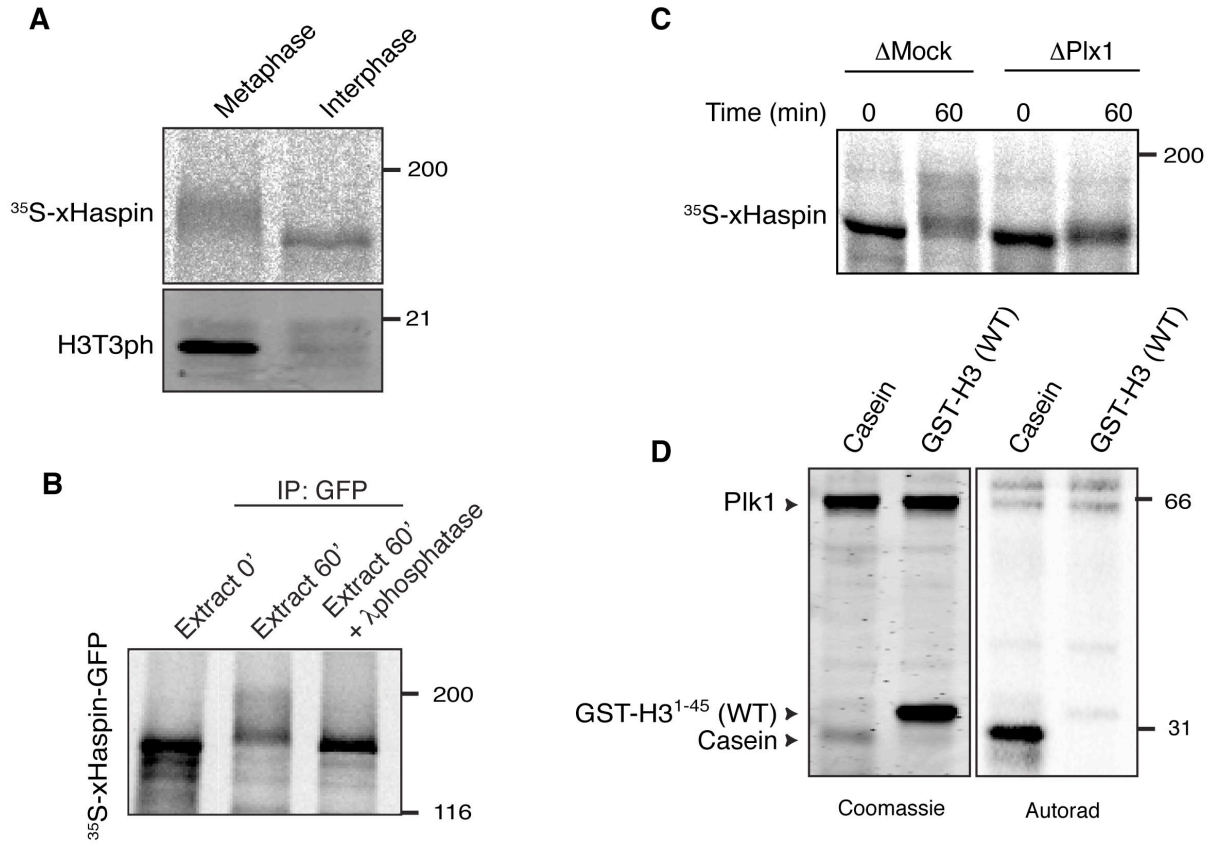


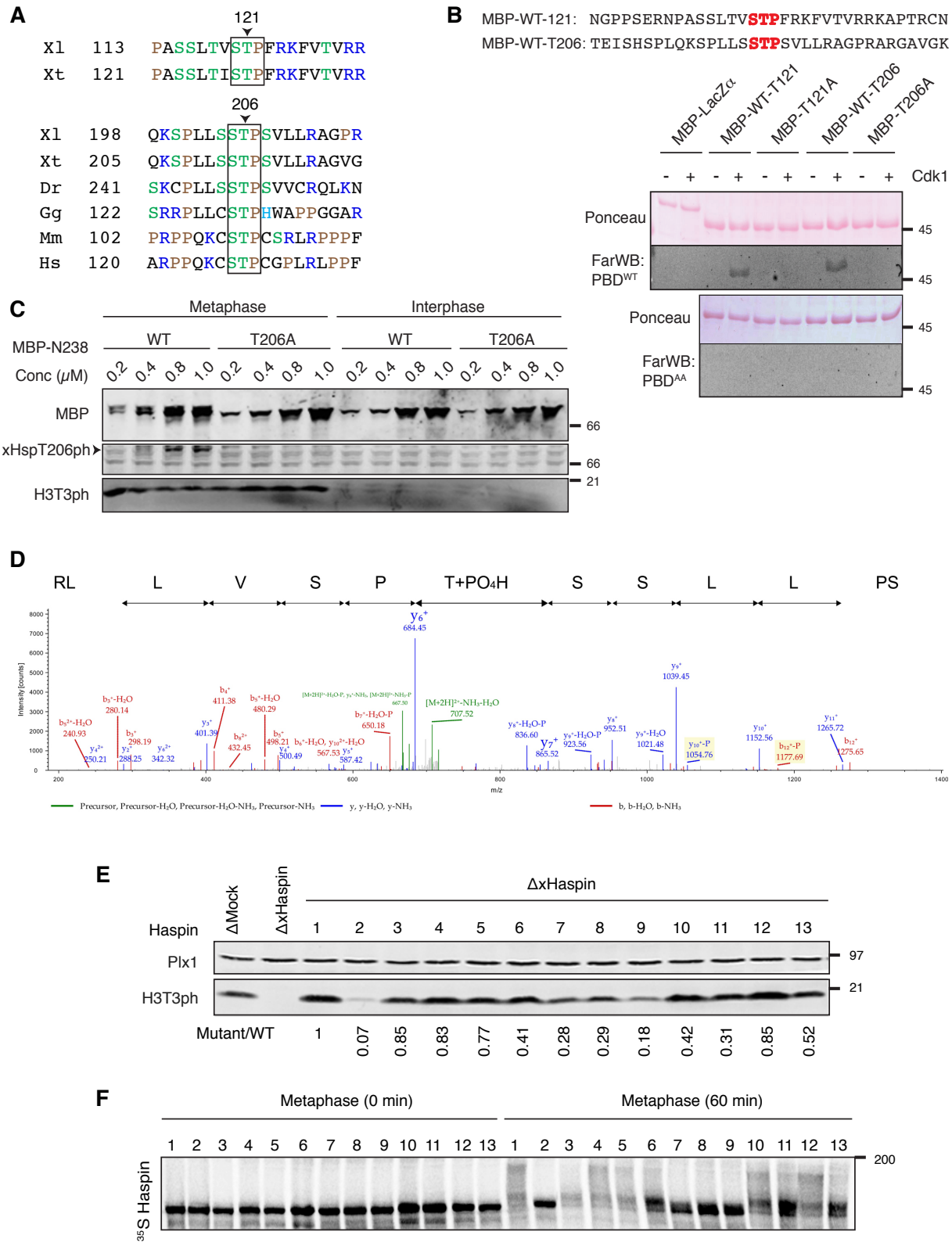
Figure S1. Plx1-dependent phosphorylation of xHaspin in metaphase egg extracts, related to Figure 1.

