The differential effects of wildtype and mutated K-Ras on MST2 signalling are determined by K-Ras activation kinetics

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Abstract

K-Ras is frequently mutated in human cancers. Mutant (mt) K-Ras can stimulate both oncogenic transformation and apoptosis through activation of ERK and AKT pathways and the MST2 pathway, respectively. The biological outcome is determined by the balance and crosstalk between these pathways. In colorectal cancer (CRC) K-Ras mutation is negatively correlated with MST2 expression, as mt K-Ras can induce apoptosis by activating the MST2 pathway. However, wildtype (wt) K-Ras can prevent the activation of the MST2 pathway upon growth factor stimulation and enable transformation by mt K-Ras in CRC cells that express MST2. Here we have investigated the mechanism how wt and mt K-Ras differentially regulate the MST2 pathway and MST2 dependent apoptosis. The ability of K-Ras to activate MST2 and MST2 dependent apoptosis is determined by the differential activation kinetics of mt K-Ras and wt K-Ras. Chronic activation of K-Ras by mutation or overexpression of Ras exchange factors results in the activation of MST2 and LATS1, increased MST2-LATS1 complex formation and apoptosis. In contrast, transient K-Ras activation upon EGF stimulation prevents the formation of the MST2-LATS1 complex in an AKT dependent manner. Our data suggest that the close relationship between Ras pro-survival and pro-apoptotic signalling is coordinated via the differential regulation of the MST2-LATS1 interaction by transient and chronic stimuli.

Introduction

The Ras family of small GTPases comprises three isoforms H-Ras, N-Ras, and K-Ras (7). Mutations of these proteins, in particular K-Ras, are present in more than 30 % of cancers making Ras mutations one of the most frequent events in cancer (14). Upon growth factor stimulation Ras proteins cycle between an inactive state bound to GDP and an active state bound to GTP. Ras activation is induced by guanidine exchange factors (GEFs) and inactivation is catalysed by GTPase activating proteins (GAPs), which enhance the intrinsic ability of Ras to hydrolyse GTP (7). Activated Ras proteins bind to several effector proteins that mediate a range of different biological processes such as proliferation, differentiation and apoptosis. The best characterised Ras effectors are Raf-1, phosphoinositide-3 kinase (PI3K), and RalGDS (8). Raf-1 and PI3K pathways play a central role in the regulation of pro-survival and proliferation signals, and aberrant activation of these pathways is observed in most transformed cells (10, 35). These pathways interact at different levels through various positive and negative feedback loops upstream and downstream of Ras. Hence, the crosstalk between these important pathways plays a central role in cell fate decisions (2).

Recently the tumour suppressor RASSF1A was recognized as Ras effector, specifically of K-Ras (24, 29) .The expression of RASSF1A is frequently suppressed in cancer due to gene silencing by promoter methylation (1, 13). RASSF1A is a member of the Ras associated family of proteins that comprises ten genes, each featuring several splice variants. RASSF1A function is further regulated by phosphorylation (42) and is involved in the control of cell cycle progression, microtubule stability and apoptosis (13). How RASSF1A regulates most of its biological effects is not well understood, as it lacks

71 catalytic activity. However, its role in apoptosis is better studied. RASSF1A can trigger
72 apoptosis through at least two pathways. One involves binding of RASSF1A to the Bax
73 binding protein MOAP-1/MAP-1adaptor, which induces a conformational change that
74 activates the proapoptotic function of Bax (6, 40). The other mechanism relies of the
75 stimulation of the MST/Hippo pathway (5, 18), which is emerging as a central regulator
76 of organ size, cell polarization and apoptosis (25, 27). RASSF1A dissociates MST2
77 from the inhibitory complex with Raf-1 and stimulates MST2 kinase activity as well as
78 binding to its substrate LATS1 (26, 28). Activated LATS1 can phosphorylate different
79 effectors including YAP1. LATS1 phosphorylates YAP1 on several residues (44), which
80 have different functions. The phosphorylation of S127 plays a role in cell growth and
81 size control and inactivates the transcriptional function of YAP1 by promoting its
82 retention in the cytosol and degradation (30, 44). However, RASSF1A stimulation
83 causes LATS1 to phosphorylate YAP1 on a different residue(s), which is yet to be
84 identified, enabling YAP1 to translocate to the nucleus and bind p73 (26). The
85 YAP1/p73 complex activates the transcription of several pro-apoptotic genes (26, 38). In
86 addition, we have recently demonstrated that mt K-Ras regulates the MST2 – LATS1
87 pathway through RASSF1A in colorectal cancer (CRC) cells (3). In this case apoptosis
88 is due to LATS1 binding to and sequestering the ubiquitin ligase Mdm2 from p53, which
89 results in the stabilisation and activation of p53 and subsequent apoptosis (3). Thus,
90 depending on the mode of upstream activation the MST2-LAST1 pathway can utilise
91 different downstream effectors. Another intricacy of this pathway, at least in mammalian
92 cells, is its differential regulation by mt versus wt K-Ras. Whereas mt K-Ras stimulates
93 apoptotic signalling through this pathway, wt K-Ras can inhibit it (3).

Here, we have investigated the mechanistic basis for the differential regulation of the MST2 pathway by wt and mt K-Ras. We show that the different activation dynamics of mt K-Ras and RASSF1A are responsible for the differential effects of mt K-Ras and wt K-Ras on the MST2 pathway. We also present evidence that in response to growth factor stimulation RASSF1A specifically interacts with K-Ras but not H- or N-Ras. Furthermore, our data indicate that mt and wt K-Ras differ in their abilities to activate AKT, and that AKT activation is central for inhibiting the MST2-LATS1 pathway.

