



Title	Mutant K-Ras Activation of the Proapoptotic MST2 Pathway Is Antagonized by Wild-Type K-Ras
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Title: Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wildtype K-Ras

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Abstract: K-Ras mutations are frequent in colorectal cancer (CRC), albeit K-Ras is the only Ras isoform that can elicit apoptosis. Here we show that mutant K-Ras directly binds to the tumour suppressor RASSF1A to activate the apoptotic MST2-LATS1 pathway. In this pathway LATS1 binds to and sequesters the ubiquitin ligase Mdm2 causing stabilisation of the tumour suppressor p53 and apoptosis. However, mutant Ras also stimulates autocrine activation of the EGFR which counteracts mutant K-Ras induced apoptosis. Interestingly, this protection requires the wildtype K-Ras allele, which in part via AKT activation inhibits the MST2 pathway. Confirming the pathophysiological relevance of the molecular findings we find a negative correlation between K-Ras mutation and MST2 expression in human CRC patients and CRC mouse models. The small number of tumours with co-expression of mutant K-Ras and MST2 has elevated apoptosis rates. Thus, in CRC mutant K-Ras transformation is supported by the wildtype allele.

Suggested Reviewers:

Opposed Reviewers:

RE: MOLECULAR-CELL-D-11-00147

"Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wildtype K-Ras"

Dear Dr. Evans,

Thank you for the reviewers' comments and your invitation to submit a revised version of the paper. We have performed additional experiments as advised by the reviewers, and revised the paper according to their suggestions. We are now in a position to present a manuscript that fully addresses the reviewers' concerns. A detailed response is included below. I hope the reviewers will find the revisions satisfactory, and we are looking forward to hearing from you.

Best wishes,

Walter Kolch

MOLECULAR-CELL-D-11-00147

Response to Reviewers' Comments

Our responses are in blue. Figures for the reviewers are at the end.

Reviewer #1:

In this manuscript the authors claim that:

- * The RASSF1A-MST2-LATS1 pathway is regulated by mutant K-Ras
- * The effectors of this pro-apoptotic pathway are Mdm2 and p53 and not YAP and p73
- * wt K-Ras is activated by EGF and EGFR to regulate MST2 and therefore MST2-dependent apoptosis
- * MST2 is downregulated in colorectal carcinomas expressing mutant K-Ras, especially at advanced stages.

There are few major points which make the findings unclear and/or controversial. This consequently makes the manuscript not suitable for acceptance in Mol Cell ,at least, in its present form.

Major points:

- * In Fig.1 the authors show that "MST2 exists in two distinct protein complexes": a complex with RASSF1A and another with Raf-1. MST2 bound to RASSF1A is the active MST2 while inactive MST2 would be bound to Raf-1. However, since the levels of MST2 coimmunoprecipitated with RASSF1A are much higher than the levels coimmunoprecipitated with Raf-1, it is not possible to claim that MST2 complexed with RASSF1A is more active than MST2 bound to Raf-1.

The reviewer seems to have missed the data presented in Fig. S1D. We wrote in the text: *"A quantitative comparison of the MST2 activities associated with Raf-1 and RASSF1A in HCT116 cells showed that MST2 is >5fold more active when bound to RASSF1A (Fig. S1D)."* RASSF1A co-immunoprecipitates more MST2 than Raf-1. This was shown in Fig. 1D. In order to be able to compare the kinase activities of MST2 bound to RASSF1A and Raf-1 we had included Fig. S1D, where we quantitated MST2 kinase activity in Raf-1 immunoprecipitates and a titration curve of RASSF1A immunoprecipitates, where we could directly compare kinase activity based on equal amounts of MST2 protein contained in Raf-1 and RASSF1A immunoprecipitates. These results showed that MST2 associated with RASSF1A is >5fold more active than MST2 associated with Raf-1. Thus, our original conclusion is valid. We also now have included a new, extended Fig. 1D which shows the reciprocal immunoprecipitations and contains additional control IPs with irrelevant antibodies as requested by reviewer 2. A quantitation of the sequential IPs in the new Fig. 1D also shows that MST2 kinase activity (normalized to the amount of co-precipitating MST2 protein) is >5fold higher in RASSF1A IPs than in Raf-1 IPs. These results are fully consistent with our original conclusions.

- * In Fig.2A the authors show that HCT cells grown in low serum undergo apoptosis, which is mediated in part by the RASSF1A pathway. Moreover they claim that neither p73 nor YAP knock down (kd) reduce apoptosis (Fig.S2A-B). Instead it is evident in Fig.S2B that YAP1 kd induces apoptosis in presence of low serum. How do the authors explain this induction of apoptosis when YAP is silenced? It should be taken into consideration that YAP has been shown to

both sustain proliferation and induce apoptosis. Is YAP sustaining proliferation in low serum condition? If so, are the authors looking at a pro-apoptotic pathway or at a pro-survival pathway?

The dual role of YAP as oncogene and tumour suppressor gene is now rapidly emerging (Bertini et al., 2009; Pan, 2010; Zeng and Hong, 2008; Zhao et al., 2010). In the context of this manuscript, it was our priority to assess whether YAP1 is a pro-apoptotic effector of the mutant K-Ras stimulated RASSF1A pathway or not. In Fig.S2B we showed that YAP1 is not an effector of mutant K-Ras induced apoptosis, as siRNA downregulation of YAP1 did not rescue apoptosis in HCT116 cells. Therefore, we did not analyse the YAP1 pathway further. Instead, we concentrated on identifying the relevant effector of the mutant K-Ras activated MST2 pathway as p53.

We now have performed additional experiments in regard to the reviewer's question to distinguish between YAP1 effects sustaining proliferation or inducing apoptosis. First, we analysed cell cycle progression (Fig. A). The results show that knockdown of either MST2 or YAP1 in serum starved HCT116 cells has no effect on cell cycle progression. Similar results also were recently reported by Vigneron et al. (Vigneron et al., 2010). Thus, YAP1 is not required for sustaining proliferation in mutant K-Ras cells. Second, we analysed whether the enhancement of apoptosis in HCT116 due to downregulation of YAP1 is related to the expression of a mutant K-Ras allele in these cells (Fig. B). For this purpose we compared the effects of YAP1 downregulation on apoptosis in HCT116 (KRAS^{wt/mt}) and the isogenic Hke3 (KRAS^{wt/-}) cell line, as well as in Hke3 reconstituted with a mutant K-Ras. The results show that YAP1 downregulation increased apoptosis in both HCT116 and Hke3 cells. Importantly, reconstitution of Hke3 cells with mutant K-Ras increased apoptosis to the level of HCT116, but this increase was unaffected by downregulation of YAP1 (Fig. B). In addition, we also have obtained biochemical evidence suggesting that YAP1 is not engaged by the mutant K-Ras activated MST2 pathway (Fig. C). Expression of mutant K-Ras does not affect the interaction between LATS1 and YAP1, which we previously showed to be disrupted by pro-apoptotic MST2 signalling that uses YAP1 as effector (Matallanas et al., 2007). Furthermore, mutant K-Ras does not change the phosphorylation of YAP1 at S127, which was previously shown to be critical for its oncogenic and growth promoting functions (Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007). In summary, these results fully corroborate our conclusion that YAP1 is not an effector of the MST2 pathway activated by mutant K-Ras.

* In Fig.2 the authors show that LATS1 kd and MST2 kd enhance Mdm2 protein levels in HCT cells but not in Hke3 cells. Moreover, in Fig.2D, they claim that both LATS1 and MST2 downregulation enhances Mdm2 binding to p53 only in HCT cells. Since the levels of Mdm2 increase in these cells and the levels of coimmunoprecipitated Mdm2 resemble total Mdm2 levels, the immunoprecipitation results not convincing.

We have clarified this issue in the revised paper by calculating the recruitment ratio (RR) of Mdm2 to p53 in HCT116 and Hke3 based on quantitation of the experiments. The RR relates the amount of Mdm2 co-immunoprecipitated with p53 to the amount of Mdm2 (normalised to the tubulin loading control) in the lysates of HCT116 and Hke3 cells, respectively. The results clearly show that the downregulation of LATS1 or MST2 induces a 2-4fold enhanced recruitment of Mdm2 to p53 in HCT116 cells, but not in Hke3 cells. These data are now included in Fig. 2D.

* In Fig.S4C, the authors claim that MST2 and LATS1 "phosphorylations were at basal levels in cells expressing wt K-Ras and did not change when wt K-Ras expression was downregulated". From the figures they show, it seems instead that wt K-Ras downregulation diminishes the levels of phosphorylated LATS1 and MST2, with equal protein loading. It is therefore not possible to conclude that only mutant K-Ras but not wt K-Ras regulates LATS1 and MST2 phosphorylation.

We now provide a more accurate description of the result based on the quantitation of these experiments. At equal expression levels of wt and mt K-Ras LATS1 phosphorylation is ~3fold and MST2 phosphorylation is ~6 times higher in mt K-Ras than in wt K-Ras cells. MST2 phosphorylation in wt Ras cells is low and does not decline further as wt K-Ras expression is downregulated. However, the low LATS1 phosphorylation in wt K-Ras cells diminishes further as K-Ras expression decreases. We have changed the text accordingly, and show the quantitation in Fig. S4C.

* The authors state that in Fig.5A "BIBX 1382 and gefitinib enhanced apoptosis in growing HCT116 but not Hke3 cells". By looking carefully at Fig.5A, one can see that both gefitinib and BIBX 1382 almost double the percentage of apoptotic cells in HCT116 cell line but, despite the high values on the Y-axis, the same phenomenon is visible in Hke3 cells, both in presence and in absence of MST2. Therefore, it cannot be concluded that EGFR function is required to [what?] of cells with K-Ras mutations, since data in Hke3 cells are not clear. Moreover, MST2 kd in Hke3 cells induces apoptosis upon EGFR inhibition, suggesting a role for MST2 (and the pathway under study as well) in promoting cell proliferation rather than in promoting apoptosis in this cell line.

We now have included p-values in Fig. 5A to clarify this issue. Treatment of HCT116 cells with EGFR inhibitors causes a significant increase in apoptosis. In contrast, the slight enhancement of apoptosis in Hke3 cells the reviewer refers to is not statistically significant. Conversely, the downregulation of MST2 in HCT116 leads to a significant reduction of apoptosis even below the rate of untreated cells under all conditions, confirming that the MST2 pathway is conferring the sensitivity to apoptosis. In Hke3 cells EGFR inhibitors, MST2 knockdown, or the combination of both did not cause a significant change in apoptosis, except with gefitinib treatment where MST2 knockdown increased apoptosis. For the reviewers we are also including a bar graph showing the apoptosis in Hke3 cells from Fig. 5A in a higher resolution (Fig. D). In addition, we are also including a different set of experiments (n=3) where we have compared the apoptosis of HCT116 and Hke3 to BIXB 1382 fully confirming that HCT116 are sensitive while Hke3 are not (Fig. E).

To address the question whether the MST2 pathway controls proliferation we have measured cell cycle progression in HCT116 cells transfected with control or MST2 siRNAs (Fig. A). Knocking down MST2 had no impact on cell cycle progression arguing against MST2 regulating proliferation.

Minor points:

* Fig.4B: total LATS1 protein levels should be shown.
A blot showing total LATS1 protein level has been included.

* Fig.7A is not clear: it would be more immediate to show the percentage of patients rather than the number.
We have added percentages to Figs. 7A and 7C.

* Spelling and punctuation should be checked.

We have done that.

Reviewer #2:

The mechanism through which Ras can stimulate apoptosis and the role of wild-type Ras in cells transformed by mutant Ras are two questions at the forefront of Ras biology. Matallanas et al. tackle both of these questions in this new study in which the authors conclude that oncogenic K-Ras stimulates apoptosis in an isoform-specific fashion by acting through RASSF1A, MST2, LATS1, Mdm2 and p53 and that autocrine growth factor signaling through endogenous K-Ras mitigates the pro-apoptotic response. The strength of the study lies in the interesting results that tie these two pathways together and in the data that relate the signaling pathways to human colorectal carcinoma. The work is interesting and significant but some of the data presented suffer from methodologic weaknesses detailed below.

Much of the work relies on siRNA silencing of components of the RASSF1A, MST2, LATS1 or Raf-1 pathways. But the knockdowns are quite variable and several of the blots that show them are poor. For example RASSF1A is knocked down to undetectable levels in HCT116 cells in Fig. 2A but is much less affected in Fig. 1E and 5D. Under these circumstances one must worry about off target effects of the siRNAs and reversal with siRNA-resistant expression constructs can be useful as the authors employed for their knockdown of K-Ras.

Some variability in knockdown efficiency between different experiments is normal, most likely due to differences in transfection efficiency. However, much of the apparent variation is actually due to different exposures of the Western blots. We are now providing shorter exposures of the relevant Western blots for Figs. 1E and 5D, which now show similar extents of knockdown. All the siRNAs used were extensively validated in a previous paper (Matallanas et al., 2007). In addition, as suggested by the reviewer we have done rescue experiments with siRNA resistant RASSF1A and MST2 mutants, which are included as Fig. S2A. They fully confirm the specificity of the siRNAs used and results obtained with them. We also used siRNA resistant LATS1 mutants, but found that the exogenous expression of these mutants or wildtype LATS1 induced high levels of apoptosis which made the interpretation of rescue experiments difficult. We have previously shown that LATS1 knockout cells are resistant to Fas induced apoptosis, and that re-expression of LATS1 sensitizes the cells to apoptosis (Matallanas et al., 2007). We have included this figure for the convenience of the reviewers as Fig. F.

It is not clear how the measurements of apoptosis were made or why the basal apoptosis rate of HCT116 cells in low serum varies from 13% (Fig. 3A) to 45% (Fig. 2A).

While there is some normal variation in apoptosis assays, the main reason for the variations pointed out by the reviewer is simply differences in the timepoints when apoptosis was measured. As stated in the respective figure legends the apoptosis measurements were taken at different timepoints after serum deprivation. For some experiments shorter timepoints were chosen in order to enable biochemical experimentation on the same population of cells, which otherwise would not have been possible due to extensive cell death. While these differences pertain to the absolute rates of apoptosis, they do not change any of the interpretations in our paper. Fig. G shows the effect of different timepoints on the apoptosis measurements under unperturbed conditions as well as downregulation of RASSF1A pathway components by siRNA.

The methods state that apoptosis was measured by quantifying "DNA fragmentation by FACS" as described in O'Neill et al 2004, but in this study apoptosis was scored either as cells with sub-genomic DNA content as assessed by propidium iodide staining or by annexin-V staining of cells that are negative for propidium iodide uptake.

We have added further detail to the Methods Section clarifying that we have used the DNA fragmentation assay as measured by subgenomic DNA content.

In addition to clarifying the method used the authors should consider independent confirmation of apoptosis with alternate methods such as caspase or PARP cleavage or cytochrome c release since apoptosis is so central to the author's thesis.

As requested by the reviewer we are now providing additional types of apoptosis assays for the salient data. In Fig. S2B we show caspase 3/7 activity measured using a fluorescent substrate (z-VAD-FMK-FITC) and FACS analysis in serum starved (0.1% serum for 24 hours) HCT116 cells. In Fig. S3C we show the processing of pro-caspase 3 into the active forms induced by the downregulation of wt K-Ras by three different siRNAs in serum starved HCT116 cells. The results fully support our originally reported data.

The figures that show an in-gel MST2 kinase assay of immunoprecipitates lack a critical control: IP with an irrelevant antibody to determine non-specific background.

This control has been included in the revised Fig. 1D.

Figure 1D is seriously flawed and without several additional controls should be omitted. The authors measure MST2 protein (immunoblot) and kinase activity (in gel MBP phosphorylation) in RASSF1A immunoprecipitates from whole cell lysate and from Raf-1 immunoprecipitates from RASSF1A-immunodepleted cell lysates. The author's interpret the results as revealing that more MST2 protein, and in particular active kinase, is complexed with RASSF1A than with Raf-1. This presupposes stable complexes rather than equilibria binding within the cytosol and ex-vivo after cell lysis.

Our interpretation is not dependent on the assumption of stable protein complexes, but only on the assumption of equilibrium binding. The equilibrium binding is given by the dissociation constant K_d , which is the ratio between the off and on rate constants of the binding reaction ($K_d = k_{off}/k_{on} = [A][B]/[AB]$). As the protein complexes are subjected to the same treatment during cell lysis, immunoprecipitation and washing, K_d is a thermodynamic constant, and the experimental manipulations will change the free protein ($[A]$, $[B]$) and protein complex ($[AB]$) concentrations to maintain the constant K_d relationship. Thus, as long as protein complexes are treated under the exact same conditions (as is the case here), the resulting changes after experimental manipulation are (i) related to the original state of the complexes in the cell, and (ii) can be compared directly. If this were not the case, actually all co-immunoprecipitation experiments would be invalid.

Moreover, many more controls are needed to interpret the results. These include first and second IP with irrelevant antibodies and reciprocal immunodepletion.

We have replaced Fig. 1D with a new figure which contains control IPs with irrelevant antibodies and reciprocal IPs.

Finally, since no IP for MST2 was used what we are shown is MBP kinase activity somewhere in the gel. With the myriad of sticky kinases in cell

lysates it would be very surprising if the band shown was the only one in the gel. The authors need to show that authentic MST2 migrates with the band shown in the figure and should show how many bands appear in the gel after incubation with [³²P]ATP.

As stated in the Methods section the MST2 kinase assay is performed by first immunoprecipitating MST2, and then subjecting the MST2 IP to an in gel kinase assay. An autoradiogram of an in gel kinase assay showing the whole gel is shown in Fig. H. It shows that total lysates are assayed there are two bands: MST2 and a ca. 80kDa band. When MST2 IPs are used, the MST2 band is the only prominent band on the gel, even after long exposure.

In Fig. 1E why is the lysate not blotted for LATS1?

Sorry for the oversight. This control is included now.

In Fig. 1F the authors show that expressing ectopic oncogenic K-Ras in Hke3 cells stimulates apoptosis and that Raf-1 mitigates the effect. Although the authors established in Fig. 2 that the parental HCT116 cells have significantly more basal apoptosis than the Hke3 derivatives that have lost K-Ras12D, it would nonetheless be informative to determine if ectopic HA-K-Ras12V or siRNA for Raf-1 have any effect on apoptosis in HCT116 cells.

Overexpression of K-RasV12 slightly enhances apoptosis in serum starved but not growing HCT11 cells (Fig. I).

It would also be informative to confirm the Raf-1 siRNA results with a MEK inhibitor.

Both the downregulation of Raf-1 and to a lesser extent the pharmacological inhibition of MEK cause apoptosis (Fig. K). Combining Raf-1 knockdown with MEK inhibition only slightly enhances apoptosis above the levels observed with Raf-1 knockdown alone. As the Raf-1 knockdown also reduces ERK activation, these results suggest that in terms of apoptosis regulation Raf-1 has two functions. One is the suppression of MST2, and another the activation of ERK pathway.

Finally, given the resistance of Hke3 cells to the modulation of MST2 kinase activity by RASSF1A knockdown it would be important to confirm that RASSF1A knockdown has no effect on Hke3 apoptosis unless HA-K-Ras12V is ectopically expressed.

The increase in apoptosis triggered by K-RasV12 expression in Hke3 cells can be abrogated by downregulation of RASSF1A. These data are now included as Fig. 1G. Please note that the difference in apoptosis rate is due to the different periods of serum starvation.

The specific silencing of wild-type K-Ras with three carefully designed siRNAs is not convincingly demonstrated. Reuven Agami first demonstrated that oncogene-specific silencing of K-Ras was possible (Cancer Cell, 2:243, 2002) however many have since had difficulty reproducing this result. To prove that their siRNAs are truly specific for wt K-Ras the authors must show that they cannot knock down endogenous oncogenic K-Ras with their reagents. This is easily accomplished. HCT116/Hke3 are not the only isogenic pairs generated by Kinsler and Vogelstein. They also generated lines that are hemizygous for either wt K-Ras or K-Ras12D. It is these hemizygous cell lines that would provide the proof needed. An alternative would be to use the MEFs from Mariano Barbacid's Ras-less ES cells that express only wt or oncogenic K-Ras.

We have performed this experiment as suggested using the Rasless MEFs. The experiment fully confirms the specificity of the siRNAs and is included as Fig. S3A.

The immunoblot shown in Fig 3B does not support the authors' interpretation of the data. It shows that the "rescue" siRNA-resistant wild-type HA-K-Ras is expressed far below the residual levels of endogenous K-Ras after knockdown making it difficult to understand why this would act to reverse the effect of K-Ras silencing on apoptosis of HCT116 cells.

The experiment shown in Fig. 3B is representative of 4 independent experiments, all with similar results. The apparently lower expression of the rescue HA-K-Ras construct is due to the fact that the transfection efficiency is not 100%. Thus, also the rescue is not complete.

Minor point: all of the data shown as blots or in-gel kinase assays are presumably representative of several. Nowhere does it state how many times the experiments were performed independently. When data are shown as bar graphs, error bars are drawn and p values are given indicating that the experiment was performed independently at least three times but this information is not included.

The number of independent experimental repeats is no less than n=3. We have now included the number of repeats for each experiment in the figure legends.

Figures for Reviewers

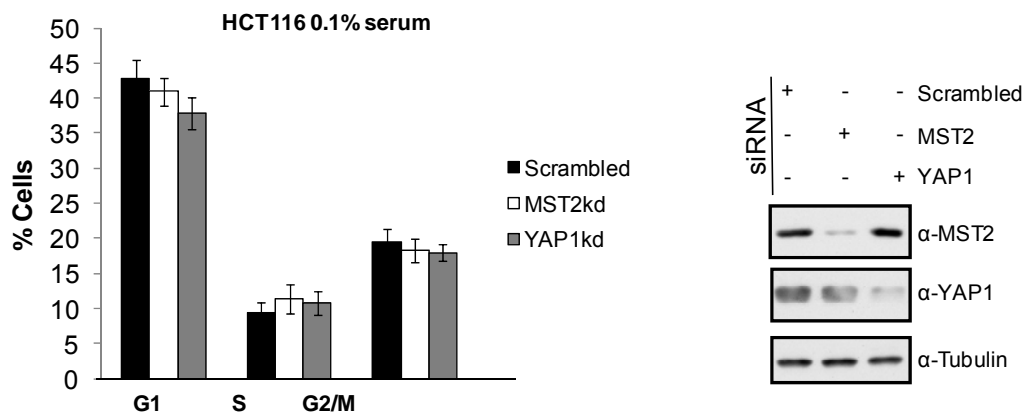


Fig. A. Downregulation of YAP1 or MST2 does not affect cell cycle progression. Cells were transfected with siRNAs against MST2, YAP1 (MST2kd, YAP1kd) or scrambled control siRNA. Downregulation of MST2 and YAP1 was ascertained by Western blotting. Cells were serum starved for 16 hours and treated with 5-BrdU for 30 minutes. Cells were collected and incubated with anti-BrdU antibody, and cell cycle was analysed by flow cytometry (n=3).

