

1 **An initial phase of JNK activation inhibits cell death early in the endoplasmic**
 2 **reticulum stress response**

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22 **Running Title:** JNK signalling in the early UPR

23 **Key words:** Apoptosis, endoplasmic reticulum, IRE1, JNK, stress response, unfolded
 24 protein response

25 **Abbreviations:** ER – endoplasmic reticulum, JC-1 - 5,5',6,6'-tetrachloro-1,1',3,3'-
 26 tetraethylbenzimidazolylcarbocyanine iodide, MEF – mouse embryonic fibroblast,
 27 qPCR – quantitative PCR, RT – reverse transcriptase, UPR – unfolded protein
 28 response

29 **Summary statement**

30 Activation of JNK by endoplasmic reticulum stress kinetically precedes activation of
31 XBP1 by IRE1 α . JNK-dependent induction of several inhibitors of apoptosis inhibits
32 apoptosis early in the endoplasmic reticulum stress response.

33 **Abstract**

34 Accumulation of unfolded proteins in the endoplasmic reticulum (ER) activates the
35 unfolded protein response (UPR). In mammalian cells, UPR signals generated by
36 several ER membrane resident proteins, including the bifunctional protein kinase
37 endoribonuclease IRE1 α , control cell survival and the decision to execute apoptosis.
38 Processing of *XBP1* mRNA by the RNase domain of IRE1 α promotes survival of ER
39 stress, while activation of the mitogen-activated protein kinase JNK by IRE1 α late in
40 the ER stress response promotes apoptosis. Here we show that activation of JNK in
41 the ER stress response precedes activation of XBP1. This activation of JNK is
42 dependent on IRE1 α and TRAF2 and coincides with JNK-dependent induction of
43 expression of several antiapoptotic genes, including *cIAP1*, *cIAP2*, *XIAP*, and *BIRC6*.
44 ER-stressed *jnk1*^{-/-} *jnk2*^{-/-} mouse embryonic fibroblasts (MEFs) display more
45 pronounced mitochondrial permeability transition and increased caspase 3/7 activity
46 compared to wild type MEFs. Caspase 3/7 activity is also elevated in ER-stressed
47 *ciap1*^{-/-} *ciap2*^{-/-}, and *xiap*^{-/-} MEFs. These observations suggest that JNK-dependent
48 transcriptional induction of several inhibitors of apoptosis contributes to inhibiting
49 apoptosis early in the ER stress response.

50 **Introduction**

51 Perturbation of protein folding homeostasis in the endoplasmic reticulum (ER)
52 activates several signal transduction pathways collectively called the unfolded protein
53 response (UPR) (Ron and Walter, 2007; Walter and Ron, 2011). In mammalian cells,
54 the UPR is initiated by several ER membrane resident proteins, including the protein
55 kinase-endoribonuclease (RNase) IRE1 α (Tirasophon et al., 1998; Wang et al., 1998),
56 the protein kinase PERK (Harding et al., 1999; Shi et al., 1999; Shi et al., 1998), and
57 several type II transmembrane transcription factors such as ATF6 α (Yoshida et al.,
58 2000) and CREB-H (Zhang et al., 2006). All of these signalling molecules activate
59 prosurvival, but also proapoptotic responses to ER stress.

60 These opposing signalling outputs are exemplified by IRE1 α . The RNase activity
61 of IRE1 α initiates non-spliceosomal splicing of the mRNA for the transcription factor

62 XBP1 (Calfon et al., 2002; Lee et al., 2002; Shen et al., 2001; Yoshida et al., 2001),
63 which in turn induces transcription of genes encoding ER-resident molecular
64 chaperones (Lee et al., 2003), components of the ER-associated protein degradation
65 machinery (Oda et al., 2006; Yoshida et al., 2003), and several phospholipid
66 biosynthetic genes (Lee et al., 2003; Lee et al., 2008) to promote cell survival. The
67 IRE1 α RNase activity also initiates the decay of several mRNAs encoding proteins
68 targeted to the ER (Gaddam et al., 2013; Han et al., 2009; Hollien et al., 2009; Hollien
69 and Weissman, 2006), which decreases the protein folding load of the stressed ER.
70 Degradation of *DR5* mRNA by IRE1 α contributes to establishment of a time window
71 for adaptation to ER stress (Lu et al., 2014). On the other hand, IRE1 α promotes
72 apoptosis via both its RNase and protein kinase domains. Cleavage of several
73 miRNAs, including miRNA-17, -34a, -96, and -125b, by the RNase domain of IRE1 α
74 stabilises and promotes translation of *TXNIP* and *caspase-2* mRNAs (Lerner et al.,
75 2012; Osowski et al., 2012; Upton et al., 2012). *TXNIP* promotes apoptosis through
76 activation of caspase-1 and secretion of interleukin 1 β (Lerner et al., 2012). The role
77 of caspase-2 in ER stress-induced apoptosis has recently been questioned (Lu et al.,
78 2014; Sandow et al., 2014). The kinase domain of IRE1 α activates the mitogen-
79 activated protein (MAP) kinase JNK through formation of a complex with the E3
80 ubiquitin ligase TRAF2 and the MAP kinase kinase kinase (MAPKKK) ASK1
81 (Nishitoh et al., 2002; Urano et al., 2000). Sequestration of TRAF2 by IRE1 α may
82 also contribute to activation of caspase-12 in murine cells (Yoneda et al., 2001).
83 Pharmacologic (Chen et al., 2008; Huang et al., 2014; Jung et al., 2014; Jung et al.,
84 2012; Smith and Deshmukh, 2007; Teodoro et al., 2012; Wang et al., 2009; Zhang et
85 al., 2001) and genetic (Arshad et al., 2013; Kang et al., 2012) studies have provided
86 evidence that activation of JNK 12 h or later after induction of ER stress is
87 proapoptotic.

88 Much less is known about the role of JNK at earlier time points in the ER stress
89 response. In tumour necrosis factor (TNF)- α -treated cells two phases of JNK
90 activation can be distinguished (Lamb et al., 2003; Roulston et al., 1998), an early and
91 transient antiapoptotic and a later phase, that coincides with activation of caspases
92 (Roulston et al., 1998). In the early phase JNK induces expression of JunD and the
93 antiapoptotic ubiquitin ligase cIAP2/BIRC3 (Lamb et al., 2003). Furthermore,
94 phosphorylation of Bad at T201 and subsequent inhibition of interaction of Bad with

95 Bcl-x_L underlies the antiapoptotic role of JNK in interleukin (IL)-3-dependent
96 hematopoietic cells (Yu et al., 2004), while JNK mediates IL-2-dependent survival of
97 T cells through phosphorylation of MCL1 (Hirata et al., 2013). This functional
98 dichotomy of transient and persistent JNK signalling prompted us to investigate
99 whether an initial phase of JNK activation exists in the ER stress response and to
100 characterise the functional significance of such an initial phase of JNK activation in
101 ER-stressed cells.

102 **Results**

103 *ER stress activates JNK before XBP1 splicing reaches maximal levels*

104 To investigate how early JNK is activated in the ER stress response we characterised
105 JNK activation over an 8 h time course by monitoring phosphorylation of JNK in its
106 T-loop on T183 and Y185 by Western blotting with antibodies against phosphorylated
107 and total JNK. In mouse embryonic fibroblasts (MEFs), phosphorylation of JNK in its
108 T-loop increased as early as 10 min after addition of 1 μ M thapsigargin (Fig. 1A,C) or
109 10 μ g/ml tunicamycin (Fig. 1D,F). JNK phosphorylation returned to near basal levels
110 8 h after addition of thapsigargin or tunicamycin to cells. The ability of these two
111 mechanistically different ER stressors to elicit rapid phosphorylation of JNK, which
112 over several hours declines to near basal levels, suggests that this initial phase of JNK
113 activation is caused by ER stress invoked by these two chemicals and not a response
114 to secondary effects of these compounds. To compare the kinetics of JNK activation
115 to the kinetics of the *XBP1* splicing reaction and phosphorylation of the PERK
116 substrate eIF2 α we monitored *XBP1* splicing by using reverse transcriptase (RT)-PCR
117 and phosphorylation of eIF2 α on S51 by Western blotting. Spliced *XBP1* mRNA
118 differs from unspliced *XBP1* mRNA by lacking a 26 nt intron. Hence, the presence of
119 a shorter RT-PCR product on agarose gels is indicative of activation of the IRE1 α
120 RNase activity and processing of *XBP1* mRNA. In thapsigargin-treated MEFs ~45%
121 of *XBP1* mRNA were spliced 20 min after addition of thapsigargin (Fig. 1B,C). *XBP1*
122 splicing reached maximal levels only after several hours of thapsigargin treatment,
123 suggesting that activation of JNK precedes maximal activation of *XBP1*.
124 Phosphorylation of eIF2 α was observed within 10 min after induction of ER stress
125 with 1 μ M thapsigargin, which indicates that both eIF2 α and JNK are phosphorylated
126 before significant levels of *XBP1* mRNA are spliced (Fig. 1B,C). When ER stress was
127 induced with 10 μ g/ml tunicamycin, phosphorylation of JNK and eIF2 α also preceded

128 splicing of *XBPI* (Fig. 1D-F). Furthermore, *XBPI* splicing reached maximal levels
129 only after JNK phosphorylation returned to near basal levels in tunicamycin-treated
130 MEFs. In both thapsigargin- and tunicamycin-treated MEFs phosphorylation of eIF2 α
131 declined towards the end of the time course, which is consistent with the transient
132 nature of the translational arrest mediated by eIF2 α S51 phosphorylation (Kojima et
133 al., 2003; Novoa et al., 2003).

134 To investigate whether a similar kinetic relationship between phosphorylation of
135 JNK and eIF2 α and *XBPI* splicing exists in other cell types, we repeated these
136 experiments with Hep G2 hepatoma cells, 3T3-F442A adipocytes, and C₂C₁₂
137 myotubes. In Hep G2 cells, JNK phosphorylation increased 30 min after addition of 1
138 μ M thapsigargin to the cells and then declined to near resting levels after ~120 min of
139 thapsigargin exposure (Fig. S1A,C). By contrast, 30 min after addition of thapsigargin
140 only ~7% of *XBPI* mRNA were spliced, and after another 15 min *XBPI* splicing was
141 approximately half maximal (Fig. S1B,C). *XBPI* splicing reached maximal levels
142 only after 6 h of thapsigargin treatment. In 3T3-F442A adipocytes phosphorylation of
143 JNK reached a maximum as early as 10 min after application of 1 μ M thapsigargin,
144 then returned to basal levels before increasing again towards the end of the time
145 course (Fig. S1D,F). *XBPI* splicing, however, was not detectable until 45 min after
146 addition of thapsigargin, required 4 h to reach maximal levels, and remained at this
147 level for at least another 4 h (Fig. S1E,F). Thus, activation of JNK also precedes
148 activation of *XBPI* in Hep G2 cells and 3T3-F442A adipocytes and also returns to
149 near basal levels of JNK activity after several hours of ER stress. We made the same
150 observations in C₂C₁₂ myotubes. In these cells an increase in JNK phosphorylation
151 was detected as early as 10 min after induction of ER stress with 1 μ M thapsigargin
152 (Fig. S1G,H,J), while the earliest time point at which an increase in *XBPI* splicing
153 was detected was 20 min (Fig. S1I,J). At the same time, activation of JNK diminished
154 over time in C₂C₁₂ myotubes, while the level of *XBPI* splicing remained at maximal
155 levels (Fig. S1H-J). In all three cell lines, phosphorylation of both eIF2 α and JNK
156 preceded splicing of *XBPI* (Fig. S1). We conclude that activation of JNK preceding
157 induction of *XBPI* splicing and leading to an initial phase of JNK activity are
158 phenomena that can be observed in several ER-stressed murine and human cell types.
159 *The initial phase of JNK activation in ER-stressed cells requires IRE1 α and TRAF2*

160 Several different stresses activate JNK (Kyriakis et al., 1994). To examine if the rapid
161 JNK activation seen upon thapsigargin or tunicamycin treatment is in response to ER
162 stress and thus mediated via IRE1 α and TRAF2, we characterised whether this rapid
163 JNK activation is IRE1 α - and TRAF2-dependent. Activation of JNK in the first ~60
164 min after induction of ER stress with 1 μ M thapsigargin was decreased in *ire1 α ^{-/-}* and
165 *traf2^{-/-}* MEFs compared to WT MEFs and did no longer reach statistical significance
166 (Figs 1, 2). In both *ire1 α ^{-/-}* and *traf2^{-/-}* MEFs JNK activation was delayed and reached
167 maximal levels only towards the end of the 8 h time course (Fig. 2). This delayed
168 activation of JNK may be explained by stresses other than and possibly secondary to
169 ER stress, for example oxidative stress (Mauro et al., 2006). Before the onset of the
170 delayed phosphorylation of JNK in *ire1 α ^{-/-}* and *traf2^{-/-}* MEFs, phosphorylation of JNK
171 was higher in WT MEFs than in the *ire1 α ^{-/-}* or *traf2^{-/-}* MEFs (Fig. 2G), suggesting that
172 the early JNK activation in ER-stressed cells requires both IRE1 α and TRAF2.

