Exploring the regulation and function of human Lats1 and Aurora A kinases in mitosis

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Sämtliche Experimente sind von mir selbst durchgeführt worden, falls nicht explizit auf dritte verwiesen wird. Ich versichere, daß ich weder versucht habe, eine Dissertation oder Teile einer Dissertation an einer anderen Stelle einzureichen, noch eine Doktorprüfung durchzuführen.

Eunice H.Y. Chan
München, den 31-05-2007

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Summary

Mitosis is the process by which sister chromatids are equally segregated into two daughter cells. Tight control in various events during mitotic progression is essential for maintaining chromosome stability. Mitotic kinases including Cyclin dependent kinase 1 (Cdk1) and Aurora family are required for regulating proper mitotic progression by phosphorylating mitotic substrates thereby, controlling their activities, localization or abundance. On the other hand, these mitotic kinases are modulated by de-novo synthesis, activators, phosphorylation and ubiquitin-dependent proteolysis. A thorough understanding of the function and regulation of mitotic kinases could further our knowledge on mitotic progression.

In the first part of the thesis, we investigated the expression, localization and regulation of human Lats1 kinase, which is a close homologue of the yeast Dbf2 kinase family involved in the mitotic exit network (MEN). Despite the fact that Lats1 has been suggested to be a spindle protein that binds and inactivates Cdk1, we found that Lats1 is mainly cytoplasmic throughout the cell cycle by immunofluorescence microscopy. Both yeast two-hybrid and coimmunoprecipitation showed no significant interaction between Lats1 and Cdk1. Although Lats1 was highly phosphorylated during mitosis, no detectable kinase activity was observed. However, we identified Ste20 like kinase MST2 as the upstream regulator of human Lats1. Phosphorylation of Lats1 by Mst2 resulted in the activation of Lats1 kinase activity both *in vivo* and *in vitro*. This kinase-substrate relation was proven to be specific, as another distant Mst2 homolog, Mst4, did not possess this ability. Subsequent mass-spectrometry-based phosphosites analysis revealed that Mst2 phosphorylates Lats1 on more than five residues. Alanine mutations on Lats1T1079 and S909 impaired Lats1 kinase activity. Thus, we could not confirm the suggested role of Lat1 in mitosis. Instead, we show that similar to its *Drosophila* ortholog, Lats1 is involved in the Mst2 signaling pathway and might control developmentally regulated cell proliferation and apoptosis in mammals.

In the second part of this thesis, we characterized hBora, a novel Aurora A interactor originally found in *Drosophila*. We show that hBora is upregulated and phosphorylated during mitosis. siRNA-mediated knockdown of hBora led to spindle

formation defects and aneuploidy. hBora overexpression caused monoastral spindle formation and mislocalization not only of Aurora A but also Plk1. Further investigations showed that Cdk1 phosphorylation on hBoraSer252 leads to Plk1 binding and this may promote the SCF-mediated proteolysis of hBora. Indeed, Plk1 depletion led to an increase in hBora levels. Interestingly, the co-depletion of both hBora and Plk1 (to lower hBora levels in Plk1 depleted cells) rescued the localization of Aurora A to the centrosomes and bipolar spindle formation. Thus, we propose that hBora is a functional link between Plk1 and Aurora A and that by modulating the proteolysis of hBora, Plk1 could regulate Aurora A localization and activity. At the end, we also investigated the function of Aurora A and could show that Aurora A is required for centriole cohesion and centrosome separation.

Introduction

An overview of the cell cycle

The cell cycle is an ordered set of events that leads to the reproduction of two identical cells. The events culminating in cell duplication and division are in order: G1 (Gap phase1), S (Synthesis phase), G2 (Gap phase2) and M (Mitosis and cytokinesis) phase (Fig. 1). G1, S and G2 phases are collectively known as interphase, in which the cell spends most of its time. DNA replication occurs in S phase and the two gap phases, G1 (between M phase and S phase) and G2 (between S phase and M phase) allow the cell to grow and to prepare for the next phase. The M phase comprises the segregation of duplicated chromosomes (mitosis) and the distribution of chromosomes into two daughter cells (cytokinesis).

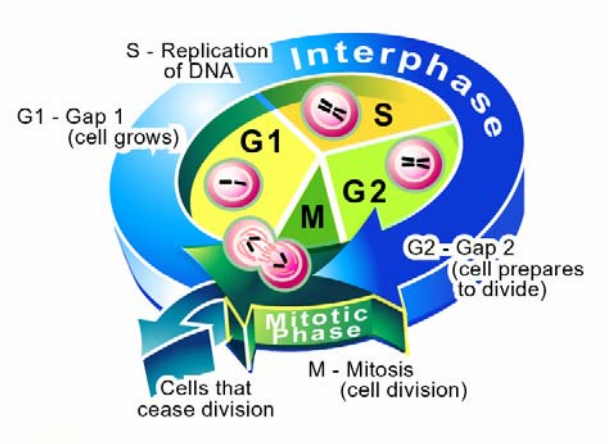

Figure 1. The cell cycle.
Cell cycle begins with duplication of the cell´s components, including exact duplication of each chromosome in S phase. These components are then divided equally between two daughter cells in M phase. Image adapted from "The Science Creative Quarterly", URL (scq.ubc.ca), artist: Jane Wang.

An overview of mitosis

Although being relatively brief, mitosis is the most dramatic event during the cell cycle. Mitosis is divided into 5 stages: prophase, prometaphase, metaphase, anaphase and telophase (Fig. 2). At prophase, the chromosomes undergo condensation. The two centrosomes, the major microtubule-organizing centres (MTOC) in animal cells (duplicated previously in S phase), increase the nucleation of highly dynamic microtubules (MTs). This leads to the separation of centrosomes and spindle aster formation (Doxsey, 1998; Luders and Stearns, 2007; Meraldi and Nigg, 2002). During

prometaphase, the nuclear envelope is broken down. MTs are captured by kinetochores situated on the centromeres of the mitotic chromosomes (Rieder, 2005). The capture of MTs emanating from opposite poles by sister chromatids promotes the congression of chromosomes, which then align at the equator of the spindle to form the metaphase plate. Once each sister-chromatid pair is attached to the opposite poles to form a bipolar mitotic spindle, the spindle checkpoint is inactivated which then leads to anaphase onset. At anaphase, the paired chromatids synchronously separate due to sudden loss in sister chromatid cohesion and each chromatid is then pulled towards the poles by shortening of kinetochore MTs (Anaphase A). The centrosomes move towards the cell cortex assisting further sister chromatid separation. (Anaphase B). During telophase, the chromosomes arrive at the poles of the spindle, the nuclear envelope reforms around the daughter chromosomes, and chromatin decondensation begins. Cytokinesis, the division of the cytoplasm, starts with the contraction of an actomyosin-based contractile ring, which assembles at the site of the spindle midzone and pinches into the cell to create two daughters, each with one nucleus and one centrosome (Pines and Rieder, 2001)

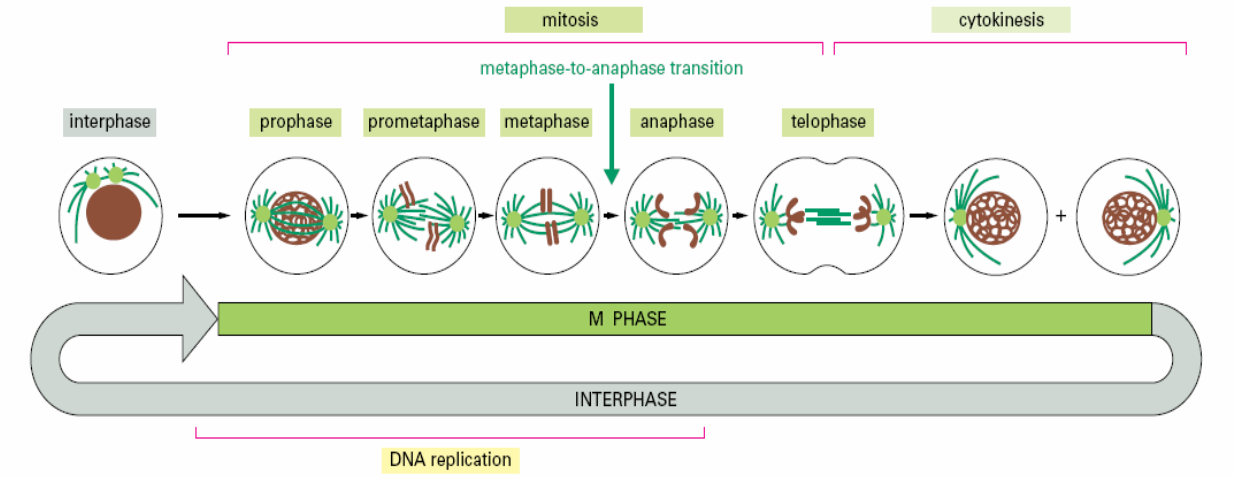

Figure 2. M phase progression in animal somatic cells.

Schematic representation of different stages of mitosis and cytokinesis. Mitosis is broadly divided into prophase, prometaphase, metaphase, anaphase and telophase. Cytokinesis is closely linked to mitosis. The colours shown here are brown for DNA, light green for centrosomes and dark green for MTs. Image adapted from Alberts et al., Molecular Biology of the Cell, fourth edition, 2002.

Regulation of mitotic progression by kinases

Rigorous control of mitotic events is essential for the successful completion of cell division and it is mediated by two major regulatory mechanisms: phosphorylation and proteolysis. These two mechanisms are interdependent as the proteolytic machinery is controlled by phosphorylation and many mitotic kinases are downregulated by degradation. Figure 3 summarizes the role of different mitotic kinases at different stages of mitosis.

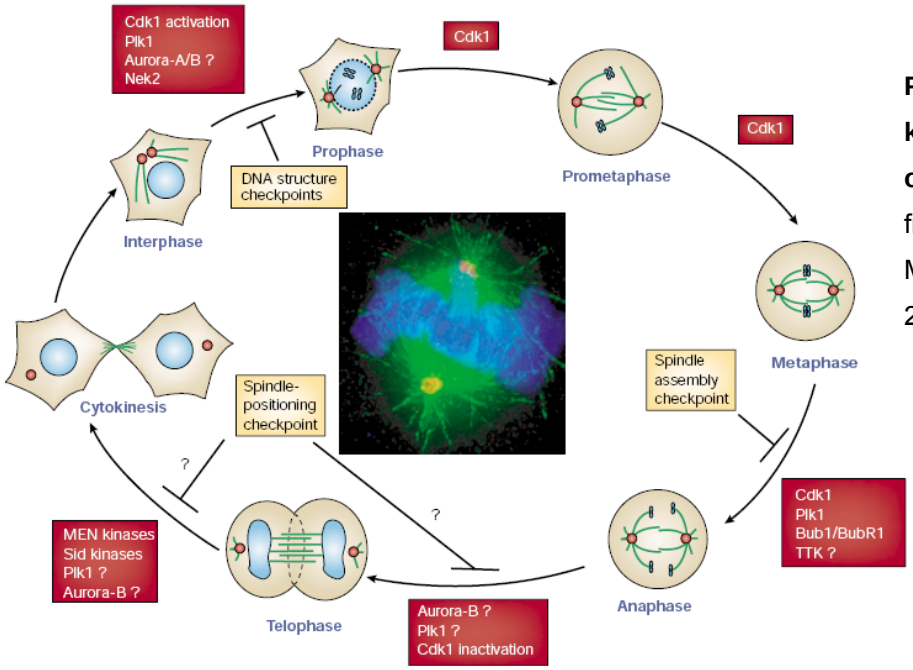

Figure 3. Role of mitotic kinases at different stages of mitosis. Image adapted from Nigg, Nature Reviews, Molecular Cell Biology, Vol. 2, January, 2001.

Cyclin-dependent kinase 1

Intense studies from the past decades had brought to light a number of kinases involved in the control of mitosis including the Polo and Aurora family kinases. Nevertheless, Cyclin-dependent kinase 1 (Cdk1), which is a founding member of a family of heterodimeric serine/threonine protein kinases termed Cdks (Cyclin-dependent kinases) (Morgan, 1997; Murray, 2004; Nigg, 2001) remains the most prominent mitotic kinase. Similar to other Cdks, Cdk1 consists of a catalytic subunit that has to bind to a regulatory subunit (called cyclin) in order to become enzymatically active (Hunt, 1991; Nigg, 1995). The protein levels of cyclins fluctuate during the cell cycle in a controlled manner (Evans et al., 1983) and this then directly regulates Cdk's activities. In

mammals, the activation of Cdk1 at the G2/M transition depends on the binding of cyclin A/B and dephosphorylation of two neighbouring residues in the ATP-binding site (threonine 14 and tyrosine 15) by Cdc25C which antagonizes the actions of Wee1 and Myt1 kinases (Ohi and Gould, 1999). Moreover, complete activation of the Cdk1 kinase is accomplished by phosphorylation of threonine 161 on the activation loop of Cdk1 (Makela et al., 1994; Nigg, 1996) by the Cdk-activating kinase (CAK) (Harper and Adams, 2001). Active Cdk1 first appears predominantly on centrosomes in prophase cells (Jackman et al., 2003). Its phosphorylation of numerous substrates, including nuclear lamins, condensins and microtubule-binding proteins, is essential for nuclear envelope breakdown, chromosome condensation, and spindle assembly, respectively (Andersen, 1999; Nigg, 1995). Furthermore, Cdk1-cyclin A/B complexes regulate the anaphase-promoting complex/cyclosome (APC/C), the major ubiquitin-dependent proteolytic machinery, which controls the timely degradation of critical mitotic regulators such as cyclin B (Peters, 2006). Thus, upon cyclin B destruction, Cdk1 becomes inactive, and Cdk1 substrates are dephosphorylated by counteracting phosphatases, which promotes mitotic exit and cytokinesis by facilitating nuclear envelope reformation, spindle disassembly and chromosome decondensation.

Polo-like kinase 1 (Plk1)

Polo-like kinases (Plks) have drawn much attention recently because of their close collaboration with Cdk1 in regulating mitotic events and the uncovering of Plk regulatory mechanisms. Polo-like kinase 1 (Plk1) is the most well-characterized Plk among the 4 family members in mammals and is highly conserved from yeast to human (Barr et al., 2004). The localization of Plk1 undergoes a highly dynamic change throughout mitosis, from the centrosomes, spindle poles and kinetochores to the central spindle and postmitotic bridge (Fig. 4).

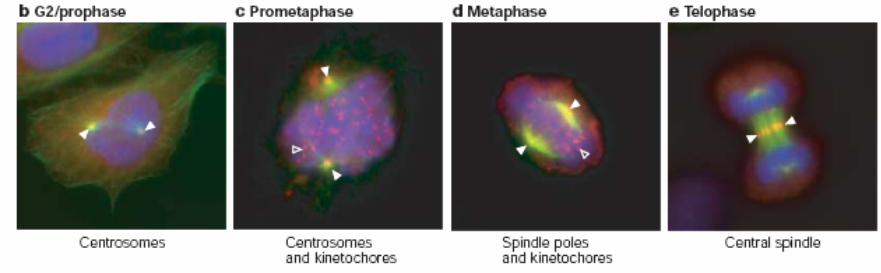

Figure 4. Localization of Plk1 (in red, arrows) during mitosis. Images adapted from Barr et al., Nature Reviews on Molecular Cell Biology, 2004.

Structurally, Plk1 features a C-terminal polo-box domain (PBD), which functions as a phosphopeptide-binding motif (Fig. 4) (Elia et al., 2003a). The PBD has been shown to be required for Plk1's targeting to substrates and subcellular localization (Lee et al., 1999; Reynolds and Ohkura, 2003; Seong et al., 2002). The PBD binds to phoshopeptides containing the consensus sequence S-pS/pT-P/X and the two residues His538 and Lys540 of PB2 are responsible for the binding (Cheng et al., 2003; Elia et al., 2003a; Elia et al., 2003b). Interestingly, the PBD can also interact with the kinase domain of Plk1, resulting in an inhibition of function, at least *in vitro* (Jang et al., 2002). This thus led to the hypothesis that upon prior phosphorylation by proline-directed serine/threonine kinase, the so-called priming kinases, phosphoproteins dock to the PBD. This liberates the catalytic domain of Plk1 due to a conformational change and thus promotes Plk1 kinase activation. Active Plk1 could then phosphorylate either the docking protein itself or other downstream targets (Fig. 5). Current evidence shows that Cdk1/Cyclin B is the most prominent priming kinase that phosphorylates Plk1 docking proteins. Nevertheless, MAP kinase Erk2 (Fabbro et al., 2005), Calmodulin dependent kinase II (CaMKII) (Rauh et al., 2005) and Plk1 itself (Neef et al., 2003) have also been shown as priming kinases.

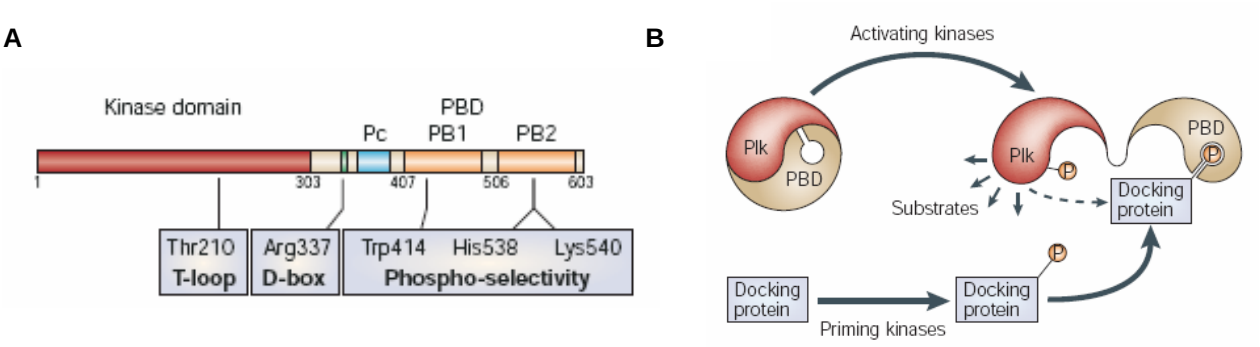

Figure 5. Plk1 domain structure and its regulation model.

A) Plk1 N-terminal habours the catalytic domain whereas the C-terminal PBD is required for targeting. Residues essential for activation, destruction and phosphopeptide binding are indicated. B) Model of Plk1 targeting to a docking protein prephosphorylated by a priming kinase, which then induces Plk1 kinase activity. Illustrations adapted from Barr et al., Nature Reviews on Molecular Cell Biology, 2004.

Plk1 has been implicated in regulating various stages of mitosis. Evidence suggests that together with Cdk1/Cyclin B, Plk1 is part of the amplification loop to trigger mitotic entry by regulating Cdc25C or Wee1 (van Vugt and Medema, 2005). In accordance with its localization to the centrosome and spindle poles, Plk1 is involved in centrosome maturation and separation at early mitosis (Barr et al., 2004; Glover, 2005). For instance, the phosphorylation of ninein-like protein (Nlp) and Kizuna by Plk1 has been shown to be required for the centrosomal MT nucleation process and the maintenance of spindle pole integrity, respectively (Casenghi et al., 2003; Oshimori et al., 2006). In addition, the centrosomal localization of another mitotic kinase, Aurora A, has also been shown to be dependent on Plk1 (De Luca et al., 2006; Hanisch et al., 2006). At metaphase-anaphase transition, Plk1 promotes the dissociation of chromosome cohesion by regulating cohesin, which holds the two sister chromatids together and shugoshin, which acts as a guardian for cohesion (Uhlmann, 2004; Watanabe, 2005). As mentioned previously, APC/C is essential for the timely degradation of numerous mitotic players for mitotic exit (Peters, 2006). At prometaphase, Emi1 is targeted for $SCF^{\beta\text{-}TrCP}$ mediated degradation after Plk1 phosphorylating its degron motif, which thus activates APC/C (Moshe et al., 2004; Schmidt et al., 2006). Together with Cdk1, Plk1 has also been shown to directly phosphorylate and activate different APC/C subunits at anaphase onset (Barr et al., 2004; Kraft et al., 2003) and further investigation is required to elucidate the role of Plk1 in this activation process. Finally, Plk1 modulates cytokinesis by phosphorylating other targets such as MKlp2 and Ect2 (Neef et al., 2003; Niiya et al., 2006).

Aurora kinase family

Aurora kinases were first identified in *Drosophila,* in a screen for mutated genes that leads to mitotic spindle and centrosome abnormalities (Glover et al., 1995). There are three Aurora kinases in mammals (Meraldi et al., 2004). Aurora A was found to be associated predominantly with the centrosomes and spindle from prophase to telophase (Berdnik and Knoblich, 2002). Aurora A localization and kinase activity is controlled by TPX2, a microtubules binding protein involved in the Ran-GTP mediated spindle assembly pathway. TPX2 targets Aurora A to the spindle and, moreover, TPX2 binding

keeps the phosphorylated activation segment (containing T288) of Aurora A in a conformationally active state, thus protecting Aurora A from inactivation by protein phosphatase 1 (PP1) (Fig. 6) (Bayliss et al., 2003; Kufer et al., 2003). Two other proteins, Ajuba and Bora have also been implicated in Aurora A kinase activation (Hirota et al., 2003; Hutterer et al., 2006), but their precise roles in Aurora A regulation require further study.

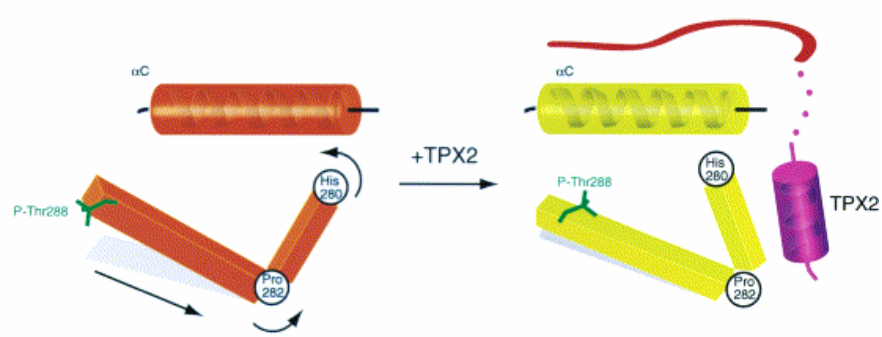

Figure 6. Schematic representation of the molecular mechanism of TPX2-mediated activation of Aurora A. The upstream stretch of TPX2 (red) anchors the TPX2 to the N-terminal lobe of Aurora A. The downstream stretch (pink helix) hooks the activation segment triggering a lever-arm-like movement, where rotations at His280^{AUR} and Pro282^{AUR} pull on Thr288^{AUR}, thus preventing the action of PP1. Figure adapted from Bayliss et al., Molecular Cell, 2003.

Aurora A activity is closely correlated with mitotic entry, the maturation of mitotic centrosomes and spindle assembly. Moreover, Aurora A controls the timely mitotic entry by modulating nuclear envelope breakdown (Hachet et al., 2007; Portier et al., 2007). It assists in the centrosome maturation by recruiting proteins such as γ-tubulin (Berdnik and Knoblich, 2002), D-TACC *(Drosophila*-Transforming, Acidic, Coiled Coil containing protein*)* (Giet et al., 2002), SPD-2 (Kemp et al., 2004), centrosomin (Terada et al., 2003) and chTOG (colonic and hepatic tumour overexpressed protein) (Conte et al., 2003) and, consequently, participates in spindle assembly and stability. Nevertheless, the molecular mechanisms of Aurora A function still remain obscure. A number of proteins have been identified to be Aurora A binding partners or substrates, but it is unclear whether all of these protein substrates actually are phosphorylated by Aurora A *in vivo* (Table 1) (Li and Li, 2006).

Protein	Characteristic	Function
Ajuba	Cell-cell adhesion protein	Activates Aurora A in G2
BRCA1	Breast cancer susceptibilty gene	BRCA1 phosphorylation by Aurora A plays a role in G2/M transtiton
CDC25B	Phosphotase activating Cdk1/CyclinB	Key activator of cell cycle
Cdh-1	E-Cadherin	Adaptor of APC/C
CPEB	Cytoplasmic polyadenylation eternal binding protein	Controls polyadenylation induced translation in germ cells
Eg5	Mitotic kinesin	Centrosome seperation and spindle bipolarity
Lats2	Tumor suppressor gene	Cell cyle regulation
p53	Transcritpion factor, tumor suppressor	Centrosomal p53 when phosphorylated promotes its degradation by MDM2
TPX2	Microtubule-associated Protein	Recruits Xklp2 kinesin to microtubules, activates Aurora A targeting the mitotic spindle
TACC1, 2, 3	Transforming acidic coiled coil	Regulates microtubule dynamics ,localizes D-TACC and its binding
PP1	Protein phosphatase 1	Regulator of cellular functions such as division, homeostasis and apoptosis
Bora	Cytoplasmic and nuclear protein	Activates Aurora A in G2
Histone H3	DNA-associated protein	Together with other histones associates with DNA to form the nucleosome

Table 1. Candidate substrates of Aurora A (modified from Li et al., Pharmacology & therapeutics, 2006).

In contrast, the function and mode of action of another Aurora family member, Aurora B, is relatively clear when compared with Aurora A. Aurora B is a chromosome passenger protein that forms a complex with INCENP, survivin and Borealin (Gassmann *et al.*, 2004; Sampath *et al.*, 2004). It localizes to kinetochores from prophase to metaphase, and to the central spindle and midbody in anaphase and telophase (Carmena and Earnshaw, 2003). The kinase activity of Aurora B is activated by INCENP, which itself is also an Aurora B substrate. Aurora B is required for spindle checkpoint signaling (Giet and Glover, 2001), central spindle formation and cytokinesis (Giet and Glover, 2001). A number of substrates of Aurora B have been discovered, including CENP-A required for chromosome condensation (Zeitlin *et al.*, 2001), MCAK (mitotic centromere associated kinesin) required for correcting the improper attachment of MTs to kinetochores (Andrews *et al.*, 2004; Lan *et al.*, 2004), MgcRacGAP, a GTPase activating protein required for cytokinesis (Hirose *et al.*, 2001; Minoshima *et al.*, 2003) and MKlp1 (mitotic kinesin-like protein), which is also required for cytokinesis (Guse *et al.*, 2005).

MEN/SIN kinases?

In budding yeast and fission yeast, a conserved signaling cascade known as mitotic-exit network (MEN) and septation-initiation network (SIN), respectively, controls key events during exit from mitosis and cytokinesis (Bardin and Amon, 2001). In higher eukaryotes, several kinases (Ndr/LATS family) are structurally related to a yeast SIN/MEN kinase (budding yeast Dbf2p/Mob1p and fission yeast Sid2p/Mob1p), but no functional homologies have yet been shown (Bardin and Amon, 2001; Nigg, 2001). Human Lats1 and Lats2 kinases have been implicated in regulating G1/S progression, cytokinesis and apoptosis, but the molecular pathways in which these kinases function remain to be clarified (Bothos et al., 2005; Li et al., 2003; Tao et al., 1999; Yang et al., 2004).

Aim of this thesis

The aim of this thesis has been to study the role of different kinases in mitotic progression. The thesis has been structured in two parts. In the first part, we explored the possible role of human Lats1 kinase in mitosis, mainly because of its close homology with the yeast Dbf2 kinase, which is involved in the mitotic exit network. We also studied its regulation by Ste20 like kinase Mst2, based on the fact that Lats has been shown to interact with Mst2 in *Drosophila*. In the second part, we turned to study the function and regulation of Aurora A kinase, by focusing on novel binding partners. We studied the interaction between Aurora A and hBora, a Aurora A activator originally identified in *Drosophila*. Our finding that hBora interacts with Aurora A and also another mitotic kinase, Plk1, then prompted us to study the regulation of Aurora A by Plk1 via hBora and their role in spindle assembly. At the end, we investigated the function of Aurora A by siRNA mediated depletion and overexpression study.

Part I: Basic characterization of human Lats1/2 kinases and their regulation by Ste20-like kinases Mst1/2

Introduction I

LATS: a tumor suppressor gene

The *Drosophila melanogaster warts* (*wts*) gene, also known as *large tumor suppressor* (*lats*), encodes a putative serine/threonine protein kinase. This gene was originally identified in two independent searches for loss of function mutants that gave rise to tissue overgrowth in flies (Justice et al., 1995; Xu et al., 1995). Two homologues genes were subsequently identified in mammals, named *LATS1* and *LATS2 (KPM)* (Hori et al., 2000; Nishiyama et al., 1999; Tao et al., 1999; Yabuta et al., 2000) . The human *LATS1* gene was able to rescue the *Drosophila wts/lats* mutant phenotype, arguing that it is a genuine orthologue of *Drosophila wts/lats* (Tao et al., 1999). Importantly, mammalian *LATS1* displays properties of a tumor suppressor gene. Mice with a disrupted *LATS1* gene showed ovarian stromal cell tumors and an increased incidence of soft tissue sarcomas (St John et al., 1999). Moreover, *LATS1* expression is reduced or absent in a number of human soft tissue sarcomas, suggesting that altered Lats1 levels might contribute to tumor formation also in human (Hisaoka et al., 2002).

Proposed mitotic function of human Lats

Concerning the cellular function of Lats kinases, two schools of thoughts have emerged, that do not have to be mutually exclusive. One proposed idea is that Lats plays a pivotal role during mitosis of the cell cycle. Based on the high homology of the Lats kinase domain with the yeast Dbf2 kinase family, a function of Lats1 during mitosis has been proposed. *Saccharomyces cerevisiae* Dbf2 is a component of the so-called mitotic exit network (MEN), which ensures proper chromosome segregation during mitosis. A number of other Dbf2 related kinases of various organisms have been implicated to function in diverse aspects of cell proliferation and morphogenesis. In human the closest Dbf2 and Lats homologs are the Ndr kinases, of which the functions are presently not known. Experimental evidence supporting a role for Lats1 in mitosis came with the observation that human Lats1 is a mitotic phospho-protein that could interact with the mitotic cyclin dependent kinase1 (Cdk1) during early mitosis (Tao et al., 1999). Cdk1 bound to Lats1 was devoid of cyclin A and cyclin B and hence in an

inactive state. Moreover, *Drosophila lats* phenotypes could be suppressed by mutations in Cdc2 and Cyclin A and based on these findings it was suggested that Lats1 might negatively regulate cell cycle progression by inhibiting Cdk1 (Tao et al., 1999). Additional evidence supporting a role for Lats1 in mitosis came from the observation that human Lats1 localizes to the mitotic spindle (Morisaki et al., 2002; Nishiyama et al., 1999). The role of Lats1 at the mitotic spindle is not known, but it has been proposed to play a role in targeting the focal adhesion protein, zyxin, to the spindle (Hirota et al., 2000).

Drosophila Lats is required for cell cycle exit and apoptosis

Another view on Lats functioning has come from studies on eye imaginal disc development in *Drosophila* embryos. During retinal development, *Drosophila wts/lats* mutants showed a delayed cell cycle exit and an absence of the normally occurring apoptotic cell death (Tapon et al., 2002). Further inspection revealed increased levels of cyclin E and DIAP1 (*Drosophila* inhibitor of apoptosis 1) in these mutant cells. Based on these observations it was proposed that *Drosophila* Wts/Lats regulates developmentally controlled cell cycle exit and apoptosis. Such a dual function could readily explain the tissue overgrowth phenotype observed in *wts/lats* mutants. Mutations in two additional genes were recently shown to produce phenotypes that are very similar to those seen in *wts/lats* mutants. One of these genes, termed *salvador* (*sav*) (also named *shar-pei*), codes for a protein with two WW domains and a predicted coiled coil, suggesting that it may function as an adaptor (Kango-Singh et al., 2002; Tapon et al., 2002). The other, termed *hippo* (*hpo*), codes for a protein kinase of the Ste20-family (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003). Reminiscent of *wts/lats* mutants, mutations in either *sav* or *hpo* also resulted in delayed cell cycle exit, reduced apoptosis, and increased levels of cyclin E and DIAP1. This genetic evidence strongly suggested a functional link between the proteins encoded by *hpo*, *wts* and *sav*, and in support of this view, these *Drosophila* proteins could be shown to interact with each other (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003) . Moreover, Hpo was able to phosphorylate both Wts/Lats and Sav, and the phosphorylation of Wts/Lats by Hpo was enhanced by the

presence of Sav (Pantalacci et al., 2003; Wu et al., 2003) . These data suggested that Wts/Lats, Sav and Hpo might form a trimeric complex in which Sav functions as an adaptor protein to bring Wts/Lats in close proximity to Hpo (Harvey et al., 2003).

Putative orthologs of *Drosophila* Sav and Hpo are also present in mammals. Although little is known about the putative human Sav ortholog, hWW45, this gene was found to be mutated in a number of cancer cell lines (Tapon et al., 2002). The likely human orthologs of *Drosophila* Hpo are the Mst2 and Mst1 protein kinases, with 60 % and 58 % sequence identity, respectively. When expressed in *Drosophila*, Mst2 was able to rescue the *hpo* mutant phenotype, showing that it can act as a functional orthologue (Wu et al., 2003). The molecular function of Mst2 is not known, but the related Mst1 kinase was reported to induce apoptosis upon overexpression (Graves et al., 1998; Lee et al., 2001) . In addition, both Mst1 and Mst2 are substrates of caspase 3. Thus, both Mst1 and Mst2 appear to be involved in apoptosis.

Inspired by the above two models of Lats1 functioning, we decided to explore the expression, localization and regulation of human Lats1. Surprisingly, we could not confirm previous reports suggesting a role for Lats1 in mitosis, despite Lats1 being phosphorylated during this stage of the cell cycle. Interestingly, however, we found that Mst2 and hWW45 interact with each other in human cells and that both Mst2 and Mst1 are able to phosphorylate Lats1 and Lats2, thereby stimulating Lats kinase activity. Detailed studies revealed that the activation of Lats1 by Mst2 results from the phosphorylation of two essential and highly conserved residues. From these data we conclude that Wts/Lats, Hpo/Mst2 and Sav/hWW45 form an evolutionary conserved regulatory module. The precise function(s) of this module remain to be unraveled but the available data point to a signal transduction pathway involved in controlling cell proliferation and apoptosis.

