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The BRCA1-binding protein BRAP2 can act as a cytoplasmic retention factor for nuclear and nuclear envelope-localizing testicular proteins

Rebecca G. Davies [a], Kylie M. Wagstaff [a], Eileen A. McLaughlin [c], Kate L. Loveland [a,b], David A. Jans [a,*]

[a] Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia
[b] Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria, Australia
[c] Priority Research Centre in Chemical Biology and Reproductive Science, University of Newcastle, Callaghan, NSW, Australia

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ABSTRACT

Regulation of nuclear protein import is central to many cellular processes such as development, with a key mechanism being factors that retain cargoes in the cytoplasm that normally localize in the nucleus. The breast cancer antigen BRCA1-binding protein BRAP2 has been reported as a novel negative regulator of nuclear import of various nuclear localization signal (NLS)-containing viral and cellular proteins, but although implicated in differentiation pathways and highly expressed in tissues including testis, the gamut of targets for BRAP2 action in a developmental context is unknown. As a first step towards defining the BRAP2 interactome, we performed a yeast-2-hybrid screen to identify binding partners of BRAP2 in human testis. Here we report characterization for the first time of three of these: the high mobility group (HMG)-box-domain-containing chromatin component HMG20A, nuclear mitotic apparatus protein NuMA1 and synaptic nuclear envelope protein SYNE2. Co-immunoprecipitation experiments indicate association of BRAP2 with HMG20A, NuMA1, and SYNE2 in testis, underlining the physiological relevance of the interactions, with immunohistochemistry showing that where BRAP2 is co-expressed with HMG20A and NuMA1, both are present in the cytoplasm, in contrast to their nuclear localization in other testicular cell types. Importantly, quantitative confocal microscopic analysis of cultured cells indicates that ectopic expression of BRAP2 inhibits nuclear localization of HMG20A and NuMA1, and prevents nuclear envelope accumulation of SYNE2, the first report of BRAP2 altering localization of a non-nuclear protein. These results imply for the first time that BRAP2 may have an important role in modulating subcellular localization during testicular development.

1. Introduction

Protein transport into and out of the nucleus through the nuclear pore complexes embedded in the nuclear envelope [1–5] is a highly regulated process that is central to many cellular processes, including development. Proteins greater than 40–60 kDa in size require a nuclear localization signal (NLS) in the cargo protein that is recognized by members of the importin superfamily of protein transporters [2,4], generally either the importin α/β heterodimer [2,5], or one of the many importin β homologues independent of importin α. Nuclear import can be tightly regulated through a number of mechanisms, including cytoplasmic retention, whereby cargo proteins are bound by cytoplasmic factors and as a result, are retained within the cytoplasm [4].

A cytoplasmic retention factor of interest in this context is the BRCA1-associated binding protein 2 (BRAP2; also known as Impedes Mitogenic Signal Propagation or Imp), first identified as a cytoplasmic protein recognizing the NLS of the breast cancer antigen BRCA1 [3]. Consistent with a role in cellular differentiation, BRAP2 appears to interact with the cyclin-dependent-kinase-inhibitor p21 during monocyte differentiation. Both nuclear and cytoplasmic forms of p21 appear to carry out distinct functions; nuclear p21 acts as a cell cycle brake, whereas cytoplasmic p21 has various roles, including protection against apoptosis, promotion of neurite growth in developing neurons, and facilitation of assembly and nuclear translocation of cyclin D/Cdk4 complexes [6]. BRAP2 has also been shown to act as a cytoplasmic retention factor for specific viral proteins such as the SV40-Large Tumor-Antigen (T-ag) and human cytomegalovirus processivity factor ppUL44 [1,7] in a phosphorylation-dependent fashion, as well as inhibiting nuclear transport of other cellular proteins such as p53 [1].

BRAP2 has more recently been implicated in a diverse range of signaling pathways, in part involving ubiquitinylation/neddylation. Through the E3 ubiquitin ligase activity of its RING domain, for example,

Abbreviations: NLS, nuclear localization signal; BRAP2/Imp, BRCA1 Associated Protein 2/Impedes Mitogenic Signal Propagation; BRCA1, Breast Cancer Type I Susceptibility Protein; HMG20A, High Mobility Group Protein 20A; NuMA1, Nuclear Mitotic Apparatus Protein 1; SYNE2, Synaptic Nuclear Envelope Protein 2; T-ag, Simian Virus 40 Large Tumor Antigen; CLSM, confocal laser scanning microscopy; NCBI, National Centre for Biotechnology Information

* Corresponding author at: Department of Biochemistry and Molecular Biology, Monash University, Wellington Road, Clayton, Victoria 3800, Australia. Tel.: +61 3 9902 9341.
E-mail address: david.jans@monash.edu (D.A. Jans).

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BRAP2 appears to facilitate Lys-63 linked ubiquitin modification of the Cell division cycle gene 14 (HsCdc14A) [8]; BRAP2 has also been shown to co-localize with HsCdc14A on mitotic spindle poles, suggesting a potential role for it in cell cycle regulation [8]. Alterations in BRAP2 have been observed in conditions such as myocardial infarction [9] and carotid atherosclerosis, where a buildup of lipid components within the arterial walls leads to nuclear factor-κB (NF-κB)-dependent recruitment of inflammatory cytokines. This appears to relate in part to BRAP2's ability to interact with both IκB (Inhibitor of κB) β and IκB Kinase (IKK) β, both of which are components of the IKK-signalosome, which is responsible for phosphorylation/degradation of IκB [10], the canonical cytoplasmic retention factor for NF-κB [11]. IκBβ sequesters NF-κB in the cytoplasm, while IKKβ stimulates Iκβ degradation in response to the secretion of inflammatory cytokines, to allow NF-κB to translocate to the nucleus; upregulation of BRAP2, dependent on interaction with IKK, can enhance NF-κB nuclear translocation [10]. BRAP2 has also been shown to interact with Cul1, a cullin family protein that undergoes the ubiquitin-like modification neddylation, and makes up part of the Skp, Cullin, F-box containing multi-protein E3 ubiquitin ligase SCF complex, responsible for mediating degradation of Iκβ [11]. Interestingly, BRAP2 possesses a consensus neddylation sequence similar to those possessed by cullin family proteins, and is able to bind Cul1 itself, in response to Tumor necrosis factor-α stimulation, to help suppress NF-κB nuclear translocation in response to cytokine stimulation [11]. This effect of BRAP2 on the NF-κB pathway is antagonistic to that which occurs as a result of its interaction with IKK (above), presumably a result of the complex interaction between BRAP2 and Cul1 [11]. Finally, BRAP2 is known to inhibit the ERK signal transduction pathway following lytic cycle activation post Epstein–Barr virus infection, by interacting with KSRI (kinase suppressor of Ras 1) [12], a scaffold protein responsible for mediating complexation between Raf, MEK (mitogen activated protein kinase) and ERK (extracellular signal-regulated kinase) [13]. BRAP2/Imp can also suppress interferon-γ secretion in response to T-cell receptor activation [14].

