

1 **Comprehensive Evaluation of Rapamycin's Specificity**
2 **as an mTOR Inhibitor**

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25 **Running Title:** Rapamycin specifically inhibits mTOR

26

27 **Abstract**

28 Rapamycin is a macrolide antibiotic that functions as an immunosuppressive and anti-cancer
29 agent, and displays robust anti-ageing effects in multiple organisms including humans.
30 Importantly, rapamycin analogs (rapalogs) are of clinical importance against certain cancer
31 types and neurodevelopmental diseases. Although rapamycin is widely perceived as an
32 allosteric inhibitor of mTOR (mechanistic target of rapamycin), the master regulator of cellular
33 and organismal physiology, its specificity has not been thoroughly evaluated so far. In fact,
34 previous studies in cells and in mice suggested that rapamycin may be also acting independently
35 from mTOR to influence various cellular functions. Here, we generated a gene-edited cell line,
36 that expresses a rapamycin-resistant mTOR mutant (mTOR^{RR}), and assessed the effects of
37 rapamycin treatment on the transcriptome and proteome of control or mTOR^{RR}-expressing
38 cells. Our data reveal a striking specificity of rapamycin towards mTOR, demonstrated by
39 virtually no changes in mRNA or protein levels in rapamycin-treated mTOR^{RR} cells, even
40 following prolonged drug treatment. Overall, this study provides the first comprehensive and
41 conclusive assessment of rapamycin's specificity, with important potential implications for
42 ageing research and human therapeutics.

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50 **Keywords**

51 rapamycin / sirolimus / mTORC1 / ageing / proteomics

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54 **Introduction**

55 Rapamycin (also known as Sirolimus) is a naturally-occurring macrolide compound which was
56 originally isolated from soil bacteria on Easter Island (Rapa Nui) in 1972 (Benjamin *et al*, 2011;
57 Arriola Apelo & Lamming, 2016; Li *et al*, 2014). Rapamycin was primarily used in the clinic
58 as an anti-fungal agent until 1999 when it was approved by the FDA for the prevention of
59 kidney transplant rejection and later for the treatment of advanced kidney cell carcinoma. Its
60 immunosuppressive and anti-proliferative properties are thought to be largely mediated by
61 inhibition of mTOR, a serine/threonine kinase that functions as the master regulator of most
62 cellular functions, including immune cell activation and cell growth control (Arriola Apelo &
63 Lamming, 2016; Benjamin *et al.*, 2011; Fernandes & Demetriades, 2021; Li *et al.*, 2014). At
64 the molecular level, rapamycin inhibits mTORC1 (mTOR complex 1) activity as a complex
65 with the cytosolic immunophilin FKBP12 (FK506-binding protein 12). Binding of this drug-
66 protein complex to mTOR blocks access to its catalytic site and prevents the phosphorylation
67 of key substrates like S6K (ribosomal protein S6 kinase) (Chung *et al*, 1992). Additional
68 immunophilins such as FKBP12.6, FKBP51, and FKBP52 have also been reported to bind and
69 shape the pharmacology of rapamycin (Marz *et al*, 2013).

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71 Over the last two decades, rapamycin has gained renewed interest after multiple studies
72 uncovered its powerful anti-ageing properties (Arriola Apelo & Lamming, 2016; Benjamin *et*
73 *al.*, 2011; Fernandes & Demetriades, 2021; Li *et al.*, 2014). Rapamycin has repeatedly been
74 shown to extend lifespan and/or healthspan in worms (Robida-Stubbs *et al*, 2012), flies (Bjedov
75 *et al*, 2010; Castillo-Quan *et al*, 2019; Schinaman *et al*, 2019), and mice (Bitto *et al*, 2016; Fok
76 *et al*, 2014; Harrison *et al*, 2009). It extends chronological age in yeast (Powers *et al*, 2006) and
77 reduces markers of senescence in cultured cells (Wang *et al*, 2017). More recently, studies
78 conducted on marmoset monkeys have demonstrated rapamycin to have a good safety and
79 tolerability profile (Lelegren *et al*, 2016; Tardif *et al*, 2015) thus paving the way for human

80 trials. Currently, trials are underway on both companion dogs (Creevy *et al*, 2022) and humans
81 (NCT04488601) with preliminary evidence suggesting improvement in cardiac function in
82 middle-aged dogs who received rapamycin for 10 weeks (Urfer *et al*, 2017). Notably,
83 rapamycin extends lifespan at doses much lower than the ones used to achieve
84 immunosuppression in transplant patients thus minimizing potential side effects. Finally, even
85 transient, intermittent, or late-life rapamycin administration has been shown to extend lifespan
86 in flies and mice thus making Rapamycin an attractive option for human use as an anti-ageing
87 compound (Bitto *et al.*, 2016; Harrison *et al.*, 2009; Partridge *et al*, 2020).

88
89 The target specificity of rapamycin has recently been debated. On one hand, *in vitro* kinase
90 activity assays showed other serine/threonine kinases, besides mTOR, to be largely insensitive
91 to rapamycin up to concentrations at the micromolar range. Likewise, receptor-binding assays
92 indicated that many ligand-receptor interactions remain unaffected by rapamycin, with the
93 possible exception of histamine I binding (European Medicine Agency, 2005). On the other
94 hand, accumulating evidence in the literature suggests that rapamycin may exert some of its
95 effects via mTOR-independent mechanisms. This is also supported by the fact that most kinase
96 inhibitors demonstrate very low target selectivity (Hantschel, 2015; Hantschel *et al*, 2012;
97 Karaman *et al*, 2008). For instance, rapamycin was suggested to block the exercise-induced
98 accumulation of ribosomal RNA (rRNA) in the skeletal muscle of both wild-type and mice
99 containing a rapamycin-resistant mTOR allele (Goodman *et al*, 2011). Moreover, rapamycin
100 and other rapalogs were shown to directly bind and activate the lysosomal mucolipin TRP
101 channel (TRPML1; also known as MCOLN1) independently of mTOR inhibition (Zhang *et al*,
102 2019). Finally, since FKBP's serve as chaperones for proper folding of several proteins (Bonner
103 & Boulianne, 2017; Bultynck *et al*, 2001; Galfre *et al*, 2012; Vervliet *et al*, 2015; Wang *et al*,
104 1996), it can be speculated that their binding to rapamycin may be influencing cellular
105 physiology via altering the interaction of FKBP's to their client proteins.

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107 Here, to comprehensively address this important unresolved issue, we generated a rapamycin-
108 resistant cell line by editing a single base in the *MTOR* gene at its endogenous locus (Choi *et*
109 *al*, 1996; Hosoi *et al*, 1999; Lorenz & Heitman, 1995) and assessed how rapamycin affected
110 the cellular transcriptome and proteome in an unbiased manner. These experiments unraveled
111 an impressive specificity of rapamycin towards mTOR, with the rapamycin effects on gene
112 expression and protein levels being virtually non-existent in the mTOR-mutant cells.

113

114 **Results**

115 **Generation and characterization of a gene-edited cell line that expresses rapamycin-** 116 **resistant mTOR**

117 Mutations in the mTOR FRB (FKBP-rapamycin-binding) domain that disrupt its interaction
118 with FKBP12 and confer rapamycin resistance to mTOR have been described almost 30 years
119 ago (Brown *et al*, 1995; Chen *et al*, 1995; Choi *et al.*, 1996; Lorenz & Heitman, 1995; Hara *et*
120 *al*, 1997; Hosoi *et al.*, 1999). Although such mTOR mutants have been used in previous studies,
121 usually expressed exogenously in cells or via transgenic expression in mouse tissues, the
122 presence of endogenous wild-type mTOR has complicated the interpretation of such results (Ge
123 *et al*, 2009; Goodman *et al.*, 2011; Luo *et al*, 2015; Zhang *et al*, 2000). Moreover, a
124 comprehensive analysis of rapamycin's effects in such cellular models, beyond single readouts,
125 is lacking. Therefore, to probe whether rapamycin also acts through mTOR-independent
126 mechanisms or exclusively via mTOR inhibition, we used CRISPR/Cas9-mediated gene editing
127 to generate a HEK293FT cell line that expresses a Ser2035Thr mTOR mutant (Fig. 1A and Fig.
128 EV1) that was previously described to be rapamycin-resistant (Brown *et al.*, 1995; Chen *et al.*,
129 1995; Choi *et al.*, 1996; Lorenz & Heitman, 1995)(Hara *et al.*, 1997; Hosoi *et al.*, 1999).
130 Intuitively, we called this mutant and the associated cell line, mTOR^{RR} (mTOR rapamycin-
131 resistant). Importantly, as we mutated *MTOR* in the endogenous locus, no wild-type mTOR is

132 expressed in these cells, verified by genomic DNA sequencing (Fig. EV1). Consistent with
133 previous reports, the rapamycin-induced interaction of FLAG-tagged FKBP12 with wild-type
134 mTOR, was completely abrogated in mTOR^{RR} (Fig. 1B). Of note, the catalytic activity of
135 mTORC1 containing mTOR^{RR}, as assessed by the phosphorylation of its direct substrate S6K,
136 was indistinguishable from that in control cells and was diminished by treatment with Torin1,
137 an ATP-competitive mTOR inhibitor (Fig. 1C). In contrast, mTOR^{RR} demonstrated complete
138 resistance to rapamycin even when cells were treated with this compound at micromolar
139 concentrations (Fig. 1C), or when the treatment was extended to 24 or 48 hours (Fig. 1D).
140 Similar to rapamycin, mTOR^{RR} also exhibited full resistance to other rapalogs like everolimus
141 and temsirolimus (Fig. 1E), but responded properly to amino acid (AA) starvation, showing
142 that the regulation of mTORC1 by other inhibitory stimuli is unperturbed in mTOR^{RR} cells (Fig.
143 1F). In sum, the mTOR^{RR} HEK293FT cell line is a ‘clean’, reliable and robust model to
144 investigate rapamycin’s specificity towards mTOR.

145

146 **Rapamycin alters gene expression exclusively via mTOR inhibition**

147 Having validated our experimental model, we then treated control (WT) and mTOR^{RR} cells
148 with rapamycin for 24 hours and performed RNA-seq experiments to investigate its effects on
149 global gene expression. In line with mTOR—directly or indirectly—regulating the activity of
150 several transcription factors (Hardwick *et al*, 1999; Laplante & Sabatini, 2013), we detected
151 more than 5000 genes whose expression changed significantly in WT cells upon rapamycin
152 treatment (Fig. 2A,B and Suppl. Table 1). Our analysis identified several genes that are known
153 to be affected by mTOR inhibition (e.g., HMOX1, RHOB, MYC) (Bayeva *et al*, 2012; Gordon
154 *et al*, 2015; Jin *et al*, 2013; Sun *et al*, 2022; Visner *et al*, 2003) (Fig. 2B) thus validating our
155 experimental setup. Similarly, rapamycin decreased the expression of AA transporters like
156 SLC7A5 and SLC7A11, which are known to be regulated downstream of an mTORC1-ATF4
157 axis (Torrence *et al*, 2021) (Fig. 2B and Suppl. Tables 1, 2).

