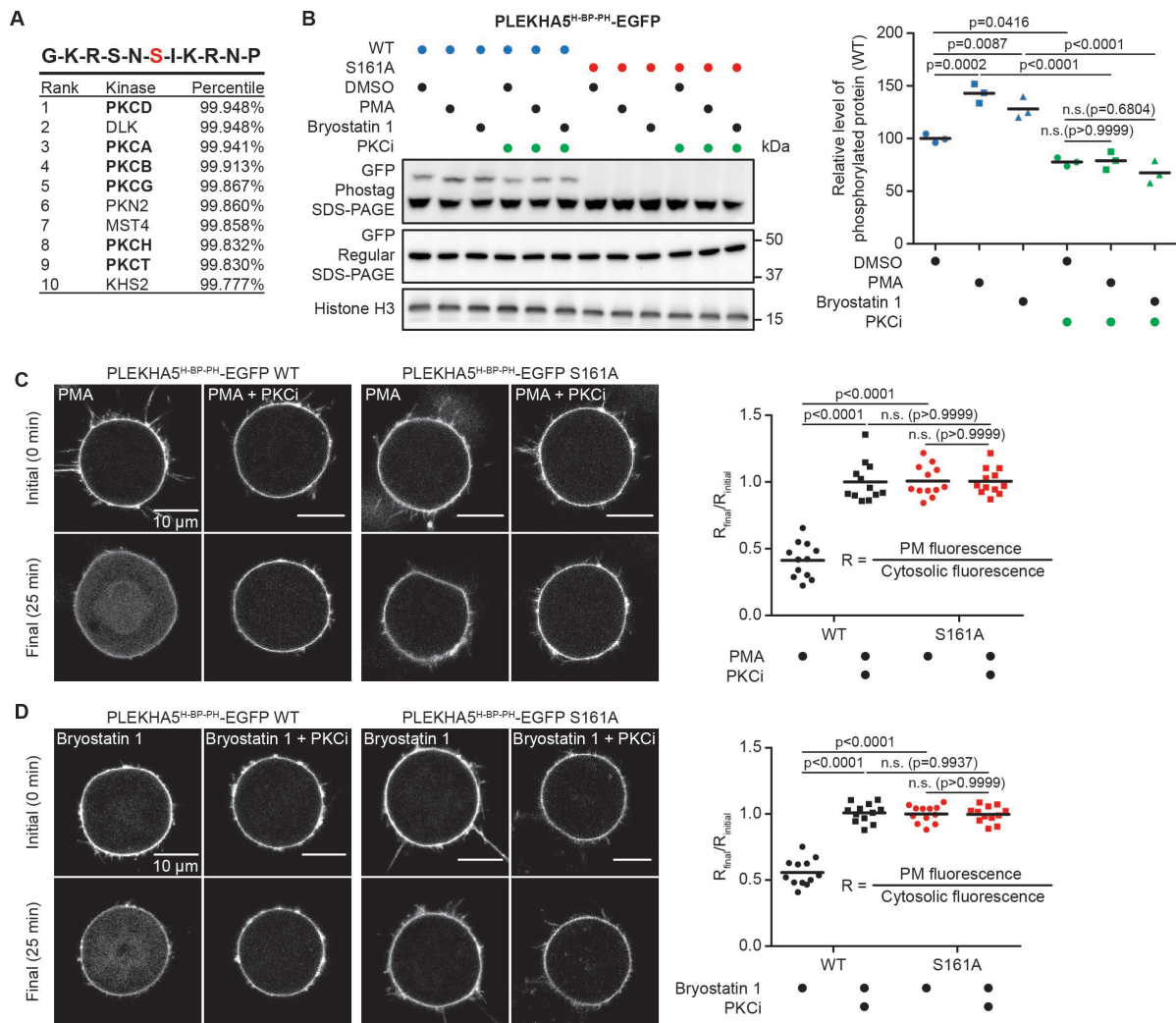


Figure 2.5. S161 phosphorylation and PLEKHA5 PM localization are modulated by protein kinase C (PKC) activity. (A) Top 10 kinase candidates for phosphorylating S161. PKC isoforms are bolded. (B) S161 phosphorylation increased upon stimulation of PKC activity, and the increase was attenuated with pre-treatment of PKC inhibitors. Representative western blots (left) and quantification (right) from Phos-tag and regular SDS-PAGE analysis probing

PLEKHA5^{H-BP-PH}-GFP are shown (n=3 biological replicates, one-way ANOVA with Sidak post hoc test). (C-D) PM localization of PLEKHA5^{H-BP-PH} was decreased upon activation of PKC by PMA (C) or Bryostatin 1 (D). Representative confocal micrographs before and after agonist treatment for each condition are shown at left, with quantification of the ratios of PM to cytosolic fluorescence before and after treatment are shown at right (n=12 cells, one-way ANOVA with Sidak post hoc test).

Finally, we examined whether the PPP family of phosphoprotein phosphatases dephosphorylate PLEKHA5 phospho-S161, because members of this serine/threonine phosphatase family carry out numerous dephosphorylation events related to mitosis²³. Pan inhibition of PPP phosphatases using okadaic acid induced elevation of phospho-S161 levels, but treatment of PP1- or PP2A/4-selective inhibitors caused minimal effects on S161 phosphorylation, indicating the involvement of multiple phosphatases in this dephosphorylation event (Appendix Figure A6e).

2.3 DISCUSSION

PLEKHA5 is a previously understudied PH domain-containing protein within the WW-PLEKHA subfamily¹³. Although the protein predominantly localizes to the microtubule network during interphase, a pool PLEKHA5 translocate to the PM specifically during mitosis, beginning from NEBD and ending at telophase. Mechanistic studies revealed that PI(4,5)P₂ binding mediates this PM localization but that it is critically regulated by the phosphorylation status of a single residue, S161, whose phosphorylation antagonizes PM recruitment and is decreased during mitosis. Although several kinase families are predicted to phosphorylate S161²², we found PKC to contribute substantially to this phosphorylation, raising the question of

how S161 dephosphorylation might accompany mitosis onset. Interestingly, two PKC isozymes, PKC α and PKC β II, which were among the most highly predicted kinases for S161 phosphorylation, accumulate in the nucleus during the G2-to-M transition, where they phosphorylate lamin B1 to drive its disassembly and promote NEBD^{24–26}. The timing of the nuclear accumulation of these kinases — and therefore their concomitant depletion from the cytoplasm, where PLEKHA5 resides in interphase on the microtubule cytoskeleton — correlates with the onset of PLEKHA5 recruitment to the PM. Therefore, we propose a model wherein reductions in cytoplasmic PKC activity (and potentially that of CK1 and/or additional kinases), coupled to activity of PPP family phosphatases during the G2-to-M transition and in early mitosis, cooperate to reduce S161 phosphorylation and enhance PLEKHA5 PM localization.

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CHAPTER 3

PLEKHA5 FUNCTIONS AS A MICROTUBULE ADAPTOR FOR THE SPATIAL REGULATION OF APC/C ACTIVITY

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“Proximity Labeling Reveals Spatial Regulation of the Anaphase-Promoting Complex/Cyclosome by a Microtubule Adaptor”

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3.1 INTRODUCTION

Progression through mitosis involves spatiotemporal coordination of cytoskeletal and membrane dynamics with changes in protein activities, chromosomal dynamics, and signaling events¹. A major role of the ubiquitin–proteasome system is to orchestrate cell cycle progression through temporally controlled protein degradation programs². The anaphase-promoting complex/cyclosome (APC/C) is the primary E3 ubiquitin ligase controlling mitotic events including G2/M transition, early mitosis, the metaphase–anaphase transition, and mitotic exit³.

APC/C is a 14-subunit complex and conserved member of the Cullin-RING E3 ligase family that mediates K11- and K48-linked polyubiquitination of several proteins involved in mitotic progression⁴, including the cyclin-dependent kinase

inhibitor p21 and cyclin A in early mitosis, cyclin B1 and the separase inhibitor securin at the metaphase–anaphase transition, and the DNA replication inhibitor geminin in early G1^{5–9}. Its ubiquitin ligase activity requires the association of one of two structurally related coactivators, CDC20 and CDH1, which interacts with APC/C during mitosis and in G1 phase, respectively¹⁰. The temporal control of APC/C-mediated ubiquitination in these processes is well established, including identities of ubiquitination substrates and accessory factors that modulate E3 ligase activity^{11,12}. However, there are major open questions concerning the spatial organization of the APC/C¹¹. These include an incomplete understanding of its subcellular localizations, what factors influence changes in its localization, and how compartmentalization of APC/C at different subcellular locations affects its activity toward specific substrates.

Here, we identified a link between the spatial organization of APC/C and the cellular functions of PLEKHA5 as a microtubule adaptor protein. The incomplete understanding of the spatial organization of APC/C is partly due to the challenges associated with effectively determining the subcellular localizations of this large, multi-subunit protein complex with conventional imaging methods. The deficiencies of these conventional tools motivated us to develop an alternative strategy harnessing TurboID-based proximity labeling^{13–15} to assess APC/C localization, and we reveal a general mechanism by which the spatial organization of APC/C is coordinated by PLEKHA5 to ensure its proper activity during mitosis. We propose that PLEKHA5 functions as a microtubule-specific APC/C adaptor to recruit a pool of APC/C to the microtubule cytoskeleton, promoting its loading with CDC20 and

enabling the APC/C^{CDC20}-mediated ubiquitination of key mitotic effector proteins, thus facilitating mitosis progression.

3.2 RESULTS

3.2.1 PLEKHA5 Interacts with APC/C, a Key E3 Ubiquitin Ligase Regulating Mitosis

To explore the molecular functions of PLEKHA5, we identified potential protein–protein interaction partners using stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative mass spectrometry. We performed anti-GFP immunoprecipitation (IP) from HeLa cells stably expressing EGFP-PLEKHA5 or EGFP and subjected the immunoprecipitates to bottom-up proteomics analysis. Interestingly, among the most strongly enriched proteins were several subunits of the anaphase-promoting complex/cyclosome (APC/C) (Figure 3.1B and Appendix Table B1).

We validated the interaction of endogenous PLEKHA5 with two representative APC/C subunits, ANAPC4 and ANAPC8, by co-IP, followed by western blotting (Figure 3.1C, D). To map the interacting regions, we performed co-IP of APC/C subunits with several GFP-tagged constructs corresponding to truncated forms of PLEKHA5 or isolated PLEKHA5 domains (Figure 3.1E and Appendix Figure B1a). The GFP fusion to the proline-rich domain (PRD) (EGFP-PLEKHA5^{PRD}) exhibited a strong interaction with APC/C (Figure 3.1E), whereas a construct lacking the PRD (EGFP-PLEKHA5^{ΔPRD}), despite its high expression levels, failed to interact with

APC/C (Appendix Figure B1a). These results suggest that the PRD of PLEKHA5 is sufficient and necessary for the interaction with APC/C.

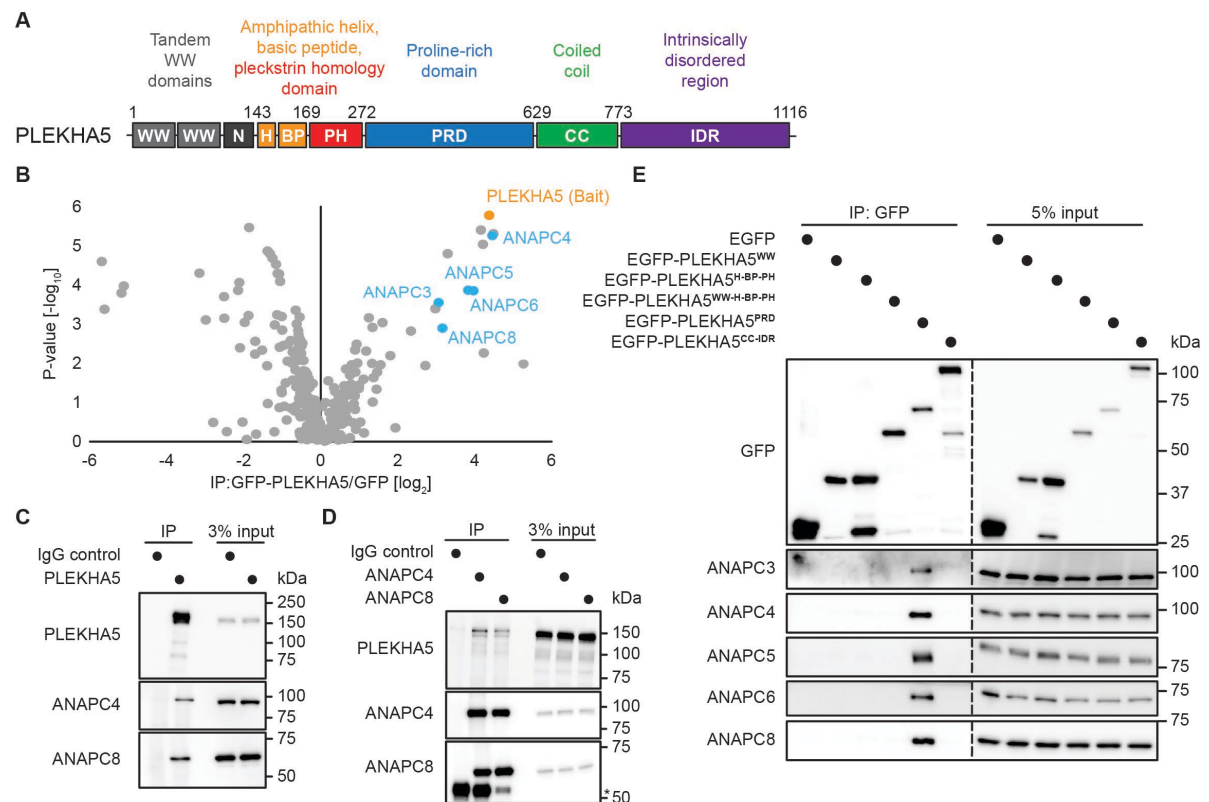


Figure 3.1. PLEKHA5 interacts with several APC/C subunits. (A) Domain map of PLEKHA5. (B) Volcano plot from SILAC MS proteomics experiments to determine the PLEKHA5 interactome, where proteins that are statistically significantly enriched in α-GFP immunoprecipitates from GFP-PLEKHA5-expressing HeLa cells compared to the IPs from GFP-expressing cells, including several APC/C subunits, appear on the top right. Statistical significance was determined by a Mann–Whitney *U* test. (C, D) Western blot analysis of co-IP experiments for endogenous PLEKHA5 (C) and the APC/C subunits ANAPC4 and ANAPC8 (D) to demonstrate the interaction between endogenous PLEKHA5 and the APC/C. IgG heavy chain from the IP is marked by an asterisk. (E) Truncation studies to reveal the PLEKHA5 PRD as sufficient to interact with APC/C.

One consequence of the PLEKHA5–APC/C interaction could be that PLEKHA5 is a ubiquitination substrate of APC/C. To test this possibility, we monitored PLEKHA5 protein levels over the cell cycle by western blotting in HeLa cells that were first

synchronized to the S-phase by double thymidine block (DTB)¹⁶ and then released from synchronization, with samples collected every 2 h for 24 h (Appendix Figure B1b). Unlike other known protein substrates of APC/C, such as cyclin A and cyclin B1, which show oscillating expression patterns, PLEKHA5 protein levels were remarkably stable over the experimental timeframe (Appendix Figure B1c, d), indicating that PLEKHA5 levels are not regulated via cell cycle-dependent proteolysis and thus that PLEKHA5 is likely not an APC/C substrate.

3.2.2 Development of a Proximity Biotinylation Tool to Probe the Microtubule Localization of Endogenous PLEKHA5, APC/C, and Its Substrates

If PLEKHA5 is not a ubiquitination substrate of APC/C, we next hypothesized that it may function as an adaptor protein to recruit this E3 ligase to the microtubule network as a site of action because the microtubule cytoskeleton is a major cellular structure that organizes mitotic events. To test this hypothesis, we needed to examine the colocalizations of PLEKHA5, APC/C, CDC20, and the substrates on microtubules. Previous studies using immunofluorescence have reported multiple localizations of APC/C, including the cytosol, the prophase nucleus, and, critically, numerous parts of the microtubule network, including spindle poles/centrosomes, kinetochores, and chromosomes^{11,17–19}. However, commercially available antibodies for different APC/C subunits have failed to consistently mark the subcellular localizations of the complex by immunofluorescence, and exogenous overexpression of GFP fusions to individual APC/C subunits may not reliably assemble into functional APC/C complexes^{18,20}. A recent high-throughput effort

(OpenCell) has generated CRISPR knock-in cell lines with GFP fusions to >1300 human proteins expressed endogenously²¹, yet for several APC/C subunits in this collection, no reliable localization was observed to intracellular organelles or structures.

Fortunately, recent developments of enzyme-mediated proximity labeling techniques such as BioID/TurboID and APEX provide new avenues for mapping the subcellular localizations of untagged, endogenous proteins with near-nanometer spatial resolution²². In these strategies, an organelle-tethered enzyme releases a highly reactive intermediate, typically a biotin derivative, enabling covalent biotinylation of nearby proteins within a limited radius. As such, proximity biotinylation has been widely applied for mapping the proteomes of organelles and other discrete structures such as membrane contact sites and synaptic clefts. Notably, the covalent nature of the labeling allows for sensitive detection and quantification of even minor or transient spatially restricted pools of proteins that exhibit dynamic behavior and/or can exist in multiple subcellular localizations, including large cytosolic pools, all of which are very challenging to visualize with standard imaging-based methods.

Therefore, we developed an alternative strategy harnessing TurboID-based proximity labeling^{13–15} to ascertain the microtubule localization of endogenous PLEKHA5, APC/C, and other proteins (Figure 3.2A). Proximity labeling-based tools exist for tagging specific subsets of the microtubule network, for example, the centrosome–cilium interface, noncentrosomal microtubule organizing centers, kinetochores, and dynein-associated proteins^{23–26}. To our knowledge, however,

A

DCX/EMTB
V5
TurboID
AMP
ATP + B
Immunofluorescence for V5 and biotin
Streptavidin-agarose pulldown
Confocal microscopy to image localization (V5) and activity (Strep-568) of TurboIDs (B-E)
Western blot to identify biotinylated proteins (F-G)

B

DCX-TurboID-V5
Streptavidin-568
Merge
10 μm

C

EMTB-TurboID-V5
Streptavidin-568
Merge
10 μm

D

DAPI
DCX-TurboID-V5
Streptavidin-568
Merge
10 μm

E

DAPI
EMTB-TurboID-V5
Streptavidin-568
Merge
10 μm

F

DCX-TurboID-V5

IP: Streptavidin 5% input

async prometa biotin

MAP4 250

Tubulin 50

CD44 100

Actin 75

Actin 50

Histone H3 15

PLEKHA5 150

ANAPC3 100

ANAPC5 75

ANAPC6 75

CDC20 50

Cyclin A 50

Cyclin B1 50

p21 20

Securin 25

V5 75

Streptavidin 250

Streptavidin 150

Streptavidin 100

Streptavidin 75

Streptavidin 50

Streptavidin 37

G

EMTB-TurboID-V5

IP: Streptavidin 5% input

async prometa biotin

MAP4 250

Tubulin 50

CD44 100

Actin 75

Actin 50

Histone H3 15

PLEKHA5 150

ANAPC3 100

ANAPC5 75

ANAPC6 75

CDC20 50

Cyclin A 50

Cyclin B1 50

p21 20

Securin 25

V5 75

Streptavidin 250

Streptavidin 150

Streptavidin 100

Streptavidin 75

Streptavidin 50

Streptavidin 37

36

network. (A) Schematic representation of proximity biotinylation using MT-TurboID for visualization and identification of microtubule-associated proteins. (B–E) Both microtubule-targeting TurboID constructs exhibit the expected cellular localization and are active, as shown by staining against V5 (green) and biotin (streptavidin-Alexa Fluor 568, magenta) during interphase (B, C) and prometaphase (D, E). For (D, E), DAPI staining is indicated at left to show the expected chromosomal morphology of prometaphase, but for clarity, the merged image only contains the anti-V5 and streptavidin channels. (F, G) PLEKHA5, APC/C subunits, and APC/C substrates, but not markers of other subcellular locations, were biotinylated by microtubule-targeting TurboID constructs in both asynchronous and prometaphase-synchronized HeLa cells.

We first validated the localization and biotinylation activity of the two MT-TurboID constructs, DCX-TurboID-V5 and EMTB-TurboID-V5, by performing IF against the V5 tag and biotin (Figure 3.2B–E and Appendix Figure B2). We then validated the specificity of the MT-TurboIDs using streptavidin-agarose enrichment of biotinylated proteins, followed by western blot analysis (Figure 3.2F, G). Both MT-TurboID constructs successfully labeled microtubule-associated protein 4 (MAP4), α -tubulin, and PLEKHA5 upon the addition of exogenous biotin but showed negligible biotinylation of markers of the plasma membrane (CD44), the actin cytoskeleton (actin), and the nucleus (histone H3), demonstrating that the activity of MT-TurboIDs was selectively confined to proteins on microtubules. Importantly, in the same experiment, representative APC/C subunits ANAPC3, ANAPC5, and ANAPC6, the coactivator CDC20, and APC/C substrates including cyclin A, cyclin B1, p21, and securin^{5–8} were biotinylated by both MT-TurboIDs in either asynchronous or prometaphase-synchronized cells by a 16 h treatment of S-trityl-L-cysteine (STLC)²⁹ (Figure 3.2F, G). These results reveal that APC/C structural subunits, CDC20, and several APC/C substrates all exhibit microtubule localization

both in interphase and in M phase and further validate MT-TurboIDs as general “in vivo biochemistry” tools for interrogating the microtubule localization of target proteins of interest.

3.2.3 PLEKHA5 Depletion Caused a Decrease in the Microtubule Localization of APC/C Subunits but Not CDC20

Next, to elucidate the requirement for PLEKHA5 in promoting APC/C localization to microtubules, we evaluated the extent of microtubule association of APC/C in control versus siPLEKHA5 cells using MT-TurboID proximity biotinylation, enrichment of biotinylated proteins, and western blot (Figure 3.3A). We confirmed that treating cells with siPLEKHA5 did not alter the expression level nor the activity of MT-Turbos in cells stably expressing these constructs (Figure 3.3C, E, V5 and streptavidin blots). Upon knockdown of PLEKHA5 in either asynchronous populations or cells synchronized to prometaphase, several representative subunits of the APC/C complex exhibited a significant decrease in microtubule localization marked by both MT-TurboID constructs (Figure 3.3B–E). However, in these same experiments, there was no significant change in levels of microtubule-associated CDC20 upon PLEKHA5 knockdown (Figure 3.3B–E). These data suggest that PLEKHA5 plays a role in the recruitment of a pool of apo APC/C complexes to microtubules and are consistent with the hypothesis, wherein PLEKHA5 functions as an APC/C adaptor to promote its localization to microtubules.

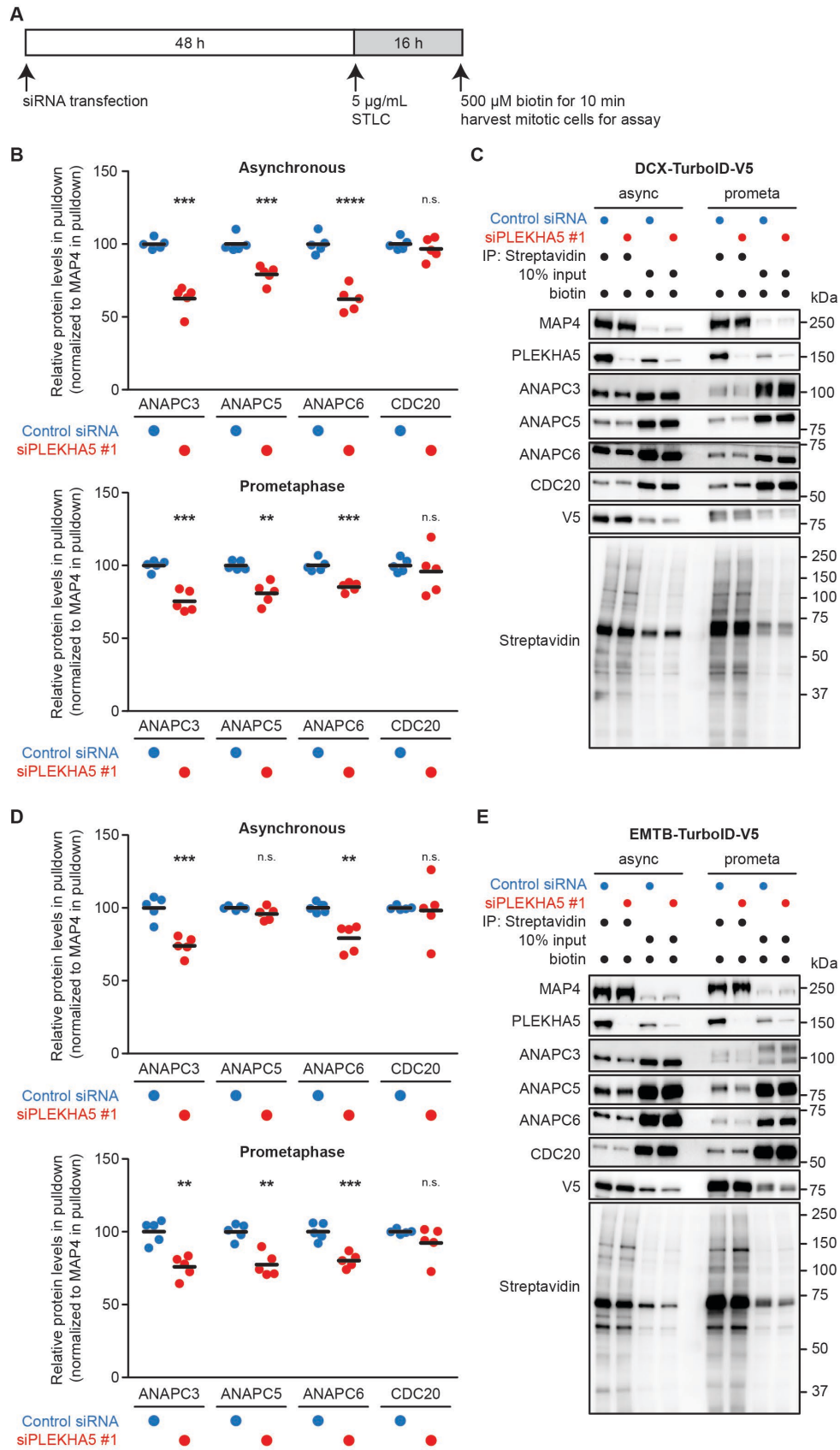


Figure 3.3. PLEKHA5 knockdown causes a decrease in the association of APC/C, but not CDC20, with microtubules. (A) Schematic representation of the experimental timeline. (B–E) Knockdown of PLEKHA5 leads to reduced levels of APC/C subunits in streptavidin pulldowns from HeLa cells stably expressing DCX-TurboID-V5 (B, C) or EMTB-TurboID-V5 (D, E). Shown are the representative blots for the assay (C, E) and quantifications of protein levels (B, D) in streptavidin pulldowns ($n = 5$). Student's t -test: n.s. not significant; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

3.2.4 PLEKHA5 Promotes APC/C Association with CDC20 and Activation in Prometaphase

Based on the above data showing decreased microtubule-associated APC/C but not CDC20 upon PLEKHA5 depletion, we tested if PLEKHA5 knockdown impairs the interaction of APC/C and CDC20 for the assembly of active E3 ligase complexes at microtubules and thus affects its catalytic activity. We first examined the composition of APC/C in mitosis. We isolated endogenous APC/C via anti-ANAPC3 IP from HeLa cells that were either asynchronous or synchronized to prometaphase by STLC, and we confirmed that several representative structural subunits and its key mitotic coactivator CDC20 were enriched from the cell lysates (Appendix Figure B3a). We then compared the APC/C enriched from prometaphase cells that had been transfected with control or two different PLEKHA5 siRNA duplexes (Figure 3.4A). PLEKHA5 knockdown did not affect the assembly of the structural components of APC/C, but the association of APC/C with CDC20 was significantly reduced, although the overall level of CDC20 was not affected by siPLEKHA5 treatment (Figure 3.4B, C and Appendix Figure B3b, c).

