

Phosphorylation of human Sgo1 by NEK2A is essential for chromosome congression in mitosis

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Chromosome segregation in mitosis is orchestrated by the interaction of the kinetochore with spindle microtubules. Our recent study shows that NEK2A interacts with MAD1 at the kinetochore and possibly functions as a novel integrator of spindle checkpoint signaling. However, it is unclear how NEK2A regulates kinetochore-microtubule attachment in mitosis. Here we show that NEK2A phosphorylates human Sgo1 and such phosphorylation is essential for faithful chromosome congression in mitosis. NEK2A binds directly to HsSgo1 *in vitro* and co-distributes with HsSgo1 to the kinetochore of mitotic cells. Our *in vitro* phosphorylation experiment demonstrated that HsSgo1 is a substrate of NEK2A and the phosphorylation sites were mapped to Ser¹⁴ and Ser⁵⁰⁷ as judged by the incorporation of ³²P. Although such phosphorylation is not required for assembly of HsSgo1 to the kinetochore, expression of non-phosphorylatable mutant HsSgo1 perturbed chromosome congression and resulted in a dramatic increase in microtubule attachment errors, including syntelic and monotelic attachments. These findings reveal a key role for the NEK2A-mediated phosphorylation of HsSgo1 in orchestrating dynamic kinetochore-microtubule interaction. We propose that NEK2A-mediated phosphorylation of human Sgo1 provides a link between centromeric cohesion and spindle microtubule attachment at the kinetochores.

Keywords: Sgo1, NEK2A, phosphorylation, microtubule, kinetochore

Cell Research (2007) 17:608-618. doi: 10.1038/cr.2007.55; publication online 10 July 2007

Introduction

Chromosome movements during mitosis are governed by the interaction of spindle microtubules with a specialized chromosome domain located within the centromere. This specialized region, called the kinetochore (e.g. Ref [1]), is the site for spindle microtubule-centromere association. In addition to providing a physical link between chromosomes and spindle microtubules, the kinetochore plays an active role in chromosomal segregation through microtubule motors and spindle checkpoint sensors located at or near it [2-4]. Several elegant studies demonstrate an essential role for the Shugoshin (Sgo) family of proteins in temporal control of centromeric separation in eukaryotic cells

[5-10]. Interestingly, vertebrate Sgo1 interacts strongly with microtubules *in vitro* and regulates kinetochore microtubule stability *in vivo* [6]. Thus, Sgo1 provides a novel link between sister centromere cohesion and microtubule interactions at kinetochores. However, it has remained elusive as to the precise function and regulation of Sgo1 in kinetochore-microtubule association.

Mitosis is orchestrated by signaling cascades that coordinate mitotic processes and ensure accurate chromosome segregation. The key switch for the onset of mitosis is the archetypal cyclin-dependent kinase, Cdc2. In addition to the master mitotic kinase Cdc2, mitosis also involves the actions of three other protein serine/threonine kinase families, the Polo kinases, Aurora kinases, and the NIMA-related kinases (NEK) [11, 12]. The latter family has proven the most enigmatic in function, although recent advances from several sources are beginning to reveal a common functional theme. Our recent study demonstrates that NEK2A is a kinetochore-associated protein kinase essential for faithful chromosome segregation [13]. However, it remains

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Received 9 May 2007; revised 11 May 2007; accepted 14 May 2007; published online 10 July 2007

unknown how NEK2A governs dynamic kinetochore-microtubule association.

To delineate the molecular regulation of NEK2A in kinetochore-microtubule interaction, we immobilized recombinant kinetochore proteins onto a nitrocellulose membrane for assessing NEK2A-binding activity, which led to the identification of an inter-relationship between NEK2A and HsSgo1. Our biochemical characterization demonstrates that HsSgo1 is a novel substrate of NEK2A. Most importantly, the phosphorylation of HsSgo1 by NEK2A is essential for faithful chromosome congression and the proper attachment of spindle microtubule to the kinetochore. We propose that NEK2A-mediated phosphorylation of HsSgo1 links kinetochore-microtubule attachment to chromosome segregation dynamics.

Materials and Methods

DNA construction

Full-length human *Sgo1* gene (NM_001012409) was generated by PCR from a human Testis cDNA library (Clontech) and cloned into T-vector (Takara Biotechnology, Dalian, China). To construct GFP-HsSgo1, GST-HsSgo1 and FLAG-HsSgo1 plasmids, the full-length HsSgo1 cDNA was subcloned into pEGFP-C3 vector (Clontech, Palo Alto, CA, USA) digested with *Pst*I and *Bam*HI, pGEX-6P-1 vector (Amersham Biosciences Corp., Piscataway, NJ, USA) digested with *Bam*HI and *Sal*I, and p3×FLAG-myc-CMV-24 vector (Sigma) digested with *Hind*III and *Bam*HI. GFP-tagged and GST-tagged phospho-mutants HsSgo1^{S14A}, HsSgo1^{S507A}, HsSgo1^{S14/S507A}, and HsSgo1^{S14/S507D} were created by standard PCR methods with a site-directed mutagenesis kit (Takara Biotechnology, Dalian, China). All constructs were sequenced in full.

Reagents

Monoclonal antibody against GFP was purchased from CLONTECH (Palo Alto, CA, USA); FLAG antibody (M2) and tubulin antibody were obtained from Sigma Chemical (St Louis, MO, USA); SGO1L1 monoclonal antibody was ordered from Abnova Inc. (Taiwan); and anti-NEK2A antibody was purchased from BD Biosciences (La Jolla, CA, USA). Recombinant active NEK2 kinase was purchased from Upstate Biotechnology.

In vitro phosphorylation of HsSgo1 by NEK2A

The GST-tagged wild-type HsSgo1 and non-phosphorylatable (HsSgo1^{S14A}, HsSgo1^{S507A} and HsSgo1^{S14/S507A}) mutants were expressed in *Escherichia coli* strain BL21 (DE3) and purified by using glutathione-agarose beads (Sigma) as previously described [13]. Briefly, 1 liter of LB media was inoculated with bacteria transformed with GST-HsSgo1. The expression of protein was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 3 h. After the induction, bacteria were harvested by centrifugation and re-suspended in phosphate-buffered saline (PBS) containing proteinase inhibitors (leupeptin, pepstatin, and chymostatin; 5 μg/ml), and sonicated for four bursts of 10 s each by using a probe-tip sonicator. The lysis solution was cleared by centrifugation for 20 min at 10 000 × g. The soluble fraction was applied to a column packed with glutathione-agarose beads, followed by extensive washes with PBS.

For *in vitro* phosphorylation assay, aliquots of wild-type GST-HsSgo1 and mutant HsSgo1 proteins (20 μg each) were incubated with 200 ng of active NEK2A in kinase buffer (25 mM HEPES, pH 7.2, 1 mM DTT, 50 mM NaCl, 2 mM EGTA, 5 mM MgSO₄) with 50 μM ATP and 0.5 μCi of [³²P]-ATP. The reaction mixtures (50 μl) were incubated at 30 °C for 30 min and terminated by adding SDS-PAGE sample buffer. Proteins were then fractionated on SDS-PAGE. The gel was stained with Coomassie Brilliant Blue and quantified by a PhosphorImager (Amersham Biosciences) as previously described [14].

