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RING1B ubiquitination and stability are regulated by ARF

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ABSTRACT

The activity and stability of the E3 ubiquitin ligase RING1B are controlled by the ubiquitin system. Self-ubiquitination of RING1B, generating K6, K27 and K48-based mixed polyubiquitin chains, is a prerequisite for its activity as an E3 ligase for histone H2A. Monoubiquitination of histone H2A is one of the hallmarks of Polycomb-mediated gene silencing. The destruction of RING1B however, is mediated through K48 polyubiquitination catalyzed by the ubiquitin ligase E6-AP. Both forms of ubiquitination of RING1B are mutually exclusive and therefore the balance between them may constitute a point of regulation of Polycomb-mediated gene repression. Here we identify ARF as a regulator of RING1B ubiquitination. ARF appears to selectively prevent RING1B self-ubiquitination, probably allowing more efficient E6-AP-mediated ubiquitination and subsequent degradation of RING1B. By binding to the RING domain of RING1B, ARF disrupts RING1B homodimerization, providing a potential mechanism for its effect on RING1B self-ubiquitination.

1. Introduction

Histone H2A was the first protein shown to be ubiquitinated, and remains one of the most abundant among the ubiquitinated proteins in the cell. Monoubiquitination of histone H2A on lysine 119 is a hallmark of gene silencing mediated by Polycomb Group (PcG) Repressive Complexes (PRCs) [1,2]. Through this modification, PRCs control embryogenesis, differentiation, stem cell pluripotency and X chromosome inactivation [3]. The importance of proper PRCs function is underlined by severe developmental defects that result from their decreased activity [3,4]. On the other hand, overexpression of PcG proteins, particularly BMI1 and EZH2, is associated with increased invasiveness and poor clinical outcome of various cancers [5].

As the active E3 ubiquitin ligase of the PRC1, RING1B is responsible for the monoubiquitination of histone H2A and thereby plays a central role in PcG-mediated gene repression. At present, little is known about the regulation of the activity of RING1B. Recently, we established that RING1B self-ubiquitination, generating mixed and multiply branched K6, K27 and K48-based polyubiquitin chains, is an essential prerequisite for its ability to ubiquitinate histone H2A [6]. The ubiquitin system also plays a role in the degradation of RING1B, in which E6-associated protein (E6-AP) mediates K48-based polyubiquitination that targets RING1B to proteasome-mediated destruction [7]. These two modes of ubiquitination, with opposing outcomes, target the same lysine residues on RING1B and are therefore mutually exclusive [7]. Following these observations, we aimed to identify factors that regulate the two modes of ubiquitination of RING1B, and therefore affect PcG-mediated gene repression. We identified USP7 (ubiquitin specific protease 7) as a deubiquitinating enzyme that targets RING1B. USP7 hydrolyzes both self- and E6-AP-catalyzed ubiquitin chains suggesting that it functions as a switch that resets RING1B back to its native base state [8]. As USP7 brings RING1B to a state where it can be subjected again to each of the two modes of ubiquitination, we hypothesized that at least one mode of RING1B ubiquitination should be regulated by an upstream factor, which we aimed to identify in the present study.

One of the loci of particular interest that is subject to repression by the PRC1 complex is the cyclin-dependent kinase 2A (*CDKN2A*) locus [4,9]. This locus encodes for the tumor suppressor proteins $p16^{INK4a}$ and ARF (alternate reading frame). Overexpression of these proteins appears to be one of the main underlying causes for the deleterious effects of deficiency of PRC1 proteins. Interestingly, reactivation of the *CDKN2A* locus in mouse embryonic fibroblasts lacking the PRC1 subunit *Mel18* coincides with an increase in the expression of RING1B regulated post-transcriptionally [10]. This observation opens a possibility that the expression levels of proteins encoded by the *CDKN2A* locus are inversely correlated with

Abbreviations: ARF, alternate reading frame; CDKN2A, cyclin-dependent kinase inhibitor 2A; CHX, cycloheximide; COMMD1, copper metabolism gene MURR1 domain 1; E6-AP, E6-associated protein; MDM2, mouse double minute 2; NIAM, nuclear interactor of ARF and MDM2; PcG, polycomb group; PRC1, polycomb repressive complex 1; USP7, ubiquitin specific protease 7; XIAP, X-linked inhibitor of apoptosis; WT, wild type.

