Supporting Information

Sasaki *et al.* 10.1073/pnas.0807899106

SI Text

Cell Culture. HeLa, HEK293, MCF7, and NIH 3T3 cells were grown in DMEM supplemented with 10% FBS (Cell Culture Technologies) at 37 °C and 5% CO_2 in a humidified incubator. MRC5 and T24 cells were grown in MENα (GIBCO) and McCoy's 5A medium (GIBCO), respectively. Some cells were treated with actinomycin D (0.03 or 0.3 μg/mL, 4 h) or with DRB (100 μM, 6 h) (Sigma).

Cell Fractionation. We followed the nucleolar isolation protocol developed by the Lamond laboratory. (www.lamondlab.com/f7nucleolarprotocol.htm). HeLa nuclei were prepared from 1×10^8 cells, washed 3 times with PBS, resuspended in 5 mL of buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT], and homogenized for 10 strokes by using a Dounce homogenizer with a tight pestle. An aliquot of the cell homogenate was removed as a source for total RNA. The rest of the cell homogenate was centrifuged at 218 $\times$ *g* for 5 min at 4 °C, yielding the cytosolic (supernatant) and nuclear (pellet) fractions. The pellet was resuspended in 3 mL of S1 solution (0.25 M sucrose, 10 mM $MgCl_2$), overlaid onto 3 mL of S2 solution (0.35 M sucrose, 0.5 mM $MgCl_2$), and centrifuged at 1,430 $\times$ *g* for 5 min at 4 °C. The pellet was resuspended in 3 mL of S2 solution and an aliquot was removed as the nuclear fraction. The rest of the suspension was sonicated by 5-s pulses repeated 20 times by using a hand-held sonicator equipped with a microprobe (UR-20P; TOMY Seiko) at 80% of the maximum output. The sonicate was overlaid onto an equal volume of S3 solution (0.88 M sucrose, 0.5 mM $MgCl_2$). Before centrifugation, the border of the 2 layers was marked on the tube. The solution was then centrifuged at 5,000 $\times$ *g* for 10 min at 4 °C, resulting in 2 layers of supernatant (Np1 and Np2) and 1 pellet (No). The pellet was rinsed with S2 solution and recovered by centrifugation. The resulting pellet and the previously obtained fractions were subjected to RNA extraction.

RNA FISH. FISH was performed basically as described (1). In brief, cells were seeded onto a multichamber culture slide (BD Falcon) and fixed with 4% paraformaldehyde in PBS. After deproteination and dehydration, the slides were incubated with prehybridization solution (2× SSC, 1× Denhardt solution, 50% formamide, 10 mM EDTA, 100 μg/mL yeast tRNA, and 0.01% Tween 20) at 55 °C for 2 h. RNA probes were prepared by using a DIG/FITC RNA labeling kit (Roche). Prehybridized slides were incubated with a hybridization solution (the prehybridization solution plus 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 then 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. Probe detection was carried out as follows. The slides were blocked with 1% Roche Blocking Reagent in TBST (Tris-buffered saline, 0.01% Tween-20) at room temperature (RT) for 1 h and then incubated with anti-DIG and/or anti-FITC antibodies diluted with a blocking reagent for 1 h. Unbound antibodies were removed by washing 3 times in TBST for 15 min. The slides were incubated with a fluorophore-conjugated secondary antibody for 1 h. After washing, the slides were coverslipped with Vectashield (Vector Laboratories) containing 4′, 6-diamidino-2-phenylindole (DAPI).

Immunofluorescence. Cells were seeded onto culture slides and fixed with 4% paraformaldehyde/PBS. The cells were permeabilized with 0.2% Triton X-100/PBS for 5 min, rinsed, and then blocked with 10% normal horse serum (Vector Laboratories) in PBS for 1 h. Primary antibodies against proteins and/or against the hapten of the RNA probes were applied overnight at 4 °C and then washed 3 times with PBST (PBS, 0.01% Tween-20) for 15 min. Alexa-conjugated secondary antibodies (Molecular Probes, Invitrogen) were applied for 1 h at RT. After washing, the samples were counterstained with DAPI.

Fluorescence Microscopy. Immmunostained cells were examined by using an LSM 510 META laser-scanning microscope with a C-Apochromat 40X/1.2W lens (Carl Zeiss). Each data series (samples on a single 8-chambered culture slide probed with the same probe and/or antibodies) was processed with fixed parameters so that the signal intensities could be compared. Acquired confocal images were pseudocolored and merged by using an image analysis software (LSM Image Browser).

Quantitative Reverse Transcription-PCR (qRT-PCR). qRT-PCR was carried out as described (2). Briefly, total RNA was prepared from cell cultures, fractionated cell lysates, and knockdown oligo-treated cells using a PARIS kit (Ambion). RNA (1 μg) was reverse transcribed using a QuantiTect reverse transcription kit (Qiagen). Primers were designed using the Primer3 software (see Table S3) and purchased from Invitrogen. Aliquots of cDNA were subjected to real-time PCR, performed using a Lightcycler 480 SYBR green I Master (Roche Diagnostics) according to the manufacturer's protocol.

Northern Blotting. A 10-μg aliquot of total RNA per lane was separated on a 0.8% agarose gel containing 2% formaldehyde and was blotted to a positively charged nylon membrane (Hybond N+; Amersham Biosciences). After UV cross-link, the blots were hybridized overnight to ^{32}P-labeled cDNA probes at 68 °C in an ExpressHyb Hybridization solution (Clontech). Autoradiography was processed using a Fuji BAS2500 Bioimaging analyzer.

RNase Protection Assay. The RNase protection assay was performed as described previously (3). The probes used were internally ^{32}P-labeled antisense MENε/β RNAs covering the sequences shown in Fig. S1. The protected RNA fragments were separated on 8% denaturing polyacrylamide gels.

RNAi. RNAi was carried out as described (4). The siRNAs for p54 were synthesized by Sigma Genosys, Inc., and those for PSP1 were purchased from Ambion (#116209 and #132412). For PSF, stealth siRNAs were synthesized by Invitrogen. HeLa cells were grown to 30–50% confluency in 6-well tissue culture dishes. The siRNA duplexes (50 nM) were administered to the HeLa cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 48 h, total RNA was prepared using an RNAeasy kit (Qiagen). qRT-PCR was carried out as described previously (2).