(A) Full-length xHaspin, translated and labeled with ^{35}S in reticulocyte lysates, was incubated with either metaphase or interphase *Xenopus* egg extracts. Protein samples were analyzed by SDS-PAGE, followed by autoradiography (top), or by Western blot using anti-H3T3ph antibody (bottom).

(B) ^{35}S-labled xHaspin tagged with GFP was incubated with metaphase extract for 0 or 60 min, and immunoprecipitated with an anti-GFP antibody. The antibody beads were subjected to mock or lamda-phosphatase treatment. Lamda-phosphatase collapsed the shifted forms into a lower band, indicating that the metaphase-associated mobility shift is caused by phosphorylation.

(C) ^{35}S-labled xHaspin was incubated with metaphase ΔMock (control) extracts or ΔPlx1 extracts for 0 or 60 min. The metaphase-specific mobility shift of Haspin was reduced in ΔPlx1 extracts.

(D) The N-terminal tail (1-45 aa) of histone H3 tagged with GST ($H3^{1-45}$-GST) (Dai et al., 2005), or the dephosphorylated Casein protein were subjected to an *in vitro* kinase assay using purified Plk1 with γ-^{32}P-ATP. Coomassie and autography of the reaction products is shown. Plk1 effectively phosphorylated the dephosphorylated casein protein but not GST-H3.


A
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Xl 113 PASSLTVSTPFRKFVTVRR
Xt 121 PASSLTISTPFRKFVTVRR
206
Xl 198 QKSPLLSSTPSVLLRAGPR
Xt 205 QKSPLLSSTPSVLLRAGVG
Dr 241 SKCPLLSSTPSVVCRQLKN
Gg 122 SRRPLLCSTPHWAPPGGAR
Mm 102 PRPPQKCSTPCSRLRPPPF
Hs 120 ARPPQKCSTPCGPLRLPPF
B
MBP-WT-121: NGPPSERNPASSLTVSTPFRKFVTVRRKAPTRCN
MBP-WT-T206: TEISHSPLQKSPLLSSTPSVLLRAGPRARGAVGK
MBP-LacZα
MBP-WT-T121
MBP-T121A
MBP-WT-T206
MBP-T206A
- + - + - + - + - + Cdk1
Ponceau
FarWB: PBDWT
Ponceau
FarWB: PBDAA
45
C
Metaphase
Interphase
MBP-N238
WT
T206A
WT
T206A
Conc (μM) 0.2 0.4 0.8 1.0 0.2 0.4 0.8 1.0 0.2 0.4 0.8 1.0 0.2 0.4 0.8 1.0
MBP
xHspT206ph
H3T3ph
66
66
21
D
RL L V S P T+PO4H S S L L PS
y6+ 684.45
y7+ 865.52
Intensity [counts]
m/z
Precursor, Precursor-H2O, Precursor-H2O-NH3, Precursor-NH3
y, y-H2O, y-NH3
b, b-H2O, b-NH3
E
ΔMock
ΔxHaspin
ΔxHaspin
Haspin 1 2 3 4 5 6 7 8 9 10 11 12 13
Plx1
H3T3ph
97
21
Mutant/WT 1 0.07 0.85 0.83 0.77 0.41 0.28 0.29 0.18 0.42 0.31 0.85 0.52
F
Metaphase (0 min)
Metaphase (60 min)
1 2 3 4 5 6 7 8 9 10 11 12 13 1 2 3 4 5 6 7 8 9 10 11 12 13
35S Haspin
200

Figure S2. H3T3 phosphorylation in metaphase extracts requires T206-dependent Plx1-docking and multisite phosphorylation at the Haspin N terminus, related Figure 2.

(A) Multisequence alignment of Haspin sequences adjacent to *Xenopus laevis* (Xl) $ST^{121}P$ (left) and $ST^{206}P$ (right). Xt, *Xenopus tropicalis*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Mm, *Mus musculus*; Hs, *Homo sapiens*.

(B) Far Western analysis detecting the phospho-dependent interaction between xHaspin N-terminal constructs and the PBD of Plx1. MBP-lacZα (control) or MBP fused to the 34 amino acids surrounding T121 or T206 of xHaspin (top) were phosphorylated by Cdk1-cyclin B, and transferred to a nitrocellulose membrane (Ponceau), followed by incubation with recombinant PBD of Plx1. A $PBD^{H532A/K534A}$ mutant defective in phospho-dependent binding (PBD^{AA}) was used as a negative control. Binding of the PBD on the membrane was monitored by anti-Plx1 antibody (FarWB). Binding of PBD^{WT} to both Haspin peptides upon Cdk1-dependent phosphorylation at T121 and T206 sites was detected.

(C) T206 of xHaspin is phosphorylated in metaphase egg extract. A recombinant protein of the N-terminal 238 amino acids of xHaspin fused to MBP (MBP-xHaspin N238), with or without a T206A mutation, was incubated with metaphase or interphase egg extracts for 60 min at the indicated concentrations. Samples were subjected to Western blot analysis. The anti-phospho xHaspinT206 antibody reacted with MBP-xHaspin N238 after incubation with metaphase extracts, but not with interphase extracts, in a T206-dependent manner.

(D) Fragment annotated tandem MS (ion trap) spectrum of the doubly charged phosphorylated peptide (SPLLSST^{206}PSVLLR) obtained from MBP-xHaspin-N520 incubated with metaphase egg extracts. The difference between the y-ion fragment 6 and 7 is 181.07, which is in agreement with a threonine phosphorylated residue (average theoretical mass: 181.08 Da), demonstrating that T206 is phosphorylated in metaphase egg extracts.

(E) Representative Western blot of xHaspin phosphorylation mutants, quantified in Figure 2D. The mutants were produced and labeled with ^{35}S in reticulocyte lysates and subsequently incubated with metaphase ΔHaspin extracts for 60 min. The samples were processed for Western blot analysis. Plx1 was used as a loading control. The H3T3ph activity was normalized to each mutant's expression level, and the data is presented as a percentage of the wild-type control under the gel after normalization by the input ^{35}S Haspin levels shown in E.

(F) An autoradiograph of samples in Figure S2E, 0 or 60 min after incubation with metaphase extracts, showing the impact on the xHaspin phoshorylation-sensitive shift when various amino acids in its N terminus are mutated.

