THE RASSF1A TUMOR SUPPRESSOR REGULATES A CASCADE OF ONCOGENIC SIGNALS THAT ARE RESTRAINED BY G1 CHECKPOINT MECHANISMS

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The RASSF1A tumor suppressor is one of the most commonly inactivated genes in cancer. To understand why epigenetic silencing of RASSF1A promotes tumorigenesis, I employed a loss of function approach to elucidate the role of RASSF1A in cancer. RASSF1A is reported to regulate apoptosis, cell cycle progression, and microtubule dynamics. Disruption of these processes by RASSF1A loss may disrupt cellular integrity and promote oncogenesis. I found that RASSF1A depletion elevated oncogenic signaling pathways; however, RASSF1A depletion also induced cell cycle arrest. RASSF1A is a critical regulator in maintaining the balance between pro-growth and anti-growth signals. RASSF1A suppresses proliferative signaling pathways such as the MAPK pathway, promotes apoptosis through MST2, but paradoxically, promotes G1/S progression through modulation of the ubiquitin ligase $SCF^{\beta TrCP}$. Thus, RASSF1A represents a critical line of defense against tumorigenesis as its loss triggers cell arrest; however, loss of RASSF1A also promotes proliferative signaling events, and additional malfunctions in cell cycle regulation will likely drive tumorigenesis.

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LIST OF ABBREVIATIONS

APC/C	Anaphase promoting complex/cyclosome
BTrCP	Beta-transducin repeat containing protein
Cdc20	Cell division cycle 20
Cdh1	Cdc20 homolog 1
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
Emi1	Early mitotic inhibitor-1
ERK	Extracellular signal-related kinase
EWS	Ewing's sarcoma protein
LATS1	Large antigen tumor suppressor 1
MAPK	Mitogen-activated protein kinase
MST2	Mammalian STE20-like kinase 2
OIS	Oncogene induced senescence
PICS	PTEN induced cellular senescence
RASSF1A	Ras association domain family 1, isoform A
RASSF1C	Ras association domain family 1, isoform C
Rb	Retinoblastoma protein
REST	RE1-silencing transcription factor
SCF	Skp1-Cullin-Fbox
SKP2	S-phase associated kinase protein 2
VHL	Von Hippel-Lindau

CHAPTER ONE:

RASSF1A regulates diverse pathways for cellular homeostasis. Inactivation of RASSF1A compromises cellular integrity, which can promote oncogenesis.

RASSF1A is a tumor suppressor that is lost in many types of cancers. Despite the prevalence of RASSF1A silencing in cancers, the mechanism by which RASSF1A functions as a tumor suppressor is not well understood. Many papers have been published over the past decade describing RASSF1A functions, but several of these studies are controversial. The discrepancy between results from different labs may be due to artifacts of overexpression assays. To investigate RASSF1A function under physiological context, we focused our studies on knocking down RASSF1A using RNAi. By utilizing multiple RASSF1A oligos, we can account for off-target effects and hope to gain a better understanding of the role of RASSF1A during the development of cancer.

RASSF1A is a tumor suppressor

Epigenetic silencing has emerged as a key factor in cancer development. RASSF1A (Ras association [RalGDS/AF-6] domain family 1) is one of the most altered genes found in cancer and the majority of RASSF1A silencing is due to hypermethylation of CpG islands within its promoter (Figure 1). Although RASSF1A inactivation is due mainly to epigenetic mechanisms, RASSF1A mutations were found at a rate of 15% in human nasopharyngeal carcinoma [1] and deletions in numerous cancer types have also been reported [2]. Thus, both RASSF1A alleles can be inactivated by epigenetic and genetic mechanisms. The first evidence that

RASSF1A was a tumor suppressor was uncovered in breast and lung cancer lines that frequently harbored a 120kb deletion in the chromosomal region 3p21.3 [3]. The RASSF1 gene was identified within this region and eight RASSF1 isoforms have been detected (RASSF1A-H). Only the major transcripts, RASSF1A and RASSF1C, have been studied in much detail, and in contrast to RASSF1A, the RASSF1C isoform is expressed in normal and cancer cell lines [3].

To test whether RASSF1A could act as a tumor suppressor, many studies have been focused on re-introducing RASSF1A into cancer cell lines that no longer express RASSF1A. It was reported that overexpression of RASSF1A could reduce oncogenic properties, such as anchorage independent proliferation, colony formation, proliferation, migration, invasion [4] and tumor volume *in vivo* [3]. RASSF1A -/- mice are viable and grow normally, but exhibit an increase in spontaneous and carcinogen-induced tumor susceptibility [5]. Around 18-20 months, RASSF1A -/- mice developed a noticeable increase in spontaneous tumors (31.7%) compared to RASSF1A +/- (17.1%) and RASSF1A +/+ (4.2%). Tumors from RASSF1A -/- mice include lymphoma, leukemia, lung adenoma, breast adenocarcinoma, lung papillary tumor, and rectal papilloma [5]. RASSF1A deficient mice also developed skin and lung tumors at a significantly higher rate than wild-type mice upon exposure to carcinogens [5].

P53 is also known to be a potent tumor suppressor, and Tommasi et al. found that RASSF1A -/- p53 -/- double knockout mice had enhanced tumor formation, tetraploidy, cytokinesis defects, and chromosomal abnormalities compared to RASSF1A -/- p53 +/- mice [6]. Their work may suggest that RASSF1A inactivation is

an early event in oncogenesis, and this hypothesis is corroborated by previous studies which described RASSF1A loss in neoplastic tissue of renal cell carcinoma [7], breast [8], melanoma [9], and testicular germ cell tumors [10]. Clinical evidence from a patient study in NSCLC (non-small cell lung cancer) predicted that RASSF1A methylation is an indicator of poor survival when compared to tumors with unmethylated RASSF1A [3]. In this study, resected lung tumors were taken from patients and the methylation status of RASSF1A was determined. The mean overall survival rate of patients whose tumors were unmethylated was 52 months compared to 37 months for patients with tumors with methylated RASSF1A. Interestingly, treatment of 5'azacitidine, which blocks DNA methyltransferases, therefore preventing methylation of the RASSF1A promoter, can improve progression-free and overall survival of NSCLC patients [94]. Collectively, these findings suggest that RASSF1A is an important tumor suppressor.

RASSF1A structure and regulation

As shown in Figure 2, RASSF1A is a 340 amino acid protein that contains a C1/DAG (diacylglycerol) domain that is similar to the C1 domain in PKC (protein kinase C) for membrane binding; an ATM (ataxia telangiectasia, mutated) domain that is phosphorylated by ATM upon DNA damage; a RA (Ras association) domain; and a SARAH (Sav/RASSF/Hpo) protein-protein interaction domain, which binds to MST1/2 (Mammalian STE20-like kinase). For many years, it was controversial whether Ras could bind RASSF1A directly. Some labs have failed to see an interaction [11] while others could show an interaction i*n vitro* or by overexpression

[12], [13]. Recently however, mutant K-Ras was discovered to bind RASSF1A by endogenous co-immunoprecipitation [14].

RASSF1A does not possess catalytic activity but is thought to be a scaffolding protein, which brings together different complexes. As shown in Figure 2, RASSF1A interacts with many different proteins related to the cell cycle, which will be discussed in more detail. In addition, several kinases phosphorylate RASSF1A, and these post-translational modifications are crucial for its function [15, 16]. RASSF1A and RASSF1C share ATM, RA, and SARAH domains, but have different promoters so RASSF1C lacks the C1/DAG domain, which may account for observed differences in function. In the RASSF1A promoter, a p53 binding site has been identified and p53 has been shown to negatively regulate RASSF1A expression [17].

Two E3 ubiquitin ligases have been reported to promote RASSF1A degradation during the cell cycle. Song et al. suggest that during the G1/S transition, CDK4-Cyclin D phosphorylates RASSF1A, which triggers RASSF1A for ubiquitination by SKP2 and degradation by the proteasome to promote proliferation [18]. Also, the APC/Cdc20 complex may ubiquitinate RASSF1A, which in turn, allows for increased APC/C degradation of Cyclin A and mitotic exit [19]. However, the data from this paper is not entirely convincing due to what appears to be modest differences. Methylation of the RASSF1A promoter is thought to be partly mediated by the DNA methyltransferase, DNMT3B [20]. The homeobox protein, HOX3B, increases DNMT3B expression, which leads to hypermethylation of the RASSF1A promoter. DNMT3B recruitment to RASSF1A promoter is facilitated by MYC and the Polycomb

receptor complex 2. Thus, RASSF1A methylation by HOX3B may contribute to the oncogenic activity of HOX3B.

The Cell Cycle

For proliferation, cells must undergo replication and division through a process known as the cell cycle (Figure 3). The cell cycle can be divided into two major phases: interphase and mitosis. Interphase is a period of growth and DNA replication while mitosis is the process of cell division into two daughter cells. Interphase can be further subdivided into 3 distinct phases: G1, S, and G2. Cells in G1 (GAP1) increase in size and prepare for the next phase. During S phase, DNA replication occurs and chromosomes double to prepare for division. G2 (GAP2) is an additional period of growth as cells prepare to enter mitosis, when identical pairs of chromosomes divide into each daughter cell. Mitosis is separated into 4 phases: prophase, metaphase, anaphase, and telophases. In prophase, chromatin condenses into chromosomes and centrosomes appear. Metaphase consists of the nuclear membrane disintegrating, microtubules attaching to kinetochores, and chromosomes aligning along the metaphase plate. Two homologous chromosomes, known as sister chromatids, separate in anaphase towards opposites poles to provide an identical pair of chromosomes to each new cell. In telophase, the nuclear envelope and nucleoli reform as chromosomes unwind back into chromatin. Cytokinesis quickly follows and divides cytoplasm, organelles, and cell membrane into two daughter cells to complete the cell cycle.

Each phase of the cell cycle is driven by distinct CDK (Cyclin-dependent kinase) and cyclin complexes [21]. Cyclins are the regulatory subunit and CDKs are the catalytic component of the complexes. Depending on the phase of the cell cycle, different cyclins are expressed at different times to activate CDKs, which are expressed constitutively. Activated CDK-cyclin complexes can then phosphorylate proteins necessary for cell cycle progression. CDK-cyclin complexes become inactivated when cyclins are degraded, upon inhibitory phosphorylation or upon dephosphorylation of activation phosphorylation sites, or binding with CDK inhibitor proteins. In G1, Cyclin D levels increase and CDK4/6-CyclinD complexes phosphorylate the retinoblastoma protein (Rb), which releases inhibition on the E2F transcription factor. E2F is key to initiating entry into S phase by increasing expression of genes, such as Cyclin E, Cyclin A, DNA polymerase, and thymidine kinase. CDK2-Cyclin E complexes are therefore activated, and the cell enters S phase. Cyclin A levels rise during S phase and associate with CDK2 to promote completion of DNA synthesis. At the G2/M transition, Cyclin B levels increase and Cyclin B activates CDK1 to enter mitosis. At the end of metaphase, Cyclin B is degraded, triggering exit from mitosis.

To ensure that cell cycle progression proceeds faithfully, the cell has developed a series of checkpoints for protection against genetic and chromosomal aberrations. The first checkpoint occurs at the G1/S transition where the cell decides between division, delaying division, or entering a resting stage known as G_0. The cell must irreversibly commit to cell division to proceed to S phase. The next checkpoint occurs at the G2/M transition. At this

checkpoint, the cell confirms that DNA synthesis is complete and no DNA damage has occurred during the process. The last checkpoint occurs during mitosis, and is known as the mitotic spindle checkpoint. Here, the cell ensures that kinetochores are properly attached to microtubules for proper sister chromatid separation.

The G1/S checkpoint is a critical decision-making point, where the cell irreversibly commits to replication. This checkpoint is highly regulated by many proteins that can sense unfavorable conditions for proliferation. There are two main types of CDKIs (CDK inhibitors) known as Cip (CDK interacting protein)/Kip (Kinase interacting protein) or INK4 (Inhibitory of kinase 4)/ARF (Alternative reading frame) proteins [22]. The Cip/Kip family includes p21, p27, and p57. These CDKIs arrest cells in G1 by directly inhibiting a broad range of CDK-cyclin complexes upon anti-proliferative signals. Specifically, p27 and p21 inhibit CDK4/6-Cyclin D complexes while p21 can also inhibit CDK2 complexes. P57 is thought to bind to and block the activity of CDK4 and CDK2. INK4/ARF proteins are expressed from the same CDKN2A locus and target CDK4/6 for inhibition. INK4a (p16) binds CDK4 to induce G1 arrest while p19ARF protein prevents p53 degradation. P53 is a transcription factor that is a master regulator of G1. Upon DNA damage or other anti-proliferative stimuli, p53 directly increases p21 transcription to induce cell cycle arrest or apoptosis if DNA is irreparable.

The cell cycle is also tightly regulated by a series of E3 ubiquitin ligases to ensure a one-way, irreversible progression through each phase. The cullin-based class of E3 ubiquitin ligases are found in either SCF (Skp1, Cullin, F-Box,

Rbx1) or APC/C (Anaphase promoting complex/cyclosome) complexes, and these complexes polyubiquitinate substrates to mark them for degradation by the proteasome [23]. The F-box proteins of the SCF complex give substrate specificity to each unique complex. In contrast, Skp1, Cullin, and Rbx1 proteins are present in every SCF complex. SCF complexes require substrates to be phosphorylated prior to recognition; in contrast, APC/C substrates do not. The APC/C complex does require co-activation by either Cdh1 (Cdc20 homology 1) or Cdc20 (Cell division cycle protein 20) [24]. APC/Cdh1 complexes are predominantly active during late mitosis and early G1 to enhance degradation of SKP2 (S phase kinase-associated protein 2) and mitotic cyclins, such as Cyclin A and Cyclin B [25]. SKP2 is an F-box protein of the SCF^{SKP2} E3 ubiquitin ligase that promotes entry into S phase and targets many substrates, reviewed in [23]. Two key SCF^{SKP2} substrates are CDK inhibitors, p27 and p21, which serve as G1 checkpoint gatekeepers. Upon degradation of p27 and p21, the cell is permitted to enter into S phase. Cdc20 associates with APC/C to promote further degradation of Cyclin B and securin to trigger exit form mitosis. Securin binds to separase, an enzyme that is responsible for degradation of the cohesin rings that bind two sister chromatids. The destruction of cohesin is crucial to initiate anaphase, where the sister chromatids separate to different poles.

At the G1/S transition, the F-box protein, βTrCP (βeta-transducin repeat containing protein), associates with the SCF complex to regulate entry into S phase by targeting, Emi1 (Early mitotic inhibitor-1), one of its many substrates (complete list of substrates reviewed in [23]). At the G1/S transition, decreased

activity of the E3 ubiquitin ligase complex, $SCF^{\beta TrCP}$ allows for accumulation of Emi1 after phosphorylation by Plk1 (Polo-like kinase 1) to signal ubiquitination. Emi1 binds to and suppresses the activity of the APC/Cdh1 complex. Inhibition of APC/Cdh1 then allows for increased accumulation of its substrates, including SCF^{SKP2}, which triggers the degradation of the CDK (cyclin dependent kinases) inhibitors, p27 and p21, after CDK2 phosphorylation. In an anti-proliferative signaling environment, these CDK inhibitors accumulate and induce cell cycle arrest by suppressing CDK activity at G1.

Cell cycle regulation and cancer

Improper cell cycle progression can lead to accumulation of genetic aberrations that lead to cancer. If DNA is damaged during the replication process, cells normally undergo either cell cycle arrest to allow for correction of the problem or the cell may undergo apoptosis if damage is too detrimental. However, if the cell does not receive cues that DNA damage has occurred, these mutations can result in additional defects, which may promote oncogenesis. A key mediator of DNA damage is the transcription factor, p53, and p53 is one of the most commonly mutated genes in cancers. Cancerous cells may arise due to p53 deficiency because the cell cannot engage p53 downstream transcriptional targets, such as p21, that induce arrest; therefore, the cell cannot arrest to correct genetic damage and additional mutations accumulate.

In addition, dysfunction of E3 ubiquitin ligases, such as $SCF^{\beta TrCP}$ and SCF^{SKP2}, can lead to cancer [23]. High βTrCP levels have been observed in

melanoma, breast, colon, and pancreatic cancer. Presumably, increased $SCF^{\beta TrCP}$ promotes increased degradation of Emi1 and more rapid G1/S transition due to increased cyclin degradation. SKP2 is misregulated in many types of cancer, including breast, cervical, colon, glioma, lung cancer, melanoma, ovarian, and prostate. Increased SKP2 expression leads to decreased levels of p27 and p21, which normally serve as checkpoints during the cell cycle. Without these crucial CDKIs, the cell loses control of proliferation and may propagate with mutations that might lead to oncogenesis. Indeed, downregulated p27 and p21 expression is associated with some cancers [26, 27]. In addition, deficient APC/C ubiqutin ligase activity can lead to premature exit from mitosis, resulting in genomic instability [24].

Senescence

Senescence is an irreversible form of cell cycle arrest, which can be induced by telomere shortening, DNA damage, or oncogenic and stress signals. Initially, senescence was thought to be an artifact of cell culture, however recent data suggests that senescence does occur *in* vivo [28-30]. Neoplasms with activated oncogenes have been shown to contain senescence cells, suggesting senescence may serve as a barrier to transformation. In addition, inactivation of Ras-induced senescence is reported to transform lymphocytes into invasive T-cell lymphomas [31]. Typically, both Rb and p53 pathways must be activated for senescence to occur in humans while mice require only one pathway to be activated. Cellular senescence is characterized by flattened morphology; cell cycle arrest; SAHF (Senescence-

associated heterochromatin foci); increased autophagy; increased γ-H2AX and 53BP1; and will stain positive for SA-β-gal (Senescence associated acidic β-galactosidase).

Activation of oncogenic signals may also trigger senescence. Expression of constitutively active HRas12V in primary fibroblasts elicited cell cycle arrest and a senescence phenotype [32], which was termed OIS (Oncogene induced senescence). Their work suggested that cells induced senescence as a protective mechanism in response to oncogenic signals to suppress hyper-proliferation; therefore, additional mutations would need to be acquired to escape this checkpoint mechanism. Other oncogenes, such as B-Raf, c-Raf, and MEK/MAPK have been described to induce senescence [28, 33, 34].

Loss of tumor suppressors may also activate senescence as described in studies of VHL and PTEN loss. The VHL (von Hippel-Lindau) tumor suppressor is an E3 ubiquitin ligase that regulates destruction of HIF (Hypoxia-inducible factor) under oxygenated conditions. Mutations in VHL can cause renal cell carcinoma. In MEFs, loss of VHL induced senescence in a p53 and HIF-indepependent manner [35]. VHL deficiency led to an increase in the chromatin remodeler, p400, and also downregulated SKP2 levels, which led to increased accumulation of p27 and activation of Rb protein. The signaling pathways that regulate PICS (PTEN induced cellular senescence) involve both p53-dependent and p53-independent pathways (Figure 4). Loss of PTEN activates ARF, which inhibits MDM2, resulting in stabilization of p53. Increased p53 levels lead to increased expression of its target gene, p21, to induce senescence. In a p53-independent manner, PTEN loss coupled

with SKP2 loss may also promote senescence. MEFs taken from SKP2-/- PTEN+/- mice had increased β-galactosidase staining when compared to SKP2-/- or PTEN+/- MEFs alone. Moreover, suppression of p27 or p21 could overcome senescence, illustrating the importance of CDKIs in the senescence program. In addition, Lin et al. discovered that SKP2 inhibition could induce senescence in cells that contained oncogenic signals, such as Ras and E1A [30]. In normal cells, inhibition of SKP2 showed a decrease in proliferation rates but did not induce senescence. Because SKP2 inhibition can induce senescence in cancer cells but not in normal cells, some attention has been given to SKP2 inhibitors for use as pro-senescence therapy for cancer patients [30]. Importantly, SKP2 inhibition is reported to induce senescence independently of p53 status, which is critical since many cancers are p53 deficient. SCF^{SKP2} complex inhibitors are currently in Phase I development.

Known RASSF1A Functions (Figure 5)

Numerous studies have sought to understand why RASSF1A is silenced at such a high frequency in tumors. One advantage tumors may have by silencing RASSF1A is gaining a pro-survival phenotype. RASSF1A has been shown to induce apoptosis upon TNFα (Tumor necrosis factor α) stimulation or Fas ligand binding [36]. TNFα binds to the TNFR1 (Tumor necrosis factor receptor 1), which recruits RASSF1A for the activation of MOAP-1 (Modulator of apoptosis 1). MOAP-1 then triggers Bax to induce mitochondrial cell death [37]. RASSF1A is also described to activate apoptosis through the MST2/LATS1 pathway (similar to the pro-apoptotic Hippo pathway in *Drosophila* [38]). RASSF1A activates MST2 by promoting

autophosphorylation of MST2 (Mammalian Ste-20 like kinase 2). MST2 then interacts with LATS1 (Large antigen tumor suppressor 1), and LATS1 phosphorylates Yap1 (Yes associated protein 1) to promote p73 expression and puma to induce apoptosis [36]. Recently, RASSF1A has been shown to modulate mutant K-Ras induction of apoptosis which is deflected by the wild-type K-Ras allele in colorectal cancer [14]. Instead of MST2/LATS1 activation of p73 and puma, RASSF1A associates with MST2/LATS1 to stabilize levels of p53 by inhibiting its E3 ubiquitin ligase, MDM2. Mutant K-Ras induced apoptosis is suppressed by the remaining K-Ras wild-type allele, which engages c-Raf, an inhibitor of MST2 signaling. Thus, inactivation of the RASSF1A pro-apoptotic pathway may enhance survival conditions for tumors. RASSF1A has been shown to play a role in DNA damage [39]. Upon exposure to ionizing radiation or UV damage, ATM phosphorylates RASSF1A at Ser131 to induce MST2/LATS1 activation of apoptosis. With RASSF1A knockdown, the DNA damage response (DDR) was impaired. Within the ATM binding domain, RASSF1A polymorphism A133S is described to promote earlier onset of breast cancer in BRCA1/2 mutant carriers. Polymorphism at this site disrupted cell cycle regulation and showed an increased risk of breast cancer [40].

Overexpression studies show RASSF1A binds to microtubules during interphase and localizes to centrosomes during mitosis [41-44]. RASSF1A association with microtubules may be explained by yeast two-hybrid studies, which found RASSF1A interacts with MAP1b [42] and C19ORF5 [41]. MAP1b and C19ORF5 are MAPs (Microtubule associated proteins) that bind tubulin and stabilize microtubules. Destabilization of microtubules with RASSF1A loss may lead to

mitotic abnormalities and genetic instability, facilitating acquisition of mutations. Indeed, loss of RASSF1A did increase mitotic aberrations, cytokinesis failure, and tetraploidization [38, 45]. However, there is not much data to support that endogenous RASSF1A binds to microtubules. RASSF1A contains a polybasic region, which may bind microtubules indiscriminantly when overexpressed. More studies are merited to determine the importance of RASSF1A binding to microtubules.

