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p38α MAP Kinase as a Sensor of Reactive Oxygen Species in Tumorigenesis

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SUMMARY

p38α is a stress-activated protein kinase that negatively regulates malignant transformation induced by oncogenic H-Ras, although the mechanisms involved are not fully understood. Here, we show that p38α is not a general inhibitor of oncogenic signaling, but that it specifically modulates transformation induced by oncogenes that produce reactive oxygen species (ROS). This inhibitory effect is due to the ROS-induced activation of p38α early in the process of transformation, which induces apoptosis and prevents the accumulation of ROS and their carcinogenic effects. Accordingly, highly tumorigenic cancer cell lines have developed a mechanism to uncouple p38α activation from ROS production. Our results indicate that oxidative stress sensing plays a key role in the inhibition of tumor initiation by p38α.

INTRODUCTION

Cancer is a complex disease that involves the disruption of cell and tissue homeostasis via a series of successive genetic changes (Hanahan and Weinberg, 2000). These include activating mutations in the *H-*, *N-*, and *K-ras* proto-oncogene family members, which have been found to be mutated or overexpressed in more than 30% of human tumors (Bos, 1989).

Ras-induced tumorigenesis is accompanied by a number of biochemical changes, including the activation of the ERK MAP kinase (MAPK)-, PI3K-, and RalGDS-signaling pathways (Downward, 2003). Furthermore, increased intracellular levels of reactive oxygen species (ROS) have also been reported to mediate some biological effects of oncogenic H-Ras, such as mitogenesis in fibroblasts (Irani et al., 1997), the onset of premature senescence in primary cells (Lee et al., 1999; Nicke et al., 2005), the generation of genomic instability (Woo and Poon, 2004), and malignant transformation (Mitsushita et al., 2004). In contrast, N-Ras has not been linked to oxidative stress yet, whereas K-Ras has been reported to either increase or decrease intracellular ROS levels depending on the cellular context (Maciag and Anderson, 2005; Santillo et al., 2001). The ability of other oncogenes, apart from Ras, to induce ROS production has not been described; however, BCR/ABL (Sattler et al., 2000) and several growth factor receptors that signal through Ras, such as the transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) receptors, have all been reported to raise intracellular ROS levels in hematopoietic cells (Sattler et al., 1999).

Oxidative stress has been traditionally considered as a toxic by-product of cellular metabolism, but it has been recently appreciated that ROS are actively involved in the regulation of signal-transduction pathways (Hancock et al., 2001), and that they can also cooperate with oncogenic signaling in cellular transformation and cancer (Suh et al., 1999; Woo and Poon, 2004). The carcinogenic effects of ROS accumulation have been proposed to operate at various levels, including changes in gene expression (Allen and Tresini, 2000), increased proliferation and DNA-mutational rates (Irani et al., 1997; Toyokuni, 2006), and

SIGNIFICANCE

The characterization of tumor suppressors whose activity could be stimulated for cancer therapy is an area of intense research. We show that the ability of p38 MAPK to induce apoptosis in response to the detection of reactive oxygen species (ROS) plays an important inhibitory role in tumor initiation. This activity is likely to be relevant for human cancer, as the tumorigenicity of cancer cell lines correlates with increased levels of glutathione S-transferase (GST) proteins that specifically desensitize p38α activation from ROS accumulation. Our results illustrate a mechanism used by cancer cells for the inactivation of tumor-suppressor pathways and suggest that restoring the ROS-induced activation of p38 MAPK, for example by targeting GST proteins, may be of potential therapeutic interest.

genomic instability (Woo and Poon, 2004). Furthermore, high levels of ROS have been detected in several human cancer cell lines (Szatrowski and Nathan, 1991), as well as in human tumors from different tissues (Toyokuni et al., 1995). ROS have also been implicated in the proliferation of melanoma, breast carcinoma, and fibrosarcoma human tumor cell lines (Church et al., 1993; Fernandez-Pol et al., 1982). Taken together, these reports support the causal link between oxidative stress and cancer, which was proposed 20 years ago (Ames, 1983).

p38α MAPK plays an important role in the coordination of the cellular responses to many stress stimuli. The signaling pathways leading to the activation of p38α involve several upstream MAP3Ks, with apoptosis signal-regulating kinase 1 (ASK1) (MAP3K5) playing a major role in p38α activation by oxidative stress (Tobiume et al., 2001). ASK1 activation is thought to involve both oligomerization and autophosphorylation, which is prevented in nonstressed cells by the binding of stress-sensitive proteins. Two of these ASK1-binding proteins are thioredoxin (Trx) and glutathione S-transferase Mu-1 (Gstm1), which dissociate from ASK1 after oxidative stress and heat shock, respectively (Dorion et al., 2002; Matsukawa et al., 2004). Interestingly, overexpression of Gstm1 inhibits p38α activation by oxidative stress, which might be accounted for by the binding of both Gstm1 and Trx to the same N-terminal region of ASK1 (Cho et al., 2001).

In addition to its key role as a coordinator of the cellular responses to stress, p38α has also been shown to regulate other cellular processes in a cell-type-specific manner (Nebreda and Porras, 2000). Of note, p38α negatively regulates the malignant transformation induced by oncogenic H-Ras, and several mechanisms have been proposed to explain this putative tumor-suppressor role, including inhibition of the ERK pathway (Li et al., 2003), induction of premature senescence (Wang et al., 2002) or of a p53-dependent cell cycle arrest (Bulavin et al., 2002), and upregulation of cell cycle inhibitors, such as $p16^{INK4a}$ (Bulavin et al., 2004) and $p21^{Cip1}$ (Nicke et al., 2005). Other reports indicate that p38α may also antagonize malignant transformation induced by N-Ras in fibroblasts (Wolfman et al., 2002) and by K-Ras in colon cancer cells (Qi et al., 2004), although the mechanisms involved are poorly understood.

Here, we show that p38α is not a general inhibitor of oncogenic signaling, but that it specifically modulates malignant transformation induced by oncogenes that produce ROS. Interestingly, some human cancer cells can bypass the inhibitory role of p38α on ROS accumulation, and this leads to enhanced tumorigenicity. Thus, oxidative stress sensing by p38α MAPK is an important mechanism by which to negatively regulate the onset of cancer.

RESULTS

p38α-Deficient MEFs Are Sensitized to H-RasV12-Induced Transformation

To investigate the effect of p38α on H-Ras-induced transformation, fibroblasts derived from wild-type (WT) and $p38\alpha^{-/-}$ mouse embryos were immortalized by the 3T3 protocol. Consistent with previous reports (Brancho et al., 2003; Chen et al., 2000; Faust et al., 2005), we found that immortalized WT and $p38\alpha^{-/-}$ mouse embryo fibroblasts (MEFs) proliferated with comparable rates, although upon H-RasV12 transduction (Figure 1A) or under low-serum conditions (Figure S1; see the Supplemental Data available with this article online), $p38\alpha^{-/-}$ MEFs proliferated faster than WT MEFs. This was consistent with higher levels of cyclin D1 in exponentially proliferating $p38\alpha^{-/-}$ MEFs expressing H-RasV12 (Figure 1B), as expected from the known ability of p38α to downregulate cyclin D1 expression (Lavoie et al., 1996). However, in contrast to the described role of p38α as a modulator of the $p16^{Ink4a}/p19^{Arf}$ pathways in primary stem cells and breast tumorigenesis (Bulavin et al., 2004; Ito et al., 2006), we observed no differences in the levels of $p16^{Ink4a}$ between H-RasV12-expressing WT and $p38\alpha^{-/-}$ MEFs (Figure 1C), whereas $p19^{Arf}$ was not detected by immunoblotting (data not shown).

We also analyzed the ability of p38α-deficient cells to grow in soft agar, which is considered a better marker for in vivo tumorigenesis than the rates of proliferation. We found that H-RasV12-expressing $p38\alpha^{-/-}$ MEFs were 40% less adherent (data not shown) and showed a more refringent morphology than H-RasV12-transduced WT MEFs (Figure 1D). Moreover, H-RasV12-transformed $p38\alpha^{-/-}$ MEFs formed bigger foci and were able to produce about 9-fold more colonies in soft agar than H-RasV12-transduced WT MEFs (Figures 1E and 1F). Importantly, the differences between WT and $p38\alpha^{-/-}$ MEFs could be rescued by reintroduction of p38α in H-RasV12-expressing $p38\alpha^{-/-}$ cells (Figures 1G and 1H), arguing that the observed differences are directly due to the absence of p38α, and not to secondary genetic alterations. Of note, we could not detect p53 protein expression in immortalized $p38\alpha^{-/-}$ and WT MEFs, either when proliferating or after stress treatments (Figure 1B and data not shown), suggesting that p38α inhibits H-RasV12-induced transformation of fibroblasts by p53-independent mechanisms, in agreement with previous work (Bulavin et al., 2004). The in vivo relevance of these observations was confirmed by injecting nude mice subcutaneously with H-RasV12-transformed $p38\alpha^{-/-}$ MEFs, which gave rise to tumors significantly faster than H-RasV12-transformed WT MEFs (Figure 1I).

