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1 **Splice variants of protein disulfide isomerase - identification, distribution and**
2 **functional characterization in the rat**

3

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15

16 **Abstract**

17 **Background:** Protein Disulfide Isomerase (PDI) enzyme is an emerging therapeutic
18 target in oncology and hematology. Although PDI reductase activity has been studied
19 with isolated fragments of the protein, natural structural variations affecting reductase
20 activity have not been addressed.

21 **Methods:** In this study, we discovered four coding splice variants of the *Pdi* pre-mRNA
22 in rats. In vitro Michaelis constants and apparent maximum steady-state rate constants
23 after purification and distribution in different rat tissues were determined.

24 **Results:** The consensus sequence was found to be the most expressed splice variant
25 while the second most expressed variant represents 15 to 35 % of total *Pdi* mRNA.

26 The third variant shows a quasi-null expression profile and the fourth was not
27 quantifiable. The consensus sequence splice variant and the second splice variant are
28 widely expressed (transcription level) in the liver and even more present in males.
29 Measurements of the reductase activity of recombinant PDI indicate that the
30 consensus sequence and third splice variant are fully active variants. The second most
31 expressed variant, differing by a lack of signal peptide, was found active but less than
32 the consensus sequence.

33 **General significance:** Our work emphasizes the importance of taking splice variants
34 into account when studying PDI-like proteins to understand the full biological
35 functionalities of PDI.

36

37 **Keywords**

38 Protein disulfide isomerase (PDI), Splice variant, Protein variant, Electron transfer
39

39

40 **1. Introduction**

41 Protein Disulfide Isomerase (PDI, EC.5.3.4.1, NC_005109.4) also known as Prolyl 4-
42 Hydroxylase Beta subunit (P4HB) is a thiol oxidoreductase and molecular chaperone
43 studied since the 1980s [1,2]. PDI is the founding enzyme of the so-called Protein
44 Disulfide Isomerase-like (PDI-like) family [3–6]. PDI is an emerging pharmacological
45 target [7,8] as it appears to be implicated in a variety of pathophysiological processes
46 such as cancer [9], neurodegenerative diseases [10], blood coagulation, [11–13] and
47 oxidative stress [14,15]. Sharing common ancestral features with thioredoxin, the
48 structure of PDI is composed of two thioredoxin-like (TRX-like) domains with active
49 CXXC motif sites (both CGHC) traditionally referred to as “a and a’ domains”, and two
50 substrate-binding domains referred to as “b and b’ domains” [16]. PDI is an

51 endoplasmic reticulum (ER) luminal protein captured by ER-resident receptors via a
52 C-terminal tetrapeptide retention signal (the KDEL-retention signal)[17]. Depending on
53 the context, the biological catalytic functions of PDI are reduction, oxidation or
54 isomerization of thiol-proteins [18–21]. Functional investigation of the structural
55 domains of PDI has been performed in several *in vitro* [18,22–25] and *in vivo* studies
56 [12,14,26–28].

57 Like many eukaryotic proteins, *Pdi* appears to display splice variants based on whole
58 exome sequencing or computed predictions [29–31]. Nevertheless, no systematic
59 study on the functionality (enzyme activity) of these splice variants is currently
60 available. Their functional importance remains to be elucidated while they might have
61 a significant impact on the design of medical treatments targeting PDI. Thus, in this
62 study, our objective was to identify natural variants of the PDI resulting from alternative
63 splicing in rats and to functionally characterize them to estimate their biological
64 significance.

65

66 **2. Materials and methods**

67

68 **2.1 Isolation of total RNA and cDNA synthesis**

69 Total RNA was isolated from 100 mg of rat tissues (8-week-old male and female
70 Sprague Dawley rats - n=4 for both sexes) using the Trizol[®] reagent (Invitrogen) and
71 the SV Total RNA Isolation System[®] kit (Promega, France) according to
72 manufacturer's recommendations. Reverse transcription was performed using the
73 Prime Script RT reagent Kit, Perfect Real Time[®] (Clontech), according to the
74 manufacturer's recommendations. One μL of total RNA at 100 ng/ μL was mixed with
75 2 μL of Prime Script buffer, 0.5 μL of oligodT primer (50 μM), 0.5 μL random hexameric

76 primers (100 μ M) and 0.5 μ L of enzyme in a total volume of 10 μ L for 15 minutes at 37
77 $^{\circ}$ C and 5 seconds at 85 $^{\circ}$ C. Complementary cDNA was stored for less than 10 days at
78 -80 $^{\circ}$ C.

79

80 **2.2 Identification of *Pdi* splice variants by semi-nested PCR and TOPO cloning**

81 Amplification of rat *Pdi* (NM_012998.2) was performed using a two-step PCR
82 procedure with a high-fidelity polymerase (Accuprime Taq DNA Polymerase System®,
83 Thermo Fisher) (0.2 μ L) in a 50 μ L reaction volume composed of 10 μ M of each primer
84 (10 μ M) and 5 μ L of buffer II. In the first PCR step, 5 μ L of total cDNA from male rat
85 liver was amplified with primer P1 (forward) and P2 (reverse) (Figure 1, Figure 2A and
86 Table 1). P1 and P2 primers were designed to bind to untranslated transcribed regions
87 (UTR3' and UTR5') to amplify canonical *Pdi* mRNA but also hypothetical alternative
88 splice *Pdi* mRNAs. After an initial denaturation (94 $^{\circ}$ C for 2 minutes), 10 PCR cycles
89 were performed (94 $^{\circ}$ C for 2 minutes, 94 $^{\circ}$ C for 30 seconds, 60 $^{\circ}$ C for 30 seconds and
90 68 $^{\circ}$ C for 50 seconds). Then, this first PCR product was used as a template to explore
91 alternative splicing of PDI by amplifying either the 5' or 3' part to visualize minority
92 variants. For the second PCR step, 1 μ L of the first step PCR product was amplified
93 using P1 and P4 primers for amplification of the 5' part, and P3 and P2 primers for
94 amplification of the 3' part. For both parts, 30 PCR cycles were performed (94 $^{\circ}$ C for 2
95 minutes, 94 $^{\circ}$ C for 30 seconds, 55 $^{\circ}$ C for 30 seconds and 68 $^{\circ}$ C for 50 seconds) (Figure
96 1A and Table 1).

97 After DNA electrophoresis, the amplicons were selectively extracted and directly
98 cloned with the Zero Blunt Topo PCR Cloning Kit (Thermo Fisher Scientific), according
99 to the manufacturer's recommendations. Two apparent profiles were found after DNA
100 electrophoresis (long and short splice variants according to the full size of PDI). Five

101 plasmids from each of the two profiles (long and short) containing splice variants of rat
 102 PDI, were sequenced by Sanger sequencing (Biofidal, France) using SP6 and T7
 103 primers. Sequence analysis and multiple sequence alignment were performed using
 104 CLC Sequence Viewer 8.0.0. The EMBOSS Needle online software URL:
 105 https://www.ebi.ac.uk/Tools/psa/emboss_needle/, was used to determine sequence
 106 identity and similarity after pairwise sequence alignment (Needleman-Wunsch
 107 algorithm).

108

109 **Table 1:** List of primer sequences for nested PCR of rat *PDI*.

Primer name	Seminested PCR primer sequences (5'-3')
P1	CGCTGCCGACGTCCGAC
P2	CACCGAGGTTTTGGGTTTCAGG
P3	CAAAGATTTTCGGAGGTGAAA
P4	AAGCCCTCGGCCGCTTT

