SAM68-Specific Splicing Is Required for Proper Selection of Alternative 3’ UTR Isoforms in the Nervous System

Yoko Iijima, Masami Tanaka, Satoko Suzuki, ..., Masato Ohtsuka, Peter Scheiffele, Takatoshi Iijima
takatoshi.iijima@tokai-u.jp

HIGHLIGHTS
SAM68 and the related protein SLM1 exhibit distinct alternative splicing activity
SAM68 specifically controls 3’ UTR selection of multiple neuronal genes
Proper 3’ UTR selection is necessary for IL1RAP neuronal function
Neuronal expression of SAM68 requires proper 3’ UTR selection in the nervous system

DATA AND CODE AVAILABILITY
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Thus the SAM68-specific splicing program provides a mechanism for neuronal selection of alternative secreted type in Tor accessory protein (selection or alternative polyadenylation. The altered ALE usage of a novel target, interleukin 1 receptor isoform switch of a number of neuronal targets through the alteration in alternative last exon (ALE) switch during neural development (Kalsotra and Cooper, 2011; Vuong et al., 2016) and show distinct patterns in a neuronal tissue- or cell type-specific manner (Iijima et al., 2016; Nguyen et al., 2016; Li et al., 2007; Raj and Blencowe, 2015). Furthermore, neuronal activity modulates alternative splicing of neural genes via Ca2+-dependent signaling pathways (Razanau and Xie, 2013). Thus, neuronal alternative splicing is dynamically controlled in a spatiotemporal manner, which likely contributes to brain function complexity and diversity (Li et al., 2007; Raj and Blencowe, 2015). However, the RNA regulatory mechanisms underlying spatiotemporal and dynamic alternative splicing in neurons are only now being uncovered.

Neuronal alternative splicing is dynamically exerted by regulatory activity and unique expression patterns of RNA-binding proteins (RBPs). We previously identified SAM68 (Src-associated in mitosis of 68-kDa protein, khdbs1) as a critical regulator of neuronal activity-regulated alternative splicing (Iijima et al., 2011). Moreover, two related proteins, SLM1 and SLM2 (SAM-like molecule 1 and 2), have been implicated in neuronal cell-type-specific splicing (Ehrmann et al., 2013; Iijima et al., 2014; Nguyen et al., 2016). SAM68, SLM1, and SLM2 belong to the STAR (signal transduction and activation of RNA) family of proteins, which share 70%–80% of amino acid sequence identities in their KH-type RNA-binding domains (Di Fruscio et al., 1999). Important targets of SAM68, SLM1, and SLM2 are the mRNAs encoding Neurexin (Nrxn) proteins (Iijima et al., 2016). Neurexins are synaptic cell surface receptors extensively regulated at alternative splicing level (Missler and Sudhof, 1998). All three STAR family proteins induce skipping of exon 20 at the Nrxn alternatively spliced segment 4 (AS4). The splicing decision at AS4 is critical for differential interactions with several ligands that are essential mediators of synaptic properties, including neuroligins, leucine-rich repeat proteins, and the Cbln1-GluD2 complex (Baudouin and Scheiffele, 2010; Boucard et al., 2005; Ko et al., 2009; Krueger et al., 2012; Matsuda and Yuzaki, 2011; Uemura et al., 2010). Indeed, the Nrxn AS4 is particularly important for synaptic strength and plasticity regulation (Aoto et al., 2013; Traunmuller et al., 2016), which is dynamically controlled by STAR family proteins in neuronal

**SUMMARY**

Neuronal alternative splicing is a core mechanism for functional diversification. We previously found that STAR family proteins (SAM68, SLM1, SLM2) regulate spatiotemporal alternative splicing in the nervous system. However, the whole aspect of alternative splicing programs by STARs remains unclear. Here, we performed a transcriptomic analysis using SAM68 knockout and SAM68/SLM1 double-knockout midbrains. We revealed different alternative splicing activity between SAM68 and SLM1; SAM68 preferentially targets alternative 3’ UTR exons. SAM68 knockout causes a long-to-short isoform switch of a number of neuronal targets through the alteration in alternative last exon (ALE) selection or alternative polyadenylation. The altered ALE usage of a novel target, interleukin 1 receptor accessory protein (IL1RAP), results in remarkable conversion from a membrane-bound type to a secreted type in Sam68 KO brains. Proper ALE selection is necessary for IL1RAP neuronal function. Thus the SAM68-specific splicing program provides a mechanism for neuronal selection of alternative 3’ UTR isoforms.

**INTRODUCTION**

Alternative pre-mRNA splicing is a powerful mechanism that generates molecular diversity from a limited number of genes and is therefore thought to be essential for biological complexity and diversity in mammals. In particular, the regulation is highly dynamic and complex in the central nervous system (CNS) (Barbosa-Morais et al., 2012; Merkin et al., 2012). Alternative splicing decisions are known to be dynamically switched during neural development (Kalsotra and Cooper, 2011; Vuong et al., 2016) and show distinct patterns in a neuronal tissue- or cell type-specific manner (Iijima et al., 2016; Nguyen et al., 2016; Li et al., 2007; Raj and Blencowe, 2015). Furthermore, neuronal activity modulates alternative splicing of neural genes via Ca2+-dependent signaling pathways (Razanau and Xie, 2013). Thus, neuronal alternative splicing is dynamically controlled in a spatiotemporal manner, which likely contributes to brain function complexity and diversity (Li et al., 2007; Raj and Blencowe, 2015). However, the RNA regulatory mechanisms underlying spatiotemporal and dynamic alternative splicing in neurons are only now being uncovered.

Neuronal alternative splicing is dynamically exerted by regulatory activity and unique expression patterns of RNA-binding proteins (RBPs). We previously identified SAM68 (Src-associated in mitosis of 68-kDa protein, khdbs1) as a critical regulator of neuronal activity-regulated alternative splicing (Iijima et al., 2011). Moreover, two related proteins, SLM1 and SLM2 (SAM-like molecule 1 and 2), have been implicated in neuronal cell-type-specific splicing (Ehrmann et al., 2013; Iijima et al., 2014; Nguyen et al., 2016). SAM68, SLM1, and SLM2 belong to the STAR (signal transduction and activation of RNA) family of proteins, which share 70%–80% of amino acid sequence identities in their KH-type RNA-binding domains (Di Fruscio et al., 1999). Important targets of SAM68, SLM1, and SLM2 are the mRNAs encoding Neurexin (Nrxn) proteins (Iijima et al., 2016). Neurexins are synaptic cell surface receptors extensively regulated at alternative splicing level (Missler and Sudhof, 1998). All three STAR family proteins induce skipping of exon 20 at the Nrxn alternatively spliced segment 4 (AS4). The splicing decision at AS4 is critical for differential interactions with several ligands that are essential mediators of synaptic properties, including neuroligins, leucine-rich repeat proteins, and the Cbln1-GluD2 complex (Baudouin and Scheiffele, 2010; Boucard et al., 2005; Ko et al., 2009; Krueger et al., 2012; Matsuda and Yuzaki, 2011; Uemura et al., 2010). Indeed, the Nrxn AS4 is particularly important for synaptic strength and plasticity regulation (Aoto et al., 2013; Traunmuller et al., 2016), which is dynamically controlled by STAR family proteins in neuronal
activity- and cell-type-specific fashions (Ehrmann et al., 2013; Iijima et al., 2011, 2014; Nguyen et al., 2016; Traunmuller et al., 2016).

Several groups have previously identified additional substrates for SAM68 and SLM2 (Chawla et al., 2009; Ehrmann et al., 2013, 2016; Huot et al., 2012; La Rosa et al., 2016; Traunmuller et al., 2016). Knockout mice of SAM68, SLM1, and SLM2 exhibit several morphological and functional defects in adult brains (Ehrmann et al., 2016; Iijima et al., 2011, 2014; Lukong and Richard, 2008; Traunmuller et al., 2016). We previously found that Sam68 and Slm1 KO mice particularly have cerebellar malformation and motor deficits (Iijima et al., 2011, 2014). Nevertheless, most neuronal functions of STAR family proteins in the mature brain remain unresolved. However, given that SAM68 and SLM1 are widely expressed in the brain throughout life, spatiotemporal regulation of alternative splicing by SAM68/SLM1 could play a critical role in multiple aspects of neuronal development, differentiation, and function. Thus, the recent findings pave the way to uncover and characterize novel targets for spatiotemporal alternative splicing programs by SAM68/SLM in the nervous system. Here we reveal that SAM68 shapes neuronal diversity of alternative 3’ UTR isoforms and demonstrate the critical role of the SAM68 splicing program in the proper 3’ UTR selection.

RESULTS

Characterization of SAM68/SLM1-Dependent Alternative Splicing Programs

To decipher alternative splicing programs encoded by SAM68 and SLM1 proteins, we attempted to locate new candidate RNA substrates by microarray-based screening using SAM68/SLM1 knockout mice. We utilized the exon array on the primary experiments, the dataset was validated by RT-qPCR, and the altered exons were further confirmed by RNA sequencing (RNA-seq). We previously showed that SLM1 protein acts as a heteromeric complex with SAM68 in co-expressing neurons (Iijima et al., 2014). Given that STARs share 70%–80% of amino acid sequence identity in their RNA-binding domains (Di Fruscio et al., 1999), it is expected that SAM68 would share a significant amount of RNA substrates with SLM1 with functional redundancy. Therefore, for the initial transcriptomic analysis, we attempted to identify candidate RNA substrates in the midbrain of both SAM68/SLM1 double-knockout (Sam68/Slm1 DKO) mice and SLM1 single-knockout (Slm1 KO) mice. We focused this analysis on the midbrain because this area is a site of prominent co-expression of SAM68 and SLM1. Initially, we compared the levels of gene expression between wild-type (WT), Slm1 KO, and Sam68/Slm1 DKO mice. A scatterplot showed that the gene expression profiles of Slm1 KO and Sam68/Slm1 DKO mice were highly similar to those of WT mice (correlated efficiency: 0.996–0.997) (Figure S1A), indicating that knockout of SAM68 and/or SLM1 did not significantly influence overall transcript levels. In fact, the volcano plots showed that there were only 10–12 genes that are significantly altered in both Sam68/Slm1 DKO and Slm1 KO mice compared with WT (corrected p values < 0.05; threshold set: fold change [FC] ≥ 2.0) (Figure S1B, and Table S1). Validation by RT-qPCR showed that the gene alterations are partially shared between both genotypes (Figure S1C), but others are unique for either SAM68 or SLM1 (Figure S1D). However, Slm1 transcripts were not listed in the altered genes on the exon array. Although we previously confirmed that SLM1 protein is completely lacking in Slm1 KO mice (Iijima et al., 2014), the RNA-seq data exhibited that the transcripts lacking exon 2 remain expressed (data not shown). That is why Slm1 transcripts were not listed in the downregulated genes. Reportedly, SAM68 has multiple functions on RNA metabolism, and a multitude of RNA substrates including non-coding RNAs have been identified using other approaches (Li et al., 2017; Sanchez-Jimenez and Sanchez-Margalet, 2013; Vogel and Richard, 2012). Therefore the very modest number of transcriptomic changes identified in our sample was surprising. The results were largely confirmed by RNA-seq analysis in Sam68/Slm1 DKO mice (cor. efficiency: 0.986) (Figure S2A). Nevertheless, our results suggest that, even in Slm1/Sam68 double-knocked mice, the effect on total transcript levels is likely to be only minor in the mouse midbrain.

