

# Specific interaction with the nuclear transporter importin $\alpha 2$ can modulate paraspeckle protein 1 delivery to nuclear paraspeckles

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**ABSTRACT** Importin (IMP) superfamily members mediate regulated nucleocytoplasmic transport, which is central to key cellular processes. Although individual IMP $\alpha$  proteins exhibit dynamic synthesis and subcellular localization during cellular differentiation, including during spermatogenesis, little is known of how this affects cell fate. To investigate how IMP $\alpha$ s control cellular development, we conducted a yeast two-hybrid screen for IMP $\alpha 2$  cargoes in embryonic day 12.5 mouse testis, a site of peak IMP $\alpha 2$  expression coincident with germ-line masculinization. We identified paraspeckle protein 1 (PSPC1), the original defining component of nuclear paraspeckles, as an IMP $\alpha 2$ -binding partner. PSPC1-IMP $\alpha 2$  binding in testis was confirmed in immunoprecipitations and pull downs, and an enzyme-linked immunosorbent assay-based assay demonstrated direct, high-affinity PSPC1 binding to either IMP $\alpha 2$ /IMP $\beta 1$  or IMP $\alpha 6$ /IMP $\beta 1$ . Coexpression of full-length PSPC1 and IMP $\alpha 2$  in HeLa cells yielded increased PSPC1 localization in nuclear paraspeckles. High-throughput image analysis of >3500 cells indicated IMP $\alpha 2$  levels can directly determine PSPC1-positive nuclear speckle numbers and size; a transport-deficient IMP $\alpha 2$  isoform or small interfering RNA knockdown of IMP $\alpha 2$  each reduced endogenous PSPC1 accumulation in speckles. This first validation of an IMP $\alpha 2$  nuclear import cargo in fetal testis provides novel evidence that PSPC1 delivery to paraspeckles, and consequently paraspeckle function, may be controlled by modulated synthesis of specific IMPs.

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Abbreviations used: ADAR, adenosine deaminases acting on RNA; BSA, bovine serum albumin; CI, confidence interval; co-IP, coimmunoprecipitation; DAPI, 4',6-diamidino-2-phenylindole; DBHS, *Drosophila* behavior, human splicing; dpp, days postpartum; DTT, dithiothreitol; E, embryonic day; ELISA, enzyme-linked immunosorbent assay; ESC, embryonic stem cell; GEE, generalized estimating equation; GFP, green fluorescent protein; GM, geometric mean; GS4B, glutathione

Sepharose 4B slurry; GST, glutathione S-transferase; IMP, importin; mAb, monoclonal antibody; MMI, Monash Micro Imaging; NEAT1, nuclear-enriched abundant transcript 1; NLS, nuclear localization signal; NONO, non POU domain-containing, octamer-binding protein; NOPS, NONA/paraspeckle; NPC, nuclear pore complex; O/N, overnight; PFA, paraformaldehyde; PIC, protease inhibitor cocktail; PSF, splicing factor proline/glutamine rich; PSPC1, paraspeckle protein 1; RRM, RNA recognition motifs; RT, room temperature; SCRAM, scrambled; siRNA, small interfering RNA; TBS, Tris-buffered saline; Y2H, yeast two-hybrid.

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## INTRODUCTION

Cellular differentiation processes, including spermatogenesis, are mediated by an ordered series of gene expression and cell cycle events (reviewed in Eddy, 2002; Eddy and O'Brien, 1998) that require regulated trafficking of specific proteins between the nucleus and cytoplasm (Hogarth *et al.*, 2005; Major *et al.*, 2011). Passage between these two compartments occurs only through the nuclear envelope-localized nuclear pore complexes (NPCs), which permit active transport of cargo proteins >45 kDa by a group of transport proteins known as the importins (IMPs) and exportins (Macara, 2001; Major *et al.*, 2011).

IMP $\alpha$  proteins are adaptor molecules in the classical nuclear import pathway, linking a cargo protein bearing a nuclear localization sequence (NLS) to an importin  $\beta$  1 (IMP $\beta$ 1) molecule (Macara, 2001). IMP $\beta$ 1 facilitates movement of this trimeric complex into the nucleus through its transient interactions with the nucleoporins lining the NPC. There are six known IMP $\alpha$  proteins in mice; these are categorized into three groups based on their structural similarities, with most known to have binding specificity for different cargoes (Miyamoto *et al.*, 1997; Nigg, 1997; Tsuji *et al.*, 1997; Jans *et al.*, 2000; Goldfarb *et al.*, 2004; Mason *et al.*, 2009; Kelley *et al.*, 2010). Here we employ the mouse nomenclature for naming IMP $\alpha$ s, with IMP $\alpha$ 2 the product of the *Kpna2* gene (Major *et al.*, 2011). Using embryonic stem cells (ESCs) as a model differentiation system, IMP $\alpha$  subtype switching was previously shown to drive major differentiation outcomes through its impact on the nuclear import of specific transcription factor subsets (Yasuhara *et al.*, 2007; Young *et al.*, 2011). IMP $\alpha$ 2 is essential for maintenance of ESCs in an undifferentiated state (Yasuhara *et al.*, 2007) through its capacity to restrict the nuclear import of key transcription factors, such as OCT6, which drive differentiation into neural lineages (Yasuhara *et al.*, 2013). Accumulating evidence that regulated synthesis of IMP $\alpha$  proteins is also of fundamental importance to spermatogenesis includes the identification of distinct cargoes bound to individual IMP $\alpha$ s in progressively maturing male germ cells of the mammalian testis (Miyamoto *et al.*, 2013; Arjomand *et al.*, 2014).

IMP $\alpha$ 2 is required for germ cell differentiation in *Drosophila melanogaster* (Miyamoto *et al.*, 2012). Mutation of *IMP $\alpha$ 2* in *Drosophila* causes a block in transport between nurse cells and the developing oocyte that results in female sterility (Gorjanacz *et al.*, 2002), and IMP $\alpha$ 2 is expressed in male mitotic and meiotic germ cells (Giarre *et al.*, 2002). Despite recent establishment of genetically modified mouse lines targeting components of the nucleocytoplasmic transport system (Miyamoto *et al.*, 2012), none is reported for *IMP $\alpha$ 2* at present. The IMP $\alpha$ 2 expression profile during postnatal murine testis development is distinct from that of other IMP $\alpha$  mRNAs and proteins (Hogarth *et al.*, 2006, 2007). Two peaks are apparent when *IMP $\alpha$ 2* mRNA levels are analyzed across a developmental murine testis time course, one at the onset of gonad differentiation, embryonic day (E) 12.5, and the second in the adult testis (Shima *et al.*, 2004; Small *et al.*, 2005; Major *et al.*, 2011). These peaks coincide with important differentiation transitions in the male germ line, and identification of IMP $\alpha$  cargoes is predicted to reveal key control elements required for spermatogenesis.

The mammalian testis provides an excellent model in which to explore the relationship between selective cargo protein binding to IMPs and cellular differentiation outcomes. In mice, the bipotential primordial germ cells complete their migration into the urogenital ridge by E11.5, where their cell cycle and differentiation fate is determined by the surrounding somatic environment (Adams and McLaren, 2002; Bowles *et al.*, 2006; Koubova *et al.*, 2006). Embryonic testicular germ cells, termed "gonocytes," proliferate until

approximately E14.5, become quiescent, and then reenter the mitotic cell cycle shortly after birth. This last transition marks the beginning of spermatogenesis, a complex process involving three key stages: 1) mitotic divisions of spermatogonial stem cells and spermatogonia, 2) meiotic division of spermatocytes, and 3) morphological changes that transform haploid, round spermatids into the elongated, motile mature spermatozoa that deliver the male's genetic material to the oocyte (Kerr *et al.*, 2006).

IMP $\alpha$ 2 was used in our previous yeast two-hybrid (Y2H) analysis of the adult mouse testis, which identified chromatin remodeling factors in haploid spermatids as IMP $\alpha$ 2 cargo (Ly-Huynh *et al.*, 2011). Focus on the peak in *IMP $\alpha$ 2* mRNA level at E12.5 in the present study was chosen, as it corresponds to the time when the sexually indifferent gonad is first masculinized. This process is critical for establishment of the secondary sexual features by which an individual's gender is identified at birth, and its disturbance is considered to underlie testicular dysgenesis syndrome and an increased risk of testicular germ cell cancer developing in young adults (Looijenga *et al.*, 2011). The expanding interest in IMP $\alpha$ 2 as a biomarker for cancer reflects its strong expression, particularly within the nucleus, in many tumor types (Christiansen and Dyrskjot, 2013). Our screen has identified the first five candidate IMP $\alpha$ 2 cargoes in the fetal testis. Among these, paraspeckle protein 1 (PSPC1) was selected for further analysis due to its previous characterization as an integral component of nuclear paraspeckles and its detection within germ cells of the adult testis in which IMP $\alpha$ 2 is also present (Fox *et al.*, 2002; Kuwahara *et al.*, 2006).

Paraspeckles are currently understood to arise from the assembly of PSPC1 and at least two other core *Drosophila* behavior, human splicing (DBHS) proteins: PSF (splicing factor proline/glutamine rich [also named SFPO and REP1]) and NONO (the non POU domain-containing, octamer-binding protein [also named nonA, NRB54, and P54NRB]) (Fox *et al.*, 2002, 2005) around the specific long noncoding RNA transcript, nuclear-enriched abundant transcript 1 (NEAT1) (Chen and Carmichael, 2009; Clemson *et al.*, 2009; Sasaki *et al.*, 2009; Sunwoo *et al.*, 2009). Paraspeckles are one of many distinct subnuclear bodies identified as containing discrete constituents (Fong *et al.*, 2013). Our measurement of preferential binding by PSPC1 for IMP $\alpha$ 2 and IMP $\alpha$ 6 in the presence of IMP $\beta$ 1 documents the importance of classical nucleocytoplasmic transport for paraspeckle formation. This study offers the new insight that PSPC1 delivery to paraspeckles can be controlled through regulated synthesis of nuclear transport machinery during development, with broader implications for cell biology and RNA metabolism based on the functional importance of this distinct subnuclear structure.

## RESULTS

### PSPC1 is a cargo of IMP $\alpha$ 2

To identify IMP $\alpha$ 2 cargoes in the fetal testis, we screened a Y2H library constructed using E12.5 mouse testes for binding partners of full-length IMP $\alpha$ 2. We identified five candidate cargoes: PSPC1; rRNA-processing 1 homologue; Maged1 melanoma antigen, family D, 1; heterogeneous nuclear ribonucleoprotein C; and biorientation of chromosomes in cell division 1-like. All candidate IMP $\alpha$ 2 cargoes were determined to contain putative NLSs using the cNLS Mapper program (Kosugi *et al.*, 2009), and each was predicted to localize in the nucleus using PSORT II (Nakai and Horton, 1999; Supplemental Table S1). Publicly accessible microarray data (www.ncbi.nlm.nih.gov/geo; Barrett *et al.*, 2013) indicate that the expression profile of each candidate IMP $\alpha$ 2 cargo coincides with IMP $\alpha$ 2 in the fetal and adult mouse testis (Supplemental Figure S1).