Results I

LATS1 is ubiquitously expressed in contrast to LATS2

To characterize the expression of the human *LATS1* and *LATS2* genes, cDNA panels (Clontech) of various human tissues were used. To distinguish between *LATS1* and *LATS2* expression a PCR based approach, with specific primer combinations, was used to survey these panels. Whereas *LATS1* turned out to be ubiquitously expressed, *LATS2* expression was limited to a small number of tissues and maximal expressions of *LATS2* were observed in leukocytes, lung, pancreas and placenta (Fig. 7A). No obvious correlation between *LATS1* and *LATS2* expression and mitotic activity of the different organs could be established. A relatively high number of PCR amplifications was required for *LATS2* detection (45 as compared to 35 for *LATS1*), suggesting that its expression is relatively low in comparison to *LATS1*. Examination of a cDNA panel of established human cell lines (Clontech) showed similar results (Fig. 7B), with relatively low *LATS2* expression levels (Fig. 7B). Although *LATS1* and *LATS2* expressions have been investigated separately before (Hori et al., 2000; Tao et al., 1999; Yabuta et al., 2000), this is the first direct comparison between *LATS1* and *LATS2* expression. Based on our results, indicating that *LATS1* is expressed more ubiquitously and to higher levels than *LATS2*, we decided to focus our research primarily on the analysis of Lats1.

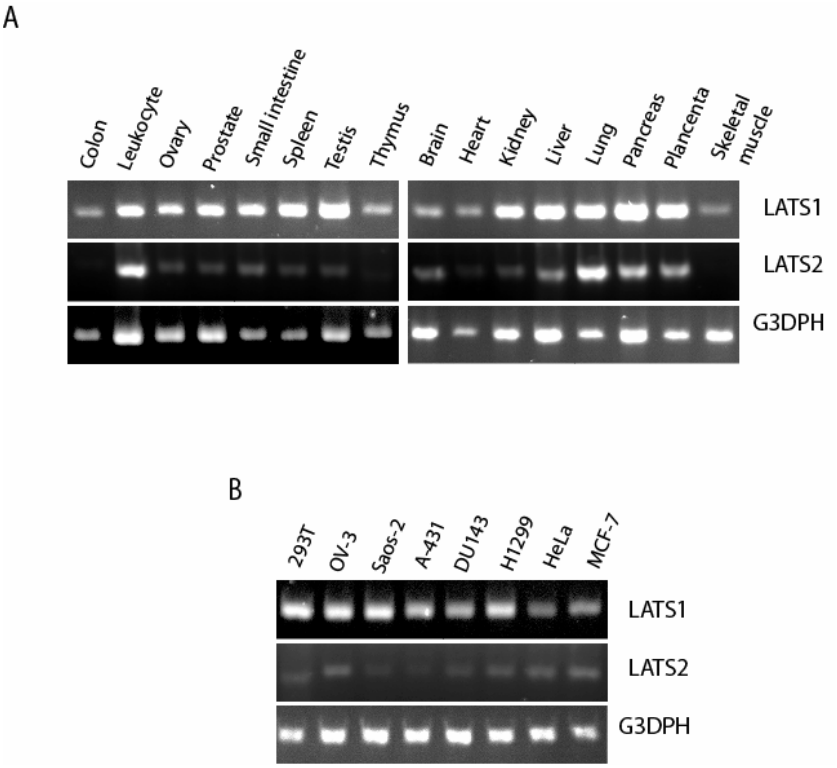

Figure 7. Expression profile of *LATS1* and *LATS2.*

(A) *LATS1* is widely expressed in various tissues. PCR was performed on cDNA panels from Clontech using primers described in MATERIALS AND METHODS. 35 and 45 cycles were used for amplification of *LATS1* and *LAT2*, respectively. The PCR products were then subjected to agarose gel electrophoresis, stained by ethidium bromide and visualized by UV exposure. *G3PDH* was used for normalization. (B) Ubiquitous expression of *LATS1* in established cell lines. See (A).

Lats1 is phosphorylated during mitosis

For investigating Lats1, a specific polyclonal antiserum against the N-terminal Lats1 domain (aa 267 to 403) was raised. This affinity purified anti-Lats1 antibody recognized a single band at about 110kDa on Western blots of HeLaS3 cell lysates (Fig. 8A). No signal was observed when blots were probed with the corresponding pre-immune serum. Depletion of Lats1 by siRNA showed a strong diminishment of the 110 kDa band, confirming that this band represents the Lats1 protein (Fig. 8B). To examine the cell cycle regulation of Lats1, synchronized HeLaS3 cells obtained by different drug arrest-release protocols were used. In a first experiment, cells were released from a double aphidicolin block at the G1/S phase and samples were taken at regular intervals. Western blot analysis showed that Lats1 levels remained fairly constant during the cell cycle (Fig. 8C), but that part of Lats1 showed a slightly retarded mobility (upshift) at T=4-10 hours. This was coinciding with maximal expression of cyclin B1, indicating that Lats1 was phosphorylated during mitosis. This effect was even more pronounced when nocodazole blocked and released cells were investigated, reflecting the higher percentage of mitotic cells obtained by this method. The retarded electrophoretic mobility of Lats1 clearly paralleled the expression of cyclin B1 (Fig. 8D). The upshift was also observed in non-drug treated mitotic shake off cells indicating that this is not a drug-based artefact (Fig. 8D). Our results are consistent with a previous observation, showing a phosphorylation mediated upshift of Lats1 in nocodazole blocked and released mitotic cells (Tao et al., 1999). That Lats1 phosphorylation parallels cyclin B1 expression suggests that Cdk1/cyclin B1 could be responsible for this upshift.

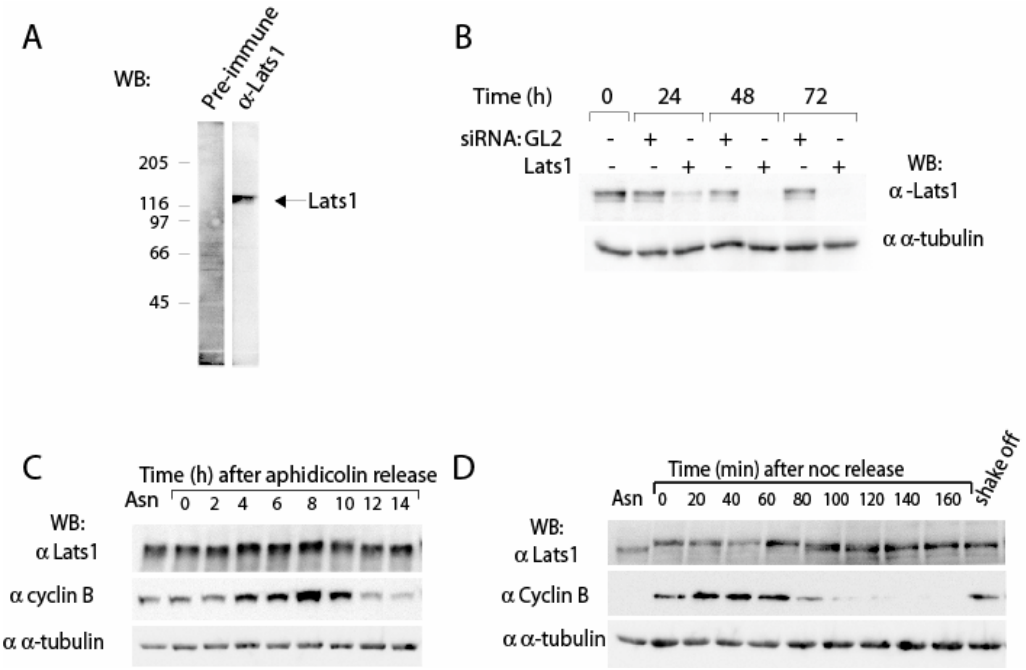

Figure 8. Characterization of the Lats1 antibodies and cell cycle profiles of Lats1.

(A) Lats1 polyclonal antibodies recognize endogenous Lats1 protein. HeLaS3 cell extracts were immunoblotted by pre-immune IgG or affinity purified anti-Lats1 antibodies. Arrow represents endogenous Lats1. (B) Effective silencing of endogenous Lats1 upon siRNA treatment. HeLaS3 cells were transfected with siRNA duplex. Total cell extracts were harvested at the indicated time points and subjected to immunoblotting by anti Lats1 and α-tubulin antibodies. (C) Constants levels of Lats1 protein throughout the cell cycle and upshift during mitosis. HeLaS3 cells were synchronized by aphidicolin for 14 h. Then cells were released into fresh medium and harvested at the indicated time points. Cell extracts were then subjected to immunoblotting analysis by anti-Lats1 and α-tubulin antibodies. (D) Lats1 gets highly upshifted during mitosis. HeLaS3 cells were arrested in prometaphase by pre-synchronization with thymidine and subsequently nocodazole as described in MATERIALS AND METHODS. Cell extracts were resolved by SDS-PAGE and probed with anti Lats1, cyclin B1 and α-tubulin antibodies.

Lats1 and Cdk1 do not interact in either coimmunoprecipitation or yeast two-hybrid

In support of the above possibility, it has been reported that Lat1 is a Cdk1/cyclin B1 substrate. Surprisingly, though, it also has been reported that Lats1 can bind to and inhibit Cdk1 during early mitosis when it is in its phosphorylated form. This is difficult to reconcile with the observation that Cdk1/cyclin B1 is probably responsible for Lats1 phosphorylation and we therefore tested this possible interaction between Cdk1 and Lats1. According to our results, we could not establish any interaction by either two-hybrid analysis (Fig. 9A) or co-immunoprecipitation (Fig. 9B). Thus Lats1 is a mitotic phosphoprotein, but a role for Cdk1 inhibition could not be confirmed.

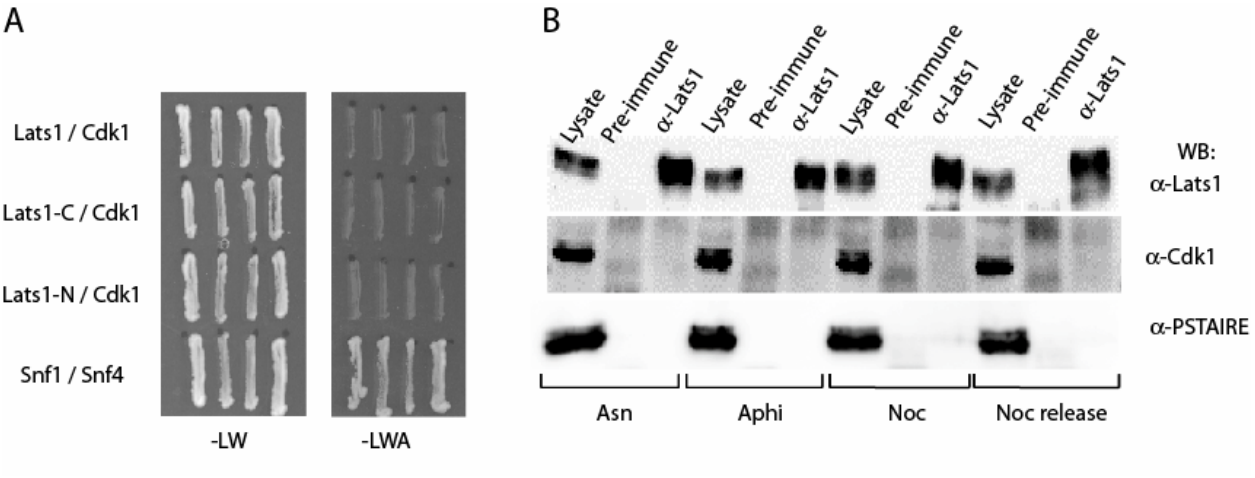

Figure 9. No significant interaction between Lats1 and Cdk1 by yeast two-hybrid and coimmunoprecipitation.

(A) Yeast two-hybrid indicated the absence of Lats1 and cdk1 interaction. Full-length, C-terminal and N-terminal of Lats1 in pGAD was transformed together with cdk1 in pGBD. Then the colonies were grown on minus leu, trp and ade plates for selection. (B) Cdk1 does not coimmunoprecipitate with Lats1 throughout the cell cycle. HeLaS3 cells were synchronized by aphidicolin (lanes 4-6), nocodazole (lanes 7-9) for 14 h and released from nocodazole for 90 min (lanes 10-12). Then cell extracts were prepared and used for immunoprecipitating endogenous Lats1 (upper panel). Coimmunoprecipitated complexes were then probed with anti-Cdk1 (middle panel) and anti-pistaire antibodies (lower panel).

Lats1 shows a diffuse cytoplasmic staining throughout the cell cycle

Previous studies had indicated that Lats1 localizes to the mitotic spindle (Hirota et al., 2000; Nishiyama et al., 1999), suggesting a role for Lats1 during mitosis. To substantiate these findings we used our specific polyclonal Lats1 antibody to investigate Lats1 localization in HeLaS3 cells. In interphase cells this antibody showed a diffuse cytoplasmic staining throughout the cytoplasm, but no staining from the nucleus (Fig. 10A, upper panels). In HEK293T and U2OS cells Lats1 staining was also diffuse throughout the cytoplasm, but also faint staining could be observed at cell-cell contacts (data not shown). The significance of the latter observation has so far not been further investigated. In all experiments the control pre-immune serum showed a significant by lower cytoplasmic staining (Fig. 10A, lower panels). Depletion of Lats1 by siRNA in HeLaS3 cells showed a significant decrease of the Lats1 cytoplasmic staining (Fig. 10B, lower panel), which was not observed in a control siRNA experiment with GL2 duplexes (Fig. 10B, upper panel). This clearly indicates that the observed cytoplasmic staining corresponds to Lats1 protein. This was further confirmed by expression studies of myc-Lats1 and GFP-Lats1 that also both resided in the cytoplasm (Fig. 10C, data not shown). The Lats1 antibody readily detected these expressed tagged proteins, confirming once more that this antibody recognized Lats1 in cells (Fig. 10C). To our surprise no co-localization of endogenous Lats1 was observed with spindle microtubules in mitotic cells (Fig. 10D). Instead a diffuse cellular staining was seen. Similar results were obtained when we investigated expressed myc- or GFP- tagged Lats1 in mitotic cells (Fig. 10E). In previous studies, Lats1 spindle staining was observed in cells that were first pre-permeabilized in microtubule stabilizing buffer, before fixation and staining (Hirota et al., 2000; Nishiyama et al., 1999). In contrast we used standard paraformaldehyde and methanol fixation procedures. Pre-permeabilization obviously leads to loss of proteins from the cell and moreover could lead to re-localization of proteins. This might explain why in these former studies weak spindle localizations were observed.

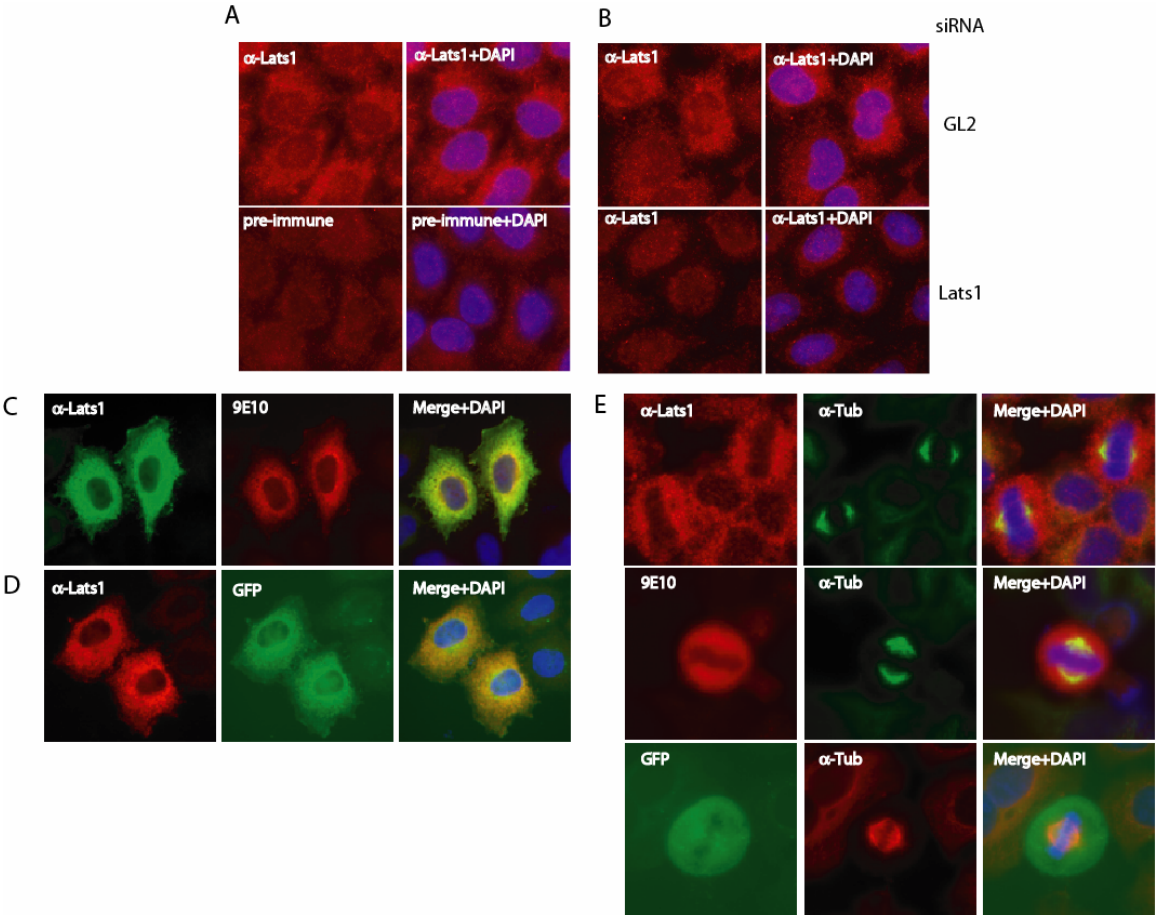

Figure 10. Lats1 shows a cytoplasmic staining throughout the cell cycle.

(A) Cytoplasmic staining of endogenous Lats1 in both interphase and mitotic cells. Methanol-fixed HelaS3 cells were co-stained with α-tubulin (green) and α-Lats1 or pre-immune serum (red). DNA was visualized by DAPI (blue). (B) Lats1 siRNA treatment results in decreased cytoplasmic Lats1 levels. HeLaS3 cells were subjected to Lats1 and GL2 (as control) siRNA treatment for 48 h. Cells were then fixed and the levels of endogenous Lats1 were monitored with Lats1 (red), α-tubulin (green) and DNA (blue). (C) Myc and GFP-tagged Lats1 showed cytoplasmic straining. HeLaS3 cells were transiently transfected with GFP-Lats1 (right) or myc-Lats1 (left). Cells were fixed and labeled with Lats1 (green) and α-myc antibodies (red, for myc-transfection only) and DAPI (blue). (D) Endogenous Lats1 does not localize to the spindle. Methanol-fixed cells were labeled with Lats1 (red), α-tubulin (green) and DAPI (blue). (E) Cytoplasmic localization of overexpressed myc-Lats1 during mitosis. HeLaS3 cells transfected with myc-Lats1 for 24 h were assayed by labeling with myc (red) and α-tubulin (green). DNA is strained in blue with DAPI.

Lats1 is absent from a spindle preparation

To circumvent the problem that absence of staining never proves absence of protein, we resorted to biochemistry. Based on a previously published method (Sillje and Nigg, 2006; Zieve and Solomon, 1982), we isolated mitotic spindles from HeLaS3 cells. The success of this isolation of mitotic spindles was verified by visualization of the spindles by differential interference contrast (DIC) light microscopy (Fig. 11A). To further validate the purification of these spindles, we compared samples prepared in the presence of taxol (microtubule stabilizing) or in the presence of nocodazole (microtubule destabilizing). Western blot analysis revealed that spindle components, including α-tubulin, Plk1, Aurora A and TPX2 were present in samples prepared in the presence of taxol, but not in the presence of nocodazole, (Fig. 11B and data not shown). As anticipated from the immunofluorescence data, Lats1 could not be detected in these spindle preparations by Western blotting. Although we cannot exclude that Lats1 might be lost during the isolation procedure, we like to note that all known spindle proteins tested so far could be readily detected in these isolates. Together with the immunofluorescence microscopy results, the most straightforward interpretation is that low levels of Lats1 show primarily a diffuse cellular distribution during mitosis.

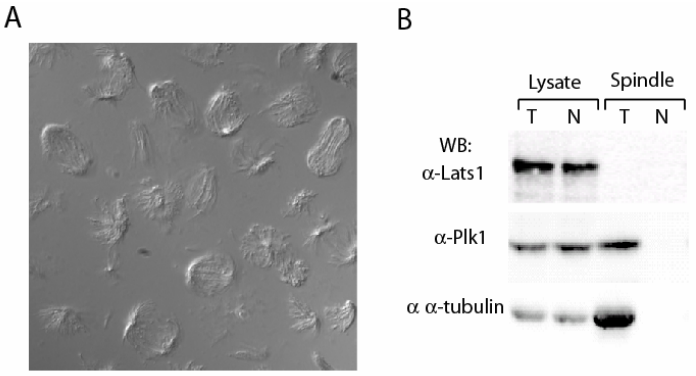

Figure 11. No cofractionation of Lats1 with spindles.

(A) Photo of isolated spindles. Spindles were prepared as described in MATERIALS AND METHODS. Isolated spindles were then visualized by differential interference contrast (DIC) light microscopy. (B) Lats1 is absent from the spindles preparation. HeLaS3 cells were synchronized by aphidicolin and, subsequently, taxol or nocodazole (as negative control) were used for isolating the spindles. The resulting spindles were subjected to immunoblotting by anti-Lats1, Plk1, α-tubulin antibodies.

Lats1 is active in okadaic acid (OA) treated cells, but not in mitotic cells

Many kinases are controlled by reversible phosphorylation, and we therefore next asked whether the observed Lats1 phosphorylation during early mitosis would regulate Lats1 kinase activity. Previously, no significant kinase activity measurements for Lats1 had been reported for any species, and indeed we found it difficult to measure Lats1 kinase activity. No good exogenous substrates for Lats1 could be found, suggesting that Lats1 is not a promiscuous kinase but only phosphorylates a very limited number of physiological substrates. Lats1 showed, however, clear auto-phosphorylation, and we therefore relied mostly on this to attest Lats1 kinase activity (Fig. 12A). The endogenous Lats1 kinase activities of interphase and nocodazole-treated mitotic HeLaS3 cells were determined by *in vitro* kinase assays with immunoprecipitated endogenous Lats1 in the presence of [γ-^{32}P]ATP. No significant kinase activities were observed in either asynchronous cells or nocodazole-treated mitotic cells (Fig. 12A). Thus Lats1 phosphorylation during mitosis did not correlate with an increased kinase activity. Interestingly, however, treatment of cells with the PP1 and PP2A serine/threonine protein phosphatase inhibitor okadaic acid (OA) (Cohen et al., 1990) showed a marked increase in Lats1 kinase activity as shown by the appearance of a radioactive band at 110 kDa (Fig. 12A). No radioactive bands were observed in immunoprecipitates with pre-immune IgG´s, indicating that this was Lats1 specific (Fig. 12A). A similar activation by OA has previously been reported for the homologues Ndr1 kinase, indicating that this family of Lats and Ndr kinases is regulated by reversible serine/threonine phosphorylation (Millward et al., 1999). As shown by Western blotting, OA treatment resulted in a more pronounced upshift of Lats1 as compared to nocodazole treatment, indicating that distinct or additional serine or threonine residues are phosphorylated in the presence of OA that contribute to Lats1 activation.

To corroborate these findings, myc tagged Lats1 wildtype (WT) and a catalytically inactive kinase dead (KD) mutant, containing a mutation changing the conserved aspartate in subdomain VII into alanine (D846A), were transiently expressed in HEK293T cells. After treatment of these cells with nocodazole, OA, or nothing, these recombinant proteins were immunopurified, using anti-myc 9E10 beads, and used in *in vitro* kinase assays. Again, significant Lats1 kinase activity was observed only in

immunoprecipitates from OA treated cells (Fig. 12B). No activity was observed under any of these conditions with the myc-Lats1KD mutant (Fig. 12B), excluding the possibility that the observed phosphorylation of Lats1 could be attributed to co-precipitating kinases. During the course of this study it was reported that Lats1 could be slightly activated upon release from a nocodazole block for 10-20 min in Rat1 cells (Iida et al., 2004). To test this *in vitro* kinase assays were performed on myc-Lats1WT and KD immunoprecipitates from nocodazole blocked and released HEK293T cells. In contrast to what has been reported previously, we did not observe any significant increase in Lats1 activity in nocodazole released cells as compared to OA induced activation (Fig. 12C). Altogether our data show that despite being phosphorylated during mitosis, mitotic Lats1 does not contain significant kinase activity. Lats1 was however strongly activated by OA treatment, suggesting that serine/threonine phosphorylation is important for Lats1 activity.

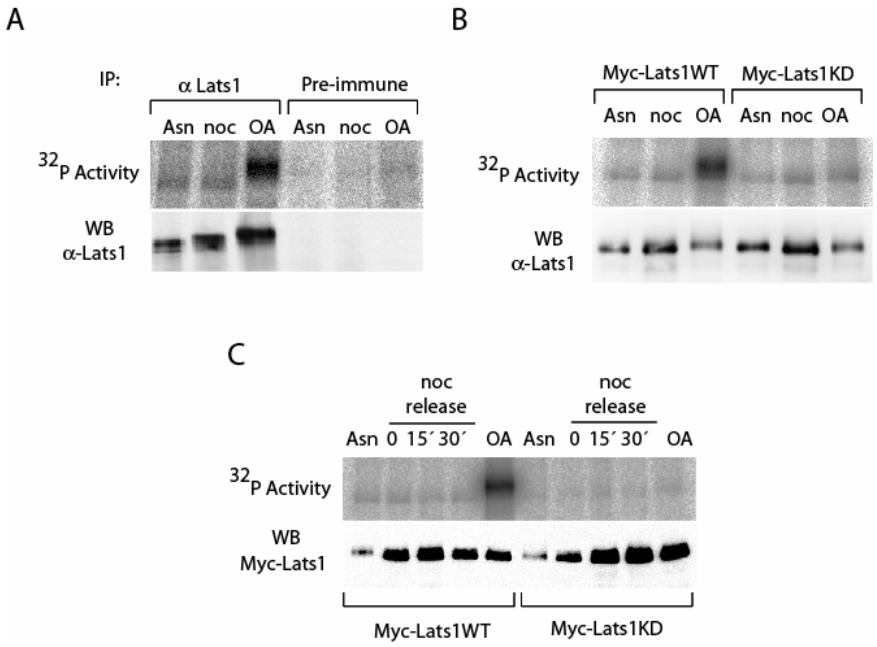

Figure 12. Activation of Lats1 kinase activity by okadaic acid (OA).

(A) *In vivo* activation of Lats1 kinase by okadaic acid (OA). HeLaS3 cells were treated with nocodazole for 14 h or 1 μM OA for 1 h and HEPES cell extracts were prepared. Endogenous Lats1 was then pulled down and kinase activities were detected by *in vitro* kinase assay. Levels of immunoprecipitated Lats1 protein were confirmed by Western blotting. Pre-immune IgG pull down was used as a negative control. (B) Overexpressed Lats1 can be activated by okadaic acid. HEK293T cells were transiently transfected

with either myc-Lats1WT or KD for 24 h, before cells were treated with OA for 1 h or blocked by 200 ng/ml nocodazole for 14 h and released from prometaphase. Cell extracts were then prepared at the indicated time points. Kinase activities of the myc-immunoprecipitates were assayed and detected by autoradiography. Western blot was performed to check the levels of myc-Lats1. (C) No significant Lats1 activities were detected during mitosis when compared with OA treated cells. The kinase activities of overexpressed Lats1 were examined as in (B) while nocodazole treated cells were also released into fresh medium for 15 min and 30 min before harvesting.

Mst2 interacts with hWW45

Although a mitotic modification of Lats1 could be confirmed, previously reported spindle association and Cdk1 interaction could not be reproduced. Hence, we next turned to explore the possible interaction between human Lats1, hWW45 and Mst2 kinase, as described for the purported respective homologues in *Drosophila* (Wts, Sav and Hpo). To explore possible *in vivo* interactions between human Lats1, hWW45 and Mst2, the three proteins were epitope-tagged and co-expressed in HEK-293T cells. FLAG-Mst2 was then immunoprecipitated with anti-FLAG antibody and co-precipitation of hWW45 or Lats1 was assessed by Western blotting. In FLAG-Mst2 immunoprecipitates, GFP-tagged hWW45 could readily be detected, indicating that these two proteins are able to form a complex (Fig. 13A). No interaction was observed between GFP-hWW45 and an unrelated FLAG-tagged protein (FLAG-Ect2), demonstrating that the Mst2-hWW45 interaction was specific (Fig. 13B). Myc-Lats1, on the other hand, could not be detected in Mst2 immunoprecipitates, regardless of whether or not GFP-hWW45 was co-expressed (Fig. 13A). Similarly, in a reciprocal experiment, both FLAG-Mst2 and GFP-hWW45 were absent from myc-Lats1 immunoprecipitates (Fig. 13C). Thus, under the experimental conditions used here, human Lats1 did not stably interact with either hWW45 or Mst2. To corroborate these results and map the interaction domains between Mst2 and hWW45, yeast two-hybrid assays were performed. Supporting the results of the co-immunoprecipitation experiments, a yeast-two hybrid interaction could be demonstrated between Mst2 and hWW45 (Fig. 13D, upper panel), and this interaction required the C-terminal halves of the two proteins (Fig. 13D, lower panel). In contrast, no interaction could be detected between Lats1 and either Mst2 or hWW45, regardless of whether full-length Lats1 or Lats1 domains were used (Fig. 13D, upper panel and data not shown). Results were independent of whether the proteins were

fused to the Gal4 DNA-binding or activation domains (data not shown). Taken together, these experiments indicate that Mst2 and hWW45 are able to form a stable complex *in vivo*. In contrast, no stable interaction could be observed between either Mst2 or hWW45 and Lats1.

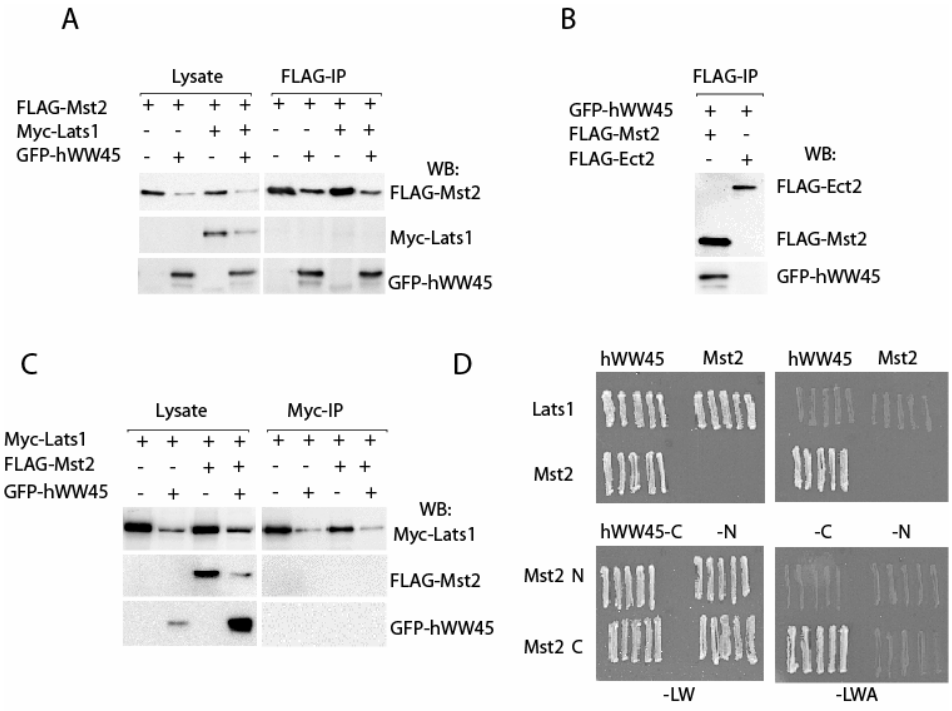

Figure 13. Mst2 interacts with hWW45.

(A) HEK293T cells were co-transfected with plasmids expressing myc-Lats1, FLAG-Mst2 and GFP-hWW45, as indicated. Cell lysates (left) and FLAG-Mst2 immunoprecipitates (right) were immunoblotted (WB) with antibodies against FLAG, myc and GFP. (B) GFP-hWW45 was co-expressed with FLAG-Mst2 or FLAG-Ect2 (negative control). FLAG-immunoprecipitates were subsequently probed with antibodies against FLAG and GFP (C) Experiment as described under A, except that myc-Lats1 was immunoprecipitated with anti-myc 9E10 beads. (D) Yeast two-hybrid analysis with full-length Lats1, Mst2 and hWW45 proteins (upper panel) or with N- and C-terminal domains of Mst2 and hWW45 (lower panel). Interactions were reflected by growth on selective plates (-LWA) (right). For control, growth on non-selective plates (-LW) is shown (left).

