Charles University in Prague

Faculty of Science

Developmental and Cell Biology

Mina Rajković

Functional significance of DHX38 in pre-mRNA splicing and its role in the mechanism of retinitis pigmentosa

Funkční význam DHX38 při splicování pre-mRNA a jeho role v mechanismu retinitis pigmentosa.

Doctoral Thesis

Supervisor: Prof. Mgr. David Staněk, Ph.D.

Department of RNA Biology, Institute of Molecular Genetics of the Czech Academy of Sciences

Prague, 2024.

I hereby declare that I wrote the thesis independently and I cited all informational sources. This work or a substantial part of it was not presented to obtain another academic degree or equivalent.

Prague, 2024. Mina Rajković

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 2024. Mina Rajković

It was exciting, hard and full of RNA and life knowledge journey.

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To my friends, Mum, Dad, Azra, and Stefan, thank you for your endless support, motivation and love. Bojana, my comrade!

In the end, I would like to dedicate all the next pages to my biggest life inspirations and loves, Radoslav and Vukan. ❤️

I did it!

Sincerely, your Mina.

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Abstract

Retinitis pigmentosa (RP) is an eye disorder impacting around two million people worldwide. In this work, we analyzed the effect of an amino acid substitution within the RNA helicase DHX38 (Prp16), which gives rise to RP. In yeast, Prp16 has been identified as a helicase necessary for the second step of splicing. Our research showed DHX38's interaction with crucial splicing components relevant to both steps of splicing, although we did not observe that the RP mutation changes these interactions. We then downregulated DHX38 and monitored splicing changes. While we noted only minor changes in overall splicing, we detected 71 altered alternative splicing events. We then investigated the role of DHX38 in the splicing of genes specific to the retina. Our results spotlighted the dependence of FSCN2 splicing on DHX38. Intriguingly, the DHX38 RP variant exhibited an inhibition on RHO splicing. Lastly, we showed that the overexpression of DHX38 promoted the usage of both canonical and cryptic 5' splice sites within the HBB splicing reporter. In summary, our data show that DHX38 is a splicing factor that promotes the splicing of cryptic splice sites and it is involved in alternative splicing. Furthermore, we provide evidence suggesting that the RP-associated substitution G332D influences the splicing activity of DHX38.

Abstrakt

Retinitis pigmentosa (RP) je oční porucha, která postihuje přibližně dva miliony lidí po celém světě. V této práci jsme analyzovali vliv záměny aminokyselin v RNA helikáze DHX38 (Prp16), která způsobuje RP. V kvasinkách byla Prp16 identifikována jako helikáza nezbytná pro druhý krok sestřihu. Naše výzkumy ukázaly interakci DHX38 s klíčovými složkami sestřihu relevantními pro oba kroky sestřihu, i když jsme nepozorovali, že by mutace způsobující RP tyto interakce měnila. Poté jsme snížili expresi DHX38 a sledovali změny v sestřihu. Zatímco jsme zaznamenali pouze menší změny v celkovém sestřihu, detekovali jsme 71 změněných alternativních sestřihových událostí. Dále jsme zkoumali roli DHX38 v sestřihu genů specifických pro sítnici. Naše výsledky ukázaly závislost sestřihu FSCN2 na DHX38. Dále jsme zjistili, že DHX38 RP varianta inhibuje sestřih RHO. Nakonec jsme ukázali, že nadměrná exprese DHX38 podporuje použití jak kanonických, tak kryptických 5' sestřihových míst v HBB sestřihovém reportéru. Souhrně naše data ukazují, že DHX38 je sestřihový faktor, který podporuje sestřih kryptických sestřihových míst a je zapojen do alternativního sestřihu. Navíc poskytujeme důkazy naznačující, že substituce G332D spojená s RP ovlivňuje sestřihovou aktivitu DHX38.

Literature review

History of splicing

A new era in molecular biology began when Sydney Brenner posited messenger RNA (mRNA) as an intermediate between genes and proteins (Brenner et al., 1961). Around the same time, Hoagland and his colleagues identified transfer RNA (tRNA) in bacteria (Hoagland et al., 1958). In the following years, hetero-nuclear hnRNA, small-nuclear snRNAs and restriction endonucleases were described (Warner et al., 1966; Weinberg and Penman, 1968; Holmes et al., 1972; Robertson, et al., 1972). With these new insights, RNA became a rising star in the field of research.

The next step was to describe the synthesis of this molecule. Researchers used adenoviruses as a model system (Berge et al. 1977; Chow and Broker, 1978). Infection of eukaryotic cells with this adenovirus resulted in long primary RNA transcripts containing numerous segments between coding regions, which were called introns (Berget et al., 1977; Chow and Broker, 1978).

A year later, introns were discovered in hemoglobin and immunoglobin genes (Darnell, 1978). The phenomenon of removing introns from tRNA was characterized and the splicing reaction was described. Introns are excised as a unique linear molecule with 5′-hydroxyl and 3′-phosphate termini (Knapp et al., 1979).

The complex containing elements for intron removal is called the spliceosome and was discovered by Brody and Abelson in 1985 when they conducted in vitro splicing reactions of pre-mRNA in a yeast extract by glycerol gradient centrifugation (Brody and Abelson, 1985).

The assembly of this machinery on pre-mRNA is a stepwise process (Tardiff and Rosbash, 2006). This unique feature depends on intron sequences within pre-mRNA and particles called U snRNPs.

Splicing elements

Introns

We distinguish two types of introns on messenger RNA.

Major or **U2-dependent introns** (U2 intron) are ubiquitously expressed. The boundaries of the U2 intron follow a GT-AG rule, where the GT dinucleotide marks the start of the intron and is called the **5' splice site** (5'SS) while the AG marks its end and is called the **3' splice site** (3'SS) (Levine and Durbin, 2001). Usually, 18-44 nucleotides upstream of the 3' splice site is located **branch point** and its motif has conserved adenine (A) and thymine (T) in a short sequence of CTRAY (Mercer et al., 2015). The efficiency using the branch point is increased by the **polypyrimidine tract** which also aids 3'SS recognition (Figure 1). Experiments have shown that the strongest polypyrimidine tracts contain 11 continuous uridines, whereas a smaller number of repeated uridines are less effective in 3'SS selection (Coolidge, et al., 1997).

The other class of introns are the **minor** or **U12-dependent introns** (U12 intron), which follow the AT-AC rule. This type of introns has highly conserved 5'SS as well as branch point sequences lacking polypyrimidine (Burge et al., 1998).

These two classes of introns differ in the composition of spliceosomal proteins, the rate of intron removal and the abundance of snRNP, with the number favoring major introns and snRNP (Montzka and Steitz, 1988; Pessa et al., 2006; Tarn and Steitz, 1996). Despite structural differences and an "alternative" spliceosomal machinery that splices U12 introns, the splicing mechanism and co-transcriptional nuclear splicing are shared features (Singh and Padgett, 2009).

In cells, minor introns are present in less than 1% of cases. Despite their low abundance, U12 introns are found in genes with very important functions such as genes belonging to the voltage-gated I on channel superfamily (Wu and Krainer, 1999; Yeo et al., 2007). Between 700-800 genes carry one U12-type intron (Turunen et al., 2013), about 50 genes contain two U12-type introns and a few of them have three U12-type introns. In the genes containing U2 and U12 together, the removal of this type of intron can be limiting (Patel et al., 2002).

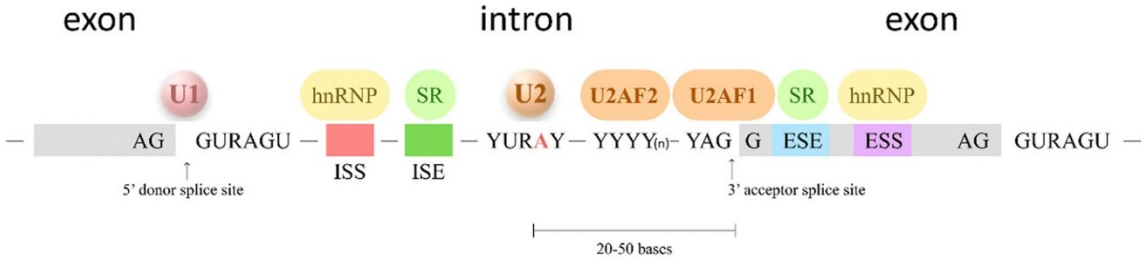


Figure 1. Conserved sequences at exon/intron boundaries in U2 intron.

Evolutionarily conserved sequences are recognized by RNAs and specific proteins. 5' donor splice site and 3' acceptor splice sites, R – purine, Y – pyrimidine, red A represents the base mediating branching of intron lariat, YYYY(n) - polypyrimidine tract. ESE, ESS- exonic splicing enhancer and silencer, ISS, ISE- intron splicing silencer and enhancer are necessary for splice site selection (further in the text).

U snRNP

U snRNPs are a symbiosis between uridine-rich small nuclear RNA and many proteins. The U snRNAs involved in splicing are U1, U2 U4, U5 and U6 snRNA where the numbers before U roughly correlate with the abundance of these RNAs in the cells. U snRNPs are a symbiosis between uridine-rich small nuclear RNA and many proteins. The U snRNAs involved in splicing are U1, U2 U4, U5 and U6 snRNA where the numbers before U roughly correlate with the abundance of these RNAs in the cells (Figures 2, 3). To form a functional complex in the snRNP, the U snRNA undergoes trafficking between the nucleus and the cytoplasm (reviewed in Patel and Bellini, 2008).

First, RNA polymerase II in the nucleus transcribes U1, U2, U4, and U5 snRNAs and when 7-methylguanosine (monomethyl-cap) is added, it signals snRNA export (Hamm and Mattaj, 1990). In the cytoplasm, in an ordered way, snRNA joins with Sm proteins (E, F, G, D1, D2, D3 and B/B') (Shaw et al., 2008). The next step is the import of U snRNPs into the nucleus, where they are targeted for modification in Cajal bodies (pseudouridylation and 2′-O-methylation) (Karijolich and Yu, 2010; Bohnsack and Sloan, 2018).

U6 is transcribed by RNA polymerase III (Reddy et al., 1987) and does not leave the nucleus thanks to Lsm proteins (Lsm 2, 3, 4, 5, 6, 7 and 8), which act as retention signals (reviewed in Didychuk et al., 2018). Modification of the U6 snRNA occurs during its transition through the nucleolus (Lange and Gerbi, 2000).

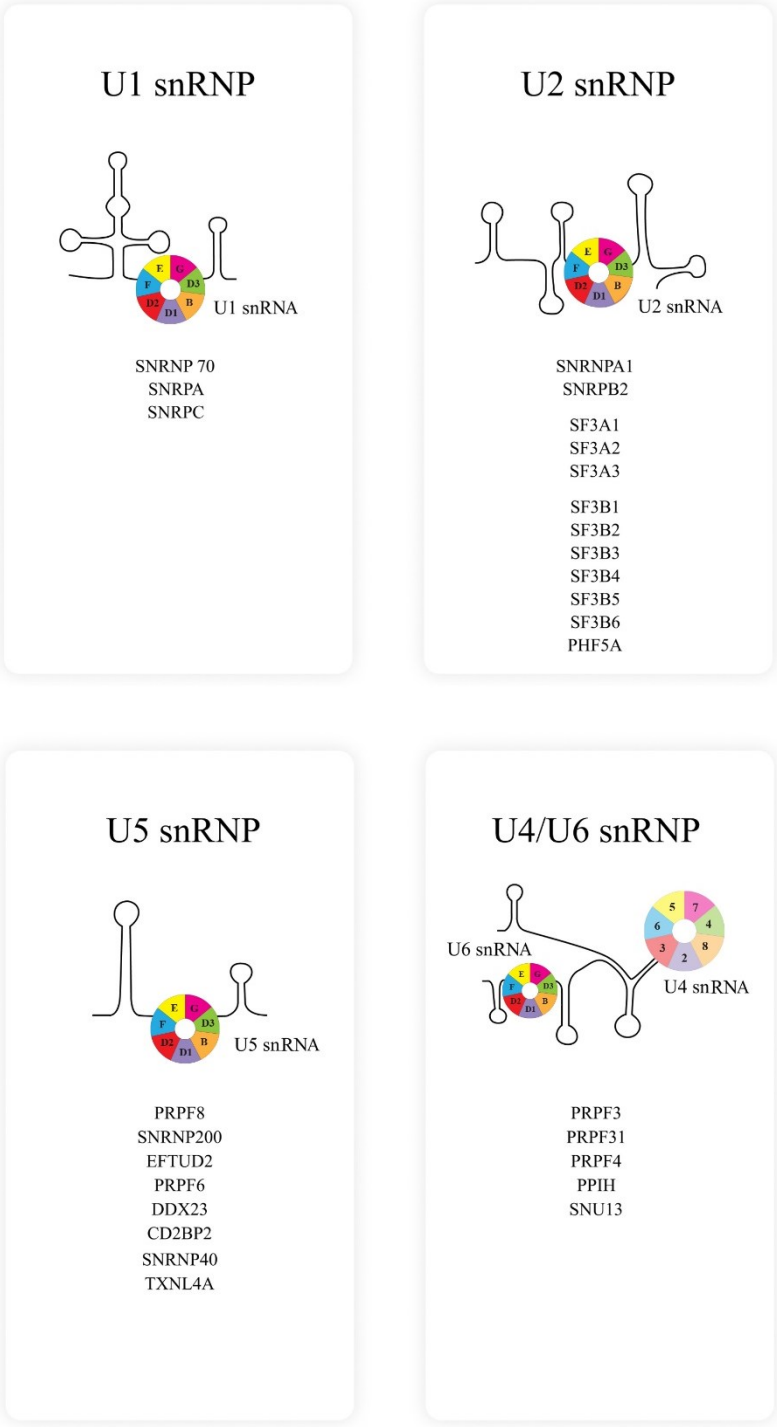
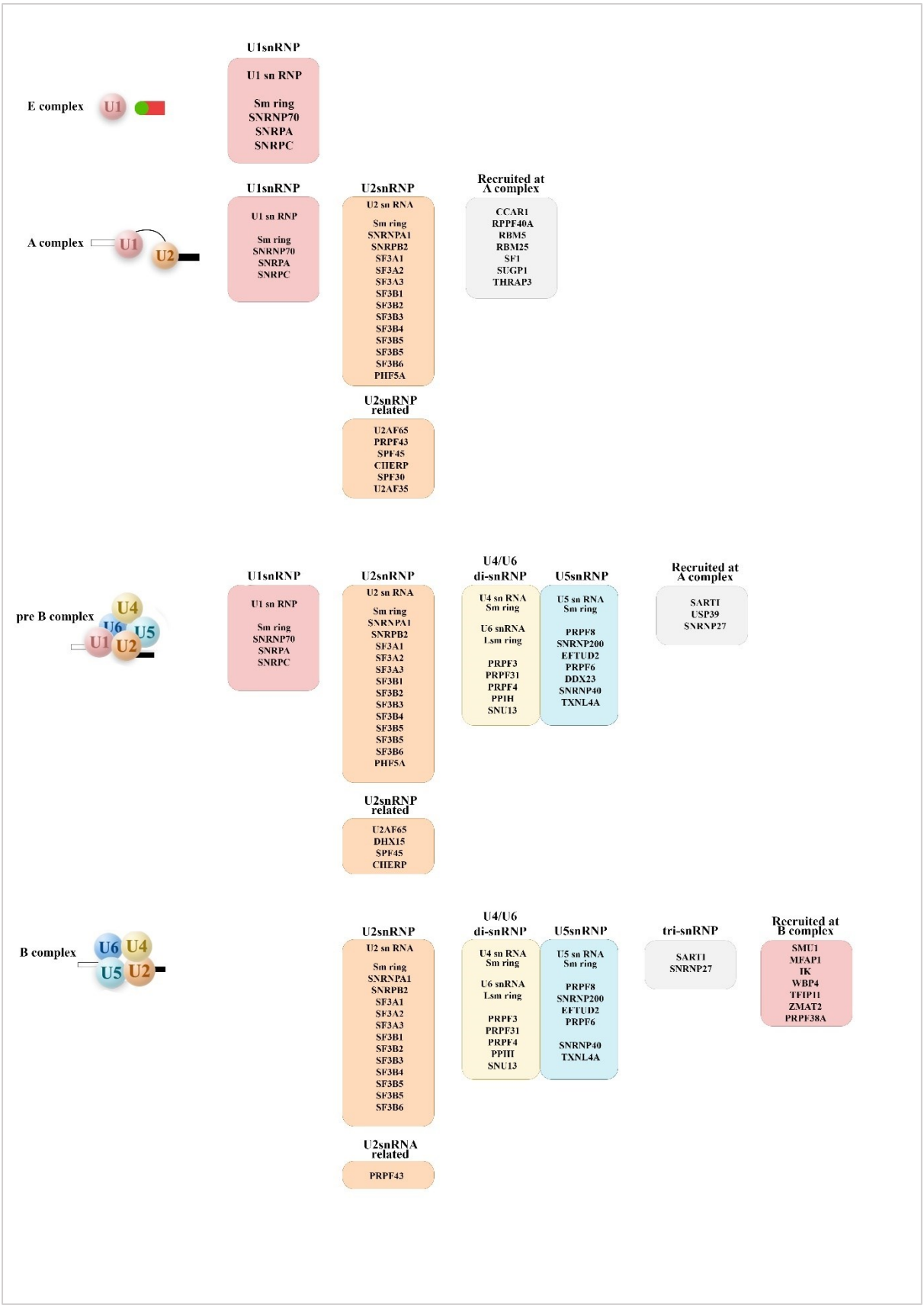


Figure 2. Basic components of the major U snRNP complex.

Individual snRNPs are composed of U snRNA (U1 snRNA, U2 snRNA, U5 snRNA, U4 snRNA, U6 snRNA), a common core of seven Sm proteins (B/B', D3, D2, D1, E, F, and G) and Lsm proteins (2, 3, 4, 5, 6, 7, 8), U snRNP specifically-bound proteins. U snRNAs are shown in their presumed secondary structures. Based on (Will and Lührmann, 2011).

Splicing proteins are highly conserved between Saccharomyces pombe (fission yeast), Saccharomyces cerevisiae (budding yeast) and humans (Käufer and Potashkin, 2000) with the highest similarity between fission yeast and humans (Käufer and Potashkin, 2000; Fair and Pleiss, 2017). In terms of the mechanism of splicing, fission yeast is thought to be an intermediate between S. cerevisiae and humans, with an evolutionary advance towards alternative splicing. Overall, splicing in yeasts is simpler due to the small number of intron-containing genes, poor intron number per gene, the lack of SR protein (in the case of budding yeast) and the very low number of hnRNPs (Davis et al., 2000). The splicing mechanism between yeast and humans is conserved through evolutionary history (Figure 4).


E complex
U1snRNP
U1 sn RNP
Sm ring
SNRNP70
SNRPA
SNRPC
A complex
U1snRNP
U1 sn RNP
Sm ring
SNRNP70
SNRPA
SNRPC
U2snRNP
U2 sn RNA
Sm ring
SNRNPA1
SNRPB2
SF3A1
SF3A2
SF3A3
SF3B1
SF3B2
SF3B3
SF3B4
SF3B5
SF3B5
SF3B6
PHF5A
U2snRNP related
U2AF65
PRPF43
SPF45
CHERP
SPF30
U2AF35
Recruited at A complex
CCAR1
RPPF40A
RBM5
RBM25
SF1
SUGP1
THRAP3
pre B complex
U1snRNP
U1 sn RNP
Sm ring
SNRNP70
SNRPA
SNRPC
U2snRNP
U2 sn RNA
Sm ring
SNRNPA1
SNRPB2
SF3A1
SF3A2
SF3A3
SF3B1
SF3B2
SF3B3
SF3B4
SF3B5
SF3B5
SF3B6
PHF5A
U2snRNP related
U2AF65
DHX15
SPF45
CHERP
U4/U6 di-snRNP
U4 sn RNA
Sm ring
U6 snRNA
Lsm ring
PRPF3
PRPF31
PRPF4
PPIH
SNU13
U5snRNP
U5 sn RNA
Sm ring
PRPF8
SNRNP200
EFTUD2
PRPF6
DDX23
SNRNP40
TXNL4A
Recruited at A complex
SARTI
USP39
SNRNP27
B complex
U2snRNP
U2 sn RNA
Sm ring
SNRNPA1
SNRPB2
SF3A1
SF3A2
SF3A3
SF3B1
SF3B2
SF3B3
SF3B4
SF3B5
SF3B5
SF3B6
U2snRNA related
PRPF43
U4/U6 di-snRNP
U4 sn RNA
Sm ring
U6 snRNA
Lsm ring
PRPF3
PRPF31
PRPF4
PPIH
SNU13
U5snRNP
U5 sn RNA
Sm ring
PRPF8
SNRNP200
EFTUD2
PRPF6
SNRNP40
TXNL4A
tri-snRNP
SART1
SNRNP27
Recruited at B complex
SMU1
MFAP1
IK
WBP4
TFIP11
ZMAT2
PRPF38A

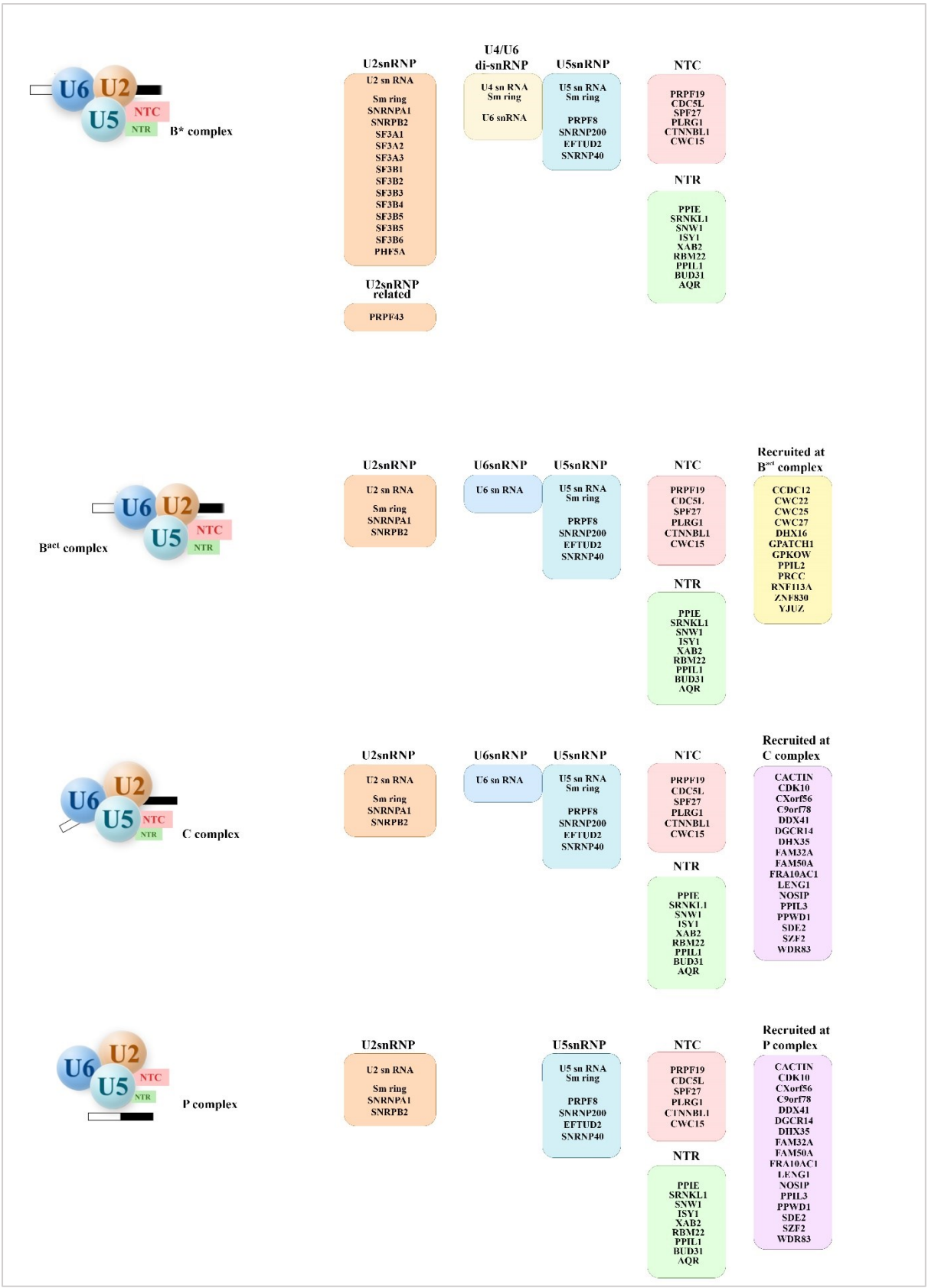


Figure 3. Detailed composition of snRNP and snRNP-associated components in the spliceosome.

Based on (Agafonov et al., 2011) (Kastner et al., 2019).

