## Figure S4

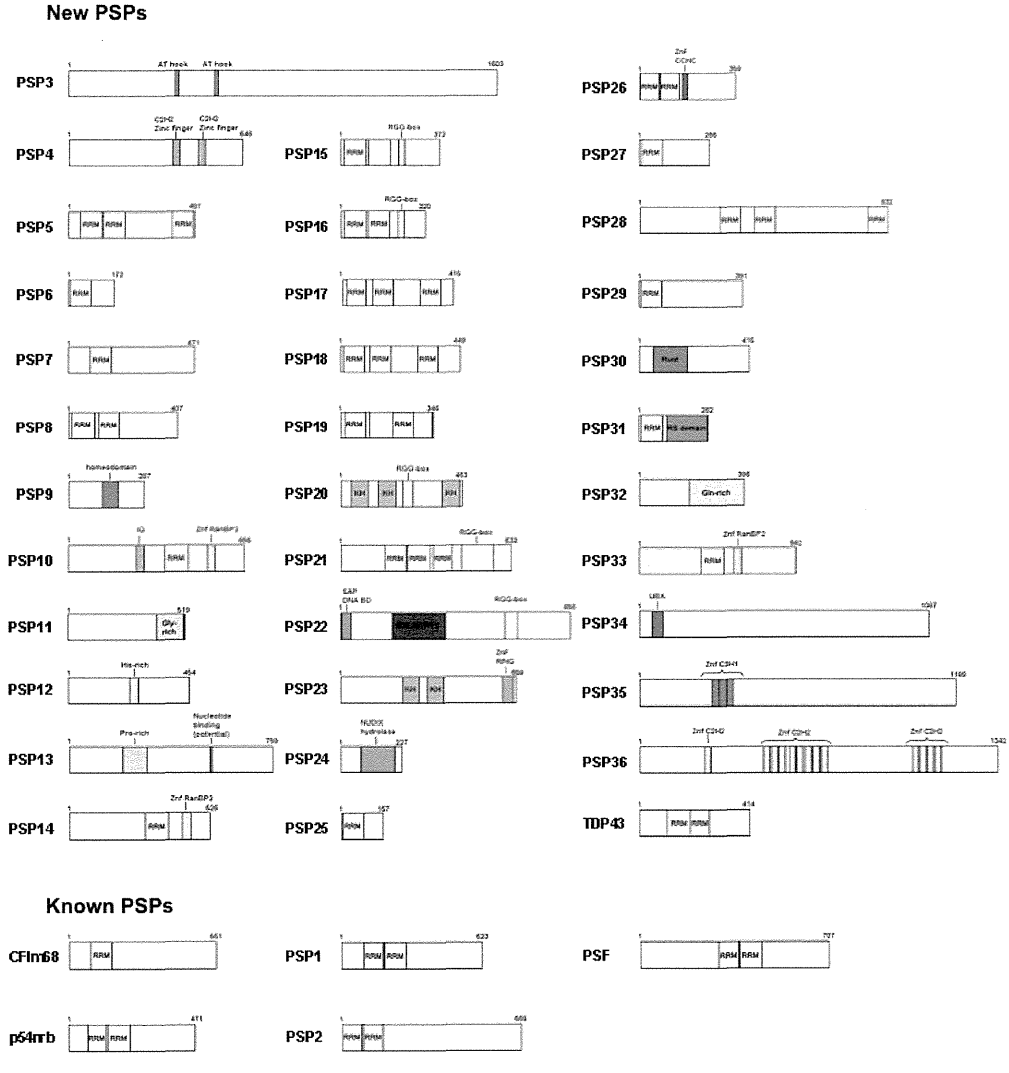
New PSPs
PSP3
AT hook
AT hook
PSP4
CCHC Zinc finger
CCHC Zinc finger
PSP5
RRM
RRM
RRM
PSP6
RRM
PSP7
RRM
PSP8
RRM
RRM
PSP9
homeodomain
PSP10
IQ
Znf RanBP2
RRM
PSP11
Gly-rich
PSP12
His-rich
PSP13
Pro-rich
Nucleotide binding (potential)
PSP14
Znf RanBP2
RRM
PSP15
RGG-box
RRM
PSP16
RGG-box
RRM
RRM
PSP17
RRM
RRM
RRM
PSP18
RRM
RRM
RRM
PSP19
RRM
RRM
PSP20
RGG-box
KH
KH
KH
PSP21
RGG-box
RRM
RRM
RRM
PSP22
EAR DNA BD
RGG-box
PSP23
Znf RING
KH
KH
PSP24
NUDIX hydrolase
PSP25
RRM
PSP26
Znf CCHC
RRM
RRM
PSP27
RRM
PSP28
RRM
RRM
RRM
PSP29
RRM
PSP30
Runt
PSP31
RRM
RS domain
PSP32
Gln-rich
PSP33
Znf RanBP2
RRM
PSP34
UBA
PSP35
Znf CSH1
PSP36
Znf C2H2
Znf C2H2
Znf C2H2
TDP43
RRM
RRM
Known PSPs
CFIm68
RRM
PSP1
RRM
RRM
PSF
RRM
RRM
p54nrb
RRM
RRM
PSP2
RRM
RRM

**Figure S4. Compilation of the new PSPs, Related to Figure 2 and Table 1.** Schematics of the major domains are shown. Domain structures of five known PSPs are shown below as references.

