Mammalian sterile 20–like kinase 1 (Mst1) suppresses lymphoma development by promoting faithful chromosome segregation

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Abstract

The mammalian Hippo signaling pathway has been implicated in oncogenesis in the context of solid tumors such as hepatocellular carcinoma. MST1 kinase, the core component of the Hippo signaling pathway, is highly expressed in hematopoietic cells. However, its possible impact on tumorigenesis in this setting is unknown. In this study, we provide evidence that Mst1 loss in the mouse enhances chemically and genetically induced lymphoma development by inducing chromosomal instability. Mst1 deficiency increased susceptibility to T-cell acute lymphoblastic leukemia induced by mutagen exposure. Notably, before transformation Mst1−/− normal thymocytes showed no changes in proliferation or apoptosis in vitro and in vivo, but they displayed elevated levels of abnormal mitotic chromosomes and aneuploidy, conditions known to promote tumorigenesis. Mst1−/− mice also showed accelerated formation of spontaneous lymphomas in a p53 deficient background, accompanied by severe aneuploidy. In clinical specimens of lymphoma and leukemia, we documented frequent downregulation of MST1 expression, consistent with our findings. Taken together, our findings reveal a tumor suppressive function of Mst1 based on its ability to prevent chromosomal instability in lymphocytes.

Introduction

Mst1 (also known as Stk4) is a serine/threonine kinase that belongs to the mammalian-Ste20-kinase family (1). Most previous studies have focused on the apoptosis-related functions of Mst1. During apoptosis, Mst1 is cleaved by activated caspase 3 to yield a catalytically active N-terminal peptide. This cleaved N-terminal kinase domain mediates chromatin condensation by phosphorylating histone H2B. Furthermore, overexpression of either Mst1 or its closest homolog, Mst2, induces apoptosis in many cancer cell lines. The mitogen-activated protein...
kinases (MAPKs), c-Jun N-terminal kinase (JNK) and p38, are known to be downstream effectors of activated Mst1 during apoptosis [reviewed in (2, 3)]. More recently, several reports have revealed other roles of Mst1 that are unrelated to cell death. For example, Mst1 is activated during mitosis and regulates cell-cycle progression via phosphorylation of MOBKL1A and MOBKL1B (4). Interestingly, Mst1 is also involved in regulating intracellular vesicle trafficking of integrin beta 2 (Itbg2/LFA-1) in the context of T cell activation (5).

Genetic screening studies in Drosophila have defined the Hippo signaling pathway, showing that this pathway is an important developmental program that controls proliferation and apoptosis for proper organ development (6, 7). According to the current model, Hippo, the Drosophila homolog of Mst1/2, phosphorylates and thereby activates the Warts kinase (Lats1/2 in mammals) with the help of Salvador (Sav1 in mammals). Warts activity is further enhanced by binding to Mats (Mob1 in mammals), another substrate of Hippo. Activated Warts, in turn, phosphorylates and inactivates the transcriptional co-activator Yorkie (Yap1 in mammals) (8). Importantly, Drosophila mutants for hippo, salvador, or warts show a severe overgrowth phenotype in mutant tissues. By extension, the mammalian Hippo pathway likely represents a new tumor-suppressor pathway (6, 7).

Consistent with this assumption, genetically engineered mouse models of mammalian Hippo pathway components frequently develop spontaneous tumors (9-16). Of note, liver-specific Mst1/2- or Sav1-knockout mice eventually develop liver cancers accompanied by Yap1 upregulation (12-15); and Yap1-overexpressing transgenic mice show enlarged livers and ultimately succumb to liver cancer (16). These observations clearly demonstrate that the mammalian Hippo pathway has a crucial role in preventing tumorigenesis though inactivation of Yap1. However, whether Mst1 has tumor-suppressive functions in organs other than the
liver and intestine, and whether Mst1 can function as a tumor suppressor independently of the canonical Hippo signaling pathway (i.e., inactivation of Yap1) remains to be answered.

