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Review Article

Alternative splicing of *P2RX7* pre-messenger RNA in health and diseases: Myth or reality?

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ABSTRACT

Alternative splicing (AS) tremendously increases the use of genetic information by generating protein isoforms that differ in protein–protein interactions, catalytic activity and/or subcellular localization. This review is not dedicated to AS in general, but rather we focus our attention on AS of *P2RX7* pre-mRNA. Whereas *P2RX7* mRNA is expressed by virtually all eukaryotic mammalian cells, the expression of this channel receptor is restrained to certain cells. When expressed at the cell membrane, P2RX7 controls downstream events including release of inflammatory molecules, phagocytosis, cell proliferation and death and metabolic events. Therefore, P2RX7 is an important actor of health and diseases. In this review, we summarize the general mechanisms leading to AS. Further, we recapitulate our current knowledge concerning the functional regions in P2RX7, identified at the genetic or exonic levels, and how AS may affect the expression of these regions. Finally, the potential of *P2RX7* splice variants to control the fate of cancer cells is discussed.

Splicing and alternative splicing

The first description of alternative splicing (AS) was reported by Chow and colleagues in 1977 who described a new mechanism for the biosynthesis of mRNA in mammalian cells [1]. For this discovery, Richard Roberts and Philips Sharp were awarded the Nobel Prize in 1993. Since then, more than 32,000 articles can be found on Pubmed using "alternative splicing" as key word. Of importance, AS is linked to diseases and cancer, with 6269 and 7553 publications found at the beginning of 2019.

General mechanisms leading to splicing

The regulation of splicing is a complex mechanism that requires several factors involved in the precise selection of splicing sites and subsequent splicing processes [2]. As illustrated in [Fig. 1A], the splicing consists of six sequential steps

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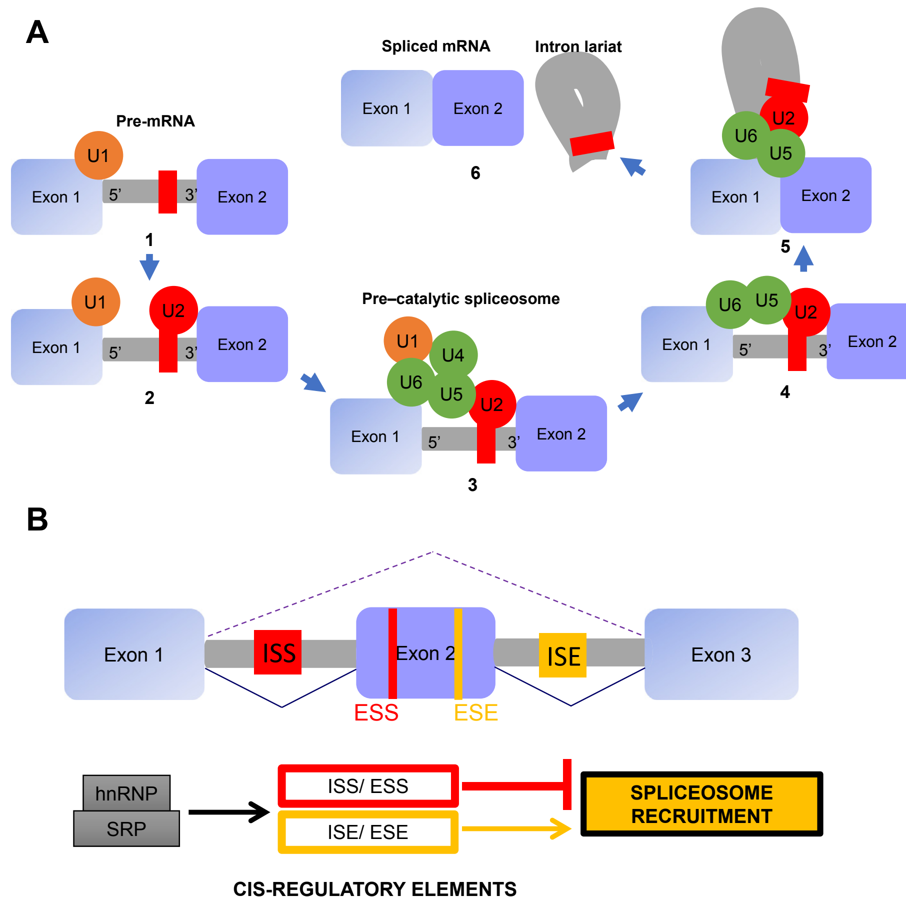

Fig. 1 Alternative Splicing requires two levels of regulation. (A) *Pre-mRNA splicing* require 6 sequential steps: 1: Pre-mRNA splicing starts with U1 snRNP (yellow circle) binding to the 5′-splice site; 2: U2 snRNP binds to the branch point (red rectangle) of the intronic sequence (gray bar); 3: Pre-assembled U4/U5/U6 snRNP (green circles) bind to U1 and U2 forming the pre-catalytic complex, and U1 snRNA 5′ site is transferred to U6; 4: Spliceosome activation starts with U1 and U4 snRNP exclusion; 5: Intramolecular stem-loop between U2 and U6 allows transesterifications steps which lead to exons linking and 6: Spliced mRNA is released as a ribonucleoproteic particle; intronic sequence is released as a « lariat » (gray loop). Note: The protein composition of each spliceosomal complex is not shown in this figure for the purpose of simplification. (B) *pre-mRNA splicing regulation*. Cis-regulatory elements are sequences localized in exonic or intronic pre-mRNA which regulate alterative splicing and spliceosome recruitment. ESS (in red letters) and ISS (highlighted in red), exonic splicing silencer and intronic splicing silencer inhibit the splicing of pre-mRNA. ESE (in yellow letters) and ISE (highlighted in yellow), exonic splicing enhancer and intronic splicing enhancer are sequences which promote the splicing of pre-mRNA and will conduct in the present illustration to Exon 2 skipping (broken line). This splicing mechanism involves the recruitment of regulation proteins such as hnRNPs (Heterogeneous nuclear ribonucleoproteins), and SRP (Serine-Arginine rich proteins), which can bind on cis-regulatory elements and regulate splicing.