We next examined exon alteration between WT, Slm1 KO, and Sam68/Slm1 DKO mice. We observed that 122 and 172 exons were altered by more than 2.4-fold in Slm1 KO and Sam68/Slm1 DKO mice, respectively (Figure 1A). Given that the whole gene expression profiles were almost unchanged (Figure S1), the majority of the exon alterations were likely due to the change in splicing events. We then compared the altered profiles at the exon level between Slm1 KO and Sam68/Slm1 DKO midbrains. The Venn diagram exhibited that 66 of 228 exons overlapped between the genotypes. We also found that 106 exons were altered only in Sam68/Slm1 DKO mice (Figure 1B). These exons are likely to contain SAM68-specific targets. We also observed that 56 exons were altered only in Slm1 KO mice. Indeed, given our previous finding that splicing activity of Nrxn3 exon20 is quite opposite between the two proteins (Iijima et al., 2014), these could also include exons that are regulated differentially between SAM68 and SLM1. Gene ontology (GO) analysis of the
Figure 1. Comprehensive Comparison of Altered Exon Profiles between Slm1 KO and Sam68/Slm1 DKO Mice

Total RNAs from midbrains of WT, Slm1 KO, and Sam68/Slm1 DKO mice were subjected to data analyses on exon array (Agilent, Sure Print G3 Mouse Exon Microarray 2x400 K) (n = 3 animals/genotype).

(A) Scatterplots showing fold change for exons (Slm1 KO versus WT, Sam68/Slm1 DKO versus WT) (total 122 and 172 exons, respectively, threshold set: FC ≥ 2.4, raw probe signal intensity ≥ 100 in either of the two genotypes, normalized gene expression > C0/3 in either of the two genotypes) (n = 3 per genotype) (red and blue dots).

(B) Venn diagram showing the numbers of altered exons (total 228 exons; threshold set: FC ≥ 2.4, raw probe signal intensity ≥ 100 in either of the two genotypes, normalized gene expression > C0/3) in both Slm1 KO and Sam68/Slm1 DKO mice.

(C) SLM1/SAM88-targeted exons (FC ≥ 2.4)

SLM1-targeted genes (Genes that include altered exons in Slm1 KO)

| GOID     | GO Term              | GO category       | Count | %   | PValue   | Fold Enrichment |
|----------|----------------------|-------------------|-------|-----|----------|-----------------|
| GO:001787 | hydrolase activity   | Molecular function| 14    | 1.66 | 0.033291 | 2.3430295       |
| GO:0003676 | nucleic acid binding | Molecular function| 11    | 1.09 | 0.0190269 | 2.2314467       |
| GO:0031917 | TACR1 complex        | Cellular component| 2     | 2.38 | 0.0274451 | 71.110074       |
| GO:0004519 | endonuclease activity | Molecular function| 3     | 3.57 | 0.0451911 | 9.27321049      |
| GO:0005774 | extracellular region | Cellular component| 13    | 15.47 | 0.0436320 | 44.3186275      |
| GO:0043202 | negative regulation  | Biological process | 2     | 2.38 | 0.0436320 | 44.3186275      |

SLM1/SAM88-targeted genes (Genes that include altered exons in Sam68:Slm1 DKO)

| GOID     | GO Term             | GO category       | Count | %   | PValue   | Fold Enrichment |
|----------|---------------------|-------------------|-------|-----|----------|-----------------|
| GO:0003076 | nucleic acid binding | Molecular function| 14    | 1.29 | 0.0480342 | 2.3789995       |
| GO:0005332 | GABA-ammonium transporter | Molecular function| 2     | 1.85 | 0.0167030 | 105.90638       |
| GO:0015812 | GABA transporter    | Biological process | 2     | 1.85 | 0.0169078 | 103.19154       |
| GO:0006366 | neurotransmitter transport | Biological process| 3     | 2.78 | 0.0193901 | 13.855387       |
| GO:0033841 | 1-acetylserine-3-phosphatase | Molecular function| 2     | 1.85 | 0.0452465 | 46.7055047      |
| GO:0046053 | regulation of RNA splicing, via spliceosome | Biological process | 2     | 1.85 | 0.0420050 | 46.184623       |
| GO:0002059 | extracellular region | Cellular component| 10    | 13.09 | 0.0401459 | 1.7344834       |
| GO:0006810 | transport           | Biological process | 15    | 13.58 | 0.0491519 | 1.71107915      |
| GO:0007268 | chemical synaptic transmission | Biological process| 3     | 3.70 | 0.0480126 | 4.8334699       |
altered exons in each genotype showed that major subsets were enriched for similar terms, but those in Sam68/Slm1 DKO mice were much more enriched for the neuronal terms (Figure 1C, red terms). Therefore, these results imply that SAM68 and SLM1 encode overlapping but distinct alternative splicing programs.

The SAM68-Specific Splicing Program Preferentially Regulates Alternative 3’ UTR Exons of Neuronal Genes

To further pursue the potential difference in the splicing program between SAM68 and SLM1, we then classified significantly altered exons into five categories (coding sequence [CDS], 5’ untranslated region [5’ UTR], 3’ untranslated region [3’ UTR], duplicated [containing both CDS and UTR], and unknown [not annotated in refseq] exons), and compared the relative percentage of each altered exon between Slm1 KO and Sam68/Slm1 DKO mice. Interestingly, we noticed that there was a remarkable difference in the pattern of the exon alteration between Slm1 KO and Sam68/Slm1 DKO mice; 3’ UTR exons were preferentially altered in Sam68/Slm1 DKO mice (Figure 2A), although RNA-seq data in Sam68/Slm1 DKO mice showed that these exon alterations largely included all alternative exon events (i.e., cassette exons, mutually exclusive exons, alternative 5’ splice site, alternative 3’ splice site, and retained introns) (Figure S2B). Indeed, 3’ UTR exons were frequently observed in the top lists of significantly altered exons in Sam68/Slm1 DKO mice (Figure 2B).

We listed 35 genes whose 3’ UTR exons were significantly altered (threshold set: FC > 2.4, p < 0.05) (Table S2). Twenty of 35 genes were unique for Sam68/Slm1 DKO mice. Importantly, arranged scatterplots of all exons (251 exons) in 35 genes showed that the alteration in 3’ UTR exons likely did not follow the change in their neighboring coding exons within their encoding genes (Figure 2C), indicating a specific alteration in alternative 3’ UTR isoform choice of these genes.

Interestingly, GO analyses of the altered 3’ UTR exons in Sam68/Slm1 DKO mice predicted that significant numbers of these targets might include transcripts encoding transmembrane or secreted proteins with neuronal function (Figure 2D). Intriguing examples were exon 8b of Il1rap (interleukin 1 receptor accessory protein, synaptic adhesion protein), exon 26b of Pcdh15 (protocadherin-15, cell adhesion protein that plays an essential role in maintenance of normal retinal and cochlear function), exon 19 of Cp (ceruloplasmin, iron transporter), and exon 4b of Glra3 (glycine receptor alpha 3, glycnergic ion channel) (see Figure 2C). Indeed, RNA-seq analysis showed that the proximal 3’ UTR exons of these transcripts were markedly included in Sam68/Slm1 DKO mice, whereas these were almost excluded in WT mice (Figures 2E and S2C), resulting in a long-to-short isoform switch of several neuronal targets through alteration in alternative last exon (ALE) selection in Sam68/Slm1 DKO mice. In addition, because preferential alteration in 3’ UTR exons occurred in Sam68/Slm1 DKO, but not particularly in Slm1 KO mice (Figures 2A and 2B), we hypothesized that the aberrant choice of alternative 3’ UTR isoforms was largely caused by the single-knockout effect of SAM68. To clarify the possibility, the altered 3’ UTR exon events observed in the exon array were validated in Sam68 KO, Slm1 KO, and Slm2 mutant (Slm2 MT) brains separately by RT-qPCR analysis (Figure 3). Slm2 MT mice expressed SLM2 protein that lacks a first QUA domain (Figure S3), which results in a significant reduction in SLM2 activity toward alternative splicing of Nrxn AS4, a major SLM2 target in the brain (Figures S3E and S3F). In this analysis, we focused on eight genes (Il1rap, Cp, Pcdh15, Lrcc1, Pcdh17, Dlgap1, Sema3a, and Fbxl3) observed only in Sam68/Slm1 DKO on the exon array. The RT-qPCR analyses revealed that these exon alterations did not occur in Slm1 KO and Slm2 MT mice, except for Fbxl3, and were specifically caused by single loss of Sam68 (Figure 3). The Sam68 KO-specific alteration included all three types of alternative 3’ UTR splicing events (ALE type [Figure 3A], ALE type with alternative 5’ splice site [Figure 3B], and alternative polyadenylation type [APA] [Figure 3C]). Thus, these data show that the Sam68-specific splicing program controls alternative 3’ UTR isoform selection.

To further investigate the ALE choice by SAM68, we focused on alternative splicing of Il1rap (ALE with alternative 5’ splice site), Pcdh15 (ALE), Cp (ALE), and Glra3 (ALE). The RT-qPCR analyses revealed that short-form (SF) variants of Il1rap, Pcdh15, Cp, and Glra3 including proximal 3’ UTR exons were dramatically increased in the midbrain of Sam68 KO and Sam68/Slm1 DKO mice, whereas the long-form (LF) variant was reciprocally reduced (Figures 4A–4D, S4, and S5A). Notably, whereas >90% of Il1rap transcripts account for an LF variant in WT mice, >50% of these transcripts were occupied by the atypical SF variant in
### SLM1/SAM68-targeted genes (including 3' UTR exons)

#### Key word category

| Enrichment terms | Category | Count | % | p-value | Fold enrichment |
|------------------|----------|-------|---|---------|----------------|
| Hematopoietic    | Keywords | 11    | 3.950 | 0.006  | 2.142          |
| Pyrimidine/urate/acid | Keywords | 3    | 5.950 | 0.006  | 2.142          |
| Transmembrane helix | Keywords | 24   | 44.444 | 0.003  | 1.493          |
| Transmembrane | Keywords | 24   | 44.444 | 0.004  | 1.493          |

### GO-enriched terms

| GO-ID   | Term                                | GO category                  | GO term                           | Count | p-value | Fold enrichment |
|---------|-------------------------------------|------------------------------|-----------------------------------|-------|---------|----------------|
| GO:000576 | extracellular region                | Cellular component           | 19                               | 0.052 | 2.277E-04 | 2.599          |
| GO:0007968 | chemical synaptic transmission    | Biological processes        | 39                               | 5     | 0.005  | 7.100          |
| GO:0005184 | neuropeptide hormone activity      | Molecular function           | 3                                | 0.006 | 0.006  | 35.006         |
| GO:0006786 | neurotransmitter transport         | Biological processes        | 3                                | 0.006 | 0.006  | 35.006         |
| GO:0032463 | protein-coding binding             | Molecular function           | 6                                | 0.016 | 0.016  | 4.061          |
| GO:015512 | GABA transport                     | Biological processes        | 2                                | 0.016 | 0.016  | 32.175         |
| GO:0005330 | GABA transporter                   | Molecular function           | 3                                | 0.016 | 0.016  | 32.175         |
| GO:0006854 | phosphohydrolase lysosomal process | Biological processes        | 3                                | 0.004 | 0.004  | 12.152         |
| GO:0030441 | 1-acylglycerol-3-phosphohydrolase O-acyethyltransferase activity | Molecular function | 2                                | 0.026 | 0.026  | 53.949         |

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**Il1rap**

**wild-type**

**Sam68_Slm1**

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**Cp**

**wild-type**

**Sam68_Slm1**

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Figure 2. Altered 3′ UTR Exon Events Of Neuronal Genes in Sam68/Slm1 DKO Brains

(A) Classification of exons altered in Slm1 KO and Sam68/Slm1 DKO mice on exon array datasets. Exons are classified into the following five categories: CDS, 5′ UTR, 3′ UTR, duplicated, and unknown exons. (threshold set: raw probe signal intensity ≥100 in either of the two genotypes, FC ≥ 2.0). The x axis represents the percentage of altered exons per classified exon. Annotation was referenced on Mouse July 2007 (NCBI37/mm9).