**Figure S5**

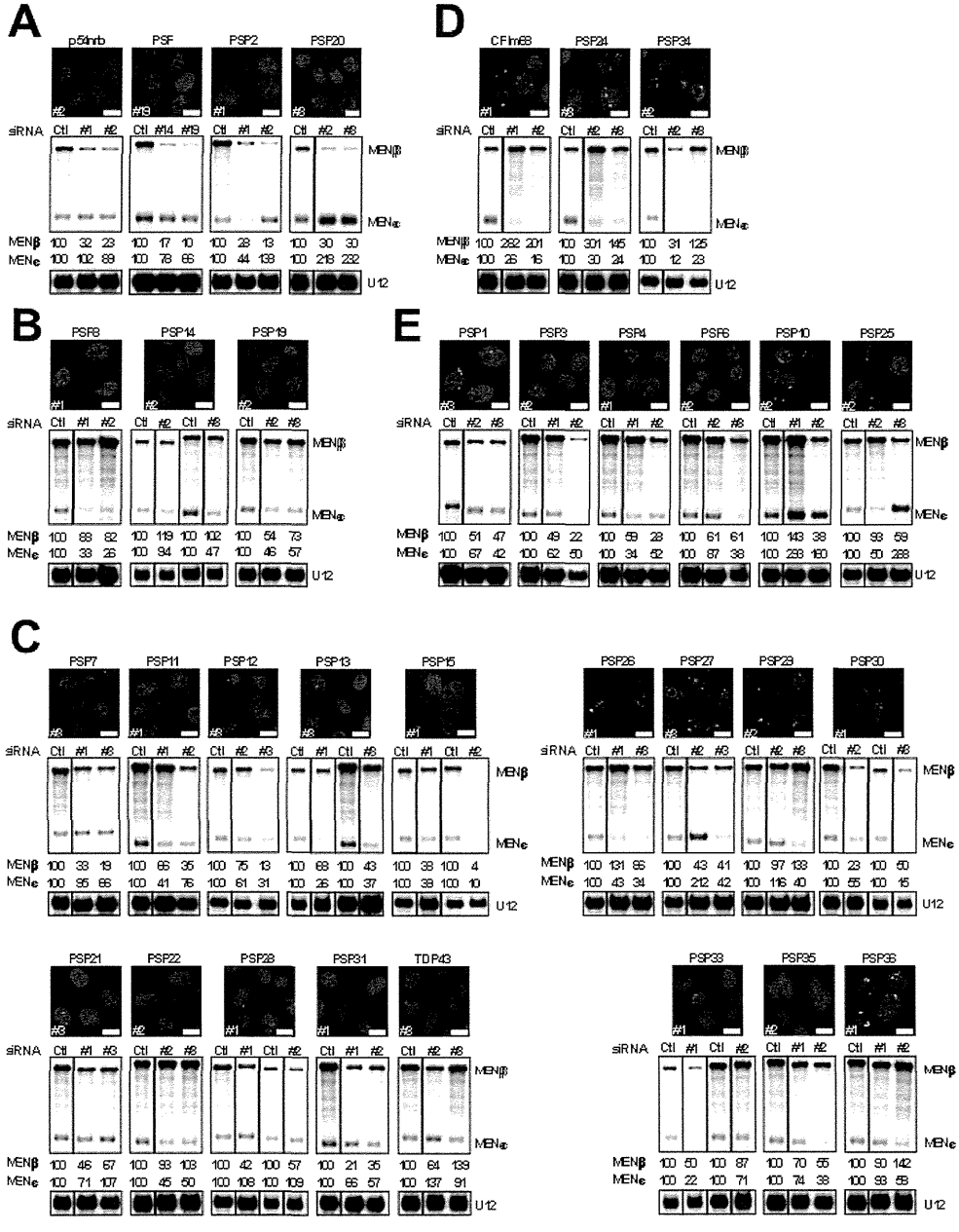
A
p54nrb
PSF
PSP2
PSP20
siRNA
Ctl
#1
#2
#19
#3
MENβ
MENε
U12
D
CFIm68
PSP24
PSP34
B
PSP8
PSP14
PSP19
E
PSP1
PSP3
PSP4
PSP6
PSP10
PSP25
C
PSP7
PSP11
PSP12
PSP13
PSP15
PSP26
PSP27
PSP29
PSP30
PSP21
PSP22
PSP28
PSP31
TDP43
PSP33
PSP35
PSP36

**Figure S5. Compiled data for the RNAi of paraspeckle proteins, Related to Figure 3 and Table 1.** Shown are the raw data on paraspeckle appearance detected by RNA-FISH and MENε/β ncRNA accumulation, as detected by RPA. Data are grouped according to their categories shown in Tables 1 and S3: Category 1A (**A**), Category 1B (**B**), Category 2 (**C**), Category 3A (**D**), and Category 3B (**E**). Representative data are also shown in Figures 3 and 4.

Figure S6

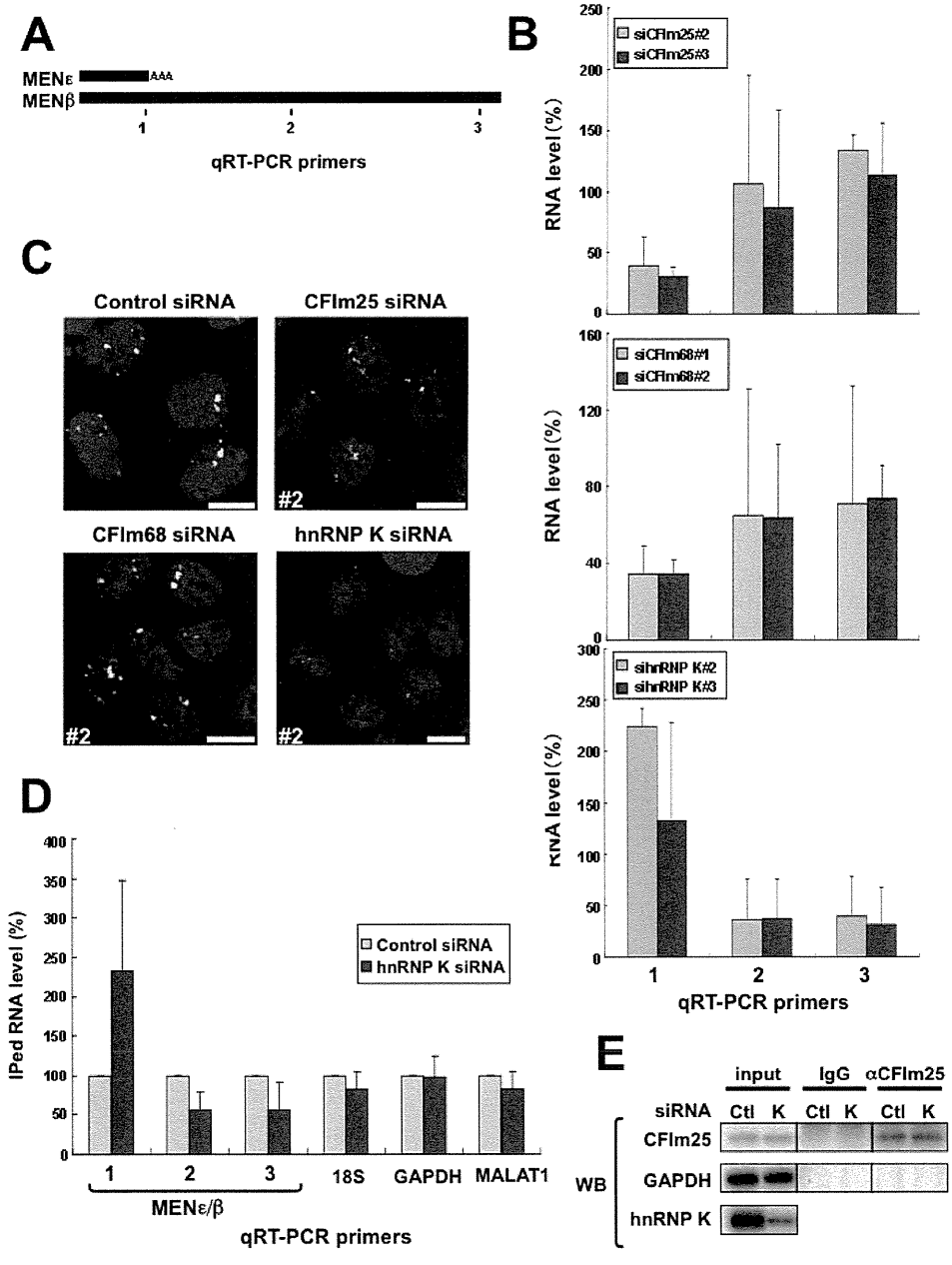
A
MENε
AAA
MENβ
1
2
3
qRT-PCR primers
B
siCFIm25#2
siCFIm25#3
RNA level (%)
siCFIm68#1
siCFIm68#2
RNA level (%)
sihnRNP K#2
sihnRNP K#3
RNA level (%)
1
2
3
qRT-PCR primers
C
Control siRNA
CFIm25 siRNA
#2
CFIm68 siRNA
hnRNP K siRNA
#2
#2
D
IPed RNA level (%)
Control siRNA
hnRNP K siRNA
1
2
3
18S
GAPDH
MALAT1
MENε/β
qRT-PCR primers
E
input
IgG
αCFIm25
siRNA
Ctl
K
Ctl
K
Ctl
K
CFIm25
GAPDH
hnRNP K
WB