Splicing dynamics: Assembly, Catalysis and Disassembly

Constitutive splicing

The earliest start of splicing process begins with the recognition of the 5'SS, 3'SS and branch point. This boundary marking can be attributed to either exon definition or intron definition, depending on the size of the exons and introns.

In the case of exon definition, splicing proteins first identify pairs of splice sites located on exons. This phenomenon is mainly observed in vertebrate genes, which are characterized by short exons and large introns. In contrast, the definition of an intron is more common in the lower eukaryotes, where small introns are flanked by long exons (De Conti et al., 2013).

In a highly orchestrated process, approximately 150 splicing proteins dynamically engage in the splicing cycle to excise introns. The initial complex formed, known as the commitment **E complex**, prepares the pre-mRNA for spliceosome assembly (Figure 4) (Kondo et al., 2015). U1C plays a key role in strengthening the base-pair interaction between the U1 RNA and the 5' splice site (Heinrichs et al., 1990; Zhuang and Weiner, 1986). Recognition of the branch point by SF1, PYT by U2AF2 and 3'SS by U2AF1 completes this step.

Upon the formation of the **A complex**, splice sites are stabilized and the U2 snRNP recognizes the branch site (Query et al., 1997). Prp5 and Sub2 helicases facilitate the displacement of SF1 (Zhuang and Weiner, 1986; Konarska and Sharp, 1987). The subsequent phase involves the incorporation of the pre-formed U4/U6.U5 tri-snRNP, leading to the formation of the **pre-B complex** (Boehringer et al., 2004; Nguyen et al., 2015).

Following this, U1snRNP is released with the assistance of Prp28, and the pre-B complex transitions into the stable **B complex** (Staley and Guthrie, 1999; Boesler et al., 2016). The translocation of Brr2 from one end of the U4 snRNA to the other initiates spliceosome activation. This unwinding of the U4/U6 duplex exposes the central region of the U6 snRNA, which then forms a helical structure with the U2 snRNA, resulting in the U2/U6 helix (Madhani and Guthrie, 1992). This conformation adopts a catalytically active conformation (Legrain et al., 1988; Datta and Weiner, 1991). Both the nineteen complex (NTC) and the NTC-related complex (NTR) contribute to a catalytic complex and the formation of the **B* complex** (reviewed in Hogg et al., 2010).

The importance of these factors in stabilizing the $\mathbf{B^{act}}$ complex was further validated by functional experiments (Fica et al., 2013).

The initial transesterification reaction occurs during a step in which a conserved adenosine located at the branch point initiates an attack on the nucleotide at the 5' end of the intron, forming an intron lariat while exposing the 3' end of the upstream exon. The helicase Prp2 promotes the binding of the branch factors and the spatial alignment of the 5'SS and the BP for branching (Semlow et al., 2016). These rearrangements within the spliceosome mark the start of the first splicing step, referred to as the branching reaction.

After the first transesterification reaction, the **C complex** is established. Comprehensive analyses of the protein composition of the C complex indicate an association with over 150 proteins (Jurica et al., 2002; Zhou et al., 2002).

The transition from the C complex to the **C* complex** involves the helicase Prp16, which orchestrates the remodelling of the spliceosome. This process enables the dissociation of branch factors and facilitates the precise alignment of the 3'SS into the active site. This alignment is guided by the U5 snRNA, which promotes the juxtaposition of the 5' and 3' exons (Newman and Norman, 1992). These cumulative actions culminate in the occurrence of the second transesterification reaction, often referred to as the ligation reaction. During this step, the free 3' hydroxyl group of the 5' exon attacks the 5' end of the 3' exon, forming a functional mRNA molecule and an intron-lariat structure.

The completion of these events leads to the formation of the **P complex**. The release of exons is facilitated by the Prp22 helicase, leading to the formation of the intron-lariat spliceosome (ILS) complex. The subsequent disassembly of the ILS complex is orchestrated through the activity of the helicase Prp43 (Liu et al. 2017) (Figure 4).

Each of these successive steps is monitored several times. Specific sites on the pre-mRNA are recognized by different factors, helping to ensure splicing accuracy.

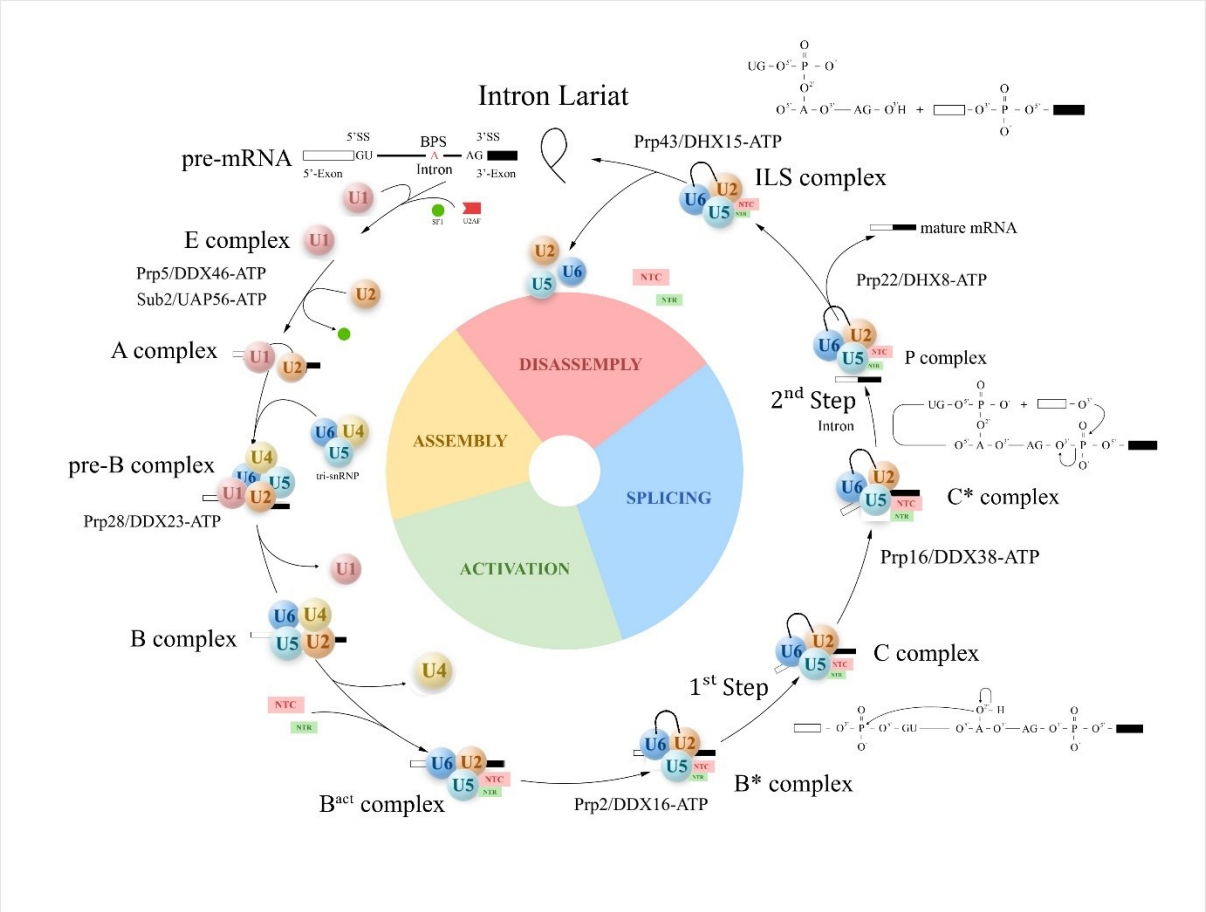


Figure 4. Simplified scheme of the splicing process.

Exon and intron sequences are indicated by boxes and lines, respectively. U snRNPs are represented as coloured circles. Multiple RNA ATPases/helicases are indicated (annotation in yeast slash in humans). Based on (Borišek et al. 2021).

Alternative splicing

The idea of alternative splicing came in 1978 when scientists were trying to understand why cells have about 25,000 protein-coding genes but produce more than three times as many proteins. This discrepancy arises because the final splicing products of many human genes encode multiple RNA variants, which give rise to different protein isoforms in terms of structure and function.

The main categories of alternative splicing (AS) events include “cassette” exon skipping, alternative 5’ and 3’ splice site selection, alternatively retained introns and mutually exclusive exons (Figure 5). These AS events depend on the complexity of the organism, type of cell,

stage of development and cellular differentiation. The mechanism itself occurs in different frequencies between different living kingdoms.

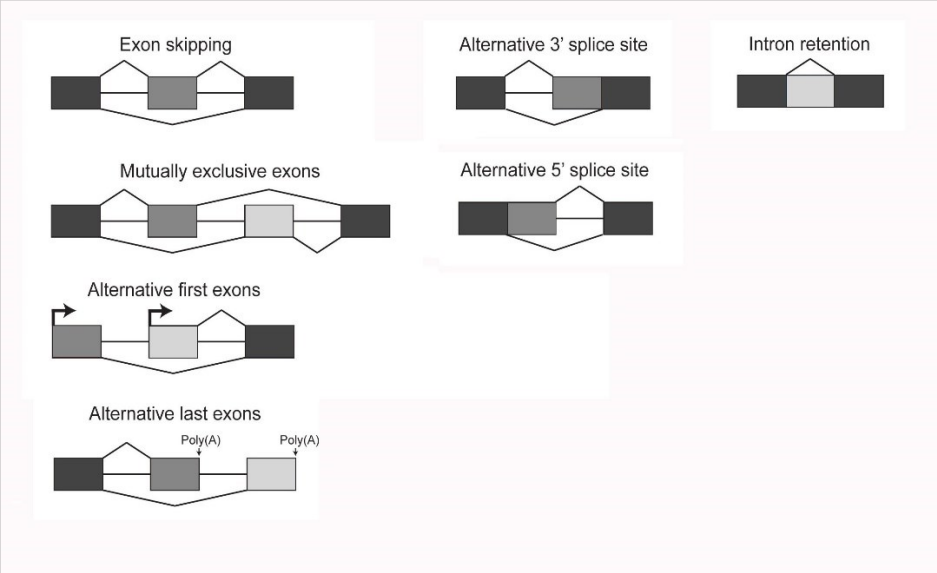


Figure 5. Seven major types of alternative splicing events.

Constitutive exons are represented as dark grey boxes, alternatively spliced regions in lighter greys, introns are represented by solid lines in between and solid lines above indicate splicing activities. The frequency of the specific type of AS varies among species. Exon skipping is more abundant in eukaryotes and intron retention is most common in lower metazoans, fungi and protozoa. Based on (Park et al., 2018).

Elements on the pre-mRNA that are necessary for the AS process (cis-acting elements) are exonic splicing enhancers (ESEs) and intronic splicing enhancers (ISEs) and they are bound by positive trans-acting factors, such as SR proteins (serine/arginine-rich family of nuclear phosphoproteins). In contrast, exonic splicing silencers (ESSs) and intronic splicing silencers are bound by negative trans-acting factors, such as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Figure 1). Enhancers tend to be more prominent in constitutive splicing, whereas silencers are comparatively more important in alternative splicing (reviewed in Wang and Burge, 2008). The partnership between these elements results in the promotion or inhibition of spliceosome assembly of the weak splice sites, respectively (reviewed in Wang et al., 2015).

SR proteins are named after an arginine/serine-enriched domain. Phosphorylation and dephosphorylation of these serines facilitate the trafficking of these proteins between the nucleus and the cytoplasm ensuring their role in the regulation of alternative splicing (reviewed in Ghosh and Adams, 2011). In addition to the 12 evolutionary conserved splicing factors that

belong to this family, there are a large number of RS domain-containing proteins also known as SR-like or SR-related proteins. Typically, they contain one or two copies of an RRM (RNA recognition motif) at the N-terminal domain that confers RNA-binding specificity and a C-terminal RS domain that promotes protein-protein interactions that facilitate recruitment of the spliceosome and contact to the pre-mRNA (van Der Houven et al., 2000; Zhu and Krainer, 2000). Its role in splicing activation, coupling of splicing to transcription, modulation of protein SUMOylation, splicing regulation, chromatin remodelling, transcription and cell cycle has been well established (reviewed in Long and Caceres, 2009; Twyffels, et al., 2011).

Heterogeneous nuclear ribonucleoproteins (hnRNPs), a diverse family of proteins, have no universal definition describing their general structure or function. However, they share common features such as their association with nascent pre-mRNAs and the presence of RNA-binding domains (Piñol-Roma et al., 1988). This family comprises around 16 types of hnRNPs, named alphabetically from hnRNP A1 to U (reviewed in Geuens et al., 2016). The functions of these proteins depend on their cellular localization. They are involved in a wide range of cellular activities such as transcription, pre-mRNA processing, translation and turnover (reviewed in Han et al., 2010).

RNA Helicases

Omnipresent and heterogenous groups of RNA helicases are central RNA-modulating molecules in cells. They accompany RNA throughout the entire life cycle.

The presence of seven to eight conserved motifs in the sequence identifies RNA helicases (de la Cruz et al., 1999). They are classified into six families, with superfamilies SF3, SF4, and SF5 being oligomeric proteins found in viruses or bacteria (reviewed in Singleton et al., 2007). The majority of RNA helicases belong to the SF1 and SF2 superfamilies. These non-oligomeric proteins are further divided into subfamilies based on sequence, structural, and mechanistic differences (reviewed in Fairman-William et al., 2010:1). The SF1 family includes subfamilies such as the Upf1-like subfamily, which are primarily involved in splicing and nonsense-mediated decay. The DEAD-box helicases, DEAH-RHA helicases, RIG-I related proteins, Ski2-like proteins and the NS3/NPH-II subfamily, which consists exclusively of proteins of viral origin, are the five RNA helicase subfamilies that make the SF2 superfamily (reviewed in Leitão et al., 2015).

In addition to their structural classification, RNA helicases are classified based on the mechanism by which they unwind nucleic acid.

The first group, so-called canonical RNA helicases, unwind RNA duplexes by binding to a single-stranded region of the RNA and moving along the duplex. The energy for this process depends on ATP.

The second group belongs to the DEAD-box family and consists of monomeric helicases that locally separate RNA duplexes. The mechanism involves loading the enzyme onto RNA duplexes, which is s facilitated by ATP binding. Once bound, the helicase can locally open RNA duplexes, promoting the formation of single strands. Importantly, ATP is required for the release of the free enzyme along the RNA strand after the local unwinding process (reviewed in Jankowsky, 2011; Leitão et al., 2015).

Since the first description of the role of RNA helicases in ribosome biogenesis and rRNA maturation (de la Cruz et al., 1999), numerous papers have been published linking these proteins to every step of the translation process. Only in ribosome biogenesis have more than ten RNA helicases been described to play a role (reviewed in Westhof and Fritsch, 2000; Martin et al., 2013). Prp43/DHX15 is the pioneer RNA helicase linked with ribosome biogenesis with all binding sites on the pre-rRNA being identified (Bohnsack et al., 2009; Lebaron et al., 2009). To ensure the correct position of the ribosome entry, DHX29 binds directly to the 40S ribosome subunit, stimulating the initiation of translation (Pisareva et al., 2008; Dhote et al., 2012). During the later stages of translation initiation, DHX33 has been reported to promote 80S ribosome assembly (Zhang et al., 2015). In the complex translation process DDX5, DDX7 and DDX36 are well-studied RNA helicases. They act as facilitators or repressors of G4 quadruplex secondary structure resolution (Vaughn et al., 2005; Dardenne et al., 2014; Sun et al., 2022).

With regard to mRNA decay, DHX36 has been shown to be involved in AU-rich element-mediated mRNA decay (Tran et al., 2004). The helicase Upf1 is a central component of the NMD machinery (Franks et al., 2010). In the decapping mRNA decay pathway, the RNA helicase has been shown to impede ribosome transit rates on the transcript making them vulnerable to decapping (Sweet et al., 2012).

RNA helicases have many roles in cellular stress responses, with helicases such as p54, DDX3, and eIF4A, PHAU being associated with stress granules (Briolat and Reysset, 2002; Wilczynska et al., 2005; Mazroui et al., 2006; Chalupníková et al. 2008). Human RCK/p54 facilitates P-body formation (Chu & Rana, 2006) (Figure 6).

Additionally, RNA helicases play a role in developmental processes such as spermatogenesis where DDX4 or Vasa is indispensable (Shibata et al., 2004; Costa et al., 2006) and in embryonic and ovarian development is DP103 (Mouillet et al., 2008).

By utilizing viral RNA helicases or hijacking cellular RNA helicases, these molecules play a pivotal role in detecting viral RNA, monitoring its replication, and facilitating antiviral immune responses (reviewed in Steimer and Klostermeier, 2012). In addition, RNA helicase function includes nucleation of protein-protein interactions and maintenance of the RNP complex in a long-lived, stable configuration in a multistep process called RNA clamping (Ballut et al., 2005).

Finally, RNA helicases have a role in viral infection (Cheng et al., 2018), ageing (Park et al., 2017), neurological diseases such as ALS, PD, and AD (reviewed in Castelli et al., 2022), DNA damage repair as well as tumorigenesis (reviewed in Xie et al., 2022) and cancer (reviewed in Fuller-Pace and Moore, 2011).

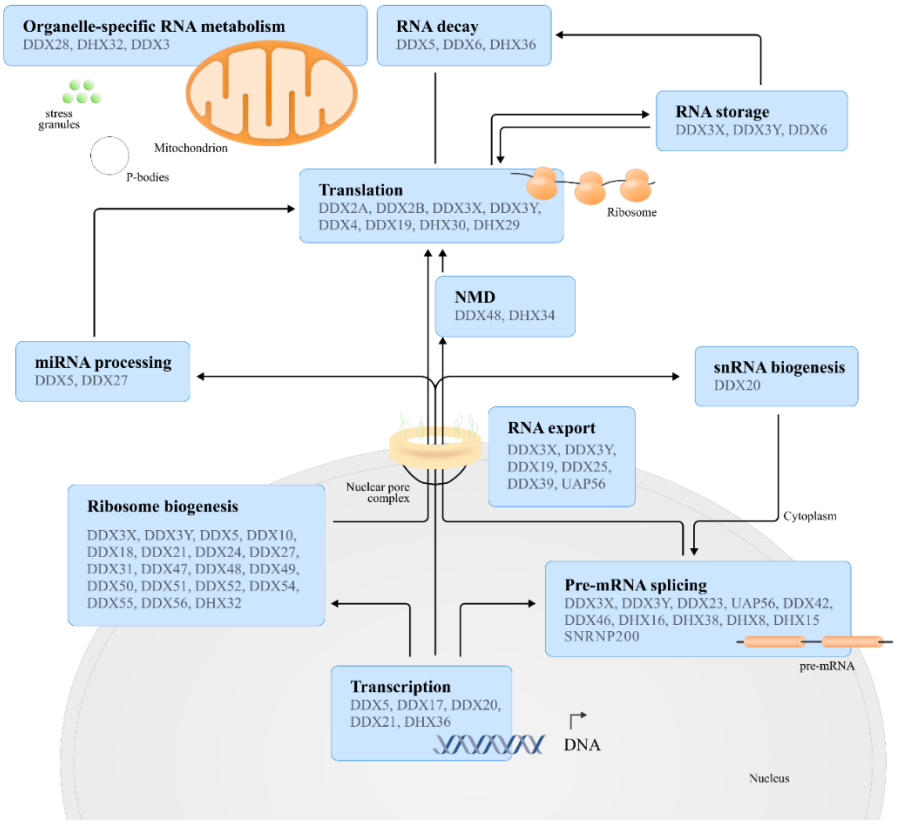


Figure 6. Role of RNA helicase in a cellular process.

In the nucleus, the described role of RNA helicases includes ribosome biogenesis, transcription and pre-mRNA splicing. In the cytoplasm, these include processes like microRNA (miRNA) processing, nonsense-mediated decay (NMD), and protein translation, as well as organelle-specific RNA metabolism. These enzymes are required for the directional transport of mRNA molecules between the nucleus and cytoplasm. Based on (Linder and Jankowsky, 2011).

In prokaryotes, these helicases are less abundant but highly required for cellular adaptation to abiotic stress, RNA turnover, transcription, ribosome biogenesis, initiation and stimulation of translation, and small RNA metabolism (reviewed in Owttrim, 2013).

There are indications that RNA helicase functions are conserved pathways between bacteria and yeast (reviewed in Kelley et al., 2003).

DExD/H helicase: Key players in the dynamic of RNA splicing

DExD/H helicase is the join name for the DEAD-box and the related DEAH, DExH and DExD families that shear structurally conserved motifs (reviewed in Tanner and Linder, 2001; Caruthers and McKay 2002). The distinctive feature for which these proteins are named consists of conserved motifs made of the amino-acid sequence aspartate-glutamate-alanine-aspartate, abbreviated D-E-A-D. These characteristic motifs for DEAD-box proteins are found in several hundred proteins (Linder et al., 1989; Silverman et al., 2003).

In addition to this domain, they have the N and C-terminal domains, variable in size and composition and give specificity to each enzyme (reviewed in Caruthers and McKay, 2002). The most conserved and studied domain is the helicase core domain. It contains two domains called RecA-like due to their structural similarity to the bacterial recombinase A protein (RecA). These domains change orientation with respect to each other. This change is critical for the enzyme function, where ATP hydrolysis occurs in closed states (Andreou and Klostermeier, 2012). The helicase motifs involved in RNA binding are located on the opposite side of the helicase core. These two domains are further structurally and functionally subdivided (reviewed in Caruthers and McKay, 2002). In 1982, Walker and colleagues identified and defined motifs I or Walker A and motif II or Walker B in their role in NTP binding (Walker et al., 1982). Subsequent motifs, Ia, Ib and Ic, participate in RNA-binding and structural rearrangements during ATP binding and hydrolysis (reviewed in Schwer and Meszaros, 2000; Cordin et al., 2006). Motif II is characterized by four amino acids. Together with motif I, is required for ATP binding and hydrolysis. Motif III is necessary for the coordination of the nucleic acid binding site and the NTP binding site. Motif IV is thought to bind ssRNA. Together with motif V, it is involved in substrate binding, while motif VI links NTP binding and hydrolysis with conformational changes needed for helicase activity (reviewed in Hernández-Díaz et al., 2021) (Figure 7).

The DEAD subfamily has an additional 9 amino acid sequence called the Q motif, named after an invariant glutamine that is essential for guiding duplex unwinding and RNA-stimulated hydrolysis of the triphosphate (Tanner et al., 2003; Cordin et al., 2004). On the other hand, the DEAH-box helicase subfamily has other unique domains such as winged helix (WH), ratchet-like, and oligosaccharide-binding fold (OB fold) domains (reviewed in De Bortoli et al., 2021). The C-terminal domain (CTD) domain, which is specific to DEAH-box helicases makes a difference in the unwinding mechanism compared to DEAD-box helicases. Together, CTD and the helicase core form an RNA-binding tunnel that helps DEAH-box helicases to translocate on long RNA segments. The CTD of the DEAH box helicases closely interacts with the RecA1 domains, which help other proteins regulate the helicase activity by binding to the CTD. DEAD-box helicases do not have this domain and they efficiently unwind duplexes of up to ~10 to 12 bp (Bottaro et al., 2014). Regardless of the duplex length, these helicases use one ATP per unwinding. In addition, most DEAD-box helicases do not favour specific RNA sequences or structures (reviewed in Putnam and Jankowsky, 2013).

There are 25 of these proteins in yeast, 13 fewer than in humans. They show conservation and role in almost all aspects of RNA life (reviewed in Linder, 2006).

Figure 7. Schematic illustration of the splicing helicase organization.

(A) DEAD helicase organization showing N, C terminal domains and the conserved helicase core. The catalytic core consists of two RecA-like domains with functional motifs involved in ATP and RNA binding. **(B)** DEAH helicase organization showing N and C terminal domains containing winged helix (WH), helical bundle (HB), oligosaccharide (OB) domains and the conserved helicase core. **(C)** Ski2-like helicase organization showing N and C terminal domains containing WH, HB, OB, helix-loop-helix (HLH) and immunoglobulin-like (IG) domains and the conserved helicase core. The catalytic core consists of two RecA-like domains which contain functional motifs involved in ATP and RNA binding. Motifs Q, I, II and VI are involved in ATP binding, and motifs Ia, Ib, Ic, IV, IVa and V are involved in RNA binding. Motifs III and Va are related to the linking of ATP hydrolysis and double-strand unwinding. Based on (Hernández-Díaz et al., 2021).

Functional role of DExD/H helicase in pre-mRNA processing

Helicases are involved in the highly dynamic and acrobatic movements of splicing.

Members of the DEAD-box family involved in pre-mRNA processing are Prp5/DDX46, Sub2/DDX39 and Prp28/DDX23, representatives of the Ski2-like family are Brr2/SNRNP200 and Prp2/DHX16, Prp16/DHX38, Prp22/DHX32 and Prp43/DHX37 belong to the DExD/H family (Figure 4).

The interaction of spliceosomal helicases with the spliceosome which manipulates mRNA, U snRNAs and snRNPs is only transient. The only exception is SNRNP200/Brr2, which is an essential component of the U5 snRNP.