(B) The list of top 20 list exons that were significantly increased or decreased in Sam68/Slm1 DKO midbrains (excluding unknown genes and genes including exons altered at the gene level) (threshold set: FC ≥ 2.4, raw probe signal intensity ≥100 in either of the two genotypes, normalized gene expression > 3 in either of the two genotypes, p < 0.05 [compared to WT]). SKO: Slm1 KO, DKO: Sam68/Slm1 DKO.

(C) Arranged scatterplots of all exons (total 251 exons) in 35 genes that include the significantly altered 3′ UTR exons in Sam68/Slm1 DKO mice. CDS, 5′ UTR, 3′ UTR, duplicated, and unknown exons.

(D) GO analyses of genes that include altered 3′ UTR exons in Sam68/Slm1 DKO mice; 78 genes that include significantly altered 3′ UTR exons (FC ≥ 2.0, p < 0.05) were subjected to GO analysis. Enrichment was thresholded by p value (p < 0.05). Keyword category (left). GO-enriched terms (right). Red represents the neuronal terms.

(E) Aberrant 3′ UTR exon selection of the representative genes, Il1rap and Cpx, in Sam68/Slm1 DKO brains shown by RNA-seq (Illumina Hiseq). The alignment of RNA-seq was based on the UCSC genome browser Mouse NCBI37/mm10 assembly.

Sam68 KO and Sam68/Slm1 DKO mice (Figure 4C). By contrast, knockout of Slm1 did not affect any isoform levels of these transcripts and did not have additive effects with loss of Sam68 (Figures 4A–4D and S4). In addition, the isoform alteration in other analyzed transcripts as shown in Figure 3 (Lrrc1, Pcdh17, Dlgap1, sema3e, and Fbxl3) also had no additive effects with the double knockout (Figures S5B–SSD). Thus, we confirmed that these ALE selections are specifically controlled by SAM68. Interestingly, at the protein level, inclusion of Il1rap exon 8b, Pcdh15 exon 26b, Cpx exon 17, and Glra3 exon 4b results in production of soluble forms, lacking transmembrane domains or glycosylphosphatidylinositol anchor (Figure S6A). Indeed, when these soluble-form variants were expressed in HEK293T cells, significant amounts of protein products were detected in the cultured medium (Figure S6B). The majority of Il1rap, Pcdh15, Cpx, and Glra3 transcripts are LF variants encoding transmembrane proteins in WT brains (Figures 4A–4D and S4). There are three ALEs in Il1rap, which produce two transmembrane isoforms (isofor 1 and 3) and one soluble isoform (isoform 2) (Figure 4E, illustration). Consistent with the altered ALE selection at the transcript level, protein analysis by parallel reaction monitoring exhibits significant reduction in transmembrane protein isoform 1 in Sam68 KO brains relative to overall Il1rap protein levels (isofor 1, 2, 3 [total]) (Figure 4E). These results indicate that aberrant ALE selection of these transcripts in Sam68 KO causes marked conversion into atypical secreted type of proteins in the nervous system.

Soluble IL1RAP Influences Synaptogenic Signaling through Transsynaptic IL1RAP-PTPd Interaction

Our data indicated that single loss of SAM68 caused aberrant ALE selection of Il1rap, Pcdh15, and Glra3 in Sam68 KO, resulting in marked conversion into atypical secreted type of proteins in the nervous system. Therefore, we then tested the influence of short/secreted isoforms on neuronal functions. A previous study revealed that IL1RAP and the paralog IL1RAP-like 1 (IL1RAP-L1) organize excitatory synapses through transsynaptic interaction with the protein tyrosine phosphatase d (PTPd), a member of the presynaptic cell adhesion molecule, in the nervous system (Yoshida et al., 2011, 2012) (Figure 5A). We examined the mRNA expression of Il1rap and of the related molecules in various brain regions and the developing cortex. The transcripts were ubiquitously expressed in whole brain tissues and throughout development (Figures S7A and S7B). In addition, ALE choice of IL1RAP in Sam68 KO and Sam68/Slm1 DKO mice was altered at the same level between the cortex, midbrain, and cerebellum (Figure S7C). Here, we tested the effect of soluble IL1RAP (sIL1RAP) on IL1RAP-induced presynaptic organization and PTPd-induced postsynaptic organization. To this end, we employed a co-culture system wherein primary cerebellar neurons are combined with non-neuronal cells expressing a single synaptogenic molecule (Scheiffele et al., 2000) (Figures S5B and S7D). Cerebellar culture is a highly homogeneous neuron culture, which is appropriate for this assay. IL1RAP-hemagglutinin (HA)-expressing HEK293T cells triggered robust levels of presynaptic differentiation, as measured by recruitment of the presynaptic marker synaptobrevin (VAMP2) (Figure 5E). By contrast, co-expression with sIL1RAP-HA in HEK293T cells or introduction of sIL1RAP-HA into the cultured neurons with lentivirus significantly reduced the recruitment of the presynaptic marker, demonstrating the competitive effect of sIL1RAP on synapse organization mediated by IL1RAP-PTPd interaction. The paralog IL1RAP-L1-expressing HEK293T cells also triggered presynaptic differentiation (Figure 5E middle). Similar to IL1RAP, co-expression with sIL1RAP-HA in HEK293T cells significantly reduced IL1RAP-L1-induced recruitment of the presynaptic marker (Figure 5E middle) but did not affect neuroligin-1-induced recruitment (Figure 5E right), confirming the competitive effect of sIL1RAP on other PTPd-mediated synapse organization. We next examined the influence of sIL1RAP on postsynaptic recruitment onto PTPd-expressing
SAM68 is required for synaptogenic signaling through transsynaptic IL1RaCP/IL1RAP-L1-PTPβ interaction.

To address the molecular mechanism by which SAM68 targets the significant number of ALEs, we attempted to identify the recognition element of SAM68 for ALE splicing. Reportedly, the canonical poly(A) signal (PAS) sequences (AAUAAA) are optimal binding sites for SAM68 (Feracci et al., 2016; Ray et al., 2013). A recent study suggested that SAM68 masks this intronic PAS to prevent premature termination of the transcript through aberrant alternative polyadenylation (La Rosa et al., 2016). Therefore, to identify the cryptic PAS at the intronic sequence of Il1rap, we performed 3' rapid amplification of cDNA ends analysis from Sam68 KO brains and detected two major transcripts of Il1rap exon 8b (Figure S8A, arrows). The sequence analyses confirmed that the two transcripts were the full-length of exon 8b (exon 8b LF) and shorter ones (exon 8b SF) (Figures 6A and S8B). Actually, we found that the 3' UTR of exon 8b contains two putative PAS sites (PAS1 and PAS2) (Figure 6A, blue boxes). Here, RNA-seq showed that most of the transcript reads were terminated around PAS1 in Sam68/Slm1 KO mice (Figure 6B). Although an RT-qPCR study detected
Figure 4. Sam68 KO Causes Atypical Long-to-Short Isoform Conversion of Il1rap and Cp via Aberrant Usage of ALEs

(A and B) Schematic illustration of alternative exon choice at Il1rap exon 8 and Cp at exon 13 (top panel) and the representative gel images of semi-quantitative RT-PCR with these 3' UTR exon choices in midbrains from WT, Slm1 KO, and Sam68/Slm1 DKO mice (bottom panel). (A) Exon 8b on Il1rap and (B) exon 13 on Cp.

(C and D) Relative levels of total mRNA and two alternative isoforms (LF and SF variants) and abundance ratio of SF (red) to LF (blue) between midbrains from WT, Sam68 KO, Slm1 KO, and Sam68/Slm1 DKO mice by RT-qPCR. The RO value of total transcripts was normalized to that of Gapdh, whereas the RO value of each alternative isoform was normalized to that of the total transcripts. For the abundance ratio of SF to LF, the percentage of the SF variant was largely estimated from the CT value (CTSF) directly compared with that of LF (CTLF) at the same threshold set for the CT value. RO LF + RO SF values for the total transcript level were set to 100%. RO value of two transcripts was normalized to that of Gapdh. (C) Il1rap (D) Cp (n = 3–6 animals per genotype).

(E) Quantification of IL1RAP protein isoforms by parallel reaction monitoring (PRM). To quantify low-abundant protein isoforms, heavy reference peptides for isoform 1/2/3 (total), isoform 1, and isoform 3 of IL1RAP were used in PRM-liquid chromatography-mass spectrometry. Plots show normalized endogenous (light) to reference (heavy) peak intensities of WT and Sam68 KO hippocampal samples (n = 5 per genotype) or average changes between genotypes for isoform 1 and 3 (isoform 1/2/3 [total] set as reference). Data are presented as the mean ± SEM. Significance is indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05. One-way ANOVA followed by Bonferroni’s test.

The transcripts of exon 8b LF in Sam68 KO brains by using LF-unique primer set (primer 3) when compared with those of WT, it appeared that the amount was very small (only 2-fold higher compared with that of WT) (Figure S8C). These data indicate that the major transcripts in Sam68 KO brains are exon 8b SF. Indeed, the sequences of PAS1 were completely conserved between humans and mice (Figure S8D). Therefore PAS1 is possibly the most actionable in the absence of Sam68.

Expectedly, UV cross-linked RNA immunoprecipitation with Sam68 antibody in WT brains showed the assembly of Sam68 near PAS1, whereas binding in other regions was much weaker (Figures 6Ca and 6D). To further test the direct binding to PAS1, we examined the binding of Sam68 to synthetic RNA oligonucleotides spanning 30 bases of the PAS1 region (Figure 6E). The Il1rap 8b UTR WT probe (PAS1 WT) yielded efficient binding of endogenous Sam68 from brain extracts in the pull-down assays (Figure 6F). Furthermore, mutation of two nucleotides in a presumptive Sam68-binding PAS site (PAS1 a/c mutating) significantly reduced the recovery of Sam68 (Figures 6E and 6F), demonstrating that endogenous Sam68 can directly recognize the PAS1 sequence. Under the same conditions, the other RBP, Rbfox1, was not recovered in the precipitates. Therefore, these data suggest that Sam68 regulates ALE selection through direct binding to the cryptic PAS in intron 8 of WT brains.

