# Demystifying MST Family Kinases in Cell Death

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**Abstract:** The MST family of protein kinases plays a critical role in the regulation of cell death in diverse organisms including mammals. The intracellular signaling pathways that regulate MST-driven cell death in mammalian cells are the subject of intense investigation. Stress stimuli including oxidative stress and DNA damaging agents trigger the activity of MST in cells. Although the mechanisms by which oxidative stress and DNA damage trigger MST activation remain to be identified, MST activity can be regulated by caspase-induced cleavage as well as interactions with other proteins in cells. Once activated upon oxidative stress, MST induces cell death *via* phosphorylation and activation of the transcription factor FOXO3 or the histone protein H2B. This review focuses on the currently known upstream activating mechanisms for MST, and explores the downstream signaling pathways that mediate MST's principal function in cell death. Elucidation of MST functions and their regulatory mechanisms in cell death have important implications for our understanding of cellular homeostasis as well as the pathogenesis of diverse diseases.

## INTRODUCTION

The family of Sterile 20-like kinases consists of serine/threonine kinases that are conserved from *Saccharomyces cerevisiae* to *Homo sapiens*. In the budding yeast, Sterile 20 (Ste20) was originally found to link pheromone signaling of the mating pathway to mitogen-activated protein kinase (MAPK) signaling [1, 2]. In this pathway, Ste20 functions as a MAP4K that activates the MAP3K, Ste11 [3]. Since these early studies, our knowledge of signaling by Ste20 and its orthologs has expanded to include diverse biological processes in metazoans ranging from cell survival and death signaling, to the control of organ size, and lifespan regulation.

The founding members of the mammalian Ste20 orthologs, the mammalian Sterile 20-like kinases (MST), were originally identified by two separate approaches. Amplification of a human lymphocyte cDNA library by degenerate oligonucleotide primers corresponding to conserved regions of serine/threonine kinases yielded two highly similar proteins homologous to Ste20, MST1 and MST2 [4]. MST1 and MST2 were also identified in 'in-gel' kinase assays as kinases responsive to stress and were originally named Krs-2 and Krs-1, corresponding to MST-1 and MST-2, respectively [5]. The MST family of kinases now includes MST1 [4, 5], MST2 [4, 5], MST3 [6], and MST4 [7, 8]. Each one of these proteins harbors a N-terminal kinase domain and a C-terminal regulatory domain, rendering them structurally as members of the Class II germinal center kinase family of Sterile 20-like kinases [3, 9]. However, despite this structural similarity, the MSTs exhibit differences in kinase activation. For example, MST1, MST2, and MST4 can be phosphorylated *in vitro* by the addition of ATP and $Mg^{2+}$, while MST3 prefers $Mn^{2+}$ as a divalent cation [6]. Of the MST kinases, MST1 and MST2 share the highest sequence homology (78% identity, 88% similarity) and are the best understood members of the MST family kinases to date.

In this review, we will focus on the principal roles of MST1 and MST2 (herein called MST) and touch upon the roles of their orthologs across species, including Ste20 in *S. cerevisiae* and *Caenorhabditis elegans* Sterile 20-like kinase 1 and -2 (Cst-1 and Cst-2). However, progress in elucidating Ste20 signaling has also been made in *Drosophila melanogaster*, where the Ste20 ortholog Hippo (Hpo) is critical to the regulation of cell death and organ size [10]. Hpo associates with the tumor suppressor proteins Salvador and Warts (Wts) [11-14], and together, their activities culminate in controlling cell growth, survival, and proliferation through phosphorylation-mediated regulation of the transcriptional coactivator Yorkie 1 [15, 16]. Due to the focus of this review on mammalian cell death, please refer to [10] for an up-to-date review of Hpo signaling in *Drosophila*.

In mammalian systems, MST transcripts are ubiquitously expressed and are best known for their function in promoting cell death. However, emerging evidence points to new roles for MSTs that reach far beyond the regulation of cell death. In the immune system, the Rap1-RAPL-MST1 signaling complex facilitates the mobilization of intracellular vesicle pools required for the assembly of the leading edge in lymphocytes [17]. In the nervous system, the neuron specific isoform MST3b plays a key role in neurotrophin-induced axon growth [18]. Overall, the functions of MSTs have been characterized in cell culture models. Therefore, clearly defined roles of MST kinases *in vivo* and their contribution to development and disease remain unclear. To date, knockouts of members of the MST family of kinases have not been reported. Therefore, it will be important in future studies to employ mouse genetic approa-

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ches to determine the *in vivo* roles of distinct MST family members and to elucidate the full range of MST biology.

