Chapter 10
Hippo in Cell Cycle and Mitosis

Norikazu Yabuta and Hiroshi Nojima

Abstract The Hippo pathway regulates cell growth and cell cycle-dependent processes, including mitosis, cell cycle checkpoints, mitotic checkpoints, and DNA damage response (DDR) checkpoints, thereby preventing the accumulation of abnormal cells with aneuploidy and polyploidy. Moreover, Mst1/2, Lats1/2, Mob1, and Rassf1A primarily colocalize with mitotic regulators, such as Aurora A and Polo, at the centrosome, and then dynamically translocate to the nucleus or the central spindle and the midbody in response to various stimuli. In particular, Lats1/2 play various roles in the DDR checkpoint, maintaining centrosome integrity, mitotic checkpoints (including the spindle assembly checkpoint (SAC)), mitotic exit, cytokinesis, EMT, and cellular senescence. Lats2 also plays a pivotal role in the cell cycle checkpoint via the p53 pathway, thereby functioning as another "guardian" of genome integrity. Therefore, the machinery and related molecules within the Hippo pathway may be potent and promising cancer therapy targets, which may arrest or kill malignant tumor cells without the side effects associated with commonly used treatments.

Keywords Hippo • Cell cycle • Checkpoint • Mitosis • Lats1/2 • Chromosomal instability

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10.1 Introduction

Human malignant cancer cells exhibit two major hallmarks: abnormal cell proliferation and chromosomal instability (CIN) (Hanahan and Weinberg 2011; Gordon et al. 2012). The main causes of CIN are aberrant regulation of cell cycle checkpoints and abnormal mitosis, both of which lead to the accumulation of genetic errors and to the loss or gain of genetic material, which are then passed on to the next generation. In particular, malfunction of mitotic progression or the spindle assembly checkpoint (SAC) results in centrosome hyperamplification, defective centrosome separation, and incorrect microtubule-kinetochore attachments. This leads to chromosome missegregation or failure of cytokinesis, followed by the generation of aneuploid cells and genetically unstable tetraploid cells, which facilitate tumor progression (Storchova and Kuffer 2008; Davoli and de Lange 2011; Vitale et al. 2011). Therefore, the cellular machinery is stringently organized by the actions of various molecules: cell cycle and checkpoint regulators, such as Cyclins/Cdks, Cdk inhibitors, pRb, E2F, Cdc25, and p53; DNA damage checkpoints, such as ATM (Ataxia-Telangiectasia muted), ATR (Ataxia-Telangiectasia and Rad-3-related), Chk1, and Chk2; mitotic kinases, such as Cdk1, Aurora, Polo, NIMA, and BubR1; and apoptosis regulators, such as Bax, Bcl, and caspases.

The Hippo signaling pathway, which is highly conserved in fruit flies and higher eukaryotes, regulates organ size by controlling cell proliferation and apoptosis, and plays an essential role in the suppression of tumor cell growth and the self-renewal of stem cells (Pan 2010; Zhao et al. 2011). In mammalian cells, four kinds of serine (S)/threonine (T) kinases, Mst1 (mammalian sterile 20-like kinase 1), Mst2, Lats1 (large tumor suppressor 1), and Lats2; two kinds of adaptor protein, hWW45/hSav1 and Mob1 (*M*ps *o*ne-*b*inder 1); and two kinds of the transcriptional coactivator, Yap (yes-associated protein) and Taz (transcriptional coactivator with PDZ-binding motif) form the core components of the canonical Hippo pathway. Mst1/2, Lats1/2, WW45, Mob1, and Yap/Taz are functionally equivalent to their fruit fly homologs, Hpo (Hippo), Wts (Warts/Lats), Mats (mob as tumor suppressor) and Yki (Yorkie), respectively (see Fig. 10.5, left panel).

These core components comprise the main phosphorylation signaling cascade in response to upstream activation signals, such as cell–cell contact. In human cells, activated Mst1/2 kinases, along with their activator, WW45, cooperatively phosphorylate both Lats1/2 kinases (at S909* and T1079 at the C-terminus of Lats1, and at S872* and T1041 at the C-terminus of Lats2; *these sites possibly function as autophosphorylation sites) and their regulator, Mob1 (at T12 and T35), thereby inducing formation of activated Lats1-Mob1 and/or Lats2-Mob1 complexes. This results in phosphorylation of Yap/Taz (at S127 and S381 in Yap, and at S89 and S311 in Taz), preventing their nuclear translocation and the subsequent induction of cell-proliferative and anti-apoptotic gene expression (Pan 2010; Zhao et al. 2011). Phosphorylation of Yap-S127 sequesters Yap within the cytoplasm via binding to 14-3-3 proteins, whereas phosphorylation of Yap-S381 targets Yap for degradation via ubiquitin-mediated proteolysis (Zhao et al. 2007, 2010). Moreover, the Hippo core pathway engages a variety of upstream regulators and modulators, such as Fat1/4, Merlin/

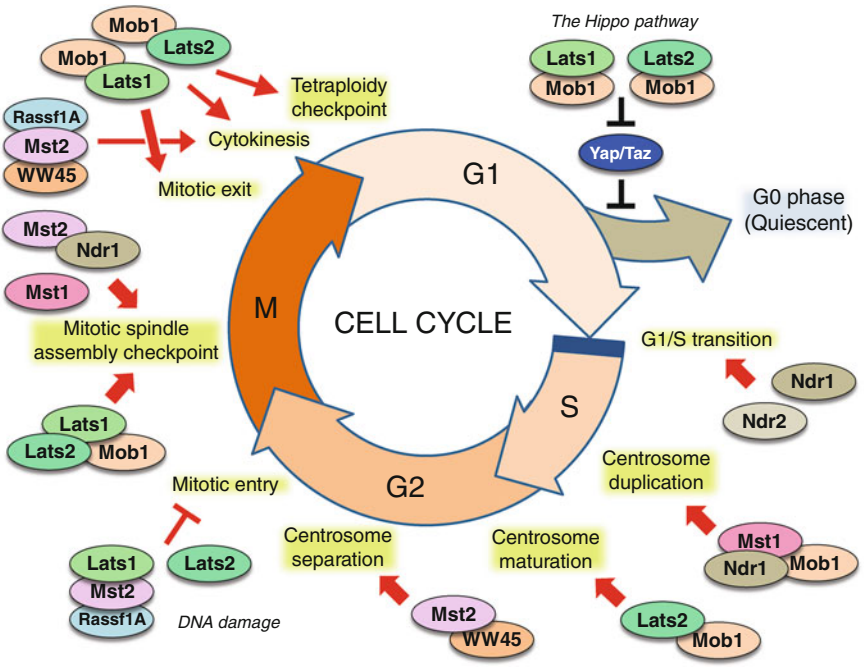

Fig. 10.1 Roles of the different Hippo pathway components in cell cycle regulation. The core Hippo pathway inhibits the function of Yap/Taz and increases the expression of genes involved in cell proliferation. In other words, the core Hippo components induce growth arrest via the canonical Hippo pathway, whereas they play pivotal roles in controlling the cell cycle and cell cycle checkpoints. Because Kibra is phosphorylated by Aurora A, and contributes to Lats2 stabilization, Kibra may regulate Aurora A/Lats2-mediated cell cycle functions (not shown here)

Nf2, Kibra, Tao-1/TAOK, Willin/FRMD6, Rassf, Ajuba (a fruit fly homolog of dJub), Itch, and Angiomotin/Amot (Zhao et al. 2011; Boggiano and Fehon 2012).