Lats1 is phosphorylated by Mst2

In a next series of experiments, recombinant Lats1, Mst2 and hWW45 were produced by *in vitro* coupled transcription translation (IVT) and interactions were explored by co-immunoprecipitation experiments. Myc-hWW45, but not myc-Lats1, could be co-

immunoprecipitated with FLAG-Mst2 (Fig. 14A, right panel), confirming and extending the results shown in Figure 13. Most interestingly, however, these experiments also revealed that co-translation with FLAG-Mst2 resulted in an upshift of myc-Lats1 in SDS-PAGE (Fig. 14A, left panel), suggesting that Mst2 could cause Lats1 phosphorylation in the lysates. To further examine this possibility, both wild-type (WT) and catalytically inactive (kinase dead; KD) mutants of Lats1 and Mst2 were expressed individually in HEK293T cells. After immunopurification of the proteins via their myc- (Lats1) or FLAG- (Mst2) tags, *in vitro* kinase assays were carried out in the presence of [γ-^{32}P]ATP. Substantial incorporation of ^{32}P into myc-Lats1WT and KD proteins could be seen following incubation with FLAG-Mst2WT but not FLAG-Mst2KD (Fig. 14B). In contrast, although FLAG-Mst2WT appeared to undergo autophosphorylation, no phosphorylation of FLAG-Mst2KD proteins by myc-Lats1WT could be detected (Fig. 14B). Thus, Mst2 could clearly phosphorylate Lats1, at least *in vitro.* These data also indicate that the presence of hWW45 was not required for phosphorylation of Lats1 by Mst2.

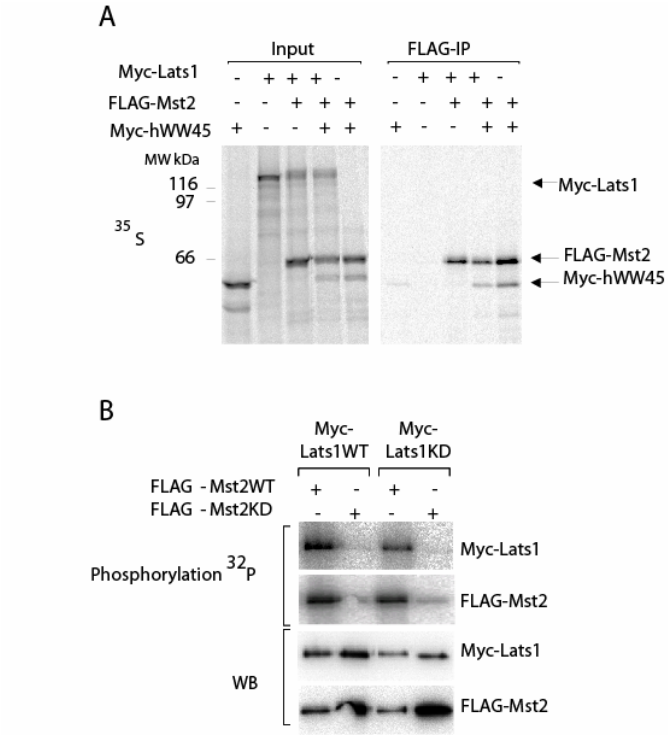

Figure 14. Mst2 phosphorylates Lats1 *in vitro*. (A) Myc-Lats1, FLAG-Mst2 and myc-hWW45 were produced in different combinations by IVT in the presence of ^{35}S methionine. Flag-Mst2 was subsequently immunoprecipitated and IVT input (left) and FLAG-immunoprecipitates (right) were analysed by SDS-PAGE followed by autoradiography. (B) Immunopurified myc-Lats1WT or KD (on beads) and FLAG-Mst2WT or KD (in solution) were mixed in different combinations in Lats1-kinase buffer in the presence of [γ-^{32}P]ATP, as indicated. Kinase reactions were analysed by SDS-PAGE followed by autoradiography. Western blotting with anti-myc 9E10 and FLAG antibodies confirmed the presence of myc-Lats1 and FLAG-Mst2 proteins.

Lats1 is activated by Mst2 -mediated phosphorylation

Next, we asked whether phosphorylation by Mst2 would lead to activation of the Lats1 kinase. It proved difficult to find an exogenous substrate for measuring Lats1 kinase activity, which probably indicates that Lats kinases phosphorylate only a limited number of physiological substrates. However, Lats1 WT was found to auto-phosphorylate, which made it possible to use autophosphorylation as a read-out for Lats1 kinase activity. To determine the effect of Mst2 phosphorylation on Lats1 activity, immobilized myc-Lats1WT or KD proteins were phosphorylated by pre-incubation with soluble FLAG-Mst2WT in the presence of non-radioactive ATP. As negative controls, myc-Lats1 proteins were incubated in parallel with FLAG-Mst2KD. Subsequently, FLAG-Mst2 proteins were removed by extensive washing and myc-Lats1 proteins were incubated for autophosphorylation to occur in the presence of radioactive [γ-^{32}P]ATP. After pre-phosphorylation by FLAG-Mst2WT, myc-Lats1WT showed strong autophosphorylation activity, whereas the activity of the same protein pre-incubated with FLAG-Mst2KD was barely detectable (Fig. 15A). No incorporation of ^{32}P could be observed into myc-Lats1KD proteins, demonstrating that FLAG-Mst2 kinase had been effectively removed prior to the addition of [γ-^{32}P]ATP (Fig. 15A). These results clearly show that Mst2-mediated phosphorylation stimulates the *in vitro* kinase activity of Lats1.

To extend these findings to an *in vivo* situation, myc-Lats1WT and KD were co-expressed with FLAG-Mst2WT or KD in HEK293T cells. Then, myc-Lats1 kinases were immunoprecipitated and the associated activities determined. Strong stimulation of myc-Lats1WT kinase activity could be observed upon co-expression with FLAG-Mst2WT, but not with FLAG-Mst2KD (Fig. 15B). No significant activities were associated with myc-Lats1KD proteins, ruling out the presence of co-precipitating kinases. Moreover, Western blot analyses showed the absence of contaminating FLAG-Mst2 proteins (Fig. 15B). We conclude that Mst2 is able to activate Lats1 kinase also *in vivo*.

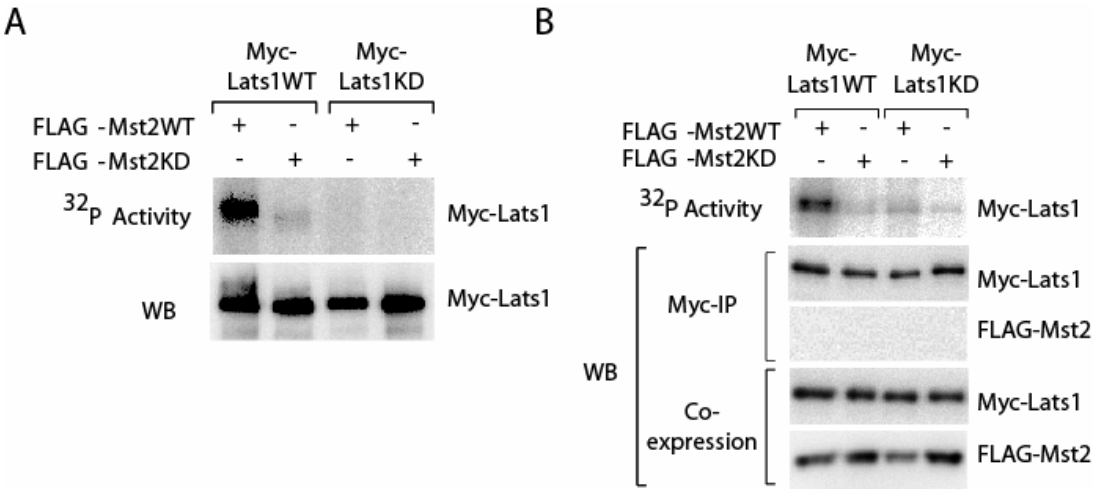

Figure 15. Mst2 activates Lats1 both *in vitro* and *in vivo*.

(A) Myc-Lats1WT or KD proteins immunopurified from IVT reactions (on beads) were mixed with equal amounts of soluble FLAG-Mst2WT or KD isolated from HEK293T cells. These proteins were pre-incubated for 30 min at 30°C in the presence of 10 μM ATP, after which the FLAG-Mst2 proteins were washed away. The myc-Lats1 beads were then used for kinase assays in the presence of [γ-^{32}P]ATP. After SDS-PAGE, Lats1 autophosphorylation was visualized by autoradiography. Western blotting revealed that similar amounts of myc-Lats1 proteins were recovered after washing. (B) Myc-Lats1WT or KD was expressed in HEK293T cells together with FLAG-Mst2WT or KD, as indicated. Myc-Lats1 proteins were immunoprecipitated with anti-myc 9E10 beads and used for *in vitro* kinase assays. After SDS-PAGE, Lats1-autophosphorylation was visualized by autoradiography. Western blot analysis confirmed equal expression levels (co-expression). Moreover, equal amounts of myc-Lats1 proteins were recovered by immunoprecipitation and these precipitates were devoid of residual FLAG-Mst2 proteins (Myc-IP).

Specific activation of Lats1 and Lats2 by Mst2/1 kinases

Considering the extensive sequence homology between human Lats1 and Lats2, we asked whether Mst2 could also activate Lats2. Myc-Lats2WT and KD proteins were prepared by IVT, together with either FLAG-Mst2WT or KD. Then, myc-Lats proteins were immunoprecipitated, washed free of Mst2, and subjected to kinase assays. For comparison, myc-Lats1WT and KD were analyzed in parallel. FLAG-Mst2WT, but not KD, could readily stimulate the auto-phosphorylation activity of both myc-Lats1 and -Lats2WT proteins (Fig. 16A, upper panel). Although ^{32}P incorporation into myc-Lats2 was lower than that into myc-Lats1, this can be attributed to a difference in the corresponding protein levels, as shown by Western blotting (Fig. 16A, lower panel). Thus, Mst2 is clearly able to activate both Lats1 and Lats2.

Mst2 belongs to a kinase family that comprises several members. Although Mst2 is most closely related to *Drosophila* Hpo and capable of functional complementation (Wu et al., 2003), it seemed possible that other Mst family members could carry out similar functions. We therefore tested two additional Mst kinases for their ability to activate Lats kinases *in vitro*, notably Mst1, a close homologue of Mst2, and Mst4, a more distant family member implicated in cell migration and polarization (Preisinger et al., 2004). Using the IVT assay described above, we found that both FLAG-Mst2 and Mst1 were able to activate myc-Lats1, whereas Mst4 produced little, if any, activation (Fig. 16B, top row). As judged by their ability to incorporate ^{32}P through autophosphorylation, all FLAG-MstWT kinases were similarly active, whereas the corresponding KD mutants were inactive (Fig. 16B, second row). Recovery of the myc-Lats1 protein and the various FLAG-Mst proteins was monitored by Western blotting (Fig. 16B, bottom rows). These results show that Mst2 and Mst1 can activate Lats1, whereas a more distant family member, Mst4, displays little, if any, activity.

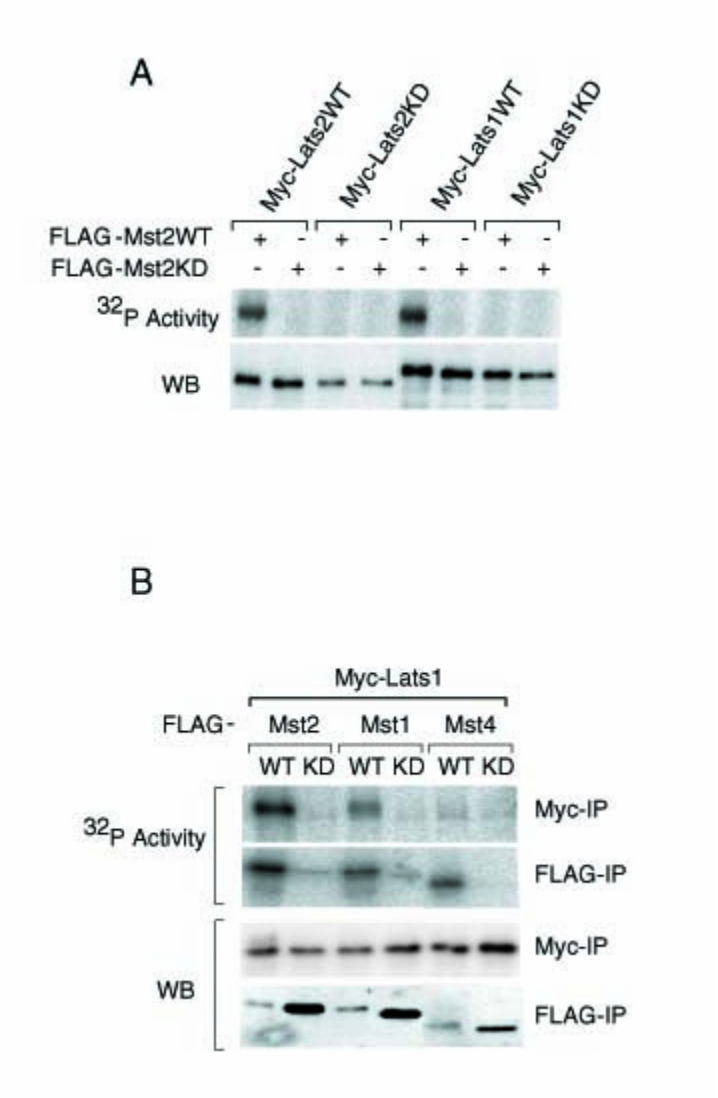

Figure 16. Lats kinases are specifically activated by Mst1 and Mst2.

(A) The indicated myc-Lats proteins were co-expressed by IVT together with FLAG-Mst2WT or KD. They were then immunopurified and used for *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. After SDS-PAGE, Lats autophosphorylation was visualized by autoradiography. Western blotting of the immunoprecipitates with anti-myc 9E10 antibodies, showed lower levels of myc-Lats2 as compared to myc-Lats1. (B) Myc-Lats1WT or KD proteins were co-expressed by IVT together with the indicated FLAG-Mst proteins. They were then immunopurified and used for *in vitro* kinase assays as described above. In parallel, FLAG-Mst proteins were immunopurified with anti-FLAG M2 beads and used for *in vitro* autophosphorylation assays to determine their relative kinase activities. Western blot analysis confirmed recovery of Lats and Mst proteins.

The Lats1 activation segment resides in the C-terminal (catalytic) domain

The above data are consistent with a model according to which Mst2 phosphorylates and activates Lats1 not only *in vitro* but also *in vivo*. To explore the mechanism underlying Lats1 activation, several myc-tagged Lats1 deletion constructs were generated (Fig. 17A). To determine which of these Lats1 deletion mutants could still be regulated by Mst2, they were then produced by IVT together with FLAG-Mst2WT or KD, immunoprecipitated and tested for activity. A construct lacking the N-terminal 588 residues was activated almost to the same extent as the Lats1WT protein, indicating that the entire N-terminal half of Lats1 was dispensable for its activation by Mst2 (Fig. 17B). Further deletion of N-terminal sequences, up to residue 662, resulted in a kinase that could not be regulated by Mst2, although it displayed some basal activity (Fig. 17B). We conclude that Mst2 can still regulate a fragment of Lats1 encompassing residues 589-1130 and that removal of residues 589-662 abolishes this response. Remarkably, neither the N-terminally located putative UBA domain, a structure believed to bind ubiquitin moieties (Buchberger, 2002), nor the PPXY motifs, previously implicated in mediating the interaction between Wts/lats and Sav in *Drosophila* (Tapon et al., 2002), were required for this regulation.

We have previously shown that Lats1 was mainly present in an inactive state in cells, but could be readily activated upon treatment of cells with okadaic acid (OA) (Fig. 12). This afforded the interesting possibility to investigate the *in vivo* activation of the different Lats1 protein fragments. HEK293T cells were therefore transiently transfected with the various Lats1 constructs and subsequently treated with OA or solvent, before the myc-Lats1 proteins were immunopurified and analyzed for activity. In excellent agreement with the results obtained *in vitro*, the mutant lacking the first N-terminal 588 residues was still fully activated by OA, whereas further removal of residues up to amino acid 662 resulted in a non-responsive kinase (Fig. 17C). Thus, activation of Lats1 mutants by Mst2 *in vitro* and by OA *in vivo* displays the same structural requirements. This congruence strongly supports the notion that Mst2 acts directly upstream of Lats1 in OA-treated cells.

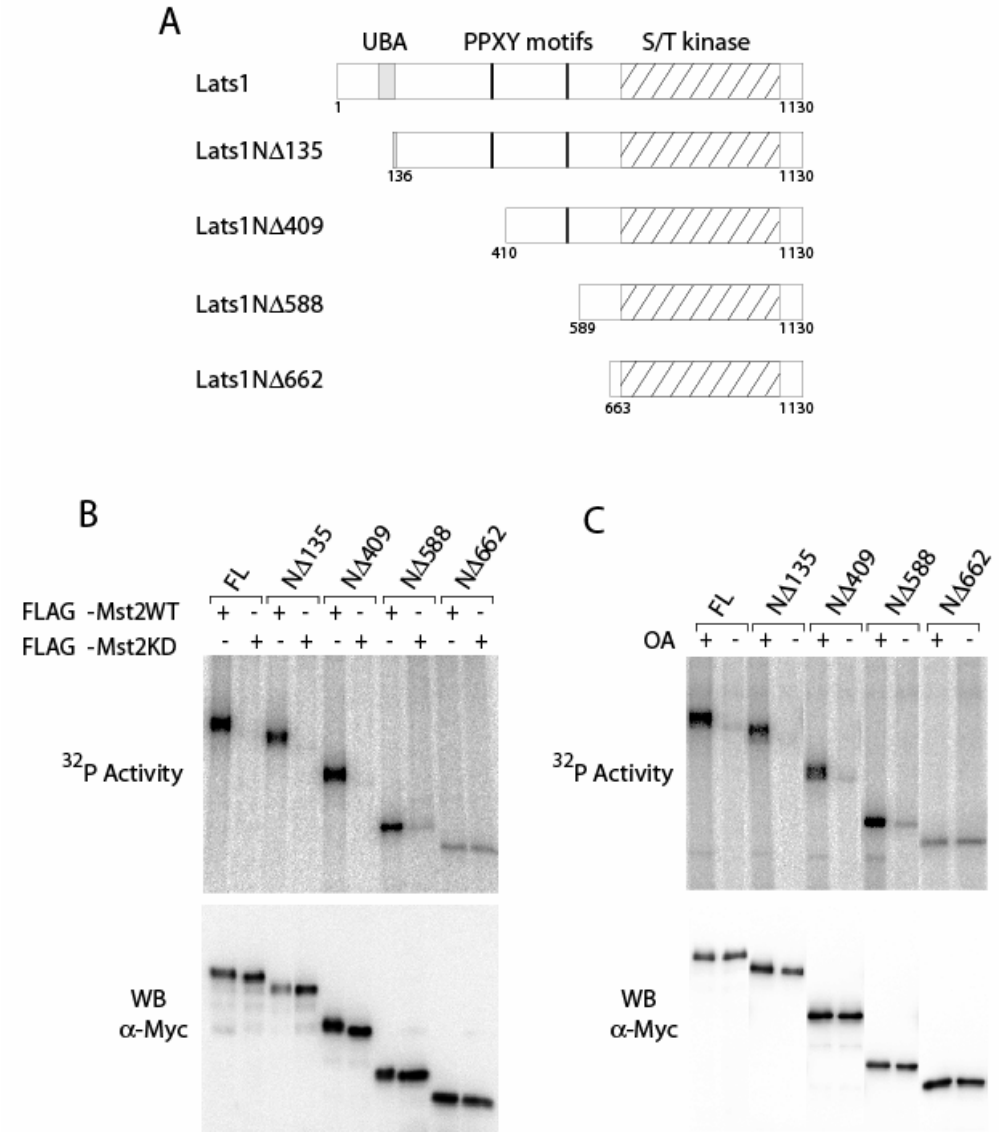

Figure 17. Identification of a Lats1 activation segment in the C-terminal domain.

(A) Schematic diagram showing different Lats1 deletion constructs with numbers denoting amino acids. Shown are a putative UBA domain in grey, two PPXY motifs in black and the kinase domain as a striped box. (B) Myc-tagged Lats1 constructs were co-expressed by IVT together with FLAG-Mst2WT or KD. Following immunoprecipitation they were used for *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. Autophosphorylation of Lats1 was visualized by autoradiography. Western blot analysis confirms recovery of similar amounts of myc-Lats1 proteins. (C) HEK293T cells were transfected for 24 h with myc-tagged Lats1 constructs and then treated for 1 h with 1 μM of okadaic acid (OA) or ethanol (solvent for OA). Myc-Lats1 proteins were immunoprecipitated and their activities determined as described above. Western blotting shows equal recovery.

Phosphorylation of S909 and T1079 is essential for Lats1 kinase activity

To identify the phosphorylation sites involved in Lats1 regulation, the smallest regulated Lats1 kinase fragment identified above (myc-Lats1NΔ588WT) was phosphorylated *in vitro* with FLAG-Mst2WT. To distinguish sites phosphorylated by Mst2 from those modified through Lats1 autophosphorylation this fragment was also incubated with FLAG-Mst2KD and a corresponding inactive mutant (myc-Lats1NΔ588KD) was incubated with both Mst2WT and Mst2KD. All samples were then subjected to extensive analysis by mass spectrometry performed by Dr. M. Nousiainen. These studies resulted in the identification of 11 phosphorylation sites within myc-Lats1NΔ588WT. Representative of these data, Figure 18 illustrates the ion scans allowing the identification of two particularly relevant sites (serine (S) 909 and threonine (T) 1079), whereas Figure 19 summarizes all results in schematic form. Three sites (S613, S633 and S1111) were phosphorylated in all samples, indicating that they were already occupied prior to the isolation of the myc-Lats1 proteins from HEK293T cells (Fig. 19). As these sites do not appear to contribute directly to the Lats1 kinase regulation by Mst2, they were not further investigated. Similarly, since Mst2 can activate both Lats1 and Lats2, three phosphorylation sites (S674, T1012 and S1049) present in Lats1 but absent from Lats2 were considered unlikely to constitute important regulatory sites. This left a total of five sites (S872, S909, T967, T1060 and T1079) for further investigation (Fig. 19).

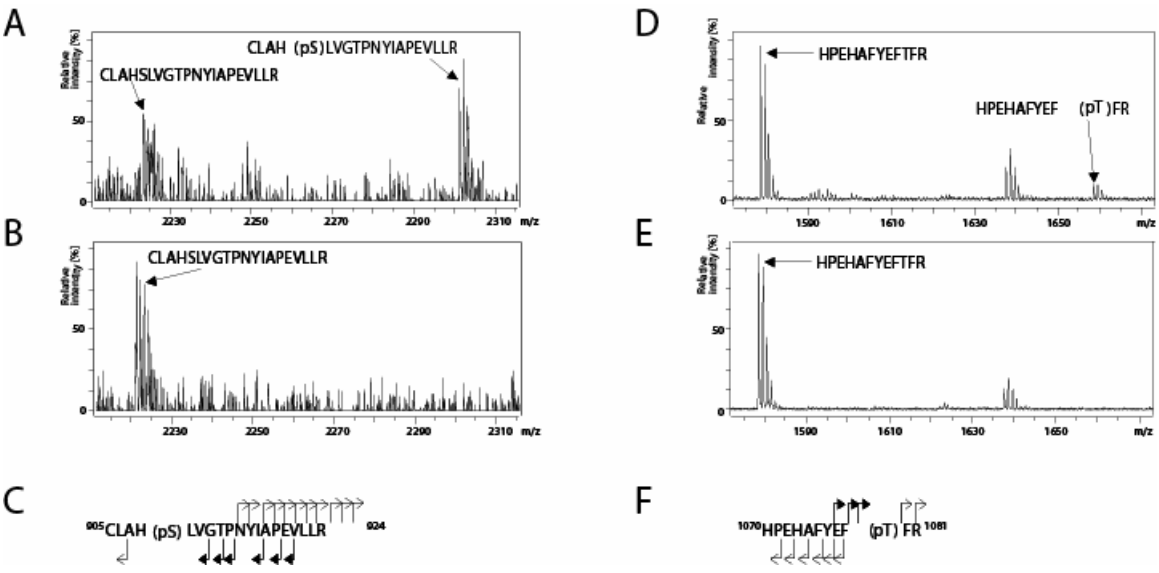

Figure 18. MS profiles of Lats1-derived tryptic peptides.

Shown are MALDI-TOF spectra, acquired in negative ion mode, of tryptic digests of myc-Lats1NΔ588WT treated with either FLAG-Mst2WT or KD. The selected mass ranges show the peaks originating from the unphosphorylated and phosphorylated peptides. Left panels (A-C) show the peptide comprising S909 and right panels (D-F) show the peptide containing T1079. Myc-Lats1NΔ588WT was treated with FLAG-Mst2WT (spectra A and D) or KD (spectra B and E). The exact phosphorylation sites were localized through fragmentation of the phosphorylated peptides by nanospray ionization Q-TOF mass spectrometry in positive ion mode (C, F). Open and bold arrows indicate unphosphorylated and phosphorylated fragments, respectively.

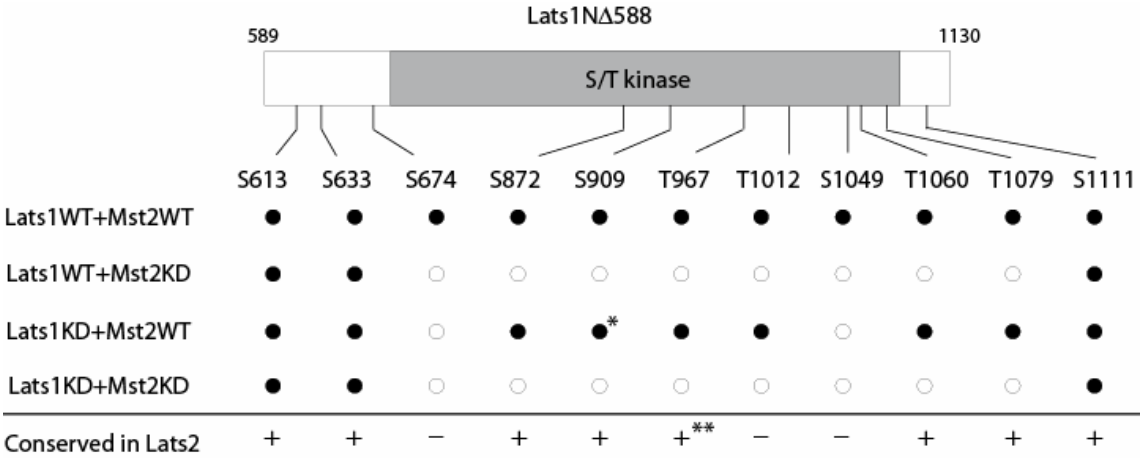

Figure 19. Schematic summary of the phosphorylation-sites identified in Lats1.

Myc-tagged Lats1NΔ588WT or KD and FLAG-Mst2 WT and KD were purified from HEK293T cells and incubated in different combinations in the presence of 10 μM ATP. Sites phosphorylated in the myc-Lats1NΔ588 proteins were then identified by mass spectrometry. Filled circles refer to phosphorylated residues, whereas open circles indicate absence of detectable phosphorylation. The bottom row indicates whether (+) or not (-) the relevant residues are conserved in Lats2. * = This site could not be phosphorylated by Mst2 in the D846A mutant, but could be phosphorylated in another Lats1KD mutant (T1079A). Myc-Lats1KD mutants. ** = not strictly conserved but neighboring threonine present.

To explore the functional significance of these five sites, they were individually mutated to alanine (A), thus mimicking an unphosphorylated state. The S872A, S909A, T967A, T1060A and T1079A mutant proteins were produced by IVT, together with either FLAG-Mst2WT or KD, and after isolation by immunoprecipitation, their kinase activities were determined (Fig. 20A). Three of the mutants, S872A, T967A and T1060A, could still be activated by Mst2 to a similar extent as myc-Lats1WT. In contrast, the S909A and T1079A mutants could not be activated (Fig. 20A). This suggested that phosphorylation at both of these sites is required for Lats1 kinase activation. To examine whether acidic charges at these sites might be sufficient to confer activation, S909 and T1079 were mutated to glutamate (E) or aspartate (D), either individually or in combination. Neither S909D (or E) nor T1079D (or E) mutant proteins showed significant activity when produced in the presence of FLAG-Mst2KD (Fig. 20B, C). However, co-expression with FLAG-Mst2WT resulted in the activation of

both the T1079D and the T1079E proteins (Fig. 20C), whereas the S909D or E mutants proved refractory (Fig. 20B). Mutants carrying acidic residues at both S909 and T1079 did not show any activity and could not be activated (data not shown). Thus, phosphorylation of T1079 seems to be necessary but not sufficient for Lats1 activation. Although the S909D or E mutants could not be activated by FLAG-Mst2WT (Fig. 20B), it should be noted that the S909 residue is located within the activation loop and that there are strong precedents of kinase regulation by activation-loop phosphorylation (Johnson et al., 1996). It appears that phosphorylation at S909, in addition to phosphorylation at T1079, is required for Lats1 activation, but that substitution of the S909 phosphorylation site by acidic residues is not sufficient to mimic a corresponding phosphorylation event.

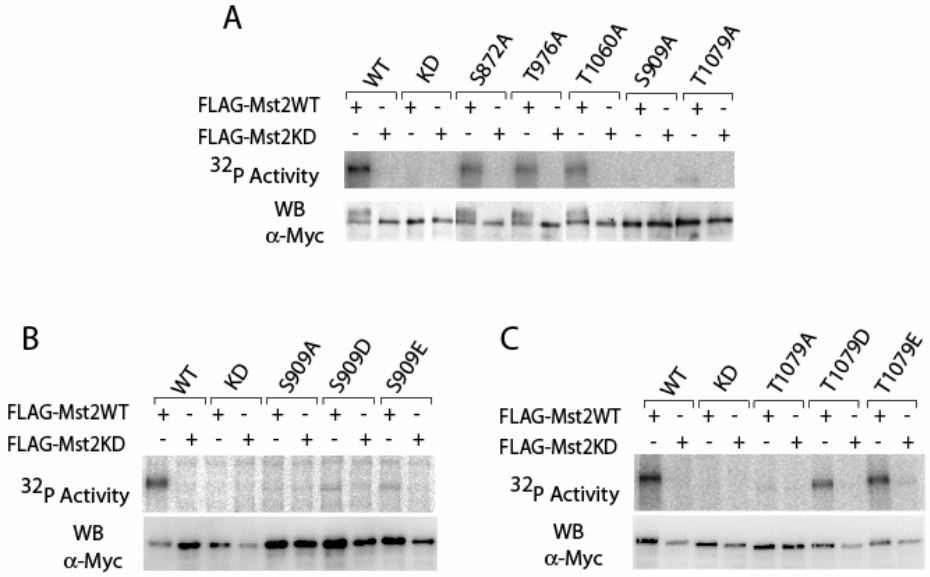

Figure 20. Phosphorylation of S909 and T1079 is essential for Lats1 kinase activation.

(A) The indicated myc-Lats1 mutants were expressed by IVT together with FLAG-Mst2WT or KD. Myc-Lats1 proteins were then immunoprecipitated and used for *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. Proteins were subjected to SDS-PAGE and autophosphorylation activity visualized by autoradiography. (B) The indicated myc-Lats1 mutants, as well as the WT and KD kinases, were expressed by IVT, together with FLAG-Mst2WT or KD and used for *in vitro* kinase assays, as described above. (C) The indicated myc-Lats1 mutants, as well as WT and KD kinases, were expressed by IVT together with FLAG-Mst2WT or KD and used for *in vitro* kinase assays, as described above. Western blotting in A-C shows equal protein recovery.

Discussion I

Lats (Wts) kinases have been implicated in the regulation of cell cycle progression and apoptosis. Recent genetic studies in *Drosophila* have shown that Lats/Wts kinases interact with two other proteins termed Salvador (Sav) and Hippo (Hpo) (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003). This raised the question of how exactly these proteins cooperate to form a signaling module, and how they are regulated at a molecular level. Furthermore, proteins structurally related to *Drosophila* Lats, Sav and Hpo have also been described in mammals, but no functional interactions between these proteins have been previously reported. Here we have shown that human Lats1 forms a signaling module with Mst2 and hWW45, the putative human homologs of *Drosophila* Hpo and Sav, respectively. Specifically, our data show that Mst2 phosphorylates Lats1 on two critical residues, identified as S909 and T1079, respectively. These phosphorylations result in Lats1 kinase activation, demonstrating that the Ste20-like kinase Mst2 functions as an upstream activator of Lats1. The WW protein hWW45 was not required for the phosphorylation and activation of Lats1 by Mst2 Although this protein could not be shown to interact with Lats1, it was clearly able to bind Mst2. Taken together with recent results from *Drosophila*, these data provide strong evidence for the evolutionary conservation of a signaling module comprising Lats(Wts) kinase, a Ste20-like upstream regulatory kinase (Mst2/Hpo), and a WW protein (hWW45/Sav). Evidence for a mitotic role of Lats1 was not obtained, suggesting that the original findings supporting a role for Lats1 in mitosis need to be reevaluated. Although we cannot rigorously exclude possible mitotic functions, our observation that Lats1 is part of a conserved signal transduction pathway, points to other roles for human Lats1, in analogy to *Drosophila* Wts.

Ste20 family members as upstream regulators of Lats/Dbf2-related kinases

Our biochemical studies demonstrate an enzyme-substrate relationship between human Mst2 and Lats1, confirming and extending previous data on *Drosophila* Hpo and Lats (Pantalacci et al., 2003; Wu et al., 2003) . In addition, we found that Mst2 was able to phosphorylate and activate not only Lats1 but also the related kinase Lats2. Moreover, Mst1 could also activate Lats1, but Mst4, a more distantly related Mst family

member, could not. Thus, the activation of Lats1/2 kinases by Mst family members displays specificity. Most importantly, co-expression experiments indicate that Mst2 can also activate Lats1 *in vivo* and treatment of cells with the phosphatase inhibitor OA, a potent activator of Mst1/2 (Lee et al., 2001; Taylor et al., 1996), also resulted in activation of Lats1. This *in vivo* activation showed the exact same requirements for a C-terminal Lats1 domain that had been inferred from *in vitro* activation studies with recombinant Mst2 and Lats1 proteins. Taken together, these data strongly suggest that Mst2 is a direct upstream activator of Lats1.

