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MST1/MST2 Protein Kinases: Regulation and Physiologic Roles

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Abstract

The MST1 and MST2 protein kinases comprise the GCK-II subfamily of protein kinases. In addition to their amino-terminal kinase catalytic domain, related to that of the *Saccharomyces cerevisiae* protein kinase Ste20, their most characteristic feature is the presence near the carboxy terminus of a unique helical structure called a SARA domain; this segment allows MST1/MST2 to homodimerize and to heterodimerize with the other polypeptides that contain SARA domains, the noncatalytic polypeptides RASSF1–6 and Sav1/WW45. Early studies emphasized the potent ability of MST1/MST2 to induce apoptosis upon being overexpressed, as well as the conversion of the endogenous MST1/MST2 polypeptides to constitutively active, caspase-cleaved catalytic fragments during apoptosis initiated by any stimulus. Later, the cleaved, constitutively active form of MST1 was identified in nonapoptotic, quiescent adult hepatocytes as well as in cells undergoing terminal differentiation, where its presence is necessary to maintain those cellular states. The physiologic regulation of full length MST1/MST2 is controlled by the availability of its noncatalytic SARA domain partners. Interaction with Sav1/WW45 recruits MST1/MST2 into a tumor suppressor pathway, wherein it phosphorylates and activates the Sav1-bound protein kinases Lats1/Lats2, potent inhibitors of the Yap1 and TAZ oncogenic transcriptional regulators. A constitutive interaction with the Rap1-GTP binding protein RASSF5B (Nore1B/RAPL) in T cells recruits MST1 (especially) and MST2 as an effector of Rap1's control of T cell adhesion and migration, a program crucial to immune surveillance and response; loss of function mutation in human MST1 results in profound immunodeficiency. MST1 and MST2 are also regulated by other protein kinases, positively by TAO1 and negatively by Par1, SIK2/3, Akt, and cRaf1. The growing list of candidate MST1/MST2 substrates suggests that the full range of MST1/MST2's physiologic programs and contributions to pathophysiology remains to be elucidated.

Graphical Abstract

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Notes

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MST1(STK4/KRS2/YSK3) and MST2(STK3/KRS1) are closely related protein (ser/thr) kinases, initially identified in a search for protein kinases with catalytic domains related to that of the *Saccharomyces cerevisiae* kinase Ste20 (hence mammalian Ste20-related).¹ Similar efforts uncovered numerous kinases with Ste20-related catalytic domains, situated either very near the polypeptide carboxy terminus, i.e., the P21 activated kinase (PAK)-related subfamilies 1–6, or near the polypeptide amino terminus, i.e., the germinal center kinase (GCK)-related subfamilies 1–8.² Each of these kinase subfamilies is distinguished by its structurally distinct noncatalytic segments; the *Drosophila melanogaster* and *Caenorhabditis elegans* genomes usually encode a single representative. In early studies, overexpression of MST1 and MST2, which comprise the GCK-2 subfamily,² was accompanied by activation of the stress-related MAPKs, Jnk and p38, suggesting that, like some other Ste20-related kinases, MST1/MST2 served as MAP4Ks.³ To date, however, evidence that MST1 and MST2 interact directly and promote the activation of specific MAP3Ks is lacking, nor has MST1/MST2 deficiency been associated with defective MAPK activation. MST1 or MST2 overexpression was also found to consistently induce apoptosis; moreover, apoptosis activated the endogenous MST1/MST2 kinases,^{4–9} responses suggestive of a physiologic role for these kinases in effecting apoptosis. The discovery of “Hippo”,^{10–14} the *Drosophila* ortholog of MST1/MST2, provided a distinctive identity for these kinases. Hippo is the core upstream element of a tumor suppressor kinase cascade whose sole function in the control of cell proliferation and survival in the fly is to phosphorylate and negatively regulate the transcriptional coactivator yorkie;¹⁵ inactivation of Hippo or its downstream substrate, the yorkie kinase Warts/Lats, allows yorkie to enter the nucleus and bind the TEAD family protein Scalloped. The yorkie/Scalloped complex drives a transcriptional program that promotes cell proliferation and suppresses developmental apoptosis.^{16,17} Mammalian MST2, but not MST1, can complement Hippo loss of function.¹⁰

Unexpectedly and in contrast to the *Drosophila* loss-of-function phenotypes, biallelic LOF mutations in human MST1 cause a complex mixed immunodeficiency syndrome,^{18–22} the phenotype also seen in mice lacking MST1;^{23–26} moreover, the major effects of MST1/MST2 deficiency in the mouse lymphoid system do not involve disinhibition of the yorkie homologue Yap.²³ Mice lacking MST2 alone have normal lymphoid function²⁷ and no obvious phenotype, but global deficiency of both MST1 and MST2 gives embryonic lethality.^{27,28} Hippo-like phenotypes emerge when dual inactivation of MST1/MST2 is effected in a tissue specific manner, but only in some tissues (e.g., liver²⁷ and intestinal mucosa²⁹ but not in skin³⁰); the variable requirement for MST1/MST2 for Yap inhibition probably reflects the ability of other mammalian GCK family kinases to serve as the core upstream element in the “Hippo” kinase cascade.³¹ In addition, the transcriptional outputs

directed by Yap (and, to a lesser extent, TAZ) are also regulated by inputs (e.g., angiotensin or NF2) that are parallel to or bypass entirely the Hippo/MAP4K input. Excellent recent reviews of the varied regulation of Yap and TAZ, i.e., mammalian Hippo pathway, are available.^{32–34} Here we focus on MST1 and MST2, their structure, scaffolds, mechanisms of regulation, and physiologic substrates known thus far.

