

1 **G3BP1 tethers the TSC complex to lysosomes and suppresses mTORC1 in the absence**
2 **of stress granules**

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56 **Summary**

57 G3BP1 (Ras GTPase-activating protein-binding protein 1) is widely recognized as a core
58 component of stress granules (SG), non-membranous RNA-protein-assemblies required for
59 cellular survival under stress. We report that in the absence of SG, G3BP1 acts as lysosomal
60 anchor of the Tuberous Sclerosis Complex (TSC) protein complex. By tethering the TSC
61 complex to lysosomes, G3BP1 suppresses signaling through the metabolic master regulator
62 mTORC1 (mechanistic target of rapamycin complex 1). Like the known TSC complex
63 subunits, G3BP1 suppresses phenotypes related to mTORC1 hyperactivity in the context of
64 tumors and neuronal dysfunction. Thus, G3BP1 is not only a core component of SG but also
65 a key element of lysosomal TSC-mTORC1 signaling.

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68 **Highlights**

69 The *bona fide* stress granule component G3BP1

- 70 • is a key element of the TSC-mTORC1 signaling axis.
- 71 • tethers the TSC complex to lysosomes.
- 72 • prevents mTORC1 hyperactivation by metabolic stimuli.
- 73 • suppresses mTORC1-driven cancer cell motility and epileptiform activity.

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76 **Keywords**

77 TSC complex, mTORC1, G3BP1, G3BP2, lysosome, stress granule, metabolism, cancer,
78 epilepsy

79 Introduction

80 The TSC complex suppresses signaling through the mechanistic target of rapamycin complex
81 1 (MTOR complex 1, mTORC1), a multiprotein kinase complex that constitutes a metabolic
82 master regulator (Kim and Guan, 2019; Liu and Sabatini, 2020; Tee, 2018). mTORC1
83 promotes virtually all anabolic processes (Hoxhaj and Manning, 2019; Mossmann et al., 2018),
84 and its hyperactivity is associated with metabolic imbalance and human diseases related to
85 cellular overgrowth, migration, and neuronal excitability (Condon and Sabatini, 2019).
86 Consequently, mTORC1 is recognized as an important driver of tumorigenesis as well as
87 epilepsy (Crino, 2016; LiCausi and Hartman, 2018; Tee et al., 2016). The cause of mTORC1
88 hyperactivity is often related to a disturbance of the TSC multiprotein complex, known to
89 consist of the subunits TSC1 (hamartin), TSC2 (tuberin), and TBC1D7 (Dibble et al., 2012).
90 The central role of the TSC complex as a tumor suppressor is highlighted by the fact that
91 mutations in the *TSC1* and *TSC2* genes frequently occur in cancer (Huang and Manning,
92 2008; Kwiatkowski, 2003) and cause tuberous sclerosis complex (TSC), an autosomal
93 dominant disorder, which leads to benign tumors in almost all organ systems and represents
94 one of the most frequent genetic causes of epilepsy (Borkowska et al., 2011; Curatolo et al.,
95 2008; Jozwiak et al., 2019; Marcotte and Crino, 2006; Orlova and Crino, 2010).

96 In healthy cells, nutritional inputs such as insulin (Menon et al., 2014) and amino acids
97 (Carroll et al., 2016; Demetriades et al., 2014) inhibit the TSC complex, resulting in the de-
98 repression of mTORC1 (Kim and Guan, 2019). The TSC complex acts as a GTPase-activating
99 protein (GAP) towards the small GTPase Ras homolog-mTORC1 binding (RHEB) (Garami et
100 al., 2003; Inoki et al., 2003; Tee et al., 2003; Zhang et al., 2003). RHEB directly binds and
101 activates mTORC1 at lysosomes (Avruch et al., 2006; Long et al., 2005; Sancak et al., 2010;
102 Sancak et al., 2007). Thus, RHEB inactivation by the TSC complex restricts the activity of
103 mTORC1 and its multiple anabolic outcomes (Condon and Sabatini, 2019; Kim and Guan,
104 2019; Rabanal-Ruiz and Korolchuk, 2018). Suppression of RHEB and mTORC1 by the TSC
105 complex takes place at mTORC1's central signaling platform – the lysosomes (Demetriades
106 et al., 2014; Menon et al., 2014). Thus, recruitment to the lysosomal compartment is crucial

107 for the TSC complex to act on RHEB and mTORC1. The molecular mechanism anchoring
108 mTORC1 at the lysosomes via the LAMTOR-RAG GTPase complex is understood in much
109 detail (Condon and Sabatini, 2019; Kim and Guan, 2019; Rabanal-Ruiz and Korolchuk, 2018).
110 Furthermore, RHEB is known to directly associate with lysosomes via its farnesyl-moiety
111 (Rabanal-Ruiz and Korolchuk, 2018). However, the TSC complex lacks a clear lipid-targeting
112 signal (Kim and Guan, 2019) and it is not yet known how the TSC complex is recruited to
113 lysosomes. Identifying the lysosomal anchor for the TSC complex is important to understand
114 the molecular basis of mTORC1 suppression by the TSC complex. In addition, a tether of the
115 TSC complex is likely to be of high biomedical relevance because of its possible involvement
116 in diseases driven by TSC-mTORC1 dysregulation.

117 In this study, we identify G3BP1 as a lysosomal tether of the TSC complex. G3BP1 is
118 primarily recognized as an RNA-binding protein that constitutes a core component of SG
119 (Alam and Kennedy, 2019; Reineke and Neilson, 2019), cytoplasmic RNA-protein assemblies
120 formed upon stresses that inhibit protein synthesis (Anderson and Kedersha, 2002; Buchan
121 and Parker, 2009). They are sites of stress-induced mRNA triage that sort transcripts for
122 maintenance or decay and adapt cellular signaling to stress (Anderson and Kedersha, 2008;
123 Anderson et al., 2015). G3BP1 is best described as a SG nucleating protein (Alam and
124 Kennedy, 2019; Kedersha et al., 2016; Mahboubi and Stochaj, 2017; Tourriere et al., 2003),
125 and is widely used as a marker to monitor SG assembly (Kedersha et al., 2008; Moon et al.,
126 2019). G3BP1's function in SG has also been linked with its involvement in neurological
127 diseases and cancer (Alam and Kennedy, 2019). Only few SG-independent functions of
128 G3BP1 have been proposed. As a protein with RNA binding properties, G3BP1 was suggested
129 to bind to mRNAs of oncogenes and tumor suppressors (Alam and Kennedy, 2019). In its
130 initial report, G3BP1 was proposed to act as a Ras GTPase-activating protein (Ras GAP)
131 binding protein (Gallouzi et al., 1998; Kennedy et al., 2001; Parker et al., 1996) and thus a
132 protein binding property gave rise to its name, although this putative function has since been
133 challenged (Annibaldi et al., 2011). Thus, at present we know little about potential protein
134 binding properties of G3BP1 and putative functions that do not involve SG.

135 **Results**

136 **G3BP1 inhibits mTORC1 in the absence of stress granules.**

137 In a proteomic analysis of the MTOR interactome (Schwarz et al., 2015), we discovered that
138 G3BP1 was significantly enriched with high sequence coverage, along with MTOR and the
139 mTORC1-specific scaffold protein regulatory-associated protein of MTOR complex 1
140 (RPTOR) (**Figure 1A, S1A, B**). We confirmed the mass spectrometry data by co-
141 immunoprecipitation and found that G3BP1 interacts with MTOR and RPTOR in MCF-7 breast
142 cancer cells (**Figure S1C, D**). G3BP1 is well known for its role in SG assembly (Alam and
143 Kennedy, 2019; Reineke and Neilson, 2019), and SG inhibit mTORC1 (Thedieck et al., 2013;
144 Wippich et al., 2013). To test whether G3BP1 inhibits mTORC1 under conditions that induce
145 SG, we treated MCF-7 cells with arsenite, a frequently used inducer of SG (Anderson et al.,
146 2015). After 30-minute exposure to arsenite, a cytoplasmic punctate pattern of the SG markers
147 G3BP1 and eukaryotic translation initiation factor 3 subunit A (EIF3A) (Kedersha and
148 Anderson, 2007) indicated the presence of SG (**Figure 1B**). Arsenite stress also enhanced
149 the inhibitory phosphorylation of the eukaryotic translation initiation factor 2 alpha (EIF2S1) at
150 Ser51 (**Figure 1C**), which serves as a marker for conditions that inhibit translation and induce
151 SG (Anderson and Kedersha, 2002). In agreement with earlier reports (Heberle et al., 2019;
152 Thedieck et al., 2013; Wang and Proud, 1997), arsenite exposure for 30 minutes enhanced
153 the phosphorylation of the mTORC1 substrate ribosomal protein S6 kinase B1 (RPS6KB1)
154 (Holz and Blenis, 2005) at T389 (RPS6KB1-pT389) (**Figure 1C, E**). G3BP1 knockdown by
155 short hairpin RNA (shG3BP1, **Figure 1D, S1E**) reduced the G3BP1 protein levels, but did not
156 alter RPS6KB1-T389 phosphorylation (**Figure 1C, E**). Also, upon arsenite exposure for
157 various time periods up to 60 minutes, G3BP1 knockdown by shRNA or siRNA (**Figure S1E**)
158 did not alter RPS6KB1-pT389 levels (**Figure S1F-K**). Therefore, we conclude that in the
159 presence of SG, G3BP1 does not affect mTORC1 activity.

160 We next tested whether G3BP1 influences mTORC1 activity under conditions that are
161 not associated with the formation of SG. For this purpose, we starved MCF-7 cells and then
162 restimulated them with insulin and amino acids to activate metabolic signaling through

163 mTORC1. G3BP1 was targeted by two different shRNA sequences (**Figure S1E**). Insulin and
164 amino acids enhanced phosphorylation of RPS6KB1-T389 and of its substrate ribosomal
165 protein S6 (RPS6-pS235/236) (Pende et al., 2004), indicative of mTORC1 activation (**Figure**
166 **1F, H, I and S2A, C, D**). Of note, G3BP1 knockdown led to a further increase in RPS6KB1-
167 pT389 and RPS6-pS235/236 (**Figure 1F-I and S2A-D**). In triple negative MDA-MB-231 cells
168 (Neve et al., 2006), shG3BP1-mediated knockdown enhanced RPS6KB1-pT389 and RPS6-
169 pS235/236 as well (**Figure 1J-M and S2E-H**). Targeting G3BP1 by siRNA knockdown (**Figure**
170 **S2I-L**) or CRISPR/Cas9 knockout (**Figure 1N-Q and S2M**) also resulted in RPS6KB1-T389
171 and RPS6-S235/236 hyperphosphorylation. To test whether enhanced RPS6KB1-pT389 and
172 RPS6-pS235/236 in G3BP1-deficient cells is mediated by mTORC1, we used the allosteric
173 mTORC1 inhibitor rapamycin, which potently inhibited RPS6KB1-T389 and RPS6-S235/236
174 phosphorylation in G3BP1-deficient cells (**Figure 1R, S**). Thus, we conclude that G3BP1
175 restricts mTORC1 activation by amino acids and insulin.

176 As G3BP1 is a core component of SG (Alam and Kennedy, 2019; Reineke and Neilson,
177 2019), which are known to inhibit mTORC1 under stress (Thedieck et al., 2013; Wippich et al.,
178 2013), we wondered whether SG were also present in metabolically stimulated cells. To test
179 this, we performed immunofluorescence (IF) experiments in which we analysed the
180 distribution patterns of endogenous G3BP1 and EIF3A in cells stimulated with insulin and
181 amino acids, or upon arsenite stress as a positive control (**Figure S2N, O**). G3BP1 knockdown
182 reduced G3BP1 levels, as expected, but SG remained present in the arsenite treated cells
183 (further discussed below). While arsenite induced SG, no puncta indicative of SG became
184 visible in insulin and amino acid stimulated cells, and G3BP1 and EIF3A were distributed
185 throughout the cytoplasm. Thus, mTORC1 inhibition by G3BP1 occurs in the absence of SG.

186 **G3BP1 resides at lysosomes.**

187 To identify the subcellular compartment where G3BP1 acts in the absence of SG, we
188 fractionated lysates of starved cells by sucrose density gradient centrifugation (**Figure 2A**).
189 The TSC complex components TSC1, TSC2, and TBC1D7 were predominantly detected in

190 the fractions containing the lysosome associated membrane proteins 1 and 2 (LAMP1,
191 LAMP2) (Eskelinen, 2006). This is in line with earlier biochemical and IF-based studies
192 demonstrating that the TSC complex inhibits mTORC1 at lysosomes when cells lack amino
193 acids or growth factors (Carroll et al., 2016; Demetriades et al., 2014; Menon et al., 2014). In
194 the absence of SG inducers, G3BP1 exhibits a ubiquitous cytoplasmic localization (**Figure**
195 **S2N**) (Irvine et al., 2004), but so far no specific sub-cellular enrichment has been identified.
196 We found that G3BP1 resides in the lysosomal fractions (**Figure 2A**). Thus, in the absence of
197 SG, G3BP1 co-fractionates with the TSC complex and lysosomal proteins. We demonstrated
198 the lysosomal association of G3BP1 further *in situ* by proximity ligation assays (PLA) of G3BP1
199 with LAMP1 (**Figure 2B, C**). Thus, we propose that G3BP1 localizes to lysosomes, in close
200 proximity to LAMP1.

201 **G3BP1 tethers the TSC complex to lysosomes.**

202 G3BP1 co-fractionates with the TSC complex (**Figure 2A**), and we investigated whether they
203 physically interact. Indeed, as TSC1 and TBC1D7, G3BP1 co-immunoprecipitated with TSC2
204 (**Figure 2D**). PLA supported the association of G3BP1 with TSC2 *in situ* (**Figure 2E, F**),
205 indicative of a distance between the two proteins of less than 40 nm (Debaize et al., 2017).
206 Thus, G3BP1 is a novel interactor of the TSC complex.

207 Interestingly, TSC2 and G3BP1 both co-immunoprecipitated with MTOR (**Figure 2G-**
208 **I**). This physical interaction likely reflects the lysosomal localization of G3BP1, the TSC
209 complex, and mTORC1. G3BP1 deficiency significantly reduced TSC2-MTOR association
210 (**Figure 2G-I**), suggesting that G3BP1 is required for the TSC complex to act on MTOR. As a
211 likely scenario, we hypothesized that G3BP1 might inhibit mTORC1 by mediating the
212 localization of the TSC complex to lysosomes. We first tested this assumption in IPs of TSC2,
213 which co-immunoprecipitated not only TSC1 and G3BP1 but also the lysosomal proteins
214 LAMP1 and 2 (**Figure 2J, K**). G3BP1 deficiency significantly reduced the physical interaction
215 of TSC2 with LAMP1 (**Figure 2K-N**), indicative of a role of G3BP1 as a lysosomal tether for
216 the TSC complex.

217 To further address the requirement of G3BP1 for the lysosomal localization of the TSC
218 complex, we analyzed TSC2-LAMP2 association *in situ* by PLA in G3BP1-proficient
219 and -deficient cells (**Figure 3A, B**). As reported earlier (Carroll et al., 2016; Demetriades et
220 al., 2014; Demetriades et al., 2016; Menon et al., 2014), TSC2-LAMP2 association was
221 highest in starved cells and decreased upon stimulation with amino acids and insulin. In
222 starved cells, G3BP1 knockdown significantly reduced TSC2-LAMP2 association, to a similar
223 level as observed upon insulin and amino acid stimulation. This result was corroborated by IF
224 analysis of TSC2 and LAMP1 co-localization in G3BP1 CRISPR/Cas9 KO cells (**Figure 3C,**
225 **D**). G3BP1 KO reduced TSC2-LAMP1 co-localization in starved cells to the same extent as
226 metabolic stimulation with insulin and amino acids. Thus, G3BP1 mediates lysosomal
227 localization of the TSC complex in cells deprived of insulin and nutrients. In agreement with
228 this, we observed a significant induction of RPS6KB1 and RPS6 phosphorylation not only in
229 metabolically stimulated cells, but also when inhibiting G3BP1 in starved cells (**Figure 3E-H**).
230 The signals under starvation had been quenched in earlier experiments by the much stronger
231 signals upon metabolic stimulation (**Figure 1F-I**). Thus, we propose that in G3BP1 deficient
232 cells, impaired lysosomal recruitment of the TSC complex under starvation enhances
233 mTORC1 activity. This results in faster phosphorylation of mTORC1 substrates upon
234 metabolic stimuli.

235 The TSC complex acts as a GAP for RHEB, and their interaction contributes to the
236 lysosomal localization of the TSC complex (Carroll et al., 2016; Menon et al., 2014). A similar
237 function has been suggested for RAG GTPases upon depletion of amino acids (Demetriades
238 et al., 2014). To test whether the mechanisms via which G3BP1 and RHEB target the TSC
239 complex to lysosomes are interdependent, we compared the effects of RHEB and G3BP1
240 inhibition on TSC2-LAMP1 co-localization (**Figure 3C, D**). We found that G3BP1 KO and
241 RHEB knockdown reduced TSC2-LAMP1 co-localization to a similar extent, and they did not
242 exert an additive effect. Thus, G3BP1 and RHEB are both necessary for the lysosomal
243 recruitment of the TSC. In other words, the association with its target GTPase is not sufficient
244 for the lysosomal localization of the TSC complex as it requires G3BP1 as an additional tether.

245 **G3BP1 suppresses mTORC1 via the TSC complex.**

246 Our data so far showed that G3BP1 recruits the TSC complex to lysosomes and inhibits
247 mTORC1. We tested next if G3BP1's function as an mTORC1 suppressor depends on the
248 TSC complex. For this purpose, we conducted an epistasis experiment in which we analyzed
249 the effect of G3BP1 inhibition on mTORC1 activity in the presence or absence of TSC2
250 (**Figure 3I-L**). We had previously stimulated cells with insulin and amino acids, as they both
251 signal through the TSC complex (Carroll et al., 2016; Demetriades et al., 2014; Demetriades
252 et al., 2016). Amino acids also signal to mTORC1 via TSC complex-independent routes (Liu
253 and Sabatini, 2020; Rabanal-Ruiz and Korolchuk, 2018). Thus, for the epistasis experiment,
254 we opted to stimulate the cells exclusively with insulin to only assess mTORC1 inactivation
255 via the TSC complex. As expected, RPS6KB1-T389 was hyperphosphorylated to a similar
256 extent in starved or insulin-stimulated TSC2 CRISPR/Cas9 KO cells, as the TSC complex was
257 absent. G3BP1 inhibition induced RPS6KB1-T389 hyperphosphorylation in starved control
258 cells, and this effect was further enhanced by insulin. However, G3BP1 inhibition did not
259 further enhance RPS6KB1-pT389 in TSC2 KO cells (**Figure 3I, L**). Thus, we propose that
260 G3BP1 and the TSC complex act in the same signaling pathway to suppress mTORC1.

