Silencing of the Lats2 tumor suppressor overrides a p53-dependent oncogenic stress checkpoint and enables mutant H-Ras-driven cell transformation

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Abstract

The Lats2 tumor suppressor protein has previously been implicated in promoting p53 activation in response to mitotic apparatus stress, by preventing Mdm2-driven p53 degradation. We now report that Lats2 also plays a role in an ATR-Chk1-mediated stress checkpoint in response to oncogenic H-Ras. Activated mutant H-Ras triggers the translocation of Lats2 from centrosomes into the nucleus, coupled with an increase in Lats2 protein levels. This leads to induction of p53 activity, upregulation of proapoptotic genes, downregulation of antiapoptotic genes and eventually apoptotic cell death. Many of the cells that survive apoptosis undergo senescence. However, a fraction of the cells escape this checkpoint mechanism, despite maintaining high mutant H-Ras expression. These escapers display increased genome instability, as evidenced by a substantial fraction of cells with micronuclei and cells with polyploid genomes. Interestingly, such cells exhibit markedly reduced levels of Lats2, in conjunction with enhanced hypermethylation of the *Lats2* gene promoter. Our findings suggest that Lats2 might play an important role in quenching H-Ras-induced transformation, while silencing of Lats2 expression might serve as a mechanism to enable tumor progression.

Keywords

Ras; p53; Lats2; apoptosis; polyploidy

Introduction

Cancer arises through the accumulation of genetic changes that enhance the growth and survival of developing tumor cells. Activating mutations in the Ras pathway occur in approximately 30% of all human cancers (Ding *et al.* 2008). However, mutation of Ras alone is insufficient to induce tumor development. To a large measure, this is due to the fact that aberrant activation of oncogenic Ras triggers a series of protective cellular responses aimed at curtailing its potential cancer-promoting effects. This protective response is dependent on, and often orchestrated by, a number of pivotal tumor suppressor proteins. For instance, deregulated Ras activity can induce cellular senescence, a process shown to depend

at least in part on p53 and on the products of the INK4a tumor suppressor locus (Serrano 1997). More recent evidence implicates Ras-induced p53-dependent senescence as a pivotal cancer-inhibitory mechanism also in animal models and in humans (Bartek *et al*. 2007; Ventura *et al*. 2007; Xue *et al*. 2007; Halazonetis *et al*. 2008). Consistent with this view, the tumor suppressor p53 is mutated at a high frequency in many tumor types. Even in those cancers that retain wild-type p53, its activity is likely to be compromised by genetic or epigenetic deficiencies in upstream pathways (Vazquez *et al*. 2008; Wang and El-Deiry 2008).

Recently, we described a novel tumor suppressor axis, Lats2 *(Large Tumor Suppressor 2)*-Mdm2-p53, which is critical for the maintenance of proper chromosome number in the face of mitotic apparatus stress (Aylon *et al*. 2006). In response to such stress, Lats2 protein departs the centrosomes, where it normally resides, and translocates to the nucleus. In the nucleus, Lats2 binds the negative regulator of p53, Mdm2, leading to inhibition of p53 degradation and induction of a p53-driven transcriptional response. Within this process is also embedded a positive feedback loop, wherein the *Lats2* gene itself is directly transcriptionally activated by p53, leading to a gradual and continuous increase in Lats2 protein levels. This axis underpins a checkpoint mechanism that acts to prevent the proliferation of cells with polyploid genomes.

A number of studies suggest a specific involvement of Lats2 in protecting cells from Ras driven transformation and tumorigenesis. Using a system of V-Ras-transformed NIH3T3, Li et al (2003) found that overexpression of Lats2 could suppress tumorigenesis in nude mice. Subsequently, Voorhoeve et al (2006) reported that downregulation of Lats2 via overexpression of miR-372/3 could bypass H-Ras-induced senescence in primary human fibroblasts.

We now provide evidence that H-Ras activation affects the Lats2 tumor suppressor in a three-pronged manner. Initially, acute signaling propagated from oncogenic Ras leads to a pronounced upregulation of Lats2, through a combination of transcriptional and posttranscriptional mechanisms. This underpins an ATR-Lats2-p53-dependent replicative stress checkpoint response that promotes apoptosis. Following this wave of apoptosis, Lats2-dependent senescence acts as a second line of defense against H-Ras activation. Finally, cells surviving sustained oncogenic H-Ras activity are found to have neutralized the Lats2-p53 tumor suppressor pathway by hypermethylation of the *Lats2* gene promoter. These cells emerge with features characteristic of transformation, such as polyploidy, enhanced cell migration and anchorage-independent growth. Remarkably, reconstitution of Lats2 expression leads to a p53-dependent reversal of these transformed features and leads to induction of apoptosis. These findings illustrate the importance of Lats2 in quenching H-Ras-induced transformation and provide experimental evidence that silencing of Lats2 expression may serve as a mechanism to enable tumor progression.

Materials and Methods

Plasmids

The plasmids used are summarized in Supplemental Table 1.

RNA analysis

Total RNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel) or mirVana miRNA isolation kit (Ambion). qRT-PCR was performed as described (Aylon *et al*. 2006). For small RNAs (5S and miR-373), 2.5 µg total RNA was converted to cDNA using specific primers for reverse transcription (Ambion) and MMLV reverse transcriptase. Expression levels were determined using miR-373 specific primers relative to 5S RNA (Ambion). Primer sequences are listed in Supplemental Table 2.

Protein analysis

Western blot analysis was performed using standard procedures, employing the following antibodies: Lats2 (LA-2; Yabuta *et al*. 2007), GAPDH (Chemicon), H-Ras (sc-520, Santa Cruz); p53 (mix of DO1+PAb1801) and p21 (sc-397, Santa Cruz). Immunofluorescence was performed as described (Aylon *et al*. 2006). Lats2 was detected with anti-KPM/Lats2 (Santa Cruz) or LA-2 (Yabuta *et al*. 2007). Protein signals were quantified using ImageJ software and normalized to the corresponding GAPDH signals.