leading to the removal of specific sequences in a vast genomic sequence background. This “scissor mechanism” relies on five small nuclear ribonucleoproteins (snRNP U1, U2, U4, U5 and U6) which together form the core of the spliceosome. In brief, pre-mRNAs splicing is initiated by the binding of U1 on the 5′-splice site (step 1) and U2 on the branch point (step 2). Then, U4, U5 and U6 associate with U1 and U2 to form the pre-catalytic spliceosome complex (step 3). Further, U1 5′ snRNA site is transferred to U6, leading to the activation of the spliceosome and to the removal of U1 and U4 from the complex (step 4). Consequently, U6 replaces U1 location and the interaction between U6 and U2 gathers the 5′-splice site and the branch point allowing the 5th transesterification step. The two exons are then brought to close proximity by U5 and finally joint ending the splicing procedure (step 6). The splicing regulation does not only depend on the composition of splice sites, indeed, ribonuclear–protein complexes, referred as RNPs in [Fig. 1B], associate with pre-mRNA to facilitate exon recognition [3]. These regulatory proteins are classified in two major classes: heterogeneous nuclear ribonucleoproteins (hnRNPs) which bind to RNA, and SR proteins which contain serine and arginine rich protein regions involved in protein recognition. Both families regulate splicing via binding of intronic or exonic splicing silencers or enhancers as illustrated in [Fig. 1B] [2,4–6]. Finally, additional levels of regulation are sustained by the secondary structure

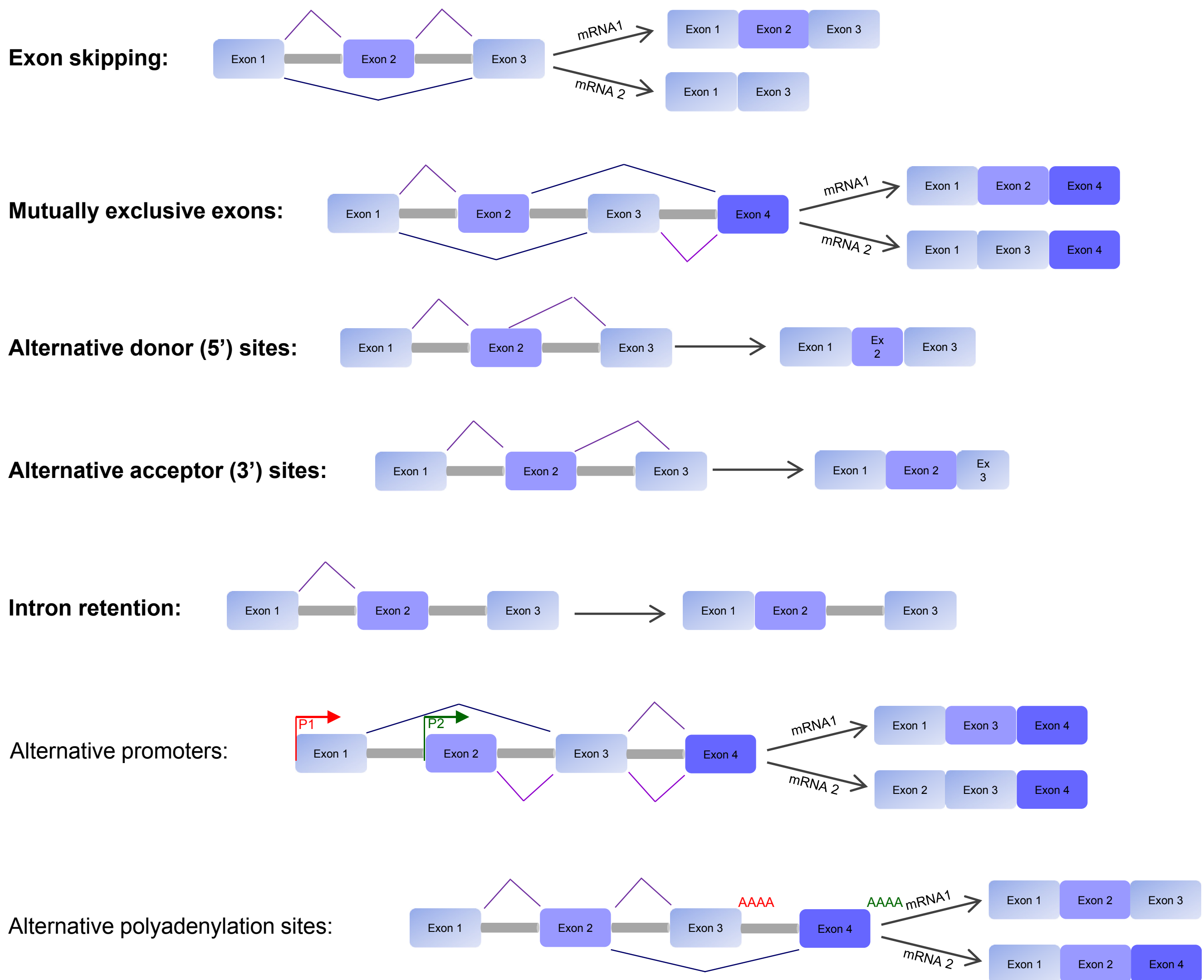

Fig. 2 From one pre-mRNA to various mRNAs. The seven described modes of alternative splicing increase the mRNA diversity. Bold characters are for the most common AS modes. Exon skipping, also named cassette exons, allows the exclusion of a full exon. Mutually exclusive exons lead to the egress of one exon out of two. Alternative donor 5′ site uses an alternative 5′ splice donor site to generate mRNA. Alternative acceptor 3′ site uses an alternative 3′ splice acceptor site. Intron retention corresponds to the addition of an intronic sequence within mRNA. Alternative promoter mode describes the use of different promoters resulting in a different start site of the mRNA transcript. An alternative promoter can be located at the 5′ exon location or within an exon. Alternative polyadenylation sites refer to a splicing based on the presence and recognition of different polyadenylation sites.

of the pre-mRNA that can influence the recognition of splice sites [6], but also by cellular signal transduction pathways inducing phosphorylation events as reviewed by Stamm [7].

Alternative splicing

Most of protein-encoding genes have multiple exons that are alternatively combined to form distinct mRNAs [8,9]. As illustrated in [Fig. 2], several patterns of alternative splicing (AS) with varying complexity exist and participate in the expansion of genetic diversity. These alternative combinations can be organ-specific, tissue-specific or cell-type specific [2,10].

