Supplementary Figure Legends

Suppl. Figure 1. Mean cluster size estimation.

A. Example input image after Z-stack projection and background correction. Red square, the region of interest (ROI) to be analyzed.

B. The ROI is sub-divided into 32x32 pixel areas.

C. The auto-correlation product of a single 32x32 pixel area corresponding to the square marked in white in B.

D. The intensity profile of the auto-correlation product after radial averaging. The full width at half maxima (FWHM) represents the typical size of an object in the original 32x32 pixel square. Histogram representation of typical size values of all sub-regions (black columns), super-imposed by the fitted gaussian function (red line). The mean and standard deviation of the fitted distribution are used to estimate the mean cluster size and standard deviation for the entire ROI.

Suppl. Figure 2. Complementation of IRE1α-deficient HAP1 cells with WT IRE1-GFP.

A. IRE1α protein expression. Total cell extracts containing 90 μg of proteins each were resolved by SDS-PAGE, and western blotted with anti-IRE1α antibody. HAP1-/ and +/ indicate IRE1α-deficient and –sufficient haploid cell lines, respectively.

B. Exogenous WT IRE1GFP can splice XBP1 mRNA. HAP1KO and HAP1KO WT IRE1GFP cells were treated with Tm (4 μg/ml) for the indicated times. RNA was extracted, XBP1 splicing assayed by RT-PCR and run on agarose gel.

C. Comparisons of IRE1α protein expression levels. Stable HAP1KO sub-lines expressing the indicated IRE1GFP constructs were compared for IRE1α expression. Exogenous IRE1GFP expression was induced by 24 hr incubation with 1 μg/ml dox and assessed by western blot analysis as above. IRE1α bands were quantified and normalized to the expression of the irrelevant cytosolic protein 14.3.3. End., expression of endogenous IRE1α in parental HAP1 cells. Overexp., level of WT IRE1GFP expressed in HEK 293T cells under control of a CMV promoter.

D. IRE1GFP protein levels remain constant during the Tm treatment. HAP1KO WT IRE1GFP cells were induced with dox (1 μg/ml) for 24 hr, dox was then washed out and cells treated with Tm (4 μg/ml) for the indicated times. Cells were lysed and proteins analyzed by western blot

using anti-HA and anti-tubulin antibodies. Arrow indicates full length IRE1GFP. §: lower molecular weight bands that appear to be IRE1α specific and size-sensitive to Tm treatment.
E. XBP1 splicing activities of endogenous IRE1α or WT IRE1GFP are comparable. IRE1α-sufficient HAP1 (HAP1 +/) and HAP1KO WT IRE1GFP cells were treated with TG at the indicated concentrations and times, RNA was extracted and XBP1 splicing assayed as above.
F. Overexpression of IRE1α leads to spontaneous activation. HEK 293T cells were treated with TG (0.2 μM) for the indicated times and XBP1 mRNA splicing was assayed as above. End., endogenous IRE1α in parental 293T cells. Overexp., WT IRE1GFP expressed off a CMV promoter in HEK 293T cells.