261 **TSC2 mediates the formation of the G3BP1-TSC complex.**

262 To further understand the molecular makeup of the TSC-G3BP1 complex, we next determined
263 which of the known subunits mediates G3BP1 binding. For this purpose, we analyzed G3BP1
264 binding to TSC1 in TSC2 KO or control cells (**Figure 4A**). TSC2 KO resulted in a complete
265 loss of G3BP1 from the TSC1-TBC1D7 complex, indicating that G3BP1 binds TSC2. We next
266 aimed to determine the TSC2-binding domain of G3BP1. A C-terminal fragment of G3BP1,
267 consisting of amino acids 333-466, co-immunoprecipitated with GFP-TSC2 to a similar extent
268 as full-length G3BP1 (**Figure 4B, C**). This indicates that G3BP1 binds TSC2 mainly via its C-
269 terminus, harboring RNA recognition motifs (RRM) and arginine-glycine-glycine repeats
270 (RGG) (Tourriere et al., 2003) (**Figure S1A**). In contrast, the middle part (amino acids 183-
271 332; containing the proline rich domain) and the N-terminal region (amino acids 1-182;

272 harboring the NTF2-like domain) of G3BP1 exhibited faint or no interaction with TSC2,
273 respectively. Thus, we conclude that the G3BP1-TSC2 interaction is mainly mediated by
274 G3BP1's C-terminus. Of note, overexpression of C-terminal G3BP1 (lacking the NTF2-like
275 domain) cannot induce SG (Reineke and Lloyd, 2015; Takahashi et al., 2013; Tourriere et al.,
276 2003; Zhang et al., 2019). This further supports that C-terminal G3BP1 interacts with TSC2 in
277 a SG-independent manner. We propose that the C-terminal region of G3BP1 has a dual
278 function in mediating the interaction with RNA in SG (Reineke and Neilson, 2019), and with
279 the TSC complex under non-stress conditions.

280 The known members of the TSC complex are resistant to high salt and detergent
281 conditions, indicative of their high binding affinity (Dibble et al., 2012; Nellist et al., 1999). The
282 complex formed by TSC1, TSC2, and TBC1D7 remains stable at 1.5 M NaCl and 0.1%
283 (3.5 mM) sodium dodecyl sulfate (SDS) (Dibble et al., 2012). To obtain information about the
284 affinity of the TSC2-G3BP1 interaction, we incubated TSC1 IPs with up to 1.5 M NaCl or up
285 to 3.5 mM SDS (**Figure 4D**). While the TSC1-TSC2 interaction was resistant to 1.5 M NaCl,
286 the binding to G3BP1 was lost at 0.5 M NaCl. This salt sensitivity suggests that the complex
287 is formed via electrostatic interactions. In line with this, the G3BP1 C-terminus harbors an
288 intrinsically disordered region (IDR) (Panas et al., 2019), which – as is typical for IDRs
289 (Forman-Kay and Mittag, 2013) – contains a high density of positively charged arginine
290 residues that mediate electrostatic interactions. Importantly, the interaction of TSC2 with
291 G3BP1 was highly stable against denaturation by SDS that preferentially disrupts hydrophobic
292 interactions at the concentration used in this experiment (3.5 mM) (Hojgaard et al., 2018).
293 Thus, upon SDS exposure, G3BP1 exhibits high affinity to the TSC complex, which is in a
294 similar range as that between TSC1 and TSC2 (Dibble et al., 2012). We conclude that the
295 TSC complex and G3BP1 form a highly stable complex that requires electrostatic interactions.

296 **G3BP1 bridges TSC2 to LAMP1/2.**

297 We next assessed the proximity of the G3BP1 association with TSC2, the LAMP1/2 proteins,
298 and MTOR. Bimolecular fluorescence complementation (BiFC) assays detect protein-protein

299 interactions in living cells with a maximum distance of 10 nm (Hu et al., 2002) (**Figure 4E, F**
300 and **S3A**), and are thus indicative of close, likely direct contact between proteins. While all
301 BiFC fusion proteins were expressed (**Figure S3B**), no BiFC signal was observed for cells in
302 which G3BP1 was co-expressed with MTOR (**Figure 4E, F**). Thus, their interaction detected
303 in IPs may not be direct, but is possibly mediated by their common association with lysosomes.
304 In contrast, we did detect BiFC signals for G3BP1 with LAMP1, LAMP2, and TSC2, indicative
305 of a close interaction between them. Based on this, and on our findings that G3BP1
306 knockdown impedes TSC2-LAMP1/2 binding (**Figure 2K-N and 3A, B**) and TSC2 KO
307 prevents G3BP1 binding to TSC1-TBC1D7 (**Figure 4A**), we propose that G3BP1 bridges
308 TSC2 to the lysosomal proteins LAMP1 and LAMP2, thereby mediating the lysosomal
309 localization of the TSC complex.

310 **G3BP1 co-appears with the TSC complex during evolution.**

311 As our analyses established G3BP1 as a key component of mammalian TSC-mTORC1
312 signaling, we asked whether G3BP1 appeared during evolution together with the other
313 subunits of the TSC complex and its targets. Therefore, we analyzed the phylogenetic
314 distribution of G3BP1, TSC1, TSC2, TBC1D7, RHEB, and MTOR (**Figure 4G**). While MTOR
315 and RHEB are present in the yeast *S. cerevisiae*, G3BP1 appears together with the other TSC
316 complex components in the clade of Deuterostomia. Although G3BP1 orthologues have been
317 proposed in *S. cerevisiae* (Yang et al., 2014) and in the nematode *C. elegans* (Jedrusik-Bode
318 et al., 2013), evidence for their functional homology with G3BP1 is scarce. Our sequence
319 similarity analyses (BLASTP, NCBI NR database, BLOSUM45 matrix; 19.02.2020) showed
320 that the human protein with the highest similarity to the proposed G3BP1 orthologue Bre5
321 (UniProt ID P53741) in *S. cerevisiae* is a *C. elegans* UNC-80 like protein that is functionally
322 unrelated to G3BP1. And although the *C. elegans* protein GTBP-1 (UniProt ID Q21351)
323 exhibits the highest sequence similarities to human G3BP1 and 2, the similarities are low (e-
324 values 4-e7 and 0.12) and are restricted to the NTF2 and RRM domains of which they cover
325 only 23%, thus not matching the thresholds for our phylogenetic analysis. In summary, while

326 SG existed already in low eukaryotes, including *S. cerevisiae* (Hoyle et al., 2007), we propose
327 that a functional G3BP1 orthologue emerged later together with the TSC complex.

328 **G3BP2 is a functional paralogue of G3BP1 in mTORC1 signaling.**

329 G3BP2 exhibits high identity and similarity with G3BP1 (**Figure S4A, B**) (Kennedy et al.,
330 2001), and can substitute for G3BP1 in SG assembly (Kedersha et al., 2016; Matsuki et al.,
331 2013). Thus, G3BP1 and 2 might be redundant for many functions, and we asked whether
332 G3BP2 might also compensate for G3BP1 in mTORC1 signaling. Indeed, phylogenetic
333 analysis suggests that G3BP2 emerged together with G3BP1 indicating that they both evolved
334 from a common ancestor gene as functional components of the TSC-mTORC1 axis
335 (**Figure 5A**). Like G3BP1, G3BP2 co-immunoprecipitated with the TSC complex and MTOR
336 (**Figure 5B, Figure S4C**). G3BP2 co-fractionated with G3BP1 and lysosomal proteins in
337 sucrose gradients, identifying the lysosome as their primary localization site when SG are
338 absent (**Figure 5C**). G3BP2 gave rise to BiFC signals with LAMP1, LAMP2, and TSC2
339 (**Figure 5D, E and S4D, E**), suggesting that G3BP2 binds to TSC2 and the LAMP1/2 proteins
340 directly. G3BP2 knockdown enhanced RPS6KB1-T389 and RPS6-S235/236 phosphorylation,
341 indicative of mTORC1 hyperactivity (**Figure 5F-I**). In agreement with previous data (Kedersha
342 et al., 2016), G3BP2 expression was enhanced in G3BP1 KO cells (**Figure 5J, K**) and less so
343 upon G3BP1 knockdown (**Figure 5L, M**). This suggests that indeed G3BP2 induction may
344 partially compensate for G3BP1 KO, highlighting the strength of the effect of G3BP1 on
345 mTORC1 activity (**Figure 1N-Q**). Thus, we conclude that G3BP2 is a functional paralogue of
346 G3BP1 in TSC-mTORC1 signaling.

347 **G3BP1 suppresses mTORC1-driven migration in breast cancer cells.**

348 We next investigated the consequences of G3BP1-mediated mTORC1 suppression in the
349 context of cancer. In migration assays, G3BP1 deficiency resulted in faster wound closure,
350 which was abrogated by rapamycin (**Figure 6A, B**). This suggests that G3BP1 restricts
351 mTORC1-driven cell motility. As changes in proliferation might confound cell motility assays,
352 we analyzed proliferation by real-time cell analysis (RTCA). In line with previous findings

353 (Winslow et al., 2013), G3BP1-deficiency reduced cell proliferation (**Figure 6C, D**), indicating
354 that mTORC1-driven cell motility in G3BP1-deficient cells was not a result of enhanced
355 proliferation. Analysis of RNASeq data from invasive breast cancer revealed G3BP1 mRNA
356 expression levels to be similar in the four breast cancer subtypes defined by the PAM50
357 classification (Koboldt et al., 2012) (**Figure 6E**). Analysis across all subtypes showed that
358 patients with *G3BP1* mRNA or protein expression below the median exhibited significantly
359 shorter relapse free survival (RFS) than those with expression above the median
360 (**Figure 6F, G**). Our observations phenocopied the shorter RFS in patients with low TSC1 or
361 TSC2 levels (**Figure 6H, I**). This suggests that G3BP1 and the two core TSC complex
362 components could be used as subtype-independent prognostic markers in breast cancer
363 patients and indicators of mTORC1 activity and cancer cell motility.

364 **Brain G3BP1 suppresses mTORC1-driven epileptogenic events.**

365 Next to its importance as a tumor suppressor, the TSC complex has crucial neuronal functions
366 and epilepsy is a hallmark of TSC. Therefore, G3BP1 may play a similar role in the brain. To
367 test this, we conducted TSC1 IPs from rat brain lysates (**Figure 6J**). Together with TSC2,
368 G3BP1 co-immunoprecipitated with TSC1, indicating that G3BP1 binds the TSC complex in
369 the brain. To explore the impact of G3BP1 in epilepsy, we used a zebrafish model in which
370 *tsc2* KO elicits pronounced epileptiform events and which is thus suitable to recapitulate the
371 human TSC disease (Scheldeman et al., 2017). The zebrafish G3BP1 orthologue exhibits
372 67.8% sequence identity with the human protein (**Figure S5A**). We targeted zebrafish *g3bp1*
373 with morpholino oligonucleotides (G3BP1 MO) (**Figure S5B**). Efficient *g3bp1* knockdown was
374 evaluated by RT-PCR (**Figure 6K**). In agreement with our observations in human cell lines,
375 *g3bp1* inhibition enhanced mTORC1 activity, as determined by RPS6-pS235/236 levels, in
376 the zebrafish larvae (**Figure 6L, M**). Recordings of non-invasive local field potentials (LFP)
377 from larval optic tecta (**Figure 6N, O** and **S5C, D**) revealed that *g3bp1* deficiency elicits
378 epileptiform events. We tested whether the increased number of epileptiform events was due
379 to hyperactive mTORC1. To reduce mTORC1 hyperactivity, we treated control and G3BP1

380 MO injected larvae with rapamycin prior to brain activity recordings. Rapamycin fully
381 suppressed the epileptiform events in *g3bp1*-deficient larvae to the level in control animals
382 (**Figure 6N**). We confirmed this result by power spectral density (PSD) analysis (**Figure 6P**),
383 an automated method to quantify the spectral power across multiple LFP recordings (Hunyadi
384 et al., 2017). We found that *g3bp1* deficiency enhanced the LFP power in the frequency range
385 between 20-80 Hz, an effect that was fully rescued by rapamycin (**Figure 6P**). Taken together,
386 we conclude that *g3bp1* deficiency elicits mTORC1-driven epileptiform events. Thus, *g3bp1*
387 inhibition phenocopies the effect of a *tsc2* KO (Scheldeman et al., 2017), highlighting the
388 importance of *g3bp1* as a suppressor of neuronal mTORC1 *in vivo*.

389 **Discussion**

390 In this study, we demonstrate that G3BP1 acts outside of SG as a lysosomal tether of the TSC
391 complex (**Graphical Abstract**). G3BP1 directly interacts with TSC2 and LAMP1/2, thus
392 securing the TSC complex to lysosomes. Similar to the known TSC complex subunits, G3BP1
393 suppresses mTORC1. TSC2 and G3BP1 do not exert additive effects on mTORC1 activity in
394 insulin-stimulated cells, highlighting that they act together in the insulin-mTORC1 axis. G3BP1
395 deficiency leads to mTORC1-driven phenotypes in both cancer and neuronal dysfunction.
396 Thus, we propose that G3BP1 is not only a core SG component but also a key element of
397 mTORC1 signaling on lysosomes.

398 G3BP1 was identified over two decades ago as a RasGAP binding protein, and thus a
399 role of G3BP1 in the RAS pathway was proposed (Gallouzi et al., 1998; Kennedy et al., 2001;
400 Parker et al., 1996). However, this hypothesis has been questioned (Annibaldi et al., 2011)
401 and present research primarily focuses on the role of G3BP1 in SG formation and RNA
402 metabolism (Alam and Kennedy, 2019; Reineke and Neilson, 2019). In line with the initial
403 reports, we demonstrate that G3BP1's identification as a GAP-binding protein was correct -
404 although for a different GAP - as it exerts this role by binding TSC2, the GAP component of
405 the TSC complex (Inoki et al., 2003). It therefore may be rewarding to revisit whether G3BP1
406 also binds to other RAS-related GAPs. Our data indicate that, at least in the insulin-mTORC1

407 axis, G3BP1 exerts its suppressor function through the TSC complex. However, this does not
408 exclude involvement in other signaling pathways such as RAS (Parker et al., 1996), NFKB1
409 (Prigent et al., 2000), WNT (Bikkavilli and Malbon, 2011), and TGFB (Zhang et al., 2015). As
410 they all crosstalk with mTORC1 via the TSC complex (Ghosh et al., 2006; Inoki et al., 2006;
411 Ma et al., 2005; Thien et al., 2015), the observations implicating G3BP1 in these pathways
412 might in fact result from its function within the TSC complex; which will be an intriguing
413 direction for future research.

414 Why does G3BP1 inhibit mTORC1 upon metabolic starvation and restimulation, but
415 not under stress conditions that promote SG formation? It is well documented that arsenite
416 and other SG-inducing stressors enhance TSC2 degradation (Heberle et al., 2019; Huang and
417 Manning, 2008; Orlova and Crino, 2010; Thedieck et al., 2013). Without TSC2, G3BP1 cannot
418 bind to the TSC complex (**Figure 4A**) and thus cannot inhibit mTORC1. Another mechanism
419 by which G3BP1 might inhibit mTORC1 under stress is through its role as a nucleator of SG,
420 which restrict mTORC1 activity (Thedieck et al., 2013; Wippich et al., 2013). However,
421 previous studies (Bley et al., 2015; Kedersha et al., 2016; Matsuki et al., 2013) and our own
422 results (**Figure S2N, O**) show that SG are present in G3BP1-deficient cells. SG formation in
423 the absence of G3BP1 is mediated by other SG factors such as T cell internal antigen 1 (TIA1)
424 (Kedersha et al., 2016) or the G3BP1-paralogue G3BP2 (Kedersha et al., 2016; Kennedy et
425 al., 2001; Matsuki et al., 2013), and thus SG remain to inhibit mTORC1. Hence, the absence
426 of G3BP1's inhibitory effect on mTORC1 in arsenite-stressed cells is likely due to (i) the
427 degradation of TSC2 and (ii) the presence of SG in the absence of G3BP1.

428 By means of biochemical approaches, we identify the lysosome as the primary site of
429 G3BP1 localization when SG are absent (**Figure 2A and 5C**). This is in agreement with the
430 major function of the TSC complex and mTORC1 at lysosomes, and this view is further
431 supported by the appearance of G3BP1 in a recently published study on the lysosomal
432 proteome (Wyant et al., 2018). Interestingly, SG have recently also been reported to hitchhike
433 on lysosomes with annexin A11 (ANXA11) acting as a tether (Liao et al., 2019). The proximity
434 of SG to lysosomes might allow G3BP1 shuttling, enabling rapid switching between its two

435 functions. Despite the strong biochemical evidence for its lysosomal localization, we do not
436 exclude that G3BP1 controls signaling at other subcellular sites. IF data show a ubiquitous
437 cytoplasmic distribution of G3BP1 in the absence of SG (**Figure S2N**) (Irvine et al., 2004),
438 reminiscent of the IF patterns for the TSC complex (Carroll et al., 2016; Demetriades et al.,
439 2014) and MTOR (Betz and Hall, 2013). Indeed, next to lysosomes, MTOR has been proposed
440 to localize to multiple subcellular sites (Betz and Hall, 2013), and accumulating evidence
441 suggests that both RHEB and the TSC complex can reside at sites other than lysosomes (Hao
442 et al., 2018; Zhang et al., 2013). Thus, both biochemical data and imaging results correlate
443 with our suggestion of a functional connection between G3BP1, the TSC complex and
444 mTORC1 at lysosomes and, likely, other subcellular loci (Kim and Guan, 2019).