It is generally accepted that these helicases bind in an ATP-independent manner to the spliceosome and are released from the spliceosome by ATP hydrolysis. In cases where ATP is absent or its function is compromised, the helicases are retained on the spliceosome (Liu et al., 2008).

Despite decades of intensive study in yeast, defining the precise targets, functions, and mechanisms of action of these enzymes remains a challenge. Moreover, each helicase has a role not only in one process but in multiple steps during splicing and spliceosome maturation. Long ago, Hopfield and Ninio proposed the kinetic proofreading mechanism of helicases as a competition between two reactions in which the main reaction proceeds with the optimal product while the secondary reaction discards suboptimal substrates (Hopfield, 1974; Ninio, 1975).

For most splicing helicases their role in increasing splicing fidelity is thought to follow the timer or sensor model of proofreading. In the timer model, the helicase provides a limited time window for a specific event during the splicing cycle. If the optimal substrate completes the proofreading step faster than the helicase can act, it will proceed to the product (Semlow and Staley, 2012). In the sensor model, the helicase rejects the slower suboptimal substrate as a consequence of a difference in the stability of the spliceosome containing a substrate (Semlow and Staley, 2012).

Splicing helicase

DDX39B/ Sub2

It has been suggested that Mud2 or U2AF65 which binds to 3'SS as a heterodimer with BBP, is replaced by Sub2 to enable U2snRNP association with the pre-mRNA (Fleckner et al., 1997; Kistler and Guthrie, 2001). DDX39B or U2AP56 in humans also initially interacts with U2AF65 in an ATP-dependent manner to facilitate spliceosome assembly (Shen et al., 2008). A delayed intermediate containing U2 snRNP and accumulation of the CC2 complex has been shown in studies using Sub2-deficient extracts. (Libri et al., 2001). Depletion or inactivation of SUB2 also affects the mature mRNA levels of intron-containing genes *in vivo* (Libri et al., 2001).

DDX46/ Prp5

In the fission yeast and humans, Prp5 has been shown to associate with U1 snRNPs and U2 snRNPs via its N-terminal domain, bridging U1 and U2 snRNPs to form the prespliceosome (Xu et al., 2004).

Prp5 is stalled on the spliceosome when the branch point is mutated, preventing the recruitment of the tri-snRNP to the spliceosome (Liang and Cheng, 2015). Recent data have shown that a lack of pseudouridines at certain positions of the U2 snRNA leads to reduced affinity of Prp5 for the U2 snRNP and ATPase activity of the helicase (Wu et al., 2016). Prp5 has been proposed to play a role in splicing fidelity control and branch site proofreading by competing with the base pairing between the U2 snRNA and the branch site sequence (Xu and Query, 2007; Liang and Cheng, 2015). Prp5 mutations affect BS detection accuracy (Beier et al., 2019). Structural data showed that when the spliceosome stalls due to a branch point mutation or before the pre-spliceosome complex is formed, Prp5 blocks the next step (Zhang et al., 2021).

DDX23/ Prp28

Guthrie and Straus were the first to identify Prp28 in splicing (Strauss and Guthrie, 1991). It was initially described as a protein necessary for the fidelity of 5'SS recognition by displacing U1 and promoting U1/U6 switching at the 5'SS (Staley and Guthrie, 1999). Years later, its role

was expanded to include proofreading of the 5'SS during spliceosome assembly (Yang et al., 2013).

It has been proposed that Prp28 may destabilize U1-5'SS interactions by affecting protein binding to the U1 snRNA or the 5'SS. Mutations in several U1 snRNP components can overcome the need for Prp28 (Chen et al., 2001; Hage et al., 2009).

In humans, DDX23 has an additional N-terminal domain with RS repeats that are phosphorylated by SRPK2 which helps this helicase to regulate the recruitment of the tri-snRNP to the spliceosome (Mathew et al., 2008).

New data showed its involvement in a stage of spliceosome activation and interaction with the GU dinucleotide on 5'SS by making contact with the U1C, Prp8, Brr2 and Snu114. They showed that the Prp28 ATPase activity is potentiated by the phosphorylated Npl3 (Yeh et al., 2021).

SNRNP200/ Brr2

Brr2 was one of the first RNA helicases to be shown to have unwinding activity (Laggerbaue et al., 1998, Raghunathan and Guthrie, 1998). In dissecting the function of Brr2, several works have revealed that the target for this unwinding activity is the U4/U6 duplex (Raghunathan and Guthrie, 1998; Kim and Rossi, 1999) and it is thought that this unwinding event occurs during the activation of spliceosome directly (Laggerbauer et al., 1998).

SNRNP200 is a component of the U5snRNP and its activity is controlled by the other two members of U5snRNP, Snu114 and Prp8. The tight structural connection between these three proteins has been visualized by cryo-electron microscopy (Häcker et al., 2008). Distinct domains within Prp8 play a crucial role in controlling the unwinding activity of Brr2, RNA binding and ATPase activity (Maeder et al., 2009; Mozaffari-Jovin et al., 2012, 2013). The second protein, Snu114, acts as a signal-dependent switch. It transmits signals to Brr2 to control spliceosome dynamics (Small et al., 2006).

A unique characteristic of Brr2 is its two domains, the N-terminal domain responsible for unwinding and the C-terminal domain serving as an interaction platform for Prp2, Prp16, Slu7 and Ntr2 required for spliceosome disassembly indicating its role in the final step of splicing (van Nues and Beggs, 2001).

Cvačková with her co-workers found that mutations in SNRNP200 reduced splicing of the optimal substrate and enhanced the recognition of cryptic 5'SS (Cvačková et al., 2014a). New

data supports its role in proofreading by preserving 5'SS identity (Cartwright-Acar et al., 2022).

DHX16/Prp2

DHX16 has a significant role in the B^{act} spliceosome and the first catalytic reaction (Warkocki et al., 2009: Agafonov et al., 2011). Prp2 is functionally dependent on Cwc22, as its absence results in the dissociation of Prp2 from the spliceosome (Yeh et al., 2011). Another factor, Spp2 influences the ATPase activity of the helicase in transforming the spliceosome into a state of catalytic activity (Warkocki et al., 2015).

Prp2 translocate to the pre-mRNA in an ATP-dependent manner and this activity destabilizes SF3a/b making the BS adenosine accessible for a nucleophilic attack at the 5'SS (Warkocki et al., 2009; Liu and Cheng, 2012). Recent work has shown that after the catalytic activation, Prp2 dissociates from the spliceosome, along with SF3A and SF3B, facilitating the relocation of the branch duplex to enable catalysis (Schmitzová et al., 2023).

Liu and Cheng were able to show that Prp2 interacts with the intron within a defined segment of between 23 and 33 nucleotides downstream of the branch point (Liu and Cheng, 2012).

Additionally, Prp2's role extends to the displacement of NTC-associated proteins, such as Cwc24, Cwc27, and the RES complex protein Bud13, preventing the premature exit of unspliced pre-mRNAs from the nucleus (Ohrt et al., 2012). It is hypothesized that the ATP-dependent activity of Prp2 drives the activation of spliceosome protein dissociation. At the same time, it creates high affinity binding sites for catalytic step 1 factors such as Yju2 and Cwc2 (Ohrt et al., 2012).

New experiments in S. pombe have shown that most introns require Prp2 for efficient splicing. The degree of Prp2 dependence is intron-specific. They show that dependence on Prp2 for efficient splicing can be either increased or decreased by 5'SS and BP sequences (Hümmer et al., 2021).

DHX38/PRP16

An additional helicase that plays a vital role in enhancing the efficiency of splicing is Prp16. This helicase was initially classified as a splicing protein in the late 1980s in two parallel experiments. In one study a mutant Prp16 in cells resulted in a significant change in the splicing of two transcripts, MATa1 and rp51a (Couto et al., 1987). In the second study, researchers

induced an A to C mutation at the conserved intron branch point within the actin-HIS4 gene, where splicing defects were suppressed by Prp16 (Vijayraghavan et al., 1986).

A few years later, Schwer and Guthrie conducted functional experiments that elucidated the role of Prp16 in the ATP-dependent reaction, necessary for the second step of splicing. They also showed that Prp16 associates transiently with the spliceosome and that protein release from the spliceosome needs ATP hydrolysis by Prp16 (Schwer and Guthrie, 1991).

Notably, a significant decrease in protein ATPase activity in vitro correlated with less efficient branch point identification (Burgess and Guthrie, 1993). It was hypothesized that proofreading is regulated by the rate of ATP hydrolysis by Prp16 with Prp16 influencing whether the lariat continues to splice or is discarded and degraded (Burgess and Guthrie, 1993).

Recent studies show that Prp16 effectively delays the progress of slower substrates by catalyzing the cleavage of the 5' splice site at a reduced rate (Koodathingal et al., 2010). In budding yeast, a function of Prp16 is to enhance the efficiency of the spliceosome by rejecting suboptimal choices before the initiation of a reaction. Additionally, Prp16 plays a crucial role in repositioning substrates for exon ligation following the selection of optimal splice sites (Semlow et al., 2016).

Although Prp16 in yeast is well studied, this is not the case for the human homologue DHX38. A few studies have shown its localization to nuclear speckles and revealed that partial deletion reduces splicing activity (Ortlepp et al., 1998). DHX38 has been identified in B^{act} (Bessonov et al., 2010) and it has been shown that DHX38 cooperates with PRP2 through interactions with GPKOW (Hegele et al., 2012).

DHX8/ Prp22

The first described role of Prp22 was in the release of mature mRNA (Company et al., 1991). Within the C* complex, Prp22 replaces Prp16 and together with Slu7 and Prp18 promotes exon ligation for the majority of pre-mRNA substrates (Schwer and Gross, 1998; Mayas et al., 2006). Schwer and Gross concluded that the involvement of this helicase in the second catalytic step depends on the distance between the BP and the 3' splice site (Schwer and Gross, 1998).

A decade later, it was shown that this is not the only function of Prp22. Mutations in the ATP and RNA unwinding domains of Prp22 allowed the formation of mRNA from aberrant intermediates (Mayas et al., 2006).

A proposed model for the proofreading mechanism of Prp22 suggests that Prp22 can distinguish between complexes that are unable to complete exon ligation before Prp22 action.

Prp22 rejects lariat intermediates that have not been able to reach the exon-ligation conformation in a stable manner by binding to the 3' exon (Strittmatter et al., 2021).

DHX15/ PRP43

Prp43 is a multitasking helicase, present in the A, B, B^{act}, and C complexes of the spliceosome, with moderate abundance in the A and B complexes and fewer in others (Bessonov et al., 2010; Agafonov et al., 2011).

More than two decades ago, the initial role of Prp43 in splicing was described as facilitating the disassembly of the spliceosome (Arenas and Abelson, 1997). More detailed work has shown that at this final stage, Prp43 is required for the ATP-dependent production of lariat and the U2.U5.U6 snRNPs recycling (Martin et al., 2002; Boon et al. 2006; Fourmann et al. 2013). In addition, the potential role of Prp43 in a U4/U6.U5 tri-snRNP reassembly and assembly pathway in Cajal bodies has been suggested (Chen et al. 2017).

Like most members of the family, Prp43 also monitors splicing fidelity. In the process of discarding suboptimal substrates, Prp43 cooperates with Prp16 (Koodathingal et al. 2010b; Mayas et al., 2010). A new study shows that Prp43 is involved in disassembling complexes that are unable of forming a productive branch helix (Maul-Newby et al., 2022).

The role of splicing factors in disease pathogenesis

In the early 1980s, when a retained intron was found in β-thalassemia patients, researchers started to intensively study RNA splicing errors in diseases (Ley et al., 1982).

A frequently cited paper from 2014 predicts that approximately one-sixth of all human genetic diseases resulting from point mutations are from splicing defects (Jian et al., 2014).

Splicing alterations influence every cellular aspect such as transcription, alternative splicing, nonsense-mediated decay, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), post-translational modifications…

Mutations affecting splicing can be categorized as **cis-acting** or **trans-acting**, based on the site of impact (Figure 8).

Cis-mutations are changes within the pre-mRNA sequence. Prominent examples include alterations in the 5' donor or 3' acceptor splice sites, which have been described in detail in cystic fibrosis and familial dysautonomia (reviewed in Daguenet et al., 2015). Due to the difficulty in identifying the precise location of branch point and polypyrimidine tract mutations,

a small number of diseases are connected with mutations in these sequences. The best described are Marfan syndrome with a branch point mutation in the fibrillin 1 and 2 genes and fish-eye disease with LCTA gene mutations. In cystic fibrosis, patients exhibit a short polypyrimidine tract, while transversions in the polypyrimidine tract of the FIX gene result in hemophilia B (reviewed in Lewandowska, 2013).

On the other hand, trans mutations involve changes in UsnRNP proteins, RNA binding proteins and regulators. Mutations in UsnRNP´s biogenesis and assembly have implications for spinal muscular atrophy, Alzheimer's disease, poikiloderma with neutropenia, Prader-Willi syndrome, and retinitis pigmentosa (reviewed in Daguenet et al., 2015).

SF3B1 stands out as the most frequently mutated spliceosome component in cancer, with its mutations identified across various myeloid malignancies (Yoshida et al., 2011). Cancer is connected with both cis and trans mutations and the progression of cancer is often a consequence of aberrant splicing (reviewed in Rahman et al., 2020).

The pathophysiology of tauopathies and neurodegenerative diseases involves dysfunction of RNA binding proteins and RNA splicing (Apicco et al., 2019). Misregulation of SR and hnRNP is connected to cancers, MLS, autism and Hunting disease (reviewed in Cáceres and Kornblihtt, 2002; Cherry and Lynch, 2020).

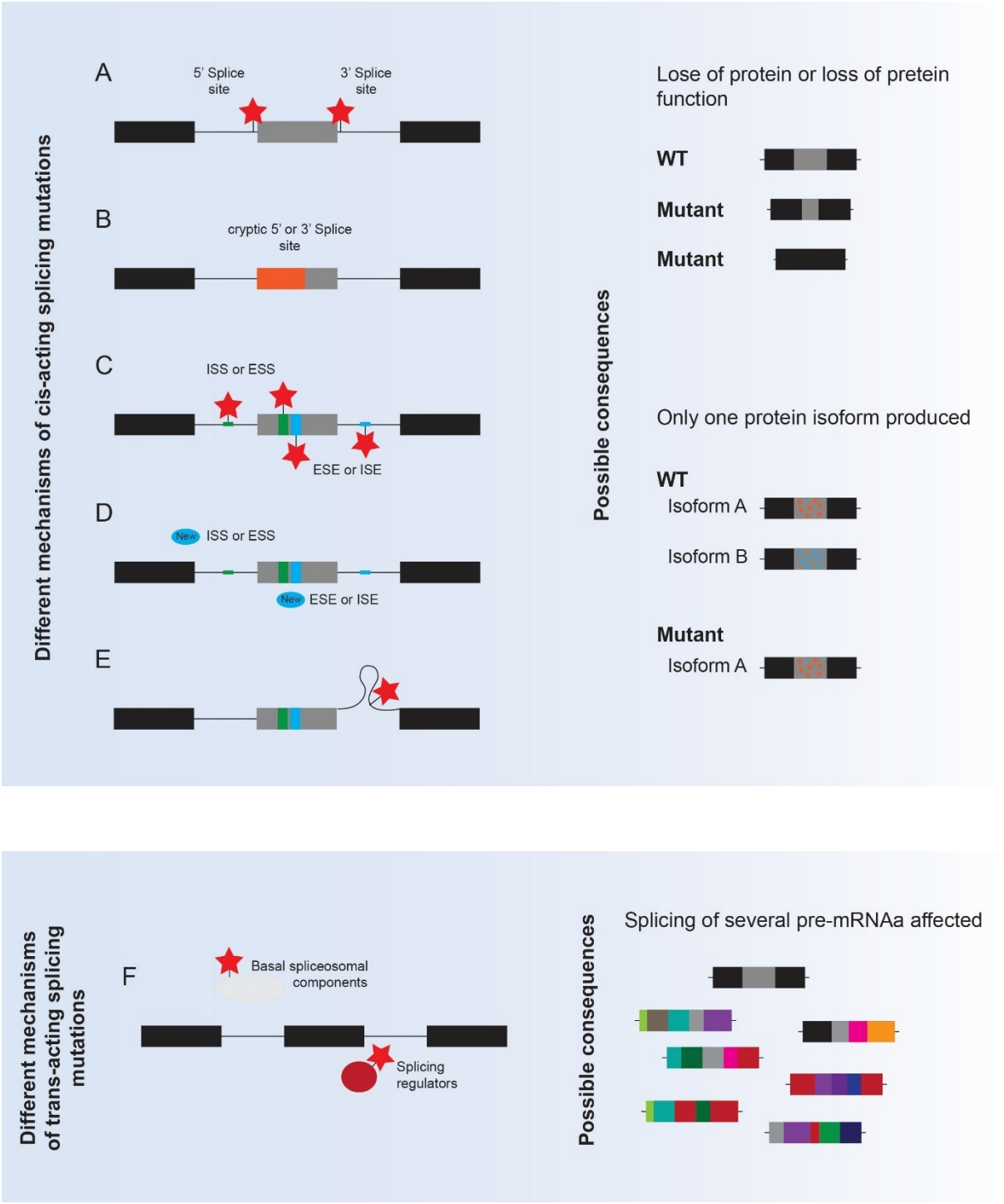


Figure 8. Examples of splicing connected mutations and their possible consequences.

Exons are in the form of boxes, introns are in the form of lines, and mutations are represented as asterisks. **(A–E)** Cis-acting mutations on a pre-mRNA. **(A)** Mutations disrupt 5' or 3' splice sites. **(B)** Mutations create cryptic 5' or 3' splice sites. **(C)** Mutations disrupting splicing regulatory sequences-intronic (ISS) or exonic (ESS) splicing silencers or intronic (ISE) or exonic (ESE) splicing enhancers. **(D)** Mutations create new ISS, ESS, ISE or ESE. **(E)** Mutations affect the secondary structure of the pre-mRNA. Possible consequences are complete loss of a protein, creation of a mutant protein, production of a protein lacking a domain or unbalanced protein production. **(F)** Trans-acting mutations

of a specific splicing regulator can lead to aberrant splicing of several different pre-mRNAs. Based on (Pistoni et al., 2010).

Retinitis pigmentosa

Retinitis pigmentosa (RP) is one of the most intriguing and widespread disorders connected to splicing. Despite this, it is classified as a rare disease and its prevalence varies, for example, from almost 1 in 7000 in Spain to 1 in 3000-4000 in Norway and Denmark (Perea-Romero et al., 2021).

As early as 1857, F.C. Donders, a Dutch physician introduced the term ‘retinitis pigmentosa’ and this terminology has been maintained.

Even though inflammation plays a modest role during the progression of the disease, the term has persisted. Clinically, patients with retinitis pigmentosa exhibit bone-spicule deposits on the interior eye surface, along with weakened retinal vessels and diminished visual fields (reviewed in Hamel, 2006). In most cases, symptoms emerge in the early teens and progress until the age of 40 to 50 when severe visual impairment occurs. A rare case is the early-onset form manifests as Leber congenital amaurosis, leading to blindness by the tender age of 1 year (Tsang and Sharma, 2018).

The term "rod-cone dystrophy" is often used in RP to describe the order of photoreceptor degeneration that occurs in this retinal disease. The human retina has one type of rod cell, which is responsible for seeing in dim light vision and three types of cone cells which absorb red, blue and green light (Figure 9). Rod accounts for around 91 million per retina and cones roughly 4.5 million (reviewed in Kawamura and Tachibanaki, 2008; reviewed in Kefalov, 2012). They all share the same mechanism of light detection. Rod apoptosis led to impaired night vision followed by the death of cones resulting in a reduction in the retina's outer nuclear layer's thickness, and highly structurally transformed retinal pigments (reviewed in Hamel, 2006). Besides the photoreceptor layer, the retinal pigment epithelium (RPE) is also affected in RP. Cells in the RPE have a role in the maintenance of the blood-retinal barrier, the transport of metabolites, phagocytosis of shed photoreceptors and the recycling of pigments necessary for the visual cycle (reviewed in Strauss, 2005). Rods, cones and the retinal pigment epithelium (RPE) are metabolically coupled and are dependent on each other structurally and functionally. After the loss of photoreceptors, the signature genes the RPE involved in these processes are also changed.

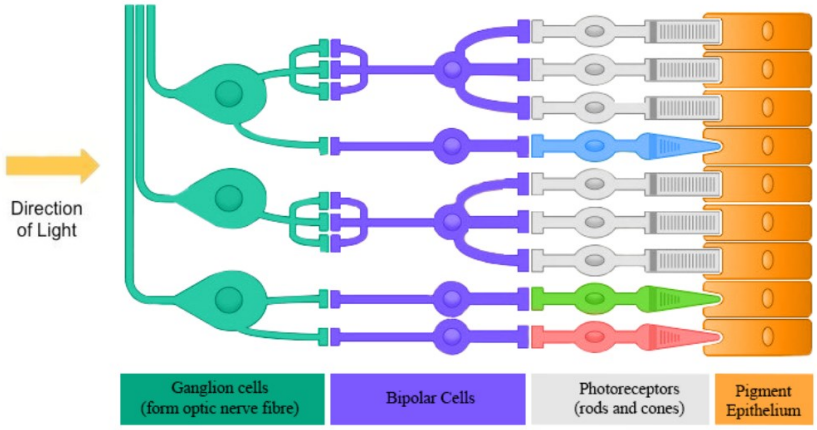


Figure 9. Simplified diagram of the basic structure of the retina.

Light first goes through ganglion cells which convey the information to the brain in the form of an electrical signal. Bipolar cells connect the photoreceptor cell with the ganglionic cells and transmit the electrical information to several other cells of the retina. Photoreceptors are cones represented as blue, green and red (depending on absorbing light) and rods represented in grey color. Retinal epithelium serves as a blood-retina barrier, transport of metabolites, phagocytosis of shed photoreceptors and recycling of pigment. Based on (https://thesciencecodex.wordpress.com/tag/the-brain/).

Retinitis pigmentosa displays all forms of inheritance patterns (Hamel 2006) and although patients may have uncommon biochemical processes, their symptoms and outcomes are crisscrossing (Marano et al., 2000). To date, around 90 genes have been involved in the pathogenesis of RP (https://web.sph.uth.edu/RetNet/) which is nearly one-third of the summarized 230 genes associated with photoreceptor cell loss (reviewed in Winkler et al. 2020).

The RP phenotype is restricted to the retinal tissue, so the causative mutations are in genes expressed and required specifically in the retina and retinal epithelium. A quarter of adRP cases are due to mutations in rhodopsin (RHO), a rod-specific pigment, and the second most common cause of adRP accounting for 15-20% of adRP cases is linked to mutations in the pre-mRNA processing factor (PRPF) genes, which encodes a core component of the spliceosome (reviewed in Růžičková and Staněk, 2017; reviewed in Yang et al., 2021).

It remains unclear why mutations in these splicing factors, which are universally present in all cell types, manifest as tissue-specific phenotypes.

For more than two decades, scientists have utilized a heterogeneous array of cell lines and animal models to reveal the mechanism behind PRPF mutations.

In particular, the dominant ARPE-19 cell line, derived from human retinal pigment epithelial cells, has played a pivotal role in these investigations (Dunn et al., 1996).

In the simplest organism, yeast, most of the proteins have been identified as essential splicing factors with orthologs of all the human splicing factors responsible for retinitis pigmentosa.

The cone-rich retina of the zebrafish is an excellent model for exploring retinal metabolism and morphology especially since it differentiates together with the RPE within 72hpf (Jaroszynska et al., 2021). RP-associated splicing genes, mainly PRPF4 and PRPF31 have been studied in these animal models for the study of RP pathogenesis and drug screening (Yin et al., 2011; Chen et al., 2014).

The complexity of the human retina makes it difficult to find a suitable model that fully recapitulates the disease. However, a dog model has been used to treat LCA due to a mutation in an RPE65. That resulted in Luxturna, the first therapy product approved for this treatment (reviewed in Winkler et al., 2020).

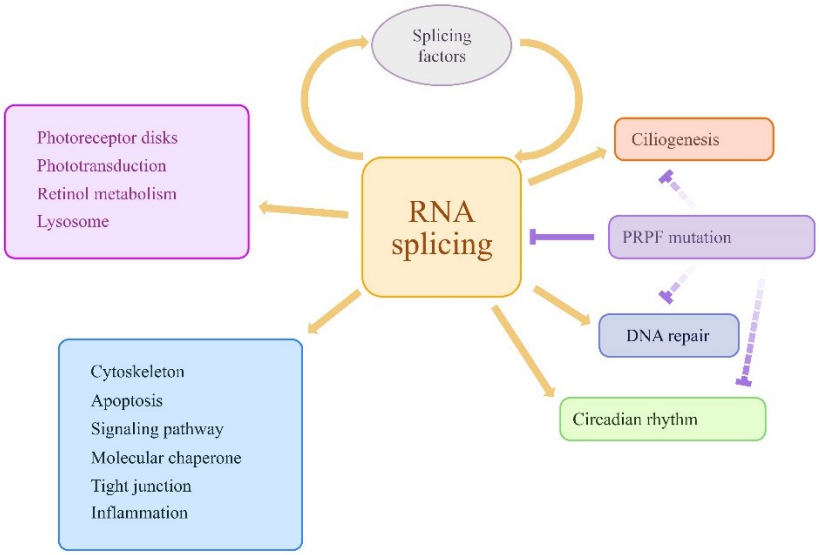


Figure 10. Effects of mutations in PRPF proteins on biological processes in the retina.