110

111 **2.3 Absolute quantification of splice variants by RT-qPCR**

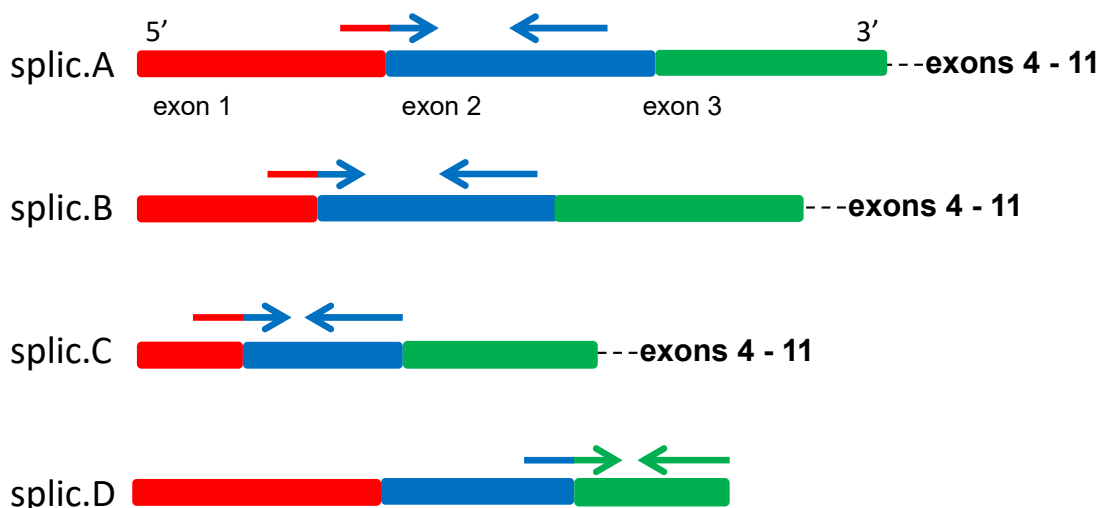
112 A quantitative reverse transcription PCR (RT-qPCR) was performed for relative
 113 quantification of *Pdi* variants in rats using specific primers designed to quantify spliced
 114 variants (Figure 1): splice A primers (forward and reverse) were designed to bind to
 115 the exon 6 region; splice B, C and D forward primers were designed to bind to a specific
 116 sequence from alternative splice (to the overlap of the splice area) and reverse primers
 117 to bind to the exon 2 region. The reaction mixture contained 1 μ L of cDNA (2 ng), 4 μ L
 118 of 5X HOT BIOAmp® EvaGreen qPCR Mix (Biofidal, France) and 15 nM of each
 119 forward and reverse primer in a final volume of 20 μ L. The qPCRs were performed with
 120 the MX3000P qPCR Machine (Stratagene). After an initial enzyme activation step at
 121 95°C for 15 min, 45 PCR cycles were performed (95° for 30 seconds, 60°C for 40
 122 seconds and 72°C for 30 seconds). Three replicates per organ per rat (n=4 for each
 123 sex) were performed.

124 The specificities and efficiencies of the primers under our experimental conditions were
 125 evaluated by qPCR amplicon sequencing (Biofidal, France) and the cDNA dilution
 126 method (standard curves). All efficiencies were close to 100 %. *Gapdh* amplification in
 127 all experiments was established as a quality control for cDNA. No significant
 128 differences were found in its expression and *Gapdh* amplification was used as a
 129 housekeeping gene for qPCR normalization in all rat tissues. Absolute quantification
 130 was obtained using a standard curve from serial dilution of the plasmid obtained by
 131 TOPO cloning.

A.

Primer name	qPCR primer sequences (5'-3')	Target	Amplicon size (bp)
splicA - forward	GGTGGAGTTCTATGCCCAT	Splice variant A	176
splicA - reverse	ACTTGATTGTGGGGTAGCCA		
splicB -forward	ACATGCTGAAGAAGAGC	Splice variant B	275
splicB - reverse	AGCTGTATATTCCTTTGGGG		
splicC -forward	CATGCTGGCCCA	Splice variant C	184
splicC- reverse	AGCTGTATATTCCTTTGGGG		
splicD -forward	GACCTGGCCCAGCAGTC	Splice variant D	190
splicD- reverse	GGTACTTGAAAAACACATCGCT		

B.



132
 133 **Figure 1: Primer sequences for qPCR of rat *Pdi* splice variants.** (A) Table of qPCR
 134 primers used to selectively amplify rat *PDI* splice variants. (B) Selective quantification
 135 strategy. Arrow indicates the qPCR primers positions. Note that each forward primer
 136 overlaps specific the specific sequence of the considered splicing variant.
 137

138 **2.4 Expression and purification of recombinant PDI splice variants**

139 *Pdi* splice variants were cloned into the bacterial expression vector pET-28b+ (*Bam*HI
140 restriction site). The N-terminal hexahistidine-tagged PDI variants were expressed in
141 Rosetta™ II (Novagen) cells grown in terrific broth medium at 37 °C and induced, when
142 the optical density at 600 nm (OD_{600nm}) reached 0.7, by 0.250 mM Isopropyl β-d-1-
143 thiogalactopyranoside (VWR) for 20 hours at 25 °C. After centrifugation (5000 x g for
144 20 minutes), bacterial spheroplasting (approximately 2.5 g of pellet) was performed in
145 HEPES buffer (50 mM HEPES, 500 mM NaCl and 0.5 mg/L DTT) with lysozyme (0.5
146 mg/L) and 2 mM phenylmethylsulfonyl fluoride (PMSF), for 20 minutes at 4°C. Lysis
147 was achieved by rapid disruption with a Potter-Elvehjem followed by sonication at 4
148 °C. All protein variants were purified by HisTrap™ affinity chromatography (Cytiva).
149 Protein concentration was determined using the Pierce™ BCA protein assay kit,
150 according to the manufacturer's recommendations.

151

152 **2.5 Insulin reduction assay**

153 The insulin turbidity test was performed according to Holmgren [32] with slight
154 modifications. In 1 mL cuvettes, 1 μM of recombinant PDI protein was mixed in 20 mM
155 phosphate buffer, 200 mM NaCl, 1 mM EDTA pH 7, with 16.6 mM of reduced
156 glutathione (GSH) (Sigma Aldrich), 2 units of glutathione reductase (Sigma Aldrich,
157 G3664), 150 μM of NADPH (VWR, PanReac AppliChem A1395). After a pre-incubation
158 time of approximately 2 min to reduce PDI and potential traces of oxidized glutathione
159 (GSSG), various concentrations of human insulin (Sigma Aldrich, 91077C) were added
160 to initiate the reaction. Precipitation due to the reduction of insulin by PDI was
161 monitored at 600 nm, and NADPH consumption at 340 nm, using a spectrophotometer
162 UVmc² (SAFAS) at 34 °C. Rutin (PhytoLab) was dissolved in DMSO for the inhibition

163 experiments. NADPH was dissolved in ultra-pure water, its integrity was checked using
164 the A_{260}/A_{340} absorbance ratio [33,34] and its concentration was measured
165 spectrophotometrically at 340 nm using a molar extinction coefficient of $6200 \text{ M}^{-1}\cdot\text{cm}^{-1}$
166 at 340 nm, 25 °C in water [35,36]. The kinetics of NADPH consumption showed a lag
167 phase. The rate was measured using the derivative of the kinetic curve at the maximum
168 slope point, and this value was divided by the molar extinction coefficient of NADPH
169 and the concentration of PDI to obtain a steady-state rate constant.

170

171 **2.6 N,N-di(thioamido-fluoresceinyl)-cystine (DTFCys2) reduction assay**

172 Synthesis of N,N-di(thioamido-fluoresceinyl)-cystine (DTFCys2) was performed
173 according to the literature with slight modifications[37] : L-Cystine (12.3 mg, 0.051
174 mmol) and FITC (59.7 mg, 3 eq.) were dissolved in a 0.1 M aqueous solution of sodium
175 carbonate solution (3 mL) and acetone (3 mL). The solution was stirred at 50°C for 24
176 hours in a sealed tube protected from light. After concentration, the crude product was
177 purified by a column of Sephadex G-50 eluted with water affording DTFCys2 as a pure
178 solid (20.3 mg, 39%). The ^1H NMR spectrum was in accordance with the literature.

179 PDI splice variant reductase activities were determined using DTFCys2 as substrate.
180 In 400 μL reaction mix, variable concentrations of recombinant PDI proteins in 20 mM
181 phosphate buffer, NaCl 100 mM pH 7.4, were mixed with 10 μM DTFCys2 and 10 μM
182 DTT (20 μM DTFCys2 and 20 μM DTT for Splic.B) and the increase in fluorescence
183 due to the release of N,N-di(thioamido-fluoresceinyl)-cysteine was followed in a
184 SAFAS Xenius fluorimeter with excitation and emission wavelengths set at 494 and
185 530 nm, respectively. DTT is required to allow the recycling of oxidized PDI and
186 maintain a constant active concentration of PDI[37]. A blank was systematically
187 recorded to account for the reduction of DTFCys2 by DTT by preincubation of 1 min

188 and the reaction was initiated by the addition of the PDI. The rate of the reaction in
189 $\mu\text{M}\cdot\text{s}^{-1}$ was deduced by calibrating the fluorescence emission of N,N-di(thioamido-
190 fluorescéinyl)-cysteine. The rates were linearly dependent on the PDI concentrations
191 and the rate constant characterizing each splice variant was deduced from the slope
192 of the rate vs. PDI concentration measured between 0.25 and 1 μM for Splic.A and
193 Splic.C, and 1 to 5 μM Splic.B. Rutin inhibition was evaluated by measuring the rate of
194 the reaction catalyzed by 1 μM Splic.A and 0.5 μM Splic.C.

195

196 **2.7 Circular dichroism**

197 Purified splice variants were first desalted on PD MiniTrap™ G-25 (GE Healthcare).
198 CD spectra were recorded on a Chirascan Plus spectrometer (Applied Photophysics,
199 Ltd, UK) with 10 μM protein desalted into sodium phosphate buffer (NaPi 10 mM, NaF
200 100 mM pH7.5). Quartz cells with 1 mm pathlength were filled with 200 μL of the
201 sample, and the final result is the average of 3 independent runs. CD spectra were
202 analyzed using the CDNN software (Boehm, 1992).

