NRDE2 Interacts with an Early Transcription Elongation Complex and Widely Impacts Gene Expression

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Abstract: NRDE2 is a highly conserved protein that is implicated in post-transcriptional gene silencing in S. pombe and C. elegans while in mammals, it has been shown to modulate splicing. To determine whether NRDE2 may be implicated in other processes in humans, we performed tandem affinity purification followed by proteomic analysis of NRDE2 from nuclear extracts in HEK293T and HeLa cells. Our data show that, in addition to its well-characterized partner, MTR4 helicase (MTREX), as well as several splicing factors, NRDE2 also interacts with chromatin-associated factors involved in transcription, including the Polymerase-Associated Factor 1 (PAF1) complex and elongating forms of RNA polymerase II (RNAPII). To further investigate the function of NRDE2 in gene expression, we performed RNA-seq following its transient depletion. Differential expression analysis showed that loss of NRDE2 modulated the expression of thousands of genes. While effects on splicing, including intron retention, were detected, as described previously, our analysis revealed that the impact of NRDE2 on intron retention is more widespread than previously thought. Moreover, intron retention was also highly associated with down-regulation of mRNA expression. Taken together, these results suggest that NRDE2 associates with the transcription and splicing machineries and affects RNA processing.

Keywords: NRDE2; transcription; intron retention; splicing

1. Introduction

NRDE2 (nuclear RNAi-defective 2) is a conserved protein with homologs detected in eukaryotes ranging from fission yeast to human but absent in budding yeast. NRDE2 was initially discovered in *Caenorhabditis elegans* where it is involved in nuclear RNAi, a process that regulates gene silencing by small RNA molecules, including siRNAs (small interfering RNAs) [1]. The NRDE pathway maintains heritable RNAi silencing in *C. elegans*. Nuclear-localized siRNAs direct NRDE-2 factors to nascent pre-messenger RNAs (pre-mRNAs), which leads to H3K9Me3 formation and inhibition of RNA polymerase II (RNAPII) during the elongation phase of transcription [1-3]. NRDE-2, together with NRDE-3, is required for the association of NRDE-1 with pre-mRNA and chromatin and is required for small-RNA dependent silencing via H3K9 methylation [4]. Interestingly, the evolutionarily conserved MTR4 helicase was shown to interact with NRDE2 and contribute to NRDE2-dependant co-transcriptional gene silencing [5].

The fission yeast homolog Nrl1 has been shown to form a complex with Mtl1 (MTREX in mammals) and Ctr1 proteins and interact with core splicing factors [6] [7]. The Nrl1–Ctr1–Mtl1 complex has been proposed to regulate the splicing of cryptic introns and target unspliced transcripts for degradation by the nuclear exosome. Nrl1 targets transcripts with cryptic introns to form heterochromatin at developmental genes and retrotransposons [6].

In mammals, NRDE2 has been implicated in several cellular processes, including splicing and the maintenance of genome integrity. Proteomic studies have identified interactions with numerous splicing factors and loss of NRDE2 induces splicing defects, particularly retention of weakly spliced introns, in mouse and human models [8,9]. Loss of NRDE2 also induces DNA damage and formation of R-loops [10]. Recent studies have indicated that NRDE2 is involved in DNA damage responses, possibly through interactions with repair pathways that ensure proper gene expression under stress conditions [11]. Like its counterparts in *S. pombe* and *C. elegans*, a major partner of mammalian NRDE2 is the MTR4 helicase, MTREX [5,6,10,12], which will be referred to as MTR4. Indeed, interaction with MTR4 appears to be required for stability of NRDE2 protein [5]. NRDE2 has been suggested to reduce exosome activity by inhibiting MTR4 function [12]. NRDE2 interaction with MTR4 locks it in a closed conformation and inhibits MTR4 interaction with the exosome as well as partner proteins, such as the cap-binding complex (CBC) and ZFC3H1 [12]. Interestingly, the interaction between NRDE2 and MTR4 has been shown to be required for self-renewal in mouse embryonic stem cells [12]. Moreover, NRDE2 has been implicated in several human diseases. NRDE2 was identified as a biomarker in circulating tumor cells found in patients with metastatic melanoma, based on analysis of genome-wide copy-number aberration and loss-of-heterozygosity [13]. More recently, NRDE2 was identified as a novel susceptibility gene for hepatocellular carcinoma (HCC) [11]. NRDE2 was shown to bind to subunits of casein kinase 2 (CK2) and facilitate the assembly and activity of the CK2 holoenzyme. NRDE2-mediated enhancement of CK2 activity enhanced the phosphorylation of MDC1 and facilitated the DNA damage response. This function was almost completely abrogated by a rare-variant, NRDE2-p.N377I, which sensitized HCC cells to poly(ADP-ribose) polymerase (PARP) inhibitors [11]. NRDE2 was also shown to play a role during infection by Kaposi's Sarcoma-Associated Herpesvirus (KSHV). KSHV late transcripts evade degradation through the activity of the NRDE2 that inhibits RNA degradation pathways [14].

Thus, NRDE2 appears to have a conserved role in the regulation of splicing. However, its precise molecular mechanisms, particularly in mammalian systems, remain under investigation. Here, we have identified novel interactants of NRDE2. We identified not only proteins implicated in splicing, but many novel chromatin-associated partners that are associated with early elongating RNAPII complexes. RNA-seq analysis revealed that loss of NRDE2 impacts the expression of many genes. Defects in splicing, particularly intron retention, were identified. However, many effects on gene

expression appear to occur independently of splicing defects. These findings may point to additional roles for NRDE2 in the regulation of gene expression.

2. Results

2.1.1. NRDE2 interacts with transcription-associated proteins and RNA processing factors

To gain insight into NRDE2 function, we created Hela S3 and HEK293T cell lines stably expressing NRDE2 with tandem Flag and HA tags on the N-terminus (F.H-NRDE2). In HeLa S3 cells, F.H-NRDE2 was expressed following transduction with a lentiviral vector, as described previously [15] while Crispr-Cas9 technology was used to insert the tag at the endogenous NRDE2 locus in HEK 293T cells, as described previously [16]. We first verified that each cell line expressed F.H-tagged NRDE2, and that a known partner of NRDE2, MTR4, could be detected in F.H-NRDE2 immunoprecipitates (Supplementary Fig. 1a, b). Nuclear extracts of F.H-NRDE2-HEK293T or F.H-NRDE2- HeLa S3 were immunoprecipitated using anti-Flag antibody. Immunoprecipitates were immunoblotted using anti-HA and anti-MTR4 antibodies (Supplementary Fig. 1a, b). Thus, F.H-NRDE2 was expressed and interacted with a known partner in both HeLa S3 and HEK293T cells.