**Figure S6. Regulatory factors of alternative 3' processing of MENε/β ncRNAs, Related to Figure 4. A, B.** MENε/β levels were quantified by RT-qPCR (B) with the primer pairs shown in A. The RNA levels in cells treated with control siRNA were considered to represent 100%. **C.** Detection of paraspeckles in siRNA-treated cells. The siRNAs used are shown on the top of each panel and are different from those in Figure 4B. **D.** CFIm25 association with MENε/β ncRNAs in the control (Control siRNA) and hnRNP K-eliminated cells (hnRNP K siRNA). Coimmunoprecipitated (co-IPed) MENε/β ncRNAs were monitored by RT-qPCR with the primers shown in A. 18S rRNA (18S), GAPDH mRNA, and Malat-1 ncRNA are controls. Data are normalized by the levels of immunoprecipitated CFIm25 detected by Western blot (in E). **E.** Western blotting to measure the levels of CFIm25, GAPDH, and hnRNP K in input and immunoprecipitation fractions used in D.

Figure S7

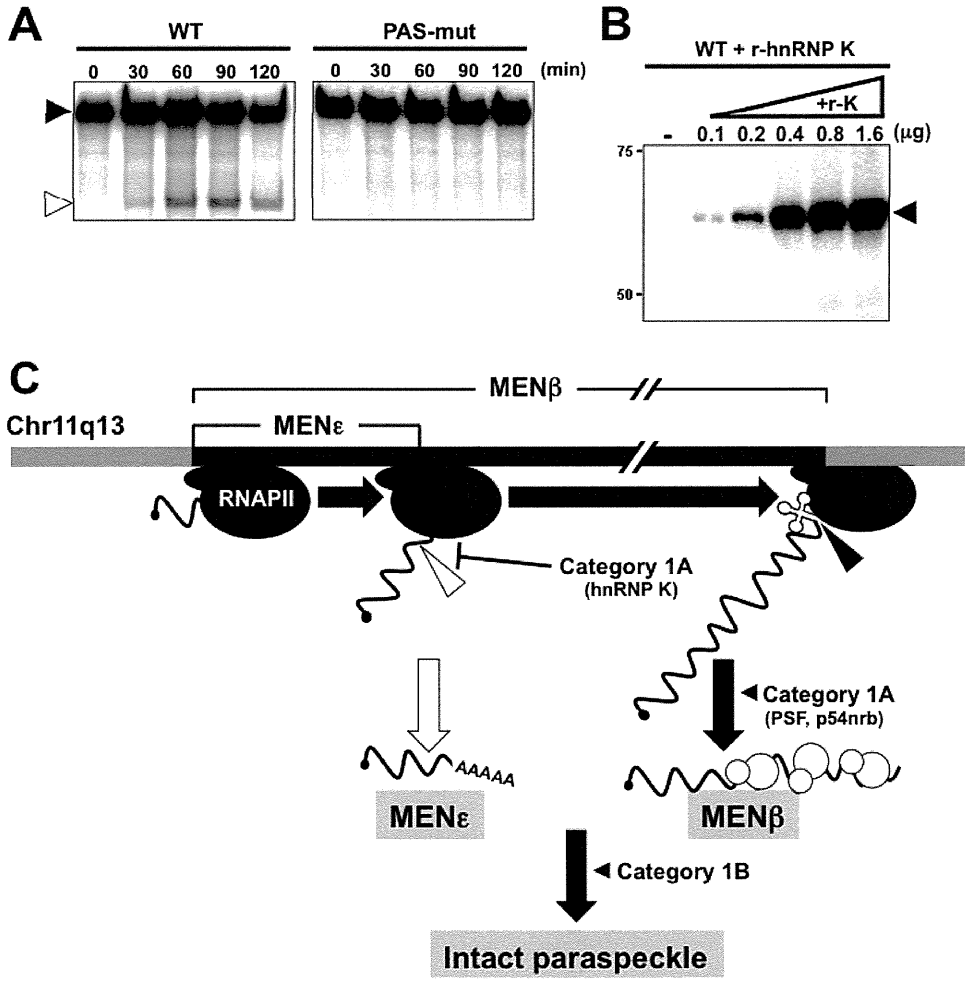
A
WT
PAS-mut
0 30 60 90 120
0 30 60 90 120 (min)
B
WT + r-hnRNP K
+r-K
- 0.1 0.2 0.4 0.8 1.6 (μg)
75
50
C
Chr11q13
MENβ
MENε
RNAPII
Category 1A
(hnRNP K)
Category 1A
(PSF, p54nrb)
AAAAA
MENε
MENβ
Category 1B
Intact paraspeckle

**Figure S7. Supplemental information for in vitro processing experiments, Related to Figures 5 and 6. A.** Accurate occurrence of in vitro 3'-end processing. In vitro processing was performed as in Figure 5B with WT and PAS-mut substrates. **B.** Binding of the recombinant hnRNP K protein to the substrate RNA. The $^{32}$P-labeled substrate RNA was mixed with increasing amounts of the recombinant hnRNP K protein shown above. Binding was monitored by UV-crosslinking, and $^{32}$P-labeled proteins were detected by SDS-PAGE. **C**. Current model of intact paraspeckle formation. The essential steps, including 1) ongoing transcription of MENε/β by RNA polymerase II (RNAPII), 2) MENβ synthesis by alternative 3'-end processing, 3) MENβ stabilization by category 1a proteins such as PSF and p54nrb, and 4) subsequent assembly step(s), are schematized and represented by bold black arrows. Category 1B proteins act in an essential step other than MENβ accumulation. MENε synthesis is dispensable; therefore, it is shown by a white arrow. The 3'-ends of MENε and MENβ are formed by distinct mechanisms: canonical polyadenylation (open triangle) and RNase P cleavage (closed triangle). The significance of the noncanonical 3'-end processing of MENβ remains uncertain. Our preliminary results show that the noncanonical processing is not necessary for the rescue activity of MENβ in Figure 1A.

