Misregulation of human sortilin splicing leads to the generation of a nonfunctional progranulin receptor

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Edited* by Susan Lindquist, Whitehead Institute for Biomedical Research, Cambridge, MA, and approved November 12, 2012 (received for review July 7, 2012)

Sortilin 1 regulates the levels of brain progranulin (PGRN), a neurotrophic growth factor that, when deficient, is linked to cases of frontotemporal lobar degeneration with TAR DNA-binding protein-43 (TDP-43)–positive inclusions (FTLD-TDP). We identified a specific splicing enhancer element that regulates the inclusion of a sortilin exon cassette (termed Ex17b) not normally present in the mature sortilin mRNA. This enhancer element is consistently present in sortilin RNA of mice and other species but absent in primates, which carry a premature stop codon within the Ex17b sequence. In the absence of TDP-43, which acts as a regulatory inhibitor, Ex17b is included in the sortilin mRNA. In humans, in contrast to mice, the inclusion of Ex17b in sortilin mRNA generates a truncated, nonfunctional, extracellularly released protein that binds to but does not internalize PGRN, essentially acting as a decoy receptor. Based on these results, we propose a potential mechanism linking misregulation of sortilin splicing with altered PGRN metabolism.

dementia | neurodegeneration | ALS

Sortilin (SORT) 1, primarily expressed in neurons, is a member of a family of cellular vacuolar protein sorting 10 (VSP10)-domain receptors, and a key player in regulating neuronal viability and function (1, 2). SORT1 is a receptor of neurotrophic factors and neuropeptides, as well as a coreceptor to cytokine receptors, tyrosine receptor kinases, G protein–coupled receptors, and ion channels (2).

Since the discovery that SORT1 is a neuronal progranulin (PGRN) receptor that mediates PGRN endocytosis and delivery to lysosomes (3, 4), it has garnered a great deal of interest from the neurodegeneration field. Mutations in the progranulin gene (*GRN*) cause *GRN* haploinsufficiency and frontotemporal lobar degeneration with TAR DNA-binding protein-43 (TDP-43)–positive inclusions (FTLD-TDP) (5, 6), highlighting the importance of proper PGRN homeostasis. TDP-43 abnormal cytoplasmic redistribution and sequestration into inclusions in FTLD-TDP and other TDP-43 proteinopathies (5, 6) are expected to adversely affect TDP-43 nuclear function. TDP-43 regulates many facets of RNA metabolism, such as exon cassette splicing for a variety of transcripts (7, 8). Although the link between PGRN and TDP-43 pathology has yet to be elucidated, it is intriguing that Tdp-43 regulates *Sort1* splicing in mouse brain (8) and may, thus, influence Pgrn metabolism.

Herein, we explore the role of TDP-43 in inhibiting the inclusion of a sortilin exon cassette (termed Ex17b) in the mature sortilin mRNA. We provide evidence linking loss of nuclear TDP-43 function and the consequent misregulation of *SORT1* splicing with changes in PGRN metabolism. Shedding new light on SORT1 biology, and how different SORT1 isoforms influence PGRN trafficking and metabolism, may aid in the development of therapies for diseases in which PGRN levels are altered.

Results

Sortilin 1 Splicing Variants. The full-length sortilin transcript encodes widely conserved functional domains across species, including an endoplasmic reticulum (ER) import signal, a VSP10 luminal domain, a transmembrane domain, and a cytosolic domain (Fig. S1*A*). The majority of sortilin ligands bind the VSP10 domain, and the cytosolic domain contains the active sorting signal to direct sortilin and its ligands to lysosomes. With regards to full-length and alternatively spliced sortilin isoforms, the Ensembl Genome Browser (www.ensembl.org) recognizes transcripts of a variety of species (Table S1). Of interest, an alternatively spliced sortilin transcript having an extra coding exon between the VSP10 and transmembrane domains is present in some species (e.g., mice, rats) but not reported in humans (Table S1 and Fig. S1 *A–C*); however, the biological significance of this sortilin isoform remains unknown.

Polymenidou et al. demonstrated that Tdp-43 binds, and regulates splicing of, *Sort1* mRNA in mouse brain (8). They showed that, the mouse *Sort1* sequence chr3:108483527–108483626 (which they denote as exon 18), is normally absent in the mature *Sort1* transcript, while its inclusion is favored upon mouse Tdp-43 down-regulation (8). Because chr3:108483527–108483626 corresponds to the above mentioned extra exon located between the VSP10 and transmembrane domains in *Sort1*, and because this extra exon is not included in the predominant sortilin transcripts in mammals (Table S1), we propose that *Sort1* transcripts lacking this extra exon would be more accurately termed $Sort1_{WT}$. Furthermore, *Sort1* transcripts containing this extra exon, such as those generated when Tdp-43 levels are down-regulated, should be termed $Sort1_{+Ex17b}$, given that this additional exon cassette is located within intron 17 of $Sort1_{WT}$, and immediately follows exon 17 in the mature transcript (Fig. S1*D*).

A Protein Encoding Mouse $Sort1_{+Ex17b}$ Is Generated upon Tdp-43 Down-Regulation. We confirmed $Sort1_{WT}$ as the main isoform generated in the presence of normal Tdp-43 levels in mouse N2A cells, with minimal mouse $Sort1_{+Ex17b}$ produced under these conditions (Fig. 1*A*). Conversely, down-regulation of Tdp-43 expression in cells treated with a siRNA against *Tardbp* (si*Tardbp*), led to the accumulation of $Sort1_{+Ex17b}$ (Fig. 1*A*). Quantification of mouse *Sort1* RNA levels by quantitative (q) RT-PCR demonstrated the accumulation of $Sort1_{+Ex17b}$ upon Tdp-43 depletion is statistically significant (Fig. 1*B*).

