

PSF: nuclear busy-body or nuclear facilitator?

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PTB-associated splicing factor (PSF) is an abundant and essential nucleic acid-binding protein that participates in a wide range of gene regulatory processes and cellular response pathways. At the protein level, PSF consists of multiple domains, many of which remain poorly characterized. Although grouped in a family with the proteins p54nrb/NONO and PSpC1 based on sequence homology, PSF contains additional protein sequence not included in other family members. Consistently, PSF has also been implicated in functions not ascribed to p54nrb/NONO or PSpC1. Here, we provide a review of the cellular activities in which PSF has been implicated and what is known regarding the mechanisms by which PSF functions in each case. We propose that the complex domain arrangement of PSF allows for its diversity of function and integration of activities. Finally, we discuss recent evidence that individual activities of PSF can be regulated independently from one another through the activity of domain-specific co-factors. © 2015 The Authors. *WIREs RNA* published by John Wiley & Sons, Ltd.

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INTRODUCTION

PTB-associated splicing factor/splicing factor proline-glutamine rich (PSF or SFPQ), as the names imply, was first identified as a protein required for pre-mRNA splicing that interacts with the splicing regulatory protein polypyrimidine tract-binding protein (PTB).¹ Cloning and sequencing of this protein also revealed it to be markedly enriched for proline and glutamine residues.¹ However, these names belie the complexity of protein domains, interacting partners, and cellular activities that have subsequently been ascribed to PSF in the 20+ years since its initial characterization. Indeed, we are only just beginning to

fully appreciate the broad importance of this protein, and much remains to be understood about how PSF carries out its many roles in DNA and RNA stability and expression.

PSF belongs to a conserved family of multifunctional nuclear factors termed DBHS (Drosophila behavior human splicing) proteins, which also includes human p54nrb (also known as NONO) and PSpC1 (paraspeckle protein component 1).^{2,3} All three of these DBHS proteins are conserved throughout vertebrate species, while flies, worms, and yeast express a single DBHS protein. DBHS proteins are defined by a core domain arrangement consisting of tandem RNA-recognition motifs (RRMs), a ~100 amino acid coiled-coil domain, and a conserved intervening sequence referred to as a NONA/ParaSpeckle (NOPS) domain. Importantly, the NOPS and RRM2 domains mediate the formation of homodimers and heterodimers among the DBHS proteins⁴ (see below). Not surprisingly, therefore, all three DBHS proteins often co-localize and co-purify together.^{5,6}

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In terms of subcellular localization, PSF and the other DHBS proteins can be found in the nucleoplasm and nucleolar caps as well as in paraspeckles.^{2,3,7} Although not completely understood, the partitioning of PSF into these various compartments may be controlled by cellular environment and/or post-translational modifications (PTM; see *Regulation*). Of particular interest is the location of PSF in paraspeckles, as this is a definitive feature of DHBS proteins.³ Paraspeckles are subnuclear bodies that are often present adjacent to, but distinct from, speckles and are defined by the presence of the NEAT1 (nuclear-enriched abundant transcript 1) noncoding RNA (ncRNA) and the DHBS proteins.^{3,8} Knockdown studies have shown that both PSF and p54nrb/NONO are required for the formation of paraspeckles, while PSPC1 is less critical for paraspeckle formation and may localize to these structures as a consequence of the protein–protein interactions among all the DHBS proteins.⁹ The structure, regulation, and function of paraspeckles have been reviewed elsewhere,^{3,7} so are not covered in depth here.

In addition to the common DHBS core, PSF features additional domains (see below) that are not present in p54nrb/NONO or PSPC1.² These PSF-specific domains confer unique functions and regulatory sites to PSF that are not matched in the other DHBS proteins. Notably, PSF, but not the other vertebrate DHBS proteins, is essential for cellular viability. In cultured human cells, reduction of PSF expression by as little as twofold to threefold induces rapid apoptosis.^{10,11} Conversely, p54nrb/NONO is readily knocked down with little phenotype, and some mammalian cell types do not express detectable amounts of PSPC1.^{11–13} In zebrafish, PSF is necessary for general cell survival and for neuronal development, while in mice even modest depletion of PSF in thymocytes is sufficient to block T-cell development.^{10,14} Moreover, somatic mutations in the gene encoding PSF, or gene fusion events between PSF and other proteins, have been linked to multiple diseases including autism,¹⁵ Alzheimer's disease,¹⁶ renal cell carcinoma,¹⁷ acute myeloid and lymphoblastic leukemia,^{18,19} and prostate cancer.²⁰ Whether the expression or function of PSF is altered in these disease states remains to be determined.

In sum, PSF is a unique multidomain protein that is essential to the viability of many, if not all, eukaryotic cells. However, the precise reason PSF is required for cell growth and development remains unknown. As described below, PSF has been shown to play a role in many aspects of nucleic acid biology, from genome stability to RNA processing. Moreover, PSF

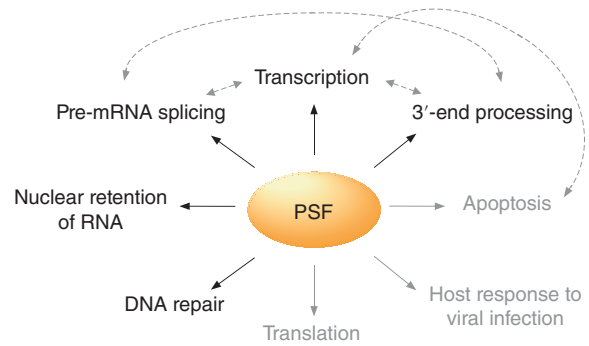


FIGURE 1 | Cellular activities of PTB-associated splicing factor (PSF). A schematic highlighting the cellular activities which PSF has thus far been reported to regulate. Black activities are those for which the mechanism of PSF activity is best understood. Gray activities are those with less experimental support or unclear mechanism. Dotted double arrow indicates coordination of activities through PSF.

has the potential to serve as a bridge between nuclear processes, a critical consequence of PSF's multifaceted existence and a theme highlighted by several studies. Is there one cellular process for which PSF is particularly indispensable, or does the loss of PSF result in cell death through partial compromise or disconnection of many processes simultaneously? In order to answer these questions, we need a better understanding of the mechanism by which PSF contributes to each of its known activities and of how the participation of PSF in this assortment of activities is regulated.

CELLULAR ACTIVITIES OF PSF

A confounding issue in the study of PSF is the fact that this protein has been implicated in such a wide range of cellular activities (Figure 1). To begin to understand PSF, one must first consider what is known about its biochemical and cellular functions and determine which activities are clearly direct functions of PSF, and which may be indirect consequences of other mechanisms or have been less convincingly demonstrated.

