# Bypass of activation loop phosphorylation by aspartate 836 in activation of the endoribonuclease activity of Ire1

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27

28 **Abstract**

29 The bifunctional protein kinase-endoribonuclease Ire1 initiates splicing of the mRNA for the

30 transcription factor Hac1 when unfolded proteins accumulate in the endoplasmic reticulum.

31 Activation of *Saccharomyces cerevisiae* Ire1 coincides with autophosphorylation of its

32 activation loop at S840, S841, T844, and S850. Mass spectrometric analysis of Ire1 expressed

33 in *Escherichia coli* identified S837 as another potential phosphorylation site *in vivo*. Mutation

34 of all five potential phosphorylation sites in the activation loop decreased, but did not

35 completely abolish, splicing of *HAC1* mRNA, induction of *KAR2* and *PDI1* mRNAs, and

36 expression of a β-galactosidase reporter activated by Hac1[i]. Phosphorylation site mutants

37 survive low levels of ER stress better than *IRE1* deletions strains. *In vivo* clustering and

38 inactivation of Ire1 are not affected by phosphorylation site mutants. Mutation of D836 to

39 alanine in the activation loop of phosphorylation site mutants nearly completely abolished

40 *HAC1* splicing, induction of *KAR2*, *PDI1*, and β-galactosidase reporters, and survival of ER

41 stress, but had no effect on clustering of Ire1. By itself, the D836A mutation does not confer a

42 phenotype. These data argue that D836 can partially substitute for activation loop

43 phosphorylation in activation of the endoribonuclease domain of Ire1.

44 **Introduction**

45 Accumulation of unfolded proteins in the endoplasmic reticulum (ER) activates the

46 bifunctional transmembrane protein kinase-endoribonuclease Ire1 (1, 2). In ER-stressed cells

47 Ire1 oligomerises (3, 4) and concentrates in clusters at the ER membrane (5, 6) independent

48 of its protein kinase activity (6, 7). In the yeast *Saccharomyces cerevisiae*, activated Ire1

49 cleaves the mRNA for the transcription factor *HAC1* to initiate removal of a 252 nt intron

50 from *HAC1* mRNA (8-11) that inhibits translation of unspliced *HAC1* (*HAC1*[u]) mRNA (12).

51 Ligation of the 5' and 3' exons of *HAC1* mRNA by tRNA ligase produces spliced *HAC1*

52 mRNA (*HAC1*[i]) (13), which is readily translated. Hac1[i] induces transcription of genes

53 encoding ER-resident molecular chaperones and protein foldases, such as *BiP/KAR2* and

54 *PDI1*, to alleviate the unfolded protein burden in the ER (14, 15). Many Hac1[i] target genes

55 contain a short promoter element, termed the unfolded protein response element (UPRE)

56 (16), to which Hac1[i] binds as a heterodimer with the transcription factor Gcn4 (17).

57     The N-terminal lobe of protein kinases harbours an activation segment, whose start and

58 end points are defined by the conserved amino acid sequence motifs DFG and APE in *S.*

59 *cerevisiae* Ire1. The activation segment encompasses the $Mg^{2+}$-binding loop, a short β strand,

60 β9 [β10 in Ire1, (18)], the activation loop, and the P+1 loop (Fig. 1) (19). Phosphorylation in

61 the activation loop activates many protein kinases (20). Phosphorylation-dependent protein

62 kinases often display an invariant arginine (R796 in Ire1) that precedes the catalytic aspartate,

63 D797 in Ire1 (20). Crystallographic examination of active conformations of these RD kinases

64 has revealed that phosphoamino acids in the activation loop are in contact with a basic pocket

65 formed by the invariant arginine, a basic amino acid located in β9 (β10 in Ire1), and often a

66 third basic amino acid located in helix αC in the N-terminal lobe (19, 20). The invariant

67 arginine, R796, preceding the catalytic aspartate, D797, and a lysine in β10, K833, have been

68 conserved throughout evolution in Ire1 (Fig. 1). Mass spectrometric mapping of tryptic

69 peptides revealed four potential phosphorylation sites in Ire1, S840, S841, T844, and S850

70 (18). Mutational analyses illustrate the importance of phosphorylation of Ire1 *in vivo*. A

71 S840A S841A double mutant displayed a large defect in induction of both *KAR2* and *PDI1*

72 mRNAs (3), while *HAC1* splicing by a S840A S841A T844A triple mutant and survival of

73 ER stress by a S840A S841A T844A S850A quadruple mutant were severely decreased (18,

74 21). In addition, phosphorylation of human IRE1α increased its RNase activity towards a

75 short, fluorescently-labelled stem loop substrate ~100-fold (22).

76     However, *HAC1* splicing in cells expressing S840A-S841A-T844A-Ire1 nevertheless

77 reached ~20% of the level of *HAC1* splicing in cells expressing WT Ire1 (21), which suggests

78    that the S840A S841A T844A mutant transduces a weak ER stress signal. In addition,

79    mutation of catalytic amino acids in the protein kinase active site, for example in D828A and

80    D797A K799A mutants, decreases, but does not abolish, processing of *HAC1* mRNA by Ire1

81    (7, 21). Finally, addition of the non-phosphorylatable ATP analogue 1-*tert*-butyl-3-

82    naphthalen-1-ylmethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylemine to cells expressing protein

83    kinase deficient mutants restored the ability to process *HAC1* mRNA (23). These

84    observations suggest that activation loop phosphorylation is dispensable for, at least partial,

85    activation of Ire1. The purpose of this study was to elucidate mechanisms that allow Ire1 to

86    transduce an ER stress signal in the absence of phosphorylation in its activation segment. Our

87    work identifies an additional potential phosphorylation site in the activation segment, S837.

88    A mutant lacking all phosphorylation sites in the activation segment, including S837, still

89    transduces a weak ER stress signal. The ability to transduce this weak ER stress signal

90    requires the presence of an aspartate, D836, in the activation loop.

91    **Materials and Methods**

92    **Plasmid constructions.** Plasmids were maintained in *Escherichia coli* XL10-Gold cells

93    (Agilent Technologies, Stockport, UK, cat. no. 200314). Standard protocols were used for

94    plasmids constructions. The single copy *URA3* plasmid pJK59 (24) for expression of Sec63-

95    GFP was obtained from W. A. Prinz (National Institute of Diabetes and Digestive and Kidney

96    Diseases, National Institutes of Health, Bethesda).

97        YCplac33-S840A-S841A-T844A-S850A-*IRE1*-HA (brief YCplac33-Q-A-*IRE1*-HA) was

98    generated by cloning the 2,002 bp *Afe*I-*Msc*I fragment of pC3060-S840A-S841A-T844A-

99    S850A (18) into *Afe*I- and *Msc*I-digested YCplac33-*IRE1*-HA (23). To generate YCplac33-

100    S837A-S840A-S841A-T844A-S850A-*IRE1*-HA (brief YCplac33-P-A-*IRE1*-HA) the 310 bp

101    *Pst*I-*Sac*I fragment of YCplac33-Q-A-*IRE1*-HA was inserted into *Pst*I- and *Sac*I-digested

102    pUC18 (25) and the S837A point mutation introduced by QuikChange site-directed

103  mutagenesis (Agilent Technologies) with primers H8293 and H8294 (Table 1). After

104  confirmation of the mutagenised sequence by Sanger sequencing, the 3,103 bp *Pst*I-*Sac*I

105  fragment was inserted into *Pst*I- and *Sac*I-digested YCplac33-Q-A-*IRE1*-HA (23). To

106  introduce the D836A mutation into wild type (WT) Ire1 and S840A-S841A-Ire1 the 1,405 bp

107  *Hin*dIII-*Sac*I fragments of YCplac33-*IRE1*-HA and YCplac-S840A-S841-*IRE1*-HA (23)

108  were cloned into *Hin*dIII- and *Sac*I-digested pUC18, followed by introduction of the D836A

109  mutation by Quik-Change site-directed mutagenesis with primers H8623 and H8624 in the

110  case of WT Ire1 and primers H8625 and H8626 in the case of S840A-S841A-Ire1. After

111  confirmation of the mutagenised sequence by Sanger sequencing, the 1,405 bp *Hin*dIII-*Sac*I

112  fragments of the two pUC18 plasmids were cloned into YCplac33-*IRE1*-HA to produce

113  YCplac33-D836A-*IRE1*-HA and YCplac33-D836A-S840A-S841A-*IRE1*-HA, respectively.

114  To introduce the D836A mutation into Q-A- and P-A-Ire1, the 1,578 bp *Bam*HI-*Kpn*I

115  fragment of pGEX-1λT-Q-A-C'*IRE1* (Šestak *et al.*, unpublished) was cloned into *Bam*HI-

116  and *Kpn*I-digested pUC18 and the 69 bp *Eco*RV-*Pst*I fragment replaced with annealed and

117  phosphorylated deoxyoligonucleotides H8627 and H8628 or annealed and phosphorylated

118  deoxyoligonucleotides H8629 and H8630. The 1,578 bp *Kpn*I-*Bam*HI fragments of the

119  resulting plasmids were cloned into *Bam*HI- and *Kpn*I-digested pAW42 (26). From the two

120  resulting plasmids, pGEX-1λT-Q-A-C'*IRE1* and pGEX-1λT-P-A-C'*IRE1*, the 310 bp *Pst*I-

121  *Sac*I fragments were cloned into *Pst*I- and *Sac*I-digested YCplac33-Q-A-*IRE1*-HA to

122  generate YCplac33-D836A-Q-A-*IRE1*-HA and YCplac33-D836A-P-A-*IRE1*-HA.

123  For tagging Ire1 with mCherry the 2,013 bp *Afe*I-*Sac*I fragment of pEvA97 (5) was cloned

124  into the corresponding *Afe*I- and *Sac*I-digested YCplac33-*IRE1*-HA plasmids. Then the 3,356

125  bp *Hin*dIII-*Nhe*I fragments of the resulting YCplac33-*IRE1*-HA plasmids were reinserted into

126  pEvA97 because mCherry fluorescence was not detectable in unstressed cells when the

127  mCherry-Ire1 fusion protein was expressed from YCplac33 in initial experiments. pGEX-

128    1λT-L745A-C'IRE1 was constructed by amplifying a 1,277 bp PCR product with primers

129    8619G and H4075A04 from YCplac33-L745A-*IRE1* (23), and cloning the *Bam*HI- and *Pml*I-

130    digested PCR product into *Bam*HI- and *Pml*I-digested pAW42.

131        For galactose-inducible expression of HA- and protein A-tagged Dcr2 and Ptc2, the 2,777

132    bp and 2,435 *Bss*HII fragments of BG1805-*DCR2* and BG1805-*PTC2* (27) were ligated to the

133    6,692 bp *Bss*HII fragment of pRSII422 (28) or the 6,676 bp *Bss*HII fragment of pRS425 (29).

134    H338A-Dcr2 (30) and E37A-D38A-Ptc2 (31) were generated by QuikChange site-directed

135    mutagenesis with primer pairs DCR2-H338A-F and DCR2-H338A-R and PTC2-E37A-

136    D38A-F and PTC2-E37A-D38A-R (Table 1) on BG1805-DCR2 and BG1805-*PTC2*,

137    respectively. After confirmation of the mutagenised sequences by Sanger sequencing, the

138    1,070 bp *Cla*I-*Eag*I fragment of BG1805-H338A-*DCR2* was cloned into *Cla*I- and *Eag*I-

139    digested pRSII422-*DCR2* and the 1,153 bp *Sac*I-*Xho*I-fragment of BG1805-E37A-D38A-

140    *PTC2* was cloned into *Sac*I- and *Xho*I-digested pRSII422-*PTC2* and from there the 2,777 bp

141    *Bss*HII fragments of pRSII422-DCR2 and pRSII422-H338A-*DCR2* and the 2,435 bp *Bss*HII-

142    fragments of pRSII422PTC2 and pRSII422-E37A-D38A-*PTC2* were cloned into *Bss*HII-

143    digested pRS425.

144    **Yeast methods.** Yeast strains (Table 2) were transformed by the LiOAc method (32). Ire1

145    was deleted in W303-1A (33) as described previously (34). *DCR2* and *PTC2* were deleted by

146    PCR-based gene disruption (35) with primer pairs H9327 and H9328 and H9329 and H9330

147    (Table 1) and pFA6a-*kan*MX2 (35) or pFA6a-*hph*NT1 (36) as template. The *GAL1* promoter

148    and a N-terminal T7 epitope tag were introduced by transforming PWY 260 with PCR

149    constructs amplified with primer pairs H9328 and H9331 and H9330 and H9332 from

150    plasmid pFA6a-*kan*MX6-$P_{GAL1}$-T7. pFA6a-*kan*MX6-$P_{GAL1}$-T7 was constructed by inserting

151    annealed and kinased oligonucleotides U5803H01 and U5803H02 into *Pac*I- and *Bam*HI-

152    digested pFA6a-*kan*MX6-$P_{GAL1}$ (37). The T7 sequence in pFA6a-*kan*MX6-$P_{GAL1}$-T7 was

153    confirmed by Sanger sequencing. In frame fusion of the T7 epitope tag to the *DCR2* and

154    *PTC2* open reading frames was confirmed by Sanger sequencing.

