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Breast cancer metastasis suppressor 1 (BRMS1) is stabilized by the Hsp90 chaperone

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Abstract

Breast cancer metastasis suppressor 1 (*BRMS1*) is a member of the mSin3-HDAC transcription co-repressor complex. However, the proteins associated with *BRMS1* have not been fully identified. Yeast two-hybrid screen, immuno-affinity chromatography, and co-immunoprecipitation experiments were performed to identify *BRMS1* interacting proteins. In addition to known core mSin3 transcriptional complex components RBBP1 and mSDS3, *BRMS1* interacted with other proteins including three chaperones: DNAJB6 (MRJ), Hsp90, and Hsp70. Hsp90 is a known target of HDAC6 and reversible acetylation is one of the mechanisms that is implicated in regulation of Hsp90 chaperone complex activity. *BRMS1* interacted with class II HDACs, HDAC 4, 5, and 6. We further found that *BRMS1* is stabilized by Hsp90, and its turnover is proteasome dependent. The stability of *BRMS1* protein may be important in maintaining the functional role of *BRMS1* in metastasis suppression.

Keywords

Metastasis suppressor gene; *BRMS1*; Protein–protein interaction; Chromatin modulation; Yeast two-hybrid; Heat shock protein; Immunoprecipitation; MALDI-TOF-MS; Chromatography

The metastatic cascade is a highly inefficient process that requires coordinated expression of a large assortment of specific genes [1-3]. As such, proteins that regulate transcription may dramatically impact this process. Breast cancer metastasis suppressor 1 (*BRMS1*), which suppresses metastasis without blocking tumorigenesis in multiple human and murine

#Abbreviations: *BRMS1*, breast cancer metastasis suppressor gene 1; CMF-DPBS, calcium- and magnesium-free Dulbecco's phosphate-buffered saline; DMEM-F12, mixture (1:1) Dulbecco's modified minimum essential medium and Ham's F-12 medium; HBSS, Hank's balanced salt solution; SDS sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TTBS, Tris-buffered saline with 0.05% Tween 20.

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carcinomas [4-8], is a predominantly nuclear protein that has two coiled-coil domains which are suggested to maintain multiple protein–protein interactions; and, it has been shown to regulate the expression of specific genes [9,10]. This transcriptional regulation is, at least in part, due to association of BRMS1 with a mSin3/histone deacetylase complex [11-13]. The core mSin3/HDAC complex is associated with multiple other molecules which provide specificity of interaction with DNA.

The goal of the current study was to identify BIPs as a first step toward determining BRMS1/HDAC/mSin3 mechanism of action. We identified additional BIPs that are not known to be part of the core mSin3 complex, suggesting alternative roles for BRMS1. The interactors identified are involved in transcriptional regulation, cell cycle progression, cytoskeletal rearrangement, and chaperone function. These results have important implications for the multiple roles of BRMS1 in normal cell functions as well as in suppression of metastasis. Also, this study highlights the fact that, in addition to correlating expression levels of BRMS1 with metastatic potential, the stability of BRMS1 protein must also be considered.

Experimental procedures

Cell lines and cell culture

The metastatic human breast carcinoma cell line, MDA-MB-231, was transduced with a HIV type 1-based, lentiviral vector system to constitutively express BRMS1 [14-16]. The BRMS1 coding sequence was inserted into the vector 5' of the internal ribosome entry site and puromycin sequences, each of which were under the control of the early cytomegalovirus promoter. Infectious stock was prepared by transfection of 293T cells and used at a multiplicity of infection of ~10. The monkey kidney cell line, COS7, was used for transient transfections to validate protein–protein interactions. All cells were maintained in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium (DMEM-F12; Invitrogen, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 0.02 mM non-essential amino acids, and 1 mM sodium pyruvate with no antibiotics nor antimycotics were passaged at 80–90% confluence using a 0.125% trypsin and 2 mM EDTA solution (231-BRMS1) or a 2 mM EDTA solution (COS7) in Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline (CMF-DPBS). Cells were maintained on 100 mm tissue culture dishes (Corning, Corning, NY) at 37 °C with 5% CO₂ in a humidified atmosphere. All cell lines were found to be negative for *Mycoplasma* spp. contamination using a PCR-based method (TaKaRa, Madison, WI).