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the stability of RING1B. The tumor suppressant functions of ARF have mainly been attributed to its involvement in the p53 pathway. ARF prevents the degradation of p53 by inhibiting its ubiquitination by MDM2 (mouse double minute 2) or ARF-BP1 [11,12]. In addition, ARF affects the ubiquitination and degradation of several other proteins, both in negative {e.g. NIAM (nuclear interactor of ARF and MDM2) [13], E7-mediated proteolysis of pRB [14]}, and positive {e.g. B23 [15], COMMD1 (copper metabolism gene MURR1 domain 1) [16]} manners. In the case of COMMD1, ARF promotes K63-linked ubiquitination, which appears to stabilize the protein, whereas XIAP (X-linked inhibitor of apoptosis) promotes K48-linked ubiquitination that results in degradation of COMMD1 [17]. These observations suggest that in the case of COMMD1, ARF is capable of directing its mode of ubiquitination it. Taken together, these studies implicate ARF as a regulator of the ubiquitin system. We therefore investigated whether ARF has a regulatory role in the ubiquitination and degradation of RING1B as well.

2. Materials and methods

2.1. Antibodies

Antibodies against c-Myc (9E10), E6-AP (H-182) and GST (B-14) were from Santa Cruz. Anti-Flag (M2) and anti-tubulin (DM1A) were from Sigma. Anti-HA was from Covance. Monoclonal anti-Ring1B (3–3) was from MBL.

2.2. Plasmids and siRNAs

RING1B in pCS2+ for *in vitro* translation, 6Myc-RING1B in pCAGGS, and RING1B (full length and deletion mutants) in pEBB for expression in cells were described elsewhere [6,8]. Full length cDNA coding for ARF was kindly provided by Dr. Moshe Oren (Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel) and subcloned into pEBB with either a Flag or GST epitope. Deletion mutants of ARF were generated by PCR and subcloned into pEBB with a GST epitope. HA-ubiquitin in the F373.SEQ vector [6], E6-AP in pCS2 [7], and COMMD1-Flag in pEBB [17] were described elsewhere. siRNA oligonucleotides that specifically target *ARF* but not $p16^{INK4a}$ were ordered from Sigma (SASI_Hs01_00075731).

2.3. Cell lines and transfection

Cultured 293T cells were transiently transfected using the jet-PEI™ transfection reagent (Polyplus Transfection), and cells were harvested for analysis after 24–72 h. For experiments monitoring stability of proteins, cycloheximide (CHX; 100 μg/ml) was added for the indicated time periods.

2.4. Isolation of ubiquitinated RING1B from cells

293T cells were transfected with the different cDNAs as indicated for each experiment. Cells were lysed in a buffer containing 1% SDS, 1 mM EDTA, 2 mM Na_3VO_4 (in PBS), and lysates were heated to 95 °C (5 min). Benzonase (Sigma) was added to digest DNA. Following centrifugation of the lysates for 15 min at room temperature, four volumes of lysis buffer (25 mM HEPES, pH7.9, 1% Triton X-100, 10% Glycerol, 100 mM NaCl, and 1 mM EDTA) were added to the supernatant, after which it was subjected to immunoprecipitation with anti-Myc. Following SDS–PAGE, ubiquitinated proteins were visualized via Western blotting using anti-HA antibody.

2.5. Protein interaction analyses

For detection of RING1B-ARF complexes, 293T cell lysates (prepared in Triton lysis buffer) were subjected to GST pull-down using immobilized glutathione (Sigma). The precipitated complexes were resolved via SDS–PAGE and proteins visualized via Western blotting.

