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Parkinson's disease–related gene variants influence pre-mRNA splicing processes

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ABSTRACT

We have analyzed the impact of Parkinson's disease (PD)–related genetic variants on splicing using dedicated minigene assays. Out of 14 putative splicing variants in 5 genes (*PINK1*, [PTEN induced kinase 1]; *LRPPRC*, [leucine-rich pentatricopeptide repeat containing protein]; *TFAM*, [mitochondrial transcription factor A]; *PARK2, [parkin RBR E3 ubiquitin protein ligase]*; and *HSPA9, [heat shock protein family A (Hsp70) member 9]*) 4 *LRPPRC* variants, (IVS32−3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G) influenced pre-messenger RNA splicing by modulating the inclusion of the respective exons. In addition, 1-Methyl-4-phenylpyridinium ion–induced splicing changes of endogenous *LRPPRC* messenger RNA, reproduced the effect of the *LRPPRC* IVS35+14C>T mutation. Using silencing and overexpression methods, we show that *LRPPRC* exon 33 splicing is negatively regulated by heterogeneous nuclear ribonucleoprotein A1 both in a minigene and endogenous context. Furthermore, exon 33 exclusion due to PD-associated mutation IVS32−3C>T or heterogeneous nuclear ribonucleoprotein A1 overexpression and exon 35 exclusion due to IVS35+14C>T can be rescued by co-expression of modified U1 small nuclear RNAs, providing a potentially useful therapeutic strategy. Our results indicate for the first time that *LRPPRC* intronic variants can affect normal splicing of this gene and may influence disease risk in PD and related disorders.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, defined by cardinal motor features: tremor, rigidity, bradykinesia, and postural instability (Samii et al., 2004). Both genetic and environmental factors are thought to contribute to its pathogenesis (Polito et al., 2016). To date approximately 10% of PD cases arise from mutations in known genes, whereas the remaining cases are idiopathic. Although many variants within splicing regions have been identified in PD patients, very rarely their function has been addressed (Cruts et al., 2012; Samaranch et al., 2010). In this article we analyze functionally selected intronic or synonymous exonic variants identified in PD patients in genes coding for PTEN induced kinase 1 (*PINK1*), heat shock protein family A (Hsp70) member 9 (*HSPA9*), leucine-rich pentatricopeptide repeat containing protein (*LRPPRC*), mitochondrial transcription factor A (*TFAM*), and parkin RBR E3 ubiquitin protein ligase (*PARK2*) (Biswas et al., 2010; De Mena et al., 2009; Gaweda-Walerych et al., 2010; Rakovic et al., 2011; Samaranch et al., 2010; Valente et al., 2004; Zou et al., 2004). All the analyzed variants encode proteins related to mitochondrial maintenance. Mitochondrial dysfunction has been implicated in both sporadic and genetically caused PD, as reviewed by (Gaweda-Walerych and Zekanowski, 2013b). More specifically, PINK1 along with parkin are crucial for mitochondrial biogenesis, transport, and dynamics, whereas TFAM is responsible for mitochondrial DNA (mtDNA) replication, transcription, and packaging, as reviewed by (Gaweda-Walerych and Zekanowski, 2013a). *HSPA9*-encoded mortalin aids in (re)folding of mitochondrial polypeptides (Deocaris et al., 2006). Its levels are downregulated in PD brains, animal and cellular PD models (Chiasserini et al., 2011; Jin et al., 2006; Shi et al., 2008) and correlate with PD progression (Shi et al., 2008).

The LRPPRC localizes predominantly to mitochondria where it stabilizes mitochondrial messenger RNAs (mRNAs), promotes their polyadenylation, and coordinates mitochondrial translation (Chujo et al., 2012; Mili and Pinol-Roma, 2003; Ruzzenente et al., 2012). Mutations in *LRPPRC* cause the French-Canadian type of Leigh syndrome (LSFC), an autosomal, recessive, neurodegenerative disease with the onset in infancy (Mootha et al., 2003) and presence of parkinsonian features (Cooper et al., 2006). At the molecular level, PD and LSFC share deficiency of mitochondrial complex I and IV (Mootha et al., 2003; Mourier et al., 2014; Sasarman et al., 2015; Xu et al., 2004). For this reason, Rakovic et al. sequenced all the 38 exons

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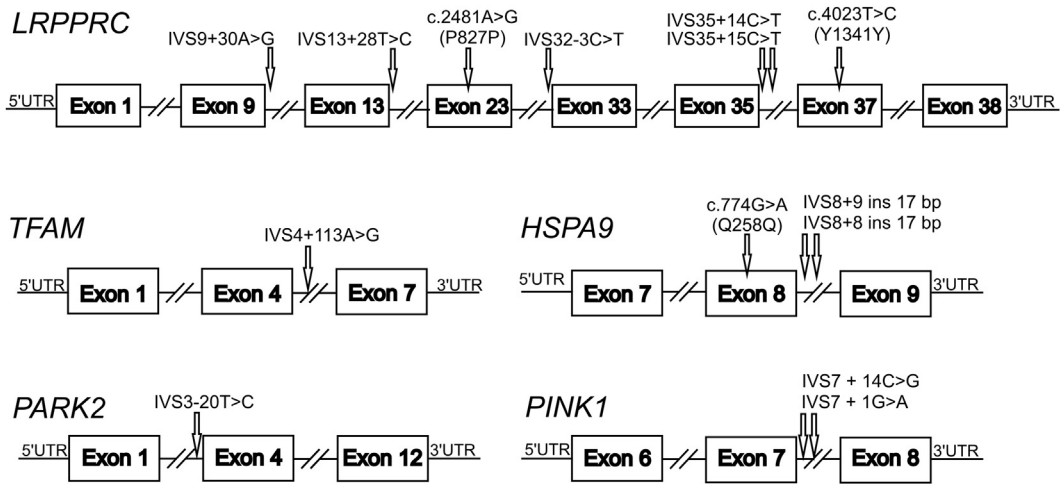

Fig. 1. Schematic representation of the analyzed variants reported in PD patients in the 5 PD-related genes, *LRPPRC, TFAM, HSPA9, PARK2,* and *PINK1*. Exonic sequences are boxed, introns are lines, whereas the arrows indicate the position of genomic variants relative to the splice sites. Abbreviations: *HSPA9*, heat shock protein family A (Hsp70) member 9; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; *PARK2*, parkin RBR E3 ubiquitin protein ligase; *PINK1*, PTEN induced kinase 1; *TFAM*, mitochondrial transcription factor A.

with flanking intronic regions of *LRPPRC* gene in 46 PD patients, revealing over 20 variants (Rakovic et al., 2011) from which we have chosen 7 for further analysis in minigene assay (Fig. 1).

Proteomic analyses showed that mortalin, LRPPRC, and TFAM interact with some of the well-known PD-related proteins such as PINK1, parkin, and DJ-1, indicating that they operate within the same molecular pathway, as reviewed by (Gaweda-Walerych and Zekanowski, 2013b). Thus, it is possible that variants influencing splicing in the previously mentioned genes could ultimately lead to deregulation of various mitochondrial parameters and contribute in this way to PD pathogenesis.

All the variants analyzed in this article can potentially change splicing regulatory sequences such as 5′ and 3′ splice site (5′ss, 3′ss, respectively) or exonic splicing enhancers and silencers leading to aberrant splicing pattern. Our goal was to determine, using minigene assays, whether these mutations influence pre-mRNA processing, for example, through increased exclusion of the analyzed exon or creation of aberrant mRNA isoforms (Baralle and Baralle, 2005; Baralle et al., 2009). Such events could lead to changed mRNA or protein properties or even result in protein depletion.

Our second aim was to pinpoint major regulatory-splicing factors regulating splicing of the exons influenced by the analyzed mutations, through silencing and overexpression assays.

Finally, we also outline possible new therapeutic venues for rescuing mutation-related aberrant splicing through the expression of adapted U1 small nuclear RNAs (U1 snRNAs) (Fernandez Alanis et al., 2012). The U1 snRNAs along with specific U1 proteins form the U1 small nuclear ribonucleoprotein complex crucial for recognizing the 5′ splice site during the first step of the splicing reaction. It is the 5′tail of U1 snRNA that interacts with the 5′ss through its 9-bp tail and recruits the spliceosome machinery on the exon (Aebi et al., 1986). Recently, a novel strategy to correct exon skipping has been developed based on adapted U1 snRNAs with a 5′ tail sequence modified so that it is 100% complementary to exon-intron boundary or intronic sequences downstream of canonical donor splice site (Fernandez Alanis et al., 2012). Their overexpression in vitro can successfully correct not only 5′ss mutations where wt U1snRNAs naturally bind but also 3′ acceptor splice site mutations (Fernandez Alanis et al., 2012).