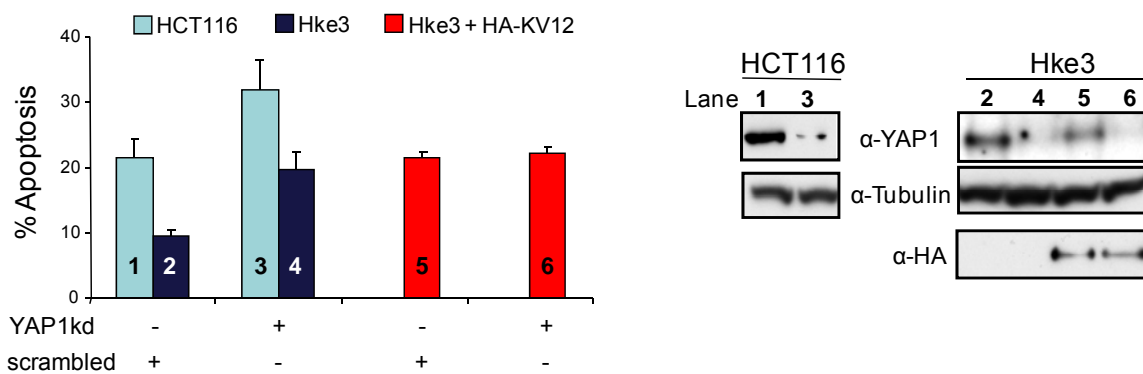


Fig. B. Apoptosis induced by downregulation of YAP1 is independent of mutant K-Ras. HCT116 ($KRAS^{wt/G13D}$) and isogenic Hke3 ($KRAS^{wt/-}$) cells were transfected with siRNA against YAP1 or scrambled siRNA as indicated. Hke-3 were also transfected with HA-tagged K-RasV12 where indicated. The cells were serum starved for 16 hours, and apoptosis was measured by PI staining and flow cytometry (n=3). Error bars show standard deviation. Protein expression was assayed by Western blotting with the indicated antibodies. Lane numbering of bars corresponds to the lane numbering in the Western blot.

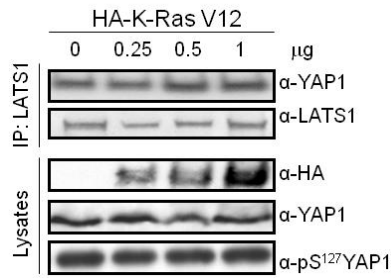


Fig. C. Mutant K-Ras does not affect LATS1-YAP1 association and phosphorylation of YAP1 at S127. Endogenous LATS1 was immunoprecipitated from cells transiently transfected with increasing amounts of HA-tagged K-RasV12. LATS1 immunoprecipitates were blotted for associated endogenous YAP1. Total lysates were analysed in parallel for the expression of endogenous YAP1, HA-K-RasV12, and phosphorylation of S127 in YAP1

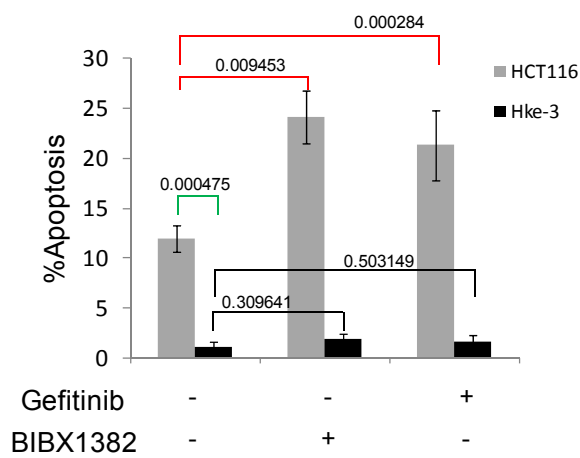


Fig. D. EGFR inhibitors do not induce significant apoptosis in Hke3 cells. Data from Fig. 5A (n=6) are shown on a higher resolution scale. Student's t-test was used to assess statistical significance. P-values are shown.

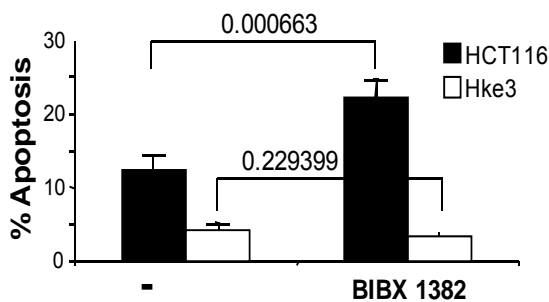


Fig. E. HCT116 cells, but not Hke3 cells, are sensitive to BIBX 1382 induced apoptosis in Hke3 cells. This experiment (n=3) was performed independently from Fig. 5A. Cells were treated with BIBX 1382 under the same conditions as in Fig. 5A. p values were calculated by Student's t-test (n=3).

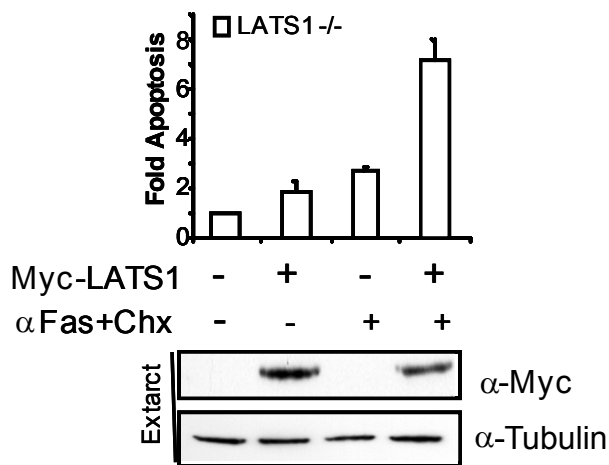


Fig. F. Re-expression of LATS1 restores the resistance of LATS1 knockout cells to apoptosis. This is Fig S6C from one of our previous publications (Matallanas et al., 2007). LATS1 knockout (MEF LATS1^{-/-}) were transfected with myc-tagged LATS1 and treated with anti-FAS JO2 plus CHX where indicated. Reconstitution of LATS1^{-/-} cells with restores their sensitivity to Fas induced apoptosis. Levels of apoptosis in were measured by FACS analysis using zVAD-fmk-FITC. Expression of endogenous and transfected proteins was analysed by

Western blotting of cell lysates with the indicated antibodies. Error bars indicate standard deviation.

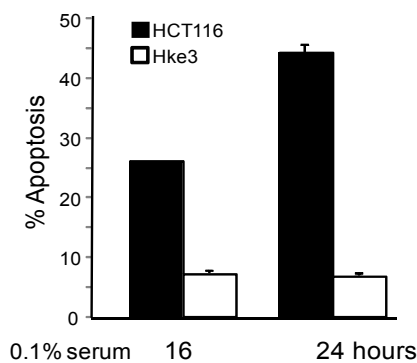
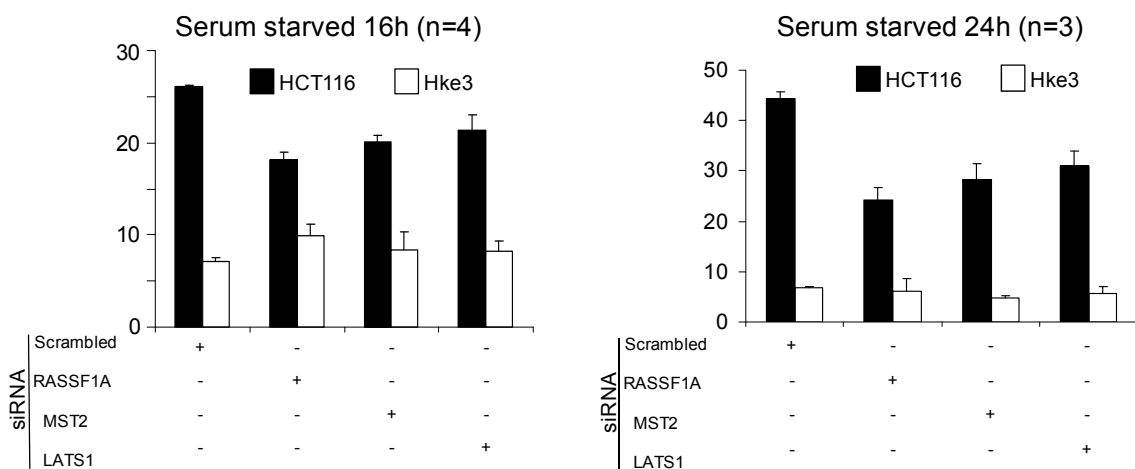


Fig. G. Apoptosis rates in HCT116 cells are dependent on the time of measurement after serum deprivation.

Apoptosis induced by different periods of serum starvation (as indicated) was measured by assessing DNA fragmentation as described in the Methods section (n=3).

Left panel, Prolonging serum starvation increases apoptosis in HCT116 cells, but does not affect the low basal rate of apoptosis in Hke3 cells. **Lower panels,** the high rate of apoptosis in HCT116 can be decreased by downregulation of RASSF1A, MST2 or LATS1.



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Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wildtype K-Ras

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Running Title: K-Ras transformation requires the suppression of MST2 signalling

Abstract

K-Ras mutations are frequent in colorectal cancer (CRC), albeit K-Ras is the only Ras isoform that can elicit apoptosis. Here we show that mutant K-Ras directly binds to the tumour suppressor RASSF1A to activate the apoptotic MST2-LATS1 pathway. In this pathway LATS1 binds to and sequesters the ubiquitin ligase Mdm2 causing stabilisation of the tumour suppressor p53 and apoptosis. However, mutant Ras also stimulates autocrine activation of the EGFR which counteracts mutant K-Ras induced apoptosis. Interestingly, this protection requires the wildtype K-Ras allele, which in part via AKT activation inhibits the MST2 pathway. Confirming the pathophysiological relevance of the molecular findings we find a negative correlation between K-Ras mutation and MST2 expression in human CRC patients and CRC mouse models. The small number of tumours with co-expression of mutant K-Ras and MST2 has elevated apoptosis rates. Thus, in CRC mutant K-Ras transformation is supported by the wildtype allele.

Highlights

- Mutant K-Ras induces apoptosis by engaging the RASSF1A – MST2 –LATS1 pathway
- Apoptosis is mediated by Mdm2 sequestration resulting in p53 stabilisation and activation
- EGF receptor activated wildtype K-Ras protects against MST2 mediated apoptosis
- Colon cancers with K-Ras mutations retain the wildtype K-Ras allele or downregulate MST2 expression

Introduction

Mammals possess three *RAS* genes that encode four proteins, H-Ras, K-Ras4A, K-Ras4B and N-Ras. The Ras proteins cycle between inactive Ras.GDP and active Ras.GTP forms (Barbacid, 1987; Malumbres and Pellicer, 1998). Receptors activate Ras by recruiting guanine-nucleotide exchange factors (GEFs) to the membrane, where they convert Ras.GDP to Ras.GTP. Mutations in codons 12, 13 or 61 render Ras constitutively GTP bound and activated. Such mutations occur in more than 30% of human tumours, with K-Ras mutations accounting for ~85% of all Ras mutations in human cancers. They are frequent in epithelial cancers of the lung, pancreas and colon. N-Ras is mutated in ~15% of Ras tumours usually melanoma, liver and leukaemias. H-Ras mutations are rare (>1%) and occur in bladder cancer (Downward, 2003; Karnoub and Weinberg, 2008).

Ras proteins are molecular switches regulating fundamental biological functions such as differentiation, proliferation, senescence, motility and cell apoptosis. This plethora of effects is achieved through the engagement of diverse downstream effectors, which are defined by binding specifically to active Ras.GTP (Buday and Downward, 2008; Karnoub and Weinberg, 2008). The best characterized Ras effectors comprise Raf family proteins, phosphoinositide-3 kinase (PI3K), and RalGDS GEFs. These *bona fide* effectors are implicated in pro-survival and proliferation signalling and contribute to oncogenic Ras cell-transformation. However, in certain situations Ras also can be growth inhibitory and pro-apoptotic. The pathways that mediate these effects are not well understood (Cox and Der, 2003), but may involve specific Ras effectors that trigger apoptosis. One candidate is Bcl-X_L, an anti-apoptotic mitochondrial protein that is specifically engaged by mt K-Ras phosphorylated by protein kinase C resulting in the promotion of apoptosis (Bivona et al., 2006). Other candidates are NORE1A and RASSF1A, members of the RASSF family. RASSF1A is the most frequently altered tumour suppressor gene in human cancer. Usually,

its expression is silenced by promoter hypermethylation (Avruch et al., 2009; Richter et al., 2009). Most RASSF family members contain a predicted Ras association (RA) domain whose functionality is unclear in most cases. NORE1A can bind K-Ras.GTP through its RA domain and regulate the pro-apoptotic activity of MST1 and MST2 kinases (Khokhlatchev et al., 2002; Ortiz-Vega et al., 2002). RASSF1A can regulate MST2 kinase activity upon K-Ras activation although evidence for a direct interaction between these proteins is lacking. It was proposed that K-Ras regulates RASSF1A via heterodimerisation of RASSF1A with NORE1A, which provides the Ras binding function (Ortiz-Vega et al., 2002). *In vitro* studies suggested that the RASSF1A RA domain could directly interact with Ras (Stieglitz et al., 2008). However, it is unknown whether this association also occurs *in vivo*.

RASSF1A regulates different biological processes including cell cycle and mitotic arrest, migration, DNA damage and apoptosis (Donninger et al., 2007; Richter et al., 2009). We recently described that in mammalian cancer cells RASSF1A can promote apoptosis by activating the MST2 kinase. MST2 in turn activates the LATS1 kinase, which induces the formation of a complex between YAP1 and p73 transcription factors that elevates the expression of the pro-apoptotic gene *PUMA* (Hamilton et al., 2009; Matallanas et al., 2007). A critical step is the formation of the RASSF1A-MST2 complex, which requires the release of MST2 from an inhibitory complex with Raf-1 (O'Neill et al., 2004; Romano et al., 2010). However, the upstream activator of RASSF1A is unknown.

Here, we show that RASSF1A is a selective K-Ras effector that can induce apoptosis by activating the MST2 pathway. However, the downstream effector is not p73 – YAP1, but p53, which is stabilized and activated by LATS1 sequestering the p53 ubiquitin ligase Mdm2. Our results further indicate that mutant (mt) K-Ras induces autocrine loops that use the EGFR and the wildtype (wt) K-Ras allele to prevent apoptosis. Thus, oncogenic transformation by mt K-Ras requires wt K-Ras, EGFR signalling and suppression of the MST2 pathway.

Results

K-Ras activates MST2 via RASSF1A

Previous results demonstrated that RASSF1A can engage the MST2 pathway and that this contributes to the tumour suppressor functions of RASSF1A (Khokhlatchev et al., 2002; Ortiz-Vega et al., 2002) (Matallanas et al., 2007; O'Neill et al., 2004; Romano et al., 2010). However, the upstream regulation of RASSF1A remained unclear. In order to address whether K-Ras plays a role, we used the isogenic cell line pair HCT116 and Hke3. HCT116 are colorectal cancer cells with a wt *K-Ras* and an mt *K-RasD13* allele. Somatic deletion of the *K-RasD13* allele caused reversion of the oncogenic phenotype and generated the Hke3 cells (Shirasawa et al., 1993). Both cell lines express endogenous RASSF1A making them ideally suited to study differential effects of wt and mt K-Ras in an endogenous setting. MST2 kinase activity was higher in HCT116 than in Hke3. This elevation was dependent on RASSF1A and was abrogated by RASSF1A downregulation (Fig. 1A). Conversely, overexpression of RASSF1A increased MST2 kinase activity in Hke3 to a similar level as in HCT116, where MST2 was constitutively active and not further stimulated by RASSF1A expression (Fig. S1A).

These results suggested that mt K-Ras activates MST2 in a RASSF1A dependent manner. Therefore, we tested whether endogenous RASSF1A, K-Ras and MST2 proteins can interact (Fig. 1B). RASSF1A co-immunoprecipitated both K-Ras and MST2. The interaction was specific, as it was not observed with a control antibody, and was further enhanced by serum showing that the association requires K-Ras activation. The interaction observed in unstimulated cells is likely due to the presence of the mt K-Ras protein. In addition, the ability to activate MST2 was specific to K-Ras, as neither H-Ras nor N-Ras could stimulate MST2 (Fig. 1C). Furthermore, increasing the expression of mt K-Ras enhanced MST2 kinase

activity and binding to its substrate LATS1 in a dose dependent manner (Fig. S1B). By contrast, mt H-Ras suppressed MST2 kinase activity and LATS1 binding (Fig. S1C).

As MST2 can bind to Raf-1 or RASSF1A we used sequential immunoprecipitations in order to assess relative MST2 activities in these two fractions. We immunoprecipitated RASSF1A or Raf-1 from HCT116 cells, and then used the respective supernatants to immunoprecipitate the other protein and assay the associated MST2 kinase activity and protein. A larger fraction of MST2 was bound to RASSF1A than to Raf-1. Importantly, the MST2 kinase activity associated with RASSF1A was >5fold higher than the MST2 activity bound to Raf-1 (Figs. 1D). A titration of RASSF1A immunoprecipitates to contain similar amounts of MST2 as Raf-1 immunoprecipitates confirmed that MST2 is >5fold more active when bound to RASSF1A (Fig. S1D).

In summary, these results show that active MST2 is associated with RASSF1A and that mt K-Ras enhances the stimulation of MST2 by RASSF1A. In order to examine the role of Raf-1 in K-Ras mediated MST2 activation, we knocked down Raf-1 by siRNA in HCT116 (Fig. 1E). Raf-1 depletion increased MST2 kinase activity and association with LATS1, whereas depletion of RASSF1A had the opposite effect indicating that Raf-1 can counteract MST2 activation by mt K-Ras. The observed biochemical effects also have biological consequences. Expression of mt K-RasV12 in Hke3 cells doubled the rate of apoptosis. This increase was exacerbated when Raf-1 was downregulated (Fig. 1F). In addition, the increase in apoptosis triggered by K-RasV12 expression in Hke3 cells could be abrogated by downregulation of RASSF1A (Fig. 1G).

The mutant K-Ras activated MST2 pathway induces apoptosis by stabilizing p53

Our previous work has shown that RASSF1A can activate a proapoptotic pathway consisting of RASSF1A -> MST2 -> LATS1 -> p73-YAP (Matallanas et al., 2007).

Therefore, we investigated whether mt K-Ras activates apoptosis using this pathway. To this end, we compared how knocking down individual pathway components by siRNA affected apoptosis in HCT116 versus Hke3 cells (Fig. 2A). The specificity of the siRNAs has been established before (Matallanas et al., 2007). We further ascertained their specificity by performing rescue experiments with silent point mutants of RASSF1A, MST2, and LATS1 mutants, which made them siRNA resistant (Fig. S2A). Serum deprivation induced considerable levels of apoptosis in HCT116 cells, which was reduced by knocking down RASSF1A, MST2, or LATS1. By contrast, Hke3 cells exhibited low apoptosis levels, which were not affected by the knockdown of MST2 pathway components (Fig. 2A). Measuring apoptosis by an independent method fully confirmed these results (Fig. S2B). These results show that the mutation of endogenous K-Ras predisposes cells to apoptosis, which in part is mediated by the MST2 pathway. Surprisingly, downregulation of p73 or YAP1 did not reduce apoptosis of HCT116 cells (Fig. S2C,D) suggesting that the effector of the MST2 apoptosis pathway induced by mt K-Ras branches off the known pathway downstream of LATS1. In a proteomics screen for LATS1 binding proteins we identified Omi/Htra2, a proapoptotic serine protease (O'Connell and Stenson-Cox, 2007), as LATS1 associated protein. However, Omi siRNA did not affect apoptosis in HCT116 and Hke3 cells (Fig. S2E), and therefore was not pursued further.

However, LATS1 bound to Mdm2 preferentially in HCT116 cells (Fig. 2B). Mdm2 is an E3 ubiquitin ligase that targets the p53 tumour suppressor for degradation (Wade et al., 2010). Downregulation of MST2 or LATS1 augmented Mdm2 protein levels and diminished p53 levels in HCT116 cells, but had no effects in Hke3 cells (Fig. 2C). In addition, downregulation of LATS1 or MST2 enhanced recruitment of Mdm2 to p53 2-4 fold in HCT116 cells but not in Hke3, where p53-Mdm2 binding was constitutively high and actually downregulated by MST2 or LATS1 knockdown (Fig. 2D). These results suggest that

the mt K-Ras activated MST2-LATS1 pathway causes the sequestration of Mdm2 by LATS1 resulting in the stabilisation of p53. This stabilisation correlated with enhanced p53 activity as evidenced by the induction of the p53 target gene p21^{WAF} (Fig. S2F). A prediction of these findings is that the downregulation of p53 should reduce apoptosis in HCT116 but not in Hke3 cells. Indeed, knocking down p53 decreased apoptosis of HCT116 more than twofold, but had no effect in Hek3 cells (Fig. 2E). Taken together, these data indicate that that p53 is an effector of proapoptotic mt K-Ras signalling.

Wildtype K-Ras antagonizes mutant K-Ras induced apoptosis in CRC cells

The above results show that mt K-Ras engages the MST2 pathway to promote apoptosis, whereas the wt K-Ras allele did not. In order to investigate the role of wt K-Ras we downregulated its expression in HCT116 cells. Three different siRNAs designed to specifically target wt K-Ras reduced the expression of endogenous K-Ras protein in HCT116 cells. A co-expressed Flag-tagged K-Ras D13 mutant protein was not affected ascertaining that the siRNAs specifically reduced the levels of the wt K-Ras protein (Fig. S3A). All three wt K-Ras specific siRNAs enhanced apoptosis of HCT116 cells under conditions of serum starvation and growth in full serum (Fig. 3A). The relative changes in apoptosis rates caused by depletion of wt K-Ras were higher in the presence of full serum, but the results suggest that even the basal activity of wt K-Ras without growth factor stimulation provides apoptosis protection. Importantly, the increase of apoptosis caused by downregulation of wt K-Ras was rescued by a wt K-Ras (K-Raswt#) with a silent mutation that renders it insensitive to the siRNAs under conditions of growth in full serum (Fig. 3B) and serum starvation (Fig. S3B). Measuring apoptosis by assessing caspase 3 cleavage confirmed the protective effect of wt K-Ras (Fig. S3C). Blocking the activation of wt K-Ras by expression of a dominant negative K-RasN17 mutant also promoted apoptosis (Fig. S3D). K-RasN17 is quite specific for K-Ras

and only slightly inhibits H-Ras or N-Ras activation (Matallanas et al., 2003). In addition, downregulation of wt K-Ras had no effect on apoptosis in Hke3 cells, but significantly enhanced apoptosis caused by the re-expression of mt K-RasV12 (Fig. 3C). In summary, these data make the intriguing suggestion that wt K-Ras can counteract the pro-apoptotic effects of mt K-Ras.

Analysis of mutant and wildtype K-Ras signalling in genetically engineered cell systems

To corroborate these conclusions we used a cell system where the functions of mt and wt K-Ras can be genetically dissected and analysed in isolation and without any possibly confounding contributions of other Ras genes. This system is mouse embryonic fibroblasts (MEF) where all three Ras genes were deleted, and either wt K-Ras or mt K-RasV12 are expressed from a doxycycline (Dox) repressible promoter (Drosten et al., 2010). The expression of K-RasV12 in serum starved cells efficiently induced apoptosis, while wt K-Ras was ineffective (Fig 4A). The same results were obtained when the cells were kept in 10% serum (Fig. S4A). Importantly, K-RasV12 mediated apoptosis was reduced by MST2 downregulation (Fig 4A). Furthermore, MST2-LATS1 association and MST2 kinase activity were selectively enhanced by mt but not wt K-Ras (Fig. 4B). Both wt and mt K-Ras were expressed to similar levels (Fig. 4C). When we monitored MST2 kinase activity and LATS1 association during the induction of mt or wt K-Ras expression by doxycycline withdrawal, we observed an increase in MST2 kinase activity and MST2-LATS1 association only when mt K-Ras was induced (Fig. S4B). In the reverse situation, when K-Ras expression was switched-off by doxycycline (Fig. S4C), we found that the activating sites in MST2 and LATS1 were phosphorylated in the presence mt K-Ras and declined to basal levels when mt K-Ras expression was repressed. These phosphorylations were at or close to basal levels in cells expressing wt K-Ras and did not change when wt K-Ras expression was downregulated.

Notably, MST2 protein levels rose when mt K-Ras expression was reduced. This observation is consistent with our hypothesis that MST2 serves as a proapoptotic effector of mt K-Ras and therefore cells can only tolerate a limited level of MST2 expression in the presence of mt K-Ras. This observation is also consistent with our finding that MST2 protein levels are reduced in human CRC specimens expressing mt K-Ras (Fig. 7A).