STRUCTURE

The human MST1 and MST2 polypeptides consist of 487 and 491 amino acids, respectively, sharing >95% identical catalytic domains and being ~75% identical overall. The next most similar segment and most distinctive feature of these kinases is their “SARAH” domain, a unique coiled-coil structure situated near the polypeptide carboxy terminus, named for the three gene (families) that bear homologous structures: the *Drosophila* scaffold protein Salvador^{35,36} and its mammalian ortholog Sav1/WW45, the RASSF 1–6 polypeptides, and Hippo/MST1/MST2³⁷ (Figure 1). The SARAH domains are ~50 amino acids in length and mediate MST1/MST2 homo- and heterodimerization.³⁸ Whereas the MST1 SARAH monomer is relatively unstable, homodimerization creates a highly stable pair of long antiparallel helices that share a strongly hydrophobic interface with a low nanomolar K_D ; each long helix has a short 3_{10} helix at its amino-terminal end that is kinked back toward the opposite long helix.³⁹ The segment linking the catalytic domain to the SARAH domain is unstructured;³⁹ however, deletion of this domain (e.g., MST1[331–394]) increases MST1 kinase activity after transient expression ~9-fold compared with that of wild-type MST1, as judged by the phosphorylation of myelin basic protein, a nonphysiologic substrate.⁴⁰ The notable features of this central region include a caspase 3 cleavage motif (DEMD₃₂₆ for MST1 and DELD₃₂₂ for MST2) situated just beyond the catalytic domain, a more distal caspase 6/7 cleavage motif (TMTD₃₄₉) in MST1 only, and several phosphorylation sites, discussed below.

REGULATION OF CATALYTIC ACTIVITY

Extracted after transient expression, MST1 or MST2 is recovered as a homodimer, and incubation in vitro with Mg and ATP results in activation of their catalytic activity toward exogenous substrates.⁴¹ Activation requires phosphorylation within the “activation” loop at MST1[Thr183]/MST2-[Thr180], which occurs in trans within the MST1/MST2 dimer; conversion of these activation loop Thr residues to Ala largely eliminates activity toward exogenous substrates, although a slight ability to mediate transautophosphorylation remains. Mutation of the critical lysine within the ATP binding site (K59 for MST1 and K56 for MST2) or the catalytic aspartate (D149 for MST1 and D146 for MST2) abolishes catalytic activity and creates dominant inhibitors. Deletion or mutation of the MST1/MST2 SARAH domain yields a polypeptide with greatly reduced kinase activity in vitro in the presence of Mg and ATP, due to loss of homodimerization and conversion of activation loop phosphorylation from a zero-order reaction to a first-order reaction;⁴¹ thus, efficient activation loop phosphorylation, either in trans within the dimer or by an upstream kinase, is critical for MST1/MST2 kinase activity. The transiently expressed recombinant MST1 homodimer exhibits approximately 2–3% of its maximal activity upon being extracted, indicating that endogenous protein (ser/thr) phosphatase activity is sufficient to maintain the

MST1 homodimer in a largely dephosphorylated state. Incubation of cells with protein (ser/thr) phosphatase inhibitors such as okadaic acid causes a time-dependent activation of endogenous MST1/MST2;⁴¹ the extent to which this reflects autophosphorylation or an upstream kinase is not known. The MAP4K and group 8 GCK, TAO, has been shown to be capable of phosphorylating and activating Hippo as well as mammalian MST2.^{42,43} Conversely, the *Drosophila* kinase Par-1,^{44,45} a microtubule binding, Snf1/AMPK family kinase,⁴⁶ associates with Salvador and Hippo and catalyzes an inhibitory phosphorylation at Hippo[Ser30]; the mammalian homologues MARK1 and MARK4 can also induce a mobility shift in MST2.⁴⁴ TAO1 is known to phosphorylate and activate Par-1,⁴⁶ suggesting that complex regulation upstream of MST1/MST2 remains to be elucidated. In addition to MST1/MST2 homodimers, these kinases can also heterodimerize via their SARAH domains, and the K_d for the heterodimer is ~6-fold lower than for an MST2 homodimer. Heterodimer formation is promoted by Ras-GTP in an Erk-dependent manner, and the MST1/MST2 heterodimer exhibits kinase activity weaker than that of either homodimer. The ability of Ras-GTP to stimulate the formation of foci and growth in soft agar of MST1/MST2 DKO MEFs is strongly suppressed by the presence of either MST1 or MST2, but less so if both are present, suggesting that Ras-GTP may retard the antiproliferative effects of MST1/MST2 by promoting their heterodimerization.⁴⁷

Each of the noncatalytic SARAH domain-containing polypeptides, i.e., SAV1/WW45 and RASSF1–6, is capable of heterodimerizing with MST1 and MST2³⁸ to alter their regulation; for example, addition of an equimolar amount of Nore1/RASSF5 to MST1 in vitro results in the displacement of all MST1 from homodimers into RASSF5/MST1 heterodimers,^{48,49} and the MST1/RASSF5 heterodimer, like the monomeric variants of MST1, exhibits little or no kinase activity in vitro in the presence of Mg and ATP.⁴¹ Once MST1 is activated, the binding of RASSF5 does not suppress MST1 activity.⁵⁰ Thus, the mode of MST1/MST2 regulation will be specified by the composition and architecture of MST1/MST2 complexes, and this will be determined by the abundance of the various noncatalytic SARAH domain-containing polypeptides relative to MST1/MST2 and to each other and their relative affinity for the MST1/MST2 SARAH domains compared to that of MST1/MST2 homo- and heterodimers. In general, the relative affinities and tissue specific abundance of the SARAH domain partners in vivo have not as yet been determined. In addition, most cancer-derived cell lines exhibit very low levels of expression of the RASSF polypeptides,^{51–53} so that the regulation of endogenous and especially overexpressed MST1/MST2 in such lines should be considered provisional, or at best specific to that cellular context until confirmed in in vivo settings.

REGULATION BY RASSF PROTEINS

There are six genes encoding RASSF polypeptides, each of whose carboxy-terminal ~220 amino acids consists of a Ras-Rap association (RA) domain followed immediately by a SARAH domain.^{51–53} Co-crystals of Nore1/RASSF5 with Ras-GTP demonstrate that the Nore1 RA domain is amino-terminally extended as compared with the Ras binding domain of cRaf1;⁵⁴ a structural characterization of the interaction of other RASSF proteins with Ras-like GTPases is not available. The SARAH domains of RASSF1–6 are each able to heterodimerize with MST1/MST2;³⁸ the proteins called RASSF7–10 contain an RA domain,

