Supplementary Information

Title: Decoding Polo-like kinase 1 signaling along the kinetochore-centromere axis

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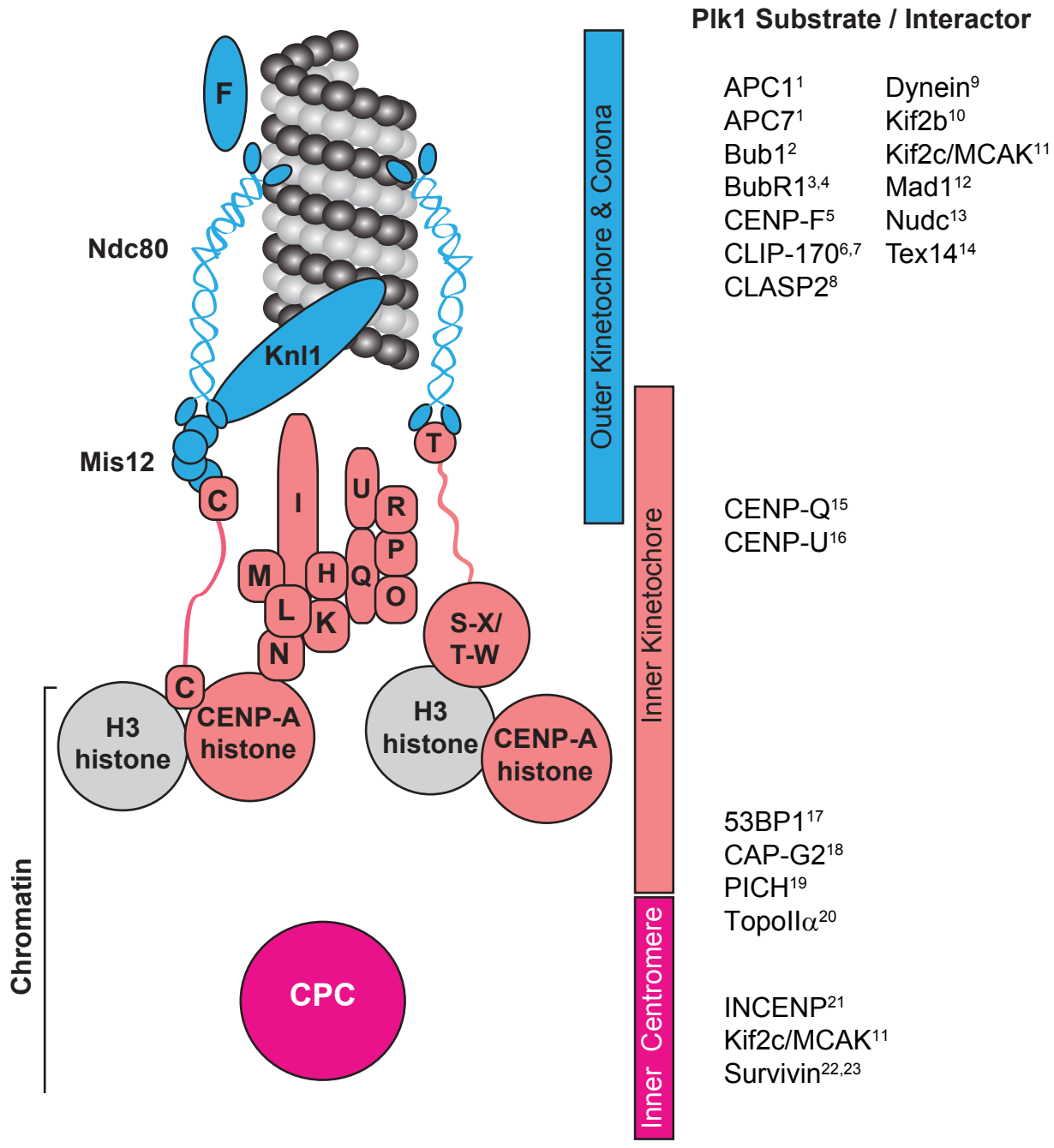
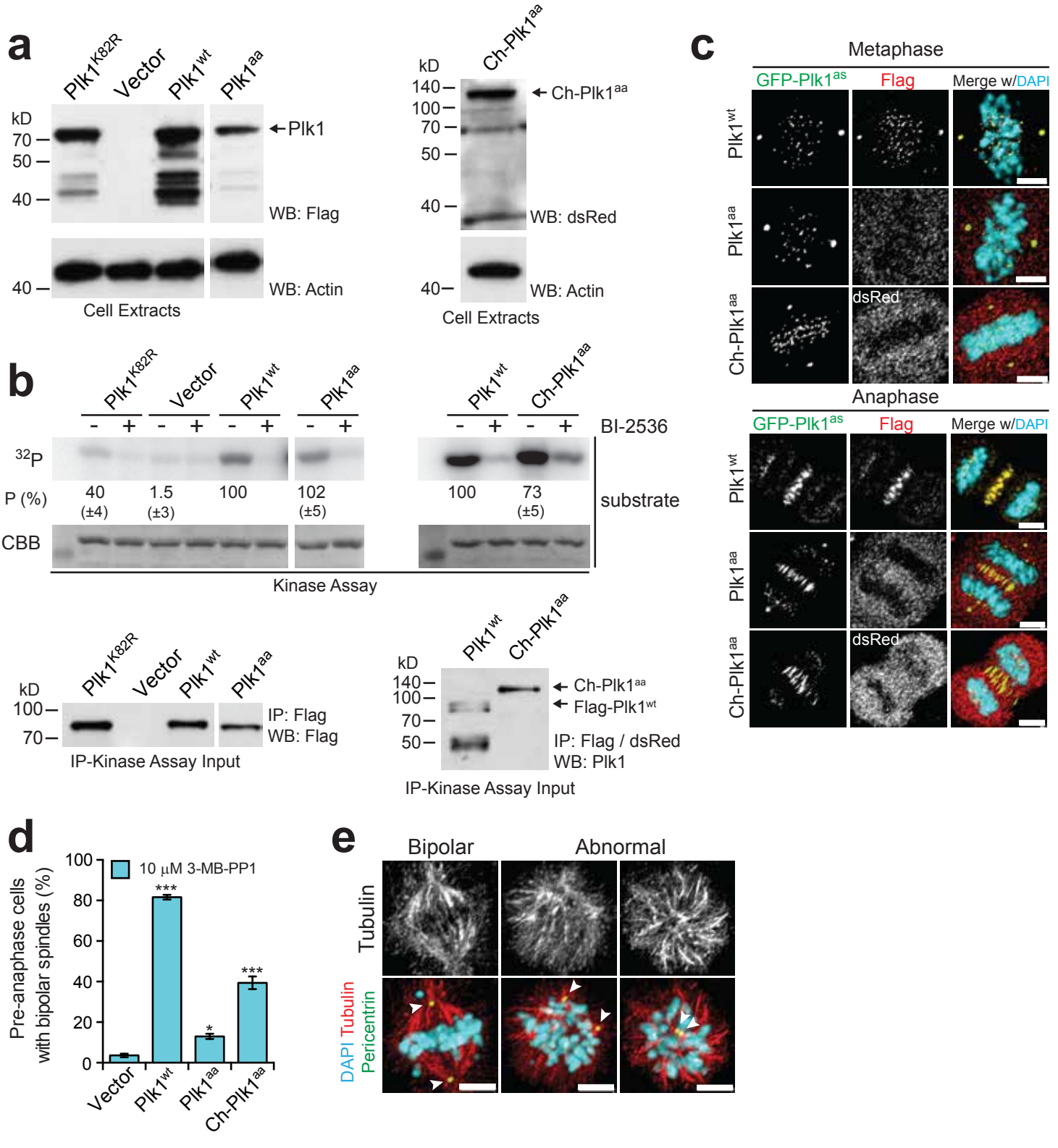
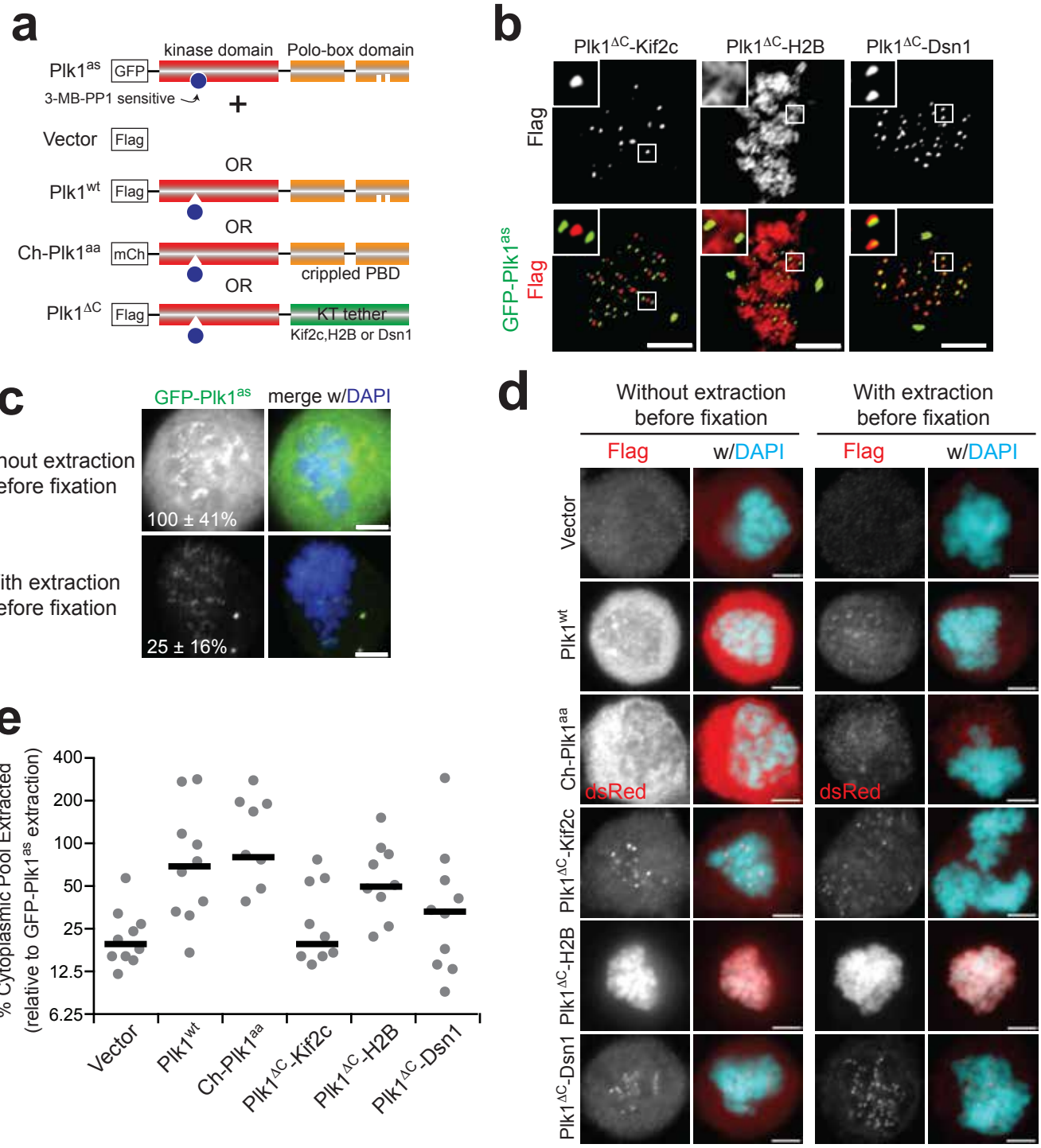
Supplementary Results