The Ensembl Genome Browser recognizes a mouse Ex17b-containing *Sort1* variant (ENTUMUST00000090564) believed to encode an 858-aa protein (33 aa longer than $Sort1_{WT}$) (Table S1). To determine whether an Ex17b-containing protein isoform is generated upon mouse Tdp-43 depletion, we assessed protein

Author contributions: M.P., K.R.J.-W., W.C.L., T.F.G., E.B., and L.P. designed research; M.P., K.R.J.-W., W.C.L., T.F.G., C. Stuani, and E.B. performed research; M.P., W.C.L., Y.-J.Z., Y.-F.X., J.G., C. Stuani, R.R., D.W.D., and L.P. contributed new reagents/analytic tools; M.P., W.C.L., T.F.G., E.B., and L.P. analyzed data; and M.P., T.F.G., C. Stetler, E.B., and L.P. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1211577110/-/DCSupplemental.

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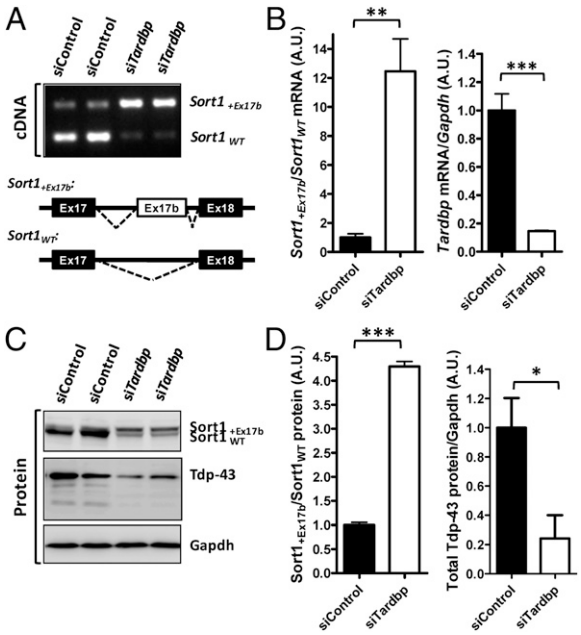

Fig. 1. Mouse *Sort1*$_{+Ex17b}$ is generated and translated into protein upon Tdp-43 down-regulation. Endogenous Tdp-43 was knocked down in mouse N2A cells using siRNA targeting *Tardbp* (si*Tardbp*). RNA and protein were collected 72 h later. (*A*) cDNA prepared from the RNA was subjected to PCR using primers in exons 17 and 19 of mouse *Sort1*$_{WT}$. A ∼350- and a ∼250-bp band products were produced. Sequencing of the PCR products confirmed that the ∼250-bp product corresponds to sortilin exons 17, 18, and 19 (*Sort1*$_{WT}$), whereas the ∼350-bp product (predominant in TDP-43–depleted mouse cells) contains the same exons as *Sort1*$_{WT}$, and an additional 99 bp from intron 17 (*Sort1*$_{+Ex17b}$). (*B*) qRT-PCR was performed to determine the mouse *Sort1*$_{+Ex17b}$/*Sort1*$_{WT}$ ratio, and relative *Tardbp* mRNA levels under control and knockdown conditions. (*C*) Western blots of total protein lysates from siControl and si*Tardbp*-treated cells were probed with an antibody recognizes both Sort1 isoforms. Tdp-43 protein expression was analyzed to confirm knockdown efficiency. (*D*) Band intensities for mouse Sort1$_{+Ex17b}$ and Sort1$_{WT}$ were quantified and presented as a ratio. Tdp-43 protein levels were also quantified relative to Gapdh levels. Statistical differences were calculated by Student *t* tests. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$ ($n = 3$). A.U., arbitrary units.

lysates of N2A cells treated with si*Tardbp*. Cells treated with control siRNA (siControl) expressed a Sort1-immunopositive band corresponding to Sort1$_{WT}$ (Fig. 1*C*), whereas si*Tardbp*-treated cells showed a reduction in Sort1$_{WT}$ expression allowing the visualization of a higher molecular weight Sort1 isoform, Sort1$_{+Ex17b}$ (Fig. 1*C*). Densitometric analysis of Sort1-immunopositive bands demonstrated that the changes in mouse Sort1 protein expression (Fig. 1*D*) corresponded with the observed changes in *Sort1* mRNA (Fig. 1*B*).

Note that the generation of *Sort1*$_{+Ex17b}$ is specifically regulated by Tdp-43, because down-regulation of fused in sarcoma (Fus), an RNA binding protein also implicated in alternative splicing, failed to produce significant levels of *Sort1*$_{+Ex17b}$ RNA or protein (Fig. S2). Furthermore, we confirmed the generation of *Sort1*$_{+Ex17b}$ in primary cultures and in vivo when *Tardbp* is down-regulated (Fig. S3 *A* and *B*). Conversely, mouse *Sort1*$_{+Ex17b}$ levels were decreased in a dose-dependent manner in transgenic mice constitutively overexpressing human TDP-43 (Fig. S3*C*).