Far-western blotting analysis and pull-down assay

The far-western blotting assay was carried out as described previously [13]. In brief, aliquots (~0.5 μg) of recombinant kinetochore proteins such as MAD1, MAD2, BUB1, BUB3, CENP-E and HsSgo1 were dipped onto nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 2% BSA in PBS containing Tween-20 and incubated with ~1 μg/ml of His-tagged NEK2A. After 2 h incubation, the membrane was washed four times with PBS and incubated with NEK2A antibody for 1 h. Alkaline phosphatase-conjugated secondary antibody was used to detect the primary antibody, and the blot was developed using NBT and BCIP as substrates.

The NEK2A affinity pull-down was previously described [13]. In brief, His-tagged NEK2A immobilized on the Ni-NTA agarose beads (Qiagen) was used as an affinity matrix to incubate with purified GST-HsSgo1. The agarose beads were washed with PBS five times before being boiled in SDS-PAGE sample buffer. The proteins were then fractionated on SDS-PAGE and the gel was stained with Coomassie brilliant blue.

Cell culture and transfection

HeLa and 293T cells, from American Type Culture Collection (Rockville, MD, USA), were maintained as subconfluent monolayers in DMEM growth medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen) at 37 °C with 10% CO₂. Transfections were performed using Lipofectamine 2000 (Invitrogen) exactly as described [13].

Chromosome squashes

Chromosome squashes were prepared essentially as previously described [15]. In brief, GFP-HsSgo1-transfected HeLa cells were treated with 10 ng/ml nocodazole (Sigma) for 18 h. After arrest, mitotic HeLa cells were harvested by mitotic shake-off and washed with ice-cold PBS. Mitotic HeLa cells were hypotonically swollen for 5 min at room temperature in 10 vol of PEM buffer containing 5 mM Pipes, pH 7.2, 0.5 mM EDTA, 5 mM MgCl₂, 5 mM NaCl, and a protease inhibitor cocktail (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin) before squashed onto coverslips using Cytospin 4 cytocentrifuge (1 000 rpm for 2 min; Thermo Scientific). The chromosomes on the coverslips were then fixed with 1% formaldehyde followed by three washes with PBS. The squashed chromosomes were then stained with anti-centromere antibody (ACA) and counterstained with DAPI (Sigma).

Immunoprecipitation

To test whether HsSgo1 forms a complex with NEK2A *in vivo*, 293 cells were transfected with FLAG-HsSgo1 and GFP-NEK2A plasmid. Twenty-four hours after the transfection, cells were collected

and solubilized in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, and 10 g/ml pepstatin A). Lysates were cleared by centrifugation at $16\,000 \times g$ for 10 min at 4 °C. FLAG-tagged fusion proteins were precipitated by incubating with anti-FLAG antibody coupled agarose beads (Sigma). Beads were washed five times with lysis buffer and then boiled in protein sample buffer for 2 min. After SDS-PAGE, proteins were transferred to nitrocellulose membrane. The membrane was divided into three strips and probed with antibodies against the FLAG tag, GFP tag and tubulin, respectively. Immunoreactive signals were detected with ECL kit (Pierce) and visualized by autoradiography on Kodak BioMax film.

Western blotting

Samples were subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Proteins were probed by appropriate primary and secondary antibodies at concentrations recommended by the manufacturer. Proteins were detected using ECL (Pierce). The band intensity was then scanned using a PhosphorImager (Amersham Biosciences).

To determine whether NEK2A-mediated phosphorylation of HsSgo1 contributes to its mobility shift in SDS-PAGE, GFP-HsSgo1 transfected cells or NEK2A siRNA-treated cells were synchronized with 10 ng/ml nocodazole 24 h post-transfection. At the end of synchronization, cells were collected and solubilized in SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE and transferred onto nitrocellulose membrane for western blotting analysis with an anti-HsSgo1 antibody.

Immunofluorescence microscopy

For immunofluorescence analysis, cells were seeded onto sterile, acid-treated 12 mm coverslips in 24-well plates (Corning Glass). Twenty-four hours after transfection with various GFP-tagged HsSgo1 plasmids, HeLa cells were pre-extracted with PHEM (100 mM PIPES, 20 mM HEPES, pH 6.9, 5 mM EGTA, 2 mM MgCl₂, and 4 M glycerol) plus 0.2% Triton X-100 followed by fixation and blocking as described previously [4, 15]. Cells were then incubated with primary antibody against centromere (ACA) followed by a rhodamine-conjugated secondary antibody. DNA was stained with DAPI (Sigma). Fluorescence labeling was examined under a laser scanning confocal microscope LSM510 (Carl Zeiss) and images were collected and analyzed with Image-5 (Carl Zeiss, Germany).

Results

HsSgo1 is a novel NEK2A-binding partner

Our previous study demonstrated that NEK2A is also located to the kinetochore during chromosome congression in mitosis [13]. To delineate the molecular mechanism underlying NEK2A regulation in kinetochore protein-protein interaction networks, we immobilized bacterially expressed recombinant kinetochore proteins onto a nitrocellulose membrane to conduct a quick search for NEK2A-binding proteins using a “high-content” far-western assay [13]. The NEK2A-binding activity was then detected by an anti-NEK2A monoclonal antibody. One advantage of such a “high-content” assay is to screen a large number of