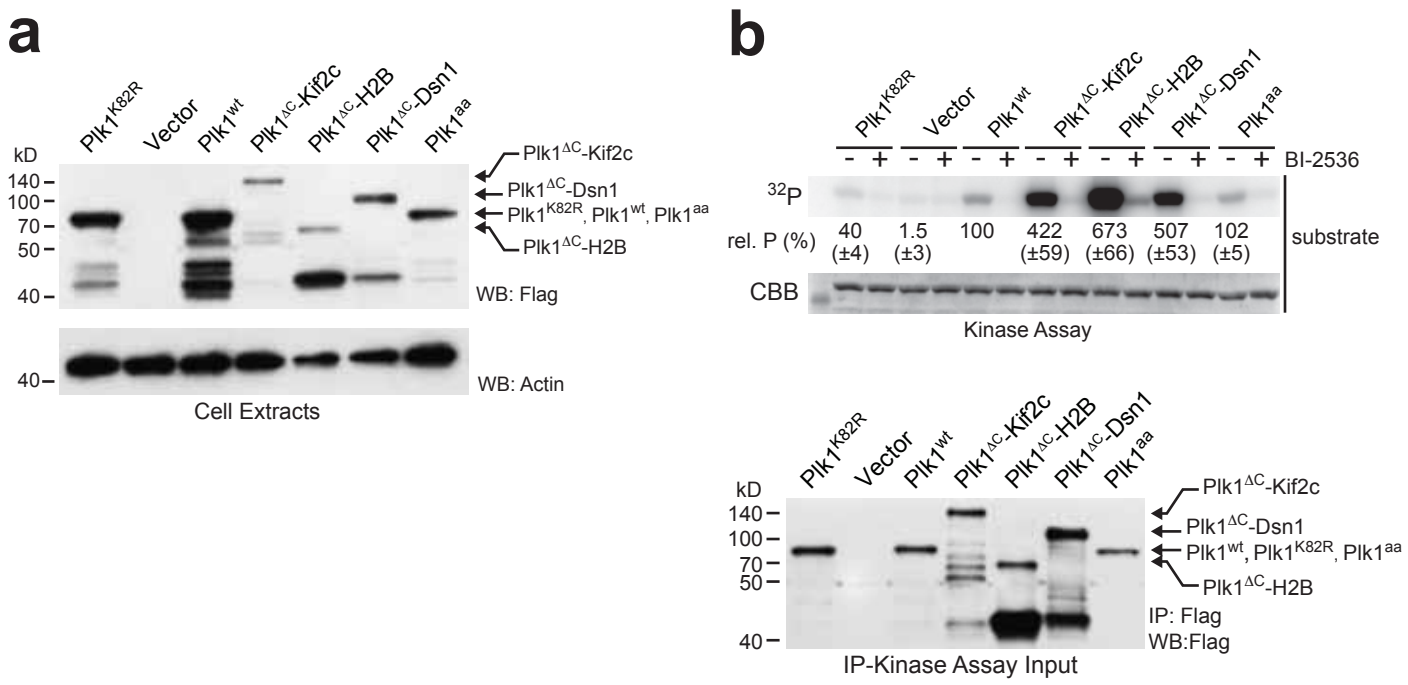
Supplementary Figure 1. Plk1 substrates and interactors are found along the entire kinetochore-centromere axis. *Left*, Schematic of the human kinetochore and centromere. Centromere proteins are indicated by their respective letters. CPC, Chromosomal Passenger Complex. *Right*, List of known mitotic Plk1 substrates and interactors based on reference articles. Locales were determined by referenced articles or inferred based on localization or known binding partners mapped in additional reports, but not referenced here.


a
Plk1K82R
Vector
Plk1wt
Plk1aa
kD
70
50
40
Plk1
WB: Flag
40
WB: Actin
Cell Extracts
Ch-Plk1aa
kD
140
100
70
50
40
Ch-Plk1aa
WB: dsRed
40
WB: Actin
Cell Extracts
b
Plk1K82R
Vector
Plk1wt
Plk1aa
- + - + - + - +
32P
rel. P (%)
40 (±4)
1.5 (±3)
100
102 (±5)
CBB
Plk1wt
Ch-Plk1aa
- + - +
BI-2536
100
73 (±5)
substrate
Kinase Assay
Plk1K82R
Vector
Plk1wt
Plk1aa
kD
100
70
IP: Flag
WB: Flag
IP-Kinase Assay Input
Plk1wt
Ch-Plk1aa
kD
140
100
70
50
Ch-Plk1aa
Flag-Plk1wt
IP: Flag / dsRed
WB: Plk1
IP-Kinase Assay Input
c
Metaphase
GFP-Plk1as
Flag
Merge w/DAPI
Plk1wt
Plk1aa
Ch-Plk1aa
dsRed
Anaphase
GFP-Plk1as
Flag
Merge w/DAPI
Plk1wt
Plk1aa
Ch-Plk1aa
dsRed
d
Pre-anaphase cells with bipolar spindles (%)
100
80
60
40
20
0
10 μM 3-MB-PP1
***
***
*
Vector
Plk1wt
Plk1aa
Ch-Plk1aa
e
Bipolar
Abnormal
Tubulin
DAPI Tubulin Pericentrin

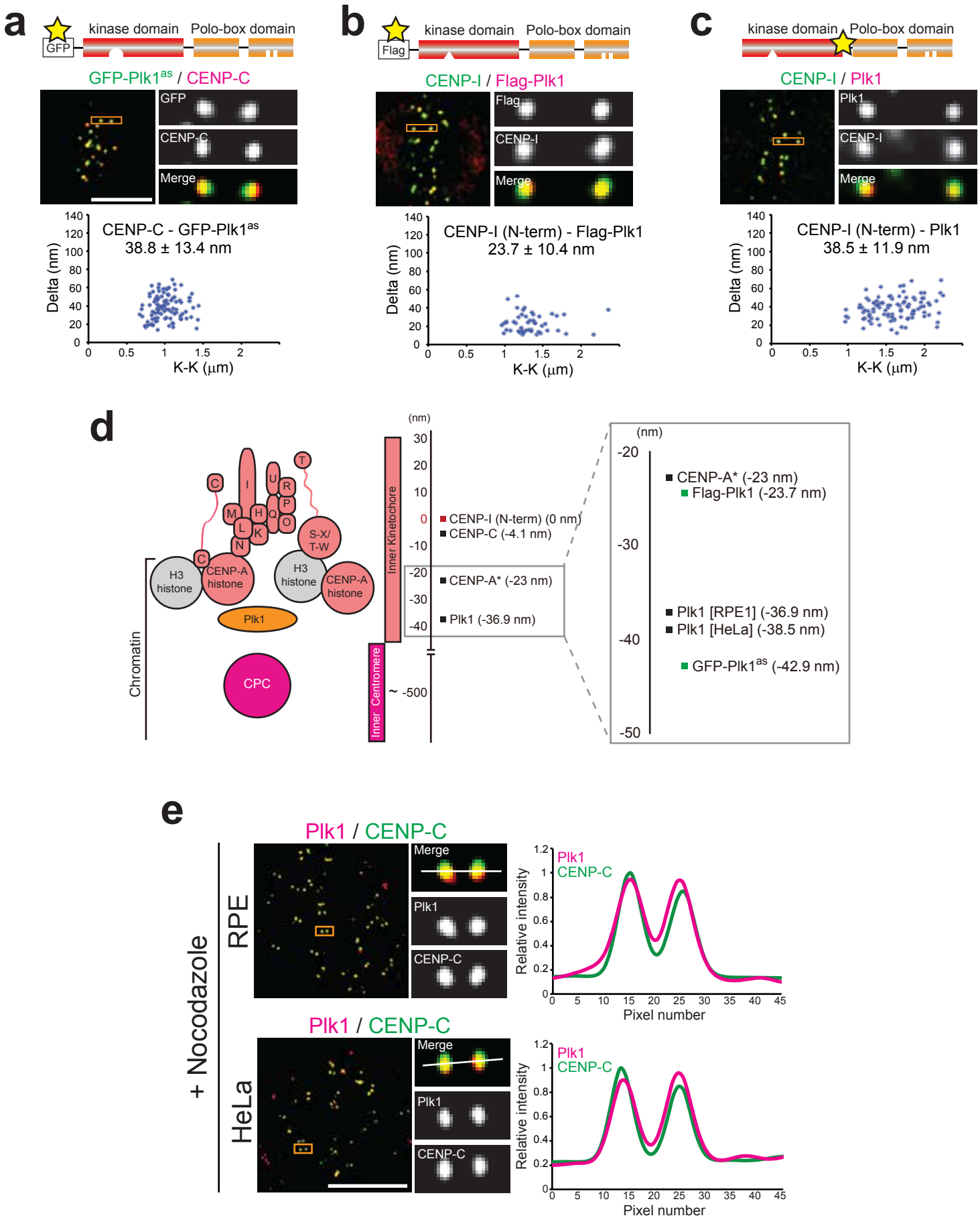
Supplementary Figure 2. Plk1 signaling at the kinetochore requires binding via its PBD (companion to Fig. 1) (a) Immunoblot of mitotic protein extracts from RPE1 cell lines co-expressing GFP-Plk1^{as} and the Flag tag (Vector), wildtype Plk1 (Plk1^{wt}), wildtype Plk1 with a mutated PBD (Plk1^{aa} and Ch-Plk1^{aa}) or catalytically inactive Plk1 (Plk1^{K82R}). Membranes probed for Flag-tagged (Flag blot) or mCherry-tagged (dsRed) Plk1 and Actin (loading control). **(b)** Plk1 construct activity determined by Flag- or dsRed-immunoprecipitation from mitotic protein lysates, followed by incubation with [γ-^{32}P]-ATP and Plk1-specific substrate. Where indicated, 200 nM BI-2536 added to reactions to inhibit Plk1. *Top*, autoradiograph (^{32}P) and Coomassie gel (CBB) of substrate from kinase reactions. Average substrate labeling (± SEM) reported relative to Plk1^{wt}. *Bottom*, immunoblot of kinase reaction input after immunoprecipitation (IP). Plk1 detected by Flag (left) or Plk1 (right) antibodies. **(c)** Representative maximal intensity projection micrographs demonstrating normal localization of Plk1^{wt}, but impaired localization of Plk1^{aa}/Ch-Plk1^{aa}, to centrosomes and kinetochores during metaphase and the central spindle during anaphase. **(d-e)** Delocalized Plk1 (Ch-Plk1^{aa} > Plk1^{aa}) partially restores mitotic bipolar spindles. **(d)** Graph shows average percentage (± SEM) of pre-anaphase cells with bipolar spindles for each cell line challenged with 10 μM 3-MB-PP1 (n=150 cells/experiment; 5 independent experiments). **(e)** Representative maximal intensity projection micrographs from (d). Arrowheads highlight pericentrin-decorated centrosomes. *P<.05, ***P<0.0001 by one-way ANOVA (panel d). Scale bar, 5 μm (panels c,e). Uncropped gels presented in Supplementary Figure 10.