Plasmids and constructs

The plasmids containing FLAG-epitope tagged HDAC2 and 3 were generous gifts of S. L. Schreiber [17,18]. The plasmids containing FLAG-epitope tagged HDAC1, 4, 5, and 6 were generous gifts of Seto [19]. cDNA for mSDS3, BAF57, and MRJ were generously provided by Ayer [20], Weissman [21], and Sung [22], respectively. A NMI cDNA clone from pACT (breast) library and BRMS1 cDNA were cloned into the constitutive mammalian expression vector pcDNA3 (Invitrogen) under control of the cytomegalovirus promoter. To detect BRMS1 protein expression, a chimeric molecule, pcDNA3-901-BRMS1, was constructed with an N-terminal epitope tag (SV40T epitope 901) [23].

Yeast two-hybrid screen

The yeast two-hybrid screen to isolate cDNA encoding BRMS1 interacting proteins was performed essentially as described [24,25]. We used pDBTrp and cloned the entire coding frame of human BRMS1 in frame with GAL4 DNA-binding domain to obtain pDB-BRMS1. This construct was used to transform yeast strain AH 109 (*MATa*, *trp-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*,

URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ, and *MEL1*) (BD Biosciences, Clontech, CA) to obtain a GAL4DB-BRMS1 fusion (bait) expressing strain of AH109. This strain was used to screen mammary gland, prostate and placenta MATCHMAKER™ cDNA libraries in pACT2 (BD Biosciences, Clontech). Transformants were selected by the use of appropriate media (yeast drop out minimal medium lacking histidine, tryptophan, and leucine). His⁺ colonies were tested for growth on minimal medium lacking adenine, tryptophan, leucine, and β-galactosidase activity as described previously [26]. cDNA plasmids were isolated from each positive yeast clone using Zymoprep™ (Zymo Research, Orange, CA) and sequenced. The interaction was reconfirmed by plasmid loss experiment for the bait as well as prey protein coding vectors. Introduction of the missing partner recovered the growth on minimal medium lacking histidine, tryptophan, and leucine. This was also confirmed by growth on minimal medium lacking adenine, tryptophan, and leucine and restoration of β-galactosidase activity.

Transient transfection

Transient transfection studies in COS7 were performed using pcDNA3-901-BRMS1, and the plasmids containing the cDNA of the interactors. The transfections were performed using Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. Briefly, COS7 cells were plated on to 100 mm tissue culture plates one day before the transfection, to achieve a confluence of 80–90%. The cells were transfected using ~24 μg plasmid DNA/plate under serum-free conditions. The serum containing medium was added 4–6 h after transfection. The proteins were harvested after 40–48 h for co-immunoprecipitation studies.

Antibodies, immunoprecipitation, and Western blotting

An antibody directed against the 901 epitope was provided by Satvir Tevethia and the anti-MRJ antibody was obtained from Chin-Hua Sung. Other antibodies used in this study were purchased: anti-human BAF57 (Active Motif, Carlsbad, CA), anti-SDS3 (Bethyl Laboratories Inc., Montgomery, TX), anti-NMI (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-FLAG M2 (Sigma, Saint Louis, MO). A monoclonal antibody directed against BRMS1 (3a1.21) was generated and validated by MALDI-TOFMS as described below.