Interestingly, BRAP2 is expressed in the testis to a much higher extent than in other tissues [15], implying a potentially critical role in testicular germ cell development. Essentially nothing, however, is known of BRAP2's binding partners, or its functional role in the testis. Here we address this question for the first time, performing a yeast 2-hybrid screen using a human testis cDNA library to begin to define the BRAP2 interactome. Of a number of interacting partners identified, three are characterized in detail; the high mobility group (HMG)-box-domain-containing chromatin component HMG20A, the nuclear mitotic apparatus protein NuMA1 and synaptic nuclear envelope protein SYNE2. The results not only validate interaction with BRAP2 in testis, but implicate particular testicular germ cells, such as the pachytene spermatocytes, as a key site of BRAP2 action for the first time. Most importantly, BRAP2 is shown for the first time to be functional as a cytoplasmic retention factor for HMG20A, NuMA1 and SYNE2, and hence likely to play a critical developmental role in the testis.

2. Materials and methods

2.1. Yeast two-hybrid screen

Yeast 2-hybrid screening was performed by Hybrigenics Inc. (Paris, France). Briefly, amino acids 343–592 of human BRAP2 were cloned into vector pB27 (derived from pBTM116) to encode the C-terminal LexA fusion protein, N-LexA-BRAP-C, which was used as a bait to screen a cDNA library from human testes, constructed in plasmid p6 (derived from plasmid pGADGH) [16]. A mating strategy [17] was used to screen 146 million clones, using yeast strains Y187 (matα) and L40ΔGal4 (mata) and 179 HIS positive colonies were selected from a medium lacking tryptophan, leucine and histidine, but substituted with 5 mM 3-aminotriazole to prevent bait autoactivation. Prey fragments of the positive clones were amplified at their 3′ and 5′ ends and the sequences produced from PCR were used to identify interacting proteins from the GenBank™ database (NCBI). A confidence score (PBS, Predicted Biological Score) was allocated to each interaction, to enable interacting clones to be prioritized.

2.2. Expression plasmid construction

GFP-fused BRAP2 (encoding amino acids 2–592 and 343–592) and coilin (amino acids encoding 2–112) expression vectors were constructed using the Gateway system (Invitrogen, Carlsbad, CA, USA) [1], in the plasmid pDONR207 or pDONR222 expression vectors respectively. LR recombination reactions were subsequently performed using the Gateway-compatible destination vector pEPI-RfC [18] to generate GFP-fusion protein-encoding constructs for mammalian cell expression, as previously described [19]. An additional construct was generated by PCR amplification of the region encoding BRAP2(343–592) and cloned into the expression vector pHM830 [20] between the *Afl*II and *Age*I restriction endonuclease sites to encode the fusion protein GFP-BRAP2(343–592)-β-galactosidase. The integrity of all constructs was verified by DNA sequencing.

2.3. Cell culture

Cells of the COS-7 African green monkey kidney or HeLa human cervical cancer lines were maintained in DMEM containing 10% fetal calf serum in a 5% CO_2 humidified incubator at 37 °C [1].

2.4. Transfection/immunofluorescence

Cells seeded onto glass coverslips were transfected using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's specifications. 16 or 40 h post transfection, cells were fixed [1] and incubated in anti-SV40 T-ag (Santa Cruz, 1:750) or anti-SYNE2 (Sapphire Bioscience, 1:100) monoclonal antibodies, or anti-HMG20A (ProteinTech Group, 1:100) or anti-NuMA1 (Abcam, 1:100) polyclonal antibodies, followed by incubation with Alexa 568-labeled goat anti-rabbit secondary antibody or Alexa 568-labeled rabbit anti-mouse secondary antibody (Invitrogen, 1:1000). Coverslips were mounted onto slides using ProLong® Gold Anti-Fade Reagent (Invitrogen), containing the DNA-specific dye 4,6-diamidino-2-phenylindole (DAPI) or 4% propyl gallate, as appropriate.

2.5. CLSM and image analysis

Cells immunostained for endogenously expressed proteins or transfected to express GFP-fusion proteins were imaged on a Nikon C1 inverted microscope as previously described [1] using a Nikon 100× oil immersion lens. The nuclear to cytoplasmic fluorescence ratio (Fn/c) was determined as previously described [1] from digitized images using the ImageJ 1.43r public domain software (NIH), statistical analysis performed using a 2-tailed unpaired *t*-test and the GraphPad Prism 5.0c software.