158

159 Gene ontology (GO) term enrichment analysis, using the differentially regulated genes that are
160 strongly down- or upregulated by rapamycin ($\text{Log}_2\text{FC} < -0.75$ and $\text{Log}_2\text{FC} > +0.8$,
161 respectively), revealed a strong enrichment of ribosome-related Biological Process (BP) and
162 Cellular Component (CC) terms (e.g., BP:GO:0042254~ribosome biogenesis;
163 CC:GO:0030684~preribosome) (Fig. 2C,D and Suppl. Table 2). Confirming the well-known
164 role of mTOR in promoting ribosome biogenesis and positively regulating rRNA expression
165 (Mayer & Grummt, 2006; Powers & Walter, 1999), all genes that fall under these terms (e.g.,
166 RRP12, RRP9, RRS1, RRP1, RPF2, NOP16, NOL6, DDX21, MRTO4, MRM1) were found to
167 be downregulated by rapamycin (Fig. 2C,D and Suppl. Table 2). Interestingly, we also observed
168 a strong enrichment of terms related to mitochondria-resident proteins (e.g.,
169 CC:GO:0005739~mitochondrion; CC:GO:0005759~mitochondrial matrix) and associated
170 mitochondrial functions (e.g., BP:GO:0019752~carboxylic acid metabolic process) among the
171 genes that are differentially regulated by rapamycin (Fig. 2C,D and Suppl. Table 2). Similar
172 results were obtained when performing the GO analysis with more relaxed criteria including all
173 significantly down- and upregulated genes, instead of setting cut-offs for those that change
174 robustly upon rapamycin (Fig. EV2A,B and Suppl. Table 3). Strikingly, unlike the massive
175 transcriptional effects of rapamycin in control cells, we found only 3 genes whose expression
176 was altered in mTOR^{RR} cells treated with rapamycin (Fig. 2A,B and Suppl. Table 1). These
177 data indicated that rapamycin practically regulates transcription exclusively via its direct
178 inhibitory effect on mTOR.

179

180 **Rapamycin alters the cellular proteome exclusively through mTOR**

181 In addition to their involvement in transcriptional regulation, the best-described role of
182 rapamycin and mTORC1 is in the control of *de novo* protein synthesis via controlling the
183 phosphorylation and activity of—direct or indirect—mTORC1 targets like S6K, 4E-BP1, and

184 S6, with only certain 4E-BP1 phospho-sites being rapamycin-sensitive (Thoreen *et al*, 2012;
185 Thoreen *et al*, 2009). Therefore, we next sought to investigate the rapamycin-induced changes
186 in the cellular proteome and to explore how much of this happens due to mTOR inhibition.

187

188 To this end, we treated control and mTOR^{RR} cells with rapamycin for 24 or 48 hours and
189 performed whole-proteome quantitative mass spectrometry experiments. Out of a total of
190 approximately 7500 proteins that were detected and quantified, the levels of more than 2500
191 and 2300 proteins changed significantly in WT cells treated with rapamycin for 24 or 48 hours,
192 respectively (Fig. 3A-C and Suppl. Table 4). Similar to what we observed in transcriptomic
193 analyses, these hits include multiple proteins belonging to pathways that have been previously
194 described to be regulated by rapamycin and mTOR (e.g., PDCD4, RHOB, HMOX1, SQSTM1,
195 PRELID1, SCD, SESN2) (Bayeva *et al.*, 2012; Dorrello *et al*, 2006; Gordon *et al.*, 2015; Jin *et*
196 *al.*, 2013; Ko *et al*, 2017; Mauvoisin *et al*, 2007; Sun *et al.*, 2022; Visner *et al.*, 2003; Wall *et*
197 *al*, 2008; Zhu *et al*, 2020) as well as several amino acid transporters (SLC7A11, SLC38A10,
198 SLC3A2, SLC7A5) (Graber *et al*, 2017; Nachev *et al*, 2021; Torrence *et al.*, 2021; Zhang *et al*,
199 2021) (Fig. 3B,C and Suppl. Table 4). Remarkably, however, none of the 7574 detected proteins
200 were differentially regulated in rapamycin-treated mTOR^{RR} cells, even after prolonged drug
201 treatment (Fig. 3A-C and Suppl. Table 4), again showing complete absence of mTOR-
202 independent effects by rapamycin.

203

204 Consistent with our observations from RNA-Seq experiments, GO analysis using all proteins
205 that are significantly down- or upregulated ($p < 0.05$) upon 24-hour rapamycin treatment in WT
206 cells showed strong enrichment of terms related to ribosomes and translation (e.g.,
207 BP:GO:0022613~ribonucleoprotein complex biogenesis; BP:GO:0042254~ribosome
208 biogenesis; BP:GO:0006412~translation) (Fig. 3D and Suppl. Table 5) with the majority of
209 proteins that belong to this category being downregulated (e.g., CDC123, CCRN4L, LTV1,

210 RRN3, EIF1AD, SESN2, GNL3L, CCDC86, WDR74, TNRC6A) (Fig. 3B,D and Suppl. Tables
211 4, 5). Another prominent group of GO terms that is enriched in the proteomic analysis includes
212 terms related to the cell cycle (e.g., BP:GO:0007049~cell cycle, BP:GO:0000278~mitotic cell
213 cycle) (Fig. 3D and Suppl. Tables 4, 5) with proteins being either down- or upregulated upon
214 rapamycin treatment (e.g., CDC123, CDC6, ASNS, ECD, CEP72, PDCD4, RHOB, HMGN3,
215 DHFR, TRIOBP) (Fig. 3D). Of note, this is in line with the well-established role of rapamycin
216 and mTOR in the regulation of cell proliferation (Dowling *et al*, 2010). Similar results were
217 obtained when analyzing the respective dataset (all significantly-regulated proteins; $p < 0.05$)
218 from 48-hour rapamycin treatment in WT cells (Fig. 3A,C, Fig. EV3 and Suppl. Table 6).
219
220 Furthermore, in line with the robust expression changes in genes related to mitochondria (Fig.
221 2C,D), we observed similar effects in the levels of mitochondrial proteins, as shown by the
222 strong enrichment of related GO terms (e.g., CC:GO:0005739~mitochondrion;
223 CC:GO:0005759~mitochondrial matrix) (Fig. 3E and Suppl. Tables 4, 5). Interestingly, when
224 performing the GO term analysis using only the strongly up- or downregulated proteins, the
225 enrichment of terms related to mitochondrial proteins became even more prominent (Fig. EV4
226 and Suppl. Table 7) with most mitochondria-related proteins being upregulated by rapamycin
227 (e.g., BNIP3, BNIP3L, CPS1, TXNRD2, SDSL, ACSF2, BPHL, HMGCL, HSD17B8, SFN)
228 (Fig. EV4 and Suppl. Tables 4, 7).
229
230 Finally, although a connection between mTOR activity and cell adhesion has been described
231 before, the underlying mechanisms are less clear (Asrani *et al*, 2017; Chen *et al*, 2015).
232 Interestingly, our proteomics analysis showed that rapamycin treatment led to significant
233 changes in the levels of a large number of proteins that are related to adherens and anchoring
234 junctions (CC:GO:0005912~adherens junction; CC:GO:0070161~anchoring junction), most of
235 which are downregulated under these conditions (e.g., GJA1, SLC3A2, RANGAP1, DSP,

236 FASN, APC, EEF2, EIF2A, TNKS1BP1, ATXN2L) (Fig. 3B,E and Suppl. Tables 4, 7). This
237 suggests that some of the effects of rapamycin/mTOR on cell adhesion may stem from changes
238 in the junctional proteome.

239

240 In sum, our unbiased interrogation of rapamycin's effects in cells reveals an extraordinary
241 specificity of this compound towards mTOR, and—at the same time—provides a
242 comprehensive evaluation of its role on the cellular transcriptome and proteome.

243

244 **Discussion**

245 According to previous studies, the majority of inhibitory compounds that are used in research
246 or in therapeutics demonstrate off-target effects, influencing the activity of additional signaling
247 molecules that can also be structurally-unrelated to their presumed targets. Sometimes,
248 inhibitors even show higher potency towards off-targets compared to on-target effects (Davies
249 *et al*, 2000; Bain *et al*, 2003; Bain *et al*, 2007; Fedorov *et al*, 2007; Davis *et al*, 2011). In fact,
250 very few FDA-approved kinase inhibitors have demonstrated high target selectivity, whereas
251 the majority influences the activity of 10-100 off-target kinases (Hantschel, 2015; Hantschel *et*
252 *al*, 2012; Karaman *et al*, 2008). Importantly, the low specificity and selectivity of most kinase
253 inhibitors greatly complicates the interpretation of data originating from their use and has
254 important implications for their applicability in both research and therapeutics.

255

256 Surprisingly, although rapamycin has been used as an mTOR inhibitor for more than three
257 decades, its specificity towards this key signaling hub has not been thoroughly evaluated so far.
258 In fact, previous studies have hinted at the existence of mTOR-independent functions of
259 rapamycin in cells and in transgenic mouse models thus underscoring the need for a detailed
260 evaluation of its specificity. In transgenic mice overexpressing a rapamycin-resistant mTOR
261 mutant in skeletal muscles, rapamycin was still able to partially suppress mechanical-loading-

262 induced ribosome biogenesis (Goodman *et al.*, 2011). It is worth noting however, that these
263 transgenic mice were maintained as hemizygotes with the mTOR^{RR} allele expressed on top of
264 endogenous wild-type mTOR (Ge *et al.*, 2009; Goodman *et al.*, 2011), which does not allow
265 for safe conclusions to be drawn from such experiments. Indeed, the observed rapamycin effects
266 that were previously interpreted as mTOR-independent can also be ascribed to the inhibition of
267 endogenous wild-type mTOR molecules. This is also supported by our findings, using a gene-
268 edited cell line that expresses mutant, rapamycin-resistant mTOR from the endogenous *MTOR*
269 locus, while lacking expression of wild-type mTOR: unlike the previously-described mTOR^{RR}
270 transgenic mice, our mTOR^{RR} cells are fully resistant to rapamycin, which is demonstrated by
271 unaffected mTORC1 activity, diminished FKBP12-mTOR binding, and complete lack of
272 transcriptional or proteomic changes in response to rapamycin treatment.

