Hsp72 is targeted to the mitotic spindle by Nek6 to promote K-fiber assembly and mitotic progression

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Hsp70 proteins represent a family of chaperones that regulate cellular homeostasis and are required for cancer cell survival. However, their function and regulation in mitosis remain unknown. In this paper, we show that the major inducible cytoplasmic Hsp70 isoform, Hsp72, is required for assembly of a robust bipolar spindle capable of efficient chromosome congression. Mechanistically, Hsp72 associates with the K-fiber-stabilizing proteins, ch-TOG and TACC3, and promotes their interaction with each other and recruitment to spindle microtubules (MTs). Targeting of Hsp72 to the mitotic spindle is dependent on phosphorylation at Thr-66 within its nucleotide-binding domain by the Nek6 kinase. Phosphorylated Hsp72 concentrates on spindle poles and sites of MT–kinetochore attachment. A phosphomimetic Hsp72 mutant rescued defects in K-fiber assembly, ch-TOG/TACC3 recruitment and mitotic progression that also resulted from Nek6 depletion. We therefore propose that Nek6 facilitates association of Hsp72 with the mitotic spindle, where it promotes stable K-fiber assembly through recruitment of the ch-TOG–TACC3 complex.

Introduction

Heat shock proteins (HSPs) are molecular chaperones that use ATP hydrolysis to aid the folding of nascent polypeptides, maintain proteins in unstable conformations, and prevent protein denaturation. These functions are essential in many biological contexts, including assembly of macromolecular complexes, protein trafficking, and regulation of enzyme activity (Bukau et al., 2006). HSPs are particularly important in cells subject to proteotoxic stress and are attracting considerable interest as potential targets for cancer therapy (Powers and Workman, 2007; Jego et al., 2013). The Hsp70 proteins represent a major family of HSPs that are frequently overexpressed in human cancers (Rohde et al., 2005; Daugaard et al., 2007; Kampina and Craig, 2010). Their overexpression correlates with poor prognosis and drug resistance, whereas blocking Hsp70 function gives a therapeutic response in tumor models (Nylandsted et al., 2000; Schmitt et al., 2006; Leu et al., 2009; Massey et al., 2010; Powers et al., 2010; Rérole et al., 2011; Balaburski et al., 2013; Murphy, 2013).

In humans, there are eight canonical members of the Hsp70 family, as well as more distantly related members such as Hsp110 (Rohde et al., 2005; Daugaard et al., 2007). Some are expressed in a constitutive manner, such as Hsc70 (encoded by the HSPA1A gene), whereas others are induced upon stress, such as Hsp72 (encoded by the HSPA1A gene). Rapidly dividing cancer cells frequently express high levels of both Hsc70 and Hsp72 as a result of oncogenic stress. Although some Hsp70 proteins are restricted to membranous compartments, such as Grp78/BiP in the endoplasmic reticulum and Grp75/mortalin in the mitochondria, Hsc70 and Hsp72 are present throughout the cytoplasm and nucleus. Hsp70 proteins also associate with the microtubule (MT) cytoskeleton, including the mitotic spindle, although to date, their only described function at this site is in protecting spindle pole integrity after heat shock (Liang and MacRae, 1997; Mack and Compton, 2001; Hut et al., 2005; Sauer et al., 2005; Elsing et al., 2014).

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Abbreviations used in this paper: HSP, heat shock protein; IF, immunofluorescence; IP, immunoprecipitate; KESTREL, kinase substrate-tracking and elucidation; LSB, low salt buffer; MT, microtubule; NEBD, nuclear envelope breakdown.

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Results and discussion

Hsp72 is a novel mitotic substrate of the Nek6 kinase

Several members of the NEK protein kinase family contribute to mitotic progression (O’Connell et al., 2003; Quarmby and Mahjoub, 2005; Moniz et al., 2011; Fry et al., 2012). One of these is Nek6, which plays an essential role in spindle assembly and cytokinesis (Yin et al., 2003; O’Regan and Fry, 2009). Phosphorylation by Nek6 targets the Eg5/Kif11 motor protein to spindle MTs to promote centrosome separation (Rapley et al., 2008). However, this alone is unlikely to explain the fragile spindles and mitotic arrest that arise from blocking Nek6 function. Here, we show that Hsp72 is a novel mitotic substrate of Nek6 and that together these proteins play an essential role in assembly of robust mitotic spindles capable of efficient chromosome congression through K-fiber (kinetochore fiber) recruitment of the ch-TOG (colonic and hepatic tumor overexpressed protein) and TACC3 complex.

