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Coordinated regulation of p31^{Comet} and Mad2 expression is required for cellular proliferation

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p31^{Comet} is a well-known interacting partner of the spindle assembly checkpoint (SAC) effector molecule Mad2. At the molecular level it is well established that p31^{Comet} promotes efficient mitotic exit, specifically the metaphase–anaphase transition, by antagonizing Mad2 function. However, there is little knowledge of how p31^{Comet} is regulated or the physiological importance of controlling p31^{Comet}. Here, we show that the Rb–E2F pathway regulates p31^{Comet} expression. In multiple tumor types (including breast and lung) p31^{Comet} expression is increased along with Mad2. Expression of this antagonist–target pair is coordinated in cells and correlated in cancer. Moreover, a narrow range of p31^{Comet}:Mad2 ratios is compatible with cellular viability. Our data suggest that coordinate regulation is important for the outgrowth of oncogenic cell populations. Our findings suggest that altered p31^{Comet}:Mad2 expression ratios may provide new insight into altered SAC function and the generation of chromosomal instability in tumors.

Introduction

The spindle assembly checkpoint (SAC) is an essential, evolutionarily conserved cellular surveillance mechanism, which ensures that chromosomes segregate with high fidelity during mitosis. In prometaphase (early mitosis), chromosomes lacking bipolar microtubule attachments activate the SAC. The Mad1–Mad2 heterodimer binds the kinetochores of unattached chromosomes and catalyzes the activation of free Mad2. Active Mad2, along with the BubR1-Bub3 heterodimer, bind and inhibit the ubiquitin ligase, APC^{Cdc20}, triggering mitotic arrest. Upon bipolar attachment of all chromosomes, the SAC is satisfied and APC^{Cdc20} is released to drive chromosome segregation and mitotic exit, allowing continued cellular proliferation.

Chromosomal instability is widespread in cancer. Deficient SAC function has long been suspected to underlie this hallmark trait in tumors. Indeed, loss or mutation of SAC components clearly drives chromosomal instability in both animal and cell culture models.[1-9] However, evidence for a cancer association of such defects in SAC function is lacking, likely due to the essential nature of the SAC.[10-15] In contrast, many SAC components, including the effector molecules Mad2 and BubR1 are overexpressed in tumors.[16,17] In particular, recent work in cell and animal models has demonstrated that hyperactivation of the SAC, caused by abnormally high levels of Mad2, drives chromosomal instability leading to tumorigenesis.[9,16-18] However, Mad2-mediated hyperactivation of the SAC also leads to increased mitotic duration, reduced proliferation, and decreased viability.[18] The mechanism that allows Mad2-overexpressing cells to escape the potentially tumor-suppressive characteristics of the hyperactive SAC remains to be determined.

The p31^{Comet} protein was identified as a binding partner of Mad2.[19] Overexpression of p31^{Comet} bypasses hyperactivation of the SAC caused by microtubule poisons.[20-23] Depletion of p31^{Comet} delays the release of APC^{Cdc20} from the Mad2–BubR1 complex after drug-induced activation of the SAC.[22-25] p31^{Comet} functions by antagonizing Mad2 and mutants that fail to bind Mad2 fail to silence SAC activity.[20] The exact mechanism that p31^{Comet} employs to silence SAC activity, e.g., preventing the generation of active Mad2 by the Mad1–Mad2 heterodimer, removing Mad2 from the inhibited Cdc20 complex, facilitating Mad2-mediated destruction of Cdc20, or all three, is still being delineated.[20,22,23,26-28] Regardless of the mechanism, while many analyses of p31^{Comet} function have been performed after treatment with microtubule poisons, which hyperactivate the SAC, we know relatively little about the biological importance of p31^{Comet} in the absence of drug. In addition, regulation of p31^{Comet} is essentially unknown at all levels. We therefore asked whether p31^{Comet} is abnormally expressed in human cancers and whether this might be important for the growth of cells with intrinsically high SAC activity due to Mad2 overexpression. Here, we have analyzed the expression of p31^{Comet} and examined the importance of coordinately increasing p31^{Comet} expression along with Mad2 for cellular survival.

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Results

p31^{Comet} expression is upregulated in cancer

p31^{Comet} is a well-known antagonist of the SAC effector Mad2, yet the biological importance of SAC silencing is largely uncharacterized.[19-28] We queried the Oncomine database to examine the expression of p31^{Comet} in human cancers (**Fig. 1A**). p31^{Comet} expression is upregulated in tumors originating from multiple tissues. Notably, p31^{Comet} expression is increased in tumors of breast and lung, where upregulation of Mad2 is known promote to tumorigenesis. To extend these analyses to changes in protein expression, we employed a highly characterized in vitro model of human mammary epithelial cell (HMEC) immortalization and transformation.[29-38] HMECs undergo 2 major events, stasis and crisis, en route to full immortalization and susceptibility to transformation. Overcoming these hurdles is tightly associated with the silencing or functional loss of the tumor suppressors p16^{Ink4A} and p53, respectively.[34,39,40] We mimicked successful navigation of stasis and crisis by sequential introduction of p16^{Ink4A}- and p53-targeting shRNAs (for full characterization of these cells, see ref. 29). p31^{Comet} protein levels were barely detectable in primary HMECs (**Fig. 1B** and data not shown). In contrast, loss of p16^{Ink4A} triggered a dramatic increase in p31^{Comet} protein that was unaffected by the introduction of the oncogenes c-Myc and Ras^{G12V}. Mad2 protein levels exhibited a similar trend of increased expression, whereas Cyclin A levels exhibited a modest increase at this stage (**Fig. 1B**). These data suggest that increased p31^{Comet} expression is not due to dramatic increase in proliferation, as Cyclin A is also required for proliferation. Thus, our data suggest that p31^{Comet} increases along with its target Mad2, perhaps to attenuate the deleterious effects associated with Mad2 overexpression (see below).