RASSF1A functions in the cell cycle by regulating genes that promote cell cycle progression. It was previously shown that knockdown and overexpression of RASSF1A causes cell cycle arrest by inhibiting Cyclin D accumulation [46]. The APC/Cdc20 complex degrades Cyclin B levels to exit mitosis, and RASSF1A is reported to inhibit the APC/Cdc20 complex to allow for adequate completion of mitosis [45]. Loss of RASSF1A led to increased APC/Cdc20-mediated degradation of Cyclin B, which resulted in premature exit from mitosis and mitotic abnormalities. Another lab was unable to reproduce these results, suggesting more studies need to be performed to validate RASSF1A interacts with the APC/Cdc20 complex [47]. RASSF1A is described to interact with $p120^{E4F}$ and promote the inhibition of Cyclin A2 to cause cell cycle arrest [48, 49]. However, Song et al. reported that RASSF1A increased Cyclin A levels, suggesting other factors may be involved. In addition, RASSF1A is required during cytokinesis where Aurora B phosphorylates RASSF1A triggering recruitment of Syntaxin16 to the midzone and midbody during telophase [50]. Syntaxin16 is part of the t-SNARE family and is important for completion of cytokinesis. Cytokinesis defects were observed in cells expressing a mutant non-

phosphorylated RASSF1A. Impairment of pro-apoptotic pathways, DDR, microtubule stability, and cell cycle defects may all contribute to tumorigenesis when RASSF1A is lost.

Summary

RASSF1A is a tumor suppressor that is epigenetically modified in cancer. Because the majority of RASSF1A silencing is due to hypermethylation, the RASSF1A alleles remain intact, which makes reactivating this gene a therapeutically attractive target. Despite the discoveries of RASSF1A biological functions, it is not entirely clear how RASSF1A functions as a tumor suppressor. The functions of RASSF1A are controversial perhaps because most studies have been performed by overexpressing RASSF1A, which may lead to overexpression artifacts. This may account for the conflicting data between labs. To circumvent these overexpression caveats, I have focused on depleting endogenous RASSF1A to determine loss of function phenotypes. Surprisingly, I discovered that RASSF1A silencing leads to G1 cell cycle arrest. To gain further understanding of why loss of a tumor suppressor would elicit such a paradoxical response, I investigated the effects of RASSF1A on the cell cycle, focusing on the G1 checkpoint. Our studies suggest that inactivation of RASSF1A leads to increased oncogenic potential, which activates a G1 checkpoint to restrict the growth of hyper-proliferative cells.

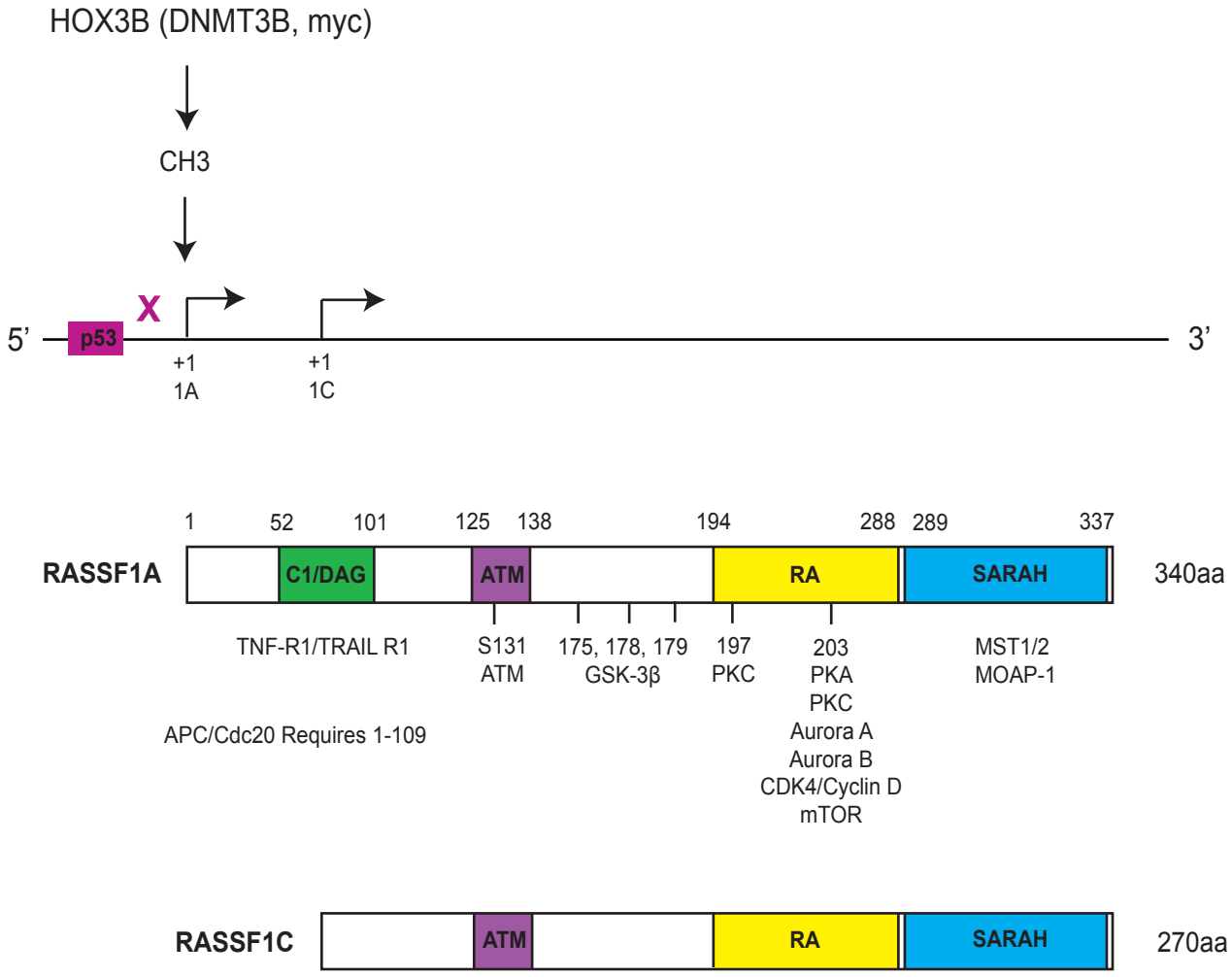
Figure 1. RASSF1A methylation status in primary tumors.

Adapted from Donninger, et al. [2]

Primary Tumor Type	Frequency	Reference
Bladder	30-50%	Marsit et al., 2006
Breast	95% 81% 85%	Yeo et al., 2005; Shinozaki et al., 2005, Cho et al., 2011
Cervical Adenocarcinoma	45% 35%	Cohen et al., 2003; Mitra et al., 2011
Cholangiocarcinoma	67%	Tischoff et al., 2005
Colorectal	20% 52%	Miranda et al., 2006; Oliveira et al., 2005
Ependymoma	36% 86%	Michalowski et al., 2006; Hamilton et al., 2005
Esophageal	34% 64%	Wong et al., 2006; Li et al., 2011
Gastric	44%	Oliveira et al., 2005
Glioma	57% 54%	Hesson et al., 2004; Horiguchi et al., 2003
Head and neck	15% 17%	Dong et al., 2003; Hogg et al., 2002
Hepatocellular	75%	Katoh et al., 2006; 64% Hua et al., 2011
Hodgkin Lymphoma	65%	Murray et al., 2004
Leukemia	0% 15%	Johan et al., 2005, Harada et al., 2002
Lung: NSCLC	39% 28% 15%	Chen et al., 2006; Grote et al., 2006; Safar et al., 2005
Lung: SCLC	88%	Grote et al., 2006
Medulloblastoma	79%	Lusher et al., 2002
Melanoma	41% 83%	Spugnardi et al., 2003; Calipel et al., 2011
Nasopharyngeal	68%	Tan et al., 2006
Neuroblastoma	83%	Lazcoz et al., 2006
Ovarian	26% 30% 51%	Teodoridis et al., 2005; Makarla et al., 2005; Bondurant et al., 2011
Pancreatic	63%	Liu et al., 2005
Pheochromocytomas	22%	Astuti et al., 2001
Prostate	99%	Jeronimo et al., 2004
Renal	56-91%	Yoon et al., 2001; Dreijerink et al., 2001
Retinoblastoma	59%	Harada et al., 2002
Rhabdomyosarcoma	61%	Harada et al., 2002
Testicular Nonseminoma	83%	Honorio et al., 2003
Testicular Seminoma	40%	Honorio et al., 2003
Thyroid	71% 35%	Schagdarsurengin et al., 2006; Nakamura et al., 2005
Wilms tumor	54%	Wagner et al., 2002

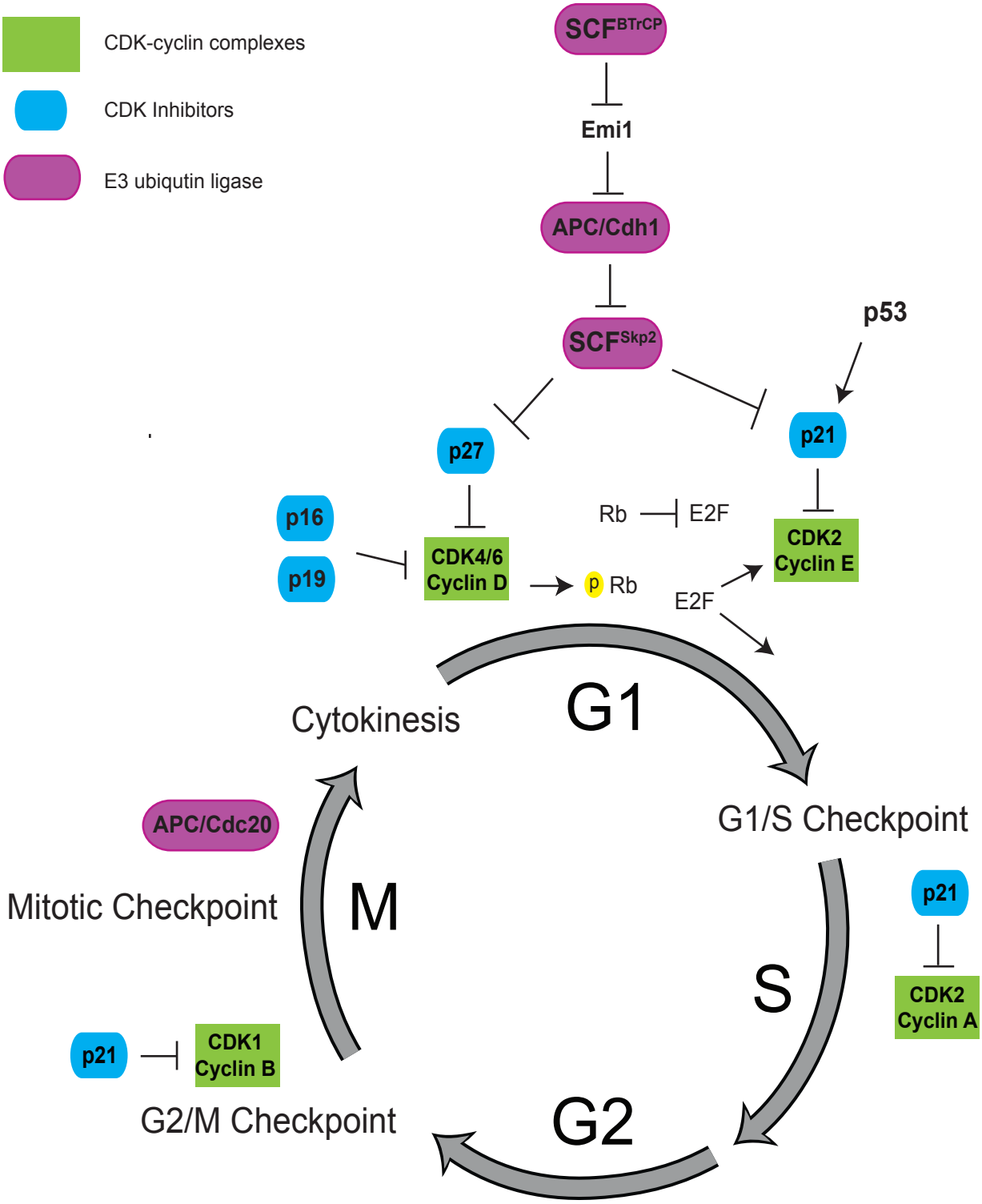
RASSF1A is frequently hypermethylated in many primary tumors.

Figure 2: RASSF1A structural domains and regulation



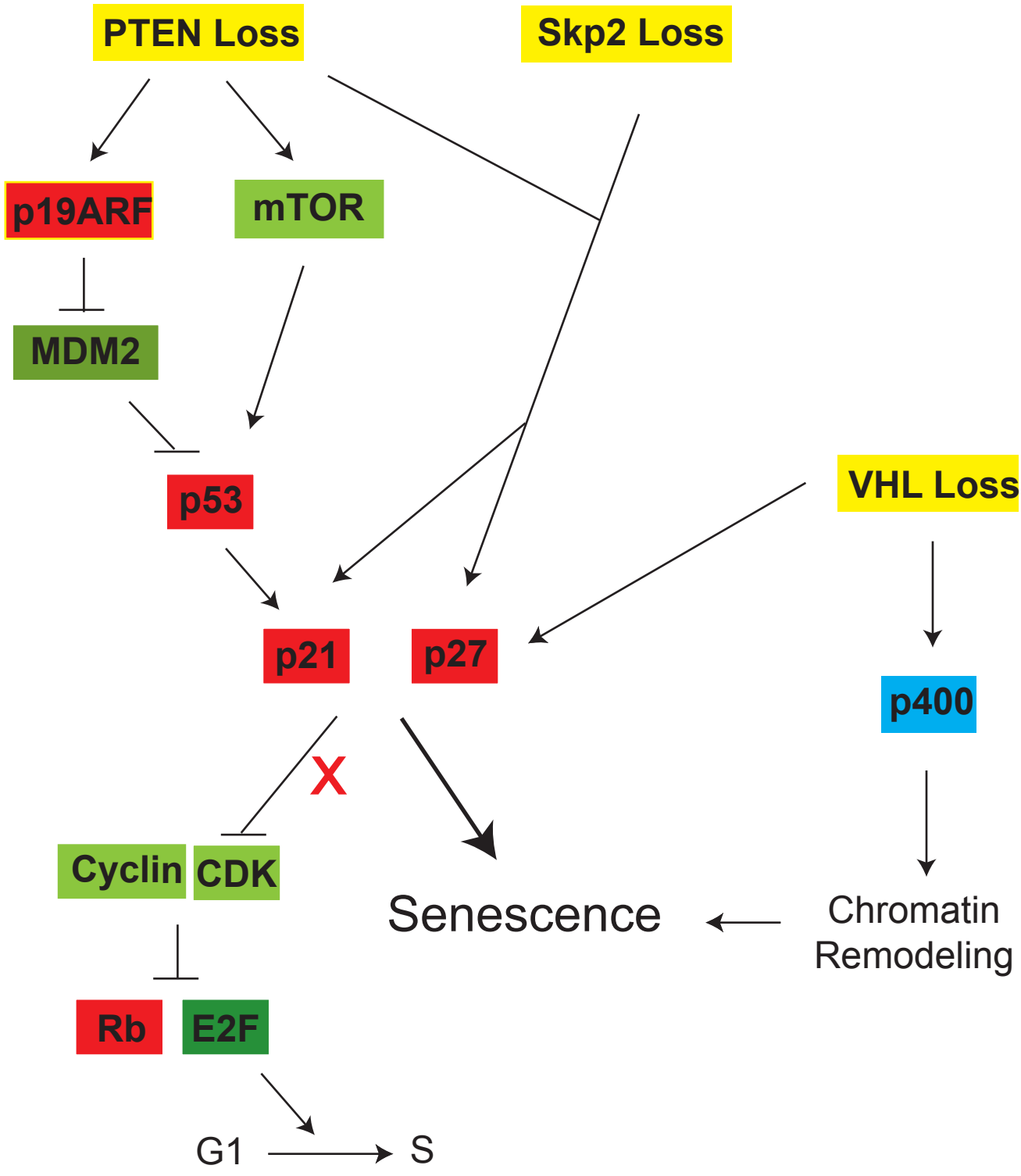
RASSF1A and RASSF1C isoforms contain an ATM, RA, and SARAH domain. RASSF1A additional contains a C1/DAG domain due to different promoters for each isoform. RASSF1A is phosphorylated by many kinases to regulate cellular homeostasis. Hypermethylation of the RASSF1A promoter can occur through HOX3B-mediated recruitment of DNMT3B.

Figure 3: The Cell Cycle



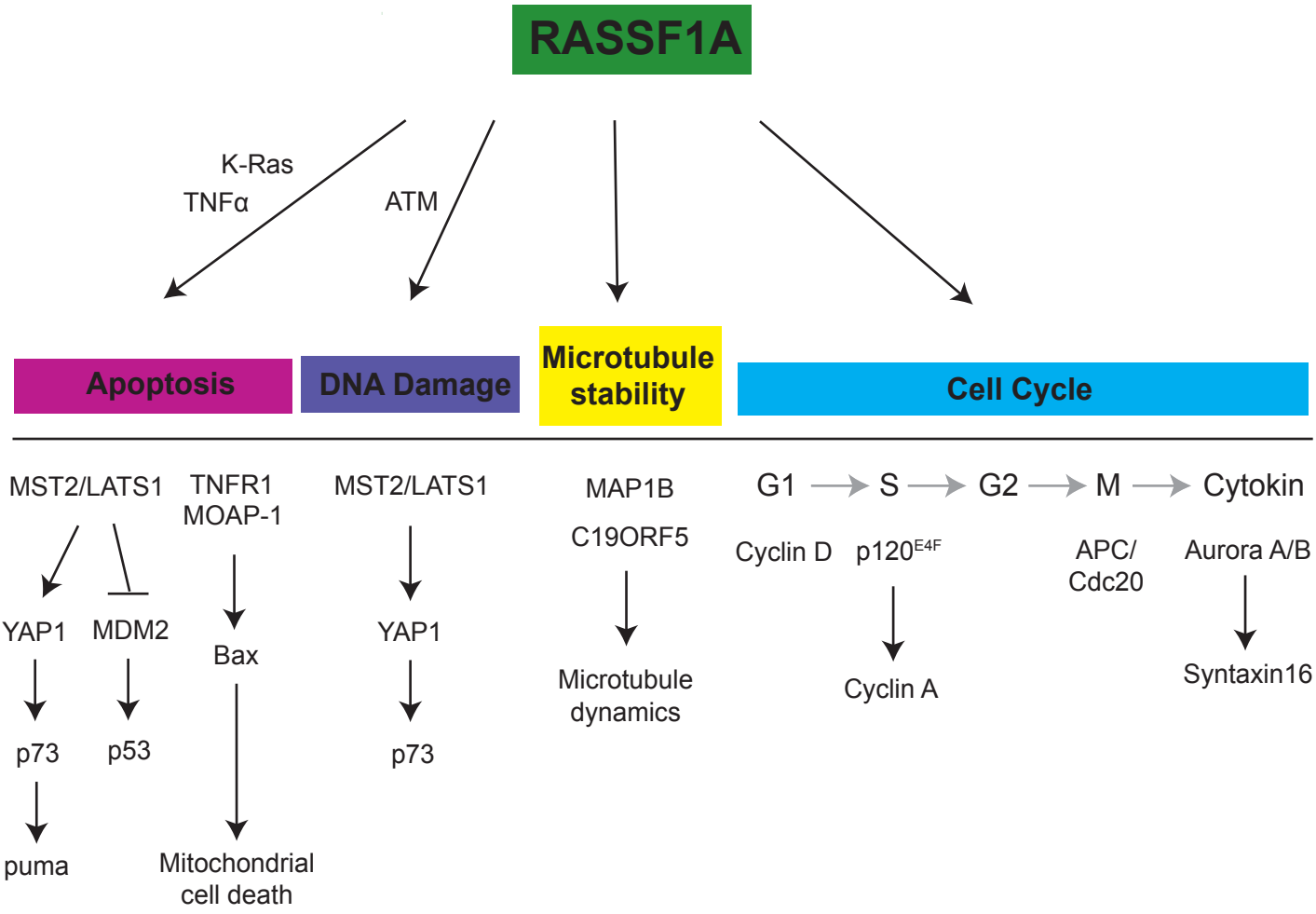
Our studies focus on the G1 phase of the cell cycle. G1 phase is regulated by multiple E3 ubiquitin ligases to ensure a one-way progression through the cell cycle.

Figure 4: Tumor Suppressor Loss Induced Senescence



Loss of PTEN can induce senescence in a p53-dependent manner. Collaborative loss of PTEN and SKP2 or loss of VHL can induce p53-independent senescence by upregulating levels of p27 and p21.

Figure 5: Known RASSF1A functions



RASSF1A regulates many proteins involved in maintaining cellular integrity, including: apoptosis, the DNA damage response, microtubule dynamics, and the cell cycle.

CHAPTER TWO

The RASSF1A Tumor Suppressor Restrains APC/C Activity During the G1/S Phase Transition to Promote Cell Cycle Progression in Human Epithelial Cells (Angelique W. Whitehurst, Rosalyn Ram, Latha Shivakumar, Boning Gao, John D. Minna and Michael A. White, *Mol Cell Bio* 2008)

ABSTRACT

Multiple molecular lesions in human cancers directly collaborate to deregulate proliferation and suppress apoptosis to promote tumorigenesis. The candidate tumor suppressor RASSF1A is commonly inactivated in a broad spectrum of human tumors and has been implicated as a pivotal gatekeeper of cell-cycle progression. However, a mechanistic account of the role of RASSF1A gene inactivation in tumor initiation is lacking. Here I have employed loss-of-function analysis in human epithelial cells for a detailed investigation of the contribution of RASSF1 to cell cycle progression. I find that RASSF1A has dual opposing regulatory connections to G1/S phase cell cycle transit. RASSF1A associates with the Ewing's Sarcoma breakpoint protein, EWS, to limit accumulation of cyclin D1 and restrict exit from G1. Surprisingly, I find that RASSF1A is also required to restrict $SCF^{\beta TrCP}$ activity to allow G/S phase transition. This restriction is required for accumulation of the anaphase promoting complex/cyclosome (APC/C) inhibitor EMI1 and the concomitant block of APC/C-dependent cyclin A turnover. The consequence of this relationship is inhibition of cell cycle progression in normal epithelial cells upon RASSF1A depletion despite elevated Cyclin D1 concentrations. Progression to

tumorigenicity upon RASSF1A gene inactivation should therefore require collaborating genetic aberrations that bypass the consequences of impaired APC/C regulation at the G1/S phase cell cycle transition.

INTRODUCTION

Normal cellular proliferation proceeds through a regimented surveillance of proliferative and apoptotic checkpoints, that integrate pro- and anti-growth signals. It is the responsibility of so called “tumor suppressor proteins” to regulate these checkpoints; their loss facilitates, and is likely required for, the development of the semi-autonomous proliferative capacity of cancer cells. Recently, RASSF1A has emerged as a candidate tumor suppressor protein that may play a crucial role in mechanisms that curb aberrant, proliferative signals. RASSF1A is found in the 3p21.3 chromosomal region, which commonly exhibits loss of heterozygosity in lung, breast, ovarian, nasopharyngeal and renal tumors [51]. Although expressed in ‘normal’ epithelial cells, RASSF1A is absent in many cancer cells due to a high level of methylation at the CpG sites in its promoter [52]. A splice variant of RASSF1A regulated by an independent promoter, RASSF1C, is expressed in both normal and cancer cells and does not have a methylated promoter [51]. RASSF1A inactivation is an extremely common event in many human cancers including 80-100% of small cell lung cancer cell lines and tumors, 30-40% of non-small cell lung cancer cell lines and tumors, 49-62% of breast cancer cell lines and tumors, 67%-70% of primary nasopharyngeal cancers, and 91% of primary renal cell carcinomas [51-53]. Furthermore, evidence suggests that RASSF1A is silenced during early neoplastic

changes in the breast, including intraductal papillomas and epithelial hyperplasia, indicating that its activation is an early event in cancer progression [54]. Mice engineered to lack expression of RASSF1A are normal, however they are more susceptible to spontaneous and radiation-induced tumorigenesis [5].