## MST ACTIVATION AND LOCALIZATION

The mechanisms leading to MST activation and cell death vary depending on stimulus specificity and cell type. Exposure of cells to stress and apoptosis-inducing stimuli such as staurosporine, Fas ligation, and oxidative stress activates MST. Once active, MSTs dimerize *via* the SARAH (Salvador/Rassf/Hippo) domain at the extreme C-terminal region of MST and autophosphorylate on specific threonine residues in the activation loop of subdomain VIII (MST1 T183 and T187; MST2 T180 [19-22]. Autophosphorylation of MST has also been described to occur at Serine 327 *in vitro* [23]; however the biological impact of this event remains to be understood. Upon activation, MST promotes cell death in part through the activation of caspases [24] and other downstream signaling pathways including c-Jun N-terminal kinase (JNK) [24, 25], Histone H2B [26, 27], the transcription factor FOXO3 [28], and LATS-Yes-associated protein (YAP) signaling [29].

Since MSTs are potent regulators of cell death, a major question in the field involves understanding the upstream signaling mechanisms that regulate MST activity. Several distinct modes of MST regulation have been suggested. Early work revealed that caspases cleave MST1 at DEMD$^{326}$S [23, 24, 30] and TMTD$^{349}$G [23], thereby activating MST. However, MST can also be regulated *via* association and/or relocalization by upstream interacting proteins including Nore1 (novel Ras effector 1) [31], RAPL (Regulator of cell adhesion and polarization enriched in lymphoid tissues) (see above) [17], or Raf-1 [32]. Finally, a recent study has reported that Akt negatively regulates MST1 through phosphorylation [33].

Caspases induce MST cleavage in a number of cell types in response to CD95/Fas activation, stress stimuli, or survival factor deprivation [23, 24, 34-36]. In-gel kinase assays initially revealed 36kDa and 41kDa MST1 fragments, which were identified as portions of the N-terminal kinase domain released by caspase cleavage from the non-catalytic C-terminal domain and nuclear export signals [23, 24, 34-36]. Following caspase cleavage, the N-terminal portion of MST translocates to the nucleus and promotes cell death *via* activation of caspases and caspase-activated DNAse [25]. Thus, MST can be both activated by caspases and lead to caspase activation, and in this manner, MST may amplify its own apoptotic potential. In this model, nuclear translocation also facilitates MST autoactivation [34, 37]. Consistent with this idea, cleaved autophosphorylated MST2 is more resistant than full-