Material and methods

Constructs and siRNA. Constructs encoding pCEFL-HA-H-Ras, -K-Ras, -N-Ras, -K-RasV12, -H-RasV12 and –N-RasV12; pCEFL-Flag-GRF2, -K-RasV12, -H-RasV12 and –N-RasV12; pCEFL-AU5-SOS1 and pGEX-4T-RBD have been described before (4, 23). HA-RASSF1A and Flag-RASSF1A constructs have been described before (29). Myc-K-RasV12 was cloned in the pEF6 plasmid. siRNAs against MST2, RASSF1A, LATS1, wt K-Ras, YAP1 and p73 have been described and validated before (3, 26).

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Sub-confluent cells were transfected with LipofecAMINE 2000 (invitrogene) following manufacturer's instructions.

*Immunoprecipitation and immunoblotting***.** Immunoprecipitations were performed as described before (26). Briefly, cells were lysed in 20mM HEPES pH7.5, 150mM NaCl, 1% NP40, 2mM NaF, 10mM β-glycerophosphate, 2mM $Na_4P_2O_4$ and protease and phosphatase inhibitors. After incubation at 4^{o}C for 2 hours, immunoprecipitates were washed 3 times with lysis buffer containing 0.5 % NP-40, separated by SDS-PAGE and analysed by Western blotting.

Antibodies and reagents. All antibodies were from commercial sources: mouse monoclonal anti-HA (Santa Cruz); anti-HA-HRP 3F10 (Roche); rabbit polyclonal anti-MST2 (Epitomics); goat polyclonal anti-MST2 (C-19 Santa Cruz); mouse monoclonal anti-C-Raf (BD transduction laboratories); goat polyclonal anti-LATS1 (n-18 and g-16 Santa Cruz); rabbit polyclonal anti-YAP1 (Santa Cruz); mouse monoclonal anti-RASSF1A (ebioscience); rabbit polyclonal anti-RASSF1 (Santa Cruz); anti-p73 mAb (Ab4) (Neomarkers); anti-H-Ras mouse monoclonal, anti-K-Ras Mouse-monoclonal, anti-N-Ras rabbit polyclonal (Santa Cruz); mouse monoclonal ppErk and rabbit polyclonal Erk (Sigma); Rabbit polyclonal Phospho-YAP S127 (New England biosciences); mouse monoclonal Myc-Tag (Upstate); mouse monoclonal anti-AU5 (Covance); Rabbit polyclonal AKT, p-S308-AKT, p-S473-AKT and p-AKT-substrate cell signalling; rabbit polyclonal p-T180-MST2 cell signalling and (New England Biolabs); Mouse monoclonal GSK3B (Santa Cruz); Rabbit monoclonal Phospho-GSK3α/β (Ser21/9) (Cell signalling). LY294002, Akt inhibitor IV and EGF are from Calbiochem.

MST2 in gel kinase activity. MST2 kinase activity was measured as before (28). Briefly, cell lysates were divided in half and MST2 was immunoprecipitated from both fractions as above. For each sample one immunoprecipitate aliquot was Western blotted for MST2 as a loading control for the experiment, while the other immunoprecipitate aliquot was subjected to an in-gel kinase assay. For this purpose, immunoprecipitates were resolved on a SDS-PAGE gel containing myelin basic protein. The gel was washed 3 times to remove SDS with 20% propanol, 50mM Tris pH 8.0. and equilibrated with kinase buffer(40mM HEPES pH8.0, 10mM $MgCl_2$, 0.5mMEGTA and 50mM ATP 25mCi) [γ-^{32}P]ATP and then incubated in kinase buffer with 25 mCi ^{32}P-γ-ATP for 2

hours. After several washes with of 5% TCA and 1% sodium pyrophosphate the gel was dried and exposed to X-Ray film.

Ras pull down activation assay. Ras activation was measured as described previously (23). Briefly cells were lysed using MLB buffer (25mM HEPES pH 7.5, 150mM NaCl, 1% NP-40, 1% Na deoxycholate, 10% glycerol, 10mM $MgCl_2$ and protease and phosphatase inhibitors). Active (i.e. GTP-loaded) Ras was affinity purified using a recombinant GST-Raf RBD (Ras binding domain, amino acids 1–149) protein and detected by immunoblotting with anti-K-Ras antibody. Three experiments were quantified using ImageJ.

Apoptosis assays. Apoptosis levels were measured by assessing DNA fragmentation using PI staining by FACS as described before (28). The graphs show the quantitation of cells with fragmented, i.e. sub G1, DNA content from at least 3 independent experiments. Error bars represent standard deviation.

Results

RASSF1A selectively interacts with K-Ras in a growth factor regulated manner.