Although the expression levels of Dox-regulated K-Ras in the Rasless MEFs is similar to endogenous K-Ras levels of the parental MEFs (Drosten et al., 2010), we sought to confirm these findings by expressing mt K-Ras from its own endogenous promoter. For this purpose we used MEF K-Ras (+/LSLG12Vgeo) [MEF wt/KV12] a cell line that has wt KRAS and oncogenic KRAS alleles expressed from their endogenous promoters, and MEF K-Ras (lox/LSLG12Vgeo) cells [MEF -/KV12] (Puyol et al., 2010) that upon OHT treatment express the mt K-RasV12 from its own endogenous promoter while losing the wt KRAS allele by concomitant excision. OHT treatment enhanced the apoptosis rates in MEF K-Ras (lox/LSLG12Vgeo) cells grown in 10% serum and under conditions of serum withdrawal but not in the MEF +/+ control cells or in the MEF K-Ras (+/LSLG12Vgeo) (Fig. 4D). This result confirms that expression of wt K-Ras protein is necessary to prevent activation of apoptosis caused by oncogenic K-Ras. Again, MST2 downregulation rescued K-RasV12 induced apoptosis in MEF K-Ras (lox/LSLG12Vgeo) (Fig. 4E). Taken together, these data from genetically engineered cell systems that allowed us to study the biochemical and biological effects of mt and wt K-Ras in isolation fully confirm the findings in the CRC cells. They show that mt K-Ras activates the MST2 pathway to induce apoptosis. By contrast, wt K-Ras not only failed to engage the proapoptotic MST2 pathway, but when stimulated by growth factors counteracted MST2 activation (Fig. 4B)

EGF receptor inhibition and depletion of wildtype K-Ras enhance the ability of mutant K-Ras to activate the RASSF1A - MST2 pathway

The results presented in Figs. 3 and 4 raised the intriguing hypothesis that wt K-Ras can interfere with the activation of the MST2 pathway by mt K-Ras. K-Ras induces the expression of autocrine EGF receptor (EGFR) ligands in different cell types (Gangarosa et al., 1997; Watanabe et al., 1996). Whereas EGFR phosphorylation was barely detectable in Hke3 cells, it was easily observable in serum starved HCT116 (Fig. S5A), indicating that mt K-Ras stimulates EGFR signalling. Therefore, we examined whether the EGFR was involved in apoptosis protection mediated by wt K-Ras.

Two structurally different EGFR inhibitors, BIBX 1382 (Solca et al., 2004) and gefitinib (Hegymegi-Barakonyi et al., 2009), enhanced apoptosis in growing HCT116 but not Hke3 cells (Fig 5A). The increase in apoptosis was thoroughly suppressed even below basal levels by downregulation of MST2. Furthermore, HT29, a CRC line with mt B-Raf, and wt K-Ras, and dks8ND12 (Kreeger et al., 2009), a CRC line with mt N-Ras, were insensitive to EGFR inhibitors. These data demonstrate that EGFR function is selectively required to sustain the viability of cells with K-Ras mutations, but not of cells with N-Ras or B-Raf mutations. The latter two cell lines and Hke3 were also resistant to apoptosis induction by serum withdrawal while HCT116 were sensitive (Fig. S5B), confirming that mt K-Ras transformed cells need growth factor signalling to maintain full viability. Therefore, we analysed the biochemical effects of EGFR inhibitors on the MST2 pathway. In HCT116 cells BIBX 1382 promoted MST2-LATS1 interactions in a RASSF1A dependent manner (Fig. 5B). In Hke3 cells the MST2-LATS1 interaction was low, but it could be substantially enhanced by expression of mt K-Ras and simultaneous inhibition of the EGFR (Fig. 5C). These results suggest that in the presence of mt K-Ras EGFR inhibitors induce activation of the MST2 pathway. In order to investigate the biological consequences we assayed the role of

the MST2 pathway in apoptosis caused by EGFR inhibition. BIBX1382 induced apoptosis could be completely rescued by knocking down RASSF1A, MST2 or LATS1 (Fig. 5D). The proapoptotic effect of EGFR inhibition was augmented by downregulation of endogenous wt K-Ras in HCT116 cells, and this increase could be completely rescued by expression of the siRNA resistant wt K-Ras# (Fig. 5E). These results indicate that the wt K-Ras allele is critical for transducing anti-apoptotic EGFR signals.

Mutant K-Ras induced apoptosis is counteracted by autocrine activation of the EGF receptor and AKT signalling

What activates the EGFR? Treatment of Hke3 cells with conditioned medium from HCT116 stimulated EGFR activation and the activation of downstream signalling pathways such as ERK (Fig. 6A). Both processes were inhibited by BIBX1382. In addition, HCT116 conditioned medium efficiently activated endogenous wt K-Ras in Hke3 in an EGFR dependent manner (Fig. 6B). These results suggest that mt K-Ras initiates an autocrine loop that activates the EGFR and the endogenous wt K-Ras, which then may inhibit the MST2 pathway and apoptosis. Therefore, we investigated downstream pathways that could mediate this effect. K-Ras activates multiple pathways including the anti-apoptotic AKT pathway, and also induces the expression of EGFR feedback inhibitors (Amit et al., 2007), which conceivably could modulate the role of EGFR - K-Ras axis in apoptosis regulation. Examining EGFR feedback inhibitors showed that Sprouty-2 expression was not altered by mt K-Ras (Fig. S6A), and therefore was not pursued further. Another EGFR feedback inhibitor, Mig-6, was constitutively expressed in HCT116 cells, but EGF inducible in Hke3 cells (Fig. S6A). Transfection of Mig-6 slightly increased MST2 kinase activity in HCT116 but not Hke3 cells (Fig. S6B). However, neither downregulation of endogenous Mig-6 nor

overexpression of Mig-6 significantly affected apoptosis (Fig. S6C), suggesting that Mig-6 does not play a significant role in the MST2 apoptosis pathway described here.

We previously observed that AKT can inhibit MST2 signalling (Romano et al., 2010), and that AKT activity is substantially lower in Hke3 compared to HCT116 (Pollock et al., 2005). Therefore, we investigated the effects of K-Ras and EGF on the AKT pathway (Fig. 6C). EGF stimulated AKT and suppressed MST2 activity in Hke3 cells. In contrast, a PI3K inhibitor (LY294002) or AKT inhibitor activated MST2 and suppressed AKT activity. Both inhibitors induced apoptosis (Fig. 6D), which was further augmented by knocking down wt K-Ras. By contrast, MST2 depletion reduced apoptosis. Importantly, MST2 depletion also diminished apoptosis caused by knocking down wt K-Ras. EGF decreased PI3K or AKT inhibitor induced apoptosis. This decrease was completely dependent on wt K-Ras and enhanced by knocking down MST2. These data suggest that (i) the AKT pathway is important to mediate cell survival in response to EGF; (ii) EGF uses wt K-Ras to engage the AKT pathway; and (iii) MST2 counteracts the survival functions of EGF, wt K-Ras and the AKT pathway.

KRAS mutation is associated with MST2 silencing or retention of the wt KRAS allele in human and murine colorectal cancers

KRAS is commonly mutated in CRC (Downward, 2003). Recent results also show frequent epigenetic silencing of RASSF1A (Agathangelou et al., 2005; Donniger et al., 2007) and MST1 (Mino0 et al., 2007). MST2 was not investigated so far, but our results predict that oncogenic K-Ras mutations should be counterselected when MST2 signalling is intact. To test this hypothesis, we examined a cohort of 173 human CRC patients (cohort 1) for KRAS gene mutations and MST2 protein expression (Fig. 7A). MST2 expression was assessed by immunohistochemistry (IHC) and categorized as positive, weak positive and

negative (Fig. 7B). Weak positive and negative samples were combined (termed negative from here onwards) and compared to positive samples. MST2 expression was decreased or lost in more than half of CRCs. KRAS mutations were significantly increased in MST2 negative samples, while CRCs with wt KRAS exhibited loss or retention of MST2 expression to equal parts (Fig. 7A). Analysis of a different cohort of 60 CRC patients (cohort 2) confirmed these results (Fig. S7A). In this cohort we also assessed mutations in the BRAF gene. There was no correlation between BRAF gene mutations and MST2 protein expression, indicating that it is not the chronic stimulation of the ERK pathway that is incompatible with MST2 expression but a mt K-Ras specific signal.

A critical question is whether MST2 positive CRCs with KRAS mutations retain the wt KRAS allele. KRAS sequence analysis in general supported our hypothesis that MST2 negative tumours can lose the wt KRAS allele, while MST2 positive tumours retain it (Fig. 7B). In order to exclude that this finding was due to contamination of the tumour material with normal cells we used laser capture microdissection (LMD) to isolate pure tumour tissue. We took advantage of the fact that many CRCs show heterogenous MST2 expression to isolate MST2 positive and negative areas of the same tumour for sequencing of the KRAS alleles thereby providing a stringent control for possible contaminations. The results showed that KRAS mutations are significantly enriched in MST2 negative areas (Fig. S7B). LMD performed on 10 independent CRC samples demonstrated a significant positive correlation between the MST2 negative tumour area and KRAS mutations (Fig. S7C). Unfortunately, there is no mouse model available where the effects of a *KRas* gene mutation could be compared in the absence versus the presence of a wt allele. Therefore, we analysed MST2 expression in tumours arising in the *APC^{Flox}/KRas^{G12D}* mouse model of intestinal adenocarcinoma (Haigis et al., 2008) and the *KRas^{G12D}* model of pancreatic ductal adenocarcinoma (Morton et al., 2010). These tumours exhibit rather homogenous MST2

protein expression and are very amenable to isolate pure tumour tissue. LMD isolated tumour material exhibited equal ratios of wt and mt *KRas* alleles (Fig. S7D). These results support our hypothesis that MST2 expression and K-Ras mutations are counterselected and require the retention of wt K-Ras to be compatible.

As *KRAS* mutations occur early in the pathogenesis of CRC (Takayama et al., 1998) these results also suggest that loss of MST2 expression may be part of the selection process towards more malignant behaviour. Therefore, we stratified cohort 1 according to cancer progression as measured by Dukes' stage (Fig. 7C). There was no significant association between *KRAS* mutations and loss of MST2 expression in early stages Dukes' A and B, which are devoid of metastasis, whereas a highly significant association emerged in Dukes' C and D, which designate advanced disease with local or distant metastases, respectively.

In addition, our hypothesis suggested that apoptosis should be enhanced in the few CRCs, which have *KRAS* mutations and retain MST2 expression. Therefore, we measured apoptosis rates by TUNEL staining, and found that the apoptotic index was significantly elevated in tumours with mt *KRAS* and MST2 expression (Fig. 7D). In summary, the clinical data support our hypothesis that MST2 transduces a pro-apoptotic signal from mt K-Ras, and that, therefore, MST2 expression is silenced or balanced by expression of a wt *KRAS* allele in CRCs driven by mt K-Ras. In addition, the data indicate that the reduction of MST2 expression is a frequent event in CRC, but preferentially occurs in advanced stages.

Discussion

K-Ras is the most commonly mutated Ras family gene in cancer playing important roles in prevalent human malignancies such as pancreatic, lung and colorectal cancers (Downward, 2003). Yet, it is the only family member that can also activate apoptosis (Cox and Der, 2003). This paradox has not been solved. Here, we provide evidence for a

mechanism how mt K-Ras can activate apoptosis via engaging the RASSF1A – MST2 – LATS1 pathway, but also counterbalance the proapoptotic signal by activating the EGFR and wt K-Ras through induction of an autocrine loop (Fig. S8). Our data suggest that RASSF1A is a direct effector of K-Ras, but not other Ras isoforms. Mt K-Ras can promote MST2 activation in two ways, one is by direct disruption of the inhibitory Raf-1 – MST2 complex (Matallanas et al., 2008), the other by forming an activating RASSF1A – MST2 complex as reported here. Mt K-Ras activated MST2 can initiate a proapoptotic pathway that culminates in the stabilization of p53 by LATS1 restricting the recruitment of the E3 ubiquitin ligase Mdm2 to p53. Interestingly, a previous report showed that LATS2 can bind to and inhibit Mdm2 activity (Aylon et al., 2006). LATS1 was not examined in this study, but our results are consistent with such a role where LATS1 sequesters and inhibits Mdm2. As KRAS mutations are frequent in human cancer (Downward, 2003) such a mechanism that automatically couples oncogenic signalling to the risk of apoptosis could be an intrinsic defence against unlicensed proliferation. The observation that the frequency of KRAS mutations in human CRC actually decreases from 80-92% in nondysplastic crypt foci to 57% in dysplastic crypt foci (Takayama et al., 1998) is in line with this interpretation and suggests that there is a selection pressure on cells with mt K-Ras during tumorigenesis.

Our data also suggest that the retention of a wt KRAS allele could be part of this selection process. Specifically, our results reveal an unexpected synergism between mt and wt K-Ras in transformation (Fig. S8). Mt K-Ras entertains an autocrine loop that activates the EGFR system and through wt K-Ras signalling counterbalances the apoptosis induced by mt K-Ras. A critical part of this protective effect is contributed by AKT. We have previously shown that AKT can phosphorylate MST2 blocking MST2 activity and binding to RASSF1A, while promoting MST2 interacting with Raf-1 (Romano et al., 2010). This mechanism can dynamically change the ratio between the inactive and active populations of MST2 that are

bound to Raf-1 and RASSF1A, respectively. This mechanism also seems critical for the functional balance between the mt and wt K-Ras proteins, as either downregulation of Raf-1 or inhibition of AKT derails the balance in favour of MST2 activation and apoptosis. In this scenario not only the relative abundance of mt and wt K-Ras proteins is important, but equally important are the abundances of Raf-1, RASSF1A and the posttranslational modifications that change the ability of MST2 to enter inactive complexes with Raf-1 or activating complexes with RASSF1A. Thus, it is plausible that tissue specific differences in the expression of these components or activities in modulatory pathways can shift the biochemical balance to generate biologically different outcomes. This hypothesis will need to be tested in a broad range of cell lines and tissues, but will help to elucidate some of the tissue specific effects of K-Ras.

Mouse models of chemically induced lung tumours suggest that wt K-Ras acts as tumour suppressor in lung (To et al., 2006; Zhang et al., 2001), while our data suggest that wt K-Ras facilitates transformation by mt K-Ras in colon. The mechanism of the tumour suppressor function of wt K-Ras has not been elucidated, but may be related to differential expression or activity of modulator pathways discussed above. There is increasing evidence that oncogenic effects are highly context and tissue dependent. The classic example is TGF β , which can have both tumour suppressing and promoting effects (Pardali and Moustakas, 2007). More recent examples of proteins that can have both oncogenic and tumour suppressive functions are E2F (Johnson and Degregori, 2006), Sprouty-2, which functions as tumour suppressor in lung (Shaw et al., 2007) and oncogene in colon cancer (Holgren et al., 2010), and YAP, which accelerates hepatocellular carcinoma (Lu et al., 2010; Zhou et al., 2009) but suppresses breast cancer (Matallanas et al., 2007; Yuan et al., 2008).

Our clinical data show that downregulation of MST2 expression is frequent in human CRCs and coincides with K-Ras mutations. In contrast, we observed no correlation between

MST2 expression and B-Raf mutations. This is consistent with the inability of B-Raf to regulate MST2 (O'Neill et al., 2004). These data also indicate that it is not high ERK pathway activity that selects against MST2 expression, but a mt K-Ras specific function, i.e. apoptosis induction. This interpretation is supported by the observation that the few CRCs, which feature both KRAS mutations and MST2 expression have elevated rates of apoptosis. Interestingly, Liu et al have recently described a correlation between mt K-Ras and spontaneous apoptosis in CRC (Liu et al., 2011). Our results suggest that mt K-Ras stimulates the secretion of autocrine growth factors that signal via the EGFR and the wt K-Ras protein to counteract MST2 induced apoptosis. This mechanism seems unexpected in the light of recent clinical observations that KRAS mutations in CRC correlate with resistance to monoclonal antibodies (cetuximab and panitumumab) that inhibit the EGFR (Normanno et al., 2009; Siena et al., 2009). The simplest assumption is that K-Ras is a critical effector of the EGFR, and that a mutation in K-Ras renders an upstream blockade of the EGFR futile. However, only 40-70% of CRCs with wt K-Ras respond to cetuximab and panitumumab (Normanno et al., 2009; Siena et al., 2009), and there is no correlation between K-Ras mutation status and response of CRCs to gefitinib, a chemical EGFR inhibitor (Ogino et al., 2005). Moreover, a small sub-set of patients with K-Ras mutations (~3% in CRC) responds to EGFR inhibitor treatment (Linardou et al., 2008), and the lack of response may be related with the K-RasG12V mutation since K-RAS G13D seem to respond to cetuximab mutation (De Roock et al., 2010). Thus, the situation is more complex. Our findings may contribute to clarify the situation. The observation of KRAS mutations correlating with resistance to EGFR inhibitory antibodies was made in metastatic CRC (De Roock et al., 2010). Our data show that MST2 expression is preferentially lost in metastatic CRC (Dukes' C and D stages) suggesting that the ability of mt K-Ras to induce apoptosis in late stage CRC is already blunted by abolishing the effector and, therefore, no longer requires the protective function of wt-Ras and EGFR. A

provocative conclusion from these findings may be to use EGFR inhibitory antibodies in early stage CRC. Although Dukes A stage is sufficiently curable by surgery alone, 20-30% of Dukes B CRC relapse (Johnston, 2005). They may be candidates for EGFR inhibitory therapy.

Experimental procedures

Further details are described in the extended experimental procedures.

Reagents and cells. HCT116, Hke3, MCF7, HeLa, MEF-K-RasV12 Tet-Off, and MEF-K-Ras wt Tet-Off, MEF K-Ras (+/+);RERT (ert/ert) and MEF K-Ras (lox/LSLG12Vgeo) ;RERT (ert/ert) [-/V12] cells were grown in DMEM plus 10% foetal calf serum.

Plasmids and siRNA. siRNAs targeting RASSF1A, p73, Raf-1, LATS1 and MST2 have been described before (Matallanas et al., 2007). Specific siRNAs against K-Ras wt were designed against codon 13 of K-Ras and custom made by Dharmacon. pCEFL-Flag-K-Raswt# is a wt K-Ras containing a conservative point mutation that confers resistance to the wt K-Ras specific siRNAs, and was generated by site directed mutagenesis. Expression plasmids for HA-RASSF1A, Flag-RASSF1A (Matallanas et al., 2007) and wt HA-H-Ras, wt HA-K-Ras, wt HA-N-Ras, HA-K-RasV12, Flag-K-RasV12 are cloned in pCEFL and were described before (Matallanas et al., 2003).

Immunoprecipitation and immunoblotting was performed as previously described (Chen et al., 1993; Matallanas et al., 2007).

Ras activation assay. Ras activation was measured as described before.(Matallanas et al., 2003). Active, i.e. GTP loaded Ras was affinity purified using GST-Raf RBD (amino acids 1 to 149) and detected by immunoblotting with anti-K-Ras antibody.

MST2 kinase activity was measured by subjecting MST2 immunoprecipitates to in gel kinase assays as described before (O'Neill et al., 2004). For assaying MST2 kinase activity in

Raf-1 or RASSF1A protein complexes, cell lysates were immunoprecipitated first with anti-Raf-1 for two hours. The Raf-1 immunoprecipitate was collected by centrifugation. The supernatant was transferred to another tube and immunoprecipitated with anti-RASSF1A for two hours. Both immunoprecipitates were assayed for MST2 kinase activity by an in gel kinase assay or immunoblotted.

Apoptosis was assayed by quantifying DNA fragmentation as measured by the sub-genomic DNA content assessed by propidium iodide staining and FACS analysis as previously described (O'Neill et al., 2004). YO-PRO®-1 (Invitrogen)/PI was used following manufacture instructions to measure apoptosis in MEF^{-K-RASV12} cells.

Analysis of tumours. Clinical data of the colorectal cancer patient cohort were previously described (Al-Mulla et al., 2006). *K-RAS* codon 12 & 13 mutations were sequenced (Al-Mulla et al., 1998), and *BRAF* V600E mutations were detected by real-time PCR (Benlloch et al., 2006). MST2 immunohistochemistry was performed as previously described (Al-Mulla et al., 2006) using the monoclonal rabbit anti-MST2 antibody (Cell Signaling) and biotinylated anti-Rabbit IgG (Vector Laboratories) as secondary antibody. Negative control sections were incubated without the primary antibody. Two-sided Fisher's exact test was used to calculate correlations between mutations of *BRAF*, *K-RAS* genes and MST2 expression. The *APC^{Flox}/K-Ras^{G12D}* model of intestinal adenocarcinoma was described previously (Haigis et al., 2008). Tumour tissue was isolated by laser capture microdissection.

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Figure legends

Fig. 1. K-Ras activates MST2 via RASSF1A. (A) Hke3 and HCT116 were transfected with RASSF1A or control (scrambled) siRNA. MST2 immunoprecipitates (IPs) were assayed for kinase activity (KA, relative values as determined by laser scanning densitometry are given below the lanes). (B) Endogenous K-Ras-RASSF1A co-immunoprecipitation in HCT116 cells kept in 10% or 0.1% serum. RASSF1A or GFP-control IPs were blotted with the indicated antibodies. (C) Hke3 cells were transfected with HA-tagged K-RasV12, H-RasV12 or N-RasV12 mutants. Cells were serum starved for 16 hours before MST2 kinase activity was measured. Extracts were blotted with the indicated antibodies. (D) MST2 exists in two protein complexes with high and low kinase activity. RASSF1A or Raf-1 were immunoprecipitated from HCT116 cells serum starved for 16 hours, and the supernatants of each IP were with the reciprocal antibody. The IPs were assayed for associated MST2 kinase activity (KA) and blotted with the indicated antibodies. Blots were scanned by laser densitometry, and adjusted MST2 kinase activity was calculated as normalized ratio between MST2 kinase activity bands and MST2 protein levels. As positive control total MST2 was immunoprecipitated and assayed. IPs with irrelevant antibodies (GFP, GST) served as negative control. (E) HCT116 cell were transfected with RASSF1A, Raf-1 or non-targeting siRNAs (scrambled). MST2 IPs were assayed for kinase activity and LATS1 association. (F) Hke3 cells were transfected with HA-K-RasV12 (HA-KV12) and siRNA against Raf-1 as indicated, and apoptosis was determined in cells serum starved for 48 hours. Error bars show standard deviation. (G) Hke3 cells were transfected with HA-K-RasV12 (HA-KV12) and siRNA against RASSF1A as indicated. Cells were serum starved for 14 hours before apoptosis was determined. Error bars

show standard deviation. All experiments represent at least three independent repeats, with 1G n=4.

Fig. 2. The mutant K-Ras activated MST2 pathway induces apoptosis by stabilizing p53.

(A) HCT116 and Hke3 cells were transfected with RASSF1A, MST2, LATS1 or scrambled control siRNAs as indicated. Cells were starved for 24 hours and apoptosis levels were measured. Error bars show standard deviation. Aliquots of cell extracts were assayed by Western blot for downregulation of the indicated proteins. Tubulin was used as loading control. (B) Mutant K-Ras induces Mdm2 binding to LATS1. HCT116 and Hke3 cells were grown in 10% serum or serum starved. Endogenous Mdm2 was immunoprecipitated and Western blotted with the indicated antibodies. (C) Cells were transfected with siRNA against MST2, LATS1 or scrambled control, and the levels of p53 and Mdm2 expression were assessed by Western blotting. (D) Mdm2 binding to p53 was assayed in p53 IPs (adjusted to equal loading) prepared from cells transfected with MST2, LATS1 or control (scrambled) siRNAs. The recruitment ratio (RR) is the ratio of Mdm2 detected in p53 IPs in each cell line divided by the total amount of Mdm2 in lysates normalized to tubulin expression. (E) Cells were transfected with p53 or scrambled siRNAs and serum starved for 20 hours. Apoptosis was assayed by DNA fragmentation. Error bars show standard deviation. All experiments represent at least three independent repeats, with 2B n=4, and 2C n=6.