Splicing

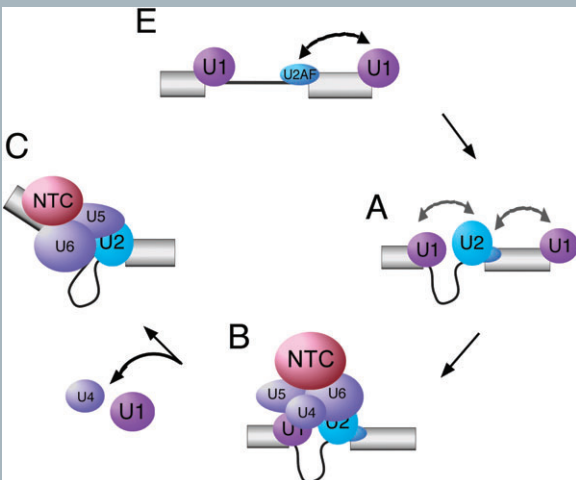
The first activity attributed to PSF was pre-mRNA splicing. In 1991, Patton et al. demonstrated that a complex containing the RNA-binding protein PTB and an unknown splicing factor of apparent molecular weight of 100 kDa was required to splice an α -tropomyosin pre-mRNA substrate in nuclear extracts.²¹ They went on to clone this unknown protein in 1993 and coined the name PSF.¹ In nuclear extract, pre-mRNA splicing requires the step-wise assembly of multiple spliceosomal subunits and

co-factors to form the final enzymatic spliceosome complex²² (Box 1). Immunodepletion of nuclear extract with antibodies to PSF was shown to block spliceosome assembly at the earliest steps.¹ These *in vitro* experiments were interpreted to suggest that PSF had a general and essential role in early spliceosome formation; however, with hindsight it seems likely that many PSF-co-associated proteins were also lost during immunodepletion of PSF. Moreover, it is now known that individual pre-mRNA substrates often have distinct sensitivities to even ‘core’ splicing factors.^{23,24} Accordingly, caution should be taken in overinterpreting these initial experiments. Indeed, later proteomic studies have identified PSF in catalytic or immediately precatalytic spliceosomal complexes,^{25–27} and biochemical studies have demonstrated a role for PSF in the second catalytic step of splicing (i.e., exon joining) of some,²⁸ but not all,²⁹ pre-mRNA substrates.

BOX 1

SPLICEOSOME ASSEMBLY AND FUNCTION

The spliceosome consists of five U snRNPs (U1, U2, U4, U5, and U6), each comprised of a small RNA and associated proteins, plus additional protein such as in the NineTeen complex (NTC). These subunits associate with the pre-mRNA in an ordered fashion to form the catalytic core in which intron removal and exon joining take place. Assembly of the spliceosome involves a network of dynamic protein and/or RNA interactions, in which exons are first recognized and then paired together within the catalytic core. E and A complexes are considered to be early complexes, while C complex is the final catalytically active spliceosome.



These early studies of PSF function, together with two decades of increased knowledge of splicing regulation, suggest a model in which PSF is loosely associated with the spliceosome in such a way that it can impact spliceosome assembly in a substrate-dependent manner. Such activity is typical of proteins we now call splicing regulators, which are broadly defined as any protein that controls alternative splicing.³⁰ PSF has recently been shown to influence alternative splicing of both the CD45 (cluster of differentiation 45) and Tau genes through direct interaction with specific RNA sequences. In the Tau gene, PSF interacts with a stem-loop structure at the exon–intron boundary downstream of exon 10 to repress inclusion of this exon in the final mRNA.³¹ Similarly, PSF represses inclusion of exon 4 of the human CD45 gene by binding to a pyrimidine-rich region within this exon.^{11,32}

PSF can also promote exon inclusion. For example, PSF induces the neural-specific inclusion of the N30 exon of nonmuscle myosin heavy-chain II-B by promoting binding of the splicing regulator Rbfox3 (RNA-binding protein, fox-1 homolog 3) to the substrate pre-mRNA through protein–protein interactions.³³ Furthermore, Cho et al. demonstrated that the inclusion of exon 7 of SMN2 (survival of motor neuron 2) in neuroblastoma cells is induced by binding of PSF to a purine-rich sequence in the exon.³⁴ Interestingly, we note that mis-splicing of SMN2, like Tau, has been implicated in neurologic pathology,^{35,36} lending further support to a possible role for PSF in human disease.

Despite more than two decades of study of PSF in splicing, the exact mechanism(s) through which PSF regulates exon use remains unknown. The case of Tau exon 10 likely represents an example of direct steric hindrance, in which the binding of PSF to the hairpin structure precludes binding of the U1 snRNA component of the spliceosome to the 5' splice site embedded within this hairpin.³¹ However, it is less clear how binding of PSF within an exon may repress (CD45) or enhance (SMN2) exon inclusion. One intriguing possibility in the case of SMN2 is that PSF may aid in the recruitment of the U4/U5/U6 tri-snRNP (small nuclear ribonucleoprotein) subunit of the spliceosome (Box 1), as it has been shown to interact directly with stem-loop 1 of the U5 snRNA component of the tri-snRNP.³⁷ Finally, we note that although there is much evidence to support direct regulation of the spliceosome by PSF, it may also impact splicing through its effect on transcription and/or polyadenylation. These additional activities, and the potential PSF-mediated coupling of transcription, splicing, and polyadenylation, are described below.

3'-End Processing

Similar to many proteins initially characterized as splicing factors, PSF is now known to be involved in many aspects of mRNA biogenesis. Indeed, within a few years of the initial discovery of PSF, evidence began to emerge that this protein might also regulate the 3' polyadenylation of mRNAs. The role of PSF in 3'-end formation was first identified as part of a search for protein components of a complex (SF-A) containing the U1A protein distinct from the U1snRNP.³⁸ Sucrose gradient fractionation and immunoprecipitation of HeLa cell extracts suggested that the SF-A complex contained five proteins in addition to U1A, the largest of which was identified as PSF.³⁹ Furthermore, the SF-A complex was found to contain additional splicing factors such as p54nrb/NONO, and antibodies to the complex affected coupled splicing and polyadenylation at suboptimal polyadenylation sites.^{40,41} Finally, tethering of PSF adjacent to the suboptimal polyadenylation signal from the COX-2 (cyclooxygenase 2) 3'UTR was shown to activate use of this polyadenylation site in the absence of any other regulatory sequences, demonstrating a direct role of PSF in 3' processing site choice.⁴⁰ Additional reports have also shown an effect of PSF in stimulating cleavage and polyadenylation at a weak site in the prothrombin F2 gene and in reporter constructs containing the SV40 polyadenylation site,^{42,43} and have observed PSF within the 3'-end processing complex purified from mammalian cells.⁴⁴ Taken together, these studies suggest that PSF and associated protein factors may help ensure that polyadenylation at non-canonical or suboptimal polyadenylation signals can take place. The PSF-driven mechanisms that promote the use of polyadenylation signals have not yet definitively been elucidated, but may include recruitment or stabilization of the basal polyadenylation machinery.