Sustained Activation of p38α Inhibits H-RasV12-Induced Transformation, but Not ERK Activation

NIH3T3 fibroblasts are immortalized, highly contact-inhibited cells that carry a homozygous deletion in the entire *INK4a/ARF* locus. To confirm whether p38α could negatively regulate H-RasV12-induced transformation independently of $p16^{INK4a}$ and $p19^{ARF}$, NIH3T3 fibroblasts were transfected with H-RasV12, either alone or together with the specific p38 MAPK activator MKK6DD. Expression of MKK6DD resulted in efficient activation of endogenous p38α (Figure 2A), which correlated with the inhibition of H-RasV12-induced transformation, as determined by the less refringent morphology and the reduced

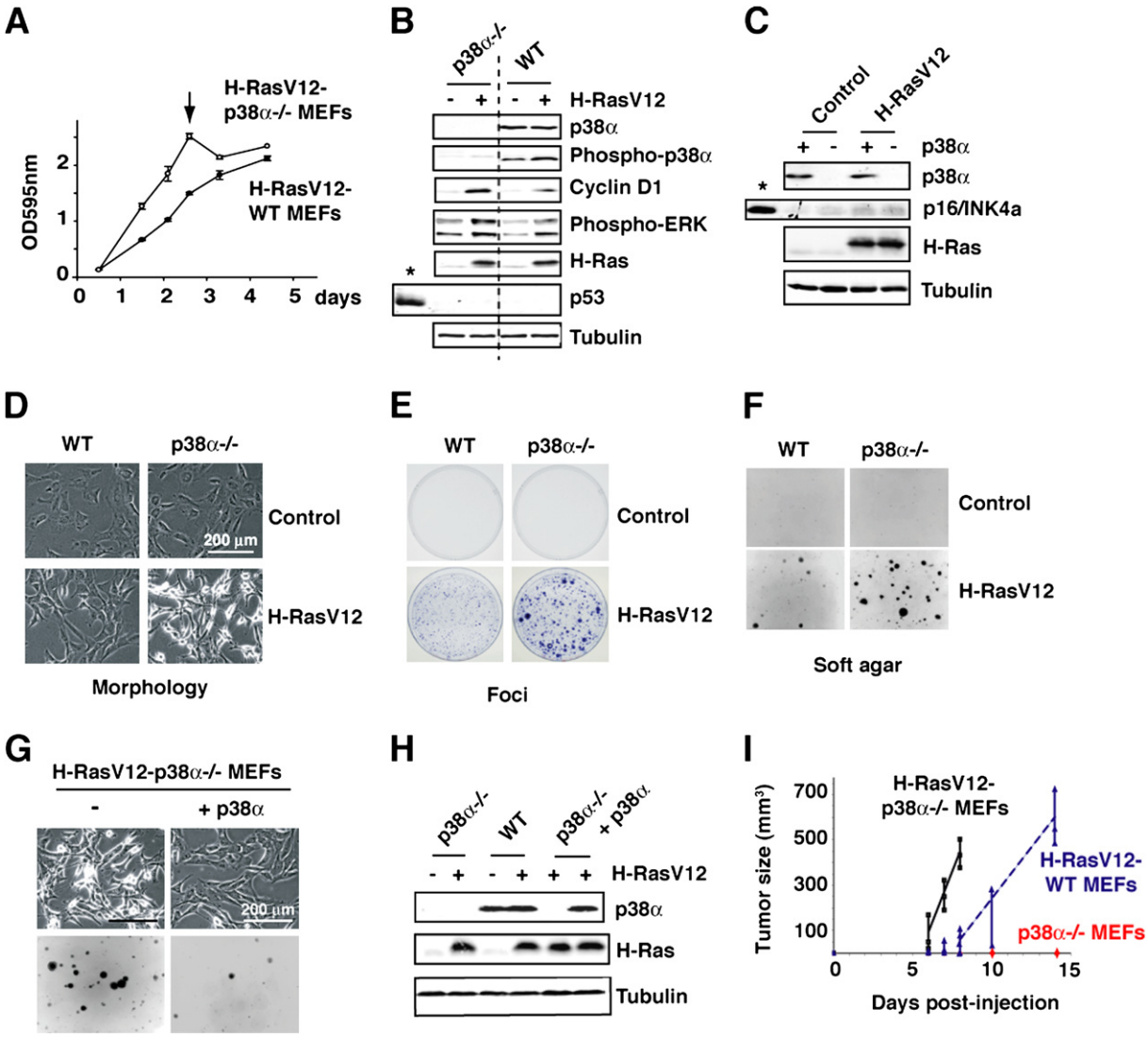

Figure 1. p38α Negatively Regulates H-RasV12-Induced Malignant Transformation

(A) Proliferation rates of H-RasV12-expressing WT and p38α$^{-/-}$ MEFs. The arrow indicates when H-RasV12-expressing p38α$^{-/-}$ cells achieved confluency. The error bars show SD.

(B and C) Total cell lysates from exponentially proliferating WT and p38α$^{-/-}$ MEFs (50 μg total protein) were analyzed by immublotting with the indicated antibodies. Primary and SV40 LT-Ag-immortalized MEFs were used as controls for p53 and p16^{INK4a} immunoblotting, respectively (indicated by asterisks).

(D–F) H-RasV12-expressing WT and p38α$^{-/-}$ MEFs as well as control cells transduced with the empty vector were selected with puromycin (1.5 μg/ml) for 1 week and then compared in terms of (D) morphology, (E) ability to form foci, and (F) anchorage-independent growth in soft agar.

(G and H) p38α$^{-/-}$ MEFs were rescued by forced expression of p38α and then analyzed for (G) anchorage-independent growth and morphology, as well as by (H) immublotting with the indicated antibodies.

(I) Immunodeficient nude mice were injected subcutaneously with control p38α$^{-/-}$ (rhombus) and H-RasV12-expressing WT (triangles) or p38α$^{-/-}$ (squares) MEFs, and tumor size was measured periodically. Error bars show SD.

number of both foci formation and anchorage-independent growth (Figure 2B).

The ability of p38 MAPK to inhibit ERK activation has been previously documented (Li et al., 2003). We therefore investigated whether the negative effect of p38α on H-RasV12-induced transformation could be accounted for by interfering with the activation of the ERK pathway, since this has been shown to be important for the H-Ras-induced transformation of mouse fibroblasts (Cowley et al., 1994; Mansour et al., 1994). We found that exponentially proliferating H-RasV12-WT MEFs contained similar phospho-ERK levels as H-RasV12-p38α$^{-/-}$ cells (Figure 1B). Moreover, kinetic analysis of ERK activation in response to serum stimulation showed no differences between p38α$^{-/-}$ and WT MEFs, either in the presence or absence of H-RasV12 expression (Figure 2C). We also

