

1 **Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly**
2 **synthesised insulin receptors to the cell surface**

3 Revised Version

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19

20 ABSTRACT

21 Accumulation of unfolded proteins in the endoplasmic reticulum (ER) causes ER stress
22 and activates a signalling network known as the unfolded protein response (UPR). Here
23 we characterise how ER stress and the UPR inhibit insulin signalling. We find that ER
24 stress inhibits insulin signalling by depleting the cell surface population of the insulin
25 receptor. ER stress inhibits proteolytic maturation of insulin proreceptors by interfering
26 with transport of newly synthesised insulin proreceptors from the ER to the plasma
27 membrane. Activation of AKT, a major target of the insulin signalling pathway, by a
28 cytosolic, membrane-bound chimera between the AP20187-inducible F_v2E dimerisation
29 domain and the cytosolic protein tyrosine kinase domain of the insulin receptor was not
30 affected by ER stress. Hence, signalling events in the UPR, such as activation of the JNK
31 MAP kinases or the pseudokinase TRB3 by the ER stress sensors IRE1 α and PERK, do
32 not contribute to inhibition of signal transduction in the insulin signalling pathway.
33 Indeed, pharmacologic inhibition and genetic ablation of JNKs, as well as silencing of
34 expression of TRB3, did not restore insulin sensitivity or rescue processing of newly
35 synthesised insulin receptors in ER-stressed cells.

36 HIGHLIGHT SUMMARY

37 ER stress inhibits activation of AKT by insulin by depleting insulin receptors and interferes
38 with delivery of newly synthesised insulin receptors to the cell surface. Bypass of the
39 secretory pathway in synthesis of the cytosolic protein tyrosine kinase domain of the insulin
40 receptor negates the effects of ER stress on activation of AKT by insulin.

41 INTRODUCTION

42 In mammalian cells, most secreted proteins and proteins residing in the plasma membrane or
43 the secretory pathway are transported into the ER while their polypeptide chains are being
44 assembled by translating ribosomes (Walter and Lingappa, 1986). In the ER, newly
45 synthesised proteins fold into their native three-dimensional structures, undergo multiple post-
46 translational modifications including asparagine (N)-linked glycosylation (Hubbard and Ivatt,

47 1981; Kornfeld and Kornfeld, 1985) and the formation of disulphide bonds (Fewell et al.,
48 2001). Interaction of newly synthesised proteins with several chaperone systems facilitates
49 their productive folding, but also serves as a quality control mechanism to retain newly
50 synthesised proteins in the ER until they have completed their folding and maturation
51 processes (Hebert and Molinari, 2007). Consequently, unfolded or only partially folded
52 proteins are prevented from exiting the ER until they have completed their folding process or
53 are targeted to degradation mechanisms if they fail to fold productively, such ER-associated
54 degradation (ERAD) (Meusser et al., 2005) or ER-phagy (Bernales et al., 2006).

55 The accumulation of unfolded and partially folded proteins in the ER activates a
56 signalling network termed the unfolded protein response (UPR) (Schröder and Kaufman,
57 2005; Walter and Ron, 2011). Three ER transmembrane proteins, the membrane-bound
58 transcription factor ATF6 (Yoshida et al., 2000, 2001b), the protein kinase PERK (Shi et al.,
59 1998; Harding et al., 1999; Shi et al., 1999), and the protein kinase-endoribonuclease (RNase)
60 IRE1 α (Tirasophon et al., 1998) initiate signalling in the UPR. After cleavage from the
61 endomembrane system ATF6 translocates to the nucleus and activates transcription of genes
62 encoding ER-resident molecular chaperones and components of the ER-associated protein
63 degradation machinery (Ye et al., 2000; Wu et al., 2007; Yamamoto et al., 2007). PERK
64 transiently attenuates general translation in ER-stressed cells by phosphorylating the α subunit
65 of eIF2 (Shi et al., 1998; Harding et al., 1999). Phosphorylation of eIF2 α also promotes
66 translation of mRNAs with several short upstream open reading frames leading to induction
67 of the transcription factor CHOP (Harding et al., 2000) and the pseudokinase TRB3 (Ohoka et
68 al., 2005).

69 IRE1 α is a bifunctional protein kinase-RNase (Tirasophon et al., 1998; Tirasophon et al.,
70 2000). The IRE1 α RNase activity initiates splicing of *XBP1* mRNA which encodes a bZIP
71 transcription factor (Shen et al., 2001; Yoshida et al., 2001a; Calton et al., 2002; Lee et al.,
72 2002). Spliced XBP1 (XBP1^s) is a more potent transcriptional activator than unspliced XBP1
73 (XBP1^u) for genes encoding ER resident molecular chaperones, phospholipid biosynthetic
74 enzymes, and proteins involved in ER-associated protein degradation (Shen et al., 2001;

75 Yoshida et al., 2001a; Calfon et al., 2002; Lee et al., 2002). In addition, relaxed specificity of
76 the RNase activity mediates decay of many mRNAs encoding proteins targeted to the
77 secretory pathway (Hollien and Weissman, 2006; Hollien et al., 2009; Gaddam et al., 2013).
78 Through association with the E3 ubiquitin ligase TRAF2 IRE1 α activates the JNK family of
79 mitogen-activated protein kinases (Urano et al., 2000).

80 Insulin signaling is initiated by binding of insulin to the insulin receptor, activation of the
81 protein tyrosine kinase domain and tyrosine autophosphorylation of the insulin receptor, and
82 extensive tyrosine phosphorylation of insulin receptor substrate (IRS) proteins [reviewed in
83 (Saltiel and Kahn, 2001)], including phosphorylation of Y612, Y632, Y896, Y941, Y1173,
84 and Y1229 in human IRS1 (Shoelson et al., 1992; Sun et al., 1993; Rocchi et al., 1995; Xu et
85 al., 1995; Esposito et al., 2001; Hers et al., 2002). Phosphorylated Y612, Y632, and Y941 are
86 binding sites for the Src homology 2 (SH2) domain of the p85 α subunit of
87 phosphatidylinositol (PI) 3-kinase (PI3K) (Sun et al., 1993; Rocchi et al., 1995; Esposito et
88 al., 2001). After formation of PI-3,4-bis- and PI-3,4,5-trisphosphate by PI3K,
89 phosphoinositide-dependent kinases (PDKs) and isoforms of the protein serine/threonine
90 kinase AKT are recruited to the plasma membrane. Colocalization of PDKs and AKT to the
91 plasma membrane facilitates phosphorylation of AKT on T308 by PDK1 (Alessi et al., 1996),
92 and on S473 by mTORC2 (Sarbasov et al., 2005; Guertin et al., 2006; Jacinto et al., 2006),
93 PAK1 (Mao et al., 2008), and ILK (McDonald et al., 2008) leading to activation of AKT.
94 Activated AKT facilitates glucose transport, protein and glycogen synthesis, and inhibits
95 gluconeogenesis. Recruitment of GRB2 to IRS1 phosphorylated at Y896 via its SH2 domain
96 (Sun et al., 1993; Myers et al., 1994) activates mitogen-activated protein kinases, such as
97 p42/p44, and contributes to generating a mitogenic signal in insulin-stimulated cells
98 (Valverde et al., 2001).

99 Inhibition of signal transduction in the insulin signalling pathway is the cause for insulin
100 resistance when cells fail to respond normally to insulin. Insulin resistance can be caused by a
101 decrease in insulin receptors or defects in signal transduction downstream of the insulin
102 receptor. Activation of JNKs and TRB3 by the UPR has been implicated in the inhibition of

103 insulin signalling downstream of the insulin receptor (Özcan et al., 2004; Koh et al., 2006;
104 Koh et al., 2013). JNKs inhibit tyrosine phosphorylation of IRS1 by the insulin receptor by
105 phosphorylating S307 in murine IRS1 and S312 in human IRS1 (Aguirre et al., 2000; Aguirre
106 et al., 2002). Consequently, phosphorylation and activation of AKT by insulin is inhibited by
107 JNKs (Lee et al., 2003; Nguyen et al., 2005; Emanuelli et al., 2008). TRB3 interacts with and
108 inhibits phosphorylation of AKT (Du et al., 2003) and also interacts with IRS1 and inhibits its
109 phosphorylation at Y612 by the insulin receptor (Koh et al., 2013).

110 JNKs become rapidly and transiently activated in ER-stressed cells to promote an
111 adaptive response to ER stress (Brown et al., 2016). The motivation for this study was to
112 characterise whether this rapid, initial JNK activation in the first 10 - 120 min of the ER stress
113 response causes insulin resistance. However, we find no evidence for inhibition of insulin-
114 stimulated AKT phosphorylation or IRS1 tyrosine phosphorylation in cells exposed to ER
115 stress for up to ~8-12 h despite activation of JNKs and induction of TRB3. Only ER stress
116 lasting for more than ~8-12 h inhibited insulin-stimulated AKT phosphorylation, but did so
117 independent of JNKs and TRB3, and correlated with depletion of β chains of the mature
118 insulin receptor, accumulation of unprocessed α - β precursors of the insulin receptor in the
119 ER, and depletion of GFP-tagged insulin receptors from the plasma membrane. Moreover,
120 phosphorylation of AKT at S473 in response to activation of a cytosolic, membrane-bound
121 chimera between the AP20187-inducible F γ 2E dimerisation domain (Clackson et al., 1998;
122 Yang et al., 2000) and the protein tyrosine kinase domain of the insulin receptor is not
123 affected by ER stress lasting for 24 h. We propose that inhibition of trafficking of newly
124 synthesised insulin receptors to the plasma membrane suffices and is necessary to inhibit
125 activation of AKT by insulin in ER-stressed cells by depleting the plasma membrane
126 population of the insulin receptor.

127 **RESULTS**

128 *ER stress for up to 8 h does not inhibit insulin-stimulated AKT activation.* We used *in vitro*
129 differentiated C₂C₁₂ myotubes, 3T3-F442A preadipocytes, and Hep G2 hepatoma cells to

130 characterise the effects of ER stress on insulin signalling, because these cell types are cell
131 culture models of the main tissues and organs contributing to glucose homeostasis, muscle,
132 adipose tissue, and the liver (Saltiel and Kahn, 2001). We first characterised whether ER
133 stress induced with different ER stressors for up to 8 h inhibits signal transduction
134 downstream of the insulin receptor by monitoring insulin-stimulated phosphorylation of AKT
135 at T308 and S473 in *in vitro* differentiated C₂C₁₂ myotubes. C₂C₁₂ myotubes were serum-
136 starved for 18 h, treated with ER stressors for the last 1 to 8 h of serum starvation and then
137 stimulated with 100 nM insulin for 15 min in the continued presence of ER stressors. 100 nM
138 insulin was chosen, because inhibition of insulin signalling downstream of the receptor
139 manifests independent of insulin concentration (Olefsky and Kolterman, 1981). To exclude
140 drug specific effects on insulin signalling, we used three different ER stressors, the SERCA
141 pump inhibitor thapsigargin (Thastrup et al., 1990), the N-glycosylation inhibitor tunicamycin
142 (Kuo and Lampen, 1976; Lehle and Tanner, 1976), and the protease SubAB, which cleaves
143 and inactivates the ER resident HSP70 class molecular chaperone BiP/GRP78 (Paton et al.,
144 2006). We also titrated the concentrations of both thapsigargin and tunicamycin in the culture
145 medium over a 10- or 100-fold concentration range, respectively. AKT phosphorylation was
146 chosen as readout, because its dynamic range is larger than the dynamic ranges of many
147 physiological responses to insulin such as translocation of GLUT4 to the plasma membrane
148 (Hoehn et al., 2008), uptake of 2-deoxyglucose (Whitehead et al., 2001), or glucose oxidation
149 (Kono and Barham, 1971). Induction of ER stress with 0.1-1.0 μ M thapsigargin, 0.1-10 μ g/ml
150 tunicamycin, or SubAB for up to ~8 h in C₂C₁₂ myotubes, however, did not decrease insulin-
151 stimulated phosphorylation of AKT at T308 (Figure 1, A and B) or S473 (Figure 1, A and C).

152 To confirm that treatment of serum-starved C₂C₁₂ cells with ER stressors induces ER
153 stress, we monitored *XBPI* splicing using reverse transcriptase PCR. The IRE1 α -initiated
154 *XBPI* splicing reaction removes a 26 nt intron from *XBPI* mRNA. Therefore, the appearance
155 of a shorter reverse transcriptase PCR product on 2% (w/v) agarose gels indicates activation
156 of the RNase activity of IRE1 α . Upon exposure of serum-starved C₂C₁₂ cells to 0.3 μ M
157 thapsigargin, 1 μ g/ml tunicamycin, or 1 μ g/ml SubAB a shorter reverse transcriptase PCR

158 product appeared (Figure 1, D), which represents spliced *XBPI* mRNA. Strong induction of
159 *TRB3* mRNA after induction of ER stress for 4 or 8 h was also detected (Figure 1, E and F),
160 which suggests that serum-starved C₂C₁₂ cells experience ER stress when challenged with
161 thapsigargin, tunicamycin or SubAB. Furthermore, serum starvation did not decrease *XBPI*
162 splicing in cells exposed to 1 μM thapsigargin for 1 h (Figure S1, A), which argues against
163 the possibility that induction of ER stress is blunted by decreased protein synthesis rates in
164 serum-starved cells. Thapsigargin-, tunicamycin-, or SubAB-induced ER stress for up to 12 h
165 also did not inhibit insulin-stimulated AKT activation in 3T3-F442A adipocytes or Hep G2
166 hepatoma cells, or over a period of 4 h in Fao rat hepatoma cells cultured in RPMI 1640 or
167 Coon's modification of Ham's F12 medium (data not shown).

168 JNKs are activated as early as 10 min after induction of ER stress in C₂C₁₂ myotubes and
169 3T3-F442A adipocytes, and after 30 min in Hep G2 cells (Brown et al., 2016), which raises
170 the possibility that ER stress may inhibit the insulin signalling pathway around these times in
171 the ER stress response. However, 30 min of thapsigargin-induced ER stress did not decrease
172 insulin-stimulated phosphorylation of AKT on S473 in 3T3-F442A adipocytes, C₂C₁₂
173 myotubes, or Hep G2 cells (Figure S1, B and C). Induction of ER stress for 30 min with
174 tunicamycin or SubAB also did not decrease insulin-induced phosphorylation of AKT (Figure
175 S1, B and C). These results suggest that activation of JNKs by ER stress does not inhibit
176 signal transduction in the insulin signalling pathway. To characterise whether lower, more
177 physiologic insulin concentrations (Cryer and Polonsky, 1998; Unger and Foster, 1998)
178 unmask effects of ER stress on insulin-stimulated AKT phosphorylation, we stimulated cells
179 with 10 nM insulin for 15 min. ER stress induced with 0.3 μM thapsigargin, 1 μg/ml
180 tunicamycin or SubAB for 30 min up to 8 h had no effect on phosphorylation of AKT on
181 S473 in 3T3-F442A, C₂C₁₂, or Hep G2 cells stimulated with 10 nM insulin for 15 min (Figure
182 2). In summary, these data establish that short periods of ER stress lasting for up to ~8 h, in
183 which JNKs are activated (Brown et al., 2016) and *TRB3* is induced (Figure 1, E and F), do
184 not inhibit insulin-dependent AKT phosphorylation in 3T3-F442A, C₂C₁₂, Fao, and Hep G2
185 cells.

186 *ER stress for up to 30 min does not inhibit IRS1 tyrosine phosphorylation.* Phosphorylation of
187 AKT is downstream of tyrosine phosphorylation of IRS1 by the activated insulin receptor in
188 the insulin signalling pathway (Backer et al., 1992; Franke et al., 1995). The absence of
189 effects of heterozygosity for IRS1 in lean mice on control of blood glucose levels (Shirakami
190 et al., 2002) and the lack of effects of partial shRNA-mediated knockdown of IRS1 in skeletal
191 muscle on local glucose clearance (Cleasby et al., 2007) suggest that IRS1 is available in
192 excess over the amounts needed for full activation of downstream events in the insulin
193 signalling pathway. To address the possibility that decreases in IRS1 tyrosine phosphorylation
194 in ER-stressed cells are not reflected at the level of AKT phosphorylation, we directly
195 examined the effects of ER stress on IRS1 tyrosine phosphorylation. First, we characterised
196 whether within the initial 30 min time window after induction of ER stress, in which ER
197 stress activates JNKs in 3T3-F442A, C₂C₁₂, and Hep G2 cells (Brown et al., 2016), a decrease
198 in tyrosine phosphorylation of specific, well-characterised insulin-responsive tyrosine
199 phosphorylation sites, such as Y608 (mouse)/Y612 (human; from here on abbreviated as
200 Y608/612), Y628/632, Y891/896, and Y935/941 (Shoelson et al., 1992; Sun et al., 1993; Xu
201 et al., 1995; Hers et al., 2002), could be observed. We could extract intact IRS1 from 3T3-
202 F442A, C₂C₁₂, and Hep G2 cells only under strongly denaturing conditions such as 8 M urea,
203 2.5% (w/v) SDS, or 7 M urea, 2 M thiourea, 2.5% (w/v) SDS, or 8 M guanidinium
204 hydrochloride, 1% (v/v) Triton X-100, or 4 M guanidinium thiocyanate, 1% (v/v) Triton X-
205 100, or 10-20% (w/v) trichloroacetic acid (TCA, data not shown). In addition, detection of
206 full-length IRS1 by Western blotting required electrotransfer onto PVDF membranes at pH
207 ~10 in the presence of SDS as described in “Materials and Methods”. Under these conditions,
208 Western blots displaying one band at ~180 kDa, the migration position of IRS1 in SDS-
209 PAGE (Sun et al., 1991), were obtained with all four single tyrosine phosphorylation site
210 antibodies and anti-IRS1 antibodies (Figures 3, S2, and S3). Antibodies against
211 phosphorylated tyrosine phosphorylation sites gave much stronger signals on samples isolated
212 from insulin-treated cells at ~180 kDa (Figures 3, S2, and S3). Induction of ER stress with 1
213 μ M thapsigargin for up to 30 min or with 0.1 μ g/ml, 1 μ g/ml, or 10 μ g/ml tunicamycin for 30

214 min did not affect phosphorylation of Y608/612, Y628/632, Y891/896, or Y935/941 when
215 cells were stimulated with 10 or 100 nM insulin for 5 min (Figures 3, S2, and S3). An ~3-fold
216 increase in IRS1 levels upon insulin stimulation in 3T3-F442A cells (Figure S2) and an ~2-
217 fold increase in C₂C₁₂ myotubes and Hep G2 cells (Figures 3 and S3) were also not affected
218 by thapsigargin or tunicamycin.

219 Human IRS1 has 32 tyrosyl residues, while murine IRS1 has 34. Phosphorylation of at
220 least 19 tyrosines in human IRS1 and 13 tyrosines in murine IRS1 has been shown
221 experimentally (Hornbeck et al., 2015). An additional eight tyrosines in human IRS1 and nine
222 tyrosines in murine IRS1 feature at least one acidic amino acid in the six immediately
223 upstream amino acids. Upstream acidic amino acids can be a feature of tyrosine
224 phosphorylation sites (Neil et al., 1981; Smart et al., 1981; Baldwin et al., 1982; Hunter,
225 1982; Patschinsky et al., 1982; Pike et al., 1982; Baldwin et al., 1983) and are, for example,
226 enriched in the experimentally confirmed tyrosine phosphorylation sites of human and murine
227 IRS1 (human IRS1, upstream positions -1 to -3, χ^2 p value < 0.05; murine IRS1, upstream
228 positions -1 to -6, χ^2 p value < 0.01). Given the large number of confirmed and putative
229 tyrosine phosphorylation sites in IRS1, it is possible that individually surveying a subset of
230 tyrosine phosphorylation sites may not uncover effects of ER stress on tyrosine
231 phosphorylation of IRS1. To address this concern, we immunoprecipitated IRS1 from cell
232 lysates prepared from unstressed cells, or cells that were exposed to 1 μ M thapsigargin or 10
233 μ g/ml tunicamycin for 30 min and then stimulated with 10 or 100 nM insulin for 5 min in the
234 continued presence of thapsigargin or tunicamycin, and Western blotted the
235 immunoprecipitates with a pan-phosphotyrosine antibody (clone 4G10[®] Platinum), and an
236 anti-IRS1 antibody (Figure 4). We observed a strong increase in tyrosine phosphorylation
237 after stimulation with either 10 or 100 nM insulin for 5 min, but neither thapsigargin nor
238 tunicamycin had any effect on the level of tyrosine phosphorylation of IRS1 (Figure 4). In
239 summary, these data show that ER stress lasting for up to 30 min does not affect insulin-
240 stimulated IRS1 tyrosine phosphorylation.

241 *ER stress does not elicit serine 307/312 phosphorylation of IRS1.* JNKs inhibit tyrosine
242 phosphorylation of IRS1 by the activated insulin receptor by phosphorylating IRS1 at
243 S307/312 (Aguirre et al., 2000; Aguirre et al., 2002). Unaltered tyrosine phosphorylation of
244 IRS1 in ER-stressed cells (Figures 3, 4, S2, and S3) suggested that JNKs, despite being
245 activated by ER stress (Brown et al., 2016), do not phosphorylate IRS1 at S307/312 or that
246 phosphorylation of IRS1 at S307/312 by JNKs does not inhibit tyrosine phosphorylation of
247 IRS1 by the insulin receptor in ER-stressed cells. To distinguish between these possibilities,
248 we measured IRS1 S307/312 phosphorylation in serum-starved cells and standardised the
249 phospho-S307/S312 IRS1 signal to the signal for total IRS1 (Figure 5). We included
250 treatment with 5 µg/ml anisomycin for 30 – 60 min, which has been reported to elicit
251 phosphorylation of IRS1 at S307/312 (Aguirre et al., 2000; Aguirre et al., 2002; Werner et al.,
252 2004), as a positive control, because we obtained only faint signals with the anti-pS307/S312
253 antibody with cell lysates prepared from cells exposed to 1 µM thapsigargin for up to 2 h
254 (Figure 5). In 3T3-F442A and Hep G2 cells, anisomycin retarded migration of IRS1 in 7.5%
255 SDS-PAGE gels (Figure 5, A and E). This suggests that IRS1 becomes phosphorylated at
256 additional sites than those reported in the literature in response to anisomycin, S302/307
257 (Werner et al., 2004), S307/312 (Aguirre et al., 2000; Aguirre et al., 2002; Werner et al.,
258 2004), and possibly S632/636 and S635/639 (Hiratani et al., 2005), to explain the shift in
259 migration position. Retardation of IRS1 in SDS-PAGE was not seen in thapsigargin-treated
260 cells (Figure 5). These data argue that ER stress induced with thapsigargin does not elicit
261 S307/312 phosphorylation of IRS1 and that phosphorylation of IRS1 at other sites remains
262 below the threshold necessary to affect retardation of IRS1 in SDS-PAGE.

263 *ER stress for ≥ 12 h inhibits insulin-stimulated AKT phosphorylation.* Several studies have
264 reported that ER stress lasting for 12 h or longer causes insulin resistance (Zhou et al., 2009;
265 Avery et al., 2010; Xu et al., 2010; Tang et al., 2011; Hassan et al., 2012; Jung et al., 2013;
266 Panzhinskiy et al., 2013). Such long periods of ER stress may cause insulin resistance by
267 depleting the plasma membrane population of the insulin receptor, because the insulin
268 receptor has a half-life of 7-13 h (Reed and Lane, 1980; Kasuga et al., 1981; Reed et al.,

1981a; Reed et al., 1981b; Capeau et al., 1985; Savoie et al., 1986; Grako et al., 1992). For this reason, we characterised whether ER stress for ≥ 12 h decreases insulin-stimulated phosphorylation of AKT. 12 h after induction of ER stress, insulin-stimulated S473 phosphorylation of AKT was decreased in C₂C₁₂ cells exposed to 10 μ g/ml of tunicamycin (Figure 6, A and B). 18 h after induction of ER stress, several ER stressors and markedly lower tunicamycin concentrations decreased insulin-stimulated AKT phosphorylation at S473 (Figure 6, A and B). After 24 h of ER stress, all ER stress-inducing conditions decreased insulin-stimulated AKT phosphorylation at S473 in C₂C₁₂ cells. 24 h of ER stress induced with thapsigargin, tunicamycin, or SubAB decreased cell numbers (Figure S4, A and B), but did not affect the activity of mitochondrial redox chains in the remaining cells (Figure S4, C), suggesting that remaining cells were viable and that loss of viability cannot explain the decrease in S473 phosphorylation of AKT. We made similar observations in Hep G2 and 3T3-F442A cells. In Hep G2 cells, induction of ER stress for 18 h did not affect insulin-stimulated phosphorylation of AKT, except when cells were exposed to 10 μ g/ml tunicamycin (Figure S5, A and B). After 24 h of ER stress a ten-fold lower tunicamycin concentration also reduced insulin-stimulated AKT phosphorylation, and after 36 h all ER stress inducing conditions decreased insulin-stimulated AKT phosphorylation (Figure S5, A and B). 36 h of ER stress decreased the number of Hep G2 cells remaining in culture dishes (Figure S4, D and E), but did not affect the viability of the cells remaining in culture dishes (Figure S4, F). In 3T3-F442A cells, insulin-stimulated S473 phosphorylation of AKT started to decline after 12 h of ER stress and continued to decline over the next 12 h (data not shown). These data confirm that periods of ER stress that exceed the half-life of the insulin receptor decrease insulin-stimulated AKT phosphorylation.