2.6. Co-immunoprecipitation/Western analysis

All studies that were carried out complied with the NHMRC Code of Practice for the Care and Use of Animals for Experimental Purposes, and were confirmed by the Monash University Standing Committee on Ethics in Animal Experimentation. Wild type adult mouse testes from inbred mice (C57/BL6-Jx129SV) were obtained from Monash University Central Animal Services. The animals were killed by cervical dislocation prior to dissection and decapsulation of the testes. Following PBS washes, testes were homogenized using RIPA buffer (150 mM sodium chloride, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8) containing a protease inhibitor cocktail (Roche). Cellular debris was removed by centrifugation at 20,000 ×*g*

for 45 min at 4 °C. Protein concentration of the cleared lysate was determined by Bradford assay (Biorad). Co-immunoprecipitation was performed using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Upstate Cell Signalling Solutions) according to the manufacturer's instructions, using 4 μg of anti-HMG20A, anti-NuMA1, anti-SYNE2, anti-BRAP2 (Sigma), anti-$(HIS)_6$ (BD Pharmingen) or anti-GFP (Roche) antibodies. Following overnight incubation, proteins were eluted from the beads with 70 μl or 50 μl, as appropriate, 1×, 2× and 4× non-denaturing buffer and 1× denaturing buffer, respectively, and subjected to SDS-polyacrylamide gel electrophoresis (12% gel, with 8% for NuMA1).

GFP-fusion proteins were immunoprecipitated from HEK293T cell lysates using the Protein A/G PLUS-Agarose immunoprecipitation reagent (Santa Cruz Biotechnology) as previously described [1]; 30 μl of the eluate was subjected to SDS-polyacrylamide gel electrophoresis (12% gel).

After electrophoresis, proteins were transferred to Polyvinylidene Fluoride (PVDF) membranes (PALL Corporation) preactivated in isopropanol (Merck) and Western blotting carried out as previously described [1]. Briefly, the membrane was blocked in 5% skim milk powder in PBS/0.05% Tween 20 (Amresco) and incubated for 1 or 2 h with rabbit primary anti-BRAP2 (1:1000), anti-NuMA1 (1:1000 or 1:100, as appropriate), anti-HMG20A (1:1000 or 1:200, as appropriate) or anti-SYNE2 (Sigma, 1:300) antibodies. Subsequently, blots were incubated in HRP-coupled goat anti-rabbit secondary antibody (Millipore, 1:10,000), for 1 h and detected using enhanced chemiluminescence (ECL) (Perkin Elmer) according to the manufacturer's instructions.

2.7. Lysate preparation/Western analysis

Adult rat testis lysates were prepared from 60 to 90 day-old Sprague Dawley outbred rats, while adult mouse testis lysate was prepared from C57 black mice, as per Section 2.6. Isolated rodent spermatogonia, round spermatids, and pachytene spermatocytes were extracted and isolated as previously described [21].

Lysates (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis (8% gel) and Western transfer, blocking, incubation with antibodies and ECL detection performed essentially as above (Section 2.6).

2.8. Immunohistochemistry

Immunohistochemistry on Bouins-fixed paraffin-embedded day 15 and 30 and adult mouse testes from Asmu:Swiss mice was performed as previously described [22]. The tissues were incubated with anti-BRAP2 (1:500), anti-HMG20A (1:100) or anti-NuMA1 (1:100) antibodies made up in TB, overnight in a humid chamber at 4 °C, and subsequently incubated with a biotin-conjugated sheep anti-rabbit antibody (Millipore, 1:500) at room temperature. Following incubation with Vectastain® (Vector Laboratories), washing, treatment with 3,3′-diaminobenzidine/activation with H_2O_2, and counterstaining with Harris' Hematoxylin (BioRad), slides were mounted onto coverslips using DPX (di-n-butyl-phthalate in xylene) mounting solution (Sigma). Samples were imaged using a bright field microscope (Provis) with either a 40 or 100× oil immersion lens.

3. Results

3.1. Identification of testicular binding partners of BRAP2

Although BRAP2 is highly expressed in testis, there is no information as to its binding partners in the testis/potential targets for regulation of nuclear localization. To address this, a human testis cDNA library was screened using human BRAP2 in the yeast 2-hybrid system. Of a number of high confidence potential binding partners with 7–30 individual clones identified for each, three were selected for further investigation: the HMG-box-domain-containing chromatin component HMG20A [23], NuMA1, a structural nuclear protein expressed during interphase that is thought to function in linking microtubules to the spindle poles during mitosis, and the nuclear envelope protein SYNE2, which is believed to play a role in linking the nucleus and cytoskeleton [24].

3.2. BRAP2 interacts with HMG20A, NuMA1 and SYNE2 in mouse testis

To confirm interaction, co-immunoprecipitation was performed from lysates from adult mouse testis using specific antibodies to HMG20A, NuMA1, SYNE2 or BRAP2, with anti-$(HIS)_6$ or anti-GFP antibodies as controls. Western analysis was used to detect co-immunoprecipitated protein and its binding partners (Fig. 1). Endogenous BRAP2 co-immunoprecipitated with the anti-HMG20A, -NuMA1 and -SYNE2 antibodies, but failed to be pulled down using the anti-$(HIS)_6$ antibody (Fig. 1A), indicating that BRAP2 is indeed found in complexes with these proteins in adult mouse testis. The co-immunoprecipitations were performed using anti-BRAP2 antibodies, endogenous SYNE2 and NuMA1 being able to be co-immunoprecipitated with the anti-BRAP2 antibody but not control antibodies (anti-$(HIS)_6$ or anti-GFP antibodies) respectively (Fig. 1B). Detection of HMG20A in anti-BRAP2 immunoprecipitates did not prove possible for technical reasons, but immunoprecipitation using anti-GFP antibody after ectopic expression of the fusion protein GFP-BRAP2(343–592) in HEK293T cells enabled detection of endogenous HMG20A, in contrast to cells expressing GFP alone as a control (Fig. 1C), thus confirming the interaction. Clearly, BRAP2 is able to interact with HMG20A, NuMA1 and SYNE2.