but no SARAH domain, and do not interact with MST1/MST2.⁵⁵ Among RASSF1–6, the founding member Nore1/RASSF5 and RASSF1 have been studied in the most depth. The relationship between MST1/MST2 and its RASSF partners is best characterized in murine T cells, wherein MST1 is much more abundant than MST2 and the shorter Nore1B/RASSF5B isoform, also called RAPL,^{56,57} is the predominant noncatalytic SARAH domain-containing polypeptide. There MST1 is found in a 1:1 complex with Nore1B/RAPL,^{23,58} and in unstimulated T cells, MST1 is inactive, as judged by the phosphorylation state of an endogenous substrate Mob1.²³ The Nore1/RASSF5 RA domain binds with high affinity to Ras-GTP and with lower affinity to the GTP-charged forms of other Ras-like GTPases, including Rap1 GTP.^{59,60} In the T cell, stimulation of the T cell receptor or certain chemokine receptors causes rapid activation of MST1 that is dependent on both RAPL and Rap1-GTP; the RAPL/MST1 complex binds Rap1-GTP and is recruited to the “immune synapse” at the cell surface where MST1 undergoes activation.^{23,58} Whether this occurs because of the ability of clustered RAPL/MST1 heterodimers to mediate transphosphorylation or through an upstream activating kinase is not known. Elimination of either RAPL or MST1 has similar effects on T cell behavior^{23,24,56–58} in fact, elimination of MST1 reduces RAPL polypeptide (but not mRNA) levels.²³ This provides a model for the dual nature of the RASSF polypeptides in MST1/MST2 regulation; Nore1B/RASSF5B maintains the kinase in an inactive state but is indispensable for MST1 activation by upstream signals (here an activated Ras-like GTPase). This scenario is broadly analogous to the regulation of the cAMP-dependent protein kinase, where an upstream signal (cAMP) binds to the regulatory subunits (R) promoting the disinhibition of the catalytic subunits. Considerable evidence indicates that Nore1A/RASSF5A functions as a tumor suppressor;^{52,53} however, in contrast to Nore1B/RASSF5B/RAPL, the physiological regulation of Nore1A/RASSF5A and the contribution of MST1/MST2 to its tumor suppressor effector mechanisms are as yet unclear.⁶¹

The other RASSF polypeptide thus far studied in detail in relationship to MST1/MST2 is RASSF1.^{51,62} Expressed as two major isoforms, the longer RASSF1A and the shorter RASSF1C, like Nore1A/B, differ in their amino-terminal segments but share identical carboxy termini encompassing the RA and SARAH domains. RASSF1A is a tumor suppressor of modest potency^{63,64} whose expression is epigenetically inactivated at an especially high frequency in many human cancers, whereas expression of RASSF1C is usually unaffected.^{51,62} As with Nore1A/Nore1B, both RASSF1A and RASSF1C dimerize with MST1/MST2; coexpression of RASSF1A or 1C in excess of MST1 suppresses MST1 activity,⁴¹ suggesting that, as with Nore1B/RASSF5b/RAPL-MST1, the inactive RASSF1A/MST1 or RASSF1A/MST2 complex awaits an upstream activating input. Nevertheless, the nature of the putative upstream activating input to RASSF1A/C in MST1/MST2 regulation is uncertain. In *Drosophila*, genetic and biochemical evidence indicates that the single dRASSF polypeptide is inhibitory to the Hippo kinase;⁶⁵ however, conditions under which dRASSF mediates an activating input have not been identified. In mammalian systems, several reports identify endogenous Ki-Ras-GTP as an activating input.^{66,67} However, when compared in parallel with RASSF5/Nore1A for their ability to bind Ras-GTP, either in vitro⁵⁹ or with cooverexpression,⁶⁰ RASSF1A exhibits very much weaker binding. Moreover, RASSF1A and RASSF5A can heterodimerize through their nonhomologous

amino-terminal segments, and the level of binding of recombinant RASSF1A to coexpressed Ras-GTP is increased by concurrent expression of full length Nore1A and abolished by expression of Nore1A that has had its RA domain deleted.⁶⁰ Thus, the slight binding of RASSF1A to Ras-GTP may reflect the dimerization of RASSF1A with Nore1A. Nevertheless, it remains feasible that Ki-Ras-GTP, in a tissue specific manner, perhaps assisted by Nore1A or as yet unidentified interacting polypeptides, can effectively recruit an endogenous RASSF1A/MST1-2 complex.⁶⁸ TNF family receptors have also been reported to recruit RASSF1A in a ligand-stimulated manner and thereby activate the RASSF1A/MST1 complex;^{69–71} however, recruitment and activation are usually delayed for some hours after ligand addition, suggesting some indirect mechanism. RASSF1A overexpression itself has been observed to cause MST1/MST2 activation,^{72,73} and one report proposes that activation occurs through the ability of RASSF1A to disrupt an inhibitory interaction between MST2 and cRaf1⁷³ (see below). Thus, the regulation of RASSF1A-MST1/MST2 complexes and the extent to which MST1/MST2 contributes to the tumor suppressor function of RASSF1A require further investigation.

SALVADOR-SAV1-WW45

Genetic screens in *Drosophila* identified Salvador as a necessary component of the Hippo pathway,^{35,36} and biochemical studies show that this is accomplished through the ability of Salvador to bind both Hippo (via their respective SARAH domains) and the Hippo substrate and Yap kinase Warts/Lats (through the interaction of Warts PPXY sequences with the Salvador's tandem WW domains).^{10–14} These features are preserved in the interactions among MST2, WW45, and Lats1. In mouse liver⁷⁴ or intestine,⁷⁵ the Yap-dependent proliferative phenotypes of WW45 deficiency resemble those caused by combined MST1/MST2 deficiency^{27,29} but are much less pronounced, suggesting that some level of MST1/MST2-catalyzed phosphorylation and activation of Lats1/Lats2 persists in the absence of WW45. In mouse embryonic keratinocytes undergoing growth arrest and differentiation in response to the addition of millimolar Ca²⁺ concentrations, a complex of MST1/MST2, WW45, and Lats1/Lats2 assembles, concomitant with an increase in the level of MST1[Thr183]/MST2[Thr180] phosphorylation; keratinocytes lacking WW45 fail to activate MST1/MST2.⁷⁶ Although WW45 appears to be required for MST1/MST2 activation in this cellular context, it is not known whether this involves a WW45-stimulated MST1/MST2 autophosphorylation or the recruitment of an upstream kinase such as TAO1. The AMPK/SNF1-related Par-1 kinase, which phosphorylates and inactivates Hippo/MST2, does bind to Salvador;^{44,45} moreover, the AMPK/SNF1-related kinases Sik2 and Sik3 have also been identified as negative regulators of the Hippo-Warts/Lats interaction that act by phosphorylating Salvador.⁷⁷ How these kinase loops are regulated and play out in various cellular contexts remains to be uncovered.