Senescence was quantified by staining with a β-gal activity kit (Cell Signaling) following 4 or 10 days of hygromycin and blasticidin selection.

Cell culture, transfections, viral infections, Cell cycle analysis, Promoter methylation, Cell migration and Soft agar colony assays are described in Supplementary Materials and Methods.

Results

To explore the functional links between oncogenic H-Ras activation and Lats2, we made use of WI-38 human lung embryonic fibroblasts that had been immortalized with telomerase and subsequently subjected to long term passaging in culture (WI-38/hTERT[fast]; Milyavsky *et al*. 2003). Four days after infection of these cells with a recombinant retrovirus encoding constitutively activated mutant H-RasV12, a marked increase in endogenous Lats2 protein was evident (Fig 1A). *Lats2* mRNA increased nearly twofold (Fig 1B, black bars), partially accounting for the observed elevation in Lats2 protein. We also quantified unspliced, intron-containing *Lats2* precursor mRNA, expected to more reliably capture differences in transcription rates (Phelps *et al*. 2006; Ponzio *et al*. 2007). This analysis revealed a nearly 4.5 fold increase in *Lats2* precursor mRNA in response to H-RasV12 expression (Fig 1B, gray bars). A similar trend was observed also in non-immortalized WI-38 cells (Supplementary Fig S1). Hence, oncogenic H-Ras stimulates *Lats2* gene transcription and accumulation of Lats2 protein in both primary and immortalized WI-38 cells.

The microRNA miR-373 can target directly *Lats2* mRNA (Voorhoeve *et al*. 2006). Indeed, H-RasV12 overexpression downregulated miR-373 levels (Supplementary Fig S2), suggesting that repression of miR-373 expression by activated H-Ras also might, in principle, promote Lats2 induction. However, inhibition of miR-373 by a specific locked nucleic acid (LNA) oligonucleotide or overexpression of a pre-miR-373 precursor had very little effect on Lats2 protein levels in these cells (data not shown). It remains possible that,

in other cell types with higher levels of endogenous miR-373, repression of miR-373 by activated H-Ras does play a role in modulating Lats2.

The fold increase of Lats2 protein (Fig. 1A) exceeded that of *Lats2* mRNA (Fig. 1B). Therefore, to determine whether H-RasV12 also had an effect on Lats2 protein stability, a cycloheximide chase experiment was performed. As seen in Fig. 1C, endogenous Lats2 protein stability was indeed elevated by H-RasV12. Whereas the half-life of Lats2 was approximately 5 hours in control cells, it was extended to about 8 hours in cells infected with H-RasV12. Moreover, in human breast cancer-derived MCF7 cells, transient transfection of a Lats2 expression plasmid together with increasing amounts of an H-RasV12 plasmid led to a dose-dependent increase in the levels of the exogenous Lats2 protein (Supplementary Fig. S3). Together, these data indicate that multiple mechanisms, transcriptional as well as post-transcriptional, contribute to Lats2 protein upregulation by oncogenic H-Ras.

The protein kinases ATM and Chk2 recently have been proposed to mediate cellular senescence in response to DNA damage occurring downstream to aberrant oncogene activation, thereby setting a barrier against oncogene-driven transformation (reviewed in Bartek *et al*. 2007). However, Lats2 is not activated in response to overt DNA damage (Aylon *et al*. 2006). On the other hand the ATM-related kinase, ATR, known to play an important role in the response to DNA replication stress, has also been implicated in H-Ras signaling (Abulaiti *et al*. 2006; Fikaris *et al*. 2006; Mallette *et al*. 2007). We therefore investigated whether ATR might be involved in relaying the H-Ras-driven signal for Lats2 induction. Indeed, expression of H-RasV12 caused a marked increase of phosphorylated ATR (Fig 2A, lanes 1,5), confirming that H-Ras-induced oncogenic stress leads to ATR activation in these cells. Strengthening the possibility that ATR mediates the pertinent signal, treatment of H-RasV12 expressing cells with caffeine, a compound that inhibits ATR (as well as ATM, although less specifically), decreased basal levels of Lats2 and impeded Lats2 accumulation (Fig 2A, compare lanes 5,7). ATR-mediated phosphorylation of the Chk1 kinase on serine 345 is key to the propagation of replication stress signals (Jiang *et al*. 2003). Indeed, oncogenic H-Ras led to a substantial induction of Chk1 Ser345 phosphorylation, suggested by the appearance of a slower migrating form of Chk1 and formally proven with phosphospecific antibodies (Fig 2A, compare lanes 1 and 5). These phosphorylation events were abrogated by treatment with caffeine (compare lanes 5 and 7). Importantly, treatment with the Chk1 inhibitor UCN-01 (lane 8) significantly diminished basal as well as H-RasV12-induced Lats2 protein. Similar effects were observed in HCT116 colorectal cancer-derived cells (Supplementary Fig S4), supporting the generality of this signaling pathway. In contrast, the involvement of the stress kinase p38 varied among different cell lines; whereas treatment with the p38 inhibitor SB203580 abrogated H-RasV12-induced Lats2 accumulation in HCT116 cells (Supplementary Fig. S4), it failed to do so in the non-transformed WI-38 cells (Figure 2A). It is also noteworthy that both these cell types lack functional p14ARF tumor suppressor protein (Burri *et al*. 2001; Milyavsky *et al*. 2003), indicating that, at least in WI-38 and HCT116 cells, ARF is dispensable for the induction of Lats2 by oncogenic Ras. Together, these findings imply that oncogenic H-Ras generates ATR-Chk1-dependent stress, which induces Lats2 accumulation.

