Ubiquitin-dependent and Ubiquitin-independent Control of Subunit Stoichiometry in the SWI/SNF Complex

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The mammalian SWI/SNF chromatin remodeling complex is a key player in multiple chromatin transactions. Core subunits of this complex, including the ATPase, Brg-1, and various Brg-1-associated factors (BAFs), work in concert to maintain a functional remodeling complex. This intra-complex regulation is supervised by protein-protein interactions, as stoichiometric levels of BAF proteins are maintained by proteasomal degradation. We show that the mechanism of BAF155-mediated stabilization of BAF57 involves blocking its ubiquitination by preventing interaction with TRIP12, an E3 ubiquitin ligase. Consequently, as opposed to complexed BAF57, whose principal lysines are unavailable for ubiquitination, uncomplexed BAF57 can be freely ubiquitinated and degraded by the proteasome. Additionally, a BAF57 mutant, which contains no lysine residues, was found to retain its ability to be stabilized by interaction with BAF155, suggesting that in addition to the ubiquitin-dependent mechanism of BAF57 degradation, there exists a ubiquitin-independent mechanism that may involve the direct interaction of BAF57 with the proteasome. We propose that this regulatory mechanism exists to ensure functional fidelity of the complex and prevent the accumulation of uncomplexed proteins, which may disrupt the normal activity of the complex.

Mammalian DNA is packaged into chromatin with the help of nucleosomes as a means to regulate transcription, replication, DNA repair, and recombination, all highly dynamic processes (1). When compacted into chromatin, genes are often inactive due in part to the inability of the cell transcription machinery to gain access to regulatory regions (1). This access and subsequent gene activation is achieved partly with the help of ATP-dependent chromatin remodeling complexes (2). The mammalian SWI/SNF chromatin remodeling complex is a large multiprotein machine that functions to decondense or loosen DNA, which is tightly wrapped around nucleosomes. The major catalytic component of the complex can be either Brg-1 or Brm, both ATPases that use the energy from ATP hydrolysis to expose gene promoters (3). By doing so, SWI/SNF chromatin remodeling complexes have the ability to regulate transcription and, thus, control the vast majority of cellular processes. To obtain full functionality, however, the SWI/SNF complex requires the presence of Brg-1-associated factors or BAFs (2). At least 10 BAFs exist, including but not limited to BAF47, BAF53, BAF57, BAF60, BAF155, BAF170, BAF180, BAF200, and BAF250 (4, 5). These BAFs are present in different combinations depending on the specific complex(es) of which they are a part (5–7).

Although the functions of all the BAFs have not been fully elucidated, a number of specific roles/functions have been proposed. BAF47 is a known interacting partner with ALL-1 via its SET domain (8). BAF53 is a known c-Myc-interacting nuclear cofactor and forms into complexes with histone acetyltransferase activity (9). BAF57 interacts with estrogen receptor α (10) and androgen receptor (11) to recruit the SWI/SNF complex. BAF60α mediates the interaction between GR and the SWI/SNF complex (12) and also interacts with p53 to recruit the SWI/SNF complex (13). Finally, although BAF155/BAF170 have not been shown to interact with chromatin-modifying enzymes or be involved in SWI/SNF complex recruitment, they have been implicated as scaffolding proteins for the complex and have been shown to regulate the expression levels of other BAFs (14, 15). In the case of BAF155 and BAF57, it was observed that BAF155 interacts with and stabilizes BAF57 (14). Subsequently, it was revealed that BAF57 is regulated at the level of the proteasome and that either inhibiting the proteasome or expressing BAF155 resulted in an increase in BAF57 (14). The importance of BAF155 in SWI/SNF subunit regulation was further defined, as it was demonstrated that SRG3, the murine homolog of BAF155, also interacts with and stabilizes BAF47, BAF60α, and Brg-1 (15).

Because the SWI/SNF complex functions in the regulation and fidelity of such tightly controlled cellular processes such as growth, development, mitosis, cell cycle control, differentiation, hormonal response, apoptosis, retroviral infection, and carcinogenesis (16–19), understanding the regulation and fidelity of the SWI/SNF complex itself is of the utmost importance. Given the diverse components and proposed roles for the complex, the understanding of SWI/SNF subunit stoichiometry may be central to understanding the control of the complex.

Although it is clear that BAF155 plays a role in SWI/SNF complex stoichiometry, to what extent and the mechanism(s) by which it is achieved are not fully understood. Biochemical

2 The abbreviations used are: BAF, Brg-1-associated factor; TRIP12, thyroid hormone receptor-interacting protein 12; LMO, LIM-only; SSBP, single-stranded DNA-binding protein; GR, glucocorticoid receptor; ESI, electrospray ionization; Ub, ubiquitin; NLS, nuclear localization signal; CLIM, cofactor of LIM-HD protein; RLIM, RING finger LIM-domain binding protein.
analyses suggest that the presence of free unbound SWI/SNF complex subunits is unlikely (4, 20). This would suggest that the cell exercises cellular mechanisms by which protein subunit expression and/or degradation are controlled. Indeed, stable cell lines overexpressing BAF57 mutants actually down-regulate the endogenous BAF57 protein levels in the cell such that the total amount of cellular BAF57 remained the same as in parental cells (14).

