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The Bmi-1 helix–turn and ring finger domains are required for Bmi-1 antagonism of (−) epigallocatechin-3-gallate suppression of skin cancer cell survival

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ABSTRACT

The Bmi-1 Polycomb group (PcG) protein is an important epigenetic regulator of chromatin status. Elevated Bmi-1 expression is observed in skin cancer and contributes to cancer cell survival. (−) Epigallocatechin-3-gallate (EGCG), an important green tea-derived cancer prevention agent, reduces Bmi-1 level resulting in reduced skin cancer cell survival. This is associated with increased $p21^{Cip1}$ and $p27^{Kip1}$ expression, reduced cyclin, and cyclin dependent kinase expression, and increased cleavage of apoptotic markers. These EGCG-dependent changes are attenuated by vector-mediated maintenance of Bmi-1 expression. In the present study, we identify Bmi-1 functional domains that are required for this response. Bmi-1 expression reverses the EGCG-dependent reduction in SCC-13 cell survival, but Bmi-1 mutants lacking the helix–turn–helix–turn–helix–turn (Bmi-1ΔHT) or ring finger (Bmi-1ΔRF) domains do not reverse the EGCG impact. The reduction in Ring1B ubiquitin ligase activity, observed in the presence of mutant Bmi-1, is associated with reduced ability of these mutants to interact with and activate Ring1B ubiquitin ligase, the major ligase responsible for the ubiquitination of histone H2A during chromatin condensation. This results in less chromatin condensation leading to increased tumor suppressor gene expression and reduced cell survival; thereby making the cells more susceptible to the anti-survival action of EGCG. We further show that these mutants act in a dominant-negative manner to inhibit the action of endogenous Bmi-1. Our results suggest that the HT and RF domains are required for Bmi-1 ability to maintain skin cancer cell survival in response to cancer preventive agents.

1. Introduction

Epigenetic regulatory events are important as they influence the open and closed status of chromatin and thereby influence gene expression and cell survival. The polycomb group (PcG) genes encode a family of proteins which regulate cell survival via epigenetic mechanisms [1]. PcG proteins operate as two classes of multimeric chromatin binding complexes — polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) [2]. The PRC1 complex includes Bmi-1, Ph1, CBX and Ring1A/B, while the PRC2 complex contains Ezh2, EED, Suz12, and RbAp46 [3]. As an initial step in regulation, trimethylation of lysine 27 of histone H3 (H3K27me3) occurs via the action of the Ezh2 PcG protein [4,5]. H3K27me3 then serves as a binding site for the CBX protein of the PRC1 complex [4]. Once bound, the Ring1B protein of the PRC1 complex catalyzes ubiquitination of histone H2A at lysine 119 (H2AK119ubi) [3,4,6]. These sequential trimethylation and ubiquitination events result in chromatin condensation leading to gene silencing [2,5].

Bmi-1 is an important member of the PcG family. It is a small 324 amino acid protein that has no known enzymatic activity, but serves as the key regulatory component of the PRC1 complex. Bmi-1 binds to Ring1B and the resulting interaction enhances Ring1B E3 ligase activity and H2AK119ubi formation [7]. Several domains of the Bmi-1 protein are conserved among species, including the ring finger (RF), helix–turn–helix–turn–helix–turn (HT) and proline/glutamic acid/serine/threonine rich (PEST) motifs [7–9]. A few studies have addressed the role of these domains. For example, Bmi-1 oncogenic activity requires the N-terminal ring finger (RF) domain and the (HT) domain [7,8]. The RF domain is also required for Bmi-1 synergy with the c-myc oncogene [8]. The HT domain is required for transcriptional repression in rat

Abbreviations: Bmi-1, B-cell-specific Moloney murine leukemia virus integration site 1; HT, helix–turn; RF, ring finger; Ezh2, enhancer of zeste homolog 2; PcG, polycomb group; H2AK119ub, histone H2A ubiquitinated on lysine 119; H3K27me3, histone H3 trimethylation on lysine 27; Cdk, cyclin dependent kinase; Wt, wild-type.

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embryo fibroblasts, but is not required for cell transformation [10]. In human diploid fibroblasts both the RF and HT domains are required for $p16^{INK4A}$ suppression that leads to bypass of senescence [11]. The HT and RF domains are also required for Bmi-1 immortalization of normal human mammary epithelial cells [12].

Several PcG genes are recognized as oncogenes and their products are found to be deregulated in cancer cells [13,14,14–21], and Bmi-1 is an important example. Elevated Bmi-1 expression is associated in cancer development [14,20–22]. Bmi-1 is also required for stem cell survival. For example, maintenance of hematopoietic stem cell [11,12] involves Bmi-1 suppression of the Ink4A locus [23].

Recent studies suggest a role for Bmi-1 in skin disease and skin cancer. Bmi-1 expression is increased in skin cancer tissues and skin cancer cell lines [24], and elevated expression of Bmi-1 is associated with resistance of skin cancer cells to cancer prevention agents [1,25,26]. Green tea polyphenols are important chemopreventive agents that are active in preventing skin cancer [27–29]. The major active constituent is (−) epigallocatechin-3-gallate (EGCG) [28,30]. Our previous studies show that EGCG treatment reduces skin cancer cell expression of Bmi-1 and other PcG proteins, and that this is associated with reduced cell cycle protein level and activation of apoptosis [1,25]. Dose response studies indicate that loss of Bmi-1 occurs at EGCG concentrations of 20–60 μM [25]. Moreover, we found that forced vector-mediated expression of Bmi-1 can reverse these actions [25]. Despite this confirmed survival role of Bmi-1 in skin cancer cells, the importance of specific Bmi-1 functional domains in this process has not been examined. The ability to force expression of Bmi-1 to reverse the anti-survival actions of EGCG is an important tool [31], as it permits us to express Bmi-1 mutants where specific functional domains are mutated and thereby examine the role of such domain in survival. In this present study, we characterize the requirement for the RF and HT domains for Bmi-1 activity in countering the anti-cancer impact of EGCG in skin cancer cells.

