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4 # The influence of 4-thiouridine labeling on pre-mRNA splicing outcomes
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36 # Abstract

37

38 Metabolic labeling is a widely used tool to investigate different aspects of pre-mRNA
39 splicing and RNA turnover. The labeling technology takes advantage of native cellular
40 machineries where a nucleotide analog is readily taken up and incorporated into nascent RNA.
41 One such analog is 4-thiouridine (4sU). Previous studies demonstrated that the uptake of 4sU at
42 elevated concentrations (>50μM) and extended exposure led to inhibition of rRNA synthesis and
43 processing, presumably induced by changes in RNA secondary structure. Thus, it is possible
44 that 4sU incorporation may also interfere with splicing efficiency. To test this hypothesis, we
45 carried out splicing analyses of pre-mRNA substrates with varying levels of 4sU incorporation
46 (0-100%). We demonstrate that increased incorporation of 4sU into pre-mRNAs decreased
47 splicing efficiency. The overall impact of 4sU labeling on pre-mRNA splicing efficiency
48 negatively correlates with the strength of splice site signals such as the 3' and the 5' splice sites.
49 Introns with weaker splice sites are more affected by the presence of 4sU. We also show that
50 transcription by T7 polymerase and pre-mRNA degradation kinetics were impacted at the
51 highest levels of 4sU incorporation. Increased incorporation of 4sU caused elevated levels of
52 abortive transcripts, and fully labeled pre-mRNA is more stable than its uridine-only counterpart.
53 Cell culture experiments show that a small number of alternative splicing events were modestly,
54 but statistically significantly influenced by metabolic labeling with 4sU at concentrations
55 considered to be tolerable (40 μM). We conclude that at high 4sU incorporation rates small, but
56 noticeable changes in pre-mRNA splicing can be detected when splice sites deviate from
57 consensus. Given these potential 4sU artifacts, we suggest that appropriate controls for
58 metabolic labeling experiments need to be included in future labeling experiments.

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60 # Introduction

61 4-thiouridine (4sU), a uridine analog, is a commonly used metabolic label that can be
62 employed to investigate a wide variety of topics that range from pre-mRNA generation to mRNA
63 degradation. When added to cell medium, 4sU is readily taken up and incorporated into newly
64 transcribed RNA [1,2]. In mammalian cells, this uptake results in a 0.5% to 2.3% median 4sU
65 incorporation rate [3,4] when in the presence of 500μM 4sU for 2hr and 100μM for 24hr. 4sU
66 differs from uridine by the existence of a sulfur atom rather than an oxygen atom in the 4th
67 carbon position on the nitrogenous base [5]. 4sU incorporation into RNA assists in the physical
68 or computational isolation of RNA species. The modified nucleotide can be biotinylated to allow
69 for bead capture and subsequent isolation [1,6]. The presence of 4sU in RNA also induces a
70 single nucleotide sequencing artifact that can later be used to identify the presence of 4sU in an
71 RNA strand [3,6]. Despite its widespread use, not much is known about the functional
72 implications of 4sU incorporation in downstream RNA processes. Recent studies suggested that
73 labeling at 4sU concentrations >100μM, concentrations commonly used across many different
74 cell types[2], may elicit adverse effects, especially during extended labeling times (>12hrs) [7].
75 Indications of toxicity were also observed at concentrations as low as 50μM [7], presumably
76 because ribosomal RNA synthesis is inhibited, triggering cellular stress response [7]. These
77 observations suggested that 4sU incorporation can negatively impact translation and cellular
78 homeostasis. Given the extensive use of 4sU labeling in the study of nascent pre-mRNA
79 splicing, we set out to determine if intron removal efficiency is influenced by 4sU incorporation.
80

81 Pre-mRNA splicing is an essential biological process that allows for proteomic
82 complexity, despite the relatively small number of protein-coding genes within the human
83 genome [8]. This process is carried out by the spliceosome, a multiprotein/snRNA complex that
84 recognizes and binds to hallmark pre-mRNA sequences. These sequences include both the 5'

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85 and 3' splice sites (ss), the branch point, and the polypyrimidine tract [9]. A single nucleotide
86 change in any of these sequences can alter the efficiency of pre-mRNA recognition by the
87 spliceosome. Thus, it is possible that 4sU incorporation impacts pre-mRNA splicing.
88
89 Using complementary *in vitro* and cell culture experiments, we show that pre-mRNA
90 splicing can be impacted by the incorporation of 4sU into pre-mRNAs. The extent of splicing
91 interference depends on 4sU incorporation levels and the intrinsic ability of introns to be
92 recognized by the spliceosome. Thus, at high levels of 4sU incorporation, weaker exons are
93 more likely to be differentially processed. 4sU labeling also interfered with *in vitro* transcription
94 and *in vitro* pre-mRNA stability at elevated levels of incorporation. We conclude that the
95 presence of 4sU in nascent RNA can interfere with pre-mRNA splicing. However, the negative
96 effects are only detectable at elevated levels of 4sU incorporation levels and in the context of
97 weak splicing events.

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98 # Materials and Methods

99

100 ## *In vitro* transcription of radiolabeled pre-mRNA

101 ADML and β-Globin, two well-studied DNA constructs that are spliced with ease by the
102 spliceosome when transcribed under normal conditions, were used to generate pre-mRNA for *in*
103 *vitro* splicing[10]. Splice site strengths for each of the constructs were determined using
104 MaxEntScanner [11]. After linearizing with BamHI (**Promega, R6021**) and
105 cleaning/concentrating (**Zymo, D4013**) ADML and β-Globin, a 10μL radioactive *in vitro*
106 transcription reaction was performed using T7 polymerase, [α-^{32}P] CTP (**Perkin Elmer,**
107 **BLU008H250UC**), and phosphorylated 4-thiouridine (**TriLink, N-1025**). Different strands of pre-
108 mRNA were generated for each construct that included different quantities of 4sU. The amounts
109 of 4sU relative to the total amount of rUTP used in the transcription reaction mixture were 0%,
110 2.5%, 15%, 30%, and 100%. The resulting pre-mRNA was PAGE purified, eluted, precipitated,
111 and resuspended at 10μL per 100,000cpm on the Geiger counter.