For co-immunoprecipitation studies, after transfection with appropriate vectors, cells were washed twice with ice-cold PBS and lysed with 1% Triton X-100 (Sigma) lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 2.0 mM EDTA), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 μg/mL aprotinin, 50 mM NaF, 0.2 mM Na₃VO₄, and 10 μL/mL of a protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanido) butane (E-64), bestatin, leupeptin, and aprotinin (Sigma). Lysate was passed through a 21 g needle several times, incubated on ice for 2.5 h, then centrifuged for 10 min at 18,000 rcf at 4 °C to remove insoluble debris. Lysates were then rocked gently in the presence of appropriate antibody (1–2 μg) for 1 h at 4 °C. Twenty microliters of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added and lysates were rocked overnight at 4 °C. Agarose beads were washed twice with PBS and boiled in SDS-sample buffer. The solubilized protein was resolved by SDS-PAGE and transferred to PVDF membrane. Proteins were fixed by air drying for 15 min at room temperature. The membrane was then wetted in methanol, rinsed in distilled water and blocked in a TTBS solution (0.05% Tween 20, 20 mM Tris, and 140 mM NaCl, pH 7.6) containing 5% non-fat milk for 1 h followed by incubation with appropriate primary antibody for 1 h at room temperature or overnight at 4 °C under constant agitation. Membranes were then washed with TTBS and probed with 1:10,000 dilution of sheep anti-mouse or goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham-Pharmacia Biotech, Buckinghamshire, UK) in a solution of 5% non-fat milk/TTBS for 1 h at room temperature before washing in TTBS. Bound secondary antibodies were detected using ECL™ (Amersham-Pharmacia Biotech).

Immuno-affinity purification

The 3a1.21 monoclonal BRMS1 antibody was *N*-linked to agarose beads using the AminoLink Plus Immobilization kit (Pierce, Rockford, IL). Briefly, 5 mg of purified 3a1.21 in 3 mL of 0.1 M phosphate, 0.15 M NaCl, pH 7.2, was incubated with 2 mL 50% agarose slurry and 40 μ L sodium cyanoborohydride overnight at 4 °C. The gel was washed with 5 mL PBS and remaining active sites were blocked with quenching buffer (1 M Tris-HCl, pH 7.4). Whole cell lysate from 231-BRMS1 cells was incubated in the column overnight at 4 °C. BRMS1 and putative interactors were eluted with 0.15 M glycine, pH 2.5. Fractions positive for BRMS1 as determined by Western blot were combined and electrophoresed on a 12% SDS-PAGE followed by Coomassie staining. Visible bands were excised and submitted to the UAB Mass Spectrometry and Proteomics Shared Facility where they were digested with trypsin and identified by MALDI-TOF-MS.

³⁵S protein labeling

231-BRMS1 cells were grown to 80–90% confluence in 100 mm tissue culture plates. Media were removed and replaced with cysteine- and methionine-free media containing 5% fetal bovine serum and 50 μ Ci/mL [³⁵S]methionine (TransLabel, ICN Pharmaceuticals, Costa Mesa, CA) for 1 h. Cells were then washed with PBS and placed in original (unlabeled) media. Cells were lysed and immunoprecipitated with 3a1.21 mAb as described above at selected time points. PVDF membranes were exposed on X-ray film for 14 days. The blots were also probed with 3a1.21 mAb as described above.

Inhibitor treatment

The 231-BRMS1 cells were treated with selected concentrations of the Hsp90 inhibitor geldanamycin (GA; Sigma) alone or in combination with 10 μ M of the proteasome inhibitor, MG-132 (Sigma), for 6 h in serum-free media. Cells were lysed and probed with 3a1.21 mAb as described above. The blots were also probed with Hsp90 (Cell Signaling) and β -actin (Sigma) antibodies for comparison.

Results

BRMS1 binds to multiple proteins

BRMS1 has been previously reported to interact with mSin3/HDAC complex and suppress transcription [11–13]. In order to further characterize this complex with the goal of elucidating the mechanism(s) underlying the ability of BRMS1 to suppress metastasis, a yeast two-hybrid screen was performed as described above. Full-length BRMS1 was used as bait and human mammary gland (or prostate or placenta) cDNA library as prey. Six genetic interactors of BRMS1 were identified in addition to the previously reported mSDS3 (SAP45, suppressor of defective silencing, Sin3 associated protein) and RBBP1 (Rb-binding protein) [11]. The six novel interactors are not part of the mSin3 core complex and include NMI (N-myc interactor), MRJ (Hsp40-related chaperone) [27], CCG1 (TAFII250, a protein essential for progression of G phase), SMTN (Smoothelin, a cytoskeletal protein specific to smooth muscles) [28], KPNA5 (karyopherin α 5) [29], and BAF 57 (BRG1 associated factor) (Table 1). Interestingly, immuno-affinity chromatography identified two other chaperone proteins known to form a complex with Hsp40; Hsp90 and Hsp70 (Fig. 1).