2. Materials and methods

2.1. Selection of the variants for the analysis

The variants have been selected based on their association with PD risk (association studies, mutational screening), the lack of detailed functional analysis of their potential influence on splicing (except for *PINK1* IVS7+1G>A for which we try rescue approach), and they have usually higher frequency than in control groups or database (Biswas et al., 2010; Cruts et al., 2012; De Mena et al., 2009; Gaweda-Walerych et al., 2010; Rakovic et al., 2011; Valente et al., 2004). For bioinformatic analysis of splicing impact, we used Human Splicing Finder (Desmet et al., 2009) and Clone Suite Manager v. 9.0.

2.2. Hybrid minigene and U1 snRNA expression constructs

In the case of each wild-type (WT) minigene construct, the appropriate exon with circa 200 bp of flanking intronic sequences was amplified from human DNA using primers with NdeI restriction sites (Table S1). Polymerase chain reaction (PCR) products were passed through Sephacryl S-400 HR gel filtration media (GE Healthcare Life Sciences), cloned blunt end into pUC19 plasmid and subsequently excised and subcloned into pTB plasmid via NdeI restriction site (Baralle and Baralle, 2005). Respective mutations were introduced into WT pTB plasmids by site-directed mutagenesis using specific primers (Table S2). Exon-specific U1 snRNAs were created by site-directed mutagenesis reaction (primers are enlisted in Table S3) on a vector that includes the sequence coding for human snRNA U1 (*RNU1-1*, NCBI ref seq NC_000001.11), kindly provided by Dr F. Pagani (Fernandez Alanis et al., 2012; Pagani et al., 2002). The expected sequence changes for all constructs were confirmed by sequencing.

2.3. Cell culture, transient transfection, silencing, RNA extraction, reverse transcription, and PCR

Hela, HEK293T, and SH-SY5Y cells were grown in standard conditions described previously (Mohagheghi, 2016). Hela and SH-SY5Y cells were treated with 2.5 mM 1-Methyl-4-phenylpyridinium iodide (MPP+) (Sigma), dissolved in standard medium, for 24 hours. For minigene transfection, 0.5–2 μg of pTB constructs (WT or

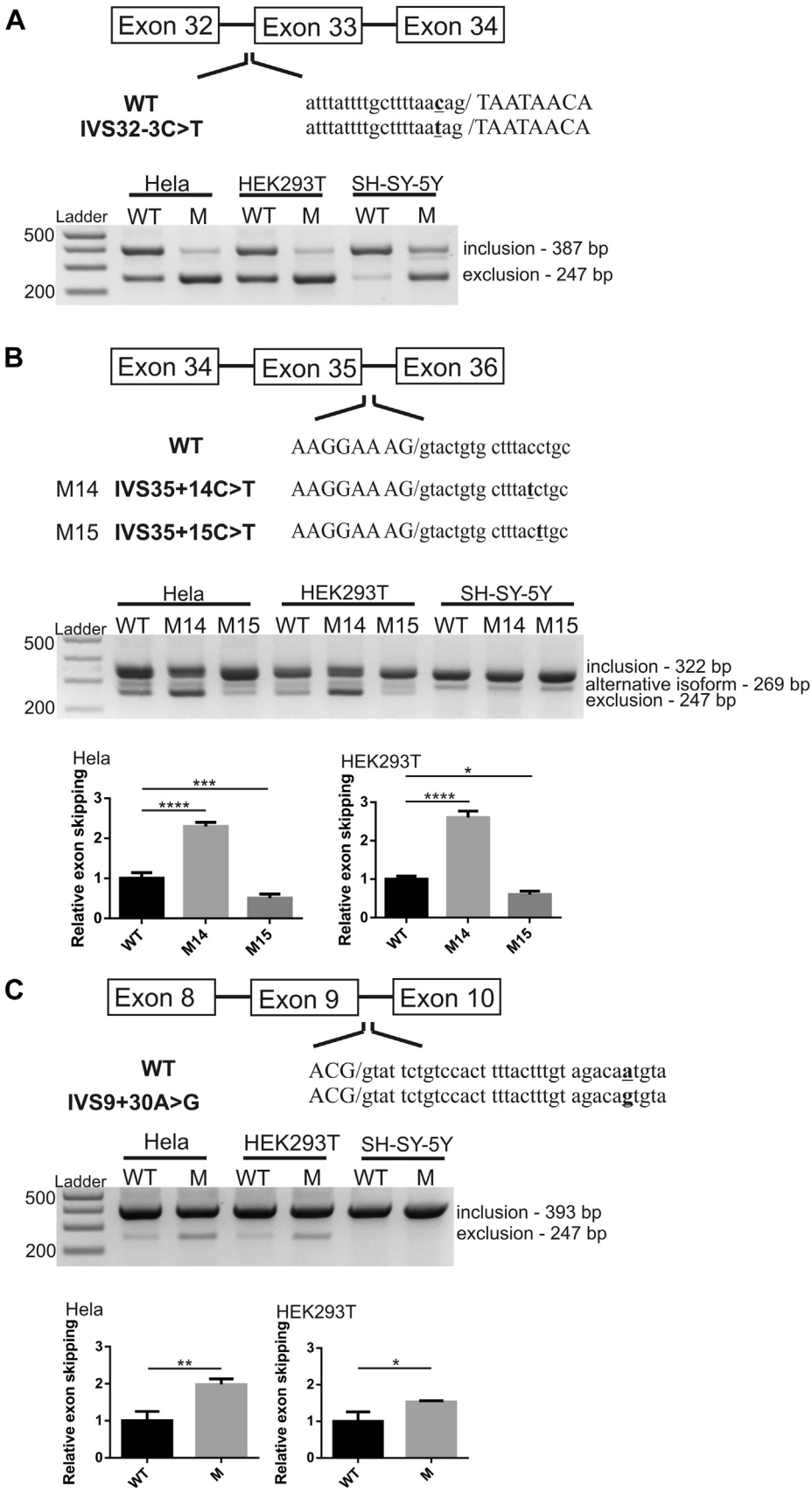

Fig. 2. *LRPPRC* intronic variants (IVS32−3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G) influence splicing in minigene system. In upper panels (A), (B), and (C), there is a schematic representation of *LRPPRC* regions affected by splicing mutations. Exonic and intronic sequences are in upper and lower case, respectively. Lower (A), middle (B), and middle (C) panels represent RT-PCR analysis of *LRPPRC* mRNA splicing pattern 24 hours after transfection of WT and MUT minigenes (IVS32−3C>T, IVS35+14C>T, IVS35+15C>T, and

mutated [MUT]) were transfected with Effectene (QIAGEN) or Lipofectamine 2000 (Invitrogen). For co-expression experiments, 0.25–2 μg of each plasmid were co-transfected (Dardis et al., 2014). When required, the amount of DNA was adjusted with the respective empty vectors or pEGFP-C1 vector. Each transfection experiment was performed at least 3 times, and a representative gel is shown. For silencing, Hela or SH-SY5Y cells were transfected with appropriate siRNA for 2 consecutive days. Then, 6 hours after the second silencing, WT or mutated (MUT) minigenes were transfected. RNA and proteins were collected 24 hours after minigene transfection. Reverse transcription PCR (RT-PCR) analysis of minigene-derived mRNA was done with primers alpha23 and Bra2 (Table S4). RNA extraction (Eurogold Trifast, Euroclone), reverse transcription, PCR with pTB-specific primers (Table S4) or primers for endogenous *LRPPRC* mRNA (Table S5), were performed by standard protocols (Mohagheghi et al., 2016). siRNA sense sequences used for silencing the expression of luciferase (control), human heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), human hnRNPA2 (Darmacon); human SRSF1 (Thermo Scientific) are enlisted in Table S6.

2.4. Inhibition of nonsense-mediated decay

Nonsense-mediated decay (NMD) inhibition was incurred through administration of 50 μg/ml of cycloheximide (CHX) for 3 hours before the collection of RNA after 24 hours and verified by RT-PCR of Down syndrome critical region 1 (*DSCR1*) mRNA. GAPDH expression was used as a reference (Table S7).

2.5. Western blot

Western blot was performed as previously described (Mohagheghi et al., 2016). Proteins were probed with primary antibodies against: hnRNPA1, hnRNPA2 (already available in-house), ASF/SF2 (SRSF1) (anti-mouse, Invitrogen) LRPPRC (anti-rabbit, GeneTex Inc), and housekeeping p84 (anti-mouse, Abcam) or tubulin (anti-mouse, Sigma).