Together with the correlative observations described above, RASSF1A was implicated as a tumor suppressor gene through studies in which its re-expression in lung carcinoma cells reduced colony formation, suppressed anchorage–independent growth and inhibited tumor formation in nude mice [52]. Previously, I have found that RASSF1A overexpression blocks proliferation and decreases the levels of cyclin D1, presumably preventing cells from passing through the Rb-family cell cycle restriction point and entering S-phase. Similarly, the reduction of RASSF1A protein levels by siRNA increased cyclin D1 protein levels. Overexpression of the viral oncoprotein, E7, which inhibits the interaction between Rb and E2F, produced proliferative cells resistant to RASSF1A induced cell cycle arrest, placing RASSF1A's antiproliferative effect prior to the Rb checkpoint [46]. A supporting clinical correlation comes from studies of cervical cancer in which there is an inverse correlation between HPV infection (E7 expression) and RASSF1A methylation status, indicating that these two oncogenic changes disable similar tumorigenic pathways [55, 56].

A variety of interacting proteins have been characterized that may participate in RASSF1A-dependent regulatory events [41, 45, 57, 58]. However, a mechanistic account of the consequences of RASSF1A loss on tumor progression remains elusive. Here, we describe a detailed loss of function analysis to directly evaluate the impact of RASSF1A depletion on the molecular changes required for cell cycle progression. We

find that RASSF1A inhibits cyclin D1 accumulation through an association with the Ewings Sarcoma Protein. In addition, RASSF1A restricts APC/C activity in G1-S through a functional interaction with βTRCP. Together, this data suggests that RASSF1A has both positive and negative inputs into cell cycle progression that may represent a fail-safe relationship. As a consequence, multiple genetic lesions would be required to overcome RASSF1A function during tumor progression. While loss of RASSF1A may not directly promote oncogenic transformation, it may provide a permissive environment for acquiring additional genetic lesions that lead to tumorigenesis.

RESULTS

RASSF1A Interacts with the Ewing Sarcoma Breakpoint Protein (EWS).

Our previous observations had suggested that RASSF1A expression in tumor cells that lack RASSF1A inhibits cyclin D1 accumulation and cell cycle progression [46]. To identify proteins that may participate in RASSF1A-dependent cell cycle modulation, we performed a yeast two-hybrid screen with an N-terminal fragment of RASSF1A (Figure 6A). From this screen we isolated the zinc finger domain of the Ewing Sarcoma Breakpoint protein as a specific RASSF1A binding partner that could be recapitulated by overexpression co-immunoprecipitation (Figure 6B). The Ewing Sarcoma Breakpoint protein has been characterized for the fusion it forms with transcription factors such as Fli1 to cause Ewing's Sarcoma [59]. The full-length protein has both a transcriptional activation domain as well as a predicted zinc finger RNA binding domain. HeLa cells retain expression of RASSF1A, presumably

because E7, which directly inactivates Rb, allows G1 progression independently of a requirement for cyclin D1 accumulation. Therefore, we used these cells to examine native complexes of RASSF1A without potential complications from overexpression artifacts. Immunoprecipitates from whole cell lysates using an antibody specific for the RASSF1A isoform co-precipitated endogenous EWS protein (Figure 6C). We also identified the EWS family member FUS/TLS and a previously described RASSF1A interacting protein, MST2, in the RASSF1A immunoprecipitates [58]. Our previous studies had indicated that loss of RASSF1A induced an accumulation of cyclin D1 protein levels, without a detectable change in cyclin D1 mRNA levels. Further observations found that neither cyclin D1 transcription nor protein degradation rates are altered in cells overexpressing RASSF1A, suggesting that cyclin D1 translation rates may be changing [46]. While little is known about the function of the wild type EWS protein, there are a number of RNA binding motifs indicating that EWS may be involved in translation. The identification of EWS in the polysome fraction of HEK293 cells further supports a role for EWS in active translation [60]. Cyclin D1 has a complex promoter and multiple regulatory elements have been implicated in its translational regulation. Given the role of RASSF1A in the regulation of cyclin D1 accumulation, we examined the contribution of EWS to this phenotype. We first compared cyclin D1 protein accumulation upon siRNA mediated RASSF1A depletion with or without co-depletion of EWS (Figures 7A-C). As expected, cyclin D1 levels were elevated in cells depleted of RASSF1A alone. However in either HeLa or MCF10A cells co-depletion of EWS and RASSF1A resulted in levels of cyclin D1 similar to control, suggesting that EWS is a positive regulator of cyclin D1 protein

accumulation. To test this directly, HeLa cells depleted of RASSF1A and/or EWS were synchronized at G_0 by overnight serum starvation followed by a 6-8 hours exposure to FBS. As shown in Figure 7D, EWS depletion significantly impaired serum-induced cyclin D1 accumulation relative to control. Previous reports have suggested that the EWS protein can positively regulate c-fos transcription. As c-fos is an immediate early gene product that can positively regulate cyclin D1 transcription, this could account for the consequence of EWS depletion on Cyclin D1 accumulation. However, we saw no consequence of EWS depletion on c-fos expression following 45 minutes of serum stimulation (data not shown). To determine if the impact of EWS depletion on cyclin D1 was significant enough to impact cell cycle progression, we analyzed cells for BrdU incorporation. HeLa cell proliferation was only slightly affected by EWS knockdown, probably because HeLa cell cycle progression is independent of cyclin D1 regulation, as a consequence of Rb inactivation (Figure 7E). Similarly, the A549 non-small cell lung cancer cell line, which lacks RASSF1A and has bypassed Rb-checkpoint control through p16 inactivation, was insensitive to EWS depletion. In contrast, proliferation in non-tumorigenic MCF10A cells, which have an intact Rb checkpoint, is inhibited upon EWS depletion (Figure 7D,E). This result suggests that in established tumor cell lines, which presumably have acquired multiple genetic alterations (including the loss of p16), EWS function is uncoupled from cell cycle progression (Figure 7E). These phenotypes were also validated by a second, independent EWS siRNA (data not shown). Thus, it appears that EWS is a positive regulator of cyclin D1, perhaps at the level of translation, and is required for RASSF1A to modulate cyclin D1 accumulation.

RASSF1A is required for cell cycle progression.

Surprisingly, BrdU labeling also demonstrated that depletion of RASSF1A from either HeLa, MCF10A or Normal Human Bronchial Epithelial cells impaired proliferation (Figure 7E and data not shown). This observation is an apparent paradox given that RASSF1A is a candidate tumor suppressor that limits cyclin D1 accumulation and it suggests that RASSF1A may have multiple roles in the regulation of cell cycle progression.

To determine where in the cell cycle RASSF1A depleted cells were arrested, we treated control siRNA transfected and RASSF1A siRNA transfected cells with nocodazole for 18 hours. Nocodazole interferes with microtubule dynamics and through activation of the spindle assembly checkpoint. FACS analysis for DNA content indicated that while control transfected cells treated with nocodazole accumulated in G2/M as expected, cells depleted of RASSF1A and treated with nocodazole retained a significant population of cells in G1 (Figure 8A). Similar results were observed with for MCF10A cells (data not shown). This analysis suggests RASSF1A positively contributes to G1/S phase progression.

Progression through G1 requires accumulation of cyclin D1 to inactivate Rb, followed by activation of cyclin E and cyclin A. Despite an accumulation of cylin D1 in RASSF1A depleted cells, we found that cyclin A was dramatically downregulated and cyclin E levels remained unchanged (Figure 8B and data not

shown). In addition, Cyclin B1, which is required for G2 progression, was also reduced in RASSF1A depleted cells (Figure 8B). We did not see an impact of EWS depletion on cyclin A or B accumulation (data not shown). Given that cyclins A and B are both substrates of APC/C, while cyclin E is not, we examined an additional substrate of APC/C, SKP2 [61]. This APC/C substrate was also dramatically reduced in RASSF1A depleted cells as compared to controls, and a SKP2 client protein, the cyclin-dependent kinase inhibitor p27, was concomitantly elevated (Figure 8B and Figure 9A). Given that APC/C inhibition is required at the G1/S transition to allow for stabilization of proteins required for S-phase progression [62], RASSF1A depletion may result in unrestrained activation APC/C at the G1/S transition, therefore arresting cells in late G1.

Inactivation of APC^{CDH1} Restores Proliferation in RASSF1A Depleted Cells.

Inhibition of APC/C can occur through the cyclin A/CDK2 phosphorylation induced inhibition of the APC co-activator, CDH1, or by direct binding of the APC inhibitor, Emi1 [61]. To determine if unrestrained APC/C activity is responsible for the decreased cyclin A and SKP2 protein levels in RASSF1A depleted cells, we inactivated APC by co-depleting CDH1. As shown in Figure 9, co-depletion of CDH1 and RASSF1A restored cyclin A and SKP2 levels, reversed p27 accumulation and restored BrdU incorporation, suggesting that RASSF1A contributes to APC^{CDH1} inactivation during the G1/S transition.

RASSF1A mediates cell cycle regulation through βTRCP.

Our observation that inactivation of the APC/C could reengage cell cycle progression in RASSF1A depleted cells prompted us to examine the mechanism by which RASSF1A impacts the APC/C activity. Emi1 is an APC/C inhibitor that has been found to function at the G1/S transition and during G2 [63]. Consistent with the hyperactive APC/C phenotype we saw a significant decrease in Emi1 expression in RASSF1A knockdown cells (Figure 10A). Transient overxpression of RASSF1A was also sufficient to induce a modest but reproducible increase in Emi1 accumulation (Figure 10B). Surprisingly, we found that direct siRNA mediated depletion of Emi1 resulted in apparent cell cycle arrest in S-phase together with massive endoreduplication, a phenotype that was not altered by co-depletion of RASSF1A (Figure 10C,D). A very recent observation has demonstrated that Emi1 is required to allow cyclin A accumulation and S-phase exit [64]. To test the possibility that RASSF1A dependent accumulation of Emi1 is required for G1/S phase transition, we examined the consequence of depleting βTRCP, the F-box protein required for SCF mediated ubiquitination of Emi1 [65]. We found that the depletion of either βTRCP1, 2 or both was sufficient to rescue Emi1 expression in the absence of RASSF1A expression (Figure 11A and data not shown). Furthermore, siRNA of βTRCP1, 2 or both was sufficient to rescue cell cycle progression (Figure 11B and data not shown). βTRCP knockdown was confirmed by qPCR (Figure 11C). In combination with a recent report that RASSF1 proteins can bind directly to βTRCP, these observations indicate that RASSF1A plays a pivotal role in restricting βTRCP during G1/S phase transition to allow APC/C inactivation by Emi1. In contrast to depletion or RASSF1A,

depletion of EWS had no effect on Emi1 expression, suggesting the βTRCP arm of RASSF1A regulation is distinct from consequences on EWS function (Figure 11D).

DISCUSSION

RASSF1A gene inactivation has been established as a common event in many cancers. A number of studies have implicated RASSF1A function in the regulation of transcription, chromosome segregation and apoptosis [41, 45, 46, 57, 58]. However, a mechanistic account of the contribution of RASSF1A inactivation to cancer cell proliferation and survival has not been developed. Through a detailed loss of function of analysis we find that RASSF1A has both positive and negative inputs into cell cycle progression (Figure 12). RASSF1A association with EWS restricts cyclin D1 accumulation while interaction with βTRCP is necessary for G1/S phase transition and cell cycle progression. These observations introduce an apparent paradox in which a tumor suppressor is both positively and negatively coupled to proliferation. This framework implies that the function of RASSF1A may be context dependent. In normal cells, this coupling may represent a tumor progression checkpoint that generates the necessity for multiple genetic lesions to release restraints on proliferation and survival. On the other hand, the loss of RASSF1A may result in a transient cell cycle arrest that provides a permissive environment for the accumulation of additional oncogenic insults that would otherwise engage an apoptotic response in cycling cells. Future studies that determine which genetic alterations occur after RASSF1A

inactivation will be required to delineate the order of specific genetic changes that must occur to cause tumorigenesis.

Mechanistically, a molecular coupler between RASSF1A and cell cycle progression appears to be regulation of Emi1 accumulation in late G1 through restriction of βTRCP activity. While we found that loss of RASSF1A results in βTRCP dependent Emi1 loss and G1 arrest, when Emi1 is silenced directly an S-phase arrest occurs concomitant with endoreduplication [64, 66]. This observation further supports the notion that Emi1 is important in S phase, not only to restrict APC/C as UBCH10 levels begin to increase but also to ensure proper regulation of DNA replication [62, 64]. This highlights the critical role of Emi1 in multiple cell cycle control points and suggests that distinct cell cycle transitions require different stoicheometric ratios between APC/C and Emi1. These ratios are likely dependent on additional APC regulatory molecules, such as the availability of UBCH10, cyclin A and CDK1/2.

$SCF^{\beta TRCP}$ has been implicated as a regulator of Emi1 during prophase [65]. Here, we show that an inhibition of $SCF^{\beta TRCP}$ during G1 is an additional control point for Emi1 regulation. Recently, the RASSF1A family member, RASSF1C, has been shown to interact directly with $SCF^{\beta TRCP}$ and regulates the degradation of β-catenin and IκB [67]. Given the observation that RASSF1A family members, including RASSF1C can function as a complex ([68] and our unpublished observations), $SCF^{\beta TRCP}$ likely represents the proximal molecular entry point for RASSF1A control of G1/S phase transitions; perhaps through deflection of the capacity of RASSF1C to activate $SCF^{\beta TRCP}$ [67]. This scenario

would predict that any protumorigenic consequences of unrestrained $SCF^{\beta TRCP}$ activity, bestowed upon loss of the RASSF1A tumor suppressor, would require the continued expression of RASSF1C. In fact, RASSF1C is rarely if ever lost in human cancers despite frequent LOH at this locus coupled to selective inactivation of the RASSF1A splice form [5, 69].

Acknowledgements

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Figure 6

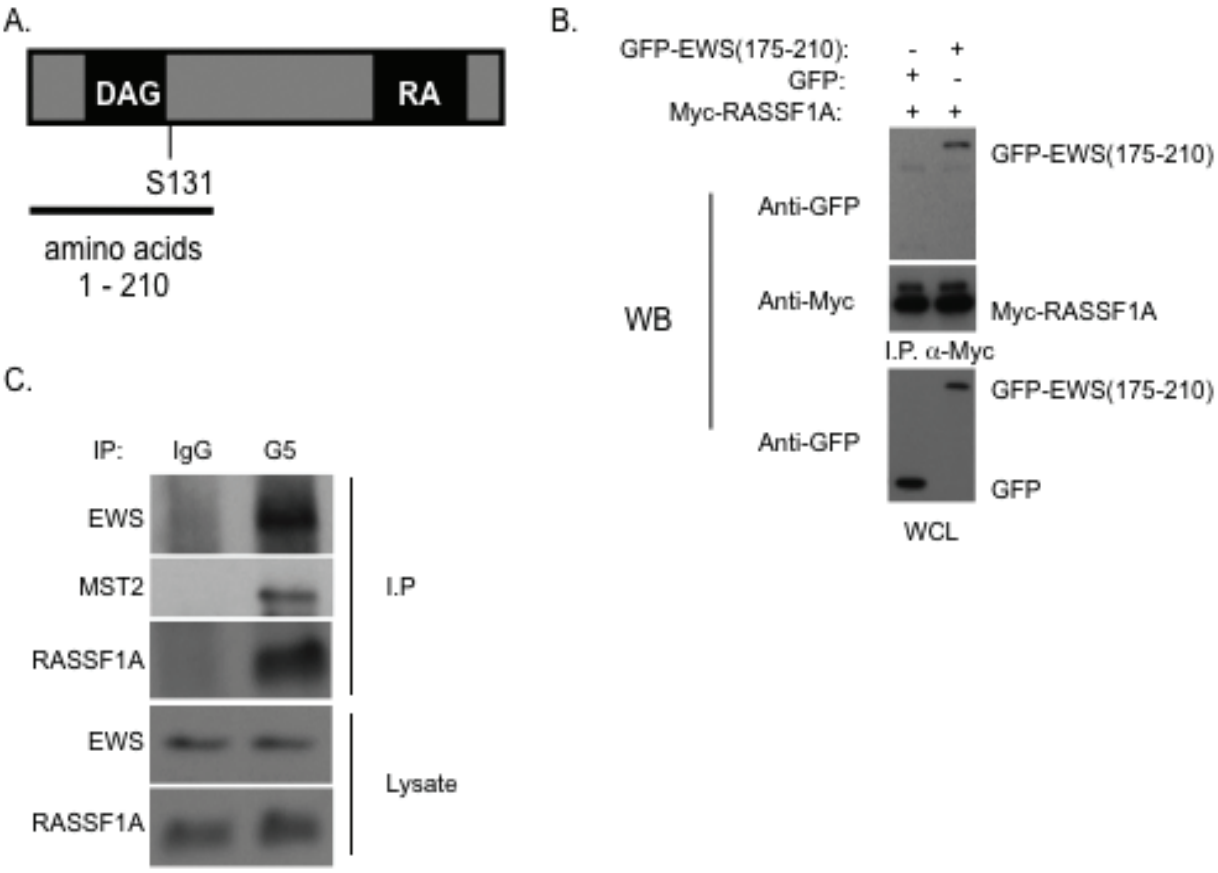

A.
DAG
RA
S131
amino acids
1 - 210
B.
GFP-EWS(175-210):
GFP:
Myc-RASSF1A:
GFP-EWS(175-210)
Anti-GFP
Anti-Myc
Myc-RASSF1A
WB
I.P. α-Myc
GFP-EWS(175-210)
Anti-GFP
GFP
WCL
C.
IP:
IgG
G5
EWS
MST2
RASSF1A
I.P
EWS
RASSF1A
Lysate

Figure 6: RASSF1A interacts with the Ewings Sarcoma Breakpoint Region Protein.

A: Schematic of RASSF1A protein. The region (a.a. 1-210) used in a yeast 2-hybrid screen for interacting proteins is indicated.

B: 293 cells were transfected with Myc-tagged RASSF1A and a GFP-tagged EWS zinc finger domain (a.a. 175-210). After 48 hours, cells were lysed and a-myc coupled agarose was used to immunoprecipitate RASSF1A. Lysates and immunoprecipitates were resolved on SDS-PAGE gel and immunoblotted with myc and GFP antibodies as indicated.

C: HeLa cell lysates were immunoprecipitated with a monoclonal RASSF1A specific antibody, G5. Lysates and immunoprecipitates were resolved on an SDS-PAGE gel, transferred to PVDF membrane and immunoblotted with a polyconal RASSF1A-specific antibody, 4169, and an EWS antibody as indicated.

Figure 7

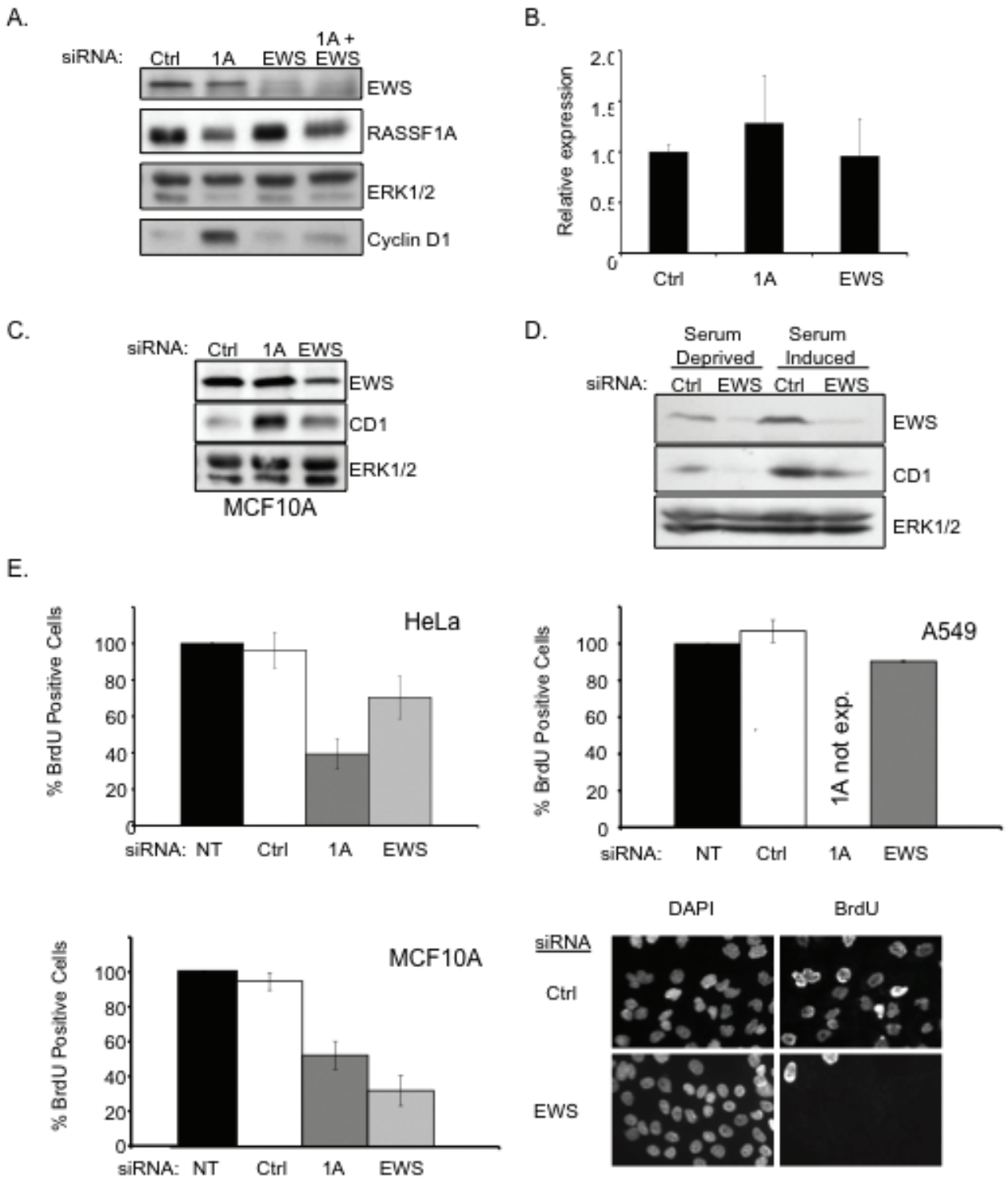

A.
siRNA:
Ctrl
1A
EWS
1A + EWS
EWS
RASSF1A
ERK1/2
Cyclin D1
B.
Relative expression
Ctrl
1A
EWS
C.
siRNA:
Ctrl
1A
EWS
EWS
CD1
ERK1/2
MCF10A
D.
Serum Deprived
Serum Induced
siRNA:
Ctrl
EWS
Ctrl
EWS
EWS
CD1
ERK1/2
E.
% BrdU Positive Cells
HeLa
siRNA:
NT
Ctrl
1A
EWS
% BrdU Positive Cells
A549
1A not exp.
siRNA:
NT
Ctrl
1A
EWS
% BrdU Positive Cells
MCF10A
siRNA:
NT
Ctrl
1A
EWS
DAPI
BrdU
siRNA
Ctrl
EWS

Figure 7: EWS regulates cyclin D1 expression.