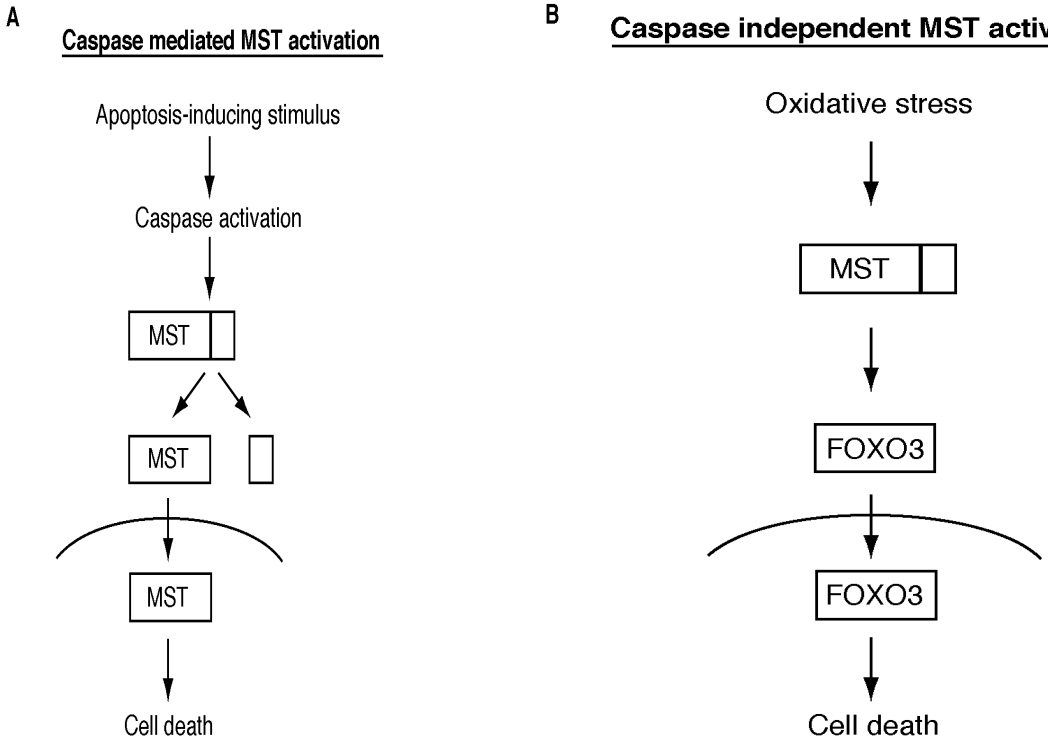


**Fig. (1).** MST can be activated by both caspase-dependent and caspase-independent mechanisms. (**A**) Apoptosis inducing stimuli trigger the activation of caspases, which in turn cleave MST. Caspase cleavage releases the N-terminal kinase domain from the C-terminal regulatory domain. The N-terminal domain then translocates into the nucleus where it promotes cell death [23, 24, 34-36]. (**B**) Oxidative stress promotes MST activation in the absence of caspase cleavage. In this example, oxidative-stress-activated MST interacts with and phosphorylates FOXO3, which translocates into the nucleus and promotes cell death through the transcription of cell death genes, such as *bim* [28]. In this manner, MST propagates the oxidative-stress-induced signal to the nucleus independently of proteolysis.

length MST2 to dephosphorylation by phosphatases [21]. Interestingly, the two MST1 caspase cleavage sites may be targeted by different caspases, raising the possibility of differential regulation of MST *in vivo*. Specifically, MST1 is cleaved at $DEMD^{326}S$ by caspases 3, 6, 7, and 9, while only caspases 6 and 7 cleave MST1 at the $TMTD^{349}G$ site [23]. While the $DEMD^{326}S$ site is conserved both in MST1 and MST2, $TMTD^{349}G$ is not found in MST2, raising the possibility that MST activation may be fine-tuned *via* distinct mechanisms.

While caspase cleavage and nuclear translocation were initially thought to be required for MST activation and cell death, growing evidence suggests that full length MST also promotes cell death independently of proteolysis. In *S. cerevisiae*, hydrogen peroxide induces the translocation of full-length Ste20 to the nucleus, leading to Histone H2B phosphorylation and cell death [27]. In mammalian cells, oxidative stress also activates MST, but MST remains in the cytoplasm [28]. Oxidative-stress-activated MST phosphorylates the transcription factor FOXO3 (see below) leading to the nuclear translocation of FOXO3 and induction of transcription. Thus, the stress-induced MST signal is propagated to the nucleus *via* the transcription factor FOXO3 [28]. Finally, the caspase cleavage site is not conserved in *Drosophila*, where Hpo plays an important role in the regulation of cell death and organ size [12-14, 38]. Thus, several mechanisms of MST activation have evolved in signaling cell death, suggesting that MST plays a pivotal role in cell death (Fig. (**1**)).

The first evidence pointing to protein-protein interactions regulating MST activity came from the identification of the Nore1-MST1 association in a yeast two hybrid screen [31]. As with MST dimerization, heterotypic interactions of MST such as with Nore1 and other members of the closely related RASSF (Ras association domain family protein) family of tumor suppressors are mediated by the SARAH domain [22]. Since Nore1 is a Ras effector protein, the Nore1-MST1 association was proposed to promote Ras-mediated apoptosis [31, 39]. However, co-expression of Nore1 (or the closely related RASSF1) with MST1 decreases MST1 autophosphorylation at S183 and MST1's subsequent activation [37]. A current model suggests that interaction with Nore1 relocalizes MST1 to putative sites of activation. Consistent with this model, membrane targeting of MST1 by N-terminal myristoylation or co-transfection with Nore1CAAX increases MST1 activity and cell death [31, 37].

