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6 Interaction between the assembly of the ribosomal subunits: Disruption of 7 40S ribosomal assembly causes accumulation of extra-ribosomal 60S 8 ribosomal protein uL18/L5

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14 Running title: Effect of 40S assembly on extra-ribosomal uL18/L5

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24 # Abstract

25 Inhibition of the synthesis of a ribosomal protein (r-protein) abrogates the assembly of
26 its cognate subunit, while assembly of the other subunit continues. Ribosomal
27 components that are not stably incorporated into ribosomal particles due to the
28 disrupted assembly are rapidly degraded. The 60S protein uL18/L5 is an exception,
29 because this protein accumulates extra-ribosomally during inhibition of 60S assembly.
30 Since the r-proteins in each ribosomal subunit are essential only for formation of their
31 own subunit, it would be predicted that accumulation of extra-ribosomal uL18/5 only
32 occurs during restriction of 60S assembly, and not during abolition of 40S assembly.
33 Contrary to this prediction, we report here that repression of 40S r-protein genes does in
34 fact lead to accumulation of uL18/L5 does outside the ribosome due modified 60S
35 assembly. Furthermore, the effect varies depending on which 40S ribosomal protein is
36 repressed. We propose that disruption of early steps in the 40S subunit assembly
37 changes the kinetics of 60S subunit assembly resulting in a buildup of extra-ribosomal
38 uL18/L5, even though 60S formation continues. Finally, our results show that
39 maintenance of the pool of extra-ribosomal uL18/L5 requires continual protein synthesis
40 showing that extra-ribosomal protein is not stable, but is slowly “consumed” by
41 incorporation into 60S subunits and/or turnover.

42

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43 # Introduction

44 The ribosome biogenesis process is preserved throughout eukaryotic evolution,
45 although the complexity has evolved from yeast to humans [1, 2]. It begins with RNA
46 polymerase I transcription of a long 18S-5.8S-25S/28S precursor rRNA and RNA
47 polymerase III transcription of precursor 5S rRNA [3-6]. The precursor transcripts are
48 processed into the mature rRNA components concurrently with the incorporation of
49 ribosomal proteins (r-proteins) into the emerging ribosomal subunits. Ribosomal
50 proteins are translated in the cytoplasm and chaperoned into the nucle(o)lus where
51 most of the ribosome formation takes place. Besides the synthesis of the components of
52 the mature ribosomes, the construction of ribosomes also requires in excess of 250
53 ribosomal assembly factors, a number of which are important for the assembly of both
54 the 40S and the 60S ribosomal subunits, while others are specific to the formation of
55 one the ribosomal subunits [7].

56 Assembly of each subunit requires production of a full set of the r-proteins found
57 in the mature subunit. Significant reduction of the production of just one r-protein or
58 assembly factor prevents completion of the assembly process. If the perturbation is
59 limited to protein(s) required for only one or the other subunit, only the assembly of the
60 cognate subunit is abolished, while the assembly of the other subunit continues (e.g. [8,
61 9]). The abolishment of the assembly of a ribosomal subunit does not stop the synthesis
62 of its r-proteins, but proteins that fail to become incorporated into stable ribosomal
63 particles are rapidly eliminated by proteasomal turnover [8, 10, 11].

64 Nevertheless, one 60S protein, uL18, evades rapid degradation and accumulates
65 in a complex with 5S rRNA outside of the ribosome during repression of the gene for the

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66 60S r-protein uL5 [12], previously named L11 and L16 [13, 14]. Furthermore, distortion
67 of cell fate in metazoans has been attributed to r-protein mediated regulation of factors
68 controlling the growth [15, 16]. It is therefore important to understand the mechanisms
69 for the build-up of extra-ribosomal r-protein pools. Since the r-proteins in each ribosomal
70 subunit are essential only for the assembly of their cognate subunit, it would be
71 expected that interruption of the assembly of one subunit only affects accumulation of
72 extra-ribosomal r-proteins for that subunit. We tested this expectation by repressing
73 several 40S r-protein genes and measuring the buildup of extra-ribosomal r-proteins.
74 Surprisingly, and in contrast to the prediction, extra-ribosomal uL18 accumulates when
75 the synthesis of either 40S or 60S r-proteins is constrained. Moreover, the amount of
76 extra-ribosomal uL18 accumulating depends on which 40S r-protein gene is repressed.
77 We interpret these results to mean that disruption of the assembly of the 40S subunit
78 can affect the kinetics of assembly of the 60S subunit. Furthermore, we show that
79 buildup of extra-ribosomal uL18 does not require formation of the complex of uL18, uL5,
80 5S rRNA and the Rrs1 and Rpf2 assembly factors, which is an intermediate in the
81 normal 60S subunit assembly.
82

83 Materials and methods

84 Nomenclature for r-proteins.

85 We use the 2014 universal nomenclature [13]. In the figures, the classic protein names
86 are also indicated after a slash.
87

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88 ## Strains and growth conditions.

89 All strains are derived from BY4741. In each strain one r-protein gene encoding eS4,
90 eS6, uS17, eS19, eS31, eL40, or eL43, was expressed exclusively from the *Gal1/10*
91 promoter. These strains are referred to as P_{gal}-xx where xx is the protein expressed
92 from the galactose promoter (Table S1). In the experiment in Fig 1B described below,
93 P_{gal}-eL43 was transformed with a second plasmid carrying a gene for uL18-FLAG
94 expressed from the constitutive RpS28 promoter (Philipp Milkereit, personal
95 communication).

96 Cells were grown at 30°C with shaking in YEP-galactose media. At OD_{600} of 1.0
97 (about $2x10^7$ cells per ml), the culture was shifted to YPD (glucose) media for 6-21
98 hours. All strains had a doubling time of 1.5-2.0 hours in galactose. After the shift,
99 growth of the P_{gal}-xx strains gradually decreased [9]. Cells were harvested at 8000 rpm
100 for 10 minutes and washed once with 10 mL ice cold RNase free water and stored at -
101 20°C until further use. Procedures for lysis and sucrose gradient centrifugation were
102 described previously [9].

103

104 ## Western analysis and antisera.

105 Western blots [9] were probed with rabbit polyclonal antisera prepared for our laboratory
106 by Covance (Princeton, New Jersey, USA) using synthetic peptides with the sequence
107 of 20–22 N-terminal amino acids of uS4, uL4, uL5, and uL18 as antigens. Monoclonal
108 anti-FLAG antibody was purchased from Thermo-Fisher (catalogue number MA1-
109 91878).

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110 As described in Results, western blots probed anti-uL18 revealed a band co-
111 migrating in electrophoresis with the ribosomal uL18 band in fractions close to the top of
112 sucrose gradients loaded with extracts of specific strains shifted to glucose medium. To
113 determine if this band indeed represents uL18 we analyzed P_{gal}-uL18 13.5 hours after
114 shifting the culture to glucose medium. In this experiment, we saw no band co-migrating
115 with the ribosomal uL18 band at the top of the gradient, consistent with the fact the
116 uL18 synthesis was abolished and confirming that the band at the top of the gradient,
117 which co-migrates in gel electrophoresis and is stained with our anti-uL18, in fact is
118 uL18 (Fig 1A). However, our anti-uL18 also reacted with a second band at the top of the
119 sucrose gradients (marked with asterisk in all figures). To determine if this band is
120 related to uL18, we transformed P_{gal}-eL43 with a second plasmid constitutively
121 expressing FLAG-tagged uL18 (see above) in addition to the native uL18 chromosomal
122 gene. After the shift from galactose to glucose medium, a single band of FLAG-tagged
123 uL18, comigrating with the FLAG-uL18 band in the ribosomal fractions, appeared at the
124 top of the gradient (Fig 1B), but no band corresponding asterisked band in blots stained
125 with anti-uL18 was seen. Furthermore, the asterisked band was present after repressing
126 uL18 synthesis in Pgal-uL18 (Fig 1A). Based on the experiments in Fig 1, we conclude
127 that the band marked with asterisk in the blots stained with anti-uL18 is not related to
128 uL18, but must represent an unspecific protein that cross-reacts with our uL18
129 antiserum. This is supported by the presence of the asterisked band after shifting the
130 parent strain to glucose medium (Fig 2C).
131
132 **Fig 1**. Analysis of the specificity of anti-uL18/L5. (A) P_{gal}-uL18/L5 was grown in