A: HeLa cells were transfected with the indicated siRNAs in the presence of serum. 72 hours later, lysates were resolved on SDS-PAGE and immunoblotted for indicated proteins.

B: Cyclin D1 mRNA concentrations from cells treated as in (a) were evaluated by quantitative rtPCR. Error bars represent standard deviation from the mean (s.d.m.) from 3 biological replicates.

C: MCF10A cells were transfected with the indicated siRNAs. 72 hours later whole cell lysates were immunoblotted to visualize the indicated proteins. ERK1/2 is included as a loading control.

D: HeLa cells were transfected as in (a) with the exception that cells were incubated in serum-free media for 24 hours prior to lysate collection as indicated. ERK1/2 is included as a loading control.

E: The indicated cell lines were transfected as in (a). 48 hours following transfection, BrdU was added to media for an additional 24 hours. BrdU incorporation was detected with an anti-BrdU antibody, nuclei were counter stained with DAPI, and % of BrdU positive cells was calculated by microscopic observation. A minimum of 100 cells were analyzed for each condition in each experiment. Values are normalized to BrdU incorporation frequencies observed in untransfected cells arbitrarily set to 100 %. Error bars indicate s.d.m. from 3 biological replicates. Representative micrographs are shown for MCF10A cells at the bottom left.

Figure 8

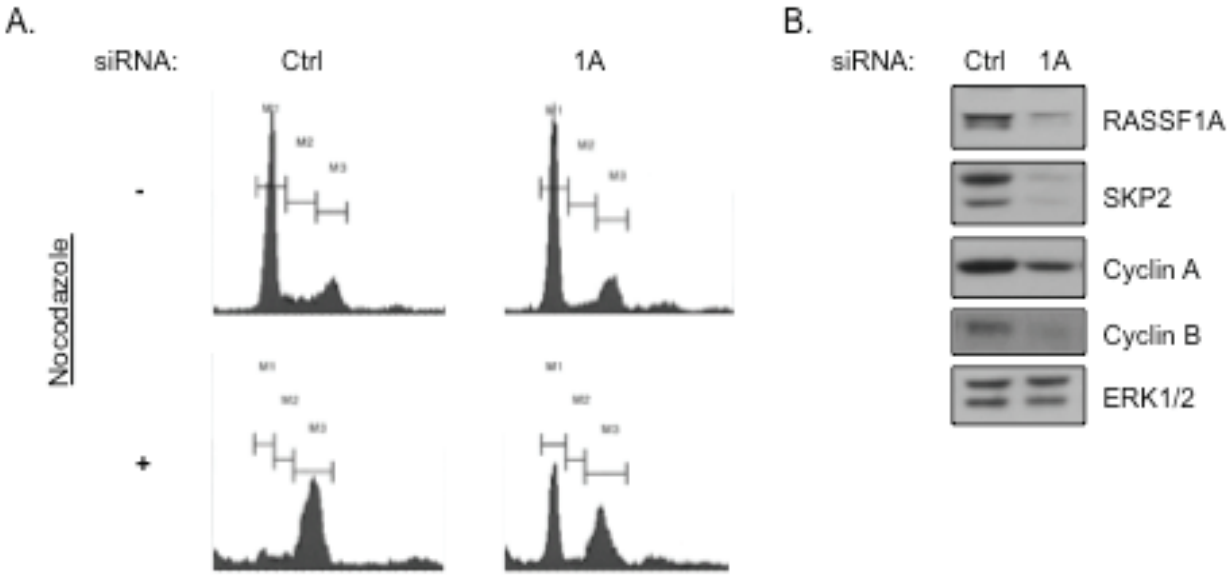

A.
siRNA:
Ctrl
1A
-
+
Nocodazole
M1
M2
M3
B.
siRNA:
Ctrl
1A
RASSF1A
SKP2
Cyclin A
Cyclin B
ERK1/2

Figure 8. siRNA of RASSF1A induces a G1 arrest.

A: HeLa cells were transfected with the indicated siRNAs. 48 hours post-transfection, 100 ng/mL nocodazole was added to media and cells were incubated for an additional 18 -20 hours. Cells were then trypsinized, fixed and stained with propidium iodide (P.I.). FACS profiles of P.I. intensity are shown. M1 indicates 2N; M2 indicates >2N and <4N; and M3 indicates 4N DNA content.

B: Whole cell lysates from cells treated as in (a) were immunoblotted to visualize the indicated proteins. ERK1/2 is included as a loading control.

Figure 9

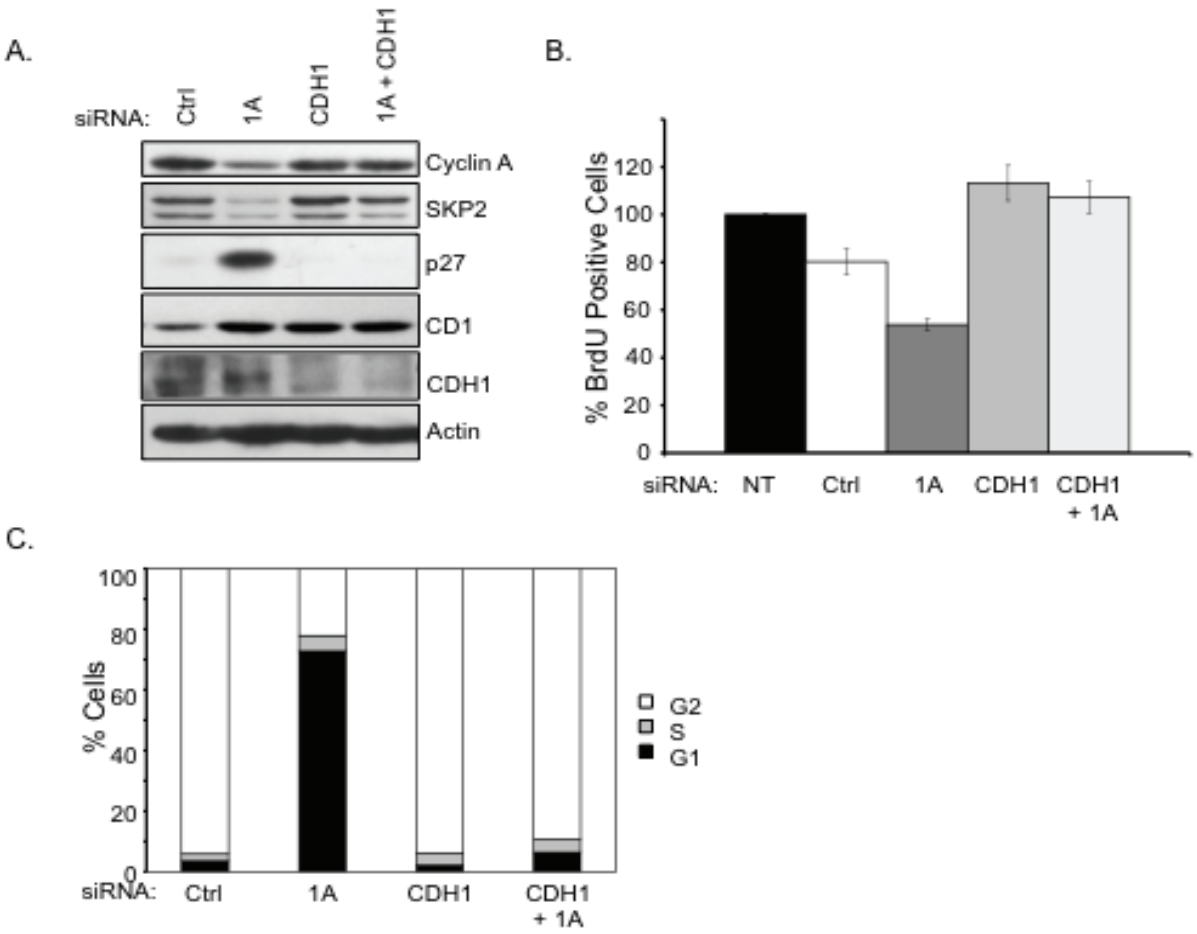

A.
siRNA:
Ctrl
1A
CDH1
1A + CDH1
Cyclin A
SKP2
p27
CD1
CDH1
Actin
B.
% BrdU Positive Cells
0
20
40
60
80
100
120
siRNA:
NT
Ctrl
1A
CDH1
CDH1 + 1A
C.
% Cells
0
20
40
60
80
100
siRNA:
Ctrl
1A
CDH1
CDH1 + 1A
G2
S
G1

Figure 9. CDH1 depletion rescues G1 arrest induced by siRNA of RASSF1A.

A: Hela cells were transfected with the indicated siRNAs and whole cell lysates were immunoblotted to visualize the indicated proteins.

B: Cells treated as in (a) were analyzed for BrdU incorporation as in Figure 2d.

C: HeLa cells were transfected with the indicated siRNAs. 48 hours post-transfection, 100 ng/mL nocodazole was added to media and cells were incubated for an additional 18 -20 hours. FACS analyses was performed as in Figure 3B. Population distributions are shown relative to DNA content. Results are representative of three independent experiments.

Figure 10

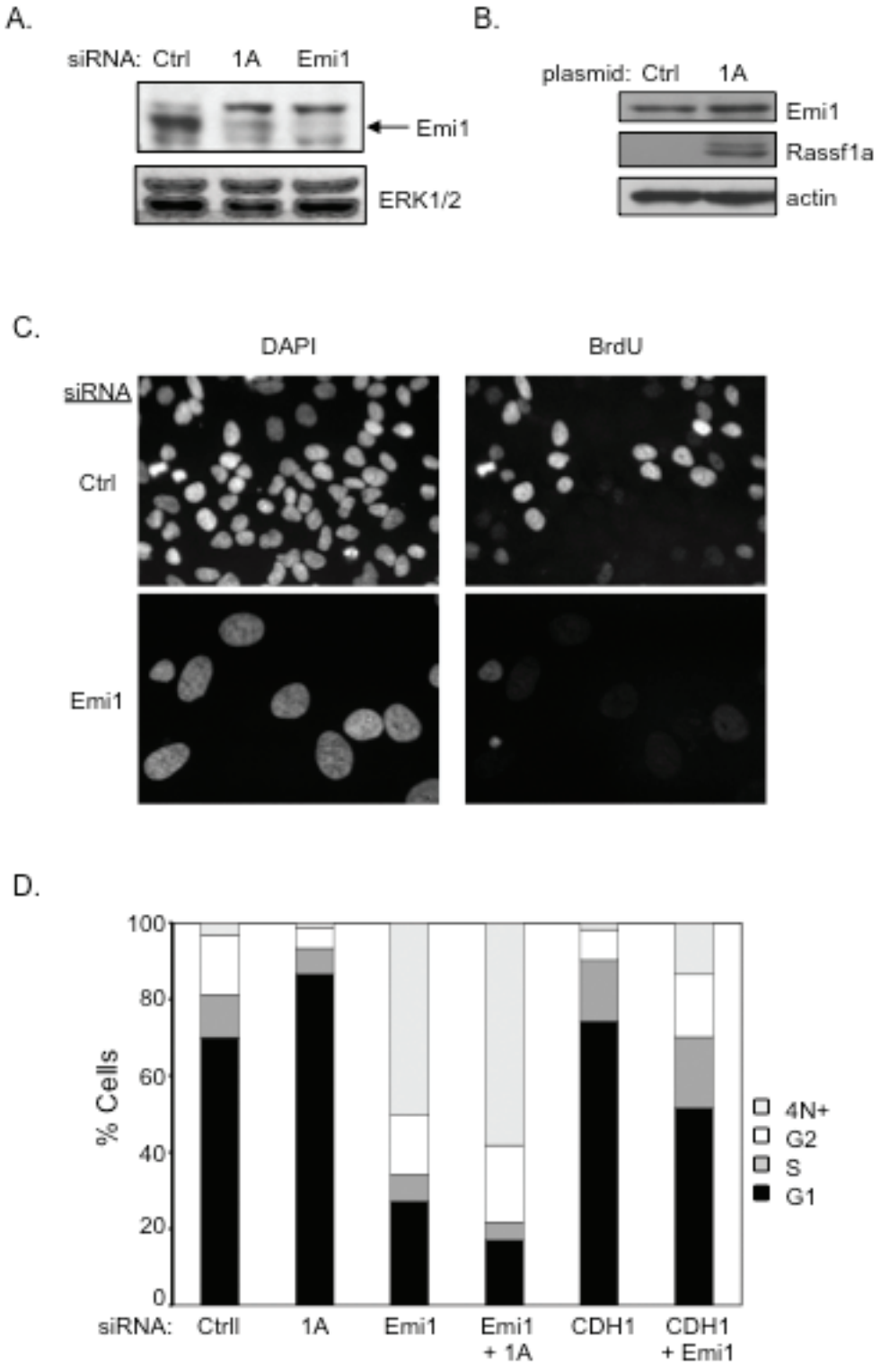

A.
siRNA: Ctrl 1A Emi1
Emi1
ERK1/2
B.
plasmid: Ctrl 1A
Emi1
Rassf1a
actin
C.
DAPI
BrdU
siRNA
Ctrl
Emi1
D.
% Cells
100
80
60
40
20
0
siRNA: Ctrll 1A Emi1 Emi1 + 1A CDH1 CDH1 + Emi1
4N+
G2
S
G1

Figure 10. Emi1 mediates the RASSF1A depletion induced G1 arrest.

A: HeLa cells were transfected and immunoblotted for indicated proteins as in Figure 7a.

B: Cells were transfected with the indicated plasmids. 48 hours post transfection, whole cell lysates were immunoblotted for the indicated proteins.

C: BrdU incorporation was assayed as in Figure 7d. Representative micrographs are shown.

D: FACS analysis of HeLa transfected with indicated siRNAs. Population distributions are shown relative to DNA content. Results are representative of three independent experiments.

Figure 11

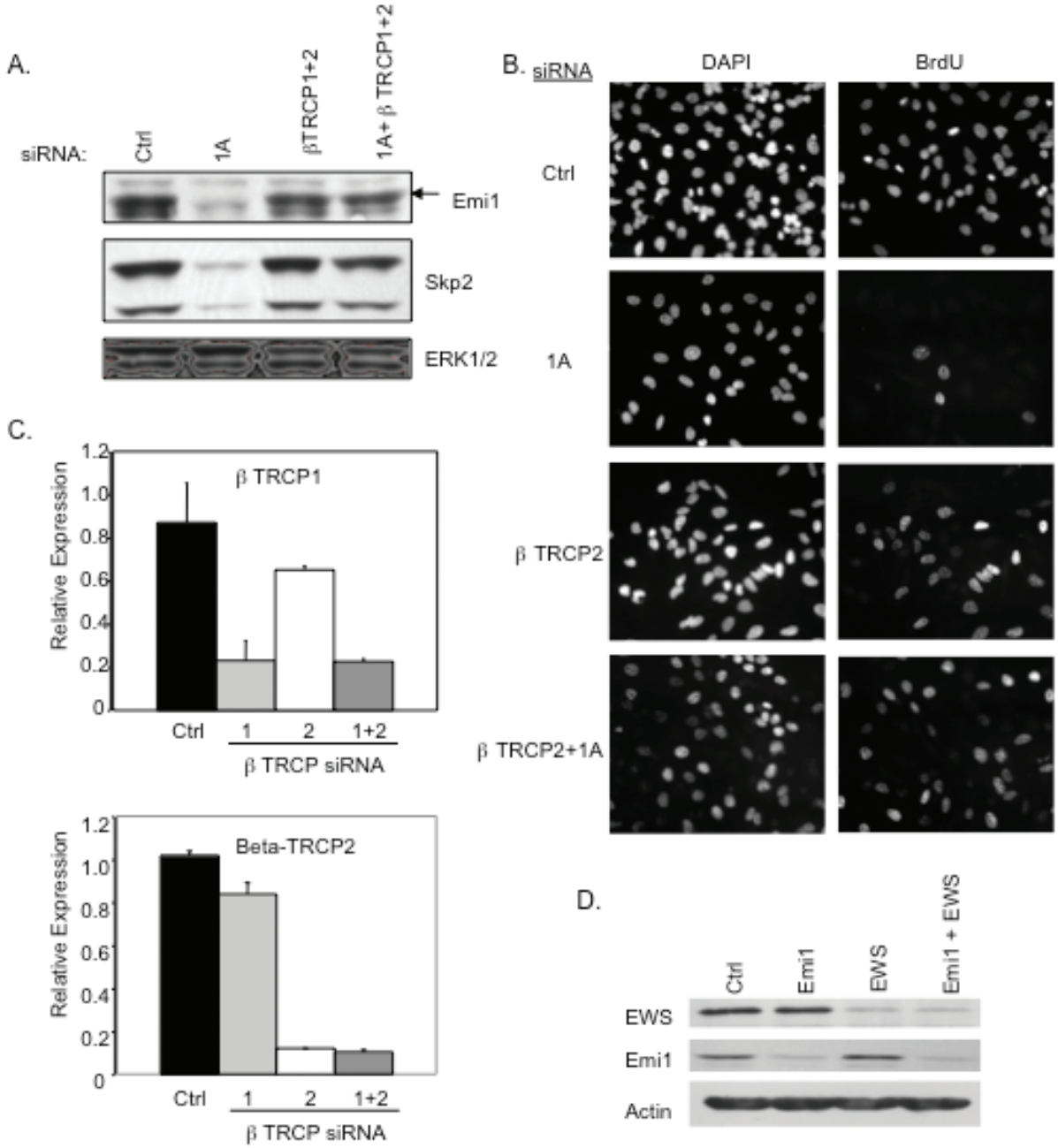

A.
siRNA:
Ctrl
1A
βTRCP1+2
1A+β TRCP1+2
Emi1
Skp2
ERK1/2
B.
siRNA
DAPI
BrdU
Ctrl
1A
β TRCP2
β TRCP2+1A
C.
β TRCP1
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Ctrl
1
2
1+2
β TRCP siRNA
Beta-TRCP2
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Ctrl
1
2
1+2
β TRCP siRNA
D.
Ctrl
Emi1
EWS
Emi1 + EWS
EWS
Emi1
Actin

Figure 11. βTRCP siRNA restores Emi1 levels and rescues G1 arrest.

A: 72 hours post transfection with the indicated siRNAs, HeLa cell lysates were immunoblotted to visualize indicated proteins.

B: BrdU incorporation was assayed as in Figure 7d.

C: bTRCP1 and 2 mRNA concentrations were measured by qPCR from samples treated as in (a).

D: 72 hours post transfection with the indicated siRNAs, HeLa cell lysates were immunoblotted to visualize indicated proteins.

Figure 12

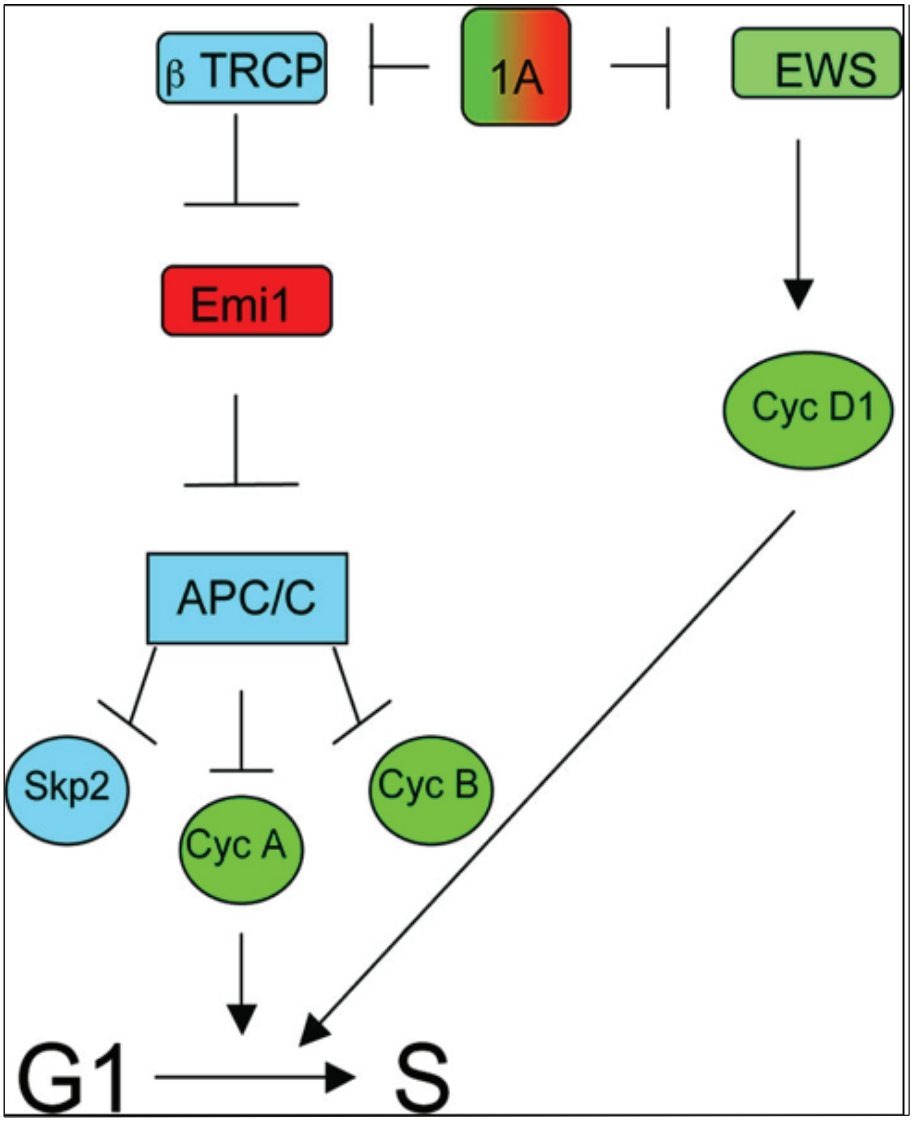


Figure 12. A model for dual opposing regulatory connections of RASSF1A to cell cycle progression. The presence of RASSF1A provides a regulatory input for restriction of EWS-dependent cyclin D1 accumulation (antiproliferative) and for restriction of bTRCP-dependent Emi1 inactivation (proproliferative). Loss of this regulatory input allows aberrant cyclin D1 accumulation, but also restricts cyclin A accumulation as a consequence of aberrant APC/C activity during the G1-S phase transition.

FIGURE CREDITS

I performed all of the experiments presented in Chapter 2 with the following exceptions:

Figure 6A, B, C: Latha Shivakumar, Angelique Whitehurst

Figure 7A, C, D, E: Angelique Whitehurst

Figure 8A: Angelique Whitehurst

CHAPTER THREE:

RASSF1A-Deficiency Releases an Oncogenic Signaling Cascade that is Suppressed by G1 Checkpoint Mechanisms

INTRODUCTION

RASSF1A is one of the most commonly inactivated genes in cancer. To understand why RASSF1A is frequently lost in cancers, I employed loss of function assays using RNAi to examine the role of RASSF1A in cancer. Surprisingly, I found that RASSF1A loss led to cell cycle arrest, induced by G1 checkpoint mechanisms. The G1 checkpoint was activated by decreased levels of SKP2, which allowed for increased levels of CDK inhibitors, p27 and p21. Additionally, I found that RASSF1A led to an increase in oncogenic signals, including: increased miR-21 expression, ERK activation, Cyclin D accumulation, and a decrease in REST protein accumulation. To explain this apparent paradox, I propose that RASSF1A loss triggers oncogenic signals that are suppressed by checkpoint mechanisms to protect the cell from uncontrolled proliferation. Additional mutations would be required to escape this checkpoint and could promote transformation.