Nuclear Retention

As mentioned above, PSF is often localized within the nucleus to structures known as paraspeckles. Paraspeckles are built around the long ncRNA NEAT1, and are directly involved in regulating nuclear retention of mRNAs.^{7,8,45} This signal for nuclear retention appears to be the inclusion of the atypical nucleobase inosine in messages to be retained.⁴⁶ Inosine (I) is the product of deamination of adenosine (A). Such A-to-I deamination is catalyzed by the ADAR (Adenosine De-Aminase RNA-specific) RNA-editing enzymes, which preferentially bind to double-stranded RNA.⁴⁷ Most paraspeckle-retained messages appear to contain long inverted repeats that are predicted to form extended RNA duplexes, which

are then extensively edited by ADAR.^{8,47} PSF, together with Matrin 3, PSPC1, and p54nrb/NONO, binds with high affinity to hyper A-to-I edited mRNAs, presumably through specific recognition of the inosines.⁴⁶ This high-affinity interaction anchors hyperedited RNAs within paraspeckles and prevents their export to the cytoplasm.^{8,46} Notably, such PSF-dependent nuclear retention has been observed to be relieved either by loss of NEAT1 expression and concomitant dissociation of paraspeckles,⁸ or by specific cleavage of the inosine-containing portion of the message, typically in an extended 3'UTRs.⁴⁸ Thus, nuclear retention by PSF is an important regulatory layer in determining the export and expression of mammalian mRNAs.

Translation

Although PSF is strongly nuclear localized under normal circumstances, a few reports have suggested a role for PSF in cytoplasmic IRES (internal ribosome entry site)-mediated translation.^{49,50} Translation typically initiates at the capped 5' end of mRNAs. However, translation can also initiate at internal ribosome entry sites, or IRESs, which are complex secondary or tertiary RNA structures internal to the mRNA that facilitate ribosome assembly.⁵¹ In one study, Sharathchandra et al. demonstrated that in cell lysates and purified *in vitro* assays PSF can bind directly to an IRES element located within the p53 gene.⁴⁹ Whether PSF interacts with the p53 IRES in cells remains unclear; however, knockdown of PSF in H1299 cells decreased both IRES- and non-IRES-dependent expression of p53, suggesting an indirect effect.⁴⁹ More convincing evidence for a role of PSF in translation comes from a study showing that PSF participates in IRES-mediated translation of a set of apoptotic-regulated genes during TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis.⁵⁰ Importantly, this IRES-activity correlated with mis-localization of PSF to the cytoplasm in response to TRAIL-induced apoptosis.⁵⁰ These results suggest that cytoplasmic localization and activity of PSF might be used by cells as a gauge of cellular crisis, a hypothesis that is further supported by the observed cytoplasmic accumulation of PSF in Alzheimer's and Pick's disease.¹⁶

Transcription

Importantly, PSF impacts not only RNA biogenesis but also DNA-mediated processes. The first DNA-related role to be uncovered for PSF was the regulation of transcription. Indeed, it is now known that PSF can act as both a positive and a negative transcriptional

regulator. Roepcke et al. showed that in HEK293 cells PSF and p54nrb/NONO bind to a tandem sequence motif that serves as an enhancer and positively regulates expression of ribosomal protein genes such as RPL18 (60S ribosomal protein L18).⁵² The PSF and p54nrb/NONO complex can also enhance transcription by serving as a bridge between RNA polymerase II (Pol II) transcription complex and other nuclear proteins. PSF and p54nrb/NONO specifically bind to the Pol II C-terminal domain (CTD), and recruit other splicing and/or polyadenylation factors to the site of transcription.^{43,53} Given the extensive interplay of transcription and RNA processing,⁵⁴ such physical nucleation is predicted to facilitate the overall efficiency of transcription. PSF has also been shown to enhance transcription by linking Pol II to nuclear actin and/or through the recruitment of gene-specific co-enhancers.^{55,56}

As an aside we note that the ability of PSF to interact with the transcription machinery is also necessary for transcription-coupled stimulation of splicing and 3'-end processing.⁴³ Along this same theme, PSF may also serve as a bridge to couple 3'-end processing and/or the Pol II complex to transcription termination. Specifically, PSF associates with the 5' to 3' exonuclease XRN2/Rat1 (5'-3' exoribonuclease 2), which is essential for transcription termination.¹³ Immunodepletion of PSF causes an accumulation of 3' cleaved RNA *in vitro*, consistent with a loss of XRN2/RAT1 activity,¹³ although it is unknown whether association of PSF to Pol II is required for recruitment of XRN2/RAT1 or whether this activity entails only the involvement of PSF with the 3'-end processing machinery.

The ability of PSF to function as an adaptor also allows it to repress transcription by recruiting histone deacetylases (HDACs) to force 'repressive' chromatin marks at targeted genes. For example, PSF has been shown to recruit HDACs to DNA-bound nuclear hormone receptors or circadian rhythm-controlling factors, often through interaction with the HDAC-associated protein SIN3A (SIN3 transcription regulator family member A).⁵⁷⁻⁵⁹ In another example, PSF represses STAT6 (signal transducer and activator of transcription 6)-mediated transcription of IgE by binding to STAT6 and recruiting HDAC1 to the IgE promoter.⁶⁰ The interaction of STAT6 and PSF is dependent on IL-4-induced phosphorylation of STAT6, demonstrating the potential for condition-specific regulation of transcription through PSF.⁶⁰ Finally, PSF can also directly interact with DNA to repress the transcription of neighboring genes, as has been observed for IL-8 and RAB23 (Ras-related protein Rab-23),^{61,62} although the mechanism of this

repression remains unknown. Interestingly, in both of these latter two instances, transcriptional repression by PSF is regulated by competition with ncRNAs, as will be discussed further below.

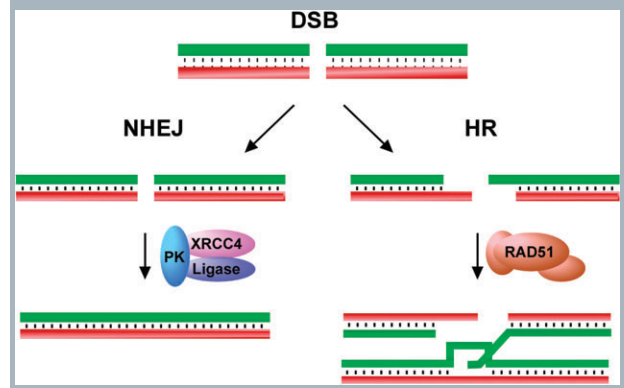
DNA Repair

A second well-documented role of PSF in DNA biology is in the repair of damaged DNA. The molecular response to DNA damage involves a highly orchestrated set of events in which multiple protein complexes sense and repair the lesion or break (Box 2). Many RNA-binding proteins impact the DNA damage response (DDR) indirectly through controlling the expression of DDR proteins.⁶³ However, several studies have demonstrated a direct role for PSF in the DDR, specifically in the recognition and repair of DNA DSBs.^{12,64-66} PSF binds directly to DSBs both *in vitro* and in cells through part of its N-terminal region encompassing the RGG box through the proline-rich domain (see below).^{12,64,66} This same N-terminal domain of PSF also mediates interaction of PSF with the recombinase RAD51D (DNA repair protein RAD51 homolog 4).^{64,65} Importantly, RAD51D is part of a complex that plays an essential role in HR, one of the main paths to DSB repair (Box 2). Remarkably,

BOX 2

DOUBLE-STRAND BREAK REPAIR

Repair of DNA double-strand breaks (DSBs) occurs by one of two mechanisms. Nonhomologous end-joining (NHEJ, left) is rapid but error prone and involves the activity of a DNA-dependent protein kinase (PK) and ligase in complex with XRCC4 to seal the breaks. By contrast, homologous recombination (HR, right) utilizes the RAD51 complex to promote strand invasion repair by templating on the sister chromosome.