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133 galactose medium and shifted to glucose medium. A lysate prepared after repression of
134 uL18/L5 gene for 13.5 hours was fractionated on a sucrose gradient. Fractions from the
135 top of the gradient and the 60S-80S ribosome peaks were analyzed by western blot
136 stained with anti-uL18/L5. (B) P_{gal}-eL43/L43 was transformed with a plasmid harboring a
137 constitutively expressed gene for uL18/L5-FLAG. The culture was grown in galactose
138 medium and shifted to glucose medium for 16 hours. Lysates prepared from cells before
139 and after repressing uL43/L43 synthesis were fractionated on sucrose gradient and
140 aliquots of fractions from the top of the gradient and the 60S-80S peaks were analyzed
141 for content of FLAG-tagged protein by western blot.
142

143 Statistics.

144 Pairwise t-test was used.
145

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146 # Results

147 ## Disruption of ribosome assembly

148 To specifically abolish assembly of 60S or 40S subunits we repressed individual r-
149 proteins genes cognate to one or the other ribosomal subunit. This was accomplished
150 by using yeast strains in which the only gene for a given r-protein is transcribed from the
151 *Gal1/10* promoter. We refer to these strains as P_{gal}-xx, where xx is the name of the
152 protein encoded by the gene under galactose control. In galactose medium a full set of
153 r-proteins is synthesized, but shifting the cells to glucose medium abrogates synthesis
154 of r-protein xx, which prevents assembly of the cognate ribosomal subunit.
155

156 ## Extra-ribosomal uL18 accumulates during repression of
157 ## some 40S r-protein genes

158 To measure extra-ribosomal accumulation of uL18 and several other r-proteins upon
159 repression of specific r-protein genes, we fractionated crude cell extracts on sucrose
160 gradients and analyzed the sucrose gradient fractions on western blots probed with
161 antisera specific to the 60S r-proteins uL18, uL5, uL4 and the 40S r-protein uS4. Fig 2A
162 shows a western blot stained with anti-uL18 of fractions from across a sucrose gradient
163 loaded with an extract of P_{gal}-eL43 prepared 6 hours after the shift to glucose medium.
164 A band co-migrating with the ribosomal uL18 band was observed close to the top of the
165 sucrose gradient. A second protein marked with an asterisk and moving slightly slower
166 also appeared. As described in Materials and Methods we confirmed that band that
167 comigrates with the ribosomal uL18 band indeed represents uL18, while the slightly

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168 slower moving band is not related to uL18 (Fig 1). In an experiment with P_{gal}-eL43
169 expressing FLAG-tagged uL18 a band comigrating with ribosomal FLAG-uL18 was also
170 seen at the top of the sucrose gradient after the shift from galactose to glucose medium,
171 but not before the shift (Fig 1A). Furthermore, no uL18 band was seen at the top of the
172 gradient after shifting the parent strain to glucose medium (Fig 2B). Thus, the
173 experiment in Fig 2A shows that uL18 accumulates outside ribosomal particles during
174 repression of uL43 synthesis. This was anticipated, since repression of the 60S r-
175 protein uL5 is known to provoke a buildup of extra-ribosomal uL18 [12].
176
177 **Fig 2**. Repression of several 40S r-protein genes causes accumulation of extra-
178 ribosomal uL18/L5, but not extra-ribosomal uL5/L11, uL4/L4, or uS4/S9. Whole cell
179 lysates of glucose cultures of P_{gal}-eL43/L43, P_{gal}-eS4/S4, and the parent strain BY4741
180 were fractionated on sucrose gradients and the indicated fractions were analyzed by
181 western blots probed with anti-sera for r-proteins uL18/L5, uL4/L4, eS4/S4, and uL5/L11
182 as indicated at each blot. (A) Pgal-eL43/L43 after 6 hours in glucose medium. (B) The
183 parent strain (BY4741) after 0 and 16 hours in glucose. Image (i) shows a blot probed
184 with a mixture of antisera for uL4/L4 and uS4/S9. Panel (ii) shows the same blot after it
185 was probed further with a mixture of antisera for uL18/L5 and uL5/L11. The bands
186 marked with a blue asterisk in some panels are not related to uL18/L5 (see Material and
187 Methods). (C) Northern blots of fractions from a sucrose gradient loaded with P_{gal}-
188 eS4/S4 after 8 hours in glucose medium were probed with anti-sera for uS4/S9, uL4/L4,
189 uL5/L11, and uL18/L5.
190

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191 We next asked if abolishment of 40S r-protein genes also triggered extra-
192 ribosomal uL18 accumulation, we analyzed extracts of P_{gal}-eS4 that had been shifted
193 from galactose to glucose medium for 8 hours. Unexpectedly, we found a build-up of
194 extra-ribosomal uL18 at the top of the sucrose gradient. In contrast, no uL4, uL5 or uS4
195 was found outside in the ribosome peaks (Fig 2C). Additionally, the parent strain
196 BY4741 did not accumulate extra-ribosomal r-proteins whether grown in galactose or
197 glucose, as expected since assembly of both subunits proceeds uninterrupted in the
198 parent whether it grows glucose and galactose medium (Fig 2B). Overall the results in
199 Fig 2 shows that disruption of 40S assembly can generate a pool of extra-ribosomal
200 uL18. Extra-ribosomal accumulation of uL18 is thus not specific to interference with 60S
201 assembly.

202 We then tested if repression of other 40S r-protein genes also cause formation of
203 extra-ribosomal uL18. Sucrose gradients were loaded with whole-cell extracts after
204 repressing the genes for uS4, eS6, uS17, eS19, or eS31. Extracts prepared after
205 repression of the 60S genes for eL40 or eL43 were used as controls. In all cases, we
206 found uL18 bands at the top of the gradient (Fig 3A), although the strength of the bands
207 varied. Quantification of uL18 in the top and the ribosome fractions showed that the
208 fraction of total uL18 found outside ribosomal subunits varied by several fold with the
209 protein whose synthesis was abolished (Fig 3B). Repression of eS4 synthesis
210 generated as much extra-ribosomal uL18 as did the repression of the two 60S r-protein
211 genes. In contrast, the fraction of uL18 found in the extra-ribosomal fractions after
212 abolishing eS31 synthesis was borderline visible. The extra-ribosomal uL18 in strains
213 repressed for other 40S proteins was at intermediate levels.

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214

215 **Fig 3**. Quantification of extra-ribosomal uL18/L5 pool after repression of several 40S

216 and 60S r-protein genes. P_{gal}-eS4/S4, -eS6/S6, -uS17/S11, -eS19/S19, -eS31/S31, -

217 eL40/L40, and -eL43/L43 were grown in galactose medium and shifted to glucose

218 medium. (A) Sucrose gradients were loaded with lysates prepared after the shift and

219 fractions from the top of the gradient and from the 60S-80S ribosomal peaks were

220 analyzed on western blots probed with anti-uL18/L5. The proteins whose synthesis is

221 repressed by the shift to glucose medium is indicated to the left of the western blots. (B)

222 Quantification of uL18/L5 at the top of the sucrose gradient. The western blots in panel

223 (A) were quantified using ImageJ and the fraction of the total uL18/L5 (sum of uL18/L5

224 in both top and ribosome fractions) present at the top of the gradient is shown. The

225 average of three biological repeats is shown for repression eS4/S4, uS17/S11,

226 eS31/S31, eL43/L43 genes and the average of two biological replicates is shown for the

227 repression of the eS6/S6, eS19/S19, eL40/L40 genes. The error bars indicate standard

228 error of the mean. The data for each gene repression experiment was compared to the

229 results from the parent strain (BY4741) harvested after a shift to glucose medium by

230 pairwise t-test. +++ indicates $p<0.001$, ++ $p<0.005$, + $p<0.01$ (C) The bands marked

231 with a blue asterisk in some panels are not related to uL18/L5 (see Materials and

232 Methods).

233

234 The differences among strains in the extra-ribosomal uL18 pool size led us to

235 question whether more extra-ribosomal uL18 would accumulate with longer times of

236 40S r-protein gene repression. We used the Pgal-eS31 strain to address this question,

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237 because the accumulation of extra-ribosomal uL18 was barely visible in this strain.
238 However, comparing lysates prepared after incubation in glucose medium for 8, 9.5 or
239 17 hours did not reveal an increase in extra-ribosomal uL18 with time, making it unlikely
240 that the amount of extra-ribosomal uL18 changes with time (Fig 3C). Rather, the results
241 suggest that the pool of extra-ribosomal uL18 reaches a steady-state level. Together
242 the results in Fig 3 show that the size of the pool of extra-ribosomal uL18 during
243 disruption of 40S assembly varies with the 40S gene repressed.
244