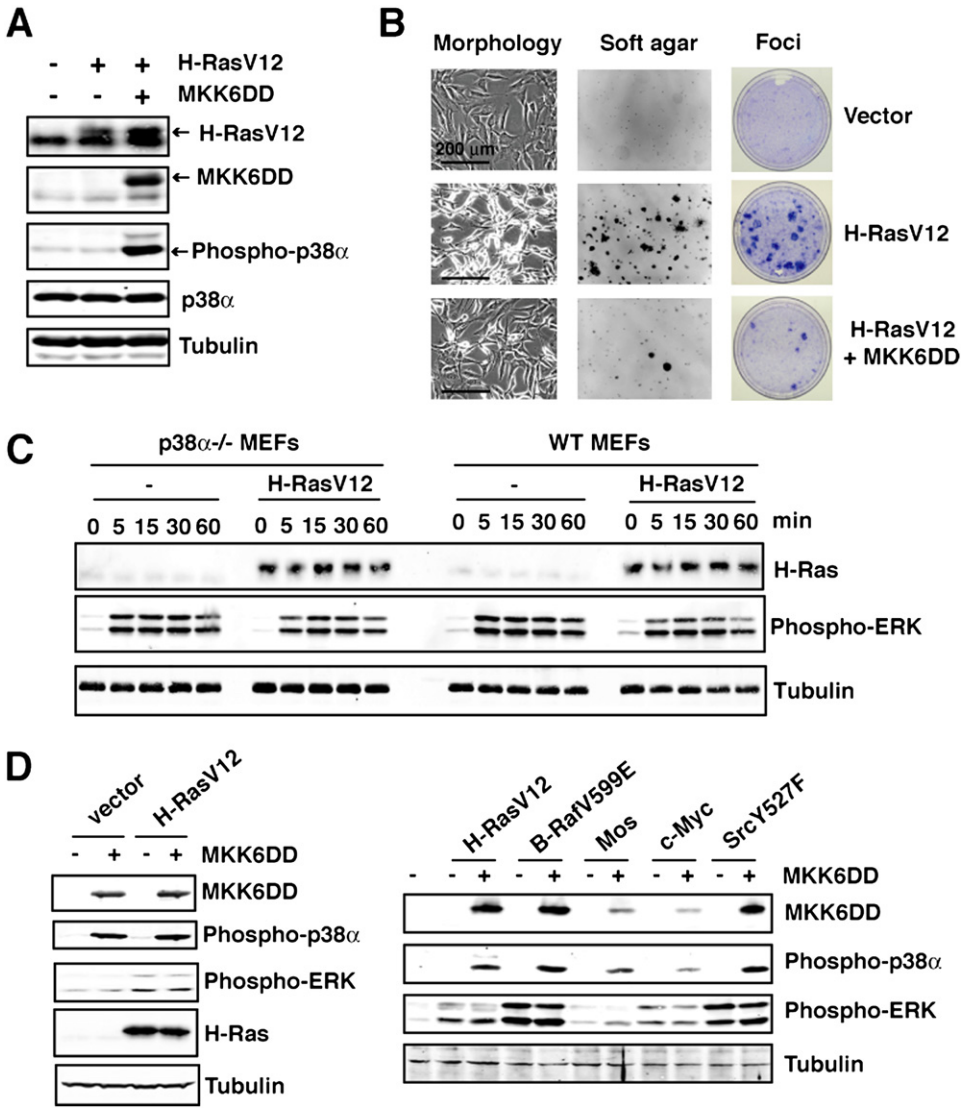

Figure 2. p38α Regulates H-RasV12-Induced Transformation Independently of the *INK4a/ARF* Locus and the ERK Pathway

(A) NIH3T3 fibroblasts were transfected with H-RasV12 in combination with MKK6DD or an empty vector and 48 hr later were analyzed by immunoblotting with the indicated antibodies.

(B) NIH3T3 cells stably expressing H-RasV12 alone or in combination with MKK6DD were analyzed for transformation-associated morphological changes (left panels) and anchorage-independent growth in soft agar (middle panels). NIH3T3 cells were also transiently transfected with H-RasV12 or H-RasV12 plus MKK6DD and were analyzed for foci formation during the course of 3 weeks (right panels).

(C) Kinetics of ERK activation in the indicated cell lines after incubation in 0.5% serum for 60 hr, followed by stimulation with 10% FBS.

(D) NIH3T3 cells were transiently transfected with the indicated oncogenes, together with MKK6DD or an empty vector, and 48 hr later were analyzed by immunoblotting.

confirmed that MKK6DD expression affected neither the basal nor the oncogene-induced levels of active ERK in NIH3T3 fibroblasts (Figure 2D). These results indicated that p38α was not inhibiting H-RasV12-induced transformation of MEFs and NIH3T3 cells by interfering with ERK activation.

p38α Inhibits H-RasV12-Induced ROS Accumulation by Triggering Apoptosis

Our results indicated that p38α was able to inhibit H-RasV12-induced transformation of premalignant fibroblasts independently of both p53 and the *INK4a/ARF* locus, and without interfering with ERK activation. Next, we investigated the effect of p38α on the production of ROS, a well-known biological consequence of oncogenic H-Ras expression (Irani et al., 1997).

We found that H-RasV12-p38α$^{-/-}$ MEFs accumulated significantly higher levels of intracellular ROS than H-RasV12-WT MEFs (Figure 3A). Quantitative analysis showed that H-RasV12-WT MEFs contained 2- to 3-fold higher ROS levels than nontransformed WT or p38α$^{-/-}$ cells, whereas ROS levels detected in p38α$^{-/-}$ MEFs

expressing H-RasV12 were ~25-fold higher (Figure S2). Interestingly, the higher levels of ROS in H-RasV12-p38α$^{-/-}$ MEFs not only correlated with their enhanced transformed phenotype, but also with two known outcomes of oxidative stress, which are reduced intracellular phosphatase activity and high levels of genomic instability (Figures S3A and S3B). In support of the idea that high intracellular ROS levels could play a causal role in the malignant phenotype, we were able to inhibit the more dramatic transformed morphology of H-RasV12-expressing p38α$^{-/-}$ MEFs by incubation with the antioxidant glutathione (Figure 3B).

To further analyze the interplay between p38α, ROS accumulation, and H-RasV12-induced transformation, we used a 4-hydroxytamoxifen (OHT)-inducible ER-HRasV12 system (De Vita et al., 2005). Surprisingly, we found that, early in H-RasV12 induction with OHT, both p38α$^{-/-}$ and WT MEFs contained comparable ROS levels (Figure 3C, upper panel). However, long-term accumulation of ROS was only observed in p38α$^{-/-}$ cells. In order to elucidate why WT cells were not able to accumulate ROS, we monitored the time-dependent appearance of the ER-HRasV12-induced transformed morphology in WT and p38α$^{-/-}$ MEFs (Figure 3C, lower panel). We observed that both WT and p38α$^{-/-}$ MEFs acquired a similar transformed morphology shortly after OHT treatment. However, WT cells undergo up to 10-fold more apoptosis than p38α$^{-/-}$ cells when treated with OHT for 1 week (Figure 3C, arrows, and Figure 3D), which occurs in parallel with a drop in their ROS levels between 7 and 10 days after OHT treatment, depending on the experiment. The occurrence of apoptosis was confirmed biochemically by the accumulation of processed p85 PARP (Figure 3E), and its key role for ROS downregulation in WT MEFs was further supported by incubation with the pan-caspase inhibitor ZVAD-fmk. Indeed, the inhibition of apoptosis by ZVAD-fmk (Figure 3F) interfered with ROS downregulation in WT MEFs at late times after ER-HRasV12-induction, without affecting ROS levels in p38α$^{-/-}$ cells (Figure S4). Extended treatment with OHT for up to 3 weeks did not have any further effect on the surviving cells, which showed sustained low levels of ROS. Moreover, no apoptotic crisis was observed in p38α$^{-/-}$ MEFs expressing ER-HRasV12 or in control WT and p38α$^{-/-}$ MEFs treated with OHT, arguing in favor of an early p38α-mediated inhibitory mechanism in response to oncogenic H-RasV12-induced ROS. In agreement with this, phospho-p38α levels transiently increased after OHT treatment, in parallel with p85 PARP accumulation (Figure 3E), and decreased after 2 weeks of treatment.

Next, we studied whether the observed increase in p38α activity was necessary for H-RasV12-induced apoptosis. WT MEFs expressing ER-HRasV12 were incubated with the p38 MAPK inhibitor SB203580, and apoptosis was quantified 8 days after OHT treatment. As expected, SB203580 strongly impaired the H-RasV12-induced activation of the p38 MAPK pathway and the subsequent apoptotic response (Figure 3F), without affecting the H-RasV12-induced accumulation of ROS (data not shown), which indicates that p38α activation lays downstream of ROS and is required for apoptosis induction by H-RasV12.

H-RasV12-Induced ROS Accumulation Is Mediated by the ERK and Rac1 Pathways and Involves NADPH Oxidases

To elucidate which pathways mediate the long-term accumulation of ROS in H-RasV12-transformed p38α$^{-/-}$ MEFs, we used rotenone, an inhibitor of the mitochondrial electron transport chain, and diphenylene iodonium chloride (DPI), an inhibitor of NADPH oxidase (NOX) enzymes that are major mediators of the nonmitochondrial ROS production (Kamata and Hirata, 1999). Incubation with rotenone did not affect H-RasV12-induced ROS accumulation in p38α$^{-/-}$ MEFs, whereas DPI basically abolished it (Figure S5A). *Nox* genes have been previously associated with cellular transformation and cancer (Suh et al., 1999). In agreement with this, we found that the *Nox1* and *Nox4* mRNAs were upregulated in H-RasV12-transformed p38α$^{-/-}$ MEFs, suggesting that these two NOX family members may be involved in H-RasV12-induced ROS production in fibroblasts (Figure S5B).

We also used chemical inhibitors to investigate the contribution of different Ras-activated signaling pathways to ROS production. Our results indicated that H-RasV12-induced ROS accumulation in p38α$^{-/-}$ MEFs was mediated by cooperative action of the ERK and Rac1 pathways, but did not require PI3K activity (Figures S5C and S5D).