203

204 **2.8 Data analysis**

205 For gene expression analysis (absolute quantification of mRNA): the mean amount of
206 each splice variant mRNA from each tissue (or sex) was compared to the mean of the
207 transcripts of the others using an unpaired moderate t-test with Benjamini-Hochberg
208 correction to minimize the false discovery rate. The Mann-Whitney U test was used to
209 compare the apparent kinetics parameters obtained with the insulin reduction assay
210 (apparent Michaelis constant K_M^{app} and apparent maximum rate constant $k_{\text{MAX}}^{\text{app}}$). P-value
211 ≤ 0.05 was considered statistically significant. All statistics were performed using R
212 software (R Development Core Team (2005). R: A language and environment for

213 statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-
214 900051-07-0, URL: <http://www.R-project.org> (August 2020)).

215

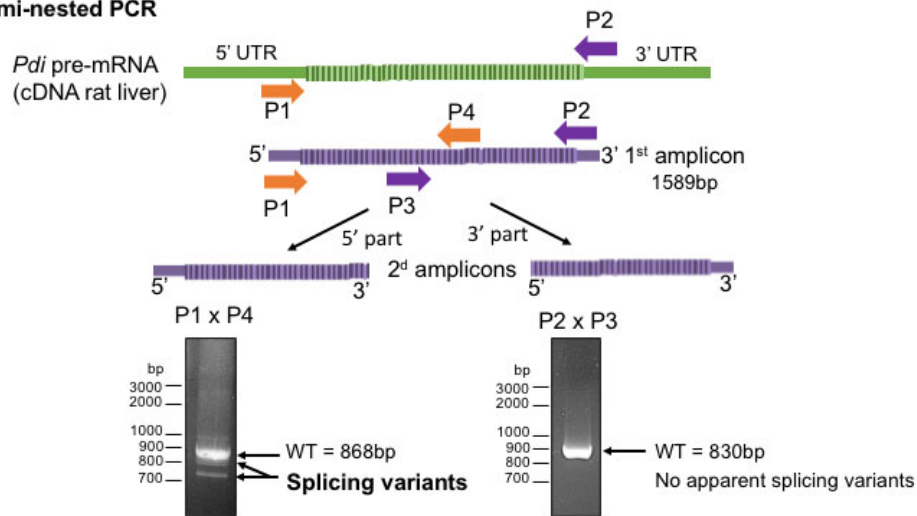
216 **3. Results**

217

218 **3.1 *Pdi* presents different splice variants in rat liver**

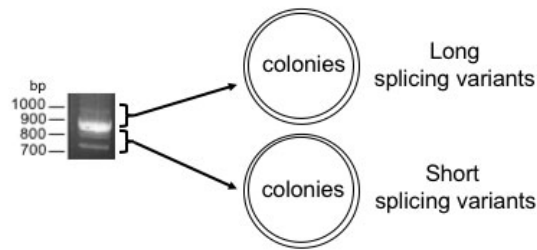
219 The semi-nested PCR approach followed by the cloning and sequencing of the PCR
220 products revealed the expression of at least five different *Pdi* transcripts in rat liver
221 (Figure 2). The nucleotide and protein sequences of the detected splice variants are
222 summarized in Figure 2C and Figure 3, respectively. Transcript A corresponded to the
223 transcript described in the genome databases (CCDS: NM_012998.1). The other
224 transcripts (Splic.B to E) were all shorter. One of these transcripts (Splic.E) showed
225 splicing of a large portion of exon 1 including the initiation codon (from nucleotide 1 to
226 nucleotide 60) and did not exhibit alternative initiation codons. All the other splice
227 variants were coding variants. The longest coding variant exhibited a truncation of the
228 last part of the first exon (from nucleotides 8 to 94), corresponding to a loss of 29 amino
229 acids (Splic.B) (from amino acid 2 to 30). Another splice variant had a sequence loss
230 overlapping exons 1 and 2 (from nucleotides 4 to 180), corresponding to a loss of 59
231 amino acids containing the first active site of the consensus coding sequence (Splic.C)
232 (from amino acid 2 to 60 of the consensus sequence). The alignment of Splic.B, C,
233 and D with the computational predicted splice variants of P4hb in rat is shown in Figure
234 4.

A. Semi-nested PCR

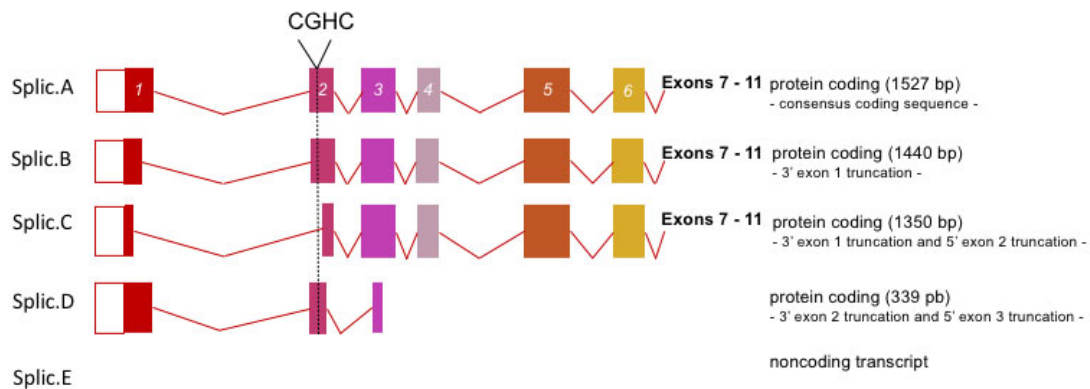


B. Topoisomerase based cloning (TOPO cloning)

Zero Blunt® TOPO® PCR Cloning, ThermoFisher scientific

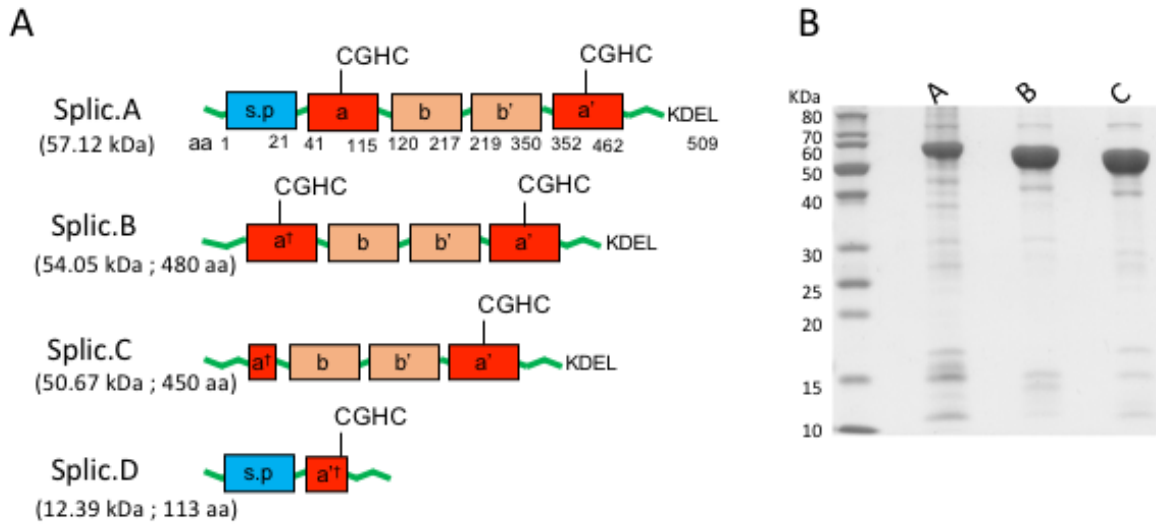


C. Nucleotide sequence of the Pdi splicing variants



235

236 **Figure 2: Major splice variants of *Pdi* in rat liver identified by the semi-nested PCR approach**
 237 **followed by TOPO cloning of the PCR products.** (A) Semi-nested PCR strategy, P1-4: primers, WT:
 238 consensus coding sequence of *Pdi*. (B) Topoisomerase-based cloning of PCR products from P1 X P4
 239 nested PCR. (C) Alignment of simplified splice variant structures from 5'UTR to exon 6: exons (boxes);
 240 introns (lines connecting boxes); coding sequences (filled boxes) and untranslated region (unfilled
 241 boxing).
 242



243

244 **Figure 3: Protein variants of PDI.** (A) Alignment of structural protein variants of PDI: catalytically active
 245 TRX-like domains (red boxes a and a'); inactive TRX domains (blue boxes b and b'); coding sequences
 246 (red boxes) and predicted signal peptide (blue box s.p); partial homology or truncated sequence
 247 compared to Splic.A (†). (B) Denaturing SDS-PAGE of His-tagged recombinant PDI protein variants
 248 expressed by *E. coli* Rosetta II cells and isolated by affinity chromatography (SDS-PAGE: 15 µg of
 249 proteins/well; colloidal aluminium-sulfate based Coomassie blue coloration, sensitivity 1 ng/band). A:
 250 Splic.A, B: Splic.B, C: Splic.C.