p31^{Comet} expression is regulated by Rb-E2F

p16^{Ink4A} is a Cdk inhibitor and its loss leads to aberrant phosphorylation of the Rb tumor suppressor and subsequent activation of E2F family transcription factors. Consistent with its increased expression in this cell population, Mad2 is a direct target of the Rb–E2F pathway.[9,17] We therefore investigated whether p31^{Comet} might also be regulated by this pathway. To determine if p31^{Comet} expression is directly regulated by E2F activity rather than indirectly via an E2F target, we sought to determine whether E2Fs act on the p31^{Comet} promoter. We probed a repository of ChIP–chip data in the ENCODE database at UCSC (http://genome-preview.ucsc.edu/ENCODE/) to determine whether E2Fs bind to the p31^{Comet} promoter. Indeed, all of the E2Fs tested in the ENCODE project, E2F1, E2F4, and E2F6, as well as exogenous HA-E2F1, bound to regions proximal to the transcription start sites of both p31^{Comet} variants 1 and 2 (**Fig. 2A** and not shown).

The ENCODE data supported E2Fs as drivers of p31^{Comet} expression. To test this possibility, we asked whether modulating E2F activity alters p31^{Comet} expression. We expressed HA-E2F1 in HCT116 cells, where E2F1 has been shown to modulate Mad2 expression.[17] p31^{Comet} expression was increased by E2F1 (**Fig. 2B**). As controls, we monitored the protein levels of additional E2F1 targets, Mad2 and Cyclin A, which increased as expected (**Fig. 2B**). Because exogenous E2F activity drives p31^{Comet} expression, we expected that cells with intrinsically higher endogenous E2F activity would also have higher p31^{Comet} expression. We found that Rb$^{-/-}$ 3T3 cells express higher levels of p31^{Comet} than wild-type 3T3 cells (**Fig. 2C**). Similar results were obtained in cells that have been deleted for the Rb family members p107 and p130 (**Fig. 2C**). These results are in good agreement with our initial observation that p31^{Comet} is increased upon depletion of p16 in HMECs, which affects the activity of all 3 Rb family proteins (**Fig. 1B**). In contrast, reduction of E2F activity by expression of tetracycline-inducible Rb in the Rb$^{-/-}$ cell line SAOS-2, results in a loss of p31^{Comet} as well as Cyclin A (**Fig. 2D**).[41] Together with the ENCODE data, our results support p31^{Comet} as an E2F target gene. Moreover, the ENCODE data represents E2F family binding to the p31^{Comet} promoter in cells derived from non-transformed breast, breast cancer, cervical cancer, B lymphocytes, and testicular cancer, indicating that p31^{Comet} is regulated by E2F in multiple tissues and tumor types. Indeed, correlation analyses of p31^{Comet} expression with that of E2F family members indicate that multiple E2Fs target p31^{Comet} (**Fig. 2E and F**). We analyzed the correlation between p31^{Comet} expression and E2F family members in expression data for invasive ductal breast cancer and lung adenocarcinoma obtained from the Oncomine database. In breast cancer samples, p31^{Comet} expression correlated most significantly with E2F5 (**Fig. 2E**). In contrast p31^{Comet} expression correlated most highly with E2F4 in lung cancer (**Fig. 2F**). Similar results were obtained with Mad2 (not shown). Notably the regulation of p31^{Comet} by multiple E2Fs is consistent with regulation by multiple Rb family members, which exhibit differential association with E2F proteins. These data indicate that regulation of p31^{Comet} (as well as Mad2) by E2Fs may be stochastic, and that allowing multiple family members to drive expression would ensure coordinate regulation of protein levels.

p31^{Comet} expression is cell cycle-regulated

Regulation by Rb-E2F suggests that p31^{Comet} expression may be cell cycle-regulated. We examined p31^{Comet} protein levels in HeLa cells that were synchronized at the G_1/S by a double-thymidine block and followed through to the next S phase. Levels of p31^{Comet} and Mad2 proteins did not oscillate dramatically during the cell cycle (**Fig. 3A**). In contrast, levels of the additional E2F targets and mitotic regulators Securin and Cyclin B dropped precipitously after mitosis and then re-accumulated as cells entered the next S phase (**Fig. 3B**). We reasoned that this discrepancy might reflect differences in the requirements to destroy these proteins in order to complete mitosis. Because rapid destruction of p31^{Comet} and Mad2 is not required, residual protein in cycling cells may mask the appearance of newly synthesized proteins as cells progress toward the next mitosis. Moreover, as Rb function is compromised in HeLa cells, the window of p31^{Comet} expression during the cell cycle may be less defined, contributing to the lack of oscillating protein levels. We therefore examined the expression of p31^{Comet} in Rb-proficient T98G cells that had been blocked in G_0 by serum starvation and stimulated to enter the cell cycle by addition of serum. Under these conditions we were able to observe an increase in p31^{Comet} expression as cells progressed through the cell cycle. The increase in expression occurred at