The primary results of RP-related PRPF mutations are impaired spliceosome assembly and defective RNA splicing. This alters the RNA splicing defect in the retina. Most of the affected genes are important for retinal functions (retinol metabolism, phototransduction, photoreceptor disk, lysosome). Also, mis-spliced genes cluster in general biological processes, such as inflammation, apoptosis, cytoskeleton, tight junction, molecular chaperone and signalling pathway. The interrupted line indicates the effects of PRPF mutations on ciliogenesis, DNA repair and circadian rhythm. Based on (Yang et al., 2021).

Research efforts are currently intensive in finding effective drugs and therapies for retinitis pigmentosa. Nutritional supplements targeting the vitamin A pathway in the retina may

potentially slow the progression of diseases. Neuroprotective reagents like neurotrophic factors CNTF and agents such as NAC and its potent variant NACA have shown efficacy in animal models. Promising results in preclinical and clinical studies have been observed with stem cell therapy, gene augmentation therapy, and CRISPR/Cas9-based correction of retinitis pigmentosa mutations, whereas retinal implants are currently successful primarily for patients with little to no visual function (reviewed in Nguyen et al., 2023).

Mutations in splicing proteins connected to retinitis pigmentosa

Mutation in di-U4/U6 snRNP proteins

Since the initial discovery of the mutation of **PRPF3** in RP (Chakarova et al., 2002), 10 heterozygous mutations have been described. In particular, the conserved C-terminal domain, crucial for the interaction between this protein and the di-snRNP U4/U6 serves as a hotspot for RP-related mutations. A homozygous PRPF3 knockout in zebrafish is lethal and in the knockout embryos there is noted higher cell death level in the developing eyes with no similar effect in heterozygous knockouts (Amsterdam et al., 2004; Graziotto et al., 2008). Among the mutations found in unrelated families, the most studied has been Thr494Met. Comitato and colleagues have demonstrated that this mutation alters the localization of the protein in cultured differentiated photoreceptors, causing it to aggregate in the nucleolus (Comitato et al., 2007). This amino acid substitution results in a reduction in protein phosphorylation, affecting its association with PRPF4, and U4/U6 snRNA (Gonzalez-Santos et al., 2008). Clinical examination of the retina revealed differences in phenotypes in a five-generation Swiss family with the absence of rod-specific waves (Vaclavik et al., 2010). Transgenic mice carrying this heterozygous mutation showed late-onset degenerative morphology of the RPE. The homozygous state was more severe (Farkas et al., 2014; Graziotto et al., 2011).

To date, a total of 5 mutations in **PRPF4** have been linked with RP (Chen et al., 2014; Linder et al., 2014). PRPF4 is a component of the tri-snRNP. Codon-changing mutations are usually located in the highly conserved C-terminal part of PRPF4, a region that interacts with PRPF3. Linder and colleagues identified an Arg192His amino acid exchange in this region that disrupts the PRPF3-PRPF4 interaction (Linder et al., 2014). In fibroblasts from an affected individual with the change of amino acid from Pro to Leu, several tri-snRNP components changed the expression levels, including PRPF4. The expression pattern of SC35 was altered. Furthermore,

This mutation led to systemic malformations in zebrafish embryos, particularly in the retina (Chen et al., 2014).

Another di-snRNP protein associated with RP is **PRPF31**, a protein essential for the assembly and stability of U4/U6.U5 tri-snRNPs (Weidenhammer et al., 1996; Makarova et al., 2002). The first mutation was reported by Vithana and colleagues about 20 years ago (Vithana et al., 2001). Since then, 229 PRPF31 mutations of various types have been identified mainly located in the exons 6-10. These mutations have been implicated as a major cause of adRP, which is the second most common cause of adRP in multiple populations, accounting for 5-10% of cases in the USA, Spain, France, North America, China, and Belgium (reviewed in Wheway et al., 2020). A consistent feature of these mutations is their incomplete penetrance. Most of the reported PRPF31 mutations are presumed to be loss-of-function variants that lead to the complete loss of protein expression from the mutated allele.

Primary mouse retinal cell cultures carrying point mutations at positions 256 and 371 result in apoptosis (Yuan et al., 2005). Knockdown of PRPF31 and several RP mutations in zebrafish leads to alternations in retina-specific genes and aberrant cytoplasmic localization in rod cells (Yin et al., 2011). In another study, reduced levels of PRPF31 primarily affect the outer segments of the photoreceptors in the retina and impair visual function (Linder et al., 2011). A non-synonymous nucleotide exchange which leads to the change of Alanine to Proline on position 216, destabilizes the protein and expression of this mutant leading to changes in cell proliferation and structural modification of Cajal bodies. The same paper showed a significant reduction in the association between this mutant and snRNPs in the nucleus that association between this mutant and snRNPs (Huranová et al., 2009). In a heterozygous knock-in mouse model carrying the same mutation, photoreceptor loss was observed within 18 months (Valdés-Sánchez et al. 2019). Given that PRPF31 plays a significant role in ciliogenesis (Wheway et al., 2015) much of the research move focuses on studying cilia and phagocytosis, where mutations and decreased levels of this protein result in defects in both processes (Buskin et al., 2018). PRPF31 mutated patient-derived retinal organoids and RPE and PRPF31 +/- mouse tissues showed that nearly all processes involving RNA are intensely affected with the spliceosome complex being one of the most affected cellular components in the transcriptome (Buskin et al., 2018).

Mutation in U5snRNP proteins

The mutation in the **PRPF6** protein Tanackovic and colleagues first identified in 2011 (Tanackovic et al., 2011). 18 PRPF6 mutations have been identified since then. Some of the mutations showed suppressed tri-snRNP accumulation (Gamundi et al., 2008) and RP patient lymphoblasts containing the Arg729Trp mutation displayed defective pre-mRNA splicing with retained introns (Tanackovic et al., 2011). An iPSC model was created by reprogramming peripheral blood mononuclear cells from an adRP patient with PRPF6. The results showed aberrant morphology, disordered microvilli and reduced expression of genes in RPE. In addition, RPE cells with the PRPF6 mutation had reduced phagocytosis in the outer segments of the photoreceptors, as well as disturbed cell polarity and barrier function (Liang et al., 2022).

The connection between **PRPF8** mutations and adRP was first revealed in 2001 (McKie et al., 2001). A total of 64 PRPF8 mutations have been listed. Most of the mutations are clustered in the C-terminal region, which is functionally conserved and interacts with SNRNP200. The interaction between PRPF8 and SNRNP200 is affected by RP-associated mutations (van Nues and Beggs, 2001; Pena et al., 2007). The effects of RP-related mutations have been studied in yeast, where splicing and growth defects have been observed in different mutants (Boon et al., 2007; Graziotto et al., 2011). In cells, mutant PRPF8 proteins display reduced interaction with SNRNP200, reducing the functional U5 snRNP levels and further suppressing splicing efficiency (Mozaffari-Jovin et al., 2013; Mayerle and Guthrie, 2016). In homozygotic mice, decreased phagocytosis is observed in primary RPE cultures with weakened adhesion between RPE apical microvilli and photoreceptor outer segments. The mislocalization of receptors in the photoreceptor outer segments has also been detected (Farkas et al., 2014). Malinova and colleagues showed that PRPF8 mutants assemble less efficiently with the U5 snRNP (Malinová et al., 2017). Recently, it has been shown that PRPF8 mutation leads to general changes in splice site selection and exon inclusion. This is particularly true for genes involved in splicing and ribosome function (Arzalluz-Luque et al., 2021). Homozygous mice with early expression of the abnormal PRPF8 variants had progressive cerebellar atrophy and reduced splicing protein expression (Krausová et al., 2023).

There are 45 mutations in the **SNRNP200** helicase connected to RP. Usually, these mutations impair the unwinding function of this protein (Zhao et al., 2009). It has also been shown that mutations resulting in amino acid changes from Ser to Lys at position 1087 and Arg to Lys at position 1090 disturb the ability of BRR2 to recognize the 5’ splice site (Cvačková et al., 2014a). Ledoux and Guthrie demonstrated that the RP mutations within the ratchet domain

impair BRR2 translocation through RNA helices (Ledoux and Guthrie 2016). Overexpression of the mutation Arg2030C mutation causes abnormalities in zebrafish. C6088T mutation leads to severe defects in the systemic phenotype of zebrafish embryos, with a drastic reduction in the expression of rhodopsin in injected zebrafish. (Zhang et al., 2020).

Mutation in U2 snRNP protein

New research has shown that zebrafish lacking **SF3B4** exhibit signs similar to RP. These zebrafish have degeneration of the rod, serious defects of retinal pigment epithelium, altered retinol metabolism and retinoic acid signalling (Ulhaq et al., 2023).

Mutation in non-snRNP proteins linked to RP

Limited research on **RP9** has shown that it is localized to nuclear speckles and interacts with the splicing factor U2Af35 (Maita et al., 2004). Two detected mutations in the RP9 gene have been connected with RP. Only one missense mutation D170G resulted in a defect in splicing activity and a reduced proportion of phosphorylated PAP-1, a protein connected with pap activity (Maita et al., 2004).

In 2014, a missense mutation altering Gly at position 332 in both alleles of **DHX38** was identified in four individuals from Pakistani families (Ajmal et al., 2014). Four years later, a new mutation, Arg324Gln, was described in two Pakistani families. Carriers of both mutations developed night blindness aged 3-4, progressing to complete blindness aged 7-8 (Ajmal et al., 2014; Latif et al., 2018). Computational analysis suggested that Gly332 is the most flexible of all residues and that this flexibility may be necessary for the normal function of the protein. It was speculated that the insertion of aspartic acid would lead to protein misfolding, causing protein degradation or impaired function (Ajmal et al., 2014). Using whole exome sequencing in 2022, a novel DHX38 mutation causing an arRP variant was identified in two sisters from Saudi Arabia. This mutation leads to poor visual and extensive retinal pigmentary changes (Al-Johani et al., 2022).

While the above mutations are associated with "simple" forms of RP, RP can also be part of syndromic forms that involve multiple organs and exhibit pleiotropic effects. Retinal disease may result from a systemic pathology. Among the syndromic forms, Usher syndrome is the most frequent. This syndrome typically presents in childhood and is characterized by congenital hearing loss, vestibular dysfunction, and retinal degeneration (Keats and Savas,

2004). The second most common syndromic form is Bardet-Biedl syndrome, which not only includes retinal degeneration but also features such as obesity, polydactyly, genital and renal malformations, and behavioural and developmental disabilities (Katsanis, 2004).

Material and methods

Cloning of plasmids

Complementary DNA (cDNA) of human DHX38 was ordered from Open Biosystems (catalogue: MHS6278-202828190, clone ID 3504632).

The first vector into which we inserted this cDNA was the pEGFP-C2 vector, and the cDNA was inserted between the KpnI and HindIII restriction sites. Additionally, the DHX38 cDNA was cloned into the 3xFLAG vector (EGFP-C3 vector portion GFP is replaced with 3xFLAG), utilizing the EcoRI and KpnI restriction sites.

Site-directed mutagenesis PCR was used to introduce the point mutation G995A, which results in the substitution of the amino acid p.G332D, site-directed mutagenesis PCR was performed. The constructs were confirmed by DNA sequencing. For purification and concentration, the Clean and Concentrate kit from Zumo Research was utilized following the recommended manufacturer's protocol.

The **LCTA** minigene (exon 4_inton 4_exon 5) was amplified from genomic DNA and subsequently cloned into the pcDNA 3.1 plasmid between the restriction sites KpnI and EcoRI. To generate a specific branch point substitution, a point mutation was introduced using site-directed mutagenesis PCR (Li and Pritchard, 2000).

DHX38 forward	5’ ATGGACGAGGACTATGACGAG 3’	cloning
DHX38 reverse	5’ CATGTACCAATCCCGATC 3’	cloning
DHX38 forward	5’ ATGGACGAGGACTATGACGAG 3’	SDM
DHX38 reverse	5’ CATGTACCAATCCCGATC 3’	SDM
LCTA forward	5’ GGGTACCTGCACACACTGGTGCAGAACC 3’	cloning
LCTA reverse	5’ GGAAGGTGCCACTCCCACTGTC 3’	cloning
LCTA forward	5’ AGCTGCCCTGTCCCCTTCCACC 3’	SDM
LCTA reverse	5’ GGGGTCTGGGGCACCTGC 3’	SDM

Table 1. List of primers used for cloning and site-directed mutagenesis (SDM) of DHX38 and LCTA.

Plasmid cloning was done using a competent strain of DH5α bacteria. These bacteria were subsequently cultured in an LB medium supplemented with the necessary antibiotics.

For the isolation of plasmid DNA, we used the QIAprep Spin Miniprep kit (Qiagen) as described in the manufacturer's protocol.

1%	tryptone
0.5%	yeast extract
1%	NaCl

Table 2. Composition of LB medium.

Cell cultivation, siRNA treatment and transfections

HEK293 cells were cultured in high glucose (4500 mg/l) DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin. Cells were maintained in a controlled environment at 37 °C in a 5% CO_2 atmosphere.

To downregulate endogenous DHX38 expression, siRNA duplex (Ambion) and negative control (#5, Ambion) were utilized. These were introduced into cells at a final concentration of 50nM, along with the Oligofectamine reagent (Invitrogen), following the manufacturer's instructions.

For RNA-seq analysis, siRNA SMART (ThermoFisher Scientific) was transfected into cells using an Oligofectamine reagent (Invitrogen) at the final concentration of 50nM following the manufacturer's protocol.

Post-transfection, cells were incubated for 72 hours, with a medium change after 48 hours.

All plasmids were transfected into cells using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Analysis of the cells was performed 24 hours after the transfection.

The β-globin M1 reporter, the M2 reporter and the β-globin WT constructs were used for the cryptic splice site usage experiment (Cvačková et al., 2014b). For the splicing efficiency of weak and strength splice sites, splicing reporters derived from the non-coding RNA-a2 with splice strength 5' SS 7.53, 5' SS 9.60, 5' SS 11.08, WT and reporter with deleted Ts in PPT (Krchnáková et al., 2019).

For microscopic observations, cells were transfected using the Lipofectamine LTX reagent (Invitrogen) in a ratio of 3 µl of the reagent to 2 µg of plasmid DNA. The reagent, DNA, and serum-free DMEM medium were mixed and incubated at room temperature for 20 minutes before being added to the cell medium. Cells were used for experiments 24 hours after transfection.

For splicing assays, cells were transfected with the Lipofectamine LTX reagent (Invitrogen) at a ratio of 1.5 µl of the reagent to 1 µg of plasmid DNA. The transfection reagent, DNA, and serum-free DMEM medium were combined and incubated at room temperature for 20 minutes before being added to the cell medium. These cells were used for experiments 24 hours after transfection.

siRNA duplex for knockdown of DHX38	5’ UCAGCUCACAGACCAAAGCGGtt 3’
negative control	# 5
siSMART pool targeting sequences:	5’ GCACUGAUCUGGACUGUCA 3’ 5’ GAUCGGGAUUGGUACAUGA 3’ 5’ AUGCUAAGGCCAUGCGGAA 3’ 5’ CCACUCAGCUGACGCAGUA 3’

Table 3. List of sequencing for knockdown of endogenous DHX38.

RNA isolation, cDNA synthesis, RT PCR and qPCR

Cells were cultivated until they reached a 90% confluency and subsequently, RNA was isolated using either the TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer´s protocol. The isolated RNA was then precipitated using isopropanol, resuspended in Nuclease-Free Water (Ambion) and treated with Turbo DNase (Ambion) following instructions of the manufacturer.

cDNA was synthesised using SuperScript III (Thermo Fisher Scientific). In a total reaction of 13µl, we used 500ng of total RNA and RT-specific primers or random hexamers (Sigma Aldrich).

For the PRC reaction, a mixture of 10µl was prepared, using the Physion enzyme and 2.5µl of cDNA. The products of the PCR reaction were separated on a 2% agarose gel and the intensity of individual bands was quantified using ImageJ software.

Quantitative PCR was performed using LightCycler® 480 SYBR Green I Master (Roche) on a LightCycler 480 system (Roche). The reaction volume of 5 µl contained 2 µl of template cDNA (diluted to 1:10) and 500nM of each primer. Splicing efficiency was calculated as 2(Ct[mRNA]–Ct[pre-mRNA]).

Random hexamers	NNNNNN	RT
pre-mRNA GAPDH forward	5'ACATCGCTCAGACACCATGG3'	PCR
pre-mRNA GAPDH reverse	5'CAGGGAAGCTCAAGGGAGAT 3'	
mRNA GAPDH forward	5' ACATCGCTCAGACACCATGG 3'	
mRNA GAPDH reverse	5'GTTAAAAGCAGCCCTGGTGA 3'	
pre-mRNA LDHA forward	5'TGGCAGCCTTTTCCTTAGAA 3'	
pre-mRNA LDHA reverse	5' TGTGCAACTGCACTCTACCC 3'	
mRNA LDHA forward	5' TGGCAGCCTTTTCCTTAGAA 3'	
mRNA LDHA reverse	5' CTTTCTCCCTCTTGCTGACG 3'	
RHO forward	5' CGGAGGTCAACAACGAGTCT 3'	
RHO reverse	5' TCTCTGCCTTCTGTGTGGTG 3'	
FSCN forward	5' TGCCAACACCATGTTTGAGA 3'	
FSCN reverse	5' GCTTGAGGGTGAACTCTTCG 3'	
β globin forward	5' CAAGGTGGACGTGGATGAAG 3'	
β globin reverse	5' GGACAGATCCCCAAAGGACT 3'	
LCTA forward	5' TACCTGCACACACTGGTGCAGAAC 3'	
LCTA reverse	5' AGGCCAAGACCAGCATGGGCTTGA 3'	
ncRNA-a2 spliced variant 1 forward	5' CTCATTCGGTCCATCCAACT 3'	splicing efficiency
ncRNA-a2 spliced variant 1 reverse	5' ACCTGGAGCCCGGAAGGAAC 3'	
BBS1 NSS forward	5' GCTGGCAGATTTACATGGGGAT 3'	
BBS1 NSS unspliced reverse	5' GAATTCCAGATATGCTGGGCC 3'	
BBS1 NSS spliced reverse	5' CAGTGGTCCTTTGAGCACCTTC 3'	
BBS1 LSS forward	5' GCTGGAGCTAAGTGAAATGGAGGC 3'	
BBS1 LSS unspliced	5' TGGCATGGCTGGAAGGGATATAGC 3'	
RPE65 NSS forward	5' CCAGGAGCTCCTTGTTCTTGTTCTCGGTGC 3'	
RPE65 NSS spliced reverse	5' CCTGCTGGTGGTTACAAGAA 3'	
RPE65 NSS unspliced reverse	5' CTTGTGCAGGAGGGCTTGCC 3'	
RPE65 LSS forward	5' CACTGAGAGACGCCAAGGAA 3'	
RPE65 LSS unspliced reverse	5' CAACCGCTCCAGCTATGACGTTCTT 3'	
RPE65 LSS spliced reverse	5' CAGGCTTTGGGCCCCACTTGATAA 3'	
	5' ATGGCCACCTTGTTATAGTCGAG 3'	

Table 4. List of primers used for reverse transcription and splicing efficiency.

Immunoprecipitation

Lipofectamine 3000 (Invitrogen) was used for the transfection of 7 µg of DNA according to the manufacturer's protocol. Twenty-four hours after the transfection, cells were washed 3x with PBS buffer, harvested into PBS and then centrifuged at 1 000 g and 4 °C for 10 minutes. The harvested cells were subsequently resuspended in NET2 buffer with the addition of Recombinant RNasin (Promega) and Protease Inhibitor Cocktail Set III (Calbiochem). The samples were sonicated in an ice bath (3x30 pulses; 0.5 s for each pulse at 60% of maximum energy) and then centrifuged at 20 000 g and 4 °C for 10 minutes and 20 µl of the supernatant was saved as 'input' samples. For immunoprecipitation, the remaining supernatant was pre-cleaned by incubating on a rotator with 10 µl Protein G Sepharose beads (GE Healthcare) at 4 °C for 2 hours. For the immunoprecipitation step, 30 µl Protein G Sepharose beads were washed 3 times with NET2 buffer and incubated on a rotator with NET2 buffer and 0.4 µl GFP or 1.4 µl anti-FLAG M2 (Sigma Aldrich, slbx2256) antibody at 4 °C for 4 hours. Beads were then washed 3x with PBS and incubated on a rotator with pre-cleaned cell lysates at 4 °C overnight. The next day, lysates were discarded, the beads were washed 4x with NET2 buffer and resuspended with 30 µl 2x concentrated sample buffer.

50 mM	Tris–HCl pH 7.5
150 mM	NaCl or 300mM NaCl
0.05%	IGEPAL CA-630

Table 5. Composition of NET2 buffer.

20%	glycerol
4%	SDS
2%	2-mercaptoethanol 250mM Tris-HCl pH 6,8
0,02%	bromphenol blue

Table 6. Composition of 2x Sample Buffer.

Western Blotting

Proteins were separated by 10% SDS-PAGE and transferred to a protran 0.45 µm nitrocellulose membrane (GE Healthcare). The blotting sandwich was made with gel and the membrane in the middle with 3 layers of filter paper and one sponge on each side. All components were soaked in a transfer buffer. A BioRad wet blotting apparatus was used for western blotting. The next step was membrane washing in PBST (PBS supplemented with 0.05% Tween) and blocked by incubation on the shaker in 10% low-fat milk/PBST.

The listed primary and secondary antibodies were diluted in 1% low-fat milk/PBST and AffiniPure goat secondary antibodies conjugated with horse radish peroxidase (Jackson ImmunoResearch) were used.

The final step was the incubation of the membrane with SuperSignal Femto or Pico West (Thermo Fisher Scientific) and the images were developed in an LAS-3000 imager (Fujifilm).

40% acrylamide/N,N'-methylene bisacrylamide 37.5:1	1.36 ml	40% acrylamide/N,N'-methylene bisacrylamide 37.5:1	272 µl
1.5M Tris-HCl pH 8.8	1.3 ml	1M Tris-HCl pH 6.8	260 µl
10% SDS	50 µl	10% SDS	20 µl
dH2O	2.24 ml	dH2O	1.426 ml
10% APS	50 µl	10% APS	20 µl
TEMED	2 µl	TEMED	2 µl

Table 7. Composition of 10% separating gel (5ml) and stacking gel (2ml) for western blot.

25mM Tris-HCl
192mM glycine
0.1% SDS

Table 8. Composition of SDS running buffer.

25mM Tris-HCl 192mM glycine 20% methanol

Table 9. Composition of transfer buffer.

mouse anti-DHX38	Santa Cruz/ sc-81081
rabbit anti-SNRNP200	Sigma Aldrich/ HPA029321
rabbit anti-U2A	Abcam/ab128937
mouse anti-PRPF8 (E-5)	Santa Cruz/ sc-55533
mouse anti-PRP19	Santa Cruz/ sc-135879
mouse anti-DHX16	Santa Cruz/sc-514338
mouse anti-SLU7 (B-11)	Santa Cruz/sc-376985
mouse anti-FLAG M2	Sigma Aldrich/slbx2256
mouse anti-GAPDH	Abcam/AB9484
mouse anti-GFP	Santa Cruz/ sc-9996
goat anti-GFP	kindly provided by Pavel Tomancak and Pavel Mejstrik, Max Planck Institute for Molecular Cell biology and Genetics, Dresden, Germany
mouse anti-tubulin TU-01	kindly provided by Pavel Dráber, Institute of Molecular Genetics, Prague, Czech Republic
rabbit anti-EFTUD2	kindly provided by Reinhard Lührmann, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany
rabbit anti-RBM39	Sigma Aldrich/ HPA001591
rabbit anti-U1C	Abcam/ ab192028
mouse anti-SF3B4	Abcam/ ab104226
goat anti-mouse and goat anti-rabbit	conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories)

Table 10. List of antibodies used for western blotting.

RNA Silver staining

RNA was analyzed by separation on a denaturing polyacrylamide gel. The amount of 5 µl of IP and 5 µl of input RNA samples were mixed with 10 µl of 0.5x concentrated sample buffer and evaporated to the final volume of 5 µl. During this step, 4M urea from the 0.5x sample buffer was concentrated to 8M. Samples were then incubated at 65 °C for 15 minutes.

4M urea
10mM Tris-HCl pH 8.0
0.1% xylene blue

Table 11. Composition of 0.5x sample buffer.