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Figure 1. Nek6 interacts with and phosphorylates Hsp72. (A) KESTREL analysis of HEK 293 cytosolic extracts. Substrate-containing Superdex 200 fractions 3–7 were pooled, incubated with or without Nek6 and \( \gamma^{[32P]}ATP \), and analyzed by Coomassie blue stain (CB) and autoradiography (\( 32P \)). (B) Flag IPs were prepared from HEK 293 cells transfected with Flag-Nek6 and synchronized in S or M. As controls, asynchronous cells (As) transfected with Flag-Nek6 were subject to IP with rabbit IgGs or no antibodies (Ab). IPs were incubated with \( \gamma^{[32P]}ATP \) before analysis by Coomassie blue stain and autoradiography (\( 32P \)). Lines have been added to top blots to indicate where intervening lanes were spliced out for presentation purposes. In A and B, molecular masses (kilodaltons) and proteins identified by mass spectrometry are indicated. (C) HEK 293 cells were transfected with wild-type (WT) or catalytically inactive (K75M and K64M) Flag-Nek6 or -Nek7 for 24 h before synchronization in S or M. Flag IPs were analyzed by Western blotting with the antibodies indicated. (D) Lysates (inputs), Hsp72 IPs, and IPs prepared with rabbit IgGs from asynchronous, S-phase, or M-phase arrested HEK 293 cells were analyzed by Western blotting with the antibodies indicated. (E) Schematic representation of Nek7, Nek6, and Nek6-\( \Delta \)NTE. N, N terminus; C, C terminus. (F) In vitro kinase assays using purified wild-type Nek6, Nek6-\( \Delta \)NTE, or no kinase with \( \beta \)-casein and Hsp72 protein substrates in the presence of \( \gamma^{[32P]}ATP \). (G) Substrate phosphorylation was quantified by scintillation counting of dried gels shown in F. Error bars show means ± SD.
Hsp72, identified using antibodies that do not cross-react with Hsc70 (Fig. S2 A), coprecipitated with wild-type and catalytically inactive Nek6 from S- and M-phase cells but not with the closely related Nek7 kinase (Fig. 1 C). In contrast, both Nek6 and Nek7 coprecipitated with their upstream activator, Nek9 (Roig et al., 2002).

Endogenous Nek6, but not Nek7, also coprecipitated with Hsp72, indicating that this interaction was not a result of Nek6 overexpression. Indeed, Nek6 coprecipitated more efficiently from cells arrested in M than S phase (Fig. 1 D). As Nek6 and Nek7 differ significantly only in the short (∼30–40 amino acid) extensions N-terminal to the catalytic domains, a Nek6 truncation mutant lacking this N-terminal extension (Nek6-ΔNTE) was generated (Fig. 1 E). Although retaining significant activity against β-casein, truncated Nek6 barely phosphorylated Hsp72 (Fig. 1, F and G). This agrees with previous suggestions that the N-terminal extensions of these two kinases dictate their substrate specificity at least in the context of the full-length proteins (Richards et al., 2009; Vaz Meirelles et al., 2010). It also confirms that Nek6 and Nek7 almost certainly play distinct roles in cell division (O’Regan and Fry, 2009).

Hsp72 is required for spindle assembly and chromosome congression

To determine what function Hsp72 may have in mitotic progression in the absence of heat shock, it was depleted for 72 h with two independent siRNAs (Fig. S2 B) or inhibited for 4 h with the ATP-competitive small molecule, VER-155008 (Massey et al., 2010). HeLa cells were used for these experiments as they express high levels of Hsp72 (Rohde et al., 2005). Time-lapse imaging of EGFP-lamin A and H2B-mCherry revealed that depletion or inhibition of Hsp72 did not interfere with...
orientation from the horizontal plane were also indicative of defective spindle–cortex attachment (Fig. 2, D, F, and G). Addition of the Hsp70 inhibitor to preformed spindles assembled in MG132-arrested mitotic cells led to release of chromosomes from the metaphase plate, indicating that Hsp70 is required to maintain, as well as establish, chromosome alignment (Fig. 2 H and Videos 4 and 5). Staining for the spindle assembly checkpoint protein, BubR1, revealed intense kinetochore staining upon either depletion or inhibition of Hsp72 (Fig. S2, D and E). Hence, Hsp72 is essential for HeLa cells to generate a robust mitotic spindle capable of cortical attachment, chromosome congression, and satisfaction of the spindle assembly checkpoint. Although the phenotypes of Hsp72 depletion and Hsp70 inhibition were remarkably similar, some differences were noted (e.g., more consistent displacement of spindle orientation from the horizontal plane were also indicative of defective spindle–cortex attachment (Fig. 2, D, F, and G). Ad-