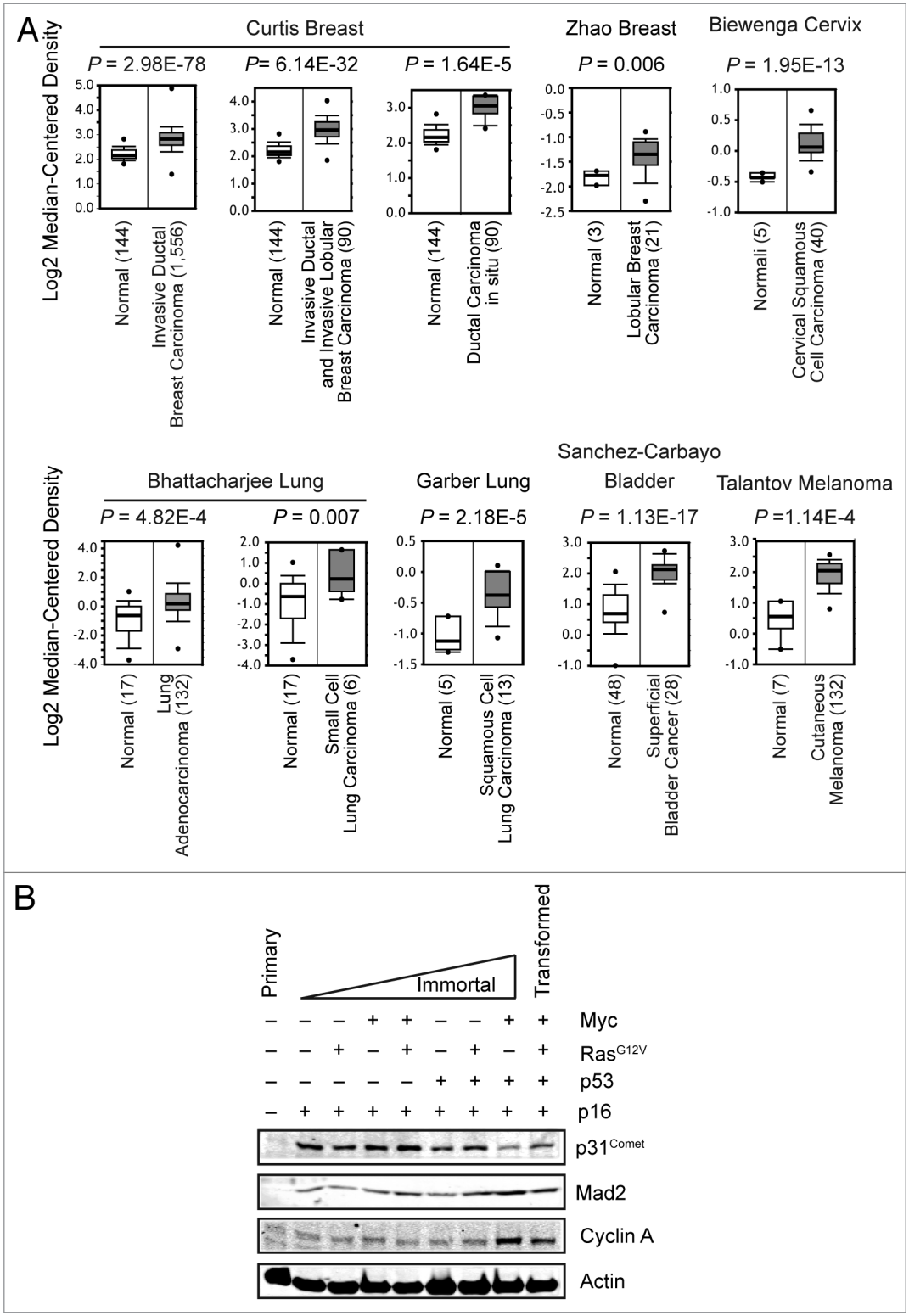

Figure 1. Expression levels of p31^{Comet} increase in cancer. (**A**) Comparison of p31^{Comet} mRNA expression differences between normal and tumor samples for multiple tissues. The mean expression for each sample group is represented. The number of samples in each group are indicated in parentheses. Data were obtained from the Oncomine database. (**B**) p31^{Comet} protein levels increase during the immortalization/transformation of HMECs. Cell lysates of primary HMECs and derivatives expressing the indicated shRNAs or oncogenes were probed for the indicated proteins by immunblot analysis.

both the mRNA and protein levels. Moreover, p31^{Comet} expression kinetics mirrored those of additional E2F targets, Mad2 and Cyclin A (**Fig. 3B and C**). Together these data support p31^{Comet} as a cell cycle-regulated E2F target gene.