2.6. Real-time PCR

For minigene assays, exon exclusion was measured by quantitative real-time PCR using SYBR Green I Master (Roche) and primers complementary exclusively to vector-derived hybrid complementary DNA without the insert (Table S8), using a Biorad Real-Time PCR System, as per manufacturer's instructions. Changes in gene expression were determined with the ΔCt method using alpha-hemoglobin for normalization (Table S8).

2.7. Statistical analysis

For quantitative densitometric analysis of splicing pattern on agarose gels we used ImageJ software (Schneider et al., 2012). For each band in a gel line, ImageJ assigns intensity value. These values obtained from different biological repeats of the same experiment ($n \geq 3$) were used to calculate the mean percentage of inclusion or exclusion of the analyzed exons. The percentage values obtained from different biological replicates were used to calculate standard deviations and statistical significance (*t*-test and 1-way ANOVA test—posttest Tukey, GraphPad Prism 6.0). $p < 0.05$ was considered significant. For densitometric analysis of WB results, intensity value of each LRPPRC band was normalized to respective p84 value, then mean, standard deviation, and statistical significance values were calculated based on the individual ones ($n \geq 3$).

3. Results

A schematic diagram of all the variants analyzed in this study is shown in Fig. 1. Each variant was analyzed by preparing a suitable minigene as described in Section 2. Minigenes were then transfected in human cervical cancer-derived Hela, embryonic kidney HEK293T, and neuroblastoma SH-SY5Y cells to highlight any tissue-specific differences. In all cases, spliced products were detected using minigene-specific primers and RT-PCR. Spliced products were sequenced to confirm their identity.

3.1. LRPPRC *variants*

In the *LRPPRC* gene, (Fig. 1) 4 variants were found to alter the RNA splicing pattern in the minigene assay: IVS32−3C>T, IVS35+14C>T, IVS35+15C>T, and IVS9+30A>G (Fig. 2).

Intron 32 variant (IVS32−3C>T, c.3492−3C>T, rs35113761) has been found in 3 out of 46 patients with PD, a frequency almost 4 times higher than the one reported in 1000G (1000 Genomes project) CEU (Utah residents of northern and western European ancestry) database (0.065 vs. 0.017) (Lill et al., 2012; Rakovic et al., 2011). This substitution, localized within the 3'ss, results in almost complete skipping of exon 33 in all 3 tested cell lines (Fig. 2A). Bioinformatic analysis (reference mRNA NM_133259.3) indicated that skipping of exon 33 would result in frameshift of the *LRPPRC* ORF with the introduction of a premature stop codon likely leading either to NMD or the expression of a truncated protein (1204 aa) (Fig. S1C).

The 2 adjacent variants in intron 35 IVS35+14C>T (c.3900+14C>T, rs3795859), and IVS35+15C>T (c.3900+15C>T, rs76850904) were found in PD patients/1000G CEU database with frequency of 0.15/0.075 and 0.086/0.092, respectively (Rakovic et al., 2011) (Lill et al., 2012). IVS35+14C>T variant led to increased exclusion of exon 35, whereas IVS35+15C>T, on the contrary, caused decreased exclusion compared with WT minigene (Fig. 2B). Real-time PCR with primers designed to measure exclusion in minigene plasmid showed that IVS35+14C>T resulted in 2.3 and 2.6 higher exclusion of exon 35 compared to WT minigene, in Hela and HEK293T cells, respectively, (Fig. 2B, lower panel). On the contrary, IVS35+15C>T led to lower relative exclusion levels, 0.5 and 0.6 in Hela and HEK293T cells, respectively, (Fig. 2B, lower panel). Interestingly, in SH-SY5Y cells in minigene system, we observed a predominant band corresponding to exon inclusion (322 bp) but also a lower band (269 bp) corresponding to alternative isoform lacking the first 53 bp of exon 35, confirmed by sequencing (Fig. S1E). Bioinformatic analysis showed that lack of the first 53 bp of exon 35 results in a frameshift and introduction of a premature stop codon (TAG at 3890-92 bp) possibly resulting in NMD or truncated protein (1278 aa) (Fig. S1E). On the other hand, it is worth noting that the entire exon 35 deletion would lead to shorter protein lacking 25 aa (1369 aa) without any frame change

IVS9+30A>G, respectively) in Hela, HEK293 T or SH-SY5Y cells. Lower (B) and (C) panels represent real-time PCR analysis with primers specifically measuring the levels of exclusion in minigene system. IVS35+14C>T (M14) causes increase in exon exclusion (2.3 and 2.6 times), whereas IVS35+15C>T (M15) lowers exclusion levels by about half in comparison to IVS35WT, in Hela and HEK293T cells, respectively. IVS9+30A>G (M) presence led to 1.98 and 1.53 times higher skipping of exon 9 than WT minigene (WT), in Hela and HEK293T cells, respectively (C, lower panel). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Abbreviations: *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

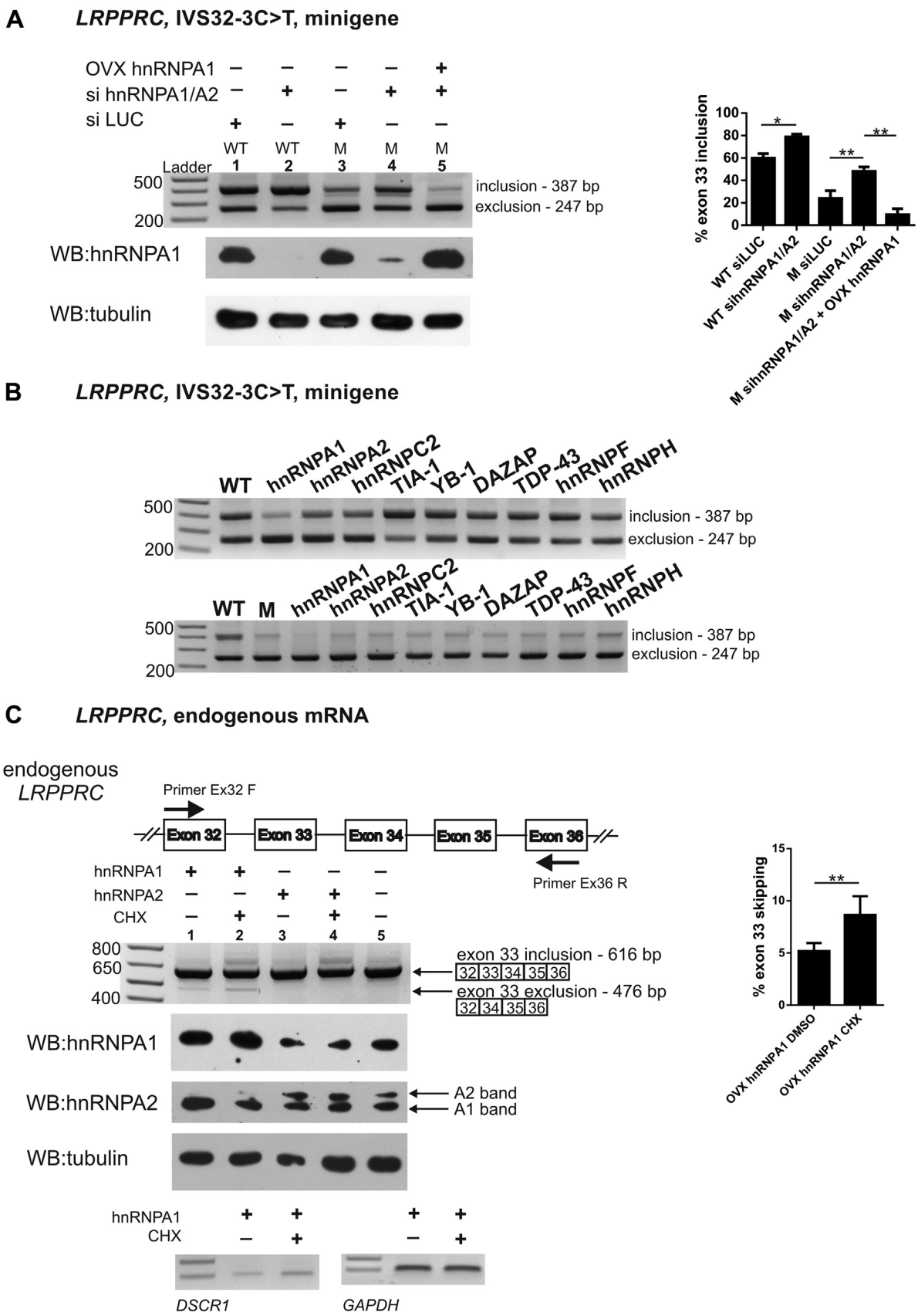

Fig. 3. Negative regulation of *LRPPRC* exon 33 by hnRNPA1 in minigene and endogenous context. (A) Silencing of endogenous hnRNPA1/A2 rescues exon 33 skipping in MUT minigene carrying IVS32−3C>T. WT minigenes (lane 1, 2) and MUT minigenes (lanes 3, 4, 5) were transfected in HeLa cells in the presence of siLUC (lanes 1, 3) or si hnRNP A1/A2 (lanes 2, 4, 5). In addition, overexpression of hnRNP A1 was performed in lane 5. Right panel shows the percentage of exon 33 inclusion determined densitometrically. $^{*}p < 0.05$, $^{**}p < 0.01$. Left middle and lower panels represent Western blot for hnRNPA1 protein and tubulin, respectively. (B) Overexpression of hnRNPA1 increases exclusion of exon 33 in WT and