ACTIVATION DURING APOPTOSIS AND IN NORMAL LIVER

Activation of MST1 and MST2 occurs uniformly in cells undergoing apoptosis, as judged by the marked increase in the level of phosphorylation at MST1 and MST2 activation loop sites Thr183 and Thr180, respectively.⁴¹ In addition, concomitant with the apoptotic activation of caspase 3, a substantial portion of both MST1 and MST2 undergoes cleavage after Asp326

and Asp322, respectively (DEM/LD_{326/322}).^{4–9} This removes the autoinhibitory and SARAH domains, freeing MST1/MST2 of all regulatory inputs apart from phosphatase-catalyzed inactivation. Caspase 3 cleavage also removes the nuclear export signals,^{9,78} giving this 35–40 kDa catalytic fragment unrestricted access to the nucleus, in contrast to full length MST1/MST2, which resides almost entirely in the cytoplasm.⁷⁸ Given the potent proapoptotic capacity of recombinant full length MST1/MST2, these active catalytic fragments seem to be likely to contribute to the progression of apoptosis and have been implicated, e.g., in the apoptotic phosphorylation of histone H2B[Ser14].⁷⁹

In view of the evidence pointing to active MST1/MST2 as an apoptotic effector, the finding that a substantial fraction of MST1 and some MST2 in normal mouse liver are present as catalytically active 35–40 kDa fragments indistinguishable in size from those observed in cells undergoing apoptosis was completely unexpected.²⁷ No such fragment is seen in mouse T cells where MST1 is most abundant and is present entirely as a full length polypeptide, both before and after activation.²³ With regard to the mechanism of MST1 activation in normal liver, the very limited ability of recombinant MST1[1–326] to catalyze autophosphorylation *in vitro*⁴¹ suggests that activation occurs prior to caspase 3 cleavage; in addition, the ability of the 35 kDa MST1 fragment to maintain Thr183 phosphorylation once the fragment is cleaved suggests either protection from phosphatase action, ongoing phosphorylation by an upstream kinase, or both. However, the identity of the proteins that interact with the full length, as yet unactivated MST1 in liver and whether TAO1 kinase 1 or another kinase participates in the regulation of MST1/MST2 are not known. With regard to the mechanism of MST1/MST2 cleavage, apart from the presence of the caspase 3 recognition motifs, evidence directly implicating caspase 3 is lacking. Nevertheless, MST1/MST2 can be linked to caspase 3 in HeLa cells using *in situ* proximity ligation,⁸⁰ and nonapoptotic functions of caspase 3 are known;^{81–83} e.g., myoblast differentiation requires the caspase 3 cleavage of several substrates, including MST1.^{84,85} Possible roles for the constitutively active MST1/MST2 catalytic fragment include maintenance of replicative quiescence and/or maintenance of the differentiated state. Hepatocyte renewal normally results from the division of differentiated adult hepatocytes, which occurs at random approximately once per year;⁸⁶ within 3 days of inactivation of hepatic MST2 on an MST1 null background, all adult hepatocytes enter the cell cycle.²⁷ Intestinal epithelial cells, which arise from a stem cell compartment and turn over daily, also contain the active MST1/2 fragment; there dual inactivations of MST1 and MST2 expand the stem cell compartment and inhibit the progression of enterocytes into differentiated forms.²⁹ Thus, as in skeletal muscle, a cleaved MST1/MST2 may serve primarily to enforce the differentiated state.^{87,88}

INHIBITION BY AKT

Cross regulation between insulin-IGF signaling and the Hippo pathway is clear-cut in both *Drosophila* and mammalian systems. In mammalian cells, cross regulation occurs at the level of Akt and MST1/MST2; MST1 and MST2 both contain a classical Akt phosphorylation site (RXXRXXT_{120/117}) that is phosphorylated in response to IGF1.⁸⁹ MST1[Thr120Asp] exhibits diminished activity, cleavage, and nuclear localization, effects also elicited by overexpression of PI-3 kinase.^{89,90} MST2[Thr117] phosphorylation is also reported to reduce the level of binding of MST2 to RASSF1A while increasing the level of

binding to Raf1⁹¹ (see below). MST1 is phosphorylated in vitro by Akt at KRRDET₃₈₇, a noncanonical Akt site that in cells is phosphorylated in a PI-3 kinase-dependent manner in response to EGF. An MST1[Thr387Glu] mutant exhibits reduced proapoptotic efficacy and weakened ability to catalyze the activating phosphorylation of FOXO3,⁹² a putative apoptotic effector of MST1.^{93,94} Interestingly, the protein phosphatase PHLPP, which dephosphorylates Akt[Ser473]⁹⁵ and thereby interferes with the ability of Akt to phosphorylate and negatively regulate Foxo,⁹⁶ also promotes the dephosphorylation of MST1[Thr387], thus enhancing MST1-catalyzed FOXO activation.⁹⁷ Reciprocal with Akt inhibition of MST1/MST2, the latter have been shown to be capable of binding Akt and, in a kinase-independent manner, inhibiting Akt activity;⁹⁸ whether this mechanism operates in vivo is not known.

In *Drosophila*, yorkie is a positive regulator of Akt abundance⁹⁹ and Akt is required for the proliferative response of cells with deficient Hippo signaling.¹⁰⁰ Although Hippo Thr132 is homologous to the canonical Akt recognition motif present at MST1[Thr120]/MST2[Thr117], the functional significance of this site has not been evaluated in the *Drosophila* system.