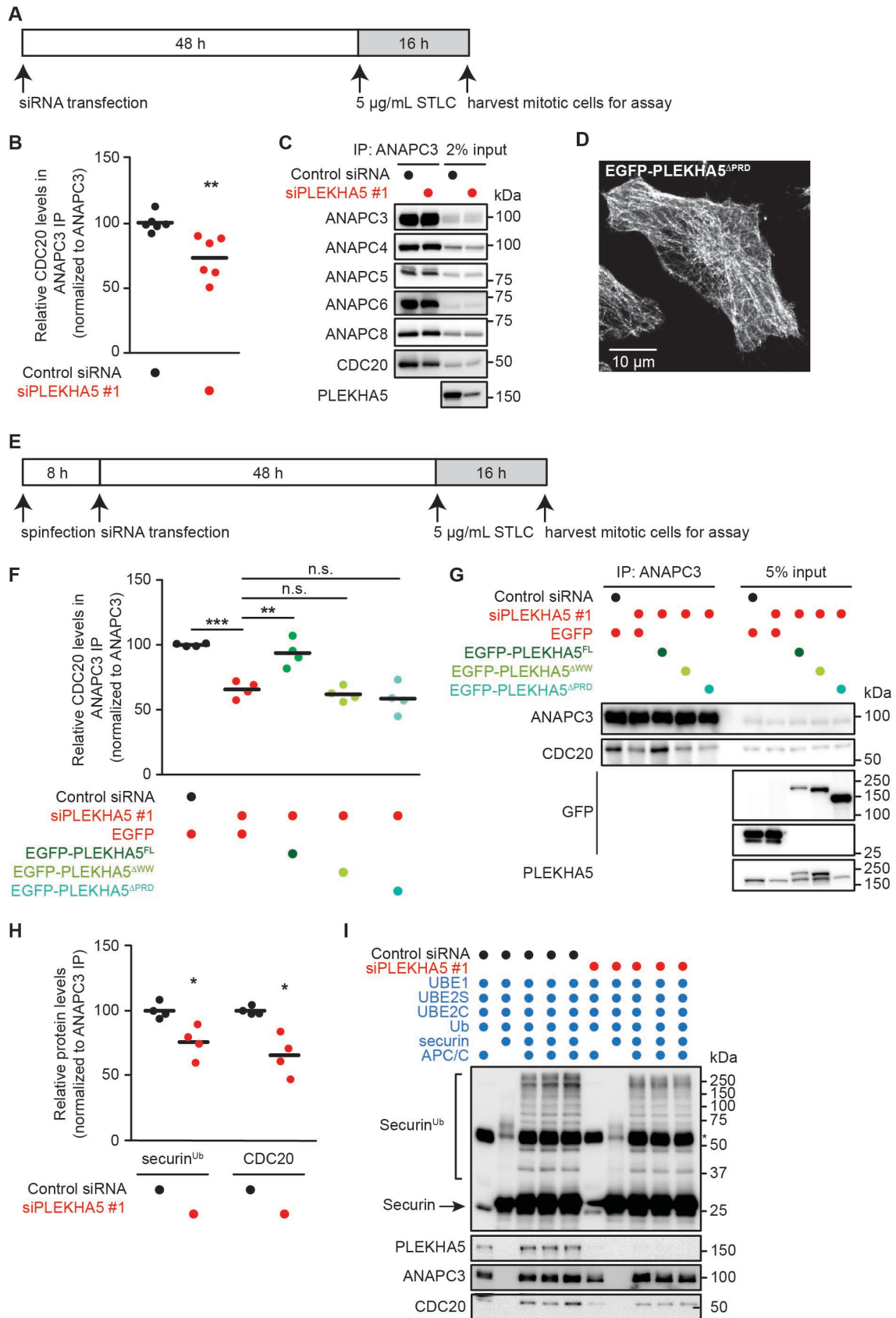


Figure 3.4. PLEKHA5 knockdown reduces CDC20 loading of APC/C in prometaphase and diminishes the catalytic activity of APC/C. (A) Schematic representation of experimental timeline. Prometaphase cells were detached from the plate by gentle tapping and collected for use in assays. (B, C) siPLEKHA5 #1 treatment causes a decrease in APC/C association with CDC20. Shown are the representative western blot analysis of co-IP experiments for endogenous ANAPC3 (C) and quantification of CDC20 levels in IP samples (B) ($n = 6$). Student's t-test: $**p < 0.01$. (D) Confocal microscopy analysis of EGFP- $\text{PLEKHA5}^{\Delta\text{PRD}}$, showing its microtubule localization. (E) Schematic representation of the experimental timeline for rescue experiments. (F, G) APC/C-CDC20 interaction can only be rescued by GFP- $\text{PLEKHA5}^{\text{FL}}$. Shown are quantification of CDC20 levels in IP samples (F) and representative western blots (G) ($n = 4$). We note that EGFP- $\text{PLEKHA5}^{\Delta\text{PRD}}$ expression was verified by the anti-GFP western blot because this construct does not contain the epitope recognized by the PLEKHA5 antibody. ANOVA (one-way, Tukey): n.s. not significant, $**p < 0.01$, $***p < 0.001$. (H, I) In vitro ubiquitination assays indicate that PLEKHA5 siRNA causes a reduction in APC/C E3 ligase activity. Shown are quantification of polyubiquitinated securin ($\text{securin}^{\text{Ub}}$) and CDC20 levels in the reactions (H) and representative western blots for the assay (I). IgG heavy chain from the APC/C purification is marked by an asterisk, and complete reactions (lanes 3–5 and 8–10) are shown in technical triplicate for each biological replicate ($n = 4$ biological replicates plotted in H). Student's t-test: $*p < 0.05$.

The reduced APC/C–CDC20 interaction was rescued upon expression of an siRNA-resistant EGFP- $\text{PLEKHA5}^{\text{FL}}$ construct, but minimal rescue was observed using two truncation constructs, EGFP- $\text{PLEKHA5}^{\Delta\text{WW}}$ or EGFP- $\text{PLEKHA5}^{\Delta\text{PRD}}$ (Figure 3.4E–G). EGFP- $\text{PLEKHA5}^{\Delta\text{WW}}$ retained its interaction with APC/C (Appendix Figure B1a) yet could not localize to the microtubule network (Figure 2.1C), whereas EGFP- $\text{PLEKHA5}^{\Delta\text{PRD}}$ localized to microtubules correctly (Figure 3.4D) but failed to interact with APC/C (Appendix Figure B1a). The results from these rescue experiments demonstrate that both the microtubule localization of PLEKHA5 and its interaction with APC/C are critical to restoring normal APC/C–CDC20 association in prometaphase cells that is perturbed upon PLEKHA5 knockdown. These data indicate that the recruitment of a pool of APC/C to microtubules by PLEKHA5

promotes efficient loading of CDC20 onto APC/C to form active E3 ligase complexes.

To measure the effect of this decreased CDC20 association on APC/C function, we assessed the E3 ligase activity of APC/C^{CDC20} using in vitro ubiquitination assays³⁰. We reconstituted the ubiquitination cascade using the enriched APC/C and other required components, including E1, E2s, ubiquitin, ATP, and recombinantly purified securin as the APC/C substrate. Western blot analysis of the reaction mixtures revealed that the in vitro ubiquitination of securin was dependent upon the presence of all components, and the APC/C purified by IP was indeed active (Appendix Figure B3d). Next, we evaluated the catalytic activities of APC/C^{CDC20} purified from control or siPLEKHA5 cells synchronized to prometaphase. Upon PLEKHA5 knockdown, the in vitro polyubiquitination of securin by APC/C was significantly decreased, which was consistent with a decrease in APC/C loading with CDC20 caused by siPLEKHA5 (Figure 3.4H, I). These results indicate that depletion of PLEKHA5 from prometaphase cells antagonizes the loading of CDC20 onto APC/C and thus impairs the catalytic activity of the E3 ubiquitin ligase toward its substrates.

3.2.5 PLEKHA5 Knockdown Attenuates Mitotic Progression and Causes Increases in the Fraction of Cells in G2 and M Phases

The modulation of APC/C activity by PLEKHA5 suggests potential functions of PLEKHA5 in mitosis, as APC/C is the primary E3 ligase controlling mitotic entry, progression, and exit. We monitored mitosis progression by performing

temperature- and CO₂-controlled live-cell imaging over extended time periods of asynchronous cells expressing fluorescent markers of chromatin, H2B-mCherry, and quantified the time from nuclear envelope breakdown (NEBD) to anaphase onset, signified by chromosomal segregation³¹ (Figure 3.5A). PLEKHA5 knockdown caused a minor but significant increase in time for cells to enter anaphase (Figure 5.3B and Appendix Figure B4a). The modest nature of this delay suggests an effect of PLEKHA5 on only a subset of APC/C complexes. Nevertheless, we note that the effect on mitosis in unperturbed, asynchronous cells seen here is similar in magnitude to depletion of the protein phosphatase PP2A-B55, a nonessential but important regulator of mitosis³¹.

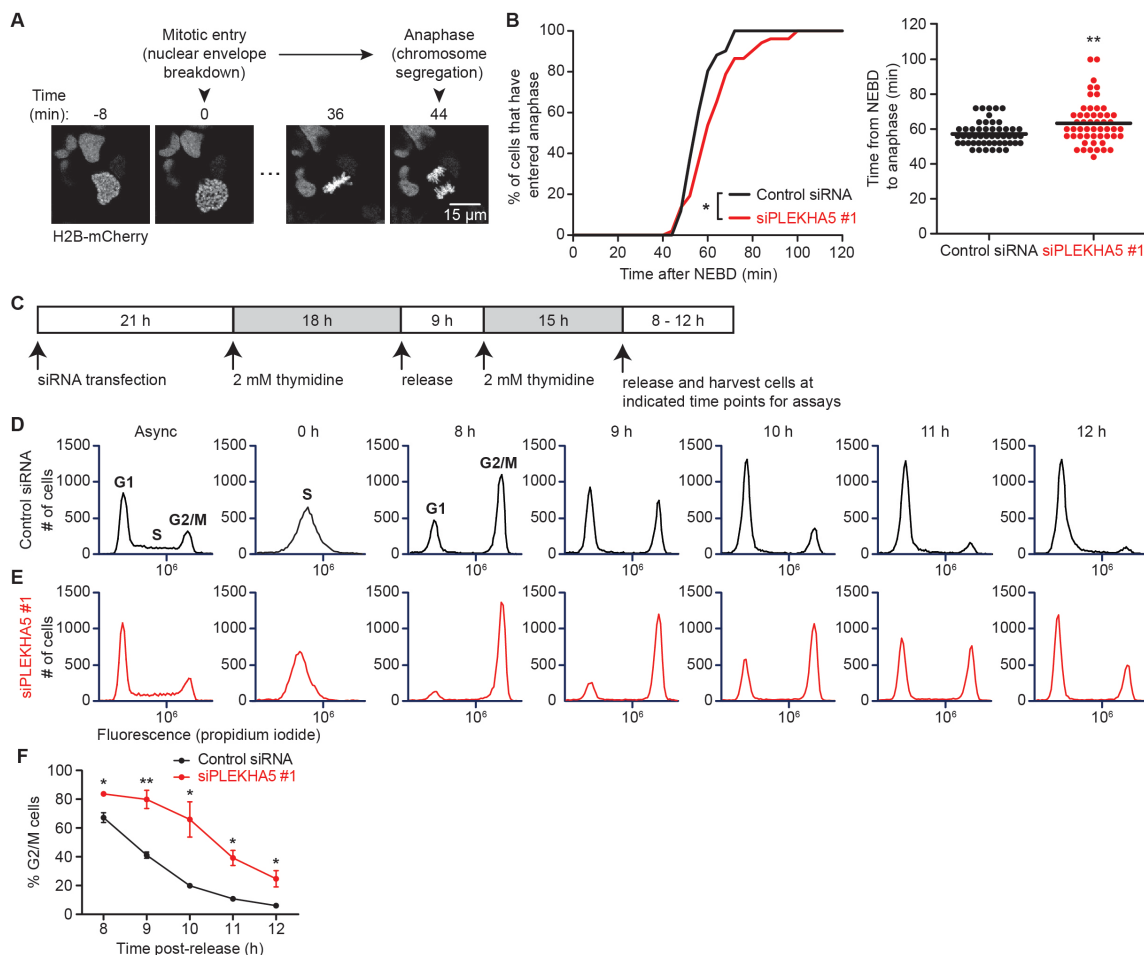


Figure 3.5. PLEKHA5 knockdown delays mitotic progression and increases the fraction of cells in G2 and M phases. (A) Schematic representation of mitotic progression analysis in asynchronous HeLa cells by time-lapse, live-cell imaging of H2B-mCherry. (B) Quantification of mitosis progression in asynchronous HeLa cells, with time from NEBD to anaphase plotted as cumulative frequency (left, Kolmogorov–Smirnov test) and scatter plot (right, Mann–Whitney U test) ($n = 50$ – 51 cells): $*p < 0.05$; $**p < 0.01$. (C) Schematic representation of the experimental timeline using a double thymidine block (DTB). (D–F) siPLEKHA5 #1 treatment leads to the accumulation of HeLa cells in the G2 and M phases. DNA content of asynchronous (async) or DTB-synchronized/released HeLa cells transfected with control siRNA (D) or siPLEKHA5 #1 (E) were evaluated by PI staining and flow cytometry analysis, and the percentage of cells in G2 and M phases was quantified and shown in (F) ($n = 3$). Student's t -test: $*p < 0.05$; $**p < 0.01$.

We also analyzed the cell-cycle phase of HeLa cells subject to DTB synchronization and release by using propidium iodide staining of fixed cells, followed by flow cytometry (Figure 3.5C). We found that depletion of PLEKHA5 by two different siRNAs led to an increased percentage of cells retained in G2 and M phases after release from DTB (Figure 3.5D–F and Appendix Figure B4b-d).

3.2.6 PLEKHA5 Knockdown Increases the Levels of Mitotic APC/C Substrates

To further understand the effect of PLEKHA5 knockdown in APC/C-mediated polyubiquitination and mitosis progression on a molecular level, we analyzed the protein levels of several APC/C ubiquitination substrates and mitosis markers following DTB synchronization and release via western blotting (Figure 3.6A). Consistent with previous results wherein APC/C isolated from siPLEKHA5-treated cells exhibited lower in vitro catalytic activity, several APC/C substrates, including cyclin A, cyclin B1, securin, and p21, all showed aberrant accumulation upon PLEKHA5 knockdown using two different siRNA duplexes (Figure 3.6B, C

and Appendix Figure B5a, b). These results indicate that PLEKHA5 depletion results in a defect in APC/C-mediated polyubiquitination of mitotic effector proteins and subsequent proteasomal degradation.

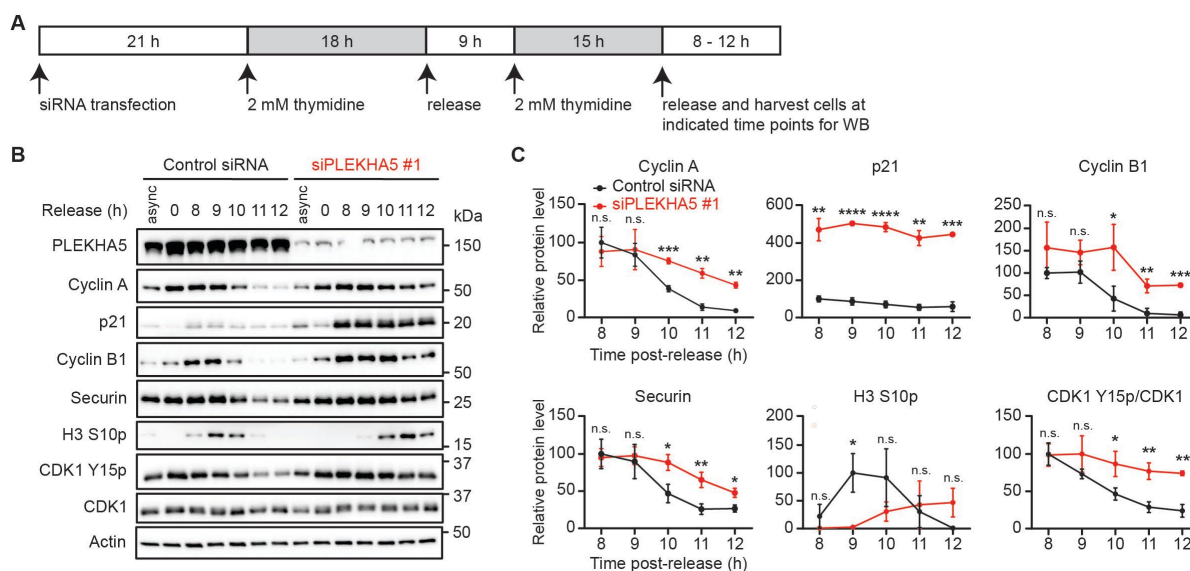


Figure 3.6. PLEKHA5 knockdown causes a buildup of APC/C substrates. (A) Schematic representation of the experimental timeline. (B, C) APC/C substrates and G2/M protein markers persist in cells treated with siPLEKHA5 #1. Shown are representative western blots (B) and quantification of protein levels (C) of lysates from HeLa cells subject to control siRNA or siPLEKHA5 #1 ($n = 3$). Student's t -test: n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

However, the substantial accumulation of p21 at early timepoints caught our attention because p21, in addition to being an APC/C substrate, is also an inhibitor of the CDK1–cyclin B1 complex, the major kinase complex that positively regulates mitosis onset and advance³². The accumulation of p21 may be due to insufficient APC/C activity, but it can also suggest the activation of the G2/M DNA damage checkpoint, which leads to prolonged G2/M arrest³³. Thus, we analyzed the levels of a mitosis protein marker, phospho-histone H3 at serine 10 (H3 S10p), and CDK1. PLEKHA5 knockdown caused a delay in the appearance and the peak expression

of H3 S10p (Figure 3.6B, C and Appendix Figure B5a, b). Furthermore, the analysis of CDK1 revealed prolonged expression of its inactive form, which bore inhibitory phosphorylation at tyrosine 15 (CDK1 Y15p)³⁴, at later timepoints in PLEKHA5-depleted cells compared to the control (Figure 3.6B, C and Appendix Figure B5a, b). These results, along with the induction of p21 expression, indicate that siPLEKHA5 potentially caused a partial G2-to-M transition arrest prior to its function in mitosis and that PLEKHA5 may play additional roles in cell cycle regulation beyond a role in mitosis.

To explore the potential effects of PLEKHA5 depletion prior to mitotic entry, we analyzed the G2-to-M transition by imaging cells expressing GFP-tagged proliferating cell nuclear antigen (EGFP-PCNA) and H2B-mCherry and measuring the time between the disappearance of PCNA puncta and NEBD³⁵ (Figure 3.7A). In the asynchronous population of cells, we found no statistically significant difference between siPLEKHA5 and control in the G2-to-M transition period (Figure 3.7B and Appendix Figure B6). Additionally, we examined the levels of the same G2/M markers and APC/C substrates after a DTB synchronization and release but in the presence of the phosphodeficient CDK1 T14A/Y15F (AF) mutant (Figure 3.7C). This CDK1 AF mutant, which cannot possess inhibitory phosphorylation marks, is constitutively active, and its expression induces cells to partially bypass the G2/M checkpoint^{34,36–38}. Compared to control, cells expressing CDK1(AF)-3xFLAG with control siRNA treatment showed increased mitosis progression, signified by earlier timepoints for H3 S10p peak expression, a faster decrease in its levels, and reduced accumulation of APC/C substrates (Figure 3.7D, E, compare the gray and

black lines in quantification plots, significance marked by *). These results confirm that expressing the CDK1(AF) mutant indeed permits cells to partially bypass the G2/M checkpoint and promote mitosis entry in our system. We then compared the effect of PLEKHA5 knockdown versus control siRNA in CDK1(AF)-expressing cells. Again, we observed a delay in H3 S10p disappearance and elevated levels of endogenous CDK1 Y15p and APC/C substrates upon PLEKHA5 knockdown (Figure 3.7D, E, compare the black and red lines in quantification plots, significance marked by #). Collectively, these studies support a major role for PLEKHA5 in regulating mitosis.

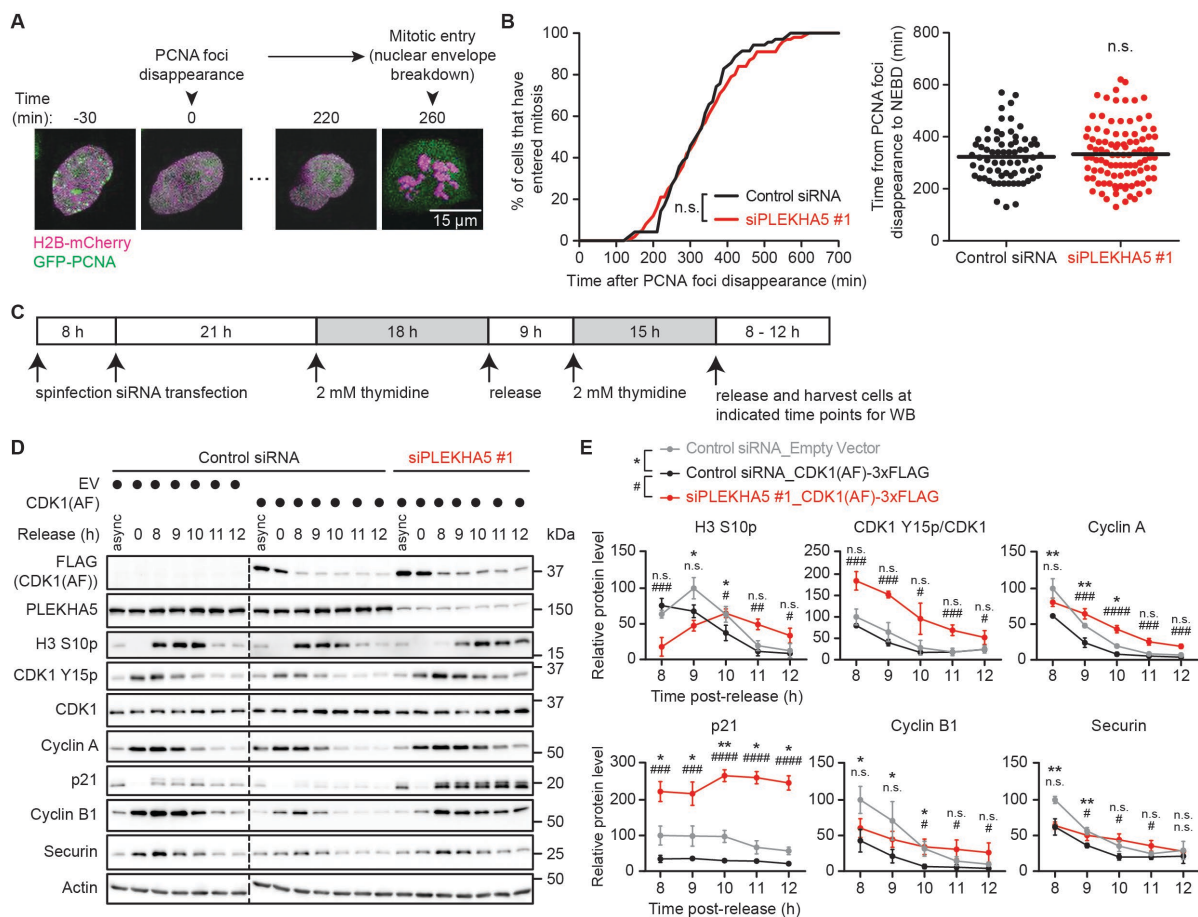


Figure 3.7. Knockdown of PLEKHA5 has little effect in G2-to-M transition in asynchronous cells and still results in buildup of mitosis markers and APC/C

substrates in S phase-synchronized HeLa cells expressing a phosphodeficient CDK1 T14A/Y15F (AF) mutant that allows partial G2/M checkpoint bypass. (A) Schematic representation of G2/M transition analysis in asynchronous HeLa cells by time-lapse, live-cell imaging of H2B-mCherry and EGFP-PCNA. The time between disappearance of PCNA puncta and NEBD allows for quantitation of delays in the G2/M transition in single cells. (B) Quantification of G2/M transition in asynchronous HeLa cells, with time from PCNA foci dissolution to NEBD plotted as cumulative frequency (left) and scatter plot (right) (n=70-100 cells). Kolmogorov-Smirnov test for cumulative frequency and Mann-Whitney *U* test for duration: n.s. not significant. (C) Schematic representation of experimental timeline. HeLa cells were transduced with lentivirus encoding CDK1(AF)-3xFLAG or empty virus (EV) via spinfection. Eight h after the spinfection, cells were treated with an siRNA targeting PLEKHA5 or a control siRNA and subjected to DTB. Cells were rinsed and released into fresh medium for indicated amount of time before harvest for Western blot analysis. Representative Western blots (D) and quantification plots (E) show the levels of protein markers analyzed in the experiment (n=3). ANOVA (one-way, Tukey): n.s. not significant; * and # *p* < 0.05; ** and ## *p* < 0.01; *** and ### *p* < 0.001; **** and #### *p* < 0.0001.

3.2.7 Interaction of PLEKHA5 with APC/C at Microtubules Is Required to Rescue Mitotic Defects Caused by PLEKHA5 Knockdown

To further investigate the mechanism of the PLEKHA5–APC/C interaction in regulating mitosis, we performed rescue experiments with siPLEKHA5-resistant EGFP, EGFP-PLEKHA5^{FL}, EGFP-PLEKHA5^{ΔWW}, and EGFP-PLEKHA5^{ΔPRD} constructs (Figure 3.8A). In accordance with previous results, control rescue (with EGFP) in siPLEKHA5-treated cells led to a significant increase of APC/C substrates and G2- and M-phase protein markers (Figure 3.8B, C, compare the first and second bands in groups of five in representative blots, e.g., lanes 16 vs 17, and the black and red lines in quantification plots, significance marked by *). Importantly, the changes in protein expression patterns induced by PLEKHA5 depletion were partially restored by the introduction of an siRNA-resistant form of full-length PLEKHA5 protein (Figure 3.8B, C, compare the second and third bands

in groups of five in representative blots, e.g., lanes 17 vs 18, and the red and dark-green lines in quantification plots, significance marked by #). For example, the peak expression of H3 S10p shifted earlier to 9 h post-release, and its levels decreased to a greater extent at later time points. Similarly, the buildup of other markers and APC/C substrates was partially reduced by the expression of EGFP-PLEKHA5^{FL} in cells treated with siPLEKHA5.

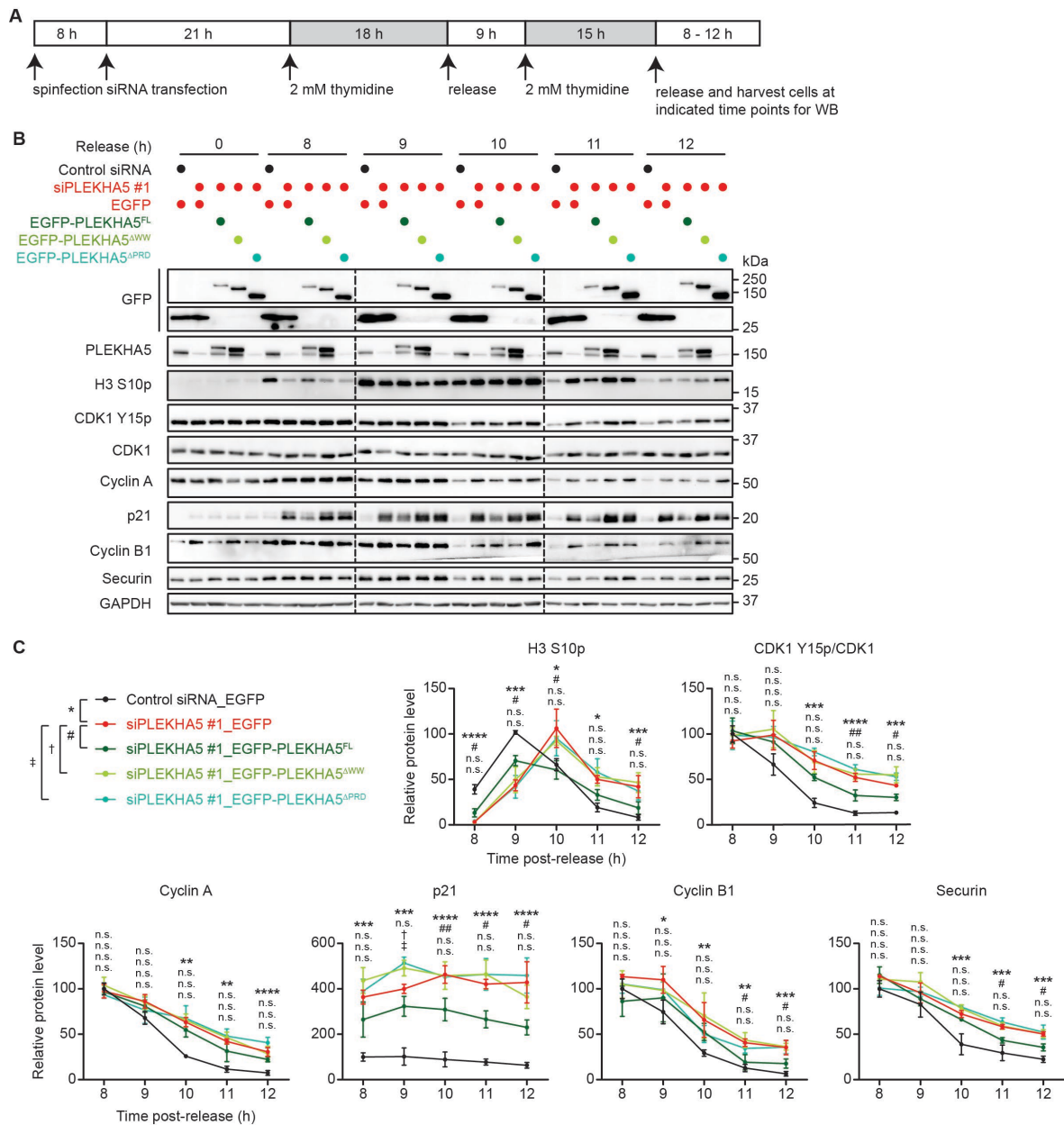


Figure 3.8. PLEKHA5–APC/C interaction at microtubules is required to rescue the accumulation of mitosis markers and APC/C substrates induced by PLEKHA5 knockdown. (A) Schematic representation of the experimental timeline. (B, C) Accumulation of mitosis markers and APC/C substrates in siPLEKHA5 samples is partially rescued by exogenous expression of EGFP-PLEKHA5^{FL} but not EGFP-PLEKHA5^{ΔWW} or EGFP-PLEKHA5^{ΔPRD}. Shown are representative western blots (B) and quantification of protein levels (C) in HeLa cells transduced with conditioned media containing lentivirus encoding EGFP, siRNA-resistant EGFP-PLEKHA5^{FL}, siRNA-resistant EGFP-PLEKHA5^{ΔWW}, and siRNA-resistant EGFP-PLEKHA5^{ΔPRD} by spinfection ($n = 3$). Notes: EGFP-PLEKHA5^{ΔPRD} expression is verified by GFP blot because this construct does not contain an epitope recognized by the PLEKHA5 antibody. ANOVA (one-way, Tukey): n.s. not significant; *, #, † and ‡ $p < 0.05$; **, ##, †† and ‡‡ $p < 0.01$; ***, ###, ††† and ‡‡‡ $p < 0.001$; ****, ####, †††† and ‡‡‡‡ $p < 0.0001$.

We note that complete rescue was not achieved, likely due to both the relatively poor expression of EGFP-PLEKHA5^{FL} and the maximal transduction efficiency with the rescue construct of ~60% (Appendix Figure B7). Nevertheless, minimal rescue was observed in cells expressing siRNA-resistant forms of the truncation constructs, EGFP-PLEKHA5^{ΔWW} or EGFP-PLEKHA5^{ΔPRD}, which are unable to recruit APC/C to microtubules, despite their much higher expression in rescue experiments compared to the full-length construct (Figure 3.8B,C, compare the second and fourth or fifth bands in groups of five in representative blots, e.g., lanes 17 vs 19 and 20, and the red and yellow–green or blue–green lines in quantification plots, significance marked by † and ‡, respectively, and Appendix Figure B7). These rescue experiments reveal that the recruitment of APC/C to microtubules by PLEKHA5 is critical for the proteolysis of APC/C ubiquitination substrates and proper advancement of mitosis. Collectively, even though we cannot fully exclude the possibility that PLEKHA5 exerts additional regulation of the cell cycle prior to mitosis, which will be discussed further, our data suggest that PLEKHA5 ensures

proper mitotic progression by recruiting a pool of APC/C complexes to microtubules and are consistent with a model wherein PLEKHA5 functions as an APC/C adaptor to promote its localization to microtubules and thus enhance the efficiency of CDC20 loading onto this pool of APC/C to promote substrate ubiquitination during mitosis (Figure 3.9).