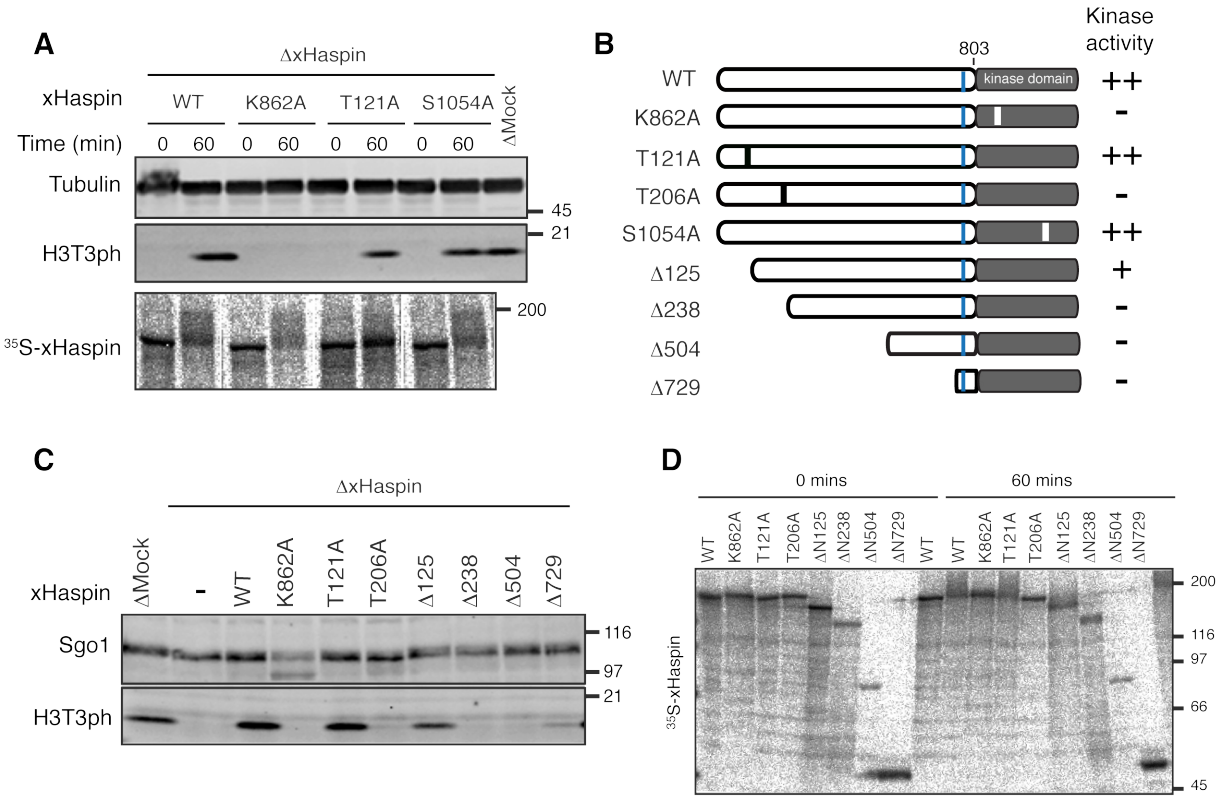

Figure S3. Deletion analysis of xHaspin, related to Figure 3.
(A) Full-length xHaspin with K862A (kinase-dead), T121A, or S1054A (Polo-target consensus site within activation loop) mutations was labeled with ^{35}S in reticulocyte lysates and incubated with metaphase ΔHaspin extracts. Western blots using anti-H3T3ph and anti-tubulin antibodies (top), and autoradiograph are shown.
(B) Schematic of Haspin constructs. Vertical bars mark the position of alanine mutations. Blue bars mark the position of the HBIS.
(C and D) ^{35}S-labeled Haspin constructs shown in B were incubated with metaphase ΔHaspin extracts for 60 min.
(C) Western blots using anti-H3T3ph antibody and anti-Sgo1 antibody (loading control).
(D) An autoradiograph showing the protein level of the various Haspin constructs.

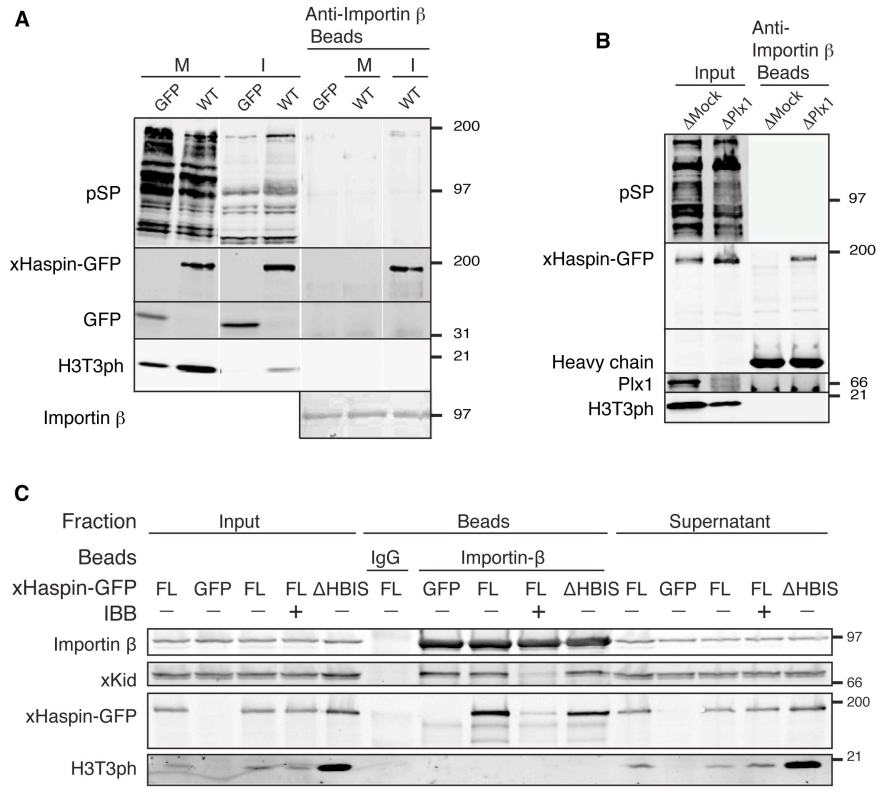

Figure S4. Importin β interacts with xHaspin in a cell cycle- and Plx1-dependent manner but does not inhibit xHaspin activity, related to Figure 4.

(A) Interphase-specific binding of xHaspin to Importin β. GFP (control) or Haspin-GFP were translated from exogenously added mRNA for 1 hr in metaphase egg extracts, and then either maintained in M phase (M) or released into interphase (I) by calcium addition. Cycloheximide was then added to stop translation. Anti-Importin β-beads were incubated with these extracts for 1hr at room temperature. Total fractions and bead fractions were analyzed by Western blot. The anti-pan phospho SP site (Cdk1 substrate) antibody was used to confirm the maintenance of cell cycle stages. The blots were probed with anti-GFP (detect GFP and xHaspin-GFP) and anti-H3T3ph antibodies. The immunoisolated Importin β was visible by Ponceau stainining.

(B) Binding of xHaspin to Importin β is inhibited by Plx1 in metaphase. GFP or xHaspin GFP translated in control or Plx1-depleted metaphase egg extracts were subjected to immunoisolation with anti-Importin β-beads.

(C) Excess IBB dissociates xHaspin from Importin β in interphase extracts but does not re-activate Haspin kinase activity. Full-length xHaspin-GFP (FL), xHaspin ΔHBIS-GFP (ΔHBIS), or GFP (GFP) were translated in metaphase egg extracts for 1 hr released into interphase for 30 min, then treated with either 20 μM of IBB or buffer (-) for 1 hr while incubated with anti-Importin β beads. Beads were then subjected to Western blot analysis. Addition of IBB effectively inhibited binding of xHaspin-GFP and xKid to Importin β (beads), but did not promote H3T3 phosphorylation (input).