INHIBITION BY CRAF1

cRaf1 and B Raf are the primary activators of the MEK-Erk module downstream of Ras-GTP; Araf exhibits little MAP3K activity.¹⁰¹ Genetic inactivation of cRaf1 in the mouse results in embryonic lethality accompanied by massive apoptosis.¹⁰² Genetic replacement of cRaf1 with a cRaf1 mutant (Y340F/Y341F) that lacks the ability to phosphorylate MEK1/2 and activate Erk nevertheless fully rescues the lethality.¹⁰³ This unexpected finding provoked a search for Erk-independent, antiapoptotic functions of cRaf1. Proteomic analyses of cRaf1 pull downs identified several associated protein kinases, including MST2, ASK1 (both capable of initiating apoptosis), and Roka.¹⁰² Raf1 (and Araf but not B Raf) was shown to bind the MST2 SARAH domain, inhibiting MST2 homodimerization and activation; this inhibition does not require Raf1 kinase activity, providing a mechanism by which a kinase-inactive cRaf1 could suppress apoptosis, i.e., by binding and suppressing MST2 activation.¹⁰⁴ In a subsequent series of studies, MST2–Raf1 binding was shown to be enhanced by the inhibitory Raf1[Ser259] phosphorylation, which can be catalyzed by Akt or in a feedback mode by Lats1; loss of Ser259 phosphorylation activates both Raf1 and MST2.^{105,106} Reciprocally, the MST2 binding site on cRaf1 overlaps the Ras binding domain, suppressing Raf1 activation; MST2 depletion was observed to relieve this suppression. Overexpression of RASSF1A disrupted the Raf1/MST2 complex, and activation of Ras, especially Ki-Ras, not only activated Raf1 directly but also activated MST2 through a Ki-Ras-GTP/RASSF1A complex.⁶⁶ In addition, Abl phosphorylation of MST2[Y81], a conserved site, is reported to disrupt the Raf1–MST2 interaction, favoring MST2 homodimerization and activation.¹⁰⁷ The complexity of the regulatory interactions proposed for Ki-Ras/RASSF1A/Raf1/MST2 is considerable, and the reader is referred to detailed descriptions of the supporting data and computational models derived from these studies.^{105,108} It should be noted, however, that differing, partially conflicting results have been reported; e.g., although mitogens were observed to increase the level of Raf1[Ser259] phosphorylation and promote a Raf1-MST2 interaction, depletion of MST2 or Lats1 in those

experiments reduced the extent of Raf1 activation.¹⁰⁹ The extent to which the functional relationships reported between Raf1 and MST2 are dependent on the specific cell lines or culture conditions employed is unclear. Moreover, the circumstances wherein Raf1/MST2 cross regulation operates in vivo are not known; BRaf does not bind MST2/Hippo, interdicting evaluation through *Drosophila* genetics. In the mouse, crosses of MST2^{-/-}/Raf1^{+/-} mice do not yield live MST2^{-/-}/Raf1^{-/-} offspring (D. Zhou and J. Avruch, unpublished observations), suggesting that MST2 deficiency does not ameliorate the embryonic lethality of Raf1 deficiency.

ACTIVATION BY REACTIVE OXYGEN SPECIES

Extracellular H₂O₂ (at levels of 0.1–10 mM) and other oxidants cause rapid activation of MST1/MST2.^{41,92} Although widely recognized to be an unphysiologic stimulus, H₂O₂ is frequently used to mimic the consequences of excess ROS that occur under many pathophysiologic circumstances when the extent of generation of free radicals exceeds the available antioxidant capacity. One reliable cellular response to H₂O₂ is a widespread increase in the level of Ser/Thr/Tyr phosphorylation, generally attributed to direct oxidative inactivation of Tyr and Ser/Thr specific protein phosphatases and the PIP3 phosphatase, PTEN.¹¹⁰ In addition, some enzymes are modified through a more specific mechanism involving the thioredoxins, as first demonstrated for ASK1. The reduced form of thioredoxin1 or -2, ~12 kDa cysteine-containing polypeptides, in their reduced form binds ASK1 specifically and inhibits its ability to autophosphorylate and autoactivate; H₂O₂ oxidizes thioredoxin, causing its dissociation and allowing ASK1 activation.¹¹¹ Reduced thioredoxin also binds MST1 (but not MST2) through the SARAH domain and inhibits MST1 activity probably by interdicting homodimerization. H₂O₂-induced thioredoxin oxidation causes its dissociation from MST1;¹¹² H₂O₂ concomitantly promotes the binding of TRAF2, through its central zinc finger region, to the MST1 SARAH domain. TRAF2 somehow promotes MST1 self-association and activation and thus appears to serve as a scaffold function for MST1 in response to H₂O₂.¹¹³ Notably, antioxidants such as *N*-acetylcysteine prevent the TNF α activation of MST1, suggesting a role for ROS in the delayed activation of MST1 by TNF α .¹¹² The mechanism by which H₂O₂ activates MST2, which does not bind thioredoxin, remains to be elucidated.

REGULATION BY TYROSINE PHOSPHORYLATION

Several phosphoproteomic surveys detected the phosphorylation of MST1[Tyr433], a site not conserved in MST2 or Hippo. The first recognition of this phosphorylation in a regulatory context emerged from the finding that the apoptosis of hippocampal neurons induced by H₂O₂ could be ameliorated by STI571/Imatinib inhibition of the Abl tyrosine kinase.⁹⁴ Previous studies had identified MST1 as a mediator of H₂O₂-induced neuronal apoptosis,⁹³ and depletion of Abl in cultured primary neurons was thereafter shown to ameliorate the apoptosis induced by MST1 overexpression. Abl phosphorylates MST1[Tyr433] in vitro, and H₂O₂ induces MST1-[Tyr433] phosphorylation in neurons in an STI571-sensitive and Abl-dependent manner. Coexpression with Abl modestly increased the abundance and reduced the level of polyubiquitination of MST1 (but not of MST1[Tyr433Phe]), which is mediated predominantly by the ubiquitin ligase/cochaperone

CHIP/STUB1. Concomitant with Tyr433 phosphorylation, MST1-catalyzed phosphorylation of the Foxo3 forkhead domain is enhanced, a modification that promotes the nuclear entry and transcriptional activation of Foxo1/3, an important contributor to H₂O₂-induced apoptosis.¹¹⁴ Whether the enhanced Foxo phosphorylation reflects increased MST1 abundance, kinase activation, or enhanced MST1–Foxo3 association is unclear. A similar H₂O₂–Abl–MST1–Foxo3 pathway was identified in astrocytes;¹¹⁵ however, a somewhat different situation is seen in microglia, the brain's scavenger system. Transient middle cerebral artery occlusion causes cortical damage by both ischemic and ROS-induced neuronal death, the latter releasing elements that activate microglia; in turn, microglial activation, in addition to initiating clearance of cellular debris, results in the production of inflammatory cytokines, e.g., TNF α . Infarct size and the level of cytokine production are substantially reduced in MST1^{−/−} mice; moreover, selective inactivation of MST1 in microglia and macrophages also significantly reduces infarct size. Activated microglia exhibit MST1[Tyr433] phosphorylation, and transient expression in microglia of MST1 but not MST1-[Tyr433Phe] greatly enhances LPS-induced TNF α and IL-6 expression. MST1[Tyr433] phosphorylation in activated microglia is catalyzed by Src rather than Abl, and the Src inhibitor AZD0530 reduced the level of Tyr433 phosphorylation, the level of LPS-induced microglial cytokine production in vitro, and infarct size in vivo. TNF α and IL-6 transcription in microglia is likely mediated by NF κ B, and H₂O₂-stimulated I κ B α phosphorylation in microglia is enhanced in an MST1-dependent manner.¹¹⁶ Altogether, these reports establish that MST1[Tyr433] phosphorylation has important functional consequences, including the stabilization of the MST1 polypeptide; however, whether and how this modification alters the MST1 activation state and the interaction with scaffolds and substrates remains to be elucidated. As noted above, Abl can also phosphorylate MST2[Tyr81], a modification reported to disrupt the inhibitory Raf1–MST2 interaction.¹⁰⁷