We recently have demonstrated that K-Ras binds RASSF1A in a GTP dependent manner, and that K-Ras can upregulate MST2 signalling through RASSF1A (3). This effect seemed to be specific for K-Ras since H-Ras and N-Ras failed to activate MST2 signalling. Moreover, while oncogenic mt K-RasV12 stimulated MST2 kinase activity and binding to its substrate LATS1, oncogenic mt H-RasV12 or mt N-RasV12 exerted

an inhibitory effect upon MST2 activation. These observations indicated that Ras isoforms differentially regulate the MST2 pathway. This differential effect of Ras isoforms may be due to direct regulation of RASSF1A signalling by the three isoforms or by indirect regulation of the MST2 pathway through crosstalk with other signalling pathways regulated by N- and H-Ras. A possible interaction of Ras with RASSF1A proteins has been described in the literature (15). However, the reports were contradictory. For instance, it has been reported that RASSF1A binds preferentially to H-Ras (21, 33) or K-Ras (13). These discrepancies likely arise from the use of overexpression systems, different assays or different cell types. Therefore, in order to clarify this issue we re-evaluated this issue using physiological growth factor stimulation in two experimental systems, i.e. MCF7 breast carcinoma and HeLa cervical carcinoma cells. In these cells we have a wealth of data on the biochemical and biological consequences of MST2 signalling, in particular the induction of apoptosis (26). HeLa is one of the few immortalized cell lines that retain RASSF1A expression, while MCF7 cells lack endogenous RASSF1A expression.

Therefore, we tested whether H-Ras and K-Ras can bind to RASSF1A upon serum stimulation of MCF7 cells transfected with a HA-RASSF1A expression vector (Fig. 1A). Endogenous K-Ras co-immunoprecipitated with exogenously expressed RASSF1A, and RASSF1A co-immunoprecipitated with endogenous K-Ras. Serum increased this interaction and also induced the co-precipitation of MST2 suggesting that RASSF1A can bind to K-Ras and MST2 in a growth factor induced manner. Both the RASSF1A and MST2 interaction with K-Ras increased steadily over a timecourse of 45 minutes serum stimulation (Fig. S1) K-Ras also co-immunoprecipitated with endogenous Raf-1

in a serum stimulated fashion, but this interaction preceded the interaction of K-Ras with MST2 showing that K-Ras engages the Raf-1 and MST2 pathways with different kinetics. By contrast, in the same type of experiment we did not observe any interaction of RASSF1A with endogenous H-Ras or N-Ras (Fig. 1A and data not shown), indicating that K-Ras is the only member of the Ras family that can bind to RASSF1A. It is possible that this observation was due to different levels of expression of the Ras isoforms in MCF7 cells or to the different affinity of the Ras isoform specific antibodies. In addition, binding of RASSF family members to different Ras isoforms has been reported, but only investigated in depth for RASSF5 (37). RASSF1A was previously reported to associate with K-Ras proteins using heterodimerization with RASSF as intermediate (29). Using purified protein fragments the isolated Ras association domain of both RASSF5 and RASSF1A also were shown to directly bind to recombinant H-Ras protein in vitro (37, 41). In transfection experiments RASSF1A co-immunoprecipitated with activated K-Ras (33), and one study reported as unpublished results that RASSF1A binds better to K-Ras than H-Ras (13). Our own data showing that mt K-Ras, but not mt H-Ras or mt N-Ras can activate MST2 (24) suggested that RASSF1A binding may be selective for Ras isoforms. However, it is problematic to draw definitive conclusions from the results of the previous experiments as they are difficult to compare having been performed in very different experimental settings. Therefore, we directly compared the capacities of H-, K-, and N-Ras isoforms to associate with RASSF1A under the same experimental conditions (Fig. 1B). For this purpose we co-expressed HA-tagged-Ras family members with Flag-RASSF1A and stimulated the cells with EGF, which is a known regulator of Ras and MST2 (3, 12, 39). Only K-Ras co-precipitated

with RASSF1A, confirming that K-Ras selectively associates with RASSF1A (Fig. 1B). This result is further supported by the observation that K-RasV12 co-immunoprecipitated with RASSF1A, while no mt H-RasV12 or N-RasV12 could be co-immunoprecipitates with RASSF1A (Fig. S2).

Interestingly, EGF regulated the interaction of K-Ras with RASSF1A in a bimodal way, showing a transient peak at 15 minutes and a second peak at the 45 and 60 minutes timepoints. This binding pattern followed the activation kinetics of K-Ras in MCF7 cells transfected with RASSF1A and stimulated with EGF, where K-Ras activation occurred biphasically with a first peak at 5 minutes and a second smaller peak commencing after 45 minutes of stimulation (Fig. 1C). Interestingly, the first peak coincided with K-Ras binding to Raf-1, while Raf-1 binding was back to basal levels at the second peak of K-Ras activation These results show that EGF induces the formation of K-Ras-RASSF1A and K-Ras-Raf-1 complexes in a GTP dependent, but kinetically different manner. They further suggest that K-Ras may coordinate different biological effects resulting from the differential activation of the Raf-1 versus the RASSF1A pathway as specified by the K-Ras activation kinetics.

EGF suppresses the pro-apoptotic MST2 pathway downstream of RASSF1A

In light of the above data and our previous work showing that activation of the EGF receptor can protect against MST2 induced apoptosis (3), we studied how EGF stimulation regulates the MST2 pathway in HeLa cells. These cells express endogenous RASSF1A and allowed us to investigate endogenous protein complexes (Fig. 2A). In unstimulated cells MST2 kinase activity was low, and MST2 co-immunoprecipitated with Raf-1 and LATS1 but not with RASSF1A. EGF stimulation rapidly disrupted the

association between MST2 and LATS1, and subsequently induced binding of MST2 to RASSF1A and Raf-1. Concomitant with these changes in association MST2 kinase activity was first reduced and then enhanced by EGF. We have previously shown that MST2 bound to Raf-1 is inhibited, while MST2 bound to RASSF1A is activated (3). As Raf-1 and RASSF1A compete for MST2 binding, the MST2-Raf-1 and MST2-RASSF1A complexes are mutually exclusive but coexist as separate complexes (3), Thus, albeit EGF increases the sequestration of MST2 into the inhibitory complex with Raf-1, it also enhances the formation of the activating MST2-RASSF1A complex resulting in an overall increase in MST2 kinase activity at later time points (Fig. 2A). These observations suggested an intricate coordination of the kinetics of MST2 activity by EGF.