251

A

	Signal peptide								
P4hb-203 (canonical)	MLSRALLCLA	LAWAARVGAD	ALEEEEDNVLV	LKKSNFAEAL	AAHNYLLVEF	Y-APW	CGHC	K	59
P4hb-201	MKE-----	-----	-----	VQREEIDLDDG	GLPSSVLSVF	TDAPW	CGHC	K	33
Splic.B	ML-----	-----	-----	-KKSNFAEAL	AAHNYLLVEF	Y-APW	CGHC	K	30
P4hb-203 (canonical)	ALAPEYAKAA	AKLKAEGSEI	RLAKVDATEE	SDLAQQYGVR	GYPTIKFFKN	GDTASPKEYT			119
P4hb-201	ALAPEYAKAA	AKLKAEGSEI	RLAKVDATEE	SDLAQQYGVR	GYPTIKFFKN	GDTASPKEYT			93
Splic.B	ALAPEYAKAA	AKLKAEGSEI	RLAKVDATEE	SDLAQQYGVR	GYPTIKFFKN	GDTASPKEYT			90
P4hb-203 (canonical)	AGREADDIVN	WLKKRTGPAA	TTLSDTAAAE	SLVDSSEVTV	IGFFKDAGSD	SAKQFLAAE			179
P4hb-201	AGREADDIVN	WLKKRTGPAA	TTLSDTAAAE	SLVDSSEVTV	IGFFKDAGSD	SAKQFLAAE			153
Splic.B	AGREADDIVN	WLKKRTGPAA	TTLSDTAAAE	SLVDSSEVTV	IGFFKDAGSD	SAKQFLAAE			150
P4hb-203 (canonical)	AVDDIPFGIT	SNSDVFSKYQ	LDKDGVVLFK	KFDEGRNFFE	GEITKEKLLD	FIKHNQLPLV			239
P4hb-201	AVDDIPFGIT	SNSDVFSKYQ	LDKDGVVLFK	KFDEGRNFFE	GEITKEKLLD	FIKHNQLPLV			213
Splic.B	AVDDIPFGIT	SNSDVFSKYQ	LDKDGVVLFK	KFDEGRNFFE	GEITKEKLLD	FIKHNQLPLV			210
P4hb-203 (canonical)	IEFTEQTAPK	IFGGEIKTHI	LLFLPKSVSD	YDGKLSNFKK	AAEGFKGKIL	FIFIDSDHTD			299
P4hb-201	IEFTEQTAPK	IFGGEIKTHI	LLFLPKSVSD	YDGKLSNFKK	AAEGFKGKIL	FIFIDSDHTD			273
Splic.B	IEFTEQTAPK	IFGGEIKTHI	LLFLPKSVSD	YDGKLSNFKK	AAEGFKGKIL	FIFIDSDHTD			270
P4hb-203 (canonical)	NQRILEFFGL	KKEECPAVRL	ITLEEEMTKY	KPESDELTAE	KITQFCHHFL	EGKIKPHLMS			359
P4hb-201	NQRILEFFGL	KKEECPAVRL	ITLEEEMTKY	KPESDELTAE	KITQFCHHFL	EGKIKPHLMS			333
Splic.B	NQRILEFFGL	KKEECPAVRL	ITLEEEMTKY	KPESDELTAE	KITQFCHHFL	EGKIKPHLMS			330
P4hb-203 (canonical)	QELPEDWDKQ	PVKVLVGKNF	EEVAFDEKKN	VFVEFYAPWC	GHCKQLAPIW	DKLGETYKDH			419
P4hb-201	QELPEDWDKQ	PVKVLVGKNF	EEVAFDEKKN	VFVEFYAPWC	GHCKQLAPIW	DKLGETYKDH			393
Splic.B	QELPEDWDKQ	PVKVLVGKNF	EEVAFDEKKN	VFVEFYAPWC	GHCKQLAPIW	DKLGETYKDH			390
P4hb-203 (canonical)	ENIVIAKMDS	TANEVEAVKV	HSFPTLKFFP	ASADRTVIDY	NGERTLDGFK	KFLESGGDG			479
P4hb-201	ENIVIAKMDS	TANEVEAVKV	HSFPTLKFFP	ASADRTVIDY	NGERTLDGFK	KFLESGGDG			453
Splic.B	ENIVIAKMDS	TANEVEAVKV	HSFPTLKFFP	ASADRTVIDY	NGERTLDGFK	KFLESGGDG			450
P4hb-203 (canonical)	AGDNDLDLE	EALEPDMEED	DDQKAVKDEL						* 510
P4hb-201	AGDNDLDLE	EALEPDMEED	DDQKAVKDEL						* 484
Splic.B	AGDNDLDLE	EALEPDMEED	DDQKAVKDEL						* 481

B

	Signal peptide								
P4hb-203 (canonical)	MLSRALLCLA	LAWAARVGAD	ALEEEEDNVLV	LKKSNFAEAL	AAHNYLLVEF	Y-APW	CGHC	K	59
P4hb-201	M-----	---KEVQRE	EIDLDDGGL--	-----	--PSSVLSVF	TDAPW	CGHC	K	33
Splic.C	M-----	-----	-----	-----	-----	-----	-----	-----	1
P4hb-203 (canonical)	ALAPEYAKAA	AKLKAEGSEI	RLAKVDATEE	SDLAQQYGVR	GYPTIKFFKN	GDTASPKEYT			119
P4hb-201	ALAPEYAKAA	AKLKAEGSEI	RLAKVDATEE	SDLAQQYGVR	GYPTIKFFKN	GDTASPKEYT			93
Splic.C	-LAPEYAKAA	AKLKAEGSEI	RLAKVDATEE	SDLAQQYGVR	GYPTIKFFKN	GDTASPKEYT			60
P4hb-203 (canonical)	AGREADDIVN	WLKKRTGPAA	TTLSDTAAAE	SLVDSSEVTV	IGFFKDAGSD	SAKQFLAAE			179
P4hb-201	AGREADDIVN	WLKKRTGPAA	TTLSDTAAAE	SLVDSSEVTV	IGFFKDAGSD	SAKQFLAAE			153
Splic.C	AGREADDIVN	WLKKRTGPAA	TTLSDTAAAE	SLVDSSEVTV	IGFFKDAGSD	SAKQFLAAE			120
P4hb-203 (canonical)	AVDDIPFGIT	SNSDVFSKYQ	LDKDGVVLFK	KFDEGRNFFE	GEITKEKLLD	FIKHNQLPLV			239
P4hb-201	AVDDIPFGIT	SNSDVFSKYQ	LDKDGVVLFK	KFDEGRNFFE	GEITKEKLLD	FIKHNQLPLV			213
Splic.C	AVDDIPFGIT	SNSDVFSKYQ	LDKDGVVLFK	KFDEGRNFFE	GEITKEKLLD	FIKHNQLPLV			180
P4hb-203 (canonical)	IEFTEQTAPK	IFGGEIKTHI	LLFLPKSVSD	YDGKLSNFKK	AAEGFKGKIL	FIFIDSDHTD			299
P4hb-201	IEFTEQTAPK	IFGGEIKTHI	LLFLPKSVSD	YDGKLSNFKK	AAEGFKGKIL	FIFIDSDHTD			273
Splic.C	IEFTEQTAPK	IFGGEIKTHI	LLFLPKSVSD	YDGKLSNFKK	AAEGFKGKIL	FIFIDSDHTD			240
P4hb-203 (canonical)	NQRILEFFGL	KKEECPAVRL	ITLEEEMTKY	KPESDELTAE	KITQFCHHFL	EGKIKPHLMS			359
P4hb-201	NQRILEFFGL	KKEECPAVRL	ITLEEEMTKY	KPESDELTAE	KITQFCHHFL	EGKIKPHLMS			333
Splic.C	NQRILEFFGL	KKEECPAVRL	ITLEEEMTKY	KPESDELTAE	KITQFCHHFL	EGKIKPHLMS			300
P4hb-203 (canonical)	QELPEDWDKQ	PVKVLVGKNF	EEVAFDEKKN	VFVEFYAPWC	GHCKQLAPIW	DKLGETYKDH			419
P4hb-201	QELPEDWDKQ	PVKVLVGKNF	EEVAFDEKKN	VFVEFYAPWC	GHCKQLAPIW	DKLGETYKDH			393
Splic.C	QELPEDWDKQ	PVKVLVGKNF	EEVAFDEKKN	VFVEFYAPWC	GHCKQLAPIW	DKLGETYKDH			360
P4hb-203 (canonical)	ENIVIAKMDS	TANEVEAVKV	HSFPTLKFFP	ASADRTVIDY	NGERTLDGFK	KFLESGGDG			479
P4hb-201	ENIVIAKMDS	TANEVEAVKV	HSFPTLKFFP	ASADRTVIDY	NGERTLDGFK	KFLESGGDG			453
Splic.C	ENIVIAKMDS	TANEVEAVKV	HSFPTLKFFP	ASADRTVIDY	NGERTLDGFK	KFLESGGDG			420
P4hb-203 (canonical)	AGDNDLDLE	EALEPDMEED	DDQKAVKDEL						* 510
P4hb-201	AGDNDLDLE	EALEPDMEED	DDQKAVKDEL						* 484
Splic.C	AGDNDLDLE	EALEPDMEED	DDQKAVKDEL						* 451