245 uL18 accumulates due to interference with subunit assembly, 246 not degradation of mature subunits

247 We have previously shown that the mature 40S and 60S ribosomal subunits depend on
248 each other for stability and accumulation [9]. Thus, there are two possible principle
249 sources of extra-ribosomal L18: modification of 60S assembly and breakdown of mature
250 60S subunits. To distinguish these possibilities, we investigated if blocking protein
251 synthesis with cycloheximide changed the amount of extra-ribosomal uL18. If the extra-
252 ribosomal uL18 stems from degradation of preexisting ribosomes, cycloheximide should
253 not affect the pool of extra-ribosomal uL18, but if the extra-ribosomal uL18 depends on
254 continual protein synthesis, addition of cycloheximide should reduce the pool of extra-
255 ribosomal uL18. Accordingly, we grew P_{gal}- eL43 in galactose and shifted it to glucose
256 medium for 6 hours. At this time approximately 50% of the total uL18 was found at the
257 top of the gradient (Fig 4A(i)). The culture was then split and cycloheximide (100 µg/ml)
258 was added to one aliquot, while nothing was added to the other part. After 4 hours of
259 additional culturing, both aliquots were harvested and analyzed for extra-ribosomal

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260 uL18. No uL18 band was seen at the top of the gradient after cycloheximide inhibition of
261 protein synthesis (Fig 4A(ii)), while the level of extra-ribosomal uL18 was unchanged in
262 the sample without the drug (Fig 4A(iii)).
263
264 **Fig 4**. Mechanism of accumulation of extra-ribosomal uL18/L5. Cultures were grown in
265 galactose medium and shifted to glucose medium. (A) Cycloheximide decreases the
266 pool of extra-ribosomal uL18/L5 during repression of a 60S r-protein gene. Six hours
267 after the shift of P_{gal}-eL43/L43 from galactose to glucose medium, cycloheximide was
268 added to an aliquot of the culture (final concentration100 µg/ml), while incubation of
269 another aliquot was continued without the drug. Both aliquots were harvested at 10
270 hours after the shift from galactose to glucose. Whole cell extracts were analyzed by
271 sucrose gradients and western blots. (i) P_{gal}-eL43/L43 6 hours after the shift of media,
272 (ii) Pgal-eL43/L43 incubated with cycloheximide added 6 hours after the media shift and
273 harvested 10 hours after the shift. (iii) P_{gal}-eL43/L43 incubated for 10 hours in glucose
274 without cycloheximide. (B) Cycloheximide also decreases the pool of extra-ribosomal
275 uL18/L5 during inhibition of 40S assembly. P_{gal}-eS4/S4 was grown in galactose medium
276 and shifted to glucose medium. After 4 hours in glucose medium, cycloheximide (100
277 µg/ml) was added and cells were harvested 0, 15 and 45 minutes after the inhibition of
278 protein synthesis. (C) Inhibition of TOR and repression of rRNA synthesis does not
279 result in accumulation of extra-ribosomal uL18/L5. Rapamycin was added to Pgal-
280 eL43/L43 growing in **galactose** medium (**no** shift to glucose) and 4 hours later an
281 extract was analyzed by sucrose gradient centrifugation and western blots developed
282 with antisera specific to uL18/L5 and uL4/L4. (D) Repression the genes for the 60S

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283 ribosomal assembly factors Rrs1 and Rpf2 results in accumulation of extra-ribosomal
284 uL18/L5. P_{gal}-Rrs1 and -Rpf2 were grown in galactose medium and shifted to glucose
285 medium for 16 hours. Whole cell extracts were analyzed by sucrose gradient
286 centrifugation and western blots developed with antiserum specific to uL18/L5. The
287 bands marked with a blue asterisk is not related to uL18/L5; see Material and Methods.
288
289 Inhibition of protein synthesis in P_{gal}-eS4 gave a similar result. Cycloheximide
290 was administrated for 15 and 45 minutes to a culture four hours after the shift from
291 galactose to glucose. While no change was seen after 15 minutes, the extra-ribosomal
292 uL18 level was reduced by about 50% after 45 minutes with cycloheximide (Fig 4B).
293 Together the experiments in Fig 4A-B show that the extra-ribosomal uL18 is depleted, if
294 it is not replenished by new synthesis, indicating that extra-ribosomal uL18 is generated
295 during 60S assembly rather than degradation of mature 60S subunits.
296 To investigate if extra-ribosomal uL18 also accumulates when rRNA synthesis th
297 is inhibited by the TOR-targeting drug rapamycin, we grew Pgal-eL43 in **galactose**
298 medium (i.e. eL43 synthesis is **not** interrupted) and added rapamycin for 4 hours at a
299 final concentration of 0.2 µg/ml. Fig 4C shows that no uL18 is seen at the top of the
300 gradient after blocking rRNA transcription for 6 hours. This is consistent with the
301 conclusion that the extra-ribosomal uL18 cannot come from degradation of mature 60S
302 subunits, but requires continual synthesis of ribosomal components and failing
303 assembly of ribosomal particles.
304 We further investigated the origin of extra-ribosomal r-proteins by depleting each
305 of the ribosomal assembly factors Rrs1 and Rpf2 that combine with uL18, uL5, and 5S

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306 rRNA prior to docking in the precursor 60S particle [17]. Fig 4D shows that depleting
307 either Rrs1 or Rpf2 increased the pool of extra-ribosomal uL18 in agreement with the
308 effect of mutating the *RRS1* gene [18]. This further supports our conclusion that build-up
309 of extra-ribosomal uL18 is caused by inhibition of ribosomal assembly rather than
310 degradation of mature ribosomes. Furthermore, we conclude that accumulation of extra-
311 ribosomal uL18 does not require formation of the complete uL18-uL5-5S rRNA-Rrs1-
312 Rpf2 particle, since depletion of the assembly factors does not prevent the formation of
313 an extra-ribosomal uL18 pool. This is also supported by the fact extra-ribosomal uL5
314 does not accumulate proportionally to uL18 during abrogation of eS4 synthesis (Fig 2B).
315

316 # Discussion

317 We have shown that the 60S r-protein uL18 accumulates extra-ribosomally during
318 repression of several 40S r-protein genes (Figs 2 and 3). Although it was known that
319 uL18 evades rapid degradation and accumulates outside of the ribosome during
320 abrogation of 60S assembly [12], it was unexpected that extra-ribosomal uL18 also
321 builds up during inhibition of 40S assembly, because
322 repression of an r-protein gene specifically halts the assembly of their own subunit,
323 while assembly of the other subunit continues [9]. We further showed that maintenance
324 of the pool of extra-ribosomal uL18 requires continual protein synthesis, whether
325 provoked by disruption of the formation of the 60S or the 40S subunit (Fig 4A-B). Thus,
326 the extra-ribosomal r-proteins must be a product of failing or distorted ribosomal
327 assembly rather than degradation of mature 60S subunits. This conclusion is supported
328 by the fact that uL18 accumulates outside ribosomes in response to depletion of Rrs1

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329 and Rpf2 (Fig 4D), both of which are involved in the incorporation of uL18 into the 60S
330 precursor particle [17].
331 The fraction of uL18 found outside ribosomal subunits varies with the 40S r-
332 protein whose synthesis is abrogated. In the extremes, abolishment of eS4synthesis
333 generates a response similar to that seen during repression of two 60S r-protein genes,
334 while extra-ribosomal uL18 is borderline detectable during abrogation of eS31 synthesis
335 (Fig 3). This gradient correlates with the abundance of 40S r-proteins in the 90S
336 ribosomal particle, an early 40S assembly intermediate [19], suggesting that preventing
337 early steps of pre-40S precursor assembly have the strongest effect on accumulation of
338 extra-ribosomal uL18. This can be rationalized in the context of our recent finding that
339 the 40S r-protein eS7 and the 60S r-protein uL4 coprecipitate in immune-purifications of
340 ribosomal precursor complexes that also contain the ITS1 sequence upstream of the
341 cleavage site [20]. This suggests that early intermediates in the 40S and 60S subunits
342 may interact, because they co-assemble on the emerging precursor rRNA before the
343 pre-rRNA is cleaved between the 18S and 5.8S parts. Moreover, it is known that
344 approximately 80% of the transcripts in rapidly growing yeast cells are cleaved between
345 the 18S and 5.8S parts while transcription is still ongoing ("co-transcriptional rRNA
346 processing") [21], but the cleavage is prevented by the assembly factor Rrp5 until
347 Domain 1 of the 60S part of the transcript is completed [22]. Since two 60S r-proteins
348 (uL4 and uL24) bind to Domain 1, the delay of pre-rRNA cleavage until Domain 1 is
349 synthesized is compatible with co-assembly of the early 40S and early 60S precursor
350 particle.
351 We therefore posit that the simplest explanation for the buildup of extra-