Therefore, we analysed other EGF effector pathways that are known to impinge on the regulation of MST2. AKT can phosphorylate MST2 on Thr^{117} and Thr^{384}, in a PI3K dependent manner, inhibiting MST2 kinase activity by preventing its auto-phosphorylation on Thr^{180} and enhancing its binding to Raf-1 (34). Hence, we analysed EGF mediated AKT activation and MST2 phosphorylation in more detail. For this purpose we used an antibody specific for AKT substrate phosphorylation motifs that allowed us to monitor the AKT-directed phosphorylation of MST2 (34). In parallel, we measured MST2 activation by performing in gel kinase assays or using an anti-T180-MST2 specific antibody, which detects an auto-phosphorylation event that is essential for MST2 activation (32). While EGF activated AKT rapidly, coinciding with the disruption of the MST2-LATS1 complex, MST2 kinase activation occurred later when AKT activity was declining (Fig. 2A). Chemical inhibitors of AKT (AKT I) or its upstream

activator PI3K (LY294002) completely reverted the EGF mediated suppression of MST2 auto-phosphorylation and therefore its activation (Fig. 2B). These results indicate that the high level of AKT activity triggered by EGF early after stimulation promotes sequestration of MST2 into the inactive complex with Raf-1, while the decline of AKT activity at later time points permits MST2 activity to rise. Interestingly, the increase in MST2 kinase activity occurs at a time when MST2 is dissociated from its substrate LATS1, suggesting that it cannot translate into downstream effects mediated by LATS1. Consequently, EGF activates MST2 but interferes with its pro-apoptotic functions by disrupting MST2 binding to its substrate LATS1. By contrast, mt K-Ras enhances both MST2 kinase activity and binding to LATS1 leading to an increase in apoptosis (3). These results suggest that K-Ras promotes pro-apoptotic signalling through the MST2 pathway when constitutively activated by mutation, but interferes when transiently activated by physiological growth factors. We currently do not know the mechanistic basis for this differential effect of EGF and mt K-Ras, but this finding is consistent with our previous results showing that EGF activation of wt K-Ras interferes with MST2 mediated apoptosis triggered by mt K-Ras (3).

To confirm that the effect of EGF on the MST2-LATS1 interaction is mediated by K-Ras we used the dominant inhibitory mutant K-RasN17 (Fig. 2C). This mutant selectively inhibits the activation of endogenous K-Ras with minor effects on H-Ras and N-Ras activation (23). Expression of K-RasN17, but not of H- and N-RasN17 (Fig. S3), prevented the decrease of the MST2-LATS1 interaction caused by EGF supporting the hypothesis that the inhibitory effect of EGF on MST2 signalling involves the K-Ras dependent uncoupling of MST2 from LATS1. K-RasN17 also impaired the activation of

277 AKT by EGF as well as the phosphorylation of MST2 by AKT (Fig. 2C). As previously
278 shown this phosphorylation inhibits MST2 (34), suggesting that K-Ras can inhibit MST2
279 via activation of AKT. We confirmed this hypothesis by expressing the FLAG-tagged
280 MST2 (T117/384AA) double mutant. Contrary to what happened to FLAG-tagged MST2
281 wt, the interaction of this mutant with LATS1 was not affected by EGF stimulation
282 confirming that AKT mediates this effect (Fig. 2D).

283 In order to compare the role of different Ras isoforms in the activation of AKT upon EGF
284 stimulation we used dominant inhibitory mutants of each Ras isoform. H-RasN17 is able
285 to inhibit the activation of all three Ras isoforms, while K-RasN17 and N-Ras specifically
286 inhibit the activation of their cognate wild type proteins and to a lesser degree the
287 activation of H-Ras, allowing to discriminate which Ras isoform are mediating the
288 activation of their different effectors (23). We expressed the three Ras dominant
289 inhibitory mutants and treated the cells with EGF for 10 minutes (Fig 2E). EGF
290 activation of AKT was severely reduced by the expression of the K-RasN17 (~60%
291 reduction) and H-RasN17 (~43% reduction) while N-RasN17 (~26% reduction) was less
292 effective, indicating that K-Ras is the main Ras isoform mediating AKT activation upon
293 EGF stimulation.

294 These data suggested that EGF activated wt K-Ras should have an inhibitory effect on
295 the activation of the MST2 pro-apoptotic signalling pathway initiated by RASSF1A. In
296 order to test this hypothesis we assessed how EGF affected the effects of RASSF1A
297 expression on downstream signalling events. As previously reported, RASSF1A causes
298 the release of YAP1 from LATS1 and binding of YAP1 to p73, which together activate
299 expression of the pro-apoptotic BH3 domain protein Puma (26).The decrease of LATS1-

YAP1 interaction caused by RASSF1A was rescued by treatment with EGF (Fig. 2F). These finding reveal opposite effects of EGF and stimulation of the Fas death receptor, which elevated MST2 kinase activity, interaction with its substrate LATS1, and promoted YAP1-p73 binding (3). This is likely due to the activation of AKT antiapoptotic signal mediated by EGF-activated K-Ras.