155        To induce ER stress, cells were grown to mid-exponential growth phase on synthetic

156    dextrose (SD) medium lacking uracil or leucine (38). 1,4-DL-Dithiothreitol (DTT) was added

157    to a final concentration of 2 mM from a 1 M stock solution made in water. To wash out DTT,

158    cells treated with 2 mM DTT for 2 h were washed once with SD medium lacking uracil and

159    then resuspended in the same culture volume of fresh SD medium lacking uracil. For

160    fluorescence microscopy the concentrations of adenine and L-tryptophan were raised to 100

161    mg/l. Survival of ER stress was scored by spotting 2 µl of serial ten-fold dilutions of fresh

162    overnight cultures onto freshly prepared synthetic media agar plates lacking uracil and

163    containing 2% (w/v) glucose as carbon source. Expression of *DCR2* and *PTC2* from the

164    *GAL1* promoter was induced by growing the cells from single colonies in a small volume of

165    synthetic medium with 1% (w/v) raffinose and 2% (w/v) galactose as carbon source lacking

166    uracil, both uracil and adenine, or both uracil and leucine. Cells were then spotted onto

167    synthetic media plates containing 1% (w/v) raffinose and 2% (w/v) galactose for ER stress

168    survival assays. All overnight cultures were adjusted to optical densities at 600 nm of 3.0

169    before preparing serial ten-fold dilutions. To semiquantitatively evaluate growth assays,

170    threshold dilutions, $D_T$, were defined as the maximum dilutions at which growth could be

171    observed and were determined with an accuracy limit of ~0.5. The difference between the

172    two negative decadic logarithms of the threshold dilutions for tunicamycin exposed cells and

173    untreated cells, $\Delta\log D_T$, is defined as $\Delta\log D_T = -\log D_{T,Tm} - (-\log D_{T,u})$, where $D_{T,Tm}$ is the

174    threshold dilution of tunicamycin-exposed cells and $D_{T,u}$ the threshold dilution of untreated

175    cells. Only differences in $\Delta\log D_T \geq 0.5$ were considered to be significant.

176    **Northern analysis.** Northern blotting was performed as described previously (34), except

177    that cells were lysed by bead-beating with 0.5 mm diameter glass beads (Stratech Scientific,

178     Newmarket, UK, cat. no. 11079105z) in a Precellys 24 instrument (Bertin Technologies,

179     Montigny-le-Bretonneux, France) at 4°C with two cycles of 30 s at 6,500 rpm separated by a

180     30 s break. The probes for *HAC1* (34), *KAR2* (39), *PDI1* (39), and the loading control pC4/2

181     (40) were described previously. All mRNAs were quantified by phosphorimaging on a

182     Typhoon 9400 system (GE Healthcare, Little Chalfont, UK). Volumetric measurements were

183     normalised to the loading control pC4/2. The percentage of *HAC1$^i$* (% *HAC1$^i$*) is defined as

$$\% \, HAC1^i = \frac{HAC1^i}{HAC1^u + HAC1^i + 1^{st} \text{ exon \& intron} + 1^{st} \text{ exon}}$$

184     The percentage of *HAC1* cleavage (% cleavage) is defined as

$$\% \text{ Cleavage} = \frac{HAC1^i + 0.5 \cdot (1^{st} \text{ exon \& intron} + 1^{st} \text{ exon})}{HAC1^u + HAC1^i + 1^{st} \text{ exon \& intron} + 1^{st} \text{ exon}}$$

185     **β-Galactosidase reporter assays** were performed as described before (39). Cells were grown

186     to mid-exponential phase, a zero hour sample collected, and the remainder of the culture

187     exposed to 2 mM DTT and further samples collected after 1 h and 2 h. After washing cells

188     with ice-cold water, protein extracts were produced in 1 x reporter lysis buffer (Promega,

189     Southampton, UK, cat. no. E3971) by bead-beating with 0.5 mm diameter glass beads in a

190     Precellys 24 instrument at 4°C with 3 cycles of 10 s at 6,500 rpm. Between each cycle

191     samples were cooled for 5 min on ice. The lysates were centrifuged at 12,000 g and 4°C for

192     10 min and supernatants were transferred into fresh microcentrifuge tubes to obtain protein

193     extracts. β-Galactosidase activity was standardised to total protein determined with the DC

194     protein assay from Bio-Rad Laboratories (Hemel Hempstead, UK, cat. no. 500-0116).

195     **Protein extraction and Western blotting.** Mid-exponential growth phase cells were washed

196     once with 5 ml of ice-cold water , once with 1 ml ice-cold water and then resuspended in 200

197     μl 8 M urea, 2.5 % (w/v) SDS, 50 mM Tris·HCl, pH 7.5 (at 4°C), 6 mM EDTA, 5 mM

198     β-mercaptoethanol, 2 mM phenylmethanesulphonyl fluoride (PMSF), and 6 mM 4-(2-

199     aminoethyl)-benzenesulphonyl fluoride (AEBSF). The cells were lysed by bead-beating as

200    described for the β-galactosidase reporter assays. Protein concentrations were quantified with

201    bicinchoninic acid (41) after alkylation of β-mercaptoethanol with 0.1 M iodoacetamide in

202    0.1 M Tris·HCl, pH 8.0 at 37°C for 15 min.

203        Proteins (50 μg) were separated on 8% SDS-PAGE gels (42) and transferred to

204    polyvinylidene difluoride (PVDF) membranes (Amersham HyBond[TM]- P, pore size 0.45 μm,

205    GE Healthcare, Little Chalfont, UK, cat. no. RPN303F) by semi-dry electrotransfer in 0.1 M

206    Tris, 0.192 M glycine and 5% (v/v) methanol at 2 mA/cm$^2$ for 75 min. Membranes were

207    stained with 0.5% (w/v) Ponceau S in 1% (v/v) acetic acid for 5 min at RT, destained 3 x 2

208    min with water, dried on air, and photographed. Membranes were then blocked overnight in

209    5% (w/v) skimmed milk powder in TBST [20 mM Tris·HCl, pH 7.6 (at RT), 137 mM NaCl,

210    and 0.1% (v/v) Tween-20] at 4°C and then incubated with a 1:1,000 dilution of rabbit anti-

211    HA antibody (Sigma-Aldrich, Poole, UK, cat. no. H6908, batch no. 015M4868V) in TBST +

212    5% (w/v) skimmed milk powder for 2 h at RT. Blots were washed four times with TBST and

213    then probed with a 1:2,000 dilution of anti-rabbit IgG (H+L)-peroxidase conjugate (Cell

214    Signaling Technology Inc., Danvers, MA, USA, cat. no. 7074S, batch no. 24) in TBST + 5%

215    (w/v) skim milk powder for 1 h at RT. Blots were then washed four times with TBST and

216    developed by enhanced chemiluminescence as described before (43). To probe blots for the

217    actin loading control, blots were stripped after enhanced chemiluminescence detection by

218    washing them twice for 5 min with 100 mM Tris·HCl, pH 8.5 (at RT) and 0.1% (v/v) Tween-

219    20, twice for 15 min with 100 mM Tris·HCl, pH 8.5 (at RT), 200 mM β-mercaptoethanol,

220    and 0.1% (v/v) Tween-20, twice for 5 min with TBST, twice for 15 min with 100 mM

221    glycine, pH 2.5 (at RT) and 0.1% (w/v) Tween-20 at 37°C, and twice for 15 min with TBST.

222    Blots were then blocked overnight at 4°C with 5% (w/v) skimmed milk powder in TBST and

223    then incubated for 1 h at RT with a 1:1·10$^5$ dilution of a mouse anti-β-actin antibody (Abcam,

224    Cambridge, UK, cat. no. ab170825, batch no. GR184354-8) in TBST + 5% (w/v) skimmed

225 milk powder. Blots were washed four times with TBST, incubated for 1 h at RT with a

226 $1:2\cdot10^5$ dilution of a goat anti-mouse IgG (H+L) peroxidase conjugate (Thermo Fisher

227 Scientific, Loughborough, UK, cat. no. 31432, batch no. OE17149612) in TBST + 5% (w/v)

228 skimmed milk powder, washed again four times with TBST, and developed by enhanced

229 chemiluminescence. Films were scanned on a MFC-5320DW all-in-one printer (Brother

230 Industries, Manchester, UK) and saved as tif files. Bands were quantified using CLIQS 1.1

231 (Totallab, Newcastle upon Tyne, UK).

232 **Protein expression and purification.** The cytosolic domains of WT and L745A-Ire1 starting

233 at amino acid 556 were expressed as *N*-terminal GST fusion proteins from plasmid pGEX-

234 1λT (Genbank accession no. U13849) by autoinduction (44) in *E. coli* BL21-

235 CodonPlus(DE3)-RIL cells (Agilent Technologies, cat. no. 230240). These constructs

236 encompass the complete linker, serine/threonine protein kinase, and RNase domains of Ire1.

237 A ZYM-5052 culture containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol was

238 inoculated with 1/1000 volume of a fresh overnight culture grown in MDG medium, grown at

239 37°C for 5 h and then a further 28 h at 20°C (44). The cells were harvested by centrifugation

240 at 4,750 g and 4°C for 15 min and washed twice with ice-cold 0.2 M Tris·HCl, pH 8.0 (at

241 4°C), and finally resuspended in 0.2 M Tris·HCl, pH 8.0 (at 4°C), 0.5 M sucrose, one tablet

242 of Roche complete protease inhibitors lacking EDTA (Roche Applied Sciences, Burgess Hill,

243 UK, cat. no. 11873580001), 2 mM PMSF, 6 mM AEBSF, and 1 μg/ml pepstatin. The cells

244 were lysed by addition of 10 $\mu g/OD_{600\,nm}$ chicken lysozyme (Sigma-Aldrich, cat. no. 62971),

245 addition of EDTA to a final concentration of 1 mM, dilution of the cell suspension with one

246 volume of $H_2O$, and incubation of the cell suspension on a roller mixer for 10 min at room

247 temperature. After addition of 1/10 Vol. of 10% (v/v) Triton X-100 the cell suspension was

248 sonicated in a Soniprep 150 sonicator (MSE Ltd., London, UK) with a 19 mm diameter probe

249 in an ice/NaCl bath for twelve cycles of 1 min sonication with an amplitude of 0.22 microns

250 followed by a cooling period, in which the cell suspension was allowed to cool to 4°C. The

251 lysate was then cleared by centrifugation at 40,000 g and 4°C for 15 min. The GST-Ire1

252 fusion protein was purified by affinity chromatography on GSTrap 4B sepharose columns

253 (GE Healthcare, cat. no. 28-4017-45) and eluted from the column with 20 mM Tris·HCl, pH

254 7.5 (at 4°C), 100 mM NaCl, 5% (v/v) glycerol, 0.1% (w/v) CHAPS, 1 mM EDTA, 2 mM

255 PMSF, and 10 mM glutathione. The eluted protein was concentrated in Amicon Ultra-15

256 centrifugal filters (MWCO 50 kDa, Merck Millipore, cat. no. UFC905008) and desalted on a

257 HiTrap desalting column (GE Healthcare, cat no. 17-1408-01).

258 **Mass spectrometric (MS) identification of Ire1 autophosphorylation sites.** 5 μg of the

259 cytosolic domains of WT and L745A-Ire1 expressed as GST fusion proteins in *E. coli* and

260 purified by affinity chromatography on GSTrap 4B columns as described above were run on

261 an 8% SDS-PAGE gel and stained with Coomassie Brilliant Blue R250. Coomassie-stained

262 protein bands were excised from the gel, transferred to a microtitre plate and destained, buffer

263 exchanged, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin using

264 an automated digestion robot (DiGilab, Genomic Solutions, Ann Arbor, MI, USA) as

265 described before (45).

266 MS/MS analysis of the cytosolic domains of WT and mutant Ire1 expressed in *E. coli* was

267 carried out on an LTQ XL orbitrap mass spectrometer (Thermo Scientific) coupled to an

268 Ultimate 3000 nano HPLC system. The RP-LC system consisted of a desalting column (300

269 μm x 5 mm, PepMap C18 3 μm, 100 Å) and an analytical column (75 μm x 250 mm,

270 PepMap C18 3 μm, 100 Å) with split solvent delivery (split ratio 1:300). A Thermo

271 nanospray II source was fitted with a 30 μm silica emitter tip (PicoTip, New Objective, US)

272 and maintained at 1100 V ion spray voltage. Peptide samples (3 μl) were loaded onto the trap

273 column in 0.1% (w/v) trifluoroacetic acid at 20 μl/min for 3 min and eluted at 300 nl/min

274 using a gradient from 0.05% (v/v) formic acid in water (A) to 0.05% formic acid in 80% (v/v)

275    acetonitrile (B). The gradient profile was as follows: 4% buffer B for 3 min, 4% B to 40% B

276    in 40 min, 40% B to 65% B in 6 min, 65% B to 95% B in 1 min, and 95% B for 6 min. Using

277    Excalibur 2.0.1, intact peptides were detected between $m/z$ 400 and $m/z$ 1,600 in the orbitrap

278    at a resolution of 60,000. Internal calibration was performed using the ion signal of

279    $(Si(CH_3)_3O)_6H^+$ at $m/z$ 445.120025 as a lock mass (46). Maximum ion accumulation time

280    allowed on the LTQ orbitrap was 1 s for all scan modes. Automatic gain control was used to

281    prevent over-filling of the ion trap. Collision induced dissociation (CID) spectra of the top 5

282    peptide ions (minimum intensity 10,000 counts, rejection of singly charged precursors) were

283    acquired at a normalised collision energy of 35. Dynamic exclusion was set with a repeat

284    count of 1, a repeat time of 30 s and, an exclusion time of 3 min and an exclusion list size of

285    50. The chromatography feature was enabled with a correlation area ratio of 1.0. Activation

286    Q was set to 0.25 with 30 ms activation time.

287      XITandem tornado version 2009.04.01.1 (47) was used to search the ENSEMBL yeast

288    proteome database (version SGD1.01.55) and the common repository of adventitious proteins

289    (cRAP, http://www.theqpm.org/crap/index.html) for the identification of proteins and the

290    reversed version of both databases. Precursor mass accuracy was set to 20 ppm and fragment

291    mass error to 0.6 Da. Carbamidomethylation of cysteine was set as a fixed modification and

292    phosphorylation of serine, threonine and tyrosine as a variable modification. At the

293    refinement step, oxidation of methionine and tryptophan, double oxidation of methionine and

294    tryptophan, deamidation of asparagine and glutamine, methylation of aspartate, glutamate,

295    histidine, lysine, arginine and asparagine, carbamidomethylation of lysine, histidine, aspartate

296    and glutamate and dehydration of serine and threonine were included in the search

297    parameters. At a cutoff $\log_e$ value of 1.0, searching the decoy databases indicated a false

298    discovery rate between 1.18% and 1.39% for protein identification. Detailed results and all

299    spectra can be accessed via the following links: WT - http://human.thegpm.org/thegpm-

300    cgi/plist.pl?path=/tandem/archive/GPM28700000061.xml            and         L745A         -

301    http://human.thegpm.org/thegpm-cgi/plist.pl?path=/tandem/archive/GPM28700000059.xml.