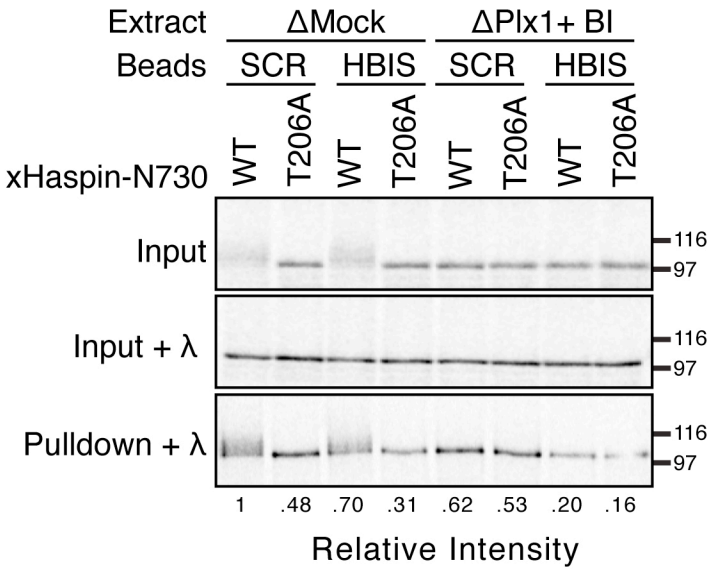

Figure S5. Plx1-dependent phosphorylation of the Haspin N-terminus facilitates charge-mediated, sequence-independent binding to the HBIS, related Figure 5. Plx1 was inhibited by partial immunodepletion (ΔPlx1) from and by adding the Plx1 inhibitor Bi2536 to metaphase egg extracts. Extract that was mock depleted (ΔMock) is shown as a control. ^{35}S-xHaspin N730 (WT) or xHaspin N730^{T206A} (T206A) was incubated to these extracts. Autoradiographs of crude extracts (Input), crude extracts treated with lambda phosphatase to reduce the phospho-dependent mobility shift and to compare protein loading (Input + λ), and pulldowns of ^{35}S-labeled proteins from the input samples using either scrambled sequence-peptide (SCR) or HBIS-peptide coated beads followed by lambda phosphatase treatment (pulldown + λ). Relative intensity of the pulldown was calculated by integrating the total intensity of ^{35}S protein in each lane, and dividing it by the total intensity in the sample representing WT/SCR/ΔMock.

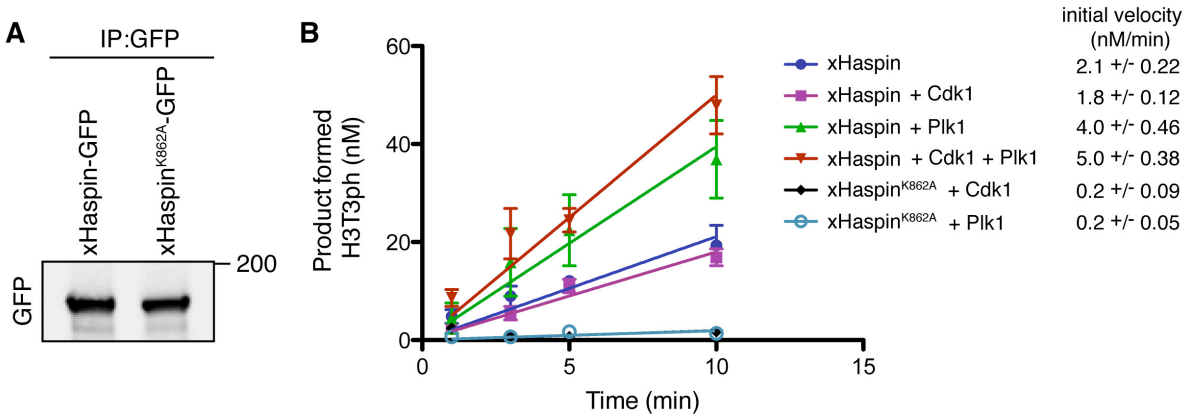

Figure S6. xHaspin immunoprecipitated from interphase extracts can be further activated after incubation with Plk1, related to Figure 6.
(A) xHaspin-GFP, either wild-type or kinase inactive K862A, was translated in interphase egg extracts and immunoprecipiated using an anti-GFP antibody. An anti-GFP Western blot of the immunoprecipitated xHaspin proteins is shown.
(B) The beads coated with xHaspin-GFP were incubated with buffer, Cdk1/Cyclin B, Plk1 or both Cdk1/Plk1 and ATP. Following this pre-phosphorylation step, the beads were used in an *in vitro* kinase assay with 200 nM GST-H3$^{1-45}$ and 750 µM ATP. The initial velocities and the reaction curves of the various conditions are shown. Bars represent ranges of two independent experiments.

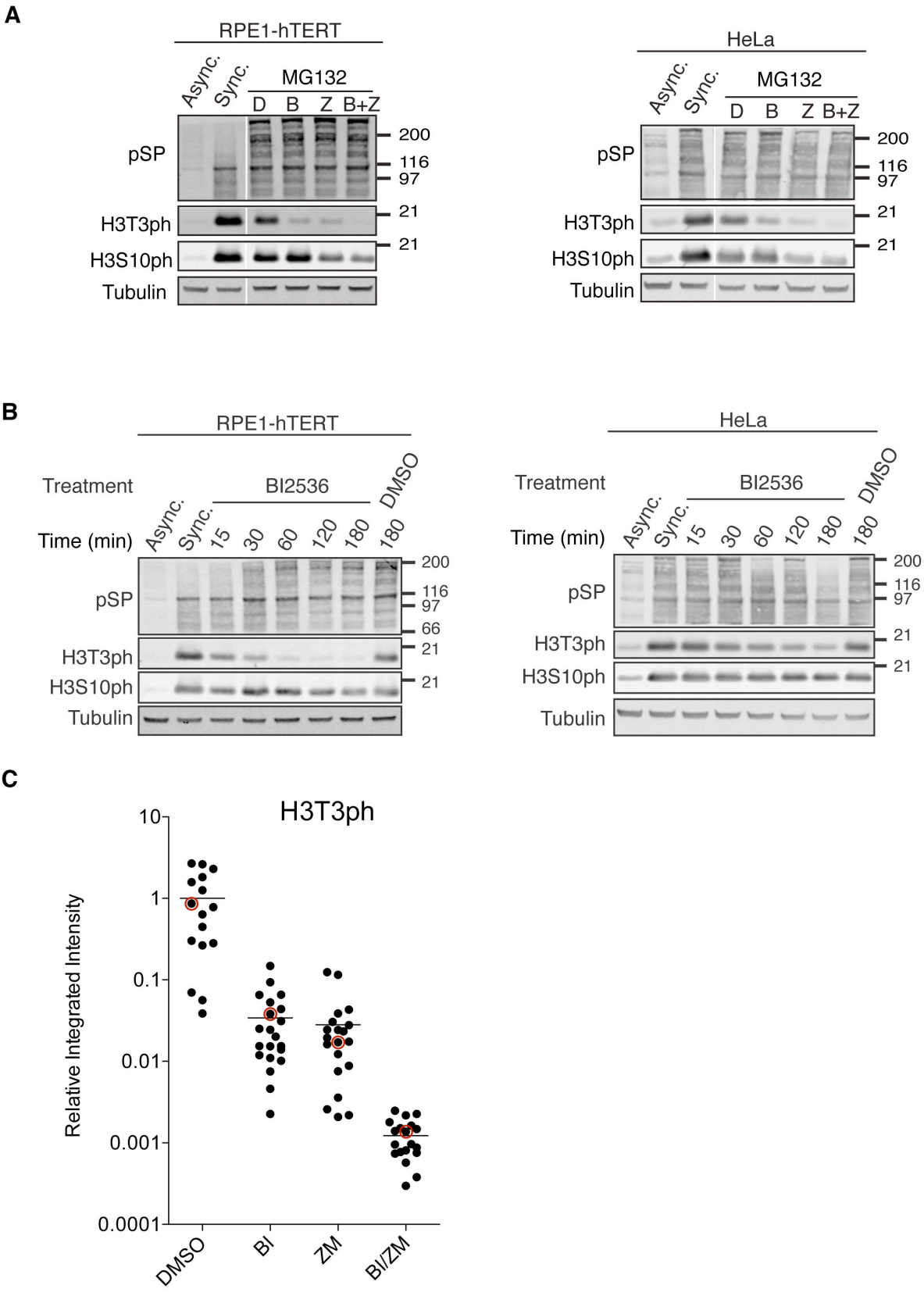
A
RPE1-hTERT
Async.
Sync.
MG132
D
B
Z
B+Z
pSP
H3T3ph
H3S10ph
Tubulin
200
116
97
21
21
HeLa
B
RPE1-hTERT
Treatment
BI2536
DMSO
Time (min)
15
30
60
120
180
66
HeLa
C
H3T3ph
Relative Integrated Intensity
10
1
0.1
0.01
0.001
0.0001
DMSO
BI
ZM
BI/ZM