a
kinase domain
Polo-box domain
Plk1as
GFP
3-MB-PP1 sensitive
+
Vector
Flag
OR
Plk1wt
Flag
OR
Ch-Plk1aa
mCh
crippled PBD
OR
Plk1ΔC
Flag
KT tether
Kif2c,H2B or Dsn1
b
Plk1ΔC-Kif2c
Plk1ΔC-H2B
Plk1ΔC-Dsn1
Flag
GFP-Plk1as
Flag
c
GFP-Plk1as
merge w/DAPI
Without extraction before fixation
100 ± 41%
With extraction before fixation
25 ± 16%
d
Without extraction before fixation
With extraction before fixation
Flag
w/DAPI
Flag
w/DAPI
Vector
Plk1wt
Ch-Plk1aa
dsRed
dsRed
Plk1ΔC-Kif2c
Plk1ΔC-H2B
Plk1ΔC-Dsn1
e
% Cytoplasmic Pool Extracted (relative to GFP-Plk1as extraction)
400
200
100
50
25
12.5
6.25
Vector
Plk1wt
Ch-Plk1aa
Plk1ΔC-Kif2c
Plk1ΔC-H2B
Plk1ΔC-Dsn1

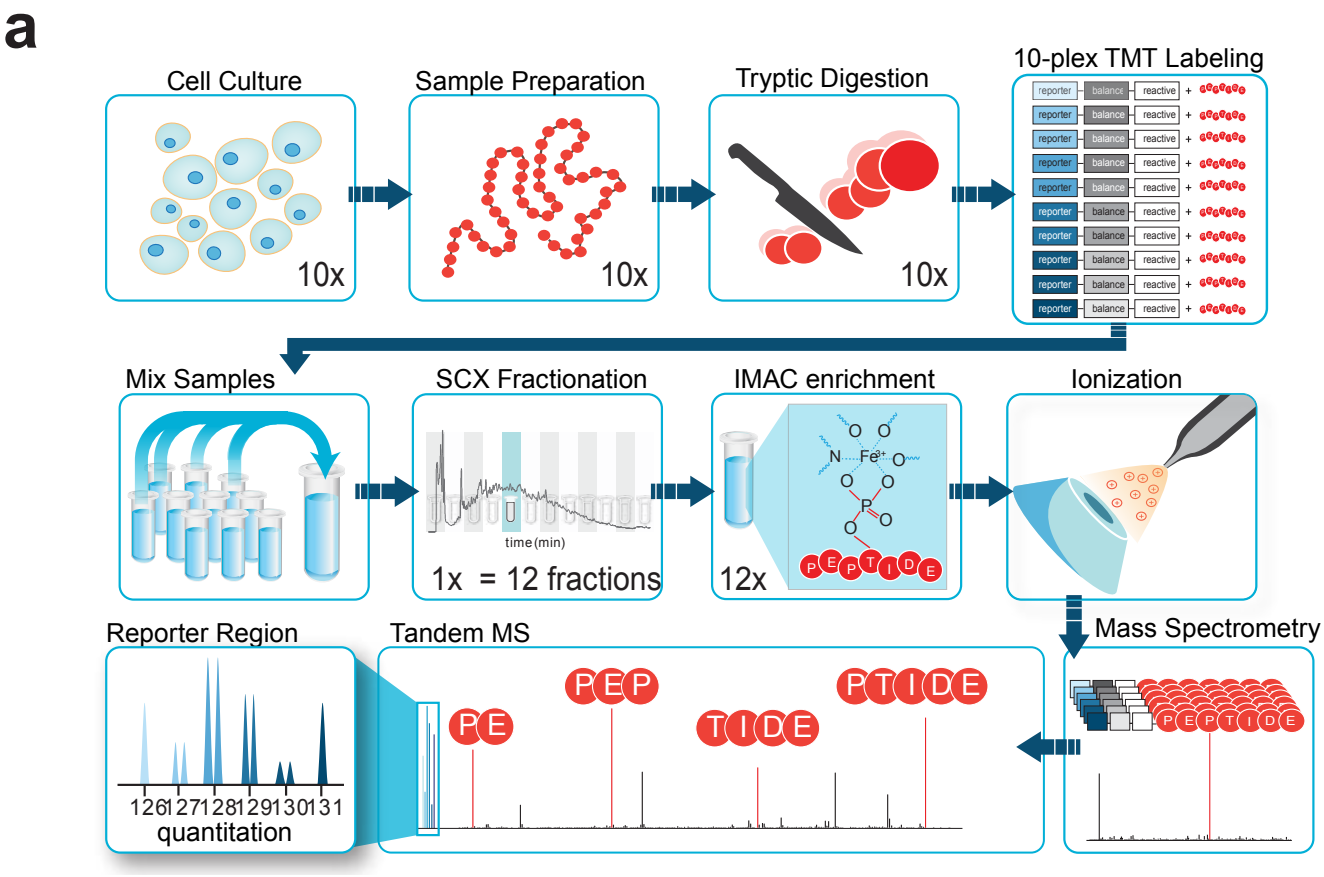
Supplementary Figure 3. Localization analysis of kinetochore-tethered Plk1 constructs (companion to Fig. 2) (a) Schematic of complementation strategy. As in Figure 1b, except additional Flag-tagged lines were generated to localize the wild-type Plk1 kinase domain (Plk1ΔC) to discrete locales at the kinetochore. Flag-Plk1ΔC was tethered to proteins localizing to the inner centromere (Kif2c), chromatin (H2B), or to the outer kinetochore (Dsn1). **(b)** Representative single plane micrographs depicting localization of Flag-tagged Plk1 localized to the inner centromere (Plk1ΔC-Kif2c), to chromatin (Plk1ΔC-H2B), or to the outer kinetochore (Plk1ΔC-Dsn1). **(c-e)** To quantitate the fraction of unbound cytoplasmic Plk1 and tethered Plk1, cells were processed for immunofluorescence with and without a 15 s extraction step (removes soluble protein) prior to fixation. **(c)** Representative single-plane, non-deconvolved micrographs of GFP-Plk1^{as} with and without 15 s extraction step prior to fixation. Numbers represent average fluorescent intensity (± SD) as a percent of the average intensity in non-extracted cells (n=60 cells/condition). **(d)** Representative single-plane, non-deconvolved micrographs of Flag-tagged or mCherry-tagged (dsRed) Plk1 constructs with and without 15 s extraction step prior to fixation. **(e)** Graph indicates percentage of soluble tethered construct extracted relative to amount of soluble GFP-Plk1^{as} extracted in cells. Dots represent single cells from one replicate and bars indicate median values. Log2 scale used for Y-axis. Scale bars, 5 μm (panels b,c,d).

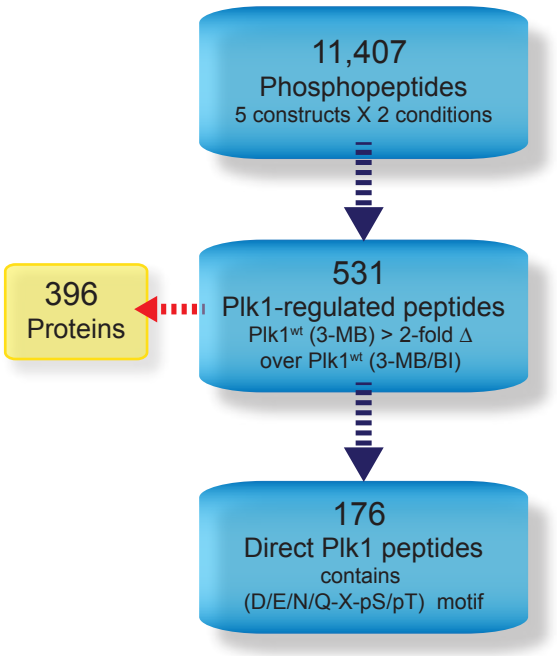


Supplementary Figure 4. Activity analysis of kinetochore-tethered Plk1 constructs (companion to Fig. 2). (a) Immunoblot of mitotic protein extracts from RPE1 cell lines co-expressing GFP-Plk1^{as} and the Flag tag (Vector), wildtype Plk1 (Plk1^{wt}), catalytically inactive Plk1 (Plk1^{K82R}), delocalized Plk1 (Plk1^{aa}) or the kinetochore-tethered Plk1 constructs. Membranes probed for Flag-tagged Plk1 (Flag blot) and Actin (loading control). **(b)** Plk1 construct activity determined by Flag-immunoprecipitation from mitotic protein lysates, followed by incubation with [γ-^{32}P]-ATP and Plk1-specific substrate. Where indicated, 200 nM BI-2536 added to reactions to inhibit Plk1. *Top*, autoradiograph (^{32}P) and Coomassie gel (CBB) of substrate from kinase reactions. Average substrate labeling (± SEM) reported relative to Plk1^{wt}. *Bottom*, immunoblot of kinase reaction input after immunoprecipitation (IP). Plk1 detected by Flag antibody. Uncropped gels presented in Supplementary Figure 11.


a
GFP
kinase domain
Polo-box domain
GFP-Plk1as / CENP-C
GFP
CENP-C
Merge
CENP-C - GFP-Plk1as
38.8 ± 13.4 nm
Delta (nm)
K-K (μm)
b
Flag
kinase domain
Polo-box domain
CENP-I / Flag-Plk1
Flag
CENP-I
Merge
CENP-I (N-term) - Flag-Plk1
23.7 ± 10.4 nm
Delta (nm)
K-K (μm)
c
kinase domain
Polo-box domain
CENP-I / Plk1
Plk1
CENP-I
Merge
CENP-I (N-term) - Plk1
38.5 ± 11.9 nm
Delta (nm)
K-K (μm)
d
Chromatin
H3 histone
CENP-A histone
Plk1
CPC
Inner Kinetochore
Inner Centromere
(nm)
CENP-I (N-term) (0 nm)
CENP-C (-4.1 nm)
CENP-A* (-23 nm)
Plk1 (-36.9 nm)
~ -500
CENP-A* (-23 nm)
Flag-Plk1 (-23.7 nm)
Plk1 [RPE1] (-36.9 nm)
Plk1 [HeLa] (-38.5 nm)
GFP-Plk1as (-42.9 nm)
e
+ Nocodazole
RPE
HeLa
Plk1 / CENP-C
Merge
Plk1
CENP-C
Relative intensity
Pixel number