112

113 ## *In vitro* pre-mRNA splicing

114 *In vitro* splicing was carried out by following a well-established protocol [12] in either
115 12.5μL or 25μL reactions for 90min or the indicated time for the experiment. The splicing
116 reactions were run out on 6% polyacrylamide (19:1 acrylamide: bisacrylamide) 7 M urea gels
117 and imaged using a GE Typhoon imager. Splicing efficiency was determined by following the
118 "percent spliced" equation in the *in vitro* splicing protocol [12]. Splicing rates were determined by
119 fitting data points to pseudo-first-order decay equations. All resulting gels were analyzed using
120 GelAnalyzer 19.1 software [13], visualized using GraphPad Prism9 software [14] and all
121 statistical analyses were performed with Prism9's built-in statistical analysis tools. The

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122 degradation assays were performed identically to the *in vitro* splicing assays, except we created
123 degradation conditions by omitting the addition of RNasin, CP, and ATP from the reaction
124 mixture and by depleting the nuclear extract of ATP by incubation at room temperature for
125 30min prior to the addition of the labeled RNA. Normalized degradation profiles were calculated
126 by dividing the band intensity of the intact pre-mRNA by the band intensity of the degraded pre-
127 mRNA along the time points of the reaction.
128

129 HEK293 metabolic labeling

130 HEK293 cells (**ATCC, CRL-1573**) were grown in 6-well plates at 37°C, 5% CO_2 in a
131 humidified incubator to 80% confluency for the start of the 2hr labeling experiments and 40%
132 confluency for the start of the 24hr labeling experiments. The cells were grown in DMEM/High
133 Glucose (**HyClone, SH30022.01**) plus 10% FBS (**GeneClone, 25-550**). A metabolic labeling
134 protocol was followed[6] and the experimental HEK293 cells were incubated in the presence of
135 40µM unphosphorylated 4-thiouridine (**Cayman Chemical, CAY16373-5**). Total RNA was
136 harvested from the cells using Trizol (**Ambion, 15596018**) and processed per the metabolic
137 labeling protocol. The resulting pure total RNA was stored in TE at -80°C for further
138 experimentation.
139

140 Endpoint PCR

141 Using 1µg of total RNA harvested from the metabolic labeling experiments, cDNA was
142 generated using a first-strand cDNA synthesis kit (**Invitrogen, 18080-044**) by following the
143 manufacturer-provided protocol. Oligo $d(T)_{16}$ (**IDT, 51-01-15-06**) was used as the primer for the
144 cDNA reaction. PCR primers were designed that flank the exons/regions of interest or using
145 previously described primer sets [15]. Primer sequences are as follows: **F_RIOK3_Ex5**

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146 CCGGTTCCCACTCCTAAAAAGGGC; **R_RIO-K3_Ex10**
147 CCAGCATGCCACAGCATGTTATACTCAC; **F_TRA2B_Ex1** AGGAAGGTGCAAGAGG TTGG;
148 **R_TRA2B_Ex3** TCCGTGAGCACTTCCACTTC; **F_CLK2_Ex3** CCGGACATTTAGCCGC TCAT;
149 **R_CLK2_Ex6** TGGCCATGGTAGTCAAACCA; **F_DDX5_Ex11** ATTGCTACAGATGTGG
150 CCTCC; **R_DDX5_Ex12** TGCCTGTTTTGGTACTGCGA. We designed additional sets of
151 primers to investigate both alternative ‘Alt’ splicing events and constitutive ‘Const’ splicing
152 events: **F_ADAP2_Alt_Ex2** CAGCAGAGTTAAATCTGTGCGAC; **R_ADAP2_Alt_Ex4**
153 CTTAGCTCGAATCCATTGTTCC; **F_ADAP2_Const_Ex6** AATGCCACCTTCCAGACAGA;
154 **R_ADAP2_Const_Ex8** TGGCCCAGTCTTTTCCATGA; **F_DOLPP1_Alt_Ex5**
155 GCACCAAACAAACAACGCCA; **R_ DOLPP1_Alt_Ex6** GGAAGAACTCGGAGACAGGC; **F_**
156 **DOLPP1_Const_Ex2** GTCGGTTTCGTGACCCTCATC; **R_ DOLPP1_Const_Ex5**
157 CAGGAACCTGGCGTTGTTTG; **F_ZNF711_Alt_Ex6** CACCAGTGGACATTCAGTAGC; **R_**
158 **ZNF711_Alt_Ex8** GCTTGACAATCTTCATACCTTCG; **F_ ZNF711_Const_Ex4**
159 GTGATTCAAGCAGCTGGAGG; **R_ ZNF711_Const_Ex6** CATCTTCTCCCGCTGCATTC. PCR
160 was performed after appropriate adjustments in annealing temperature and elongation time
161 were made for each primer set using an Eppendorf Mastercycler Nexus thermal cycler. PCR
162 reactions were run out on 2.0% agarose gels at 120V for 40min and imaged using a Bio-Rad gel
163 doc imager.

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164 # Results

165

166 ## The impact of 4sU on *in vitro* splicing efficiencies

167 To measure the effect of 4sU incorporation on pre-mRNA splicing we carried out *in vitro*
168 and cell culture pre-mRNA splicing analyses. For the *in vitro* approach, we evaluated the pre-
169 mRNA splicing activities of two well-studied minigene constructs, ADML and β-globin, by
170 generating and testing pre-mRNAs with varying amounts of 4sU incorporation (0%, 2.5%, 30%,
171 and 100% 4sU incorporation). ADML and β-globin minigenes are gold standard *in vitro* splicing
172 constructs that have been shown to splice efficiently in splicing extract. Both minigenes harbor
173 strong splice sites, with ADML containing a 5' splice site with a MaxEnt score (MES) [11] of 7.9
174 and a 3' splice site MES of 12.5, and β-Globin containing a 5' splice site MES of 8.1 and 3'
175 splice site MES of 9.5 (Fig 1A). Given its higher 3' splice site score, the ADML minigene is
176 considered to be more efficiently spliced than the β-globin minigene.

177

178 **Fig 1. *In vitro* splicing kinetics of 4sU labeled RNA.** (A) Schematic depictions of the ADML
179 and β-Globin minigenes used. Exon and intron sizes and splice site strengths (MaxEnt score)
180 are indicated. (B) Autoradiogram representing time course behavior of 0%, 2.5%, 30%, and
181 100% 4sU containing β-Globin pre-mRNAs. Precursor RNA and spliced products are defined on
182 the right of the gel. The numbers under each lane represents the efficiency of splicing. (C)
183 Graphical representation of β-Globin time-course splicing assay splicing efficiencies over time
184 (n=3) and the corresponding splicing rate (ordinary one-way ANOVA, P=0.0031). (D) Graphical
185 representation of ADML time-course splicing assay splicing efficiencies over time (n=3) and the

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186 corresponding splicing rate (ordinary one-way ANOVA, P=0.14)

187

188 To determine if 4sU incorporation influences rates of *in vitro* splicing, splicing efficiencies
189 after 1hr and 2hrs were compared between minigene constructs with incorporation rates of 0%,
190 2.5%, 30%, and 100% 4sU. For β-globin we observe a gradual decrease in the average splicing
191 efficiency as the level of 4sU incorporation in the pre-mRNA increases (Figs 1B and C). While
192 4sU incorporation rates of 2.5% did not change observed splicing rates significantly, higher
193 levels of 4sU incorporation led to an up to a 3-fold reduction in splicing (Fig 1C). The
194 observations are qualitatively similar for the tested ADML pre-mRNA (Fig 1D). However,
195 considering the range of splicing reduction and variation of the independent experiments, the
196 differences observed did not meet the cutoff for statistical significance.