PSPC1 was selected for further investigation. It is a DBHS-containing protein family member; the full-length 523-amino-acid (aa) protein contains two RNA recognition motifs (RRMs), a NONA/paraspeckle (NOPS) domain, and two putative NLSs (Myojin *et al.*, 2004; Passon *et al.*, 2012; Figure 1A). The span of these domains was determined by alignment of the mouse PSPC1 sequence with the human sequence for which the PSPC1/NONO heterodimer structure has been reported (Passon *et al.*, 2012). The two putative NLSs were previously identified in PSPC1 (Myojin *et al.*, 2004) through homology with candidate NLSs identified in PSF (Dye and Patton, 2001). One NLS corresponds to two independent but overlapping potential bipartite NLSs in the aa 331–358 region, and the second is a monopartite NLS identified at the C-terminus (aa 517–525). A shorter PSPC1 isoform (391 aa) lacks the C-terminal putative NLS and is highly expressed in the kidney (Myojin *et al.*, 2004). The longer isoform is expressed at low levels in many adult mouse tissues but is enriched in the adult testis and present in round spermatids, pachytene spermatocytes, and Sertoli cells (Myojin *et al.*, 2004). The clone identified in this screen encodes a sequence lacking the first 105 aa (Figure 1A) but includes both putative NLSs, indicating it corresponds to the long PSPC1 isoform. The interaction between IMP $\alpha$ 2 and PSPC1 was first tested in yeast, using Y2H library PSPC1 clone (Figure 1B) in addition to full-length PSPC1. Both interact with IMP $\alpha$ 2 and with IMP $\alpha$ 6 (Figure 1B).

Two approaches demonstrated that IMP $\alpha$ 2 and PSPC1 associate in testis cells. Endogenous IMP $\alpha$ 2 and PSPC1 proteins were coimmunoprecipitated from a day 28 mouse testis lysate (Figure 1C, left-hand side). In addition, purified recombinant glutathione S-transferase (GST)-tagged IMP $\alpha$ 2 proteins pulled down endogenous PSPC1 from a day 28 mouse testis lysate, while GST alone did not (Figure 1C, right-hand side). Two additional control proteins (IMP $\alpha$ 2-ED and IMP $\alpha$ 2 $\Delta$ IBB) were used for these experiments. The IMP $\alpha$ 2-ED mutant contains two point mutations within the NLS-binding groove, a lysine at amino acid 192 and an arginine at amino acid 396, severely decreasing the ability of IMP $\alpha$ 2 to bind cargo protein NLSs, while IMP $\beta$ 1-binding capacity is maintained (Giesecke and Stewart, 2010; Yasuda *et al.*, 2012). The truncated IMP $\alpha$ 2 $\Delta$ IBB protein lacks the IMP $\beta$ -binding domain (Yasuda *et al.*, 2012) and is considered to bind to cargo proteins more effectively than full-length IMP $\alpha$ 2 protein, because it lacks the autoinhibitory activity present in the IBB domain (Giesecke and Stewart, 2010). The IMP $\alpha$ 2 $\Delta$ IBB protein pulled down more endogenous PSPC1 than the native protein did, while the IMP $\alpha$ 2-ED protein pulled down less (Figure 1C), as predicted.

To further characterize the interaction between PSPC1 and IMP $\alpha$ s from each of the three structural clades (Kelley *et al.*, 2010), we performed an enzyme-linked immunosorbent assay (ELISA)-based IMP-binding assay using affinity-purified HIS-tagged recombinant PSPC1 protein with GST-tagged or GST-void IMP $\alpha$  (Supplemental Figure S2, A–C). Data from a representative experimental replicate are presented in Figure 1D; mean  $K_d$  and  $B_{max}$  values from three independent experiments are shown in Table 1. Using GST-IMP $\alpha$  proteins, we measured the binding affinities of IMP $\alpha$ 2 and IMP $\alpha$ 6 (mean  $K_d$  values of 15.0 nM and 5.9 nM, respectively) and found them to be greater than that of IMP $\alpha$ 4 (mean  $K_d$  of 57.9 nM). The GST-alone control displayed negligible binding to PSPC1. Predimerization of GST-void IMP $\alpha$ 2, IMP $\alpha$ 4, or IMP $\alpha$ 6 with GST-IMP $\beta$ 1 to form a canonical nuclear transport heterodimer increased the affinity of each IMP $\alpha$  for PSPC1. The observed mean  $K_d$  values for IMP $\alpha$ 2 and IMP $\alpha$ 6 were 1.9 nM and 2.1 nM, respectively, while the affinity of IMP $\alpha$ 4 was lower, with a much larger mean  $K_d$  value of 21.3 nM. GST-IMP $\beta$ 1 on its own displayed low to negligible PSPC1 binding.

Together these findings demonstrate that PSPC1 and IMP $\alpha$ 2 interact and provide strong evidence that PSPC1 nuclear import is preferentially mediated through IMP $\alpha$ 2/IMP $\beta$ 1 or IMP $\alpha$ 6/IMP $\beta$ 1 heterodimer complexes using the classical nucleocytoplasmic transport pathway.

### PSPC1 is expressed in distinct cell populations during testis development

We next set out to document the pattern of PSPC1 expression throughout testis development, as this has only been shown previously in adult mouse testes (Myojin *et al.*, 2004; Kuwahara *et al.*, 2006). Western blotting was performed on mouse testis lysates from animals aged E13.5 through to adulthood using a mouse monoclonal antibody (mAb) that specifically binds the long, testis-enriched PSPC1 isoform (Figure 2A). The detected band of ~60 kDa matches that previously reported for the longer isoform in adult mouse testis (Myojin *et al.*, 2004). No band was detected in either 8 or 15 d postpartum (dpp) lysates, indicating up-regulation of PSPC1 occurs in postmitotic germ cells. The potential for the shorter PSPC1 isoform to be present in the testis remains to be established.

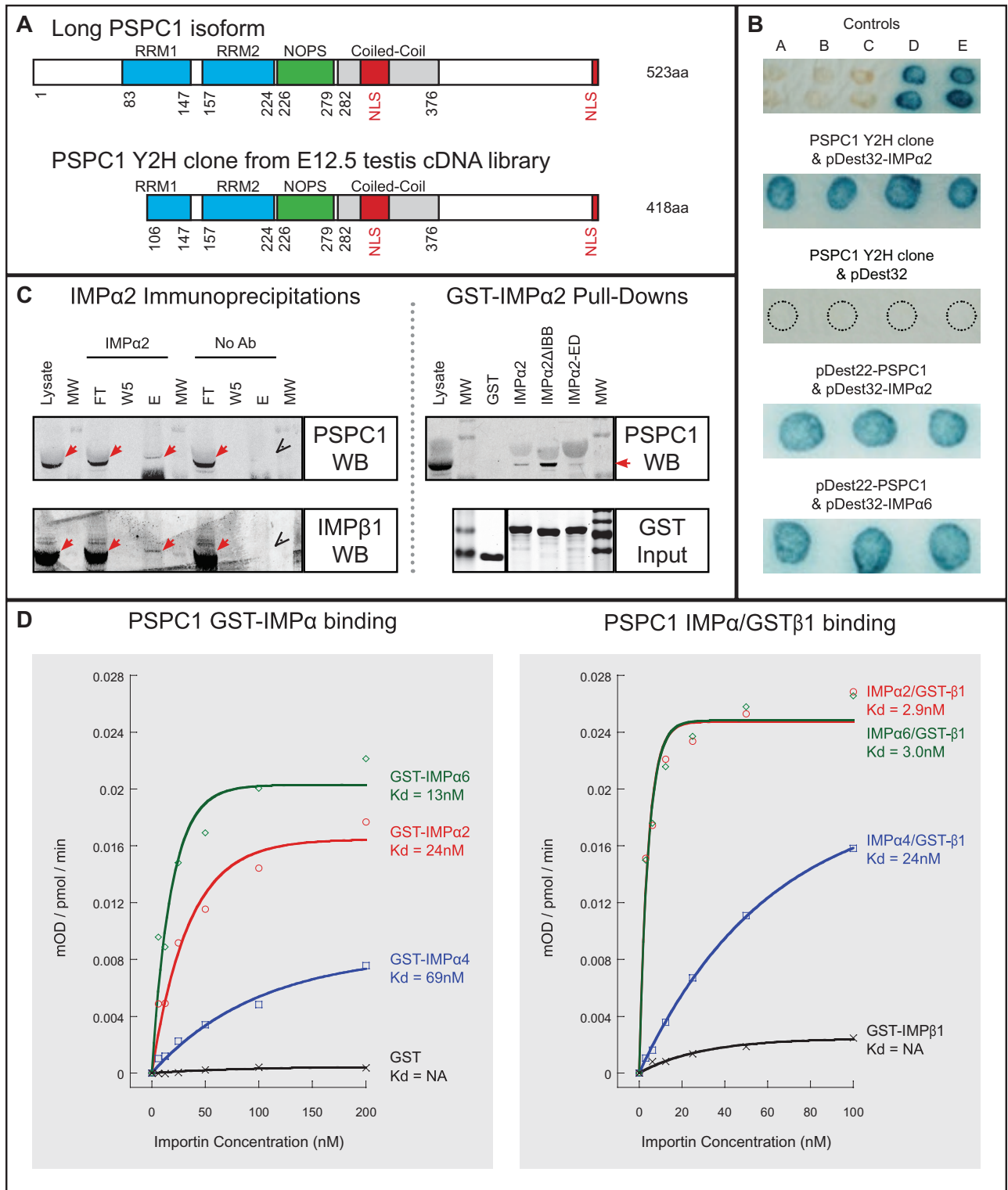
Whole-mount immunofluorescence identified IMP $\alpha$ 2 and PSPC1 within overlapping germ cell subpopulations in the adult mouse testis (Figure 2B). Germ cell subtypes were identified by their size and shape, distinct chromatin morphology, and position within the seminiferous epithelium. PSPC1 was observed within the nucleus of pachytene spermatocytes, round spermatids, and their supporting Sertoli cells (Figure 2B). The IMP $\alpha$ 2 signal was strongest within the pachytene spermatocyte cytoplasm and was readily detected in both cytoplasm and nucleus of round spermatids (Figure 2B).

Visualization of IMP $\alpha$ 2 (Figure 2C) and PSPC1 (Figure 2D) cellular distribution used a newly available antibody for IMP $\alpha$ 2 detection, yielding a more distinct and stronger signal than previously visualized by us in fixed-tissue sections (Ly-Huynh *et al.*, 2011). In the E12.5 mouse testis, IMP $\alpha$ 2 was detected in the cytoplasm and nucleus of gonocytes, the most primitive male germ cells. In adult mouse testis, the IMP $\alpha$ 2 signal was most intense within the nucleus of pachytene spermatocytes and early round spermatids; a weaker, predominantly cytoplasmic signal was evident within other germ cell types and in Sertoli cells. PSPC1 was detected in the gonocyte nucleus and cytoplasm at E12.5 (Figure 2D) and in the nucleus of spermatocytes, round spermatids, and Sertoli cells in the adult testis (Figure 2D; as reported in Myojin *et al.*, 2004; Kuwahara *et al.*, 2006).

These results delineate a substantial overlap in the cellular and subcellular distribution of PSPC1 and IMP $\alpha$ 2 in adult and embryonic mouse testes, supporting the hypothesis that IMP $\alpha$ 2 serves as a transport factor for cargoes expressed in a developmentally regulated manner. We therefore assessed the potential for IMP $\alpha$ 2 to influence PSPC1 nuclear import and paraspeckle formation.

