inconsistent paraspeckle phenotypes between treatments with the two siRNAs (PSP16, 17, 18, and 32). Several PSPs were tentatively categorized according to the consistent paraspeckle phenotypes between treatments with two siRNAs, although the RPA data were highly variable (PSP10, 15, 25, and 27).

Three PSPs are involved in MENε/β isoform synthesis by modulating alternative 3'-end processing

Because the MENε/β isoforms share an identical 5' terminus, they are likely produced by alternative 3'-end processing. The 3'-ends of MENε and MENβ are formed by two distinct mechanisms: canonical polyadenylation and RNase P cleavage, respectively. The above RNAi experiments identified factors that are involved in this alternative 3'-processing event.

Two PSPs, CFIm68 and PSP24/CFIm25, form a heterodimer (CFIm complex) to facilitate the 3'-end processing of alternatively processed mRNAs (Kim et al., 2010). These PSPs also appear to act in MENε 3'-end processing. We observed that the RNAi of CFIm25 or CFIm68 markedly diminished MENε levels and simultaneously increased the MENβ level (Figures 4A and S6B). The CFIm complex binds to UGUA sequences located upstream of the canonical polyadenylation signal and recruits the general 3'-end processing machinery to polyadenylation sites (Venkataraman et al., 2005). Sequence searches revealed that five UGUA sequences are clustered 42–169 nt upstream of the polyadenylation signal (PAS) (AAUAAA) for MENε 3'-end processing (Figure 5A). This result strongly suggests that CFIm facilitates the 3'-end processing of MENε through binding to the UGUA sequences. Intact paraspeckles remained

detectable after treatment with RNAi for either CFIm25 or CFIm68 (Figures 4B and S6C), even though MENε was obliterated (Figures 4A and S6B). This result confirms that MENε is dispensable for paraspeckle formation.

PSP20/hnRNP K is a new member of category 1A that is required for MENβ accumulation. Treatment with hnRNP K RNAi disrupted the paraspeckles and concomitantly decreased the MENβ level, but simultaneously elevated the MENε level (>2-fold) (Figures 4A, 4B, S6B, and S6C). This finding was not observed with an RNAi of any other category I protein (Figure S5A), which suggests that hnRNP K facilitates MENβ synthesis, rather than its stabilization, by modulating MENε 3'-end processing.

RT-qPCR measurement of MENε/β ncRNAs coimmunoprecipitated with anti-CFIm25 antibody (αCFIm25) revealed that the MENε/β-overlapped region, but not the MENβ-specific region, was markedly increased with hnRNP K RNAi (Figures S6D and S6E). This finding indicates that the association of CFIm25 with MENε was accelerated by hnRNP K elimination in vivo.

The hnRNP K-eliminated cells were transfected with a plasmid for siRNA-resistant hnRNP K (K#2 and K#3 in Figure 4C), and the ratio of the MENε and MENβ isoforms (MENε/β) was measured. As the amount of exogenous hnRNP K increased (lower panels of Figure 4C), the MENε/β ratio proportionally decreased (Figure 4C). Moreover, exogenous hnRNP K rescued the defect of paraspeckle formation (Figures 4D and 4E). These results indicate that hnRNP K is responsible for MENβ synthesis, which determines the MENε/β ratio and consequent paraspeckle formation.

Previous SELEX analyses have identified CU-rich stretches as preferred binding sequences for hnRNP K (Thisted et al., 2001). We identified a CU-rich stretch (UCCCCUU) that perfectly matched a SELEX-derived sequence, which was present in the region adjacent to the canonical polyadenylation signal (blue box in Figure 5A) that is conserved in rodents (data not shown). Therefore, hnRNP K likely binds to the CU-rich stretch and interferes with MENε 3'-end processing, resulting in the preferential synthesis of MENβ. These data indicate that hnRNP K modulates the alternative 3'-end processing for MENβ synthesis that initiates paraspeckle formation.