197

198 It is likely that the more striking differences in splicing efficiencies observed for the β-
199 globin analysis are the consequence of its intrinsically weaker splice site signals when
200 compared to ADML. We conclude that elevated levels of 4sU incorporation into pre-mRNAs can
201 reduce splicing efficiencies and the resulting splicing rates, at least within the context of the
202 time-course *in vitro* splicing assays used.

203

204 Stability of 4sU Containing pre-mRNA

205 Modified RNAs are known to be more stable than unmodified RNAs [16,17]. To evaluate
206 whether the incorporation of 4sU into RNA alters the stability of the resulting RNA we carried out
207 *in vitro* degradation assays comparing the fate of fully modified or unmodified RNA samples
208 over a 90 min period. Unmodified ADML RNAs displayed measurable degradation over the 90
209 min incubation (Fig 2A). Interestingly, fully modified ADML RNAs did not undergo any
210 measurable degradation over the same time frame. Linear regression of the profiles indicates

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211 statistically significant differences between the degradation behaviors (Fig 2B, simple linear
212 regression, $P < 0.05$). Analysis revealed a 9-fold difference in degradation kinetics between
213 unmodified and fully modified RNA. These results suggest that the presence of 4sU promotes
214 RNA stability, similar to other RNA modifications [18]. Just as 2'-O-methyl RNA and
215 phosphorothioate RNA modifications resist nuclease activity [19], 4sU has the potential to limit
216 nuclease attack.
217
218 **Fig 2. *In vitro* degradation assay of ADML minigene.** (A) Representative autoradiograph of
219 degradation time-course for unmodified and fully modified 4sU ADML pre-mRNA. t=0 represents
220 input RNA. Full-length input RNA is defined by the cartoon to the right of the gel. (B)
221 Quantitation of the data shown in (A). Simple linear regression of time course data was used for
222 statistical analysis, with unmodified RNA P=0.024 and fully modified RNA P=.0092.
223

224 The effects of 4sU on in vitro transcription

225 *In vitro* transcription of ADML and β-globin at the defined concentrations of 4sU resulted
226 in variable accumulation of abortive transcripts. For example, transcription of ADML lead to a
227 marked increase of an abortive transcript with higher incorporation rates of 4sU (Fig 3A). At
228 100% 4sU incorporation, the majority of transcripts were abortive, resulting in a truncated RNA.
229
230 **Fig 3. *In vitro* transcription of ADML and β-Globin**. Autoradiograph depicting the T7
231 polymerase transcription profiles of A) ADML and (B) β-Globin. Four transcripts of each
232 minigene were created with varying amounts, 0%, 2.5%, 30%, and 100%, of 4sU incorporated.
233 The full pre-mRNA strands and abortive transcripts in each lane are labeled.
234

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235 Qualitatively similar trends were observed when transcribing β-globin as multiple
236 abortive transcripts are observed in the 100% 4sU lane (Fig 3B). Apparently, T7 RNA
237 polymerase faced similar, but less extreme difficulties in generating the full β-globin pre-mRNA.
238 These observations demonstrated that 4sU can negatively impact *in vitro* transcription
239 processivity leading to pre-maturely terminated RNA products.
240

241 The impact of 4sU on splicing in Hek293 Cells

242 To investigate the impact of 4sU incorporation on the splicing of endogenous genes we
243 metabolically labeled HEK293 cells. Based on previous reports we used a 4sU concentration
244 (40μM) that does not elicit toxic translation effects and we evaluated splicing after 2hrs and
245 24hrs of labeling. These 4sU metabolic labeling conditions are representative of the
246 experimental approaches taken when evaluating nascent pre-mRNA splicing. They are also
247 known to result in a 4sU incorporation rate of 0.5% to 2.3% [3,4]. In our analysis, we focused on
248 evaluating the effects of 4sU on the splicing efficiency of constitutively spliced exons
249 (considered efficient splicing events) and alternatively spliced exons (considered less efficient
250 splicing events).
251

252 Our results demonstrate that 4sU metabolic labeling does not significantly interfere with
253 the splicing of constitutive exons. For all examples tested we observed efficient exon inclusion
254 regardless of the presence or absence of 4sU in either the 2hr or 24hr time points (Fig 4A).
255 These observations suggest that efficient splicing events are not influenced by 4sU at
256 incorporation rates typically used in cell culture experiments. To determine whether less efficient
257 splicing events are affected by 4sU labeling we tested a select number of exon skipping events,
258 alternative splice site selection events, and intron retention events. Most of the exon skipping
259 events evaluated did not reveal any significant changes in splicing patterns between the control

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260 and 4sU labeled samples in either the 2hr or the 24hr samples (Fig 4B), even though some
261 exon inclusion levels were intrinsically low (Fig 4B, ZNF711). As the only exception we detected
262 a small, but statistically significant decrease in *DOLPP1* exon 6 inclusion (Fig 4B). We also
263 detected small, but statistically significant changes in intron retention or alternative 5' splice site
264 choice after 24hrs of 4sU incubation (Figs 5A, B). At longer exposure to 4sU labeling, the level
265 of *DDX5* intron 11 retention increased from 8% to 12%. While the magnitude of these changes
266 is small, the increase in intron retention indicates that at the conditions used 4sU labeling can
267 have an inhibitory effect on intron excision. Similarly, minor changes in alternative splice site
268 choice are observed for *RIOK3* exon 8 after 24hrs of incubation (Fig 5B).
269
270 **Fig 4. Splicing behavior in cell culture in the presence of 4sU.** PCR analysis of (A)
271 constitutive exons within the genes *ADAP2*, *DOLPP1*, and *ZNF711* and (B) alternatively
272 included exons in genes *ADAP2*, *CLK2*, *TRA2B*, *ZNF711*, and *DOLPP1*. Alternative exon
273 inclusion in *DOLPP1* (24hr sample t-test, P=0.0024). The cartoon to the right of each image
274 indicates exon inclusion or exclusion. cDNA samples were analyzed after 2 and 24 hours of 4sU
275 incubation.
276
277 **Fig 5. Alternative splicing behavior in cell culture in the presence of 4sU.** PCR analysis of
278 (A) intron retention in *DDX5* (2hr sample t-test, P=0.08; 24hr sample t-test, P=0.0045) and (B)
279 alternative 5'ss selection in *RIOK3* (2hr sample t-test, P=0.012; 24hr sample t-test, P=0.042).
280 The cartoon to the right of each image defines the alternative splicing products. cDNA samples
281 were analyzed after 2 and 24 hours of 4sU incubation.
282
283 Consistent with our *in vitro* 4sU data we conclude that efficiently spliced introns are not
284 significantly affected by the presence of 4sU, at least not at the conditions used. With a few