MST2 was also found to form a physical complex with the Ras effector protein Raf-1 in a biochemical screen of Raf-1 interacting proteins [32]. In this signaling pathway, apoptosis inducing conditions including staurosporine and Fas ligation reduce the interaction of MST2 and Raf-1, and concomitantly increase MST2 activity. In addition, Raf-1 depletion from tumor lines promotes MST2 activation and apoptosis [32]. Consistent with these results, expression of Raf-1 in Raf-1-deficient cells inhibits MST2 dimerization and autophosphorylation [32]. Surprisingly, a Raf-1 mutant lacking full activation (Raf-1 YY340-341FF) retains the ability to regulate MST2 activity. Thus, Raf-1 may regulate MST2 signaling independently of Raf-1's kinase activity, perhaps through the recruitment of phosphatases [32]. Taken together, these findings suggest that MST may be sequestered in an inactive state through interaction with upstream regulatory proteins.

Interestingly, the Raf-1-MST2 interaction was recently shown to be regulated by RASSF1A [29]. In response to Fas ligation, RASSF1A disrupted the association of Raf-1 and MST2, leading to MST-mediated LATS activation and subsequent phosphorylation of YAP1 [29]. Further, MST2-LATS signaling was found to promote YAP1 nuclear translocation, association with p73, and p73-dependent transcription of the pro-apoptotic gene *puma* in MCF7 and HeLa cells [29]. Intriguingly, the *Drosophila* MST ortholog Hpo reportedly triggers the Wts-induced phosphorylation of the YAP ortholog Yorki and thereby promotes Yorki's interaction with 14-3-3 proteins, leading to Yorki's cytoplasmic retention and inactivation [29]. Thus, critical differences in MST signaling mechanisms regulating YAP localization and subsequent transcriptional regulation persist. It will be important in future studies to define the molecular features underlying these biological distinctions, which may arise from subtleties in stimulus specificity, cell type, and choice of target gene.

Finally, MST activity may also be regulated by Akt-induced phosphorylation [33]. Oxidative stress activates MST (see below), and promotes cell death through the phosphorylation and activation of FOXO3 [28]. Building on this model, Jang and colleagues found that Akt interacts with and phosphorylates MST1 at threonine 387 [33]. Akt-mediated phosphorylation of MST1 is associated with both decreased caspase cleavage of MST1 and MST1 activation. Further, Akt inhibition of MST1 activity prevents phosphorylation and nuclear accumulation of FOXO3, leading to cell survival [28, 33].

## DOWNSTREAM OF MST

MST activates a number of signaling pathways that promote cell death, including caspases, JNK [20, 24, 25, 40], Histone H2B [26, 27], the transcription factor FOXO3 [28], and LATS (see above) [29]. As stress activated kinases with a broad range of substrates, JNK and p38MAPK were considered good candidates for amplifying MST-induced apoptotic signals in cells [24, 25]. Of these two kinases, JNK has emerged as an important mediator of MST-induced cell death [20, 25, 34]. While it remains unclear how MST activates the JNK pathway, expression of dominant negative JNK inhibits MST1-induced caspase activation, death-associated cell morphological changes, and cell death [25]. Further, MST1-mediated apoptosis is blocked in MKK 4/7 double knockout embryonic stem cells lacking functional JNK signaling [40]. Surprisingly, while JNK activation may be required for MST-mediated caspase-activated apoptosis in stem cells and cell lines, caspase inhibitors do not always rescue the morphological

changes typically associated with apoptosis, such as membrane blebbing and chromatin condensation [25, 40]. Thus, JNK signaling may also play an important role in MST-activated cell shape changes that typically go hand-in-hand with cell death.