To identify interactants of NRDE2 in each cell type, nuclear extracts obtained from each cell line were subjected to tandem affinity purification using anti-Flag followed by anti-HA antibodies, as described previously [15,16]. Eluates were analyzed by tandem mass spectrometry (Taplin facility, Harvard University, Boston) and interactants having 2 or more peptides and FC>2 compared to the control HeLa S3 or HEK293T cells were selected. Flag.HA-NRDE2 specifically interacted with 102 proteins in HeLa S3 cells while 81 specific interactants were detected in HEK293T (Supplementary Table 1). Among these, 20 interactants were detected in both cell lines (Figure 1a and Supplementary Table 2). Gene ontology (GO) analysis showed that NRDE2 interacted with proteins involved in RNA processing and splicing in both HeLa S3 (Figure 1b) and HEK293T cells (Figure 1c). Interestingly, more interactants associated with chromatin were detected in HeLa S3 cells compared to HEK293T. GO network analysis furthermore showed that NRDE2 interactants are implicated in RNA processing and, in particular, RNA splicing (Supplementary Figure 2).

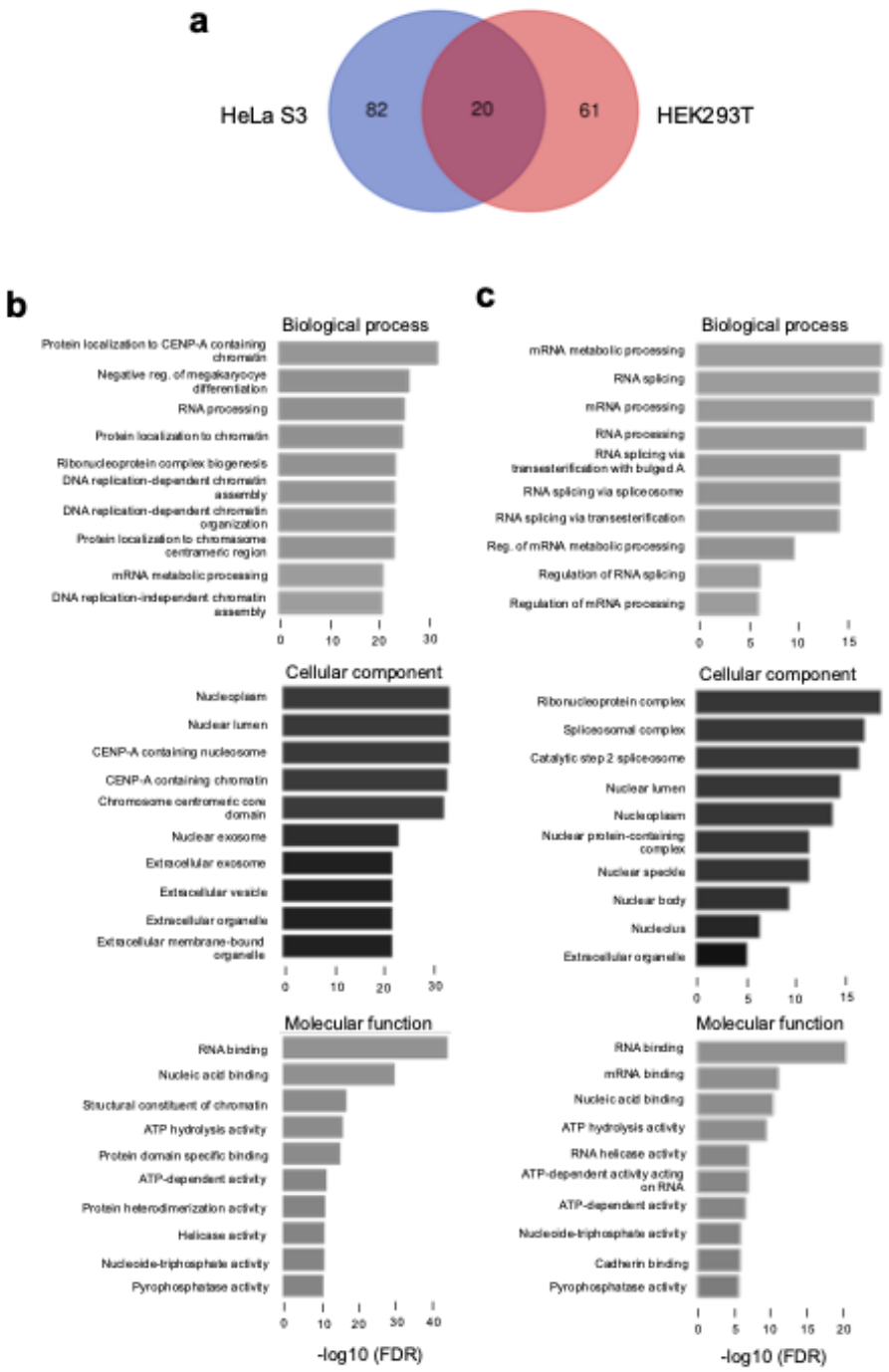

Figure 1. NRDE2 interacts with proteins found in the nucleus and chromatin-associated proteins that are implicated in RNA processing. a. Venn diagram showing the overlap between NRDE2 interactants detected in FH-NRDE2-expressing HeLa S3 or FH-NRDE2-expressing HEK293T cells, as indicated. b. GO analysis of NRDE2 interactants detected in FH-NRDE2-expressing HeLa S3. c. GO analysis of NRDE2 interactants detected in FH-NRDE2-expressing HEK293T.

