Supplementary legends:

Supplementary figure 1. Structure of the Haspin inhibitor 5-ITu

Supplementary figure 2. Haspin inhibition upon 5-ITu treatment

HeLa cells were arrested in mitosis for 16 hours with nocodazole (0.33 µM) and successively 10 µM MG132 was added for 15 min to prevent mitotic exit. The cells were treated with increasing concentration of 5-ITu to hinder Haspin kinase activity. After 1,5 hours the mitotic cells were processed for immunofluorescence. The cells were stained with DAPI (DNA, blue), CREST sera (kinetochores, red), H3Thr³ phosphorylation (green)

Supplementary figure 3. Haspin consensus motif matches amino acid sequence around Histone H3Thr³.

Haspin consensus motif matches the amino acid sequence around Thr³ of the histone H3. Spot intensities from oriented peptide library screening blots were quantified and used to generate the Haspin motif logo. Hydrophilic residues are in blue, neutral in green and hydrophobic in black

Supplementary figure 4. Crystal structure of Haspin kinase domain in complex with Histone H3 tail. Shown are details of the substrate interaction with the catalytic domain.
Supplementary figure 5. *In vitro* kinase reaction with predicted Haspin peptides substrates.

*In vitro* kinase reaction with predicted Haspin peptides substrates. The NetPhorest algorithm was used to predict Haspin substrates. 101 peptides substrates were chemically synthetized and tested for *in vitro* kinase reaction with Haspin and identified by LC-MS/MS. The spectra of the 35 phosphorylation sites identified with the highest phosphorylation sites localization probability (PTMprophet probability=1) are presented.

Supplementary figure 6. Sequence alignments of the four Haspin *in vitro* phosphorylation substrate sites on CENP-T.

Sequence alignments of the four Haspin *in vitro* phosphorylation substrate sites on CENP-T. The four sites present all an Arg residue in position -1. Basic residues are colored blue, polar residues in green, hydrophobic residues in black.

Supplementary figure 7. Annotated consensus fragmentation spectra of the phosphorylated peptides identified in this study.

Supplementary table 1. Peptides identified by LC-MS/MS of mitotic chromatin proteins.

Mitotic chromatin samples were digested with trypsin and successively the phosphorylated peptide were enriched by titanium dioxide chromatography and identified by LC-MS/MS.
**Supplementary table 2. Peptides quantified upon Haspin inhibition treatment of mitotic cells.**

Precursor ion intensities for the quantification of phosphopeptides were extracted using openMS. Statistically regulated peptide were determined using calculated by a linear model using the publicly available R-based Limma package (80). The calculated P-values were adjusted for multiple comparisons using Benamini and Hochberg correction.

**Supplementary table 3. Gene Ontology enriched terms within the regulated phosphoproteins upon Haspin inhibition treatment.**

The enrichment of gene ontology terms within the subset of regulated peptides was determined using the annotation tools GOrilla (23).

**Supplementary table 4. Kinase target predictions within the downregulated phosphorylation sites upon Haspin inhibition.**

Kinase targets were predicted using the iGPS (28) and NetworKin (29) algorithms. Enrichment tests used the hypergeometric distribution(15). P value cutoff for enrichment is set at $5 \times 10^{-4}$.

**Supplementary table 5. Aurora kinase substrate predictions.**

Aurora substrates downregulated upon Haspin inhibition.

**Supplementary table 6. CLK substrate predictions.**
CLK substrates downregulated upon Haspin inhibition.

Supplementary table 7. RSK kinase substrate predictions.
RSK substrates downregulated upon Haspin inhibition.

Supplementary table 8. Data collection and refinement statistics of the Haspin-HistoneH3 tail crystal structure.

Supplementary table 9. Predicted Haspin substrates within the downregulated phosphorylation sites on chromatin proteins.
NetPhorest predictions of Haspin substrates on chromatin proteins, the results were filtered based on probability higher than 0.1, which led a false positive rate (FPR) of the Haspin substrate predictions lower than 10%.

Supplementary table 10. NetPhorest prediction of Haspin substrate phosphorylation sites identified on Human proteins.
NetPhorest predictions of Haspin substrates in the SwissProt database, the results were filtered based on probability higher than 0.1, which led a false positive rate (FPR) of the Haspin substrate predictions lower than 10%.

Supplementary table 11. Unphosphorylated peptide sequence used as substrates for the in vitro kinase reaction with Haspin.
Peptides selected for in vitro kinases reaction with Haspin kinase.
**Supplementary table 12. Phosphorylated peptides identified by LC-MS/MS after in vitro kinase reaction with Haspin.**

Peptides phosphorylated *in vitro* by Haspin were enriched using immobilized metal affinity chromatography and identified by LC-MS/MS.

**Supplementary table 13. List of the confident Haspin interacting partners.**

Haspin was expressed in HEK-293 cells as tagged bait and purified using tandem affinity chromatography approach. The copurified proteins were analysed by LC-MS/MS and the results were filtered to determine the most confident Haspin interacting partners.

**Supplementary table 14. Phosphorylation sites on Haspin interacting proteins quantified upon Haspin inhibition treatment.**

**Supplementary table 15. Raw intensities measured for each of the phosphorylated peptides quantified in this study.**