hnRNP K-binding arrests the CFIm-dependent 3'-end processing of MENε in vitro

To investigate how hnRNP K controls alternative 3'-end processing, the MENε 3'-end processing reaction was recapitulated in HeLa cell nuclear extract (HNE). A ^{32}P-radiolabeled RNA substrate that contained 303 nt spanning the processing site of MENε (Figure 5A) was incubated in HNE. Because no Mg^{2+} was added to the in vitro reaction, the endonucleolytic cleavage solely produced the 209-nt processed RNA without subsequent polyadenylation. The cleavage product was detectable after incubation for 30 min (Figures 5B and S7A). Processed RNA was not generated from an RNA substrate with a mutated polyadenylation signal (PAS-mut in Figure S7A), which indicated that accurate RNA processing was recapitulated in vitro.

To check the roles of CFIm and hnRNP K in the 3'-end processing of MENε in vitro, RNA substrates in which the putative CFIm-binding sequences

(CFBS) or hnRNP K-binding sequence (KBS) were mutated (CFIm-mut and K-mut, respectively, in Figure 5A) were applied to the in vitro processing reaction. Time-course experiments revealed that CFIm-mut exhibited marked deceleration of the processing compared to the wild-type substrate (WT) (Figures 5B and 5C), confirming the reported evidence that CFIm facilitates 3'-end processing through its association with CFBS (e.g., Venkataraman et al., 2005). By contrast, K-mut accelerated in vitro processing (Figures 5B and 5C), which supported our in vivo results that hnRNP K acts to suppress the 3'-end processing of MENε. The results of a gel mobility shift assay with recombinant hnRNP K protein (r-K) confirmed that r-K binding was mostly abolished by KBS mutation (Figure 5D), indicating that hnRNP K binds to KBS and arrests the CFIm-dependent 3'-end processing of MENε.

hnRNP K arrests the RNA-binding of CFIm for MENε 3'-end processing

We examined the effect of hnRNP K on the RNA-binding of CFIm during in vitro processing. Proteins that bound to WT during in vitro processing were visualized by UV-crosslinking (Figure 6A lanes 1 and 4). Immunoprecipitation with αCFIm25 and αCFIm68 revealed that the UV-crosslinked ~68 and ~25 kDa proteins were efficiently precipitated by each antibody (Figure 6A), indicating that they corresponded to CFIm68 and CFIm25, respectively.

To assess whether the extra hnRNP K interferes with the RNA-binding of CFIm, r-K was added to the in vitro processing. On the WT substrate, r-K markedly interfered with the UV-crosslinking of CFIm68 and CFIm25 in a concentration-dependent manner (Figure 6B, lanes 1-4). Immunoprecipitation of

UV-crosslinked CFIm68 and CFIm25 clearly showed that the RNA-bindings of CFIm68 and CFIm25 were diminished (~50%) in the presence of r-K (Figures 6C-6E). The mutation of KBS (K-mut) substantially elevated the UV-crosslinking of CFIm68 and CFIm25 (Figure 6B, lanes 2 and 6). The interfering effect of r-K on CFIm68-binding was milder on K-mut compared to WT (Figure 6B, lanes 6-8). These data indicate that hnRNP K-binding to KBS results in the arrest of CFIm-binding.

Finally, to obtain further mechanistic insights into the hnRNP K-dependent arrest of the 3'-end processing of MENε, the interaction between hnRNP K and CFIm was investigated. Endogenous hnRNP K was coimmunoprecipitated with CFIm25 in the presence of RNase A (Figure 6F, lanes 5 and 6) but not with CFIm68 (Figure 6F, lanes 7 and 8). This interaction was confirmed during the in vitro processing, in which the supplemented r-K prominently coprecipitated with αCFIm25 (Figure 6G). This result was supported by protein-interaction data in the public database, in which CFIm25 but not CFIm68 was listed as an hnRNP K interactor (data not shown). Importantly, the CFIm25-CFIm68 interaction was markedly weakened (~50%) in the presence of excess r-K (Figures 6G and 6H). This latter finding strongly suggests that hnRNP K competes for the binding of CFIm25 with CFIm68, which is a possible underlying mechanism for the arrest of CFIm-binding by hnRNP K (Figure 6I).