445 The proposed function of G3BP1 and the TSC complex in the same pathway would
446 suggest that deficiency of either factor affects mTORC1-driven phenotypes in a similar way.
447 Ablation of the *TSC1* or *TSC2* tumor suppressor genes results in increased cancer cell motility
448 and metastasis (Astrinidis et al., 2002; Goncharova et al., 2006). Similarly, G3BP1 deficiency
449 enhances cancer cell motility in an mTORC1-dependent manner (**Figure 6A, B**), and low
450 G3BP1 mRNA and protein levels correlate with a poor outcome in breast cancer (**Figure 6F,**
451 **G**). Conflicting observations on the effect of G3BP1 on cell motility (Alam and Kennedy, 2019)
452 may arise from the growth defect, which we (**Figure 6C, D**) and others observe upon G3BP1
453 inhibition (Alam and Kennedy, 2019; Dou et al., 2016; Huang et al., 2016; Wang et al., 2018).
454 This growth defect has been attributed to the de-repression of cell cycle arrest factors whose
455 mRNAs are bound and inhibited by G3BP1 (Alam and Kennedy, 2019). Such cell cycle defects
456 can mask G3BP1's inhibitory effect on migration, depending on the cell context and type of
457 assay.

458 The opposite effects of G3BP1 on migration and proliferation may also limit its potential
459 as a therapeutic target in cancer. In addition, the dual roles of G3BP1 in oncogenic mTORC1
460 signaling versus SG formation argue against G3BP1 as an anti-tumor target, as G3BP1
461 inhibition alone is not sufficient to inhibit SG (**Figure S2N, O**; and (Kedersha et al., 2016)), but
462 results in mTORC1 hyperactivation. G3BP1 may, however, be a promising marker to guide

463 drug therapies targeting mTORC1 and its upstream cues. Such compounds have been
464 approved for several tumor entities including metastatic ER-positive breast cancer (Baselga
465 et al., 2012; Paplomata and O'Regan, 2014), but their clinical success so far remained limited
466 (Friend and Royce, 2016). At first glance, our finding that low G3BP1 levels correlate with a
467 shorter progression-free survival in breast cancer seems at odds with reports on sarcoma
468 (Somasekharan et al., 2015), colon (Zhang et al., 2012), and gastric cancer (Min et al., 2015),
469 in which high G3BP1 expression positively correlates with tumor size, invasion, and
470 metastasis. Yet, SG were found to be critical for G3BP1-mediated oncogenicity in these
471 entities, suggesting that the function of G3BP1 as a SG nucleator may dominate in these
472 cases. This effect likely is less important in tumors addicted to hyperactive mTORC1, in which
473 G3BP1 may act as a tumor suppressor. This suggests that G3BP1 is a poor prognostic marker
474 across different cancer entities as both high and low levels can be oncogenic. However, low
475 G3BP1 levels are likely a good indicator of mTORC1 hyperactivity, which correlates with tumor
476 sensitivity to mTORC1 inhibitors (Grabiner et al., 2014; Kwiatkowski and Wagle, 2015; Meric-
477 Bernstam et al., 2012; Wagle et al., 2014). Therefore, low G3BP1 levels might enable the
478 stratification of patients to clinical inhibitors of mTORC1 and its upstream cues.

479 Also neuronal G3BP1 phenotypes deserve evaluation as to whether they are mediated
480 by the TSC-mTORC1 axis. G3BP1 deficiency impairs synaptic transmission (Martin et al.,
481 2013; Zekri et al., 2005) and there is evidence for a linkage with early-onset epilepsy in
482 humans (Appenzeller et al., 2014; Heyne et al., 2018). Our finding that *g3bp1* inhibition elicits
483 epileptogenic events in zebrafish (**Figure 6N, O**) supports a link between G3BP1 deficiency
484 and epilepsy. G3BP1 down-regulation inactivates the TSC complex, and *TSC1* and *TSC2*
485 mutations - leading to de-repression of mTORC1 - frequently cause epilepsy (Curatolo et al.,
486 2015; Jozwiak et al., 2019; Roach and Kwiatkowski, 2016). Consistent with a common
487 mechanism, rapamycin suppresses the epileptogenic events in *g3bp1* deficient zebrafish
488 larvae (**Figure 6N**). G3BP1's function via the TSC complex, the insulin responsive GAP of
489 RHEB, is mirrored by the KICSTOR complex (Peng et al., 2017; Wolfson et al., 2017). The
490 KICSTOR complex is the lysosomal tether for the GATOR1 subcomplex, which is the GAP for

491 the RAG GTPases that activate mTORC1 in response to amino acids. Like mutations in the
492 genes encoding the components of the TSC complex, mutations in genes encoding the
493 KICSTOR complex (Wolfson et al., 2017) and GATOR1 subcomplex (Baldassari et al., 2016)
494 components have been associated with neuronal malformation and epilepsy, referred to as
495 “mTORopathies” (Crino, 2015; Wong and Crino, 2012). mTORC1 inhibitors show encouraging
496 results for the treatment of TSC-related epilepsy (van der Poest Clement et al., 2020) and
497 have been proposed to benefit epilepsy patients with alterations in KICSTOR or GATOR1
498 (Baulac, 2016; Crino, 2015; Sadowski et al., 2015). Our findings suggest that also epilepsy
499 patients with G3BP1 alterations may benefit from treatment with mTORC1 inhibitors, which
500 will add G3BP1 to the family of genes whose mutations cause mTORopathies.

501 In conclusion, we identify G3BP1 as an essential lysosomal tether of the TSC complex
502 that suppresses mTORC1 at lysosomes. Future research will reveal whether this dual role in
503 nutrient signaling and SG formation is specific to G3BP1, or whether also other SG
504 components have non-granule functions to orchestrate cellular responses to environmental
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560 last authors, all other author names are listed in alphabetical order.

561 **Declaration of Interests.**

562 The authors declare no competing interests.

563

564 **Figure Legends**

565 **Figure 1. G3BP1 suppresses mTORC1 reactivation by insulin and nutrients.**

566 **(A)** Re-analysis of the MTOR interactome data reported by Schwarz et al. (2015). Volcano
567 plot showing the mean \log_{10} ratios of proteins detected by tandem mass spectrometry in
568 MTOR *versus* mock immunoprecipitation (IP) experiments. Proteins quantified in at least two
569 out of three biological replicates were plotted against the negative \log_{10} p-value (Student's t-
570 test). Proteins with a mean ratio > 5 and a p-value < 0.01 (sector highlighted in dark gray)
571 were considered significantly enriched. G3BP1 is marked in green, the mTORC1 core
572 components MTOR and RPTOR are marked in blue.

573 **(B)** Immunofluorescence (IF) analysis of MCF-7 cells, serum starved, and treated with 500 μM
574 arsenite for 30 minutes. Cells were stained with G3BP1 and EIF3A antibodies. Scale bar
575 10 μm . Representative images shown for $n = 3$ biological replicates.

576 **(C)** MCF-7 cells stably transduced with shG3BP1 #1 or shControl were serum starved and
577 treated with 500 μM arsenite for 30 minutes. Data shown are representative of $n = 4$ biological
578 replicates.

579 **(D)** Quantitation of G3BP1 immunoblot data shown in **(C)**. Data are shown as the mean \pm SEM
580 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
581 green bars) were compared between shControl and shG3BP1 #1 cells, using a one-way
582 ANOVA followed by a Sidak's multiple comparisons test across $n = 4$ biological replicates. p-
583 values of the Sidak's multiple comparisons test are presented above the bar graphs.

584 **(E)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(C)**. RPS6KB1-pT389 levels
585 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
586 cells as described in **(D)**.

587 **(F)** shG3BP1 #1 or shControl MCF-7 cells were serum and amino acid starved, and stimulated
588 with 100 nM insulin and amino acids (insulin / aa) for the indicated time periods. Data shown
589 are representative of $n = 7$ biological replicates.

590 **(G)** Quantitation of G3BP1 immunoblot data shown in **(F)**. Data are shown as the mean \pm SEM
591 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
592 green bars) were compared between shControl and shG3BP1 #1 cells, using a one-way
593 ANOVA followed by a Sidak's multiple comparisons test across n = 7 biological replicates. p-
594 values of the Sidak's multiple comparisons test are presented above the corresponding bar
595 graphs.

596 **(H)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(F)**. RPS6KB1-pT389 levels
597 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
598 cells as described in **(G)**.

599 **(I)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(F)**. RPS6-pS235/236 levels
600 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
601 cells as described in **(G)**.

602 **(J)** shG3BP1 #1 or shControl MDA-MB-231 cells were serum and amino acid starved, and
603 stimulated with 100 nM insulin / aa for the indicated time periods. Data shown are
604 representative of n = 5 biological replicates.

605 **(K)** Quantitation of G3BP1 immunoblot data shown in **(J)**. Data are shown as the mean \pm SEM
606 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
607 green bars) were compared between shControl and shG3BP1 #1 cells, using a one-way
608 ANOVA followed by a Sidak's multiple comparisons test across n = 5 biological replicates. p-
609 values of the Sidak's multiple comparisons test are presented above the corresponding bar
610 graphs.

611 **(L)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(J)**. RPS6KB1-pT389 levels
612 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
613 cells as described in **(K)**.

614 **(M)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(J)**. RPS6-pS235/236 levels
615 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
616 cells as described in **(K)**.

617 **(N)** G3BP1 CRISPR/Cas9 KO or control MCF-7 cells were serum and amino acid starved, and
618 stimulated with 100 nM insulin / aa for the indicated time periods. Data shown are
619 representative of n = 3 biological replicates.

620 **(O)** Quantitation of G3BP1 immunoblot data shown in **(N)**. Data are shown as the mean \pm
621 SEM and overlaid with the single data points represented as dot plots. G3BP1 levels (black
622 and green bars) were compared between control and G3BP1 KO cells, using a one-way
623 ANOVA followed by a Sidak's multiple comparisons test across n = 3 biological replicates. p-
624 values of the Sidak's multiple comparisons test are presented above the corresponding bar
625 graphs.

626 **(P)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(N)**. RPS6KB1-pT389 levels
627 (black and blue bars) are represented and compared between control and G3BP1 KO cells as
628 described in **(O)**.

629 **(Q)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(N)**. RPS6-pS235/236 levels
630 (black and blue bars) are represented and compared between control and G3BP1 KO cells as
631 described in **(O)**.

632 **(R)** shG3BP1 #1 or shControl MCF-7 cells were serum and amino acid starved, and stimulated
633 with 100 nM insulin / aa for the indicated time periods. The rapamycin treatment started
634 30 minutes before insulin / aa stimulation. Data shown are representative of n = 4 biological
635 replicates.

636 **(S)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(R)**. Data are shown as the
637 mean \pm SEM and overlaid with the single data points represented as dot plots. RPS6KB1-
638 pT389 (black and blue bars) was compared between shControl and shG3BP1 #1 cells, using
639 a one-way ANOVA followed by a Sidak's multiple comparisons test across n = 4 biological
640 replicates. p-values of the Sidak's multiple comparisons test are presented above the
641 corresponding bar graphs.

642 **Figure 2. G3BP1 resides at lysosomes.**

643 **(A)** Separation of MCF-7 cell lysates by sucrose density gradient. Cells were serum and amino
644 acid starved. Samples were separated in a 10 to 40% sucrose gradient and analyzed by
645 immunoblot. TSC2, TSC1 and TBC1D7, TSC complex; LAMP1 and LAMP2, lysosomal
646 proteins; CDC37, cytoplasmic marker; RAB5A and RAB7A, early and late endosomal marker
647 proteins, respectively; Histone H3 and LMNA, nuclear markers. Data shown are
648 representative of n = 3 biological replicates.

649 **(B)** PLA analysis of G3BP1-LAMP1 association in serum and amino acid starved MCF-7
650 G3BP1 CRISPR/Cas9 KO and control cells. Data shown are representative of n = 3 biological
651 replicates. PLA puncta, white dots; nuclei, blue (DAPI). Scale bar 10 μ m.

652 **(C)** Quantitation of data shown in **(B)**. Data are shown as the mean \pm SEM and overlaid with
653 the single data points represented as dot plots. The number of PLA puncta per cell was
654 normalized to 1 for the mean of control cells. Control and G3BP1 KO cells were compared
655 using a paired two-tailed Student's t-test across n = 8 technical replicates. The p-value is
656 presented above the graph. Data shown are representative of n = 3 biological replicates.

657 **(D)** IPs from MDA-MB-231 cells with antibodies against TSC2 (TSC2 #1) or mock (mouse
658 IgG). Data shown are representative of n = 3 biological replicates.

659 **(E)** PLA analysis of G3BP1-TSC2 association in serum and amino acid starved MCF-7 G3BP1
660 CRISPR/Cas9 KO and control cells. Data shown are representative of n = 4 biological
661 replicates. PLA puncta, white dots; nuclei, blue (DAPI). Scale bar 10 μ m.

662 **(F)** Quantitation of data shown in **(E)**. Data are shown as the mean \pm SEM and overlaid with
663 the single data points represented as dot plots. The number of PLA puncta per cell was
664 normalized to 1 for the mean of control cells. Control and G3BP1 KO cells were compared
665 using a paired two-tailed Student's t-test across n = 8 technical replicates. The p-value is
666 presented above the graph. Data shown are representative of n = 4 biological replicates.

667 **(G)** IPs from MCF-7 cells with antibodies against MTOR or mock (rat IgG). shG3BP1 #1 or
668 shControl cells were serum and amino acid starved, and stimulated with 100 nM insulin / aa
669 for 15 minutes. Data shown are representative of n = 4 biological replicates.

670 **(H)** Quantitation of G3BP1 immunoblot data shown in **(G)**. The ratios of G3BP1/ MTOR (black
671 and green bars) are shown as the mean \pm SEM and overlaid with the single data points
672 represented as dot plots. All data were normalized to 1 for shControl. shControl and
673 shG3BP1 #1 cells were compared using a paired two-tailed Student's t-test across n = 4
674 biological replicates. p-values are presented above the corresponding bar graphs.

675 **(I)** Quantitation of TSC2 immunoblot data shown in **(G)**. The ratios of TSC2/ MTOR (black and
676 orange bars) are represented and compared between shControl and shG3BP1 #1 cells as
677 described in **(H)**.

678 **(J)** IPs from MCF-7 cells with antibodies against TSC2 (TSC2 #2 or #3) or mock (rabbit IgG).
679 Data shown are representative of n = 3 biological replicates.

680 **(K)** IPs from MCF-7 cells with antibodies against TSC2 (TSC2 #2) or mock (rabbit IgG).
681 shG3BP1 #1 or shControl cells were serum and amino acid starved, and stimulated with
682 100 nM insulin / aa for 15 minutes. Data shown are representative of n = 4 biological replicates.

683 **(L)** Quantitation of TSC1 immunoblot data shown in **(K)**. The ratios of TSC1/ TSC2 (black and
684 orange bars) are shown as the mean \pm SEM and overlaid with the single data points
685 represented as dot plots. All data were normalized to 1 for shControl. shControl and
686 shG3BP1 #1 cells were compared using a paired two-tailed Student's t-test across n = 4
687 biological replicates. p-values are presented above the corresponding graphs.

688 **(M)** Quantitation of G3BP1 immunoblot data shown in **(K)**. The ratios of G3BP1/ MTOR (black
689 and green bars) are represented and compared between shControl and shG3BP1 #1 cells as
690 described in **(L)**.

691 **(N)** Quantitation of LAMP1 immunoblot data shown in **(K)**. The ratios of LAMP1/ TSC2 (black
692 and grey bars) are represented and compared between shControl and shG3BP1 #1 cells as
693 described in **(L)**.

694 **Figure 3. G3BP1 tethers the TSC to lysosomes.**

695 **(A)** PLA analysis of TSC2-LAMP2 association in si*Renilla* luciferase (Control) or siG3BP1
696 transfected MCF-7 cells. Cells were serum and amino acid starved, and stimulated with 1 μ M
697 insulin / aa for 15 minutes. Data shown are representative of n = 4 biological replicates. PLA
698 puncta, white dots; nuclei, blue (DAPI). Scale bar 100 μ m.

699 **(B)** Quantitation of data shown in **(A)**. Data are shown as the mean \pm SEM and overlaid with
700 the single data points represented as dot plots. The number of PLA puncta per field was
701 normalized to the number of DAPI-positive nuclei, and the mean of serum and amino acid
702 starved control cells was set to 1. Control and siG3BP1 cells were compared using a one-way
703 ANOVA followed by a Sidak's multiple comparisons test across n = 12 technical replicates. p-
704 values are presented above the graphs. Data shown are representative of n = 4 biological
705 replicates.

706 **(C)** IF analysis of LAMP1-TSC2 co-localization in MCF-7 G3BP1 CRISPR/Cas9 KO and
707 control cells. Cells transfected with either siControl or siRHEB were serum and amino acid
708 starved, and stimulated with 1 μ M insulin / aa for 15 minutes. Scale bar 10 μ m. White regions
709 in overlay, co-localization of LAMP1 and TSC2. Insert, magnification of the area in the yellow
710 square. Nuclei were stained with DAPI. Images are representative of n = 4-5 distinct fields of
711 view/ replicate and n = 3 biological replicates.

712 **(D)** Quantitation of data shown in **(C)**. The Manders' correlation coefficient for TSC2 and
713 LAMP1 is represented as mean \pm SEM, which was calculated across n = 3 biological replicates
714 with 4-5 distinct fields of view in each. The single data points are overlaid as dot plots. The
715 differences among all conditions were assessed by a one-way ANOVA followed by a Sidak's
716 multiple comparisons test. p-values are presented above the graphs.

717 **(E)** shG3BP1 #1 or shControl MCF-7 cells were serum and amino acid starved. The arrow
718 indicates the specific RPS6KB1-pT389 signal. Data shown are representative of n = 8
719 biological replicates.

720 **(F)** Quantitation of G3BP1 immunoblot data shown in **(E)**. Data are shown as the mean \pm SEM
721 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and

722 green bars) were compared between shControl and shG3BP1 #1 cells, using a paired two-
723 tailed Student's t-test across n = 8 biological replicates. p-values are presented above the
724 corresponding bar graphs.

725 **(G)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(E)**. RPS6KB1-pT389 levels
726 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
727 cells as described in **(F)**.

728 **(H)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(E)**. RPS6-pS235/236 levels
729 (black and blue bars) are represented and compared between shControl and shG3BP1 #1
730 cells as described in **(F)**.

731 **(I)** Control or TSC2 CRISPR/Cas9 KO MDA-MB-231 cells, transfected with either siControl or
732 siG3BP1 were serum starved, and stimulated with 100 nM insulin for 15 minutes. Data shown
733 are representative of n = 4 biological replicates.