Figure 3. Hsp72 promotes recruitment of ch-TOG–TACC3 complexes to K-fibers. [A] HeLa cells treated as indicated were placed on ice for 10 min before IF with α-tubulin and CENP-A antibodies to reveal K-fibers. [B] K-fiber intensity is plotted relative to that in mock-treated cells. [C] HeLa cells treated as indicated were incubated with 50 µM monastrol for 4 h, placed on ice for 10 min, and then processed for IF with α-tubulin and CENP-A antibodies. [D] The length of K-fibers in the monopolar spindles is plotted. For box and whisker plots, boxes represent the 25th and 75th percentile, the green and white lines represent the medians and means, respectively, and whiskers show the 10th and 90th percentiles. [E and F] HeLa cells treated as indicated were processed for IF with antibodies against α-tubulin and ch-TOG [E] or TACC3 [F]. [G and H] The intensity of ch-TOG [G] and TACC3 [H] relative to tubulin from cells in E and F is indicated. (I) The amount of proteins precipitated in J is quantified relative to that in DMSO-treated Hsp70 IPs. [J] Flag IPs prepared from mitotic cells induced to express Flag-TACC3 with doxycycline and treated with Hsp70i were analyzed by Western blotting with the antibodies indicated. [K] The amount of proteins precipitated in L is quantified relative to DMSO-treated Flag IPs. In A, C, E, and F, DNA was stained with Hoechst 33258. Data are means ± SD of 100–300 cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bars, 10 µm.

chromatin condensation or nuclear envelope breakdown (NEBD). However, it strongly perturbed chromosome congression and anaphase onset (Fig. 2 A, Fig. S2 C, and Videos 1–3). In some cells, chromosomes rapidly detached from the metaphase plate, whereas in others, chromosomes failed to congress. Quantification confirmed that depletion or inhibition of Hsp72 extended the time in prometaphase by delaying the time from NEBD to last chromosome congressed and, in metaphase, by delaying the time from last chromosome congressed to anaphase onset (Fig. 2, B and C).