Oncogenic K-Ras activates the MST2 pathway pro-apoptotic signal in HeLa cells.

In CRC cells oncogenic mt K-Ras promotes apoptosis by activating the MST2 pathway, and this requires EGF receptor activity and a wt K-Ras allele (3). The above data suggest that in HeLa cells wt K-Ras can suppress MST2 pathway activation by activating AKT and disrupting the interaction between MST2 and LATS1. These findings prompted us to perform a detailed study of how oncogenic mt K-Ras affects the MST2 pathway in HeLa cells. Over-expression of increasing amounts of mt K-RasV12 activated MST2 kinase activity and increased MST2-RASSF1A and Raf-1-MST2 interactions in a dose dependent manner (Fig. 3A). In contrast, expression of oncogenic mt H- or N-RasV12 mutants did not enhance MST2 kinase activity but reduced MST2 activation (Fig. 3B and Sup, Fig S4). This effect is probably due to an indirect effect of this mutants on the regulation of the MST2 pathway since only wt and mt-K-Ras, but not H- or N-Ras can bind RASSF1A in these cells (Fig. 1 and Fig S2). Overall, these results showed that amongst the different oncogenic Ras isoforms the MST2 pathway is specifically activated by mt K-Ras. Therefore, these observations are similar to what we observed when wt K-Ras was activated by physiological stimuli, such as EGF (Fig. 1). In order to track down the differences between wt and mt K-Ras signalling we investigated downstream MST2 signalling. K-RasV12 but not H-RasV12 and N-RasV12

increased the binding of MST2 to LATS1 (Fig. 3C and Fig. S4B). By contrast, EGF disrupted the MST2-LATS1 complex (Fig. 2). In MCF7 cells, which do not express endogenous RASSF1A, both MST2 kinase activity and LATS1binding were dramatically enhanced by the co-expression of RASSF1A and K-RasV12 (Fig.3D and Fig S5), indicating that K-RasV12 cooperates with RASSF1A to activate LATS1.

Further downstream, K-RasV12 did not affect the association of LATS1 with YAP1 or YAP1 phosphorylation on S127 (Fig. 3E), which is thought to be a critical phosphorylation site for enabling the oncogenic potential of YAP1. This observation also indicated that mt K-Ras activation of apoptosis through the MST2 pathway was not mediated by YAP1-p73, as we had previously observed when the MST2 pathway was activated by Fas or overexpression of RASSF1A (26). Therefore, we tested whether mt K-Ras induced apoptosis of HeLa cells was dependent on the other components of the MST2 pathway (Fig. 3F and Fig S6). siRNA mediated down-regulation of RASSF1A, MST2 and LATS1, but not of YAP1 and p73 rescued mt K-Ras induced apoptosis confirming that mt K-Ras induced apoptosis in HeLa is dependent on the kinase core of the pathway, but uses an effector different from p73.

Differential activation of AKT by mt K-Ras and wt K-Ras explains their distinct regulation of the MST pathway.

These results confirmed the fundamental differences in the signalling properties of wt versus mt K-Ras. While mitogen activated wt K-Ras inhibits the MST2 pathway, mt K-Ras activates it. This functional divergence centres on the regulation of MST2-LATS1 and LATS1-YAP1 binding. Both the formation of MST2-LATS1 and disruption of LATS1-YAP1 complexes are critical for the induction of apoptosis by RASSF1A (26). EGF

disrupts MST2-LATS1 complexes, whereas mt K-Ras promotes MST2-LATS1 binding. In addition, EGF, but not mt K-Ras, antagonises the RASSF1A induced decrease of LATS1-YAP1 binding.

We have previously shown that AKT can inactivate MST2 by direct phosphorylation (34), and that the antiapoptotic effect of wt K-Ras in CRC cells requires the activation of AKT signalling (24). Now we observed that upon EGF stimulation K-Ras dependent activation of AKT inhibits MST2 activation (Fig. 2D). Our previous findings also indicated that in CRC the inhibition of MST2 by AKT was dependent on the presence of wt K-Ras, as mt K-Ras was not able to exert this inhibition when HCT116 cells were depleted of wt K-Ras. We observed the same paradoxical interaction between wt K-Ras and mt K-Ras in HeLa cells (Fig 4A) where the mt K-Ras induction of MST-LAST1 interaction was rescued by EGF stimulation. This regulation of the MST2 pathway is mediated by AKT as PI3K inhibition increases the MST2-LAST1 interactions and rescued MST2 inhibition caused by EGF (Sup. Fig S7). This made us wonder whether mt K-Ras and EGF-activated wt K-Ras could differentially regulate AKT activation. To test this hypothesis we transfected all three oncogenic mt Ras isoforms into HeLa cells and compared their effects on AKT activation with EGF (Fig. 4B). The expression of mt Ras isoforms resulted in a modest activation of AKT with N-RasV12 being the strongest activator, H-RasV12 the weakest, and K-RasV12 intermediate. By contrast, EGF treatment caused a much higher activation of AKT than any of the oncogenic RasV12 proteins (~ 3fold > mt K-Ras). Similarly, we observed that EGF activated ERK1/2 stronger than mt RasV12, although in this case H-, K- and N-RasV12 activated ERK at similar levels. Importantly, the different abilities of EGF and mt RasV12 proteins to activate AKT