(Fig. S1D). Interestingly, NCBI database annotates *LRPPRC* transcript variant X1 (NCBI: XM_011532473.1) with exon 35 skipped, which suggests this exon is alternatively spliced; however, the existence of the truncated protein has been predicted merely bioinformatically (NCBI: XP_011530775.1).

Finally IVS9+30A>G (c.1077+30A>G, rs7593842) in *LRPPRC* resulted in exon 9 skipping (Fig. 2C), 1.98 and 1.53 times higher than in WT minigene, in Hela and HEK293T cells, respectively, as demonstrated with real-time PCR (Fig. 2C, lower panel). According to bioinformatic analysis, skipping of exon 9 would result in a frameshift change and the introduction of a premature stop codon (TAA, at nucleotide residue 1094–96) likely leading to NMD-mediated degradation of the mRNA or the expression of a truncated protein (346 aa) (Fig. S1B).

The other *LRPPRC* variants analyzed: IVS13+28T>C (c.1582+28T>C, rs62135104); exon 23 c.2481A>G, (P827P, rs115993634); exon 37 c.4023T>C, (Y1341Y) did not have any effect on splicing in our minigene system and were not further investigated (Fig. S2).

3.2. Functional investigation and rescue of the LRPPRC IVS32−3C>T variant

Despite of the strong effect of acceptor IVS32−3C>T mutation (almost complete skipping of exon 33 in the minigene system) (Fig. 2A), splicing prediction programs failed to detect a highly significant change in the strength of acceptor splice site following this substitution (HSF score: WT = 84.77, MUT = 77.09; MaxEnt score: WT = 6.92, MUT = 5.26). Thus, we reasoned that in addition to acting on 3′ss strength, the −3C>T substitution may have created or affected an existing splicing regulatory element. One possibility according to HSF prediction is that IVS32−3C>T may have created a new hnRNPA1 site. It can be envisaged that hnRNPA1 binding to this position could compete with U2AF35 binding to the acceptor site. To test this hypothesis, we silenced hnRNPA1 and hnRNPA2 (hnRNPA1/A2). Although we recovered exon 33 recognition to WT level (Fig. 3A, lane 4), we also observed improved exon recognition in the WT minigene (Fig. 3A, lane 2). In case of the mutated minigene, the increased inclusion was reversed on simultaneous coexpression of si-resistant hnRNPA1 after hnRNPA1/A2 silencing (Fig. 3A, lane 5). Silencing results (Fig. 3A) are in agreement with the results of overexpression of different hnRNPs where we observed that only hnRNPA1 decreased exon 33 inclusion in both WT and MUT minigene (Fig. 3B). Finally, a very similar rescue effect on WT and MUT minigene on silencing of hnRNPA1/A2 could also be observed in the SH-SY5Y cell line, indicating that the negative regulation by hnRNPA1 is common to different tissues (data not shown).

These results suggest that in addition to the putative motif created by IVS32−3C>T there are other hnRNPA1-binding sites already present either within exonic or flanking intronic sequences contributing to the control of exon 33 splicing. Because of the negative regulation of exon 33 inclusion by hnRNPA1 in minigene context (Fig. 3A and B), we further tested if hnRNPA1 could also influence endogenous *LRPPRC* mRNA splicing. Only on overexpression of hnRNPA1, but not other hnRNPs or SRs, for example, hnRNPA2 (or TDP-43, TIA-1, SRSF1, not shown), an additional band appeared corresponding to an isoform with exon 33 skipped, confirmed by sequencing of PCR product (Fig. 3C, lane 1, 2). The isoform underwent degradation by NMD as its expression levels increased following treatment with CHX (Fig. 3C, lane 1, 2). As a positive control for NMD, we used the *DSCR1* gene, a known NMD target (Mendell et al., 2004; Wittmann et al., 2006) and the *GAPDH* gene as a control for equal complementary DNA input (Fig. 3C, lower panel).

Based on the involvement of hnRNPA1 in exon 33 splicing regulation, we decided to evaluate different therapeutic strategies to correct the effects of this mutation (Buratti et al., 2006). One of them is overexpression of SR proteins that usually promote exon inclusion and act as general antagonists of hnRNPs repressor functions. To investigate this possibility, we overexpressed several of the major SR protein family members (SRSF1 to SRSF6, and SRSF9) in the presence of the minigene carrying the IVS32−3C>T substitution. However, none of the tested SR proteins was able to increase exon 33 inclusion in MUT minigene (Fig. S3).

An alternative therapeutic strategy would be the coexpression of adapted recombinant U1snRNA that could improve the recognition of the donor site of exon 33 in the presence of the IVS32−3C>T substitution. To achieve this, we designed 3 modified U1 snRNAs either fully complementary to 5′ss donor site (U1LRP33wt) or to downstream intronic positions +8 (U1LRP33+8) and +12 (U1LRP33+12) in intron 33 as indicated in Fig. 4A. Hela cells were then cotransfected with WT or MUT minigene and the various U1 constructs (Fig. 4B). As expected, the overexpression of WT U1snRNA did not have any effect on WT and MUT minigene (Fig. 4B, line 2, 7). Overexpression of (1) U1LRP33wt; and (2) U1LRP33+12 successfully rescued defective splicing in the mutated minigene to WT levels (Fig. 4B, lines 8, 10) whereas U1LRP33+8 gave only partial rescue (Fig. 4B, line 9). Likewise, all the adapted U1snRNAs improved inclusion of exon 33 in WT minigene, with similar strength as observed for MUT minigene (Fig. 4B, lines 3, 4, 5). In addition, co-expression of U1LRP33+12 was able to reverse the effects of hnRNPA1 overexpression in minigene system (Fig. 4C). Most importantly, overexpressing of hnRNPA1 in Hela cells along with U1LRP33+12 in the endogenous context caused the disappearance of the lower band corresponding to the *LRPPRC*-splicing isoform lacking exon 33 (476 bp) (Fig. 4D, lane 3, 4). This suggests that the U1snRNA constructs can overcome the effects of eventual hnRNP A1 overexpression as well as the presence of the acceptor site mutation, IVS32−3C>T.

3.3. Functional investigation and rescue of the LRPPRC IVS35+14C>T mutation

In parallel with the *LRPPRC* IVS32−3C>T rescue experiments, we also attempted to correct the effects of the *LRPPRC* IVS35+14C>T mutation.