Table S1. Localization of paraspeckle proteins

| PSP # | Proteins | Paraspeckle localization | | |
|---|---|---|---|---|
| | | Venus* | +Act D** | Endogenous*** |
| **New paraspeckle proteins** | | | | |
| PSP3 | AHDC1 | Yes | Yes | N.D. |
| PSP4 | AKAP8L | Yes | Yes | Yes |
| PSP5 | CELF6 | Yes | Yes | N.D. |
| PSP6 | CIRBP | Yes | Yes | Yes |
| PSP7 | CPSF7 | Yes | Yes | Yes |
| PSP8 | DAZAP1 | Yes | Yes | Yes |
| PSP9 | DLX3 | Yes | Yes | N.D. |
| PSP10 | EWSR1 | Yes | Yes | Yes |
| PSP11 | FAM98A | Yes | Yes | Yes |
| PSP12 | FAM113A | Yes | Yes | Yes |
| PSP13 | FIGN | Yes | Yes | Yes |
| PSP14 | FUS | Yes | Yes | Yes |
| PSP15 | HNRNPA1 | Yes | Yes | Yes |
| PSP16 | HNRNPA1L2 | Yes | Yes | Yes |
| PSP17 | HNRNPF | Yes | Yes | Yes |
| PSP18 | HNRNPH1 | Yes | Yes | Yes |
| PSP19 | HNRNPH3 | Yes | Yes | Yes |
| PSP20 | HNRNPK | Yes | Yes | Yes |
| PSP21 | HNRNPR | Yes | Yes | Yes |
| PSP22 | HNRNPUL1 | Yes | Yes | Yes |
| PSP23 | MEX3C | Yes | Yes | N.D. |
| PSP24 | NUDT21 | Yes | Yes | Yes |
| PSP25 | RBM3 | Yes | Yes | N.D. |
| PSP26 | RBM4B | Yes | Yes | Yes |
| PSP27 | RBM7 | Yes | Yes | Yes |
| PSP28 | RBM12 | Yes | Yes | Yes |
| PSP29 | RBMX | Yes | Yes | Yes |
| PSP30 | RUNX3 | Yes | Yes | N.D. |
| PSP31 | SRSF10 | Yes | Yes | Yes |
| PSP32 | SS18L1 | Yes | Yes | Yes |
| PSP33 | TAF15 | Yes | Yes | Yes |
| PSP34 | UBAP2L | Yes | Yes | Yes |
| PSP35 | ZC3H6 | Yes | Yes | N.D. |
| PSP36 | ZNF335 | Yes | Yes | Yes |
| TDP43 | TARDBP | N.D. | N.D. | Yes |
| **Known paraspeckle proteins** | | | | |
| CFIm68 | CPSF6 | N.D. | N.D. | Yes |
| p54nrb | NONO | Yes | Yes | Yes |
| PSP1 | PSPC1 | Yes | Yes | Yes |
| PSP2 | RBM14 | Yes | Yes | Yes |
| PSF | SFPQ | Yes | Yes | Yes |

*Yes: Paraspeckle localization of Venus-fusion protein, **Yes: Relocation of Venus-fusion protein to perinucleolar caps,

***Yes: Paraspeckle localization of endogenous proteins.

Table S2. RNAi knockdown of paraspeckle proteins

| PSP # | Proteins | Confirmation of RNAi knockdown | | |
|---|---|---|---|---|
| | | RNAi* | Detection | |
| | | | RT-qPCR** | Western** |
| **New paraspeckle proteins** | | | | |
| PSP3 | AHDC1 | 2 siRNAs | 32% | N.D. |
| PSP4 | AKAP8L | 2 siRNAs | 7% | <1% |
| PSP5 | CELF6 | N.D. | N.D. | N.D. |
| PSP6 | CIRBP | 2 siRNAs | 16% | N.D. |
| PSP7 | CPSF7 | 2 siRNAs | 9% | 24% |
| PSP8 | DAZAP1 | 2 siRNAs | 12% | 22% |
| PSP9 | DLX3 | N.D. | N.D. | N.D. |
| PSP10 | EWSR1 | 2 siRNAs | 14% | <1% |
| PSP11 | FAM98A | 2 siRNAs | 3% | 22% |
| PSP12 | FAM113A | 2 siRNAs | 34% | N.D. |
| PSP13 | FIGN | 2 siRNAs | 35% | 29% |
| PSP14 | FUS | 2 siRNAs | 24% | 14% |
| PSP15 | HNRNPA1 | 2 siRNAs | 4% | 8% |
| PSP16 | HNRNPA1L2 | 2 siRNAs | 39% | N.D. |
| PSP17 | HNRNPF | 2 siRNAs | 6% | 25% |
| PSP18 | HNRNPH1 | 2 siRNAs | 18% | 4% |
| PSP19 | HNRNPH3 | 2 siRNAs | 3% | 15% |
| PSP20 | HNRNPK | 2 siRNAs | 7% | 7% |
| PSP21 | HNRNPR | 2 siRNAs | 7% | 10% |
| PSP22 | HNRNPUL1 | 2 siRNAs | 5% | 1% |
| PSP23 | MEX3C | N.D. | N.D. | N.D. |
| PSP24 | NUDT21 | 2 siRNAs | 2% | <1% |
| PSP25 | RBM3 | 2 siRNAs | 4% | <1% |
| PSP26 | RBM4B | 2 siRNAs | 20% | 4% |
| PSP27 | RBM7 | 2 siRNAs | 4% | 27% |
| PSP28 | RBM12 | 2 siRNAs | 18% | 7% |
| PSP29 | RBMX | 2 siRNAs | 5% | 13% |
| PSP30 | RUNX3 | 2 siRNAs | 29% | N.D. |
| PSP31 | SRSF10 | 2 siRNAs | 9% | 10% |
| PSP32 | SS18L1 | 2 siRNAs | N.D. | N.D. |
| PSP33 | TAF15 | 2 siRNAs | 16% | 12% |
| PSP34 | UBAP2L | 2 siRNAs | 5% | <1% |
| PSP35 | ZC3H6 | 2 siRNAs | 27% | N.D. |
| PSP36 | ZNF335 | 2 siRNAs | 43% | 11% |
| TDP43 | TARDBP | 2 siRNAs | 17% | 2% |
| **Known paraspeckle proteins** | | | | |
| CFIm68 | CPSF6 | 2 siRNAs | 1% | 1% |
| p54nrb | NONO | 2 siRNAs | *** | *** |
| PSP1 | PSPC1 | 2 siRNAs | 8% | *** |
| PSP2 | RBM14 | 2 siRNAs | 19% | 5% |
| PSF | SFPQ | 2 siRNAs | 4% | *** |

*2 siRNAs: Consistent results of RNAi with two siRNAs, **: Residual amounts (%) are shown, ***: Successful elimination was confirmed by Sasaki et al. (2009).