Uncoupling p38α Activation from Oxidative Stress Enhances H-RasV12-Induced Transformation

We have recently identified *gstm2* as one of the genes that may be regulated by p38α in H-RasV12-transformed MEFs (unpublished data). Gstm2 was more than 90% homologous to Gstm1 (Figure 4A), another member of the same GST family whose overexpression has been reported to inhibit the oxidative stress-induced activation of p38 MAPK by binding to and inhibiting ASK1 (Cho et al., 2001; Dorion et al., 2002). *gstm1* has been associated with elevated breast cancer risk (Parl, 2005) and is also overexpressed in tumors from brain, skin, and kidney, according to the Cancer Genome Anatomy Project database (http://cgap.nci.nih.gov/Tissues). To address the putative role of Gstm2 as a modulator of p38α function in H-RasV12-induced tumorigenesis, we investigated whether Gstm2 overexpression could affect the activation of p38α by H-RasV12. In agreement with a recent report detailing the use of ovarian epithelial cells (Nicke et al., 2005), we found that the H-RasV12-induced activation of p38α was impaired in the presence of the antioxidant N-acetyl-L-cysteine (NAC), suggesting that ROS are also key mediators of the activation of p38α by H-RasV12 in fibroblasts. Interestingly, Gstm2 overexpression inhibited the activation of p38α (and its activator MKK6) by H-RasV12, but interfered neither with the activation of other H-Ras-regulated signaling pathways, such as the PI3K/Akt pathway, nor with the activation of p38α by other stimuli such as UV (Figure 4B). This result argues that Gstm2

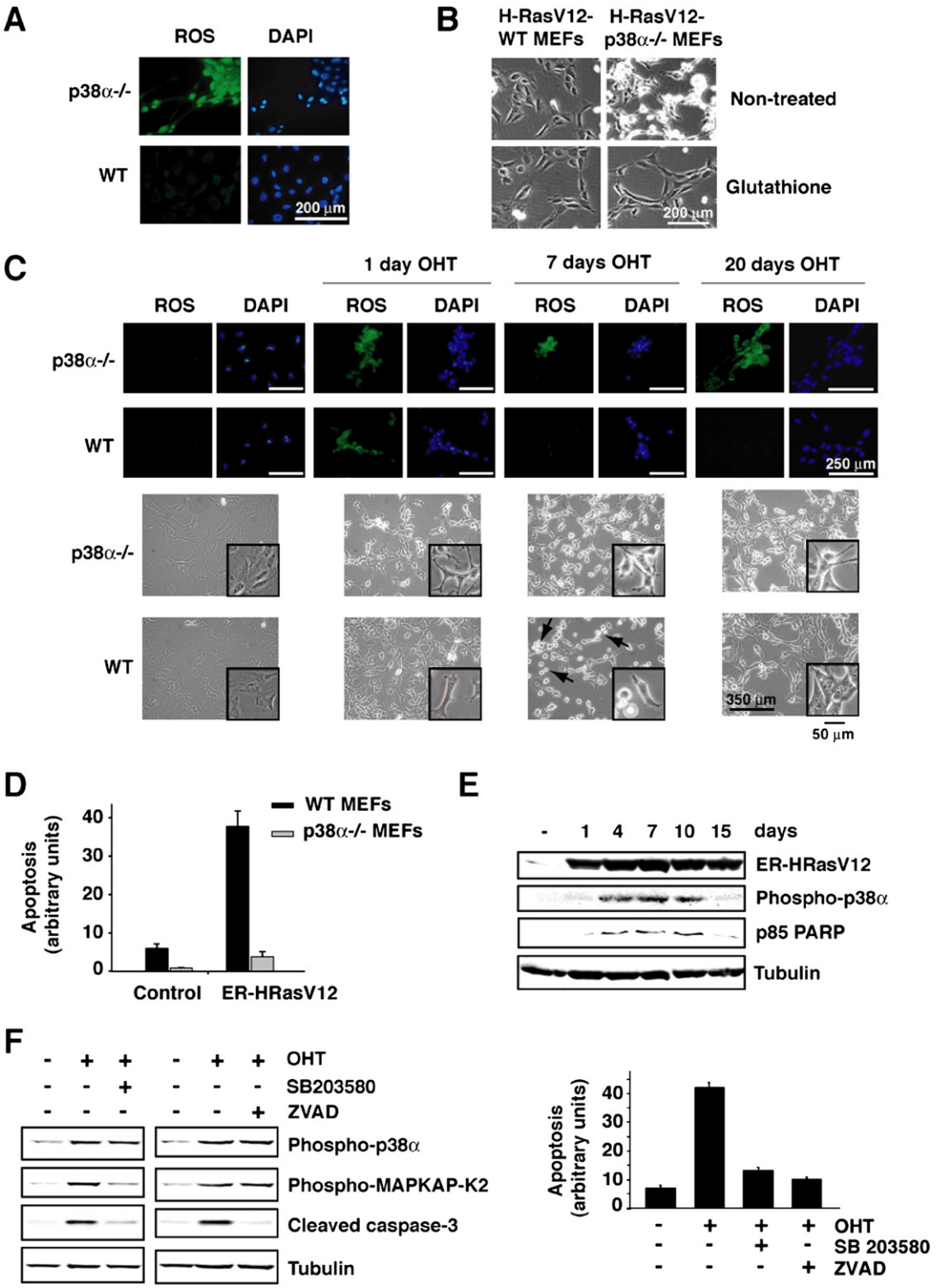

Figure 3. p38α Prevents the Long-Term Accumulation of H-RasV12-Induced High Levels of ROS through an Apoptotic Response

(A) WT and p38α$^{-/-}$ MEFs stably expressing H-RasV12 were analyzed for intracellular ROS levels by immunofluorescence.

(B) WT and p38α$^{-/-}$ MEFs were transduced with H-RasV12 and then selected for 1 week with either 1 μg/ml puromycin alone (top panels) or in combination with reduced glutathione (10 mM for 3 days, followed by 5 mM for 4 days; bottom panels) before the pictures were taken.

(C) WT and p38α$^{-/-}$ MEFs expressing an OHT-inducible H-RasV12 construct (ER-HRasV12) were treated for different times with 1 μM OHT, and ROS accumulation was visualized by immunofluorescence (top panel). Transformation-associated morphological alterations were also monitored in parallel (bottom panel). The insets show magnifications of representative fields.

specifically uncouples p38 MAPK activation from H-RasV12-induced ROS accumulation. We confirmed that Gstm2 can also interact with ASK1 (Figure 4C), which probably accounts for its ability to inhibit ROS-induced p38 MAPK activation, as it has been proposed for Gstm1 (Cho et al., 2001). Consistent with the ability of Gstm2 to interfere with p38α activation, we detected long-term ROS accumulation in H-RasV12-WT MEFs expressing Gstm2 (Figure 4D). Most importantly, accumulation of ROS in H-RasV12-WT MEFs after Gstm2 overexpression correlated with a stronger transformed phenotype, similar to that observed in H-RasV12-p38α$^{-/-}$ MEFs, both at the morphological level and by the enhanced ability of the cells to grow in soft agar (Figure 4E). Of note, the growth of H-RasV12-p38α$^{-/-}$ MEFs in soft agar was not affected by Gstm2 overexpression (data not shown), suggesting that p38α was an important target for the Gstm2 effect observed in WT cells. These results strongly support the hypothesis that p38α functions as a key oxidative stress sensor in oncogenic transformation by H-RasV12.

p38α Specifically Regulates Malignant Transformation by ROS-Inducing Oncogenes

We next investigated whether the role of p38α as a ROS sensor in H-RasV12- induced transformation could be extended to other oncogenes. Thus, WT and p38α$^{-/-}$ MEFs were transduced with a panel of oncogenes, covering different pathways and subcellular localizations, and were analyzed in terms of anchorage-independent growth, focus formation, and intracellular ROS levels (Table 1). As with H-RasV12, none of the oncogenes tested were able to induce long-term accumulation of ROS in WT MEFs. However, the oncogenes Neu V664E and N-RasV12 did induce high ROS levels in p38α$^{-/-}$ MEFs that were comparable to those observed for H-RasV12 (Table 1). Interestingly, ROS accumulation in p38α$^{-/-}$ MEFs expressing N-RasV12 or Neu V664E correlated with a more dramatic transformed phenotype in these cells than in WT MEFs expressing the same oncogenes. No differences in soft agar growth were observed between WT and p38α$^{-/-}$ MEFs with the other oncogenes, including K-RasV12, which was consistent with their inability to induce long-term accumulation of high ROS levels in MEFs (Table 1). These results suggest that p38α functions as an oxidative stress sensor in tumorigenesis, with the capacity to downregulate malignant transformation by oncogenes that induce ROS production. Of note, p38α negatively regulated the induction of focus formation by oncogenic forms of Raf-1, B-Raf, and K-Ras, which do not produce high ROS levels (Table 1), suggesting that p38α may also have ROS-independent antioncogenic functions.