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285 exceptions, the alternative splicing events evaluated did not indicate altered splicing outcomes

286 in the presence of 4sU. Thus, 4sU labeling at the 40 μM conditions used herein does not trigger

287 drastic, widespread, and general changes in alternative splicing.

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288 # Discussion

289

290 It is important to investigate whether the tools the scientific community use unintentionally
291 create biases and side effects on the experiments being performed. 4sU labeling is a widely
292 used tool in the RNA biology field to capture the processing of nascent RNAs and to evaluate
293 RNA half-lives that is thought to have no consequence on the experimental outcome, aside from
294 the previously reported inhibition of tRNA synthesis and nucleolar stress [7] at elevated
295 concentrations. Here, we show that the incorporation of 4sU into pre-mRNA strands is not
296 simply inert to the metabolism of an expressed RNA. At elevated levels of 4sU incorporation
297 RNA transcription, pre-mRNA splicing, and RNA stability can be altered. However, at labeling
298 concentrations typically used in cell culture experiments, the inhibitory effects of 4sU
299 incorporation on pre-mRNA splicing are minimal, affecting only introns that are already weakly
300 recognized by the spliceosome. Collectively, this data demonstrates that 4sU is not completely
301 inert when employed as an experimental tool.

302

303 Interestingly, at high levels of 4sU incorporation transcription by T7 RNA polymerase
304 resulted in increased levels of abortive transcripts. This was particularly striking for the ADML
305 minigene (Fig 3A). To identify potential causes for the marked transcription interference we
306 evaluated the RNA sequence of each transcript. The ADML minigene encodes a run of eight
307 consecutive uridines towards the 3' end of the intron. Thus, it is possible that when the
308 polymerase encounters a run of consecutive uridines, the repeated incorporation of 4sU could
309 interfere with polymerase processivity, resulting in increased abortive transcripts. The expected
310 size of the ADML abortive transcript is consistent with this possible explanation. The longest
311 stretch of consecutive uridines encoded by the β-globin minigene is only six nucleotides long.

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312 These observations suggest that longer runs of consecutive uridine induce abortive transcription
313 when the RNA is labeled exclusively with 4sU. The potential implications of this transcription
314 interference are straightforward. Incomplete pre-mRNA strands may not be stable, they may not
315 be fully transcribed, and if they are, they could not have proper function.
316
317 The results of our experiments demonstrate that 4sU incorporation can affect pre-mRNA
318 splicing. To what degree do these observations imply that the use of 4sU in metabolic labeling
319 experiments should be viewed critically? We suggest considering the following aspects when
320 designing metabolic labeling experiments. First, the concentration of 4sU used in the labeling
321 approach. Second, the length of metabolic labeling, and third, the splice potential of the exons
322 and introns investigated.
323
324 To evaluate the influence of 4sU labeling on pre-mRNA splicing we designed experiments
325 that directly measure altered splicing outcomes using *in vitro* splicing assays. These
326 investigations were complemented with 4sU labeling approaches of cell cultures, followed by
327 mRNA analysis. Differences in intron excision, exon skipping, or alternative splice site selection
328 rates in cell culture experiments can certainly be triggered by direct splicing effects, however,
329 they can also be representative of altered mRNA stabilities and/or transcription outputs. With
330 these interpretation limitations in mind, our data provide clear guidance when designing and
331 interpreting splicing analyses using 4sU labeling. Increased levels of 4sU incorporation can lead
332 to measurable defects in pre-mRNA splicing, preferentially for weaker splicing events. This is
333 demonstrated in our *in vitro* splicing assays when pre-mRNAs of different incorporation levels
334 were evaluated. However, our data also demonstrate that for the minigenes used, 4sU
335 incorporation levels >30% need to be achieved to interfere with splicing efficiency. Such
336 elevated levels of 4sU incorporation cannot be reached when performing cell culture metabolic

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337 labeling experiments. The length of 4sU incubation time is also an important factor when
338 carrying out cell culture metabolic labeling. This is because longer incubation increases the
339 fraction of mRNAs labeled with 4sU, allowing the establishment of steady-state levels of 4sU
340 mRNAs. An important consideration when analyzing 4sU labeling experiments is the identity of
341 the exon or intron evaluated. By definition, constitutive exons are always included in the final
342 mRNA while alternative exons are not. As such, perturbations in recognizing exons, such as the
343 presence of 4sU within the transcript, could have varying influences on alternative and
344 constitutive exon inclusion levels. We observed that constitutive splicing is not affected by the
345 presence of 4sU at the 40 μM concentration used. Most of the alternatively spliced exons
346 evaluated were also not significantly affected by 4sU incorporation. Only a limited number of
347 splicing events appeared to indicate altered splice site selection or reduced intron excision.
348 Furthermore, the magnitudes of the observed splice changes were small, suggesting that while
349 statistically significant, biological consequences are expected to be minimal. In summary, 4sU
350 metabolic labeling of cell cultures has the potential to induce altered splicing. While infrequent,
351 at small magnitudes, and only in the context of inefficiently spliced events, appropriate control
352 experiments need to be included to deconvolute splicing differences of tested experimental
353 conditions from those elicited by 4sU labeling.
354
355 It is known that naturally occurring 4-thiouridine modifications increase the stability of some
356 organismal tRNAs[20] and it has been suggested that the incorporation of 4sU changes the
357 structure of the transcribed RNA. The potential to form secondary structures has been shown to
358 greatly impact exon recognition by the spliceosome [21], so it is entirely possible that 4sU
359 incorporation into RNAs interferes with efficient exon recognition through inducing altered RNA
360 structures.
361