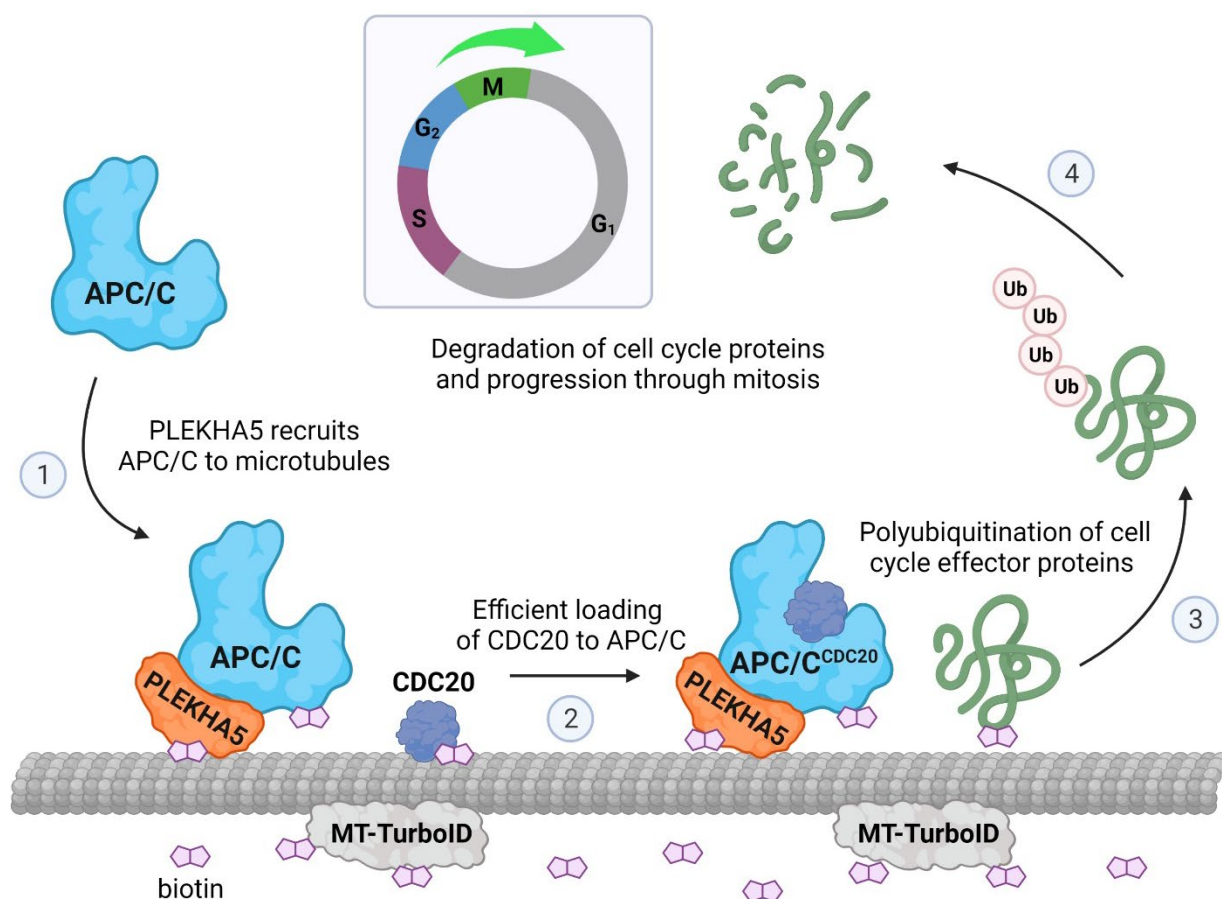


Figure 3.9. Model for PLEKHA5 function in mitosis as a microtubule-localized adaptor that regulates spatial organization of the APC/C E3 ubiquitin ligase. PLEKHA5 is a microtubule-resident protein that recruits a pool of APC/C to the microtubule cytoskeleton, as demonstrated by its biotinylation by microtubule-localized proximity labeling tools (MT-TurbolID), thus increasing the local concentration of APC/C along the microtubule network. Upon mitotic phosphorylation, this pool of APC/C can efficiently interact with its coactivator CDC20 and subsequently polyubiquitinate its mitotic substrates. In this manner, PLEKHA5 organizes APC/C in space to facilitate the timely turnover of key mitotic proteins to promote progression through mitosis (Figure created with BioRender).

3.3 DISCUSSION

PLEKHA5 belongs to the PLEKHA4–7 protein family, members of which serve as intracellular adaptor proteins to promote specific subcellular localization of their interacting proteins to carry out various functions^{39,40}. PLEKHA5 is critical for proliferation and invasiveness of melanoma cells with a high propensity for brain metastasis⁴¹. Other studies have implicated PLEKHA5 in the regulation of malignant progression in diffuse-type gastric carcinoma and breast cancer metastasis^{42,43}. These reports underscore the importance of elucidating the fundamental molecular and cellular functions of PLEKHA5, particularly those that impact the regulation of the cell cycle.

We found that PLEKHA5 exhibits a microtubule localization in HeLa cells and interacts with the E3 ubiquitin ligase APC/C. We reasoned that PLEKHA5 is likely not a substrate for APC/C during mitosis because PLEKHA5 levels remained stable throughout the cell cycle, and it does not possess APC/C destruction motifs such as the D-box, KEN-box, or ABBA motif⁴⁴ within its primary amino acid sequence. Instead, we hypothesized that PLEKHA5 might be a microtubule-localized APC/C adaptor.

To overcome the challenges associated with determining APC/C localization through imaging and to test the hypothesis that PLEKHA5 mediates the spatial organization of APC/C, we developed a proximity biotinylation approach using microtubule-targeted TurboID constructs to assess endogenous protein localizations to the microtubule cytoskeleton. Indeed, we detected biotinylation of PLEKHA5, APC/C, CDC20, and relevant APC/C substrates, confirming that these

proteins localize to or very near to microtubules. The recruitment of APC/C to microtubules was partly dependent on PLEKHA5, as PLEKHA5 depletion reduced microtubule-associated APC/C and further impaired the loading of CDC20 onto this pool of APC/C and exhibited a decrease in catalytic activity.

The connection to APC/C prompted us to explore the potential roles of PLEKHA5 in regulating mitosis. Depletion of PLEKHA5 antagonized mitotic progression and led to accumulations of APC/C^{CDC20} substrates in a manner dependent upon the PLEKHA5–APC/C interaction at microtubules. Unexpectedly, we also observed a delay in mitotic entry from the DTB experiment, although the analysis of G2-to-M transition in asynchronous HeLa cells by imaging showed little difference in control versus siPLEKHA5 cells. Still, the induction of high p21 expression may indicate the presence of DNA damage upon PLEKHA5 knockdown. One possible explanation involves CDH1, the coactivator for APC/C from mitotic exit to the end of G1. Our proposed model of PLEKHA5 as a microtubule adaptor protein for APC/C also applies, in principle, to facilitate the formation of the active APC/C^{CDH1} complex, which has been shown to respond to DNA damage and regulate DNA repair⁴⁵. In the context of PLEKHA5 knockdown, the APC/C–CDH1 interaction may decrease and therefore impair APC/C^{CDH1} catalytic activity, similar to what we have shown for APC/C^{CDC20}, which would lead to unresolved DNA damage and activation of the checkpoint machinery to delay mitotic entry. Testing of this hypothesis will require future studies into the potential effects of PLEKHA5 on APC/C^{CDH1} activity and function.

Collectively, this study reveals a new framework for the spatial regulation of APC/C in mitosis. In our model, PLEKHA5 interacts with and recruits a pool of APC/C to the microtubule cytoskeleton. This interaction would increase the local concentration of APC/C along the network and effectively reduce the dimensionality of its search for coactivators and substrates from three dimensions (in the cytosol) to one dimension (along microtubule tracks). Upon mitotic phosphorylation of its subunits and relief of the spindle assembly checkpoint, this pool of APC/C would efficiently encounter its coactivator CDC20 and subsequently be capable of ubiquitinating key mitotic effector proteins. Our study not only reveals a mechanism controlling the spatial regulation of the major E3 ubiquitin ligase in mitosis but also provides a means for understanding how such spatial regulation could be intimately linked to the established temporal regulation of this enzyme that is critical for progression through mitosis. Given the localization of PLEKHA5 and the large number of PLEKHA5-interacting proteins identified in our proteomics experiments, it is possible that PLEKHA5 acts more generally as an adaptor to facilitate protein recruitment to the microtubule cytoskeleton in both interphase and mitosis. Furthermore, our studies provide a mechanism that accounts for a role for PLEKHA5 in promoting progression through mitosis that may be operational in settings where elevated PLEKHA5 levels have been linked to cancer progression. Finally, the MT-TurboIDs developed herein have the potential to serve as broadly useful tools for interrogating the dynamic association of diverse proteins with the microtubule cytoskeleton in many physiological contexts.

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CHAPTER 4

DEVELOPMENT OF A REVERSIBLE, MITOSIS-SPECIFIC PLASMA MEMBRANE PROTEIN RECRUITER

4.1 INTRODUCTION

Extracellular cues are converted into intracellular signaling cascades via regulatory mechanisms that frequently involve conditional recruitment of key enzymes to the plasma membrane (PM) to access substrates, activators, and/or inhibitors^{1,2}. Synthetic biology approaches can short-circuit these natural systems, bypassing cell-surface receptors by enabling spatiotemporal control over the localizations and activities of these signaling enzymes. Most commonly, these tools involve conditional protein dimerization following an exogenous trigger such as a small molecule (chemogenetics) or visible light (optogenetics)³⁻⁶.

Though these systems are widely applied, their limitations include, for chemogenetics, lack of reversibility⁷ and undesired physiological effects of rapamycin⁸, the most frequently used trigger, or, for optogenetic tools, phototoxicity, loss of microscopy channels for observation, and challenges with multiplexing^{4,9}. Critically, these methods all involve application of an exogenous perturbation, adding complexity and motivating searches for new triggers (e.g., heat¹⁰). An alternate approach for non-perturbative conditional protein activation would involve no exogenous trigger at all, instead harnessing an endogenous stimulus.

A natural system in all dividing eukaryotic cells exhibiting strict temporal control over protein activation and deactivation is the cell cycle, which features regulated changes

in protein posttranslational modification and proteolysis caused by the oscillatory activities of kinases, phosphatases, and ubiquitin ligases^{11–13}. Indeed, the oscillating patterns of enzymatic activities and protein levels have been exploited as endogenous triggers that control levels of different fluorescent proteins to enable visualization of cell cycle stage^{14–16}. However, this approach only allows for observation, not manipulation, of cellular processes.

Here, we report the design, optimization, and application of a Mitosis-enabled Anchor-away/Recruiter System (MARS) that enables conditional localization of target proteins to the PM exclusively during mitosis. MARS comprises an engineered 129 amino acid (15 kDa) module from pleckstrin homology domain-containing A5 (PLEKHA5) that localizes to the cytosol during interphase and the PM during mitosis. We apply MARS to facilitate recruitment of various protein cargoes, including an endogenously tagged protein kinase, a phospholipase, and a lipid kinase. These studies point to roles for excess phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃) at the PM in hindering progression through anaphase. MARS represents the first example of harnessing mitosis as an endogenous stimulus to mediate reversible protein recruitment without exogenous triggers, and we anticipate it will be useful for manipulating mitosis-specific biological activities without the necessity for synchronization.

4.2 RESULTS

4.2.1 Engineering of a Mitosis-specific PM-binding Motif Based on PLEKHA5

To complement chemogenetic- and optogenetic-based platform for conditional protein recruitment, we pursued to harness the cell cycle-dependent PM binding capability of PLEKHA5 (characterized in Chapter 2) to develop a tool called Mitosis-enabled Anchor away/Recruiter System (MARS), which uses mitosis as an endogenous, natural trigger for reversible protein recruitment at the plasma membrane. Based on the H-BP-PH fragment of PLEKHA5, we aimed to engineer a mitosis-specific PM binding motif that resides in the cytosol during interphase and translocates to the PM during mitosis. We first had to eliminate any PM localization during interphase while retaining PM association during mitosis. Previously, mutation of four basic residues in the PLEKHA4 BP motif decreased PM localization in cells and PI(4,5)P₂ binding in vitro¹⁷. As these residues were conserved in PLEKHA5 (Appendix Figure C1a and Figure 4.1A), we analyzed the localizations of several single and double mutants of these residues within PLEKHA5^{H-BP-PH}. Only K163A/R164A retained mitotic PM localization while exhibiting nucleocytoplasmic localization in interphase (Figure 4.1B and Appendix Figure C1b). Addition of an internal or C-terminal nuclear export signal (NES) eliminated the nuclear localization during interphase (Figure 4.1C), allowing for better accessibility to cytosolic signaling protein cargoes.

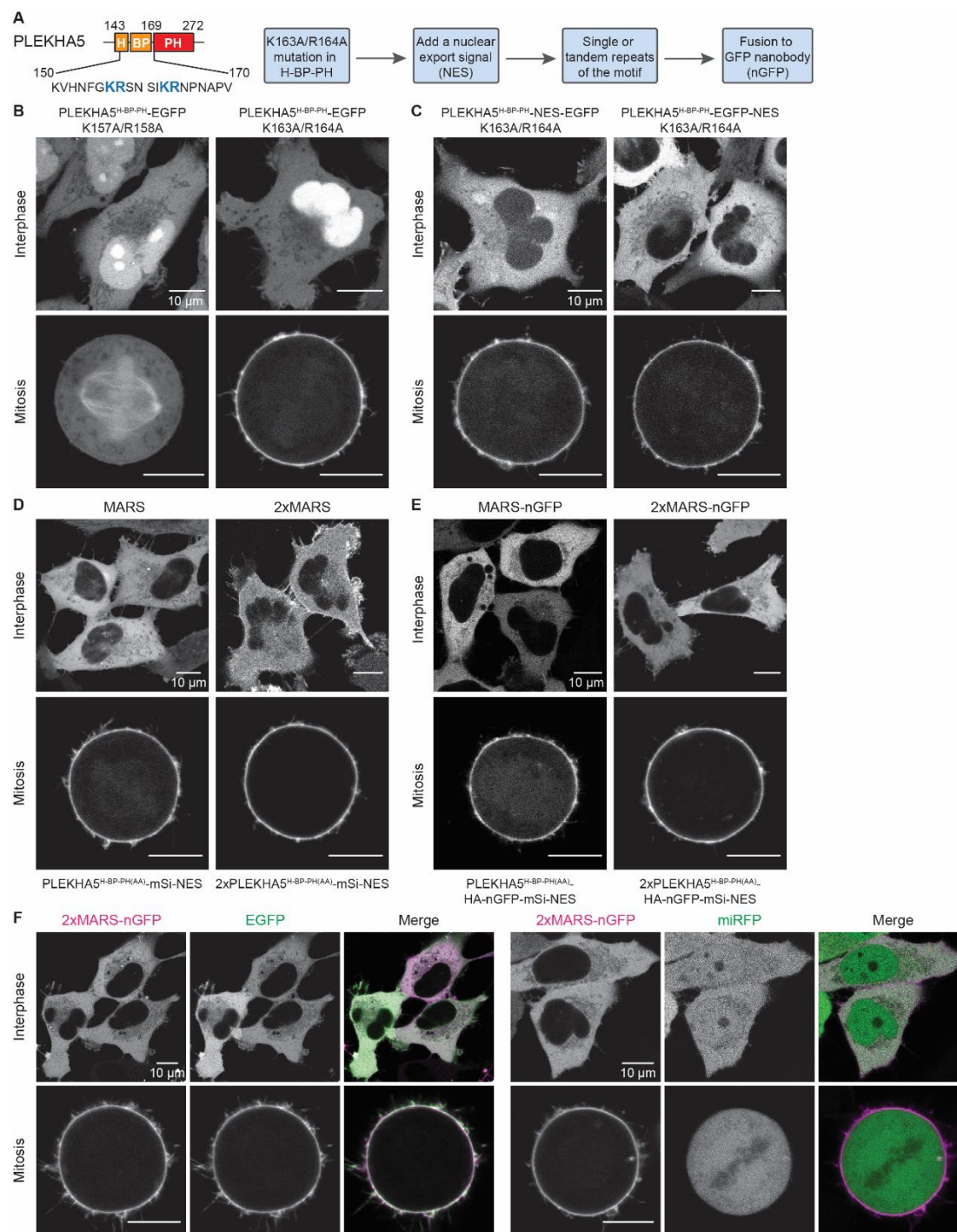


Figure 4.1. Design and engineering of Mitosis-enabled Anchor-away/Recruiter System (MARS), based on the H-BP-PH motifs of PLEKHA5. (A) Domain map of the PLEKHA5 H-BP-PH motifs with K157, R158, K163, and R164 highlighted in blue, and flow chart of protein engineering workflow to generate MARS. (B) Live-cell imaging of HeLa cells expressing different mutants of PLEKHA5. (C) Localization of PLEKHA5^{H-BP-PH}-GFP with addition of a nuclear export signal (NES) at two different

positions. (D, E) Live-cell confocal microscopy to examine subcellular localization of the single and tandem MARS constructs without (D) or with (E) fusion to a GFP nanobody. (F) 2xMARS-nGFP is capable of recruiting GFP (left panel) but not mRFP (right panel) to the PM during mitosis.

The module can be fused directly to protein cargoes or to a GFP nanobody (nGFP) for the recruitment of GFP-tagged proteins. Because of the latter option, we replaced the GFP visualization tag with the red fluorescent protein mScarlet-i (mSi) in the optimized constructs, termed MARS (PLEKHA5^{H-BP-PH(AA)}-mSi-NES) and MARS-nGFP (PLEKHA5^{H-BP-PH(AA)}-HA-nGFP-mSi-NES). Finally, to enhance avidity for the PM and allow for potential alterations in affinity conferred by cargo fusion/binding, we also constructed tandem repeats termed 2xMARS and 2xMARS-nGFP. Imaging analysis established the expected cytosolic-to-PM shift in localization between interphase and mitosis (Figures 4.1D, E and Appendix Figure A1c, d). Finally, we demonstrated the functionality of 2xMARS-nGFP, which recruited soluble GFP but not monomeric infrared fluorescent protein (mRFP) to the PM during mitosis (Figure 4.1F).

4.2.2 MARS Can Recruit Multiple Functional Enzymatic Cargoes to the PM during Mitosis

We next evaluated the general ability of MARS to mediate mitosis-conditional recruitment of functional enzyme cargoes to the PM (Figure 4.2A). First, we selected polo-like kinase 1 (PLK1), a critical regulator of mitosis with multiple important non-PM localizations during both interphase and mitosis. We expressed 2xMARS-nGFP,

chosen over MARS-nGFP due to its low cytoplasmic background during mitosis (Figure 4.1E), in cells expressing endogenously tagged PLK1-GFP. Control cells transfected with nGFP-free MARS exhibited expected PLK1-GFP localization at centromeres, midbody, mitotic kinetochores, and spindle poles¹⁸ (Figure 4.2B). Expression of 2xMARS-nGFP, however, induced PM recruitment of a substantial pool of PLK1-GFP exclusively during mitosis and not during interphase (Figure 4.2B).

We next tested a phospholipase D from *Streptomyces sp.* PMF (PLD_{PMF}), which converts the abundant phospholipid phosphatidylcholine (PC) to the signaling lipid phosphatidic acid (PA) and which we have harnessed for use as an optogenetic membrane editor for controlled phospholipid modification in mammalian cells^{19–21}. Direct fusion of 2xMARS to PLD_{PMF} (with mCherry in place of mSi) led to complete PM recruitment during mitosis (Figure 4.2C), whereas fusion of a single MARS motif led to only partial PM recruitment (Appendix Figure C2a). We then assessed whether such PLD recruitment induced membrane editing of PC to PA in mitosis by measuring the enzymatic activity of 2xMARS-PLD_{PMF} in asynchronous or mitotic cells using our previously developed chemoenzymatic method, IMPACT, coupled to flow cytometry^{20,22}.

Indeed, 2xMARS-PLD_{PMF} exhibited significantly higher IMPACT fluorescence in prometaphase cells compared to asynchronous cells, and a catalytically dead form (bearing the H170A mutation in PLD_{PMF}) exhibited low IMPACT signal at all cell cycle stages, demonstrating the specificity of the IMPACT assay to MARS-associated PLD

activity (Figure 4.2D). These results show that direct fusion of 2xMARS to PLD_{PMF} does not impair its catalytic function and permits mitosis-specific PM recruitment.

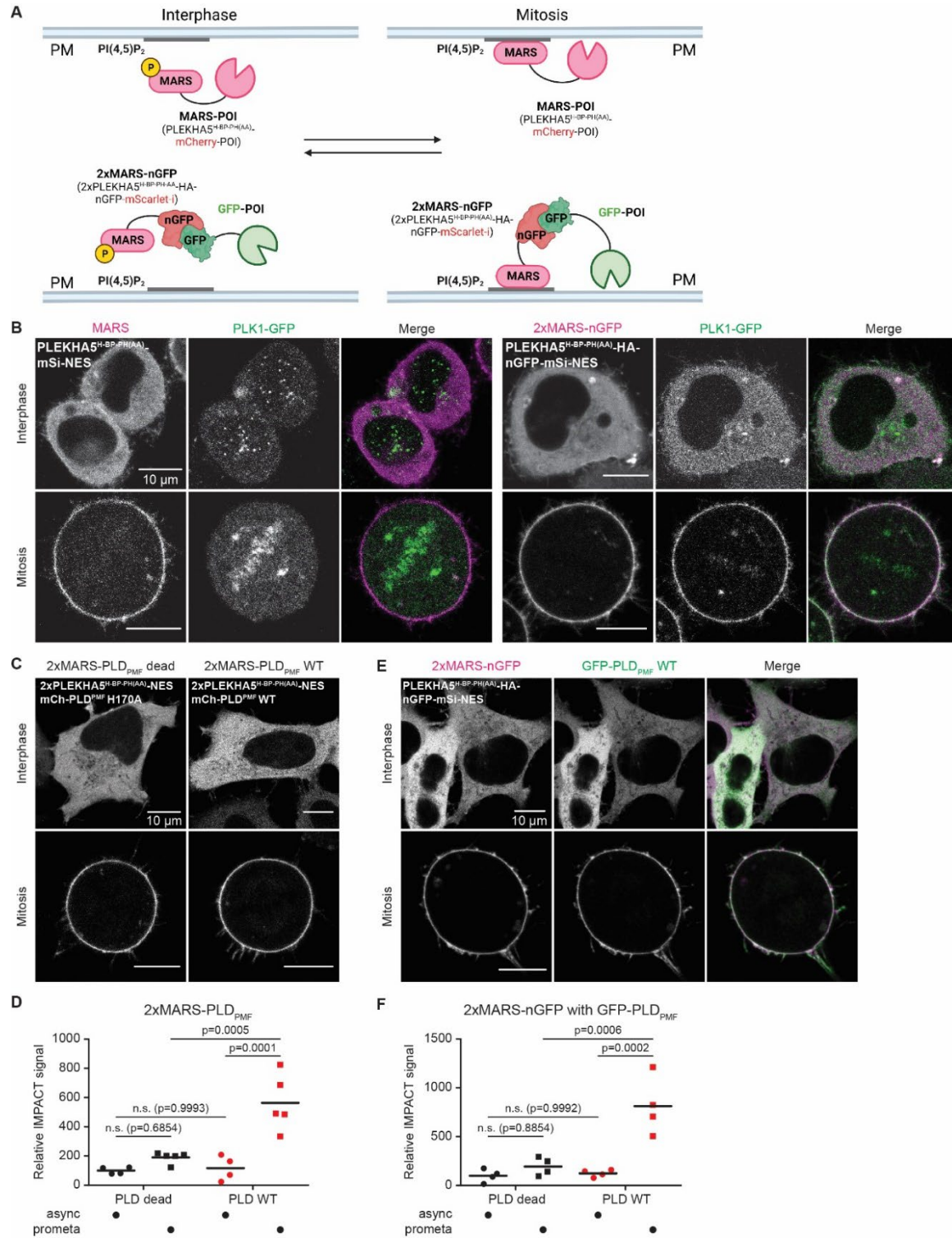


Figure 4.2. MARS recruits polo-like kinase 1 (PLK1) and phospholipase D (PLD) to the PM specifically during mitosis. (A) Cartoon illustration of the working principle of MARS for either direct fusion (top) or nGFP-based recruitment (bottom) of proteins of interest (POI) to the PM during mitosis. Cartoon is generated using BioRender. (B) 2xMARS-nGFP (magenta) anchored PLK1-GFP (green, endogenously tagged) at the PM during mitosis in HeLa cells. (C-D) Tandem MARS fusion to PLD from *Streptomyces* sp. PMF (PLD_{PMF}) enabled mitosis-induced PM recruitment of functional PLD_{PMF}. (C) Live-cell confocal microscopy of HeLa cells expressing catalytically dead (H170A) or WT 2xMARS-PLD_{PMF} in interphase and mitosis. (D) Measurements of relative activity of 2xMARS-PLD_{PMF} variants in asynchronous or isolated prometaphase cells measured by IMPACT labeling followed by flow cytometry demonstrate elevated activity of WT but not catalytically dead PLD concomitant with its selective recruitment to the PM during mitosis (n=4 biological replicates, one-way ANOVA with Sidak post hoc test). (E-F) 2xMARS-nGFP recruited active GFP-PLD_{PMF} to the PM during mitosis. (E) Representative confocal micrographs of HeLa cells transfected with 2xMARS-nGFP (magenta) and WT GFP-PLD_{PMF} (green). (F) Measurements of relative activity of GFP-PLD_{PMF} variants in asynchronous or isolated prometaphase cells measured by IMPACT labeling followed by flow cytometry demonstrate elevated activity of WT but not catalytically dead PLD concomitant with its selective recruitment to the PM during mitosis (n=4 biological replicates, one-way ANOVA with Sidak post hoc test).

Alternatively, the use of 2xMARS-nGFP to recruit exogenously expressed GFP-PLD_{PMF} to the PM during mitosis was also successful (Appendix Figure C2b and Figure 4.2E). Again, PLD_{PMF} exhibited mitosis-specific membrane editing activity as assessed by IMPACT (Figure 4.2F), illustrating effective recruitment of a functional GFP-tagged lipid-modifying enzyme by 2xMARS-nGFP.

A third protein cargo we applied MARS to was Class I phosphoinositide 3-kinase (PI3K), which phosphorylates PI(4,5)P₂ to form PI(3,4,5)P₃²³. Following precedent for optogenetic PI3Ks²⁴, we fused MARS to an mCherry-tagged inter-Src homology 2 (iSH2) domain of the PI3K regulatory subunit p85 (MARS-p85^{iSH2}), which can heterodimerize the endogenous PI3K catalytic subunit, p110⁴. MARS-p85^{iSH2} expression recruited a functional Class I PI3K to the PM and induced formation of

PI(3,4,5)P₃ at this membrane only during mitosis, visualized using the PI(3,4,5)P₃ biosensor GFP-Akt^{PH}, with MARS-p85^{iSH2}-induced PI(3,4,5)P₃ levels significantly higher than basal PI(3,4,5)P₃ levels in control cells expressing MARS only (Figures 4.3A, B). In contrast, the tandem 2xMARS-p85^{iSH2} exhibited undesired PM association during interphase, illustrating the importance of having both the single and tandem constructs as options for MARS-cargo design (Appendix Figure C2c). Again, the nGFP-based system worked as an alternate method for PM recruitment of PI3K during mitosis. Here, we co-expressed GFP-p85^{iSH2} or GFP only with 2xMARS-nGFP and a PI(3,4,5)P₃ biosensor, Akt^{PH}-mRFP670 and observed expected mitotic cargo recruitment for GFP-p85^{iSH2} and GFP but PI(3,4,5)P₃ synthesis only upon mitotic GFP-p85^{iSH2} recruitment (Figures 4.3C, D). These studies demonstrate that both direct fusion and the nGFP-based system can enable conditional PI3K recruitment and PI(3,4,5)P₃ synthesis at the PM during mitosis.

4.2.3 MARS-p85^{iSH2} Reveals an Inhibitory Effect of a PM PI(3,4,5)P₃ Pool on Early Mitosis Progression

PM-localized phosphoinositides (PI4P, PI(4,5)P₂, and PI(3,4,5)P₃) play several key roles throughout cell division, including cell rounding beginning in prophase, spindle orientation during metaphase, and furrow ingression during cytokinesis²⁵. These functions have been investigated largely by genetic, pharmacological, and chemogenetic methods for chronically or acutely manipulating PIP levels that are often employed in conjunction with harsh cell synchronization methods that can

perturb physiology^{26–28}. MARS presents an opportunity to manipulate PM PI(3,4,5)P₃ levels in bulk cell populations without synchronization, prompting us to investigate whether MARS-p85^{SH2} might reveal new mitotic roles for PI(3,4,5)P₃.