3.3. HMG20A and NuMA1 are cytoplasmic in mouse testicular cell types expressing BRAP2

mRNA expression data from a mouse testis age series for BRAP2 and its three binding partners indicate dynamic changes in expression levels (Fig. 2A); BRAP2 increases in expression from c. day 14/16, whereas HMG20A, NuMA1 and SYNE2 all show decreasing expression post day 14/16. Western analysis for BRAP2 protein levels confirmed this, with higher expression evident in adult (mouse and rat) testis lysates and isolated pachytene spermatocytes and round spermatids, compared to spermatogonia (see Fig. 2B). Immunohistochemistry to assess protein localization *in situ* was compared in testis sections from day 15 and day 30 as well as adult mice [15], revealing BRAP2 to be present in the cytoplasm of the pachytene spermatocytes in all samples, but largely absent from the earlier cell types (Fig. 2C). HMG20A was present in the nuclei of some of the early spermatogonia in 15-day- and 30-day-old mouse testes, but was predominantly in the cytoplasm of the pachytene spermatocytes from both day 15 and day 30 mice (Fig. 2D). Similarly, NuMA1 was present in the cytoplasm of the pachytene spermatocytes in 15-day-old mouse testis and in the nuclei of some of the early spermatogonia and the nuclei and cytoplasm of the round spermatids in day 30 mouse testis (Fig. 2D).

The results indicate that in the germ cell types where BRAP2 appears to be expressed to reasonable levels, its interactors HMG20A and NuMA1 are cytoplasmic, in contrast to their predominantly nuclear localization in the earlier germ cell types where BRAP2 expression is low. The clear implication is that interaction of BRAP2 with HMG20A and NuMA1 in the testis (see Fig. 1) has functional consequences, resulting in cytoplasmic retention of HMG20A and NuMA1 to prevent their nuclear action in the later stages of spermatogenesis.

3.4. Ectopically expressed BRAP2 can act as a cytoplasmic retention factor for HMG20A and NuMA1

To confirm the ability of BRAP2 to have an effect of cytoplasmic retention on HMG20A and NuMA1 implied by the results above, the effect of BRAP2 on subcellular localization of endogenous HMG20A

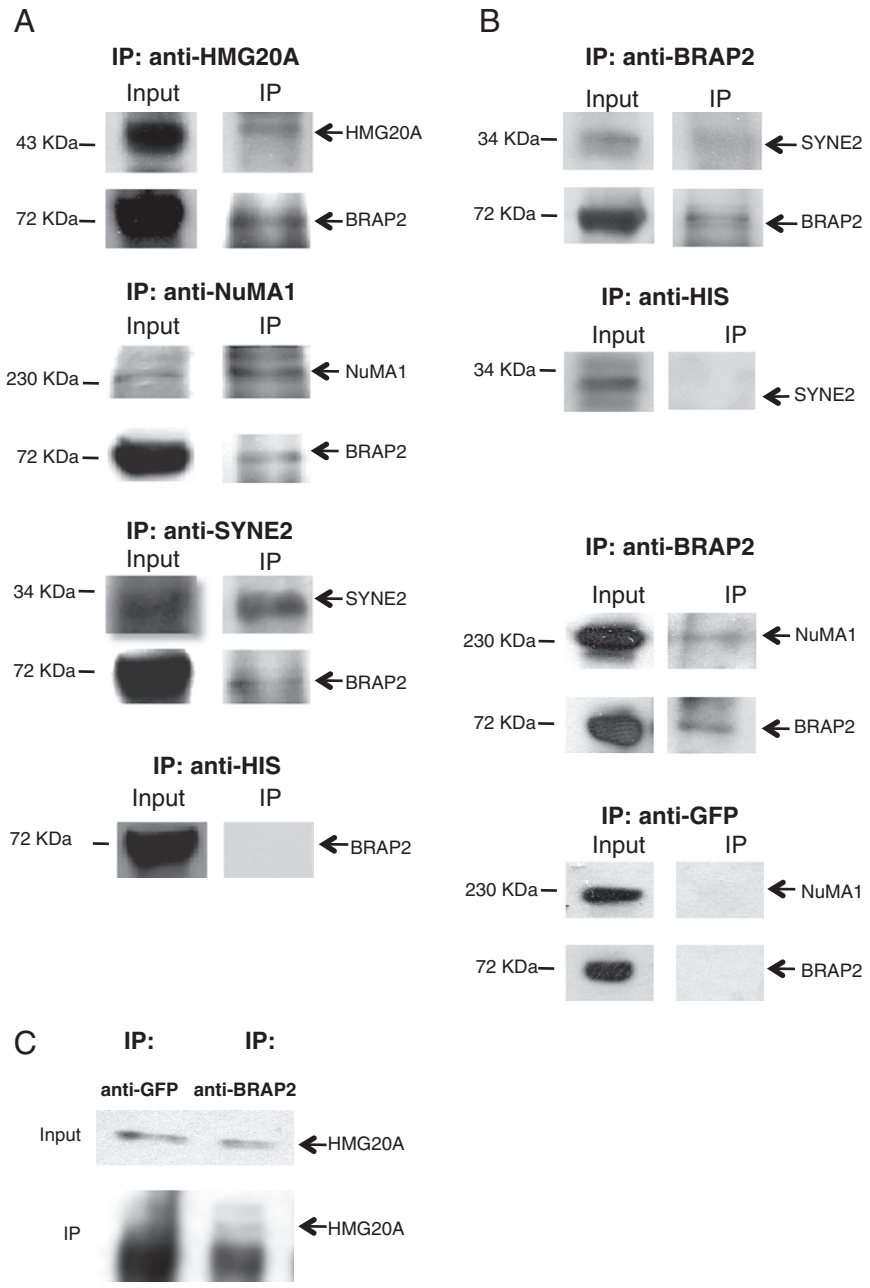
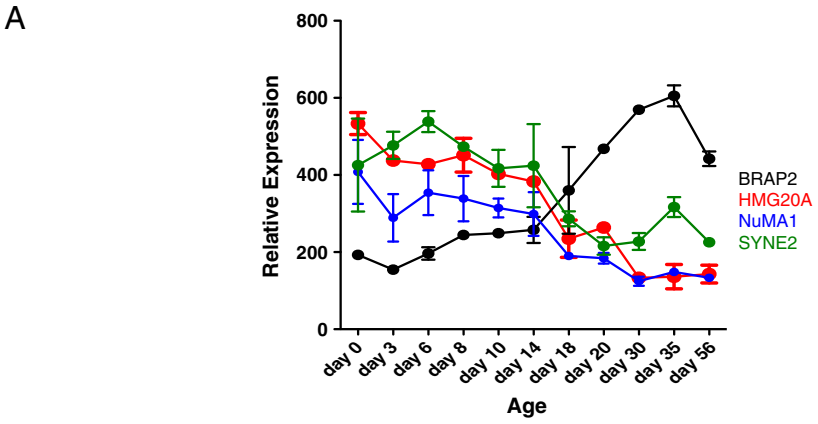