Gstm2 Impairs p38α Activation by Oxidative Stress in Human Epithelial Cells

Gstm2 was able to specifically inhibit p38α activation after oncogene-induced oxidative stress, without affecting other H-Ras-activated pathways in murine fibroblasts (Figure 4B). To complement these observations, we overexpressed Myc-tagged Gstm2 in HEK293 human epithelial cells, which then were stimulated with H_2O_2, sorbitol, UV irradiation, and cisplatin, or cotransfected with H-RasV12. We confirmed that Gstm2 efficiently inhibited p38α activation induced by H-RasV12 or H_2O_2 (Figures S6A and S6B). However, the Gstm2 inhibitory effect was more modest in the cases of osmotic shock, UV irradiation, and cisplatin treatment (Figure S6C). On the other hand, Gstm2 overexpression did not affect ERK activation by any of these stimuli and either only partially inhibited or had no effect on JNK activation induced by H_2O_2 and H-RasV12, respectively (data not shown). These results indicate that Gstm2 targets a key regulator of the oxidative stress-induced activation of p38 MAPK (i.e., ASK1), whereas additional pathways may contribute to JNK activation by oxidative stress.

ROS Accumulation in Human Cancer Cell Lines Correlates with Enhanced Tumorigenicity

Our results indicated that oncogene-induced ROS accumulation correlated with enhanced tumorigenicity in fibroblasts. However, many human neoplasms originate from epithelial cells, in which little is known about ROS levels. Thus, we compared intracellular ROS levels and tumorigenic potential in a panel of human epithelial cell lines derived from colon, prostate, breast, and lung tumors. We observed a strong correlation in all tissues between high levels of ROS and efficient anchorage-independent growth (Figure 5). These results suggest that ROS accumulation may enhance the malignant phenotype of cancer cells, in agreement with the procarcinogenic effects mediated by oxidative stress. In contrast, we observed no correlation between oxidative stress accumulation and the invasivity of these cancer cell lines (Figure 5). This was confirmed by the lack of effect of antioxidant treatment on the invasivity of MDA-MB-231 cells (data not shown). Thus, intracellular ROS levels seem to correlate with the tumorigenic potential of human cancer cells, but not with their invasive capacity.

Cancer Cell Lines with High ROS Levels Are Partially Impaired in p38α Activation

The identification of cancer cell lines that contained high levels of ROS despite expressing normal levels of p38α (Figure 6A) was intriguing, given the ability of p38α to sense oxidative stress and negatively regulate ROS

(D) Apoptosis was determined in empty vector- and ER-HRasV12-transduced WT and p38α$^{-/-}$ MEFs after 8 days of treatment with 1 μM OHT. Error bars show SD.

(E) ER-HRasV12-expressing WT MEFS were treated with 1 μM OHT and analyzed by immunoblotting with the indicated antibodies.

(F) ER-HRasV12-expressing WT MEFs were treated with 1 μM OHT for 4 days and then incubated for another 4 days with OHT together with SB203580 (10 μM) or ZVAD-fmk (20 μM). Cell lysates were analyzed by immunoblotting with the indicated antibodies. Apoptosis was quantified by an ELISA assay (right panel). Error bars show SD.

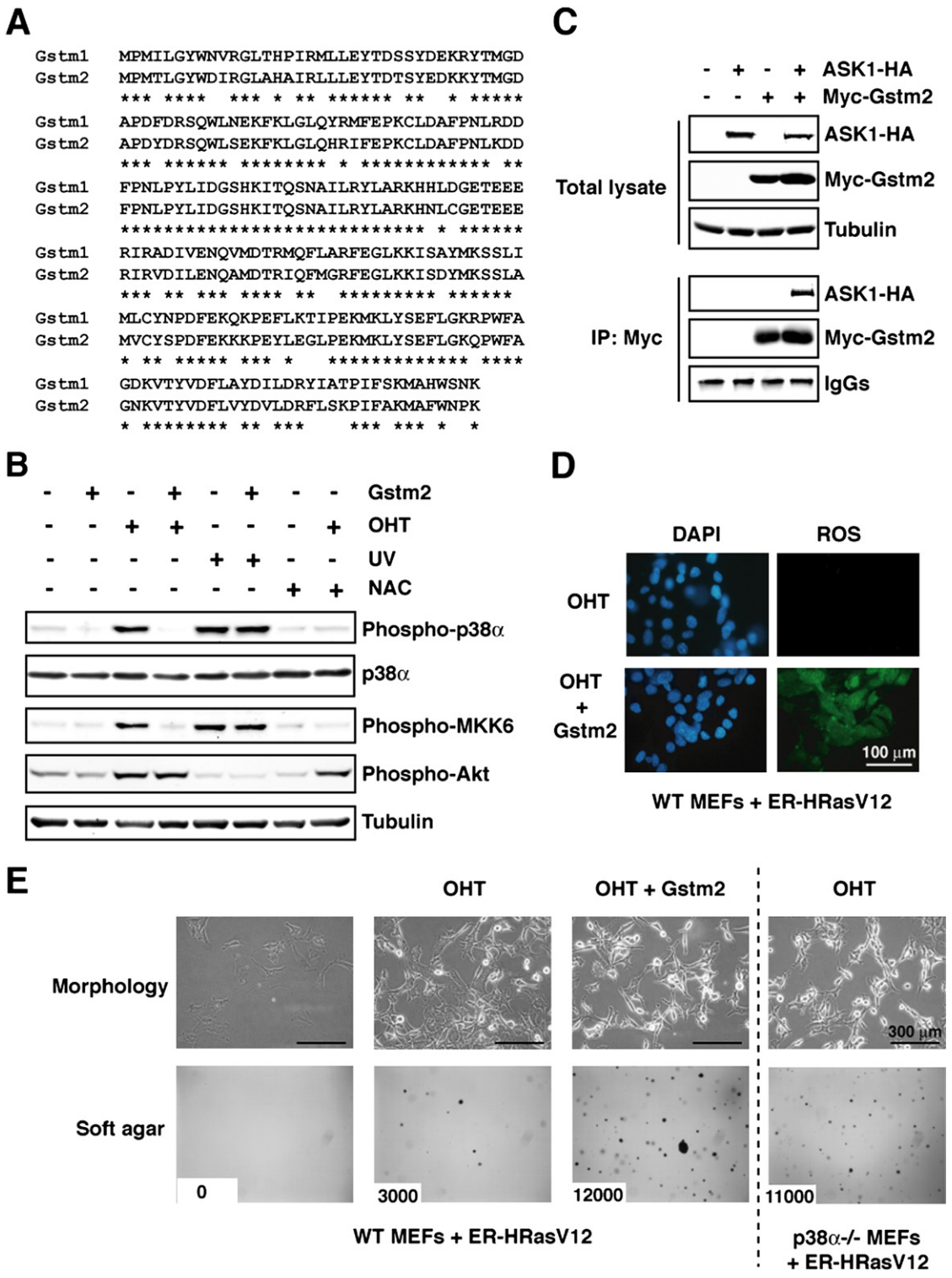

Figure 4. Uncoupling ROS Accumulation from p38α Activation Results in Enhanced Tumorigenicity

(A) Amino acid sequence alignment of murine Gstm1 (NP_034488) and Gstm2 (NP_032209). Asterisks indicate identical amino acids.

(B) ER-HRasV12-expressing WT MEFs were transduced with murine Gstm2 or an empty vector and then either stimulated with UV irradiation or treated with 1 μM OHT for 5 days. One sample was coincubated with 5 mM NAC during the last 16 hr of OHT treatment. Total cell lysates were analyzed by immunoblotting with the indicated antibodies.

(C) Lysates from 293T cells transfected with plasmids expressing ASK1-HA and Myc-Gstm2 were subjected to immunoprecipitation with Myc antibody. The total lysates and the Myc immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

Table 1. Effect of p38α on Anchorage-Independent Growth and Focus Formation Induced by Different Oncogenes in Mouse Fibroblasts

Oncogene	ROS[a] (p38α$^{-/-}$ MEFs)	Soft Agar[b] (p38α$^{-/-}$ MEFs)	Focus Formation	
			p38α$^{-/-}$ MEFs[c]	NIH3T3 + MKK6DD[d]
Neu V664E	+	Enhanced	Enhanced	Reduced
H-RasV12	+	Enhanced	Enhanced	Reduced
N-RasV12	+	Enhanced	Enhanced	Reduced
K-RasV12	–	As WT	Enhanced	ND
B-Raf V599E	–	As WT	Enhanced	Reduced
Raf-1 22W	–	As WT	Enhanced	Reduced
RalGDS-CAAX	–	As WT	As WT	ND
Rac1 N115I	–	As WT	As WT	ND
MEK1 ΔN	–	As WT	As WT	As NIH3T3
v-Mos	–	As WT	As WT	ND
c-Src Y527F	–	As WT	As WT	As NIH3T3
SV40 LT-Ag	–	As WT	As WT	As NIH3T3
v-Jun	–	As WT	As WT	As NIH3T3
c-Myc	–	As WT	As WT	As NIH3T3

[a] ROS levels were visualized by immunofluorescence; "+" indicates ROS accumulation to high levels.
[b] Soft agar was used to measure anchorage-independent growth in p38α$^{-/-}$ MEFs as compared to WT MEFs.
[c] Focus formation in p38α$^{-/-}$ MEFs as compared to WT MEFs.
[d] Focus formation in NIH3T3 fibroblasts expressing the p38 MAPK activator MKK6DD versus NIH3T3 fibroblasts. ND, not determined.

accumulation. Thus, we investigated the pattern of p38α activation in response to H_2O_2-induced oxidative stress in colon and breast cancer cell lines, which contained various levels of ROS. As shown in Figures 6A and 6B, H_2O_2 treatment activated p38α about 2-fold more efficiently in ROS-negative than in ROS-positive cancer cells. Interestingly, no differences in the activation of p38α were observed when cells were exposed to other stresses such as UV irradiation and osmotic shock (Figure 6C) or cisplatin treatment (data not shown). Our results therefore indicate that cancer cell lines with high ROS levels have developed specific mechanisms by which to desensitize p38α activation from oxidative stress, most likely in order to tolerate the high levels of ROS. Of note, whereas p38α activation was partially uncoupled from oxidative stress in ROS-producing cancer cells, JNKs, particularly the p54 JNK isoform, appeared to be more efficiently activated (Figure 6B). On the other hand, the ERK pathway was similarly activated in ROS-positive and ROS-negative cancer cell lines (Figure 6B).