Staining of fixed cells with α-tubulin and CENP-A antibodies after either depletion or inhibition of Hsp72 revealed the presence of poorly organized spindles with reduced MT density (Fig. 2, D and E). A shortened interpolar distance, fewer astral MTs in the vicinity of poles and displacement of spindle orientation from the horizontal plane were also indicative of defective spindle–cortex attachment (Fig. 2, D, F, and G). Addition of the Hsp70 inhibitor to preformed spindles assembled in MG132-arrested mitotic cells led to release of chromosomes from the metaphase plate, indicating that Hsp70 is required to maintain, as well as establish, chromosome alignment (Fig. 2 H and Videos 4 and 5). Staining for the spindle assembly checkpoint protein, BubR1, revealed intense kinetochore staining upon either depletion or inhibition of Hsp72 (Fig. 2, D and E). Hence, Hsp72 is essential for HeLa cells to generate a robust mitotic spindle capable of cortical attachment, chromosome congression, and satisfaction of the spindle assembly checkpoint. Although the phenotypes of Hsp72 depletion and Hsp70 inhibition were remarkably similar, some differences were noted (e.g., more consistent displacement of spindle orientation in...
control of cancer cell division. HSP inhibitors are being developed as anticancer drugs with Hsp90 inhibitors in advanced clinical trials.
Figure 4. Nek6 phosphorylation targets Hsp72 to the mitotic spindle. (A) Schematic representation of Hsp72 showing nucleotide (NBD) and substrate (SBD) binding domains and sequence alignment of the human Hsp70 family around threonine-66. Bold indicates conserved residues. N, N terminus; C, C terminus. (B) Purified Hsp72 wild-type (WT) or T66A proteins incubated with or without purified Nek6 were subjected to Western blotting with total Hsp70 and Hsp70-pT66 (pHsp70) antibodies. (C) HEK 293 cells were either untransfected (lane 1) or transfected with Flag-Hsp72 wild-type (lanes 2–4) and left asynchronous (As) or synchronized at S or M before immunoprecipitation with Flag antibodies. Lysates and Flag IPs were analyzed by Western blotting with Flag and pHsp70 antibodies. (D) Lysates and Hsp72 IPs from HEK 293 cells that were either asynchronous or synchronized in different cell cycle stages were analyzed by Western blotting with Flag and pHsp70 antibodies. (E) Lysates and Hsp72 IPs from HEK 293 cells transfected with Nek6, Nek7, or GAPDH siRNAs were analyzed by Western blotting as indicated. (F) HeLa cells were processed for IF with α-tubulin and Hsp72 antibodies. (G) HEK 293 cells transfected with GFP-tagged wild-type and catalytically inactive (K71E) Hsp72 were analyzed by IF with α-tubulin (red) and GFP (green) antibodies. (H and I) HeLa cells were processed for IF with pHsp70 and α-tubulin, CenpA, or CenpE antibodies, as indicated. (J) HeLa cells were mock- or Nek6-depleted before IF with Hsp72 and α-tubulin antibodies. (K) The intensity of Hsp72 at metaphase spindle poles was quantified relative to α-tubulin. In F–J, DNA was stained with Hoechst 33258 (blue). siMock, mock siRNA. Bars: (F–I [left images] and J) 10 µm; (I, right images) 0.5 µm.
Figure 5. **Hsp72-T66E rescues mitotic defects that arise upon Nek6 depletion.** (A) HeLa cells were mock or Nek6 depleted for 48 h before transfection with Flag-Hsp72 constructs as indicated for 24 h and IF with Flag and phospho-H3 antibodies. The mitotic index of transfected cells was counted by microscopic analysis of chromatin. (B) HeLa cells transfected with Flag-Hsp72 constructs indicated for 24 h were stained with Flag and α-tubulin antibodies and scored by IF for the occurrence of nuclear defects. Data in A and B are means ±SD of 100–300 cells. (C) HeLa cells were treated as in A, placed on ice for 10 min, and analyzed by IF with antibodies against α-tubulin to reveal K-fibers (green) and Flag to detect transfected cells. (D) Total K-fiber intensity from C is plotted relative to that in mock-treated samples. (E and F) HeLa cells were treated and processed as in C but with ch-TOG (E) and TACC3 (F) antibodies. (G and H) Total ch-TOG and TACC3 intensity from E and F is plotted relative to that in mock-treated samples. Error bars show means ± SD. In C, E, and F, DNA was stained with Hoechst 33258 [blue]. siMock, mock siRNA; WT, wild type. *, P < 0.05. Bars, 10 µm.
(Neckers and Workman, 2012). Hsp70 works in concert with Hsp70, and dual depletion of Hsc70 and Hsp72 blocks not only Hsp90 function but also the Hsp70-mediated inhibition of apoptosis that can otherwise confer resistance to Hsp90 inhibitors (Powers et al., 2008). Hence, considerable effort is now being put into development of Hsp70 inhibitors (Lee et al., 2009; Patury et al., 2009; Massey et al., 2010; Powers et al., 2010; Balaburski et al., 2013; Budina-Kolometes et al., 2014). Non-cancer-derived cells tend to have reduced transcription of Hsp72 in mitosis (Martinez-Balbás et al., 1995). This results from HSF2 (heat shock factor 2) acting as a transcriptional repressor in mitosis, blocking expression of Hsp72 by HSF1. However, in some cancer cells, HSF2 is repressed in mitosis, allowing HSF1 to maintain elevated Hsp72 (Elsing et al., 2014). The dependence on Hsp72 that this implies may provide a therapeutic window for targeted inhibitors in these cancer types. Hsp72 is a novel mitotic substrate of Nek6, and there is growing support for Nek6 as an anticancer target (Capra et al., 2006; Chen et al., 2006; Takeno et al., 2008; Nassirpour et al., 2010). The efficacy of Hsp70 inhibitors, both as single agents and in combination with Hsp90 or Nek6 inhibitors, will be exciting to test in different cancer settings.