The Hippo pathway is an important piece of cellular machinery that induces cell growth arrest in response to cell–cell contact (known as "contact inhibition"), which is thought to function during oncogenic suppression. Studies of human tumor samples show that many regulators of the Hippo pathway appear to be dysfunctional; for example, Fat1/4, Merlin/Nf2, Kibra, Mst1/2, and Lats1/2 are downregulated, whereas Yap/Taz are overexpressed (Pan 2010). This suggests that the Hippo pathway plays a crucial role in the tumor suppressor pathways in human malignant cancers. However, it is unclear how the Hippo pathway is involved in suppressing and eliminating malignant tumor cells showing aneuploidy or polyploidy.

Recently, some components of the Hippo pathway were found to regulate CIN by interacting with the cell cycle checkpoint machinery and through mitotic regulation (Fig. 10.1). This chapter provides an overview of the current knowledge about the role played by the Hippo pathway in regulating the cell cycle and mitosis, with particular focus on the function of Lats1/2 kinases and their regulators as central players in the cell cycle, mitosis, and checkpoint regulation.

10.2 Mst1 and Mst2

10.2.1 Mst1 and Mst2 as Members of the MEN/SIN Family

The mammalian Hippo homologs, Mst1 and Mst2, belong to the sterile 20-like kinase family, and have some biological functions that are independent of the canonical Hippo pathway (namely Yap/Taz-independent growth control). For example, they are involved in stress-induced apoptosis via the cleavage and subsequent nuclear translocation of their proteins (Ling et al. 2008; Matallanas et al. 2008). Of note, recent reports show that both Mst1 and Mst2 play important roles in both mitosis and apoptosis by taking advantage of some Hippo pathway components; this function appears to be evolutionarily conserved from yeast to humans (Bardin and Amon 2001; Hergovich and Hemmings 2012). Both Cdc15 in budding yeast and Sid1 in fission yeast, which are functional counterparts of Mst1/2, localize to the nascent centrosome (the so-called spindle pole body [SPB] in yeast) at anaphase, and to the contractile ring (the so-called bud neck and septum site in budding and fission yeast, respectively) at cytokinesis (Guertin et al. 2000; Menssen et al. 2001). At the onset of late mitosis, activated Cdc15 and Sid1 kinases directly phosphorylate and activate the Dbf2/Mob1 and Sid2/Mob1 kinase complexes (Lats1/2-Mob1 complexes in mammals), which in turn inhibit mitotic CDK1 through the release of Cdc14 phosphatase (budding yeast Cdc14 and fission yeast Clp1) from the nucleolus into the nucleoplasm and cytoplasm. Eventually, these signaling cascades, which are known as the mitotic exit network (MEN) in budding yeast and the septation initiation network (SIN) in fission yeast, allow the cells to exit mitosis, complete cytokinesis, and then execute a successful transition from mitosis into the next G1 phase (Bardin and Amon 2001). On the other hand, there are no reports that *hippo* mutant fruit flies show a failure of cytokinesis, although RNAi-mediated *hippo*-knockdown cells do show severe defects in mitotic spindle and central spindle formation (Bettencourt-Dias et al. 2004).

10.2.2 Centrosome Regulation by Mst1 and Mst2

Like Cdc15/Sid1 in yeast, mammalian Mst2 localizes to the centrosomes during interphase, at the spindle poles during mitosis, and at the midbody (the final convergent structure of the contractile ring) during cytokinesis (Guo et al. 2007). Moreover, Mst1/2 kinases are activated by increasing protein levels and kinase activity in mitotic cells treated with nocodazole, a molecule that depolymerizes the microtubules (Praskova et al. 2008). In cells arrested at M phase by treatment with nocodazole, Mst1 and Mst2 are phosphorylated on the activation loop at threonine 183 (T183) and T180, respectively; activated Mst2 then phosphorylates both Lats1 and Mob1 to activate the Lats1/Mob1 complex (Praskova et al. 2008). Interestingly,

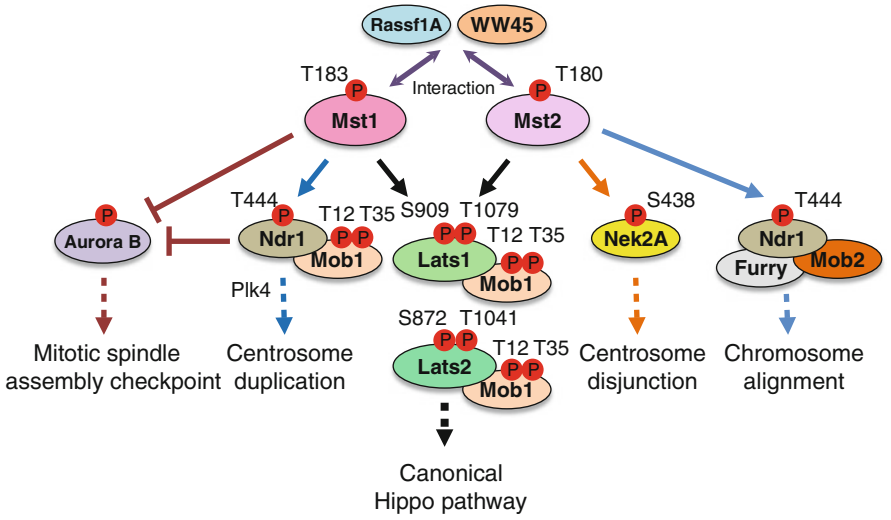

Fig. 10.2 Mst1/2 kinases regulate the Hippo pathway and the cell cycle by phosphorylating different downstream targets. Mst1/2 are activated by binding to Rassf1A and/or WW45. Five axes (the Mst1/2-Lats1/2 axis, the Mst1-Aurora B axis, the Mst1-Ndr1 axis, the Mst2-Nek2A axis, and the Mst2-Ndr1 axis) regulate five subcellular events: the canonical Hippo pathway, the mitotic spindle assembly checkpoint, Plk4-driven centrosome duplication, centrosome disjunction, and chromosome alignment, respectively

association of Mst1/2 to two functional scaffold proteins, RASSF1A and WW45 (also known as hSav1 [a Salvador homolog] in mammals) within the Hippo pathway is initially required for mitotic activation of the Mst1/2 kinases, which takes place at the centrosome (Guo et al. 2007). Moreover, Mst2 promotes the association between RASSF1A and WW45 via their C-terminal coiled-coil motifs, known as Sav/Rassf/Hpo (SARAH) domains (Guo et al. 2007). Thus, it is suggested that Mst1/2 is efficiently activated in response to mitotic signaling by a positive feedback loop between Mst1/2 and RASSF1A/WW45 on the centrosome.

Indeed, Mst1 and Mst2 appear to play key roles in centrosomal integrity. Mst1, together with Mob1, regulates centrosome duplication (Hergovich et al. 2009). SiRNA-mediated downregulation of Mst1 or Mob1 inhibits centriole duplication in human osteosarcoma U2OS cells. Moreover, Mst1 directly phosphorylates Ndr1 kinase (the second member of the Dbf2 kinase family, which is equivalent to Tricornered [Trc] in fruit flies) at T444 on the hydrophobic motif to activate the Ndr1/Mob1 complex. This is essential for Polo-like kinase 4 (Plk4)-driven centriole duplication via localization of the distal end-capping protein, CP110, to the centriole, but not that of the cartwheel protein HsSAS-6 (Habedanck et al. 2005). Notably, the other Hippo pathway components, WW45 and RASSF1A, appear to be dispensable for centrosome duplication mediated by the Mst1-Ndr1-Mob1 pathway (Hergovich et al. 2009) (Fig. 10.2).