MUT minigenes. Panels represent RT-PCR analysis of minigene-derived mRNA. Hela cells were cotransfected with vectors expressing different hnRNPs and either WT (upper panel) or MUT minigene constructs (lower panel). (C) Overexpression of hnRNPA1 causes skipping of exon 33 in endogenous *LRPPRC* mRNA. Vectors expressing hnRNPA1 (lane 1, 2) or hnRNPA2 (lane 3, 4) or control vector were transfected in Hela cells. Cells were treated with dimethyl sulfoxide (DMSO) (line 1, 3, 5) or cycloheximide (CHX) (line 2, 4). Upper left panel represents fragment of *LRPPRC* pre-mRNA (exonic sequences are boxed, intronic sequences represented by lines) amplified with primers Ex32F and Ex36R (indicated by the arrows), specific to endogenous mRNA. Middle left panel shows RT-PCR analysis of endogenous *LRPPRC* mRNA. Skipping of exon 33 produces a band shorter by 140 bp, indicated by the arrow. Right panel shows percentage of exon 33 exclusion determined densitometrically; $^{**}p < 0.01$. hnRNPA1 and hnRNPA2 protein levels were monitored by Western blot, whereas tubulin was used as a protein level control. Membrane was first blotted with antibody against hnRNPA1 (upper WB panel). Because membrane was not stripped before blotting with antibody against hnRNPA2, the middle WB panel shows both the expression of hnRNPA2 (upper band) and hnRNPA1 (lower band). hnRNPA2 is overexpressed in the lane 3, compared to the normal levels in the lane 5—taking into consideration lower tubulin signals in lane 3 versus lane 5. As a positive control for NMD, the Down syndrome critical region 1 (*DSCR1*) gene was used, as a control for even complementary DNA input in RT-PCR *GAPDH* gene was used (lower left and right panel, respectively). Abbreviations: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; NMD, nonsense-mediated decay; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

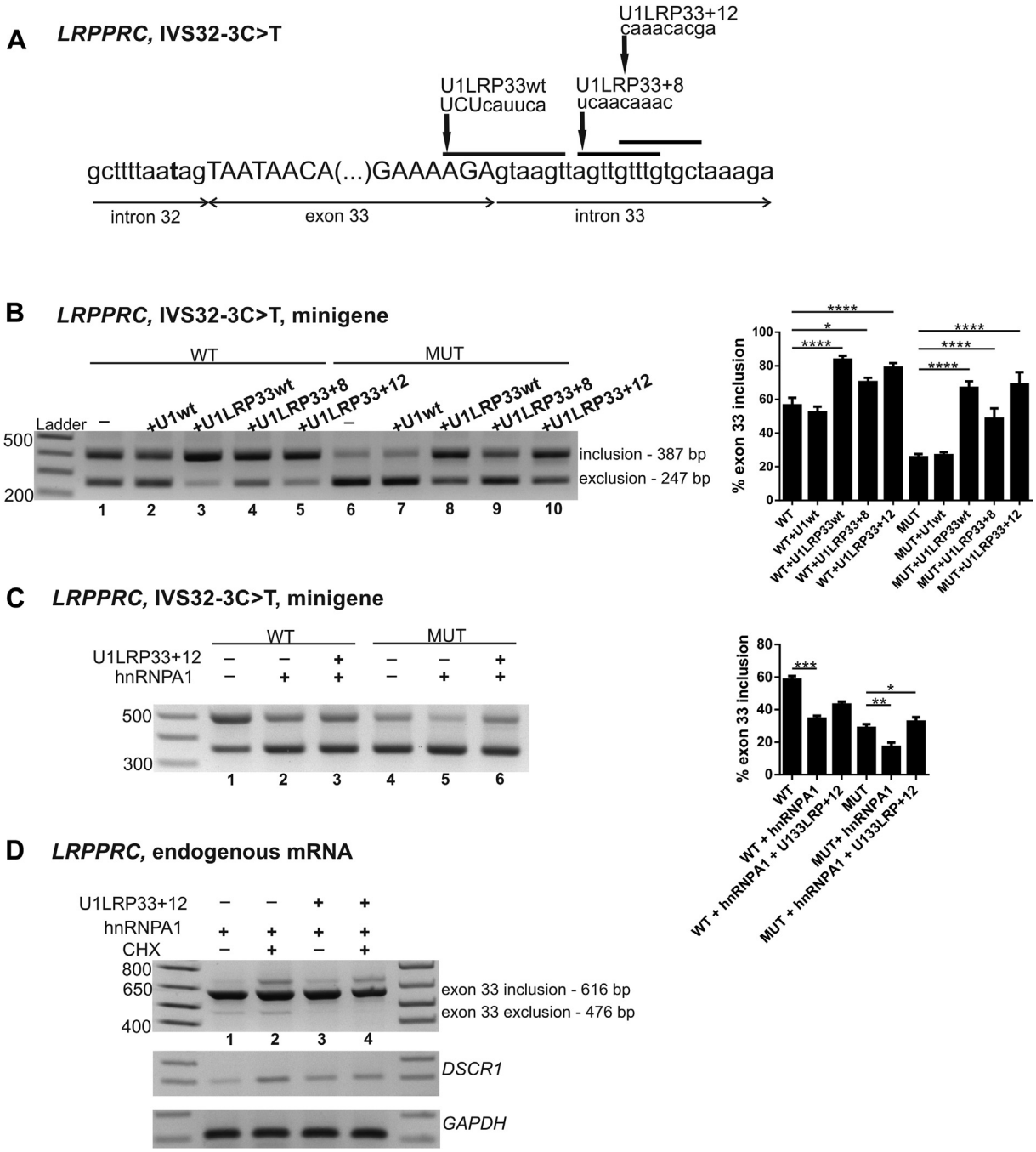

Fig. 4. Overexpression of adapted snRNAU1 complementary to the 5′ss of *LRPPRC* exon 33 can rescue both the minigene and endogenous defective splicing. (A) Schematic representation of the *LRPPRC* pre-mRNA fragment comprising exon 33 (upper case) with flanking intronic sequence (lower case). Arrows indicate the positions to which complementary snRNAU1 has been designed; U1LRP33wt, U1LRP33+8, U1LRP33+12, and IVS32−3C>T mutations are in bold; (B) RT-PCR analysis of splicing pattern in Hela cells cotransfected with WT or MUT minigene vector (carrying IVS32−3C>T mutation) and different snRNAU1 constructs (complementary to the positions shown in A). Right panel shows percentage of exon 33 inclusion determined densitometrically. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. (C) RT-PCR analysis of splicing pattern in Hela cells cotransfected with WT (line 1,2,3) or MUT minigene vector (carrying IVS32−3C>T mutation, line 4,5,6) and vector overexpressing hnRNPA1 alone or together with snRNAU1-carrying plasmid (U1LRP33+12). Right panel shows percentage of exon 33 inclusion determined densitometrically. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (D) U1LRP33+12 rescued skipping of exon 33 in endogenous context. RT-PCR analysis of the endogenous *LRPPRC* mRNA splicing pattern in Hela cells on overexpression of hnRNPA1 and cotransfection with snRNAU1-carrying vector (designated as U1LRP33+12) complementary to intronic position IVS33+12. Cells were treated with DMSO (line 1, 3) or cycloheximide (CHX) (line 2, 4) for 3 hours before collecting RNA (after 24 hours). Abbreviations: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

In search for possible auxiliary splicing factors that could regulate exon 35 inclusion, we overexpressed selected hnRNP and SR proteins (Fig. 5A and B). Among hnRNP proteins, only TIA-1 overexpression resulted in increased inclusion of exon 35 in the context of the MUT minigene (IVS35+14C>T), (Fig. 5A, lower panel). Interestingly, TIA-1 overexpression in Hela cells also increased the level of a truncated alternative isoform lacking first 53 bp of exon 35 as confirmed by sequencing (Fig. 5A, 269 bp band, indicated with an arrow; Fig. S1E).

Among the serine/arginine-rich (SR) proteins tested, overexpression of SRSF1 (ASF/SF2) and SRSF9 (SRp30c) could rescue