Validation of the protein interactions by co-immunoprecipitation

In order to confirm the interaction between the various interactors and BRMS1, cDNA of the interactors and BRMS1 were co-transfected in COS7 and the immunoprecipitations were performed by interactor-specific antibodies. RBBP1 [11], mSDS3, BAF 57, NMI, and MRJ were confirmed to interact with BRMS1 (Fig. 2A). mSDS3, BAF57, and NMI interaction was

confirmed by reverse co-immunoprecipitation as well (data not shown). The interaction with Hsp90 was validated by co-immunoprecipitation in both directions using the 231-BRMS1 cells and the 3a1.21 mAb or Hsp90 pAb (Fig. 2B).

BRMS1 is a dynamic protein that is stabilized by Hsp90

To address the importance of chaperone interaction with BRMS1, the half life of BRMS1 in cells was tested. The 231-BRMS1 cells were labeled with [³⁵S]methionine and BRMS1 was immunoprecipitated at selected time points. A significant decrease in labeled BRMS1 was noted after only 2 h following the radioactive pulse and almost complete disappearance at 8 h. However, total BRMS1 (labeled and unlabeled) showed no detectable changes (Fig. 3A). The proteasome inhibitor, MG-132, blocked the degradation of BRMS1 compared to vehicle control (DMSO) or untreated cells (Fig. 3B). These results show that BRMS1 is a dynamic protein that is degraded through the proteasomal pathway.

To further characterize the functional interaction of BRMS1 with Hsp90, 231-BRMS1 cells were treated with selected concentrations of the Hsp90 inhibitor geldanamycin (GA). Inhibition of Hsp90 by GA prevents stabilization of client proteins and leads to degradation of the client protein by the proteasome [30,31]. As such, BRMS1 was shown to be degraded in a dose-dependent manner and this degradation could be rescued by combining the treatment with MG-132 (Fig. 4). No significant change in the levels of Hsp90 or β -actin was noted. These results demonstrate that BRMS1 is a client protein of the Hsp90 chaperone complex.

BRMS1 binds to class I and class II HDACs

The mSin3/HDAC core complex includes HDAC1 and 2 which belong to class I HDACs. HDAC3, another class I HDAC, shares high sequence similarity to HDAC1 and 2 and we therefore tested whether BRMS1 could interact with this HDAC. HDAC3 is typically found in distinct complexes from HDAC1 and 2 [32,33]. Additionally, class II HDACs (HDAC4, 5, and 6) that are predominantly localized in the cytoplasm and specifically HDAC6 which has been demonstrated to regulate the Hsp90 chaperone complex [34-36] were tested for interaction with BRMS1. BRMS1 and FLAG-epitope tagged HDAC-cDNAs were co-transfected in COS7. The immunoprecipitations by anti-FLAG antibodies showed BRMS1 co-immunoprecipitation (Fig. 5A). The reverse co-immunoprecipitation further confirmed these interactions (Fig. 5B). These results further demonstrate that BRMS1 binds to multiple protein complexes including the Hsp90 chaperone complex.

Discussion

BRMS1 is a predominantly nuclear protein that suppresses metastasis in multiple systems without blocking tumorigenesis. BRMS1 protein contains several putative protein-binding domains including coiled-coil and imperfect leucine zippers. Previously we identified that BRMS1 was part of the core mSin3 complex [11] that was subsequently confirmed by two other groups [12,13]. The goals of this study were to further characterize the BRMS1 interacting proteins that may be important for metastasis suppression. The yeast two-hybrid screen showed interesting candidate proteins. In addition to RBBP1 and mSDS3, BAF57, NMI, and CCG1 (TAFII250) are associated with protein complexes involved in regulation of transcription [21,37-39]. BAF57 and CCG1, although involved in transcriptional regulation, are distinct from the mSin3 core complex. BAF57 is a member of the SWI/SNF complex that has recently been found to associate with mSin3 [40,41]. CCG1 is part of the TFIID complex that includes Rb [42]. The functional role for BRMS1 interacting with these proteins is currently under investigation.