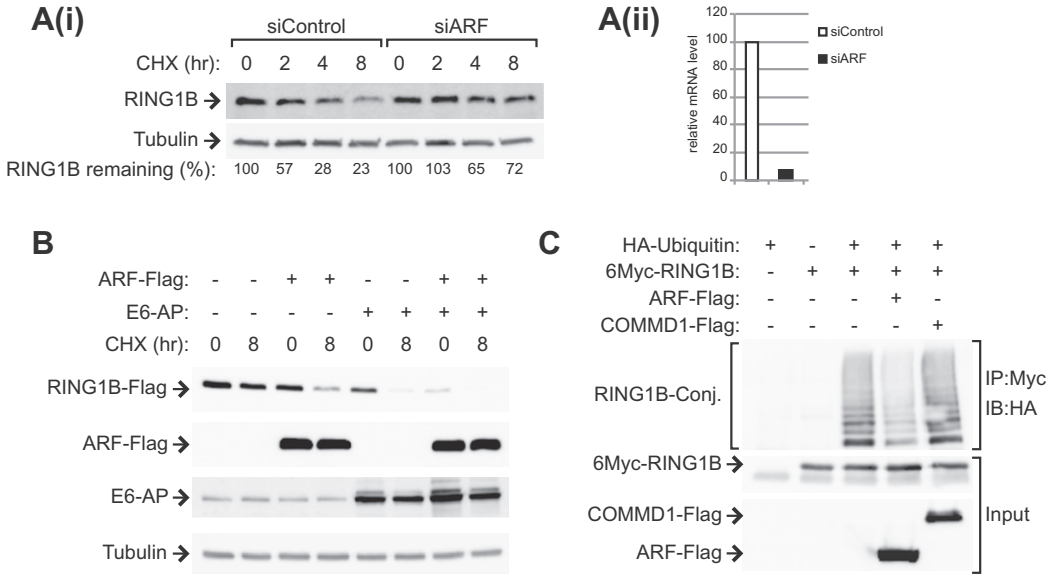

Fig. 1. ARF modulates the ubiquitination and stability of RING1B. (A, B) Time-dependent stability of RING1B was monitored in 293T cells after the addition of CHX and following silencing of endogenous ARF (A), or following overexpression of ARF, E6-AP, or both (B). The SDS–PAGE-resolved proteins were detected following Western blotting, using antibodies against RING1B, Flag, E6-AP or Tubulin (used as a loading control). ARF mRNA level was measured to ascertain it was silenced efficiently (Aii). (C) ARF affects the ubiquitination of RING1B. 293T cells were transfected with cDNAs coding for 6Myc-RING1B, HA-ubiquitin, and either ARF-Flag, or COMMD1-Flag, as indicated. Ubiquitinated RING1B was isolated by immunoprecipitation using anti-Myc antibody. The conjugates were visualized using anti-HA antibody following SDS–PAGE of the immunoprecipitated complexes.

2.6. RNA extraction and real time PCR analysis

After trypsinization at 37 °C, 293T cells were collected and total RNA was extracted using the Nucleospin RNA II kit (Macherey–Nagel). The isolated RNA was subjected to first strand cDNA synthesis using the RevertAid H minus kit (Fermentas). Quantitative analysis of *ARF* transcripts was done by real time PCR with Sybr Green (Thermo Scientific) using an ABI 7000 thermocycler. The sequences of the primers used were as followed: ARF sense GAGAACATGGTGCGCAGGT; ARF antisense GATGTGAACCACGAAAACCC TC; GAPDH sense GCACCAGGTGGTCTC CTC; GAPDH antisense TGACAAAGTGGTCGT TGA.