Glycine-Rich Domain and RNA Recognition Motifs of Human TDP-43 Are Required to Inhibit Ex17b Inclusion in Mouse *Sort1*. To determine whether human TDP-43 proteins inhibit Ex17b inclusion in mouse *Sort1*, we performed experiments in which siRNA-resistant human wild-type (TDP-43$_{WT}$) or mutant TDP-43$_{5F\rightarrow L}$ were expressed before siControl or si*Tardbp* treatment. TDP-43$_{5F\rightarrow L}$ contains 5 phenylalanine to leucine substitutions within the RNA recognition motifs (RRMs) of TDP-43 that abolish its ability to bind RNA (9). Efficiency of mouse Tdp-43 down-regulation and human TDP-43 overexpression was confirmed by densitometric analyses of immunoblots (Fig. S4 *A* and *B*). As expected, increased levels of mouse *Sort1*$_{+Ex17b}$ mRNA were observed in si*Tardbp*-treated cells compared with siControl-treated cells, despite overexpression of a control plasmid encoding yellow fluorescent protein (YFP) (Fig. 2 *A* and *B*). Overexpression of human TDP-43$_{WT}$ in si*Tardbp*-treated cells partially inhibited the inclusion of Ex17b in mouse *Sort1*, and similar changes were reflected in Sort1$_{+Ex17b}$ protein expression (Fig. 2 *A*–*C*). Similar to TDP-43$_{WT,}$ TDP-43 ALS-

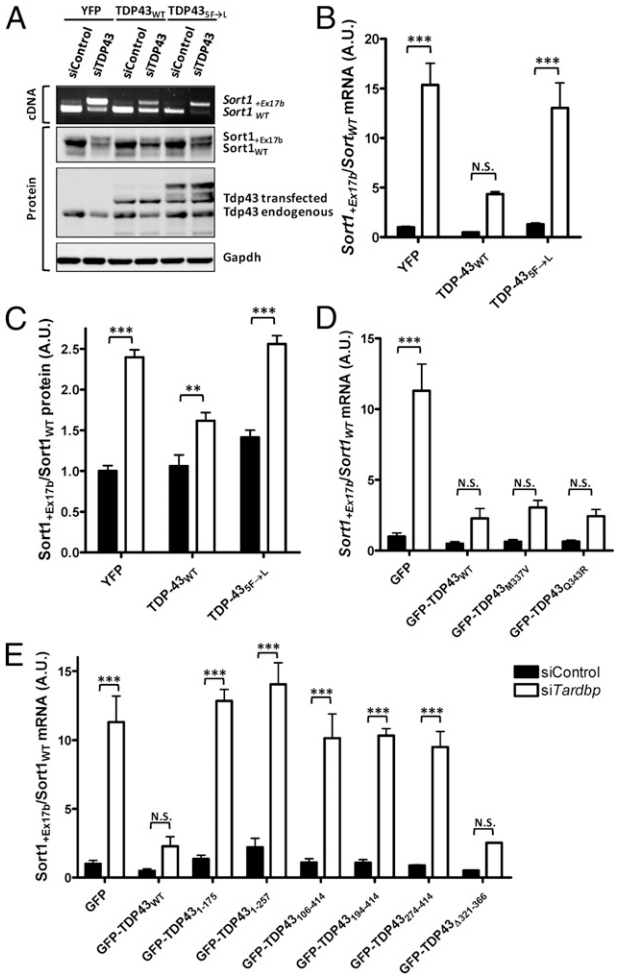

Fig. 2. RRMs and glycine-rich region of human TDP-43 are required to inhibit Ex17b inclusion in mouse *Sort1*. Mouse N2A cells were transfected to overexpress YFP, or the indicated human TDP-43 constructs, followed by treatment with siControl or si*Tardbp*. Because si*Tardbp* targets the 3′UTR of mouse *Tardbp*, it does not affect the expression of human TDP-43. RNA and protein were isolated. (*A*–*C*) Compared with si*Tardbp*-treated cells overexpressing YFP, human TDP-43$_{WT}$ overexpression blocked the accumulation of mouse *Sort1*$_{+Ex17b}$ mRNA (*A* , *Upper*, and *B*) and Sort1$_{+Ex17b}$ protein (*A* , *Lower*, and *C*). In contrast, human TDP-43$_{5F\rightarrow L}$ was unable to inhibit Ex17b inclusion (*A*–*C*). (*D*) Human TDP-43$_{M337V}$ and TDP-43$_{Q343R}$ overexpression attenuated mouse *Sort1*$_{+Ex17b}$ splicing in si*Tardbp*-treated cells to the same degree as human TDP-43$_{WT}$, as assessed by qRT-PCR. (*E*) qRT-PCR was performed to measure the effect of the deletion constructs on mouse *Sort1*$_{+Ex17b}$ levels upon depletion of Tdp-43. Only expression of human TDP-43$_{WT}$ and TDP-43$_{\Delta 321–366}$ significantly reduced the levels of mouse *Sort1*$_{+Ex17b}$, generated in response to a decrease in endogenous mouse Tdp-43. Statistical differences were calculated by two-way ANOVA followed by the Bonferroni posttest. N.S.: nonsignificant, $^{**}P < 0.005$, $^{***}P < 0.0001$, $n = 3$. A.U., arbitrary units.

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associated mutant proteins normally localize to the nucleus (Fig. S4*C*) (10), thus it was not surprising that TDP-43_{M337V} and TDP-43_{Q343R} mutant proteins attenuated Ex17b splicing in si*Tardbp*-treated mouse cells to the same degree as human TDP-43_{WT} (Fig. 2*D* and Fig. S4 *C–E*). Furthermore, mice overexpressing human TDP-43_{M377V} mutant protein present reduced levels of $Sort1_{+Ex17b}$ (Fig. S3*D*), as do mice overexpressing human TDP-43_{WT}. These data indicate that, similar to CFTR exon 9 splicing regulation by mutant TDP-43 (11), disease-associated human TDP-43 proteins do not adversely affect mouse *Sort1* Ex17b splicing. Unlike TDP-43_{WT}, however, overexpression of TDP-$43_{5F\rightarrow L}$ did not inhibit $Sort1_{+Ex17b}$ production (Fig. 2 *A–C*), suggesting that human TDP-43 directly binds mouse *Sort1* to regulate Ex17b splicing.