We next tested whether the interactants of F.H-NRDE2 identified by proteomics could be detected in interaction with endogenous, untagged NRDE2. Several interactants belonging to different nuclear

protein complexes were analyzed by co-immunoprecipitation using nuclear extract of parental HEK293T cells. The interaction with MTR4, the most abundant NRDE2 interactant identified in both cell types, was confirmed (Figure 2a). In addition, interaction with several RNA helicases, DDX3, DDX15 and DDX17, was also confirmed. The NRDE2 interactome in both cell types contained numerous splicing factors, albeit with a low number of peptides. Interaction between NRDE2 and splicing factors such as EFTUD2, SFPQ and SNRNP200, was also confirmed. Interactions with RNA-binding proteins, such as SAF-B, ILF2 and ILF3, were also confirmed. The DNA gyrase, Topoisomerase I (TOP1) was identified as an interactant of NRDE2 and its interaction with endogenous NRDE2 was also confirmed (Figure 2a).

We noticed that both HeLa S3 and HEK293T FH-NRDE2 interactomes each contained subunits of the multi-subunit Polymerase-Associated Factor 1 complex (PAF1c) although the subunits identified in each cell type were not identical. FH-NRDE2 HEK293T interactome contained the CTR9 subunit, whereas PAF1, CDC73 and WDR61 subunits of PAF1c were identified in the FH-NRDE2 HeLa S3 interactome (Supplementary Table 2). Interaction between endogenous NRDE2 and subunits of PAF1c was analyzed by co-immunoprecipitation. Indeed, NRDE2 interacted with PAF1, CDC73, and CTR9 subunits while an interaction with the highly labile subunit RTF1 could not be detected (Figure 2B), suggesting that NRDE2 interacts with the core PAF1 complex. Since PAF1 interacts directly with transcribing RNA polymerase II (RNAPII) [17,18], we tested whether NRDE2 could also be detected in interaction with the different forms of RNAPII involved in transcription. NRDE2 interacted modestly with total RNAPII, that includes both actively transcribing and non-transcribing forms of RNAPII. Interestingly, NRDE2 interacted more robustly with RNAPII involved in the initiation and early steps of transcription that is phosphorylated on serine 5 of the carboxy terminal domain (CTD) of the large subunit (RNAPII pS5) as well as with the form involved in the elongation of transcription, that is phosphorylated on serine 2 of the CTD (RNAPII pS2) (Figure 2B). Thus, NRDE2 interacts with actively transcribing RNAPII as well as the PAF complex that is known to associate with RNAPII and co-ordinates many histone modifications that are necessary for active transcription.

Since an interaction between NRDE2 and actively transcribing forms of RNAPII was detected, we next tested whether NRDE2 might interact with other factors known to be implicated in the elongation of transcription. Interestingly, endogenous NRDE2 was found to interact with CDK9 and cyclin T1 subunits of the positive transcription elongation complex, P-TEFb, that are thought to act in the early steps of transcription elongation (Figure 2c). Notably, however, NRDE2 did not interact with CDK12, that is thought to control elongation throughout the body of genes (Figure 2c). P-TEFb is sequestered in an inactive complex by 7SK RNA and HEXIM1 [19]. Notably, although NRDE2 interacted with CDK9 and cyclin T1, no interaction could be detected with HEXIM1 (Figure 2c). This

could suggest that NRDE2 interacts with the active form of P-TEFb. An interaction was also detected between NRDE2 and ELOA subunit of the elongin complex that is involved in the elongation of transcription. An interaction was also tested between NRDE2 and subunits of the Integrator complex that is implicated in the pausing of RNAPII close to transcription start sites [20-22]. NRDE2 interacted with several subunits of the Integrator complex, INTS3, INTS9 and INTS13 (Figure 2c), which suggests that NRDE2 interacts with the holo-Integrator complex. Integrator is known to interact with the negative regulator of elongation, NELF [21,22]. Notably, although NRDE2 interacts with Integrator subunits, it does not interact with NELF-E (Figure 2c). Taken together, these results reveal interactions between NRDE2 and numerous factors involved in active RNAPII transcription, particularly at the step of early elongation.

Recently, NRDE2 has been reported to associate with subunits of casein kinase 2 (CK2) and facilitate the assembly of the CK2 holoenzyme [11]. Interestingly, CSNK2A1 and CSNK2A2 subunits were identified in the FH-NRDE2 interactome in HeLa S3 cells. However, we were unable to confirm the interaction by co-immunoprecipitation analysis in HEK293T cells (Figure 2c). This may be due to differences in extraction or immunoprecipitation methods used for mass spectrometry compared to co-immunoprecipitation analysis. In addition, we did not detect subunits of PAXT or NEXT in NRDE2 interactomes from either HeLa S3 or HEK293T cells nor we were able to detect an interaction between NRDE2 and ZFC3H1 subunit of PAXT, or ZCCHC8 subunit of NEXT by co-immunoprecipitation (Figure 2C). These results support the finding that NRDE2 prevents MTR4 helicase from interacting with exosome-associated complexes [12]. However, several subunits of nuclear exosome were detected as interactants of NRDE2 in HeLa S3 cells (Supplementary Tables 1 and 2). It should be noted that a robust interaction between NRDE2 and EXOSC10 has been reported previously [9].