Fig. 1. BRAP2 interacts with HMG20A, NuMA1 and SYNE2 in adult mouse testis lysate. A. Co-immunoprecipitation (IP) was performed using lysates from wild type C57 black adult mouse testes using the Catch and Release® v2.0 Reversible Immunoprecipitation System. Endogenous BRAP2 was co-immunoprecipitated using anti-HMG20A, anti-NuMA1 and anti-SYNE2 antibodies, but failed to co-immunoprecipitate using an anti-$(HIS)_6$ control antibody (top left). B. Endogenous SYNE2 was co-immunoprecipitated using an anti-BRAP2 antibody, but failed to co-immunoprecipitate using an anti-$(HIS)_6$ antibody, while NuMA1 was co-immunoprecipitated using an anti-BRAP2 antibody, but failed to co-immunoprecipitate using an anti-GFP control antibody (right). C. HMG20A was co-immunoprecipitated from HEK293T cell lysates transfected to express GFP-BRAP2(343–592), but failed to co-immunoprecipitate with HEK293T cell lysates transfected with GFP alone (bottom left).

and NuMA1, with T-ag as a positive control, was assessed. GFP fused to BRAP2 residues 343–592, previously shown to be the key functional domain for BRAP2 cytoplasmic retention activity [1], was ectopically expressed in COS-7 or HeLa cells, and its effects compared to those of GFP alone. Briefly, cells were immunostained 16 h post-transfection using specific antibodies to the respective proteins and Alexa 568-coupled secondary antibodies, were imaged using CLSM (Fig. 3B/D, respectively). HMG20A resembled T-ag in showing strong nuclear staining in the absence of BRAP2 in COS-7 cells, with increased cytoplasmic staining evident in the presence of GFP-BRAP2(343–592), but not GFP alone. Quantitative analysis confirmed these results, whereby determination of the nuclear to cytoplasmic fluorescence ratio (Fn/c) revealed that nuclear accumulation of the control T-ag was significantly ($p < 0.0001$; Fig. 3B/C) c. 25–30% reduced in the presence of GFP-BRAP2(343–592), consistent with previous results [1]; GFP alone had no significant effect, confirming the specificity of the effects with respect to BRAP2. That this was a specific effect was indicated by the fact that GFP fused to the coiled-coil domain (residues 2–112) of the Cajal body component coilin [25] did not inhibit T-ag nuclear localization (Supp. Fig. 1) in contrast to GFP fused to BRAP2; the clear implication is that the BRAP2 coiled-coil domain confers specific binding/function in modulating nuclear transport, in contrast to the coiled-coil domain of coilin that presumably has more of a structural role.