Decreased insulin-stimulated AKT phosphorylation correlates with depletion of the β chain of the mature insulin receptor in ER-stressed cells. The hypothesis, that ER stress for > 12 h decreases insulin-stimulated S473 phosphorylation of AKT by depleting the insulin receptor at the plasma membrane, predicts a decrease of mature β chains of the insulin receptor over the duration of ER stress. The insulin receptor is synthesised as a proreceptor of ~ 190 kDa or

297 ~210 kDa due to alternative glycosylation (Hwang and Frost, 1999). Cleavage of the
298 proreceptor into mature α and β chains of ~135 kDa and ~95 kDa in the *trans*-Golgi network
299 by several proprotein convertases (Robertson et al., 1993; Bravo et al., 1994) yields the
300 mature insulin receptor. Western blotting of cell lysates with an antibody against the β chain
301 of the insulin receptor revealed bands representing the proreceptor at ~190 kDa and ~210 kDa
302 and the β chain at ~95 kDa (Figures 6A and S5A). C₂C₁₂ cells stressed with 10 μ g/ml
303 tunicamycin displayed a decrease in insulin receptor β chains 12 h after induction of ER stress
304 that is around the same time at which this condition decreases insulin-stimulated AKT S473
305 phosphorylation. After 18 and 24 h of ER stress, lower concentrations of ER stress and other
306 ER stressors such as thapsigargin or SubAB also decreased insulin receptor β chains (Figure
307 6, A and B). The decrease in insulin receptor β chains correlated with the decrease in insulin-
308 stimulated AKT phosphorylation (Figure 6, C). Exposure of Hep G2 cells to 18 h of ER stress
309 did not affect the abundance of β chains of the mature insulin receptor (Figure S5, A). By
310 contrast, after 24 h or 36 h of ER stress a decline in β chain abundance coincided with
311 decreased phosphorylation of AKT on S473 by insulin (Figure S5, A and B). The decrease in
312 S473 phosphorylation of AKT correlated with the decrease in β chain abundance in ER-
313 stressed Hep G2 cells (Figure S5, C) and 36 h of ER stress significantly increased
314 unprocessed α - β proreceptors in Hep G2 cells (Figure S5, D). We also observed a correlation
315 between a decrease in insulin receptor β chains and decreased, insulin-stimulated AKT
316 phosphorylation in 3T3-F442A cells (data not shown). Insulin by itself, however, did not
317 affect the abundance of β chains of the insulin receptor in any cell line (Figures 6A-B and
318 S5A-B, and data not shown). In summary, these data establish that in ER-stressed cells a
319 decrease in insulin-stimulated AKT phosphorylation correlates with a decrease in mature
320 insulin receptors.

321 Next, we characterised whether the depletion of β chains in ER-stressed cells is sufficient
322 to decrease insulin-stimulated AKT phosphorylation by silencing expression of the insulin
323 receptor gene in C₂C₁₂ myoblasts by using three small interfering (si) RNAs and comparing
324 insulin-stimulated AKT S473 phosphorylation to cells transfected with a siRNA against

325 eGFP. All three siRNAs decreased insulin receptor mRNA steady-state levels by 50-70%
326 (Figure 6, D) and mature β chains to a similar extent (Figure 6, E). Concomitant with the
327 decrease in insulin receptor levels, insulin-stimulated AKT S473 phosphorylation was
328 decreased by 50-80% (Figure 6, E). These experiments suggest that an ~50% decrease in
329 insulin receptor levels suffices to decrease insulin-stimulated AKT S473 phosphorylation to a
330 similar degree. In summary, these data suggest that depletion of β chains of the mature insulin
331 receptor suffices to decrease insulin-stimulated AKT phosphorylation in ER-stressed cells.

332 *Inhibition of protein synthesis and synthesis of α - β proreceptors cannot fully explain*
333 *decreased insulin-stimulated S473 phosphorylation of AKT in ER stress lasting for 24 h.* ER
334 stress may decrease mature insulin receptors by decreasing transcription of the insulin
335 receptor gene (Örd and Örd, 2003; Jang et al., 2010), degrading the insulin receptor mRNA
336 via the RIDD activity of IRE1 α (Hollien and Weissman, 2006; Hollien et al., 2009), inhibiting
337 translation of the insulin receptor mRNA, by interfering with folding and maturation of newly
338 synthesised insulin receptors in the ER and transport of newly synthesised insulin receptors to
339 the plasma membrane, or increasing the turnover of insulin receptors at the cell surface.
340 Therefore, we decided to determine which of these processes contribute to lower levels of
341 mature insulin receptors in ER-stressed cells.

342 Reverse transcriptase-quantitative PCR (qPCR) showed that steady-state levels of the
343 insulin receptor mRNA increase ~6 fold in ER-stressed C₂C₁₂ cells (Figure 7, A), thus making
344 it unlikely that transcriptional effects or RIDD activity of IRE1 α can explain loss of insulin
345 receptor β chains in ER-stressed cells.

346 To explore whether a translational arrest can explain the loss of β chains, we labelled
347 newly synthesised proteins for 30 min with a mix of [³⁵S]-L-methionine and [³⁵S]-L-cysteine
348 and measured incorporation of [³⁵S]-L-methionine/[³⁵S]-L-cysteine into protein by
349 scintillation counting of TCA precipitates (Figure 7, B-D) or after separating equal amounts
350 of proteins on SDS-PAGE gels by storage phosphor imaging (Figure 7, E). Storage phosphor
351 signals were normalised to the intensity of Coomassie Brilliant Blue R-250 staining of the
352 gels to account for small variations in loading of SDS-PAGE gels. Overall, scintillation

353 counting of TCA precipitates and storage phosphor imaging of gels gave very similar results
354 (Figure 7, B-E). In C₂C₁₂ and Hep G2 cells, 0.1 μ M thapsigargin decreased protein synthesis
355 rates measured by scintillation counting to $52 \pm 4\%$ and $66 \pm 4\%$ of untreated cells,
356 respectively, or $55 \pm 3\%$ and $79 \pm 2\%$ when measured by storage phosphor imaging. In
357 contrast, treatment with 0.1 μ g/ml tunicamycin for 24 h did not affect protein synthesis rates
358 (Figure 7, C-E). Surprisingly, both thapsigargin and tunicamycin increased protein synthesis
359 rates in 3T3-F442A cells 1.97 ± 0.04 and 1.61 ± 0.03 -fold when measured by storage
360 phosphor imaging, and 2.1 ± 0.1 and 2.1 ± 0.2 -fold when measured by scintillation counting.
361 These effects of both thapsigargin and tunicamycin on protein synthesis rates in 3T3-F442A
362 cells were seen in four independent experiments. To investigate if decreased protein synthesis
363 in thapsigargin-treated C₂C₁₂ and Hep G2 cells may be caused by increased phosphorylation
364 of eIF2 α at S51, we examined phosphorylation of eIF2 α at S51 by Western blotting. While
365 treatment with 0.1 μ M thapsigargin for 30 min led to a dramatic increase in phosphorylation
366 of eIF2 α in all three cell types, neither treatment with 0.1 μ M thapsigargin or 0.1 μ g/ml
367 tunicamycin for 24 h affected phosphorylation of eIF2 α at S51 (Figure 7, F). Therefore, the
368 inhibitory effect of 24 h of thapsigargin treatment of C₂C₁₂ and Hep G2 cells on protein
369 synthesis rates is independent of phosphorylation of eIF2 α at S51.

370 To directly establish whether ER stress affects synthesis of new insulin receptors, we
371 measured synthesis rates of the α - β proreceptor by incorporation of [³⁵S]-L-methionine/[³⁵S]-
372 L-cysteine into newly synthesised proteins and immunoprecipitation of the insulin receptor
373 with an antibody against the β chain of the mature insulin receptor. Immunoprecipitates were
374 resolved by SDS-PAGE after boiling for 5 min in 10% (w/v) SDS and 2.5% (v/v) β -
375 mercaptoethanol (see 'Materials and Methods' for details). Initial experiments showed very
376 faint bands for both the α - β proreceptor and β chain in cell lysates prepared from C₂C₁₂ cells
377 labelled for 8 h with $\sim 70\%$ [³⁵S]-L-methionine/ $\sim 25\%$ [³⁵S]-L-cysteine (data not shown). Much
378 stronger signals obtained with lysates prepared from Hep G2 and especially 3T3-F442A cells
379 labelled for 8 h allowed us to identify several bands that were not observed in a control
380 immunoprecipitation with normal rabbit IgG (Figure 7, G). The running positions of these

381 bands in SDS-PAGE identified two of these bands as the α - β proreceptor and the β chain of
382 the mature insulin receptor (Figure 7, G). When cells were labelled for 1 h with [35 S]-L-
383 methionine/[35 S]-L-cysteine, the band representing the β chain of the mature insulin receptor
384 was no longer detected (Figure 7, G). Quantification of storage phosphor signals revealed that
385 in 3T3-F442A cells $\leq 2.6 \pm 0.4$ % and in Hep G2 cells $\leq 9.0 \pm 0.1$ % of α - β proreceptors were
386 processed to mature insulin receptors in the 1 h label. These values likely represent an upper
387 limit of α - β proreceptor processing in the 1 h label, because of the contribution of other
388 comigrating, [35 S]-L-methionine/[35 S]-L-cysteine-labelled proteins to the storage phosphor
389 signal at the migration position of the β chain. Hence, the amount of α - β proreceptors
390 synthesised in the 1 h labelling period is representative of the synthesis rate of the α - β
391 proreceptor.

392 Consistent with total protein synthesis rates, 3T3-F442A cells treated for 24 h with 0.1
393 μ M thapsigargin showed a 2.33 ± 0.04 -fold increase in α - β proreceptor synthesis, while
394 treatment of 3T3-F442A cells with 0.1 μ g/ml tunicamycin or Hep G2 cells with either 0.1 μ M
395 thapsigargin or 0.1 μ g/ml tunicamycin for 24 h did not affect α - β proreceptor synthesis
396 (Figure 7, G and H). When cells were treated with tunicamycin, α - β proreceptors migrated
397 faster in SDS-PAGE due to their decreased molecular weights caused by inhibition of N-
398 glycosylation by tunicamycin (Figure 7, G). In summary, these experiments reveal that
399 conditions exist in which ER stress for >12 h decreases insulin-stimulated S473
400 phosphorylation of AKT without decreasing general protein synthesis or synthesis of the
401 insulin proreceptor, for example exposure of C₂C₁₂ cells for 24 h to 0.1 μ g/ml tunicamycin
402 (Figures 6 and 7), and 24-36 h exposure of Hep G2 cells to low concentrations of thapsigargin
403 or tunicamycin (Figures S5 and 7).

404 *ER stress does not increase the rate of insulin receptor turnover at the cell surface.* Another
405 possibility for how ER stress may deplete β chains of the mature insulin receptor is that
406 increased proteolytic activity associated with the secretory pathway, either lysosomal
407 proteolytic activity (Chiang et al., 2012; Imanikia et al., 2019) or proteasomal activity
408 associated with the ER (Casagrande et al., 2000; Friedlander et al., 2000; Termine et al.,

2009; Ron et al., 2011; Chiang et al., 2012), results in increased turnover of mature insulin receptors in ER-stressed cells. To address this possibility, we determined the half-life of the insulin receptor at the cell surface in unstressed cells, and cells in which ER stress was induced with 0.3 μ M thapsigargin, 1 μ g/ml tunicamycin, or 1 μ g/ml SubAB. We biotinylated surface exposed proteins with the membrane-impermeable biotinylation reagent sulphosuccinimidyl-6-(biotinamido)hexanoate, and then continued to culture cells in the presence or absence of ER stressors for up to 72 h. At several times after biotinylation of cell surface proteins, we analysed proteins isolated with streptavidin-agarose beads by SDS-PAGE and Western blotting. The stability of the streptavidin-biotin interaction in 6 M urea (Kurzban et al., 1991) allowed us to wash streptavidin-agarose beads twice with 6 M urea, 1% (v/v) Triton X-100 to remove non-biotinylated proteins. Western blotting of proteins isolated on streptavidin-agarose beads for the abundant intracellular protein GAPDH revealed that GAPDH was not retained on streptavidin agarose beads (Figure 8), showing that intracellular proteins were not biotinylated and that GAPDH was not retained on the beads via nonspecific interactions. Streptavidin-agarose beads also did not purify any insulin receptors from cells that were not exposed to the biotinylation reagent (Figure 8, A, C, and E, lanes labelled '-'), showing that insulin receptors were only retained on streptavidin-agarose beads when they were biotinylated and, hence, surface exposed. When the supernate of a pull-down reaction with streptavidin-agarose beads was used in a second pull-down reaction with a new aliquot of streptavidin-agarose beads (Figure 8, A, C, and E, lanes labelled '0' and marked with an arrowhead), no or only faint bands were obtained for the β chain of the insulin receptor, showing that the yield of the first pull-down reaction consistently was > 96%. In all cell lines and under all conditions the abundance of insulin receptors that bound to streptavidin-agarose beads decreased over time, indicating that initially surface exposed and biotinylated insulin receptor molecules were degraded. In all cases the decay of biotinylated insulin receptors followed a monoexponential relationship (Figure 8). This allowed us to calculate half-lives for the insulin receptor at the cell surface from the slopes of linearised relationships and to compare half-lives between unstressed cells and cells exposed to ER stressors (Figure 8). The

437 calculated half-lives are similar to previously reported half-lives of total cellular insulin
438 receptors (Reed and Lane, 1980; Kasuga et al., 1981; Krupp and Lane, 1981; Reed et al.,
439 1981a; Reed et al., 1981b; Grako et al., 1992) and insulin receptors at the cell surface (Rosen
440 et al., 1979; Kasuga et al., 1981; Savoie et al., 1986). In C₂C₁₂ and Hep G2 cells, induction of
441 ER stress with 0.3 μ M thapsigargin, 1 μ g/ml tunicamycin, or 1 μ g/ml SubAB did not alter the
442 half-life of the insulin receptor at the cell surface (Figure 8, D and F). By contrast, ER stress
443 increased the half-life of insulin receptors at the surface of 3T3-F442A cells (Figure 8, B).
444 Therefore, increased turnover and degradation of cell surface-exposed insulin receptors
445 cannot explain the loss of insulin receptors leading to a decrease in insulin-stimulated S473
446 phosphorylation of AKT in ER stressed cells.

447 *Unprocessed α - β proreceptors accumulate in the ER of ER-stressed cells.* Since effects on
448 transcription, translation, or degradation of insulin receptors cannot fully explain loss of
449 mature insulin receptors in ER-stressed cells, we characterised whether transport of newly
450 synthesised insulin receptors to the plasma membrane is inhibited by ER stress. Consistent
451 with this hypothesis is that while mature β chains decrease in ER-stressed cells, the levels of
452 α - β proreceptors increase relative to the levels of the β chains (Figures 9A and S5D). This
453 suggests that α - β proreceptors accumulate in an early compartment of the secretory pathway
454 such as the ER or *cis*-Golgi, because cleavage of the proreceptor into α - and β chains occurs
455 in the *trans*-Golgi network (Robertson et al., 1993; Bravo et al., 1994). To provide additional
456 evidence that proreceptors accumulate in the ER or *cis*-Golgi, we digested protein extracts
457 from un- and ER-stressed C₂C₁₂ cells with Endo H. Endo H releases high mannose and some
458 hybrid type N-linked oligosaccharides from glycoproteins by cleaving between the two N-
459 acetylglucosamine units (Maley et al., 1989). High mannose oligosaccharides are
460 characteristic of proteins that have not been processed by enzymes in the Golgi complex.
461 Endo H-digested α - β proreceptors migrated at the same position in SDS-PAGE as fully
462 deglycosylated proreceptors synthesised in tunicamycin-treated cells (Figure 9, B and D) or
463 obtained with PNGase F (Maley et al., 1989) (Figure 9, B-D), suggesting that none of the N-
464 glycans on the majority of α - β proreceptors were exposed to processing enzymes of the Golgi

465 complex. By contrast, mature α and β chains carry both Endo H sensitive and Endo H-
466 resistant *N*-linked oligosaccharides [(Heidenreich and Brandenburg, 1986; Hwang and Frost,
467 1999) and Figure 9, D]. These data are consistent with the conclusion that α - β proreceptors
468 accumulate in the ER or *cis*-Golgi of ER-stressed cells.

469 To directly establish whether insulin receptors deplete at the plasma membrane and
470 accumulate in intracellular compartments, we compared the localisation of C-terminally GFP-
471 tagged insulin receptors expressed in HEK 293 cells treated for 18 h with 0.1 μ g/ml
472 tunicamycin or 1 μ g/ml SubAB to untreated HEK 293 cells. HEK 293 cells were chosen for
473 these experiments because they can be easily transfected, in contrast to Hep G2 cells, do not
474 grow in clumps, and adhered better to culture vessels when treated with ER stressors than
475 C₂C₁₂ cells. ER stress lasting for 18 h depletes insulin receptor β chains in HEK 293 cells
476 (Figure 9, E) and slightly decreases cell numbers (Figure 9, F). In unstressed cells, the GFP-
477 tagged insulin receptor predominantly localised to the plasma membrane (Figure 9, G), which
478 is supported by the high Pearson's correlation coefficient, r_{obs} , for the GFP fluorescence and
479 the fluorescence of the CellMask Deep Red plasma membrane stain (Figure 9, H). By
480 contrast, ER-stressed HEK 293 cells displayed intracellular GFP fluorescence and decreased
481 colocalisation of GFP and CellMask Deep Red fluorescence (Figure 9, G and H). These
482 observations are consistent with the conclusion that ER stress depletes the population of
483 insulin receptors at the plasma membrane by interfering with trafficking of newly synthesised
484 insulin receptors from the ER to the plasma membrane.

485 *AKT activation by a cytosolic F_v2E-insulin receptor chimera is not affected by ER stress.* To
486 establish whether inhibition of transport of insulin receptors in the secretory pathway is
487 necessary for ER stress to cause insulin resistance, we bypassed the secretory pathway in
488 synthesis of functional insulin receptor protein tyrosine kinase domains by creating a chimera
489 in which the signal peptide, extracellular and transmembrane domains of the insulin receptor
490 have been replaced by an *N*-terminal myristoylation signal and the F_v2E domain (Figure 10,
491 A). The myristoylation signal mediates *N*-terminal myristoylation of the protein and its
492 anchoring to intracellular membranes (Maurer-Stroh et al., 2002a, b). The F_v2E domain

493 contains two binding sites for the bivalent macrolide AP20187 and binds AP20187 with
494 subnanomolar affinities (Clackson et al., 1998; Yang et al., 2000). Binding of AP20187 to the
495 F_V2E domain induces dimerisation of the chimeric protein. Dimerisation of the F_V2E-insulin
496 receptor chimera with AP20187 in stably transfected Flp-In T-Rex 293 cells increased
497 phosphorylation of the chimera at tyrosine 610, which is equivalent to tyrosine 1355 in the
498 long isoform of the human insulin receptor, showing that the chimera possesses tyrosine
499 autophosphorylation activity (Figure 10, B). Because AKT S473 phosphorylation was
500 unresponsive to serum starvation in Flp-In T-Rex 293 cells (data not shown), we transiently
501 transfected the chimera into C₂C₁₂ myoblasts. In C₂C₁₂ cells, AP20187 stimulated AKT S473
502 phosphorylation ~4.5 fold (Figure 10, C and D). Thus, activation of the F_V2E-insulin receptor
503 chimera recapitulates several events in insulin signalling. In transiently transfected C₂C₁₂ cells
504 ER stress induced for 24 h with thapsigargin, tunicamycin, or SubAB reduced endogenous β
505 chains by ~40% (Figure 10, C) but did not affect activation of AKT by the chimera (Figure
506 10, C and D). Furthermore, ER stress induced with thapsigargin, tunicamycin, or SubAB
507 increased the abundance of unprocessed endogenous α - β proreceptors (Figure 10, E) and
508 induced *TRB3* (Figure 10, F). Hence, activation of AKT by the cytosolic, AP20187-
509 stimulated insulin receptor chimera is not affected by ER stress despite induction of *TRB3*,
510 depletion of β chains of the mature, endogenous insulin receptor, and accumulation of
511 unprocessed endogenous α - β proreceptors.

512 *Pharmacologic inhibition of JNKs does not rescue insulin-stimulated S473 phosphorylation*
513 *of AKT in ER-stressed cells.* Previous work has suggested that activation of both the family of
514 JNK MAP kinases and *TRB3* by ER stress leads to a decrease in insulin-stimulated AKT
515 phosphorylation in ER-stressed cells (Özcan et al., 2004; Koh et al., 2006; Koh et al., 2013).
516 The lack of effects of 24 h of ER stress on the F_V2E-insulin receptor chimera (Figure 10)
517 prompted us to characterise whether the decrease in insulin-stimulated AKT phosphorylation
518 in cells experiencing ER stress for >12 h manifests independent of activation of JNKs or
519 *TRB3*. We first characterised JNK activation in C₂C₁₂ and Hep G2 cells exposed to ER stress
520 for 12 – 36 h using phosphorylation of JNKs at T183 and Y185 in their T-loops as a marker

521 for their activation (Lawler et al., 1998; Fleming et al., 2000). No signals for JNKs
522 phosphorylated at T183 and Y185 were obtained for C₂C₁₂ cells exposed to 0.1 – 1.0 μ M
523 thapsigargin, 0.1 – 10 μ g/ml tunicamycin, or 1 μ g/ml SubAB for 12, 18, or 24 h or Hep G2
524 cells exposed to 0.1 – 10 μ g/ml tunicamycin or 1 μ g/ml SubAB for 18, 24, or 36 h in Western
525 blots, despite detecting very strong signals in cells irradiated with UV light (400 J/m², λ = 254
526 nm; Figure 11, A and data not shown). Thus, if JNKs are activated in these conditions, the
527 levels of JNK species phosphorylated at T183 and Y185 are below levels that can be detected
528 by Western blotting. By contrast, exposure of Hep G2 cells to 0.1 – 1.0 μ M thapsigargin for
529 18 – 36 h revealed activation of JNKs (Figure 11, A). Additional bands detected with the anti-
530 phospho-T183-phospho-Y185-JNK antibody that migrate below the migration position of the
531 46 and 54 kDa isoforms of JNKs may represent phosphorylated species of other MAP
532 kinases, such as p38, p42, and p44 (Figure 11, A), suggesting that exposure to thapsigargin
533 for \geq 18 h may activate several MAP kinases in Hep G2 cells.