DISCUSSION

Expansion of paraspeckle components

In the current study, the FLJ cDNA-based localization screening revealed 34 new PSPs. Many of the new PSPs are likely present throughout the nucleoplasm, and subsets may be concentrated in paraspeckles. An analysis of the compilation of all of the PSPs (Table 1 and Figure S4) indicated that most possess canonical RNA-binding domains. CFIm25, which was found to possess no canonical RNA-binding motif, has a NUDIX hydrolase domain that acts like an authentic RNA-binding protein (Yang et al., 2010). Some of the paraspeckle-localized RNA-binding proteins (e.g., p54nrb, PSF, PSP2, EWSR1, FUS, TAF15 and TDP43) mediate both transcription and RNA processing (Auboeuf et al., 2005). Several of the new PSPs (e.g., ADHC1, DLX3, and ZNF335) are likely to be DNA-binding proteins that are involved in transcriptional control. This finding raises the possibility that paraspeckles may integrate tightly coupled transcription and posttranscriptional events. Alternatively, paraspeckles may be involved in RNA-dependent epigenetic regulation. Indeed, the PSPs, FUS and hnRNP K, act as regulators of epigenetic regulation through interacting with long ncRNAs (Huarte et al., 2010; Wang et al., 2008).

Several PSPs are disease-related. The genes for nine PSPs (p54nrb, PSF, CFIm68, EWSR1, FUS, TAF15, DAZAP1, RBM3, and SSL18L1) are the breakpoints of chromosomal translocation that result in the production of abnormal fusion proteins responsible for various cancers (Kim et al., 2006). Four of them (PSF, p54nrb, DAZAP1, and FUS) belong to category 1, suggesting that the paraspeckle structure is altered in tumor cells in which the genes have undergone translocations. FUS and TDP43 are commonly associated with a neurodegenerative disease, familial amyotrophic lateral

sclerosis (ALS) (Lagier-Tourenne and Cleveland, 2009). TDP43 was found to associate prominently with MENε/β in the brain of patients with FTLD-TDP, which is an ALS-related neurodegenerative disease with TDP43 inclusions (Tollervey et al., 2011). These evidences suggest that TDP43 associated with MENε/β is sequestered in paraspeckles, where it is functionally modulated.

Mechanism of alternative RNA processing of MENε/β ncRNAs

MENβ was found to be essential for paraspeckle formation. Therefore, the alternative 3'-end processing event that leads to MENβ accumulation is a fundamental molecular event for paraspeckle formation. Alternative 3'-end processing, which produces various mRNA isoforms with different 3'-UTR lengths, is utilized mainly for situations in which the produced mRNA isoforms are subjected to differential regulation by 3'-UTR–interacting factors. In the case of MENε/β ncRNAs, this mechanism diversifies the ncRNA functions.

The alternative 3'-end processing of MENε/β comprises two distinct 3'-end processing mechanisms: canonical polyadenylation for MENε and RNase P-mediated cleavage for MENβ. CFIm binds UGUA sequences and facilitates processing and polyadenylation at adjacent sites (Venkataraman et al., 2005). Our RNAi and in vitro analyses indicated a corresponding mechanism for the 3'-end processing of MENε.

PSP7/CFIm59 was reported to form a heterodimer with CFIm25 to facilitate 3'-end processing (Kim et al., 2010). However, we observed that CFIm59 RNAi markedly decreased the MENβ level, which was the opposite effect of CFIm25 RNAi (see Table S3). This finding suggests that CFIm59 has

an additional role in MENβ processing or stabilization. Alternatively, CFIm59 may play a counteracting role to that of CFIm68-CFIm25 in 3'-end processing under a specific (e.g., paraspeckle-localized) condition.