Importantly, HMG20A showed results similar to those for T-ag, with significantly ($p < 0.0001$) almost 50% reduced nuclear accumulation/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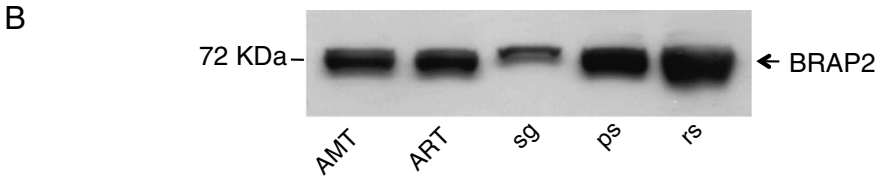
BRAP2
HMG20A
NuMA1
SYNE2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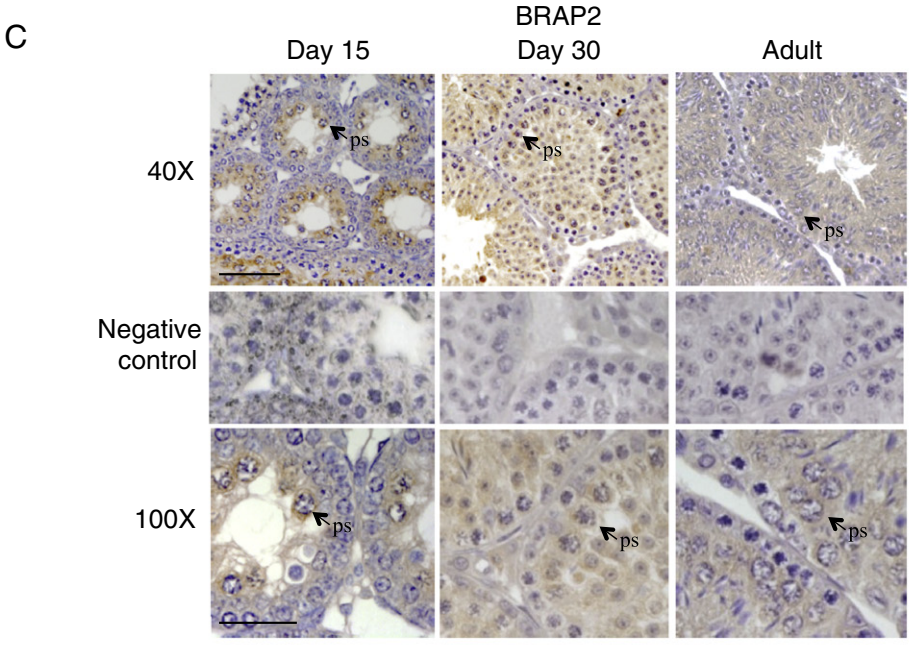
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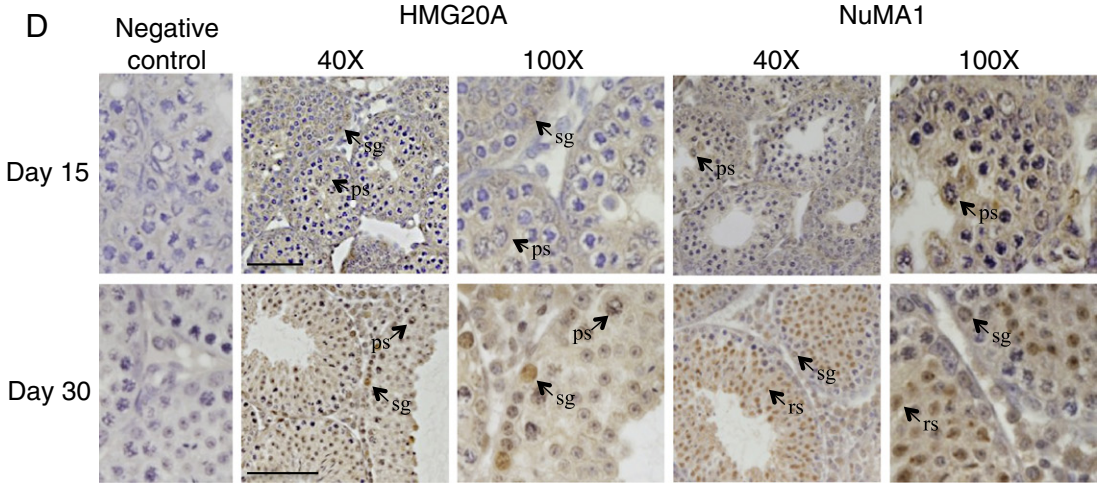
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increased cytoplasmic staining in the presence of GFP-BRAP2(343–592) but not in the presence of GFP alone (Fig. 3D/E), strongly implying that BRAP2 can act as a cytoplasmic retention factor for HMG20A in intact cells to inhibit its nuclear import. Comparable experiments for NuMA1 (Fig. 3F), revealed reduced nuclear accumulation/increased cytoplasmic localization of endogenous NuMA1 in the presence of GFP-BRAP2(343–592), with quantitative analysis (Fig. 3G) confirming significant ($p < 0.0001$) almost 50% reduced nuclear accumulation. These results clearly support the idea that like HMG20A, NuMA1 is a target of the cytoplasmic retention activity of BRAP2.

3.5. BRAP2 is a cytoplasmic retention factor for SYNE2 in COS-7 cells

Subcellular localization of SYNE2 was similarly assessed, in HeLa (data not shown) and COS-7 cells transfected to express the indicated GFP-BRAP2 fusion proteins or GFP alone at 16 (Fig. 4) or 40 h (not shown) post-transfection. In the absence of overexpressed BRAP2/in the presence of overexpressed GFP alone, SYNE2 showed distinctive nuclear rim staining (indicated by the white arrows) together with diffuse cytoplasmic staining and cytoplasmic aggregates. In the presence of GFP-BRAP2(2–592) and GFP-BRAP2(343–592), in contrast, localization of SYNE2 at the nuclear envelope was largely absent (indicated by white arrow heads), with a corresponding increase in diffuse cytoplasmic staining. These results suggest that, as for HMG20A and NuMA1, SYNE2 subcellular localization can be modulated by BRAP2's cytoplasmic retention activity.

4. Discussion

This is the first study to indicate a role for the cytoplasmic retention factor BRAP2 in the mammalian testis, importantly documenting its ability to alter subcellular localization of the nuclear proteins HMG20A and NuMA1. We also show for the first time that BRAP2 can modulate localization of the nuclear envelope protein SYNE2, implying that BRAP2 has a role in modulating subcellular localization that is not restricted to nuclear transport.

4.1. BRAP2 binds HMG20A, NuMA1 and SYNE2 in adult mouse testis

HMG20A, NuMA1 and SYNE2 are identified and validated here for the first time as binding partners of BRAP2 in the mammalian testis. HMG20A belongs to the HMG box class of proteins, which encode a DNA-binding domain that is involved in the regulation of transcription and translation and plays a role in maintaining chromatin conformation [26]. HMG20A is ubiquitously expressed in many tissues and may act as a non-histone component of chromatin or interact with tissue-specific transcription factors [23]. By inhibiting nuclear accumulation of HMG20A, BRAP2 may therefore be acting as an additional layer of transcriptional control. NuMA1 is a nuclear protein expressed during interphase, but is also abundant at the spindle poles of cells undergoing mitosis [27], where it forms complexes with dynein and dynactin [28] to help link microtubules to the spindle poles [29]; whether BRAP2 action plays a direct role in modulating spindle pole stability is unclear, but an intriguing idea in this context is that modulation of NuMA1 nuclear localization in interphase may contribute to the binding of NuMA1 to microtubules in the cytoplasm, which may subsequently be critical at the onset of mitosis. SYNE2 belongs to the nesprin family of proteins, which are characterized by the presence of multiple spectrin repeats, a bipartite NLS motif and a conserved transmembrane domain [30], which is important for nuclear envelope localization. SYNE2 also associates with F-actin through actin-binding sites [31], thereby acting to maintain the structural integrity of the nucleus [24]. By regulating the nuclear/nuclear envelope localization of SYNE2, BRAP2 may modulate nuclear integrity, which may be important during the complex rearrangements in chromatin and nuclear structure that occur in the later stages of spermatogenesis.