In contrast to the evidence from mammalian systems suggesting that MST activates JNK, the *Drosophila* JNK ortholog Basket is not required for Hpo signaling and Hpo-mediated cell death [11]. In mammalian cells, MST-induced phosphorylation and consequent activation of FOXO3 occurs in a JNK-independent manner [28]. Therefore, while the role of MST in mediating cell death is evolutionarily conserved, the exact mechanisms that control cell death downstream of MST appear to have diverged across evolution. These findings imply that multiple mechanisms of MST regulation operate in order to sustain such a tight conservation of biological function.

In response to cytotoxic agents such as UV irradiation and etoposide in mammalian cell lines and during *Xenopus laevis* tail resorbtion, MST1 phosphorylates Histone H2B serine14 (S14) [26]. Interestingly, Histone H2B phosphorylation also occurs in *S. cerevisiae*. However, the phosphorylation event in yeast takes place on serine 10, a residue distinct from mammalian Histone H2B S14 [27]. Although the modification of histones provides a means for regulating transcriptional changes on a global level, neither phosphorylation event is evolutionarily conserved. Thus, additional mechanisms coupling MST1 to the regulation of transcription have evolved.

Recent studies have led to the identification of the FOXO transcription factors as important targets of MST [28]. In both primary mammalian neurons and cell lines, oxidative stress activates MST, which in turn catalyzes the phosphorylation of FOXO3 at four conserved serine residues within the Forkhead domain of FOXO. These residues: S207, S213, S229, and S230, are conserved among FOXO family members and across species [28]. Under basal conditions, FOXOs are typically sequestered in the cytoplasm through interaction with the 14-3-3 family proteins [41]. However in response to MST-induced phosphorylation of FOXO3 at S207, the FOXO-14-3-3 interaction is disrupted and FOXO3 translocates to the nucleus [28]. Oxidative stress both induces the transcriptional activity of pro-death genes, such as *bim*, and promotes cell death in an MST- and FOXO-dependent manner [28]. A series of loss-of-function and gain-of-function experiments using RNAi further demonstrated that MST-induced phosphorylation of FOXO3 S207 drives MST-induced neuronal death in response to oxidative stress [28].

Since both MSTs and FOXOs are evolutionarily conserved, elucidation of the MST-FOXO signaling pathway in mammalian cells introduces a number of interesting questions regarding the role of this pathway in other systems. In light of these findings, the existence of MST-FOXO signaling was further tested in the model organism *C. elegans*. These nematodes possess two closely related genes to *mst*, *cst-1 and cst-2* (*C. elegans* Ste20-like kinases 1 and 2). The *C. elegans* FOXO family member DAF-16 is fundamental to the regulation of lifespan [42]. Consistent with the model that MST-FOXO signaling is evolutionarily conserved, MST phosphorylates DAF-16 serine 196, which corresponds to FOXO3 S207 [28]. Since DAF-16 regulates lifespan rather than cell death, the impact of CST-DAF-16 signaling on *C. elegans* lifespan regulation was investigated [28]. In these experiments, CST knockdown was found to decrease nematode lifespan and to correlate positively with the advanced appearance of markers of aging [28]. In support of these findings, overexpression of CST also extends nematode lifespan in a DAF-16-dependent manner. Taken together, these findings demonstrate that MST is a central regulator of a number of biological processes spanning from single cells to intact organisms.

The characterization of the MST-FOXO signaling pathway raises several fundamental questions for future investigations. First, it will be important to identify the suite of MST-regulated FOXO target genes as this will shed light on the molecular mechanisms of how MST-FOXO signaling influences diverse biological conditions such as cellular homeostasis and longevity. In addition, in mammalian systems mouse genetics approaches are required to characterize the role of MST-FOXO signaling *in vivo*. Finally, since oxidative stress is associated with diverse diseases ranging from diabetes mellitus to neurodegenerative disease, an invaluable and new direction will be to investigate the role of MST-FOXO signaling in the pathogenesis of these diseases.