C

	Signal peptide								
P4hb-203 (canonical)	MLSRALLCLA	LAWAARVGAD	ALEEEEDNVLV	LKKSNFAEAL	AAHNYLLVEF	YAPW	CGHC	KA	60
P4hb-202	MLSRALLCLA	LAWAARVGAD	ALEEEEDNVLV	LKKSNFAEAL	AAHNYLL---	YAPW	CGHC	KA	47
Splic.D	MLSRALLCLA	LAWAARVGAD	ALEEEEDNVLV	LKKSNFAEAL	AAHNYLL---	YAPW	CGHC	KA	47
P4hb-203 (canonical)	LAPEYAKAAA	KLKAEGSEIR	LAKVDATEES	DLAQQYGVRR	YPTIKFFKNG	DTASPKEYTA			120
P4hb-202	-----	-----	-----	-----	-----	-----			47
Splic.D	-----	-----	-----	-----	-----	-----			47
P4hb-203 (canonical)	GREADDIVNW	LKKRTGPAAT	TLSDTAAAES	LVDSSSEVTVI	GFFKDAGSDS	AKQFLAAEA			180
P4hb-202	-----	-----	-----	-----	-----	-----			47
Splic.D	-----	-----	-----	-----	-----	-----			47
P4hb-203 (canonical)	VDDIPFGITS	NSDVFSKYQL	DKDGVVLFK	FDEGRNFFEG	EITKEKLLDF	IKHNQLPLVI			240
P4hb-202	-----	-----	-----	-----	-----	-----			47
Splic.D	-----	-----	-----	-----	-----	-----			47
P4hb-203 (canonical)	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	DGKLSNFKKA	AEGFKGKILF	IFIDSDHTDN			300
P4hb-202	-----	-----	-----	-----	-----	-----			47
Splic.D	-----	-----	-----	-----	-----	-----			47
P4hb-203 (canonical)	QRILEFFGLK	KKEECPAVRLI	TLEEEMTKYK	PESDELTAEK	ITQFCHHFLF	GKIKPHLMSQ			360
P4hb-202	-----	-----	-----	-----	-----	-----			47
Splic.D	-----	-----	-----	-----	-----	-----			47
P4hb-203 (canonical)	ELPEDWDKQP	VKVLVGKNFE	EVAFDEKKNV	FVEFYAPWC	GHCKQLAPIWD	KLGETYKDHE			420
P4hb-202	-----	-----	-----	-VEFYAPWC	GHCKQLAPIWD	KLGETYKDHE			76
Splic.D	-----	-----	-----	-VEFYAPWC	GHCKALAPEYA	KAA-----			69
P4hb-203 (canonical)	NIVIAKMDS	ANEVEAVKVH	SFPTLKFFPA	SADRTVIDYN	GERTLDGFKK	FLESGGDGA			480
P4hb-202	NIVIAKMDS	ANEVEAVKVH	SFPTLKFFPA	SADRTVIDYN	GERTLDGFKK	FLESGGDGA			136
Splic.D	---AKLKA	GSEIRLAKV-	---	-DAT-----	---	---			87
P4hb-203 (canonical)	GDNDLDLE	ELEPDMEED	DQKAVKDEL						* 510
P4hb-202	GDNDLDLE	ELEPDMEED	DQKAVKDEL						* 166
Splic.D	---EESDLAQ	QSQPCLTLQL	QSPWWTQAK						* 114

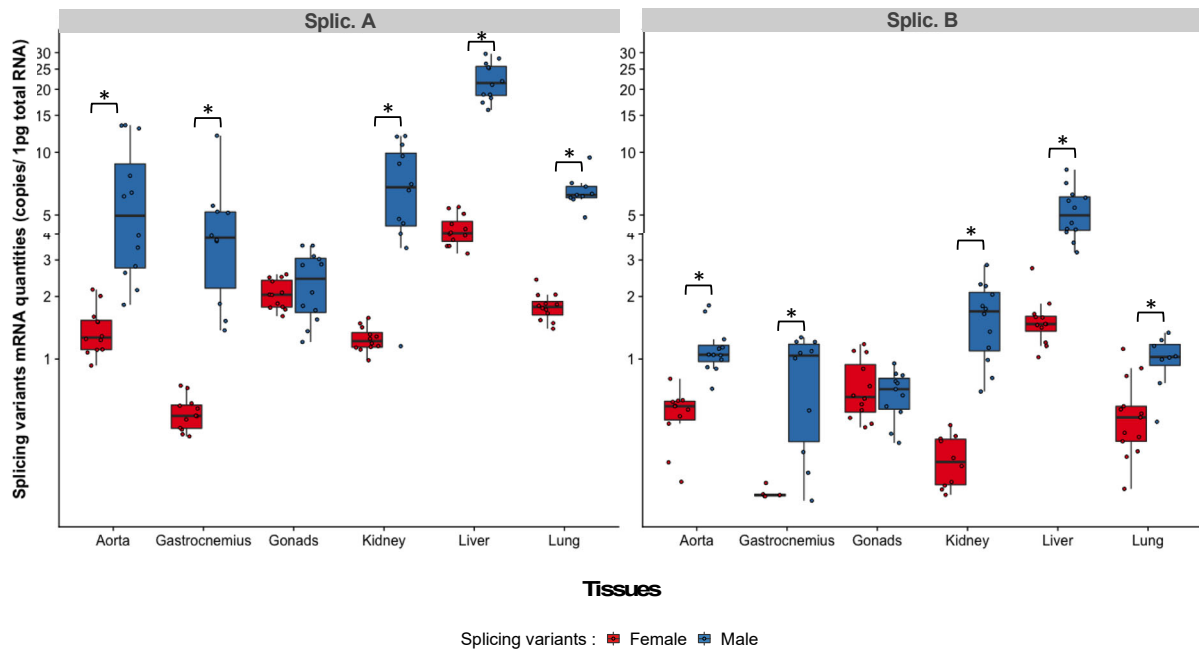
253 **Figure 4: Protein sequence alignment of Splic.B, C and D with the canonical sequence of rat**
254 **P4hb (P4hb-203) and its most similar computational predicted splice variants.:** (A) Sequence
255 alignment of Splic. B with computational predicted P4hb isoform 201 (P4hb-201:
256 ENSRNOT00000106240.1, Ensembl databank June 2022) (B) Sequence alignment of Splic. C with
257 computational predicted P4hb isoform 201(P4hb-201: ENSRNOT00000106240.1, Ensembl databank
258 june 2022) (C) Sequence alignment of Splic. D with computational predicted P4hb isoform 202 (P4hb-
259 202: ENSRNOT00000100112.1, Ensembl databank june 2022). Active site (CXXC): full lines boxes;
260 ER-retention sequence (KDEL): dashed lines boxes.
261

262 **3.2 Tissue expression of *Pdi* splice variants differs between tissues and sex in** 263 **rats**

264 The expression level of *Pdi* variants was quantified by RT-qPCR in six tissues (aorta,
265 gastrocnemius, gonads (testicles and ovaries), kidney, liver, and lung from 8-week-old
266 male and female rats (Figure 1). Regardless of the tissue considered, only Splic.A, B,
267 and C were quantifiable by quantitative RT-PCR (Figure 5). Nevertheless, since
268 Splic.A and Splic.B mRNAs together accounted for almost 100% of all *Pdi* transcripts,
269 Splic.D mRNAs could be considered negligible in all 6 tissues regardless of gender
270 (Table 2).

271 Whatever the tissue considered Splic.A mRNAs were systematically the most
272 abundant. They were 2.5 to 5.5 times (depending on the tissue) more expressed than
273 Splic.B mRNAs in males; they were 1.8 to 3 times more abundant in females (Table
274 2). With the exception of testis, Splic.B mRNAs, in proportion to total PDI mRNAs, were
275 most highly represented in liver in males and females; they were least represented in
276 lung in males and in kidney for females. The proportion of Splic.B mRNAs was
277 systematically higher in female rats than in males (except for kidney). Nevertheless,
278 the strategy to amplify Splic.A mRNAs could also amplify undetected splice variants.