It is usually accepted that events conferring biological gain of functions are conserved throughout evolution. To address whether specific AS are conserved within evolution, Merkin and collaborators performed a transcriptome sequencing (RNA Seq) analysis using paired-end short or long read sequencing of poly-A-selected RNA on nine tissues from five vertebrates [11]. Doing so, they identified almost 500 exons with conserved AS that are highly conserved in mammals. Interestingly, these exons often encode phosphorylation sites, and their tissue specific splicing may have important effect on signaling pathways. Unexpectedly, the authors described an extensive variation in the splicing of these exons between species, at a level that even exceeds intraspecies differences

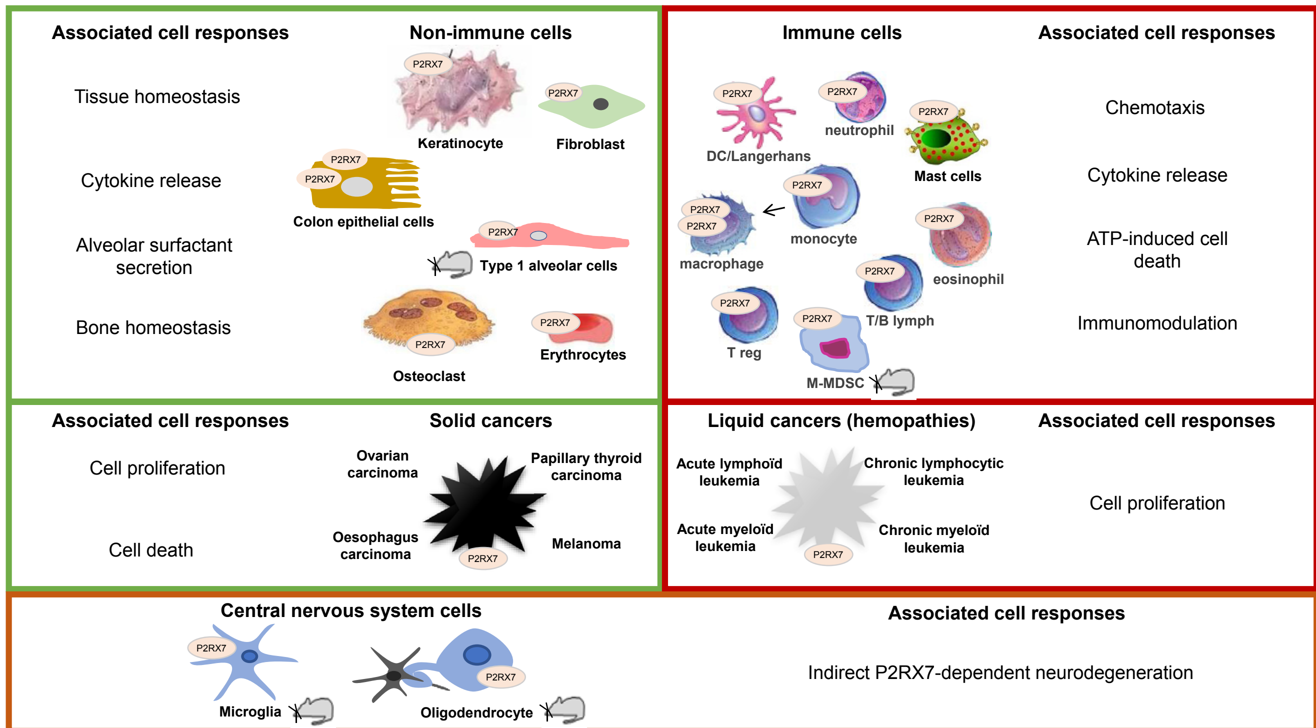

Fig. 3 P2RX7 expression in eukaryotic cells. The best way to sense cell stress is to express a receptor that is activated by molecules, like nucleotides, that are normally sequestered in the cells. Release of nucleotides, in response to mechanical injury, necrosis, apoptosis or inflammatory cell activation, depends on several molecular pathways, such as vesicular ADP release from platelets, pannexin-mediated ATP release during apoptosis, connexin- or pannexin-mediated ATP and autophagy. By sensing extracellular ATP, P2RX7 which is expressed by a large variety of cells plays such a role. In non-immune cells, functional P2RX7, (i.e. characterized by its expression at cell membrane, channel activity and/or macropore opening) has been found in colon [12] and lung epithelial cells [13], keratinocytes [14], osteoclasts [15], fibroblasts [16], and erythrocytes [17], where its activation has been shown to support tissue homeostasis, proliferation, survival and metabolism. In immune cells, expression of P2RX7 was documented in dendritic cells [18–20], neutrophils [21], mast cells [22], monocytes/macrophages [23,24], lymphocytes [25], eosinophils [26] and M-MDSC [27]. In these cells, P2RX7 expression is involved in chemotaxis, cytokine release, ATP-induced cell death and immunomodulation. In addition, P2RX7 expression was found in both solid [28–32] and liquid cancers [33–35], where it was described to sustain cell proliferation or cell death. Other publications claimed that P2RX7 was overexpressed in cancer from various origins [35,36]. Yet, these conclusions were obtained from immunohistochemistry analyses, where the identification of P2RX7 expressing cells is somehow difficult to characterize. Finally, P2RX7 was described to be expressed in microglia and oligodendrocytes [37]. Note: Here we showed P2RX7 expression reported to be functional in human cells, or in mouse cells when no data were available for human (a mouse drawing is then symbolized in this case).

between tissues [11]. In a complementary study, Barbosa-Morais and collaborators showed that in the mammalian lineage, organs of primate species have higher AS frequencies than organs of other vertebrates. They concluded that organs' AS profiles are more closely correlated to the identity of a species than to the organ, in contrast to the well conserved organ-dependent differences in mRNA expression during vertebrate evolution [38]. Together, these studies reveal that AS and transcription regulation are not subjected to the same evolutionary constraints; apparition of new mRNA variants is dominated by species-specific differences that accumulate more rapidly than transcriptional regulation even during a short period of evolution.

Whereas transcriptional regulation and constitutive splicing maintain normal cell physiology, AS can result in various human diseases by modifying, or even abrogating, some functional regions of proteins that are involved in cell homeostasis [39]. In fact, the hundreds of factors allowing the precise regulation of AS are themselves subjected to modifications, which in turn can cause deregulation of each of the signaling pathways across virtually all biological processes. For instance and as recently reviewed by Czubay and Piekiello-Witkowska, phosphorylation of splicing factors results in changed AS of tumor suppressors, regulators of cell signaling or apoptosis resulting in an impact on cancer development and progression [40]. Modification of AS is also the result of sequence variation. Indeed, it was reported that 22% of missense disease alleles alter splicing and it was predicted that one third of all mutations affect splicing [41]. To fully appreciate the relationship between missense mutation,

AS and diseases, computational approaches [42] and animal models [43] have been developed.