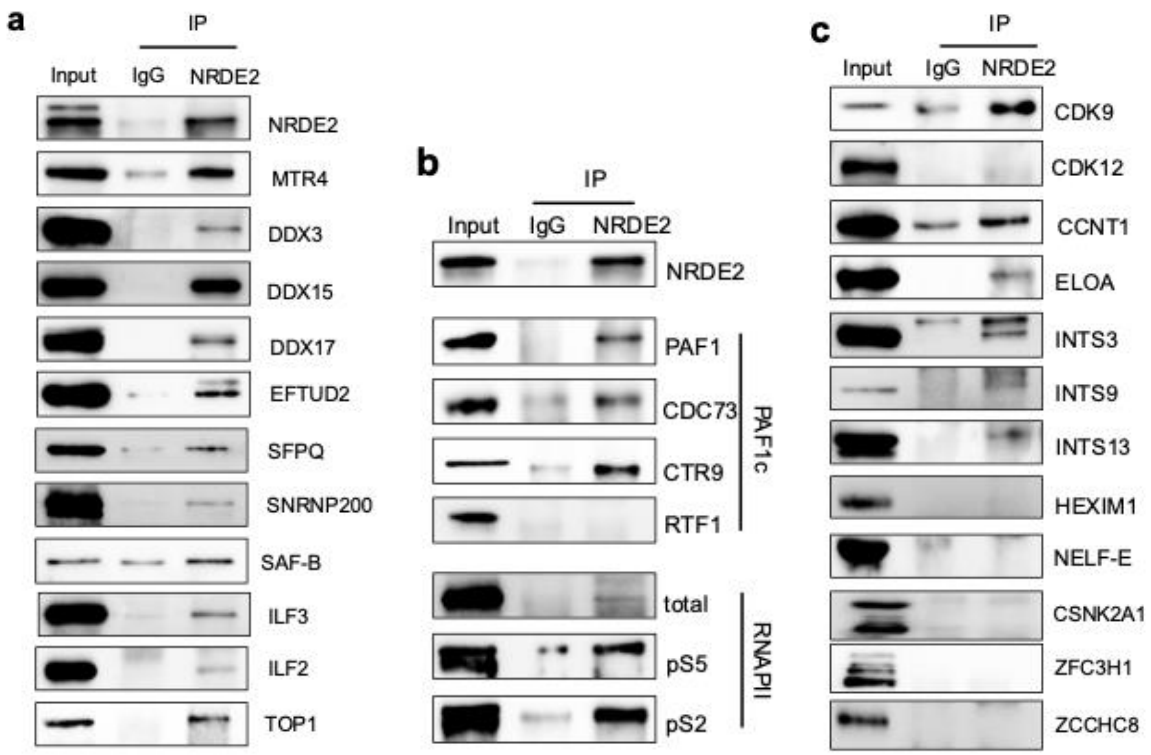

Figure 2. NRDE2 interacts with proteins implicated in transcription and mRNA processing. a. Co-immunoprecipitation analysis of endogenous NRDE2 and interactants identified by mass spectrometry in either HeLa S3, HEK293T or both cell types. HEK293T nuclear extracts were immunoprecipitated using an IgG control or NRDE2 antibodies, as indicated. Immunoprecipitates and an aliquot of nuclear extract (input, 5%) were analyzed by SDS-PAGE followed by immunoblot using the antibodies indicated on the figure. b. Co-immunoprecipitation analysis of endogenous NRDE2 and subunits of PAF1c identified by mass spectrometry, as well as with different forms of RNAPII. HEK293T nuclear extracts were immunoprecipitated using an IgG control or NRDE2 antibodies, as indicated. Immunoprecipitates and an aliquot of nuclear extract (input, 5%) were analyzed by SDS-PAGE followed by immunoblot using the antibodies indicated on the figure. c. Co-immunoprecipitation analysis of endogenous NRDE2 and RNAPII-associated proteins implicated in transcription. HEK293T nuclear extracts were immunoprecipitated using an IgG control or NRDE2 antibodies, as indicated. Immunoprecipitates and an aliquot of nuclear extract (input, 5%) were analyzed by SDS-PAGE followed by immunoblot using the antibodies indicated on the figure.

2.1.2 NRDE2 affects expression and mRNA splicing at a subset of genes

Given that NRDE2 interacts with transcription-associated proteins, and has been reported to modulate mRNA processing steps, such as splicing [8,9], we determined the effect of NRDE2 on the transcriptome. Expression of NRDE2 was diminished by RNAi (Figure 3a). Loss of NRDE2 has a very widespread effect on gene expression. Expression of approximately 13,000 genes was deregulated following depletion of NRDE2 (Figure 3b). Of these, 6239 genes were down-regulated while 6439 genes were up-regulated (FC>1.5, $p < 0.05$). Browser shots of down-regulated genes, SLC1A4 and ARPIN, and up-regulated genes, PLEKHB1 and BTN3A2, are shown in Figure 3c. Thus, NRDE2 has a significant effect on the transcriptome.