Figure S7. Both Plk1 and Aurora B kinase activity are required for robust H3T3 phosphorylation in human cells, related to Figure 7.

(A and B) RPE1-hTERT (left) or HeLa (right) cells were synchronized in mitosis with a thymidine-nocodazole block followed by mitotic shake-off. Mitotic cells were placed in media containing MG132 and nocodazole and treated with either 100 nM of the Plk1 inhibitor BI2536 (B), 2 μM of the Aurora B inhibitor ZM447439 (Z), both inhibitors (B+Z), or DMSO (D). Western blots using indicated antibodies are shown.

(C and D) RPE1-hTERT (left) or HeLa cells (right) were synchronized in mitosis by a thymidine-nocodazole block, collected by mitotic shake-off, placed in media containing nocodazole and treated with either DMSO or a Plk1 inhibitor. Samples were collected at various intervals by mitotic shake-off and levels of H3T3ph were monitored by Western blot. While levels of H3S10ph remained relatively constant, Plk1 inhibition resulted in a gradual decrease in H3T3ph levels beginning 15-30 min after treatment.

(E) Relative integrated intensity of H3T3ph for individual cells in Figure 7A and 7B. Data are plotted on a log scale to visualize the additive effect of Plk1/ Aurora B inhibition (BI/ZM) relative to Plk1 (BI) or Aurora B (ZM) inhibition alone. Red circles indicate the representative images shown in Figure 7A.

Supplemental Experimental Procedures

Antibodies

For Western blot: anti-Ku70, anti-Op18, anti-INCENP, and anti-Aurora B, were used as previously described (Kelly et al., 2007; Postow et al., 2008). Other primary antibodies were diluted at the following concentrations: 1:10,000 anti-H3T3ph antibodies (Millipore # 07-424); 1:200 anti-phospho-Aurora (#2914, Cell Signaling Technology); 1 µg/ml anti-xHaspin antibody (raised against GST-xHaspin$_{65-184}$) (Kelly et al., 2010); 0.2 µg/ml anti-Plx1 antibody (raised against a C-terminal peptide CLQSSKSAVAHVKASA); 1:200 xSgo1 antibody (gift from Ana Losada); 1:1000 Phospho-(Ser) CDKs Substrate Antibody (Cell Signaling Technology #2324); 1:1000 mouse anti-Karyopherin β, (BD Transduction Laboratories, #610560); 1:10,000 anti-MBP monoclonal antibody (NE Biolabs #E8032S); 1:400,anti-GFP (Roche #11 814 460 001). IRDye 680 goat anti-rabbit or mouse IgG (LICOR) and IR Dye 800CW goat anti-rabbit or mouse IgG (LICOR) were used as secondary antibodies. The Odyssey Infrared Imaging System (LICOR) was used for detection and quantitation.

For immunofluorescence: Primary antibodies were used for immunofluorescence at the following concentration: 1:12,500 H3T3ph (Millipore # 07-424); 1:2000 Aurora B (BD Transduction Laboratories #611082); 1:400 CREST. Secondary antibodies were used for immunofluorescence at the following concentration: 1:1000 anti-rabbit IgG Alexa 488 (Invitrogen #A-11034), 1:1000 anti-mouse IgG dylight 594 (Jackson ImmunoResearch

Laboratories #715515150), 1:400 anti-Human Ig(H+L) 649 (Jackson ImmunoResearch Laboratories #709496149).

Construction of Plasmids

cDNA encoding *Xenopus laevis* Haspin (IMAGE clone 4055317) was purchased from Open Biosystems and its full-length sequence was determined and deposited to Genbank (NP_001233237). MBP-xHapinΔN729 (previously named Haspin$_C$ see (Kelly et al., 2010)) was cloned into the pMAL-c2e vector with a C-terminal His_6 using the primers (5'-CGCGGGGTACCGCCAGGAACCTCAGGC-3') and (5'TCTAGACTAGTGGTGATGGTGATGATGCTGGAACAGCTTG-3') that amplify residues 730-1146 of xHaspin. To produce the fusion protein used for xHaspin antibody production (N terminal fragment 65-184aa), the construct was amplified using the primers (5'-CGCGGG TCGACTCCCCACAAAAAAAAG-3') and (5'-GCGGCGGCCGCCTAGTGGTGATGGTGATGATGACTACGAATGTCT-3') and cloned into pGEX-6P-2 (GE Healthcare). Deletion mutants were generated using a modified Quickchange protocol. For Far Westen analysis we produced the recombinant PBD of Plx1. We cloned the Plx1 PBD (317-598 aa) into pGEX-6P-2 (GE Healthcare) with a C-terminal His tag aided by the primers: (5' CGCGGAATTCGGTCCATTGCGCCCAGCACTATT-3') and (5'-GCGGCGGCCGCCTAGTGGTGATGGTGATGATGTGCTGAGGCCTTTAC). QuickChange mutagenesis (Stratagene) was employed to mutated H532/K534 to alanine in order to create a PBD impaired for phosphopeptide binding. To obtain the

various xHaspin phosphorylation mutants, we employed a QuickChange Multi Site Directed Mutagenesis kit (Agilent Technologies, #200514-5).

Recombinant proteins

MBP-xHaspin-ΔN729 and MBP-xHaspin-ΔN729$^{\Delta HBIS}$ were expressed in BL21 *E.coli* and purified by Ni-NTA standard methods, followed by gel filtration (S200) and dialyzed into 50 mM HEPES pH 8, 300 mM NaCl, 10 % glycerol. MBP-xHaspin-N420 and MBP-xHaspin-N520 were purified similarly to the kinase domain constructs except a higher salt concentration was used (500 mM NaCl vs. 300 mM) and an extra wash step was applied with $MgCl_2$/ATP (50 mM Tris pH 8, 500 mM NaCl, 2.5 mM ATP, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol). MBP-hHaspin was bacterially expressed using pMALc2/coHaspin (codon optimized, a generous gift from Jonathan Higgins)(Patnaik et al., 2008), and was purified following a standard MBP/amylose chromatography protocol, using 1 M NaCl column buffer. The protein was stored in 50 mM HEPES pH 8, 300 mM NaCl, 10 % glycerol. The kinase was stored at 4°C after purification and the *in vitro* kinase experiments were performed within two days of purification. We noted that liquid nitrogen freezing and storing at -80°C resulted in protein degradation upon thawing.