Tissue-Specific SAM68 Expression Determines ALE Selection in Spatial Fashion

Given that a significant number of Sam68-targeted transcripts could be expressed in tissues other than the brain, it would be of interest to explore how Sam68-dependent ALE selection is controlled in those other tissues. Therefore, we examined the expression profiles of Il1rap, Cp, Pcdh15, and Lrrcc1 in various tissues. We observed that transcripts of Il1rap, Cp, and Lrrcc1 were detected ubiquitously (Figure S9A). On the other hand, expression of Sam68 exhibited a tissue-specific pattern (Figure S9B). In particular, Sam68 expression appears to be very subtle in the liver. RT-qPCR also showed low expression of not only Sam68 but also Slm1 in the liver at the transcript level (Figure S9C). We then examined the ratio of Il1rap and Cp splicing isoforms (LF versus SF) in several tissues. The ratio was highly variable among tissues (Figure 7A). In contrast to the brain, both major transcripts were the SF variant in the liver, in which Sam68 expression is very low. Indeed, we found that the amount of Sam68 is inversely correlated with the abundance of the SF variant (Il1rap, R² = 0.85, p = 0.008; Cp, R² = 0.86, p = 0.04, Figure 7B). We also observed that the amount of the Il1rap SF variant was significantly increased in the Sam68 KO lung and brain compared with the WTs (Figure 7C), whereas ectopic expression of Sam68 in primary liver cell culture significantly reduced the SF variant (Figure 7D). These results showed that ALE selection of these Sam68 targets is highly dependent on the expression dose of Sam68.

Furthermore, we observed that although Sam68 is not expressed in the normal mouse liver, it was strongly expressed in a human hepatocarcinoma cell line, i.e., HepG2 cells (Figure 7E). In association with the strong expression of Sam68, we found that the ratio of the Il1rap SF variant in HepG2 cells was markedly lower (<40%), compared with that in the normal mouse liver (Figure 7F). To verify whether the low amount of the Il1rap SF variant in HepG2 cells is due to the aberrant expression of Sam68 in carcinoma cells, we examined the knockdown effect of human Sam68 (hsSam68) on the ratio of Il1rap splicing variants in HepG2 cells. We found that knockdown of hsSam68 partially, but significantly, increased the Il1rap SF variant (Figure 7G). These results further suggest that Sam68 is a dominant regulator for ALE selection of Il1rap throughout the whole tissue. Thus, the absence of Sam68 causes a long-to-short isoform switch of the neuronal targets in non-neuronal tissues (Figure 7H),
Figure 5. Soluble IL1RAcP Disturbs PTPδ-Induced Synaptogenic Signaling and IL-1-Mediated NMDAR Function in the Nervous System

(A) Illustration of excitatory synapse organization through synaptic interaction of IL1RAP and the related-protein IL1RAP-L1 with PTPδ, and IL-1-induced potentiation of NMDAR-mediated calcium influx through interaction with IL-1 receptor (IL1R) in the CNS.

(B) Schematic illustration of neuron-HEK293T cell co-culture assay. To examine IL1RAP-mediated postsynaptic assembly, HEK293T cells expressing PTPδ or neurexin-1β (NRX1β)-HA were co-cultured with cerebellar neurons (DIV10-14).

(C and D) Soluble IL1RAP (sIL1RAP) disturbs PTPδ-induced synaptogenic signaling. Postsynaptic assembly on HEK293T cells was detected by immunostaining with postsynaptic marker, PSD-95. (C) HEK293T cells expressing PTPδ or NRX1β-HA with or without sIL1RAP-HA (ratio 1:1). (D) HEK293T cells expressing PTPδ or NRX1β were co-cultured with...
indicating that the SAM68 expression level is critical for the tissue-specific selection of alternative 3’ UTR isoforms through ALE choice. Indeed, whereas atypical sIL1RAP could impair PTPα-mediated synapse organization in the nervous system (Figure 5), physiological sIL1RAP in plasma plays a homeostatic role in IL-1 signaling by antagonizing the interaction with IL-1R1 in the immune system (Jensen et al., 2000; Smeets et al., 2005). Therefore, SAM68-dependent ALE selection could be necessary to exert distinct functions of ubiquitously expressed molecules between the nervous and the non-nervous systems (Figure 7I).

DISCUSSION

Distinct Alternative Splicing Activity between SAM68 and the Related Proteins SLMs

We showed that neuronal alternative splicing by STAR family proteins is an important mechanism for functional diversification. Here, we conducted transcriptomic analyses using Slm1 KO and Sam68/Slm1 DKO brains and showed a different splicing activity between SAM68 and SLM1. This study focused on the neuronal isoform selection in 3’ UTR by SAM68 and demonstrated their functional aspects through the identification of a novel target IL1RAP in neurons (Figure 5). Very recently, two articles also elucidated the interaction between SAM68 and U1 small nuclear ribonucleoprotein particle (snRNP) as a global mechanism underlying ALE regulation by SAM68 (Naro et al., 2019; Subramania et al., 2019), supporting our findings in the CNS. U1 snRNP prevents premature transcript termination by inhibition of cryptic PAS formation factors have been shown to play a role in alternative splicing (Misra and Green, 2016).

In addition to the difference in splicing activities between SAM68 and SL M1, this study also suggested a difference between SL M1 and another family protein, SL M2. We newly mapped the entire SL M1-dependent program and revealed that a significant number of exons seemed to be altered in Slm1 KO brains (Figure 1 and Table S2), whereas SLM2 encodes a highly selective alternative splicing program that regulates only a few synaptic molecules (Traunnmuller et al., 2016). Regardless of the high structural homology between SLM1 and SLM2 (Di Fruscio et al., 1999), the large functional difference between the two closely related proteins is very surprising. We previously showed that SAM68 can heteromerize with SL M1, but not with SL M2 (Iijima et al., 2014), which suggests that endogenous SL M2 ordinarily exists as a homodimer. Thus dimer formation is intrinsically different between SL M1 and SL M2. Increased RNA affinity through dimer formation is a critical parameter enabling SLM proteins to select their functional targets with the transcriptome (Feracci et al., 2016). Therefore, one possibility is that the structural difference in dimer interface between SL M1 and SL M2 complexes results in distinct splicing programs. However, numerous questions on the functional difference between STARs remains to be addressed in future studies.

Critical Role of Proper ALE Selection of Il1rap between the Nervous and Other Systems by Distinct SAM68 Expression Level

This study revealed that SAM68 is a dominant factor for ALE selection of Il1rap in the nervous system. mIL1RAP is necessary for organizing excitatory synapses through transynaptic interaction with PTPα in...
the CNS (Yoshida et al., 2012). This study demonstrated that, in addition to the significant reduction in mIL1RAP (Figure 4), the competitive effect of sIL1RAP could accelerate the impairment in PTP\textsubscript{d}–mediated synapse organization (Figures 5A–5D). Reasonably, the competitive effect is supported by the X-ray structural analysis showing that the Ig domains of IL1RAP and PTP\textsubscript{d} are the elements responsible for the heterophilic interaction (Yamagata et al., 2015). Both IL1RAP and PTP\textsubscript{d} have several transsynaptic binding partners. PTP\textsubscript{d} organizes synapses through interaction with IL1RAP-L1 and Slitrk3 (Takahashi et al., 2012; Yoshida et al., 2011). In addition to IL1RAP, because we demonstrated the competitive effect of sIL1RAP on presynapse assembly onto HEK293T cells expressing another paralog, IL1RAP-L1, on co-culture assays (Figure S7E), sIL1RAP may influence several related transsynaptic types of synaptogenic signaling in the CNS. We also revealed that sIL1RAP significantly affects IL-1\textbeta–induced NMDAR activation in hippocampal neurons (Figures 5E and 5F). Thus, this study suggests that proper

Figure 6. SAM68 Directly Binds to Cryptic PAS in the Intron 8 of \textit{Il1rap}
(A) The full-length cDNA sequence of \textit{Il1rap} exon 8 (left), and the schematic illustration of the two major transcripts in Sam68\textsuperscript{KO} brains (exon 8b SF and exon 8b LF) (right). Green indicates the coding exon region, blue shows putative PAS sites on the 3' UTR.
(B) RNA-seq on \textit{Il1rap} exon 8b in wild-type and Sam68/Slm1 DKO brains. Arrowheads represent two putative PAS sites (PAS1 and PAS2).
(C–F) Mapping of SAM68 recognition elements in \textit{Il1rap}. (C and D) UV cross-linked RNA immunoprecipitation (CLIP) assay. (C) Positions of three primer sets used for the assay. (D) The representative gel loading images of the CLIP assay using anti-SAM68 antibody and the quantification by RT-qPCR analysis (n = 3 brains). (E and F) Biotinylated RNA pull-down experiments. (E) Biotinylated RNA oligonucleotide probes covering the 3' UTR sequence of \textit{Il1rap} exon 8b used in pull-down experiments. The PAS a/c mut probe contains two nucleotide changes (red). (F) The pull-down experiments with mouse adult brain extracts. Bound proteins were detected by western blot analysis with anti-SAM68 and anti-Rbfox1 antibodies. SAM68 binding was quantified by densitometric scanning of western blot signals (n = 5).
Data are presented as the mean ± SEM. Significance is indicated as follows: ***p < 0.001, **p < 0.01, *p < 0.05. Student’s t test.
Figure 7. The Distinct Amount of SAM68 Is Responsible for Proper 3′ UTR Isoform Selection of Il1rap and Cp in the Nervous and Non-nervous Systems

(A) Abundance ratio of SF to LF in the brain, lung, intestine, spleen, and liver of WT mice. For the abundance ratio of SF to LF, the percentage of the SF variant was largely estimated from the CT value (CpSF) directly compared with that of LF (CpLF) at the same threshold set for the CT value. RO LF + RO SF values for total Il1rap were set to 100%. ROQ value of two transcripts was normalized to that of Gapdh (n = 3–4 animals per group).

(B) Reciprocal correlation between SAM68 level and production of Il1rap and Cpf SFs. SAM68 was quantified by western blot analysis. The value for the cerebellum was set to 1.0. Correlation coefficients between SAM68 and the SF transcript of Il1rap and Cpf (right) were determined in the scatterplot analysis. The gray lines in the scatterplot are the 95% confidence limit of the best fit line.

(C) Quantification of the SF variant of Il1rap between brain and non-neuronal tissues (lung, intestine, liver, and spleen) from WT and Sam68 KO mice by RT-qPCR. The RO value of total transcripts was normalized to that of total Il1rap (n = 3 animals per genotype). ROQ value of SF transcripts was normalized to that of total Il1rap (n = 3 cultures per group).

(D) Quantification of the SF variant of Il1rap between the primary liver cell cultures and the ones in which SAM68 was ectopically expressed with lentiviral infection. The RO value of total transcripts was normalized to that of Gapdh. The ROQ value of SF transcripts was normalized to that of total Il1rap (n = 3 cultures per group).

(E) Representative images of western blot analysis with the α-SAM68 antibody. Human hepatoma cell line, HepG2, cells aberrantly express SAM68 at high level.

(F) Low production of the Il1rap SF variant in HepG2 cells. The abundance ratio of SF to LF was compared between the normal mouse liver and HepG2 cells (n = 3–4 cultures per group).

(G) Restoration of aberrant ALE selection in HepG2 cells by knockdown of SAM68. Knockdown of aberrantly expressing Sam68 partially but significantly increased the level of the Il1rap SF isoform in HepG2 cells The RO value of SF transcripts was normalized to that of total Il1rap (n = 3–4 cultures per group).

(H and I) Model of tissue-specific isoform selection of Il1rap and Cp through usage of ALEs between the brain and liver by physiologically expressed Sam68. (H) The neurons strongly express Sam68, so that they dominantly produce the membrane forms. In contrast to the brain, secreted forms lacking transmembrane domain or glycosylphosphatidylinositol anchor are abundantly produced in the liver. (I) SAM68-specific ALE selection is required for the organization of IL1RAP-dependent excitatory synapses through transsynaptic interaction with PTPβ in the nervous system. On the other hand, absence of SAM68 causes the release of IL1RAP into the plasma, which could be necessary for homeostatic control of IL1-mediated inflammation.