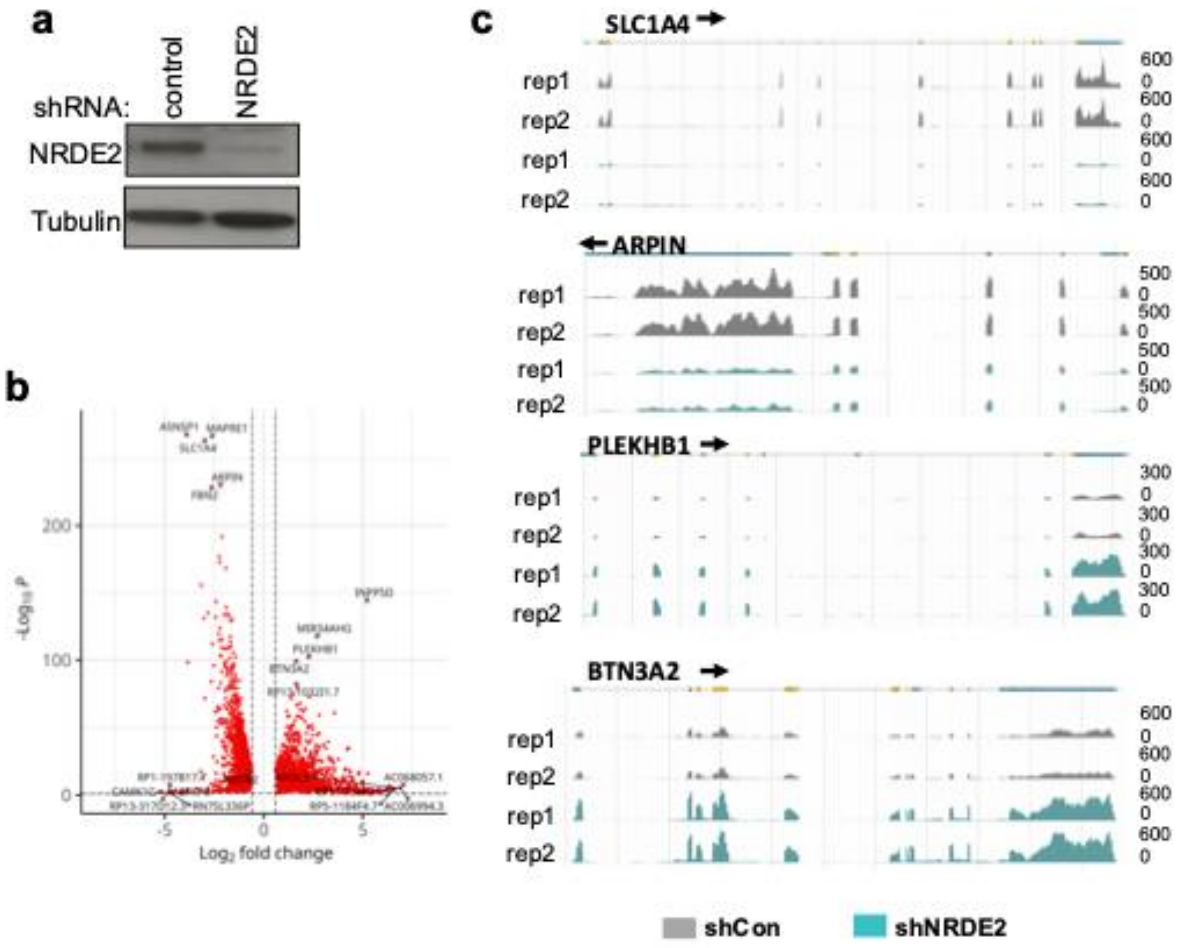

Figure 3. NRDE2 modulates the expression of a subset of genes. a. HeLa cells transduced with lentiviral particles expressing shRNA against NRDE2 or a non- targeting control were harvested 5 days post-transduction. Extracts were analyzed by immunoblot using the indicated antibodies. b. Volcano plot showing the differential expression of mRNAs in extracts of HeLa cells transduced with lentiviral particles expressing shRNA against NRDE2 or a non-targeting control. c. Browser shots of RNA-seq signal from control or NRDE2 knock-down samples, as indicated, over representative genes. A schematic representation of the gene is shown above.

NRDE2 has been shown to affect splicing of short, GC-rich introns with relatively weak 5' and 3' splice sites in both mouse and human cells [8,9]. We therefore determined whether defects in splicing could be detected in control and NRDE2 knock-down RNA-seq samples. Since NRDE2 has been shown to affect intron retention (IR), we first analyzed IR using IRFinder [23]. Similarly to previous studies, we found that NRDE2 suppressed IR at a subset of genes [8,9]. However, we detected significantly more NRDE2-regulated IR events in HeLa cells compared to MDA-MB-231 cells [9]. In HeLa cells, 2298 deregulated IR events were detected using IRFinder (using default parameters) (Figure 4a) while 178 were detected previously in MDA-MB-231 cells [9]. However, this represents 9% of all IR events in HeLa cells and 8% in MDA-MB-231 cells, suggesting that the results are qualitatively similar. Among

the deregulated IR events that occurred following loss of NRDE2 in HeLa cells, NRDE2-suppressed IR accounted for 94%, like that observed previously [9]. Distribution analysis of NRDE2-suppressed IR events showed that most genes (66%) showed retention of only 1 intron of the mRNA transcript following loss of NRDE2 (Figure 4b). Retention of up to 4 introns per gene accounted for 86% of all NRDE2-suppressed IR events. Thus, loss of NRDE2 led to retention of a small number of introns per gene and was limited to a subset of genes. Browser shots of PPP2R5C, containing a single retained intron and TMEM18, containing multiple retained introns are shown in Figure 4c.

We also determined whether loss of NRDE2 might induce other type of splicing defects in addition to intron retention. Analysis of RNA-seq samples using Suppa2 [24] revealed that NRDE2 does contribute to the regulation of additional splicing events (Supplementary Figure 3). Differential analysis of skipped exons, mutually exclusive exons, alternative last exons, alternative first exons, alternative 5′ splice site and alternative 3′ splice site usage showed that loss of NRDE2 led to deregulation of these splicing events, in addition to that of IR. These NRDE2-regulated splicing events represented approximately 5% to 7% of the total number of each event detected in control cells, whereas IR occurred more frequently, representing 12% of all IR events detected in control cells (Supplementary Figure 3).

We next determined the association of NRDE2-suppressed IR with differential gene expression (Figure 4D). Thus, differential RNA-seq analysis was compared to NRDE2-suppressed IR. NRDE2-suppressed IR was strongly associated with down-regulation of mRNA expression (Figure 4d, left Venn diagram, $p=4.2e-275$, Fisher's exact test). Notably, 57% of NRDE2-suppressed IR occurred in down-regulated genes. NRDE2-suppressed IR also occurred in up-regulated genes (Figure 4d, middle Venn diagram, $p=4.9e-10$, Fisher's exact test), representing approximately 16% of NRDE2-suppressed IR. NRDE2-suppressed IR also occurred in genes whose expression was unchanged (347 IR, 27%), although this effect was not significant (Figure 4d, right Venn diagram, $p=0.023$, Fisher's exact test). Thus, NRDE2-suppressed IR was commonly associated with differential gene expression, and in particular, down-regulation of expression. However, it should be noted that, following loss of NRDE2, the expression of nearly 12,000 genes was modified in a manner that appears to be independent of intron retention. This may suggest that NRDE2 could have additional roles in the regulation of gene expression.