Since the N-terminal half of Lats1 (aa 1-588) was dispensable for the activation of Lats1 by Mst2, mass spectrometry was used to identify phosphorylation sites within the C-terminal domain of Lats1. A total of 11 sites were identified and five sites, all of which are conserved in Lats2, could be shown to be phosphorylated by Mst2. Two of these, S909 and T1079, were required for Lats1 activation. Interestingly, these sites are located within the activation loop (S909) and a hydrophobic motif (T1079), two domains previously been implicated in the activation of other kinases (Johnson et al., 1996; Tamaskovic et al., 2003). In particular, the sequences flanking S909 and T1079 are highly conserved not only in Lats kinases, but also in families of structurally related kinases, exemplified by human Ndr1 and *Saccharomyces cerevisiae* Dbf2p (Tamaskovic et al., 2003). Much like Lats, yeast Dbf2p kinase is also regulated by a member of the Ste20 kinase family, Cdc15p (Tamaskovic et al., 2003). Moreover, human Ndr1 is phosphorylated on S281 and T444 (Millward et al., 1999), two residues corresponding to S909 and T1079 of Lats1. Thus, it is attractive to speculate that Ndr1 may also be subject to regulation by a Ste20-like kinase.

Ste20-like Mst family members are unlikely to be the only kinases acting on Lats1. In a previous study, Lats1 was shown to be phosphorylated and upshifted during M phase of the cell cycle (Tao et al., 1999). We have confirmed this observation but found that this phosphorylation does not result in enhanced autophosphorylation activity (Fig 10). Moreover, whereas phosphorylation of Lats1 by Mst2 *in vitro* caused a clear upshift, no significant upshift was seen upon co-expression of Lats1 with Mst2 *in vivo*, presumably because of phosphatase action. Yet, Lats1 kinase activity was enhanced under both conditions, indicating that a retarded electrophoretic mobility cannot be used

as a reliable indicator of Lats1 activity. Interestingly, OA treatment of Lats1-transfected cells resulted in an upshift in case of the Lats1 WT protein but not the Lats1 KD mutant, indicating that autophosphorylation can give rise to a retarded electrophoretic mobility. In nocodazole-arrested mitotic cells, Cdk1 and Aurora A kinases could be responsible for causing an upshift of Lats1, although the consequences of these phosphorylations remain to be clarified (Morisaki et al., 2002; Toji et al., 2004).

What is the role of hWW45 in the regulation of Lats kinases?

Following up on the recent identification of the WW protein, Sav, in *Drosophila*, we have also explored the role of the apparent human Sav homolog, hWW45. Our experiments concur to demonstrate that hWW45 and Mst2 are able to form a complex. In contrast, no evidence could be obtained for stable binding of either Mst2 or hWW45 to Lats1, and no ternary complexes were observed. At first glance, this appears to contrast with results obtained in *Drosophila*, where Sav (hWW45) was shown to interact with Lats (Tapon et al., 2002) and enhance its phosphorylation by Hpo (Pantalacci et al., 2003; Wu et al., 2003) . We cannot definitively explain this difference, but note that the *Drosophila* studies made use of a Sav fragment, comprising the WW domain, whereas the present study was carried out with the full-length hWW45 protein. This raises the intriguing possibility that the interaction of full-length Sav/hWW45 with Lats1/Lats is subject to regulation.

Observations made in *Drosophila* have led to the proposal that Sav could function as a scaffold for the formation of a trimeric complex (Harvey et al., 2003). This proposal is attractive, but the existence of a trimeric complex between Lats1/Lats, Mst2/Hpo and hWW45/Sav remains to be demonstrated and alternative scenarios should not be excluded. Although the PPXY motifs located in the N-terminal half of Lats have been implicated in mediating the interaction between Lats and Sav in *Drosophila* (Tapon et al., 2002), we found that these motifs were dispensable for the activation of human Lats1 by both Mst2 *in vitro* and OA *in vivo*. Thus, we presently favor a model according to which hWW45 (Sav) functions primarily as a regulator of Mst2 (Fig. 21).

Interestingly, the interaction between the C-termini of Mst2 and hWW45 may involve a motif, termed SARAH (Sav/Rassf/Hpo), that appears to function as a modular

protein-protein interaction domain (Scheel and Hofmann, 2003). A SARAH motif is also present in proteins of the Rassf family, the founding member of which is encoded by the tumor suppressor gene *RASFF1* (Dammann et al., 2000). These proteins harbor a Ras-association domain (hence their name: ras association domain family) and are believed to act in signal transduction pathways. Although the exact functions of Rassf proteins remain to be unraveled, roles in cell cycle progression and apoptosis have been proposed (Khokhlatchev et al., 2002; Song et al., 2004). Moreover, Rassf family members Rassf1 and Nore1/Rassf5 have recently been shown to interact with Mst1, and to inhibit Mst1 auto-activation *in vitro* (Praskova et al., 2004). Thus, it will be interesting to explore the possibility that Rassf proteins could attribute to the regulation of the Mst-Lats-hWW45 signaling modules.

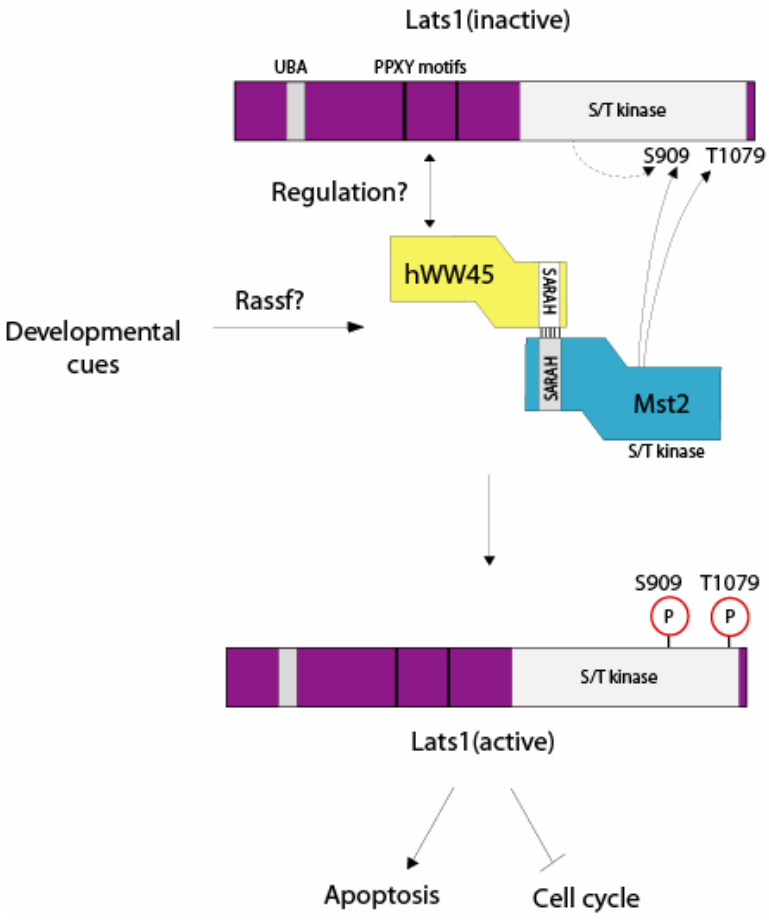

Figure 21. The hWW45-Mst2-Lats1 signal transduction module.

This model incorporates data described here for human proteins and elsewhere for Drosophila homologs Sav, Hpo, and Wts. The activation of Lats1 by Mst2 has only been shown in human cells (this study), but considering the extensive sequence similarity, is likely to represent an evolutionarily conserved mechanism. For simplicity, Mst1 and Lats2 are not shown in this model.

Emerging evidence for an evolutionarily conserved signaling pathway

Pioneering work performed in *Drosophila* provides strong evidence that the Lats-Hpo-Sav module plays a key role in regulating cell cycle exit and apoptosis during development (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003) . Here we have shown that a very similar signaling module is operating in human cells (Fig. 21). Gene disruption experiments performed in mice support a critical role of Lats kinases in tumor suppression (St John et al., 1999), and recent studies point to diverse roles of human Lats kinases in cell cycle progression (Li et al., 2003; Yang et al., 2001) , cytokinesis (Yang et al., 2004) and the induction of apoptosis (Yang et al., 2001). However, the precise roles of mammalian Lats kinases remain to be clarified. Moreover, with at least two similar Mst kinases acting on two closely related Lats kinases, the situation in human cells is expected to be more complex than in *Drosophila*.

In summary, our present data demonstrate the evolutionary conservation of a signaling module in which Lats kinases are downstream effectors of a complex comprising Mst kinases and hWW45 (Fig. 21). Combined biochemical and genetic investigations, will hopefully soon lead to the further delineation of this pathway that is likely to be important for development and homeostasis.

Summary I

In the first part of this thesis, we have investigated the possible role of Lats1 kinase in mitosis. Our data does not support the reported role of human Lats1 in mitosis. Instead, we showed that Lats1 activity is regulated by Mst2 kinase, demonstrating the evolutionary conservation of a Lats/Mst/hWW45 pathway, which is required for controlling cell size and growth in *Drosophila*. Because the research interest of our department is on kinases that play essential role in mitosis and the mitotic function of Lats1 does not seem to be promising, I then changed my project and set out to study the function of Aurora A kinase, focusing on possible interaction partners.

Part II: Exploring the function and regulation of Aurora A kinase

Introduction II

Centrosome maturation in mitotic spindle assembly

The spindle apparatus, a bipolar array of highly dynamic microtubules (MTs), is indispensable for accurate sister chromatid segregation during mitosis. Its precise regulation in time and space is critical for genome stability. Moreover, spindle positioning determines the cleavage plane during cell division and is critical for stem cell function and tissue formation. In somatic animal cells, spindle assembly is strongly influenced by centrosomes. At the G2 to M transition, these organelles undergo a maturation process that enhances the recruitment of γ-tubulin ring complexes (γTuRCs) and other MT regulatory factors. Concomitantly, the two centrosomes separate from each other to form the spindle poles. These crucial events are controlled by three highly conserved serine/threonine kinases, Cdk1, Plk1 and Aurora A (Barr et al., 2004; Blagden and Glover, 2003; Nigg, 2001; Vagnarelli and Earnshaw, 2004). Cdk1 is initially activated at the centrosome (Jackman et al., 2003) and required for centrosome separation (Blangy et al., 1995; Sawin and Mitchison, 1995). Likewise, Plk1 and Aurora A localize to centrosomes and are activated at the G2/M transition, but the coordination of their activities is not presently understood.

Plk1 and Aurora A are required for centrosome maturation and spindle assembly

When Plk1 activity is impaired by antibody injection (Lane and Nigg, 1996), RNAi-mediated Plk1 depletion (Hanisch et al., 2006; Sumara et al., 2004; van Vugt et al., 2004) or small molecule inhibitors (Lenart et al., 2007; McInnes et al., 2006; Peters et al., 2006), cells fail to recruit γ-TuRCs and often form monoastral spindles. Several candidate substrates of Plk1 have been identified, including abnormal spindle (asp) in *Drosophila* (do Carmo Avides et al., 2001) and translationally controlled tumor protein (TCTP) (Yarm, 2002), ninein-like protein (Nlp) (Casenghi et al., 2003) and Kizuna (Kiz) in vertebrates (Oshimori et al., 2006), but the exact role of Plk1 in spindle assembly remains unclear.

Interestingly, RNAi-mediated depletion of Aurora A also results in the formation of monopolar spindles, decreased accumulation of γ-tubulin and reduced density of centrosomal MTs (Ducat and Zheng, 2004). Aurora A is thought to regulate MT nucleation and spindle pole formation through the recruitment of centrosomal proteins, including transforming acidic coiled-coil protein (D-TACC) (Giet et al., 2002), minispindles (Msps) (Barros et al., 2005) and centrosomin (CNN) in *Drosophila* (Berdnik and Knoblich, 2002), and the Msps homologue XMAP215 in *Xenopus* (Kinoshita et al., 2005; Peset et al., 2005). Considering that both Plk1 and Aurora A play important roles in centrosome maturation and bipolar spindle formation, one would expect that the functions of these kinases should be coordinated. No direct interaction between the two kinases has yet been observed, but, interestingly, Plk1 is required for centrosomal localization of Aurora A (De Luca et al., 2006; Hanisch et al., 2006).

Regulation of Plk1 and Aurora A

Plk1 and Aurora A are activated through phosphorylation within their respective activation loops (T210 in human Plk1; T288 in human Aurora A (Jang et al., 2002; Littlepage et al., 2002), and both kinases are also controlled by additional mechanisms. In the case of Plk1, the C-terminal end domain (the so-called polo-box domain; PBD) functions as a phospho-peptide binding module that mediates the binding of Plk1 to proteins pre-phosphorylated by 'priming' kinases (Cheng et al., 2003; Elia et al., 2003a; Elia et al., 2003b). In the case of Aurora A, regulation appears to be conferred primarily through interactions with binding partners. Of several Aurora A interactors (Chen et al., 2002; Farruggio et al., 1999; Hirota et al., 2003; Mori et al., 2007; Ouchi et al., 2004), the role of the MT binding protein TPX2 (Targeting Protein for XKlp2) is understood best (Eyers et al., 2003; Kufer et al., 2003; Tsai et al., 2003). Binding of the N-terminus of TPX2 triggers kinase activation through a conformational change that protects the activation loop of Aurora A from dephosphorylation by protein phosphatase 1(PP1) (Bayliss et al., 2003; Eyers et al., 2003). Moreover, TPX2 targets Aurora A to the mitotic spindle (Kufer et al., 2002) and confers regulation by the Ran GTPase spindle assembly pathway (Tsai et al., 2003).

Bora is a novel Aurora A interactor and activator

Bora was recently identified as a novel binding partner of Aurora A (Hutterer et al., 2006). Its overexpression suppressed the centrosome maturation and asymmetric division defects of *Drosophila* Aurora A mutants. Furthermore, Bora was able to activate Aurora A, albeit to a modest extent. Activation of Cdk1 at the onset of mitosis triggered the translocation of *Drosophila* Bora from the nucleus to the cytoplasm, providing an attractive mechanism for the activation of cytoplasmic Aurora A. Finally, initial studies on human Bora (hBora) suggested an evolutionarily conserved function for this protein (Hutterer et al., 2006). In the following, we investigate the significance of the reported interaction between Aurora A and hBora (Hutterer et al., 2006). The results shown in the following chapter lead us to present a working model according to which Plk1 influences Aurora A function and localization through the regulation of the abundance of hBora. We emphasize that the proposed model will require further experimental testing and, for the time being, must be considered tentative.

Results II

1. hBora, a novel Aurora A binding partner links Plk1 functions with Aurora A

1.1 hBora interacts with Aurora A

To confirm the reported interaction between Aurora A and hBora (Hutterer et al., 2006), these two proteins were overexpressed in HEK293T cells and co-immunoprecipitation experiments were carried out in either direction. The binding between Aurora A and hBora could be readily detected (Fig. 22). Interestingly, Aurora A bound preferentially to the phosphorylated (upshifted) form of hBora (Fig. 22A). Moreover, little if any binding could be seen between hBora and a catalytically inactive Aurora A mutant (Fig. 22A). These observations suggest that only the mitotically phosphorylated hBora interacts with active Aurora A. Using similar co-immunoprecipitation experiments, the Aurora A binding site could be mapped to the phosphorylated N-terminal domain (1-327) of hBora, while the C-terminal domain (329-559) failed to bind (Fig. 22B). To examine the ability of hBora to activate Aurora A, recombinant proteins were mixed *in vitro* and Aurora A activity was assayed through autophosphorylation as well as phosphorylation of an exogenous substrate, myelin basic protein (Fig. 22C). Activation of Aurora A could be detected, but when compared to TPX2 (Eyers et al., 2003), the stimulatory effect of hBora appeared to be modest, in agreement with previous data (Hutterer et al., 2005). This prompts the question of whether the activation of Aurora A is the main physiological function of hBora.

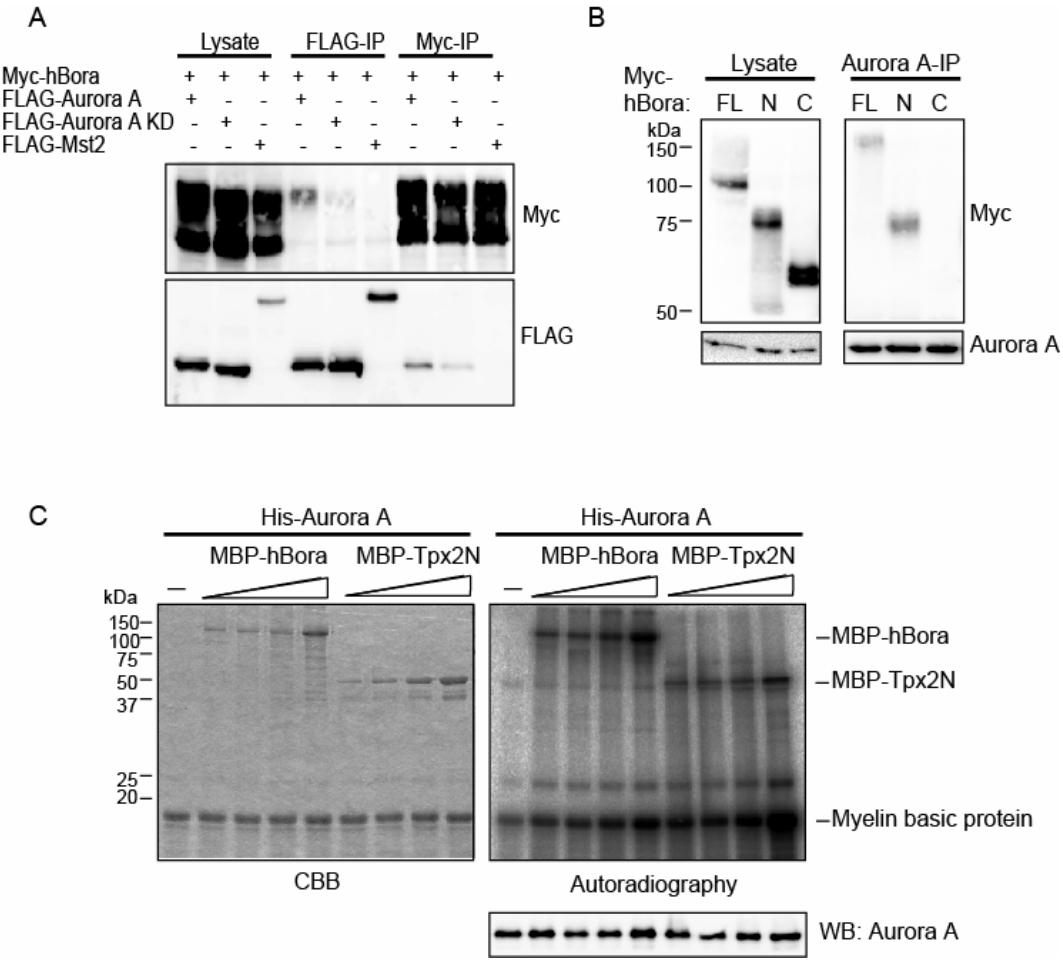

Figure 22. hBora interacts with Aurora A.

(A) HEK293T cells were co-transfected with plasmids expressing myc-hBora, FLAG-Aurora A wild type, kinase dead (KD) or FLAG-Mst2 (negative control) for 48 h and arrested by nocodazole. FLAG-immunoprecipitates were immunoblotted with antibodies against FLAG and myc. Note that the upshifted form of hBora co-immunoprecipitates preferentially with wild type Aurora A. (B) HEK293 cells were transfected with plasmids expressing myc-tagged hBora, hBoraN and hBoraC and arrested by nocodazole. Aurora A-immunoprecipitates were immunoblotted with antibodies against Aurora A and myc. Single asterisk (*) denotes unphosphorylated hBora and doube asterisk (**) indicates phosphorylated hBora. Note preferential binding of the phosphorylated, upshifted hBora N-terminus. (C) *In vitro* kinase assay was performed using His-Aurora A treated with PP1 with an increasing amount of MBP-hBora or MBP-TPX2 aa1-43 (MBP-TPX2N). Kinase reactions were performed for 30 min at 30 °C in the presence of [γ-^{32}P]ATP, using myelin basic protein (MBP) as a substrate. Phosphate incorporation was visualized by autoradiography and a Western blot demonstrates the presence of equal amounts of Aurora A in all samples.

1.2 Cell cycle expression of hBora

To study the cell-cycle regulation of hBora, HeLaS3 cells were arrested at G1/S (thymidine block) or prometaphase (nocodazole) and analyzed at different time points after release (Fig. 23A and B, respectively). As demonstrated using a specific polyclonal antibody raised against the full-length protein, hBora levels were maximal at 8 h after release from the thymidine block (Fig. 23A, t=8) and in nocodazole-arrested cells (Fig. 23B, t=0). This peak expression coincided with maximal expression of cyclin B1 and was accompanied by a retarded electrophoretic mobility (upshift), suggesting that hBora was modified during mitosis. The hBora upshift was also observed in mitotic cells that had been collected by shake off without any use of drugs, indicating that it is not a drug-induced artefact (Fig. 23B, shake off). Upon release from the nocodazole block, hBora levels gradually decreased (Fig. 23B, t=80 and t=100) before the protein reaccumulated in a faster migrating form at later times (Fig. 23B, t=140, 160). As shown in Figure 23C, the expression of myc-tagged hBora in HEK293T cells also resulted in the appearance of an upshifted band in nocodazole but not aphidicolin arrested cells, and this upshifted band was sensitive to phosphatase treatment, indicating that it reflects a phosphorylated form of hBora (see also Fig. 28A). Together, these data indicate that hBora is maximally expressed during early mitosis, similar to Aurora A and cyclin B1 (Fig. 23B), and, moreover, that hBora is phosphorylated on specific sites during early mitotic progression.

The phosphorylation of hBora is likely to be complex, as several candidate mitotic kinases act on hBora, at least *in vitro* ((Hutterer et al., 2006), Fig. 24A). In particular, hBora could be phosphorylated by Cdk1/cyclin B, Plk1 and Aurora A but not Aurora B, and both Cdk1/cyclin B and Plk1 retarded the electrophoretic mobility of this protein, albeit to a different extent (Fig. 24B)

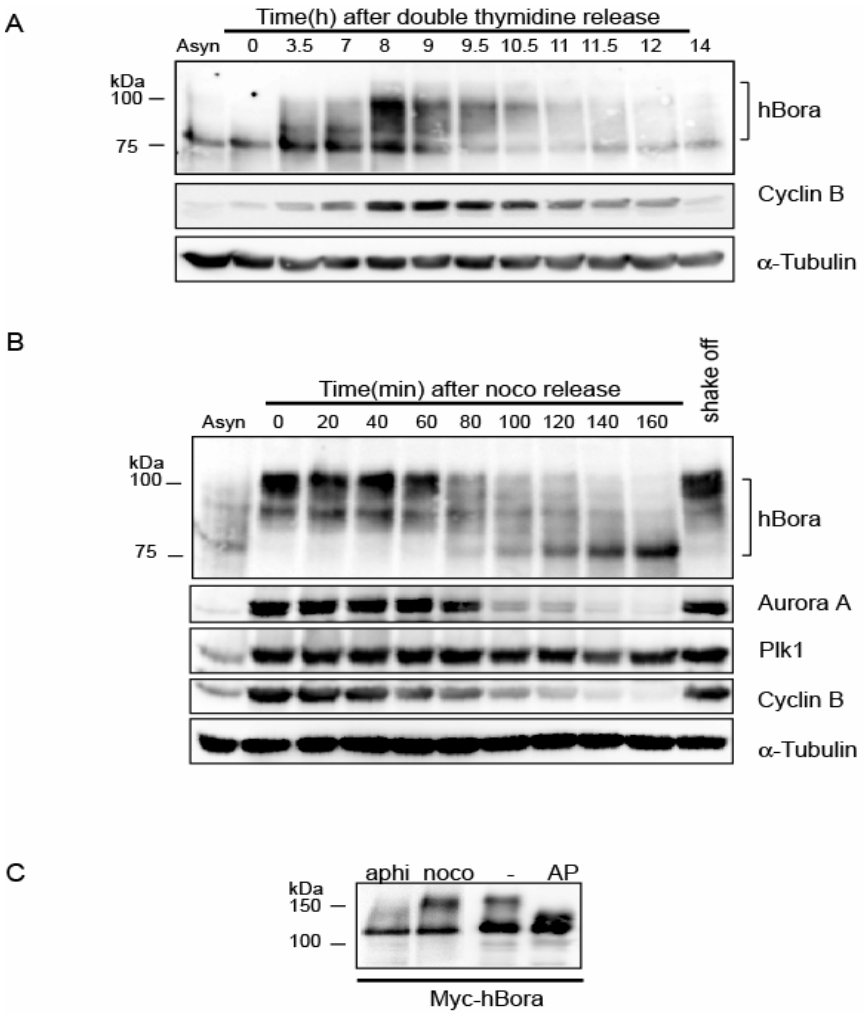

Figure 23. hBora is cell-cycle regulated and phosphorylated during mitosis.

(A) HeLaS3 cells were released from a G1/S arrest (induced by double thymidine block), samples were collected at the indicated times (hours) and probed by Western blotting, using the antibodies indicated. Cyclin B levels indicate exit from mitosis and α-tubulin levels serve as a loading control. The first lane contains a lysate prepared from an asynchronous culture (Asyn). (B) HeLaS3 cells were released from nocodazole-induced mitotic arrest (indicated as time 0), samples were collected every 20 min and probed by Western blotting, using the antibodies indicated. The electrophoretic mobility of myc-hBora overexpressed in HEK293T cells was assayed by Western blotting performed on interphase (aphi, aphidicolin-arrested) or mitotic (noc, nocodazole-arrested) lysates. Myc-hBora from mitotically arrested cells was immunoprecipitated and treated for 30 min with or without alkaline phosphatase (AP).

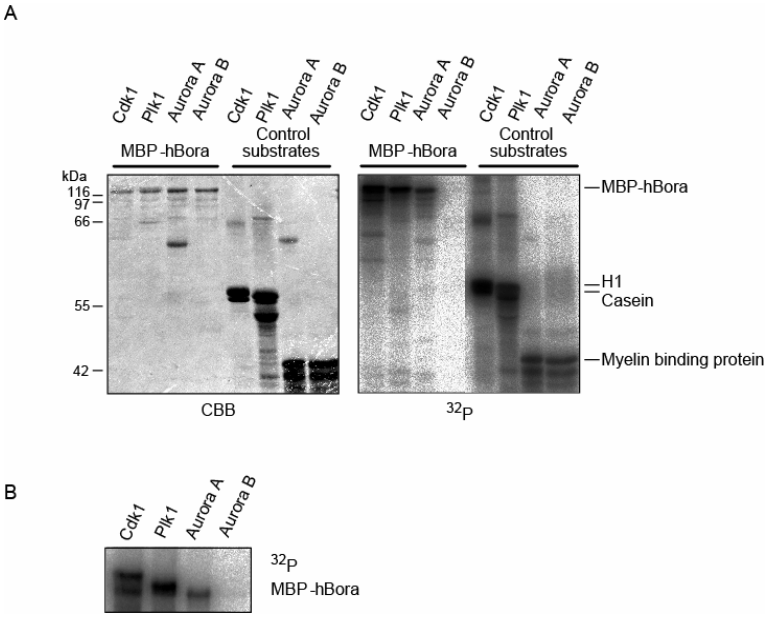

Figure 24. hBora is phosphorylated by Cdk1, Plk1 and Aurora A but not Aurora B *in vitro*.

(A) *In vitro* kinase assay were performed with Cdk1, Aurora A, Aurora B or Plk1 and MBP-hBora as a substrate in the presence of [γ-^{32}P]ATP and the samples were subjected to SDS-PAGE. Coomassie blue (CBB) staining shows protein loading. (B) Plk1 and Cdk1 phosphorylations lead to upshifts of hBora. Experiments were performed as in A, but samples were subjected to 7.5% SDS-PAGE to better reveal hBora upshifts upon phosphorylation.

1.3 Identification of multiple phosphorylation sites in hBora

The above results show that hBora gets highly phosphorylated during mitosis and can be phosphorylated by Cdk1, Plk1 and Aurora A *in vitro*. Together with Dr. Xiuling Li, we next tried to identify hBora phosphorylation sites from two different sources: first we analyzed GST-tagged hBora purified from Sf9 insect cells treated with or without okadaic acid (OA), and second, we studied MBP-tagged hBora purified from bacteria, after phosphorylation by Cdk1, Plk1 and Aurora A *in vitro.* Samples were separated by SDS-PAGE. Then, gel bands were excised and tryptic digestion was performed. For identification of phosphorylation sites within these tryptic digests (Shevchenko et al., 1996b), phosphorylated peptides were first identified by MALDI-TOF mass spectrometry (Brucker Daltonik, Bremen, Germany) and subsequently confirmed by post source decay (PSD) (Hoffmann et al., 1999a). The exact localization of the phosphorylated residues within the peptides was determined by MS-MS based sequencing using a quadrupole time-of-flight (QTOF) mass spectrometer (Table 2). These results indicate

that hBora can be phosphorylated at many different sites. The significance of one of the sites identified here (S252) will be addressed below.

No.	Site	Peptide	Sf9 w.o. OA	Sf9 with OA	Cdk1 *in vitro*	Plk1 *in vitro*	Aurora A *in vitro*
1	T12	MQIpTPETPGR	X	X			
2	T15	MQITPEpTPGR		X	X		
3	S47	(VFK) pSTKLPTPGK					X
4	T52	STKLPpTPGK		X	X		
5	S59	WpSIDQLAVINPVEIDPEDIHR		X			
6	Y82	QALpYLSHSR					X
7	S84	QALYLpSHSR		X			
8	S112	DVIVPpSPWTDHEGK		X	X		
9	T131	CpTNINSDSPVGK					X
10	S137	CTNINSDpSPVGK		X	X		
11	T144	KLpTIHSEK					X
12	S183	ADEFADQpSPGNLSSSSLR	X	X	X		
13	S188	ADEFADQSPGNLpSSSSLRR		X	X		X
14	S189	ADEFADQSPGNLSpSSSLRR		X			
15	S191	ADEFADQSPGNLSSSpSLR	X	X			
16	S239	pSPLQTPSSGQFSSSPIQApSAK	X		X		
17	T243	SPLQpTPSSGQFSSSPIQASAK					X
18	S246	SPLQTPSpSGQFSSSPIQASAK					X
19	S250	SPLQTPSSGQFpSSSPIQASAK		X	X		
20	S252	SPLQTPSSGQFSSpSPIQASAK	X	X	X		
21	S257	SPLQTPSSGQFSSSPIQApSAK			X		
22	T287	KYSLGSITSPSPISSPTFSPIEFQIGEpTPLSEQR				X	
23	T296	KFpTVHSPDASSGTNSNGITNPCIR					X
24	T299	FTVHpSPDASSGTNSNGITNPCIR	X	X	X		
25	T312	KFTVHSPDASSGTNSNGIpTNPCIR					X
26	S318	(CIR)pSPYIDGCSPIK		X	X		
27	S325	SPYIDGCpSPIK		X	X		
28	S331	NWpSPMR		X	X		
29	S339	LQMYpSGGTQYR			X		X
30	T342	LQMYSGGpTQYR			X		
31	S389	QFpSNEASTHGTHLVVTAMSVTQNQSSASEK	X	X			
32	T432	EKDNNpTVDMVDPIEIADETTWIK		X			
33	S552	TApSPFQCSpSP		X	X		
34	S558	TASPFQCSpSP		X	X		

Table 2. Summary of the phosphorylation sites identified in hBora phosphorylated by Cdk1, Plk1 and Aurora A *in vitro* and in hBora purified Sf9 insect cells treated with or without okadaic acid (OA). The phosphorylated residues are indicated in red.

1.4 Depletion of hBora causes aberrant spindle formation

To explore the consequences of hBora depletion, two siRNA duplexes targeting hBora were used. Western blots performed on mitotic cells revealed extensive depletion of hBora after 72h of siRNA treatment, whereas levels of Aurora A and Plk1 remained unchanged (Fig. 25A). Compared to a GL2 control duplex (Elbashir et al., 2001), the two hBora siRNA duplexes produced similar increases in mitotic indices (Fig. 25B) and qualitatively similar spindle abnormalities (Fig. 26A). In response to hBora depletion, many cells displayed bipolar spindles that were larger and denser than normal spindles (Fig. 26B). In addition, we observed cells with long, wavy spindles and lagging chromosomes and, as reported previously (Hutterer et al., 2006), occasional multipolar spindles (Fig. 26B). Long, wavy spindles occasionally displayed fragmented poles (not shown), suggesting that they evolved to form multipolar spindles, a conclusion supported by live cell microscopy (not shown).

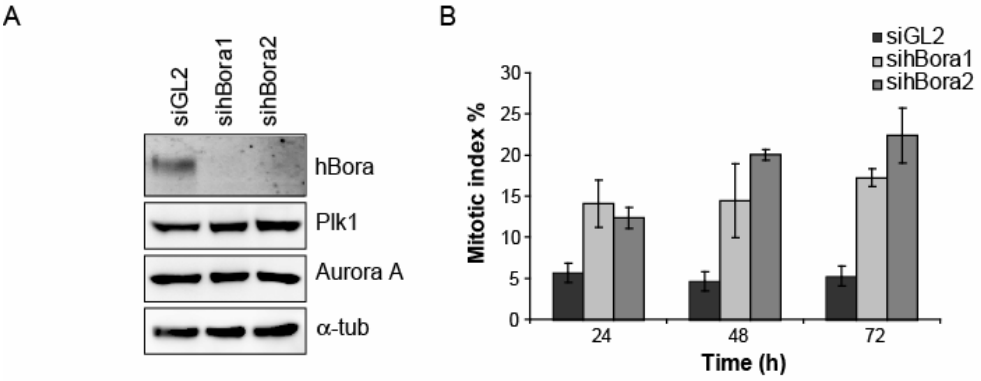

Figure 25. hBora depletion leads to an increase in mitotic indices.