In this report we set out to evaluate the mechanism(s) by which BAF155 regulates the stoichiometry of BAF57. We find that BAF155 stabilizes free and uncomplexed BAF57 by binding to it and blocking the extent to which it is ubiquitinated. In addition, we show that mutating the lysine residues within BAF57 stabilizes the protein in the absence of BAF155. Finally we propose TRIP12 as the E3 ubiquitin ligase responsible for the ubiquitination of BAF57 and demonstrate that BAF155 blocks the interaction between BAF57 and TRIP12. These results provide a mechanism describing how the mammalian SWI/SNF chromatin remodeling complex maintains its subunit stoichiometry within the cell.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Chemicals**—Antibodies to FLAG (M2, mouse), FLAG (F7425, rabbit), and β-actin (AC15) were purchased from Sigma. Nonspecific normal IgG antibodies and antibodies to myc (9E10), HA (F-7), and glucocorticoid receptor (GR; M-20) were purchased from Santa Cruz Biotechnologies. Antibodies to myc and V5 were purchased from Invitrogen. TRIP12 and GAPDH (6C5) antibodies were purchased from Bethyl Laboratories and RDI/Fitzgerald Industries, respectively. Additional nonspecific normal IgG antibodies were purchased from Millipore. MG132, epoxomicin, and cycloheximide were obtained from Calbiochem.

**Cell Culture and Transient Transfections**—Human U3 cells, a derivative of U2OS osteosarcoma cells, were created and maintained as previously described (21). U3 cells DNA transfections were performed in antibiotic-free media using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO2 for 48 h.

**Plasmids**—BAF155 and BAF57 expression constructs are inserted into pCMV/myc/nuc and pcDNA3.1(−) (Invitrogen), respectively. WT BAF155-myc, WT BAF57-FLAG, BAF57ΔPH-FLAG, and BAF57ΔNHRIL-FLAG have been described previously (14). BAF57ΔHMG2-FLAG and BAF57ΔCC2-NLS-FLAG were constructed by Dr. Jianguang Chen. pMT123-HA-Ub was a kind gift from Dr. Andrew Wallace. BAF57ΔAD1-FLAG, BAF57CC and BAF57aa190–366-FLAG, WT were created using PCR with the appropriate primers and WT BAF57-FLAG as a template. WT BAF57–3XNLS-FLAG was created by inserting the WT BAF57 gene into pCMV/myc/nuc to fuse a 3XNLS signal to the C terminus of BAF57 and then designing specific primers with a C-terminal FLAG tag to generate the BAF57–3XNLS-FLAG gene by PCR. This insert was then ligated into pcDNA3.1(−). The sequences of all final plasmids were confirmed by DNA sequencing.

**Site-directed Mutagenesis**—Lysine-to-arginine point mutants were created using the QuikChange XL site-directed mutagenesis kit (Stratagene). The initial reaction contained the WT BAF57–3XNLS-FLAG plasmid as a template, the appropriate primers (designed according to the specifications outlined in the manufacturer’s protocol), and other components provided in the kit as described by the manufacturer. Subsequent reactions adding additional point mutations contained the previously created point mutation-containing plasmid, the appropriate primers, and other components provided in the kit. Lysines were mutated one, two, or three at a time in a single reaction, depending on the proximity of the residues and the primers used. Construction of the BAF57 K→R(14)-3XNLS-FLAG plasmid required six successive reactions. BAF57aa190–366-FLAG K→R was created using PCR with the appropriate primers and the BAF57 K→R(14)-3XNLS-FLAG plasmid as a template (note: the resulting plasmid does not contain a 3XNELS sequence). The sequences of all final plasmids were confirmed by DNA sequencing.

**Western Blots**—After a 48-h transfection, cells were washed and scraped into phosphate-buffered saline containing a protease inhibitor mixture (Complete, EDTA-free, Roche Applied Science). Cells were pelleted by centrifugation, and whole cell extracts were prepared by lysis in a previously described high salt buffer (Buffer L: 20 mM Tris- HCl (pH 7.5), 400 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% Nonidet P-40) (14) containing a protease inhibitor mixture (Sigma P8340). After a Bradford protein assay (Bio-Rad) to determine protein concentrations, equal micrograms (50–100) of whole cell extracts were resolved by SDS-PAGE, transferred to a PVDF membrane (Invitrogen), and analyzed using the appropriate antibodies. Exogenous BAF57 and BAF155 were probed with FLAG and myc antibodies, respectively. Proteins were detected using horse-radish peroxidase-conjugated secondary antibodies (GE Healthcare) and Western Lighting-ECL Chemiluminescent Reagent (PerkinElmer Life Sciences). Membranes were stripped in between antibody probes using Restore Western blot Stripping Buffer (Thermo Scientific).