2. Materials and methods

2.1. Chemicals and reagents

(−) Epigallocatechin-3-gallate (EGCG) and dimethyl sulfoxide (DMSO) were procured from Sigma (St Louis, MO). A 1000-fold concentrate of EGCG was prepared in DMSO and stored at −80 °C. Dulbecco's Modified Eagle Medium (DMEM) and trypsin were purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal antibody against human Bmi-1 (ab14389) and goat polyclonal antibody against Ring1B (ab3832) was purchased from Abcam Inc. (Cambridge, MA). Mouse monoclonal anti-Ezh2 (#612667) was obtained from BD Transduction Labs (San Jose, CA). Antibodies specific for histone H3 K27-trimethyl (H3K27me3) (07-449), and ubiquitinated lysine 119 of histone H2A (H2AK119ub) (AB10029) were from Millipore (Billerica, MA). Anti-rabbit procaspase 9 (9502), and anti-mouse procaspase 8 (9746) were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-β-actin (A5441) was obtained from Sigma (St Louis, MO). Peroxidase-conjugated sheep anti-mouse IgG (NA931) and donkey anti-rabbit IgG (NA934) were purchased from GE Healthcare (Piscataway, NJ).

2.2. Adenoviruses encoding wild-type and mutant Bmi-1

Empty and human Bmi-1 (hBmi-1) expressing adenoviruses were constructed as reported previously [10]. These include Bmi-1 reading frames lacking the HT or RF domain. FLAG-Bmi-1(ΔRF) and FLAG-Bmi-1(ΔHT) cDNAs were cloned into pSHUTTLE-TET adenovirus backbone plasmid (Stratagene, La Jolla, CA; 240006) and transfected into A293 packaging cells to produce tAd5-FLAG-hBmi-1(ΔRF) and tAd5-FLAG-hBmi-1(ΔHT). Each virus encodes a protein with the FLAG epitope fused at the N-terminus of the Bmi-1 protein. SCC-13 cells were infected with Bmi-1 encoding or empty adenovirus in the presence of a tetracycline activator encoding Ad5-TA helper virus and 2.5 μg/ml polybrene [25].

2.3. Cell culture and proliferation studies

SCC-13 cells, obtained from American Type Culture Collection (ATCC; Rockville, MD), were maintained in a DMEM supplemented with D-glucose, L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, sodium pyruvate and 5% fetal calf serum [32]. For virus infection, the cells were plated in 9.6 cm^2 dishes, allowed to attach overnight, and infected with 5 MOI of tAd5-EV, tAd5-hBmi-1 or mutant Bmi-1-encoding virus in the presence of 5 MOI of Ad5-TA virus and 2.5 μg/ml polybrene in serum-free DMEM. After 6 h, the virus was removed and replaced with fresh complete medium and incubation was continued in the presence or absence of EGCG for an additional 48 h. The cells were then harvested using 0.025% trypsin containing 1 mM EDTA and counted.

2.4. Bmi-1 expression detection by immunofluorescence

Subcellular location of Bmi-1 or Bmi-1 mutant was monitored by immunostaining. SCC-13 cells were seeded onto cover slips and allowed to attach for 24 h. Cells were infected with appropriate virus and then treated without or with 60 μM EGCG for 24 to 48 h. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 (T9284, Sigma), blocked in 10% goat serum, and incubated with Cy3-conjugated mouse anti-FLAG (A9594, 1:100; Sigma-Aldrich). Cells were also incubated with Hoechst 33258 (H3569, 1:2000, Invitrogen) prior to thorough washing and mounting on slides. An Olympus IX81 spinning disk confocal microscope was used to collect the cell images.

2.5. Immunoblot analysis

Total cell extracts were prepared from SCC-13 cells and protein concentration was determined utilizing the Bradford Bio-Rad protein assay. Equal quantities of protein were electrophoresed on 8–10% denaturing SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk, incubated with an indicated primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescence detection (Amersham Biosciences) was used to visualize secondary antibody binding.

3. Results

3.1. Impact of wild-type and mutant Bmi-1 on EGCG suppression of SCC-13 cell survival

Our previous studies indicate that EGCG treatment suppresses endogenous Bmi-1 level and that this is associated with reduced skin cancer cell survival and induction of apoptosis [1,3,24–26]. Optimal inhibition of cell survival and induction of apoptosis is observed between 20 and 60 μM EGCG [25]. We have also showed that forced expression of Bmi-1 reverses these responses [31]. However, the role of specific conserved Bmi-1 protein domains in mediating this survival response is not known. The Bmi-1 RF and HT domains are conserved across species (Fig. 1A) [8,10,33]. To determine whether these domains are required for Bmi-1 pro-survival action on skin cancer cells, we used Bmi-1 encoding viruses to maintain Bmi-1 or Bmi-1 mutant expression during challenge with EGCG. We challenged with a high dose of EGCG to provide a rigorous test of the ability of Bmi-1 proteins, with specific mutations, to restore cancer cell survival. The key is that we are able to express enough Bmi-1 using adenovirus vectors to overcome the routine EGCG-dependent reduction in Bmi-1 protein level.