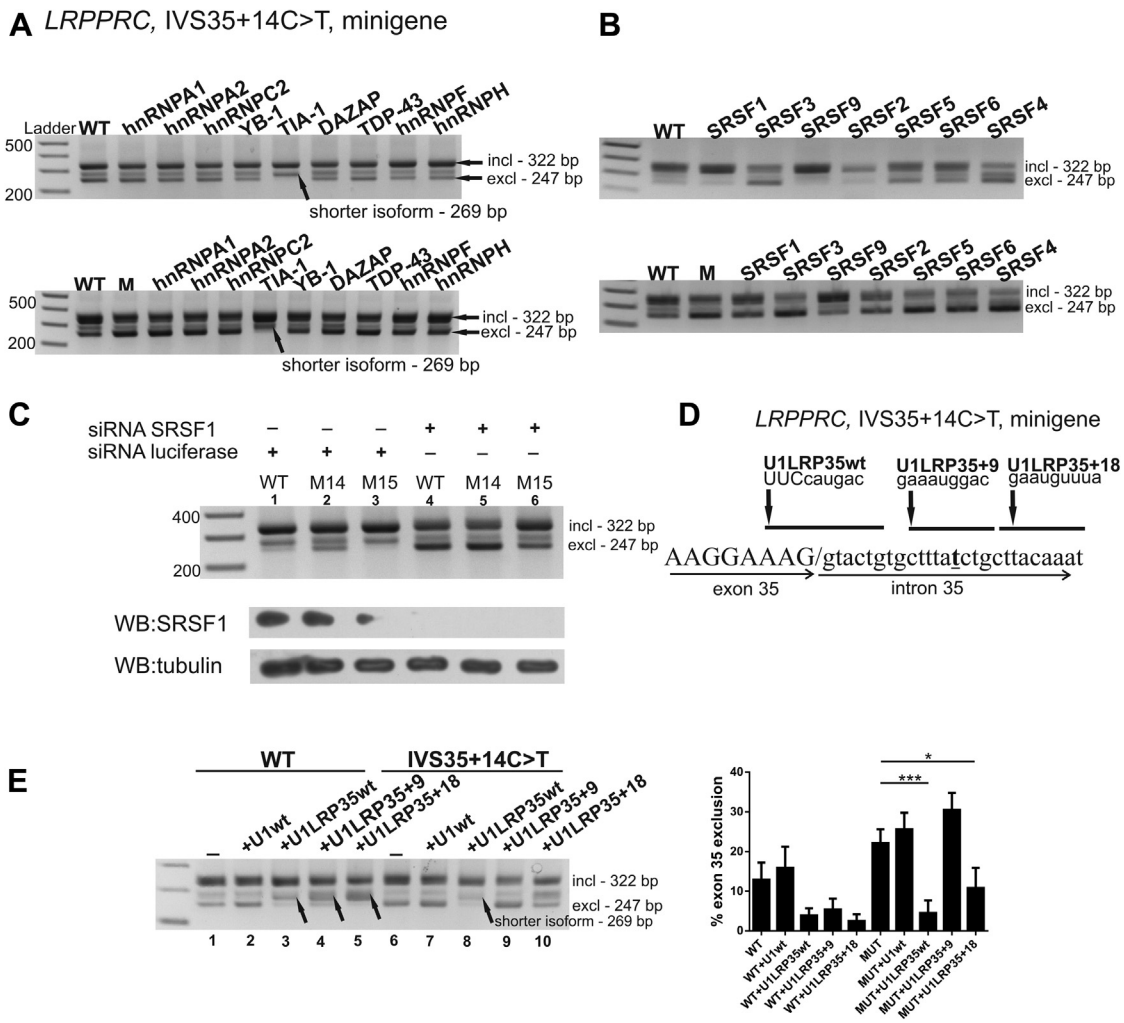

Fig. 5. Overexpression of TIA-1, SRSF1, and SRSF9 decreases exon 35 exclusion. Hela cells were cotransfected with vectors expressing different hnRNPs (A) or SR proteins (B) and either WT (upper panels) or MUT minigene constructs (lower panels). (A) Apart from increasing exon 35 inclusion, TIA-1 overexpression results in production of alternative isoform lacking first 53 bp of exon 35 (indicated by an arrow) both in the context of WT and MUT minigene; (B) overexpression of SRSF1 and SRSF9 increases exon 35 inclusion without producing the short alternative isoform; (C) Upper panel shows RT-PCR analysis of minigene-derived mRNA WT (lane 1, 4) and MUT (lane 2, 3, 5, 6) in Hela cells with silenced expression of *SRSF1* gene; SRSF1 protein level was monitored by Western blot (middle panel), whereas tubulin was used as a protein level control (lower panel); (D) Schematic representation of the *LRPPRC* pre-mRNA fragment comprising exon 35 (upper case) with flanking intronic sequence (lower case). Arrows indicate the positions to which adapted snRNAU1 has been designed, U1LRP35wt, U1LRP35+9, U1LRP33+18; (E) RT-PCR analysis of splicing pattern in Hela cells cotransfected with WT or MUT minigene vector (carrying IVS35+14C>T mutation) and different snRNAU1 constructs complementary to the positions shown in (D). Right panel shows percentage of exon 35 exclusion determined densitometrically. $^{*}p < 0.05$, $^{***}p < 0.001$. Abbreviations: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; *LRPPRC*, leucine-rich pentatricopeptide repeat containing; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

exon 35 splicing of the MUT minigene, also enhancing inclusion of WT minigene (Fig. 5B). To confirm the overexpression results, we silenced SRSF1 in Hela cells and, as expected, we noted increased exclusion for the WT, IVS35+14C>T and IVS35+15C>T minigenes compared to luciferase-silenced controls (Fig. 5C).

In addition, we observed rescue of MUT minigene IVS35+14C>T after coexpression of U1 snRNA fully complementary to 5′ss donor site (U1LRP35wt) but not to downstream intronic positions +9 and +18 (U1LRP35+9, U1LRP35+18), as indicated in Fig. 5D and E. Similarly to the case of TIA-1 overexpression, the overexpression of adapted U1 snRNAs produced also the alternative isoform containing only 22 last bp of exon 35 in addition to rescuing exon 35 splicing, (Fig. 5E, 269 bp band, indicated by arrows). In case of U1LRP35+18 overexpression, although the exclusion band diminished, the alternative band (269 bp) increased instead of WT inclusion band (322 bp) (Fig. 5E).

Taken together, these results suggest that both *LRPPRC* mutations, IVS32−3C>T. and IVS35+14C>T, may be rescued using genetic approaches that are currently being used in preclinical trials to correct splicing mutations (Rogalska et al., 2016).

3.4. MPP+ induces exon 35 skipping accompanied by decrease in LRPPRC mRNA and protein levels

Because administration of complex I inhibitor MPP+ is a well-documented model of PD pathogenesis, we wanted to assess its influence on *LRPPRC* splicing. 2.5-mM MPP+ was previously reported to cause cell death in less than 20% of SH-SY5Y cells after 24 hours (Wang et al., 2011). Indeed, both in Hela and SH-SY5Y cells, we could not see visible signs of cell death under phase-contrast microscope after MPP+ treatment (data not shown). We observed skipping of exon 35 (Fig. 6A, lines 3, 4, 7, 8) accompanied by decrease of wt isoform (Fig. 6A, compare lines 1, 5 vs. 3, 7) in Hela and SH-SY5Y cells. In Hela cells, 20% of mRNA displayed skipped exon 35. After CHX treatment, 26% of produced mRNA was WT, 16% corresponded to the isoform with exon 35 skipped, whereas 60% to