Supplementary Figure 5. Validation of high resolution Plk1 localization in cells (companion to Fig. 2). (a-c) High resolution, single-plane micrographs and delta analysis of antibodies targeting GFP tag of the GFP-Plk1^{as} construct (panel a), Flag tag of the Flag-Plk1^{wt} construct (panel b) in RPE1 cells or endogenous Plk1 in HeLa cells (panel c). K-K indicates distance between the two centroids. Yellow stars indicate protein positions targeted by antibodies. **(d)** Map of kinetochore with distances plotted relative to the N-terminus of CENP-I. **(e)** Single-plane, high-resolution micrographs of RPE1 and HeLa cells challenged with 10 μM nocodazole for 2 h to destabilize microtubules. Linescans indicate relative signal intensities from Plk1 and CENP-C antibodies of kinetochore pair highlighted in inset. Scale bars, 5 μm.





c

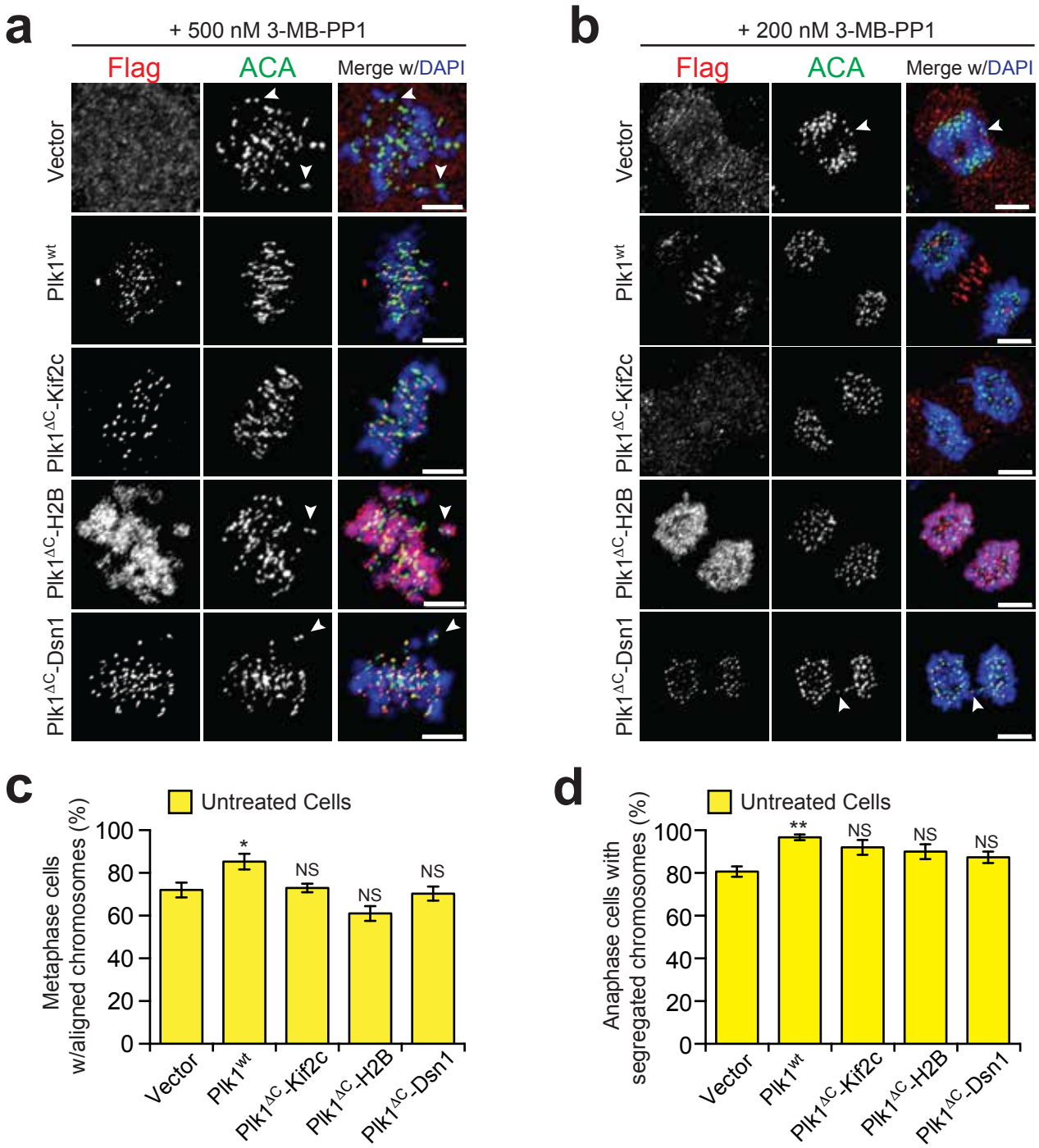
Direct Plk1 peptides at kinetochore

Gene ID (residue)	Fold Δ vs. Plk1wt (BI-2536 inhibited) WT	AA	Kif2c	H2B	Dsn1
outer kinetochore					
CENPE (T2601;S2605)	4.6	2.8			
CENPF (S1747**)	19.8	11.8	3.3	1.8	3.9
CENPF (S1770;T1781)	2.9	1.1	1.6	1.0	1.4
CDC27 (S435**)	6.4	1.8	9.2	0.8	1.2
CDC27 (S387**)	2.1	1.1		1.1	1.9
ANAPC1 (S372**;S377**)	7.1	0.5	1.0	0.4	1.0
CHAMP1 (S627)	2.3		1.8	0.9	0.3
TP53BP1 (S835;836**;839**)	10.3	1.1	2.8	1.4	1.9
TP53BP1 (S1004**;S1007**)	4.8	0.3	0.5	0.6	0.7
CHD8 (S2008)	2.0	0.9	1.1	0.3	0.9
USP16 (S415;S423)	5.7	1.4	2.0	9.5	1.9
KIF4A (S1054)	6.6	1.8	9.6	8.2	1.6
ERCC6L (S980)	2.3	0.8	1.6	0.9	1.1
ERCC6L (S1117;S1118)	2.3	0.6		0.7	1.2
INCENP (S218;T219)	3.2	2.2			2.9
inner centromere					

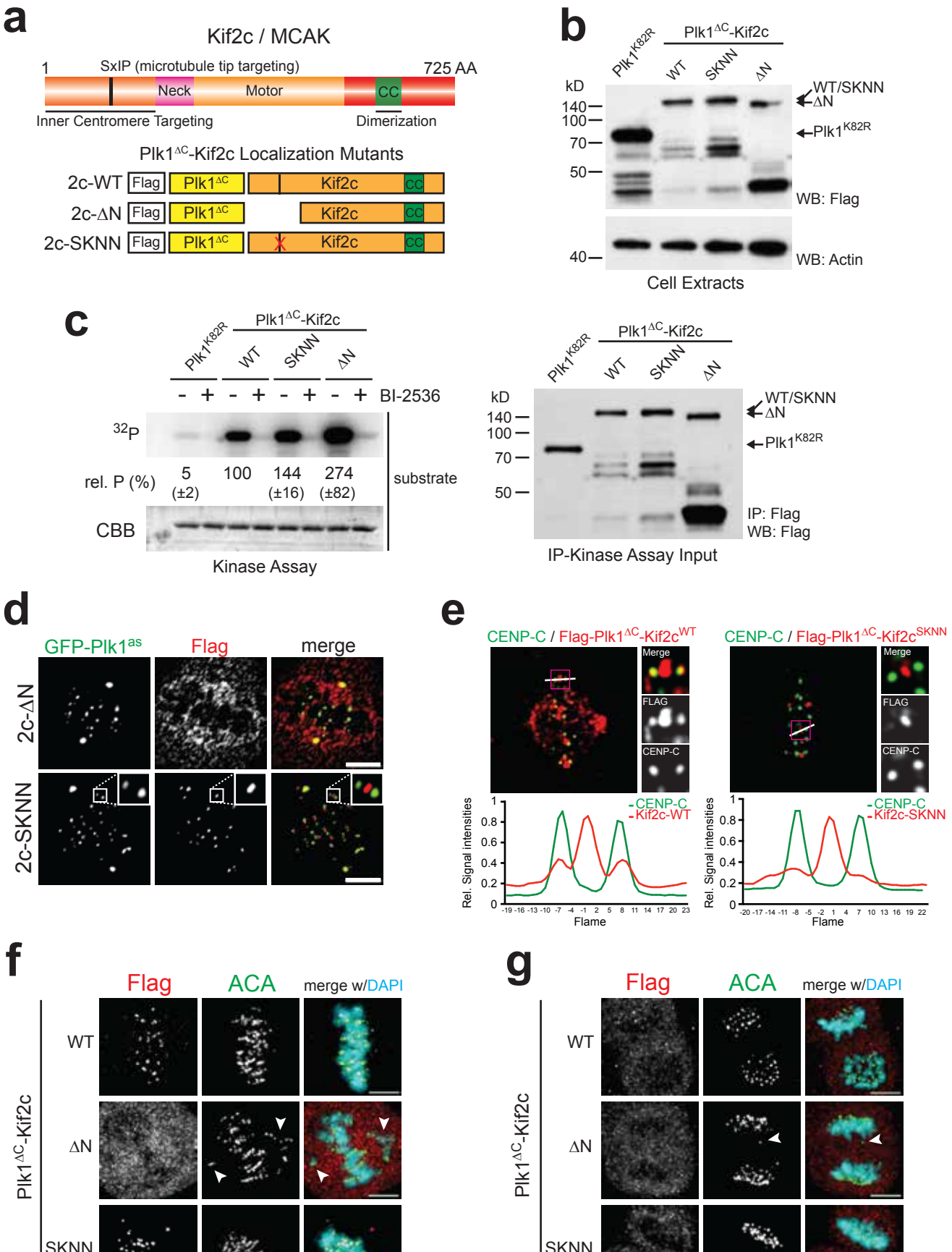
Reversibly Restored
- > 10 fold
- 5-10 fold
- 2-5 fold

Not Reversibly Restored
- 1-2 fold
- < 1 fold
- > 2 fold, but not inhibited by BI-2536