4.2. BRAP2 co-expression with HMG20A and NuMA1 in mouse testis results in cytoplasmic localization

The immunohistochemical data here clearly indicates that BRAP2 protein is present within the cytoplasm of the pachytene spermatocytes in adult mouse testis, consistent with public domain affymetrix data shown in Fig. 2A. Both HMG20A and NuMA1, normally nuclear proteins, were found to be present within the cytoplasm of the pachytene spermatocytes, suggesting an effect of BRAP2 expression on the localization of both proteins in this cell type, consistent with BRAP2 acting as a cytoplasmic retention protein in the testis. Consistent with this idea, HMG20A and NuMA1 are localized within the nucleus in cell types such as the early spermatogonia, where BRAP2 is expressed at low levels.

4.3. BRAP2 can act as a cytoplasmic retention protein for HMG20A, NuMA1 and SYNE2

In the case of HMG20A and NuMA1, BRAP2 interaction inhibits nuclear accumulation, as has been observed for viral proteins such as T-ag [1,2], but BRAP2 inhibition of nuclear envelope localization of SYNE2, represents the first time BRAP2 cytoplasmic retention activity has been shown to affect subcellular trafficking other than that of nuclear import. That the results are physiologically relevant is indicated by the fact that BRAP2, which is clearly complexed with HMG20A, NuMA1 and SYNE2 in the testis, is expressed in some of the same germ cell types (pachytene spermatocytes) and that in this cell type, the proteins in question are predominantly cytoplasmic, consistent with BRAP2's apparent cytoplasmic retention role. The clear implication is that BRAP2 may play a key role in the later stages of spermatogenesis through modulating subcellular localization of key nuclear/nuclear envelope proteins.

Of interest is the fact that the three interactors of BRAP2 characterized here for the first time, as well as BRAP2 itself, possess coiled-coil domains, implying that BRAP2-binding partner interaction may be directly dependent on coiled-coil sequences; specificity of the coiled coil interaction between BRAP2 and its binding partners is implied by the data in Supp. Fig. 1. Importantly, although association of endogenous BRAP2 with NuMA1, SYNE2 and HMG20A is clearly evident from our co-immunoprecipitation experiments, including in rodent testis, formal demonstration that binding between BRAP2 and its cargoes is direct remains to be established.

Fig. 2. Expression of BRAP2 in the same testicular germ cell types as HMG20A and NuMA1 results in cytoplasmic localization. A. Plot of affymetrix data (GEO dataset GDS605) for relative levels of BRAP2, HMG20A, NuMA1 and SYNE2 mRNA expression throughout spermatogenesis in testes from mice aged 0–56 days as indicated. B. Western analysis for BRAP2 of adult mouse/rat testis lysates, and isolated mouse testis cell types; from left to right: AMT, adult mouse testis lysate; ART, adult rat testis lysate; sg, mouse spermatogonia; ps, rat pachytene spermatocytes; rs, mouse round spermatids. C. Immunostaining for BRAP2 in Bouins-fixed, paraffin-embedded mouse testis sections from Swiss mice. Visualization using 40× and 100× objectives indicates BRAP2 expression in the cytoplasm of the pachytene spermatocytes in 15-day-old, 30-day-old and adult mouse testis sections. Panels in between 40× and 100× sections immunostained for BRAP2, represent negative controls of the relevant sections. Scale bar on 40× objective images is equivalent to 100 μm and on 100× objective images is equivalent to 50 μm. D. Immunostaining for HMG20A and NuMA1 as per C. HMG20A expression is evident in the nuclei of the early spermatogonia and the cytoplasm of the pachytene spermatocytes, in both 15-day-old and 30-day-old mouse testis sections. NuMA1 expression can be seen in the cytoplasm of the pachytene spermatocytes in 15-day-old mouse testis sections and the nuclei of the early spermatogonia and nuclei and cytoplasm of the round spermatids in 30-day-old mouse testis sections. Panels to the left of sections immunostained for HMG20A, represent negative controls of the relevant sections. Scale bars as per C.