To further evaluate regions of human TDP-43 necessary for regulating Ex17b splicing, N2A cells were transfected with plasmids encoding GFP-tagged TDP-43_{WT} or various TDP-43 deletion products (depicted in Fig. S4*F*), before treatment with siControl or si*Tardbp*. Thereafter, expression levels of mouse *Sort1* isoforms (Fig. 2*E*), as well as *Tardbp* and human GFP-tagged TDP-43 products (Fig. S4 *G* and *H*), were examined by qRT-PCR. As seen in Fig. 2*E*, overexpression of human TDP-43_{WT} significantly inhibited production of mouse $Sort1_{+Ex17b}$ upon Tdp-43 depletion, consistent with our above findings. Human TDP-$43_{\Delta 321–366}$ also inhibited $Sort1_{+Ex17b}$ production, indicating that residues 321–366 are not required for TDP-43–mediated inhibition of Ex17b splicing, in contrast to their requirement for *CFTR* exon 9 splicing inhibition (11). Unlike TDP-43_{WT} and TDP-$43_{\Delta 321–366}$, human TDP-43 proteins containing one (GFP-TDP$43_{1–175}$) or two (GFP-TDP$43_{1–257}$) RRMs, but lacking the glycine-rich region, did not inhibit Ex17b splicing (Fig. 2*E*). Additionally, human TDP-43 lacking a nuclear localization signal did not inhibit mouse Ex17b splicing, even if two RRM (GFP-TDP$43_{106–414}$), one RRM (GFP-TDP$43_{194–414}$), no RRM (GFP-TDP$43_{274–414}$), and the glycine-rich region were present (Fig. 2*E*). In conclusion, the RNA-binding ability and the glycine-rich region of human TDP-43 are required for regulating mouse *Sort1* splicing, as is the ability of TDP-43 to access the nucleus.

$SORT1_{+Ex17b}$ Is Generated in Human Cells upon TDP-43 Depletion. To determine whether human TDP-43 binds the human *SORT1* transcript to regulate splicing, we performed UV-crosslinking and RNA–TDP-43 immunoprecipitation in human M17 cells overexpressing either human TDP-43_{WT} or the TDP-$43_{5F\rightarrow L}$ RNA-binding mutant (Fig. 3*A*). qRT-PCR analyses of TDP-43–bound RNA demonstrated human *SORT1* transcripts bound to TDP-43_{WT} but not TDP-$43_{5F\rightarrow L}$ proteins, indicating that human TDP-43 does bind human *SORT1* and further confirming that intact RRMs are necessary for this interaction (Fig. 3*A*). Transcripts previously identified to be regulated by, or known to bind, mouse Tdp-43 protein served as positive controls (Table S2).

To investigate whether human TDP-43 regulates human *SORT1* splicing, using a similar strategy as above, M17 cells were treated with siControl or si*TARDBP*, and RNA was extracted for qRT-PCR analyses of human $SORT1_{+Ex17b}$. Whereas knockdown of mouse Tdp-43 resulted in a marked increase in mouse $Sort1_{+Ex17b}$ (Fig. 1*B*), TDP-43 knockdown in human cells led to a modest, yet statistically significant, increase in human $SORT1_{+Ex17b}$, despite similar knockdown efficiency (Fig. 3*B*). This was somewhat surprising given the high degree of homology between human and mouse Ex17b and flanking intronic sequences (Fig. S5).

Next, we evaluated whether changes in *SORT1* are observed in FTLD-TDP cases, which are characterized by the abnormal sequestration of TDP-43 into cytosolic inclusions. Whereas $SORT1_{+Ex17b}$ RNA could not be detected in the cerebellum (unaffected region) of either controls or pathologically confirmed FTLD-TDP cases, $SORT1_{+Ex17b}$ was significantly increased in the temporal cortex (affected region) of FTLD-TDP cases (Fig. 3*C*). Of note, whereas a decrease in $Sort1_{WT}$ levels accompanies the increase in $Sort1_{+Ex17b}$ levels in TDP-43–depleted mouse cells (Fig. S6*A*), both human sortilin isoforms were significantly elevated in si*TARDBP*-treated human cells, and in the