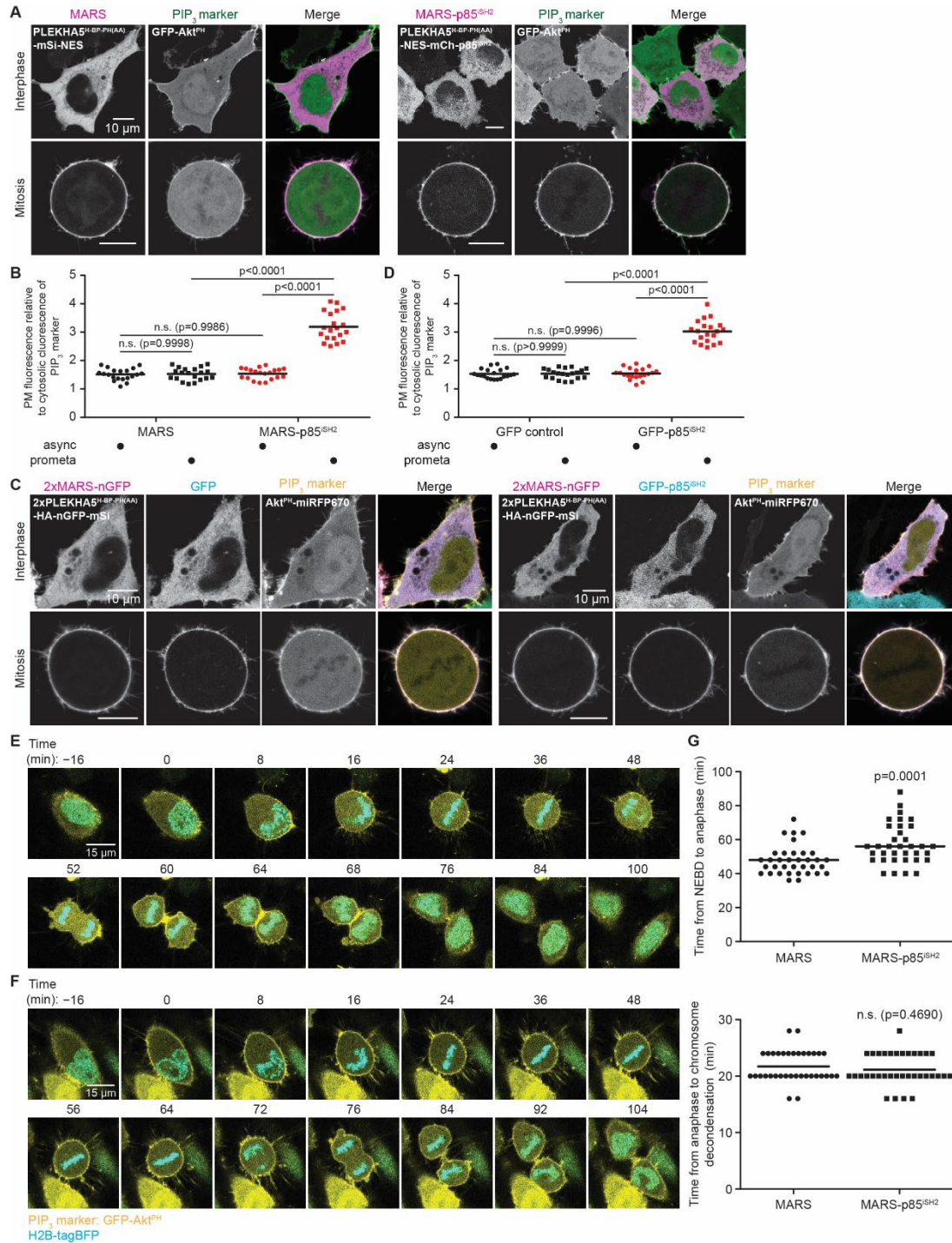


Figure 4.3. MARS-mediated mitotic PM recruitment of Class I PI3K reveals effects of excessive PI(3,4,5)P₃ production at the PM during mitosis. (A, B) Single MARS fusion to p85^{iSH2} was sufficient to recruit endogenous PI3K to the PM to generate PI(3,4,5)P₃ during mitosis. (A) Confocal microscopy of HeLa cells expressing MARS (control, magenta) or MARS-p85^{iSH2} (magenta) along with the PI(3,4,5)P₃ biosensor GFP-Akt^{PH} (green). (B) Quantification of the PM to cytosolic fluorescence ratios of GFP-Akt^{PH} as a readout for measuring PM PI(3,4,5)P₃ levels (n=20 cells, ANOVA with Sidak post hoc test). (C, D) 2xMARS-nGFP recruited GFP-p85^{iSH2} to the PM during mitosis and produced PI(3,4,5)P₃. (C) Representative micrographs of HeLa cells transfected with 2xMARS-nGFP (magenta), GFP only or GFP-p85^{iSH2} (cyan), and an mRFP-tagged PI(3,4,5)P₃ biosensor, Akt^{PH}-mRFP670 (yellow). (D) Quantification of the PM to cytosolic fluorescence ratios of Akt^{PH}-mRFP670 as the measure for PM PIP₃ level (n = 20 cells, ANOVA with Sidak post hoc test). (E-G) Excess PM PI(3,4,5)P₃ levels during mitosis caused a delay in mitosis progression through anaphase. (E-F) Representative still images from time-lapse movies in asynchronous HeLa cells stably expressing H2B-tagBFP and transfected with either MARS (E) or MARS-p85^{iSH2} (F), and GFP-Akt^{PH}. Images display merged channels of H2B-tagBFP (cyan) and GFP-Akt^{PH} (yellow). Numbers indicate time before/after nuclear envelope breakdown (defined as t=0 min). (G) Quantification of mitosis progression in asynchronous HeLa cells stably expressing H2B-BFP and transfected with GFP-Akt^{PH} and either MARS or MARS-p85^{iSH2}, with time from NEBD to chromosome separation at anaphase onset (top) and from anaphase onset to chromosome decondensation in cytokinesis (bottom) plotted as scatter plots (n=36 cells, Mann–Whitney U test).

We performed live-cell, time-lapse microscopy of HeLa cells expressing H2B-tagBFP to mark chromosomes, GFP-Akt^{PH} to visualize PI(3,4,5)P₃, and either MARS-p85^{iSH2} or MARS as a negative control, over 14 hours. As expected, MARS and MARS-p85^{iSH2} exhibited PM localization from NEBD until telophase and otherwise cytosolic localization (Appendix Figure C2d, e). GFP-Akt^{PH} localization in control cells expressing MARS revealed homeostatic PI(3,4,5)P₃ pools that did not exhibit significant PM enrichment until telophase and cytokinesis, where fluorescence was observed at the cell periphery and between the two daughter cells, consistent with previous observations (Figure 4.3E)²⁶. In MARS-p85^{iSH2}-expressing cells, GFP-Akt^{PH}

displayed elevated PM signal starting from NEBD, confirming mitosis-induced PI(3,4,5)P₃ production at the PM (Figure 4.3F).

Quantification of the duration of the early stages of mitosis, i.e., from NEBD during prophase to chromosomal separation at anaphase onset, revealed a significant increase in the time needed to enter anaphase in cells with excess PI(3,4,5)P₃ (Figure 4.3G). However, a similar delay was not observed for mitotic exit, i.e., from anaphase onset to chromosomal decondensation during telophase and cytokinesis (Figure 4.3G). Therefore, overproduction of PI(3,4,5)P₃ on the PM during mitosis impairs regular mitosis progression through anaphase, but not afterward, potentially due to improper spindle orientation triggered by the presence of excess PI(3,4,5)P₃, as first proposed by studies employing chronic perturbations such as PTEN knockdown²⁶. These results reveal distinct effects for PM PI(3,4,5)P₃ at different stages of mitosis and, more broadly, showcase the utility of MARS for conditional modulation of signaling events occurring at the PM during mitosis with high spatiotemporal precision and without synchronization or other exogenous perturbations.

4.3 DISCUSSION

Inspired by this dynamic PM localization of PLEKHA5, we engineered a Mitosis-enabled Anchor-away/Recruiter System, MARS, for conditional PM recruitment of protein cargoes during mitosis. MARS is based on a 129 amino acid (15 kDa) PM binding motif from PLEKHA5 but containing additional modifications to ensure exclusive cytosolic localization during interphase and PM localization during mitosis.

Versatility is built into MARS in two ways. First, single and tandem (2xMARS) modules were constructed to provide options for low- and high-affinity PM localization, with the tandem modules particularly useful for larger, bulkier cargoes that might attenuate membrane translocation. Second, MARS can be either fused directly to the protein cargo or a chimeric protein containing MARS fused to a GFP nanobody (MARS-nGFP) that can recruit GFP-tagged proteins. Collectively, we demonstrated the ability of various MARS constructs to efficiently recruit three different functional cargoes to the PM during mitosis, including an endogenously tagged protein kinase (PLK1) and two lipid-modifying enzymes (PLD and PI3K) capable of editing PM lipid content to produce phosphatidic acid and PI(3,4,5)P₃ respectively. Our studies in developing mitotic PM recruitable versions of these proteins highlight the importance of both the direct fusion vs. GFP nanobody and single vs. tandem MARS options in construct design.

One application of MARS is for anchor-away of endogenously tagged cytoplasmic proteins with important mitotic functions from their endogenous location(s) to the PM, as we demonstrated for PLK1. Upon mitosis onset, rapid MARS-mediated knock sideways could enable phenotypic and mechanistic investigation downstream of the mitosis-specific loss-of-function, or more accurately loss-of-localization, for these proteins. Removing the NES from MARS would expand its application to nuclear proteins while allowing such cargoes to maintain their proper nuclear localization during interphase. Therefore, MARS represents an approach for functional depletion of enzymes that complements genetic knockdown, pharmacological inhibition, and other stimulus-triggered anchor-away strategies, with the advantage that its onset is

highly temporally controlled, and it does not require perturbative synchronization methods for implementation in bulk populations.

A second type of application for MARS is to spatiotemporally modify PM lipid metabolism and signaling pathways during mitosis, e.g., by acute production of two anionic signaling lipids, PA and PI(3,4,5)P₃. Notably, our studies using MARS to specifically elevate PI(3,4,5)P₃ levels at the PM during mitosis revealed a selective effect of excess PI(3,4,5)P₃ in delaying mitotic progression up to the spindle checkpoint, but not afterward. Importantly, these cells were otherwise unperturbed, highlighting the ability of MARS to mediate acute manipulation of lipid metabolism during mitosis with a high degree of spatiotemporal control and without synchronization or other external stimuli. We envision application of MARS for spatiotemporally controlled editing of additional lipids or manipulation of signaling pathways at the PM to investigate mitosis-specific functions.

In sum, we identify and characterize a phosphorylation switch that governs the PM-cytoplasmic cycling of a multidomain adaptor, PLEKHA5, and we then engineer a minimal motif from this protein into a module termed MARS that facilitates selective recruitment of protein cargoes to the PM exclusively during mitosis in otherwise unperturbed cells. MARS represents a versatile and tunable platform for mediating protein anchor-away to rapidly extract proteins from their native localizations to the PM during mitosis, and it can also facilitate conditional recruitment of lipid-metabolizing enzymes to the PM for mitosis-selective membrane editing to precisely manipulate lipid composition and signaling. Given that many proteins exhibit dynamic

localization changes that accompany natural oscillations in their posttranslational modification and/or binding interactions with other biological molecules, the self-contained nature of MARS, whose translocation exploits endogenous signaling events, will hopefully inspire the development of additional tools for conditional protein activation as a function of changes to cellular physiological states without need for exogenous triggers.

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CHAPTER 5

CONCLUSIONS AND OUTLOOK

Phosphoinositides are a class of phospholipids with important signaling functions. Residing in the cytosolic leaflets of different cellular membranes, these molecules present their headgroups to engage various types of “reader” proteins and participate in the regulation of myriads physiological and phenotypic outcomes^{1,2}. Pleckstrin Homology (PH) domain-containing proteins are the largest family of phosphoinositide-interaction proteins with more than 250 members, but the cellular properties and molecular functions of the majority of these proteins remain to be explored²⁻⁴. The Baskin lab has focused on investigating the PLEKHA subfamily of PH domain-containing protein PLEKHA4-7. Several members of the PLEKHA subfamily have been reported to serve as adaptors for other proteins or protein complexes at the plasma membrane⁵ and the microtubule cytoskeletons⁶, regulating their subcellular localizations and biological functions. This dissertation describes the mechanistic investigations and protein engineering of a poorly studied member within this subfamily, PLEKHA5.

In chapter 2, we outlined the phenotypic characterization and mechanistic investigation governing the constitutive microtubule localization and mitosis-specific plasma membrane (PM) association of PLEKHA5. We discovered that the N-terminal tandem WW domains mediate the microtubule localization of PEKHA5, and the H-BP-PH motif binds to PI(4,5)P₂ to recruit PLEKHA5 to the PM. While mechanistically, we demonstrated that the cell cycle-dependent PM association of PLEKHA5 was

regulated by a phosphorylation switch, much is yet to be studied about the purpose of this PM translocation. One way to approach this question is to look for other proteins displaying increased PM binding during mitosis, which can be achieved via proximity labeling to probe for the changes in the PM proximal proteome as a function of different cell cycle stages. By cross checking with the PLEKHA5 interactome, we could identify protein binding partners co-enriched at the PM with PLEKHA5 during mitosis, and the revelation of such interactions could help to elicit other unidentified roles of PLEKHA5 in mitosis regulation.

Following the observation of the microtubule (MT) localization of PLEKHA5, in chapter 3 we identified PLEKHA5 as an interacting partner of APC/C and established its functional role as a spatial regulator of APC/C activity. In contrast to the temporal control of APC/C-mediated polyubiquitination, which has been extensively studied⁷⁻⁹, the factors and mechanisms that control the dynamic localizations of APC/C remain elusive¹⁰, due in part to difficulties in determining APC/C localizations using conventional imaging-based methods such as IF and (even endogenous) fluorescent protein tagging¹¹⁻¹³. To overcome these challenges and to test the hypothesis that PLEKHA5 mediates the spatial organization of APC/C, we developed a proximity biotinylation approach using microtubule targeted TurboID constructs to assess endogenous protein localizations to the microtubule cytoskeleton. Indeed, we detected biotinylation of PLEKHA5, APC/C, CDC20 and relevant APC/C substrates, confirming that these proteins localize to or very near to microtubules and demonstrating the utility of these MT-TurboIDs as versatile “in vivo biochemistry” tools for examining the microtubule localization of endogenous proteins of interest. The two

MT-TurboIDs (DCX-TurboID and EMTB-TurboID) appear to decorate the entire microtubule network rather than specific locations, such as the centrosomes/spindle poles and kinetochores, to which APC/C can localize^{14–17}. Additional TurboID constructs localized to these specific substructures within the microtubule network^{18–21}, and different types of microtubule fibers including kinetochore fibers, non-kinetochore fibers, and astral fibers, may in the future be useful for more granular sampling of APC/C localizations and APC/C composition and phosphorylation status at such locations which may be critical for modulating APC/C activity and functions in mitosis^{22,23}.

In addition to studying PLEKHA5's function in APC/C-related ubiquitination, we have started to explore additional roles of PLEKHA5 in more broadly regulating ubiquitination pathways through its interaction with two Cullin-RING E3 ligase substrate adaptors identified in the SILAC proteomics experiment, KCTD3 (K-potassium Channel Tetramerization Domain Containing 3) and its closely related paralog SHKBP1 ((SH3-Domain Kinase Binding Protein 1)-Binding Protein 1) (Figure 5.1 and Appendix Table B1).

We validated the interaction of GFP-PLEKHA5 with mScarlet-i-KCTD3 and HA-SHKBP1, by co-IP, followed by western blotting (Figure 5.1B). To map the interacting regions, we performed co-IP of endogenous KCTD3 and HA-SHKBP1 with several GFP-tagged constructs corresponding to truncated forms of PLEKHA5 or isolated PLEKHA5 domains (Figure 3.1E and Appendix Figure B1a). The GFP fusion to the tandem WW domain (WW) (EGFP-PLEKHA5^{WW}) and the N-terminal fragment WW-

H-BP-PH (EGFP- $\text{PLEKHA5}^{\text{WW-H-BP-PH}}$) exhibited interactions with KCTD3 (Figure 5.1C). For SHKBP1, the WW domain again showed the strongest association, with the C-terminal regions (EGFP- $\text{PLEKHA5}^{\text{CC-IDR}}$) also displayed decent interaction with HA-SHKBP1 (Figure 5.1D). These results suggest that the WW domain of PLEKHA5 is most likely to mediate the interaction with KCTD3 and SHKBP1.

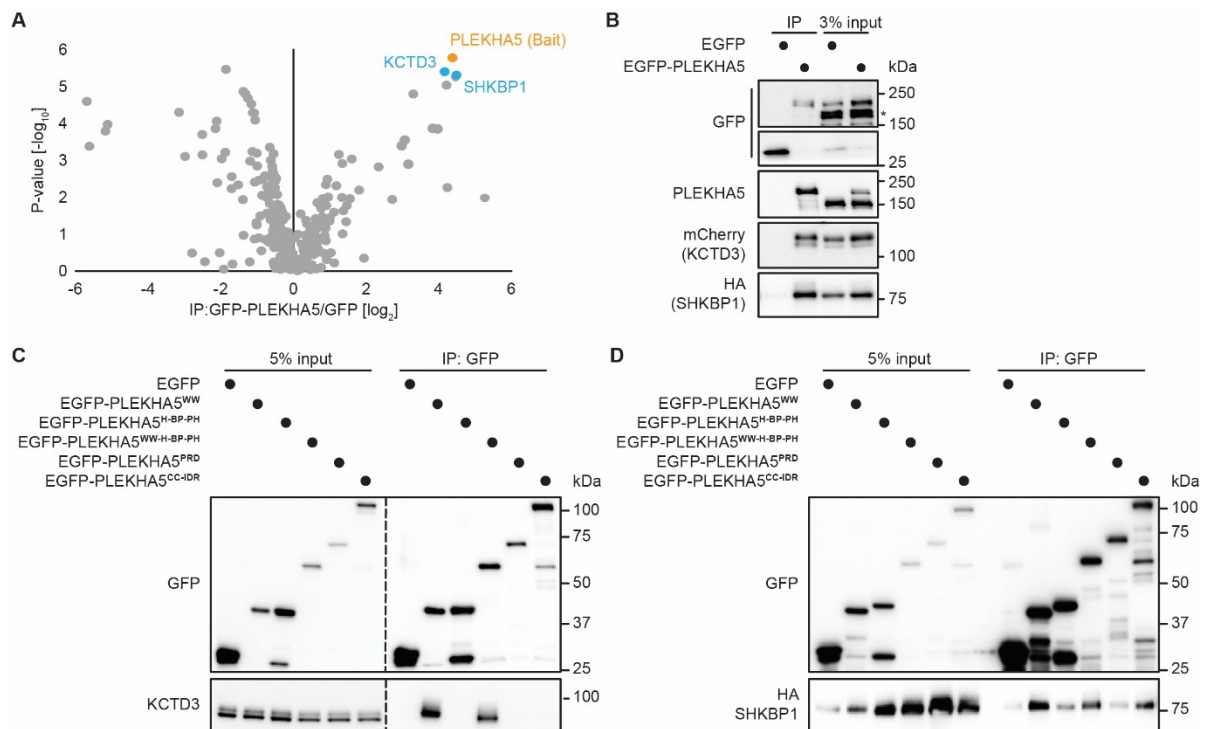


Figure 5.1. PLEKHA5 interacts with KCTD3 and SHKBP1 through the N-terminal tandem WW domain. (A) Volcano plot from SILAC MS proteomics experiments to determine the PLEKHA5 interactome, where proteins that are statistically significantly enriched in α-GFP immunoprecipitates from GFP- PLEKHA5 -expressing HeLa cells compared to the IPs from GFP-expressing cells, including KCTD3 and SHKBP1 highlighted in blue, appear on the top right. Statistical significance was determined by a Mann–Whitney U test. (B) Western blot analysis of co-IP experiments for GFP-tagged PLEKHA5 to demonstrate the interaction between GFP- PLEKHA5 and mScarlet-i-KCTD3 and HA-SHKBP1. Cells were co-transfected with all three plasmids, and the lysates were subject to anti-GFP IP. Non-specific bands from the GFP blot are marked by an asterisk. (C,D) Truncation studies to reveal the PLEKHA5 PRD as sufficient to interact with endogenous KCTD3 (C) and HA-SHKBP1 (D). Note: Data shown in part D was obtained by Shun Enomoto.

KCTD3 and SHKBP1 are members of the KCTD family of BTB (Bric-à-brac, Tramtrack, Broad complex) domain-containing proteins, which are named after the hallmark BTB fold at the N-terminus of these proteins²⁴. Of the 25 proteins within the KCTD family, KCTD3 and SHKBP1 are most closely related and they are the only two members harboring an 8x-WD40 repeats downstream of the BTB domain (Figure 5.2A)^{24,25}. One important cellular function the BTB proteins carry out are to serve as substrate recruiting adaptors for Cullin 3-RING E3 ligases (CRL3), where the BTB domains mediate the interactions with the N-terminal region of Cullin 3 (CUL3) and the rest functional domains are the substrate recognition regions that impart substrate specificity for different adaptors²⁶. Although KCTD3 and SHKBP1 are implied as substrate adaptors for CRL3, association of CUL3 with either KCTD3 or SHKBP1 has not been demonstrated, nor have their substrates been revealed²⁷.

To determine the CUL3 interactions, we performed co-IP of HA-CUL3 with GFP-tagged KCTD3 and SHKBP1, along with KLHL12 (Kelch Like Family Member 12), a BTB protein which has been shown to recruit DVL3 (Dishevelled Segment Polarity Protein 3) to CUL3 ubiquitin ligase complex and therefore serve as our positive control²⁸. Indeed, all three GFP fusion proteins demonstrated binding to HA-CUL3 (Figure 5.2C). A previous report pointed out that an aromatic residue within the BTB domain, Y74 in another BTB protein KBTBD8 (Kelch Repeat and BTB Domain Containing 8), was critical for its association with CUL3²⁹. Structural comparison in PyMOL revealed that KBTBD8 Y74 corresponds to Y57 in KLHL12, F43 in KCTD3, and F44 in SHKBP1 within the BTB folds (Figure 5.2B). Mutation of these aromatic residues to alanine reduced HA-CUL3 co-IP (Figure 5.2C), indicating that the BTB

domains were important for mediating CUL3 interactions with KLHL12, KCTD3 and SHKBP1. To further characterize KCTD3 and SHKBP1 and the potential cellular functions of their associations with PLEKHA5, we are conducting experiments to determine the identities of ubiquitination substrates for these CRL3 adaptors and examine functional phenotypes of KCTD3 and SHKBP1 knockdown/knockout.

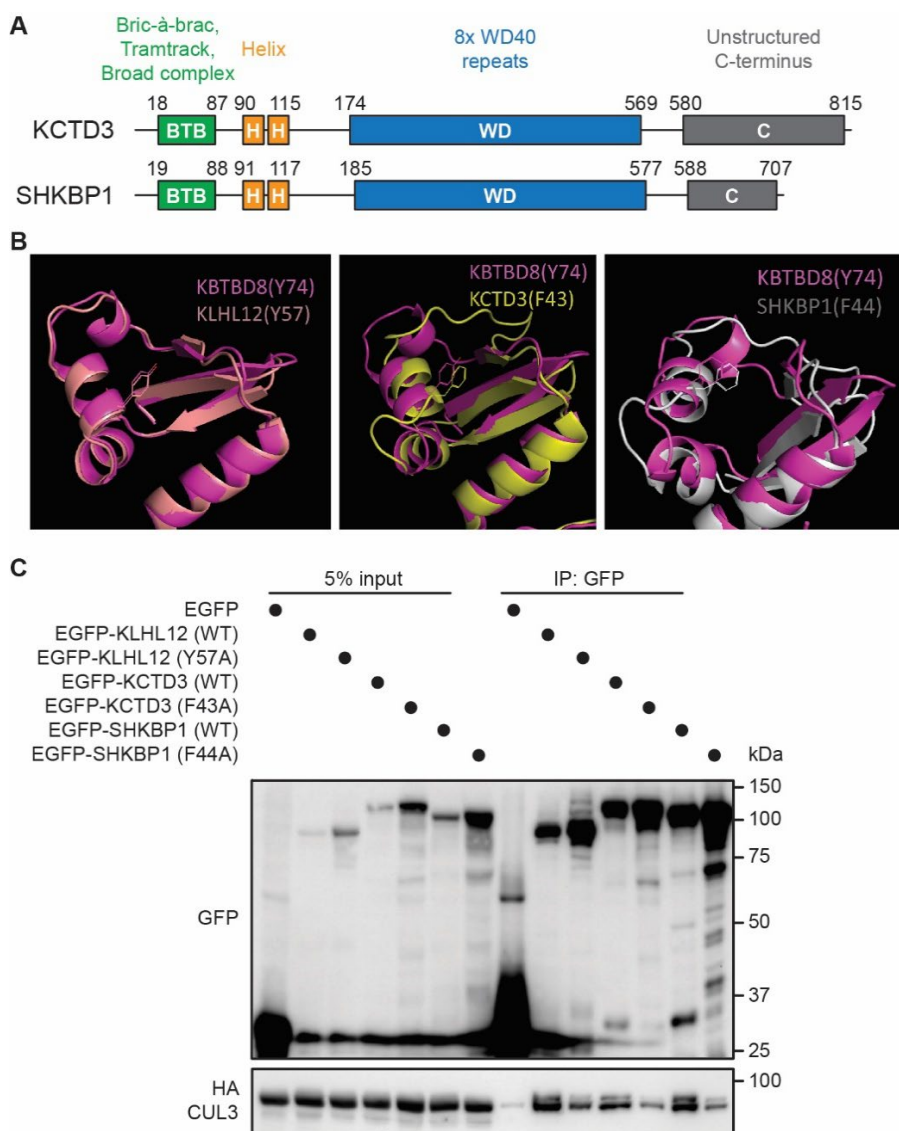


Figure 5.2. KCTD3 and SHKBP1 bind to CUL3. (A) Domain map of KCTD3 and SHKBP1. (B) Structural alignment of KBTBD8 (magenta) with KLHL12 (pink), KCTD3 (yellow), and SHKBP1 (grey). (C) Mutation of an aromatic residue in the BTB domain of KLHL12, KCTD3, and SHKBP1 decreases their interaction with CUL3. Note:

structural comparison and residue mutagenesis were accomplished by Shun Enomoto and Jingyi Hu, western blot was acquired by Lin Luan.

In chapter 4, we harnessed the dynamic PM binding capability of PLEKHA5 characterized in chapter 2, developed the Mitosis-enabled Ancho-away/Recruiter System (MARS), and demonstrated the utility of MARS with three different enzymes. MARS could serve as an alternative approach besides small molecule-based or light-activated methods for PM protein recruitment, with a unique advantage that no exogenous treatments are needed to trigger the PM translocation as mitosis is natural process occurring in every single dividing cell. Applying MARS to other signaling proteins would gain further understanding of connections between PM initiated signaling pathways and mitosis regulation. Further, MARS is compatible with additional engineering to generate other platforms to control signal transduction. For example, MARS can be turned into a “mitosis-off” system, where a MARS-protease fusion construct would release the proteins of interest from the PM and turn off the signaling activities in mitosis. Complementary with regular MARS, this “mitosis-off” platform could help understand the consequences of terminating signaling cascades specifically during mitosis.

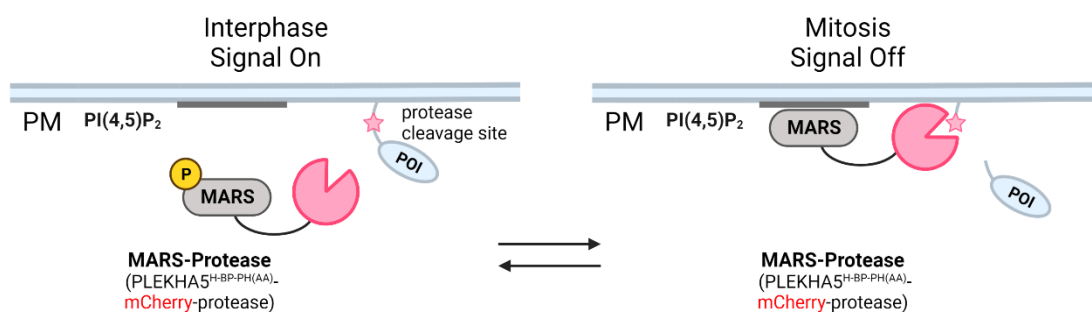


Figure 5.3. Schematic illustration of the “mitosis-off” system.

In summary, the work presented in this dissertation has contributed to a deeper understanding of a previously understudied bifunctional PH domain-containing protein PLEKHA5. We characterized the subcellular localizations of PLEKHA5 at the microtubule cytoskeleton and the plasma membrane, elucidated its cellular function as a microtubule adaptor for spatial organization of the E3 ligase activity of the Anaphase Promoting Complex/Cyclosome, and engineered a mitosis-specific reversible enzyme recruitment platform based on the PM binding motif of PLEKHA5, which represents an addition to the existing repertoire for conditional protein translocation.