Lats2 is predominantly a centrosomal protein (Toji *et al*. 2004). Upon damage to the mitotic apparatus Lats2 departs from the centrosome and accumulates in the nucleus, where it binds Mdm2 and stabilizes p53 (Aylon *et al*. 2006). Remarkably, nuclear accumulation of Lats2, which occurs only rarely and sporadically in unperturbed WI-38/hTERT[fast] cells (Fig 2B, vector/mock), was dramatically elicited by transient overexpression of H-RasV12 (Fig 2B, H-RasV12/mock). Furthermore, as previously observed (Denko *et al*. 1995; Abulaiti *et al*. 2006), cells expressing oncogenic H-RasV12 tended to form micronuclei; these micronuclei contained Lats2 (arrowhead). Interestingly, inhibition of ATR by caffeine or of Chk1 by UCN-01 completely abolished Lats2 nuclear accumulation (Fig 2B). Instead, with both inhibitors, Lats2 was predominantly distributed throughout the cytoplasm. These observations suggest that H-RasV12-induced nuclear translocation of Lats2 is a multistep process; the first step involves release of Lats2 from the centrosomes into the cytoplasm, while in the second step cytoplasmic Lats2 is transported into the nucleus. While both steps are induced by oncogenic H-Ras, only the second one appears to be dependent on ATR and Chk1. Our data also suggests that centrosome-confined Lats2 may not be readily accessible to antibodies, accounting for the very weak staining in control cells (Fig. 2B, vector/mock) as compared to the more robust signal on Western blots (Fig. 2A).

Treatment of H-RasV12-overexpressing WI-38 cells with the p38 kinase inhibitor, SB203580, elicited a mixed phenotype. While the majority of Lats2 protein became nuclear, a portion remained cytoplasmic (Fig 2B). Interestingly, in HCT116 cells, where SB203580 abrogated Lats2 upregulation in response to H-RasV12 expression (Supplementary Fig S4), it also prevented its nuclear accumulation (Supplementary Fig S5). In sum, we find that constitutively active H-Ras generates a signal that dislodges Lats2 from the centrosomes and drives it into the nucleus in an ATR-Chk1 dependent manner. Depending on cell context, p38 may also participate in this pathway.

In response to mitotic apparatus damage, Lats2 stabilizes p53 by alleviating Mdm2-dependent p53 ubiquitylation and degradation (Aylon *et al*. 2006). Likewise, oncogenic activation also caused Lats2-dependent p53 accumulation. Whereas knockdown of Lats2 did not affect basal p53 levels (Fig. 3A, lanes 1,2), it strongly attenuated p53 induction by H-RasV12 (lanes 3,4). p21 is a canonical p53-response gene that is markedly upregulated in response to H-Ras (Fig. 3B, lanes 1,3). In Lats2-depleted cells, p21 induction by H-RasV12 was completely abolished at both protein (Fig 3B, lanes 3,4) and RNA (Fig 3C) levels. Modulation of Lats2 also influenced the transcriptional program of other p53 target genes. Thus, transient overexpression of H-RasV12 resulted in increased transcription of the proapoptotic gene *Bax* and decreased expression of the antiapoptotic gene *Birc3/cIAP2* (Fig 3C). Remarkably, Lats2 knock-down (Fig. 3D) reversed this trend, restoring both *Bax* and *Birc3* mRNA to nearly basal levels (Fig. 3C). A similar picture was observed in HCT116 cells (Supplementary Fig S6). Moreover, in an isogenic HCT116 cell line lacking functional p53, knockdown of Lats2 had no effect on *p21* or *Birc3* mRNA levels (HCT-/-, Supplementary Fig S6), indicating that the transcriptional input of Lats2 downstream to H-Ras activation was strictly p53-dependent. Interestingly, similar to what has been observed in cells under mitotic apparatus stress (Aylon *et al*. 2006), induction of *Lats2* mRNA by oncogenic H-Ras was entirely dependent on p53 (Fig 3D). In all above aspects, Lats2 fits the

bill of a classic tumor suppressor protein in that it responds to H-Ras activation by enhancing the expression of cell cycle-inhibitory and proapoptotic genes and diminishing that of antiapoptotic genes.

We next explored the biological consequences of the Ras-Lats2-p53 pathway. Within 4 days, expression of H-RasV12 led to a nearly six-fold increase in apoptosis in WI-38 cells (2.3% to 13.4%), as assessed by the presence of cells with a sub-G1 (<2N) DNA content (Fig 4A, top panels). Remarkably, Lats2 knock-down reduced apoptosis to nearly basal levels, with a concurrent elevation in the number of polyploid (>4N) cells (from 10.8% to 16.6%, Fig. 4A, top panels). Treatment with caffeine, which inhibited the nuclear accumulation of Lats2 in response to H-RasV12, had a similar effect as Lats2 knock-down; specifically, caffeine diminished H-RasV12-driven apoptosis and increased the percentage of polyploid cells (Fig 4B). Hence, ATR-mediated nuclear accumulation of Lats2 is essential to efficiently induce apoptosis and prevent genomic instability in response to oncogenic H-Ras.

Similar to its effect on H-RasV12-driven induction of polyploidy, Lats2 knock-down also enhanced H-RasV12-dependent cell migration (Fig 4C), indicating that Lats2 acts to counteract also this malignancy-related effect of oncogenic H-Ras. Together, these data indicate that Lats2 obstructs the survival and propagation of genomically unstable cells and restricts their malignant potential.

Like apoptosis, senescence serves as a barrier to prevent oncogene-driven cellular transformation (Lowe 1999; Bartkova *et al*. 2006; Kim *et al*. 2007). Previously, Lats2 was found as a target of miR-372/3 in a screen for bypassing H-RasV12-induced, p53-dependent senescence in primary BJ cells (Voorhoeve *et al*. 2006), suggesting that Lats2 may mediate the H-RasV12 signal that triggers p53 also in senescence. To address this possibility, we employed senescence-associated β-galactosidase (SA β-gal) staining to monitor cell senescence at different times after infection with H-RasV12. Four days after infection, when H-RasV12 primarily elicited apoptosis, a mild induction of H-RasV12-induced senescence could be observed (Fig. 4E). Much more dramatic senescence was seen at ten days after infection with H-RasV12 (Fig. 4D,E; the siCont/H-RasV12 panel in Fig. 4D contains a much smaller total number of cells than the other 3 panels). Remarkably, the oncogene-induced senescence was completely abrogated by knockdown of Lats2. Together, these data suggest two waves of Lats2-dependent barriers to combat H-RasV12 oncogenesis. First, the bulk of cells undergo Lats2-dependent apoptosis; then, many of the survivors undergo Lats2-dependent senescence.