## CONCLUSIONS

Although progress in elucidating MST signaling pathways has been made (Fig. (**2**)), much remains to be done for a deeper understanding of MST signaling in the regulation of cell death. While several stimuli trigger MST activity in cells, the specific signaling mechanisms by which a distinct stimulus such as oxidative stress stimulates MST remain unclear. In regard to the characterized mechanisms of MST activation, the distinguishing features leading to either caspase cleavage or full-length MST activation remain unclear. Caspase-mediated cleavage suggests a “point of no return” for MST activity in the nucleus, meaning that cell death invariably ensues. In contrast, reversible activation of MST signaling such as by phosphorylation allows for dynamic regulation of MST signaling. Interestingly, MST-induced phosphorylation of FOXO3, which shuttles between the cytoplasm and nucleus, may take place in the cytoplasm or the nucleus. Thus, FOXO3 shuttling endows MST with the luxury to influence transcriptional responses even when the intracellular signals do not trigger caspase-mediated MST cleavage.

An important question for future studies is the role of protein phosphatases in the regulation of MST signaling. The observation that Raf-1 may prevent MST activation in part by recruiting protein phosphatases and thereby dephosphorylating MST raises the possibility that inhibition of protein phosphatases might be a ge-

neral reversible mechanism of MST activation [32]. Consistent with this model, inhibition of phosphatases by okadaic acid or calyulin A treatment promotes MST autophosphorylation [32, 36, 37, 43]. Interestingly, since MST is activated by oxidative stress, the reversible modification of protein phosphatase enzymatic activities by endogenous redox signaling might contribute to MST activation [44]. An analogous mode of regulation has been reported in the context of JNK signaling where TNF$\alpha$-induced oxidative stress triggers oxidation of MAPK phosphatases and thus inactivates these phosphatases leading to sustained JNK activity and cell death [45, 46].

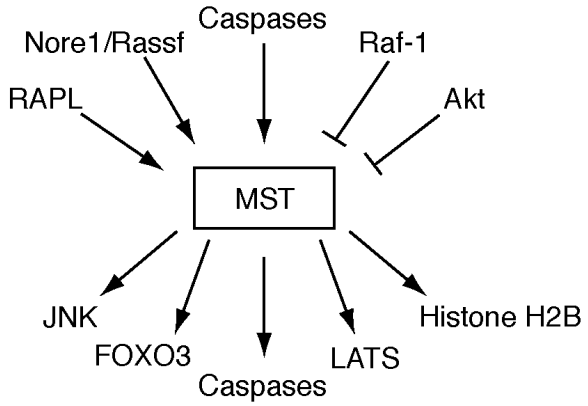


**Fig. (2).** A growing number of upstream signaling pathways regulate MST activity and direct MST-induced downstream signaling. MST can be activated through interactions with upstream regulatory proteins such as RAPL [17] and Nore1/Rassf [31, 37] that relocalize MST to sites of activation. In addition, MST can be activated by caspase cleavage [23, 24, 34-36]. Both Raf-1 and Akt negatively regulate MST; Raf-1 sequesters MST2 [32] and Akt mediated phosphorylation of MST1 T387 inhibits MST1 activation [33]. Depending on the stimulus and cell type, MST activates several downstream signaling pathways including the reciprocal activation of caspases [24], JNK [24, 25], FOXO3 [28], LATS [16], and Histone H2B [26, 27] in order to promote cell death.

Finally, a role for MST signaling in disease pathogenesis will be important to consider. The MST genes are methylated and downregulated in soft tissue sarcomas [47]. In line with these findings, MST may function as a tumor suppressor in human colorectal cancer [48]. MST also promotes cardiac myocyte apoptosis following ischemia/reperfusion [49, 50]. Finally, elucidation of MST signaling as an oxidative stress-responsive mechanism in neurons suggests that MST signaling may provide a mechanistic basis for how oxidative stress contributes to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease.

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## ABBREVIATIONS

CST = *Caenorhabditis elegans* Sterile 20-like kinase

FOXO = Forkhead transcription factor

Hpo = Hippo

JNK = c-Jun N-terminal kinase

MAPK = Mitogen-activated protein kinase

MKK = Mitogen-activated protein kinase kinase

MST = Mammalian Sterile 20-like kinase

Nore1 = Novel Ras effector 1

RAPL = Regulator of cell adhesion and polarization enriched in lymphoid tissues

RASSF = Ras association domain family protein

SARAH = Salvador/Rassf/Hippo domain

Ste20 = Sterile 20

UV = Ultraviolet

Wts = Warts

YAP = Yes-associated protein

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