The multifunctional hnRNP K protein is involved in transcriptional regulation, pre-mRNA splicing, mRNA stability, and translation (Bomsztyk et al., 2004). Our data add a new function to this list: the regulation of 3'-end processing. The results of our RNAi and immunoprecipitation experiments suggest that hnRNP K is required for MENβ accumulation through its arrest of the 3'-end processing of MENε. Meanwhile, the results of the in vitro processing and UV-crosslinking experiments revealed that hnRNP K interferes with the RNA-binding of the CFIm complex through its binding to KBS. However, the possibility that hnRNP K additionally participates in the stabilization and/or noncanonical 3'-end processing of the MENβ isoform cannot be ruled out. Mapping of the hnRNP K-binding sites may uncover additional function(s) of hnRNP K in MENε/β expression.

Our coimmunoprecipitation experiments indicated that CFIm25, but not CFIm68, interacted with hnRNP K in vivo and in vitro. The supplemented r-K interacted with CFIm25, which resulted in diminished interaction with CFIm68 and suggested the underlying mechanism. The hnRNP K-binding to KBS proximal to CFBS would provide an environment in which hnRNP K and CFIm68 effectively compete for association with CFIm25, which eventually determines the MENε/β isoform ratio. The dissection of hnRNP K and CFIm25 to identify the interacting domain(s) and further interaction studies would solidify this model.

Several RNA-binding proteins (e.g., hnRNP I, p54nrb, and HuR) reportedly bind to upstream sequences implicated in the regulation of mRNA 3'-end processing; however, their detailed mechanisms of action remain to be investigated (Millevoi and Vagner, 2010). It would be intriguing to pursue the generality of the hnRNP K-dependent regulatory mechanism in the 3'-end processing of mRNAs and other ncRNAs. The MENε/β ratio is controllable, because it is variable in different mouse tissues (Nakagawa et al., 2011). The hnRNP K protein is expressed ubiquitously, but its activity is controlled by posttranslational modification under various conditions, such as DNA damage (Chen et al., 2008). This fact raises the possibility that the MENε/β ratio is controlled through the modification status of hnRNP K.

Steps required for paraspeckle formation

Our results provide several important insights into paraspeckle formation. The plasmid rescue experiment clarified that MENβ, but not MENε, is a necessary RNA component for de novo paraspeckle formation. This evidence supports our previous observations that: 1) PSF or p54nrb RNAi leads to paraspeckle disintegration as a consequence of MENβ destabilization (Sasaki et al., 2009), and 2) paraspeckles are observable solely in the MENβ-expressing cells of mouse tissues (Nakagawa et al., 2011). By contrast, Shevtsov and Dundr (2011) reported that tethering MENε at the specific chromosomal site triggers on-site paraspeckle formation. Clemson et al. (2009) reported that MENε overexpression in a stable cell line increased the number of nuclear paraspeckles. We observed a similar effect with MENε overexpression,

although MENβ overexpression increased the paraspeckle numbers more effectively. Because these experiments were performed in cells possessing intact paraspeckles with endogenous MENβ, it is likely that locally concentrated MENε captured the preexisting paraspeckles or their subparticles containing MENβ, and resulted in the formation of paraspeckles containing exogenous MENε.

The existence of category 1B proteins argues that MENβ accumulation alone is insufficient for paraspeckle formation. An additional step involving category 1B proteins is required for intact paraspeckle formation subsequent to the assembly of the primary MENβ subcomplex with category 1A proteins. Category 1B proteins may be involved in the assembly of a higher-order paraspeckle structure that is built with multiple copies of the MENβ subcomplex, as well as with the MENε subcomplex (Figure S7C). Indeed, DAZAP1 in category 1B has been shown to interact with PSF in category 1A (Yang et al., 2009). We cannot rule out the possibility that category 1B proteins bind to unidentified essential RNA component(s) of the paraspeckle, because all category 1B proteins possess RRMs.