Ectopic Expression of MENε/β. MENε was reverse-transcribed with a dT primer and subcloned into the pMIR-REPORT Luciferase vector (Ambion) at the Mlu I/Pme I site. MENβ lacking the 3′ 10 kb was created by recombineering (5). A retrieving vector was constructed based on the pMIR-REPORT Luciferase, in which

2 500-bp fragments homologous to MENβ were inserted into the Mlu I/Pme I site and the Pme I/HindIII site, respectively. For the oligonucleotide sequences used to amplify homologous fragments, see Table S1. The retrieving vector was electroporated into *E. coli* harboring the BAC clone CITB2521O8 (Invitrogen), which contains the entire MENε/β sequence. These 2 MENε/β constructs were transfected to HeLa or NIH 3T3 cells using Lipofectamine 2000 (Invitrogen). Localization of ectopically expressed RNA was examined by double-labeling FISH for endogenous MENε/β and costained with antibodies for endogenous paraspeckle proteins.

Statistical Analyses. An unpaired, 2-tailed *t* test was carried out for the data shown in Fig. 5 *A* and *C*. We separated our observations into 2 categories by selecting 2 nonoverlapping probes among the 7 probes. Observations from these 2 probes were associated with one category, and the rest of the observations were associated with the other category. There were 21 (i.e., $7 \times (7 - 1)/2$) possible combinations of the 2 probes for all 7 probes. We then performed unpaired, 2-tailed *t* tests for the categorized observations. For the actual computation, we used the *t* test function included in the R software package. The alternative hypothesis used was that the means of the 2 samples are greater/less than the other. Table S3 shows the probe combinations that displayed the lowest *P* value. For p54 and PSF, the probe combo 1–2 showed the lowest *P* value and was found to be significant ($P < 0.01$). PSP1 did not display any significance by this *t* test. αFlag and Flag-pcDNA probes did not show significant differences, whereas the αPSF and Flag-p54 1–2 probe combo significantly differed from the 4–8 combo. For Fig. 5*D*, we carried out paired *t* tests to evaluate the statistical significance for differences of the average relative MENε/β levels between each pair of experiments (p54+UV 1, 2, 4, and 8). Table S5 shows the *P* values obtained by the *t* tests. No statistically significant differences were found for the experiments 1–2 and 4–8, whereas the other combinations yielded significant differences. The computation was performed using the *t*.test function (alternatives = "less" and paired = TRUE, whereas other options remain default values) of the open source software R (www.r-project.org/).

1. Sone M, *et al.* (2007) The mRNA-like noncoding RNA Gomafu constitutes a novel nuclear domain in a subset of neurons. *J Cell Sci* 120:2498–2506.
2. Sasaki YTF, *et al.* (2007) Identification and characterization of human non-coding RNAs with tissue-specific expression. *Biochem Biophys Res Commun* 357:991–996.
3. Hirose T, Steitz JA (2001) Position within the host intron is critical for efficient processing of box C/D snoRNAs in mammalian cells. *Proc Natl Acad Sci USA* 98:12914–12919.
4. Ideue T, Sasaki YTF, Hagiwara M, Hirose T (2007) Introns play an essential role in splicing-dependent formation of the exon junction complex. *Genes Dev* 21:1993–1998.
5. Liu P, Jenkins NA, Copeland NG (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* 13:476–484.