GST-Plx1-PBD-His was purified by Ni-NTA standard methods and dialyzed into protein binding buffer (50 mM HEPES pH 8, 300 mM NaCl, 0.1% Tween 20, 4 % w/v skim milk, 10 % glycerol, 1 mM DTT). His_6-tagged IBB was generated and purified from a plasmid generously provided by Karsten Weis following a published procedure (Weis et al., 1996).

Xenopus egg extract and Immunodepletions

Meiosis metaphase II-arrested CSF extracts were prepared using a method previously described (Murray, 1991). To make antibody-bound beads, Protein A Dynabeads (Invitrogen) were washed four times with TBS and incubated on a rotator with 10 µg antibody per 100 µl beads for 45 min at room temperature. To crosslink the antibodies to the beads, the antibody-bound beads were washed three times each with TBS and conjugation buffer (0.1M HEPES pH 8.0, 150 mM KCl), and then incubated with 6.8 mM BS_3 (Bis[sulfosuccinimidyl]suberate; Pierce) in conjugation buffer for 30-45 min at room temperature on a rotator. The cross-linking reaction was quenched with two 15 min incubations with 1 M Tris pH 7.5. The beads were then washed three times with TBS and stored at 4°C until use (up to 24 hr). The beads were washed three times with SDB (5 mM HEPES pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 150 mM sucrose) right before use. xHaspin was depleted from CSF extracts containing cycloheximide (100 µg/ml) using anti-xHaspin-coated protein A-Dynabeads (Invitrogen), following an immunodepletion protocol as described (Kelly et al., 2010). For CPC depletion, anti-INCENP antibodies were used (Sampath et al. 2004). Extracts treated with protein A-beads coated with rabbit IgG were used as control extracts.

Immunoprecipitations

RNAs encoding wild-type or various xHaspin mutants carrying a C-terminal GFP tag were synthesized using the SP6 mMessage mMachine RNA transcription kit (Ambion). These mRNAs were added at 0.1 µg/µl to CSF-arrested or interphase extracts and

incubated for 60-90 min to allow expression. Then protein G-beads coated with GFP antibody (2 µg of GFP antibody for 50 µl of beads) were incubated with the extract on ice for 1hr. The beads were removed and washed 5x with ice cold Wash buffer (1xPBS, 1% Triton X-100, 0.5 mM PMSF, 5 mM β-glycerophosphate, 1x phophatase inhibitor cocktail 2 (P5726 Sigma).

Importin β beads were prepared by coupling 12 µl of the Nuclear transport factor p97, mAB (3E9) (Enzo, ALX-804-025) to 33 µl of prewashed protein G beads. As a control we used the Mouse IgG antibody from Sigma diluted in PBS with 50% glycerol. The extract was incubated with 0.1 µg/µl of xHaspin mRNA for 45 min at room temperature. At this point the extract was divided into two tubes, one was maintained in CSF for another 60 min, while to the other we added calcium and induced interphase at room temperature. Cycloheximide was added to both samples to stop further xHaspin translation. To 40 µl of extracts we added 15 µl of Importin β coupled beads. They were incubated at room temperature with occasional mixing for 1 hr. Prior to removing the beads we took the input Western blot sample. The beads were removed by placing them on a magnet for 2 min. The supernatant Weston blot sample was taken at this point. Immediately 150 µl of wash buffer was added to the beads to avoid drying. The beads were washed 5 times in ice cold buffer containing: 1xPBS, 1% Triton, 0.5 mM PMSF, phosphatase inhibitor cocktail [Sigma, p2850], 5 mM β-glycerophosphate, 1xLPC. The beads were resuspended in an equal volume of sample buffer and boiled prior to running on a polyacrylamide gel.

MBP-xHaspin-N420 constructs were incubated with extract at 2 mM for 60 min. They were subsequently captured using protein G-Dynabeads coated with anti-MBP

antibody (NE Biolabs #E8032S), 3 µg of antibody for 30 µl of beads. They were washed and prepared as above.

Far Western Blots

Protein phosphorylation was performed at RT using 173 pmols of the various substrates, 0.2 ng/µl Cdk1/Cyclin B (Millipore #14-450), 250 µM cold ATP in a final volume of 30 µl 1x kinase buffer (10x kinase buffer: 200 mM HEPES-KOH pH 7.7, 1.5 M NaCl, 100 mM $MgCl_2$, 10 mM DTT, phosphatase inhibitor cocktail [Sigma, p2850]). The reaction was quenched by addition of 10 µl 4xSDS buffer and heat and the samples were run on a polyacrylamide gel and transferred to nitrocellulose membrane (Whatman Protran BA79). The membrane was blocked for 1 hr using Odyssey blocking buffer (LICOR 927-40000). The bait concentration (either PBD^{WT} or $PBD^{H532A/K534A}$) was set to 5 µg/ml. The bait was incubated in protein binding buffer (50 mM HEPES pH 8, 300 mM NaCl, 0.1% Tween 20, 4% w/v skim milk, 10% glycerol, 1mM DTT) for 10 hr at room temperature. The membrane was washed twice with PBST for 10 min and incubated with primary antibody (anti-Plx1) for 1 hr and after two more washes in PBST, an IR Dye 800CW goat anti-rabbit IgG (LICOR) secondary antibody was applied for 1 hr. The membrane was washed three times in PBST (10 min) and twice in PBS (10 min) before imaging it using the Odyssey LICOR system.

Kinetic Analysis

We analyzed kinetic data from anti-phospho H3T3 Western blots by adapting a method published by (Good et al., 2009). Briefly, to generate the standard H3T3ph curve, we

incubated 10 µM of $H3^{1-45}$-GST (produced from plasmids, generously provided by J. Higgins) (Dai et al., 2005) with 2 µM MBP-xHaspin-Δ729 overnight at 16 °C to ensure complete phosphorylation of the substrate. Serial dilutions of fully phosphorylated $H3^{1-45}$-GST were analyzed by quantitative Western blot with anti-H3T3ph antibody. To determine the linear range of the Westen blot signal, several dilution series of the anti-H3T3ph antibody were employed. For kinetic analysis, we set up a reaction mix that contained 1 nM of either MBP-xHaspin-ΔN729 or MBP-xHaspin-$\Delta N729^{\Delta HBIS}$, 750 µM ATP, varying concentrations of GST-H3 1 to 45 (0, 50, 100, 200, 400, 800, 1600 nM) in 1x Kinase buffer (20 mM HEPES pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) on ice. The reaction was initiated by addition of ATP and the substrate and it was allowed to proceed on ice. Samples were collected at 1, 3.5 and 7 min and quenched by adding 4x SDS sample buffer containing 50 mM EDTA followed by boiling. The samples were run on a polyacrylamide gel and subjected to Western blotting analysis using as primary antibody anti-phospho H3T3ph 1:10000 (Millipore # 07-424) that was optimized to be in the linear range and as secondary antibody goat anti-rabbit IgG LICOR IRDye 800. The blots were scanned and quantified on LICOR Odyssey Imaging system. The data was exported to Prism 5 and rate plots (AU / min) were generated, all with an $R^2 > 0.95$. Using the standard curve mentioned above, we estimated the absolute concentration of phosphorylated substrates to obtain the rate plots in nM / min. Triplicate data sets were fit to the Michaelis-Menten equation with Prism to calculate K_m and k_{cat} for each curve.