(A) Effective depletion of hBora from nocodazole arrested HeLaS3 cells arrested by 72h siRNA treatment is shown by Western blotting. Note that Plk1 and Aurora A levels are unaffected. □-tubulin levels provide a loading control. (B) Histogram summarizing the mitotic indices of cells treated with GL2 or hBora siRNA duplex for 24h, 48h and 72h in 3 independent experiments (n=200); bars indicate standard deviations.

Since hBora is a binding partner of Aurora A, we next asked whether hBora depletion would affect Aurora A localization. To facilitate comparisons with controls, we focused on those hBora-depleted cells that showed relatively normal bipolar spindles. Even though such cells may not reflect the most severe phenotype of hBora depletion, immunofluorescence microscopy readily revealed an enrichment of Aurora A on the spindles (Fig. 26C). In addition, the density of spindle MT was increased in hBora-depleted cells (Fig. 26C), whereas other proteins, notably pericentrin or Plk1, were not detectably affected (not shown)

To determine whether the increase in MT density in the spindles of hBora-depleted cells was due to an increased stability of kinetochore MTs (K-fibers), cells were subjected to cold treatment (Rieder and Borisy, 1981). After 1h of cold treatment, only centrosomal tubulin remained visible in control cells, as expected. In stark contrast, K-fibers remained largely intact in hBora-depleted cells (Fig. 26D), regardless of which hBora duplex was used for depletion (Fig. 26E). Taken together, the above results indicate that hBora depletion results in the increased association of Aurora A with the spindle apparatus and concomitant stabilization of K-fibers. This then presumably leads to the formation of dense or aberrantly shaped (long and wavy) spindles, followed by the occasional fragmentation of spindle poles and multipolar divisions.

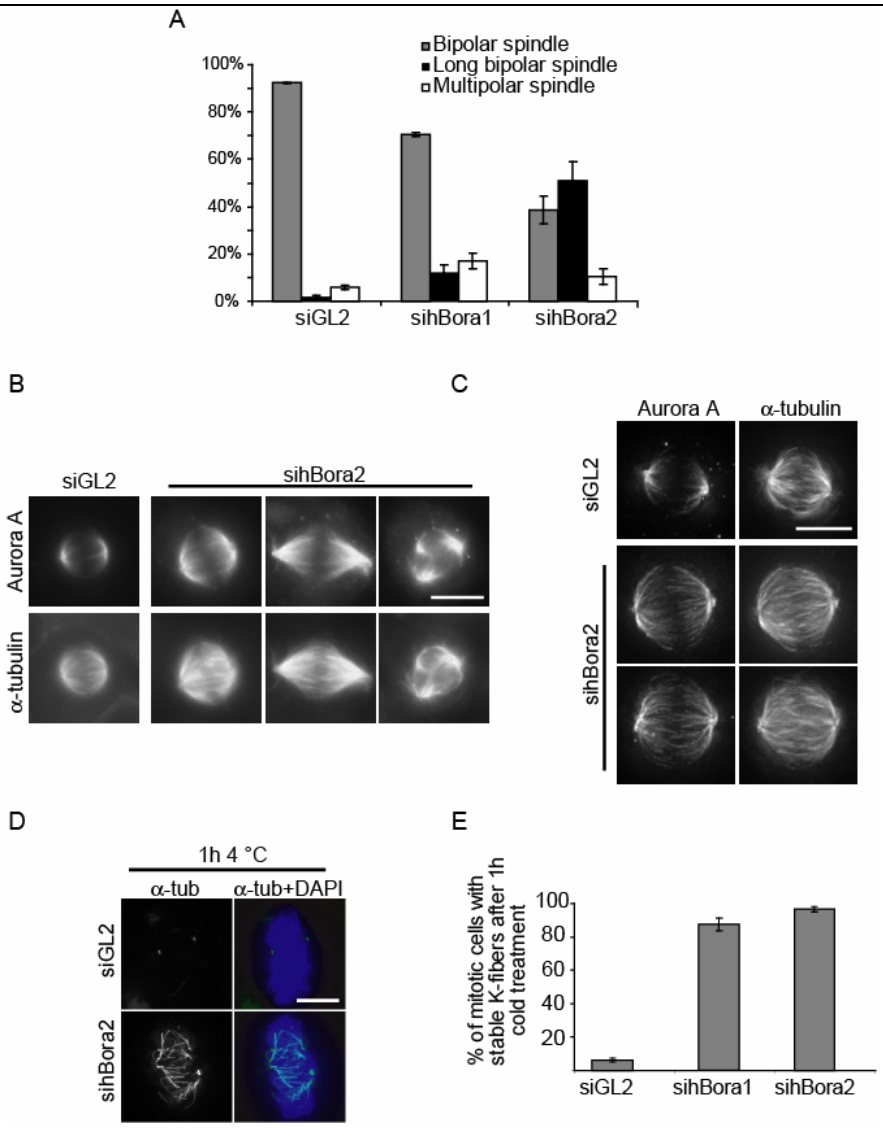

Figure 26. Aberrant spindle formation in hBora-depleted cells.

(A) Histogram illustrating percentages of mitotic cells with spindle abnormalities. HeLaS3 cells treated with GL2 or hBora siRNA duplexes for 72h and released from double thymidine block were fixed after 10h and stained with α-tubulin and DAPI for determining spindle morphology. Results are from 3 individual experiments (300-350 cells each), and bars indicate standard deviations. (B) HeLaS3 cells were treated with siRNA duplex specific for hBora or GL2 control for 72h. Aurora A and α-tubulin were visualized by immunofluorescence. Bar, 10μm. (C) HeLaS3 cells were treated as in (A) and stained for indicated proteins. Note increased microtubule density and increased Aurora A staining on the spindles of hBora-depleted cells. Bar, 10μm. HeLaS3 cells treated as in (A) were placed on ice for 1h and the presence of cold stable microtubules (green) was assessed by immunofluorescence. Bar, 10μm. (E) Histogram summarizing percentages of mitotic cells with cold-stable K-fibers. Results are from three independent experiments (n=100-150); bars indicate standard deviations.

1.5 Excess hBora causes Aurora A mislocalization and monoastral spindle formation

Staining of mitotic HeLaS3 cells with anti-hBora antibodies failed to highlight any specific structures (not shown) and immunostaining of ectopically expressed myc-tagged hBora confirmed a diffuse localization of hBora (Fig. 27A). Remarkably, nearly all mitotic cells overexpressing hBora showed monoastral spindles (Fig. 27A), indicating that excess hBora interferes with bipolar spindle formation. The same phenotype was also observed upon overexpression of the N-terminus of hBoraN, whereas the hBoraC produced no effect (Fig. 27A). This strongly suggested that the induction of monoastral spindles by excess hBora reflected interference with Aurora A function. Indeed, overexpression of either hBora or hBoraN resulted in displacement of Aurora A from the spindle, whereas the kinase localized normally to the bipolar spindles of untransfected cells or cells expressing hBoraC (Fig. 27B). Since the Aurora A binding site was mapped to the N-terminus of hBora ((Hutterer et al., 2006) Fig. 22B and Fig. 28A), the most straightforward interpretation of the above results is that excess cytoplasmic hBora interferes with bipolar spindle formation through the sequestration of Aurora A away from the spindle.

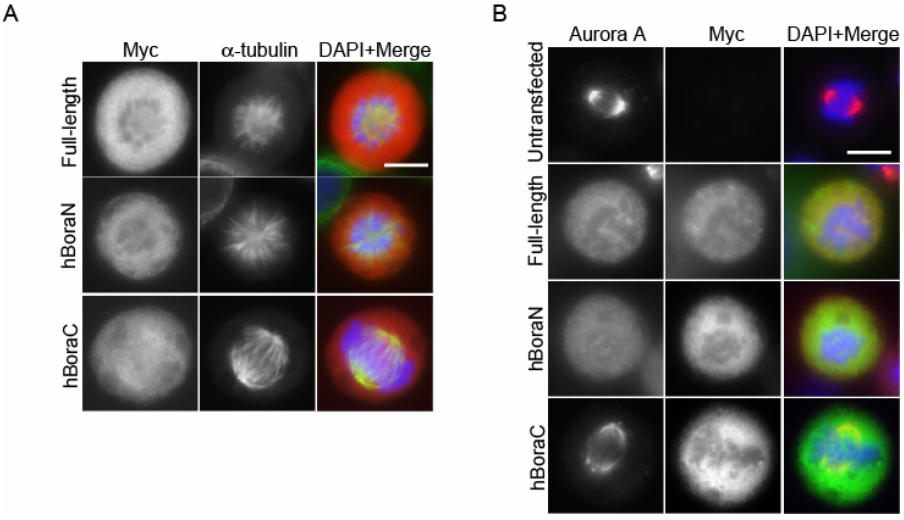

Figure 27. Overexpression of hBora leads to monoastral spindle formation and Aurora A mislocalization.

(A, B) HeLaS3 cells transfected with the indicated myc-tagged hBora constructs were fixed and stained with 9E10 anti-myc (red) and α-tubulin (green) (A) or with 9E10 anti-myc (green) and Aurora A (red) antibodies (B). DNA was stained with DAPI (blue). Bars, 10μm.

To corroborate the above conclusion, we generated a tetracycline (tet)-inducible HEK293 cell line that allows the controlled expression of myc-tagged hBoraN. As shown by Western blotting, tet-induction of hBoraN for 48h resulted in two myc-immunoreactive bands (Fig. 28A). The upper band was sensitive to alkaline phosphatase treatment, confirming that it represents a phosphorylated form of myc-hBoraN (Fig. 28A). Strikingly, more than 80% of the cells expressing hBoraN were arrested in mitosis and mostly displayed monoastral spindles (Fig. 28A) and, as seen before (Fig. 27A, B), Aurora A was displaced from these spindles (Fig. 28C). This was most obvious in comparison to the Aurora A localization in cells in which monopolar spindles were formed due to inhibition of the kinesin-related motor Eg5 by monastrol (Mayer et al., 1999) (Fig. 28C, upper row). Upon induction of hBoraN, the recruitment of γ-tubulin was also drastically impaired, demonstrating a defect in centrosome maturation, whereas TPX2 and Aurora B analyzed for control were not displaced (Fig. 28C).

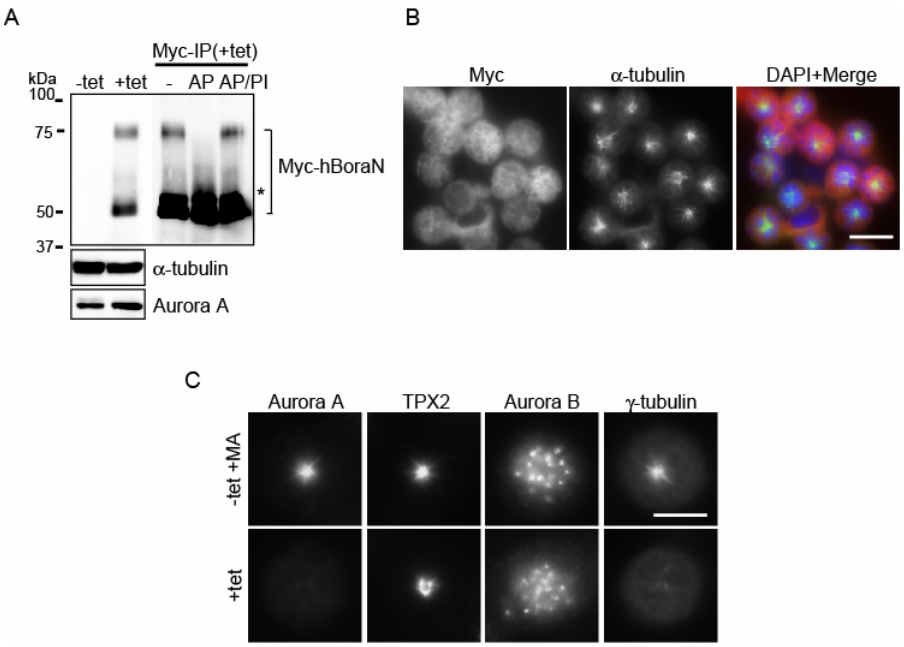

Figure 28. Tetracyclin-inducible myc-hBoraN 293 stable cell line.

(A) Expression of myc-hBoraN in a stably transfected HEK293T cell line induced for 48h with tetracyclin (+tet) was analyzed by Western blotting with anti-hBora antibodies and anti-Aurora A antibodies, α-tubulin was used as loading control. Myc-hBoraN from induced cells was immunoprecipitated and treated with alkaline phosphatase (AP) in the presence or absence of phosphatase inhibitors (PI). Note the absence of the upper band in the sample treated with AP. The asterisk denotes the IgG heavy chain. (B) Cells induced as in (B) were fixed and stained with 9E10 anti-myc (red) and α-tubulin (green) antibodies. DNA was stained with DAPI (blue). Bar, 10μm. (C) Myc-hBoraN stable cell line was induced with tetracycline (+tet) for 48h or treated with 150μM monastrol (-tet, +MA) for control. The localization of indicated proteins was monitored by immunofluorescence. Bar, 10μm.

1.6 hBora interacts with Plk1 during mitosis

We were intrigued by the remarkable similarity between the phenotype induced by excess hBora and that displayed by cells that lack Plk1 protein or activity, notably with regard to Aurora A recruitment to the centrosome (De Luca et al., 2006; Hanisch et al., 2006). This prompted us to explore a potential link between hBora and Plk1. First, we asked whether hBora overexpression would perturb Plk1 localization. Indeed, induction of myc-hBoraN led to Plk1 displacement from both the spindle poles and the kinetochores (Fig. 29A) and, as described above for Aurora A, this effect was induced by hBora and hBoraN, but not hBoraC (Fig. 29B). Next, we asked whether Plk1 and hBora might interact with each other *in vivo*. When myc-hBoraN was immunoprecipitated from the tet-inducible cell line, both Plk1 and Aurora A could readily be detected in the immunoprecipitates, whereas TPX2 and Aurora B were absent (Fig. 30A). These results are in agreement with the data shown in Figure 3E and, furthermore, show that hBoraN and TPX2 do not bind to the same pool of Aurora A.

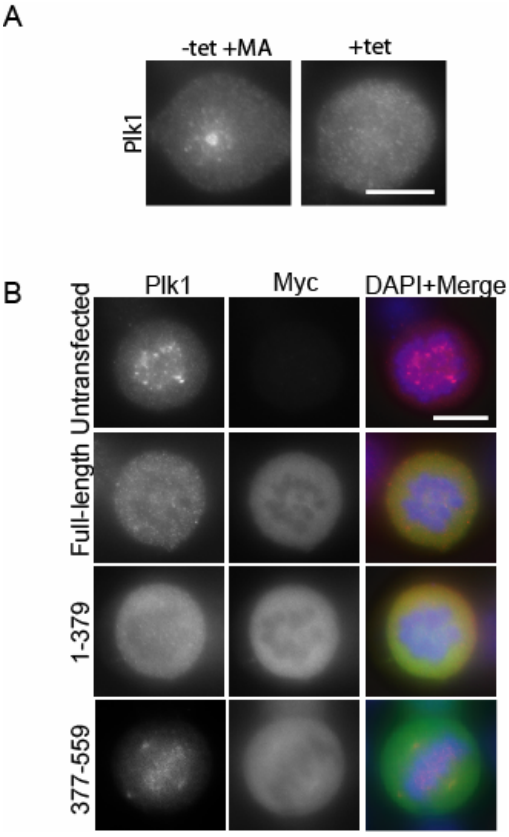

Figure 29. Overexpression of hBora displaces Plk1 from the centrosomes and kinetochores.

(A) Myc-hBoraN stable cell line was induced with tetracycline (+tet) for 48h or treated with monastrol (-tet, +MA) for control. Plk1 was visualized by immunofluorescence. Bar, 10μm. (B) HeLaS3 cells transfected with myc-tagged hBora, hBoraN and hBoraC were fixed and stained with 9E10 anti-myc (green) and Plk1 (red) antibodies. DNA was stained with DAPI (blue). Bar, 10μm.

To determine which domain of Plk1 interacts with hBora, HEK293T cells were co-transfected with myc-hBora and various GFP-Plk1 constructs and co-immunoprecipitation experiments were then performed on nocodazole-arrested cells. As shown in Figure 30B, phosphorylated forms of hBora were co-precipitated with both wild-type (WT) and catalytically inactive (KD) versions of Plk1, indicating that Plk1 activity is not required for this interaction. However, the hBora proteins co-precipiated by active and inactive versions of Plk1 displayed different electrophoretic mobilities, suggesting that they differed in phosphorylation state. Most importantly, the GFP-tagged C-terminal end domain of Plk1 (GFP-Plk1PBD) also bound to hBora, whereas the catalytic domain (GFP-Plk1Cat) did not (Fig. 30B). Taken together, the above results suggested that Plk1 interacts via its PBD with a phosphorylated form of hBora and that this binding prompts hBora phosphorylation by Plk1. Both *Drosophila* and human Bora can be phosphorylated by Cdk1 ((Hutterer et al., 2006); Fig. 24). Moreover, we could identify one potential Cdk1-induced PBD-docking site in hBora (S252), which has been conserved in evolution (Fig. 30C). Thus, we tested the functionality of this candidate PBD docking site in a Far-Western ligand binding assay (Neef et al., 2003). Without pre-phosphorylation by Cdk1, wild-type hBora showed very little binding to the GST-PBD, but strong binding was seen after phosphorylation (Fig. 30D, central panel). PBD binding to a S252A mutant of hBora was markedly reduced, albeit not completely abolished, even after pre-phosphorylation by Cdk1 (Fig. 30D, central panel). Virtually no binding to any hBora protein was seen with a PBD mutant (GST-PBD AA) that is unable to bind to phosphopeptides (Elia et al., 2003a) (Fig. 30D, right panel), and Coomassie Blue staining confirmed the presence of equal amounts of hBora (Fig. 30D, left panel). These results identify the motif centred on S252 as a major Cdk1-dependent Plk1-PBD binding site in hBora.

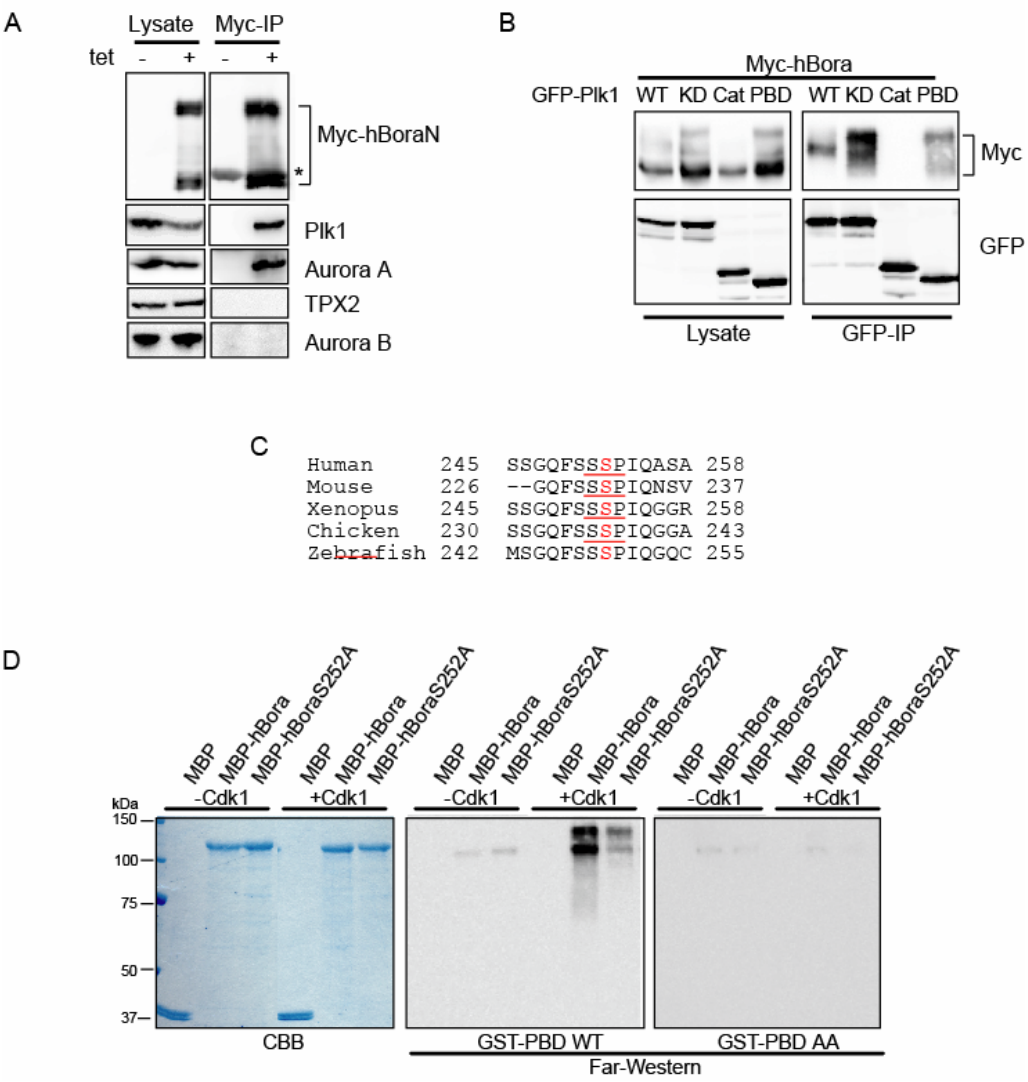

Figure 30. hBoraS252 is a PBD docking site.

(A) Myc-hBoraN was immunoprecipitated with 9E10 anti-myc antibody from nocodazole arrested cells treated with or without tetracycline (tet) for 48h. Immunoprecipitates were probed by Western blotting with antibodies against the indicated proteins. The asterisk denotes the IgG heavy chain. (B) Myc-hBora and GFP tagged-Plk1 constructs [wild-type (WT), kinase dead (KD), catalytic domain (Cat), polo-box domain (PBD)] were co-expressed in HEK293T cells for 48h and cells were arrested with nocodazole for the last 16h. The presence of myc-hBora in GFP-immunoprecipitates was assessed by Western blotting. (C) Evolutionary conservation of the putative PBD binding site in hBora (underlined). The phosphorylated serine is marked in red. Numbers refer to their position. (D) *In vitro* kinase assay were performed with Cdk1-cyclin B (or buffer for control) and the indicated proteins as substrates. The samples were subjected to SDS-PAGE and subsequently a Far Western ligand blotting assay using GST-PBD and GST-PBD-AA was performed. Coomassie blue staining (CBB) showed protein loading.

1.7 Plk1 triggers the SCF$^{\beta\text{-}TrCP}$ mediated degradation of hBora

Having shown that hBora is a binding partner (Fig. 22) and potential substrate of Plk1 (Fig. 24), we next explored the physiological significance of this interaction. We discovered that siRNA-mediated depletion of Plk1 led to the accumulation of hBora to very high levels, as compared to control or TPX2-depleted cells (Fig. 32A). This suggested that Plk1 might be required for regulating hBora stability, similar to the recently established role of this kinase in triggering the degradation of other regulators of cell cycle progression, notably Wee1 (Watanabe et al., 2004), Claspin (Mamely et al., 2006), Emi1 (Moshe et al., 2004) and Erp1/Emi2 (Liu and Maller, 2005; Rauh et al., 2005; Tung et al., 2005). This Plk1-dependent mechanism for regulated protein degradation requires the phosphorylation of a conserved motif (DpSGxxpT), a so-called phosphodegron, which then triggers the binding of the β-transducin repeat-containing protein (β-TrCP) component of a Skp1–Cul1–F-box-protein (SCF) ubiquitin ligase, followed by polyubiquitination and proteasomal degradation. Examination of the hBora sequence revealed a potential β-TrCP binding motif within the C-terminus, which is conserved amongst species (Fig. 32B). To explore the hypothesis that Plk1 might act through this putative phosphodegron, we first tested the ability of Plk1 to phosphorylate various recombinant hBora proteins *in vitro*. Plk1 readily phosphorylated full-length hBora, as well as N- and C-terminal fragments, but phosphorylation was reduced upon mutation of the putative phospho-degron (S497A/T501A) (Fig. 31). Next, we depleted Plk1 from HeLa cell lines moderately expressing either full-length hBora or hBoraN and then examined hBora proteins levels in lysates prepared from nocodazole-arrested cells. Similar to the results described above for endogenous hBora (Fig. 32A), Plk1 depletion led to an accumulation of myc-hBora (Fig. 32C). In stark contrast, hBoraN accumulated to similar levels in both control and Plk1-depleted cells (Fig. 32C), strongly supporting the hypothesis that the Plk1-dependent degradation of hBora is mediated by a phosphodegron within the C-terminal domain.

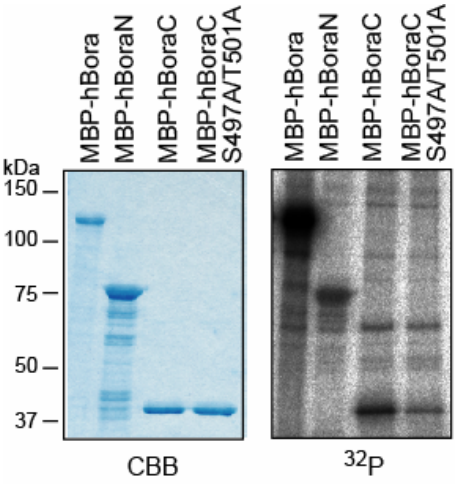

Figure 31. Plk1 phosphorylates the conserved phosphodegron of hBora *in vitro*.
Bacterially expressed MBP-tagged hBora, hBoraN, hBoraC and hBoraC S497A/T501A) were incubated together with Plk1 isolated from baculovirus in the presence of [γ-^{32}P]ATP *in vitro*. Kinase reactions were analysed by SDS–PAGE followed by autoradiography.

To further corroborate the involvement of Plk1 activity in hBora degradation, we tested the ability of catalytically inactive Plk1 (KD) to interfere with hBora degradation by acting as a dominant negative mutant. In response to co-expression of full-length myc-hBora with Plk1KD, a significant increase in hBora (phospho-) protein levels could be seen, whereas the co-expression of wild-type Plk1 (WT) had no obvious effect (Fig. 32D). Mutation of the phosphodegron (S497/T501) or the PBD-docking site (S252A) both abolished responsiveness of the mutant hBora proteins to alterations in Plk1 activity (Fig. 32D). Finally, the mitotically phosphorylated form of wild-type hBora could readily be co-immunoprecipitated with β-TrCP, whereas both the phosphodegron and the PBD-docking site mutants failed to interact (Fig. 32E). Taken together, these results demonstrate that the phosphodegron identified in hBora is functional and that Plk1 binding is essential for triggering the β-TrCP-mediated degradation of hBora. As a consequence, interference with Plk1 activity results in the accumulation of hBora (phospho-) protein.

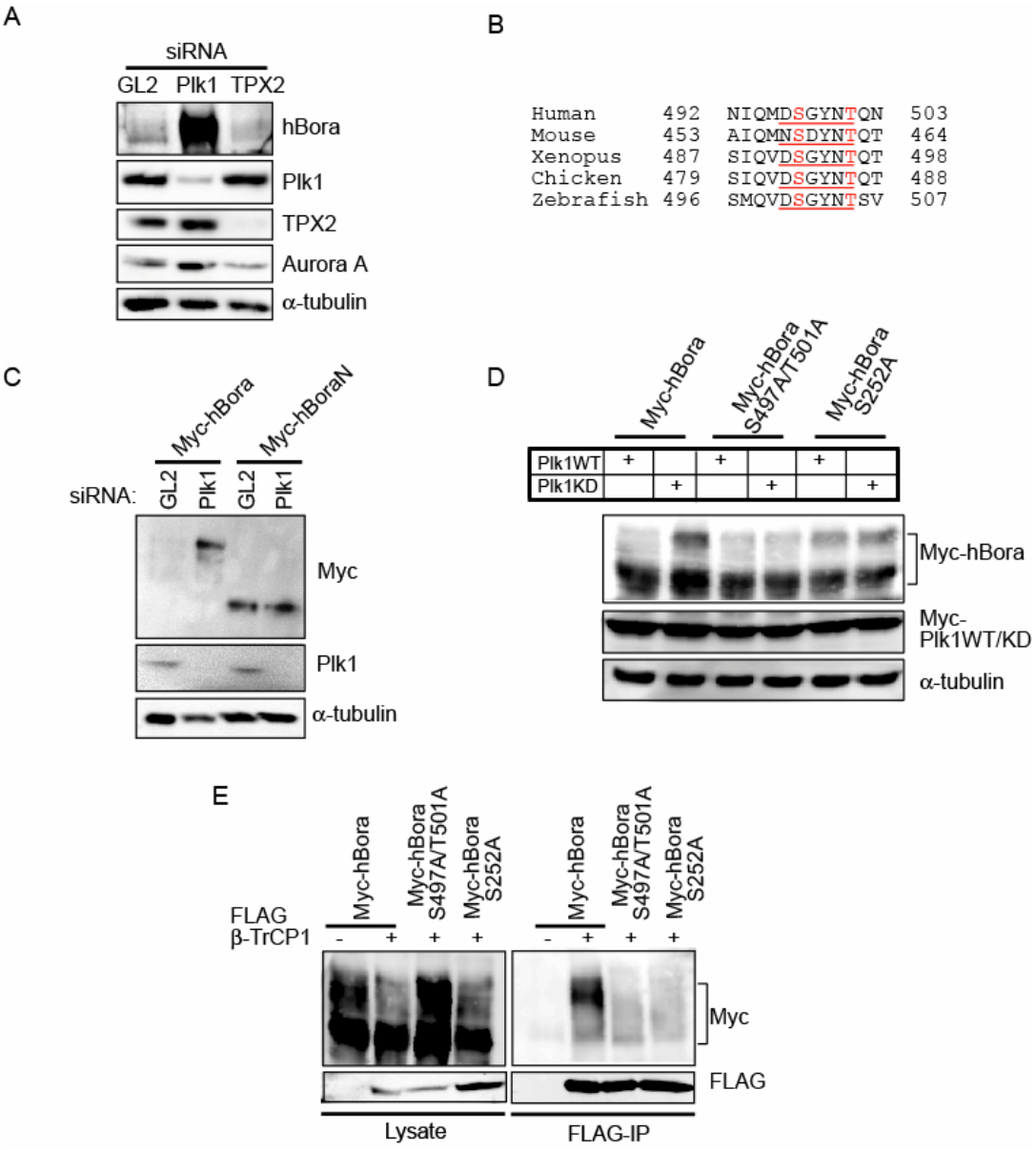

Figure 32. Mitotic degradation of hBora depends on Plk1 and β-TrCP.

(A) HeLaS3 cells were treated with siRNA duplexes specific for Plk1 for 36h or for TPX2 (or GL2 for control) for 72h. Cells were treated with nocodazole for the last 16h and collected by shake off. Cell extracts were probed by Western blotting with antibodies against the indicated proteins. α-tubulin levels provide a loading control. (B) Evolutionary conservation of the putative phosphodegron in hBora (underlined). The phosphorylated serine and threonine are marked in red. Numbers refer to their position. Note that the mouse sequence shows a slight deviation from the proposed consensus sequence (DpSGxxpT). (C) Expression of myc-hBora full-length or hBoraN in stable HeLaS3 cell lines was induced for 12h with tetracyclin before Plk1 and GL2 siRNA duplexes were added for another 36h. Cells were treated with nocodazole for the last 16h and were then collected by shake-off. Cell extracts were probed by Western blotting with antibodies against the indicated proteins and equal loading is shown by probing

for α-tubulin. (D) HEK293T cells were co-transfected with various constructs of myc-hBora (wild-type, phosphodegron mutant; S497A/T501A, and PBD docking mutant; S252A) and myc-Plk1 WT or KD for 48h. Cell extracts were probed by Western blotting with antibodies against the indicated proteins and α-tubulin levels provide a loading control. The expression between different hBora constructs is not comparable, because of variations in transfection efficiencies. Various constructs of myc-hBora (as in D) and FLAG-β-TrCP1 were coexpressed in HEK293T cells for 48h and treated with nocodazole for the last 16h. FLAG-immunoprecipitates were analyzed by immunoblotting with myc and FLAG antibodies. (E) Various constructs of myc-hBora (as in E) and FLAG-β-TrCP1 were coexpressed in HEK293T cells for 48h and treated with nocodazole for the last 16h. FLAG-immunoprecipitates were analyzed by immunoblotting with myc and FLAG antibodies.

1.8 Plk1 regulates Aurora A by controlling hBora levels

Considering that hBora acts as a regulator of Aurora A, but is itself controlled by Plk1, it follows that hBora could contribute to coordinate the functions of these two key regulators of mitotic progression. One specific prediction from our data is that Plk1 may determine Aurora A localization and activity through its ability to regulate the intracellular levels of hBora. Indeed, when Aurora A was immunoprecipitated from Plk1-depleted cells, most Aurora A was complexed to hBora, reflecting the increased levels of hBora in such cells (Fig. 33). Importantly, the amounts of TPX2 that could be co-precipitated with Aurora A were substantially reduced, although the total cellular level of TPX2 was not changed (Fig. 33). This indicates that hBora competes with TPX2 for Aurora A binding and that levels of hBora determine how much Aurora A is available for binding to other partners, notably TPX2.