RBBP1 and mSDS3 are members of the mSin3/HDAC complex. mSDS3 shares 23% identity and 49% similarity in primary amino acid sequence with BRMS1 [11]. Another component of this complex, p40, shares 58% identity and 79% similarity (comparing the C-terminal 196 AA of BRMS1 with the N-terminal 196 AA of p40) with BRMS1 [11] suggesting the existence of a family of BRMS1 proteins that together or independently form multi-molecular complexes to regulate gene expression through the mSin3/HDAC complex. Chromatin structure is dynamic and is remodeled in part by covalent modifications that change access of DNA-binding transcription factors and transcriptional machinery to particular DNA sequences. The mSin3/HDAC chromatin remodeling complex has been traditionally thought to regulate transcription by recruiting HDAC1 and 2 activity to sequence-specific transcriptional repressors [43]. Additionally, it has been found to maintain other enzymatic functions including DNA and histone methylation, *N*-acetylglucosamine transferase activity, and nucleosome remodeling [44]. The precise function and specificity of the core complex seems to be modulated by transient association with gene-specific transcription factors [45]. Therefore, each BRMS1 family member may separately dictate functionality or gene specificity of the complex.

The involvement of particular HDACs in these transcriptional complexes is not completely understood. HDAC1 and 2 have been shown to be an important component of the mSin3 complex, however, they are also part of many other molecular complexes including NuRD, CoREST, BHC110, and XFIM. HDAC3 is also a class I HDAC, however, it is more restricted to the NCOR/SMRT complex and is typically involved in repression of specific genes connected with nuclear receptor signaling [46,47]. BRMS1 was found to interact with each of these HDACs, which may suggest a diverse role for BRMS1 in multiple transcriptional regulatory pathways.

Class II HDACs include HDAC4, 5, 6, 7, 9, and 10. These are mainly cytoplasmic but have also been shown to be shuttled to the nucleus and interact with transcriptional regulating complexes [47]. Recently, HDAC6 has gained attention because it regulates the activity of the Hsp90 chaperone complex [34-36]. It was found that deacetylation of Hsp90 by HDAC6 is required to properly stabilize a client protein. However, when our cells were treated with the HDAC inhibitor trichostatin A (TSA), co-immunoprecipitation did not show any change in the interaction between BRMS1 and Hsp90 (data not shown). This may suggest that BRMS1 plays another role in this complex (such as recruiting HDAC6 to the complex) or that some client proteins are stabilized by an HDAC-independent mechanism.

We chose to follow up the Hsp40, 70, and 90 interactions with functional studies due to the implications for regulation of BRMS1 at the protein level. The Hsp90 chaperone complex is activated during cell stress (such as cancer metastasis) to associate with distinct client proteins [30]. In addition to Hsp70 and 40, this chaperone complex includes many co-chaperones and adapter proteins for proper function. The stability, localization, and function of client proteins are dependent on this complex and it has been targeted in cancer treatment because of the important roles it plays in maintaining client protein function in growth, survival, and apoptotic pathways [31,48]. Examples of client proteins involved in cancer progression and resistance to therapy include the estrogen receptor, receptor tyrosine kinases of the *erbB* family, Akt, and mutant p53 [49]. Several small molecule inhibitors of Hsp90 have been identified that disrupt client protein stabilization and target them for proteasomal-mediated degradation. Hsp90 inhibitors are currently being tested in several clinical trials for the treatment of cancer in single agent studies and in combination with conventional chemotherapy [48]. It has been hypothesized that Hsp90 inhibitors will alter the activity of the multitude of oncogenic proteins requiring Hsp90 for proper function with little or no effect on normal cells. However, implications of the destabilization of tumor or metastasis suppressors, such as BRMS1, should

be evaluated further. The interplay between BRMS1 stability and functionality as it relates to cancer metastasis is the objective for future studies.