Balanced expression of p31^{Comet} and Mad2 is required for cellular proliferation

The results of our studies are consistent with the described role of p31^{Comet} in promoting efficient mitotic exit.[22-25] However, our data also has additional implications for the biological importance of p31^{Comet}. Increased expression of Mad2 leads to hyperactivation of the SAC and diminished cellular viability and proliferation.[18] Similarly, overexpression of p31^{Comet} also results in decreased proliferative capacity.[9,18] We reasoned that, in addition to being part of a cell cycle-regulated transcriptional module, coordinate regulation of both p31^{Comet} and Mad2 by the Rb–E2F pathway ensures that the potential deleterious effects of each these proteins when overexpressed are buffered by the expression of the other. To test this hypothesis, we first determined that induction of p31^{Comet} or ectopic expression of Mad2 in tetracycline-inducible p31^{Comet} HeLa cells dramatically reduced proliferation (**Fig. 4A**). As expected, simultaneous induction of p31^{Comet} with ectopic expression of Mad2 resulted in partial restoration of cellular proliferation. Examination of the total p31^{Comet} and Mad2 levels in these cells revealed that the relative p31^{Comet}:Mad2 ratio remained altered in our coexpressing population (**Fig. 4B and C**). The partial rescue we observed in these cells is likely due to the imbalanced ratio. Indeed, the level of p31^{Comet} in this population is unable to rescue the increased mitotic index caused by Mad2 overexpression (**Fig. 4D**). To further examine the importance of the p31^{Comet}:Mad2 ratio, we co-transfected p31^{Comet} along with increasing amounts of Mad2 plasmid (**Fig. 4E and F**). Increasing Mad2 levels first rescued