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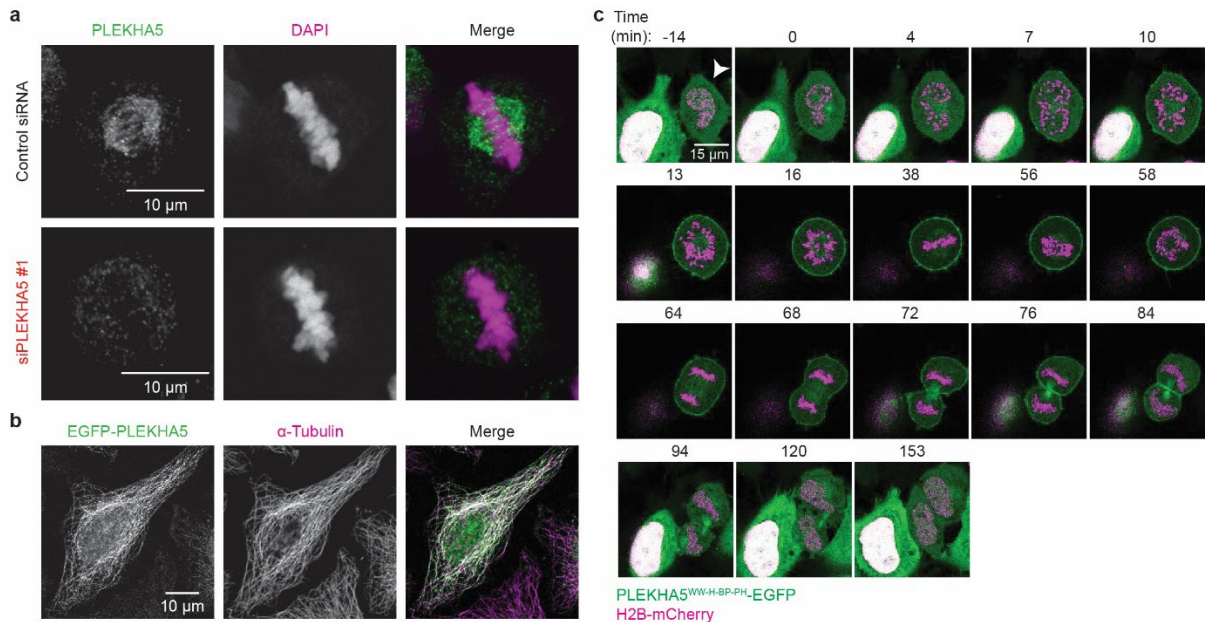
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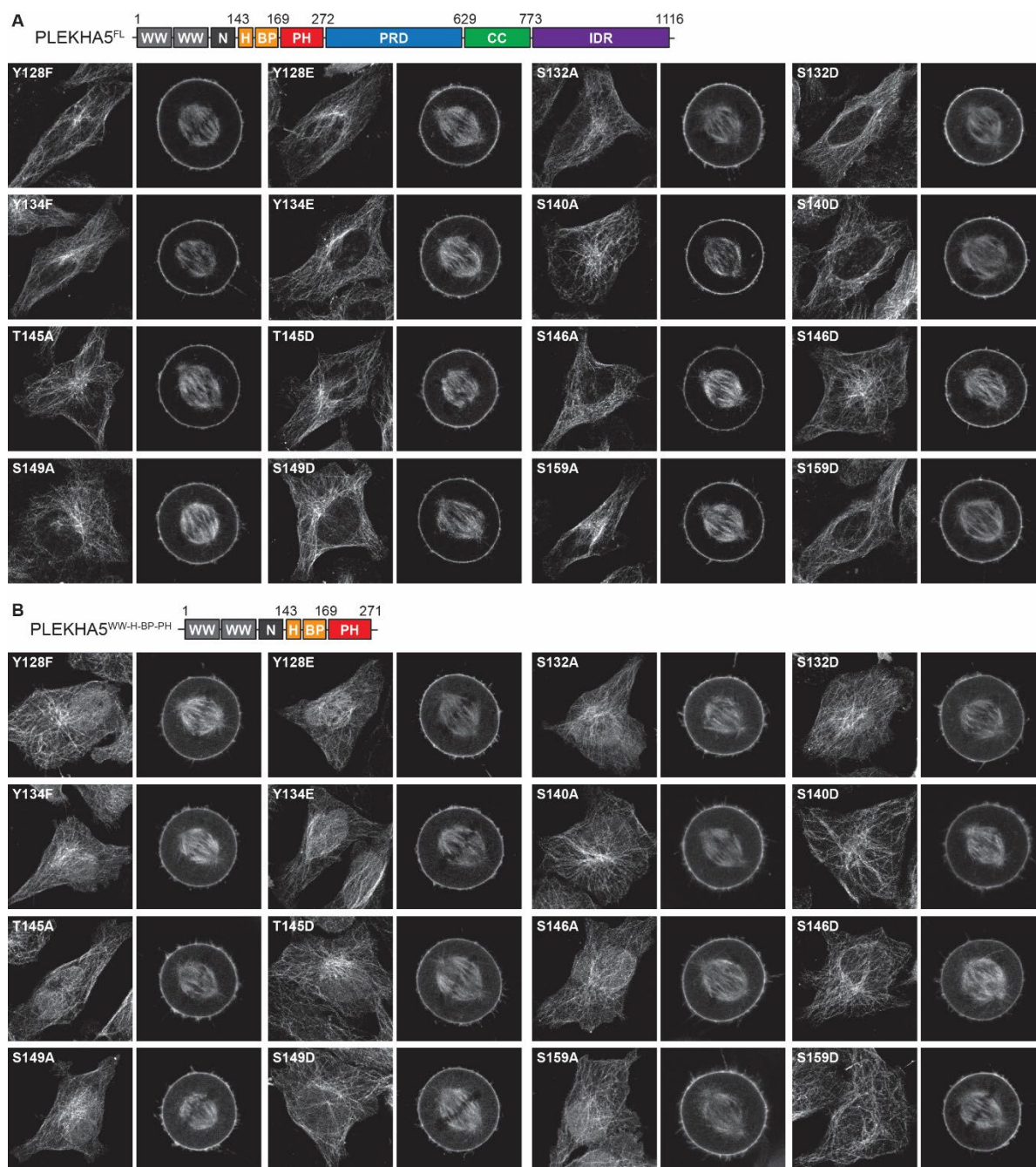
APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER 2

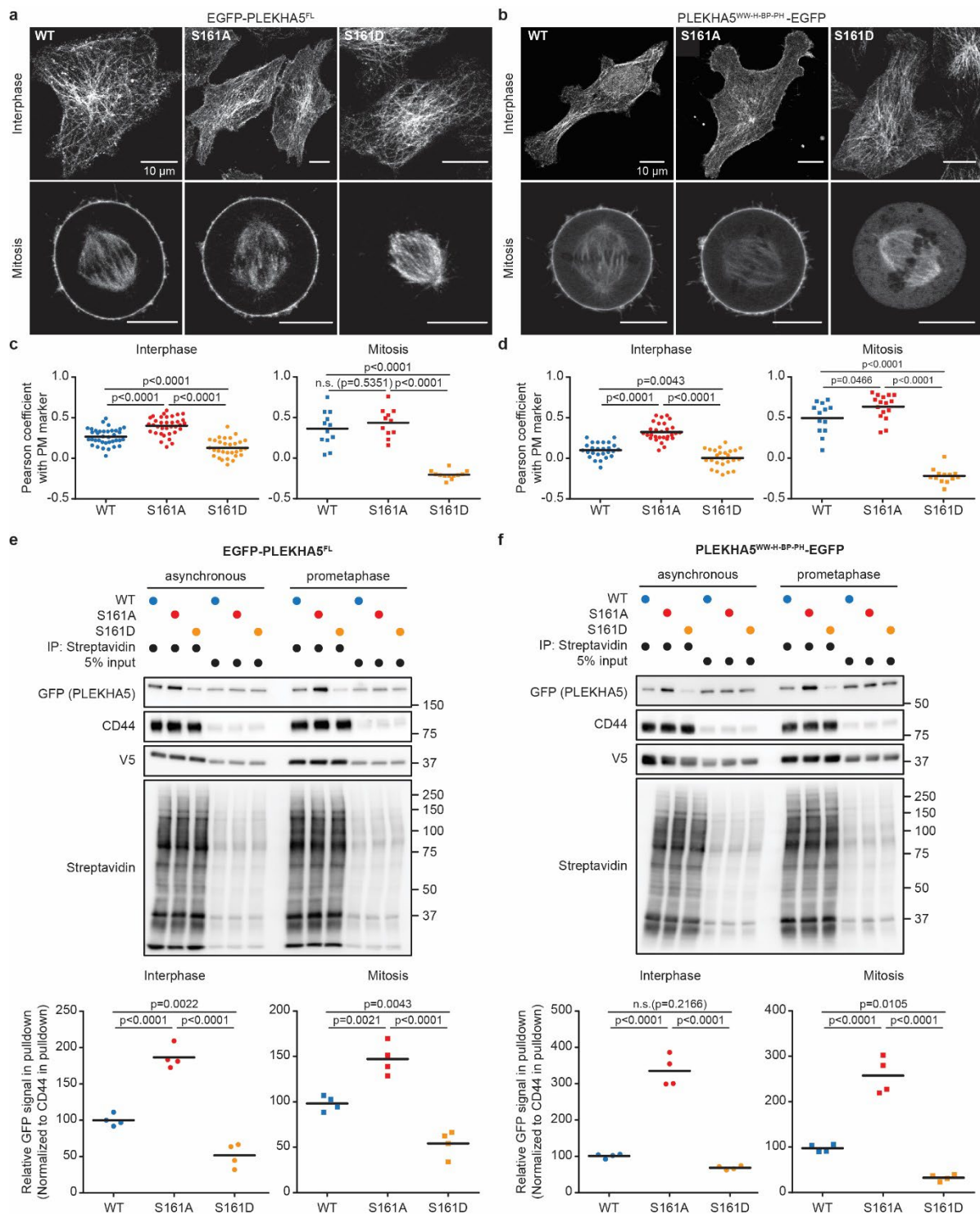
A1 SUPPLEMENTAL FIGURES



Appendix Figure A1 (Related to Figure 2.1). Analyses of PLEKHA5 localization to microtubule cytoskeleton and the plasma membrane. (a) Immunofluorescence of endogenous PLEKHA5 in HeLa cells transfected with an siRNA against PLEKHA5 (siPLEKHA5 #1) or a control siRNA. Signal of PLEKHA5 (green) diminishes upon siPLEKHA5 #1 treatment, demonstrating the efficiency of knockdown and specificity of the antibody against PLEKHA5. (b) Colocalization of GFP-PLEKHA5 with microtubules as detected by anti- α -tubulin immunofluorescence. (c) Representative series of still images from a time-lapse movie in asynchronous, H2B-mCherry-expressing (magenta) HeLa cells transfected with PLEKHA5^{WW-H-BP-PH}-EGFP (green). The numbers on top of the images are the time from nuclear envelope breakdown (t = 0 min) till the images were taken.

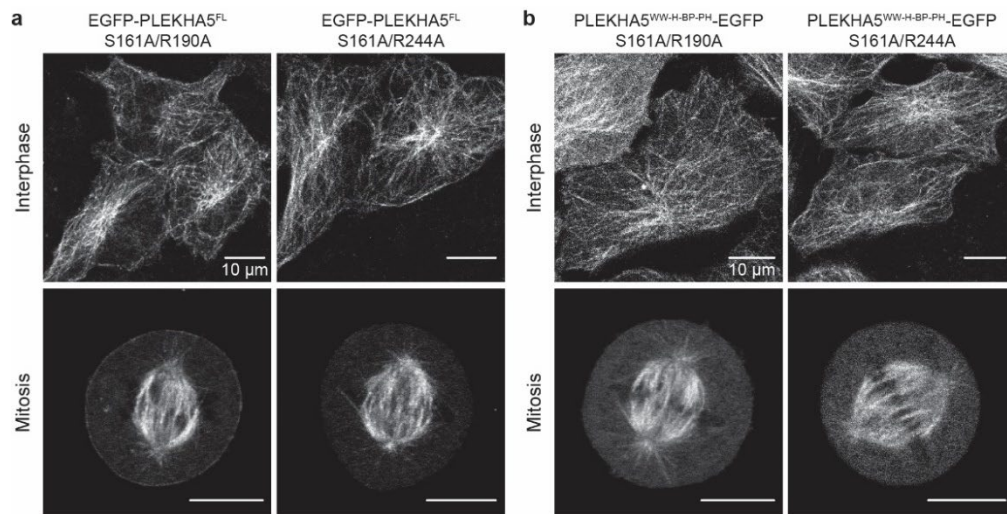


Appendix Figure A3 (related to Figure 2.3). Live-cell imaging of phosphodeficient and phosphomimetic mutants of GFP-tagged PLEKHA5 full-length protein (A) or WW-H-BP-PH fragment (B).

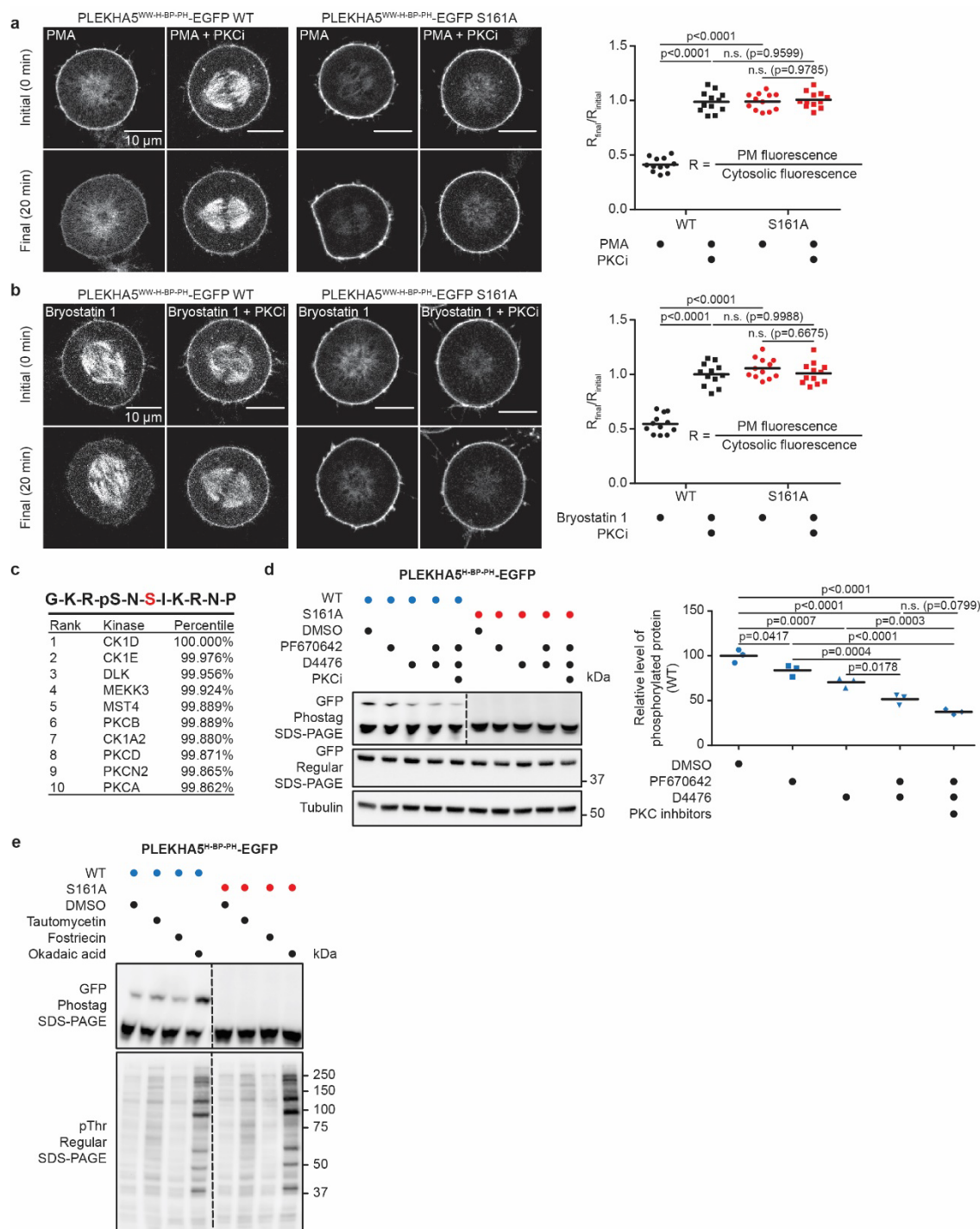


Appendix Figure A4 (Related to Figure 2.3). S161 phosphorylation status affects PM localization of EGFP- $\text{PLEKHA5}^{\text{FL}}$ and $\text{PLEKHA5}^{\text{WW-H-BP-PH}}\text{-EGFP}$. (a,b) Live-imaging of subcellular localizations of WT, S161A, and S161D variant of full-length PLEKHA5 (a) and the WW-H-BP-PH construct (b). (c,d) Quantification of PM

localization for EGFP- $\text{PLEKHA5}^{\text{FL}}$ (c) and $\text{PLEKHA5}^{\text{WW-H-BP-PH}}$ -EGFP (d) using colocalization analysis. Pearson coefficients were computed from images acquired in HeLa cells co-expressing either of the PLEKHA5 constructs with a transfectable PM marker Lyn10-LOV-mCherry. For EGFP- $\text{PLEKHA5}^{\text{FL}}$ in interphase: $n = 37$ cells for WT, 35 cells for S161A, and 30 cells for S161D; in mitosis: $n = 12$ cells for WT and S161D, and 11 cells for S161A. For $\text{PLEKHA5}^{\text{WW-H-BP-PH}}$ -EGFP in interphase, $n = 26$ cells for WT, 29 cells for S161A, and 25 cells for S161D; in mitosis: $n = 13$ cells for WT and S161D, and 16 cells for S161A (one-way ANOVA with Tukey post hoc test). (e.f) Quantification of PM association for EGFP- $\text{PLEKHA5}^{\text{FL}}$ (e) and $\text{PLEKHA5}^{\text{WW-H-BP-PH}}$ -EGFP (f) using PM-targeted proximity biotinylation. Cells stably expressing Lyn10-Turbid-V5 were transfected with WT EGFP- $\text{PLEKHA5}^{\text{FL}}$ or constructs with S161A or S161D mutations. Biotinylated proteins at or proximal to the PM were then enriched by streptavidin agarose pulldown, and the level of GFP-tagged PLEKHA5 WT or mutant proteins in the pulldown samples was analyzed by western blot. Representative western blots are shown on top with quantifications of GFP signals in the streptavidin pulldowns on the bottom ($n = 4$ biological replicates, one-way ANOVA with Tukey post hoc test).



Appendix Figure A5 (related to Figure 2.3). (a,b) Live-cell imaging of HeLa cells expressing combinational mutant of S161A with either R190A or R244A for $\text{PLEKHA5}^{\text{FL}}$ -EGFP (a) and $\text{PLEKHA5}^{\text{H-BP-PH}}$ -EGFP (b).



Appendix Figure A6 (related to Figure 2.5). PKC and CK1 kinase activities contribute to S161 phosphorylation. (a,b) PLEKHA5^{WW-H-BP-PH} displayed reduced PM localization upon activating PKC by PMA (a) and Bryostatin 1 (b). Representative micrographs before and after agonists treatment for each condition are shown on the left, with quantifications of the ratios of PM fluorescence relative to cytosolic fluorescence (excluding microtubule signal) before and after the treatment plotted on

the right (n = 12 cells, one-way ANOVA with Sidak post hoc test). (c) Top 10 kinase candidates for phosphorylating S161 (highlighted in red) if S159 serves as the priming phosphorylation site. (d) Inhibition of CK1 activity decreased S161 phosphorylation. Cells were transfected with WT or S161A mutant of PLEKHA5^{H-BP-PH}-EGFP and treated with CK1 inhibitors as indicated. PF670642 was used at 5 μ M (selective for CK1d and CK1e), and D4476 was used at 100 μ M. Shown are representative western blots and quantifications for Phos-tag and regular SDS-PAGE analysis of PLEKHA5^{H-BP-PH}-EGFP (n = 3 biological replicates, one-way ANOVA with Tukey post hoc test). (e) Phos-tag SDS-PAGE analysis of S161 phosphorylation upon treatment of inhibitors for PPP family of protein Ser/Thr phosphatases.

A2 MATERIALS AND METHODS

A2.1 Antibodies

Mouse anti-PLEKHA5, Santa Cruz Biotechnology (sc-390311, RRID: N/A); rabbit anti-EGFR, Rockland (100-401-149, RRID: AB_2293276); mouse anti-actin, MP Bio (08691001, RRID: AB_2335127); mouse anti-V5, Bio-Rad (MCA1360GA, RRID: AB_567249); mouse anti-CD44, Cell Signaling Technology (3570, RRID: AB_2076465); rabbit anti-EEA1, Thermo Fisher (PA1-063A, RRID: AB_2096819); mouse anti- α tubulin, Sigma-Aldrich (T5168, RRID: AB_477579); rabbit anti-Histone H3, Cell Signaling Technology (4499, RRID: AB_10544537); mouse anti-GFP, Santa Cruz Biotechnology (sc-9996, RRID: AB_627695); mouse anti-mCherry, Novus Biologicals (NBP1-96752, RRID: AB_11034849); rabbit anti-phospho-Threonine/Tyrosine, Cell Signaling Technology (9381, RRID: AB_330301); goat anti-mouse HRP, Jackson ImmunoResearch Labs (115-035-146, RRID: AB_2307392); goat anti-rabbit HRP, Jackson ImmunoResearch Labs (111-035-144, RRID: AB_2307391); donkey anti-mouse Alexa Fluor 488, Invitrogen (A-21202, RRID: AB_141607).

A2.2 Other reagents

Lipofectamine 2000, Invitrogen (11668019); Polybrene Infection/Transfection Reagent, Sigma-Aldrich (TR-1003); GFP-Trap magnetic agarose, ChromoTek (gtma-20); Trypsin Gold Mass Spectrometry Grade, Promega (V5280); D-biotin, Chem-Impex (00033, Cas #: 58-85-5); (+)-S-Trityl-L-cysteine, Alfa Aesar (L14384, CAS #: 2799-07-7); Puromycin dihydrochloride, Sigma-Aldrich (P8833, CAS # 58-58-2); Doxycycline hyclate, Acros (446061000, CAS #: 24390-14-5); cOmplete Protease Inhibitor Cocktail, Roche (5056489001); Sodium fluoride, Chem-Impex (01523, CAS #: 7681-49-4); Sodium orthovanadate, MP Bio (0215966410, CAS #: 13721-39-6); Oxotremorine M, Santa Cruz Biotechnology (sc-203656, CAS #: 63939-65-1); Phorbol-12-Myristate-13-Acetate, Santa Cruz Biotechnology (sc-3576, CAS #: 16561-29-8); Bryostatin 1, Cayman Chemical (14802, CAS #: 83314-01-6); Gö 6983, Cayman Chemical (13311, CAS #: 133053-19-7); Sotrastaurin, Cayman Chemical (16726, CAS #: 425637-18-9); PF 670462, Fisher Scientific (50-115-0729, CAS #: 950912-80-8); D 4476, Fisher Scientific (2902/10, CAS #: 301836-43-1); Tautomycetin, Tocris (2305, CAS #: 119757-73-2); Fostriecin, VWR (102987-176, CAS #: 87860-39-7); Okadaic acid, Cayman Chemical (10011490, CAS #: 78111-17-); Phos-tag™ Acrylamide, NARD (AAL-107); 32% Paraformaldehyde, Electron Microscopy Sciences (15714); Streptavidin Alexa Fluor 568, Invitrogen (S11226); Pierce™ High Capacity Streptavidin Agarose, Thermo Fisher (20359); Streptavidin-conjugated HRP, GeneTex (GTX85912); ProLong™ Diamond Antifade Mountant with DAPI, Thermo Fisher (P36971); Clarity™ Western ECL Substrate, Bio-Rad (1705061); BCA Protein Assay Kit, Thermo Fisher (23225).

A2.3 Plasmids and Cloning

A PLEKHA5 cDNA (Uniprot Q9HAU0, a gift from Haiyuan Yu, Cornell University) was cloned into the pEGFP-C1 vector (Clontech) using BamHI and Sall to generate EGFP-PLEKHA5^{FL}. Subsequently, fragments and deletions of PLEKHA5 were generated by standard or overlap PCR, and inserts were cloned to pEGFP-C1 vector, again using BamHI and Sall, and to pEGFP-N1-vector with NheI and Sall. Also, two deletion constructs were generated by QuikChange XL Site-directed mutagenesis kit (Agilent). The amino acid sequences of the fragments and deletions are the following: PLEKHA5^{WW} (1-105), PLEKHA5^{H-BP-PH} (143-271), PLEKHA5^{WW-H-BP-PH} (1-271), PLEKHA5^{PRD} (272-628), PLEKHA5^{CC-IDR} (629-1116), PLEKHA5^{ΔWW} (106-1116), PLEKHA5^{ΔH-BP-PH} (1-142, 272-1116), PLEKHA5^{ΔPRD} (1-271, 629-1116), PLEKHA5^{ΔCC-IDR} (1-628). For generation of stable HeLa cells used in the proteomics experiment, EGFP-PLEKHA5 was subcloned into the pCDNA5-FRT vector (Thermo Fisher) using NheI and BamHI. mScarlet-i-PLEKHA5^{WW} was made by inserting the cDNA sequence encoding tandem WW domain (residue 1-105) into the mCarlet-i-C1 vector (Addgene 85044) using BamHI and Sall. For amino acid mutagenesis, mutations were incorporated into PLEKHA4 and PLEKHA5 plasmids using the QuikChange XL Site-directed mutagenesis kit (Agilent).

For TurboID experiments, pCDNA3-Lyn10-TurboID-V5 was cloned by replacing the C1(1-29) sequence in C1(1-29)-TurboID-V5-pCDNA3 (Addgene, 107173) with a Lyn10 tag (amino acid sequence GCIKSKRKDG). Then the entire Lyn10-TurboID-V5 cassette was inserted into the pSBtet-Pur plasmid (Addgene 60507) linearized by

NcoI and BspDI to replace the firefly luciferase downstream of the Tet-responsive promoter, generating the pSBtet-Lyn10-TurboID-V5-puro plasmid used for making a HeLa cell line stably expressing Lyn10-TurboID-V5 under Doxycycline induction.

A2.4 Cell Culture

Flp-In T-Rex HeLa cells (Thermo Fisher) were cultured in DMEM (Corning) supplemented with 10% FBS (Corning) and 1% penicillin/streptomycin (P/S, Corning) at 37 °C in a 5% CO₂ atmosphere. Stable expression of H2B-mCherry was accomplished by transducing pLenti6-H2B-mCherry plasmid (Addgene, 89766) into Flp-In T-Rex HeLa cells and blasticidin (Alfa Aesar) selection at 2.5 µg/mL. To generate stable cell line expressing Lyn10-TurboID-V5 under doxycycline induction (2.5 µg/mL), pSBtet-Lyn10-TurboID-V5-puro and pCMV(CAT)T7-SB100 (Addgene 34879) were co-transfected into Flp-In T-Rex HeLa cells in a 19:1 ratio, and cells were selected with 2 µg/mL puromycin 24 h after transfection.

A2.5 Plasmid Transfection

Plasmids were transfected into mammalian cells using Lipofectamine 2000 following the manufacturer's protocol. Opti-MEM was replaced with Transfectagro. Cells were incubated with the transfection mix in Transfectagro with 10% FBS for 7–8 h, then cultured in regular growth medium until harvest.

A2.6 Cell synchronization

To synchronize cells in prometaphase, 5 µg/mL of S-trityl-L-cysteine (STLC) was added to confluent dishes of cells for 16 h. Mitotic cells were dislodged from dishes

by gentle tapping, washed with 1X DPBS, and collected. Cells pellets were stored at -80°C if not used immediately.

A2.7 Confocal Microscopy

For live-cell imaging, cells were seeded on 35 mm glass bottom culture dishes (14 mm diameter, #1.5 thickness, Matsunami Glass). For immunofluorescence, cells were seeded on 12 mm cover glass (#1.5 thickness, Fisherbrand) in 12-well plates (Corning). Cells were imaged (live cell) or fixed (immunofluorescence) 24–30 h post transfection. Images were acquired via Zeiss Zen Blue 2.3 software on a Zeiss LSM 800 confocal laser scanning microscope equipped with Plan Apochromat objectives (40x 1.4 NA) and two GaAsP PMT detectors. Solid-state lasers (405, 488, 561, and 640 nm) were used to excite DAPI/tagBFP, EGFP/Alexa Fluor 488, and mCherry/mScarlet-i/Alexa Fluor 568, and miRFP/miRFP670 respectively. Live-cell time-lapse movies were acquired using definite focus. Depending on the experiments, images and movies were acquired either at room temperature, or at 37°C with a 5% CO_2 atmosphere when indicated. Acquired images were analyzed using FIJI. To quantify PM and cytosolic fluorescence, 5 line-scans at different areas were drawn for each cell to get the average fluorescence. Coloc2 command was used to compute the Pearson Coefficients with the PLEKHA5 channel set as reference ROI or mask.

A2.7.1 Live-cell Imaging

For most live imaging experiments not involving time-lapse movies, cells were rinsed twice and then imaged in Tyrode's-HEPES buffer (T/H buffer: 135 mM NaCl, 5 mM

KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mg/mL glucose, 5 mg/mL bovine serum albumin, 20 mM HEPES, pH 7.4) at room temperature.

For PI(4,5)P₂ depletion assay, three plasmids, PLEKHA5^{H-BP-PH}-EGFP, M1R-mCherry, and iRFP-PLCd^{PH}, were co-transfected into HeLa cells for 24–30 h. Cells were rinsed with T/H buffer twice, and subjected to time-series acquisition with definite focus at room temperature. Images were acquired every 5 s for in 5 min total, and 10 μM oxotremorine-M was added 30 s after starting the acquisition to activate M1R for acute PI(4,5)P₂ depletion at the plasma membrane.

For PKC activation imaging experiments, cells were transfected with GFP-tagged PLEKHA5 constructs for 24–30 h and then treated with PKC inhibitors (100 nM Gö 6983 and 100 nM Sotrastaurin) or vehicle for 1 h. T/H buffer was used to rinse the cells twice and added to cells with fresh PKC inhibitors or vehicle. PKC agonists, PMA (100 nM) or Bryostatin 1 (200 nM), was added to the dishes, and immediately afterwards time-lapse acquisition with definite focus was started for 20-25 min at 37 °C with a 5% CO₂ atmosphere. Images were acquired every 1 min.

For long-term time-lapse imaging, cells were imaged in DMEM phenol red (Gibco) supplemented with 10% FBS and 1% P/S at 37 °C in a 5% CO₂ environment 24–30 h post transfection. For PLEKHA5^{WW-H-BP-PH}-EGFP and H2B-mCherry imaging to track PLEKHA5 PM translocation, images were acquired every 1 min for 12 h.

A2.7.2 Immunofluorescence

For immunofluorescence analysis of Lyn10-TurboID-V5, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton-X 100 in 1X PBS for 5

min, and blocked in 2% BSA and 0.1% Tween-20 in 1X PBS (blocking buffer) for 30 min. Cells were treated with primary antibody (anti-V5) in blocking buffer for 1 h, rinsed with wash buffer three times and incubated with secondary antibodies in blocking buffer for 1 h. Cells were then rinsed with wash buffer three times and with 1X PBS twice, mounted on slides in ProLong Diamond Antifade with DAPI, and incubated overnight in dark before imaging. All steps were performed at room temperature, and slides were stored at 4 °C.

A2.8 Proximity biotinylation by Lyn10-TurboID

pSBtet-Lyn10-TurboID-V5 expression were induced in HeLa cells with 2.5 µg/mL Doxycycline for 48 h, and then incubated with 500 µM biotin for 10 min at 37 °C under 5% CO₂. For localization analysis by immunofluorescence, cells were rinsed five times with 1X PBS and then prepared as described earlier (see Confocal microscopy section). For streptavidin-agarose pulldown and Western blot, cells were rinsed five times with 1X PBS and lysed with RIPA buffer (150 mM NaCl, 25 mM Tris pH 8.0, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100,) supplemented with cOmplete protease inhibitor cocktail. Cell lysates were sonicated with four pulses at 20% intensity using a tip sonicator and centrifuged at 13,000 *g* for 5 min at 4 °C to clarify lysates. Protein concentration was quantified using the BCA assay (Thermo Fisher), and a small fraction of the clarified cell lysates was saved and normalized as input. The remaining cell lysates were subjected to pulldown using streptavidin-agarose with rotation at 4 °C overnight (12-16 h). The resin was then centrifuged for 5 min at 1000 *g*, washed two times with RIPA buffer, one time with 1

M KCl, one time with 0.1 M Na₂CO₃, one time with 2 M urea in 10 mM Tris pH 8.0, and two times with RIPA buffer to eliminate non-specific binding. Samples were then denatured and analyzed by SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate.

A2.9 Western Blot

Cells were lysed with RIPA buffer (150 mM NaCl, 25 mM Tris pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with cOmplete protease inhibitor cocktail and a phosphatase inhibitors mixture (20 mM sodium fluoride, and 1 mM sodium orthovanadate) on ice. Cell lysates were sonicated for 2 pulses at 20% intensity and centrifuged at 13,000 *g* for 5 min at 4 °C. Protein concentrations of the clarified cell lysates were quantified using the BCA assay. For Phostag SDS-PAGE, ZnCl₂ solution was added to cell lysates to reach a final Zn²⁺ concentration of 1 mM to counteract the EDTA in RIPA buffer, which would interfere with electrophoresis of phostag gels. Cell lysates were normalized to 2–3 mg/mL, and denatured with 6X Laemmli Buffer (13.3% SDS, 0.067% bromophenol blue, 52.2% glycerol, 67 mM Tris pH 6.8 and 11.1% β-mercaptoethanol) at 95 °C for 5 min. Samples were analyzed by Phostag or regular SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate (Bio-Rad).