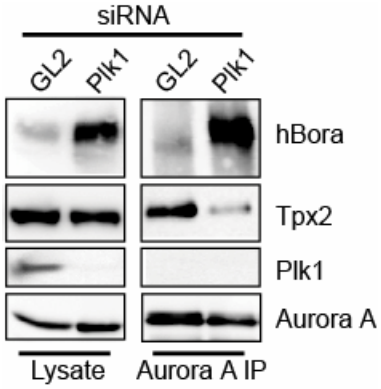

Figure 33. hBora competes with TPX2 in cells.
HeLaS3 cells were treated with Plk1 or GL2 siRNA duplexes for 36h. Cells were treated with nocodazole for 16h before harvesting them by shake-off. Cell extracts and Aurora A immunoprecipitates were probed by Western blotting with antibodies against the indicated proteins.

The above findings also suggested an attractive explanation for the observation that Plk1 is required for Aurora A localization to spindle poles (De Luca et al., 2006; Hanisch et al., 2006). Specifically, we reasoned that the requirement for Plk1 during centrosome maturation and spindle formation could reflect, at least in part, its role in lowering hBora levels below a threshold, such as to allow Aurora A to exert its functions on the centrosome and spindle. This model predicted that a reduction of hBora levels might alleviate at least some of the early mitotic defects that are typically seen in Plk1-depleted cells. To explore this possibility, we treated cells with siRNA duplexes targeting Plk1 and hBora, either in combination or singly (paired with GL2 for control), and asked whether co-depletion of hBora and Plk1 would bring Aurora A back to the centrosome and restore spindle bipolarity. Co-depletion of hBora with Plk1 suppressed the accumulation of hBora that normally results from Plk1 depletion, as expected (Fig. 34A). Both Plk1 depletion and Plk1/hBora co-depletion led to marked increases in mitotic indices (Fig. 34B), indicating that not all early mitotic functions of Plk1 can be attributed to its interaction with hBora, but the two cell populations displayed remarkably different phenotypes. Whereas the Plk1-depleted cells displayed mostly monopolar spindles, as expected, the Plk/hBora co-depleted cells showed mostly bipolar spindles, albeit with uncongressed chromosomes (Fig. 34C and D). Moreover, Aurora A was displaced from spindle poles in Plk1-depleted cells, consistent with previous data (De Luca et al., 2006; Hanisch et al., 2006), but largely restored to these structures in Plk1/hBora co-depleted cells (Fig. 34E lower panel). To facilitate the comparison with Plk1-depleted cells, Plk1/hBora co-depleted cells were also treated with monastrol to induce monopolar spindles (Fig. 34E middle panel) and, furthermore, both cell populations were exposed to 4^{o}C in order to depolymerize spindle MTs and better visualize centrosome-associated Aurora A (Fig. 34F). Taken together, these results demonstrate that co-depletion of hBora largely rescued the defects in bipolar spindle formation and Aurora A localization that normally result from Plk1 depletion.

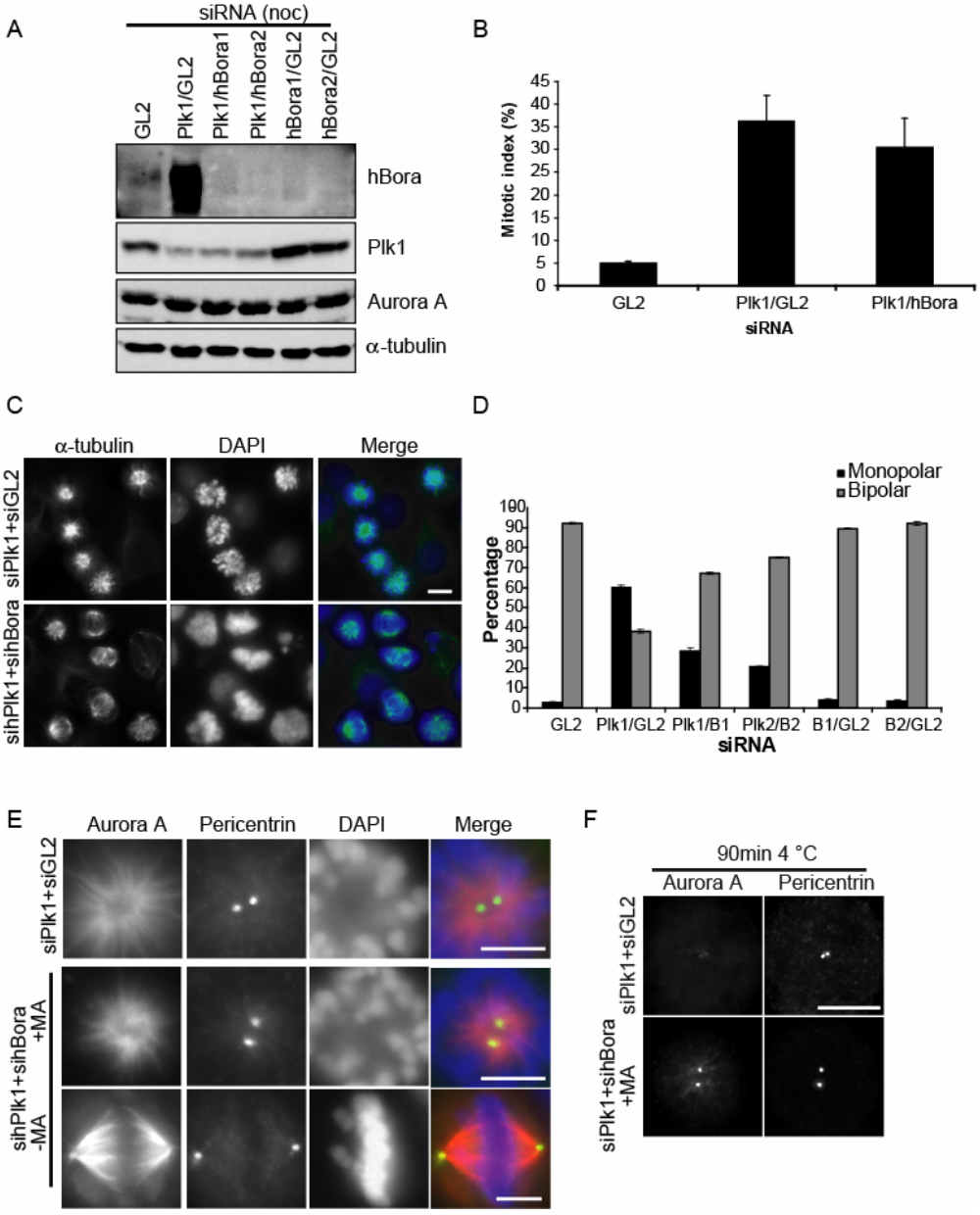

Figure 34. Plk1 regulates Aurora A localization by modulating hBora protein levels.

(A) HeLaS3 cells were treated with different combination of siRNA duplexes and arrested by nocodazole. Lysates were then assayed by immunoblotting against the indicated proteins. Total amount of duplexes were held constant at 100nM. (B) Histogram showing the mitotic indices of HeLaS3 cells treated with different siRNA duplexes. Results are from 3 individual experiments (300-350 cells each), and bars indicate standard deviations. (C) α-tubulin (green) and DAPI (blue) staining was performed on cells treated with combination of siRNA duplexes as indicated. Bar, 10μm. (D) Histogram summarizes the percentages of mitotic cells with monopolar/ bipolar spindle in experiments performed as in (B). Results are from 3 independent experiments (n=300-350), bars indicate standard deviations. (E) HeLaS3 cells

treated with Plk1/GL2 or Plk1/hBora siRNA duplexes and subsequently with or without 150μM monastrol (MA) were fixed and stained for Aurora A (red) and pericentrin (green). Bar, 10μm. (F) Same as E, but cells were subjected to cold treatment for 90min before fixation and staining.

2. Functional studies of Aurora A

2.1 Aurora A depletion leads to long/multipolar spindle formation and abnormal centriole splitting

In a final series of experiments, we have used siRNA to explore the function of Aurora A. HeLaS3 cells were treated with GL2 or Aurora A siRNA duplexes for 72h and then were released from a double thymidine block for 12h to enrich for mitotic cells. While Western blotting showed an effective depletion of Aurora A protein (Fig. 35), IF microscopy showed weak residual staining of Aurora A at the centrosome for Aurora A siRNA treated cells (not shown). Aurora A depletion led to an increase in mitotic index when compared to controls (from 10% to 30%) and to massive apoptosis. In addition, Aurora A depletion caused severe spindle abnormalities during mitosis. More than 80% of mitotic cells formed either abnormally long or multipolar spindles, which were found in fewer than 10% of control cells (Fig. 36A and C). Pericentrin staining revealed centrosome abnormalities (Fig. 36B). Aurora A depleted cells often had more than 2 centrosomes with irregular shape, indicating that the centrosome may fragment which then resulted in abnormal spindles with diffuse or multiple poles. To find out whether the formation of multipolar spindles is caused by centrosome overduplication, cells were stained with centrin antibodies to visualize centrioles (Fig. 36A and B). In control cells, each centrosome (as indicated by pericentrin staining) consists of a pair of centrioles (as indicated by centrin staining) (Fig. 37A). Aurora A depleted cells frequently carried two pairs of centrioles, indicating

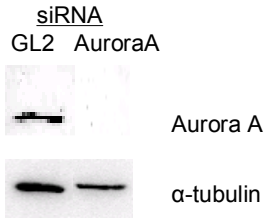

Figure 35. Depletion of Aurora A. Effective depletion of Aurora A after 72h siRNA treatment is shown by Western blotting. α-tubulin levels provide a loading control.

that the centrosome duplicated normally (Fig. 37). However, instead of centrioles being arranged pairwise, more than 60% of mitotic cells treated with the Aurora A siRNA duplexes showed split centrioles (Fig. 37). The above data showed that the multipolar spindles formed in Aurora A depleted cells may arise from abnormal centriole splitting which results in the formation of multiple microtubules organizing centres. Thus, we conclude that Aurora A is required for centriole cohesion in mitosis.

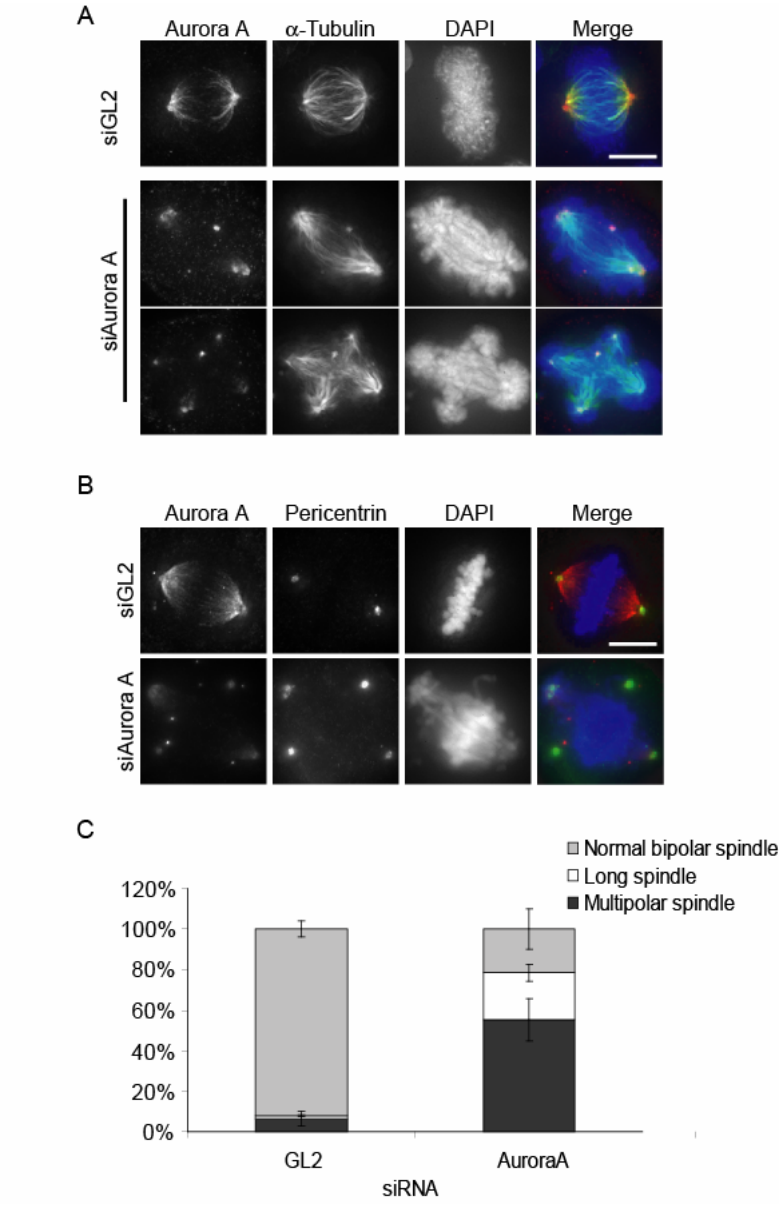

Figure 36. Aurora A depletion leads to multipolar spindle formation.
(A) HeLaS3 cells were treated with Aurora A siRNA duplexes for 72h and synchronized by double thymidine block. Cells were fixed and stained with α-tubulin (green) and Aurora A (red) antibodies. DNA was stained with DAPI (blue). Bar, 10μm. (B) Same as A, but cells were stained with pericentrin (green) and Aurora A (red) antibodies. Bar, 10μm. (C) Histogram summarizes percentage of mitotic cells with spindle abnormalities. Results from three independent experiments (n=100-150); bars indicate standard deviations.

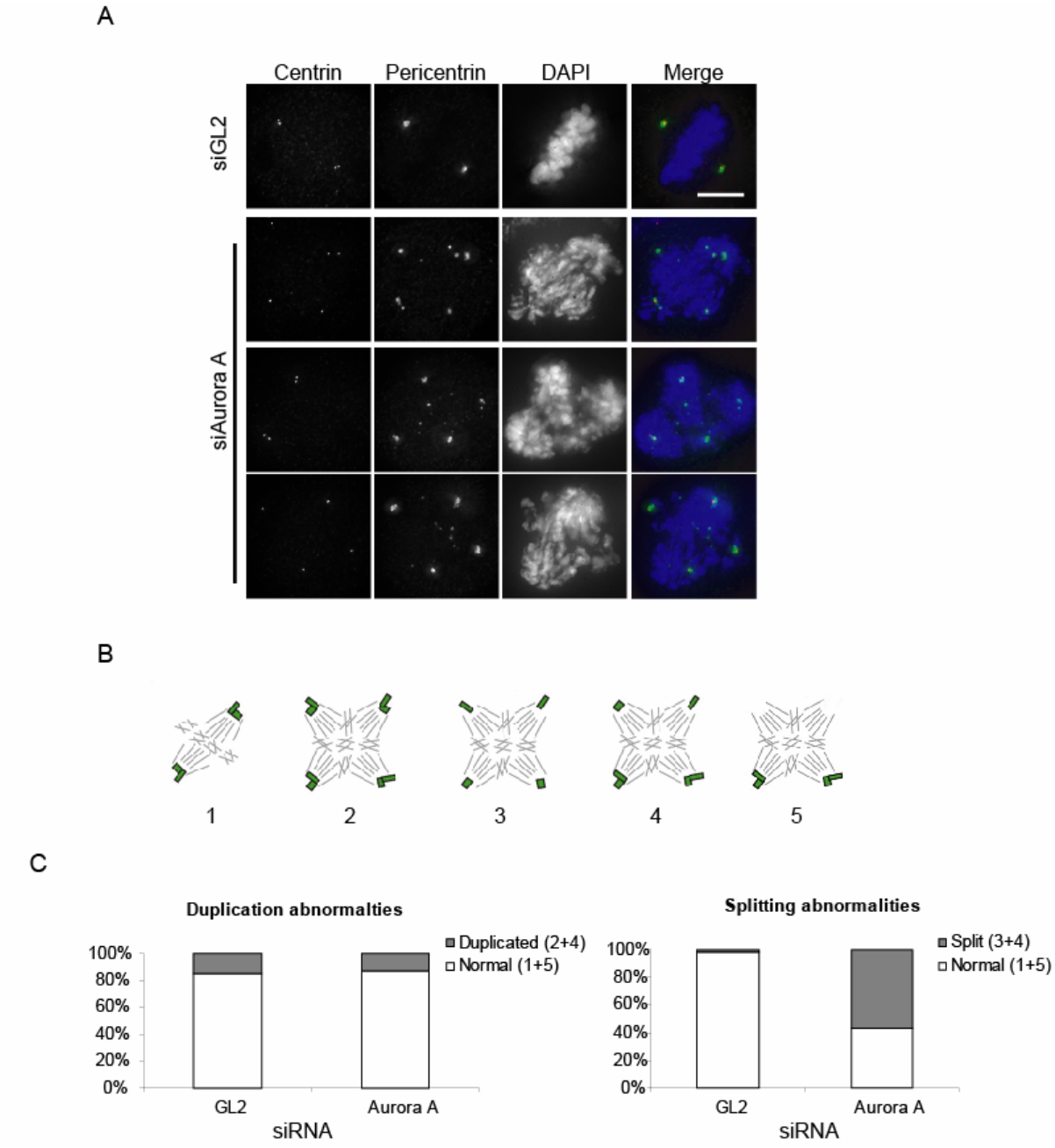

Figure 37. Aurora A depletion induces centriole splitting in mitosis.

(A) Cells were treated as described in the legend to Fig. 36 and then stained with centrin (red) and pericentrin (green) antibodies to visualize different centrosome abnormalities. (B) Diagrams depict different types of centrosome abnormalities and centrioles shown in green. Cells containing 2 pairs of centrioles are defined as normal (1, 5). Cells with more than 2 pairs of centrioles reflect centrosome overduplication (2, 4) and cells with unpaired centrioles reflect premature splitting (3+4) (C) Quantitative studies demonstrate the percentages of mitotic cells showing centrosome overduplication (left histogram) or centriole splitting (right histogram).

2.2 Aurora A activity is required for centrosome separation

We have shown above (section 1.5) that the mislocalization of both Plk1 and Aurora A in hBoraN overexpressing cells leads to monoastral spindle formation (Fig 27). To check whether we could rescue the centrosome separation defects by reintroducing Aurora A and whether the activity of Aurora A was required for such a rescue, we overexpressed wild-type (WT) or kinase dead (KD) version of Aurora A in hBoraN-expressing HEK293 cell (Fig. 38). Both GFP-tagged Aurora A WT and KD localized normally to centrosomes and spindles in mitotic cells (Fig. 38). However, only overexpression of catalytically active Aurora A, but not kinase dead Aurora A, restored spindle bipolarity in hBoraN overexpressing cells. This indicates that Aurora A kinase activity is required for centrosome separation and the maintenance of spindle bipolarity.

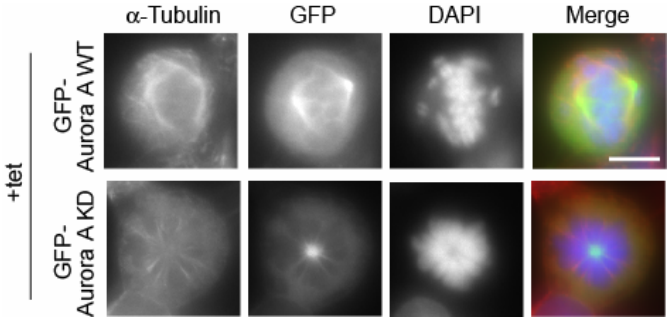

Figure 38. Overexpression of Aurora A WT but not KD restores bipolar spindle formation in hBoraN overexpressing cells.

Myc-hBoraN stable cell line was induced with tetracycline (+tet) and transfected with GFP-Aurora A WT or GFP-Aurora A KD for 48h. Cells were fixed and stained with α-tubulin (red) and DAPI (blue) to visualize the spindle and DNA morphologies, respectively.

2.3 Aurora A localization is required for centriole cohesion

Overexpression of hBora leads to monoastral spindle formation due to the mislocalization of both Plk1 and Aurora A. To specifically test the influence of Aurora A mislocalization in cells, we overexpressed hBora1-232 in HEK293 cells. As shown by immunoprecipitation, this mutants bind only to Aurora A but not to Plk1 (Fig. 39A). By immunofluorescence, we could show that Aurora A is displaced upon overexpression in Myc-hBora1-232 cells. Interestingly, cells highly overexpressing Myc-hBora1-232 formed mostly multipolar spindles (Fig. 39 upper and middle panel) with split centrioles

(Fig. 39B lower panel, note that the centrioles are disengaged when we used CAP350 as a centriole marker). This confirms that Aurora A localization at the centrosome is essential for centriole cohesion.

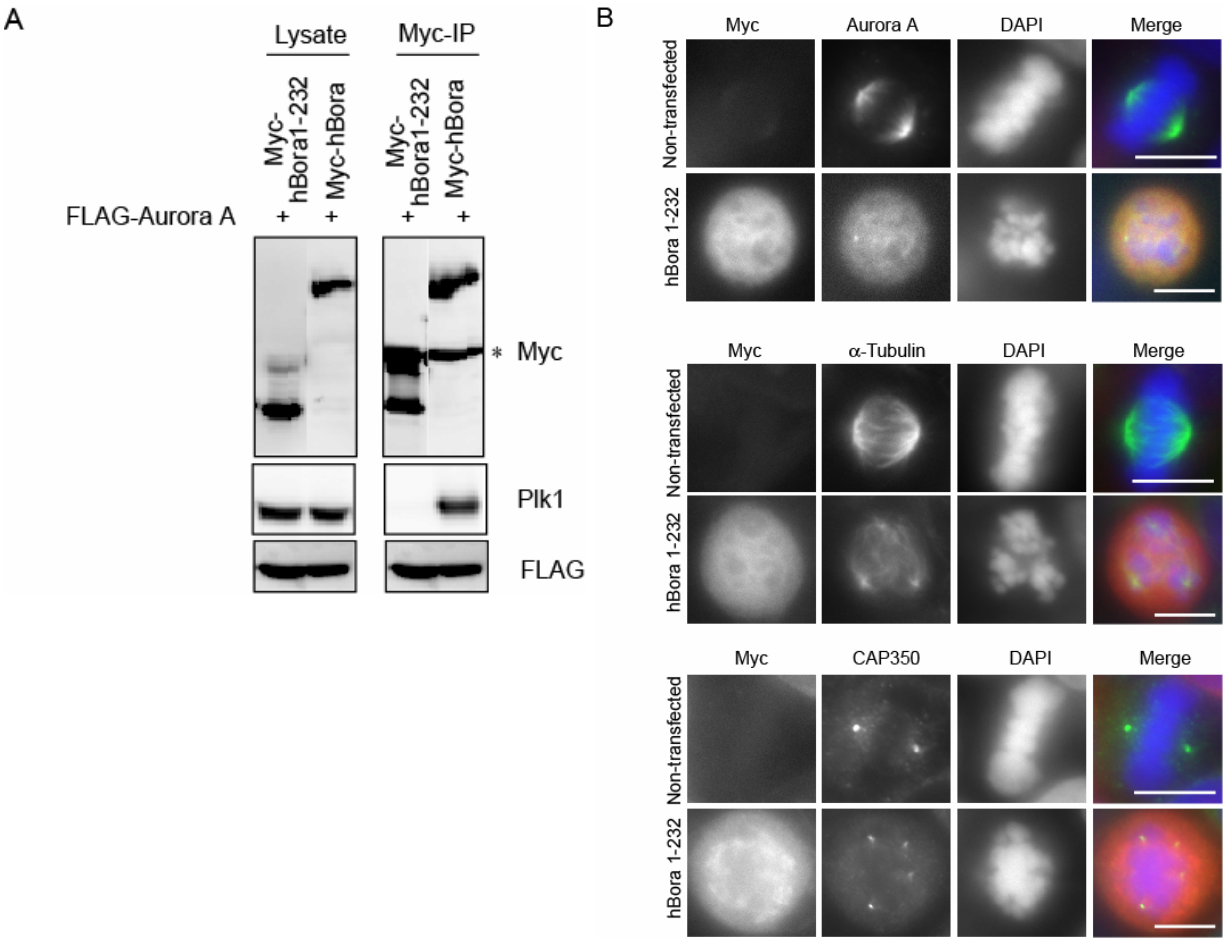

Figure 39. Displacement of Aurora A leads to multipolar spindle formation and centriole splitting.
(A) Myc-hBora or Myc-hBora1-232 and FLAG-Aurora A were overexpressed in HEK293T cells arrested by nocodazole. The presence of endogenous Plk1 and FLAG-Aurora A in the Myc-coimmunoprecipitates were detected by Western blotting. The asterisk denotes the IgG heavy chain. (B) Myc-hBora1-232 was overexpressed in HEK293T cells for 48h. Cells were fixed and stained with Myc (red) and DAPI (blue), together with Aurora A, α-tubulin (red) or CAP350.

Discussion II

1. Plk1 controls the function of Aurora A kinase by regulating the protein levels of hBora

We have studied the regulation and function of hBora, a recently described interactor of Aurora A (Hutterer et al., 2006). Our studies indicate that precise hBora levels are critical for correct Aurora A localization and spindle assembly. Furthermore, we demonstrate that hBora binds not only to Aurora A but also to Plk1. In particular, the phosphorylation of hBora on the Cdk1 site S252 triggers the recruitment of Plk1, which then phosphorylates hBora within a conserved phosphodegron motif, resulting in the $SCF^{\beta\text{-}TrCP}$- mediated degradation of hBora. In support of this scenario, interference with Plk1 activity results in a drastic upregulation of hBora and concomitant sequestration of Aurora A to the cytosol. Thus, by virtue of its ability to regulate the abundance of a cytoplasmic hBora-Aurora A complex, Plk1 controls the availability of Aurora A for interactions with spindle-associated partners such as TPX2. This mechanism also provides an attractive molecular explanation for recent data suggesting that Plk1 acts upstream of Aurora A in centrosome maturation and spindle assembly (De Luca et al., 2006; Hanisch et al., 2006). Collectively, our findings lead us to propose that hBora contributes to integrate the functions of three major mitotic kinases, Cdk1, Plk1 and Aurora A.

hBora levels are critical for proper spindle assembly

In response to siRNA-mediated depletion of hBora, we observed the formation of long and wavy spindles, which eventually progressed to form multipolar spindles. In addition, we observed unusually ‘fat’ spindles that were characterized by increased MT density, increased amounts of spindle-associated Aurora A, and increased cold stability of K-fibers. The exact mechanisms underlying these spindle defects remain to be unraveled, but since hBora and TPX2 do not bind simultaneously to Aurora A, any reduction in hBora levels is expected to favor complex formation between Aurora A and spindle-associated activators such as TPX2 (Eyers et al., 2003; Kufer et al., 2002; Tsai et al., 2003). In turn, the increased abundance of Aurora A on the spindle is likely to

cause enhanced activity of downstream effectors, notably chTOG, the human homolog of *Xenopus* XMAP215 and *Drosophila* minispindles (Barros et al., 2005; Giet et al., 2002; Kinoshita et al., 2005; Peset et al., 2005). Consistent with the above interpretation, the phenotypes seen in hBora-depleted cells resemble those of cells overexpressing Aurora A (Anand et al., 2003; Meraldi et al., 2002).

hBora interacts not only with Aurora A but also with Plk1

Striking spindle defects were also observed upon overexpression of hBora. Because excess hBora severely impaired the recruitment of both Aurora A and γ-tubulin to centrosomes, centrosome maturation and separation failed, resulting in the formation of monopolar spindles. Importantly, this phenotype was dependent on the ability of hBora to bind Aurora A. Excess hBora caused the sequestration of Aurora A into a diffusely localized pool, whereas TPX2 remained on the spindle apparatus, indicating that cytoplasmic hBora regulates the size of the Aurora A pool that is available for interactions with spindle-associated binding partners. In support of this conclusion, a rapid exchange of Aurora A between the spindle and the cytosol has previously been observed in photobleaching experiments (Stenoien et al., 2003). Considering that Aurora A displays activity when bound to hBora ((Hutterer et al., 2006), this study), it is entirely possible that this diffusely localized complex carries out important functions by acting on cytosolic substrates, perhaps regulating cell cycle progression. However, when hBora is deregulated, it perturbs Aurora A functions that are important for spindle assembly. Thus, the phenotypic consequences of hBora depletion and overexpression can largely be explained by deregulation of Aurora A complexes on mitotic structures.

The monopolar spindles produced in response to hBora overexpression are remarkably similar to those seen in Plk1-depleted cells, in that both are characterized by a conspicuous absence of Aurora A from the unseparated centrosomes. This prompted us to explore a possible relationship between hBora and Plk1. We found that hBora interacts not only with Aurora A but also with Plk1, in both cases through its N-terminal domain. The interaction between hBora and Plk1 requires the Plk1 PBD and prior phosphorylation of hBora on a Cdk1 site, S252, in line with an increasingly well established model (Elia et al., 2003a).

Plk1 regulates hBora stability

Most importantly, we discovered that Plk1 depletion causes a massive upregulation of hBora, indicating that Plk1 normally sets a threshold for the abundance of hBora. Our study further revealed a mechanistic explanation for the control of hBora levels by Plk1. After its recruitment to hBora, Plk1 in fact phosphorylates a conserved phosphodegron, which then serves as a recognition motif for the ubiquitin ligase $SCF^{\beta\text{-}TrCP}$, leading to the proteasomal degradation of hBora. At first glance, it may seem surprising that hBora levels peak during early mitosis, when both Cdk1 and Plk1 are active and thus would be expected to trigger hBora degradation. Interestingly, however, hBora is highly phosphorylated during early mitosis and appears to serve as a substrate for several mitotic kinases, including Aurora A. So, it is possible that the population of hBora that accumulates during early mitosis is protected against degradation through phosphorylation at particular sites and/or the binding of interaction partners (perhaps Aurora A itself). Furthermore, phosphatases counteracting Cdk1 and/or Plk1 are likely to contribute to the establishment of a steady-state level of hBora. Complex regulation of protein stability is not without precedent (Mailand et al., 2002) and so we anticipate the existence of multiple mechanisms to ensure physiological levels of hBora and appropriate timing of hBora degradation. As noted in the introduction, we emphasize that the proposed regulation of the hBora levels by the combined actin of Plk1 and β-TrCP awaits confirmation through continued experimentation. We also note that hBora carries within its N-terminus potential destruction motifs for yet another ubiquitin ligase, the anaphase promoting complex/cyclosome (APC/C). So, although our data point to $SCF^{\beta\text{-}TrCP}$ as the ubiquitin ligase responsible for Plk1-induced hBora degradation, it would be premature to exclude that other ligases may contribute to hBora degradation, particularly during later stages of mitosis or the subsequent G1 phase.

Through hBora Plk1 acts as an upstream regulator of Aurora A

Both Plk1 and Aurora A regulate key events during late G2 and early mitosis, including centrosome maturation and spindle assembly. Most recently, Plk1 was also found to be required for Aurora A localization to centrosomes, suggesting that it might act upstream of Aurora A (De Luca et al., 2006; Hanisch et al., 2006). In principle, this

latter observation might reflect a direct interaction between the two kinases, or a need for Plk1 to phosphorylate a particular substrate that would in turn be required for Aurora A recruitment to the centrosome. Although plausible, these two mechanisms presently lack experimental support. Our present data, centered on hBora, suggest an alternative explanation. Specifically, we propose that Plk1 activity is required to keep hBora levels below a certain threshold, such as to prevent sequestration of Aurora A into cytoplasmic complexes with hBora. In support of this view, several of the early mitotic defects typically seen in cells deprived of Plk1 protein and/or activity could be rescued by hBora co-depletion. This implies that the centrosome maturation and separation defects seen in Plk1-depleted cells may be attributed, at least in part, to the hBora-mediated impairment of Aurora A function.

We emphasize that not all early mitotic functions of Plk1 can be explained through hBora-mediated regulation of Aurora A. In particular, co-depletion of hBora did not rescue the chromosome congression defect or the mitotic arrest typically seen in Plk1-depleted cells. This notwithstanding, our studies on hBora have uncovered an important mechanistic relationship between Plk1 and Aurora A. Thus, it is interesting to consider the possible implications of this relationship for the proposed roles of mitotic kinases in tumorigenesis. Both Plk1 and Aurora A are often overexpressed in tumors (Knecht et al., 1999; Sen et al., 1997) and Aurora A is considered a cancer susceptibility gene (Ewart-Toland et al., 2003; Meraldi et al., 2004). It is intriguing, therefore, that hBora also maps to a chromosomal region (13q21) that is often altered in tumors (Rozenblum et al., 2002). In consideration of the data reported here, one would predict that deregulation of hBora should lead to similar cellular phenotypes as the aberrant expression of either Plk1 or Aurora A.

2. Functions of human Aurora A kinase

The role of Aurora A in centrosome maturation and separation, bipolar spindle assembly is mainly established from genetic studies in different organisms. (Giet and Prigent, 2000; Glover et al., 1995; Hannak et al., 2001). In contrast, results obtained through depletion or functional inhibition of Aurora A in somatic mammalian cells are confusing. Different consequences including mitotic entry delay (Marumoto et al., 2003), multipolar

spindle formation (De Luca et al., 2006), chromosome misalignment (Marumoto et al., 2003), centrosome separation defects (Hoar et al., 2007) and cytokinesis defects (Marumoto et al., 2003) have been described. This is probably due to the fact that Aurora A plays multiple roles at different stages during mitosis.

Our study suggest a role of Aurora A for centriole cohesion and spindle pole integrity, in line with the observations from (De Luca et al., 2006). It has been shown that centrioles disengage at the end of mitosis, presumably under the control of separase (Tsou and Stearns, 2006). The mother centriole then becomes mobile and moves rapidly to the midbody, which is considered to be a crucial event for cytokinesis (Piel et al., 2001). Thus, it is possible that Aurora A maintains centriole cohesion during mitosis before cytokinesis occurs. Interestingly, by making use of the observation that hBora overexpression mislocalizes Aurora A and causes monoastral spindle formation, we demonstrated that the co-overexpression of active Aurora A kinase (but not of a kinase dead mutant) could restore spindle bipolarity in hBora overexpressing cells. This implies that Aurora A kinase activity is required for centrosome separation and bipolar spindle formation. Furthermore, we explored the function of Aurora A by introducing a fragment of hBora that binds only to Aurora A but not to Plk1. This created an environment in which Aurora A activity is still present but the kinase is mislocalized. These hBora1-232 overexpressing cells formed multipolar spindle with split centrioles. Thus, we conclude that the spindle pole localization of Aurora A is required for maintaining centriole cohesion.