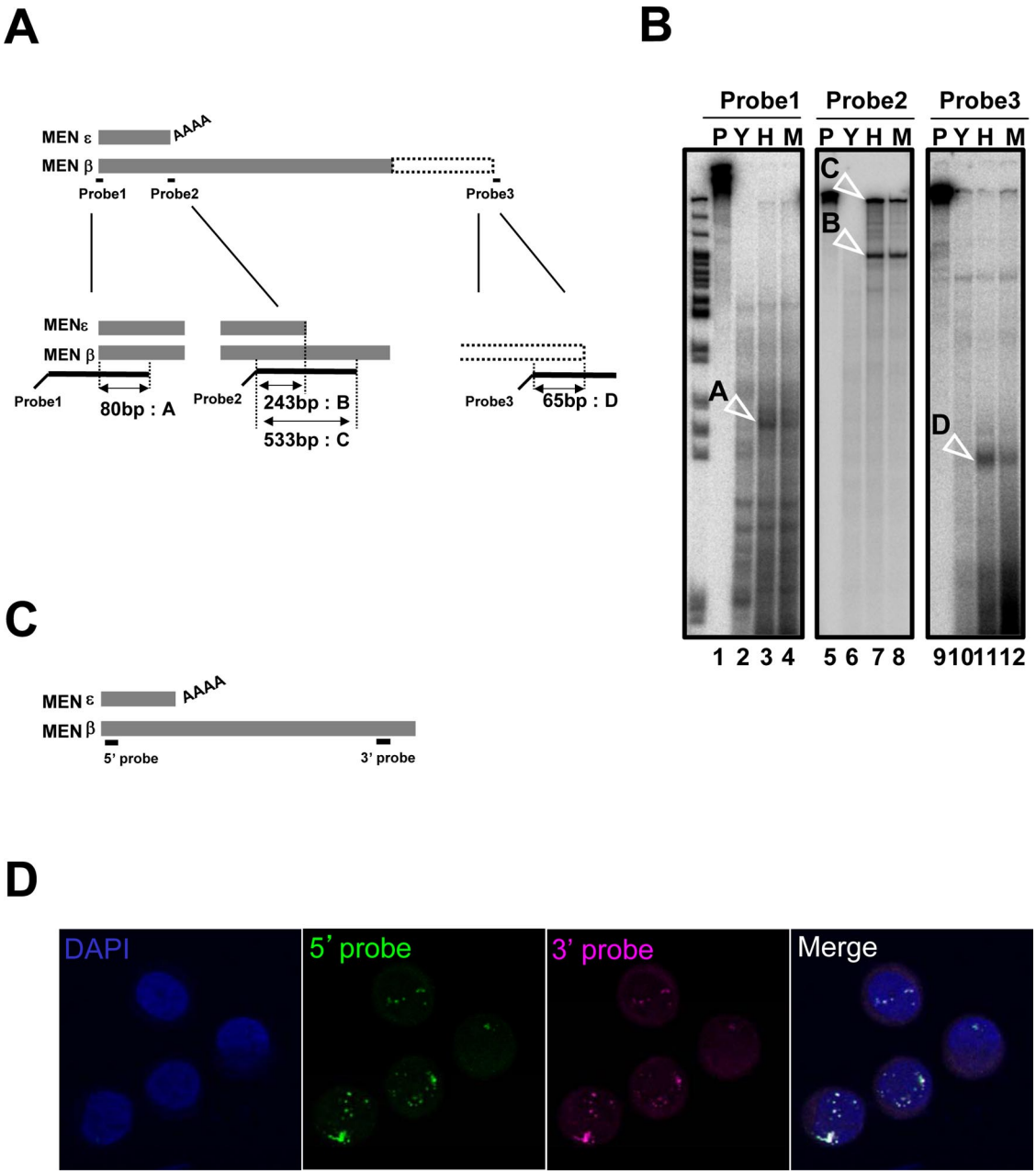

Fig. S1. Characterization of the short (MENε) and long (MENβ) isoforms of the MEN transcripts. (*A*) Schematic diagram depicting the structure of MENε/β transcripts with the mapped terminus. The positions of antisense RNA probes designed for the RNase protection assay (probes 1 $\approx$ 3) are shown below. The 5′ and 3′ terminal regions of MENε/β transcripts are enlarged in the lower figures. The sizes of the protected fragments (A–D in *B*) are also shown. (*B*) The protected fragments from RNase A/T1 digestion are indicated by the open arrowheads. RNAs prepared from HeLa (lane H), MRC5 (lane M), and yeast (lane Y; control) cells were used. The undigested probes are also shown (lane P). The molecular size marker is a ^{32}P-labeled ψX174 RF HincII digest fragment (lane MW). Quantitation of the intensities of bands B and C indicate that the accumulation ratio of MENβ to MENε is 3:7. (*C*) Positions of the 2 probes relative to MENε and -β are shown schematically. (*D*) HeLa cells were subjected to FISH with 2 different probes. The 5′ probe recognizes both MENε and -β (in green), whereas the 3′ probe recognizes only MENβ (in magenta). The signal obtained by both probes coincides completely (Merge, white). Cells were counterstained with DAPI (blue).

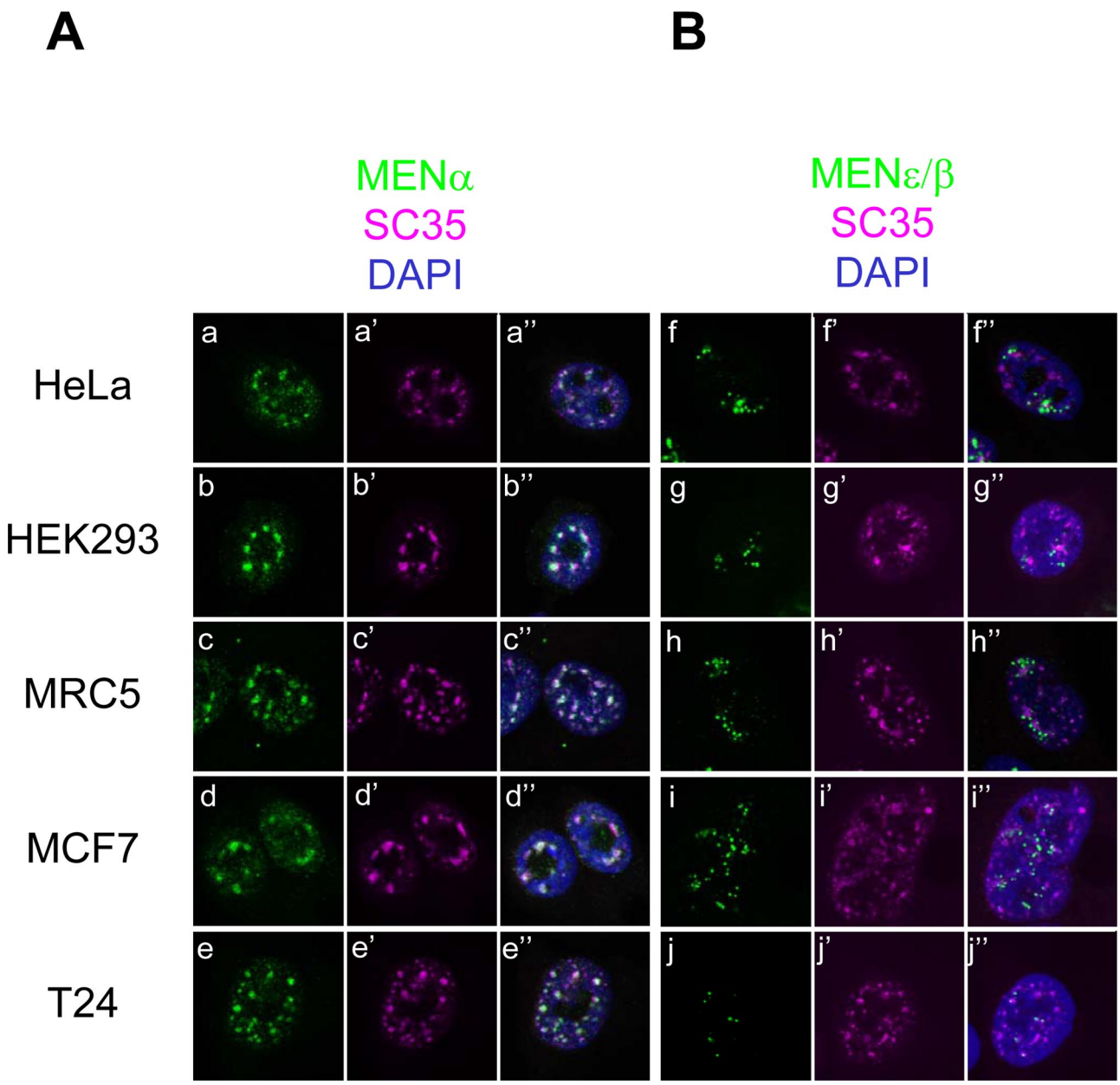

Fig. S2. MENα, but not MENε/β, colocalizes to splicing speckles. FISH-IF experiments were carried out with various human cell lines. The probes for the RNAs (green, *a*–*e*, and *f*–*j*) and the speckle marker, SC35 (magenta, *a*′–*e*′ and f′–*j*′), are indicated above each image. The names of the cell lines examined are indicated to the left of the images.