279



280

281 **Figure 5: Expression of *Pdi* splice variant mRNAs in different tissues of 8-week male and female**
 282 **rats (n=4 for both sexes). Results are presented as number of copies/pg of total RNA. *, indicates a**
 283 **statistically significant difference between males and females (p-value < 0.05). The Splic.C variant is**
 284 **not shown in the figure as its quantities were too low (between 1 to 8 copies/25 ng of total RNA).**
 285

286 **Table 2: Proportion of RNAs of each *Pdi* splice variant to total *Pdi* transcripts in different tissues**
 287 **in 8-week-old female and male rats. Results are expressed as mean \pm CI. *, significant statistical**
 288 **difference between males and females (p value < 0.05). (p-value < 0.05).**

Tissues	Splic A	Splic B	Splic C
Aorta	male: 82.1 +/- 42.2% female: 74.5 +/- 10.6%	male: 17.9 +/- 3.1%* female: 38.5 +/- 9.2%*	male: <1% female: <1%
Liver	male: 77.6 +/- 15.2% female: 67.2 +/- 9.9%	male: 22.4 +/- 4.0%* female: 36.9 +/- 6.6%*	male: <1% female: <1%
Gastrocnemius	male: 60.3 +/- 19.9% female: 71.7 +/- 10.9%	male: 16.5 +/- 6.7%* female: 31.3 +/- 7.0%*	male: <1% female: <1%
Gonad	male: 68.0 +/- 19.0% female: 74.3 +/- 5.6%	male: 28.8 +/- 4.9%* female: 36.1 +/- 7.8%*	male: <1% female: <1%
Lung	male: 83.2 +/- 8.7% female: 82.4 +/- 3.5%	male: 15.5 +/- 3.5%* female: 31.8 +/- 8.2%*	male: <1% female: <1%
Kidney	male: 76.6 +/- 31.5% female: 74.2 +/- 7.8%	male: 23.4 +/- 5.9% female: 24.1 +/- 7.1%	male: <1% female: <1%

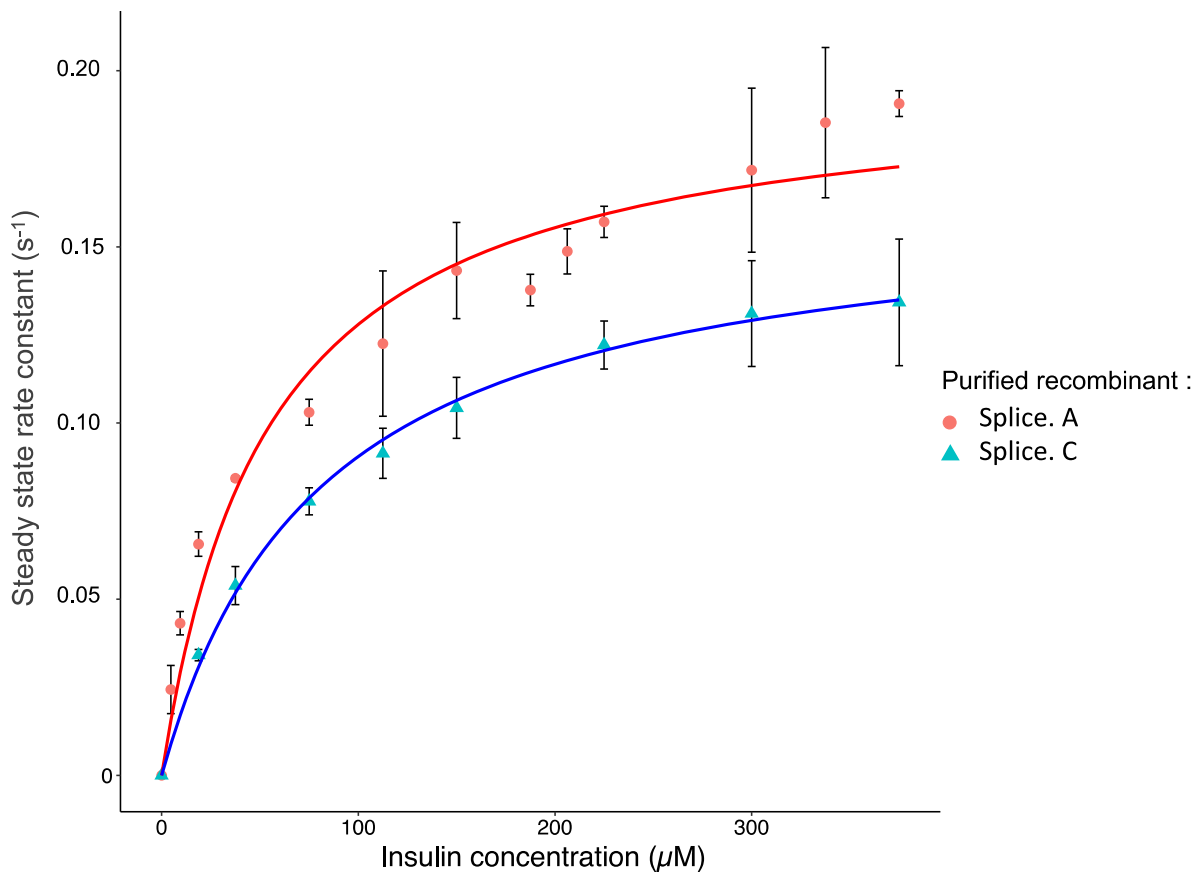
289

290 **3.3 Splic.C displays similar reductase activity to Splic.A, while Splic.B displays**
 291 **lower reductase activity.**

292 Splice variants A, B, C and D were expressed as histidine-tagged proteins in *E. coli*
 293 [38]. While Splic.A, B and C were successfully expressed and purified (Figure 3B),

294 Splic.D could not be expressed under the same experimental conditions suggesting
295 instability of the corresponding protein.

296 The functionality of the histidine tagged Splic.A, B and C was first analyzed by their
297 ability to reduce insulin. His-tagged-Splic.A and C proteins were shown to successfully
298 reduce insulin in the presence of reduced glutathione. The reaction rates of Splic.A
299 and Splic.C towards insulin followed a Michaelis-Menten model (Figure 6) allowing the
300 determination of kinetic parameters. The apparent K_m values were $56.6 \pm 7.9 \mu\text{M}$ for
301 Splic.A and $81.4 \pm 7.8 \mu\text{M}$ for Splic.C, and the apparent $k_{\text{MAX}}^{\text{app}}$ were $0.197 \pm 0.007 \text{ s}^{-1}$ for
302 Splic.A and $0.164 \pm 0.006 \text{ s}^{-1}$ for Splic.C.



304 **Figure 6: Insulin reduction by purified recombinant Splic.A and Splic.C as a function of insulin**
305 **concentration. A (in red) and C (in blue).** Measurements were done in the presence of 1 μM PDI
306 splice variant, 16,6 mM GSH, 2 U/μL glutathione Reductase in 20 mM phosphate buffer, 200 mM NaCl,
307 1 mM EDTA pH 7. The reaction was initiated by adding insulin after a pre-incubation time of around 2
308 min. NADPH consumption was measured at 340 nm and the steady state rate constant (s⁻¹) was
309 calculated as mentioned in Materials and Methods. Results are the mean ± SD of three independent
310 determinations. Data were fitted by non-linear regression to the Michaelis–Menten model using R
311 software.
312

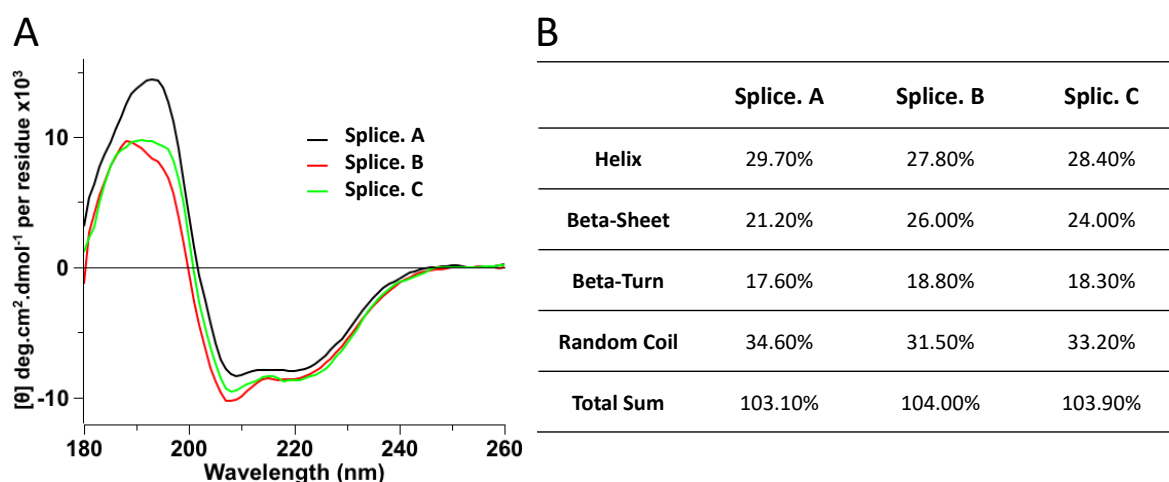
313 No reduction of insulin by Splic.B was detected under the same experimental
 314 conditions. This absence of insulin reduction was observed with two different batches
 315 of recombinant Splic.B.