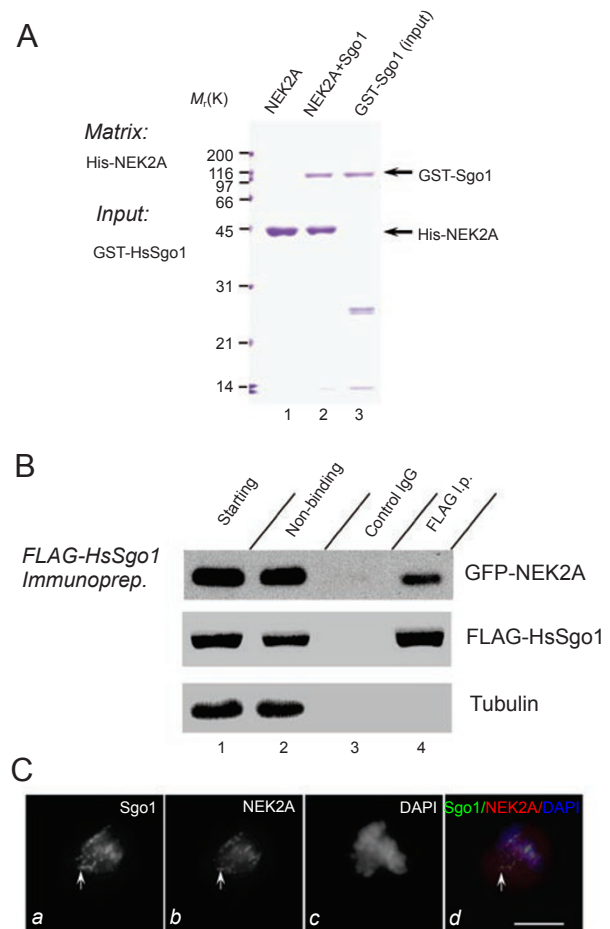


Figure 1 NEK2A interacts with HsSgo1 and co-distributes to the kinetochore. **(A)** Reconstitution of NEK2A-HsSgo1 interaction using recombinant fusion proteins. Histidine-tagged NEK2A recombinant protein purified on Ni NTA-agarose beads was used as affinity matrix for absorbing GST-tagged HsSgo1 protein purified from bacteria (lane 2). Aliquots of histidine-tagged NEK2A and GST-HsSgo1 were used as loading controls (lanes 1 and 3). **(B)** Co-immunoprecipitation of HsSgo1 and NEK2A from transfected 293T cells. 293T cells co-transfected with a FLAG-tagged hSgo1 and GFP-tagged NEK2A were extracted and subjected to immunoprecipitation by using monoclonal antibodies to FLAG, respectively. Control immunoprecipitation was performed using a non-specific mouse IgG. Starting fractions, non-binding fractions, and immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using anti-GFP antibody, anti-FLAG antibody and anti-tubulin antibody respectively. Western blotting verifies co-immunoprecipitation of GFP-NEK2A and FLAG-HsSgo1. No tubulin was detected in the FLAG immunoprecipitates. **(C)** Co-distribution of HsSgo1 and NEK2A to the kinetochore of mitotic HeLa cells. *a-d* represent optical images collected from one HeLa cell triply stained with mouse anti-HsSgo1 antibody (Sgo1, green), DAPI (DNA, blue), rabbit NEK2A antibody (NEK2A, red), and their merged images. Sgo1 antibody stained centromeres of mitotic HeLa cells as pairs of unresolved double dots (*a*; arrow). NEK2A staining appears as pairs of clearly resolved double dots (*b*; arrow). Merge shows a superimposition of NEK2A to that of Sgo1 staining. Bar, 10 μ m.

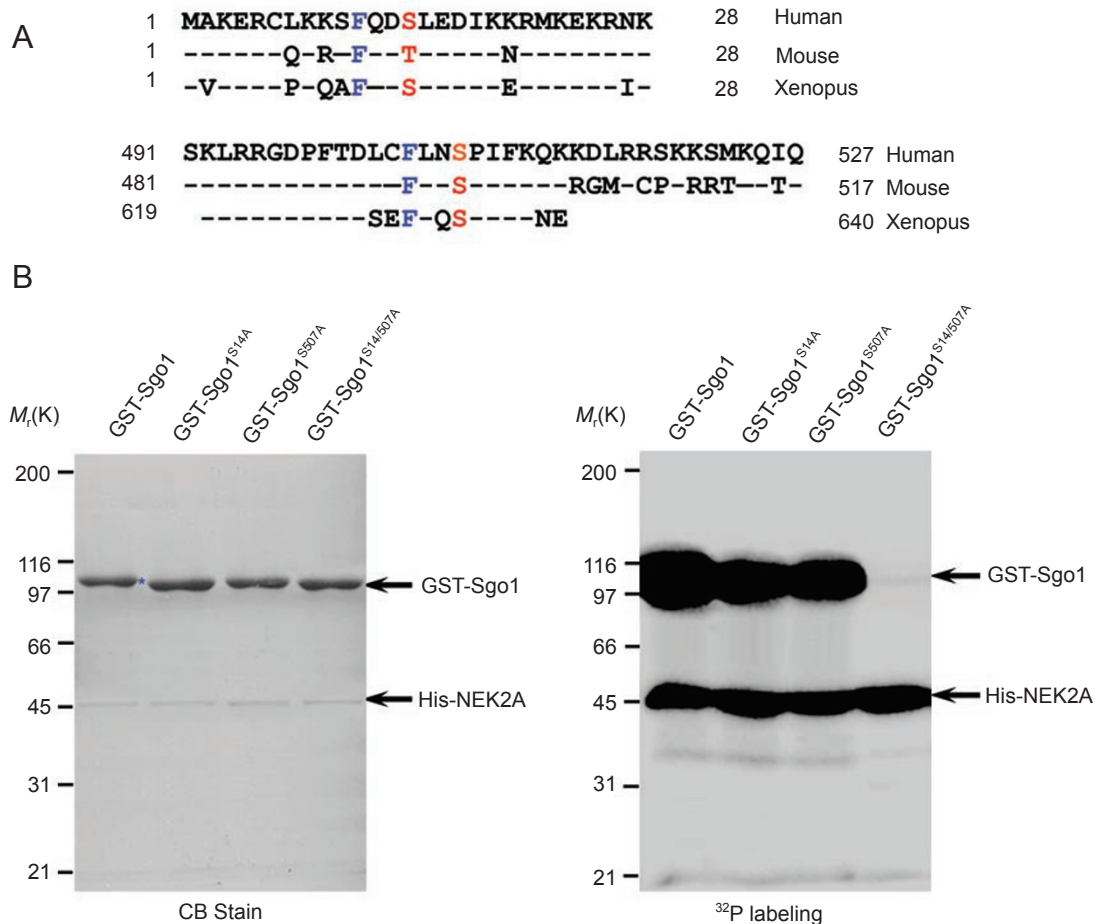


Figure 2 Ser¹⁴ and Ser⁵⁰⁷ of HsSgo1 are substrates of NEK2A. **(A)** Alignment of conserved NEK2A phosphorylation sites on *Xenopus*, mouse, and human Sgo1 proteins. Computational analyses were performed using GPS as described in “Materials and Methods”. Based on the GPS prediction, Ser¹⁴ and Ser⁵⁰⁷ are potential NEK2A-mediated phosphorylation sites of HsSgo1. **(B)** Ser¹⁴ and Ser⁵⁰⁷ of HsSgo1 are substrates of NEK2A. Bacterially expressed GST-HsSgo1 fusion proteins, both wild type and mutants (S14A, S507A and S14/507A), were purified and phosphorylated *in vitro* using [³²P]-ATP and active NEK2A as described under “Materials and Methods.” Samples were separated by SDS-PAGE. Coomassie brilliant blue-stained gel of samples of wild-type GST-Sgo1 plus NEK2A (GST-Sgo1), single mutant S14A GST-Sgo1 plus NEK2A (GST-Sgo1^{S14A}), single mutant S507A GST-Sgo1 plus NEK2A (GST-Sgo1^{S507A}), and double mutant S14/507A GST-Sgo1 plus NEK2A (GST-Sgo1^{S14/507A}). Note that roughly equivalent amounts of GST-HsSgo1 proteins were present in the four reactions (left panel). The same gel was dried and subsequently incubated with X-ray film. Note that there was dramatic incorporation of ³²P into wild type and single mutant, but not double mutant, HsSgo1 proteins (right panel). In addition, wild-type GST-Sgo1 protein (marked with blue asterisk) displayed a slower mobility compared to those of non-phosphorylatable mutants, suggesting the mobility shift is a reflection of Sgo1 protein phosphorylation.

potential interacting proteins in parallel yet avoiding initial intensive labor spent on purifying large quantities of proteins required for pull-down assays. This assay has detected the binding activity of several known NEK2A-interacting proteins, such as Hec1 and Mad1, validating the sensitivity of such an assay (Supplementary Figure 1). This assay is very specific as NEK2A was never found to be associated with GST tag, MBP tag, BSA, or kinetochore protein CENP-E. Interestingly, NEK2A was found to associate with

HsSgo1 immobilized onto the membrane (Supplementary Figure 1; B8), suggesting a potential interaction between NEK2A and HsSgo1.