Fig. 3. Wildtype K-Ras antagonizes mutant K-Ras induced apoptosis in CRC cells. (A)

HCT116 cells were transfected with three different siRNAs (K-Ras1-3) specifically targeting wt K-Ras. Apoptosis was measured by assaying DNA fragmentation in cells kept in media containing 0.1% or 10% serum for 20 hours. Error bars show standard deviation. Cell lysates were Western blotted with the indicate antibodies. The K-Ras antibody detects both wt and mt

K-Ras proteins. **(B)** HCT116 cells were transfected with two different siRNAs specific for K-Ras wt and a siRNA resistant Flag-tagged K-Ras expression vector (K-Raswt#) as indicated. Apoptosis was measured as above. Error bars show standard deviation. P-values were obtained by t-test. K-Ras expression was assayed by Western blotting; the open arrowhead indicates Flag-K-Raswt#, the filled arrowhead endogenous K-Ras. **(C)** Hke3 cells were transfected with siRNAs and HA-tagged K-RasV12 as indicated. After 20 hours of serum deprivation cell death was measured as before. Error bars show standard deviation. P-values were obtained by Student's t-test. All experiments represent at least three independent repeats, with 3B and 3C n=4.

Fig. 4. Analysis of mutant and wildtype K-Ras signalling in genetically engineered cell systems. **(A)** Ras-less MEFs reconstituted with doxycycline repressible wt K-Ras or mt K-RasV12 were transfected with MST2 siRNA. Apoptosis was measured after 24 hours of serum starvation. Where indicated 1ng/ml doxycycline was added 48 hours before starvation. Lysates were Western blotted with the indicated antibodies. **(B)** MST2 and LATS1 were immunoprecipitated from growing or serum starved MEFs expressing wt or mt K-RasV12. MST2 kinase activity (KA) and association with LATS1 was assayed by Western blotting with the indicated antibodies. **(C)** Expression levels of wt and mt K-RasV12 induced in MEFs by doxycycline withdrawal were assessed by Western blotting. Tubulin was used as control for equal loading. **(D)** K-Ras (+/+);RERT (ert/ert)[wt/wt], MEF K-Ras (lox/LSLG12Vgeo);RERT (ert/ert) MEFs [-/KV12] and K-Ras [+/LSLG12Vgeo];RERT (ert/ert) MEF (wt/V12), were treated with 600nM 4-OHT for 72 hours. The cells were serum starved for 24 hours where indicated (0.1% serum) and apoptosis was measured using YO-PRO®-1/PI as indicated in materials and methods. **(E)** K-Ras (lox/LSLG12Vgeo);RERT (ert/ert) treated with 4-OHT for 4 days where transfected with MST2 siRNA. The cells were

serum starved for 16 hours and apoptosis was measured with YO-PRO®-1/PI. All experiments represent three independent repeats.

Fig. 5. EGFR inhibition and depletion of wildtype K-Ras enhance the ability of mutant K-Ras to activate the RASSF1A - MST2 pathway. (A) HCT116 (mt K-Ras), Hke3 (wt K-Ras), DKS8 ND12 (mt N-Ras), and HT29 (mt B-Raf) cells were transfected with non-targeting or MST2 specific siRNAs, and incubated with 5 μ M Gefitinib or BIBX1382 EGFR inhibitors for 24 hours. Apoptosis was measured by assaying DNA fragmentation. Error bars show standard deviation. P-values were calculated by Student's t-test; p-values for HCT116 cells are in black, for Hke3 cells in green; - means non-significant. (B) HCT116 cells were transfected with RASSF1A siRNA and treated with 5 μ M BIBX1382 for 30 minutes as indicated. MST2 IPs and cell lysates were Western blotted with the indicated antibodies. (C) Hke3 cells were transfected with Flag-K-RasV12 and RASSF1A siRNA and treated with 5 μ M BIBX1382. MST2 IPs were Western blotted with the indicated antibodies. (D) Growing HCT116 cells were transfected with RASSF1A, MST2, LATS1 or control siRNAs as indicated. Cells were treated with 5 μ M BIBX1382 for 20 hours and apoptosis was measured. Error bars show standard deviation. (E) HCT116 cells were transfected with wt K-Ras specific siRNAs, and a siRNA resistant Flag-tagged wt K-Ras construct (KRaswt#) as indicated. 4 hours later cells were treated with 5 μ M BIBX1382 for 20 hours, and assayed for apoptosis. All experiments represent at least three independent repeats, with 5A n=4 for DKS8 ND12, and n=5 for HCT116 and Hke3; 5D and 5E n=4.

Fig. 6. Mutant K-Ras induced apoptosis is counteracted by autocrine activation of the EGF receptor and AKT signalling. (A) Serum starved Hke3 cells were incubated with 5 μ M EGFR inhibitor BIBX1382 and HCT116 conditioned medium as indicated. The EGF receptor

was immunoprecipitated and its activation status was monitored using an anti-phosphotyrosine antibody. Downstream ERK activation was assessed by blotting with phospho-ERK (pERK) specific antibodies. **(B)** Hke3 cells were serum starved for 24 hours and treated with 5 μ M BIBX 1382 for 24 hours as indicated. Then cells were treated with EGF or conditioned media obtained from HCT116 cells for the indicated timepoints. Activated K-Ras was pulled-down using GST-Ras binding domain (GST-RBD) beads. **(C)** Serum starved Hke3 cells were incubated with 5 μ M PI3K inhibitor LY294002 (LY) or 10 μ M AKT inhibitor IV (AktI) for 30 minutes and stimulated with EGF (10nM) for the indicated times. MST2 IPs were analysed for kinase activity (KA). AKT and ERK activities were assessed by Western blotting of cell lysates with phosphospecific antibodies. **(D)** Hke3 cells were transfected with control, MST2, K-Ras siRNAs or a combination of MST2 plus K-Ras siRNAs. Apoptosis was quantified by assaying DNA fragmentation. 10 μ g of cellular extracts were analyzed for MST2 and K-Ras expression by Western blotting. All experiments represent three independent repeats.

Fig. 7. K-Ras mutation correlates with MST2 silencing or retention of the wt K-Ras allele in human and murine colorectal cancers. **(A)** MST2 protein expression and KRAS gene mutations were assayed in 173 human CRC samples (cohort 1) as described in experimental procedures. The p value was calculated using the two-sided Pearson's chi square test. **(B)** Representative sections of human paraffin embedded CRCs stained for MST2 protein expression. Corresponding KRAS gene sequencing traces are shown below. **(C)** MST2 protein expression and KRAS mutations in cohort 1 stratified according to Dukes stages. The p value was calculated using the two-sided Pearson's chi square test. **(D)** KRAS status in human CRCs was compared with MST2 expression in regard to apoptosis rate measured by TUNEL assay.

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Fig. 1

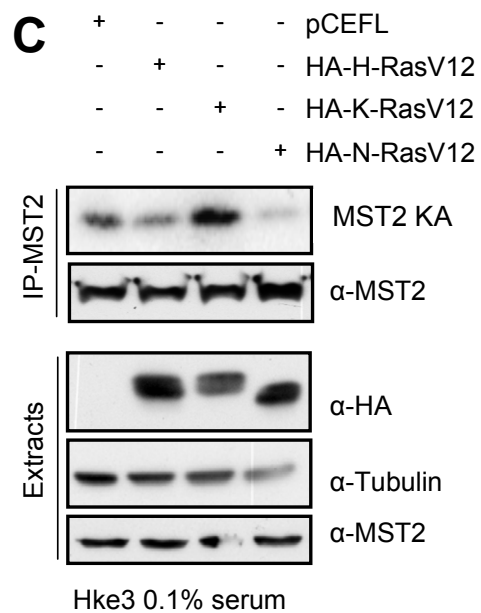
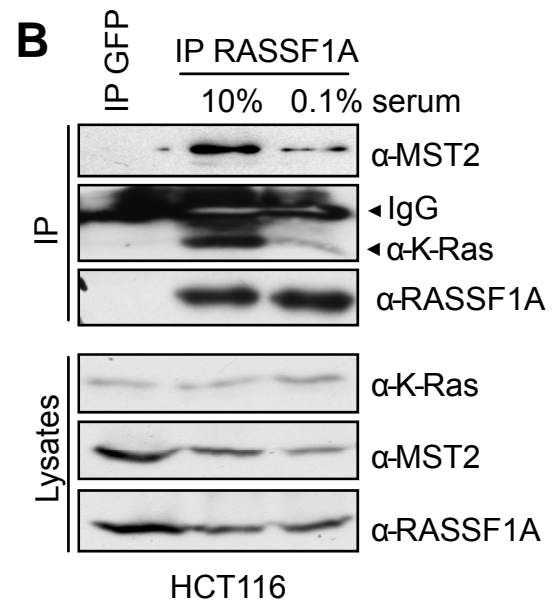
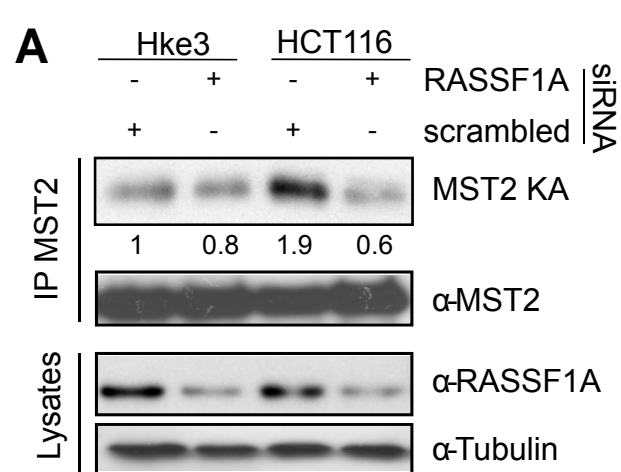


Fig. 1

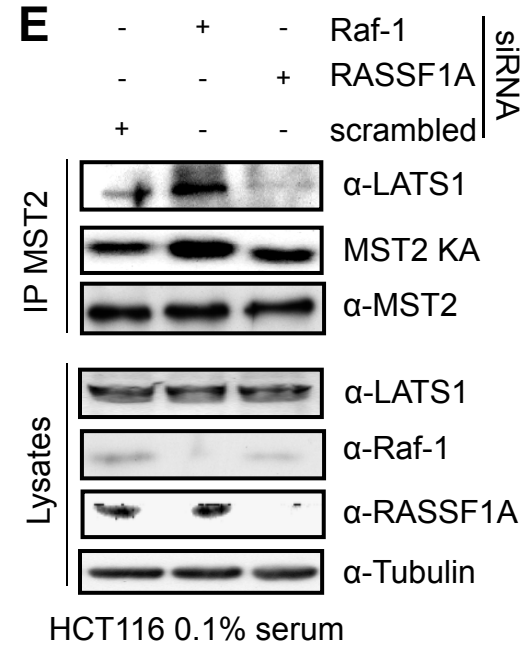
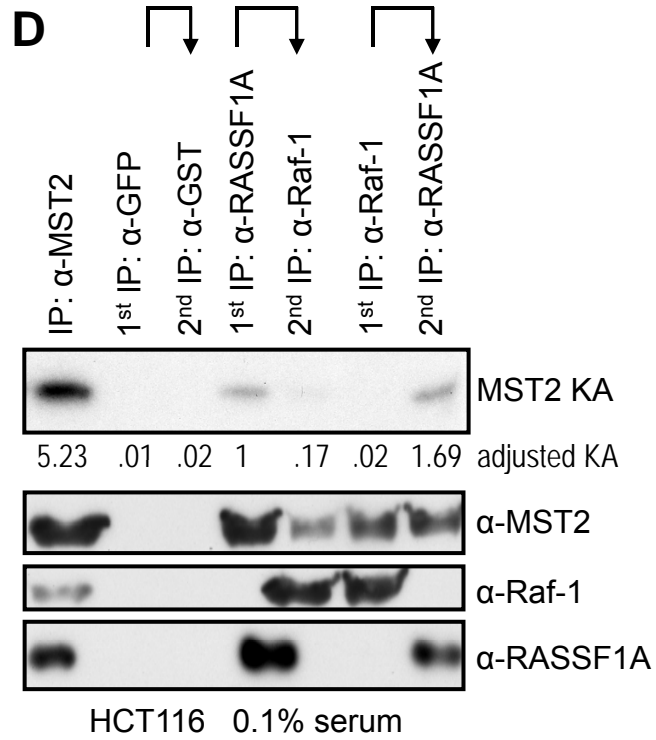


Fig. 1

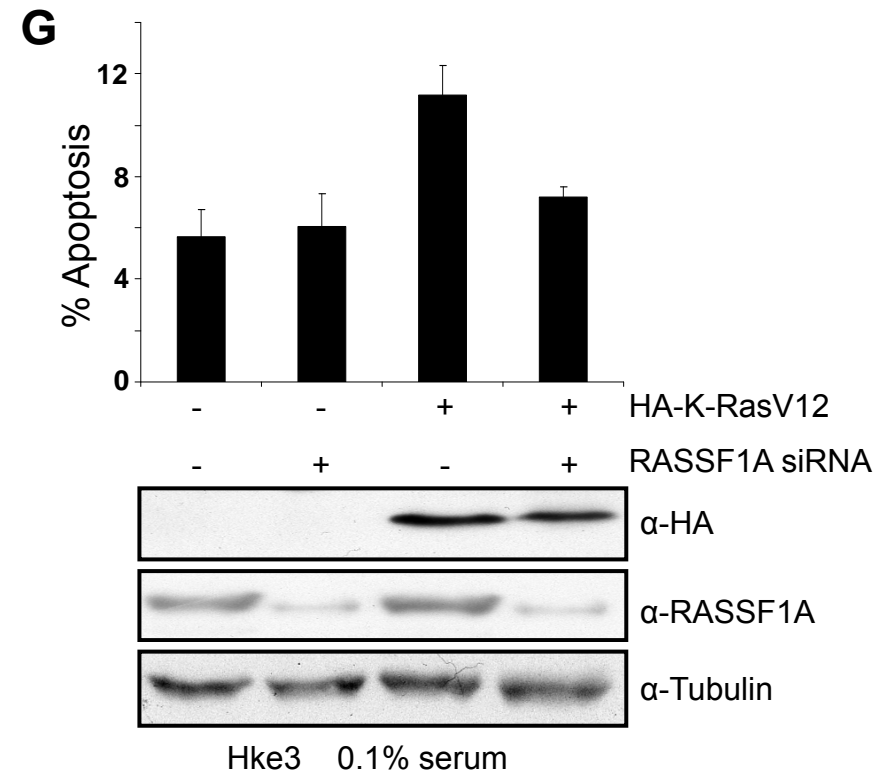
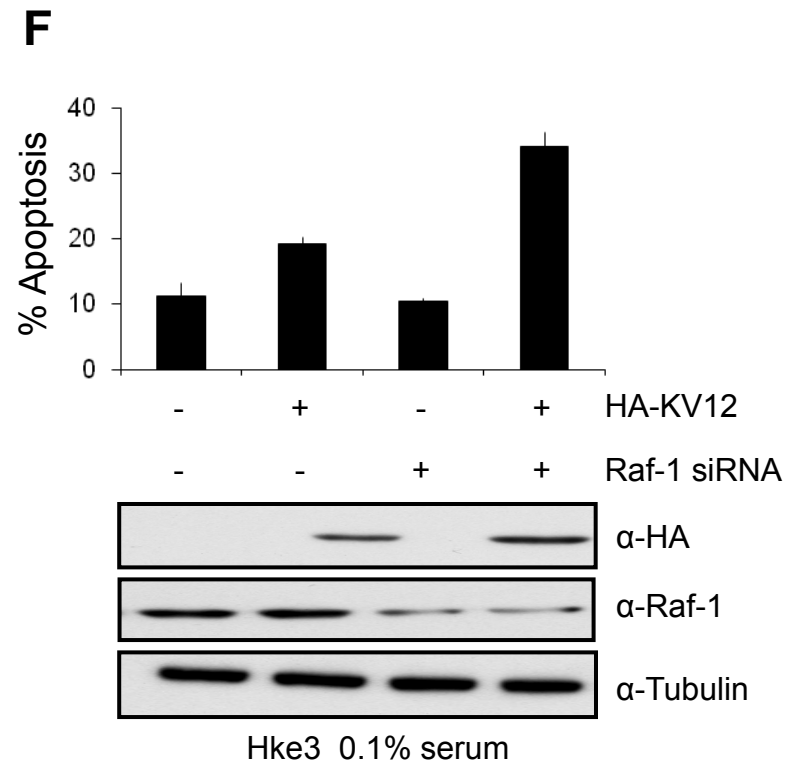


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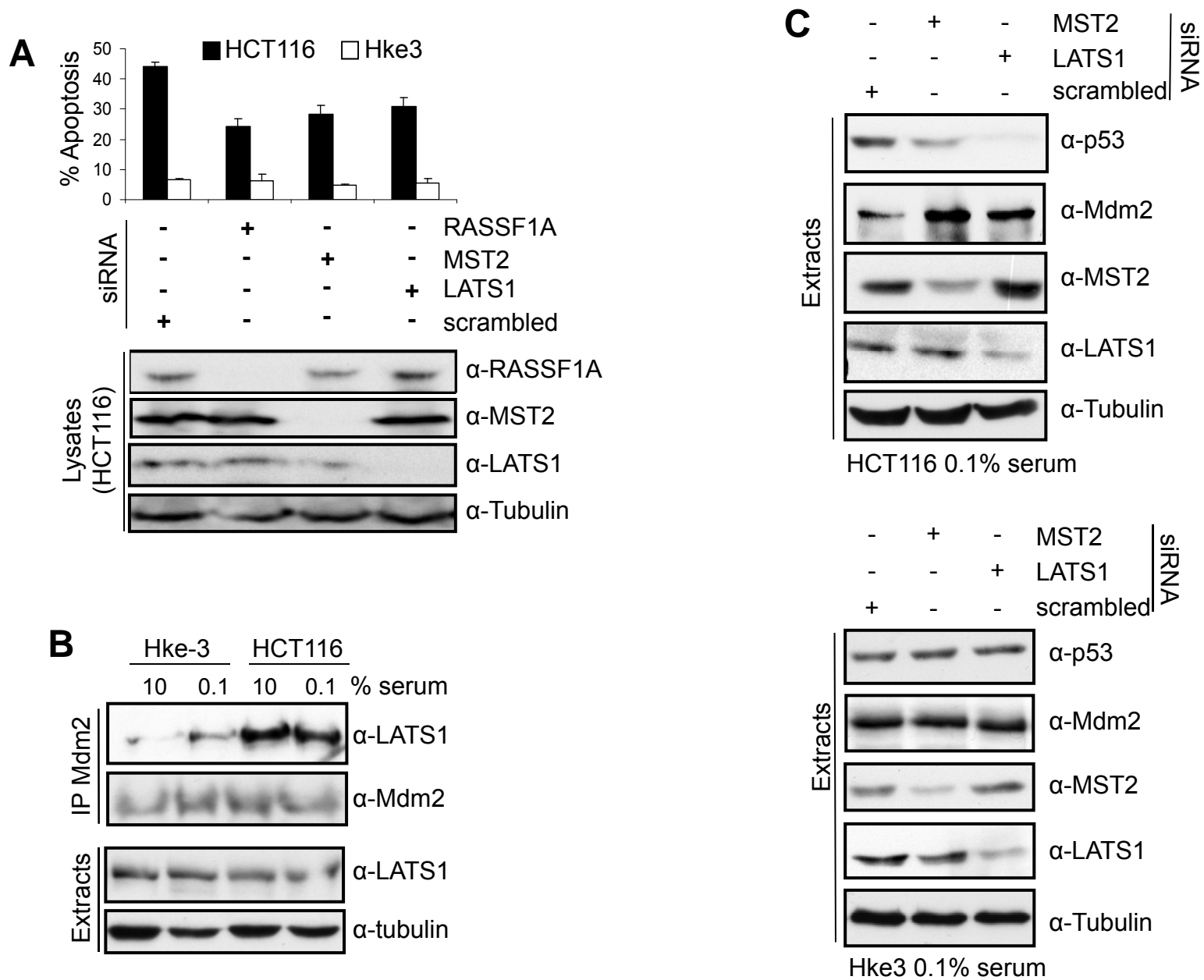


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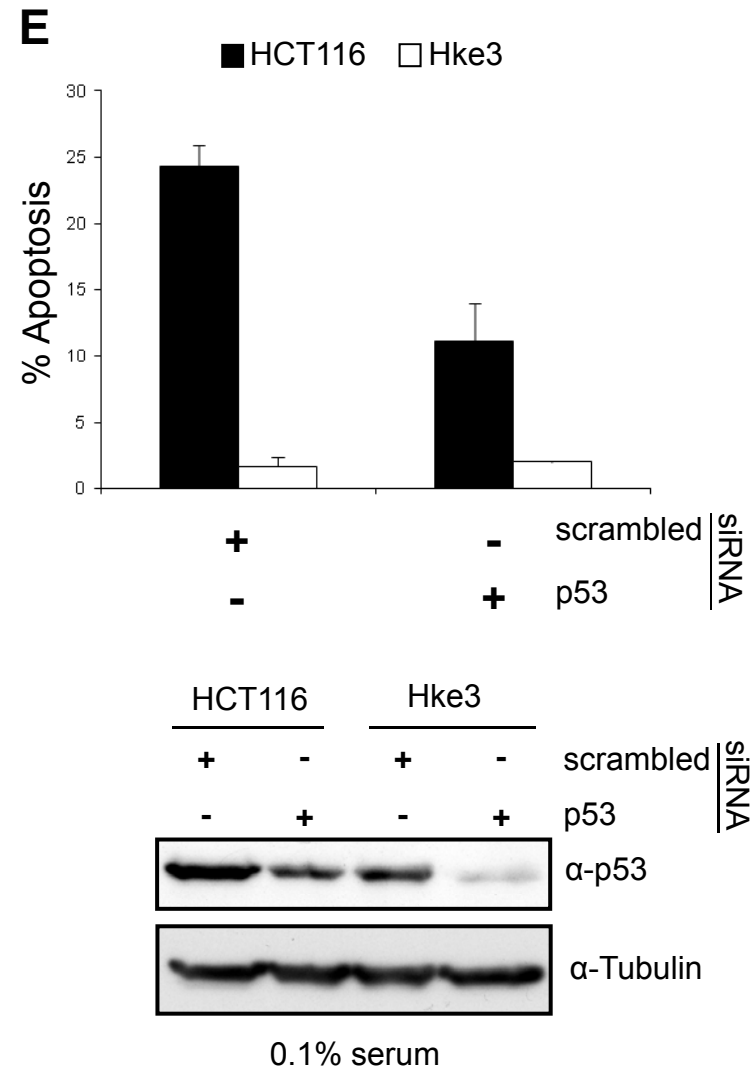
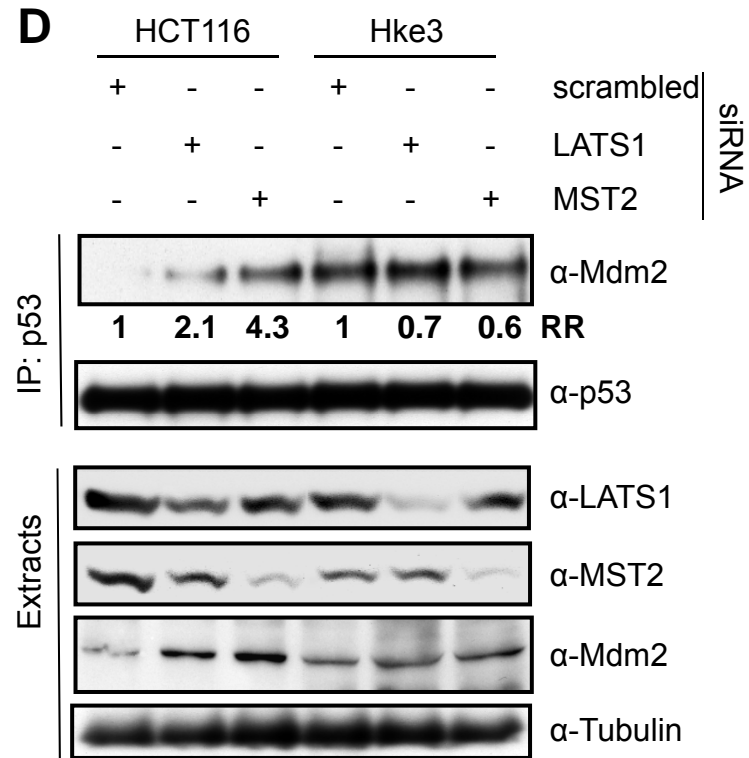