**Immunoprecipitations**—Immunoprecipitations were performed as previously described (14). Briefly, whole cell extracts were prepared as described above by lysis in Buffer L. After Bradford protein quantification, 400–500 μg of total protein was diluted with an equal volume of NaCl-free Buffer L, bringing the total NaCl concentration to 200 mM. Buffer L with 200 mM NaCl was then added to the sample to bring the total volume up to 500 μL. After the addition of 2–4 μg of the appropriate antibody, the samples were mixed overnight at 4 °C. The following day 20–25 μL of Protein A/G PLUS-agarose immunoprecipitation reagent (Santa Cruz Biotechnologies) was added, and the samples were mixed for an additional 2 h at 4 °C. The agarose beads were washed five times with Buffer L containing 200 mM NaCl, all the supernatant was removed, and 1× SDS gel-loading buffer was added to each sample. Samples were incubated at 95 °C for 3–5 min, and immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blot. When necessary, a negative control immunoprecipitation was performed using an equal amount of nonspecific normal mouse IgG antibody.

**Immunoprecipitation-Mass Spectrometry Analysis**—UL3 cells were transfected with either WT BAF57-FLAG or empty vector...
as a negative control. Cells were lysed, and immunoprecipitations were setup with buffers described above. 3 mg of total whole cell extract was used for each immunoprecipitation. The lysate was first precleared by mixing with 75 μl of Protein A/G PLUS-agarose immunoprecipitation reagent at 4 °C for 2 h. The lysates were transferred to new tubes, and 15 μg of α-FLAG (M2) antibody was added. After mixing overnight at 4 °C, 75 μl of Protein A/G PLUS-agarose immunoprecipitation reagent was added, and mixing was continued for an additional 2 h at 4 °C. The agarose beads were then washed five times, and the beads were resuspended in 1 × SDS gel-loading buffer. Samples were incubated at 95 °C for 3–5 min, immunoprecipitated proteins were resolved by 10% SDS-PAGE, and the gel was stained with SimplyBlue SafeStain (Invitrogen). Digestion and mass spectrometry was performed as each gel lane was excised manually as 24 separate samples and digested with trypsin (Promega) for 8 h in an automated fashion with a Prosect robotic digester from Genomic Solutions. Resulting peptides were lyophilized and then resuspended in 35 μl of 0.1% formic acid. NanoLC-ESI-MS and MS/MS analyses were performed using an Agilent 1100 nanoLC system on-line with an Agilent 6340 ion trap mass spectrometer with the Chip Cube Interface. Briefly, 20 μl of digest were loaded onto an Agilent C18 chip (75 μm × 43 mm) followed by a linear gradient from 5% acetonitrile, 0.1% formic acid to 50% acetonitrile, 0.1% formic acid to the column over 45 min. The mass spectrometer was used in the positive ion, standard enhanced mode and included settings of a mass range from 200 to 2200 m/z, an ionization potential of 2.1 kV, an ICC smart target of 100000 ions accumulated in the trap or 200 milliseconds of accumulation, and a 1.0-V fragmentation amplitude. MS/MS data were acquired using a data-dependent acquisition format, with the six most abundant ions from each MS scan with a threshold of 5000 counts further interrogated by MS/MS. Automated data base searching was performed as peak lists generated from the data obtained from each nanoLC-ESI-MS/MS analysis using the Data Extractor feature of the SpectrumMill software from Agilent. The resulting extracted data were then searched against the NCBI non-redundant data base using the MS/MS Search function in the SpectrumMill software. Search settings included enzyme specificity with up to two missed cleavages allowed, a precursor ion mass tolerance of 1.5 Da, a product ion mass tolerance of 1.0 Da, variable methionine oxidation, and a minimum matched spectral intensity of 70%. Sequence alignments of MS/MS spectra were manually validated.

siRNA Transfection—A small interfering RNA (siRNA) duplex targeting the mRCA coding region of TRIP12 (NCBI Accession Number NM_004238) was designed and synthesized by Dharmacon/Thermo Scientific, 5′-GCUCUAUAC-GCAAAGGUUA-3′. NTC-1, a non-targeting control siRNA (D-001810-01-20), and a siRNA targeting GAPDH (D-001830-01-20) were also purchased from Dharmacon/Thermo Scientific. siRNA transfections were performed in antibiotic-free media using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Protein levels were determined by Western blot.

RESULTS

BAF155 Blocks the Ubiquitination of BAF57—The SWI/SNF chromatin remodeling complex is composed of a number of core subunits including Brg-1, which possesses ATPase activity, and accompanying BAFs. Previous reports have shown that the stoichiometry of these BAFs is highly important to the complex and that BAF155 plays a pivotal scaffolding role in regulating the protein levels of other complex members (14). To explore the underlying mechanism, we asked if exogenous WT BAF57 would also be stabilized by exogenous WT BAF155. We observed that in UL3 cells BAF155 expression did in fact lead to elevated levels of BAF57 (data not shown).