MITOTIC ACTIVATION OF MST1/MST2

The well-studied mitotic exit network of *S. cerevisiae*¹¹⁷ and the septation initiation network of *Schizosaccharomyces pombe*¹¹⁸ are each comprised of a scaffold-bound Ste20-related, GCK-like kinase (Cdc15p or Sid1p) that phosphorylates Mob1 and an NDR family kinase (Dbf2 or Sid2p), to allow mitotic exit and cytokinesis. The noncatalytic regions of Cdc15p and Sid1p bear no resemblance to those of MST1/MST2, an indication that the upstream regulation is likely to differ; nevertheless, the participation of MST1/MST2 in cell cycle progression and mitotic exit, in part through the phosphorylation of Mob1, has been conserved. MST1/MST2 and Lats1/Lats2 are activated at some point during G2/M and are rapidly deactivated upon mitotic exit.¹²¹ Lats1/Lats2 have been associated with centrosomal duplication; however, MST1/MST2 mitotic substrates other than Mob1 and Lats and the mechanism of MST1/MST2 activation during cell cycle progression are not known and are perhaps the least studied aspects of their regulation in mammalian cells.¹¹⁹

MST1/MST2 SUBSTRATES

Mob1 and Lats1/Lat2

The best characterized MST1/MST2 substrates are Mob1A/Mob1B and Lats1/Lats2, elements immediately downstream of MST1/MST2 in the Hippo pathway. MST1/MST2 catalyzes Lats phosphorylation at sites within a hydrophobic motif (FYEFTFRRFF) located toward the carboxy-terminal end with respect to the catalytic domain (Lats1[Thr1079] and Lats2[Thr1041]).¹²⁰ Early studies showed that okadaic acid-induced phosphorylation of Mob1 promoted its binding to Lats, and the ability of Lats to catalyze autophosphorylation of its activation loop (Lats1-[Ser909] and Lats2[Ser872]).^{121,122} MST1/MST2-catalyzed Lats phosphorylation and Lats autophosphorylation are both required absolutely for Lats1/Lats2 kinase activation.

Mob1A and Mob1B consist of 216 amino acids and are 95% identical. MST1 and MST2 phosphorylate Mob1A/B[Thr35] and -[Thr12], the latter phosphorylation possibly requiring the former; Mst3 cannot catalyze Mob1 phosphorylation in vitro, and the ability of other Ste20-related kinases has not been reported.¹²¹ Side-by-side comparisons in vitro show that MST1 and MST2 catalyze the phosphorylation of Mob1 much faster than the phosphorylation of Lats1/2. This difference reflects two factors; one is a requirement for binding of Mob1 to Lats for efficient Lats phosphorylation by MST1/MST2. Keratinocytes from mice lacking Mob1A/Mob1B exhibit markedly diminished levels of Lats1/Lats2 hydrophobic motif phosphorylation.¹²³ Thus, Mob1 binding to Lats, in addition to promoting Lats autophosphorylation, is critical for MST1/MST2-catalyzed Lats phosphorylation. The second factor is the requirement for a scaffold that approximates MST1/MST2 with Lats; the ability of MST1/MST2 to phosphorylate Lats1 in vitro is greatly stimulated by the presence of Sav1/WW45, which has little or no effect on Mob1 phosphorylation. MST1/MST2 also phosphorylates the Lats kinase paralogs NDR1/NDR2,¹²⁴ probably through the mediation of a different scaffold, i.e., the protein Furry (in yeast) or Furry-like.¹²⁵ Whether these scaffolds participate in MST1/MST2-catalyzed substrate phosphorylation by mechanisms in addition to their ability to approximate the kinase-substrate pair is not known.

The very rapid phosphorylation of Mob1 in vitro may also reflect the fact that activated MST2 itself serves as a scaffold for Mob1. Initial studies observed that transiently expressed MST2 bound Mob1 in vitro much more strongly when MST2 was fully autophosphorylated.¹²¹ Subsequently, Ni et al.¹²⁶ showed that Mob1 binds specifically to a region in the MST2 linker that contains multiple autophosphorylation sites; deletion of the linker region, although preserving the catalytic and SARAH domains, virtually abolished MST2's ability to phosphorylate Mob1 in vitro. The MST2 autophosphorylated linker contains seven phospho-Thr-Met motifs; elimination of all seven abolishes Mob1 binding, but four (Thr349, -356, -364, and -378), when present as the only Thr-Met motif, can rescue Mob1 binding and phosphorylation. A synthetic peptide whose sequence is centered around the phospho-Thr₃₇₈-Met motif can bind Mob1[1-50] efficiently through a two-site mechanism involving an indispensable phospho-Thr/Mob1[Lys₁₅₃-Arg₁₅₄-Arg₁₅₇] interaction and a weaker second site involving an MST2 hydrophobic motif further toward

the carboxy-terminal end (FMDYF₃₉₇); inasmuch as the three nonsufficient Thr-Met motifs (Thr325, -336, and -342) are those most distant from the MST2 hydrophobic site, the hydrophobic interaction may confer important additional binding energy. Importantly, a similar bipartite interaction was observed for the interaction between Hippo and the *Drosophila* Mob1 ortholog Mats. The MST1 linker region contains six Thr-Met motifs and a downstream hydrophobic FLEYF₃₉₉ segment. Whether the MST1/MST2 Thr(P)-Met motifs function to dock substrates other than Mob1 has not been determined, although retention of the linker segment reduces the K_m for FoxO1 phosphorylation (see below).