Chromosomal instability is a phenomenon in which whole chromosomes are mis-segregated during mitosis. Chromosomal instability, as well as its consequence, aneuploidy, are common characteristics of most cancers (17) and correlate with poor prognosis in some types of cancers (18). Many studies have provided evidence that chromosomal instability participates in tumor initiation and progression (19). In fact, a series of genetic studies in mice with defects in the spindle assembly checkpoint suggested that aneuploidy or chromosomal instability promotes the development of tumors (20-24). Interestingly, MST1 and MST2 have been shown to be crucial for precise alignment of mitotic chromosomes. HeLa cells depleted of MST1, MST2, or its downstream kinase NDR1, show unaligned metaphase chromosomes, which can potentially lead to aneuploidy (25, 26). Intriguingly, Ndr1-knockout mice developed T-cell lymphomas at their old age (27).

Here, for the first time, we demonstrate that Mst1 functions in suppressing tumor development in lymphocytes in vivo. Lymphoma development is accelerated in Mst1-deficient mice upon mutagen treatment or p53 deletion. Furthermore, Mst1-deficient lymphocytes are prone to mis-segregate chromosomes during mitosis and show increased levels of aneuploidy, which likely contributes to tumorigenesis. These data suggest that Mst1 functions as a tumor suppressor in lymphocytes by maintaining genomic integrity during mitosis.

Materials and Methods

Mice

Mst1−/− mice (28) were backcrossed to C57BL/6 mice seven times before being used in
experiments. For N-ethyl-N-nitrosourea (ENU)-induced lymphoma development, 3- to 8-week-old mice were given 100 mg/kg ENU (Sigma) via intraperitoneal injection, and observed for 8 months. Mice observed to be moribund during the course of the study were euthanized and subjected to a complete necropsy. Mst1−/− mice were bred to p53−/− mice (29) to generate cohorts of p53−/−Mst1+/+ and p53−/−Mst1−/− mice. Mice care and experiments were performed in accordance with procedures approved by the Korea Advanced Institute of Science and Technology-Animal Care and Use Committee.

### Flow cytometry

For double-negative (DN) subset analysis, thymocytes were stained with antibodies against the following lineage-specific markers: CD4 (RM4-5), CD8a (53-6.7), TCRβ (H57-597), Gr1 (RB6-8C5), CD11b (3A33), B220 (RA3-6B2), Nk1.1 (PK136), TER119, CD44 (IM7), CD25 (PC61.5), and c-Kit (2B8). All antibodies were purchased from BD Biosciences or eBioscience. Flow cytometric analyses were performed using a FACS Calibur or LSR II (BD Biosciences) system.

### Cell proliferation

For *in vitro* proliferation assays, thymocytes were placed in 96-well plate with RPMI-1640 medium at a density of 2x10⁵ cells per well. Cells were stimulated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 250 ng/ml ionomycin (Sigma) for 72 hours. Cell numbers were determined by measuring absorbance at 450 nm after incubating with WST-1 reagent (Daeil Lab Services). Average absorbance values of triplicate samples were used. For *in vivo* 5-bromo-2′-deoxyuridine (BrdU) incorporation assays, wild-type and Mst1−/− mice were intraperitoneally injected with 50 mg/kg of BrdU. After 1 hour, thymocyte were isolated and immunostained with antibodies against CD4, CD8, and TCRβ to determine thymocyte subsets. Fixation and staining for BrdU detection were performed using the BrdU Flow Kit.
(BD Biosciences) according to the manufacturer’s instructions.

**Histological analyses**

Tissues were fixed in 10% formalin at 4 °C, embedded in paraffin, sectioned (4 μm thickness), and stained with hematoxylin and eosin (H&E).

**Immunofluorescence**

Freshly isolated thymocytes were incubated with RPMI-1640 medium supplemented with 5 ng/ml PMA and 250 ng/ml ionomycin for 60 hours. Cytospin preparations of cultured thymocytes were fixed, permeabilized, and stained with antibodies against phosphorylated histone H3 (Cell Signaling) and α-tubulin (Calbiochem), and then counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Confocal images were obtained using a LSM710 laser-scanning confocal microscope (Carl Zeiss).

**Metaphase chromosome spreads**

Freshly isolated splenocytes were incubated with RPMI-1640 medium containing 5 μg/ml lipopolysaccharide (Sigma) and 5 μg/ml concanavalin A (Sigma) for 48 hours before used in karyotyping. Karyotyping procedures were described previously (21).