Data are presented as the mean ± SEM. Significance is indicated as follows: ***p<0.001; *p < 0.05; Student’s t test.

ALE usage by SAM68-specific splicing is critical for both aspects of synaptic organization and plasticity in the CNS.

In contrast to the brain, the liver is thought to be a major source of sIL1RAP, which is suggested to play an important role in the homeostasis of IL-1 signaling by antagonizing the interaction of IL1RAP with IL-1R1 in the immune system (Jensen et al., 2000; Smee et al., 2005). The reduced level of physiological sIL1RAP in the plasma is in fact implicated in several diseases (Bozaoglu et al., 2014; Michaud et al., 2014). Thus, tissue-specific SAM68 expression could play a critical role in distinct functions of ubiquitously expressed proteins between the nervous and non-nervous systems through ALE selection (Figures 7H and 7I).

Regulatory Functions of the SAM68 Splicing Program Dedicated to Alternative 3′ UTR Isoform Diversity

Thousands of mammalian genes encode alternatively spliced isoforms in their 3′ UTR (Miura et al., 2013; Tian et al., 2005). Here, we demonstrated that SAM68 is required for the spatial control of alternative 3′ UTR isoforms between the nervous and the other systems by identification of new SAM68 targets (Figure 7). Importantly, GO analyses implied that SAM68 targets the 3′ UTR exons of multiple transcripts that encode neuronal membrane or secreted proteins (Figures 1E and 2D). The biochemical studies indeed found drastic shift to short isoforms by aberrant ALE selection in Sam68 KO brains, which could result in membrane-to-secreted isoform conversion at the protein level. Thus the findings strongly suggest that SAM68 is a key regulator for shaping the diversity of neuronal 3′ UTR isoforms in the nervous system.

The other intriguing point regarding alternative 3′ UTR selection is the molecular control at the transcript level. This study also found that SAM68 regulates not only ALE but also APA (Figures 2 and 3C), which alters the length of the 3′ UTR itself. Such alternative 3′ UTR diversity by APA and ALE contributes to the posttranscriptional processes such as translation, mRNA stability, and subcellular localization during development (Di Giammartino et al., 2011; Taliaferro et al., 2016) and dendritic localization and the local translation in the
nervous system (Tushev et al., 2018). Therefore, it would be of interest to explore how the ALE/APA-mediated mechanism by SAM68 contributes to molecular functions at the transcript level in a future study. Overall, although the mechanism by which a specific subset of 3’ UTR exons is controlled by SAM68-specific splicing should be examined, our findings could provide a general principle underlying the control of alternatively spliced 3’ UTR isoforms.

Limitations of Study
In this study, we performed transcriptomic analysis using SAM68 knockout and SAM68/SLM1 double-knockout midbrains and revealed a different alternative splicing activity between SAM68 and SLM1; we characterized alternative 3’ UTR selection by SAM68-specific splicing in the nervous system. However, the open questions on the mechanism underlying the differential splicing activity between SAM68 and the related family proteins remains to be addressed in future studies.

Our findings extend the understanding on the neuronal function of SAM68, in particular through the identification of IL1RAP as a new SAM68 target. However, the physiological consequences were mainly obtained by neuronal culture system. Further studies are needed to confirm the functional relevance in vivo.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY
The data presented in this article have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE110258.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.11.028.

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AUTHOR CONTRIBUTIONS
Y.I., Masami Tanaka, and T.I. conceived and designed the experiments; Y.I., Masami Tanaka, S.S., D.H., Y.S., N.A., C.O., T.I., and M.I. performed the experiments; Y.I., Masami Tanaka, S.S. D.H., Masayuki Tanaka, C.O., and T.I. analyzed the data; S.S., C.O., M.T., Y.I., M.O., P.S., and T.I. contributed reagents/materials/analysis tools; Y.I., Masami Tanaka, S.S., and T.I. wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

SAM68-Specific Splicing Is Required for Proper Selection of Alternative 3' UTR Isoforms in the Nervous System

Yoko Iijima, Masami Tanaka, Satoko Suzuki, David Hauser, Masayuki Tanaka, Chisa Okada, Masatoshi Ito, Noriko Ayukawa, Yuji Sato, Masato Ohtsuka, Peter Scheiffele, and Takatoshi Iijima
Supplementary information

Transparent Methods

Animals All procedures related to the care and treatment of animals were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of Tokai University. All mice were maintained under specific pathogen-free conditions at the Laboratory Animal Center, Tokai University. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tokai University (permit number 141018). All surgeries were performed under sodium pentobarbital anesthesia, with all efforts made to minimize animal suffering. For the transcriptomic analyses, LC-MS analysis and splicing assays, we largely used more than three male animals at 2-3 months old.

Information of animals used in this study

| Animals: organism/strains | Source |
|--------------------------|--------|
| Mouse: Sam68/Slm1 double knockout: B6.129 (SJL) | Iijima et al., J.Cell Biol., 2011 |
| Mouse: Slm1 knockout: B6.129 (SJL) | Iijima et al., J.Cell Biol., 2011 |
| Mouse: Slm2 mutant: B6 | This paper (see Figure S3) |
| Mouse: Sam68 knockout: B6.129 (SJL) | Richard et al., PLOS Genet., 2005 |

Mouse exon array Total RNA was prepared using NucleoSpin RNA XS (Takara, Japan) and quantified using a NanoDrop-1000 spectrophotometer. The quality was monitored with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). One hundred nanograms of RNA was labeled with the Low Input Quick Amp WT Labeling Kit, One-Color*3 and hybridized using the SurePrint G3 Mouse Exon Microarray 2x400K (Agilent Technologies, Folsom, CA, USA) according to the manufacturer’s protocol. Hybridization signals were detected using the DNA microarray scanner G2600D (Agilent Technologies), and all scanned images were analyzed using Agilent feature extraction software (v11.5.1.1). Raw data were imported with the GeneSpring GX software (v13.0, Agilent Technologies, CA, USA). After 75 percentile shift normalization, unsupervised analysis was performed by hierarchical clustering on genes and samples: Euclidean distance, and Wards linkage clustering. To locate genes that are differentially expressed in each knockout mouse group compared with WT, genes with normalized gene expression values averaged for each genotype less than -3 and raw probe signal intensity values averaged for each genotype less than 100 across three genotypes and probe’s genomic coordinates located in sex chromosomes were eliminated from further analysis. We constructed scatter plots with Pearson’s correlation coefficient and volcano plot conducted with moderate t-test with Benjamini-Hochberg multiple testing correction for the remaining genes. The data presented in this manuscript have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE110258.

RNA-seq After the QC procedures, mRNA from eukaryotic organisms is enriched using oligo(dT) beads. For prokaryotic samples, rRNA is removed using the Ribo-Zero rRNA Removal Kit (Illumina) that leaves the mRNA. First, the mRNA is fragmented randomly by adding fragmentation buffer, then the cDNA is synthesized.
using an mRNA template and random hexamer primers, after which a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H, and DNA polymerase I are added to initiate the second-strand synthesis. Second, after a series of terminal repair, a ligation, and sequencing adaptor ligation, the double-stranded cDNA library is completed through size selection and PCR enrichment. The qualified libraries are fed into HiSeq PE150 sequencers (Illumina) after pooling according to the manufacturer’s protocol: effective concentration and expected data volume. The filtering processes to clean reads are as follows: (1) Remove reads containing adapters. (2) Remove reads containing N > 10% (N represents the base and cannot be determined). (3) Remove reads containing low quality (Qscore <= 5) base which is over 50% of the total base. Cleaned reads were aligned to mouse reference genome mm9 with the hisat2 program (Kim et al., 2015). Aligned reads were counted in every region (gene, 5’-UTR, CDS and 3’-UTR from RefSeq mm9 version) by the featureCounts program (Liao et al., 2014). To detect the exons differentially regulated between DKO and WT, the Mixture-of-ISOforms (MISO v0.5.3) program was used. The BAM files produced by the hisat2 program and the files for alternative events for the mm9 annotation version created by rnaseqlib (https://github.com/yarden/rnaseqlib) were used as inputs. To identify DKO-regulated events, we used the criteria: the Bayes factor is >10. The raw data obtained in this study can be accessed at the DDBJ database (6781), with accession number of DRA6781, with bioproject accession number of PRJDB6781.

**Antibodies and DNA constructs** For immunoblot and immunostaining analyses, the following commercially available antibodies were used: mouse anti-VAMP2 (clone 69.1, Synaptic Systems, Göttingen, Germany), rabbit anti-GAPDH (G9545, Sigma–Aldrich, St Louis, MO, USA), rat anti-HA (clone 3F10, Roche Applied Science), and mouse anti-PSD-95 (1D10, Neuromab, Davis, CA, USA). Rabbit anti–SAM68, guinea pig anti-SLM1 and anti-SLM2 have been described previously (Iijima et al., 2014; Iijima et al., 2011). Secondary antibodies with minimal interspecies cross-reactivity conjugated to cyanine and Alexa 633, 546 or 488 (dyes were obtained from Jackson ImmunoResearch, Westgrove, PA, USA and Invitrogen, Carlsbad, CA, USA) were used for visualization in the immunostaining.

Expression vectors for NL1, NRX1β, IL1PAcP, IL1RAP-L1, PTPs, Pcdh15, SLM1 and SLM2 have been previously described (Chih et al., 2006; Kazmierczak et al., 2007; Yoshida et al., 2012; Yoshida et al., 2011). For construction of expression vectors for soluble IL1PAcP, soluble Pcdh15, Cp and GlyRα3, these cDNAs were subcloned into the multi-cloning site of the pCAGGS vector.

**Antibodies used in this study**

| Antibodies                          | SOURCE        | IDENTIFIER       |
|-------------------------------------|---------------|------------------|
| Mouse monoclonal anti-HA (clone HA-7) | Sigma–Aldrich | Product No: H9658 |
| Mouse monoclonal anti-FLAG (M2)     | Sigma–Aldrich | Product No: F1804 |
| Rabbit polyclonal anti-SAM68        | iijima et al., Cell., 2011 | N/A             |
| Rabbit polyclonal anti-SLM1         | iijima et al., Cell., 2011 | N/A             |
| Antibodies                                      | SOURCE                     | IDENTIFIER |
|------------------------------------------------|----------------------------|------------|
| Rabbit polyoclocal anti-SLM2                   | Iijima et al., Cell., 2011 | N/A        |
| Rabbit polyoclocal anti-GAPDH                  | Sigma-Aldrich              | Product No: G9545 |
| Mouse monoclocal anti-VAMP2 (synaptobrevin2) (clone 69.1) | Synaptic Systems           | Cat#104 211 |
| Mouse monoclocal anti-PSD95 (clone K28/43)     | NeuroMab                   | Cat#75-028 |

Recombinant used in this study

| Antibodies                                      | SOURCE                     | IDENTIFIER |
|------------------------------------------------|----------------------------|------------|
| pFLAG-IL1RAcP                                   | Yoshida et al., J.Neurosci., 2012 | N/A        |
| pFLAG-IL1RAP-L1                                 | Yoshida et al., J.Neurosci., 2011 | N/A        |
| pFLAG-PTPδ                                      | Yoshida et al., J.Neurosci., 2011 | N/A        |
| pCAG Neurexin1-HA                               | Chih et al., Neuron, 2005    | N/A        |
| pCAG Neurexin1β-HA                              | Chih et al., Neuron, 2005    | N/A        |
| pCAG kGRIR                                      | Hanawa et al., Mol Ther., 2002 | N/A        |
| pCAG VSVG                                       | Hanawa et al., Mol Ther., 2002 | N/A        |
| pCAG RTR2                                       | Hanawa et al., Mol Ther., 2002 | N/A        |
| pCAGGS slL1RAP-HA                               | This paper                 | N/A        |
| pCAGGS mPcdh15-HA                               | This paper                 | N/A        |
| pCAGGS sPcdh15-HA                               | This paper                 | N/A        |
| pCAGGS GlyRa3-HA                                | This paper                 | N/A        |
| pCAGGS GlyRa3 N’ter-HA                          | This paper                 | N/A        |
| pCAGGS mCp-HA                                   | This paper                 | N/A        |
| pCAGGS sGp-HA                                   | This paper                 | N/A        |
| pUC57-sgRNA                                     | Addgene                    | Addgene No.#51132 |

**Generation of Slm2 mutant animals by genome editing with CRISPR/CAS9 system** All procedures were performed as described previously (Miura et al., 2015). For preparation of sgRNA and CAS9 mRNA, the sgRNA against downstream of the translation start site in exon 1 of the Khdrbs3 gene was designed using CRISPR and CHOPCHOP (see the table below). The templates for sgRNA synthesis were PCR amplified with primer sets using pUC57-sgRNA vector (Addgene number: #51132) as
a template (see the table below). Four hundred ng of gel-purified PCR products were subjected to RNA synthesis with MEGAscript™ T7 Kit (Ambion) and DNase treatment followed by purification of mRNA using the MEGAclear Kit. The synthesis and purification of Cas9 mRNA was performed as described for the RNA synthesis steps of ssDNA synthesis.