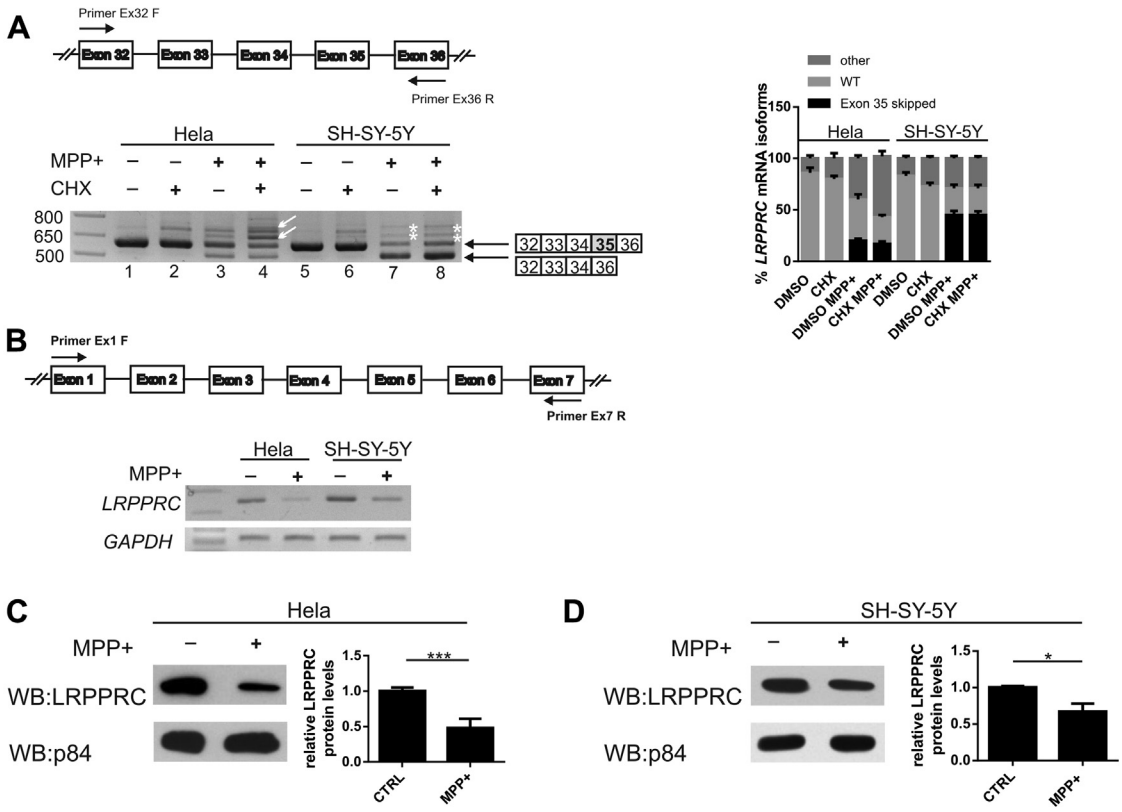

Fig. 6. MPP+ treatment causes skipping of exon 35 in endogenous *LRPPRC* mRNA and decrease in protein levels. (A) Hela and SH-SY5Y cells were treated with MPP+ (2.5 mM) for 24 hours and with DMSO (line 1, 3, 5, 7) or cycloheximide (CHX) (line 2, 4, 6, 8) for 3 hours before collecting RNA. Upper panel represents fragment of *LRPPRC* pre-mRNA amplified with primers specific to endogenous mRNA (indicated by the arrows). Lower panel shows RT-PCR analysis of endogenous *LRPPRC* mRNA. Skipping of exon 35 produces a band shorter by 75 bp, indicated by the arrow; right panel shows proportions of *LRPPRC* mRNA isoforms generated after MPP+ treatment, with inhibition of NMD by CHX, determined densitometrically; (B) Upper panel represents fragment of *LRPPRC* pre-mRNA (exonic sequences boxed, intronic—lines) amplified with primers Ex1F and Ex7R (indicated by the arrows), specific to endogenous mRNA. Lower left panel shows RT-PCR analysis of endogenous *LRPPRC* mRNA, *GAPDH* was used as even complementary DNA input control; (C) and (D) LRPPRC protein levels were monitored by Western blot, in Hela and SH-SY5Y cells, respectively, whereas p-84 was used as a protein level control. Right (C) and (D) panels show densitometric measurement of LRPPRC protein levels obtained from 3 experiments (n = 3). $^{*}p < 0.05$, $^{***}p < 0.001$. Abbreviations: hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; LRPPRC, leucine-rich pentatricopeptide repeat containing protein; MPP+, 1-Methyl-4-phenylpyridinium ion; mRNA, messenger RNA; MUT, mutated; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

other alternative isoforms (Fig. 6A, line 4, white arrows) that undergo NMD (compare Fig. 6B). In SH-SY5Y cells after MPP+ treatment, 26% of WT mRNA was produced, 45% corresponded to exon 35–skipped mRNA, and only 29% to other isoforms (Fig. 6A, line 7 and 8, white asterisks). None of these isoforms underwent NMD, as shown by the same proportions without and with CHX treatment. Total mRNA level of *LRPPRC* decreased after MPP+ treatment as shown by RT-PCR of its 5′ region (exons 1–7) (Fig. 6B). For this reason, we expected decreased LRPPRC protein levels, after treatment with MPP+, more pronounced in Hela than those in SH-SY5Y cells (also due to degradation by NMD), which, indeed, was confirmed by Western blot (Fig. 6C and D).

However, on Western blots, we could not detect 2 bands, corresponding to wt and exon 35–skipped LRPPRC (predicted difference of 2.7 kDa corresponding to 25 aa), even if it is not supposed to undergo NMD and the polyclonal antibody, theoretically, should have recognized both isoforms (the immunogen is within the N-terminal and central part of the protein) (Fig. 6B).

These results suggest that the decrease of LRPPRC protein is due to reduced levels of its mRNA associated with exon 35 skipping.

Furthermore, we checked whether MPP+-induced exon 35 skipping or decrease in LRPPRC protein could be rescued by overexpression of SRSF1, SRSF9, or TIA-1, the splicing factors that we observed to improve exon 35 inclusion in minigene system (Fig. 5A and B). None of these factors could decrease substantially (more than 10%) exon 35 mRNA skipping or increase LRPPRC protein levels (Fig. S7A and B). However, it must be also taken into account that MPP+ exerts its effect on all the cells in culture, whereas protein overexpression will have lower efficiency. Furthermore, MPP+ influences a plethora of cellular-signaling pathways in parallel: oxidative stress, DNA and protein damage, cell-cycling arrest, apoptosis, autophagy, to name a few (Dagda et al., 2013; Krug et al., 2014; Xu et al., 2005). That is why targeting only one of them might not be sufficient to obtain rescue effect.

3.5. PINK1 *variants*

PINK1 intronic mutation IVS7+1G>A (g.16378G>A; c.1488 + 1G>A) segregating with PD was detected in a large family pedigree (Samaranch et al., 2010) (Fig. 1). IVS7+1G>A was present either in a compound heterozygous (together with exon 7 deletion, c.1252_1488del) or in a homozygous state. Our minigene vectors transfected in cultured cells reproduced closely the abnormal splicing pattern observed in leukocytes of PD patients by Samaranch et al.: exon 7 skipping and truncated splicing isoform lacking 33 bp due to the use of a cryptic exonic 5′ss (Fig. S4A and B) (Samaranch et al., 2010).

Notwithstanding the G nucleotide in position +1 within 5′ss is highly conserved in humans and aberrant splicing due to its mutation is very difficult to rescue through adapted U1 snRNAs

overexpression in minigene system (Fernandez Alanis et al., 2012; Hartmann et al., 2010; Matos et al., 2014; Schmid et al., 2013), we decided to analyze if this was possible to explore potential therapeutic options. Adapted U1 snRNAs were designed to be either fully complementary to the mutated donor site or to downstream intronic positions as indicated in Fig. S4C. In keeping with previous reports (Fernandez Alanis et al., 2012; Hartmann et al., 2010; Matos et al., 2014; Schmid et al., 2013), we observed that upon co-expression of U1PINK1Ex7WT, and to lower extent U1PINK1Ex7+16, exclusion of exon 7 decreased with simultaneous increase of the alternative inclusion band corresponding to an isoform lacking 33 bp, originating from the use of a cryptic 5′ss with almost as high HSF score (90.18) as WT donor site (95.95). This is in accordance with previous observations suggesting that the ability to rescue 5′ss mutations in position +1 could depend on the strength of adjacent alternative donor sites (Matos et al., 2014). These authors noticed about 50% rescue for *HGSNAT* c.234 +1G>A mutation with traces of alternative isoform being produced, only when the alternative 5′ss had null HSF score compared with the WT one (Matos et al., 2014). So far, U1 snRNA–mediated rescue of mutations in position +1 was successful only in patient's fibroblasts, whereas in minigene system, it led exclusively to nondesired alternative splicing pattern, possibly due to the lack of endogenous regulatory context (Hartmann et al., 2010; Matos et al., 2014). Thus, our adapted U1 snRNAs should be tested in fibroblasts from PD patients to find out whether they represent a viable option to rescue this mutation or alternative strategies should be considered. It also remains to be established whether PINK1 protein lacking 11 aa might be harmful for a cell due to, for example, dominant negative effect or it still preserves some residual function. In the latter case, rescue with snRNAU1 might be the way to help patients with compound heterozygous or homozygous IVS7+1G>A mutation. In the analyzed family among 2 carriers of homozygous IVS7+1G>A mutation, one was asymptomatic, whereas the other one had PD (Samaranch et al., 2010). To clear this issue, functional analysis of truncated PINK1 protein is necessary.

Finally, with regards to *PINK1,* we also analyzed variant IVS7+14C>G (c.1488 + 14C>G) detected in 1 early-onset PD patient and absent among 200 control individuals (Valente et al., 2004). However, this mutation did not have any effect on splicing in our minigene system (Fig. S4A and B) and the sequenced band corresponded to correctly spliced exon 7 flanked by minigene sequences.

3.6. TFAM *and* PARK2 *variants*

The *TFAM* variant IVS4+113 A>G (c.441+113A>G, rs2306604) that we found previously to be a risk factor for PD (Gaweda-Walerych et al., 2010) has been postulated to create a putative new splice donor and a cryptic splice acceptor in in silico analysis, possibly resulting in the production of a truncated TFAM protein of 102 amino acids (Gunther et al., 2004). A minigene analysis, however, failed to demonstrate any aberrant splicing (Fig. S5A).

The *PARK2* variant IVS3−20T>C (c.413-20T>C, rs4709583) has been detected in a homozygous state in 1 Polish PD patient (personal communication, prof. J. Limon) and was found more frequent in early-onset Parkinson's disease patients than that in control subjects in Chinese population (Zou et al., 2004). Minigene analysis showed mRNA carrying this variant to be processed in an analogous way as to the WT minigene (Fig. S5B). For this reason, none of above changes was further investigated.