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352 ribosomal uL18 during inhibition of 40S assembly is that the folding of the 60S part of
353 the early rRNA transcript is influenced by 40S r-proteins that bind to rRNA prior to
354 separation of the subunit moieties of the emerging rRNA transcript. Even though 40S r-
355 proteins are not required for 60S formation, such changes to early 60S folding may
356 affect the path used for downstream 60S assembly and thereby change the kinetics of
357 uptake of newly synthesized 60S proteins into the precursor 60S. Ultimately, this could
358 change the propensity for accumulation of specific extra-ribosomal 60S proteins.
359 While the primary function of r-proteins is as components of the ribosome, r-
360 proteins also have important extra-ribosomal functions, at least in metazoan cells. Thus,
361 r-proteins from both ribosomal subunits have been identified as cancer drivers [23]. The
362 mechanism for r-protein-mediated regulation of growth and cell fate presumably
363 involves binding of r-proteins to several regulators of growth and the progression of the
364 cell cycle during distortion of ribosomes biogenesis (“ribosomal or nucleolar stress”) [24-
365 27]. While these functions of extra-ribosomal proteins have been intensely investigated,
366 little is known about the origin of the extra-ribosomal r-protein pools. Since the major
367 features of pathways for ribosomal assembly evolutionarily conservation, we suggest
368 that our analysis in the yeast model organism contributes to understanding the
369 complexity how ribosome assembly impacts regulation of growth. In fact, interactions
370 between 40S and 60S incorporation of r-proteins is likely stronger in metazoans than in
371 yeast, because a larger fraction of pre-rRNA is cleaved into subunit-specific pieces after
372 completion of transcription (“post-transcriptionally rRNA processing”) in metazoans than
373 in fast-growing yeast cells. The difference between the ratio of co-transcriptional and
374 post-transcriptional is evident from Northern blots of rRNA processing intermediates in

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375 the two types of organisms: full length rRNA precursor transcript is more prevalent
376 relative to other processing intermediates in mammalian cells (e.g. [28]) than it is in fast-
377 growing yeast cells (e.g. [9]).
378
379
380

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387

388 # Conflict of interest

389 The authors declare that they have no conflict of interest.
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393 # References

394 1. Tomecki R, Sikorski PJ, Zakrzewska-Placzek M. Comparison of preribosomal
395 RNA processing pathways in yeast, plant and human cells - focus on coordinated action
396 of endo- and exoribonucleases. FEBS Lett. 2017;591(13):1801-50. Epub 2017/05/20.
397 doi: 10.1002/1873-3468.12682. PubMed PMID: 28524231.
398 2. Tafforeau L, Zorbas C, Langhendries JL, Mullineux ST, Stamatopoulou V, Mullier
399 R, et al. The Complexity of Human Ribosome Biogenesis Revealed by Systematic
400 Nucleolar Screening of Pre-rRNA Processing Factors. Mol Cell. 2013;51(4):539-51.
401 Epub 2013/08/27. doi: 10.1016/j.molcel.2013.08.011. PubMed PMID: 23973377.
402 3. Piazzi M, Bavelloni A, Gallo A, Faenza I, Blalock WL. Signal Transduction in
403 Ribosome Biogenesis: A Recipe to Avoid Disaster. Int J Mol Sci. 2019;20(11). Epub
404 2019/06/06. doi: 10.3390/ijms20112718. PubMed PMID: 31163577.
405 4. Cerezo E, Plisson-Chastang C, Henras AK, Lebaron S, Gleizes PE, O'Donohue
406 MF, et al. Maturation of pre-40S particles in yeast and humans. Wiley interdisciplinary
407 reviews RNA. 2019;10(1):e1516. Epub 2018/11/09. doi: 10.1002/wrna.1516. PubMed
408 PMID: 30406965.
409 5. Bassler J, Hurt E. Eukaryotic Ribosome Assembly. Annu Rev Biochem.
410 2019;88:8.1–8.26. doi: 10.1146/annurev-biochem-013118-110817. PubMed PMID:
411 30566372.
412 6. Pena C, Hurt E, Panse VG. Eukaryotic ribosome assembly, transport and quality
413 control. Nat Struct Mol Biol. 2017;24(9):689-99. Epub 2017/09/08. doi:
414 10.1038/nsmb.3454. PubMed PMID: 28880863.

bioRxiv preprint doi: https://doi.org/10.1101/756114; this version posted September 3, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

415 7. Klinge S, Woolford JL, Jr. Ribosome assembly coming into focus. Nat Rev Mol
416 Cell Biol. 2019;20(2):116-31. Epub 2018/11/24. doi: 10.1038/s41580-018-0078-y.
417 PubMed PMID: 30467428.
418 8. Gorenstein C, Warner JR. Synthesis and turnover of ribosomal proteins in the
419 absence of 60S subunit assembly in *Saccharomyces cerevisiae*. Mol Gen Genet.
420 1977;157:327-32.
421 9. Gregory B, Rahman N, Bommakanti A, Shamsuzzaman M, Thapa M, Lescure A,
422 et al. The small and large ribosomal subunits depend on each other for stability and
423 accumulation. Life Sci Alliance. 2019;2(2). Epub 2019/03/07. doi:
424 10.26508/lsa.201800150. PubMed PMID: 30837296; PubMed Central PMCID:
425 PMCPMC6402506.
426 10. Lam YW, Lamond AI, Mann M, Andersen JS. Analysis of nucleolar protein
427 dynamics reveals the nuclear degradation of ribosomal proteins. Curr Biol.
428 2007;17(9):749-60. PubMed PMID: 17446074.
429 11. Sung MK, Porras-Yakushi TR, Reitsma JM, Huber FM, Sweredoski MJ, Hoelz A,
430 et al. A conserved quality-control pathway that mediates degradation of unassembled
431 ribosomal proteins. Elife. 2016;5. Epub 2016/08/24. doi: 10.7554/eLife.19105. PubMed
432 PMID: 27552055; PubMed Central PMCID: PMCPMC5026473.
433 12. Deshmukh M, Tsay Y-F, Paulovich AG, Woolford JL, Jr. Yeast ribosomal protein
434 L1 is required for the stability of newly synthesized 5S rRNA and the assembly of 60S
435 ribosomal subunits. Mol Cell Biol. 1993;13:2835-45.
436 13. Ban N, Beckmann R, Cate JH, Dinman JD, Dragon F, Ellis SR, et al. A new
437 system for naming ribosomal proteins. Current opinion in structural biology.

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438 2014;24:165-9. doi: 10.1016/j.sbi.2014.01.002. PubMed PMID: 24524803; PubMed
439 Central PMCID: PMCPMC4358319.
440 14. Mager WH, Planta RJ, Ballesta JG, Lee JC, Mizuta K, Suzuki K, et al. A new
441 nomenclature for the cytoplasmic ribosomal proteins of *Saccharomyces cerevisiae*.
442 Nucleic Acids Res. 1997;25(24):4872-5.
443 15. Pelava A, Schneider C, Watkins NJ. The importance of ribosome production, and
444 the 5S RNP-MDM2 pathway, in health and disease. Biochem Soc Trans.
445 2016;44(4):1086-90. doi: 10.1042/BST20160106. PubMed PMID: 27528756; PubMed
446 Central PMCID: PMCPMC4984446.
447 16. Liao JM, Cao B, Zhou X, Lu H. New insights into p53 functions through its target
448 microRNAs. J Mol Cell Biol. 2014;6(3):206-13. Epub 2014/04/18. doi:
449 10.1093/jmcb/mju018. PubMed PMID: 24740682; PubMed Central PMCID:
450 PMCPMC4034730.
451 17. Zhang J, Harnpicharnchai P, Jakovljevic J, Tang L, Guo Y, Oeffinger M, et al.
452 Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and
453 rpL11 into nascent ribosomes. Genes Dev. 2007;21(20):2580-92. PubMed PMID:
454 17938242.
455 18. Gomez-Herreros F, Rodriguez-Galan O, Morillo-Huesca M, Maya D, Arista-
456 Romero M, de la Cruz J, et al. Balanced production of ribosome components is required
457 for proper G1/S transition in Saccharomyces cerevisiae. J Biol Chem.
458 2013;288(44):31689-700. Epub 2013/09/18. doi: 10.1074/jbc.M113.500488. PubMed
459 PMID: 24043628; PubMed Central PMCID: PMCPMC3814764.