translated into a marked disparity of MST2 phosphorylation. Only EGF stimulated the phosphorylation of MST2 on AKT consensus sites, while RasV12 proteins were ineffective. The different levels of AKT and ERK1/2 activation caused by EGF and transfected RasV12 constructs are not due to poor transfection, as the transfection efficiency was ~90%. In order to assess for dosage effects, we transfected increasing amounts of K-RasV12 showing that under the conditions used the activation of ERK and AKT is saturated (Fig. 4C). Thus, our data suggest that EGF interrupts MST2 signalling by stimulating the inhibitory phosphorylation of MST2 by AKT, whereas mt Ras is ineffective. The lower level of ERK1/2 and AKT1 activation caused by oncogenic Ras isoforms is likely due to the activation of negative feedback loops that downregulate these pathways in response to chronic stimulation (2). To test this hypothesis in our cell systems, we performed EGF stimulation time course experiment in MCF7 and monitored the effect of chemical inhibition of the AKT/ERK pathways. Our data indicate that in MCF7 cells there is an ERK-dependent negative regulation of AKT activation (Fig 4D and sup fig S8). Furthermore, this feedback loop is also modulated by RASSF1A (Fig. 4E and Fig. S9), indicating that the regulation of AKT and ERK signalling is also regulated by the MST2 pathway.

Mutant K-Ras induced apoptosis is due to chronic K-Ras activation

The above results suggested that the sustained activation of mt Ras may be responsible for the inability to constrain MST2 activity. Another explanation for this paradoxical effect of K-Ras signalling upon MST2 may be due to qualitatively different biochemical properties of oncogenic mt K-Ras and wt K-Ras. To distinguish between

these two scenarios we used Hke3 cells ($KRAS^{-/wt}$), which are an isogenic derivative of HCT116 ($KRAS^{mt/wt}$) cells where the mt KRAS allele has been knocked out (36). We previously used this cell line pair to show that mt K-Ras induces apoptosis via activation of the MST2 pathway (24). We over-expressed two Ras guanidine exchange factors (RasGEFs), SOS1 and GRF2 that cause chronic activation of endogenous K-Ras (23), or stimulated the cells with EGF for 5 minutes or 16 hours. Over-expression of SOS1 and GRF2 resulted in a constitutive activation of endogenous K-Ras (Fig. 5A) while EGF stimulation resulted in a transient activation of K-Ras, which had returned to basal levels at the 16 hour time point. Importantly, the overexpression of SOS1 or GRF2 induced apoptosis (Fig. 5B) suggesting that apoptosis is a consequence of the chronic hyperactivation of K-Ras, i.e. a quantitative trait rather than a qualitative difference between mt and wt K-Ras signalling.

We also confirmed this hypothesis by experiments in HeLa cells. EGF activated both ERK and AKT. Over-expression of SOS1 and GRF2 induced a slight stimulation of ERK activity, but no AKT activation was observable as detected by the phosphorylation of AKT substrates or AKT itself (Fig. 5C). Furthermore, overexpression of SOS and GRF2 strongly increased the MST2-LATS1 interaction (Fig. 5D), Their effect on MST2 autophosphorylation was less dramatic and rather subtle with GRF2 as compared to SOS. The reason for this differential behaviour is unclear at present. present, As seen in Hke3 cells, SOS1 and GRF2 over-expression increased apoptosis, which could be abrogated by siRNA mediated downregulation of K-Ras or MST2 (Fig. 5E). These results strongly indicated that the chronic activation of K-Ras either by mutation or

overexpression of RasGEFs is sufficient for the activation of apoptosis and that the MST2 pathway is mediating this effect.

Discussion

K-Ras mutation is one of the most frequent events in cancer (14), but paradoxically K-Ras is the only member of the Ras family that can activate apoptosis (11). Our recent work has helped to identify the MST2 pathway as one of the signalling pathway that mediates K-Ras pro-apoptotic effects and shown that this apoptosis signal is repressed to allow CRC progression (3). Oncogenic K-Ras activates the RASSF1A-MST2-LATS1 pathway resulting in the stabilisation and activation of the tumour suppressor p53 due to LATS1 mediated sequestration of the p53 ubiquitin ligase MDM2. We also have shown a statistically significant inverse correlation between MST2 expression and KRAS gene mutation in human CRC patients with metastatic disease (3), suggesting that shutting down the pro-apoptotic signal mediated by MST2 is necessary for mt K-Ras driven cancer progression. Interestingly, we also observed that wt K-Ras can prevent mt K-Ras induced apoptosis by inhibiting MST2 signalling. This anti-apoptotic effect of wt K-Ras requires EGFR signalling and the activation of AKT. The evidence described here provides a mechanistic explanation of the differences in MST2 pathway regulation by wt K-Ras and mt K-Ras.

RASSF1A is a specific effector of K-Ras. Both mt K-Ras and wt K-Ras bind to RASSF1A promoting the formation of the RASSF1A-MST2 interaction. However, they have very different effects on the downstream regulation of the pathway. Wt K-Ras activated by EGF interrupts the pathway by physically decoupling MST2 from LATS1.

On the other hand, mt K-Ras increases the formation of the MST2-LAST1 complex leading to enhanced apoptosis. These differences are encoded by the activation kinetics rather than qualitative differences in signalling between mt and wt K-Ras, as the chronic activation of wt K-Ras by over-expression of RasGEFs also induces apoptosis.

