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SRG3 Interacts Directly with the Major Components of the SWI/SNF Chromatin Remodeling Complex and Protects Them from Proteasomal Degradation*

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The mammalian SWI/SNF complex is an evolutionarily conserved ATP-dependent chromatin remodeling complex that consists of nine or more components. SRG3, a murine homologue of yeast SWI3, *Drosophila* MOIRA, and human BAF155, is a core component of the murine SWI/SNF complex required for the regulation of transcriptional processes associated with development, cellular differentiation, and proliferation. Here we report that SRG3 interacts directly with other components of the mammalian SWI/SNF complex such as SNF5, BRG1, and BAF60a. The SWIRM domain and the SANT domain were required for SRG3-SNF5 and SRG3-BRG1 interactions, respectively. In addition, SRG3 stabilized SNF5, BRG1, and BAF60a by attenuating their proteasomal degradation, suggesting its general role in the stabilization of the SWI/SNF complex. Such a stabilization effect of SRG3 was not only observed in the *in vitro* cell system, but also in cells isolated from SRG3 transgenic mice or knock-out mice haploinsufficient for the *Srg3* gene. Taken together, these results suggest the critical role of SRG3 in the post-transcriptional stabilization of the major components of the SWI/SNF complex.

The mammalian SWI/SNF complexes are evolutionarily conserved ATP-dependent chromatin remodeling complexes, which use the energy of ATP hydrolysis to mobilize nucleosomes and remodel chromatin structure (1, 2). These complexes play important roles in transcriptional regulation, thereby controlling diverse cellular processes including proliferation, differentiation, cell death, and tumorigenesis (3–6). The mammalian SWI/SNF complexes are multisubunit complexes that consist of invariant core components and variable components (7). The subunit diversity of mammalian SWI/SNF complexes suggests that different complexes might have tissue-specific roles during development (8). The core components of the mammalian SWI/SNF complexes are BRG1 or hBRM, SNF5/INI1/BAF47, BAF155/SRG3, and BAF170 (9). BRG1 and BRM are DNA-dependent ATPase homologous to yeast SWI2/SNF2. Biochemical experiments have shown that although BRG1 or BRM alone can remodel nucleosomal arrays, the addition of other core components (BAF155, BAF170, and SNF5) to BRG1 stimulates the remodeling activity of BRG1 at a rate that is comparable with the entire complex *in vitro* (10).

Human SNF5 was initially identified by the yeast two-hybrid system through its interaction with human immunodeficiency virus type 1 integrase (11). It was shown that human SNF5 interacts with c-Myc, thereby enhancing c-Myc-mediated transactivation by recruiting the SWI/SNF complex to the E-box (12). Furthermore, human SNF5 is known as a tumor suppressor in atypical teratoid and malignant rhabdoid tumors and the majority of these tumors have deletion or point mutations in SNF5 leading to disruption of normal function of SNF5 (13, 14).

Srg3 (*Swi3-related gene*), a murine homologue of yeast *Swi3*, *Drosophila Moira*, and human *Baf155*, was initially isolated as a gene highly expressed in the thymus but at a low level in the peripheral lymphoid organ (15). It is a core component of the mouse SWI/SNF complex that is required for the regulation of transcriptional processes associated with development, cellular differentiation, and proliferation (10, 16–18). We found that the expression level of SRG3 is down-regulated after positive selection of developing thymocytes and this is critical in determining glucocorticoid sensitivity in T cells (15, 19, 20). Furthermore, T cell receptor and Notch1 signaling inhibits glucocorticoid-induced apoptosis of developing thymocytes by down-regulating the SRG3 expression (20, 21). Whereas the function of BRG1 and SNF5 as a component of the SWI/SNF complex has been revealed (10, 12, 22), the function of SRG3 as a component of the SWI/SNF complex has not yet known.

SRG3 contains several highly conserved domains such as the SANT (SWI3, ADA2, N-CoR, and TFIIIB) domain, the SWIRM (SWI3, RSC8, and MOIRA) domain, and the leucine-zipper motif. It was reported that the SANT domain of MOIRA is important to the interaction with BRM (23) and the leucine-zipper motif of BAF155 is required for interaction with BAF57 (24). The direct interactions between SRG3 homologues and other components of the SWI/SNF complex have been

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reported. In yeast, SWI3 interacts directly with SWI2/SNF2 and SNF11 (25). Direct interactions between components of the RSC complex, a SWI/SNF-related complex, have also been reported. RSC8, STH1, and RSC6 components of the RSC complex are homologues of SRG3, BRG1, and BAF60a, respectively (26). RSC8 interacts directly with STH1 and RSC6 and can self-associate to form a dimer through the leucine-zipper motif (27, 28). These characteristics in the structure of the SRG3 protein and protein-protein interactions between SRG3 homologues and other components suggest a possibility that SRG3 may interact directly with other components of the mammalian SWI/SNF chromatin remodeling complex.

The free subunits of the multisubunit complex tend to be degraded by protein quality control systems to ensure proper stoichiometry if they are not assembled into complex (29). It has been reported that the stability of components of the SWI/SNF complex is mutually dependent. The stability of SWI3 is decreased in the absence of SWI1 and SWI2 (30), and BAF155 stabilizes the BAF57 protein (24). However, the protein-protein interactions among the components of the mammalian SWI/SNF complex and control of the stability of the complex are poorly understood. Here we describe the critical role of SRG3 in protein stability, overall stoichiometry, and subcellular localization of the SWI/SNF complex.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Plasmids—Cycloheximide (Sigma) and MG132 (Calbiochem) were purchased commercially. Antibodies to the Myc epitope (9E10) and HA[3] epitope (12CA5) were purchased from Roche Applied Science. Antibodies to the FLAG epitope (M2 or polyclonal) and β-actin (AC-15) were purchased from Sigma. Antibodies to BAF60a, BAF47, and TBP (TATA-binding protein) were purchased from Transduction Laboratories, BD Biosciences, and Santa Cruz, respectively. Antisera against SRG3 and BRG1 were raised from rabbits in our laboratory.

For construction of plasmids used in yeast two-hybrid assay, baits were cloned in-frame into the pGBKT7 vector and preys were cloned in-frame into the pACT2 vector (Clontech). The cDNA fragments encoding amino acid 183–541 of wild-type SRG3 or the SWIRM domain mutants of SRG3 were inserted in-frame into the pGEX-4T-2 vector for the expression of glutathione *S*-transferase (GST) fusion proteins. The mouse cDNAs for mouse *Srg3*, *Brg1*, or *Snf5* were subcloned into pCAGGSBS using EcoRI for the expression of Myc epitope- or FLAG epitope-tagged proteins. pCAGGSBS expression vector was constructed by inserting the 2.3-kb fragment of the pCAGGS vector containing the cytomegalovirus immediate-early enhancer linked to the chicken β-actin promoter and rabbit β-globin poly(A) into the SalI and PstI sites of the pBluescript vector. All cDNAs and mutants of *Srg3* were generated by PCR amplification with proper primers. The PCR products were verified by sequencing.

Yeast Two-hybrid Assay—Yeast transformation was performed using the lithium acetate method. Yeast strain AH109 (genotype: *MATa, trp1–901, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, MEL1, GAL2*$_{UAS}$*GAL2*$_{TATA}$*-ADE2, URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ*) was co-transformed with plasmids encoding GAL4BD and GAL4AD fusion proteins and the interactions of these recombinant proteins were tested by evaluating the ability of co-transformants to grow on SD-agar plates without Leu, Trp, His, and Ade. The interactions between SRG3 mutants and SNF5 were confirmed by β-galactosidase assay as described previously (31).