302    The mass spectrometry proteomics data have been deposited to the ProteomeXchange

303    Consortium via the PRIDE (48) partner repository with the dataset identifier PXD004924.

304    **Confocal fluorescence microscopy.** Cells grown to mid-exponential growth phase in SD

305    medium lacking leucine and uracil and supplemented with 100 mg/l adenine and 100 mg/l L-

306    tryptophan were concentrated ~60 fold in SD medium lacking leucine and uracil and

307    supplemented with 100 mg/l adenine and 100 mg/l L-tryptophan before addition of 2 mM

308    DTT. 5 μl of untreated cell suspensions or cell suspensions exposed to 2 mM DTT were

309    examined on a Zeiss LSM 880 with Airyscan confocal inverted microscope (Carl Zeiss Ltd.

310    Cambridge, UK) using a Plan-Apochromat 63x/1.4 Oil DIC M27 objective and a MDS

311    488/594 beam splitter. GFP fluorescence was excited with a 488 nm laser at a power setting

312    of 5 – 8% and its fluorescence emission was collected between 498 to 564 nm with a

313    photomultiplier tube. mCherry fluorescence was excited with a 594 nm laser at a power

314    setting of 28 – 40% and its fluorescence emission was collected at 694 – 754 nm with a

315    gallium arsenide phosphide detector. Gain settings between 650 and 900 were used to collect

316    images.

317    **Statistical analyses.** Experimental data are presented as the mean and its standard error. For

318    composite parameters, errors were propagated using the law of error propagation for random,

319    independent errors (49). All data were analysed for normality using the D'Agostino-Pearson

320    omnibus normality test (50), equality of variances (homoscedasticity) using the Brown-

321    Forsythe test (51) or Bartlett's test (52), and for additivity of means, treatment effects, and

322    errors using Tukey's test (53, 54) before ANOVA. Heteroscedastic or nonadditive data were

323    ln-transformed before ANOVA (53) or analysed by Welch's test (55) followed by the

324    Games-Howell post-hoc test (56). Normality was examined on the pooled data for the *ire1Δ*

325    deletion strain transformed with YCplac33 or, alternatively, on all pooled data points before

326    induction of ER stress. In all analyses $P \leq 0.05$ was considered to be statistically significant.

327    Brown-Forsythe tests for equality of variances, Tukey's test for additivity, Welch's tests, and

328    Games-Howell post hoc tests were performed in Microsoft Excel 2010 (Microsoft

329    Corporation, Redmond, WA, USA) using the Real Statistics plug-in for Microsoft Excel

330    (http://www.real-statistics.com/). All other statistical calculations were performed in

331    GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA, USA).

332    **Results**

333    *S837 is a potential autophosphorylation site.* To identify autophosphorylation sites in the

334    activation segment of Ire1, we expressed the cytosolic portion of Ire1 starting at Q556 as an

335    *N*-terminal glutathione *S* transferase fusion protein in *E. coli*, purified it by affinity

336    chromatography on glutathione sepharose beads and analysed tryptic digests by tandem mass

337    spectrometry. Four peptides that cover the activation segment from L835 to R856 carrying

338    one phosphoryl group at S841 (spectrum 1155, data not shown), two phosphoryl groups at

339    S841 and T844 (spectrum 1475, data not shown), three phosphoryl groups at S840, S841, and

340    S850 (spectrum 1976, Fig. 2A), and three phosphoryl groups at S840, T844, and S850

341    (spectrum 2031, Fig. 2B) were identified. In addition, a shorter peptide starting at T844 and

342    carrying one phosphoryl group at S850 was identified (spectra 760 and 816, data not shown).

343    These autophosphorylation sites in the activation segment of WT Ire1 correspond to

344    previously reported autophosphorylation sites in the activation segment (18).

345    We also characterised autophosphorylation of an L745A mutant of Ire1, which possesses

346    an enlarged ATP binding pocket to accommodate chemically-modified, bulkier adenine

347    moieties (57, 58). *In vivo*, L745A-Ire1 supports induction of an UPRE-*lacZ* reporter to ~60%

348    of WT levels (23), which suggests that this mutant possesses significant protein kinase and

349    RNase activities. MS/MS analyses of tryptic peptides derived from the L745A mutant

350 identified several peptides that cover the activation segment from L835 to R856. One carried

351 three phosphoryl groups at S840, S841, and T844 (spectrum 1583, data not shown), another

352 carried four phosphoryl groups at S840, S841, T844, and S850 (spectrum 2251, data not

353 shown), and a third one also carried four phosphoryl groups (spectrum 2263, data not shown).

354 Of these, three could be unambiguously assigned to S840, S841, and T844, while the forth

355 phosphoryl group is located on S850 or T852. No peptide allowed unambiguous assignment

356 of a phosphoryl group to T852. A peptide that covers the activation segment from K834 to

357 R856 carried two phosphoryl groups at S837 and T844, one phosphoryl group at S840 or

358 S841 and another phosphoryl group at S850, T852, or S853 (spectrum 1462, Fig. 2C).

359 Evidence for phosphorylation at S837 is provided by the $m/z$ ratio of the $^{+1}$b ion of $m/z$

360 709.033 Da, which exceeds the $m/z$ ratio for the unphosphorylated peptide by 79.7 Da. This

361 difference matches, within the measurement error of 0.6 Da, the mass difference between a

362 phosphorylated and unphosphorylated peptide. In addition to peptides carrying multiple

363 phosphoryl groups two monophosphorylated peptides, one covering the activation segment

364 from L835 to R843 and being phosphorylated at S841 (spectrum 152, data not shown) and

365 one covering the activation segment from T844 to R856 and being phosphorylated at S850

366 (spectra 488 and 540, data not shown) were detected. These data show that

367 autophosphorylation of WT and L745A Ire1 in the activation segment strongly overlap and

368 raise the possibility that S837 is an additional autophosphorylation site in the activation

369 segment of Ire1.

370 *Mutation of all phosphorylation sites in the activation segment decreases, but does not*

371 *eliminate, HAC1 splicing, induction of ER chaperone genes, and survival of ER stress.* To

372 evaluate the contribution of S837 to activation of Ire1 we characterised the ER stress

373 response of three Ire1 mutants, S840A S841A, S840A S841A T844A S850A (brief: Q-A),

374 and S837A S840A S841A T844A S850A (brief: P-A). A time course experiment revealed

375    that *HAC1* splicing reaches steady-state levels as early as 15 min after induction of ER stress

376    with 2 mM DTT in cells in the mid-exponential growth phase (Fig. 3A-C). An increase in

377    *HAC1* splicing 15 min after induction of ER stress was detectable in all three mutants, but

378    was quantitatively lower than in cells expressing WT Ire1. Differences between the three

379    phosphorylation site mutants were not statistically significant (Fig. 3B, C). 2 h after induction

380    of ER stress the difference between cells lacking Ire1 and cells expressing either S840A-

381    S841A-Ire1 or Q-A-Ire1 were statistically significant (Fig. 3B, C), whereas the difference

382    between cells lacking Ire1 and cells expressing P-A-Ire1 did not become statistically

383    significant (Fig. 3B, C). These two comparisons, cells expressing P-A-Ire1 to cells expressing

384    S840A-S841A- or Q-A-Ire1 and cells expressing P-A-Ire1 to cells lacking Ire1, suggest that

385    S837 may make a minor contribution to activation of Ire1.

386        The differences in *HAC1* splicing were reflected by the induction of *KAR2* and *PDI1*

387    mRNAs. Induction of both mRNAs kinetically trailed *HAC1* splicing and reached a

388    maximum only after ~1 h of ER stress (Fig. 3A, D-E). All three phosphorylation site mutants

389    induced both *KAR2* and *PDI1* mRNAs to similar levels, which remained, especially in the

390    case of *KAR2* mRNA, below the level of induction reached in cells expressing WT Ire1. 2 h

391    after induction of ER stress with 2 mM DTT *KAR2* mRNA, but not *PDI1* mRNA levels, were

392    significantly increased in all autophosphorylation site mutants when compared to cells

393    lacking Ire1, suggesting that even P-A-Ire1 can partially activate expression of *KAR2* in ER-

394    stressed cells.

395        Next, we compared expression of an UPRE-*lacZ* reporter between cells expressing the

396    different autophosphorylation site mutants, because accumulation of the comparatively stable

397    protein β-galactosidase (59) may reveal subtle differences in transduction of the ER stress

398    signal. All three autophosphorylation site mutants induced expression of the reporter, but to a

399    lower degree than cells expressing WT Ire1 (Fig. 4A, B). This observation suggests that Ire1

400 can transduce an ER stress signal in the absence of autophosphorylation in its activation loop.

401 Induction of the *lacZ* reporter was stronger in cells expressing S840A-S841A-Ire1 than in

402 cells expressing either Q-A- or P-A-Ire1 (Fig. 4A), which supports the conclusion that

403 phosphorylation of S837, T844, or S850 contributes to activation of Ire1 at least when

404 phosphorylation at S840 and S841 is no longer possible.

405 To explore the possibility that decreased *HAC1* splicing and *KAR2* and *PDI1* mRNA

406 induction by activation loop mutants is caused by a defect in clustering of Ire1 *in vivo* (5), we

407 monitored foci formation by WT and mutant Ire1 fused to the fluorescent protein mCherry

408 before and after induction of ER stress with 2 mM DTT for 15 min or 60 min. Before

409 induction of ER stress Ire1-mCherry displayed a distribution characteristic for an ER protein

410 with areas of fluorescence around the nucleus and the cell surface, which are indicative of the

411 perinuclear and cortical ER (Fig. 5). This distribution also overlapped with the distribution of

412 a fluorescent marker for the ER, a fusion of GFP to the C-terminus of the Sec63 subunit of

413 the protein translocation channel of the ER membrane (24). In addition to this ER

414 localisation, cells carrying WT and mutant *IRE1* alleles also showed a distinct intracellular

415 mCherry fluorescence that filled most of the cell body (Fig. 5). This mCherry fluorescence

416 colocalises with the fluorescence of the vacuolar stain 7-amino-4-chloromethylcoumarin

417 (data not shown). As early as 15 min after induction of ER stress with 2 mM DTT, a

418 characteristic punctuate fluorescence developed for the mCherry, but not the GFP

419 fluorescence in cells expressing WT Ire1-mCherry. This punctuate fluorescence is indicative

420 of clustering of Ire1 *in vivo* in the ER membrane (5). 1 h after DTT treatment most of the

421 mCherry fluorescence localises to clusters (Fig. 5). There were no noticeable differences in

422 the development of punctuate mCherry fluorescence in cells expressing activation loop

423 mutants of Ire1 fused to mCherry (Fig. 5). These experiments suggest that activation loop

424 mutants do not display a defect in clustering *in vivo*.

425      To evaluate whether the weak ER stress response of the phosphorylation site mutants

426    suffices to survive ER stress, we characterised the survival of these mutants under conditions

427    of low levels of ER stress. Consistent with the gene expression data, all phosphorylation site

428    mutants displayed a small degree of protection against ER stress, as evidenced by their

429    improved growth on plates containing 0.4 μg/ml tunicamycin when compared to cells lacking

430    Ire1 (Fig. 6). At higher concentrations of tunicamycin this growth advantage of the

431    phosphorylation site mutants over the *IRE1* deletion strain was diminished. Activation loop

432    mutants express to levels comparable to WT Ire1 in both the S288C and W303 genetic

433    backgrounds (Fig. 7 and data not shown), which suggests that the partial defect in responding

434    to ER stress displayed by activation loop mutants cannot be explained by decreased

435    intracellular abundance of mutant Ire1 proteins. In summary, these data show that all three

436    autophosphorylation site mutants can activate a weak, but physiologically significant, ER

437    stress response.

438    *D836 is required for the ER stress response mediated by phosphorylation site mutants.* The

439    activation loop of Ire1 features an aspartate that has been functionally conserved throughout

440    evolution in fungal and plant Ire1 (Fig. 1). Phosphorylation-independent RD kinases can

441    employ a negatively charged glutamate located in the activation loop to stabilise the basic

442    pocket formed by the invariant arginine and the basic amino acid located in strand β9 (20).