Altogether, deregulation of AS has been described to play a role in diseases as diverse as diabetes, neurological diseases, cardiovascular diseases, immunological and infectious diseases as well as cancer. As this topic is far beyond the scoop of this review, we invite interested readers to refer to an excellent review published by Kim and collaborators [10].

Alternative splicing of *P2RX7*

The purinergic P2RX7 receptor

Half a century was necessary to link purine nucleotides and nucleosides as extracellular ligands [44] to the existence of two families of purinergic receptors [45]. It is now well described that the P1 [46] and the P2 receptors comprise numerous members and that the P2 family is divided in two subgroups P2Y and P2X receptors [47]. The purinergic P2X receptors are activated by extracellular adenosine 5′-triphosphate (eATP) [47,48]. This family includes seven members which all share a common structure based on two transmembrane spanning regions, a large extracellular loop and intracellular N- and C-termini [48]. Within this family, sequence identity from the N terminus to the second transmembrane domain is relatively high. By contrast, their C-termini are specific to the individual P2X receptors and contain consensus binding motifs for protein kinases and other regions that may be involved in cell-signaling. This is particularly true for the seventh member of the P2X family, previously described as P2Z receptor [49], which is characterized by the presence of a long intracytoplasmic C-terminal sequence [48] and now referenced as P2RX7.

In the presence of eATP, P2RX7 trimeric receptors form a poorly-selective channel leading to membrane depolarization, potassium efflux and calcium and sodium influx [48]. Whereas *P2RX7* mRNA is expressed by virtually all mammalian cells, the expression of the protein was described in immune but also in non-immune cells [Fig. 3]. In cells expressing P2RX7 at the membrane, its expression was thought to induce various downstream events including cell proliferation and cell death, metabolic events, phagocytosis and release of inflammatory molecules [50–53]. As discussed later in this manuscript, P2RX7 could be expressed but maintained within the cytoplasm. In this condition protein expression is dissociated from protein activation. In addition, it was shown in mouse CD8 T cells that the sensitivity to eATP depends more on the stage of cell differentiation than on the level of P2RX7 expression [54]. Within the P2RX family, P2RX7 is unique. First, it is activated by relatively unusual high concentration of eATP (1 mM under physiological concentration), second it allows the permeation of large molecules up to 900 Da and third it is responsible for eATP-dependent cell lysis, a response that depends on the presence of the C-terminal sequence [49]. The recently described crystal structure of mammalian P2RX7 (from panda) gave new insights on the architecture of this receptor [55]. Importantly, the trimeric association of P2RX7 was confirmed by this study; the same conclusion was drawn by Kasuya and collaborators studying the crystal structure of the chicken P2RX7 [56]. As previously described for P2RX4 and P2RX3, the single subunit of a C-terminal truncated P2RX7 resembles a "dolphin-like" shape [57–59]. In the absence of eATP, the transmembrane helices constrict the channel gate at residues G338, S339 and S342. Hence, this conformation may represent the closed state. Unfortunately, the crystal structure in the presence of eATP was not sufficiently stable to be studied and the proposed mechanism is yet to be confirmed. By contrast, the crystal structure of P2RX7 in the presence of five different antagonists highlighted the existence of a drug binding pocket in the upper body domain which is close to the eATP binding pocket. Therefore, available antagonists act as non-competitive inhibitors. Combining different analyses, the following mechanism for P2RX7 activation and inhibition was proposed: In the presence of eATP, both the drug binding pocket and the inter-subunit cavity in the upper body domain (also called turret) of P2RX7 undergo conformational rearrangements. This leads to the narrowing of these domains, which consequently allows the enlargement of the lower body domain resulting in the opening of the channel. When allosteric antagonists are bound to P2RX7, turret closure is restrained and the channel remains closed [55]. This mechanism implies that when eATP is within its binding pocket, the drug binding pockets are inaccessible, preventing the binding of allosteric inhibitors.

Noteworthy, the crystal structure has been obtained using a C-terminally truncated version of P2RX7 which may represent a caveat in the understanding of P2RX7 biology. Indeed, the long C-terminal tail of P2RX7 has been described to be essential to sustain permeation of large molecules and cytolysis [49]. Here again, the group of Kawate improved understanding on the role of the C-termini of P2RX7 by investigating the effect of the membrane's lipid composition on P2RX7 functionality [60]. Using purified panda P2RX7 reconstituted into liposomes, it was elegantly demonstrated that the dye-permeant pore opens independently of the long C-terminal tail. Further, the authors highlighted the capacity of phosphatidylglycerol and sphingomyelin to facilitate channel activity by modifying the lipid composition of the liposomes, whereas cholesterol drastically attenuated dye-uptake by directly acting on P2RX7 transmembrane domains. So, if the dye-permeable pore of P2RX7 is independent of the N- and C-terminal extremity, do these intracytoplasmic sequences have a biological function? Part of the answer was brought by Robinson and collaborators who linked cholesterol sensitivity of P2RX7 channel activity to the presence of multiple cholesterol recognition amino acid consensus motifs within both N-terminal and proximal C-terminal regions of P2RX7 [61,62]. Additionally, it was shown that the juxta-transmembrane amino and carboxyl termini are involved in the regulation of P2RX7 gating [63]. And finally, it was demonstrated that the C-terminal Cysteine-rich region of P2RX7 counteracts the inhibitory effect of cholesterol [60]. All these evidences, combined with new technologies to analyze dilatation of P2RX7 eATP-induced pore opening [64,65], push aside the old dogma postulating that the formation of the large pore is a consequence of P2RX7 cation-selective channel dilatation and rather

highlight the importance of lipid composition to modulate the size of the pore. In agreement with this, it was shown that P2RX7 is associated with lipid rafts, which are dependent of palmitoylation of Cys residues located in the C-terminal region [61]. Yet, as nicely discussed by Di Virgilio and collaborators, we cannot exclude that consequently to P2RX7 stimulation, other permeability pathways are activated [66].

P2RX7: one gene, many proteins

P2RX7 gene was investigated in mammalian and non-mammalian species, the more studied species being human, rat and mouse, as detailed in the well documented review published elsewhere [53]. We will focus here on human and mouse, with an emphasis on *P2RX7* splice variants.