The role of MENε remains obscure, despite its higher abundance compared to MENβ. The RNAi results indicate that category 3A and 1B proteins contribute to MENε accumulation, suggesting that MENε forms subcomplexes with these proteins. Recent electron microscopic observations have shown that MENε localizes at the paraspeckle periphery (Souquere et al., 2010). Paraspeckles presumably are involved in the nuclear retention of specific mRNAs, which raises an interesting possibility: the more-conserved MENε RNA

may serve as a functional unit for paraspeckle-conducting events (such as nuclear mRNA retention), rather than for its structural maintenance. In this way, MENε synthesis could account for the amplification of the functional units at the paraspeckle periphery. Our trials to identify PSPs that mediate the nuclear retention of mRNAs by RNAi were unsuccessful, which suggests functional redundancy within the PSPs or the presence of additional unidentified factors.

We have constructed a model of paraspeckle formation on the basis of the data presented in this manuscript (Figure S7C). To understand the details of each process, it is important to map the RNA-protein and protein-protein interactions in this structure. Further studies will identify additional PSPs and RNAs, some of which may be critical for paraspeckle structure. Indeed, additional paraspeckle-localized proteins that are not included in our list were reported recently (Bond and Fox, 2009). It will be important to investigate the connections among chromatin structure, transcription machinery, and paraspeckle formation. The ongoing transcription of MENε/β recently was found to be a prerequisite for paraspeckle formation (Mao et al., 2011). Therefore, the initial step of paraspeckle formation may occur cotranscriptionally (Figure S7C). Further mechanistic investigations of MENε/β ncRNAs and PSPs should elicit a novel view of the formation of these tremendously large ribonucleoprotein particles and their linkage to function.

EXPERIMENTAL PROCEDURES

Cell cultures and transfection

HeLa, MEF, and NIH3T3 cells were grown in DMEM (10% FBS). Some cells were treated with actinomycin D (0.3 μg/mL, 4 h). Transfection of MEF cells was performed with the Nucleofector MEF starter kit and the Nucleofector device (Ronza) or FuGene HD (Promega). Expression from the MENε or MENβ construct in MEF was confirmed by RT-qPCR. Transfection of other cell lines was performed with Lipofectamine 2000 or Lipofectamine LTX (Invitrogen).

In vitro 3'-end processing assay

The ^{32}P-labeled RNA substrate was synthesized with SP6 RNA polymerase (TaKaRa). HNEs were prepared according to Dignam et al. (1983). The in vitro RNA processing reaction was performed as described, with minor modifications (Takagaki et al., 1988). Briefly, ^{32}P-labeled RNA (2×10^4 cpm, ~5 fmol) was incubated in a 12.5-μL reaction mixture containing 8 mM HEPES (pH 7.9), 8% glycerol, 40 mM KCl, 0.2 mM PMSF, 0.4 mM DTT, 2.08 mM EDTA, 40 mM creatine phosphate, 40 μg/mL *E. coli* tRNA, 0.25 U RNasin (Promega), 2.5% polyvinyl alcohol, and 4 μL of HNE (32%). After the solution was reacted at 30 °C for the indicated time, RNA was extracted and separated by 6% PAGE containing 7 M urea.

UV-crosslinking

UV-crosslinking was performed as described (Hirose et al., 2006) after incubating the samples under conditions of in vitro 3'-end processing. UV light (1.8 J/cm^2) was applied to an open-top reaction tube on ice with a UV-crosslinking device (CL-1000, UVP). RNase A and RNase T1 were added and

incubated for 15 min at 37 °C, and precipitation with 50% acetone was performed. Precipitated proteins were fractionated by SDS-PAGE. Substrate RNA was preincubated with r-K for 15 min at 30 °C, after which HNE was added for an additional 15-min incubation. The r-K was expressed in *E. coli* cells [BL21(DE3)-CodonPlus RILP (Stratagene)] and purified with Ni affinity chromatography.

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