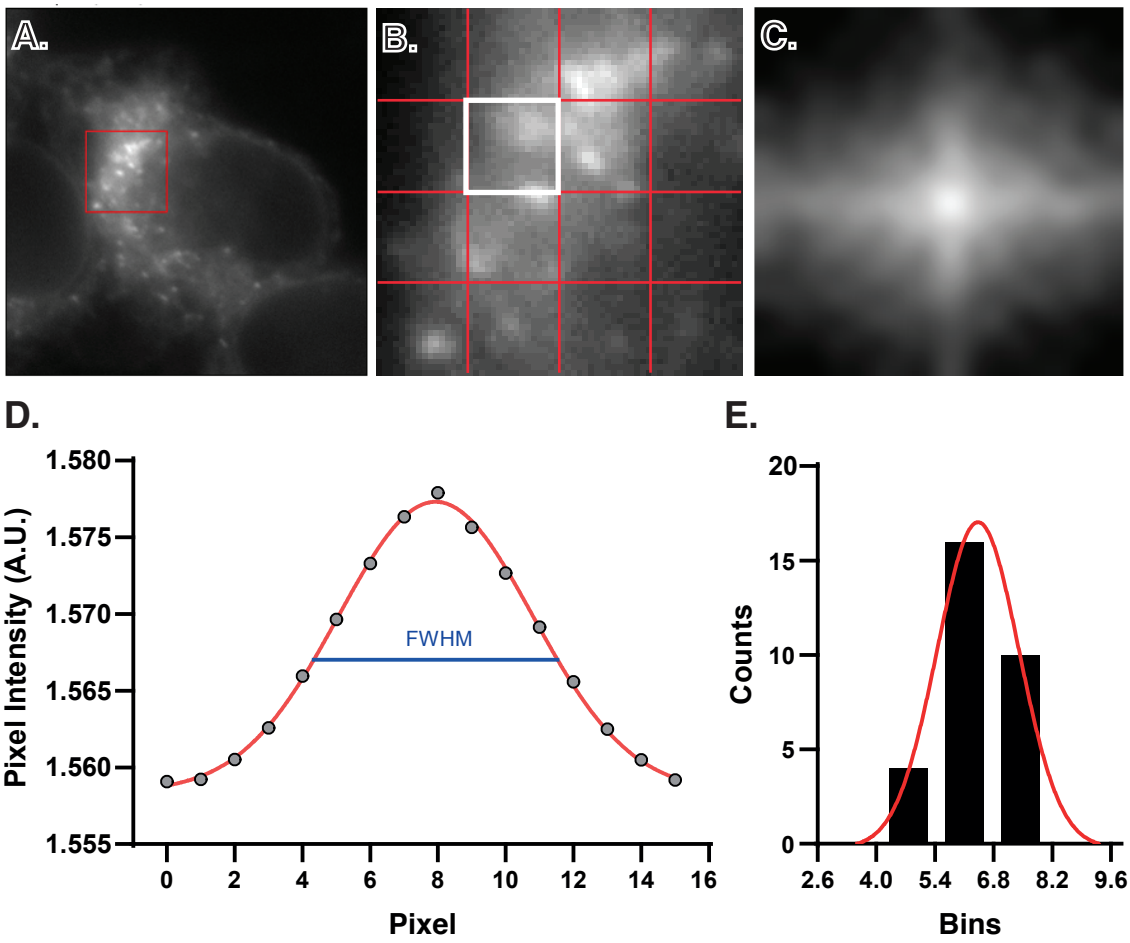
Suppl. Figure 3. <u>XBP1 splicing and clustering behavior of K907A IRE1GFP</u>.
A. K907A mutation inactivates IRE1α RNase activity. HAP1KO IRE1GFP cells were treated with Tm (4 μg/ml) for the indicated times, XBP1 splicing was assessed by RT-PCR as before.
B. K907A induces fewer clusters per cell following ER stress. Histogram distribution of clusters per cell following Tm treatment (4 μg/ml) at the indicated times.
C. K907A induces more intense clusters following ER stress. Histogram distribution of cluster intensity (a.u.) following Tm treatment (4 μg/ml) at the indicated times**.**

Suppl. Figure 4. <u>Ablation of BiP triggers IRE1α clustering</u>.
A. SubAB cleaves BiP in a concentration- and time-dependent manner. HAP1KO IRE1GFP WT were treated with SubAB (0.1 μg/ml) or (0.3 μg/ml) for the indicated times. Proteins were extracted and analyzed by western blot with anti-BiP antibody. 14.3.3 was used as housekeeping. Arrows indicate full length (~78 KDa) and cleaved BiP (~28 KDa).
B. SubAB-induced and K907A IRE1α hyper clusters are approximately the same size. Comparison of clusters sizes (μm^2) throughout treatments. Histogram distribution of WT IRE1GFP expression cells following SubAB depletion of BiP, and K907A IRE1GFP cells treated with Tm (4μg/ml) for the indicated times.
C. Absence of spliced XBP1 still induces BiP mRNA expression after ER stress. HAP1KO IRE1GFP WT or K907A were treated with Tm (4 μg/ml) and/or 4μ8c (16 μM) for the indicated times and BiP mRNA levels were assessed by qRT-PCR using Rpl19 as housekeeping.

Supplementary Video Legends

1. Suppl. Video 1. Time course of IRE1 clustering.
Composite of time-lapse images taken every 30 min over an 8 hr treatment with Tm (4 μg/ml).