As mentioned above, Gstm1 has been reported to inhibit the activation of p38 MAPK by oxidative stress, a function that we have shown is also shared by Gstm2. Interestingly, Gstm1 mRNA and protein levels were very high in most ROS-positive cancer cell lines, while they were absent or expressed at very low levels in ROS-negative cells (Figure 7A).

Next, we analyzed whether higher expression levels of Gstm proteins could account for the differences in ROS accumulation and p38α activation observed in cancer cell lines. First, we found that p38α activation was enhanced by siRNA-mediated knockdown of Gstm1 in the ROS-producing cancer cell lines MDA-MB-231 and A549 (Figure 7B) as well as in DU145 (data not shown). Similar results were obtained upon knockdown of Gstm2 in the cancer cell lines MCF7 (Figure 7B) and SW620 (data not shown), which express Gstm2, but not Gstm1 (Figure 7A and Figure S7). Interestingly, the activation of p38α observed upon knockdown of Gstm1 and Gstm2 correlated in all cases with enhanced apoptosis (Figure 7B). Conversely, overexpression of Gstm2 in MCF7 and SW620 cells resulted in reduced basal levels of activated p38α, as well as in the desensitization of p38α to oxidative stress (Figure 7C). Finally, overexpression of Gstm2 led to the accumulation of higher levels of ROS and the acquisition of a more malignant

(D and E) ER-HRasV12-expressing WT MEFs were transduced with murine Gstm2 or an empty vector, treated with 1 μM OHT for 3 weeks, and then analyzed for (D) intracellular ROS levels and (E) transformation-associated morphological alterations and anchorage-independent growth in soft agar.

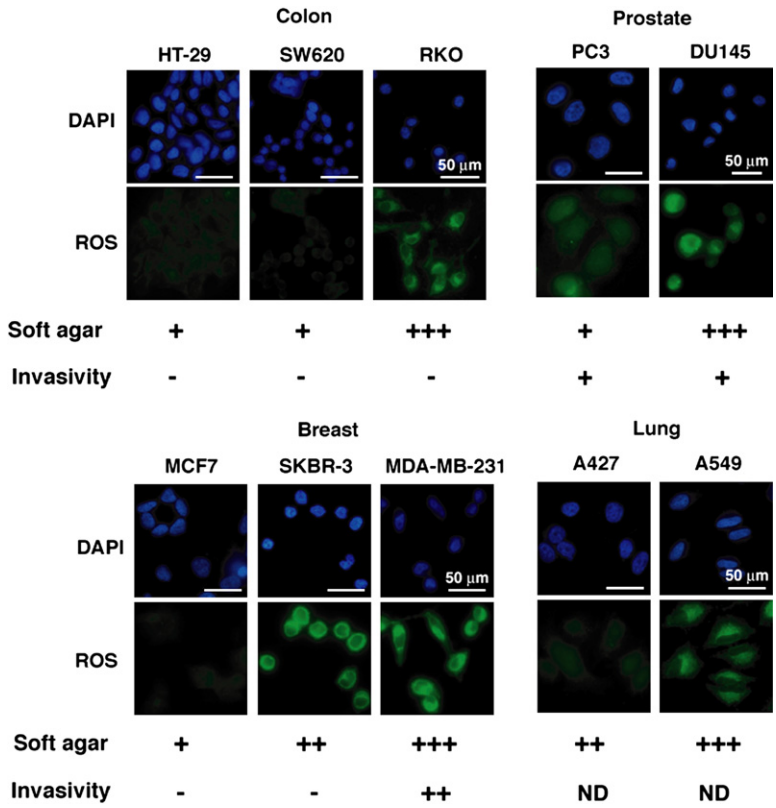

Figure 5. High Levels of ROS Correlate with Enhanced Tumorigenicity, but Not Invasivity, in Human Cancer Cell Lines from Different Tissues

The indicated human cancer cell lines were analyzed for intracellular ROS levels by immunofluorescence, for anchorage-independent growth in soft agar (+, 2,000–4,000; ++, 5,000–7,000; +++, 10,000–17,000 colonies), and for invasivity in matrigel chambers (–, <15; +, 25–50; ++, >90 arbitrary units). ND, not determined.

phenotype in MCF7 cells (Figure 7D), as well as in SW620 cells (data not shown). Of note, we did not observe changes in ROS levels after Gstm downregulation in the cancer cell lines mentioned above (data not shown), suggesting that Gstm proteins function downstream of ROS.

Taken together, these results argue that upregulation of Gstm proteins may be responsible for the partially impaired activation of p38α in ROS-producing cancer cells. Thus, Gstm1 and Gstm2 may inhibit the ROS-sensing and tumor-suppressor function of p38α in human epithelial cells, which is in agreement with their association with increased malignancy of several types of cancer.

DISCUSSION

p38α MAPK was identified as a protein kinase that coordinates the cellular responses to many types of stresses, including those that trigger oxidative stress production. In addition, p38α has been recently shown to mediate physiological processes in response to endogenous ROS, such as the regulation of the lifespan of murine hematopoietic stem cells (Ito et al., 2006). Here, we show that the ability of p38α to trigger apoptosis in response to oncogene-induced ROS accumulation plays a key role in the regulation of malignant transformation. Interestingly, highly tumorigenic human cancer cells can override this p38α function.

p38α as a Negative Regulator of Malignant Transformation

Previous studies have established p38α as a negative regulator of H-RasV12-induced cellular transformation, an effect that can be mediated by p53 and the $p16^{INK4a}$ and $p19^{ARF}$ pathways (Bulavin et al., 2002, 2004). Our results indicate that p38α can also inhibit H-RasV12-induced tumorigenesis in the absence of a functional p53 response and independently of $p16^{INK4a}$/$p19^{ARF}$. It therefore appears that the mechanisms by which p38α can impinge on malignant transformation may vary depending on the cell type and, probably, also between primary and immortalized cells (Ito et al., 2006; Li et al., 2003).

We show here that the ability of p38α to detect oxidative stress production early in the process of oncogenic H-Ras-induced transformation is important for its inhibitory effect on tumorigenesis. We have also extended this p38α-mediated inhibitory mechanism to other oncogenes, providing a molecular basis for the specificity of p38α as a tumor suppressor. Namely, we found that p38α functions as a tumor surveillance system activated by ROS, which, in turn, inhibits tumor initiation, at least in part, by inducing apoptosis. In agreement with this, ROS-induced sustained activation of p38α has been implicated in apoptosis induction (Tobiume et al., 2001), which can be mediated by both transcriptional and posttranscriptional mechanisms (Porras et al., 2004; Wada and Penninger, 2004), although low levels of oxidative stress can also induce a p38 MAPK-dependent cell cycle arrest (Kurata, 2000). Oxidative stress sensing, therefore, represents a

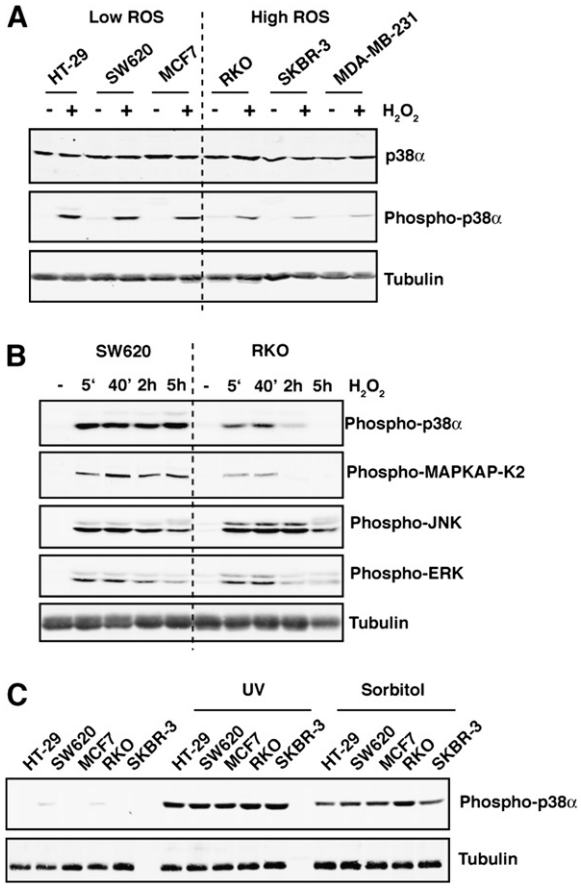

Figure 6. Human Cancer Cell Lines with High ROS Levels Are Partially Impaired in p38α Activation

(A) Cell lines derived from human colon and breast tumors, which contained either low or high ROS levels, were exposed to 1 mM H_2O_2 for 30 min and analyzed by immunoblotting with the indicated antibodies.

