Oncogene. Author manuscript; available in PMC 2010 February 25.

Published in final edited form as:
Oncogene. 2009 August 20; 28(33): 2988–2998. doi:10.1038/onc.2009.152.

RASSF2 associates with and stabilises the proapoptotic kinase MST2

Wendy N Cooper[1], **Luke B Hesson**[1,2], **David Matallanas**[3], **Ashraf Dallol**[1], **Alex von Kriegsheim**[3], **Robyn Ward**[2], **Walter Kolch**[3,4], and **Farida Latif**[1]

[1]Section of Medical and Molecular Genetics, Institute of Biomedical Research, University of Birmingham, Birmingham B15 2TT. UK

[2]Department of Medical Oncology, St. Vincent's Hospital, Victoria Street, Darlinghurst, New South Wales 2010, Australia

[3]Proteomics and Signalling Networks Group, The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK

[4]Integrative and Systems Biology, Institute for Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

Abstract

RASSF2 is a tumour suppressor that in common with the rest of the RASSF family contains Ras association and SARAH domains. We identified the proapoptotic kinases MST1 and MST2 as the most significant binding partners of RASSF2, confirmed the interactions at endogenous levels and demonstrated that RASSF2 immunoprecipitates active MST1/2. We then demonstrated that RASSF2 can be phosphorylated by a co-immunoprecipitating kinase which is likely to be MST1/2. Furthermore, we demonstrated that RASSF2 and MST2 do indeed colocalise, but whilst RASSF2 alone is nuclear, the presence of MST1 or MST2 results in colocalisation in the cytoplasm. Expression of RASSF2 (stably in MCF7 or transiently in HEK-293) increases MST2 levels and knockdown of RASSF2 in HEK-293 cells reduces MST2 levels, additionally colorectal tumour cell lines and primary tumours with low RASSF2 levels show decreased MST2 protein levels. This is likely to be mediated by RASSF2-dependent protection of MST2 against proteolytic degradation. Our findings suggest that MST2 and RASSF2 form an active complex in vivo where RASSF2 is maintained in a phosphorylated state and protects MST2 from degradation and turnover. Thus we propose that the frequent loss of RASSF2 in tumours results in destabilisation of MST2 and thus decreased apoptotic potential.

Keywords

RASSF2; MST2; MST1; proteomics; epigenetics

Introduction

The RASSF family of proteins is comprised of 10 members all sharing a region of homology, the ras association domain. The classical RASSFs (RASSF1-6) contain in addition a SARAH domain, so named for its original identification in Sav (the *Drosophila* WW45 orthologue), RASSF and Hippo (the *Drosophila* MST orthologue) (Scheel and

Requests for reprints to Professor Farida Latif at Section of Medical and Molecular Genetics, The Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom. Tel: (44)(0) 121 627 2741; Fax: (44)(0) 121 414 2538; f.latif@bham.ac.uk.

Europe PMC Funders Author Manuscripts

Hofmann, 2003). The best studied member of the RASSF family, RASSF1A is important for cell cycle regulation, apoptosis and microtubule stability (Agathanggelou *et al.*, 2005). Though rarely mutated, RASSF1A is frequently inactivated by promoter methylation in a wide range of tumour types (Agathanggelou *et al.*, 2005) and has recently been demonstrated to be degraded by calpain mediated proteolysis (Kuznetsov and Khokhlatchev, 2008). The more recently discovered and less well characterised RASSF2 has also been shown to be frequently inactivated by promoter methylation in a wide range of tumour types including colorectal (Hesson *et al.*, 2005), gastric (Endoh *et al.*, 2005), oral (Imai *et al.*, 2008), endometrial (Liao *et al.*, 2008), breast and lung (Cooper *et al.*, 2008; Kaira *et al.*, 2007). RASSF2 has been shown to inhibit the growth of lung (Vos *et al.*, 2003) and breast (Cooper *et al.*, 2008) cancer cell lines, both *in vitro* and *in vivo.* We and others have described the RASSF2 nuclear localisation signal (Cooper *et al.*, 2008; Kumari *et al.*, 2007; Maruyama *et al.*, 2008) and demonstrated that a functional NLS is required for full tumour suppressor activity. In order to further understand the function of RASSF2 we made use of proteomic approaches to determine RASSF2 binding partners.

MST1 (STK4/KRS2) and MST2 (STK3/KRS1) are members of the GCKII subclass of sterile 20 proapoptotic kinases, they contain an amino terminal kinase domain separated from a C terminal inhibitory domain by caspase cleavage sites. Full length MST1 and 2 are predominantly cytoplasmic due to the presence of carboxy terminal nuclear export signals (Ura *et al.*, 2001), but nuclear accumulation is observed following treatment with Leptomycin B (a nuclear export inhibitor) demonstrating that MSTs perpetually shuttle through the nucleus (Lee *et al.*, 2001; Lee and Yonehara, 2002; Lin *et al.*, 2002; Ura *et al.*, 2001). Kinase activity is regulated by autophosphorylation, caspase cleavage and possibly dimerisation and co-factor binding. Autophosphorylation at threonine in the context KRNT (MST1 T183, MST2 T180) is essential for full kinase activity (Deng *et al.*, 2003; Glantschnig *et al.*, 2002) and may influence susceptibility to cleavage (Graves *et al.*, 2001). MST1 contains caspase cleavage sites at amino acids 326 and 349 (Graves *et al.*, 2001; Lee *et al.*, 2001), cleavage can be induced by CD95/Fas (Graves *et al.*, 1998), anti-cancer drugs (Kakeya *et al.*, 1998), or biphosphonates (inducers of osteoclast apoptosis) (Reszka *et al.*, 1999). Cleavage by caspases separates the MST kinase and inhibitory domains (Graves *et al.*, 2001; Graves *et al.*, 1998; Lee *et al.*, 2001) resulting in nuclear accumulation of the kinase domain (Lee *et al.*, 2001; Ura *et al.*, 2001) where it can phosphorylate histone H2B at Ser 14 (Cheung *et al.*, 2003) resulting in chromatin condensation and apoptosis, however recent reports suggest that JNK activation not H2B phosphorylation is essential for chromatin condensation (Hu *et al.*, 2007; Ura *et al.*, 2007). It has been proposed (Deng *et al.*, 2003) that in normal cells autophosphorylation is reversible by protein phosphatases resulting in the majority of MST protein being unphosphorylated. However in cells undergoing apoptosis, caspase 3-dependent cleavage of MST and irreversible autophosphorylation results in an accumulation of active MST.

In common with the RASSF family of proteins, MST1 and MST2 contain a SARAH domain located at the C terminus. This domain is essential for dimerisation (Creasy *et al.*, 1996) and binding to RASSF1 or NORE1 (Khokhlatchev *et al.*, 2002; Oh *et al.*, 2006; Rabizadeh *et al.*, 2004). Addition of purified RASSF1A or NORE1A to MST1 inhibits kinase activity in vitro (Guo *et al.*, 2007; Oh *et al.*, 2006; Praskova *et al.*, 2004; Rabizadeh *et al.*, 2004). In contrast in cells induction of MST1 membrane localisation via membrane bound NORE or myristoylation augments its apoptotic efficiency (Khokhlatchev *et al.*, 2002; Praskova *et al.*, 2004). However the picture in cells without forced membrane localisation is less clear; decreased MST1 phosphorylation in cells following coexpression of MST1 with RASSF1A, F1C, NORE1A or 1B was observed (Khokhlatchev *et al.*, 2002; Praskova *et al.*, 2004) however increased phosphorylation of MST2 or MST1 following RASSF1A transfection has also been demonstrated (Guo *et al.*, 2007; Oh *et al.*, 2006; Rabizadeh *et al.*, 2004). These

differences could be due to differing experimental protocols, but may well reflect the presence of other factors that are available to be recruited. Depending on which factors are in the complex RASSF1A may be able to switch between activation and inhibition. Thus RASSF family members may act as versatile scaffolds for bringing together MSTs, their regulators and substrates, and possibly different RASSFs may bring along different regulators and or substrates. Therefore after we identified MST1 and MST2 as significant binding partners of RASSF2 we deemed it important to determine the specific properties of the RASSF2 MST interacting unit.