### IMP $\alpha$ 2 targets PSPC1 to paraspeckles

For modulation of both IMP $\alpha$ 2 levels and functionality, constructs encoding green fluorescent protein (GFP)-tagged proteins corresponding to GST-IMP $\alpha$ 2 isoforms used in pull-down experiments were transfected into COS-7 and HeLa cells. Predicted nuclear transport outcomes for IMP $\alpha$ 2 cargoes arising from overexpression of these proteins are outlined in Figure 3A. Full-length IMP $\alpha$ 2 (GFP-IMP $\alpha$ 2-FL) was expected to increase nuclear accumulation of IMP $\alpha$ 2 transport cargoes, while GFP-IMP $\alpha$ 2 $\Delta$ IBB protein was predicted to compete with endogenous IMP $\alpha$ 2 for cargo binding and reduce cargo nuclear transport. Finally, GFP-IMP $\alpha$ 2-ED was expected to have minimal or no effect on endogenous cargo transport



**FIGURE 1: PSPC1 identified as a selective cargo of IMP $\alpha$ 2.** (A) Mouse PSPC1 schematics. Full-length PSPC1 (long isoform, 523 aa) contains two RRM1s (blue), a NOPS (green), a coiled-coil domain (gray), and two putative NLSs (red). The first putative NLS is within the coiled-coil domain and the second is at the C-terminus (adapted from Myojin *et al.*, 2004; Passon *et al.*, 2012). The E12.5 testis cDNA library clone identified in this Y2H screen encodes aa 106–523 of the long PSPC1 isoform. (B) PSPC1 interacts with IMP $\alpha$ 2 and IMP $\alpha$ 6 in the Y2H system, as assessed by X-gal substrate blue signal intensity. Controls: yeast X-gal controls (A: no interaction; B: weak; C: intermediate; D: strong; E: very strong). PSPC1 Y2H clone + IMP $\alpha$ 2: four blue spots indicate strong interactions between the PSPC1 Y2H clone and IMP $\alpha$ 2 in cotransformants. PSPC1 Y2H Clone + pDEST32 plasmid (autoactivation control): four black dotted circles denote cotransformants in yeast showing no interaction. PSPC1 + IMP $\alpha$ 2 and PSPC1 + IMP $\alpha$ 6: X-gal signals illustrate strong interaction using full-length PSPC1. (C) Left, IMP $\alpha$ 2 IPs using day 28 testis lysate. Samples are lysate, size markers (MW),

	GST-IMP $\alpha$ 2	GST-IMP $\alpha$ 4	GST-IMP $\alpha$ 6	GST	IMP $\alpha$ 2/ GST-IMP $\beta$ 1	IMP $\alpha$ 4/ GST-IMP $\beta$ 1	IMP $\alpha$ 6/ GST-IMP $\beta$ 1	GST-IMP $\beta$ 1
$K_d \pm \text{SEM}$	15.0 $\pm$ 4.3	57.9 $\pm$ 7.5	5.9 $\pm$ 3.3	NA	1.9 $\pm$ 0.5	21.3 $\pm$ 10.8	2.1 $\pm$ 0.5	35.5 $\pm$ 9.3
$B_{\text{max}} \pm \text{SEM}$	81.1 $\pm$ 7.3	52.4 $\pm$ 9.3	93.3 $\pm$ 5.8	NA	100.0 $\pm$ NA	85.4 $\pm$ 3.2	95.0 $\pm$ 2.8	16.3 $\pm$ 3.1

Mean  $K_d$  and  $B_{\text{max}} \pm \text{SEM}$  from three independent ELISA-based IMP-binding assay experiments are shown. The  $B_{\text{max}}$  values are displayed as comparative percentages relative to IMP $\alpha$ 2/GST-IMP $\beta$ 1 (set to 100%).

**TABLE 1:** PSCP1 ELISA-based binding assay average estimated  $K_d$  and  $B_{\text{max}}$  values.

and was considered as a control for nonnuclear transport-related effects of GFP-IMP $\alpha$ 2 isoform overexpression.

The impact of each isoform on ectopic PSCP1 localization into paraspeckles was assessed following cotransfection with a DsRed2-tagged PSCP1 construct. Immunofluorescence to detect endogenous PSCP1 was performed for cells transfected with only GFP-IMP $\alpha$ 2 variants. Constructs in HeLa cells yielded qualitatively similar outcomes to those in COS-7 cells (Figure 3B and Supplemental Figure S2D), with GFP-IMP $\alpha$ 2-FL protein increasing speckle number and size. Fluorescent PSCP1-positive nuclear speckles were visible in 68% of COS-7 cells transfected with PSCP1 alone and in 64% of cells cotransfected with IMP $\alpha$ 2-FL and PSCP1, suggesting that paraspeckle formation was not augmented by ectopic PSCP1. However, IMP $\alpha$ 2-FL and PSCP1 cotransfection in COS-7 cells resulted in qualitatively larger and more abundant PSCP1 nuclear speckles than did transfection with PSCP1 alone.

Several major experimental and analytical refinements enabled quantification of these outcomes in HeLa cells, the most common model for paraspeckle analyses (Fox and Lamond, 2010). Owing to variability in paraspeckle numbers detected within nontransfected HeLa cells, a minimum of 100 cells per sample was examined. Confocal three-dimensional z-series images permitted identification and quantification of immunofluorescent PSCP1 punctate nuclear foci over the full cell volume. Imaris software image analysis was performed on samples into which only GFP-IMP $\alpha$  constructs were introduced, with immunofluorescence used to detect endogenous PSCP1 (examples in Figure 3C).

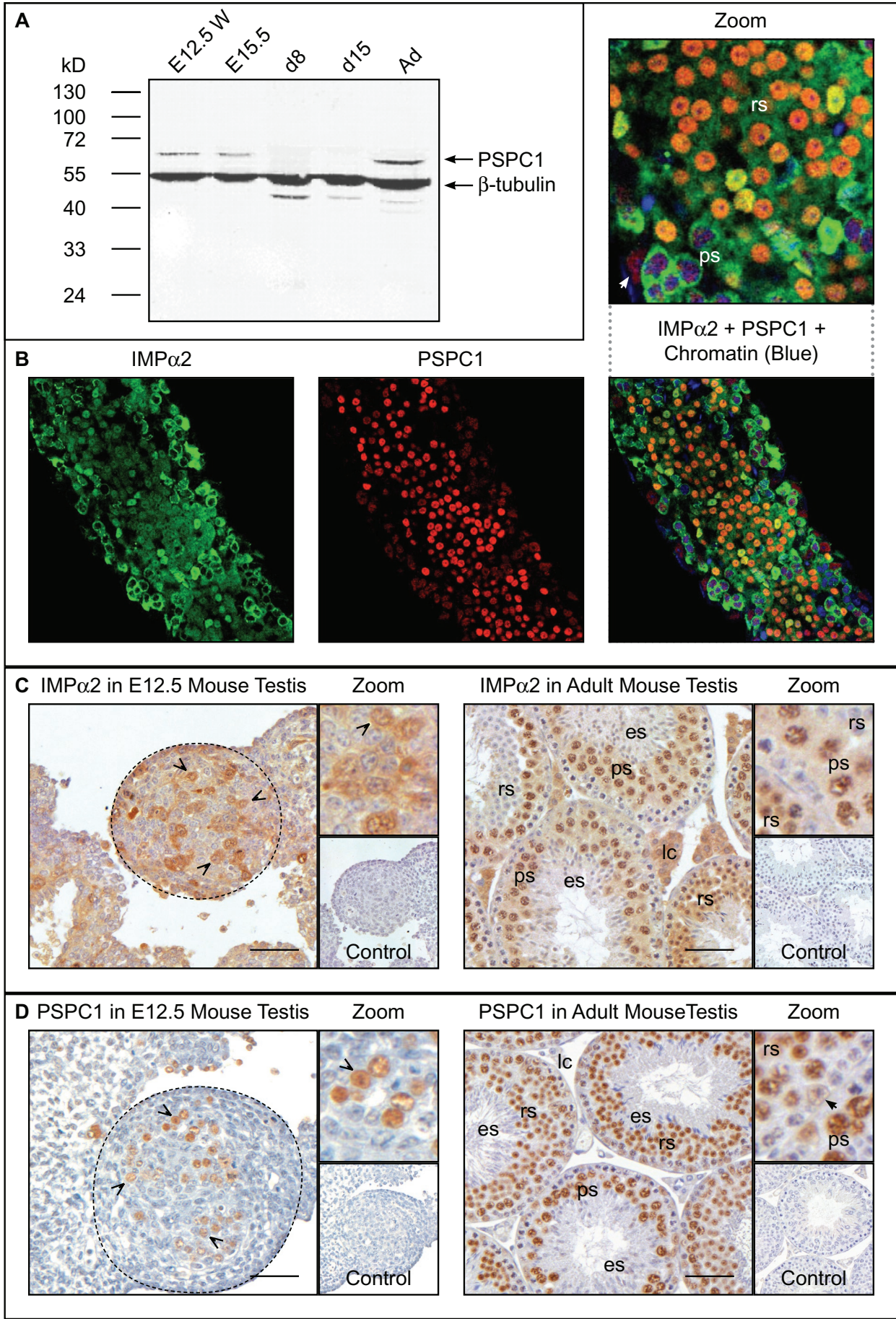
Quantitative outcomes are summarized in Table 2; Supplemental Figures S3 and S4 contain detailed analyses. All data analyses assumed that outcomes for each cell arose from independent events. Because the data were skewed, the geometric mean (GM) rather than the arithmetic mean was examined. Nonparametric Mann-Whitney *U*-tests were initially used to test for significant differences between transfection groups on a per-cell basis, and all PSCP1 nuclear speckle-negative cells were included (Supplemental Figure S3). These pairwise comparisons identified significant differences in immunofluorescent PSCP1 nuclear speckles arising from transfection with IMP $\alpha$ 2 constructs corresponding to differing

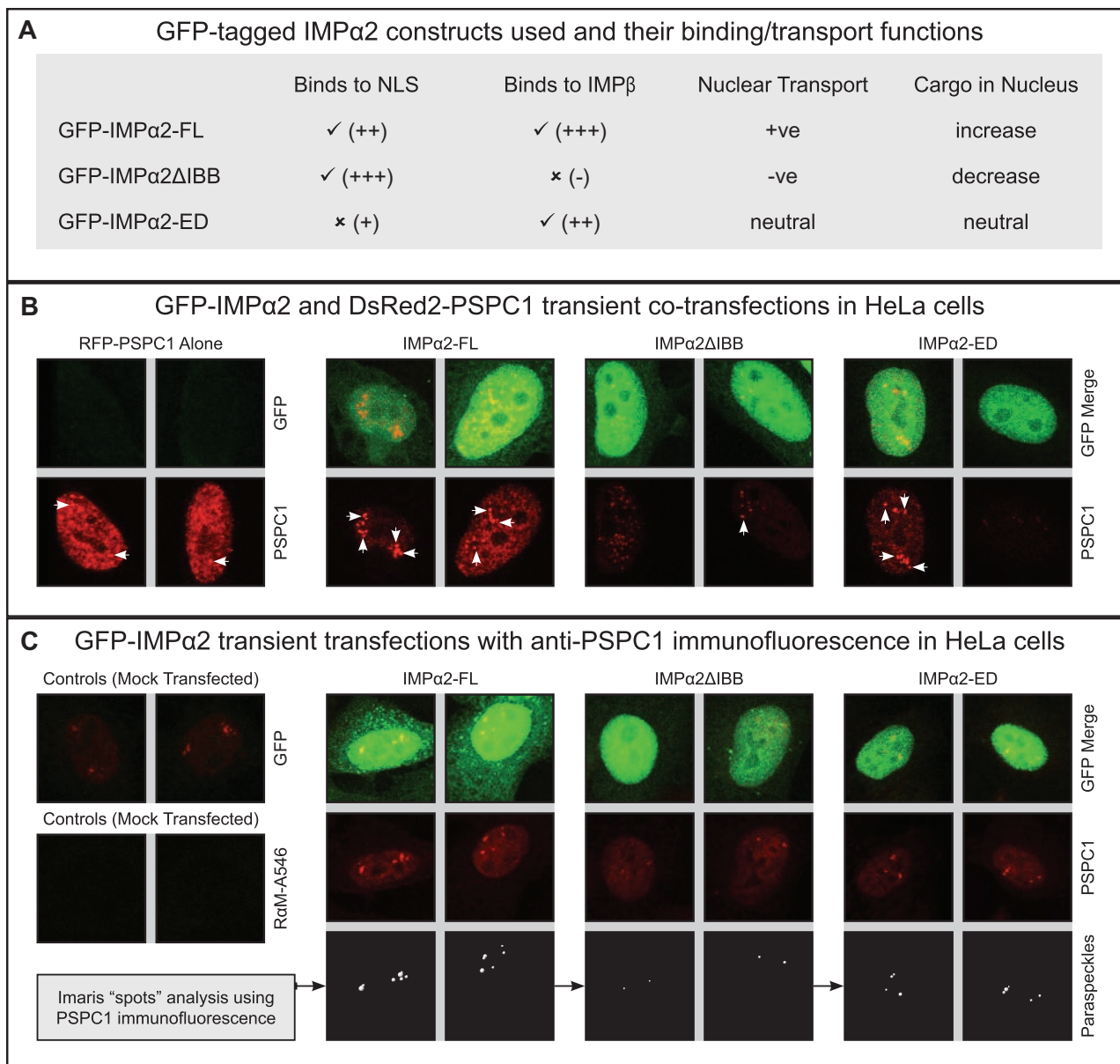
nuclear transport capacities. Significantly lower numbers of immunofluorescent PSCP1 nuclear speckles, total volume of these nuclear foci, and total intensity of PSCP1 associated with nuclear speckles was measured within cells of the GFP-IMP $\alpha$ 2 $\Delta$ IBB group in comparison with either GFP-IMP $\alpha$ 2-FL or GFP-IMP $\alpha$ 2-ED samples. Analysis using a parametric test, an analysis of variance with Games-Howell post hoc test, yielded similar outcomes (Supplemental Figure S3).