Previously, I have found that RASSF1A modulates $SCF^{\beta TrcP}$ activity towards Emi1 to regulate p27 levels to induce G1 arrest. Here, I have extended these observations and found that RASSF1A loss increases $SCF^{\beta TrcP}$ activity towards another $SCF^{\beta TrcP}$ substrate, REST (RE-1 silencing transcription factor), leading to its degradation. REST, also known as NRSF (neural-restrictive silencer factor), is mainly known for its role in the repression of neuronal genes. REST binds to RE-1 sites in promoter regions of non-neuronal genes. Although REST has been shown to

contribute to oncogenesis in neuronal tumors by suppressing neuronal differentiation, REST has also been identified as a tumor suppressor. In an shRNA screen for tumor suppressors, loss of REST leads to transformation of HMECs [70]. In mouse embryonic stem cells, REST was found to inhibit miR-21 expression [71].

MiR-21 was one of the first microRNAs to be designated as an oncomir. MiR-21 is overexpressed in multiple types of cancers (reviewed in [72]) and has been shown to support tumorigenesis *in vivo*. In a mouse model of pre-B-cell lymphoma, expression of miR-21 was sufficient to induce tumor formation [73]. In addition, although miR-21 expression had no effect, co-expression of miR-21 with K-Ras could enhance tumor burden compared to K-Ras mice alone in a model of lung cancer [74]. Studies suggest miR-21 may promote proliferation, invasion, survival, and anchorage-independent proliferation [72]. Mir-21 has been shown to support these oncogenic events by targeting numerous tumor suppressors, including PTEN, PDCD4, Spry1/2, TPM1, and RECK (reviewed in [72]). However, one study suggested that miR-21 may target the oncogene CDC25A in colon cancer cells, which implicates that miR-21 can inhibit proliferation in some cells [75].

RASSF1A contains an RA (Ras association) domain, but for many years, it was debated whether RASSF1A interacted with Ras endogenously. Recently, RASSF1A has been described as an effector of mutant K-Ras in colon cancer [14]. RASSF1A activates MST2 to induce apoptosis; therefore, binding of RASSF1A to K-Ras may explain the apoptotic effects of K-Ras. Ras is a small GTP-ase that responds to mitogenic cues to promote proliferation. Ras effectors include PI3K, Ral, and the MAPK pathway. Ras can regulate the MAPK pathway through c-Raf and

MEK. C-Raf has been described to bind and inactivate MST2 to prevent MST2-induced apoptosis and support proliferation. Upon apoptotic signals, MST2 dissociates with c-Raf and interacts with RASSF1A, which activates MST2. Therefore, the status of Ras activity may mediate the association of MST2 with either RASSF1A or c-Raf to induce apoptosis or cell proliferation.

Depletion of RASSF1A appears to unleash pro-proliferative events, such as increased miR-21, MAPK activation, and Cyclin D accumulation and concurrently, decreased levels of the tumor suppressor REST. However, despite the accumulation of oncogenic signals incurred upon RASSF1A loss, I observed cell cycle arrest. Similar to RASSF1A, induction of cell cycle arrest and senescence has been reported with loss of other tumor suppressors, such as PTEN [29, 30] and VHL [35]. Senescence may serve as a protective mechanism to prevent hyper-proliferation upon aberrant oncogenic signals. Reminiscent of PICS (PTEN induced cellular senescence), loss of RASSF1A induces activation of the G1 checkpoint in a SKP2-p27-p21 dependent manner. Here, I show that RASSF1A deficiency releases oncogenic signals that are restrained by CDK inhibitors.

RESULTS

Both RASSF1A and RASSF1C isoforms have been shown to regulate the activity of the $SCF^{\beta TrcP}$ complex [67] and recent data suggests $SCF^{\beta TrcP}$ directly targets RASSF1C for degradation [76]. To investigate whether RASSF1A could directly interact with βTrCP, I perfomed endogenous co-immumoprecipitation assays with two different RASSF1A antibodies and found that RASSF1A associates with

βTrCP (Figure 13A). Previous work in our lab has shown that RASSF1A regulates Emi1 protein levels in a $SCF^{\beta TrcP}$ -dependent manner [77]. RASSF1A depletion led to increased $SCF^{\beta TrcP}$ activity, allowing for increased degradation of its substrate, Emi1. Due to the frequency of RASSF1A loss in cancers, I investigated whether RASSF1A regulation of other $SCF^{\beta TrcP}$ substrates could explain the tumor suppressive nature of RASSF1A. Of the known $SCF^{\beta TrcP}$ substrates, I focused on REST due to its described function as a tumor suppressor. I tested whether RASSF1A depletion could affect levels of REST protein. Interestingly, I found with RASSF1A knockdown, REST levels were decreased (Figure 13B) in both HeLa and HBEC30 cell lines with multiple RASSF1A siRNA oligos (Figure 13C). Because RASSF1A knockdown has been shown to induce G1 arrest, I tested whether knockdown of RASSF1A would have a similar effect on REST levels in cells synchronized by a double thymidine block, which arrests cells at the G1/S transition. I observed a similar decrease in REST levels (Figure 13D), suggesting the effect of RASSF1A loss on REST levels was not simply a consequence of G1 arrest. Furthermore, a time course following RASSF1A and REST levels revealed an inverse correlation of the two genes that suggests when RASSF1A is expressed, REST levels are down and alternatively, when RASSF1A are lowest, REST levels are highest (Supplementary Figure 13).

RASSF1C has been implicated in both pro-tumor [78, 79] and anti-tumor [80] effects. Due to lack of commercial antibodies for RASSF1C, RASSF1C mRNA was measured by qPCR. I observed that RASSF1A oligos also increase (si-RASSF1A-1) and decrease (si-RASSF1A-3, 4, pool) RASSF1C levels (Figure 13E). Oligos targeting either RASSF1A alone or both RASSF1A and RASSF1C have similar

effects on downstream targets that were tested (Figure 13C, 14A, 16E). This suggests my results do not appear to be RASSF1C dependent. Surprisingly, RASSF1A knockdown significantly increased RASSF1C mRNA expression (Figure 13E). Recently, a study revealed an inverse correlation between RASSF1A and RASSF1C expression in pancreatic endocrine tumors [81], which further supports the notion of a reciprocal regulatory relationship. However, our results suggest that RASSF1A regulation of RASSF1C does not affect the signaling pathways in our studies.

To test whether changes in REST levels were due to protein degradation via the $SCF^{\beta TrcP}$ complex, MG132 (proteasome inhibitor) was added to RASSF1A depleted cells. At time zero, si-RASSF1A treated cells have decreased REST expression, but by six hours, REST protein accumulates more than control (Figure 13F). Since RASSF1A negatively regulates $SCF^{\beta TrcP}$, I suspect that the increase in REST levels with MG132 seen in RASSF1A depleted cells may be due to increased βTrCP activity. Next, I wanted to verify that REST protein levels could be reciprocally affected by overexpression of RASSF1A. Upon expression of RASSF1A but not RASSF1C, levels of REST protein were increased similar to that observed with MG132 treatment (Figure 13G). These data further support distinct roles of RASSF1A and RASSF1C isoforms in the regulation of βTrCP and give credence to my hypothesis that the RASSF1A regulation of REST protein levels are RASSF1C independent.

REST has been shown to inhibit miR-21 expression in mouse ES cells, so I tested to see whether RASSF1A knockdown affected levels of miR-21. Upon

RASSF1A depletion using multiple oligos, miR-21 expression increased by two and three-fold (Figure 14A). Mir-21 levels are thought to be lowest in G1 [82], which emphasizes the significance of these results as RASSF1A depletion arrests cells in G1. As previously reported, si-REST also resulted in an increase in miR-21 expression. To test whether the increase in miR-21 expression with RASSF1A loss was due to an increase in $SCF^{\beta TrcP}$ activity, I codepleted RASSF1A and βTrCP and found that this abolished the increase in miR-21 observed with RASSF1A knockdown alone (Figure 14B). In addition, βTRrCP loss alone could decrease miR-21, which further supports the existence of a βTrCP-REST-miR-21 signaling pathway. Our observations indicate that RASSF1A modulates miR-21 levels in a βTrCP-REST dependent manner.

Examination of the literature for miR-21 molecular targets, revealed a glioma microarray data set that described expression of a miR-21 agonist decreased SKP2 mRNA while a miR-21 antagonist increased SKP2 levels [83]. Because I previously described that RASSF1A can regulate SKP2 levels through Emi1, I wondered whether both $SCF^{\beta TrcP}$ substrates, Emi1 and REST, affected SKP2 in a similar manner. Comparable to RASSF1A loss, expression of a miR-21 mimic decreased levels of SKP2 while a miR-21 inhibitor, increased SKP2 levels in HeLa cells (Figure 14C) and numerous other cell lines (unpublished data – U2OS, A549, HCC15, HBEC30). In addition, miR-21 overexpression altered SKP2 mRNA levels in a similar pattern to the protein, validating results in glioma cells. Expression and inhibition of miR-21 levels were confirmed by qPCR (Supplementary Figure 14). Moreover,

REST knockdown led to decreased SKP2 levels (Figure 14D), which adds further support to the hypothesis
that REST regulates SKP2 by modulating miR-21.

SKP2 regulates the degradation of numerous proteins involved in cell cycle progression. To determine whether miR-21 might affect SKP2 substrates, I tested p21, p27, and Cyclin E. P21 and p27 are CDK (cyclin dependent kinase) inhibitors that block CDK/cyclin complexes at various stages of the cell cycle (described in Chapter 1). Cyclin E and p21 levels were significantly upregulated by miR-21 expression, and miR-21 inhibition decreased these proteins (Figure 14E). When cells were synchronized with nocodazole, p27 protein levels were increased by miR-21 overexpression and decreased by miR-21 inhibition. Cyclin A is decreased with RASSF1A knockdown [77], but is not a SKP2 substrate. Importantly, Cyclin A was not affected by miR-21 expression or inhibition, illustrating miR-21 specificity towards SKP2 substrates but not all regulators of the cell cycle. Cyclin B is not a known SKP2 substrate but was modulated by miR-21 (Figure E). Cyclin D is not a direct substrate of SKP2, but a negative correlation between SKP2 levels and Cyclin D has been described, in which SKP2 -/- MEFS have increased Cyclin D protein accumulation [84]. Depletion of both RASSF1A and REST increased levels of Cyclin D, as did overexpression of miR-21 (Figure 14F). These data support a hypothesis that miR-21 regulate downstream targets of SKP2 by modulating SKP2 levels.

RASSF1A depletion promotes G1 arrest through increased CDKI stability so I performed FACS analysis to determine whether miR-21 overexpression induced p27 and p21 expression to similarly halt cell cycle progression. I observed no significant

difference in the percentage of G1 positive cells between control and miR-21 agonist (Figure 15A). This result is perhaps unsurprising given that SKP2 depletion alone is reported to only slow cell cycle progression rather than cause G1 arrest. As previously reported, RASSF1A loss resulted in the accumulation of G1 positive cells. These data suggest that RASSF1A regulation of SKP2 mRNA levels alone is insufficient to engage the G1/S checkpoint, and that the coordinate regulation of multiple $SCF^{\beta TrcP}$ targets, APC/C, and both SKP2 mRNA and protein is required for RASSF1A to regulate G1/S transition.

To determine whether RASSF1A-depletion induced G1 arrest was, in part, mediated by miR-21, I codepleted RASSF1A and miR-21 in HeLa and HBEC30 cells and measured cell proliferation by BrdU incorporation. MiR-21 inhibition rescued si-RASSF1A induced G1 arrest in both cell lines (Figure 15B). In accordance with these results, codepletion of RASSF1A and miR-21 rescued SKP2 protein and decreased the stability of SKP2 substrates (Figure 15C). In addition, these data suggest that miR-21 inhibitors in cancer therapy should be considered with caution. In cells which have been depleted of RASSF1A, miR-21 inhibition allows for increased SKP2 expression, resulting in decreased CDK inhibitor stability, facilitating enhanced proliferation in both a cancer and normal cell line. It may, therefore, be important to screen tumors for RASSF1A expression to determine whether a drug targeting miR-21 would be of therapeutic value.

As previously reported, RASSF1A regulates SKP2 in a βTrCP-Emi1-APC/C-dependent manner. To test whether miR-21 regulates SKP2 in a similar manner, I investigated Emi1 levels upon miR-21 expression and inhibition and observed no

change (Figure 15D). In addition, codepletion of RASSF1A and CDH1 rescued SKP2 protein levels [77]; however codepletion of CDH1 with either miR-21 agonist or antagonist did not affect levels of SKP2 protein compared to miR-21 agonist and antagonist alone (Figure 15E). These data suggests that miR-21 acts independently of Emi1 and APC/CDH1 to regulate SKP2. No apparent miR-21 seed sequence was identified in the 3'UTR of SKP2 mRNA, so further studies are merited to determine whether miR-21 directly or indirectly regulates SKP2 mRNA levels.

In a mouse model of cardiac hypertrophy, RASSF1A was described to regulate the MAPK pathway [85]. These results were further supported by studies in uveal melanocytes where RASSF1A depletion led to increased ERK activation [9]. In concordance with these studies, I observed that RASSF1A loss in HeLa cells resulted in increased phospho-ERK (T202/Y204) (Figure 16A). To determine how RASSF1A might be regulating ERK activation, I investigated known RASSF1A interacting proteins. MST2 (Mammalian STE20-like kinase 2) is described to shuttle between either RASSF1A or c-Raf complexes [86] (Figure 16B). RASSF1A directs MST2 to induce apoptosis while the interaction of MST2 with c-Raf is described to activate the MAPK pathway through stabilization of the phosphatase, PP2A, which removes an inhibitory phosphorylation site on c-Raf (S259) [87]. It is reported that disrupting c-Raf and MST2 complex formation can increase the interaction between MST2 and RASSF1A [36]. Therefore, I proposed that the opposite could be true, by which disruption of RASSF1A and MST2 complexes could promote c-Raf and MST2 complexes. Conceivably, loss of RASSF1A may increase ERK activity by enhancing MST2 and c-Raf interaction. Indeed, I observed that RASSF1A depletion decreased

levels of inhibitory phospho-cRaf (S259) and increased activating phosphorylation on ERK (Figure 16C).

Consistent with this model, codepletion of RASSF1A and MST2 resulted in decreased ERK activation compared to RASSF1A knockdown alone (Figure 16D). Thus, RASSF1A regulates MAPK activation in a MST2 dependent manner. Of note, I observed that MST2 knockdown decreased levels of RASSF1A, suggesting these two proteins may stabilize each other. Ras is a small GTPase that controls ERK activation; however, Ras activity was not increased when RASSF1A was depleted (Figure 16E). These data add further support to a model where RASSF1A opposes Raf-MST2 complex formation and subsequent downstream MAPK activation.

As previously shown, loss of RASSF1A leads to G1 arrest/delay [77]. I tested multiple siRNAs that target the RASSF1A isoform and observed G1 arrest (Figure 17A). Previous work in our lab has demonstrated that loss of RASSF1A decreases levels of SKP2 and with nocodazole treatment, increases levels of p27. Here, I observed that p21, a SKP2 substrate, is upregulated with RASSF1A depletion, presumably through increased protein stabilization (Figure 17B). Since p21 is a direct transcriptional target of p53, I tested to see whether changes in p53 and p21 were coupled. However, with RASSF1A knockdown, I see no change in p53 levels despite an increase in p21 protein. Interestingly, p53 knockdown does decrease levels of p21, but with codepletion of p53 and RASSF1A, p21 levels are higher relative to p53 knockdown alone. These data suggest that the observed increase in p21 is due to protein stability and not increased p53-mediated expression of p21 mRNA.

To determine whether G1 arrest induced by RASSF1A loss was dependent on either p21 or p27, I performed knockdown of both CDKIs to investigate whether proliferation could be rescued. Co-depletion of RASSF1A with p27, p53, or p21 was sufficient to rescue cell proliferation as measured by BrdU incorporation (Figure 17C). Notably, p53 and p21 both rescued proliferation significantly more than control, which may be unsurprising given that p53 regulates p21 levels. Moreover, RASSF1A loss promoted stabilization of p21 levels whether codepleted with control, p27, or p53. Contrary to other reports that p53 negatively regulates RASSF1A [17], I did not see an increase in RASSF1A levels with p53 knockdown. To further validate these findings, I tested the effect of RASSF1A loss on a normal cell line, HBEC30 (human bronchial epithelial cells), and received similar results. Interestingly, HBEC30 cells exhibited increased sensitivity to p53 and p21 loss, compared to Hela (Figure 17C). This difference could be attributed to the E6 and E7 viral oncoproteins that disrupt the Rb pathway in HeLa cells. Possessing an already aberrant Rb pathway may explain why HeLa cells are less sensitive to perturbation within this pathway. Multiple CDK/Cyclin complexes can phosphorylate Rb to promote its dissociaton from E2F to facilitate G1/S transition. Unphosphorylated Rb levels indicate a cell cycle block, which is observed with RASSF1A knockdown in both HBEC30 and HeLa cells (Figure 17D) and is in accordance with cell proliferation data (Figure 17C). To determine whether RASSF1A loss could inhibit growth in 3D culture, I transfected HeLa cells with shRASSF1A and plated cells in soft agar to allow for colony formation. I found that RASSF1A-deficient cells had reduced colony formation compared to control (Figure 17E). Moreover, codepletion of RASSF1A and p27

could rescue soft agar colony formation, supporting our hypothesis that cell cycle arrest upon RASSF1A loss is dependent on CDK inhibitors.

Knockdown of RASSF1A in multiple cell lines (HeLa, HBEC, MCF10A, BJ fibroblasts - data not shown) induces cell cycle delay, but not in all cell lines tested. In HCEC cell lines expressing either CDK4/hTERT for immortalization (Supplemental Figure 17A), CDK4/hTERT/Kras (Figure 17F), CDK4/hTERT/shp53 (Supplemental Figure 17B) or CDK4/hTERT/shp53/Kras (Supplemental Figure 17C), RASSF1A depletion enhanced proliferation as measured by BrdU incorporation. Moreover, with codepletion of RASSF1A and p21, I observed enhanced proliferation compared to either RASSF1A or p21 knockdown alone. Despite decreased SKP2 and elevated p21 levels, cells did not undergo cell cycle arrest in stark contrast to previous results in other cell lines. Since the RASSF1A-βTrCP-Emi1 pathway mediates the cell cycle, I investigated whether RASSF1A regulation of Emi1 is uncoupled in these cells. In opposition to what I observed in HeLa cells, I observed that RASSF1A depletion in HCECs led to an increase in Emi1 protein levels and an increase in phosphorylated Rb protein. To determine whether increased proliferation with RASSF1A knockdown was dependent on increased Emi1 levels, I codepleted RASSF1A and Emi1. Knockdown of Emi1 with RASSF1A abolished the increased proliferation observed with RASSF1A knockdown alone (Figure 17G), suggesting that enhanced proliferation is dependent on Emi1. These findings suggest that the RASSF1A-βTrCP-Emi1 arm of SKP2 regulation is disengaged in HCECs. However, RASSF1A depletion in HCECs still decreased REST (Figure 17H) and SKP2 (Figure 17F) protein levels, which suggests that the RASSF1A-βTrCP-REST-miR-21-SKP2

pathway is intact in HCECs. However, HCECs are still insufficient to engage G1/S arrest in the absence of destabilization of Emi1 and its inhibition of APC/C.

DISCUSSION

RASSF1A regulates multiple pathways involved in normal cell cycle progression (Figure 18). Epigenetic silencing of this gene may therefore disrupt many downstream signals affecting proliferation rates. A multitude of cancer cell lines and tumors show increased silencing of the RASSF1A promoter. Here, I describe that RASSF1A depletion can upregulate many oncogenic signals such as miR-21 expression and ERK activation and downregulate REST, a known tumor suppressor. Despite elevated growth signals observed with RASSF1A knockdown, I observed a decrease in proliferation accompanied by G1 cell cycle arrest. These results are corroborated by studies in uveal melanocytes in which RASSF1A loss promoted cell cycle arrest and senescence [9]. Also, in an shRNA screen, shRASSF1 was found to decrease proliferation of oncogenic Ras transformed cells and reduce tumor burden when these cells were transplanted into mice [88]. It is conceivably that acute silencing of RASSF1A engages a checkpoint mechanism because of mitogenic cues, which are aberrantly upregulated with RASSF1A loss. This checkpoint may prevent oncogenesis by blocking proliferation, and similar mechanisms of tumor suppression have been seen with PTEN Induced Cellular Senescence (PICS). Similar to PICS, RASSF1A-deficiency may engage a tumor suppressor phenotype by modulating SKP2 thereby upregulating its senescence associated downstream targets, p27 and p21.

Although I observed G1 arrest but not senescence in RASSF1A depleted BJ fibroblasts, recent studies in uveal melanocytes reported β-galactosidase staining (a hallmark of cellular senescence) in cells depleted of RASSF1A [9]. This senescence phenotype required increased p21 and ERK activity, which is strikingly reminiscent of our observations of increased p21 levels and MAPK activity when RASSF1A was transiently depleted from HeLa and HBEC cells. The discrepancy in β-galactosidase staining might be attributed to the role of MAPK regulating proliferation in fibroblasts but differentiation and pigement production in melanocytes. Therefore, RASSF1A loss may contribute to engagement of senescence, but is insufficient to engage all senescence characteristics in some cells. This is not unusual as a recent paper suggests there exists both weak and potent inducers of senescence, in which the PIK3CA/AKT pathway was a weak inducer of senescence [89]. Importantly, a weak inducer of senescence may have powerful effects on transformation.

RASSF1A may mediate a balance between pro and anti-proliferative signals. I propose when RASSF1A is silenced, that the balance between these opposing signals is disrupted. To counter the oncogenic signals that are released with RASSF1A loss, the cell engages checkpoint mechanisms to arrest the cell and protect from transformation, as I described with HeLa and HBEC30 cells. However, when the checkpoint is disengaged, as occurs in HCEC cells, I observed increased proliferation with RASSF1A depletion. Presumably, HCECs are not restricted by the G1 checkpoint due to uncoupling from the βTrCP-Emi1 arm of RASSF1A signaling. These results support our hypothesis that both arms of RASSF1A signaling must be

coupled to maintain cellular homestasis, and disruption in this coupling may, in part, explain the high frequency of RASSF1A inactivation in tumors.

Previous data suggest that RASSF1A loss is an early event in cancer progression. In concordance with Knudsen's "two-hit" hypothesis, I propose that early RASSF1A loss leads to upregulated mitogenic signals, which engages a G1 checkpoint (Figure 19). A cancer cell may acquire a second mutation to overcome the G1 checkpoint block, leading to tumorigenesis. Possible second hits may involve the AKT/PTEN pathway, SKP2 amplification, or SKP2 overexpression, which occur in many types of cancer. The PI3KCA/AKT pathway has already been described to suppress Ras induced senescence in human fibroblasts and may also provide an escape for cancer cells deficient in RASSF1A.