Results

In a yeast two hybrid screen, RASSF2 interacts with several proteins including MST1 implicated in mediating apoptosis

A yeast two hybrid screen was performed with RASSF2 as bait. Interactions that were validated by yeast co-transformations included those with members of the RASSF family (RASSF3 and NORE1), Filamin C (an actin binding protein that when mutated can cause autosomal dominant myofibrillar myopathy), C14ORF2 (a protein of unknown function), Secretogranin III (a neuroendocrine secretory protein), and MST1 (a proapoptotic kinase also known as STK4). The MST1/2 association was the most interesting given the roles of MST1 and MST2 in apoptosis. However there was little information about the other interacting proteins to help placing the putative association in the context of apoptotic cell signalling. Therefore, we performed the FLAG-immunoprecipitation/mass spectrometry analysis in an effort to find more RASSF2-interacting proteins.

MST 1 and 2 are the most significant binding partners of RASSF2 in a FLAG immunoprecipitation/ mass spectrometry analysis

We have generated clones stably expressing FLAG tagged RASSF2 (Cooper *et al.*, 2008) in the MCF7 cell background where endogenous RASSF2 expression is silenced via promoter methylation. From these cells we used FLAG immunoprecipitation to enrich for proteins binding to RASSF2. The immunoprecipitate was eluted with FLAG peptide and resolved through a polyacrylamide gel (Fig 1a). The gel was then cut into sections and each section was digested with trypsin and peptides were analysed by mass spectrometry. The list of potential interactors was headed by the mammalian ste20 like kinases 1 and 2 (MST1 and MST2). Next to the bait protein they were the strongest band and peptide coverage of these proteins was 29.8% for MST1 and 52.1% for MST2 (Fig 1b).

Endogenous MST1 and MST2 and RASSF2 coimmunoprecipitate, and their interaction is mediated via the RASSF2 SARAH domain

In order to verify that the interaction between MST1 or MST2 and RASSF2 was not an experimental artefact of FLAG-RASSF2 overexpression, the data was confirmed in reverse using FLAG tagged MST1 or MST2 to immunoprecipitate endogenous RASSF2 (data not shown). To demonstrate that these interactions were not mediated by the FLAG tag, endogenous RASSF2 was immunoprecipitated from A549 cells (a cell line that we have demonstrated expresses high levels of RASSF2 by RT-PCR, and that by RT-PCR the levels of RASSF2 do not change with treatment with the demethylating agent 5-aza-2′deoxycytidine) and endogenous MST1 and MST2 shown to coprecipitate (Fig 2a). The reciprocal experiment showed that antibodies against endogenous MST1 or MST2, but not IgG alone, precipitated RASSF2 (Fig 2b). Immunoprecipitation of RASSF2 from A549 demonstrates that the majority of MST1 and a large fraction of MST2 present in the cell were found in complex with RASSF2 (Fig 2c). Similar data were also obtained in HEK-293 cells.

In order to map the interaction domains constructs of RASSF2 were generated that deleted amino acids 249-end, or 108-253. These constructs partially delete the RA domain and all of the SARAH domain or the NLS and most of the RA domain respectively (Fig 3a). Cotransfection of GFP-tagged RASSF2 with FLAG-tagged MST1 or MST2 into HEK-293 cells followed by FLAG immunoprecipitation demonstrated interactions between MST1 or MST2 and full length RASSF2 and RASSF2 Δ108-253, but not with amino acids 1-248 (Fig 3b-c) suggesting that the interaction is likely to be mediated via the RASSF2 SARAH domain. Therefore we mutated a leucine (conserved between RASSF family members and MSTs from a variety of species including human, mouse and *Xenopus*) within the SARAH domain (L290) to proline, this alteration greatly reduced the interaction between RASSF2 and MST2, and abolished the interaction between RASSF2 and MST1 (Fig 3d). This residue is also important for RASSF2 dimerisation as RASSF2 L290P is substantially less able to immunoprecipitate GFP tagged RASSF2 (Fig S1). In addition to wildtype MST1/2, kinase inactive (K56R) and non-phosphorylatable (T183A, T180A) mutants of MST1 and MST2 also immunoprecipitated RASSF2 (Fig 3b-c). Thus RASSF2 interacts with both MST1 and MST2, and kinase null and non-phosphorylatable mutants of MST1/2, and the interaction is mediated by the RASSF2 SARAH domain.

RASSF2 immunoprecipitates active MST

Immunoprecipitation of RASSF2 demonstrated that RASSF2 interacts with both wild-type MST1 and MST2 and kinase-inactive (MST2 K56R) and non-activatable mutants (MST1 T183A, MST2 T180A), suggesting that full activity of MST1/2 is not a prerequisite for the interaction to occur. In order to determine if RASSF2 was sequestering MST1 and 2 in an inactive form and as such involved in the regulation of MST activity, we transfected HEK-293 cells with FLAG tagged RASSF2 then treated the cells with the phosphatase inhibitor okadaic acid (OA) which is known to activate MST. Incorporation of γ-^{32}P ATP was used to determine the FLAG associated kinase activity. Phosphorimaging of the gel demonstrated two differently sized substrates for ^{32}P incorporation corresponding in size to MST1/2 and RASSF2 (Fig 4a,b) indicating that RASSF2 was a substrate for MST1/2 phosphorylation. The band corresponding to the autoradiograph was excised (Fig 4b) and subject to Electron Capture Dissociation FT-ICR mass spectroscopy this confirmed the presence of both phosphorylated and unphosphorylated peptides of RASSF2. Subdivision of the kinase reaction into two fractions corresponding to the portion of proteins that remained bound to MST2 throughout the kinase reaction and those proteins that dissociated during the reaction indicated that phosphorylation of RASSF2 does not induce its dissociation from MST2 (Fig S2). Similar experiments performed with cotransfected FLAG-MST2-K56R (kinase dead mutant) resulted in undetectable levels of ^{32}P incorporation (data not shown) indicating that MST2 is inducing the phosphorylation.

RASSF2 can stabilise MST2

Interestingly whilst the amount of MST2 immunoprecipitating with FLAG-RASSF2 remains constant (or perhaps increases, Fig 4a) total MST2 diminishes in lysates following OA treatment, suggesting that activation of MST2 may induce its degradation. In addition cotransfection of GFP-RASSF2 with FLAG-MST2 resulted in much higher levels of FLAG-MST2 expression than when FLAG-MST2 was cotransfected with RASSF2 deletions (Fig 3c). Thus we hypothesised that RASSF2 was protecting MST2 protein. In order to test this hypothesis we compared MCF7 cells that lack RASSF2 protein with MCF7 cells stably expressing RASSF2.