In Vitro Binding Assay—All recombinant GST fusion proteins were purified as previously described (15). For the *in vitro* binding assay, cells were lysed with buffer X (100 mM Tris-HCl (pH 8.5), 250 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 2 mg/ml bovine serum albumin) in the presence of complete EDTA-free protease inhibitor mixture tablets (Roche). Extracts were incubated with 30 μl of glutathione-Sepharose beads (GE Healthcare) preincubated with purified GST fusion proteins. After incubation for 30 min at 4 °C, the beads were washed with buffer X and the eluted proteins were used for immunoblot analysis and Coomassie Blue stain.

Mice, Cell Culture, and Transfections—Transgenic mice overexpressing SRG3 and *Srg3* heterozygote (+/−) knock-out mice were previously described (17, 19). Mice were bred and maintained under pathogen-free conditions, and experiments were performed in accordance with institutional and national guidelines.

COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (WelGENE). NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% bovine calf serum (WelGENE). Typical transfections were performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions and cells were incubated for 48 h after transfection.

Immunoprecipitation and Immunoblot Analysis—Cells were lysed in buffer X and immunoprecipitated with appropriate antibodies as previously described (19). For immunoblot analysis, proteins were resolved on SDS-PAGE gels (7.5 or 10%) and transferred to Immobilon-P membrane (Millipore). Densitometric band intensity was determined using Gel-Pro Analyzer software (Media Cybernetics).

RNA Interference in Mammalian Cells—siRNAs with the following sense and antisense sequences were used: *Brg1*, 5′-CCGUCAAGGUGAAGAUCAATT-3′ (sense), 5′-UUGAUCUUCACCUUGACGGTT-3′ (antisense); *Srg3*, 5′-CAUCCUGGUUUGAUUAUAATT-3′ (sense), 5′-UUAUAAUCAAACCAGGAUGTT-3′ (antisense); *GFP*, 5′-GUUCAGCGUGUCCGGCGAGTT-3′ (sense), 5′-CUCGCCGGACACGCUGAACTT-3′ (antisense). All siRNAs were purchased from Samchully Pharm. NIH3T3 cells were plated in a 60-mm dish, 2×10^5 cells per well, 18–24 h before transfection. Cells were transfected with 200 μM siRNAs by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Northern Blot Analysis—Total RNAs were prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Northern blot analysis was performed as previously described (20). The blots were hybridized with specific

[3] The abbreviations used are: HA, hemagglutinin; TBP, TATA-binding protein; GST, glutathione *S*-transferase; siRNA, small interfering RNA; NLC, normal littermate control; NLS, nuclear localization signal; AB, activation domain; BD, binding domain; TRITC, tetramethylrhodamine isothiocyanate.

TABLE 1

Interactions between SRG3 and other major components of the SWI/SNF complex

The interactions between SRG3 and other major components of the SWI/SNF complex were analyzed by yeast two-hybrid assay. The interactions of SRG3 fused to the GAL4 DNA-binding domain (GAL4BD) with other components fused to the GAL4 activation domain (GAL4AD) were tested by evaluating the ability of co-transformants to grow on SD agar plates without Leu, Trp, His, and Ade (SD/−Leu/−Trp/−His/−Ade).

GAL4BD fusions	GAL4AD fusions	Interaction[a]
GAL4BD	GAL4AD	−
GAL4BD-SRG3	GAL4AD	−
GAL4BD-SRG3	GAL4AD-SNF5	+
GAL4BD-SRG3	GAL4AD-BRG1	+
GAL4BD-SRG3	GAL4AD-BAF60a	+

[a] +, interacting protein; −, non-interacting protein.

murine *Brg1*, *Srg3*, and *Snf5* probes. The 1-kb *Brg1* probe or the 1.2-kb *Snf5* probe was generated by random priming using the EcoRI fragment of the FLAG-BRG1 or FLAG-SNF5 expression vectors, respectively, and the specific probe for *Srg3* was generated as described previously (20).

Immunofluorescence Analysis—COS-1 cells grown on coverslips were fixed for 30 min in phosphate-buffered saline containing 3.7% paraformaldehyde, washed three times with phosphate-buffered saline, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 2% normal donkey serum for 30 min. Subsequently, cells were incubated at room temperature with primary and secondary antibodies for 3 and 1 h, respectively. The following antibodies were used: mouse anti-Myc (1:200), rabbit anti-FLAG (1:200), TRITC-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch), and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch). Fluorescent images were obtained by confocal microscopy (Radiance 2000, Bio-Rad).

RESULTS

SRG3 Interacts Directly with Major Components of the SWI/SNF Complex—To study the role of SRG3 in the protein-protein interactions among the components of the mammalian SWI/SNF complex, we analyzed the direct interactions between SRG3 and other major components of the SWI/SNF complex by yeast two-hybrid assay. Plasmid encoding SRG3 protein fused to the GAL4 DNA binding domain (GAL4BD) was co-transformed into yeast with plasmid encoding SNF5, BRG1, or BAF60a fused to the GAL4 activation domain (GAL4AD). The interactions of these recombinant proteins were tested by evaluating the ability of co-transformants to grow on SD agar plates without Leu, Trp, His, and Ade. As shown in Table 1, SRG3 interacted directly with SNF5, BRG1, and BAF60a. BAF155 was also shown to interact with BAF57 and BAF60a by *in vitro* binding assays (24, 32). The co-immunoprecipitations between SRG3 and other components (SNF5, BAF60a, and BAF57) of the SWI/SNF complex are also carried out to result in the same conclusions (Fig. 5*H*). All of these results suggest that SRG3 may function as a scaffold protein that interacts directly with other major components including SNF5, BRG1, BAF60a, and BAF57.

Next, we identified the domain requirements for the SRG3-SNF5, SRG3-BRG1, and SRG3-BAF60a interactions. A series of deletion mutants of SRG3 were generated and analyzed by a yeast two-hybrid assay (Fig. 1). The N-terminal region of SRG3 (SRG3-N) spanning amino acid residues 1–710 bound to SNF5, but not the C-terminal region of SRG3 (SRG3-C) spanning