On the other hand, Mst2 plays a pivotal role in centrosome disjunction. Specifically, Mst2 and WW45 directly interact with the NIMA-related kinase Nek2A (but not with Nek2B) via the SARAH domains in their C-terminal regions, and recruit it to the centrosome (Mardin et al. 2010) (Fig. 10.2). Moreover, Mst2 phosphorylates Nek2A at S438, which promotes the recruitment of Nek2A to the centrosome, but does not increase its kinase activity. Simultaneously, the Mst2-WW45 complex contributes to the phosphorylation of two centrosomal linker proteins, c-Nap1 and Rootletin (both major Nek2A phosphorylation targets, which bridge the gap between the two centrosomes). Interestingly, other Hippo pathway components, such as Lats1/2, Rassf1A, and Yap, are dispensable for the centrosomal targeting of Nek2A. Downregulation of Mst1/2, as well as Nek2 and WW45, prevents centrosome splitting during late G2 phase, which can be rescued by overexpression of a constitutively phosphorylated Nek2A mutant (Nek2A-4D, harboring a S438D mutation). Furthermore, Mst2 is directly phosphorylated by Polo-like kinase 1 (Plk1), which prevents the association between Mst2-Nek2A and protein phosphatase 1γ (PP1γ), thereby promoting Nek2A kinase activity (Mardin et al. 2011). Therefore, Mst2 and WW45 are key regulators of centrosome disjunction downstream of Plk1 via Nek2A-induced c-Nap1/Rootletin dissociation and subsequent bipolar spindle formation. In support of this, evidence from budding yeast shows that a Polo-like kinase homolog, Cdc5, is required for the recruitment of Cdc15 (Mst1/2) onto the SPB (Rock and Amon 2011).

10.2.3 Chromosome Regulation by Mst1 and Mst2

Importantly, siRNA-mediated downregulation of Mst1 frequently causes chromosome misalignment and anaphase delay in human cervical cancer HeLa cells through Mad2- and BubR1-dependent spindle checkpoint activation. In particular, Mst1 associates with, and directly phosphorylates, Aurora B, a mitotic spindle checkpoint kinase. This phosphorylation negatively modulates Aurora B kinase activity and stabilizes the kinetochore-microtubule attachments (Oh et al. 2010). Like Mst1 depletion, downregulation of Mst2 causes chromosome misalignment, although it is unclear whether Mst2 directly phosphorylates Aurora B and/or regulates its activity (Oh et al. 2010). Instead, Mst2 contributes to the mitotic activation of Ndr1 kinase in cooperation with its activator, Mob2, and a scaffold protein called Furry (Fry) (present in the spindle microtubules), thereby regulating the precise alignment of mitotic chromosomes (Chiba et al. 2009).

Taken together, it is likely that mammalian Mst1 and Mst2 are essential regulators of centrosomal integrity (Mst1 regulates centrosome duplication, whereas Mst2 regulates centrosome disjunction), and of accurate chromosome alignment and segregation during mitosis. However, although yeast homologs Cdc15/Sid1 are mainly involved in mitotic exit or cytokinesis, the precise role of Mst1/2 during cytokinesis is not fully understood (apart from its ability to restore cytokinesis in Rassf1A-deficient mouse embryo fibroblasts (Guo et al. 2007)).

10.3 Lats1 and Lats2

10.3.1 Protein Structures of Lats1/2

Lats1 and Lats2 (Lats1/2) are mammalian homologs of yeast mitotic kinases: Dbf2/Dbf20 in budding yeast and Sid2 in fission yeast (Tao et al. 1999; Yabuta et al. 2000). Lats1 and Lats2 are also known as Warts/h-warts and Kpm, respectively (Nishiyama et al. 1999; Hori et al. 2000). Compared with the primary structures of their yeast homologs, Lats kinases in higher eukaryotes, including fruit fly Warts/Lats, nematode Lats1, and mammalian Lats1/2, possess a long, stretched N-terminal non-kinase region (approximately 700 amino acids) upstream of the highly conserved serine/threonine kinase domain (the kinase activity of which is regulated by Mst1/2). However, this region is not present in yeast Dbf2/Sid2 and mammalian Ndr1/2 (Visser and Yang 2010). Because organ size and tumor formation are not a concern for unicellular organisms such as yeast, the N-terminal regions of Lats kinases in higher eukaryotes may play various roles in tumor development and malignancy, not only through the canonical Hippo pathway but also through the other mechanisms, such as cell cycle and checkpoint controls. Moreover, a comparison between the N-terminal halves of mammalian Lats1 and Lats2 reveals that they share low sequence similarity, apart from Lats conserved domain 1 (LCD1) and LCD2, which may be functionally important for tumor suppression. By contrast, their C-terminal kinase domains are highly conserved (85% and 80% sequence identity between human and mouse, respectively) (Li et al. 2003). It is suggested that Lats1 and Lats2 possess both common and mutually exclusive physiological functions.

Mammalian Lats1/2 kinases are involved in cell cycle regulation and malignancy (Fig. 10.1). Although the N-terminal regions of Lats1/2 interact with some Hippo pathway regulators, such as Mob1, Yap, Taz, and Kibra (Pan 2010; Sudol and Harvey 2010; Visser and Yang 2010), they also interact with various cell cycle regulators. For example, the N-terminus of Lats1 physically interacts with the mitotic kinase, Cdc2 (Cdk1), the actin filament assembly factor, Zyxin, and a regulator of actin dynamics, LIM kinase 1 (LIMK1) (Tao et al. 1999; Hirota et al. 2000; Yang et al. 2004), whereas the N-terminus of Lats2 interacts with centrosomal proteins such the LIM protein, Ajuba, and Aurora A kinase (Abe et al. 2006; Toji et al. 2004). Moreover, a variety of protein kinases phosphorylate the N-terminal non-kinase regions of Lats1/2, although their C-termini are phosphorylated by Mst1/2 kinases (S909 and T1079 of Lats1, and S872 and T1041 of Lats2) (Fig. 10.5). The N-terminal region of Lats1 is phosphorylated by Cdc2/cyclin B (at S613), NUAK1 (at S464), and PKCδ (at S464), whereas that of Lats2 is phosphorylated by Aurora A (at S83 and S380) and Chk1/2 (at S408); these phosphorylations regulate their subcellular localization, protein stability, and/or enzymatic activity (Morisaki et al. 2002; Chan et al. 2005; Takahashi et al. 2006; Humbert et al. 2010; Okada et al. 2011; Yabuta et al. 2011).