PSF promotes HR both by directly activating strand invasion and by stimulating the repair activity of the RAD51 complex.^{64,67} Moreover, binding of PSF to DSBs also recruits p54nrb/NONO, which, in turn, recruits machinery involved in NHEJ, the other major branch of DSB repair (Box 2). Consistently, cellular depletion of PSF results in defects in both HR and NHEJ, resulting in a delay in DSB repair, accumulation in S phase, chromosomal instability, sister chromatid cohesion defects, and sensitivity to DNA-damaging agents.^{65,66}

Viral Infection

In addition to the above-mentioned roles of PSF in specific cellular processes, PSF has also been reported to regulate replication and infectivity of several mammalian viruses, including HIV,^{68,69} hepatitis delta virus (HDV),⁷⁰ and influenza.⁷¹ One potential mechanism by which PSF may accomplish this is through regulation of viral RNA processing. PSF appears to promote production of viral transcripts through its interaction with HIV-encoded Rev, as siRNA depletion of PSF results in a decrease in unspliced viral RNAs.⁶⁹ PSF can also bind to HIV-1 viral mRNA through the *cis*-acting regulatory elements (INS) in the gag mRNA, resulting in decreased expression of Rev-dependent transcripts, including gag-pol and env.⁶⁸ In the case of gag-pol and env, PSF has been proposed to function at an mRNA degradation step⁶⁸; however, no evidence was provided to support this notion.

Influenza virus appears to require PSF as an essential host factor in order to ensure proper viral RNA multiplication and replication.⁷¹ Depletion of PSF by siRNA in influenza-infected A549 cells resulted in a robust decrease in viral yields, as well as reduced and temporally delayed flu virus gene expression and decreased overall viral transcription.⁷¹ These effects of PSF were specific to influenza virus as neither adenovirus replication nor VSV (vesicular stomatitis virus) replication was affected by siRNA to PSF. Curiously, viral splicing was not affected by PSF depletion, suggesting that viral RNA splicing is regulated by a subset of host factors.⁷¹ Finally, PSF interacts with the terminal stem-loop domains of HDV RNA in both polarities, and is thought to be involved here as a host factor in the life cycle of HDV.⁷⁰ The authors speculate that this interaction disrupts the host cell processes in which PSF is a major player. They also hypothesized that PSF may be involved in HDV RNA transcription and/or replication. The authors showed no evidence for this, but cite PSF's key role in HIV-1 vRNA regulation in support of their hypothesis.⁷⁰

Apoptosis

Apoptosis, or programmed cell death, is a normal physiological process by which damaged or superfluous cells are eliminated. When apoptosis is impeded, as in many cancers, uncontrolled cell proliferation ensues. Tsukahara et al. showed that PSF interacts with peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear receptor involved in cell proliferation and apoptosis.⁷² Knockdown of PSF in PPAR γ -expressing DLD-1 colon cancer cell lines resulted in loss of the autophagic marker LC3B (microtubule-associated proteins 1A/1B light chain 3B) and a corresponding induction of apoptosis via caspase-3.^{72,73} Interestingly, the same consequence of PSF knockdown was not observed in PPAR γ -low HT-29 cells.^{72,73} Taken together, these studies suggested that PSF is a regulator of cell death in some colon cancer cells, and the relative expression levels of PPAR γ appear to play an important role.⁷² Loss of PSF activity has also been shown to induce apoptosis in zebrafish and in murine T cells.^{10,14} Although the mechanism of apoptosis induction in these systems is not well understood, at least in T cells loss of PSF-dependent expression of histone genes has been suggested as a contributor to apoptosis.¹⁰

Other studies have examined the changes in subcellular localization of PSF during apoptosis. Shav-Tal et al. showed that nuclear detection of PSF was reduced during apoptosis as visualized by monoclonal antibody staining.⁷⁴ They went on to demonstrate that PSF is not degraded, but is hyperphosphorylated during apoptosis. The authors demonstrate that hyperphosphorylation does not directly preclude antibody recognition, but suggest that phosphorylation induces changes to the conformation of PSF and/or its association with new protein partners, such as U1-70K and SR (serine/arginine-rich splicing factor) proteins, which in turn cause epitope masking.⁷⁴ They were not able, however, to demonstrate a mechanism for the association of these new partners with PSF during apoptosis, nor were they able to distinguish whether the association was direct or indirect.

DOMAINS OF PSF

PSF encompasses 707 amino acids and has a molecular weight of 76 kDa, although it typically migrates on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel at an apparent molecular weight of ~100 kDa. Proteolytic cleavage products of apparent molecular weights of 47 and 68 kDa, and an alternatively spliced form of 669 amino acids, have also been described in various cell types.^{1,28,75} These truncated forms differ from the full-length

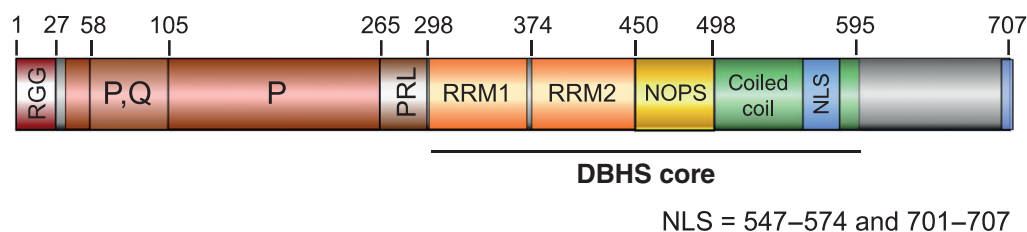


FIGURE 2 | Domain structure of PTB-associated splicing factor (PSF). A schematic of the domains of PSF along the primary sequence of the protein. Numbers indicate amino acid. Domains are as discussed in the text. RGG, RGG box; P, proline-rich domain including proline/glutamine-rich subdomain (P,Q); PRL, PR linker; NLS, nuclear-localization sequence. RRM1, RRM2, NOPS, and coiled-coil domains are as listed. The portion of PSF that comprises the DBHS core region is noted. Exact amino acid boundaries of the NLS are also given below.

protein in the fraction of the C-terminus included; however, differential functions or regulated expression have not been ascribed to these truncations. At least seven distinct domains have been defined within PSF by sequence, function, and/or structural analysis (Figure 2). Below, we describe what is known, and what still remains unknown, about each of these domains.