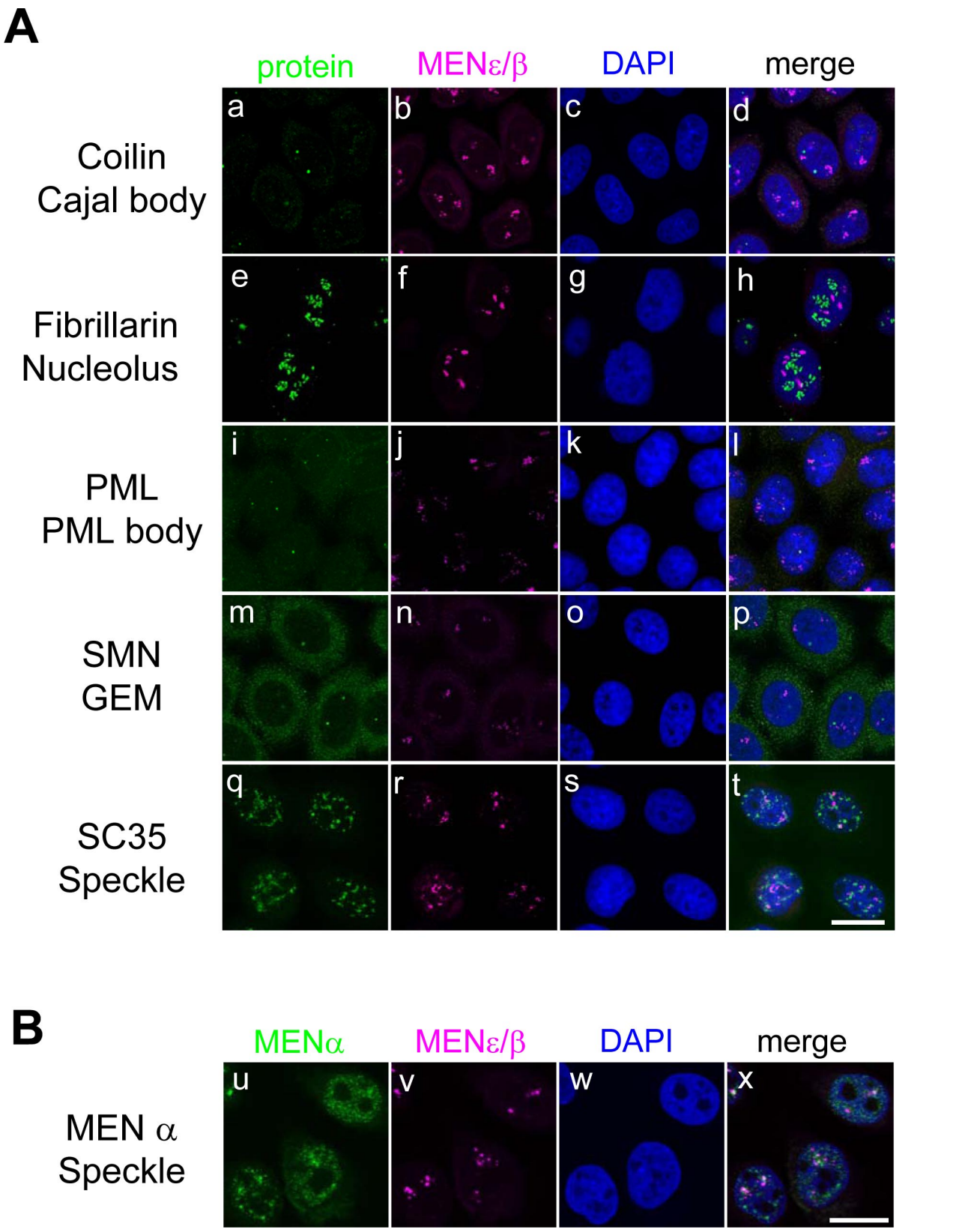

Fig. S3. Subnuclear localization of the MENε/β ncRNAs. FISH-IF experiments were carried out with a MENε/β probe in combination with antibodies against various marker proteins for known nuclear bodies (*A*) or with another ncRNA, MENα (*B*). The names of the nuclear bodies and their marker proteins are indicated to the left of the images. (Scale bars: 10 μm.)

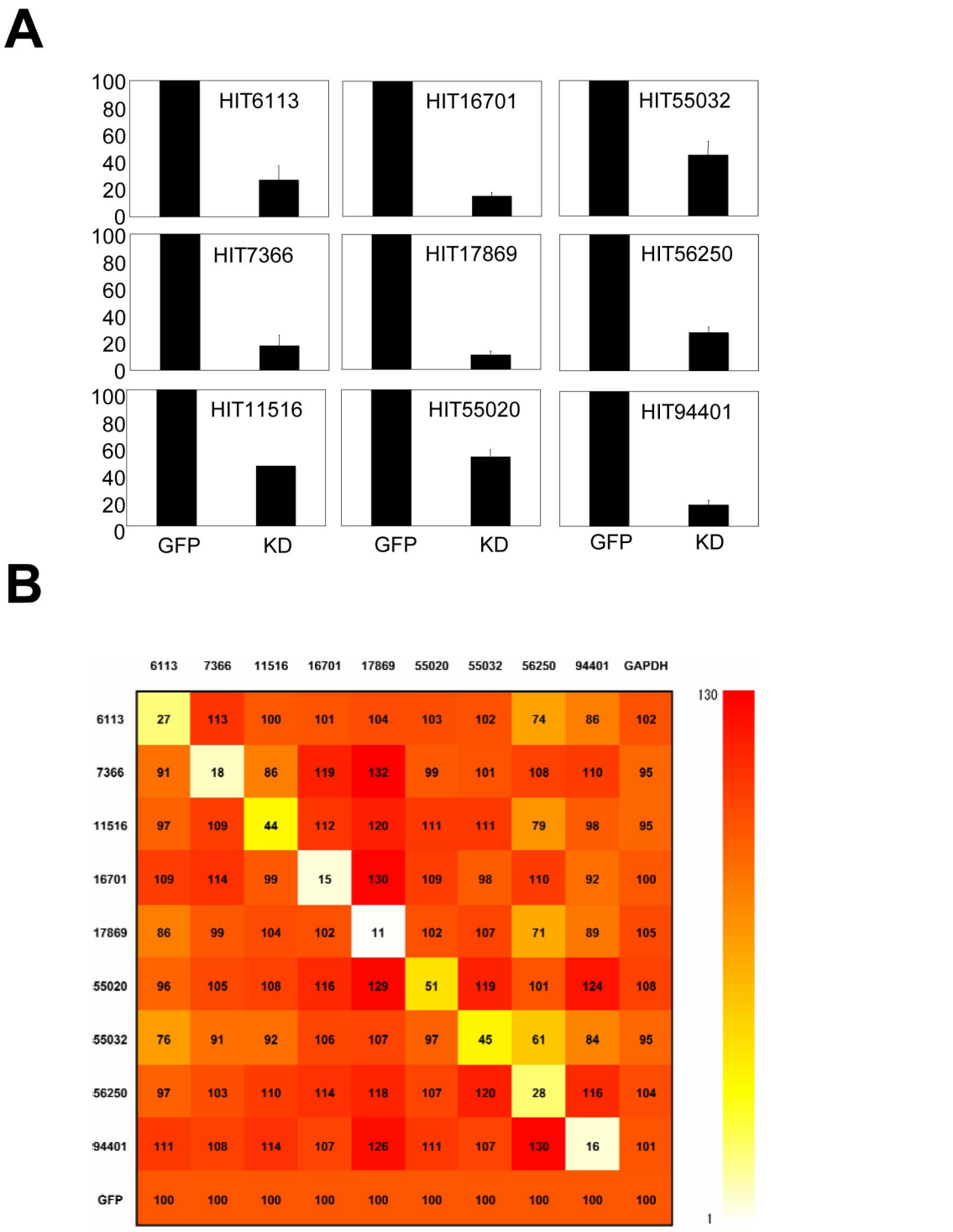

Fig. S4. Knockdown of nuclear-localized mRNA-like ncRNAs. (*A*) The RNA levels in cells treated with control oligonucleotide (GFP) or target oligonucleotide (KD) are shown. (*B*) The matrix depicts the target specificity of each knockdown oligonucleotide. The knockdown oligonucleotides are aligned on the vertical axis, and the residual ncRNA levels upon knockdown are aligned on the horizontal axis.