3. Results

3.1. ARF regulates the ubiquitination and stability of RING1B

To assess a possible role of ARF in the regulation of RING1B stability, we transfected 293T cells with siRNAs that silence ARF and monitored the degradation of endogenous RING1B using the protein synthesis inhibitor cycloheximide [CHX; Fig. 1A(i)]. The extent of silencing of the *ARF* transcript was confirmed by quantitative real time PCR [Fig. 1A(ii)]. Downregulation of ARF resulted in a notable stabilization of RING1B. In the reciprocal experiment, overexpression of ARF resulted in both a reduction in the steady state level of RING1B and in its accelerated degradation (Fig. 1B). The effect of ARF was stimulated when E6-AP was also overexpressed (Fig. 1B), suggesting a possible cooperation between E6-AP and ARF in the degradation of RING1B. Next, we investigated if ARF has an effect on the ubiquitination status of RING1B in cells. As can be seen in Fig. 1C, following overexpression of ARF the level of ubiquitinated RING1B was dramatically decreased. Overexpression of the unrelated protein COMMD1 did not affect RING1B ubiquitination. It is possible that ARF inhibits self-ubiquitination of RING1B, which appears to be quantitatively dominant, thus allowing a more efficient ubiquitination mediated by E6-AP.

3.2. ARF interacts with the RING finger of RING1B via its N-terminal domain

Next, we investigated if RING1B and ARF interact physically in 293T cells. As can be seen in Fig. 2A, ARF-Flag co-precipitated with