MCF7 cells stably expressing RASSF2 have consistently higher levels of MST2 than MCF7 cells transfected with empty vector (Fig 5a). Treatment with agents that induce apoptosis (staurosporine or okadaic acid, OA) could increase this difference by reducing the levels of

MST2 by a greater extent in cells lacking RASSF2 than in cells expressing RASSF2. Thus RASSF2 may protect MST2 from degradation or RASSF2 may induce MST2 expression. Next we looked at HEK293 cells transiently transfected with RASSF2 and/or MST2 (Fig 5b). In these experiments RASSF2 was able to increase MST2 protein levels of both endogenous and CMV promoter driven HA tagged MST2 demonstrating that this effect was not mediated at the level of transcription. Additionally the mutant of RASSF2 with diminished ability to bind MST2 (RASSF2 L290P) demonstrated a reduced ability to stabilise MST2 (data not shown). Knockdown of RASSF2 expression in HEK-293 and HCT116 cell lines with siRNA directed against RASSF2 resulted in decreased levels of MST2 and also MST1 (Fig 5c). Thus RASSF2 may play a role in protection of MST2 in cells and loss of RASSF2 by promoter methylation as is frequently observed in human tumours (Hesson *et al.*, 2005; Cooper *et al.*, 2008) may result in destabilisation of MST2. Therefore we analysed a series of human colon cancer cell lines for expression of RASSF2 and MST2. We observed a correlation between the levels of RASSF2 and MST2 protein expression, for example the cell line HCT116 that expresses RASSF2 also expresses higher levels of MST2 than the cell lines that do not express RASSF2 (Fig 5d). In addition we investigated whether loss of RASSF2 protein correlated with reduced levels of MST2 in primary colorectal carcinomas (CRC). In all 12 samples were analysed. We found four tumours expressed higher levels of both RASSF2 and MST2 (for example, tumours 39 and 97, Fig 5e), whilst seven tumours displayed vastly reduced or undetectable levels of either protein (for example, tumours 14, 13 and 43, Fig 5e). In only one of the twelve tumours were there high levels of MST2 in the absence of RASSF2.

In order to study the effect of the protein phosphatase inhibitor OA, HEK-293 cells were treated with OA for various periods of time (Fig 6a). Treatment with OA substantially diminished total MST2 by 3 hours. This was not accompanied by appearance of the N-terminal cleavage product (Fig 6a) suggesting that this reduction was not a result of MST2 cleavage. Therefore we postulated that activation of MST2 by OA can induce its degradation and that RASSF2 may protect MST2 from this degradation. One potential mediator of this degradation is the proteasome, and to test whether OA was inducing proteasome mediated degradation of MST2 we pre-treated HEK-293 cells with the proteasome inhibitor MG132 for 1h, followed by treatment with OA for a further 3h. Treatment with MG132 at concentrations between 10 and 50μM could restore MST2 to levels similar to that observed without OA (Fig 6b) indicating that degradation of MST2 in response to OA is indeed mediated via the proteasome. Transfection of FLAG-RASSF2 was also able to partially rescue the OA-induced decrease in levels of MST2, again suggesting that RASSF2 has a protective effect on MST2 (Fig 6b). The effect of expression of FLAG-RASSF2 is weaker in HEK-293 cells than in MCF7 cells and this is probably due to the presence of sufficient endogenous RASSF2 in HEK-293 cells to stabilise the majority of endogenous MST2.

RASSF2 and MST1 or MST2 colocalise in the cytoplasm

In order to better understand the functional relevance of these interactions we cotransfected RASSF2 and MST1/2 into Cos7 cells. MST1 or MST2 alone were predominantly cytoplasmic, and RASSF2 alone was predominantly nuclear. However when cotransfected with MST1 or MST2, RASSF2 became relocalised to the cytoplasm ($P<0.0001$ for FLAG vs FLAG-MST1; $P<0.0001$ for FLAG vs FLAG-MST2, Fig 7). The RASSF2 mutant with the SARAH domain deleted (RASSF2 1-248) was resistant to this translocation (Fig 7). Interestingly mutants of MST2 were unable to induce this relocalisation with the kinase dead mutant K56R and the non-phosphorylatable mutant T180A leaving RASSF2 predominantly nuclear ($P<0.0001$ for FLAG-MST2 vs FLAG-MST2 K56R; $P<0.0001$ for FLAG-MST2 vs FLAG-MST2 T180A, Fig 7). Immunoprecipitation (Fig 3b-c) showed that these mutants

bound RASSF2 equally well suggesting that the mutants are not held in an alternative conformation that binds RASSF2 less well. This suggests that only fully active MST can induce translocation of RASSF2. As kinase assay suggests that MST1/2 can phosphorylate RASSF2 we postulate that phosphorylation of RASSF2 may alter its properties overcoming the function of the RASSF2 nuclear localisation signal (Cooper *et al.*, 2008; Kumari *et al.*, 2007; Maruyama *et al.*, 2008) and enabling RASSF2 to be translocated to the cytoplasm.

Discussion

Yeast two hybrid and FLAG-immunoprecipitation mass spectrometry based interaction screens indicated that the proapoptotic kinases MST1 and MST2 were the most significant RASSF2-interacting partners. Whilst it had previously been demonstrated that MST1 and 2 can interact with NORE1A, NORE1B, RASSF1A and RASSF1C (Hwang *et al.*, 2007; Khokhlatchev *et al.*, 2002; Praskova *et al.*, 2004), no previous characterisation of the interaction between endogenous RASSF2 and endogenous MST1/2 has been reported. First we showed (Fig 2) that RASSF2 can co-immunoprecipitate endogenous MST1 and MST2, and also that antibodies against MST1 or MST2 can coprecipitate endogenous RASSF2. Deletion and mutagenesis of RASSF2 demonstrated that this interaction was indeed mediated via the RASSF2 SARAH domain as is the case for RASSF1A and NORE1 (Khokhlatchev *et al.*, 2002).

Inspection of the Coomassie stained gel of proteins coprecipitating with RASSF2 (Fig 1a) suggested that two of the three most intensely staining bands were the correct sizes to be RASSF2 and MST1/2. This and the huge significance with which MST1 and MST2 peptides were identified by mass spectroscopy suggested that the RASSF2/MST complex was very abundant and possibly that the majority of MST1 and MST2 present in the cells was in complex with RASSF2. Thus we compared the amount of MST1 and MST2 present in lysates from untransfected cells with the amount remaining unbound in the supernatant after immunoprecipitation of RASSF2. This demonstrated that the vast majority of MST1 and most of the MST2 in A549 or HEK-293 cells was present in complex with RASSF2. This suggests that RASSF2 is a more significant binding partner of MST1 and MST2 than NORE1 or RASSF1, because MST1 only immunoprecipitates substoichiometric amounts of NORE1/RASSF1A (Praskova *et al.*, 2004) perhaps this is because the rest of the MST1 is bound to RASSF2. This demonstration that the majority of MST1 and MST2 in cells are present in complex with RASSF2 is in accordance with size exclusion chromatography which indicated that wild type MST1 and and monomeric MST1 (ie SARAH domain mutant L444P) were present in higher molecular weight complex (250kDa and 130kDa respectively) than would be predicted from their amino acid composition (predicted monomer 55.6 kDa) (Creasy *et al.*, 1996; Lee and Yonehara, 2002).

Because RASSF2 was present in complex with the majority of cellular MST1 and MST2, it was important to determine whether RASSF2 was specifically sequestering an inactive subset of MST, thus we used kinase assay to demonstrate that the RASSF2 associated MST1/2 was both able to phosphorylate itself and interestingly could phosphorylate RASSF2 in a manner analogous to that seen for Sav (Callus *et al.*, 2006).