A2.10 Anti-GFP Immunoprecipitation

Cells expressing GFP fusion proteins were harvested and lysed in IP lysis buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 5 mM EDTA, 50 mM Tris pH 7.5) supplemented with cOmplete protease inhibitor cocktail on ice. Cell lysates were

sonicated for 4 pulses at 20% intensity and centrifuged at 13,000 *g* for 5 min at 4 °C. Protein concentration of the clarified cell lysates was quantified using the BCA assay, and a small fraction of the clarified cell lysates was separated and normalized as input. The remaining cell lysates were subjected to immunoprecipitation using GFP-Trap Magnetic Agarose with rotation at 4 °C overnight (12–16 h). The magnetic resin was then separated by a magnetic tube rack, washed three times with lysis buffer (at least 10 times the bead volume), denatured and analyzed by SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate, or by mass spectrometry-based phosphoproteomics analysis, as described in detail in the next section.

A2.11 Mass spectrometry analysis of phosphopeptides

The GFP-Trap Magnetic resin was washed four times with IP lysis buffer and incubated with elution buffer (100 mM Tris pH 8.0, 1% SDS) at 65 °C for 15 min. Collected supernatant were reduced with 10 mM DTT at 65 °C for 10 min and then alkylated with 25 mM iodoacetamide (in 50 mM Tris pH 8.0) at room temperature for 15 min in dark. Proteins from the mixed sample were precipitated in a solution containing 50% acetone, 49.9% ethanol and 0.1% acetic acid (PPT solution) in the –20 °C freezer for 1 h and pelleted at 17,000 *g* for 10 min. The protein pellet was washed with PPT solution once, pelleted again, air-dried, and resuspended in urea/Tris solution (8 M urea, 50 mM Tris pH 8.0) and NaCl/Tris solution (150 mM NaCl, 50 mM Tris pH 8.0) in a ratio of 1:3 respectively, for a final concentration of 2 M urea. One µg of mass spectrometry grade trypsin (Trypsin Gold, Promega; 1

mg/mL in 0.03% acetic acid), was added and proteins were trypsinized at 37 °C overnight on a nutator.

Protein digests were acidified with 0.25% formic acid (FA) and 0.25% trifluoroacetic acid (TFA), desalted using the Sep-Pak C18 resin (Waters; WAT054945), and dried. The sample was then suspended in 70% acetonitrile with 1% formic acid and subjected to hydrophilic interaction chromatography (HILIC) prefractionation using a high-performance liquid chromatography (HPLC) UltiMate3000 system (Thermo Fisher Scientific). Fractions were then analyzed via nanoLC-MS/MS using an UltiMate 3000 system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Reverse phase chromatography was conducted using a C18 resin (Reposil Pur C18AQ 3 μ m) packed in-house into a 35 cm long column (100 μ m inner diameter) using a 70-minute linear gradient from 8% to 30% acetonitrile with 0.1% FA at a flow rate of 300 nL/min. The Xcalibur 2.2 software (Thermo Fisher Scientific) was used for data-dependent acquisition, with a scan range of 380 to 1800 m/z and a mass resolution of 60,000. The automatic gain control target was set to 3e6 and the maximum injection time to 30 ms. MS² was performed by the acquisition of the 20 most abundant ions per cycle fragmented with a normalized collision energy of 28. The isolation window for the fragmented ions was set to 2 m/z with a mass resolution of 15,000, a maximum injection time of 100 ms, and an automatic gain control target of 1e5.

For data analysis and identification of phosphopeptides, raw data was processed through the Trans Proteomic Pipeline package¹ (ver. 6.0.0) with spectral data files

searched through the Comet search engine². Parameters were set to the mass tolerance of 10 ppm, and a semi-tryptic digest requirement with 2 allowed miscleavages. Data was further validated using PeptideProphet and PTMProphet with a dynamic mass modification of 79.966331 Da for phosphorylated serines, threonines, and tyrosines, and a static modification of 57.021464 Da for alkylated cysteines.

A2.12 Phostag SDS-PAGE

A2.12.1 Phostag gel preparation

Phostag gels were prepared as described³ with slight modification. Briefly, to make 8 mL of 9% resolving phostag gel solution (enough for one 1.5 mm Bio-Rad mini gel cast), the following reagents were mixed together evenly: 3.28 mL of deionized water, 2 mL of 1.4 M Bis-Tris·HCl (pH 6.8), 40 µL of 5 mM Phos-tag solution, 40 µL of 10 mM ZnCl₂ solution, 2.4 mL of 30% Acrylamide/0.8% Bis-acrylamide, 240 µL 10% APS (w/v), and 8 µL of TEMED. 3 mL of stacking gel solution (enough for one 1.5 mm Bio-Rad mini gel cast) was prepared by mixing together: 1.76 mL of deionized water, 0.75 mL of 1.4 M Bis-Tris·HCl (pH 6.8), 400 µL of 30% Acrylamide/0.8% Bis-acrylamide, 90 µL of 10% APS (w/v), and 3 µL of TEMED.

A2.12.2 Phostag gel electrophoresis

Running buffer (0.1 M Tris, 0.1 M MOPS, 5 mM sodium bisulfite, and 0.1% SDS, pH 7.8) for Phostag gel electrophoresis was prepared fresh every time. Gels were run under constant current (30 mA per gel) for 3 h, then rocked in methanol-free transfer buffer (25 mM Tris·HCl, 96 mM glycine, pH 8.2; 50 mL per gel) plus 1 mM EDTA (pH

8.0) twice for 10 min each to remove Zn^{2+} ions, and once in methanol-free transfer buffer for 10 min before proteins were transferred to nitrocellulose membranes.

A 2.13 Kinase and phosphatase agonists and inhibitors treatment summary

The following compounds were added to cells in conditions as summarized:

PKC agonists: PMA, 100 nM, 20-30 min; Bryostatin 1, 200 nM, 20-30 min.

PKC inhibitors: Gö 6983 (100 nM) and Sotrastaurin (100 nM), 1 h.

CK1 inhibitors: PF670642 (selective for CK1d and CK1e), 5 μM , 16 h; D4476, 100 μM , 8 h.

Phosphatase inhibitors: Tautomycin (selective for PP1), 1 μM , 6 h; Fostriecin (targets PP2A and PP4), 1 μM , 1 h; Okadaic acid, 1 μM , 30 min.

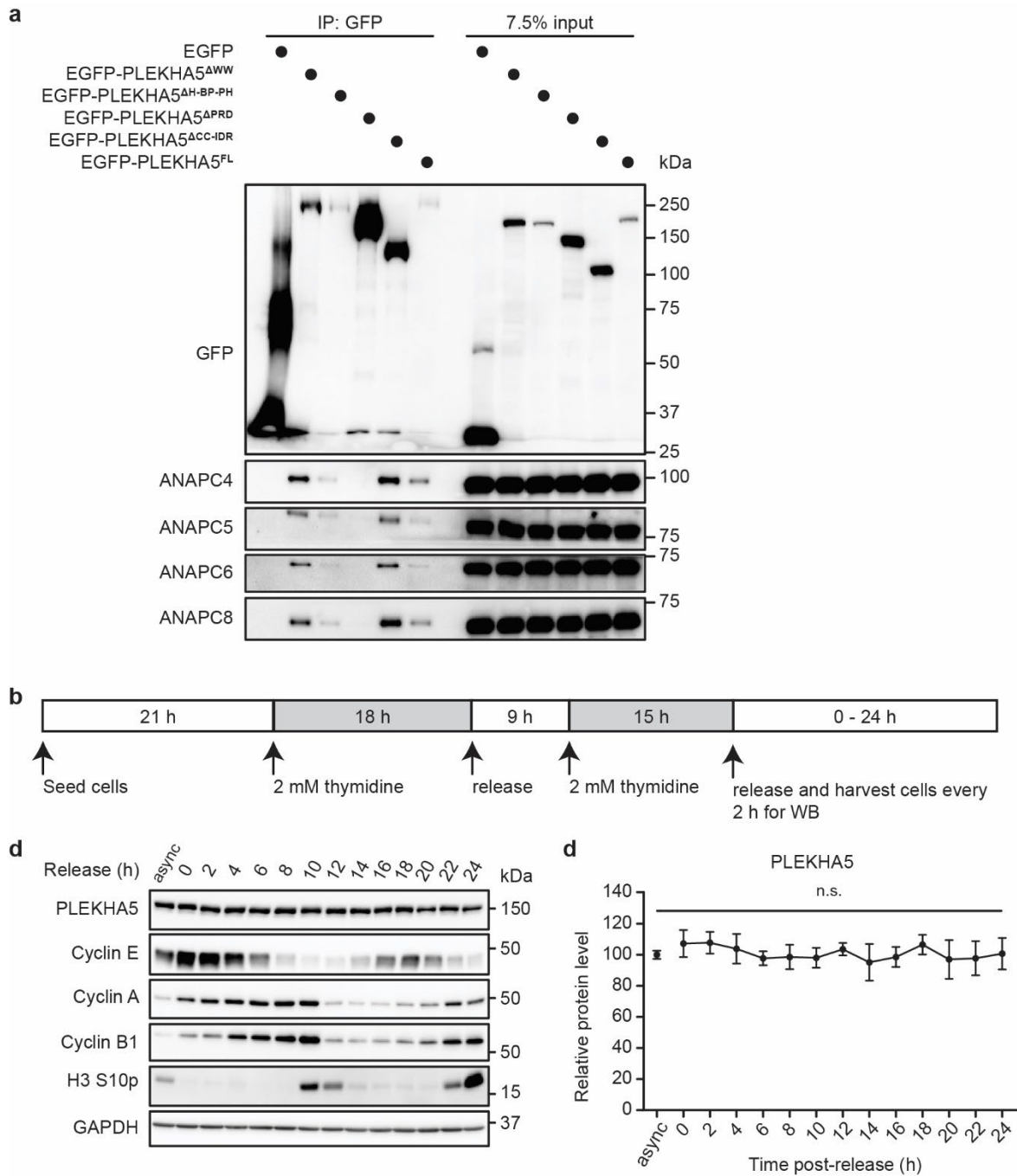
A2.14 Statistical analysis

For all experiments involving quantification, statistical significance was calculated in GraphPad Prism using tests as indicated in the figure legend, including an unpaired two-tailed t-test with unequal variance or a one-way ANOVA with Tukey or Sidak post-hoc test. P values for each comparison are reported, and the number of biological replicates or cells analyzed is stated in the legend. All the raw data were plotted into graphs using GraphPad Prism. For all scatter plots, the black line indicates the mean, and each dot represents an individual biological replicate.

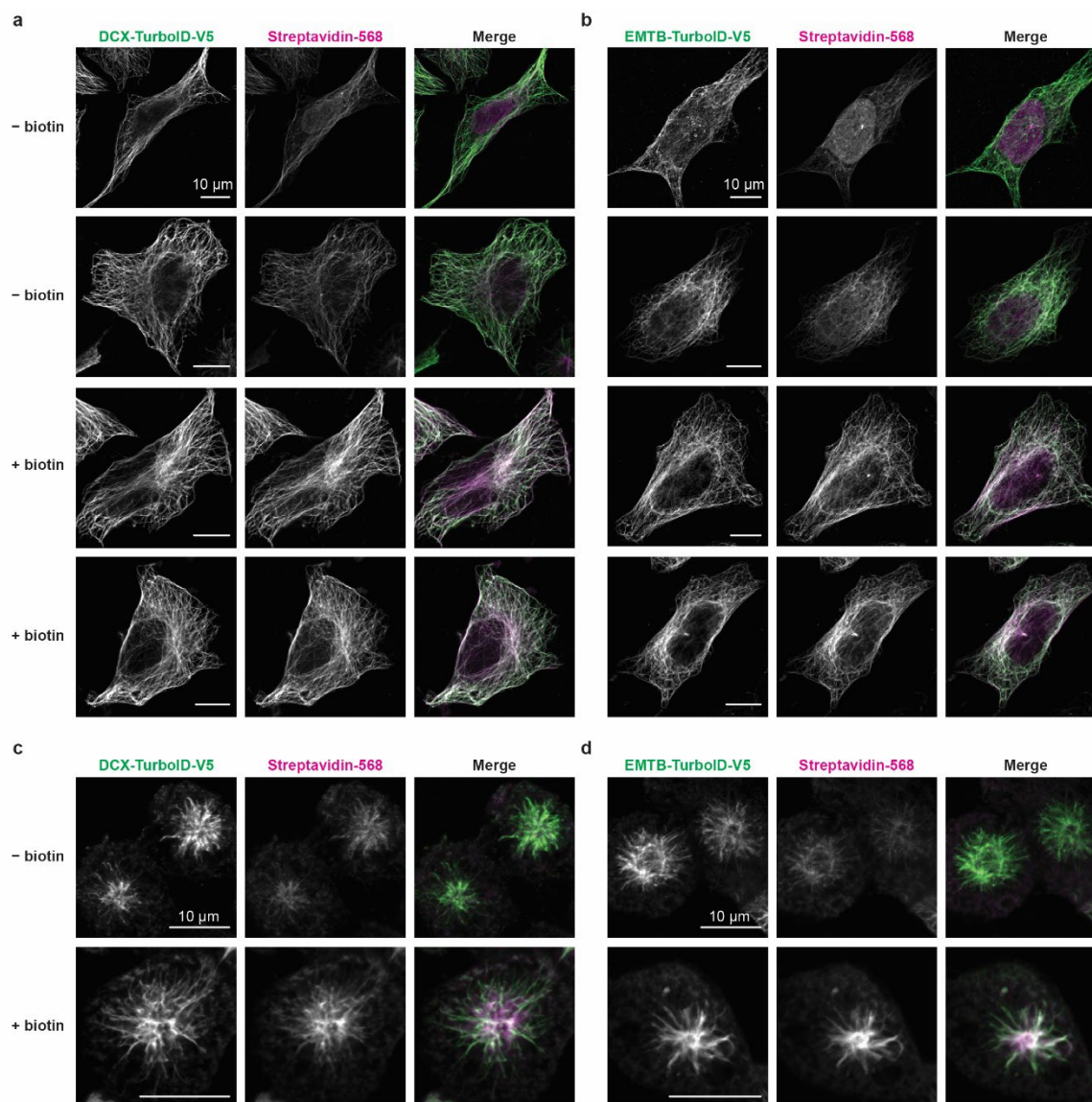
APPENDIX B

SUPPLEMENTARY INFORMATION FOR CHAPTER 3

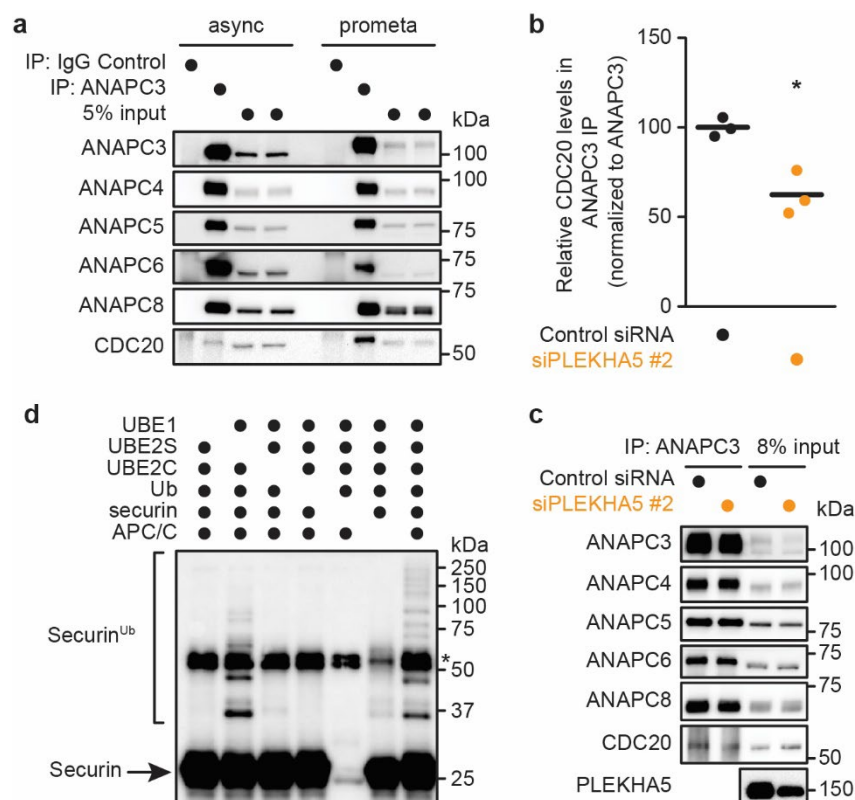
B1 SUPPLEMENTAL FIGURES AND TABLE



Appendix Figure B1 (Related to Figure 3.1). Domain mapping of the PLEKHA5–APC/C interaction and PLEKHA5 protein levels throughout the cell cycle. (a) The proline-rich domain (PRD) of PLEKHA5 is necessary for the interaction with APC/C. Shown is western blot analysis of α -GFP IP from HeLa cells transfected with GFP or the indicated GFP-*PLEKHA5* truncation constructs and blotted for endogenous APC/C subunits. (b) Schematic representation of experiment timeline. HeLa cells were synchronized to S phase by a double thymidine block (DTB). Cells were then rinsed, released into fresh growth medium, and harvested at indicated time points after the release. (c, d) *PLEKHA5* protein level is relatively stable over different stages of the cell cycle. Shown are representative Western blots (c) and quantification of *PLEKHA5* level (d) of lysates from asynchronous or DTB-synchronized/released HeLa cells ($n=4$). ANOVA (one-way, Tukey): n.s. not significant ($p > 0.05$).

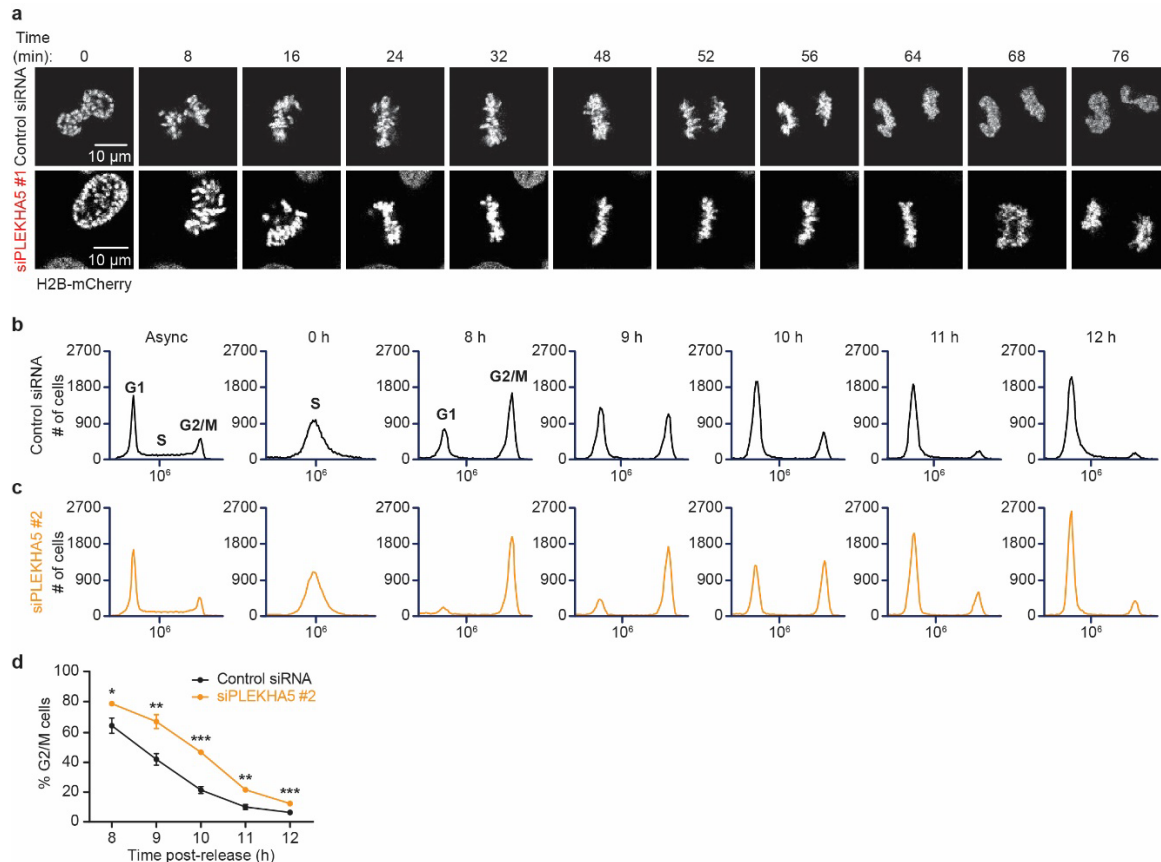


Appendix Figure B2 (Related to Figure 3.2). Immunofluorescence analysis of MT-TurboID at interphase and prometaphase. (a, b) During interphase, both microtubule targeting TurboID constructs localize correctly and the Streptavidin-568 (magenta) signals on microtubules increase upon addition of 500 μ M biotin. (c, d) At prometaphase, both MT-TurboID constructs show specific biotinylation activity along the microtubule network.

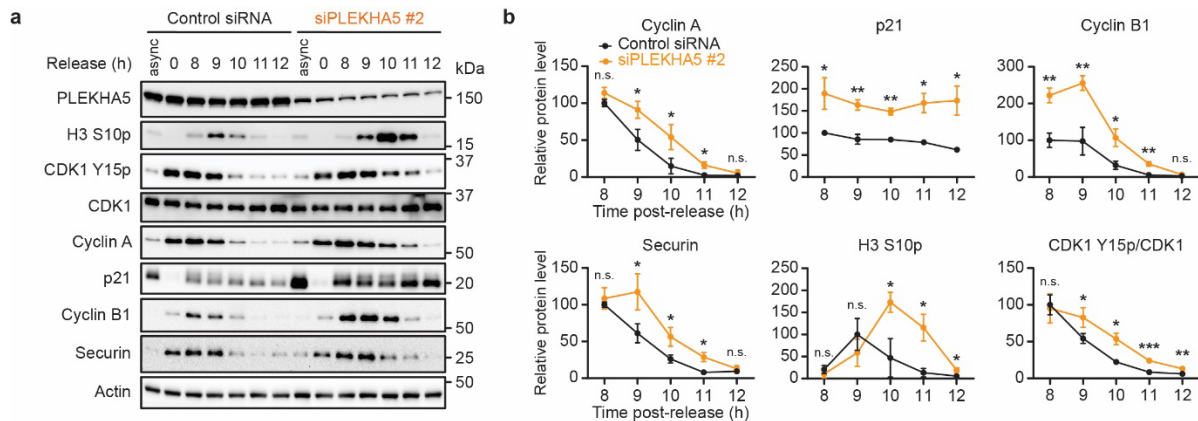


Appendix Figure B3 (Related to Figure 3.4). Effect of PLEKHA5 knockdown on APC/C composition and in vitro E3 ligase activity. (a) Endogenous APC/C can be purified using α -ANAPC3 IP from cells that were either asynchronous (async) or synchronized to prometaphase (prometa) by treatment with 5 μ g/mL STLC for 16 h. Shown is Western blot characterization of APC/C enriched from HeLa cells compared to whole cell lysate (input). (b, c) siPLEKHA5 #2 causes a decrease in APC/C association with its key mitotic CDC20. Shown are representative Western blots from co-IP experiments for endogenous ANAPC3 (c) and quantification of CDC20 levels in the IP samples (b) (n=3). (d) APC/C was purified from HeLa cells by incubation of lysates (pre-cleared with protein G Sepharose) with α -ANAPC3 antibody and then protein G Sepharose, followed by rinses to yield APC/C on resin. In vitro ubiquitination assays with the APC/C substrate securin were set up by addition of E1 (UBE1), two E2s (UBE2S and UBE2C), ubiquitin (Ub), ATP, and APC/C (on resin), at 30 $^{\circ}$ C for 30 min with gentle agitation, followed by denaturation and Western blot, probing for securin. Indicated on the blot is non-ubiquitinated securin (~25 kDa), mono- and poly-

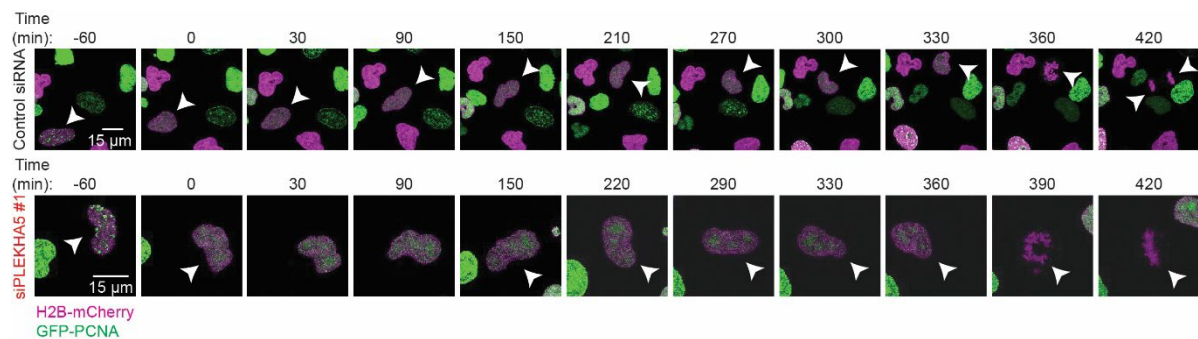
ubiquitinated securin (securin^{Ub}), and, with an asterisk (~55 kDa), IgG heavy chain from the APC/C purification. Note appearance of securin^{Ub} only in lane 7, which contains all reaction components (as well as some in lane 2, containing UBE2C, which supports multi-monoubiquitination and/or generation of short ubiquitin chains, even in the absence of UBE2S, which generates longer polyubiquitin chains). Student's t-test: * $p < 0.05$.



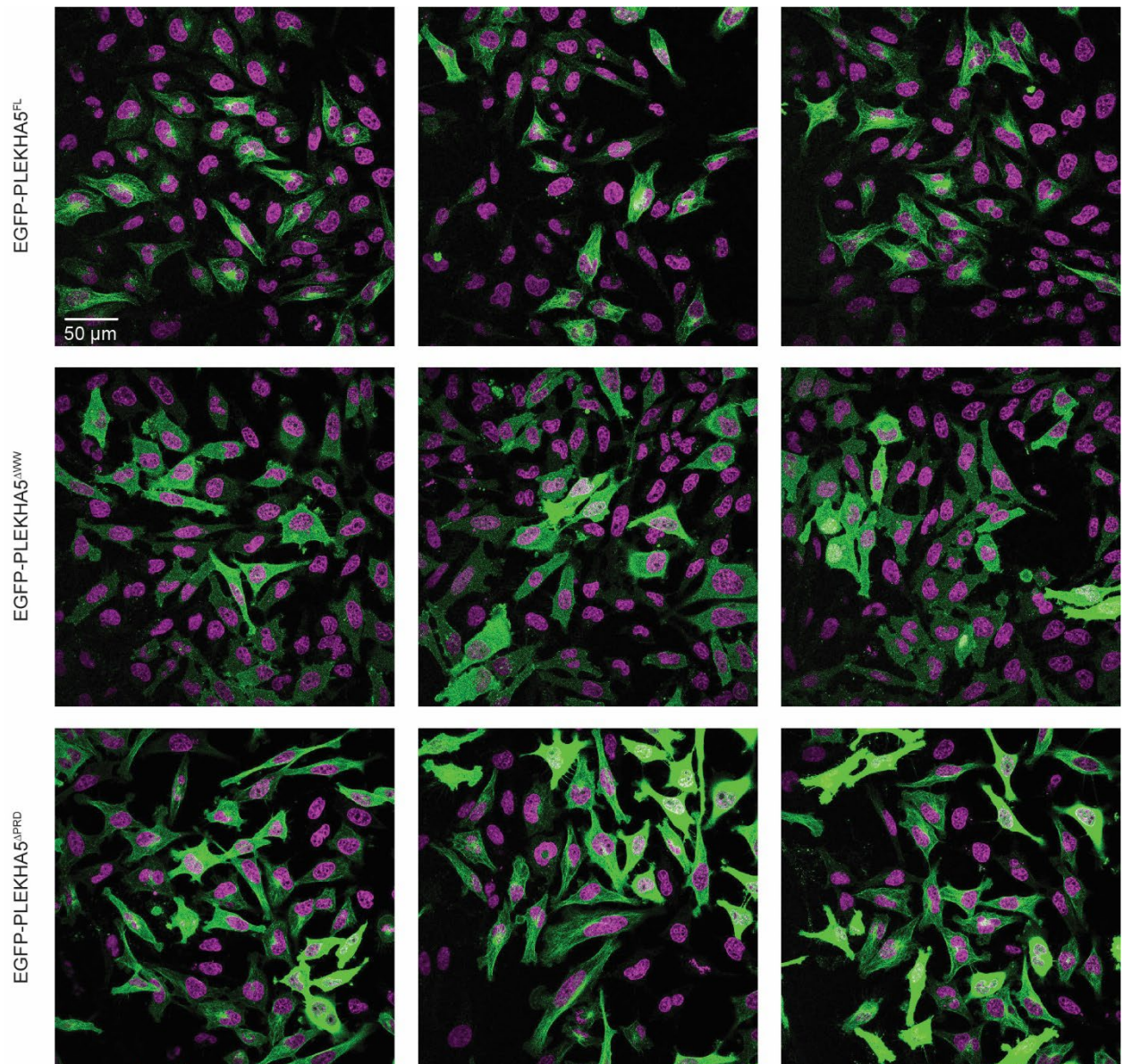
Appendix Figure B4 (Related to Figure 3.5). PLEKHA5 knockdown causes delayed mitotic progression and accumulation of cells in G2/M assessed by flow cytometry. (a) Representative series of still images from a time-lapse movie of the nucleus in asynchronous, H2B-mCherry-expressing HeLa cells treated with a control siRNA for siPLEKHA5 #1. Numbers on top of the images are the time from nuclear envelop breakdown ($t = 0$ min) till the images were taken. (b-d) siPLEKHA5 #2 treatment leads to accumulation of HeLa cells in G2/M phase. DNA content of asynchronous (async) or DTB synchronized HeLa cells treated with the control siRNA (b) or siPLEKHA5 #2 (c) were evaluated by propidium iodide staining and flow cytometry analysis, and the percentage of cells in G2/M phase were quantified and shown in (d) ($n=3$). Student's t-test: n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Appendix Figure B5 (Related to Figure 3.6). PLEKHA5 depletion led to accumulation of APC/C substrates during mitosis. G2/M protein markers and APC/C substrates persist in cells treated with siPLEKHA5 #2. Shown are representative western blots (a) and quantifications of protein levels (b) in lysates from DTB synchronized HeLa cells subject to control siRNA or siPLEKHA5 #2 (n=3). Student's t-test: n.s. not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Appendix Figure B6 (Related to Figure 3.7). Still images from a time-lapse movie of the nuclear region in a single cell for the G2/M transition analysis in asynchronous HeLa cells expressing H2B-mCherry and EGFP-PCNA.