734 **(J)** Quantitation of TSC2 immunoblot data shown in **(I)**. Data are shown as the mean \pm SEM
735 and overlaid with the single data points represented as dot plots. TSC2 levels (black and
736 orange bars) were compared between control and TSC2 KO cells using a one-way ANOVA
737 followed by a Sidak's multiple comparisons test across n = 4 biological replicates. p-values
738 are presented above the corresponding bar graphs.

739 **(K)** Quantitation of G3BP1 immunoblot data shown in **(I)**. Data are shown as the mean \pm SEM
740 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
741 green bars) were compared between siControl and siG3BP1 in control or TSC2 KO cells,
742 using a one-way ANOVA followed by a Sidak's multiple comparisons test across n = 4
743 biological replicates. p-values are presented above the corresponding bar graphs.

744 **(Q)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(I)**. RPS6KB1-pT389 levels
745 (black and blue bars) are represented and compared between siControl and siG3BP1 in
746 control or TSC2 KO cells as described in **(K)**.

747 **Figure 4. Properties of the TSC2-G3BP1 interaction.**

748 **(A)** IPs from TSC2 KO or control MDA-MB-231 cells with antibodies against TSC1 (TSC1 #1)
749 or mock (rabbit IgG). Data shown are representative of n = 3 biological replicates.

750 **(B)** IPs with antibodies against GFP or Flag from HEK293- β_2 AR cells co-transfected with
751 TSC2-GFP and full length G3BP1₁₋₄₆₆-MYC or truncated G3BP1-MYC versions (G3BP1₁₋₁₈₂,
752 G3BP1₁₈₃₋₃₃₂, G3BP1₃₃₃₋₄₆₆). Data shown are representative of n = 5 biological replicates.

753 **(C)** Quantitation of G3BP1-myc immunoblot data shown in **(B)**. The ratios of G3BP1-myc/
754 TSC2-GFP are shown. All data were normalized to 1 for G3BP1₁₋₄₆₆. Data are shown as the
755 mean \pm SEM and overlaid with the single data points represented as dot plots. The ratios were
756 compared between full length G3BP1₁₋₄₆₆ and the truncated versions (G3BP1₁₋₁₈₂, G3BP1₁₈₃₋
757 ₃₃₂, G3BP1₃₃₃₋₄₆₆), using a one-way ANOVA followed by a Sidak's multiple comparisons test
758 across n = 5 biological replicates. p-values are presented above the corresponding bar graphs.

759 **(D)** Resistance of the TSC-G3BP1 complex against high salt or detergent. IPs from MDA-MB-
760 231 cells with antibodies against TSC1 (TSC1 #2) or mock (mouse IgG) were incubated with
761 the indicated concentrations of NaCl and SDS. Data shown are representative of n = 3
762 biological replicates.

763 **(E)** Bimolecular fluorescence complementation (BiFC) analysis of HEK293T cells transfected
764 with plasmids carrying G3BP1 fused to a C-terminal mLumin fragment, together with an N-
765 terminal mLumin fragment only (Control), or an N-terminal mLumin fragment fused to MTOR,
766 LAMP1, LAMP2 or TSC2. Scale bar 100 μ m. One representative image of each channel is
767 shown for at least n = 3 biological replicates. A scheme depicting the fusion constructs is
768 shown in **Figure S3A**.

769 **(F)** Quantitation of data shown in **(E)**. Data are shown as the mean \pm SEM and overlaid with
770 the single data points represented as dot plots. The percentages of mLumin fluorescence
771 intensity (RFP) / picture were compared between G3BP1-Control and the different plasmid
772 combinations (G3BP1-MTOR, G3BP1-LAMP1, G3BP1-LAMP2, G3BP1-TSC2), using a one-
773 way ANOVA followed by a Sidak's multiple comparisons test across at least 22 biological fields

774 of view from at least $n = 3$ biological replicates. p-values are presented above the
775 corresponding bar graphs.

776 **(G)** Excerpt of a phylogenetic Blast analysis of G3BP1, TSC1, TSC2, TBC1D7, RHEB, and
777 MTOR. A black square depicts the presence of the protein in the respective species, based
778 on blastp+ search against NCBI nr protein database (e-value < $1e-30$; for details see materials
779 and methods).

780 **Figure 5. G3BP2 shares the function of G3BP1 in the TSC-mTORC1 axis.**

781 **(A)** Reanalysis of phylogenetic Blast analysis presented in **Figure 4G** including G3BP2 in
782 addition.

783 **(B)** IPs from HEK293T cells with antibodies against TSC2 (TSC2 #1) or mock (mouse IgG).
784 Data shown are representative of n = 3 biological replicates.

785 **(C)** Separation of MCF-7 cell lysates by a 10 to 40% sucrose density gradient. Cells were
786 serum and amino acid starved. Data shown are representative of n = 3 biological replicates.

787 **(D)** BiFC analysis of HEK293T cells transfected with plasmids carrying G3BP2 fused to a C-
788 terminal mLumin fragment, together with an N-terminal mLumin fragment only (Control), or an
789 N-terminal mLumin fragment fused to MTOR, LAMP1, LAMP2 or TSC2. Scale bar 100 μ m.
790 One representative image of each channel is shown for n = 4 biological replicates. A scheme
791 depicting the fusion constructs is shown in **Figure S4D**.

792 **(E)** Quantitation of data shown in **(D)**. Data are shown as the mean \pm SEM and overlaid with
793 the single data points represented as dot plots. The percentages of mLumin fluorescence
794 intensity (RFP)/ picture were compared between G3BP2-Control and the different plasmid
795 combinations (G3BP2-MTOR, G3BP2-LAMP1, G3BP2-LAMP2, G3BP2-TSC2), using a one-
796 way ANOVA followed by a Sidak's multiple comparisons test across at least 15 biological fields
797 of view from n=4 biological replicates. p-values are presented above the corresponding bar
798 graphs.

799 **(F)** MCF-7 cells transfected with siControl or siG3BP2 were serum and amino acid starved,
800 and stimulated with 100 nM insulin / aa for the indicated time periods. Data shown are
801 representative of n = 4 biological replicates.

802 **(G)** Quantitation of G3BP2 immunoblot data shown in **(F)**. Data are shown as the mean \pm SEM
803 and overlaid with the single data points represented as dot plots. G3BP2 levels (black and
804 green bars) were compared between siControl and siG3BP2 cells, using a one-way ANOVA
805 followed by a Sidak's multiple comparisons test across n = 4 biological replicates. p-values
806 are presented above the corresponding bar graphs.

807 **(H)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(F)**. RPS6KB1-pT389 levels
808 (black and blue bars) are represented and compared between siControl and siG3BP2 cells as
809 described in **(G)**.

810 **(I)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(F)**. RPS6-pS235/236 levels
811 (black and blue bars) are represented and compared between siControl and siG3BP2 cells as
812 described in **(G)**.

813 **(J)** G3BP1 CRISPR/Cas9 KO or control MCF-7 cells were serum and amino acid starved.
814 Data shown are representative of n = 4 biological replicates.

815 **(K)** Quantitation of data shown in **(J)**. Data are shown as the mean \pm SEM and overlaid with
816 the single data points represented as dot plots. G3BP2 levels (black and green bars) were
817 compared between control and G3BP1 KO cells, using a paired two-tailed Student's t-test
818 across n = 4 biological replicates. The p-value is presented above the bar graph.

819 **(L)** MCF-7 cells, transfected with siControl or siG3BP1 were serum and amino acid starved.
820 Data shown are representative of n = 3 biological replicates.

821 **(M)** Quantitation of data shown in **(L)**. Data are shown as the mean \pm SEM and overlaid with
822 the single data points represented as dot plots. G3BP2 levels (black and green bars) were
823 compared between siControl and siG3BP1 cells, using a paired two-tailed Student's t-test
824 across n = 3 biological replicates. The p-value is presented above the bar graph.

825

826 **Figure 6. G3BP1 inhibits mTORC1-driven cancer cell motility and epileptogenic events.**

827 **(A)** Scratch assay in shG3BP1 #1 or shControl MCF-7 cultures. Pictures were taken at 0, 24,
828 and 48 hours. Rapamycin was added 24 hours prior to the 0 h time point. The scratch was
829 highlighted using the TScratch software (Geback et al., 2009). A representative image for each
830 condition is shown. Data shown are representative of n = 3 biological replicates.

831 **(B)** Quantitation of data shown in **(A)**. Data are shown as the mean \pm SEM and overlaid with
832 the single data points represented as dot plots. Percentage of wound closure at 48 h was
833 normalized to the initial wound area (0 h), and compared between shControl and shG3BP1 #1
834 cells, using a one-way ANOVA followed by a Sidak's multiple comparisons test across n = 12
835 scratches from n = 3 biological replicates. p-values are presented above or below the
836 corresponding bar graphs.

837 **(C)** RTCA proliferation analysis of shG3BP1 #1 or shControl MCF-7 cells. The impedance was
838 measured every 30 minutes for 5 days. Displayed is the relative confluence of cells normalized
839 to 1 for the maximum value. Data are shown as the mean \pm SEM for n = 6 biological replicates.

840 **(D)** Quantitation of data shown in **(C)**. The proliferation (slope/ hour) was compared between
841 shControl and shG3BP1 #1 cells using a paired two-tailed Student's t-test across n = 6
842 biological replicates. Data were normalized to the shControl condition, which was set to 1.
843 Data are shown as the mean \pm SEM with the corresponding dot plots overlaid. p-values are
844 presented above the corresponding bar graphs.

845 **(E)** *G3BP1* mRNA expression analysis. RNA seq V2 RSEM values from TCGA invasive breast
846 cancer (TCGA, provisional) were classified according to PAM50 and analysed regarding
847 *G3BP1* mRNA expression. Expression of *G3BP1* in luminal A (n = 231), luminal B (n = 127),
848 HER2-enriched (n = 58) and basal-like (n = 97) breast cancer samples was analysed using a
849 Kruskal-Wallis ANOVA by ranks. Data are shown as boxplots, representing the median with
850 25th and 75th percentiles as boxes and 5th and 95th percentiles as whiskers. The p-value of
851 the Kruskal-Wallis ANOVA by ranks is shown.

852 **(F)** Relapse-free survival of breast cancer patients based on *G3BP1* mRNA expression
853 (probelID: 225007_at). Patients with high *G3BP1* mRNA expression (n=1224) were compared

854 to patients with low expression (n=409). Breast cancer patients were divided based on the
855 best performing threshold. The survival period was assessed using the log-rank test and the
856 p-value is presented.

857 **(G)** Relapse-free survival comparing patients with high G3BP1 protein levels (n=57, probeID:
858 Q13283) to those with low (n=67) G3BP1 protein expression. Breast cancer patients were
859 divided based on the best performing threshold. The survival period was assessed using the
860 log-rank test and the p-value is presented.

861 **(H)** Relapse-free survival of breast cancer patients based on *TSC1* mRNA expression
862 (probeID:209390_at). Patients were split into those with high expression (n= 2541) and low
863 expression levels (n=1030). Breast cancer patients were divided based on the best performing
864 threshold. The survival period was assessed using the log-rank test and the p-value is
865 presented.

866 **(I)** Relapse-free survival of breast cancer patients based on *TSC2* mRNA expression (probeID:
867 215735_s_a). Patients were split into those with high expression (n=1712) and low expression
868 levels (n= 1859). Breast cancer patients were divided based on the best performing threshold.
869 The survival period was assessed using the log-rank test and the p-value is presented.

870 **(J)** IPs from brain tissue of rats with antibodies against TSC1 (TSC1 #3) or mock (rabbit IgG).
871 Data shown are representative of n = 2 biological replicates.

872 **(K)** PCR of control (control MO) and G3BP1 (G3BP1 MO) morpholino-injected zebrafish larvae
873 at 2 and 3 days post fertilization (dpf). 10 larvae per condition were pooled. Data shown are
874 representative of n = 3 biological replicates.

875 **(L)** Zebrafish larvae, injected with control MO or G3BP1 MO for 2 or 3 days were analyzed by
876 immunoblot. Data shown are representative of n = 4 biological replicates.

877 **(M)** Quantitation of RPS6-pS235/236 immunoblot data shown in (L). Data are shown as the
878 mean \pm SEM and overlaid with the single data points represented as dot plots. Protein levels
879 were normalized to the loading control GAPDH and then to the intensity of the control MO.
880 The normalized RPS6-pS235/236 values were pooled for day 2 and 3. Control and G3BP1

881 MO (black and blue bars) were compared using a paired two-tailed Student's t-test across
882 $n = 4$ biological replicates. The p-value is presented above the bar graph.

883 **(N)** Control and G3BP1 MO injected zebrafish larvae were treated on 3 dpf for 24 h with
884 rapamycin or left untreated. Non-invasive local field potentials were recorded for 10 minutes
885 from larval optic tecta at 4 dpf. Epileptiform events are represented as the mean \pm SEM, and
886 were compared between control and G3BP1 MO using a one-way ANOVA followed by a
887 Sidak's multiple comparisons test across 20 larvae per condition. p-values are presented
888 above the corresponding bar graphs.

889 **(O)** Non-invasive local field potentials in control and G3BP1 MO (quantified and described in
890 **(N)**). Three representative 10 minutes recordings are shown for control and G3BP1 MO.

891 **(P)** Power spectral density (PSD) estimation for data shown in **(N)**. Data are represented as
892 mean \pm SEM. The PSD was compared, using a two-way ANOVA followed by a Sidak's multiple
893 comparison test across 20 larvae per condition. p-values are presented for the comparisons
894 between control MO versus G3BP1 MO, G3BP1 MO versus G3BP1 MO + rapamycin, and
895 control MO + rapamycin versus G3BP1 MO + rapamycin.

896 **Star Methods**

897 **Contact for Reagent and Resource Sharing**

898 Further information and requests for resources and reagents should be directed to and will
899 be fulfilled by the Lead Contact, Kathrin Thedieck (kathrin.thedieck@uibk.ac.at).

900 **Method Details**

901 **Cell culture conditions and cell treatments**

902 Experiments were performed in HeLa alpha Kyoto cells, MCF-7 cells (ACC115), MCF-7 cells
903 expressing GFP-LC3 (MCF-7-LC3), MDA-MB-231, HEK293T, and HEK293- β_2 AR cells. All
904 cells, except of HEK293- β_2 AR, were cultivated in Dulbecco's modified Eagle's medium
905 (DMEM) with 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS) and 3 mM L-
906 glutamine (termed full DMEM medium) if not indicated otherwise. HEK293- β_2 AR were cultured
907 in DMEM with 4.5 g/L glucose and 0.584 mM L-glutamine, supplemented with 10% FBS and
908 1% penicillin and streptomycin. All cell lines were maintained at 37°C in a 5% CO₂ incubator
909 and regularly tested for mycoplasma infection.

910 SG formation was induced with arsenite at a final concentration of 500 μ M for the indicated
911 time periods. Prior to arsenite stress, cells were washed with phosphate-buffered saline (PBS)
912 and serum starved for 16 hours.

913 Metabolic stimulation experiments: for serum and amino acid starvation, cells were washed in
914 PBS and cultured for 16 hours in Hank's balanced salt solution (HBSS). For stimulation with
915 insulin and amino acids (insulin / aa), the medium was exchanged to DMEM supplemented
916 with 3 mM L-glutamine and 100 nM or 1 μ M insulin, as indicated in the figure legends.

917 For serum starvation, cells were washed in PBS and cultured for 16 hours in DMEM with 4.5
918 g/L glucose, supplemented with 3 mM L-glutamine. For stimulation with insulin alone, insulin
919 was directly added to the starvation media for the time periods indicated in the figure legends.

920 Lyophilized rapamycin was dissolved in methanol to a concentration of 1 nmol / μ L and
921 aliquoted to 5 μ L per tube. 5 μ L aliquots were dried with open lids under a sterile cell culture

922 hood and deep frozen at -80° degrees. Aliquots were thawed immediately before an
923 experiment and methanol-dried rapamycin was directly dissolved in HBSS or DMEM to a final
924 concentration of 20 or 100 nM, as indicated. Hence, no carrier was used in experiments with
925 rapamycin.

926 **RNA knockdown experiments**

927 siRNA knockdown of G3BP1, G3BP2 and RHEB was induced for two days using ON-TARGET
928 plus SMARTpool siRNA at a final concentration of 40 nM. As a negative control, a non-
929 targeting scrambled siRNA pool (siControl) was used at the same concentration. siRNA
930 transfection was performed using Lipofectamine 3000 or RNAiMAX transfection reagents
931 according to the manufacturer's protocols. The medium containing the transfection mix was
932 replaced 6 hours after transfection. For PLA analysis in **Figure 3A**, siRNA knockdown of
933 G3BP1 was induced for five days using siGENOME SMARTpool siRNA at a final
934 concentration of 15 nM. Here siRNA against *Renilla* luciferase (Control) was used as a control.

935 Doxycyclin-inducible shRNA knockdown cell lines for G3BP1 were generated using the
936 pTRIPZ system using the Trans-Lentiviral shRNA Packaging Mix (Horizon Discovery). Viral
937 particles were produced using shRNA constructs targeting G3BP1 (shG3BP1 #1 or shG3BP1
938 #2) or a non-targeting scrambled control sequence (shControl) according to the
939 manufacturer's protocol. MCF-7-LC3 and MDA-MB-231 cells were transduced in three rounds.
940 The cells were incubated with the viral supernatant containing 8 $\mu\text{g}/\text{mL}$ polybrene for 16 hours,
941 followed by 6 hours of fresh full medium. Antibiotic selection was carried out 48 hours post-
942 transduction with 2 $\mu\text{g}/\text{mL}$ puromycin for 7 days. Expression of the shRNA was induced with
943 2 $\mu\text{g}/\text{mL}$ doxycycline for 4 days. Monoclonal cell populations were obtained by limiting
944 dilutions. Knockdown efficiency was tested at protein level by immunoblotting.

945 **Knockout cell lines**

946 CRISPR/Cas9 knockout cell lines for G3BP1 and TSC2 were generated using a two-vector
947 system as previously described (Sanjana et al., 2014). First, doxycyclin-inducible Cas9

948 expressing MDA-MB-231 and MCF-7 cell lines were generated by lentiviral transduction using
949 the pCW-Cas9-Blast vector (Addgene plasmid # 83481) and thereafter selected with 5 µg/mL
950 blasticidin for 48 hours. Next, the Cas9 expressing cells were transduced with the lentiGuide-
951 Puro vector (Addgene plasmid # 52963) containing either no sgRNA (control), or sgRNA
952 targeting G3BP1 (G3BP1 KO) or TSC2 (TSC2 KO). These cells were selected with 2 µg/mL
953 puromycin for 48 hours. Monoclonal cell populations were obtained by limiting dilutions. Cas9
954 expression was induced with 2 µg/mL doxycycline for 48 hours. Knockout efficiency was
955 tested at protein level by immunoblotting.