Our findings imply that acute signaling, propagated from oncogenic H-Ras, evokes a Lats2-p53-dependent checkpoint response that triggers apoptosis and senescence. It is conceivable that continued expression of activated H-Ras might exert a selective pressure favoring cells that have inactivated this checkpoint response. Such "escapers" are more likely to emerge with features of transformation, e.g. genomic instability, increased proliferation and augmented migration and invasiveness. Indeed, when H-RasV12-infected WI-38 cells were maintained in culture for more extended periods, they exhibited increased genome instability. As illustrated in Fig. 4A, cultures surviving 2 weeks of H-RasV12 expression

bear a larger portion of polyploid cells (from 4.5% to 17.2% >4N DNA content without or with H-RasV12, respectively; bottom panels). In these cells, apoptosis levels were slightly lower and, notably, were unaffected by Lats2 knock-down. It is conceivable that such "escapers" might already constitute the majority of cells that have failed to undergo senescence by day 10 (see Fig. 4D). Together, these observations suggest that "escaper" cells might have successfully downregulated the Lats2 response, resulting in enhanced survival and increased genome instability.

To explore more directly the notion that Lats2 dysfunction might underlie the "escaper" phenotype, we examined microscopically the pattern of Lats2 protein distribution in such cells. Indeed, whereas H-Ras activation led to extensive nuclear accumulation of Lats2 in the days immediately following infection (see Fig. 2B), this effect was lost upon more prolonged cultivation (Fig. 5A, third row). Furthermore, such cells exhibited persistent centrosomal Lats2, which failed to translocate to the nucleus even after nocodazole treatment (Fig. 5A, fourth row), unlike the typical response (Aylon *et al*. 2006; see also Fig. 5A, second row). Hence, prolonged expression of oncogenic H-Ras selects for cells in which the Lats2 response has been numbed. As previously reported (Abulaiti *et al*. 2006), long-term H-RasV12 expressors displayed an elevated incidence of micronuclei (Fig 5A, DAPI), which became even more pronounced following nocodazole treatment. This further argues that numbing of the Lats2 response enables the survival and propagation of genomically unstable cells.

How is the Lats2 response dampened in cells that have evaded the selective pressure imposed by persistent H-RasV12 expression? We observed no significant difference in miR-373 expression between short and long-term H-RasV12 expressors (Supplementary Fig S7A), nor was there a change in Lats2 protein half-life, which remained approximately 8 hours (Supplementary Fig S7B). In contrast, *Lats2* transcription was markedly downregulated in the "escaper" cells, as evident from the quantitative analysis of mature as well as precursor intronic *Lats2* mRNA (Fig 5B). Numbing of the Lats2-p53 axis by long-term H-RasV12 activation could be confirmed also at the protein level (Fig 5C). Furthermore, diminished Lats2 expression resulted in reduced levels of p53 and p21 (Fig 5C, compare lane 2 and 4). Remarkably, once cells had switched off the Lats2 axis, even reintroduction of extra H-RasV12 failed to upregulate Lats2 again, despite a clear increase in H-Ras protein (Fig 5D, compare lane 4 and 6). Likewise, Abulaiti *et al*. (2006) reported that chronic overexpression of Ras in cultured rat thyroid cells selects for the emergence of survivors that have switched off the apoptotic response to excess Ras; such cells were shown to display compromised p53 activity and to have become refractory to a second challenge with activated Ras.

Promoter hypermethylation plays a significant role in gene inactivation in various human cancers (reviewed in Esteller 2008). Moreover, *Lats2* promoter hypermethylation in tumors has been documented (Jiang *et al*. 2006; Shema *et al*. 2008). We therefore asked whether elevated *Lats2* promoter hypermethylation might underlie also the downregulation of *Lats2* transcription in this in vitro "tumor progression" model. To this end, we performed methylation-specific qPCR analysis on genomic DNA. As seen in figure 5D, selection for survival with chronic expression of H-RasV12 resulted in a pronounced increase of *Lats2*

promoter hypermethylation. This suggests that *Lats2* promoter hypermethylation enables the emergence of transformed cells harboring activated oncogenic H-Ras.

If the silencing of *Lats2* expression contributes to the establishment of the H-Ras-driven transformed phenotype, it is conceivable that coexpression of ectopic *Lats2* might attenuate this phenotype. One characteristic often associated with oncogenic H-Ras is acquisition of anchorage-independent growth, manifested experimentally as an ability to form colonies in soft agar. Importantly, overexpression of *Lats2* dramatically decreased the ability of H-RasV12 cells to form agar colonies (Fig 6). Notably, knockdown of p53 in conjunction with *Lats2* overexpression restored the ability of H-RasV12 expressing cells to form colonies (Fig. 6A, right panels), indicating that inhibition of anchorage-independent cell growth by Lats2 is p53-dependent.

In Fig. 6A, ectopic Lats2 was co-introduced together with H-RasV12 infection, before selection of H-RasV12 "escapers" took place. Since "escapers" presumably underwent strong selective pressure to subvert the Lats2 response, subsequent reintroduction of ectopic Lats2 might be expected to exert even more profound inhibitory effects on those cells. Indeed, whereas overexpression of ectopic *Lats2* led to only a modest increase in apoptosis (10.6% to 17.1%) in control (vector) cells (Fig. 6B, upper panels), it had a dramatic effect on the "escapers", massively inducing apoptosis (10.0% to 50.5%, lower panels), even though the extent of Lats2 overexpression was comparable in the vector and H-Ras "escapers" (Supplementary Fig S8). Moreover, Lats2 expression almost completely eliminated the polyploid subpopulation, suggesting that these cells were preferentially shunted to apoptosis. Thus, restoration of high Lats2 levels in the "escapers" can effectively reverse the outcome of the long-term selection process and curtail the cancer-promoting consequences of chronic H-RasV12 expression.