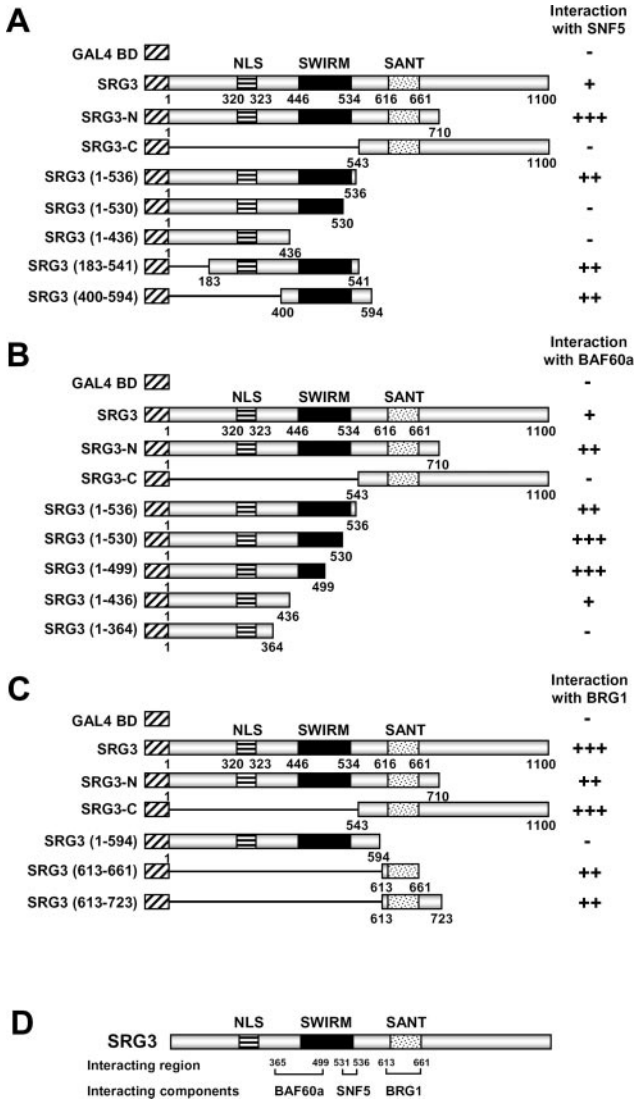

FIGURE 1. **SRG3 interacts directly with major components of the SWI/SNF complex.** *A*, the interactions between deletion mutants of SRG3 and murine SNF5 were analyzed by the yeast two-hybrid assay. The schematic representation of deletion mutants of SRG3 and their binding to murine SNF5 fused to GAL4AD is depicted. The interactions of each SRG3 protein with SNF5 were tested by evaluating the ability of co-transformants to grow on SD/−Leu/−Trp/−His/−Ade plates and represented as negative (−), weak (+) to strong (+++) relative to the binding strength of SRG3-N to SNF5. The interactions are also confirmed by β-galactosidase assay. The *thin line* indicates the deleted region and the *solid black box* indicates the SWIRM domain. The *horizontally striped box* and *dotted box* indicate the NLS sequence and the SANT domain, respectively. The *diagonally striped box* indicates a GAL4BD in the pGBKT7 vector and the *numbers below bars* represent amino acid residues. The *numbers* at the *beginning* and *ends* of the *boxes* represent the position of the amino acid residue at each edge of the domain. *B*, the interactions between deletion mutants of SRG3 and BAF60a were analyzed by yeast two-hybrid assay. The interactions of each SRG3 protein with BAF60a were tested as described above and represented as negative (−), weak (+) to strong (+++) relative to the binding strength of SRG3-N to BAF60a. *C*, the interactions between deletion mutants of SRG3 and BRG1 were analyzed by the yeast two-hybrid assay. The interactions of each SRG3 protein with BRG1 were tested as described above and represented as negative (−), weak (+) to strong (+++) relative to the binding strength of SRG3-N to BRG1. *D*, the schematic representation of interacting components and their interacting region in SRG3 is depicted.

amino acid residues 543–1100 (Fig. 1*A*). The minimal region required for SNF5 binding was deduced as amino acid residues 437–536, where amino acid residues 531–536 were especially critical. Intriguingly, this region contains the SWIRM domain (amino acid residues 446–534), which is required for binding to other proteins (33, 34). Among the fragments tested, the shortest SNF5-binding region of SRG3 (SRG3-(400–594)) includes the entire SWIRM domain and the deletion of this domain disrupted the interaction with SNF5. Likewise, deletion of the SWIRM domain significantly reduced SRG3-BAF60a interaction, although deletion of the upstream region of the SWIRM domain was also required for complete disruption of this interaction (Fig. 1*B*). These results indicate that the SWIRM domain is important for SRG3-SNF5 and SRG3-BAF60a interactions, and the upstream region of the SWIRM domain may also function as BAF60a-binding region.

The domain requirements for the SRG3-BRG1 interaction were also analyzed by yeast two-hybrid assay. As shown in Fig. 1*C*, SRG3-N and SRG3-C, which have an intact SANT domain interacted with BRG1, but the deletion of this domain disrupted SRG3-BRG1 interaction. The shortest BRG1-binding region of SRG3 (SRG3-(613–661)) also includes the entire SANT domain. These results show that the SANT domain is necessary and sufficient for SRG3-BRG1 interaction. Taken together, SRG3 has specific domains that are important for direct interactions between SRG3 and major components of the SWI/SNF complex, which enables SRG3 to function as a scaffold protein of the SWI/SNF complex (Fig. 1*D*).

The Amino Acid Residues Leu-531 and Asn-533 on the SWIRM Domain Are Important for the SRG3-SNF5 Interaction—We further examined which residues of the SWIRM domain are crucial for the SRG3-SNF5 interaction. Because differential binding properties were observed between SRG3-(1–536) and SRG3-(1–530), and amino acid residues 531–536 of SRG3 are highly conserved in the SWIRM domains of SRG3 homologues (35), a deletion mutant of SRG3 at positions 531–536 and substitution mutants were constructed and interactions of these SRG3 mutants analyzed with SNF5 (Fig. 2*A*). A deletion mutant of SRG3 (SRG3-N-(Δ531–536)) did not interact with SNF5, showing that the region of amino acid residues 531–536 is necessary for the SRG3-SNF5 interaction. Substitution of Val-532 with alanine (V532A) had little effect on SRG3-SNF5 interaction, but mutations on Leu-531 (L531A) or Asn-533 (N533A) significantly reduced this interaction. Moreover, double substitutions on Leu-531 and Asn-533 (L531A/N533A) completely disrupted SRG3-SNF5 interaction. Such results were reconfirmed by the *in vitro* binding assay (Fig. 2*C*). The amino acid residue 183–541 region of wild-type SRG3 or SWIRM domain mutant SRG3 was fused to GST (Fig. 2*B*). FLAG-SNF5 protein was expressed in COS-1 cells and examined for interaction with GST fusion proteins. As shown in Fig. 2*C*, the SNF5 protein could bind to wild-type SRG3 (GST-SRG3), but not to the deletion mutant (GST-Δ531–536) nor the double point mutant (GST-L531A/N533A). Taken together, these results demonstrate that SRG3 interacts directly with SNF5 through the SWIRM domain and amino acid residues 531–536 (especially Leu-531 and Asn-533) are important for this interaction.

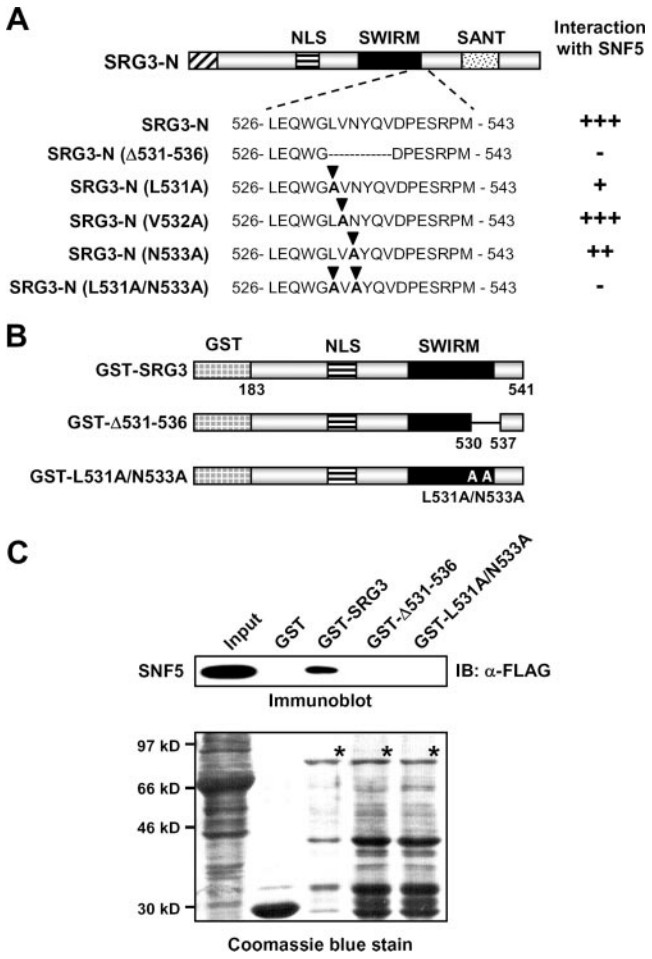

FIGURE 2. **The amino acid residues Leu-531 and Asn-533 on the SWIRM domain are important for the SRG3-SNF5 interaction.** *A*, deletion of amino acid residues 531–536 or alanine substitution at Leu-531 and Asn-533 of SRG3 disrupts the SRG3-SNF5 interaction. The schematic representation of SRG3 mutants and their binding to murine SNF5 in the β-galactosidase assay is depicted. The binding strength reported as negative (−), weak (+) to strong (+++) is the same as described in the legend to Fig. 1*A*. *B*, the schematic representation of fragments spanning amino acid residues 183–541 of wild-type SRG3 and the SWIRM domain mutants fused to GST is depicted. The *checked box* indicates GST and the *numbers below bars* represent amino acid residues. *C*, GST fusion proteins containing amino acid residues 183–541 of wild-type SRG3 (*GST-SRG3*), mutant SRG3 with a deletion at amino acid residues 531–536 (*GST-Δ531–536*), or mutant SRG3 with point mutations at amino acid residues Leu-531 and Asn-533 (*GST-L531A/N533A*) were subjected to *in vitro* binding assay. Immunoblot analysis was performed with anti-FLAG antibody (*upper panel*). The membrane was stained by Coomassie Brilliant Blue for detection of the GST fusion proteins (*lower panel*). The *asterisks* next to the bands in the *lower panel* indicate the position of the GST fusion proteins.