956 **Cloning**

957 The coding sequences (CDS) of G3BP1, G3BP2, LAMP1 and LAMP2 were obtained from the
958 clone repository of the DKFZ Genomics and Proteomics Core Facility (GPCF) as Gateway®
959 compatible clones in pENTR221 or pENTR223. The CDS of MTOR and TSC2 were gifts from
960 Dominic Esposito (Addgene plasmids # 70422 and # 70640) and obtained as Gateway®
961 compatible clones in pDonor-255. After sequence verification, the CDS were cloned into the
962 BiFC destination vectors pGW-MYC-LC151 for G3BP1 and G3BP2, and pGW-HA-LN151 for
963 LAMP1, LAMP2, MTOR and TSC2 by Gateway®-specific LR-reaction following the
964 manufacturer's protocol (Invitrogen). Previously, the vectors bFos-MYC-LC151 and bJun-HA-
965 LN151 (Chu et al., 2009) were adapted for Gateway cloning. MYC-LC151 and HA-LN151
966 PCR-fragments were generated and cloned into modified pDEST26 vectors resulting in pGW-
967 MYC-LC151 and pGW-HA-LN151, as previously described (Weiler et al., 2014). Using the
968 Gateway®-specific LR reaction, TSC2 was also cloned into pEGFP-C. Three G3BP1
969 truncation constructs in pGW-MYC-LC151 were generated with primers placed at the end or
970 start positions of each construct, respectively: G3BP1₁₋₁₈₂-MYC, G3BP1₁₈₃₋₃₃₂-MYC and
971 G3BP1₃₃₃₋₄₆₆-MYC. AttB sites were added to the CDS by a two-step PCR. The first PCR was
972 performed with hybrid primers, consisting of half of the AttB sites and the other half being gene
973 specific. The second PCR was done with primers covering the complete AttB sites (see key
974 resources table for more details).

975 **Cell lysis and immunoblotting**

976 For lysis, cells were washed with PBS and lysed with radio immunoprecipitation assay (RIPA)
977 buffer (1% IGEPAL CA-630, 0.1% SDS, and 0.5% sodium deoxycholate in PBS)
978 supplemented with Complete Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2 and
979 Cocktail 3. Protein concentration was measured using Protein Assay Dye Reagent
980 Concentrate and adjusted to the lowest value. Cell lysates were mixed with sample buffer
981 (10% glycerol, 1% beta-mercaptoethanol, 1.7% SDS, 62.5 mM TRIS base [pH 6.8], and
982 bromophenol blue), and heated for 5 minutes at 95 °C. Cell lysates were then loaded on SDS
983 polyacrylamide gel electrophoresis (PAGE) gels with a concentration of 8%, 10%, 12% or 14%
984 polyacrylamide. Polyacrylamide gels were prepared consisting of two distinct layers: a
985 stacking and a separation gel. For the lower separation gel, polyacrylamide was diluted with
986 375 mM TRIS base [pH 8.8] to the respective percentage. For the upper stacking gel,
987 polyacrylamide was mixed with 0.125 M TRIS base [pH 6.8] to a final concentration of 13%.
988 Electrophoresis was carried out with a Mini-PROTEAN Tetra Vertical Electrophoresis Cell
989 system that was filled with electrophoresis buffer (0.2 M glycine, 25 mM TRIS base, and 0.1%
990 SDS), and an applied voltage of 90 to 150 V. Subsequently, proteins were transferred to
991 polyvinylidene difluoride (PVDF) membranes by using a Mini-PROTEAN Tetra Vertical
992 Electrophoresis Cell system filled with blotting buffer (0.1 M glycine, 50 mM TRIS base, 0.01%
993 SDS, [pH 8.3], and 10% methanol) and an applied voltage of 45 V for 2 hours. Afterwards,
994 membranes were blocked in 5% bovine serum albumin (BSA) – TRIS-buffered saline tween
995 (TBST) buffer (0.15 M NaCl, 60 mM TRIS base, 3 mM KCl, and 0.1% Tween-20, [pH 7.4]).
996 Membranes were incubated overnight with primary antibodies at 4 °C, following the
997 manufacturer's instructions for the respective antibodies. The next day, membranes were
998 washed in TBST buffer and incubated for at least one hour with the corresponding horseradish
999 peroxidase (HRP) coupled secondary antibodies. For detection, Pierce ECL western blotting
1000 substrate or SuperSignal West FEMTO were used to detect chemiluminescence using a LAS-
1001 4000 camera system, a ChemiDoc XRS+ camera or a Fusion Fx camera. For graphical
1002 presentation, raw images taken with the LAS-4000 or Fusion camera were exported as RGB

1003 color TIFF files using ImageJ version 1.50b, and further processed with Adobe Photoshop
1004 version CS5.1. Raw images taken with a ChemiDoc XRS+ camera were processed with Image
1005 Lab version 5.2.1 and exported for publication as TIFF files with 600 dpi resolution.

1006 **Immunoprecipitation (IP)**

1007 For IP experiments, cells were washed three times in ice-cold PBS and then harvested in
1008 CHAPS based IP lysis buffer (40 mM HEPES, 120 mM NaCl, and 0.3% CHAPS, [pH 7.5])
1009 supplemented with Complete Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2 and
1010 Cocktail 3. The lysate volume was adjusted to 1 - 2.5 mL per 15 cm cell culture plate,
1011 depending on the cell density. The lysate was incubated under gentle agitation for 20 minutes
1012 at 4 °C, centrifuged for 3 minutes at 600 g at 4 °C, the pellet was discarded and the supernatant
1013 was transferred to fresh tubes. In case of multiple samples, the protein concentration was
1014 measured using Protein Assay Dye Reagent Concentrate and all samples were adjusted to
1015 the lowest value. The lysates were pre-incubated with 10 µL pre-washed Protein G covered
1016 Dynabeads per mL of lysate for 30 minutes at 4 °C under gentle agitation. A fraction of each
1017 lysate was mixed with 5 x sample buffer, referred to as 'lysate' input in the figure panels. For
1018 IP, the pre-cleaned lysates were subdivided, and specific antibodies or isotype control IgG
1019 antibodies (mock condition) were added using 7.5 µg antibody per mL of pre-cleaned lysate.
1020 Isotype control IgG antibodies (mock antibodies) were used in the same concentration as the
1021 protein-specific antibodies. After 30 minutes at 4 °C under gentle agitation, 37.5 µL pre-
1022 washed Protein G covered Dynabeads / mL lysate were added, and the incubation was
1023 continued for 90 minutes at 4 °C under gentle agitation. Finally, beads were washed with
1024 CHAPS lysis buffer three times shortly and three times for 10 minutes at 4 °C under gentle
1025 agitation, and taken up in 1 x sample buffer. Samples were heated for 5 minutes at 95 °C and
1026 separated by SDS PAGE. For IP experiments with TSC2 and respective mock antibodies, the
1027 samples were heated for 10 minutes at 70 °C.

1028 For TSC1-IPs with NaCl and SDS washes, the IP was performed as detailed above but with
1029 a CHAPS-based IP lysis buffer without NaCl (40 mM HEPES, and 0.3% CHAPS, [pH 7.5]).

1030 Before the final washing steps, the TSC1-IP was subdivided into six tubes. Each IP was
1031 washed with CHAPS-based lysis buffer supplemented with the indicated NaCl or SDS
1032 concentrations three times shortly and three times for 10 minutes at 4 °C under gentle
1033 agitation, and taken up in 1 x sample buffer. Samples were heated for 10 minutes at 70 °C
1034 and separated by SDS PAGE.

1035 For GFP-IP experiments, 1.7×10^6 HEK293- β_2 AR cells per dish were seeded on 10 cm dishes
1036 (2 dishes per condition). 24 hours after seeding, the cells were co-transfected with 2 μ g TSC2-
1037 GFP (full length) and 1 μ g G3BP1-myc constructs (full-length or truncated versions) using
1038 Transfectin (ratio 2:1) in FBS-free DMEM, following the manufacturer's protocol. After
1039 48 hours of transient overexpression, cells were washed once in ice-cold PBS and pooled into
1040 one tube per condition. Cells were centrifuged at 16000 g for 1 minute at room temperature
1041 and resuspended in 1 mL of CHAPS-based IP lysis buffer, supplemented with protease
1042 inhibitors (100 μ M Leupeptin, 100 μ M Aprotinin, 1 μ g / mL Pepstatin A) and phosphatase
1043 inhibitors (1 mM Sodium orthovanadate, 1 mM Sodium pyrophosphate, 1 mM sodium fluoride).
1044 The cells were disrupted and the DNA was sheared through the repeated use of a syringe
1045 with a 21G x 0.80 mm needle. Afterwards, the lysate was incubated on ice for 15 minutes at
1046 4 °C, centrifuged for 45 minutes at 16000 g at 4 °C, the pellet was discarded and the
1047 supernatant was transferred to fresh tubes. If the supernatants appeared viscous the DNA
1048 shearing was repeated. Otherwise, the lysates were pre-incubated with 12 μ L Protein G
1049 sepharose beads per mL of lysate for 60 minutes at 4 °C under gentle agitation. A fraction of
1050 each lysate was mixed with 5 x sample buffer (25 mM Tris-HCl pH 6.8; 4% (w/v) SDS; 3%
1051 (w/v) DTT; 0.02% (v/v) bromophenol blue), referred to as 'lysate' in the figure panels. For IP,
1052 the pre-cleared lysates were subdivided, and 1 μ g/mL of anti-GFP antibody or anti-Flag
1053 antibody were added. After 3 hours at 4 °C under gentle agitation, 12 μ L Protein G sepharose
1054 beads per mL lysate were added, and the incubation was continued for 60 minutes at 4 °C
1055 under gentle agitation. Finally, beads were washed with CHAPS-based lysis buffer five times
1056 shortly and once for 5 minutes at 4 °C under gentle agitation. In-between the samples were

1057 centrifuged for 1 minute at 9600 g to remove the supernatant. Finally, the IP samples were
1058 dissolved in 30 μ L 1 x sample buffer. Samples were heated for 5 minutes at 95 °C and
1059 separated by SDS PAGE.

1060 The animals that were used to obtain brain tissue for IP of endogenous TSC1 were sacrificed
1061 according to protocol, which complied with European Community Council Directive
1062 2010/63/EU. The cerebral cortex from one hemisphere of a rat brain was homogenized in 4
1063 mL lysis buffer (40 mM Tris-HCl pH 7.5, 120 mM NaCl) containing 0.3 % CHAPS,
1064 supplemented with protease and phosphatase inhibitors, using a glass teflon homogenizer.
1065 The homogenate was diluted 1:1 with lysis buffer containing 0.1 % CHAPS and incubated
1066 under gentle agitation for 90 minutes at room temperature. The brain lysate was centrifuged
1067 at 1000 g, 4 °C for 10 minutes, the pellet was discarded and the supernatant was transferred
1068 to fresh tubes. A fraction of each lysate was mixed with 4 x sample buffer, referred to as 'lysate'
1069 input in the figure panels. 30 μ L of Protein G covered Dynabeads were pre-conjugated in lysis
1070 buffer containing 0.1 % CHAPS with 4 μ g of TSC1 antibody or isotype control rabbit IgG (mock
1071 condition) for 2 hours at 4 °C. For IP, the pre-conjugated beads were incubated with the lysate
1072 at 4 °C overnight under gentle agitation. Finally, beads were washed with lysis buffer
1073 containing 0.1 % CHAPS four times for 3 minutes at 4 °C under gentle agitation, and taken up
1074 in 1 x sample buffer. Samples were heated for 10 minutes at 95 °C and separated by SDS
1075 PAGE.

1076 **Sucrose gradients**

1077 Cells were lysed in homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 25 mM
1078 KCl, 5 mM MgCl₂, 3 mM imidazole), supplemented with Complete Protease Inhibitor Cocktail
1079 and Phosphatase Inhibitor Cocktail 2 and Cocktail 3 on a rocking platform for 30 minutes at
1080 4 C. Subsequently, cells were scraped and centrifuged at 12,000 g for 10 minutes at 4°C. The
1081 pellet was discarded, the supernatant was transferred to a fresh tube and the absolute protein
1082 concentration was determined with Protein Assay Dye Reagent Concentrate by calculating a
1083 BSA adjustment curve ranging from 0.5 mg / mL to 7.5 mg / mL BSA. 1.5 mg protein was

1084 loaded on 4 mL of a continuous sucrose gradient (10% to 40% sucrose) and centrifuged
1085 194,000 x g for 16 hours. Each sample was divided into 26 fractions and 5 x sample buffer
1086 was added to a final concentration of 1 x. Every second fraction was analyzed by immunoblot.

1087 **Immunofluorescence (IF)**

1088 In order to analyze SG assembly, cells were grown on coverslips and treated as indicated in
1089 the respective figure captions. Cells were washed with PBS and fixed with ice-cold methanol
1090 for 5 minutes on ice. After fixation, cells were washed three times with PBS, and permeabilized
1091 with 0.1% Triton X-100 in PBS for 60 seconds. Cells were washed with PBS and blocked with
1092 3% FCS in PBS for 30 minutes at room temperature, and incubated with primary antibodies
1093 against G3BP1 and EIF3A at 4 °C overnight. The cells were washed three times with PBS and
1094 incubated with Alexa Fluor 568 and Alexa Fluor 488 labeled secondary antibodies and
1095 Hoechst 33342 at room temperature for 30 minutes in the dark. Afterwards, cells were washed
1096 three times with PBS and twice in deionized water. The cells were mounted with Mowiol 4-88,
1097 including DABCO (1,4-diazabicyclo[2.2.2]octane) and supplemented with 10% NPG (n-propyl-
1098 gallate). Cells were analyzed by fluorescence microscopy. Images were taken using a wide-
1099 field AxioObserver Z1 microscope equipped with an Apotome, a 63x / 1.4 oil objective, and an
1100 AxioCamMRm CCD camera. For each experimental setup, the magnification and exposure
1101 times were adjusted to the condition with the brightest signal, and the settings were retained
1102 throughout for all conditions. For presentation in figures, single layers of Z-stacks were
1103 exported as TIFF with no compression using Zen2012 blue edition software, and brightness
1104 and contrast were adjusted for better visibility.

1105 The number of SG / cell was analyzed on unprocessed image raw files without any adjustment
1106 using Fiji software version 1.49v, creating maximum intensity projections of all Z-stacks. We
1107 used a background subtraction of 1, threshold adjustment with the intermodes function, and
1108 the 'Analyze Particles' function with a particle size from 0.2-infinity and a circularity from 0.5-
1109 1. SG were counted using the EIF3A channel. The number of SG / image was then normalized

1110 to the number of cells by counting the nuclei in the Hoechst channel and analyzed using a
1111 one-way ANOVA followed by a Sidak's multiple comparisons test.

1112 TSC2-LAMP1 co-staining was performed as described previously (Carroll et al., 2016). Briefly,
1113 cells were grown on coverslips and treated as indicated in the figure. The medium was
1114 removed and cells were fixed with 4% formaldehyde in PBS for 10 minutes at room
1115 temperature. After fixation, cells were permeabilized with 0.5 % Triton X-100 in PBS for 10
1116 minutes at room temperature. Cells were blocked with 5 % normal goat serum in PBS and
1117 0.05 % Tween-20 for 1 hour at room temperature, and incubated with primary antibodies
1118 against TSC2 and LAMP1 at 4 °C overnight. The following day, cells were washed and
1119 incubated with the appropriate secondary antibodies for 1 hour at room temperature.
1120 Afterwards, the cells were washed and coverslips were mounted on slides with Prolong Gold
1121 antifade reagent with 4',6-Diamidin-2-phenylindol (DAPI).

1122 Cells were analyzed by fluorescence microscopy. Z-stack images were taken using a Leica
1123 SP8 microscope, a 63x objective, 1.5x digital zoom and filters suitable for the used
1124 fluorophores. Identical settings were used to capture images across five to six separate fields
1125 (20 to 40 cells) of view. For presentation in figures, pictures were opened in Fiji (version 1.52p)
1126 and Z-stacks were projected (max). Channels were split and brightness and contrast were
1127 adjusted for better visibility. Afterwards channels were converted to RGB colour. Regions of
1128 interest (ROI) were selected and coordinates were copied to maintain the same ROI in the
1129 different channels. For single channel images, channels were pseudo-coloured grey, for
1130 merge images, the Alexa 488 channel was left green and the Alexa 555 channel was pseudo-
1131 coloured magenta. All images were exported as TIFF with no compression.

1132 For TSC2-LAMP1 co-staining, the Manders' coefficient was calculated using the Coloc2 plug-
1133 in of the ImageJ software (v1.51r). Prior to running the plug-in, a mask was made of the DAPI
1134 channel and subtracted from the other channels. A constant threshold was applied to all the
1135 images in the Z-stack, and for every image within each experiment and the Manders'

1136 colocalization coefficient was calculated. Differences in the tested conditions were analyzed
1137 using a one-way ANOVA followed by a Sidak's multiple comparisons test across n = 5-6 fields
1138 of view from one dataset representative of at least three independent experiments.