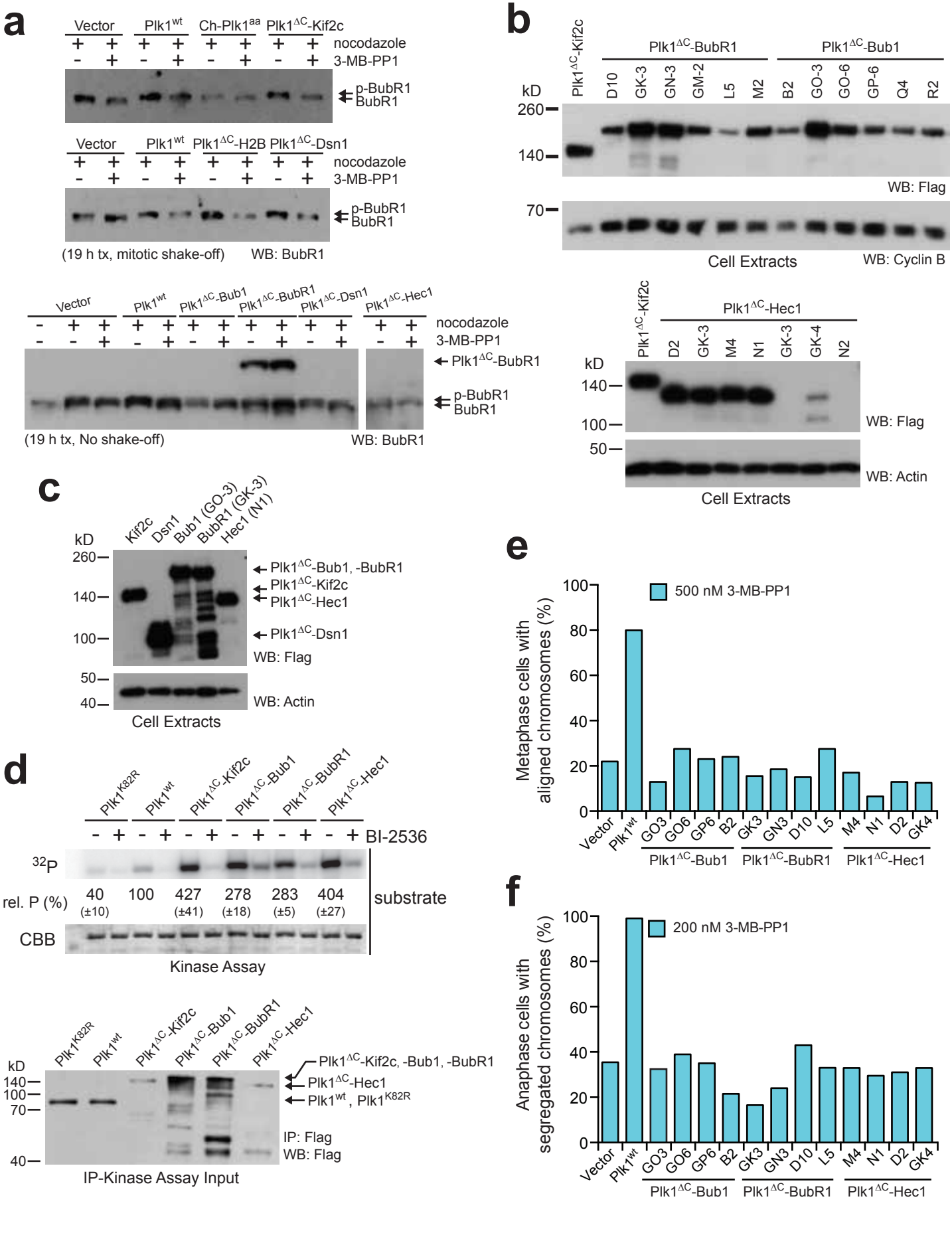
Supplementary Figure 6. Sample preparation and results for mass spectrometry (MS) of Plk1 cell lines (companion to Figs. 3-4). RPE1 cells co-expressing GFP-Plk1^{as} and Flag-Plk1^{wt}, Ch-Plk1^{aa}, Flag-Plk1ΔC-Kif2c, Flag-Plk1ΔC-H2B, or Flag-Plk1ΔC-Dsn1 were challenged with 10 μM 3-MB-PP1 (inhibits Plk1^{as}) +/- 200 nM BI-2536 (inhibits complementing wildtype Plk1 allele) for a total of 10 samples. Mitotic cells were collected by shake-off. **(a)** Pellets were lysed, and their resultant proteins were denatured, reduced, alkylated, and tryptically digested. The 10 samples were each chemically labeled with isobaric 10-plex TMT tags and mixed in equal ratios. The single pooled sample was fractionated by Strong Cation Exchange (SCX) chromatography, and the 12 resultant SCX fractions were enriched for phosphopeptides by immobilized metal affinity chromatography (IMAC). Phosphopeptides from each fraction were chromatographically separated and ionized for MS analysis. Isobaric, chemically labeled peptide species were isolated and fragmented to produce sequencing ions. MS fragmentation also releases non-isobaric reporter ions from the TMT tags, which reveal the relative abundance of each peptide across the 10 samples (inset). **(b)** A total of 11,407 phosphopeptides were encountered, of which 531 (translating to 396 proteins) were regulated by Plk1 (Flag-Plk1^{wt} cell line exhibited > 2-fold change when comparing 3-MB-PP1 challenged cells with those challenged both with 3-MB-PP1 and BI-2536). 176/531 peptides also contained the D/E/N/Q-X-pS/pT motif, indicating that these were likely direct Plk1 targets. **(c)** Quantitative analysis of mitotic kinetochore phosphopeptides directly targeted by wild-type Plk1 (WT) and relative targeting abilities of the PBD mutant (AA) or kinetochore-tethered Plk1 (Kif2c, H2B, or Dsn1). Values indicate fold-change (> 2-fold considered significant) in phosphopeptide abundance of the uninhibited Plk1 constructs compared to inhibition of WT. Phosphopeptides arranged from outer kinetochore to inner centromere.



Supplementary Figure 7. Plk1 activity tethered to chromatin or the inner centromere promotes chromosome alignment and segregation (companion to Fig. 4) (a) Representative maximal intensity projection micrographs of pre-anaphase mitotic cells challenged with 500 nM 3-MB-PP1. Arrowheads indicate misaligned chromosomes. **(b)** Representative maximal intensity projection micrographs of anaphase cells challenged with 200 nM 3-MB-PP1. Arrowheads indicate lagging chromosomes. ACA, Anti-Centromere Antibody. Scale bars, 5 μm. (c-d) Plk1 tethered to kinetochore proteins Kif2c, H2B, or Hec1 do not produce mitotic errors in untreated cells. **(c)** Graph shows average percentage (±SEM) of pre-anaphase mitotic cells at metaphase with fully aligned chromosomes for each cell line (n=150 cells/experiment; 3 independent experiments). **(d)** Graph shows average percentage (±SEM) of anaphase cells with fully segregated chromsomes for each cell line (n≥50 cells/experiment; 3 independent experiments). *P<.05, **P<0.005 by one-way ANOVA; NS, not significant.

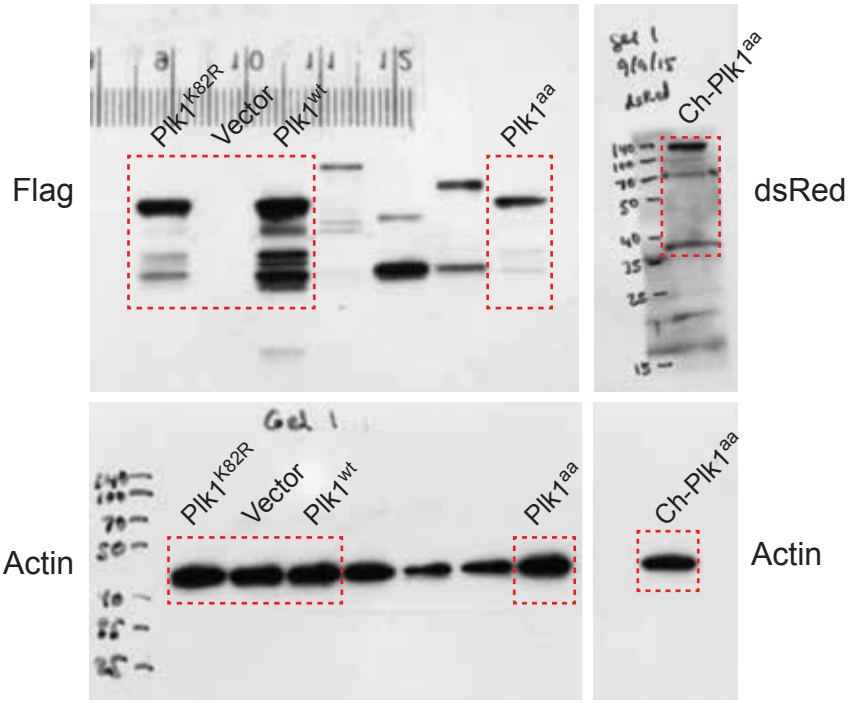

a
Kif2c / MCAK
1
SxIP (microtubule tip targeting)
725 AA
Neck
Motor
CC
Inner Centromere Targeting
Dimerization
Plk1ΔC-Kif2c Localization Mutants
2c-WT
2c-ΔN
2c-SKNN
Flag
Plk1ΔC
Kif2c
CC
b
Plk1K82R
Plk1ΔC-Kif2c
WT
SKNN
ΔN
kD
140
100
70
50
40
WT/SKNN
ΔN
Plk1K82R
WB: Flag
WB: Actin
Cell Extracts
c
Plk1K82R
Plk1ΔC-Kif2c
WT
SKNN
ΔN
- + - + - + - + BI-2536
32P
rel. P (%)
5 (±2)
100
144 (±16)
274 (±82)
substrate
CBB
Kinase Assay
kD
140
100
70
50
WT/SKNN
ΔN
Plk1K82R
IP: Flag
WB: Flag
IP-Kinase Assay Input
d
GFP-Plk1as
Flag
merge
2c-ΔN
2c-SKNN
e
CENP-C / Flag-Plk1ΔC-Kif2cWT
CENP-C / Flag-Plk1ΔC-Kif2cSKNN
Merge
FLAG
CENP-C
Rel. Signal intensities
-CENP-C
-Kif2c-WT
-Kif2c-SKNN
Flame
f
Flag
ACA
merge w/DAPI
Plk1ΔC-Kif2c
WT
ΔN
SKNN
g
Flag
ACA
merge w/DAPI
Plk1ΔC-Kif2c
WT
ΔN
SKNN

Supplementary Figure 8. Plk1 activity tethered to the inner centromere pool of Kif2c rescues chromosome alignment and segregation (companion to Fig. 5). (a) *Top*, Schematic of Kif2c (also known as mitotic centromere-associated kinesin, MCAK) highlighting important domains. CC, coiled coil. *Bottom*, Schematic of Plk1-tethered to Kif2c localization mutants: ΔN disrupts targeting to both the inner centromere and microtubule tips, whereas SKNN disrupts targeting to the tips only. **(b)** Immunoblot of mitotic protein extracts from RPE1 cell lines co-expressing GFP-Plk1^{as} and catalytically inactive Plk1 (Plk1^{K82R}), or wildtype Plk1 tethered to wildtype Kif2c (WT), Kif2c with an N-terminal deletion that prevents inner centromere and (+)tip targeting (ΔN), or Kif2c with a mutated SxIP domain that prevents (+)tip targeting (SKNN). Membranes probed for Flag-tagged Plk1 (Flag blot) and Actin (loading control). **(c)** Plk1 construct activity determined by Flag-immunoprecipitation from mitotic protein lysates, followed by incubation with [γ-^{32}P]-ATP and Plk1-specific substrate. Where indicated, 200 nM BI-2536 added to reactions to inhibit Plk1. Left, autoradiograph (^{32}P) and Coomassie gel (CBB) of substrate from kinase reactions. Average substrate labeling (± SEM) reported relative to WT. Right, immunoblot of kinase reaction input after immunoprecipitation (IP). Plk1 detected by Flag antibody. **(d)** Representative single-plane micrographs highlighting diffuse spindle localization of the ΔN mutant and discrete targeting of the SKNN mutant to the inner centromere (insets). **(e)** Single-plane, high-resolution micrographs of Kif2c-WT and SKNN mutant showing lack of outer kinetochore Kif2c signal. Linescans indicate relative signal intensities from the CENP-C and Flag antibodies of kinetochore pair highlighted in inset. **(f)** Representative maximal intensity projection micrographs of pre-anaphase mitotic cells challenged with 500 nM 3-MB-PP1. Arrowheads indicate misaligned chromosomes. **(g)** Representative maximal intensity projection micrographs of anaphase cells challenged with 200 nM 3-MB-PP1. Arrowheads indicate lagging chromosomes. ACA, Anti-Centromere Antibody. Scale bars, 5 μm (panels d, f, g). Uncropped gels presented in Supplementary Figure 11.