316 Reductase activity of splice variants was thus explored using the N,N-di(thioamido-
 317 fluoresceinyl)-cystine (DTFCys2) substrate [37]. Consistent results with those obtained
 318 with insulin were obtained using the DTFCys2 fluorescent substrate regarding Splic.A
 319 and C. These PDI variants were more active with this substrate, with reductase rate
 320 constants of 3.3 and 6.8 s⁻¹ for Splic.A and Splic.C, respectively. In contrast, using the
 321 DTFCys2 substrate, reductase activity of Splic.B was detected. This activity was 30 to
 322 50-fold lower than that of Splic.A and Splic.C, with a rate constant of 0.12 ± 0.01 s⁻¹.

323

324 **3.4 The reductase activity of Splic.B is low although its conformation is similar** 325 **to that of Splic.A and B.**

326 Because Splic.B presented low reductase activity compared to Splic.A and C while its
 327 sequence was similar to theirs, the conformation of this variant was analyzed by
 328 circular dichroism and compared to the results obtained for Splic.A and C (Figure 7).



329

330 **Figure 7: Conformation analysis by circular dichroism.** A/ Superimposed circular dichroism spectra
 331 of Splic.A, B, and C were determined at 10 μM of each protein. CD spectral range 180-260 nm,
 332 temperature: 20°C, step size: 1nm, bandwidth: 1nm; B/ fractions of the secondary structure components
 333 drawn from the spectra with CDNN software.

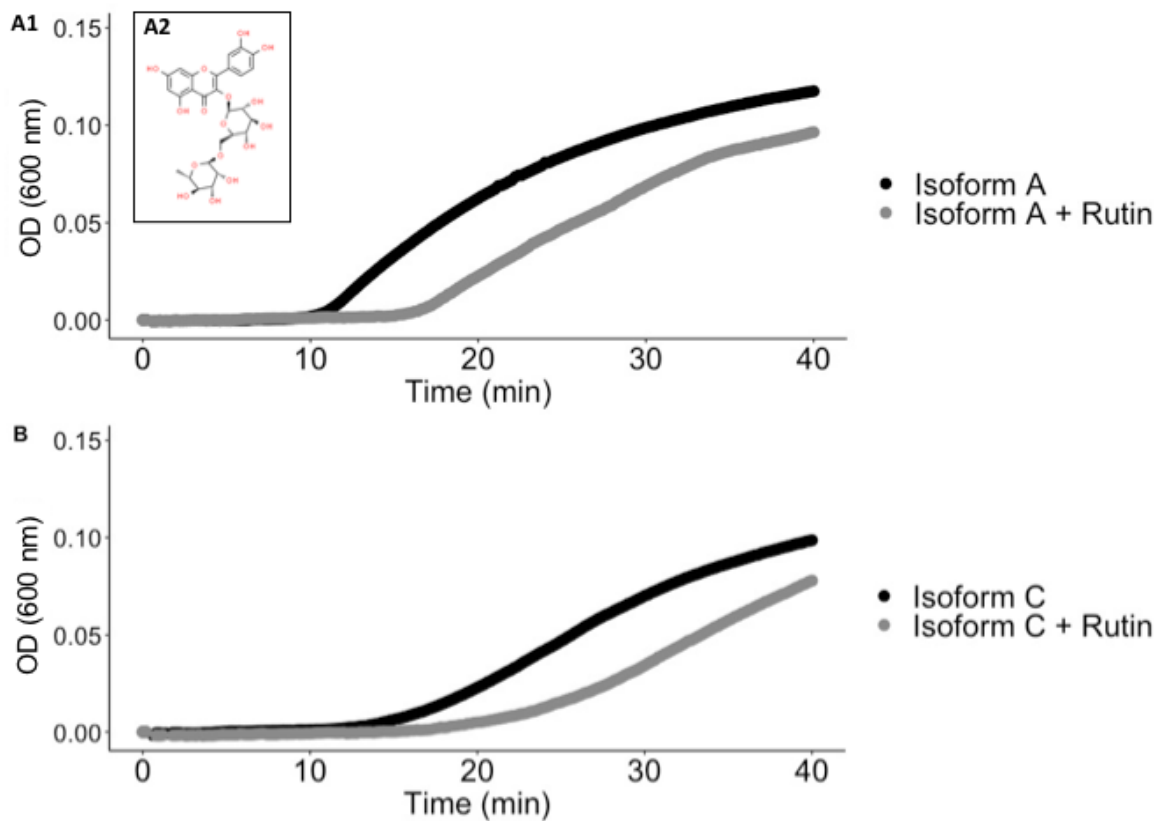
334

335 No significant differences in spectra were noticed between the three splice variants
336 (Figure 7A), although Splic.A appeared to be the most structured protein with a
337 stronger peak at 192 nm. Alpha helix secondary structures were present for all splice
338 variants and accounted for nearly 30% of the signal (Figure 7B). However, a higher
339 proportion of beta sheets was observed for Splic.B compared to Splic.A and to a lesser
340 extent Splic.C.

341

342 **3.5 Rutin similarly inhibits reductase activities catalyzed by Splic.A and C.**

343 Inhibition of Splic.A and Splic.C-catalyzed insulin reduction by a classical inhibitor used
344 in PDI studies, i.e. rutin (quercetin 3-rutinoside) was analyzed. For this purpose, the
345 precipitation of insulin due to the reduction of disulfide bonds by PDI variants was
346 measured at 600 nm [38] in the presence of 100 μ M of rutin as described by Lin et al.
347 [39]. Absorbance threshold of 0.003 was considered a significant threshold for insulin
348 precipitation. Whether the reaction was catalyzed by Splic.A or Splic.C, the reduction
349 of insulin (Figure 8) was inhibited by the addition of rutin. The inhibitory efficacy of 100
350 μ M rutin on Splic.C was similar to that observed for Splic.A (i.e., 94% of that observed
351 for Splic.A). Similar results were obtained using the fluorescent DTFCys2-based
352 assay. Rutin inhibited Splic.A and Splic.C activities by 13 and 25% at 50 μ M, and by
353 68 and 76% at 200 μ M, respectively.



354

355 **Figure 8: Inhibition of insulin reductase activity catalyzed by recombinant Pdi splice variants by**
 356 **rutin.** The measurements were done in the presence of 1 μ M PDI variant, 16,6 mM GSH, 2 U/ μ L
 357 glutathione reductase in 20 mM phosphate buffer, 200 mM NaCl, 1 mM EDTA pH 7, with or without 100
 358 μ M Rutin (structure in the box) and the reaction was initiated by adding 37.5 μ M of insulin after a pre-
 359 incubation time of around 2 min. Insulin precipitation was monitored at 600 nm for at least 40 minutes.
 360 A/ Effect of rutin on Splic.A, B/ Effect of rutin on Splic.C.

361

362 4. Discussion

363 Alternative splicing of pre-mRNA is an important component of gene expression
 364 regulation and increases the complexity of the proteome. Numerous studies have
 365 proven the fundamental role of alternative splicing, for example, on proteins sub-
 366 cellular localization or enzymatic properties [29,40]. PDI is a thiol and chaperone
 367 oxidoreductase of the thioredoxin superfamily and displays multiple biological
 368 functions. Therefore, the study of PDI alternative splicing is particularly relevant. In
 369 humans, the splice variant landscape of the PDI has been described [31], and would
 370 produce 17 coding proteins (Ensembl database, *P4hb*: ENSG00000185624 - June
 371 2022). In rats, a traditional laboratory animal used in pharmacological studies, while

372 three variants were computationally predicted (Ensembl database, P4hb-203 (2411
373 bp, 509 aa): ENSRNOT00000054958.3; P4hb-201 (3123 bp, 482 aa):
374 ENSRNOT00000106240.1; P4hb-202 (1331 bp, 165 aa): ENSRNOT00000100112.1 -
375 June 2022), our experimental study allows to describe four coding transcripts (Splic.A,
376 B, C and D) and one noncoding transcript (Splic. E). Alignment of their sequence with
377 computational predictions from the Ensembl data bank (Figure 4) indicated that Splic.A
378 matches the consensus sequence of rat PDI (P4hb-203) (100% identity and
379 homology). Splic.B presents 93.5% identity and similarity regarding P4hb-201 and
380 three amino acids less than P4hb-201; Splic.C, 93.0% identity and similarity regarding
381 P4hb-201 and 33 amino acids less than P4hb-201; Splic.D, 41.6% identity and 44.9%
382 similarity regarding P4hb-202 and 53 amino acids less than P4hb-202. Therefore, the
383 computational predictions in rat are close to our findings for Splic.B and C. Our results
384 are also consistent with the RNA-seq data analysis and computational predictions of
385 *Pdi* splice variants in humans [31] (Ensembl database, *P4hb*: ENSG00000185624 -
386 June 2022) and mice (Ensembl database, *P4hb*: ENSMUSG00000025130 - June
387 2022), because, in both cases, N-terminal splice variants were predominant.