RGG Box

The first N-terminal 27 amino acids of PSF are highly enriched in arginine and glycine residues, including multiple trimeric RGG repeats, a motif known as an RGG box.⁷⁶ RGG boxes are relatively rare, being present in only about 100 proteins in humans; however, proteins that contain RGG motifs are highly enriched for RNA-binding activity.⁷⁷ In cases in which the RNA-binding specificity has been studied, the RGG motif mediates association with G-quartet structures.⁷⁷ RGG boxes have also been shown to mediate interaction with DNA and protein partners, as well as to control protein localization.⁷⁷ In PSF, the N-terminal RGG box is essential for cleavage and polyadenylation, but is dispensable for interaction with the Pol II CTD, and has only a minor effect on splicing.⁴³ The mechanism by which the RGG box of PSF influences 3'-end processing remains unknown.

Notably, RGG motifs are substrates for several arginine methyltransferases (PRMTs), and methylation of RGG repeats has been shown to influence the interaction of RGG boxes with other proteins.⁷⁷ Therefore, it is possible that methylation may toggle any given RGG motif between nucleic acid and protein binding. Evidence for methylation of the RGG box of PSF exists^{78,79}; however, relevant PRMTs have not been identified nor has the functional relevance of PSF methylation been established.

Proline-Rich

The ~200 amino acids following the N-terminal RGG box are characterized by an enrichment of proline

and glutamine residues. This includes a ~50-amino acid stretch where all but 6 amino acids are proline or glutamine, followed by an additional ~150 amino acids where over a third are prolines. From a practical standpoint, this proline-rich region can sometimes hinder expression and solubility of the full-length protein, precluding a detailed understanding of the functional aspects of the proline-rich domain of PSF.

Based on our understanding of short proline-rich domains within signaling proteins, it is likely that the proline-rich region of PSF plays an important role in mediating protein–protein interactions, particularly with non-DBHS proteins. PSF has been shown to interact with the proline-binding SH3 domain of the T-cell signaling molecule Nck, at least within cell lysates.⁸⁰ Furthermore, deletion of the proline-rich domain abolished the ability of PSF to associate with the strong transcriptional enhancer VP16.⁴³ Finally, at least one report has convincingly demonstrated that this domain contributes to DNA-binding activity.⁶⁴ It has thus far not been determined whether all of the prolines are required for all function ascribed to this domain, or if there is redundancy or separable activities within this peptide region.

RNA-Recognition Motifs

Without question the best characterized domains of PSF are the RNA-recognition motifs, or RRM, a motif first described in the early 1990s.^{81,82} As the name implies, this protein motif is frequently involved in RNA recognition and binding. RRMs are found in ~500 human proteins, and have been extensively reviewed elsewhere.^{83,84} RRMs are often found in multiple copies in a single protein. In the case of PSF, two tandem RRMs are present roughly in the middle of the primary sequence of the protein, where they are separated from each other by a seven-amino acid alanine-rich linker (FATHAAA). By comparison, the RRMs of PSF themselves are each ~70–80 amino acids, as is typical of this domain.

The first structure of an RRM domain was reported in 1994 by the group of Nagai.⁸⁵ As of 2014, more than 150 additional structures for RRM domains from a wide range of proteins have been deposited in the protein data bank (PDB). The RRM domain folds in a highly defined and stable structure in which two α helices are packed up against four β -strands configured in an antiparallel β -sheet. Although the structure of the RRM domains of PSF has not yet been published, a structure of the RRM domains of the other human DBHS proteins was reported in 2012 from a co-crystal of p54nrb/NONO and PSPC1, which included both RRM domains of each protein plus additional sequences that comprise the DBHS core⁴ (NOPS and coiled-coil, see below). In this structure, the RRM domains adopt a general canonical fold, and the two RRM domains of each monomer are held in a rigid extended conformation owing to interactions between the NOPS domain of one monomer (see below) and RRM2 of the other. Notably, the crystallized NOPS–RRM2 interaction leaves the putative RNA-binding face of RRM2 accessible, so this interaction is compatible with RNA binding of RRM2. Given the high degree of homology between p54/NONO, PSPC1, and PSF (80–90% over the RRM domains and 100% in the linker), and the general structural similarity of all RRM domains, the PSPC1 and p54nrb/NONO structures can be reasonably assumed to be a good proxy for the RRM domain region of PSF.

The most canonical mode of RNA–RRM interaction involves stacking of aromatic residues on the β -sheet of the RRM with nucleobases or sugar moieties of the RNA.⁸⁴ Remarkably, however, RRM domains can associate with RNA using a range of strategies, including specific interactions of RNA with loops at the base of the β -sheet and/or with the α -helices.^{86–88} Preliminary studies from one of our groups have revealed that the more C-terminal RRM (RRM2) contains the primary RNA-binding activity of PSF, while the more N-terminal RRM (RRM1) has little ability to bind RNA on its own (C.A.Y. and K.W.L., unpublished data). This is surprising given that RRM1 contains the above-mentioned conserved aromatic groups on the β -sheet, whereas RRM2 lacks such residues at appropriate positions. Therefore, we predict that RRM2 of PSF binds to RNA by a noncanonical mode. This perhaps provides a mechanistic explanation for why the RNA binding of PSF is more promiscuous or poorly defined than that of many other RRM proteins. Indeed, various studies have reported specific binding of PSF to pyrimidine-rich RNAs,^{1,11} GA-rich sequences,^{34,37} and GU-rich sequences⁸⁹ as well as structured RNAs.^{31,37,70}

Importantly, while RRM domains are named for their ability to bind RNA, studies of numerous RRM domains have shown them to also participate in protein–protein interactions.^{83,90} Perhaps because of this multiplicity and complexity of function, the two RRM domains of PSF are not interchangeable. For example, structure–function studies have shown RRM1 to be required for association with VP16 but dispensable for interaction with the Pol II CTD, whereas the converse is observed for RRM2⁴³ (Figure 3(a)). By contrast, both RRM domains are required for splicing activity⁴³ and for stable association with NEAT1,⁶¹ even though, as discussed above, only RRM2 is likely required for direct RNA binding, and only RRM2 is required for subnuclear localization.⁹¹ The precise division of labor for RRM1 versus RRM2, and the sequence or structural features that dictate the observed functional differences, remain to be determined for most activities of PSF.

NOPS

Immediately following RRM2 is the novel NOPS domain (NONA/ParaSpeckle domain), defined by virtue of its homology and structure in the p54nrb/NONO/PSPC1 dimer. In older literature, a portion of the NOPS domain was denoted as an extension of RRM2⁴³; however, as mentioned above, the crystal structure of the p54nrb/NONO/PSPC1 heterodimer clearly reveals that the 52 amino acids following RRM2 fold into a distinct domain that interacts extensively with RRM2 of the dimeric partner.⁴ This NOPS domain also makes contacts with the coiled-coil domain (see below) of the crystal partner.⁴ Strikingly, mutation of residues of the NOPS domain that interact with the RRM2 or the coiled-coil domain abolish the ability of PSPC1 to interact with wild-type DBHS proteins or localize to speckles in cells.⁴ Therefore, the NOPS domain can best be described as a protein–protein interaction domain that is essential for the formation of functional dimers in the cell.