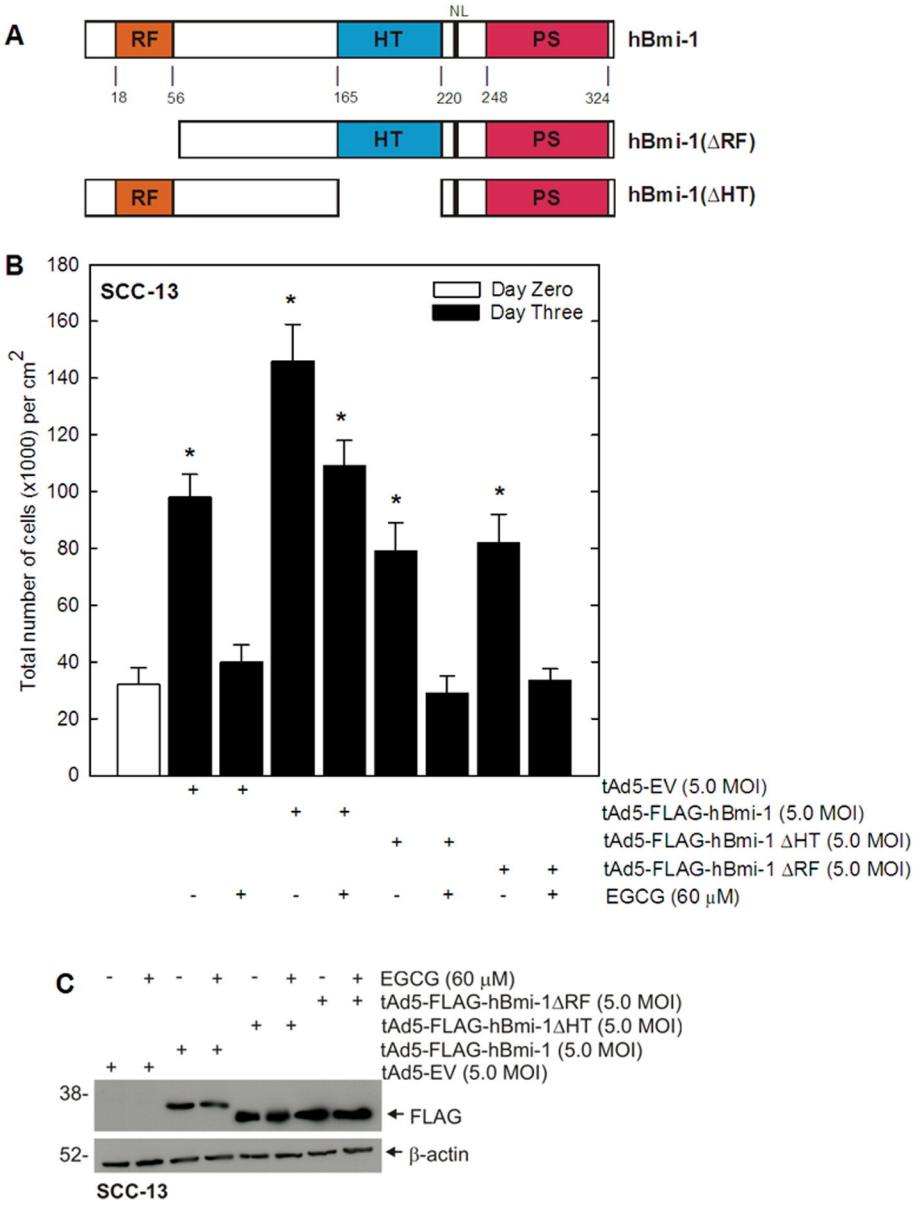

Fig. 1. Wild-type and mutant Bmi-1 impact on EGCG suppression of SCC-13 cell survival. A) Schematic of Bmi-1 showing the ring finger (RF, amino acids 18–56), helix–turn–helix–turn–helix–turn–helix (HT, amino acids 165–220) domains, nuclear localization signal (NL, amino acids 232–235) and proline/serine-rich sequence (PS, amino acids 248–324). Bmi-1 is a 326 amino acid protein. B) SCC-13 cells, growing at low confluence, were infected with 5.0 MOI of indicated FLAG-Bmi-1 adenovirus in the presence of 5.0 MOI of tAd5-TA helper virus. At one day post-infection, cells were treated with 0 or 60 μM EGCG for two days and the cells were harvested and counted. The values are mean ± SEM, n = 3. Asterisks indicate a significant increase over control (day zero, open bar), $p < 0.05$. C) Expression of FLAG-Bmi-1 proteins. SCC-13 cells were infected with 5.0 MOI of indicated FLAG-Bmi-1 adenovirus in the presence of 5.0 MOI of tAd5-TA helper virus. At one day post-infection, cells were treated with 0 or 60 μM EGCG for two days and the cells were then harvested and extracts were prepared for anti-FLAG detection of FLAG-Bmi-1. Similar blots were obtained in each of three experiments.

Cells were infected with tAd5-EV, tAd5-hBmi-1, tAd5-hBmi-1(ΔHT) or tAd5-hBmi-1(ΔRF) and after 24 h treated with 0 or 60 μM EGCG for 48 h. Total cell number increases three-fold during three days of growth in empty vector-infected cells (Fig. 1B) and this increase is eliminated when cells are treated with EGCG. Bmi-1 expression enhances basal growth and also inhibits the ability of EGCG to reduce cell number. In contrast, Bmi-1(ΔHT) or Bmi-1(ΔRT) expressing cells grow at a similar rate to tAd5-EV infected cells. Moreover, these mutant proteins do not counter the EGCG-dependent suppression of growth. Fig. 1C confirms that wild-type and mutant forms of Bmi-1 are expressed at comparable levels, indicating that the absence of activity of the mutants is not due to differences in expression. Endogenous Bmi-1 is not detected in this analysis because the antibody is directed against the FLAG-epitope attached to the expressed Bmi-1 proteins. Fig. 2 shows the morphology of the cells and confirms that Bmi-1 reduces the cell loss observed with EGCG. However, Bmi-1(ΔRT) and Bmi-1(ΔHT) are not able to reduce cell loss. Moreover, only wild-type Bmi-1 prevents the EGCG-associated change in cell morphology. These results suggest that both RF and HT domains of Bmi-1 are required for Bmi-1-dependent survival of EGCG-treated SCC-13 cells.

3.2. Altered subcellular localization does not explain response

We next monitored the subcellular distribution of mutant and wild-type Bmi-1. Bmi-1 is a nuclear-acting protein and we would expect nuclear localization. As shown in Fig. 3 (upper panels) Bmi-1, Bmi-1(ΔHT) and Bmi-1(ΔRF) all localize in the nucleus in a generally similar pattern. Moreover, the lower panels in Fig. 3 show that EGCG does not cause a differential redistribution of the wild-type or mutant proteins. Thus, the action of EGCG to reduce cell survival in the presence of