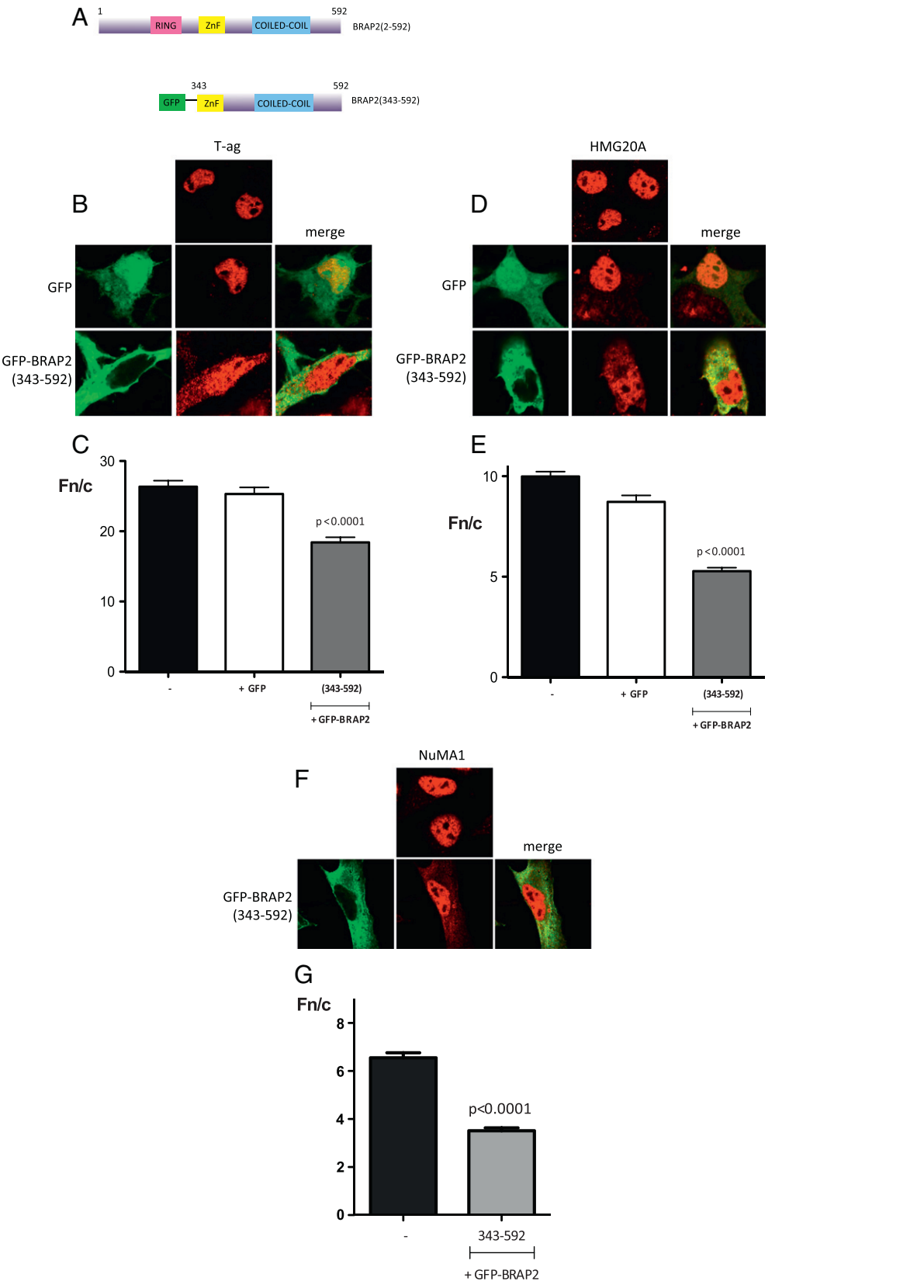

Fig. 3. BRAP2 can act as a negative regulator of nuclear import of HMG20A and NuMA1. A. Schematic diagram for the domain structure of BRAP2 and the GFP-BRAP2 fusion construct used. B. COS-7 cells were transfected to express GFP-BRAP2 or GFP alone as indicated (left panels) and fixed and stained for endogenous T-ag (middle panels). C. Digitized images such as those in B were analyzed to determine the nuclear to cytoplasmic fluorescence ratio of the endogenous T-ag protein in the absence (−) or presence of the indicated GFP-fusion protein. Results are for the nuclear to cytoplasmic ratio (Fn/c: mean +/− SEM, n = 50). p Values are indicated. D. As for B for CLSM images of COS-7 cells stained with a HMG20A antibody in the absence or presence of ectopically expressed GFP-BRAP2 or GFP alone. E. Analysis of digitized images such as those in D to determine the nuclear to cytoplasmic fluorescence ratio of the endogenous HMG20A protein as per C. F. As for B and D for CLSM images of HeLa cells stained with a NuMA1 antibody in the presence of ectopically expressed GFP-BRAP2. G. Analysis of digitized images such as those in F to determine the nuclear to cytoplasmic fluorescence ratio of the endogenous NuMA1 protein as per C. ZnF; Zinc finger, RING; ring finger.

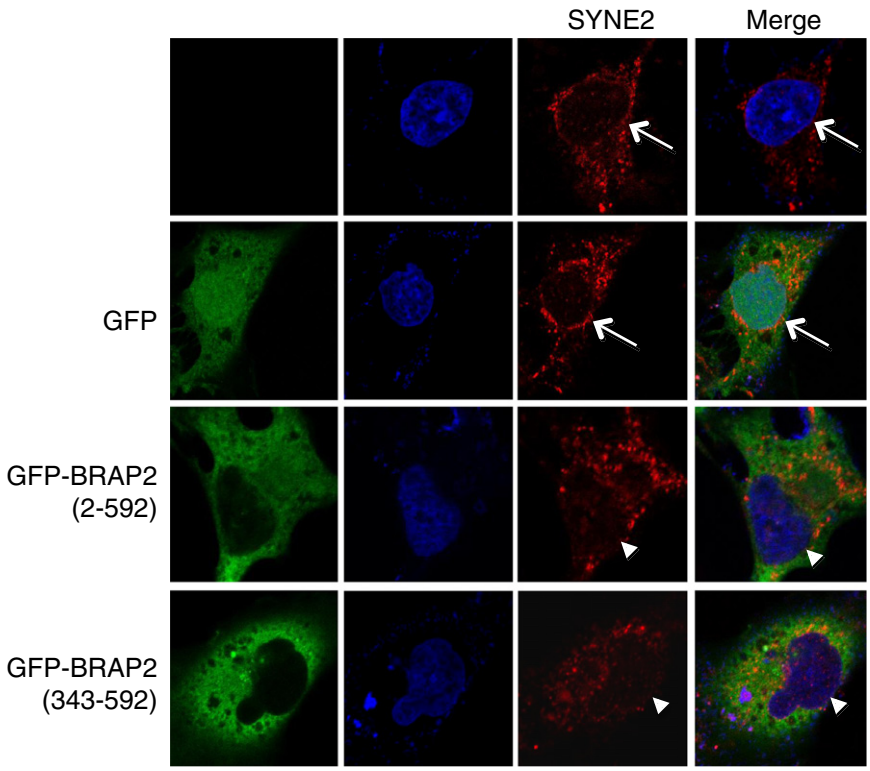

Fig. 4. BRAP2 can reduce nuclear envelope localization of SYNE2. COS-7 cells were transfected to express the indicated GFP-fusion proteins (left panels) and fixed and stained for endogenous SYNE2 (middle right panels). Arrows indicate nuclear rim staining and arrowheads indicate lack of nuclear rim staining as a result of BRAP2 over-expression.

5. Conclusion

In summary, this study implicates BRAP2 as playing an important role in the testis in modulating subcellular localization of a range of testicular proteins, which have roles in the nucleus and/or the nuclear envelope. Further characterization of its novel binding partners such as those identified and validated here, will help establish BRAP2's potential key role as a regulator of important cellular processes such as spermatogenesis and other developmental processes [1].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.05.015.

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