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460 19. Zhang L, Wu C, Cai G, Chen S, Ye K. Stepwise and dynamic assembly of the
461 earliest precursors of small ribosomal subunits in yeast. Genes Dev. 2016;30(6):718-32.
462 Epub 2016/03/17. doi: 10.1101/gad.274688.115. PubMed PMID: 26980190; PubMed
463 Central PMCID: PMCPMC4803056.
464 20. Fox JM, Rashford RL, Lindahl L. Co-Assembly of 40S and 60S Ribosomal
465 Proteins in Early Steps of Eukaryotic Ribosome Assembly. Int J Mol Sci. 2019;20(11).
466 Epub 2019/06/12. doi: 10.3390/ijms20112806. PubMed PMID: 31181743.
467 21. Osheim YN, French SL, Keck KM, Champion EA, Spasov K, Dragon F, et al.
468 Pre-18S ribosomal RNA is structurally compacted into the SSU processome prior to
469 being cleaved from nascent transcripts in Saccharomyces cerevisiae. Mol Cell.
470 2004;16(6):943-54. Epub 2004/12/22. doi: S1097276504007233 [pii]
471 10.1016/j.molcel.2004.11.031. PubMed PMID: 15610737.
472 22. Khoshnevis S, Liu X, Dattolo MD, Karbstein K. Rrp5 establishes a checkpoint for
473 60S assembly during 40S maturation. RNA. 2019;25(9):1164-76. Epub 2019/06/21. doi:
474 10.1261/rna.071225.119. PubMed PMID: 31217256.
475 23. Fancello L, Kampen KR, Hofman IJ, Verbeeck J, De Keersmaecker K. The
476 ribosomal protein gene RPL5 is a haploinsufficient tumor suppressor in multiple cancer
477 types. Oncotarget. 2017;8(9):14462-78. Epub 2017/02/02. doi:
478 10.18632/oncotarget.14895. PubMed PMID: 28147343; PubMed Central PMCID:
479 PMCPMC5362418.
480 24. Deisenroth C, Zhang Y. The Ribosomal Protein-Mdm2-p53 Pathway and Energy
481 Metabolism: Bridging the Gap between Feast and Famine. Genes Cancer.

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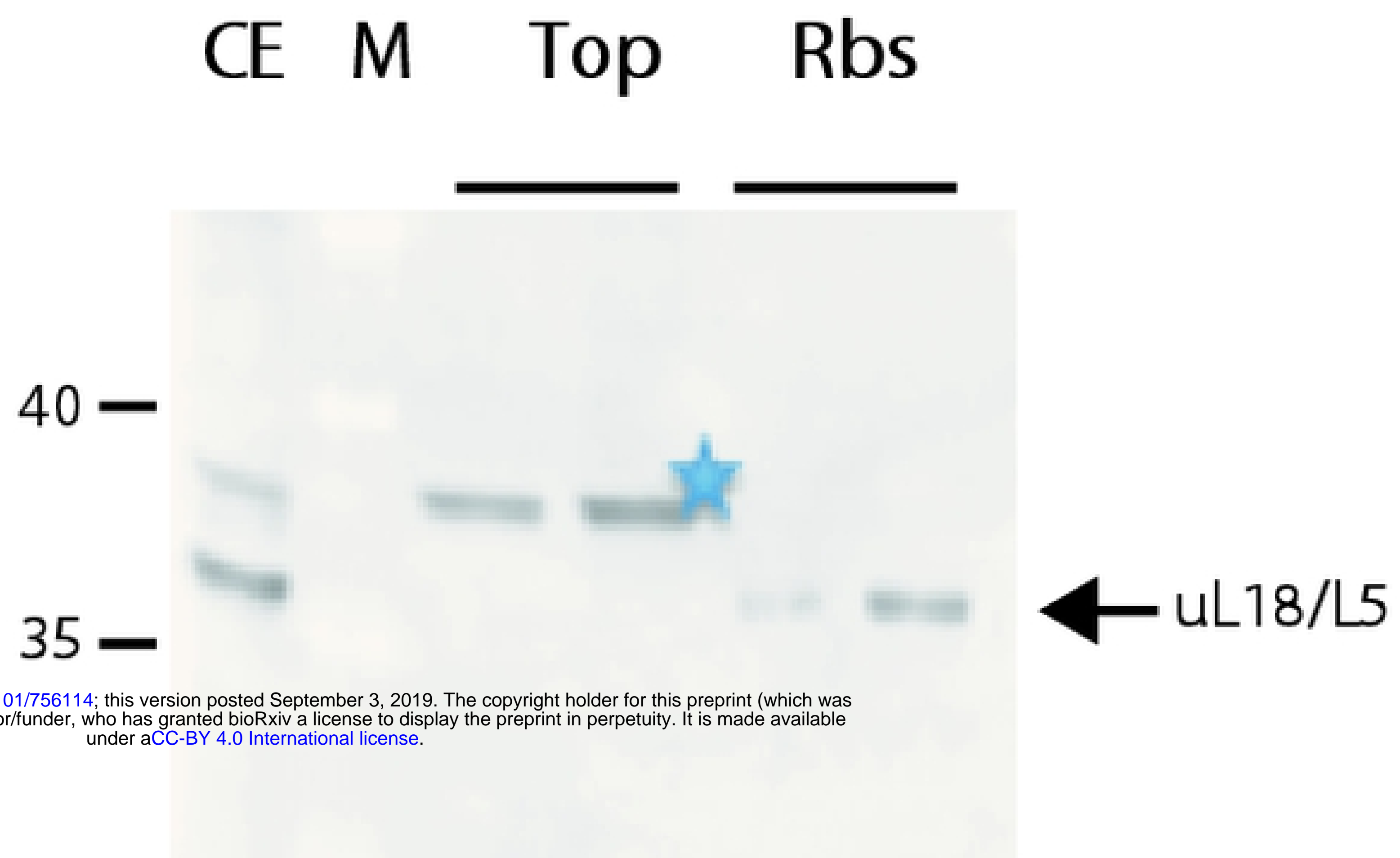
482 2011;2(4):392-403. Epub 2011/07/23. doi: 10.1177/1947601911409737. PubMed PMID:
483 21779508; PubMed Central PMCID: PMC3135641.
484 25. James A, Wang Y, Raje H, Rosby R, DiMario P. Nucleolar stress with and
485 without p53. Nucleus. 2014;5(5):402-26. doi: 10.4161/nucl.32235. PubMed PMID:
486 25482194; PubMed Central PMCID: PMCPMC4164484.
487 26. Bursac S, Brdovcak MC, Donati G, Volarevic S. Activation of the tumor
488 suppressor p53 upon impairment of ribosome biogenesis. Biochim Biophys Acta.
489 2014;1842(6):817-30. doi: 10.1016/j.bbadis.2013.08.014. PubMed PMID: 24514102.
490 27. Zhou X, Liao WJ, Liao JM, Liao P, Lu H. Ribosomal proteins: functions beyond
491 the ribosome. J Mol Cell Biol. 2015;7(2):92-104. doi: 10.1093/jmcb/mjv014. PubMed
492 PMID: 25735597; PubMed Central PMCID: PMCPMC4481666.
493 28. Goldfarb KC, Cech TR. Targeted CRISPR disruption reveals a role for RNase
494 MRP RNA in human preribosomal RNA processing. Genes Dev. 2017;31(1):59-71.
495 Epub 2017/01/25. doi: 10.1101/gad.286963.116. PubMed PMID: 28115465; PubMed
496 Central PMCID: PMCPMC5287113.
497