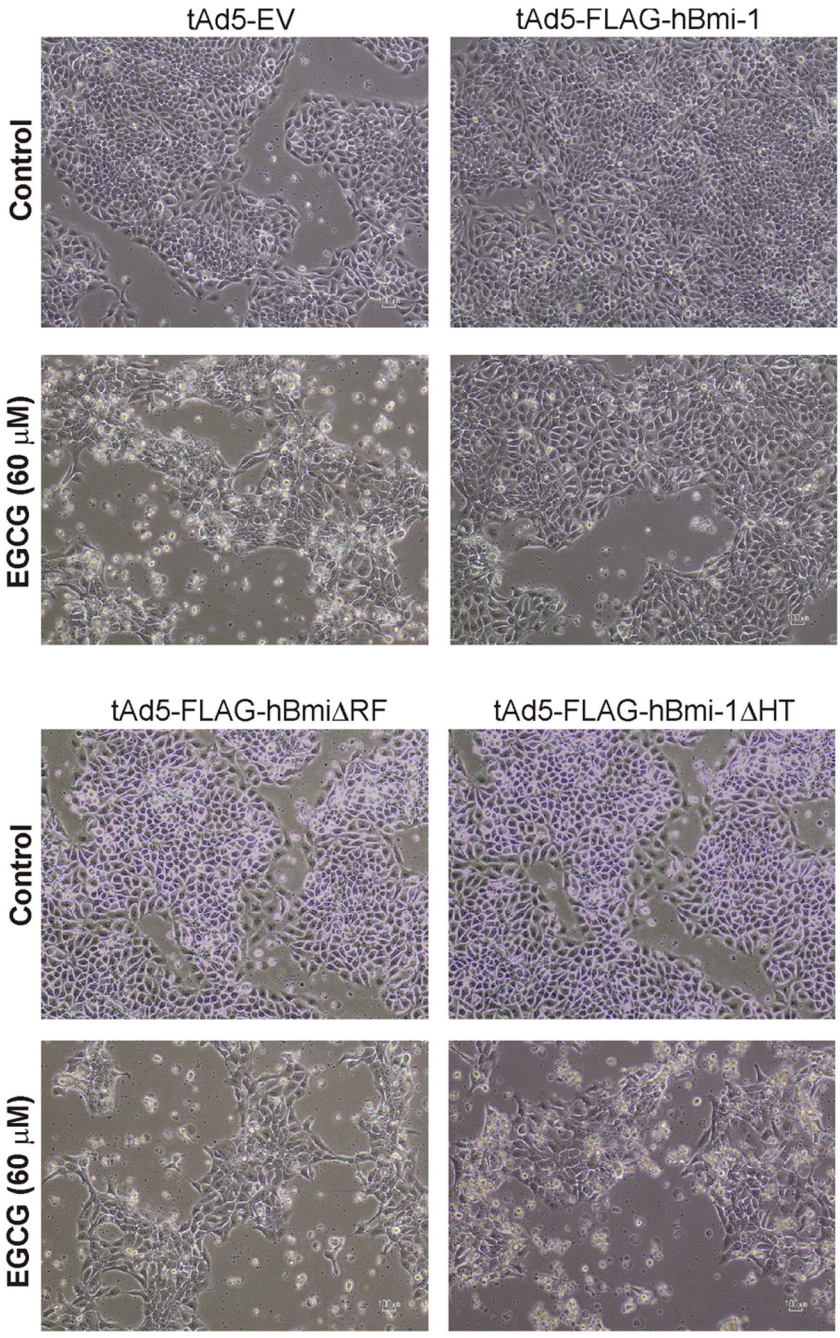

Fig. 2. Impact of EGCG and Bmi-1 on cell morphology. SCC-13 cells, growing at low confluence, were infected with 5.0 MOI of indicated FLAG-Bmi-1 adenovirus in the presence of 5.0 MOI of tAd5-TA helper virus. At one day post-infection, cells were treated with 0 or 60 μM EGCG for two days and the cells were photographed. Only wild-type Bmi-1 prevents the EGCG-associated morphological changes. Similar results were observed in each of three independent experiments.

mutant Bmi-1 is not due to the EGCG-dependent movement of these proteins out of the nuclear compartment, nor does deletion of the HT or RF domains alter location. This is consistent with the fact that the nuclear localization signal (Fig. 1A) is intact in all three proteins [10].

3.3. Differential regulation of PcG protein function by Bmi-1 mutants

The polycomb group (PcG) proteins sequentially methylate and ubiquitinate histones to produce condensed chromatin to suppress gene expression [34,35]. The PRC2 and PRC1 complexes suppress gene expression via a two-step mechanism [34]. In the initial step, the Ezh2 subunit of the PRC2 complex catalyzes trimethylation of lysine 27 of histone H3 (H3K27me3) [36,37]. Following this, the CBX protein, of the PRC1 complex, interacts with H3K27me3 to anchor the PRC1 complex to chromatin and the Ring1B subunit of this complex ubiquitinates lysine 119 of histone H2A (H2AK119ub) [38]. The ultimate effect is to silence tumor suppressor gene expression and enhance cell survival. Since the PRC2 and PRC1 complexes work together, we explored whether altering the level of Bmi-1, a PRC1 component, would change the level of EZH2, a PRC2 component. SCC-13 cells were treated with Bmi-1, Bmi-1(ΔRF) or Bmi-1(ΔHT) encoding virus and then challenged with EGCG. Fig. 4A shows that Ezh2 level is reduced in EGCG-treated cells. This reduction is also observed in cells expressing Bmi-1(ΔHT) and Bmi-1(ΔRF), but is attenuated in cells expressing wild-type Bmi-1. These studies confirm that EGCG reduces Ezh2 expression and shows that this response can be attenuated by expression of Bmi-1 [1,25]. It further shows that mutating the HT or RF domain obviates the ability of Bmi-1 to prevent Ezh2 loss. In addition, we examined