273

274 A more recent study reported that micromolar concentrations of rapamycin (or of other rapalogs
275 like everolimus and temsirolimus) can directly bind to TRPML1/MCOLN1, the primary
276 lysosomal calcium release channel, and enhance its activity in an mTOR-independent manner
277 (Zhang *et al.*, 2019). Although rapalogs may indeed act on additional substrates at such
278 concentrations, these are two to three orders of magnitude higher than those classically used in
279 cell culture experiments (2-20 nM; like those we used for the transcriptomic and proteomic
280 analyses described here) or—most importantly—those that are detected in the blood of patients
281 that take rapamycin. More specifically, whole-blood concentrations (WBC) of
282 rapamycin/sirolimus are in the 10-20 nM range in renal transplant patients to prevent graft
283 rejection (Meier-Kriesche & Kaplan, 2000). In patients treated with everolimus against
284 metastatic renal cell carcinoma, the median WBC is approximately 15 nM (Takasaki *et al.*,
285 2019), while plasma concentrations of temsirolimus for relapsed/refractory multiple myeloma
286 average around 9 nM (Farag *et al.*, 2009). Higher doses of temsirolimus have only been used as
287 a last resort in clinical trials against aggressive forms of lymphoma with WBC values reaching

288 500-600 nM (Hudes *et al.*, 2007), which is still more than 25 times lower than the rapamycin
289 concentration required to half-maximally activate TRPML1 (Zhang *et al.*, 2019). Thus, the
290 rapamycin-mediated TRPML1 activation, while observed *in vitro*, is not likely to be
291 physiologically relevant for research and therapeutics. Furthermore, rapamycin doses aimed at
292 slowing or reversing ageing are generally even lower than those used for immunosuppression
293 and cancer treatment (Bitto *et al.*, 2016; Bjedov *et al.*, 2010; Castillo-Quan *et al.*, 2019; Fok *et*
294 *al.*, 2014; Harrison *et al.*, 2009; Robida-Stubbs *et al.*, 2012; Schinaman *et al.*, 2019), further
295 suggesting that TRPML1 activation is unlikely to be involved in rapamycin-mediated lifespan
296 extension.

297

298 Rapamycin acts an allosteric mTOR inhibitor in a complex with FKBP12 and other FKBP
299 family members (Chung *et al.*, 1992; Marz *et al.*, 2013). Most FKBP family members possess peptidyl-prolyl
300 isomerase (PPIase) activity, hence functioning as protein folding chaperones for a variety of
301 different proteins (Harrar *et al.*, 2001; Kolos *et al.*, 2018). For instance, FKBP12 and FKBP12.6
302 have been shown to bind and modulate the activity of ryanodine receptors (RyRs) and inositol
303 1,4,5-trisphosphate receptors (IP3Rs) (Bultynck *et al.*, 2001; Galfre *et al.*, 2012; Vervliet *et al.*,
304 2015), which are channels involved in intracellular calcium release. FKBP12 has also been
305 shown to inhibit TGF β family type I receptors (Wang *et al.*, 1996). Likewise, the larger
306 FKBP51 and FKBP52 chaperones control glucocorticoid receptor (GR) localization and
307 activity (Fries *et al.*, 2017) as well as the protein levels of Argonaute 2 (AGO2) (Martinez *et al.*,
308 2013), an essential component of the RNA-induced silencing complex (RISC). Hence, it would
309 be reasonable to speculate that binding of rapamycin to different FKBP proteins may influence
310 the folding—and thus function—of client proteins, also beyond mTOR inhibition. Because any
311 changes in receptor or signaling pathway activities, organelle function, metabolism, or other
312 cellular processes are eventually translated to changes in gene or protein expression, we here
313 analyzed the cellular transcriptome and proteome to interrogate the effects of rapamycin

314 treatment in an unbiased manner. These experiments revealed a striking dependence of
315 rapamycin on mTOR inhibition to influence cells, with mTOR^{RR}-expressing cells
316 demonstrating virtually no effects upon treatment with this drug. In sum, although rapamycin
317 could—in theory—affect cells also independently from mTOR inhibition (via TRPML1 or
318 through FKBP-dependent mechanisms), this is not the case, at least for rapamycin
319 concentrations that are within the nanomolar range used in research or found in the blood of
320 patients treated with this drug or its analogs.

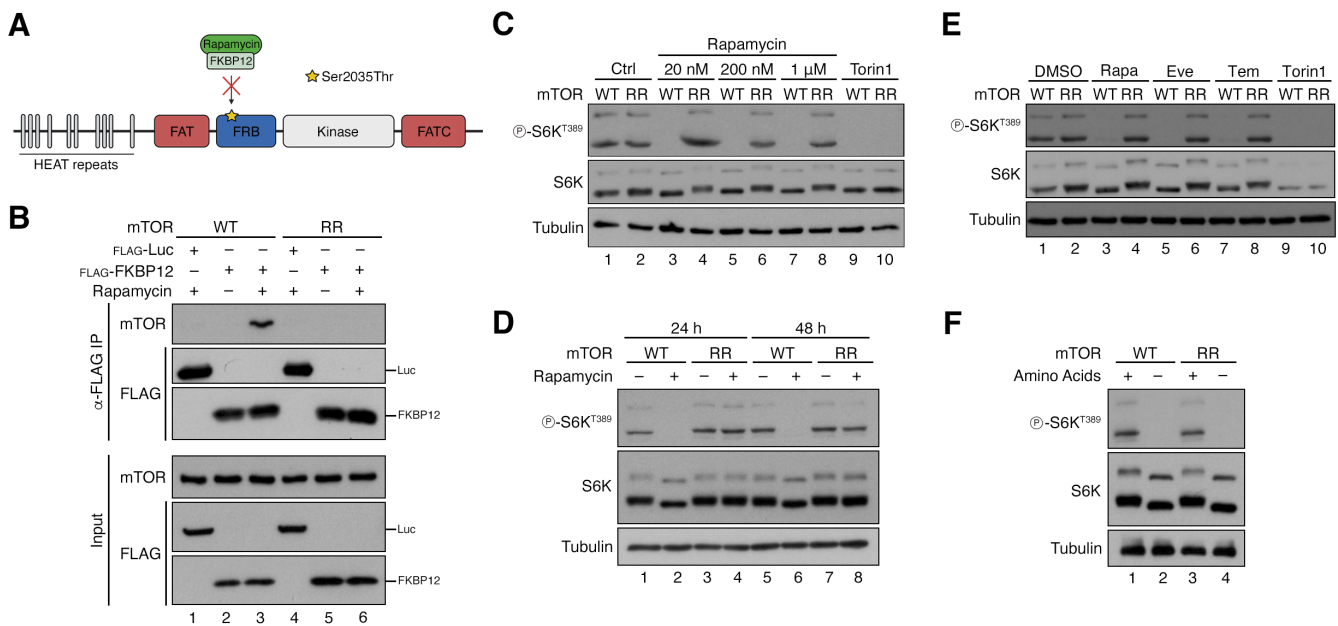
321
322 In addition to assessing rapamycin's specificity towards mTOR, we here also investigated how
323 rapamycin influences gene expression and whole-cell protein levels in control cells that express
324 wild-type mTOR. Consistent with the role of mTORC1 in regulating cap-dependent translation
325 (Fonseca *et al*, 2014; Holz *et al*, 2005; Liu & Sabatini, 2020; Ma & Blenis, 2009; Thoreen *et*
326 *al.*, 2012) and with rapamycin's ability to repress both global and specific translation of key
327 subsets of transcripts (Dickinson *et al*, 2011; Huo *et al*, 2011; Nandagopal & Roux, 2015;
328 Tsukumo *et al*, 2016; Wang *et al*, 2007), these transcriptomics and proteomics datasets
329 identified several thousands of genes and proteins whose expression changes in rapamycin-
330 treated cells. For instance, highlighting the well-described role of rapamycin/mTOR on
331 ribosomal biogenesis, rapamycin treatment strongly downregulated the expression of genes
332 encoding for ribosomal proteins, tRNA synthetases (Kim *et al*, 2017; Lee *et al*, 2012) and other
333 accessory proteins involved in this process (Mayer & Grummt, 2006; Powers & Walter, 1999)
334 (Fig. 2 and EV2). Accordingly, we observed a strong enrichment of proteins related to non-
335 coding RNA metabolic processes and ribonucleoprotein complex biogenesis among those
336 downregulated by rapamycin (Fig. 3 and EV3). Furthermore, both our RNA-seq and proteomics
337 data also confirm previous studies about rapamycin's role in regulating mitochondrial function
338 (Morita *et al*, 2017; Ramanathan & Schreiber, 2009; Rosario *et al*, 2019; Schieke *et al*, 2006;
339 Villa-Cuesta *et al*, 2014) (Fig. 2, 3 and EV2, 3). Interestingly, we find that proteins that are

340 associated with adherens/anchoring junctions are enriched among those downregulated by
341 rapamycin, which may suggest a role for mTOR in the regulation of cell-cell and cell-matrix
342 contacts (Fig. 3E and EV3B). Finally, we observe an enrichment of terms associated with cell
343 cycle and mitosis in the rapamycin-dependent proteome, possibly reflecting the anti-
344 proliferative effects of rapamycin (Zaragoza *et al*, 1998) and the role of mTOR in cell
345 proliferation (Dowling *et al.*, 2010).

346

347 Overall, we here provide an unbiased, comprehensive evaluation of rapamycin's specificity
348 towards mTOR in mammalian cells, in unprecedented depth. Given the immense potential that
349 rapamycin and its analogs have as anti-ageing compounds or in therapeutics, these findings
350 provide important insight for both basic and translational research and aim at improving the
351 applicability and specificity of its use in humans.

352



353

354 **Figure 1. Generation and characterization of a gene-edited cell line that expresses**
 355 **rapamycin-resistant mTOR.**

356 **(A)** Schematic model of the mTOR protein depicting key domains and the location of the
 357 Ser2035Thr substitution in the FRB domain that prevents its inhibition by rapamycin/FKBP12.

358 **(B)** Diminished binding of FKBP12 to the mTOR^{RR} mutant protein. Co-immunoprecipitation
 359 experiments in control (WT) or mTOR^{RR} HEK293FT cells, transiently expressing FLAG-
 360 tagged FKBP12 or Luciferase (Luc) as negative control, treated with rapamycin (20 nM, 1 h)
 361 or DMSO. Binding of mTOR to FKBP12 was analyzed by immunoblotting as indicated.