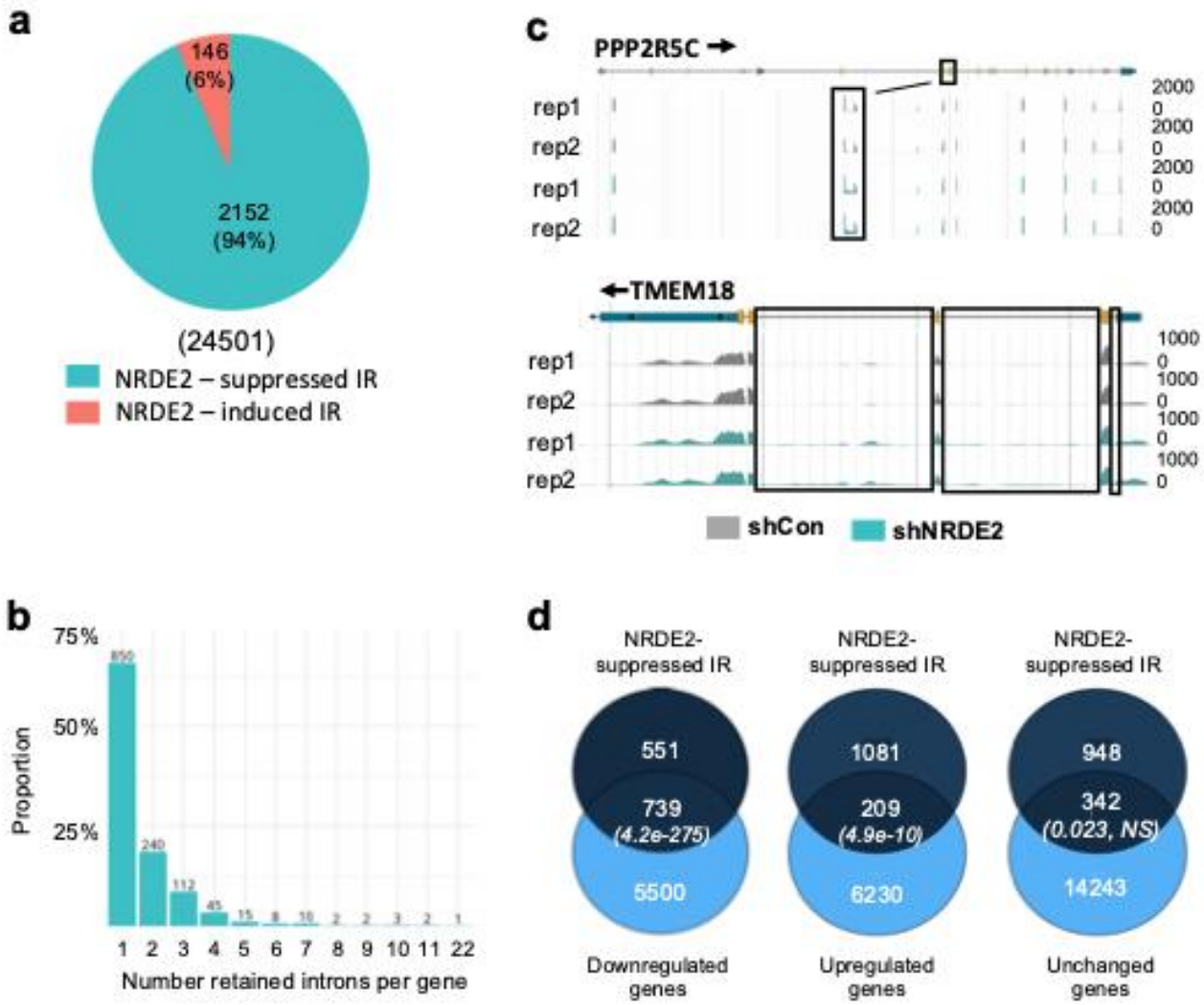

Figure 4. NRDE2 affects splicing at a subset of genes. a. Piechart showing the number of differentially retained introns (NRDE2-suppressed or NRDE2-induced) detected by IRFinder in RNA-seq samples from control compared to NRDE2 knock-down cells. b. Distribution analysis of NRDE2-suppressed IR showing the number of retained introns per gene. Numbers above the bars show the number of NRDE2-suppressed IR events. c. Browser shots of RNA-seq signal from control or NRDE2 knock-down samples, as indicated, over representative genes. A schematic representation of the gene is shown above. Boxed regions show NRDE2-suppressed IR. d. Venn diagrams showing the overlap between genes exhibiting NRDE2-suppressed IR and down-regulation (left), upregulation (middle) or unchanged (right) expression. P-values of the overlap (Fisher's exact test) are shown in brackets on the Venn diagrams.

3. Discussion

NRDE2 is a highly conserved protein that has been implicated in the control of splicing in fission yeast through to humans, and in small RNA-dependent gene silencing in lower organisms. To further characterize human NRDE2, we characterized its interactome in 2 human cell types, HeLa S3 and HEK293T. As described in previous studies [5,10,12], the major interactant of NRDE2 in both cell types

was the RNA helicase, MTR4 (MTREX). Many NRDE2 interactants were detected in both cell types, while others were detected in either HEK293T or HeLa S3. Gene ontology (GO) analysis of NRDE2 interactants identified in HEK293T or Hela S3 revealed that many are implicated in RNA processing and splicing. This result corroborates previous reports showing that NRDE2 interacts with numerous splicing factors [8,9]. NRDE2 interactants identified in HeLa S3 were also implicated in chromatin-associated pathways and biological processes. This may reflect differences in protein extraction efficiency from chromatin in HeLa S3 compared to HEK293T. Of interest, several subunits of the RNAPII-associated complex, PAF1, were identified in HeLa S3 while one PAF1c subunit was identified in HEK293T. The interactions were validated by co-immunoprecipation analysis in HEK293T (Figure 2b). Interestingly, RTF1 that is not considered to be a stable component of PAF1c in humans [25-27], could not be detected in interaction with NRDE2 (Figure 2b). We then extended our co-immunoprecipitation analysis to test interactions with RNAPII, as well as proteins known to be implicated in transcription. NRDE2 interacted with RNAPII, particularly with phosphorylated forms that are involved in the initiation and elongation of transcription (pS5 and pS2, respectively, Figure 2b). Moreover, NRDE2 interacted with CDK9 and cyclin T1 subunits of pTEFb but did not interact with CDK12 (Figure 2c). Phosphorylation of the RNAPII CTD is tightly regulated during the transcription cycle. The transition from promoter-proximal pausing to transcription elongation is controlled by pTEFb while CDK12, in association with cyclin K, regulates elongation and termination of transcription [28]. Thus, NRDE2 interaction with pTEFb but not CDK12 could suggest that it may be implicated in an early step of transcription elongation occurring close to the promoter region. Consistent with this idea, NRDE2 was found to interact with several subunits of Integrator complex (Ints3, Ints9 and Ints11, Figure 2c) that terminates RNAPII transcription in the promoter-proximal region of genes. NRDE2 was also found to interact with ELOA subunit of the Elongin complex, a heterotrimeric elongation factor required for RNAPII-dependent transcription (Figure 2c). Notably, it has been shown that RNAPII cannot bind the Elongin and PAF complexes simultaneously [29], suggesting that NRDE2 may bind an RNAPII complex undergoing remodeling during the early elongation steps of transcription. Consistent with the idea that NRDE2 interacts with actively transcribing RNAPII, no interaction could be detected with HEXIM1, a subunit of inactive pTEFb [19], or the NELF-E subunit of the negative elongation factor that is associated with paused RNAPII [30].