Further studies will focus on addressing these potential scenarios of tumor progression and may facilitate our understanding of the extremely high frequency of RASSF1A silencing in tumors. Importantly, future studies should focus on the therapeutic dangers of targeting miR-21, specifically in cells, which have lost RASSF1A. My findings strongly suggest that inhibition of miR-21 may be detrimental due to its ability to upregulate levels of Skp2 and downregulate CDKIs, p27 and p21, which could enhance growth. My results also support the use of SKP2 inhibitors to treat RASSF1A-deficient tumors. To test this hypothesis, a study could be performed in which RASSF1A -/- mice are treated with SKP2 inhibitors to determine whether SKP2 treatment reduces the rate of tumor formation. SKP2 inhibitors (MLN4924) are currently being tested in clinical trials [90] and it is of great interest to know if patient benefit will depend on RASSF1A methylation status.

Figure 13

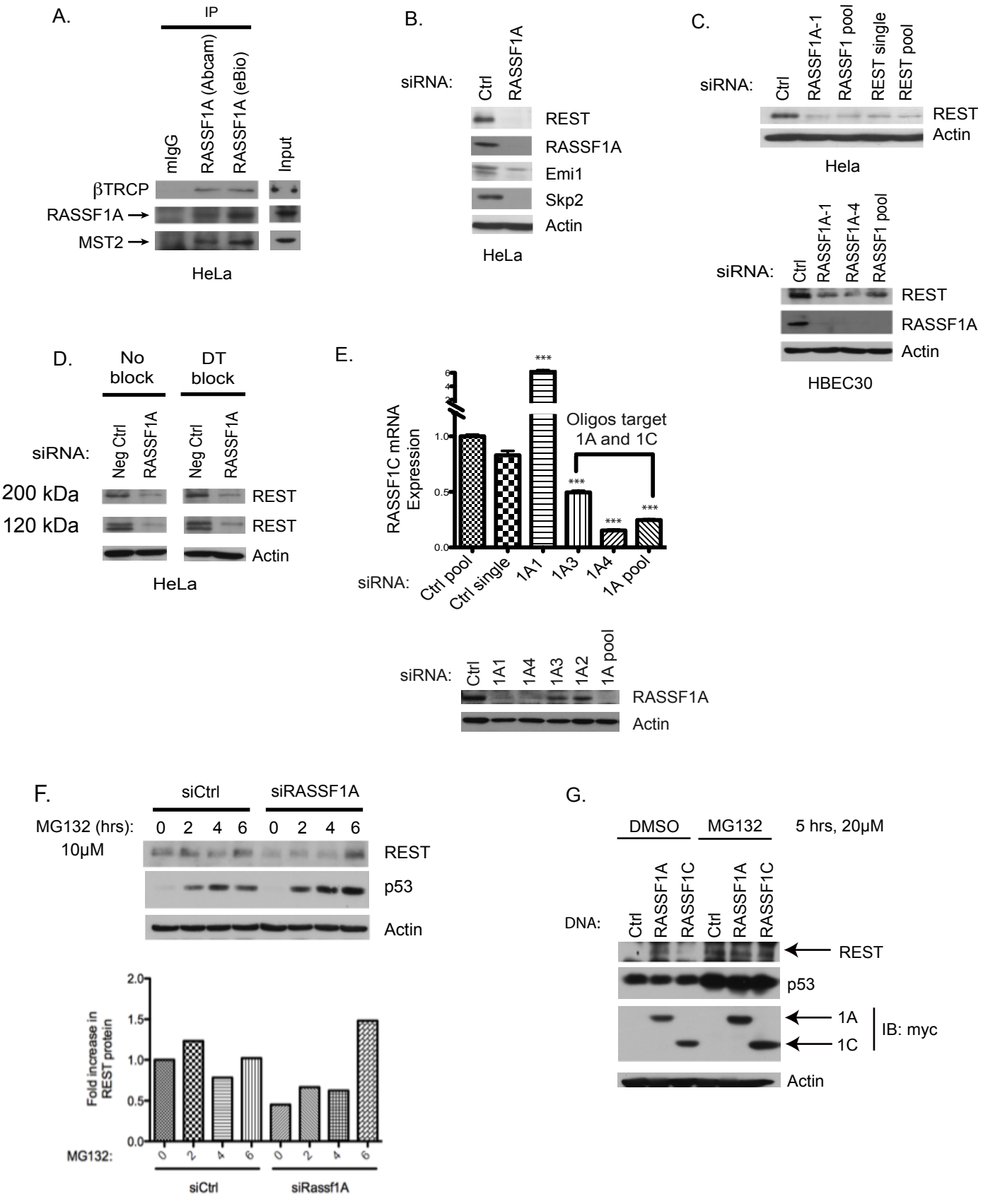

A.
IP
mIgG
RASSF1A (Abcam)
RASSF1A (eBio)
Input
βTRCP
RASSF1A
MST2
HeLa
B.
siRNA:
Ctrl
RASSF1A
REST
RASSF1A
Emi1
Skp2
Actin
HeLa
C.
siRNA:
Ctrl
RASSF1A-1
RASSF1 pool
REST single
REST pool
REST
Actin
Hela
siRNA:
Ctrl
RASSF1A-1
RASSF1A-4
RASSF1 pool
REST
RASSF1A
Actin
HBEC30
D.
No block
DT block
siRNA:
Neg Ctrl
RASSF1A
200 kDa
120 kDa
REST
REST
Actin
HeLa
E.
RASSF1C mRNA Expression
Oligos target 1A and 1C
siRNA:
Ctrl pool
Ctrl single
1A1
1A3
1A4
1A pool
siRNA:
Ctrl
1A1
1A4
1A3
1A2
1A pool
RASSF1A
Actin
F.
siCtrl
siRASSF1A
MG132 (hrs):
0 2 4 6 0 2 4 6
10μM
REST
p53
Actin
Fold increase in REST protein
MG132:
siCtrl
siRassf1A
G.
DMSO
MG132
5 hrs, 20μM
DNA:
Ctrl
RASSF1A
RASSF1C
REST
p53
1A
1C
IB: myc
Actin

Figure 13: RASSF1A regulates the REST tumor suppressor.

A: Hela cells were lysed and mouse IgG (control) or RASSF1A antibodies were used for co-immunoprecipitation assay. Immunoprecipitates were blotted for indicated proteins.

B: siRNAs targeting Control or RASSF1A were transfected into HeLa cells. 72 hours after transfection, lysates were immunoblotted with indicated antibodies. Actin was blotted as a loading control.

C: Multiple siRNAs targeting RASSF1A or REST were transfected into HeLa and immunblotted with indicated antibodies.

D: Asynchronous and HeLa cells synchronized by double thymidine block were transfected with siCtrl and siRASSF1A. Two REST bands appeared at 200 and 120 kDa and were confirmed by siREST (data not shown).

E: si-RASSF1A oligos were transfected into HeLa cells. RASSF1C mRNA was measured by qPCR (top panel) and RASSF1A levels were measured by western blot (bottom panel).

F: HeLa cells were transfected with siCtrl or siRASSF1A. At the indicated times prior to lysis, MG132 (10μM) was added to cells. Quantification of protein levels was performed using Image J analysis.

G: RASSF1A-myc and RASSF1C-myc plasmids were expressed in HeLa cells and treated with DMSO (control) or MG132 (20μM) for 5 hours prior to lysis. Lysates were blotted for indicated proteins.

Figure 14

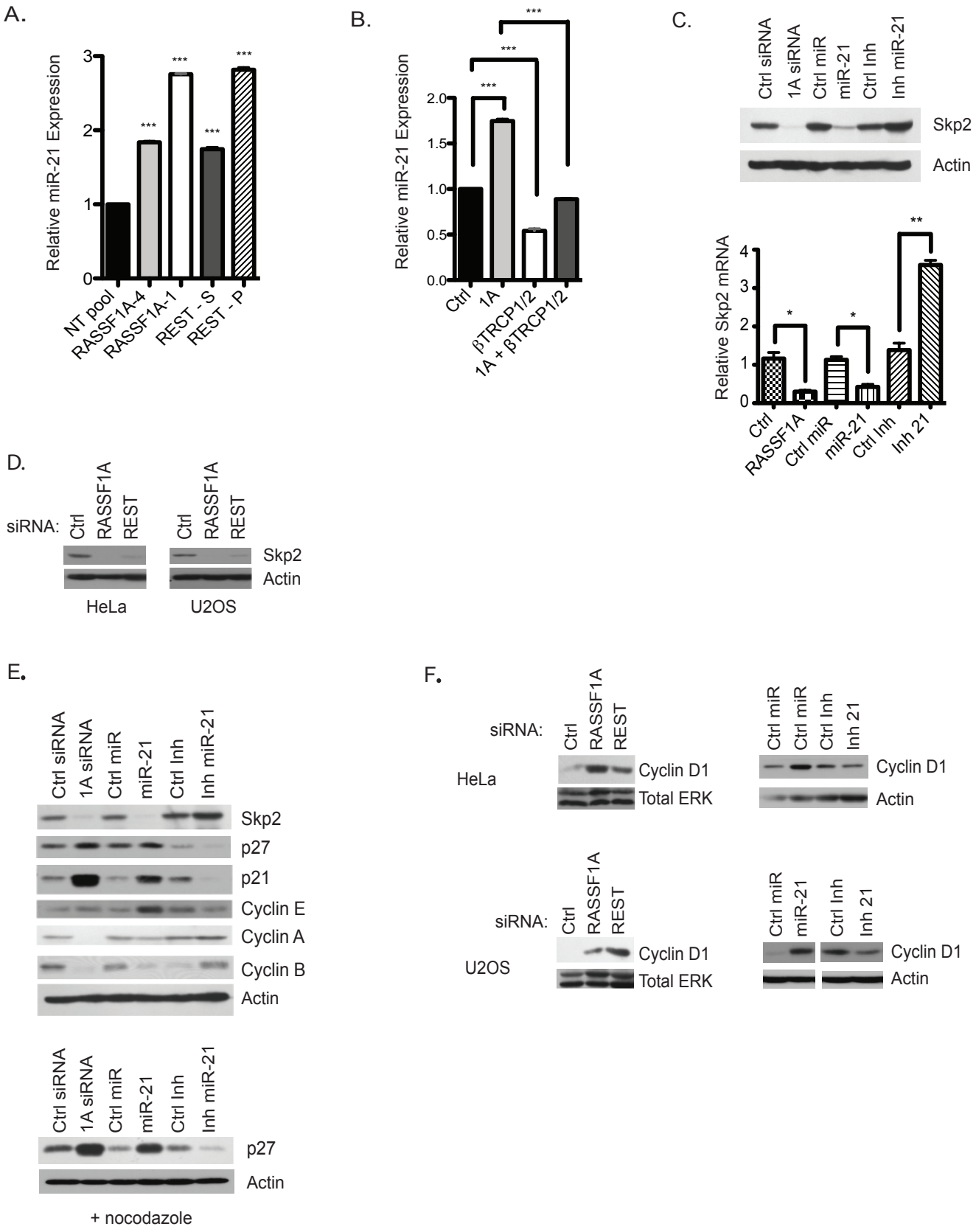

A.
Relative miR-21 Expression
NT pool
RASSF1A-4
RASSF1A-1
REST - S
REST - P
B.
Relative miR-21 Expression
Ctrl
1A
βTRCP1/2
1A + βTRCP1/2
C.
Ctrl siRNA
1A siRNA
Ctrl miR
miR-21
Ctrl Inh
Inh miR-21
Skp2
Actin
Relative Skp2 mRNA
Ctrl
RASSF1A
Ctrl miR
miR-21
Ctrl Inh
Inh 21
D.
siRNA:
Ctrl
RASSF1A
REST
Skp2
Actin
HeLa
U2OS
E.
Ctrl siRNA
1A siRNA
Ctrl miR
miR-21
Ctrl Inh
Inh miR-21
Skp2
p27
p21
Cyclin E
Cyclin A
Cyclin B
Actin
p27
Actin
+ nocodazole
F.
siRNA:
Ctrl
RASSF1A
REST
HeLa
Cyclin D1
Total ERK
Ctrl miR
Ctrl miR
Ctrl Inh
Inh 21
Cyclin D1
Actin
siRNA:
U2OS
Cyclin D1
Total ERK
Ctrl miR
miR-21
Ctrl Inh
Inh 21
Cyclin D1
Actin

Figure 14: RASSF1A suppresses miR-21 expression; RASSF1A and miR-21 regulate SKP2 and SKP2 substrates.

A: HeLa cells were transfected with indicated siRNAs. MiR-21 levels were measured by qPCR.

B: Same as in (A).

C: HeLa cells were transfected with indicated siRNA or miRNA. Ctrl miR was used as a control miRNA mimic and Ctrl Inh was used as a control miRNA inhibitor. Lysates were collected for protein analysis by western blot (top panel) and mRNA analysis by qPCR (bottom panel).

D: Indicated siRNAs were transfected into HeLa or U2OS cells and lysates were blotted with indicated antibodies.

E: Indicated siRNAs or miRNAs were transfected into HeLa cells and lysates were collected for western blot analysis with indicated antibodies (top panel). HeLa cells were treated with nocodazole (100ng/ml) for 16 hours prior to lysis (bottom panel) and collected for western blot for indicated antibodies.

F: HeLa or U2OS cells were transfected with indicated siRNAs or miRNAs and harvested for western blot.

Figure 15

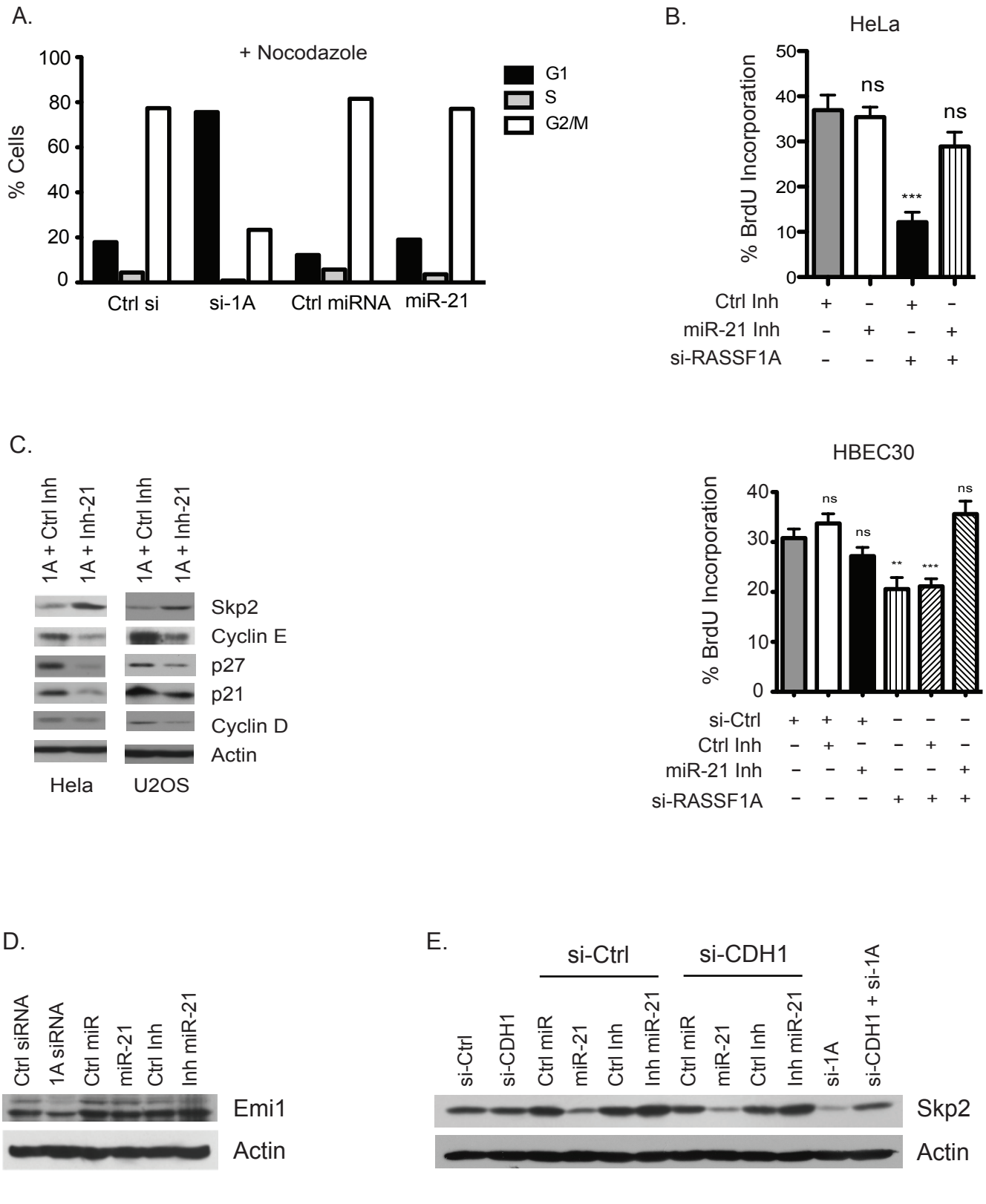

A.
+ Nocodazole
% Cells
100
80
60
40
20
0
G1
S
G2/M
Ctrl si
si-1A
Ctrl miRNA
miR-21
B.
HeLa
% BrdU Incorporation
50
40
30
20
10
0
ns
ns
***
Ctrl Inh + – + –
miR-21 Inh – + – +
si-RASSF1A – – + +
C.
1A + Ctrl Inh
1A + Inh-21
1A + Ctrl Inh
1A + Inh-21
Skp2
Cyclin E
p27
p21
Cyclin D
Actin
Hela
U2OS
HBEC30
% BrdU Incorporation
40
30
20
10
0
ns
ns
ns
**
***
si-Ctrl + + + – – –
Ctrl Inh – + – – + –
miR-21 Inh – – + – – +
si-RASSF1A – – – + + +
D.
Ctrl siRNA
1A siRNA
Ctrl miR
miR-21
Ctrl Inh
Inh miR-21
Emi1
Actin
E.
si-Ctrl
si-CDH1
si-Ctrl
si-CDH1
Ctrl miR
miR-21
Ctrl Inh
Inh miR-21
Ctrl miR
miR-21
Ctrl Inh
Inh miR-21
si-1A
si-CDH1 + si-1A
Skp2
Actin

Figure 15: Mir-21 mediates RASSF1A regulation of SKP2 and proliferation.

A: HeLa cells were transfected and treated with nocodazole (100ng/ml) for 16 hours prior to lysis. Cells were stained with propidium iodide and collected for FACS analysis 72 hours after transfection.

B: HeLa and HBEC30 cells were transfected and incubated with BrdU for 5 hours (HeLa) or 20 hours (HBEC30). BrdU assay was performed and BrdU incorporation was counted using Image J software.

C: HeLa and U2OS cells were co-transfected with si-RASSF1A and and miR-21 inhibitor. Cells were lysed 72 hrs after transfection and blotted for indicated antibodies.

D: HeLa cells were transfected with siRNA or miRNA and blotted for indicated antibodies.

E: Same as in (D).

Figure 16

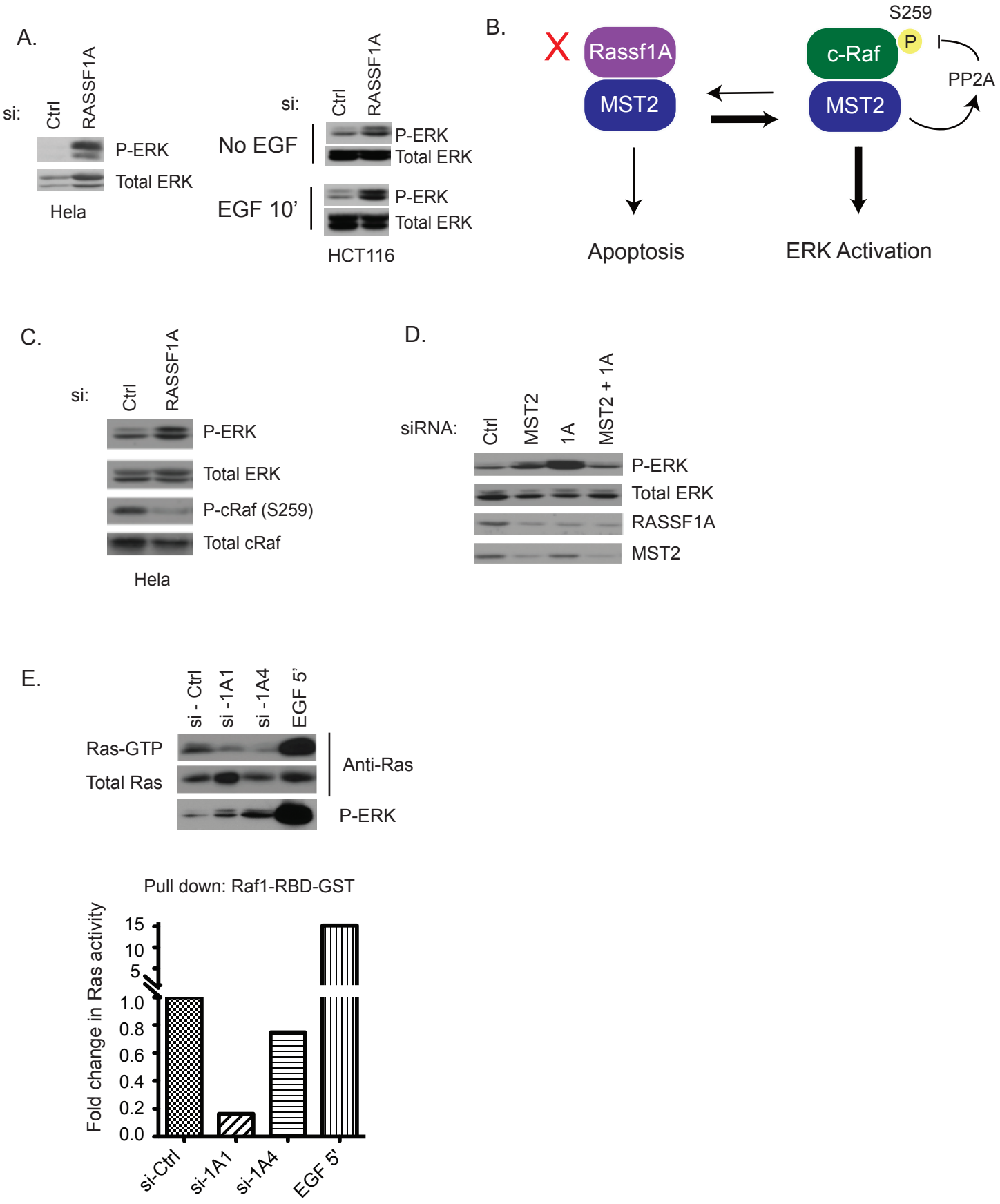

A.
si:
Ctrl
RASSF1A
P-ERK
Total ERK
Hela
si:
Ctrl
RASSF1A
No EGF
P-ERK
Total ERK
EGF 10'
P-ERK
Total ERK
HCT116
B.
X
Rassf1A
MST2
Apoptosis
S259
c-Raf
P
PP2A
MST2
ERK Activation
C.
si:
Ctrl
RASSF1A
P-ERK
Total ERK
P-cRaf (S259)
Total cRaf
Hela
D.
siRNA:
Ctrl
MST2
1A
MST2 + 1A
P-ERK
Total ERK
RASSF1A
MST2
E.
si - Ctrl
si -1A1
si -1A4
EGF 5'
Ras-GTP
Total Ras
Anti-Ras
P-ERK
Pull down: Raf1-RBD-GST
Fold change in Ras activity
15
10
5
1.0
0.8
0.6
0.4
0.2
0.0
si-Ctrl
si-1A1
si-1A4
EGF 5'

Figure 16: RASSF1A suppresses ERK activation by binding to MST2.