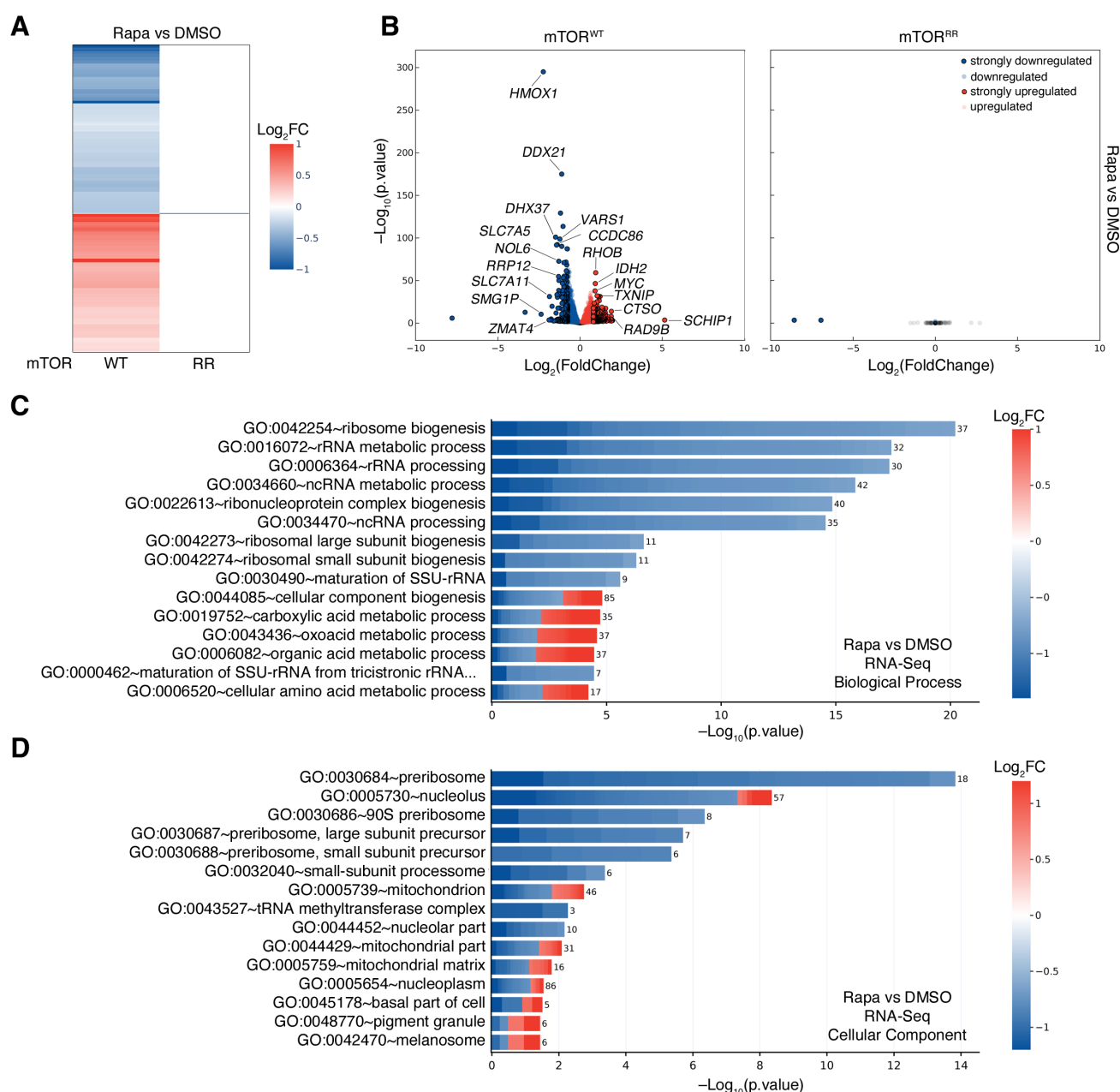
362 **(C)** mTOR^{RR} does not respond to rapamycin even at extremely high concentrations.
 363 Immunoblots with lysates from HEK293FT WT and mTOR^{RR} cells treated for 1 h with 20 nM
 364 to 1 μM rapamycin as indicated, 250 nM Torin1, or DMSO as control. mTORC1 activity was
 365 assessed by S6K phosphorylation.

366 **(D)** mTOR^{RR} is resistant to long-term rapamycin treatment. Immunoblots with lysates from
 367 HEK293FT WT and mTOR^{RR} cells treated with DMSO or rapamycin (20 nM) for 24 or 48 h,
 368 probed with the indicated antibodies.

369 **(E)** mTOR^{RR} shows resistance to multiple rapalogs. Immunoblots with lysates from HEK293FT
370 WT and mTOR^{RR} cells treated with DMSO, rapamycin (Rapa; 20 nM), everolimus (Eve; 20
371 nM), temsirolimus (Tem; 20 nM), or Torin1 (250 nM) for 1 h, probed with the indicated
372 antibodies.

373 **(F)** Cell expressing mTOR^{RR} have active mTORC1 that responds properly to AA starvation.
374 Immunoblots with lysates from HEK293FT WT and mTOR^{RR} cells treated with control (+) or
375 AA-free media (-) for 1 h, probed with the indicated antibodies.

376



377

378 **Figure 2. Rapamycin alters gene expression exclusively via mTOR inhibition.**

379 **(A)** Heatmap depicting the rapamycin-induced changes in gene expression in HEK293FT WT
 380 and mTOR^{RR} cells. RNA-Seq data from cells treated with rapamycin (20 nM, 24 h) expressed
 381 as log-transformed fold change (Log₂FC). Only statistically-significant changes (p < 0.05) are
 382 shown.

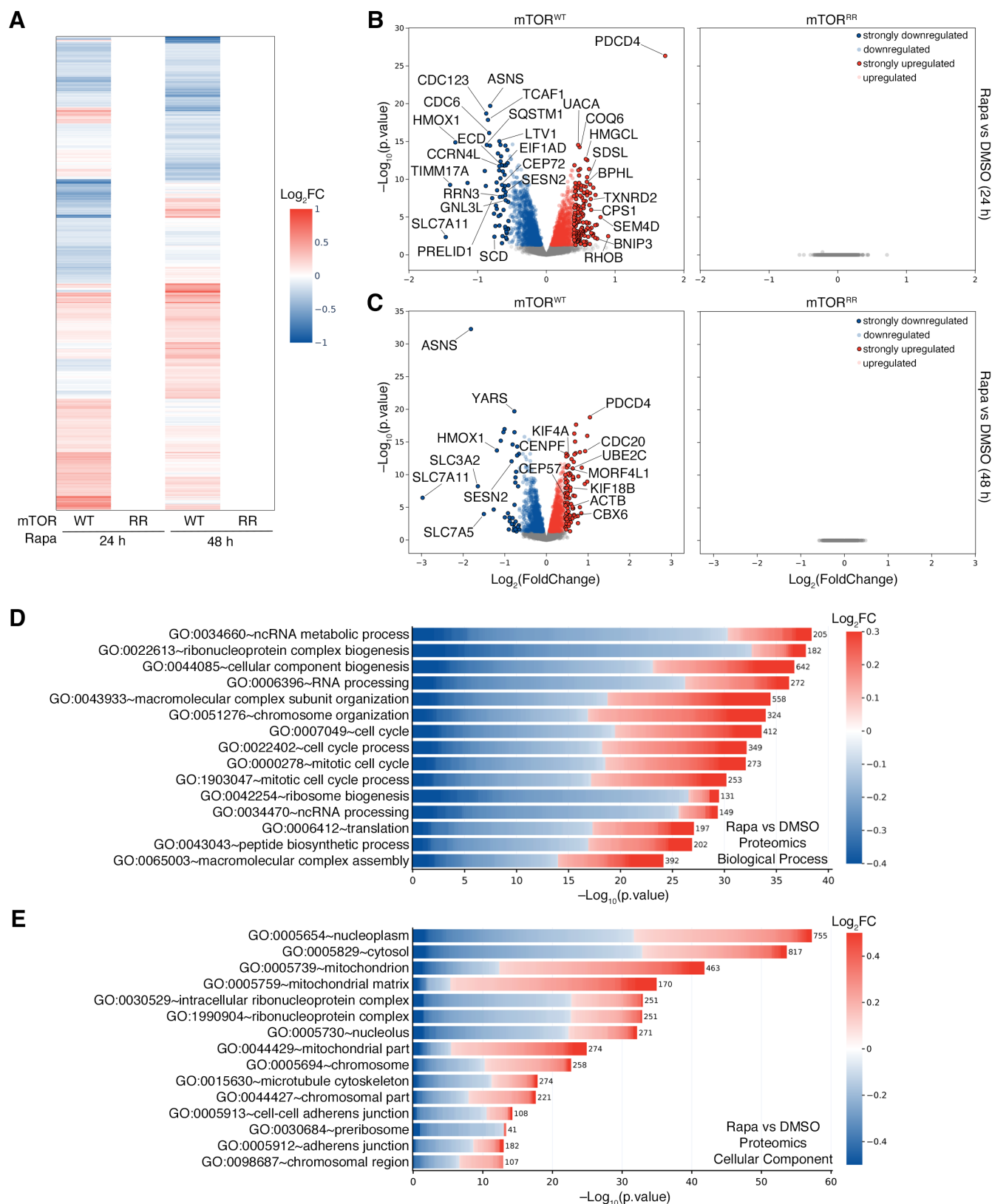
383 **(B)** Volcano plots showing the rapamycin-induced changes in gene expression in HEK293FT
 384 WT (left) and mTOR^{RR} (right) cells from the RNA-Seq experiment described in (A). Genes
 385 that are significantly (p < 0.05) down- or upregulated by rapamycin are shown in blue or red

386 respectively. Strongly down- ($\text{Log}_2\text{FC} < -0.75$) or upregulated ($\text{Log}_2\text{FC} > +0.8$) genes are
387 shown with black outline. Unaffected genes ($p > 0.05$) shown as grey dots. Selected genes are
388 marked in the plot.

389 **(C)** Biological process (BP) GO term analysis using the genes that are strongly down- (blue) or
390 upregulated (red) by rapamycin in WT cells as described in (B). The color of each box in the
391 cell plot represents log-transformed fold change values for each gene in rapamycin- vs DMSO-
392 treated cells. The number of genes in the selected dataset for each GO term is shown on the
393 right side of each bar.

394 **(D)** As in (C), but for Cellular Component (CC) GO term analysis.