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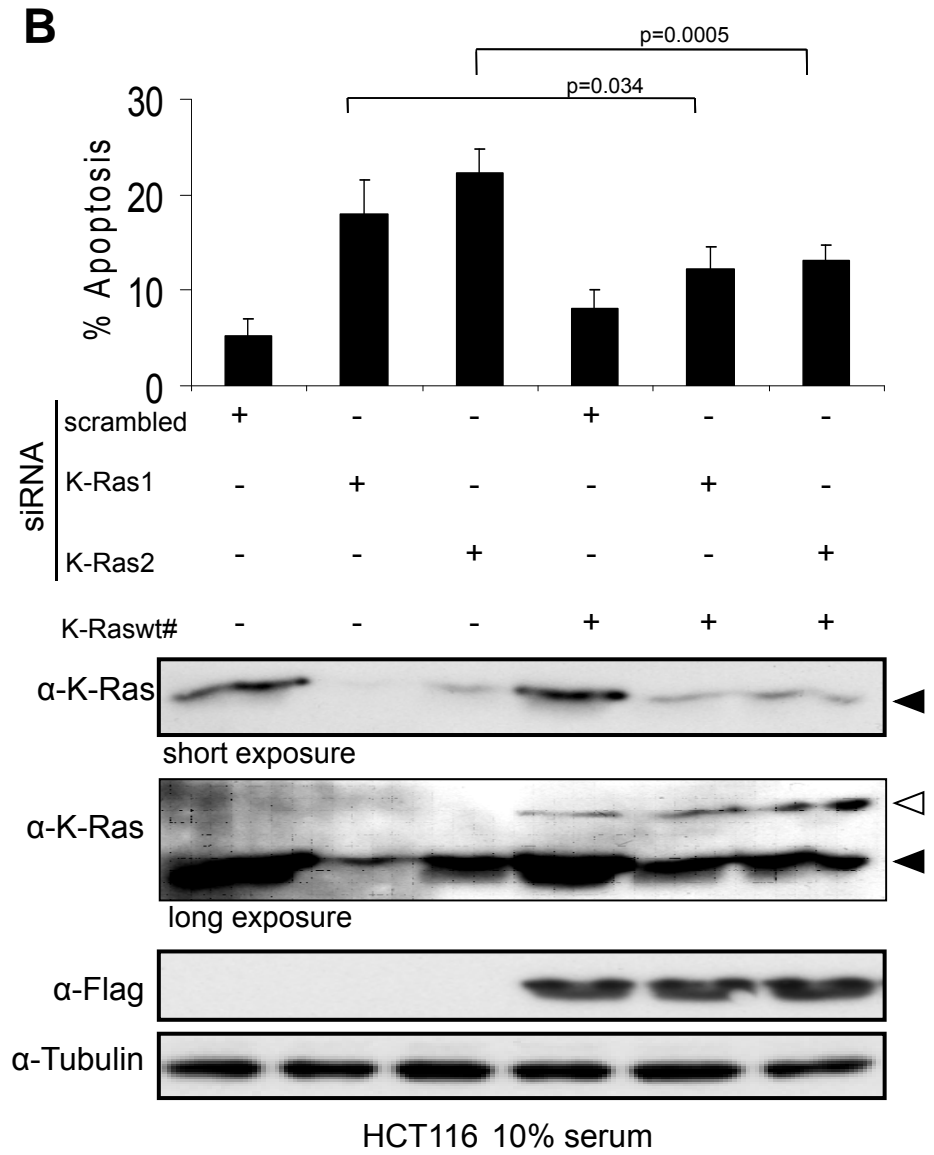
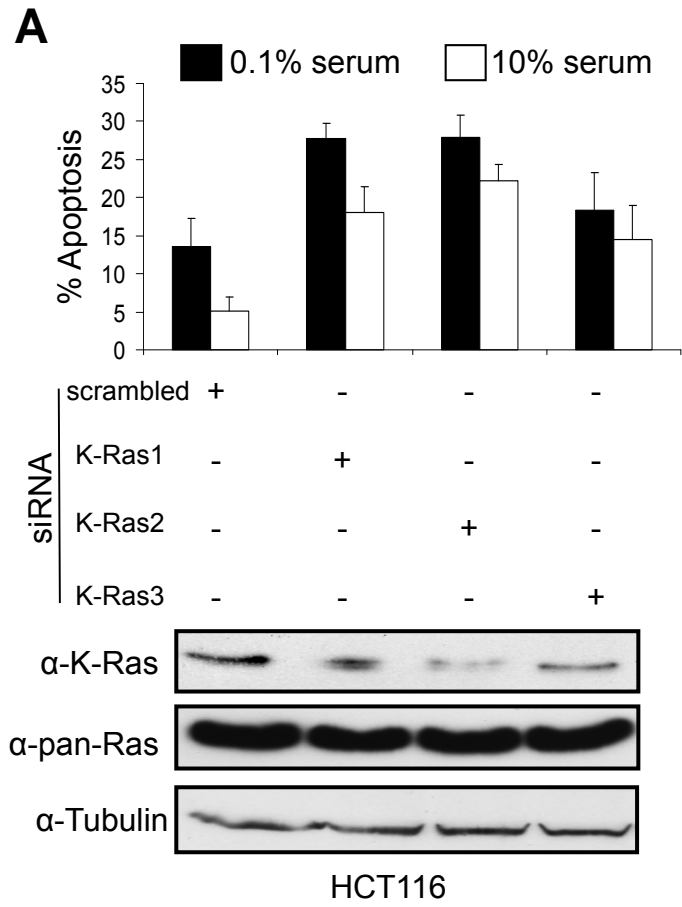


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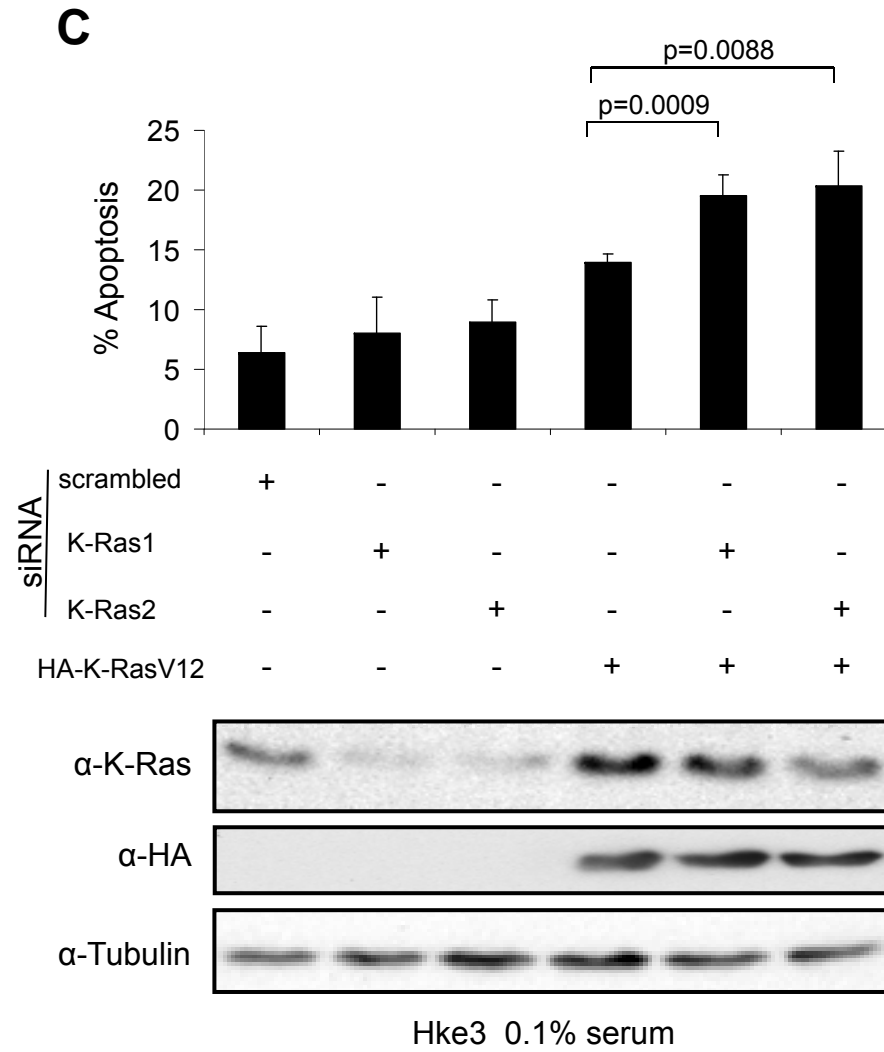


Fig. 4

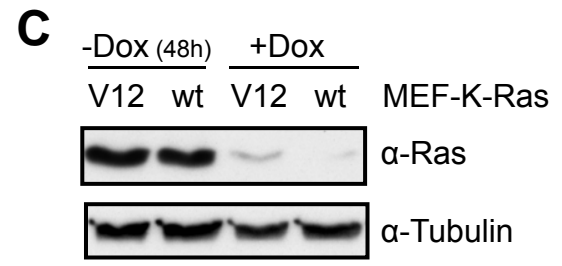
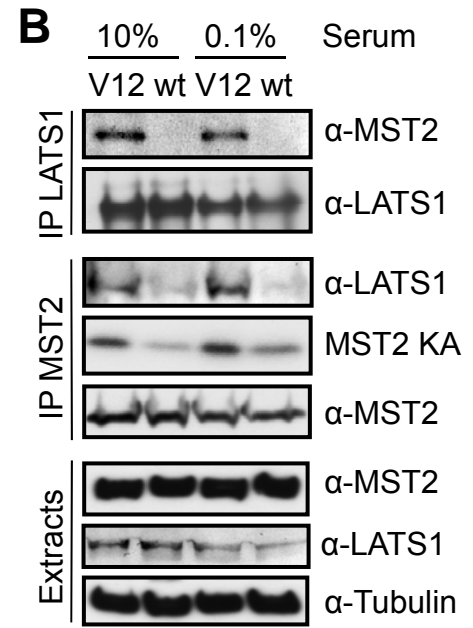
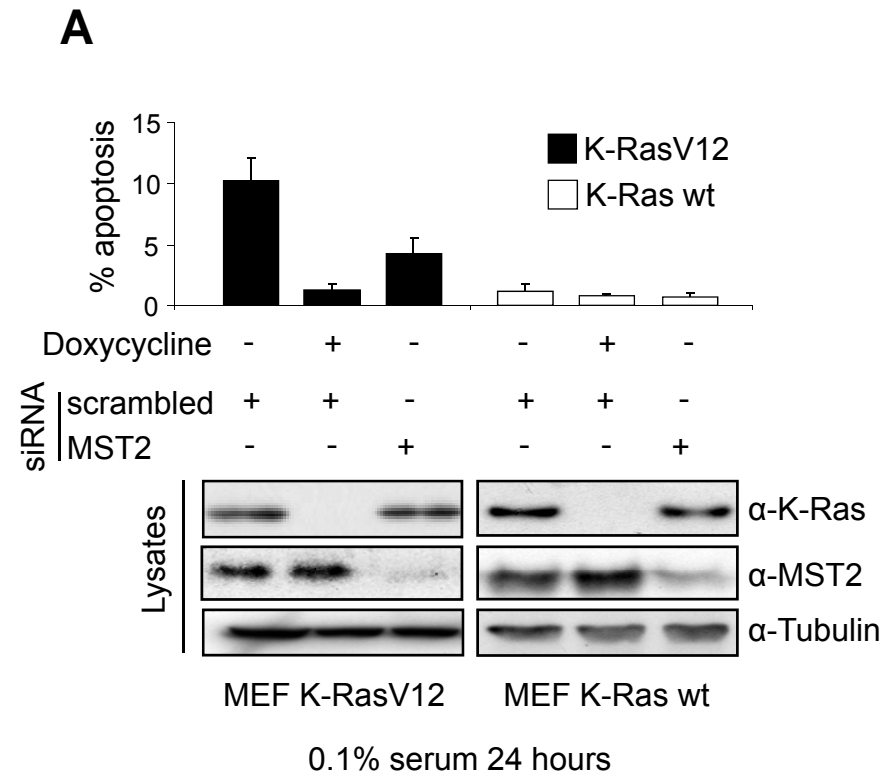


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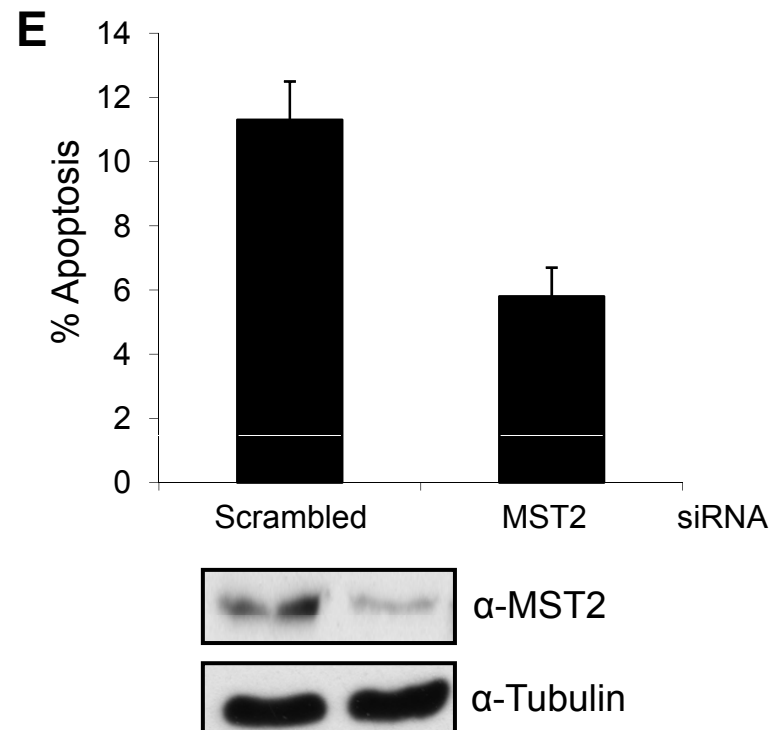
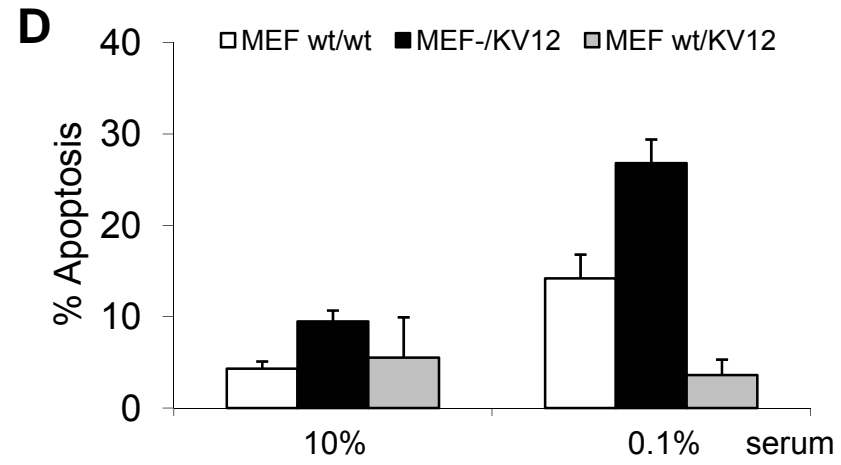


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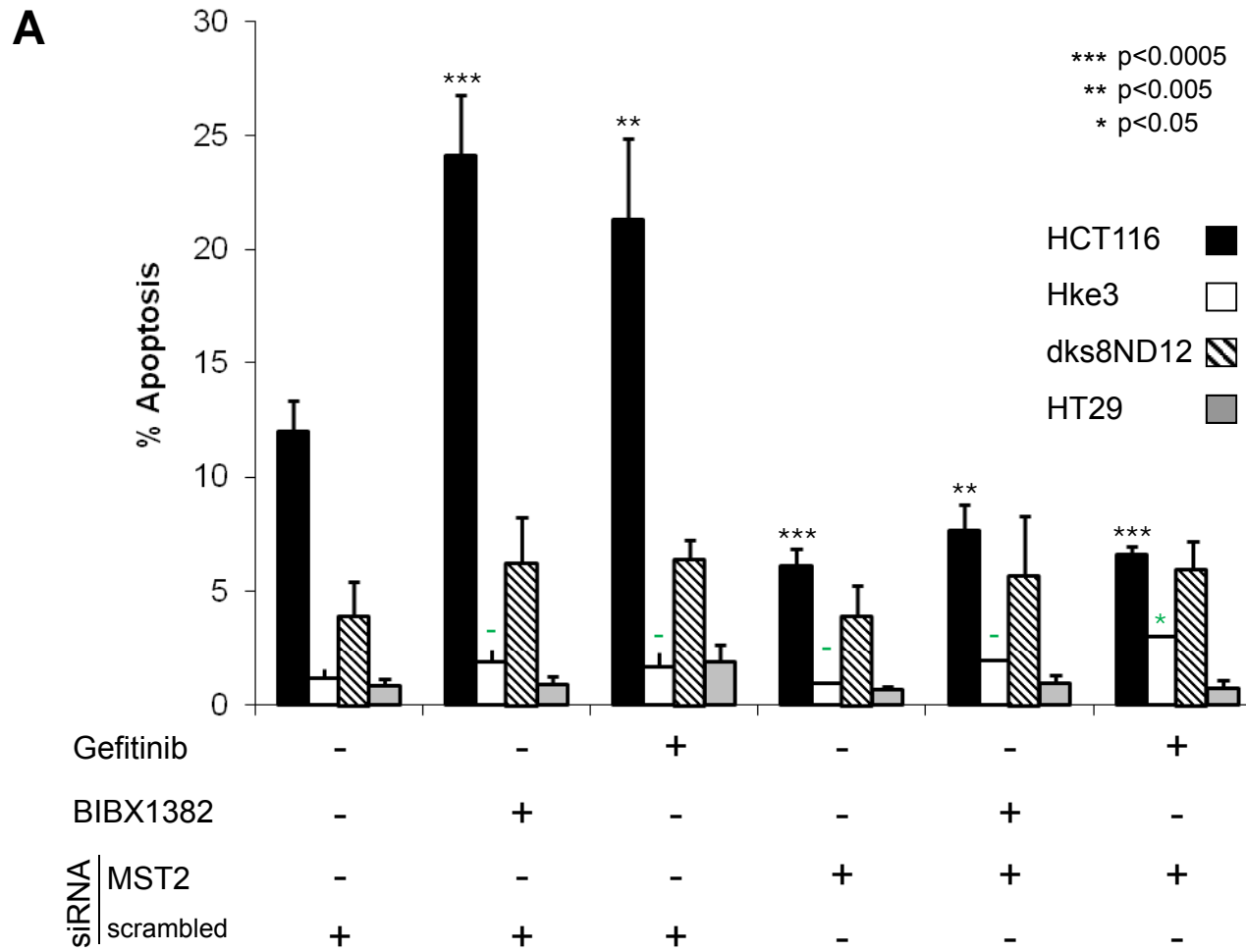


Fig. 5

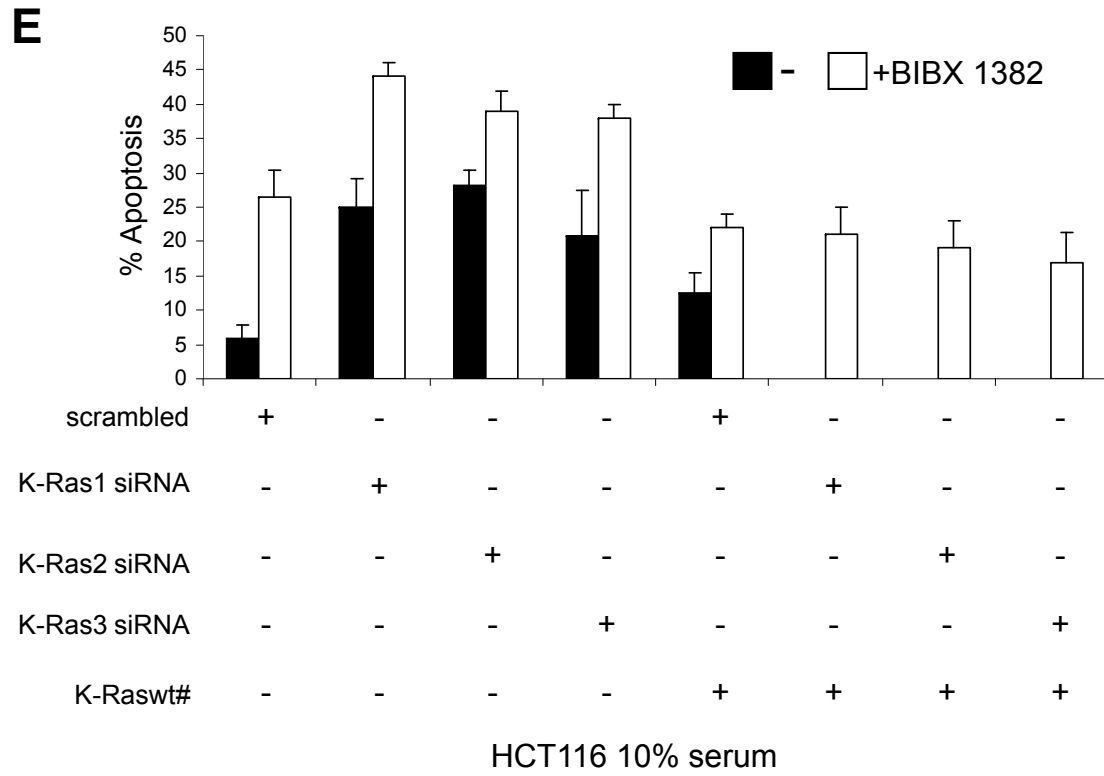


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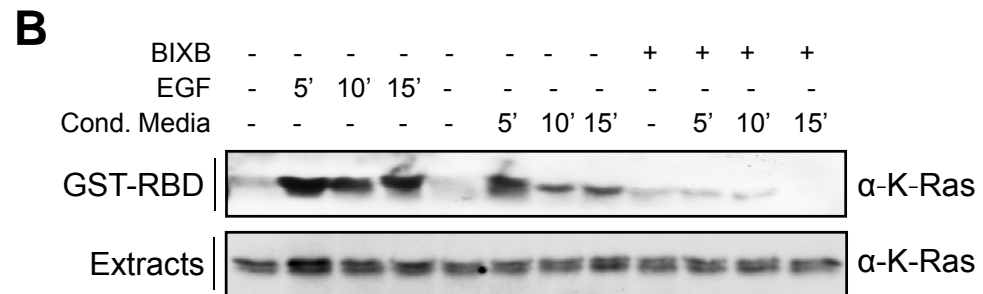
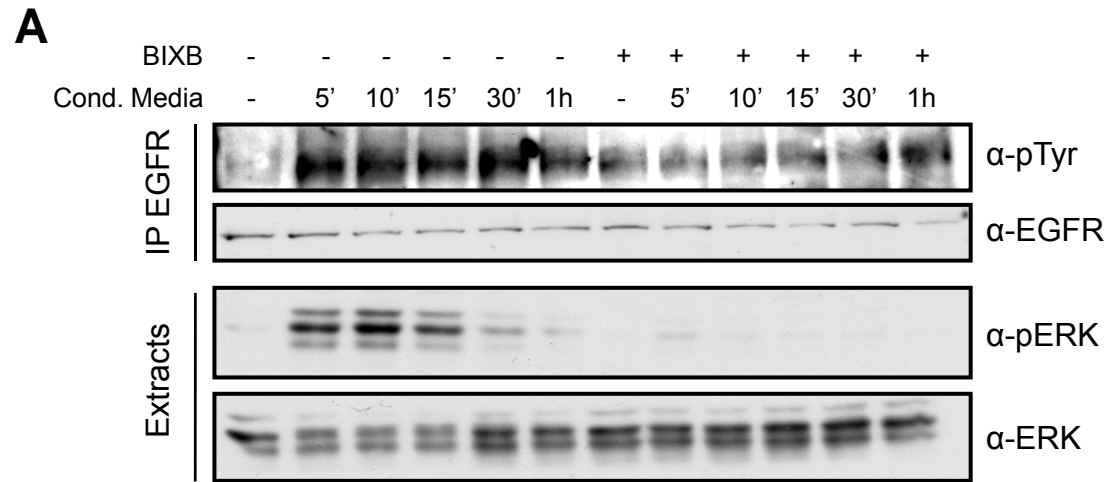