**Array comparative genomic hybridization (array CGH)**

Array CGH studies were performed using the SurePrint G3 mouse CGH Microarray 180k Kit (Agilent Technologies), according to the manufacturer’s protocol. See Supplementary Materials and Methods for details.

**In silico analysis of MST1 expression in hematological tumors**

Comparative analyses between normal and tumor cells were performed with the use of publically available microarray data from the Oncomine database (30). Gene expression microarray data of T-cell acute lymphoblastic leukemia (T-ALL) cohort were downloaded from the Gene Expression Omnibus (GEO) database (GSE26713). See Supplementary
Materials and Methods for details.

Results

Mst1 deficiency increases susceptibility to ENU-induced lymphomagenesis.

Mst1 is most abundantly expressed in hematopoietic cells, including lymphocytes, and plays a role in regulating peripheral T cell proliferation and adhesion (31, 32). However, Mst1-/- mice rarely develop spontaneous tumors in lymphoid organs (15). To investigate whether Mst1 plays a role in inhibiting mutagen-induced tumorigenesis in lymphocytes of these mice, we injected wild-type and Mst1-/- mice with the same amount of ENU, which is a highly potent mutagen that induces mostly thymic lymphomas in mice (33). During the 8-month observation, only 5% of wild-type mice died at this dose of ENU, whereas 50% of Mst1-/- mice became moribund with symptoms of cachexia and dyspnea (Fig. 1A, Supplementary Table S1). In most cases, the thymus and spleen were massively enlarged in these moribund Mst1-/- mice (Fig. 1B). Stains of peripheral blood smears revealed the existence of large blast cells in Mst1-/- mice in contrast to the small lymphocytes present in wild-type mice (Fig. 1C). Histological analyses of moribund Mst1-/- mice further revealed that tumor cells had spread to many organs, including the thymus, spleen, bone marrow, liver, kidney, and lung (Fig. 1D). Intriguingly, in some cases, tumor cells were found only in the thymus, suggesting that tumors in Mst1-/- mice could have originated in the thymus and progressed to widespread leukemic stages in some mice. We further analyzed immunophenotypes of tumor cells in various hematopoietic tissues using flow cytometry. Ectopic CD4+/-CD8+ cells were detected in thymus, spleen, bone marrow, and peripheral blood of moribund Mst1-/- mice (Fig. 1E), indicating that the tumor cells belonged to the T-cell lineage and were similar to immature double-positive (DP) T cells. The tumors were mono- or oligo-clonal in origin, and
transplantable to irradiated recipient mice (Supplementary Fig. S1A-C). Taken together, these data indicate that mice injected with ENU develop thymic lymphomas or precursor T-cell lymphomas/leukemias, as has been previously reported, and Mst1−/− mice are more prone to ENU-induced lymphomagenesis.

**Mst1 is not required for T-lineage development.**

To elucidate the mechanism by which Mst1 deficiency accelerates T lymphoma development, we first investigated T lymphocyte development in Mst1−/− mice. T lymphocytes develop in the thymus and progress through double-negative (DN), intermediate-single-positive (ISP), double-positive (DP) and, finally, single-positive (4SP or 8SP) stages. Developmental arrest at certain stages, such as DN3 or DP, has been reported to accelerate lymphoma development in several mouse models (34, 35). Mst1−/− mice showed no significant differences in thymocyte composition except for a mild increase in SP cells, as previously reported (Fig. 2A, B). Increases in CD4 and CD8 SP subsets are thought to be due to a defect in thymic egress (31, 36). A more detailed analysis of DN populations also revealed normal development through DN1 to DN4 stages in these mutant animals (Fig. 2C, D). Furthermore, BrdU pulse-chase experiments revealed that Mst1−/− thymocytes that incorporated BrdU during their DN stages progressed to DP (day 2) and SP (day 5) cells with similar kinetics to wild-type cells (Supplementary Fig. S2A-B). Thus, these data suggest that Mst1 deficiency does not induce overt defects in thymocyte development that might contribute to lymphomagenesis.