395



396

397 **Figure 3. Rapamycin alters the cellular proteome exclusively via mTOR inhibition.**

398 **(A)** Heatmap depicting the rapamycin-induced changes in the proteome of HEK293FT WT and

399 mTOR^{RR} cells. Shown are whole-proteome quantitative mass-spectrometry data from cells

400 treated with rapamycin (20 nM) for 24 or 48 h expressed as log-transformed fold change
401 (Log_2FC). Only statistically-significant changes ($p < 0.05$) are shown.

402 **(B)** Volcano plots showing the rapamycin-induced proteomic changes in HEK293FT WT (left)
403 and mTOR^{RR} (right) cells from the mass-spectrometry experiment described in (A). Proteins
404 that are significantly ($p < 0.05$) down- or upregulated by rapamycin (20 nM, 24 h) are shown
405 in blue or red respectively. Most strongly down- ($\text{Log}_2\text{FC} < -0.55$) or upregulated ($\text{Log}_2\text{FC} >$
406 $+0.4$) proteins are shown with black outline. Unaffected proteins ($p > 0.05$) shown as grey dots.
407 Selected proteins are marked in the plot.

408 **(C)** As in (B), but for cells treated with 20 nM rapamycin (or DMSO) for 48 h. Strongly down-
409 ($\text{Log}_2\text{FC} < -0.65$) or upregulated ($\text{Log}_2\text{FC} > +0.45$) proteins are shown with black outline.

410 **(D)** Biological process (BP) GO term analysis using the proteins that are significantly down-
411 (blue) or upregulated (red) by 24 h rapamycin in WT cells as described in (B). The color of
412 each box in the cell plot represents log-transformed fold change values for each protein in
413 rapamycin- vs DMSO-treated cells. The number of proteins in the selected dataset for each GO
414 term is shown on the right side of each bar.

415 **(E)** As in (D), but for Cellular Component (CC) GO term analysis.

416

417

418 **Methods**

419 **Cell culture**

420 All cell lines were grown at 37 °C, 5% CO₂. Human female embryonic kidney HEK293FT cells
421 (#R70007, Invitrogen; RRID: CVCL_6911) were cultured in high-glucose Dulbecco's
422 Modified Eagle Medium (DMEM) (#41965-039, Gibco) supplemented with 10% fetal bovine
423 serum (FBS) (#F7524, Sigma, or #S1810, Biowest) and 1% Penicillin-Streptomycin (#15140-
424 122, Gibco).

425

426 HEK293FT cells were purchased from Invitrogen. The identity of the HEK293FT cells was
427 validated by the Multiplex human Cell Line Authentication test (Multiplexion GmbH), which
428 uses a single nucleotide polymorphism (SNP) typing approach, and was performed as described
429 at www.multiplexion.de. All parental and edited cell lines were regularly tested for
430 *Mycoplasma* contamination using a PCR-based approach and were confirmed to be
431 *Mycoplasma*-free.

432

433 **Cell culture treatments**

434 To allosterically inhibit mTOR, rapamycin (#S1039, Selleckchem), everolimus (#S1120,
435 Selleckchem) or temsirolimus (# S1044, Selleckchem) were dissolved in DMSO and added
436 directly into full cell culture media at a final concentration of 20 nM unless otherwise indicated
437 in the figure legends. Treatments were performed for the times described in the figure legends.
438 DMSO was used as a negative control for all treatments. Torin1 (#4247, Tocris) was used as
439 an ATP-competitive mTOR inhibitor and added in the culture media at a final concentration of
440 250 nM for 1 hour.

441

442 Amino acid (AA) starvation experiments were performed as described previously (Demetriades
443 *et al*, 2014; Demetriades *et al*, 2016). In brief, custom-made starvation media were formulated

444 according to the Gibco recipe for high-glucose DMEM specifically omitting all AAs. The
445 media were filtered through a 0.22- μ m filter device and tested for proper pH and osmolality
446 before use. For the respective AA-replete (+AA) treatment media, commercially available high-
447 glucose DMEM was used (#41965039, Thermo Fisher Scientific). All treatment media were
448 supplemented with 10% dialyzed FBS (dFBS) and 1x Penicillin-Streptomycin (#15140-122,
449 Gibco). For this purpose, FBS was dialyzed against 1x PBS through 3,500 MWCO dialysis
450 tubing. For basal (+AA) conditions, the culture media were replaced with +AA treatment media
451 1 hour before lysis. For amino-acid starvation (-AA), culture media were replaced with
452 starvation media for 1 hour.

453

454 **Antibodies**

455 Antibodies against phospho-p70 S6K (Thr389) (#9205), p70 S6K (#9202 for Fig. 1C,E; #97596
456 for Fig. 1D,F), FLAG (#2368) and mTOR (#2983) were purchased from Cell Signaling
457 Technology. The anti-human tubulin (#T9026) antibody was purchased from Sigma.

458

459 **Plasmid DNA transfections**

460 Plasmid DNA transfections in HEK293FT cells were performed using Effectene transfection
461 reagent (#301425, QIAGEN) according to the manufacturer's instructions.

462

463 **Generation of the gene-edited mTOR^{RR} cell line**

464 The rapamycin-resistant mTOR (mTOR^{RR}) HEK293FT cell line was generated by gene-editing
465 using the pX459-based CRISPR/Cas9 method as described elsewhere (Ran *et al*, 2013). The
466 sgRNA expression vectors were generated by cloning appropriate DNA oligonucleotides
467 (Suppl. Table 8) in the BbsI restriction sites of pX459 (#62988, Addgene). In brief, transfected
468 cells were selected with 3 μ g/ml puromycin (#A11138-03, Thermo Fisher Scientific) 48 hours

469 post-transfection. Single-cell clones were generated by FACS sorting and individual clones
470 were validated by genomic DNA sequencing and functional assays.

471

472 **Gene expression analysis (RNA-Seq)**

473 To analyze gene expression changes via RNA-seq experiments, total mRNA was isolated using
474 QIAshredder columns (#79656, QIAGEN) and the RNeasy Plus Mini Kit (#74034, QIAGEN)
475 according to the manufacturer's instructions. RNA-seq experiments were performed by the
476 Max Planck Genome Centre (MPGC) Cologne, Germany (<https://mpgc.mpipz.mpg.de/home/>).
477 RNA quality was assessed with an Agilent Bioanalyzer (Nanochip). Library preparation was
478 done according to NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina
479 (#E7760L, New England Biolabs) including polyA enrichment and addition the of ERCC RNA
480 spike-ins. Libraries were quality controlled by Agilent TapeStation or LabChip GX or GX
481 Touch (PerkinElmer). Sequencing-by-synthesis was performed on a HiSeq 3000 (Illumina)
482 with single read mode 1 x 150 bp. Data from one representative RNA-seq experiment, out of
483 two independent replicates, are shown in this manuscript. Each experiment was performed from
484 3 independent biological replicates. The raw data from both RNA-seq experiments are available
485 in the NCBI Sequence Read Archive (see also the Data Availability section).

486

487 **Cell lysis and immunoblotting**

488 For standard SDS-PAGE and immunoblotting experiments, cells from a well of a 12-well plate
489 were treated as indicated in the figures and lysed in 250 µl of ice-cold Triton lysis buffer (50
490 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 2 mM Na-vanadate, 0.011
491 gr/ml beta-glycerophosphate) supplemented with 1x PhosSTOP phosphatase inhibitors
492 (#4906837001, Roche) and 1x cOmplete protease inhibitors (#11836153001, Roche) for 10
493 minutes on ice. Samples were clarified by centrifugation (15000 rpm, 10 min, 4 °C) and
494 supernatants were boiled in 1x SDS sample buffer (5x SDS sample buffer: 350 mM Tris-HCl

495 pH 6.8, 30% glycerol, 600 mM DTT, 12.8% SDS, 0.12% bromophenol blue). Protein samples
496 were subjected to electrophoretic separation on SDS-PAGE and analyzed by standard Western
497 blotting techniques. In brief, proteins were transferred to nitrocellulose membranes (#10600002
498 or #10600001, Amersham) and stained with 0.2% Ponceau solution (#33427-01, Serva) to
499 confirm equal loading. Membranes were blocked with 5% skim milk powder (#42590, Serva)
500 in PBS-T [1x PBS, 0.1% Tween-20 (#A1389, AppliChem)] for 1 hour at room temperature,
501 washed three times for 10 min with PBS-T and incubated with primary antibodies [1:1000 in
502 PBS-T, 5% bovine serum albumin (BSA; #10735086001, Roche)] rotating overnight at 4 °C.
503 The next day, membranes were washed three times for 10 min with PBS-T and incubated with
504 appropriate HRP-conjugated secondary antibodies (1:10000 in PBS-T, 5% milk) for 1 hour at
505 room temperature. Signals were detected by enhanced chemiluminescence (ECL) using the
506 ECL Western Blotting Substrate (#W1015, Promega) or SuperSignal West Pico PLUS
507 (#34577, Thermo Scientific) and SuperSignal West Femto Substrate (#34095, Thermo
508 Scientific) for weaker signals. Immunoblot images were captured on films (#28906835, GE
509 Healthcare; #4741019289, Fujifilm).

510

511 **Co-immunoprecipitation (co-IP)**

512 For co-IP experiments, 1.5×10^6 cells were transiently transfected with the indicated plasmids
513 and lysed 36 h post-transfection in IP lysis buffer (50 mM Tris pH 7.5, 0.3% CHAPS, 150 mM
514 NaCl, 50 mM NaF, 2 mM Na-vanadate, 0.011 g/ml β -glycerophosphate, 1x PhosSTOP
515 phosphatase inhibitors and 1x cOmplete protease inhibitors). FLAG-tagged proteins were
516 incubated with 30 μ l pre-washed anti-FLAG M2 affinity gel (Sigma, #A2220) for 3 h at 4 °C
517 and washed four times with IP wash buffer (50 mM Tris pH 7.5, 0.3% CHAPS, 150 mM NaCl
518 and 50 mM NaF). Samples were then boiled for 6 min in 1x SDS sample buffer and analysed
519 by immunoblotting using appropriate antibodies.

520

521 **Quantitative whole-cell proteomics**

522 For mass-spectrometry experiments, HEK293FT cells were cultured in 10 cm dishes in 10 ml
523 of complete culture media as described above. Rapamycin (or DMSO as negative control) were
524 added directly in the culture media for 24 or 48 hours. Experiments were performed with 5
525 independent biological replicates per condition (1x 10 cm dish per replicate). In brief, cells
526 were scraped, collected in 1.5 ml tubes on ice, washed in serum-free media, pelleted by
527 centrifugation (500 x g, 3 min), and cell pellets were snap-frozen in liquid nitrogen and stored
528 at -80 °C.