***In vitro* kinase assays with recombinant proteins**

To check the ability of Cdk1 and Plk1 to phosphorylate the xHaspin N terminus (MBP-xHaspin-N520) *in vitro*, we set up 25 µl reactions, at room temperature (RT), in the presence of 250 µM cold ATP and 2.5 µCi per reaction of γ-^{33}P ATP. The substrate, MBP-xHaspin-N520 was added to the reaction at 0.3 µM. Cdk1/Cyclin B (Millipore #14-450) was added to the reaction at 0.2 ng/µl. Plk1 (SignalChem # P41-10H-10) was added to the reaction at 32 ng/µl. Co-incubation of Cdk1/Cyclin B and Plk1 was performed as follows: MBP-xHaspin-N520 was allowed to pre-incubate with Cdk1/CyclinB in the presence of cold ATP only, for 1 hr at RT. Then, RO-3306 (Enzo, ALX-270-463), a Cdk1 inhibitor was added to the reaction to 10 µM final concentration and allowed to incubate for 30 min. To this reaction we added Plk1 together with γ-^{33}P ATP.

To phosphorylation recombinant hHaspin by Plk1 and Cdk1/Cyclin B, 1 µM MBP-hHaspin was incubated in a mixture of 1x Kinase buffer with either 3 ng/µl Cdk1/Cyclin B (Millipore #14-450), 32 ng/µl Plk1 (SignalChem # P41-10H-10), or both kinases, 250 µM cold ATP, 3.75 µCi per reaction of γ-^{33}P ATP for 2 hr at RT. Then, the reaction was diluted in 1x Kinase buffer to 25 nM MBP-hHaspin and placed on ice for 30 min to cool. This pre-phosphorylated MBP-hHaspin was added in 1:10 (2.5 nM final) to another in vitro kinase reaction that was kick started by the addition of 300 nM GST-H3 and 750 µM cold ATP. At 8, 12 and 20 min, 20 µl were removed and added to 8 µl of 4xSDS buffer. These samples were boiled and processed for Western blot analysis. The LICOR Odyssey Imaging system was used to quantify the H3T3ph signal.

The ability of Plk1 to phosphorylate either the N-terminal tail (1-45 aa) of histone H3 or a positive control, namely dephosphorylated Casein (SignalChem, C03-54BN),

was assessed in the presence of 250 µM cold ATP and 2.5 µCi per reaction of γ-^{33}P ATP (Figure S1D).

Antibody beads kinase assay

To perform the *in vitro* kinase assay involving full length Xenopus Haspin (Figure S6), wild-type or kinase inactive xHaspin K862A, tagged with GFP, were expressed and purified from interphase *Xenopus* egg extracts following the protocol described in the Immunoprecipitations section. After washing, the beads were resuspended in an equal volume of sperm dilution buffer (SDB: 1 mM $MgCl_2$, 100 mM KCl, 150 mM Sucrose, 5 mM HEPES, 100 µg/ml cytochalasin B), 10 µl of these beads were pre-incubated with either Cdk1, Plk1 or both. 25 µl reactions were assembled to contain 250 µM cold ATP, 10 µl of xHaspin beads, 2 ng/µl Cdk1/Cyclin B (Millipore #14-450), 32 ng/µl Plk1 (SignalChem # P41-10H-10). These reactions were incubated for 1.5 hr at RT. Following this incubation, the 25 µl reaction was placed on ice for 30 min. Then to start the reaction, the 25 µl were added to 115 µl of an ice cold 1x Kinase buffer mixture containing 200 nM GST-H3$^{1-45}$, 750 µM cold ATP. At 0, 1, 3, 5, and 10 min, 20 µl were removed and added to 8 µl of 4xSDS buffer. These samples were boiled and processed for Western blot analysis. The LICOR Odyssey Imaging system was used to quantify the H3T3ph signal.

Quantification of H3T3ph activity exhibited by xHaspin phosphorylation mutants

The expression level of Haspin phosphorylation mutants (figures 2E and S2E,F) were quantified based on the ^{35}S intensity present in the 0 min input sample by using the

Odyssey 2.1.10 quantification software (LICOR). In multiple experiments we confirmed that performing the quantification using the ^{35}S intensity present at the 60 min gave the same result, and since quantifying a sharp band was less technically challenging, we reported the results using the 0 min input. The H3T3ph levels were quantified in a similar manner. The data was processed by first normalizing the H3T3ph level to the ^{35}S level of each mutant. Subsequently, the mutant activity was expressed as a percentage of the wild-type activity. P-values were determined using a one-way ANOVA followed by a Bonferroni post-hoc test.

Human tissue culture

HeLa cells were cultured in DMEM (Gibco) with 10% FBS and non-essential amino acids. RPE1-hTERT cells were cultured in DMEM:F12 (ATCC) with 10% FBS. For thymidine-nocodazole synchronization, cells at 40 % confluency were grown in 2 mM thymidine for 24 hr, released into fresh media for 3 hr, then arrested in mitosis with 100 ng/mL nocodazole for 12-16 hr. Cells were collected by mitotic shake-off. For inhibitor experiments, mitotic cells were incubated for 1 hr in media with 10 μM MG132, then treated with either 0.1 μM BI2536, 2 μM ZM447469, both or DMSO for 1-3 hr.

Generation of LAP-hHaspin cell lines

Stable, single site integration of LAP-hHaspin constructs was achieved using the Flp-In T-REx tissue culture system (Invitrogen). Parental DLD1 Flp-In cell lines and a pcDNA5/FRT/TO integration vector containing LAP-tagged CENP-E were a generous gift from Arshad Desai, Reto Gassmen, and Stephen Taylor. hHaspin cDNA was a

generous gift from Jonathan Higgins. CENP-E was removed from the pcDNA5/FRT/TO/LAP vector by restriction enzyme digestion, and hHaspin cDNA PCR amplified with 5'-ATCATCCTCGAGATGGCGGCTTCGCTCC (forward) and 5'-ATCATCGCGGCCGCTTACTTAAACAGACTGTGCTGGCAG (reverse) was cloned in frame with the LAP-tag following digestion with the same enzymes. The hHaspin-T128A and hHaspin-ΔHBIS mutants were made using QuickChange mutagenesis (Agilent). pcDNA5/FRT/TO/LAP-hHaspin constructs were integrated into parental DLD1 Flp-In lines using the manufacturers protocol and selected for by growth in media containing 400 μg/ml Hygromycin. Resultant colonies were expanded clonally and LAP-hHaspin integration was verified by Western blot analysis following doxycyclin-induction.