Using the BAF155-BAF57 relationship as a model, we hypothesized that because BAF155 stabilizes BAF57 by rescuing it from proteasomal degradation, perhaps BAF155 blocks the ubiquitination of BAF57. To test this idea, we set up a ubiquitination assay in which an HA-tagged ubiquitin construct was co-expressed with BAF57 in the absence or presence of BAF155 in UL3 cells (Fig. 1A). Whole cell extracts were then immunoprecipitated with a FLAG antibody, which would pull down any and all BAF57. The HA-ubiquitin construct was also transfected alone (lane 1) to determine the levels of background binding by any non-BAF57 ubiquitinated proteins to the antibody or beads. As seen in Fig. 1A, in the presence of HA-ubiquitin and BAF57 alone (lane 2), we observe a ladder of ubiquitinated BAF57 species. Co-expressing BAF155 (lane 3) reveals a significant decrease in the amount of BAF57 ubiquitination as compared with lane 2. This result suggested that BAF155 blocked and/or prevented the ubiquitination of BAF57. Importantly, the FLAG (BAF57) probe revealed a ubiquitinated BAF57 species. This species is diminished in the presence of BAF155 as compared with BAF57 alone. As a control, we co-expressed Brg-1 instead of BAF155 with BAF57 to make sure that it is specifically the presence of BAF155 and not the presence of any SWI/SNF complex member that caused the drop in BAF57 ubiquitination (lane 4). As predicted, Brg-1 had no effect on BAF57 ubiquitination. As another control, to determine whether the presence of BAF155 affected the cell ubiquitination machinery in general rather than specifically blocking BAF57 ubiquitination, we tested whether BAF155 influenced the ubiquitination of GR, another readily ubiquitinated protein (Fig. 1B). Again, as predicted, the ubiquitination pattern of GR was unaffected by the expression of BAF155. These findings support our hypothesis that BAF155 specifically stabilizes BAF57 by blocking its ubiquitination.

Amino Acids That Lie within and Flank the Coiled-coil Region of BAF57 Are Critical for Stabilization—In an attempt to narrow down the region of BAF57 that may contain key ubiquitinated lysines responsible for its proteasomal degradation, we created a series of BAF57 truncation and deletion mutants (Fig. 2A). We then expressed each of these BAF57 mutants in either the absence or presence of BAF155 in UL3 cells to determine their ability to be stabilized (Fig. 2B). The result that BAF57ΔPH could still be stabilized by BAF155 suggested that the proline-rich and HMG domains are not critical for BAF57 stabilization. BAF57ΔHMG2 and BAF57ΔHRL1 were also stabilized by BAF155, revealing that the regions in between
BAF155 Blocks BAF57 Ubiquitination

**A.**

| Input | IP |
|-------|----|
| BAF57-FLAG: BAF155-myc: Brg-1-V5: | + + + |
| BAF155-myc: BAF155-FLAG: Brg-1-V5: | - + + |
| BAF155-myc: BAF155-FLAG: | + + |

**B.**

| Input | IP |
|-------|----|
| GR: BAF155-myc: | + |
| BAF155-myc: | + |
| BAF155-FLAG: | + |

**FIGURE 1.** BAF155 decreases the ubiquitination of BAF57 but does not affect the ubiquitination of GR. A, UL3 cells were transiently co-transfected with an HA-tagged ubiquitin construct (pMT123-HA-Ub), BAF57-FLAG, and either empty vector, BAF155-myc, or Brg-1-V5. Lane 1 was transfected with only the HA-ubiquitin construct, and empty vector and served as a negative control. Whole cell extracts were immunoprecipitated with a FLAG (BAF57) antibody. Both inputs and precipitated products (IP) were resolved by 10% SDS-PAGE and were analyzed by Western blot using antibodies to HA (ubiquitin), FLAG (BAF57), myc (BAF155), and V5 (Brg-1). Solid arrows indicate the position of non-ubiquitinated BAF57. Dashed arrows indicate ubiquitinated BAF57 bands on corresponding blots. Notably, lane 4 was spliced to lanes 1–3; however, all figure panels were from the same blots and from the same exposures. B, UL3 cells were transiently co-transfected with an HA-tagged ubiquitin construct, GR, and either empty vector or BAF155-myc. Whole cell extracts were immunoprecipitated with a GR antibody. Both inputs and precipitated products (IP) were resolved by 10% SDS-PAGE and analyzed by Western blot using antibodies to HA (ubiquitin), GR, and myc (BAF155).

Amino acids 132–172 and 173–219, respectively, also do not contribute to the elevated BAF57 levels seen. Finally, BAF57ΔAD1, being stabilized by BAF155, suggests that amino acids 300–411 are likewise not required for the increase seen for BAF57. Therefore, we found that BAF57CC, a mutant truncated down to amino acids 220–299, is stabilized by BAF155, indicating that this protein fragment may contain potentially ubiquitinated lysines. We did not truncate this fragment farther on the N-terminal side because previous reports indicate that amino acids 220 through the coiled-coil are important for binding to BAF155 (14). Accordingly, BAF57ΔCC2, a protein that does not bind BAF155 (data not shown), is, thus, not stabilized by BAF155. Additionally, we did not truncate it farther on the C-terminal side because cutting off the entire acidic domain resulted in a significant decrease in the expression of the protein (data not shown).