Materials and methods

KESTREL analysis

600 confluent 15-cm dishes of HEK 293 cells were collected, washed in PBS, and lysed in 50 mM Tris.HCl, pH 7.5, 5% glycerol, 14 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM Pefabloc. Insoluble material was sedimented by centrifugation at 15,000 g for 25 min at 4°C. The supernatant was filtered and degassed and stored at −80°C. Buffer was exchanged into MOPS−low salt buffer (LSB; 30 mM MOPS, pH 7.0, 5% glycerol, 7 mM 2-mercaptoethanol, and 0.03% Brij) by chromatography over a 480-ml Sephadex G-25 fine column. The desalted extract was applied to a 25-ml Heparin Sepharose High Performance column. The column was washed in 125 ml MOPS-LSB and then eluted over a 400-ml gradient to 1 M NaCl in MOPS-LSB while 40 10-ml fractions were collected. 20 µl of each fraction were diluted 1:10 in KESTREL test buffer (50 mM Tris.HCl, pH 7.5, 7 mM 2-mercaptoethanol, 1 mM EGTA, 10 µg/ml leupeptin, and 1 mM Pefabloc) and incubated for 5 min with 3 mM MnCl2 and 1 kBq/vial of [32P]-ATP in the absence or presence of 1 µg/ml active recombinant Nek6 (Invitrogen). Reaction products were analyzed by SDS-PAGE, transfer to Immobilon P (EMD Millipore), and subsequent autoradiography. Heparin Sepharose fractions 16−18 contained potential Nek6 substrates and so were pooled, desalted, separated along a 10-ml gradient to 1 M NaCl on a 1-ml Source 15Q column, and analyzed for potential Nek6 substrates as described in the previous paragraph. Substrate containing Source 15Q fractions 16−18 were pooled, separated by size using a 120 µl Superdex 200 column, and analyzed as in this paragraph. Superdex 200 fractions 3−7 were pooled, concentrated by filtration, and incubated with either 3 mM MnCl2 or 10 mM Mg-EGTA in the presence of γ32P-ATP, with or without 1 µg/ml Nek6. The reactions were denatured, alkylated with 50 mM iodoacetamide, and separated by SDS-PAGE and the gels were stained with colloidal Coomassie (Invitrogen) and subsequently analyzed by autoradiography. Protein bands that were visible with Colloidal Coomassie staining and had also radiolabeled in the presence of Nek6 were excised from the gels, destained, digested with trypsin, and subjected to mass spectrometry fingerprinting. Matrix-assisted laser desorption/ionization–tandem mass spectrometry (MALDI-TOF/TOF) analysis was performed using a Quadrupole Time-of-Flight mass spectrometer (Bruker Daltonics) for acquisition and the Mascot search engine (Matrix Science) to search for matches in the NCBI nr database.

Plasmid construction, mutagenesis, and recombinant protein expression

Full-length cDNAs expressing human Hsp72 and Hsc70, provided by R. van Montfort (Institute of Cancer Research, Sutton, England, UK), were amplified by PCR and inserted into pLEICS-12 for expression with an N-terminal Flag tag from a constitutive cytomegalovirus promoter in mammary cells. They were then subcloned into the pTWO-E plasmid, expressed as His fusions from a T7 promoter, and purified on 5-ml HiTrap columns (GE Healthcare). Columns were washed with 50 mM imidazole, and protein was eluted on a gradient of 50–500 mM imidazole. The eluted protein was dialyzed against 20 mM Tris, pH 8.0, 100 mM NaCl, and 5 mM β-mercaptoethanol and passed over a 5-ml Q Sepharose column to remove contaminants by the Escherichia coli Hsp70 homologue Dnak. The Q column flow-through was dialyzed into 100 mM Tris, pH 7.5, 150 mM NaCl, 6 mM MgCl2, and 10% glycerol and concentrated to 5 mg/ml. The absence of Dnak from Hsp70 samples was confirmed by Western blotting with SPA-880 mouse antibody (Enzo Life Sciences). Flag-Nek6 and Flag-Nek7 plasmids are as previously described (O’Regan and Fry, 2009). In brief, full-length cDNAs of Nek6 and Nek7 were subcloned into the pFLAG-CMV2 vector as PCR fragments using NotI and XbaI sites. Flag-TACC3 was inserted in p2eThyHyg by subcloning a Flag-TACC3 PCR fragment with Nhel and Not sites. Mutations were introduced using the GeneTailer Site-Directed Mutagenesis System (Invitrogen), and all constructs were confirmed by Sanger sequencing.