Human splice variants

The P2RX7 gene, which is localized on chromosome 12q24, consists of 13 exons. Constitutive splicing leads to the common *P2RX7-A* mRNA, but 12 additional transcripts have been described due to alternative splicing [67]. Among those splice variants, nine were studied in more detail [Fig. 4A]. Three of them, the variants *-C*, *-E* and *-G*, code for very short proteins and are assumed to be unable to form a channel receptor. By contrast, the variants *-B* and *-J* have been described to be expressed under normal and/or pathological conditions and to interfere with P2RX7-induced biological responses [67]. The *P2RX7-B* variant, which is expressed in almost all eukaryotic cells and appears to be regulated in immune cells, is characterized by the deletion of the last 249 amino acids from the C-terminal extremity that have been replaced by 18 different amino acids [67]. The homotrimer was described to retain ion–channel activity when expressed in a heterologous cell system. However, the EC50 for BzATP reaches 500 μM, which questions its ability to really channel Ca^{2+}. In addition, P2RX7-B homotrimers were described to be non-permeant to dyes, such as Yo-pro-1. On the contrary, the heterotrimer P2RX7-A/P2RX7-B was described to be more efficient than P2RX7-A, in particular by supporting cell growth [68]. With the C-terminal extremity of P2RX7 being involved in trafficking and cell surface expression, it could be that the increased efficiency of the heterotrimer is correlated with an improved expression at the plasma membrane [69–71]. Alternative splicing of exon 8 which is leading to a frameshift mutation with a new stop codon produces the *P2RX7-J* variant. The resulting protein is composed of the N-terminal extremity, the first transmembrane domain and 2/3 of the extracellular loop. The P2RX7-J receptor is inactive alone, but when co expressed with P2RX7-A, the association of -A and -J isoforms has a dominant negative effect over the P2RX7-A homotrimer which may protect certain cell types from eATP-induced cell death [72,73]. *P2RX7-H, -D and -F* result from alternative splicing leading to the insertion of an extra exon, or deletion of exons 5 and 8, respectively. The corresponding proteins are missing the N-terminal sequence, the first transmembrane domain and part of the cytoplasmic loop [Fig. 4B]. Given the fact that normal trafficking toward the cell surface requires the N-terminal sequence, it is likely that these isoforms are not correctly inserted in cell membrane. However, if expressed, these isoforms can be localized in the cytoplasm or in intracellular vesicles. Yet, only few *P2RX7-H* mRNA are expressed in tissues from different origins [67]. It is important to keep in mind that all these results were obtained after overexpression of splice variants in the HEK293 heterologous cell system. Undeniably, additional studies are required to examine the endogenous expression of P2RX7 isoforms, and their functional consequences, in human cells.

Mouse splice variants

The murine gene of *P2rx7*, which is located on chromosome 5, maintains the same genomic organization as human *P2RX7*. The common *P2rx7-a* mRNA is made of 13 exons. Four alternative splice variants have been described in mice so far, *P2rx7-b*, *-c*, *-d* and *-k* [Fig. 4A]. Two of them, *P2rx7-b*, *-c*, correspond to deletions within exon 13 and give rise to shorten proteins which are characterized by lower channel activity [53]. The P2RX7-d isoform corresponds to the shortest form of the protein with a large truncation in the extra-cytoplasmic loop, loss of both the second transmembrane domain and the intracytoplasmic C-terminal tail [74]. Interestingly, this largely truncated protein retains the ability to be correctly inserted within the membrane when co-transfected with P2RX7-a. It is likely that the heterotrimer is formed during vesicle trafficking towards the cell membrane, and once inserted in the membrane, P2RX7-d downregulates eATP-induced macropore formation. On the contrary, P2RX7-k protein, which results from an alternative splicing of exon 1, differs from P2RX7-a in the N-terminal cytoplasmic extremity and most of the first transmembrane domain [75]. *P2rx7-k* mRNA is expressed in all tissues but more expressed than *P2rx7-a* in spleen and liver. P2RX7-k homotrimer is more sensitive than P2RX7-a to ATP and is more potent to permeate ions and large cations. In addition, it was shown that P2RX7-k could be activated by ADP-ribosylation, which correspond to a second mode of activation [76]. P2RX7-k seems to be exclusively expressed by T cells and no co-expression of *P2rx7-k* and *P2rx7-a* was described. This mutually exclusive expression suggests a regulation at the level of the promoter. Whether this regulation is due to epigenetic modifications (such as methylation), specific expression of transcription enhancers/inhibitors or both remains to be determined. *P2rx7-k* mRNA escapes gene deletion in the Glaxo $P2rx7^{-/-}$ mice, where *LacZ* gene was inserted after ATG at the 5′ end [75]. The existence of these splice variants led to a careful re-evaluation of the expression of P2RX7 receptor isoforms in the Pfizer transgenic *P2rx7* KO mice, where region encoding Cys^{506} to Pro^{532} was replaced with the neomycin resistant gene [76,77]. While P2RX7-k protein is expressed in T cells from the “Glaxo mice”, Pfizer KO mice express ΔC isoforms (namely P2RX7-b, -c, -d). However, these proteins were inefficiently trafficked to the cell surface and consequently not responsive to agonistic stimulation [78]. Together, these results indicate that the Pfizer model is the preferential one to evaluate the functional role of P2RX7 in homeostasis and pathological conditions. In the same line, the third *P2rx7* KO model, in which the beta galactosidase/Neomycin genes were inserted within the exon 2 [79], is expected to be a good model, with none of the variant being expressed. In addition, two additional knock-in