SRG3 Regulates the Protein Levels of Major Components of the SWI/SNF Complex—SRG3 interacts directly with SNF5, BRG1, and BAF60a, thus, there is a possibility that SRG3 regulates their protein stability. To test this possibility, we analyzed the effect of SRG3 expression on the protein level of SNF5, BRG1, and BAF60a. First, we co-transfected a fixed amount of FLAG-SNF5 expression vector with increasing amounts of Myc-SRG3 expression vector and determined the protein level of SNF5 by immunoblot analysis. We found that the protein level of SNF5 is up-regulated by SRG3 in a dose-dependent manner, suggest-

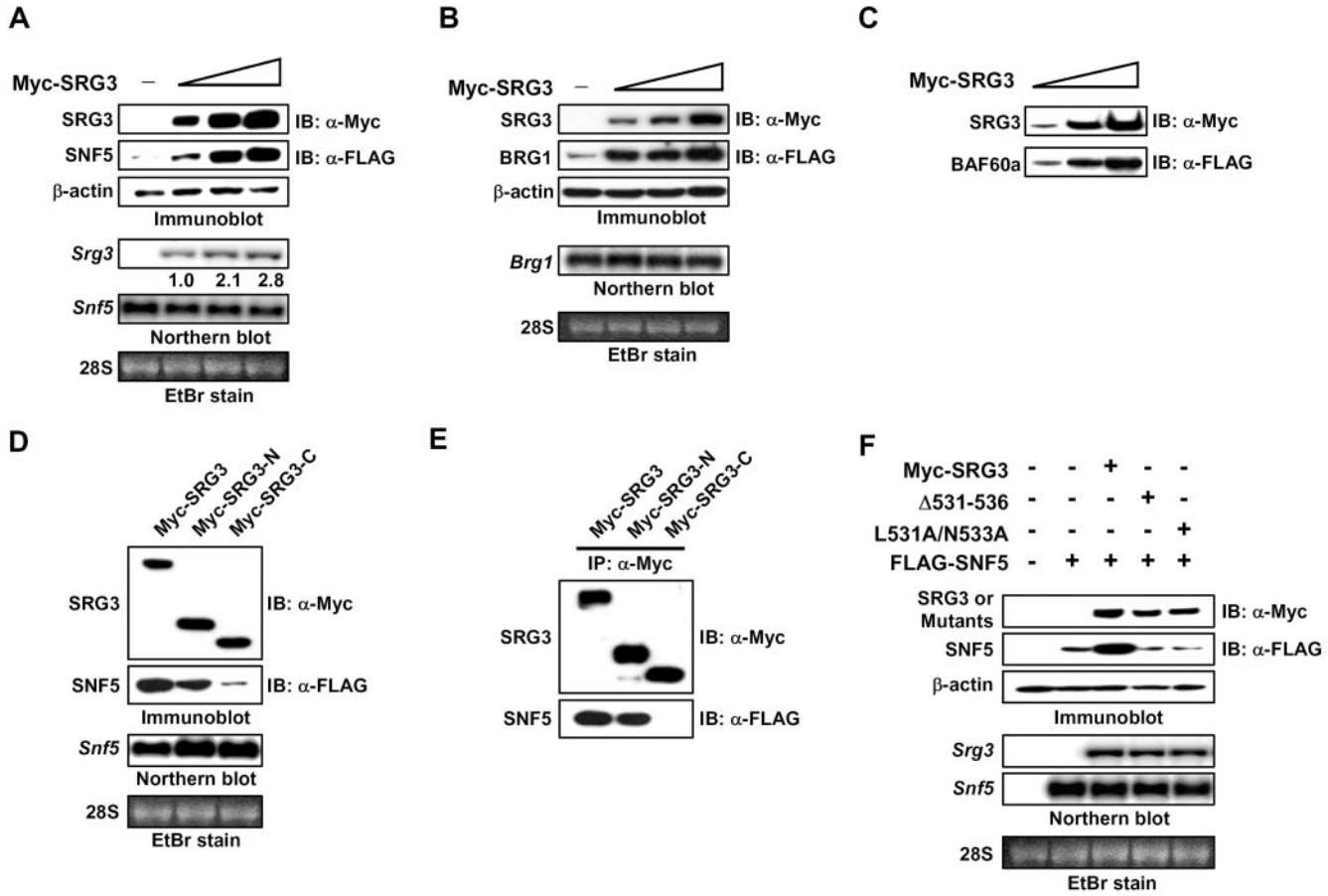

FIGURE 3. **SRG3 regulates the protein levels of major components of the SWI/SNF complex.** *A* and *B*, COS-1 cells were co-transfected with a fixed amount of FLAG-SNF5 (*A*) or FLAG-BRG1 (*B*) expression vector along with increasing amounts of Myc-SRG3 expression vector, and subsequently subjected to immunoblot (*IB*) and Northern blot analyses. The *numbers* in *A* represent the relative value of the mRNA level of Srg3. β-Actin and 28 S rRNA were shown as a control. *C*, COS-1 cells were co-transfected with a fixed amount of FLAG-BAF60a expression vector along with increasing amounts of Myc-SRG3 expression vector, and subsequently subjected to immunoblot analysis. *D*, COS-1 cells were co-transfected with Myc-SRG3, Myc-SRG3-N, or Myc-SRG3-C expression vector along with FLAG-SNF5 expression vector. Whole cell extracts (50 μg) were analyzed by immunoblot analysis with anti-Myc (*upper panel*) or anti-FLAG antibodies (*middle panel*). For Northern blot analysis, total RNAs were isolated from the same pool of cells described above. *E*, whole cell extracts (300 μg) used in *D* were immunoprecipitated with anti-Myc antibody and subjected to immunoblot analysis with anti-Myc (*upper panel*) or anti-FLAG antibodies (*lower panel*). *F*, FLAG-SNF5 expression vector was co-transfected into COS-1 cells along with empty vector, Myc-SRG3, or the SWIRM domain mutants of SRG3 (Δ531–536, L531A/N533A) expression vectors. Whole cell extracts were analyzed by immunoblot analysis with anti-Myc or anti-FLAG antibodies. For Northern blot analysis, total RNAs were isolated from the same pool of cells described above.

ing that SNF5 expression is dependent on the expression level of SRG3 (Fig. 3*A*). The mRNA level of *Snf5* was not significantly changed in each transfection, as shown by Northern blot analysis that it is likely that the SRG3 effect on the protein level of SNF5 does not occur at the transcriptional level. Similar results were obtained with BRG1 and BAF60a, showing that the protein levels of BRG1 and BAF60a were also augmented by SRG3 in a dose-dependent manner (Fig. 3, *B* and *C*). These results imply that SRG3 regulates the protein levels of major components of the SWI/SNF complex.