A. uL18/L5 repressed 13.5 hours



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B. FLAG-tagged uL18/L5

eL43/L43

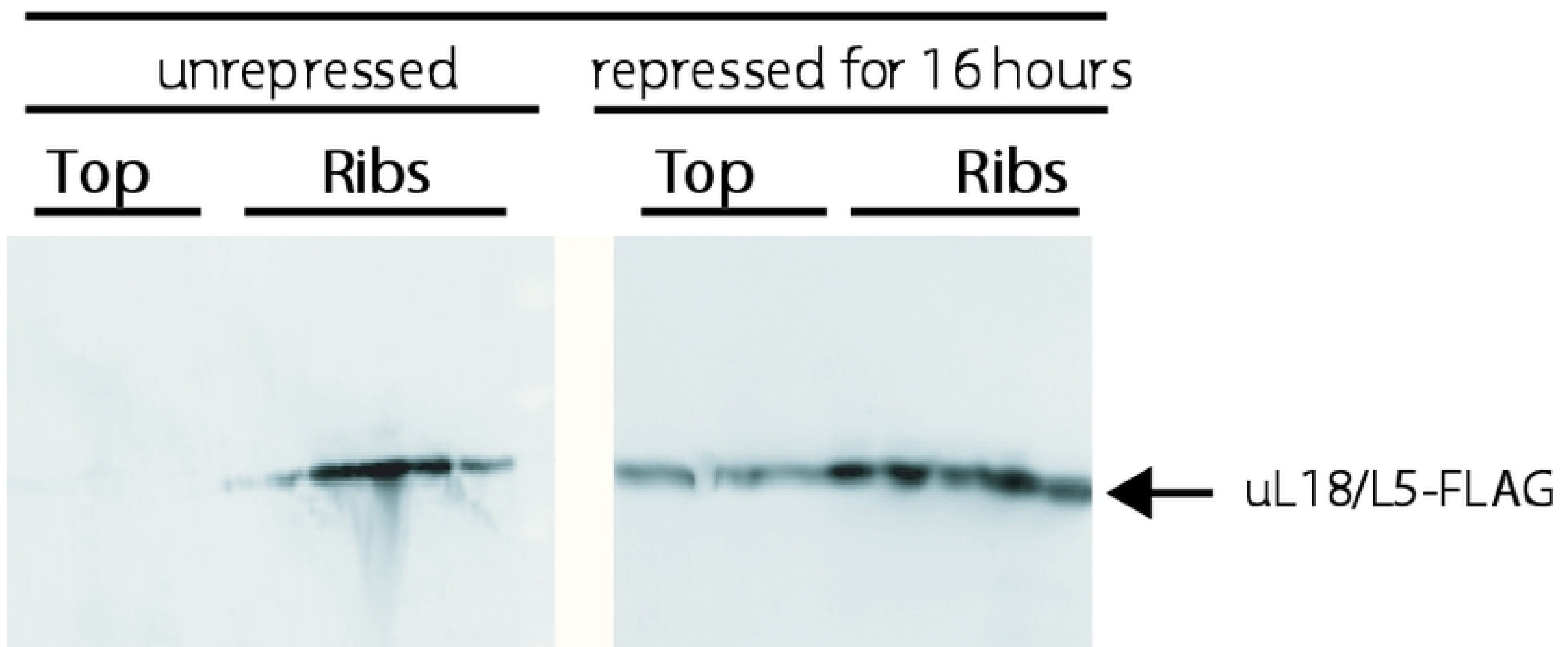
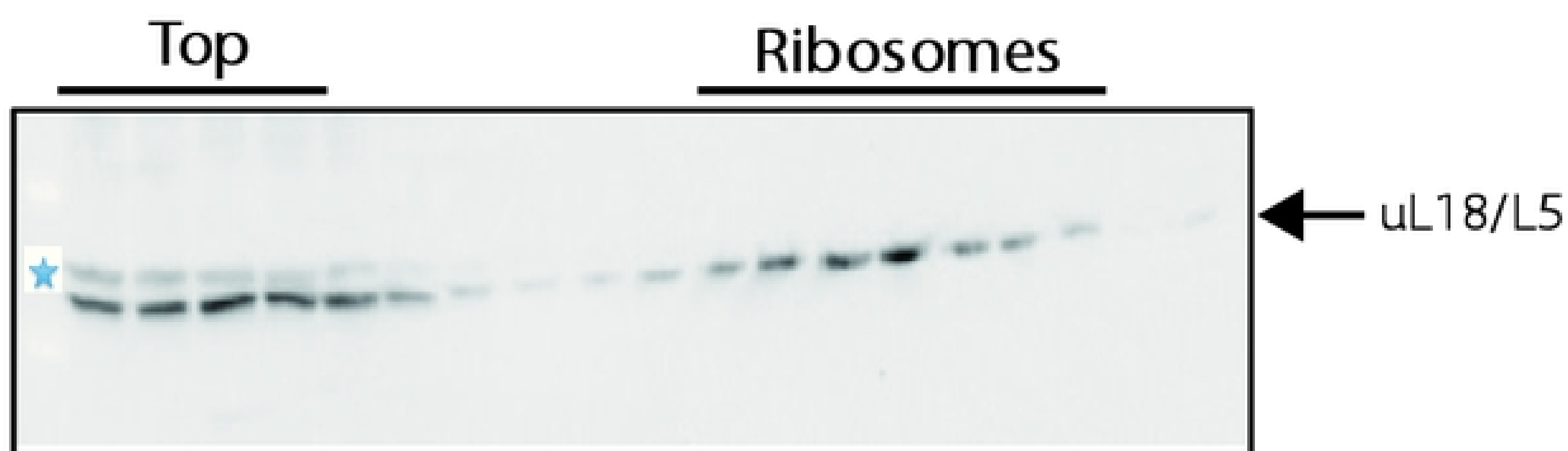
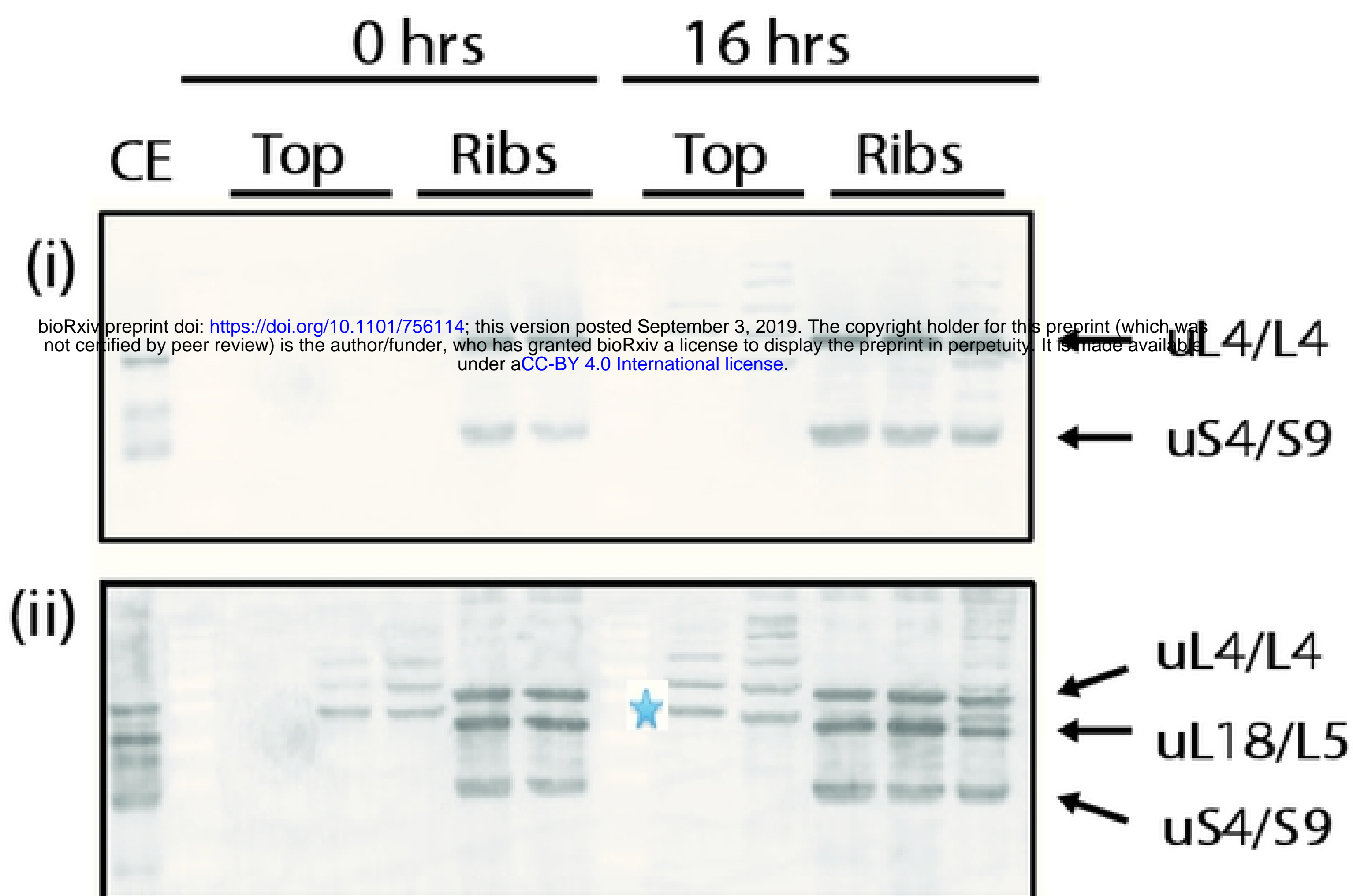