Discussion

H-Ras is among the most frequently mutated genes in cancer. Intrinsically, H-Ras activation delivers potent oncogenic signals and contributes richly to deregulation of cell proliferation and establishment of transformed features. Luckily, cellular transformation is a relatively rare event. This is thanks to the action of checkpoint mechanisms that eliminate potentially dangerous cells from the replicative pool by shunting them to cell cycle arrest, apoptosis or senescence. Hence, tumor cells must cope with heavy selective pressure to undergo adaptations that allow them to neutralize or bypass these barriers.

The observations described in the present report provide additional insights into the mechanisms that underlie the H-Ras-triggered protective checkpoint response as well as the escape of mutant H-Ras-expressing cells from this response en route to becoming fully transformed. Our findings are consistent with a model wherein activation of oncogenic H-RasV12 results in a stress that is sensed by the ATR-Chk1 checkpoint pathway. Activation of ATR and Chk1 leads to nuclear accumulation and stabilization of Lats2 protein. Lats2 subsequently mediates the p53 response to H-Ras oncogene activation, directing cells towards an antiproliferative transcriptional program as well as inducing p53-dependent transcription of the *Lats2* gene itself. Thus, the Lats2-p53 tumor suppressor positive

feedback pathway acts to combat the potentially harmful outcome of oncogenic activation. However, rare cells might escape this checkpoint block by inactivating Lats2, as well as other components of the pathway, such as p53. In the case of Lats2, such bypass can be achieved epigenetically by promoter hypermethylation and subsequent quenching of *Lats2* gene expression.

At which point in the history of the cells does the *Lats2* promoter become hypermethylated? A number of studies suggest that oncogenic Ras can induce the expression of several DNA methyltransferases (Pruitt *et al*. 2005; Chang *et al*. 2006), hence, it is possible that the silencing of *Lats2* occurs only after H-RasV12 introduction. Yet, given the short duration of the experiment shown in Fig. 5D and the fact that the WI-38/hTERT[fast] cells underwent extensive passaging *in vitro* (Milyavsky *et al*. 2003) prior to H-RasV12 infection, it appears more likely that the stress response elicited through activation of the ATR-Chk1-Lats2-p53 pathway selects for cells with preexisting *Lats2* promoter hypermethylation, rather than inducing multiple independent *de novo* methylation events. In fact, the extent of hypermethylation in the control WI-38/hTERT[fast] cells (Fig. 5D, vector), albeit low, is already significantly higher than in earlier passage WI-38 cells (data not shown). This scenario is reminiscent of observations made in human breast tissue, where preexistent silencing of the INK4A/ARF tumor suppressor locus by promoter hypermethylation in seemingly normal tissue might provide the setting for the emergence of tumor initiating cells upon subsequent oncogene activation (Bean *et al*. 2007). Thus, it is tempting to speculate that, likewise, cells with silenced *Lats2* may also be present sporadically in normal tissue, offering ready soil for oncogene-driven transformation and tumor development. Notably, loss of p16(INK4A) expression, which took place in the course of WI-38/hTERT[fast] cell propagation in culture (Milyavsky *et al*. 2003), may have accelerated DNA methylation (Reynolds *et al*. 2006), including that of the *Lats2* promoter, thus generating a larger pool of cells to select from in response to H-RasV12-induced stress. Furthermore, by creating centrosome dysfunction (McDermott *et al*. 2006), prior loss of p16 might facilitate Lats2 activation through chronic mitotic apparatus stress (Aylon *et al*. 2006). This may account for the occasional activation of Lats2 prior to H-RasV12 overexpression (Fig. 2B, upper left).

It still remains to be determined exactly how activation of the Ras-ATR-Chk1 axis leads to Lats2 induction. The data presented in this study implies that this induction is driven at multiple levels. On the one hand, there is an increase in the rate of *Lats2* gene transcription, which is dependent on p53 (Fig. 3D); this is reminiscent of the response to mitotic apparatus stress, where p53 is also required for induction of *Lats2* gene expression (Aylon *et al*. 2006). On the other hand, the Ras-ATR-Chk1 axis alters the stability and subcellular localization of the Lats2 protein. Activated H-Ras triggers the departure of Lats2 from centrosomes and its subsequent translocation into the nucleus; whereas the former is not dependent on ATR and Chk1, the latter is. Interestingly, the increased stability of Lats2 protein appears to be correlated with its nuclear translocation, since treatment with compounds such as caffeine and UCN-01, which prevent Lats2 nuclear translocation, also abolish the increase in steady state Lats2 levels. These events might be regulated, at least in part, by putative covalent modifications on the Lats2 protein. Such modification might differ between the different states of Lats2: centrosomal (non-stressed), non-centrosomal cytoplasmic (activated H-Ras

together with ATR-Chk1 pathway inhibition, or nuclear (activated H-Ras and functional ATR-Chk1 pathway). Perhaps relevant to this notion is the observation by Toji *et al*. (2004) that preventing the phosphorylation of Lats2 by Aurora A kinase releases Lats2 from the centrosome, but is not sufficient for nuclear accumulation. We propose that generation of mitotic stress, either by treatment with nocodazole (Aylon *et al*. 2006) or oncogene activation (this work) is necessary for Lats2 nuclear import.