173 To establish if the initial phase of JNK activation is IRE1 α - and TRAF2-
174 dependent in cells other than MEFs we characterised whether small interfering (si)-
175 RNA-mediated knockdown of IRE1 α or TRAF2 reduces JNK activation by ER stress.
176 Two *IRE1 α* siRNAs (#2 and #3, Table S1) reduced *IRE1 α* mRNA levels to ~40% of
177 control eGFP siRNA transfected cells 72 h post-transfection (Fig. S2A) and decreased
178 activation of JNK to 60 \pm 17% and 30 \pm 9% of eGFP siRNA-transfected cells,
179 respectively (Fig. S2B,C). Likewise, two siRNAs against human or murine TRAF2
180 blunted the ER stress-dependent JNK activation in Hep G2 cells, 3T3-F442A
181 fibroblasts, and C₂C₁₂ myoblasts (Figs S2D-F, S3). Furthermore, a dominant negative
182 mutant of TRAF2, TRAF2 Δ 1-86 (Hsu et al., 1996; Reinhard et al., 1997), which lacks
183 the RING domain (Fig. S4A) inhibited TNF- α -induced JNK activation (Fig. S4B) and
184 blunted the initial phase of JNK activation upon induction of ER stress with 1 μ M
185 thapsigargin in 3T3-F442A preadipocytes (Fig. S4C,D) and C₂C₁₂ myoblasts (Fig.
186 S4E,F). Taken together, these data demonstrate that the initial phase of JNK
187 activation upon induction of ER stress is mediated by both IRE1 α and TRAF2.

188 *The initial phase of JNK activation in ER-stressed cells inhibits cell death via*
189 *induction of inhibitors of apoptosis (IAPs)*

190 An initial phase of JNK activation by stresses other than ER stress is viewed as being
191 antiapoptotic (Chen et al., 1996a; Lee et al., 1997; Nishina et al., 1997; Raingeaud et
192 al., 1995; Sluss et al., 1994; Traverse et al., 1994). To characterise whether JNK

193 activation early in the ER stress response is also antiapoptotic, we studied whether
194 mitochondrial permeability transition (MPT) is more pronounced in JNK-deficient
195 MEFs than WT MEFs, because MPT is often observed in apoptotic cells (Bradham et
196 al., 1998; Fulda et al., 1998; Narita et al., 1998; Scorrano et al., 1999). After exposure
197 of cells to 1 μ M thapsigargin or 10 μ g/ml tunicamycin for up to 4 h MPT was
198 revealed by staining cells with the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-
199 tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Reers et al., 1991; Smiley et al.,
200 1991) (Fig. 3A). MPT inhibits accumulation of JC-1 in mitochondria and blue-shifts
201 its fluorescence emission from a punctuate orange to a green fluorescence (Reers et
202 al., 1991). After induction of ER stress with 1 μ M thapsigargin for 45 min or 4 h MPT
203 was observed in a greater percentage of *jnk1^{-/-} jnk2^{-/-}* MEFs than WT MEFs (Fig.
204 3A,B). Similar results were obtained when ER stress was induced with 10 μ g/ml
205 tunicamycin for 4 h (Fig. 3A,C). To provide further evidence for increased apoptotic
206 cell death in JNK-deficient cells we measured caspase 3/7-like protease activities
207 early in the ER stress response (Fig. 3D,E). Two ER stressors, thapsigargin and
208 tunicamycin, elicited a more pronounced increase of caspase 3/7-like protease
209 activities in *jnk1^{-/-} jnk2^{-/-}* MEFs than in WT MEFs 4 h after induction of ER stress
210 (Fig. 3D,E). These data suggest that JNK signalling early in the ER stress response
211 inhibits apoptosis.

212 In the early antiapoptotic response to TNF- α JNK is required for expression of the
213 mRNA for the antiapoptotic ubiquitin ligase cIAP2/BIRC3 (Lamb et al., 2003). This
214 motivated us to compare the expression of mRNAs for antiapoptotic genes including
215 *cIAP1*, *cIAP2*, *XIAP*, and *BIRC6* at the onset of activation of JNK with 1 μ M
216 thapsigargin in WT and *jnk1^{-/-} jnk2^{-/-}* MEFs. Expression of the mRNAs for cIAP1,
217 cIAP2, XIAP, and BIRC6 increased in WT cells in the first 45 min of ER stress. By
218 contrast, *cIAP1*, *cIAP2*, and *BIRC6* mRNA levels decreased in *jnk1^{-/-} jnk2^{-/-}* cells (Fig.
219 4). The increase in *XIAP* mRNA was more pronounced in WT than in *jnk1^{-/-} jnk2^{-/-}*
220 MEFs, suggesting that JNK positively regulates expression of *XIAP* mRNA (Fig. 4C).
221 To establish whether mammalian inhibitors of apoptosis (IAPs) delay the onset of
222 apoptosis in the early ER stress response we compared caspase 3/7-like protease
223 activity in WT, *ciap1^{-/-} ciap2^{-/-}*, and *xiap^{-/-}* MEFs. Both *ciap1^{-/-} ciap2^{-/-}* and *xiap^{-/-}*
224 MEFs displayed 4.4 ± 1.2 fold higher caspase 3/7-like protease activities than WT
225 MEFs under unstressed conditions (Fig. 5A), which is consistent with increased

226 susceptibility of these cells and cells treated with IAP antagonists to undergo
227 apoptosis (Conte et al., 2006; Geserick et al., 2009; Schimmer et al., 2004; Vince et
228 al., 2007; Yang and Du, 2004). ER stress induced for 4 h with thapsigargin or
229 tunicamycin resulted in a greater increase in caspase 3/7-like protease activities in
230 *ciap1^{-/-} ciap2^{-/-}* and *xiap^{-/-}* MEFs than in WT MEFs (Fig. 5B,C). Taken together, the
231 decreased transcriptional induction of several IAPs in *jnk1^{-/-} jnk2^{-/-}* MEFs, increased
232 MPT and increased caspase 3/7-like protease activities in JNK-deficient MEFs,
233 *ciap1^{-/-} ciap2^{-/-}*, and *xiap^{-/-}* MEFs suggest that JNK-dependent transcriptional
234 induction of several IAPs inhibits apoptosis early in the ER stress response.

235 **Discussion**

236 We show that JNK is activated early in the mammalian UPR and that this immediate
237 JNK activation is antiapoptotic. Activation of JNK early in the UPR by two
238 mechanistically distinct ER stressors, thapsigargin and tunicamycin (Figs 1, S1), and
239 its dependence on IRE1 α and TRAF2 (Figs 2, S2-S4) provides evidence that the early
240 JNK activation is in response to ER stress. Greater activation of caspase 3/7-like
241 protease activities and a more rapid MPT were observed in ER-stressed JNK-deficient
242 MEFs than in WT MEFs (Fig. 3). These data support the view that early JNK
243 activation protects ER-stressed cells from executing apoptosis prematurely and are
244 consistent with the observation that *traf2^{-/-}* MEFs are more susceptible to ER stress
245 than WT MEFs (Mauro et al., 2006). Early JNK activation coincides with induction of
246 several antiapoptotic genes (Figs 1, 4). Maximal expression of these mRNAs was
247 JNK-dependent (Fig. 4). MEFs lacking several IAPs, such as *ciap1^{-/-} ciap2^{-/-}* and
248 *xiap^{-/-}* MEFs, displayed greater caspase 3/7-like protease activities than WT MEFs
249 during short periods of ER stress (Fig. 5). These observations support the view that
250 IAPs, whose transcriptional induction is JNK-dependent in the early ER stress
251 response, protect cells against apoptosis early in the ER stress response.

252 Mostly pharmacologic data support that activation of JNK late in the ER stress
253 response promotes cell death (Arshad et al., 2013; Chen et al., 2008; Huang et al.,
254 2014; Jung et al., 2014; Jung et al., 2012; Kang et al., 2012; Smith and Deshmukh,
255 2007; Tan et al., 2006; Teodoro et al., 2012; Wang et al., 2009; Zhang et al., 2001).
256 Our work suggests that two functionally distinct phases of JNK signalling exist in the
257 ER stress response - an early prosurvival phase and a late phase that promotes cell
258 death. Biphasic JNK signalling with opposing effects on cell viability exists also in

259 other stress responses. Transient activation of JNK in response to several other
260 stresses is antiapoptotic (Chen et al., 1996a; Lee et al., 1997; Nishina et al., 1997;
261 Raingeaud et al., 1995; Sluss et al., 1994; Traverse et al., 1994), while persistent JNK
262 activation causes cell death (Chen et al., 1996a; Chen et al., 1996b; Guo et al., 1998;
263 Sanchez-Perez et al., 1998). These opposing functional attributes of transient and
264 persistent JNK activation have also been causally established by using JNK-deficient
265 MEFs reconstituted with 1-*tert*-butyl-3-naphthalen-1-ylmethyl-1*H*-pyrazolo[3,4-
266 *d*]pyrimidin-4-ylamine (1NM-PP1)-sensitised alleles of JNK1 and JNK2 (Ventura et
267 al., 2006). Hence, the antiapoptotic function of the initial phase of JNK activation in
268 the ER stress response is another example for the paradigm that the duration of JNK
269 activation controls cell fate. Identification of *ciAP1*, *XIAP*, and *BIRC6* as genes whose
270 expression required JNK in the early response to ER stress (Fig. 4) has allowed us to
271 extend the repertoire of antiapoptotic JNK targets. These, and possibly other genes,
272 may also contribute to how JNK inhibits cell death in other stress responses.

273 The existence of an initial antiapoptotic phase of JNK signalling in the ER stress
274 response raises at least two questions: 1) What are the molecular mechanisms that
275 define this initial phase as antiapoptotic? 2) Which mechanisms may restrict
276 antiapoptotic JNK signalling to the early response to ER stress? While future
277 experiments will be necessary to answer these questions, possible explanations may
278 be that the duration of activation affects the subcellular localisation of JNKs, that JNK
279 signalling outputs are controlled by molecular determinants, or that the JNK
280 signalling pathway functionally interacts with other signalling pathways, for example
281 the NF- κ B pathway.

282 Opposing signalling outputs of extracellular signal-regulated kinases (ERKs) in
283 PC12 cells have been explained by different subcellular localisations of ERKs
284 (Marshall, 1995). JNK, however, does not appear to relocalise upon stimulation,
285 either in response to transient or persistent activation (Chen et al., 1996a; Sanchez-
286 Perez et al., 1998). This is also the case for JNK activated early in the ER stress
287 response (Fig. 6). An alternative possibility is that JNK substrates function as
288 molecular determinants of the biological functions of transient and persistent JNK
289 activation, respectively. This is, for example, the case for the ERK substrate c-Fos
290 (Murphy et al., 2002).

291 In the ER stress response NF- κ B activation is transient and displays kinetics in
292 several cell lines that are reminiscent of the initial phase of antiapoptotic JNK
293 signalling reported in this study (Deng et al., 2004; Jiang et al., 2003; Wu et al., 2002;
294 Wu et al., 2004). In TNF- α signalling JNK functionally interacts with the NF- κ B
295 pathway. JNK activation in the absence of NF- κ B is apoptotic (Deng et al., 2003; Guo
296 et al., 1998; Liu et al., 2004; Tang et al., 2002) or necrotic (Ventura et al., 2004),
297 while NF- κ B transduces an antiapoptotic response to TNF- α (Devin et al., 2000;
298 Kelliher et al., 1998). At the transcriptional level NF- κ B cooperates with JunD
299 (Rahmani et al., 2001), whose phosphorylation is decreased in *jnk1*^{-/-} *jnk2*^{-/-} MEFs
300 (Ventura et al., 2003). NF- κ B induces *cIAP1*, *cIAP2*, and *XIAP* (Stehlik et al., 1998).
301 JunD contributes to the transcriptional induction of *cIAP2* in TNF- α -stimulated cells
302 (Lamb et al., 2003). This collaboration between NF- κ B and transcription factors
303 controlled by JNK, such as JunD, may explain the JNK-dependent induction of
304 *cIAP1*, *cIAP2*, *XIAP*, and *BIRC6* (Fig. 4), and potentially other antiapoptotic genes,
305 early in the ER stress response.

306 Transient activation of NF- κ B in the ER stress response may also contribute to
307 control of the duration of antiapoptotic JNK signalling. NF- κ B inhibits JNK
308 activation by TNF- α (De Smaele et al., 2001; Papa et al., 2004; Reuther-Madrid et al.,
309 2002; Tang et al., 2002; Tang et al., 2001) through induction of XIAP (Tang et al.,
310 2002; Tang et al., 2001) and GADD45 β (De Smaele et al., 2001; Papa et al., 2004).
311 TNF- α also induces the dual specificity phosphatase MKP1/DUSP1 (Guo et al.,
312 1998). In murine keratinocytes *cis*-platin induces persistent JNK activation but
313 induces MKP1 only weakly, while transient JNK activation by *trans*-platin correlated
314 with strong induction of MKP1 (Sanchez-Perez et al., 1998). shRNA-mediated knock-
315 down of MKP1 elevated JNK phosphorylation by tunicamycin in C17.2 neural stem
316 cells, which correlated with increased caspase-3 cleavage and decreased cell viability
317 (Li et al., 2011). These observations suggest that MKP1 is a negative regulator of
318 JNK in ER-stressed cells. However, it remains unresolved if the effects of the MKP1
319 knock-down on caspase-3 cleavage and cell viability are causally mediated via JNK
320 or other MKP1 substrates, such as the p38 MAP kinases (Boutros et al., 2008). In
321 tunicamycin-, but not DTT-treated cerebellar granule neurons S359 phosphorylation
322 and stabilisation of MKP1 were observed, which correlated with short-term JNK
323 activation in tunicamycin-treated cells and prolonged JNK activation in DTT-treated

324 cells (Li et al., 2011). While these results suggest that MKP1 may control the duration
325 of JNK activation in ER-stressed cells, they may also be the result of different
326 pharmacokinetics or secondary effects of the two ER stressors, especially as JNK is
327 activated by diverse stresses (Kyriakis et al., 1994). For example, DTT chelates heavy
328 metal ions, including Zn^{2+} ions, with pK values of ~10-15 (Cornell and Crivaro, 1972;
329 Gnonlonfoun et al., 1991; Krężel et al., 2001) and thus may affect many metal-
330 dependent proteins. DTT can also alter proton gradients over membranes (Petrov et
331 al., 1992), because of its pK_a of ~9.2 (Whitesides et al., 1977), and may reduce
332 lipoamide and through this affect pyruvate dehydrogenase and ATP generation,
333 because its standard redox potential is more negative than the standard redox potential
334 of lipoamide (Cleland, 1964; Massey, 1960). Hence, additional experimentation is
335 required to characterise the role of MKP1 in the ER stress response.