Summary II

Aurora A kinase has been implicated in regulating mitotic entry, centrosome maturation, and spindle assembly, but the molecular mechanisms underlying its regulation and mode of action still remain largely unknown. In this study, we investigated the interaction between Aurora A and hBora, a protein which was originally identified as an Aurora activator in *Drosophila*. We show that hBora is a cell-cycle regulated protein required for proper spindle assembly. While hBora can activate Aurora A *in vitro*, it also interacts with Plk1. The interaction between hBora and Plk1 is promoted by Cdk1 phosphorylation and is required for degradation of hBora, possibly mediated by the $SCF^{\beta\text{-}TrCP}$. Most importantly, we demonstrate that Plk1 regulates Aurora A function during centrosome maturation and assembly by modulating the protein levels of hBora.

After characterizing the interaction between Aurora A and hBora, we also tried to investigate the function of Aurora A by siRNA mediated depletion and overexpression studies. Aurora A depletion led to multipolar spindle formation due to abnormal centriole splitting. Interestingly, overexpression of a fragment of hBora which mislocalizes Aurora A but not Plk1 showed the same phenotype. In addition, since only the kinase active version of Aurora A could rescue bipolar spindle formation in hBora overexpressing cells, we speculate that Aurora A may have dual functions at centrosomes. We suggest that the kinase activity of Aurora A is required for centrosome maturation and separation, while the localization of Aurora A is required for maintaining centriole cohesion.

Materials and Methods

Plasmid constructions and site directed mutagenesis

Human *LATS1* cDNA was a kind gift from Hideyuki Saya and *MST1* and *MST4* plasmids were kindly provided by Francis Barr. *LATS2* was assembled from three expressed sequence tags (ESTs) (ID: IMAGp998K2462472Q2, IMAGp998M236303Q2, IMAGp998F098723Q2), hWW45 from EST (ID: IMAGp998J155583), hBora from EST (ID: IRAKp961O2411Q2), β-TrCP1 from EST (ID: HU3_p983C04289D). All ESTs were obtained from the "Deutsches Ressourzentrum für Genomforschung" (RZPD). Mst2 was obtained from a λgt11 cDNA library (Stratagene). All cDNAs were cloned into pRcCMV vectors in-frame with sequences that encode amino-terminal FLAG, EGFP or 3x myc tags. Site-directed mutagenesis was performed by PCR with oligonucleotides containing appropriate mutations. Constructs encoding catalytically inactive kinases were created by changing a conserved aspartate in subdomain VII, to an alanine (Lats1D846A, Lats2D809A, Mst2D164A). Mutations in hBora phosphodegron and PBD docking site were created by changing conserved serine or threonine residues to alanine (S252A, S497A/T501A. All constructs were verified by sequencing.

Cell culture, synchronization, and transfection

HeLaS3 and HEK293T cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco-BRL), supplemented with 10% heat inactivated fetal calf serum (FCS) and penicillin-streptomycin (100 IU/ml and 100 mg/ml, respectively). Okadaic acid (OA) treatment of cells was performed by adding 1μM OA (Alexis) to the culture medium for 1h at 37°C.

293T cells were arrested at prometaphase stage by 500ng/ml nocodazole for 18h. HeLaS3 cells were synchronized at the G1/S phase border by a double aphidicolin or thymidine block as follows. Cells were first treated for 12h with 1.6μg/ml aphidicolin (Sigma) or 1mM thymidine, then washed 3 times with PBS and released for 12h into fresh medium before drug was added again for another 12h. Cells were subsequently washed three times in PBS and released into fresh medium after which samples were

taken at regular intervals. To arrest HeLaS3 cells at prometaphase, cells were first pre-synchronized at the G1/S phase border by treatment with 1.6μg/ml aphidicolin for 12h. Cells were then washed three times in PBS and released into medium containing 50 ng/ml nocodazole (Sigma) and culturing was continued for an additional 14h. After that, mitotic cells were collected by mechanical shake-off, washed twice with PBS, and released into normal growth medium. Samples were taken either from the arrested cells or after release at various time intervals. Alternatively, cells were treated with 150 μg/ml Monastrol (Sigma) for 16h.

Transfection of HEK293T cells for purifying recombinant proteins were performed using the calcium phosphate method as described by (Seelos, 1997). HeLaS3 cells were transfected for immunofluorescence microscopy by using Fugene (Roche), according to the manufacturer's instructions.

Generation of stable cell lines

To generate the myc-hBora and myc-hBora(1-327) Tet-on inducible stable cell lines, Tet-on HeLaS3 and HEK293 TrexTM cells were transfected in 15 cm dishes with 5μg plasmids by Fugene. After 48h, transfected cells were selected by 1.5μl/ml puromycin for 48h and diluted for single cell colony formation. The stable cell lines were then grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco-BRL), supplemented with 10% heat inactivated fetal calf serum (FCS) and penicillin-streptomycin (100IU/ml and 100mg/ml, respectively) in the presence of 2μg/ml blasticidin and 1.5μg/ml puromycin for selection. Expression of myc-hBora and myc-hBora(1-327) were induced by 1μg/ml tetracycline.

Cell extracts and Western blot analysis

Cells were washed with ice cold PBS containing 1mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in ice cold HEPES lysis buffer (50 mM Hepes pH7.4, 150mM NaCl, 0.5% Triton X-100) containing 1μM OA, 30μg/ml RNase A, 30μg/ml DNase and protease and phosphatase inhibitors (Sillje et al., 1999). After 15min on ice, cells were collected by scraping and centrifuged at 13000 RPM for 15min at 4°C. Protein concentrations were determined using the Dc protein assay (Bio-Rad).

For immunoblotting, equal amounts of protein were separated by SDS-PAGE followed by transfer to nitrocellulose membranes (Schleicher & Schuell). Membranes were probed with the following antibodies: affinity-purified rabbit anti-Lats1 (1μg/ml), affinity-purified rabbit anti-hBora (1μg/ml), anti-FLAG antibodies (1:1000; rabbit; sigma), 9E10 anti-myc (1:5), anti-Plk1 (1:5), anti-GFP (1:5), Aurora A antibodies (1:1000; mouse; BD Transduction Laboratories), anti-Aurora B (AIM1) (1:500; mouse; BD Transduction Laboratories), anti-TPX2 (1:1000; mouse; Abcam, Cambridge, UK), anti α-tubulin (1:3000; mouse; Sigma-Aldrich), anti-cyclin B1 (1:1000; mouse; Sigma). Primary antibodies were detected with HRP-conjugated anti-mouse or anti-rabbit antibodies (Pierce). Signals were detected by ECL SuperSignal (Pierce Chemical Co.).

Spindle preparation

For mitotic spindle isolations, HeLaS3 cells were firstly arrested in G1/S by the addition of 1.6μg/ml aphidicolin for 12h. Upon release from this block, 50ng/ml nocodazole was added and cells were grown for another 12h. Mitotic cells were obtained by shake-off, washed and released into fresh medium for 1h. At this time point the mitotic spindles had reformed and after addition of 5μg/ml taxol or 100ng/ml nocodazole (negative control) for 5min. cells were harvested. The subsequent spindle isolation procedure was essentially as described before (Sillje and Nigg, 2006).

Preparation of Baculoviruses, Sf9 cell culture, and purification of recombinant proteins

Baculovirus expressing GST-hBora and His-hBora were produced with appropriate pVL1393 constructs, using the BaculoGold transfection kit (BD PharMingen). Sf9 cells (Invitrogen) were grown in T.C.100 medium supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Invitrogen) at 27 °C prior to infection with baculoviruses. 48 h post infection, proteins were purified as described previously (Kelm et al., 2002) and subjected to SDS-PAGE or used for kinase assays. MBP-tagged proteins were purified from *E. coli* as described (Baumann et al., 2007)

Antibody production

For Lats1 and hBora antibody production, the specific N-terminal Lats1 fragment (aa 267-403) and full length hBora were expressed and purified as His_6 tagged fusion proteins in *Escherichia coli*, using the bacterial expression vector pQE30 (Qiagen) and pET28(Clontech), respectively. Antibodies against these antigens were raised by immunization of rabbits with 250μg antigen for four times (Charles River Laboratories). For immuno-purification serum was applied to a Ni^{2+} resin column containing the antigen used for immunization. After washing and elution from the column, antibodies were dialyzed against PBS, essentially as described before (Chalamalasetty et al., 2006).

Immunofluorescence microscopy

HeLaS3 cells were grown on HCl-treated coverslips while HEK293T cells were grown on fibronectin coated coverslips. For paraformaldehyde fixation, cells were fixed for 10 min at RT in a 3% paraformaldehyde/2% sucrose solution before permeablizing by ice-cold PBS with 0.5% Triton-X-100. To visualize kinetochores staining, cells were fixed and permeabilized by PTEMF solution containing 4% formaldehyde; 0.2% Triton-X-100; 10mM EGTA and 1mM MgCl in 20mM PIPES, pH6.8 for 15min at room temperature. For centrosomal protein labeling, cells were fixed by cold methanol at -20°C for 5min. Coverslips were then washed with PBS and blocked with 1 % BSA in PBS. Incubation of both primary and secondary antibodies was done in PBS containing 1% BSA, inside a humidified chamber for 1-2h at RT. Primary antibodies used were: anti-Lats1 (1μg/ml; rabbit) and the corresponding pre-immune antibodies (1μg/ml), anti-hBora (1μg/ml; rabbit) and the corresponding pre-immune antibodies (1ug/ml), anti-Plk1 (1:5), anti-Aurora A (1:1000; mouse; BD Transduction Laboratories), anti-Aurora B (AIM1) (1:500; mouse; BD Transduction Laboratories), anti-TPX2 (1:1000; mouse; Abcam), anti-γ-tubulin (Sigma), anti-pericentrin (Abcam), FITC-labeled anti-α-tubulin (1:500; Sigma) and TRITC or FITC-labeled anti-myc 9E10 (1:1000; Santa Cruz Biotechnology), anti-α-tubulin (1:200; Sheep; cytoskeleton); monoclonal anti-γ-tubulin (1:3000; Sigma); polyclonal Ch-Tog antibodies (1μg/μl; rabbit; kindly provided by Dr.

Xiumin Yan). polyclonal CAP350 antibodies (1μg/μl; goat; kindly provided by Dr. Xiumin Yan). Secondary antibodies used were: Alexa488- and Alexa568-conjugated goat α-mouse IgG (Sigma-Aldrich, 1:1000) and Alexa488- and Alexa568-conjugated goat α-rabbit IgG (Molecular Probes 1:1000). DNA was strained with 2μg/ml diamidino-2-phenylindole DAPI (Sigma-Aldrich).

Immunofluorescence microscopy was performed with a Zeiss Axioplan II microscope (Carl Zeiss, Jena, Germany) equipped with a 63x oil immersion objective and images were acquired using a Micromax charge couple device (CDD) camera (Princeton Instruments, Trenton, NJ). Alternatively, a Deltavision microscope on a Nikon Eclipse TE200 base (Applied Precision, Issaquah, WA) equipped with an Apo 60_/1.4 oil immersion objective and a CoolSnap HQ camera (Photometrics) was used for collecting 0.15-μm distanced optical sections in the z-axis. Images at single focal planes were processed with a deconvolution algorithm depending on the objective used. Settings were conservative, with noise filtering set to low and 2 deconvolution cycles. The number of z-stacks collected was variable (between 5 and 20), depending on the height of the individual cell. Images were projected into one picture using the Softworx software (Applied Precision). Exposure times and settings for image processing (deconvolution) were constant for all samples to be compared within any given experiment. Images were processed with Adobe Photoshop CS (AdobeSystems).

siRNA transfection

The following siRNA sequences were used for silencing of different genes and a GL2 duplex targeting luciferase was used as a control.

Lats1: 5’ AATCCACAAATGCTTCAAGAC 3’
hBora1: 5’ CCGGTTGATAATGGCAGTTTA 3’
hBora2: 5’ TAACTAGTCCTTCGCCTATTT 3’
Plk1: 5’ CGAGCTGCTTAATGACGAGTT 3’
Aurora A: 5’ CAGGGCTGCCATATAACCTGA 3’
Aurora A2: 5’ 725 ATGCCCTGTCTTACTGTCATT 3’
TPX2: 5’ GAA TGG AAC TGG AGG GCT TTT 3’

Annealing of the siRNAs and transfections using oligofectamine (Life Technologies) were performed as described (Elbashir et al., 2001). For co-depletion experiments, HeLaS3 cells were treated with GL2 or hBora siRNA for 36h. Subsequently fresh media was replaced containing combinations of Plk1/GL2 or Plk1/hBora siRNA duplexes for another 36h.

Far Western ligand binding assays

hBora pre-phosphorylated by Cdk1 in the presence of cold ATP was separated by 7.5% SDS-PAGE, Western blotted, then probed with GST-tagged PBD (1µg/ml) for 2h at 4°C. Bound proteins were then detected by monoclonal anti-Plk1 antibody.

Immunoprecipitation

For immunoprecipitation, cell lysates, prepared as described above, or *in vitro* coupled transcription translation (IVT) mixtures, prepared with the TNT-coupled reticulocyte lysate system (Promega) were incubated with: immobilized rabbit anti-Lats1 antibodies, rabbit anti-hBora antibodies, rabbit anti-Aurora A antibodies, rabbit anti-GFP(kindly provided by Dr. Francis Barr), monoclonal 9E10 anti-myc antibodies or anti-FLAG®M2 Affinity gel (Sigma). In each case 1µg of antibody was coupled to 1µl Sepharose-G or A beads (20µl beads in total) (Pierce).

To elute FLAG-tagged proteins, the immune complexes were washed twice with buffer D (20mM Hepes, pH7.8, 250mM KCl, 0.2mM EDTA and 0.1% NP40) and then incubated for 30min at room temperature in the same buffer including 100µg/ml FLAG-peptide (Sigma).

In vitro kinase assays

In vitro kinase assays for immune complexes or recombinant proteins were performed in the corresponding kinase buffer.

Lats1: Hepes kinase buffer (25mM Hepes pH7.4, 50mM NaCl, 5mM $MgCl_2$, 5mM $MnCl_2$, 5mM β-glycerophosphate and 1mM DTT)

Plk1 and Cdk1: BRB80 kinase buffer (80 mM Pipes, pH 6.8/0.5 mM, MgCl2/1 mM

EGTA, 5mM β-glycerophosphate and 1mM DTT)

Aurora A: Aurora A kinase buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 5 mM $MnCl_2$, 5 mM NaF, 1 mM DTT)

Kinase reactions were carried out at 30°C for 30min in buffer supplemented with 10μM ATP and 2μCi [γ-^{32}P] ATP (Amersham Corp.). Reactions were stopped by the addition of SDS sample buffer and heating to 95°C. Reaction products were visualized by SDS-PAGE followed by autoradiography.

For testing the activation of Aurora A by TPX2 or hBora, Aurora A isolated from bacteria were pre-treated with protein phosphatase 1 (PP1) for 30min at 30°C. The reaction was then stopped by the addition 0.1μM microcystin.

PCR on cDNA panels

The mRNA levels of *LATS1* and *LATS2* were detected by PCR on cDNA panels from Clontech with two pairs of specific oligos. For *LATS1*, oligos (LATS1-For) 5'-GAATCCTTAGTTCCTCAGAGGCATGGC-3' and (LATS1-Rev) 5'-ACTCTGCATGATTGGTTGTCTGCC-3' were used and for *LATS2*, we used oligos (LATS2-For) 5'-GGCTCTACGTGCCGCACCCACACCACAAGCAG -3' and (LATS2-rev) 5'-GCATGCTCCTCCTTGGCGTCCAAGCCCTCCGCA- 3'. PCR cycles were 35 and 45 for *LATS1* and *LATS2*, respectively. As to *G3DPH*, the control oligos from the cDNA panels (Clontech) were used for 16 PCR cycles. PCR products were then subjected to electrophoresis in agarose gels and visualized by ethidium bromide staining.

Mass spectrometry

Coomassie-stained protein bands were in-gel digested by modified trypsin (sequencing grade; Promega) (Shevchenko et al., 1996a) and desalted using homemade miniaturized reversed-phase columns (Gobom et al., 1999). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Reflex III instrument (Bruker Daltonik, Bremen, Germany) in both positive and negative reflector modes. 2,5-dihydroxybenzoic acid (Bruker Daltonik) was used as a matrix. Mass spectra were searched for peptides showing a difference of 80 mass units, and candidate phosphopeptides were submitted to post-source decay fragment ion analysis

(Hoffmann et al., 1999b). Peptides showing the typical losses of 98 mass units (phosphoric acid) and 80 mass units (phosphate) were accepted as phosphopeptides. For peptide sequencing by tandem mass spectrometry, samples were dissolved in water/methanol (1/1, v/v) containing 2% formic acid, filled into nanospray needles (Protana), and analyzed on a Q-TOF Ultima mass spectrometer (Micromass).

Yeast two-hybrid studies

Yeast two-hybrid assays were performed using a system described previously (James et al., 1996). Full cDNAs as well as fragments of Lats1 (aa 1-587 or 588-1130), Mst2 (aa 1-312, 312-491) and hWW45 (aa 1-194, 194-384) were cloned into pGAD and pGBD vectors. *S. cerevisiae* strain *PJ69-4A* transformants were selected on YNB medium without Leu and Trp and then streaked onto selective YNB plates, lacking Leu, Trp and Ade, to reveal potential interactions.

Abbreviations

AA	amino acid
ATP	adenosine 5´-triphosphate
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CMV	cytomegalo virus
CO_2	carbon dioxide
DAPI	4´,6-diamidino-2-phenylindole
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene dinitrilo tetraacetic acid
EGTA	ethylene gycol tetraacetic acid
FCS	fetal calf serum
HEPES	N-2-Hydroxyethylpiperazine-N`-2-ethane sulfonic acid
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactopyranoside
IU	international unit
KD	catalytically dead
kDa	kilo-daltons
LB	luria-broth
mAb	monoclonal antibody
MALDI	Matrix assisted laser desorption ionisation
MT	microtubules
MS	mass spectrometry
MW	molecular weight marker
OA	okadaic acid
OD	optical density
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PIPES	1,4-Piperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
Rpm	revolutions per minute
RT	room temperature
SDS-PAGE	sodium-dodecylsulfate polyacrylamid gelelectrophoresis
siRNA	small interference Ribonucleic Acid
WT	wild-type

List of plasmids

Name	Tag	Gene	Insert	Species	Vector
EC 1	Myc	syntenin	full-length	human	pcDNA3.1/3xmyc-C
EC 2		smurf2	full-length	Human	pBSKS+
EC 3	Myc	smurf2	full-length	Human	pcDNA3.1/3xmyc-C
EC 4	Myc	lats1	C-term	Human	pcDNA3.1/3xmyc-B
EC 5	Myc	lats1KD	C-term	Human	pcDNA3.1/3xmyc-B
EC 6	Myc	lats1	C-term	Human	pcDNA3.1/3xmyc-A
EC 7	Myc	lats1KD	C-term	Human	pcDNA3.1/3xmyc-A
EC 8	GST	Ndr-substrate	38bp	Human	pGEX-6P-3
EC 9	myc-His	lats1	full-length	Human	pcDNA4/TO/myc-HisB
EC 10	myc-His	lats1KD	full-length	Human	pcDNA4/TO/myc-HisB
EC 11		lats1	S635A	human	pBSSK+
EC 12		lats1	S635E	human	pBSSK+
EC 13		lats1	S635D	human	pBSSK+
EC 14	Myc	lats1	S635A	human	pcDNA3.1/3mycC
EC 15		lats1	S635E	human	pcDNA3.1/3mycC
EC 16		lats1	S635D	human	pcDNA3.1/3mycC
EC 17	GST	lats1	aa625-639	human	pGEX-6P-3
EC 18	GST	lats1	aa680-694	human	pGEX-6P-3
EC 19	GST	lats1	aa694-709	human	pGEX-6P-3
EC 20	GST	Alg2	Alg2	human	pGEX-6P-3
EC 21	AD	Kiaa0008	Kiaa0008	human	pGAD-C1
EC 22	BD	Kiaa0008	Kiaa0008	human	pGBD-C1
EC 23	AD	Kiaa0008	aa 442-920	human	pGAD-C2
EC 24	BD	Kiaa0008	aa 442-920	human	pGBD-C2
EC 25	AD	Kiaa0008	aa 1-441	human	pGAD-C1
EC 26	BD	Kiaa0008	aa 1-441	human	pGBD-C1
EC 27		Omi	Omi	human	pCR4-TOPO
EC 28		Omi	aa133-459	human	pCR4-TOPO
EC 29	Myc-His	Omi	Omi	human	pcDNA/TO/myc-HisA
EC 30	His	Omi	aa133-459	human	pET-28a(+)
EC 31	Flag	Mst2	Mst2	human	pcDNA3.1+ N-Flag

EC 32	Flag	-	-	-	pcDNA3.1+ N-Flag
EC 33	Myc	lats1	T1079A	human	pcDNA3.1/3mycC
EC 34		Omi	Omi	human	pOTB7
EC 35		FAM	FAM	human	pT7T3D-PacI
EC 36		FAM	FAM	human	pT7T3D-PacI
EC 37		Smurf2	Smurf2	human	pT7T3D-PacI
EC 38		Smurf2	Smurf2	human	pT7T3D-PacI
EC 39	Flag	Smurf2	Smurf2	human	?
EC 40	Flag	Mst2	Mst2	human	pcDNA3.1+ N-Flag
EC 41	AD	Mst2	full-length	human	pGAD-C2
EC 42	BD	Mst2	full-length	human	pGBD-C2
EC 43	BD	hWW45	aa190-384	human	pGBD-C1
EC 44	BD	hWW45	aa1-190	human	pGBD-C1
EC 45	Myc	Lats1	S909A	human	pcDNA3.1 Myc-C
EC 46	Myc	Mst1	full-length	human	pcDNA3.1 Myc-A
EC 47		Mst2	full-length	human	pCR4-TOPO
EC 48		Mst2	aa1-311	human	pCR4-TOPO
EC 49		Mst2	aa312-488	human	pCR4-TOPO
EC 50	BD	Mst2	aa1-311	human	pGBD-C1
EC 51	BD	Mst2	aa312-488	human	pGBD-C1
EC 52		DJ1042	EST of DJ1042	human	pOTB7
EC 53	Myc	lats1	full length S872A	human	pcDNA3.1MycC
EC 54	Myc	lats1	full length S872E	human	pcDNA3.1MycC
EC 55	Myc	lats1	full length S872D	human	pcDNA3.1MycC
EC 56		Mst1	full length	human	pCR4-TOPO
EC 57	Myc	Mst1	D167A	human	pcDNA3.1MycA
EC 58	Myc	lats1	S909A/T1079A	human	pcDNA3.1MycC
EC 59	AD	Mst2	aa1-311	human	pGAD-C1
EC 60	AD	Mst2	aa312-488	human	pGAD-C1
EC 61	Myc	DJ1042	full length	human	pcDNA3.1MycC
EC 62	GST	Lats1	aa626-644	human	pGEX-6P-3
EC 63	GST	Lats1	aa1066-1085	human	pGEX-6P-3
EC 64	Flag	Mst1	D167A	human	pcDNA3.1Flag
EC 65	Flag	Mst1	full length	human	pcDNA3.1Flag

EC 66	Myc	Lats1	T1036A/S1037A	human	pcDNA3.1MycC
EC 67	GST	Lats1	aa590-640	human	pGEX-5X-2
EC 68	GST	Lats1	aa590-755	human	pGEX-5X-2
EC 69	Myc	Lats1	S690A	human	pcDNA3.1MycC
EC 70	Myc	Lats1	S818A	human	pcDNA3.1MycC
EC 71	Myc	Lats1	S909D	human	pcDNA3.1MycC
EC 72	Myc	Lats1	S909E	human	pcDNA3.1MycC
EC 73	Myc	Lats1	T1079D	human	pcDNA3.1MycC
EC 74	Myc	Lats1	T1079E	human	pcDNA3.1MycC
EC 75	Myc	Lats1	S690D	human	pcDNA3.1MycC
EC 76	Myc	Lats1	S690E	human	pcDNA3.1MycC
EC 77	Myc	Lats1	T967A	human	pcDNA3.1MycC
EC 78	Myc	Lats1	T967D	human	pcDNA3.1MycC
EC 79	Myc	Lats1	T967E	human	pcDNA3.1MycC
EC 80	Flag	Lats1	aa663-1130	human	pcDNA3.1FlagC
EC 81	Flag	Lats1	aa663-1130 KD	human	pcDNA3.1FlagC
EC 82	Flag	Lats1	aa558-1130	human	pcDNA3.1FlagA
EC 83	Flag	Lats1	aa558-1130 KD	human	pcDNA3.1FlagA
EC 84	Myc	Lats1	T1060A	human	pcDNA3.1MycC
EC 85	Myc	Lats1	T1060D	human	pcDNA3.1MycC
EC 86	Myc	Lats1	T1060E	human	pcDNA3.1MycC
EC 87	Myc	Ysk1	full length	human	pcDNA3.1/3xmyc-A
EC 88	Myc	Mst4	full length	human	pcDNA3.1/3xmyc-A
EC 89	Myc	Ysk1	D158A	human	pcDNA3.1/3xmyc-A
EC 90	myc	Mst4	D162A	human	pcDNA3.1/3xmyc-A
EC 91	flag	Ysk1	full length	human	pcDNA3.1/3xFLAG-A
EC 92	flag	Mst4	full length	human	pcDNA3.1/3xFLAG-A
EC 93	flag	Ysk1	D158A	human	pcDNA3.1/3xFLAG-A
EC 94	flag	Mst4	D162A	human	pcDNA3.1/3xFLAG-A
EC 95	myc	Lats1	aa588-1130, S909T	human	pcDNA3.1/3xmyc-C
EC 96	myc	Lats1	aa588-1130, T1079A	human	pcDNA3.1/3xmyc-A
EC 97	myc	Lats1	aa588-1130, T1079D	human	pcDNA3.1/3xmyc-A
EC 98	myc	Lats1	aa588-1130, T1079E	human	pcDNA3.1/3xmyc-A
EC 99	myc	Lats1	T1012A	human	pcDNA3.1/3xmyc-C

EC100	myc	Lats1	T1031A	human	pcDNA3.1/3xmyc-C
EC101	myc	Lats1	aa588-1130,S909T,D50D846A	human	pcDNA3.1/3xmyc-C
EC102	flag	hWW45	full length	human	pcDNA3.1/3xFLAG-C
EC103	myc	Lats1	S909E, T1079E	human	pcDNA3.1/3xmyc-C
EC104		D19SH3	EST full length	human	pDNR-LIB
EC105		D19SH3	EST full length	human	pcmv-SPORT 6
EC106	myc	D19SH3	full length	human	pcDNA3.1/3xmyc-C
EC107	flag	Rassf1A	full length	human	pCMV-Tag1
EC108	myc	AuroraA	full length	human	pcDNA3.1/3xmyc-C
EC109	myc	AuroraA	D274A	human	pcDNA3.1/3xmyc-C
EC110	myc	Rassf1A	full length	human	pcDNA3.1/3xmyc-A
EC111	Flag	AuroraA	WT	human	pcDNA3.1/Flag-A
EC112	Flag	AuroraA	D274A	human	pcDNA3.1/Flag-A
EC113	HA	Rassf1A	full length	human	pcDNA3.1/3xmyc-A
EC114	AD	Rassf1A	full length	human	pGAD-C2
EC115	BD	Rassf1A	full length	human	pGBD-C2
EC116	HA	Lats1	full length	human	pcDNA3.1/HA-C
EC117	Flag	hWW45	full length	human	pcDNA3.1/Flag-C
EC118	Flag	Rassf1A	full length	human	pcDNA3.1/FlagA
EC119	HA	Mst2	full length	human	pcDNA3.1/HA-C
EC120	GST	hWW45	a.a.197-221	human	pGEX-6P-3
EC121	GST	hWW45	full length	human	pGEX-6P-3
EC122		CMT2	EST full length	human	
EC123	Myc	CMT2	full length	human	pcDNA3.1/Myc-C
EC124		DDX3	EST full length	human	
EC125	Myc	DDX3	full length	human	pcDNA3.1/Myc-C
EC126	His	Rassf1A	full length	human	pQE-32
EC127	His	Rassf1A	a.a.1-105	human	pQE-32
EC128	Myc	Mst2	full length	human	pcDNA3.1/Myc-C
EC129		Mst1	full length	human	pBSSK
EC130		Mst2	full length	human	pBSKS
EC131		Rassf1A	full length	human	pBSKS
EC132	FLAG	Rassf1A	full length	human	pcDNA4/TO Puromycin

EC133	His	Rassf1A	full length	human	pET28c
EC134	Myc	Mst2	full length	human	pcDNA4/TO Puromycin
EC135	FLAG	Mst1	full length	human	pcDNA3.1-C-FLAG
EC136	FLAG	Mst2	full length	human	pcDNA3.1-C-FLAG
EC137	FLAG	Lats1	full length	human	pcDNA3.1-C-FLAG
EC138	FLAG	Rassf1A	full length	human	pcDNA3.1-C-FLAG
EC139		Kiaa0949	EST C-terminal	human	
EC140		CRIK	EST N-terminal	human	
EC141	FLAG	CRIK	full length	Mouse	
EC142	FLAG	CRIK	KD	Mouse	
EC143	FLAG	CRIK-SK	short form	Mouse	
EC144	FLAG	CRIK-SK KD	short form	Mouse	
EC145		hBora	EST full length	human	
EC146	Myc	hBora	full length	human	pcDNA3.1/Myc-C
EC147	FLAG	hBora	full length	human	pcDNA3.1/Flag-C
EC148	AD	hBora	full length	human	pGAD-C(1)
EC149	BD	hBora	full length	human	pGBD-omega-C(1)
EC150	N-termHis	hBora	full length	human	pET-28b-HS2
EC151	C-term His	hBora	full length	human	pET-28b-HS1
EC152	Myc	hBora	a.a.1-267	human	pcDNA3.1/Myc-C
EC153	Myc	hBora	aa268-559	human	pcDNA3.1-Myc
EC154	C-term Myc	hBora	full-length	human	pcDNA4/TO/myc-His A
EC155	BD	hBora	aa1-267	human	pGBD-omega-C(2)
EC156	BD	hBora	aa268-559	human	pGBD-omega-C(2)
EC157	AD	hBora	aa1-267	human	pGAD-C(2)
EC158	AD	hBora	aa268-559	human	pGAD-C(2)
EC159	Myc	hBora	full-length	human	pcDNA4/TO Puro
EC160	MBP	hBora	full-length	human	pMALpFN
EC161		Kiaa0097	EST	human	
EC162	Myc	hBora	aa1-327	human	pcDNA3.1-Myc
EC163	Myc	hBora	aa329-559	human	pcDNA3.1-Myc
EC164	Myc	RanBP1	full-length	human	pcDNA3.1-Myc
EC165	GFP	hBora	full-length	human	pEGFP T7/C1
EC166	Myc	beta-TrCP	full-length	mouse	

EC167	GST	hBora	full-length	human	pVL13GST92
EC168	His	hBora	full-length	human	pVL1393N-termPreScission
EC169	Myc	hBora	aa1-329	human	pcDNA4/TO Puro
EC170	Myc	hBora	aa1-80	human	pcDNA3.1-Myc
EC171	Myc	hBora	aa80-327	human	pcDNA3.1-Myc
EC172	Myc	hBora	T52A	human	pcDNA3.1-Myc
EC173	Myc	hBora	R193A/L196A	human	pcDNA3.1-Myc
EC174	Myc	hBora	T15A	human	pcDNA3.1-Myc
EC175	Myc	hBora	S252	human	pcDNA3.1-Myc
EC176	Myc	hBora	S188A	human	pcDNA3.1-Myc
EC177	Myc	hBora	S262A	human	pcDNA3.1-Myc
EC178	Myc	hBora	S298A	human	pcDNA3.1-Myc
EC179	Myc	hBora	T144A	human	pcDNA3.1-Myc
EC180	Myc	hBora	S497A/T501A	human	pcDNA3.1-Myc
EC181	Myc	hBora	S274A/S558A	human	pcDNA3.1-Myc
EC182	Myc	hBora	T296A	human	pcDNA3.1-Myc
EC183	Myc	hBora	T296A, T144A	human	pcDNA3.1-Myc
EC184	Myc	hBora	T296A, S262A	human	pcDNA3.1-Myc
EC185	Myc	hBora	S274A	human	pcDNA3.1-Myc
EC186	Myc	hBora	S558A	human	pcDNA3.1-Myc
EC187	Myc	hBora	S252A, S274A	human	pcDNA3.1-Myc
EC188	Myc	hBora	S252A, S558A	human	pcDNA3.1-Myc
EC189	Myc	hBora	S325A	human	pcDNA3.1-Myc
EC190	MBP	hBora	S252A	human	pMALpFN
EC191	MBP	hBora	S497A/T501A	human	pMALpFN
EC192	GFP	beta-TrCP	full-length	mouse	pEGFP T7/C1
EC193	Myc	hBora	aa1-327, S252A	human	pcDNA3.1-Myc
EC194	Myc	hBora	aa329-559, S497A/T501A	human	pcDNA3.1-Myc
EC195	Myc	hBora	aa1-232	human	pcDNA3.1-Myc
EC196	MBP	hBora	aa1-327	human	pMALpFN
EC197	MBP	hBora	aa329-559	human	pMALpFN
EC198	MBP	hBora	aa329-559, S497A/T501A	human	pMALpFN
EC199	Myc	hBora	S252E	human	pcDNA3.1-Myc

EC200	Myc	hBora	S497E/T501E	human	pcDNA3.1-Myc
EC201	Myc	hBora	S251A/S252A	human	pcDNA3.1-Myc
EC202	myc	hBora	S251A	human	pcDNA3.1-Myc
EC203	FLAG	beta-TrCP	full-length	human	pcDNA3.1-Flag-C

References

Anand, S., S. Penrhyn-Lowe, and A.R. Venkitaraman. 2003. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell*. 3:51-62.