40 ml of gel mixture was used. Urea, 10x TBE and acrylamide were first mixed and heated on a stirrer at 75-90°C until the urea dissolved. The gel was then cooled at room temperature, mixed with APS and TEMED and water was added to the final volume. The gel was immediately poured into the apparatus. After polymerisation, the gel was heated by pre-run at 500 V for around 15 minutes. 2.5 µl of input RNA and 5 µl of IP RNA were loaded and separated at 500 V for 2 h 40 minutes.

urea	19.2 g
30% acrylamide/bis-acrylamide 19:1	13.3 ml
10x TBE	4 ml
10% APS	400 µl
TEMED	25 µl
dH_2O	to 40 ml

Table 12. Composition of 7M urea/10% polyacrylamide RNA gel.

0.89M Tris base
0.89M boric acid
20mM EDTA

Table 13. Composition of 10x TBE buffer.

When the RNA was sufficiently separated, the RNA gel was fixed with 40% methanol + 10% acetic acid for 30 minutes. It was then treated with 3.4mM K2Cr2O7 + 3.2mM HNO3 for 10

minutes, washed 3 times briefly with water and silver stained (12mM AgNO3) for 30 minutes. After staining, the gel was washed again with water and the image was developed with 280mM Na2CO3 + 0.02% formaldehyde. The reaction was stopped by washing in 1% acetic acid for at least 10 minutes.

Immunofluorescent staining

Cells were cultured in a petri dish of 6cm diameter on coverslips (Marienfeld Superior). After 24 hours of transfection, cells were washed with 1xPBS, fixed for 10 minutes in 4%PFA/PIPES in RT and again washed with 1xPBS. Permeabilization was done with 0.5% Triton X-100/PBS at RT for 5 minutes. The next step was to wash cells 3x with 1xPBS. Coverslip was then blocked for 10 minutes in 5% (v/v) normal goat serum (Jackson ImmunoResearch Laboratories). After further 3 times washing with 1xPBS the samples were incubated with appropriate primary and secondary antibodies diluted in PBS, 1 hour each antibody at RT. The list of antibodies is in Table 12. Following three more washes with 1xPBS, the coverslips were mounted to microscope slides. Using 3.5µl DAPI Fluoromount-G containing 4,6-diamidino-2-phenylindole (Southern Biotech) for staining of DNA.

Cells were imaged by the DeltaVision microscope system (Applied Precision) coupled to an Olympus IX70 microscope. The microscope was equipped with an oil immersion objective of a 60x and a numerical aperture of 1.4.

2 g	Paraformaldehyde
25 ml	0.2M PIPES pH 6.9
100 µl	1M $MgCl_2$
125 µl	0.5M EGTA pH 8
25 ml	dH_2O

Table 14. Composition of 4% paraformaldehyde/PIPES.

mouse anti-DHX38 mouse anti-FLAG M2 mouse anti-GFP	
goat anti-mouse	conjugated with DyLight488 (Jackson ImmunoResearch Laboratories)

Table 15. List of antibodies for immunofluorescent staining.

RNA seq and data analysis

HEK293 cells from a 100mm in diameter petri dish were resuspended in 1ml of TRIZOL (Invitrogen) and total RNA was isolated using a Direct-zol microprep kit (Zymo Research) including DNase I treatment. Good quality RNA (RIN > 9.2) was ribodepleted and used for library preparation. Libraries were prepared using the KAPA RNA HyperPrep Kit with RiboErase (Roche) and sequenced on an Illumina NextSeq 500 instrument with 2x75 bp configuration. For subsequent data processing, the bioinformatic pipeline nf-core/RNAseq was used (version 1.4.2; Zenodo; https://doi.org/10.5281/zenodo.3503887).

Individual steps included the removal of sequencing adaptors with Trim Galore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), mapping with HISAT2 (Kim et al., 2015) and gene expression quantification using Salmon (both based on reference genome GRCh38, Ensembl annotation version 101(Yates et. al., 2020)). Quantified expression data served as input for differential gene expression analysis using the DESeq2 R Bioconductor package. Genes exhibiting |Log2 Fold change|>1 and statistical significance (FDR < 0.05) between compared groups of samples (DHX38 knockdown vs. cells treated with negative control siRNA) were considered as differentially expressed. Furthermore, based on alternative splicing analysis of mapping data with ASpli 2.0 R Bioconductor package (Love et. al., 2014), genes containing introns exhibiting a percentage of intron retained (PIR) difference > 10 % were considered as differentially spliced. RNA-seq data are accessible via the ArrayExpress database under accession E-MTAB-11127.

CHAC1 forward	5’ GAGACACCTTCCATCGGG 3’
CHAC1 reverse	5’TACTTCAGGGCCTTGCTTAC 3’
DDR2 forward	5’ TCTATGATGGAGCTGTTGGA 3’
DDR2 reverse	5’ TTCATGGGTCTGGGTGAAAT 3’
ASNS forward	5’ TGGCTCTGTTACAATGGTGA 3’
ASNS reverse	5’ GCTCAATTCCTCCTTTGTCA 3’
SLC7A11 forward	5’ GTGGGGTCCTGTCACTATTT 3’
SLC7A11 reverse	5’ AGACTCGTACAAAAGCTGGT 3’
TRIB3 forward	5’ CTGTGTCGCTTTGTCTTCG 3’
TRIB3 reverse	5’ AGTATCTCAGGTCCCACGTA 3’
PCK2 forward	5’ GGTGTTACTGTGACCTCCTG 3’
PCK2 reverse	5’ GCACAAAATCGAGAGTTGGG 3’
NTS forward	5’ TGTATGCATGCTACTCCTGG 3’
NTS reverse	5’ GAAATCTGCTTCTAATGCTTTCA 3’
RAI2 forward	5’ ATCCTGTAATCGTCCCCTTG 3’
RAI2 reverse	5’ ACTGGAGCTGAACTTGGATT 3’
BAX forward	5’ CATGTTTTCTGACGGCAACT 3’
BAX reverse	5’ ATGGTTCTGATCAGTTCCGG 3’
TP53I3 forward	5’ GCCCCTGTTTTCAAAGCTAC 3’
TP53I3 reverse	5’ TGGAGAAGTGAGGCAGAATT 3’
TNFRSF10D forward	5’ AAGTTCGTCGTCTTCATCGT 3’
TNFRSF10D reverse	5’ GACACTCCTCCTCCTTGAG 3’
SYP forward	5’ GAGTTCGAGTACCCCTTCAG 3’
SYP reverse	5’ GGAGTAGAGGAAGGCAAACA 3’

Table 16. List of primers used for qPCR/RNA sequencing analysis.

PCR reactions

Real-time PCR

Initial denaturation	95°C 7min 1 cycle
Quantification – Denaturation	95°C for 20s, 61°C for 20s, 72°C for 35s for 40 cycles
Melting curve analysis	95°C 15s, 55°C 1min 1 s, 37 °C 1min, 1s for 1 cycle

Site-directed mutagenesis

1:30 98°C

0:15 98°C
0:20 56 C
10:00 68 C
} 27x

RT-PCR

65°C and ice 3min

+ SSIII enzyme for 50 C 1h

70°C 15 min

Splicing efficiency

1:30 98℃

0:10 98℃
0:10 58℃
2:30 72℃
} 28x

10:00 72℃

Splicing efficiency of LCTA minigene

1:30 98 C

0:10 98 C
0:10 58 C
2:30 72 C
} 33x

10:00 72 C

Results

The first experiment is part of a published work Královicová J, Ševcíková I, Stejskalová E, Obuća M, Hiller M, Stanek D, Vorechovský I. PUF60-activated exons uncover altered 3' splice-site selection by germline missense mutations in a single RRM. Nucleic Acids Res. 2018 Jul 6;46(12):6166-6187 where we analyzed the role of PUF60 and RBM39 in alternative splicing. I tested the interaction of RBM39 with U1 snRNP and U2 snRNP and U2AF by immunoprecipitations and showed RBM39 was specifically pulled down by U1-specific protein U1 70K and U2AF35. Our data suggested that RBM39 might participate in the formation of the A complex in a gene-specific manner.

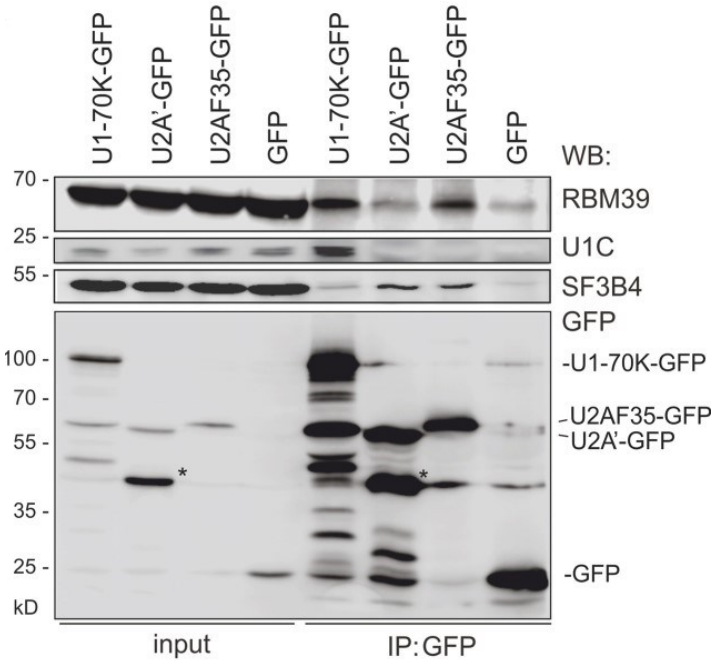


Figure 11. RBM39 interactions with proteins of the spliceosome.

The interaction of RBM39 with the U1-specific protein U1-70K, the U2-specific protein U2A' and the small subunit of U2AF was assayed by immunoprecipitations. HeLa cells were transiently transfected with U1-70K-GFP, U2A-GFP or U2AF35-GFP, immunoprecipitated with anti-GFP antibodies and probed with RBM39, U1C, SF3B4, U1-70K, U2AF35, U2A' and GFP antibodies. For positive controls, we used U1C for immunoprecipitations for U1-70K-GFP and SF3B4 for U2A'-GFP. Asterisks represent a partially degraded U2A'-GFP.

The second part consists of my work published in Obuća M, Cvačková Z, Kubovčiak J, Kolář M, Staněk D. Retinitis pigmentosa-linked mutation in DHX38 modulates its splicing activity. PLoS One. 2022 Apr 6;17(4): e0265742 and unpublished experiments and results. Part of the work was funded by Charles University Grant Agency (number 1270217), which was successfully completed.

DHX38 is a crucial RNA helicase with a conserved role in various organisms. The gene encoding DHX38 shows length conservation across species, and a high degree of sequence conservation at the C-terminal domain suggests functional importance. The N-terminal region is the least conserved part (reviewed in Cordin and Beggs, 2013). Moreover, on the cryo-EM maps that allowed detailed insights into Prp16 in yeast, the N-terminal region could not be seen (Galej et al., 2013; Yan et al., 2017; Wilkinson et al., 2021; Garbers et al., 2023).

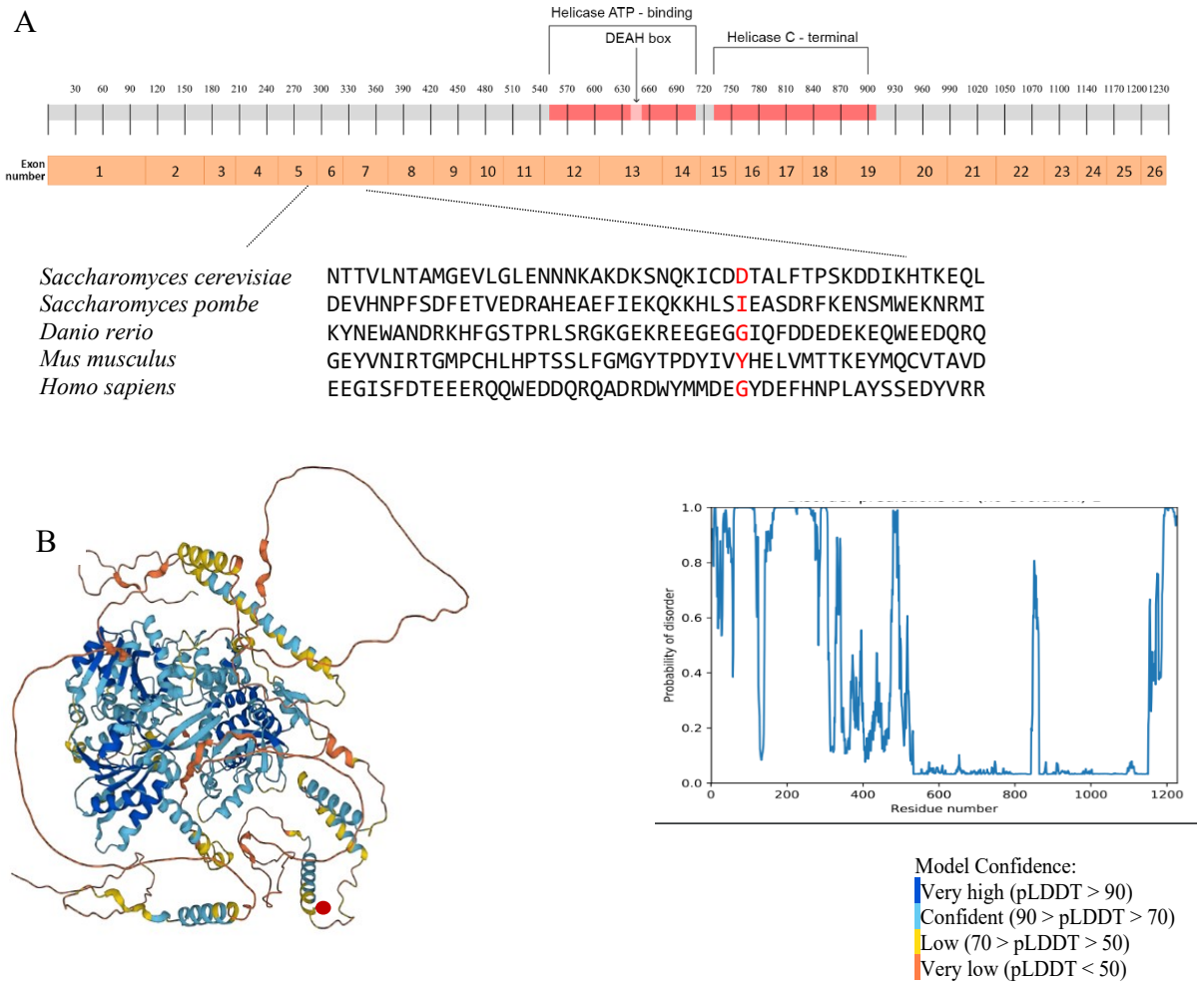


Figure 12. Predicted structure of DHX38.

(A) Conserved domain of DHX38 and sequence around RP mutation G332D across species. (**B)** Alpha fold predicted 3D structure of DHX38. Red circle marks the position where localise G332D mutation.

We cloned human complementary DNA into a vector containing the CMV promoter and GFP at the N-terminus of DHX38. The transfected construct did not alter cell proliferation but transient expression of the GFP-DHX38^{WT} construct showed disrupted localization in the cytoplasm (Figure 13A). Furthermore, transiently expressed GFP-DHX38 did not interact with basic components of the B and C spliceosome complex, PRPF8 and SNRNP200 (Figure 13B). This result suggests that the GFP tag might have disrupted DHX38 localization and interaction profile.

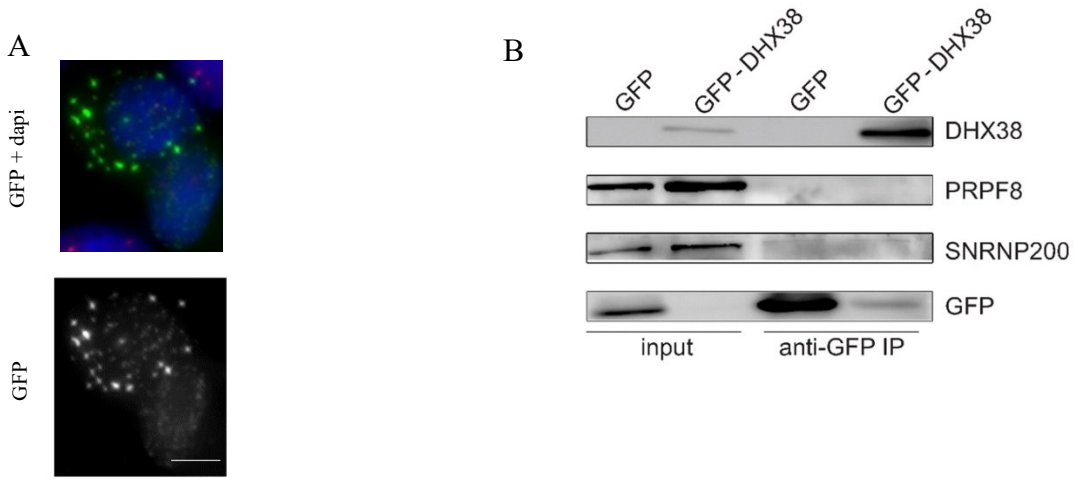


Figure 13. Coprecipitation and localization of GFP-DHX38.

(A) GFP-DHX38 does localise in nucleus in very small amount and forms aggregates in cytoplasm GFP (green), DAPI (blue). (**B)** GFP-DHX38 does not associate with PRPF8 and SNRNP200, U5-specific proteins. GFP-DHX38 was expressed in HEK293 cells, immunoprecipitated and co-purification of PRPF8 and SNRNP200 monitored by Western blotting. Scale bar -10µm.

The next step was to tag DHX38 with a smaller FLAG tag at the N-terminus. We introduced a mutation that changed glycine into aspartic acid using site-directed mutagenesis PCR (SDM).

Localization of DHX38WT and DHX38G332D

Transient expression of both vectors, DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG, in HEK293 cells, showed cytoplasmic and nuclear localization (Figure 14A). Interestingly, the same

localization pattern was observed when cells were stained the cells with a commercial antibody against DHX38 (Figure 14B).

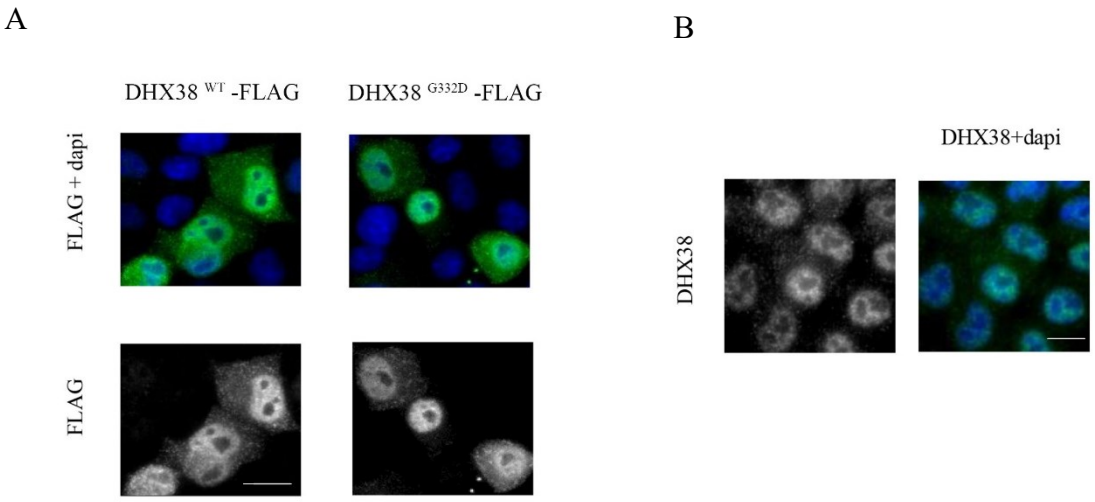


Figure 14. The localization of DHX38WT-FLAG, DHX38G332D-FLAG and endogenous DHX38 proteins in HEK293 cells.

(A) DHX38-WT and DHX38^{G332D}-FLAG have nuclear and cytoplasmic localisation. GFP (green), DAPI (blue). **(B)** DHX38 localisation in cell. DHX38 (green), DAPI (blue). Scale bar -10μm.

Characterization of DHX38 interaction profile with splicing factors

The G332D mutation is located within a protein region mediating interactions with other splicing factors in yeast Prp16. However, the binding partners of human DHX38 were unknown.

To shed light on this, we performed immunoprecipitation experiments with DHX38-FLAG. Our results showed that DHX38^{WT}-FLAG interacts with several U2 snRNP proteins including SNRNPA1 and U2A', along with U5 snRNP proteins EFTUD2, SNRNP200 and PRPF8. Interactions with the PRPF19 protein, a member of the RPR19 complex, as well as the splicing step 1 factor DHX16/PRP2 and the splicing step 2 factor SLU7, were also observed. These results suggest that DHX38 is involved in multiple complexes during the splicing process.

Comparing the coprecipitated proteins between WT and mutated DHX38, we did not detect any altered interaction patterns between DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG proteins (Figure 15A).

To understand better the impact of the G332D mutation on interaction strength, we conducted immunoprecipitation under more stringent conditions, utilizing a buffer with 300 mM NaCl and compared to immunoprecipitation under the standard 150 mM NaCl concentration. Notably, while many interactions observed in prior experiments were lost under these stringent conditions, a weak interaction between SNRNP200 and both DHX38^{WT} and DHX38^{G332D} persisted (Figure 15B).

Based on our immunoprecipitation experiments, we concluded that DHX38 interacts with an active spliceosome during the first and second steps of splicing. Furthermore, it was concluded that the identified point mutation does not affect its association with proteins within splicing complexes. However, loss of association with splicing proteins indicates that DHX38 is not a stable component of the spliceosome and interacts with it weakly or transiently.

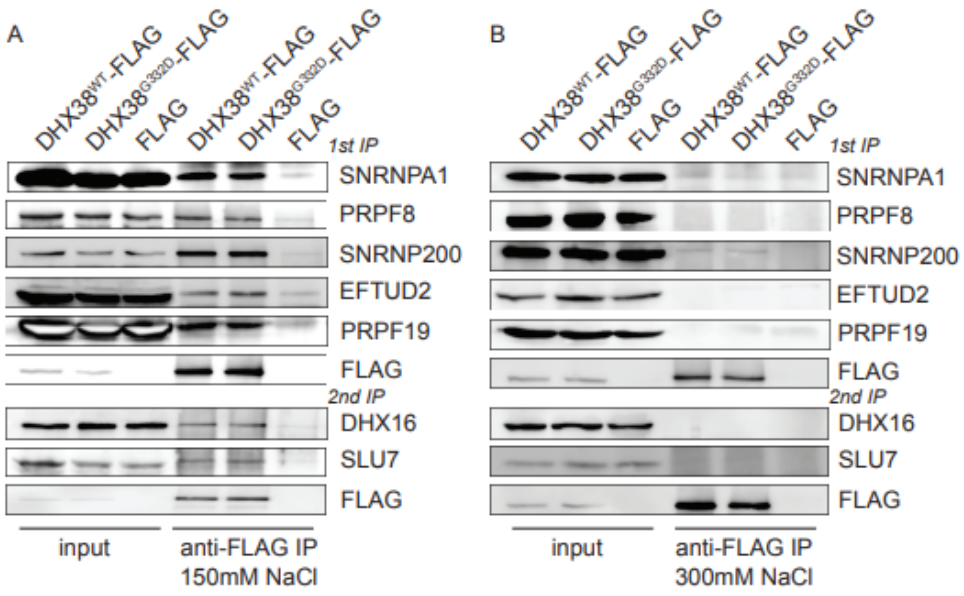


Figure 15. DHX38 is associated with multiple spliceosomal components.

(A) DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG were transiently expressed in HEK293 cells, immunoprecipitated and the copurification of selected spliceosomal proteins was monitored. Immunoprecipitation was performed in normal NET2 buffer containing 150 mM NaCl **(B)** or in stringent NET2 buffer containing 300 mM NaCl.

DHX38 does not associate with snRNAs.

RNA helicases are defined as enzymes involved in the unwinding of double-stranded RNA. We therefore tested whether DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG pull-down splicing snRNAs. However, we did not observe any interactions (Figure 16).