10.3.2 Lats1/2 Are Human Tumor Suppressors

The *LATS1/2* genes are candidate human tumor suppressor genes. Human *LATS2* maps to the chromosome 13q11-q12 region, in which loss of heterozygosity has been frequently observed in various primary cancers (Yabuta et al. 2000). In fact, expression of *LATS1/2* is downregulated in a variety of human malignant tumors, such as aggressive breast cancers, acute lymphoblastic leukemia, non-small cell lung cancer, and astrocytoma, by hypermethylation of their promoter regions (Takahashi et al. 2005; Jiménez-Velasco et al. 2005; Jiang et al. 2006). In human breast and prostate cancer samples, *LATS2* expression is downregulated by a defect in the transcriptional factor, FOXP3 (Li et al. 2011), and inactivating mutations within the *LATS2* coding region have also been reported in human malignant mesothelioma (Murakami et al. 2011). In particular, expression of *LATS2* in human testicular germ cell tumors, colorectal cancer, gastric cancer cell lines, and esophageal cancer cell lines is downregulated by micro (mi)RNAs (miR-372 and/or miR-373) (Voorhoeve et al. 2006; Cho et al. 2009; Lee et al. 2009; Yamashita et al. 2012). Moreover, Lats1 knockout (*Lats1*$^{-/-}$) mice are susceptible to soft-tissue sarcomas and ovarian tumors, although Lats2 knockout (*Lats2*$^{-/-}$) mice are embryonic lethal before embryonic day 12 (St. John et al. 1999; McPherson et al. 2004; Yabuta et al. 2007). Notably, disruption of *Lats2* in MEFs (mouse embryonic fibroblasts) causes centrosomal fragmentation, abnormal mitotic spindle formation, chromosomal missegregation, and failure of cytokinesis, thereby inducing the CIN that is a hallmark of malignant tumor cells (McPherson et al. 2004; Yabuta et al. 2007). Taken together, these reports suggest that Lats1 and Lats2 cooperatively regulate the cell cycle and tumor suppression in mammalian cells.

10.3.3 Lats1/2 Play Pivotal Roles in the Cell Cycle Checkpoint

Lats1/2 play important roles in cell growth and the regulation of organ size through the Hippo pathway, and have a role in chromosomal stability through cell cycle checkpoint regulation. Specifically, enforced expression of Lats1 in MEFs and human cancer cells harboring a nonfunctional p53 tumor suppressor (such as HeLa and C33A cells) causes G2/M arrest via the binding and inactivation of Cdc2 kinase, whereas it triggers apoptosis in human cancer cells harboring wild-type p53 (such as A549 and HCT116 cells) via induction of p53 or proapoptotic Bax (Tao et al. 1999; Yang et al. 2001; Xia et al. 2002). By contrast, exogenous expression of Lats2 in mouse NIH3T3/*v-ras* cells causes G1/S arrest by inactivating Cdk2/Cyclin E (which express wild-type p53), but causes G2/M arrest in functional p53-deficient HeLa cells by inactivating Cdc2/Cyclin B, leading to apoptosis (Kamikubo et al. 2003; Li et al. 2003). In lung cancer cells such as A549 and H1299, overexpression of Lats2 causes apoptosis by downregulating expression of the anti-apoptotic proteins, Bcl-2 and Bcl-X_L, a mechanism that is independent of p53 status (Ke et al. 2004).

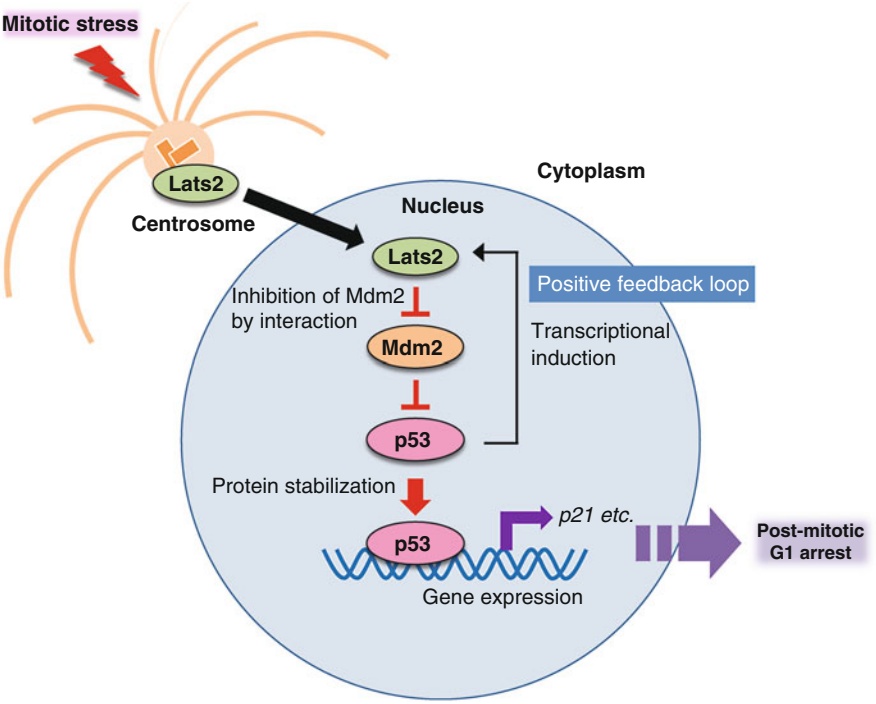

Fig. 10.3 The Lats2-Mdm2-p53 pathway regulates the G1 tetraploidy checkpoint. In response to mitotic stress, the Lats2-Msm2-p53 axis forms a positive feedback loop and subsequently promotes post-mitotic G1 arrest by inducing the expression of *p21* (see text)

The p53 tumor suppressor is a key regulator of the cell cycle checkpoint that activates and functions as a transcription factor to induce cell cycle arrest and apoptosis in response to various stimuli and stresses (Aylon and Oren 2011). Importantly, Oren's group showed that Lats2 positively regulates p53 at the cell cycle checkpoint via its involvement in a novel tumor suppressor axis, Lats2-Mdm2-p53 (Aylon et al. 2006) (Fig. 10.3). When cells are exposed to microtubule poisons, such as nocodazole, which elicit mitotic stress in cells, Lats2 translocates from the centrosome to the nucleus, where it accumulates. Nuclear Lats2 directly binds to and inhibits the E3 ubiquitin ligase, Mdm2, thereby stabilizing and activating the p53 protein; p53 then selectively binds to the promoter region of *LATS2,* rapidly upregulating Lats2 expression via a positive feedback loop. As a result, an abundance of p53 upregulates expression of the Cdk inhibitor, p21, thereby inducing G1 arrest of aberrant cells that exit mitosis without proper sister chromatid segregation or cytokinesis (mitotic slippage), and preventing tetraploidization (the G1 tetraploidy checkpoint) (Aylon et al. 2006). Moreover, an E3 ubiquitin ligase, Fbw7, also cooperatively regulates p53-dependent induction of Lats2 and p21 expression by degrading Cyclin E and Aurora A (Finkin et al. 2008).