336 The duration of JNK activation may also be regulated at the level of the ER stress
337 perceiving protein kinase IRE1 α . Activation of JNK by IRE1 α requires interaction of
338 TRAF2 with IRE1 α (Urano et al., 2000). This interaction has not been observed in
339 cells expressing kinase and RNase-defective K599A-IRE1 α (Urano et al., 2000). JNK
340 activation precedes *XBPI* splicing (Figs 1, S1). *XBPI* splicing by mammalian IRE1 α
341 is stimulated by phosphorylation of IRE1 α (Prischi et al., 2014). Hence, overall
342 phosphorylation of IRE1 α seems to be an unlikely explanation for the transiency of
343 JNK activation. It is, however, possible that the specific pattern of phosphorylation of
344 the ~10 phosphorylation sites in IRE1 α (Itzhak et al., 2014) controls its affinity
345 towards TRAF2 and the activation of JNK by IRE1 α .

346 In conclusion, we show that an initial phase of JNK activation produces
347 antiapoptotic signals early in the ER stress response. Our work also identifies JNK-
348 dependent expression of several antiapoptotic genes, including *cIAP1*, *cIAP2*, and
349 *XIAP*, as a mechanism through which JNK exerts its antiapoptotic functions early in
350 the ER stress response.

351 **Materials and Methods**

352 **Antibodies and reagents.** Rabbit anti-phospho-S51-eIF2 α (cat. no. 9721S, batches
353 10-12), rabbit anti-JNK (cat. no. 9252, batch 15) rabbit anti-JNK2 (cat. no. 9258,
354 batch 9), rabbit anti-phospho-JNK (cat. no. 4668, batches 9 and 11) antibodies, and
355 human recombinant TNF- α (cat. no. 8902) were purchased from Cell Signaling
356 Technology Inc. (Danvers, MA, USA). The mouse anti-GAPDH antibody (cat. no.

357 G8795, batch 092M4820V) was purchased from Sigma-Aldrich (Gillingham, UK),
358 the rabbit anti-eIF2 α antibody (cat. no. sc-11386, batch G1309) and the rabbit anti-
359 TRAF2 antibody (cat. no. sc-876, batches G1508 and J2009) from Santa Cruz
360 Biotechnology (Santa Cruz, CA, USA), and the mouse anti-emerin antibody (cat. no.
361 ab49499) from Abcam (Cambridge, UK). siRNAs against TRAF2, IRE1 α , and eGFP
362 were obtained from Sigma-Aldrich. siRNA sequences are listed in Table S1.
363 Tunicamycin was purchased from Merck Chemicals (Beeston, UK) and thapsigargin
364 from Sigma-Aldrich (Gillingham, UK).

365 **Plasmids.** Plasmids were maintained in *Escherichia coli* XL10-Gold cells (Agilent
366 Technologies, Stockport, UK, cat. no. 200314). Standard protocols for plasmid
367 constructions were used. Plasmid pMT2T-TRAF2 Δ 1-86 was generated by amplifying
368 a 1,327 bp fragment from pMT2T-HA-TRAF2 (Leonardi et al., 2000) with primers
369 H8215 and H8216 (Table S2). The PCR product was cleaved with *Cla*I and *Not*I and
370 cloned into *Cla*I- and *Not*I-digested pMT2T-HA-TRAF2 to yield pMT2T-TRAF2 Δ 1-
371 86. The TRAF2 region in pMT2T-TRAF2 Δ 1-86 was confirmed by sequencing.

372 **Cell culture.** WT, *ire1 α* ^{-/-} (Lee et al., 2002), *jnk1*^{-/-} *jnk2*^{-/-} (Tournier et al., 2000),
373 *traf2*^{-/-} (Yeh et al., 1997), *ciap1*^{-/-} *ciap2*^{-/-} (Geserick et al., 2009), and *xiap*^{-/-} (Vince et
374 al., 2008) MEFs were provided by R. J. Kaufman (Sanford Burnham Medical
375 Research Institute, La Jolla, CA, USA), R. Davis (University of Massachusetts,
376 Worcester, MA, USA), T. Mak (University of Toronto, Ontario Cancer Institute,
377 Toronto, Ontario, Canada), and J. Silke (Walter+Eliza Hall Institute for Medical
378 Research, Victoria, Australia), respectively. 3T3-F442A preadipocytes (Green and
379 Kehinde, 1976), C₂C₁₂ myoblasts (Blau et al., 1985), and Hep G2 cells (Knowles et
380 al., 1980) were obtained from C. Hutchison (Durham University), R. Bashir (Durham
381 University), and A. Benham (Durham University), respectively. All cell lines were
382 tested for mycoplasma contamination upon receipt in the laboratory with the EZ-PCR
383 mycoplasma test kit from Geneflow (cat. no. K1-0210, Lichfield, UK). Mycoplasma
384 testing was repeated every ~3 months with all cells in culture at that time.
385 Contaminated cultures were discarded.

386 All cell lines were grown at 37°C in an atmosphere of 95% (v/v) air, 5% (v/v)
387 CO₂, and 95% humidity. Hep G2 cells were grown in minimal essential medium
388 (MEM) (Eagle, 1959) supplemented with 10% (v/v) foetal bovine serum (FBS) and 2
389 mM L-glutamine. All other cell lines were grown in Dulbecco's modified Eagle's

390 medium (DMEM) containing 4.5 g/l D-glucose (Morton, 1970; Rutzky and Pumper,
391 1974), 10% (v/v) FBS, and 2 mM L-glutamine. The medium for *ire1 α* ^{-/-} and
392 corresponding WT MEFs was supplemented with 110 mg/l pyruvate (Lee et al.,
393 2002). To differentiate C₂C₁₂ cells 60-70% confluent cultures were shifted into low
394 mitogen medium consisting of DMEM containing 4.5 g/l D-glucose, 2% (v/v) horse
395 serum, and 2 mM L-glutamine and incubated for another 7-8 d while replacing the
396 low mitogen medium every 2-3 d (Bains et al., 1984). Differentiation of C₂C₁₂ cells
397 was assessed by microscopic inspection of cultures, staining of myotubes with
398 rhodamine-labelled phalloidin (Amato et al., 1983), and reverse transcriptase PCR for
399 transcription of the genes encoding *S*-adenosyl-homocysteine hydrolase (*AHCY*),
400 myosin light chain 1 (*MYL1*), and troponin C (*TNNC1*, Fig. S1G). 3T3-F442A
401 fibroblasts were differentiated into adipocytes as described before (Mihai and
402 Schröder, 2015). Adipocyte differentiation was assessed by analysing Nile red-stained
403 cells by flow cytometry as described before (Mihai and Schröder, 2015). ER stress
404 was induced with 1 μ M thapsigargin or 10 μ g/ml tunicamycin, if not stated otherwise.

405 Hep G2 cells were transfected with plasmids using jetPRIME (Polyplus
406 Transfection, Illkirch, France, cat. no. 114) and with siRNAs using INTERFERin
407 (Polyplus Transfection, cat. no. 409) transfection reagents. Plasmids and siRNAs were
408 transfected into all other cell lines by electroporation with a Neon electroporator (Life
409 Technologies, Paisley, UK) using a 10 μ l tip. Manufacturer-optimised electroporation
410 conditions were used for 3T3-F442A preadipocytes and C₂C₁₂ myoblasts. MEFs were
411 electroporated with one pulse of 1200 V and a pulse width of 30 ms. 10-20 nM of
412 each siRNA were transfected. The control siRNA was designed against the enhanced
413 green fluorescent protein (eGFP) from *Aequora victoria*. Transfection efficiencies
414 were determined by transfection of 2 μ g of pmaxGFP (Lonza Cologne GmbH,
415 Cologne, Germany) and detection of GFP-expressing cells with a Zeiss ApoTome
416 fluorescence microscope. Transfection efficiencies were >80%. 24 h after transfection
417 cells were analysed or time courses initiated, if not stated otherwise.

418 **RNA extraction and RT-PCRs.** RNA was extracted with the EZ-RNA total RNA
419 isolation kit (GeneFlow, cat. no. K1-0120) and reverse transcribed with oligo-dT
420 primers (Promega, Southampton, cat. no. C1101) and Superscript III reverse
421 transcriptase (Life Technologies, cat. no. 18080044) as described previously (Cox et
422 al., 2011). Protocols for detection of splicing of murine and human *XBPI* have been

423 described previously (Cox et al., 2011). In brief, 2.5 μ l of the cDNA synthesis
424 reaction were amplified with 1 μ M of primers H8289 and H8290 for human *XBPI*
425 and primers H7961 and H7962 for murine *XBPI* in a 50 μ l reaction containing 1 x
426 GoTaq reaction buffer (Promega, cat. no. M7911), 1.5 mM $MgCl_2$, 200 μ M dNTPs,
427 and 0.05 U/ml GoTaq hot start polymerase (Promega, cat. no. M5001). The reaction
428 was incubated for 2 min at 94°C, and then cycled for 35 cycles consisting of
429 subsequent incubations at 94°C for 1 min, 59°C for 1 min, and 72°C for 30 s, followed
430 by a final extension step at 72°C for 5 min. *ACTB* was amplified under the same
431 conditions as described for *XBPI* except that GoTaq G2 Flexi DNA polymerase
432 (Promega, cat. no. M7801) was used. Human *ACTA1* was amplified with primers
433 H8287 and H8288 and murine *ACTB* with primers H7994 and H7995. Primer
434 sequences are listed in Table S2. Band intensities were quantified using ImageJ
435 (Collins, 2007) and the percentage of *XBPI* splicing calculated by dividing the signal
436 for spliced *XBPI* mRNA by the sums of the signals for spliced and unspliced *XBPI*
437 mRNAs. Quantitative PCRs (qPCRs) were run on a Rotorgene 3000 (Qiagen,
438 Crawley, UK). Amplicons were amplified with 0.5 μ l 5 U/ μ l GoTaq[®] Flexi DNA
439 polymerase (Promega, cat. no. M8305), 2 mM $MgCl_2$, 200 μ M dNTPs, and 1 μ M of
440 each primer and detected with a 1:167,000 fold dilution of a SybrGreen stock solution
441 (Life Technologies, cat. no. S7563) or the GoTaq qPCR Master Mix from Promega
442 (cat. no. A6002). Primers for qPCR are listed in Table S2. qPCR using GoTaq DNA
443 polymerase were performed as follows. After denaturation for 2 min at 95°C samples
444 underwent 40 cycles of denaturation at 95°C for 30 s, primer annealing at 58°C for 30
445 s, and primer extension at 72°C for 30 s. After denaturation at 95°C for 2 min qPCRs
446 with the GoTaq qPCR Master mix were cycled 40 times at 95°C for 15 s, 60°C for 15
447 s, and 72°C for 15 s for *cIAP1*, *cIAP2*, *XIAP*, and *BRUCE* and 40 times at 95°C for 15
448 s, 60°C for 60 s for *ACTB*. Fluorescence data were acquired during the annealing step
449 or in case of qPCR amplification of *ACTB* with the GoTaq qPCR Master Mix during
450 the first 30 s at 60°C. Amplification of a single PCR product was confirmed by
451 recording the melting curves after each PCR run. Average amplification efficiencies
452 in the exponential phase were calculated using the comparative quantification analysis
453 in the Rotor Gene Q software and were between 0.6 and 0.7 for all qPCRs. C_T values
454 were calculated and normalised to *GAPDH*, *ACTA1*, or *ACTB* mRNA levels as
455 described by Pfaffl (Pfaffl, 2001) taking the average amplification efficiencies into

456 account. Results represent the average and standard error (s.e.m.) of three technical
457 repeats. qPCR results were confirmed by at least one other biological replicate.
458 Murine *AHCY*, *MYL1*, and *TNNC* qPCRs were standardised to *GAPDH*, murine
459 *BIRC6*, *cIAP1*, *cIAP2*, *TRAF2*, and *XIAP* qPCRs to *ACTB*, the human *IRE1 α* qPCR to
460 *GAPDH* and the human *TRAF2* qPCR to *ACTA1*.

461 **Cell lysis and Western blotting.** Cells were washed three times with ice-cold
462 phosphate-buffered saline (PBS, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 27 mM KCl,
463 137 mM NaCl, pH 7.4) and lysed in RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM
464 NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS]
465 containing Roche complete protease inhibitors (Roche Applied Science, Burgess Hill,
466 UK, cat. no. 11836153001) as described before (Cox et al., 2011).