**Lysine-to-arginine Mutations Stabilize BAF57 and Reduce the Impact of BAF155-mediated Stabilization.** To gauge the significance of these residues, we mutated the lysines in between amino acids 220 and 299 of BAF57 within the context of the full-length protein. We first constructed three point mutants, K→R(5), K→R(7) and K→R(10), with the number of lysines mutated to arginines equaling 5, 7, and 10, respectively (Fig. 3A). We expressed these mutants in UL3 cells in the presence or absence of BAF155 and found that as the lysines were mutated, the BAF57 protein became more stable in the absence of BAF155 (Fig. 3B, lanes 1–8). Fig. 3C graphically depicts the -fold change in the amount of BAF57 protein expression upon the addition of BAF155. Notably, the K→R(10) mutant with all lysines mutated within amino acids 220–299 was still able to be stabilized by BAF155, suggesting that additional lysines outside this range are ubiquitinated. Consequently, we constructed two additional lysine-to-arginine point mutants, K→R(13) and K→R(14), with lysine mutations on both the N- and C-terminal sides of the 220–299 segment (Fig. 3A). Interestingly, neither of these two new mutants conferred any significant additional stability to BAF57 (Fig. 3, B and C). Not surprisingly, therefore, there was a negative (−0.67), although statistically significant (p = 0.001) Spearman’s ρ correlation between the -fold change in BAF57 expression upon the addition of BAF155 and the number of lysines mutated to arginine. However, from these data we obtain two important results; 1), that mutating BAF57 lysines stabilizes the protein in the absence of BAF155 (Fig. 3B), and 2), that mutating BAF57 lysines affects the extent to which BAF155 can stabilize BAF57 (Fig. 3C).

**Lysine-to-arginine Mutations Decrease BAF57 Ubiquitination.** To verify that the mutations in BAF57 from lysine to arginine resulting in its stabilization (Fig. 3B) were due to decreasing the protein ubiquitination, we performed a ubiquitination assay with the five point mutants we generated (Fig. 4). As evidenced by the HA (ubiquitin) probe, as the number of mutated lysines increases from left to right, the amount of ubiquitinated BAF57 concomitantly decreases. Even more striking is the result with the FLAG (BAF57) probe. On this blot, two separate ubiquitinated BAF57 species are visible, and their expression, as with the HA (ubiquitin) probe, diminishes from left to right, indicating that as the number of lysines in each successive mutant decreases, so does their level of ubiquitination.

**The Mutant BAF57 K→R(14) Interacts with BAF155.** To address the possibility that mutating the lysines in BAF57 could alter the protein native folding in such a way that its interaction specificity for BAF155 is diminished, we performed a co-immunoprecipitation between BAF57 K→R(14) and BAF155 (Fig. 5A). The results show that even with 14 lysines mutated to arginine, the interaction between BAF57 K→R(14) and BAF155 is just as strong and specific as the interaction between WT BAF57 and BAF155 (Fig. 5A, compare lanes 3 and 6).

A BAF57 Mutant Containing No Lysines Is Stabilized by BAF155. Having confirmed that BAF57 stability is regulated by an ubiquitin-dependent pathway, we sought to determine whether there were additional pathways by which the protein
was regulated. To do so, we created the largest BAF57 fragment we could that contained no lysine residues from the BAF57 K→R(14) point mutant. Using specifically designed PCR primers, we created BAF57aa190–366 K→R, a truncation mutant in which all lysines are mutated to arginine (Fig. 5B). Using WT BAF57 as a template, we also created BAF57aa190–366 WT, a truncation mutant that contains all endogenous lysine residues. These two new proteins were tested for their ability to be stabilized by BAF155. As expected, the mutant with the WT sequence was stabilized by BAF155 (Fig. 5C). However, surprisingly, the expression of the truncation protein that contained no lysines and, therefore, could not be ubiquitinated, was also greatly increased upon the addition of BAF155 (Fig. 5C). These results suggest that there exists an alternate, ubiquitin-independent mechanism of BAF57 degradation, a mechanism that, like BAF57 ubiquitination, is also thwarted by the interaction of BAF155 with BAF57.