443    For example, in phosphorylase kinase glutamate 182 neutralises the invariant arginine that

444    precedes the catalytic aspartate (60). Mutation of this glutamate to serine decreases catalytic

445    efficiency ~20 fold (61). In partially active human CDK2-cyclin A complexes the basic

446    pocket is stabilised by interaction with glutamate 162 (62). For these reasons, we introduced a

447    D836A mutation into WT Ire1 and the three phosphorylation site mutants and compared the

448    ER stress response of these mutants to the ER stress response of WT Ire1, S840A-S841A-

449    Ire1, Q-A-Ire1, and P-A-Ire1.

450      Splicing of *HAC1* mRNA and induction of both *KAR2* and *PDI1* mRNA were monitored

451    in time course experiments similar to the experiments described above. Introduction of the

452    D836A mutation into WT Ire1 had no effect on *HAC1* splicing or induction of *KAR2* and

453    *PDI1* mRNAs (Fig. 8). By contrast, introduction of the D836A mutation into S840A-S841A-

454    Ire1, Q-A-Ire1, or P-A-Ire1 resulted in a virtually complete loss of *HAC1$^i$* mRNA and

455    abrogated induction of *KAR2* mRNA (Fig. 8). *PDI1* mRNA no longer increased to levels

456    significantly above levels seen in cells deleted for *IRE1*. Despite of the absence of *HAC1*$^i$

457    mRNA, faint bands representing cleavage intermediates, such as the 1$^{st}$ exon of *HAC1*$^u$

458    mRNA plus the intron and the 1$^{st}$ exon of *HAC1*$^u$ mRNA could still be observed in cells

459    expressing D836A-S840A-S841A-, D836A-Q-A-, or D836A-P-A-Ire1 (Fig. 8A).

460      Introduction of the D836A mutation nearly completely eliminated expression of

461    β-galactosidase from UPRE-*lacZ* reporters when introduced into S840A-S841A- and Q-A-

462    Ire1 (Fig. 9). Introduction of the D836A mutation into P-A-Ire1 further decreased expression

463    of β-galactosidase 2 h after induction of ER stress with 2 mM DTT (from $11.2 \pm 1.6$ U/g to

464    $5.6 \pm 1.4$ U/g) to levels very close to and statistically undistinguishable from levels seen in

465    *IRE1* deletion strains exposed to ER stress for 2 h ($2.7 \pm 0.4$ U/g), but this decrease did not

466    reach statistical significance. Differences in expression levels of the β-galactosidase reporter

467    in *IRE1* deletion cells, cells expressing D836A-S840A-S841A-, D836A-Q-A-, or D836A-P-

468    A-Ire1 were not statistically significant (Fig. 7). The D836A mutation, however, did not

469    affect expression of β-galactosidase when introduced into WT Ire1 (Fig. 9). The D836A

470    mutation did also not affect clustering of Ire1 *in vivo*, either in the context of otherwise WT

471    Ire1 or the activation loop mutants (Fig. 10), or steady-state expression levels of Ire1 (Fig. 7).

472    Introduction of the D836A mutations into the phosphorylation site mutants resulted in growth

473    phenotypes very similar to the growth phenotypes of *IRE1* deletion cells (Fig. 11), but, as in

474    the case of the gene expression data the D836A mutation by itself did not decrease survival of

475　ER stress induced with tunicamycin or DTT (Fig. 11). Taken together, these data support the

476　conclusion that D836 is required for the residual ER stress response only when activation

477　loop phosphorylation is impaired.

478　*Activation loop mutants do not affect inactivation of Ire1.* To investigate whether mutations

479　in the activation loop affect the inactivation of Ire1, we characterised the decay of *HAC1*

480　splicing, *KAR2* and *PDI1* mRNA after washout of DTT from cells exposed to 2 mM DTT for

481　2 h. The percentage of *HAC1^i* mRNA, the percentage of *HAC1* mRNA cleavage, *KAR2* and

482　*PDI1* mRNA levels decayed with first order kinetics (Fig. 12). The lower maximal responses

483　of the S840A S841A, Q-A, and P-A mutants to 2 mM DTT (Figs. 3, 8, and 12) allow these

484　mutants to return to basal levels of *HAC1* splicing, *KAR2* and *PDI1* mRNA levels earlier than

485　cells expressing WT and D836A-Ire1 (Fig. 12). We did not find any statistically significant

486　differences in the decay rates for any of the Ire1 mutants in an ordinary one way ANOVA

487　with Tukey's correction for multiple comparisons. Likewise, no obvious differences between

488　WT Ire1 and any Ire1 mutants in the dissolution of Ire1 clusters at the ER membrane were

489　observed (Fig. 5 and Fig. 10). These data suggest that inactivation of Ire1 is independent of

490　D836 and phosphorylation sites in the activation loop.

491　*Negative regulation of Ire1 by the phosphatase Ptc2 requires phosphorylation sites in the*

492　*activation loop of Ire1.* The identical inactivation kinetics for WT Ire1 and activation loop

493　mutants prompted us to characterise whether two negative regulators of Ire1, the

494　phosphatases Dcr2 (30) and Ptc2 (31), negatively regulate Ire1 through its activation loop.

495　Overexpression of Ptc2 from the *GAL1* promoter on a 2 μ plasmid inhibited growth (31).

496　Overexpression of WT Ptc2, but not catalytically inactive E37A-D38A- or D234A-Ptc2, also

497　inhibited growth of cells exposed to a low concentration tunicamycin (31). Consistent with

498　this earlier report, we find that overexpression of Ptc2 from the *GAL1* promoter on the 2 μ

499　plasmid pRSII422 inhibited growth of unstressed cells (Fig. 13A). Overexpression of

500    catalytically inactive E37A-D38A-Ptc2 also inhibited growth of unstressed cells, but to a

501    lesser extent than overexpression of WT Ptc2 (Fig. 13A). Deletion of *IRE1* slightly impaired

502    growth of unstressed cells on raffinose and galactose, but also masked the negative effects of

503    expression of WT or E37A-D38A-Ptc2 on growth of unstressed cells (Fig. 13A). Likewise,

504    expression of D836A-P-A-Ire1 masked the negative effects of expression of WT or E37A-

505    D38A-Ptc2 on growth of unstressed cells, suggesting that WT and E37A-D38A-Ptc2 act

506    through Ire1 to inhibit growth of unstressed cells.

507         These genetic interactions between *IRE1* and *PTC2* in unstressed cells complicate the

508    interpretation of effects of overexpression of Ptc2 in ER-stressed cells expressing different

509    *IRE1* alleles. Therefore, we semiquantitatively scored the effects of overexpression of Ptc2 on

510    survival of ER stress by calculating the differences between the maximum dilutions at which

511    growth of more than two cells can be observed for ER-stressed and unstressed cells. This

512    scoring system revealed that expression of WT Ptc2, but not E37A-D38A-Ptc2, in cells

513    expressing WT or D836A-Ire1 impaired growth in the presence of ER stress induced with 0.8

514    µg/ml tunicamycin (Fig. 13A). This observation is consistent with the earlier finding that

515    overexpression of WT, but not catalytically inactive Ptc2, inhibited growth of ER-stressed

516    cells (31). The negative effects of overexpression of WT Ptc2 on growth of ER-stressed cells

517    were abrogated in *ire1Δ* cells or cells expressing P-A- or D836A-P-A-Ire1 (Fig. 13A). These

518    data show that deletion of *IRE1* or expression of a mutant in which all potential

519    phosphorylation sites in the activation loop have been mutated to alanine mask the effects of

520    overexpression of WT Ptc2 on growth of ER-stressed cells.

521         Overexpression of Dcr2 by placing the *GAL1* promoter in front of the chromosomal

522    *DCR2* gene was reported to inhibit growth when ER stress was induced with tunicamycin

523    (30). In contrast to this finding, we did not observe any inhibition of growth by

524    overexpression of Dcr2 using a similar expression system (Fig. 13B). We then explored

525  whether further elevation of Dcr2 levels by expressing Dcr2 from the *GAL1* promoter on the

526  2 μ plasmid pRSII422 affects growth of ER-stressed cells, because overexpression of Ptc2

527  from the *GAL1* promoter on a 2 μ plasmid inhibited growth of ER-stressed cells (Fig. 13A)

528  while overexpression of Ptc2 by placing the *GAL1* promoter in front of the chromosomal

529  *PTC2* gene had no effect of growth of ER-stressed cells (data not shown). Overexpression of

530  WT Dcr2 and catalytically inactive H338A-Dcr2 (63) from the *GAL1* promoter on a 2 μ

531  plasmid inhibited growth of unstressed WT cells to the same extent, but did not affect

532  survival of ER stress (Fig. 13C). Deletion of *IRE1* slightly impaired growth on raffinose and

533  galactose (Fig. 13C) and largely masked the negative effects of expression of WT and

534  H338A-Dcr2 on growth of unstressed cells (Fig. 13C). Expression of WT or H338A-Dcr2 did

535  not alter survival of ER stress by *ire1*Δ cells (Fig. 13C). These data suggest that

536  overexpression of Dcr2 does not affect survival of ER stress.

537  To characterise whether deletion of *DCR2* or *PTC2* affects survival of ER stress we

538  constructed *dcr2*Δ and *ptc2*Δ strains, and double *dcr2*Δ *ptc2*Δ strains. Survival of ER stress

539  was not altered by deletion of *DCR2*, deletion of *PTC2*, or by simultaneous deletion of both

540  *DCR2* and *PTC2* (Fig. 13D). These data suggest that negative regulation of Ire1 by Dcr2 and

541  Ptc2 to optimally tune the amplitude of the Ire1 signalling output is not required to survive

542  ER stress or that other phosphatases exist that can compensate for the loss of both Dcr2 and

543  Ptc2 in *dcr2*Δ *ptc2*Δ cells.

544  **Discussion**

545  The data presented here show that Ire1 can transduce a partial ER stress signal in the absence

546  of phosphorylation in its activation loop (Figs. 3 and 4). The partial activation of *HAC1*

547  splicing by activation loop mutants resulted in partial induction of *KAR2* and *PDI1* mRNAs

548  (Fig. 3D, E) and partial protection from ER stress (Fig. 6). In addition, we show that

549  transduction of this partial ER stress signal by activation loop mutants relies on the presence

550 of a negatively charged amino acid such as an aspartate or a glutamate in the activation loop,

551 which has been conserved throughout evolution in fungal and plant Ire1 (Fig. 1). Mutation of

552 this aspartate, D836 in *S. cerevisiae* Ire1, to alanine in activation loop mutants nearly

553 completely eliminated production of *HAC1ⁱ* mRNA, induction of *KAR2* and *PDI1* mRNA, of

554 an UPRE-*lacZ* reporter, and survival of ER stress (Figs. 8, 9 and 11). In this way,

555 introduction of the D836A mutation into activation loop mutants is reminiscent of the *in vitro*

556 behaviour of human IRE1α. IRE1α lacks negatively charged amino acids in its activation

557 loop (Fig. 1A) and displayed a ~100-fold increase of its $V_{max}$ and $k_{cat}$ upon phosphorylation

558 (22). By contrast, the D836A mutation by itself had no effect, because the levels of *HAC1*

559 splicing, induction of *KAR2* and *PDI1* mRNA and of the UPRE-*lacZ* reporter, and survival of

560 ER stress were indistinguishable from cells expressing WT Ire1. These observations suggest

561 that D836 can partially substitute for activation loop phosphorylation in the activation loop

562 mutants.

563 The role of activation loop phosphorylation in activation of Ire1 is thought to lie in

564 conformational changes in the activation loop that result in opening of the ATP binding

565 pocket (18). Binding of ATP then induces oligomerisation of Ire1 and RNase activity (18). In

566 this model for activation of Ire1, point mutations in the protein kinase domain, that largely

567 inactivate the protein kinase activity of Ire1, should display a significant loss of RNase

568 activity. However, mutation of D828, which contributes to coordinating two $Mg^{2+}$ ions

569 important for catalysis of the γ-phosphoryl transfer reaction, or mutation of the catalytic

570 aspartate, D797, did not destroy RNase activity (7, 21). Retention of RNase activity by these

571 protein kinase mutants may, as in the case for the activation loop mutants, be explained by

572 the presence of D836 in the activation loop.

573 Based on its primary amino acid sequence, Ire1 belongs to the family of RD kinases and

574 its reliance on activation loop phosphorylation for full activity [Figs. 2 and 3 and (22)]

575    suggests that Ire1 belongs to the family of phosphorylation-dependent RD protein kinases. In

576    these protein kinases phosphoamino acids in the activation loop move into a basic pocket

577    formed by the invariant arginine that precedes the catalytic aspartate (R796 in *S. cerevisiae*

578    Ire1) and a second basic amino acid located in strand β9 (K833 on strand β10 in Ire1). The

579    crystal structure of oligomeric Ire1 shows phosphorylated T844 in contact with this basic

580    pocket (4), as would be expected for a phosphorylation-dependent RD kinase. However,

581    phosphorylation at T844 is of lesser importance than phosphorylation at S840 or S841,

582    because induction of both *KAR2* and *PDI1* mRNAs was more severely affected in the S840A

583    S841A double mutant than the T844A single mutant (3). The importance of S840 or S841 in

584    activation of Ire1 is further supported by the observation that all phosphorylation site mutants

585    show a similar decrease in *HAC1* splicing and induction of both *KAR2* and *PDI1* (Fig. 3).