Our data predict that inactivation of the Lats2 tumor suppressor might facilitate tumor progression. Indeed, this conjecture is further supported by the analysis of human tumors. Thus, in breast cancers, decreased *Lats2* expression is linked to biologically aggressive phenotypes, such as large tumor size, high lymph node metastasis and estrogen- and progresterone-receptor negativity (Takahashi *et al*. 2005). Similarly, in astrocytoma, hypermethylation of the *Lats2* promoter was detected in over 70% of the cases and associated with higher tumor grade and increased overall malignancy (Jiang *et al*. 2006). Moreover, treatment of astrocytoma cell lines with a DNA methyltransferase inhibitor restored *Lats2* levels and caused apoptosis, indicating that silencing of *Lats2* is an important step in tumor cell resistance to apoptosis (Jiang *et al*. 2006). Downregulation of *Lats2* via promoter hypermethylation is also associated with poor prognosis in acute lymphoblastic leukemia (Jimenez-Velasco *et al*. 2005). Furthermore, 50% of non-small cell lung carcinoma tumors exhibit decreased *Lats2* expression (Strazisar *et al*. 2008). Interestingly, diminished *Lats2* levels are highly correlated with lower expression of the p53 target gene *Mdm2* (Strazisar *et al*. 2008), supporting the notion that downregulation of Lats2 might negatively affect p53 transcriptional activity. Together, these studies are consistent with the view that evasion of the Lats2-p53 tumor suppressor pathway is an important contributor to tumor progression. Reconstitution of Lats2 expression, for instance by erasure of *Lats2* promoter hypermethylation, might thus qualify as a potential means to combat tumors in which Lats2 has been silenced but the p53 gene remains intact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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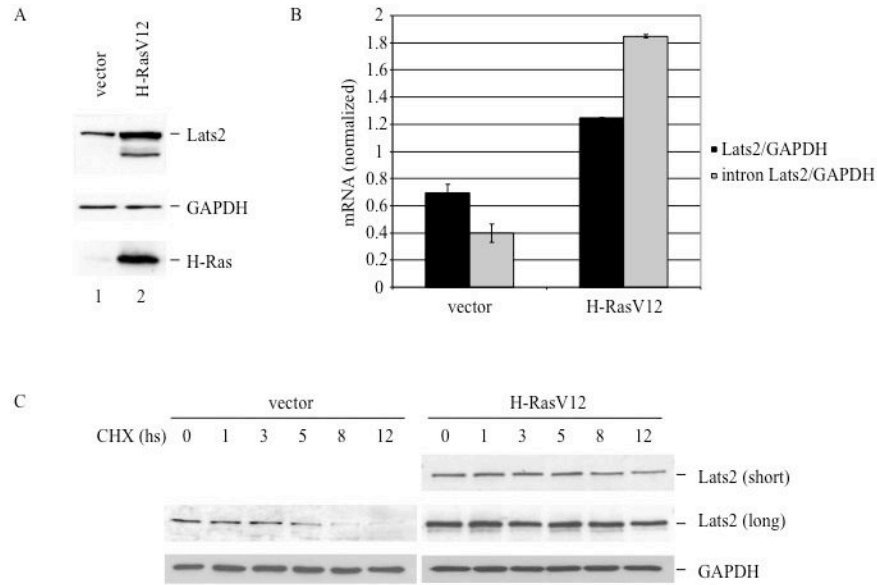
Figure 1. H-Ras overexpression causes an increase in endogenous Lats2
(A) WI-38 cells were infected with H-RasV12 or vector only. Lysates of hygromycin-resistant cells were analyzed four days after infection by Western blot to visualize Lats2 protein, GAPDH and H-Ras. (B) Cells were infected as in (A). RNA was prepared from each culture four days after infection and analyzed by qRT-PCR. Values were normalized to *GAPDH* mRNA. "Lats2" refers to the product of primers amplifying the exon3-exon4 junction whereas "intron Lats2" amplifies a region within intron 3 of Lats2. (C) Cells were infected as in (A), treated four days after infection with 80µg/ml cycloheximide (CHX) for the indicated time periods, and then harvested for Western blot analysis. For easier comparison, both a short and long exposure of the Lats2 blot are presented.
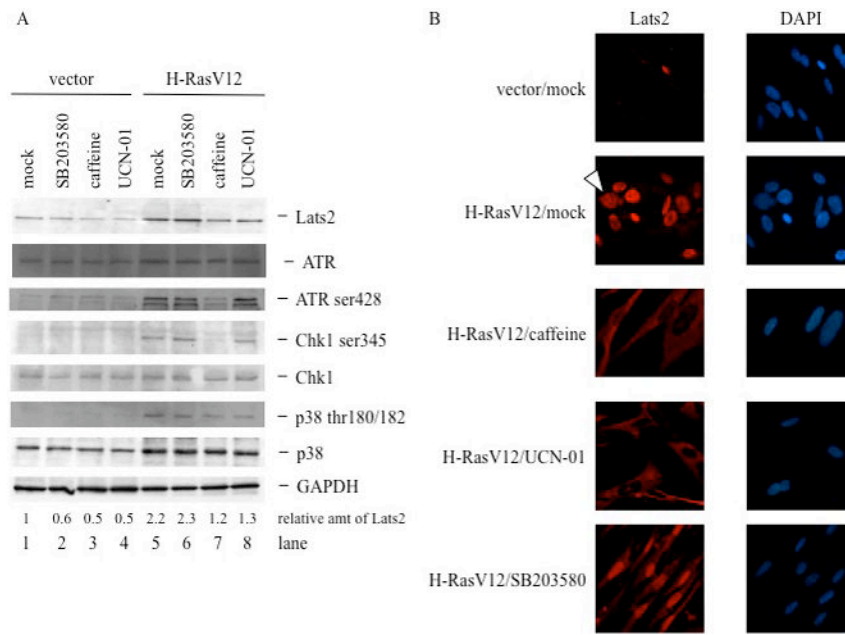
Figure 2. Chk1 and ATR are implicated in Lats2 accumulation in response to activated H-Ras
(A) Cells were infected as in Fig. 1A. Four days after infection, hygromycin-resistant cultures were treated overnight with either 10μM SB 203580, 0.5μM UCN-01, or 100μM caffeine, or left non-treated (mock). Cell lysates were subjected to Western blot analysis to visualize ATR, phospho-ATR, total Chk1, phospho-Chk1, p38 and phopho-p38. (B) Cells were processed and treated as in (A), fixed and immunostained to visualize Lats2. Nuclear DNA was visualized by DAPI staining. Arrowhead denotes cell with micronuclei. Protein signals were quantified using ImageJ software and normalized to the corresponding GAPDH signals.
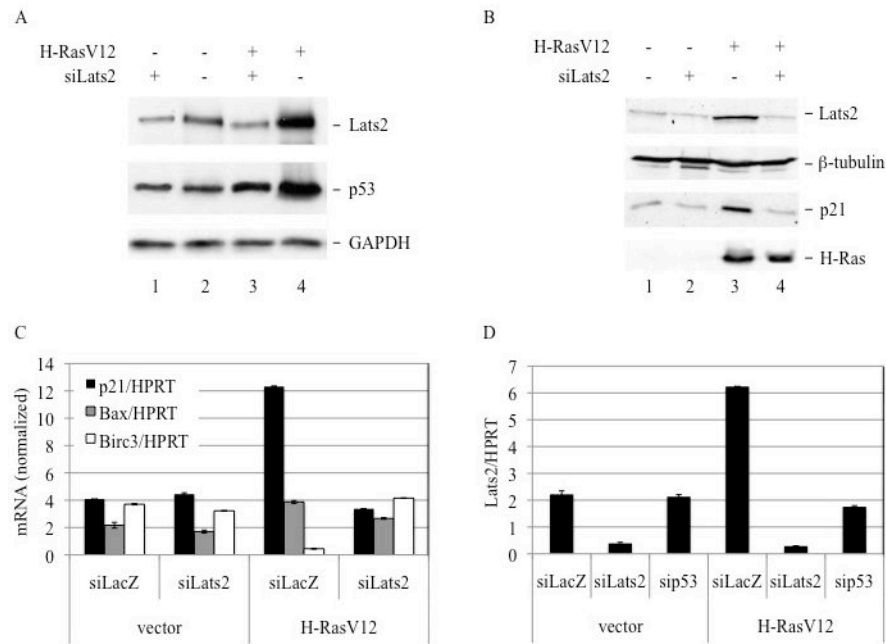
Figure 3. p53 response to H-RasV12 overexpression is dependent on Lats2