Cell culture, synchronization, and transfection

HeLa and HEK 293 cells were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO2 atmosphere. For in vivo induction of expression of Flag-TACC3, TetOn HeLa cells (Takara Bio Inc.) were used. TetOn HeLa cells with stably integrated pTRE2hyg-Flag-TACC3 plasmid were maintained with 300 µg/ml G418 and 200 µg/ml Hygromycin B. Expression of Flag-TACC3 was induced with 0.5 µg/ml doxycycline, 24 h before analysis. Cells were synchronized in S phase by incubation for 16 h with either 1 mM hydroxyurea or 2 mM thymidine. Mphase arrested cells were prepared by shake-off after 16 h treatment with either 50 ng/ml nocodazole or 100 µM monastrol. For G1 cells, a mitotic population was collected as described for M phase–arrested cells and replaced into fresh medium for 6 h. G2 cells were obtained by placing thymidine-treated cells into fresh medium for 8 h. Synchronization was confirmed by flow cytometry. To re-veal K-fibers, synchronized cells were replaced into chilled media and incubated on ice for 10 min before methanol fixation. For IPs from mitotic cells, nocodazole was washed out, and cells were replaced into fresh media for 45 min to allow spindle MTs to form before cell lysis. Transient transfections were performed with FuGENE HD reagent (Promega) according to manufacturer’s instructions, and cells were transfected for 24 h, unless stated otherwise. To inhibit Hsp70, cells were treated with 5 µM VER-155008 (Tocris Bioscience) for 4 h; control cells were treated with the same volume of DMSO. To depolymerize MTs, cells were incubated with 3 µM nocodazole for 4 h. HeLa-EGFP-LaminA/mCherry-H2B cells were provided by Kleiman (European Molecular Biology Laboratory, Heidelberg, Germany) and grown in DMEM supplemented with 10% FBS, 500 µg/ml G418, and 0.5 µg/ml puromycin.

Fixed and live-cell microscopy

Cells grown on acid-etched glass coverslips were fixed with ice-cold methanol and processed as previously described (O’Regan and Fry, 2009). In brief, media were aspirated, and cells were fixed by incubation in ice-cold methanol at −20°C for a minimum of 10 min. Cells were then blocked in PBS supplemented with 3% BSA before incubation with the appropriate antibody diluted as required in PBS supplemented with 3% BSA. For Hsp72 staining, cells were preextracted for 30 s with 60 mM Pipes, 25 mM Hepes, pH 7.4, 10 mM EGTA, 2 mM MgCl2, and 1% Triton X-100 before methanol fixation, whereas for ch-TG and TACC3 staining, cells were fixed in 3.7% formaldehyde for 30 min. Cells were permeabilized with 0.5% Triton X-100. Primary antibodies were 1 µg/ml mouse Hsp72 (Enzo Life Sciences), 0.3 µg/ml mouse α-tubulin (Sigma-Aldrich), 2 µg/ml rabbit α-tubulin (Abcam), 0.5 µg/ml mouse Flag (Sigma-Aldrich), 2 µg/ml mouse phosho–histone H3 (EMD Millipore), 2 µg/ml mouse CenpA (Abcam), 1 µg/ml rabbit ch-TG (Bethyl Laboratories, Inc.), 2 µg/ml mouse TACC3 (Abcam), 1 µg/ml mouse BubR1 (EMD Millipore), 0.5 µg/ml rabbit GFP (Abcam), 1 µg/ml mouse α-tubulin (Santa Cruz Biotechnology, Inc.), 2 µg/ml rabbit Nek6 (O’Regan and Fry, 2009), and 1 µg/ml rabbit pHsp70 (this study). Nek6 antibodies were raised against a peptide corresponding to amino acid 1–15 conjugated to keyhole limpet hemocyanin via a N-terminal cysteine (CMAGQPHGMPHGGSSN), whereas Hsp70 antibodies were raised against a phosphopeptide corresponding to amino acid residues 60–72 conjugated to Neuraminidase A (ALNPQpVpVDFAKRC). Secondary antibodies were used Alexa Fluor 488 and 594 goat anti-rabbit and goat anti-mouse IgGs (1 µg/ml; Invitrogen).
Imaging was performed on a confocal microscope (TCS SP5; Leica) equipped with an inverted microscope (DM6000 B; Leica) using a 63x oil objective (numerical aperture, 1.4). Z stacks comprising 30–50 0.3-µm sections were acquired using LAS-AF software (Leica), and deconvolution of 3D image stacks were performed using Huygens software (Scientific Volume Imaging). To quantify spindle and K-fiber MIs, the mean pixel intensity of the α-tubulin antibody signal over a half-spindle was measured, and cytoplasmic background was subtracted. Intensities were scaled so that the control intensity was 100%. To quantify recruitment of TACC3 and ch-TOG, the mean pixel intensity over a half-spindle was measured in both the spindle and TACC3 or ch-TOG channels, and cytoplasmic background was subtracted. The intensity of TACC3 or ch-TOG was then normalized to the spindle intensity.