## Table S3. Summary of RNAi phenotypes

| RNAi targets | | MENε/β levels* | | Paraspeckles (%)** | Category |
|---|---|---|---|---|---|
| PSP# | Proteins | MENε (%) | MENβ (%) | | |
| **New paraspeckle proteins** | | | | | |
| PSP3 | AHDC1 | 56 ± 8 | 35 ± 19 | 80.7 | 3B |
| PSP4 | AKAP8L | 43 ± 12 | 43 ± 21 | 88.1 | 3B |
| PSP5 | CELF6 | N.D. | N.D. | N.D. | N.D. |
| PSP6 | CIRBP | 62 ± 34 | 61 ± 0 | 85.8 | 3B |
| PSP7 | CPSF7 | 80 ± 20 | 26 ± 9 | 69.9 | 2 |
| PSP8 | DAZAP1 | 29 ± 4 | 85 ± 4 | 27.8 | 1B |
| PSP9 | DLX3 | N.D. | N.D. | N.D. | N.D. |
| PSP10 | EWSR1 | 227 ± 94 | 90 ± 74 | 79.5 | 3B |
| PSP11 | FAM98A | 58 ± 24 | 50 ± 21 | 47.7 | 2 |
| PSP12 | FAM113A | 46 ± 21 | 44 ± 43 | 64.2 | 2 |
| PSP13 | FIGN | 31 ± 7 | 55 ± 17 | 35.2 | 2 |
| PSP14 | FUS | 70 ± 33 | 111 ± 12 | 28.8 | 1B |
| PSP15 | HNRNPA1 | 24 ± 20 | 21 ± 24 | 63.6 | 2 |
| PSP16 | HNRNPA1L2 | N.D. | N.D. | N.D. | N.D. |
| PSP17 | HNRNPF | N.D. | N.D. | N.D. | N.D. |
| PSP18 | HNRNPH1 | N.D. | N.D. | N.D. | N.D. |
| PSP19 | HNRNPH3 | 51 ± 7 | 63 ± 13 | 12.2 | 1B |
| PSP20 | HNRNPK | 225 ± 9 | 30 ± 0 | 23.9 | 1A |
| PSP21 | HNRNPR | 89 ± 25 | 56 ± 14 | 58.5 | 2 |
| PSP22 | HNRNPUL1 | 47 ± 3 | 98 ± 7 | 42.6 | 2 |
| PSP23 | MEX3C | N.D. | N.D. | N.D. | N.D. |
| PSP24 | NUDT21 | 27 ± 4 | 223 ± 110 | 79.0 | 3A |
| PSP25 | RBM3 | 169 ± 168 | 76 ± 24 | 85.8 | 3B |
| PSP26 | RBM4B | 38 ± 6 | 109 ± 31 | 75.0 | 3B |
| PSP27 | RBM7 | 127 ± 120 | 42 ± 1 | 105 | 3B |
| PSP28 | RBM12 | 109 ± 0 | 49 ± 10 | 44.3 | 2 |
| PSP29 | RBMX | 78.0 ± 53 | 115 ± 25 | 93.2 | 3B |
| PSP30 | RUNX3 | 35 ± 28 | 36 ± 19 | 78.4 | 3B |
| PSP31 | SRSF10 | 61 ± 6 | 28 ± 9 | 37.5 | 2 |
| PSP32 | SS18L1 | N.D. | N.D. | N.D. | N.D. |
| PSP33 | TAF15 | 46 ± 34 | 68 ± 26 | 54.5 | 2 |
| PSP34 | UBAP2L | 17 ± 7 | 78 ± 66 | 109 | 3A |
| PSP35 | ZC3H6 | 56 ± 25 | 62 ± 10 | 77.8 | 3B |
| PSP36 | ZNF335 | 75 ± 24 | 116 ± 36 | 101 | 3B |
| TDP43 | TARDBP | 114 ± 32 | 101 ± 53 | 32.5 | 2 |
| **Known paraspeckle proteins** | | | | | |
| CFIm68 | CPSF6 | 21 ± 7 | 242 ± 57 | 83.5 | 3A |
| p54nrb | NONO | 95 ± 9 | 27 ± 6 | 9.9 | 1A |
| PSP1 | PSPC1 | 54 ± 17 | 49 ± 2 | 83.5 | 3B |
| PSP2 | RBM14 | 91 ± 66 | 20 ± 10 | 7.7 | 1A |
| PSF | SFPQ | 72 ± 8 | 13 ± 4 | 23.3 | 1A |

*Average of two siRNAs (Ctl was defined as 100%), **Relative percentage for cell with Ctl [88% of cells treated with control siRNA (n > 50) possessed paraspeckles; therefore, Ctl was defined as the standard (100%)].

Table S4. Antibodies used in this study

| Antigen | Host | Supplier | Application | Dilution used |
|---|---|---|---|---|
| AKAP8L | rabbit | Abcam | WB, IF | 1:2000, 1:100 |
| CIRBP | rabbit | Abcam | IF | 1:100 |
| CPSF6 (CFIm68) | rabbit | Bethyl Laboratories | WB, IF, IP | 1:2000, 1:100, 2 μg/reaction |
| CPSF7 | rabbit | Abcam | WB, IF | 1:2000, 1:100 |
| DAZAP1 | rabbit | Abcam | WB, IF | 1:500, 1:100 |
| EWS | mouse | Santa Cruz | WB, IF | 1:1000, 1:200 |
| FAM98A | rabbit | Abcam | WB, IF | 1:2000, 1:100 |
| FAM113A | rabbit | Abcam | WB, IF | 1:2000, 1:100 |
| FIGN | rabbit | Santa Cruz | WB, IF | 1:2000, 1:100 |
| FUS | mouse | Santa Cruz | WB, IF | 1:1000, 1:1,000 |
| HNRNPA1 | mouse | ImmuQuest | WB, IF | 1:20000, 1:1,000 |
| HNRNPA1L2 | rabbit | Abcam | IF | 1:100 |
| HNRNPF | mouse | Abcam | WB | 1:2000 |
| | rabbit | Abcam | IF | 1:100 |
| HNRNPH1 | rabbit | Abcam | WB | 1:30000 |
| | rabbit | Bethyl Laboratories | IF | 1:100 |
| HNRNPH3 | rabbit | Abcam | WB, IF | 1:1000, 1:100 |
| HNRNPK | mouse | Abcam | WB, IF | 1:10000, 1:1,000 |
| HNRNPR | rabbit | Abcam | WB, IF | 1:2000, 1:1,000 |
| HNRNPUL1 | rabbit | Abcam | WB, IF | 1:20000, 1:100 |
| NONO (p54nrb) | mouse | BD Biosciences | WB, IF | 1:2500, 1:1,000 |
| NUDT21 (CFIm25) | mouse | Bio Matrix Research | IP | 1 μg/reaction |
| | rabbit | Proteintech Group | WB, IF | 1:1000, 1:100 |
| PSPC1 (PSP1) | rabbit | Generated by MBL | WB, IF | 1:10000, 1:1,000 |
| RBM4B | rabbit | Abcam | WB, IF | 1:2000, 1:100 |
| RBM7 | rabbit | Atlas Antibodies | WB, IF | 1:1000, 1:100 |
| RBM12 | rabbit | Abcam | WB | 1:2000 |
| | rabbit | Abcam | IF | 1:100 |
| RBM14 | rabbit | Bethyl Laboratories | WB, IF | 1:10000, 1:100 |
| RBMX | rabbit | Abcam | WB, IF | 1:400, 1:100 |
| SFPQ (PSF) | mouse | Sigma | WB, IF | 1:2000, 1:100 |
| SRSF10 | rabbit | Santa Cruz | WB, IF | 1:200, 1:100 |
| SS18L1 | rabbit | Abcam | IF | 1:100 |
| TAF15 | rabbit | Abcam | WB, IF | 1:1000, 1:100 |
| TARDBP (TDP43) | rabbit | Proteintech Group | WB, IF | 1:1000, 1:100 |
| UBAP2L | rabbit | Abcam | WB, IF | 1:5000, 1:100 |
| ZNF335 | rabbit | Abcam | WB | 1:1000 |
| | rabbit | Bethyl Laboratories | IF | 1:100 |
| Digoxygenin | mouse | Roche | FISH | 1:1000 |
| FITC | rabbit | Abcam | FISH | 1:1000 |
| FLAG (M2) | mouse | Sigma | IF | 1:1000 |
| GAPDH | mouse | Abcam | WB | 1:10000 |