Appendix Figure B7 (Related to Figure 3.8). Confocal imaging of cells transduced with siPLEKHA5 #1-resistant rescue constructs to show transduction efficiency. Three representative images were shown for each PLEKHA5 rescue construct used. Transduction efficiency was ~60% for EGFP-PLEKHA5^{FL} and ~80% for EGFP-PLEKHA5^{ΔWW} and EGFP-PLEKHA5^{ΔPRD}. The expression level of the full-length protein was also lower (note lower GFP fluorescence intensity in the images and lower intensity of anti-GFP bands in representative Western blots in Figure 7). Magenta: Hoechst 33342 (nuclei); Green: GFP fluorescence.

APPENDIX TABLE B1 Raw data from SILAC mass spectrometry experiments

For a complete list of hits from the SILAC MS proteomics experiment to determine the PLEKHA5 interactome, please refer to Table S1 in **Cao, X. et al. *ACS Chem Bio* (2022).**

B2 MATERIALS AND METHODS

B2.1 Antibodies

Mouse anti-PLEKHA5, Santa Cruz Biotechnology (sc-390311, RRID: N/A); mouse anti-ANAPC3, Santa Cruz Biotechnology (sc-9972, RRID: AB_627228); rabbit anti-ANAPC4, Bethyl Laboratories (A301-176A, RRID: AB_2227071); rabbit anti-ANAPC5, ABclonal (A7109, RRID: AB_2767664); mouse anti-ANAPC6, Santa Cruz Biotechnology (sc-376091, RRID: AB_10988885); rabbit anti-ANAPC8, Cell Signaling Technology (15100, RRID: AB_2798708); mouse IgG2b isotype control, Cell Signaling Technology (53484, RRID: AB_2799435); normal rabbit IgG control, Cell Signaling Technology (2729, RRID: AB_1031062); mouse anti-GFP, Santa Cruz Biotechnology (sc-9996, RRID: AB_627695); mouse anti-phospho-Histone H3 (Ser10), Cell Signaling Technology (9706, RRID: AB_331748); rabbit anti-phospho-CDK1 (Tyr15), Cell Signaling Technology (9111, RRID: AB_331460); mouse anti-CDK1, Santa Cruz Biotechnology (sc-54, RRID: AB_627224); mouse anti-cyclin A, Santa Cruz Biotechnology (sc-239, RRID: AB_627334); mouse anti-cyclin B1, Santa Cruz Biotechnology (sc-245, RRID: AB_627338); rabbit anti-p21, Cell Signaling

Technology (2947, RRID: AB_823586); mouse anti-securin, Santa Cruz Biotechnology (sc-56207, RRID: AB_785382); mouse anti-actin, MP Bio (08691001, RRID: AB_2335127); mouse anti-GAPDH, GeneTex (GTX78213, RRID: AB_625368); rabbit anti-FLAG, Sigma-Aldrich (F7425, RRID: AB_439687); rabbit anti-CDC20, Cell Signaling Technology (4823, RRID: AB_10549074); mouse anti-CDC20, Santa Cruz Biotechnology (sc-13162, RRID: AB_628089); mouse anti-MAP4, Santa Cruz Biotechnology (sc-390286, RRID: N/A); mouse anti- α tubulin, Sigma-Aldrich (T5168, RRID: AB_477579); mouse anti-CD44, Cell Signaling Technology (3570, RRID: AB_2076465); rabbit anti-Histone H3, Cell Signaling Technology (4499, RRID: AB_10544537); mouse anti-V5, Bio-Rad (MCA1360GA, RRID: AB_567249); goat anti-mouse HRP, Jackson ImmunoResearch Labs (115-035-146, RRID: AB_2307392); goat anti-rabbit HRP, Jackson ImmunoResearch Labs (111-035-144, RRID: AB_2307391); donkey anti-mouse Alexa Fluor 488, Invitrogen (A-21202, RRID: AB_141607).

B2.2 Chemicals and recombinant proteins

Lipofectamine 2000, Invitrogen (11668019); Lipofectamine RNAiMAX, Invitrogen (13778150); Polybrene Infection/Transfection Reagent, Sigma-Aldrich (TR-1003); GFP-Trap magnetic agarose, ChromoTek (gtma-20); Trypsin Gold Mass Spectrometry Grade, Promega (V5280); Protein G Sepharose, BioVision (6511); Thymidine, Chem-Impex (00306); Propidium iodide, Sigma-Aldrich (P4170); RNase A, Research Products International (R21750); Nocodazole, AdipoGen (AG-CR1-0019); (+)-S-Trityl-L-cysteine, Alfa Aesar (L14384); Puromycin dihydrochloride,

Sigma-Aldrich (P8833); Blastidicin S hydrochloride, 10 mg/ml in HEPES buffer, Alfa Aesar (J67216); Hygromycin B, 50 mg/mL in PBS, Invitrogen (10687010); cOmplete Protease Inhibitor Cocktail, Roche (5056489001); Sodium β -glycerophosphate pentahydrate, Alfa Aesar (L03425); Sodium fluoride, Chem-Impex (01523); Sodium pyrophosphate decahydrate, Fisher (S390); Sodium orthovanadate, MP Bio (0215966410); UBE1, R&D Systems (E-304); UBE2C, R&D Systems (E2-654); UBE2S, R&D Systems (E2-690); securin, Abcam (ab87664); D-biotin, Chem-Impex (00033); Streptavidin Alexa Fluor 568, Invitrogen (S11226); Pierce™ High Capacity Streptavidin Agarose, Thermo Fisher (20359); Streptavidin-conjugated HRP, GeneTex (GTX85912); ProLong™ Diamond Antifade Mountant with DAPI, Thermo Fisher (P36971); Clarity™ Western ECL Substrate, Bio-Rad (1705061).

B2.3 siRNA duplexes

Negative control siRNA

Sense: 5'-rCrGrUrUrArArUrCrGrCrGrUrArUrArArUrArCrGrCrGrUAT-3'

Antisense: 5'-rArUrArCrGrCrGrUrArUrUrArUrArCrGrCrGrArUrUrArArCrGrArC-3'

siPLEKHA5 #1

Sense: 5'-rArGrArCrCrUrGrArArGrArArGrUrArGrArUrArUrUrGrATG-3'

Antisense: 5'-rCrArUrCrArArUrArUrCrUrArCrUrUrCrUrUrCrArGrGrUrCrUrGrU-3'

siPLEKHA5 #2

Sense: 5'-rGrUrUrGrCrArArCrCrArUrGrArCrArUrCrUrGrArArGrAAA-3'

Antisense: 5'-rUrUrUrCrUrUrCrArGrArUrGrUrCrArUrGrGrUrUrGrCrArArCrArG-3'

B2.4 Plasmids and cloning

A PLEKHA5 cDNA (Uniprot Q9HAU0, a gift from Haiyuan Yu, Cornell University) was cloned into the pEGFP-C1 vector (Clontech) using BamHI and Sall to generate EGFP-PLEKHA5^{FL}. Subsequently, fragments and deletions of PLEKHA5 were generated by standard or overlap PCR, and inserts were cloned to pEGFP-C1 vector, again using BamHI and Sall, and to pEGFP-N1-vector with NheI and Sall. Also, two deletion constructs were generated by QuikChange XL Site-directed mutagenesis kit (Agilent). The amino acid sequences of the fragments and deletions are the following: PLEKHA5^{WW} (1-105), PLEKHA5^{H-BP-PH} (143-271), PLEKHA5^{WW-H-BP-PH} (1-271), PLEKHA5^{PRD} (272-628), PLEKHA5^{CC-IDR} (629-1116), PLEKHA5^{ΔWW} (106-1116), PLEKHA5^{ΔH-BP-PH} (1-142, 272-1116), PLEKHA5^{ΔPRD} (1-271, 629-1116), PLEKHA5^{ΔCC-IDR} (1-628). For generation of stable HeLa cells used in the proteomics experiment, EGFP-PLEKHA5 was subcloned into the pCDNA5-FRT vector (Thermo Fisher) using NheI and BamHI.

For rescue experiments, silent mutations were incorporated into EGFP-PLEKHA5^{FL} and EGFP-PLEKHA5^{ΔPRD} to generate siPLEKHA5 #1-resistant constructs using the QuikChange XL Site-directed mutagenesis kit. The following codons within the siRNA targeting region were mutated: R629, E631, E632, V633, I635. The siRNA-resistant EGFP-PLEKHA5^{FL} and EGFP-PLEKHA5^{ΔPRD} were then subcloned to an engineered lentiviral transduction vector pCDH-CMV-MCS using NheI and BamHI. The pCDH-CMV-MCS vector was generated by removing the puromycin resistance expression

cassette from the pCDH-CMV-MCS-EF1 α -Puro vector (a gift from Jan Lammerding, Cornell University) using NotI and SalI, followed by blunting the sticky ends with T4 DNA polymerase (NEB). The blunted DNA fragment was then phosphorylated by T4 Polynucleotide Kinase (NEB) and re-ligated with T4 DNA Ligase (Thermo Fisher). Furthermore, the NotI restriction digestion site was restored, and an AscI site was added upstream of the XbaI site using the QuikChange XL Site-directed mutagenesis kit to generate the final pCDH-CMV-MCS vector.

To generate the lentiviral plasmid encoding CDK1(AF)-3xFLAG, a CDK1(AF) cDNA (Addgene, 39872) was cloned into p3xFLAG-CMV-14 vector (Sigma-Aldrich) using NotI and KpnI. Subsequently, CDK1(AF)-3xFLAG was inserted into the pCDH-CMV-MCS vector for lentivirus production. EGFP-PCNA was cloned by swapping out mTagBFP2 in mTagBFP2-PCNA (Addgene, 85725) for EGFP (digested from EGFP-C1 vector) using AgeI and BspEI, and then EGFP-PCNA was cloned into the pCDH-CMV-MCS-EF1 α -Puro vector by XbaI and NotI to yield the lentiviral plasmid.

For TurboID experiments, mammalian-expression plasmids DCX-TurboID-V5 and EMTB-TurboID-V5 were cloned by replacing the C1(1-29) sequence in C1(1-29)-TurboID-V5-pCDNA3 (Addgene, 107173) with DCX (amplified from pLenti6-DCX-EGFP, Addgene, 80599) and EMTB (amplified from EMTB-mCherry, Addgene, 26742) respectively, using HindIII and NotI. The lentiviral plasmid for EMTB-TurboID-V5 was made by cloning the EMTB-TurboID-V5 cassette into the pCDH-CMV-MCS-EF1 α -Puro vector via EcoRI and BamHI. The Lentiviral plasmid for DCX-TurboID-V5 was

cloned by inserting the DCX-TurboID-V5 cassette into NotI-digested pCDH-CMV-MCS-EF1 α -Puro vector via Gibson assembly.

B2.5 Cell culture

Flp-In T-Rex HeLa (Thermo Fisher) and HEK 293TN cells (Anthony Bretscher, Cornell University) were cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (FBS) (Corning) and 1% penicillin/streptomycin (P/S, Corning) at 37 °C in a 5% CO₂ atmosphere. HEK 293TN cells were also supplemented with 1% sodium pyruvate (Corning). Stable GFP-PLEKHA5 expression was achieved by transfecting Flp-In T-Rex HeLa cells with flippase (pOG44, Thermo Fisher) and pCDNA5-FRT-EGFP-PLEKHA5 following the manufacturer's protocol (Thermo Fisher). After 36 h, cells were selected with 200 μ g/mL hygromycin B (Invitrogen). Stable expression of H2B-mCherry was accomplished by transducing pLenti6-H2B-mCherry plasmid (Addgene, 89766) into Flp-In T-Rex HeLa cells and blasticidin (Alfa Aesar) selection at 2.5 μ g/mL. Subsequently, lentiviral transduction of pCDH-CMV-EGFP-PCNA-EF1 α -Puro followed by puromycin (Sigma-Aldrich) selection at 2 μ g/mL yielded the HeLa cell line stably expressing both H2B-mCherry and EGFP-PCNA. HeLa cells stably expressing DCX-TurboID-V5 and EMTB-TurboID-V5 were generated by lentiviral transduction of pCDH-CMV-DCX-TurboID-V5-EF1 α -Puro and pCDH-CMV-EMTB-TurboID-V5-EF1 α -Puro respectively into Flp-In T-Rex HeLa cells and puromycin selection. Cell lines were used without further authentication, and mycoplasma testing (MycoSensor PCR assay, Agilent) was performed yearly.

B2.6 Transfection of plasmids and siRNAs

Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol but replacing Opti-MEM with Transfectagro (Corning). Cells were incubated with the transfection mix in Transfectagro with 10% FBS for 7–8 h, then the media was exchanged back to regular growth medium. DsiRNA duplexes were purchased from IDT. siRNA transfections were achieved using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's procedures except using Transfectagro instead of Opti-MEM. Twelve to 16 h after incubation with the transfection mix in Transfectagro with 10% FBS, cells were supplemented with the regular growth medium.

B2.7 Confocal Microscopy

For live-cell imaging, cells were seeded on 35 mm glass bottom culture dishes (14 mm diameter, #1.5 thickness, Matsunami Glass). For immunofluorescence, cells were seeded on 12 mm cover glass (#1.5 thickness, Fisherbrand) in 12-well plates (Corning). Cells were imaged (live cell) or fixed (immunofluorescence) 24–30 h post transfection. For immunofluorescence analysis of PLEKHA5 and MT-TurboID constructs, cells were extracted in Microtubule Stabilizing Buffer (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 5 mM EGTA pH 6.8, 0.5% Triton-X 100) for 30 s and fixed in methanol prechilled to $-20\text{ }^{\circ}\text{C}$ for 3 min. The cells were then rehydrated in wash buffer (0.1% Tween-20 in 1X PBS) for 5 min three times, further permeabilized with 0.5% Triton-X 100 in 1X PBS for 5 min and blocked in 2% BSA and 0.1% Tween-20 in 1X PBS (blocking buffer) for 30 min. Cells were incubated with primary antibody in

blocking buffer for 1 h, rinsed with wash buffer three times and treated with secondary antibody in blocking buffer for 1 h. Cells were then rinsed with wash buffer three times, with 1X PBS twice, mounted on slides in ProLong Diamond Antifade with DAPI (Thermo Fisher) and incubated overnight in dark before imaging. All steps were performed at room temperature, and slides were stored at 4 °C for long-term storage.

Images were acquired via Zeiss Zen Blue 2.3 software using a Zeiss LSM 800 confocal laser scanning microscope equipped with Plan Apochromat objectives (40× 1.4 NA) and two GaAsP PMT detectors. Solid-state lasers (405, 488, and 561 nm) were used to excite DAPI, EGFP/Alexa Fluor 488, and mCherry/Alexa Fluor 568, respectively. Live-cell time-series movies were acquired using Definite Focus. Depending on the experiments, images and movies were acquired either at room temperature or at 37 °C with a 5% CO₂ atmosphere. Acquired images were analyzed using FIJI.

B2.8 Immunoprecipitation and Western blots

Cells were harvested and lysed in IP lysis buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 5 mM EDTA, 50 mM Tris pH 7.5) supplemented with cOmplete protease inhibitor cocktail (Roche) on ice. Cell lysates were sonicated for 6 pulses at 20% intensity and centrifuged at 13,000 *g* for 5 min at 4 °C. Protein concentration of the clarified cell lysates was quantified using the BCA assay (Thermo Fisher), and a small fraction of the clarified cell lysates was separated and normalized as input. The remaining cell lysates were subjected to immunoprecipitation using GFP-Trap Magnetic Agarose (Chromotek) with rotation at 4 °C overnight (12–16 h). The

magnetic resin was then separated by a magnetic tube rack, washed three times with lysis buffer (at least 10 times the bead volume), denatured and analyzed by SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate (Bio-Rad), or by mass spectrometry-based proteomics analysis, as described in detail in the next section. For immunoprecipitation against endogenous proteins using soluble antibodies, the clarified cell lysates were incubated with primary antibodies (2 µg antibody per mg of input for IgG control, PLEKHA5, ANAPC3 and ANAPC4, and 1:150 dilution for ANAPC8) for 2 h at 4 °C with rotation, followed by addition of Protein G Sepharose (BioVision) and rotation at 4 °C overnight. The resin was then centrifuged for 5 min at 1000 *g*, washed three times with lysis buffer (at least 10 times of the bead volume), denatured and analyzed by SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate.

B2.9 SILAC Labeling and Mass Spectrometry-Based Proteomics Analysis

For quantitative proteomics, Flp-In HeLa cells stably expressing EGFP-PLEKHA5 were cultured in “heavy” SILAC DMEM media (Thermo) supplemented with 10% dialyzed FBS (Corning) and 1% P/S for at least five passages to allow full labeling of cells before they were used in experiments. “Light” EGFP-expressing HeLa cell line was generated previously⁴. “Heavy” SILAC DMEM contained L-lysine ¹³C₆, ¹⁵N₂ and L-arginine ¹³C₆, ¹⁵N₄. “Light” SILAC DMEM contained L-lysine ¹²C₆, ¹⁴N₂ and L-arginine ¹²C₆, ¹⁴N₄. Cells were lysed and immunoprecipitated with GFP-Trap magnetic agarose.

The GFP-Trap Magnetic resin was washed four times with lysis buffer and incubated with elution buffer (100 mM Tris pH 8.0, 1% SDS) for 5 min at 95 °C. The supernatant from “light” and “heavy” samples were collected and mixed in a 1:1 ratio. The mixed sample was then reduced with 10 mM DTT (65 °C, 10 min) and alkylated with 25 mM iodoacetamide (in 50 mM Tris pH 8.0, room temperature, 15 min in dark). Proteins from the mixed sample were precipitated in a solution containing 50% acetone, 49.9% ethanol and 0.1% acetic acid (PPT solution) on ice for 1 h and pelleted at 17,000 *g* for 10 min. The protein pellet was washed with PPT solution once, pelleted again, air-dried and resuspended in urea/Tris solution (8 M urea, 50 mM Tris pH 8.0) and NaCl/Tris solution (150 mM NaCl, 50 mM Tris pH 8.0) in a ratio of 1:3 respectively, for a final concentration of 2 M urea. One µg of mass spectrometry grade trypsin (Trypsin Gold, Promega; 1 mg/mL in 0.03% acetic acid), was added and proteins were trypsinized at 37 °C overnight on a nutator. The next day, the digested peptides were acidified to a final concentration of 0.25% formic acid and 0.25% trifluoroacetic acid. Samples were desalted using a 50 mg C18 Sep-Pak column (Waters), and dried using a SpeedVac vacuum concentrator.

Next, peptides were subject to MS/MS analysis as described previously^{5,6}. Briefly, samples were resuspended in 90 µL of 65% acetonitrile and 1% formic acid and subjected to hydrophilic interaction chromatography (HILIC) using a TSKgel amide 80 column with a 5 µm particle size (Tosoh Bioscience). Ten fractions were collected at 1-min intervals with the fraction collection gradient starting at 92% solvent A (80% acetonitrile, 0.005% trifluoroacetic acid) and 8% solvent B (0.025% trifluoroacetic acid). The fraction collection gradient ended at 80% solvent A and 20% solvent B.

Fractions were dried in a vacuum concentrator and resuspended in a solution of 0.1% trifluoroacetic acid and 0.1 picomole/ μ L angiotensin II peptide (Sigma-Aldrich).

Each fraction was analyzed using a ThermoFisher Q Exactive HF Orbitrap mass spectrometer coupled to an UltiMate 3000 HPLC system and a C18 capillary column (20 cm length, 125 μ m inner diameter) packed in-house with 3- μ m, 200-A C18AQ particles (Prontosil). Peptides were separated over a 60-min linear gradient from 8% to 40% acetonitrile in 0.1% formic acid at a flow rate of 200 nL/min. The mass spectrometer was operated in data-dependent mode, and survey scans were acquired in the Orbitrap mass analyzer over the range of 380 to 2,000 m/z with an MS1 mass resolution of 60,000. The maximum ion injection time for the survey scan was 80 ms with a 3×10^6 automatic gain-control target. Tandem mass spectrometry was performed by selecting up to the 10th most abundant ion (top10 method) with a charge state of 2, 3, or 4 and with an isolation window of 2.0 m/z. Selected ions were fragmented by higher energy collisional dissociation (HCD) with a normalized collision energy of 27, and the tandem mass spectra were acquired in the Orbitrap mass analyzer with a mass resolution of 15,000. For the database search, raw spectra were searched in a SORCERER system (Sage-N Research, Inc.) using SEQUEST software (Thermo Fisher Scientific), and all entries were from the human UniProt proteome database. The following parameters were used in the database search: semitryptic requirement, a mass accuracy of 15 ppm for the precursor ions, a differential modification of 8.0142 Da for lysine and 10.00827 Da for arginine, and a static mass modification of 57.021465 Da for alkylated cysteine residues. The XPRESS software, part of the Trans-Proteomic Pipeline (Seattle Proteome Center),

was used to quantify all the identified peptides. A Wilcoxon rank-sum test was used to measure statistical significance.

B2.10 Lentivirus production

Lentivirus was produced in HEK 293TN cells, which were seeded two days before transfection to achieve about 60% confluency on the day of transfection with viral plasmids. Packaging plasmids VSVg and PAX2, along with the lentiviral plasmid encoding proteins of interest, were co-transfected into HEK 293TN cells in the ratio of 1:3.1:4.2 using Lipofectamine 2000 overnight in Transfectagro. Fresh growth media was changed the next morning (14–16 h post transfection) to replace Transfectagro. The virus-containing medium was collected 4 times every 12 h starting from 24–30 h post transfection. Collected virus medium was filtered through a 0.45 μm syringe filter and stored at 4 °C for up to 48 h if not used immediately.

B2.11 Lentivirus transduction

Most cell lines stably expressing proteins of interest were generated via traditional lentivirus transduction. Cells were seeded on 6-well plates one day before transduction to achieve around 80% confluent on the day of transduction. One well of “kill control” cells was included in the selection process where cells in this well would not be treated with virus. During viral transduction, cells in each well were treated with 1 mL of fresh growth medium, 2 mL of virus-containing medium and 8 $\mu\text{g/mL}$ of polybrene (Sigma-Aldrich). The transduction process was repeated three times every 12 h, and after the last transduction, fresh medium was supplemented for 12 h before the selection process. Cells in 6-well plates were trypsinized, seeded

into 10-cm dishes and incubated with appropriated selection drugs until all the kill control cells died. During the selection, drug-containing media was changed every two days. Stable expression of proteins of interest was examined by confocal microscopy or Western blot analysis. Transient virus transduction for exogenous protein expression was achieved by spinfection where indicated. On the day of spinfection, 1 million cells were seeded into each well in 6-well plates and spread evenly, and each well contained 0.5 mL of fresh growth medium, 3.5 mL of virus medium and 8 µg/mL of polybrene. After seeding the cells, the 6-well plates were immediately centrifuged at 930 *g* for 2 h at 33 °C. After centrifugation, without aspirating the transduction medium, 2 mL of fresh growth medium was added to each well, and cells were incubated in a 5% CO₂ atmosphere at 37 °C until use.

B2.12 Cell synchronization

B2.12.1 S-phase synchronization by double thymidine block: When cells reached 60–70% confluency, 2 mM thymidine was added to the medium for 18 h. Thymidine-containing medium was then discarded, and cells were washed with 1X DPBS (Corning) once and supplemented with fresh growth medium for 9 h. Again, 2 mM thymidine was added and left in medium for 15 h. Cells were washed with 1X DPBS once and released into fresh medium for indicated time before harvest for assays.

B2.12.2 Prometaphase synchronization by S-trityl-L-cysteine: To synchronize cells in prometaphase, 5 µg/mL of S-trityl-L-cysteine (STLC) was added to confluent dishes of cells for 16 h. Mitotic cells were dislodged from dishes by gentle tapping, washed

with 1X DPBS, and collected. Cells pellets were stored at -80°C if not used immediately.

B2.13 Proximity Biotinylation by MT-TurboIDs

HeLa cells transfected with pCDNA3-DCX-TurboID-V5 or pCDNA3-EMTB-TurboID-V5 for 30 h were incubated with 500 μM biotin for 10 min at 37°C under 5% CO_2 . For localization by immunofluorescence, cells were rinsed five times with $1\times$ PBS and processed for immunofluorescence analysis as described earlier (see section B3.7). For streptavidin-agarose pulldown and western blot, cells were rinsed five times with $1\times$ PBS and lysed with RIPA buffer (150 mM NaCl, 25 mM Tris pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche). Cell lysates were sonicated with four pulses at 20% intensity and centrifuged at 13,000g for 5 min at 4°C . The protein concentration of the clarified cell lysates was quantified using the bicinchoninic acid (BCA) assay (Thermo Fisher), and a small fraction of the clarified cell lysates was reserved and normalized as input. The remaining cell lysates were subjected to immunoprecipitation using streptavidin-agarose (Thermo Fisher) with rotation at 4°C overnight. The resin was then centrifuged for 5 min at 1000g, washed two times with RIPA buffer, one time with 1 M KCl, one time with 0.1 M Na_2CO_3 , one time with 2 M urea in 10 mM Tris pH 8.0, and two times with RIPA buffer to eliminate nonspecific binding. Samples were then denatured and analyzed by SDS-PAGE and western blot with detection by chemiluminescence using the Clarity Western ECL substrate.

B2.14 Isolation of endogenous APC/C for in vitro ubiquitination assays

Endogenous APC/C was isolated from HeLa cells synchronized to prometaphase by STLC. Briefly, collected prometaphase HeLa cells were lysed in ubiquitination assay lysis buffer (150 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 20 mM HEPES pH 7.4, 0.1% NP-40) supplemented with cOmplete protease inhibitor cocktail (Roche) and 1 µL of benzonase (Millipore) per 15-cm plate on ice. Cell lysates were vortexed vigorously for 10 s, sonicated with four pulses at 20% intensity and spun at 13,000 g for 5 min at 4 °C. Clarified cell lysates were pre-cleared with washed Protein G Sepharose (BioVision) for 1 h with rotation at 4 °C. Protein concentration of the pre-cleared cell lysates was quantified using the BCA assay (Thermo Fisher), and a small fraction of the pre-cleared cell lysates was reserved and normalized as input. The remaining cell lysates were incubated with anti-ANAPC3 antibody (Santa Cruz Biotechnology, 2 µg antibody per mg of input) for 1 h at 4 °C with rotation, and then washed Protein G Sepharose was added to the sample for incubation at 4 °C with rotation for 2 h. The APC/C coupled beads were washed 5 times with ubiquitination assay lysis buffer (at least 10 times of bead volume) before use in the in vitro ubiquitination assay, which was performed immediately after isolation of APC/C on beads.

B2.15 In vitro ubiquitination assays

The assays were carried out in a 20 µL reaction volume, and components of complete reactions included: 0.5 µL of 5 µM UBE1 (125 nM final, R&D Systems), 1 µL of 10 µM UBE2S (0.5 µM final, R&D Systems), 1 µL of 10 µM UBE2C (0.5 µM final, R&D Systems), 1 µL of 10 mg/mL His-TEV-ubiquitin (0.5 µg/µL final, prepared as described

below), 2 μ L of 0.5 mg/mL securin (0.5 μ g/ μ L final, Abcam), 1 μ L of 100 μ M DTT, 1.5 μ L of energy mix (150 mM creatine phosphate, 20 mM ATP, 20 mM MgCl₂, 2 mM EGTA, pH to 7.5), 2 μ L of 10X assay buffer (500 mM NaCl, 100 mM MgCl₂ and 250 mM Tris pH 7.5), 5 μ L of PAC/C coupled resin and 5 μ L of 1X PBS. For control reactions that lacked a certain component, the mixture was supplemented accordingly with 1X PBS. Reactions were performed at 30 °C with shaking for 30 min and stopped by addition of 6X Laemmli Buffer and incubation at 95 °C for 5 min. Samples were analyzed by SDS-PAGE and Western blot.

B2.16 Protein expression and purification from *E. coli*

To generate 6xHis-TEV-ubiquitin, *E. coli* BL21-pRosetta2 transformed with pET-His-TEV-Ub plasmid (a gift from Yuxin Mao, Cornell University) was cultured as previously described⁴. Briefly, a single colony was picked and grown in terrific broth (TB) supplemented with potassium phosphate buffer (0.17 M monobasic potassium phosphate, 0.17 M dibasic potassium phosphate), ampicillin, and chloramphenicol for 14–18 h as a starter culture. 1 L of TB containing phosphate buffer and antibiotics was inoculated with 25 mL of the starter culture and grown for 6–8 h at 37 °C until OD₆₀₀ was between 2 and 3. The temperature was then decreased to 18 °C for 1 h, and protein expression was induced by adding 0.25 mM isopropylthio- β -galactosidase (IPTG) to the cultures and for at least 18–20 h at 18 °C. Cells were harvested (2100 g, 15 min, 4 °C) and stored at –80 °C until use.

Frozen cell pellets were thawed in bacterial lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at

31,000 *g* for 45 min at 4 °C. The supernatant was incubated with washed TALON metal affinity resin (Takara) for 1.5 h under nutation at 4 °C. After incubation, the resin with bound protein was washed extensively with 150 mM NaCl, 50 mM Tris pH 8.0 to get rid of non-specifically bound proteins. His-TEV-Ub was eluted by incubation of the washed resin with elution buffer (300 mM imidazole, 150 mM NaCl, 50 mM Tris pH 8.0) for 10 min at 4 °C. Elution was continued until a Bradford assay showed no more protein was eluting from the resin. All the eluant fractions were pooled and buffer-exchanged to 150 mM NaCl, 50 mM Tris pH 8.0 to remove most of the imidazole in the eluant, concentrated in 3K Amicon concentrators (Millipore), quantified using a Bradford assay, and flash frozen for storage at –80°C until use. Samples were analyzed by SDS-PAGE to verify yield and purity. In some instances, His-TEV-Ub was further purified by size exclusion chromatography with 150 mM NaCl, 20 mM Tris pH 7.5, with fractions collected based on molecular weight and verified by SDS-PAGE, followed by concentration and flash freezing for storage at –80°C.