Next, Myc-SRG3 or its Myc-tagged deletion mutants (Myc-SRG3-N, Myc-SRG3-C) were overexpressed with FLAG-SNF5 in COS-1 cells and the protein level of SNF5 determined by immunoblot analysis. As shown in Fig. 3*D*, wild-type SRG3 and the N-terminal region of SRG3 were able to up-regulate the SNF5 protein level, but the C-terminal region of SRG3 could not. The protein level of SNF5 observed in Myc-SRG3-C was similar to the level of SNF5 alone (data not shown). The expression level of each SRG3 protein was similar and the mRNA level of *Snf5* showed no difference (Fig. 3*D*, *lower panel*). To test whether wild-type SRG3 and the N-terminal region of SRG3 interact with SNF5 *in vivo*, total cell lysates were immunoprecipitated with anti-Myc antibody followed by immunoblot analysis. As shown in Fig. 3*E*, wild-type SRG3 and the N-terminal region of SRG3 were co-immunoprecipitated with SNF5, but the C-terminal region of SRG3 was not. These results suggest that the SRG3-SNF5 interaction contributes to the up-regulated protein level of SNF5.

We further investigate whether the protein level of SNF5 is dependent on direct interaction with SRG3 through the SWIRM domain. Strong expression of the SNF5 protein was observed only with co-expression of wild-type SRG3, which is capable of directly binding to SNF5 (Fig. 3*F*). In contrast, low expressions of SNF5 protein were yielded upon the co-expression of the SWIRM domain mutants of SRG3 (Δ531–536, L531A/N533A) that do not bind to SNF5. Again, such differences in the expressions of the SNF5 protein occurred at the post-transcriptional level. These results suggest that SRG3 regulates the protein level of SNF5 by direct interaction through the SWIRM domain.

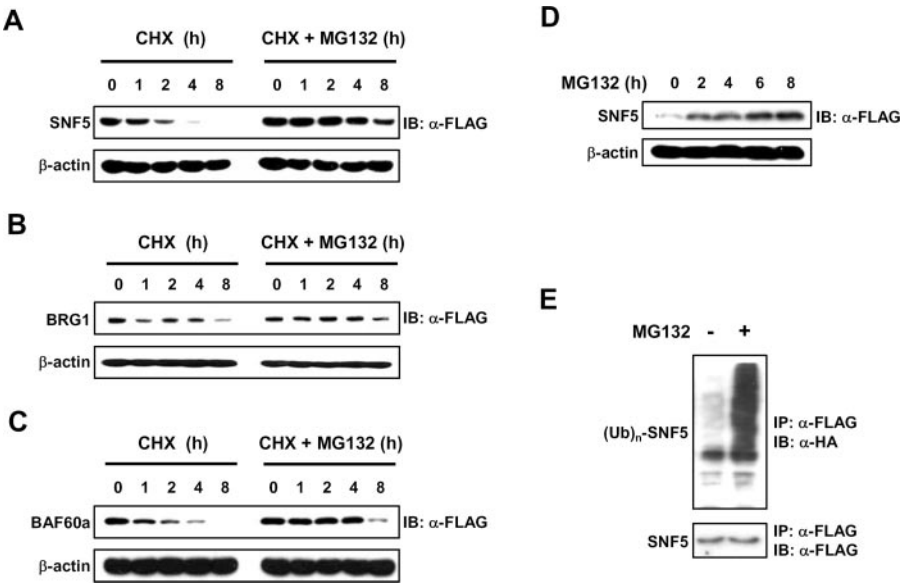

FIGURE 4. **The components of the SWI/SNF complex are degraded by proteasome pathway.** *A–C*, COS-1 cells transfected with FLAG-SNF5 (*A*), FLAG-BRG1 (*B*), or FLAG-BAF60a (*C*) expression vectors were treated with cycloheximide (*CHX*, 200 μg/ml) in the absence or presence of MG132 (20 μM) for 0, 1, 2, 4, and 8 h before cell harvest. Whole cell extracts were analyzed by immunoblot analysis with anti-FLAG antibody for determining the relative protein levels of SNF5, BRG1, and BAF60a present at each time point. *D*, COS-1 cells transfected with FLAG-SNF5 expression vector were treated with MG132 (20 μM) for 0, 2, 4, 6, and 8 h before cell harvest. Whole cell extracts were analyzed by immunoblot analysis with anti-FLAG antibody for determining the relative protein level of SNF5 present at each time point. *E*, COS-1 cells were co-transfected with FLAG-SNF5 expression vector along with HA-ubiquitin expression vector. After incubation for 40 h, MG132 (10 μM) or vehicle (Me_2SO) were treated for 6 h. Whole cell extracts were immunoprecipitated with anti-FLAG antibody and subjected to immunoblot analysis with anti-HA (*upper panel*) antibody. After stripping, the blot was reprobed with anti-FLAG antibody (*lower panel*).

observed in the absence of cycloheximide. Inhibition of the proteasome pathway by MG132 leads to a rapid increase of the SNF5 protein, showing again that degradation of the SNF5 protein is mediated by the proteasome pathway (Fig. 4*D*).

Proteins that are degraded by the proteasome are first marked by polyubiquitination to specific lysine residues (36, 37). To assess whether SNF5 undergoes covalent ubiquitination, COS-1 cells co-transfected with FLAG-SNF5 and HA-tagged ubiquitin expression vectors were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblot analysis with anti-HA antibody. A significant increase in SNF5-ubiquitin conjugates was detected in the presence of MG132, indicating that the SNF5 protein is polyubiquitinated (Fig. 4*E*). These results suggest that the SNF5 protein is specifically degraded by the ubiquitin-dependent proteasome pathway.

Next, we examined the influence of SRG3 on the degradation kinetics and half-lives of major components of the SWI/SNF complex in the absence of *de novo* protein synthesis (Fig. 5). The FLAG-SNF5 expression vector was co-transfected into COS-1 cells along with the Myc-SRG3 expression vector or empty vector. The half-life of SNF5 was measured by treating the cells with cycloheximide at various time intervals and detecting the remaining SNF5 protein level by immunoblot analysis (Fig. 5, *A* and *B*). Rapid degradation of the SNF5 protein was observed in the absence of SRG3 co-expression upon treatment with cycloheximide as described in the legend to Fig. 4*A*. On the other hand, co-expression of SRG3 significantly extended the half-life of the SNF5 protein, approximately by 6-fold, when measured by plotting the band intensities (Fig. 5*B*). Similar results were obtained with BRG1 and BAF60a (Fig. 5, *C–F*). Co-expression of SRG3 significantly extended the half-lives of the BRG1 and BAF60a proteins, although relatively slow degradation of the BRG1 protein was observed even in the absence of SRG3 co-expression (Fig. 5, *D* and *F*). However, unlike other components, SRG3 protein remained stable up to 8 h after cycloheximide treatment (Fig. 5*G*). Taken together, these results suggest that SRG3 stabilizes major components of the SWI/SNF complex by attenuating their proteasomal degradation, resulting in an increase of their half-lives.

We also investigated whether SRG3 can stabilize other components of the SWI/SNF complex as a co-immunoprecipitating complex. As seen in Fig. 5*H*, the co-precipitated amounts of BAF60a, BAF57, and SNF5 with SRG3 increased in a dose-dependent manner. Such results show that SRG3 not only stabi-

SRG3 Stabilizes Major Components of the SWI/SNF Complex by Attenuating Their Proteasomal Degradation—Because the mRNA levels of *Snf5* and *Brg1* were not affected by the expression of SRG3, it is unlikely that SRG3 controls the transcription of *Snf5* or *Brg1* from their expression vector. To investigate the role of SRG3 on the degradation of major components of the SWI/SNF complex, we examined the degradation kinetics of exogenous SNF5 protein in the absence of *de novo* protein synthesis (Fig. 4*A*). COS-1 cells transfected with the FLAG-SNF5 expression vector were treated with the protein synthesis inhibitor, cycloheximide, for 0, 1, 2, 4, and 8 h. The relative protein level of SNF5 present at each time point was determined by immunoblot analysis. Rapid degradation of the SNF5 protein was observed upon treatment with cycloheximide. Thus, we examined whether the degradation of the SNF5 protein is proteasome-mediated. COS-1 cells transfected with the FLAG-SNF5 expression vector were treated with cycloheximide plus MG132, a potent proteasome inhibitor, for the same time point as described above, and the relative protein level of SNF5 present at each time point was determined by immunoblot analysis. As shown in Fig. 4*A*, degradation of exogenous SNF5 protein was inhibited by treatment with MG132. Similar results were obtained with BRG1 and BAF60a, although relatively slow degradation of the BRG1 protein was observed upon treatment with cycloheximide (Fig. 4, *B* and *C*). These results suggest that degradation of major components of the SWI/SNF complex is mediated by the proteasome pathway. The proteasome-mediated degradation of SNF5 was also