534 To evaluate whether the JNK activation brought about by thapsigargin in Hep G2 cells
535 contributes to the loss of insulin-stimulated S473 phosphorylation of AKT, we employed two
536 selective JNK inhibitors, *N*-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-
537 dimethoxyphenyl)acetamide (JNKi VIII) and (*E*)-3-(4-(dimethylamino)but-2-enamido)-*N*-(3-
538 methyl-4-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)benzamide (JNKi XVI). Both
539 inhibitors inhibited phosphorylation of the JNK substrate c-Jun in Hep G2 cells stimulated
540 with UV or 5 μ g/ml anisomycin for 30 min with submicromolar *EC*₅₀ values that are
541 comparable to previously reported *EC*₅₀ values for inhibition of c-Jun phosphorylation in
542 TNF- α treated Hep G2 or HeLa cells (Szczepankiewicz et al., 2006; Zhang et al., 2012)
543 (Figure S6). Both inhibitors also inhibited c-Jun phosphorylation at S63 in Hep G2 cells
544 exposed to 0.1 – 1.0 μ M thapsigargin for 36 h (Figure 11, B). Thapsigargin increased steady-
545 state levels of c-Jun (Figure 11, B), which is consistent with c-Jun autoregulating its own
546 expression (Angel et al., 1988). 8 μ M of JNKi XVI decreased the increase in c-Jun levels,
547 while the same concentration of JNKi VIII had no effect on the increase in c-Jun levels in
548 thapsigargin-treated Hep G2 cells (Figure 11, B), which correlates with the stronger inhibition

549 of c-Jun S63 phosphorylation elicited by thapsigargin by JNKi XVI. Both JNK inhibitors,
550 however, did not reverse inhibition of phosphorylation of S473 of AKT in Hep G2 cells that
551 were exposed to 0.1 – 1.0 μ M thapsigargin for 36 h, and then stimulated with 10 or 100 nM
552 insulin for 15 min in the continued presence of thapsigargin (Figure 11, C-E). Both JNK
553 inhibitors decreased the insulin-stimulated phosphorylation of AKT at S473 (Figure 11, C and
554 D). Normalisation of S473 phosphorylation of AKT to the sample not exposed to thapsigargin
555 within each group (no JNKi, treatment with JNKi VIII or XVI) reinforced the conclusion that
556 both inhibitors do not reverse the inhibitory effect of thapsigargin on insulin-stimulated S473
557 phosphorylation of AKT (Figure 11, E). Consistent with this observation and the proposed
558 role for depletion of insulin receptors in ER-stressed cells as the cause for inhibition of
559 insulin-stimulated S473 phosphorylation of AKT, both JNK inhibitors also did not rescue
560 levels of β chains of the mature insulin receptor or α - β proreceptor processing in Hep G2 cells
561 exposed to 0.1 – 1.0 μ M thapsigargin for 36 h (Figure 11, F).

562 *Genetic ablation of JNK1 and JNK2 does not protect mouse embryonic fibroblasts (MEFs)*
563 *from inhibition of insulin-stimulated AKT phosphorylation by ER stress.* To confirm the
564 results obtained with pharmacologic inhibitors of JNKs we compared the effects of 24 h of
565 ER stress on insulin-stimulated S473 phosphorylation of AKT in wild type (WT) MEFs and
566 MEFs deficient in the JNK1 and JNK2 isoforms of the JNK kinases. These *jnk1^{-/-} jnk2^{-/-}*
567 MEFs lack detectable JNK activity because these cells do not express the neuronal isoform of
568 the JNKs, JNK3 (Tournier et al., 2000). Exposure of WT and *jnk1^{-/-} jnk2^{-/-}* MEFs to
569 thapsigargin, tunicamycin or SubAB for 24 h to elicit ER stress decreased insulin-stimulated
570 AKT phosphorylation in both cell types to the same degree (Figure 12, A-C), despite a $6.1 \pm$
571 0.6 increase in phosphorylation of JNKs in ER-stressed WT MEFs ($p < 0.0001$ in an ordinary
572 one-way ANOVA, Figure 12, D and E). ER stress also caused similar increases in the
573 abundance of unprocessed α - β insulin proreceptors in WT and *jnk1^{-/-} jnk2^{-/-}* MEFs (Figure 12,
574 F), which suggests that activation of the JNK kinases in the ER stress response does not affect
575 the protein folding capacity of the stressed ER.

576 *siRNA-mediated silencing of expression of TRB does not protect from inhibition of insulin-*
577 *stimulated AKT phosphorylation by ER stress.* To characterise whether TRB3 contributes to
578 the decrease in insulin-stimulated AKT phosphorylation in cells that experience ER stress for
579 24 h, we used two siRNAs to knock-down expression of *TRB3*. 48 h after transfection of
580 C₂C₁₂ myoblasts, both siRNAs decreased *TRB3* mRNA and protein levels to a similar degree
581 (Figure 13, A and B). However, the knock-down of *TRB3* mRNA and protein levels did not
582 affect insulin-stimulated phosphorylation of AKT (Figure 13, C and D) or the accumulation
583 of unprocessed α - β insulin proreceptors (Figure 13, E) in cells exposed to thapsigargin,
584 tunicamycin, or SubAB to elicit ER stress. These data argue that TRB3 does not contribute to
585 decreased insulin-stimulated AKT phosphorylation or restoration of the protein folding
586 capacity of cells that experience ER stress for 24 h. Taken together, the dispensability for
587 JNKs and TRB3, as well as the absence of effects of ER stress on activation of AKT by the
588 F_V2E-insulin receptor chimera, argue that ER stress decreases insulin-stimulated AKT
589 phosphorylation independent of signal transduction events.

590 **DISCUSSION**

591 The data presented here establish that ER stress interferes with insulin-stimulated
592 phosphorylation of AKT by inhibiting the processing of newly synthesised insulin receptors
593 in the secretory pathway, which will deplete the cell surface population of the receptor over
594 time (Figure 14, A). Several lines of evidence support this conclusion. ER stress extending
595 over several half-lives of the insulin receptor at the plasma membrane inhibits insulin-
596 stimulated AKT phosphorylation (Figures 6 and S5). Periods of ER stress shorter than the
597 half-life of 6-12 h for the insulin receptor at the plasma membrane in ER-stressed cells
598 (Figure 8) do not affect S473 phosphorylation of AKT by insulin (Figures 1, 2, and S1) or
599 IRS1 tyrosine phosphorylation (Figures 3, 4, S2, and S3). The inhibition of insulin-stimulated
600 AKT phosphorylation in ER-stressed cells coincides with a decrease in the abundance of
601 mature insulin receptors (Figures 6A-C and S5A-C). Colocalisation experiments revealed that
602 while GFP-tagged insulin receptors localise to the plasma membrane of unstressed cells, this

603 localisation of insulin receptors to the plasma membrane decreases in ER-stressed cells
604 (Figures 9, G and H). At the same time GFP-tagged insulin receptors accumulate within the
605 cell (Figure 9, G). Unprocessed α - β proreceptors, whose N-glycans remained sensitive to
606 Endo H (Figure 9, B), accumulate in ER-stressed cells (Figures 9A and S5D), suggesting that
607 unprocessed α - β proreceptors do not reach the *trans*-Golgi where they are processed to
608 mature receptors (Robertson et al., 1993; Bravo et al., 1994). siRNA-mediated knock-down of
609 expression of the insulin receptor confirmed that a ~50% decrease in insulin receptor levels
610 suffices to cause a similar decrease in insulin-stimulated AKT phosphorylation (Figure 6, E).
611 Finally, the absence of effects of ER stress on phosphorylation by the activated chimera of the
612 F_V2E domain and protein tyrosine kinase domain of the insulin receptor (Figure 10) showed
613 that processing of insulin receptors in the secretory pathway is necessary for ER stress to
614 inhibit insulin signalling. These experiments also suggested that signalling events in the UPR,
615 such as activation of JNKs or TRB3, do not affect insulin signalling downstream of the
616 insulin receptor. Pharmacologic inhibition and genetic ablation of JNKs (Figures 11 and 12)
617 and siRNA-mediated silencing of TRB3 (Figure 13) confirmed these conclusions.

618 In previous research tunicamycin was nearly exclusively used to inhibit trafficking of
619 newly synthesised insulin receptors (Keefer and De Meyts, 1981; Reed et al., 1981b; Ronnett
620 and Lane, 1981; Boyd and Raizada, 1983; Ercolani et al., 1984; Kadle et al., 1984; Ronnett et
621 al., 1984; Capeau et al., 1985; Goldstein and Kahn, 1988), which led to the conclusion that
622 the underlying cause for the trafficking defect is the lack of N-glycosylation of newly
623 synthesised insulin receptors. We show that two other ER stressors, thapsigargin and SubAB,
624 also inhibit processing of α - β proreceptors to mature receptors (Figures 9A and S5A)
625 suggesting that newly synthesised α - β proreceptors do not reach the *trans*-Golgi to be cleaved
626 into mature α - and β -chains. This conclusion is supported by the observation that all N-
627 glycans of α - β proreceptors in cells treated with thapsigargin or SubAB remain sensitive to
628 Endo H (Figure 9, B). The migration of α - β proreceptors synthesised in the presence of
629 thapsigargin or SubAB in SDS-PAGE suggests that these proreceptors are N-glycosylated to
630 the same extent as proreceptors synthesised by unstressed cells (Figures 6A, 7G, 9B, and

631 S5A), suggesting that both thapsigargin and SubAB do not inhibit N-glycosylation of newly
632 synthesised insulin receptors. Therefore, events other than lack of N-glycosylation can inhibit
633 transport of newly synthesised insulin receptors from the ER to the *trans* Golgi. Cleavage and
634 inactivation of the ER luminal HSP70 chaperone BiP by SubAB (Paton et al., 2006) may
635 interfere with correct folding of newly synthesised insulin receptors, leading to their retention
636 in the ER by the quality control machinery of the ER (Bass et al., 1998) and, to some extent,
637 targeting of unfolded proreceptors to ERAD machinery for degradation. The effects of
638 thapsigargin on protein folding in the ER are less well understood, but depletion of the ER
639 luminal Ca^{2+} store by thapsigargin (Thastrup et al., 1990) may inhibit several molecular
640 chaperones of the ER, because many of these bind Ca^{2+} ions with high capacities (Macer and
641 Koch, 1988; Fliegel et al., 1989; Wada et al., 1991). Indirect effects resulting from depletion
642 of proteins functioning in vesicular trafficking and sorting may also account for some of the
643 effects on insulin receptor trafficking, and may, for example, explain the increased half-life of
644 the insulin receptor at the plasma membrane in ER-stressed 3T3-F442A cells (Figure 8, A-B).

645 Insulin resistance can be a consequence of decreased insulin receptor levels, inhibition of
646 transduction of the insulin signal downstream of the insulin receptor (sometimes referred to as
647 ‘post-receptor events’), or a combination of the two (Olefsky and Kolterman, 1981). The
648 experiments with the F_v2E-insulin receptor chimera (Figure 10) show that ER stress does not
649 affect insulin signalling downstream of the receptor. Our work also establishes that plasma
650 membrane levels of the receptor decrease (Figures 9, G and H). Therefore, we propose that
651 ER stress primarily causes insulin resistance by decreasing the levels of insulin receptors at
652 the cell surface. Decreases in insulin receptors affect insulin sensitivity of cells, in other
653 words, shift the response curve to insulin towards higher insulin concentrations, but only
654 decrease the response to insulin when receptors become severely depleted (see, for example,
655 Figure 14, B and C, curves labelled ‘0’ and ‘1’ in B) (Olefsky and Kolterman, 1981).
656 Elevated insulin concentrations can compensate for decreased insulin sensitivity, because
657 many cell types possess ‘spare’ receptors, which allows them to mount maximal responses to
658 insulin even when only a small fraction of receptors have bound to insulin, possibly as low as

659 a few percent (Kono and Barham, 1971; Olefsky, 1975; Le Marchand-Brustel et al., 1978;
660 Hofmann et al., 1980; Frank et al., 1981). A decrease in insulin receptors will diminish the
661 response to insulin at one insulin concentration, but because insulin, the insulin receptor, and
662 insulin-insulin receptor complexes are in a dynamic equilibrium, increases in the insulin
663 concentration will compensate for the decrease in insulin receptor concentration, and allow
664 for formation of a sufficient number of insulin-insulin receptor complexes to elicit a maximal
665 response to insulin. By contrast, a decrease in the response to insulin at all insulin
666 concentrations is often indicative of inhibition of signal transduction downstream of the
667 insulin receptor (Olefsky and Kolterman, 1981). Hence, when high insulin concentrations are
668 employed, as is often done in *in vitro* studies, this is expected to primarily reveal post-
669 receptor events on insulin signalling because these affect insulin signalling independent of
670 insulin concentration, and the high insulin concentration is thought to compensate for
671 decreases in insulin sensitivity.

672 This, however, changes when insulin receptor levels decrease to such an extent that the
673 concentration of insulin-insulin receptor complexes approaches the value of K_E , the
674 concentration of the hormone, or more precisely hormone-receptor complexes, at which the
675 response to the hormone is half-maximal. At this point, the response to insulin will decrease
676 over the whole insulin concentration range (Figure 14, B and C), and complete compensation
677 by increasing insulin concentrations may, depending on the magnitude of the decrease in
678 insulin receptor levels, no longer be possible. It then also becomes difficult, if not impossible,
679 to distinguish between an effect on insulin sensitivity (due to decreased receptor levels) and
680 post-receptor events, even if dose-response curves are recorded or high insulin concentrations
681 are employed. Therefore, while previous studies have largely interpreted effects of ER stress
682 for > 12 h as post-receptor events (Avery et al., 2010; Xu et al., 2010; Tang et al., 2011;
683 Hassan et al., 2012; Jung et al., 2013; Panzhinskiy et al., 2013), it is also possible that the
684 effects of long periods of ER stress are, at least in part, the consequence of profound
685 decreases in insulin receptors and subsequently insulin sensitivity. The F_v2E-insulin receptor

686 chimera described in this work may prove to be a valuable tool to distinguish more rigorously
687 between receptor and post-receptor events in future work.

688 The interpretation of effects of ER stress on insulin signalling is further complicated by
689 the gradual decrease of insulin receptors at the plasma membrane over time until receptor
690 levels fall below the critical level, at which cells do no longer retain their complete
691 responsiveness to insulin. The time it takes for insulin receptor levels to fall to this critical
692 level depends on (a) the half-life of the receptor at the cell surface, which is affected by cell
693 type and can be affected by ER stress (Figure 8), (b) the number of cell surface insulin
694 receptors in unstressed cells, which can vary widely between cell types (Bar et al., 1976; Reed
695 et al., 1977; Capeau et al., 1978), (c) the sizes of intracellular insulin receptor pools,
696 including, for example, receptors that are transiting through the Golgi complex and receptors
697 that are recycled at the plasma membrane at the time ER stress is induced, (d) the degree of
698 completeness of inhibition of transport of newly synthesised receptors from the ER to the cell
699 surface, and (e) the fraction of newly synthesised receptors that is targeted for degradation by
700 ERAD in ER-stressed cells. For example, short periods of ER stress (< 1-2 half-lives), will
701 not deplete receptors to levels where the response to insulin decreases measurably, while long
702 periods (> 2 half-lives) will cause severe receptor depletion, severe loss of insulin sensitivity,
703 and a loss in insulin responsiveness (Figure 14, B). Likewise, a ~10-fold lower plasma
704 membrane insulin receptor population may result in measurable loss of insulin responsiveness
705 after ER stress lasting for ~3 half-lives of the receptor at the plasma membrane, while in cells
706 that possess ten times more receptors, ER stress has to last for several additional half-lives
707 before similar effects on insulin responsiveness manifest (Figure 14, B and C).

708 ER stress is present in muscle, liver, and adipose tissue of obese individuals (Özcan et al.,
709 2004; Özcan et al., 2006; Hosogai et al., 2007; Boden et al., 2008; Sharma et al., 2008;
710 Sreejayan et al., 2008). Patients with impaired glucose tolerance or overt type II diabetes
711 show a ~50% decrease in insulin receptors (Olefsky and Reaven, 1974; Goldstein et al., 1975;
712 Olefsky, 1976; Olefsky and Reaven, 1977; Pagano et al., 1977; Robinson et al., 1979;
713 Helderman and Raskin, 1980; Kobayashi et al., 1980; Kolterman et al., 1980; Kolterman et

714 al., 1981). This ~50% decrease in insulin receptors at the cell surface accounts for the
715 decreased insulin sensitivity and abnormal glucose tolerance of patients with impaired
716 glucose tolerance (Olefsky and Reaven, 1977; Pagano et al., 1977; Kolterman et al., 1980;
717 Kolterman et al., 1981). ER stress may decrease the fraction of newly synthesised receptors
718 that reach the plasma membrane or increase the transit time through the secretory pathway for
719 all or some of the newly synthesised insulin receptors and through this may contribute to the
720 decrease in the size of the steady-state cell surface population of receptors in obesity.
721 Internalisation of the insulin receptor is necessary for its degradation (Desbuquois et al., 1982;
722 Knutson et al., 1983; Wang et al., 1983; Draznin et al., 1984). A decrease in the insulin
723 receptor population at the cell surface is expected to decrease its internalisation and
724 degradation rates, and together with decreased synthesis rates may establish a new, smaller
725 steady-state population of the receptor at the plasma membrane. Hyperinsulinemia in obesity
726 may further aggravate decreases of the insulin receptor at the plasma membrane, because,
727 insulin stimulates internalisation and degradation of its receptor (Kasuga et al., 1981; Knutson
728 et al., 1982; Heidenreich et al., 1983; Ronnett et al., 1983; Freychet, 1984; Reed et al., 1984),
729 and, in adipocytes, induces ER stress (Boden et al., 2014). Thus, the effects of ER stress on
730 delivery of newly synthesised insulin receptors to the plasma membrane may contribute to
731 decreased steady-state plasma membrane levels of the receptor in obesity.

732 In conclusion, we provide evidence that ER stress requires processing of insulin receptors
733 in the secretory pathway to inhibit signal transduction in the insulin signalling pathway
734 (Figure 14, A). The effects of ER stress on trafficking of newly synthesised insulin receptors
735 to the cell surface may account for the decrease in insulin receptors in patients with impaired
736 glucose tolerance and patients with overt type II diabetes, and underlie the decreased insulin
737 sensitivity of patients with impaired glucose tolerance. Depending on the half-lives of
738 individual plasma membrane receptors, analogous effects of ER stress on other plasma
739 membrane receptors and transporters may exist.

740 MATERIALS AND METHODS

741 *Antibodies and reagents.* The mouse anti- β -actin antibody (clone 8F10-G10, cat. no.
742 ab170325, batches GR184354-8 and GR327417-1) was purchased from Abcam (Cambridge,
743 UK) and the rat anti- α -tubulin antibody (clone YOL1/34, cat. no. MCA78G, batch 1703) from
744 Bio-Rad Laboratories (Hemel Hempstead, UK). Rabbit anti-phospho-T308-AKT (clone
745 244F9, cat. no. 4056S, batches 13 and 17), anti-phospho-S473-AKT (clone D9E, cat. no.
746 4060S, batches 16, 23, and 24), rabbit anti-AKT (clone C67E7, cat. no. 4691S, batches 11,
747 17, and 20), rabbit anti-phospho-S63-c-Jun (clone 54B3, cat. no. 2361S, batch 7), rabbit anti-
748 c-Jun (clone 60A8, cat. no. 9165S, batch 11), rabbit anti-phospho-S51-eIF2 α (cat. no. 9721S,
749 batch 21), mouse anti-eIF2 α (clone L57A5, cat. no. 2103S, batch 5), rabbit anti-insulin
750 receptor β chain phosphorylated at Y1355 (clone 14A4, cat. no. 3026S, batch 1), rabbit anti-
751 insulin receptor β chain (clone 4B8, cat. no. 3025S, batches 8 and 10), rabbit anti-phospho-
752 Y891 (mouse)/Y896 (human)-IRS1 (cat. no. 3070S, batch 4), rabbit anti-IRS1 (clone
753 D23G12, cat. no. 3407S, batch 6; clone D5T8J, cat. no. 95816S, batch 1), rabbit anti-phospho-
754 T183-phospho-Y185-JNK (clone 81E11, cat. no. 4668S, batches 9, 12 and 15), and rabbit
755 anti-JNK (cat. no. 9252S, batches 15 and 17) antibody were purchased from Cell Signaling
756 Technology (Danvers, MA, USA). The mouse anti-phospho-S307 (mouse)/S312 (human)-
757 IRS1 (clone 24.6.2, cat. no. 05-1087, batch 3063387), rabbit anti-phospho-Y608
758 (mouse)/Y612 (human)-IRS1 (cat. no. 09-432, batch 3018885), rabbit anti-phospho-Y628
759 (mouse)/Y632 (human)-IRS1 (cat. no. 09-433, batch 3023592), rabbit anti-phospho-Y935
760 (mouse)/941 (human)-IRS1 (cat. no. 07-848-I, batch Q2766987), mouse anti-phosphotyrosine
761 (clone 4G10[®] Platinum, cat. no. 05-1050X, batches 2967319 and 3256630), and the rabbit
762 anti-TRB3 (cat. no. 07-2160, batch 2716134) antibody were purchased from Merck Millipore
763 (Watford, UK). Anti-insulin receptor β chain antibody (cat. no. sc-711, batch G0511) was
764 purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the mouse anti-
765 GAPDH antibody (clone GAPDH-71.1, cat. no. G8795, batches 092M4820V and
766 067M4785V) from Sigma-Aldrich (Gillingham, UK).

767 Normal rabbit IgG was purchased from Fisher Scientific (Loughborough, UK, cat. no.
768 10312573, batch UA276761) and Santa Cruz Biotechnology (cat. no. sc-2027, batches C2712

769 and D1415). SureBeadsTM protein A magnetic beads were purchased from Bio-Rad
770 Laboratories (cat. no. 1614013) and streptavidin agarose from Fisher Scientific (cat. no.
771 10302384, batch SJ523686). Goat anti-mouse IgG(H+L)-peroxidase (cat. no. 10330974,
772 batch OE17149612) and goat anti-rat IgG(H+L)-peroxidase (cat. no. 11889140, batch
773 PK209942) were purchased from Fisher Scientific, and goat anti-rabbit IgG(H+L)-peroxidase
774 from Cell Signaling Technology (cat. no. 7074S, batches 26-28).

775 Sulphosuccinimidyl-6-(biotinamido)hexanoate [EZ-link-sulfo-NHS-biotin, cat. no.
776 11851185] was purchased from Fisher Scientific. JNK inhibitor VIII [*N*-(4-amino-5-cyano-6-
777 ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide, cat. no. 420135-5MG], JNK
778 inhibitor XVI [(*E*)-3-(4-(dimethylamino)but-2-enamido)-*N*-(3-methyl-4-((4-(pyridin-3-
779 yl)pyrimidin-2-yl)amino)phenyl)benzamide, cat. no. 420150-10MG], and tunicamycin (cat.
780 no. 654380) were purchased from Merck Millipore. Endoglycosidase H (EndoH) and peptide-
781 N-glycosidase (PNGase) F were obtained from New England Biolabs (Hitchin, UK). Bovine
782 insulin (cat. no. I0516), bovine serum albumin (BSA, cat. no. A2153), dexamethasone, 3-
783 isobutyl-1-methylxanthine, and thapsigargin (cat. no. T9033) were purchased from Sigma-
784 Aldrich. Subtilase cytotoxin (SubAB) and catalytically inactive SubA_{A272}B were purified
785 from *Escherichia coli* as described before (Paton et al., 2004; Talbot et al., 2005). siRNAs
786 against murine *INSR* mRNA and *Aequora victoria* enhanced green fluorescent protein (eGFP)
787 were purchased from Sigma-Aldrich and siRNAs against murine *TRB3* from Fisher Scientific.
788 siRNA sequences are listed in Table 1.

789 *Plasmids.* Plasmids were maintained in *Escherichia coli* XL10-Gold cells (Agilent
790 Technologies, Stockport, UK, cat. no. 200314). Standard protocols for plasmid constructions
791 were used (Ausubel et al., 2017). Plasmid pmaxGFP was obtained from Lonza Cologne AG
792 (Cologne, Germany). Plasmid pEGFP-N2-hINSR encodes a fusion of the human insulin
793 receptor to eGFP (Bass et al., 2000) and was obtained from Addgene (Cambridge, MA, USA,
794 Addgene ID 22286). Plasmid pcDNA5/FRT/TO-F_v2E-INSR β was generated by cloning the
795 1,430 bp *Bsi*WI-*Xma*I fragment of pCLFv2IRE (Cotugno et al., 2004) into *Bsi*WI- and *Xma*I-
796 digested pcDNA5/FRT/TO-F_v2E-C'hIRE1 α (D. Cox and M. Schröder, unpubl.). Plasmid

797 pcDNA5/FRT/TO-MyrF_v2E-INSR β was generated by cloning the 501 bp *EcoRI-XmaI*
798 fragment of pC₄M-F_v2E (Ariad Pharmaceuticals, Cambridge, MA, USA) into *HindIII*- and
799 *XmaI*-digested pcDNA5/FRT/TO-F_v2E-INSR β after blunting the *EcoRI* and *HindIII* sites
800 with Klenow enzyme.