529 *Sample preparation*

530 For sample preparation for quantitative proteomic analysis, cell pellets were lysed in 6 M
531 guanidinium chloride (GdmCl) supplemented with 2.5 mM TCEP (tris(2-
532 carboxyethyl)phosphine), 10 mM CAA (chloroacetamide) and 100 mM Tris-HCl at room
533 temperature. Samples were boiled at 95 °C for 10 min and sonicated for 30 sec for 10 cycles,
534 with 30 sec breaks on high-performance mode with Bioruptor Plus (#B01020001, Diagenode).
535 Samples were centrifuged at 20000 x g for 20 min at RT, supernatants were diluted 10 times
536 with 20 mM Tris and protein concentration was measured using Nanodrop 2000 (#ND-2000,
537 Thermo Fischer Scientific). Three hundred micrograms of each sample were diluted 10 times
538 with 20 mM Tris and digested with 1.5 µl of Mass Spectrometry Grade Trypsin Gold (#V5280,
539 Promega) at 37 °C overnight. Digestion was stopped by adding 50% FA to the reaction at a
540 final concentration of 1%. Samples were centrifuged at 20000 x g for 10 min at RT and
541 supernatants were collected. C-18-SD StageTips were washed and equilibrated sequentially
542 with 200 µl methanol, 200 µl 40% ACN (acetonitrile) / 0.1% FA (formic acid) and 200 µl 0.1%
543 FA by centrifugation, each step for 1 min at RT. Samples were diluted with 0.1% FA, loaded
544 in StageTips and centrifuged for 1-2 min at RT. StageTips were then washed twice with 200 µl
545 0.1% FA. Tryptic peptides were eluted from StageTips with 100 µl 40% acetonitrile (ACN) /
546 0.1% formic acid (FA) by centrifugation (300 x g, 4 min, RT). Eluates were dried in a Speed-

547 Vac at 45 °C for 40-45 min and resuspended in 20 µl 0.1% FA. Four micrograms of the peptides
548 were dried in a Speed-Vac and stored at -20°C.

549 *TMT10plex labelling*

550 The dried tryptic peptides were reconstituted in 9 µl of 0.1 M TEAB (triethylammonium
551 bicarbonate). Tandem mass tag (TMT10plex; #90110, Thermo Fisher Scientific) labelling was
552 carried out according to the manufacturer's instructions with the following changes: 0.8 mg of
553 TMT10plex reagent was re-suspended with 70 µl anhydrous ACN. Seven microliters of
554 TMT10plex reagent in ACN were added to 9 µl of clean peptide in 0.1 M TEAB. The final
555 ACN concentration was 43.75% and the ratio of peptides to TMT10plex reagent was 1:20.
556 After 60 min incubation, the reaction was quenched with 2 µl 5% hydroxylamine. Labelled
557 peptides were pooled, dried, re-suspended in 200 µl 0.1% FA, split in two equal parts and
558 desalted using home-made STAGE tips (Li *et al*, 2021).

559 *Fractionation of TMT10plex-labeled peptide mixture*

560 One of the two parts was fractionated on a 1 mm x 150 mm ACQUITY column, packed with
561 130 Å, 1.7 µm C18 particles (#186006935, Waters) using an Ultimate 3000 UHPLC (Thermo
562 Fisher Scientific). Peptides were separated at a flow of 30 µl/min with an 88 min segmented
563 gradient from 1% to 50% buffer B for 85 min and from 50% to 95% buffer B for 3 min; buffer
564 A was 5% ACN, 10 mM ammonium bicarbonate, buffer B was 80% ACN, 10 mM ammonium
565 bicarbonate. Fractions were collected every three minutes, pooled in two passes (fraction 1 +
566 17, fraction 2 + 18, ... , etc.) and dried in a vacuum centrifuge (Eppendorf).

567 *LC-MS/MS analysis*

568 Dried fractions were re-suspended in 0.1% FA, separated on a 50 cm, 75 µm Acclaim PepMap
569 column (#164942, Thermo Fisher Scientific) and analyzed on a Orbitrap Lumos Tribrid mass
570 spectrometer (Thermo Fisher Scientific) equipped with a FAIMS device (Thermo Fisher
571 Scientific). The FAIMS device was operated in two compensation voltages, -50 V and -70 V.
572 Synchronous precursor selection based MS3 was used for the acquisition of the TMT10plex

573 reporter ion signals. Peptide separations were performed on an EASY-nLC1200 using a 90 min
574 linear gradient from 6% to 31% buffer; buffer A was 0.1% FA, buffer B was 0.1% FA, 80%
575 ACN. The analytical column was operated at 50 °C. Raw files were split based on the FAIMS
576 compensation voltage using FreeStyle (Thermo Fisher Scientific).

577 *Data analysis*

578 Proteomics data was analyzed using MaxQuant, version 1.6.17.0 (Cox & Mann, 2008). The
579 isotope purity correction factors, provided by the manufacturer, were included in the analysis.
580 Differential expression analysis was performed using limma, version 3.34.9 (Ritchie *et al*,
581 2015) in R, version 3.4.3 (Team, 2017).

582

583 **Gene Ontology analysis and Data presentation**

584 Gene Ontology (GO) term enrichment analysis was performed using the Database for
585 Annotation, Visualization and Integrated Discovery (DAVID) tool (Huang *da et al*, 2009a, b)
586 of the Flaski toolbox (Iqbal, 2021) (<https://flaski.age.mpg.de>, developed and provided by the
587 MPI-AGE Bioinformatics core facility). For the RNA-Seq experiments, either all significantly
588 changing genes ($p < 0.05$) or selected genes, whose expression levels changed significantly
589 between rapamycin- and DMSO-treated cells with Log_2 -transformed fold change (Log_2FC)
590 values less than -0.75 (downregulated by rapamycin treatment) or higher than +0.8 (upregulated
591 by rapamycin treatment), roughly corresponding to the top and bottom 5% of the dataset, were
592 used for the DAVID GO analysis (for GOTERM_CC_FAT, GOTERM_BP_FAT). The
593 selection criteria for each analysis are described in the respective figure legends.

594

595 For the DAVID GO analysis of quantitative proteomics experiments either all significantly
596 changing proteins ($p < 0.05$) or selected proteins whose intensity changes significantly between
597 rapamycin- and DMSO-treated cells with $\text{Log}_2\text{FC} < -0.55$ and $\text{Log}_2\text{FC} > +0.4$ (24 h treatments)

598 or $\text{Log}_2\text{FC} < -0.65$ and $\text{Log}_2\text{FC} > 0.45$ (48 h treatments) were used (for GOTERM_CC_FAT,
599 GOTERM_BP_FAT). The human proteome was used as reference list for all analyses.

600

601 Cell plots were generated using the DAVID and Cell plot apps in Flaski and include the 15
602 most significant GO terms from each analysis. The full list of genes/proteins that were detected
603 in each experiment (gray dots) was used for generating the Volcano plots. The respective graphs
604 were prepared using the Scatter plot app in Flaski and labelled in Adobe Photoshop (v. 23.4.2).
605 Significantly-changing genes/proteins are represented by blue (downregulated by rapamycin)
606 or red (upregulated by rapamycin) dots. Proteins/genes whose levels change strongly upon
607 rapamycin treatment (based on the selection criteria described above for each experiment) are
608 shown as blue or red dots with black outline.

609

610

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623

624 **Author Contributions**

625 Experimental work: FA, NG; data analysis: FA, CD; project design & conceptualization: CD;
626 project supervision: CD; funding acquisition: CD; figure preparation: FA, NG, CD; manuscript
627 draft: FA, CD. All authors approved the final version of the manuscript and agree on the content
628 and conclusions.

629

630 **Declaration of interests**

631 The authors declare no competing interests.

632

633 **Data availability**

634 The data that support the findings of this study (uncropped immunoblots) or any additional
635 information required to reanalyze the data reported in this paper are available from the

636 corresponding author upon reasonable request. Other datasets that are produced in this study
637 are available in the following databases:

- 638 • RNA-Seq data: NCBI Sequence Read Archive (SRA) [PRJNA872474](https://www.ncbi.nlm.nih.gov/sra/PRJNA872474)
639 (www.ncbi.nlm.nih.gov/sra/PRJNA872474).
- 640 • Mass spectrometry proteomics: PRIDE (Perez-Riverol *et al*, 2022) [PXD038051](https://www.ebi.ac.uk/pride/archive/projects/PXD038051)
641 (www.ebi.ac.uk/pride/archive/projects/PXD038051).

642

643 **Code availability**

644 No code was generated in this study.

645

646 **Additional Information**

647 Supplementary Information (Expanded View Figures 1-4 and Supplementary Tables 1-8) is
648 available for this paper.

649

650 Correspondence and requests for materials should be addressed to Constantinos Demetriades
651 (Demetriades@age.mpg.de).

652

653 **References**

- 654 (EMA) EMA, 2005. Rapamune: EPAR - Scientific Discussion.
- 655 Arriola Apelo SI, Lamming DW (2016) Rapamycin: An InhibiTOR of Aging Emerges From the Soil of
656 Easter Island. *J Gerontol A Biol Sci Med Sci* 71: 841-849
- 657 Asrani K, Sood A, Torres A, Georgess D, Phatak P, Kaur H, Dubin A, Talbot CC, Jr., Elhelu L, Ewald
658 AJ *et al* (2017) mTORC1 loss impairs epidermal adhesion via TGF-beta/Rho kinase activation. *J Clin*
659 *Invest* 127: 4001-4017
- 660 Bain J, McLauchlan H, Elliott M, Cohen P (2003) The specificities of protein kinase inhibitors: an
661 update. *Biochem J* 371: 199-204
- 662 Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR,
663 Cohen P (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408: 297-315
- 664 Bayeva M, Khechaduri A, Puig S, Chang HC, Patial S, Blackshear PJ, Ardehali H (2012) mTOR
665 regulates cellular iron homeostasis through tristetraprolin. *Cell Metab* 16: 645-657
- 666 Benjamin D, Colombi M, Moroni C, Hall MN (2011) Rapamycin passes the torch: a new generation of
667 mTOR inhibitors. *Nat Rev Drug Discov* 10: 868-880
- 668 Bitto A, Ito TK, Pineda VV, LeTexier NJ, Huang HZ, Sutlief E, Tung H, Vizzini N, Chen B, Smith K
669 *et al* (2016) Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice.
670 *Elife* 5
- 671 Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L (2010) Mechanisms of life
672 span extension by rapamycin in the fruit fly *Drosophila melanogaster*. *Cell Metab* 11: 35-46
- 673 Bonner JM, Boulianne GL (2017) Diverse structures, functions and uses of FK506 binding proteins.
674 *Cell Signal* 38: 97-105
- 675 Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL (1995) Control of p70 s6 kinase by kinase
676 activity of FRAP in vivo. *Nature* 377: 441-446
- 677 Bultynck G, De Smet P, Rossi D, Callewaert G, Missiaen L, Sorrentino V, De Smedt H, Parys JB (2001)
678 Characterization and mapping of the 12 kDa FK506-binding protein (FKBP12)-binding site on different
679 isoforms of the ryanodine receptor and of the inositol 1,4,5-trisphosphate receptor. *Biochem J* 354: 413-
680 422
- 681 Castillo-Quan JI, Tain LS, Kinghorn KJ, Li L, Gronke S, Hinze Y, Blackwell TK, Bjedov I, Partridge
682 L (2019) A triple drug combination targeting components of the nutrient-sensing network maximizes
683 longevity. *Proc Natl Acad Sci U S A* 116: 20817-20819