A: HeLa cells were transfected with siRNA and immunoblotted with indicated antibodies (left panel). HCT116 cells were transfected with indicated siRNAs and stimulated with and without EGF for 10 minutes prior to lysis. Lysates were immunoblotted for indicated antibodies (right panel).

B: Model of MST2 interaction with RASSF1A and c-Raf.

C: HeLa cells were transfected with indicated siRNAs and immunoblotted for indicated antibodies.

D: Same as in (C).

E: Ras-GTP levels were measured by performing Ras activity assay in HeLa cells. Raf1-RBD-GST beads were incubated with lysates transfected with indicated siRNAs and blotted for Ras. Cells were stimulated with EGF for 5 minutes as a positive control (top panel). Quantification of proteins was measured using Image J software (bottom panel).

Figure 17

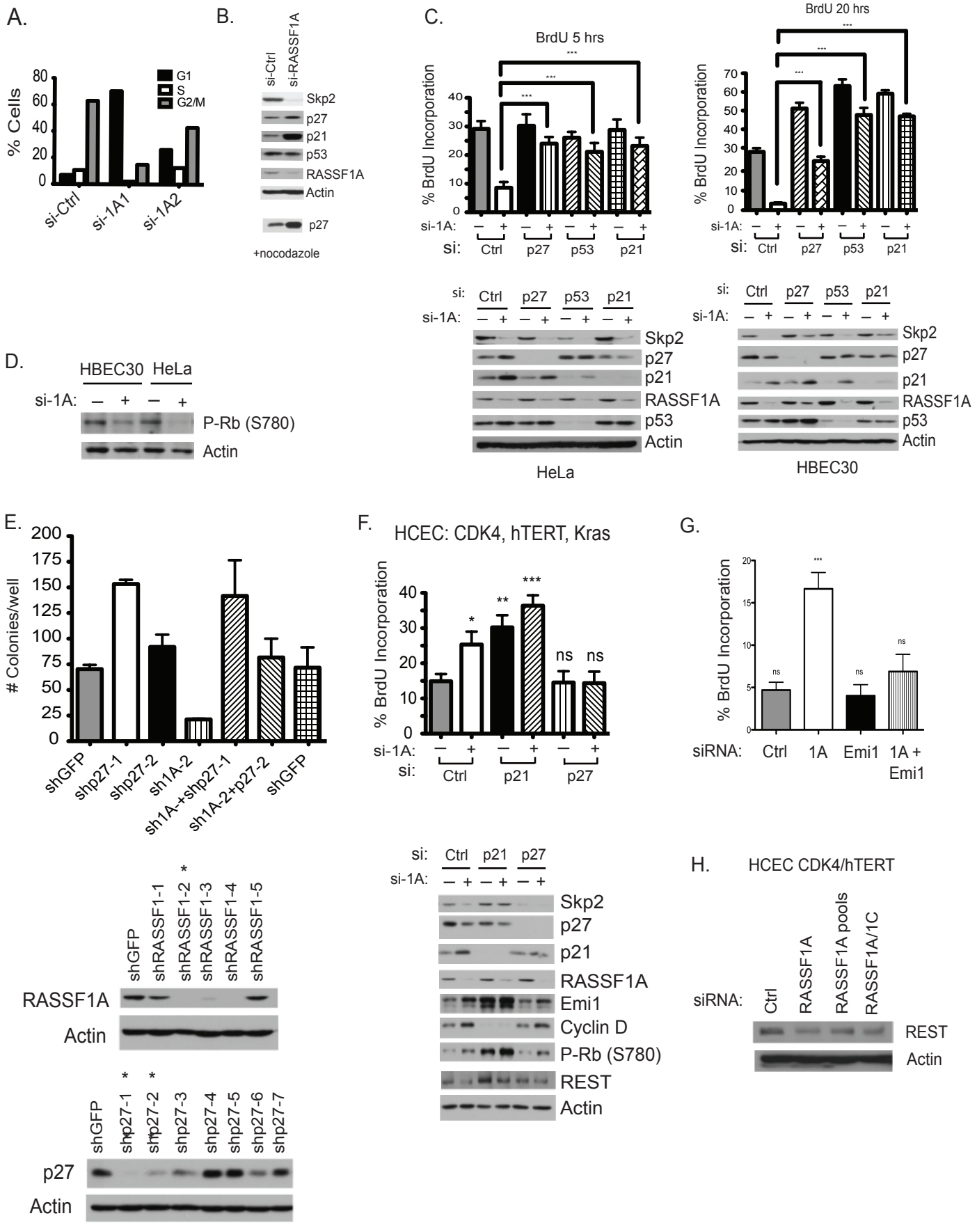

A.
% Cells
G1
S
G2/M
si-Ctrl
si-1A1
si-1A2
B.
si-Ctrl
si-RASSF1A
Skp2
p27
p21
p53
RASSF1A
Actin
p27
+nocodazole
C.
BrdU 5 hrs
BrdU 20 hrs
% BrdU Incorporation
si-1A:
si:
Ctrl
p27
p53
p21
Skp2
p27
p21
RASSF1A
p53
Actin
HeLa
HBEC30
D.
HBEC30
HeLa
si-1A:
P-Rb (S780)
Actin
E.
Colonies/well
shGFP
shp27-1
shp27-2
sh1A-2
sh1A-+shp27-1
sh1A-2+p27-2
shGFP
shGFP
shRASSF1-1
shRASSF1-2
shRASSF1-3
shRASSF1-4
shRASSF1-5
RASSF1A
Actin
shGFP
shp27-1
shp27-2
shp27-3
shp27-4
shp27-5
shp27-6
shp27-7
p27
Actin
F.
HCEC: CDK4, hTERT, Kras
% BrdU Incorporation
si-1A:
si:
Ctrl
p21
p27
Skp2
p27
p21
RASSF1A
Emi1
Cyclin D
P-Rb (S780)
REST
Actin
G.
% BrdU Incorporation
siRNA:
Ctrl
1A
Emi1
1A + Emi1
H.
HCEC CDK4/hTERT
siRNA:
Ctrl
RASSF1A
RASSF1A pools
RASSF1A/1C
REST
Actin

Figure 17: RASSF1A promotes cell cycle progression by promoting degradation of CDKIs.

A: HeLa cells were transfected with indicated siRNA. 72 hours after transfection, cells were stained with propidium iodide for FACS analysis.

B: HeLa cells were transfected with indicated siRNAs and immunoblotted with indicated antibodies (top panel). Cells were treated with nocodazole (100ng/ml) for 16 hours before lysis and blotted for p27.

C: HeLa and HBEC30 cells were transfected with indicated siRNAs. BrdU was added 5 hours (HeLa) and 20 hours (HBEC30) prior to fixation and lysis. BrdU assay was performed and BrdU incorporation was measured using Image J software (top panels). Cells were transfected and and blotted for indicated antibodies.

D: HBEC30 and HeLa cells were transfected and immunoblotted with indicated antibodies.

E: HeLa cells were infected with indicated shRNAs and plated in soft agar. Two weeks after plating, colonies were counted (top panel). Western blots of knockdown efficiency of shRNAs used for soft agar colony formation assay. ShRASSF1A-2 was used to stably knockdown RASSF1A. Shp27-1 and shp27-2 were used to stably knockdown p27 (bottom panels).

F: HCECs (CDK4, hTERT, Kras) were transfected with indicated siRNAs. BrdU was added to cells for 5 hours prior to harvest. BrdU assay was performed and BrdU incorporation was measured using Image J software (top panel). Lysates (from top panel) were immunoblotted for indicated antibodies.

G: HCEC (CDK4, hTERT) cells were treated as in (F).

H: HCEC (CDK4, hTERT) cells were transfected with si-RASSF1A oligos and blotted for indicated antibodies.

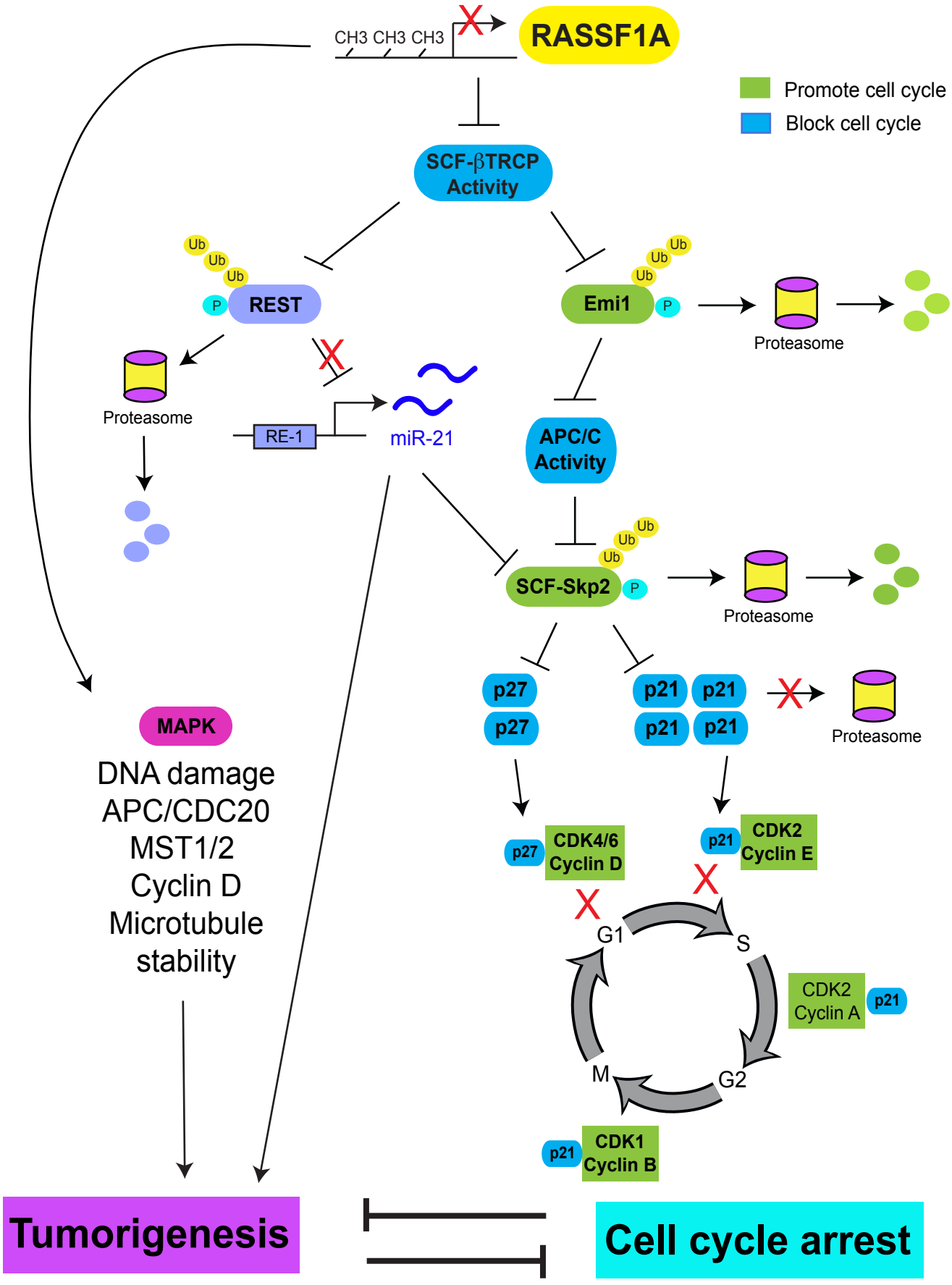

CH3 CH3 CH3
RASSF1A
Promote cell cycle
Block cell cycle
SCF-βTRCP Activity
Ub
P
REST
Emi1
Proteasome
RE-1
miR-21
APC/C Activity
SCF-Skp2
p27
p21
MAPK
DNA damage
APC/CDC20
MST1/2
Cyclin D
Microtubule stability
CDK4/6 Cyclin D
CDK2 Cyclin E
CDK2 Cyclin A
CDK1 Cyclin B
G1
S
G2
M
Tumorigenesis
Cell cycle arrest

Figure 18: RASSF1A regulates multiple signaling pathways to maintain cellular homeostasis. Imbalance of this homeostasis may lead to tumorigenesis if not regulated by checkpoint mechanisms. I hypothesize that RASSF1A loss releases oncogenic signals that are suppressed by RASSF1A regulation of βTRCP-Emi1 to restrain cells from unregulated growth.

Figure 19

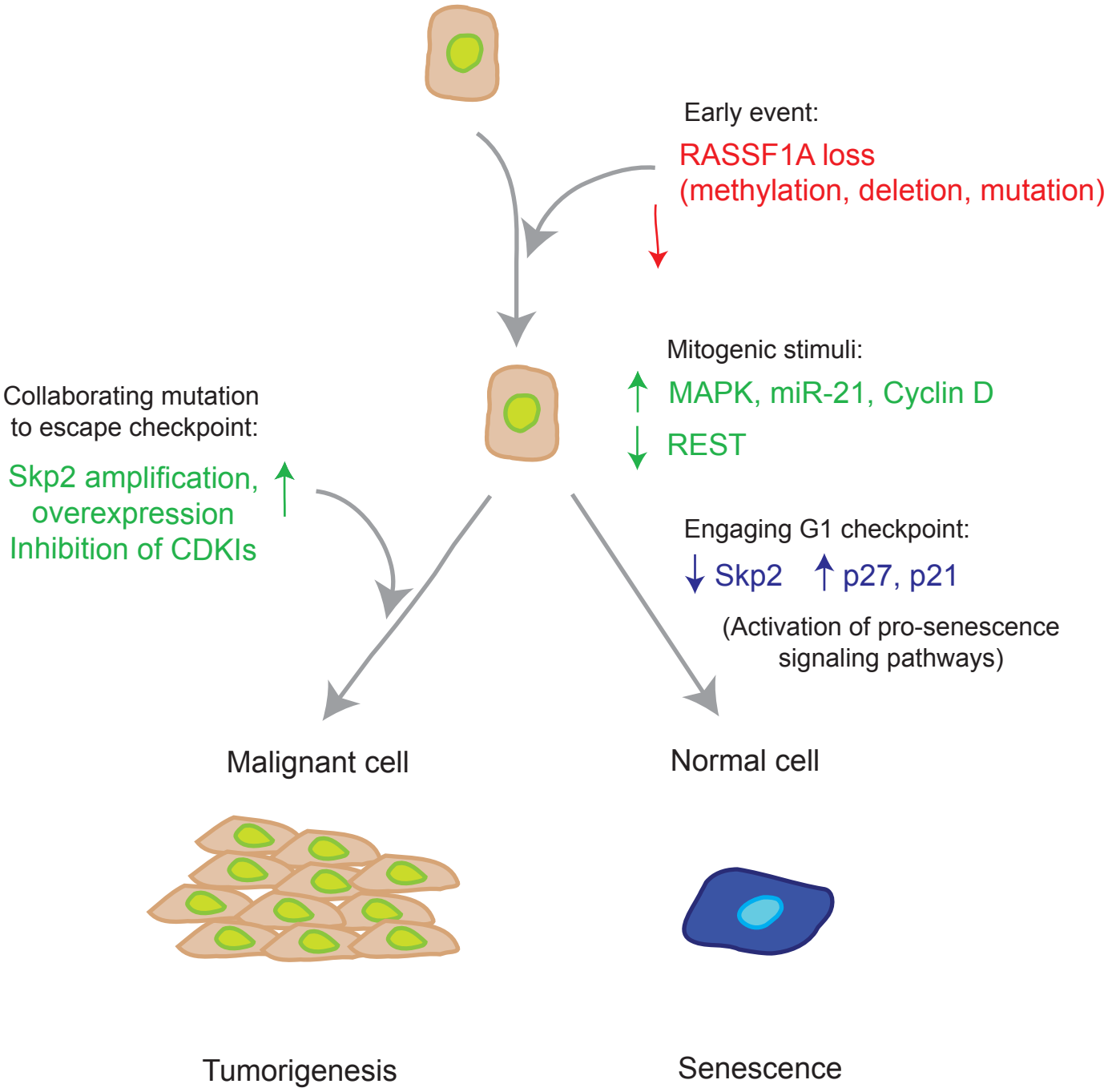
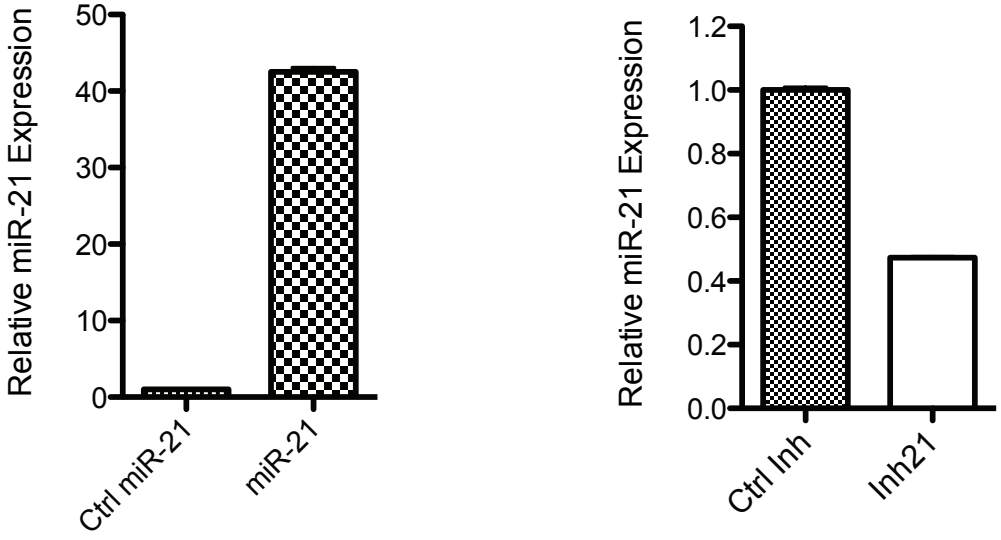

Early event:
RASSF1A loss
(methylation, deletion, mutation)
Mitogenic stimuli:
MAPK, miR-21, Cyclin D
REST
Collaborating mutation
to escape checkpoint:
Skp2 amplification,
overexpression
Inhibition of CDKIs
Engaging G1 checkpoint:
Skp2
p27, p21
(Activation of pro-senescence
signaling pathways)
Malignant cell
Normal cell
Tumorigenesis
Senescence

Figure 19: A model for RASSF1A tumor progression.

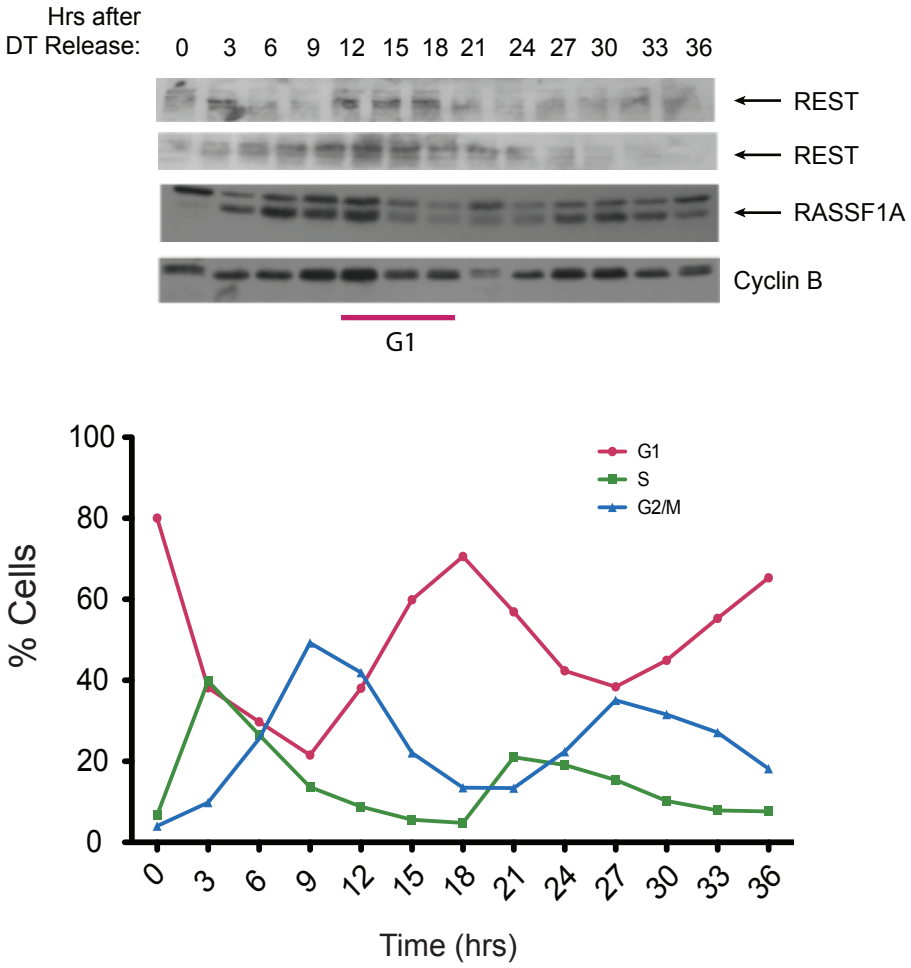
RASSF1A epigenetic silencing is thought to be an early event in tumorigenesis. With RASSF1A loss, cells possess increased mitogenic stimuli, which can engage the G1 checkpoint. However, if a cell acquires an additional mutation, such as SKP2 overexpression or inhibition of CDKIs, to escape the checkpoint, cells that have oncogenic properties may be unleashed and develop into cancer.

Supplemental Figure 3.