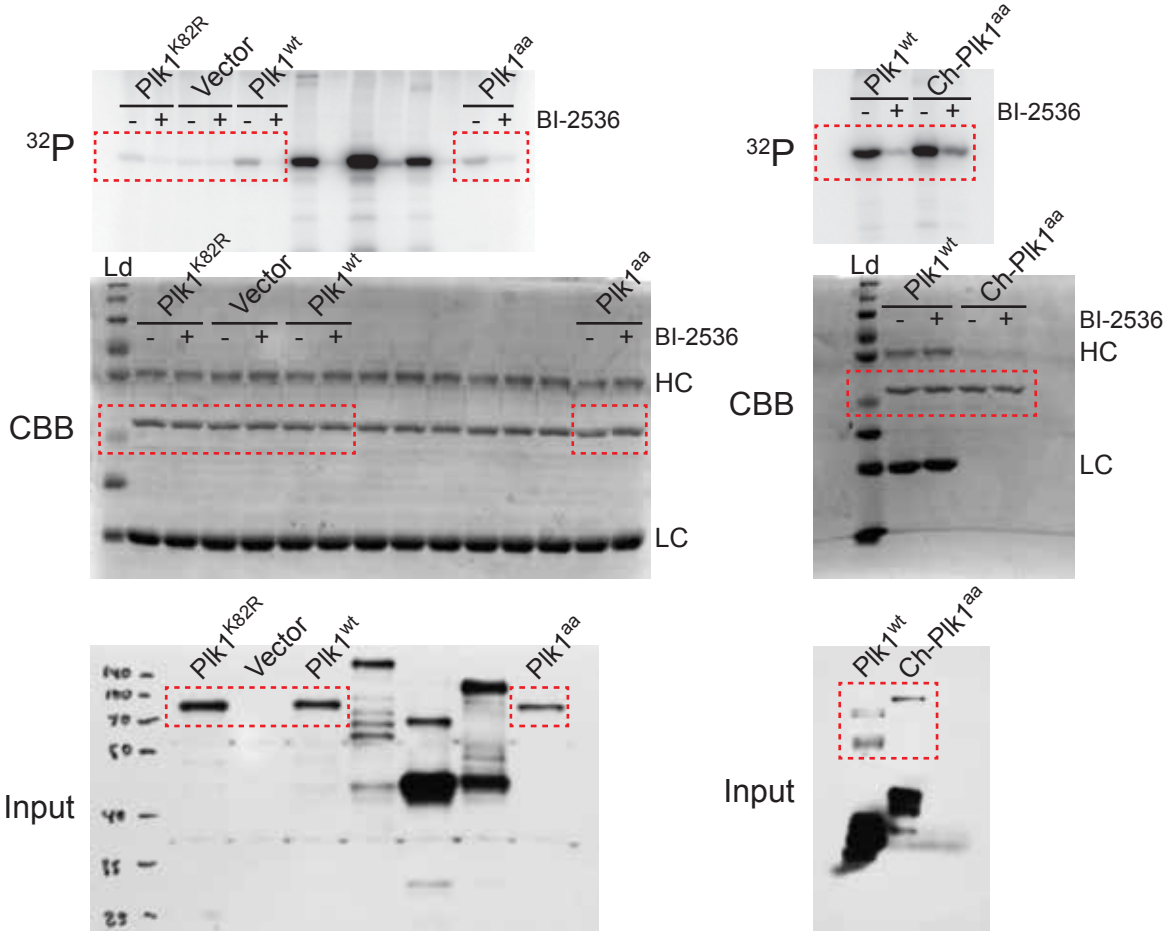

a
Vector
Plk1wt
Ch-Plk1aa
Plk1ΔC-Kif2c
nocodazole
3-MB-PP1
p-BubR1
BubR1
Plk1ΔC-H2B
Plk1ΔC-Dsn1
(19 h tx, mitotic shake-off)
WB: BubR1
Plk1ΔC-Bub1
Plk1ΔC-BubR1
Plk1ΔC-Dsn1
Plk1ΔC-Hec1
(19 h tx, No shake-off)
b
Plk1ΔC-Kif2c
Plk1ΔC-BubR1
Plk1ΔC-Bub1
D10
GK-3
GN-3
GM-2
L5
M2
B2
GO-3
GO-6
GP-6
Q4
R2
kD
260
140
70
WB: Flag
WB: Cyclin B
Cell Extracts
D2
M4
N1
GK-4
N2
100
50
WB: Actin
c
Kif2c
Dsn1
Bub1 (GO-3)
BubR1 (GK-3)
Hec1 (N1)
Plk1ΔC-Bub1, -BubR1
Plk1ΔC-Kif2c
Plk1ΔC-Hec1
Plk1ΔC-Dsn1
40
d
Plk1K82R
Plk1wt
BI-2536
32P
rel. P (%)
40 (±10)
100
427 (±41)
278 (±18)
283 (±5)
404 (±27)
substrate
CBB
Kinase Assay
Plk1ΔC-Kif2c, -Bub1, -BubR1
Plk1ΔC-Hec1
Plk1wt, Plk1K82R
IP: Flag
WB: Flag
IP-Kinase Assay Input
e
500 nM 3-MB-PP1
Metaphase cells with aligned chromosomes (%)
Vector
Plk1wt
GO3
GO6
GP6
B2
GK3
GN3
D10
L5
M4
N1
D2
GK4
f
200 nM 3-MB-PP1
Anaphase cells with segregated chromosomes (%)

Supplementary Figure 9. Analysis of outer kinetochore-tethered Plk1 constructs (companion to Fig. 5). (a) Immunoblot for BubR1 in mitotic extracts from cell lines co-expressing GFP-Plk1^{as} and the Flag tag (Vector), wildtype Plk1 (Plk1^{wt}),delocalized (Ch-Plk1^{aa}) or kinetochore-tethered Plk1 constructs (*Top:* Kif2c; *Middle*: H2B, Dsn1; *Bottom:* Bub1, BubR1, Hec1) challenged with nocodazole ± 10 μM 3-MB-PP1. In the bottom blot, the Hec1 construct is from the same gel and membrane, but exposed for a longer duration **(b)** Immunoblot of mitotic protein extracts from RPE1 cell lines co-expressing GFP-Plk1^{as} and Plk1 tethered to Kif2c, Bub1, BubR1 or Hec1. Each lane represents a clonal cell line. Membranes probed for Flag-tagged Plk1 (Flag blot) and Cyclin B (mitotic fraction, *Top*) or actin (loading control, *Bottom*). **(c-d)** Highest expressing cell lines, Bub1 (GO-3), BubR1 (GK-3), and Hec1 (N1) were selected for further characterization. **(c)** Immunoblot of mitotic protein extracts comparing expression of kinetochore-tethered Plk1 constructs. **(d)** Plk1-tethered construct activity determined by Flag-immunoprecipitation from mitotic protein lysates, followed by incubation with [γ-^{32}P]-ATP and Plk1-specific substrate. Where indicated, 200 nM BI-2536 added to reactions to inhibit Plk1. *Top*, autoradiograph (^{32}P) and Coomassie gel (CBB) of substrate from kinase reactions. Average substrate labeling (± SEM) reported relative to Plk1^{wt}. *Bottom,* immunoblot of kinase reaction input after immunoprecipitation (IP). Plk1 detected by Flag antibody. **(e-f)** Moderate to low expressing outer kinetochore tethers also fail to restore metaphase chromosome alignment or anaphase chromosome segregation when GFP-Plk1^{as} is inhibited with 3-MB-PP1. **(e)** Graph shows average percentage (± SEM) of pre-anaphase cells with bipolar spindles for each cell line challenged with 10 mM 3-MB-PP1 (n=100 cells/experiment; 2 independent experiments). **(f)** Graph shows average percentage (± SEM) of anaphase cells with fully segregated chromosomes for each cell line (n≥30 cells/experiment; 2 independent experiments). Uncropped gels presented in Supplementary Figure 12.

Supplementary Fig. 2a blots

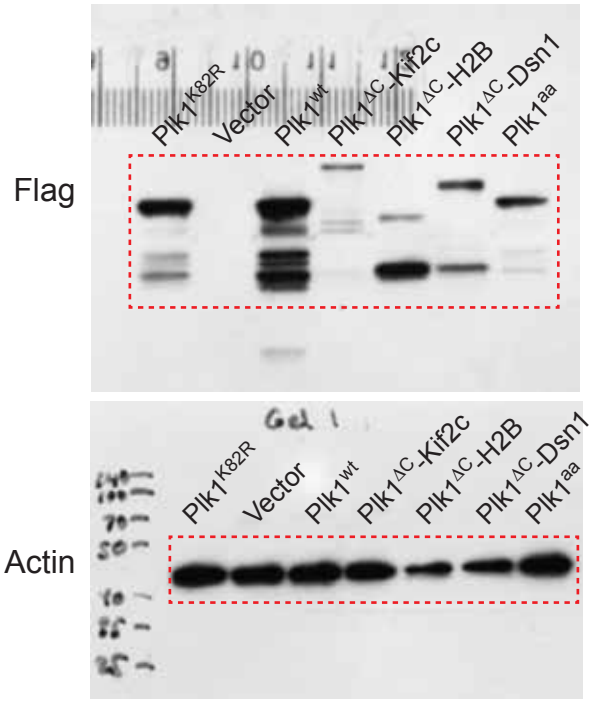


Supplementary Fig. 2b blots

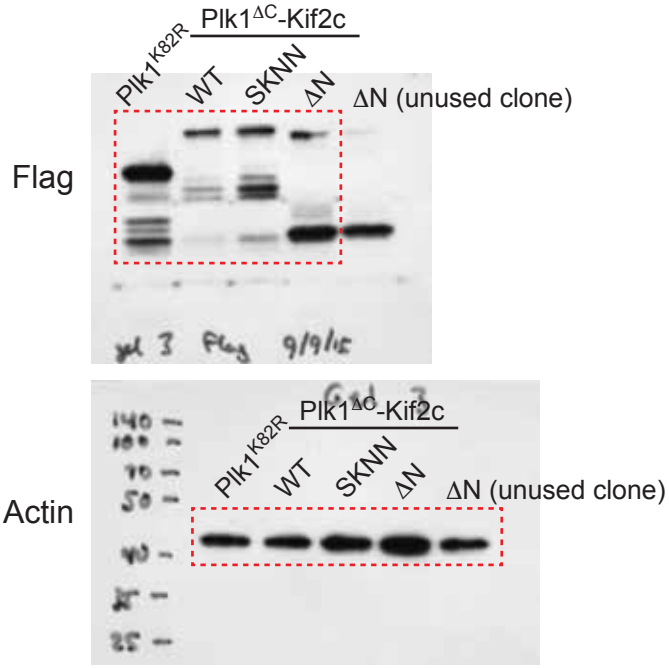


Supplementary Figure 10. Full scans of electrophoretic data from Supplementary Figure 2. Red dashed lines indicate cropped areas. Ld, ladder. HC, heavy chain. LC, light chain.