Figure 1

Fig 1





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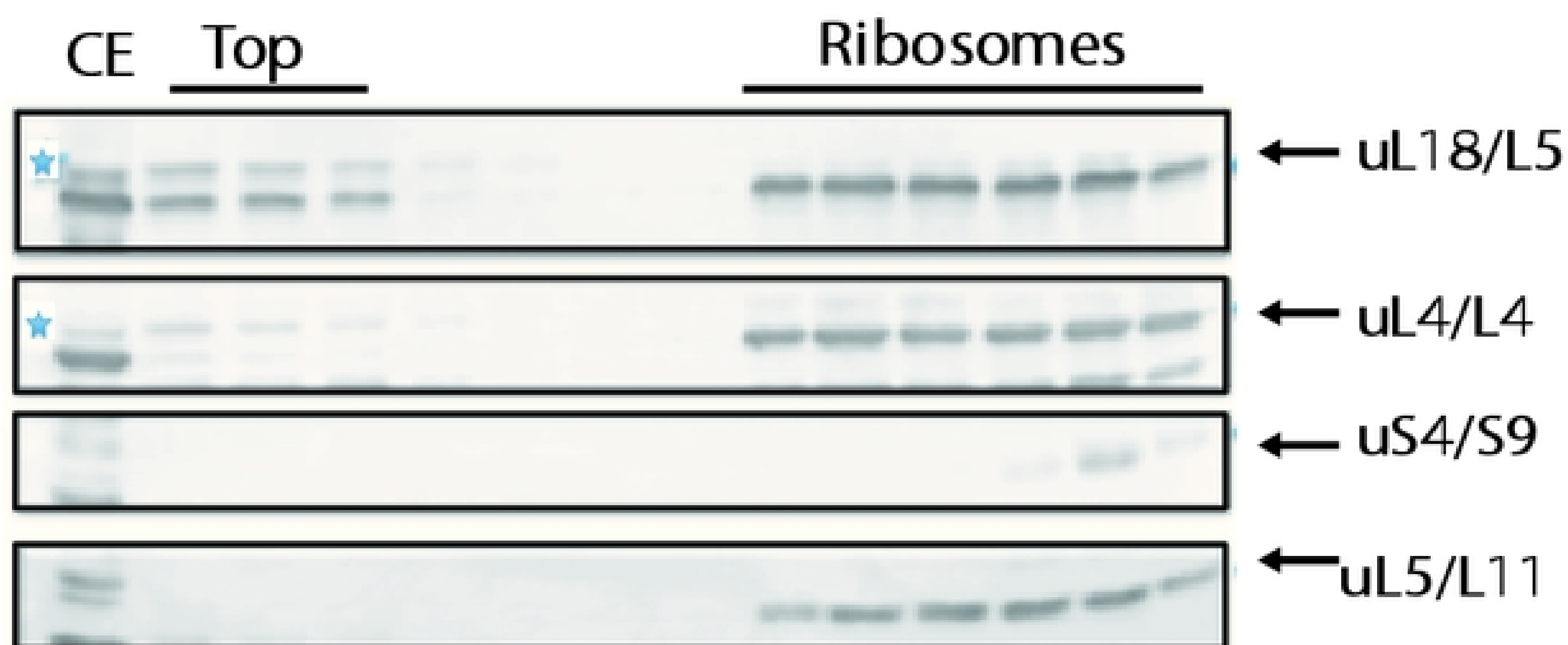