(A) WI-38 cells were infected as in Fig. 1A. Four days after infection, hygromycin-resistant cells were transiently transfected with Lats2 siRNA or control siRNA. Cell lysates were prepared 24 hours after transfection and analyzed by Western blot to visualize Lats2, p53 and GAPDH. (B) Cells were processed as in (A) and subjected to Western blot analysis with antibodies against Lats2, β-tubulin, p21 and H-Ras. (C) Cells were processed as in (A). RNA was prepared 24 hours after transfection and subjected to qRT-PCR analysis with primers specific for the indicated genes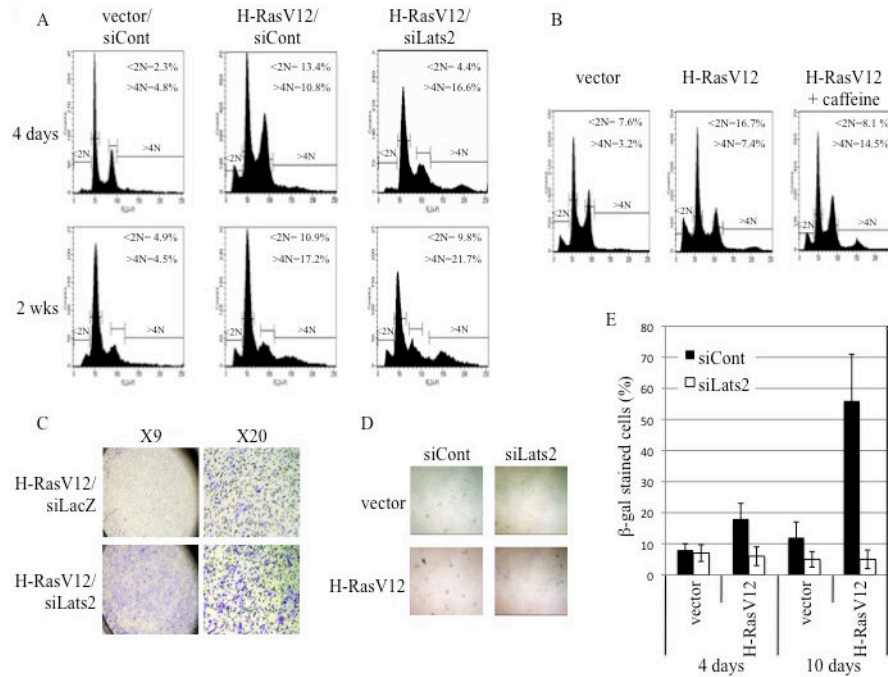. Values were normalized to *HPRT* mRNA. (D) Cells were processed as in (A), except that transfection was with either LacZ siRNA, Lats2 siRNA or p53 siRNA. RNA was prepared 24 hours after transfection and subjected to qRT-PCR analysis with primers specific for the indicated genes. Values were normalized to *HPRT* mRNA.