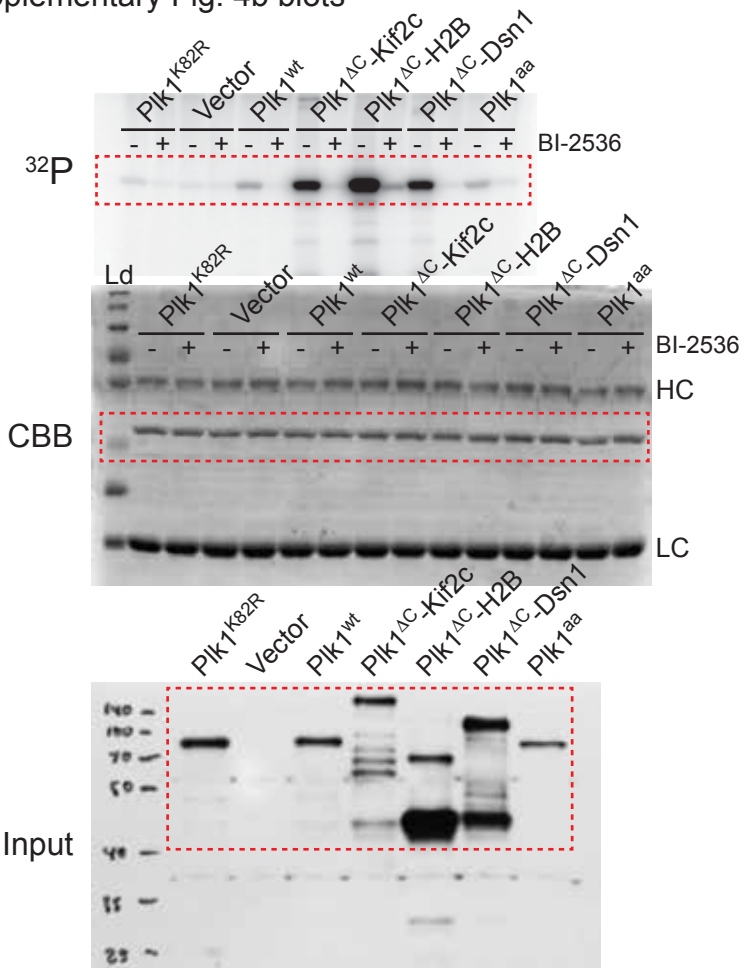
Supplementary Fig. 4a blots



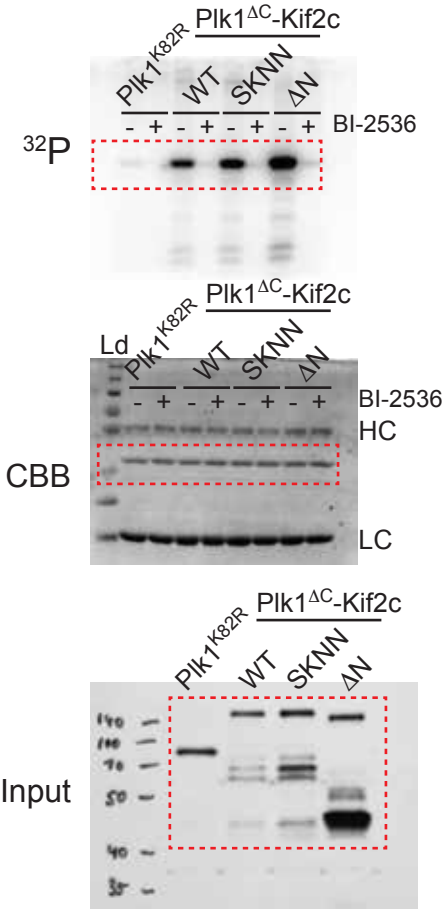
Supplementary Fig. 8b blots



Supplementary Fig. 4b blots



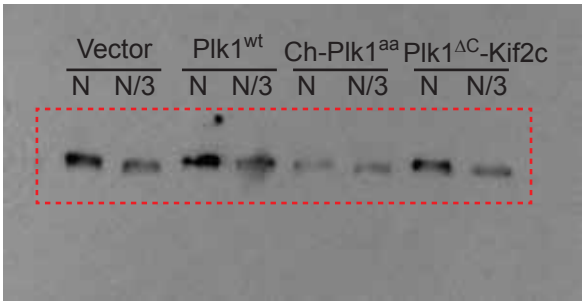
Supplementary Fig. 8c blots

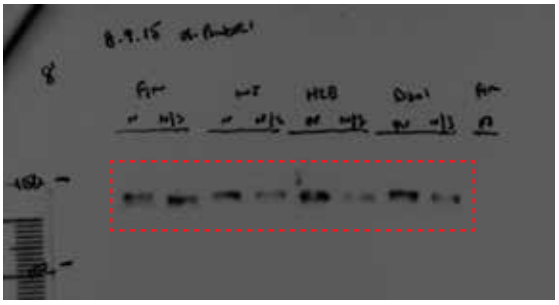


Supplementary Figure 11. Full scans of electrophoretic data from Supplementary Figures 4 & 8. Red dashed lines indicate cropped areas. Ld, ladder. HC, heavy chain. LC, light chain.

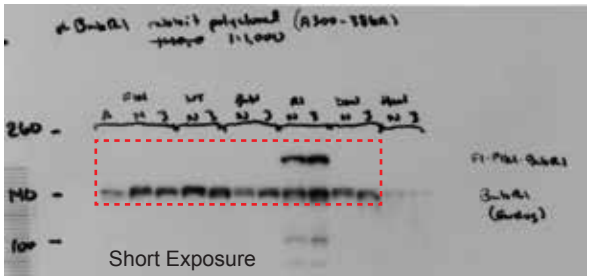
Supplementary Fig. 9a blots

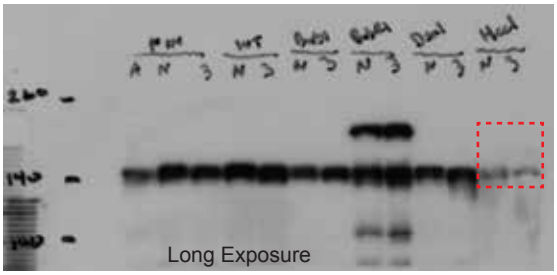
BubR1 (Taylor Ab)



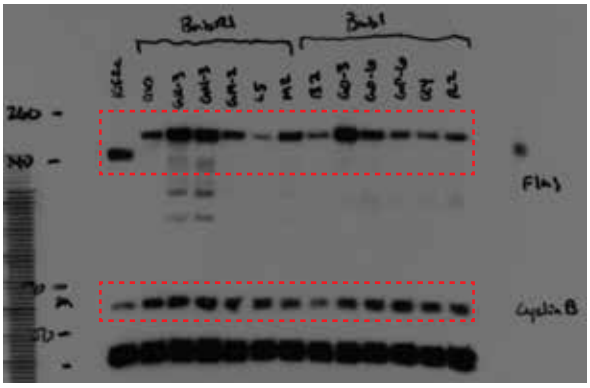


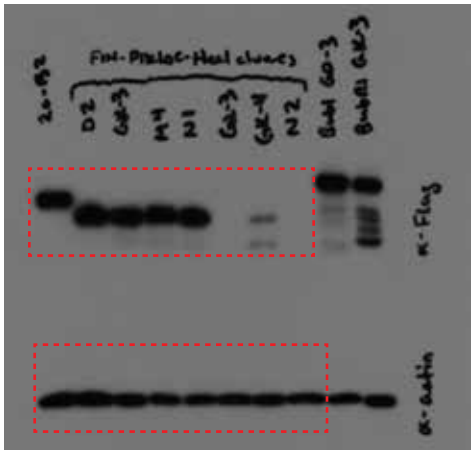
BubR1 (Bethyl Ab)