Given the documented cellular localisation of RASSF2 as nuclear (Cooper *et al.*, 2008; Kumari *et al.*, 2007; Maruyama *et al.*, 2008) and MST2 as cytoplasmic (Lee *et al.*, 2001; Lee and Yonehara, 2002; Ura *et al.*, 2001), it was perhaps surprising that that they coimmunoprecipitated. However, we found that cotransfection of RASSF2 with MST2 resulted in the translocation of RASSF2 from the nucleus to the cytoplasm, and that this relocalisation was dependent on the ability of the two proteins to interact because deletion of the SARAH domain from RASSF2 (which abolished their interaction) prevented the MST

induced relocalisation of RASSF2. The MST kinase activity was also essential for this relocalisation suggesting that perhaps MST1/2 bind to RASSF2 through their common SARAH domains and phosphorylate RASSF2 inducing RASSF2 relocalisation. Therefore we postulate that hyperphosphorylation of RASSF2 induces its nuclear-cytoplasmic translocation. Whilst MST1 and MST2 are known to cycle through the nucleus (Lee et al., 2001; Lee and Yonehara, 2002; Lin et al., 2002; Ura et al., 2001), it is possible that they can encounter RASSF2 in the nucleus, bind to it and induce its translocation to the cytoplasm where RASSF2 remains only when it becomes hyperphosphorylated. This phosphorylation of RASSF2 may be facilitated by apoptotic activation of MST1/MST2 either physiologically or constitutively by overexpression-induced autophosphorylation (Deng et al., 2003; Glantschnig et al., 2002). Therefore it is possible that RASSF2 may act as a scaffold maintaining things in the nucleus and transporting them to the cytoplasm during MST mediated apoptosis.

Stable expression of RASSF2 in the MCF7 cell line (that lacks endogenous RASSF2 expression due to promoter methylation) caused an increase in MST2 protein levels when compared with equivalent cells transfected with the empty vector, whereas treatment with OA decreased the levels of MST2. Furthermore, we demonstrated that knockdown of RASSF2 in cell lines resulted in decreased levels of MST2 protein and in colorectal tumours reduced RASSF2 protein levels correlated with reduced MST2 proteins levels. The decrease in MST2 levels was not due to cleavage of MST2, and was instead likely to be due to proteasome-mediated degradation because pretreatment with the inhibitor of proteasome mediated degradation MG132 could in part prevent the decrease in MST2 levels. In addition, expression of RASSF2 could partially protect MST2 from OA mediated degradation. We present evidence that; i). RASSF2 is a major interacting partner of both MST1 and MST2; ii). Activation of MST2 results in its increased turnover by proteasomal degradation; iii). The presence of RASSF2 protects MST2 from degradation; iv). Active MST2 can phosphorylate RASSF2; v). Active MST2 can relocalise RASSF2 from the nucleus to the cytoplasm and the kinase activity of MST2 is essential for this relocalisation. From these data we propose the following hypothesis. In the presence of RASSF2, pro-apoptotic stimuli that result in the activation of MST2 allow the cytoplasmic accumulation of the RASSF2-MST complex. In the absence of RASSF2, activation of MST2 results in its rapid degradation, nullifying its effects as a proapoptotic protein. This may explain the induction of apoptosis observed upon reexpression of RASSF2 in cell lines lacking expression (Vos *et al.*, 2003). Therefore, loss of RASSF2 in cancer cells following promoter hypermethylation can cause a decrease in MST2 stability and thus decreased apoptotic potential.

Materials and Methods

Expression constructs

Full length RASSF2 (NM_014737) was amplified by PCR using the primers CTT ATC GCG GCC GCG **atg** gac tac agc cac caa acg and CT TAT TCT AGA **tca** gat tgt tgc tgg ggt ctc and cloned between the NotI and XbaI sites of p-FLAG-CMV4 vector (Sigma-Aldrich). RASSF2 was also cloned between the BglII and SalI sites of EGFP-C2 (destroying the BglII site in the process). Deletion constructs were then generated by restriction digestion and re-ligation; ApaI was used to delete amino acids from 249 to end and BglII deleted amino acids 108-253. The QuikChange Lightning site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) was used to mutate pFLAG-RASSF2. To generate the L290P mutation, the mutagenic primers were gcttcattcagaagcCccaggaggaagaaga and tcttcttcctcctggGgcttctgaatgaagc. Expression constructs for FLAG-MST1 or MST2 and mutants of MST1 and MST2 were described previously (O'Neill *et al.*, 2004). The sequence

of constructs were verified by sequencing using fluorescent dye terminators (BigDye V3.1 Applied Biosystems).

Antibodies

Mouse monoclonal antibodies against FLAG (M2, F-1804), β-actin (A-5441) and HA (H-9658) were from Sigma-Aldrich. Rabbit polyclonal anti-HA (sc-805), anti-GFP (sc-8334) and goat polyclonal anti RASSF2 (sc-46475 for characterisation see Fig 5c and S3) and anti MST1 (sc-6088) antibodies were from Santa Cruz. Rabbit polyclonal antibody to α-tubulin (ab18251), and rabbit monoclonal antibody against STK3 (MST2, ab52641) were from Abcam. Rabbit polyclonal antibodies against MST1 (#3682), MST2 (#3952) and phosphorylated MST (#36815) were from Cell Signaling. HRP-conjugated Rabbit anti-mouse IgG (P0161) and normal control goat and rabbit IgG were purchased from DAKOCytomation. HRP-conjugated goat anti-rabbit IgG (31462) was from Pierce. Alexafluor-594-conjugated rabbit anti-mouse IgG (A11005) was purchased from Invitrogen.

Cell lines and transfection

HEK-293, Cos7, MCF7 and colorectal tumour (DLD1, HCT116, SW620, 147T, LS411) cell lines were grown in DMEM supplemented with 10% FCS and L-glutamine and were routinely maintained at 37°C and 5% CO_2. Clones of the breast tumour cell line MCF7 stably expressing FLAG-RASSF2 have been described previously (Cooper *et al.*, 2008).

Cos7 cells seeded on glass coverslips in six-well plates were transfected with lug plasmid DNA using 3ul FuGENE 6. Fortyeight hours later, cells were fixed and immunostained according to (Dallol *et al.*, 2004). Images of at least 25 randomly selected cells stained for both GFP and FLAG were captured then scored for the localisation of the green fluorescence according to the following criteria:“Mostly nuclear” indicates that either all or most of the green signal colocalised with the blue DAPI nuclear stain. “Equal” signified equal staining in nucleus and cytoplasm. “Mostly cytoplasmic” indicated that either all or most of the green signal localised in the cytoplasm.

RNAi was performed using LipoFECTAMINE (Invitrogen) with 5ng of RASSF2 siRNA (Ambion oligo ID number 21809), or scrambled oligo following the manufacturer’s instructions. Twentyfour hours later the cells were re-transfected with HiPerFect (QIAGEN) again with 5 ng of siRNA. The cells were lysed the following day.

HEK-293 cells were transfected using polyethylenimine (Polysciences; Warrington PA) according to (Durocher *et al.*, 2002). Forty-eight hours post-transfection where indicated cells were treated with 1μM Staurosporine (Sigma-Aldrich), 1μM Okadaic acid and/or MG132 (Merck Biosciences) for the times described and then lysed in 1% Triton x100, 50mM NaF, 50mM Tris pH7.5, 1mM EDTA, 1mM EGTA, 5mM Na pyrophosphate, 1mM Na vanadate, 0.27M sucrose with complete, mini, EDTA-free protease inhibitor cocktail tablet (Roche).