To validate whether HsSgo1 binds to NEK2A in solution, we carried out a pull-down assay in which histidine-tagged recombinant NEK2A was purified on Ni-NTA agarose beads and used as an affinity matrix to absorb purified GST-HsSgo1 in test tubes. As shown in Figure 1A, histidine-tagged NEK2A pulled down GST-HsSgo1, suggesting

a physical interaction between NEK2A and HsSgo1 *in vitro*. To examine whether HsSgo1 forms a complex with NEK2A in cells, we carried out an immunoprecipitation assay using mitotic lysates from 293 T cells transiently transfected to express FLAG-HsSgo1 and GFP-NEK2A. As shown in Figure 1B, western blot using GFP antibody confirmed that GFP-NEK2A is pulled down by FLAG-HsSgo1 (upper panel; lane 4) while FLAG antibody blotting confirmed a successful precipitation of HsSgo1 (middle panel; lane 4). GFP-tagged NEK2A was not precipitated with control IgG (lane 3), nor was tubulin detected in any of the immunoprecipitates. Thus, we conclude that HsSgo1 interacts with NEK2A *in vitro* and *in vivo*.

If HsSgo1 is a cognate binding partner of NEK2A, they should co-distribute to the kinetochore of prometaphase cells. To test this, we performed an immunocytochemical staining in which an anti-HsSgo1 mouse antibody and an anti-NEK2A rabbit antibody were employed to mark their kinetochore distribution. As shown in Figure 1C, HsSgo1 and NEK2A displayed a typical kinetochore distribution (arrows a and b) and the merged image confirmed their co-distribution to the kinetochore of mitotic cells (arrow d).

HsSgo1 is a novel substrate of NEK2A

Our previous studies show that mitotic kinase NEK2A functions as a novel integrator of the spindle checkpoint signaling [13]. Since HsSgo1 binds to NEK2A *in vitro* and *in vivo*, we sought to test whether HsSgo1 is a substrate of NEK2A. Our computational analysis suggests that Ser¹⁴ and Ser⁵⁰⁷, which are conserved among vertebrate HsSgo1 proteins, are potential substrates of NEK2A [16] (Figure 2A). Our mass spectrometric analysis of HsSgo1 immunoprecipitates also pointed to the possibility of Ser¹⁴ and Ser⁵⁰⁷ phosphorylation in nocodazole-synchronized HeLa cells. To test whether Ser¹⁴ and Ser⁵⁰⁷ are substrates of NEK2A, we performed an *in vitro* phosphorylation assay on recombinant GST-HsSgo1 fusion proteins, including both wild-type protein and non-phosphorylatable mutants in which Ser¹⁴ and Ser⁵⁰⁷ were either individually or both replaced by alanine (HsSgo1^{S14A}, HsSgo1^{S507A}, and HsSgo1^{S14/507A}). Both GST fusion proteins, wild type and mutant HsSgo1^{S14/507A}, migrate at about the predicted 105 kDa as shown in Figure 2B (left panel). Incubation of the fusion proteins with [³²P]-ATP and His-NEK2A resulted in the incorporation of ³²P into wild type, HsSgo1^{S14A} and HsSgo1^{S507A} proteins but not HsSgo1^{S14/507A} mutant (Figure 2B, right panel). This NEK2A-mediated phosphorylation is specific, since incubation of HsSgo1 with [³²P]-ATP in the absence of NEK2A resulted in no detectable incorporation of radioactivity into the wild-type protein (data not shown). Thus, we conclude that both Ser¹⁴ and Ser⁵⁰⁷ on HsSgo1 are substrates of NEK2A *in vitro*.

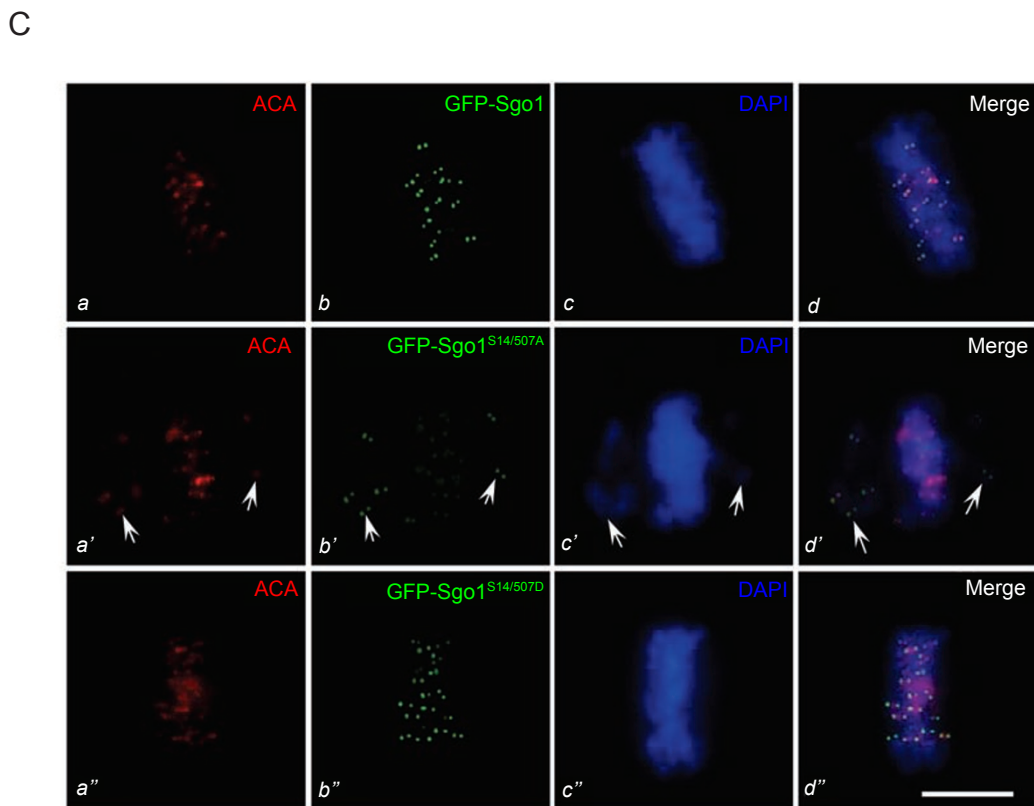
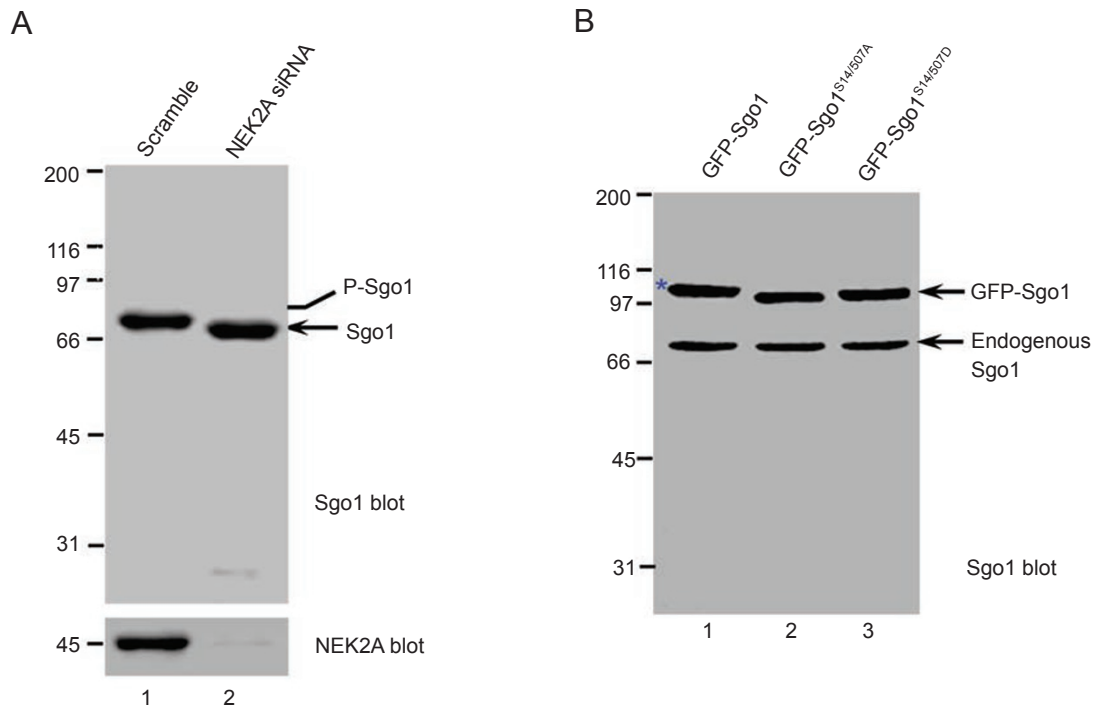
Localization of HsSgo1 to the kinetochore does not require NEK2A