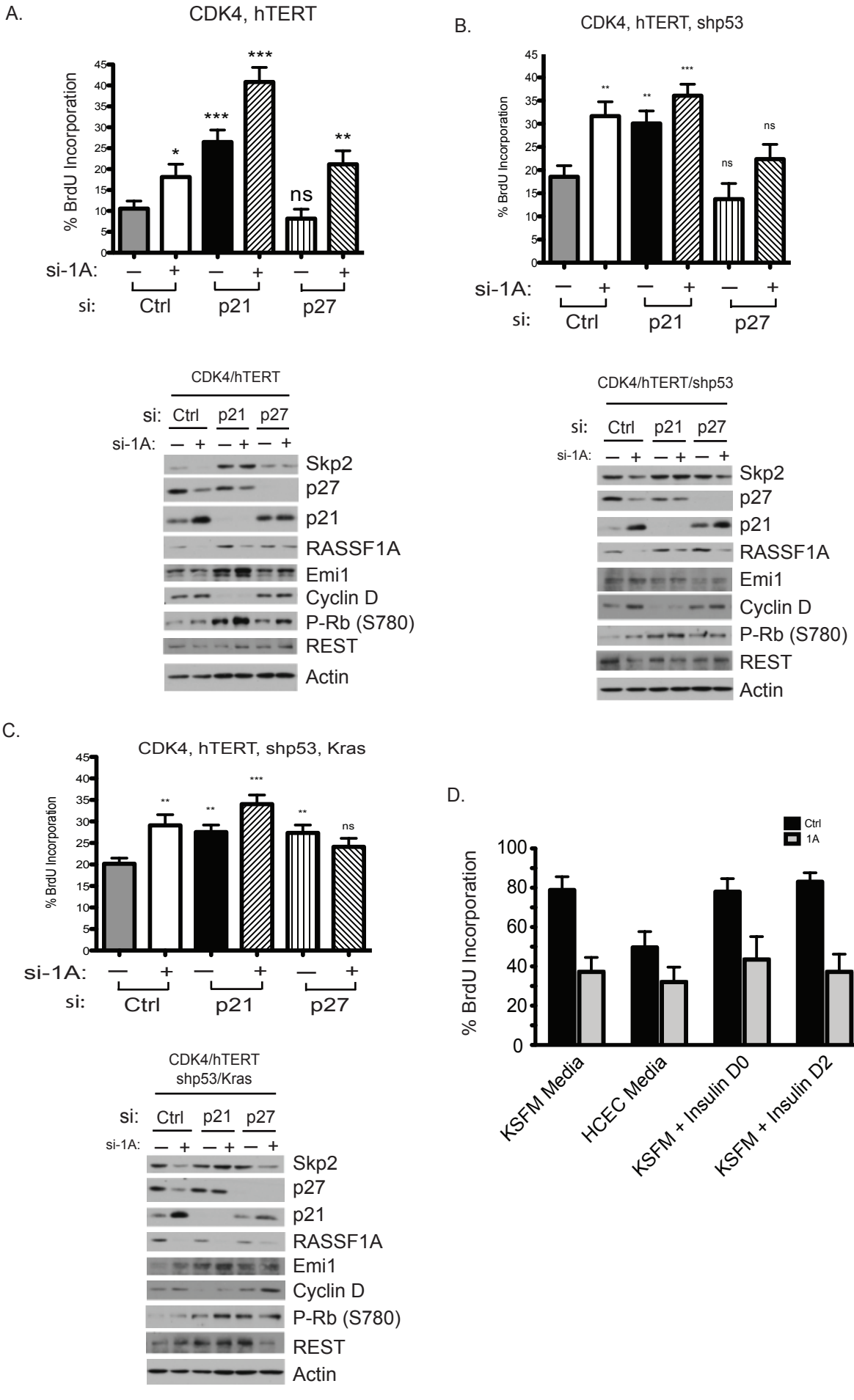
MiR-21 mimic and inhibitor were transfected into HeLa cells.
72 hours post transfection, miR-21 levels were measured by qPCR.

Supplemental Figure 13.



HeLa cells were synchronized at the G1/S transition by double thymidine block.
Cells were released and harvested every 3 hours.
Cells were immunoblotted for indicated antibodies and stained with propidium iodide for FACS analysis.

Supplemental Figure 17.



Supplemental Figure 17: Disengagement of the βTRCP-Emi1 pathway in HCECs, allows for enhanced proliferation in RASSF1A-deficient cells.

A: HCEC (CDK4, hTERT) cells were transfected with indicated siRNAs. BrdU was added to cells 5 hours prior to harvest. BrdU assay was performed and BrdU incorporation was measured using Image J software (top panel). Lysates (from top panel) were lysed and immunoblotted with indicated antibodies (bottom panel).

B: HCEC (CDK4, hTERT, shp53) cells were treated as in (A).

C: HCEC (CDK4, hTERT, shp53, Kras) cells were treated as in (A).

D: HBEC30 cells were transfected with siRNA and grown in KSFM (HBEC30 media), HCEC media, KSFM + Insulin (10μg/ml) added from Day 0 after transfection, or with KSFM + Insulin (10μg/ml) added on Day 2 after transfection. BrdU was added 20 hours prior to harvest and BrdU incorporation was measured using Image J software.

CONCLUDING REMARKS AND FUTURE STUDIES

RASSF1A mediates a balance between pro and anti-proliferative signals. Here, I describe RASSF1A suppression of growth signals, including: inhibition of Cyclin D and miR-21 expression, MAPK activation, and promoting apoptosis and microtubule stabilization (Figure 20). At the same time, RASSF1A promotes cell cycle progression by restraining the $SCF^{\beta TrCP}$ complex to stabilize Emi1 and SKP2 levels, which mediate degradation of CDKIs to allow for unimpeded G1/S transition. Upon RASSF1A loss, which is frequent in many tumors, the balance between these opposing signaling pathways may disrupt cellular integrity. Therefore, maintaining a balanced, well-regulated G1/S checkpoint may be crucial to prevent tumor formation. When this balance is uncoupled, as seen in HCEC cells, RASSF1A deficiency can enhance proliferation and further studies should be done to determine whether other tumorigenic properties are additionally enhanced. To investigate how RASSF1A loss increases proliferation in HCECs, miR-21 levels should be checked as well as APC/C substrates. With RASSF1A deficiency, increased Emi1 levels may inhibit APC/C activity allowing for increased stability of APC/C substrates, including Cyclin A and B. Increased cyclin accumulation may promote enhanced proliferation when RASSF1A is lost in HCECs. These results could help our understanding of the tumorigenic nature of RASSF1A deficiency in cancer development.

I have shown that RASSF1A associates with βTrCP by co-immunoprecipitation assay. Subsequent studies should identify which domains of RASSF1A and βTrCP bind and how this may impair $SCF^{\beta TrCP}$ activity. As mentioned in Chapter 1, mTOR has been described to phosphorylate the RA domain of RASSF1A. It is conceivable

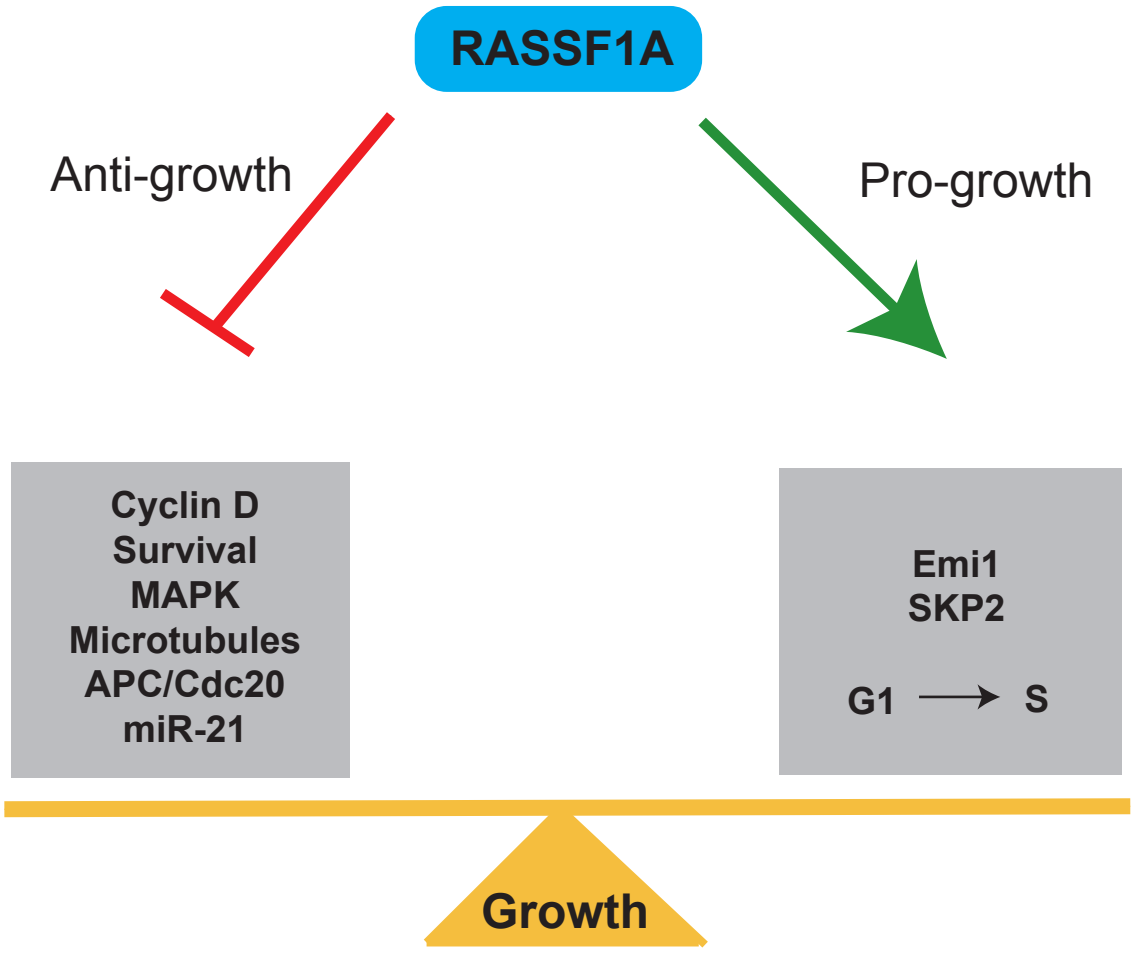
that mTOR may signal through RASSF1A to mediate cellular adaptation to nutrient rich conditions. To determine whether RASSF1A may mediate mTOR nutrient sensing, one could do co-immunoprecipitation assays with amino acid stimulation to see if this would enhance RASSF1A-βTrCP interaction. In addition, $SCF^{\beta TrCP}$ has been described to ubiquitinate the mTOR inhibitor, DEPTOR. Conceivably, RASSF1A may restrain $SCF^{\beta TrCP}$ activity towards DEPTOR, allowing for increased inhibition of mTOR. Because mTOR was shown to phosphorylate the RA domain of RASSF1A, mTOR may provide positive or negative feedback on RASSF1A restraint of $SCF^{\beta TrCP}$. Subsequent studies should be done to determine whether phosphorylation of RASSF1A by mTOR activates or inhibits the mTOR signaling pathway. Conceivably, RASSF1A may bind βTrCP upon serum-rich conditions to promote G1/S progression. Additional experiments could be done by performing Co-IP under serum-starved versus serum-fed conditions to see whether the interaction between RASSF1A-βTrCP is dependent on the presence of serum.

Currently, miR-21 inhibition is being sought as a treatment for cancer. Despite evidence that miR-21 does promote oncogenic properties *in vitro* and *in vivo*, here I show that miR-21 inhibition may enhance proliferation in RASSF1A depleted cells. In my hands, miR-21 inhibition upregulated SKP2 expression to allow for increased CDKI degradation. Because RASSF1A is frequently lost in cancers, targeting miR-21 should be approached with caution until further studies may be performed to determine the therapeutic benefits of miR-21 inhibition.

Future studies should focus on determining what other collaborating mutations may occur with RASSF1A loss to cause transformation. I wondered whether possible

second hits could include mutation or amplification of RAS, AKT, or SKP2. RAS is an upstream regulator of AKT, which has been described to phosphorylate p21 in order to re-localize p21 to the cytoplasm where it no longer inhibits CDK activity. SKP2 is overexpressed in cancers due to gene amplification and may counteract RASSF1A loss by downregulating CDKI levels, which are induced upon RASSF1A loss. To test this hypothesis, I depleted RASSF1A and expressed RAS, AKT, or SKP2 in HBEC30 cells and assayed proliferation by measuring BrdU incorporation. I observed no rescue of cell proliferation of RASSF1A-deficient cells when RAS, AKT, or SKP2 were expressed. It may be that overexpression of RAS, AKT, or SKP2 is insufficient to overcome the G1 arrest and other additional hits may cooperate with RASSF1A inactivation. However, I did not check p21 localization to determine whether RAS and AKT did in fact re-localize p21 to the cytoplasm. SKP2 was overexpressed, but there was little downregulation of p27 and p21 levels. SKP2 may need to be expressed at higher levels to get sufficient inhibition of p27 and p21 levels. Another possibility is that the kinase that phosphorylates p27 and p21, for recognition and subsequent ubiquitination by SKP2, may be limiting. Instead of using siRNA to knockdown RASSF1A, it may be necessary to stably knockdown RASSF1A with shRNAs to see a cooperating rescue in proliferation. In addition, ID-1 (Inhibitor of DNA binding 1) is reported to inhibit p21 activity despite elevated p21 levels although the mechanism is unclear [91]. ID-1 might account for the increase in proliferation seen in HCECs with RASSF1A loss despite possessing elevated p21 levels. ID-1 protein levels could be measured in HCEC cells with RASSF1A knockdown to investigate this hypothesis.

In vivo experiments should also be performed to test whether tumors from RASSF1A-/- mice are sensitive to SKP2 inhibitors. RASSF1A-/- mice develop spontaneous and carcinogen-induced tumors at a higher rate compared to RASSF1A+/+. RASSF1A-/- mice may overcome G1 checkpoint mechanisms induced upon RASSF1A loss by downregulating CDK Inhibitors through SKP2 overexpression. To test this, RASSF1A-/- could be treated with SKP2 inhibitors to determine whether these mice develop tumors at a lower frequency compared to untreated mice. If these results suggest that SKP2 inhibitors are useful in preventing transformation in RASSF1A-/- mice, then more clinical studies could be done. By screening tumors for RASSF1A inactivation, we may target signaling pathways, such as SKP2, that help to overcome cell cycle checkpoints in RASSF1A-deficient cells. By identifying potential vulnerabilities within individual tumors, I hope my work contributes to improving clinical outcome for cancer patients.



RASSF1A modulates a balance between pro and anti-growth signals.
Tipping the scale in one direction may be counterbalanced by opposing signals.

MATERIALS AND METHODS

Yeast two-hybrid library screening and pairwise interaction assay. RASSF1A (amino acids 1-210), lacking the Ras Association domain was used as bait in a yeast two-hybrid screen of a HeLa cDNA library in the yeast reporter strain L40 using standard methods [92].

Cell Culture. HeLa cells were maintained in Dulbecco's Modified Eagle Serum (DMEM) supplemented with 10 % FBS. MCF10A cells were maintained in Mammary Epthileal Cell Basal Media (MEBM) supplemented with EGF and Bovine Pituitary Extract (Cambrex). A549 cells were maintained in RPMI + 5 % FBS. U2OS and HCT116 cells were purchased from ATCC and grown in 10% McCoy's media. HBEC30 (Human bronchial epithelial cells) cells were grown in Keratinocyte-SFM media plus supplements. HCEC (Human colonic epithelial cells) cells were grown as described elsewhere [93].

siRNAs. siRNA sequences were as follows: RASSF1A-1: GACCUCUGUGGCGACUUCATT and RASSF1A-2: CACGUGGUGCGACCUCUGU; EWS: GACUCUGACAACAGUGCAATT, and AAUGGCGUCCACGGAUUAC; Emi1: GAUGCUCAAACCAAGUUAU, CDH1: GAAGGGUCUGUUCACGUAU. For βTRCP silencing, the SMARTpool™ was obtained from Dharmacon (Lafayette, CO). The following siRNAs were custom ordered from Dharmacon: RASSF1A-3: 5'-UGUGGAGUGGGAGACACCUUU-3', RASSF1A-4 [67]: 5'-UCUUCUGCUCAAUCUCAGC-3', REST single [71]: 5'-GGGCCUAAACCUCUUAAUU-3'; MST2 (ref): 5'-UUGCGACAACUUGACCGGAUU-3'. SiGenome pools fo the following genes

were ordered from Dharmacon: RASSF1, CDH1, p27 pools, p21 pools, p53 pools, REST pools. For negative control siRNA, I used: Non-targeting pool #2 (Catalog # D-001206-14-20) from Dharmacon.

MicroRNA sequences: Mimic Negative Control #1: Catalog #CN-001000-01-05; miR-21 mimic: 5'- UAGCUUAUCAGACUGAUGUUGA – 3'; Hairpin Inhibitor Negative Control #1: Catalog IN-001005-01-05; miR-21 Hairpin inhibitor: 5'-UAGCUUAUCAGACUGAUGUUGA-3'

Reagents. MG132 (C2211) was purchased from Sigma. Rabbit Antibodies against RASSF1A and C were generated at previously described[3]. Polyclonal antibody 4169 was generated using a combination of genetic and peptide immunization procedures. cDNA encoding amino acid 1-119 of RASSF1A was inserted into mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). 1mg of the plasmid DNA was initially intra-splenic (IS) injected into the rabbit. Subsequently, 1 mg of the DNA was intra-muscular (IM) injected 30 and 45 days after the IS injection. The rabbit was subcutaneously (SC) injected with a KLH conjugated peptide PAGRAGKGRTRLERANALRIA corresponding to amino acid 15-35 of RASSF1A 14 days after the second IM injection. Monoclonal antibody G5 was generated using a peptide specific to RASSF1A (SGEPELIELRELAPAGRAGKGR, corresponding to amino acid 2-22 of RASSF1A). BALB/C mice were injected IS with 100ug of KLH-conjugated peptide. The mouse was boosted four times with 60ug of the KLH-conjugated peptide by SC injection during a 90 days period. Mouse anti-RASSF1A was purchased from Abcam (ab23950) and eBioscience (#14-6888-82). Antibodies

for cyclin A, cyclin B, EWS, FUS/TLS, p53 (sc-126), and ERK2 were from Santa Cruz. Anti-SKP2 and Emi1 were from Zymed and anti-CDH1 was from LabVision Corporation. Mouse anti-BTRCP (#37-3400) was purchased from Zymed. Mouse anti-SKP2 (#32-3300) and Rabbit anti-Emi1 (#38-5000) were purchased from Zymed (Invitrogen). Rabbit anti-REST (#07-579) was purchased from Millipore. Mouse anti-Actin (A1978) was from Sigma. Mouse anti-Ras was purchased from BD Transduction Labs. Rabbit anti-MST2, Rabbit anti-p27 (#3686), Rabbit anti-p21 (#2947), Rabbit anti-p16 (#4824), Mouse anti-Cyclin E (#4129), Mouse anti-Cyclin D1 (#2926), Mouse anti-Cyclin B (#4135), Rabbit anti-phospho-ERK (#9101), and Rabbit anti-ERK (#9102) were purchased from Cell Signaling. For immunofluorescence, anti-BrdU-488 (B35130) was purchased from Invitrogen. Monoclonal Anti-BrdU was obtained from Becton Dickinson. Alexa 488 (Molecular Probes) was used as a secondary antibody for fluorescent labeling.

Transfection. Cells were transfected with RNAiMAX (Invitrogen) according to manufacturer's protocol and harvested 72 hours later.

Immunoblotting. Cells were lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, protease inhibitiors, B-mercaptoethanol) or SDS buffer (100mM Tris pH 6.8, 4% SDS, 20% glycerol, protease inhibitors, B-mercaptoethanol). Samples were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene (PVDF) membranes. Immunoblot analysis was performed with the indicated antibodies and visualized with SuperSignal West Pico chemiluminescent substrate (Pierce Chemical).

Immunoprecipitation. HeLa cells grown to confluence on 60 mm^2 dishes were lysed in buffer containing 0.5 % Triton X-100 and 150 mM NaCl and 0.5 % Deoxycholate. Soluble lysate was incubated with protein A beads for 30 minutes followed by incubation overnight with the monoclonal RASSF1A antibody G5 or rabbit IgG and protein A beads. Beads were washed 3 times in lysis buffer with 500 mM NaCl. Following washes, sample buffer was added and lysates were boiled and separated on a 10 % SDS-polyacrylamide gel followed by immunoblotting. For immunoprecipitation of RASSF1A and βTrCP, HeLa cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 137mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10mM $MgCl_2$, 2mM EGTA) plus protease and phosphatase inhibitors (Roche EDTA-free protease inhibitor cocktail, 1mM PMSF, 50mM NaF, 1mM $NaVO_4$, 80mM β-glycerosphosphate). Cells were lysed for 15 min, then cleared at 20,000 X g for 10 min at 4°C. 800µg of lysate was diluted with lysis buffer to a concentration of 1µg/µL. Complexes were immunoprecipitated with 2µg of the indicated antibody for 1 hr. Antibody-antigen complexes were precipitated with ProteinA/G-agarose beads for 1 hr. Complexes were washed in lysis buffer 3-4 times for 5 min at 4°C.

Immunofluorescence. For BrdU visualization, cells were treated with 30uM BrdU for 24 hours and then fixed in 3.7 % HCHO. Cells were permeabilized with MeOH for 10 minutes at -20C and then blocked in PBS, 5% BSA and 1% Tween for a minimum of 15 minutes. Anti-BrdU was used at a dilution of 1:4. Cells were visualized on an Axiovert upright microscope (Zeiss) equipped with a Hamamatsu black and white camera.

FACS Analysis. 72 hours post transfection cells were trypsinized and resuspended in a 50:50 mixture of EtOH and PBS. Following fixation for 30 minutes, cells were washed and labeled with propidium iodide (Sigma) at 40 ug/ml for 30 minutes at 37C. For each analysis, 10,000 cells were collected by FACScan and analyzed with the CellQuest program (Becton Dickinson).

qPCR: HeLa cells were transfected in 35 mm^2 dishes with 100 nM siRNA. 72 hours post transfection, RNA was extracted from cells with High Pure RNA Isolation Kit (Roche Applied Science) or Trizol (Invitrogen) according to the manufacturer's protocol. CDNA was synthesized with Super Script II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. For cDNA synthesis, 1 ug of RNA and oligo$(dT)_{12\text{-}18}$ primers were used. One fifteenth of the cDNA reaction was used with the Roche Light Cycler System and the Light Cycler FastStart DNA Master SYBR Green I (Roche Applied Systems). Primers were chosen to flank at least two siRNA target sequences and lie on separate exons. The primers for cyclin D1 are: 5'CCAGCTCCTGTGCTGCGAAG3' (forward) and 5'GCGGCCAGGTTCCAC3' (reverse). The primers for βTRCP1 are: 5'AGCTGTGCCAGACTCTGCTT 3' (forward) and 5'GCTGGCAGAGCAGTTATGAA3' (reverse). The primers for βTRCP2 are: 5'TGCAGCGGGACTTTATTACC 3' (forward) and 5'TCTCGTAGGCCACTGATAATTT (reverse). Primers for SKP2 are: 5'TGAGCTGAACCTCTCCTGGT 3' (forward) and 5'CTGGCACGATTCCAAAAACT 3' (reverse). Primers for RASSF1C are: 5'CTGCAGCCAAGAGGACTCGG 3' (forward) and

5'GGGTGGCTTCTTGCTGGAGGG 3' (reverse). Values were normalized using GAPDH and analyzed using the relative quantification mathematical model (Pfaffl). QPCR on miR-21 was performed using Taqman miRNA assay kit (Applied Biosystems) according to manufacturer's protocol. GAPDH was used as a loading control.

Ras activity assay. Ras activity assay was performed using Raf1-RBD-GST beads according to manufacturer's protocol (Upstate).

Soft agar colony formation. Soft agar assays were performed as described elsewhere [93].

β-galactosidase assay. β-galactosidase was measured using β-galactosidase staining kit from Cell Signaling.

Quantification and Statistics. Quantification was analyzed using Image J software. Statistics were performed using student's unpaired t-test.

FIGURE CREDITS

I performed all of the experiments presented in Chapter 3.

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