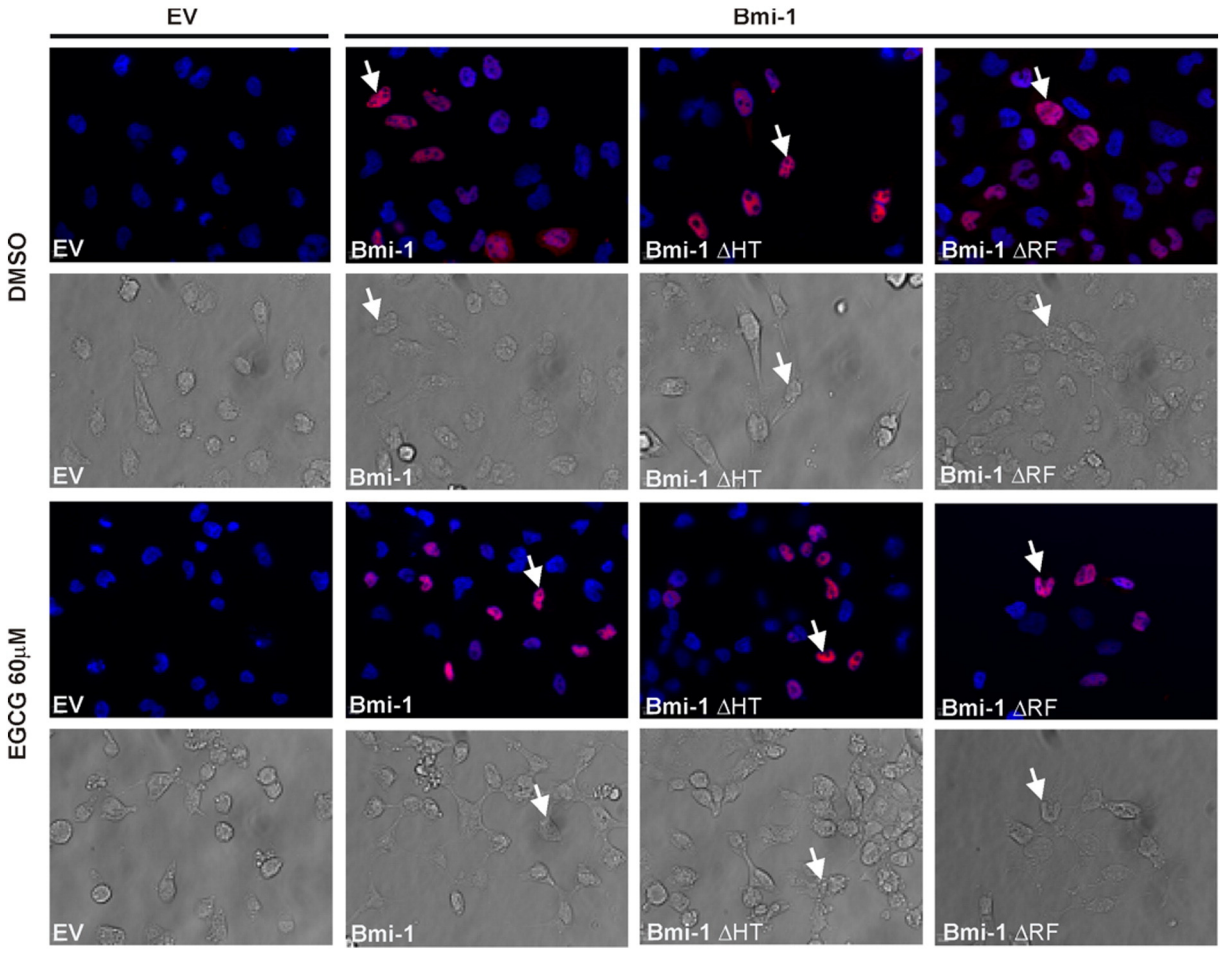

Fig. 3. Immunolocalization of FLAG-Bmi-1. Sub-confluent SCC-13 cells were grown on glass cover slips, and then infected with 5 MOI of the indicated virus. After 24 h, the cells were treated with 0 or 60 μM EGCG for an additional 48 h. At 72 h post-infection, the cells were washed twice in PBS, fixed and stained with anti-FLAG-Cy3 (red) and the nuclei with Hoechst (blue). The arrows indicate nuclear Bmi-1 staining. Similar results were observed in each of the three separate experiments.

an endpoint of Ezh2 action, methylation of histone H3 at lysine 27 [6,34,39]. Fig. 4A shows that H3K27me3 level is reduced in parallel with the reduction in Ezh2 level in EGCG-treated cells, and that this decrease is reversed by expression of wild-type Bmi-1 but not by the Bmi-1 mutants.

3.4. Mutations reduce Bmi-1 activity

Ring1B is an important histone ubiquitin ligase, which like Bmi-1, is part of the PRC1 complex [7,40]. It catalyzes H2AK119ub formation as a required step in chromatin condensation [41]. Fig. 4A shows that EGCG treatment reduces H2AK119ub formation and that this is reversed by forced expression of wild-type but not mutant Bmi-1. These studies suggest that the Bmi-1 RF and HT domains are required for Bmi-1 restoration Ring1B-dependent formation of H2AK119ub in EGCG-treated cells. Fig. 4B is a composite plot of data from three separate experiments and shows that only wild-type Bmi-1 reverses the impact of EGCG on Ezh2, H3K27me3 and H2AK119ub level.

We next examined the impact of EGCG and Bmi-1 on expression of Bmi-1 downstream targets. These targets include proteins that control cell cycle and apoptosis effectors [1,3,24–26,42,43]. EGCG treatment reduces expression of cyclin D1, cyclin E, cdk2 and cdk4, and increases expression of $p21^{Cip1}$ and $p27^{Kip1}$ [1,25,26]. Fig. 5 confirms these changes and also shows that these EGCG-stimulated events are reversed by Bmi-1. This study also shows that neither Bmi-1(ΔHT) nor Bmi-1(ΔRF) antagonizes this action of EGCG. We also monitored apoptosis markers. Consistent with a reduction in cell survival, EGCG treatment reduces the level of the Bcl-xL pro-survival protein, and activates cleavage of procaspases 8 and 9, and poly(ADP-ribose) polymerase (PARP) (Fig. 5). These effects are also reversed in the presence of the wild-type but not mutant forms of Bmi-1.

3.5. Interaction of mutant Bmi-1 with Ring1B

Bmi-1/Ring 1B heterodimer formation, in the PRC1 complex, is a required event that drives a substantial increase in Ring1B E3 ubiquitin ligase activity [44]. To understand the impact of the Bmi-1 HT and RF mutations on Bmi-1/Ring1B complex formation, we examined the ability of each mutant to co-precipitate Ring1B. FLAG-Bmi-1, FLAG-Bmi-1(ΔHT) or FLAG-Bmi-1 (ΔRF) were expressed in SCC-13 cells and extracts were prepared for anti-FLAG immunoprecipitation and immunoblot detection of Ring1B. Fig. 6 shows that only wild-type Bmi-1 is competent to precipitate Ring1B, and much reduced Ring1B precipitation is observed for the mutant Bmi-1 proteins. Anti-FLAG immunoblot shows that equal quantities of each protein are expressed in these experiments; thus, differences in Bmi-1 protein expression cannot explain the differences in interaction with Ring1B. These studies suggest that Bmi-1(ΔHT) and Bmi-1(ΔRF) interact less efficiently with Ring1B.