- 684 Chen J, Zheng XF, Brown EJ, Schreiber SL (1995) Identification of an 11-kDa FKBP12-rapamycin-
685 binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a
686 critical serine residue. *Proc Natl Acad Sci U S A* 92: 4947-4951
- 687 Chen L, Xu B, Liu L, Liu C, Luo Y, Chen X, Barzegar M, Chung J, Huang S (2015) Both mTORC1
688 and mTORC2 are involved in the regulation of cell adhesion. *Oncotarget* 6: 7136-7150
- 689 Choi J, Chen J, Schreiber SL, Clardy J (1996) Structure of the FKBP12-rapamycin complex interacting
690 with the binding domain of human FRAP. *Science* 273: 239-242
- 691 Chung J, Kuo CJ, Crabtree GR, Blenis J (1992) Rapamycin-FKBP specifically blocks growth-dependent
692 activation of and signaling by the 70 kd S6 protein kinases. *Cell* 69: 1227-1236
- 693 Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range
694 mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26: 1367-1372
- 695 Creevy KE, Akey JM, Kaeberlein M, Promislow DEL, Dog Aging Project C (2022) An open science
696 study of ageing in companion dogs. *Nature* 602: 51-57
- 697 Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some
698 commonly used protein kinase inhibitors. *Biochem J* 351: 95-105
- 699 Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallares G, Hocker M, Treiber DK, Zarrinkar
700 PP (2011) Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol* 29: 1046-1051
- 701 Demetriades C, Doumpas N, Teleman AA (2014) Regulation of TORC1 in response to amino acid
702 starvation via lysosomal recruitment of TSC2. *Cell* 156: 786-799
- 703 Demetriades C, Plescher M, Teleman AA (2016) Lysosomal recruitment of TSC2 is a universal response
704 to cellular stress. *Nat Commun* 7: 10662
- 705 Dickinson JM, Fry CS, Drummond MJ, Gundermann DM, Walker DK, Glynn EL, Timmerman KL,
706 Dhanani S, Volpi E, Rasmussen BB (2011) Mammalian target of rapamycin complex 1 activation is
707 required for the stimulation of human skeletal muscle protein synthesis by essential amino acids. *J Nutr*
708 141: 856-862
- 709 Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M (2006) S6K1- and
710 betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science* 314:
711 467-471
- 712 Dowling RJ, Topisirovic I, Alain T, Bidinosti M, Fonseca BD, Petroulakis E, Wang X, Larsson O,
713 Selvaraj A, Liu Y *et al* (2010) mTORC1-mediated cell proliferation, but not cell growth, controlled by
714 the 4E-BPs. *Science* 328: 1172-1176
- 715 Farag SS, Zhang S, Jansak BS, Wang X, Kraut E, Chan K, Dancey JE, Grever MR (2009) Phase II trial
716 of temsirolimus in patients with relapsed or refractory multiple myeloma. *Leuk Res* 33: 1475-1480

- 717 Fedorov O, Marsden B, Pogacic V, Rellos P, Muller S, Bullock AN, Schwaller J, Sundstrom M, Knapp
718 S (2007) A systematic interaction map of validated kinase inhibitors with Ser/Thr kinases. *Proc Natl*
719 *Acad Sci U S A* 104: 20523-20528
- 720 Fernandes SA, Demetriades C (2021) The Multifaceted Role of Nutrient Sensing and mTORC1
721 Signaling in Physiology and Aging. *Front Aging* 2: 707372
- 722 Fok WC, Chen Y, Bokov A, Zhang Y, Salmon AB, Diaz V, Javors M, Wood WH, 3rd, Zhang Y, Becker
723 KG *et al* (2014) Mice fed rapamycin have an increase in lifespan associated with major changes in the
724 liver transcriptome. *PLoS One* 9: e83988
- 725 Fonseca BD, Smith EM, Yelle N, Alain T, Bushell M, Pause A (2014) The ever-evolving role of mTOR
726 in translation. *Semin Cell Dev Biol* 36: 102-112
- 727 Fries GR, Gassen NC, Rein T (2017) The FKBP51 Glucocorticoid Receptor Co-Chaperone: Regulation,
728 Function, and Implications in Health and Disease. *Int J Mol Sci* 18
- 729 Galfre E, Pitt SJ, Venturi E, Sitsapesan M, Zaccai NR, Tsaneva-Atanasova K, O'Neill S, Sitsapesan R
730 (2012) FKBP12 activates the cardiac ryanodine receptor Ca²⁺-release channel and is antagonised by
731 FKBP12.6. *PLoS One* 7: e31956
- 732 Ge Y, Wu AL, Warnes C, Liu J, Zhang C, Kawasome H, Terada N, Boppart MD, Schoenherr CJ, Chen
733 J (2009) mTOR regulates skeletal muscle regeneration in vivo through kinase-dependent and kinase-
734 independent mechanisms. *Am J Physiol Cell Physiol* 297: C1434-1444
- 735 Goodman CA, Frey JW, Mabrey DM, Jacobs BL, Lincoln HC, You JS, Hornberger TA (2011) The role
736 of skeletal muscle mTOR in the regulation of mechanical load-induced growth. *J Physiol* 589: 5485-
737 5501
- 738 Gordon EB, Hart GT, Tran TM, Waisberg M, Akkaya M, Skinner J, Zinocker S, Pena M, Yazew T, Qi
739 CF *et al* (2015) Inhibiting the Mammalian target of rapamycin blocks the development of experimental
740 cerebral malaria. *mBio* 6: e00725
- 741 Graber TG, Borack MS, Reidy PT, Volpi E, Rasmussen BB (2017) Essential amino acid ingestion alters
742 expression of genes associated with amino acid sensing, transport, and mTORC1 regulation in human
743 skeletal muscle. *Nutr Metab (Lond)* 14: 35
- 744 Hantschel O (2015) Unexpected off-targets and paradoxical pathway activation by kinase inhibitors.
745 *ACS Chem Biol* 10: 234-245
- 746 Hantschel O, Grebien F, Superti-Furga G (2012) The growing arsenal of ATP-competitive and allosteric
747 inhibitors of BCR-ABL. *Cancer Res* 72: 4890-4895
- 748 Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, Kasuga M, Nishimoto I,
749 Avruch J (1997) Regulation of eIF-4E BP1 phosphorylation by mTOR. *J Biol Chem* 272: 26457-26463

- 750 Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF, Schreiber SL (1999) Rapamycin-modulated
751 transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor
752 proteins. *Proc Natl Acad Sci U S A* 96: 14866-14870
- 753 Harrar Y, Bellini C, Faure JD (2001) FKBP: at the crossroads of folding and transduction. *Trends Plant*
754 *Sci* 6: 426-431
- 755 Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel
756 K, Carter CS *et al* (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.
757 *Nature* 460: 392-395
- 758 Holz MK, Ballif BA, Gygi SP, Blenis J (2005) mTOR and S6K1 mediate assembly of the translation
759 preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell*
760 123: 569-580
- 761 Hosoi H, Dilling MB, Shikata T, Liu LN, Shu L, Ashmun RA, Germain GS, Abraham RT, Houghton
762 PJ (1999) Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent
763 apoptosis in human rhabdomyosarcoma cells. *Cancer Res* 59: 886-894
- 764 Huang da W, Sherman BT, Lempicki RA (2009a) Bioinformatics enrichment tools: paths toward the
765 comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37: 1-13
- 766 Huang da W, Sherman BT, Lempicki RA (2009b) Systematic and integrative analysis of large gene lists
767 using DAVID bioinformatics resources. *Nat Protoc* 4: 44-57
- 768 Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Staroslawska E, Sosman J,
769 McDermott D, Bodrogi I *et al* (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell
770 carcinoma. *N Engl J Med* 356: 2271-2281
- 771 Huo Y, Iadevaia V, Proud CG (2011) Differing effects of rapamycin and mTOR kinase inhibitors on
772 protein synthesis. *Biochem Soc Trans* 39: 446-450
- 773 Iqbal A, Duitama, Camila, Metge, Franziska, Roskopp, Daniel, & Boucas, Jorge. (2021) Flaski (2.0.0).
774 *Zenodo*
- 775 Jin C, Zhao Y, Yu L, Xu S, Fu G (2013) MicroRNA-21 mediates the rapamycin-induced suppression
776 of endothelial proliferation and migration. *FEBS Lett* 587: 378-385
- 777 Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P,
778 Davis MI, Edeen PT *et al* (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol*
779 26: 127-132
- 780 Kim JH, Lee C, Lee M, Wang H, Kim K, Park SJ, Yoon I, Jang J, Zhao H, Kim HK *et al* (2017) Control
781 of leucine-dependent mTORC1 pathway through chemical intervention of leucyl-tRNA synthetase and
782 RagD interaction. *Nat Commun* 8: 732