The properties of the identified nuclear PSCP1 speckles were analyzed in more detail, considering individual PSCP1 speckle-positive cells (Table 2 and Supplemental Figure S4). The most striking outcome was that GFP-IMP $\alpha$ 2 $\Delta$ IBB-transfected cells had significantly fewer endogenous PSCP1 nuclear foci per cell; these nuclear speckles were significantly smaller (volume), and the sum intensity of immunofluorescent PSCP1 signal present in nuclear speckles was lower, both within individual foci and as a cumulative value for each cell. The data summarized in Table 2 (extended in Supplemental Figures S3 and S4) highlight key impacts of IMP $\alpha$ 2 transport capacity on PSCP1 localization to nuclear bodies. First, the proportion of cells containing immunofluorescent PSCP1 nuclear speckles was lower in the IMP $\alpha$ 2 $\Delta$ IBB (32.6%) in comparison with FL and ED isoform (51.1% and 46.7%, respectively) samples, as highlighted in the calculated odds ratio when the IMP $\alpha$ 2-ED value was set at 1.000 and IMP $\alpha$ 2-FL and IMP $\alpha$ 2 $\Delta$ IBB groups were at 1.194 and 0.552, respectively. Second, when exclusively PSCP1 nuclear speckle-positive cells were examined on a per-cell basis to compare the ratio of GMs, with the IMP $\alpha$ 2-ED value set to 1.000, the IMP $\alpha$ 2 $\Delta$ IBB sample value was consistently lower for the number of speckles per cell (FL: 0.983;  $\Delta$ IBB: 0.703), sum speckle volume per cell (FL: 1.164;  $\Delta$ IBB: 0.543), and sum of the immunofluorescence intensity associated with PSCP1 nuclear speckles (FL: 1.194;  $\Delta$ IBB: 0.518). Finally, the same reduction in the ratio of GMs within the IMP $\alpha$ 2 $\Delta$ IBB group was identified for individual immunofluorescent PSCP1 nuclear speckle parameters of volume (FL: 1.024;  $\Delta$ IBB: 0.747), sum PSCP1 intensity (FL: 1.014;  $\Delta$ IBB: 0.700), and PSCP1 voxel intensity (FL: 0.986;  $\Delta$ IBB: 0.967). These differences were all significant (Table 2).

For investigating the impact of modulating IMP $\alpha$ 2 levels and functionality on another core paraspeckle component, PSF-positive

flowthrough (FT), wash no. 5 (W5), and elution (E). PSCP1 detected by Western blotting (PSCP1 WB) is ~59 kDa within the IMP $\alpha$ 2 IP elution lane (red arrows) and is not detected in the IP-negative control ("V"-shaped arrowhead, lacking IP antibody; No Ab), demonstrating that PSCP1 is coimmunoprecipitated with IMP $\alpha$ 2. IMP $\alpha$ 2 IPs were confirmed by detection of IMP $\beta$ 1, a known IMP $\alpha$ 2 interactor (IMP $\beta$ 1 WB). Red arrows indicate IMP $\beta$ 1 (detected at ~98 kDa); "V"-shaped arrowhead indicates the absence of IMP $\beta$ 1 in negative control. Right, GST-tagged IMP $\alpha$ 2 proteins "pull down" a PSCP1 band (detected at ~57 kDa) from day 28 testis lysate (GST-IMP $\alpha$ 2 pull downs). Western blotting detects PSCP1 (PSCP1 WB), sample lanes are day 28 testis lysate (lysate), size markers (MW), GST control (GST), GST-tagged full-length IMP $\alpha$ 2 (IMP $\alpha$ 2), GST-tagged truncated IMP $\alpha$ 2 (IMP $\alpha$ 2 $\Delta$ IBB), and GST-tagged mutated IMP $\alpha$ 2 (IMP $\alpha$ 2-ED). Red arrow highlights the PSCP1 band present in GST-IMP $\alpha$ 2 samples that is absent from the GST control. The GST-tagged input proteins (3  $\mu$ g) were separated by SDS-PAGE and stained with Coomassie (GST input), to show relative input equivalence (bands at ~24, 77, 73, and 77 kDa, respectively). (D) Representative PSCP1 ELISA-based IMP-binding assays using GST-IMP $\alpha$  proteins and GST alone (left) and using tag-free IMP $\alpha$ s predimerized with GST-IMP $\beta$ 1 or GST-IMP $\beta$ 1 alone (right).  $K_d$  estimates are indicated.





**FIGURE 3:** IMP $\alpha$ 2 targets PSPC1 to paraspeckles in HeLa cells. Transient transfection with plasmids encoding GFP-tagged IMP $\alpha$ 2-FL, IMP $\alpha$ 2 $\Delta$ IBB, or IMP $\alpha$ 2-ED. Images are flattened Z-series captured via confocal laser-scanning microscopy 2 d posttransfection. White arrows mark several paraspeckles (PSPC1 positive nuclear bodies). (A) GFP-tagged IMP $\alpha$ 2 isoforms and their functional properties. (B) HeLa cells transfected to express DsRed2-PSPC1 alone or cotransfected to express DsRed2-PSPC1 with GFP-IMP $\alpha$ 2 constructs. Bottom images depict DsRed2-PSPC1 localization alone, top panels depict both IMP $\alpha$ 2 (green) and PSPC1 (red) localization. The DsRed2-PSPC1 alone control is shown, with the red signal excluded from the top panel to clearly show the absence of a green signal. Each image corresponds to a single cell nucleus, with two separate images shown for each experimental group. (C) HeLa cells either mock transfected (controls) or transfected to express GFP-IMP $\alpha$ 2 constructs. Endogenous PSPC1 was visualized using immunofluorescence, with two cell images shown for each experimental group. The “GFP” control sample shows no detectable signal in mock-transfected cells, while the “R $\alpha$ M-A546” control lacking primary antibody shows the absence of secondary antibody binding. Examples of “spots” analysis using Imaris software to identify and quantify PSPC1 nuclear speckles are shown in the bottom panels.

**FIGURE 2:** PSPC1 coexpression with IMP $\alpha$ 2 in germ cells. (A) Western blot detection of PSPC1 protein (60 kDa) and  $\beta$ -tubulin (protein loading control; 55 kDa) in E12.5 whole-embryo lysate (E12.5 WE) and in whole testis lysates at E15.5, 8 dpp (d8), 15 dpp (d15), and adult (Ad). (B) Confocal laser-scanning microscopy images of whole-mount immunofluorescence showing PSPC1 and IMP $\alpha$ 2 in adult testis seminiferous tubules. Left-hand image, IMP $\alpha$ 2 (green); middle, PSPC1 alone (red); right, merged image with TO-PRO staining (chromatin). ps: pachytene spermatocytes; rs: round spermatids; white arrow: Sertoli cell nucleus. (C) IMP $\alpha$ 2 immunohistochemistry on E12.5 (1:50) and adult testes (1:100); control lacked primary antibody. (D) PSPC1 immunohistochemistry on E12.5 testis (1:50) and adult mouse testis (1:100). (C and D) ps: pachytene spermatocytes; rs: round spermatids; es: elongating spermatids; lc: Leydig cell; black arrow: Sertoli cell nucleus; “V”-shaped arrowhead: gonocyte (E12.5). Dashed line circles testis within E12.5 samples. Scale bar: 50  $\mu$ m.

	IMPα2-FL	IMPα2ΔIBB	IMPα2-ED	Totals			
Number of cells analyzed	133	482	287	902			
Number of PSPC1 foci-positive cells	68	157	134	359			
Number of PSPC1 foci detected	461	831	1013	2305			
% PSPC1 foci-positive cells (95% CI)	51.1 (33.7↔68.5)	32.6 (26.4↔38.8)	46.7 (35.9↔57.5)	<b>Lg Reg Sig (vs. ED)</b>			
Odds ratio (PSPC1 foci-positive cells) (95% CI)	1.194 (0.792↔1.803)	0.552 (0.409↔0.745)	1.000 (Control)	0.397 (FL) 0.000 (ΔIBB)*			
<b>Mean per-cell values (PSPC1 foci-positive cells)</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>Ln Reg Sig (vs. ED)</b>
Number of PSPC1 foci	5.05 (4.09↔6.22)	0.983 (0.760↔1.270)	3.61 (3.14↔4.14)	0.703 (0.574↔0.860)	5.13 (4.42↔5.97)	1.000 (Control)	0.895 (FL) 0.001 (ΔIBB)*
PSPC1 foci volume sum	1.09 (0.78↔1.52)	1.164 (0.772↔1.757)	0.51 (0.41↔0.63)	0.543 (0.392↔0.752)	0.93 (0.74↔1.19)	1.000 (Control)	0.469 (FL) 0.000 (ΔIBB)*
Foci PSPC1 intensity sum	4176 (2904↔6004)	1.194 (0.765↔1.866)	1812 (1427↔2301)	0.518 (0.364↔0.737)	3496 (2699↔4528)	1.000 (Control)	0.435 (FL) 0.000 (ΔIBB)*
<b>Mean values per PSPC1 foci</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GEE Sig (vs. ED)</b>
Volume	0.206 (0.169↔0.251)	1.024 (0.813↔1.290)	0.150 (0.132↔0.170)	0.747 (0.628↔0.888)	0.201 (0.178↔0.226)	1.000 (Control)	0.840 (FL) 0.001 (ΔIBB)*
PSPC1 intensity sum	766 (610↔963)	1.014 (0.776↔1.324)	529 (461↔607)	0.700 (0.576↔0.851)	756 (659↔868)	1.000 (Control)	0.920 (FL) 0.000 (ΔIBB)*
PSPC1 voxel intensity	90.6 (88.4↔92.8)	0.986 (0.957↔1.017)	88.8 (87.7↔89.9)	0.967 (0.946↔0.988)	91.8 (90.2↔93.5)	1.000 (Control)	0.371 (FL) 0.002 (ΔIBB)*
Trend relative to IMPα2-ED	Unchanged or small increase (-↑-)		Decreased (↓↓↓)		Normalizing control (—)		

The analyzed cell numbers for each group, number of detected PSPC1-positive nuclear foci, and proportion of cells determined to contain PSPC1 nuclear foci are presented. Values are normalized against the IMPα2-ED control (values set to 1), an "odds ratio" identifies the fold difference in number of PSPC1 nuclear foci-positive cells compared with the IMPα2-ED sample. Data assessed on a per-cell or PSPC1 nuclear foci basis include GMs with 95% Wald confidence intervals (95% CI, range in italics) and the ratio of GMs. For determining significant differences between groups, a logistic regression (Lg Reg) model was used for PSPC1 foci-positive/negative cells, linear regression (Ln Reg) models were used for per-cell data (PSPC1 foci-positive cells) and GEEs were used for per-PSPC1 nuclear foci data. Significance (Sig) values compared with the IMPα2-ED control are shown. Using Bonferroni correction, the significance threshold was reassigned from 0.05 to 0.016 (0.05 + 3 transfection groups). Even with these stringent conditions, a significantly lower value is recorded for each assessed parameter within the IMPα2ΔIBB transfection group data set (\*). Further details are provided in Supplemental Figures S3 and S4.