467 For isolation of cytosolic and nuclear fractions cells were washed two times with
468 ice-cold PBS and gently lysed in 0.32 M sucrose, 10 mM Tris-HCl pH 8.0, 3 mM
469 CaCl₂, 2 mM Mg(OAc)₂, 0.1 mM EDTA, 0.5% (v/v) NP-40, 1 mM DTT, 0.5 mM
470 PMSF. Nuclei were collected by centrifugation for 5 min at 2,400 g, 4°C. The
471 supernatant was used as the cytosolic fraction. The nuclear pellet was resuspended in
472 0.32 M sucrose, 10 mM Tris HCl pH 8.0, 3 mM CaCl₂, 2 mM Mg(OAc)₂, 0.1 mM
473 EDTA, 1 mM DTT, 0.5 mM PMSF by flipping the microcentrifuge tube. The nuclei
474 were collected by centrifugation for 5 min at 2,400 g, 4°C. After aspiration of all of
475 the wash buffer the nuclei were resuspended in 30 μ l low salt buffer [20 mM HEPES
476 (pH 7.9), 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM
477 DTT, 0.5 mM PMSF] by flipping the microcentrifuge tube. One volume of high salt
478 buffer [20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 800 mM KCl, 0.2 mM EDTA, 25%
479 glycerol (v/v), 1% NP-40, 0.5 mM DTT, 0.5 mM PMSF] was added drop wise while
480 continuously mixing the contents of the microcentrifuge tube by flipping. The tubes
481 were then incubated for 45 min at 4°C on an end-over-end rotator. The tubes were
482 centrifuged at 14,000 g for 15 min at 4°C and the supernatant transferred into a fresh
483 microcentrifuge tube to obtain the nuclear extract.

484 Proteins were separated by SDS-PAGE and transferred to polyvinylidene
485 difluoride (PVDF) membranes (Amersham HyBondTM-P, pore size 0.45 μ m, GE
486 Healthcare, Little Chalfont, UK, cat. no. RPN303F) by semi-dry electrotransfer in 0.1
487 M Tris, 0.192 M glycine, and 5% (v/v) methanol at 2 mA/cm² for 60-75 min.
488 Membranes were blocked for 1 h in 5% (w/v) skimmed milk powder in TBST [20

489 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% (v/v) Tween-20] or 5% bovine serum
490 albumin (BSA) in TBST and then incubated overnight with the primary antibody at
491 4°C and gentle agitation. Blots were washed three times with TBST and then probed
492 with secondary antibody for 1 h at room temperature. The anti-eIF2 α , anti-phospho-
493 S51-eIF2 α , anti-JNK, anti-phospho-JNK, and anti-TRAF2 antibodies were used at a
494 1:1,000 dilution in TBST + 5% (w/v) BSA. Membranes were then developed with
495 goat anti-rabbit-IgG (H+L)-horseradish peroxidase (HRP)-conjugated secondary
496 antibody (Cell Signaling, cat. no. 7074S, batch 24) at a 1:1,000 dilution in TBST +
497 5% (w/v) skimmed milk powder. The mouse anti-GAPDH antibody was used at a
498 1:30,000 dilution in TBST + 5% (w/v) skimmed milk powder and developed with
499 goat anti-mouse IgG (H+L)-HRP-conjugated secondary antibody (Thermo Scientific,
500 cat. no. 31432, batch OE17149612) at a 1:20,000 dilution in TBST + 5% (w/v)
501 skimmed milk powder. For signal detection Pierce ECL Western Blotting Substrate
502 (cat. no. 32209) or Pierce ECL 2 Western Blotting Substrate (cat. no. 32132) from
503 Thermo Fisher Scientific (Loughborough, UK) were used. Blots were exposed to CL-
504 X PosureTM film (Thermo Fisher Scientific, Loughborough, UK, cat. no. 34091).
505 Exposure times were adjusted on the basis of previous exposures to obtain exposures
506 in the linear range of the film. Films were scanned on a CanoScan LiDE 600F scanner
507 (Canon) and saved as tif files. Bands were quantified using ImageJ exactly as
508 described under the heading “Gels Submenu” on the ImageJ web site
509 (<http://rsb.info.nih.gov/ij/docs/menus/analyze.html#plot>). In case of unphosphorylated
510 proteins intensities for the experimental antibody were divided by the intensities
511 obtained with the antibody for the loading control in the same lane to correct for
512 differences in loading between lanes. Intensities for phosphorylated eIF2 α were
513 divided by the intensities obtained for total eIF2 α in the same lane. For
514 phosphorylated and total JNK, the sums of the intensities at 54 kDa and 46 kDa,
515 which both represent several JNK1 and JNK2 isoforms (Gupta et al., 1996), were
516 used to calculate the fraction of phosphorylated JNK in a similar way as described for
517 phospho-eIF2 α . Normalisation of phospho-JNK signals to JNK2 or GAPDH gave
518 qualitatively the same results. All loading control- or unphosphorylated protein-
519 corrected intensities obtained for one Western blot were then expressed relative to the
520 loading control-corrected intensity of the 0 h sample in this Western blot. To reprobe
521 blots for detection of nonphosphorylated proteins, membranes were stripped using

522 Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, cat. no. 21059) and
523 blocked with 5% (w/v) skimmed milk powder in TBST.

524 **Caspase 3 and 7-like activities** were determined with the Caspase-Glo 3/7 kit from
525 Promega (cat. no. G8091). Luminescence was read with a Synergy H4 Multi-Mode
526 Microplate Reader (BioTek, Swindon, UK) and standardised to total protein
527 concentrations determined with the *DC* protein assay from Bio-Rad Laboratories
528 (Hemel Hempstead, UK, cat. no. 500-0116).

529 **Fluorescence microscopy.** For confocal microscopy cells were grown in lumox
530 dishes (Sarstedt, Leichestet, UK, cat. no. 94.6077.331). After incubation with 1 μ M
531 thapsigargin cells were incubated with 2 μ g/ml JC-1 (Life Technologies, cat. no.
532 T3168) at 37°C for 20 min (Ankarcrona et al., 1995; Cossarizza et al., 1993; Reers et
533 al., 1991; Smiley et al., 1991). The cells were washed twice with PBS before addition
534 of fresh medium for live cell imaging on a Leica TCS SP5 II confocal microscope
535 (Leica Microsystems, Mannheim, Germany). JC-1 fluorescence was excited at 488
536 nm with an argon laser set at 22% of its maximum power. Green fluorescence
537 between 515-545 nm was collected with a photomultiplier tube and orange
538 fluorescence between 590-620 nm with a HyD 5 detector. Cells showing fluorescence
539 emission between 515-545 nm only were counted as having undergone MPT, while
540 cells that displayed punctuate fluorescence emission between 590-620 nm were
541 counted as not having undergone MPT.

542 **Error and statistical calculations.** Samples sizes (*n*) were derived from experiments
543 with independent cell cultures. Experimental data are presented as the mean and its
544 s.e.m. For composite parameters, errors were propagated using the law of error
545 propagation for random, independent errors (Ku, 1966). Statistical calculations were
546 performed in GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA, USA).

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557 **Author contributions**

558 M.Sc. conceived the project, M.B., N.S., and M.Sc. designed the experiments, M.B.,
559 N.S., M.Su, L.K.S., A.D.M., A.A.A., and J.N.W. performed experiments, and M.Sc.,
560 M.B., and N.S. analysed and interpreted the data. M.Sc. wrote the manuscript. All
561 authors reviewed and approved the manuscript.

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950 **Figure Legends**

951 **Fig. 1.** JNK activation precedes activation of *XBPI* splicing in MEFs. **(A)** Kinetics of
952 JNK and eIF2 α phosphorylation and **(B)** *XBPI* splicing in MEFs exposed to 1 μ M
953 thapsigargin. **(C)** Quantification of JNK (white circles, solid line, $n = 4$) and eIF2 α
954 (white squares, dotted line) phosphorylation from panel (A) and of *XBPI* splicing
955 (black circles, dashed line, $n = 2$) from panel (B). **(D)** Kinetics of JNK and eIF2 α
956 phosphorylation and **(E)** *XBPI* splicing in MEFs exposed to 10 μ g/ml tunicamycin.
957 **(F)** Quantification of JNK (white circles, solid line, $n = 3$) and eIF2 α (white squares,
958 dotted line) phosphorylation from panel (D) and of *XBPI* splicing (black circles,
959 dashed line) from panel (E). p values for comparison of the JNK phosphorylation after
960 addition of the drugs to the cells to JNK phosphorylation in the untreated cells were
961 obtained from an ordinary one way analysis of variance (ANOVA) with Dunnett's
962 correction for multiple comparisons (Dunnett, 1955; Dunnett, 1964). * - $p < 0.05$, ** -
963 $p < 0.01$, *** - $p < 0.001$, and **** - $p < 0.0001$. A repeat of the eIF2 α Western blots
964 gave qualitatively similar results.

965 **Fig. 2.** IRE1 α and TRAF2 are required for the initial phase of JNK activation in
966 MEFs. **(A)** Kinetics of JNK and eIF2 α phosphorylation and **(B)** *XBPI* splicing in
967 *ire1 α ^{-/-}* MEFs exposed to 1 μ M thapsigargin. For eIF2 α phosphorylation qualitatively
968 similar data were obtained in one repeat of the experiment. **(C)** Quantification of JNK
969 (white circles, solid line, $n = 3$) and eIF2 α (white squares, dotted line)
970 phosphorylation from panel (A) and of *XBPI* splicing (black circles, dashed line)
971 from panel (B). **(D)** Kinetics of JNK and eIF2 α phosphorylation and **(E)** *XBPI*
972 splicing in *traf2^{-/-}* MEFs exposed to 1 μ M thapsigargin. eIF2 α phosphorylation was
973 expressed relative to the 480 min time point. **(F)** Quantification of JNK (white circles,
974 solid line, $n = 3$) and eIF2 α phosphorylation (white squares, dotted line, $n = 2$) from
975 panel (D) and *XBPI* splicing (black circles, dashed line) from panel (E). **(G)**
976 Comparison of phosphorylation of JNK in WT, *ire1 α ^{-/-}*, and *traf2^{-/-}* MEFs before the
977 onset of elevated JNK phosphorylation after 240 min of ER stress in *ire1 α ^{-/-}* and

978 *traf2*^{-/-} MEFs. The bars represent the relative JNK phosphorylation before, 10, 20, 30,
 979 45, 60, 120, and 240 min after addition of thapsigargin to the cells. *p* values for
 980 comparison of JNK phosphorylation in treated cells to the JNK phosphorylation in
 981 untreated cells were calculated with an ordinary one way ANOVA with Dunnett's
 982 correction for multiple comparisons.

983 **Fig. 3.** JNK inhibits cell death early in the ER stress response. **(A)** WT and *jnk1*^{-/-}
 984 *jnk2*^{-/-} MEFs were treated with 1 μ M thapsigargin (Tg) or 10 μ g/ml tunicamycin (Tm)
 985 for 4 h and stained with JC-1 as described in Materials and Methods. Scale bar – 10
 986 μ m. **(B, C)** Quantification of the confocal fluorescence microscopy data shown in
 987 panel A for **(B)** thapsigargin- and **(C)** tunicamycin-treated cells. At least 600 cells
 988 were counted for each sample. **(D)** Combined activities of caspases 3 and 7 in WT and
 989 *jnk1*^{-/-} *jnk2*^{-/-} MEFs treated for 4 h with 1 or 2 μ M thapsigargin (Tg) or **(E)** 10 μ g/ml
 990 tunicamycin (Tm). The combined caspase activities are expressed relative to the
 991 untreated cells. *p* values were calculated with an ordinary two way ANOVA with
 992 Šidák's correction for multiple comparisons (Šidák, 1967) (*n* = 3 for panels D and E).

993 **Fig. 4.** JNK is required for transcriptional induction of antiapoptotic genes early in the
 994 ER stress response. **(A)** *cIAP1* (*BIRC2*), **(B)** *cIAP2* (*BIRC3*), **(C)** *XIAP* (*BIRC4*), and
 995 **(D)** *BIRC6* (*BRUCE*) steady-state mRNA levels were quantified by RT-qPCR in WT
 996 and *jnk1*^{-/-} *jnk2*^{-/-} MEFs exposed to 1 μ M thapsigargin for the indicated times. The *p*
 997 values for the genotype comparisons of an ordinary two way ANOVA with Šidák's
 998 correction for multiple comparisons are shown (*n* = 3).

999 **Fig. 5.** cIAP1, cIAP2, and XIAP protect against apoptosis early in the ER stress
 1000 response. **(A)** Combined activities of caspases 3 and 7 in untreated WT, *ciap1*^{-/-}
 1001 *ciap2*^{-/-}, and *xiap*^{-/-} MEFs and after exposure to **(B)** 2 μ M thapsigargin (Tg) or **(C)** 10
 1002 μ g/ml tunicamycin (Tm) for 4 h. *p* values were calculated with an ordinary two way
 1003 ANOVA with Dunnett's (panel A) or Tukey's (Tukey, 1949) (panels B and C)
 1004 correction for multiple comparisons (*n* = 3).