Primary tumour samples

For analysis of protein expression in primary colorectal tumours we homogenised each 100mg of tissue in the presence of 300μL cell lysis buffer (as above, but supplemented with 200mM NaCl). Samples were then incubated at 4°C for 30mins with end-over-end mixing followed by centrifugation for 10mins at 13,000rpm at 4°C. Thirty micrograms of total protein (equivalent to 1.6mg of tissue) was then subjected to SDS-PAGE, transferred to PVDF membrane and probed for the presence of RASSF2, MST2 and β-actin. Tumour samples were collected with approval from local ethics committee following institutional guidelines.

Immunoprecipitation

Lysate (1mg) was incubated with 4μg of antibody or an equivalent amount of the appropriate preimmune IgG and 8ul Protein G Sepharose (Sigma-Aldrich) or anti-FLAG M2 affinity gel (Sigma-Aldrich). After 1h30min lysate was transferred to a SpinX column (Corning) and beads washed 3x with lysis buffer supplemented with 150mM NaCl. A final wash was performed with 50mM Tris, 0.1mM EGTA, before elution with gel loading buffer.

Kinase assay

Using anti-FLAG M2 affinity gel (Sigma-Aldrich) FLAG-RASSF2 was immunoprecipitated from 1mg cell lysate. After washing the FLAG-agarose beads were incubated with kinase assay buffer (50mM TrisHCl pH 7.5, 0.1% β-mercaptoethanol, 1mM EGTA, 10mM magnesium acetate and 1mM ATP containing 0.5uCi γ^{32}P) and incubated at 30°C for 30min with vigorous shaking. The reaction was then terminated by addition of SDS sample loading buffer and subjected to SDS-PAGE. The gel was then fixed and ^{32}P detected by phosphorimaging. Alternatively the kinase assay was performed in the absence of ^{32}P, bands excised from Coomassie stained gel and phosphorylation identified by FT-ICR mass spectrometry at University of Birmingham Functional Genomics and Proteomics Unit.

Identification of novel interacting partners

Yeast two hybrid screen was performed as described previously (Fenton *et al.*, (2004). Immunoprecipitation and identification using 2D-LC/MS/MS was done as described (von Kriegsheim *et al.*, 2006) with minor modifications.

Statistical analysis

Statistical significance was assessed using the chi squared test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank David Sumpton and Willy Bienvenut for the mass spectrometry. This work was supported by Breast Cancer Campaign, Cancer Research UK, Sport Aiding Medical Research for Kids (to F.L) and The European Union FP6 project “Interaction Proteome” (LSHG-CT-2003-505520) and Cancer Research UK (to W.K).

References

Agathanggelou A, Cooper WN, Latif F. Role of the ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Research. 2005; 65:3497–3508. [PubMed: 15867337]

Callus BA, Verhagen AM, Vaux DL. Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. Febs J. 2006; 273:4264–76. [PubMed: 16930133]

Cheung WL, Ajiro K, Samejima K, Kloc M, Cheung P, Mizzen CA, et al. Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. Cell. 2003; 113:507–17. [PubMed: 12757711]

Cooper WN, Dickinson RE, Dallol A, Grigorieva EV, Pavlova TV, Hesson LB, et al. Epigenetic regulation of the ras effector/tumour suppressor RASSF2 in breast and lung cancer. Oncogene. 2008; 27:1805–11. [PubMed: 17891178]

Creasy CL, Ambrose DM, Chernoff J. The Ste20-like protein kinase, Mst1, dimerizes and contains an inhibitory domain. J Biol Chem. 1996; 271:21049–53. [PubMed: 8702870]

Dallol A, Agathanggelou A, Fenton SL, Ahmed-Choudhury J, Hesson L, Vos MD, et al. RASSFIA interacts with microtubule-associated proteins and modulates microtubule dynamics. Cancer Research. 2004; 64:4112–4116. [PubMed: 15205320]

Deng Y, Pang A, Wang JH. Regulation of mammalian STE20-like kinase 2 (MST2) by protein phosphorylation/dephosphorylation and proteolysis. J Biol Chem. 2003; 278:11760–7. [PubMed: 12554736]

Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 2002; 30:E9. [PubMed: 11788735]

Endoh M, Tamura G, Honda T, Homma N, Terashima M, Nishizuka S, et al. RASSF2, a potential tumour suppressor, is silenced by CpG island hypermethylation in gastric cancer. British Journal of Cancer. 2005; 93:1395–9. [PubMed: 16265349]

Fenton SL, Dallol A, Agathanggelou A, Hesson L, Ahmed-Choudhury J, Baksh S, et al. Identification of the E1A-regulated transcription factor p120(E4F) as an interacting partner of the RASSF1A candidate tumor suppressor gene. Cancer Research. 2004; 64:102–107. [PubMed: 14729613]

Glantschnig H, Rodan GA, Reszka AA. Mapping of MST1 kinase sites of phosphorylation. Activation and autophosphorylation. J Biol Chem. 2002; 277:42987–96. [PubMed: 12223493]

Graves JD, Draves KE, Gotoh Y, Krebs EG, Clark EA. Both phosphorylation and caspase-mediated cleavage contribute to regulation of the Ste20-like protein kinase Mst1 during CD95/Fas-induced apoptosis. Journal of Biological Chemistry. 2001; 276:14909–14915. [PubMed: 11278782]

Graves JD, Gotoh Y, Draves KE, Ambrose D, Han DKM, Wright M, et al. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. EMBO Journal. 1998; 17:2224–2234. [PubMed: 9545236]

Guo C, Tommasi S, Liu L, Yee JK, Dammann R, Pfeifer GP. RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats tumor-suppressor network. Curr Biol. 2007; 17:700–5. [PubMed: 17379520]

Hesson LB, Wilson R, Morton D, Adams C, Walker M, Maher ER, et al. CpG island promoter hypermethylation of a novel Ras-effector gene RASSF2A is an early event in colon carcinogenesis and correlates inversely with K-ras mutations. Oncogene. 2005; 24:3987–3994. [PubMed: 15806169]

Hu Y, Liu Z, Yang SJ, Ye K. Acinus-provoked protein kinase C delta isoform activation is essential for apoptotic chromatin condensation. Cell Death Differ. 2007; 14:2035–46. [PubMed: 17721436]

Hwang E, Ryu KS, Paakkonen K, Guntert P, Cheong HK, Lim DS, et al. Structural insight into dimeric interaction of the SARAH domains from Mst1 and RASSF family proteins in the apoptosis pathway. Proc Natl Acad Sci U S A. 2007; 104:9236–41. [PubMed: 17517604]

Imai T, Toyota M, Suzuki H, Akino K, Ogi K, Sogabe Y, et al. Epigenetic inactivation of RASSF2 in oral squamous cell carcinoma. Cancer Sci. 2008; 99:958–66. [PubMed: 18294275]

Kaira K, Sunaga N, Tomizawa Y, Yanagitani N, Ishizuka T, Saito R, et al. Epigenetic inactivation of the RAS-effector gene RASSF2 in lung cancers. Int J Oncol. 2007; 31:169–73. [PubMed: 17549418]

Kakeya H, Onose R, Osada H. Caspase-mediated activation of a 36-kDa myelin basic protein kinase during anticancer drug-induced apoptosis. Cancer Res. 1998; 58:4888–94. [PubMed: 9809995]