**Mst1 deficiency does not significantly affect thymocyte proliferation or apoptosis.**

Given that Mst1 mediates crucial apoptotic functions, we investigated whether Mst1 deficiency affected basal apoptotic levels in thymocytes. However, annexin-V staining revealed no significant reduction in the apoptotic levels of Mst1−/− thymocytes compared to wild-type thymocytes both in freshly isolated and *in vitro* cultured conditions (Fig. 3A,
Supplementary Fig. 2C-G). Of interest, we observed that Mst2 levels were not different between wild-type and Mst1−/− thymocytes (Supplementary Fig. S2H), suggesting that Mst2 did not compensate for Mst1 deficiency during apoptosis. Thus, the loss of Mst1 does not appear to contribute to tumorigenesis by affecting apoptosis in thymocytes.

We next attempted to examine the cell proliferation rate in Mst1−/− thymocytes. To do this, we injected BrdU into wild-type and Mst1−/− mice and examined the percentage of BrdU-positive cells in each thymocyte subset. BrdU incorporation rates in DN, ISP, and DP subsets were similar between the two genotypes (Fig. 3B). Interestingly, CD4SP and CD8SP subsets in Mst1−/− mice showed significantly reduced levels of BrdU incorporation. *In vitro* cultured wild-type and Mst1−/− thymocytes also showed similar degrees of proliferation, as revealed by WST assays and BrdU incorporation (Supplementary Fig. S2I-J). Taken together, these data indicate that it is unlikely that tumor acceleration in Mst1−/− mice was due to decreased apoptosis or increased proliferation of mutant thymocytes.

**Mst1−/− lymphocytes display both chromosomal instability and aneuploidy.**

How might the loss of Mst1 accelerate lymphomagenesis? On the basis of previous studies showing that MST1 promotes precise alignment of mitotic chromosomes (25), we hypothesized that the loss of Mst1 in thymocytes causes chromosomal instability during mitosis and promotes tumorigenesis. To test this hypothesis, we cultured wild-type and Mst1−/− thymocytes *in vitro* and stimulated them with PMA and ionomycin to promote their entry into the cell cycle. After 2 days of culture, cells were fixed and stained with antibodies against α-tubulin and phospho-histone H3, and counterstained with DAPI. We focused on metaphase to telophase cells since aberrantly positioned chromosomes, if present, are most easily recognized during these phases (inspected mitotic cells: n=1182 for WT; n=1616 for Mst1−/−). Notably, abnormal mitotic patterns were observed at a higher rate in Mst1−/− cells.
than in wild-type cells (Fig. 4A-E, Supplementary Fig. S3). The percentage of cells having misaligned metaphase chromosomes, lagging chromosomes, or chromosome bridges was all increased in Mst1−/− cells compared to wild-type cells (Fig. 4B-D). Furthermore, micronuclei were more frequently observed in Mst1−/− cells after cytokinesis (Fig. 4Am-p).

Next, we investigated whether the observed chromosomal instability in Mst1−/− cells could contribute to the formation of aneuploidy in primary lymphocytes. Chromosome spreads from wild-type and Mst1−/− splenocytes showed that the latter contained a higher percentage of aneuploid cells whose chromosome numbers deviated from the normal modal number of 40 (Fig. 4F-H). These results indicate that Mst1−/− lymphocytes showed increased levels of chromosomal instability, which might contribute to accelerated lymphoma formation in ENU-treated Mst1−/− mice.

**Loss of Mst1 accelerates lymphoma development in p53−/− mice.**

The p53 pathway has recently been shown to inhibit proliferation of aneuploid cells in culture (17) and suppress tumor development in aneuploidy-generating mouse models (37). These facts led us to hypothesize that aneuploid cells induced by the loss of Mst1 would be able to proliferate and contribute to lymphomagenesis in the absence of functional p53. To test this hypothesis in vivo, we examined tumor-free survivals of p53−/−Mst1+/+ and p53−/−Mst1−/− mice. As reported in previous study (29), all p53−/−Mst1+/+ mice developed tumors within 8 months of age (Fig. 5A). The tumors that developed in these mice included thymic lymphoma (50%), sarcoma (25%), and one brain tumor (Supplementary Table S2). Notably, p53−/−Mst1−/− mice showed accelerated lymphoma development; of these, 70% developed lymphomas with a reduced latency compared to p53−/−Mst1+/+ mice (113 days vs. 172 days; Fig. 5B). These tumor cells were either CD4−CD8+ or CD4+CD8+ (Fig. 5C).