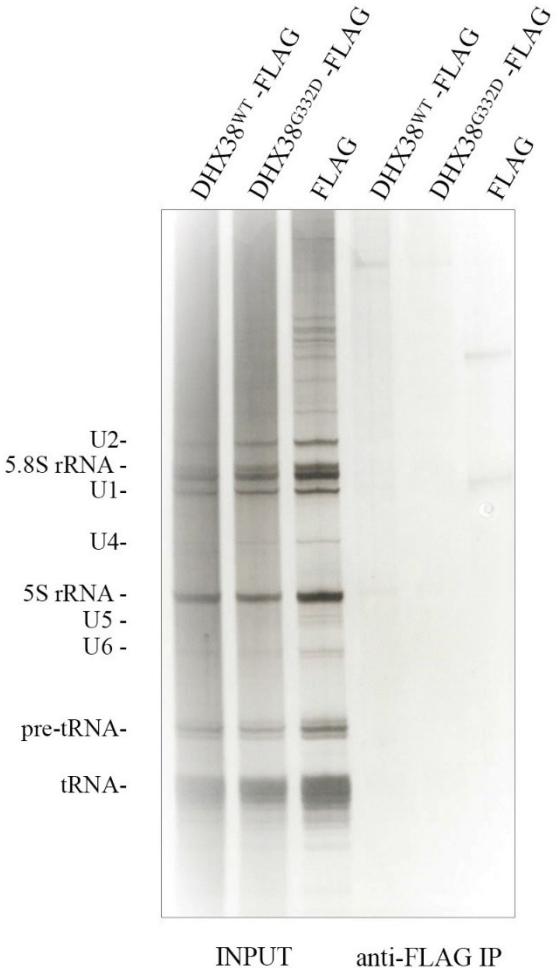


Figure 16. DHX38^{wt}-FLAG and DHX38^{G3332D}-FLAG do not associate with snRNAs.

snRNAs are resolved on denaturing urea gel and stained by silver.

Downregulation of DHX38 effects on splicing

An important question we wanted to address was how the RP mutation in DHX38 affects splicing. To answer this question, we used a small interfering RNA (siRNA) specifically designed to know down endogenous DHX38. The siRNA was targeted against the stop codon region, allowing specific downregulation of endogenous DHX38 without affecting the ectopically expressed DHX38 with the FLAG tag (Figures 17A and 17C). Initially, we knocked down DHX38 and analyzed the splicing of two housekeeping genes GAPDH and LDHA by RT-PCR (Figure 17B). We did not observe any changes in the splicing of these genes, which indicated that DHX38 is not a ubiquitous splicing factor but may function in a gene-specific manner. To test this hypothesis, we downregulated DHX38 in HEK293 cells and analyzed global changes in cellular RNA using next-generation sequencing (RNA-seq). Despite efficient DHX38 knockdown (Figure 17A), we identified only a handful of genes that showed significant changes in splicing efficiency, as measured by reads over exon-intron junctions (Table 17, Figures 18A, 19A). This is consistent with a model that human DHX38 is not an essential component of the spliceosome but is rather required for the splicing of selected genes. Surprisingly, several introns showed enhanced splicing efficiency after DHX38 knockdown (Table 17), suggesting that in some cases, DHX38 acts as splicing suppressor.

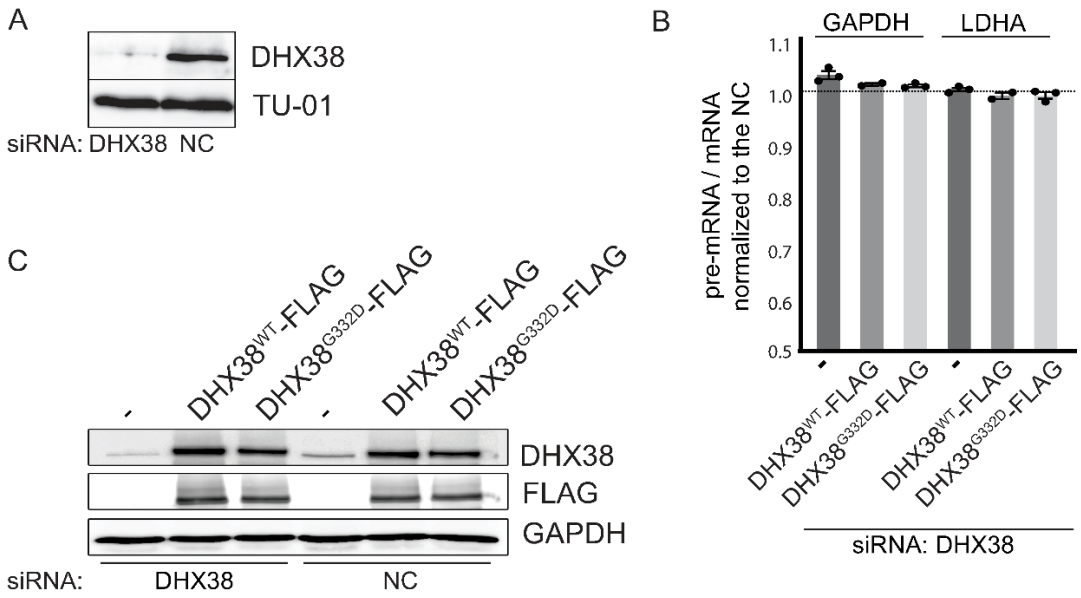


Figure 17. Downregulation of DHX38 by RNAi was monitored by Western blotting and its effect on GAPDH and LDHA.

(A) Downregulation of DHX38 by RNAi was monitored by Western blotting. TU-01 (tubulin) served as a loading control (bottom panel) **(B)** Analysis of LDHA and GAPDH splicing efficiency using RT-qPCR after downregulation of DHX38 and overexpression of DHX38WT-FLAG and DHX38G332D-FLAG proteins. The results of three independent biological experiments are shown together with SEM. **(C)** siRNA does target siRNA-resistant DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG proteins. Expression of DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG proteins was monitored by Western blotting using the anti-DHX38 antibody (top panel) and the anti-FLAG antibody (middle panel). GAPDH served as a loading control (bottom panel).

In addition, we determined genes that exhibited modified alternative splicing upon downregulation of DHX38. We specifically focused on exon inclusion/skipping and identified 71 events where exon inclusion was significantly altered upon DHX38 downregulation (Table 18, Figure 18B, 19B). These data suggest that DHX38 may function as an alternative splicing regulator. Finally, we analyzed genes differentially expressed in cells treated with anti-DHX38 and negative control siRNAs (Figure 19C). We identified 34 genes whose expression significantly changed (FDR<0.05) more than twice (Table 19, Figure 19C). From this set, we selected 6 up-regulated and 6 down-regulated genes for further investigation, probing their expression levels after DHX38 knockdown by RT-qPCR (Figures 19D and 19E).

OGA	sensing nutrient availability, metabolic and oxidative stress
RBBP7	regulation of cell proliferation and apoptosis
EPB41L2	positive regulation of protein localization to the cell cortex
APP	regulation of synaptic transmission, plasticity and calcium homeostasis
LUC7L3	regulation of gene transcription and pre-mRNA splicing
OGT	regulator of cellular homeostasis and adaptation
RPS14	protein synthesis
HNRNPH1	pre-mRNA processing
SNHG5	lncRNA
EIF4A2	protein synthesis
HMGN1	chromatin structure and gene regulation
SNHG29	snoRNA
RACK1	shuttling proteins around the cell
SNHG1	snoRNA
PPIA	activation and aggregation in platelet
RPL3	role in cell response to ribosome stress
RPS9	provides instructions for making ribosomal protein
PPM1K	regulation of the mitochondrial permeability transition pore
DDIT3	response to a variety of cell stresses and inducement of cell cycle arrest and apoptosis
SRP9	ribosomal protein
RPL31	ribosomal protein
SAT1	polyamine metabolism pathway
JMJD8	histone demethylation and other chromatin-related processes

DMXL1	cytoskeleton organisation and cell motility

Table 17. List of genes with differentially spliced introns in cells with DHX38 downregulation.

In the light blue section are genes with better-spliced introns following the knockdown of DHX38, whereas in the darker blue section are genes with less efficiently spliced introns after the knockdown of DHX38. Genes highlighted in red signify their association with neurodegenerative diseases.

MAST2	cytoskeleton regulation
BPTF	regulation of gene expression by remodeling chromatin structure
KIF23	movement and positioning of organelles and other components along microtubules
LRRFIP2	modulation of the activity of immune signaling pathways
PRR3	
CSNK1G3	regulation of processes of cell division, circadian rhythm, Wnt signalling, DNA repair and apoptosis
ACTR3B	roles in cytoskeleton dynamics and cell motility
TBC1D23	regulation of intracellular membrane trafficking
SLC50A1	transport of monocarboxylates across the cell membrane
NCOR2	transcriptional corepressor and regulation of gene expression
SBDSP1	iron metabolism and homeostasis
EPB41L2	cytoskeletal organization and cell membrane dynamics
AFMID	metabolism of the amino acid tryptophan
FOXM1	regulation of cell cycle progression and cell proliferation
TEX30	development and function of spermatozoa
GOLIM4	role in intracellular trafficking and glycosylation processes
SS18	role in the pathogenesis of synovial sarcoma
PSAP	role in lysosomal metabolism and cellular homeostasis
SMPD4	sphingolipid metabolism
MRRF	mitochondrial translation and ribosome recycling
HRAS	cellular signalling pathways that regulate cell growth, proliferation, differentiation and survival
SEC31A	role in the process of protein trafficking from the endoplasmic reticulum to the Golgi apparatus
ASPH	significant role in the post-translational modification of specific proteins
PNISR	coactivator for nuclear receptors
SLIT2	axon guidance during embryonic development
C1orf52	enables RNA binding activity
RPS24	ribosome assembly and function
UPF3B	cellular quality control
LGR5	role in tissue homeostasis, stem cell maintenance, and tissue regeneration
CIAO2A	biogenesis of iron-sulphur clusters in the cell
SRRM1	pre-mRNA processing
IPO9	nuclear import
ENAH	remodelling of actin cytoskeleton and dynamics
SAT1	polyamine metabolism pathway
ITGAE	cell adhesion and cell signalling
SPAG9	signal transduction, cell proliferation and survival, cell migration and invasion
NID1	tissue development, homeostasis, and function
SFPQ	biological role in RNA processing and gene regulation

RAC1	cellular processes involved in cell signalling, cytoskeletal dynamics, and cell migration
PHPT1	reversible dephosphorylation of phosphohistidine residues in protein
SRRM1	regulation of alternative splicing
APP	neuronal development
HMGN1	chromatin organization and gene regulation
PPP4C	cellular signal transduction and regulation
FUBP1	regulation of gene expression, cell proliferation, cell cycle control, RNA metabolism
SNHG6	lncRNA
NT5DC2	nucleotide metabolism
DHX9	pre-mRNA processing
HNRNPA2B1	RNA metabolism
UBA2	SUMOylation
CSDE1	RNA metabolism and gene regulation
PPIF	protein folding and mitochondrial function
UQCRB	mitochondrial electron transport chain
PRKDC	DNA repair and maintenance of genome stability
HDGF	cell growth, proliferation, survival, and development
PRMT1	metabolism of arginine
YWHAE	coordinator of signal transduction pathways, cell cycle regulation, apoptosis, DNA damage response
EIF5A	protein synthesis and cellular physiology
RPL38	protein synthesis and cellular processes (growth, proliferation and translational regulation)
RPS24	protein synthesis, cellular growth and translational regulation
ENSA	signal transduction pathways
ATP5F1C	cellular energy metabolism
LARP4	post-transcriptional gene regulation and protein synthesis
CD47	maintaining tissue homeostasis and preventing unwanted immune responses against healthy cells
ZNF507	regulation of gene expression
MPHOSPH9	cell division and the regulation of the mitotic spindle assembly
CASK	neuronal development, synaptic function, and signal transduction
PRPF39*	pre-mRNA processing
WIPI2	role in autophagy

Table 18. List of genes with alternatively spliced exons in cells with DHX38 downregulation.

The bolded names of genes signify alternative exon has a variant. Genes highlighted in red signify their association with neurodegenerative diseases and genes marked with an asterisk (*) are associated with retinopathy.

RAPGEF3	nucleotide exchange for the Rap subfamily of RAS-like small GTPase
CHAC1	promotion of neuronal differentiation by deglycination of the Notch receptor
INHBE	inhibition of the secretion of follitropin by the pituitary gland
DDR2	signal transduction pathways involved in cell adhesion, proliferation, extracellular matrix remodelling
FUT1	regulation of angiogenesis
SLC6A9	modulation of glycine level in the synapse by transporting glycine from the synaptic cleft into cells

SLC7A11	roles in glutamine metabolism and regulates the glucose and glutamine dependency of cancer cells
TRIB3	metabolism, cell growth, survival, apoptosis, ER stress responses, and inflammation
DDIT3	dominant-negative regulator of C/EBP-induced transcription
ASNSP1	transcribed unprocessed pseudogene
ATF5	participates in transcriptional responses of multiple cellular stressors
PCK2	opposes mitochondrial respiration and maintains the redox balance in starved lung cancer cells
NUPR1	promotion of the cancer cell proliferation
AKNA	immune system regulation, embryonic development and gene expression
STC2	cell proliferation, calcium regulation, and cancer development
BEST1*	regulation of voltage-gated Ca2+ channels to maintain calcium homeostasis
AL590617.2	lncRNA
VLDLR-AS1	lncRNA
AC026801.2	lncRNA
SCG3	regulation of the biogenesis of secretory granules
NTS	feeding regulation and metabolic control
TP53I3	role in DNA damage response and reactive oxygen species-induced apoptosis
U3	snoRNA
AC007952.4	lncRNA
NMNAT2	essential role in axon growth and survival
RAI2	role in development, cellular growth, and differentiation
TNFRSF10D	inhibitory role in TRAIL-induced cell apoptosis
HSPA1B	cellular protection and stress response
SNORD3B-2	snoRNA
CPLX2	regulation of ferroptosis and apoptosis
BAX	key role in intrinsic apoptotic signalling in neurons
SYP	crucial regulator of adrenal hyperfunction
HBA1	provides instructions for making a protein called alpha-globin
MT-RNR2	Mt_rRNA

Table 19. List of all genes differently expressed in cells with DHX38 downregulation.

In the light grey box, less prominently expressed genes are listed, while in the dark grey box, genes with more pronounced expression are listed. Genes marked with an asterisk (*) are associated with retinopathy, and those with red letters are linked to neurodegenerative diseases.

Summarization of genes that are changed after the knockdown after the RNA sequencing is represented in the form of a pie chart. They are grouped according to their known functions and biological pathways.

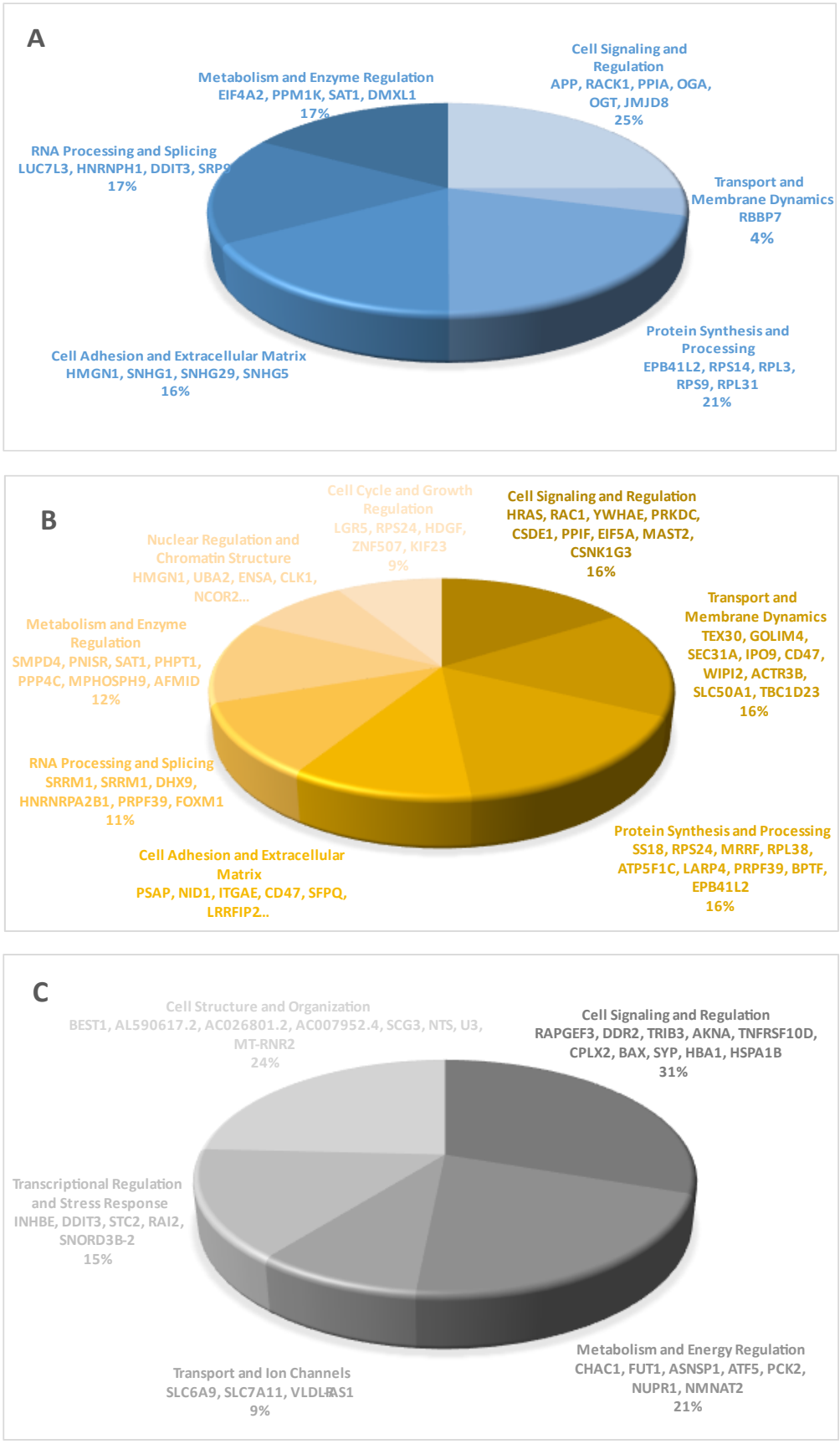


Figure 18. Altered genes after the DHX38 knockdown are grouped by combinations of their function and involvement in biological pathways.

(A) genes with differentially spliced introns in cells with DHX38 downregulation **(B)** alternatively spliced exons in cells with DHX38 downregulation **(C)** genes differently expressed in cells with DHX38 downregulation.

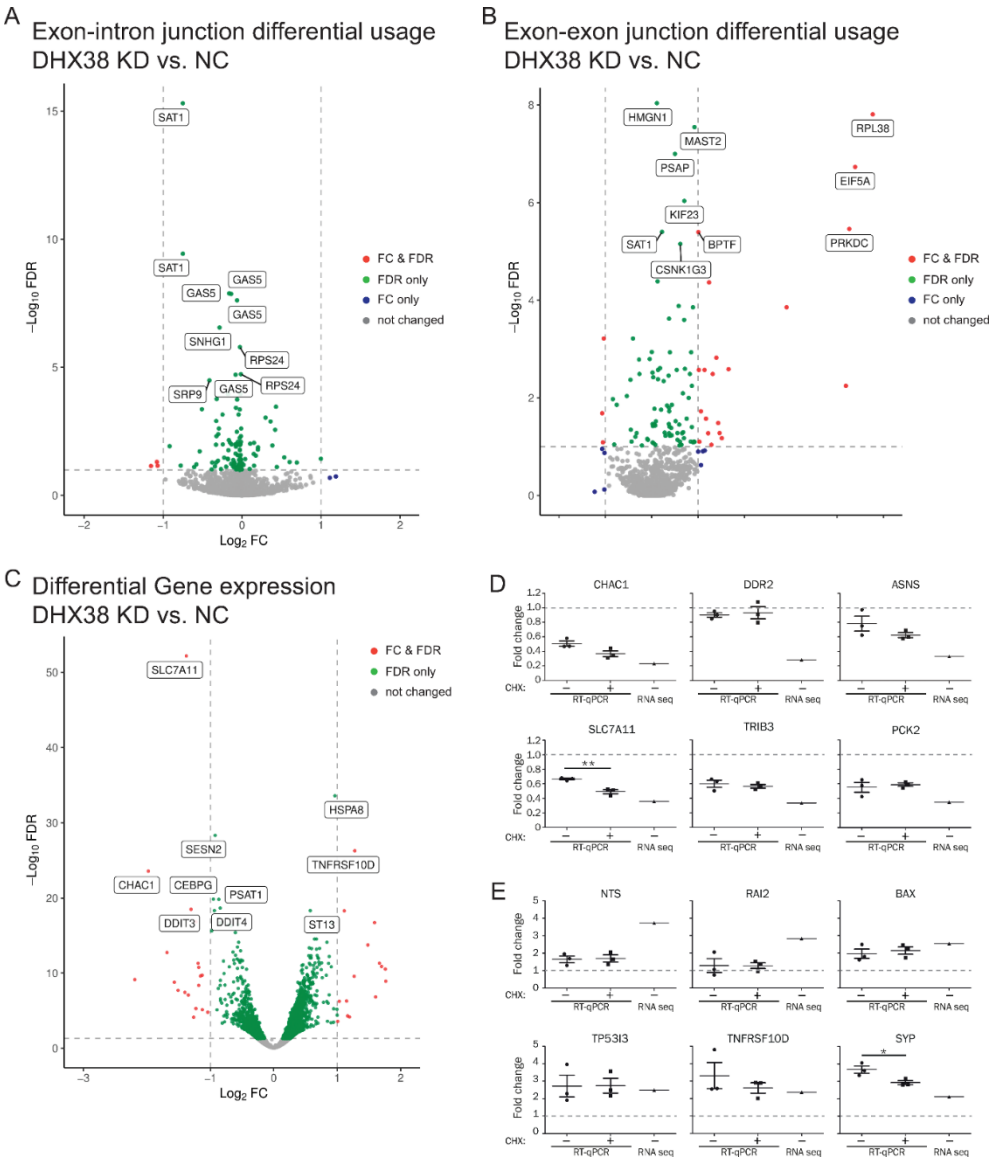


Figure 19. DHX38 downregulation changes primarily gene expression.

Summary of three independent RNA-seq analyses of HEK293 cells treated either with anti-DHX38 siRNA or negative control siRNA. **(A)** Analysis of splicing efficiency, **(B)** alternative splicing and **(C)** gene expression. **(D)** Expression of selected genes that were downregulated or **(E)** Expression of selected genes that were upregulated after DHX38 downregulation was analyzed by RT-qPCR. RNA was isolated from control cells or cells treated with cycloheximide (CHX) to reveal potential targets of non-sense mediated decay. The average of three independent experiments with SEM is shown. The

expression value determined by RNA-seq analysis is shown in the same graph for comparison (RNA seq).

Gene ontology (GO) analysis of the differentially spliced and expressed genes revealed that DHX38 knockdown triggered pathways involved in the stress response including apoptotic pathways and the response to unfolded proteins (Table 20).

Only one gene, DNA damage-inducible transcript 3 (DDIT3), exhibited reduced splicing and expression. This indicates that changes in gene expression are not primarily driven by differences in their splicing and subsequent degradation through the nonsense-mediated decay (NMD) pathway.

To test this prediction, we treated cells with cycloheximide, a potent NMD inhibitor (Carter et al., 1995) (Figures 19D and 19E). However, none of the 6 downregulated genes showed an increase in expression upon cycloheximide treatment, suggesting that their reduction is not caused by degradation via the NMD pathway.

GO analysis of biological processes of differentially expressed genes			
GO term ID	*GO term*	*Enrichment*	*p-value*
GO:0070059	intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	124	1.75e-07
GO:0006986	response to unfolded protein	57.9	6.85e-05
GO:0034620	cellular response to unfolded protein	78.6	7.11e-04
GO:0043525	positive regulation of neuron apoptotic process	62.6	5.46e-05
GO:2001244	positive regulation of intrinsic apoptotic signaling pathway	85.2	1.91e-05
GO:0009636	response to toxic substance	39.8	2.21e-04
GO:0048147	negative regulation of fibroblast proliferation	78.6	7.13e-04
GO:0010506	regulation of autophagy	45.1	1.52e-04
GO analysis of biological processes of differentially spliced genes			
GO term ID	*GO term*	*Enrichment*	*p-value*
GO:1903575	cornified envelope assembly	168	4.50e-04
GO:0051090	regulation of DNA-binding transcription factor activity	45	8.82e-04
GO:0031116	positive regulation of microtubule polymerization	43.4	9.79e-04
GO:0006470	protein dephosphorylation	15.6	8.16e-04

Table 20. GO analysis of differentially spliced and expressed genes after DHX38 knockdown.

DHX38 function in the splicing of retina-specific genes: FSCN2 and RHO

Rhodopsin is a splicing target for PRPF31, and mouse models with FSCN2 mutations exhibit photoreceptor degeneration and significant changes in rod and cone physiology (Yokokura et al., 2005). To investigate the effect of the RP mutation in DHX38 on the splicing of retina-

specific genes (rhodopsin/RHO and fascin actin-bundling protein 2/FSCN2), we downregulated endogenous DHX38 by RNAi and transiently expressed splicing reporters derived from these two retina-specific genes together with $DHX38^{WT}$-FLAG or $DHX38^{G332D}$-FLAG proteins.

Depletion of endogenous DHX38 led to reduced splicing of the FSCN2 gene, but the expression of either the WT or mutated protein ($DHX38^{G332D}$-FLAG) rescued the splicing of the FSCN2 reporter. Moreover, the splicing efficiency was partially improved compared to cells treated with the negative control siRNA (Figure 20A).