586    This suggests that the other phosphorylation sites may only play minor roles or require the

587    presence of S840 or S841 to mediate activation of the endoribonuclease domain. Data

588    obtained from the β-galactosidase reporter assays (Fig. 4) indicate that the other

589    phosphorylation sites can mediate some activation of the endoribonuclease domain

590    independent of S840 or S841. Introduction of the D836A mutation into any of the three

591    phosphorylation site mutants nearly completely abolished *HAC1* splicing, induction of *KAR2*,

592    *PDI1*, and the β-galactosidase reporter, and survival of ER stress (Figs. 8, 9, and 11). This

593    behaviour of the D836A mutation suggests that the residual ER stress response transduced by

594    S840A-S841A-Ire1 relies on D836 and that D836 can partially substitute for functions

595    provided by S840 or S841 in WT Ire1. In the absence of D836, S840 and S841 the other

596    phosphorylation sites can no longer mediate effective activation of Ire1 (Figs. 8, 9, and 11),

597    which suggests that a function provided by D836, S840 or S841 is necessary for activation of

598    Ire1. At the same time, induction of the β-galactosidase reporter decreases from S840A-

599    S841A-Ire1, to Q-A-Ire1, and P-A-Ire1 (Fig. 4), which suggests that the ability of D836 to

600 mediate activation of Ire1 requires at least one of the other phosphorylation sites. These data

601 are consistent with the view that the activation loop makes at least two contacts necessary for

602 activation of Ire1, one mediated by S840 or S841, and in their absence, and to a lesser degree,

603 by D836, and a second one mediated by one of the other phosphorylation sites.

604 The crystal structure of oligomeric Ire1 suggests that two contacts of the activation loop

605 necessary for activation of Ire1 are made between phosphorylated S840 and S841 and R896

606 of the same molecule and K678 of an adjacent Ire1 molecule, and between phosphorylated

607 T844 and the RD pocket (4). D836 may simply substitute for S840 and S841 by contacting

608 the same basic pocket, while phosphorylated T844 still remains in contact with the RD

609 pocket, but it is also possible that D836 contacts the RD pocket and that phosphorylated

610 T844, phosphorylated S837, or phosphorylated S850 contact the basic pocket formed by

611 R896 and K678 in S840A S841A mutants. Contacts made between the phosphorylated

612 activation loop and basic pockets have been proposed to facilitate oligomerisation of Ire1 (4).

613 *In vivo* clustering of Ire1, however, was not affected by the activation loop mutants (Fig. 5) or

614 by introduction of the D836A mutation into any of the activation loop mutants (Fig. 10).

615 These observations suggest that surfaces other than the phosphorylated activation loop suffice

616 to mediate efficient clustering of Ire1 *in vivo*. Therefore, it seems that the critical roles of

617 activation loop phosphorylation in activation of Ire1 may not lie in facilitating clustering of

618 Ire1.

619 D836 can only partially substitute for phosphorylation of S840 or S841, because all

620 activation loop mutants can only support levels of *HAC1* splicing that are significantly lower

621 than in cells expressing WT Ire1. This may be due to the decreased negative charge of a

622 carboxylate when compared to a phosphate and subsequent partial neutralisation of positive

623 charges in basic pockets, such as the basic pocket formed by R896 and K678 of an adjacent

624 Ire1 molecule, resulting in decreased stability of active conformations. In addition, steric

625    constraints may prevent the smaller aspartate to move as close to positive charges as

626    phosphoamino acids might, which again may destabilise active conformations. Furthermore,

627    D836 can only make one contact to basic pockets, whereas, for example, phosphorylated

628    S840 and S841 can make two contacts to the same basic pocket (4). The lack of a phenotype

629    for cells expressing D836A-Ire1 suggests that conformations formed by phosphorylated Ire1

630    are more stable or longer-lived than any conformations in which D836 attempts to take over

631    any roles of phosphorylation. Alternatively, phosphorylation may be kinetically

632    outcompeting formation of any conformations in which D836 replaces phosphoserines or

633    phosphothreonines.

634        Inactivation of Ire1 is thought to involve its dephosphorylation (21, 22), but also its

635    autophosphorylation in its αEF insertion loop (7, 21). The similar inactivation kinetics of WT

636    Ire1 and activation loop mutants (Fig. 12) suggests that inactivation of Ire1 is a twostep

637    process in which fast dephosphorylation of Ire1 precedes slower, phosphorylation-

638    independent steps (Fig. 12). In the DTT wash-out experiments (Fig. 12) only the slower

639    phosphorylation-independent steps may have been observed. These phosphorylation-

640    independent steps may represent the reassociation of Kar2 with the luminal domain of Ire1

641    (64-67), the disassembly of Ire1 oligomers, or the clearance of misfolded and damaged

642    proteins from the ER via ER-associated degradation or dilution by growth. We observed no

643    noticeable differences in the disassembly of Ire1 foci in cells expressing WT Ire1,

644    phosphorylation site mutants (Fig. 5), or a combination of phosphorylation site mutants and

645    the D836A mutation (Fig. 10). Thus, while activation of Ire1 leading to *HAC1* splicing

646    requires at least one negative charge in the activation loop, inactivation of Ire1 and

647    dissolution of Ire1 clusters proceeds independent of negative charges on the activation loop.

648        The initial, fast dephosphorylation in the inactivation of Ire1 may be mediated by the

649    phosphatases Dcr2 (30) or Ptc2 (31). Overexpression of Ptc2 in cells expressing different

650     *IRE1* alleles revealed that deletion of *IRE1* or expression of *IRE1* alleles that lack all potential

651     phosphorylation sites mask the effects of overexpression of Ptc2 (Fig. 13A). These findings

652     are consistent with the view that Ptc2 may attenuate Ire1 signalling by dephosphorylating the

653     activation loop of Ire1, resulting in decreased levels of *HAC1* splicing (31). However,

654     decreased phosphorylation of the activation loop in activation loop mutants may impair ATP

655     binding (18) and decrease the protein kinase activity of Ire1 leading to decreased

656     phosphorylation of other regions of Ire1 such as its αEF insertion loop. Therefore, the

657     epistatic relationship between Ire1 and Ptc2 may also be explained by dephosphorylation of

658     regions other than the activation loop by Ptc2. In contrast to Ptc2, we found no evidence that

659     overexpression of Dcr2 affects survival of ER stress (Fig. 13B, C). We also do not observe a

660     synthetic growth defect in *ire1Δ dcr2Δ* cells (Fig. 13D) or that deletion of *DCR2* impairs

661     survival of ER stress which contrasts to previously reported results (30). While these different

662     results may be explained by the different genetic backgrounds, W303 and S288c, used in the

663     two studies, we also did not observe any effects of overexpression of Dcr2 from the *GAL1*

664     promoter on the 2 µ plasmid pRS425 in the S288c genetic background (data not shown).

665        Deletion of *IRE1* or expression of D836A-P-A-Ire1 also masks the toxic effects of

666     overexpression of WT and catalytically inactive Ptc2 and Dcr2 on growth of unstressed cells

667     (Fig. 13A, C). For *PTC2* similar epistatic relationships to *IRE1* seem to exist in unstressed

668     and ER-stressed cells, suggesting that Ptc2 may act through the same mechanism in

669     unstressed and ER-stressed cells. It is unlikely that unmasking of the inositol auxotrophy of

670     *ire1Δ* cells (68) by overexpression of Ptc2 can explain the growth inhibitory effects of Ptc2,

671     because increasing the inositol concentration did not mitigate the growth inhibitory effects of

672     overexpression of Ptc2 (data not shown). Deletion of *IRE1* is also epistatic to overexpression

673     of Dcr2 in unstressed cells (Fig. 13C). The lack of any effect of overexpression of *DCR2* on

674     growth in the presence of ER stress suggests another genetic relationship between *IRE1* and

675 *DCR2* than between *IRE1* and *PTC2*. Deletion of *IRE1* may, for example, perturb a secretory

676 pathway function that is located upstream of a secretory pathway function of Dcr2.

677     In summary, our work shows that yeast Ire1 retains the ability to transduce a weak ER

678 stress signal when all its phosphorylation sites in its activation have been mutated to alanine.

679 The ability of these activation loop mutants to respond to ER stress relies on the presence of a

680 negatively charged amino acid in the activation loop. These findings provide a molecular

681 explanation for some of the differences between yeast and mammalian Ire1.

697 **Author contributions**

698 MCA, SS, AAA, AT, and MS designed the experiments and analysed the data. MCA, SS,

699 AAA, HAMS, MB, KB, and MS performed the experiments. MS devised the study and wrote

700 the manuscript. All authors read and approved of the manuscript. The authors declare no

701 competing or financial interests.

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884 **Figure legends**

885 **Figure 1. Sequence alignment of the activation segment of Ire1.** The GenBank accession

886 numbers are: *Arabidopsis thaliana* 1 - NP_568444, *A. thaliana 2* - NP_565419, *Ashbya*

887 *gossypii* - NP_984389, *Aspergillus fumigatus* - XP_749922, *Caenorhabditis elegans* -

888 AAL30828, *Candida albicans* - XP_717532, *Drosophila melanogaster* - NP_001097839,

889 *Homo sapiens* α - NP_001424; *H. sapiens* β - NP_150296, *Saccharomyces cerevisiae* -

890 NP_011946, and *Trichoderma reesei* - AAP92915. The sequence for *Saccharomyces bayanus*

891 Ire1 was obtained from the *Saccharomyces genome* database. Amino acids in the activation

892 loop are shown in blue, potential phosphoacceptor sites in red, and aspartic and glutamic acid

893 in orange.

894 **Figure 2. Ire1 autophosphorylates at S837. (A)** Fragmentation spectrum 1976 for the

895 peptide [835]LDSGQpSpSFRTNLNNPpSGTSGWR[856] derived from WT Ire1. Detected mass to

896 charge ratios for the [+1]y (red) and [+2]y (blue) ion series are shown. Brackets highlight

897 fragmentations that are explained by the presence of phosphoryl groups. The difference

898    between the observed mass to charge ratio and the monoisotopic mass to charge ratio for the

899    unphosphorylated ions are indicated. **(B)** Fragmentation spectrum 2031 for the peptide

900    $^{835}$LDSGQpSSFRpTNLNNPpSGTSGWR$^{856}$ derived from WT Ire1. **(C)** Fragmentation

901    spectrum 1462 for the peptide $^{834}$KLDpSGQp(SS)FRpTNLNNPp(SGTS)GWR$^{856}$ derived

902    from L745A-Ire1. Detected mass to charge ratios for the $^{+1}$y (red) and $^{+1}$b (oranges) ion series

903    are shown. In addition, one phosphoryl group is bound to S840 or S841, another phosphoryl

904    group to S850, T852, or S853.

905    **Figure 3. Mutation of phosphorylation sites in the activation loop decreases, but does**

906    **not abolish, cleavage of *HAC1* mRNA by Ire1. (A)** Northern blots for *HAC1*, *KAR2*, *PDI1*,

907    and the loading control pC4/2 (40) on RNA extracted from *ire1Δ* strains expressing the

908    indicated *IRE1* alleles from YCplac33 or carrying empty vector ('-'). Mid-exponential growth

909    phase cells were treated with 2 mM DTT for the indicated times. Abbreviations: *HAC1*$^{u}$ -

910    unspliced *HAC1* mRNA, *HAC1*$^{i}$ - spliced *HAC1* mRNA, 1$^{st}$ + i. - 1$^{st}$ exon of *HAC1*$^{u}$ mRNA

911    plus the intron, and 1$^{st}$ - 1$^{st}$ exon of *HAC1*$^{u}$ mRNA. **(B)** Quantification and 95% (open

912    symbols), 99% (half-filled symbols), or 99.9% (filled symbols) confidence intervals of the

913    percentage of *HAC1*$^{i}$ mRNA (% *HAC1*$^{i}$), **(C)** the percentage of *HAC1* mRNA cleavage (%

914    Cleavage), **(D)** induction of *KAR2* and of **(E)** *PDI1* mRNAs. Bars represent the standard error

915    (*n* = 8 for the WT, *n* = 5 for all other strains). The confidence intervals for % *HAC1*$^{i}$, %

916    cleavage, ln-transformed *KAR2* and *PDI1* mRNA levels were calculated using an ordinary

917    two-way ANOVA with Tukey's correction for multiple comparisons (69). Abbreviations: 2S-

918    A = S840-A S841A, P-A = S837A S840A S841A T844A S850A and Q-A = S840A S841A

919    T844A S850A.

920    **Figure 4. Mutation of all phosphorylation sites in the activation loop decreases, but does**

921    **not abolish, induction of UPRE-*lacZ* reporters. (A)** β-Galactosidase activity standardised

922    to total cellular protein before, 1 h, and 2 h after induction of ER stress with 2 mM DTT in

923 mid-exponential *ire1Δ* cells expressing the indicated *IRE1* alleles from YCplac33 or carrying

924 empty vector ('-'). Bars represent standard errors (*n* = 3 for all strains). **(B)** 95% (open

925 symbols), 99% (half-filled symbols), or 99.9% (filled symbols) confidence intervals (CI)

926 were calculated for the ln-transformed data with an ordinary two-way ANOVA with Tukey's

927 correction for multiple comparisons.

928 **Figure 5. Activation loop phosphorylation is dispensable for clustering of Ire1 *in vivo*.**

929 Location of Sec63-GFP and Ire1-mCherry in unstressed cells and cells exposed to 2 mM

930 DTT for 15 min, 1 h or after wash out of DTT for 1 h from cells treated with 2 mM DTT for

931 2 h. Sec63-GFP was expressed from the single copy *URA3* plasmid pJK59 in *ire1Δ* cells

932 transformed with single copy *LEU2* plasmids derived from pEvA97 that carry the indicated

933 *IRE1* alleles. Images covering ~100 cells were taken, except for the DTT wash out

934 experiment in which ~20 cells were analysed. Representative images are shown. Scale bar – 5

935 μm.