To further investigate the effects of Mst1 deficiency on chromosomal instability, we
performed chromosome counts on wild-type, Mst1−/−, p53−/−Mst1+/+, and p53−/−Mst1−/− MEFs (Supplementary Table S3). Mst1−/− MEFs showed increased number of aneuploid cells compared to wild-type (35% vs. 22%). Notably, aneuploidy rate became higher in p53−/−Mst1−/− MEFs (49%), whereas p53−/−Mst1+/− MEFs exhibited similar rate of aneuploidy to wild-type.

We next performed array CGH to compare the genomes of thymic lymphoma cells from p53−/−Mst1+/+ and p53−/−Mst1−/− mice. Lymphoma cells from both genotypes showed considerable genomic abnormalities, including whole chromosomal gains and losses in various chromosomes (Fig. 5D). However, we could not observe more gross abnormalities in p53−/−Mst1−/− lymphoma cells than in p53−/−Mst1+/+ lymphoma cells. This is likely due to the oligoclonal nature of p53−/−Mst1−/− lymphomas, since CGH signals from the gain or loss of certain chromosomes in one clone may be hidden by signals from other clones. These data, taken together, suggest that loss of Mst1 significantly promotes lymphoma development in the absence of p53, possibly through generation of chromosomal instability and genomic aberrations.

**Lymphoma acceleration by Mst1-deficient mice is not dependent on Yap1 activation.**

Given that the inactivation of Yap1 by Mst1/2 signaling is a key mechanism for tumor suppression in the liver (38), we sought to examine whether accelerated lymphoma development in Mst1-deficient mice is caused by activation of Yap1. To test this, we examined Yap1 expression according to Mst1 genotypes. Importantly, Yap1 expression in lymphoid tissues (thymus, spleen, and lymph node) from wild-type mice was below the limit of detection by ordinary immunoblotting, whereas large amounts of Yap1 were expressed in most epithelial tissues (lung, liver, and pancreas) (Supplementary Fig. S4A). Yap1 was also barely detectable in lymphoid tissues from Mst1−/− mice (data not shown). Although Yap1 is not expressed in normal lymphoid tissues, it is possible that Yap1 is specifically upregulated...
during tumorigenesis. However, ENU-induced lymphomas from Mst1−/− mice rarely expressed Yap1 (Supplementary Fig. S4B). Specifically, only two out of seven lymphoma samples showed mild Yap1 expression. These data suggest that Yap1 upregulation is not critical for ENU-induced lymphoma development in Mst1−/− mice.

Unlike ENU-induced lymphoma cells, spontaneous lymphomas from p53-null background mice usually exhibit Yap1 upregulation, as determined by immunoblotting and RT-PCR (Supplementary Fig. S4C, D). Both p53−/−Mst1+/+ and p53−/−Mst1−/− lymphomas expressed Yap1 to a similar extent. More importantly, the level of Yap1 Ser127 phosphorylation, which is considered to be mediated by Mst-Lats kinase signaling, was not different between p53−/− Mst1+/+ and p53−/−Mst1−/− samples. These data indicate that (i) Yap1 upregulation is independent of Mst1 deficiency, and (ii) Mst1 is not a crucial upstream regulator of Yap1 Ser127 phosphorylation, at least not in p53−/−Mst1+/+ lymphoma cells.