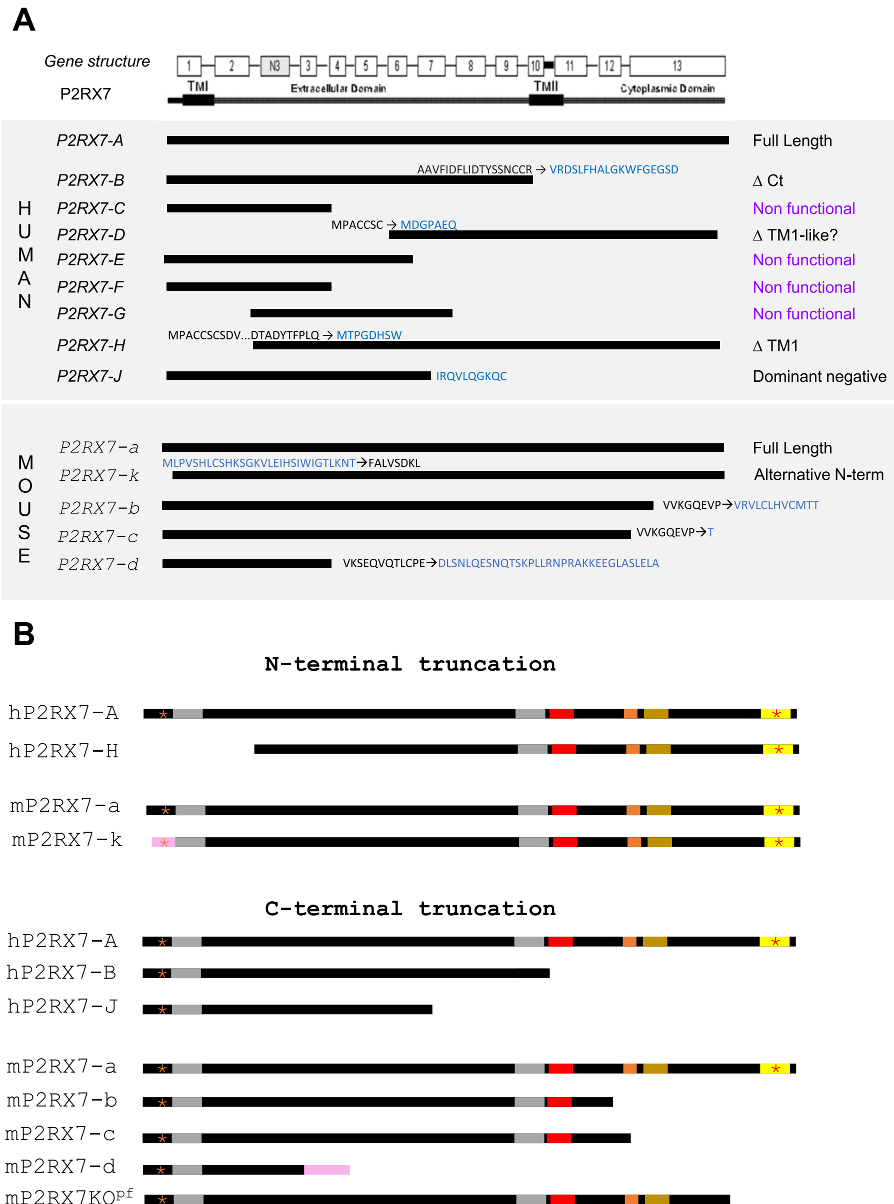

Fig. 4 Human and mouse P2RX7 splice variants. (A) Genomic organization. New sequences resulting from AS are shown in blue. Protein isoforms corresponding to P2RX7-C, -E, -F and -G were described to be nonfunctional. Yet, P2RX7-E and -F isoforms are structurally closed to the mouse P2RX7-d protein, which has been described to down regulate P2RX7 activity when co-expressed with P2RX7-A [Table 2]. By the same way, P2RX7-D corresponds to a shorter version of P2RX7-H, which has been described to be a non-functional receptor. (B) Schematic illustration showing the overall organization and the functional regions in isoforms identified at the genetic or exonic levels. Grey box: transmembrane domain 1 and 2; red box: Cys rich regions (including C371,373, 374- C477, 479, 482-C498,499, 506 and C572, 573); orange box: A actinin binding sequences; brown box: SH3 binding domain and TNF death receptor; yellow box: LPS binding domain; orange star: cholesterol sensitizing; red star: amino acids involved in trafficking and cholesterol sensitizing. The pink boxes highlighted the presence of new sequences due to splicing.

transgenic mice were generated. The first one corresponds to humanized P2RX7 mice in which the mouse exon 2 was substituted by the human cDNA covering exons 2 to 13 [80]. The second one relies on the introduction of an EGFP sequence in the 5' of exon 13 [37]. Both models were useful to characterize the expression of P2RX7 in the central nervous system (see [Fig. 3]).

Expression of splice variants: a way to regulate P2RX7 activity?

As a receptor, P2RX7 has to be correctly inserted into the cell membrane to be functional. Indeed, assembly and trafficking to the plasma membrane has been described as a regulatory mechanism, at least in some immune cells [23,81]. In addition to membrane receptor density, the concentration of extracellular ions was demonstrated to be crucial for the regulation of IL-1β

Table 1 Description of Single Nucleotide Polymorphisms of the Human *P2RX7* gene that could affect alternative splicing of variant B, H and J.

dbSNP ID	Localization	“Affected” variant	Major allele	Minor allele Frequency
rs1397153443	−3, exon 11	Variant B	C	T = 0.0000
rs778937864	−4, exon 11	Variant B	C	T = 0.0000
rs756931719	−10, exon11	Variant B	T	G = 0.00001
rs190080059	−20/−40, exon 11	Variant B	G	A/T = 0.00002
rs373584182	−20/−40, exon 11	Variant B	G	T = 0.00004
rs1352183219	−20/−40, exon 11	Variant B	G	A = 0.0000
rs760740502	−20/−40, exon 11	Variant B	G	A = 0.00002
rs916695614	−20/−40, exon 11	Variant B	C	C *none*
rs201650139	−20/−40, exon 11	Variant B	A	G *none*
rs368885357	−20/−40, exon 11	Variant B	G	T = 0.0000
rs1376166550	+2, exon 2	Variant H	T	C *none*
rs754634787	+3, exon 2	Variant H	G	A/T = 0.0000
rs965276754	−10, exon 3	Variant H	T	A *none*
rs201960043	−20/−40, exon 3	Variant H	T	Inscc = 0.0537 (InsDel)
rs200429438	−20/−40, exon 3	Variant H	C	A *nd*
rs1373724698	−20/−40, exon 3	Variant H	T	G = 0.0001
rs186332279	−20/−40, exon 3	Variant H	G	C = 0.00002
rs757343268	−20/−40, exon 3	Variant H	TTCAAA	Del, 0.00001
rs1475267143	+2, exon 7	Variant J	T	C = 0.0000
rs764748109	−8, exon 8	Variant J	T	C = 0.00001
rs1239860541	−20/−40, exon 8	Variant J	A	G = 0.0000
rs28360455	−20/−40, exon 8	Variant J	T	C = 0.0021
rs774175036	−20/−40, exon 8	Variant J	C	G/T = 0.00002
rs771002311	−20/−40, exon 8	Variant J	A	G = 0.0000
rs1210563466	−20/−40, exon 8	Variant J	C	T = 0.0000
rs1488167709	−20/−40, exon 8	Variant J	C	T = 0.00001
rs1239860541	−20/−40, exon 8	Variant J	A	G = 0.0000

secretion [82]. As discussed above, a third mode of regulation could result from the expression of homotrimer isoforms but also from the formation and expression of heterotrimeric isoforms, where the full-length form is associated with truncated splice variants.