1005 **Fig. 6.** Immediately activated JNK localizes to the cytosol during ER stress. Serum-
 1006 starved Hep G2 cells were treated for 45 min with 1 μ M thapsigargin or left untreated
 1007 before isolation of the cytosolic and nuclear fractions. The cytosolic (C) and nuclear
 1008 (N) fractions were analysed by Western blotting. The asterisk (*) indicates a non-
 1009 specific band recognised by the anti-emerin antibody. Emerin was used as a nuclear

1010 marker and GAPDH as a cytoplasmic marker. The experiment was repeated once with
1011 qualitatively similar results.

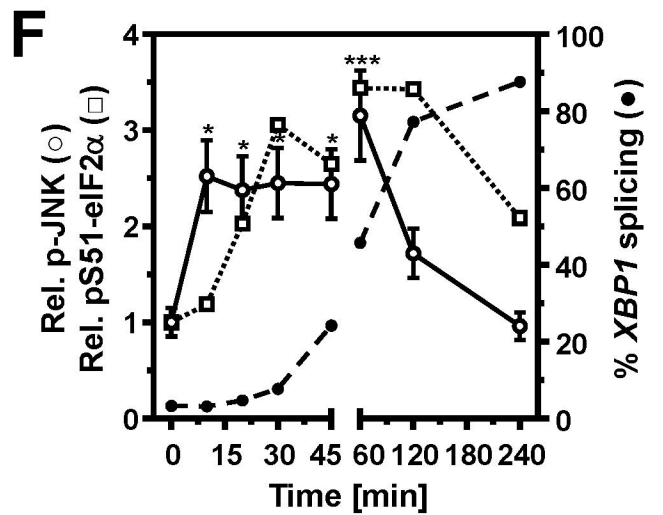
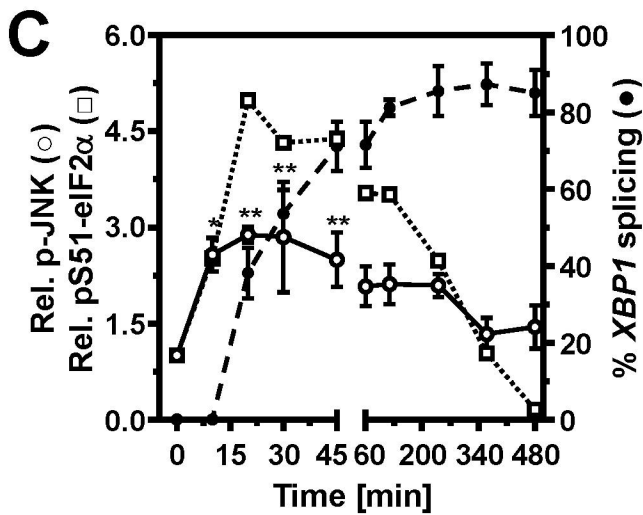
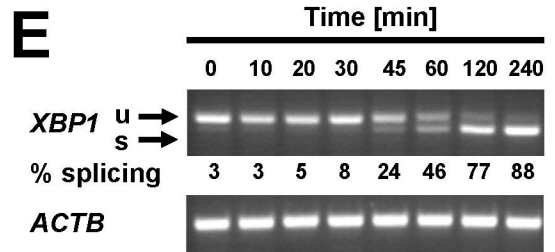
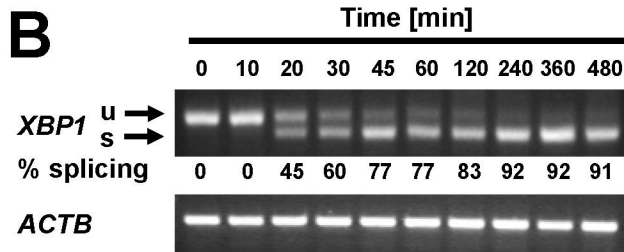
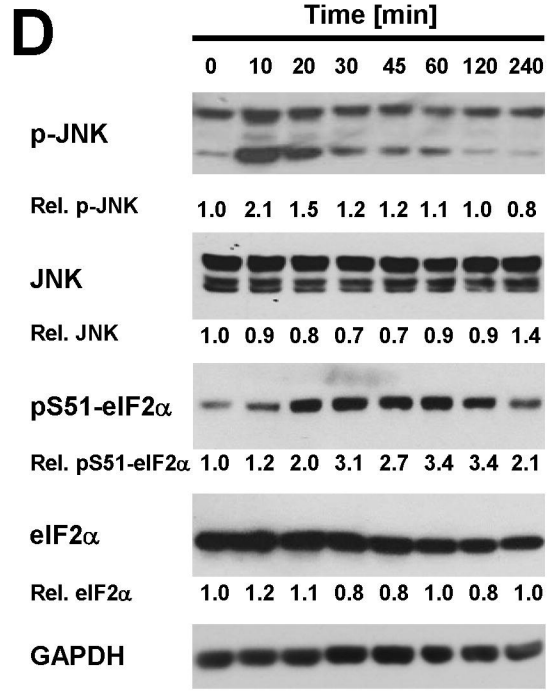
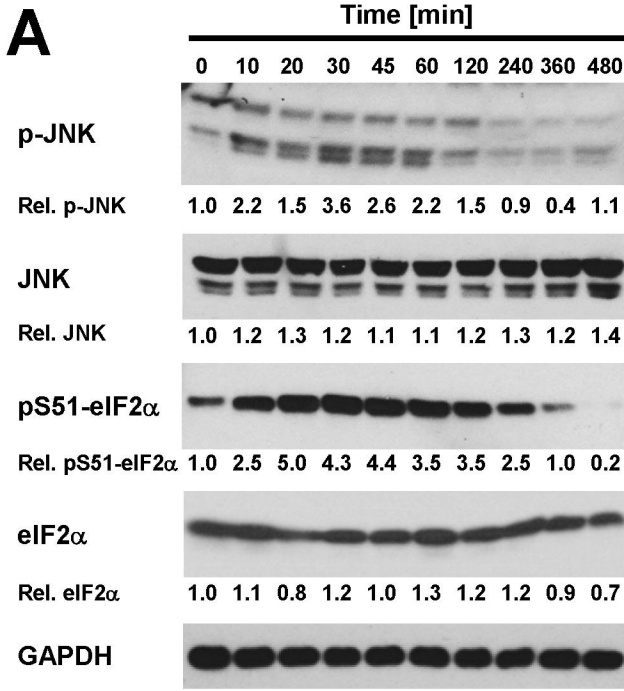


Figure 1, Brown *et al.*

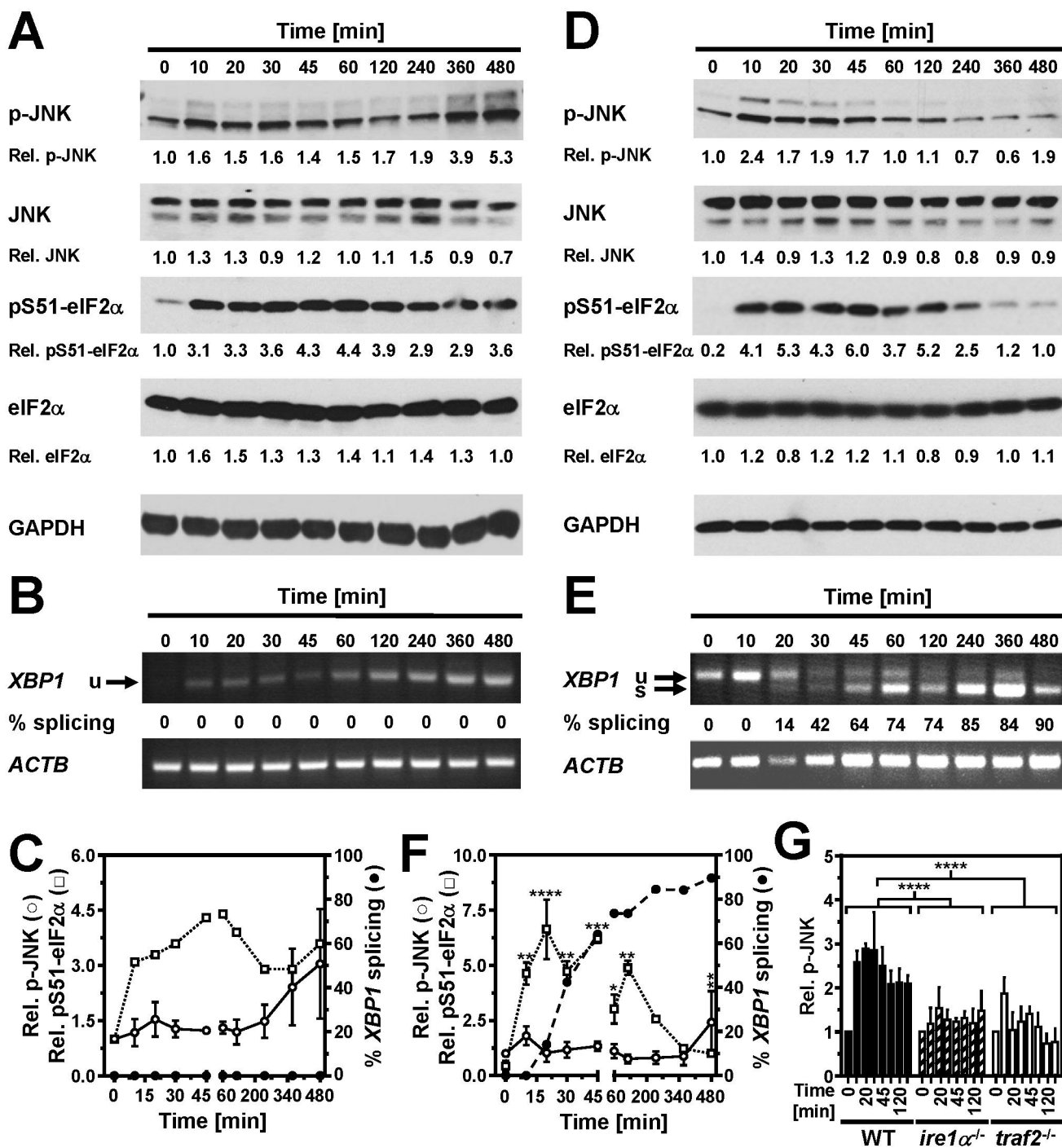


Figure 2, Brown *et al.*

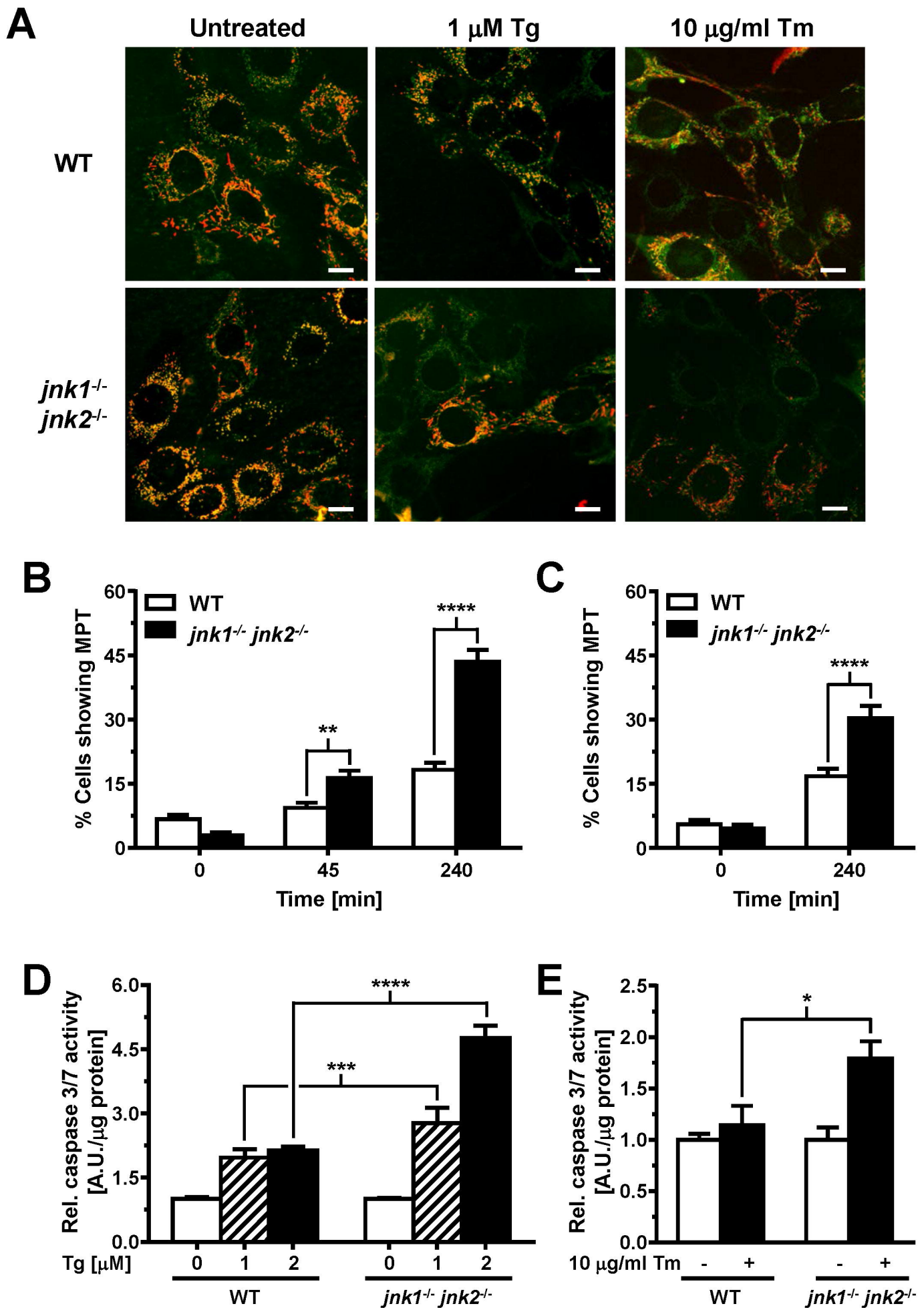


Figure 3, Brown et al.