To determine whether the proteasome is still involved in the degradation of this BAF57aa190–366 K→R truncation mutant, we performed time course experiments with MG132. To be sure that this truncated and mutated protein behaves as the wild-type protein would in these time course experiments, the same treatments were performed for both WT BAF57 and BAF57aa190–366 WT. Upon MG132 treatment, the protein levels accumulated for all three proteins (Fig. 6), indicating that the proteasome is in fact still involved in the degradation of BAF57aa190–366 K→R (Fig. 6C). Because MG132 also inhibits some non-proteasomal enzymes, we also treated cells in a time course with epoxomicin, which inhibits only proteasomal enzymes, and observed similar trends of protein accumulation (Fig. 6). To determine the stability of the protein, protein synthesis was inhibited by cycloheximide in a time course (Fig. 6). The results indicate that BAF57aa190–366 K→R, like the other two WT proteins, is highly unstable with a very short half-life (Fig. 6C). A second cycloheximide time course was performed with BAF155 co-transfected to demonstrate the stability that it confers to the BAF57 proteins. As predicted, the addition of BAF155 greatly stabilized all three proteins and drastically lengthened their half-lives (Fig. 6).

BAF57 Interacts with the E3 Ubiquitin Ligase TRIP12—In an attempt to identify the E3 ubiquitin ligase responsible for BAF57 ubiquitination, we performed an immunoprecipitation–mass spectrometry experiment. WT BAF57-FLAG or empty vector was expressed in UL3 cells and immunoprecipitated with FLAG (BAF57) antibody, and the resulting pulldown assay was resolved by SDS-PAGE and subjected to mass spectrometry analysis. The HECT domain-containing E3 ubiquitin ligase TRIP12 was putatively identified as binding to BAF57 (supplemental Table 1). This result was then verified by Western blot (Fig. 7A). After immunoprecipitation of exogenous BAF57-FLAG (lanes 1–3), we observed the pulldown of endogenous TRIP12. We next tested the idea that TRIP12 and BAF155 might be in competition for BAF57, and the outcome of this competition could determine the resulting protein levels. Indeed, immunoprecipitation of BAF57-FLAG in the presence of BAF155-FLAG (lanes 4–6) revealed a loss of BAF57-TRIP12 interaction which suggested that BAF155 interacts with and decreases BAF57 ubiquitination by preventing the interaction between BAF57 and TRIP12.

TRIP12 siRNA Knockdown Stabilizes BAF57—To further verify that TRIP12 contributes to the degradation of BAF57, we decided to knockdown TRIP12 protein levels to observe any change in exogenous BAF57 protein levels (Fig. 7B). We observed that, as compared with the protein level in the mock-treated sample (lane 1), there was an increase in BAF57 protein expression upon TRIP12 knock-down (lane 3). As negative controls, GAPDH (lane 2) or NTC-1 (lane 4) siRNA treatment did not alter the TRIP12 or BAF57 protein levels as compared with those in the mock-treated sample.

**DISCUSSION**

The supervision of SWI/SNF complex stoichiometry remains a relatively ill-defined process, and the regulation of SWI/SNF subunits within the cell has the potential to influence...
the precise control of many cellular processes. Multiple instances exist in which the strict expression levels of certain BAFs are altered in various cancers. Full-length wild-type BAF57 has been found to be reduced or lost in certain breast cancer cell lines such as BT549 cells (22). BAF57 has also been found to be elevated in a subset of prostate cancers (23). Human ovarian (SKOV3) and colorectal (SNUC2B) carcinomas are null for BAF155 expression (24). BAF47, now classified as a tumor suppressor, is mutated or truncated in a number of cancers including malignant rhabdoid tumors, choroid plexus carci-

mas, medulloblastomas, and central primitive neuroectodermal tumors (25–30). In addition, mouse model deletions of BAF47 and Brg-1 are embryonic lethal (31–33). In each of these cases, in which complex subunit stoichiometry is changed, the endogenous functions of the SWI/SNF complex may be altered. By elucidating the mechanisms by which the stoichiometry of these proteins is regulated, we may be able to better understand the pathways that lead to cancer and perhaps other disease states upon their misregulation.

In this report we examined the mechanism by which BAF155 stabilizes and maintains the stoichiometry of BAF57. This effect, however, is not limited to BAF57, as SRG3, a murine homolog of BAF155, interacts with and stabilizes BAF47, BAF60a, and Brg-1 (15). We have also verified that the same stabilization is seen with BAF155 and these SWI/SNF complex members (supplemental Fig. 1). Correspondingly, siRNA knockdown of BAF155 and BAF170 leads to decreased protein expression of BAF47, BAF60a, and Brg-1 (supplemental Fig. 2). These results suggest that BAF155 possesses a significant and critical role in maintaining the integrity and balance of this chromatin remodeling complex.

We hypothesized that because previous reports determined that excess BAF57 was degraded by the proteasome (14), that BAF155 stabilizes BAF57 by blocking its ubiquitination. We also tested the ability of BAF155 to block the ubiquitination of Brg-1, as this core SWI/SNF complex member has also been shown to be stabilized by BAF155. Interestingly, we found that the ubiquitination of Brg-1 was also diminished in the presence of BAF155 (supplemental Fig. 3), suggesting that this may be a general mechanism functioning to maintain the stoichiometry of the SWI/SNF complex.