Fig. 6

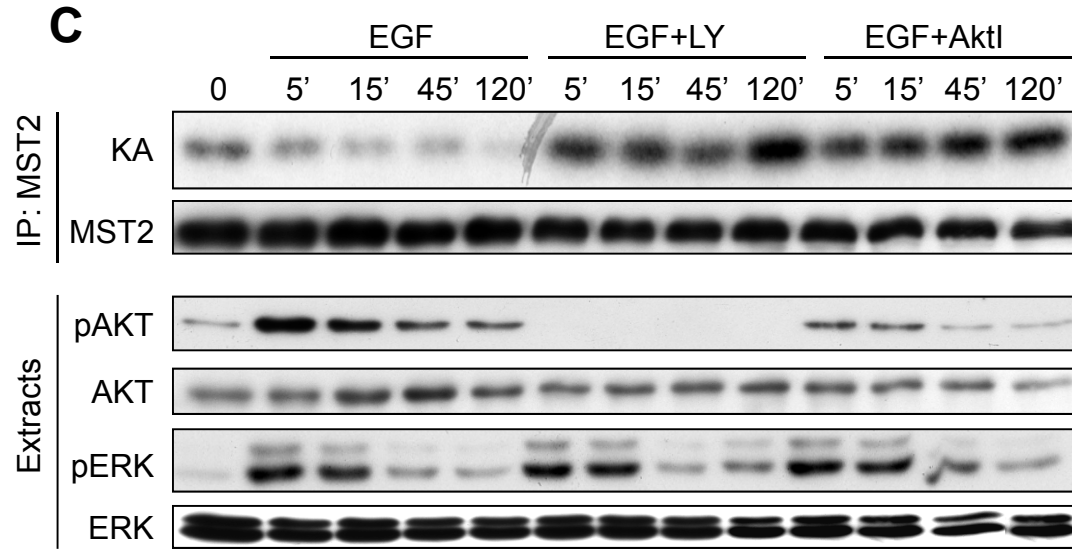


Fig. 6

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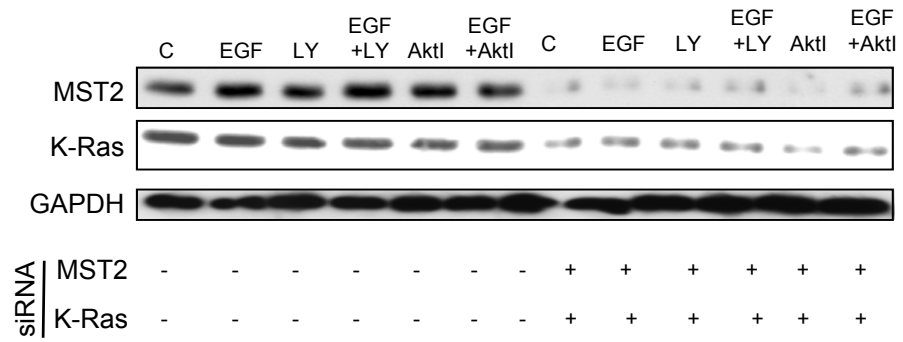
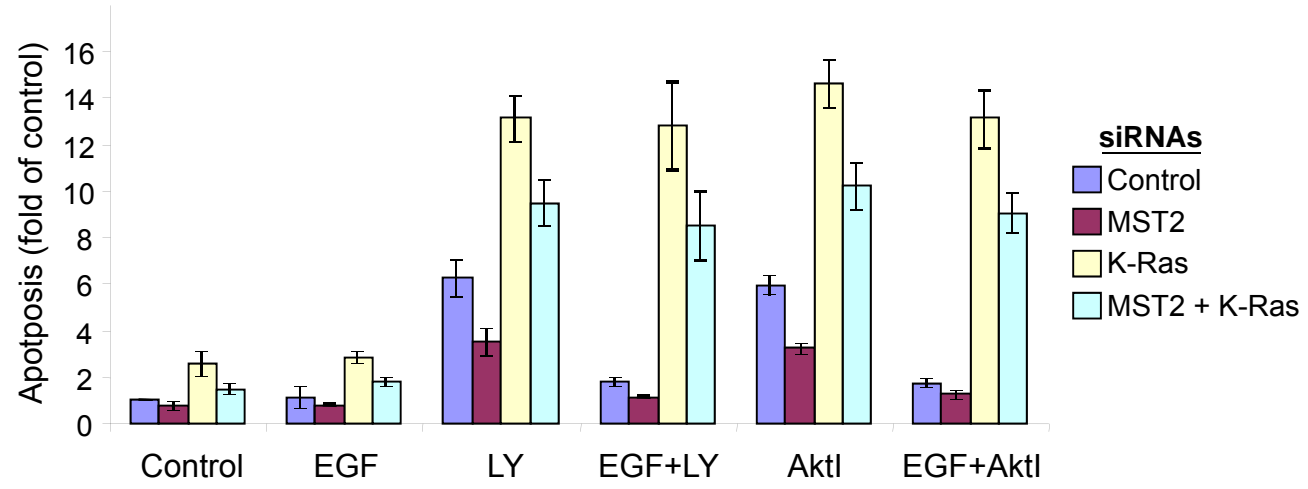


Fig 7

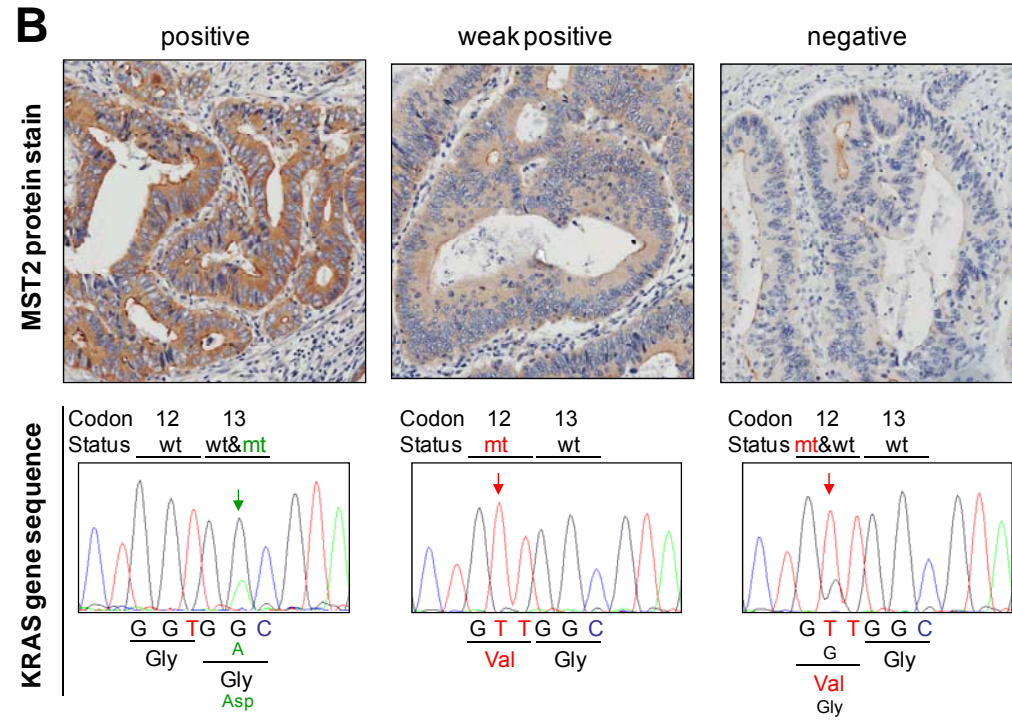
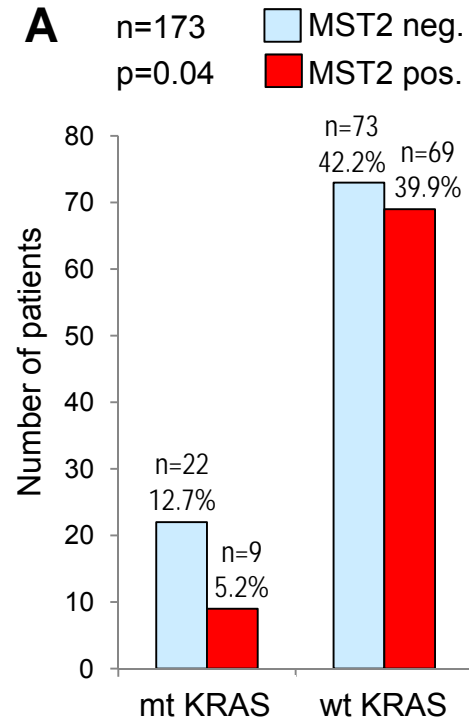
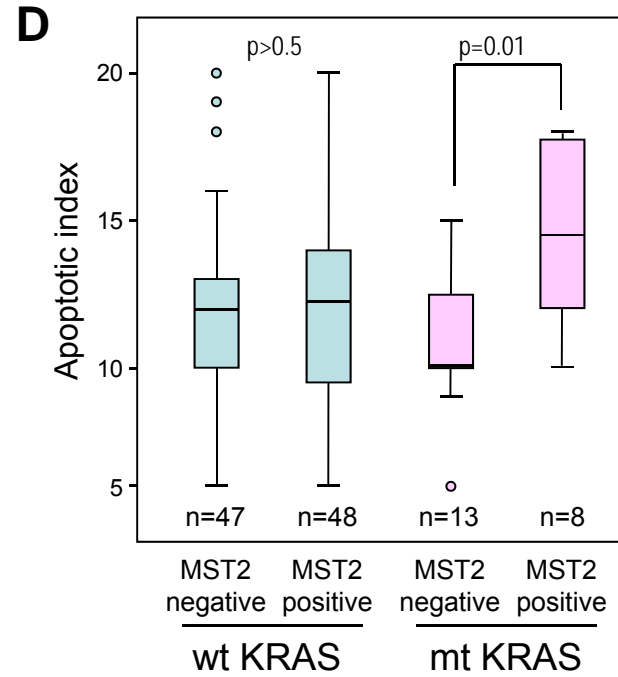
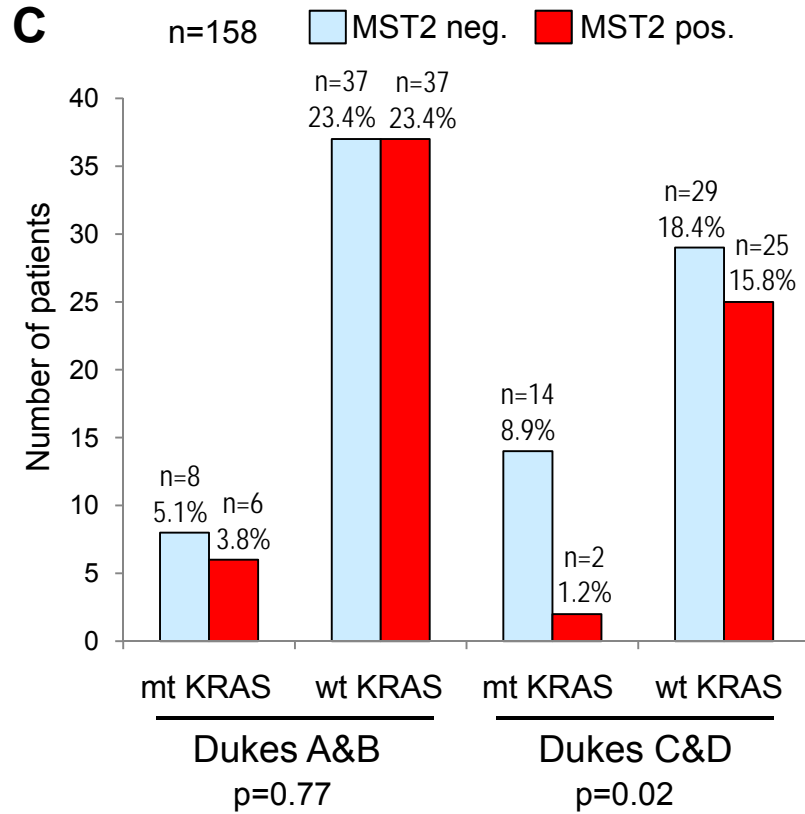


Fig. 7



INVENTORY - SUPPLEMENTAL INFORMATION

Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wildtype K-Ras

David Matallanas, David Romano, Fahd Al-Mulla, Eric O'Neill, Waleed Al-Ali, Piero Crespo, Brendan Doyle, Colin Nixon, Owen Sansom, Matthias Drosten, Mariano Barbacid, Walter Kolch

Extended experimental procedures

- Cells
- Antibodies and inhibitors
- siRNAs
- Plasmids
- Alternative apoptosis assays
- Immunoprecipitation and immunoblotting
- Laser capture microdissection (LMD) and pyrosequencing
- Apoptosis in human CRCs

Supplemental References

Supplemental Figures

Figure S1, related to Figure 1

Figure S2, related to Figure 2

Figure S3, related to Figure 3

Figure S4, related to Figure 4

Figure S5, related to Figure 5

Figure S6, related to Figure 6

Figure S7, related to Figure 7

Figure S8, Summary of paper: schematic model of the differential roles of wt and mt K-Ras in transformation.

SUPPLEMENTAL INFORMATION

Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wildtype K-Ras

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Extended experimental procedures

Cells. HCT116, Hke3, MCF7, HeLa, MEF-K-RasV12 Tet-Off, MEF-K-Ras wt and Hek293, K-Ras (+/+);RERT (ert/ert) [wt/wt], MEF K-Ras (lox/LSLG12Vgeo);RERT (ert/ert) MEFs [-/KV12] and K-Ras (+/LSLG12Vgeo);RERT (ert/ert) MEF [wt/V12] cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (Gibco-BRL). The K-RasV12 Tet-Off and K-Ras wt Tet-Off MEFs were generated from *H-Ras*^{-/-}; *N-Ras*^{-/-}; *K-Ras*^{lox/lox}; *RERT*^{ert/ert} MEFs (Drosten et al., 2010). In brief, MEFs have been co-infected with pBABEpuro tTA and pRevTRE K-RasV12 or K-Ras wt. Endogenous K-Ras was eliminated by treatment with 4-Hydroxytamoxifen. To render cells Ras-less, K-RasV12 Tet-Off and K-Ras wt Tet-Off MEFs were treated for at least 48 hours with 1ng/ml doxycycline. To allow the re-expression of the K-Ras proteins we followed the protocol recommended by Clontech. Briefly, cells were washed twice with PBS and trypsinized. Cells were collected by centrifugation and the pellets were washed with PBS. After centrifugation the cells were resuspended in DMEM and counted. 1×10^6 cells were plated in 10cm Petri dishes and allowed to adhere. After 4-6 hours the cells were washed one more time with PBS and new media was added. Re-expression of K-Ras proteins was observed after 24 hours. Transient transfections were done with LipofectAMINE (Invitrogen) according to manufacturer's instructions.

Antibodies and inhibitors 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 were from 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); goat polyclonal Hsp70 (Santa Cruz); rabbit polyclonal Phospho-EGFR and EGFR (New England); EGF receptor inhibitorII BIBX 1382, LY294002 and Akt inhibitor IV (Calbiochem); gefitinib (American Custom Chemicals Corporation).

siRNAs against RASSF1A, p73, Raf-1, LATS1 and MST2 siRNAs were from Dharmacon, and sequences have been described before (Matallanas et al., 2007).

MST2 siRNAs specifically targeting wt K-Ras were designed against codon 13 of K-Ras and custom made by Dharmacon. Sequences are

K-Ras1: 5'-GUU GGA GCU GGU GGC GUA G -3/ 5'-CAA CCU CGA CCA CCG CAU C-3';

K-Ras2 5'-GGA GCU GGU GGC GUA GGC-3'/5'-CCU CGA CCA CCG CAU CCG-3'.

K-Ras3 5'- GCU GGU GGC GUA GGC AAG-3'/5'- CGA CCA CCG CAU CCG UUC-3'.

Plasmids. HA-RASSF1A, Flag-RASSF1A, Flag-LATS1 are cloned in pcDNA3 and were described before (Matallanas et al., 2007). HA-H-Ras wt, HA-K-Ras wt, HA-N-Ras wt, HA-K-RasV12, Flag-

K-RasV12, and HA-K-RasN17 are cloned in pCEFL and were described before (Matallanas et al., 2003). pCEFL-Flag-K-Raswt# is a wt K-Ras containing a conservative point mutation that renders it resistant to the specific siRNAs against wt K-Ras. The mutation was generated by direct mutagenesis PCR using a mutagenesis kit from Stratagene, with the following oligonucleotides:

5'-GTT GGA GCT GGT GGA GTA GGC AAG AGT GCC -3' and

3'-GGC ACT CTT GCC TAC TCC ACC AGC TCC AAC - 5' (Invitrogen).

pCDNA-Flag-LATS1# is a wt LATS1 containing a conservative point mutation resistant to the single LATS1 siRNA J-004632-06 (Matallanas et al., 2007) from Dharmacon generated by directed mutagenesis using the following oligonucleotides :

5'-CGG CAA GAT AGC ATG GAC TTC AGT AAT GAA TGG GGG G -3' and

3'-CCC CCA TTC ATT ACT GAA GTC CAT GCT ATC TTG CCG - 5' (MWG).

pCDNA-Flag-MST2# resistant mutant to siRNA J-004874-07-0010 from Dharmacon (Matallanas et al., 2007) generated as above using these oligonucleotides:

5'-CGG GCC ACA AGT ACG ATG AGT GAA GGG GCC -3' and

3'-GGC CCC TTC ACT CAT CGT ACT TGT GGC CCG - 5' (MWG).

pCDNA-Flag-RASSF1A# is wt RASSF1A mutant resistant to siRNA custom made by MGW (Matallanas et al., 2007), and was generated by direct mutagenesis using the following oligonucleotides:

5'-CCC CGG GAC CTG GGA TGG GAA CCC GCG -3' and

3'-CGC GGG TTC CCA TCC CAG GTC CCG GGG - 5' (MWG).

Alternative apoptosis assays. Caspase activity was measured to confirm apoptosis by using the fluorescent cleavable substrate of caspases zVAD-fmk-FITC (Promega), according to the manufacturer's instructions and as previously described (Matallanas et al., 2007).

Immunoprecipitation and immunoblotting. Immunoprecipitations were performed as described before (Matallanas et al., 2007). Briefly, cells were lysed in 20mM HEPES pH7.5, 150mM NaCl, 1% NP-40, 2mM NaF, 10mM β -glycerophosphate, 2mM $\text{Na}_4\text{P}_2\text{O}_4$ and protease and phosphatase inhibitors. Immunoprecipitates were washed 3 times with lysis buffer containing 0.5 % NP-40, separated by SDS-PAGE and analysed by Western blotting. For YAP1-p73 co-immunoprecipitations cells were lysed in 50mM TrisHCl pH8, 100mM NaCl, 10% glycerol, 1% Triton X-100, 1mM MgCl_2 , 2mM PMSF, protease and phosphatase inhibitors. These immunoprecipitates were washed three times with NET-buffer (50mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 0.25% gelatine, 0.1% Nonidet P-40) and immunoblotted. To co-immunoprecipitate Mdm2 with p53 or LATS1 HCT116 or Hke3 cells were lysed and immunoprecipitated as previously described (Chen et al 1993). Briefly, the cells were lysed in lysis buffer containing 50mM TrisCl pH8, 5mM EDTA 150mM NaCl, 0.5% NP40 plus inhibitors. The lysates were sonicated once and the supernatants were incubated with anti-p53 (a kind gift from Karen Vousden), or a mix of anti-Mdm2 (ab-1 and ab-2; Calbiochem) antibodies and protein-G beads. After 2-3 hours incubation at 4°C the beads were washed 3 times with the lysis buffer and analysed by Western blotting. Blots were quantitated by densitometry using the program NIH Image 1.60.

Laser capture microdissection (LMD) and pyrosequencing. Human colorectal cancer samples (Al-Mulla et al., 2006) were paraffin embedded and tumour specific and normal tissue sections were isolated by LMD. Mouse tumours arising in the $\text{APC}^{\text{Flox}} / \text{KRas}^{\text{G12D}}$ model of intestinal adenocarcinoma (Haigis et al., 2008) and the $\text{KRas}^{\text{G12D}}$ model of pancreatic ductal adenocarcinoma (Morton et al., 2010) were fixed in 10% neutral buffered formalin prior to standard histological processing and paraffin embedding. For LMD 10 μm sections were mounted on Leica PET membrane Frameslides and stained with Haematoxylin and Eosin. LMD was performed on the Leica LMD7000 (Leica) according to the manufacturer's instructions. DNA was extracted using the

QIAmp DNA FFPE Tissue kit (Qiagen) according to the manufacturer's instructions. The region of the G12D mutation was amplified using the following primers:

Forward primer: ATCGTCAAGGCGCTCTTGC;

Reverse primer: GGCCTGCTGAAAATGACTGAGT.

The reverse primer was biotinylated. Successful amplification was confirmed by the presence of a band of appropriate size on a 2% agarose gel and pyrosequencing was carried out on the PSQ96MA pyrosequencing machine (Qiagen), according to the manufacturer's instructions using the sequencing primer GCGCTCTTGCCTACG. The data were analysed using the PSQ96MA software (Qiagen).

Apoptosis in human CRCs from an extended cohort (Al-Mulla et al., 2006) was measured using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as described previously (Al-Mulla et al., 2006).