**MST1 (STK4) expression is reduced in various human lymphoma/leukemia samples.**

Finally, we sought to determine whether our genetic studies in mice have clinical relevance to human hematopoietic malignancies. We thus performed *in silico* screening of the microarray database at the Oncomine website to examine expression levels of MST1 (STK4) in human hematopoietic malignancies. The human disease that most closely corresponds to our mouse lymphoma models is T-ALL, since both originate from immature precursor cells, involve upregulated Notch1, and are spread throughout the body (Fig. 1, 5, Supplementary Fig. S1D-E) (39). Hence, we examined acute lymphoblastic leukemia (ALL) samples for MST1 expression. The median expression values of MST1 in both T-ALL and B-ALL samples were significantly reduced compared to normal in two independent analyses (Fig. 6A, B) (40, 41). Similarly, remarkable down-regulation of MST1 was further confirmed in an independent cohort consisting of 117 T-ALL tissues (Fig. 6C) (42).
We then examined MST1 expression in lymphomas, which are believed to originate from more mature lymphocytes. The expression of MST1 was reduced in multiple mature B-cell lymphomas, including diffuse large B-cell lymphoma and follicular lymphoma (Fig. 6D) (43-45). These results suggest that MST1 insufficiency is likely an important contributing factor in the pathogenesis of precursor lymphoblastic leukemia as well as mature lymphocyte neoplasms in humans.

**Discussion**

Here, using two well-known mouse lymphoma models—ENU-induced lymphoma and p53 knockout-driven lymphoma—we report for the first time that loss of Mst1 accelerates tumor development. Although Mst1 deficiency accelerated tumor development in both settings, it had little, if any, effect on thymocyte proliferation or apoptosis. Instead, the accelerated tumorigenesis was accompanied by increased levels of chromosomal instability and aneuploidy in Mst1−/− lymphocytes.

The rate of spontaneous apoptosis in Mst1−/− thymocytes was comparable to that in wild-type thymocytes. Furthermore, *in vitro* culture with various apoptosis-inducing drugs revealed no differences in the rate of cell death between wild-type and Mst1−/− thymocytes. These results were quite surprising given the demonstrated pro-apoptotic role of Mst1 in many *in vitro* settings. Since we could not detect any increase of Mst2 expression in Mst1−/− cells, it is not likely that Mst2 compensates for Mst1 deficiency in the regulation of thymocyte apoptosis. Moreover, Mst1/2 double-knockout MEFs do not exhibit impaired apoptosis in response to various apoptotic signals (M.J. Kim and D.S. Lim, unpublished data), indicating that Mst1 may not be crucial for mediating apoptotic signaling, at least not in untransformed primary MEFs.
Both ENU-induced lymphoma and p53 knockout-driven lymphoma developed after long latencies (>4 months) and were clonal in origin. These characteristics suggest a requirement for additional genetic mutations in tumor development. In such circumstances, gain of a single genetic mutation that can impair the integrity of genome may accelerate tumor development by increasing the probability of acquiring additional mutations. In line with this argument, we found strong evidence that loss of Mst1 could trigger genomic instability in thymocytes. Mst1−/− thymocytes displayed an increased number of mitotic cells with abnormally positioned chromosomes compared to wild-type cells. Karyotypic analyses also revealed an increased level of aneuploidy in Mst1−/− splenocytes. The elevated level of aneuploidy in Mst1−/− lymphocytes might be caused by hyperactivation of Aurora B kinase, as in HeLa cells (25). However, we failed to detect increase in the activating phosphorylation of Aurora B or phosphorylation of its substrate, histone H3 (Supplementary Fig. S5). It is possible that our experimental methods used in HeLa cells (25) might be unsuitable for the detection of phospho-Aurora B in suspension cells. Or there might be an unrevealed aneuploid-generating mechanism induced by loss of Mst1 in lymphocytes. Recently, MST2-activated NDR1 has been implicated in the regulation of microtubule dynamics and precise alignment of mitotic chromosomes (26). It is also noted that Ndr1-null mice were prone to the development of T-cell lymphoma at their old age (27). Thus, Mst1 and/or Mst2 might relay signals to Ndr1 kinase regulating spindle microtubule dynamics and, consequently, chromosomal instability.

The increase in the percentage of aneuploid cells in Mst1−/− splenocytes was less dramatic than that reported for cells defective in spindle assembly checkpoint (38% in Bub1−/H vs. 15% in Mst1−/−) (23). Because the spindle assembly checkpoint functions normally in Mst1−/− cells (data not shown), some portion of mitotic Mst1−/− cells with misaligned chromosomes could
be corrected during the mitotic checkpoint activation. This could also provide a plausible explanation for the observation that Mst1 single-knockout mice do not develop spontaneous tumors, whereas mice lacking the spindle assembly checkpoint gene do develop various kinds of tumors at an advanced age.