936 **Figure 6. Survival of ER stress by activation loop mutants.** Survival of ER stress induced

937 with 0.4 and 0.8 μg/ml tunicamycin (Tm). Serial 10-fold dilutions of fresh overnight cultures

938 of *ire1Δ* cells expressing the indicated *IRE1* alleles from YCplac33 or carrying empty vector

939 ('-') were spotted on SD minus uracil plates containing 0.4 or 0.8 μg/ml Tm and allowed to

940 grow for 2-3 d before taking photographs. #1 and #2 indicate two independent transformants

941 for the S840A S841A and Q-A mutants. The experiment was repeated three times with

942 qualitatively similar results.

943 **Figure 7. Expression of WT and mutant Ire1 proteins. (A)** Western blots for HA-tagged

944 Ire1 and Act1 isolated from mid-exponential growth phase *ire1Δ* strains expressing the

945 indicated *IRE1* alleles from YCplac33 or carrying empty vector ('-'). Cells were treated for 2

946 h with 2 mM DTT where indicated ('+'). PVDF membranes were stained with Ponceau S

947 after electrotransfer of proteins from 8% SDS-PAGE gels. **(B)** Quantification of Ire1-HA

948    levels relative to the Act1 loading control. The expression level of Ire1-HA in untreated WT

949    cells was arbitrarily set to 1.0. The data were analysed with an ordinary two-way ANOVA

950    with Tukey's correction for multiple comparisons. No significant differences in Ire1-HA

951    expression levels were detected, except for the negative control strain transformed with

952    empty vector. Bars represent standard errors ($n = 7$ for the WT, $n = 4$ for the Q-A, D836A Q-

953    A, P-A, and D836A P-A mutants, and $n = 3$ for the D836A, S840A S841A, and D836A

954    S840A S841A mutants and the strain transformed with empty vector).

955    **Figure 8. D836 is required for cleavage of *HAC1* mRNA by activation loop mutants. (A)**

956    Northern blots for *HAC1*, *KAR2*, *PDI1*, and the loading control pC4/2 (40) on RNA extracted

957    from *ire1Δ* strains expressing the indicated *IRE1* alleles from YCplac33 or carrying empty

958    vector ('-'). Mid-exponential growth phase cells were treated with 2 mM DTT for the

959    indicated times. **(B)** Quantification of the percentage of $HAC1^i$ mRNA (% $HAC1^i$), **(C)** the

960    percentage of *HAC1* mRNA cleavage (% Cleavage), **(D)** induction of *KAR2* and of **(E)** *PDI1*

961    mRNAs. Bars represent standard errors. * - $P \le 0.05$, ** - $P \le 0.01$, *** - $P \le 0.001$, and

962    **** - $P \le 0.0001$. *P* values for % $HAC1^i$ and % cleavage were determined by Welch's test

963    followed by a Games-Howell post-hoc test. ($n = 12$ for the WT, $n = 4$ for the empty vector

964    transformed *ire1Δ* strain, and $n = 6$ for all other strains). *P* values for *KAR2* and *PDI1*

965    induction were obtained from an ordinary two-way ANOVA with Tukey's correction for

966    multiple comparisons on the ln-transformed data.

967    **Figure 9. D836 is required for induction of UPRE-*lacZ* reporters by activation loop**

968    **mutants.** β-Galactosidase activity standardised to total cellular protein before, 1 h, and 2 h

969    after induction of ER stress with 2 mM DTT in mid-exponential *ire1Δ* cells expressing the

970    indicated *IRE1* alleles from YCplac33 or carrying empty vector ('-'). Bars represent standard

971    errors ($n = 12$ for WT Ire1 and cells transformed with empty vector, $n = 9$ for all other

972    strains). * - $P \leq 0.05$ and **** - $P \leq 0.0001$. *P* values were obtained from an ordinary two-

973    way ANOVA with Tukey's correction for multiple comparisons on the ln-transformed data.

974    **Figure 10. D836 is not required for clustering of Ire1 *in vivo*.** Location of Sec63-GFP and

975    Ire1-mCherry in unstressed cells and cells exposed to 2 mM DTT for 15 min, 1 h or after

976    wash out of DTT for 1 h from cells treated with 2 mM DTT for 2 h. Sec63-GFP was

977    expressed from plasmid pJK59 in *ire1Δ* cells transformed with single copy *LEU2* plasmids

978    derived from pEvA97 that carry the indicated *IRE1* alleles. Images covering ~100 cells were

979    taken, except for the DTT wash out experiment in which ~20 cells were analysed.

980    Representative images are shown. Scale bar – 5 μm.

981    **Figure 11. Survival of ER stress by activation loop mutants requires D836.** Survival of

982    ER stress induced with 0.8 μg/ml Tm or 1.5 mM DTT. Serial 10-fold dilutions of fresh

983    overnight cultures of *ire1Δ* cells expressing the indicated *IRE1* alleles from YCplac33 or

984    carrying empty vector were spotted on SD minus uracil plates containing 0.8 μg/ml Tm or 1.5

985    mM DTT and allowed to grow for 2-3 d before taking photographs. The experiment was

986    repeated three times with qualitatively similar results.

987    **Figure 12. Mutation of phosphorylation sites in the activation does not alter inactivation**

988    **of Ire1. (A)** Northern blots for *HAC1*, *KAR2*, *PDI1*, and the loading control pC4/2 (40) on

989    RNA extracted from *ire1Δ* strains expressing the indicated *IRE1* alleles from YCplac33 or

990    carrying empty vector ('-'). Mid-exponential growth phase cells were treated with 2 mM

991    DTT for 2 h, before washing the cells once with culture medium and resuspending the cells in

992    fresh, DTT-free medium. **(B)** Plot of the natural logarithm of the percentage of *HAC1^i* mRNA

993    over time. **(C)** Plot of the natural logarithm of the percentage of *HAC1* mRNA splicing over

994    time. **(D)** Plot of the natural logarithm of *KAR2* mRNA over time. **(E)** Plot of the natural

995    logarithm of *PDI1* mRNA over time. Dotted lines represent the 95% confidence intervals of

996    the linear regression models. The first order rate constants, $k_{\text{off}}$, were calculated from the

997    slopes of the linear regression models.

998    **Figure 13. Mutation of all phosphorylation sites in the activation loop of Ire1 is epistatic**

999    **to overexpression of Ptc2. (A)** Effect of overexpression of WT and catalytically inactive

1000   E37A-D38A Ptc2 from the *GAL1* promoter on a 2 μ plasmid on survival of ER stress induced

1001   with 0.8 μg/ml Tm. Fresh overnight cultures of *ire1Δ* cells expressing the indicated *IRE1*

1002   alleles from YCplac33 and the indicated $P_{GAL1}$-*PTC2* alleles from pRSII422 were grown on

1003   1% (w/v) raffinose and 2% (w/v) galactose and spotted in 10 fold serial dilutions onto plates

1004   containing 1% (w/v) raffinose and 2% (w/v) galactose and, where indicated, 0.8 μg/ml Tm.

1005   Plates were incubated for 7 d at 30 °C. The negative decadic logarithms of the dilutions *D* of

1006   the 10-fold dilution series are shown on top of the plates. The threshold dilutions for

1007   untreated cells, $D_{T,u}$, and cells exposed to tunicamycin, $D_{T,Tm}$, and the difference between

1008   both threshold dilutions, $\Delta\log D_T$, are shown to the right of the plates. **(B)** Effect of

1009   overexpression of Dcr2 by placing the *GAL1* promoter in front of the endogenous *DCR2* gene

1010   on survival of ER stress. Serial 10-fold dilutions of fresh overnight cultures of *ire1Δ* cells and

1011   *ire1Δ* $P_{GAL1}$-*T7-DCR2* cells expressing WT *IRE1* from YCplac33 grown on 1% (w/v)

1012   raffinose and 2% (w/v) galactose were spotted onto plates containing 1% (w/v) raffinose and

1013   2% (w/v) galactose and, where indicated, 0.4 μg/ml or 0.8 μg/ml Tm. Plates were incubated

1014   for 4 d at 30 °C. **(C)** Effect of overexpression of WT and catalytically inactive H338A Dcr2

1015   from the *GAL1* promoter on a 2 μ plasmid on survival of ER stress. Fresh overnight cultures

1016   of *ire1Δ* cells expressing the indicated *IRE1* alleles from YCplac33 and the indicated $P_{GAL1}$-

1017   *DCR2* alleles from pRSII422 were grown on 1% (w/v) raffinose and 2% (w/v) galactose and

1018   spotted in 10 fold serial dilutions onto plates containing 1% (w/v) raffinose and 2% (w/v)

1019   galactose and, where indicated, 0.4 μg/ml Tm. Plates were incubated for 7 d at 30 °C. **(D)**

1020   Deletion of both *DCR2* and *PTC2* does not affect survival of ER. Serial 10-fold dilutions of

1021   fresh overnight cultures of *ire1Δ* cells, *ire1Δ dcr2Δ* cells, *ire1Δ ptc2Δ* cells, and *ire1Δ dcr2Δ*

1022   *ptc2Δ* cells expressing the indicated *IRE1* alleles from YCplac33 were spotted onto SD minus

1023   uracil plates containing 0.4 μg/ml Tm, 1 mM DTT, or 2 mM 2-deoxy-D-glucose (2-DOG) to

1024   induce ER stress. Plates were incubated for 3 d at 30 °C. All spotting assays were repeated at

1025   least once with qualitatively similar results.

1026

1027 **Table 1.** Oligodeoxynucleotides. Restriction sites are underlined. Mutagenic base

1028 substitutions in oligodeoxynucleotides used for site-directed mutagenesis are shown in bold.

| Name | Sequence |
| --- | --- |
| 8691G | TGTGCAGGATCCCAAAGATTCAAAATTTTGCCGCC |
| DCR2-H338A-F | CAATGGTATGGGGAAAT**GC**CGACGACGAGGGAAGCT |
| DCR2-H338A-R | AGCTTCCCTCGTCGTCG**GC**ATTTCCCCATACCATTG |
| H4075A04 | TGCCTTAGAACTTTCATAGC |
| H8293 | GGTCTTTGCAAAAAACTAGAC**GCCGGC**CAGGCAGCATTTAGAGCAAAT |
| H8294 | ATTTGCTCTAAATGCTGCCTG**GCCGGC**GTCTAGTTTTTTGCAAAGACC |
| H8623 | TTGATATCAGACTTTGGTCTTTGCAAAAAACTAG**CTAGC**GGTCAGTCTTCATTTAGAACAAATTTGAATAACC |
| H8624 | GGTTATTCAAATTTGTTCTAAATGAAGACTGACC**GCTAG**CTAGTTTTTTGCAAAGACCAAAGTCTGATATCAA |
| H8625 | TTTGATATCAGACTTTGGTCTTTGCAAAAAACTAG**CTAGC**GGTCAGGCTGCATTTAGAACAAATTTG |
| H8626 | CAAATTTGTTCTAAATGCAGCCTGACC**GCTAG**CTAGTTTTTTGCAAAGACCAAAGTCTGATATCAAA |
| H8627 | ATCAGACTTTGGTCTTTGCAAAAAACTAG**CTAGC**GGTCAGGCAGCATTTAGAGCAAATTTGAATAACCCTGCA |
| H8628 | GGGTTATTCAAATTTGCTCTAAATGCTGCCTGACC**GCTAG**CTAGTTTTTTGCAAAGACCAAAGTCTGAT |
| H8629 | ATCAGACTTTGGTCTTTGCAAAAA**G**CTAG**C**CGCCGGCCAGGCAGCATTTAGAGCAAATTTGAATAACCCTGCA |

H8630          GGGTTATTCAAATTTGCTCTAAATGCTGCCTGGCCGGCG**G**CTAG**C**T

TTTTGCAAAGACCAAAGTCTGAT

H9327          TCATAAATACGGATACGTCTTTCTGTACCTCCATAGCCAGCATAAC

CACCAAGCTTCGTACGCTGCAGG

H9328          AGTTTTATACTTAAGTATCGAAGACCAGCACCGTGGTTAAAAATCT

TAACAGGCCACTAGTGGATCTG

H9329          GCCGGAGGTCTTGCTCTTGGATTGGCTGGAAGGGTCAAGATTTTCT

GCATAAGCTTCGTACGCTGCAGG

H9330          TCCCTAGGATTTTGACTATTCCATTGTTGTATAAAATATAGAGAAC

CAGAAGGCCACTAGTGGATCTG

H9331          ACTACCAAGTATAATAGGTACCTTTGATACAGCCTCGGTAACCGGA

TCATACCCATTTGCTGTCCACCAG

H9332          CCGGAGTGGCTCTCTTTATCAATTACCGGGTTTGATAGAATTTGTC

CCATACCCATTTGCTGTCCACCAG

PTC2-E37A-D38A-F  ACATTAGGCTCTAGAATGTGTGAA**G**CC**G**CCAT**G**GACATCCGCCACC

PTC2-E37A-D38A-R  GGTGGCGGATGTC**C**ATGG**C**GG**C**TTCACACATTCTAGAGCCTAATGT

U5803H01       TAAATGGCTAGCATGACTGGTGGACAGCAAATGGGTG

U5803H02       GATCCACCCATTTGCTGTCCACCAGTCATGCTAGCCATTTAAT

1029

1030    **Table 2**. *Saccharomyces cerevisiae* strains. All strains, except Y01907, carry the alleles