3.6. Bmi-1 mutants inhibit wild-type Bmi-1 function

We next explored whether the mutant Bmi-1 proteins influence action of wild-type Bmi-1. Wild-type and mutant Bmi-1 were expressed in SCC-13 cells in various combinations and impact on cell number was

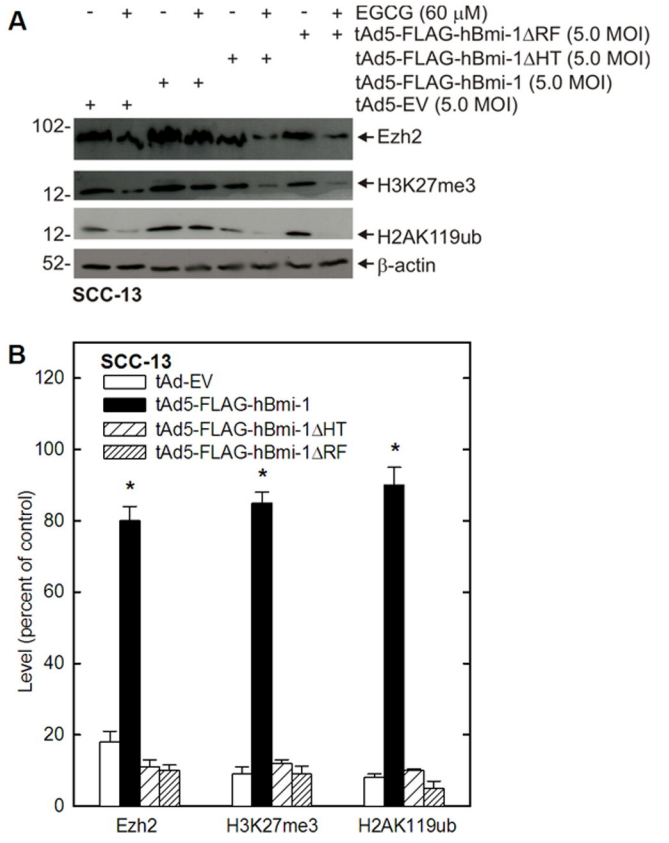

Fig. 4. Impact of Bmi-1 mutants on EGCG suppression of PcG action. SCC-13 cells were infected with 5 MOI of the indicated virus and after 24 h the cells were treated with 0 or 60 μM EGCG for an additional 48 h. A) Protein extracts were prepared for immunoblot detection of the indicated proteins. B) The values EGCG challenge experiments are plotted. The values indicate percent reduction in endpoint in EGCG-treated cells versus control as assessed by densitometry. The values represent mean + SEM, n = 3. The tAd5-hBmi-1 values are significantly elevated ($p < 0.05$) as compared to the other values.

measured. Fig. 7A shows that cell number increases over the four day growth period and expression of Bmi-1 further enhances the increase. Parallel studies reveal that co-expression of Bmi-1(ΔHT) or Bmi-1(ΔRF) with Bmi-1 inhibits the Bmi-1-associated increase in cell number. We also examined the impact of Bmi-1 mutant/wild-type co-expression on p21, as a representative biological end response. These studies show that wild-type Bmi-1 suppresses p21 expression, and that co-expression of the mutants with wild-type Bmi-1 attenuates this suppression (Fig. 7B). These findings suggest that the Bmi-1(ΔHT) and Bmi-1(ΔRF) proteins function as dominant-negative inhibitors of Bmi-1.

4. Discussion

4.1. Polycomb proteins are epigenetic regulators

Polycomb group (PcG) proteins are epigenetic regulators that were discovered in *Drosophila*. In mammals PcG proteins regulate development, differentiation and survival by modifying chromatin to silence gene expression [5,45]. The PRC1 and PRC2 complexes mediate gene expression silencing. The four protein PRC2 core complex acts to methylate chromatin. The catalytic subunit is Ezh2, a methyltransferase that trimethylates lysine 27 of histone H3 [35,37,46]. The PRC1 core complex interacts at site of H3K27me3 formation to ubiquitinate the surrounding chromatin. This ubiquitination is accomplished by the Ring1B protein of this complex [34]. Ring1B, the catalytic subunit, ubiquitinates K119 of histone H2A and activity is enhanced by association with Bmi-1 [7,38]. These events result in chromatin compaction and suppression of transcription. The combination of PRC2/PRC1 action results in the stable suppression of gene expression. In skin cancer cells, and other cancer cell types, this pathway results in silencing of tumor suppressor gene expression leading to increased cell proliferation/survival [2,5].

4.2. Polycomb proteins and chemoprevention by green tea

Prevention of skin cancer is an important goal. Of particular interest in this regard are the polyphenols present in green tea. The major active constituent is EGCG which has been shown to be an active preventive agent in cell culture and animal models of skin cancer [28]. Understanding the mechanism of EGCG action is an important goal and our recent studies indicate that polycomb proteins are important targets of EGCG action [25,26]. These studies show that EGCG reduces the level of endogenous Bmi-1, Ezh2 and several other PcG proteins via mechanisms that involve enhanced proteasome processing and that this reduces the pro-survival impact of Bmi-1 in skin cancer cells [1,24–26,32,47]. The reduction in PcG protein level is also associated with reduced histone H3 trimethylation and reduced H2A ubiquitination [26].

We focus on Bmi-1 and Ezh2, as these proteins are overexpressed in skin cancer cells and are the catalytic subunits involved in chromatin modification [3]. Key studies show that EGCG suppression of skin cancer cell survival and can be reversed by maintenance of Bmi-1 by vector medicated delivery [25,26]. However, there is no information available regarding the Bmi-1 protein domains required for this action. Bmi-1 encodes several conserved motifs, including an N-terminal ring finger (RF) domain and a helix–turn (HT) domain [7,8]. These domains are highly conserved suggesting their functional significance. The RF domain is responsible for Bmi-1 heterodimer formation with Ring1B in the PRC1 complex [7] and mutation of this domain results in reduced activity of the PRC1 complex [8]. The HT domain is required for transcriptional repression in rat embryo fibroblasts [10], and both the RF and HT domains are required for Bmi-1 dependent senescence bypass in fibroblasts [11].

4.3. Role of the Bmi-1 RF domain

In the present study we examined whether the Bmi-1 RF domain is required for Bmi-1 activity in skin cancer cells [25,26]. We show that EGCG treatment suppresses skin cancer cell survival and that this is associated with reduced expression of pro-proliferation cell cycle regulators (cyclins E and D1, cdk2 and cdk4) and increased expression of cell cycle inhibitors ($p21^{Cip1}$, $p27^{Kip1}$). We also show that EGCG treatment increases apoptosis, including procaspase 8 and 9, and PARP cleavage. It is interesting that all of the responses are partially reversed by maintaining wild-type Bmi-1 expression.