801 *Cell culture*. Wild type (WT), *ire1a*^{-/-} (Lee et al., 2002), *jnk1*^{-/-} *jnk2*^{-/-} (Tournier et al., 2000),
802 and *traf2*^{-/-} (Yeh et al., 1997) mouse embryonic fibroblasts (MEFs) were obtained from
803 Randal J. Kaufman (Sanford Burnham Medical Research Institute, La Jolla, CA), Roger
804 Davis (University of Massachusetts, Worcester, MA, USA), and Tak Mak (University of
805 Toronto, Ontario Cancer Institute, Toronto, Ontario, Canada), respectively. 3T3-F442A
806 preadipocytes (Green and Kehinde, 1976), C₂C₁₂ myoblasts (Blau et al., 1985), HEK 293 cells
807 (Graham et al., 1977; Harrison et al., 1977; Graham et al., 1978), and Hep G2 cells (Knowles
808 et al., 1980) were obtained from C. Hutchison (Durham University), R. Bashir (Durham
809 University), M. Cann (Durham University), and A. Benham (Durham University),
810 respectively. Fao rat hepatoma cells (Deschatrette and Weiss, 1974) were obtained from
811 Public Health England (Salisbury, UK). The Flp-In T-Rex 293 cell line was obtained from
812 Life Technologies (Paisley, UK). All cell lines were tested for mycoplasma contamination
813 upon receipt in the laboratory with the EZ-PCR mycoplasma test kit from Geneflow (cat. no.
814 K1-0210, Lichfield, UK). Mycoplasma testing was repeated every ~3 months with all cells in
815 culture at the time. Contaminated cultures were discarded.

816 All cell lines were grown in an atmosphere of 95% (v/v) air, 5% (v/v) CO₂, and 95%
817 humidity. Fao rat hepatoma cells were grown in Roswell Park Memorial Institute (RPMI)
818 1640 medium (Moore et al., 1967) or in Coon's modification of Ham's F12 medium (Coon
819 and Weiss, 1969) containing 10% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine.
820 Hep G2 cells were cultured in minimum essential medium (MEM) (Eagle, 1959)
821 supplemented with 10% (v/v) FBS and 2 mM L-glutamine. All other cell lines were cultured
822 in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l D-glucose (Morton,
823 1970; Rutzky and Pumper, 1974), 10% (v/v) FBS, and 2 mM L-glutamine. The medium for
824 *ire1a*^{-/-} and corresponding WT MEFs was supplemented with 110 mg/l pyruvate (Lee et al.,

825 2002). The medium for the Flp-In T-Rex 293 cells was supplemented with 100 µg/ml zeocin
826 and 15 µg/ml blasticidin and the medium for Flp-In T-Rex 293 cells stably expressing the
827 F_V2E-insulin receptor chimera with 100 µg/ml hygromycin B and 15 µg/ml blasticidin. For
828 immunoprecipitation of IRS1, [³⁵S]-L-methionine pulse labelling experiments, and
829 determination of the half-life of the insulin receptor at the cell surface 3T3-F442A cells were
830 cultured in gelatinised tissue cultures dishes (Schröder and Friedl, 1997b).

831 siRNAs and plasmids were transfected with INTERFERin or jetPRIME transfection
832 reagent (Polyplus Transfection, Illkirch, France) following the manufacturer's instructions,
833 respectively. The stably transfected Flp-In T-Rex 293 cell lines expressing a fusion of the
834 F_V2E drug-inducible dimerisation domain (Clackson et al., 1998) to the β chain of the human
835 insulin receptor with an *N*-terminal myristoylation signal were generated by transfection of
836 the Flp-In T-Rex 293 cell line with pOG44 (Life Technologies) and pcDNA5/FRT/TO-
837 MyrF_V2E-INSRβ. Selection of stably transfected clones was initiated 24 h after transfection
838 by using 50 µg/ml hygromycin B. After two days the hygromycin B concentration was
839 increased to 100 µg/ml.

840 C₂C₁₂ myoblasts were differentiated into myotubes and 3T3-F442A preadipocytes into
841 adipocytes as described before (Mihai and Schröder, 2015; Brown et al., 2016). ER stress was
842 induced with 0.1 to 1 µM thapsigargin, 0.1 to 10 µg/ml tunicamycin, or 1 µg/ml SubAB. To
843 stimulate cells with insulin, cells were serum-starved for 18 h, followed by addition of fresh
844 serum-free culture medium containing 10 nM or 100 nM insulin for 5 min or 15 min. Cells
845 were serum-starved during the last 18 h of treatments with ER stressors lasting for more than
846 18 h. When cells were ER-stressed for shorter periods, the ER stressors were applied towards
847 the end of the serum starvation, for example for the last 12 h of serum starvation in case of
848 treatment with ER stressors for 12 h. Expression of the F_V2E-insulin receptor chimera was
849 induced for 24 h with 1 µg/ml tetracycline in stably-transfected Flp-In T-Rex 293 cells. The
850 chimera was dimerised by treating cells with 100 nM AP20187 (B/B homodimeriser, TaKaRa
851 Bio Europe, Saint-Germain-en-Laye, France, cat. no. 635058) for the times indicated in the
852 text.

853 Crystal violet staining was used as a proxy to determine the number of cells remaining in
854 culture dishes after exposure to ER stressors. Cultures were washed with phosphate-buffered
855 saline (PBS, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4) at
856 RT and stained for 10 min with 0.2% (w/v) crystal violet in 2% (v/v) ethanol at RT. After
857 washing with tap water to remove unbound crystal violet, the crystal violet was dissolved
858 with 1% (w/v) SDS and the absorbance at 570 nm read in a Spectramax 190 microplate reader
859 (Molecular Devices, San Jose, CA, USA). The activity of mitochondrial redox chains was
860 determined using thiazolyl blue tetrazolium bromide (MTT) as described previously (Mihai
861 and Schröder, 2015), except that the absorbance at 690 nm was subtracted from the
862 absorbance at 590 nm. Corrected MTT absorbances were normalised to the crystal violet
863 absorbance of corresponding, identically treated cultures, and expressed relative to the
864 absorbance of cells exposed to 0.1% (v/v) DMSO, which was arbitrarily set to 1.0.

865 *RNA extraction and reverse transcriptase PCRs.* RNA was extracted and reverse transcribed
866 as previously described (Cox et al., 2011). Protocols for quantification of splicing of *XBPI*
867 have been described previously (Cox et al., 2011; Brown et al., 2016). qPCRs were run on a
868 Rotorgene 3000 (Qiagen, Crawley, UK) using GoTaq G2 Flexi DNA polymerase (Promega,
869 Southampton, UK, cat. no. M7801) and analysed as described before (Brown et al., 2016).
870 Primer sequences are listed in Table 2 or have been reported before (Brown et al., 2016).
871 Amplification of a single PCR product was confirmed by recording the melting curves after
872 each PCR. Amplification efficiencies for all qPCRs were $\sim 0.75 \pm 0.05$. Results represent the
873 average and standard error of three technical repeats. qPCR results were confirmed by at least
874 one other biological replicate.

875 *Cell lysis and Western blotting.* Protein extracts for Western blotting except for extraction of
876 IRS1 were produced as previously described (Brown et al., 2016). In brief, cells were washed
877 three times with ice-cold PBS (pH 7.4) and lysed in RIPA buffer [50 mM Tris-HCl, pH 8.0 at
878 4 °C, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, 0.1% (w/v)
879 SDS] containing Roche complete protease inhibitors (Roche Applied Science, Burgess Hill,
880 UK, cat. no. 11836153001) or 10 mM EDTA, 2 mM PMSF, 6 mM 4-(2-

881 aminoethyl)benzenesulphonyl flouride (AEBSF), 5 mM benzamidine, 10 µg/ml aprotinin, and
882 each 1 µg/ml of antipain dihydrochloride, chymostatin, leupeptin, and pepstatin A. To inhibit
883 protein phosphatases, PhosSTOP phosphatase inhibitors (Sigma-Aldrich, cat. no. 04 906 837
884 001) were added when Roche complete protease inhibitors were used. When individual
885 protease inhibitors were used, 1 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 10
886 mM sodium pyrophosphate, and 200 nM okadaic acid were included to inhibit protein serine
887 and threonine phosphatases. To preserve protein serine, threonine, and tyrosine
888 phosphorylation when individual protease inhibitors were used, cells were lysed in 50 mM
889 HEPES-NaOH (pH 8.0 at 4 °C), 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v)
890 Triton X-100, 0.1% (w/v) SDS, 6 mM EGTA, 1 mM sodium fluoride, 1 mM sodium
891 vanadate, 10 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM PMSF,
892 6 mM AEBSF, 5 mM benzamidine, 10 µg/ml aprotinin, and each 1 µg/ml of antipain
893 dihydrochloride, chymostatin, leupeptin, and pepstatin A, 200 nM okadaic acid, 200 µM 2-
894 bromo-4-methoxyacetophenone (Arabaci et al., 1999; Arabaci et al., 2002) and 20 µM ethyl
895 3,4-dephostatin (Watanabe et al., 2000; Suzuki et al., 2001). Protein concentrations were
896 determined with the DC protein assay from Bio-Rad Laboratories (cat. no. 500-0116).
897 Samples were denatured for 5 min at 100 °C after addition of 1/6 volume of 6 x SDS-PAGE
898 sample loading buffer [350 mM Tris·HCl (pH 6.8 at RT), 30% (v/v) glycerol, 10% (w/v)
899 SDS, 0.5 g/l bromophenol blue, 2% (v/v) β-mercaptoethanol]. 10 - 100 µg total protein were
900 separated by SDS-PAGE (Laemmli, 1970) and transferred onto polyvinylidene difluoride
901 (PVDF) membranes (Amersham HyBond™-P, pore size 0.45 µm, GE Healthcare, Little
902 Chalfont, UK, cat. no. RPN303F) by semi-dry blotting in 0.1 M Tris, 0.192 M glycine, 5%
903 (v/v) methanol at 2 mA/cm² for 75 min.

904 Protein extracts for Western blotting for IRS1 and phosphorylated species of IRS1 were
905 prepared from cells serum-starved with media lacking phenol red. Media were aspirated and
906 cells lysed in 8 M urea, 2.5% (w/v) SDS, 50 mM Tris·HCl (pH 7.5 at 4 °C), 6 mM EDTA, 2
907 mM PMSF, 40 mM AEBSF, 5 mM benzamidine, and 10 µM E-64. Lysates were cleared by
908 centrifugation at 627,000 g for 2 h at 4 °C. Protein concentrations were determined with

909 bicinchoninic acid (Smith et al., 1985). Samples were denatured for 5 min at 70 °C after
910 addition of 1/6 volume of 6 x SDS-PAGE sample loading buffer. 50 – 200 µg protein were
911 separated on 7.5% SDS-PAGE gels and transferred to PVDF membranes by wet transfer in 10
912 mM NaHCO₃, 3 mM NaCO₃, 0.025% (w/v) SDS (Dunn, 1986) at 50 V for 18 h (for a 1 mm
913 thick gel) at 4 °C after equilibration of gels for 3 x 20 min at 4 °C in 10 mM NaHCO₃, 3 mM
914 NaCO₃, 0.025% (w/v) SDS.

915 Membranes were blocked for 1 h at RT or overnight at 4 °C in 5% (w/v) skimmed milk
916 powder in TBST [20 mM Tris-HCl (pH 7.6 at RT), 137 mM NaCl, and 0.1% (v/v) Tween-20]
917 or in TBST + 3% (w/v) skimmed milk powder when the anti-phosphotyrosine antibody was
918 used. The anti-phospho-T308-AKT, anti-phospho-S473-AKT, anti-AKT, anti-phospho-S63-
919 c-Jun, anti-c-Jun, anti-phospho-S51-eIF2 α , anti-eIF2 α , anti-phospho-Y1355-insulin receptor
920 β chain, anti-insulin receptor β chain (clone 4B8), anti-phospho-Y891 (mouse)/Y896
921 (human)-IRS1, anti-IRS1 (clones D23G12 and D5T8J), anti-phospho-T183-phospho-Y185-
922 JNK, and anti-JNK antibodies were incubated with membranes at a 1:1,000 dilution in TBST
923 + 5% (w/v) BSA over night at 4°C with constant rotation. For the rabbit anti-IRS1 antibody,
924 clone D23G12 was used to develop Western blots of cell lysates, while clone D5T8J was used
925 to develop Western blots of immunoprecipitates of IRS1. The anti-phospho-Y608
926 (mouse)/Y612 (human)-IRS1 and anti-phospho-Y628 (mouse)/Y632 (human)-IRS1
927 antibodies were incubated with membranes at a 1:4,000 dilution in TBST + 5% (w/v)
928 skimmed milk powder for 2 h at RT. The anti-phospho-Y935 (mouse)/941 (human)-IRS1
929 antibody was incubated with membranes at a 1:1,000 dilution in TBST + 5% (w/v) skimmed
930 milk powder for 2-4 h at RT or overnight at 4 °C. The anti phospho-S307 (mouse)/S312
931 (human)-IRS1 antibody was incubated with membranes at a 1:1,000 dilution in TBST + 5%
932 (w/v) skimmed milk powder overnight at 4 °C, and the anti-phosphotyrosine antibody at a
933 1:1,000 dilution in TBST + 3% (w/v) skimmed milk powder for 4 h at RT. The polyclonal
934 anti-INSR β chain antibody was used at a 1:200 dilution and the anti-TRB3 antibody at a
935 concentration of 0.1 µg/ml in TBST + 5% (w/v) skimmed milk powder overnight at 4 °C. The
936 anti- β -actin antibody was used at a dilution of 1:10,000 – 1:20,000 in TBST + 5% (w/v)

937 skimmed milk powder for 2 h at RT, the anti-GAPDH antibody at a dilution of 1:10,000 –
938 1:30,000 in TBST + 5% (w/v) skimmed milk powder for 2 h at RT or overnight at 4 °C, and
939 the anti- α -tubulin antibody at a dilution of 1:1,000 – 1:2,000 in TBST + 5% (w/v) skimmed
940 milk powder for 2 h at RT. Membranes were washed four times with TBST for 5 min at RT
941 and then probed with goat anti-mouse IgG(H+L)-peroxidase at a dilution of 1:5,000 in TBST
942 + 5% (w/v) skimmed milk powder for 2 h at RT to detect the anti-phospho-S307 (mouse)/S312
943 (human)-IRS1 antibody and at a dilution of 1:10,000 in TBST + 5% (w/v) skimmed milk
944 powder for 2 h at RT to detect all other murine antibodies. Goat anti-rabbit IgG(H+L)-
945 peroxidase was used at a dilution of 1:2,000 in TBST + 5% (w/v) skimmed milk powder for 2
946 h at RT, and goat anti-rat IgG(H+L)-peroxidase at a dilution of 1:5,000 in TBST + 5% (w/v)
947 skimmed milk powder for 2 h at RT. Membranes were washed four times for 5 min at RT and
948 then developed with Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Fisher
949 Scientific, cat. no. 10455145), Thermo Scientific™ Pierce™ ECL 2 Western Blotting
950 Substrate (Fisher Scientific, cat. no. 11517371) or by enhanced chemiluminescence as
951 described before (Schröder and Friedl, 1997a). Blots were exposed to Thermo Scientific™
952 CL-X Posure™ film (Fisher Scientific, cat. no. 10696384) or Thermo Scientific™ CL-X
953 Posure™ film pre-flashed with a Sensitize pre-flash unit (GE Healthcare, Little Chalfont, UK,
954 cat. no. RPN 2051) following the manufacturer's instructions to capture weak signals.
955 Exposure times were adjusted based on previous exposures to obtain exposures in the linear
956 range of the film. Films were scanned on a CanoScan LiDE 600F scanner (Canon Europa,
957 Amstelveen, The Netherlands) or a MFC-5320 DW all-in-one printer (Brother Industries,
958 Manchester, UK) and saved as tif files. Bands were quantified with CLIQS 1.1 (Totallab,
959 Newcastle upon Tyne, United Kingdom). Intensities for phosphorylated proteins were
960 normalised to the intensity of the unphosphorylated species of the protein. Changes in protein
961 abundance are expressed relative to the loading control, β -actin, GAPDH, or α -tubulin.
962 Variation of normaliser samples was preserved for statistical analyses by normalising all
963 samples within one experiment to all samples with similar intensities or by normalising all
964 samples of one experimental repeat to the normaliser samples of all repeats for this type of

965 experiment and using the average of these normalisations for graphing and statistical
966 analyses.

967 To reprobe blots for detection of nonphosphorylated proteins and loading controls,
968 membranes were stripped using Thermo Scientific™ Restore™ Western Blot Stripping
969 Buffer (Fisher Scientific, cat. no. 10057103) or as described before (Armstrong et al., 2017),
970 except that the pH of the 100 mM glycine·HCl, 0.1% (v/v) Tween 20 solution was dropped to
971 1.5 and the incubation temperature with this buffer raised to 65 °C. Membranes were then
972 blocked with 5% (w/v) skimmed milk powder in TBST as described above.

973 *Immunoprecipitation of IRS1.* Protein extracts were prepared as described under “Cell lysis
974 and Western blotting” for extraction of IRS1. 2 mg of total protein were diluted with 50 mM
975 Tris·HCl (pH 7.5 at 4 °C), 1% (v/v) Triton X-100, 10 mM EDTA, 0.1% (w/v) BSA to a final
976 SDS concentration of 0.1% (w/v). All immunoprecipitations in one experiment were adjusted
977 to the same final volume and a SDS concentration of 0.1% (w/v). Lysates were precleared
978 with 10 µl SureBeads™ protein A magnetic beads at 4 °C with constant, slow rotation of the
979 tubes. Samples were centrifuged at 500 g and 4 °C for 10 s, magnetic beads collected in
980 magnetic racks, and supernates centrifuged at 100,000 g, 4°C for 30 min and transferred to
981 new tubes. After addition of 2 µl of anti-IRS1 antibody (clone D23G12), samples were
982 incubated at 4 °C overnight with constant, slow rotation. Samples were then centrifuged at
983 100,000 g, 4°C for 30 min, supernates transferred to new tubes, and immune complexes
984 collected by addition of 30 µl SureBeads™ protein A magnetic beads for 2 h at 4 °C with
985 constant, slow rotation. Samples were centrifuged at 500 g and 4 °C for 10 s and
986 immunoprecipitates collected in magnetic racks. Immunoprecipitates were washed three times
987 with 50 mM Tris·HCl (pH 7.5 at 4 °C), 1% (v/v) Triton X-100, 10 mM EDTA, 0.1% (w/v)
988 BSA, and once with 0.1% (w/v) BSA. The last wash solution was completely aspirated, the
989 beads resuspended in 58.3 mM Tris·HCl (pH 6.8 at RT), 10% (w/v) SDS, 8 M urea, 2.5% (v/v)
990 β-mercaptoethanol, 0.083 g/l bromophenol blue, and denatured at 80 °C for 5 min. Denatured
991 samples were allowed to cool to RT for 5 min, centrifuged at 17,000 g and RT for 5 min, and
992 separated on 7.5% SDS-PAGE gels. Proteins were transferred onto PVDF membranes and

993 membranes blotted with anti-phosphotyrosine and anti-IRS1 (clone D5T8J) antibodies as
994 described under “Cell lysis and Western blotting”.

995 *[³⁵S]-L-methionine/[³⁵S]-L-cysteine pulse labelling and immunoprecipitation of the insulin*
996 *receptor.* 70-80% confluent cultures were treated with 0.1 μM thapsigargin, 0.1 μg/ml
997 tunicamycin or 0.1% (v/v) DMSO for 22.5 h. Cultures were washed twice with methionine
998 and cysteine free culture medium pre-warmed to 37 °C and incubated with methionine and
999 cysteine free culture medium containing 2% (v/v) dialysed FBS in the continued presence of
1000 0.1% (v/v) DMSO, 0.1 μM thapsigargin or 0.1 μg/ml tunicamycin for 20 min at 37 °C. The
1001 medium was then replaced with fresh methionine and cysteine free culture medium
1002 containing 2% (v/v) dialysed FBS and DMSO, thapsigargin, or tunicamycin, and [³⁵S]-L-
1003 methionine/[³⁵S]-L-cysteine. 3T3-F442A cells were labelled with 250 μCi of 70% [³⁵S]-L-
1004 methionine, 25% [³⁵S]-L-cysteine (1000 Ci/mmol, Hartmann Analytic, Braunschweig,
1005 Germany, cat. no. SCIS-103) and Hep G2 cells with 125 μCi of 70% [³⁵S]-L-methionine, 25%
1006 [³⁵S]-L-cysteine (1000 Ci/mmol) per 10 cm dish for 1 or 8 h. Dishes were placed on ice, the
1007 medium aspirated and cultures washed three times with ice-cold PBS (pH 7.4), and lysed in
1008 125 μl of ice-cold 50 mM HEPES-NaOH (pH 7.5 at 4 °C), 150 mM NaCl, 0.5% (w/v) sodium
1009 deoxycholate, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS, 10 mM EDTA, 2 mM PMSF, 6 mM
1010 AEBSF, 5 mM benzamidine, 10 μg/ml aprotinin, and each 1 μg/ml of antipain
1011 dihydrochloride, chymostatin, leupeptin, and pepstatin A/10 cm dish. Lysates were cleared by
1012 centrifugation at 16,100 g, 4 °C for 30 min, and the protein concentration of lysates
1013 determined with the Bio-Rad Laboratories DC protein assay. For each immunoprecipitation
1014 2.4 mg of total cell protein were diluted to a final concentration of 4.8 mg/ml with ice-cold 50
1015 mM HEPES-NaOH (pH 7.5 at 4 °C), 150 mM NaCl, 10 mM EDTA, 0.5% (w/v) sodium
1016 deoxycholate, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS. 3T3-F442A cell lysates were
1017 precleared by incubation with 6.24 μl 100 ng/μl of normal rabbit IgG and 60 μl of
1018 SureBeadsTM protein A magnetic beads for 2 h at 10 °C with overhead rotation. For Hep G2
1019 cell lysates, 3.12 μl 100 ng/μl of normal rabbit IgG and 30 μl of SureBeadsTM protein A
1020 magnetic beads were used in the preclearing step. SureBeadsTM protein A magnetic beads

1021 were collected in magnetic racks, supernates centrifuged at 16,100 g, 4 °C for 15 min, and
1022 supernates transferred to new tubes. 6 µl of 52 ng/µl anti-insulin receptor β chain antibody
1023 (clone 4B8) or 3.12 µl 100 ng/µl normal rabbit IgG were added and samples incubated
1024 overnight at 10 °C with overhead rotation. Samples were centrifuged at 16,100 g, 4 °C for 15
1025 min, supernates transferred to new tubes, and 30 µl SureBeads™ protein A magnetic beads
1026 added to collect immune complexes while incubating samples for 2 h at 10 °C with overhead
1027 rotation. Immune complexes were collected in magnetic racks. For immunoprecipitation of
1028 the insulin receptor from 3T3-F442A cell lysates, immunoprecipitates were washed three
1029 times with ice-cold 50 mM HEPES-NaOH (pH 7.5 at 4 °C), 500 mM NaCl, 10 mM EDTA,
1030 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1 M urea, and
1031 once with ice-cold water. For immunoprecipitation of the insulin receptor from Hep G2 cell
1032 lysates, immunoprecipitates were washed three times with ice-cold 50 mM HEPES-NaOH
1033 (pH 7.5 at 4 °C), 2 M NaCl, 10 mM EDTA, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v)
1034 Triton X-100, 0.1% (w/v) SDS, and once with ice-cold water. The water was completely
1035 aspirated, immunoprecipitates resuspended in 58.3 mM Tris·HCl (pH 6.8 at RT), 5% (v/v)
1036 glycerol, 10% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 0.083 g/l bromophenol blue, and
1037 denatured at 100 °C for 5 min. Denatured samples were allowed to cool to RT for 5 min,
1038 centrifuged at 17,000 g, RT for 15 min, and separated on 7.5% SDS-PAGE gels, in which the
1039 SDS concentration had been raised to 1.25 g/l in both the stacking and separating gels. Gels
1040 were washed three times with water for 15 min at RT and dried for autoradiography with
1041 filter paper packing on a GelAir Drying System (Bio-Rad Laboratories, cat. no. 1651772)
1042 following the manufacturer's instructions (Krishnan and Nguyen, 1990). Gels were exposed
1043 to Kodak storage phosphor SD230 screens for 12 – 16 d and scanned on a Typhoon 9400
1044 system (GE Healthcare). The abundance of ³⁵S-labelled α-β proreceptors was expressed
1045 relative to one randomly selected sample treated with 0.1% (v/v) DMSO.