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Fig. 1.

Identification of chaperone protein interactors. Whole cell lysate from 231-BRMS1 was partially purified with the 3a1.21 mAb that was *N*-linked to an agarose matrix. Specific fractions of the eluate contained BRMS1 and potential interactors that were combined and electrophoresed on a 12% SDS-PAGE followed by Coomassie staining. Visible bands were excised, trypsin digested, and identified by MALDI-TOF-MS by the UAB MS core facility.

Fig. 2.

Validation of BRMS1 interactors. (A) BRMS1 was co-immunoprecipitated with mSDS3, NMI, MRJ, and BAF57 from whole cell lysates (1 mg) of COS7 transient co-transfections. Lane 1, beads with no antibody added, lane 2, whole cell lysate (80 μ g), lane 3, immunoprecipitation using an irrelevant antibody (β -actin), lane 4, immunoprecipitation with respective interactor antibody. Western blot (WB) was performed with the ant-901 epitope (BRMS1) antibody. (B) Whole cell lysate (W) from 231-BRMS1 cells were co-immunoprecipitated (IP) with either the BRMS1 3a1.21 mAb or anti-Hsp90 pAb. The blots were probed consecutively with both Abs. S is the supernatant from the IP.

Fig. 3.

BRMS1 turnover is proteasome dependent. (A) The half life of BRMS1 is approximately 2 h. The 231-BRMS1 cells were pulsed with media containing [^{35}S]Met for 1 h and collected at selected time points. The lysates were immunoprecipitated with 3a1.21 mAb and the blots were exposed on X-ray film for 14 days. Significant degradation of labeled BRMS1 is noted after 2 h. The blots were re-probed with the 3a1.21 mAb to show no change in total BRMS1. (B) BRMS1 responds to proteasome inhibition. The 231-BRMS1 cells were treated with 20 μM MG-132 (proteasome inhibitor) and compared to the controls (DMSO and untreated). A significant increase in BRMS1 levels is noted in the MG-132 treated cells demonstrating that BRMS1 protein is degraded, probably through proteasomal degradation.

Fig. 4.

BRMS1 is stabilized by Hsp90. Inhibition of Hsp90 promotes BRMS1 degradation which is rescued by inhibition of the proteasome. The 231-BRMS1 cells were treated with selected concentrations of the Hsp90 inhibitor geldanamycin (GA) alone for 6 h or in combination with 10 μ M MG-132. Significant degradation is noted for BRMS1 in the GA only treated cells. The same blot was probed with antibodies against Hsp90 and β -actin for comparison.

Fig. 5.

BRMS1 interacts with class I and class II HDACs. (A) Immunoprecipitation (IP) of BRMS1 from transiently co-transfected COS7 cells and (B) IP of indicated HDAC with anti-FLAG M2 Ab. Class I HDACs, HDAC1 and 2 (represented as H1 and H2) and class II HDACs, HDAC4, 5, and 6 (represented as H4, H5, and H6) were immunoprecipitated by BRMS1 (anti-901 mAb) from whole cell lysates (1 mg) of COS7 cells after transient co-transfections. The top panel in (A) and (B) is whole cell lysate (50 μ g) showing levels of various HDACs or BRMS1 after transient transfection. Lane M indicates lane corresponding to the molecular weight marker.

Table 1

Genetic interactors of BRMS1 in Y2H

Gene	Accession No.	Cellular location	Description
RBBP1 [11]	NM_002892	Nuclear	Binds to Rb and mSin3/HDAC
mSDS3 [11]	NM_022491	Nuclear	Member of mSin3/HDAC
BAF57	NM_003079	Nuclear	BRG associated, member of SWI/SNF
CCG1 (TAFII250)	NM004606	Nuclear	Transcription factor
NMI	NM_004688	Nuclear/cytoplasmic	Transcription factor
MRJ	NM_005494	Nuclear	Heat shock protein (Hsp40)
SMTN	NM_006932	Cytoplasmic	Actin cytoskeleton, stress fiber
KPNA5	NM_002269	Nuclear/cytoplasmic	Protein transport