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362 The results presented here are not damning evidence against the use of 4sU, rather
363 they are akin to a cautionary statement. 4sU does not broadly impact all aspects of pre-mRNA
364 generation and processing, but it does impact those events that are more easily susceptible to
365 change. If 4sU is to be used in an experiment, it is important to be mindful of the pre-mRNA
366 splicing events that are being analyzed because a small number of event changes may be
367 caused by the incorporation of 4sU itself.
368

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370

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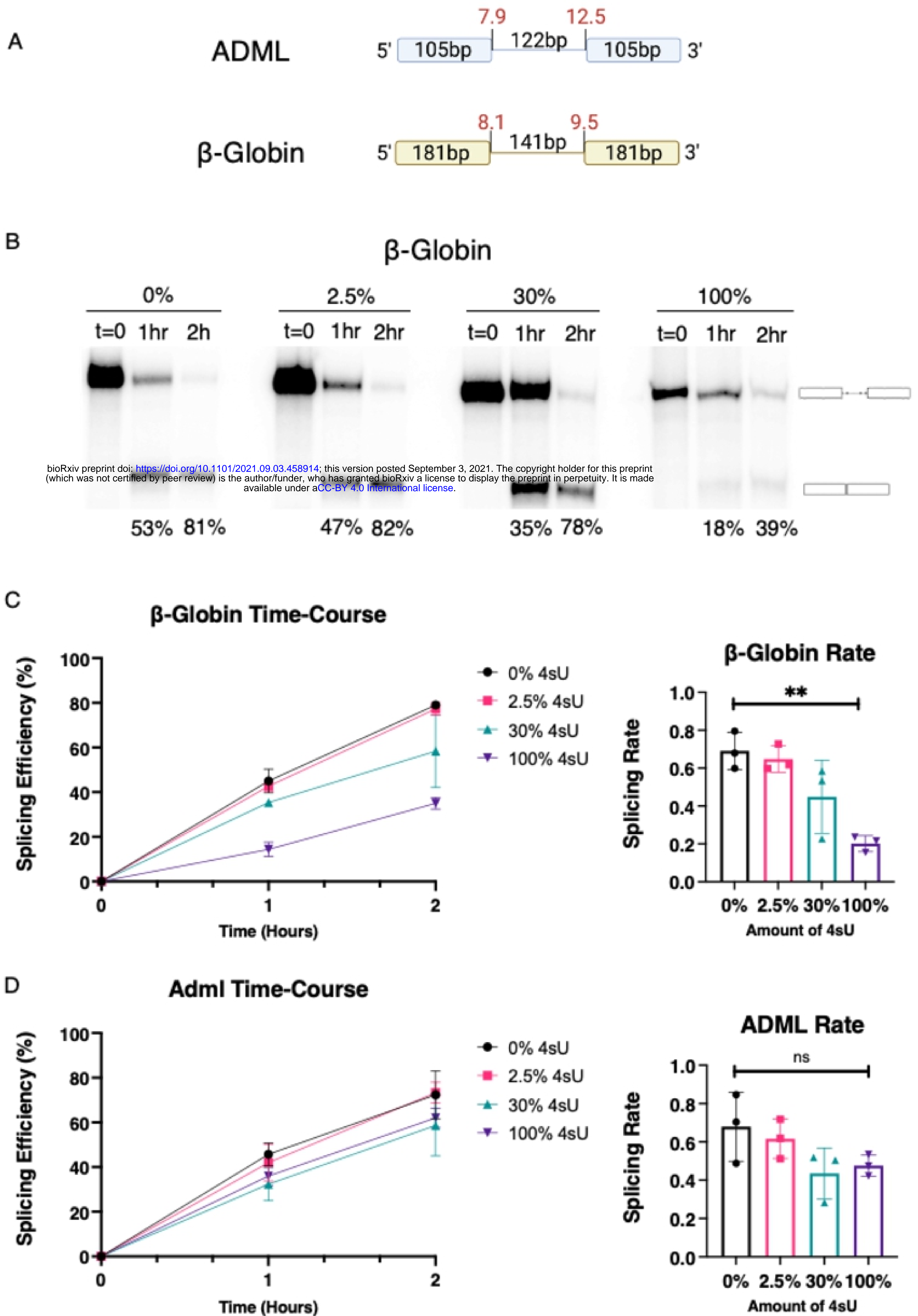

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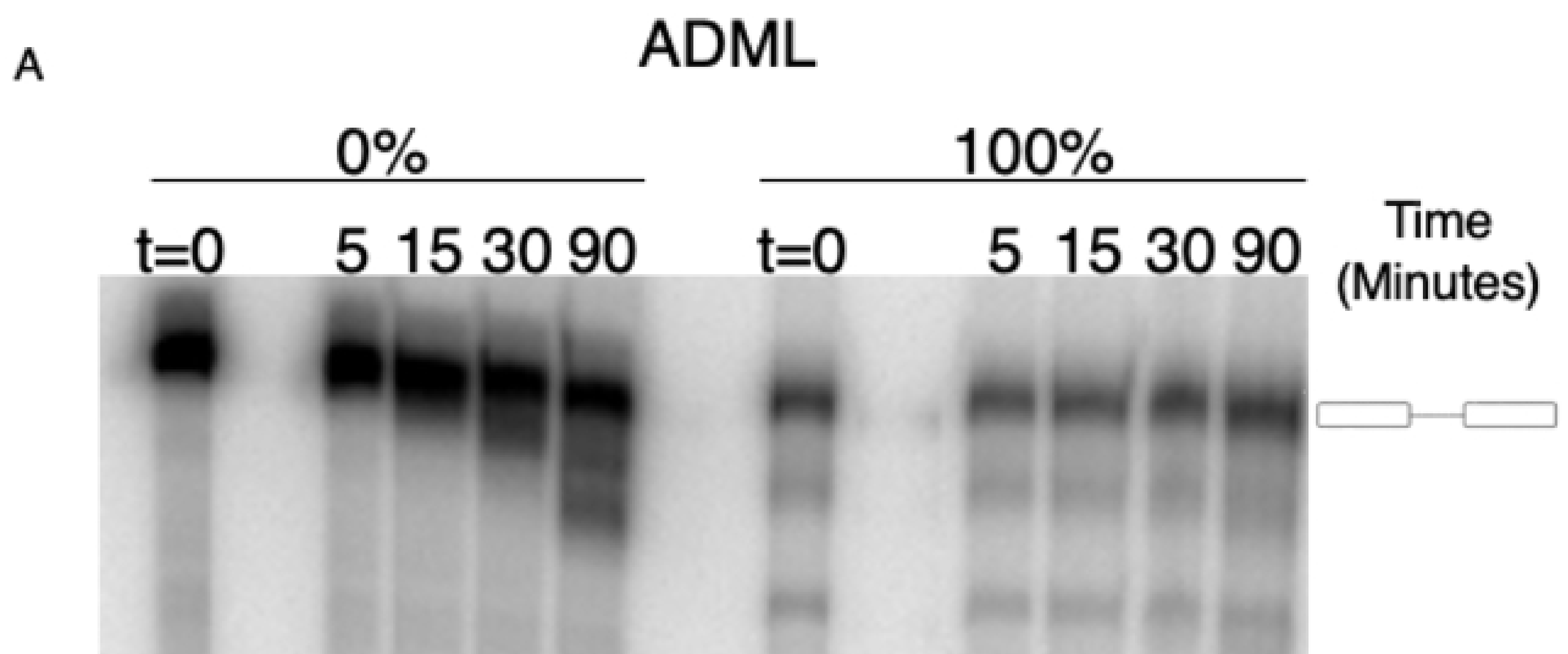
Figure 1