1046 *Measurement of protein synthesis rates by [³⁵S]-L-methionine/[³⁵S]-L-cysteine pulse*
1047 *labelling.* Cells were treated with 0.1% (v/v) DMSO, 0.1 µM thapsigargin, or 0.1 µg/ml
1048 tunicamycin and labelled with 70% [³⁵S]-L-methionine, 25% [³⁵S]-L-cysteine and protein

1049 lysates prepared as described for “[³⁵S]-L-methionine/[³⁵S]-L-cysteine labelling and
1050 immunoprecipitation of the insulin receptor”, except that cells were labelled in 6 well plates
1051 with 0.35 µCi of 70% [³⁵S]-L-methionine, 25% [³⁵S]-L-cysteine (1000 Ci/mol) per cavity for
1052 30 min, and that lysates were prepared with 50 µl of lysis buffer per cavity. 50 µg of total
1053 protein were denatured after addition of 1/6 volume of 6 x SDS-PAGE sample loading buffer
1054 and heating to 100 °C for 5 min and resolved on 12% SDS-PAGE gels. Gels were stained
1055 with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic
1056 acid for 30 min at RT, destained in 8% (v/v) acetic acid, 7% (v/v) methanol at RT, washed
1057 three times with water for 15 min at RT, and dried for autoradiography with filter paper
1058 packing as described above. Gels were exposed to Fujifilm BAS-IP multipurpose standard
1059 (MS) storage phosphor screens for 4 d or 16 d and scanned on a Typhoon 9400 system (GE
1060 Healthcare) to record the ³⁵S autoradiography signal and on a MFC-5320 DW all-in-one
1061 printer to record images of the Coomassie Brilliant Blue R-250-stained gels. Protein synthesis
1062 rates were expressed relative to the average of all cultures for one cell line treated with 0.1%
1063 (v/v) DMSO.

1064 To measure [³⁵S]-L-methionine/[³⁵S]-L-cysteine incorporation rates by scintillation
1065 counting of TCA precipitates, 50 µg of protein lysate were added to 100 µl 1 mg/ml BSA on
1066 ice, followed by 1 ml of ice-cold 10% (w/v) TCA. Samples were incubated on ice for 30 min.
1067 TCA precipitates were collected on GF/C glass fibre filters (GE Healthcare, cat. no. 1822-
1068 025) prewetted with ice-cold 10% (w/v) TCA. The TCA precipitates were washed twice with
1069 ice-cold 10% (w/v) TCA, twice with ethanol, and allowed to dry at RT. Precipitated
1070 radioactivity was measured by scintillation counting in a Tri-Carb 2200 CA Liquid
1071 Scintillation Analyzer (Canberra Packard, Pangbourne, UK) in 20 ml plastic scintillation vials
1072 with 10 ml Ecoscint A (National Diagnostics, Hesse, UK). Counts per minute were converted
1073 into disintegrations per minute by using a prerecorded ³⁵S quench curve for the transformed
1074 spectral index of the external standard constructed by adding increasing amounts of
1075 chloroform to vials each containing 1·10³ Bq of 70% [³⁵S]-L-methionine, 25% [³⁵S]-L-
1076 cysteine (1000 Ci/mol) in a total volume of 10 ml Ecoscint A/chloroform mixtures.

1077 *Measurement of the half-life of the insulin receptor at the cell surface.* 70-80% confluent
1078 cultures were washed three times with ice-cold PBS (pH 8.0; 10 mM Na₂HPO₄, 2.0 mM
1079 KH₂PO₄, 2.7 mM KCl, 137 mM NaCl), labelled for 5 min at 4 °C in PBS (pH 8.0), 1 g/l D-
1080 glucose, 100 mg/l CaCl₂, 100 mg/l MgCl₂, 36 mg/l sodium pyruvate, 2 mM
1081 sulphosuccinimidyl-6-(biotinamido)hexanoate, washed three times with PBS (pH 8.0), 100
1082 mM glycine at RT, three times with PBS (pH 7.4) at RT, replenished with culture medium
1083 containing 0.1% (v/v) DMSO, 0.3 µM thapsigargin, 1 µg/ml tunicamycin, or 1 µg/ml SubAB
1084 prewarmed to 37 °C, and incubated at 37 °C, 5% (v/v) CO₂, and 95% relative humidity for up
1085 to 72 h. To prepare protein lysates, cultures were placed on ice, washed three times with ice-
1086 cold PBS (pH 7.4), and lysed in RIPA buffer containing 10 mM EDTA, 2 mM PMSF, 6 mM
1087 AEBSF, 5 mM benzamidine, 10 µg/ml aprotinin, and each 1 µg/ml of antipain, chymostatin,
1088 leupeptin, and pepstatin A. Protein concentrations were determined with the Bio-Rad
1089 Laboratories DC protein assay. Biotinylated proteins were collected with 10 µl streptavidin-
1090 agarose beads from 100 µg total protein for 3T3-F442A cells, 200 µg total protein for C₂C₁₂
1091 cells, and 50 µg total protein for Hep G2 cells for 1 h at 4 °C. The streptavidin-agarose beads
1092 were washed once with ice-cold 10 mM HEPES (pH 7.5 at 4 °C), 1% (v/v) Triton X-100, 10
1093 mM EDTA, twice with ice-cold 10 mM HEPES (pH 7.5 at 4 °C), 1% (v/v) Triton X-100, 10
1094 mM EDTA, 6 M urea, and once with ice-cold water. The water was completely aspirated and
1095 beads resuspended in 15 µl of 6 x SDS-PAGE sample loading buffer. After denaturation at
1096 100 °C for 5 min, purified proteins were resolved on 10% SDS-PAGE gels, transferred by
1097 semi-dry blotting onto PVDF membranes, developed with anti-insulin receptor β chain (clone
1098 4B8) and anti-GAPDH antibodies, and chemiluminescence signals quantified as described
1099 under “Cell lysis and Western blotting”. To determine the half-life of the insulin receptor at
1100 the cell surface, the intensity of biotinylated insulin receptors purified on streptavidin-agarose
1101 beads immediately after labelling of cells with sulphosuccinimidyl-6-(biotinamido)hexanoate
1102 (‘0 h time point’) was arbitrarily set to 1.0. Levels of biotinylated insulin receptors purified 6
1103 h, 12 h, 24 h, 36 h, 48 h, and 72 h after labelling of cells with sulphosuccinimidyl-6-
1104 (biotinamido)hexanoate were expressed relative to the level of biotinylated insulin receptors

1105 purified at the 0 h time point. Half-lives were calculated from the slopes of linear decay
1106 curves of the natural logarithm of the relative abundance of biotinylated insulin receptors over
1107 time. For each condition, half-lives were determined in at least three independent
1108 experiments. Runs tests (Wald and Wolfowitz, 1940) did not reveal significant deviations
1109 from a linear relationship between the natural logarithm of the relative abundance of
1110 biotinylated insulin receptors and the time after the labelling reaction. Pilot experiments
1111 established that labelling of surface exposed insulin receptors is maximal and that
1112 biotinylation pull-down reactions are quantitative under the conditions described above (data
1113 not shown).

1114 *Endo H and PNGase F digests.* 8 µg of protein were denatured in 0.5% (w/v) SDS, 40 mM
1115 DTT at 100°C for 10 min. Samples were then incubated with 1000 U of Endo H in 50 mM
1116 sodium citrate, pH 5.5 (at 25 °C) at 37°C for 2 h. For PNGase F digests denatured samples
1117 were incubated with 1000 U of PNGase F in 50 mM sodium phosphate pH 7.5 (at 25 °C), 1%
1118 (v/v) NP-40 at 37 °C for 2 h.

1119 *Fluorescence microscopy.* Images of GFP-tagged insulin receptors expressed in HEK 293
1120 cells were taken on a Zeiss ApoTome microscope (Carl Zeiss, Cambridge, UK) equipped with
1121 a Zeiss 63x Plan-APOCHROMAT Oil PH3 objective with a numerical aperture of 1.4 18 h
1122 after induction of ER stress with 1 µg/ml tunicamycin or 1 µg/ml SubAB. The cell membrane
1123 was visualised by staining cells for 5 min at RT with 5 µg/ml CellMask Deep Red (Life
1124 Technologies). GFP fluorescence was observed using a band pass (BP) 450-490 filter (Carl
1125 Zeiss, FITC/GFP, filter set 9, cat. no. 488009-000) and a long pass (LP) 515 filter. CellMask
1126 Deep red fluorescence was observed using a BP546/12 filter (Carl Zeiss, Rhodamine, filter set
1127 15, cat. no. 488015-0000) and a LP 590 filter. To quantify colocalisation of the GFP-tagged
1128 insulin receptors and CellMask Deep Red signals, individual cells were defined as regions of
1129 interest (ROI) in Image J 1.47 (Schneider et al., 2012), and background-corrected for the
1130 intracellular fluorescence of CellMask Deep Red using the Background Subtraction from the
1131 ROI plug-in. The Pearson correlation coefficient between the INSR-GFP and CellMask Deep
1132 Red Fluorescence was determined in individual cells using the Colocalization Test plug-in

1133 and Costes' image randomization (Costes et al., 2004) and a point spread function (PSF)
1134 width of 0.453 μm as a quantitative measure of colocalization of both fluorescence signals
1135 (Manders et al., 1992; Manders et al., 1993).

1136 *Statistical calculations.* Experimental data are presented as means and standard errors. For
1137 composite parameters, errors were propagated using the law of error propagation for random,
1138 independent errors (Ku, 1966). Data were analysed for normality using the D'Agostino-
1139 Pearson omnibus normality test (D'Agostino and Pearson, 1973) or Shapiro-Wilk test
1140 (Shapiro and Wilk, 1965), equality of variances (homoscedasticity) using the Brown-Forsythe
1141 test (Brown and Forsythe, 1974), and, for additivity of means, treatment effects, and errors
1142 using Tukey's test (Tukey, 1949b; Little and Hills, 1972) before ordinary one- or two-way
1143 analysis of variance (ANOVA). Heteroscedastic data were transformed before ANOVA
1144 (Little and Hills, 1972) or examined by Welch's test (Welch, 1947) and a Games-Howell post
1145 hoc test (Games and Howell, 1976) or Dunnett's T3 multiple comparisons test (Dunnett,
1146 1980). Kruskal-Wallis one-way ANOVA on ranks (Kruskal and Wallis, 1952) with Dunn's
1147 post-hoc test (Dunn, 1964) was used to analyse data that are not normally distributed or
1148 heteroscedastic. Transformed data were reexamined for normality, equality of variances, and
1149 additivity. In all analyses, a familywise p value of < 0.05 was considered statistically
1150 significant. Brown-Forsythe tests for equality of variances, Tukey's test for additivity, and
1151 runs tests (Wald and Wolfowitz, 1940) were performed in Microsoft Excel 2010 or Microsoft
1152 365 Excel (Microsoft Corporation, Redmond, WA, USA) using the Real Statistics plug-in
1153 (<http://www.real-statistics.com/>). All other statistical calculations, linear and non-linear
1154 regressions were performed in GraphPad Prism 6.0.7 or 8.4.3 (GraphPad Software, La Jolla,
1155 CA, USA).

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1166 **CONFLICT OF INTEREST**

1167 The authors declare that they have no conflicts of interest with the contents of this article.

1168 **AUTHOR CONTRIBUTIONS**

1169 MS conceived and coordinated the study and wrote the paper. MB, SD, NS, ADM, JNW, and
1170 MS designed and performed the experiments. RD performed experiments. AWP and JCP
1171 prepared SubAB and SubA_{A272}B. MB, SD, NS, ADM, and MS analysed the results and
1172 prepared the figures. All authors approved the final version of the manuscript.

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1753

1754 TABLES

1755 Table 1. siRNAs.

Species	Gene	#	Sequence, sense strand	Sequence, antisense strand
<i>Mus musculus</i>	<i>INSR</i>	1	GAGAUCUCCUGGGAUUCA	AUGAAUCCCAGGAGAUCU
			UdTdT	CdTdT
<i>M. musculus</i>	<i>INSR</i>	2	CCUUAUCAAGGCCUGUCU	UAGACAGGCCUUGAUAAG
			AdTdT	GdTdT
<i>M. musculus</i>	<i>INSR</i>	3	GAAACUCUGCUUGUCUGA	UUCAGACAAGCAGAGUUU
			AdTdT	CdTdT
<i>M. musculus</i>	<i>TRB3</i>	1	GGCAGAAGCUGUGUGGAG	CUCCACACAGGCCUUCUGCd
			dTdT	TdT
<i>M. musculus</i>	<i>TRB3</i>	2	GGACAAUCCCUUUCACAA	UUUGUGAAAGGGAUUGUC
			AdTdT	CdTdT
<i>Aequora victoria</i>	eGFP		GCAAGCUGACCCUGAAGU	GAACUUCAGGGUCAGCUU
			UCAU	GCCG

1756

1757 **Table 2.** Oligodeoxynucleotides.

Name	Purpose	Sequence
H8962	<i>TRB3</i> real time PCR, forward	TTTGGAACGAGAGCAAGGCA
H8963	<i>TRB3</i> real time PCR, reverse	CCACATGCTGGTGGGTAGG
H9044	<i>INSR</i> real time PCR, forward	CTTCTCTCCGTGTCTATGG
H0945	<i>INSR</i> real time PCR, reverse	GACCATCTCGAAGATAACCA

1758

1759

1760 **FIGURE LEGENDS**

1761 **Figure 1.** Acute ER stress does not inhibit phosphorylation of AKT on T308 or S473 in C₂C₁₂
 1762 myotubes stimulated with 100 nM insulin for 15 min. (A) C₂C₁₂ myotubes were serum-
 1763 starved for 18 h and treated with the indicated concentrations of thapsigargin (Tg),
 1764 tunicamycin (Tm), 1 µg/ml SubAB, or 1 µg/ml catalytically inactive SubA_{A272}B during the
 1765 last 1-8 h of serum starvation and then stimulated with 100 nM insulin for 15 min where
 1766 indicated. Cell lysates were analysed by Western blotting. Quantification of phosphorylation
 1767 of AKT on (B) T308 and (C) S473. Bars represent standard errors ($n = 5$ for S473
 1768 phosphorylation of AKT at 8 h in unstressed, insulin-stimulated cells, $n = 6$ for all other
 1769 unstressed, insulin-stimulated samples, and $n = 3$ for all other treatments). p values for
 1770 comparison of ER-stressed samples and samples not stimulated with 100 nM insulin to
 1771 samples stimulated with 100 nM insulin were calculated by ordinary two-way ANOVA with
 1772 Dunnett's multiple comparisons test (Dunnett, 1955, 1964). (D) Detection of *XBPI* splicing
 1773 by reverse transcriptase PCR. PCR products derived from unspliced (u) and spliced (s) *XBPI*
 1774 mRNA are indicated by arrows. β -Actin (*ACTB*) was used as a loading control. (E-F)
 1775 Induction of *TRB3* in C₂C₁₂ cells by ER stress. C₂C₁₂ cells were treated with 300 nM
 1776 thapsigargin, 1 µg/ml tunicamycin, SubAB (labelled 'WT'), or SubA_{A272}B (labelled 'mt.') for
 1777 (E) 4 h and (F) 8 h. *TRB3* mRNA levels were determined by reverse transcriptase-qPCR and
 1778 standardised to the loading control *ACTB*. Bars represent standard errors ($n = 2$ for the
 1779 samples treated with thapsigargin or tunicamycin for 8 h, $n = 3$ for all other samples). p values
 1780 for comparison of treated samples to the untreated sample ('-') were calculated by ordinary
 1781 two-way ANOVA with Dunnett's multiple comparisons test taking data shown in Fig. 10F
 1782 into account. Abbreviations: *ns* – not significant, * or # - $p < 0.05$, ** or ## - $p < 0.01$, *** or
 1783 ### - $p < 0.001$, and **** or ##### - $p < 0.0001$.

1784 **Figure 2.** Acute ER stress does not inhibit phosphorylation of AKT on S473 stimulated with
 1785 10 nM insulin for 15 min. (A) 3T3-F442A cells, (B) C₂C₁₂ myotubes, and (C) Hep G2 cells
 1786 were serum-starved for 18 h and treated with 0.3 µM thapsigargin, 1 µg/ml tunicamycin, 1

1787 $\mu\text{g/ml}$ SubAB, or 1 $\mu\text{g/ml}$ catalytically inactive SubA_{A272}B during the last 30 min of serum
1788 starvation and then stimulated with 10 nM insulin for 15 min where indicated. Cell lysates
1789 were analysed by Western blotting. Bars represent standard errors ($n = 3$). p values for
1790 comparison of ER-stressed samples and samples not stimulated with 10 nM insulin to samples
1791 stimulated with 10 nM insulin were calculated by ordinary two-way ANOVA with Dunnett's
1792 multiple comparisons test.

1793 **Figure 3.** Acute ER stress does not inhibit insulin-stimulated phosphorylation of IRS1 at four
1794 specific tyrosine phosphorylation sites in C₂C₁₂ myotubes. Tyrosine phosphorylation at (A)
1795 Y608, (B) Y628, (C) Y891, and (D) Y935 was analysed by Western blotting. C₂C₁₂ myotubes
1796 were serum-starved for 18 h before exposure to 1 μM thapsigargin for 10, 20, or 30 min (left
1797 side of figure panels) or to 0.1, 1.0, or 10 $\mu\text{g/ml}$ tunicamycin for 30 min (right side of figure
1798 panels), followed by stimulation with the indicated concentrations of insulin for 5 min in the
1799 continued presence of thapsigargin or tunicamycin. Bars represent standard errors ($n = 3$). p
1800 values for comparison of effects of thapsigargin or tunicamycin within one insulin
1801 concentration and for comparison of effects of different insulin concentrations were
1802 calculated by ordinary two-way ANOVA with Tukey's multiple comparisons test (Tukey,
1803 1949a). Abbreviation: α -Tub – α -tubulin.

1804 **Figure 4.** Acute ER stress does not inhibit insulin-stimulated tyrosine phosphorylation of
1805 IRS1. Serum-starved cells were exposed to (A) 1 μM thapsigargin or (B) 10 $\mu\text{g/ml}$
1806 tunicamycin for 30 min, followed by stimulation with the indicated concentrations of insulin
1807 for 5 min in the continued presence of thapsigargin or tunicamycin. After
1808 immunoprecipitation of IRS1, Western blots were probed with an anti-phosphotyrosine ('pY')
1809 and an anti-IRS1 antibody. Bars represent standard errors ($n = 3$ for thapsigargin-treated
1810 C₂C₁₂ myotubes, $n = 4$ for thapsigargin-treated 3T3-F442A cells and tunicamycin-treated Hep
1811 G2 cells, $n = 5$ for tunicamycin-treated 3T3-F442A cells and thapsigargin-treated Hep G2
1812 cells, and $n = 6$ for tunicamycin-treated C₂C₁₂ myotubes). p values for comparison of effects
1813 of thapsigargin or tunicamycin within one insulin concentration and for comparison of effects

1814 of different insulin concentrations were calculated by ordinary two-way ANOVA with
 1815 Tukey's multiple comparisons test. For 3T3-F442A cells and Hep G2 cells treated with
 1816 tunicamycin data were square root transformed before statistical analyses.

1817 **Figure 5.** ER stress does not elicit IRS1 S307/S312 phosphorylation. (A, B) Serum-starved
 1818 3T3-F442A cells, (C, D) C₂C₁₂ myotubes, and (E, F) Hep G2 cells were treated with 1 μ M
 1819 thapsigargin or 5 μ g/ml anisomycin (ANI) for the indicated times. (A, C, and E) Cell lysates
 1820 were analysed by Western blotting. (B, D, and F) Quantification of the Western blots shown
 1821 in panels A, C, and E. Bars represent standard errors ($n = 3$). p values for comparison of every
 1822 sample to every other sample were calculated by ordinary one-way ANOVA with Tukey's
 1823 multiple comparisons test. Anisomycin-treated samples are positive controls in the phospho-
 1824 S307/S312-IRS1 Western blots.

1825 **Figure 6.** Depletion of insulin receptors coincides and correlates with decreased AKT S473
 1826 phosphorylation in ER-stressed C₂C₁₂ myoblasts and suffices to decrease AKT S473
 1827 phosphorylation. (A) Serum-starved C₂C₁₂ cells were treated with the indicated concentrations
 1828 of thapsigargin, tunicamycin, 1 μ g/ml SubAB or 1 μ g/ml SubA_{A272}B for 12 - 24 h before
 1829 stimulation with 100 nM insulin for 15 min. Cell lysates were analysed by Western blotting
 1830 for pS473-AKT, total AKT, the insulin receptor (INSR), and GAPDH. Bands representing the
 1831 α - β proreceptor, the unglycosylated α - β proreceptor, and the β chain of the mature insulin
 1832 receptor are labelled α - β , α - β - N, and β , respectively. (B) Quantification of the
 1833 phosphorylation of AKT on S473 ('pS473-AKT') and of the relative abundance of β chains of
 1834 the insulin receptor (' β chain'). Bars represent standard errors (AKT phosphorylation at S473:
 1835 $n = 6$ for cells stimulated with 100 nM insulin at the 12 h time points, $n = 7$ and $n = 9$ for the
 1836 18 and 24 h time points, for all other samples $n = 3$ for the 12 h time point, $n = 4$ for the 18 h
 1837 time point and $n = 5$ for the 24 h time point; relative abundance of β chains: $n = 9$ for cells
 1838 stimulated with 100 nM insulin at the 12 h time point, $n = 10$ for the 18 h time point, and $n =$
 1839 12 for the 24 h time point, 12 h: $n = 5$ for the unstimulated cells and the insulin-stimulated
 1840 cells treated with 0.1 mM thapsigargin, $n = 3$ for the cells treated with SubA_{A272}B, and $n = 4$

1841 for all other samples, 18 h: $n = 6$ for the unstimulated cells, $n = 3$ for cells treated with
1842 SubA_{A272}B, and $n = 5$ for all other samples, 24 h: $n = 6$ for all samples). Phosphorylation of
1843 AKT at S473 and the relative abundance of β chains are expressed relative to unstressed cells
1844 that were stimulated with 100 nM insulin for 15 min. p values for comparison of ER-stressed
1845 to unstressed samples were calculated using ordinary two-way ANOVA with Dunnett's
1846 multiple comparisons test on the original data for AKT phosphorylation at S473 and square
1847 root-transformed data for the relative abundance of β chains. (C) Correlation of insulin-
1848 stimulated AKT phosphorylation with insulin receptor β chains ($r^2 = 0.80$, two-tailed $p <$
1849 0.0001 for a significantly non-zero slope, and $p > 0.05$ for deviation from linearity calculated
1850 by a runs test, $n = 27$). Dotted lines represent the 95% confidence interval of the linear
1851 regression line. The relative phosphorylation of AKT at S473 shown in panel (B) was plotted
1852 against the relative abundance of β chains shown in panel (B). (D) Steady-state *INSR* mRNA
1853 levels in C₂C₁₂ cells transfected with 50 nM of the indicated siRNAs for 24, 48, or 72 h. Bars
1854 represent standard errors ($n = 3$). p values for comparison of cells transfected with the three
1855 *INSR* siRNAs to the cells transfected with the eGFP siRNA were calculated with an ordinary
1856 two-way ANOVA with Tukey's multiple comparisons test. Differences in *INSR* mRNA levels
1857 between different *INSR* siRNAs within individual time points or between different time points
1858 for individual siRNAs are not significant. (E) siRNA-mediated knock-down of expression of
1859 the insulin receptor inhibits insulin-stimulated phosphorylation of AKT. Serum-starved C₂C₁₂
1860 cells were stimulated with insulin 48 h after transfection of 50 nM of the indicated siRNAs.
1861 Two unspecific bands are marked with an asterisk (*').