Figure 4. Lats2 affects the biological outcome of H-RasV12 overexpression
(A) WI-38 cells were infected with various combinations of recombinant retroviruses
encoding H-RasV12, Lats2 shRNA (siLats2) or mouse-specific PUMA shRNA (siCont) or
empty retrovirus (vector). Cells were fixed, stained with PI and taken for FACS analysis
either 4 days after infection and hygromycin and blasticidin selection or after an additional
two weeks (2 wks) of growth in culture with antibiotic selection. Positions and percentages
of sub-G1 cells (<2N) and polyploid cells (>4N) are indicated. Data is representative of at
least three independent experiments. (B) Cells were infected with empty retrovirus (vector)
or H-RasV12 and selected with hygromycin. Four days after infection, cultures were treated
for 24 hours with 100μM caffeine or left untreated. Cells were fixed, stained with PI and
subjected to FACS analysis. (C) Cells were infected and selected as in (B). Four days after
infection, cells were transiently transfected with Lats2 siRNA or lacZ siRNA. 24 hours after
transfection equal amounts of cells were plated in transwells and subjected in duplicate to
migration assays. Wells were photographed at two magnifications as indicated. Data is
representative of at least three independent experiments. (D) Cells were infected and
processed as in (A). Cultures were stained for β-gal activity after 10 days of combined
hygromycin and blasticidin selection. (E) Cells were infected and selected as in (D). Data
represents percentage of β-gal positive cells after 4 and 10 days of H-RasV12 expression,
based on counting of blue and white cells from three randomly chosen fields from each
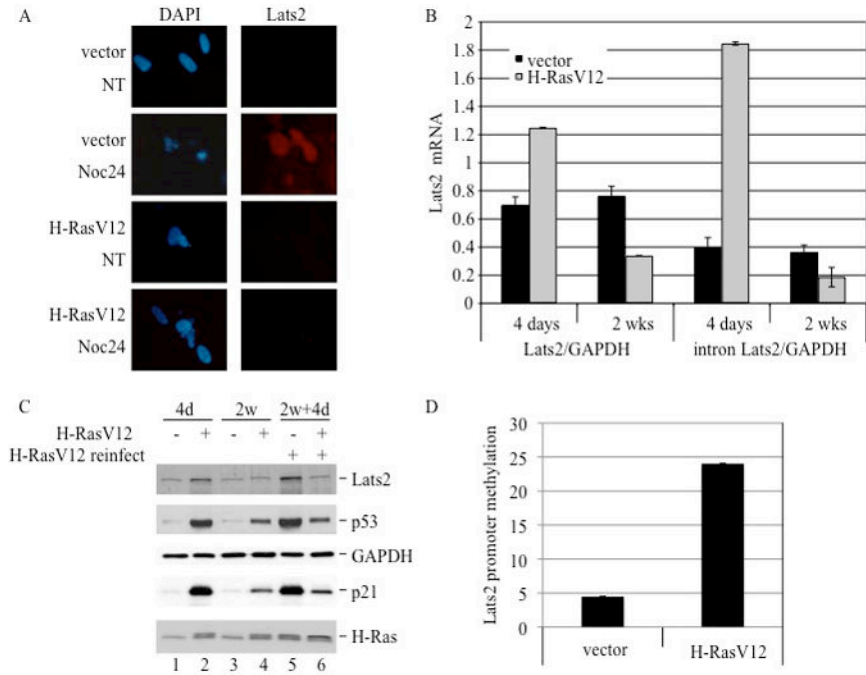condition. Error bars represent standard deviation.

Figure 5. Chronic expression of H-RasV12 selects for Lats2 silencing
(A) WI-38 cells were infected with either vector only or H-RasV12. After three weeks of selection, cells were treated overnight with 200ng/ml nocodazole (NOC) or DMSO only (NT). Fixed cells were immunostained to visualize Lats2. Nuclear DNA was visualized by DAPI staining. (B) Cells were infected as in (A). RNA was analyzed by qRT-PCR following 4 days or 2 weeks of selection, using exon-junction Lats2 primers (left) or intronic Lats2 primers (right). In both instances, values were normalized to *GAPDH* mRNA. (C) Cells were infected as in (A). After two weeks of growth under hygromycin selection, cells were infected with a recombinant retrovirus expressing H-RasV12 carrying puromycin resistance and grown for an additional 4 days (2w+4d). Cell lysates were subjected to Western blot analysis to visualize Lats2, p53, GAPDH, p21 and H-Ras. (D) Cells were infected as in (A). After two weeks of selection, high molecular weight DNA was extracted, treated with bisulfate and analyzed by qRT-PCR using methylation-specific primers. Values were normalized to non-methylated ALU sequence and a total-methylated SssI sample.
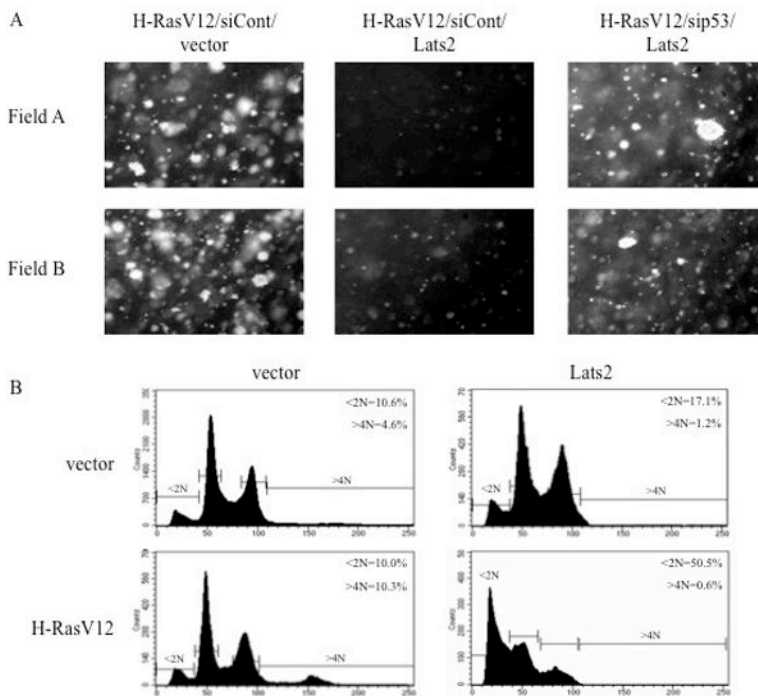
Figure 6. Overexpression of Lats2 prevents soft agar colony formation in a p53-dependent manner and triggers apoptosis

(A) WI-38 cells were infected with various combinations of recombinant retroviruses encoding H-RasV12, Myc-tagged Lats2 (Lats2), p53 shRNA (sip53), mouse-specific PUMA shRNA (siCont) or empty retrovirus (vector). Following selection for two weeks under hygromycin and blasticidin, equal amounts of cells were subjected in duplicate to a soft agar colony formation assay. Four weeks after plating, microcolonies were photographed. Two representative fields are shown for each condition. (B) WI-38 cells were infected with vector only or H-RasV12. Following three weeks of hygromycin selection, cells were infected with vector only or Myc-tagged Lats2 (Lats2) and subjected to blasticidin selection. Cells were harvested 4 days after the second infection, fixed, stained with PI and subjected to FACS analysis.