Interestingly, wild-type HsSgo1 treated with NEK2A displayed a slower mobility compared to that of non-phosphorylatable HsSgo1^{S14/507A} mutant (Figure 2B; compare lanes 1 with 4), suggesting a possible phosphorylation-mediated mobility shift induced by NEK2A. To test whether endogenous HsSgo1 mobility is a function of NEK2A, we carried out western blot of HsSgo1 from HeLa cells treated with NEK2A siRNA followed by nocodazole synchronization. As shown in Figure 3A, the mobility shift of HsSgo1 was confirmed to be NEK2A-dependent as depletion of NEK2A retained HsSgo1 to the faster migrating form, indicating that endogenous HsSgo1 is phosphorylated by NEK2A in mitosis.

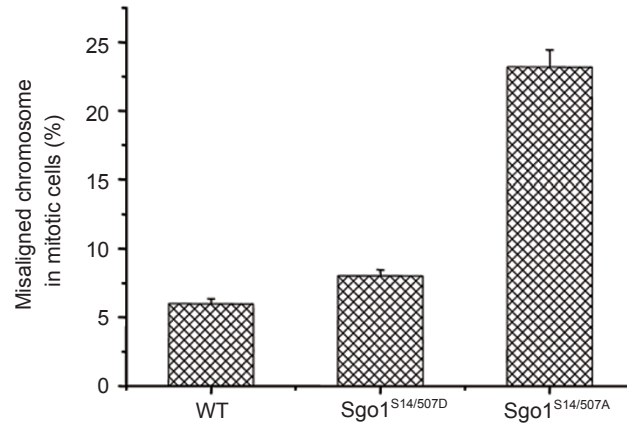
To examine whether phosphorylation of Ser¹⁴ and Ser⁵⁰⁷ is responsible for the mobility shift of HsSgo1 in mitosis, we carried out western blot analysis of HsSgo1 using HeLa cells transiently transfected to express GFP-tagged wild-type HsSgo1 and mutant HsSgo1 (HsSgo1^{S14/507A} and HsSgo1^{S14/507D}). As expected, wild-type GFP-HsSgo1 protein migrated more slowly compared to mutant proteins GFP-HsSgo1^{S14/507A} and HsSgo1^{S14/507D} while endogenous HsSgo1 proteins from these three transfectants displayed similar mobility, suggesting that the mobility shift is a function of phosphorylation on Ser¹⁴ and Ser⁵⁰⁷ (Figure 3B; lanes 1-3). Thus, we conclude that HsSgo1 is phosphorylated by NEK2A at Ser¹⁴ and Ser⁵⁰⁷ in mitosis.

To evaluate the efficacy of exogenous HsSgo1 expression, we carried out western blotting analysis using anti-HsSgo1 antibody to compare the levels of exogenously expressed GFP-HsSgo1 proteins to that of endogenous HsSgo1. As shown in Figure 3B, exogenously expressed GFP-HsSgo1 proteins were about twice the level of endogenous HsSgo1 proteins in these transfected HeLa cells. Given a typical ~50% transfection efficiency, the actual expression level of GFP-HsSgo1 in positively transfected cells is about four-fold higher than that of endogenous protein [17].

To examine whether NEK2A-mediated phosphorylation is essential for HsSgo1 localization to the kinetochore, plasmids expressing GFP-tagged wild type, non-phosphorylatable, and phospho-mimicking HsSgo1 were transfected into HeLa cells, and fusion proteins were scored for their kinetochore localization relative to the centromere marker ACA under fluorescence microscopy. The transfected cells were triply stained or analyzed for ACA (red), GFP-HsSgo1 (green) and DNA (blue). As shown in Figure 3C, the kinetochore distribution of exogenously expressed GFP-HsSgo1 proteins (wild type, HsSgo1^{S14/507A}, and HsSgo1^{S14/507D}) was readily observed as the merged images show the co-localization of GFP-HsSgo1 proteins with the



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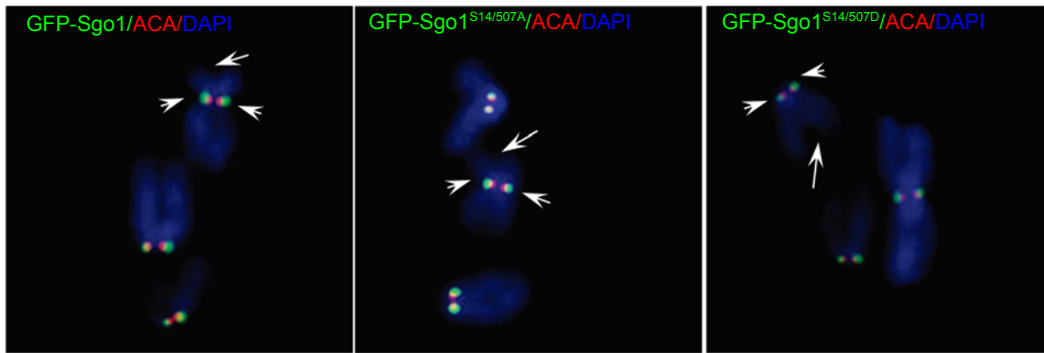