388

389 PDI has been reported to be highly concentrated in the lumen of the endoplasmic
390 reticulum and partially exported out of the cell or slightly anchored in the plasma
391 membrane [28,41]. A part of the first exon of the canonical PDI (from aa 1 to 21)
392 corresponds to the signal peptide contributing to the topological and subcellular
393 localization of the protein, especially in the ER [42]). In addition to this signal peptide,
394 PDI presents a C-terminal KDEL retention sequence in the ER inducing its retrograde
395 transport from the Golgi to the ER, and sometimes its excretion to the cell membrane
396 [17]. In our results both Splic.B and Splic.C show no signal peptide and lose some

397 amino acids from the “a” domain (11 amino acids for Splic.B; 41 amino acids for
398 Splic.C) compared to the canonical PDI. On the contrary, Splic.D contains a signal
399 peptide but does not have an ER KDEL-retention sequence. Hence, all of these splice
400 variants could display a different cellular localization compared to PDI canonical
401 isoform.

402

403 Despite its truncation, Splic.C is a functional splice variant. This result obtained using
404 two different methods is not surprising considering previous work on the
405 characterization of isolated PDI domains[39]. In fact, Splic. C is comparable to the
406 bb'xa' fragment truncated from amino acids 2 to 60, i.e. a significant portion of the a
407 domain (thioredoxin-like domain) containing the first CXXC catalytic motif[39]. The
408 bb'xa' fragment was shown to display the same reductase activity as the canonical
409 isoform (abb'xa'c)[39]. This is also the case of Splic.C which presents similar reductase
410 activity than Splic.A. As expected, our results demonstrate that the loss of part of the
411 a domain of PDI does not alter the ability of the enzyme to reduce insulin. This
412 reduction is therefore totally mediated by the second thioredoxin-like a' domain
413 (containing the second CXXC catalytic motif). Our results also confirm that the a and
414 a' domains can function independently [18,43]. Furthermore, we show that insulin
415 binding by Splic.C is not altered by the truncation of 59 amino acids because the
416 apparent Km of Splic.C towards insulin is similar to that of Splic.A. This is also
417 consistent with the literature where the b and b' domains have been described to be
418 involved in PDI substrate binding [23,39,44] and modulation of catalytic activity [25],
419 since the b and b' domains are conserved in Splic.C. In contrast, it was surprising to
420 observe that, even if Splic.B contains both active sites of canonical PDI, it shows low
421 reductase activity. Using circular dichroism, we explore the hypothesis that the

422 conformation of Splic.B impacts its reductase activity. Unfortunately, the slight
423 differences observed between variants do not explain the low activity of Splic.B.
424 Further structural studies will be necessary. The last splice variant, the Splic.D, is a
425 short isoform presenting a partial splicing of exon 2 and 3, resulting in a premature
426 stop codon (at amino acid 95) and a loss of the second catalytic domains (compared
427 to canonical isoform). Based on the literature, we can hypothesize that Splic.D could
428 act as a free thiol exchanger. Indeed, it consists of an isolated "a" PDI fragment, which
429 has been found active in previous *in-vitro* studies [25,39]. However, due to the absence
430 of the b' domain described as involved in substrate recognition[45], it would then act
431 as a free thiol exchanger without substrate specificity. Similarly, it cannot be involved
432 in the interaction with Ero1 mediating protein folding[46], since this interaction also
433 involves the b and b' domains[47].

434 As PDI inhibitors are considered as potential emerging therapeutics[8,48,49], the
435 response of splice variants to rutin, a classically used inhibitor was tested in our study.
436 Splic.C was inhibited in the same way as Splic.A. These results are consistent with the
437 results obtained in studies on artificial variants of PDI[39]. Indeed, the artificial variant
438 bb'a' comparable to Splic.C was inhibited by rutin in a similar way to canonical PDI.
439 Concerning Splic.D, only constituted by the "a" domain of PDI, and because of our
440 inability to produce this variant, we can hypothesize that this variant is probably
441 resistant to the action of rutin on the bases of the mechanism (rutin must interact with
442 the "b" domain to inhibit PDI) and of the literature (the isolated "a" domain is not
443 inhibited by rutin)[38,39]. It is therefore necessary to take into account the natural
444 isoforms of PDI when exploring the inhibitory potential of molecules.

445

446 PDI through its multiple physiological roles interacts with many proteins, i.e., proteins
447 involved in blood coagulation [8,41,50,51], hemostasis [12], vascular inflammation
448 [11], infection control [52], vitamin K metabolism [53], oxidative stress [54]. The
449 activities of Splic.A, B and C, and even D, with respect to all these targets could
450 therefore be different, considering the differences in activity observed using insulin
451 reduction or DFTCys2 assays. Moreover, cellular or extracellular localization could
452 change between splice variants as previously discussed. We report herein that
453 expression of splice variants changes according to tissue and according to gender with
454 certainly a hormonal regulation. Such differences (activity, tissue, subcellular
455 localization, gender) could be of major importance considering the involvement of PDI
456 in various crucial physiological processes. An example, PDI has been identified on the
457 surface of platelets or secreted by platelets. It contributes to the initiation of thrombus
458 formation, through a mechanism that is currently misunderstood[13,41,55]. Some
459 results suggest that PDI acts as an activator of tissue factor through the isomerization
460 of cysteine residues 186 and 209, converting the encrypted version of tissue factor to
461 active form [56]. The extracellular localization of PDI is central in this process, but little
462 is known about the mechanism leading to this extra-ER localization. As mentioned in
463 the literature, alternative splicing might be an explanation [51]. As it has already been
464 demonstrated that the KDEL ER-retention signal sequence is preserved on cell-
465 secreted PDI [57], our study leads us to question the effect of signal peptide loss on
466 the subcellular localization of PDI. *In-vitro* and *in-vivo* models have been used to
467 explore the role of PDI and should also be used to explore the biological functions of
468 PDI splice variants.
469

470 In this study the distribution of splice variants according to sex and tissue was analyzed
471 only by RT-qPCR. We attempted to do so also by western blot analysis (data not
472 shown) using an antibody targeting the C-terminal part of the human protein claimed
473 to cross-react with the rat protein (Proteintech, PDI Antibody 11245-1-AP).
474 Unfortunately, we could only detect Splic.A very weakly in the liver and traces were
475 visible in the kidney. Rat specific antibodies targeting each variant would be required
476 to achieve better sensitivity and specificity. Nevertheless, in a recent study, western
477 blot analysis on human colorectal tissue was performed [58] and different bands of
478 close molecular weight were reported. It is conceivable that these different bands may
479 correspond to splicing variants considering the differences in molecular weight
480 observed (figure 1C in [58]). These observations highlight the need for further studies,
481 to explore the protein expression of splice variants in rats. Tissue and sex-specific
482 differences in the protein expression of these variants could be of major importance
483 considering the crucial roles of PDI in many physiological processes and the possible
484 differences in activity and localization of PDI variants.

485

486 **5. Conclusion**

487 The results of the present study show that there are different splice variants of PDI in
488 rats. They exhibit reductase activity and could have biological relevance in protein thiol-
489 disulfide exchange and could constitute new pharmacological targets. Indeed, only
490 reductase activity has been explored in this study, whereas PDI also supports oxidation
491 and isomerization reactions. Characterization of the ability of splice variants to support
492 these enzymatic activities will be interesting, especially to assess the functional
493 consequences of alternative splicing. Nevertheless, only a few complex methods are
494 available to explore the isomerase and oxidase activities of PDI and are sometimes

495 difficult to analyze [59]. Whereas it has been described that both CXXC motifs are
496 required for isomerase activity, does Splic.C truncated from part of the α -domain
497 exhibits the same isomerase activity as canonical PDI isoform? While Splic.B supports
498 weak reductase activity, could it have oxidase activity?

499 On the other hand, activation of PDI requires a protein partner, the ER oxidoreduction
500 (Ero 1) protein. In this study, this partner was mimicked by glutathione or DTT.
501 Functional characterization of the variants in the presence of Ero1 would be necessary,
502 as protein-protein interactions can be modified by the different truncations. Further
503 studies will be needed to answer to all of these questions.

504 Furthermore, the PDI-like family is widely conserved in all eukaryotic species from yeast
505 to mammals [60]. Splice variants exist in other species such as humans and mice [31],
506 the same methodology used in this study should be applied to characterize splice
507 variants before designing pharmacological therapeutics.

508

509 **Declaration of competing interest:**

510 The authors have no competing interest to declare.

511

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515

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527 **Virginie Lattard**: Conceptualization, Methodology, Formal analysis, Writing - Original
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529 Supervision, Project administration, Funding acquisition.

530

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