Table S5. Primers used in this study

| Primers | Direction | Sequence |
|---|---|---|
| **Subcloning of expression vector for PSF-Flag** | | |
| | forward | CCCAAGCTTATGTCTCGGGATCGGTTC |
| | reverse | CGGGATCCAAATCGGGGTTTTTTGTTTGGGCC |
| **Subcloning of expression vector for Flag-hnRNP K** | | |
| | forward | GATTACAAGGATGACGACGATAAGGAAACTGAACAGCCAGAAGAA |
| | reverse | TTCTAACTCGAGTTAGAAAAACTTTCCAGAATACTGCT |
| **Site-directed mutagenesis to construct siRNA resistance mutant for Flag-hnRNP K** | | |
| K#2 resistant mut | forward | CCTCTTGAGTCAGACGCAGTTGAGTGCTTAAATTACCAACACTATAAAGG |
| | reverse | GAGCTGGCTGGTTGC |
| K#3 resistant mut | forward | ATACACCAAAGCCTTGCTGGTGGTATAATTGGGGTCAAAGGTGCTAA |
| | reverse | CAACAGCCTCAACTCGCA |
| **Subcloning of the template for in vitro transcription of the in vitro processing substrate** | | |
| | forward | TCGTTGGGATTTAGAGTGTATTAGTCACGC |
| | reverse | GGAGCTAGCAAATCTAGACCTAAATCTACA |
| **Site-directed mutagenesis to construct mutant substrates for in vitro RNA processing** | | |
| PAS mut | forward | TCCCCTTTACAGCACAACAAGAGTTTGAGTTCTAAA |
| | reverse | TTTAGAACTCAAACTCTTGTTGTGCTGTAAAGGGGA |
| CFIm mut1 | forward | TTGTGAAATTGAAACTCGAAAAGTAGATGGTTGAA |
| | reverse | TTCAACCATCTACTTTTCGAGTTTCAATTTCACAA |
| CFIm mut2 | forward | ACTGGTATGTTGCTCTCGATGGTAAGAACTAATTC |
| | reverse | GAATTAGTTCTTACCATCGAGAGCAACATACCAGT |
| CFIm mut3 | forward | AATTCTGTTACGTCATCGACATAATTACTAATCAC |
| | reverse | GTGATTAGTAATTATGTCGATGACGTAACAGAATT |
| hnRNPK mut | forward | ACTAATCACTTTTCTTAAAATTTACAGCACAAATAAA |
| | reverse | TTTATTTGTGCTGTAAATTTTAAGAAAAGTGATTAGT |
| **Subcloning of expression plasmid for recombinant hnRNP K protein** | | |
| | forward | TCAGTTTCCATATGGAAACTGAACAGC |
| | reverse | GTTTTTCCTCGAGGAAAAACTTTCCAGAAT |
| **MENε/β RT-qPCR** | | |
| 1 | forward | CAATTACTGTCGTTGGGATTTAGAGTG |
| | reverse | TTCTTACCATACAGAGCAACATACCAG |
| 2 | forward | CAGTTAGTTTATCAGTTCTCCCATCCA |
| | reverse | GTTGTTGTCGTCACCTTTCAACTCT |
| 3 | forward | TGTGTGTGTAAAAGAGAGAAGTTGTGG |
| | reverse | AGAGGCTCAGAGAGGACTGTAACCTG |

Table S6. siRNAs used in this study

| Target proteins | siRNAs | Sense | Antisense |
|---|---|---|---|
| **siRNAs for new paraspeckle proteins** | | | |
| PSP3 | PSP3#1 | CCGAUGGCACCUUUGGCCAAGGCUU | AAGCCUUGGCCAAAGGUGCCAUCGG |
| | PSP3#2 | GCUCCCGAGCUUGAUGGCAAGCAUU | AAUGCUUGCCAUCAAGCUCGGGAGC |
| PSP4 | PSP4#1 | CCAGAAUUAACCAGCGCUUAGAUAU | AUAUCUAAGCGCUGGUUAAUUCUGG |
| | PSP4#2 | GCCGCAUUUGGAGACAGACAUGAUG | CAUCAUGUCUGUCUCCAAAUGCGGC |
| PSP6 | PSP6#2 | GGAGGCUCCAGAGACUACUAUAGCA | UGCUAUAGUAGUCUCUGGAGCCUCC |
| | PSP6#3 | UCCUACAGAGACAGUUACGACAGUU | AACUGUCGUAACUGUCUCUGUAGGA |
| PSP7 | PSP7#1 | UCAGAAGGAGUGGACUUGAUUGAUA | UAUCAAUCAAGUCCACUCCUUCUGA |
| | PSP7#3 | GGCCCAUUCCCGAGAUUCUAGUGAU | AUCACUAGAAUCUCGGGAAUGGGCC |
| PSP8 | PSP8#1 | CCAAGAGACUCUGCGCAGCUACUUU | AAAGUAGCUGCGCAGAGUCUCUUGG |
| | PSP8#3 | UGUGGUGAGACAGAGCUCAGGGAAU | AUUCCCUGAGCUCUGUCUCACCACA |
| PSP10 | PSP10#1 | AUAGGUGUUCUGCUGAGAGUAACUG | CAGUUACUCUCAGCAGAACACCUAU |
| | PSP10#2 | AAAGAAGUCUGCCAGAUCAUCUAGA | UCUAGAUGAUCUGGCAGACUUCUUU |
| PSP11 | PSP11#1 | CCCAUUGUUGGAAGAUGGAGCGCUU | AAGCGCUCCAUCUUCCAACAAUGGG |
| | PSP11#2 | GGGAGAUGAACUGCCCGUAUCUUUC | GAAAGAUACGGGCAGUUCAUCUCCC |
| PSP12 | PSP12#2 | GCCACCUCCAAUCCCUGGCCCUAAU | AUUAGGGCCAGGGAUUGGAGGUGGC |
| | PSP12#3 | CGCUGCGAAGCGACAUGGUCCACUU | AAGUGGACCAUGUCGCUUCGCAGCG |
| PSP13 | PSP13#1 | CCCUCCUUCCUACAGUACUGCUAAA | UUUAGCAGUACUGUAGGAAGGAGGG |
| | PSP13#3 | CCCGUUACAUAUCAAGACUUUGAAA | UUUCAAAGUCUUGAUAUGUAACGGG |
| PSP14 | PSP14#2 | CGGGACAGCCCAUGAUUAAUUUGUA | UACAAAUUAAUCAUGGGCUGUCCCG |
| | PSP14#3 | GGUAAAGAAUUCUCCGGAAAUCCUA | UAGGAUUUCCGGAGAAUUCUUUACC |
| PSP15 | PSP15#1 | AUGAAGAGCUUCCUCAGCUGUUCGG | CCGAACAGCUGAGGAAGCUCUUCAU |
| | PSP15#2 | UUCAGUGUCUUCUUUAAUGCCACCA | UGGUGGCAUUAAAGAAGACACUGAA |
| PSP16 | PSP16#1 | GGCAGUUACUAAAGAAGCACGUGAA | UUCACGUGCUUCUUUAGUAACUGCC |
| | PSP16#2 | CACCUUCACUUUGGGAUAUGGAAUU | AAUUCCAUAUCCCAAAGUGAAGGUG |
| PSP17 | PSP17#2 | GCUAUGUCCAAAGACAGGGCCAAUA | UAUUGGCCCUGUCUUUGGACAUAGC |
| | PSP17#3 | GAACUUGGAUCAGAAGAUGAUGUAA | UUACAUCAUCUUCUGAUCCAAGUUC |
| PSP18 | PSP18#1 | GAACUUGAAUCAGAAGAUGAAGUCA | UGACUUCAUCUUCUGAUUCAAGUUC |
| | PSP18#3 | GGGUGUUGAAGCAUACUGGUCCAAA | UUUGGACCAGUAUGCUUCAACACCC |
| PSP19 | PSP19#2 | CCACUAAAUCCAAUACGAGUUCAUA | UAUGAACUCGUAUUGGAUUUAGUGG |
| | PSP19#3 | GGCUACGGAAGAGAUGGAAUGGAUA | UAUCCAUUCCAUCUCUUCCGUAGCC |
| PSP20 | PSP20#2 | CCGCUCGAAUCUGAUGCUGUGGAAU | AUUCCACAGCAUCAGAUUCGAGCGG |
| | PSP20#3 | UCAUCAGAGUCUAGCAGGAGGAAUU | AAUUCCUCCUGCUAGACUCUGAUGA |
| PSP21 | PSP21#1 | GAUCCAGAAGUCAUGGCUAAGGUAA | UUACCUUAGCCAUGACUUCUGGAUC |
| | PSP21#3 | AGGAUACUUAUGGGCAACAGUGGAA | UUCCACUGUUGCCCAUAAGUAUCCU |
| PSP22 | PSP22#2 | GCCCAGCAGUGGAACCAGUACUAUC | GAUAGUACUGGUUCCACUGCUGGGC |
| | PSP22#3 | GGGAACUACGACUACGGGAGCUACU | AGUAGCUCCCGUAGUCGUAGUUCCC |
| PSP24 | PSP24#2 | UGAACCUCCUCAGUAUCCAUAUAUU | AAUAUAUGGAUACUGAGGAGGUUCA |
| | PSP24#3 | GCACCAGGAUAUGGACCCAUCAUUU | AAAUGAUGGGUCCAUAUCCUGGUGC |
| PSP25 | PSP25#2 | UCAGGAGGAAAUUACAGAGACAAUU | AAUUGUCUCUGUAAUUUCCUCCUGA |
| | PSP25#3 | GCUCUUCGUGGGAGGGCUCAACUUU | AAAGUUGAGCCCUCCCACGAAGAGC |
| PSP26 | PSP26#1 | GGUUAUGGGCCAGAGAGUGAAUUAU | AUAAUUCACUCUCUGGCCCAUAACC |
| | PSP26#3 | GCAUAUGGAGCACUCGACUACUAUA | UAUAGUAGUCGAGUGCUCCAUAUGC |
| PSP27 | PSP27#2 | CAAACAUGAAGUGUCUGUUCCUUAU | AUAAGGAACAGACACUUCAUGUUUG |