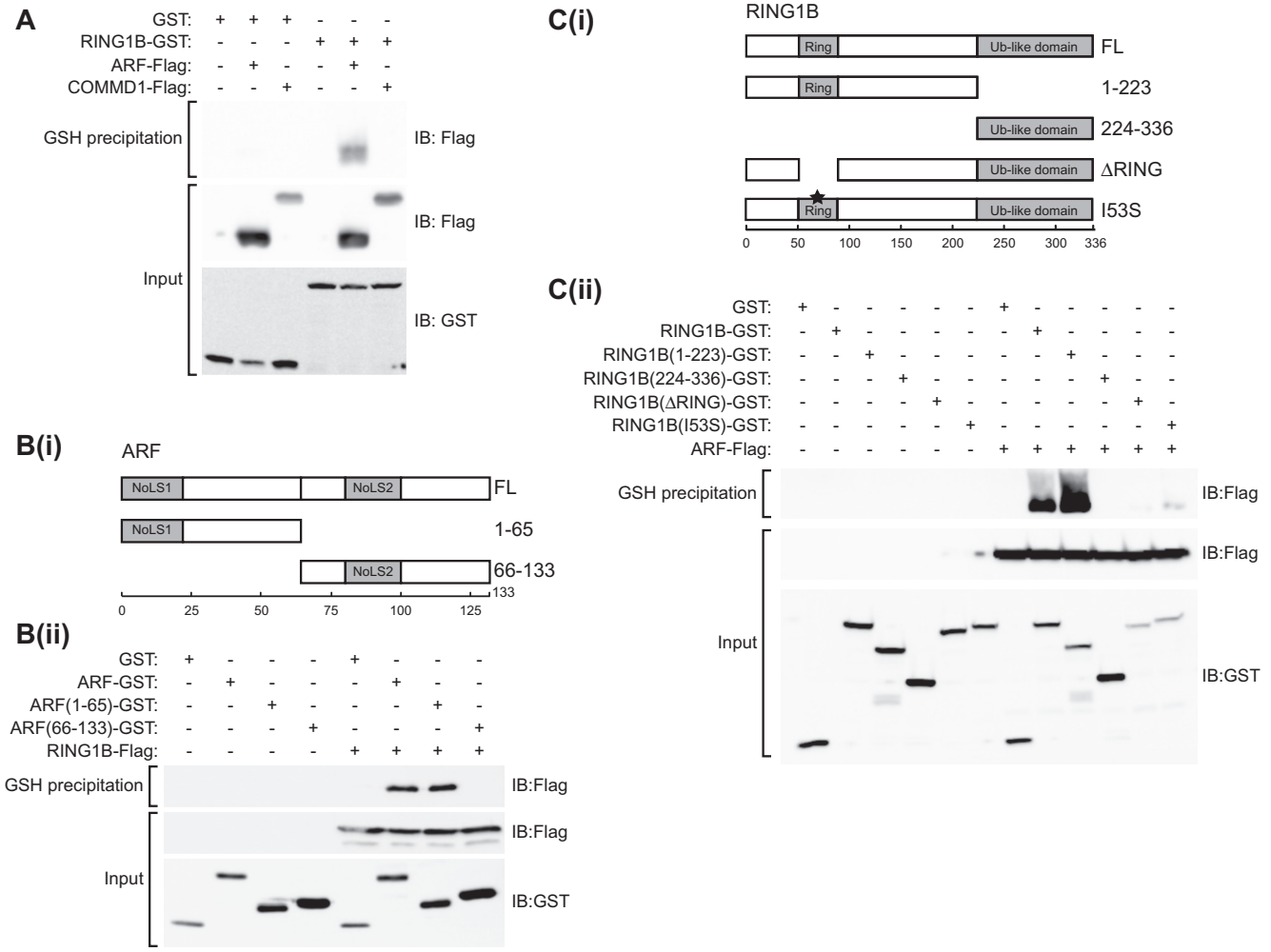

Fig. 2. ARF interacts via its N-terminal domain with the RING domain of RING1B. (A) Physical interaction between full length RING1B and ARF. 293T cells were transfected with cDNAs coding for RING1B-GST and ARF-Flag or COMMD1-Flag. Cell lysates were prepared and used for GST pull-down as described under *Materials and methods*. The SDS–PAGE-resolved proteins were detected following Western blotting using anti-Flag or anti-GST antibodies. (B) Schematic representation of ARF (i) and interaction of its different deletion derivatives with full length RING1B (ii). (C) Schematic representation of RING1B (i) and interaction of its different deletion derivatives with full length ARF (ii). GST pull down of the ARF-GST (B) and RING1B-GST (C) and visualization of the complexes were as described under A.

RING1B-GST, whereas COMMD1-Flag did not. The polypeptide sequence of ARF contains several important functional regions (Fig 2B). The N-terminal half of ARF is required for interactions with many of its binding partners such as MDM2 and COMMD1, E2F1 and p120^{E4F}, and contains a 22 residues domain that constitutes a nucleolar localization signal [16,18,19]. Within the C-terminal half, another nucleolar localization signal was identified (residues 86–100) that was shown to bind weakly to MDM2 [20,21]. GST pull-down analysis revealed that the amino terminal half of ARF is both essential and sufficient for interaction with RING1B (Fig. 2B). Structural domains within RING1B (Fig. 2C) include a RING domain, which is crucial for the recruitment of the E2 component of the conjugation machinery, and thus for its ubiquitin ligase activity. In addition, the RING domain is required for interactions with BMI1 and the deubiquitinating enzyme USP7 [8,22]. Within the C-terminus, a domain with a ubiquitin-like fold is present, which mediates interaction with CBX domain-containing PcG proteins [23–25]. As can be seen in Fig. 2C, deletion of the RING domain completely abolished the interaction with ARF. A single point mutation that inactivates the RING domain (I53S) also diminished the interaction in a dramatic way.

3.3. ARF stimulates E6-AP-mediated ubiquitination of RING1B

Next, we sought to further investigate the apparent discrepancy between the reduced ubiquitination, and the decreased stability of RING1B observed in response to ARF overexpression. As noted, RING1B undergoes two types of ubiquitination that are mutually exclusive: (1) self-ubiquitination that is required for its activity, and (2) E6-AP-mediated ubiquitination that targets it for proteasomal degradation. The destabilizing effect that ARF has on RING1B suggests that it can either inhibit RING1B self-ubiquitination or stimulate its E6-AP-mediated ubiquitination, or do both. The decrease in ubiquitination of wild type (WT) RING1B (Fig. 1C) suggests that it is the self-ubiquitination that is mostly affected. To shed additional light on the mechanism of action of ARF, we investigated its effect on the ubiquitination of both WT and self-ubiquitination-defective RING1B in the presence or absence of overexpressed E6-AP (Fig. 3). It is clear that ARF inhibits significantly the self-ubiquitination of RING1B (compare lanes 4 to 3 and 9 to 8), while it has almost no effect on the ubiquitination mediated by E6-AP (compare lanes 6 to 5 and 11 to 10). As expected, when the self-ubiquitination mutant was used, the basal level of ubiquitination was significantly reduced (compare lane 8 to 3). However, overexpression of E6-AP generated predominantly high molecular weight RING1B-ubiquitin conjugates, probably