Time lapse imaging was performed on a confocal microscope (TCS SP5) equipped with an inverted microscope (DM6000 B) using a 63x oil objective (numerical aperture, 1.4). Cells were cultured in glass-bottomed dishes (MatTek Corporation) and maintained on the stage at 37°C in an atmosphere supplemented with 5% CO2 using a microscope temperature control system (Life Imaging Services). Z stacks comprising 20 0.5-µm sections were acquired every 10 min for 218 h using LAS-AF software. Stacks were converted into maximum intensity projections using LAS-AF software, and videos were prepared using ImageJ (National Institutes of Health).

**IP and kinase assays**

Cells were harvested by incubation with PBS + 0.5 mM EDTA and pelleted by centrifugation before being lysed in Nek extraction buffer (Fry and Nigg, 1997). Antibodies used to perform IPs were 2 µg/ml rabbit Nek6 (O’Regan and Fry, 2009), 0.5 µg/ml mouse FLAG (Sigma-Aldrich), or 2 µg/ml mouse Hsp72 (Enzo Life Sciences). Control IPs were performed with rabbit or mouse IgGs (Sigma-Aldrich), as appropriate, at the same concentration. Kinase assays were performed using either 5–10 µl of washed immune complex beads or 0.1 µg of purified kinase. Proteins were incubated with 5 µg of substrate and 1 µCi γ-[32P]ATP in 40 µl kinase buffer (50 mM HEPES-KOH, pH 7.4, 5 mM MgCl2, 5 mM β-glycerophosphate, 5 mM NaF, 4 µM ATP, and 1 mM DTT) at 30°C for 30 min. Reactions were stopped with 50 µl of protein sample buffer and analyzed by SDS-PAGE and autoradiography. Substrate phosphorylation was quantified by scintillation counting of proteins excised from dried gels. Proteins that coprecipitated with and were phosphorylated by Nek6 were excised from gels and subjected to mass spectrometry fingerprinting (University of Leicester, Leicester, England, UK).

**RNAi**

Cells at 30–40% confluency were cultured in Opti-MEM Reduced Serum Medium and transfected with 50 nM ON-TARGETplus siRNA duplexes using DharmaFECT (Dharmacon) according to the manufacturer’s instructions. siRNA duplexes were as follows: Hsp72, J005168-06, -07, and -08; Nek6, J004166-06 and J004166-09; Nek7, J003795-12 (all obtained from Thermo Fisher Scientific); and GAPDH (4390850; Life Technologies). Nek6, J-004166-06 and J-004166-09; Nek7, J-003795-12 (all obtained from Cell Signaling Technology); Nek9, J-005170-06; Nek9 (sulfite), J-005170-07 and -08 (all obtained from Thermo Fisher Scientific); and GAPDH (4390850; Life Technologies). 72 h after transfection, cells were either fixed for immunocytochemistry or prepared for IP, Western blot, or flow cytometry analysis.

**Phosphomapping and phosphoantibody generation**

For phosphomapping, Hsp72 purified from HEK293 extracts using the KESTREL strategy. Fig. S2 shows additional data relating to mitotic phenotypes associated with loss of Hsp72 activity. Fig. S3 shows mapping of the Nek6 phosphorylation site on Hsp72 and the validation of Hsp72pT66 antibody specificity when used for immunofluorescence (IF). Videos 1–3 show time-lapse imaging of chromosome congression in control, Hsp72-depleted, and Hsp70-treated cells, respectively. Videos 4 and 5 show time-lapse imaging of metaphase chromosome alignment in DMSO- or Hsp70-treated cells, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201409151/DC1.