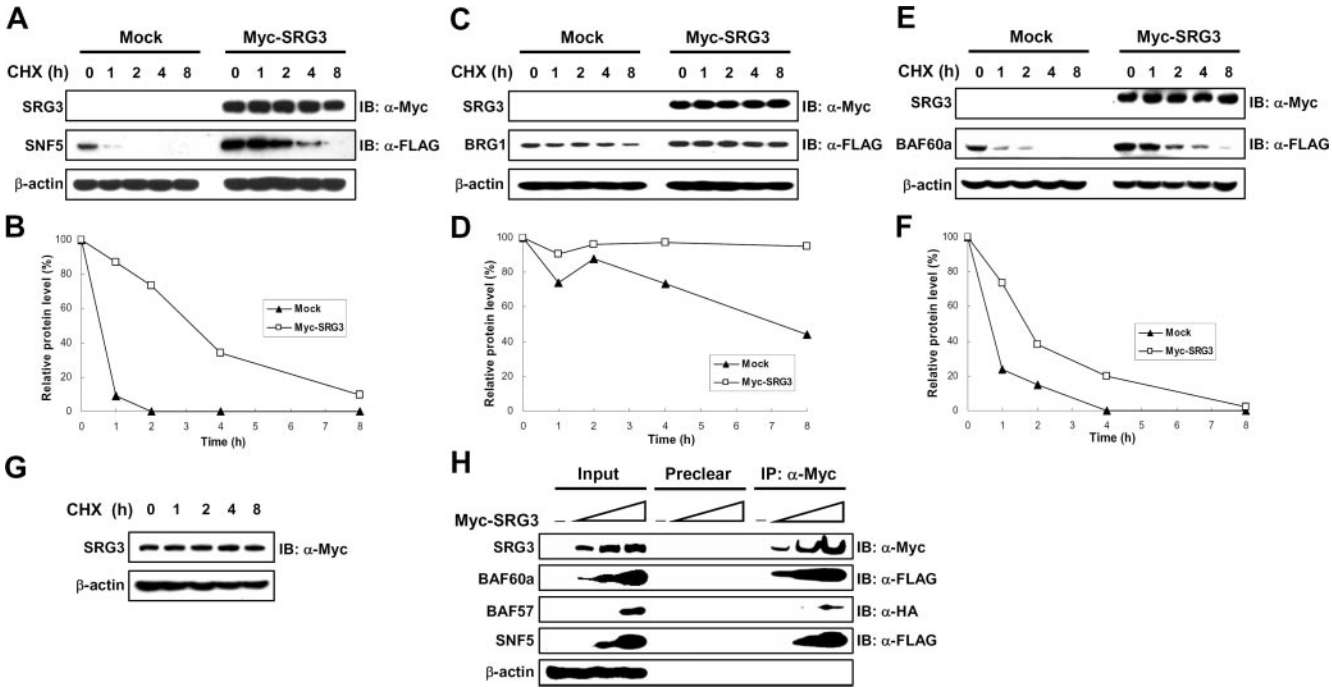

FIGURE 5. **SRG3 stabilizes major components of the SWI/SNF complex by attenuating their degradation.** *A*, *C*, and *E*, COS-1 cells were co-transfected with FLAG-SNF5 (*A*), FLAG-BRG1 (*C*), or FLAG-BAF60a (*E*) expression vector along with empty vector or Myc-SRG3 expression vector. Cells were treated with cycloheximide (200 μg/ml) at the indicated time intervals and the relative protein levels of SNF5, BRG1, and BAF60a were detected by immunoblot analysis with anti-FLAG antibody. *B*, *D*, and *F*, immunoblots of *A*, *C*, and *E* were quantified by densitometric scanning, respectively. The protein levels of SNF5, BRG1, and BAF60a were corrected with respect to the β-actin protein level and displayed relative to that observed before cycloheximide treatment (0 h). *G*, COS-1 cells transfected with Myc-SRG3 expression vector were treated with cycloheximide (200 μg/ml) at the indicated time intervals and the relative protein level of SRG3 was detected by immunoblot analysis with anti-Myc antibody. *H*, COS-1 cells were co-transfected with Myc-SRG3, FLAG-BAF60a, HA-BAF57, and FLAG-SNF5 expression vectors, and subjected to co-immunoprecipitation with anti-Myc antibody and immunoblot (*IB*) analysis with indicated antibodies.

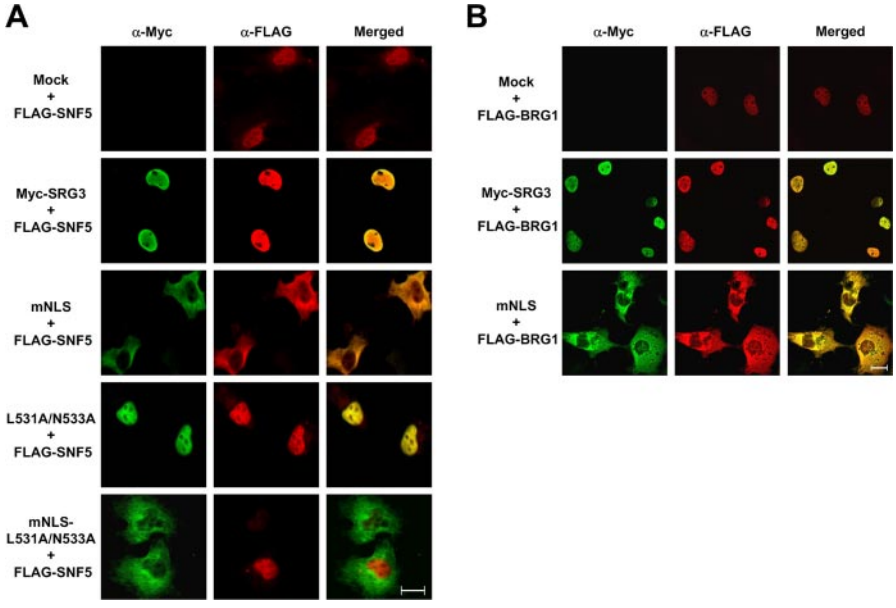

FIGURE 6. **SRG3 affects the subcellular localization of the core components of the SWI/SNF complex.** *A*, COS-1 cells grown on coverslips were co-transfected with 0.1 μg of FLAG-SNF5 expression vector along with 0.1 μg of Myc-SRG3 or mutant SRG3 expression vector. Cells were incubated for 48 h after transfection and stained as described under "Experimental Procedures." Images were obtained by confocal microscopy and co-localization was shown by a *merged view* of the *red* and *green* channels in the same field. *Bar*, 20 μm. *B*, COS-1 cells grown on coverslips were co-transfected with 0.1 μg of FLAG-BRG1 expression vector along with 0.1 μg of Myc-SRG3, or the NLS mutant of SRG3 expression vector. Staining and confocal microscopy were performed as described above. *Bar*, 20 μm.

lizes the components of the SWI/SNF complex but also increases formation of the co-immunoprecipitating complex with SRG3.