Recently, Avruch et al. suggested that Mst1 and Mst2 function as “gatekeeper” genes in the development of liver cancer (38). Liver-specific Mst1/2 homozygous mutant mice showed marked liver enlargement due to increased proliferation of hepatocytes, and eventually developed liver tumors. Thus, Mst1 and Mst2 satisfy the prerequisites of being gatekeepers in that both directly control cellular proliferation (46). Here, we showed that, unlike the case of the liver, neither cell proliferation nor apoptosis in thymocytes was affected by the loss of Mst1. Instead, our study implies that Mst1 may serve as a “caretaker” (46) to maintain the integrity of the genome during mitosis in lymphocytes. Although it is possible that Mst1 and Mst2 compensate for each other in regulating epithelial cell proliferation and tumorigenesis (10-13), we have found that deletion of both Mst1 and Mst2 specifically in hematopoietic lineages does not induce hyperplasia or spontaneous hematopoietic neoplasms over a 6-month observation period (T.S. Kim and D.S. Lim, unpublished data).

Interestingly, thymic lymphomas in a p53-null background almost always showed significant upregulation of Yap1, regardless of Mst1 genotype. Yap1 upregulation in spontaneous tumors from p53+/Mst1+/+ mice have been previously observed by others using mammary epithelial cell tumors or osteosarcomas (47, 48). Yap1 upregulation in p53−/−Mst1+/+ tumors seems to reflect an acquisition of a selective advantage by the combined p53 loss and Yap1 upregulation through an unknown mechanism. Thus, we conclude that Yap1 activation is unrelated to the accelerated lymphomagenesis caused by the loss of Mst1.

Finally, we provided evidence that the loss of MST1 is relevant to the pathogenesis of
human lymphomas/leukemias. The expression levels of MST1 were significantly reduced in various lymphoma/leukemia patient samples, supporting our notion that the loss of MST1 could contribute to tumorigenesis in hematopoietic lineages. Our results from three independent T-ALL cohorts analyzed in this study showed different degree of MST1 downregulation (Fig. 6A left, dramatic; Fig. 6A right, subtle). This discrepancy may have arisen because the diversity within T-ALLs, which can be classified into at least five subgroups with distinct gene expression pattern (49), was not taken into account.

In conclusion, our study has provided insights into the role of Mst1 in maintaining faithful segregation of chromosomes during mitosis in lymphocytes, and shown that deregulation of Mst1 accelerates lymphoma development in cooperation with p53 deficiency.

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Figure legends

**Figure 1. Mst1<sup>−/−</sup> mice are more prone to developing lymphomas/leukemias upon ENU treatment.**

* A. Tumor-free survival curves for ENU-treated wild-type (n=24) and Mst1<sup>−/−</sup> (n=26) mice. *B*. Representative pictures of wild-type and Mst1<sup>−/−</sup> mice from the analysis in A. *C*. Peripheral blood smears of wild-type and moribund Mst1<sup>−/−</sup> mice. *D*. H&E-stained histopathological sections of various tissues from wild-type and Mst1<sup>−/−</sup> mice. *Inset*: High magnification view; black asterisks indicate infiltrating tumor cells. Scale bars, 200 μm (kidney) and 500 μm (all other organs). *E*. FACS plots of cells from various hematopoietic tissues, showing CD4<sup>+</sup>/CD8<sup>+</sup> blast cells in Mst1<sup>−/−</sup> tissues.
Figure 2. Thymocyte development is relatively normal in Mst1−/− mice.
A. Representative FACS plots showing CD4 and CD8 expression in total thymocytes. Numbers indicate percentages of each subset among total thymocytes. B. Bar graph of analysis in A. Sex-matched, 6-week-old mice were used (WT, n=3; Mst1−/−, n=3). C. Representative FACS plots of DN subsets. DN (lineage− c-Kit+) cells were further divided into five subgroups according to their CD44 and CD25 expression. D. Bar graph of analysis in C. Except for slight decreases in DN1 and DN2 subsets, development through DN stages was normal in Mst1−/− mice. Sex-matched 5-week-old mice were used (WT, n=4; Mst1−/−, n=6). *p-value<0.05; **p-value<0.01.