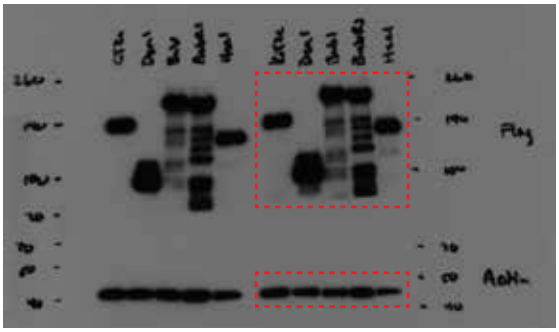


Supplementary Fig. 9b blots

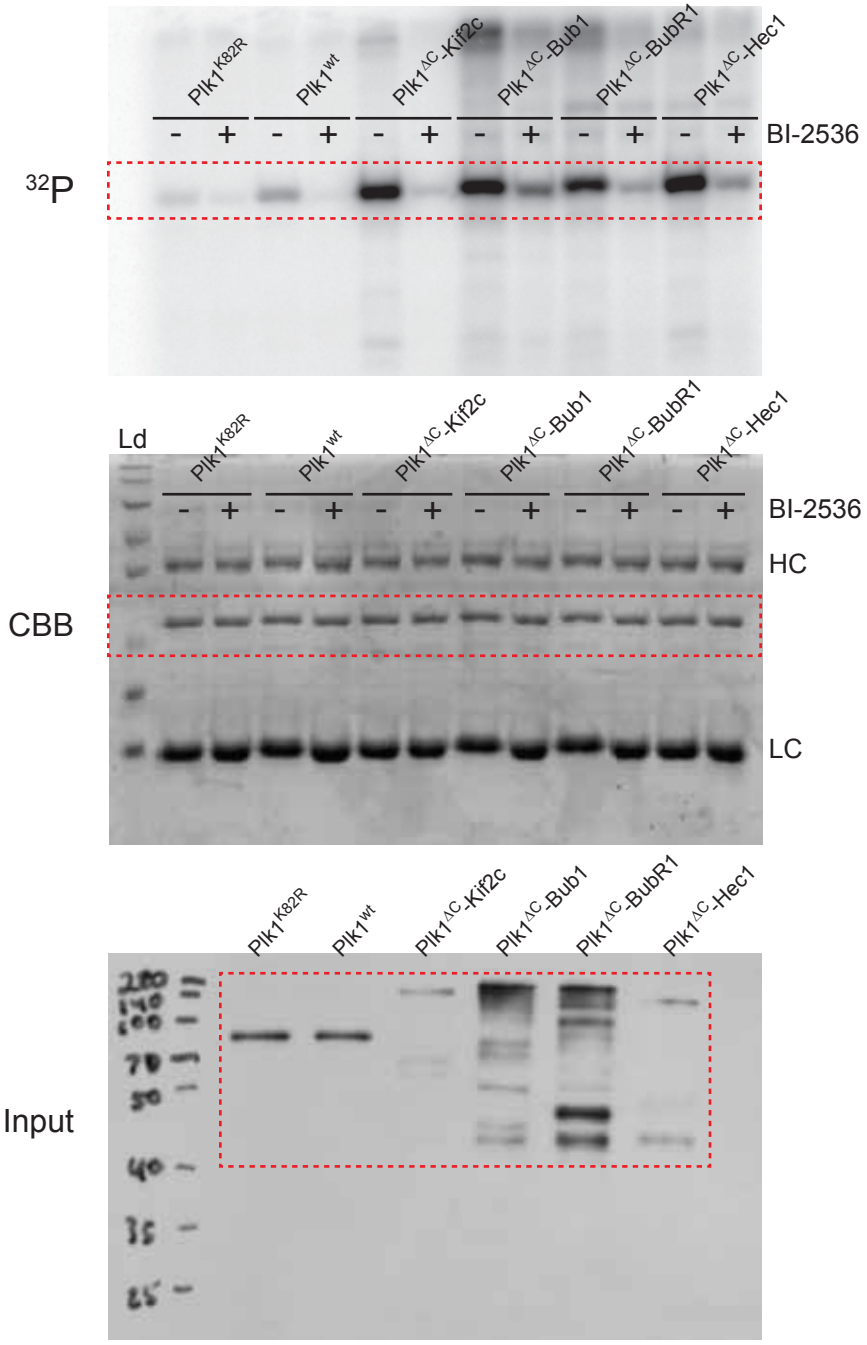




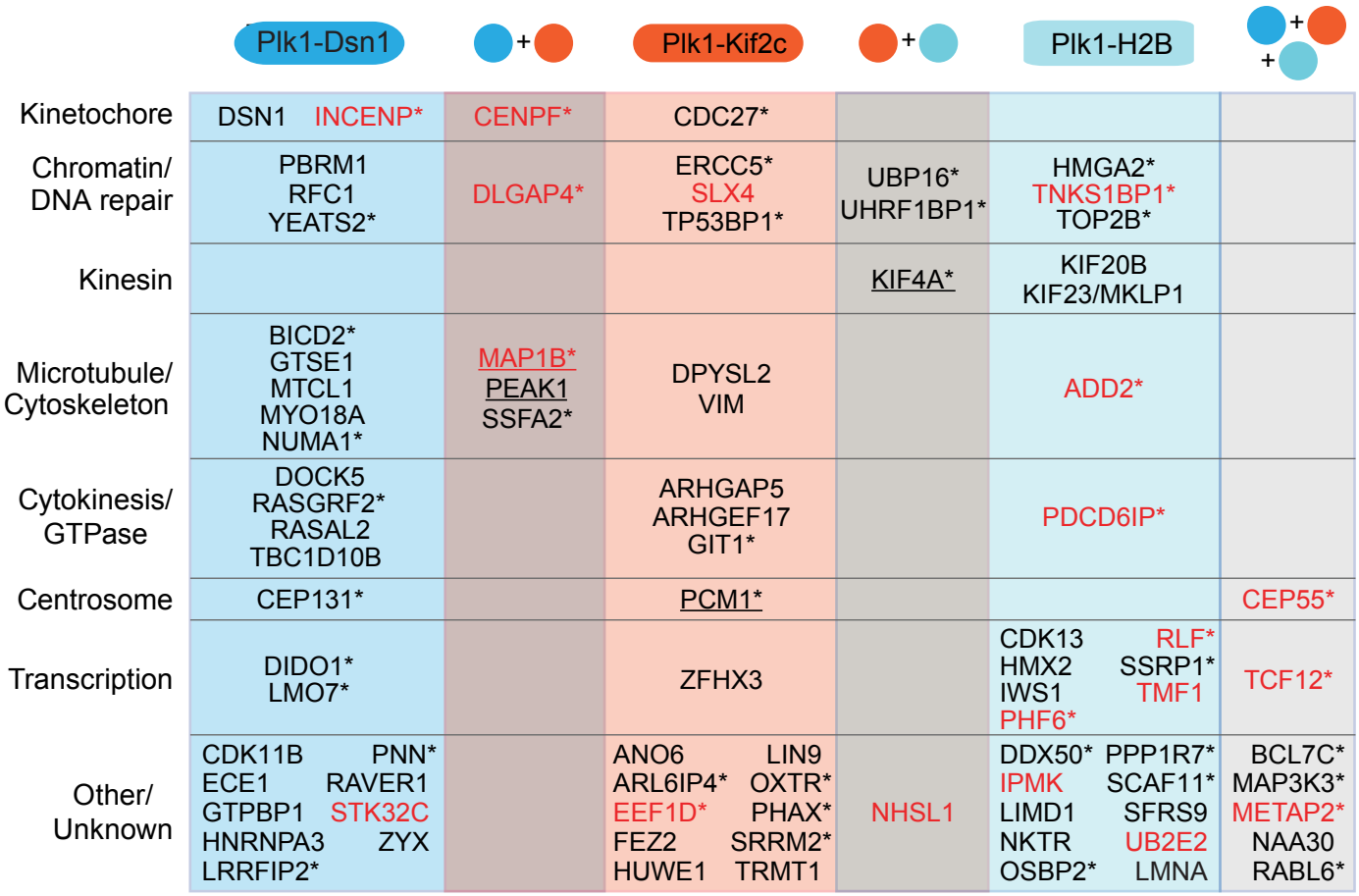
Supplementary Fig. 9c blots



Supplementary Fig. 9d blots



Supplementary Figure 12. Full scans of electrophoretic data from Supplementary Figure 9. Red dashed lines indicate cropped areas. Ld, ladder. HC, heavy chain. LC, light chain.



Supplementary Table 1. Table of Gene IDs from phosphopeptides regulated by kinetochore-tethered Plk1 constructs. Rows indicate peptide locale or cellular function. Columns indicate peptides with individual or overlapping construct regulation. Peptides also regulated by delocalized Plk1 (Ch-Plk1^{aa}) are listed in red. Asterisk(*) denotes peptides that contain the minimal Plk1 consensus (D/E/N/Q-X-pS/pT) motif. Underline denotes multiple regulated peptides encountered.

NAME	SEQUENCE
USER INSERT TOP	5'-GAATTGCTGAGGAGACATCTAGAGAATTCTCTAGACTTTCCCTCAGCAATTCCGC-3'
USER INSERT BOTTOM	5'-GCGGAATTGCTGAGGGAAAGTCTAGAGAATTCTCTAGATGTCTCCTCAGCAATTC-3'
Bub1 FWD	5'-GGAGACAUATGGACACCCCGGAAAATG-3'
Bub1 REV	5'-GGGAAAGUTTATTTTCGTGAACGCTTACATTCT-3'
BubR1 FWD	5'-GGAGACAUATGGCGGCGGTGAAGAAGG-3'
BubR1 REV	5'-GGGAAAGUTCACTGAAAGAGCAAAGCCCC-3'
Hec1 FWD	5'-GGAGACAUATGAAGCGCAGTTCAGTTTCCAG-3'
Hec1 REV	5'-GGGAAAGUTCATTCTTCAGAAGACTTAATTAGAGTAGC-3'
Kif2c FWD	5'-GGAGACAUATGGCCATGGACTCGTCGC-3'
Kif2c REV	5'-GGGAAAGUTCACTGGGGCCGTTTCTTG-3'
H2B FWD	5'-GGAGACAUATGCCAGAGCCAGCGAAGTC-3'
H2B REV	5'-GGGAAAGUTAACTTAGCGCTGGTGTACTTGGTG-3'
Dsn1 FWD	5'-GGAGACAUATGACTTCAGTGACTAGATCAGAGATCA-3'
Dsn1 REV	5'-GGGAAAGUTCACTGACAAGATCCAGATCCAGAT-3'
Kif2c-ΔN	5'-GGAGACAUCGCAAGCAGTTTTCAGTTCCTC-3'
Kif2c-SKNN TOP	5'-GTGAATTCCAAAAACAACGCTCCAAAAGAAAGTCTTCGAAG-3'
Kif2c-SKNN BOTTOM	5'-CGTTGTTTTTGGAATTCACGGATCTCCGTTTTTGTTTCTG-3'

Supplementary Table 2. Table of primers used for USER cloning and mutagenesis

Antibody (clone)	Source (Cat # or Ref.)	Dilution
Human anti-ACA	Immunovision (HCT-0100)	1:1000 (IF)
Mouse anti-β-Actin (AC-15)	Abcam (ab6276)	1:5000 (WB)
Sheep anti-BubR1 (SBR1.1)	Stephen Taylor (ref. 24)	1:1000 (IF); 1:1000 (WB)
Rabbit anti-BubR1	Bethyl Laboratories (A300-386A)	1:1000 (WB)
Rabbit anti-CENP-I	Tim Yen (ref. 25)	1:500 (IF)
Rabbit anti-CENP-C	Iain Cheeseman (ref. 26)	1:1000 (IF)
Rabbit anti-CENP-U/50	MBL (PD019)	1:200 (IF)
Mouse anti-Cyclin B1 (GNS-1)	BD Pharmingen	1:2000 (WB)
Rabbit anti-dsRed	Clontech (632496)	1:500 (IF); 1:1000 (WB)
Mouse anti-Flag (M2)	Sigma-Aldrich (F1804)	1:500 (IF)
Mouse anti-Flag (M2) HRP	Sigma-Aldrich (A8592)	1:10,000 (WB)
Mouse anti-GFP (3E6)	Invitrogen (A-11120)	1:500 (IF)
Rabbit anti-Pericentrin	Abcam (ab4448)	1:1000 (IF)
Mouse anti-Plk1 (F8)	Santa Cruz Biotechnology (sc-17783)	1:500 (IF); 1:1000 (WB)
Rat anti-Tubulin (YL1/2)	EMD Millipore (MAB1864)	1:1000 (IF)

Supplementary Table 3. Table of antibodies used for immunofluorescence and immunobloting

Supplementary Dataset 1. Results table listing phosphopeptides encountered by mass spectrometry of Plk1 cell lines labeled with tandem mass tags. A total of 11,407 phosphopeptides were encountered from the 10 cell fractions (5 cell lines +/- BI-2536). The raw 10-plex reporter ion intensities of localized phosphopeptide isoforms were $\log_2$ transformed and normalized against inhibited $Plk1^{wt}$ (+BI-2536) to obtain the relative phosphopeptide and protein quantitation for each cell line and condition. Terminal columns in the table indicate relative phosphopeptide expression for individual lines after BI-2536 treatment. See online methods section for details of analysis.

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