3.7. HSPA9 *variants*

Three variants for *HSPA9* were analyzed, a potentially pathogenic intronic insertion of 17 bp, (IVS8+8; c.879+8 ins or del of AGAGAGAGGTGAGTTAC, rs41295719), detected in 1 PD patient (age at onset 51 years, without family history) out of 330 PD subjects and 250 controls (De Mena et al., 2009); a synonymous variant c.774G>A in exon 8, (Q258Q, rs41295717) found in 1 PD patient (De Mena et al., 2009) and c.879+9 ins17 bp annotated in NCBI. None of these variants resulted in alteration of RNA processing compared to the WT sequences using the minigene splicing assay (Fig. S6).

4. Discussion

In this work, we have analyzed the impact on splicing of 14 variants detected in PD patients (Fig. 1). Four intronic substitutions in the *LRPPRC* influenced the splicing process of this gene as evidenced by minigene assays (Fig. 2). Exon 33 splicing pattern and the effect of IVS32−3C>T mutation is very similar in all 3 tested cell lines (Fig. 2A). However, the splicing pattern for exon 9 and 35 and the effects of corresponding mutations (IVS9+30A>G, IVS35+14C>T, and IVS35+15C>T) in minigene system depend on the cell line (compare Hela and HEK293T with SH-SY5Y cells, Fig. 2B and C). This could result from tissue-specific availability of some splicing factors. In addition, it suggests that also in PD patients a given mutation can have different effects depending on the tissue and it underlines the importance of analyzing splicing pattern in various disease-related cell groups.

More specifically, IVS9+30A>G and IVS32−3C>T, resulted in skipping of *LRPPRC* exon 9 and exon 33, respectively. In both cases, this would introduce a premature stop codon in the mature mRNA that could result in its degradation by NMD pathway, and as a consequence, in depletion of LRPPRC protein. Such a depletion may be linked to the origin and progression of PD and related diseases (Alzheimer's disease, French Canadian Leigh syndrome, LSFC) by mitochondrial dysfunction. Indeed, consequences of LRPPRC depletion are well documented by the analysis of tissues from LSFC patients and various knock-down or silencing approaches in cell lines. They comprise decrease in all mtDNA-encoded mRNAs and mitochondrial respiratory complexes (IV, I, and V) as low as 20%–30% of control levels in fibroblast, liver, and muscle cells from LSFC patients with the most common A354V mutation (Mourier et al., 2014; Olahova et al., 2015; Sasarman et al., 2015; Xu et al., 2004). Pathology in LSFC patients with *LRPPRC* mutations is observed mainly in organs with steady-state low level of LRPPRC, such as brain and liver (Xu et al., 2004), and mRNA levels of *LRPPRC* are known to be tissue specific (skeletal muscles>heart>placenta>kidney>liver>lung=-brain) (Mili and Pinol-Roma, 2003; Xu et al., 2004). Also, compensatory responses to LRPPRC deficiency are tissue specific (Mourier et al., 2014; Olahova et al., 2015; Sasarman et al., 2015). To date, it is not known to what extend LRPPRC is involved in post-transcriptional regulation of mitochondrial mRNAs in brain. LRPPRC has been linked recently to another late-onset neurodegenerative disease—AD, when Swedish mutant K670N/M671L of amyloid beta precursor protein (APPsw) was shown to downregulate both protein and mRNA levels of LRPPRC and its downstream target SLIRP (SRA Stem-Loop Interacting RNA Binding Protein) (Mourier et al., 2014).

Clearly, the clinical impact of *LRPPRC* variants will depend on whether the mutation occurs in a homo- or heterozygous state. It is also worth noting that mutational screening of *LRPPRC* gene in PD patients detected only intronic or exonic synonymous mutations (Rakovic et al., 2011) and none nonsynonymous mutation, the latter ones found, to date, in LSFC patients and other severe early-onset mitochondrial conditions (Mootha et al., 2003; Olahova et al., 2015). Thus, it can be envisioned that even a modest decrease of LRPPRC levels or change of its function conferred by splicing mutations (such as IVS32−3C>T, IVS9+30A>G, or IVS35+14C>T) could be further aggravated by aging-related factors (e.g., decrease

in efficiency of some molecular pathways) in the course of PD pathogenesis.

Although LRPPRC does not contain any canonical RNA-binding motifs, its ability to bind RNA has been reported. Mili and Pinol-Roma (2003) have found that the 2 last C-terminal pentatricopeptide (PPR) motifs correspond to minimal RNA-binding domain and are crucial for RNA homopolymers binding (polyU and polyC). It is worth noting that both exons 33 and 35 localize to the linker region between the 2 last PPR motifs. For this reason, lack of the C-terminal domain (due to IVS32−3C>T) or increased exclusion or inclusion of exon 35 (due to IVS35+14C>T and IVS35+15C>T, or MPP+ treatment) could interfere with the RNA-binding properties of LRPPRC. This is a possibility that should be addressed in future work on the functional properties of this protein.

The opposite effect on splicing exerted by the adjacent variants, IVS35+14C>T and IVS35+15C>T (Fig. 2B) is in accordance with higher frequency of the first one in PD patients, as mentioned in Section 3.1. Clearly, this needs to be confirmed by association studies using higher number of affected subjects and proper control groups. At the functional level, increased or decreased inclusion of the same exon has been linked to different neurodegenerative diseases (progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, or Pick's disease, respectively) as evidenced by *MAPT* mRNA isoforms containing or lacking exon 10 (Buee and Delacourte, 1999; Qian and Liu, 2014).

Importantly, we have demonstrated that mitochondrial complex I inhibitor, MPP+, causes skipping of alternatively spliced exon 35 in endogenous mRNA both in SH-SY5Y and Hela cells (Fig. 6A), which mimicks the effect exerted by IVS35+14C>T mutation in minigene system (Fig. 2B). This observation in addition supports the possible involvement of *LRPPRC* IVS35+14C>T variant in PD pathogenesis, given that administration of complex I inhibitors has become a standard approach in establishing animal and cellular PD models (Bove et al., 2005). MPP+ and annonacin were previously shown to act on splicing, increasing the levels of *MAPT* 4R isoform in human neurons, thought to contribute significantly to progressive supranuclear palsy pathogenesis (Bruch et al., 2014). Our results, therefore, highlight another possible consequence of MPP+ administration that may be related to PD pathogenesis. It remains to be established if *LRPPRC* mRNA isoform lacking exon 35 is upregulated in PD brains and whether it is accompanied by decrease in LRPPRC protein.

In addition, our study provides also some insight into the splicing regulation of exon 33 and 35. We show that hnRNPA1 is a negative regulator of exon 33 splicing both in minigene and endogenous context in Hela and SH-SY5Y cells, whereas TIA-1 exerts strong effect on exon 35. This could have important implications for neurodegenerative diseases as recent findings indicate that, for example, hnRNPA2B1 levels are upregulated in brains of patients with frontotemporal dementia with TDP-43−positive inclusions (Mohagheghi et al., 2016).

Finally, in this work, we have demonstrated that it is possible to rescue aberrant splicing caused by IVS32−3C>T and IVS35+14C>T by co-expression of adapted U1 snRNAs in a minigene system. The ability to obtain the rescue effect through engineered U1snRNAs targeting non-conserved intronic regions (in our case U1LRP33+12 recovers IVS32−3C>T minigene and more importantly corrects exon 33 skipping in the endogenous mRNA) has the advantage over mutation-adapted or exon-intron boundary complement U1s of reducing undesired off-target events (Dal Mas et al., 2015; Fernandez Alanis et al., 2012).

Although for the remaining minigene constructs we have not observed any differences between WT and MUT version in 3 different cell lines, it should be taken into account that in other tissues or in specific disease-related conditions, the splicing environment may be substantially altered and lead to different outcomes than the ones we observed.

5. Conclusions

Our results represent the first analysis of the potential functional impact of *LRPPRC* intronic variants on splicing and strongly suggest that some of these variants could be causative (susceptibility) variants leading to complete (partial) protein depletion or to its structural or functional changes. This could influence PD risk and related disorders (e.g., AD, LSFC), where mitochondrial dysfunction (deficiency of mitochondrial complex I and IV) is claimed to be the one of the main mechanisms of pathogenesis. Our results open a new avenue for future studies on the functional significance of splicing alterations for the properties of the LRPPRC protein and its role in the pathogenesis of PD and related disorders.

Disclosure statement

The authors declare there are no actual or potential conflicts of interest relating to this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neurobiolaging.2016.07.014.

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