LAP-hHaspin tissue culture experiments

For expression of LAP-hHaspin in mitosis, DLD1 T-REx cells containing LAP-tagged hHaspin, hHaspin^{T128A} or hHaspin$^{\Delta HBIS}$ were grown in 25 ng/ml doxycyclin for 8 hr to induce LAP-hHaspin expression. Cells were grown for an additional 16 hr in doxycyclin and 100 ng/ml nocodazole to synchronize them in mitosis. Cells were collected by mitotic shake-off and plated in media containing nocodazole and 10 μM MG132. For inhibitor treatments, either DMSO, 100 nM Bi2536, 2 μM ZM447439 or both inhibitors were added to the media for 1 hr. Cells were collected by mitotic shake-off and processed for Western blot analysis. For expression of LAP-hHaspin constructs in interphase, cells were grown in 2 mM thymidine for 18 hr, released into fresh media for 9 hr, then placed in media containing thymidine and 25 ng/ml doxycyclin for 24 hr. Cells were collected by trypsinization and processed for Western blot analysis.

Immunofluorescence

Cells were harvested by mitotic shake-off and resuspended in ice-cold PBS. Cells were placed on poly-L-lysine coated slides by cytocentrifugation (Stat Spin Cytofuge 2) for 4 min at 850RPM and fixed for 10 min in 2% PFA in PBS pH7.7. After rinsing in PBS, cells were permeabilized for 10 min in 0.5% Triton, rinsed twice for 5 min in PBS, then blocked for 1 hr in blocking solution (10% FBS, 10 mM Tris pH 8, 120 mM KCl, 20 mM $MgCl_2$, 0.5 mM EDTA). Cells were incubated with primary antibody for 1 hr in blocking solution with 0.1% triton X-100, rinsed three times in wash buffer (10 mM Tris pH 8, 120 mM KCl, 20 mM $MgCl_2$, 0.5 mM EDTA, 0.1% triton X-100), then incubated with secondary for 30 min in blocking solution with 0.1% triton X-100. After rinsing in wash buffer three times followed by a five-min rinse in PBS, cells were mounted with 6 μl Vectashield with DAPI. Coverslips were sealed with nailpolish and slides were stored at 4°C.

Peptides and Bead pulldowns. Biotinylated peptides corresponding to the xHaspin HBIS (biotin- HWLRLRAALSLHRKKKVQATD) or a scrambled version of the sequence (biotin- ARDQKLWSKARTHVAHLKLLR) were synthesized by the Rockefeller University Proteomics Resource Center. Peptides were >95% pure by analytical HPLC and ESI MS.

Peptide beads were prepared similar to (Kelly et al., 2010) but with the following modifications. Streptavidin-coated magnetic beads (Invitrogen) were washed three times in PBS + 0.01% triton, resuspended in PBS, then incubated for 45 min at RT

without peptide or with 10-fold excess (2 nmol peptide/mg beads) of scrambled or HBIS peptide. Beads were rinsed five times in PBS +0.01% triton and three times in sperm dilution buffer.

Peptide bead pulldowns were performed similar to (Kelly et al., 2010). For each sample, 50-100 μl beads (100-200 pmol peptide) were incubated with 30 μl CSF extract for 60 min at 4 °C mixing every 20 min. An input sample was taken before placing the extract on a magnetic rack for 10 min. Extract was removed and beads were washed three times in an equal volume of bead wash buffer (20 mM HEPES pH 8, 250 mM NaCl, 0.05 % Igpal CA-630, 10% glycerol, 0.1 mM TCEP, 0.5 mM PMSF, 10 ng/ml LPC) with phosphatase inhibitors (1 mM β-glycerophosphate, 1x phosphatase inhibitor cocktail from Sigma) and twice in plain wash buffer. All wash steps were performed at 4 °C. Beads and inputs were treated with lambda phosphatase for 45 min at 30 °C. Beads were then mixed with 4X SDS loading buffer and denatured at 100 °C for 5 min before running on a 7.5-15 % SDS-PAGE gel. Peptides and streptavidin were visualized by Coomassie.

Mass spectrometry

LC-MS/MS analysis on xHaspin fragments phospshorylated in metaphase egg extracts and in vitro with Plk1 and Cdk1-Cyclin B was performed by the Rockefeller University Proteomics Resource Center. LC-MS/MS analysis on full-length xHaspin-GFP constructs was performed by MS Bioworks (Ann Arbor, MI).

LC-MS/MS analysis on xHaspin fragments phospshorylated in metaphase egg extracts and in vitro with Plk1 and Cdk1-Cyclin B, performed by the Rockefeller University Proteomics Resource Center

Protein in-solution was reduced (Dithiothreitol) and alkylated (Iodoacetamide) prior to trypsination (Promega trypsin, Promega, Madison, WI). Generated peptides was desalted and concentrated (Rappsilber et al., 2007) prior to analysis by nano LC-MS/MS using an Q-Exactive (Thermo, San Jose, CA) mass spectrometer. MS/MS data were extracted using ProteomeDiscoverer v. 1.4 (Thermo, Bremen, Germany) and queried against a data base, containing xHaspin, common observed contaminates and background proteome (5,177 sequences), using MASCOT 2.3 (Matrixscience, London, UK). Mass tolerance of 20 ppm and 20 mDa were used for peptide precursors and peptide fragments, respectively. For peptide spectrum matches the median precursor mass accuracy was 0.46 ppm. Phosphorylations of serine, threonine and tyrosine were allowed as variable modifications together with oxidized methionine. All cysteines were treated as being iodoaceateamidated.

For the identification and validation of phosphopeptides the following strategy was followed: Phosphopeptide were not enriched prior to LC-MS/MS analysis which allowed for 1) validation of lower quality phosphopeptide spectra by comparison to high quality spectra of corresponding non-phosphorylated peptides (Supplementary Figure 1) and 2) an approximate ranking of the phosphorylated peptides based on phosphorylation level. The latter was achieved by comparing the ratio of area under curve for the phosphorylated peptide and the corresponding non-phosphorylated

peptides. Differences in ionization of phosphorylated versus non-phosphorylated peptide was not taken into account.

For peptides containing multiple potential phosphorylation sites phosphoRS was used to calculate probability for the different residues (Olsen et al., 2006).

LC-MS/MS analysis on full-length xHaspin-GFP constructs, performed by MS Bioworks (Ann Arbor, MI)

In gel digestion was performed using a ProGest robot (DigiLab) with the following protocol: 1) Washed with 25 mM ammonium bicarbonate followed by acetonitrile. 2) Reducedwith10 mMdithiothreitol at 60°C followed by alkylation with 50 mM iodoacetamide at RT. 3) Digested with sequencing grade trypsin (Promega) at 37°C for 4h. 4) Quenched with formic acid and the supernatant was analyzed directly without further processing.

The sample was analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive mass spectrometer. 30 µl of sample was loaded on a trapping column and eluted over a 75 µm analytical column at 350 nl/min; both columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data- dependent mode, with MS performed at 70,000 FWHM resolution and MS/MS performed at 17,500 FWHM. The fifteen most abundant ions were selected for MS/MS.

Data were searched using a local copy of Mascot with the following parameters:

Enzyme: Trypsin Database: NCBI *Xenopus laevis* (Appended with common

contaminants, reversed and concatenated) Fixed modification: Carbamidomethyl (C) Variable modifications: Oxidation (M), Acetyl (N-term), Pyro-Glu (N-term Q), Deamidation (N,Q), Phospho (S,T,Y) Mass values: Monoisotopic Peptide Mass Tolerance: 10 ppm Fragment Mass Tolerance: 0.015 Da Max Missed Cleavages: 2

Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a non-redundant list per sample. For protein identification the data were filtered using a minimum protein value of 80%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein. For phospho-site identification only one unique peptide was required.

Supplemental References

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