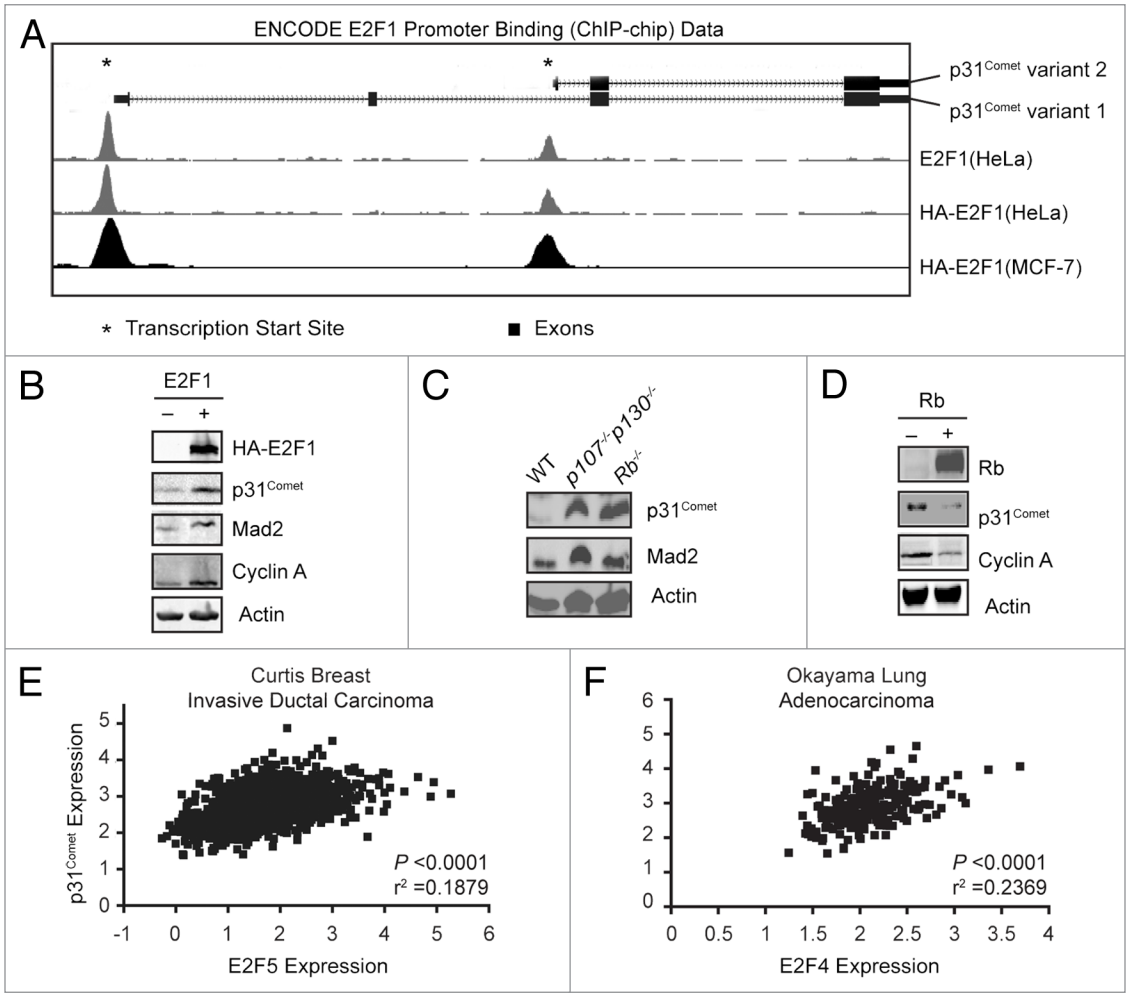

Figure 2. p31^{Comet} expression is controlled by the Rb-E2F pathway. (**A**) Schematic representation of the p31^{Comet} genomic structure, indicating the position of exons and the transcription start sites for variants 1 and 2. The positions of E2F1 binding peaks for both variants as defined by ChIP–chip data form the ENCODE project are depicted for HeLa and MCF-7 cells. Similar peaks were identified for E2F4 and E2F6. (**B**) E2F1 drives p31^{Comet} protein expression. HCT116 cells were transfected with control or HA-E2F1 constructs and harvested 24 h later for immunoblot analysis. (**C**) Loss of Rb family proteins results in increased p31^{Comet} expression. Lysates of 3T3 cells derived from MEFs with the indicated genotypes were probed for protein expression. (**D**) Rb represses the expression of p31^{Comet}. SAOS2 cells with tetracycline-inducible Rb construct were treated ± doxycycline to induce Rb expression for 24 h. Whole-cell lysates were probed with the indicated antibodies. (**E and F**) Expression data for the p31^{Comet} and the indicated E2F family member were obtained and subject to correlation analysis.

the proliferative defect resulting from exogenous p31^{Comet} expression. However, further increases in Mad2 levels were detrimental, despite the presence of exogenous p31^{Comet}. These results indicate that there is a specific range of p31^{Comet}:Mad2 ratios that are compatible with cellular proliferation.

p31^{Comet} and Mad2 are coordinately upregulated in cancer

The results of our in vitro studies support a model in which p31^{Comet} expression must increase (or potentially decrease) upon deregulation of Mad2 expression during the transformation process. To test this model, we downloaded expression data for both p31^{Comet} and Mad2 from the Oncomine database and performed correlation analyses. In data sets from breast cancer, lung cancer, and, directly in line with our analyses in HeLa cells, cervical cancer expression of p31^{Comet} is significantly correlated with Mad2 expression (**Fig. 4G**). These data are consistent with the need for coordinate regulation. Recently it was reported that the p31^{Comet}:Mad2 ratio varies among cell lines. Given that we observed increased expression of both proteins in the early steps of immortalization, we asked whether the distribution of the p31^{Comet}:Mad2 ratio may be skewed in cancer. Indeed, we found that the median p31^{Comet}:Mad2 ratio is substantially altered in tumor cells (**Fig. 4H**).

Discussion

Mad2 is a key effector molecule of the SAC and is required for inhibition of mitotic exit upon checkpoint activation. The role of p31^{Comet} in promoting efficient mitotic exit by antagonizing Mad2 is well known.[19-25,27,28] However, little is known about the regulation of p31^{Comet} or the physiological importance of p31^{Comet} function in the absence of microtubule poisons. Herein, we have provided novel insight into the regulation of p31^{Comet} expression by the Rb-E2F pathway. We have shown that multiple means of altering the function of this pathway in a panel of different cell types results in modulation of p31^{Comet} expression. We have also provided evidence that antagonism of Mad2 by p31^{Comet} is required for the long-term proliferation of cells. In line with these observations, expression of p31^{Comet} and Mad2 is coordinated in the cell cycle and during the immortalization of human mammary epithelial cells. Moreover, we found that increased expression of p31^{Comet} in tumors correlates with increased Mad2 levels. Together these data provide new insight into the regulation of SAC activity by p31^{Comet} and suggest that SAC silencing may play a role in tumorigenesis.

Deficient function of the Rb family of tumor suppressors is a frequent occurrence in cancer, including breast cancer. Silencing of p16^{Ink4A} is a key step in circumventing the first barrier toward immortalization of HMECs, termed stasis.[34,39,40] The induction of p31^{Comet} in post-stasis HMECs expressing shRNA targeting p16^{Ink4A} (shp16), which mimic selection, suggests that it may be an early event in cellular immortalization/transformation as well (**Fig. 1B**). Aberrant activation of E2F (e.g., by loss of p16^{Ink4A}) leads to induction of the SAC effector Mad2.[16,17] Indeed, we find that Mad2 is overexpressed in shp16 HMECs (**Fig. 1B**). Importantly, this change does not reflect altered proliferation, as no dramatic change in Cyclin A levels is observed (**Fig. 1B**). Furthermore, Mad2 expression does not correlate with proliferation.[9,17] Mad2 is overexpressed in breast cancer, and E2F driven upregulation of Mad2 is a major driving force in the generation of aneuploidy upon loss of the Rb pathway and is required for mammary adenocarcinoma development in *WAP-T121* (functionally deficient Rb family) mice.[16] However, overexpression of Mad2 results in hyperactivation of the SAC, which leads to aneuploidy, reduced proliferation, and decreased viability.[18,42] Nonetheless, elevated Mad2 levels are found in a number of tumor types (including breast, lung, cervix; **Fig. 4G**) and have been shown to promote chromosomal instability and tumor formation in pRb$^{-/-}$/p107$^{-/-}$/p130$^{-/-}$ (TKO) MEFs, p53 mutant mice, and mouse models of breast and lung cancer.[9,16-18]