Bmi-1 and Ring1B of the PRC1 complex are related E3 ubiquitin ligases, except that Bmi-1 has no catalytic activity. Both encode N-terminal RF domains which interact to drive Bmi-1/Ring1B heterodimer formation [7]. This is a specific interaction that is maintained during purification [7] and a core complex, including amino acids 1–102 of Bmi-1 and 5–115 of Ring1B, survives proteinase digestion and retains full ubiquitin E3 ligase activity [7]. We would predict that elimination of the RF domain from Bmi-1 would reduce its ability to interact with and activate Ring1B. Indeed, this is observed in that the Bmi-1(ΔRF) mutant does not efficiently interact with Ring1B and is unable to activate a downstream biological response. For example, unlike wild-type Bmi-1, Bmi-(ΔRF) is not able to reverse the EGCG-dependent suppression of cell viability or the EGCG associated changes in apoptotic or cell cycle marker expression. Thus, the Bmi-1 RF domain is required for Bmi-1 interaction with Ring1B, for Ring1B catalytic activity, as measured by H2AK110ub formation, and for cancer cell survival. This suggests that Bmi-1 dependent activation of Ring1B ligase activity and subsequent H2AK119ubi formation is required for cancer cell resistance to challenge with cancer prevention agent.

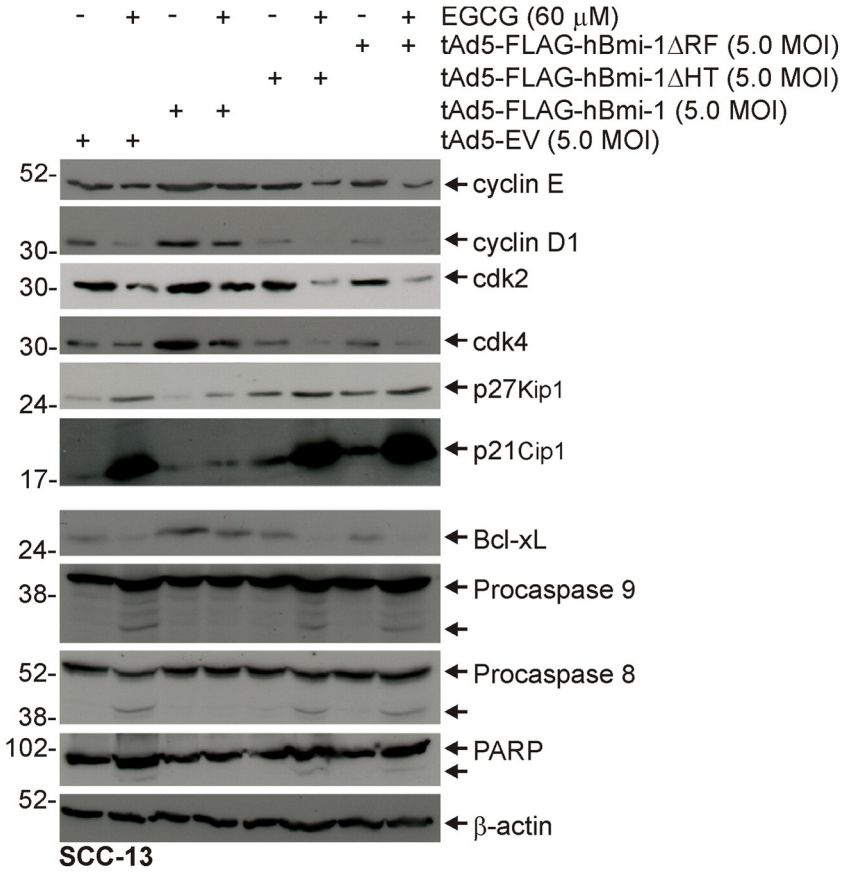

Fig. 5. Effect of Bmi-1 proteins on EGCG-dependent regulation of downstream targets. Subconfluent cultures of SCC-13 cells were infected with 5 MOI the indicated virus and 5.0 MOI of Ad5-TA helper virus. After 24 h, the cells incubated with 0 or 60 μM EGCG and after an additional 48 h and total cell lysates were prepared for immunoblot detection of the indicated epitopes. β-Actin was used as a protein loading control. The additional arrows in the procaspase 9, procaspase 8 and PARP panels indicate the apoptosis-activated cleaved forms of these proteins. Similar results were observed in three separate experiments.

4.4. Role of the Bmi-1 HT domain

We also examined the role of the Bmi-1 HT domain. Previous studies show that the HT domain is required for Bmi-1 activity in specific contexts. For example, GAL4-Bmi-1 fusion protein suppresses GAL4-luciferase reporter gene transcription, but GAL4-Bmi-1(ΔHT) is inactive [10]. Studies in other systems confirm that the HT domain is required for transcription suppression [48]. Thus, we were not surprised that the Bmi-1(ΔHT) mutant does not protect skin cancer cells against green tea challenge. What is somewhat surprising is that Bmi-1(ΔHT) mutant displays reduced interaction with Ring1B, as measured by pull-down assay. It is known that the RF domain is required for Bmi-1 interaction with Ring1B [7], but our present studies suggest that the HT domain is also required for this interaction. The mechanism whereby this domain influences this interaction is not immediately apparent. The HT domain is located at amino acids 165–220 of the Bmi-1 protein, spatially separated from the RF domain (amino acids 18–56) (Fig. 1). It is clear that the differential activity of these mutants cannot be attributed to differences in expression level or subcellular location, as the wild-type and mutant proteins are expressed at a similar level and all localize to the nucleus. Thus, it will take additional study to understand why the Bmi-1(ΔHT) mutant does not interact with Ring1B.