Figure 3. Mst1 deficiency does not promote tumorigenesis via effects on apoptosis or cell proliferation.
A. Freshly isolated thymocytes were analyzed by flow cytometry for basal apoptosis levels and expression patterns of CD4, CD8, and surface TCRβ. Results are shown in the bar graph (WT, n=5; Mst1−/−, n=5). B. In vivo BrdU incorporation of wild-type and Mst1−/− thymocytes were analyzed using flow cytometry. Results are shown in the bar graph (WT, n=7; Mst1−/−, n=7). *p-value<0.05, **p-value<0.01, ***p-value<0.001.

Figure 4. Mst1 deficiency results in increased chromosomal instability and aneuploidy in lymphocytes.
A. Representative confocal microscopic images of mitotic Mst1−/− thymocytes. a-d, metaphase cell with misaligned chromosomes; e-h, anaphase cell with lagging chromosome; i-l, telophase cell with chromosome bridge; m-p, two daughter cells with micronuclei. Scale bar, 10 μm. B-E. Bar graphs showing percentages of abnormal mitotic cells in wild-type and
Mst1<sup>−/−</sup> thymocytes.  

F. Representative images of metaphase chromosome spreads from wild-type and Mst1<sup>−/−</sup> splenocytes.  

G. Chromosome counting analysis of metaphase spreads showing an increase in aneuploid cells in Mst1<sup>−/−</sup> splenocytes. Similar results were obtained in two independent analyses.  

H. Summary of the results in G.

**Figure 5. Lymphoma development is accelerated by the loss of Mst1 in p53<sup>−/−</sup> mice.**  

A. Tumor-free survival curves for p53<sup>−/−</sup>Mst1<sup>+/+</sup> (n=9) and p53<sup>−/−</sup>Mst1<sup>−/−</sup> mice (n=7) (p-value=0.0001, Log-Rank Test).  

B. Lymphoma-free survival curves for the same cohorts in A (p-value=0.0003).  

C. Representative FACS plots of lymphoma cells from p53<sup>−/−</sup>Mst1<sup>+/+</sup> and p53<sup>−/−</sup>Mst1<sup>−/−</sup> mice compared to wild-type control thymocytes.  

D. Data from array CGH analysis of p53<sup>−/−</sup>Mst1<sup>+/+</sup> and p53<sup>−/−</sup>Mst1<sup>−/−</sup> lymphomas compared to wild-type thymocytes showing numerous whole chromosomal gains and segmental deletions. Results for four representative chromosomes are shown.

**Figure 6. Various human lymphoma/leukemia samples exhibit reduced levels of MST1 (STK4) expression.**

A, B. Relative levels of MST1 expression in T-ALL and B-ALL samples were assessed using cDNA microarray data obtained from the Oncomine database. P-values were less than 1x10<sup>−10</sup> for all analyses.  

C. MST1 expression in an independent T-ALL cohort was evaluated from the microarray data obtained from GEO.  

D. Analyses of MST1 expression in various B-cell lymphomas using the Oncomine database. MST1 expression was reduced significantly in all analyses.  

_Lanes 1, 2, 4, 6, and 11:_ diffuse large B-cell lymphoma;  

_Lanes 3, 7, and 12:_ follicular lymphoma;  

_Lane 5:_ Burkitt’s lymphoma;  

_Lane 8:_ hairy cell leukemia;  

_Lane 9:_ mantle cell lymphoma;  

_Lane 10:_ primary effusion lymphoma. Scale for relative gene expression is
shown in color bars below. *Blue*, reduced expression; *red*, increased expression.
Figure 1.
Figure 2.
Figure 4.
Figure 5.
Figure 6.

A

B

C

D

Various mature B-cell lymphomas

Median Rank p-Value Gene

Gene rank (%) indicator:

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Mammalian sterile 20-like kinase 1 (Mst1) suppresses lymphoma development by promoting faithful chromosome segregation

Tae-Shin Kim, Da-Hye Lee, Sang Kyum Kim, et al.

Cancer Res  Published OnlineFirst August 27, 2012.