If in fact BAF57 ubiquitination levels are regulated by BAF155, we postulated that mutating specific lysines within BAF57 would stabilize the protein in the absence of BAF155. As predicted, we observed an increase in the stability of the BAF57 protein as lysine residues were removed (Fig. 3B), presumably due to the loss of ubiquitinated lysines that would target the

FIGURE 3. BAF57 lysine-to-arginine mutants display increased stability in the absence of BAF155 and reduce the impact of BAF155-mediated stabilization. A, shown is a schematic representation of the WT BAF57 protein with approximate lysine positions indicated. Lysine residues mutated to arginine are indicated in red, and the resulting constructs are designated as shown. The individual construct nomenclature K→R(n) refers to the number (n) of lysine residues mutated to arginine. The precise amino acid number for each mutated lysine is indicated on the right. The residues falling within amino acids 220–299 of BAF57 are encompassed by the gray shaded box. To compensate for the loss of the endogenous NLS during lysine mutations, each construct, including WT BAF57, was C-terminal-tagged with a 3XNLS. Whole cell extracts were resolved by 10% SDS-PAGE and were analyzed by Western blot using antibodies to FLAG (BAF57), myc (BAF155), and actin. The experiment was performed in three biological replicates, and a representative blot is shown.
protein for degradation. Accordingly, the extent to which BAF155 can further stabilize these BAF57 mutants is diminished with the conversion to arginines (Fig. 3C).

A ubiquitination assay using these BAF57 lysine-to-arginine mutants provided additional confirmation of this model (Fig. 4). Thus, in the absence of BAF155, BAF57 is ubiquitinated in a lysine-dependent manner.

Given that free, uncomplexed BAF57 is degraded by the proteasome via an ubiquitin-dependent mechanism, we wanted to determine whether this was the only mechanism by which the protein is degraded. Some cellular proteins, whose cellular stoichiometry is also vital to their proper functioning, have been found to be degraded by ubiquitin-dependent and ubiquitin-independent mechanisms (34–36). One such example is p53.
BAF155 Blocks BAF57 Ubiquitination

A.

|                | BAF57-FLAG | BAF155-myc | BAF57-FLAG |
|----------------|------------|------------|------------|
| Input          |            |            |            |
| IgG            |            |            |            |
| IP             |            |            |            |
| Flag           |            |            |            |

B.

| siRNA       |          |          |          |
|-------------|----------|----------|----------|
| BAF57       |          |          |          |
| TRIP12      |          |          |          |
| GAPDH       |          |          |          |
| actin       |          |          |          |

**FIGURE 7.** BAF155 disrupts the interaction between BAF57 and TRIP12, and TRIP12 siRNA knock-down stabilizes exogenous BAF57. A, UL3 cells were transiently co-transfected with BAF57-FLAG and either empty vector (lanes 1–3) or BAF155-myc (lanes 4–6). Whole cell extracts were immunoprecipitated with either a nonspecific IgG antibody (lanes 2 and 5) or a FLAG (BAF57) antibody (lanes 3 and 6). Inputs (lanes 1 and 4) and precipitated products (IP) were resolved by SDS-PAGE and were analyzed by Western blot using antibodies to FLAG (BAF57), TRIP12, and myc (BAF155). B, UL3 cells were transiently transfected with the indicated siRNAs using Lipofectamine 2000. 48 h later, the medium was changed, and the cells were transfected with BAF57-FLAG using FuGENE 6. 48 h later, the medium was changed, and the cells were transfected with BAF57-FLAG and either empty vector (lanes 1–3) or BAF155-myc (lanes 4–6) and precipitated products (IP) were resolved by SDS-PAGE and were analyzed by Western blot using antibodies to FLAG (BAF57), TRIP12, GAPDH, and actin. Notably, lane 4 was spliced to lanes 1–3; however, all figure panels were from the same blot and from the same exposures.

However, is this ubiquitin-independent mechanism of degradation dependent on the proteasome? To answer this question, MG132 and epoxomicin time courses were performed on the BAF57aa190–366 K→R fragment, which revealed that the proteasome was in fact still involved in its degradation (Fig. 6C). One potential explanation for this result could be that BAF57, like p53 and p21, is also degraded via an indirect or direct interaction with the proteasome. Furthermore, the fact that the addition of BAF155 to the cycloheximide time course for BAF57aa190–366 K→R stabilized the protein and lengthened its half-life suggests that the protein retains sufficient native structure to bind BAF155 (Fig. 6C).

TRIP12 (thyroid hormone receptor-interacting protein 12) is a HECT domain-containing E3 ubiquitin ligase and a nuclear receptor co-regulator that interacts with the ligand binding domain of the thyroid hormone receptor and with retinoid X receptor. We identified TRIP12 in an immunoprecipitation-mass spectrometry experiment as a BAF57-interacting protein. When BAF57 is alone, it co-immunoprecipitates with TRIP12; however, in the presence of BAF155, BAF57 and BAF155 interact and the BAF57-TRIP12 interaction is lost (Fig. 7A). Furthermore, in the presence of reduced levels of TRIP12, there is a concurrent stabilization of BAF57 supporting the proposal that TRIP12 is the E3 ubiquitin ligase responsible for ubiquitinating BAF57 (Fig. 7B). Relatively little is known about TRIP12, although one ubiquitination pathway substrate has been identified when TRIP12 was shown to interact with the NEDD8 pathway-activating enzyme subunit APP-BP1 (37). Interestingly, TRIP12 will only interact with free APP-BP1 monomer but not when APP-BP1 heterodimerizes with Uba3 (37). This mechanism is analogous to what we see for the relationship between TRIP12, BAF57, and BAF155.