Supplemental References

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Supplemental Figures

Fig. S1

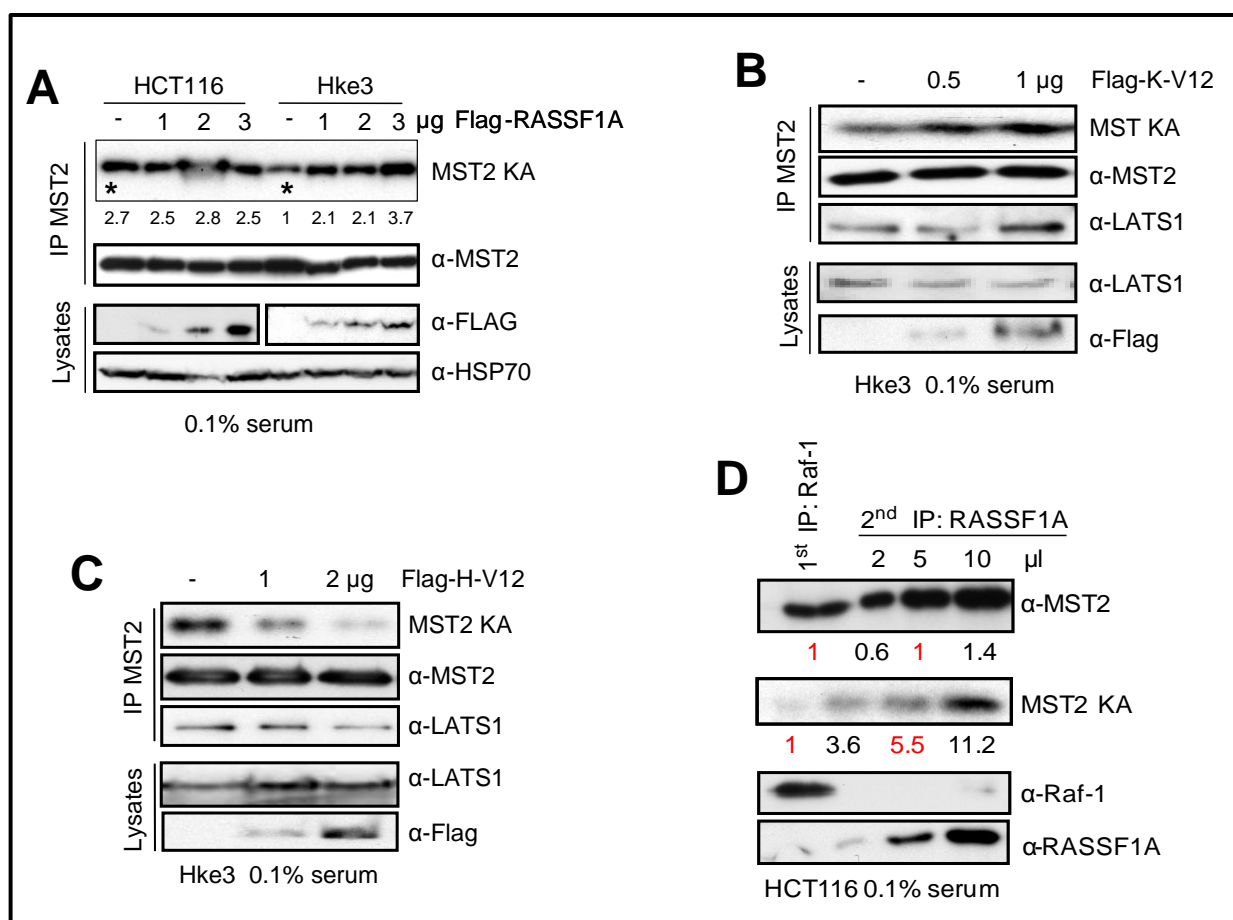


Fig. S1. Oncogenic K-Ras regulates MST2. (A) MST2 is constitutively activated in HCT116 cells due to expression of a mutated K-Ras allele. HCT116 and Hke3 cells were transfected with increasing amounts of Flag-RASSF1A. MST2 kinase activity (KA) was monitored by an in gel kinase assay. Gels were quantified using NIH image software and relative fold of activation was determined (numbers below kinase assay). Western blots were stained with the indicated antibodies. Asterisks mark activities in cells transfected with empty vector controls. HSP70 was used as a loading control.

MST2 regulation by different mutant Ras isoforms. Hke3 cells were transfected with Flag-K-Ras V12 (B) or Flag-H-Ras V12 (C). After serum deprivation endogenous MST2 was immunoprecipitated and assayed for kinase activity (KA) using an in gel kinase assay. MST2 immunoprecipitates were blotted with the indicated antibodies.

MST2 bound to RASSF1A and Raf1 have different kinase activity (D) Sequential Raf-1 and RASSF1A immunoprecipitates (IPs) from HCT116 cell lysates were assayed for MST2 kinase activity (KA). After Raf-1 was immunoprecipitated, the supernatants were incubated with anti-RASSF1A antibody. Increasing amounts of RASSF1A IPs and the amount of MST2 in these IPs were compared to the amount of MST2 in the Raf-1 IP. The IPs were assayed for MST kinase activity and blotted with the indicated antibodies. Gels were quantified using NIH image software and relative fold of activation was determined (numbers below kinase assay). The relative kinase activity of MST2 was compared when there is the same concentration of MST2 in both complexes (numbers in red).

Fig. S2

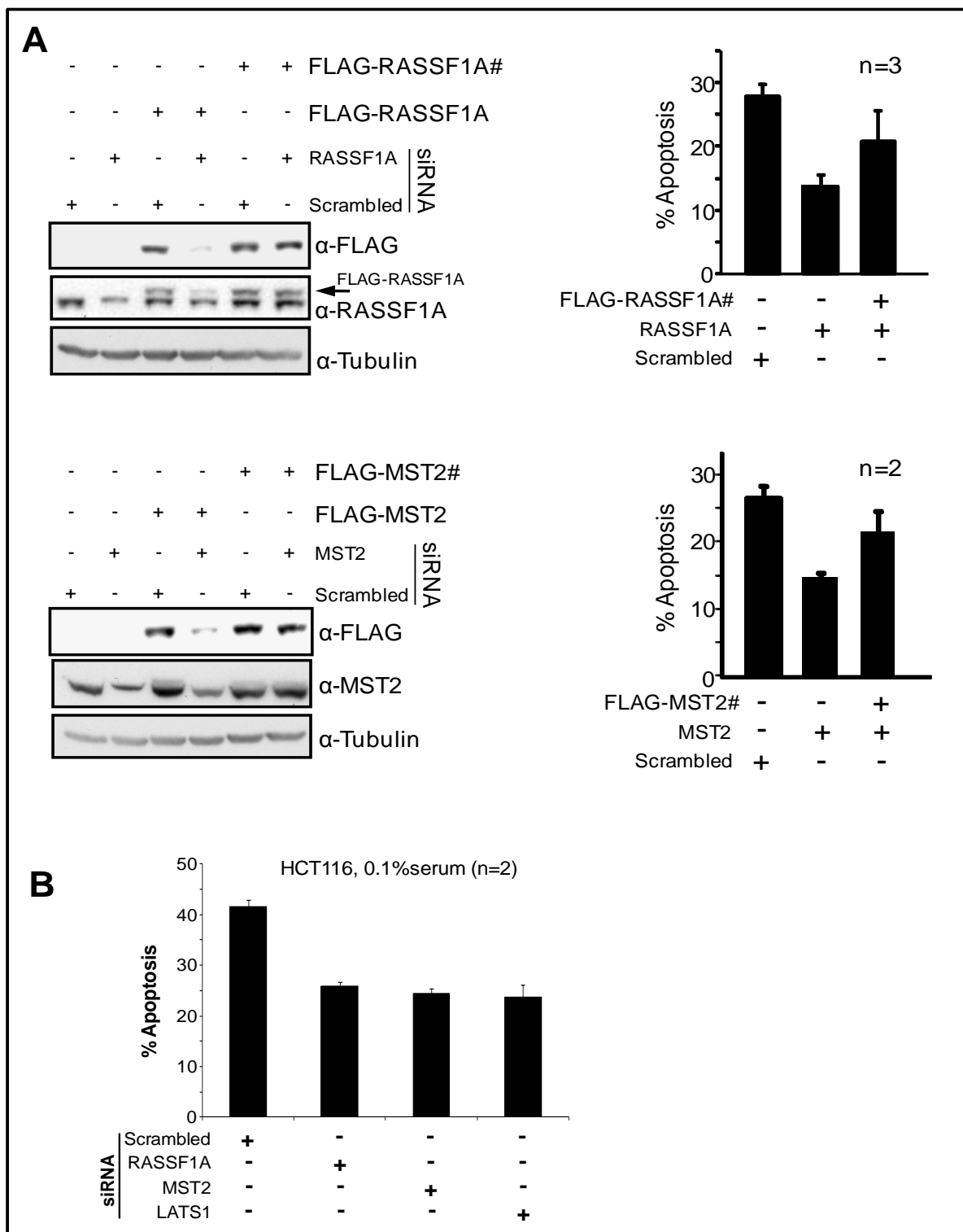


Fig. S2. (A) The specificity of siRNAs was ascertained by introducing silent point mutations into RASSF1A, MST2 and LATS1 that confer resistance to siRNAs. Mutant constructs (indicated by #) and siRNAs were transfected and protein expression and apoptosis were measured as indicated. **(B)** Apoptosis of serum starved (0.1% serum for 24 hours) HCT116 cells was measured by assessing the activation of caspases 3/7 with z-VAD-FMK-FITC as described in the Supplementary Methods.

Fig. S2 (continued)

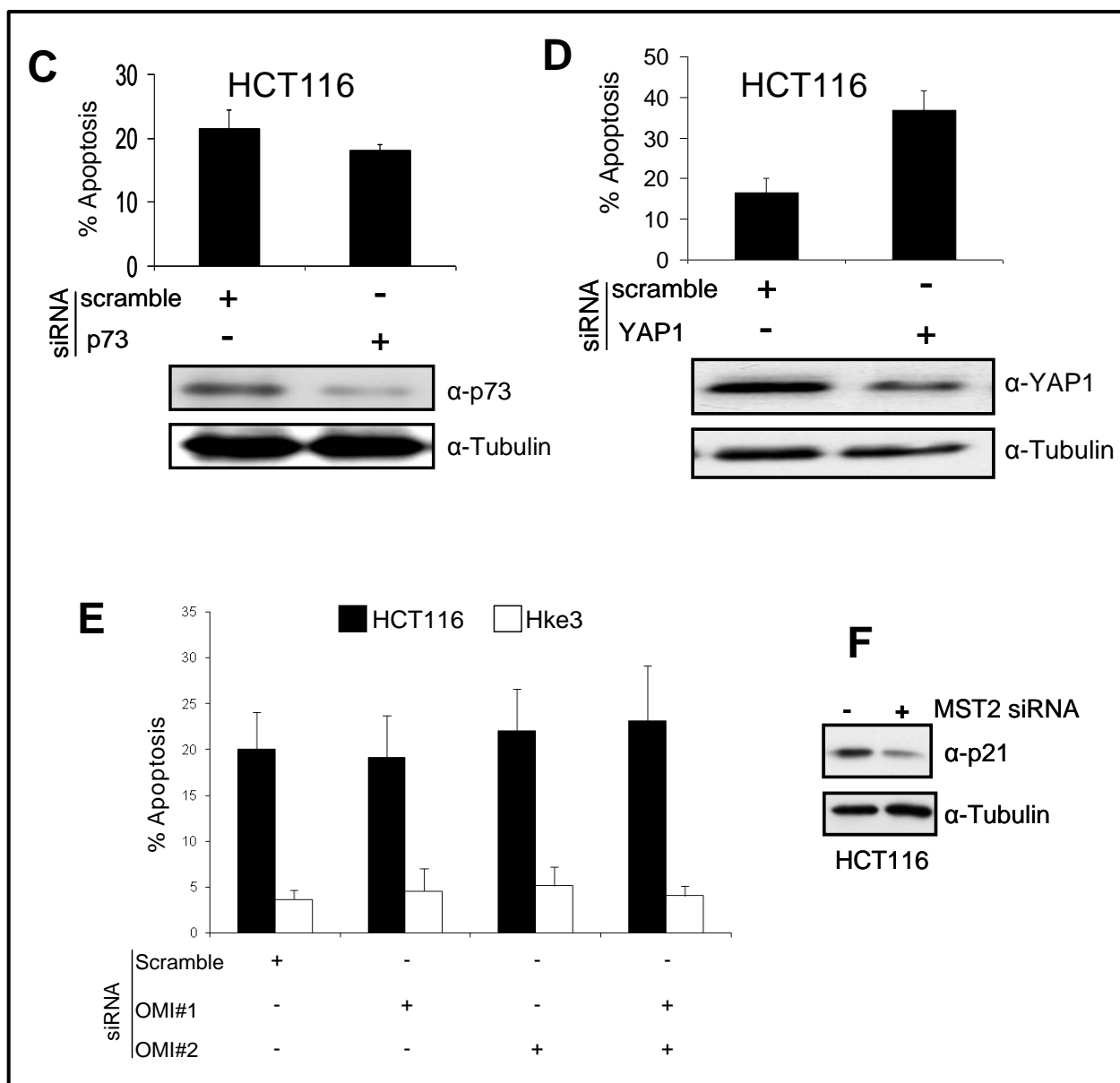


Fig. S2. Effectors of the mutant K-Ras activated apoptosis pathway. Apoptosis downstream of mutant K-RasD13 is not induced by p73, YAP1 or Omi/Htra2. HCT116 (expressing an endogenous K-RasD13 allele) were transfected with 50ng/ml of control siRNA or siRNAs against p73 (C) or YAP1 (D). Cells were serum starved for 20 hours and apoptosis was measured by DNA fragmentation. Error bars show standard deviation. (E) HCT116 and Hke3 cells were transfected with two specific siRNAs against Omi/Htra2 or non targeting siRNA as indicated, and serum starved for 20 hours. Apoptosis was assayed by DNA fragmentation. Error bars show standard deviation. (F) Expression of the p53 inducible p21^{WAF} protein was assessed by Western blotting in HCT116 cells transfected with non-targeting or MST2 siRNAs.

Fig. S3

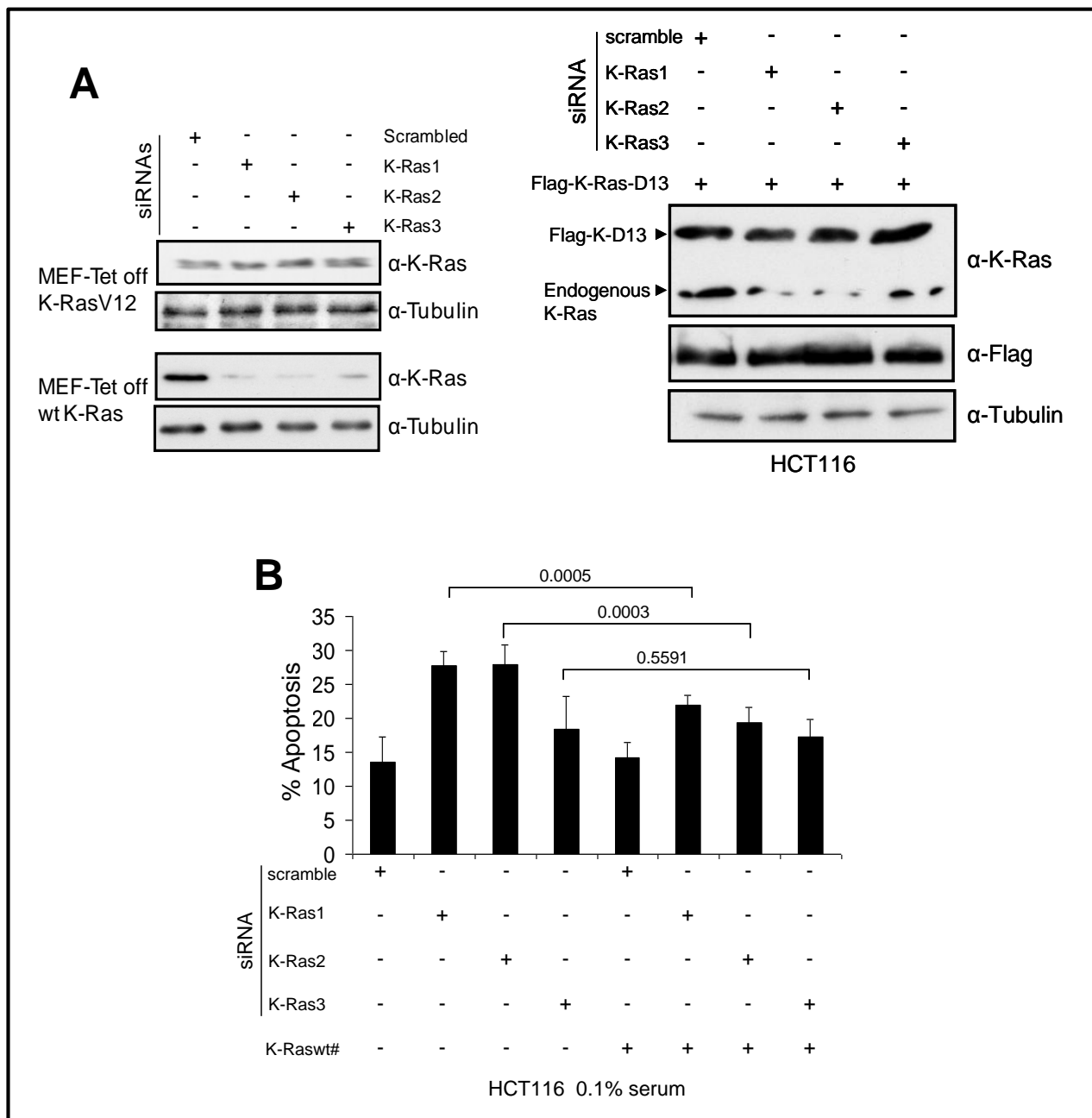


Fig. S3. Wildtype (wt) K-Ras protects against apoptosis. Specificity of siRNAs selectively targeting wt K-Ras. (A) Left panel, Rasless MEFs expressing either wt K-Ras or K-RasV12 only were transfected with three different siRNAs against wt K-Ras (K-Ras1 to 3) or non-targeting siRNA (scramble). Right panel, HCT116 cells were co-transfected with Flag-K-RasD13 and siRNAs as above. The expression of K-Ras was monitored with the indicated antibodies. Tubulin was used as loading control. (B) HCT116 cells were transfected with three different specific siRNA against wt K-Ras (K-Ras1-3). Where indicated a K-Ras expression vector was co-transfected that encodes a wt K-Ras with a silent mutation conferring resistance against the siRNAs (K-Raswt#). Apoptosis was measured by assaying DNA fragmentation after the cells had been serum starved for 16 hours. Error bars show standard deviation. P-values were obtained by T-test.

Fig. S3 (continued)

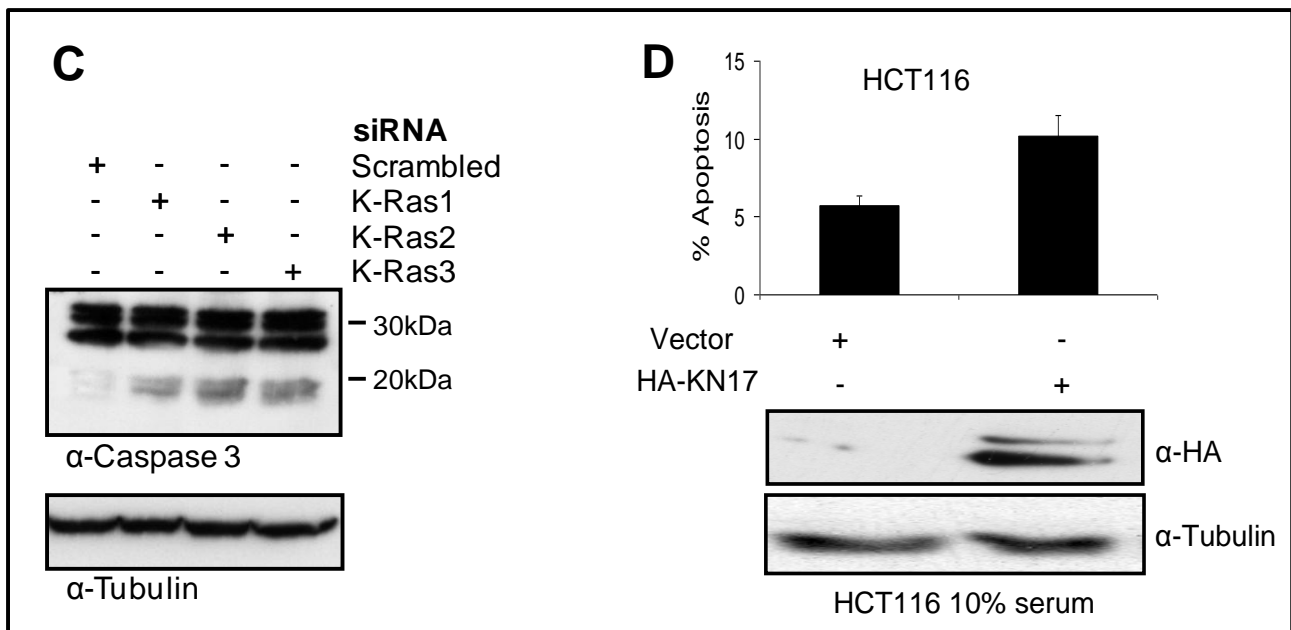


Fig. S3. (C) Activation of caspase 3 in serum starved HCT116 cells was assayed by monitoring cleavage of pro-caspase 3 into the 19kD and 17kD active forms. Cells were transfected with three different siRNAs against wt K-Ras (K-Ras 1-3) or scrambled control. Cell lysates were Western blotted with antibodies against caspase 3 or α -tubulin as loading control **(D)** HCT116 cells were transfected with empty vector or the dominant negative HA-K-RasN17 mutant (HA-KN17). Apoptosis was measured by assessing DNA fragmentation in growing cells.

Fig. S4

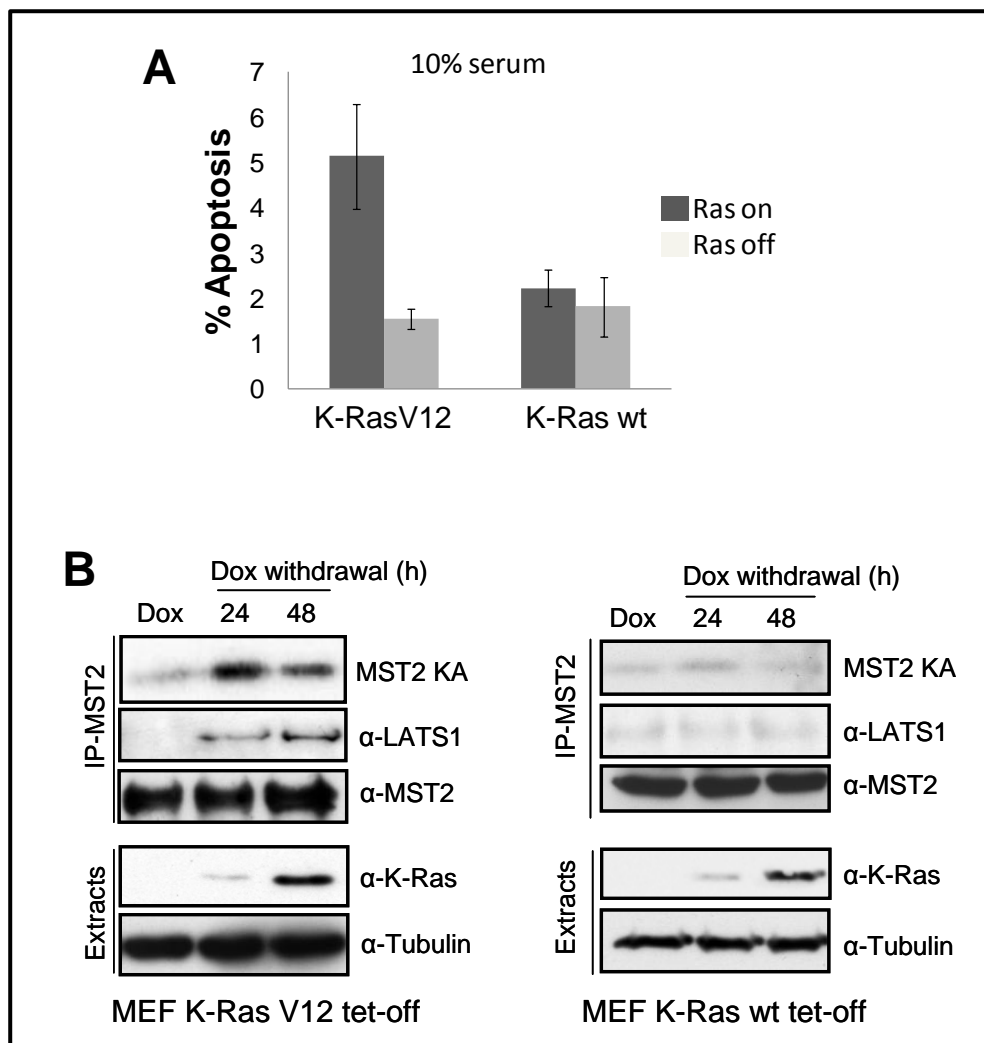


Fig. S4. Differential regulation of the MST2-LATS1 pathway by mutant and wildtype K-Ras. (A) Ras-less MEFs reconstituted with doxycycline repressible wt K-Ras or mt K-RasV12, where indicated (Ras off) 1ng/ml doxycycline was added for 48 hours. Apoptosis was measured in the presence of serum by assessing DNA fragmentation. (B) Tet-off regulatable MEF-K-Ras V12 or MEF-K-Ras wt cells were incubated for 48hours with doxycycline to silence expression of the K-Ras transgenes. After this time the cells were trypsinised and equal numbers of cell were plated with (Dox) or without doxycycline (Dox withdraw). The cells were collected at the indicated times (Dox was collected at 24 h), lysed and immunoprecipitated with anti-MST2 antibody. MST2 kinase assay was monitored by in gel kinase assay and LATS1 and Raf1 interaction with MST2 was measured by Western blotting.