Figure 3 NEK2A-mediated phosphorylation of HsSgo1 is essential for faithful chromosome congression. **(A)** HsSgo1 is a substrate of NEK2A in mitosis. Aliquots of HeLa cells were transfected with NEK2A siRNA and scramble control as described under “Materials and Methods”. Twenty-four hours after the transfection, these cells were synchronized with 10 ng/ml nocodazole for additional 18 h. Cells were then harvested for SDS-PAGE and subsequent western blotting analyses of Sgo1 (upper panel) and efficiency of NEK2A depletion (lower panel). Note that Sgo1 protein in scramble-transfected cells displayed a slower mobility compared to that of the NEK2A-depleted sample, suggesting that the mobility shift is an indicator of NEK2A-mediated phosphorylation of Sgo1. **(B)** Exogenously expressed GFP-Sgo1 protein behaves like endogenous Sgo1. Samples from both wild type and mutant Sgo1-transfected HeLa cells were prepared and separated on SDS-PAGE, blotted to nitrocellulose, and probed by an anti-HsSgo1 antibody. The Sgo1 antibody recognizes both endogenous (75 kDa) and exogenously expressed GFP-Sgo1 (105 kDa) proteins. Note that wild-type GFP-Sgo1 protein displayed a slower mobility compared to those of mutants, suggesting exogenously expressed GFP-Sgo1 protein behaves like endogenous Sgo1. **(C)** Overexpression of non-phosphorylatable HsSgo1 resulted in chromosome congression defects. This triple montage and their merges represent mitotic HeLa cells transiently transfected to express wild type and phospho-mutant HsSgo1 proteins. The transfected cells were pre-extracted before fixation as described under “Materials and Methods” and stained for ACA (red), GFP-Sgo1 (green), and DAPI (blue). Note that chromosomes failed to congress and align at the spindle equator in non-phosphorylatable Sgo1-expressing cells (arrows; *a'-d'*) and misaligned chromosome is correlated with higher expression of GFP-Sgo1^{S14/507A} compared to those near the spindle equator. Bar, 10 μm. **(D)** Phosphorylation of the HsSgo1 by NEK2A is required for faithful chromosome movement. HeLa cells were transfected with GFP-tag wild-type hSgo1, Sgo1^{S14/507A}, and Sgo1^{S14/507D} for 48 h followed by fixation and DNA staining. Cells were then examined under fluorescence microscopy to determine the phenotypes. Cells in mitosis and those that carried misaligned chromosomes were quantified and expressed as the percentage of total mitotic cells. An average of 150 mitotic cells from three separate experiments were counted. Error bars represent S.E.; *n* = 3 preparations. *p* < 0.001. **(E)** Centromeric cohesion is independent of NEK2A-mediated phosphorylation of HsSgo1. Chromosome squashes from nocodazole-treated HeLa cells transiently transfected to express wild type, non-phosphorylatable and phospho-mimicking HsSgo1 proteins were prepared as described under “Materials and Methods”. The chromosomes were fixed and stained to mark centromeres and DNA. Despite the separation of chromosome arms seen in all Sgo1-expressing cells, centromeric cohesion remained intact.

centromere marker ACA (Figure 3C, *d*, *d'* and *d''*), suggesting that exogenous HsSgo1 proteins are able to target to the kinetochore regardless of the phosphorylation status of Ser¹⁴ and Ser⁵⁰⁷. These data suggest that phosphorylation of Ser¹⁴ and Ser⁵⁰⁷ by NEK2A is not required for targeting of HsSgo1 to the kinetochore.

NEK2A-mediated phosphorylation of HsSgo1 is required for faithful chromosome congression

Interestingly, at comparable time points, many non-phosphorylatable HsSgo1^{S14/507A}-expressing cells contained misaligned chromosomes that were readily observed (Figure 3C, *a'-d'*; arrows). Examination of ~100 mitotic cells expressing HsSgo1^{S14/507A} revealed that many chromosomes in almost all positively transfected cells were positioned close to spindle poles, with some near the spindle equator (Figure 3C, *c'*). This pattern is the expected distribution for chromosomes that are actively congressing [4]. In fact, misaligned chromosomes bear double-dot staining of HsSgo1 (Figure 3C, *b'*), suggesting that these chromosomes near the poles were not separated. We surveyed approximately 150 mitotic cells, in which both sets of sister chromatids were in the same focal plane, from cells expressing the three GFP-HsSgo1 proteins (wild type, S14/507A, and S14/507D mutant). As shown in Figure 3D, expression of the non-phosphorylatable Sgo1 resulted in a significant increase in cells bearing misaligned chromosomes ($23.2 \pm 6.6\%$; $p < 0.05$). Only a minority of GFP-HsSgo1- and GFP-Sgo1^{S14/507D}-expressing cells displayed misaligned chromosomes ($6 \pm 1.9\%$ for GFP-HsSgo1 and $8 \pm 2.5\%$ for GFP-Sgo1^{S14/507D}). Thus, we conclude that phosphorylation of HsSgo1 by NEK2A is essential for faithful chromosome congression.

In vertebrates, sister chromatid cohesion is orchestrated by the associations at the centromere and chromosome arms. Depletion of HsSgo1 in HeLa cells causes massive mis-segregation of sister chromatids due to an aberrant centromeric cohesion [18]. To evaluate the functional role of NEK2A-mediated phosphorylation in centromeric cohesion, we prepared chromosome spreads of HeLa cells from GFP-HsSgo1-expressing cells synchronized by nocodazole treatment and analyzed the morphology of chromosomes whose centromeres were marked with GFP-HsSgo1 proteins. To facilitate our analysis, chromosomes were counterstained with ACA to confirm the centromeric cohesion. As shown in Figure 3E, most sister chromatids in GFP-HsSgo1 expressing cells remained attached at their centromeres while chromosome arms are separated, consistent with the fact that most of the cohesin residing along the chromosome arms is removed in prophase (e.g. Ref [19]). Thus, we concluded that phosphorylation of Sgo1 by NEK2A is not involved in centromeric cohesion.

NEK2A phosphorylation of HsSgo1 is essential for a proper chromosome-microtubule attachment

Sgo1 is a microtubule binding protein [6], and our observations of delayed chromosome congression in GFP-HsSgo1^{S14/507A}-overexpressing cells suggest that NEK2A-mediated phosphorylation is required for a stable association between the kinetochores and spindle microtubules. To validate this hypothesis, we probed for the presence of cold-stable kinetochore microtubules in GFP-HsSgo1^{S14/507A}-overexpressing cells. In brief, treatment of mitotic cells on ice during permeabilization prior to fixation destabilizes non-kinetochore microtubules, leaving behind mainly kinetochore fibers (e.g. Ref [4]). As shown in Figure 4A, the spindle remained intact after cold treatment, with microtubule fibers clearly attached to each kinetochore stained with ACA (Figure 4A, *a*, red), in cells overexpressing wild-type HsSgo1. In HsSgo1^{S14/507A}-overexpressing cells, cold-stable kinetochore-microtubule fibers were present on both aligned chromosomes and chromosomes near the pole (Figure 4A, *b'*, red). Quantitative analysis of fluorescence intensity of GFP-HsSgo1^{S14/507A} indicates that the fluorescence intensity of the kinetochore near the pole is about 5.3-fold higher than that of an aligned kinetochore, consistent with the notion that overexpression of HsSgo1^{S14/507A} produces a “dominant”-negative effect in chromosome alignment.