Figure 2

Fig 2

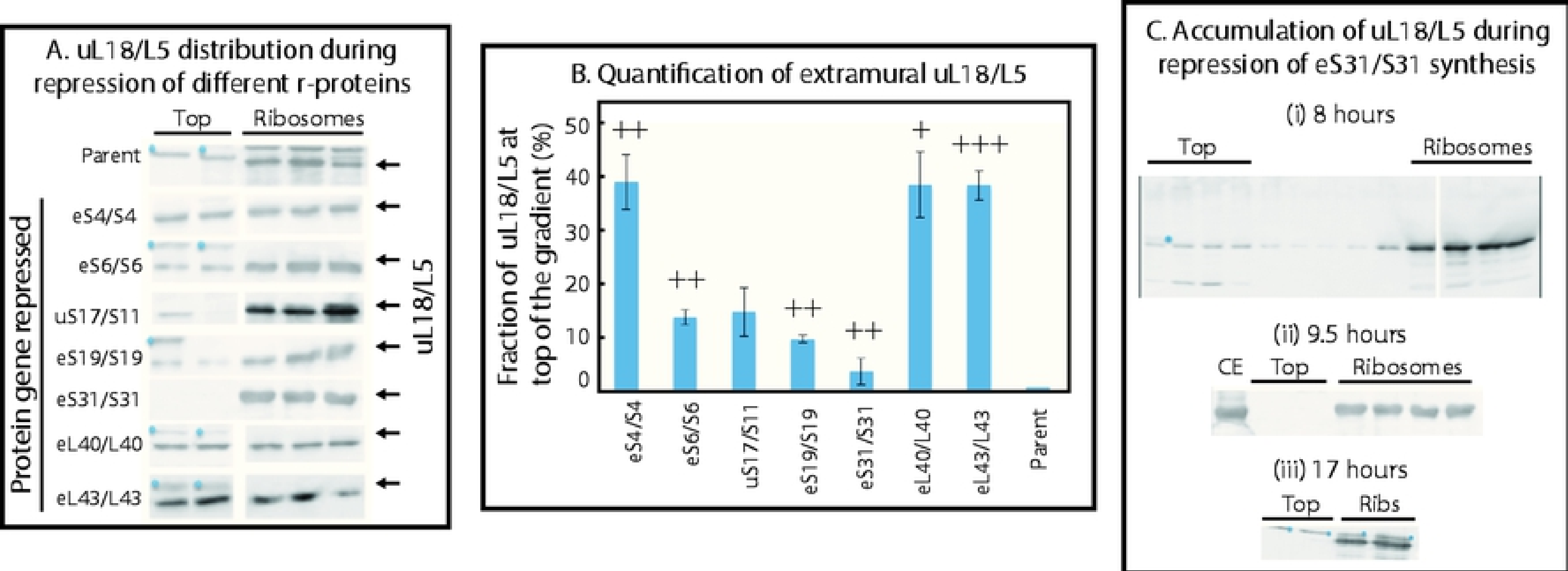
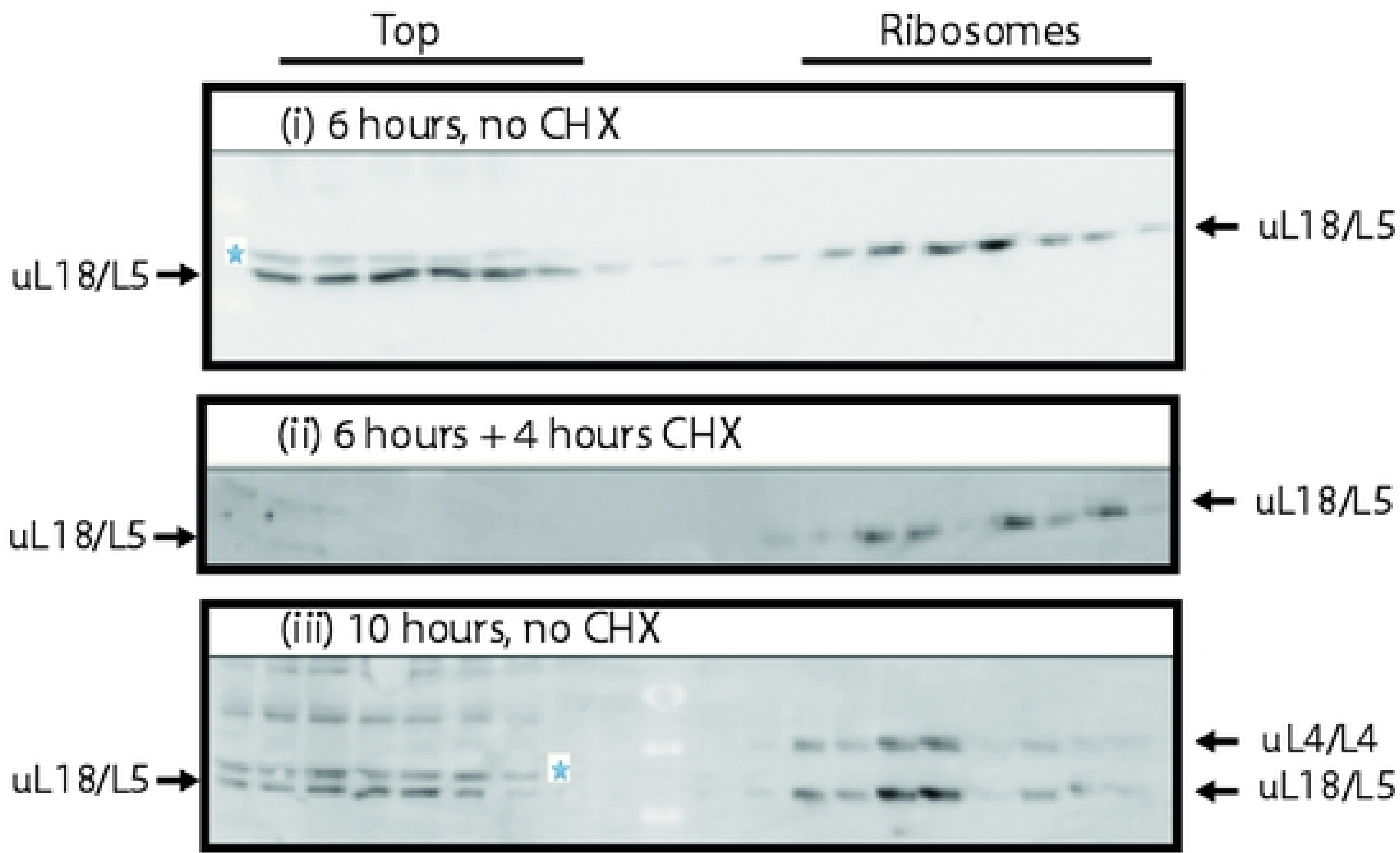
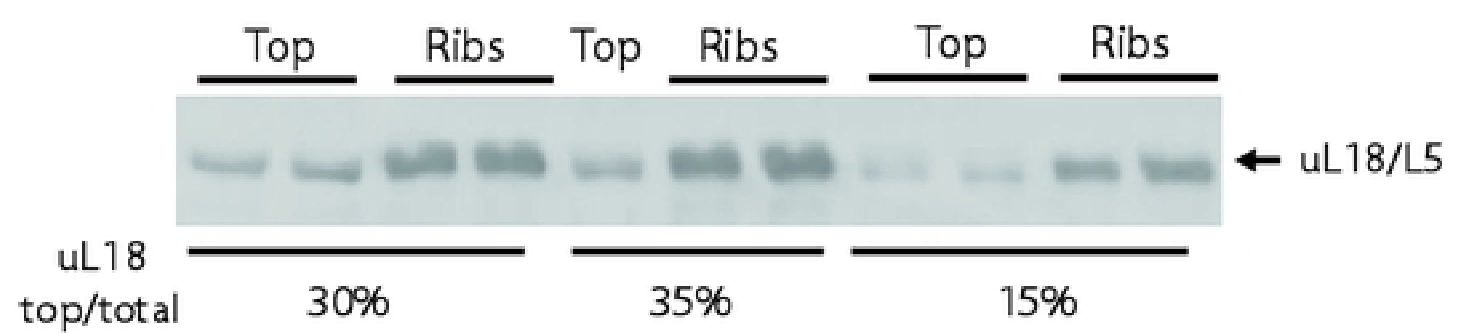


Figure 3

Fig 3





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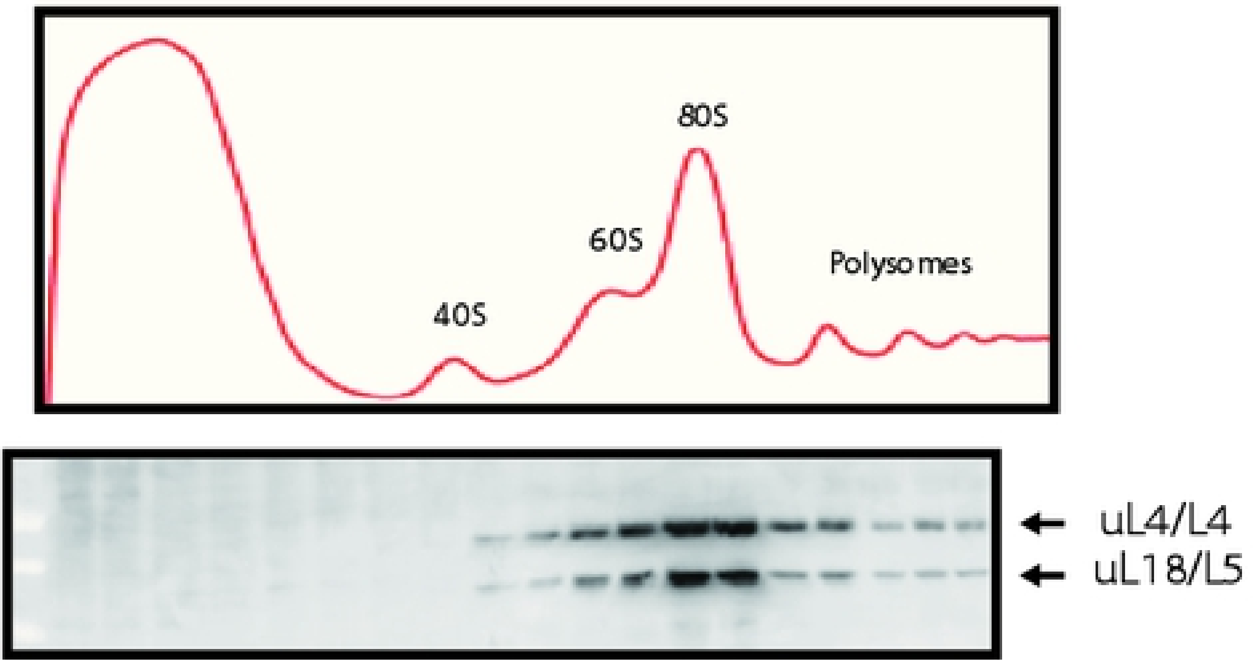

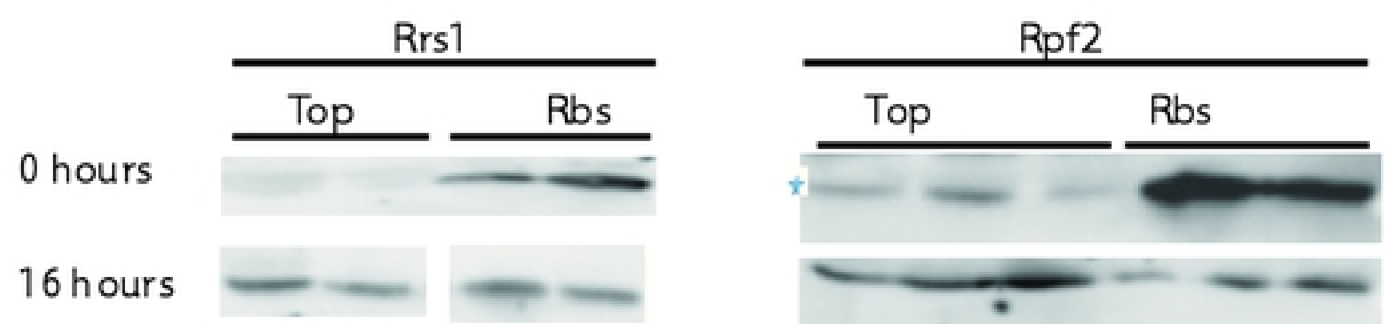

Figure 4

Fig 4