Once Mob1 is phosphorylated, its level of binding to MST2 is greatly diminished; conversely, Mob1 phosphorylation greatly improves its ability to bind Lats1/Lats2.¹²¹ This results from displacement of the Mob1 amino-terminal segment from Mob1's negatively charged Lats binding surface. Phosphorylation at Thr12, which is situated in a flexible linker segment, and at Thr35, which is located in a ("switch") helix, disorders these regions, and the two phosphoThr residues bind to separate, basically charged Mob1 surfaces, which repositions the Mob1 amino terminus away from the Lats1 binding surface. The core of the Mob1 structure is unchanged, and a functionally equivalent exposure of Mob1's Lats binding surface can be achieved by deletion of the Mob1 amino-terminal segment.¹²⁷ Phospho-Mob1 binds to a region of Lats1 (amino acids 621–703) immediately amino-terminal to the catalytic domain (amino acids 705–1010) composed of a V-shaped pair of antiparallel helices, through a mixture of charge and hydrophobic interactions; the binding of phospho-Mob1 somehow displaces the unusually long Lats activation loop from an inhibitory interaction with the ATP binding site, permitting autophosphorylation of the loop and disinhibition. The region of NDR1/NDR2 that binds phospho-Mob1 is very similar in location and sequence to that in Lats1/2. As with Lats1/Lats2, the NDR1/NDR2 hydrophobic site carboxyterminal to the catalytic domain can be phosphorylated by other Ste20 kinases, such as Mst3;¹²⁸ the latter, however, does not phosphorylate Mob1.¹²¹

The requirement for Hippo-catalyzed MATS phosphorylation to allow binding of MATS to Warts was strongly challenged by Vrabioiu and Struhl,¹²⁹ who attached fluorescent proteins to the N- and C-termini of Warts/Lats and used FRET to monitor the Warts conformation in fly wings. Deletion of MATS/Mob led to a closed, presumably inactive Warts conformation as did the morphogens, Wingless or Decapentaplegic, via activation of the protocadherins Fat and Daschous, which recruit the atypical myosin Dachs. Mutation of the Warts ATP binding site, activation loop, or Thr1083 to Ala, the site of Hippo catalyzed phosphorylation, did not alter the regulation of the Warts conformation by MATS; surprisingly, neither did the mutational inactivation of Hippo. Thus, Hippo-catalyzed MATS phosphorylation is dispensable for the ability of MATS to maintain an open conformation of Warts. The authors conclude that MATS can interact productively with Warts in the absence of Hippo, an interaction that is somehow inhibited by Dachs. Thus, MATS activation (like glycogen phosphorylase) may be regulated by a ligand (or protein-protein interaction) in a manner independent of its phosphorylation; alternatively, inasmuch as MATS phosphorylation was not examined directly in these experiments, it remains possible that MATS is phosphorylated by a kinase other than Hippo.

In addition to the NDR family of kinases, phospho-Mob1 also binds and somehow activates the DOCK6–8 subfamily of Rac1/Cdc42 guanyl nucleotide exchangers, an interaction that is critical for MST1/MST2's ability to promote the actin remodeling required for the migration and thymic egress of mature thymocytes.¹³⁰

Foxo1/3

Foxo1/3 phosphorylation confers both negative and positive regulation.¹³¹ Akt activated downstream of insulin/IGF signaling phosphorylates several sites within and adjacent to the FoxO1/3 forkhead domain that results in the binding of 14-3-3, which promotes nuclear egress. Conversely, MST1 phosphorylates other sites within the forkhead domain (FoxO1[Ser212] and FoxO3[Ser207]) that interdict 14-3-3 binding, promoting nuclear entry. An in vivo demonstration of this dual regulation is seen in *C. elegans*, where the prolonged life span caused by partial loss of function of daf2 (the insulin/IGF receptor ortholog) and by overexpression of cst-1 (the MST1/2 ortholog) is dependent on the FoxO1/3 ortholog daf16.⁹³ The effects of MST1-catalyzed FoxO1/3 phosphorylation on FoxO DNA binding vary with the specific DNA site,¹³² and thus, the transcriptional outputs are likely to vary depending on the underlying chromatin state and cell background. In neurons, MST1-catalyzed FoxO1/3 phosphorylation is proposed to serve as one of the mechanisms by which MST1 promotes apoptosis,^{93,94} whereas in T cells, MST1 activation of FoxO1/3 is proposed to ameliorate oxidative stress (through upregulation of SOD2 and catalase) and promote survival.²⁶

With regard to the interaction of MST1 with the FoxO1 forkhead domain, Anand et al.¹³³ observed that although full length MST1 and the MST1[1–334] catalytic fragment exhibited a similar k_{cat} toward the FoxO1 forkhead domain, full length MST1 had a 7-fold lower K_m and thus a 6-fold higher catalytic efficiency (k_{cat}/K_m), suggesting that the region carboxy-terminal to MST1 amino acid 334 contributed to MST1 binding to FoxO1. They identified three autophosphorylation sites within the MST1 linker segment (T340, T346, and T348), including Thr₃₄₆Met; however, it was not determined whether these contributed directly to MST1 binding of the FoxO1 forkhead domain.

Histone H2B

Chromosome condensation is one of the cardinal events during apoptosis, and histone modifications are crucial to this process. One of the key modifications is the phosphorylation of histone H2B at Ser14. Cheung et al.⁷⁹ identified a 34 kDa nuclear H2B[Ser14] protein kinase in cells undergoing apoptosis as the cleaved form of MST1. Apoptotic cleavage of MST1 and H2B[Ser14] phosphorylation exhibited a similar time course, and inhibition of caspase 3 inhibited both events. MST1 can directly phosphorylate H2B[Ser14] in vitro; in contrast to FoxO1, the cleaved catalytic fragment exhibits an ~30-fold greater catalytic efficiency toward H2B as compared with full length MST1, due to a 10-fold increase in k_{cat} as well as a 4-fold lower K_m .¹³³ The caspase cleavage also removes MST1 nuclear export signals that together with its smaller size and greater catalytic efficiency convert MST1 into an efficient H2B kinase. Subsequent work showed that cleaved MST1 is also likely to mediate the apoptotic phosphorylation of histone H2AX at Ser139.¹³⁴ There is disagreement, however, about the primacy of these modifications to MST1's ability to cause

chromosome condensation; thus, in MKK4/7 DKO MEFs, wherein MST1 overexpression fails to activate Jnk or promote chromosome condensation, expression of a constitutively active Jnk causes chromosome condensation.¹³⁵ Thus, despite its ability to directly catalyze histone phosphorylation, MST1-induced chromosome condensation may be achieved indirectly.^{136,152}

Other MST1/MST2 substrates reported thus far are listed in Table 1.