B2.17 Cell cycle analysis

B2.17.1 Propidium iodide staining and flow cytometry: Measurement of DNA content in fixed cells by propidium iodide staining and flow cytometry was performed as previously described with slight modifications. Briefly, experiments were performed in 12-well plates, and cells from each well were lifted by trypsinization and pelleted at 1000 *g* for 3 min. Cell pellets were washed with 1X DPBS once, pelleted again, and resuspended in 100 µL of 1X DPBS. One mL of ethanol prechilled to –20 °C was added to each sample slowly with vortexing, and cells were fixed at 4 °C overnight.

Next morning, fixed cells were pelleted at 1000 g for 5 min, resuspended in FACS buffer (1% FBS in 1X DPBS), transferred to a 96-well V bottom plate (Greiner Bio-One), and pelleted in the plate. Cells were washed once with FACS buffer and incubated with labeling solution (50 µg/mL propidium iodide and 1 mg/mL RNase A in FACS buffer) for 30 min at 37 °C. The plate was wrapped in foil and stored at 4 °C until samples were analyzed by flow cytometry (BD Accuri C6).

B2.17.2 Western blot: Cells were lysed with RIPA buffer (150 mM NaCl, 25 mM Tris pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with cOmplete protease inhibitor cocktail (Roche) and phosphatase inhibitors mixture (17.5 mM β-glycerophosphate, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) on ice. Cell lysates were sonicated for 2 pulses at 20% intensity and centrifuged at 13,000 g for 5 min at 4 °C. Protein concentration of the clarified cell lysates was quantified using the BCA assay (Thermo Fisher). Cell lysates were normalized to 2–3 mg/mL, and denatured with 6X Laemmli Buffer (13.3% SDS, 0.067% bromophenol blue, 52.2% glycerol, 67 mM Tris pH 6.8 and 11.1% β-mercaptoethanol) at 95 °C for 5 min. Samples were analyzed by SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate (Bio-Rad).

B2.17.3 Live-cell imaging of H2B and PCNA: Cells were transfected with siRNA duplexes as described above and seeded on 35 mm glass bottom culture dishes (14 mm diameter, #1.5 thickness, Matsunami Glass). Thirty h after siRNA transfection, regular DMEM growth medium was replaced with DMEM without phenol red (Gibco)

supplemented with 10% FBS and 1% P/S. Cells were imaged at 37 °C in a 5% CO₂ environment. H2B-mCherry was excited by the 561 nm laser, and EGFP-PCNA was excited by the 488 nm laser. For H2B-mCherry imaging to monitor mitosis progression, images were acquired every 4 min for 10–12 h. For H2B-mCherry and EGFP-PCNA imaging to measure the G2-to-M transition, images were acquired every 10 min for 30–32 h.

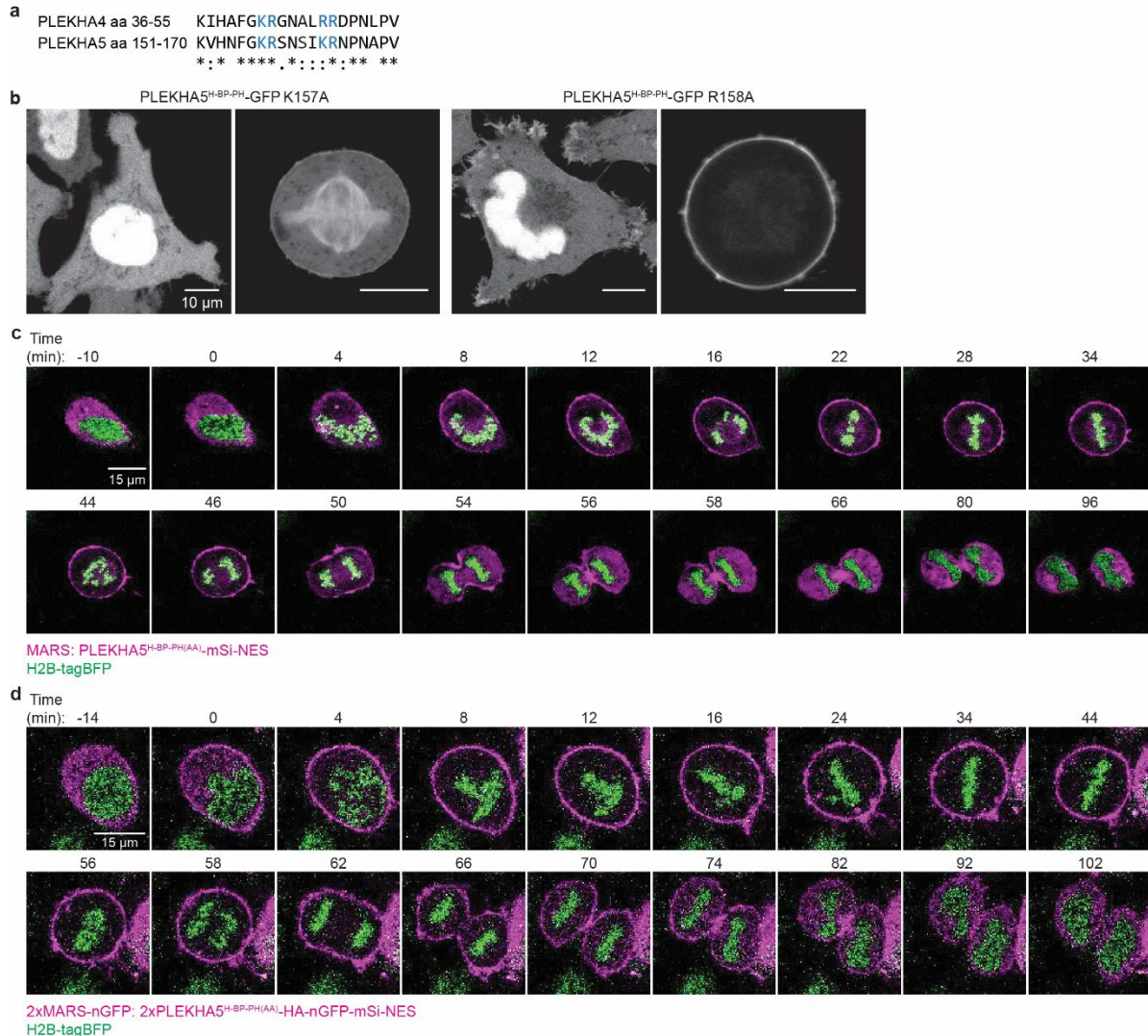
B2.18 Statistical Analysis

For all experiments involving quantification, statistical significance was calculated using an unpaired two-tailed Student's *t*-test with unequal variance in Microsoft Excel, a Mann–Whitney *U* test and a Kolmogorov–Smirnov test in GraphPad Prism, or a one-way ANOVA with Tukey post-hoc test in GraphPad Prism, as indicated in figure legends. Statistical significance of $p < 0.05$ or lower is reported, and the number of biological replicates analyzed is stated in figure legends. All raw data were plotted into graphs using GraphPad Prism. In figures containing scatter plots with straight connecting lines between markers to show changes in measurement over time, the mean at each timepoint was plotted, and the error bars represent the standard deviation. For regular scatter plots, the black line indicates the mean, and each dot represents an individual biological replicate.

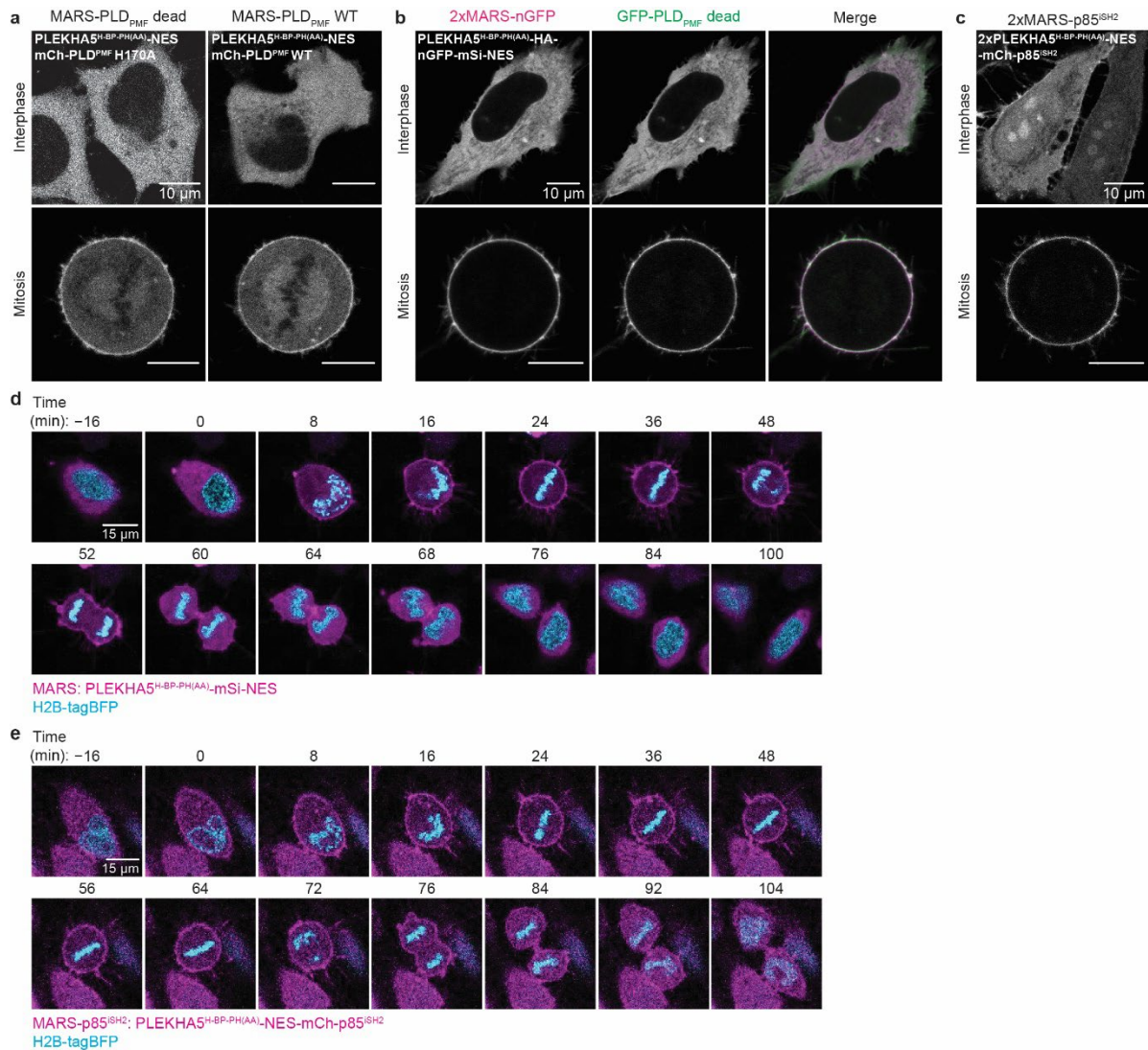
APPENDIX C

SUPPLEMENTARY INFORMATION FOR CHAPTER 4

C1 SUPPLEMENTAL FIGURES



Appendix Figure C1 (related to Figure 4.1). Design and engineering of MARS and MARS-nGFP. (a) Clustal Omega alignment of primary amino acid sequences around the “4A” region of PLEKHA4 and PLEKHA5. (b) Confocal microscopy of the K157A and R158A mutants of PLEKHA5^{H-BP-PH}-GFP. (c, d) Representative series of still images from time-lapse movies in asynchronous HeLa cells stably expressing H2B-tagBFP and transfected with either MARS (c) or 2xMARS-nGFP (d). MARS constructs are colored magenta and H2B-tagBFP is colored green. Numbers indicate time before/after nuclear envelope breakdown (defined as t=0 min).



Appendix Figure C2 (related to figure 4.2 and 4.3). Evaluation of PM localization of additional MARS constructs for PLD and PI3K and time-lapse imaging of MARS and MARS-p85^{SH2} during mitosis. (a) Live-cell confocal microscopy of HeLa cells expressing the single MARS fusion to WT or catalytic dead (H170A) forms of PLD_{PMF} revealing only partial recruitment to the PM during mitosis. (b) Representative confocal micrographs of HeLa cells transfected with 2xMARS-nGFP (magenta) and H170A mutant of GFP-PLD_{PMF} (green) showing effective PM recruitment only during mitosis. (c) Live-cell confocal microscopy of HeLa cells expressing the 2xMARS-p85^{SH2} construct, revealing undesired partial PM localization during interphase. (d, e) Representative still images from time-lapse movies in asynchronous HeLa cells stably expressing H2B-tagBFP and transfected with either MARS (d) or MARS-p85^{SH2} (e), and the PI(3,4,5)P₃ marker GFP-Akt^{PH}. Images display merged channels of H2B-tagBFP (cyan) and MARS constructs (magenta). Numbers indicate time before/after nuclear envelope breakdown (defined as t=0 min).

C2 MATERIALS AND METHODS

C2.1 Reagents

Lipofectamine 2000, Invitrogen (11668019); Polybrene Infection/Transfection Reagent, Sigma-Aldrich (TR-1003); (+)-S-Trityl-L-cysteine, Alfa Aesar (L14384, CAS #: 2799-07-7); Puromycin dihydrochloride, Sigma-Aldrich (P8833, CAS # 58-58-2); 5-gluoro-2-indolyl des-chlorohalopemide (FIPI), Cayman Chemical (13563, CAS #: 939055-18-2) 5-Hexyn-1-ol, Sigma-Aldrich (302015, CAS #: 928-90-5); AZDye 647 Azide, Click Chemistry Tools (1299); 3-azido-1-propanol⁷ and BCN-BODIPY⁸ were prepared in house as described previously.

C2.2 Plasmids and Cloning

For amino acid mutagenesis, mutations were incorporated into plasmids using the QuikChange XL Site-directed mutagenesis kit (Agilent). A nuclear export signal with amino acid sequence LQLPPLERLTLD was inserted between PLEKHA5 and GFP, or downstream of GFP into the PLEKHA5^{H-BP-PH}-EGFP plasmid, and right after mScarlet-i into the mScarlet-i-N1 vector (A gift from Barbara Baird's lab, Cornell University) using the QuikChange XL Site-directed mutagenesis kit as well.

To construct the single MARS plasmid (PLEKHA5^{H-BP-PH(AA)}-mSi-NES), H-BP-PH domain with K163A/R164A mutations were amplified from the GFP fusion construct and inserted into the EcoRI digested mScarlet-i-N1-NES vector. Additionally, PLEKHA5^{H-BP-PH(AA)}-NES-mSi was made by subcloning H-BP-PH(AA) into EcoRI digested mScarlet-i-N1 vector and then inserting the NES between PLEKHA5 and mScarlet-i using the QuikChange XL Site-directed mutagenesis kit. An additional H-

BP-PH(AA) motif was added to PLEKHA5^{H-BP-PH(AA)}-mSi-NES and PLEKHA5^{H-BP-PH(AA)}-NES-mSi at the KpnI site to generate 2xMARS tandem constructs, with the two H-BP-PH(AA) motifs separated by a flexible linker (SGGRGSGSGSGSG). Subsequently, an ORF encoding HA-nGFP (amplified from pcDNA3.1-HA-nGFP-(EAAAK)₄-OGT(4), Addgene 160739) was inserted in the PLEKHA5^{H-BP-PH(AA)}-mSi-NES plasmid using the BamHI site to yield the MARS-nGFP construct (PLEKHA5^{H-BP-PH(AA)}-HA-nGFP-mSi-NES). 2xMARS-nGFP was generated similarly by adding HA-nGFP to the 2xPLEKHA5^{H-BP-PH(AA)}-mSi-NES plasmid between the tandem H-BP-PH(AA) motifs and mScarlet-i.

Single and tandem MARS fusions to WT PLD_{PMF} were made by swapping Lyn10 in pCDNA3-Lyn10-mCherry-PLD_{PMF} (Julia Li, Baskin Lab) for either H-BP-PH(AA)-NES or 2xH-BP-PH(AA)-NES using HindIII and AgeI. Subsequently, H170A mutation of PLD was incorporated using the QuikChange XL Site-directed mutagenesis kit in these plasmids. To make MARS-p85^{iSH2} and 2xMARS-p85^{iSH2}, p85^{iSH2} sequence (amplified from pPBpuro-mNG-eDHFR(69K6)-p85^{iSH2}, Addgene 172103) was first inserted after the mCherry in pCDNA3-Lyn10-mCherry-PLD_{PMF} by BamHI and ApaI to replace PLD_{PMF}, yielding pCDNA3-Lyn10-mCherry-p85^{iSH2}. Subsequently, the Lyn10 tag was again switched to either H-BP-PH(AA)-NES or 2xH-BP-PH(AA)-NES using HindIII and AgeI. GFP-PLD_{PMF} and GFP-p85^{iSH2} were constructed by inserting the coding sequence for each gene into the EGFP-C1 expression vector (Clontech) via HindIII and KpnI.

H2B-tagBFP was cloned by inserting H2B cDNA (amplified from pLenti6-H2B-mCherry, Addgene 89766) into tagBFP-N1 vector via Sall and Apal, and subsequently H2B-tagBFP was cloned into a modified pCDH-CMV-MCS-EF1 α -Puro vector (inserted an Ascl site upstream of the Xbal site in the original plasmid) by Ascl and NotI to yield the lentiviral plasmid. Akt^{PH}-miRFP670 was generated by cloning the Akt^{PH} sequence (amplified from GFP-Akt^{PH}, a gift from the De Camilli lab at Yale University) into the miRFP670-N1 vector using Sall and Apal.

C2.3 Cell Culture

Flp-In T-Rex HeLa cells (Thermo Fisher) were cultured in DMEM (Corning) supplemented with 10% FBS (Corning) and 1% penicillin/streptomycin (P/S, Corning) at 37 °C in a 5% CO₂ atmosphere. HEK 293TN cells (gift from Anthony Bretscher, Cornell University) were cultured under the same condition with additional supplement of 1% sodium pyruvate (Corning) in the media. HeLa cell line with PLK1 tagged by GFP at the endogenous locus via CRISPR/Cas9 was a gift from Prof. Iain Cheeseman at MIT. Stable expression of H2B-tagBFP was accomplished by lentiviral transduction of pCDH-CMV-H2B-tagBFP-EF1 α -Puro into Flp-In T-Rex HeLa cells and puromycin selection at 2 μ g/mL. Cell lines were used without further authentication, and mycoplasma testing (MycoSensor PCR assay, Agilent) was performed yearly.

C2.4 Plasmid Transfection

Plasmids were transfected into mammalian cells using Lipofectamine 2000 following the manufacturer's protocol. Opti-MEM was replaced with Transfectagro. Cells were

incubated with the transfection mix in Transfectagro with 10% FBS for 7–8 h, then cultured in regular growth medium until harvest.

C2.5 Lentivirus production

HEK 293TN cells were seeded one day before transfection to achieve about 50% confluency on the day of transfection with lentiviral plasmids. Packaging plasmids PAX2 and VSVg, along with the lentiviral plasmid encoding proteins of interest, were co-transfected into HEK 293TN cells in the ratio of 3.1:1:4.2 using Lipofectamine 2000 overnight in Transfectagro. 14–16 h post transfection, Transfectagro was replaced with fresh growth medium the next morning (14–16 h post transfection) to replace Transfectagro. The virus-containing medium was collected 4 times every 12 h starting from 24–30 h post transfection, filtered through a 0.45 µm syringe filter, and stored at 4 °C for up to 48 h if not used immediately.

C2.6 Lentivirus transduction

Cells were seeded on 6-well plates one day before transduction to achieve around 70% confluency the next day. One “kill control” well was included in the selection process where cells in this well would not be treated with virus medium. During viral transduction, cells in each well were incubated with 1 mL of fresh growth medium, 2 mL of virus-containing medium, and 8 µg/mL of polybrene. The transduction process was repeated three times every 12 h, and after the last transduction, fresh medium was supplemented for 12 h before the selection process. Cells in 6-well plates were trypsinized, seeded into 10-cm dishes, and incubated with puromycin until all the kill control cells died. During the selection, drug-containing media was exchanged every

two days. Stable expression of proteins of interest was examined by confocal microscopy or Western blot analysis.

C2.7 Cell synchronization

To synchronize cells in prometaphase, 5 µg/mL of S-trityl-L-cysteine (STLC) was added to confluent dishes of cells for 16 h. Mitotic cells were dislodged from dishes by gentle tapping, washed with 1X DPBS, and collected. Cells pellets were stored at -80 °C if not used immediately.

C2.8 Confocal Microscopy

For live-cell imaging, cells were seeded on 35 mm glass bottom culture dishes (14 mm diameter, #1.5 thickness, Matsunami Glass). For immunofluorescence, cells were seeded on 12 mm cover glass (#1.5 thickness, Fisherbrand) in 12-well plates (Corning). Cells were imaged (live cell) or fixed (immunofluorescence) 24–30 h post transfection. Images were acquired via Zeiss Zen Blue 2.3 software on a Zeiss LSM 800 confocal laser scanning microscope equipped with Plan Apochromat objectives (40x 1.4 NA) and two GaAsP PMT detectors. Solid-state lasers (405, 488, 561, and 640 nm) were used to excite DAPI/tagBFP, EGFP/Alexa Fluor 488, and mCherry/mScarlet-i/Alexa Fluor 568, and mRFP/miRFP670 respectively. Live-cell time-lapse movies were acquired using definite focus. Depending on the experiments, images and movies were acquired either at room temperature, or at 37 °C with a 5% CO₂ atmosphere when indicated. Acquired images were analyzed using FIJI. To quantify PM and cytosolic fluorescence, 5 line-scans at different areas were drawn for each cell to get the average fluorescence.

For most live imaging experiments not involving time-lapse movies, cells were rinsed twice and then imaged in Tyrode's-HEPES buffer (T/H buffer: 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mg/mL glucose, 5 mg/mL bovine serum albumin, 20 mM HEPES, pH 7.4) at room temperature.

For long-term time-lapse imaging, cells were imaged in DMEM phenol red (Gibco) supplemented with 10% FBS and 1% P/S at 37 °C in a 5% CO₂ environment 24–30 h post transfection. For H2B-BFP and MARS or 2xMARS-nGFP imaging to monitor MARS localization, images were acquired every 2 min for 14 h. For H2B-BFP, GFP-Akt^{PH} and MARS or MARS-p85^{iSH2} imaging to monitor mitosis progression, images were acquired every 4 min for 14 h.

C2.9 IMPACT Labeling to Measure PLD_{PMF} Activity

C2.9.1 Azidopropanol with BCN-BODIPY labeling for 2xMARS-PLD_{PMF}

Cells were transfected with 2xMARS-PLD_{PMF} constructs for 32 h, then treated with 5 ug/mL STLC to synchronize cells to prometaphase. The same volume of DMSO was added to asynchronous populations. Cells were either incubated with a PLD inhibitor FIPI at 750 nM or vehicle for 30 min, then treated with 1 mM azidopropanol for 30 min to generate azido phosphatidyl alcohol lipid reporters for PLD activity, rinsed with 1x PBS three times, followed by incubation with 1 uM BCN-BODIPY for copper-free click chemistry tagging of the reporter lipids for 30 min. Cells were then rinsed three times with PBS, and incubated in T/H buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mg/mL glucose, 5 mg/mL bovine serum albumin, 20 mM HEPES, pH 7.4) for 15 min to rinse out unreacted, excess BCN-BODIPY. All the incubation steps

were performed at 37 °C with a 5% CO₂ atmosphere. Cells were then washed with PBS twice. Asynchronous cells were lifted by trypsin-EDTA (Corning; 0.05% trypsin and 0.1% EDTA), and prometaphase cells were dislodged by tapping plates and collected by vigorous pipetting in T/H buffer. Cells were washed with T/H buffer twice, then fixed with 4% paraformaldehyde in PBS for 15 min in dark at room temperature, rinsed with FACS buffer twice (0.1% FBS in PBS), and subjected to two-color flow cytometry analysis. 561 nm laser was used to excite the mCherry within 2xMARS-PLD_{PMF} constructs, and 488 nm laser was used to excite BCN-BODIPY as IMPACT signal for PLD activity. IMPACT signals from cells with similar expression levels of 2xMARS-PLD_{PMF} constructs were extracted for data analysis. For each experimental condition, IMPACT signals from FIPI treated samples were averaged and subtracted from each replicate, and the differences of mean fluorescence were plotted.

C2.9.2 Hexynol with AZDye 647 labeling for 2xMARS-nGFP and GFP-PLD_{PMF}

Cells were co-transfected with 2xMARS-nGFP and GFP-PLD_{PMF} constructs for 32 h, then treated with 5 ug/mL STLC to synchronize cells to prometaphase. The same volume of DMSO was added to asynchronous populations. Cells were either incubated with a PLD inhibitor FIPI at 750 nM or vehicle for 30 min, then treated with 1 mM hexynol for 30 min to generate alkyne-containing phospholipid reporters for PLD activity and rinsed with PBS three times. Incubations were performed at 37 °C with a 5% CO₂ atmosphere. Asynchronous cells were lifted by 0.05% trypsin-EDTA, and prometaphase cells were dislodged by tapping plates and collected by vigorous pipetting in T/H buffer. Cells were rinsed twice with T/H buffer, fixed with 4%

paraformaldehyde in PBS for 15 min in dark, and rinsed another two times with T/H buffer. Cells were then incubated with the AZDye 647-azide solution (100 mM Tris pH 8, 1 mM CuSO₄, 50 mM sodium ascorbate, and 5 μ M AZDye 647-azide) for click chemistry tagging of the alkyne reporter lipids for 1 h at room temperature. After being washed with FACS buffer twice, cells were subjected to three-color flow cytometry analysis. 561 nm laser was used to excite the mScarlet-i within 2xMARS-nGFP, 488 nm laser was used to excite GFP-PLD_{PMF} constructs, and 640 nm laser was used to excite AZdye 647 as IMPACT signal for PLD activity. IMPACT signals from cells with similar expression levels of 2xMARS-nGFP and GFP-PLD_{PMF} were extracted for data analysis. For each experimental condition, IMPACT signals from FIPI treated samples were averaged and subtracted from each replicate, and the differences of mean fluorescence were plotted.

C2.10 Statistical analysis

For all experiments involving quantification, statistical significance was calculated in GraphPad Prism using tests as indicated in the figure legend, including an unpaired two-tailed t-test with unequal variance, a Mann-Whitney U test, or a one-way ANOVA with Tukey or Sidak post-hoc test. P values for each comparison are reported, and the number of biological replicates or cells analyzed is stated in the legend. All the raw data were plotted into graphs using GraphPad Prism. For all scatter plots, the black line indicates the mean, and each dot represents an individual biological replicate.

APPENDIX D

SUPPLEMENTARY INFORMATION FOR CHAPTER 5

D1 MATERIALS AND METHODS

D1.1 Antibodies

Mouse anti-PLEKHA5, Santa Cruz Biotechnology (sc-390311, RRID: N/A); mouse anti-GFP, Santa Cruz Biotechnology (sc-9996, RRID: AB_627695); mouse anti-mCherry, Abcam (ab125096, RRID: AB_11133266); mouse anti-HA, Covance (MMS-101R, RRID: AB_291262); rabbit anti-KCTD3, Abcam (ab184561, RRID: N/A); goat anti-mouse HRP, Jackson ImmunoResearch Labs (115-035-146, RRID: AB_2307392); goat anti-rabbit HRP, Jackson ImmunoResearch Labs (111-035-144, RRID: AB_2307391).

D1.2 Reagents

Lipofectamine 2000, Invitrogen (11668019); GFP-Trap magnetic agarose, ChromoTek (gtma-20); cOmplete Protease Inhibitor Cocktail, Roche (5056489001); Sodium fluoride, Chem-Impex (01523, CAS #: 7681-49-4); Clarity™ Western ECL Substrate, Bio-Rad (1705061); BCA Protein Assay Kit, Thermo Fisher (23225).

D1.3 Plasmids and cloning

GFP-tagged PLEKHA5 constructs were generated previously, as described in **Appendix A2.3**. A KCTD3 cDNA (Uniprot Q9Y597, a gift from Haiyuan Yu, Cornell University) was cloned into the pEGFP-C1 (Clontech) and mScarlet-i-C1 vector (Addgene 85044) using EcoRI and KpnI to generate EGFP-KCTD3 and mScarlet-i-

KCTD3. Subsequently, the F43A mutation was incorporated into the EGFP-KCTD3 plasmid by QuikChange XL Site-directed mutagenesis kit (Agilent). A SHKBP1 cDNA (Uniprot Q8TBC3, a gift from Haiyuan Yu, Cornell University) was inserted into the EcorI and KpnI digested pEGFP-C1 vector and pCMV-HA vectors (Clontech) to construct EGFP-SHKBP1 and HA-SHKBP1. After that, F44A mutation was incorporated into EGFP-SHKBP1 by QuikChange XL Site-directed mutagenesis kit (Agilent).

D1.4 Cell Culture

Flp-In T-Rex HeLa cells (Thermo Fisher) were cultured in DMEM (Corning) supplemented with 10% FBS (Corning) and 1% penicillin/streptomycin (P/S, Corning) at 37 °C in a 5% CO₂ atmosphere. Cell lines were used without further authentication, and mycoplasma testing (MycoSensor PCR assay, Agilent) was performed yearly.

D1.5 Plasmid Transfection

Plasmids were transfected into mammalian cells using Lipofectamine 2000 following the manufacturer's protocol. Opti-MEM was replaced with Transfectagro. Cells were incubated with the transfection mix in Transfectagro with 10% FBS for 7–8 h, then cultured in regular growth medium until harvest.

D1.6 Immunoprecipitation and Western blots

Cells were harvested and lysed in IP lysis buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 5 mM EDTA, 50 mM Tris pH 7.5) supplemented with cComplete protease inhibitor cocktail (Roche) on ice. Cell lysates were sonicated for 6 pulses at

20% intensity and centrifuged at 13,000 *g* for 5 min at 4 °C. Protein concentration of the clarified cell lysates was quantified using the BCA assay (Thermo Fisher), and a small fraction of the clarified cell lysates was separated and normalized as input. The remaining cell lysates were subjected to immunoprecipitation using GFP-Trap Magnetic Agarose (Chromotek) with rotation at 4 °C overnight (12–16 h). The magnetic resin was then separated by a magnetic tube rack, washed three times with lysis buffer (at least 10 times the bead volume), denatured and analyzed by SDS-PAGE and Western blot with detection by chemiluminescence using Clarity Western ECL substrate (Bio-Rad).

APPENDIX REFERENCES

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