We next examined the microtubule attachment and the orientation of sister kinetochores on chromosomes in the cells overexpressing GFP-HsSgo1^{S14/507A} mutant. To this end, we performed confocal scanning microscopy on HeLa cells fixed and stained with anti-tubulin antibody and ACA. To show individual kinetochores more clearly, we present optical sections in insets while using maximal intensity projections of the entire Z-stack for volume views. We scored kinetochore attachment in which both kinetochores were in the same focal plane, in both wild-type GFP-HsSgo1 and GFP-HsSgo1^{S14/507A}-expressing cells. As shown in Figure 4A, it is readily apparent from the confocal projection that overexpression of the GFP-HsSgo1^{S14/507A} mutant resulted in misaligned chromosome near the poles while all chromosomes were aligned around the equator in the cells expressing wild-type HsSgo1. Enlargement of kinetochore-microtubule attachment indicates that some cold-stable microtubules display syntelic orientation and monotelic orientation (illustrated in Figure 4B). In contrast, most spindle microtubules exhibit amphitelic configuration in control cells expressing wild-type HsSgo1. Our measurement indicates no difference of the fluorescence intensity of wild-type GFP-HsSgo1 between aligned and misaligned kinetochore (data not shown). However, the misaligned chromosome is correlated with the higher expression level of HsSgo1^{S14/507A} protein as judged by the fluorescence

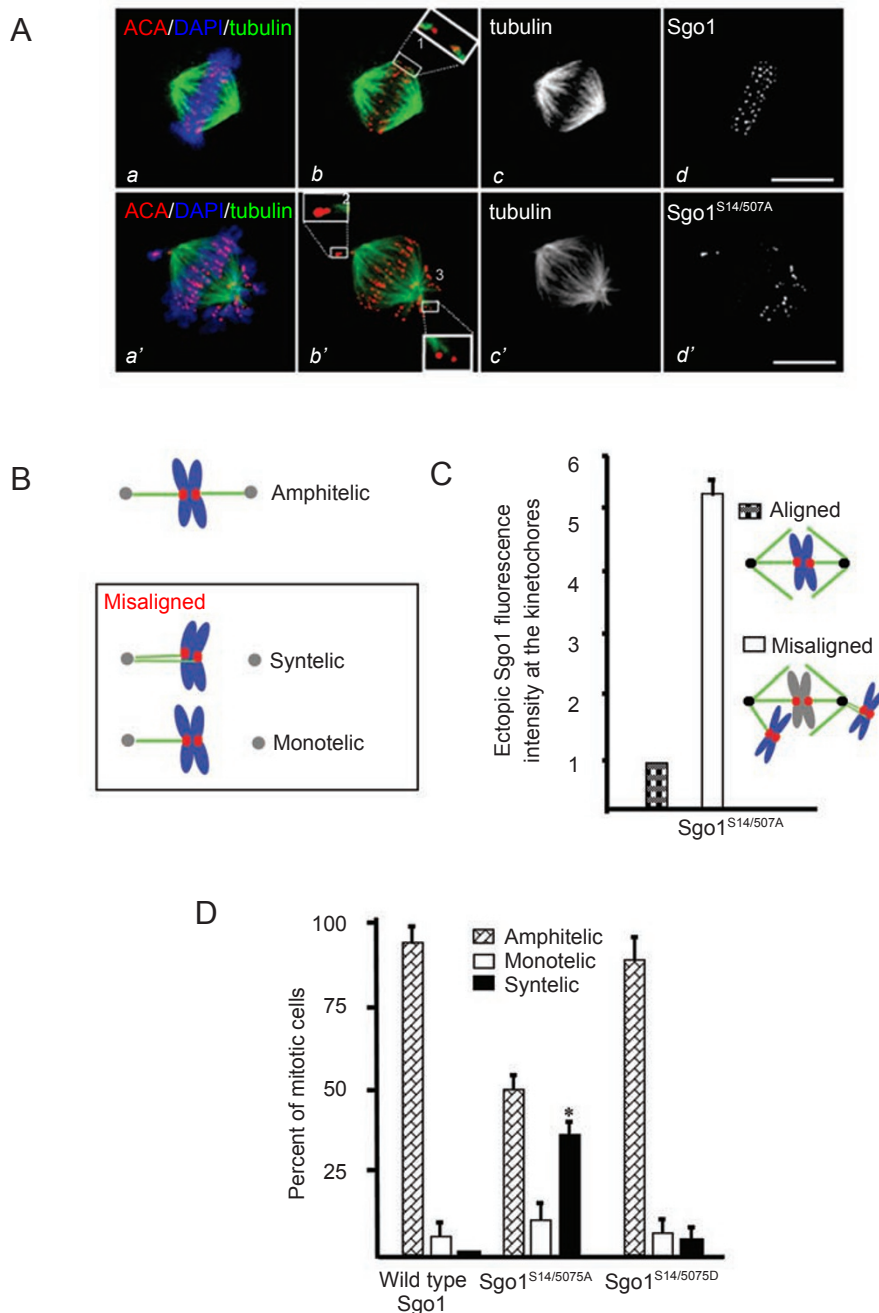


Figure 4 Inhibition of HsSgo1 phosphorylation results in aberrant kinetochore-microtubule attachments. **(A)** Indirect immunofluorescence images of HeLa cells expressing wild-type hSgo1 (*a-d*) or Sgo1^{S14/507A} (*a'-d'*). Cells were kept on ice for 7 min, permeabilized for 90 s in PHEM, then fixed and immunostained. Cold treatment selectively removes non-kinetochore microtubules. Each chromosome (blue) has two sister kinetochores (red) that are stained with the ACA. In HeLa cells expressing wild-type HsSgo1 (*a-d*), almost every pair of sister kinetochores are attached by microtubules (green) emanating from the opposing poles. However, in HsSgo1^{S14/507A}-expressing cells (*a'-d'*), several examples with both kinetochores of a sister pair attached to microtubules emanating from the same pole are observed (*b'*). 1, 2, and 3 show higher magnification micrographs for three pairs of kinetochores either properly (in 1) or aberrantly (in 2 and 3) attached to spindle microtubules. **(B)** Schematic illustration of various kinetochore-microtubule attachment orientations seen in mitosis. The boxed orientations are typical misalignment seen in HsSgo1^{S14/507A}-expressing cells. **(C)** Quantification of Sgo1^{S14/507A} fluorescence intensity at kinetochores of misaligned chromosomes compared to that of chromosomes aligned at the equator. Because of the difficulty in distinguishing syntelic orientation from monotelic orientation in some cases, we grouped these two orientations as misalignment. **(D)** Quantification of kinetochore-microtubule attachment orientation in HeLa cells expressing wild-type Sgo1, Sgo1^{S14/507A}, and Sgo1^{S14/507D} respectively. Error bars represent S.E.; *n* = 3 preparations (~100 mitotic cells per preparation), *p* < 0.001.

intensity (Figure 4C), suggesting that misaligned chromosome is a function of HsSgo1^{S14/507A} overexpression. We surveyed approximately 100 mitotic cells for kinetochore attachment orientation, in which both kinetochores were in the same focal plane, from HsSgo1^{S14/507A}-overexpressing cells and the controls. Our survey indicates that syntelic orientation is a typical feature of HsSgo1^{S14/507A}-overexpressing cells. As shown in Figure 4D, a summary from three different experiments shows that expression of non-phosphorylatable HsSgo1 resulted in significant increases in cells bearing syntelic attachment ($35.3 \pm 4.7\%$), compared to the control ($0.8 \pm 0.1\%$ for wild type). Therefore, we conclude that NEK2A-mediated phosphorylation of HsSgo1 at Ser¹⁴ and Ser⁵⁰⁷ is essential for proper attachment of the kinetochore to spindle microtubules.