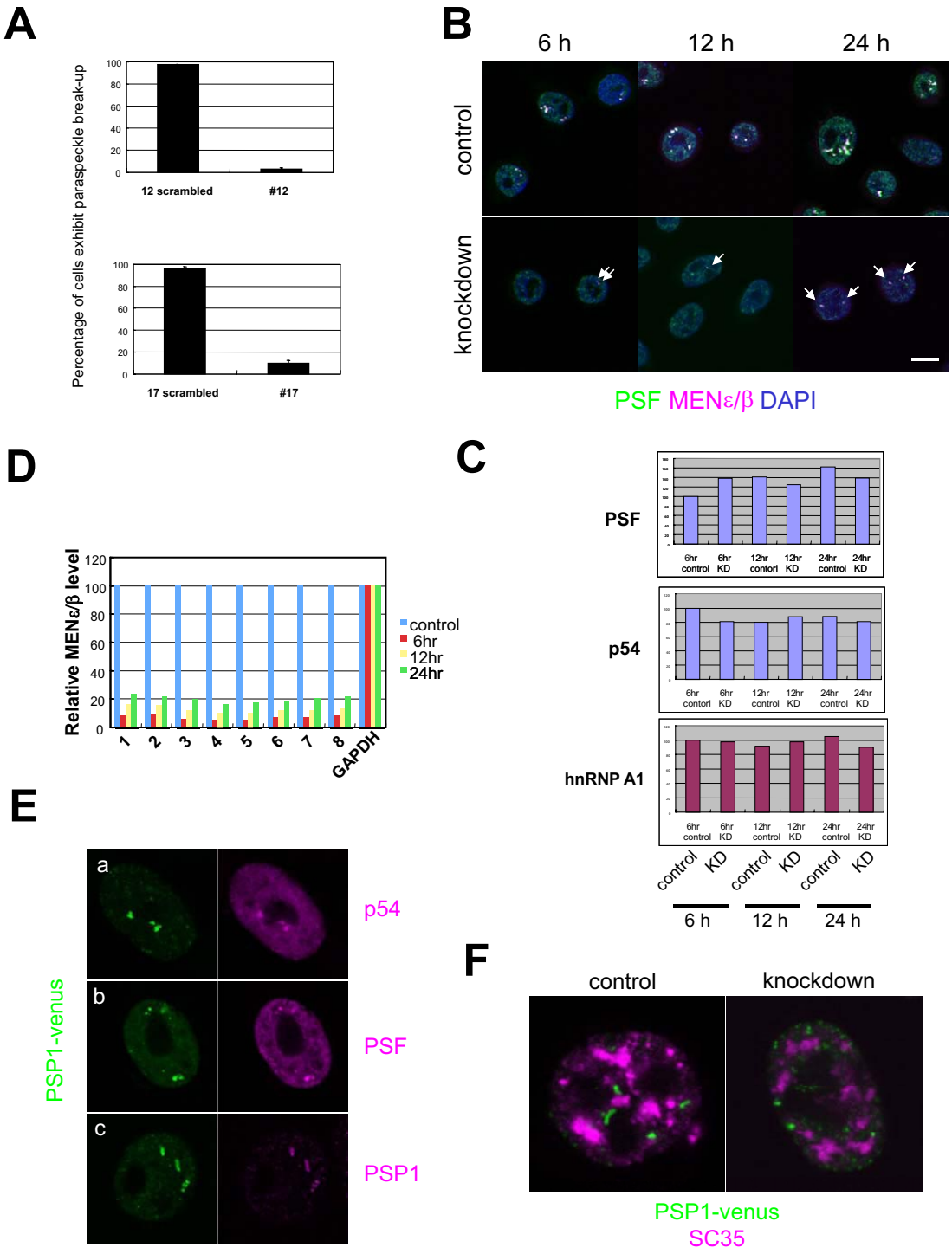

Fig. S5. Effect of MENε/β knockdown on the paraspeckle proteins. (*A*) HeLa cells were treated with either of 2 knockdown oligonucleotides (#12 or #17) or the corresponding scrambled controls (12 scrambled or 17 scrambled). Values represent the mean ± SD (n = 141–289 cells) from 2 independent experiments. (*B*) HeLa cells were treated with knockdown oligonucleotides for the indicated times (6, 12, or 24 h) and then fixed and subjected to FISH-IF with an anti-PSF antibody (green) and an MENε/β probe (magenta). (Scale bar: 10 μm.) (*C*) Knockdown efficiency was quantified by RT-qPCR. Total RNA was extracted from cells treated with control oligonucleotides (blue) or MENε/β knockdown oligonucleotides for various times (6 h, red; 12 h, yellow; 24 h, green). The amount of MENε/β transcript was measured with specific primer sets (#1 to #8, see Table S1 for further information on these primers). (*D*) The levels of the paraspeckle proteins during MENε/β knockdown were quantified by Western blot. The protein level data are normalized to the protein levels in cells treated with GFP control oligonucleotides for 6 h. The loading control for normalization was hnRNP A1. (*E*) Subnuclear distribution of the PSP1-Venus fusion protein was verified by costaining for endogenous paraspeckle proteins (*a*, p54; *b*, PSF; *c*, PSP1). (*F*) Subnuclear distribution of the PSP1-Venus fusion protein was compared with the distribution of the speckle marker protein SC35, with (knockdown, *Right*) or without (control, *Left*) MENε/β knockdown.