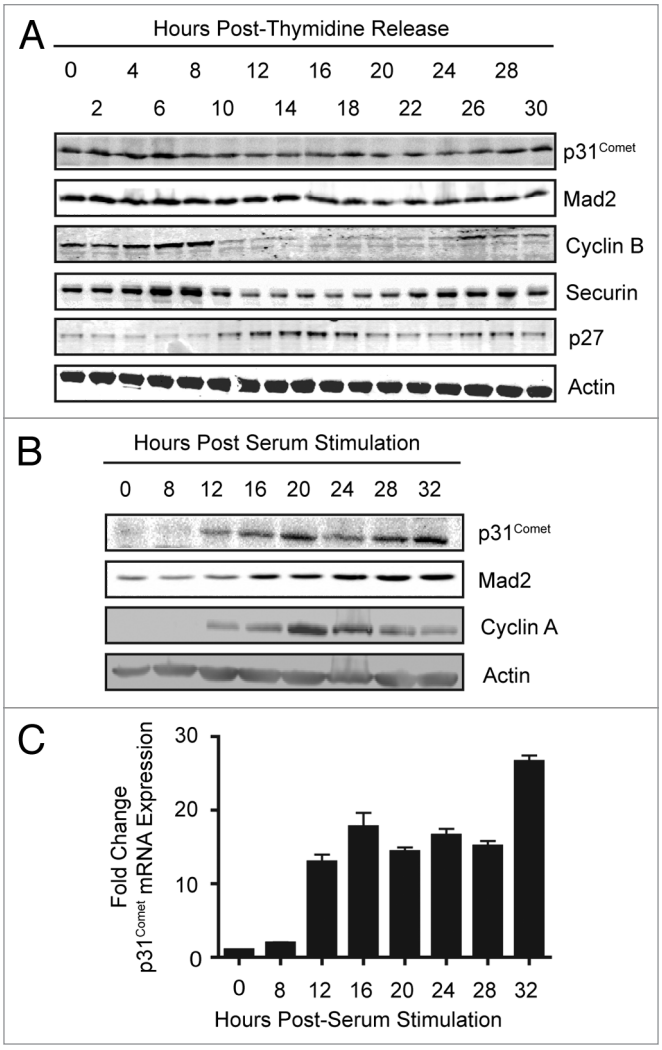

Figure 3. p31^{Comet} expression is cell cycle regulated. (**A**) HeLa cells were synchronized at G_1/S by a double thymidine block. After release into fresh media (0 h) cells were harvested at the indicated times. Whole-cell lysates were probed with antibodies against the proteins specified. (**B and C**) T98G cells were synchronized in G_0 by incubation in serum-free media for 72 h. Cells were stimulated to reenter the cell cycle by addition of media containing serum, serum stimulation. Whole-cell lysates were probed with antibodies against the specified proteins (**B**), and mRNA collected in parallel samples was analyzed by qPCR (**C**).

The results above indicate that during selection/immortalization, cells must also compensate for the effects of increased Mad2 levels. Consistent with this idea, normalizing the levels of constitutively overexpressed Mad2 in TKO MEFs to that of wild-type MEFs results in a weakened SAC.[16] These data suggest that these cells have an upregulated SAC silencing activity. Indeed, in pRb$^{-/-}$ or p107$^{-/-}$/p130$^{-/-}$ cells we find that increased p31^{Comet} levels accompany high levels of Mad2 expression (**Fig. 2D**).