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Figure S1. **Purification of Nek6 substrates using a KESTREL screen.** (A) Schematic of fractionation protocol used in KESTREL analysis. (B) Desalted HEK 293 cytosolic extract was chromatographed on heparin Sepharose. Aliquots of the fractions were incubated with Mnγ-[32P]ATP in the absence (−) or presence (+) of 1 µg/ml Nek6, denatured, and separated by SDS-PAGE. Gels were transferred to Immobilon P and analyzed by autoradiography. (C) Fractions 16–18 of the heparin Sepharose column were pooled, desalted, chromatographed on Source 15Q, and analyzed as in B. (D) Fractions 16–18 of the Source 15Q column were subjected to chromatography on a Superdex 200 column and analyzed as in B. M, molecular mass marker. ld, load.
Figure S2. Mitotic phenotypes associated with loss of Hsp72. (A) Purified Hsp72 and Hsc70 proteins were analyzed by Western blotting with the antibodies indicated to determine antibody specificity. (B) HeLa cells were either mock transfected or transfected with three distinct siRNAs directed against Hsp72 (1–3) or GAPDH for 72 h before lysates were analyzed by Western blotting with the antibodies indicated. siHsp72-2 was used in all experiments presented in this study unless indicated otherwise. (C) Time-lapse stills from videos with siHsp72-3 with the HeLa:GFP-lamin A/H2B-mCherry cell line. Time in minutes is indicated. (D) IF of HeLa cells that were mock depleted, depleted of Hsp72 for 72 h, or treated with Hsp70i for 4 h were stained with antibodies against α-tubulin and BubR1. (E) The relative intensity of BubR1 was quantified and plotted relative to that in control samples. Error bars are means ± SD. (F) HeLa cells were mock or Hsp72 depleted for 24 h before transfection with the Flag-tagged Hsp72 constructs indicated for 48 h. Cell lysates were then analyzed by Western blotting with the antibodies indicated. (G) HeLa cells were mock or Hsp72 depleted for 72 h and then analyzed by Western blotting with the antibodies indicated. (H) HeLa cells were mock or Hsp72 depleted for 72 h before IF with α-tubulin (green) and Hsp72 (red) antibodies. DNA was stained with Hoechst 33258. (I) Lysates of HeLa cells that were mock or Nek6 depleted for 48 h, before being transfected with the Flag-Hsp72 constructs indicated for 24 h, were analyzed by Western blotting with the antibodies indicated. siMock, mock siRNA; WT, wild type; WT-R, wild-type siRNA resistant. Bars, 10 µm.
Figure S3. Identification of Nek6 phosphorylation site on Hsp72. (A) Partially annotated negative precursor trace of unphosphorylated Hsp72 indicated as mass to charge ratio (m/z). Some phosphorylation on a contaminant CTP synthase 1 was detected. These data were obtained on two independent samples. (B) Purified Hsp72 was phosphorylated with Nek6 and analyzed as in A. The major new peptide (mass to charge ratio = 868.5) corresponds to Thr-66 (asterisk) of Hsp72. Hsp72 phosphomapping data were obtained on two independent samples. (C) Purified Hsp72 wild-type (WT) and T66A proteins incubated with Nek6 in the presence of γ-[\(^{32}\)P]ATP were analyzed by SDS-PAGE and autoradiography. CB, Coomassie blue. (D) IF images of mock or Hsp72-depleted HeLa cells stained with the antibodies indicated. DNA was stained with Hoechst 33258. siMock, mock siRNA. Bar, 10 µm.

Video 1. Time-lapse imaging of chromosome congression in mock-depleted cells. HeLa cells stably expressing EGFP-lamin A/H2B-mCherry were mock depleted and imaged by time-lapse confocal microscopy using a confocal laser-scanning microscope (TCS-SF5; Leica) as they progressed through mitosis. Z stacks comprising 20 0.5-µm steps were acquired every 10 min for 18 h.
Video 2. **Time-lapse imaging of chromosome congression in Hsp72-depleted cells.** HeLa cells stably expressing EGFP–lamin A/H2B-mCherry were depleted with siRNAs against Hsp72 and imaged by time-lapse confocal microscopy using a confocal laser-scanning microscope (TCS-SP5; Leica) as they progressed through mitosis. Z stacks comprising 20 0.5-µm steps were acquired every 10 min for 18 h.

Video 3. **Time-lapse imaging of chromosome congression in Hsp70i-treated cells.** HeLa cells stably expressing EGFP–lamin A/H2B-mCherry were treated with Hsp70i for 4 h and imaged by time-lapse confocal microscopy using a confocal laser-scanning microscope (TCS-SP5; Leica) as they progressed through mitosis. Z stacks comprising 20 0.5-µm steps were acquired every 10 min for 18 h.

Video 4. **Time-lapse imaging of chromosome alignment in DMSO-treated cells.** HeLa cells stably expressing H2B-mCherry were treated with MG132 for 4 h before being treated with DMSO and imaged by time-lapse confocal microscopy using a confocal laser-scanning microscope (TCS-SP5; Leica) as they progressed through mitosis. Z stacks comprising 20 0.5-µm steps were acquired every 10 min for 7 h.

Video 5. **Time-lapse imaging of chromosome alignment in Hsp70i-treated cells.** HeLa cells stably expressing H2B-mCherry were treated with MG132 for 4 h before being treated with Hsp70i and imaged by time-lapse confocal microscopy using a confocal laser-scanning microscope (TCS-SP5; Leica) as they progressed through mitosis. Z stacks comprising 20 0.5-µm steps were acquired every 10 min for 7 h.