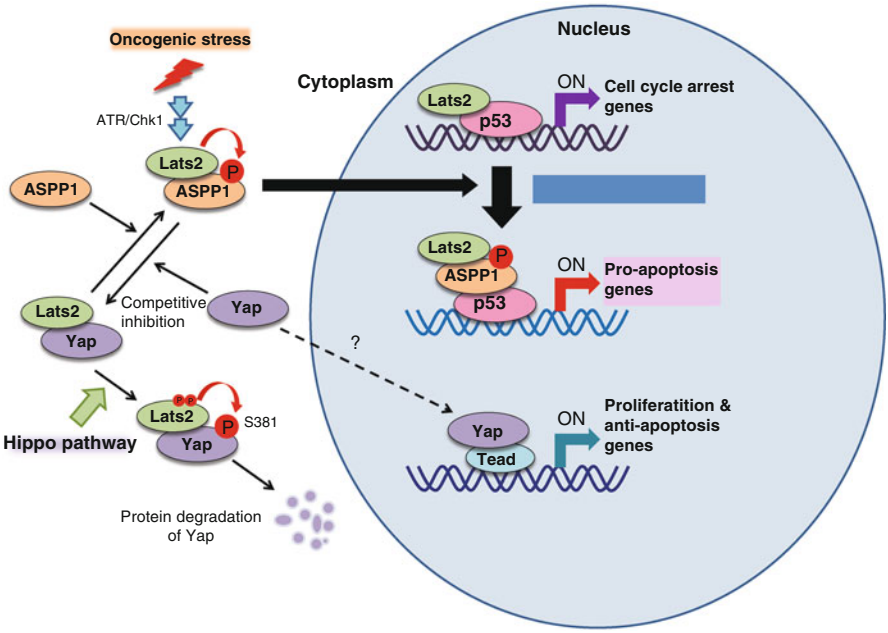

Fig. 10.4 The Lats2-ASPP1-p53 pathway regulates apoptosis. In response to oncogenic stress, probably through ATR-Chk1 signaling, the Lats2-ASPP1-p53 axis promotes the translocation of p53 from the promoters of genes involved in cell cycle arrest, such as *p21,* to the promoters of genes associated with apoptosis, such as *BAX* and *PIG3* (see text). This pathway is competitively inhibited by Yap proteins, which may be inhibited by the activated Hippo pathway

More importantly, the nuclear accumulation and translocation of Lats2 and subsequent p53 activation are also increased in response to oncogenic H-Ras via the ATR-Chk1 kinase-mediated stress checkpoint pathway (see below), which induces apoptosis or cellular senescence in polyploidy cells by quenching H-Ras-induced transformation and tumor progression (Aylon et al. 2009). So how does Lats2 efficiently promote apoptosis of transformed cells despite them being highly oncogenic? In response to oncogenic stress, Lats2 associates with, and directly phosphorylates, apoptosis-stimulating protein of p53-1 (ASPP1), which diverts p53 from the promoters of cell cycle genes such as *p21* to the promoters of proapoptotic genes such as *BAX* and *PIG3* (Aylon et al. 2010). Thus, depletion of Lats2 or ASPP1 by siRNA fails to trigger apoptosis in transformed cells, thereby increasing the number of polyploid cells. This suggests that Lats2 plays a pivotal role in the prevention of polyploidy and in the suppression of tumor malignancy. Notably, a Hippo pathway effector, Yap, competitively inhibits the interaction between Lats2 and ASPP1 and the subsequent induction of apoptosis by directly interacting with Lats2 (Aylon et al. 2010) (Fig. 10.4). Lats1 may also competitively interact with the ASPP1 or Yap proteins (Vigneron et al. 2010). Overexpression of kinase-inactivated Lats1 in Rat1 fibroblasts leads to mitotic slippage and the accumulation of tetraploidy in a

dominant-negative manner, which is accompanied by the downregulation of p53 (Iida et al. 2004). Interestingly, Lats1 can also directly interact with, and inhibit, the ability of Mdm2 to destabilize the p53 protein, thereby inducing apoptosis in colorectal cancers with a K-Ras mutation (Matallanas et al. 2011). However, it is likely that Lats1 enforces these checkpoint programs downstream of the activated Rassf1A-Mst2 pathway, whereas Lats2 takes advantage of the ATR-Chk1 pathway (Aylon et al. 2009; Matallanas et al. 2011).

Therefore, Lats1 and Lats2 are key regulators of the p53-mediated cell cycle checkpoint, which maintains the correct chromosome number through post-mitotic G1 arrest (the G1 tetraploidy checkpoint) and apoptosis. Consistent with this, fruit fly Dmp53 (the fruit fly homolog of p53)-induced apoptosis is reduced in *warts* mutants and in *hippo* and *salvador* mutants (Colombani et al. 2006).

10.3.4 Lats1/2 Also Regulate the DNA Damage Checkpoint

The DNA damage response (DDR) is crucial for maintaining genomic stability, and mainly functions through two DNA damage signaling pathways: the ATM and ATR pathways (Bartek et al. 2007; Ciccia and Elledge 2010). ATR kinase regulates the downstream effector kinase, Chk1, in response to DNA damage caused by single-strand breaks (SSBs) or stalled replication forks (referred to as the ATR-Chk1 pathway). Since Lats2 functions downstream of the ATR-Chk1 pathway in response to H-RasV12-induced oncogenic stress (Aylon et al. 2009), Lats2 may also play a role in regulating the DNA damage checkpoint.

Indeed, the authors found that Lats2 is directly phosphorylated (at S408 at its N-terminus) by Chk1 kinase in response to UV irradiation; activated Lats2 then directly phosphorylates 14-3-3 proteins at S59 (Okada et al. 2011). Interestingly, Lats2-phosphorylated 14-3-3 proteins accumulate in cytoplasmic foci known as P (processing)-bodies, in which mRNA degradation, translational repression, and mRNA surveillance are stringently regulated by miRNAs and scaffold proteins such as GW182. The accumulation of 14-3-3 proteins within the P-body, and enlargement of the P-body itself, are induced by UV damage and are dependent on Lats2, but not Lats1 (Okada et al. 2011). Thus, it is likely that the Chk1-Lats2-P-body pathway efficiently represses translation after UV damage (known as the "CLP pathway") (Fig. 10.5, middle).

However, in fruit flies, DNA damage caused by double-strand breaks (DSBs) enhances the activity of Hippo kinase in a Dmp53-dependent manner (Colombani et al. 2006). In mammals, Lats1 and Mst2 are activated downstream of Rassf1A, which is phosphorylated at S131 by ATM after DNA damage caused by DSBs, thereby inducing the stabilization of the proapoptotic transcriptional factor, p73, and subsequently triggering apoptosis (Hamilton et al. 2009). Moreover, Lats1 phosphorylates S445 of MYPT1 (myosin phosphatase-targeting subunit 1), which promotes the dephosphorylation of Plk1 by PP1C after DSBs, which results in the