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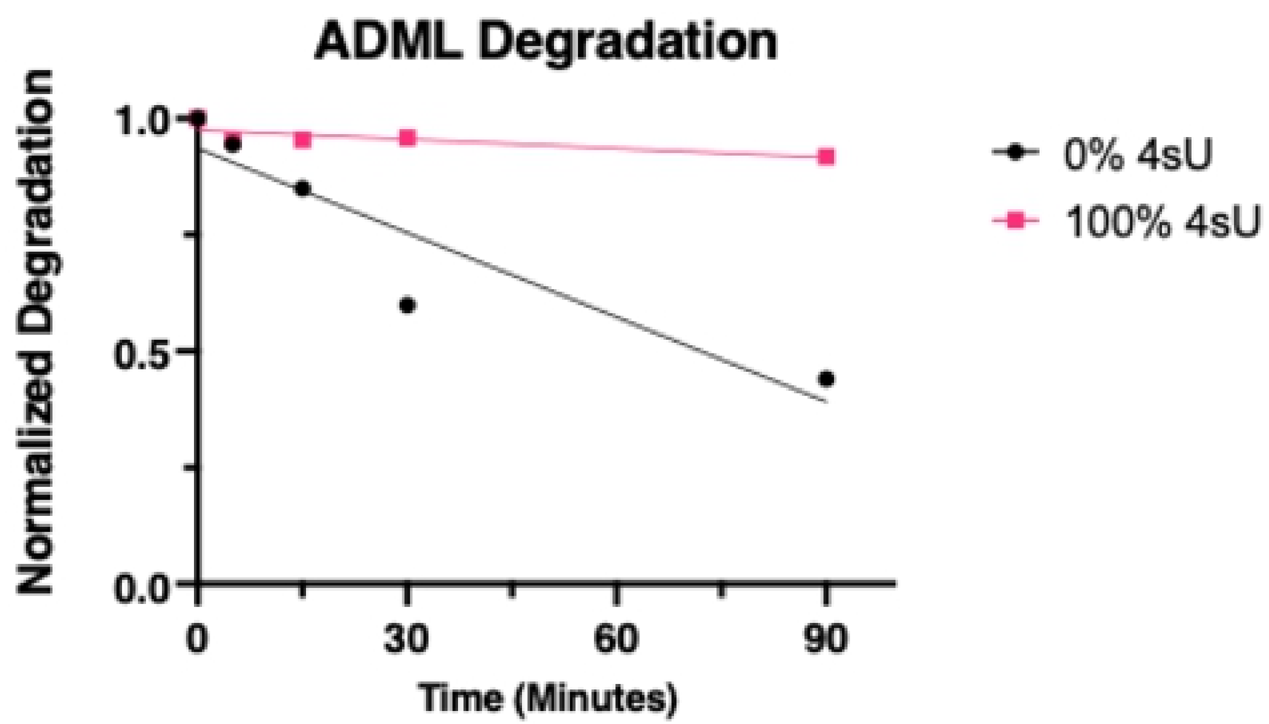
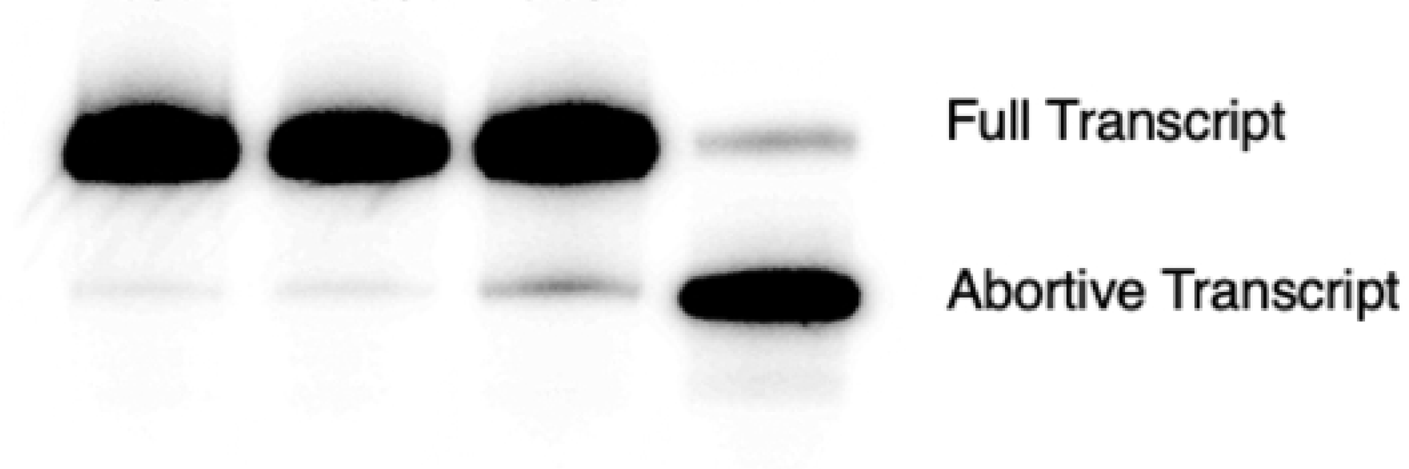