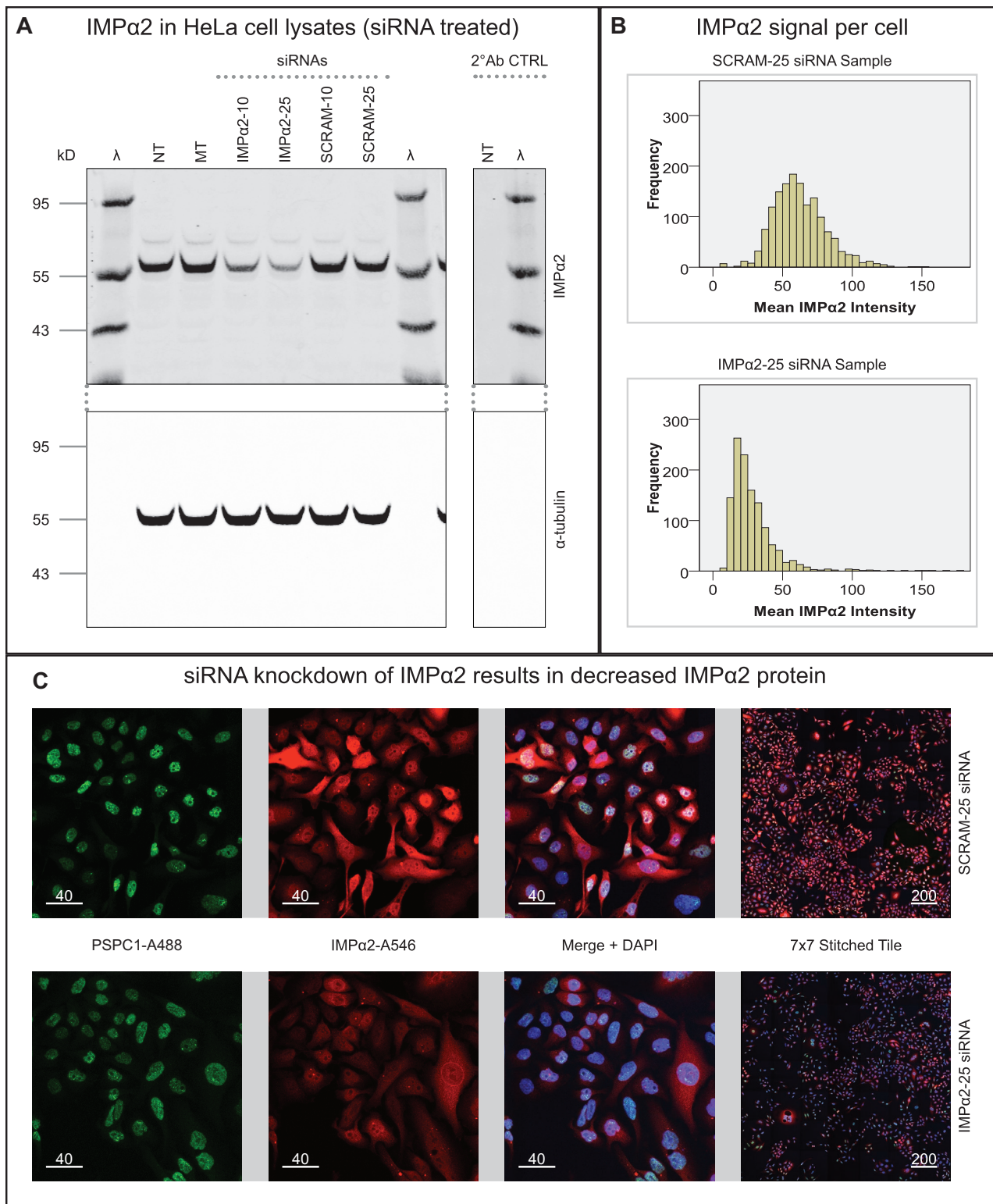
**TABLE 2: Outcomes of modulating IMPα2 expression and transport function on PSPC1-positive nuclear speckles.**

nuclear speckles were analyzed based on preliminary data collected in HeLa cells using GFP-tagged IMPα2 constructs as described above for PSPC1 nuclear speckles. Summarized in Supplemental Table S2, the proportion of PSF nuclear speckle-positive cells followed a similar trend to PSPC1 nuclear speckles, with IMPα2-FL causing a significant increase and IMPα2ΔIBB a significant decrease. No significant changes to PSF nuclear speckle parameters was observed at the per-cell or per-speckle level.

To complement these findings, we measured the impact on PSPC1-positive nuclear paraspeckles after reducing endogenous IMPα2 expression in HeLa cells using small interfering RNA (siRNA).

Effective knockdown of IMPα2 with siRNA was confirmed on a population level and for individual cells using both Western blotting and immunofluorescence (Figure 4). The GM of the IMPα2 immunofluorescence signal demonstrated that the IMPα2 protein within cells in the siRNA-treated group dropped to 53.2% of the level in the cells treated with the scrambled (SCRAM) siRNA control oligo. PSPC1 nuclear speckle numbers, size, and PSPC1 signal intensity were then assessed by immunofluorescence for endogenous PSPC1 (summarized in Table 3). While the siRNA-induced decrease in IMPα2 protein levels did not cause a significant reduction in the percentage of cells containing PSPC1 nuclear speckles, it significantly reduced the





**FIGURE 4:** siRNA knockdown of IMP $\alpha$ 2. (A) Dual fluorescent Western blot for IMP $\alpha$ 2 protein and  $\alpha$ -tubulin loading control detection. Lanes indicated are not transfected (NT), mock transfected (MT), transfected with IMP $\alpha$ 2 siRNA at 10 or 25 nM final concentration (IMP $\alpha$ 2-10 or IMP $\alpha$ 2-25), or transfected with scrambled siRNA control at 10 or 25 nM final concentration (SCRAM-10 or SCRAM-25). Lanes with size markers ( $\lambda$ ) have the molecular weight indicated on each blot in kilodaltons (kDa). (B) Histograms of the mean IMP $\alpha$ 2 intensity (detected per cell) as determined by immunofluorescence in the IMP $\alpha$ 2-25 and SCRAM-25 groups. (C) Overview of images collected from IMP $\alpha$ 2-25 and SCRAM-25 samples with immunofluorescence for PSPC1 and IMP $\alpha$ 2 with DAPI as a nuclear marker. Scale bars: 40 or 200  $\mu$ M, as indicated.

	IMPα2 siRNA		SCRAM siRNA		Totals
Number of cells analyzed	1207		1522		2729
Number of PSPC1 foci-positive cells	1144		1446		2590
Number of PSPC1 foci detected	7394		10,525		17,919
% PSPC1 foci-positive cells (95% CI)	94.8 (70.7↔118.8)		95 (73.1↔116.9)		<b>Lg Reg Sig (vs. SCRAM)</b>
Odds ratio (PSPC1 foci-positive cells) (95% CI)	0.954 (0.677↔1.345)		1.000 (Control)		0.790 (IMPα2)
<b>Mean per-cell values (PSPC1 foci-positive cells)</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>Ln Reg Sig (vs. SCRAM)</b>
IMPα2 signal intensity	25.18 (24.52↔25.85)	0.532 (0.514↔0.551)	47.29 (46.20↔48.41)	1.000 (Control)	0.000 (IMPα2)*
Number of PSPC1 foci	5.02 (4.81↔5.24)	0.889 (0.839↔0.942)	5.64 (5.43↔5.86)	1.000 (Control)	0.000 (IMPα2)*
PSPC1 foci volume sum	1.10 (1.04↔1.16)	0.818 (0.759↔0.881)	1.34 (1.56↔1.41)	1.000 (Control)	0.000 (IMPα2)*
Foci PSPC1 intensity sum	122.8 (115.2↔130.8)	0.792 (0.727↔0.862)	155.1 (146.6↔164.1)	1.000 (Control)	0.000 (IMPα2)*
<b>Mean values per PSPC1 foci</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GM and 95% CI</b>	<b>Ratio of GM and 95% CI</b>	<b>GEE Sig (vs. SCRAM)</b>
Volume	0.169 (0.166↔0.173)	0.944 (0.918↔0.971)	0.179 (0.176↔0.183)	1.000 (Control)	0.000 (IMPα2)*
PSPC1 intensity sum	805 (784↔826)	0.910 (0.880↔0.942)	884 (864↔904)	1.000 (Control)	0.000 (IMPα2)*
PSPC1 voxel intensity	118.2 (116.6↔119.8)	0.965 (0.948↔0.983)	122.4 (121.0↔123.9)	1.000 (Control)	0.000 (IMPα2)*
Trend relative to SCRAM siRNA:	Decreased (—↓—)		Normalizing control (—)		

The analyzed cell numbers for each group, number of detected PSPC1-positive nuclear foci, and proportion of cells determined to contain PSPC1 nuclear foci are presented. Values are normalized against the SCRAM siRNA control (values set to 1), an "odds ratio" identifies the fold difference in number of PSPC1 nuclear foci-positive cells compared with the SCRAM siRNA sample. Data assessed on a per-cell or PSPC1 nuclear foci basis include GMs with 95% Wald confidence intervals (95% CI, range in italics), and the ratio of GMs. For determining significant differences between groups, a logistic regression (Lg Reg) model was used for PSPC1 foci-positive/negative cells, linear regression (Ln Reg) models were used for per-cell data (PSPC1 foci-positive cells), and GEEs were used for per PSPC1 nuclear foci data. Significance (Sig) values compared with the SCRAM siRNA control are shown. Using a threshold of 0.025, a significantly lower value is recorded for each assessed parameter within the IMPα2 siRNA group data set (\*) for per-cell or per-PSPC1 foci data. The IMPα2 signal intensity per cell dropped significantly to 53.2% that of the SCRAM siRNA control sample. Further details are provided in Figure 4.

**TABLE 3: Outcomes of siRNA knockdown of IMPα2 on PSPC1-positive nuclear speckles.**

mean (geometric) number of PSPC1 nuclear speckles per cell to 88.9% that of the SCRAM control value, the sum volume of speckles per cell to 81.8% of control, and the sum of PSPC1 immunofluorescence signal associated with nuclear speckles per cell to 79.2% of control values. The measurements of individual PSPC1 nuclear speckles also revealed statistically significant parameter reductions in the IMPα2 siRNA treatment group, although these were of a smaller magnitude, with per-PSPC1 nuclear foci measurements relative to SCRAM control value at 94.4% for speckle volume, 91.0% for speckle PSPC1 intensity sum, and 96.5% for PSPC1 mean voxel intensity per speckle.