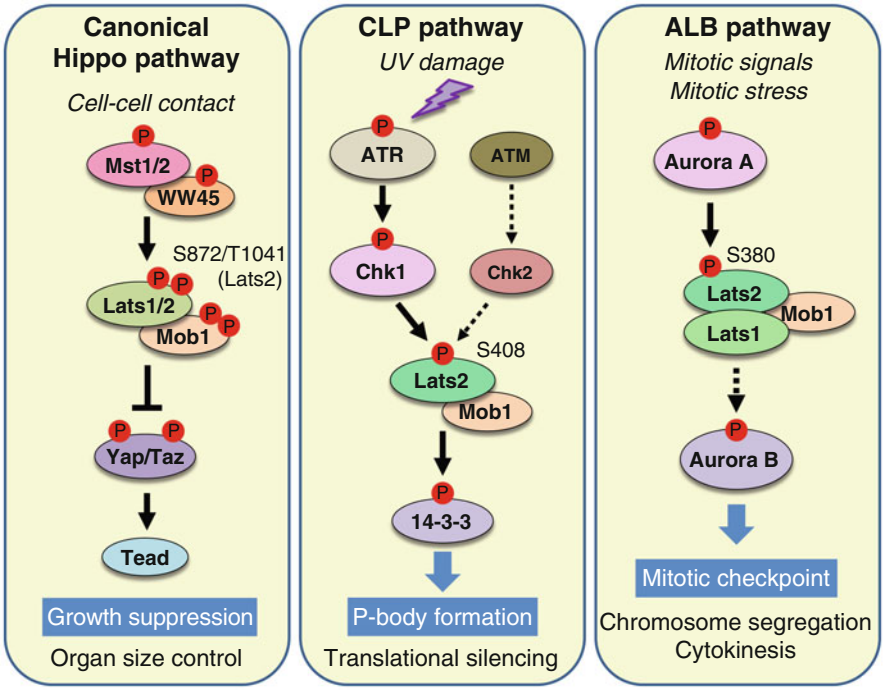

Fig. 10.5 Novel Lats2-mediated signaling pathways. The canonical Hippo pathway regulates growth suppression to control organ size by inhibiting the Yap/Taz-Tead transcriptional system (left), whereas the Chk1/2-Lats2-P-body axis (CLP pathway, middle) and Aurora A-Lats1/2-Aurora B axis (ALB pathway, right) regulate P-body formation in response to UV damage and the mitotic checkpoint, respectively

suppression of Plk1 activity during G2/M transition (Chiyoda et al. 2012). It remains unclear whether Lats2 participates in these pathways after DSBs. Future studies are needed to understand the detailed molecular mechanisms underlying the Lats1/2-mediated pathways involved in the DDR.

10.3.5 Lats1/2 Control Mitotic Progression and Cytokinesis

Dbf2, a Lats/Warts homolog in budding yeast colocalizes with its coactivator, Mob1, and the upstream kinase, Cdc15, at the SPB (or the centrosome in mammals) during anaphase. A fraction of these proteins then translocates to the bud neck during cytokinesis, where they promote MEN and cytokinesis in response to Cdc15 activation (Frenz et al. 2000; Hwa Lim et al. 2003). Another yeast counterpart, Dbf20, probably plays a minor role in the MEN (Toyn and Johnston 1994). The Lats homolog, Sid2, in fission yeast also colocalizes with Mob1 at the SPB throughout

mitosis and at the septum site during cytokinesis, where it forms an essential component of the SIN to regulate cytokinesis (Sparks et al. 1999; Hou et al. 2004). Therefore, mammalian Lats1/2 may also localize to the mitotic apparatus and actually regulate mitotic progression, mitotic exit, and cytokinesis.

In human cancer cells, Lats2 normally localizes to the centrosome, cytoplasm, and nucleus during interphase (Toji et al. 2004). *Lats2*$^{-/-}$ MEFs show amplification of pericentriolar material (PCM) such as γ-tubulin, but not of the centrioles (Yabuta et al. 2007). Moreover, siRNA-mediated depletion of Lats2 in HeLa cells also leads to loss of γ-tubulin accumulation at the centrosome and disintegration of spindle microtubules (Abe et al. 2006). These results suggest that Lats2 regulates centrosomal integrity, including centrosome fragmentation, but probably not centriole duplication. Although Lats1 also localizes to the centrosome (Nishiyama et al. 1999), there is no report to date that knockout or knockdown of Lats1 causes centrosomal abnormalities in mammalian cells. Alternatively, overexpression of the kinase-inactive form of Lats1 or Lats2 in U2OS cells seems to have no effect on centrosomal duplication, whereas overexpression of wild-type Ndr1, but not kinase dead Ndr1, leads to centrosomal overduplication, probably through the Mst1-Ndr1-Mob1 pathway (Hergovich et al. 2007, 2009). Interestingly, Lats2 associates with centrosomal proteins such as Aurora A and its activator, Ajuba (Toji et al. 2004; Abe et al. 2006). Similarly to Lats2 depletion, knockdown of Ajuba results in disintegration of the centrosome and mitotic spindle. Moreover, Ajuba physically interacts with Lats1/2, which prevents Lats1/2-mediated phosphorylation of YAP in the Hippo pathway (Das Thakur et al. 2010). Thus, the Lats1/2-Ajuba-Aurora A complex may cooperatively regulate centrosome maturation and spindle organization on the centrosome.

While studying the role of mammalian Lats2 in cell cycle regulation, the authors found that human Lats2 is multiply phosphorylated during cell cycle progression, including both at interphase and during mitosis (Toji et al. 2004). Like Lats2, Lats1 is also phosphorylated during mitosis (Tao et al. 1999; Nishiyama et al. 1999; Hori et al. 2000; Toji et al. 2004). These results suggest that Lats1/2 are stringently regulated by the Hippo pathway and by the cell cycle machinery, including the mitotic checkpoint and cytokinesis. In fact, the N-terminus of Lats2 is phosphorylated at multiple sites (at least two: S83 and S380) by Aurora A kinase during mitosis (Toji et al. 2004; Yabuta et al. 2011). Moreover, Lats2 colocalizes with Aurora A at the centrosome during mitosis (Toji et al. 2004). Aurora A is a member of the Aurora kinase family, which regulates centrosome integrity and cell division by phosphorylating numerous proteins that play a critical role in mitosis (Nigg 2001; Salaun et al. 2008). Interestingly, the subcellular localizations of phosphorylated Lats2 are different during the mitotic phase (Yabuta et al. 2011): Lats2 phosphorylated on S83 localizes to the nucleus during early mitosis and at the centrosome throughout mitosis, whereas S380-phosphorylated Lats2 localizes to the nucleus during early mitosis and to the chromosomes during metaphase. It then moves to the central spindle/spindle mid-zone during anaphase/telophase. During cytokinesis, phosphorylated Lats2 is concentrated at the midbody. It is suggested that Aurora A regulates the mitotic localization of Lats2 via different patterns of phosphorylation. On the other hand, Lats1 also localizes at the mitotic apparatus

(centrosome, mitotic spindle, central spindle, and midbody), where it associates with Zyxin to enable mitotic progression (Hirota et al. 2000). Although Lats1 may contain putative Aurora A phosphorylation sites (Sardon et al. 2010), it is unclear whether Lats1 is actually phosphorylated by Aurora A. Instead, Lats1 interacts with Cdc2 (Tao et al. 1999) and is phosphorylated at S613 by the Cdc2/Cyclin B complex during mitosis; however, the role of Cdc2/cyclin B-phosphorylated Lats1 is not clear (Morisaki et al. 2002).