1139 **Bimolecular fluorescence complementation (BiFC)**

1140 For BiFC analysis, we made use of the red fluorophore mLumin (Chu et al., 2009; Weiler et
1141 al., 2014). 24 hours prior to transfection HEK293T cells were seeded in a 24 well plate at
1142 100,000 cells / well in full medium. The cells were transiently transfected with Lipofectamine
1143 3000 following the manufacturer's protocol in the following combinations: pGW-MYC-LC151-
1144 G3BP1 (G3BP1 fused to a C-terminal mLumin fragment) with empty pGW-HA-LN151 as a
1145 negative control (a N-terminal mLumin fragment only), and pGW-MYC-LC151-G3BP1 with
1146 either pGW-HA-LN151-LAMP1, pGW-HA-LN151-LAMP2, pGW-HA-LN151-MTOR, or pGW-
1147 HA-LN151-TSC2, respectively (a N-terminal mLumin fragment fused to LAMP1, LAMP2,
1148 MTOR or TSC2, respectively) (**Figure S3A**). For G3BP2, cells were transfected with pGW-
1149 MYC-LC151-G3BP2 and either empty pGW-HA-LN151 as a negative control, or pGW-HA-
1150 LN151-LAMP1, pGW-HA-LN151-LAMP2, pGW-HA-LN151-MTOR, pGW-HA-LN151-TSC2,
1151 respectively (**Figure S4D**). In order to achieve equal expression of all plasmids, 3 times the
1152 amount of DNA was used for the MTOR, TSC2 and empty control plasmids in comparison to
1153 the G3BP1, G3BP2, LAMP1, and LAMP2 plasmids. Cells were analyzed 48 hours after
1154 transfection using a wide-field AxioObserver Z1, equipped with a 10x / 0.3 Plan-NEO objective,
1155 an AxioCamMRm CCD camera and an mPlum (64 HE) filter. mLumin fluorescence was
1156 analyzed with Fiji version 1.49 using a background subtraction of 50, threshold adjustment
1157 from 20-max, a Gaussian Blur filter of 1 and the 'Analyze Particles' function with a particle size
1158 from 20-infinity. The mLumin fluorophore signal was measured in percent / image and
1159 compared between the different combinations by a one-way ANOVA followed by a Sidak's
1160 multiple comparisons test across at least 3 independent fields of view from at least three
1161 independent datasets, respectively. In total at least 22 independent fields of view for G3BP1

1162 and 15 independent fields of view for G3BP2 were analyzed. All pictures were taken from
1163 regions with a comparable cell density.

1164 **Proximity Ligation Assay (PLA)**

1165 For TSC2-LAMP2 PLAs, 72 h after siRNA transfection, MCF-7 cells were trypsinized and
1166 seeded in a 16-well chamber slide at a density of 4×10^4 cells per well. The following day, cells
1167 were washed twice with HBSS, starved in HBSS for 16 hours, and then stimulated for
1168 15 minutes with high-glucose DMEM containing 4 mM glutamine and 1 μ M insulin. Afterwards,
1169 cells were washed once with PBS, fixed with 4 % formaldehyde for 15 minutes and
1170 permeabilized with 0.1 % Tween-20 in PBS for 5 minutes. The PLA was performed using the
1171 Duolink In Situ Red Starter Kit Mouse/ Rabbit according to the manufacturer's instructions.
1172 Briefly, after permeabilization, the samples were blocked, and then incubated overnight with
1173 antibodies against LAMP2 and TSC2. The following day, the samples were incubated with the
1174 MINUS and PLUS PLA probes corresponding to the primary antibodies used, followed by
1175 ligation and rolling-circle amplification in the presence of Texas-Red labeled oligos to generate
1176 the PLA signal. Finally, the samples were mounted with DAPI-containing mounting medium.
1177 All incubations were performed in a humidity chamber using a volume of 40 μ L per well. The
1178 experiment was imaged with a confocal microscope (SP8, Leica); twelve stacks (7-8 μ m thick
1179 with 0.3 μ m spacing between consecutive layers) per condition were acquired.

1180 For G3BP1-TSC2 and G3BP1-LAMP1 PLAs, MCF-7 CRISPR control and G3BP1 KO cells
1181 were seeded in a 16-well chamber slide at a density of 2×10^4 cells per well. The following day,
1182 cells were washed twice with PBS, and incubated with HBSS for 16 hours. Afterwards, cells
1183 were washed once with PBS and fixed with 4% formaldehyde for 5 minutes and permeabilized
1184 with 0.1 % Triton X100 in PBS for 5 minutes. The PLA was performed as described above
1185 with antibodies against G3BP1 and TSC2 or LAMP1. The slides were analyzed using an
1186 AxioObserver Z1 compound microscope equipped with an ApoTome .2 (6 pictures per slide),
1187 63x objective, and AxioCam 702mono and AxioCam 298 color cameras. Six stacks (0.5 μ m
1188 thick) per condition were acquired.

1189 For quantitation of all PLAs, the number of PLA puncta was counted across maximum intensity
1190 projections of raw files of each stack using CellProfiler and then normalized to the number of
1191 DAPI-positive nuclei on that field. For presentation in figures, maximum intensity projections
1192 were exported as TIFF, and brightness or contrast were adjusted for better visibility.

1193 **Migration assay**

1194 2-well ibidi culture-inserts were placed into 24 well plates, generating a cell-free gap of
1195 500 μ M. After knockdown induction for 4 days, 15,000 MCF-7 shControl and shG3BP1 #1
1196 cells/ well were seeded in 100 μ L full DMEM medium. 4 replicates were seeded per condition
1197 and cell line (MCF-7 shControl and shG3BP1 #1) in the presence of 2 μ g/mL doxycycline to
1198 induce shRNA expression. Where indicated, rapamycin was added during seeding to a final
1199 concentration of 20 nM. After 24 hours, ibidi culture-inserts were removed and the medium
1200 was replaced with 1 mL full DMEM medium, supplemented with 20 nM rapamycin where
1201 indicated. Pictures were taken after 0, 24, and 48 hours with a Nikon ECLIPSE Ti-E/B inverted
1202 microscope, equipped with a 4x objective, using the NIS Elements version 4.13.04 software
1203 (settings: optimal frame size 1280 x 1024, no binning, 12 bit). Pictures were taken from two
1204 different regions in an automated manner by selecting the x- and y-coordinates of the 24 well
1205 plate, assuring that the same region of the scratch was monitored across all conditions.
1206 Pictures were exported as TIFF files converting the 12 bit to 16 bit and analyzed using the
1207 TScratch software and a consistent threshold of 250. For quantitation, the width of the open
1208 wound area of the 48 hours time point was normalized to the initial scratch size and expressed
1209 as the percentage of wound closure. Data was compared using a one-way ANOVA followed
1210 by a Sidak's multiple comparisons test across n = 12 scratches from 3 independent
1211 experiments.

1212 **Proliferation assays**

1213 Cell proliferation was monitored using an xCELLigence real-time cell analysis (RTCA) system,
1214 allowing real-time, label free cellular analysis. After knockdown induction for 4 days, MCF-7
1215 cells (MCF-7 shControl and shG3BP1 #1) were seeded in duplicates at a total of 2,000 cells

1216 per E-plate 16 chamber following the manufacturer's protocol, in the presence of 2 µg/mL
1217 doxycycline. Proliferation was measured as the relative change in electrical impedance every
1218 30 minutes for 5 days until the cells reached the stationary growth phase. Proliferation was
1219 analyzed using the RTCA software 1.2. For the presentation of the growth curves, the
1220 measured impedance was normalized to the maximum value. Data was compared using a
1221 paired two-tailed Student's t-test across n = 6 independent experiments.

1222 **G3BP1 expression and survival analyses**

1223 Clinical and RNAseq data of invasive breast cancer (TCGA, provisional) were downloaded
1224 from cBio Cancer Genomics Portal (www.cbioportal.org) using the CGDS-R package (Gao et
1225 al., 2013). For 522 patients, information on the breast cancer subtype was available, of which
1226 514 had RNAseq V2 data for *G3BP1*. A Kruskal-Wallis ANOVA by ranks was applied to
1227 evaluate subtype-dependent differences in *G3BP1* transcription.

1228 The Kaplan Meier Plotter database (www.kmplot.com) (Gyorffy et al., 2010; Szasz et al., 2016)
1229 was used for survival analysis. Relapse free survival (RFS) was assessed in breast cancer
1230 patients based on gene expression of *G3BP1* (probeID: 225007_at), *TSC1* (probeID:
1231 209390_at), and *TSC2* (probeID: 215735_at). Outlier gene arrays were excluded leaving 1764
1232 patients for analysis of *G3BP1* and 3571 patients for analyses of *TSC1/TSC2*. RFS analysis
1233 in relation to G3BP1 protein expression also was based on data available in the Kaplan-Meier
1234 Plotter database, which included 126 patients. For all calculations, patients were split based
1235 on the best performing expression threshold and log-rank p-values were calculated.

1236 **Zebrafish maintenance and breeding**

1237 Adult zebrafish of the AB strain (Zebrafish International Resource Center) were maintained
1238 under standard aquaculture conditions in UV-sterilized water at 28.5 °C on a 14 hour light / 10
1239 hour dark cycle. Fertilized eggs were collected via natural spawning. Embryos and larvae were
1240 raised in embryo medium, containing 1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl,
1241 0.12 mM MgSO₄ and 0.18 mM Ca(NO₃)₂ in an incubator on a 14 hour light / 10 hour dark

1242 cycle at 28.5°C. For all experiments described, larvae at 0-4 days post fertilisation (dpf) were
1243 used. All zebrafish experiments were approved by the Ethics Committee of the University of
1244 Leuven (Ethische Commissie van de KU Leuven, approval number 150/2015) and by the
1245 Belgian Federal Department of Public Health, Food Safety and Environment (Federale
1246 Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu, approval
1247 number LA1210199).

1248 For pharmacological assessment, 3 dpf larvae were individually placed into the wells of a 96
1249 well-plate, with each well containing 100 µL of a freshly prepared 10 µM rapamycin solution in
1250 embryo medium. The untreated larvae were treated similarly with 100 µL of embryo medium.

1251 **Antisense morpholino knockdown**

1252 To achieve knockdown of *g3bp1* in zebrafish embryos, we used morpholino antisense
1253 oligonucleotides designed to target the Exon 2 – Intron 2 boundary of the *g3bp1* mRNA
1254 (G3BP1 MO, **Figure S5B**). The morpholino sequence, as synthesized by GeneTools was: 5'-
1255 TAACAAAGGGCAAGTCACCTGTGCA-3'. A randomized 25-nucleotide morpholino was used
1256 as a negative control (control MO). Embryos were microinjected at the one- or two-cell stage
1257 with 1 nL of either *g3bp1* or *control* morpholino, corresponding to 8 ng of morpholino per
1258 injection. The morpholino concentration used was defined by titration as the highest at which
1259 the larvae displayed no morphological abnormalities. The level of knockdown in the MO
1260 injected zebrafish embryos and larvae was evaluated by PCR. For each condition, 10 embryos
1261 or larvae were pooled. RNA was extracted using Trizol and treated with DNase I. 1 µg of total
1262 RNA was reverse transcribed using the “High Capacity cDNA Reverse Transcription” kit and
1263 random primers. The generated cDNA was then amplified with gene-specific primers for *g3bp1*
1264 and *β-actin*.

1265 **Zebrafish larvae lysis and immunoblotting**

1266 For RPS6-pS235/236 analysis 10 zebrafish larvae (2-3 dpf) were pooled per condition and
1267 homogenized in RIPA buffer supplemented with Complete Mini Protease Inhibitor cocktail. A

1268 Pierce BCA protein assay kit was used to determine the protein concentration of the lysates.
1269 40 µg of protein were separated on a NuPage Novex 10% Bis-Tris gel, using SDS-PAGE with
1270 NuPage MES SDS running buffer, followed by dry transfer to an iBlot gel transfer stacks
1271 nitrocellulose membrane with an iBlot Dry Blotting System, which was then blocked for 1 hour
1272 at room temperature in Odyssey blocking buffer. Overnight incubation at 4 °C with a primary
1273 antibody against RPS6-pS235/236 was followed by incubation with Dylight secondary goat
1274 antibody to rabbit IgG for 1 hour at room temperature. A rabbit antibody against GAPDH was
1275 used as a loading control. For detection, fluorescence signal was detected using an Odyssey
1276 2.1 imaging system (Li-Cor, USA). For graphical presentation, raw images were further
1277 processed with Adobe Photoshop version CS5.1.

1278 **Non-invasive local field potential (LFP) recordings**

1279 Brain activity of 4 dpf zebrafish larvae was assessed by performing non-invasive local field
1280 potential recordings, reading the electrical signal from the skin of the larva's head (Zdebik et
1281 al., 2013). A glass pipet (containing the recording electrode), filled with artificial cerebrospinal
1282 fluid (124 mM NaCl, 2 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 1.25 mM KH₂PO₄, 26 mM
1283 NaHCO₃ and 10 mM glucose), was positioned on the skin above the optic tectum using a
1284 stereomicroscope. The differential signal between the recording electrode and the reference
1285 electrode was amplified 10,000 times by DAGAN 2400 amplifier (Minnesota, USA), band pass
1286 filtered at 0.3-300 Hz and digitized at 2 kHz via a PCI-6251 interface (National Instruments,
1287 UK) with WinEDR (John Dempster, University of Strathclyde, UK). Recordings lasted for 10
1288 minutes and were analyzed with Clampfit 10.2 software (Molecular Devices Corporation,
1289 USA). A polyspiking discharge was scored positive when its amplitude exceeded three times
1290 the amplitude of the baseline and it had a duration of at least 50 ms.

1291 In addition, power spectral density (PSD) analysis of the recordings was performed using
1292 MatLab R2018 (MATrix LABoratory, USA) software (Hunyadi et al., 2017). In brief, the power
1293 spectral density of the signals were estimated using Welch's method of averaging modified
1294 periodograms with 512-point fast fourier transform of 80% overlapping 100 sample (100 ms)

1295 long segments and a Hamming window. Next, the PSD estimate of each LFP recording was
1296 summed over each 10 Hz frequency band, ranging from 0 to 100 Hz. PSD estimates were
1297 normalized against the untreated control MO injected larvae and the data were plotted as
1298 mean (\pm SEM) PSD per condition over the 20-80 Hz range. Outliers were identified via the
1299 Iterative Grubbs test ($\alpha = 0.1$).

1300 **Quantitation and Statistical Analysis**

1301 **Immunoblot quantitation**

1302 Quantitation of raw images taken with a LAS-4000 camera system or FUSION FX7 with DarQ-
1303 9 camera was performed using ImageQuant TL Version 8.1. Background subtraction was
1304 performed using the rolling ball method with a defined radius of 200 for all images. Quantitation
1305 of raw images taken with a ChemiDoc XRS+ camera system was performed using Image Lab
1306 version 5.2.1. For all images, pixel values of a single lane were normalized to the average
1307 value of all lanes, and then normalized to the loading control Tubulin if not indicated otherwise
1308 in the figure legends. Quantitation of raw images taken with an Odyssey 2.1 imaging system
1309 (Li-Cor) was performed using Image Studio Lite Version 5.2. For images from immunoblot
1310 analysis of zebrafish samples, pixel values of a single lane were normalized to the single value
1311 of the loading control GAPDH and then to the control MO within each experiment.

1312 **Protein sequence analysis**

1313 To analyse the sequence similarities between human G3BP1 (Uniprot ID: Q13283) and human
1314 G3BP2 (Q9UN86) and their domains, or between human and zebrafish G3BP1 (Q6P124)
1315 EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) with the Blosum62
1316 matrix was used. Visualization of sequence alignments was done using Texshade based on
1317 a ClustalW alignment of the whole protein sequences. The domains indicated for G3BP1 were
1318 based on Reineke and Lloyd (2015).

1319

1320 **Phylogenetic analysis**

1321 To identify possible orthologues in other species, the human proteins G3BP1 (Uniprot ID:
1322 Q13283), G3BP2 (Q9UN86), TSC1 (Q92574), TSC2 (P49815), TBC1D7 (Q9P0N9), RHEB
1323 (Q15382), and MTOR (P42345) were used as query proteins for a blastp+ search (Camacho
1324 et al., 2009) against the NCBI non-redundant protein sequence database (nr; version 2017-
1325 11). The e-value cut-off for identified proteins was 1e-30 with a maximum of 20,000 target
1326 sequences, disabled low-complexity filtering, using the BLOSUM62 matrix, a word size of 6
1327 and gap opening and extension costs of 11 and 1, respectively. The results were parsed and
1328 filtered using custom Python scripts
1329 (<https://github.com/MolecularBioinformatics/Phylogenetic-analysis>) and manually curated as
1330 described earlier (Bockwoldt et al., 2019).

1331 **Statistical analysis**

1332 GraphPad Prism version 7.04 or 8.03 was used for statistical analysis and statistical
1333 presentation of quantitations. Where two conditions were compared, a paired two-tailed
1334 Student's t-test was performed. If more than two conditions were compared, a one-way
1335 ANOVA followed by a Sidak's multiple comparisons test was applied. In the case of
1336 immunoblot time courses or PSD analysis with equal intervals more than two conditions were
1337 compared using a two-way ANOVA. For bar graphs, the corresponding dot plots were overlaid.
1338 For *G3BP1* expression analysis a Kruskal-Wallis ANOVA by ranks was performed using Dell
1339 Statistica version 13. For the analysis of relapse-free survival the Kaplan-Meier Plotter was
1340 used and a log-rank test was applied. For each experiment, the number of replicates and the
1341 statistical test applied is indicated in the figure legend.

1342 **Data availability**

1343 All data are available from the corresponding authors upon reasonable request.

1344

1345 **Supplemental figure legends**

1346 **Figure S1. G3BP1 does not alter mTORC1 activity upon arsenite stress, related to**
1347 **Figure 1.**

1348 **(A)** Amino acid sequence of G3BP1. G3BP1's five protein domains are indicated according to
1349 Reineke and Lloyd (2015) and highlighted in blue, green, brown, yellow and pink. G3BP1
1350 peptides identified in MTOR IPs by mass spectrometry (Schwarz et al., 2015) are shown in
1351 red. In total, 20 unique peptides were identified with a sequence coverage of 58.4%.

1352 **(B)** Representative annotated MS2 spectrum of the identified G3BP1 peptide
1353 DFFQSYGNVVELR. The respective peptide sequence is highlighted with a red frame in the
1354 full-length amino acid sequence of G3BP1 depicted in **(A)**.

1355 **(C)** IPs from MCF-7 cells with antibodies against MTOR or mock (rat IgG). Data shown are
1356 representative of n = 6 biological replicates.

1357 **(D)** IPs from MCF-7 cells with antibodies against RPTOR (RPTOR#1 or #2) or mock (rat IgG).
1358 Data shown are representative of n = 3 biological replicates.

1359 **(E)** Nucleotide sequence of G3BP1. The targeting sequences of the four different siRNAs from
1360 the G3BP1 siRNA pool (light green), two shRNA sequences against G3BP1 (dark green), and
1361 the sgRNA used for CRIPSR/Cas9 mediated knockout (orange) are highlighted.

1362 **(F)** Time course analysis of shG3BP1 #1 or shControl MCF-7 cells that were serum starved
1363 and exposed to 500 μ M arsenite for up to 60 minutes. Data shown are representative of n = 3
1364 biological replicates.