Overall these data indicate that the IMPα2 and PSPC1 interaction is relevant to the nuclear function of PSPC1 nucleocytoplasmic transport and subsequent localization to paraspeckles. They reveal for the first time that modulating the functional levels of IMPα2 can directly determine the number of cells that contain PSPC1 nuclear speckles and the number of speckles that these cells contain and

influence the detectable size and intensity of PSPC1 staining within individual nuclear foci.

## DISCUSSION

Changes in IMP protein levels and transport activity are now understood to be of central importance to cellular differentiation, although the mechanistic basis for this is poorly understood. As an approach to filling this knowledge gap, and because IMPα2 synthesis is dynamic during germ cell differentiation in the mammalian testis, we sought to identify IMPα2 cargoes in the E12.5 testis. Through a Y2H screen, we identified five potential IMPα2 cargoes, with each containing a cNLS for canonical nuclear transport via IMPα protein binding. Characterization of the potential interaction between PSPC1 and IMPα2 was of particular interest, because both are present in the same germ cells (Kuwahara *et al.*, 2006; Major *et al.*, 2011). Identification of PSPC1 as a component of the distinct subnuclear paraspeckle domain (Fox *et al.*, 2002) provided

a functional framework for our interest in this molecule. Paraspeckles are rich in A-to-I edited RNA transcripts; they have been implicated as a site for nuclear retention of RNAs and RNA metabolism and thereby influence cellular differentiation and stress responses (Fox and Lamond, 2010). PSPC1 contains two putative NLSs (Myojin *et al.*, 2004), and notably, the C-terminal NLS lies outside the solved structure, while the putative bipartite NLS resides in the coiled-coil domain, meaning neither of the predicted NLSs are part of the reported core globular structure (Passon *et al.*, 2012), and both are likely to be accessible to IMP $\alpha$  for binding. We demonstrated that PSPC1 and IMP $\alpha$ 2 proteins colocalize in specific male germ cell types, and we obtained biochemical evidence that PSPC1 and IMP $\alpha$ 2 interact in the testis.

The potential for IMP $\alpha$ 6 to mediate PSPC1 nuclear import was indicated by both Y2H and ELISA-based binding assays (Figure 1). While IMP $\alpha$ 2 and PSPC1 are coexpressed in several germ cell types spanning fetal through adult life, the expression of IMP $\alpha$ 6 is maximal during the latest spermatogenic stage, spermiogenesis (Hogarth *et al.*, 2006; Major *et al.*, 2011). We propose that PSPC1 and IMP $\alpha$ 6 interact during spermiogenesis, when IMP $\alpha$ 2 levels are declining. The indication that PSPC1 nuclear import is preferentially mediated by IMP $\alpha$ 2/IMP $\beta$ 1 or IMP $\alpha$ 6/IMP $\beta$ 1 heterodimer complexes reinforces the functional importance of regulated synthesis of IMP proteins, as shown for IMP $\alpha$  and IMP $\beta$  proteins in fetal and postnatal germ-line cells (Hogarth *et al.*, 2006, 2007; Loveland *et al.*, 2006; Yamaguchi *et al.*, 2006; Ly-Huynh *et al.*, 2011; Major *et al.*, 2011; Miyamoto *et al.*, 2012, 2013; Tran *et al.*, 2012; Whiley *et al.*, 2012; Arjomand *et al.*, 2014). We know that developmental switches can be affected by coordinated up- and down-regulation of specific cargoes and individual factors for nucleocytoplasmic transport. Recent studies have shown that IMP $\alpha$ 2 must be down-regulated and IMP $\alpha$ 1 up-regulated to enable ESCs to differentiate into neural lineage progenitors (Yasuhara *et al.*, 2007). This allows OCT6 and BRN2 to be released from IMP $\alpha$ 2 sequestration (via a C-terminal acidic domain) in the cytoplasm for nuclear translocation by IMP $\alpha$ 1/IMP $\beta$ 1 or IMP $\alpha$ 4/IMP $\beta$ 1 (Yasuhara *et al.*, 2013).

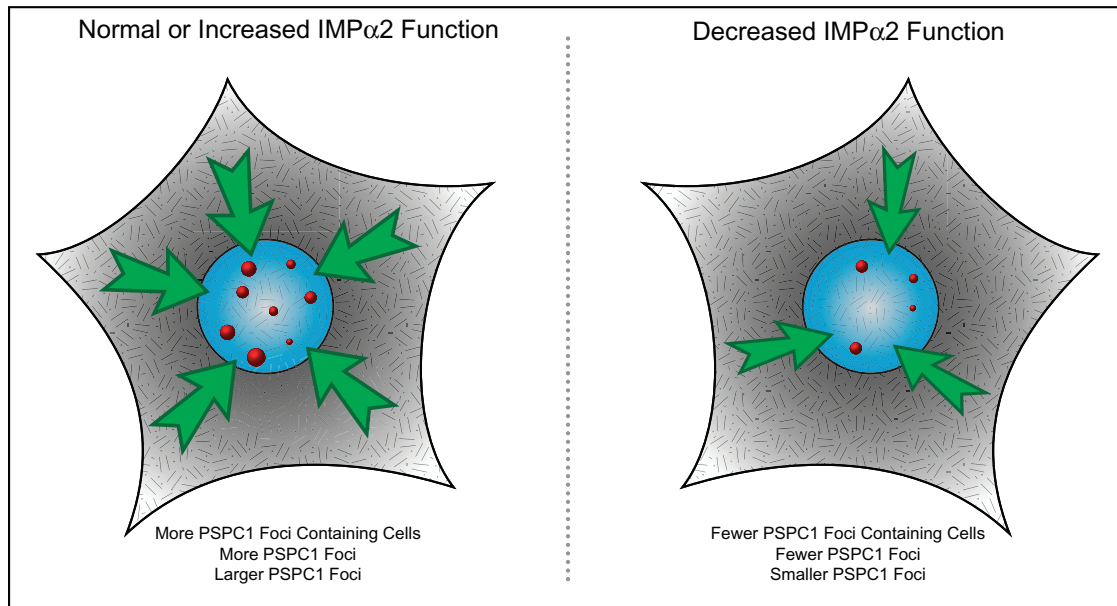
Our quantitative analysis approach has documented the highly significant effect of impaired IMP $\alpha$ 2 cargo-binding capacity on PSPC1 subnuclear targeting to paraspeckles within individual cells. The software-assisted image analysis eliminated the inherent subjectivity associated with manually assessing paraspeckle number and size, enabling quantitative analysis of 2305 PSPC1-containing nuclear speckles in 902 cells within the GFP-tagged IMP $\alpha$ 2 transfection experiment and 17,919 PSPC1 nuclear speckles in 2729 cells of the siRNA experiment. A reduced number of HeLa cells showed nuclear speckle localization of PSPC1 after transfection to express the dominant-negative IMP $\alpha$ 2 $\Delta$ IBB-encoding construct compared with the IMP $\alpha$ 2-FL or the IMP $\alpha$ 2-ED isoforms, with siRNA knockdown of IMP $\alpha$ 2 also demonstrating similar reductions in detectable PSPC1 nuclear speckle numbers and associated parameters. These outcomes provide strong evidence that the binding relationship between IMP $\alpha$ 2 and PSPC1 can modulate PSPC1 delivery to paraspeckles and thus determine paraspeckle-related RNA metabolic functions.

That IMP $\alpha$ 2 $\Delta$ IBB did not completely block paraspeckle localization of PSPC1 may reflect the presence of endogenous IMP $\alpha$ 2, so although classical nuclear transport efficiency is reduced, it is not eliminated. Consistent with this concept, siRNA knockdown of IMP $\alpha$ 2 also did not completely block PSPC1 localization to paraspeckles, although significant changes in the parameters of PSPC1 nuclear speckles were documented. While overexpression of IMP $\alpha$ 2-FL did not lead to a significant increase in any of the

assessed PSPC1 nuclear speckle attributes, small increases were measured for the proportion of speckle-positive cells and sum PSPC1 immunofluorescent speckle volume and intensity, parameters for which the odds ratio or the ratio of GMs was greater than 1.0 (Table 2). This suggests that the cellular level of PSPC1 is also an important factor limiting detectable PSPC1 nuclear body number and size. Modulating IMP $\alpha$ 2 in HeLa cells changed the detectable proportion of cells containing PSF-positive nuclear speckles, but there were no significant changes observed at the per-cell or per-PSF nuclear speckle level. We therefore hypothesize that the underlying architectural scaffold/structure of the paraspeckle is not affected by perturbations of PSPC1 delivery; rather, we suspect the functional significance of the paraspeckle is changed by dictating which RNA transcripts and other paraspeckle components are recruited. While our results indicate that IMP $\alpha$ 2 can facilitate PSPC1 nucleocytoplasmic transport, whether IMP $\alpha$ 2 specifically targets PSPC1 to nuclear paraspeckles remains to be established. The potential for IMP $\alpha$ 2 or other IMPs to deliver cargo directly to nuclear subcompartments is a focus of current work in our laboratory.

The adenosine deaminases acting on RNA (ADARs) perform A-to-I editing of selected RNA transcripts at specific residues, and the three present in mammalian cells have distinct interactions with IMP $\alpha$  proteins (Maas and Gommans, 2009a). ADAR3 and a broadly expressed ADAR2 splice variant found in testis (Maas and Gommans, 2009b) both bind to IMP $\alpha$ 2 but not to IMP $\alpha$ 1 or IMP $\alpha$ 3. In contrast, the main splice form of ADAR2 will bind to both IMP $\alpha$ 1 and IMP $\alpha$ 3 (Maas and Gommans, 2009a). This suggests that regulated IMP $\alpha$ 2 expression could modulate paraspeckle function in two ways, first directly, by mediating PSPC1 nuclear import and localization into paraspeckles, and second in an indirect manner, by controlling nuclear import of specific ADARs that impact on A-to-I editing of specific transcripts and their subsequent localization into paraspeckles. The capacity of IMP $\alpha$ 2 to facilitate the nuclear transport of other key paraspeckle components or regulate the expression of key paraspeckle components either directly or indirectly has yet to be determined. The possibility that IMP $\alpha$ 2 is regulating paraspeckles indirectly through effects upon the cell cycle or another mechanism has also not been excluded.