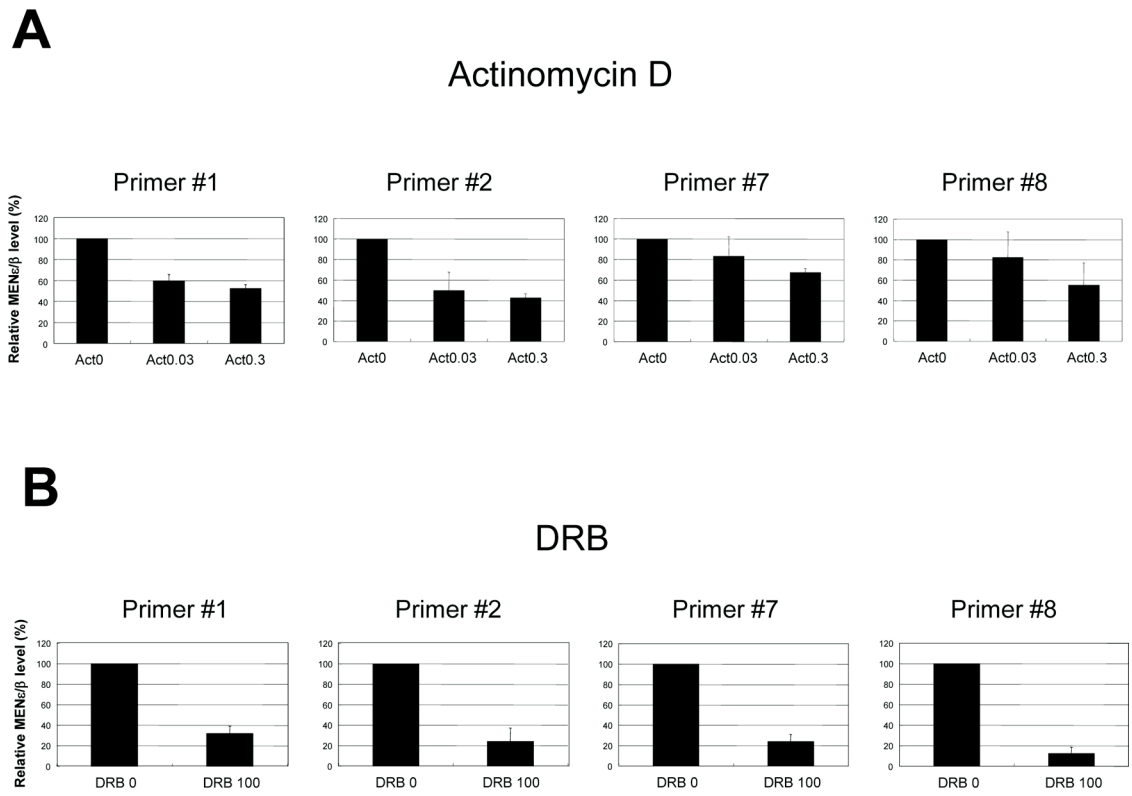

Fig. S6. Transcriptional inhibitors diminish the MENε/β level. (*A*) Total RNA was extracted from cells treated with actinomycin D and subjected to RT-qPCR with 4 different primer pairs designed for MENε/β. (*B*) RT-qPCR was performed as in *A* using total RNA extracted from DRB-treated cells. Experiments were repeated 3 times, including the experiments presented in Figs. 3 and 5 and Fig. S8.

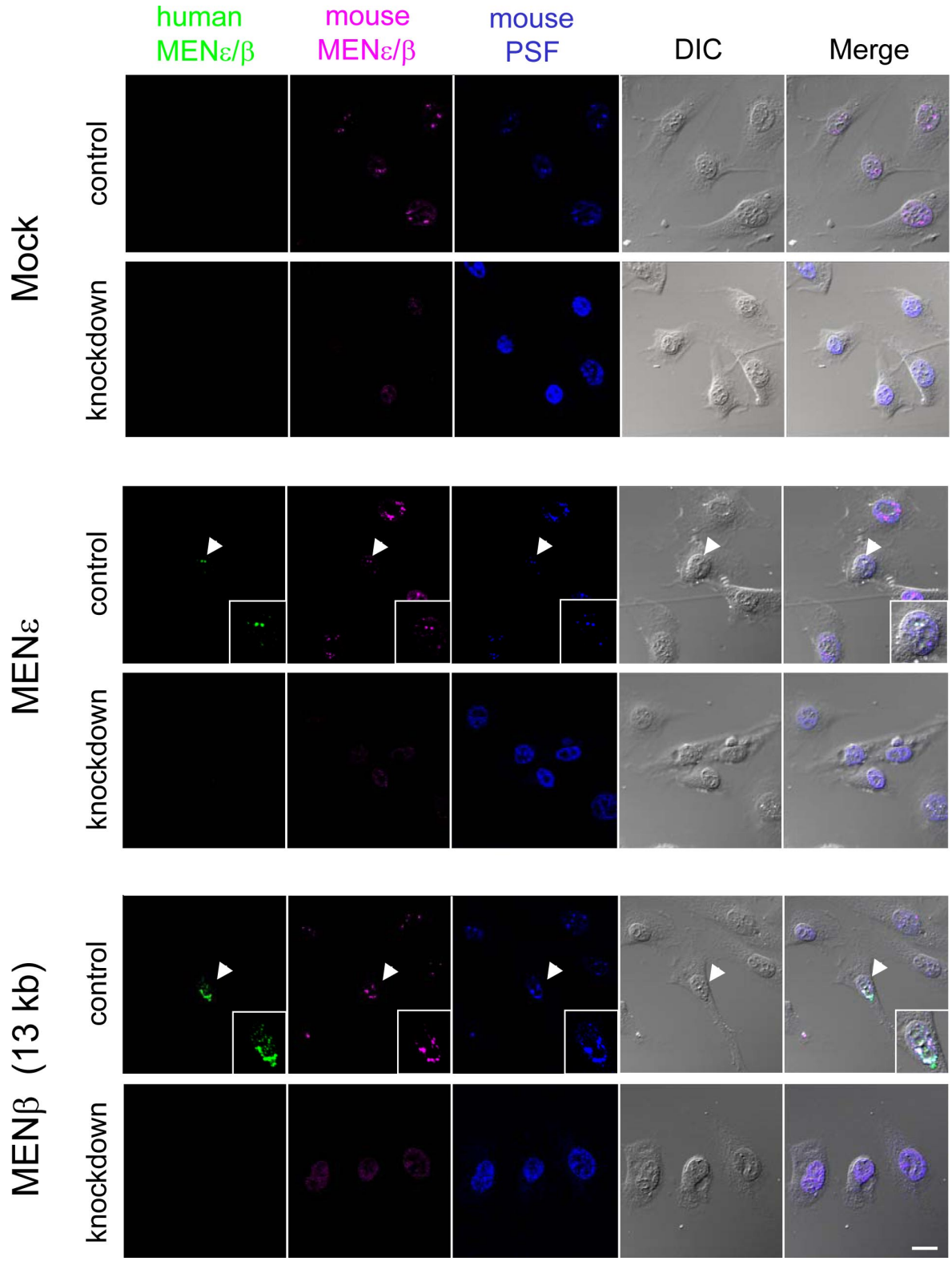

Fig. S7. Ectopically expressed MENε/β localize to paraspeckles but are incapable of rescuing disintegrated paraspeckles in MENε/β knockdown cells. Subcloned MENε/β were transfected into NIH 3T3 cells. Exogenous human MEN RNAs were detected by a human MEN probe (green) in combination with a mouse MEN probe (magenta) and an anti-PSF antibody (blue). Specimens were treated with DNase I before hybridization to minimize the background signal from plasmid templates. Mock, empty vector; DIC, differential interference contrast image. (*Upper*) Cells were treated with control antisense oligonucleotide. (*Lower*) Cells were treated with antisense oligonucleotide for mouse MENε/β. Arrowheads indicate the nuclei expressing human MENε/β RNA localized to paraspeckles. (Scale bar: 10 μm.)