Fig. S4 (continued)

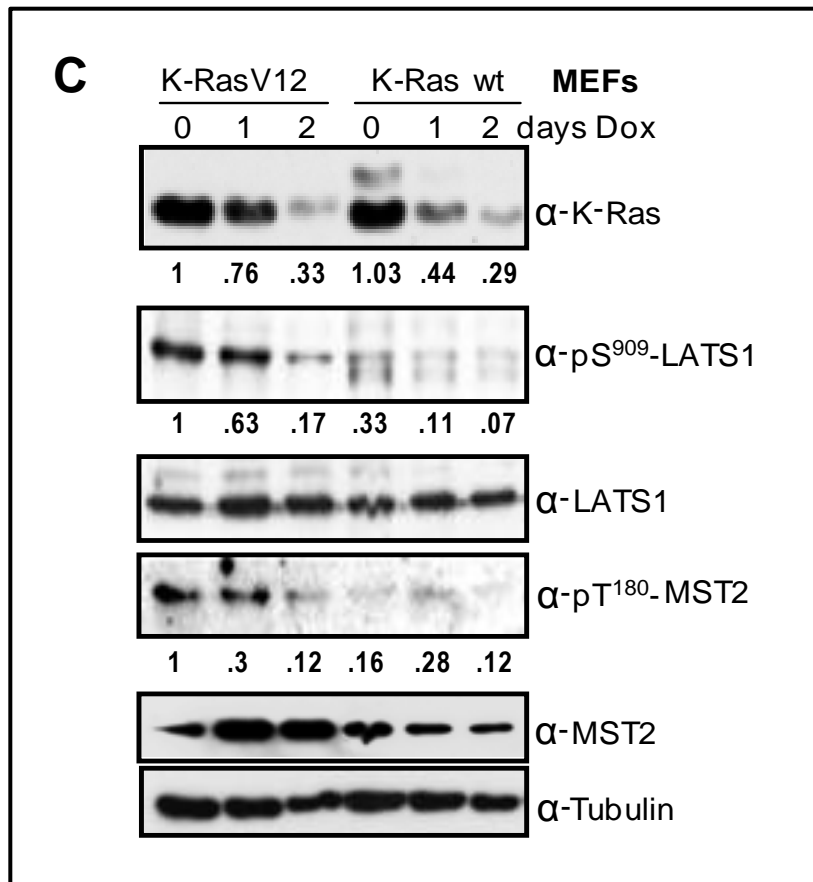


Fig. S4. (C) Tet-off MEF-K-Ras V12 and wt cells were treated with doxycycline for the indicated times to induce silencing of K-Ras expression. Lysates were blotted with the indicated antibodies. The phosphospecific MST2 and LATS1 antibodies recognise activating phosphorylation sites. The blots were quantitated by laser densitometry and Image J software. The scan units were first normalised in reference to the first lane which was set to 1. Further, K-Ras levels were normalised to tubulin levels used as loading control. The expression levels of MST2 and LATS1 were also normalised to tubulin levels, and then the level of phosphorylation was calculated as a ratio between the normalised phospho-specific signals and the corresponding normalised protein expression levels.

Fig. S5

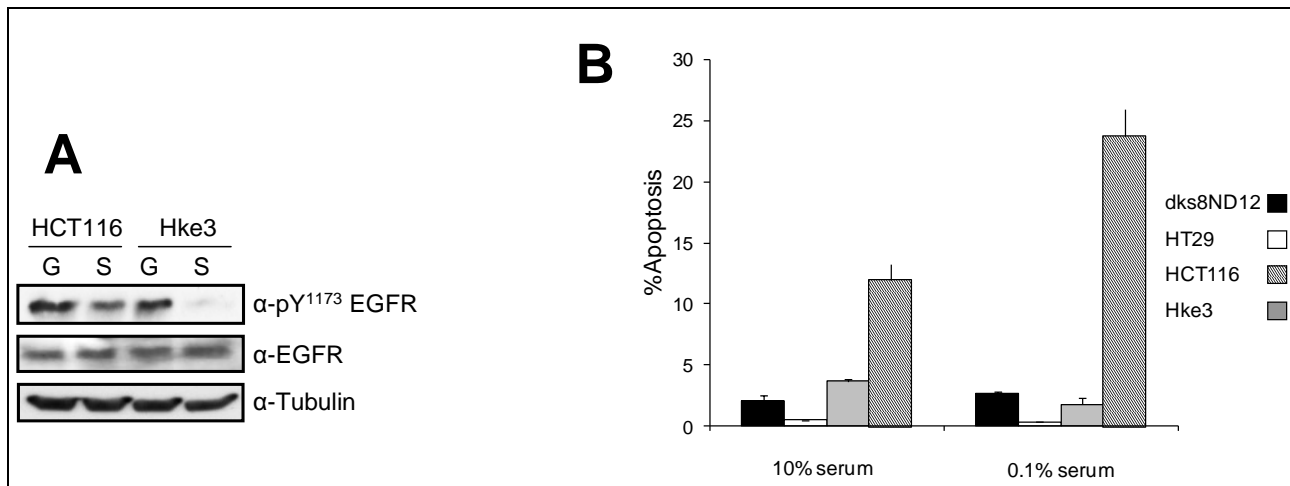


Fig. S5. Apoptosis protection is mediated by autocrine activation of the EGF receptor EGFR. (A) The EGF receptor is activated in cells expressing mutant K-Ras. Growing (G) or serum starved (S) HCT116 and Hke3 cells were examined for EGF receptor activation by Western blotting with a phospho-specific antibody recognizing phospho-Tyr1173 (53A5, Cell Signaling). HCT116 and Hke3 cells express comparable levels of EGFR. Tubulin was used as loading control. (B) Levels of apoptosis in HCT116 (mt K-Ras), Hke3 (wt K-Ras), DKS8 ND12 (mt N-Ras), HT29 (mt B-Raf) growing in the presence or absence of serum were measured by DNA fragmentation. Error bars show standard deviation.

Fig. S6

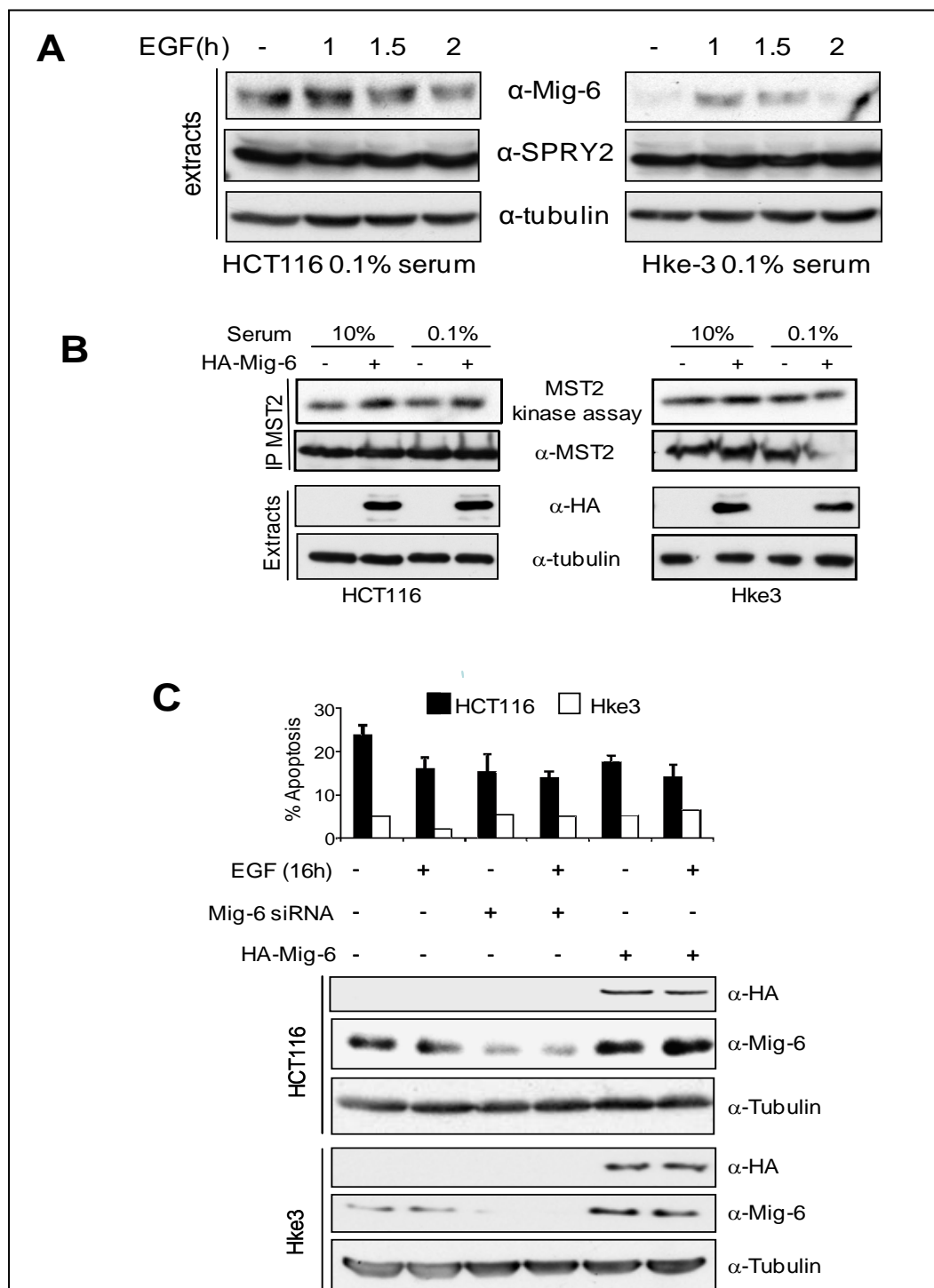


Fig. S6. Role of EGFR feedback inhibitors Sprouty-2 (SPRY2) and MIG-6. (A) HCT116 and Hke3 were serum starved for 16 hours and incubated with EGF (100ng/ml) for the indicated times. Changes of the levels of MIG-6 and SPRY2 were monitored in the lysates. (B) HCT116 and Hke3 cells were transfected with an HA-MIG-6 expression plasmid or empty vector. Cells growing in the presence or absence of serum were lysed and MST2 immunoprecipitates were assessed for kinase activity. (C) HCT116 and Hke-3 were transfected with MIG-6 siRNA, HA-MIG-6 plasmid or empty vector. Serum starved cells were treated with 100ng/ml EGF as indicated and examined for apoptosis by assaying DNA fragmentation.

Fig. S7

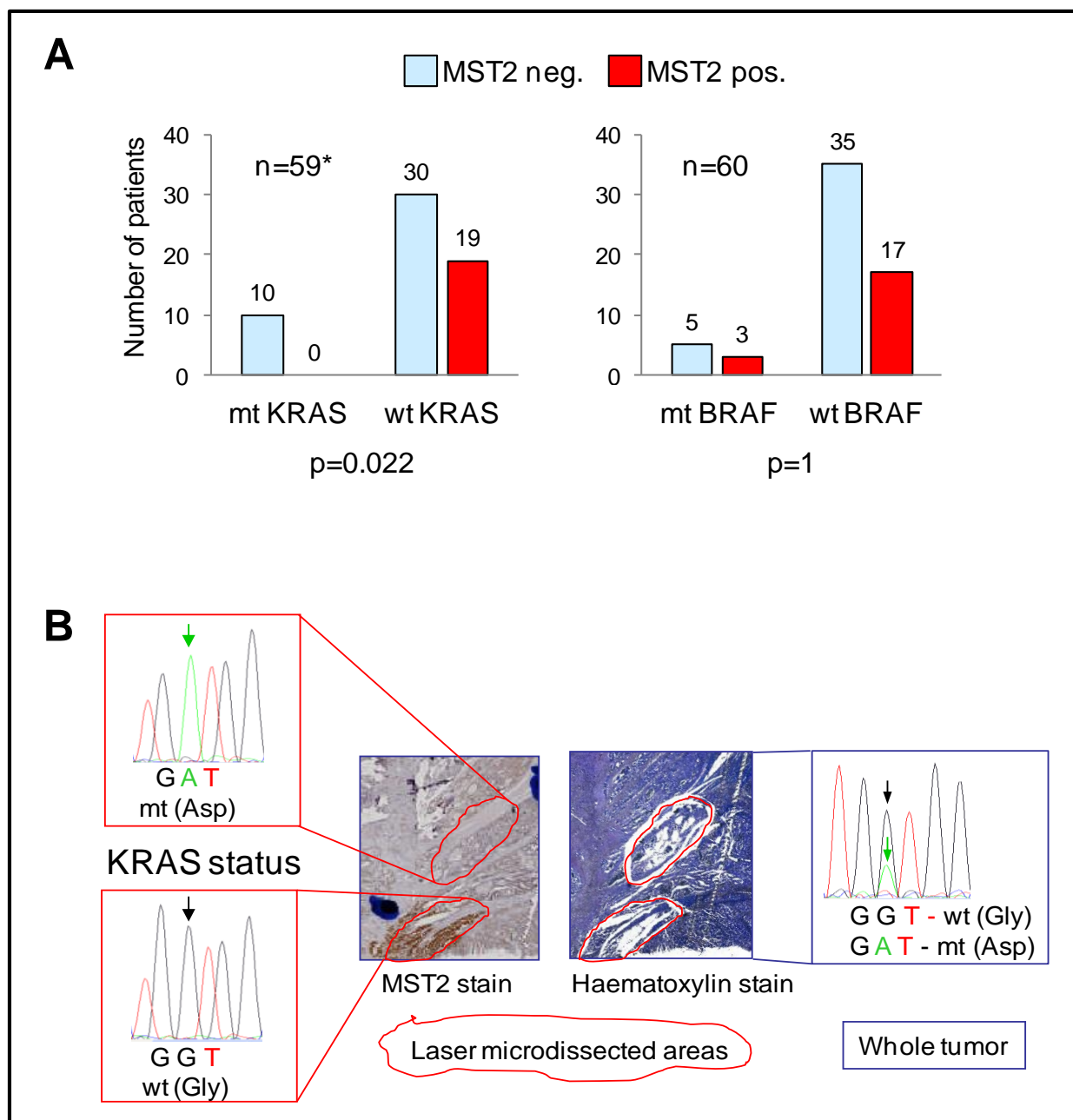


Fig. S7. Analysis of MST2 expression and KRAS mutations in colorectal cancers (CRCs). (A) MST2 expression, KRAS and BRAF mutations were assayed in 60 human CRC samples (cohort 2) as described in experimental procedures. One sample did not yield KRAS sequence (indicated by *). The p-value was calculated using the two-sided Fisher's exact test. (B) CRC tissues were laser microdissected to isolate tumour regions that express or not express MST2. KRAS was sequenced in microdissected regions and whole tumours.

Fig. S7 (continued)

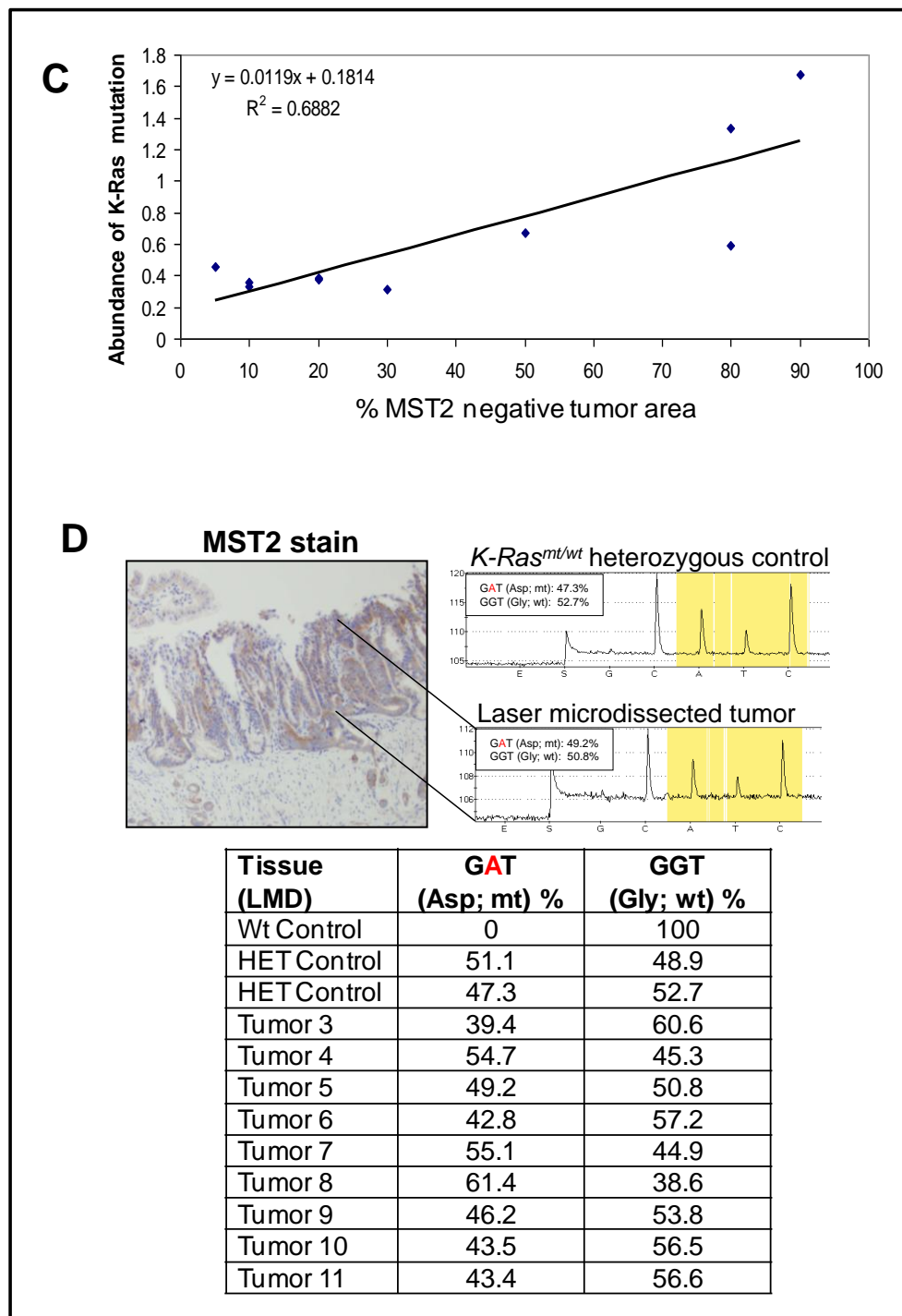


Fig. S7. (C) Correlation between percent MST2 negative tumour area and abundance of KRAS mutation (as measured by the ratio of mt/wt sequence peak heights) in 9 human CRCs. (D) Tumours arising in the $APC^{Fllox} / KRas^{G12D}$ mouse model of intestinal adenocarcinoma were stained for MST2 expression. Tumour material from these (tumors 3-7) and the $Kras^{G12D}$ model of pancreatic ductal adenocarcinoma (tumors 8-11) was isolated by laser microdissection (LMD) and examined for *K-Ras* gene mutations by pyrosequencing. The ratio between mt and wt alleles is given in the table. Tissues from wildtype (wt) $KRas^{wt/wt}$ and heterozygous (HET) $KRas^{G12D/wt}$ mice were used as controls.

Fig. S8

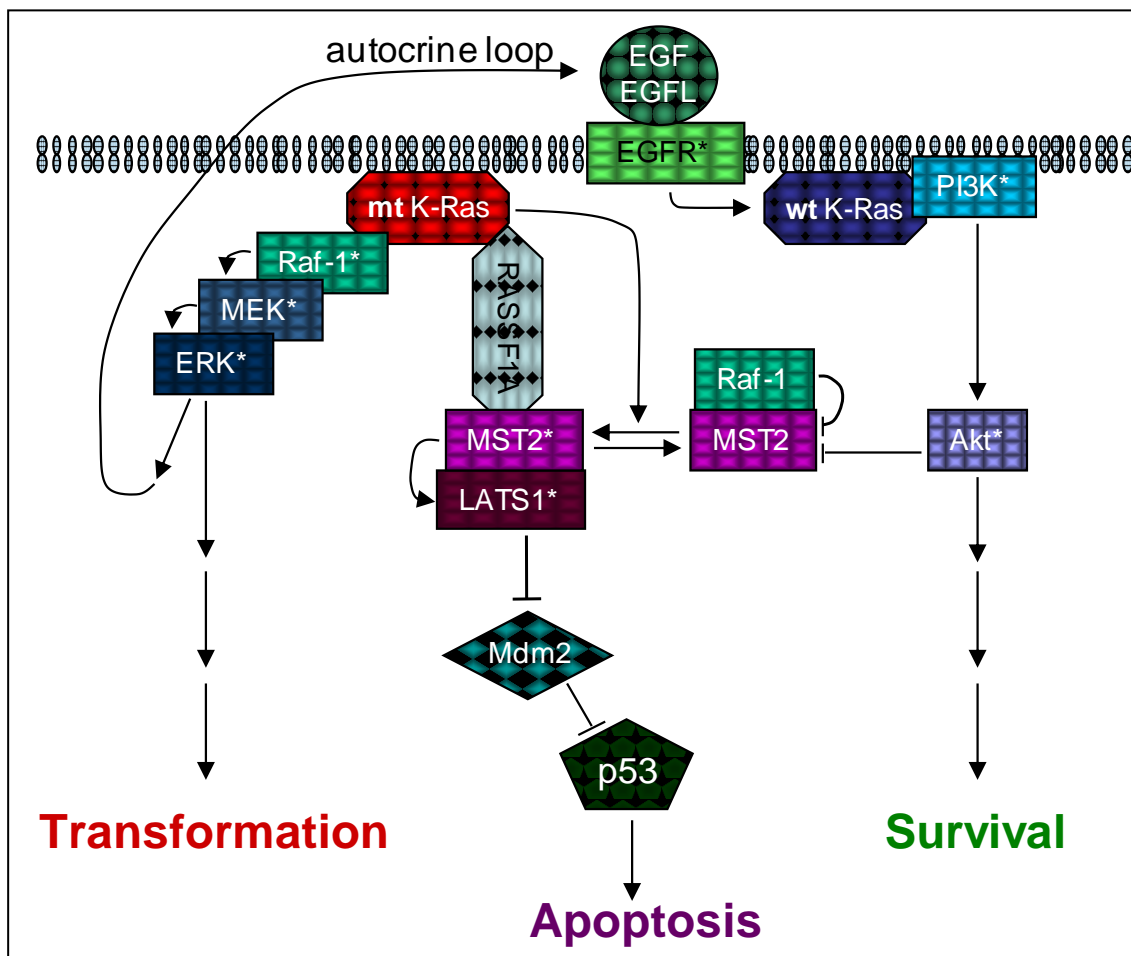


Fig. S8. Schematic model of the differential roles of wt and mt K-Ras in transformation. See text for details. Asterisks denote activated kinases. For simplicity K-Ras proteins are depicted to interact with multiple effectors simultaneously, whereas in reality K-Ras can only bind one effector at a time.