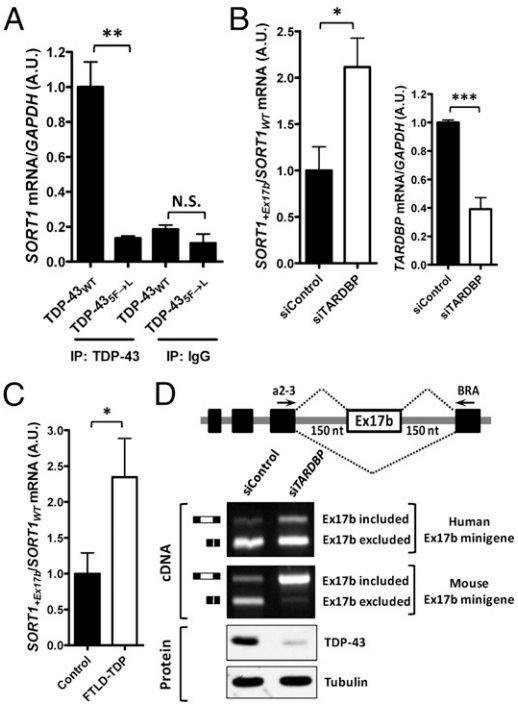

Fig. 3. $SORT1_{+Ex17b}$ splicing is strongly inhibited in human cells but occurs upon loss of TDP-43 function. (*A*) Human M17 cells were transfected to overexpress human TDP-43_{WT} or TDP-$43_{5F\rightarrow L}$. Following transfection, cells were UV-irradiated, and RNA-bound TDP-43 was immunoprecipitated (IP) as explained in *Materials and Methods*. IgG immunoprecipitation served as a negative control. qRT-PCR analyses showed significantly increased TDP-43 binding to human *SORT1* in cells expressing human TDP-43_{WT} compared with cells expressing the RNA-binding mutant TDP-$43_{5F\rightarrow L}$ ($n = 2$). (*B*) Human M17 cells were treated with siControl or si*TARDBP* for 48 h. A significant increase in $SORT1_{+Ex17b}$ mRNA levels, quantified by qRT-PCR, was found in si*TARDBP*-treated cells compared with siControl-treated cells ($n = 6$). (*C*) $SORT1_{+Ex17b}$ mRNA was also significantly elevated in the temporal cortex of FTLD-TDP compared with control cases, as evaluated by qRT-PCR. A description of cases is provided in Table S3 ($n = 20$). (*D*) Human HeLa cells were transfected with a human or mouse Ex17b minigene (illustration, sequence in Fig. S5) and treated with siControl or si*TARDBP* for 48 h. PCR using primers located in the vector backbone was performed to detect inclusion or exclusion of Ex17b. A representative blot showing TDP-43 knockdown efficiency is included. Statistical differences were calculated by Student *t* tests: N.S., nonsignificant; $*P < 0.05$; $**P < 0.005$; $***P < 0.0001$. A.U., arbitrary units.

temporal cortex of FTLD-TDP cases (Fig. S6 *B* and *C*). Furthermore, FTLD-Tau cases, which have no TDP-43 pathology, did not present altered sortilin splicing (Fig. S6*D*), suggesting that *SORT1* splicing misregulation is specific to FTLD tissue with TDP-43 pathology.

Splicing of Sortilin Ex17b in Mice and Humans Is Differentially Regulated. To compare Ex17b splicing in mice and humans, we generated minigenes that contain the 99 bp of human or mouse Ex17b, flanked by the respective 150 nt upstream and downstream of Ex17b (Fig. S5). Expression of the Ex17b minigenes in *siTARDBP*-treated cells further confirmed that mouse Ex17b is more efficiently spliced than human Ex17b (Fig. 3*D*). These findings indicate that differences in sortilin Ex17b splicing exist between mice and humans and suggest that Ex17b inclusion is repressed to a greater extent in human cells.

To further evaluate the differential regulation of Ex17b splicing between mice and humans, we overexpressed a series of mouse and human hybrid Ex17b minigenes (depicted in Fig. 4*A*) and evaluated regulatory elements upstream and downstream of the Ex17b exon cassette. As expected, when either mouse or human Ex17b minigenes were transfected into cells expressing

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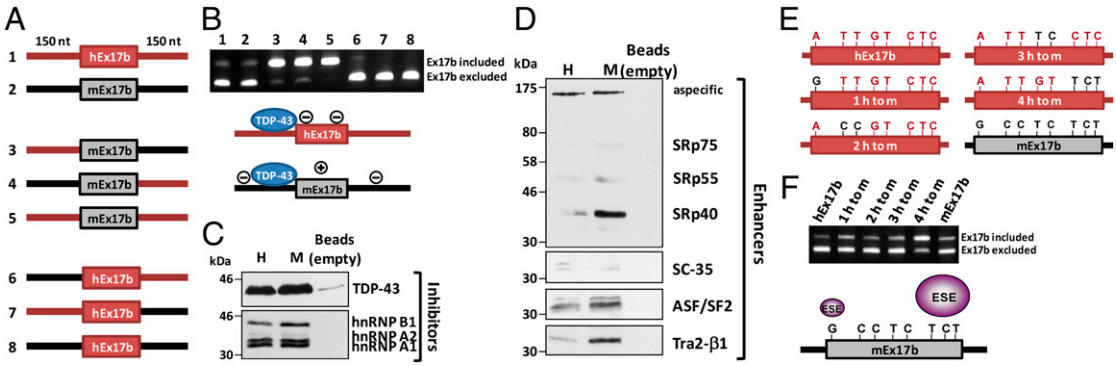

Fig. 4. Splicing enhancer sequences for Ex17b are present in mouse, but not human, sortilin. (*A*) Series of hybrid mouse and human Ex17b minigenes constructed to assess differences in the regions that control Ex17b splicing between these species. (*B*) Each minigene was expressed in human cells that expressed only endogenous TDP-43. Replacement of one or two intronic sequences flanking mouse Ex17b by the corresponding human intronic sequences promoted Ex17b inclusion (compare lanes 3, 4, and 5 to lane 2). However, when human Ex17b was flanked by mouse intronic sequences, no increase Ex17b inclusion was observed (compare lanes 6, 7, and 8 to lane 1). (*C* and *D*) In vitro–transcribed mouse and human Ex17b minigene RNA were incubated with human nuclear protein extracts to assess their ability to bind proteins known to repress (*C*) or enhance (*D*) splicing. (*E*) Several human to mouse (h to m) substitutions in the human Ex17b minigene were performed to determine which nucleotides define the splicing enhancer sequences. (*F*) h-to-m constructs (described in *E*) were expressed in human cells expressing only endogenous TDP-43. Note that the "1 h to m" substitution slightly promoted inclusion of human Ex17b, and the "4 h to m" substitution strongly promoted inclusion. $n = 3$.

endogenous TDP-43, little Ex17b inclusion occurred (Fig. 4*B*, lanes 1 and 2). The substitution of mouse introns for human introns markedly enhanced the inclusion of mouse Ex17b to almost 100% (Fig. 4*B*, lanes 3–5). In contrast, when human introns were substituted for mouse introns, virtually no inclusion of human Ex17b occurred (Fig. 4*B*, lanes 6–8). These results suggest that the introns flanking mouse Ex17b, but not human Ex17b, contain negative elements that down-regulate the inclusion of Ex17b (Fig. 4*B*, illustration). Given that the removal of these negative elements leads to an increase in mouse Ex17b inclusion, enhancer sequence(s) may exist in the mouse Ex17b sequence. Our results also suggest that the human Ex17b cassette exon is inefficiently recognized and has no need for strong silencers within its flanking intronic sequences (Fig. 4*B*, illustration).