SUMMARY

MST1 and MST2 are distinguished from members of other GCK families by the presence of a carboxy-terminal dimerization domain known as SARAH. Transiently expressed MST1 and MST2 form homodimers through their SARAH domains and are activated upon phosphorylation at a single threonine within their activation loop; this phosphorylation proceeds readily in trans within the homodimer but can also be catalyzed by the TAO kinase and perhaps by other as yet undiscovered kinases. It is not known, however, whether MST1/MST2 ever exist in tissues as homodimers, inasmuch as the noncatalytic SARAH domain polypeptides (Sav1/WW45 and the RASSF1–6 polypeptides) are each capable of heterodimerizing with MST1/MST2 to confer new regulatory behaviors and/or serve as a scaffold toward specific substrates.

The Sav1/WW45 polypeptide, a conserved component from the *Drosophila* Hippo pathway, appears to perform both functions; in mouse embryonic keratinocytes induced to differentiate in vitro by addition of Ca^{2+} , the binding of MST1/MST2 to Sav1 through a SARAH heterodimer is necessary for MST1/MST2 activation. Whether this reflects Sav1's ability to itself promote MST1/MST2 autophosphorylation or to recruit another activator, either a noncatalytic protein or another protein kinase, is unknown. As a scaffold, Sav1 also binds Lats1/Lats2, facilitating their phosphorylation by MST1/MST2. In this format, MST1 and MST2 serve as the mammalian orthologs of Hippo, a suppressor of the oncogenic and antiapoptotic transcriptional regulators Yap and TAZ.

Among the RASSF1–6 proteins, only Nore1B/RASSF5B/RAPL, the dominant MST1/MST2 partner in the lymphoid system, thus far provides a clear-cut regulatory mechanism. In resting murine T cells, full length MST1 is inactive and bound through its SARAH heterodimer to Nore1B/RASSF5B/RAPL. Activation of the T cell receptor promotes GTP charging of Ras-like GTPase Rap1, which binds to the RASSF5B Ras-Rap association (RA) domain; the Rap1-RASSF5B-MST1 complex translocates to the immune synapse at the cell surface, where MST1 is activated by T loop phosphorylation. As with Sav1, the importance of autophosphorylation versus another protein kinase for MST1/MST2 activation is unknown. T cells lack Sav1, so that MST1/MST2 phosphorylation of Lats1/Lats2 in response to physiologic stimuli is inconsequential; on the other hand, their ability to phosphorylate Mob1, which is apparently scaffolded directly by the activated MST1/MST2 kinases themselves, remains robust. In this cell background, MST1 and MST2 are crucial for the migration and adhesion of mature thymocytes and activated T cells, functions mediated in part through phospho-Mob1 activation of the DOCK6–8 subfamily of cdc42/Rac1 GEFs,

as well as through direct phosphorylation of the Rab13 GEF DENND1C and VASP, which control the recruitment of the integrin LFA-1.

MST1 (and MST2) also undergoes activation in normal hepatocytes, during myoblast differentiation and during apoptosis by undefined mechanisms, followed by a caspase 3 cleavage. This results in constitutive activation, a redistribution from a predominantly cytoplasmic localization into the nucleus, and a change in substrate preference, illustrated by a decreased affinity for FoxO1 and an increased affinity and catalytic efficiency toward histone H2B. The role of such a continuously active, nuclear predominant MST1/MST2 kinase appears to be in the enforcement of specific cellular states, e.g., mitotic quiescence, terminal differentiation, or apoptosis. The relevant substrates may be epigenetic modifiers, e.g., histones. Genetic evidence in *Drosophila* indicates that the Hippo pathway is negatively regulated by the insulin/IGF-PI3K pathway, and biochemical studies in mammalian systems show that Akt phosphorylates MST1/MST2 directly, inhibiting their catalytic activity. Conversely, although complexes containing MST2 and cRaf can be recovered from mammalian cells, the role of such complexes in the regulation of either kinase in normal or tumor cells remains incompletely defined.

Significant progress has been achieved in identifying the regulation of MST1/MST2, their physiologic substrates, and their surprisingly diverse cell roles. Nevertheless, many questions remain, including the components of the MST1/MST2-containing complexes in various normal and malignant tissues, the importance of non-SARAH domain-containing partners, such as Raf1, to MST2 regulation, the relative contributions of autophosphorylation versus upstream kinases in the physiologic regulation of these complexes, and the regulation of MST1/MST2 in mitosis and their roles in mitotic progression. The diversity of candidate substrates (Table 1) and the knowledge that virtually nothing is known about the physiologic functions of most of the SARAH domain-containing polypeptides encoded by the RASSF1–6 genes suggest that much remains to be learned about MST1/MST2.

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Figure 1. Domain structure of MST1/MST2/Hippo and representative SARAH domain-containing proteins. Proteins are listed according to Entrez Gene (<http://www.ncbi.nlm.nih.gov/genes>) with lengths of amino acids. Major isoforms and conserved protein domains are depicted with a color legend. The hatched areas in Rassf1A/C and Rassf5A/B indicate amino-terminal regions unique to each isoform.

Table 1

MST1 and/or MST2 substrates	refs
protein kinases	
Lats1, Lats2 ^a	120
NDR1, NDR2 ^b	124, 145, 154
Aurora A	140
Aurora B	141
protein kinase C α	146
Nek2A ^a	148, 153
transcriptional regulators	
Foxo1, Foxo3A	94
histone H2B	79
histone H2AX	134
IRF3	137
PDX1	147
RUNX2A ^a	149
androgen receptor	150
Sirtuin 1	151
estrogen receptor α ^a	152
cytoskeletal regulation	
DENND1C	138
VASP	138
apoptosis and/or autophagy	
Bcl-XL	143
Beclin	142
MAP1LC3A(LC3)	139
miscellaneous/multifunctional	
Mob1A, Mob1B	121
Sav1/WW45	136
peroxiredoxin-1	144

^aBound to Sav1.^bBound to Furry.