For microinjection into one-cell mouse embryos, sgRNA and Cas9 mRNA were mixed (at concentrations of 14–20 ng/μl for ssDNA, 10 ng/μl for sgRNA, and 10 ng/μl for Cas9 mRNA) and co-injected into both the pronuclei and cytoplasm of C57BL6J fertilized eggs obtained using in vitro fertilization. Injected eggs were cultured overnight in KSOM medium at 37°C with 5% CO₂, and the resulting two-cell embryos were transferred into the oviducts of pseudo-pregnant ICR females. Oligonucleotides of the primer set for the genotyping are listed below.

**Oligonucleotides for genome editing of Khdrbs3 gene with CRISPR/CAS9 system and oligonucleotide sequences of primer sets for the genotyping**

| Oligos       | Sequence (5'-3')                                               |
|--------------|---------------------------------------------------------------|
| Khdrbs3 sgRNA-F* | 5'- TAA TAC GAC TCA CTA TAG G GCG CAG GGC GTG                 |
|              | CGT GAA GG GTT TTA GAG CTA GAA ATA GCA AG -3'                |
| sgRNA-R      | 5'- AAA AAA AGC ACC GAC TCG G -3'                            |
| Khdrbs3 gt-F | 5'- TAA TAC GAC TCA CTA TAG G GCG CAG GGC GTG                |
| Khdrbs3 gt-R | CGT GAA GG GTT TTA GAG CTA GAA ATA GCA AG -3'                |

*Red shows targeted sequence of Khdrbs3 gene

**RNA isolation, quantitative PCR (qPCR) and alternative-splicing assays** RNA was isolated with RNAiso Plus reagent (TaKaRa, Tokyo, Japan), followed by removal of contaminating DNA using Turbo DNA-free (RNase-free DNase, Ambion, Austin, TX, USA). Two micrograms of total RNA was reverse transcribed using random hexamers and ImProm-II (Promega, Madison, WI, USA).

Quantitative PCR (qPCR) was performed on a StepOnePlus qPCR system (Applied Biosystems, Waltham, MS, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems) and the comparative CT method. For the relative quantification by qRT-PCR, transcript level was normalized to that of Gapdh. On the other hand, the transcript levels of each splicing isoform were normalized to that of total transcripts, to avoid confounding by differences in amount of total transcripts between groups as described previously (Suzuki et al., 2017). All the oligonucleotide primer sequences used for semi-quantitative PCR and qRT-PCR are listed below. Primers for Nrxn1/2/3 have been previously described (Iijima et al., 2011). 3' RACE was performed using the 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Cat#18373019). Two microliters of total RNA were used for first strand cDNA synthesis. Specific cDNA was then amplified by PCR using two gene-specific primers that anneal to a region of known exon sequences and an adapter primer that targets the poly(A) tail region.

**Oligonucleotide sequences of primer sets for semi-quantitative PCR**

| Forward primer | Reverse primer | Sequence | products (bp) |
|----------------|----------------|----------|---------------|
|                |                |          |               |
| Oligonucleotide sequences of primer sets for RT-qPCR |
|---------------------------------------------------|
| **Forward primer** | **Sequence** | **Reverse primer** | **products (bp)** |
| Pcdh15 ex25-F | 5'- CCG GGT ACA AGA AGA TTC TC -3' | 5'- CCG GGT ACA AGA AGA TTC TC -3' | 108/101 |
| Pcdh15 ex26a-R | 5'- CCG GGT ACA AGA AGA TTC TC -3' | 5'- CCG GGT ACA AGA AGA TTC TC -3' | 108/101 |
| Pcdh15 ex26b-R | 5'- CCG GGT ACA AGA AGA TTC TC -3' | 5'- CCG GGT ACA AGA AGA TTC TC -3' | 108/101 |
| Lrrc1 ex18-F | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 ex19a-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 ex19b-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex18)-F | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex19a)-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex19b)-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex18)-F | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex19a)-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex19b)-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex18)-F | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex19a)-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |
| Lrrc1 total (ex19b)-R | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 5'- GCC GAC CAA GAA GCC AGA GA -3' | 119/111 |

Please note that the table contains primer sequences for various gene targets, including Dlgap1, Pcdh15, and Lrrc1, among others. The sequences are given in 5' to 3' orientation, and the products are listed in base pairs (bp).
Neuronal cell culture  Cerebellar granule neuron cultures were prepared from ICR mouse pups on postnatal days 5–7 (P5-7). Cortical neuron cultures were prepared from ICR mouse pups on embryonic day 15 (E15). The tissues were dissociated with 0.05% trypsin (Sigma) in the presence of DNase I (Roche Applied Science) for 10 min at 37°C. After cell dissociation, trypsin was inactivated with soybean trypsin inhibitor (Sigma). Cortical neuron cultures (for RNA assays) and hippocampal neuron cultures (for calcium imaging) were prepared from ICR mouse pups using the same procedures on embryonic days 15 and 17 respectively. Cells were then plated into poly-D-lysine-coated dishes (2.0 × 10^5/cm^2) and maintained for 15 days in Neurobasal Medium (Invitrogen) containing 2% B27 supplement, 2 mM Glutamax, and penicillin/streptomycin (Invitrogen). For neuron-HEK293 cell co-culture assay, HEK293T cells expressing synaptogenic molecules with GFP were plated on cerebellar granule neuron culture (DIV10–12) for 1 day (2.0 × 10^4/cm^2) as described previously (Iijima et al., 2011; Sato et al., 2017). For knockdown experiments, 1–2µM of cell permeable siRNAs (Dharmacon, Lafayette, CO; see the Table below) was

| Gene   | Accession | Sequence (5') | Sequence (3') | Mismatch |
|--------|-----------|---------------|---------------|----------|
| Sema3e | ex17(LF)-F | 5'- TGT TTG GTT ATC TTA CTG TCT TGG -3' | 144 |
|        | ex17(LF)-R | 5'- GCA ATA TGG CAC ATG CTT ACA -3' | |
|        | ex17(SF)-F | 5'- TGA GAA CTT CTA ATG GAT TTC TTT -3' | 148 |
|        | ex17(SF)-R | 5'- GGA TGT CAA CAT TCT CTT TAT TCA -3' | |
| Fbxl3  | (LF)-F     | 5'- TCT GTT GCC TTT GAC ATC CA -3' | 124 |
|        | (LF)-R     | 5'- TTG CTT AGG AAA CCA TTC TAA GGA TGG -3' | |
| Fbxl3  | (SF)-F     | 5'- TCT GGA GAG ATC CGT GGA GT -3' | 139 |
|        | (SF)-R     | 5'- CCG TTA CAC ACG ATG CTT CA -3' | |
| Glra3  | ex4-F      | 5'- TGC TAA TGA GAA GGG GGC TA -3' | 129/142 |
|        | ex5a-R     | 5'- TTT AGC CCA GAG TTT GAA AA -3' | |
|        | ex5b-R     | 5'- AGC CAG CCA GAG TTC AGA AA -3' | |
| Glra3  | total(ex3)-F | 5'- TCC TCC AGT TAA TGT CAC ATG C -3' | 111 |
|        | total(ex4)-R | 5'- GGA TCA TTC CAC TCT TGA CGA -3' | |
| Abhd1  | F          | 5'- TAC TCC CAA GCT CCA CTG CT -3' | 124 |
|        | R          | 5'- AAT CCC AAC ATG CAG AGG TC -3' | |
| Padi2  | F (3UTR)   | 5'- CAG CCA TCC TCC ACC TAA AA -3' | 148 |
|        | R (3UTR)   | 5'- CCT TCC CCC TTC CCT CAT TC -3' | |
| Plac9  | F          | 5'- GTG CAA AGG CGG TTA GAC AT -3' | 108 |
|        | R          | 5'- GTT TGA AGC CAG TTC CTC CA -3' | |
| Amy1   | F          | 5'- ATC GAT GGC GTC AAA TAA GG -3' | 109 |
|        | R          | 5'- CCT CTG CCA AAA GCT ACC TG -3' | |
| Ocel1  | F (3UTR)   | 5'- GAT CAG CTA GGG CT TGA CG -3' | 112 |
|        | R (3UTR)   | 5'- CCA GCC TTG GAA AAA AAC AA -3' | |
| Gdpd3  | F          | 5'- GAT GGA TGA ACC AAT TGT CG -3' | 106 |
|        | R          | 5'- AGG CAC CAA AAT AGC ACC TG -3' | |
| Il1rap1| F          | 5'- CAG GAA TCA TTT TGG AGC TGA -3' | 107 |
|        | R          | 5'- CCC CAG TCT CTT Gat TCC AC -3' | |
| hIL1R1 | F          | 5'- GTC TTG CCT GAG GTC TTG GA -3' | 118 |
|        | R          | 5'- TTC TGC TTT CTT TTA CGT CCT CA -3' | |
| PTPRD  | F          | 5'- TGA CTT CAT TGG CCA AGT CC -3' | 100 |
|        | R          | 5'- GAA AAC TCC AGT TCT TCC AAC G -3' | |

Neuronal cell culture  Cerebellar granule neuron cultures were prepared from ICR mouse pups on postnatal days 5–7 (P5-7). Cortical neuron cultures were prepared from ICR mouse pups on embryonic day 15 (E15). The tissues were dissociated with 0.05% trypsin (Sigma) in the presence of DNase I (Roche Applied Science) for 10 min at 37°C. After cell dissociation, trypsin was inactivated with soybean trypsin inhibitor (Sigma). Cortical neuron cultures (for RNA assays) and hippocampal neuron cultures (for calcium imaging) were prepared from ICR mouse pups using the same procedures on embryonic days 15 and 17 respectively. Cells were then plated into poly-D-lysine-coated dishes (2.0 × 10^5/cm^2) and maintained for 15 days in Neurobasal Medium (Invitrogen) containing 2% B27 supplement, 2 mM Glutamax, and penicillin/streptomycin (Invitrogen). For neuron-HEK293 cell co-culture assay, HEK293T cells expressing synaptogenic molecules with GFP were plated on cerebellar granule neuron culture (DIV10–12) for 1 day (2.0 × 10^4/cm^2) as described previously (Iijima et al., 2011; Sato et al., 2017). For knockdown experiments, 1–2µM of cell permeable siRNAs (Dharmacon, Lafayette, CO; see the Table below) was
applied 5–7 days previously for harvest. Successful knockdown effects (>70%) were confirmed by RT-qPCR (data not shown).