Aurora B, another member of the Aurora kinase family, forms the chromosomal passenger complex (CPC) by interacting with the inner centromere protein (INCENP), Survivin, and Borealin/Dasra-B, enabling proper kinetochore-microtubule attachments, chromatid cohesion, spindle formation through the SAC, and cytokinesis (Ruchaud et al. 2007). Of note, a fraction of the S380-phosphorylated Lats2 in HeLa and U2OS cells colocalizes with Aurora B at the chromosome arms, the central spindle, and the midbody (Yabuta et al. 2011). Moreover, Lats2 physically interacts with Aurora B, as well as Aurora A. Interestingly, Lats2 can also interact with Lats1 in the presence of Mob1A, and Lats1 kinase plays a role in the phosphorylation of Aurora B. Indeed, the expression of a non-phosphorylated Lats2 mutant (S380A) in HeLa cells promotes lagging chromosomes, chromosome bridges, aberrant nuclear formation, micronuclear formation, and multinucleation, which are similar to the phenotypes induced by abnormal regulation of Aurora B (chromosome missegregation and cytokinesis failure). Consistent with this, Rat-1 cells overexpressing the kinase-inactive form of Lats1 show mitotic delay and SAC activation (Iida et al. 2004). Therefore, it is probable that the Aurora A-Lats1/2-Aurora B axis functions as a novel pathway to ensure accurate mitotic progression (known as the "ALB pathway") (Fig. 10.5, right). This pathway is partially conserved in budding yeast; namely yeast Mob1 (a cofactor and activator of Dbf2/Lats) allows the single yeast Aurora homolog, Ipl1p, to dissociate from the kinetochore region, and maintains the localization of CPC-containing Ipl1, Bir1 (Survivin), and Sli15 (INCENP) on the anaphase spindle (Stoepel et al. 2005). Since dysfunction of the ALB pathway promotes mitotic delay and chromosome missegregation (Yabuta et al. 2011), prolonged mitosis may be a trigger for the subsequent G1 tetraploidy checkpoint- or mitotic catastrophe-mediated cell death seen in mammalian cells expressing fully functional p53 (Vitale et al. 2011).

Lats1/2 proteins are concentrated, along with their activator Mob1A, at the midbody during cytokinesis (Bothos et al. 2005). *Lats2*$^{-/-}$ MEFs frequently show failure of cytokinesis (approximately 20–30% of primary MEFs), thereby giving rise to multinucleated cells (Yabuta et al. 2007), whereas *Lats1*$^{-/-}$ cells give rise to low numbers of multinucleated cells (approximately 5–6% of MEFs or brain cortex cells) (Yang et al. 2004). By contrast, even if wild-type Lats1 is ectopically overexpressed in human cancer cells such as HeLa, it does not affect the completion of cytokinesis. Nevertheless, Lats1 directly interacts with LIMK1, and inhibits LIMK1 kinase activity toward the actin-depolymerizing protein Cofilin, thereby preventing LIMK1-induced cytokinesis defects. Moreover, the kinase activity of Lats1 is not essential for the inhibition of LIMK1 (Yang et al. 2004). Since Lats1 knockdown, as well as Mob1A knockdown, in U2OS cells results in a prolonged telophase,

the kinase activity of Lats1 may act as a mammalian form of MEN kinase to promote exit from mitosis (Bothos et al. 2005). On the other hand, some of the *Lats2*$^{-/-}$ MEFs fail to complete cytokinesis, whereas others show accelerated exit from mitosis along with the premature downregulation of mitotic regulators (Yabuta et al. 2007). Thus, Lats2, unlike Lats1, may regulate cytokinesis by acting more like a mammalian SIN kinase than a MEN kinase.

10.3.6 Other Roles of Lats1/2: EMT and Cellular Senescence

Lats1 and 2 phosphorylate the transcriptional cofactors Yap/Taz, and transcriptionally regulate gene expression via the Hippo pathway. Lats1/2 also regulate some transcription factors or cofactors that are involved in different signaling pathways. Specifically, Lats2 interacts with, and directly phosphorylates, a zinc-finger type-transcriptional factor called Snail1, which regulates the induction of epithelial-to-mesenchymal transitions (EMT) by repressing E-cadherin expression during embryonic development and tumor progression (Zhang et al. 2011). Lats2-mediated phosphorylation of Snail1 on T203 increases the stability and nuclear retention of Snail1. Interestingly, Lats2-mediated Snail phosphorylation is induced not only by environmental EMT stimuli such as TGFβ, but also by nocodazole-induced mitotic stress, RasV12-induced oncogenic stress, and upon activation of the Hippo pathway under high cell density conditions or upon overexpression of Mst2/WW45 (Zhang et al. 2011). Overexpression of Lats1 prevents Yap-induced EMT-like phenotypes, such as cell migration and colony formation, whereas overexpression of Lats2 has little effect on Yap-mediated EMT (Zhang et al. 2008). These results suggest the possibility that two parallel pathways for EMT regulation operate in mammalian cells; namely Lats1 represses Yap-mediated EMT (the Lats1-Yap axis), whereas Lats2 promotes Snail1-mediated EMT (the Lats2-Snail1 axis).

Lats2 also directly interacts with a ligand-dependent transcription factor called the androgen receptor (AR), which regulates the development and maintenance of the male reproductive system and is implicated in the development of prostate cancer. The Lats2-AR complex represses androgen-induced expression of the prostate-specific antigen (PSA) gene by binding to its promoter and enhancer regions (Powzaniuk et al. 2004). However, it is unclear whether Lats1/2 can phosphorylate AR in vivo.

More interestingly, Lats2 also plays an important role in Retinoblastoma protein (pRB)-induced cellular senescence. Lats2 directly phosphorylates DYRK1A and activates its kinase activity toward the LIN52 subunit of the DREAM (DP, RB [retinoblastoma], E2F, and MuvB) repressor complex, which promotes the assembly of DREAM complexes at E2F-regulated promoters and the silencing of E2F target genes such as Cdc6 and Cdc25A (Tschöp et al. 2011). Thus, it is likely that Lats2 regulates G1 arrest and apoptosis through the Lats2-p53 pathway, and also regulates cellular senescence through the Lats2-pRb pathway in response to oncogenic stress, both of which are pivotal tumor suppressor mechanisms.

On the other hand, in senescent human cells, decreased Lats1 levels result in an irreversible cytokinetic block downstream of reactive oxygen species (ROS)-protein kinase Cδ (PKCδ) signaling, a positive feedback loop in which elevated ROS levels activate PKCδ, which in turn promotes further generation of ROS (Takahashi et al. 2006). Indeed, Lats1 is directly phosphorylated at S464 by both PKCδ and NUAK1/AMPK-related protein kinase 5 (ARK5) kinases, which triggers degradation of the Lats1 protein (Takahashi et al. 2006; Humbert et al. 2010).

Taken together, these studies show that Lats1/2 play a pivotal role in tumor suppression as central regulators of a wide variety of signaling pathways via cell cycle and mitotic checkpoints, the DNA damage checkpoint, apoptosis, EMT, cellular senescence, and the Hippo pathway.

10.4 Mob1

Mob1 (Mats in the fruit fly) is an evolutionarily conserved coactivator of the Dbf2 kinase family, which includes Dbf2 and Sid2 in yeast, Warts in fruit flies, and Lats1/2 and Ndr1/2 in mammals (Hergovich 2011). Mob1 interacts with, and activates, the Dbf2 kinase family in the MEN, SIN, and Hippo pathways in yeast, fruit flies, and humans. Moreover, Mob1 is phosphorylated by an upstream Hippo kinase family, which includes Cdc15 and Sid1 in yeast, Hippo in fruit flies, and Mst1/2 in mammals. Thus, since Mob1 is also a core component of the MEN/SIN pathway, it is suggested that Mob1 is profoundly implicated in Lats-mediated mitotic regulation, as well as in the Hippo pathway (Fig. 10.1).