To evaluate the role of AS in P2RX7 regulation, we focused on human and mouse P2RX7 isoforms. As shown in [Fig. 4B], two groups can be distinguished. In the first one, splicing events modify the N-terminal part of the protein, a sequence that contains amino acids critical for the localization of P2RX7 at the cell membrane. Currently, there is no evidence for a functional role of the P2RX7H isoform, which is unlikely to be trafficked to the cell membrane and does not respond to Bz-ATP [67]. However, considering the trimeric organization of P2RX7, one may speculate that a chimeric P2RX7-A/-H receptor could be assembled during ER trafficking and once correctly inserted in cell membrane, tune the amplitude of eATP-induced cell responses. However, further in-depth characterization is needed to confirm this hypothesis. On the contrary, AS in the first exon of the mouse *P2rx7* pre-mRNA produces a highly sensitive P2RX7 isoform, which is involved in immune T cells homeostasis [83]. This variant has not been described in human, even though the sequence of human intron 1-2 predicted the existence of a possible alternative splice site in exon 1 [75]. In the second group, splicing events affect exons that are crucial in mediating large cation permeation. As mentioned above, heterologous expression of human and mouse homotrimer isoforms lead to the expression of receptors characterized by functional Ca^{2+} flux and blocked permeation of large cations. Expression of these new receptors could be considered as the first level of regulation. More interestingly, the co-expression of two isoforms bring additional levels of regulation. The heterotrimer -A/-J is dominant negative in human for instance, while in mouse the co-expression of -a/-b, -a/-c, and -a/-d downregulated, or even inhibited P2RX7 function. Strikingly, co-expression of P2RX7-A/-B in HEK293 cells seems to increase the overall activity of P2RX7 as discussed earlier [68,84]. This result was unexpected considering the contrary effect observed with the mouse heterotrimeric receptors. However, one could speculate that the overall effect on heterotrimer P2RX7 activity depends on the ratio of A versus B. For instance, 2A and 1B could be more active than 3A whereas 1A and 2B could be less active. Undoubtably, additional experiments are needed to fully document in which cells and at which levels P2RX7 isoforms are expressed and what the functional consequences of heterotrimeric P2RX7 expression are? Another important question that remains to be solved is whether the non-functional P2RX7, that is expressed on cancer cells [85], is a consequence of AS leading to the formation of heterotrimeric P2RX7 that would not be recognized by the conformational antibody generated by Buell and co-workers [86], and that would alter P2RX7 activity [85]. This may be particularly relevant considering that the C-terminal truncation of P2RX7 leads to the deletion of several binding motifs involved in P2RX7-induced cell signaling pathways [50,87].

Table 2 Biological activities of human and mouse P2RX7 isoforms.

P2RX7 isoforms	Cells	Localization at the membrane	Membrane depolarization	Ca2+ influx	Macropore formation	IL1B release	Refs
P2RX7-A	HEK293,	IF	++	++	++		[49,67,68,79]
P2RX7-B	HEK293	IF	+/−	Low & transient	no	Casp1	[67,68]
P2RX7-H	HEK293		no	no	no	No casp 1	[67]
P2RX-7-J	Cervical cancer cells, MDCK	IF		low	no		[72]
P2RX7-A/-B	HEK293	IF	>-A	>-A	>-A		[67,83]
P2RX7-A/-J	HEK293	wb		<-A	<-A		[72]
P2RX7-a	Macrophages				++	++	[75]
	Brain, spleen, salivary gland, HEK293	wb	++	++	++		[76,77]
	Astrocytes	wb			++		[73,77]
	Pancreatic stellate cells	wb		++			[77]
P2RX7-b	Brain, spleen, salivary gland, HEK293	wb, ↓↓ vs P2RX7-a	↓↓ vs P2RX7-a				[77,78]
	HEK293				no		[74]
P2RX7-d	HEK293				no		[74]
P2RX7-k	T Lymphocytes						[76]
	HEK293	wb	++ (sustained vs -a)		++		[75]
P2RX7-a/-b	HEK 293	wb, b = dominant negative	b = dominant negative		↓↓ vs P2RX7-a alone		[74,78]
	Astrocytes (overexpression)				↓↓ vs P2RX7-a alone		[74]
P2RX7-a/-d	HEK293, astrocytes (overexpression)				↓↓ vs P2RX7-a alone		[74]

P2RX7 splice variants and diseases

SNPs and splice variants

On the beginning of 2019, 13,252 single nucleotide polymorphism (SNP) have been described in the *P2RX7* gene (https://www.ncbi.nlm.nih.gov/snp). While non-synonymous SNPs have been linked to pathologies as diverse as infectious diseases, bone, psychiatric, inflammatory and cardiovascular disorders or cancers, as reviewed by Sluyter [88], SNPs located within intronic regions have been poorly studied. Splicing regulatory elements can be located within intronic sequences, therefore one could assume that intronic SNPs may affect splicing and in turn cause diseases [89]. Splicing regulatory sequences include the 5′ and 3′ splice sites defining the boundary of an intron with its upstream and downstream exon, but also the branch site upstream of the 3′ splice site. Examination of the human *P2RX7* sequence indicates that the major U2 class splice site consensus sequence, including GT at the 5′ end and AG at the 3′ end, is present on the boundary of each of the P2RX7's introns.

We speculated that SNPs might lead to alternative splicing of *P2RX7* pre-mRNA. To test this hypothesis, we first listed SNPs located at the introns' boundaries. As shown in [Table 1], only one SNP, rs1475267143, changes a T to C at +2 of the 7th intron. This change, that corresponds to the most common class of non-consensus splice sites [90], may affect the splicing of exon 7, even though this hypothesis has not been tested experimentally. However, the minor allele frequency is close to zero, and therefore it is not expected that such a frequency could significantly impact the expression of *P2RX7-J*. We further extended our analysis to minus 10 to cover branchpoints of each boundaries' intron. As shown in [Table 1], we found several SNPs that can affect the splicing of *P2RX7-B*, *-H* and *-J*. Once again, all these SNPs are too sparsely expressed to envisage that they could regulate splicing events and are involved in diseases. Finally, because branchpoints within U2-type constitutive introns were located in between the position −40 to −20 from the 3' splice site, we extended our analysis to −40 nucleotides [36]. Doing so, we identified two SNPs rs28360455 and rs201960043 that could contribute to the alternative splicing of *P2RX7-J* and *P2RX7-H*, respectively. Today, no studies demonstrated that these two SNPs are linked to the expression of spliced *P2RX7* mRNA but given their relative high frequency it is tempting to speculate on the existence of such a regulation. By contrast, we were unable to identify SNPs that could modulate splicing of *P2RX7-B*. Yet, we cannot exclude that such SNPs exist, but are located within an unstudied intronic region. Alternatively, one could hypothesize that *P2RX7-B* is indeed constitutively expressed in some tissues. This idea is sustained by studies showing that P2RX7-B is expressed in various organs and resting immune cells [67], and when co expressed with P2RX7-A, may affect the binding/gating properties of P2RX7 [84]. Undoubtedly, new progresses

will be made with the implementation of novel tools to specifically assess the level of expression but also the biological activity of *P2RX7* splice variants.