To determine whether mouse and human sortilin RNA sequences possess a differential protein binding ability with respect to well-known positive and negative splicing regulators, we performed pull-down assays using in vitro transcribed Ex17b minigenes to identify factors that bind Ex17b sequences (*Materials and Methods*). These assays indicate that several negative splicing regulators, including TDP-43, bound to the human and mouse Ex17b minigene sequences with similar efficiency (Fig. 4*C*). Interestingly, positive enhancer regulators bound to the mouse Ex17b sequence much more efficiently than to human Ex17b (Fig. 4*D*), suggesting that the mouse Ex17b cassette exon contains one or more enhancer splicing sequences that are absent in human Ex17b. To test this hypothesis, we made a series of substitutions within the human Ex17b minigene, in which Ex17b human nucleotides that differed from mouse Ex17b (Fig. S5) were replaced by the respective mouse nucleotides (h-to-m; see schematic in Fig. 4*E*). Following transfection of these modified minigenes into cells, Ex17b inclusion was evaluated. Compared with the human Ex17b minigene, Ex17b inclusion was significantly enhanced in the 1 h-to-m (human A to mouse G) mutant and, especially, in the 4 h-to-m (human C-T-C to mouse T-C-T) mutant (Fig. 4*F*, compare lanes 2 and 5 with 1). These results indicate that the human-to-mouse nucleotide substitutions created otherwise absent enhancer elements within the human Ex17b cassette exon near the 3′ and 5′ splice sites (see schematic in Fig. 4*F*). Subsequent evaluation of T-C-T enhancer sortilin sequences across phyla revealed that evolutionary changes have occurred, such that T-C-T sequences are absent in primates. In general, in those families where the T-C-T enhancer sequence is lost (almost all primates), Ex17b encodes an in-frame premature stop codon or translational frame shifts (Fig. S7*A*). This evolutionary parallelism also appears to be present in other families of placental mammals where the enhancer activity is preserved, notwithstanding minor changes in the T-C-T composition (e.g., T-C-C in Lagomorpha), depending on whether Ex17b is in-frame and does not carry any premature termination codons (Fig. S7 *A–C*). Taken together, our findings demonstrate that evolutionary changes have occurred that do not favor the inclusion of Ex17b in humans.

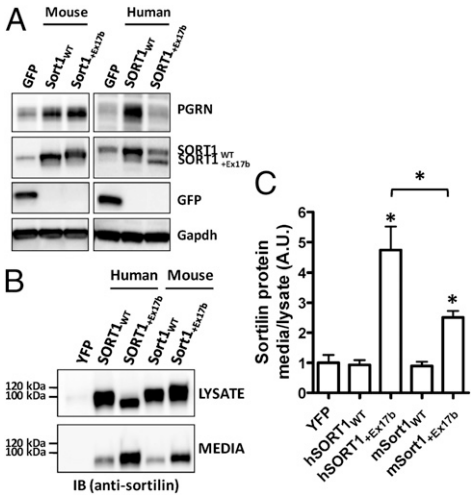

Fig. 5. Human *SORT1*$_{+Ex17b}$ proteins do not promote PGRN endocytosis and are most abundant extracellularly. (*A*) To evaluate PGRN uptake, mouse or human sortilin isoforms were expressed in N2A cells for 24 h, and cell media were supplemented with human rPGRN for 1 h prior harvest of the cells. PGRN uptake was visualized by immunoblot analyses of cell lysates. Of interest, cells expressing mouse Sort1$_{+Ex17b}$, but not human SORT1$_{+Ex17b}$, were able to uptake rPGRN. (*B* and *C*) The levels of sortilin isoforms in the media and cell lysates of human M17 cells overexpressing human or mouse sortilin proteins were evaluated by immunoblot (*B*). Note that truncated sortilin proteins (a result of human SORT1$_{WT}$, mouse Sort1$_{WT}$, and likely mouse Sort1$_{+Ex17b}$, shedding) were detected extracellularly (media). (*C*) Densitometric analyses of the immunoblots demonstrate higher levels of Ex17b-containing sortilin isoforms in the media than in cell lysates. Statistical differences were calculated by one-way ANOVA followed by Tukey posttests. $*P < 0.05$, $n = 3$. A.U., arbitrary units.

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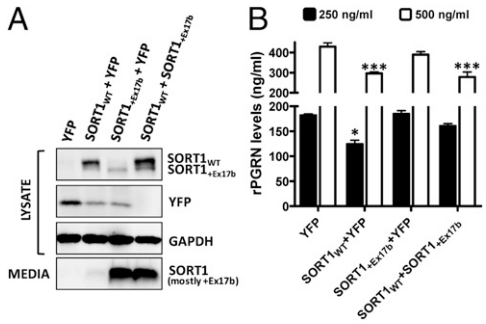

Fig. 6. $SORT1_{+Ex17b}$ proteins compete with $SORT1_{WT}$ for PGRN binding. M17 cells were transfected to overexpress equal levels of exogenous human proteins, as demonstrated by immunoblot analyses of cell lysates and media (*A*), and supplemented with two different concentrations of rPGRN. A PGRN-based immunoassay was performed to measure PGRN levels in the media (*B*). At lower rPGRN concentrations (250 ng/mL), $SORT1_{WT}$-overexpressing cells efficiently uptake rPGRN but uptake is blocked when coexpressed with $SORT1_{+Ex17b}$ proteins. At higher concentrations of rPGRN (500 ng/mL), $SORT1_{WT}$ proteins endocytose PGRN despite $SORT1_{+Ex17b}$ coexpression. Statistical differences were calculated by two-way ANOVA followed by the Bonferroni posttest (comparing to the respective YFP overexpressing control). $*P < 0.05$; $***P < 0.0001$ ($n = 2$).