In contrast, the downregulation of DHX38 did not significantly alter RHO splicing. However, overexpression of $DHX38^{G332D}$-FLAG had a negative effect on RHO splicing (Figure 20B). Similar to the FSCN2 gene, overexpression of WT DHX38-FLAG improved splicing when compared to cells treated with the negative control siRNA.

These results suggest that DHX38 has a complex role in splicing and that specific introns exhibit different sensitivities to DHX38 downregulation and overexpression. The splicing of FSCN2 appears to be dependent on DHX38, as its downregulation reduced splicing, and the expression of both WT and RP variants rescued FSCN2 splicing. On the other hand, we did not observe any significant changes in the splicing of the RHO reporter after DHX38 downregulation. However, the expression of the mutated $DHX38^{G332D}$-FLAG significantly inhibited RHO splicing, indicating a specific effect of the RP mutation on this particular splicing event.

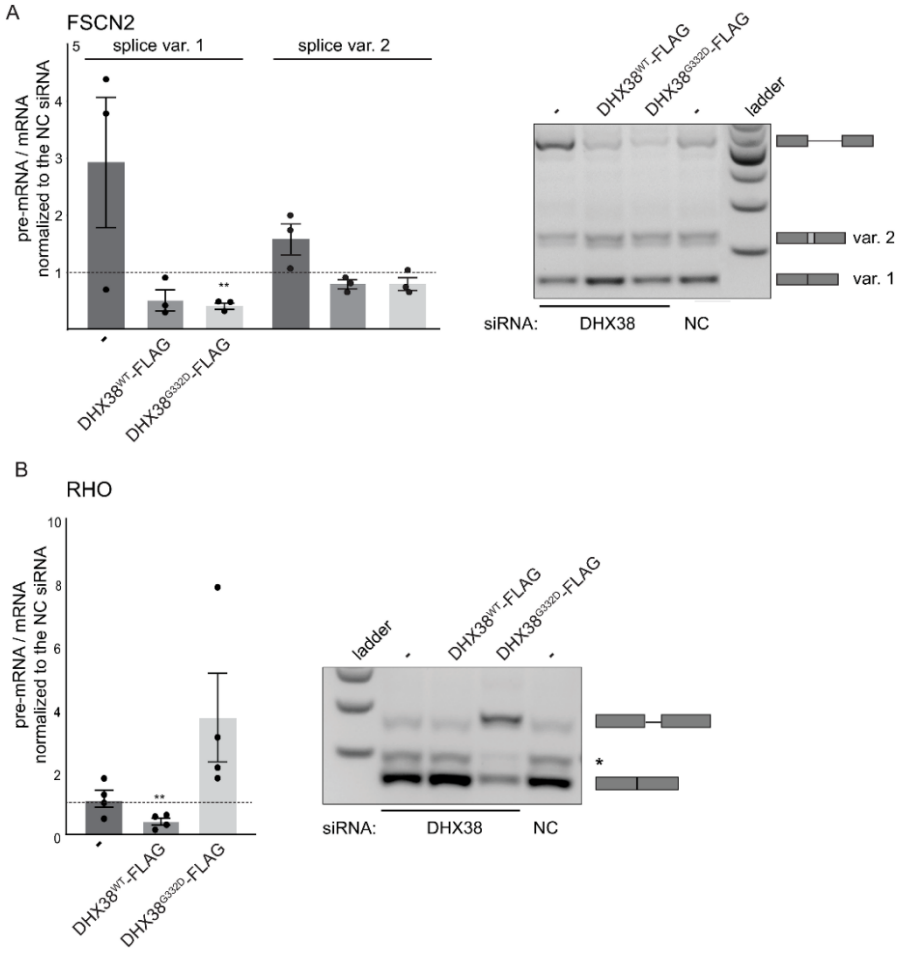


Figure 20. DHX38 is important for the splicing of retina gene-derived reporters.

Splicing of **(A)** FSCN2-derived reporter and **(B)** RHO-derived reporter was analyzed after DHX38 downregulation and after expression of siRNA-resistant DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG proteins. The normalized ratio of pre-mRNA/mRNA from at least three independent experiments is shown in a graph together with the mean and SEM. Statistical significance was assessed by a two-tail unpaired t-test (** indicates $p \leq 0.01$). Representative gels are shown. * next to RHO gel marks an unspecific product.

DHX38 function in the splicing of splice sites with different strengths.

The sequences required for normal splicing are the 5’ splice site, the branch point, the polypyrimidine tract and the 3’ splice site. These sequences are classified as 'weak' or 'strong' based on their similarity to consensus motifs. The degree to which the splice is used is assumed to increase with strength (Lim and Burge, 2001).

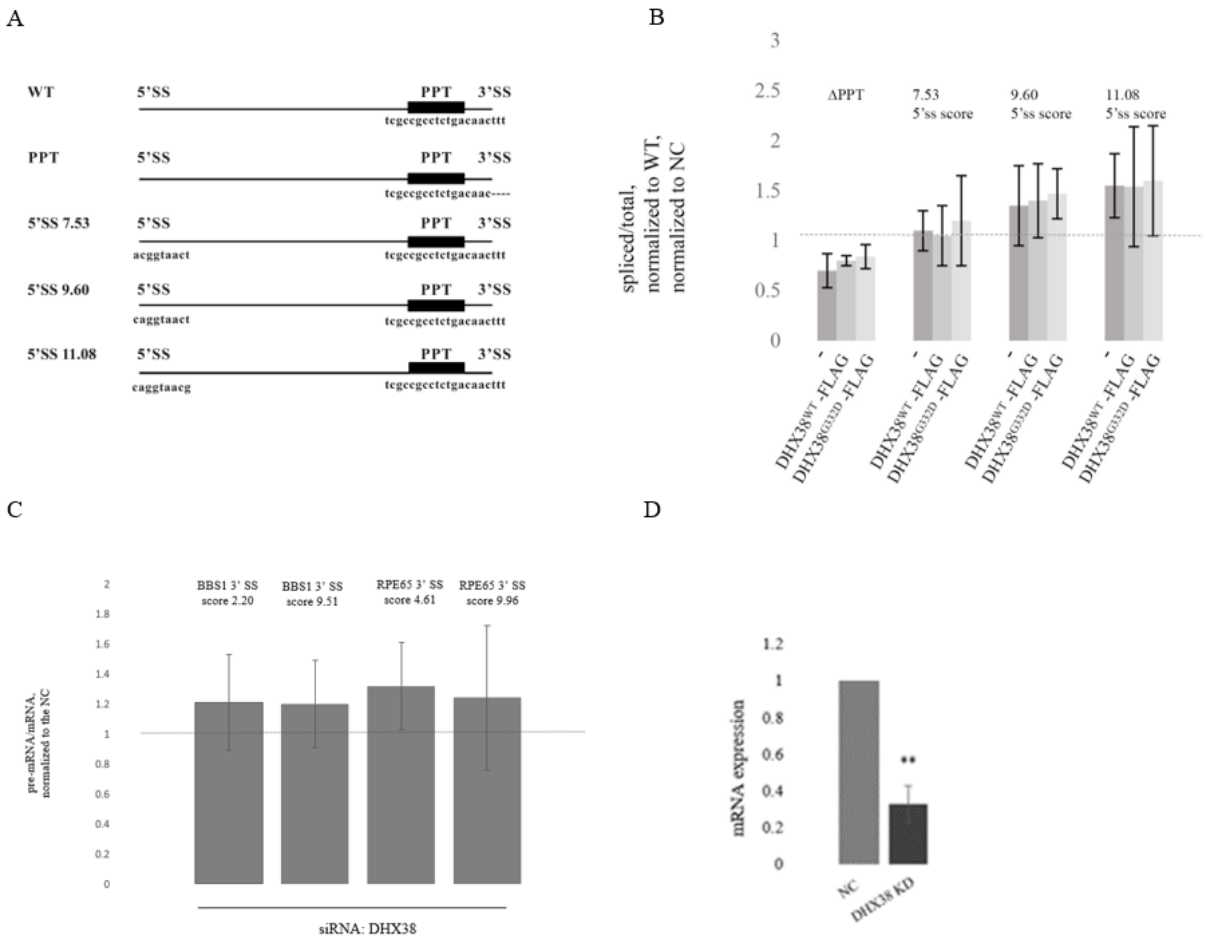


Figure 21. DHX38 does not affect the splicing of introns with changed regulatory elements 5', 3' splice sites and polypyrimidine tract.

(A) Schematic representation of the ncRNA-a2 constructs. **(B)** Splicing efficiencies of transiently expressed nc-RNA-a2s, DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG. Splicing of ncRNA-a2 is slightly less efficient after the deletion of four Ts (PPT) in PPT, strengthening of 5'SS has a small positive effect on splicing compared to WT. **(C)** Splicing efficiency of RPE65 and BBS1 with normal and low 3' splice site score in RPE cells. The 3' site score was calculated online using MaxEntScan:score3'ss for human 3'splice sites (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html). **(D)** qPCR for DHX38 mRNA in RPE cells transfected with NC and siRNA. In all experiments, the normalized ratio of pre-mRNA/mRNA or spliced/total RNA from at least three independent experiments is shown in a graph together with the mean and SEM. Statistical significance was assessed by two-tail unpaired t-test (** indicates $p \leq 0.01$).

To test whether DHX38 specifically enhances the splicing of weak splice sites, we employed splicing reporters derived from the non-coding RNA-a2 (Krchnáková et al., 2019) (Figure 21A). Endogenous DHX38 knockdown followed by overexpression of DHX38^{WT}-FLAG and DHX38^{G333D}-FLAG constructs did not affect splicing of weaker polypyrimidine tract or stronger 5' splice site (Figure 21B).

Similarly, we did not observe any significant effect on the splicing of reporters derived from RPR65 and BBS1 genes upon depletion of endogenous DHX38. In addition, changes in the strength of 3' splice sites did not affect the splicing of RPE65 and BBS1 in combination with DHX38 knockdown (Figure 21C, D). Due to poor transfection efficiency with the DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG constructs in RPE cells, it was not possible to check if the splicing is affected by overexpression of DHX38^{WT}-FLAG and DHX38^{G333D}-FLAG.

The role of DHX38 in splicing regulation: branchpoint proofreading and usage of cryptic splice site

Our previous experiments showed that DHX38 is not a ubiquitous splicing factor but is rather important for the splicing of specific genes (e.g. FSCN2) and alternative splicing regulation. Some genes showed increased splicing efficiency after DHX38 depletion, suggesting that DHX38 might act as a proofreading factor. In yeast organisms, PRP16 has been shown to play a role in proofreading of the branch point during splicing (Burgess and Guthrie, 1993). To test whether human DHX38 may have a similar function, we employed an LCTA minigene with wild-type and mutated branch point (Li and Pritchard, 2000). We applied siRNA to knockdown endogenous DHX38 and transiently coexpress DHX38^{WT}-FLAG or DHX38^{G332D}-FLAG with the LCTA splicing reporter with wild-type (LCTA-A) or mutated branch point (LCTA-A-T) LCTA splicing reporter (Figure 22A and B). Downregulation of DHX38 slightly improved the splicing of LCTA-WT (Figure 22C, line 1, Figure 22D, column 1) suggesting that DHX38 can function as a splicing suppressor of this reporter. However, overexpression of DHX38^{WT}-FLAG also enhances the splicing of the LCTA-A reporter (Figure 22C, line 2, Figure 22D, column 2) indicating that the concentration of DHX38 might be critical for splicing outcome. On the other hand, the expression of DHX38 carrying the RP mutation significantly reduced the splicing of the reporter (Figure 22C, line 3, Figure 22D, column 3) suggesting that the mutation might enhance the splicing suppressor activity of DHX38. Therefore, we used an LCTA minigene with a known branch point (Li and Pritchard, 2000) to investigate the effect of DHX38 on the splicing of mutated pre-mRNA. Knockdown of DHX38 partially improved the splicing of the mutated LCTA minigene, which is consistent with the role of DHX38 as a proofreading factor (Figure 22C, line 5, figure 22D, column 4). Overexpression of DHX38^{WT}-

FLAG and DHX38^{G332D}-FLAG restored splicing inhibition of the mutant further strengthening the function of DHX38 in proofreading the branch point of splicing substrates.

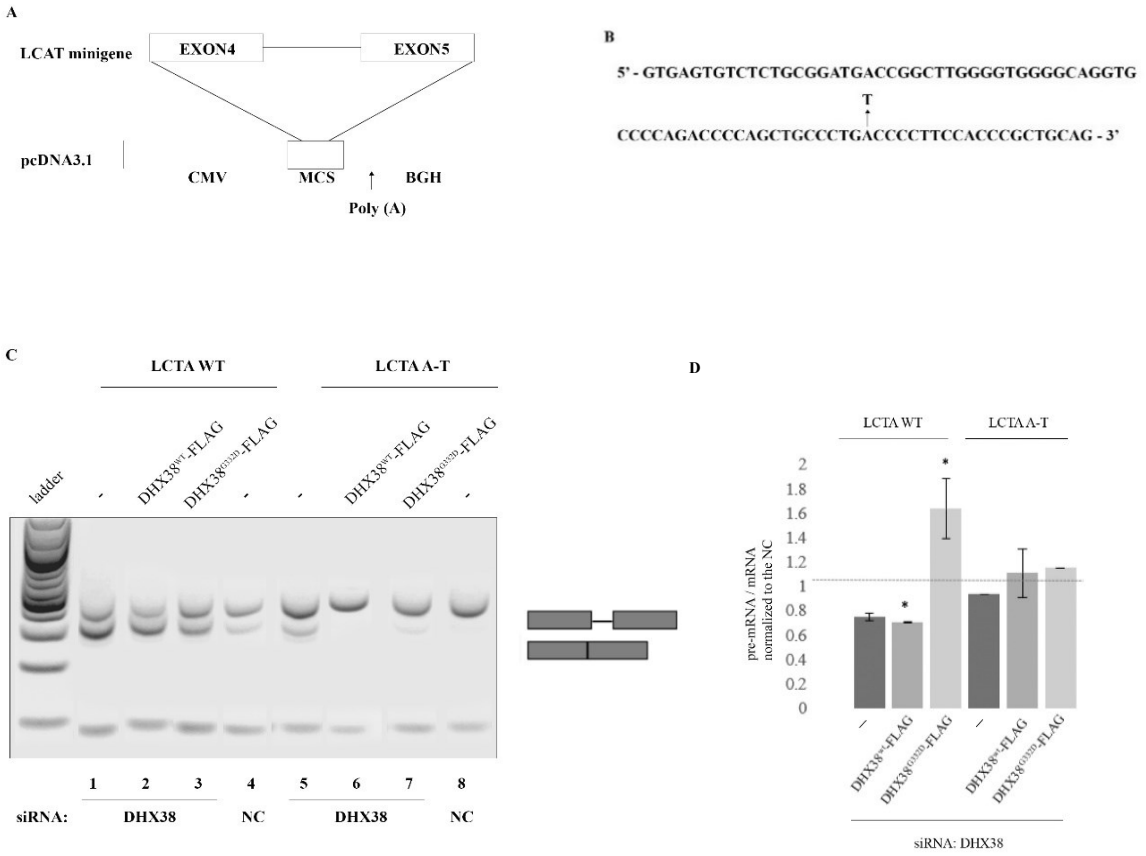


Figure 22. DHX38 does not influence the splicing of mutated branchpoint on pre-mRNA.

(A) A representative gel of LCTA-derived reporter splicing after DHX38 knockdown and expression of siRNA-resistant DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG. **(B)** Schematic presentation of the pcDNA3.1-LCAT mini gene construct, the exons 4 and 5 as well as the intron, are indicated above and below the elements, respectively. **(C)** The sequence of intron 4 of the LCTA gene, the branchpoint A is mutated to T. **(D)** Quantification of three independent experiments with the mean and SEM. Statistical significance was assessed by a two-tail unpaired t-test (* indicates $p \leq 0.05$, ** $p \leq 0.01$).

We next analyzed whether DHX38 also monitors the quality of 5' splice sites. We employed an HBB-derived splicing reporter that contains a cryptic 5' splice site 16 bp upstream of the natural 5' splice site. The usage of this cryptic splice site can be enhanced by mutation of the normal 5' splice site (Cvačková et al., 2014). Overexpression of DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG significantly stimulated splicing of normal (Figure 23, lines 2 and 3). as well as cryptic 5’ splice sites (Figure 23, lines 5-12). The data collectively indicate that

overexpression of DHX38 has a positive effect on the recognition of both normal and cryptic 5' splice sites in HBB reporter.

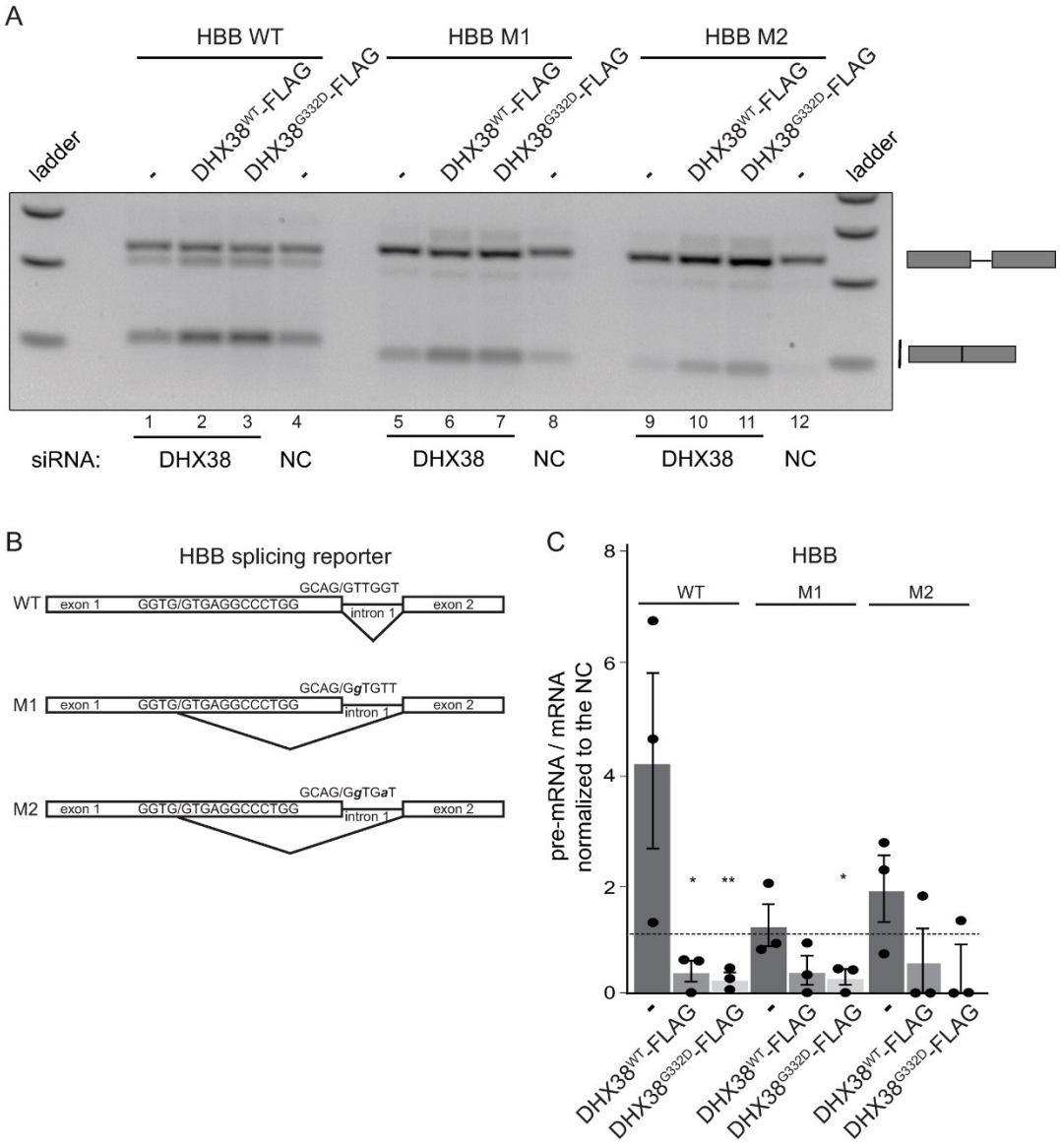


Figure 23. DHX38 promotes the splicing of the HBB-derived reporter.

(A) A representative gel of HBB-derived reporter splicing after DHX38 knockdown and expression of siRNA-resistant DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG. **(B)** A scheme of HBB-derived reporters and M1 and M2 mutants that block usage of normal splice site and promote usage of upstream 5' cryptic splice site. **(C)** Quantification of three independent experiments with the mean and SEM. Statistical significance was assessed by a two-tail unpaired t-test (* indicates $p \leq 0.05$, ** $p \leq 0.01$).

Discussion

Prp16 belongs to a family of RNA helicases that have been extensively studied in yeast. However, no structural or functional studies have been conducted on the human orthologue DHX38. The retinitis pigmentosa-associated mutation in DHX38, which is the focus of this work, is located in the N-terminal region of this protein. This region is the least conserved (reviewed in Cordin and Beggs, 2013) and on the cryo-EM maps with Prp16 in yeast, the N-terminal region could not be seen. In particular, this domain is enriched in arginine, glutamate, aspartate and serine (REDS), which accounts for more than half of all amino acids between positions 60-400 in DHX38. A similar REDS domain is found in the DHX8 spliceosomal helicase, suggesting a potential involvement for REDS in mediating protein-protein interaction (Zhou and Reed, 1998). The significance of these residues in the yeast orthologue Prp16 was assessed by alanine scanning mutagenesis (Hotz and Schwer, 1998). Additionally, the N domain of Prp16 is responsible for the interaction of this protein with other spliceosomal proteins (Wang and Guthrie, 1998).

Ajmal and colleagues did a computational simulation that indicated the presence of flexible residue glycine at position 332, which may be essential for the proper protein function. It was speculated that the insertion of aspartic acid, which is acidic compared to neutral glycine could lead to protein misfolding, potentially causing degradation or misfunction of protein (Ajmal et al., 2014b). However, we did not notice any enhanced degradation of mutated DHX38 in comparison to WT protein. DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG on the microscopy level showed the same localization pattern (Figure 14A), which indicates that the protein is properly folded. In addition, we did not detect any changes in DHX38 protein interactors with DHX38 when comparing the WT and RP variant of DHX38 (Figure 15). We did not identify interactions between DHX38^{wt}-FLAG and DHX38^{G332D}-FLAG with snRNAs (Figure 16). This is unexpected as we clearly detected an association with all major snRNP-specific markers (Figure 15). We speculate that Western blotting is more sensitive than detection of snRNAs by silver staining and we therefore did not detect snRNAs. Together our findings strongly indicate that G332D substitution does not influence the stability, localization and interaction of DHX38 with spliceosome proteins.

What we know about the function of DHX38 is mainly derived from the yeast homologue Prp16. It has been postulated that the function of Prp16 in yeast is primarily critical for the second step of splicing. However, experiments performed in nematodes, Chlamydomonas, Arabidopsis and zebrafish did not fully support this conclusion. Prp16 is not required for splicing in nematodes and Chlamydomonas but is rather involved in gene silencing and sex determination (Puoti and Kimble, 1999; Wu-Scharf et al., 2000). In Arabidopsis, the Prp16 mutation affects the splicing and expression of genes involved in auxin-mediated development (Tsugeki et al., 2015).

We therefore focused on the function of DHX38 in human splicing. We clearly showed that DHX38 interacts with multiple splicing proteins (Figure 15). However, reducing DHX38 protein expression through RNAi did not significantly inhibit splicing and only a small number of genes were affected (Figure 19). The knockout of Dhx38 in zebrafish resulted in intron retention in 150 genes, skipped exon in 685 genes and 131 alternatively spliced genes. In total, these numbers of affected genes represent only a few percent of the total number of zebrafish genes. These genes are mainly involved in processes of chromosome segregation, the microtubule cytoskeleton, cell cycle kinases and DNA damage (Tu et al., 2022). In contrast, deficiencies in the essential splicing factor PRPF31 were associated with mis-splicing of genes involved in splicing and phototransduction in the retina (Azizzadeh Pormehr et al., 2020). Furthermore, downregulation of the core splicing factor PRPF8 resulted in significantly reduced splicing of the majority of the tested genes (Klimešová et al., 2021). Differences between downregulation of DHX38 and core splicing factors indicate that vertebrate DHX38 is not an essential splicing factor but rather promotes the splicing of specific introns.

This is supported by the observation that DHX38 downregulation significantly inhibits the splicing of the FSCN2-derived splicing reporter (Figure 20A), but RPE65 and BBS1 seem to be insensitive to the downregulation of DHX38. This indicates that only certain introns are sensitive to changes in DHX38 expression or function. Similar gene-specific effects have been observed in the case of RP mutations in PRPF31, where the splicing of the RHO reporter was selectively affected but not the splicing of a reporter derived from the ROM1 gene (Yuan et al., 2005; Mordes et al., 2007). Furthermore, different RP mutations in PRPF31 and PRPF8 exhibited disparate effects on the splicing of different reporter genes (Mordes et al., 2007, Malinova et al, 2017).