(B) ROS-negative SW620 and ROS-positive RKO colon cancer cells were treated with 5 mM H_2O_2 for different times and analyzed by immunoblotting with the indicated antibodies.

(C) ROS-negative (HT-29, SW620, MCF7) and ROS-positive (RKO, SKBR-3) cancer cell lines were exposed either to UV irradiation or osmotic shock, and phospho-p38 levels were analyzed by immunoblotting.

major mechanism for the inhibitory effect of p38α on oncogene-induced transformation.

Mechanisms of p38α Activation by Oncogene-Induced ROS

Several oncogenes have been reported to induce ROS accumulation and to rely on high ROS levels for efficient transformation (Mitsushita et al., 2004), but the molecular links between oncogene activation and ROS production are not completely understood. We found that oncogenic H-Ras-induced accumulation of ROS in $p38\alpha^{-/-}$ cells requires NOX activity and is blocked by the combined use of ERK and Rac1 inhibitors. This is consistent with previous work showing that ERK can induce transcriptional upregulation of *Nox1* (Mitsushita et al., 2004), whereas Rac1 cooperates in the assembly of the fully active NOX complex at the plasma membrane (Hancock et al., 2001).

Our results are in agreement with recent work showing that activation of p38α in response to oncogenic H-Ras requires ROS production (Nicke et al., 2005). Furthermore, the slow kinetics of p38α activation, which takes 3–4 days from the onset of oncogenic H-Ras signaling, suggests that p38α is probably activated as a consequence of the high ROS levels accumulated in the cells, rather than as a direct target of H-Ras signaling. Then, how do oncogene-induced ROS lead to p38α activation? One of the key mediators of ROS-induced p38α activation is ASK1 (Matsukawa et al., 2004). Thus, it is foreseen that oncogene-induced ROS would oxidize certain cysteine residues of Trx and induce its dissociation from ASK1, hence inducing the activation of the JNK and p38 pathways. Of note, whereas ASK1 is the major mediator of p38α activation by ROS (Tobiume et al., 2001), MEKK1 may collaborate with ASK1 for the activation of JNK by oxidative stress (Xia et al., 2000; Yujiri et al., 2000).

In addition to the well-characterized role of ASK1 in the activation of p38α by oxidative stress, a recent report has also identified the Ste20 family kinase MINK (MAP4K6) as a novel mediator of the H-RasV12-induced activation of p38 MAPK (Nicke et al., 2005). The mechanism by which H-RasV12 activates MINK is unknown but requires both ERK activation and ROS production. In turn, MINK may lead to the activation of the p38 MAPK pathway through both ASK1 and Tpl2. The interplay between the direct activation of ASK1 by ROS and the participation of ASK1 in ROS-induced MINK signaling is unclear, but it might reflect different ROS-induced cellular responses dependent on signal duration or intensity. Thus, whereas ROS-induced, Trx-dependent activation of ASK1 has normally been associated with the induction of apoptosis (Tobiume et al., 2001), the MINK-mediated ASK1 activation by ROS results in cell cycle arrest (Nicke et al., 2005).

The ROS-p38α Connection in Human Cancer

Suppression of apoptosis is thought to be an important aspect of tumor development (Evan and Vousden, 2001), and multiple mechanisms for inhibition of apoptosis have been identified in human tumors. Furthermore, inactivation of apoptotic proteins potentiates malignant transformation in vitro (Kennedy and Davis, 2003). In agreement with these observations, we show that p38α can inhibit cell tumorigenicity by triggering oncogene-induced apoptosis mediated by ROS. The relevance of this finding was confirmed by the observation that human cancer cell lines have developed a mechanism by which to uncouple p38α activation from oxidative stress production, which results in enhanced tumorigenicity. This mechanism relies on the ability of the GST family members Gstm1 and Gstm2 to impair p38α activation in response to ROS accumulation (Cho et al., 2001; Dorion et al., 2002). Indeed, the ability of highly tumorigenic cancer cell lines to accumulate very high levels of ROS correlates with the upregulation of Gstm1 and Gstm2. Taken

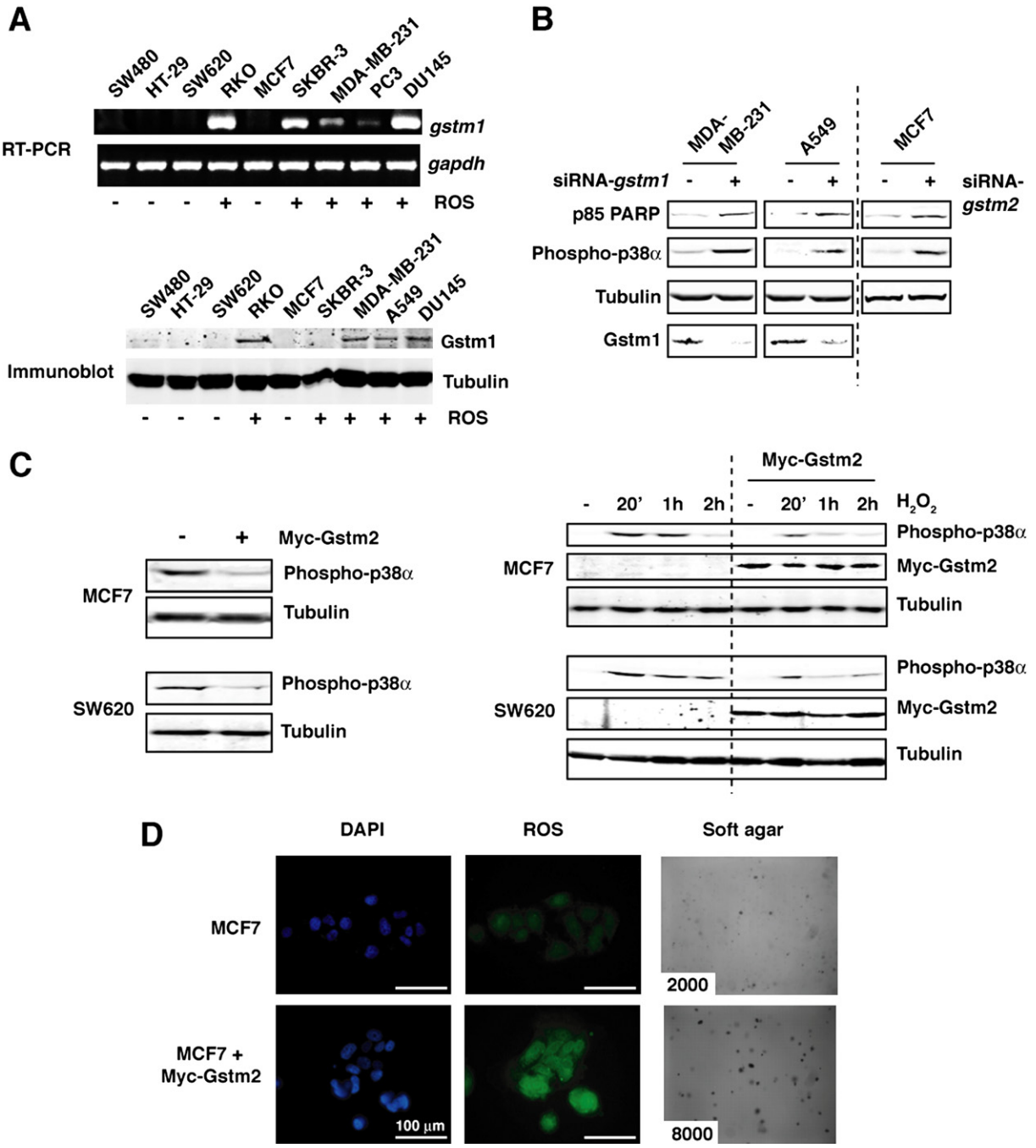

Figure 7. Gstm1 and Gstm2 Modulate the Activation of p38α by Oxidative Stress in Several Human Cancer Cell Lines

(A) *gstm1* (NM_000561) expression was analyzed by both RT-PCR (top panel) and western blot (bottom panel) in several human cancer cell lines.