P2RX7 splice variants and cancer

One of the most intriguing questions considering P2RX7 is to explain how a receptor that induces cell death in the presence of its endogenous ligand, namely eATP, could be overexpressed in some cancers [91]. Indeed, knowing that the tumor microenvironment, like the inflammatory tissue, contain hundreds of micromolar eATP [92], it is counterintuitive to imagine that a protein leading to cell death is overexpressed by cancer cells. The best way to harmonize these two observations is to hypothesize that the receptor expressed at the surface of cancer cells is unable to trigger cell death. This idea is sustained by several studies showing that P2RX7 expressed at the surface of diseased cells is a non-functional conformation [85]. The first description of the expression of a non-functional P2RX7 (nfP2RX7) was made in cancerous breast lesions where the group of Julian Barden used an antibody designed against an epitope located in the extracellular loop close to the eATP binding pocket and which is inaccessible in cells expressing normal functional P2RX7 [93,94]. Furthermore, the expression of non-functional P2RX7 on the surface of cancer cells was shown in organs as diverse as gut, ovary, cervix, lung, liver, and bladder [85]. Intriguingly, nfP2RX7 electrophoretic feature is indistinguishable from P2RX7 and today, the exact nature of this receptor channel remains a mystery. Gilbert and collaborators characterized the expression of nfP2RX7 from a panel of tumor cell lines using a new humanized antibody (BIL03) that is specific to the extracellular domain of nfP2RX7 [95]. As suggested by previous reports, they confirmed that nfP2RX7 is expressed in many cancer cell lines. They showed that nfP2RX7 is molecularly distinct from P2RX7 using specific antibodies and biological assays (EtBr uptake). Finally, the authors reported that nfP2RX7 is stored intracellularly and localized to the cell membrane upon eATP stimulation, an event that requires protein synthesis. Strikingly, it seems that expression of nfP2RX7 is mutually exclusive since its localization to the cell membrane correlates with the loss of P2RX7. As this non-functional receptor lacks apoptotic activity but retains residual non-selective cations channel, it was proposed that it promotes tumor growth. The mechanisms leading to the expression of nfP2RX7 is not fully understood nowadays, but at least two main hypotheses could be drawn. The first one, as discussed previously, relies on the formation of an heterotrimer, composed of P2RX7-A and P2RX7-B, which may lack the potential to induce cell death. In their study, Gilbert and collaborators did not check if expression of nfP2RX7 requires mRNA translation, however they hypothesized that intracellular nfP2RX7 may correspond to a misfolded protein and it is known that P2RX7-B lack C-terminal region that participates to the trafficking to the cell surface. The second one relies on the ability of cholesterol to control P2RX7-dependent large cation influx, as elegantly demonstrated by Karasawa and collaborators [60] and discussed earlier in this review. Considering that cancer cell membranes contain more saturated lipids, it is tempting to speculate that this change in lipid composition affects P2RX7 biology [96]. This hypothesis is supported by the discovery that, in a human prostate cancer cell line (LNCaP), oxidative stress-induced cell death is reversed when cells were pretreated with Soraphen A, an inhibitor of acetyl CoA carboxylase which modulates the synthesis of very long chain saturated, mono- and polyunsaturated fatty acids [97]. While this paper highlighted a direct relation between lipid composition and stress-induced cell death, no link was made with the eATP/P2RX7 signaling. By contrast, it is well established that P2RX7 activates phospholipid signaling pathways in epithelial cells through the modulation of phospholipases and sphingomyelinase [98]. Additionally, activation of P2RX7 drives the production of phosphatidic acid in RAW 264.7 macrophages which in turn delays eATP-induced pore opening and cytolysis. Therefore, a direct link between P2RX7 activation, membrane lipid composition and cell death was described at least in some immune cells [99,100]. Additional research is required to establish the relationship between eATP, expression of cell surface nfP2RX7, lipid metabolism and lack of pore opening-induced cell death. Undoubtedly, if this holds true it will reconciliate the fact that cancer cells are positively stained by antibodies directed against ubiquitously expressed P2RX7 epitopes.

Recently, a new P2RX7 splice variant was described, namely *P2RX7-V3* which contains an extra exon N3 and corresponds to a long non-coding RNA [101]. Long non-coding RNAs do not code for proteins but have been involved in diverse cellular processes by interfering with chromatin-modifying complexes and transcription factors [102]. *P2RX7-V3* is expressed at high levels in cell lines derived from uveal melanoma where it works as an oncogene. Even though the mode of action of this splice variant and its expression in other cancer tissues need to be investigated, the authors proposed that *P2RX7-V3* may be an informative biomarker of cancer. Beyond, this report suggests that at least one *P2RX7* splice variant might by itself, regulate cellular processes relevant for cancer, a field that has not yet been explored.

Conclusion

Here, we discussed the role of alternative splicing in the field of P2RX7 signaling. Our attempts were to discuss if expression of P2RX7A and its splice variants in a same cell could impact the functionality of P2RX7 and to bring new thoughts on the nature of nfP2RX7 that is expressed at the membrane of cancer cells. Despite the quantity of data published over the last two decades, there are still unresolved questions regarding the role of P2RX7 in health and diseases. Undoubtedly, development of new specific tools to unravel the expression of P2RX7 splice variants and the functionality of P2RX7 will bring new insights in biology and medicine. This is particularly relevant in the light of the recent discovery that

tumor-specific splicing produces numerous splicing associated potential neoantigens that may affect the immune response and could be exploited in the field of onco-immunology [103].

Conflicts of interest

The authors declare they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2019.05.007.

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