Khokhlatchev A, Rabizadeh S, Xavier R, Nedwidek M, Chen T, Zhang XF, et al. Identification of a novel Ras-regulated proapoptotic pathway. Current Biology. 2002; 12:253–265. [PubMed: 11864565]

Kriegsheim A, Preisinger C, Kolch W. Mapping of signaling pathways by functional interaction proteomics. Methods Mol Biol. 2008; 484:177–92. [PubMed: 18592180]

Kumari G, Singhal PK, Rao MR, Mahalingam S. Nuclear Transport of Ras-associated Tumor Suppressor Proteins: Different Transport Receptor Binding Specificities for Arginine-rich Nuclear Targeting Signals. J Mol Biol. 2007

Kuznetsov S, Khokhlatchev AV. The growth and tumor suppressors NORE1A and RASSF1A are targets for calpain-mediated proteolysis. PLoS ONE. 2008; 3:e3997. [PubMed: 19098985]

Lee KK, Ohyama T, Yajima N, Tsubuki S, Yonehara S. MST, a physiological caspase substrate, highly sensitizes apoptosis both upstream and downstream of caspase activation. J Biol Chem. 2001; 276:19276–85. [PubMed: 11278283]

Lee KK, Yonehara S. Phosphorylation and dimerization regulate nucleocytoplasmic shuttling of mammalian STE20-like kinase (MST). J Biol Chem. 2002; 277:12351–8. [PubMed: 11805089]

Liao X, Siu MK, Chan KY, Wong ES, Ngan HY, Chan QK, et al. Hypermethylation of RAS effector related genes and DNA methyltransferase 1 expression in endometrial carcinogenesis. Int J Cancer. 2008; 123:296–302. [PubMed: 18404674]

Lin Y, Khokhlatchev A, Figeys D, Avruch J. Death-associated protein 4 binds MST1 and augments MST1-induced apoptosis. J Biol Chem. 2002; 277:47991–8001. [PubMed: 12384512]

Maruyama R, Akino K, Toyota M, Suzuki H, Imai T, Ohe-Toyota M, et al. Cytoplasmic RASSF2A is a Pro-apoptotic Mediator Whose Expression is Epigenetically Silenced in Gastric Cancer. Carcinogenesis. 2008

O'Neill E, Rushworth L, Baccarini M, Kolch W. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science. 2004; 306:2267–70. [PubMed: 15618521]

Oh HJ, Lee KK, Song SJ, Jin MS, Song MS, Lee JH, et al. Role of the tumor suppressor RASSF1A in Mst1-mediated apoptosis. Cancer Res. 2006; 66:2562–9. [PubMed: 16510573]

Praskova M, Khoklatchev A, Ortiz-Vega S, Avruch J. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. Biochemical Journal. 2004; 381:453–462. [PubMed: 15109305]

Rabizadeh S, Xavier RJ, Ishiguro K, Bernabeortiz J, Lopez-Ilasaca M, Khokhlatchev A, et al. The scaffold protein CNK1 interacts with the tumor suppressor RASSF1A and augments RASSF1A-induced cell death. Journal of Biological Chemistry. 2004; 279:29247–29254. [PubMed: 15075335]

Reszka AA, Halasy-Nagy JM, Masarachia PJ, Rodan GA. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. J Biol Chem. 1999; 274:34967–73. [PubMed: 10574973]

Scheel H, Hofmann K. A novel interaction motif, SARAH, connects three classes of tumor suppressor. Curr Biol. 2003; 13:R899–900. [PubMed: 14654011]

Ura S, Masuyama N, Graves JD, Gotoh Y. Caspase cleavage of MST1 promotes nuclear translocation and chromatin condensation. Proc Natl Acad Sci U S A. 2001; 98:10148–53. [PubMed: 11517310]

Ura S, Nishina H, Gotoh Y, Katada T. Activation of the c-Jun N-terminal kinase pathway by MST1 is essential and sufficient for the induction of chromatin condensation during apoptosis. Mol Cell Biol. 2007; 27:5514–22. [PubMed: 17548476]

von Kriegsheim A, Pitt A, Grindlay GJ, Kolch W, Dhillon AS. Regulation of the Raf-MEK-ERK pathway by protein phosphatase 5. Nat Cell Biol. 2006; 8:1011–6. [PubMed: 16892053]

Vos MD, Ellis CA, Elam C, Ulku AS, Taylor BJ, Clark GJ. RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. Journal of Biological Chemistry. 2003; 278:28045–28051. [PubMed: 12732644]

Europe PMC Funders Author Manuscripts

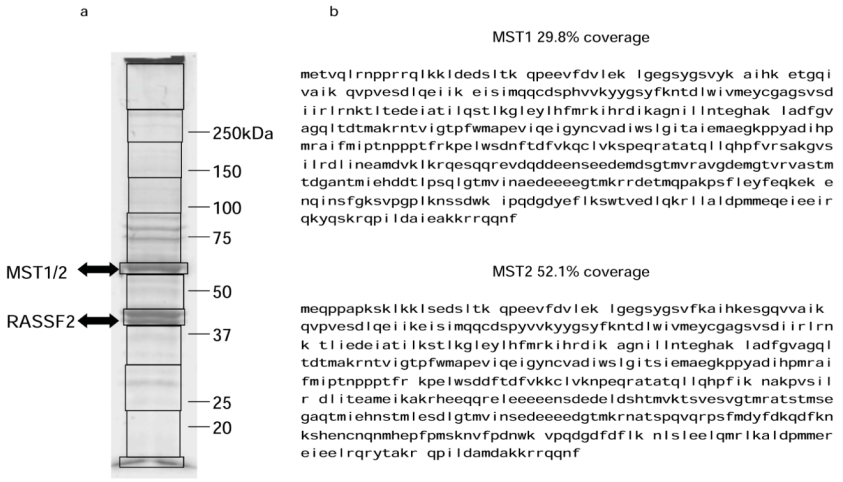

Figure 1. MST1 and MST2 are major interacting partners of RASSF2 in mammalian cells

FLAG tagged RASSF2 was immunoprecipitated from stably transfected MCF7 cells and interacting partners were resolved by electrophoresis through a 10% acrylamide gel (a). The gel was cut into 12 sections and each analysed by electrospray ESI-QUAD-TOF mass spectrometry, the resulting list of peptides was pooled and used for searching the SwissProt and NCBI databases. In addition to RASSF2 the most significant interacting partners were MST1 and MST2 with 29.8 and 52.1% peptide coverage (b), identified peptides are depicted in bold.