- 783 Ko JH, Yoon SO, Lee HJ, Oh JY (2017) Rapamycin regulates macrophage activation by inhibiting
784 NLRP3 inflammasome-p38 MAPK-NFkappaB pathways in autophagy- and p62-dependent manners.
785 *Oncotarget* 8: 40817-40831
- 786 Kolos JM, Voll AM, Bauder M, Hausch F (2018) FKBP Ligands-Where We Are and Where to Go?
787 *Front Pharmacol* 9: 1425
- 788 Laplante M, Sabatini DM (2013) Regulation of mTORC1 and its impact on gene expression at a glance.
789 *J Cell Sci* 126: 1713-1719
- 790 Lee J, Moir RD, McIntosh KB, Willis IM (2012) TOR signaling regulates ribosome and tRNA synthesis
791 via LAMMER/Clk and GSK-3 family kinases. *Mol Cell* 45: 836-843
- 792 Lelegren M, Liu Y, Ross C, Tardif S, Salmon AB (2016) Pharmaceutical inhibition of mTOR in the
793 common marmoset: effect of rapamycin on regulators of proteostasis in a non-human primate. *Pathobiol*
794 *Aging Age Relat Dis* 6: 31793
- 795 Li J, Kim SG, Blenis J (2014) Rapamycin: one drug, many effects. *Cell Metab* 19: 373-379
- 796 Li X, Franz T, Atanassov I, Colby T (2021) Step-by-Step Sample Preparation of Proteins for Mass
797 Spectrometric Analysis. *Methods Mol Biol* 2261: 13-23
- 798 Liu GY, Sabatini DM (2020) mTOR at the nexus of nutrition, growth, ageing and disease. *Nat Rev Mol*
799 *Cell Biol* 21: 183-203
- 800 Lorenz MC, Heitman J (1995) TOR mutations confer rapamycin resistance by preventing interaction
801 with FKBP12-rapamycin. *J Biol Chem* 270: 27531-27537
- 802 Luo Y, Liu L, Wu Y, Singh K, Su B, Zhang N, Liu X, Shen Y, Huang S (2015) Rapamycin inhibits
803 mSin1 phosphorylation independently of mTORC1 and mTORC2. *Oncotarget* 6: 4286-4298
- 804 Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol*
805 *Cell Biol* 10: 307-318
- 806 Martinez NJ, Chang HM, Borrajo Jde R, Gregory RI (2013) The co-chaperones Fkbp4/5 control
807 Argonaute2 expression and facilitate RISC assembly. *RNA* 19: 1583-1593
- 808 Marz AM, Fabian AK, Kozany C, Bracher A, Hausch F (2013) Large FK506-binding proteins shape
809 the pharmacology of rapamycin. *Mol Cell Biol* 33: 1357-1367
- 810 Mauvoisin D, Rocque G, Arfa O, Radenne A, Boissier P, Mounier C (2007) Role of the PI3-kinase/mTor
811 pathway in the regulation of the stearoyl CoA desaturase (SCD1) gene expression by insulin in liver. *J*
812 *Cell Commun Signal* 1: 113-125
- 813 Mayer C, Grummt I (2006) Ribosome biogenesis and cell growth: mTOR coordinates transcription by
814 all three classes of nuclear RNA polymerases. *Oncogene* 25: 6384-6391

- 815 Meier-Kriesche HU, Kaplan B (2000) Toxicity and efficacy of sirolimus: relationship to whole-blood
816 concentrations. *Clin Ther* 22 Suppl B: B93-100
- 817 Morita M, Prudent J, Basu K, Goyon V, Katsumura S, Hulea L, Pearl D, Siddiqui N, Strack S, McGuirk
818 S *et al* (2017) mTOR Controls Mitochondrial Dynamics and Cell Survival via MTFP1. *Mol Cell* 67:
819 922-935 e925
- 820 Nachef M, Ali AK, Almutairi SM, Lee SH (2021) Targeting SLC1A5 and SLC3A2/SLC7A5 as a
821 Potential Strategy to Strengthen Anti-Tumor Immunity in the Tumor Microenvironment. *Front Immunol*
822 12: 624324
- 823 Nandagopal N, Roux PP (2015) Regulation of global and specific mRNA translation by the mTOR
824 signaling pathway. *Translation (Austin)* 3: e983402
- 825 Partridge L, Fuentealba M, Kennedy BK (2020) The quest to slow ageing through drug discovery. *Nat*
826 *Rev Drug Discov* 19: 513-532
- 827 Perez-Riverol Y, Bai J, Bandla C, Garcia-Seisdedos D, Hewapathirana S, Kamatchinathan S, Kundu
828 DJ, Prakash A, Frericks-Zipper A, Eisenacher M *et al* (2022) The PRIDE database resources in 2022: a
829 hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res* 50: D543-D552
- 830 Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S (2006) Extension of chronological
831 life span in yeast by decreased TOR pathway signaling. *Genes Dev* 20: 174-184
- 832 Powers T, Walter P (1999) Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-
833 signaling pathway in *Saccharomyces cerevisiae*. *Mol Biol Cell* 10: 987-1000
- 834 Ramanathan A, Schreiber SL (2009) Direct control of mitochondrial function by mTOR. *Proc Natl Acad*
835 *Sci U S A* 106: 22229-22232
- 836 Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the
837 CRISPR-Cas9 system. *Nat Protoc* 8: 2281-2308
- 838 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential
839 expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43: e47
- 840 Robida-Stubbs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD, Neumann-Haefelin
841 E, Sabatini DM, Blackwell TK (2012) TOR signaling and rapamycin influence longevity by regulating
842 SKN-1/Nrf and DAF-16/FoxO. *Cell Metab* 15: 713-724
- 843 Rosario FJ, Gupta MB, Myatt L, Powell TL, Glenn JP, Cox L, Jansson T (2019) Mechanistic Target of
844 Rapamycin Complex 1 Promotes the Expression of Genes Encoding Electron Transport Chain Proteins
845 and Stimulates Oxidative Phosphorylation in Primary Human Trophoblast Cells by Regulating
846 Mitochondrial Biogenesis. *Sci Rep* 9: 246

- 847 Schieke SM, Phillips D, McCoy JP, Jr., Aponte AM, Shen RF, Balaban RS, Finkel T (2006) The
848 mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and
849 oxidative capacity. *J Biol Chem* 281: 27643-27652
- 850 Schinaman JM, Rana A, Ja WW, Clark RI, Walker DW (2019) Rapamycin modulates tissue aging and
851 lifespan independently of the gut microbiota in *Drosophila*. *Sci Rep* 9: 7824
- 852 Sun L, Yan Y, Lv H, Li J, Wang Z, Wang K, Wang L, Li Y, Jiang H, Zhang Y (2022) Rapamycin targets
853 STAT3 and impacts c-Myc to suppress tumor growth. *Cell Chem Biol* 29: 373-385 e376
- 854 Takasaki S, Yamaguchi H, Kawasaki Y, Kikuchi M, Tanaka M, Ito A, Mano N (2019) Long-term
855 relationship between everolimus blood concentration and clinical outcomes in Japanese patients with
856 metastatic renal cell carcinoma: a prospective study. *J Pharm Health Care Sci* 5: 6
- 857 Tardif S, Ross C, Bergman P, Fernandez E, Javors M, Salmon A, Spross J, Strong R, Richardson A
858 (2015) Testing efficacy of administration of the antiaging drug rapamycin in a nonhuman primate, the
859 common marmoset. *J Gerontol A Biol Sci Med Sci* 70: 577-587
- 860 Team RC (2017) R Core Team (2017) R: A Language and Environment for Statistical Computing.
- 861 Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, Sabatini DM (2012) A unifying model
862 for mTORC1-mediated regulation of mRNA translation. *Nature* 485: 109-113
- 863 Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray
864 NS (2009) An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant
865 functions of mTORC1. *J Biol Chem* 284: 8023-8032
- 866 Torrence ME, MacArthur MR, Hosios AM, Valvezan AJ, Asara JM, Mitchell JR, Manning BD (2021)
867 The mTORC1-mediated activation of ATF4 promotes protein and glutathione synthesis downstream of
868 growth signals. *Elife* 10
- 869 Tsukumo Y, Alain T, Fonseca BD, Nadon R, Sonenberg N (2016) Translation control during prolonged
870 mTORC1 inhibition mediated by 4E-BP3. *Nat Commun* 7: 11776
- 871 Urfer SR, Kaeberlein TL, Mailheau S, Bergman PJ, Creevy KE, Promislow DEL, Kaeberlein M (2017)
872 A randomized controlled trial to establish effects of short-term rapamycin treatment in 24 middle-aged
873 companion dogs. *Geroscience* 39: 117-127
- 874 Vervliet T, Parys JB, Bultynck G (2015) Bcl-2 and FKBP12 bind to IP3 and ryanodine receptors at
875 overlapping sites: the complexity of protein-protein interactions for channel regulation. *Biochem Soc*
876 *Trans* 43: 396-404
- 877 Villa-Cuesta E, Holmbeck MA, Rand DM (2014) Rapamycin increases mitochondrial efficiency by
878 mtDNA-dependent reprogramming of mitochondrial metabolism in *Drosophila*. *J Cell Sci* 127: 2282-
879 2290

- 880 Visner GA, Lu F, Zhou H, Liu J, Kazemfar K, Agarwal A (2003) Rapamycin induces heme oxygenase-
881 1 in human pulmonary vascular cells: implications in the antiproliferative response to rapamycin.
882 *Circulation* 107: 911-916
- 883 Wall M, Poortinga G, Hannan KM, Pearson RB, Hannan RD, McArthur GA (2008) Translational
884 control of c-MYC by rapamycin promotes terminal myeloid differentiation. *Blood* 112: 2305-2317
- 885 Wang R, Yu Z, Sunchu B, Shoaf J, Dang I, Zhao S, Caples K, Bradley L, Beaver LM, Ho E *et al* (2017)
886 Rapamycin inhibits the secretory phenotype of senescent cells by a Nrf2-independent mechanism. *Aging*
887 *Cell* 16: 564-574
- 888 Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donahoe
889 PK (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I
890 receptors. *Cell* 86: 435-444
- 891 Wang X, Yue P, Chan CB, Ye K, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Fu H, Khuri FR, Sun
892 SY (2007) Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-
893 dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. *Mol Cell Biol*
894 27: 7405-7413
- 895 Zaragoza D, Ghavidel A, Heitman J, Schultz MC (1998) Rapamycin induces the G0 program of
896 transcriptional repression in yeast by interfering with the TOR signaling pathway. *Mol Cell Biol* 18:
897 4463-4470
- 898 Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP (2000) Regulation of cellular growth by the
899 *Drosophila* target of rapamycin dTOR. *Genes Dev* 14: 2712-2724
- 900 Zhang X, Chen W, Gao Q, Yang J, Yan X, Zhao H, Su L, Yang M, Gao C, Yao Y *et al* (2019) Rapamycin
901 directly activates lysosomal mucolipin TRP channels independent of mTOR. *PLoS Biol* 17: e3000252
- 902 Zhang Y, Swanda RV, Nie L, Liu X, Wang C, Lee H, Lei G, Mao C, Koppula P, Cheng W *et al* (2021)
903 mTORC1 couples cyst(e)ine availability with GPX4 protein synthesis and ferroptosis regulation. *Nat*
904 *Commun* 12: 1589
- 905 Zhu Y, Zou R, Sha H, Lu Y, Zhang Y, Wu J, Feng J, Wang D (2020) Lipid metabolism-related proteins
906 of relevant evolutionary and lymphoid interest (PRELI) domain containing family proteins in cancer.
907 *Am J Transl Res* 12: 6015-6026
- 908