**Cell permeable siRNAs used in this study**

| siRNAs                              | SOURCE    | IDENTIFIER          |
|-------------------------------------|-----------|---------------------|
| Human Sam68 siRNA SMART pool       | Dharmaco  | E-020019-00-0005    |
| Mouse control siRNA SMART individual | Dharmaco  | D-001910-01         |
| Mouse Il1rap siRNA SMART pool      | Dharmaco  | E-042418-00-0005    |

**Protein analysis** Cells or brain tissues were lysed with RIPA buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche Applied Science). For protein interaction studies, the soluble fractions were subjected to immunoprecipitation for 24 h at 4°C and analyzed by immunoblotting. For visualization, HRP-conjugated secondary antibody and ECL detection (Pierce, Rockford, IL, USA) were used, with signals acquired with an image analyzer (LAS500; GE Healthcare, Milwaukee, WI, USA).

**Lentivirus production** The procedures of lentivirus production have been described previously (Suzuki et al., 2017). The pCL20c vectors were designed under the control of the murine stem cell virus (MSCV) promoter. The viral vector was produced by co-transfection of human embryonic kidney cells (HEK293T) with a mixture of four plasmids using a calcium phosphate precipitation method. The four-plasmid mixture consisted of 6 µg of pCAG-kGP1R, 2 µg of pCAG-4RTR2, 2 µg of pCAG-VSV-G, and 10 µg of vector plasmid pCL20c (pCL20c-MSCV-sIL1RAcP-HA-IRES-EGFP and pCL20c-MSCV-SAM68-T2A-venus. The medium containing vector particles was harvested 40 h after transfection. Medium samples were concentrated by centrifugation at 25,800 rpm for 90 min. Virus samples were then suspended in cold phosphate-buffered saline (pH 7.4), frozen in aliquots, and stored at -80°C until use. After assessing the titer in HEK293T cells, the appropriate amount of lentivirus was infected into cultured neurons 5 days before harvesting.

**Ca²⁺ imaging** Hippocampal neurons cultures were prepared from ICR mouse pups on embryonic days 16-17 (E16-17), and maintained for 19–22 days before calcium imaging. Neurons were loaded with intracellular Ca²⁺ sensitive fluorescent dye Fluo-4 AM (2–3 µM, AAT Bioquest, Cat#2551) for 30–40 min at 37°C in Neurobasal Medium containing 2% B27 supplement, 2 mM Glutamax, and penicillin/streptomycin. The dye solution was replaced with Neurobasal Medium not containing B27 supplement before recordings. Cultured neurons were stimulated with NMDA (20 µM, N-Methyl-D-aspartic acid, TOCRIS, Cat#0114) in the presence of IL-1β (0.01 ng/ml, PEPROTECH, Cat#211-11B). Recordings were performed using ArrayScan VTI HCS Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C. Fluo-4 images were acquired by excitation at 475–495 nm, and emission fluorescence was collected every 1 min at 510-531 nm. Neurons were monitored for at least 4 min prior to experiments to ensure that the Ca²⁺ fluorescence was stable. For each experiment, the intensity of fluorescence at the initial time point (–4 min) was determined as a baseline. Dye influx at each time point was quantified as a fold change of the baseline. Data were analyzed using Target Activation BioApplication.
**Immunostaining, image acquisition, and analysis** Morphometric analysis of HEK293-neuron co-culture assays was performed essentially as described (Iijima et al., 2011; Sato et al., 2017). Briefly, confocal images of GFP-positive HEK293 cells (0.40 μm optical section) were captured on a Zeiss LSM5 confocal system. The projected images were analyzed using the ImageJ software (https://imagej.nih.gov/ij/, National Institutes of Health: Bethesda, MD, USA). The VAMP2 or PSD95-positive area on GFP-positive HEK293 cells was measured using an optimal threshold for all images. Three independent experiments were quantitatively analyzed, and 20–30 of GFP-positive cells were quantified per group.

**CLIP assay** CLIP assays were performed using Magna Nuclear RIP (Cross-Linked) Nuclear RNA-Binding protein Immunoprecipitation kit (Cat. No. 17-10520, Millipore) with some modification. Adult cerebella were homogenized with PBS. After irradiation with UV light on ice (120 mJ/cm² x2), samples were lysed with Nuclei Isolation Buffer, and the nuclear fraction was isolated according to the protocol. Guinea pig anti-SAM68 antisera (Iijima et al., Cell., 2011) or preimmune ones were used for the immunoprecipitation. The isolated RNA was reverse-transcribed and used for qPCR analysis.

**Oligo-RNA pull-down experiments** Thirty mers of 5’-biotinylated 2’-OMe-RNA oligonucleotides (Eurofin genomics, Tokyo, Japan) were bound to streptavidin magnetic beads (Pierce) in RP-100 buffer (20 mM HEPES pH 7.5; 1 mM dithiothreitol, 10 mM MgCl₂, 100 mM KCl; 0.01% NP-40) overnight. The sequence of oligonucleotide was as follows; l1rap 3UTR probe (PAS WT): 5’- tgt att ttc tat aat aaa gga aaa tta caa -3’; l1rap 3UTR probe (PAS a/c mut): 5’- tgt att ttc tat act caa gga aaa tta caa -3’. Cleared brain lysates from P7-P10 mouse brains prepared in RIPA buffer were diluted with an equal volume of RP-100 buffer, and then incubated with the packed beads at room temperature for 1 h. Beads were washed three times with RP-100 buffer and the precipitate was subjected to immunoblot analysis. Signal intensities were quantified by ImageGauge software (Fujifilm).

**Sample preparation for LC-MS analysis** Hippocampal brain tissues were lysed in lysis buffer (1% sodium deoxycholate (SDC), 0.1 M TRIS, 10 mM TCEP, pH = 8.5) by homogenization with a 21G syringe followed by strong ultra-sonication (10 cycles, Bioruptor, Diagnode). After sonication sample aliquots were spun down 10min at 21000G, and the supernatant was precipitated using 20% TCA. Samples were resuspended in lysis buffer, reduced for 10 min at 95 °C and alkylated with 15 mM chloroacetamide for 30 min at 37 °C. Proteins were digested by incubation with sequencing-grade modified trypsin (1/50, w/w; Promega, Madison, Wisconsin) overnight at 37°C. To each peptide sample an aliquot of a heavy reference peptide mix containing 10 chemically synthesized proteotypic peptides (Spike-Tides, JPT, Berlin, Germany) was spiked into each sample at a concentration of 5 fmol of heavy reference peptides per 1µg of total endogenous protein mass. Then, the peptides were cleaned up using iST cartridges (PreOomics, Munich) according to the manufacturer’s instructions. Samples were dried under vacuum and stored at -80 °C until further use.
Targeted PRM-LC-MS analysis of protein isoforms

In a first step, parallel reaction-monitoring (PRM) assays (PMID: 22865924) were generated from a mixture containing 100 fmol of each heavy reference peptide and shotgun data-dependent acquisition (DDA) LC-MS/MS analysis on a Thermo Orbitrap Fusion Lumos platform (Thermo Fisher Scientific). The setup of the μRPLC-MS system was as described previously (Pubmed-ID: 27345528). Chromatographic separation of peptides was carried out using an EASY nano-LC 1000 system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 μm x 30 cm) packed in-house with 1.9 μm C18 resin (Reposil-AQ Pur, Dr. Maisch). Peptides were analyzed per LC-MS/MS run using a linear gradient ranging from 95% solvent A (0.15% formic acid, 2% acetonitrile) and 5% solvent B (98% acetonitrile, 2% water, 0.15% formic acid) to 45% solvent B over 60 minutes at a flow rate of 200 nl/min.

Mass spectrometry analysis was performed on Thermo Orbitrap Fusion Lumos mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan was followed by high-collision-dissociation (HCD) of the 10 most abundant precursor ions with dynamic exclusion for 20 seconds. Total cycle time was approximately 1 s. For MS1, 1e6 ions were accumulated in the Orbitrap cell over a maximum time of 100 ms and scanned at a resolution of 120,000 FWHM (at 200 m/z). MS2 scans were acquired at a target setting of 1e5 ions, accumulation time of 50 ms and a resolution of 30,000 FWHM (at 200 m/z). Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 30%, the mass isolation window was set to 1.4 m/z and one microscan was acquired for each spectrum.

The acquired raw-files were database searched against a mouse database (Uniprot, download date: 2017/04/18, total of 34,490 entries) by the MaxQuant software (Version 1.0.13.13). The search criteria were set as following: full tryptic specificity was required (cleavage after lysine or arginine residues); 3 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; Arg10 (R), Lys8 (K) and oxidation (M) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. The best 6 transitions for each peptide were selected automatically using an in-house software tool and imported to Skyline (version 4.1 (https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view)). A mass isolation lists containing all selected peptide ion masses were exported form Skyline and imported into the Lumos operating software for PRM analysis using the following settings: The resolution of the orbitrap was set to 120k FWHM (at 200 m/z) and the fill time was set to 246 ms to reach a target value of 1e6 ions. Ion isolation window was set to 0.4 Th and the scan range was set to 150-1500 Th. A MS1 scan using the same conditions are for DDA was included in each MS cycle. Each condition was analyzed in biological quintuplicates. All raw-files were imported into Skyline for protein / peptide quantification. To control for variation in sample amounts, the total ion chromatogram (only comprising peptide ions with two or more charges) of each sample was determined by Progenesis QI (version 2.0, Waters) and used for normalization.

| Heavy-labelled reference peptide sequences used for PRM |
|--------------------------------------------------------|
| Isoform | Peptide #1  | Peptide #2  |
| Isoform 1/2/3 (total) | VAFPLEVVQK | NYVCHAR   |
| Isoform 1 | TVLTVIK        | AGLENMASR |
**Statistical analysis**  GraphPad Prism 5 ([http://www.graphpad.com/scientific-software/prism/](http://www.graphpad.com/scientific-software/prism/), GraphPad Software, Inc., San Diego, CA, USA) was used for the statistical analysis. Pairwise comparisons were performed using Student’s t-test. For multiple comparisons, analysis of variance (ANOVA) followed by Bonferroni’s or Dunnett’s test was used.

The statistical analysis (ANOVA, Welch t tests, false discovery rate correction) and graph creations of mouse exon microarray and RNA sequencing data were performed with the R software (https://www.r-project.org/, Vienna, Austria) or GeneSpring GX software (https://www.agilent.com/en/products/software-informatics/life-sciences-informatics/genespring-gx, v13.0, Agilent Technologies).

Data are represented as the mean ± SEM. Significance is indicated as follows: ***, p < 0.001; **, p < 0.01; *, p < 0.05.