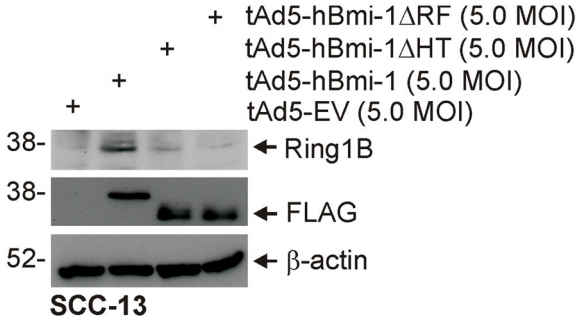

Fig. 6. Reduced interaction of Bmi-1 mutants with Ring1B. SCC-13 cells were infected with 5 MOI of empty virus (EV) or viruses encoding FLAG-tagged wild-type or mutant Bmi-1. After 24 h total cell extract was prepared for immunoprecipitation with anti-FLAG. Total protein (100 μg) was incubated with 2 μg of anti-FLAG for 2 h at 4 C followed by overnight treatment with 25 μl of protein-agarose A/B beads, and the precipitates were electrophoresed prior to immunoblot detection of Ring1B (top panel). Total extract was electrophoresed in parallel for detection of FLAG-Bmi-1 and β-actin (bottom two panels). Similar results were observed in each of three independent experiments. Anti-FLAG was used to detect each of the Bmi-1 proteins including wild-type Bmi-1, Bmi-1(ΔRF) and Bmi-1(ΔHT).

4.5. Bmi-1(ΔRF) and Bmi-1(ΔHT) act as dominant-negative regulators

As noted above, the RF domain of Bmi-1 interacts with the corresponding RF domain of Ring1B to form a heterodimer that drives a substantial increase in Ring1B activity. Our co-expression experiments indicate that Bmi-1(ΔRF) acts to inhibit Ring1B activity. This cannot be due to a loss of Bmi-1 enzymatic (ligase) activity due to the mutation, since, Bmi-1 is not an active E3 ubiquitin ligase [7,41,44]. It is also not likely due to competitive inhibition of endogenous Bmi-1 interaction with Ring1B, since the RF domain is absent in this mutant. It has recently been proposed that Bmi-1 interacts with and positions substrate for Ring1B-dependent ubiquitination [7]. Thus, it may be that mutant

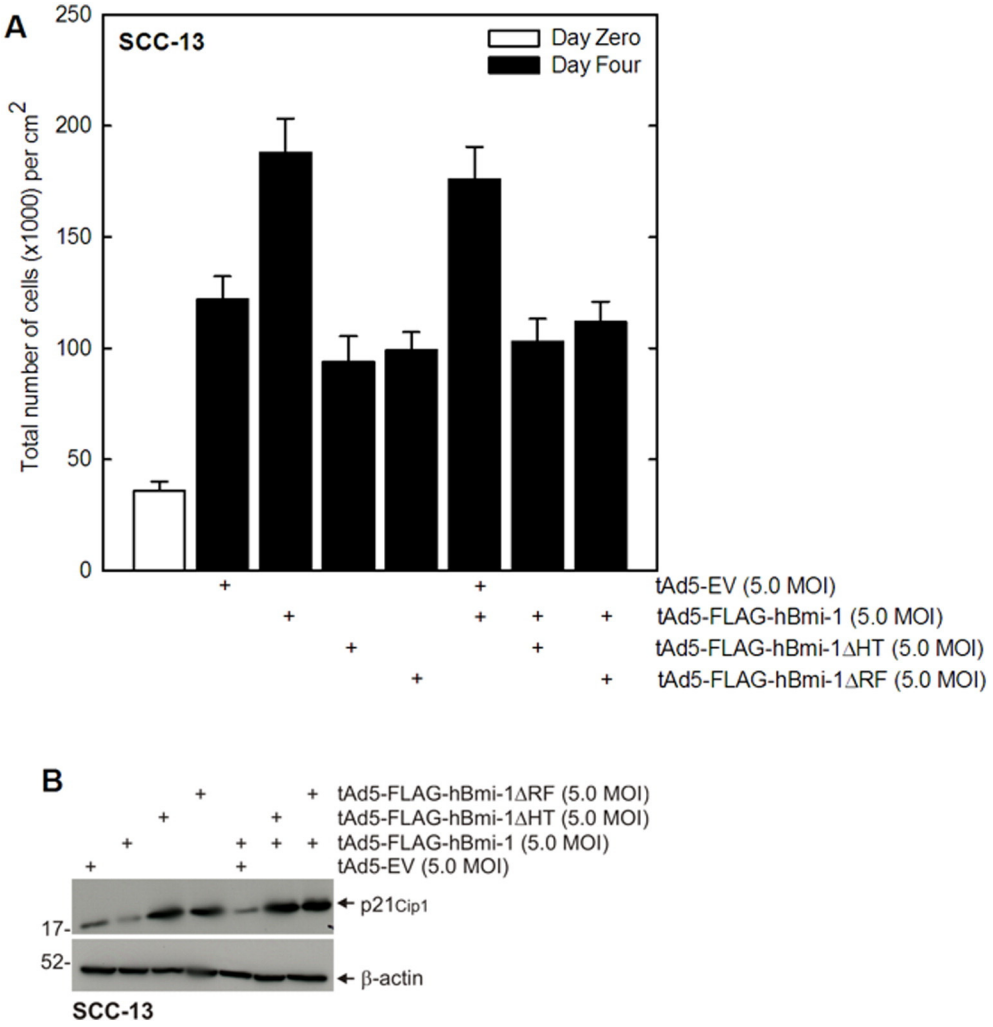

Fig. 7. Mutant Bmi-1 proteins function as inhibitors of wild-type Bmi-1. A) Subconfluent SCC-13 cell cultures were infected with 5 MOI of each indicated adenovirus. When two viruses are used, each virus is delivered at 5 MOI. At 4 d post-infection, the cells were harvested and counted. The open bar is the starting cell number at day = 0 and the closed bars are after 4 days of proliferation. The values are mean ± SEM, n = 3. B) Mutant Bmi-1 proteins inhibit Bmi-1 suppression of $p21^{Cip1}$ level. Cells were treated with virus as described above and extracts were prepared for immunoblot to detect $p21^{Cip1}$ and β-actin.

Bmi-1(ΔRF) completes with wild-type Bmi-1 for histone substrate and thereby prevents presentation of this substrate to Ring1B. It is more difficult to explain why Bmi-1(ΔHT) inhibits Ring1B activity. It is clear that this mutant inhibits Bmi-1/Ring1B heterodimer formation via a mechanism that is not known. However, how this happens requires further investigation.

In summary, we show that the Bmi-1 RF and HT domains are required for Bmi-1 protein function in skin cancer cells and that a mechanism responsible for loss of activity in these mutants is a reduced ability to interact with and activate the Ring1B ubiquitin ligase.

Author contributions

Conception and experimental design: Richard L. Eckert; data collection and interpretation: Tiffany M. Scharadin, Wen Xu, SIvaprakasam Balasubramanian.

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Competing interests

None.

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