1031    *MAT**a** ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1.*

| Name | Genotype | Reference |
|------|----------|-----------|
| W303-1A | | (33) |
| PWY 260 | *ire1Δ*::*TRP1 his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | (23) |
| MSY 14-02 | *ire1Δ*::*kan*MX2 | This study. |
| MSY 792-02 | *ire1Δ*::*TRP1 dcr2Δ*::*kan*MX2 *his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | This study. |
| MSY 793-06 | *ire1Δ*::*TRP1 ptc2Δ*::*kan*MX2 *his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | This study. |
| MSY 794-11 | *ire1Δ*::*TRP1 kanMX6-P_{GAL1,10}-T7-DCR2 his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | This study. |
| MSY 795-01 | *ire1Δ*::*TRP1 kanMX6-P_{GAL1,10}-T7-PTC2 his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | This study. |
| MSY 796-02 | *ire1Δ*::*TRP1 dcr2Δ*::*kan*MX2 *ptc2Δ*::*hph*NT1 *his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | This study. |
| MSY 797-02 | *ire1Δ*::*TRP1 dcr2Δ*::*hph*NT1 *ptc2Δ*::*kan*MX2 *his3-11,15*::*HIS3*^{+}UPRE-*lacZ leu2-3,-112*::*LEU2*^{+}UPRE-*lacZ* | This study. |
| Y01907 | *MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ire1Δ*::*kan*MX4 | (70) |

1032

1033

|                      |     |         |                   |                  |     |
|----------------------|-----|---------|-------------------|------------------|-----|
| *S. cerevisiae*      | 828 | DFGLCKK | LD-SG-QSSF-RT-NLNN | PS-GTSGWRAPE     | 859 |
| *S. bayanus*         | 828 | DFGLCKK | LD-SG-QSSF-RT-NLNN | PS-GTSGWRAPE     | 859 |
| *A. fumigatus*       | 858 | DFGLCKK | LD-D-NQSSFRAT--TAH | AA-GTSGWRAPE     | 889 |
| *A. gossypii*        | 843 | DFGLCKK | LEAE--ESSF-KT-NINN | AA-GTSGWRAPE     | 874 |
| *C. albicans*        | 928 | DFGLCKK | LEND--QSSFRAT--TQN | AASGTSGWRAPE     | 960 |
| *T. reesei*          | 953 | DFGLCKK | LE-D-RQSSFGAT--TGR | AA-GTSGWRAPE     | 984 |
| *C. elegans*         | 659 | DFGLCKR | VQP-GKNS-ISRGIASG- | LA-GTDGWIAPE     | 691 |
| *D. melanogaster*    | 690 | DFGLCKK | LNF-GKTS-FSRR--SG- | VT-GTDGWIAPE     | 720 |
| *H. sapiens* alpha   | 711 | DFGLCKK | LAV-GRHS-FSRR--SG- | VP-GTEGWIAPE     | 741 |
| H. sapiens beta      | 660 | DFGLCKK | LPA-GRCS-FSLH--SG- | IP-GTEGWMAPE     | 690 |
| *A. thaliana* 1      | 628 | DMGISKR | LPAD---TSA-KTRNST- | GLGGSSGWQAPE     | 660 |
| *A. thaliana* 2      | 590 | DMGISKR | MSRD--MSSLGH--LAT- | GS-GSSGWQAPE     | 620 |

Mg²⁺ β10    Activation loop    P+1 loop

## - DTT

| | Sec63 | Ire1 | Merge |
|---|---|---|---|
| **WT** | | | |
| **S840A S841A** | | | |
| **Q-A** | | | |
| **P-A** | | | |

## + 2 mM DTT 15 min

| | Sec63 | Ire1 | Merge |
|---|---|---|---|
| **WT** | | | |
| **S840A S841A** | | | |
| **Q-A** | | | |
| **P-A** | | | |

## + 2 mM DTT 60 min

| | Sec63 | Ire1 | Merge |
|---|---|---|---|
| **WT** | | | |
| **S840A S841A** | | | |
| **Q-A** | | | |
| **P-A** | | | |