Loss of NRDE2 induced widespread effects on gene expression. Of 27263 genes analyzed, 46.5 % (12678 genes) were differentially expressed following loss of NRDE2 (Figure 3). Among differentially regulated genes, 49.2% (6239 genes) were down-regulated while 50.8 % (6439 genes) were up-regulated following loss of NRDE2. NRDE2 has been shown to be required for suppressing intron retention in a subset of pre-mRNAs containing short, GC-rich introns with relatively weak 5' and 3' splice sites, in

both a human cell line, MDA-MB-231 cells, and murine ES cells. Analysis of splicing events in HeLa cells revealed that, like previous studies, loss of NRDE2 led to significant effects on splicing, particularly intron retention (Figure 4a). Indeed, nearly 10% of all intron retention events detected in HeLa cells were modulated by NRDE2. We next interrogated the effect of intron retention on differential gene expression. Further analysis of splicing effects compared to differential regulation of gene expression revealed that NRDE2-suppressed intron retention events were significantly associated with transcripts for which the expression was deregulated by loss of NRDE2. NRDE2-suppressed intron retention occurred most frequently in down-regulated transcripts although several hundred intron retention events were identified in transcripts that were upregulated following loss of NRDE2 (Figure 4d). Thus, NRDE2-dependent intron retention was significantly associated with differential gene expression. On the contrary, several thousand genes showed deregulated expression in a manner that was independent of intron retention (Figure 4d; 5500 down-regulated genes and 6230 up-regulated genes). It is not known whether these effects may be direct or indirect effects of NRDE2 depletion. However, the interaction of NRDE2 with an early transcription elongation complex (Figure 2) could suggest that loss of NRDE2 may induce effects on transcription and gene expression at a subset of genes. Further studies will be required to elucidate the role of NRDE2 on gene expression, independently from its role in splicing.

Taken together, our results confirm that NRDE2 interacts with RNA-processing and splicing factors and furthermore, reveal novel interactants suggesting that NRDE2 may interact with an early transcription elongation complex in human cells. Our RNA-seq analysis confirms and extends previous studies highlighting a role for NRDE2 in splicing and, particularly, intron retention and furthermore indicates that this effect may be more widespread in human cells than previously thought [9]. We demonstrate that NRDE2 has a widespread effect on gene expression, affecting nearly half of all expressed genes. While splicing defects may partially account for deregulation of gene expression, most differential expression likely occurs independently of splicing through mechanisms that are not currently known.

4. Materials and Methods

Cell Culture and Reagents

HeLa and HeLa S3 cells were grown in Dulbecco modified Eagle's minimal essential medium (DMEM) (Sigma-Aldrich D6429), supplemented with 10% FCS (Eurobio Scientific, CVFSVF00-01), and containing 1% Penicillin streptomycin (Sigma P4333). HEK-293T cells (ATCC, CRL11268) were grown in Hepes-modified DMEM (Sigma-Aldrich, D6171), supplemented with 10% FCS (Eurobio Scientific, CVFSVF00-01), 2 mM GlutaMAX (Gibco, 35050-061) and containing 1% penicillin-streptomycin (Sigma-Aldrich, P4333). Both cell lines were grown in a humidified incubator at 37 °C with 5% CO2.

Antibodies

Antibodies used in this study are shown in Table S3.

Generation of FH-NRDE2-expressing cells

FH-NRDE2-expressing HeLa S3 cells were generated by transduction of lentiviral vectors expressing FH-NRDE2, as previously described [15]. Briefly, plasmid encoding pOZ-Flag-HA-NRDE2 (pOZ-FH-NRDE2) was cloned using pOZ-N-FH vector. Lentiviral particles expressing FH-NRDE2 were produced in HEK293T cells by transfecting plasmids using calcium-phosphate. HeLa S3 were transduced using Polybrene infection/transfection reagent (Sigma-Aldrich®, TR-1003), according to the manufacturer's instructions. After 7 days, selection of transduced cells was carried out by magnetic affinity sorting with antibody against IL2 to achieve a pure population. HeLa S3 stably expressing NRDE2 (FH-NRDE2 HeLa S3) were confirmed by Western blot using anti-HA and anti-Flag antibodies.

FH-NRDE2-expressing HEK293T cells were generated by CRISPR-Cas9 Mediated Editing of endogenous NRDE2 gene.

An sgRNA targeting the NRDE2 gene around the ATG translation start site was cloned in pSpCas9 (BB)−2A-GFP plasmid (Addgene #48138). The plasmid was then transfected into HEK-293T cells along with a single-stranded oligodeoxynucleotide (ssODN) (Table S4) harboring the Flag-HA sequence flanked by homology sequences to NRDE2 around the cleavage site. Single cells were isolated and amplified. HEK- 293T clones expressing Flag-HA NRDE2 were identified by PCR and confirmed by sequencing as well as Western blot using anti-HA and anti-Flag antibodies.

RNAi

Production of short hairpin RNA (shRNA)-expressing lentiviral particles was performed as described previously [31] using plasmids expressing shRNAs targeting NRDE2 (Sigma-Aldrich MISSION shRNA, TRCN0000422527) or a non- targeting control (Addgene, plasmid #1864). For knockdown experiments, HeLa cells were transduced with lentiviral particles and harvested 5 days later.