Figure 2

A



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B

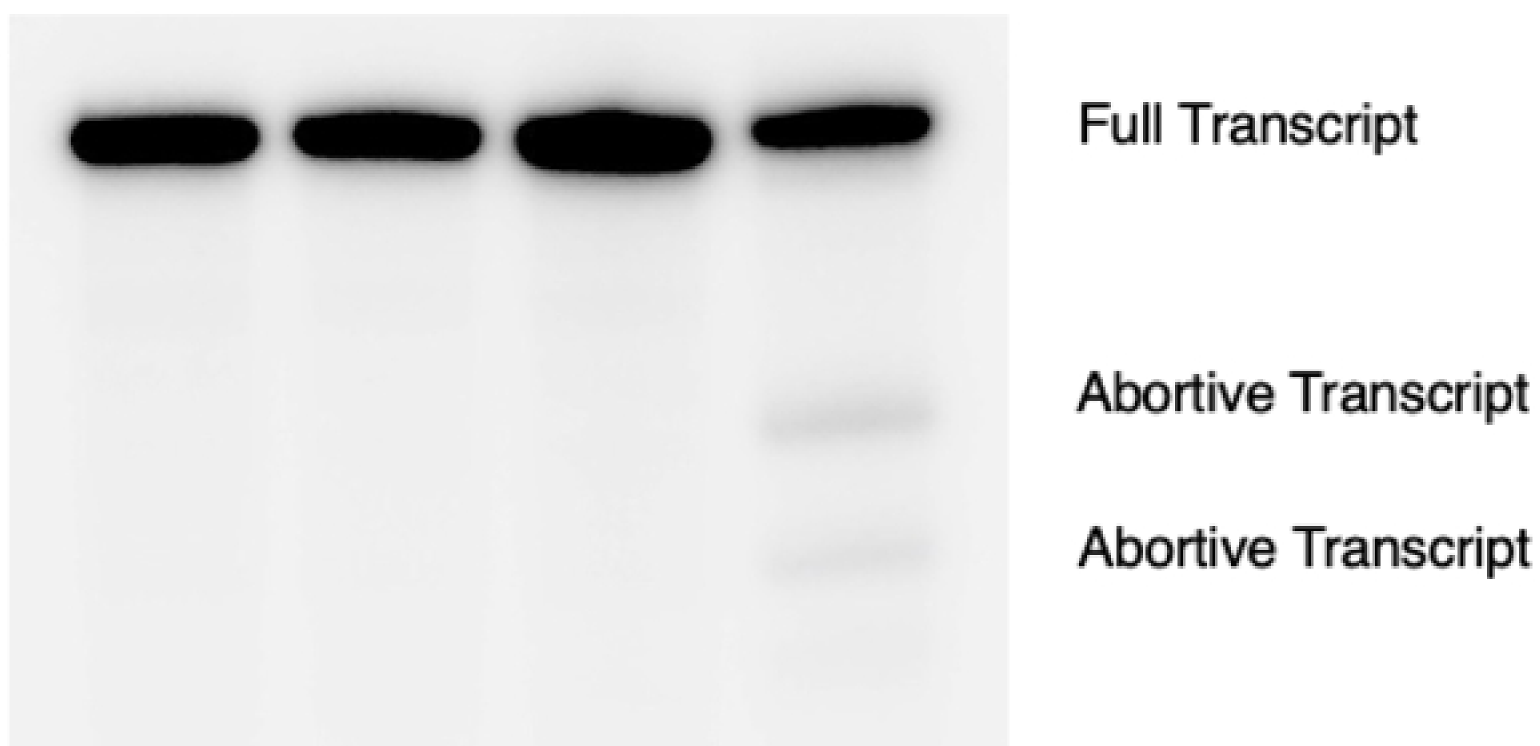

Figure 3

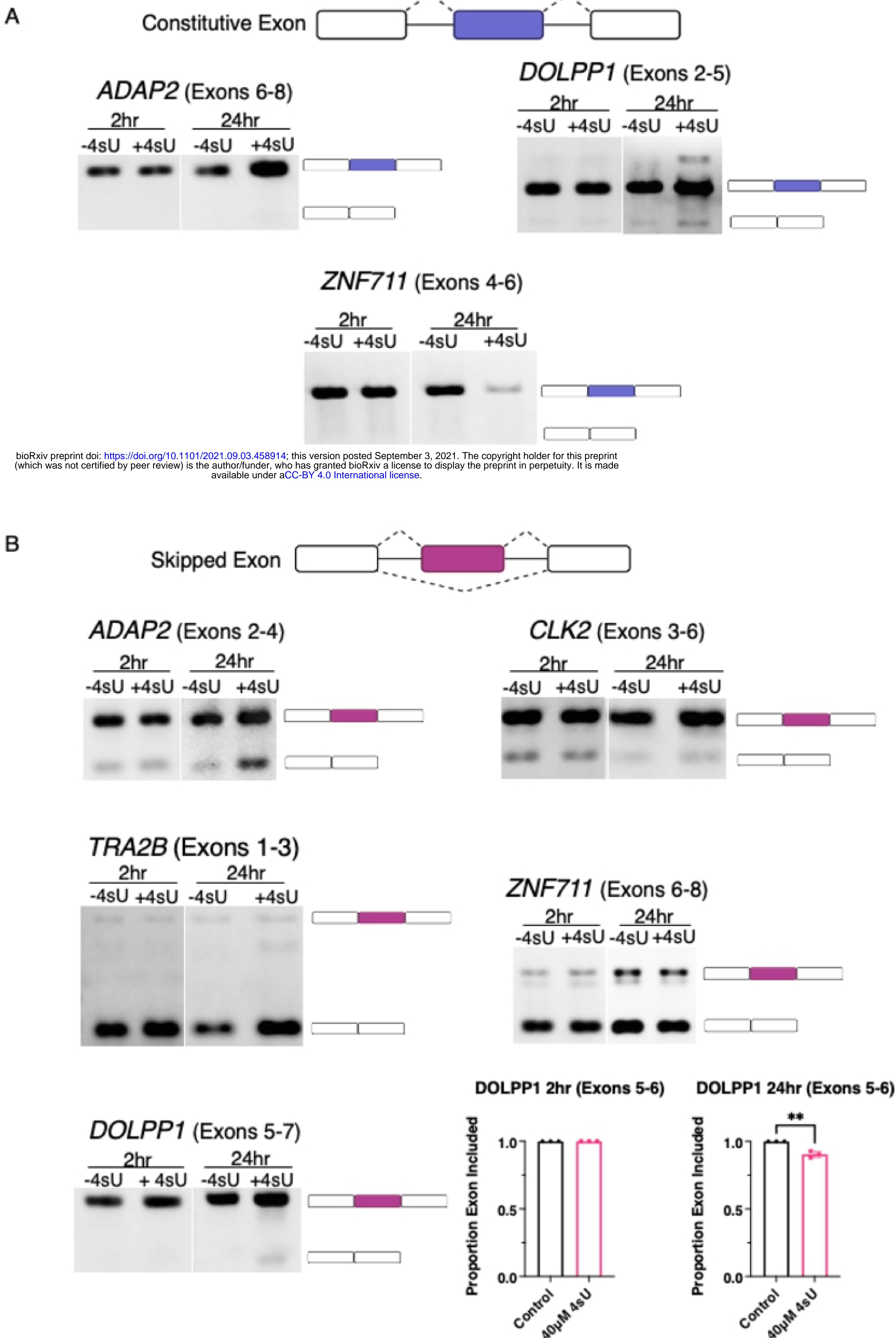

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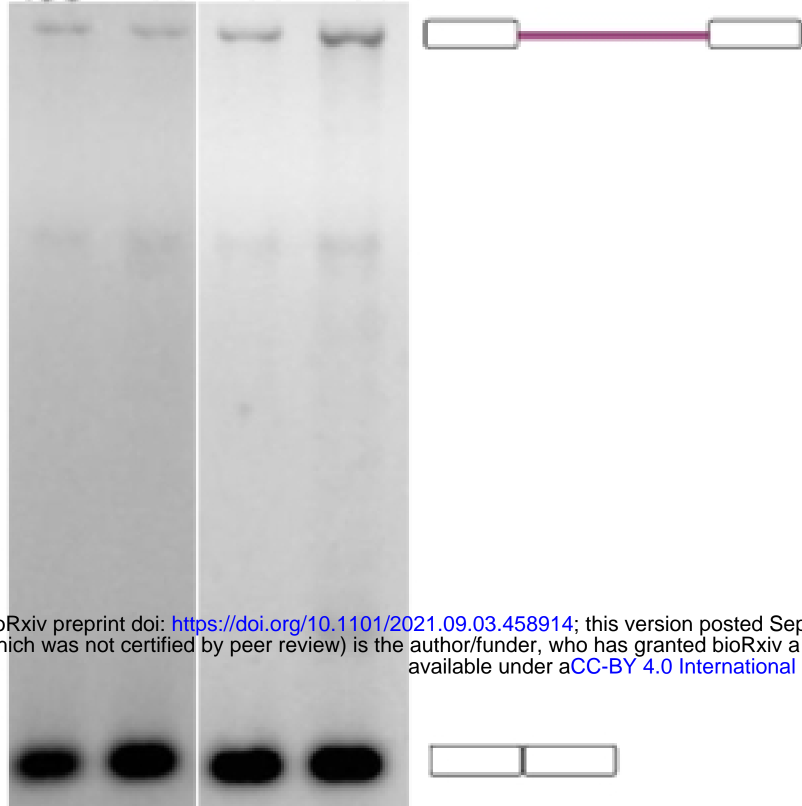
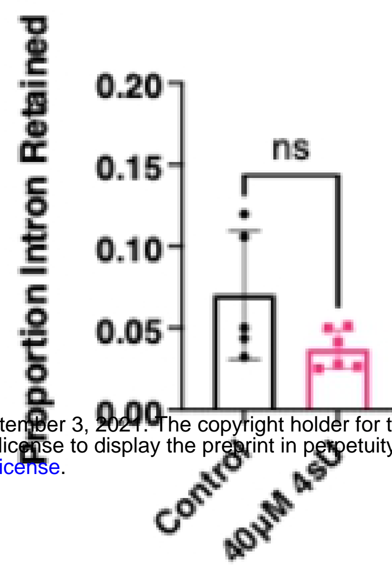