1365 **(G)** Quantitation of G3BP1 immunoblot data shown in **(F)**. Data are shown as the mean \pm
1366 SEM. G3BP1 levels (black and green curve), were compared between shControl and
1367 shG3BP1 #1 cells, using a two-way ANOVA across n = 3 biological replicates. p-values are
1368 presented at the bottom of the graph.

1369 **(H)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(F)**. RPS6KB1-pT389 levels
1370 (black and blue curve) are represented and compared between shControl and shG3BP1 #1
1371 cells as described in **(G)**.

1372 **(I)** Time course analysis of siG3BP1 and siControl transfected MCF-7 cells exposed to 500 μ M
1373 arsenite for up to 60 minutes. Data shown are representative of n = 3 biological replicates.

1374 **(J)** Quantitation of G3BP1 immunoblot data shown in **(I)**. Data are shown as the mean \pm SEM.
1375 Protein levels were normalized to the average intensity of all lanes, and then to the loading
1376 control GAPDH. G3BP1 levels (black and green curve) were compared between siControl and
1377 siG3BP1 cells, using a two-way ANOVA across n = 3 biological replicates. p-values are
1378 presented at the bottom of the graphs.

1379 **(K)** Quantitation of RPS6KB1-pST389 immunoblot data shown in **(I)**. RPS6KB1-pT389 levels
1380 (black and blue bars) are represented and compared between siControl and siG3BP1 cells as
1381 described in **(J)**.

1382 **Figure S2. G3BP1 suppresses mTORC1 activation by insulin and amino acids in the**
1383 **absence of stress granules, related to Figure 1.**

1384 **(A)** G3BP1 knockdown was induced in MCF-7 cells harboring a second shRNA sequence
1385 (shG3BP1 #2, **see Figure S1E**) targeting another exon than shG3BP1 #1. Cells were serum
1386 and amino acid starved, and stimulated with 100 nM insulin / aa for the indicated time periods.
1387 Data shown are representative of n = 5 biological replicates.

1388 **(B)** Quantitation of G3BP1 immunoblot data shown in **(A)**. Data are shown as the mean \pm SEM
1389 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
1390 green bars) were compared between shControl and shG3BP1 #2 cells, using a one-way
1391 ANOVA followed by a Sidak's multiple comparisons test across n = 5 biological replicates. p-
1392 values are presented above the corresponding bar graphs.

1393 **(C)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(A)**. RPS6KB1-pT389 levels
1394 (black and blue bars) are represented and compared between shControl and shG3BP1 #2
1395 cells as described in **(B)**.

1396 **(D)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(A)**. RPS6-pS235/236 levels
1397 (black and blue bars) are represented and compared between shControl and shG3BP1 #2
1398 cells as described in **(B)**.

1399 **(E)** G3BP1 knockdown was induced in MDA-MB-231 cells harboring a second shRNA
1400 sequence (shG3BP1 #2) targeting another exon than shG3BP1 #1. Cells were serum and
1401 amino acid starved, and stimulated with 100 nM insulin / aa for the indicated time periods.
1402 Data shown are representative of n = 4 biological replicates.

1403 **(F)** Quantitation of G3BP1 immunoblot data shown in **(E)**. Data are shown as the mean \pm SEM
1404 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
1405 green bars) were compared between shControl and shG3BP1 #2 cells, using a one-way
1406 ANOVA followed by a Sidak's multiple comparisons test across n = 4 biological replicates. p-
1407 values are presented above the corresponding bar graphs.

1408 **(G)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(E)**. RPS6KB1-pT389 levels
1409 (black and blue bars) are represented and compared between shControl and shG3BP1 #2
1410 cells as described in **(F)**.

1411 **(H)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(E)**. RPS6-pS235/236 levels
1412 (black and blue bars) are represented and compared between shControl and shG3BP1 #2
1413 cells as described in **(F)**.

1414 **(I)** siG3BP1 and siControl transfected MCF-7 cells were serum and amino acid starved, and
1415 stimulated with 100 nM insulin / aa for 15 minutes. Data shown are representative of n = 6
1416 biological replicates.

1417 **(J)** Quantitation of G3BP1 immunoblot data shown in **(I)**. Data are shown as the mean \pm SEM
1418 and overlaid with the single data points represented as dot plots. G3BP1 levels (black and
1419 green bars) were compared between siControl and siG3BP1 cells, using a one-way ANOVA
1420 followed by a Sidak's multiple comparisons test across n = 6 biological replicates. p-values
1421 are presented above the corresponding bar graphs.

1422 **(K)** Quantitation of RPS6KB1-pT389 immunoblot data shown in **(I)**. RPS6KB1-pT389 levels
1423 (black and blue bars) are represented and compared between siControl and siG3BP1 cells as
1424 described in **(J)**.

1425 **(L)** Quantitation of RPS6-pS235/236 immunoblot data shown in **(I)**. RPS6-pS235/236 levels
1426 (black and blue bars) are represented and compared between siControl and siG3BP1 cells as
1427 described in **(J)**.

1428 **(M)** Schematic diagram of sgRNA designed for the G3BP1 locus. The sequence of the sgRNA
1429 is indicated in green. The locations of the nuclease-specific protospacer adjacent motif (PAM)
1430 sequence is indicated in orange.

1431 **(N)** IF analysis of shControl and shG3BP1 #1 MCF-7 cells. Cells were either serum and amino
1432 acid starved and stimulated with 100 nM insulin / aa for 15 minutes; or serum starved and
1433 treated with 500 μ M arsenite for 30 minutes. Scale bar 10 μ m. White regions in merged
1434 images, co-localization of EIF3A and G3BP1. Inserts, magnifications of the area in the yellow

1435 squares in the merged pictures. Nuclei were stained with Hoechst. Representative images are
1436 shown for n = 3 biological replicates.

1437 **(O)** Quantitation of data shown in **(N)**. Data are shown as the mean \pm SEM and overlaid with
1438 the single data points represented as dot plots. The number of SG / cell was analyzed using
1439 the eIF3A channel. shControl and shG3BP1 #1 cells were compared using a one-way ANOVA
1440 followed by a Sidak's multiple comparisons test across n = 9 pictures from n = 3 biological
1441 replicates. p-values are presented above the corresponding bar graphs.

1442 **Figure S3: BiFC constructs and their expression, related to Figure 4.**

1443 **(A)** Scheme of the plasmids used for BiFC analyses in **Figure 4E**. If the two BiFC interaction
1444 partners A and B are in close proximity, the C-terminal and N-terminal fragments of mLumin
1445 bind and enable the fluorophore to reconstitute, which can be detected by fluorescence
1446 microscopy. A = pGW-myc-LC151 (G3BP1 fused to C-terminal mLumin). B = pGW-HA-LN151
1447 (N-terminal mLumin, control; or N-terminal mLumin fused to MTOR, LAMP1, LAMP2, or
1448 TSC2).

1449 **(B)** Immunoblot analysis of the expression of BiFC fusion proteins used in **Figure 4E**.
1450 HEK293T cells were transfected with plasmids carrying G3BP1 fused to the C-terminal
1451 mLumin fragment, together with an N-terminal mLumin fragment fused to TSC2, LAMP1,
1452 LAMP2 or MTOR, or an N-terminal mLumin fragment only (control). Data shown are
1453 representative of n = 3 biological replicates.

1454 **Figure S4. G3BP2 phenocopies G3BP1 effects in the TSC complex-mTORC1 axis,**
1455 **related to Figure 5.**

1456 **(A)** Sequence alignment of human G3BP1 and G3BP2. Protein domains are indicated
1457 according to Reineke and Lloyd (2015). High similarity is highlighted in blue. PxxP motifs are
1458 indicated in green.

1459 **(B)** Sequence similarities of human G3BP1 (Q13283) and G3BP2 (Q9UN86). Sequence
1460 alignments of the domains were done based on the domain regions (AS in G3BP1) defined
1461 for G3BP1 in Reineke and Lloyd (2015).

1462 **(C)** IPs from MCF-7 cells with antibodies against MTOR or mock (rat IgG). Data shown are
1463 representative of n = 3 biological replicates.

1464 **(D)** Scheme of the plasmids used for BiFC analyses in **Figure 5B**. If the two BiFC interaction
1465 partners A and B are in close proximity, the C-terminal and N-terminal fragments of mLumin
1466 bind and enable the fluorophore to reconstitute, which can be detected by fluorescence
1467 microscopy. A = pGW-myc-LC151 (G3BP2 fused to C-terminal mLumin). B = pGW-HA-LN151
1468 (N-terminal mLumin, control; or N-terminal mLumin fused to MTOR, LAMP1, LAMP2, or
1469 TSC2).

1470 **(E)** Expression of BiFC fusion proteins used in **Figure 5B**. HEK293T cells were transfected
1471 with plasmids carrying G3BP2 fused to the C-terminal mLumin fragment, together with an N-
1472 terminal mLumin fragment fused to TSC2, LAMP1, LAMP2 or MTOR, or an N-terminal mLumin
1473 fragment only (control). Data shown are representative of n = 3 biological replicates.

1474

1475 **Figure S5. Zebrafish G3BP1: sequence alignment, generation of G3BP1 morpholino,**
1476 **and epileptogenic events, related to Figure 6.**

1477 **(A)** Sequence alignment of human and zebrafish G3BP1. Protein domains are indicated
1478 according to Reineke and Lloyd (2015). High similarity is highlighted in blue. PxxP motifs are
1479 indicated in green. The sequences share 67,8 % similarity and 77,4 % identity.

1480 **(B)** Scheme of the generation of G3BP1 morpholino (G3BP1 MO). The G3BP1 MO was
1481 designed to target the Exon 2 – Intron 2 boundary of the *g3bp1* mRNA, interfering with normal
1482 splicing, leading to a knockdown (G3BP1 MO).

1483 **(C)** Zebrafish larvae were injected with G3BP1 MO and non-invasive local field potentials were
1484 recorded from larval optic tecta at 3 dpf for 10 minutes. Representative 10 minutes recording
1485 of G3BP1 MO with magnification of a polyspiking event is shown.

1486 **(D)** Zebrafish larvae were injected with Control MO and non-invasive local field potentials were
1487 recorded from larval optic tecta at 3 dpf for 10 minutes. Representative 10 minutes recording
1488 of Control MO with magnification of a polyspiking event is shown.

1489

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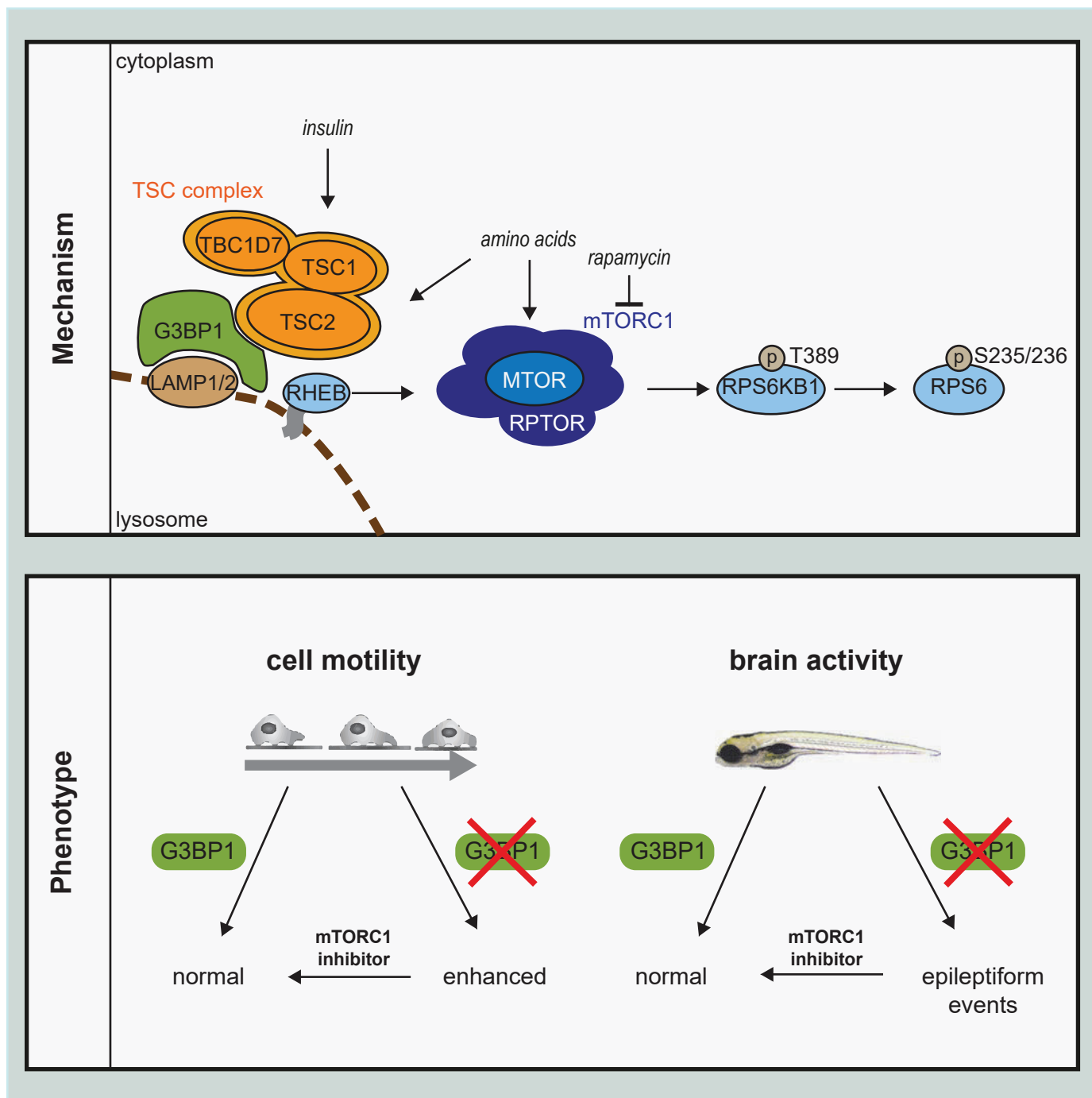
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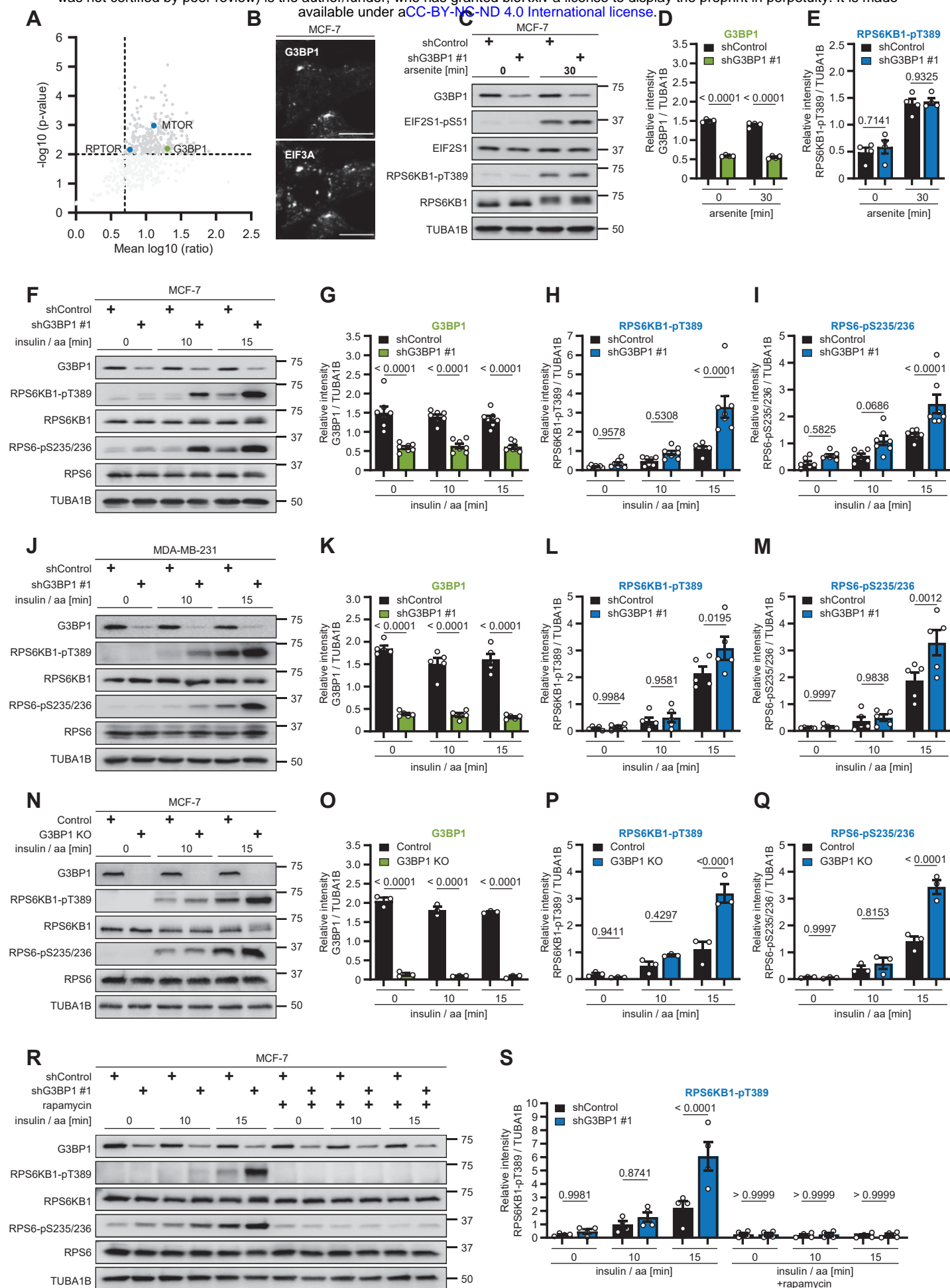