Coiled-Coil Domain

In all three DBHS proteins, the NOPS domain is followed by a highly charged sequence of ~60–100 amino acids. In the fragment of p54nrb/NONO and PSPC1 that has been crystallized, this charged sequence forms a right-handed coiled-coil structure that associates with the coiled-coil domain of the partner protein.⁴ Notably, virtually the entire coiled-coil domain is necessary for targeting of the DBHS proteins to paraspeckles, while only half of this domain is

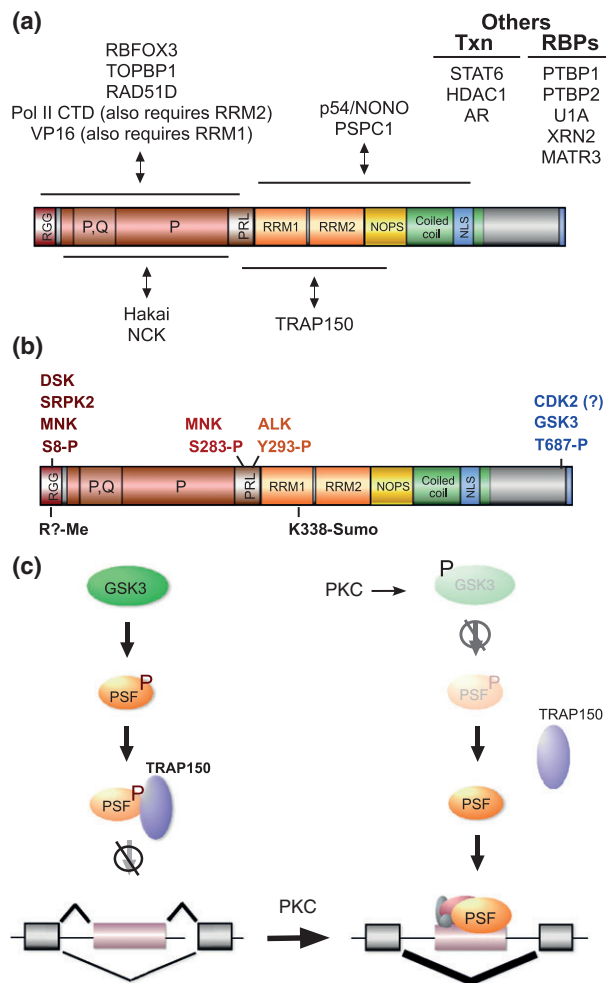


FIGURE 3 | Regulation of PTB-associated splicing factor (PSF). (a) PSF protein partners. Arrows indicate approximate domain of PSF through which partner proteins interact, in the cases where this is known. Protein partners for which no domain information is available are listed to the top right. (b) Location of mapped sites of phosphorylation on PSF for which the kinase is known. Also shown are additional mapped sites of methylation (Me) and sumoylation (Sumo). (c) Schematic of one pathway of PSF regulation for which details are known. GSK3, PSF, and TRAP-150 are as described in the text. PKC is protein kinase C, a signaling molecule known to induce inhibitory phosphorylation of GSK3, thereby reducing its activity. Red 'P' indicates GSK3-dependent phosphorylation of PSF. Gray and beige boxes and lines correspond to the exons and introns of the CD45 gene. The central beige exon corresponds to CD45 exon 4, a known target of PSF repressive activity.

needed for dimerization.⁴ On the basis of these observations, it has been proposed that the length of the full coiled-coil domains allows the DBHS proteins to form extended polymers to promote the formation of paraspeckles.⁴ While direct evidence for this model is yet to be published, it is consistent with increasing evidence that RNA-binding proteins can form subcellular

structures through self-assembly and multivalent interactions with themselves and/or RNA.^{92–94}

C-Terminus

In keeping with a model in which the C-terminus of PSF dictates protein localization, both a portion of the coiled-coil region (AA 547–574) as well as the final approximately seven amino acids of PSF mediate nuclear localization. The final seven amino acids conform to a canonical nuclear localization signal (NLS), while the internal NLS functions as a complex bipartite signal.⁹¹ No additional striking sequence or structural feature has been proposed for the C-terminal most ~100 amino acids of PSF, although the sequence is moderately enriched for glycine (~30%) suggesting flexibility of the region. Similar to the N-terminal RGG and Pro-rich sequences, the C-terminal ~100 amino acids also contain no notable homology to the other DBHS proteins.

Interestingly, there is evidence that the C-terminus plays a critical role in regulating the interaction of PSF with other molecules. In addition to the predicted flexibility of this region, the C-terminus contains sites of PTM that may alter protein function (see below). For example, phosphorylation of T687 is required for PSF to interact with its regulatory partner TRAP150 (thyroid hormone receptor-associated protein complex 150 kDa component)³² (see below). Notably, deletion of the final C-terminal amino acids (AA 667–707) also permits association of PSF with TRAP150, demonstrating that TRAP150 does not interact directly with T687, but rather that phosphorylation of this site functions as a regulatory switch.³² One specific model that has been proposed is that the C-terminus undergoes phosphorylation-dependent remodeling to alter accessibility of TRAP150 for PSF⁹⁵ (see below; Figure 3). Whether the C-terminus also regulates other protein–protein interactions of PSF, and whether additional PTMs control the activity of the C-terminus, remains an open and active area of study.

PR Linker

Finally, the most poorly understood, but potentially important domain, of PSF is a ~33-amino acid stretch between the proline-rich domain and RRM1 that we refer to here at the Proline-RRM (PR) linker. We mention this linker sequence out-of-order with the other domains discussed above, as this region has not previously been defined in the literature. However, there is evidence to suggest that the PR linker sequence confers important activity and regulation to PSF. First, this region encompasses many identified sites

of PTMs that may regulate the interactions and/or activity of PSF (see below). Secondly, work from one of our labs has suggested that this linker is required, along with the RRM for interaction of PSF with at least one co-associated protein, namely TRAP150 (see below, C.A.Y. and K.W.L., unpublished data). Further functional and structural characterization of this PR linker is necessary for a more complete understanding of how these 33 amino acids contribute to the activity and regulation of PSF.

REGULATION OF PSF

An immediate question that arises from any discussion of PSF function is how this one protein performs all its ascribed activities in a cell. Although the multiple domains of PSF described above provide the ability to execute diverse functions, clearly a single molecule of PSF is unlikely to be simultaneously engaged, for example, in splicing, transcription, and DNA repair. Thus, it is important to ask: how can the activity of PSF be partitioned among its many targets? As discussed below, an emerging theme is that the activity of PSF can be regulated by PTMs and/or co-associated factors that either recruit PSF to particular sites of action or promote or inhibit specific intermolecular interactions. Specifically, many recent data suggest that such cofactors compete with one another to bias the activity of PSF toward one target or another according to the growth conditions and needs of the cell.