(B) Downregulation of Gstm1 and Gstm2 by siRNA enhances both p38α activation and the basal apoptotic levels (as indicated by the accumulation in p85 PARP) of human cancer cell lines. Analysis by qRT-PCR confirmed that treatment with *gstm2* siRNA downregulated the *gstm2* mRNA levels to about 40% of those observed in untreated MCF7 cells.

(C) Myc-Gstm2 overexpression in the ROS-negative cancer cell lines MCF7 and SW620 reduces the basal levels of phospho-p38α (left panels) and impairs the activation of p38α by oxidative stress (right panels).

(D) MCF7 cells stably infected with Myc-Gstm2 or empty vector (EV) were analyzed for intracellular ROS levels (middle panels) and anchorage-independent growth in soft agar (right panels).

together, these results suggest that the p38 MAPK-signaling pathway may suppress tumor formation in vivo by inducing apoptosis. Furthermore, certain members of the GST family may function as potential oncogenes in human cancer, by impairing the normal inhibitory responses, such as apoptosis, triggered by p38α in response to ROS

accumulation. Consistent with this idea, a positive correlation between the expression of several GST family members and cancer progression has been recently proposed (Parl, 2005; Townsend and Tew, 2003).

Collectively, our data indicate that cancer cells may undergo positive selection for high intracellular ROS levels in their course for proliferative advantages. Thus, the carcinogenic effects associated with increased ROS levels might provide cancer cells with greater plasticity for malignant progression. Interestingly, the associated overexpression of Gstm1 and Gstm2 could be a way to specifically suppress the apoptotic effects of p38α in response to ROS, without affecting other cellular processes mediated by p38α that might be important for the viability of the cancer cell. This may explain the lack of evidence for the loss of p38α expression or activity in human cancer, and it suggests a new category of tumor-suppressor proteins in which a partial loss of function specifically impairs their negative contribution to cancer cell survival, while allowing other functions that might be important for malignant progression. This idea is in agreement with evidence indicating that p38 MAPK might, in some cases, contribute to cancer progression, for example by mediating cancer cell migration (Kim et al., 2003), by activating the transcription factor HIF-1 (Emerling et al., 2005; Nakayama et al., 2007) or by other mechanisms (Elenitoba-Johnson et al., 2003; Weijzen et al., 2002). Consistent with this idea, we have found that inhibition of p38 MAPK impairs the proliferation and anchorage-independent growth of some cancer cell lines (data not shown). Thus, whereas p38α can negatively regulate tumor initiation by triggering apoptosis in response to oncogene-induced ROS, the overall effect of p38 MAPK inhibition for human cancer is likely to be highly dependent on the tumor type and cancer stage.

EXPERIMENTAL PROCEDURES

Cell Culture

WT and p38α$^{-/-}$ primary MEFs were derived from E11.5 and E12.5 embryos (Ambrosino et al., 2003). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin (all from GIBCO-Invitrogen) and were immortalized by following the 3T3 protocol. The established cell lines represent pools of at least 100 independent clones. NIH3T3 mouse fibroblasts were maintained under subconfluent conditions in DMEM, as described above; however, in this case, DMEM was supplemented with 10% heat-inactivated newborn calf serum (NBCS). When used for focus formation assays, NBCS was reduced to 5%. The HEK293-derived, virus-packaging cell lines Ampho-Pak and 293T were obtained from Clontech and M. Serrano (CNIO), respectively. The human cancer cell lines DU145 and HT-29 were kindly provided by M. Robledo and J. Bravo (CNIO), respectively. The remaining cancer cell lines were a kind gift from A. Muñoz (Biomedical Research Institute Alberto Sols, Madrid). All human cancer cell lines were cultured in DMEM with 10% FBS.

Retroviral Infections

Expression constructs are described in Supplemental Data. Retroviruses were produced in 293T cells by transient transfection. Culture supernatants were collected 48 hr (first supernatant) and 72 hr (second supernatant) posttransfection, filtered (0.45 μm filter, PVDF, Millipore), and supplemented with 4 μg/ml polybrene (Sigma). MEFs at ~5 × 10^5 cells per 10 cm dish were infected with 6 ml of the first supernatant, supplemented 24 hr later with 3 ml of the second supernatant, and purified 48 hr postinfection with either 1–2 μg/ml puromycin for 1 week or 150–200 μg/ml hygromycin for 2 weeks. Pools of at least 10^5 independent clones were normally used. The data for H-RasV12, B-RafV599E, c-SrcY527F, SV40 LT-Ag, and v-Jun are representative of studies in which at least three different cell populations that were independently isolated were used. For other oncogenes, pools were isolated independently twice.

For the rescue experiments, two rounds of retroviral infection were performed as previously described (Ambrosino et al., 2003). MSCV or MSCV-p38α was first expressed in p38α$^{-/-}$ MEFs, followed by transduction with H-RasV12.

Retroviral transduction of MCF7 and SW620 human cancer cells with Myc-tagged Gstm2 was performed as described above, except that 293 Ampho-Pak packaging cells were used instead of 293T cells. Antibiotic selection was carried out with 100–150 μg/ml hygromycin for MCF7 and 250–300 μg/ml hygromycin for SW620.

Transformation and Tumorigenicity Assays

NIH3T3-based focus formation assays and anchorage-independent growth in soft agar were performed by following standard procedures. Tumorigenicity assays in nude mice were performed in accordance with institutional guidelines (EMBL Animal Care and Use Committee). Details are provided in Supplemental Data. For the focus formation assays with immortalized MEFs, 9 × 10^5 WT or p38α$^{-/-}$ cells were seeded per 10 cm plate and were infected with the virus-containing supernatants from 293 Ampho-Pak cells transfected with oncogene-encoding retroviral vectors. Cells were maintained in DMEM with 10% FBS, and the medium was changed every 2–3 days. Foci were counted 10–15 days after transduction.

The in vitro invasion assays were carried out in BD BioCoat Matrigel chambers (Becton Dickinson) as described in Supplemental Data.

Determination of Intracellular ROS Levels

To visualize intracellular ROS levels, proliferating cells were grown on coverslips, washed once with warm PBS, and incubated with 10 μM 2′-7′-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes D399) in warm PBS supplemented with 5.5 mM glucose. After 10 min at 37°C, PBS was replaced with complete culture medium, and cells were incubated for an additional 10–15 min, washed once again with warm PBS, and fixed in 4% formalin (Sigma). Coverslips were incubated with 1 μM 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining (Sigma) and were mounted in Mowiol (Calbiochem), and intracellular ROS levels were visualized by using an inverted fluorescence microscope, Leica DM5000B, coupled to a Leica DC500 camera. Pictures were taken at 63× magnification with the Leica IM50 software. Where indicated, cells were pretreated for 12–16 hr with rotenone (R8875, Sigma), DPI (D2926, Sigma), LY294002 (Calbiochem), NSC23766 (Calbiochem), or PD98059 (Calbiochem) before ROS visualization.

For ROS quantification, cells were treated as described above with 10 μM DCF-DA, trypsinized, and analyzed by FACS as described (Nicke et al., 2005).

Immunoblot Analysis

Cell lysates were prepared as described (Alonso et al., 2000), separated by SDS-PAGE, and analyzed by immunoblotting by using the Odyssey Infrared Imaging System (Li-Cor, Biosciences). Details on the procedure and antibodies used are described in Supplemental Data.

Cell Treatments and Assays for Survival and Proliferation

To induce p38 MAPK activation, cells were treated with 1–5 mM H_2O_2 (Sigma) for 5 min to 5 hr, 0.4 M sorbitol (Sigma) for 6 hr, and 25 μM

cisplatin (Sigma) for 8–10 hr or UV stimulated by using a Stratalinker apparatus, followed by 30 min in the 37°C incubator.

Cell proliferation assays were performed by using the MTT cell proliferation Kit I (Roche Diagnostics, Mannheim, Germany). For proliferation assays with MEFs, 1000 cells/well were seeded in triplicate, and cell numbers were monitored during the course of 5 days. Experiments were repeated at least three times.

For apoptosis assays with MEFs expressing the OHT-inducible ER-HRasV12 construct, 8000 cells/well were seeded in triplicate, and apoptosis was measured with the Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics GmbH, Germany). Experiments were performed twice.

Knockdown of Gstm1 and Gstm2 by siRNA

Human *gstm1* and *gstm2* as well as control (siGLO) siRNA oligos were obtained from Dharmacon. MDA-MB-231 and A549 cells were transfected with 200 nM and 150 nM *gstm1* siRNA, respectively. MCF7 cells were transfected with 100 nM *gstm2* siRNA. In all cases, DharmaFECT 1 buffer was used (Dharmacon). After 3 days, cells were scraped, and the lysates were analyzed by immunoblot.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and seven figures and are available at http://www.cancercell.org/cgi/content/full/11/2/191/DC1/.

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