DHX38 appears to selectively change the efficiency of splicing in only a few genes, suggesting that its role in the splicing cycle may be specific rather than global. The affected genes may possess unique features and sequence motifs that can be recognized by DHX38 or

cell-specific interaction partners that will lead to a selective effect on splicing efficiency. Interestingly, a study on human PRPF8 also revealed a weak 5' splice site element in introns as a feature that governs its splice-site utilization across the human transcriptome (Wickramasinghe et al., 2015).

The analysis sequences of introns that are dependent on Prp16 did not reveal any significant differences in the consensus sequence of 5' splice site, polypyrimidine tract, branch point, 3' splice site, intron length, AU content, 5' splice site to branch point and branch point to 3' splice site distances (Vijayakumari et al., 2019). To ascertain whether these findings apply to human DHX38, we tested the splicing of reporters derived from ncRNA-a2 (Krchnakova et al. 2019). They showed that splicing of ncRNA-a2-intron was suppressed by the deletion of four Ts in the polypyrimidine tract and enhanced by the strengthening of the 5' splice site. Following the knockdown of DHX38 and the coexpression of ncRNA-a2 constructs with DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG, splicing was observed to be little but not significantly suppressed in the case of the PPT construct, similarly what was observed in the experiments with only expressed ncRNA-a2 constructs. A small enhancement of splicing was observed in the case of ncRNA-a2-RNA with higher splice site scores. We further checked two RP-specific genes, BBS1 and RPE65 with normal 3' splice (scores 9.51 and 9.96 respectively) and weak 3' splice site (scores 2.20 and 4.41, respectively) and did not observe any splicing difference. Together these data indicate that DHX38 has little or no effect on the selection of a 3' splice site.

During pre-mRNA splicing, the splicing machinery must recognize and accurately position the appropriate substrates. A quality control mechanism ensures the accuracy of splicing reactions and degrades substrates that have bypassed the quality control. Splicing RNA helicases plays a crucial role in improving the fidelity of the splicing reaction. We therefore investigated whether DHX38 has a role in proofreading and whether RP mutation alters this function. We first employed a splicing reporter with a mutated branchpoint. Mutation completely inhibited splicing but it was partially rescued by downregulation of DHX38 (Figure 22). Furthermore, splicing inhibition was restored by the expression of DHX38^{WT}-FLAG and DHX38^{G332D}-FLAG. These data indicate that DHX38 might be involved in selection of correct branch points.

Under conditions where the normal splicing process is altered cryptic splice sites can be activated and used by the spliceosome, leading to aberrant splicing patterns. Cryptic splice sites look like normal splice sites but are typically ignored by the spliceosome. The link between neuronal disease and cryptic splicing patterns has been suggested in neuronal diseases such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (reviewed in Aldalaqan et al.,

2022). In neurons, activation of cryptic splice sites and dysregulation of splicing events can lead to protein misfolding, cellular stress and death of neurons in neurodegenerative diseases. Cvačková and colleagues have shown that two RP mutations in the core splicing protein SNRNP200 promote the utilization of a cryptic 5' splice site in an HBB splicing reporter (Cvačková et al., 2014a). It is noteworthy that a comparable enhancement of splicing from the HBB cryptic splice site was observed upon DHX38 overexpression, particularly with the RP variant of this helicase (Figure 23A, D). Likewise, overexpression of DHX38, both the wild-type and the RP variant, improved FSCN2 intron splicing (Figure 20A). These data strongly indicate that DHX38 promotes the recognition of weak 5' splice sites.

In addition to the splicing function, some researchers linked DHX38 to the stress response and DNA damage. Researchers have linked the role of DHX38 in DNA damage through the protein phosphatase 4 (PP4) complex (Sueji et al., 2015, Park and Lee, 2020), its role in the induction of apoptosis upon knockdown in ovarian clear cell carcinoma OCCC (Cona et al., 2022), and excessive accumulation of R-loops, DNA replication stress and DNA damage (Sun et al., 2023) in zebrafish. GO analysis of differentially spliced and expressed genes revealed that DHX38 knockdown triggers pathways involved in the stress response, including apoptotic pathways and the response to unfolded proteins. DDIT3, which showed reduced splicing and expression after DHX38 knockdown, is a transcription factor that plays a role in the cellular stress response and has been implicated in several diseases, including some neurodegenerative disorders. The suggestion of an association with DNA damage requires further investigation to fully elucidate the comprehensive role of DHX38 in these processes.

SMN deficiency, critical for snRNP metabolism, leads to spinal muscular atrophy (SMA), a motor neuron disease, and has been associated with decreased expression and altered splicing of a subset of genes containing the U12 intron in mammalian cells and Drosophila larvae (Lotti et al., 2012). In the list of introns altered in our DHX38 knockdown experiments, we did not find any U12 intron. Among the differentially expressed genes, only U12DB, has one U12 intron, according to the U12DB database (https://genome.crg.eu//cgi-bin/u12db/u12db.cgi).

Mutation in DHX38 may lead to general defects in splicing, although not strong, which could be specifically manifested in retinal cells. On the other hand, RP mutation might particularly affect the splicing of specific retina-specific transcripts that are crucial for proper eye function. Whether these splicing changes also apply to target retina tissues in patients expressing mutated DHX38 requires further investigation using a more retina-relevant model.

Summary

Retinitis pigmentosa (RP) is a genetically and clinically heterogeneous eye disease. Understanding its underlying mechanisms and developing effective therapies is challenging. Mutations in ubiquitous splicing proteins with tissue-specific effects add to the puzzle. DHX38, an RNA helicase involved in splicing, is an additional challenge in investigating its role in RP. Splicing helicases are characterized by weak and transient interactions with spliceosome proteins, making them difficult to study. Their absence does not usually stop the splicing process completely, although they improve the productivity and precision of splicing steps. As a result, many of these helicases and their precise mechanisms in pre-mRNA processing remain unknown. DHX38 is the human ortholog of Prp16 in yeast which has been the subject of extensive structural and functional studies. However, data for the function of human DHX38 are largely missing.

This study demonstrated the association of DHX38 with key splicing factors, various snRNP markers, and proteins involved in both steps of splicing. The amino acid substitution in DHX38 (glycine to aspartic acid) implicated in retinitis pigmentosa does not visibly alter its association with splicing complexes or its interaction with the spliceosome.

In cases where DHX38 is downregulated, only a few genes are found to show significant changes in their splicing efficiency, with the vast majority of genes showing no noticeable changes. It was observed that some introns showed increased splicing efficiency following DHX38 knockdown. No defects in the splicing efficiency of two housekeeping genes, LDHA and GAPDH, were observed, indicating that DHX38 downregulation does not inhibit general splicing.

Furthermore, the role of DHX38 in the splicing of retina-specific genes was highlighted. This was demonstrated by showing that FSCN2 splicing depended on DHX38, whereas retina-specific RHO splicing was specifically inhibited by expressing the DHX38 RP variant. Structurally, DHX38 is a member of the RNA helicase family. However, we did not observe any interaction with snRNA. In addition, we did not see any significant changes in the experiments that tested the effect of DHX38 on the splicing of the weaker 3’ splice sites, the polypyrimidine tracts and the stronger 5’ splice site.

However, the knockdown of DHX38 and overexpression of DHX38 WT or DHX38 RP influence the splicing of the reporter with a mutated branch point. DHX38 overexpression also promoted the usage of both canonical and cryptic 5' splice sites in an HBB splicing reporter. Our data suggest that DHX38 is a splicing regulator that supervises the branch point selection and enhances the splicing of specific introns with suboptimal 5' splice sites. The RP variant of DHX38 may shift the balance of the splicing regulatory network, leading to differences in the splicing of sensitive introns and exons compared to cells expressing the wild-type DHX38.

The study on DHX38 is just getting started. Our findings shed light on the intricate role of DHX38 in splicing regulation and its potential implications for retinitis pigmentosa. Further investigations are needed to fully understand the mechanisms behind these splicing changes and their contribution to the disease phenotype.

Supplementary material

Ensembl_ID	Gene symbol	J3 (exon-exon junction)	p-value	FDR	dPIR	
ENSG00000198408:Io017	OGA	10.101792943.101797980	0.000312657	0.036803466	-0.29764411	Better spliced after DHX38 KD
ENSG00000102054:I005	RBBP7	X.16852122.16852416	0.000474971	0.049200624	-0.247405625	
ENSG00000079819:Io021	EPB41L2	6.130870126.130885096	0.00025813	0.032345308	-0.246814912	
ENSG00000142192:Io010	APP	21.25982477.26021840	9.12961E-06	0.002727997	-0.200280112	
ENSG00000108848:Io016	LUC7L3	17.50750694.50752200	6.5477E-07	0.000339127	-0.162243245	
ENSG00000147162:Io021	OGT	X.71544635.71547907	3.81436E-06	0.001288424	-0.161954887	
ENSG00000164587:Io004	RPS14	5.150447735.150449492	6.7369E-05	0.01113596	-0.152915622	
ENSG00000169045:Io015	HNRNPH1	5.179614959.179616126	2.42807E-06	0.000898269	-0.140368852	
ENSG00000203875:Io004	SNHG5	6.85677492.85677954	0.000268886	0.033158324	0.109232162	Less spliced after DHX38 KD
ENSG00000156976:Io005	EIF4A2	3.186784696.186785883	5.43958E-05	0.010156266	0.114977762	
ENSG00000205581:E014	HMGN1	21.39345274.39345830	1.75984E-06	0.000683611	0.116304747	
ENSG00000175061:Io007	SNHG29	17.16439414.16439581	3.48759E-06	0.001231596	0.132296085	
ENSG00000204628:Io008	RACK1	5.181242345.181243680	1.83981E-05	0.004866004	0.133058844	
ENSG00000255717:I022	SNHG1	11.62855216.62855424	2.15702E-10	2.79298E-07	0.136407071	
ENSG00000196262:I001	PPIA	7.44796793.44798748	1.45378E-05	0.004033709	0.136957217	
ENSG00000196262:Io007	PPIA	7.44798814.44799247	7.8492E-06	0.002449651	0.139079984	
ENSG00000100316:Io015	RPL3	22.39318592.39319499	0.000134129	0.018946308	0.140580244	
ENSG00000170889:I009	RPS9	19.54206724.54207398	5.76481E-05	0.010415546	0.146914906	
ENSG00000163644:I001	PPM1K	4.88262726.88265001	0.00041148	0.043791581	0.148639095	
ENSG00000175197:Io004	DDIT3	12.57517438.57517706	2.85189E-07	0.000170434	0.161756786	
ENSG00000143742:Io006	SRP9	1.225786985.225789240	4.15866E-08	3.23087E-05	0.189702298	
ENSG00000071082:Io004	RPL31	2.101006071.101018998	9.28716E-07	0.000424423	0.21210437	
ENSG00000130066:I008	SAT1	X.23784403.23785328	9.45779E-14	3.67388E-10	0.251620445	
ENSG00000130066:I005	SAT1	X.23783883.23784294	6.33629E-20	4.92267E-16	0.252188705	
ENSG00000161999:Io010	JMJD8	16.682874.682958	0.000463398	0.048650481	0.31898686	
ENSG00000172869:I003	DMXL1	5.119071257.119071555	7.35776E-05	0.011908835	0.358168148	

Table 21. Differentially spliced introns, DHX38 KD vs NC (FDR<0.05).

Ensemble_ID	event	gene symbol	J3 (exon-exon junction)	log FC	p-value	FDR	dPSI
ENSG00000086015:E013	ES	MAST2	1.45997799.46002805	0.924	0.000	0.000	-0.393
ENSG00000171634:E011	ES	BPTF	17.67875020.67891844;17.67875020.67886128	1.008	0.000	0.000	-0.340
ENSG00000137807:E019	ES	KIF23	15.69423329.69426070	0.706	0.000	0.000	-0.282
ENSG00000093167:E023	ES	LRRFIP2	3.37083806.37094792	1.395	0.000	0.002	-0.278
ENSG00000204576:E009	ES*	PRR3	6.30557450.30561834	0.718	0.000	0.003	-0.277
ENSG00000151292:E021	ES	CSNK1G3	5.123604830.123614342	0.619	0.000	0.000	-0.265
ENSG00000133627:E009	ES*	ACTR3B	7.152801731.152814550	0.584	0.000	0.000	-0.260
ENSG00000036054:E028	ES	TBC1D23	3.100310542.100316099	1.018	0.000	0.003	-0.241
ENSG00000169241:E020	ES*	SLC50A1	1.155136376.155137561	0.755	0.001	0.027	-0.232
ENSG00000196498:E062	ES	NCOR2	12.124372610.124378237	0.543	0.000	0.003	-0.219
ENSG00000225648:E007	ES	SBDSP1	7.72829783.72831602	0.519	0.001	0.030	-0.217
ENSG00000079819:E032	ES	EPB41L2	6.130870126.130885096	1.238	0.000	0.000	-0.216
ENSG00000183077:E027	ES	AFMID	17.78191060.78206911	1.063	0.001	0.019	-0.216
ENSG00000111206:E015	ES	FOXM1	12.2864752.2866393;12.2864755.2866393	0.703	0.000	0.000	-0.200
ENSG00000151287:E004	ES	TEX30	13.102766580.102768260	0.869	0.000	0.006	-0.194
ENSG00000173905:E011	ES	GOLIM4	3.168036994.168041392	1.316	0.000	0.003	-0.188
ENSG00000141380:E014	ES	SS18	18.26035127.26038555	0.395	0.000	0.001	-0.185
ENSG00000197746:E016	ES	PSAP	10.71822007.71825837	0.504	0.000	0.000	-0.185
ENSG00000136699:E035	ES	SMPD4	2.130157396.130164374	0.368	0.000	0.004	-0.173
ENSG00000148187:E019	ES	MRRF	9.122285287.122291749	0.373	0.001	0.034	-0.173
ENSG00000174775:E007	ES	HRAS	11.532755.533453	0.381	0.001	0.019	-0.171
ENSG00000138674:E046	ES	SEC31A	4.82857764.82863318	0.377	0.001	0.016	-0.160
ENSG00000198363:E040	ES	ASPH	8.61643433.61644600	0.853	0.000	0.001	-0.160
ENSG00000132424:E012	ES*	PNISR	6.99402710.99404603	0.596	0.001	0.035	-0.159
ENSG00000145147:E023	ES	SLIT2	4.20524177.20528949	0.825	0.000	0.003	-0.158
ENSG00000162642:E004	ES	C1orf52	1.85258722.85259358	0.385	0.002	0.039	-0.153
ENSG00000138326:E031	ES*	RPS24	10.78037304.78040615	0.377	0.000	0.000	-0.151
ENSG00000125351:E017	ES*	UPF3B	X.119838527.119841076	0.424	0.002	0.041	-0.150
ENSG00000139292:E011	ES	LGR5	12.71559654.71566404	1.171	0.001	0.027	-0.146
ENSG00000166797:E008	ES	CIAO2A	15.64075537.64088687	0.675	0.000	0.008	-0.141
ENSG00000133226:E057	ES	SRRM1	1.24662804.24666815	0.438	0.000	0.014	-0.135
ENSG00000198700:E003	ES	IPO9	1.201829372.201847279	0.261	0.000	0.005	-0.124
ENSG00000154380:E010	ES	ENAH	1.225501070.225507951	0.333	0.000	0.014	-0.116
ENSG00000130066:E013	ES*	SAT1	X.23783709.23785328;X.23783883.23785328	0.225	0.000	0.000	-0.107
ENSG00000083457:E010	ES	ITGAE	17.3720402.3723688	0.205	0.001	0.017	-0.100
ENSG00000008294:E014	ES	SPAG9	17.50974947.50977108	0.183	0.000	0.002	-0.089
ENSG00000116962:E010	ES	NID1	1.236012043.236017148	1.657	0.000	0.003	-0.081
ENSG00000116560:E007	-	SFPQ	1.35177399.35189003	0.869	0.002	0.040	-0.075
ENSG00000136238:E008	ES	RAC1	7.6392041.6400126	0.138	0.000	0.004	-0.069
ENSG00000054148:E011	ES*	PHPT1	9.136850137.136850755	0.140	0.000	0.003	-0.067
ENSG00000133226:E045	ES	SRRM1	1.24653032.24654855	0.130	0.000	0.000	-0.064
ENSG00000142192:E021	ES	APP	21.25982477.26021840	0.891	0.000	0.000	-0.064
ENSG00000205581:E015	ES*	HMGN1	21.39345274.39348292;21.39345274.39347379;2	0.116	0.000	0.000	-0.056
ENSG00000149923:E018	ES*	PPP4C	16.30076475.30082484	1.141	0.000	0.003	-0.053
ENSG00000162613:E040	ES	FUBP1	1.77968203.77969925	0.095	0.001	0.030	-0.048
ENSG00000245910:E006	ES*	SNHG6	8.66922114.66922614	0.799	0.000	0.010	-0.045
ENSG00000168268:E013	-	NT5DC2	3.52525103.52527294	1.434	0.001	0.033	-0.041
ENSG00000135829:E010	ES	DHX9	1.182854178.182856532	0.080	0.002	0.037	-0.039
ENSG00000122566:E015	-	HNRNPA2B1	7.26193373.26195847	4.191	0.000	0.006	-0.033
ENSG00000126261:E007	ES	UBA2	19.34428570.34434868;19.34428570.34431861;1	0.062	0.001	0.017	-0.031
ENSG00000009307:E029	ES	CSDE1	1.114736855.114737963	0.061	0.001	0.035	-0.030
ENSG00000108179:E010	-	PPIF	10.79351583.79353707	2.913	0.000	0.000	-0.024
ENSG00000156467:E012	ES*	UQCRB	8.96231132.96231774;8.96231132.96231532	0.037	0.000	0.004	-0.019
ENSG00000253729:E057	-	PRKDC	8.47863577.47877724	4.265	0.000	0.000	-0.018
ENSG00000143321:E016	ES	HDGF	1.156744348.156745008	0.030	0.002	0.038	-0.016
ENSG00000126457:E023	ES*	PRMT1	19.49677316.49680487;19.49679925.49680487	0.025	0.001	0.034	-0.013
ENSG00000108953:E018	ES	YWHAE	17.1365058.1400047	0.022	0.000	0.003	-0.011
ENSG00000132507:E014	-	EIF5A	17.7307752.7311018	4.390	0.000	0.000	-0.005
ENSG00000172809:E011	-	RPL38	17.74203745.74204130	4.770	0.000	0.000	-0.003
ENSG00000138326:E022	ES*	RPS24	10.78037304.78040615;10.78037304.78040204;1	0.006	0.000	0.001	-0.003
ENSG00000143420:E018	ES	ENSA	1.150625688.150629550;1.150625808.15062746	-0.040	0.000	0.002	0.019
ENSG00000165629:E022	ES	ATP5F1C	10.7802854.7807659	-1.034	0.000	0.001	0.038
ENSG00000161813:E050	ES	LARP4	12.50441643.50454314	-1.068	0.001	0.021	0.124
ENSG00000196776:E006	ES	CD47	3.108047292.108057477;3.108047292.10805193	-0.263	0.000	0.003	0.126
ENSG00000196776:E005	ES	CD47	3.108047292.108057477;3.108047292.10805193	-0.272	0.000	0.002	0.131
ENSG00000168813:E005	ES	ZNF507	19.32345783.32352829	-0.838	0.000	0.011	0.153
ENSG00000051825:E046	ES*	MPHOSPH9	12.123203084.123210056	-0.537	0.000	0.009	0.181
ENSG00000147044:E029	ES	CASK	X.41553915.41559779;X.41555635.41559779	-0.404	0.000	0.001	0.183
ENSG00000185246:E015	ES*	PRPF39	14.45096228.45096887	-0.452	0.002	0.045	0.200
ENSG00000157954:E008	ES*	WIPI2	7.5190493.5199576	-0.460	0.000	0.004	0.213
ENSG00000013441:E027	ES*	CLK1	2.200859746.200861238	-0.755	0.000	0.014	0.236

Table 22. Alternatively spliced exons, DHX38 KD vs NC (FDR<0.05).

Ensembl_Gene_ID	Gene symbol	Definition	Biotype	log2FC	p-value	Adjusted p-Value	Mean expression (log10)	
ENSG00000079337	RAPGEF3	Rap guanine nucleotide exchange factor 3	protein_coding	-2.19	4.99e-12	7.62e-10	2.53	less expressed after DHX38 KD
ENSG00000128965	CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	protein_coding	-1.98	8.34e-28	2.65e-24	2.29	
ENSG00000139269	INHBE	inhibin subunit beta E	protein_coding	-1.68	3.87e-16	1.81e-13	2.07	
ENSG00000162733	DDR2	discoidin domain receptor tyrosine kinase 2	protein_coding	-1.57	1.28e-11	1.71e-09	2.68	
ENSG00000174951	FUT1	fucosyltransferase 1 (H blood group)	protein_coding	-1.51	1.99e-10	2.04e-08	2.63	
ENSG00000196517	SLC6A9	solute carrier family 6 member 9	protein_coding	-1.40	4.15e-10	3.79e-08	2.89	
ENSG00000151012	SLC7A11	solute carrier family 7 member 11	protein_coding	-1.38	4.19e-57	6.65e-53	3.20	
ENSG00000101255	TRIB3	tribbles pseudokinase 3	protein_coding	-1.35	1.04e-09	8.45e-08	3.00	
ENSG00000175197	DDIT3	DNA damage inducible transcript 3	protein_coding	-1.31	1.87e-22	3.30e-19	2.72	
ENSG00000248498	ASNSP1	asparagine synthetase pseudogene 1	transcribed_unprocessed_pseudogene	-1.26	3.42e-06	7.44e-05	2.47	
ENSG00000169136	ATF5	activating transcription factor 5	protein_coding	-1.23	1.24e-20	1.52e-17	2.86	
ENSG00000100889	PCK2	phosphoenolpyruvate carboxykinase 2, mitochondrial	protein_coding	-1.23	1.35e-07	5.25e-06	2.99	
ENSG00000176046	NUPR1	nuclear protein 1, transcriptional regulator	protein_coding	-1.19	1.70e-14	4.82e-12	2.29	
ENSG00000106948	AKNA	AT-hook transcription factor	protein_coding	-1.18	7.12e-14	1.66e-11	2.44	
ENSG00000113739	STC2	stanniocalcin 2	protein_coding	-1.18	3.89e-11	4.52e-09	3.45	
ENSG00000167995	BEST1	bestrophin 1	protein_coding	-1.16	1.46e-12	2.54e-10	2.17	
ENSG00000225177	AL590617.2	novel transcript	lncRNA	-1.13	1.11e-12	2.00e-10	2.37	
ENSG00000236404	VLDLR-AS1	VLDLR antisense RNA 1	lncRNA	-1.13	2.07e-07	7.48e-06	1.86	
ENSG00000272323	AC026801.2	novel transcript, antisense to TTC23L	lncRNA	-1.04	5.19e-07	1.63e-05	1.77	
ENSG00000104112	SCG3	secretogranin III	protein_coding	1.77	8.98e-12	1.26e-09	1.81	better expressed after DHX38 KD
ENSG00000133636	NTS	neurotensin	protein_coding	1.76	1.31e-13	2.86e-11	2.08	
ENSG00000115129	TP53I3	tumor protein p53 inducible protein 3	protein_coding	1.70	5.28e-14	1.29e-11	2.10	
ENSG00000212195	U3	Small nucleolar RNA U3	snoRNA	1.66	1.64e-14	4.73e-12	2.12	
ENSG00000262202	AC007952.4	novel transcript	lncRNA	1.60	2.13e-09	1.54e-07	1.84	
ENSG00000157064	NMNAT2	nicotinamide nucleotide adenylyltransferase 2	protein_coding	1.59	1.72e-20	1.95e-17	2.17	
ENSG00000131831	RAI2	retinoic acid induced 2	protein_coding	1.48	2.94e-17	1.79e-14	2.49	
ENSG00000173530	TNFRSF10D	TNF receptor superfamily member 10d	protein_coding	1.28	1.34e-30	5.33e-27	3.06	
ENSG00000204388	HSPA1B	heat shock protein family A (Hsp70) member 1B	protein_coding	1.27	1.62e-12	2.80e-10	4.17	
ENSG00000262074	SNORD3B-2	small nucleolar RNA, C/D box 3B-2	snoRNA	1.19	3.06e-06	6.85e-05	3.18	
ENSG00000145920	CPLX2	complexin 2	protein_coding	1.16	2.03e-06	4.92e-05	1.62	
ENSG00000087088	BAX	BCL2 associated X, apoptosis regulator	protein_coding	1.15	8.90e-09	5.28e-07	2.85	
ENSG00000102003	SYP	synaptophysin	protein_coding	1.11	3.99e-22	5.29e-19	2.57	
ENSG00000206172	HBA1	hemoglobin subunit alpha 1	protein_coding	1.03	1.01e-08	5.78e-07	2.03	
ENSG00000210082	MT-RNR2	mitochondrially encoded 16S rRNA	Mt_rRNA	1.01	1.62e-05	2.64e-04	2.93	

Table 23. Differentially expressed genes, DHX38 KD vs NC.

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