Andersen, S.S. 1999. Balanced regulation of microtubule dynamics during the cell cycle: a contemporary view. *Bioessays*. 21:53-60.

Andrews, P.D., Y. Ovechkina, N. Morrice, M. Wagenbach, K. Duncan, L. Wordeman, and J.R. Swedlow. 2004. Aurora B regulates MCAK at the mitotic centromere. *Dev.Cell*. 6:253-268.

Bardin, A.J., and A. Amon. 2001. Men and sin: what's the difference? *Nat Rev Mol Cell Biol*. 2:815-26.

Barr, F.A., H.H. Sillje, and E.A. Nigg. 2004. Polo-like kinases and the orchestration of cell division. *Nat Rev Mol Cell Bioln*. 5:429-40.

Barros, T.P., K. Kinoshita, A.A. Hyman, and J.W. Raff. 2005. Aurora A activates D-TACC-Msps complexes exclusively at centrosomes to stabilize centrosomal microtubules. *J Cell Biol*. 170:1039-46.

Baumann, C., R. Korner, K. Hofmann, and E.A. Nigg. 2007. PICH, a centromere-associated SNF2 family ATPase, is regulated by Plk1 and required for the spindle checkpoint. *Cell*. 128:101-14.

Bayliss, R., T. Sardon, I. Vernos, and E. Conti. 2003. Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Mol Cell*. 12:851-62.

Berdnik, D., and J.A. Knoblich. 2002. Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. *Curr Biol*. 12:640-7.

Blagden, S.P., and D.M. Glover. 2003. Polar expeditions--provisioning the centrosome for mitosis. *Nat Cell Biol*. 5:505-11.

Blangy, A., H.A. Lane, P. d'Herin, M. Harper, M. Kress, and E.A. Nigg. 1995. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell*. 83:1159-69.

Bothos, J., R.L. Tuttle, M. Ottey, F.C. Luca, and T.D. Halazonetis. 2005. Human LATS1 is a mitotic exit network kinase. *Cancer Res*. 65:6568-75.

Buchberger, A. 2002. From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol*. 12:216-21.

Carmena, M., and W.C. Earnshaw. 2003. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol*. 4:842-854.

Casenghi, M., P. Meraldi, U. Weinhart, P.I. Duncan, R. Korner, and E.A. Nigg. 2003. Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. *Dev Cell*. 5:113-25.

Chalamalasetty, R.B., S. Hummer, E.A. Nigg, and H.H. Sillje. 2006. Influence of human Ect2 depletion and overexpression on cleavage furrow formation and abscission. *J Cell Sci*. 119:3008-19.

Chen, S.S., P.C. Chang, Y.W. Cheng, F.M. Tang, and Y.S. Lin. 2002. Suppression of the STK15 oncogenic activity requires a transactivation-independent p53 function. *Embo J*. 21:4491-9.

Cheng, K.Y., E.D. Lowe, J. Sinclair, E.A. Nigg, and L.N. Johnson. 2003. The crystal structure of the human polo-like kinase-1 polo box domain and its phospho-peptide complex. *Embo J*. 22:5757-68.

Cohen, P., C.F. Holmes, and Y. Tsukitani. 1990. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem Sci*. 15:98-102.

Conte, N., B. Delaval, C. Ginestier, A. Ferrand, D. Isnardon, C. Larroque, C. Prigent, B. Seraphin, J. Jacquemier, and D. Birnbaum. 2003. TACC1-chTOG-Aurora A protein complex in breast cancer. *Oncogene*. 22:8102-16.

Dammann, R., C. Li, J.H. Yoon, P.L. Chin, S. Bates, and G.P. Pfeifer. 2000. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet*. 25:315-9.

De Luca, M., P. Lavia, and G. Guarguaglini. 2006. A functional interplay between Aurora-A, Plk1 and TPX2 at spindle poles: Plk1 controls centrosomal localization of Aurora-A and TPX2 spindle association. *Cell Cycle*. 5:296-303.

do Carmo Avides, M., A. Tavares, and D.M. Glover. 2001. Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. *Nat Cell Biol*. 3:421-4.\

Doxsey, S. 1998. The centrosome--a tiny organelle with big potential. *Nat Genet*. 20:104-6.

Ducat, D., and Y. Zheng. 2004. Aurora kinases in spindle assembly and chromosome segregation. *Exp Cell Res*. 301:60-7.

Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. 411:494-8.

Elia, A.E., L.C. Cantley, and M.B. Yaffe. 2003a. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science*. 299:1228-31.

Elia, A.E., P. Rellos, L.F. Haire, J.W. Chao, F.J. Ivins, K. Hoepker, D. Mohammad, L.C. Cantley, S.J. Smerdon, and M.B. Yaffe. 2003b. The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell*. 115:83-95.

Evans, T., E.T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*. 33:389-96.

Ewart-Toland, A., P. Briassouli, J.P. de Koning, J.H. Mao, J. Yuan, F. Chan, L. MacCarthy-Morrogh, B.A. Ponder, H. Nagase, J. Burn, S. Ball, M. Almeida, S. Linardopoulos, and A. Balmain. 2003. Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nat Genet*. 34:403-12.

Eyers, P.A., E. Erikson, L.G. Chen, and J.L. Maller. 2003. A novel mechanism for activation of the protein kinase Aurora A. *Curr Biol*. 13:691-7.

Fabbro, M., B.B. Zhou, M. Takahashi, B. Sarcevic, P. Lal, M.E. Graham, B.G. Gabrielli, P.J. Robinson, E.A. Nigg, Y. Ono, and K.K. Khanna. 2005. Cdk1/Erk2- and Plk1-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. *Dev Cell*. 9:477-88.

Farruggio, D.C., F.M. Townsley, and J.V. Ruderman. 1999. Cdc20 associates with the kinase aurora2/Aik. *Proc Natl Acad Sci U S A*. 96:7306-11.

Gassmann, R., A. Carvalho, A.J. Henzing, S. Ruchaud, D.F. Hudson, R. Honda, E.A. Nigg, D.L. Gerloff, and W.C. Earnshaw. 2004. Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. *J Cell Biol*. 166:179-191.

Giet, R., and D.M. Glover. 2001. Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J Cell Biol*. 152:669-682.

Giet, R., D. McLean, S. Descamps, M.J. Lee, J.W. Raff, C. Prigent, and D.M. Glover. 2002. Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. *J Cell Biol*. 156:437-51.

Giet, R., and C. Prigent. 2000. The Xenopus laevis aurora/Ip11p-related kinase pEg2 participates in the stability of the bipolar mitotic spindle. *Exp Cell Res*. 258:145-51.

Glover, D.M. 2005. Polo kinase and progression through M phase in Drosophila: a perspective from the spindle poles. *Oncogene*. 24:230-7.

Glover, D.M., M.H. Leibowitz, D.A. McLean, and H. Parry. 1995. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell*. 81:95-105.

Gobom, J., E. Nordhoff, E. Mirgorodskaya, R. Ekman, and P. Roepstorff. 1999. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J Mass Spectrom*. 34:105-16.

Graves, J.D., Y. Gotoh, K.E. Draves, D. Ambrose, D.K. Han, M. Wright, J. Chernoff, E.A. Clark, and E.G. Krebs. 1998. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *Embo J*. 17:2224-34.

Guse, A., M. Mishima, and M. Glotzer. 2005. Phosphorylation of ZEN-4/MKLP1 by aurora B regulates completion of cytokinesis. *Curr.Biol*. 15:778-786.

Hachet, V., C. Canard, and P. Gonczy. 2007. Centrosomes promote timely mitotic entry in C. elegans embryos. *Dev Cell*. 12:531-41.

Hanisch, A., A. Wehner, E.A. Nigg, and H.H. Sillje. 2006. Different Plk1 functions show distinct dependencies on Polo-Box domain-mediated targeting. *Mol Biol Cell*. 17:448-59.

Hannak, E., M. Kirkham, A.A. Hyman, and K. Oegema. 2001. Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. *J Cell Biol*. 155:1109-16.

Harper, J.W., and P.D. Adams. 2001. Cyclin-dependent kinases. *Chem Rev*. 101:2511-26.

Harvey, K.F., C.M. Pfleger, and I.K. Hariharan. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell*. 114:457-67.

Hirose, K., T. Kawashima, I. Iwamoto, T. Nosaka, and T. Kitamura. 2001. MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody. *J Biol Chem.* 276:5821-5828.

Hirota, T., N. Kunitoku, T. Sasayama, T. Marumoto, D. Zhang, M. Nitta, K. Hatakeyama, and H. Saya. 2003. Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell*. 114:585-98.

Hirota, T., T. Morisaki, Y. Nishiyama, T. Marumoto, K. Tada, T. Hara, N. Masuko, M. Inagaki, K. Hatakeyama, and H. Saya. 2000. Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. *J Cell Biol*. 149:1073-86.

Hisaoka, M., A. Tanaka, and H. Hashimoto. 2002. Molecular alterations of h-warts/LATS1 tumor suppressor in human soft tissue sarcoma. *Lab Invest*. 82:1427-35.

Hoar, K., A. Chakravarty, C. Rabino, D. Wysong, D. Bowman, N.R. D'Amore, and J.A. Ecsedy. 2007. MLN8054, a small-molecule inhibitor of Aurora A, causes spindle pole and chromosome congression defects leading to aneuploidy. *Mol Cell Biol*.

Hoffmann, R., S. Metzger, B. Spengler, and L. Otvos, Jr. 1999a. Sequencing of peptides phosphorylated on serines and threonines by post-source decay in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Mass Spectrom.* 34:1195-1204.

Hoffmann, R., S. Metzger, B. Spengler, and L. Otvos, Jr. 1999b. Sequencing of peptides phosphorylated on serines and threonines by post-source decay in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Mass Spectrom*. 34:1195-204.

Hori, T., A. Takaori-Kondo, Y. Kamikubo, and T. Uchiyama. 2000. Molecular cloning of a novel human protein kinase, kpm, that is homologous to warts/lats, a Drosophila tumor suppressor. *Oncogene*. 19:3101-9.

Hunt, T. 1991. Cyclins and their partners: from a simple idea to complicated reality. *Semin Cell Biol*. 2:213-22.

Hutterer, A., D. Berdnik, F. Wirtz-Peitz, M. Zigman, A. Schleiffer, and J.A. Knoblich. 2006. Mitotic activation of the kinase Aurora-A requires its binding partner Bora. *Dev Cell*. 11:147-57.

Iida, S., T. Hirota, T. Morisaki, T. Marumoto, T. Hara, S. Kuninaka, S. Honda, K. Kosai, M. Kawasuji, D.C. Pallas, and H. Saya. 2004. Tumor suppressor WARTS ensures genomic integrity by regulating both mitotic progression and G1 tetraploidy checkpoint function. *Oncogene*. 23:5266-74.

Jackman, M., C. Lindon, E.A. Nigg, and J. Pines. 2003. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat Cell Biol*. 5:143-8.

James, P., J. Halladay, and E.A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*. 144:1425-36.

Jang, Y.J., S. Ma, Y. Terada, and R.L. Erikson. 2002. Phosphorylation of threonine 210 and the role of serine 137 in the regulation of mammalian polo-like kinase. *J Biol Chem*. 277:44115-20.

Jia, J., W. Zhang, B. Wang, R. Trinko, and J. Jiang. 2003. The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. *Genes Dev*. 17:2514-9.

Johnson, L.N., M.E. Noble, and D.J. Owen. 1996. Active and inactive protein kinases: structural basis for regulation. *Cell*. 85:149-58.

Justice, R.W., O. Zilian, D.F. Woods, M. Noll, and P.J. Bryant. 1995. The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev*. 9:534-46.

Kango-Singh, M., R. Nolo, C. Tao, P. Verstreken, P.R. Hiesinger, H.J. Bellen, and G. Halder. 2002. Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. *Development*. 129:5719-30.

Kelm, O., M. Wind, W.D. Lehmann, and E.A. Nigg. 2002. Cell cycle-regulated phosphorylation of the Xenopus polo-like kinase Plx1. *J Biol Chem*. 277:25247-56.

Kemp, C.A., K.R. Kopish, P. Zipperlen, J. Ahringer, and K.F. O'Connell. 2004. Centrosome maturation and duplication in C. elegans require the coiled-coil protein SPD-2. *Dev Cell*. 6:511-23.

Khokhlatchev, A., S. Rabizadeh, R. Xavier, M. Nedwidek, T. Chen, X.F. Zhang, B. Seed, and J. Avruch. 2002. Identification of a novel Ras-regulated proapoptotic pathway. *Curr Biol*. 12:253-65.

Kinoshita, K., T.L. Noetzel, L. Pelletier, K. Mechtler, D.N. Drechsel, A. Schwager, M. Lee, J.W. Raff, and A.A. Hyman. 2005. Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. *J Cell Biol*. 170:1047-55.

Knecht, R., R. Elez, M. Oechler, C. Solbach, C. von Ilberg, and K. Strebhardt. 1999. Prognostic significance of polo-like kinase (PLK) expression in squamous cell carcinomas of the head and neck. *Cancer Res*. 59:2794-7.

Kraft, C., F. Herzog, C. Gieffers, K. Mechtler, A. Hagting, J. Pines, and J.M. Peters. 2003. Mitotic regulation of the human anaphase-promoting complex by phosphorylation. *Embo J*. 22:6598-609.

Kufer, T.A., E.A. Nigg, and H.H. Sillje. 2003. Regulation of Aurora-A kinase on the mitotic spindle. *Chromosoma*. 112:159-63.

Kufer, T.A., H.H. Sillje, R. Korner, O.J. Gruss, P. Meraldi, and E.A. Nigg. 2002. Human TPX2 is required for targeting Aurora-A kinase to the spindle. *J Cell Biol*. 158:617-23.

Lan, W., X. Zhang, S.L. Kline-Smith, S.E. Rosasco, G.A. Barrett-Wilt, J. Shabanowitz, D.F. Hunt, C.E. Walczak, and P.T. Stukenberg. 2004. Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. *Curr.Biol*. 14:273-286.

Lane, H.A., and E.A. Nigg. 1996. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol*. 135:1701-13.

Lee, K.K., T. Ohyama, N. Yajima, S. Tsubuki, and S. Yonehara. 2001. MST, a physiological caspase substrate, highly sensitizes apoptosis both upstream and downstream of caspase activation. *J Biol Chem*. 276:19276-85.

Lee, K.S., S. Song, and R.L. Erikson. 1999. The polo-box-dependent induction of ectopic septal structures by a mammalian polo kinase, plk, in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 96:14360-5.

Lenart, P., M. Petronczki, M. Steegmaier, B. Di Fiore, J.J. Lipp, M. Hoffmann, W.J. Rettig, N. Kraut, and J.M. Peters. 2007. The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr Biol*. 17:304-15.

Li, J.J., and S.A. Li. 2006. Mitotic kinases: the key to duplication, segregation, and cytokinesis errors, chromosomal instability, and oncogenesis. *Pharmacol Ther*. 111:974-84.

Li, Y., J. Pei, H. Xia, H. Ke, H. Wang, and W. Tao. 2003. Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene*. 22:4398-405.

Littlepage, L.E., H. Wu, T. Andresson, J.K. Deanehan, L.T. Amundadottir, and J.V. Ruderman. 2002. Identification of phosphorylated residues that affect the activity of the mitotic kinase Aurora-A. *Proc Natl Acad Sci U S A*. 99:15440-5.

Liu, J., and J.L. Maller. 2005. Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CaMK II to release metaphase arrest by cytostatic factor. *Curr Biol*. 15:1458-68.

Luders, J., and T. Stearns. 2007. Microtubule-organizing centres: a re-evaluation. *Nat Rev Mol Cell Biol*. 8:161-7.

Mailand, N., A.V. Podtelejnikov, A. Groth, M. Mann, J. Bartek, and J. Lukas. 2002. Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability. *Embo J*. 21:5911-20.

Makela, T.P., J.P. Tassan, E.A. Nigg, S. Frutiger, G.J. Hughes, and R.A. Weinberg. 1994. A cyclin associated with the CDK-activating kinase MO15. *Nature*. 371:254-7.

Mamely, I., M.A. van Vugt, V.A. Smits, J.I. Semple, B. Lemmens, A. Perrakis, R.H. Medema, and R. Freire. 2006. Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery. *Curr Biol*. 16:1950-5.

Marumoto, T., S. Honda, T. Hara, M. Nitta, T. Hirota, E. Kohmura, and H. Saya. 2003. Aurora-A kinase

maintains the fidelity of early and late mitotic events in HeLa cells. *J Biol Chem*. 278:51786-95.

Mayer, T.U., T.M. Kapoor, S.J. Haggarty, R.W. King, S.L. Schreiber, and T.J. Mitchison. 1999. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science*. 286:971-4.

McInnes, C., A. Mazumdar, M. Mezna, C. Meades, C. Midgley, F. Scaerou, L. Carpenter, M. Mackenzie, P. Taylor, M. Walkinshaw, P.M. Fischer, and D. Glover. 2006. Inhibitors of Polo-like kinase reveal roles in spindle-pole maintenance. *Nat Chem Biol*. 2:608-17.

Meraldi, P., R. Honda, and E.A. Nigg. 2002. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. *Embo J*. 21:483-92.

Meraldi, P., R. Honda, and E.A. Nigg. 2004. Aurora kinases link chromosome segregation and cell division to cancer susceptibility. *Curr Opin Genet Dev*. 14:29-36.

Meraldi, P., and E.A. Nigg. 2002. The centrosome cycle. *FEBS Lett*. 521:9-13.

Millward, T.A., D. Hess, and B.A. Hemmings. 1999. Ndr protein kinase is regulated by phosphorylation on two conserved sequence motifs. *J Biol Chem*. 274:33847-50.

Minoshima, Y., T. Kawashima, K. Hirose, Y. Tonozuka, A. Kawajiri, Y.C. Bao, X. Deng, M. Tatsuka, S. Narumiya, W.S. May, Jr., T. Nosaka, K. Semba, T. Inoue, T. Satoh, M. Inagaki, and T. Kitamura. 2003. Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. *Dev.Cell*. 4:549-560.

Morgan, D.O. 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol*. 13:261-91.

Mori, D., Y. Yano, K. Toyo-oka, N. Yoshida, M. Yamada, M. Muramatsu, D. Zhang, H. Saya, Y.Y. Toyoshima, K. Kinoshita, A. Wynshaw-Boris, and S. Hirotsune. 2007. NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment. *Mol Cell Biol*. 27:352-67.

Morisaki, T., T. Hirota, S. Iida, T. Marumoto, T. Hara, Y. Nishiyama, M. Kawasuzi, T. Hiraoka, T. Mimori, N. Araki, I. Izawa, M. Inagaki, and H. Saya. 2002. WARTS tumor suppressor is phosphorylated

by Cdc2/cyclin B at spindle poles during mitosis. *FEBS Lett*. 529:319-24.

Moshe, Y., J. Boulaire, M. Pagano, and A. Hershko. 2004. Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. *Proc Natl Acad Sci U S A*. 101:7937-42.

Murray, A.W. 2004. Recycling the cell cycle: cyclins revisited. *Cell*. 116:221-34.

Neef, R., C. Preisinger, J. Sutcliffe, R. Kopajtich, E.A. Nigg, T.U. Mayer, and F.A. Barr. 2003. Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. *J Cell Biol*. 162:863-75.

Nigg, E.A. 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays*. 17:471-80.

Nigg, E.A. 1996. Cyclin-dependent kinase 7: at the cross-roads of transcription, DNA repair and cell cycle control? *Curr.Opin.Cell Biol*. 8:312-317.

Nigg, E.A. 2001. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*. 2:21-32.

Niiya, F., T. Tatsumoto, K.S. Lee, and T. Miki. 2006. Phosphorylation of the cytokinesis regulator ECT2 at G2/M phase stimulates association of the mitotic kinase Plk1 and accumulation of GTP-bound RhoA. *Oncogene*. 25:827-37.

Nishiyama, Y., T. Hirota, T. Morisaki, T. Hara, T. Marumoto, S. Iida, K. Makino, H. Yamamoto, T. Hiraoka, N. Kitamura, and H. Saya. 1999. A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. *FEBS Lett*. 459:159-65.

Ohi, R., and K.L. Gould. 1999. Regulating the onset of mitosis. *Curr Opin Cell Biol*. 11:267-73.

Oshimori, N., M. Ohsugi, and T. Yamamoto. 2006. The Plk1 target Kizuna stabilizes mitotic centrosomes to ensure spindle bipolarity. *Nat Cell Biol*. 8:1095-101.

Ouchi, M., N. Fujiuchi, K. Sasai, H. Katayama, Y.A. Minamishima, P.P. Ongusaha, C. Deng, S. Sen, S.W.

Lee, and T. Ouchi. 2004. BRCA1 phosphorylation by Aurora-A in the regulation of G2 to M transition. *J Biol Chem*. 279:19643-8.

Pantalacci, S., N. Tapon, and P. Leopold. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat Cell Biol*. 5:921-7.

Peset, I., J. Seiler, T. Sardon, L.A. Bejarano, S. Rybina, and I. Vernos. 2005. Function and regulation of Maskin, a TACC family protein, in microtubule growth during mitosis. *J Cell Biol*. 170:1057-66.

Peters, J.M. 2006. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol*. 7:644-56.

Peters, U., J. Cherian, J.H. Kim, B.H. Kwok, and T.M. Kapoor. 2006. Probing cell-division phenotype space and Polo-like kinase function using small molecules. *Nat Chem Biol*. 2:618-26.

Piel, M., J. Nordberg, U. Euteneuer, and M. Bornens. 2001. Centrosome-dependent exit of cytokinesis in animal cells. *Science*. 291:1550-3.

Pines, J., and C.L. Rieder. 2001. Re-staging mitosis: a contemporary view of mitotic progression. *Nat Cell Biol*. 3:E3-6.

Portier, N., A. Audhya, P.S. Maddox, R.A. Green, A. Dammermann, A. Desai, and K. Oegema. 2007. A microtubule-independent role for centrosomes and aurora a in nuclear envelope breakdown. *Dev Cell*. 12:515-29.

Praskova, M., A. Khoklatchev, S. Ortiz-Vega, and J. Avruch. 2004. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *Biochem J*. 381:453-62.

Preisinger, C., Short, B., De Corte, V., Bruyneel, E., Haas, A., Kopajtich, R., Gettemans, J., Barr, F.A. 2004. YSK is activated by the Golgi matrix protein GM130 and plays a role in cell migration through its substrate 14-3-3zeta. *J Cell Biol.* 164:1009-20.

Rauh, N.R., A. Schmidt, J. Bormann, E.A. Nigg, and T.U. Mayer. 2005. Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature*. 437:1048-52.

Reynolds, N., and H. Ohkura. 2003. Polo boxes form a single functional domain that mediates interactions with multiple proteins in fission yeast polo kinase. *J Cell Sci*. 116:1329-87.

Rieder, C.L. 2005. Kinetochore fiber formation in animal somatic cells: dueling mechanisms come to a draw. *Chromosoma*. 114:310-8.

Rieder, C.L., and G.G. Borisy. 1981. The attachment of kinetochores to the pro-metaphase spindle in PtK1 cells. Recovery from low temperature treatment. *Chromosoma*. 82:693-716.

Rozenblum, E., P. Vahteristo, T. Sandberg, J.T. Bergthorsson, K. Syrjakoski, D. Weaver, K. Haraldsson, H.K. Johannsdottir, P. Vehmanen, S. Nigam, N. Golberger, C. Robbins, E. Pak, A. Dutra, E. Gillander, D.A. Stephan, J. Bailey-Wilson, S.H. Juo, T. Kainu, A. Arason, R.B. Barkardottir, H. Nevanlinna, A. Borg, and O.P. Kallioniemi. 2002. A genomic map of a 6-Mb region at 13q21-q22 implicated in cancer development: identification and characterization of candidate genes. *Hum Genet*. 110:111-21.

Sampath, S.C., R. Ohi, O. Leismann, A. Salic, A. Pozniakovski, and H. Funabiki. 2004. The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. *Cell*. 118:187-202.

Sawin, K.E., and T.J. Mitchison. 1995. Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. *Proc Natl Acad Sci U S A*. 92:4289-93.

Scheel, H., and K. Hofmann. 2003. A novel interaction motif, SARAH, connects three classes of tumor suppressor. *Curr Biol*. 13:R899-900.

Schmidt, A., N.R. Rauh, E.A. Nigg, and T.U. Mayer. 2006. Cytostatic factor: an activity that puts the cell cycle on hold. *J Cell Sci*. 119:1213-8.

Seelos, C. 1997. A critical parameter determining the aging of DNA-calcium-phosphate precipitates. *Anal Biochem*. 245:109-11.

Sen, S., H. Zhou, and R.A. White. 1997. A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines. *Oncogene*. 14:2195-200.

Seong, Y.S., K. Kamijo, J.S. Lee, E. Fernandez, R. Kuriyama, T. Miki, and K.S. Lee. 2002. A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. *J Biol Chem*. 277:32282-93.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996a. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem*. 68:850-8.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996b. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal.Chem.* 68:850-858.

Sillje, H.H., and E.A. Nigg. 2006. Purification of mitotic spindles from cultured human cells. *Methods*. 38:25-8.

Song, M.S., S.J. Song, N.G. Ayad, J.S. Chang, J.H. Lee, H.K. Hong, H. Lee, N. Choi, J. Kim, H. Kim, J.W. Kim, E.J. Choi, M.W. Kirschner, and D.S. Lim. 2004. The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. *Nat Cell Biol*. 6:129-37.

St John, M.A., W. Tao, X. Fei, R. Fukumoto, M.L. Carcangiu, D.G. Brownstein, A.F. Parlow, J. McGrath, and T. Xu. 1999. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat Genet*. 21:182-6.

Stenoien, D.L., S. Sen, M.A. Mancini, and B.R. Brinkley. 2003. Dynamic association of a tumor amplified kinase, Aurora-A, with the centrosome and mitotic spindle. *Cell Motil Cytoskeleton*. 55:134-46.

Sumara, I., J.F. Gimenez-Abian, D. Gerlich, T. Hirota, C. Kraft, C. de la Torre, J. Ellenberg, and J.M. Peters. 2004. Roles of polo-like kinase 1 in the assembly of functional mitotic spindles. *Curr Biol*. 14:1712-22.

Tamaskovic, R., S.J. Bichsel, and B.A. Hemmings. 2003. NDR family of AGC kinases--essential regulators of the cell cycle and morphogenesis. *FEBS Lett*. 546:73-80.

Tao, W., S. Zhang, G.S. Turenchalk, R.A. Stewart, M.A. St John, W. Chen, and T. Xu. 1999. Human homologue of the Drosophila melanogaster lats tumour suppressor modulates CDC2 activity. *Nat Genet*. 21:177-81.

Tapon, N., K.F. Harvey, D.W. Bell, D.C. Wahrer, T.A. Schiripo, D.A. Haber, and I.K. Hariharan. 2002.

salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell*. 110:467-78.

Taylor, L.K., H.C. Wang, and R.L. Erikson. 1996. Newly identified stress-responsive protein kinases, Krs-1 and Krs-2. *Proc Natl Acad Sci U S A*. 93:10099-104.

Terada, Y., Y. Uetake, and R. Kuriyama. 2003. Interaction of Aurora-A and centrosomin at the microtubule-nucleating site in Drosophila and mammalian cells. *J Cell Biol*. 162:757-63.

Toji, S., N. Yabuta, T. Hosomi, S. Nishihara, T. Kobayashi, S. Suzuki, K. Tamai, and H. Nojima. 2004. The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. *Genes Cells*. 9:383-97.

Tsai, M.Y., C. Wiese, K. Cao, O. Martin, P. Donovan, J. Ruderman, C. Prigent, and Y. Zheng. 2003. A Ran signalling pathway mediated by the mitotic kinase Aurora A in spindle assembly. *Nat Cell Biol*. 5:242-8.

Tsou, M.F., and T. Stearns. 2006. Mechanism limiting centrosome duplication to once per cell cycle. *Nature*. 442:947-51.

Tung, J.J., D.V. Hansen, K.H. Ban, A.V. Loktev, M.K. Summers, J.R. Adler, 3rd, and P.K. Jackson. 2005. A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of Xenopus eggs. *Proc Natl Acad Sci U S A*. 102:4318-23.

Udan, R.S., M. Kango-Singh, R. Nolo, C. Tao, and G. Halder. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol*. 5:914-20.

Uhlmann, F. 2004. The mechanism of sister chromatid cohesion. *Exp Cell Res*. 296:80-5.

Vagnarelli, P., and W.C. Earnshaw. 2004. Chromosomal passengers: the four-dimensional regulation of mitotic events. *Chromosoma*. 113:211-22.

van Vugt, M.A., and R.H. Medema. 2005. Getting in and out of mitosis with Polo-like kinase-1. *Oncogene*. 24:2844-59.

van Vugt, M.A., B.C. van de Weerdt, G. Vader, H. Janssen, J. Calafat, R. Klompmaker, R.M. Wolthuis,

and R.H. Medema. 2004. Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. *J Biol Chem*. 279:36841-54.

Watanabe, N., H. Arai, Y. Nishihara, M. Taniguchi, N. Watanabe, T. Hunter, and H. Osada. 2004. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. *Proc Natl Acad Sci U S A*. 101:4419-24.

Watanabe, Y. 2005. Shugoshin: guardian spirit at the centromere. *Curr Opin Cell Biol*. 17:590-5.

Wu, S., J. Huang, J. Dong, and D. Pan. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell*. 114:445-56.

Xu, T., W. Wang, S. Zhang, R.A. Stewart, and W. Yu. 1995. Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development*. 121:1053-63.

Yabuta, N., T. Fujii, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, H. Nishiguchi, Y. Endo, S. Toji, H. Tanaka, Y. Nishimune, and H. Nojima. 2000. Structure, expression, and chromosome mapping of LATS2, a mammalian homologue of the Drosophila tumor suppressor gene lats/warts. *Genomics*. 63:263-70.

Yang, X., D.M. Li, W. Chen, and T. Xu. 2001. Human homologue of Drosophila lats, LATS1, negatively regulate growth by inducing G(2)/M arrest or apoptosis. *Oncogene*. 20:6516-23.

Yang, X., K. Yu, Y. Hao, D.M. Li, R. Stewart, K.L. Insogna, and T. Xu. 2004. LATS1 tumour suppressor affects cytokinesis by inhibiting LIMK1. *Nat Cell Biol*. 6:609-17.

Yarm, F.R. 2002. Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. *Mol Cell Biol*. 22:6209-21.

Zeitlin, S.G., R.D. Shelby, and K.F. Sullivan. 2001. CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. *J. Cell Biol.* 155:1147-1158.

Zieve, G., and F. Solomon. 1982. Proteins specifically associated with the microtubules of the mammalian mitotic spindle. *Cell*. 28:233-42.

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SCHOLARSHIPS

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CONFERENCE ATTENDED

Poster presentation, Jacques Monod conference on Molecular Machines in Cell Division, 10–14 September 2005, Roscoff, France.

TEACHING EXPERIENCE

During my M.Phil study in the Chinese University of Hong Kong, I had given hands-on training

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LANGUAGES

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PUBLICATIONS

1. Chan, E.H.Y., Santamaria, A., Silljé, H.H.W., Nigg, E.A. Polo-like kinase(Plk1) controls Aurora A kinase function by regulating the protein level of hBora. (manuscript in preparation)
2. Leung, H.Y., Wang, Y., Chan, H.Y., Leung, L.K. (2007). Developing a high-throughput system for the screening of cytochrome P450 1A1 - Inhibitory polyphenols. *Toxicol In Vitro*. *21*, 996-1002.
3. Gruneberg, U., Neef, R., Li, X., Chan, E.H.Y., Chalamalasetty, R.B., Nigg, E.A., and Barr, F.A. (2006). KIF14 and citron kinase act together to promote efficient cytokinesis. *J. Cell Biol. 172*, 363-72.
4. Chan, E.H.Y., Nousiainen, M., Chalamalasetty, R.B., Schäfer, A., Nigg, E.A. and Silljé, H.H.W. (2005). The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene*. *24*, 2076-86.
5. Chan, H.Y., Wang, H., Tsang, D.S., Chen, Z.Y., Leung, L.K. (2003). Screening of chemopreventive tea polyphenols against PAH genotoxicity in breast cancer cells by a XRE-luciferase reporter construct. *Nutr. Cancer*. *46*, 93-100.
6. Chan, H.Y., Leung, L.K. (2003). A potential protective mechanism of soya isoflavones against 7,12-dimethylbenz[a]anthracene tumour initiation. *Br J Nutr*. *90*, 457-65.
7. Chan H.Y., Wang H., Leung L.K. (2003). The red clover (*Trifolium pratense*) isoflavone biochanin A modulates the biotransformation pathways of 7,12-dimethylbenz[a]anthracene. *Br J Nutr*. *90*, 87-92.
8. Chan, H.Y., Chen, Z.Y., Tsang, D.S., Leung, L.K. (2002). Baicalein inhibits DMBA-DNA adduct formation by modulating CYP1A1 and CYP1B1 activities. *Biomed Pharmacother*. *56*, 269-75.
9. Arooz, T., Chan, H.Y., Lau, W.S.A., Leung, K.M., Po, L.S., Siu, W.Y., Tsang, F.C., and Poon, R.Y.C. Regulation of G_2/M cell cycle DNA damage checkpoints, in *Molecular Genetic Basis of Cancer* Eds. Lung, M.L. and Hsiao, W.L.W. pp. 230-243 (2001).

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