| | | | |
|---|---|---|---|
| | PSP27#3 | UCACAGCGUAAAGUCAGAAUGAAUU | AAUUCAUUCUGACUUUACGCUGUGA |
| PSP28 | PSP28#1 | GCUUUGAAACGAAACAGAAUGCUGA | UCAGCAUUCUGUUUCGUUUCAAAGC |
| | PSP28#2 | UGCCACUGAUGAAGAUGCAAGGCUU | AAGCCUUGCAUCUUCAUCAGUGGCA |
| PSP29 | PSP29#2 | GCAGAUCGCCCAGGAAAGCUCUUCA | UGAAGAGCUUUCCUGGGCGAUCUGC |
| | PSP29#3 | CCAAGUUCUCGUGAUACUAGAGAUU | AAUCUCUAGUAUCACGAGAACUUGG |
| PSP30 | PSP30#2 | CCCUGACCAUCACUGUGUUCACCAA | UUGGUGAACACAGUGAUGGUCAGGG |
| | PSP30#3 | UCACUCAGCACCACAAGCCACUUCA | UGAAGUGGCUUGUGGUGCUGAGUGA |
| PSP31 | PSP31#1 | CCGACGACACCAGGUCUGAAGACUU | AAGUCUUCAGACCUGGUGUCGUCGG |
| | PSP31#3 | CAACUAUAGAAGAUCGUAUAGUCCU | AGGACUAUACGAUCUUCUAUAGUUG |
| PSP32 | PSP32#1 | CCAUCCAGAAGAUGCUGGACGAGAA | UUCUCGUCCAGCAUCUUCUGGAUGG |
| | PSP32#2 | GCCACGAUCGCAGACUCCAACCAGA | UCUGGUUGGAGUCUGCGAUCGUGGC |
| PSP33 | PSP33#1 | CCUUUGCCACUAGAAGACCUGAAUU | AAUUCAGGUCUUCUAGUGGCAAAGG |
| | PSP33#2 | GCUCAUAUAGCCAGCAACCAUAUAA | UUAUAUGGUUGCUGGCUAUAUGAGC |
| PSP34 | PSP34#2 | CCCGCCACAAGUAUAUGGUUAUGAU | AUCAUAACCAUAUACUUGUGGCGGG |
| | PSP34#3 | CAACACUGGCCACUUUGAACCAGAU | AUCUGGUUCAAAGUGGCCAGUGUUG |
| PSP35 | PSP35#1 | GGGAUCCCAGGAAAUUGAGAGGGAA | UUCCCUCUCAAUUUCCUGGGAUCCC |
| | PSP35#2 | GCAUCAUGCAAAUGCUGGCACUAAU | AUUAGUGCCAGCAUUUGCAUGAUGC |
| PSP36 | PSP36#1 | AGACAGCCUUGGAUCUUCUGCUGAA | UUCAGCAGAAGAUCCAAGGCUGUCU |
| | PSP36#2 | AGGAAGCCGCCUACAUCCAAGAGAU | AUCUCUUGGAUGUAGGCGGCUUCCU |
| TDP43 | TDP43#2 | UGAGCCCAUUGAAAUACCAUCGGAA | UUCCGAUGGUAUUUCAAUGGGCUCA |
| | TDP43#3 | GACAGAUGCUUCAUCAGCAGUGAAA | UUUCACUGCUGAUGAAGCAUCUGUC |
| **siRNAs for known paraspeckle proteins** | | | |
| CFIm68 | CFIm68#1 | UCCGCAUGUGAACCCAGCUUUCUUU | AAAGAAAGCUGGGUUCACAUGCGGA |
| | CFIm68#2 | ACUUCAUGGUCAGAAUCCUGUUGUA | UACAACAGGAUUCUGACCAUGAAGU |
| p54nrb | p54nrb#1 | GGGAACAGGGUUACUGUAUACUGAA | UUCAGUAUACAGUAACCCUGUUCCC |
| | p54nrb#2 | CAGUAGCUCUUAGACUCGCCUAUCU | AGAUAGGCGAGUCUAAGAGCUACUG |
| PSP1 | PSP1#2 | GCAGGUUGAUAGAAACAUCTT | GAUGUUUCUAUCAACCUGCTC |
| | PSP1#3 | GCUAGGCAUGAACACCAAUTT | AUUGGUGUUCAUGCCUAGCTG |
| PSP2 | PSP2#1 | AGUCUGCAGCCUCCUCACUAGCUUA | UAAGCUAGUGAGGAGGCUGCAGACU |
| | PSP2#3 | CCAAGGCCUCUUAAUACUUGGAAGA | UCUUCCAAGUAUUAAGAGGCCUUGG |
| PSF | PSF#14 | CAGUCAUUGUGGAACCACUUGAACA | UGUUCAAGUGGUUCCACAAUGACUG |
| | PSF#19 | CCCUAUGGUUCAGGAGGCCAGAAAU | AUUUCUGGCCUCCUGAACCAUAGGG |