**Supplementary References**

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A

**Sim1**\(^{\text{KO}}\) vs wild-type (FC > 2.0)

\[ R=0.996 \text{ (WT vs SKO)} \]
\[ n=3 \text{ (genotype)} \]

**Sam68:** Sim1\(^{\text{DKO}}\) vs wild-type (FC > 2.0)

\[ R=0.997 \text{ (WT vs DKO)} \]
\[ n=3 \text{ (genotype)} \]

B

**Sim1**\(^{\text{KO}}\) vs wild-type (FC > 2.0, \( p < 0.05 \))

**Sam68:** Sim1\(^{\text{DKO}}\) vs wild-type (FC > 2.0, \( p < 0.05 \))

C

- **Abhd1**

- **Padi2**

- **Plec9**

D

- **Amy1**

- **Ocel1**

- **Gdpd3**
Figure S1: Gene expression profiles in Sam68 KO and Sam68/Slm1 DKO brains, related to Figure 1
Total RNAs from midbrains of WT, Slm1 KO, and Sam68/Slm1 DKO mice were subjected to data analyses on exon array (Agilent, Sure Print G3 Mouse Exon Microarray 2x400K) (n=3 animals/genotype).
(A) Scatter plots of fold change for gene expression (Sam68/Slm1 DKO vs WT, Slm1 KO vs WT) (total 18,810 genes; threshold set: normalized gene expression >-3 in either of the two genotypes).
(B) Volcano plots showing fold change and p-values for genes shown in (A). Red shows genes that significantly changed by >two-fold (Sam68/Slm1 DKO vs WT, Slm1 KO vs WT, Sam68/Slm1 DKO vs Slm1 KO; threshold set: FC ≥ 2.0, normalized gene expression >-3 in either of the two genotypes, p-value<0.05).
(C,D) Validation of altered by RT-qPCR analysis using adult midbrains from WT, Sam68 KO, Slm1 KO, and Sam68/Slm1 DKO mice. Fold change (FC) and significant differences were compared to WT mice. Whereas the CT value of these transcripts was normalized to that of Gapdh. (C) Altered genes shared between Sam68 KO and Slm1 KO. (D) Altered genes unique for Sam68 KO or Slm1 KO.
Data are presented as the mean ± SEM. Significance is indicated as follows: ***, p<0.001; **, p<0.01; *, p<0.05. student's t-test.
Figure S2: Analyses by RNA-sequencing, related to Figures 1 and 2

(A) Scatter plots of fold change (≥ 2.0) for gene expression (Sam68/Slm1 DKO vs WT). Expression level was measured by fragments per kilobase of mRNA per million mapped reads (fpkm) (Filtrations: ≥ 10 reads; fpkm values ≥ 50 in 2 of 2 samples).

(B) Classification of alternative splicing events by the exon junction read in wild-type and Sam68/Slm1 DKO mice using the MISO software. The pie chart shows the percentage of five types of alternative exon changes (i.e., cassette exons, mutually exclusive exons, tandem cassette exons, alternative 5′-site, alternative 3′-site, and intron retention) in the Sam68/Slm1 DKO midbrain. The graph shows the relative ratio of altered splicing events to total events.

(C) Aberrant ALE choice of Pcdh15 and Glra3 in Sam68/Slm1 DKO brains shown in RNA-seq. Data were based on the UCSC genome browser Mouse NCBI37/mm10 assembly.
Figure S3: Generation of Slm2 mutant (Slm2\textsuperscript{MT}) mice by genome editing, related to Figure 3

(A) Illustration of Slm2 (Khdrbs3) gene disruption by genome editing with the CRISPR/CAS9 system.

(B) Images of genotyping of Slm2\textsuperscript{MT} mice by genomic PCR. Positions of the primer are shown with arrows in (A).

(C) Representative images of western blot analysis with the $\alpha$-SLM2 antibody. Whereas more than 50 kDa of SLM2 protein was expressed in wild-type’s brains, approximately 40 kDa of
SLM2 proteins lacking a first QUA domain with alternative methionine start codon were detected in Slm2 MT mice.

(D) Co-immunohistochemistry with α-SLM1 and –SLM2 antibodies in the cerebral cortex. Slm2 MT mice exhibited strong immunoreactivity for SLM2 comparable to that in wild-type mice. Consistent with a previous report that SLM1 protein level was ectopically upregulated by loss-of-function of SLM2 (Traunmuller et al., 2014), immunoreactivity for SLM1 was enhanced in the mutant mice. Scale bar = 50 µm.

(E) Representative images of semi-quantitative RT-PCR with Nrxn1-3 AS4 performed on the cortex, hippocampus, and brainstem from wild-type and Slm2 MT mice. Similar to Slm2 KO mice previously reported (Ehrmann et al., 2013; Traunmuller et al., 2016), skipping of exon 20 in all the Nrxns was dramatically impaired in the mutant mice.
Figure S4: Appearance of atypical soluble isoforms of Glra3 and Pcdh15 in Sam68KO brains by aberrant ALE usage, related to Figure 4

(A, B) Schematic illustration of alternative exon choice at Glra3 exon 4 and Pcdh15 exon 13 (top panel) the representative gel images of semi-quantitative RT-PCR with the 3'UTR exon choice in midbrains from wild-type, Slm1KO, and Sam68/Slm1DKO mice (bottom panel).

(C, D) Relative levels of total mRNA and two alternative isoforms [long form variant (LF) and short form variant (SF)] and abundance ratio of SF (Red) to LF (Blue) between midbrains from wild-type, Sam68KO, Slm1KO, and Sam68/Slm1DKO mice by qRT-PCR. Whereas the RQ value of total transcripts was normalized to that of Gapdh, the RQ value of each alternative isoform was normalized to that of total mRNA. For the abundance ratio of SF to LF, the percentage of the SF variant was largely estimated from the CT value (CtSF) directly compared to that of LF (CtLF) at the same threshold set for the CT value. RQ^LF+RQ^SF values were set to 100% (n=3 animals per each genotype).

Data are presented as the mean ± SEM. Significance is indicated as follows: **, p<0.01; *, p<0.05. One-way ANOVA followed by Dunnett’s test.
Figure S5: Altered 3’UTR isoform choice in Sam68 KO brains, related to Figure 3 and 4
(A) Representative gel images of semi-quantitative RT-PCR with the 3’UTR exon choice in midbrains from wild-type and Sam68 KO mice.
(B-D) No additive effects of aberrant 3’UTR isoform selections between Sam68 KO and
Sam68/Slm1<sup>DKO</sup> mice. Fold change (FC) and significant difference was compared to WT. Statistical differences were further tested between Sam68<sup>KO</sup> and Sam68/Slm1<sup>DKO</sup> mice to exclude any additive or synergetic effect of Sam68 and Slm1 knock out. Each alternative isoform was normalized to that of each total mRNA. (n=3–6 animals per genotype) (B) ALE type. Long form variant of Lrrcc1 (leucine-rich repeat and coiled-coil domain-containing protein 1). (C) ALE type with alternative 5′ splice sites. Two genes, short form variant of Dlgap1 (disk large-associated protein 1) and long form variant of Pcdh17 (protocadherin 17). (D) APA type. Two genes, long form variant of Fbxl3 (F-box/LRR-repeat protein3) and long form variant of Sema3e (semaphorin 3e).

Data are presented as the mean ± SEM. Significance is indicated as follows: **, p<0.01; *, p<0.05. One-way ANOVA followed by Dunnett’s test.
Figure S6: Secretion properties of the soluble isoforms of IL1RAP, GlyRa3, and Pcdh-15, related to Figure 4

(A) ALE choice of Il1rap, Cp, Glyra3, and Pcdh15 produces two types of protein products, a membrane-bound type and a soluble one.

(B) Western blot analysis using lysates of HEK293 cells expressing the soluble forms. Both membrane and soluble proteins (HA or FLAG-tagged recombinant proteins) were respectively expressed into HEK293T cells with the transfection, and cells were harvested 2 days after the transfection. Significant amounts of the soluble isoforms were released into the cultured medium.
**Figure S7: Soluble IL1RAP disturbs PTPδ-induced synaptogenic signaling, related to Figure 5**

(A, B) Expression of *Il1rap* and the related gene transcripts (*Il1rapl1, Il1, Il1r1*, and *Ptprd*) in the central nervous system by semiquantitative RT-PCR. (A) Various brain regions. (B) Developing cortex.

(C) Abundance ratio of *Il1rap* SF to LF between midbrains from wild-type, *Slm1* KO, and *Sam68/Slm1* DKO mice in three brain regions, cortex (Cx), midbrain (Mb), and cerebellum (Cb).

(D) Schematic illustration of neuron-HEK293T cell co-culture assay. To examine IL1RAP-mediated presynaptic assembly, HEK293T cells expressing IL1RAP or neurelin1 (NL1)-HA were co-cultured with cerebellar neurons (DIV10-14).

(E) Co-cultures of cerebellar granule neurons and HEK293T cells. Presynaptic assembly on HEK293T cells was detected by immunostaining with the presynaptic marker synaptobrevin (VAMP2). The overall morphology of co-cultured HEK293T cells was visualized with GFP. HEK293T cells expressing IL1RAP-FLAG, IL1RAP-L1-FLAG, or neurelin-1 (NL1)-HA with or without sIL1RAP-HA (plasmid ratio 1:1). Further, HEK293T cells expressing IL1RAP were co-cultured with cerebellar granule neurons expressing sIL1RAP-HA with lentiviral infection (n=15–27 cells per group in>10 of separated fields [see the number on each graph column]). Scale bar=5 µm.

(F) HEK293T cells expressing PTPδ were cocultured with control or IL1RAP knockdown neurons, and then immunostained with a post-synapse marker, anti-PSD-95 antibody.

(G) Detection of sIL1RAP-HA in the condition medium from cortical neuron culture by immunoprecipitation analysis with anti-HA antibody. sIL1RAP-HA was not immunoprecipitated with control IgG.
Figure S8: Identification of SAM68 recognition element in intron 8 of Il1rap, related to Figure 6

(A) Gel loading image of the 3'RACE assay from Sam68 KO mice. Arrows show two transcripts of Il1rap short isoforms. The upper band is the transcript encoding the full-length of Il1rap exon 8b (exon 8b LF). The lower band is the one lacking approximately 400 bp of the 3'end sequence (exon 8b SF). The asterisk denotes an unspecific PCR product.

(B) The cDNA sequence of Il1rap exon 8 SF. Green indicates the coding exon region. Blue shows the putative PAS site (PAS1) on the exon 8b SF UTR. Yellow shows the putative cleavage site.

(C) Survey of the 3'UTR length of Il1rap exon 8b by RT-qPCR analysis. Arrowheads show the positions of primer sets. The amount of these PCR products in Sam68 KO brains was compared to wild-type ones. All the products were significantly detectable in Sam68 KO brains compared to wild-type (n=5). RQ values for wild-type brain were set to 1.0.

(D) Conserved PAS1 sequence between humans and mice. Blue shows the PAS1 sequence. Data are presented as the mean ± SEM. Significance is indicated as follows: ***, p<0.001; **, p<0.01; *, p<0.05. student's t-test.
Figure S9: Tissue-specific splicing of ALEs of Il1rap, related to Figure 7

(A) Expression of Il1rap, Cp, Pcdh15, and Lrcc1 transcripts in various tissues by semiquantitative RT-PCR.

(B) Abundance of SAM68 protein in five tissues: brain, liver, intestine, lung, and spleen. Western blot analysis using total lysate from five tissues was performed with rabbit α-SAM68 antibody.

(C) Expression of Sam68, Slm1, and Slm2 transcripts between the brain and liver by semiquantitative RT-PCR.