According to our data a main difference between mt K-Ras and EGF activated wt K-Ras is their ability to activate AKT. While mt K-Ras only weakly activates AKT, EGF causes robust AKT activation and induces AKT mediated phosphorylation of MST2. AKT phosphorylation of MST2 blocks MST2 activity and binding to RASSF1A, but promotes MST2 interaction with Raf-1(34). This mechanism can change the ratio between the inactive and active populations of MST2 that are bound to Raf-1 and RASSF1A, respectively. Therefore, K-Ras promotes two competing pathways: one by recruiting RASSF1A and MST2, and another via AKT activation that stimulates binding of MST2 to Raf-1. Indeed, EGF enhances the formation of both MST2-Raf-1 and MST2-RASSF1A complexes. These complexes compete with each other both in terms of formation and effects. The existence of negative feedbacks from ERK1/2 and AKT is likely to explain the differential regulation of the MST2 pathway mediated by mt K-Ras and wt K-Ras. Chronic activation of K-Ras downstream pathways results in the activation of several positive and negative feedback loops (2) that may be responsible for the different biological effects mediated by this protein. The nature of the feedback loops that are regulating the crosstalk among the ERK and AKT pathways seem to be cell type specific and may explain the resistance to drugs specifically developed to target these pathways (2, 10).

458 In summary the current work explains the differential regulation by wt K-Ras and mt K-
459 Ras of the MST2 pathway. It also helps to explain how wt K-Ras collaborates with mt K-
460 Ras to facilitate CRC progression. An interesting possibility is that MST2 activity might
461 be also regulated by the other Ras isoforms. Although H-Ras and N-Ras do not interact
462 with RASSF1A, expression of their oncogenic mutants decreases MST2 kinase activity
463 (Fig. 3B). Interestingly, we also have observed that the over-expression of oncogenic H-
464 Ras and N-Ras decrease MST2 kinase activity in HCT116 CRC cells (3), pointing to the
465 possibility that these isoforms may also prevent the activation of the MST2 pathway in
466 this cell system. Although we could not detect H-RasV12 and N-RasV12 induction of
467 MST2 phosphorylation by AKT in HeLa cell, it is possible that these proteins may induce
468 such effect in other cells systems. There is clear evidence from the literature that the
469 different Ras isoforms differentially activate PI3K-AKT in a cell and tumour specific
470 fashion (9). For instance, collaboration of wt N-Ras with mt H-Ras has been proposed
471 to be necessary for the maintenance of the transformed phenotypes in different cells
472 (19). It has also been reported that a crosstalk between K-Ras and N-Ras signals may
473 be necessary for the regulation of migration and proliferation in transformed cells (16,
474 22). Two recent reports have increased the evidence for collaboration between mut Ras
475 and wt Ras isoforms in cancer, showing that SOS allosteric activation by mt-K-Ras is
476 responsible for the activation of wt Ras isoforms and necessary for tumorigenesis and
477 that the wt Ras isoform are regulating EGFR downstream signals to facilitate mut Ras
478 transformation(20, 43). Interestingly, mutations in all three Ras isoform have been
479 related with poor prognosis in thyroid tumours and concomitant mutations of K- and N-
480 Ras are observed in myeloma (17, 31). All these data strongly support the notion that

crosstalk between the different Ras isoform and their wt and mt versions can play a major role in tumour maintenance (16).

The observation that the activation of MST2 pro-apoptotic signal is closely regulated by the ERK and the AKT pathways and EGFR signalling at different levels may be of use in the design of new therapeutic treatments. Conceivably, preventing the phosphorylation of MST2 by AKT, or the inhibitory interaction of MST2 with Raf-1 would allow the activation of apoptosis by MST2. Drugs designed to activate MST2 kinase activity may help to prevent the resistance observed to the therapeutic agents specifically targeting the ERK and PI3K-AKT pathways shown in many clinical trials (3).

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Figure legends

Figure1. A) MCF7 cells were transfected with 1µg HA-RASSF1A. After 16h in 0.1% serum, cells were treated for the indicated time by re-adding serum to a final concentration of 10%. Cell extracts were split in two and immunoprecipitated with anti-HA, anti-K-Ras (upper panel) or anti-H-Ras (lower panel) and western blotted

503 with the indicated antibodies. **B)** MCF7 cells were cotransfected with FLAG-
504 RASSF1A and HA-K-, HA-H-or N-Ras wt. After 16 hour starvation (0.1% serum), the
505 cells were treated with EGF (100ng/ml) for the indicated times. HA
506 immunoprecipitates were subjected to western blotting with the indicated antibodies.
507 C) Endogenous K-Ras GTP levels were measured in EGF (100ng/ml) stimulated
508 MCF7 cells transfected with Flag-RASSF1A. Error bars show standard deviation.