Functional Differences Between Mouse and Human Ex17b-Containing Sortilin Isoforms. The *$SORT1_{+Ex17b}$* human isoform carries a premature stop codon within Ex17b sequence (Fig. S5, white font) and is thus predicted to encode a truncated protein that lacks exons 18, 19, and 20, corresponding to the transmembrane and cytosolic domains required for SORT1 function. To determine whether Ex17b inclusion affects sortilin-mediated PGRN uptake, cells were transfected to express mouse or human sortilin isoforms, and media were supplemented with exogenous recombinant human progranulin as explained in *Materials and Methods*. PGRN uptake was analyzed by immunoblot. Compared with control cells overexpressing GFP, PGRN uptake was dramatically increased in cell lysates overexpressing mouse $Sort1_{WT}$ or human $SORT1_{WT}$ (Fig. 5*A*). Similar results were observed upon overexpression of mouse $Sort1_{+Ex17b}$ (Fig. 5*A*). In contrast, PGRN levels in cells overexpressing human $SORT1_{+Ex17b}$ were no different from in GFP-expressing cells, suggesting that $SORT1_{+Ex17b}$ cannot promote PGRN endocytosis (Fig. 5*A*). This was further confirmed by immunofluorescence studies (Fig. S8 *A* and *B*). Also observed was a marked difference in the intracellular localization between $SORT1_{WT}$ and $SORT1_{+Ex17b}$ human proteins: unlike $SORT1_{WT}$, the few cells that expressed $SORT1_{+Ex17b}$ accumulated solely in the ER (Fig. S8 *C–N*).

Next, we sought to assess whether mouse and human $SORT1_{+Ex17b}$ proteins are expressed extracellularly. Thus, we overexpressed human and mouse sortilin variants in cells for 48 h and then collected cell lysates and media for immunoblot analyses. Within lysates, sortilin-immunopositive bands were observed at the expected molecular weight for full-length human $SORT1_{WT}$ and mouse $Sort1_{WT}$ (~100-kDa protein), mouse $Sort1_{+Ex17b}$ (~105 kDa), or human $SORT1_{+Ex17b}$ (~95 kDa) (Fig. 5*B*). In the media, mouse and human $SORT1_{WT}$ proteins were of 95 kDa (Fig. 5*B*), the molecular weight corresponding to the sortilin ectodomain resulting from protease cleavage within the extracellular stalk region of sortilin, an event known as shedding (12). Note that human $SORT1_{+Ex17b}$, which normally runs as a 95-kDa isoform because of its truncated nature, is most abundant in the media (Fig. 5 *B* and *C*). Although less abundant than $SORT1_{+Ex17b}$, the levels of the mouse $Sort1_{+Ex17b}$ isoform in the media were higher than mouse $Sort1_{WT}$ (Fig. 5*C*), suggesting that the extra region encoded by Ex17b in mouse $Sort1_{+Ex17b}$ facilitates the access of proteases to this region, thus increasing shedding.

Because human $SORT1_{+Ex17b}$ does not promote uptake of human PGRN, it is uncertain whether it retains the ability to bind PGRN. Thus, we immunoprecipitated either SORT1 or PGRN from media and lysates of overexpressing cells to demonstrate that, just like $SORT1_{WT}$, $SORT1_{+Ex17b}$ proteins bind intracellular, as well as extracellular, PGRN (Fig. S9). Taken together, our data demonstrate that human $SORT1_{+Ex17b}$ proteins are readily released from cells and retain their ability to bind PGRN but cannot promote PGRN uptake.

To evaluate whether $SORT1_{+Ex17b}$ expression affects PGRN uptake mediated by $SORT1_{WT}$ expression, we overexpressed cells with either human $SORT1_{+Ex17b}$, $SORT1_{WT}$, or a combination of both, and cells were supplemented with recombinant (r)PGRN at two different concentrations (Fig. 6). Equal amounts of sortilin protein were expressed in all conditions (Fig. 6*A*). The levels of rPGRN in the media were measured by a PGRN specific immunoassay. At the lower rPGRN concentration, overexpression of $SORT1_{WT}$ promotes rPGRN uptake, measured as a 25% reduction of rPGRN levels in the media, whereas $SORT1_{+Ex17b}$ overexpression did not promote rPGRN uptake (Fig. 6*B*). Overexpression of both $SORT1_{WT}$ and $SORT1_{+Ex17b}$ proteins produced a modest, but not significant, rPGRN uptake (Fig. 6*B*), suggesting that $SORT1_{+Ex17b}$ proteins compete with $SORT1_{WT}$ for PGRN binding. Supplementing cells with a higher amount of rPGRN did not reduce the levels of rPGRN in the media when $SORT1_{+Ex17b}$ and $SORT1_{WT}$ proteins were overexpressed (Fig. 6*B*). Thus, our data demonstrate that extracellular $SORT1_{+Ex17b}$ proteins compete with $SORT1_{WT}$ proteins, and possibly other PGRN receptors, for PGRN binding but do not affect normal $SORT1_{WT}$ function.

Discussion

Emerging evidence indicates that sortilin is a key player in the regulation of neuronal viability (13), as well as in a variety of other biological functions, including cholesterol and glucose metabolism (14, 15). Thus, a better understanding of the factors regulating sortilin splicing and expression is of great importance. Our studies demonstrate that the Ex17b cassette exon is not normally included in mature sortilin transcripts. However, depletion of TDP-43 leads to the accumulation of Ex17b-containing sortilin transcripts, indicating that TDP-43 is an Ex17b splicing inhibitor. This inhibitory function is dependent on the RRMs, glycine-rich domain, and nuclear localization signal of TDP-43.