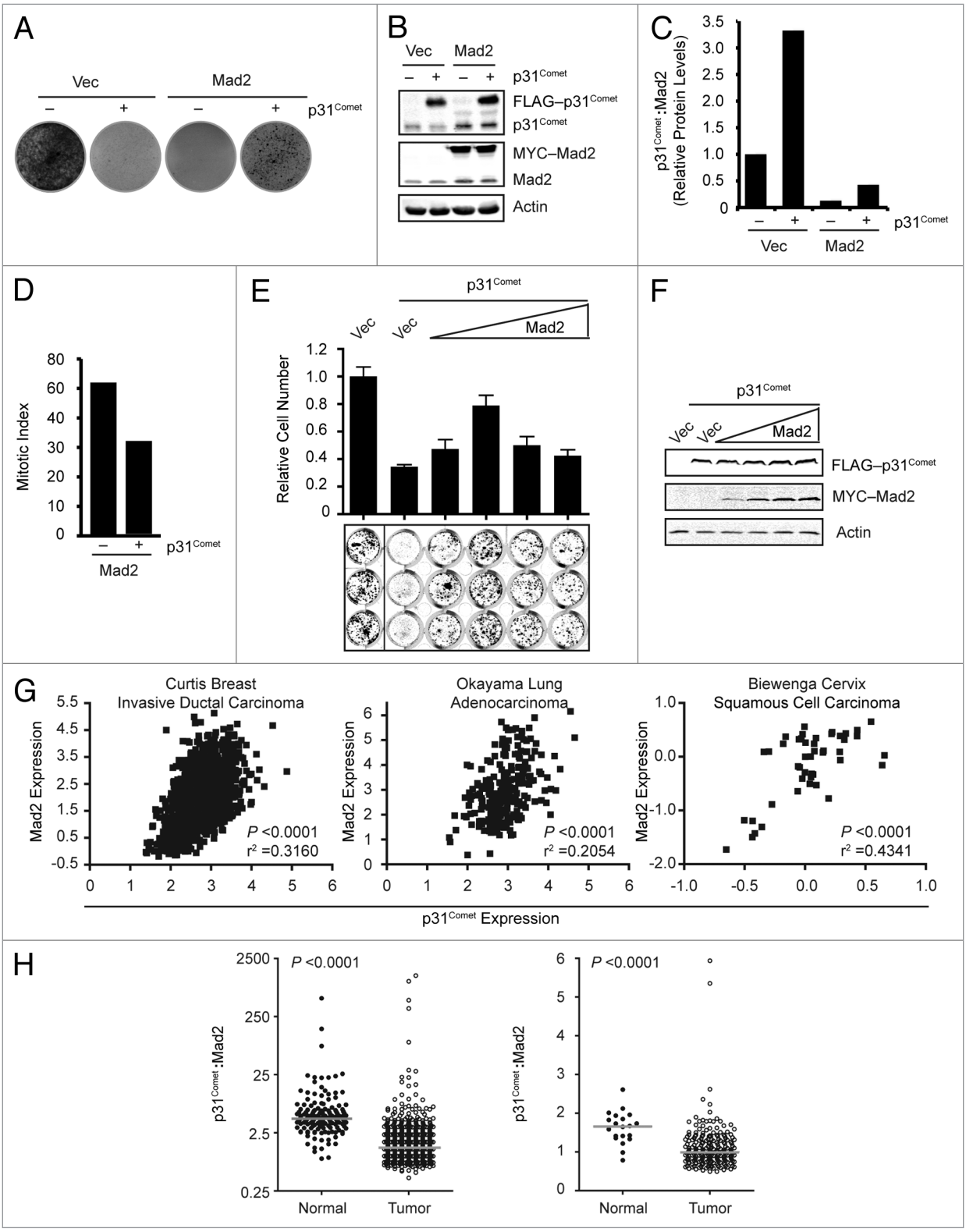

Figure 4. For figure legend, see page 3830.

Figure 4 (See previous page). Coordinated expression of p31^{Comet} and Mad2 is required for cell viability. (**A**) HeLa cells harboring a tetracycline-inducible p31^{Comet} construct were transfected with control or Mad2 containing constructs along with a plasmid encoding puromycin resistance ± doxycycline, as indicated. After selection with puromycin, cells were allowed to grow for 10 d and stained with crystal violet. (**B**) Representative western blot of cells treated as in (**A**). (**C**) Quantitation of the ratio of total p31^{Comet}:Mad2 ratios from (**B**). (**D**) Mitotic index of cells treated as in (**A**). Cells were analyzed visually for the presence of mitotic chromatin. (**E**) HeLa cells were transfected with vector or constructs encoding p31^{Comet} and Mad2 as indicated, along with a puromycin resistance plasmid. Cells were selected as in (**A**). After 10 d the cells were stained with anti-β-actin antibodies and analyzed using the LI-COR Odyssey scanner. The mean relative cell number and standard deviation from 2 experiments performed in triplicate are shown. (**F**) Representative immunoblots of cells treated in (**E**). (**G**) Expression data for p31^{Comet} and Mad2 was obtained for the indicated data sets and subject to correlation analysis. (**H**) The ratio of p31^{Comet}:Mad2 for normal and cancer subsets of the Curtis Breast and Okayama Lung datasets in (G) were calculated and compared. Gray bars indicate the median for each group.

Notably, increased p31^{Comet} expression, along with Mad2, is also found in precancerous lesions in humans; ductal carcinoma in situ (DCIS), a precursor to invasive ductal breast cancer; non-neoplastic nevi, precursor melanoma; and prostatic intraepithelial neoplasia (**Fig. 1A** and not shown). While increased expression of the Mad2 target Cdc20 in tumors could also counter elevated Mad2 in tumors, several observations argue against Cdc20 as a key buffer of Mad2. First, concomitant increase in BubR1 levels will inhibit Cdc20.[9] Second, recent studies have shown that Mad2 activation triggers the destruction of Cdc20, which is potentiated by p31^{Comet}.[25,43-45] These data, in conjunction with our observation that increased p31^{Comet} levels accompany elevated Mad2 levels in both HMECs and human tumors, indicate that induction of p31^{Comet} buffers increased Mad2 activity to allow for outgrowth of the abnormal population. Our analysis of p31^{Comet}:Mad2 ratio effects on the long-term cellular proliferation supports this indication as well (**Fig. 4A and E**). A range of p31^{Comet}:Mad2 is compatible with viability, and deviations from this range in either direction result in a significant loss of proliferative capacity (**Fig. 4E and F**). A recent study found that the p31^{Comet}:Mad2 ratio varies among cell lines and correlates with the degree of mitotic slippage during prolonged exposure to microtubule poisons.[46] We have found that the distribution of the p31^{Comet}:Mad2 ratio is significantly altered in cancer (**Fig. 4H**). Whether these altered ratios are associated with mitotic slippage in the absence of spindle poisons and may contribute to chromosomal instability will require future study.