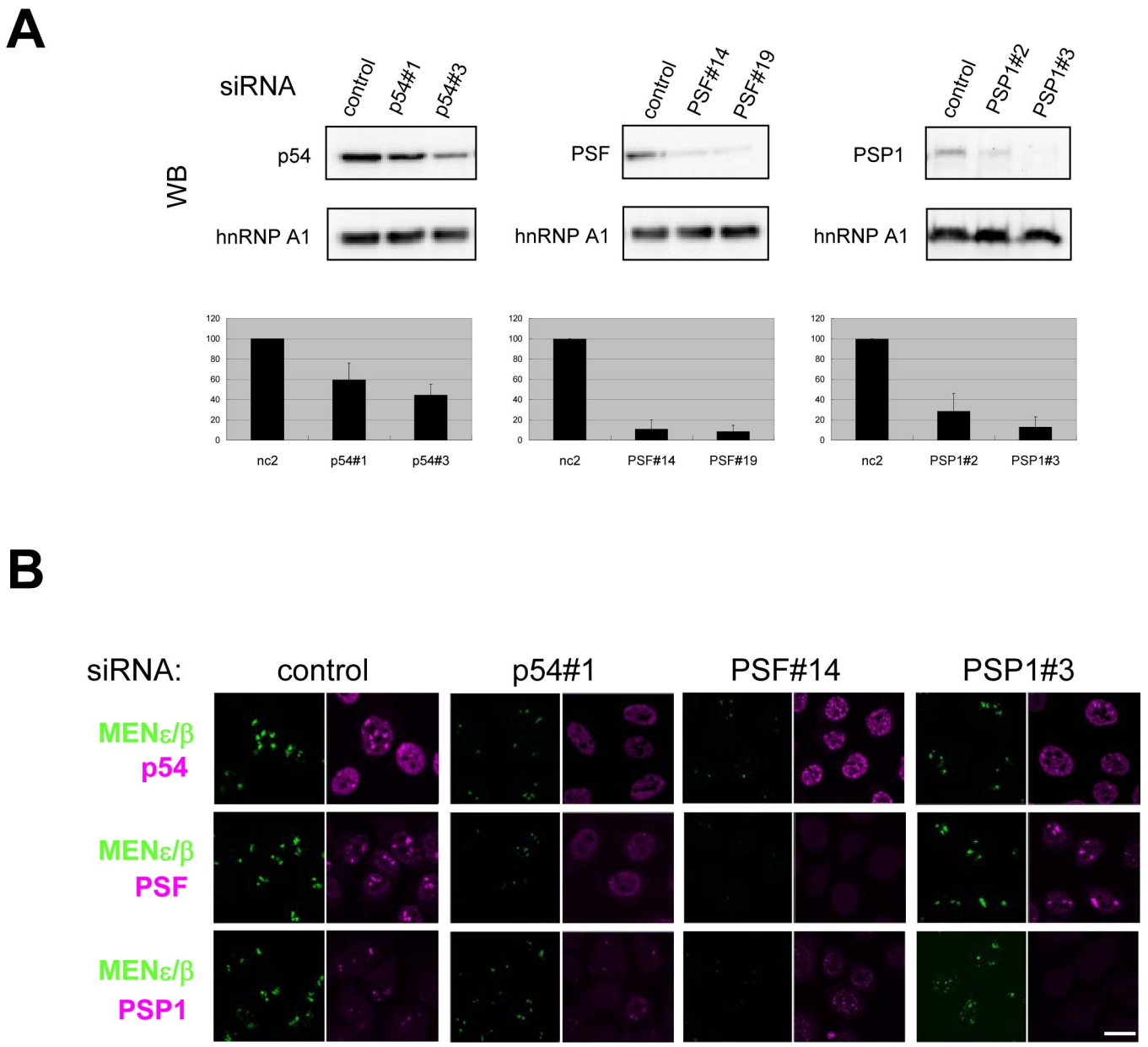

Fig. S8. Disruption of paraspeckle proteins affects paraspeckle integrity and MENε/β levels. (*A*) RNAi efficacy was confirmed by Western blotting. Blots of siRNA-treated cellular lysates were probed with antibodies for the paraspeckle proteins (p54, PSF, and PSP1) and quantified. The loading control for normalization was hnRNP A1. (*B*) FISH-IF of the paraspeckle protein knockdown cells. Cells were treated with the siRNA indicated above each image for 48 h and were then probed with MENε/β probe (green) in combination with an antibody to 1 of the 3 paraspeckle proteins (magenta: p54, PSF, or PSP1, top to bottom).