1862 **Figure 7.** Inhibition of insulin receptor synthesis at the transcriptional or translational level
1863 cannot fully account for decreased insulin-stimulated AKT S473 phosphorylation. (A) *INSR*
1864 mRNA levels measured by reverse transcriptase-qPCR in C₂C₁₂ cells treated with 300 nM
1865 thapsigargin, 1 μ g/ml tunicamycin, or 1 μ g/ml SubAB for 24 h. Bars represent standard errors
1866 ($n = 3$). p values for comparison of ER-stressed samples to unstressed samples were
1867 calculated using ordinary one-way ANOVA with Dunnett's multiple comparisons test. (B-D)
1868 Protein synthesis rates in (B) 3T3-F442A ($n = 8$), (C) C₂C₁₂ ($n = 8$), and (D) Hep G2 cells (n

1869 = 4) treated with 0.1 μ M thapsigargin or 0.1 μ g/ml tunicamycin for 24 h measured by
1870 incorporation of [35 S]-L-methionine into protein. Protein synthesis rates were determined as
1871 TCA-precipitable counts standardised to total protein. Bars represent standard errors. *p* values
1872 were calculated by ordinary one-way ANOVA with Tukey's multiple comparisons test. (E)
1873 Protein synthesis rates in cells treated for 24 h with 0.1 μ M thapsigargin or 0.1 μ g/ml
1874 tunicamycin measured by storage phosphor analysis of [35 S]-L-methionine incorporation into
1875 protein. For each cell line the storage phosphor image of the SDS-PAGE gel is shown to the
1876 left, and an image of the Coomassie Brilliant Blue R250-stained gel is shown to the right.
1877 Quantification of [35 S]-L-methionine incorporation into protein, expressed as the volume of
1878 the 35 S storage phosphor signal relative to the Coomassie Brilliant Blue R250 staining
1879 intensity is shown in the bar graphs below the gel images. Bars represent standard errors (*n* =
1880 8). *p* values were calculated by ordinary one-way ANOVA with Tukey's multiple
1881 comparisons test. (F) Phosphorylation of eIF2 α at S51 in 3T3-F442A cells (*n* = 13 for 0.5 h
1882 and *n* = 12 for 24 h), C₂C₁₂ cells (*n* = 21), and Hep G2 cells (*n* = 13 for 0.5 h and *n* = 8 for 24
1883 h) exposed for 0.5 or 24 h to 0.1 μ g/ml tunicamycin or 0.1 μ M thapsigargin. The treatment
1884 with 0.1 μ M thapsigargin for 0.5 h is a positive control for the pS51-eIF2 α Western blots.
1885 Bars represent standard errors. For 3T3-F442A cells, *p* values were calculated by Welch's
1886 ANOVA with Dunnett's T3 multiple comparisons test. For C₂C₁₂ and Hep G2 cells, *p* were
1887 calculated by a Kruskal-Wallis test with Dunn's multiple comparisons test. (G)
1888 Immunoprecipitation of the insulin receptor after a 1 h label with [35 S]-L-methionine in 3T3-
1889 F442A cells. Thapsigargin was used at 0.1 μ M and tunicamycin at 0.1 μ g/ml. Abbreviations:
1890 Mock – immunoprecipitation with non-immune IgG. 1st – immunoprecipitation of the insulin
1891 receptor after an 8 h labelling period. 2nd – immunoprecipitation of insulin receptors
1892 remaining in the supernate of the 1st immunoprecipitation. #1, #2, and #3 indicate three
1893 biological repeats. (H) Quantification of newly synthesised α - β proreceptors in 3T3-F442A
1894 cells (left) and Hep G2 cells (right). Bars represent standard errors (*n* = 3). *p* values were
1895 calculated by ordinary one-way ANOVA with Tukey's multiple comparisons test.

1896 **Figure 8.** ER stress does not increase turnover of insulin receptors at the cell surface. (A, C,
 1897 and E) Pull-down of cell surface proteins with streptavidin-agarose after biotinylation with
 1898 the cell-impermeable biotinylation reagent sulphosuccinimidy1-6-(biotinamido)hexanoate
 1899 from (A) 3T3-F442A, (C) C₂C₁₂, and (E) Hep G2 cells. Cell extracts were prepared 0 – 72 h
 1900 after labelling of cell surface proteins. Biotinylated proteins were isolated with streptavidin-
 1901 agarose, separated by SDS-PAGE, and Western-blotted for the insulin receptor and GAPDH.
 1902 ‘-’ refers to a pull-down reaction with unlabelled cell lysates. The arrows indicate that the
 1903 supernate of the pull-down reaction with the labelled 0 h sample was subjected to a second
 1904 pull-down with streptavidin-agarose. The lanes labelled ‘Input’ serve as a positive control for
 1905 the GAPDH Western blots on precipitates of the streptavidin-agarose pull down reactions and
 1906 themselves were not subjected to pull-down with streptavidin-agarose. The rows labelled
 1907 ‘Input’ show Western blots for GAPDH on equal amounts of input protein for the
 1908 streptavidin-agarose pull-down assays. The graphs show plots of the natural logarithm of the
 1909 abundance of biotinylated insulin receptors over time, the line of linear regression
 1910 (uninterrupted line), and the 95% confidence interval of the line of linear regression (dotted
 1911 lines). (B, D, and F) Comparison of the half-life, $t_{1/2}$, of the insulin receptor at the cell surface
 1912 of (B) 3T3-F442A ($n = 3$), (D) C₂C₁₂ ($n = 3$), and (F) Hep G2 (untreated $n = 7$, 0.3 μ M
 1913 thapsigargin $n = 8$, and 1 μ g/ml tunicamycin and SubAB $n = 5$) cells. Half-lives were
 1914 calculated from the slopes of linear regression lines obtained from plots of the natural
 1915 logarithm of the abundance of biotinylated insulin receptors over time. Bars represent
 1916 standard errors. p values were calculated by ordinary one-way ANOVA with Tukey’s
 1917 multiple comparisons test.

1918 **Figure 9.** Unprocessed α - β proreceptors accumulate in the ER of ER-stressed cells. (A)
 1919 Quantification of the relative abundance of α - β precursors of the insulin receptor in C₂C₁₂
 1920 cells exposed to the indicated concentrations of thapsigargin, tunicamycin, 1 μ g/ml SubAB, or
 1921 SubA_{A272}B for 24 h. Bars represent standard errors ($n = 12$ for unstressed, insulin-stimulated
 1922 cells, $n = 5$ for the samples treated with 0.3 μ M thapsigargin and SubA_{A272}B, and $n = 6$ for all
 1923 other samples). p values for comparison of ER-stressed samples and samples not stimulated

1924 with 100 nM insulin to the sample stimulated with 100 nM insulin were calculated using
1925 ordinary two-way ANOVA with Dunnett's multiple comparisons test including data for 12 h
1926 ($n = 8$ for unstressed, insulin-stimulated cells, $n = 4$ for all other samples) and 18 h ($n = 10$ for
1927 unstressed, insulin-stimulated cells, $n = 5$ for all other samples) time points. (B) Endo H
1928 digest of cell lysates prepared from C₂C₁₂ cells exposed to 1 μ g/ml SubAB, 0.3 μ M
1929 thapsigargin, or 1 μ g/ml tunicamycin. (C) Quantification of the relative abundance of β chains
1930 and α - β proreceptors from panel B. (D) The mature β chain of the insulin receptor carries
1931 Endo H sensitive N-linked oligosaccharides. Endo H and PNGase F digests of unstressed
1932 C₂C₁₂ cells were immunoblotted for the β chain of the insulin receptor. '#' indicates an
1933 unspecific band. (E) Steady-state insulin receptor levels in untreated HEK 293 cells or HEK
1934 293 cells treated for 18 h with 0.1 μ g/ml tunicamycin, 1 μ g/ml SubAB or SubA_{A272}B. (F)
1935 MTT activity of untreated HEK 293 cells and HEK 293 exposed for 18 h to 300 nM
1936 thapsigargin, 1 μ g/ml tunicamycin, or 1 μ g/ml SubAB. Bars represent standard errors ($n = 3$).
1937 p values for comparison of treated samples to the untreated sample were calculated by
1938 ordinary one-way ANOVA with Dunnett's multiple comparisons test. (G) Localisation of
1939 GFP-tagged insulin receptors in transiently transfected HEK 293 cells after 18 h treatment
1940 with 1 μ g/ml tunicamycin or 1 μ g/ml SubAB. Scale bar - 10 μ m. (H) Average Pearson's
1941 correlation coefficient r_{obs} between the GFP-tagged insulin receptor and CellMask Deep Red
1942 fluorescence determined from eleven randomly chosen cells. Bars represent standard errors (n
1943 = 10 for tunicamycin-treated samples and $n = 11$ for all other samples). p values for
1944 comparison of the treated to the untreated samples were calculated with an ordinary two-way
1945 ANOVA using Tukey's multiple comparisons test on arcsine-transformed data. The Pearson
1946 correlation coefficients for the randomised images are -0.13 ± 0.08 , -0.13 ± 0.07 , and $-0.33 \pm$
1947 0.07 for the untreated, tunicamycin-, and SubAB-treated cells and are significantly different
1948 ($p < 0.001$, calculated with a two-way ANOVA using Šidák's correction for multiple
1949 comparisons (Šidák, 1967) on arcsine-transformed data) from the corresponding Pearson
1950 correlation coefficients for the non-randomised images.

1951 **Figure 10.** Bypass of the ER in insulin receptor synthesis abrogates ER stress-induced insulin
 1952 resistance. (A) Schematic of the WT insulin receptor, the myristoylated F_V2E-insulin receptor
 1953 chimera, and activation of the chimera by AP20187. Black boxes represent the signal peptide
 1954 sequence and transmembrane domain of the insulin preproreceptor, striped boxes the protein
 1955 tyrosine kinase domain of the insulin receptor, and checkered boxes individual F_V domains.
 1956 Disulphide bonds that link α to β chains and two insulin receptor monomers are shown as
 1957 grey lines. Abbreviations: M, Myr – myristoylation signal. (B) Autophosphorylation of the
 1958 F_V2E-insulin receptor chimera in stably transfected Flp-In T-Rex 293 cells. Expression of the
 1959 chimera was induced for 27 h with 1 μ g/ml tetracycline, followed by dimerisation of the
 1960 chimera with 100 nM AP20187 for 1 or 4 h. (C) C₂C₁₂ cells were transiently transfected with
 1961 pmaxGFP or pcDNA5/FRT/TO-MyrF_V2E-INSR. 24 h after transfection, ER stress was
 1962 induced for 24 h with 0.1 μ M thapsigargin, 0.1 μ g/ml tunicamycin, or 1 μ g/ml SubAB
 1963 followed by dimerisation of the receptor chimera with 100 nM AP20187 for 4 h. ‘#’ indicates
 1964 an unspecific band. Quantification of (D) S473 AKT phosphorylation and (E) the relative
 1965 abundance of α - β proreceptors in panel C. Bars represent standard errors ($n = 3-4$). p values
 1966 for comparison of the treated to the untreated samples were calculated by ordinary one-way
 1967 ANOVA using Dunnett’s multiple comparisons test. (F) Induction of *TRB3* in C₂C₁₂ cells by
 1968 ER stress. C₂C₁₂ cells were treated with 300 nM thapsigargin, 1 μ g/ml tunicamycin, SubAB,
 1969 or SubA_{A272}B for 24 h. *TRB3* mRNA levels were determined by reverse transcriptase-qPCR
 1970 and standardised to the loading control *ACTB*. Bars represent standard errors ($n = 3$). p values
 1971 for comparison of treated samples to the untreated sample (“-“) were calculated by ordinary
 1972 two-way ANOVA with Dunnett’s multiple comparisons test taking data shown in Figures 1E
 1973 and 1F into account.

1974 **Figure 11.** JNKs are not required for insulin resistance in ER-stressed cells. (A) Activation of
 1975 JNK in Hep G2 cells exposed to different concentrations of thapsigargin, but not tunicamycin
 1976 or SubAB, for 18 – 36 h. The arrows indicate the p46 and p54 isoforms of JNKs, the three
 1977 lines phosphorylated species of p38, p42, and p44 MAP kinases. Bars represent standard
 1978 errors ($n = 3$). p values for comparison of treated samples to the untreated sample (‘-‘) were

1979 calculated by ordinary two-way ANOVA with Tukey's multiple comparisons test. (B) JNKi
1980 VIII and XVI inhibit phosphorylation of c-Jun at S63 in Hep G2 cells exposed to the
1981 indicated concentrations of thapsigargin for 36 h. Hep G2 cells were treated with 8 μ M JNKi
1982 VIII or XVI for 0.5 h before exposure to thapsigargin in the presence of 8 μ M JNKi VIII or
1983 XVI or no JNK inhibitor ('-') for 36 h. The bar graphs show S63 phosphorylation of c-Jun
1984 standardised to c-Jun levels, and c-Jun levels standardised the GAPDH levels. Bars represent
1985 standard errors ($n = 3$). p values were calculated by ordinary two-way ANOVA with Tukey's
1986 multiple comparisons test on square root or arc tangent-transformed data, respectively. (C-E)
1987 JNKi VIII and XVI do not reverse inhibition of insulin-stimulated phosphorylation of AKT at
1988 S473 by thapsigargin in Hep G2 cells. Hep G2 cells were treated for 0.5 h with 8 μ M JNKi
1989 VIII or XVI, followed by exposure to the indicated concentrations of thapsigargin in the
1990 presence of 8 μ M JNKi VIII or XVI or no JNK inhibitor ('-') for 36 h. Cells were serum-
1991 starved in the last 18 h of thapsigargin treatment and then stimulated with 10 nM or 100 nM
1992 insulin for 15 min in the continued presence of thapsigargin and JNK inhibitors, were
1993 applicable. (C) Western blots for pS473-AKT, AKT, and GAPDH. (D) Quantification of the
1994 Western blots in panel (C). Bars represent standard errors ($n = 3$). p values were calculated by
1995 ordinary two-way ANOVA with Dunnett's multiple comparisons test. (E) Reanalysis of the
1996 data in panel D after normalisation of data for each condition of JNK inhibition to the insulin-
1997 stimulated sample not exposed to thapsigargin for the corresponding condition. p values were
1998 calculated by ordinary two-way ANOVA with Dunnett's multiple comparisons test. (F) JNKi
1999 VIII and XVI do not restore levels of insulin receptor β chains or restore processing of α - β
2000 proreceptors to levels of untreated cells. Hep G2 cells were treated with 8 μ M JNKi VIII or
2001 XVI for 0.5 h before exposure to thapsigargin in the presence of 8 μ M JNKi VIII or XVI or
2002 no JNK inhibitor ('-') for 36 h. Bars represent standard errors ($n = 3$ for β chains and $n = 4$ for
2003 α - β proreceptors). p values were calculated by ordinary two-way ANOVA with Tukey's
2004 multiple comparisons test.

2005 **Figure 12.** JNK1 and JNK2 are not required for development of insulin resistance in ER-
2006 stressed cells. (A) Serum-starved WT and (B) *jnk1*^{-/-} *jnk2*^{-/-} MEFs were treated for 24 h with

2007 the indicated concentrations of thapsigargin, tunicamycin, 1 $\mu\text{g/ml}$ SubAB, or 1 $\mu\text{g/ml}$
2008 SubA_{A272}B before stimulation with 100 nM insulin for 15 min. (C) Quantification of
2009 phosphorylation of AKT on S473 relative to total AKT levels in WT and *jnk1*^{-/-} *jnk2*^{-/-} MEFs
2010 exposed to thapsigargin, tunicamycin, and 1 $\mu\text{g/ml}$ SubAB or SubA_{A272}B. Bars represent
2011 standard errors ($n = 12$ for unstressed, insulin-stimulated WT MEFs, $n = 10$ for unstressed,
2012 insulin-stimulated *jnk1*^{-/-} *jnk2*^{-/-} MEFs, $n = 6$ for all other samples for WT MEFs, and $n = 5$ for
2013 all other samples for *jnk1*^{-/-} *jnk2*^{-/-} MEFs). p values for comparison of the relative AKT S473
2014 phosphorylation between WT and *jnk1*^{-/-} *jnk2*^{-/-} MEFs were calculated using ordinary two-way
2015 ANOVA with Šidák's correction for multiple comparisons. p values for comparison of ER-
2016 stressed samples and samples not stimulated with 100 nM insulin to samples stimulated with
2017 100 nM insulin were calculated using ordinary two-way ANOVA with Dunnett's multiple
2018 comparisons test. (D) Activation of JNK in serum-starved WT MEFs exposed to the indicated
2019 concentrations of thapsigargin or tunicamycin, 1 $\mu\text{g/ml}$ SubAB, or SubA_{A272}B for 24 h before
2020 stimulation with 100 nM insulin for 15 min. (E) Quantification of the Western blots in panel
2021 D. Bars represent standard errors ($n = 8$ for insulin-stimulated cells, $n = 4$ for all other
2022 samples). p values for comparison of ER-stressed samples and samples not stimulated with
2023 100 nM insulin to samples stimulated with 100 nM insulin were calculated using ordinary
2024 one-way ANOVA with Dunnett's multiple comparisons test. (F) JNK deficiency does not
2025 protect from the effects of ER stress on insulin receptor processing in the secretory pathway.
2026 The relative abundance of α - β proreceptors was determined by Western blotting of lysates of
2027 serum-starved WT and *jnk1*^{-/-} *jnk2*^{-/-} MEFs exposed for 24 h to the indicated concentrations of
2028 thapsigargin, tunicamycin, and 1 $\mu\text{g/ml}$ SubAB or SubA_{A272}B followed by stimulation with
2029 100 nM insulin for 15 min where indicated. Bars represent standard errors ($n = 4$ for
2030 unstressed, insulin-stimulated cells, $n = 2$ for all other samples). p values for comparison of
2031 the relative abundance of α - β proreceptors between WT and *jnk1*^{-/-} *jnk2*^{-/-} MEFs were
2032 calculated by Welch's test (Welch, 1947) followed by a Games-Howell *post hoc* test (Games
2033 and Howell, 1976). p values for comparisons of treatments were calculated by ordinary two-
2034 way ANOVA with Dunnett's multiple comparisons test.

2035 **Figure 13.** TRB3 is not required for development of insulin resistance in ER-stressed C₂C₁₂
2036 cells. (A) siRNA-mediated knock-down of *TRB3* at the mRNA level 48 h after transfection of
2037 C₂C₁₂ cells with 50 nM of the indicated siRNAs. *TRB3* mRNA was determined by reverse
2038 transcriptase-qPCR and normalised to *ACTB*. Bars represent the standard error from three
2039 technical replicates. *p* values were calculated by ordinary one-way ANOVA with Dunnett's
2040 multiple comparisons test. (B) siRNA-mediated knock-down of TRB3 at the protein level 48
2041 and 72 h after transfection of C₂C₁₂ cells with 50 nM of the indicated siRNAs. (C) 24 h after
2042 transfection with the indicated siRNAs C₂C₁₂ cells were exposed to 300 μM thapsigargin, 1
2043 μg/ml tunicamycin or SubAB for 24 h and serum-starved during the last 18 h of exposure to
2044 ER stressors before being stimulated with 100 nM insulin for 15 min. Phosphorylation of
2045 AKT at S473 and insulin receptors were analysed by Western blotting. (D) Quantification of
2046 the relative phosphorylation of AKT at S473 in the Western blots of panel C. Bars represent
2047 standard errors (*n* = 5 for cells transfected with siRNAs against eGFP, *n* = 3 for all other
2048 samples). *p* values were calculated by ordinary two-way ANOVA with Dunnett's correction
2049 for multiple comparisons. (E) Quantification of the relative abundance of α-β proreceptors in
2050 the Western blots of panel C. Bars represent standard errors (*n* = 6 for cells transfected with
2051 siRNAs against eGFP, *n* = 3 for all other samples). *p* values were calculated by ordinary two-
2052 way ANOVA with Dunnett's multiple comparisons test to compare of samples to the insulin-
2053 stimulated, unstressed sample and Tukey's multiple comparisons test to compare different
2054 siRNAs.

2055 **Figure 14.** ER stress decreases insulin sensitivity by decreasing the plasma membrane
2056 population of the insulin receptor. (A) Inhibition of insulin signalling by ER stress requires
2057 transport of newly synthesised insulin receptors from the ER to the cell surface. The signal
2058 peptide sequence targets ribosomes translating the insulin receptor mRNA to the ER, where
2059 the newly synthesised polypeptide chain folds into molecules with insulin binding activity.
2060 ER stress interferes with folding of newly synthesised insulin receptor molecules, preventing
2061 its transport to the Golgi complex. The Myr-F_v2E-insulin receptor chimera is not affected by
2062 ER stress because it is translated by cytoplasmic ribosomes and folds in the cytosol into active

2063 molecules thus bypassing the ER. Abbreviation: TGN – *trans*-Golgi network. (B and C)
2064 Modelling of the response to insulin as a function of insulin and insulin receptor
2065 concentration. The response to insulin, R , is defined by the equation $R = R_{\max} \cdot [\text{INS} \cdot \text{INSR}] / (K_E$
2066 $+ [\text{INS} \cdot \text{INSR}])$, with K_E – concentration of insulin (INS)-insulin receptor (INSR) complexes
2067 at which $R = 0.5 \cdot R_{\max}$, and R_{\max} – maximal response to insulin. The concentration of insulin-
2068 insulin receptor complexes is calculated from the equilibrium $\text{INS} + \text{INSR} \leftrightarrow \text{INS} \cdot \text{INSR}$
2069 considering only high affinity binding of insulin to the insulin receptor with a dissociation
2070 constant of 200 pM (Bass et al., 1996). K_E varies widely for different physiological responses
2071 to insulin (Gammeltoft and Gliemann, 1973; Hofmann et al., 1980; Crettaz and Kahn, 1984)
2072 and has been assumed to be equal to 1 pM for illustrative purposes only. Numbers represent
2073 the half-lives of the insulin receptor at the cell surface that have elapsed since ER stress was
2074 induced.