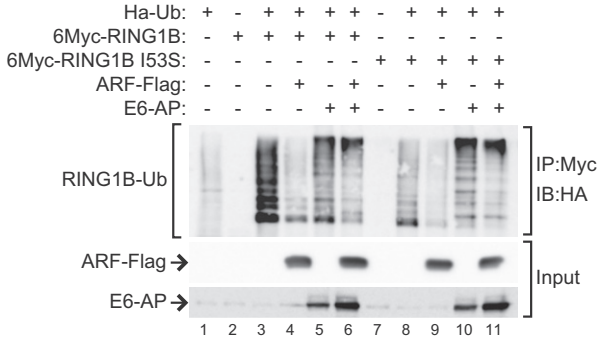

Fig. 3. ARF inhibits RING1B self-ubiquitination, but not its ubiquitination by E6-AP. 293T cells were transfected with cDNAs coding for WT or an inactive mutant of RING1B along with E6-AP and/or ARF-Flag as indicated. Conjugates were isolated and visualized as described under the legend to Fig. 1C.

reflecting the lack of competition on ubiquitination sites by the self-catalyzed process (compare lane 10 to lanes 5 and 8). Here too, ARF has almost no effect, suggesting its main effect is on self-ubiquitination, allowing E6-AP to act more efficiently.

3.4. ARF prevents RING1B homodimerization

Several RING-containing ubiquitin ligases were shown to homodimerize, which was suggested to play an import role in their ability to catalyze self-ubiquitination [26,27]. Using GST pull-down analysis, we observed that RING1B undergoes RING domain-dependent homodimerization (RING1B(ΔRING)-GST could not precipitate WT RING1B-Flag; Fig. 4A). Since ARF binds to the same region in RING1B, we hypothesized that binding of ARF to RING1B would interfere with its ability to homodimerize. As can be seen in Fig. 4B, increasing concentrations of ARF efficiently prevented co-precipitation of RING1B-Flag with RING1B-GST. As expected, a slight decrease in the levels of RING1B-Flag and RING1B-GST was observed as the concentration of expressed ARF increased. However, this mild decrease could not explain the strong decrease in RING1B-Flag co-precipitation, indicating a direct effect of ARF on RING1B homodimerization.

4. Discussion

We previously identified USP7 as a deubiquitinating enzyme targeting indiscriminately the two modes of ubiquitination of RING1B – the self-catalyzed one and the E6-AP-mediated one [8].