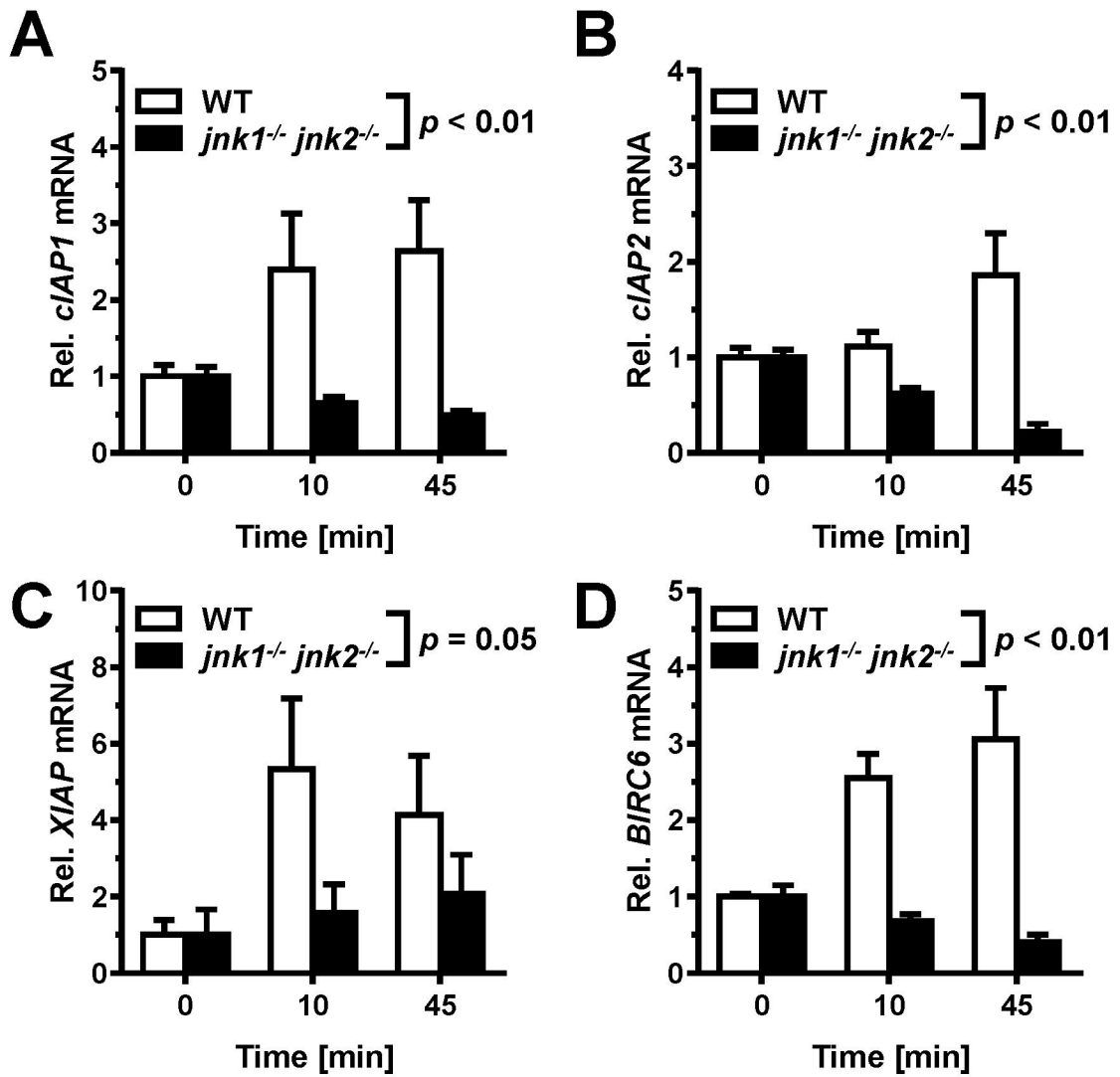


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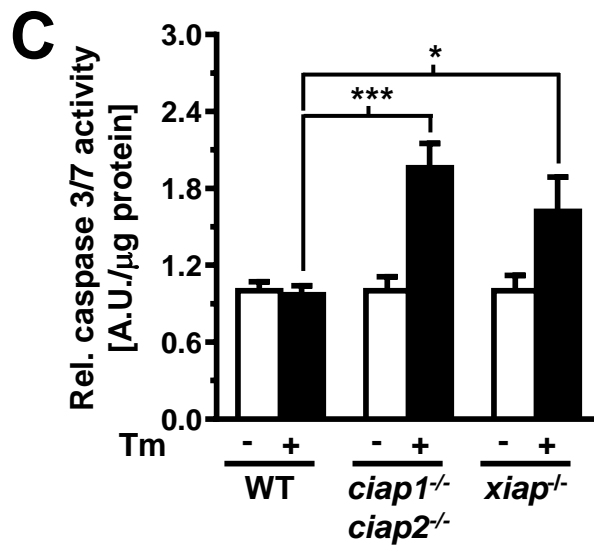
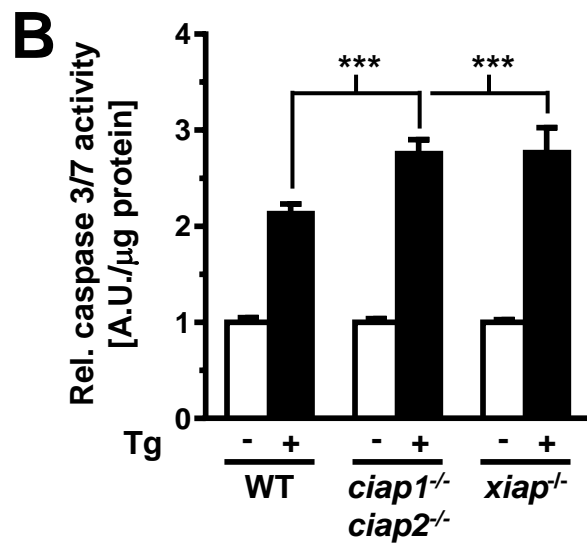
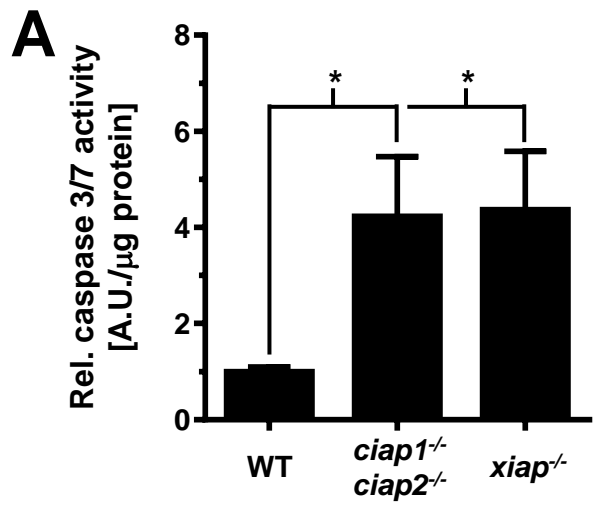


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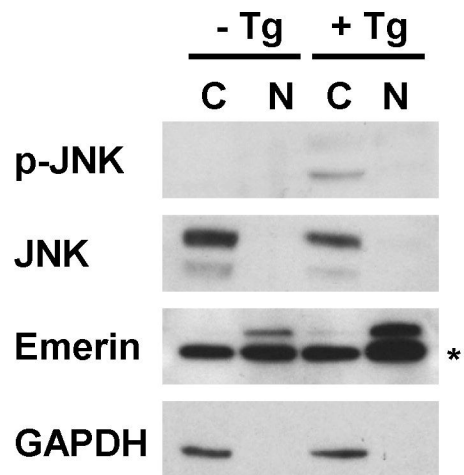


Figure 6, Brown *et al.*

1 Supplemental figure legends

2 **Fig. S1.** Kinetics of JNK and eIF2 α phosphorylation and of *XBPI* splicing in response to
 3 acute ER stress in (A-C) Hep G2 cells, (D-F) *in vitro* differentiated 3T3-F442A adipocytes,
 4 and (G-J) *in vitro* differentiated C₂C₁₂ myotubes. (A) Western blots for phospho-S51-eIF2 α
 5 (pS51-eIF2 α), eIF2 α , phospho-JNK (p-JNK), JNK, and GAPDH and (B) *XBPI* splicing in
 6 Hep G2 cells exposed to 1 μ M thapsigargin for the indicated times. (C) Quantification of the
 7 JNK (white circles, solid line, $n = 3$) and eIF2 α phosphorylation (white squares, dotted line)
 8 from panel (A) and of *XBPI* splicing (black circles, dashed line) from panel (B). (D) Western
 9 blots for pS51-eIF2 α , eIF2 α , p-JNK, JNK, GAPDH and (E) *XBPI* splicing in 3T3-F442A
 10 cells exposed to 1 μ M thapsigargin for the indicated times. (F) Quantification of JNK (white
 11 circles, solid line, $n = 2$) and eIF2 α (white squares, dotted line) phosphorylation from panel
 12 (D) and *XBPI* splicing (black circles, dashed line) from panel (E). (G) mRNA levels for the
 13 muscle differentiation markers *AHCY* encoding S-adenosyl-homocysteine hydrolase, *MYL1*
 14 encoding myosin light chain 1, and *TNNC1* encoding troponin C in differentiated C₂C₁₂ cells.
 15 The fold changes in mRNA abundance relative to undifferentiated cells (day 0) are shown.
 16 (H) Western blots for pS51-eIF2 α , eIF2 α , p-JNK, JNK, and GAPDH and (I) *XBPI* splicing
 17 in C₂C₁₂ cells exposed to 1 μ M thapsigargin for the indicated times. (J) Quantification of
 18 JNK (white circles, solid line, $n = 2$) and eIF2 α (white squares, dotted line, $n = 2$)
 19 phosphorylation from panel (H) and of *XBPI* splicing (black circles, dashed line) from panel
 20 (I). p values for comparison of the JNK phosphorylation in treated to the JNK
 21 phosphorylation in untreated cells were calculated with an ordinary one way ANOVA with
 22 Dunnett's correction for multiple comparisons. A repeat of each eIF2 α Western blot gave
 23 qualitatively similar results.

24 **Fig. S2.** The initial phase of JNK activation requires IRE1 α and TRAF2 in Hep G2 cells. (A)
 25 Hep G2 cells were transfected with 10 nM of the indicated siRNAs against human *IRE1 α* . 48
 26 h and 72 h after transfection *IRE1 α* mRNA was quantified by RT-qPCR. (B) siRNA knock-
 27 down of IRE1 α impairs ER stress-dependent activation of JNK in Hep G2 cells. 72 h after
 28 transfection with the indicated siRNAs Hep G2 cells were stimulated for the indicated times
 29 with 1 μ M thapsigargin. Cell lysates were analysed by Western blotting. (C) Quantification
 30 of JNK phosphorylation in Hep G2 cells treated for the indicated times with 1 μ M
 31 thapsigargin 72 h after transfection with the indicated siRNAs. The average and s.e.m. from
 32 two independent experiments are shown. p values for comparison of the relative JNK

33 phosphorylation in cells transfected with *eGFP* and *hIRE1 α* siRNAs at 2 and 4 h were
 34 calculated by using an ordinary two way ANOVA test with Tukey's correction for multiple
 35 comparisons. **(D)** siRNA knock-down of human TRAF2 in Hep G2 cells. Relative *TRAF2*
 36 mRNA abundance (to *ACTA1*) was measured by RT-qPCR 24 or 48 h after transfection of
 37 Hep G2 cells with the indicated siRNAs. **(E)** Knock-down of TRAF2 expression in Hep G2
 38 cells interferes with ER stress-induced JNK phosphorylation. Hep G2 cells were treated with
 39 1 μ M thapsigargin for the times indicated before protein extraction for Western blotting with
 40 antibodies against p-JNK, JNK2, TRAF2, and GAPDH. **(F)** Quantification of the JNK
 41 phosphorylation signals in the Western blots of panel (E).