The existence of the SAC recovery mechanism, mediated by p31^{Comet}, is a relatively recent discovery, and its potential involvement in cancer is essentially unknown. Our data indicate that increased p31^{Comet} expression contributes to cancer by promoting the survival of Mad2-overexpressing cells. Furthermore our findings suggest that altered p31^{Comet} expression and regulation may contribute to cancer by promoting chromosomal instability. Moreover, the potential for alterations in post-transcriptional/translational regulation of p31^{Comet} and Mad2 also exists and may further contribute to aberrant SAC function in cancer. Along this line, p31^{Comet} undergoes multiple post-translational modifications, including phosphorylation, which regulates the p31^{Comet}-Mad2 interaction (DAD, ACB, and MKS, manuscript in preparation). Mad2 is also regulated by phosphorylation. For example, phosphorylation of Mad2 by Chk1 (also an E2F target gene) stabilizes Mad2, resulting in increased protein levels.[47] Deregulation of these mechanisms may further skew the balance of p31^{Comet} and Mad2 functions, effectively altering the p31^{Comet}:Mad2 ratio and further driving tumor development. Future studies examining these aspects of p31^{Comet} and Mad2 regulation may help to reconcile in vivo function of the SAC with in silico modeling of SAC function and ultimately provide new insight into how defective SAC function drives chromosomal instability and tumor progression.[48-51]

Experimental Procedures

Cell lines and drug treatments

HeLa, HCT116 and T98G (obtained from ATCC) as well as 3T3 cells (provided by Dr William Taylor), were maintained in Dulbecco modified Eagle media (DMEM) supplemented with 10% FBS and penicillin/streptomycin in a humidified atmosphere of 10% CO_2 and a temperature of 37 °C. Tetracycline-inducible SAOS2-Rb cells were obtained from Dr Michelle Longworth and maintained in DMEM supplemented with 15% FBS minus tetracycline.[41] HMECs were cultured as described.[29] Chemicals were obtained from Sigma Aldrich unless otherwise mentioned. Doxycycline was dissolved in water and used at a concentration of 0.5 μg/μl. Puromycin was dissolved in water and used at a concentration 0.5 μg/μl.

Transfections and plasmids

Transient transfections were performed using Transit-LT1 (Mirus). The transfection reagent and DNA were used at a ratio of 3:1 and suspended in antibiotic and serum-free DMEM to form DNA and polymer complexes. The mixture was incubated at room temperature for 20 min before being added to cells. The cells were incubated at 37 °C for 24–48 h prior to harvesting. HA-tagged full-length E2F was described.[52] shRNAs and constructs used in the HMEC studies were described.[29] The cells were incubated at 37 °C for 24 to 48 h prior to harvesting or drug selection. pENTR223 p31^{Comet} variant 2 was obtained from Open Biosystems and subcloned into pGLAP2 using Gateway technology (Invitrogen).[53]

Cell synchronization, serum starvation, and colony formation

HeLa cells at 25–30% confluency were treated with 2 mM thymidine and incubated at 37 °C for 18 h. Cells were released from the block into fresh media and incubated for 9 h prior to second block in 2 mM thymidine for 18 h. Cells were released from the second block into fresh media and harvested as they progressed synchronously through G_2–M phase. T98G cells at 30–40% confluency were starved in media without serum and incubated at 37 °C for 72 h. Cells were released into media with 20% serum for stimulation and incubated at 37 °C prior to being harvested at required time points. HeLa cells were transfected as

above. Twenty-four hours after transfection, cells were split 1:3 or 1:6 and allowed to grow for an additional 24 h. Transfected cells were selected with 0.5 μg/ml puromycin for 48 h. Cells were released into fresh media and allowed to proliferate for 10 d.

Western blotting and antibodies

Cell extracts were generated in RIPA buffer composed of 10 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 1 mM DTT, protease inhibitors (Leupeptin, Pepstatin, and Chymotrypsin, each at 10 μg/ml) and phosphatase inhibitors (25 mM β-glycerophosphate, 5 mM NaF, 1 mM $NaVO_4$) or EBC buffer 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 1 mM DTT, 25 mM β-glycerophosphate, 5 mM NaF, 1 mM $NaVO_4$, and Leupeptin, Pepstatin, and Chymotrypsin, each at 10 μg/ml. Cell lysates were resolved by SDS-PAGE. The proteins were transferred to polyvinyldenefluoride (PVDF) membranes obtained from Millipore. Western blot blocking and primary antibody hybridization were performed in 5% (w/v) non-fat dry milk in phosphate-buffered saline and washed with Tris-buffered saline + 0.1% Tween-20 (TBST). Secondary antibodies (LI-COR) were incubated in TBST+ 0.02% SDS and visualized with the LI-COR Odyssey near infrared imaging system.

Commercial antibodies from Sigma were utilized for the following proteins $p31^{comet}$ (4G11), Mad1 (9B10), FLAG epitope (M2), and β-Actin (AC-15,). Antibodies to Cyclin B (GNS1), Cyclin A (H-432), RB (IF8), $p31^{Comet}$ (4RE23) were obtained from Santa Cruz Biotech. Polyclonal antibodies from Bethyl laboratories were used for Mad2 and Mad1. Antibodies against p27 and Securin were obtained from Invitrogen. Monoclonal antibody from Covance (HA.11) was used for HA tagged proteins. The anti-MYC antibody 9E10 was produced in house at the Lerner Research Institute.

Statistical methods

Correlation analyses, Mann–Whitney tests were performed using GraphPad Prism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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