Discussion

We have identified that the function of HsSgo1 is regulated by NEK2A, a kinetochore-associated kinase essential for cell division [4, 13]. Although NEK2A-mediated phosphorylation of HsSgo1 is not essential for its localization to the kinetochore, inhibition of HsSgo1 phosphorylation resulted in chromosome congression defects and aberrant kinetochore-microtubule attachments. These findings offer a mechanistic link between the NEK2A-mediated phosphorylation of HsSgo1 and proper attachment of spindle microtubules to kinetochores.

Our recent study has revealed the critical role of NEK2A in chromosome segregation in mammalian cells via its interaction with spindle checkpoint protein MAD1 [13]. To delineate the molecular mechanism underlying NEK2A regulation in kinetochore interaction, we carried a “high-content” screen for NEK2A interacting proteins and identified HsSgo1 as its cognate substrate. Despite our corroborative studies showing that HsSgo1 is phosphorylated by NEK2A at Ser¹⁴ and Ser⁵⁰⁷ *in vitro* and *in vivo*, it remains to be determined how the spatio-temporal order of this NEK2A-mediated HsSgo1 phosphorylation is orchestrated as majority of NEK2A kinase activity is minimized in prometaphase [11]. It would be of great interest to develop phosphorylation site-specific antibody to illustrate the phosphorylation and dephosphorylation dynamics of HsSgo1 in mitosis.

While NEK2A-mediated protein phosphorylation of HsSgo1 at Ser¹⁴ and Ser⁵⁰⁷ is not required for the localization of HsSgo1 at the kinetochore, overexpression of the non-phosphorylatable GFP-HsSgo1^{S14/507A} mutant results in a significant increase in attachment errors, including syntelic attachment and monotelic attachment. The aberrant attachment is a function of expression of the non-phosphorylatable GFP-HsSgo1^{S14/507A} mutant as expression of

wild-type GFP-HsSgo1 did not perturb the amphitelic attachment. However, it is unclear whether accumulation of the HsSgo1^{S14/507A} mutant protein in the kinetochore caused the chromosome congression defects or HsSgo1^{S14/507A} mutant protein became accumulated at the misaligned kinetochore as a “sensor” to the aberrant attachment. In any event, it would be of great importance to investigate whether and how overexpression of HsSgo1^{S14/507A} mutant causes the mal-orientation. In addition, it would be equally important to ascertain whether the enrichment of HsSgo1^{S14/507A} mutant protein in misaligned kinetochore is only a reporter for aberrant orientation. This will require real-time imaging of both kinetochore movements and HsSgo1^{S14/507A} molecular dynamics.

In contrast to depletion of HsSgo1 using siRNA, expression of the non-phosphorylatable HsSgo1^{S14/507A} mutant did not alter the centromeric cohesion (Figure 3E), and the localization of outer kinetochore proteins such as CENP-E and CENP-F (Fu *et al.*, unpublished observation). It remains to be established whether phosphorylation of HsSgo1 is required to regulate kinetochore microtubule dynamics in prometaphase and how such a phosphorylation regulates the kinetochore-microtubule attachment stability. Protein phosphorylation is a dynamic process and the status of phosphorylation is orchestrated by kinase and phosphatase. Despite the fact that NEK2A is degraded at prometaphase [11], the NEK2A-mediated phosphorylation of HsSgo1 remains (Figure 3A) and is essential for chromosome congression to the spindle equator at metaphase (Figure 3C). One possibility is that the NEK2A-induced phospho-epitopes remain on HsSgo1 despite the degradation of NEK2A, as the phosphatase responsible for dephosphorylation of HsSgo1 is inactive until chromosomes achieve metaphase alignment. To this end, it would be necessary to develop phospho-epitope antibody specific for Ser¹⁴ and Ser⁵⁰⁷ and illustrate the temporal profile of HsSgo1 phosphorylation. Our study here aims to provide an outline so that further mechanistic studies of phospho-regulation of HsSgo1 can be pursued.

Loss or gain of whole chromosomes, the form of chromosomal instability most commonly associated with human cancers, is expected to arise from the failure to accurately segregate chromosomes in mitosis. The mitotic checkpoint is one pathway that prevents segregation errors by blocking the onset of anaphase until all chromosomes make proper attachments to the spindle. Another process that prevents errors is stabilization and destabilization of connections between kinetochore and spindle microtubules, in which spatiotemporal regulation of kinetochore organization and microtubule attachment are integrated into mitotic events. The question remains as to how these two pathways are coordinated to ensure accurate chromosome segregation.

We observed that many chromosomes in HsSgo1^{S14/507A}-expressing cells show syntelic orientation, with both kinetochores attached to the cold-stable spindle microtubules originating from a single pole. Syntelic orientation could result from the collapse of a bipolar spindle, but our observations suggest that HsSgo1^{S14/507A}-expressing cells entering mitosis gain this syntelic orientation by *de novo* capture of microtubules by both kinetochores in a sister pair. Two mechanisms for correction of syntelic error have been proposed from meiotic studies, where the first event is the microtubule capture from the opposite pole [20] or microtubule release at the attached pole [21]. In this regard, it would be of great interest to systematically study phospho-regulation of HsSgo1 in real-time to examine whether and how syntelic orientation is corrected in mitosis.

Taken together, our finding of phospho-regulation of HsSgo1 by NEK2A demonstrates a critical role of HsSgo1 in kinetochore dynamics in addition to its function at kinetochore-microtubule attachment. The fact that suppression of HsSgo1 phosphorylation abrogates microtubule attachment to the kinetochore and induces chromosome congression defect and mis-segregation in HeLa cells prematurely exiting from anaphase (Fu *et al.*, unpublished observation), demonstrates the importance of HsSgo1 phosphorylation in faithful chromosome segregation and the maintenance of genomic stability in mitosis.

Acknowledgments

We thank members of our group for insightful discussion during the course of this study. This work was supported by grants from Chinese Academy of Science (KSCX1-YW-R65; KSCX2-YW-H10), National Basic Research Program of China (2002CB713700), Hi-Tech Research and Development Program of China (2001AA215331), Chinese Minister of Education (20020358051 to XY; PC-SIRT0413 to XD), National Natural Science Foundation of China (39925018; 30270293 to XY; 30500183 to XD; 30600222 to JY), and National Institutes of Health (USA) (DK56292; CA92080) to XY (a Georgia Cancer Coalition Eminent Scholar). JY was supported by China Postdoctor (2005037560).

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