Post-Translational Modifications of PSF

PTMs regulate the activity of virtually all classes of cellular proteins, and PSF is no exception. The most widely described modification of PSF is phosphorylation. Numerous sites of phosphorylation of PSF have been detected in proteomic studies (Phosphosite.org) and close to a dozen kinases have thus far been shown to modify or interact with PSF (Figure 3(b)). One of the earliest well-documented examples of regulation of PSF by phosphorylation was the phosphorylation of serines 8 and 283 by the MAP kinase interacting kinase MNK.⁹⁶ Both of these sites are within or adjacent to domains involved in binding of nucleic acids; S8 is within the RGGs, while S283 is within the PR linker. Phosphorylation of PSF by MNK increases the binding of PSF to at least one target RNA, the 3'UTR of TNF α (tumor necrosis factor α), as assayed by co-precipitation of TNF α mRNA with PSF.⁹⁶ Interestingly, S8 is part of an Arg-Ser dipeptide and has also been shown to be a substrate for phosphorylation by the RS kinases SRPK1 (SRSF protein kinase

1) and DSK, at least *in vitro* or when co-expressed in bacteria.⁹⁷ However, phosphorylation of PSF by SRPK1 or DSK in eukaryotic cells is yet to be demonstrated.

A second well-characterized example of phosphorylation-dependent regulation of PSF is the phosphorylation of PSF by glycogen synthase kinase 3 (GSK3), which regulates the ability of PSF to control splicing of the CD45 pre-mRNA³² (see *Splicing*). GSK3 is highly active in unstimulated T cells and phosphorylates T687 in the extreme C-terminus of PSF³² (Figure 3(b)). As mentioned above, this phosphorylation event regulates the ability of TRAP150 to bind to PSF. TRAP150, in turn, blocks the binding of PSF to RNA. Upon T-cell stimulation, GSK3 activity is attenuated, leading to an increase in PSF that lacks the phospho-T687 and is thus not bound by TRAP150.³² Once freed from TRAP150, PSF binds to target RNAs such as exon 4 of the CD45 pre-mRNA (see *Splicing*). T687 is also a putative target site for phosphorylation by cyclin-dependent kinase 2 (Cdk2). However, while Cdk2 does phosphorylate PSF in cells and *in vitro*, a version of PSF containing T687 as the only putative Cdk2 site is not a substrate for phosphorylation by this kinase.⁹⁸ Two additional serine/threonine kinases, protein kinase C α (PKC α) and the c-Jun N-terminal kinase (JNK), have been shown to associate with PSF, although it remains to be determined if PSF is a substrate for the activity of these kinases, and if so, which residues are phosphorylated.^{99,100}

While most of the studies of PSF phosphorylation have focused on serine/threonine kinases, two interesting studies have linked tyrosine phosphorylation with aberrant cytoplasmic localization of PSF in cancer cells. In anaplastic large-cell lymphomas (ALCLs) that express the fusion protein NPM/ALK (nucleophosmin/anaplastic lymphoma kinase), PSF interacts with this chimeric protein and is phosphorylated on tyrosine 293 by the kinase domain from ALK.¹⁰¹ Tyrosine 293 is within the PR linker near the site of MNK-induced phosphorylation (see above). Notably, phosphorylation of Y293 causes mislocalization of PSF from the nucleus to the cytoplasm.¹⁰¹ Whether this indicates that the PR linker is directly involved in subcellular localization, or whether the phosphorylation of Y293 induces mislocalization through altered interactions with a carrier protein remains to be determined. Similarly, PSF has also been shown to be phosphorylated by the tyrosine kinase BRK (breast tumor kinase) in response to EGF signaling.¹⁰² As in ALCL, tyrosine phosphorylation of PSF in breast cancer cells induces its cytoplasmic localization.¹⁰² The specific site of BRK-mediated phosphorylation of PSF has not been determined;

however, deletion of the C-terminus of PSF abolished phosphorylation by BRK. Because the C-terminus contains identified NLS sequences, this leads to the intriguing possibility that phosphorylation within or adjacent to the NLS regulates its activity. It has also been proposed that tyrosine phosphorylation of PSF regulates RNA and DNA binding,^{101,102} though further study is required to rigorously test these predictions. More convincingly, in the above studies, localization of PSF to the cytoplasm inhibits cell proliferation, presumably by preventing essential nuclear functions of PSF.^{101,102} An interesting area of future study will be to determine if tyrosine phosphorylation of PSF is used as a regulatory mechanism to control PSF localization and function in normal human cells.

The extensive potential for phosphorylation of PSF leads to a natural follow-up question: is dephosphorylation also regulated? Unsurprisingly, several studies have demonstrated an important role for at least one phosphatase, protein phosphatase 1 (PP1), in regulating PSF. Notably, the very C-terminal end of PSF RRM1 contains an RVxF sequence, which is the consensus for PP1 binding. Consistently, PSF interacts with PP1, as shown by two-hybrid assays, co-immunoprecipitation, and co-localization,^{103,104} though in mammalian cells this interaction appears to be indirect as it requires p54nrb/NONO, which also contains a RVxF sequence.¹⁰⁴ Regardless, PSF is clearly a target of PP1 activity. PP1 can dephosphorylate PSF *in vitro* and in cells,¹⁰⁴ and cellular inhibition of PP1 by ceramide leads to increased phosphorylation of PSF.¹⁰⁵ Furthermore, PP1 activity has been shown to influence the transcription activity of PSF in reporter assays, although the mechanism for this effect remains to be determined as PP1 has little effect on the ability of PSF to interact with transcriptional co-repressors.¹⁰⁴ An effect of PP1 on the splicing activity of PSF has also been suggested based on some subtle changes in a reporter minigene¹⁰⁴ and on the fact that some genes that exhibit altered splicing in response to ceramide treatment are also sensitive to overexpression of PSF.¹⁰⁵ However, neither of these findings strongly support a direct role of PP1-sensitive phosphorylation of PSF in splicing.

Finally, we emphasize that phosphorylation is not the only PTM that may regulate PSF. As mentioned above, two proteomic studies have demonstrated methylation of the RGG box of PSF.^{78,79} In other RGG box proteins, methylation typically alters protein–protein interactions, although the functional significance of PSF methylation has not been reported. Sumoylation of PSF within residues 337–340 (IKLE) has also been described.¹⁰⁶ Notably, this modification falls directly within RRM1, and

thus might be predicted to influence RNA binding or protein–protein interactions. In addition, co-transfection studies in mammalian cells have demonstrated that sumoylation of PSF is required for its interaction with the transcription repressor HDAC1, and increased sumoylation of PSF results in decreased histone acetylation and transcriptional activity of the human tyrosine hydroxylase promoter, a known target of PSF regulation.¹⁰⁶ Thus, there is good reason to believe sumoylation may regulate many of the central activities of PSF.