SRG3 Affects the Subcellular Localization of the Core Components of the SWI/SNF Complex—We next tested the effects of SRG3 on stabilization and subcellular localization of ectopically expressed SNF5 and BRG1 by immunofluorescence staining. We have mutated one of the putative nuclear localization signal (NLS) sequences in SRG3 and obtained a NLS mutant of SRG3 (mNLS) showing strong cytoplasmic staining signal (Fig. 6, *A* and *B*, *third row*). FLAG-SNF5 expression vector was co-transfected into COS-1 cells along with an empty vector or vectors expressing wild-type or mutant SRG3. In the absence of SRG3 co-expression, SNF5 was expressed at a very low level (Fig. 6*A*, *first row*) and localized in the nucleus as previously reported (38). In the presence of wild-type SRG3 or the NLS mutant of SRG3 co-expression, SNF5 was expressed at a very high level (Fig. 6*A*, *second* and *third rows*). SRG3 and SNF5 produced a strong fluorescence signal in the nucleus of cells co-expressing wild-type SRG3 and SNF5. On the other hand, co-expression of

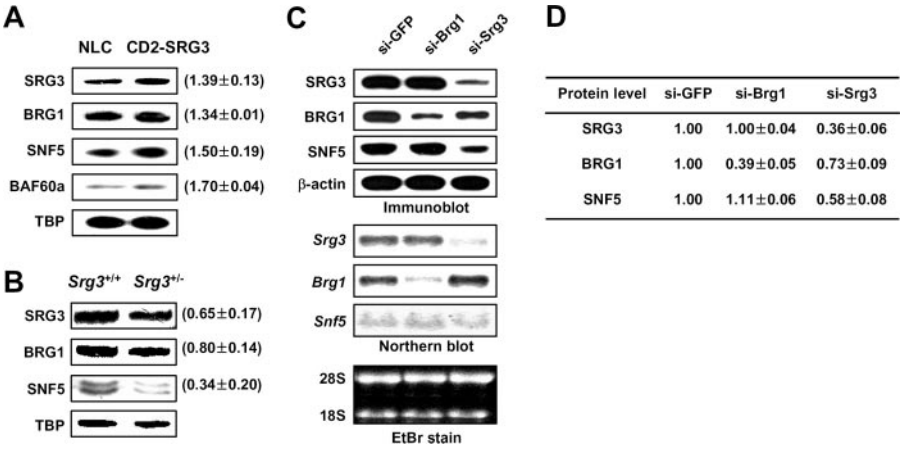

FIGURE 7. **SRG3 controls the protein level of the SWI/SNF complex *in vivo*.** *A*, nuclear extracts prepared from total thymocytes of 4-week-old CD2-SRG3 transgenic and control mice were subjected to immunoblot analysis. TBP was shown as a control, and the protein levels were quantified and normalized to TBP. *Numbers* are relative levels compared with that observed in NLC mice and represented as mean ± S.D. of three independent experiments. *B*, nuclear extracts prepared from thymocytes of $Srg3^{+/-}$ and control mice were subjected to immunoblot analysis. The protein levels were quantified and normalized to TBP. *Numbers* are relative levels compared with that observed in control mice and represented as mean ± S.D. of three independent experiments. *C*, NIH3T3 cells were co-transfected with the indicated siRNAs. Total extracts were subjected to immunoblot and Northern blot analyses. The figure is one representative of three independent experiments. *D*, immunoblots described in *C* were quantified by densitometric scanning. The protein levels of SRG3, BRG1, and SNF5 were normalized to β-actin protein. *Numbers* are mean ± S.D. of three independent experiments.

SNF5 and the NLS mutant of SRG3 displayed dramatic cytoplasmic accumulation of two proteins (Fig. 6*A*, *third row*). Interestingly, SNF5 was localized in the nucleus when co-expressed with the double mutant of both NLS and the SWIRM domain, while the mutant SRG3 produced mostly the cytoplasmic signal (Fig. 6*A*, *fifth row*). These results confirm again that the SWIRM domain is crucial to the *in vivo* SRG3-SNF5 interaction. The number of SNF5-expressing cells was small in the cell population expressing FLAG-SNF5 alone or co-expressing the SWIRM domain mutant, whereas large in cells co-expressing wild-type SRG3 or the NLS mutant of SRG3 (data not shown). These results are consistent with previous results showing that SRG3 stabilizes the SNF5 protein (Figs. 3 and 5).

We further investigated the effect of SRG3 on the stabilization and localization of BRG1. FLAG-BRG1 expression vector was co-transfected into COS-1 cells along with an empty vector or vectors expressing wild-type SRG3 or the NLS mutant of SRG3 as described in the legend to Fig. 6*A*. Confocal microscopic analysis revealed that BRG1 was localized in the nucleus when expressed alone or co-expressed with wild-type SRG3, but retained in the cytoplasm when co-expressed with the NLS mutant of SRG3 as in the case with SNF5 (Fig. 6*B*). We also confirmed that the expression level of the BRG1 protein was high in cells co-expressing wild-type SRG3 or the NLS mutant of SRG3, but low in FLAG-BRG1 alone. These results are also consistent with the biochemical results showing that SRG3 stabilizes the BRG1 protein (Figs. 3 and 5). These results show that SRG3 regulates protein stability of SNF5 and BRG1, and is also important for subcellular localization of these proteins.

SRG3 Controls the Protein Level of the SWI/SNF Complex in Vivo—Because the stabilization effect of SRG3 has been shown only in the ectopically expressed *in vitro* system so far, we next examined the role of endogenous SRG3 in stabilization of the endogenous components of the SWI/SNF complex. First, we compared expression of the SWI/SNF complex in the SRG3-overexpressing CD2-SRG3 transgenic mice with that of their normal littermate control (NLC) mice. As described previously, CD2-SRG3 mice constitutively express SRG3 in T lineage cells under control of the human *CD2* promoter and the thymic SRG3 protein expression is up-regulated by 1.3–2-fold compared with that of NLC mice (19). Such moderate augmentation of SRG3 expression was proved to be functional, as the glucocorticoid-induced apoptosis, which was reported to be regulated by the SWI/SNF complex, was enhanced in peripheral T cells of CD2-SRG3 transgenic mice (19). As shown in Fig. 7*A*, protein expressions of BRG1, SNF5, and BAF60a were all up-regulated in CD2-SRG3 transgenic mice more than in NLC mice. Thus, up-regulation of endogenous SRG3 yielded augmented expression of the endogenous SWI/SNF complex. Next, we examined expression of the SWI/SNF complex in thymocytes from the knock-out mice haploinsufficient for the *Srg3* gene. Thymocytes from the *Srg3* heterozygote (+/−) knock-out mice are known to express reduced amounts of SRG3 protein to approximately half of the NLC mice (17). In these cells, the protein levels of BRG1 and SNF5 were also down-regulated (Fig. 7*B*). This result suggests that reduced SRG3 expression resulted in down-regulation of protein levels of the SWI/SNF complex. Finally, endogenous expression of the SWI/SNF complex was examined in cells with reduced SRG3 by siRNA. The expression of SRG3 in NIH3T3 cells was specifically down-regulated by siRNA transfection. The mRNA expressions of *Brg1* and *Srg3* in the respective siRNA transfections were suppressed to ~10%, compared with control (Fig. 7*C*, *Northern blot panels*). The expression of *Srg3* siRNA (si-Srg3) down-regulated the protein levels of both BRG1 and SNF5 as well as the SRG3 protein itself (Fig. 7*C*, *third lane* in *Immunoblot panels*). However, in *Brg1* siRNA (si-Brg1)-expressing cells, only the protein level of BRG1 was down-regulated and the expression of SRG3 and SNF5 proteins was not reduced (Fig. 7*C*, *second lane* in *Immunoblot panels*). Therefore, down-regulated SRG3 expression by siRNA transfection gave the reduced SWI/SNF complex expression in NIH3T3 cells. From these three *in vivo* experimental systems, we suggest that SRG3 as a crucial protein for stable maintenance of the components of the SWI/SNF complex.