42 **Fig. S3.** The initial phase of JNK activation is TRAF2-dependent in 3T3-F442A
 43 preadipocytes and in C₂C₁₂ myoblasts. **(A, B)** *TRAF2* mRNA levels measured by real-time
 44 PCR in (A) 3T3-F442A preadipocytes and (B) C₂C₁₂ myoblasts after transfection with the
 45 indicated siRNAs. **(C)** TRAF2 protein levels relative to GAPDH in 3T3-F442A
 46 preadipocytes transfected with the indicated siRNAs against eGFP or murine TRAF2. Cells
 47 were treated with 20 ng/ml TNF- α for 20 min where indicated. **(D)** JNK phosphorylation and
 48 **(E)** *XBPI* splicing in 3T3-F442A preadipocytes transfected with a siRNA against eGFP. **(F)**
 49 Quantification of the JNK phosphorylation (white circles, solid line) from panel (D) and
 50 *XBPI* splicing (black circles, dashed line) from panel (E). **(G)** JNK phosphorylation and **(H)**
 51 *XBPI* splicing in 3T3-F442A preadipocytes transfected with murine *TRAF2* siRNA #2. **(I)**
 52 Quantification of the JNK phosphorylation (white circles, solid line, $n = 3$) from panel (G)
 53 and *XBPI* splicing (black circles, dashed line, $n = 2$) from panel (H). **(J)** JNK
 54 phosphorylation and **(K)** *XBPI* splicing in C₂C₁₂ myoblasts transfected with control siRNA
 55 against eGFP. **(L)** Quantification of the JNK phosphorylation (white circles, solid line, $n = 2$)
 56 from panel (J) and *XBPI* splicing (black circles, dashed line, $n = 2$) from panel (K). **(M)** JNK
 57 phosphorylation and **(N)** *XBPI* splicing in C₂C₁₂ myoblasts transfected with murine *TRAF2*
 58 siRNA #2. **(O)** Quantification of the JNK phosphorylation (white circles, solid line, $n = 3$)
 59 from panel (M) and *XBPI* splicing (black circles, dashed line) from panel (N). p values for
 60 comparison of the JNK phosphorylation in treated to the JNK phosphorylation in untreated
 61 cells were calculated with an ordinary one way ANOVA with Dunnett's correction for
 62 multiple comparisons.

63 **Fig. S4.** Dominant negative TRAF2 blocks initial JNK activation by ER stress in 3T3-F442A
 64 preadipocytes (C-D) and C₂C₁₂ myotubes (E-F). **(A)** Domain structures of WT and dominant-
 65 negative TRAF2 (TRAF2 Δ 1-86). **(B)** Western blots for phospho-JNK, JNK2, and TRAF2 in

66 cell lysates prepared from WT and *traf2*^{-/-} MEFs transiently transfected with 8 µg pMT2T-
67 TRAF2Δ1-86 and stimulated with 50 ng/ml TNF-α for 20 min where indicated. **(C)** JNK
68 phosphorylation in 3T3-F442A preadipocytes transfected with pMT2T-TRAF2Δ1-86 to
69 express dominant-negative TRAF2Δ1-86. **(D)** Quantification of the JNK phosphorylation
70 signals in the Western blots of panel (C). **(E)** JNK phosphorylation in C₂C₁₂ myoblasts
71 transfected with pMT2T-TRAF2Δ1-86 to express dominant-negative TRAF2Δ1-86. **(F)**
72 Quantification of the JNK phosphorylation signals.

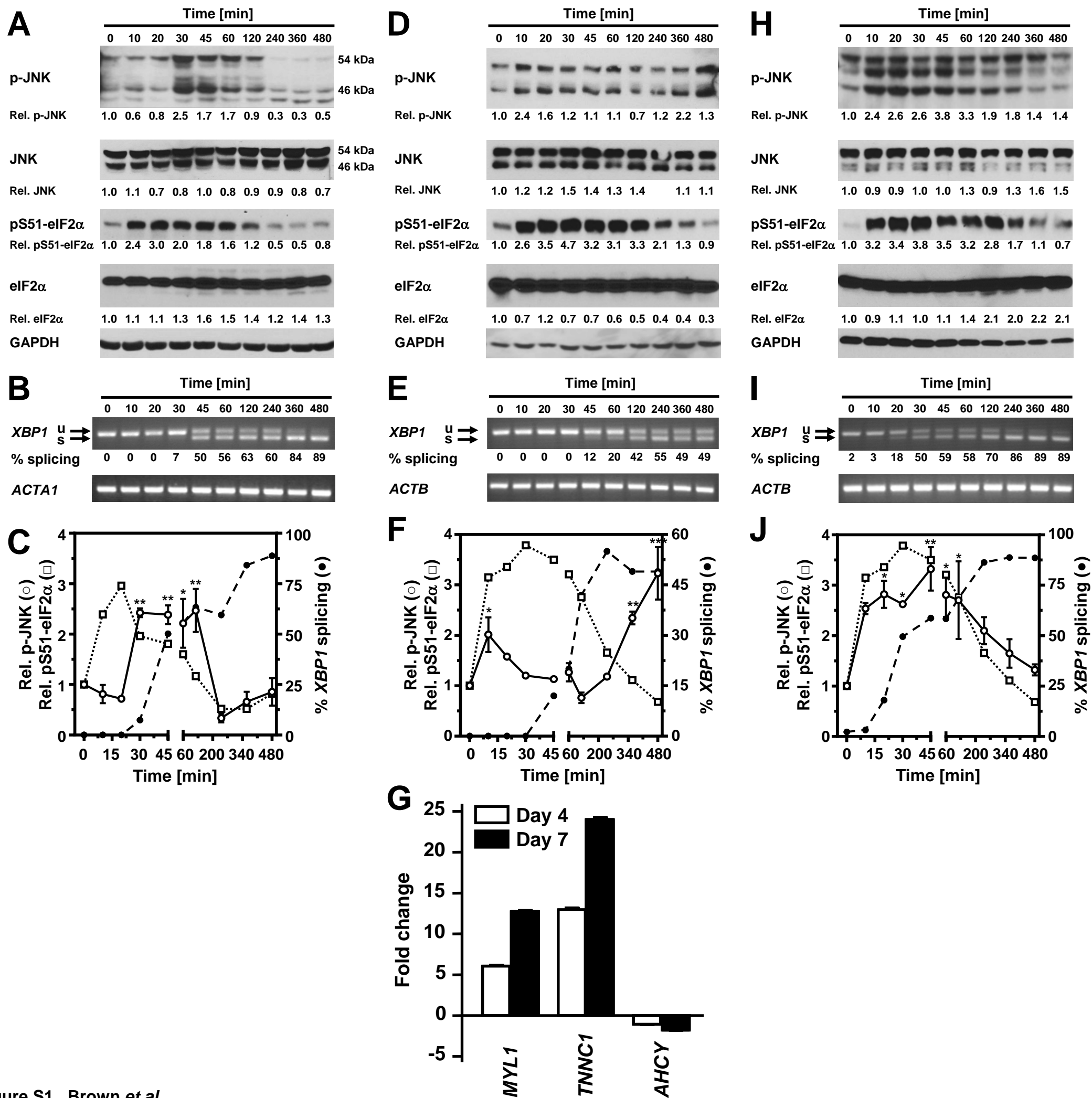


Figure S1, Brown et al.

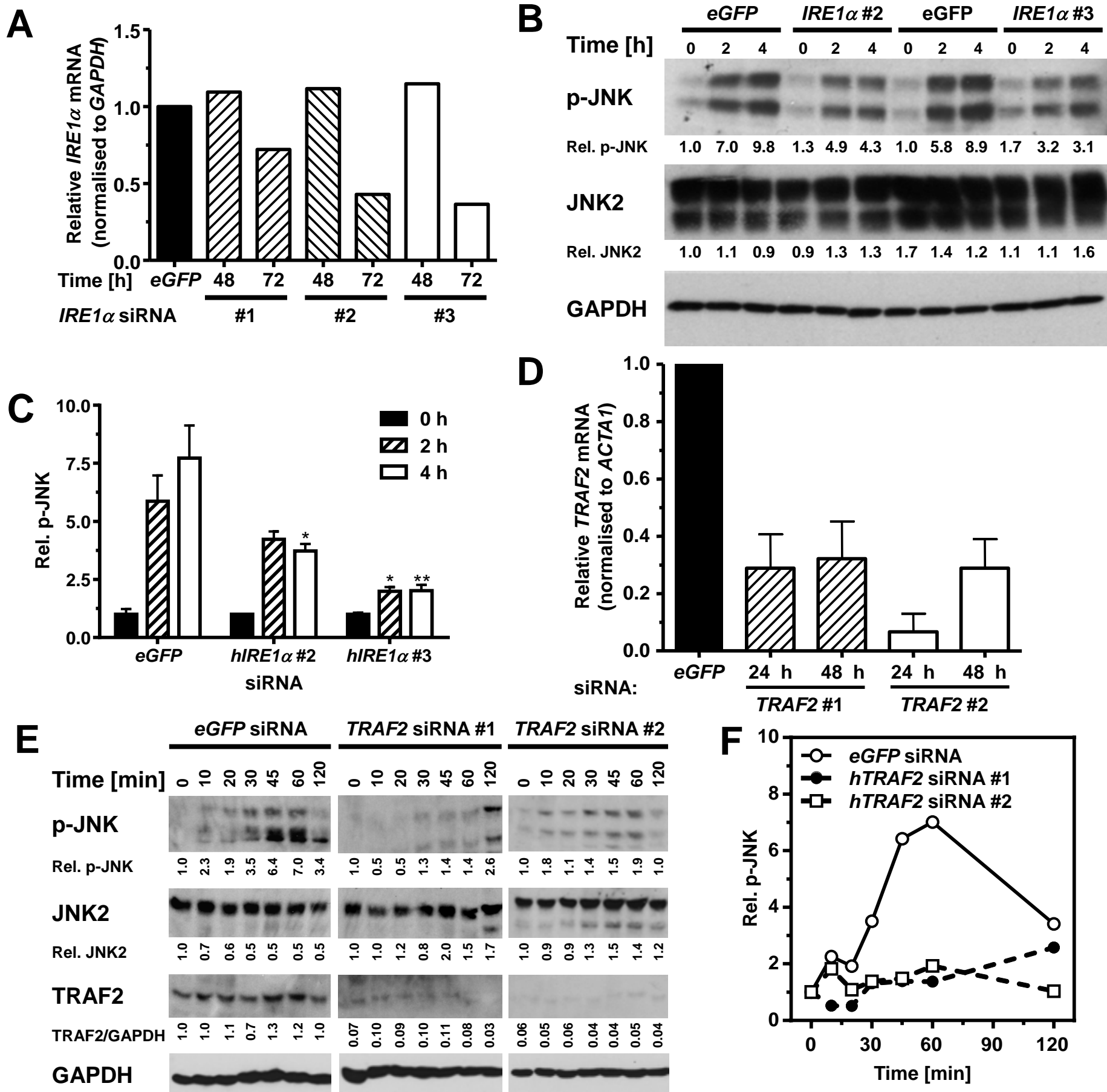


Figure S2, Brown et al.