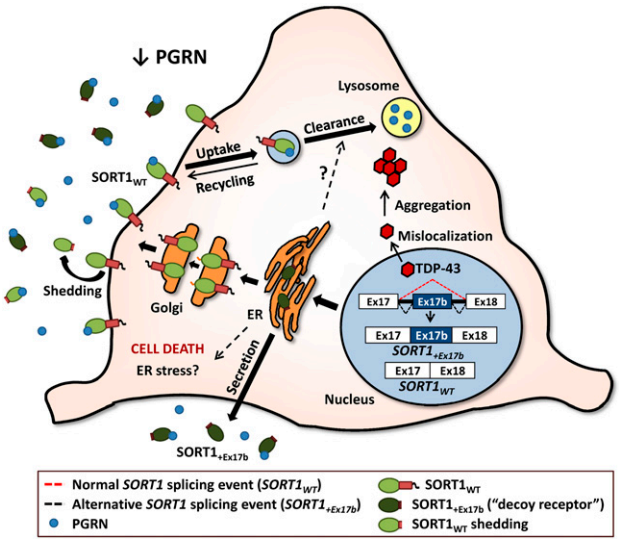

Fig. 7. Theoretical model illustrating how misregulation of human *SORT1* splicing affects PGRN levels. The absence of functional nuclear TDP-43 leads to $SORT1_{WT}$ up-regulation and generation of $SORT1_{+Ex17b}$. Both SORT1 isoforms are expected to reduce the pool of extracellular PGRN: $SORT1_{WT}$ promotes PGRN uptake, whereas extracellularly released $SORT1_{+Ex17b}$ acts as a "decoy receptor" for PGRN.

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In the absence of TDP-43, the generation of human $SORT1_{+Ex17b}$ is less pronounced than observed for mouse $Sort1_{+Ex17b}$, suggesting that human Ex17b inclusion is more tightly regulated. Indeed, we identified enhancer splicing elements within the 5′ splice site of the mouse, but not human, Ex17b sequence allowing more efficient binding of proteins that enhance splicing.

Bioinformatic analysis of the Ex17b sequence across species reveals a high degree of similarity among mammalian Ex17b sequences. Nonetheless, in humans and other primates, a nucleotide mismatch introduces an in-frame stop codon in *SORT1*. We observed that, when the stop codon is present, the 5′ splice site enhancer sequence, found in mice and other rodents, is absent. It would thus appear that evolutionary changes have occurred to repress Ex17b inclusion in those cases where it encodes a premature stop codon. The differences in sortilin splicing between mice and humans, and the subsequent functional implications of these isoforms, emphasizes the need for caution when studying human diseases/biology in nonhuman models.

The differential regulation between mouse and human sortilin may have pathological significance. Indeed, while mouse $Sort1_{+Ex17b}$ can mediate PGRN endocytosis, human $SORT1_{+Ex17b}$ is truncated and binds, but does not internalize, PGRN. Despite the tight splicing regulation of human *SORT1*, we demonstrate that levels of $SORT1_{+Ex17b}$ are significantly higher in the temporal cortex of FTLD-TDP cases compared with normal controls, presumably because of loss of functional TDP-43. Tissues lacking TDP-43 pathology, such as cerebellum in FTLD-TDP cases or brain in FTLD-Tau cases, do not present altered *SORT1* splicing.

Although *GRN* mutations lead to *GRN* haploinsufficiency in FTLD-TDP cases, the mechanism of how loss of PGRN leads to TDP-43 neuropathology remains unknown. A possible explanation is that loss of PGRN reduces chronic neurotrophic support for neurons and that compromised PGRN-mediated cell signaling leads to TDP-43 mislocalization and accumulation. The adverse relationship between loss of PGRN and TDP-43 may be reciprocal, because our data suggest that loss of nuclear TDP-43 function results in enhanced human $SORT1_{+Ex17b}$ expression, which may consequently contribute to an imbalance in extracellular vs. intracellular PGRN (Fig. 7). $SORT1_{+Ex17b}$ proteins, mainly found extracellularly, may compete with $SORT1_{WT}$ and other PGRN receptors to prevent PGRN signaling, essentially acting as a "decoy receptor" (Fig. 7). $SORT1_{WT}$ is also up-regulated in FTLD-TDP cases and TDP-43–depleted human cells, likely as a result of the "decoy effect" of $SORT1_{+Ex17b}$ proteins, and its increased expression may result in enhanced PGRN endocytosis and degradation, further reducing the pool of extracellular PGRN (Fig. 7). This may have detrimental consequences given the importance of tightly regulated PGRN levels in the brain. Overall, our studies provide valuable insight into how changes in *SORT1* splicing may affect SORT1 regulation of PGRN in the brain. Future studies should be directed toward improving our understanding of how these unfavorable *SORT1* splicing events may affect the diverse biological pathways in which SORT1 plays a role, which range from neurotrophic signaling to cholesterol and glucose metabolism.

Materials and Methods

Detailed materials and methods can be found in *SI Materials and Methods*. Procedures involving animals were performed in accordance with the Mayo Institutional Animal Care and Use Committee. Statistical differences were assessed by using GraphPad PRISM 4.0. Data on graphs is presented as mean ± SEM. The specific tests used are provided in the figure legends. Transfections were performed with Fugene6 (Roche) or Lipofectamine 2000 (Invitrogen), and knockdowns were performed using siLentfect (Bio-Rad), following the manufacturer's protocols. For cells and mouse tissues, RNA was obtained using TRIzol (Invitrogen). For human tissues, RNA was extracted using the RNeasy Plus Micro Kit (Qiagen). RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit and qRT-PCR was performed using an HT7900 analyzer (Applied Biosystems).

ACKNOWLEDGMENTS. This work was supported by the Mayo Clinic Foundation (L.P.); National Institutes of Health/National Institute of Neurological Disorders and Stroke Grants R01 NS 063964-01 (to L.P.) and R01 NS077402 (to L.P.); National Institute on Aging Grant R01 AG026251-03A2-1 (to L.P.); the ALS Association (L.P.); ALS Association Grant 771DOO (to M.P.); and Department of Defense Grants W81XWH-10-1-0512-1 (to L.P.) and W81XWH-09-1-0315AL093108 (to L.P.).

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