DISCUSSION

Biochemical studies have shown that BRG1, SNF5, and SRG3/BAF155 are core components of the SWI/SNF com-

plex (8, 10, 39). Targeted mutations of *Brg1*, *Snf5*, and *Srg3* show similar developmental defects in implantation, supporting that they work as core components *in vivo*. Whereas the function of BRG1 and SNF5 has been reported (10, 12, 22), the function of SRG3 as a component of the SWI/SNF complex has not been elucidated. Here, we show that SRG3 interacts directly with SNF5, BAF60a, and BRG1, and stabilizes them. SNF5 interacts with the SWIRM domain of SRG3 and is stabilized by the interaction. BAF60a also interacts with SRG3 through a region containing the SWIRM domain. In addition, BRG1 interacts directly with the SANT domain of SRG3. Similarly, it was shown that MOIRA, a *Drosophila* homologue of SRG3, interacts with BRM through the SANT domain (23). It was recently reported that BAF155 interacts with BAF57 through the leucine-zipper motif (24). All of these results suggest that SRG3 may act as a scaffold protein that interacts directly with other components including BRG1, SNF5, BAF60a, and BAF57. However, it is possible that BAF170 may share the scaffold function with SRG3 because the two proteins are highly homologous. It was reported that BAF57 interacts with BAF155/BAF170 and the enforced overexpression of BAF155 (SRG3) or BAF170 was able to augment the protein level of BAF57 (24). But, other core components such as BRG1 and SNF5 do not likely function as scaffold. In support of this notion, the deficiency of hSNF5/INI1, BRG1, or BRM did not impair the assembly of the remaining components of the mammalian SWI/SNF complex (8, 40, 41). Furthermore, BRG1 deficiency did not reduce the protein levels of other components, whereas SRG3 deficiency resulted in the reduction of the BRG1 and SNF5 proteins (Fig. 7, *B* and *C*).

Previous studies showed that c-Myc recruits the SWI/SNF complex through interaction with hSNF5/INI1 (12) and glucocorticoid receptor recruits the complex through BAF60a (32), implying that both SNF5 and BAF60a can function as a structural bridge between the SWI/SNF complex and transcriptional factors. In addition, BAF57 has been thought of as a component that can bind to DNA through its high mobility group domain. Therefore, it appears that SRG3 may act as a linker between BRG1 and other components interacting with specific transcriptional factors or DNA.

We have found that SRG3 contributes to the stability of the major components of the SWI/SNF complex. Cycloheximide treatment of cells ectopically expressing SNF5, BRG1, or BAF60a resulted in rapid degradation of each protein, but treatment of MG132, a proteasome inhibitor, along with cycloheximide resulted in stable expression of each protein (Fig. 4). Interestingly, ectopic co-expression of SRG3 with SNF5, BRG1, or BAF60a in the presence of cycloheximide yielded their protein stabilization (Fig. 5). However, SRG3 itself appeared to be relatively stable compared with the other components (Fig. 5). It was reported that degradation of the BAF57 protein is also mediated by a proteasome-dependent pathway and stabilized by BAF155 (24). Therefore, degradation of SNF5, BRG1, BAF60a, and BAF57 is mediated by the proteasome pathway and direct interaction of these proteins with SRG3 protects them from proteasomal degradation. Furthermore, our results suggest that *in vivo* control of the stability of the SWI/SNF complex by SRG3 is also true (Fig. 7). Transgenic overexpression of SRG3 increased protein levels of BRG1, SNF5, and BAF60a. On the other hand, protein levels of BRG1 and SNF5 were down-regulated in cells from the *Srg3* heterozygote (+/−) knock-out mice. These results strongly suggest that SRG3 is critical for stabilization of the SWI/SNF complex and that SRG3 may act as a key regulator for function of the SWI/SNF complex by regulating its protein level. There are several reports that scaffold proteins contribute to the assembly and stability of the multisubunit complex. TFIID is a multisubunit transcription factor required for RNA polymerase II-dependent transcription (42, 43). TAF1, one of the subunits of the TFIID holocomplex, has been reported to be a scaffold component of TFIID and contributes to stability of the complex (44). In addition, Aph-1, one component of the γ-secretase complex that cleaves type I transmembrane proteins, functions as a scaffold and stabilizing component of the assembly of the complex (45).

The interaction of SNF5 with SRG3 protects the protein from proteasomal degradation. Thus, cytoplasmic co-localization of mutant SRG3 and wild-type SNF5 (Fig. 6) suggests an interesting possibility that some SRG3 mutants localized in the cytoplasm can cause mislocalization of SNF5 in certain tumors. A mutant SNF5 found in malignant rhabdoid tumor lacking the 66 C-terminal amino acids unmasks the nuclear export signal sequence, thereby being mislocalized in the cytoplasm, and mutations of nuclear export signal in this mutant resulted in nuclear localization of the protein and restored its ability to cause cell cycle arrest (38). From our results, it can be postulated that mutations causing cytoplasmic localization of SRG3 could lead to tumorigenesis by mislocalization of SNF5 in the cytoplasm. This possibility and other similar phenomena need to be examined.

All SRG3 homologues contain conserved α-helical SWIRM and SANT domains that are involved in DNA-binding and histone tail-binding. Recently, the three-dimensional structures and functions of several SWIRM domains were revealed. The SWIRM domains of SWI3, ADA2α, and LSD-1 are composed of 4-, 5-, and 6 α-helices, respectively, and interact with various entities such as naked DNA, nucleosome, or histone (34, 46, 47). Moreover, SWIRM domain mutants of SWI3 that displayed *in vivo* defects also showed *in vitro* impairment of DNA-binding activity (34). The SANT domain of ISWI consists of three α-helices (48) and the domain of ADA2 is considered a histone-interaction module (49). Although these results suggested a functional role of the SWIRM and SANT domains as essential multifunctional modules in chromatin remodeling, the role of these domains in protein-protein interaction and stabilization of components of the SWI/SNF complex has not been known. We have shown here that mutations in the SWIRM domain of SRG3 abolished interaction with SNF5 and its stabilization. The deletion of the SANT domain of SRG3 also eliminated interaction with BRG1 and possibly its stabilization. Thus, mutations on the SWIRM and SANT domains may result in the defective phenotype in the function of the SWI/SNF chromatin remodeling complex. As expected,

deletion of the SWIRM domain or the SANT domain in SWI3 resulted in transcriptional defects similar to those caused by the complete deletion of the subunit (34, 49). Therefore, our results suggest that the function of the SWIRM and SANT domains of SRG3 in protein-protein interaction and stabilization of interacting components may be important for maintaining the activity of the SWI/SNF complex.

Even though we cannot exclude a possibility for another function of SRG3 in the SWI/SNF complex, the scaffold role appears to be the major function of SRG3. The expression of SRG3 is exquisitely regulated during thymocyte maturation and embryonic development (17, 21). During positive selection of developing thymocytes, T cell receptor signaling induces the expression of the Id3 protein, which blocks the activity of the E2A protein and thereby down-regulates SRG3 expression (50). We have recently found that the changes in SRG3 expression resulted in changes in the overall level of the SWI/SNF complex and its remodeling activity in developing thymocytes.[4] Furthermore, down-regulation of the SRG3 expression resulted in gaining the developing thymocytes a resistance to glucocorticoid-induced apoptosis (20, 50) and also changes in thymocyte maturation.[4] Therefore, the scaffold function of SRG3 may be importantly linked to the physiological control of the SWI/SNF complex and its activity during cell differentiation and development.

Our results provide *in vivo* as well as *in vitro* evidence supporting the scaffold function of SRG3 and the regulator of protein stability in the SWI/SNF complex. SRG3 interacts directly with BRG1, SNF5, BAF60a, and BAF57 and the interaction protected these proteins from proteasomal degradation. Reduction of the SRG3 protein decreases the protein levels of other components, whereas the reduction of the BRG1 protein does not. In addition, SRG3 is finely regulated during cell differentiation, resulting in the change in the overall activity of the SWI/SNF chromatin remodeling complex. These results imply that SRG3 may play a role as a scaffold and key regulator of the SWI/SNF chromatin remodeling complex.

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