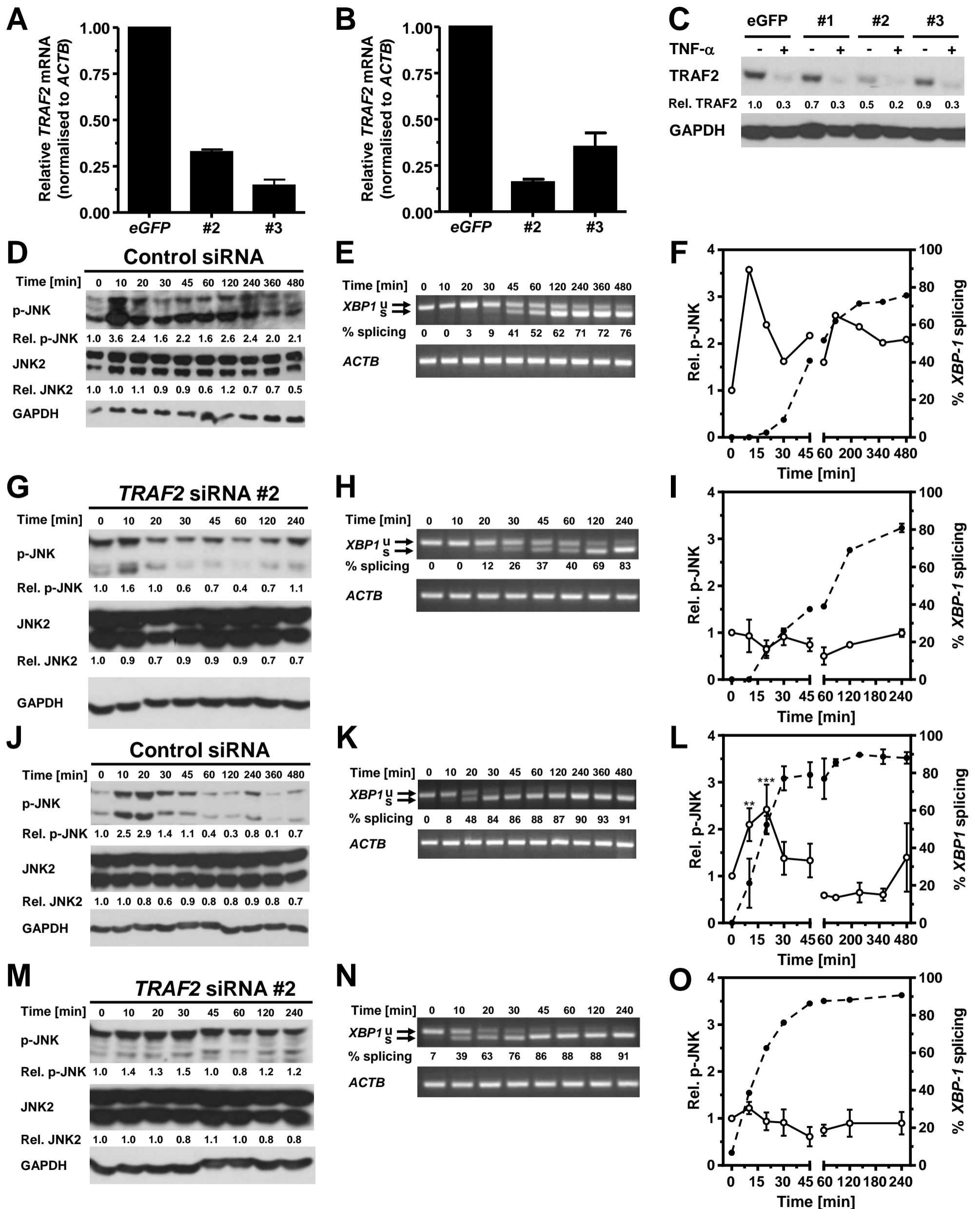


Figure S3, Brown *et al.*

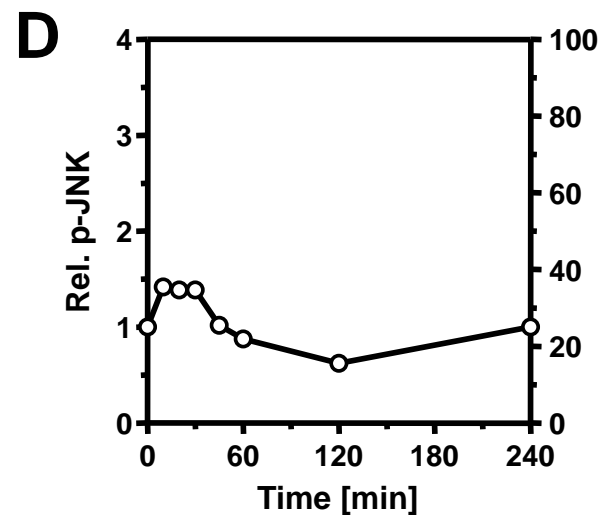
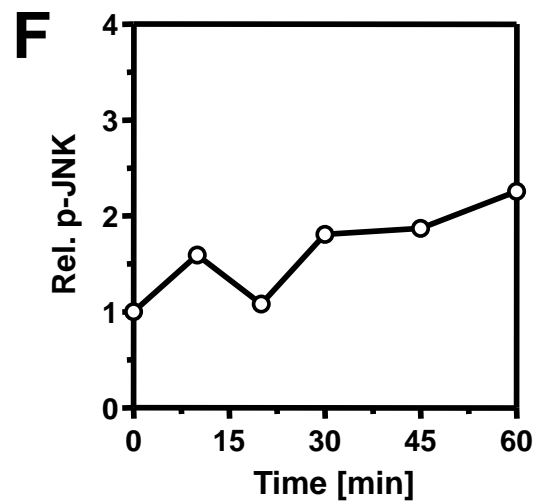
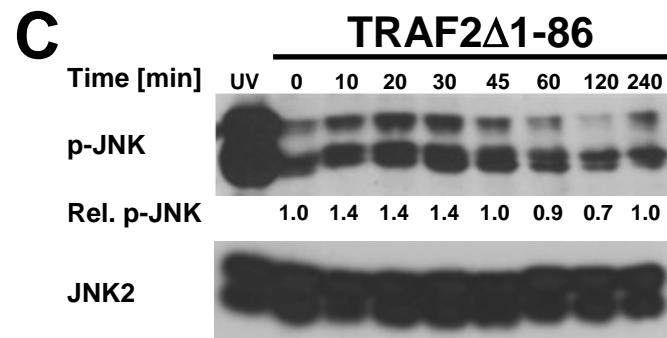
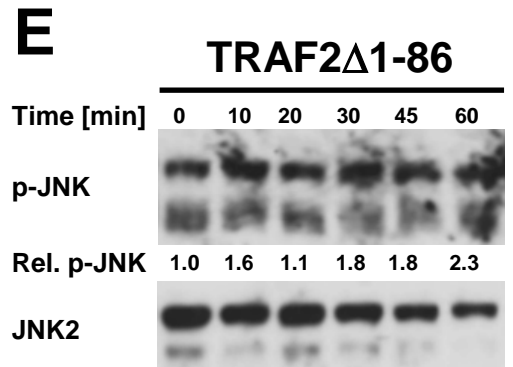
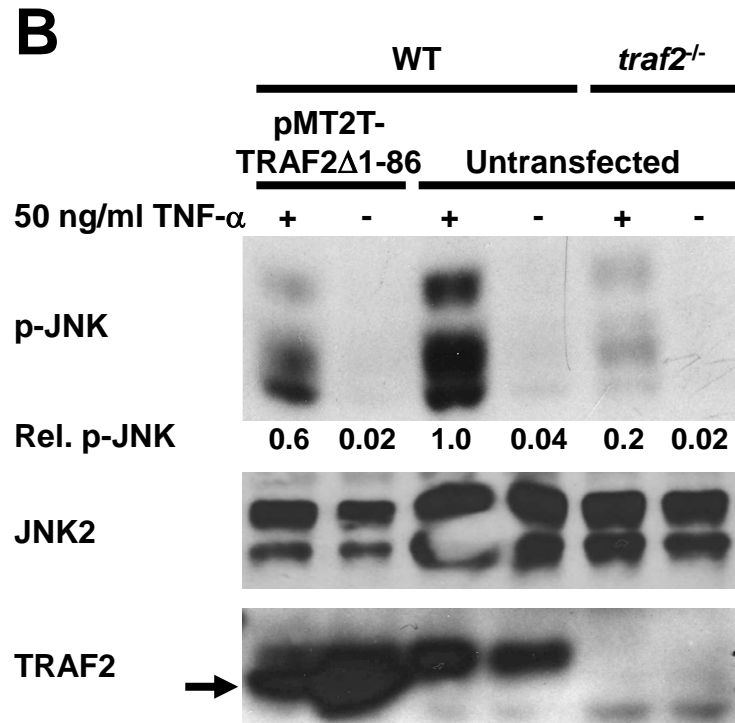
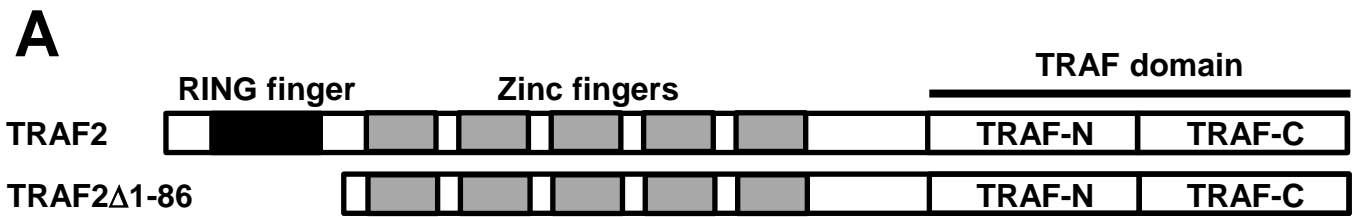


Figure S4, Brown *et al.*

1 **Table S1. siRNAs.**

Species	Gene	#	Sequence
<i>Homo sapiens</i>	<i>IRE1α</i>	1	GCGUAAAUUCAGGACCUAUdTdT
<i>H. sapiens</i>	<i>IRE1α</i>	2	GAUAGUCUCUGCCCAUCAAdTdT
<i>H. sapiens</i>	<i>IRE1α</i>	3	CAUUGCACGUGAAUUGAUAdTdT
<i>H. sapiens</i>	<i>TRAF2</i>	1	CACUCAGAGUGGGAGCACAdTdT
<i>H. sapiens</i>	<i>TRAF2</i>	2	GUCAAGACUUGUGGCAAGUdTdT
<i>H. sapiens</i>	<i>TRAF2</i>	3	GCCUUCAGGCCCGACGUGAdTdT
<i>Mus musculus</i>	<i>TRAF2</i>	1	GAAUUCCUAUGUGCGGGAUdTdT
<i>M. musculus</i>	<i>TRAF2</i>	2	GUUAGAGCAUGCAGCAAUdTdT
<i>M. musculus</i>	<i>TRAF2</i>	3	CTATGAAGGCCTGTATGAAdTdT
<i>Aequora victora</i>	eGFP		GCAAGCUGACCCUGAAGUUCAU

2

1 **Table S2. Oligodeoxynucleotides.** Restriction sites are underlined. The start codon for
 2 TRAF2 Δ 1-86 is shown in bold.

Name	Purpose	Sequence
Oligodeoxynucleotides for <i>H. sapiens</i> genes		
H8197	<i>TRAF2</i> RT-qPCR for siRNA #3, reverse	AATGGCCTTGATGAAGATGG
H8215	<i>TRAF2</i> Δ 1-86 construction, forward primer	TGCATCGAT ATG AGCAGTTCGGCCTTCCCA
H8216	<i>TRAF2</i> Δ 1-86 construction, reverse primer	CGAGCGGCCCGCCACTGTGCTGGATATCTGC
H8280	<i>TRAF2</i> RT-qPCR for siRNA #1, forward	CTTAGCCAAGGGCTGTGGT
H8281	<i>TRAF2</i> RT-qPCR for siRNA #1, reverse	AGGAATGCTCCCTTCTCTCC
H8282	<i>TRAF2</i> RT-qPCR for siRNA #2, forward	GTCCGCCTTGGTGAAAAG
H8283	<i>TRAF2</i> RT-qPCR for siRNA #2, reverse	TCTCACCTCTACCGTCTCG
H8284	<i>TRAF2</i> RT-qPCR for siRNA #3, forward	ACACCAGCAGGTACGGCTAC
H8285	<i>GAPDH</i> RT-qPCR, forward	TCACCAGGGCTGCTTTTAAAC
H8286	<i>GAPDH</i> RT-qPCR, reverse	GGCAGAGATGATGACCCTTT
H8287	<i>ACTA1</i> RT-qPCR, forward	CTGAGCGTGGCTACTCCTTC
H8288	<i>ACTA1</i> RT-qPCR, reverse	GGCATAACAGGTCCCTTCTGA
H8289	<i>XBPI</i> PCR, forward	GAGTTAAGACAGCGCTTGGG
H8290	<i>XBPI</i> PCR, reverse	ACTGGGTCCAAGTTGTCCAG
H8993	<i>IRE1</i> α RT-qPCR, forward	TGGGACAGCTAGGCTGAGAT
H8994	<i>IRE1</i> α RT-qPCR, reverse	TGGGCACATCTGTGATCAAT
Oligodeoxynucleotides for <i>M. musculus</i> genes		
H7961	<i>XBPI</i> PCR, forward	GATCCTGACGAGGTTCCAGA
H7962	<i>XBPI</i> PCR, reverse	ACAGGGTCCAAGTTGTCCAG
H7994	<i>ACTB</i> PCR, forward	AGCCATGTACGTAGCCATCC
H7995	<i>ACTB</i> PCR, reverse	CTCTCAGCTGTGGTGGTGAA

H8237	<i>TRAF2</i> RT-qPCR for siRNA #1, forward	GAACTCATCTGTCTCTCTTCTTCG
H8238	<i>TRAF2</i> RT-qPCR for siRNA #1, reverse	AGCAGGGGTGGCTAGAGTCC
H8239	<i>TRAF2</i> RT-qPCR for siRNA #2, forward	CTGCAGAGCACCCCTGTAGC
H8240	<i>TRAF2</i> RT-qPCR for siRNA #2, reverse	CCTGCAGGTTCTCAGTCTCC
H8269	<i>TRAF2</i> RT-qPCR for siRNA #3, forward	ACTGCTCCTTCTGCCTGACC
H8270	<i>TRAF2</i> RT-qPCR for siRNA #3, reverse	TTCTTTCAAGGTCCCCTTCC
H8271	<i>GAPDH</i> RT-qPCR, forward	TCGTCCCGTAGACAAAATGG
H8272	<i>GAPDH</i> RT-qPCR, reverse	CTCCTGGAAGATGGTGATGG
H8322	<i>MYL1</i> 3f RT-qPCR, forward	TGCTGACCAGATTGCCGACTTCA
H8323	<i>MYL1</i> 3f RT-qPCR, reverse	CCCGGAGGACGTCTCCCACC
H8326	<i>AHCY</i> RT-qPCR, forward	GGTGCTGAGGTGCGGTGGTC
H8327	<i>AHCY</i> RT-qPCR, reverse	GGGTCCGTCCTTGAAGTGCAGC
H8328	<i>TNNC1</i> RT-qPCR, forward	GCACCAAGGAGCTGGGCAAGG
H8329	<i>TNNC1</i> RT-qPCR, reverse	TGTGCCACTGCCATCCTCGT
H9054	<i>cIAP1 (BIRC2)</i> RT-qPCR, forward	TAGTGTTCTGTTCAGCCCG
H9055	<i>cIAP1 (BIRC2)</i> RT-qPCR, reverse	TCCCAACATCTCAAGCCACC
H9056	<i>cIAP2 (BIRC3)</i> RT-qPCR, forward	ACGATTTAAAGGTATCGCGCC
H9057	<i>cIAP2 (BIRC3)</i> RT-qPCR, reverse	CTGATACCGCAGCCCACTTC
H9076	<i>XIAP (BIRC4)</i> RT-qPCR, forward	ACGGAGGATGAGTCAAGTCAA
H9077	<i>XIAP (BIRC4)</i> RT-qPCR, reverse	AAGTGACCAGATGTCCACAAGG
H9080	<i>BRUCE (BIRC6)</i> RT-qPCR, forward	CCAGTGTGAGGAGTGGATTGC
H9081	<i>BRUCE (BIRC6)</i> RT-qPCR, reverse	CCTCAATGTCCGGATCTAAGCC