Figure 1

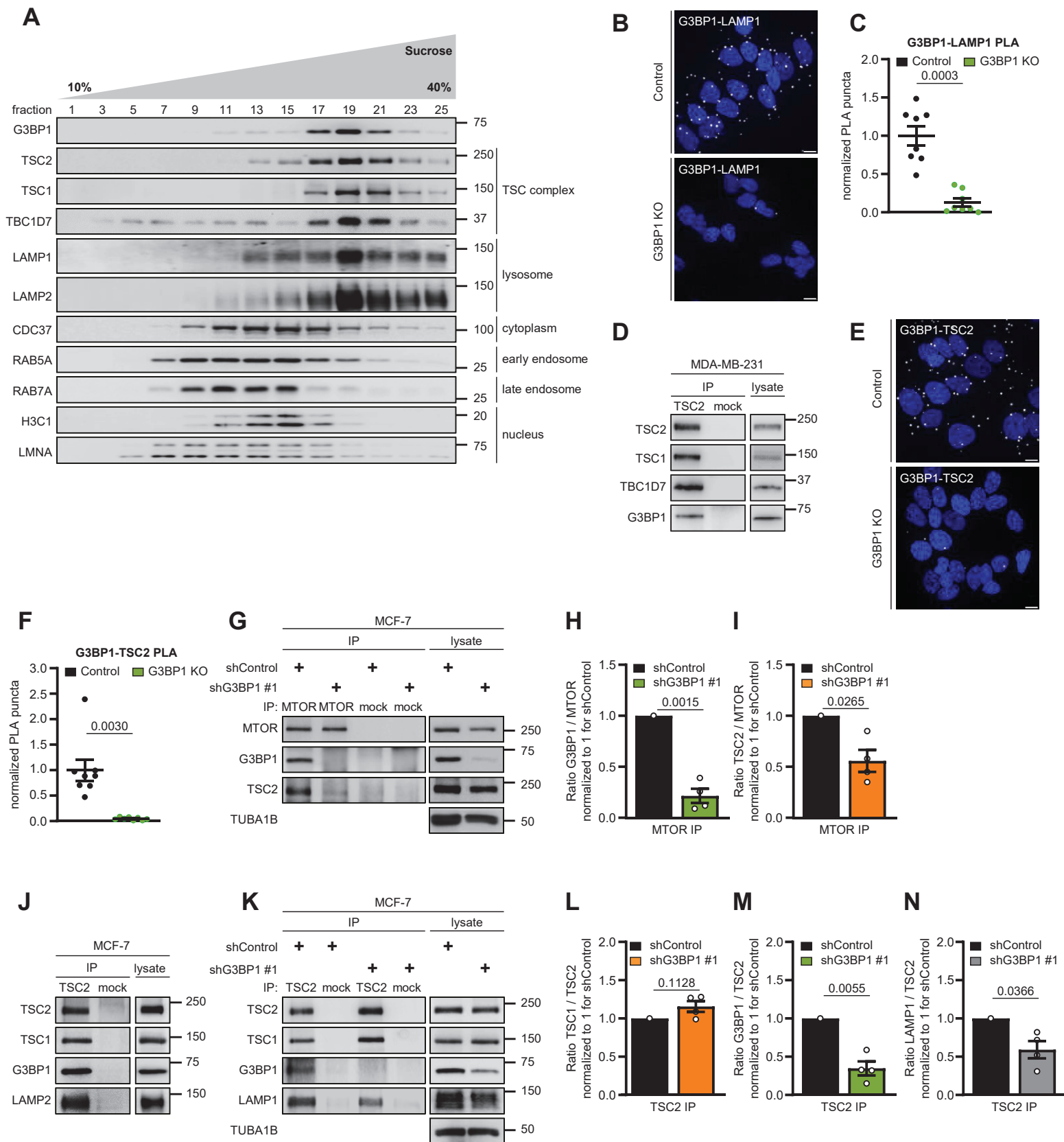


Figure 2

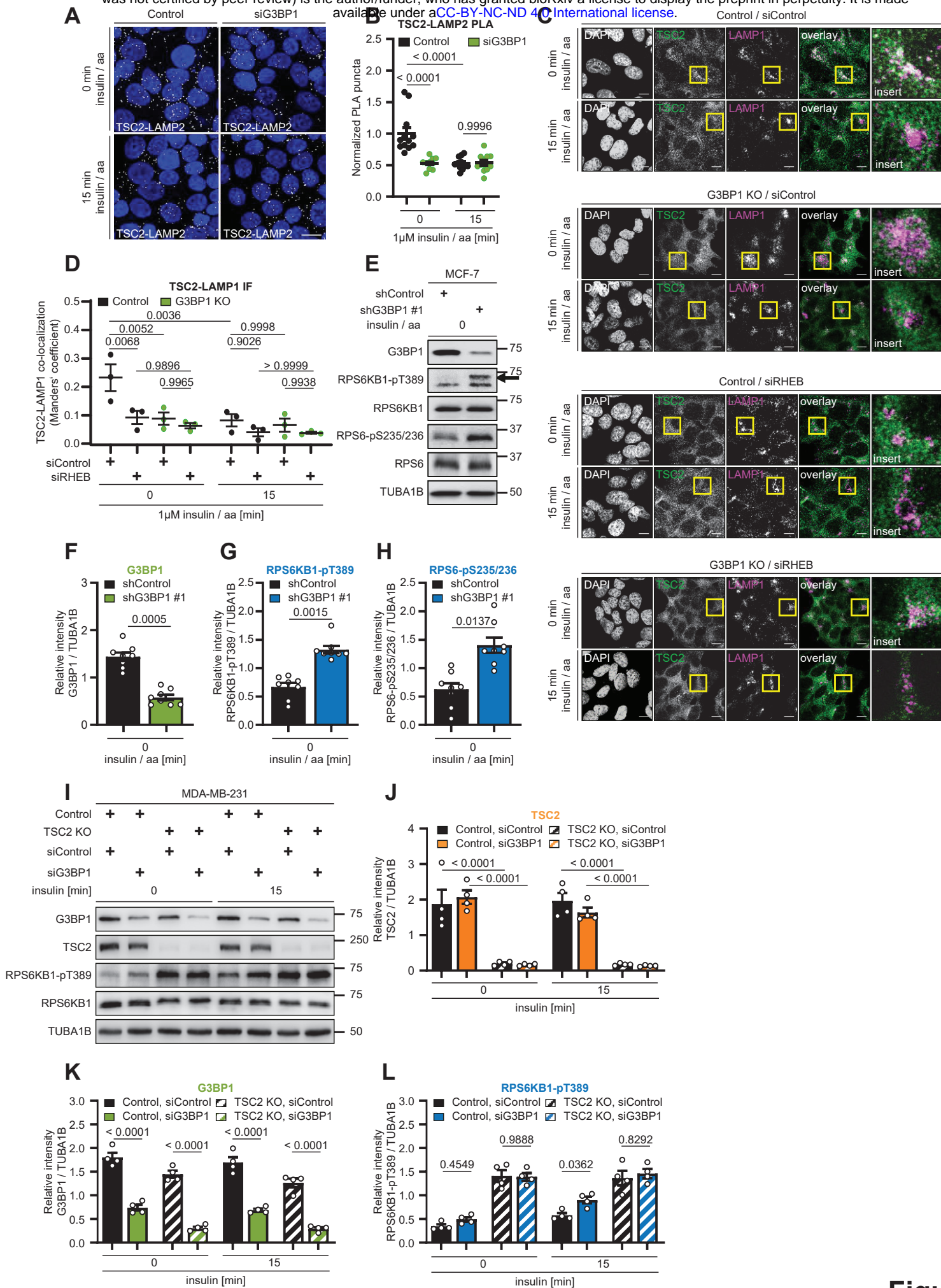


Figure 3

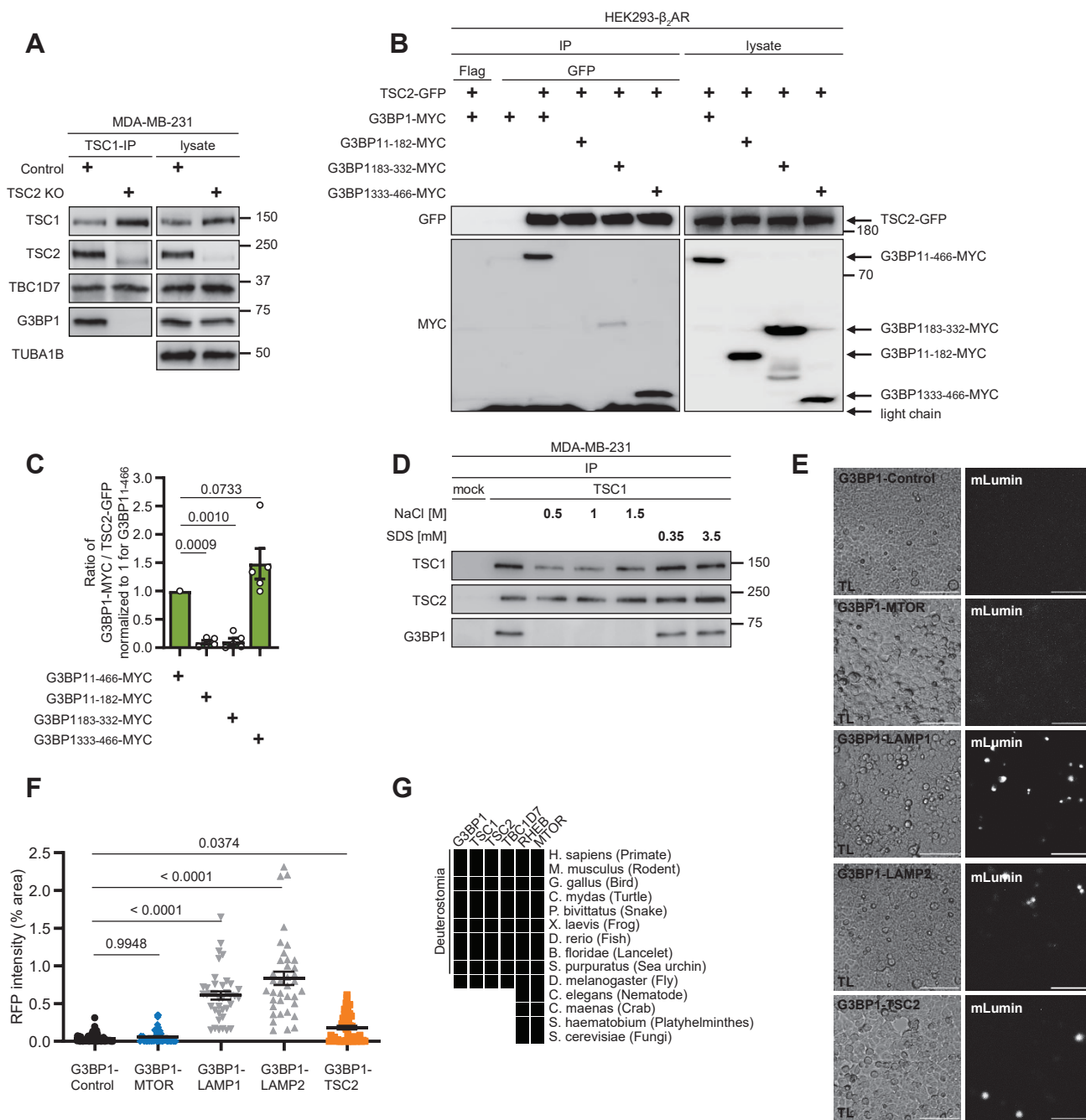


Figure 4

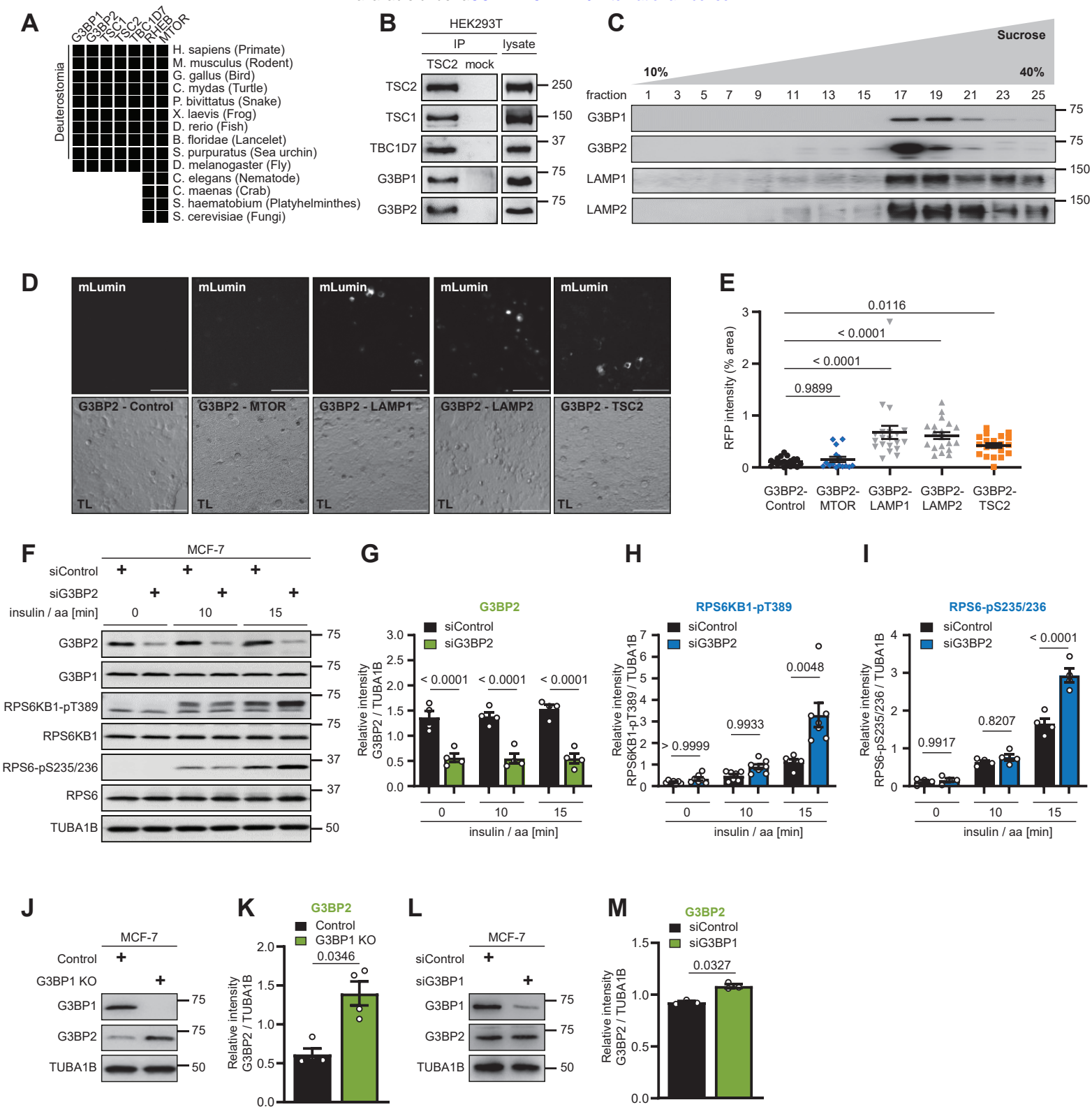


Figure 5

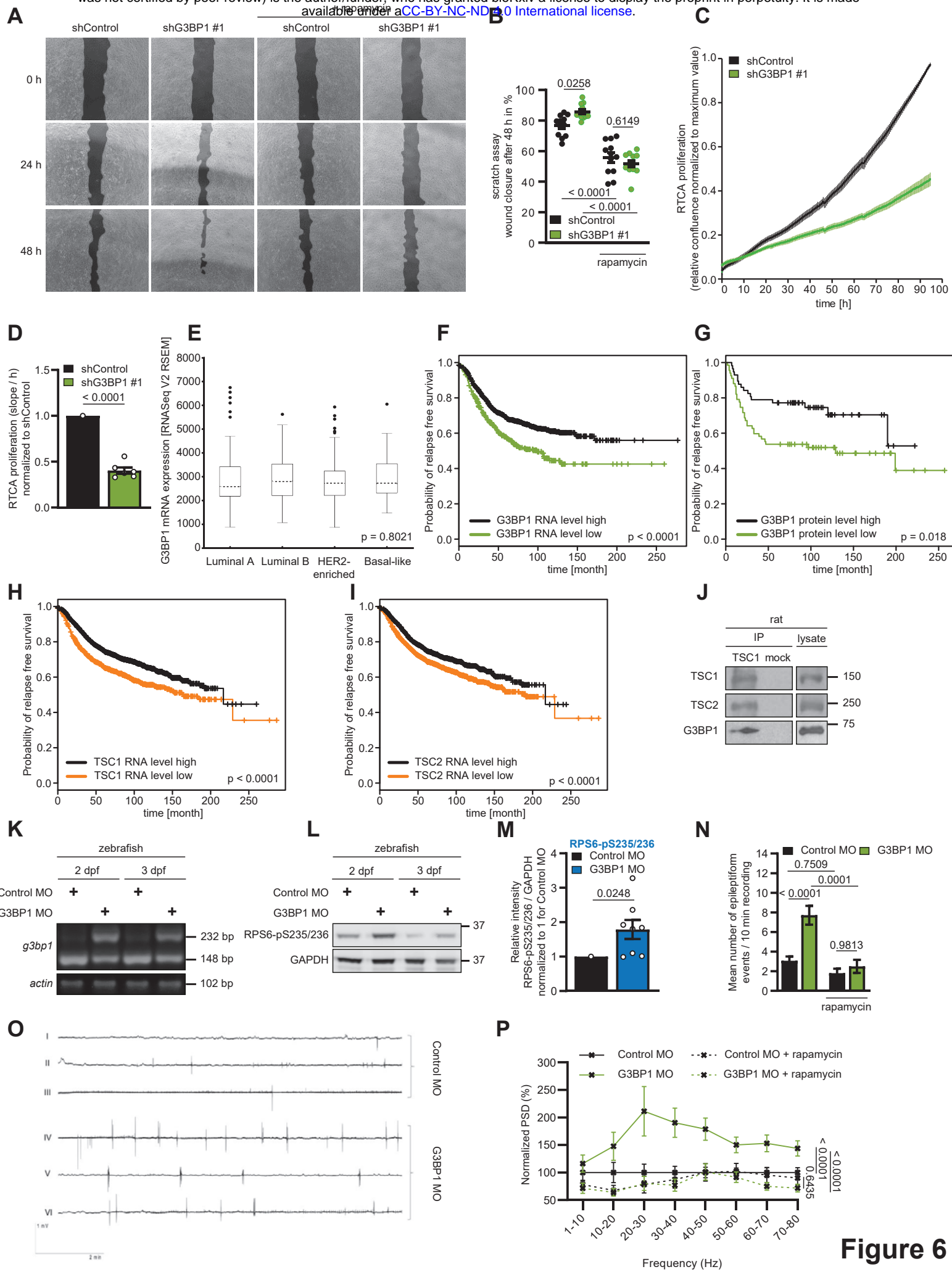


Figure 6