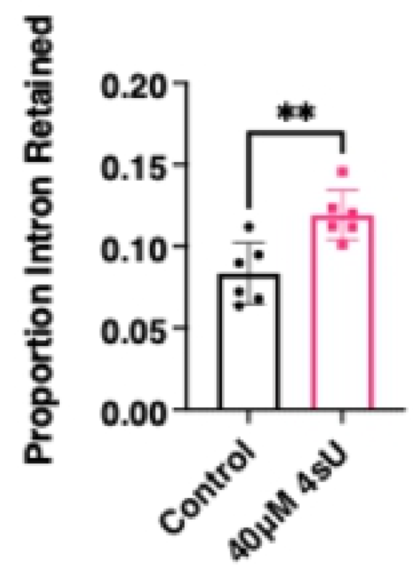
Figure 4







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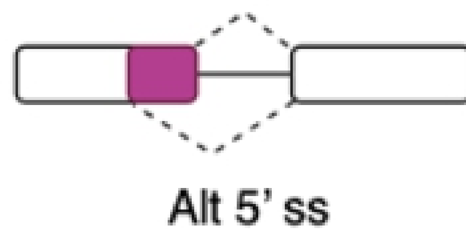

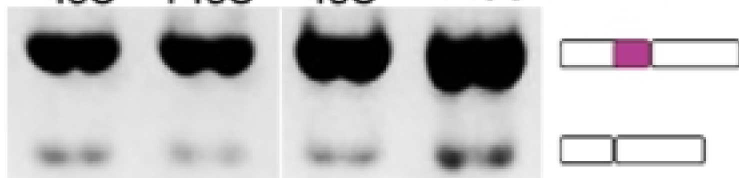

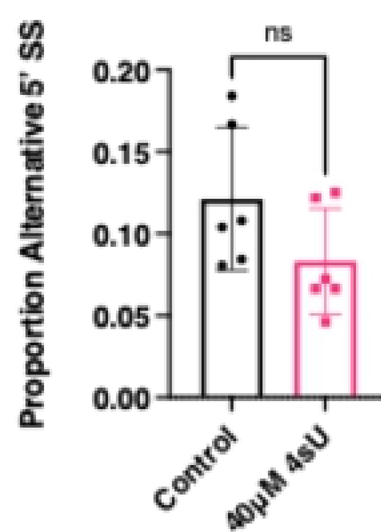

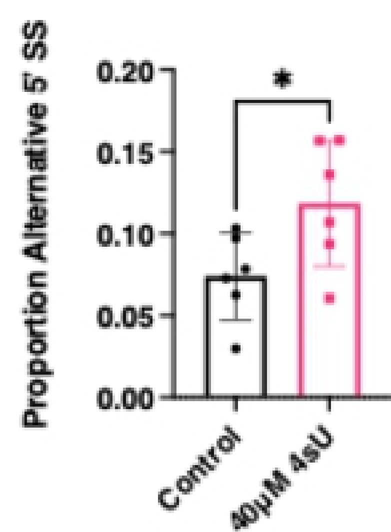

Figure 5