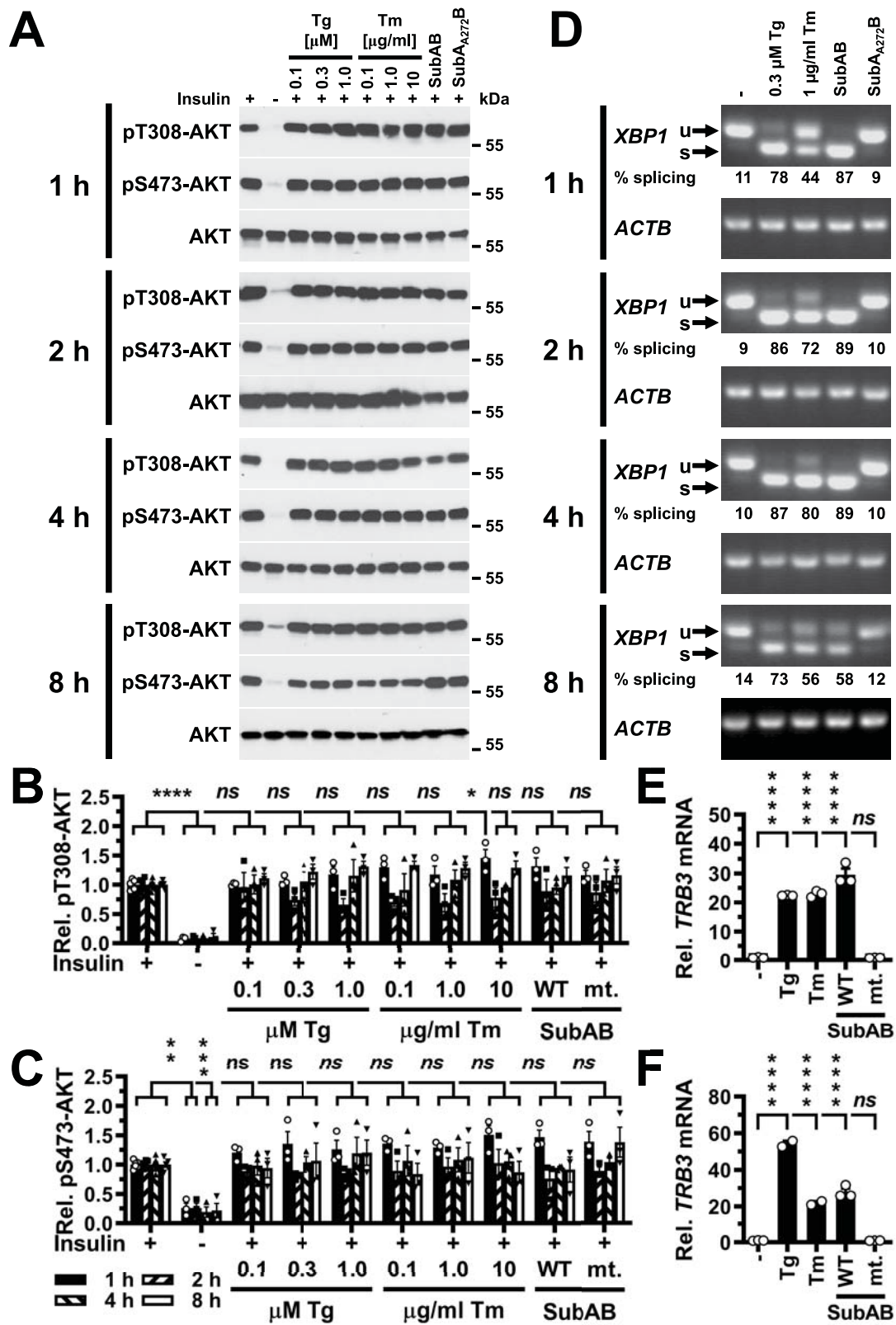


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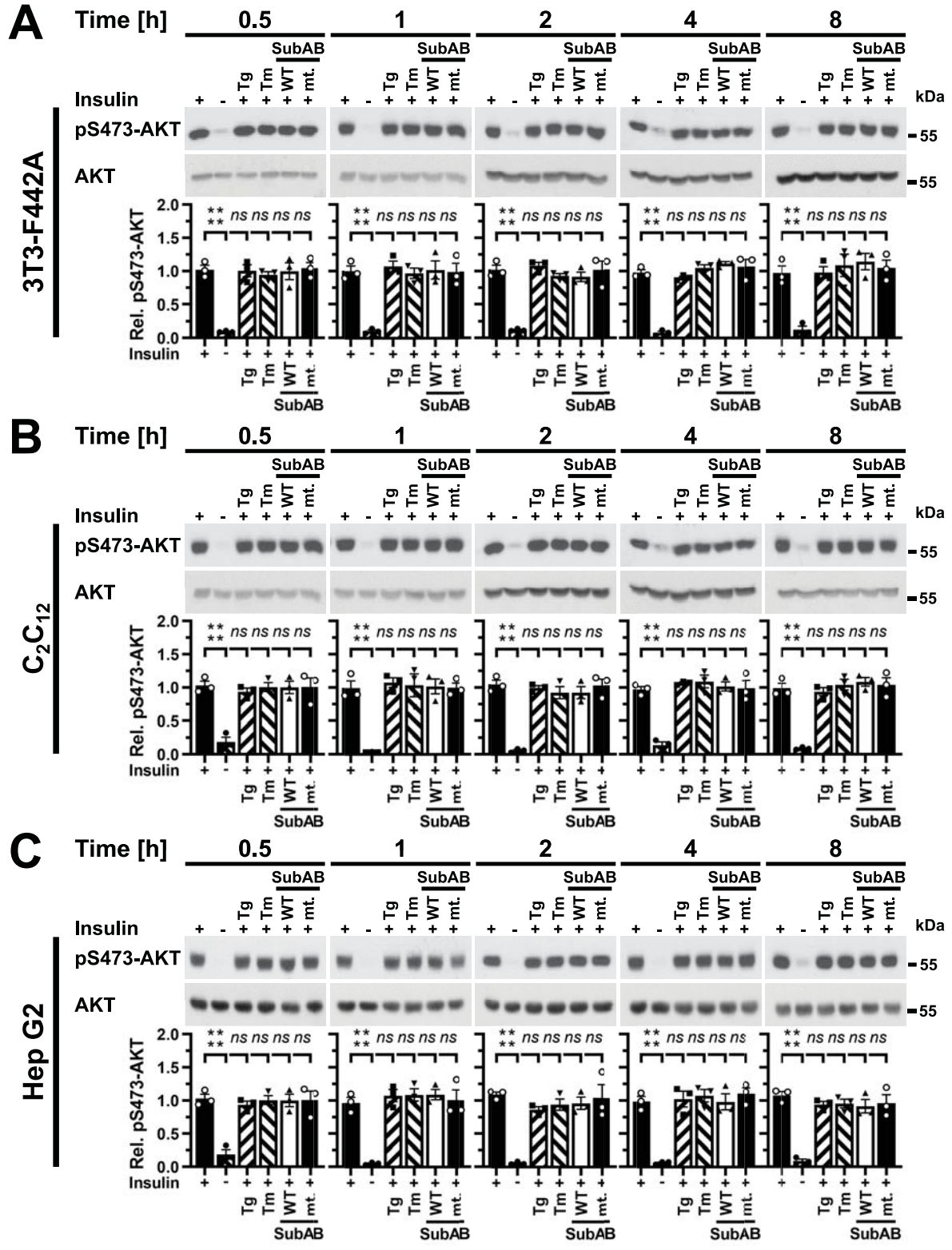


Figure 2

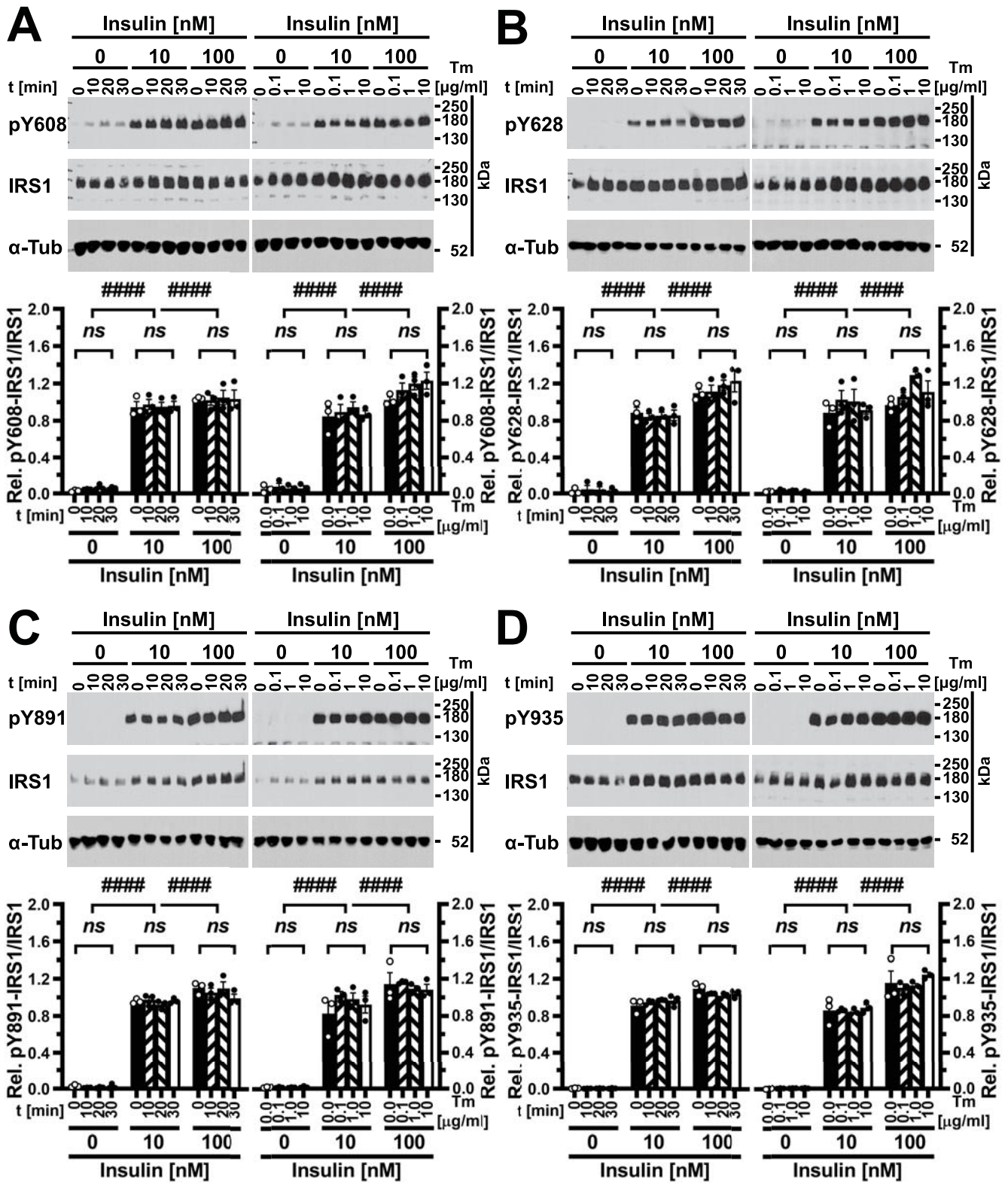


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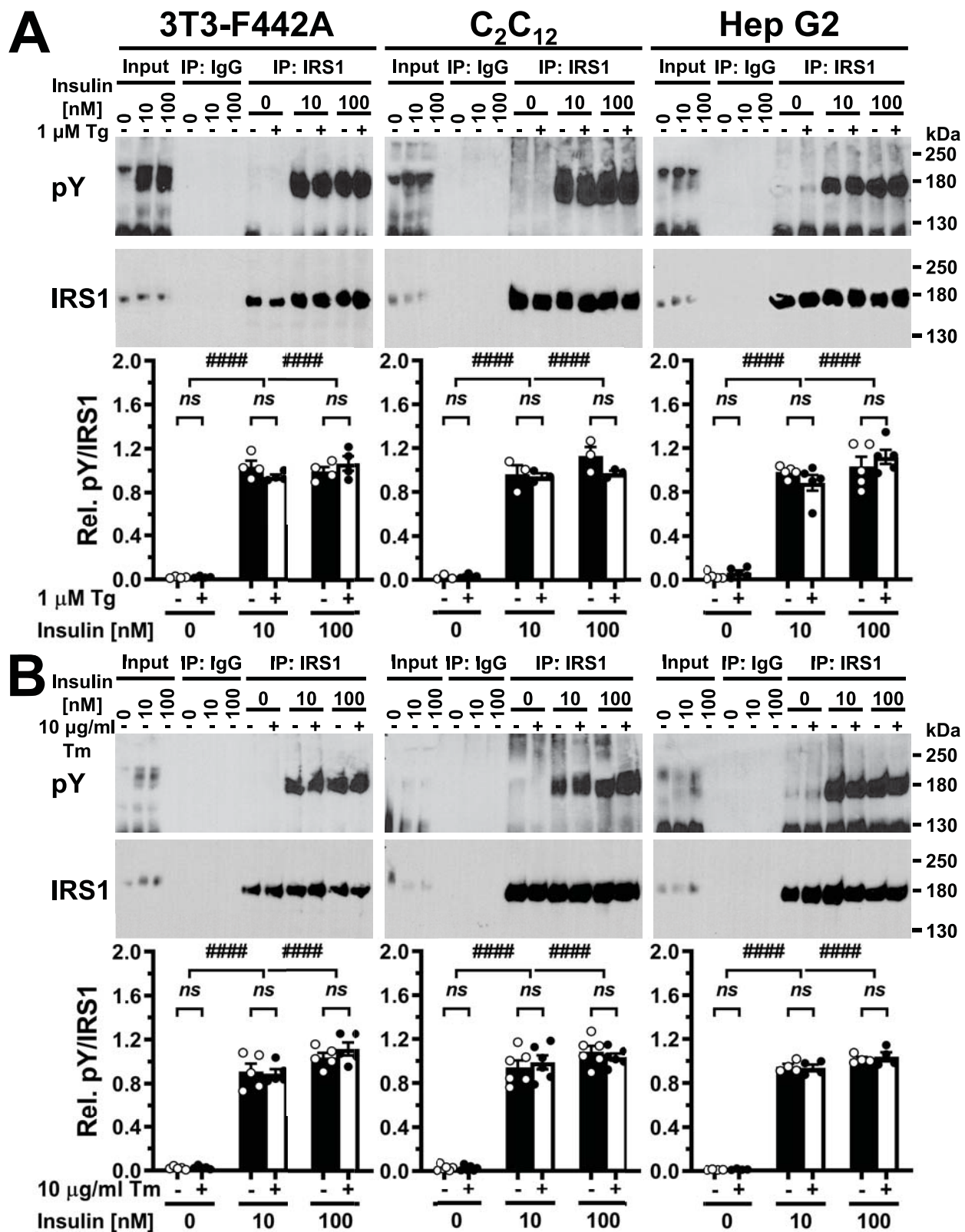


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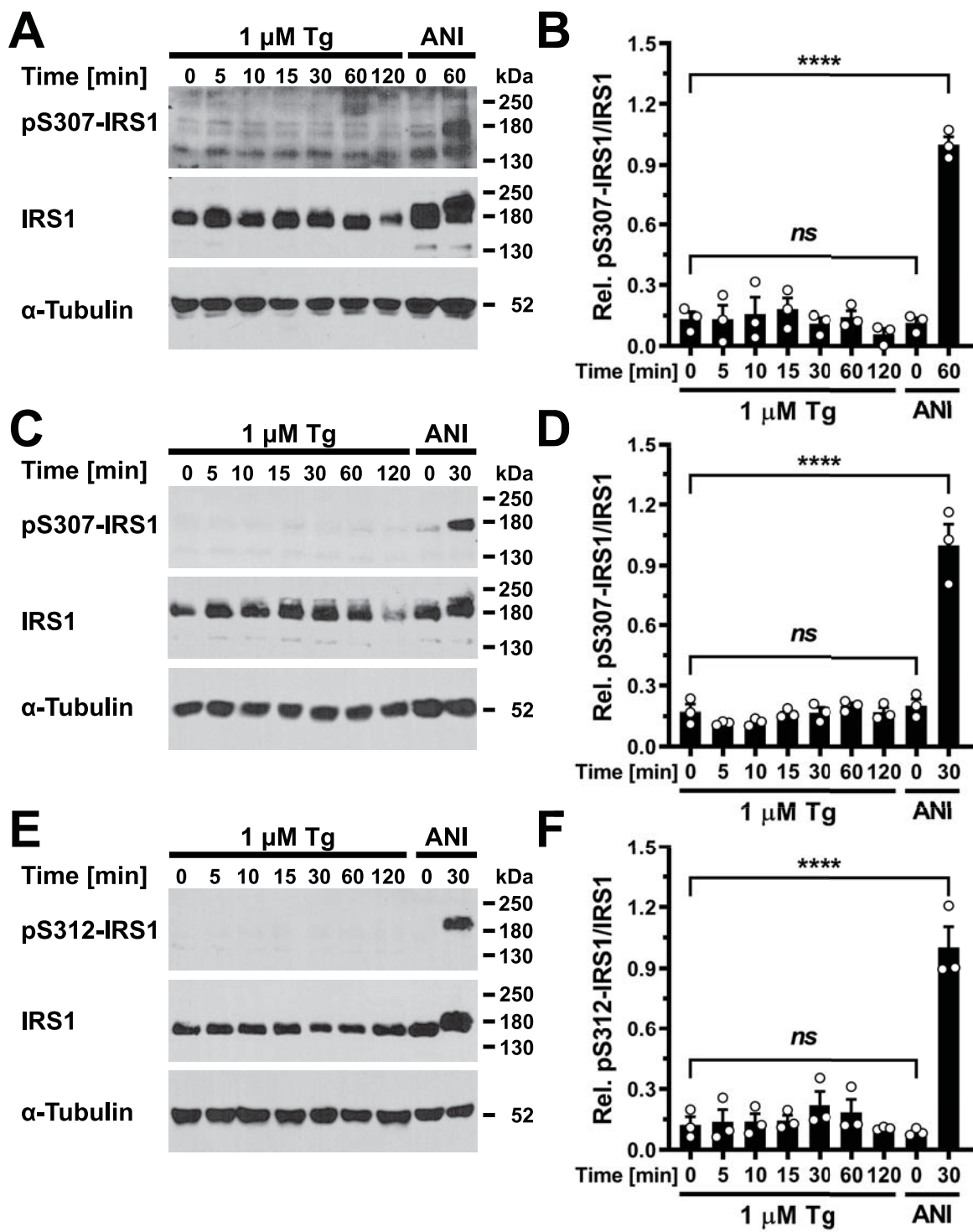


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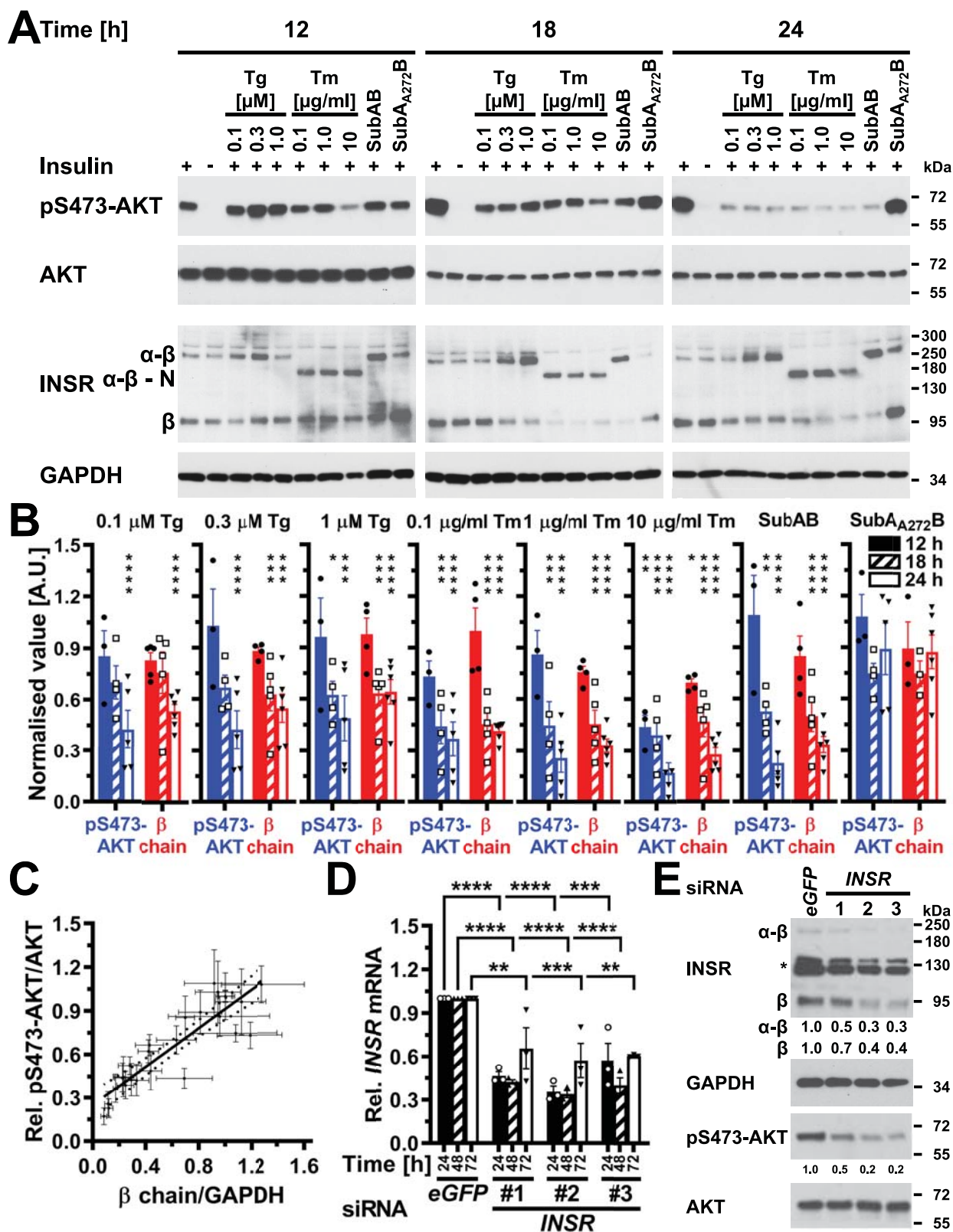


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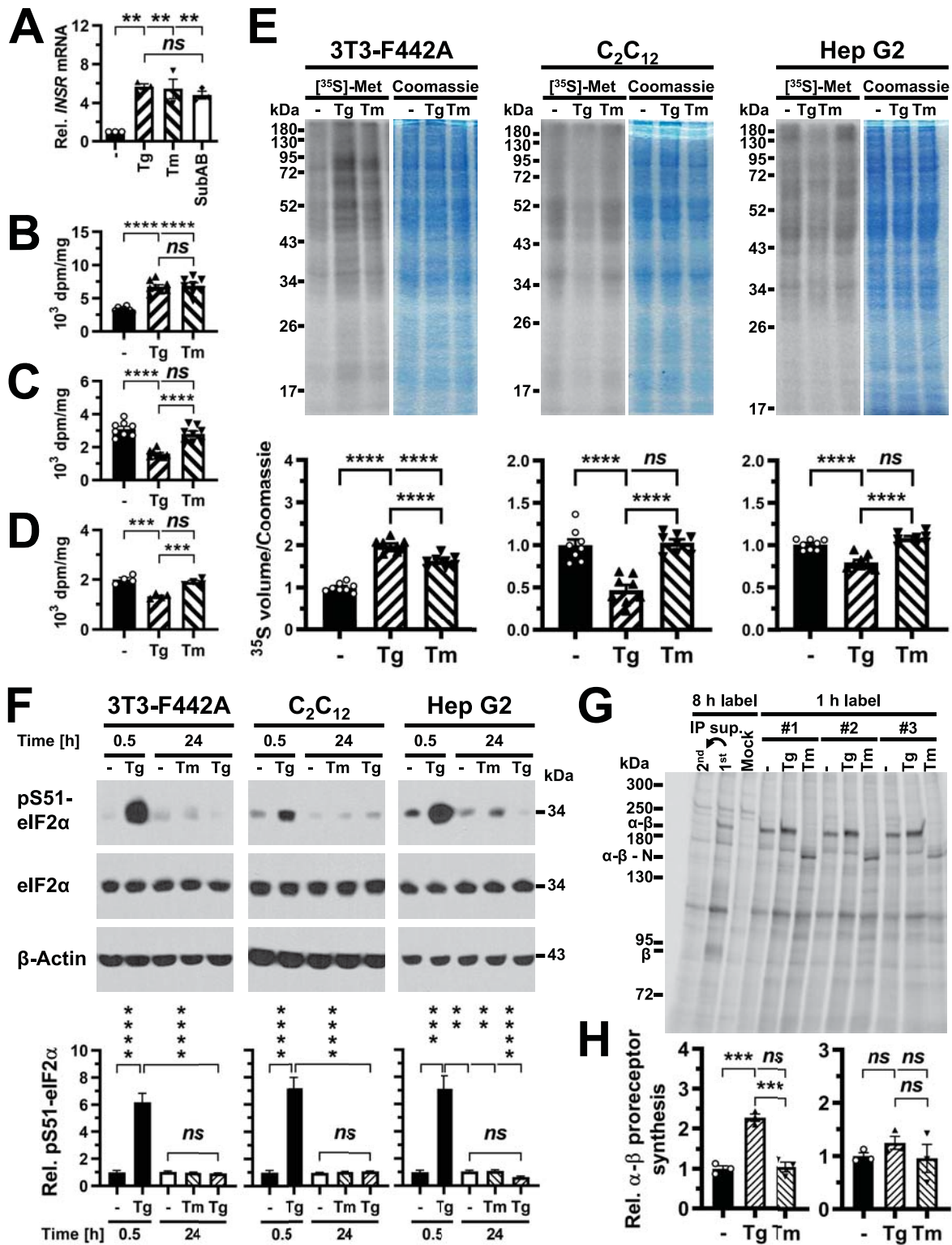