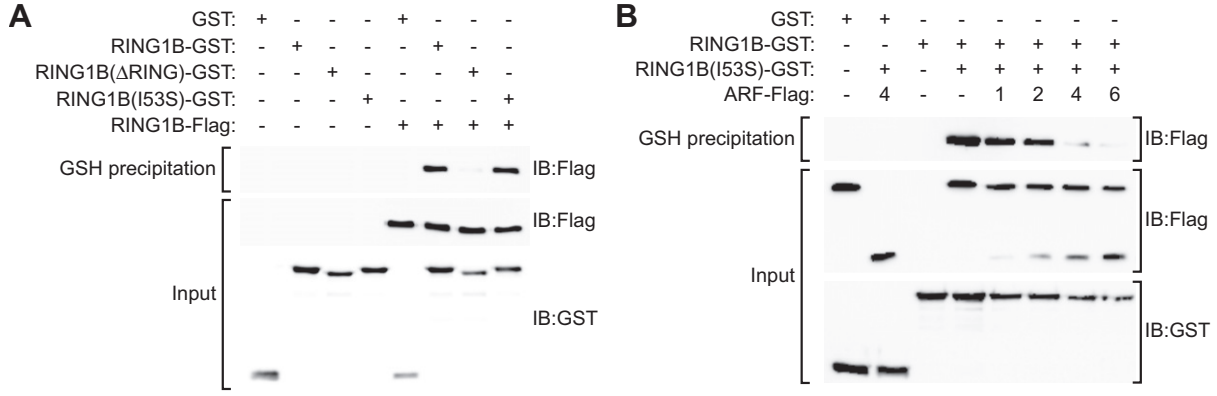

Fig. 4. ARF inhibits homodimerization of RING1B. (A) 293T cells were transfected with cDNAs coding for WT RING1B-Flag and the different RING1B-GST deletion mutants as indicated. Cell lysates were prepared for GST pull-down, and SDS–PAGE-resolved proteins were detected following Western blotting using anti-Flag or anti-GST antibodies. (B) GST pull down of RING1B-GST with RING1B-Flag in the presence of increasing concentrations of ARF-Flag.

This led us to hypothesize that USP7 serves as a switch to reset RING1B back to its naive state, and that the balance between the two types of ubiquitination is modulated by yet to be identified regulator(s) residing upstream of the ubiquitination reactions. Our current data suggest that ARF that inhibits RING1B self-ubiquitination but not E6-AP-mediated ubiquitination can be such a regulator. ARF was previously shown to affect the ubiquitination and degradation of various proteins via direct effect on ubiquitin ligases [11,12]. Taken together, these observations implicate ARF as an upstream regulator of ubiquitination by affecting directly the actual conjugation step. Our data demonstrate that ARF specifically directs RING1B into the E6-AP-mediated ubiquitination pathway by preventing its self-ubiquitination. It appears therefore that RING1B stability is inversely correlated with ARF level. Consistent with our data, it was previously shown that reactivation of ARF expression through knockout of Mel18 in mouse embryonic fibroblasts is accompanied by a dramatic decrease in RING1B levels [10].

It appears that ARF is involved with regulation of several key processes mediated by the ubiquitin system. However, a common mechanism is currently lacking. For example, none of the three ligases regulated by ARF share a common binding motive. Secondly, for some proteins (e.g. p53) ARF prevents their degradation, whereas the degradation of others (e.g. B23, RING1B) is promoted by ARF. One aspect however appears to be shared by several proteins regulated by ARF: they seem to preferentially bind to the N-terminal domain of ARF. How ARF exactly exerts its effects remains an enigma. It is possible that it induces re-localization of proteins through its N- or C-terminal nucleolar localization signals. This was previously suggested for MDM2, but later shown as not essential for the inhibitory effect of ARF on this ligase [28]. Alternatively, its highly basic nature might induce a conformational change on its binding partners. This in turn may affect the affinity of the target substrates to their ligases, or alter directly the activity of the regulated protein in case it is a ligase. Simple competitive binding may also reduce the activity or affinity of ligases, by inhibiting for example their homodimerization as we demonstrate here for RING1B. Further investigation is required to fully unveil the mechanistic nature of the functions of ARF in the ubiquitin system.

The *CDKN2A* locus, which encodes for both $p16^{INK4a}$ and ARF, is subject to repression by the PRC1 complex [9]. Although $p16^{INK4a}$ and ARF are both potent tumor suppressors, they share no sequence or functional homology, and their expression is mediated via the action of separate and different promoters. Whereas $p16^{INK4a}$ is an inhibitor of cyclin-dependent kinases and activates the pRB pathway, ARF activates the p53 pathway through inhibition of MDM2 and ARF-BP1, but also has p53-independent tumor suppressing functions [29]. The negative regulation of RING1B by ARF through inhibition of RING1B activating self-ubiquitination and decreased stability, constitutes a potential feed-forward mechanism where ARF positively regulates its own expression and that of $p16^{INK4a}$ through reactivation of the *CDKN2A* locus. Such a mechanism would ensure that once the cell commits to an apoptotic fate, it is followed through with maximum efficiency.

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