NRDE2 Protein Complex Purification

NRDE2 protein complex was extracted from nuclear extracts of HeLa S3 or HEK-293T cells stably expressing Flag-HA-NRDE2. Briefly, cells were seeded in 150 mm culture dishes the day prior to protein extraction. Cytoplasmic proteins were extracted using a mild lysis buffer [10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 2 mM MgCl2, 1 mM DTT, protease and phosphatase inhibitors (Halt™, Thermofisher 78438)]. The cell pellet was incubated for 10 min on ice, adding 0.07% NP-40 and

incubating for an additional 15 min on ice. After centrifugation (2000 x g, 5 min, 4 °C), the cytoplasmic fraction was collected and discarded. The pellet was washed with ice-cold PBS supplemented with protease and phosphatase inhibitors. After centrifugation (2000 x g, 5 min 4 °C), the supernatant was discarded leaving the packed nuclear volume (PNV). For extraction of nuclear soluble proteins, nuclei were resuspended drop-wise in 1x PNV of hypotonic buffer [20 mM Hepes pH 7.9, 20 mM NaCl, 1 mM EDTA pH 8.0, 1.5 mM MgCl2, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor (Halt™, Thermofisher 78438)], followed by the addition of 1x PNV of high salt buffer (20 mM Hepes pH 7.9, 800 mM NaCl, 1 mM EDTA pH 8.0, 1.5 mM MgCl2, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor (Halt™, Thermofisher 78438)]. Samples were rotated on a wheel for 20 min at 4 °C, followed by centrifugation (20 min 16,000 x g, 4 °C). The supernatant containing the nuclear extracts was collected.

FH-NRDE2 complex was purified by two-step affinity chromatography, as described previously (dx.doi.org/10.17504/protocols.io.kgrctv6). Sequential Flag and HA immunoprecipitations were performed on equal amounts of proteins. Silver-staining was performed according to the manufacturer's instruction (Silverquest, Invitrogen). Mass spectrometry was performed at Taplin facility, Harvard University, Boston, MA.

Coimmunoprecipitation analysis

Coimmunoprecipitation was performed using nuclear extracts of HEK-293T cells as described previously [32]. Briefly, cells were washed twice with PBS and then resuspended in Cytoplasmic Lysis Buffer (CLB) [10 mM Tris, pH = 7.4; 10 mM NaCl; 3 mM $MgCl_2$; 0.5% NP-40, complemented with protease and phosphatase inhibitors (Halt™, Thermofisher 78438)]. Nuclei were immediately pelleted by centrifugation at 300 × *g* for 4 min at 4°C. The supernatant (cytoplasmic fraction) was discarded. Another CLB wash was performed before lysing the nuclei in a Nuclear Lysis Buffer (NLB) [20 mM Tris (pH = 8), 3 mM KCl, 150 mM NaCl, 1 mM $MgCl_2$ and 0.2% Triton™ X-100, protease and phosphatase inhibitors (Halt™, Thermofisher 78438)]. After a 30 min incubation on ice, extracts were cleared by centrifugation at 14 000 rpm for 15 min at 4°C and the supernatant containing nuclear protein extracts was collected. For immunoprecipitation, nuclear protein extracts (250 μg) were incubated with antibodies (2 μg) overnight on a rotating wheel at 4°C. Protein A (Invitrogen, Dynabeads™ Protein G, 10009D) conjugated beads were added and the mixtures were incubated for 30 min at room temperature on a rotating wheel. Following 3 washes with NLB, Samples were resuspended in protein sample loading buffer (1x Laemmli buffer), incubated for 5 min at 95°C and analyzed by Western blotting using the antibodies shown in Table S2.

RNA-seq and statistical analysis

For RNA-seq, total RNA was extracted from HeLa cells using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. Paired end sequencing of polyA+ selected mRNA (20M clean reads per sample, 125 bp reads) was carried out by BGI Genomic Services (BGISEQ-500, NGS platform) in triplicates. Statistical analyses were performed as follows:

Quality Control and Pre-processing of Sequencing Data: Initial quality control of raw sequencing data was performed using FastQC (v0.11.9), and results were summarized using MultiQC (v1.11.dev0). Adapter trimming and quality filtering were conducted using fastp (v0.23.2) with default parameters. Reads were also screened against contamination sources using FastQ Screen (v0.14.1) to ensure sample integrity.

Read Alignment and Reference Genome: Processed reads were aligned to the human reference genome GRCh38 (Ensembl release 84) using STAR aligner (v2.7.10a) with a two-pass alignment strategy. The genome was indexed using STAR with the parameters --sjdbOverhang 50.

Post-alignment Processing: Duplicate reads arising from PCR amplification were removed using SAMtools rmdup (v1.12). Aligned BAM files were sorted by genomic coordinates and indexed using SAMtools index (v1.12).

Quantification of Gene Expression: Gene-level quantification was performed using featureCounts (v2.0.3) with the Ensembl GRCh38 release 84 GTF annotation file, counting reads mapping uniquely to exons.

Differential Expression Analysis: Differential gene expression analysis was carried out with DESeq2 (v1.30.1) in R/Bioconductor. Data normalization and differential expression were assessed using negative binomial generalized linear models, and results were visualized with PCA plots, MA plots, volcano plots, and heatmaps for quality control and biological interpretation.

Intron Retention Analysis: Intron retention was quantified using IRFinder (v2.0.0) [23]. Differential intron retention between experimental conditions was assessed using DESeq2 integrated within IRFinder, identifying statistically significant intron retention events. Additional differential splicing events were assessed using Suppa2 [24].

Additional Bioinformatics Tools and Analyses: Additional genomic manipulations and analyses were performed using bedtools (v2.29.1), and genomic visualization was facilitated using deepTools (v3.5.1). Workflow automation and reproducibility were managed using Snakemake (v7.32.4). Gene ontology was performed using ShinyGO (0.82) (https://bioinformatics.sdstate.edu/go/). Enrichments in Venn diagrams were performed using Fisher's exact test.

Supplementary Materials: Figure S1: FH-NRDE2 interacts with MTR4; Figure S2: NRDE2 interactants are implicated in RNA processing and chromatin-associated processes; Figure S3: NRDE2 modulates splicing. Table S1: NRDE2 interactants identified by mass spectrometry in HEK293T or HeLa S3 stably expressing Flag-HA-NRDE2.; Table S2: NRDE2 interactants identified in both HEK293T or HeLa S3 Flag-HA-NRDE2-expressing cells or in HEK293T- or HeLa S3-Flag-HA-NRDE2-expressing cells specifically. Table S3: Antibodies used in this study. Table S4: Oligonucleotides used in this study.

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Data Availability Statement: The data supporting the findings of this study are available from the corresponding authors upon request. All raw sequencing data and processed files (aligned reads, count tables, and analysis outputs) have been deposited in GEO under accession number GSE300903.

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