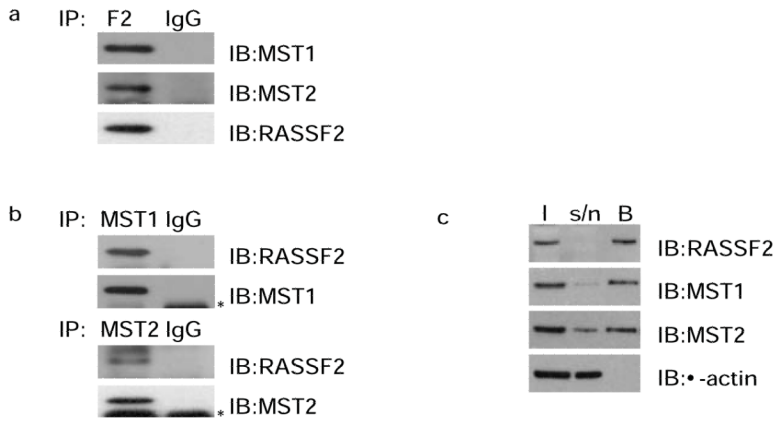

Figure 2. Endogenous MST1 and MST2 immunoprecipitate with endogenous RASSF2, and the majority of endogenous MST1 and MST2 is bound to endogenous RASSF2 in mammalian cells

To investigate whether endogenous RASSF2 interacts with endogenous MST1 or MST2 we immunoprecipitated MST1, MST2 and RASSF2 from A549 cells. In immunoprecipitates of RASSF2 both MST1 and MST2 were detected (a), and RASSF2 co-immunoprecipitated with both MST1 and MST2 (b). * corresponds to IgG heavy chain. To ascertain what fraction of endogenous MST1 or MST2 is associated with endogenous RASSF2 we immunodepleted RASSF2 from A549 protein lysates and immunoblotted the RASSF2-bound and unbound fractions (c; I is input, s/n supernatant, and B bound fraction. β-actin was included to control for equal loading of input and supernatant and to control for specificity of the immunoprecipitation). Thus the majority of both MST1 and MST2 co-immunoprecipitate with RASSF2.

Europe PMC Funders Author Manuscripts

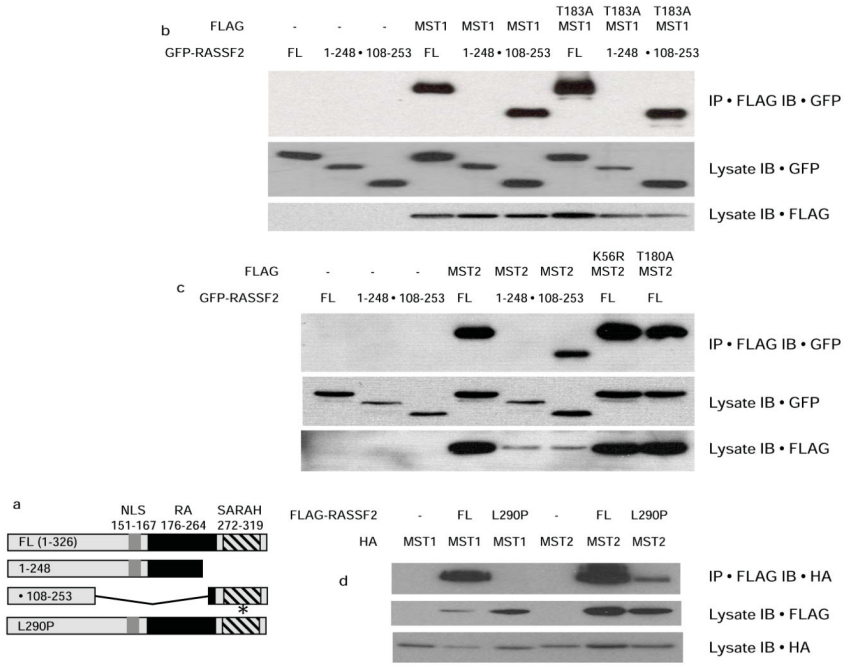

Figure 3. RASSF2 SARAH domain mediates interaction with MST1 and MST2

Deletion constructs of RASSF2 were generated deleting the SARAH domain (hatched) or RA (black) (a). HEK-293 were transfected with FLAG tagged MST1 or MST2 or mutants, and GFP tagged RASSF2. Lysates were precipitated with anti-FLAG-agarose, and the precipitating proteins immunoblotted for GFP (b, c). Alternatively FLAG tagged RASSF2 or RASSF2 L290P were cotransfected with HA tagged MST1 or MST2, precipitated with anti-FLAG-agarose, and the precipitating proteins immunoblotted for HA (d).

Europe PMC Funders Author Manuscripts

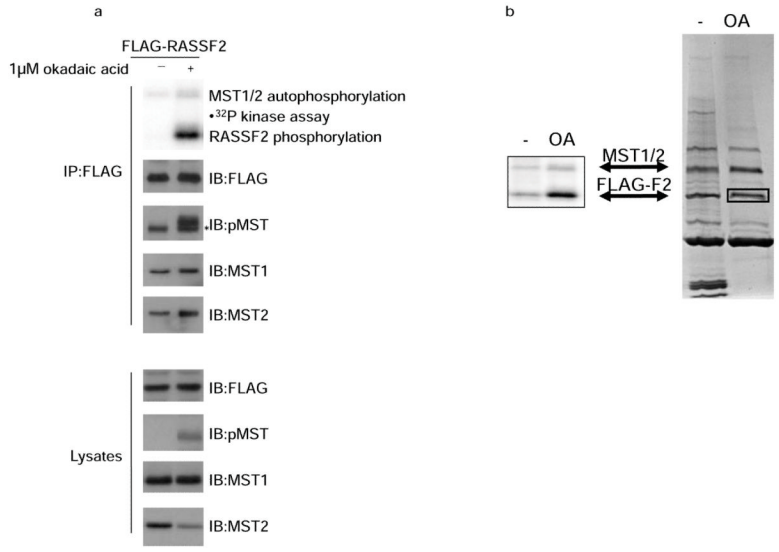

Figure 4. RASSF2 remains associated with the phosphorylated and active forms of MST1 and MST2

Lysates from HEK-293 transiently transfected with FLAG-RASSF2 and treated or not with okadaic acid as per the figure label were immunoprecipitated with FLAG agarose and the precipitating proteins subject to kinase assay (a,b). The bands with ^{32}P incorporation most probably represent autophosphorylation of MST1 or MST2, and phosphorylation of RASSF2, based on their size and intensity changes following transfection. Portions of the immunoprecipitate and the corresponding lysates were immunoblotted (a) with antibodies that recognise RASSF2, MST1, MST2 and the phosphorylated and active forms of MST1 and 2 (pMST), * corresponds to IgG heavy chain. A cold kinase assay was run in parallel, and the proteins separated through an acrylamide gel and stained with coomassie (b), the band indicated with the rectangle was excised and subject to Electron Capture Dissociation FT-ICR mass spectroscopy.

Europe PMC Funders Author Manuscripts

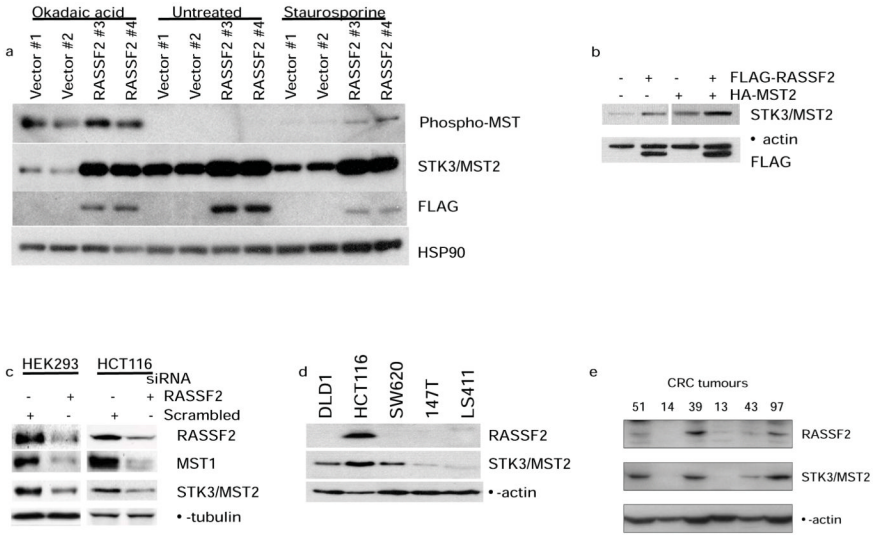

Figure 5. RASSF2 stabilises MST2 protein

(a) Stable re-expression of RASSF2 in MCF7 cells leads to an increase in MST2 levels. Treatment with OA or Staurosporine results in a decrease in MST2 levels, and the presence of RASSF2 protects MST2: MCF7 cells stably transfected with FLAG vector or FLAG tagged RASSF2, were treated for 5 hours with okadaic acid or Staurosporine, and the resulting lysates immunoblotted for phosphorylated MST, total MST2, FLAG and HSP90.