Table S1. Oligonucleotides used for PCR

Primer	Direction	Sequence
Eight primer pairs for MEN ε/β qRT-PCR		
1	Forward	CTTCCTCCCTTTAACTTATCCATTCAC
	Reverse	CTCTTCCTCCACCATTACCAACAATAC
2	Forward	AGCTGCGTCTATTGAATTGGTAAAGTAA
	Reverse	GACAGAAAGATCCCAACGATAAAAATAA
3	Forward	ACCTCTATAACCGCCTTCTCTTCTTTT
	Reverse	CTGGAGACAGCACCATTTACATACAG
4	Forward	GATCTTTTCCACCCCAAGAGTACATAA
	Reverse	CTCACACAAACACAGATTCCACAAC
5	Forward	TGAGATCCTACCTTCAGTTCTTTTGAG
	Reverse	ACTCTTGGTGAGAACGTAAAATGGTG
6	Forward	CAGTTAGTTTATCAGTTCTCCCATCCA
	Reverse	GTTGTTGTCGTCACCTTTCAACTCT
7	Forward	GGTGATTCAGTGGCATCTTTCTCTAAC
	Reverse	AGCTTTACACATACACAGGTTCTCTGG
8	Forward	GTCTTTCCATCCACTCACGTCTATTT
	Reverse	GTACTCTGTGATGGGGTAGTCAGTCAG
The other primers for qRT-PCR		
p54	Forward	TTCTACCCTGGAAAAGTGTTAGGGATT
	Reverse	CTTGTTTAATACCACCCCTCCCTTG
PSF	Forward	AGAGGAAAGTTACAGCCGAATGG
	Reverse	CATAACCTATGCCACCACCACCT
PSP1	Forward	ATTCTCAAGAGCAGACCTCTACGGATT
	Reverse	AACCACAACAGCTTTCTCTACTGGAC
GAPDH	Forward	ATGAGAAGTATGACAACAGCCTCAAGAT
	Reverse	ATGAGTCCTTCCACGATACCAAAGTT
Primer pairs for FISH probe subcloning		
5′ probe	Forward	TAGTTGTGGGGGAGGAAGTG
	Reverse	TGGCATGGACAAGTTGAAGA
3′ probe	Forward	GTCTTTCCATCCACTCACGTCTATTT
	Reverse	CACCCTAACTCATCTTACAGACCACCAG
Primer pairs for RPA probe subcloning		
Probe1	Forward	CGTCCCCCGCCCGACCTC
	Reverse	CCTGAAGCCCTGAGCTAGCCACTTC
Probe2	Forward	CAATGACTTGGGGATGATGCAAACAATTACTG
	Reverse	GTACTCCCCACCTACACACCCAC
Probe3	Forward	AGGGAAGTAAGGAAGGTAGGAGGTG
	Reverse	CAGAGGATGAGGGAGGGGATAG
Primer pair for MENε subcloning		
—	Forward	TATACGCGTGCAAAAGTTGTGGCAAGTCCA
—	Reverse	TATGTTTAAACTGAGTTTAGTCAAACTTTATTTGT
Primer pairs for MENβ subcloning		
Left arm	Forward	TATACGCGTGCAAAGTTGTGGCAAGTCCA
	Reverse	TATGTTTAAACGCATGGACAAGTTGAAGATTAGC
Right arm	Forward	TATGTTTAAACGATCTATCATGTATGCAGTGACC
	Reverse	TATAAGCTTCAGAAAATGTCTGAAGGAATATAAATG

Table S2. Primary antibodies used in this report

Antigen	Animal	Supplier	Application	Dilution	Reference
Coilin	Mouse	Abcam	IF	1:1,000	
Fibrillarin	Rabbit	Santa Cruz	IF	1:200	
hnRNP A1	Mouse	ImmuQuest	WB	1:8,000	
p54/nrb	Mouse	BD Biosciences	IF, WB	1:400, 1:2,500	
PML	Mouse	Y. Kurihara	IF	1:50	1
PSF	Mouse	Y. Kurihara	IF, WB	1:30, 1:250	1
PSF	Mouse	Sigma	IP	6 μg per reaction	
PSP1	Mouse	Y. Kurihara	IF, WB	1:2, 1:100	1
SMN	Mouse	Y. Kurihara	IF	1:10	1
SC35	Mouse	Sigma	IF	1:1,000	
Digoxygenin	Mouse	Roche	FISH	1:1,000	
Digoxygenin	Sheep	Roche	FISH	1:1,000	
FITC	Mouse	Roche	FISH	1:1,000	
FITC	Rabbit	Abcam	FISH	1:1,000	
FLAG	Mouse	Sigma	IP	2 μg per reaction	

1. Myojin R, *et al.* (2004) Expression and functional significance of mouse paraspeckle protein 1 on spermatogenesis. *Biol Reprod* 71:926.

Table S3. Probe combos that showed lowest *P* values

Dataset	Probe A	Probe B	*P*
p54–1	1	2	0.0066
p54–3	1	2	**0.0079**
PSF-14	1	2	**0.0037**
PSF-19	1	2	**0.0082**
PSP1–1	2	7	0.0709
PSP1–2	5	8	0.0136
αFlag.	1	2	0.0891
αPSF.	1	2	0.0074
Flag-pcDNA.	1	2	0.0472
Flag-p54.	1	2	0.0012

$P < 0.001$ are rendered in bold type.

Table S4. Chimeric oligonucleotides used for knockdown

Target RNA	Sequence
HIT6113	5′-mC*mG*mC*mC*mU*T*T*C*A*C*C*C*A*G*T*mC*mU*mC*mC*mC-3′
HIT7366	5′-mG*mU*mC*mC*mC*A*G*T*G*A*A*G*A*G*T*mC*mA*mG*mA*mU-3′
HIT11516	5′-mC*mG*mU*mC*mC*C*A*G*T*C*A*C*C*C*A*T*mA*mU*mU*mU*mC-3′
HIT16701	5′-mC*mU*mC*mC*mC*mU*G*G*C*T*G*T*G*T*T*C*mA*mG*mU*mC*mC-3′
HIT17869	5′-mU*mC*mC*mC*mC*mU*C*C*A*A*T*C*T*C*T*G*mC*mC*mU*mC*mC-3′
HIT55020	5′-mC*mC*mC*mC*mU*mC*T*C*T*C*T*T*C*C*C*C*T*mG*mU*mU*mU*mA*mA-3′
HIT55032	5′-mG*mU*mU*mU*mC*mC*C*A*G*C*C*G*C*C*A*T*C*mC*mC*mU*mC*mU*mG-3′
HIT94401	5′-mG*mU*mU*mU*mU*mC*A*G*G*A*C*T*C*T*A*G*G*mU*mG*mG*mG*mG*mU-3′
MENε/β#12	5′-mC*mC*mC*mC*mU*mC*T*A*G*T*C*T*T* G*G*C*mU*mC*mA*mU*mU-3′
MENε/β#12 scrambled	5′-mU*mC*mU* mG*mC*T* C*A*C* T*T*G* C*A*T*mG*mC*mC* mU*mU-3′
MENε/β#17	5′-mC*mC*mC*mC*mU*mU*C*T*C*C*T*A*G* T*A*A*mU*mC*mU*mG*mC-3′
MENε/β#17 scrambled	5′-mU*mA*mU* mC*mU*G* C*A*C* T*T*C* T*C*T* mC*mA*mC* mA*mC-3′
Mouse MENε/β	5′-mC*mC*mC*mA*mG*T*C*C*A*C*C*G*T*C*mU*mC*mC*mA*mU-3′
GFP	5′-mU*mC*mA* mC*mC*T* T*C*A* C*C*C* T*C*T* mC*mC*mA* mC*mU-3′

The asterisks represent the phosphothioate-modified backbone, and mN designates the 2′-*O*-methoxyribonucleotides

Table S5. Paired *t* test for Fig. 5*D*

Primer A	Primer B	*P*
1	2	0.520
1	4	0.021
1	8	**0.048**
2	4	**0.000**
2	8	**0.021**
4	8	0.922

$P < 0.05$ are rendered in bold type.