## 60 min after 2 mM DTT
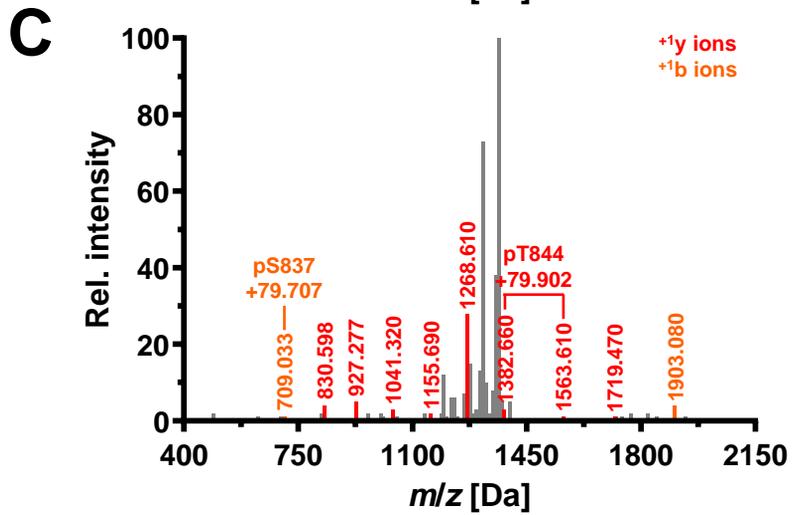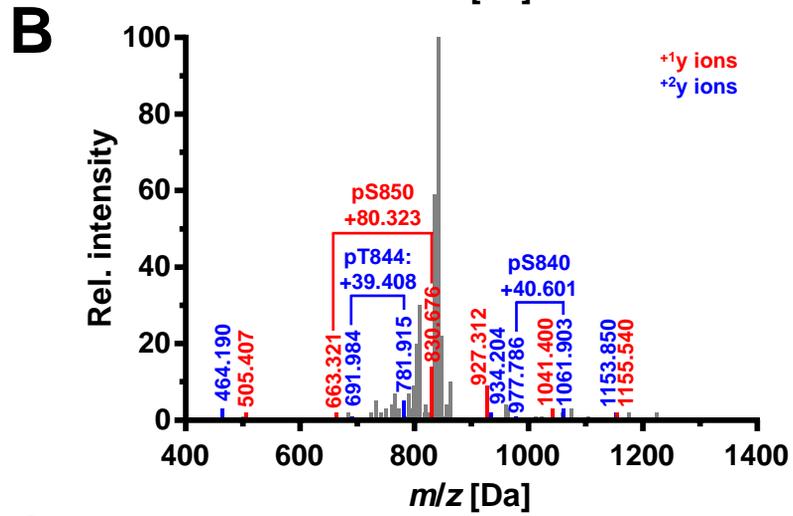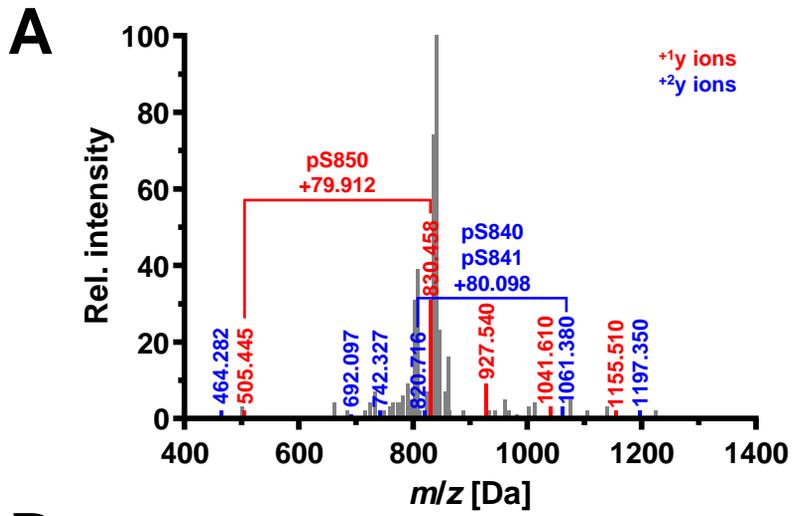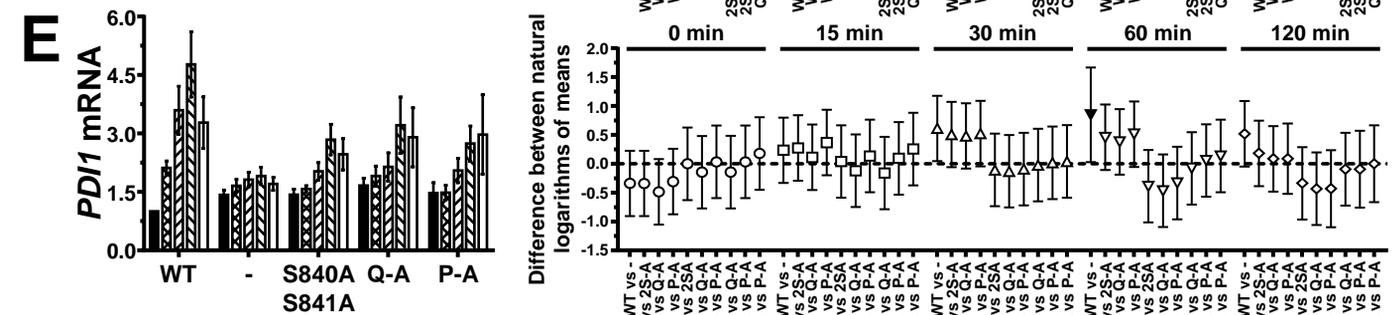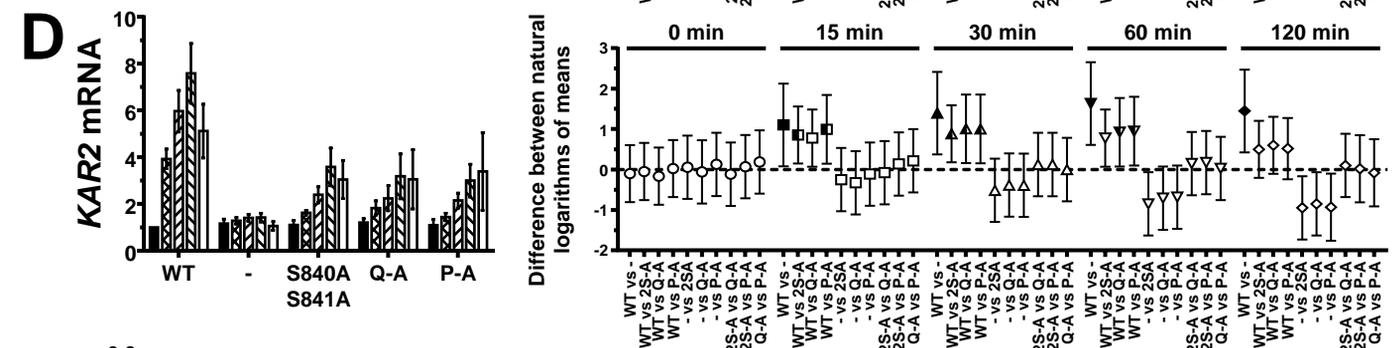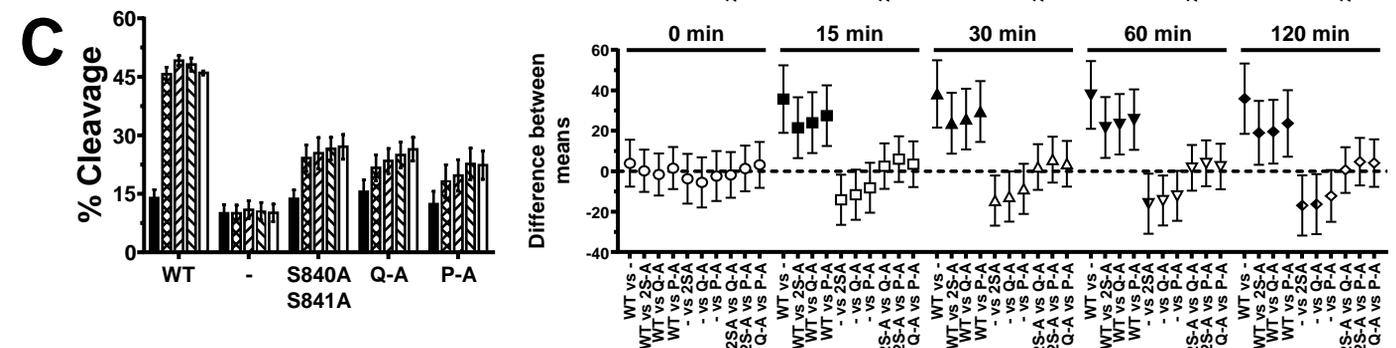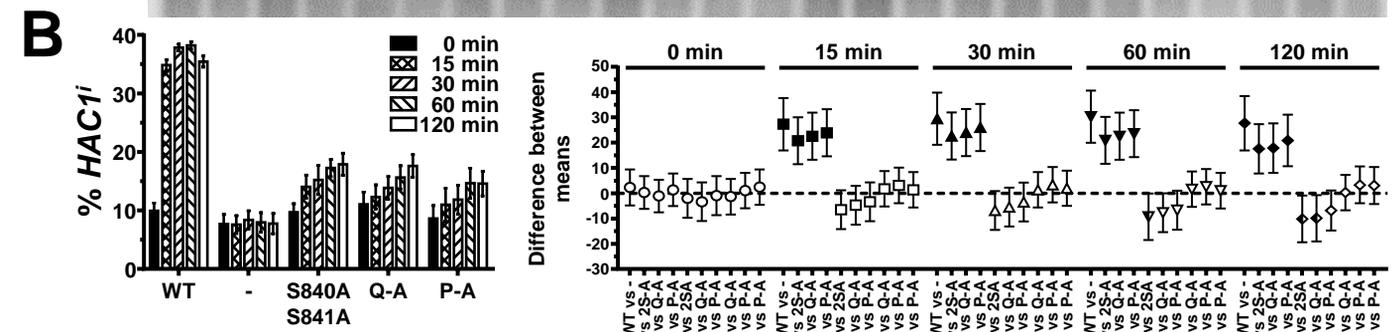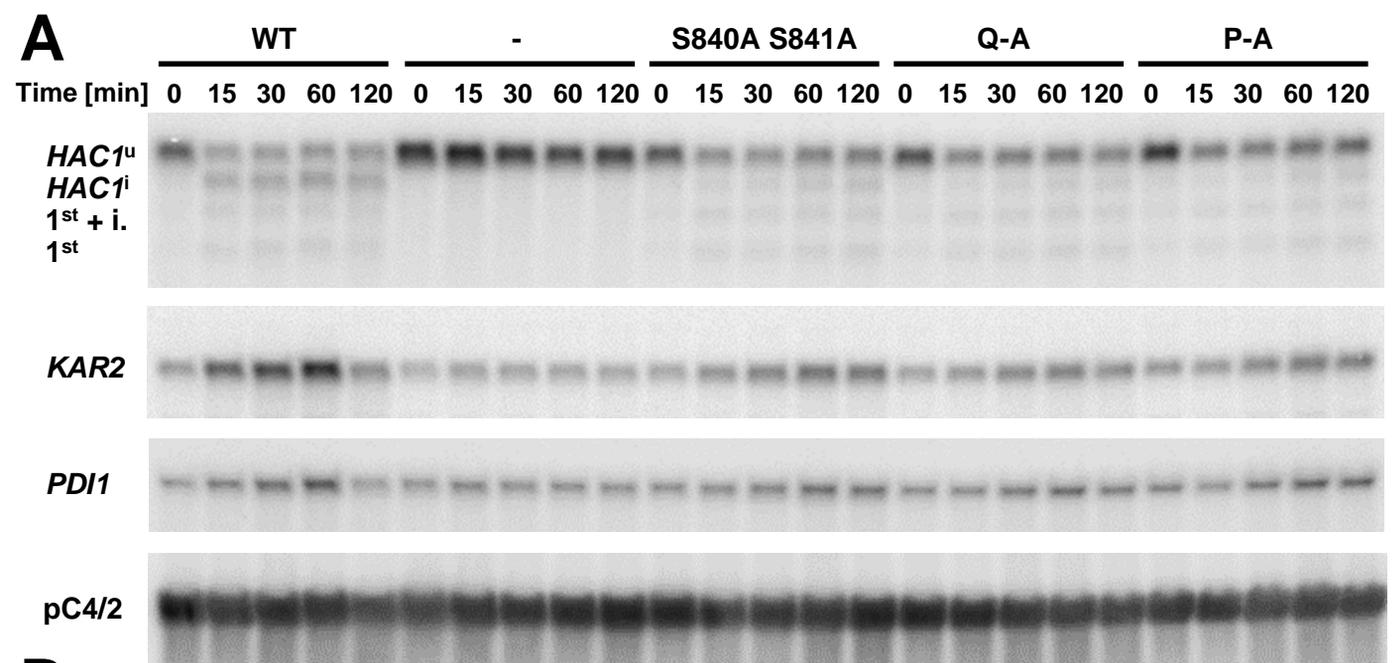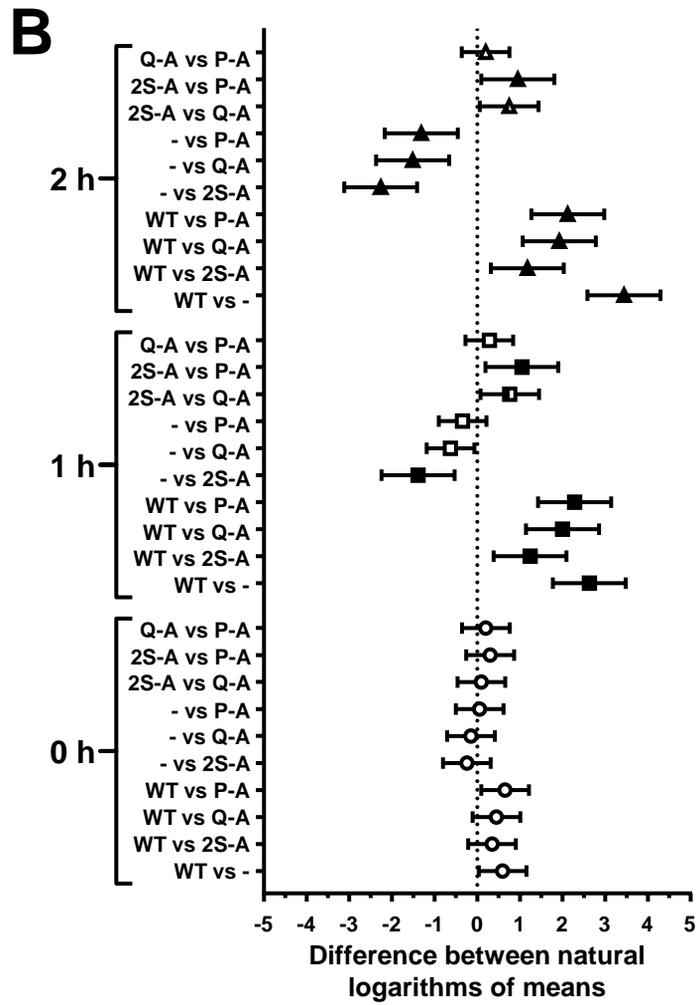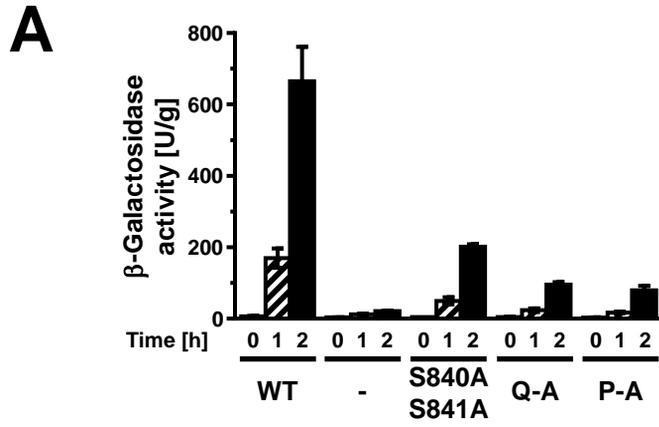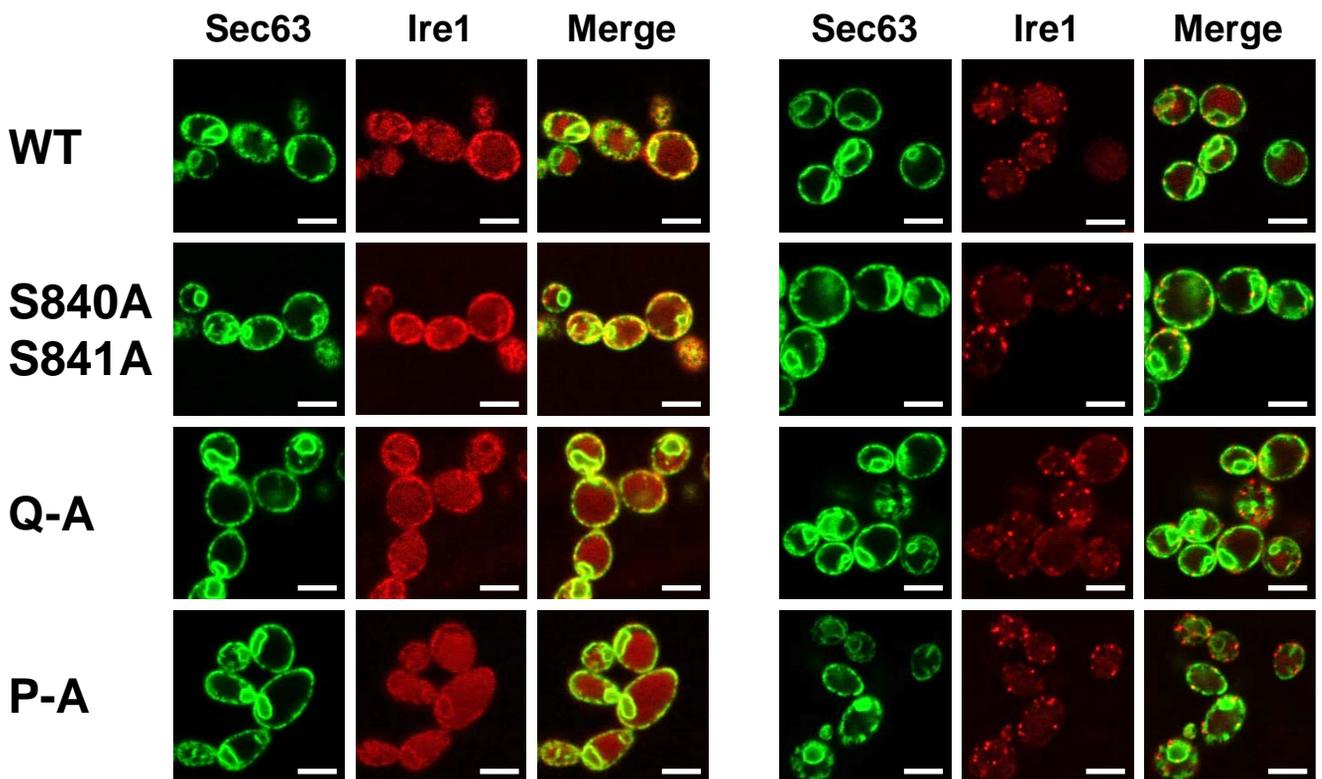
| | Sec63 | Ire1 | Merge |
|---|---|---|---|
| **WT** | | | |
| **S840A S841A** | | | |
| **Q-A** | | | |
| **P-A** | | | |

| Tm (µg/ml) | 0.0 | 0.4 | 0.8 |
|---|---|---|---|

|  | **- DTT** | | | **+ 2 mM DTT 15 min** | | |
|---|---|---|---|---|---|---|
|  | Sec63 | Ire1 | Merge | Sec63 | Ire1 | Merge |
| **WT** | | | | | | |
| **D836A** | | | | | | |
| **D836A S840A S841A** | | | | | | |
| **D836A Q-A** | | | | | | |
| **D836A P-A** | | | | | | |

|  | **+ 2 mM DTT 60 min** | | | **60 min after 2 mM DTT** | | |
|---|---|---|---|---|---|---|
|  | Sec63 | Ire1 | Merge | Sec63 | Ire1 | Merge |
| **WT** | | | | | | |
| **D836A** | | | | | | |
| **D836A S840A S841A** | | | | | | |
| **D836A Q-A** | | | | | | |
| **D836A P-A** | | | | | | |

**A**

| IRE1 | $P_{GAL1}$-PTC2 | -log$D_{\mathrm{T,u}}$ (−) | -log$D_{\mathrm{T,Tm}}$ (0.8 µg/ml Tm) | Δlog$D_{\mathrm{T}}$ |
|---|---|---|---|---|
| WT | - | 4 | 4 | 0 |
| WT | WT | 3 | 1.5 | -1.5 |
| WT | E37A-D38A | 3 | 3 | 0 |
| - | - | 3 | 0.5 | -2.5 |
| - | WT | 3 | 0.5 | -2.5 |
| - | E37A-D38A | 4 | 1 | -3 |
| D836A | - | 4 | 4 | 0 |
| D836A | WT | 3 | 1 | -2 |
| D836A | E37A-D38A | 3 | 3 | 0 |
| P-A | - | 4 | 2 | -2 |
| P-A | WT | 2 | 0 | -2 |
| P-A | E37A-D38A | 3 | 3 | -2 |
| D836A P-A | - | 3 | 0.5 | -2.5 |
| D836A P-A | WT | 3 | 0.5 | -2.5 |
| D836A P-A | E37A-D38A | 3 | 1 | -2 |

**B**

| DCR2 | - | 0.4 µg/ml Tm | 0.8 µg/ml Tm |
|---|---|---|---|
| WT | | | |
| $P_{GAL1}$-T7-DCR2 | | | |

**C**

| IRE1 | $P_{GAL1}$-DCR2 | -log$D_{\mathrm{T,u}}$ (−) | -log$D_{\mathrm{T,Tm}}$ (0.4 µg/ml Tm) | Δlog$D_{\mathrm{T}}$ |
|---|---|---|---|---|
| WT | - | 4 | 4 | 0 |
| WT | WT | 2 | 2 | 0 |
| WT | H338A | 1.5 | 1.5 | 0 |
| - | - | 3 | 1 | -2 |
| - | WT | 2.5 | 0.5 | -2 |
| - | H338A | 2.5 | 0.5 | -2 |

**D**

| IRE1 | Phosphatases | - | 0.4 µg/ml Tm | 1 mM DTT | 2 mM 2-DOG |
|---|---|---|---|---|---|
| WT | DCR2 | | | | |
| WT | dcr2Δ | | | | |
| - | DCR2 | | | | |
| - | dcr2Δ | | | | |
| WT | PTC2 | | | | |
| WT | ptc2Δ | | | | |
| - | PTC2 | | | | |
| - | ptc2Δ | | | | |
| WT | DCR2 PTC2 | | | | |
| WT | dcr2Δ ptc2Δ | | | | |
| - | DCR2 PTC2 | | | | |
| - | dcr2Δ ptc2Δ | | | | |