(b) Transient transfection of RASSF2 increases the levels of MST2 protein: HEK-293 cells were transiently transfected with FLAG vector or FLAG tagged RASSF2 with or without HA-MST2, after 48h, cells were lysed and immunoblotted for total MST2, FLAG and β-actin.

(c) Knockdown of RASSF2 decreases the levels of MST1 and MST2: HEK-293 or HCT116 cells were treated with siRNA against RASSF2, after 48h, cells were lysed and immunoblotted for RASSF2, total MST1, total MST2 and α-tubulin.

(d) Loss of RASSF2 protein expression in human colon cancer cell lines correlates with decreased levels of MST2 protein.

(e) Loss of RASSF2 protein in human colorectal carcinomas correlates with decreased levels of MST2 protein.

Europe PMC Funders Author Manuscripts

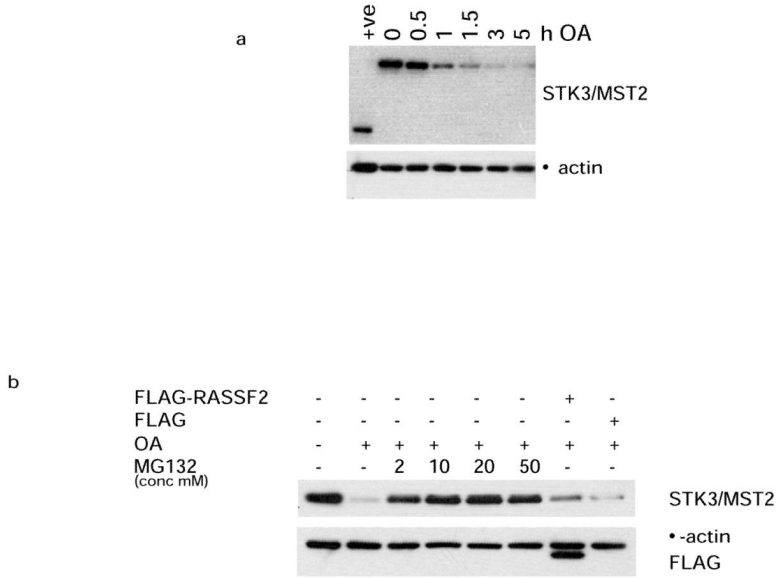

Figure 6. MG132 prevents okadaic acid induced degradation of MST2

(a) Activation of MST2 by OA results in substantial decreases in MST2 protein levels that are independent of MST2 cleavage: HEK-293 cells were treated with OA for various periods and lysates immunoblotted for MST2, and β-actin (+ve is normal human fibroblasts MRC5-VA treated with Staurosporine to induce cleavage of MST2).

(b) HEK-293 cells were treated or not with MG132 at the indicated concentration for 1h before the addition of OA for a further 3h. Additionally HEK-293 cells 48h after transient transfection with FLAG vector or FLAG tagged RASSF2 were treated with OA for 3h. Lysates were then immunoblotted for MST2, FLAG and β-actin.

Europe PMC Funders Author Manuscripts

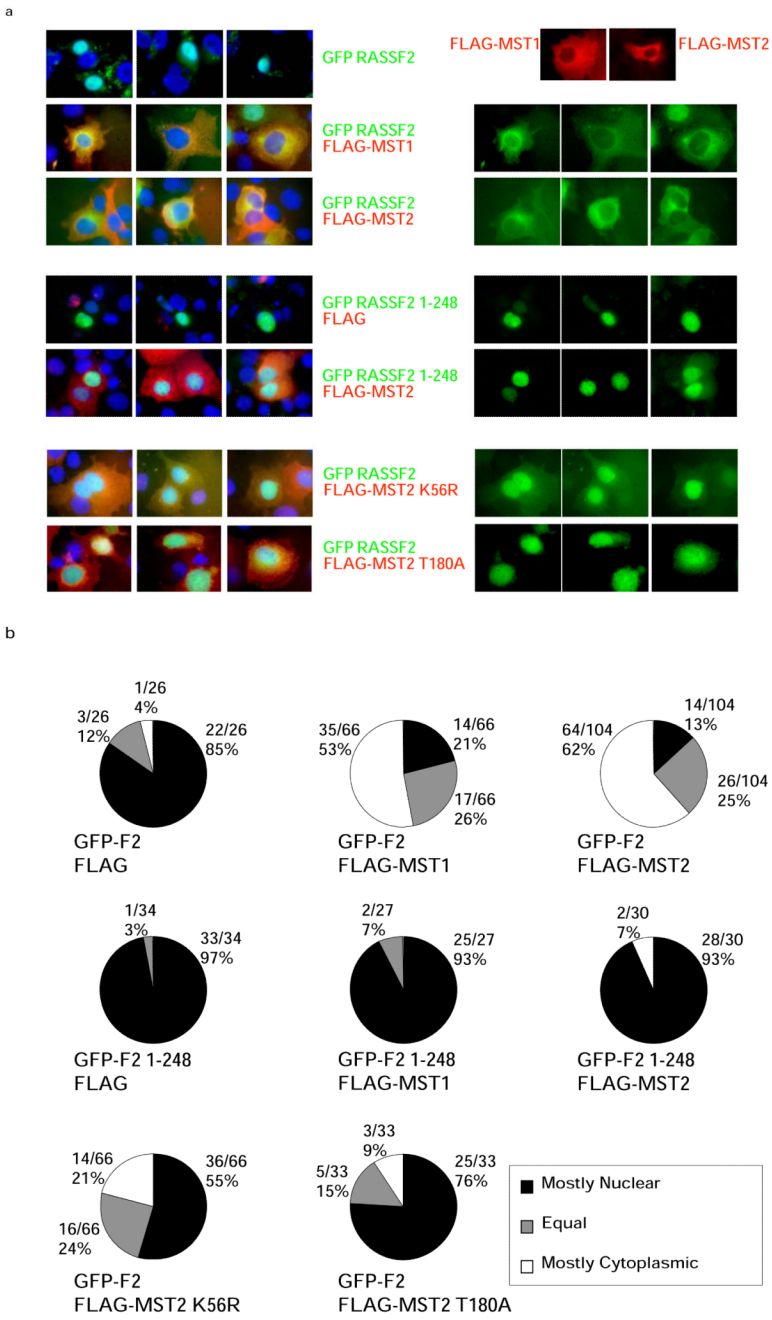

Figure 7. MST translocates RASSF2 to cytoplasm

(a) Cos7 cells were cotransfected with GFP-RASSF2 and either empty FLAG or FLAG tagged MST1 or MST2 or mutants, after 48 hours cells were fixed and stained with an anti-FLAG antibody and the number of cells exhibiting predominantly nuclear or predominantly cytoplasmic localisation determined. Three representative fields of view are shown for each transfection (b) above data depicted as a pie chart.