509 **Figure2. A)** Serum-starved HeLa cells were treated with EGF for the indicated
510 times. MST2 immunoprecipitates were subjected to an in-gel kinase assay (MST2
511 KA) or western blotted with the indicated antibodies. LATS1 immunoprecipitates
512 were blotted with the indicated antibodies. Numbers give fold of kinase activation
513 obtained by dividing kinase activity by amount of MST2 immunoprecipitated. **B)**
514 HeLa cells were treated with LY294002 (10μM) or Akt Inhibitor (10μM) for 45
515 minutes, and stimulated with 10nM EGF for a further 10minutes. Cell lysates (10μg)
516 were analysed by Western blot using antibodies against phospho- and total proteins
517 as indicated. **C)** HeLa cells were transfected with empty vector (-) or Flag-K-
518 RasN17 (+). After serum starvation the cells were treated with 10nM EGF for 5 or 45
519 minutes. The lysates were split in half and immunoprecipitated with MST2 or LATS1
520 antibodies. The immunoprecipitates were blotted with the indicated antibodies .**D)**
521 Hela cells were transfected with 1μg of wt FLAG-MST2 (left panels), or the double
522 mutant FLAG-MST2 T117/384A (Right panels), and HA-K-RasN17 where indicated.
523 After 16 hours in 0.1% serum the cells were treated with 10nM EGF for 45 min. Cells
524 lysates were split in half and immunoprecipitated with anti-MST2 or anti-LATS1
525 antibodies. The immunoprecipitates and cell extracts were western blotted with the

indicated antibodies **E**) HeLa cells were transfected with empty vector or H-RasN17, K-RasN17 or N-RasN17 respectively, then stimulated with 10nM EGF for 10 minutes. Cell lysates (10µg) were analysed by Western blotting using antibodies against phospho- and total proteins as indicated**. F)** Serum-starved MCF7 cells transfected with HA-RASSF1A or empty vector were treated with 10 nM EGF for 45 minutes. LATS1 immunoprecipitates were blotted for MST2 and YAP1 coprecipitation.

Figure3 A) HeLa cells were transfected with increasing amounts of Myc-K-RasV12. MST2 was immunoprecipitated and blotted for LATS1, Raf-1 and RASSF1A coprecipitation. In-gel kinase assay was performed for MST2 immunoprecipitates (MST2 KA). **B)** HeLa cells were transfected 0.5µg of HA-H-RasV12, HA-K-RasV12 or HA-N-RasV12 as indicated. After 16 hour in 0.1% serum the cell lysates were western blotted with the indicated antibodies **C)** LATS1 and MST2 co-immunoprecipitation in HeLa cells transfected with Myc-K-RasV12. **D)** MCF7 cells were co-transfected with HA-RASSF1A and FLAG-K-RasV12. MST2 and LATS1 immunoprecipitates were assayed for co-precipitation. Total lysates were western blotted with the indicated antibodies . **E)** HeLa cells were transfected with increasing amounts of HA-K-RasV12. After 20 hours of serum-starvation cells were lysed and LATS1 immunoprecipitates were blotted for YAP1 co-precipitation. p73 protein levels and S127 phosphorylation were monitored in the extracts using specific antibodies. **F)** HeLa cells were transfected with HA-K-RasV12 expression plasmid and 50ng/ml siRNAs against RASFF1A, MST2, LATS1, p73 as indicated. A non-targeting siRNA pool was used as control. Protein expression was monitored by

549 Western blotting. Cells were assayed for apoptosis by measuring DNA
550 fragmentation. Error bars show standard deviation (n=3).

551 **Figure 4. A)** HeLa cells were transfected with HA-K-Ras were indicated**.** After
552 serum starvation for 20 hours the cells were treated with 10nM EGF and 10 nM
553 LY294002 as indicated. MST2 was immunoprecipitated and blotted for LAST1
554 interaction. Lysates were blotted with the indicated antibodies. **B)** HeLa cells were
555 transfected with 1 µg of Flag-H-, K- or N-RasV12 expression plasmids or treated
556 with EGF for 5 minutes as indicated. MST 2 was immunoprecipitated and examined
557 for phosphorylation on AKT consensus sites by an α-AKT substrate antibody Protein
558 expression and phosphorylation in lysates were monitored with the indicated
559 antibodies. **C)**.HeLa cells were transfected with 0.5 or 1µg of Flag-K-Ras V12
560 plasmid. Cells were serum starved for 20 hours before lysates were blotted with the
561 indicated antibodies. D) MCF7 cells were serum deprived where indicated and
562 treated with 10µM UO126 for 1 hour as indicated. Cells were the incubated with
563 10nM EGF for the indicated times. Lysates protein levels and phosphorylation were
564 monitored with the indicated antibodies. D) MCF7 transfected with increasing
565 amount of HA-RASSF1A were serum starved for 20 hours and treated with 10nM of
566 EGF for the indicated times. Lysates were blotted with the indicated antibodies.)

567 **Figure 5. A)** Hke3 cells were transfected with AU5-SOS1 or Flag-GRF2
568 expression plasmids or treated with EGF (100ng/ml) for the indicated times. The
569 levels of activated K-Ras were measured by pull-down. **B**) Hke3 cells were
570 transfected with AU5-SOS1 or GRF2 or treated with EGF for 20 hours and the levels
571 of apoptosis were determined by measuring DNA fragmentation. Error bars show

standard deviation (n=6). **C)** HeLa cells were transfected as in A), and lysates were blotted with the indicated antibodies. **D**) HeLa cells were transfected with AU5-SOS1 and Flag GRF2. After 16 hours in 0.1% serum the cells were lysated and immunoprecipitated with anti-MST2. Lysates were blotted with the indicated antibodies. **E)** HeLa cells were transfected with the indicated plasmids or siRNAs, and the levels of apoptosis were measured as above. SCRB, scrambled, non-targeting siRNA. Error bars show standard deviation (n=4).

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