Our current working model, summarized in Figure 5, is that the regulated expression of IMP $\alpha$ 2 within fetal and postnatal germ-line cells will drive PSPC1 nuclear import and localization into paraspeckles. This model highlights the need to develop a better functional understanding of the downstream effects of modulating the relative proportions of PSPC1 or other core DBHS proteins localized to the paraspeckle, observed here as changes in immunofluorescent PSPC1 nuclear speckle number and size. Given that NEAT1 and paraspeckles are absent from human ESCs and that both can be induced upon differentiation, it has been suggested that paraspeckles are indicative of a differentiated cell state (Chen and Carmichael, 2009). NEAT1 transcript levels increase during muscle differentiation, and paraspeckles (punctate NEAT1 foci) are enlarged and present in greater numbers upon differentiation of myoblasts into myotubes (Sunwoo *et al.*, 2009). Interestingly, regulated expression of nuclear transport machinery is also implicated in muscle differentiation, with elevation of IMP $\alpha$ 2 during differentiation linked to myoblast proliferation, myocyte migration, and myotube size (Hall *et al.*, 2011). Thus the results of the present study, which document the capacity for IMP $\alpha$ 2 to drive an increase in PSPC1 nuclear speckle size and numbers in HeLa cells, further suggest that developmental peaks in IMP $\alpha$ 2 expression, such as in the germ cells of the E12.5 testis or during postmitotic spermatogenesis (Major *et al.*, 2011), reflect its role in determining cellular differentiation. We have



**FIGURE 5:** Proposed model of canonical nucleocytoplasmic transport role in PSC1 intracellular targeting to paraspeckles. Stylized here are the outcomes of transfection experiments performed on HeLa cells. It was observed that modulating functional IMP $\alpha$ 2 levels and, subsequently, nucleocytoplasmic transport of IMP $\alpha$ 2 cargoes (depicted as green arrows) influences the number and size of detected PSPC1 nuclear speckles (shown in red) and the proportion of cells containing these PSPC1-positive paraspeckles. We hypothesize downstream impacts on the paraspeckle functions, proposed in current literature to include the nuclear retention of RNAs, changes to RNA processing, and stress responses.

measured a decrease in IMP $\alpha$ 2 levels as meiotic male germ cells transform into haploid round spermatids (Arjomand *et al.*, 2014). This cellular transition is accompanied by major changes in RNA synthesis, storage, and turnover, and it should be informative to document what roles PSPC1 serves during these stages. The contribution of changing PSPC1/PSF levels will also be important to consider along with measuring the stoichiometry of these proteins as spermatogenesis proceeds. Given the current knowledge of paraspeckle function, we hypothesize that modulation of PSPC1 delivery to paraspeckles will manifest as changes that include nuclear retention of specific A-to-I edited RNA transcripts, altered RNA processing, and changes to general cellular stress responses (Prasanth *et al.*, 2005; Chen and Carmichael, 2009; Fox and Lamond, 2010; Nakagawa *et al.*, 2011; Nakagawa and Hirose, 2012; Naganuma *et al.*, 2012; Hirose *et al.*, 2014). This further highlights the need to understand how IMP levels and functional activities are determined.

## MATERIALS AND METHODS

### Animals and tissue sample preparation

Postnatal testis were from Swiss and C57/BL6xCBA male mice, and fetal testes were collected from embryos staged by fore- and hindlimb morphology from time-mated pregnant Swiss female mice from both Monash University and the University of Queensland Animal Services. All investigations conformed to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation or the University of Queensland Animal Ethics Committee. Lysates of fetal and juvenile-age testis samples for Western blots were pooled from different mice; each adult testis lysate was from one animal. Tissues for coimmunoprecipitations (co-IPs), pull down, or whole-mount immunofluorescence were used immediately after collection, while tissues for all other protein analyses were snap frozen and stored at

$-80^{\circ}\text{C}$ . Tissues for immunohistochemistry were fixed in Bouin's for 5 h, dehydrated through graded ethanols, paraffin embedded, and sectioned (3–5  $\mu\text{m}$ ).

### E12.5 testis cDNA library construction and yeast two-hybrid screening

Total RNA was isolated from ~500 E12.5 testes using the Microfast Track mRNA isolation kit (Invitrogen, Melbourne, Australia) according to the manufacturer's instructions. A cDNA library was generated using the CloneMiner cDNA Library Construction Kit (Invitrogen, Version B) by ligation into the pDONR222 vector. The plasmid library was transformed into ElectroMAXDH10 $\beta$  T1 cells and then recombined into the Y2H prey vector, pDEST22, using the Invitrogen Gateway system.

Putative IMP $\alpha$ 2-binding partners were identified using the Invitrogen Y2H screening protocol. The pDEST22 library clones and full-length IMP $\alpha$ 2-pDEST32 bait plasmid (Ly-Huynh *et al.*, 2011) were cotransformed into MaV203-competent yeast. Cotransformed yeasts were screened for potential interactors using three different reporter genes, and plasmids were isolated from clones passing all three tests. The interaction was validated by a second yeast cotransformation into MaV203 cells with full-length IMP $\alpha$ 2-pDEST32 or pDEST32 alone.

### Constructs and vectors

The murine PSPC1 full-length cDNA construct (encoding aa 3–523) was amplified by PCR (mRNA Accession: NM\_025682.3) with forward (5'ggggacaagttgtacaaaaagcaggcttctaagaggaacctgaagcaag3') and reverse (5'ggggaccactttgtacaagaaagctgggtcttaatatctccgaccttattag3') primers. This was recombined into pDEST22 (for Y2H system), pDEST17 (for HIS-tagged bacterial expression), and dsRED2 (DsRed2-tagged mammalian cell expression) vectors using the Invitrogen Gateway System.

The Y2H constructs encoding IMP $\alpha$ 2, IMP $\alpha$ 4, and IMP $\alpha$ 6 in the pDest32 vector (Invitrogen) were previously generated (Ly-Huynh *et al.*, 2011). GFP-IMP $\alpha$ 2 constructs for mammalian cell expression were generated previously (Ly-Huynh *et al.*, 2011; Young *et al.*, 2011) and encoded full-length IMP $\alpha$ 2 (GFP-IMP $\alpha$ 2-FL), ED mutant (GFP-IMP $\alpha$ 2-ED), and truncated IMP $\alpha$ 2 (GFP-IMP $\alpha$ 2 $\Delta$ IBB). For bacterial cell expression and affinity-based purification, the same IMP $\alpha$ 2 variants were cloned into pGEX-6P-2 (GE Life Sciences, Rydalmere, Australia) for production of GST, GST-IMP $\alpha$ 2-FL, GST-IMP $\alpha$ 2-ED, and GST-IMP $\alpha$ 2 $\Delta$ IBB (Yasuda *et al.*, 2012; Miyamoto *et al.*, 2013). The GST-IMP $\beta$ 1 plasmid used the pGEX-2T vector (GE Life Sciences; Arjomand *et al.*, 2014).

### Purification of GST/HIS-tagged and tag-void recombinant proteins expressed in *Escherichia coli*

Bacterial proteins were induced in BL21-DE3 cells with 1.0 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside; HIS-tagged: 28°C, 3.5 h; GST-tagged: 18°C, overnight (O/N). Bacteria were then harvested (6000  $\times$  g, 10 min, 4°C), washed with cold NaCl (0.9% wt/vol), and bacterial pellets stored at -80°C.

Bacterial pellets with GST-tagged proteins were resuspended on ice in GST lysis buffer (50 mM Tris-HCl, pH 8.3, 500 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], and 0.2 mM phenylmethylsulfonyl fluoride [1 M stock in EtOH]) and lysed by freeze-thawing twice (alternating between liquid nitrogen and shaking at 20°C) followed by probe sonication (5  $\times$  30 s, 1-min rests). Affinity-based purification was as previously described (Miyamoto *et al.*, 1997, 2013) using glutathione Sepharose 4B slurry (GS4B; GE Life Sciences). GST-tagged proteins were eluted with GST elution buffer (100 mM Tris-HCl, pH 8.3, 100 mM NaCl, 1 mM EDTA [3Na], 2 mM DTT, 1  $\mu$ g/ml 1:1000 protease inhibitor cocktail [PIC, Calbiochem cocktail set III, Merck-Millipore, Bayswater, Australia], 20 mM glutathione). GST-void IMP $\alpha$  proteins were generated by instead cleaving the pGEX-6P linker between the GST tag and the IMP $\alpha$  by O/N incubation with PreScission Protease (GE Life Sciences; 80 U/ml of GS4B slurry, 4°C). All proteins were reequilibrated in GST dialysis buffer (20 mM HEPES-NaOH, pH 7.3, 100 mM CH<sub>3</sub>COOK, 2 mM DTT, 1:1000 protease inhibitor cocktail [PIC]), concentrated (Amicon Ultra centrifugal filters; Merck-Millipore), and stored at -80°C. Protein concentrations were estimated by DC Protein Quant Assay (Bio-Rad, Gladesville, Australia) and SDS-PAGE against a bovine serum albumin (BSA) standard.

Bacterial pellets with HIS-tagged proteins were resuspended on ice in Ni-NTA lysis buffer (300 mM NaCl, pH 8.0, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 40 mM imidazole, 1:1000 PIC) and processed as above (freeze-thawing, sonication, centrifugation). Supernatant was rotated with prewashed Ni-NTA agarose (Qiagen, Doncaster, Australia) slurry (4°C, 2 h), washed (5 $\times$ ) with cold Ni-NTA wash buffer (300 mM NaCl, pH 8.0, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 40 mM imidazole), then eluted in Ni-NTA elution buffer (300 mM NaCl, pH 8.0, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 200 mM imidazole). HIS-tagged PSPC1 protein tended to precipitate over time, so a fresh preparation was used in every assay, and protein concentrations were reestimated immediately (DC Protein Quant Assay).

### Co-IPs

Adult mouse testis were homogenized on ice through needles (18 to 30 gauge) in IP lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, PIC 1:500) and then centrifuged. Lysate supernatant (200  $\mu$ l [-200  $\mu$ g protein]) was incubated (4°C with rotation, 2 h) with 2.0  $\mu$ g anti-IMP $\alpha$ 2 (cat. no. sc6917, lot no. c1407; Santa Cruz Biotechnology) and added to a microspin column

(GE Life Sciences) containing 60  $\mu$ l prewashed protein A/G-PLUS-Agarose (Santa Cruz Biotechnology, distributed by ThermoFisher Scientific, Scoresby, Australia), and the volume was brought to 500  $\mu$ l with IP lysis buffer for O/N incubation (4°C with rotation). Samples of flowthrough, washes (IP lysis buffer), and elution in 2 $\times$  sample buffer (4% vol/vol SDS, 25% vol/vol glycerol, 12.5% vol/vol [0.5M Tris HCl, pH 6.8], 5% vol/vol  $\beta$ -mercaptoethanol, bromophenol blue) were collected for Western blot detection of PSPC1 and IMP $\beta$ 1.

### IMP $\alpha$ 2 pull-down experiments

Testis lysates prepared as for co-IPs were precleared with GS4B slurry (60  $\mu$ l/200  $\mu$ l lysate, 4°C with rotation, ~2 h). GS4B slurry aliquots (60  $\mu$ l) were equilibrated with transport buffer (20 mM HEPES-NaOH, pH 7.3, 110 mM CH<sub>3</sub>COOK, 2 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 5 mM CH<sub>3</sub>COONa, 0.5 mM EGTA, 2 mM DTT, PIC 1:500) in microspin columns (GE Life Sciences), and then GST-tagged protein (300 pmol) was added in 300  $\mu$ l of transport buffer for binding to GS4B (4°C, 1 h with rotary agitation). Precleared testis lysate (200  $\mu$ l) was added and incubated O/N (4°C, gentle rotation). Flowthrough, washes (transport buffer), and eluate (2 $\times$  sample buffer) samples were collected for Western blot detection of PSPC1.

### ELISA-based binding assay

An ELISA-based IMP-binding assay (Hubner *et al.*, 1997) was performed using recombinant HIS-tagged PSPC1 (5 pmol/well) coated onto 96-well plates (cat. no. 260836; Nunc, distributed by ThermoFisher Scientific, Scoresby, Australia). Triplicate wells were incubated with different concentrations of GST, GST-tagged IMPs, or GST-tag cleaved IMPs preincubated with GST-IMP $\beta$ 1. After washing, IMP binding was quantitated using an anti-GST antibody (cat. no. 27457701; GE Life Sciences) and a rabbit anti-goat secondary conjugated to alkaline phosphatase (cat. no. A-4187; Sigma-Aldrich). The absorbance change at 405 nm was recorded for 90 min after *p*-nitrophenyl phosphate substrate (Sigma-Aldrich, Castle Hill, Australia) addition.  $K_d$  and  $B_{max}$  values were estimated by curve fitting using KaleidaGraph version 3.6 by Synergy.