Like yeast Mob1, mammalian Mob1 colocalizes with Lats1/2 at the centrosome, whereas fruit fly Mats colocalizes not only with Warts kinase, but also with cyclin E at the centrosome throughout the cell cycle. Moreover, *mats* mutations cause chromosome missegregation in developing fruit fly embryos. However, loss of *mats* does not affect the colcemid-induced mitotic spindle checkpoint (Shimizu et al. 2008). Furthermore, both T12 and T35 of human Mob1 are phosphorylated by Mst1/2 kinase during nocodazole-arrested mitosis and in the presence of okadaic acid (a protein phosphatase inhibitor) and H_2O_2. These two phosphorylated sites on Mob1 are crucial for binding to Lats1 and Ndr1, and for Lats1 autophosphorylation at S909, which plays an important role in the regulation of cell proliferation. Notably, cells expressing the double non-phosphorylated mutant (T12A and T35A) show accelerated G1/S progression and mitotic exit (Praskova et al. 2008).

Most interestingly, Mob1 localizes not only at the centrosome (spindle pole) but also at the kinetochores, where Plk1 and the CPC are also colocalized (Wilmeth et al. 2010). Moreover, Plk1 is required for Mob1 to localize at the centrosome, but not at the kinetochores, whereas the CPC is required for Mob1 to localize at the kinetochores, but not at the centrosome. Notably, loss of Mob1 leads to a delay in the translocation of the CPC and mitotic kinesin-like protein 2 (Mklp2) to the central spindle/spindle mid-zone during anaphase, thereby preventing recruitment of Mklp1 to the central spindle (Wilmeth et al. 2010). These results suggest that Mob1

regulates the CPC through Lats1/2 activity at the mitotic apparatus (centrosome, kinetochore, and central spindle) during metaphase/anaphase transition. Consistent with this, Lats1/2 colocalizes with Aurora B, a catalytic component of the CPC, at the central spindle and at the chromosome arms, including the kinetochores (Yabuta et al. 2011).

10.5 Rassf1A

In the Hippo pathway, the fruit fly Ras association domain family (Rassf) and its mammalian homolog, Rassf1A, modulate the Hippo and Mst1/2 kinases, respectively. Since human *RASSF1A* is often inactivated in solid tumors, Rassf1A is thought to be a predominant candidate tumor suppressor (Donninger et al. 2007; Avruch et al. 2009). Importantly, mammalian Rassf1A regulates the cell cycle and mitosis (Fig. 10.1).

Rassf1A colocalizes with Mst2, WW45, and Lats1 at the centrosome throughout the cell cycle, and at the midbody during cytokinesis. Like Lats1/2 knockout mice, MEFs from Rassf1A knockout mice (*Rassf1A*$^{-/-}$) show a failure of cytokinesis, which can be rescued by either expression of Mst2, WW45, or Rassf1A itself (Guo et al. 2007). The centrosomal localization of Rassf1A seems to be maintained via its interaction with C19ORF5/MAP1S, which contains two conserved microtubule-associated regions (Dallol et al. 2007). Interestingly, Rassf1A associates with, and is directly phosphorylated at T202 and S203 by, Aurora A during mitosis. Although T202 and S203 are located within the microtubule-binding domain of Rassf1A, it is not clear whether these phosphorylations are involved in its interaction with microtubules (Dallol et al. 2007; Song et al. 2009b). Moreover, Rassf1A interacts with Cdc20, an activator of the anaphase promoting complex/cyclosome (APC/C), thereby inhibiting APC/C-Cdc20 function during early mitosis. Aurora A-mediated phosphorylation of Rassf1A on S203 induces its dissociation from Cdc20 and the subsequent activation of the APC/C-Cdc20 complex, thereby promoting prometaphase progression (Chow et al. 2012). Alternatively, Rassf1A interacts with DNA damage-binding protein 1 (DDB1) and is degraded by the DDB1-CUL4A E3 ligase complex during mitosis (Jiang et al. 2011).

On the other hand, Aurora B also interacts with, and phosphorylates, Rassf1A on S203; however, this occurs at different times and at different subcellular locations during mitosis. S203-phosphorylated Rassf1A colocalizes with Aurora A at the centrosome during early mitosis, whereas it colocalizes with Aurora B at the central spindle/spindle mid-zone and at the midbody during late mitosis (Song et al. 2009b). Aurora B-mediated S203 phosphorylation of Rassf1A seems to regulate cytokinesis through the interaction of Rassf1A with Syntaxin16, a member of the t-SNARE family, at the spindle mid-zone and midbody (Song et al. 2009a).

Taken together, it is likely that Rassf1A is differentially regulated by Aurora A at the centrosome to progress early mitosis, and by Aurora B at the spindle mid-zone and midbody for cytokinesis.

10.6 Other Regulators: Kibra and Ndr1/2

A WW domain-containing protein called Kibra was recently identified as a new upstream regulator within the Hippo pathway (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010). Human Kibra interacts with Lats2 to positively regulate Lats2 protein stability in the Hippo pathway (Xiao et al. 2011a). In human cancer cells such as HeLa and MCF7, Kibra is phosphorylated in a cell cycle-dependent manner, and this phosphorylation is apparently enhanced during mitosis (Xiao et al. 2011b). Notably, Kibra is directly phosphorylated at S539 by the Aurora A and Aurora B kinases and dephosphorylated by PP1. Phosphorylation at S539 is required for the interaction between Kibra and Aurora A, and for its dissociation from neurofibromatosis type 2 (NF2)/Merlin during mitosis, which is involved in nocodazole-induced mitotic arrest and mitotic exit (Xiao et al. 2011b). Taken together, these studies suggest that Kibra may play a vital role as a switch between Merlin-mediated Hippo signaling and Aurora-mediated mitotic signaling.

Ndr1 (nuclear-Dbf2-related 1) and Ndr2 belong to a subfamily that is similar to, but distinct from, the Lats1/2 subfamily within the Dbf2 superfamily. The Ndr kinase subfamily is also evolutionarily conserved from yeast to humans, e.g., Cbk1 and Orb6 in budding yeast, Sax-1 in nematodes, Trc in fruit flies, and Ndr1/2 in mammals, which all play more predominant roles in maintaining cell polarity or during morphogenesis than they do in apoptosis and cell proliferation (Hergovich and Hemmings 2009). Moreover, unlike Lats1/2, Ndr1/2 cannot phosphorylate endogenous Yap/Taz proteins in cells, although they can phosphorylate recombinant Yap protein in vitro (Hao et al. 2008). Thus, Ndr1/2 are not components of the canonical Hippo pathway. Nevertheless, it is interesting that Ndr1/2, like some regulators of the Hippo pathway, are also directly phosphorylated by Mst1/2 and function in some mitotic events, such as centrosomal and mitotic chromosomal integrity, in response to Mst1/2 kinase activity (Fig. 10.2). Specifically, Ndr1 regulates centrosome duplication through Mst1-mediated phosphorylation (Hergovich et al. 2009), whereas Ndr1 regulates mitotic chromosome alignment through Mst2-mediated phosphorylation (Chiba et al. 2009). Similar to the function of Mst1, Ndr1 associates with Aurora B and inhibits its kinase activity, which promotes the destabilization of kinetochore-microtubule attachments (Oh et al. 2010). Moreover, Ndr1/2 directly phosphorylates p21, a cyclin/Cdk inhibitor, downstream of the third Mst kinase, Mst3, which plays an important role in G1/S progression (Cornils et al. 2011a, b).

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