Regulation through Protein and RNA Partners

PSF-Co-Associated Proteins

Throughout this review, we have highlighted many proteins that are known to interact with PSF, and given its size and complex domain structure, this abundance of partners should come as no surprise. In fact, the interaction of PSF with a protein partner is often the mechanism through which PSF carries out its cellular activity. As mentioned above, PSF can regulate splicing through the recruitment of Rbfox3, PTB, or the snRNPs, whereas much of the role of PSF in transcription can be attributed to its role as a bridge between transcription factors and HDACs or RNA Pol II.^{55,57} Interaction of PSF with Pol II, U1A, and the other DBHS proteins has also been implicated in transcription-coupled splicing, polyadenylation, and nuclear retention, respectively. Many questions remain, however, regarding these interactions. First, with the exception of the DHBS proteins, there is limited information regarding how PSF interacts with the above-mentioned proteins. Are these all direct interactions? What domains of PSF or the partner proteins are required for interaction? A related issue is the relative *in vitro* instability of most of the interactions mentioned, the often different subnuclear localization of these proteins, and the common sense argument against constitutive interaction of PSF with all of these protein partners. What, then, controls the interaction of PSF with each of these identified protein co-factors? One possibility is modulation of the affinity of PSF for its targets. For example, interaction of the scaffold protein hDlg with its kinase p38 γ decreases hDlg/PSF association.¹⁰⁷ Conversely, the addition of poly(ADP-ribose) (PAR) to proteins (PARylation) increases the binding affinity of NONO, and perhaps PSF, through interaction of PAR with RRM1.¹⁰⁸ PARylation of histones is often a mark for sites of DNA damage and has been linked to recruitment of PSF and p54nrb/NONO for DNA repair. PTMs of PSF itself can also influence protein–protein

interactions, as discussed above, and have been correlated with altered subnuclear localization of PSF at distinct phases of the cell cycle and in response to apoptosis and transcriptional arrest, suggesting regulated partitioning of PSF activity.^{74,109,110}

Consistent with the idea of mutually exclusive interactions contributing to the regulation of PSF, at least a few PSF-interacting proteins have been implicated not as direct partners in PSF's mechanism of action, but rather as regulatory factors that modulate the spectrum of PSF's cellular activities. In the simplest model, these proteins function by controlling the partitioning of PSF among its many cellular targets. The best characterized example of such a PSF regulatory protein is the aforementioned TRAP150 that regulates the ability of PSF to bind to the CD45 RNA (Figure 3(c)). Similarly, TRAP150 antagonizes the effect of PSF on transcription-coupled splicing of a CD44-derived minigene,¹¹¹ although it is unclear whether the primary activity of PSF in this assay is regulation of transcription or splicing. Preliminary data from our lab suggest that this regulatory activity of TRAP150 is due to the fact that it interacts directly with the RRM and PR linker of PSF, thereby sterically occluding RNA binding (C.A.Y. and K.W.L., unpublished data). Conversely, the E3 ligase Hakai promotes the interaction of PSF with RNA, specifically mRNAs of genes that promote proliferation.¹¹² Although the mechanism through which Hakai regulates PSF is unknown, it is dependent on the interaction of Hakai with the proline-rich region of PSF, but independent of the E3 ligase activity of Hakai, suggesting a model in which Hakai physically recruits PSF to particular mRNAs.

Regulation of PSF by Noncoding RNA

Strikingly, proteins are not the only molecules that have been shown to influence functional targeting of PSF. Several studies have clearly demonstrated a role for ncRNA in regulating partitioning of PSF among cellular targets. As mentioned above, PSF is often localized to paraspeckles, subnuclear bodies that are nucleated by the long ncRNA NEAT1 that is bound directly by PSF and p54nrb/NONO. Interestingly, NEAT1 expression is enhanced by several cellular stresses, including viral infection and proteasome inhibition.^{61,113} When NEAT1 is induced it recruits PSF away from DNA targets in the cell, resulting in altered expression of hundreds of PSF-dependent transcription targets.^{61,114} Of particular biologic importance, NEAT1-dependent sequestration of PSF contributes to induction of the antiviral cytokine IL-8 promoter, normally repressed by PSF, in response to viral infection.⁶¹ A similar activity has been described

for the mouse retrotransposon RNA, VL30, which relieves PSF-dependent gene repression by binding and sequestering PSF.^{62,113,115} Notably, it has been proposed that fragments of human mRNA play a similar role in sequestering PSF to regulate gene expression during tumorigenesis.¹¹⁶ Finally, given the emerging field of gene regulation by antisense transcripts, it is not surprising that in at least one case an antisense RNA has been shown to repress transcription by binding to PSF to recruit HDACs.¹¹⁷ We speculate that recruitment of PSF might be a common mechanism for the function of many antisense transcripts.

In addition to competition between RNA and DNA binding by PSF, there is also competition between RNA and protein binding. We have already seen that the protein TRAP150 competitively inhibits RNA binding by PSF. In a complementary example, binding of PSF to the speckle-related ncRNA MALAT1 dissociates the splicing factor PTBP2 from PSF.¹¹⁸ As PTBP2 has oncogenic properties, this competitive binding has been proposed as a mechanism to account for the widespread observation that increased expression of MALAT1 promotes cellular metastasis.¹¹⁸ Taken together, these examples of RNA, DNA, and proteins that compete for binding to PSF support the conclusion that the balance of expression of PSF's many co-factors and targets is likely a major control point shaping the function of PSF in any given cell.

CONCLUSION

In sum, PSF is a multidomain protein that has been implicated in a dizzying array of cellular processes (Figure 1). On the surface, the list of activities ascribed to PSF could suggest that PSF is simply a common contaminant in biochemical purifications or is just a tag-along in various cellular complexes. Undoubtedly, PSF does have the ability to interact with scores of different proteins and nucleic acids through its numerous domains. Is it also reasonable to conclude that at least some of the function of PSF is to nucleate other proteins and direct them toward a common goal, rather than PSF carrying out a specific and active task in all of the genomic and gene expression processes in which it has been implicated. However, far from being a passive bystander, we propose that a primary function of PSF is to serve as an active bridge and integrator of nuclear processes. In particular, the recent data demonstrating competition between binding partners of PSF, and/or regulation of PSF through PTMs, provide a mechanism through which one cellular activity can 'talk' to another by virtue of making PSF more

or less accessible for another cellular machine. Therefore, we conclude that PSF plays a unique and critical role in the nucleus of sensing genome integrity and balancing the activity of the various steps of gene expression appropriately. Future work to further define the regulation of PSF and to obtain the ability to selectively isolate and manipulate individual activities of PSF through domain mutations or alteration of co-regulatory factors will be tremendously valuable to

the study of nuclear processes and may have potential therapeutic value.

Note Added in Proof

Since acceptance of this review, the group of Charles Bond published a crystal structure of the DBHS domain of PSF¹¹⁹ that confirms the structural predictions made here based on studies of other DBHS proteins.

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