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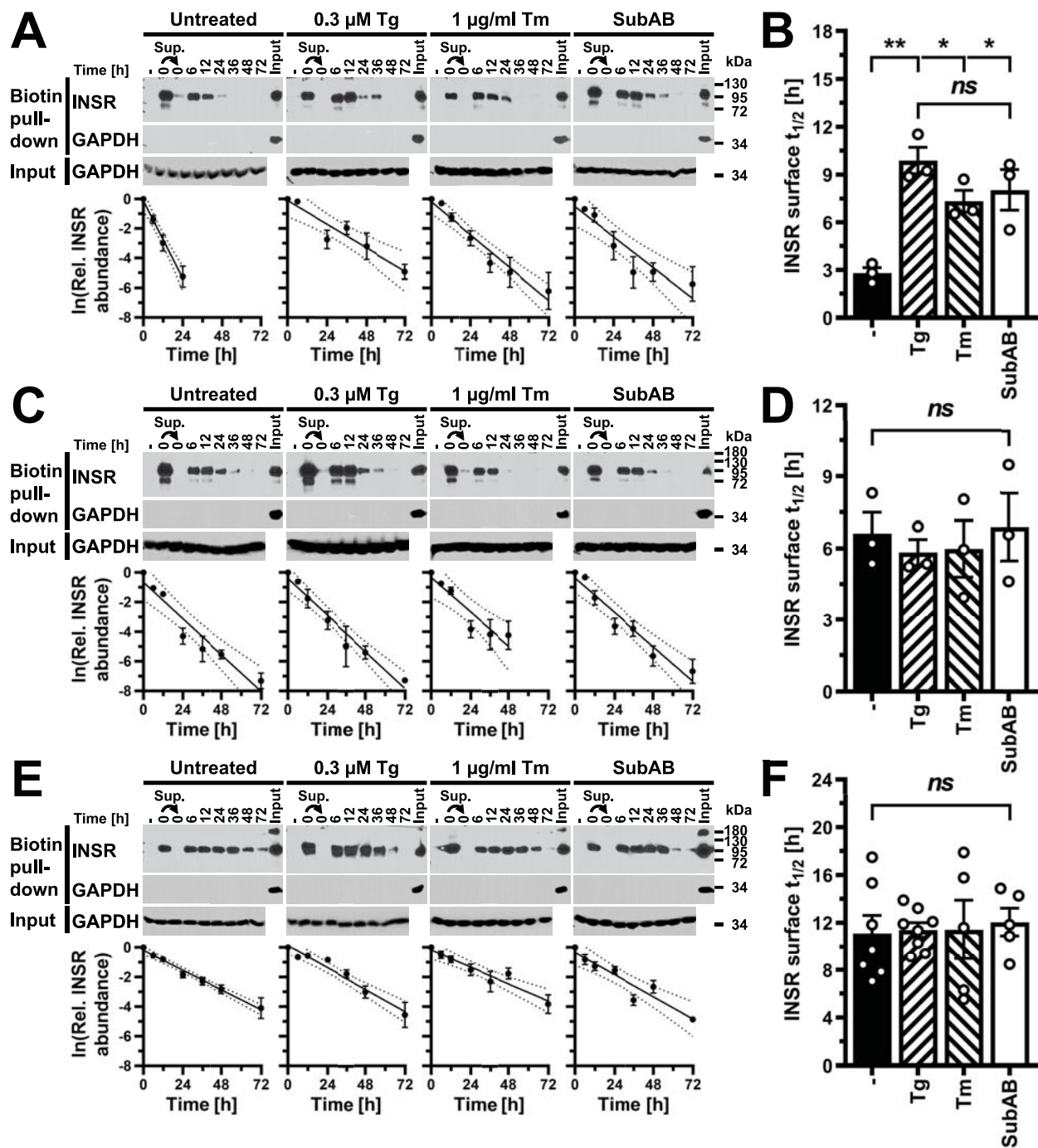


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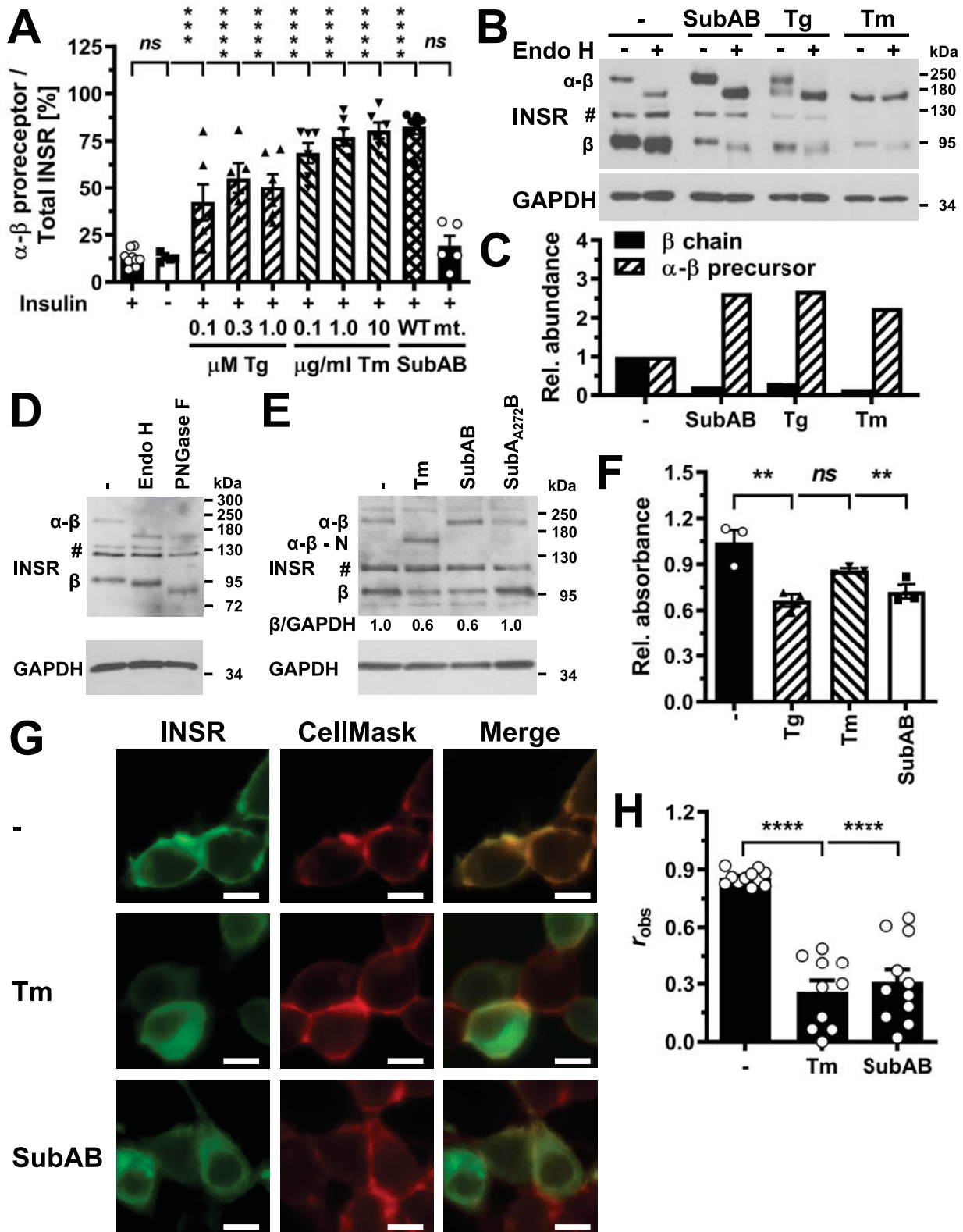


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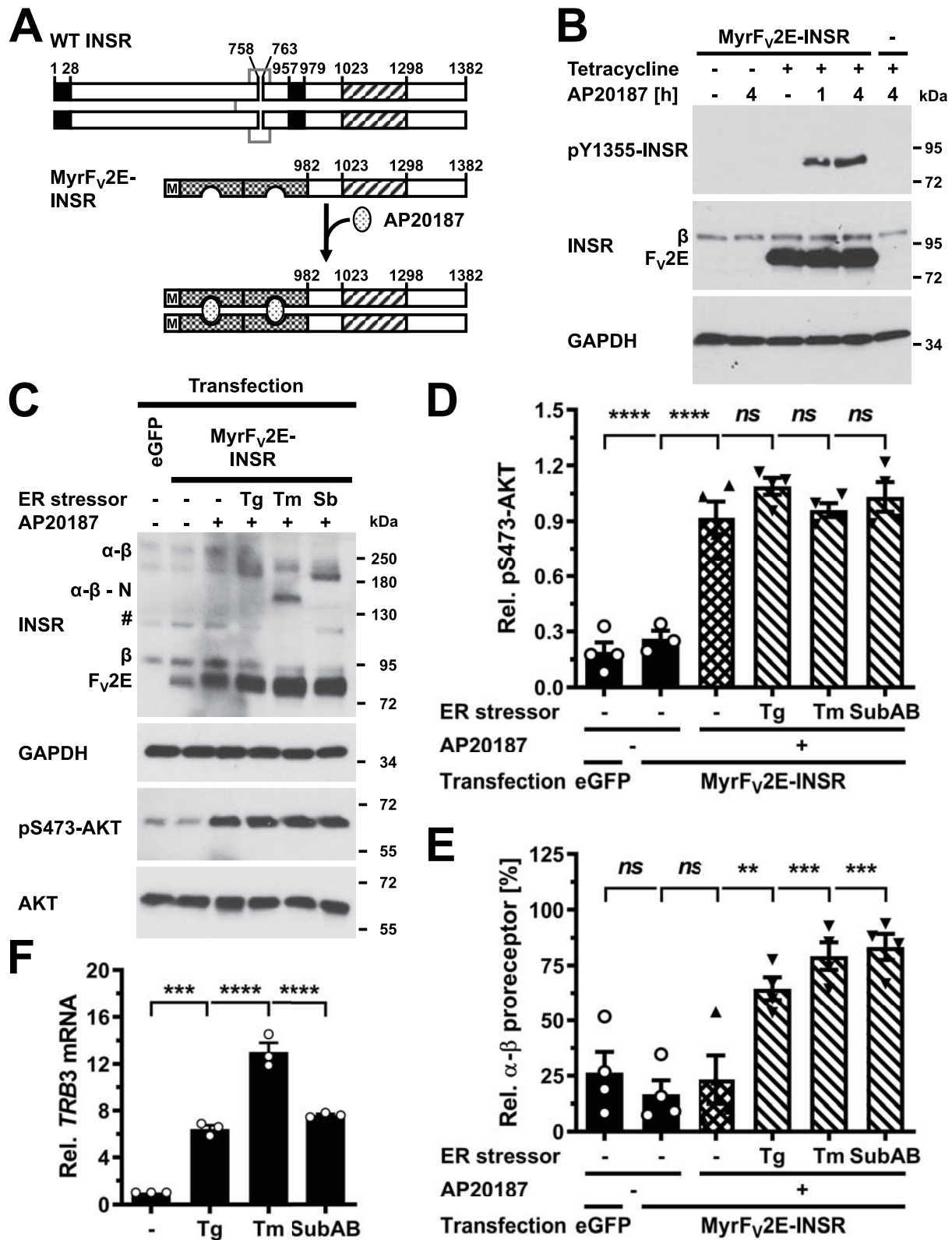


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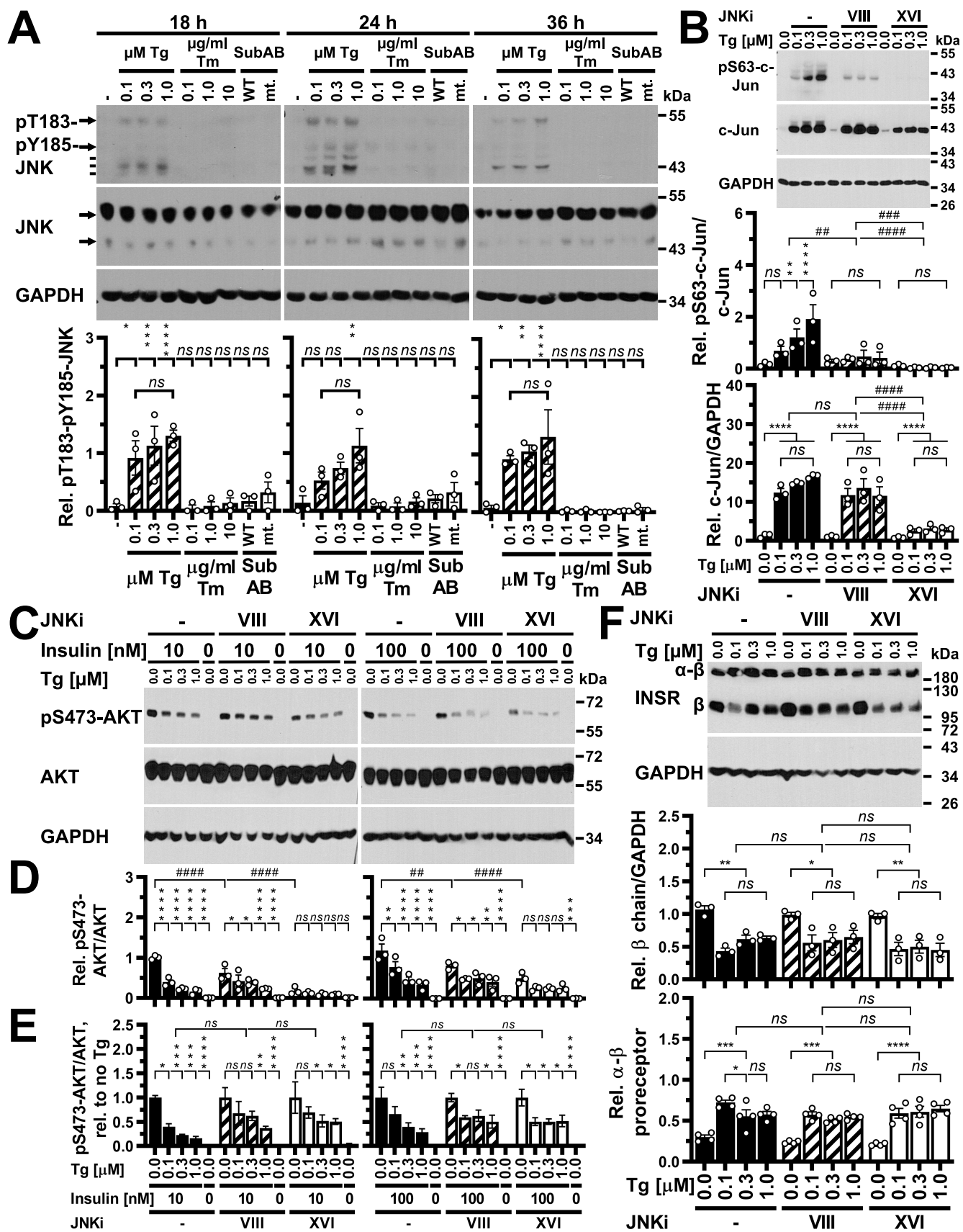


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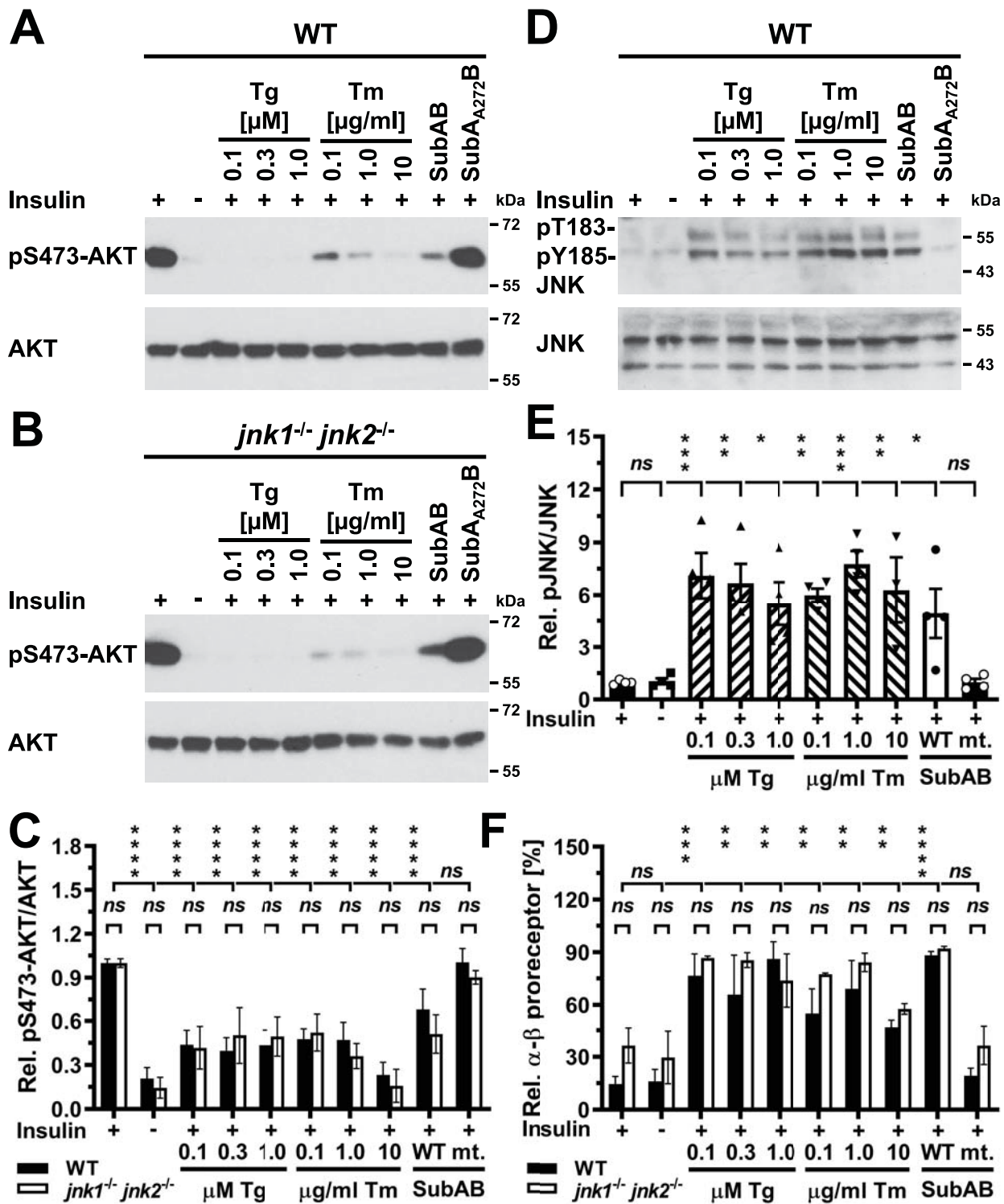


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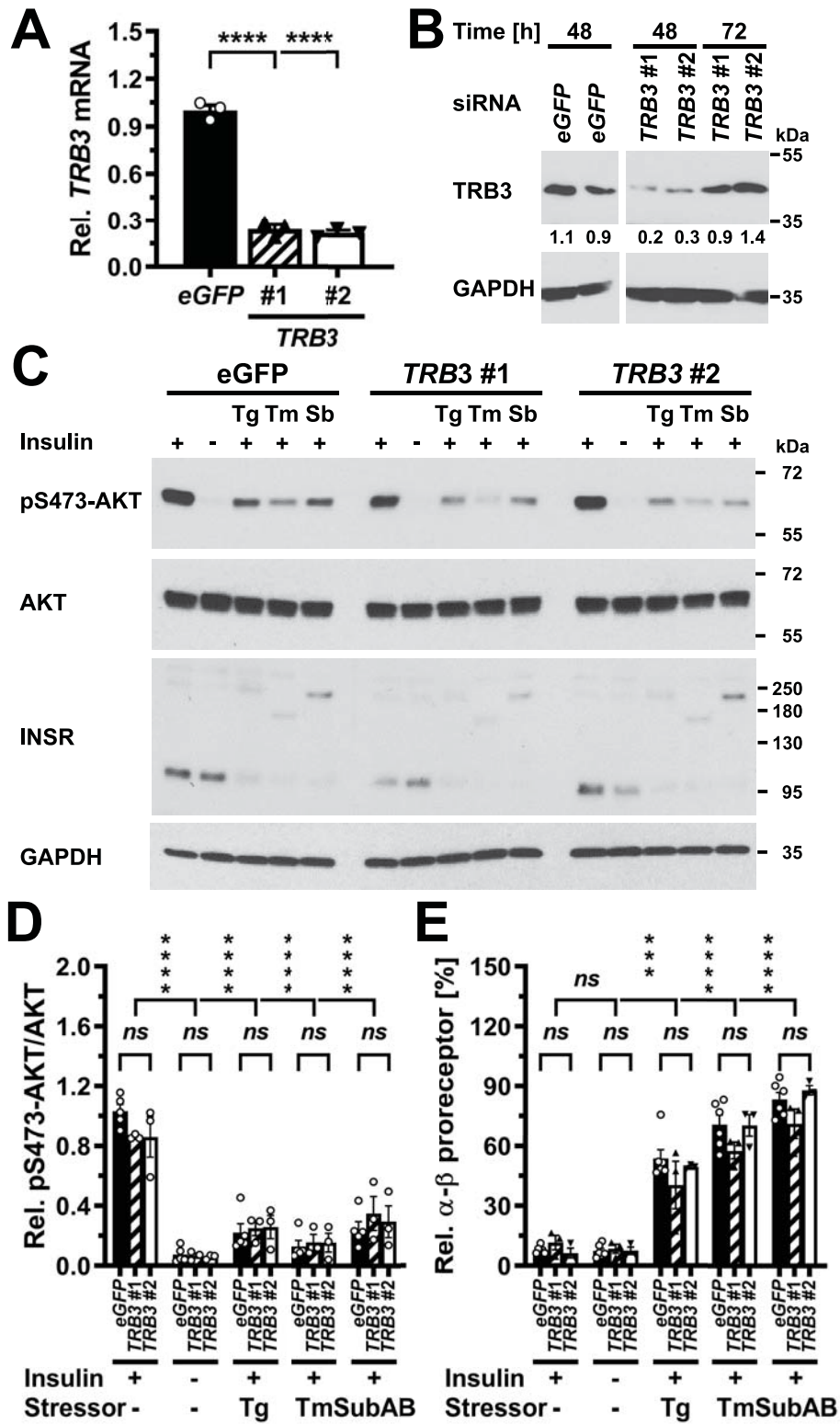


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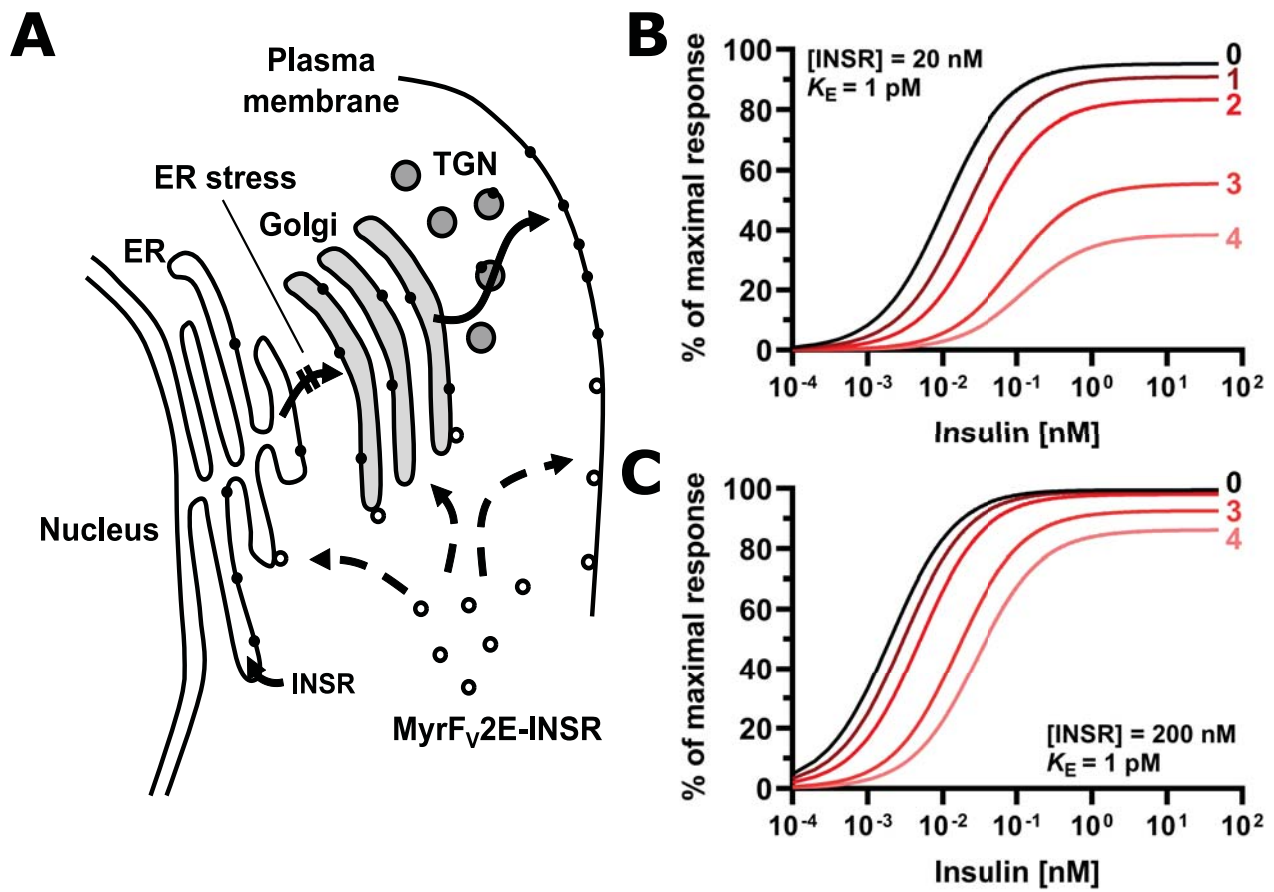


Figure 14