### Western blotting

Samples were homogenized in RIPA buffer with PIC at 4°C, as previously described (Loveland *et al.*, 1999). Tissue lysates (30  $\mu$ g per lane) were mixed with 2 $\times$  sample buffer, run on 12% SDS-PAGE gels with protein-size standards (PageRuler Prestained Protein Ladder; Fermentas-Thermo Scientific, Scoresby, Australia), transferred to Amersham Hybond-C Extra nitrocellulose membrane (GE Life Sciences), and blocked in 5% skim milk/Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.5) for 1 h. Washes were with 0.1% Tween 20/TBS, and incubations were at room temperature (RT) unless otherwise stated. The membrane was rotated with an anti-PSPC1 mouse mAb (Kawahara *et al.*, 2006; 1:200 in 5% skim milk/0.1% Tween-20/TBS, O/N, 4°C) and washed, and then rabbit anti-mouse IRDye800 secondary antibody (1:10,000; cat. no. 610431020; Rockland Immunochemicals, distributed by Jomar Bioscience, Kensington, Australia; in 5% skim milk/0.1% Tween-20/0.01% SDS/TBS) was applied (60 min) and detected using the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE). Subsequent incubation with anti  $\beta$ -tubulin mouse mAb (1:10,000; Sigma-Aldrich) provided the loading control. At least two independent lysates were examined for each age and antibody, with consistent results. The IMP $\alpha$ 2 pull-down and co-IP samples used the PSPC1 mAb diluted 1:1000 to detect PSPC1. Co-IP samples were subsequently blotted with IMP $\beta$ 1 antibody (1:1000; cat. no. sc11367; Santa Cruz Biotechnology) and goat

anti-rabbit IRDye800 secondary (1:10,000; cat. no. 611132122; Rockland Immunochemicals). The siRNA samples were simultaneously blotted with goat anti-IMP $\alpha$ 2 (1:1000; cat. no. sc-6917; Santa Cruz Biotechnology) and mouse anti- $\alpha$  tubulin (cat. no. T5168; Sigma-Aldrich) antibodies with rabbit anti-goat Alexa Fluor 680 (1:10,000; cat. no. A21088; Molecular Probes–Invitrogen) and rabbit anti-mouse IRDye800 (1:10,000; cat. no. 610431020; Rockland Immunochemicals) secondary antibodies.

### Whole-mount immunofluorescence of mouse seminiferous tubules

Tubules were collected in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) from decapsulated adult mouse testes (Szczepny *et al.*, 2009). Within 1 h of testis collection, 1-cm tubule lengths were fixed simultaneously in 3.2% paraformaldehyde (PFA; Merck; 1.5 h, RT). Processing occurred in 24-well plate wells (cat. no. 353047; BD Falcon, North Ryde, Australia) containing >3 tubule fragments with gentle rocking. Tubules were incubated in PBS/0.5% BSA (Sigma-Aldrich; 30 min, RT) and then in 0.5% Triton X-100 (Merck) in PBS (1 h, RT). Anti-PSPC1 mouse mAb (1:100) and goat polyclonal antibody to IMP $\alpha$ 2 (1:100; cat. no. sc-6917; Santa Cruz Biotechnology) in 0.5%BSA/PBS were added (O/N, 4°C). After PBS washes (4x, 1–3 h each), secondary antibodies (rabbit anti-mouse Alexa Fluor 546 [cat. no. A11060; Molecular Probes–Invitrogen], donkey anti-goat Alexa Fluor 488 [cat. no. A11055; Molecular Probes–Invitrogen] and a nuclear marker [4',6-diamidino-2-phenylindole, DAPI; Molecular Probes–Invitrogen]; TO-PRO [Molecular Probes–Invitrogen]; or Draq5 [BioStatus, distributed by Sapphire Bioscience, Waterloo, Australia]) were added (1:200 in 0.5% BSA/PBS, O/N, 4°C). Samples were washed in PBS (4x, 1–3 h, then O/N, 4°C) and then imaged either wet with an immersion lens or mounted in a “tape well” formed by placing two layers of double-sided tape with holes punched onto a slide. The well was filled to excess with mounting media (GVA Mount [Zymed–Invitrogen], SlowFade Gold, or Pro-Long Gold [Invitrogen]), and the coverslip was pressed to close the well. Excess mounting media was removed, samples were left flat to set (O/N, 4°C) and were then sealed with nail polish. Images were acquired with a Leica (Mannheim, Germany) SP5 laser-scanning confocal system with DMI6000 microscope using a 20x/1.0 NA water-immersion lens in the Monash Micro Imaging facility (MMI, Clayton, Australia).

### Immunohistochemistry

Immunohistochemistry with anti-PSPC1 (Kawahara *et al.*, 2006) and anti-IMP $\alpha$ 2 (cat. no. ab84440; Abcam) antibodies was performed as previously described (Loveland *et al.*, 1999) using 50 mM glycine for antigen retrieval (pH 3.5; >90°C for 8 min), and diluted primary antibodies (0.1% BSA/TBS) for O/N incubation at RT. Control sections lacked primary antibody. Secondary antibody incubations (1:500 in 0.1% BSA/TBS, 1 h), either biotinylated rabbit anti-mouse antibody (cat. no. E0354; DAKO, North Sydney, Australia) or biotinylated goat anti-rabbit antibody (cat. no. 656140; Invitrogen), were followed by Vectastain Elite ABC kit reagent (Vector Laboratories, distributed by Abacus Als, Waterford, Australia). Antibody binding was detected as a brown precipitate following development with 0.20 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Harris hematoxylin counterstain (Sigma-Aldrich) and DPX mountant (Sigma-Aldrich) were applied. Germ and somatic cell types were identified based on nuclear morphology and position within the developing gonad (Byskov, 1986; Russell, 1990). Slides were imaged using a Mirax Midi slide scanner (Carl Zeiss, Sydney, Australia) with a 20x objective.

### Cell culture, transfection, and indirect immunofluorescence staining

COS-7 and HeLa cells were maintained in DMEM with 5% (vol/vol) and 10% (vol/vol) fetal calf serum, respectively, penicillin-streptomycin, L-glutamine, and MEM nonessential amino acids in 5% CO<sub>2</sub> at 37°C. Twenty-four hours before transfection, cells were seeded on round coverslips in medium lacking penicillin-streptomycin in 24-well plates for GFP/red fluorescent protein (RFP)-tagged transfections or 12-well plates for siRNA knockdown. PSPC1 and IMP $\alpha$ 2 DNA constructs were transfected using Lipofectamine 2000 (Invitrogen) with 2.5  $\mu$ g (single plasmid) or 1.25  $\mu$ g (each cotransfection), following the manufacturer's method. The Dharmacon ON-TARGETplus siRNA system (GE Life Sciences) was used as per the manufacturer's instructions for siRNA knockdown of IMP $\alpha$ 2 (SMARTpool L-004702-00) with a nontargeting (SCRAM siRNA) control pool (D-001810-10) as the siRNA negative control.

Cells were fixed 48 h later in 3.2% PFA/PBS for 10 min and then washed and stored in PBS at 4°C. For indirect immunofluorescence staining, all incubations and washes (4x, 5 min each) were at RT with rocking. Cells were blocked (0.5% BSA/PBS, 30 min) and permeabilized (0.1% Triton X-100/PBS, 10 min), then anti-PSPC1/PSF (Kawahara *et al.*, 2006; 1:100 in 0.5% BSA/PBS) or anti-PSPC1/PSF and anti-IMP $\alpha$ 2 (1:100; cat. no. ab84440; Abcam, Melbourne, Australia) were applied for O/N incubation. Coverslips were washed and then incubated with secondary antibodies (rabbit anti-mouse Alexa Fluor 546 for GFP/RFP-tagged transfections and donkey anti-mouse Alexa Fluor 488 plus goat anti-rabbit Alexa Fluor 546 for siRNA knockdown samples [1:200 in 0.5% BSA/PBS, 1.5 h; cat. nos. A11060, A21202, A11010; Molecular Probes–Invitrogen]). Coverslips were washed, incubated with a nuclear marker (DRAQ5 [BioStatus] or DAPI [Invitrogen]), mounted on glass slides with GVA Mount (Zymed–Invitrogen) O/N at 4°C, and then sealed using nail polish.

### COS-7 and HeLa cell image acquisition

COS-7 cells were imaged using a Leica (Mannheim, Germany) TCS/NT laser-scanning confocal system on a DMIR microscope at 60x magnification (MMI). HeLa cell imaging was performed with a Leica SP5 laser-scanning confocal system (DMI6000 microscope, motorized stage, 63x water/glycerol objective; MMI). Images were collected as Z-series and tiled in a 7 x 7 field-of-view grid (~1.7 mm<sup>2</sup>).

### Imaris-assisted PSPC1 nuclear speckle analysis of cells transfected with GFP/RFP-tagged constructs

Individual transfected cells were three-dimensionally cropped into new files from the larger image sets described above for analysis. Cells expressing GFP or GFP-tagged IMP constructs were first identified using the Imaris software package (Bitplane, version 7). The Imaris batch processor mapped a “surface” around all cells within every image with GFP signal above a threshold level set from mock-transfected cell images. During the subsequent cell cropping, manual assessment was also performed, so cells were selected for analysis only if the entire cell nucleus was clearly defined and distinguishable within the image (x, y, and z); cells with overlapping nuclei or multinucleated cells were excluded. Rare cells containing cytoplasmic PSPC1 speckles were also excluded. To assess PSPC1 speckle number, size, and PSPC1 intensity within each cell, we used the Imaris batch processor to perform a “spots” analysis on each cell (individual image). Results were combined using the CSV Merge command, and the output files were manipulated using Excel (Microsoft, version 2007) and analyzed using SPSS software (IBM, version 17.0).

## Imaris-assisted image analysis to detect PSPC1 nuclear speckles in cells with siRNA knockdown or PSF nuclear speckles in cells transfected with GFP-tagged constructs

Individual cells, nuclei, and PSPC1/PSF nuclear foci were identified in the larger image sets described above using the Cells module of the Imaris software package (Bitplane, version 7). The results (output in CSV file formats) were combined and manipulated using Python scripts (Python Software Foundation, version 2.7) and then analyzed using SPSS software (IBM, versions 17 and 20).

### Statistical analysis

For statistical testing, individual cells were assumed to be independent, but speckles within each cell were assumed to be correlated. When analyzing the individual cell or speckle data, three outcome types were generated: 1) binary responses based on whether or not a cell was positive for speckles, 2) counts data based on the number of speckles within each cell (including/excluding zeros), and 3) continuous data based on speckle volume sum and speckle PSPC1 intensity sum.

Comparisons between groups were made using generalized linear models; logistic regression for the binary data and linear regression for the count and continuous data. As the count and continuous data were both skewed, the data were transformed using the natural logarithm to allow valid statistical inference from the linear regression models. The *p* values are based on the transformed data; however, the results were then back-transformed to give estimates in the original scale for ease of interpretation. By taking the exponent of the mean of log-transformed data, the GM (and confidence intervals [CIs]) was obtained on the original linear scale. By taking the exponent of the linear regression coefficients obtained on the log-transformed scale, the ratio of the GMs (and their 95% CIs) was obtained on the original scale. The IMP $\alpha$ 2-ED group and the SCRAM siRNA group were used as the reference groups for all models within the GFP/RFP-tagged transfections and the siRNA knockdowns, respectively. Odds ratios are given for logistic regression results. When assessing data on a per-speckle basis, continuous outcomes were examined, which again required log transformations. Generalized estimating equations (GEEs) were used to enable correlation between speckles originating from the same cell (Hanley *et al.*, 2003). Mann-Whitney *U*-tests and plots with arithmetic means (and 95% CIs) identified statistically significant differences for the same groups.

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