An intriguing possibility is that TRIP12 could be a component of a putative protein quality control system for BAF57, the entire SWI/SNF complex, or more broadly for multiprotein complexes in general. Protein quality control systems, particularly for nuclear proteins, have been found to exist in both yeast (38) and mammalian cells (39). In yeast, Sna1p, a ubiquitin ligase targets specific aberrant, misfolded, or mutant proteins to the proteasome while leaving wild-type versions of the same proteins alone (38). In mammalian cells the ubiquitin ligase UHRF-2 rids the cell of cytotoxic polyglutamine aggregates via proteasomal degradation (39). Both of these ubiquitin ligases represent key components of their respective nuclear protein quality control machinery. Consequently TRIP12 could qualify as the principal ubiquitin ligase in a protein quality control system that regulates BAF57 stoichiometry and thereby controls SWI/SNF transcriptional fidelity.

This type of mechanism to maintain protein stoichiometry in a multiprotein complex is unlikely to be unique to the SWI/SNF complex. For example, LIM homeodomain transcription factor-containing complexes can be biologically activated or repressed depending on the cofactors with which it interacts (40). Association with CLIM cofactors allows for transcriptional activation, whereas association with RILM inhibits the transcription factor function. LIM-only (LMO) proteins also negatively regulate LIM homeodomain activity by competing for CLIM cofactors. The overall control of this multifunctional,
multisubunit complex is regulated by RLIM, which is a RING H2 zinc finger ubiquitin ligase (40). RLIM ubiquitinates both CLIM cofactors and LMO proteins (LMO2 and LMO4), thus targeting them to the 26 S proteasome for degradation (40). These events of cofactor exchange, mediated by the activity of RLIM, represent a tightly controlled mechanism of transcriptional regulation.

The stoichiometry of various proteins in these LIM protein complexes has also been found to be regulated by other cellular factors. The basic helix-loop-helix factor SCL, for example, interacts with LMO2 and functions in the transcriptional control of hematopoiesis (41). By doing so, SCL protects LMO2 from proteasomal degradation mediated by RLIM. This protein-protein interaction, therefore, regulates the protein levels of LMO2 so as to maintain proper SCL/LMO2 stoichiometry and, thus, elicit the desired transcriptional response (41). Single-stranded DNA binding proteins (SSBPs) have also been found to regulate the stoichiometry of LIM complex members (42). Specifically, SSBP2, a member of an erythroid DNA binding complex, increases the endogenous protein levels of LMO2 and the LIM domain-binding protein Ldb1 by inhibiting their interaction with RLIM and, thus, preventing them from being ubiquitinated and degraded (42). It is believed that the cellular protein level of SSBP2 is so important that an increase or decrease in its expression would alter the stoichiometry of LMO2 and Ldb1 such that tumorigenesis may ensue (42).

An additional example involves p53 stoichiometry, which has also been shown to be regulated by protein-protein interactions. TAF(II)31, a coactivator protein, has been shown to bind to p53 at the same region required for its interaction with its E3 ubiquitin ligase MDM2 (43). The TAF(II)31-p53 interaction, therefore, inhibits MDM2-mediated degradation of p53, increases p53 protein levels, and activates p53-mediated transcription (43). Consequently, multiprotein complexes, which employ precise protein-protein interactions and exercise this mechanism of regulated protein degradation, may be broadly employed.

Taken together, these data suggest that BAF155 plays a significant scaffolding role within the SWI/SNF complex and is a major determinant in maintaining subunit stoichiometry. Thus, when within an intact SWI/SNF complex, the individual subunits are all protected from degradation. However, any free core subunit not protected within a SWI/SNF complex will be disposed of by the proteasome (Fig. 8). This cellular mechanism may prevent the accumulation of free BAF57, which could induce dominant negative effects. For example, BAF57 interacts with nuclear hormone receptors and is believed to interact directly with DNA, possibly at promoters (10, 12). If an excess of BAF57 exists within the cell, then uncomplexed BAF57 would, therefore, prevent fully complemented SWI/SNF complexes from accessing its targets. In certain cancers, therefore, when one of these complex members is lost, this intra-complex self-stabilization would be disrupted, thus hampering the complex remodeling ability to mediate transcriptional regulation. A comprehensive understanding of this regulatory mechanism within multiprotein complexes will help to elucidate the relationships between transcriptional mis-regulation and may have a profound impact on our understanding of disease pathogeneses.

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