**Supplemental Experimental Procedure**

**Plasmid construction**

To generate the MENε and MENβ expression vectors, the Red/ET recombination system BAC subcloning kit (Gene Bridges) was used. Initially, a low-copy expression vector, pCMV-EGFP-pA (low), was generated by blunt-ligating the expression cassette from pEGFP-N1 (Clonetech) digested with *Pci*I and *Afl*II with the plasmid backbone of pGEX-4T digested with *BspM*I and *Not*I. Gene fragments of 500 bp corresponding to the 5'- and 3'-region of MENε or MENβ were PCR-amplified and subcloned into the *Xho*I-*EcoR*I and *EcoR*I-*BamH*I sites of pCMV-EGFP-pA (low) to generate pCMV-MENε (5'-3')-EGFP-pA and pCMV-MENβ(5'-3')-EGFP-pA, respectively. The plasmid pCMV-MENε (5'-3')-EGFP-pA was digested with *BamH*I/*Not*I, blunt-ended, and self-ligated to remove the EGFP cassette, which yielded pCMV-MENε (5'-3')-pA. The plasmid pCMV-MENβ (5'-3')-EGFP-pA was digested with *Bam*/*Afl*II, blunt-ended, and self-ligated to remove EGFP and the polyadenylation signal, which yielded pCMV-MENβ (5'-3')-pA.

The above plasmids were linearized with *EcoR*I and used for subsequent BAC recombination. Red/ET-mediated recombination was performed according to the manufacturer's instructions with BAC clone RP23-209P9. The recombination generated

pCMV-MENε-pA and pCMV-MENβ, which contain mouse genomic regions chr19: 5,842,072–5,845,478 and chr19: 5,824,162–5,845,478 of the NCBI37/mm9 assembly, respectively.

The PCR-amplified PSF-Flag sequence was inserted into the pcDNA5 FRT vector (Invitrogen) to produce the PSF-Flag construct. The PCR-amplified Flag-hnRNP K sequence was inserted into pcDNA3 vector (Invitrogen), followed by production of the siRNA-resistant Flag-hnRNP K construct with the QuikChange XL site-directed mutagensis kit (Stratagene). For the template of the in vitro processing substrate, the border region of MENε/β (3521–3840 nt) was PCR-amplified and inserted into the pGEM-T easy vector (Promega). Mutant substrates were constructed with the QuikChange XL site-directed mutagensis kit (Stratagene). The bacterial expression construct for recombinant his-tagged hnRNP K protein was cloned into the pET-22b vector (Novagen). Primer information is provided in Table S5.

**RNAi**

HeLa cells were transfected with siRNAs at 33 nM (final concentration) by using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). After 48 h, the cells were again transfected and incubated for 48 h. Knockdown efficiencies were

verified by RT-qPCR or Western blotting (Table S2). The siRNAs for PSP1 were purchased from Ambion (#116209 and #132412). For other PSPs, stealth siRNAs were purchased from Invitrogen. The siRNA sequences used in this study are listed in Table S6. The effects of RNAi on paraspeckle appearance were assessed by counting the number of cells that possessed at least one paraspeckle.

**RNase protection assay**

The total RNA was prepared with Trizol reagent (Invitrogen). The RNase protection assay was performed with the RPAIII kit (Ambion), according to the manufacturer's protocol. Briefly, 3 μg of total RNA were hybridized with a $^{32}$P-labeled antisense RNA probe that was synthesized with T7 RNA polymerase (TaKaRa). RNase A/T1 digestion excluded unhybridized single-stranded RNA probes. The protected RNA fragments were separated by 6% PAGE containing 7 M urea. Radioactive RNA bands were visualized and quantified with the Bioimaging analyzer BAS3000.

**RT-qPCR**

The total RNA (1 μg) was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen). Primers were designed by Primer3 software

(http://www-genome.wi.mit.edu/ftp/distribution/software/) and purchased from Invitrogen. Aliquots of cDNA were subjected to real-time PCR with a LightCycler 480 SYBR Green I Master (Roche Diagnostics) according to the manufacturer's protocol. Primers used in this study are shown in Table S5.

**Immunofluorescence**

Immunofluorescence was performed basically as described (Sasaki et al., 2009). Briefly, cells seeded onto a multichamber culture slide (BD Falcon) were fixed with 4% paraformaldehyde/PBS. Fixed cells were permeabilized with 0.2% Triton X-100/PBS for 5 min, rinsed, and blocked with 10% normal horse serum (Vector Laboratories) in Tris-buffered saline (TBST, 0.1% Tween-20) for 1 h. Primary antibodies were applied for 1 h at room temperature (RT) or overnight at 4 °C. The samples were washed three times with TBST for 5 min each. Fluorophore-conjugated secondary antibodies were applied for 1 h at RT. After washing, the slides were mounted with Vectashield (Vector Laboratories) containing 4', 6-diamidino-2-phenylindole (DAPI). The antibodies used are shown in Table S4.

**RNA fluorescent in situ hybridization (FISH)**

The FISH was performed basically as described (Sasaki et al., 2009). Briefly, cells were seeded onto a multichamber culture slide (BD Falcon) and fixed with 4% paraformaldehyde/PBS. The fixed cells were permeabilized with 0.5% Triton X-100/PBS for 5 min, rinsed, and dehydrated. The RNA probes were prepared with a DIG/FITC RNA-labeling kit (Roche Diagnostic). Dehydrated slides were incubated with a hybridization solution (2× SSC, 50% formamide, 1× Denhardt's salt [Sigma], 10 mM EDTA, 100 μg/mL yeast tRNA, 0.01% Tween-20, and 5% dextran sulfate), containing the DIG- and/or FITC-labeled RNA probe, at 55 °C for 16 h. The slides were washed twice with prewarmed wash buffer (2× SSC, 50% formamide, and 0.01% Tween-20) at 55 °C for 30 min. Excess RNA probes were digested with 10 μg/mL RNase A in NTET (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 500 mM NaCl, and 0.1% Tween-20) at 37 °C for 1 h. The slides were washed with buffer (2× SSC, 0.01% Tween-20) at 55 °C for 30 min and twice with a second buffer (0.1× SSC, 0.01% Tween-20) at 55 °C for 30 min.

To detect RNAs that were transiently expressed from the transfected plasmid, slides were treated with RNase H in a buffer containing 20 mM HEPES, (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 100 U/mL RNaseH (TaKaRa) at 37 °C for 30 min. Subsequently, the slides were blocked with 1% Roche Blocking Reagent in TBST at RT for 1 h. The slides were incubated with anti-DIG and/or anti-FITC antibodies, which had been diluted with a