2. Suppl. Video 1. Putative fusion of IRE1 clusters.
Composite of time-lapse images taken every 3 min between 3 hr and 4 hr of treatment with Tm (4 μg/ml), near the peak of clustering.


A.
B.
C.
D.
Pixel Intensity (A.U.)
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1.575
1.570
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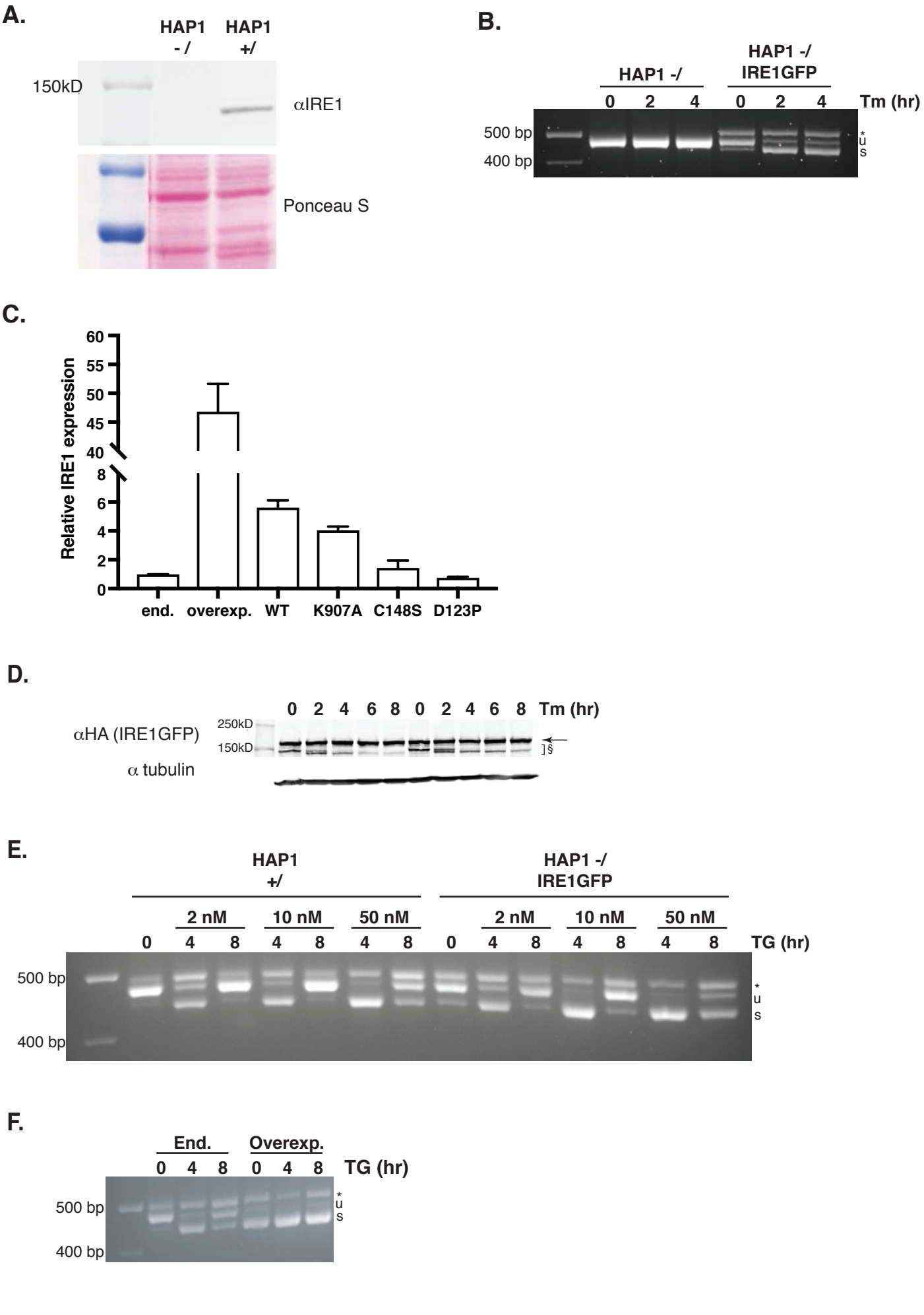
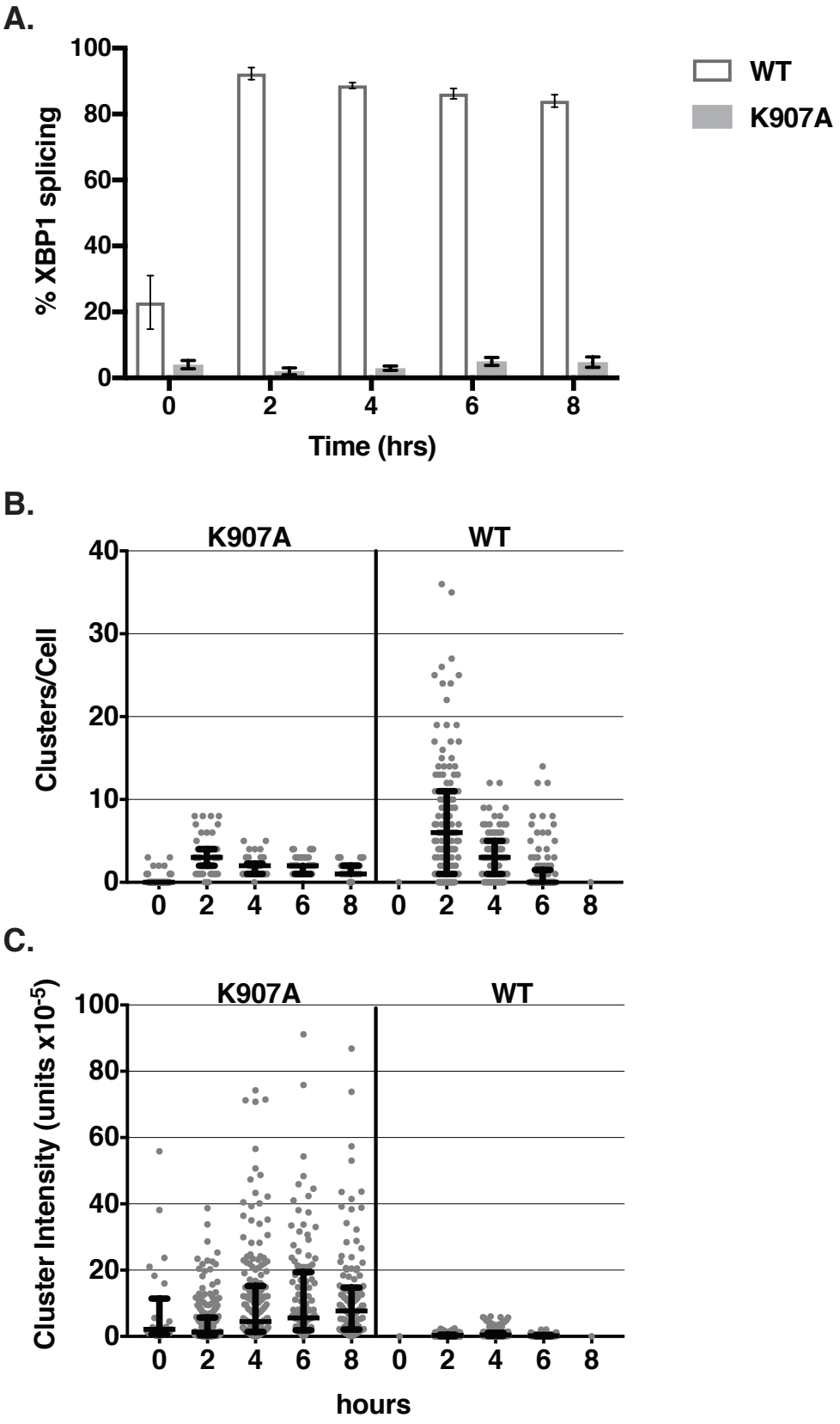
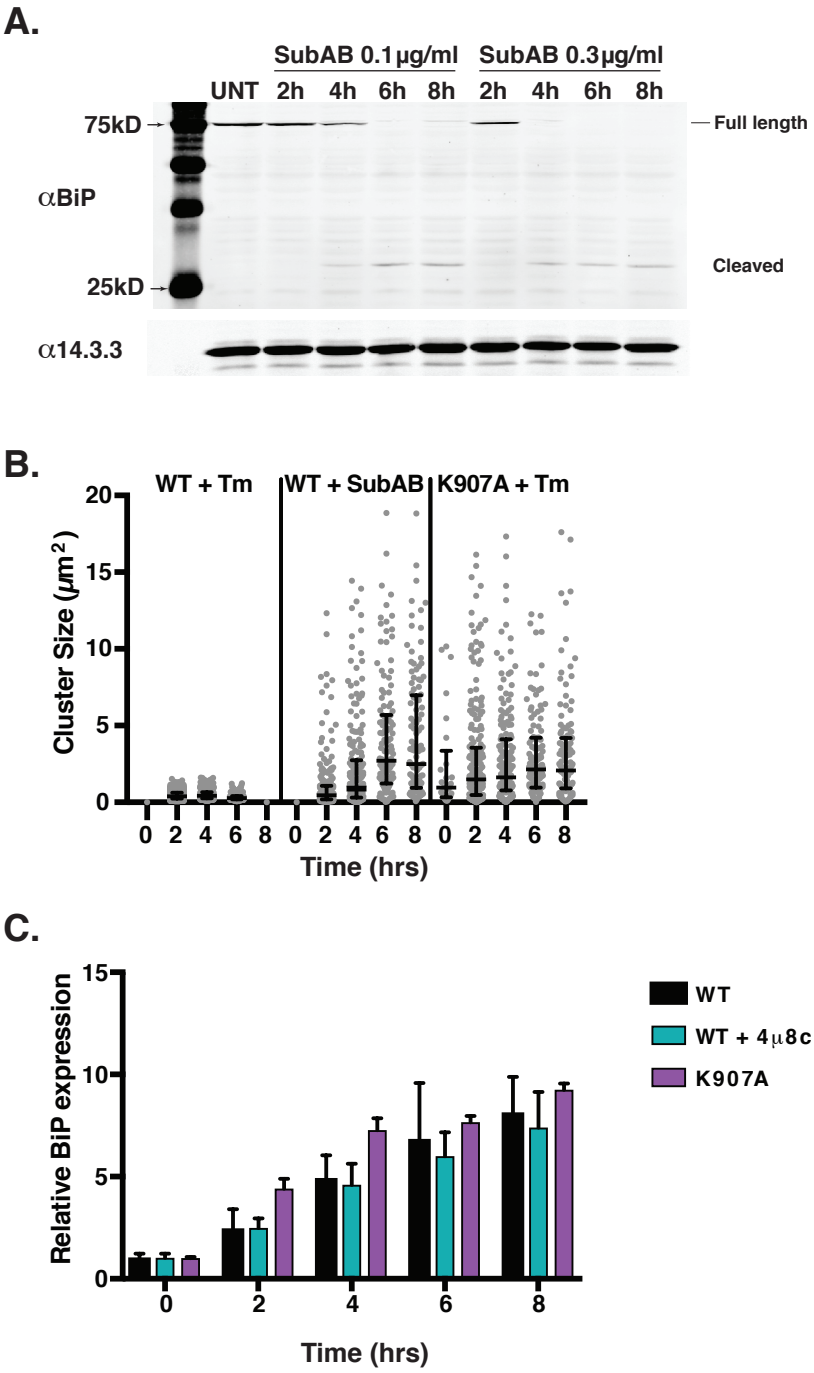
1.560
1.555
FWHM
0
2
4
6
8
10
12
14
16
Pixel
E.
Counts
20
15
10
5
0
2.6
4.0
5.4
6.8
8.2
9.6
Bins


A.
HAP1 - /
HAP1 +/
150kD
αIRE1
Ponceau S
B.
HAP1 -/
HAP1 -/ IRE1GFP
0 2 4 0 2 4 Tm (hr)
500 bp
400 bp
*
u
s
C.
Relative IRE1 expression
60
55
50
45
40
8
6
4
2
0
end.
overexp.
WT
K907A
C148S
D123P
D.
0 2 4 6 8 0 2 4 6 8 Tm (hr)
αHA (IRE1GFP)
α tubulin
250kD
150kD
]§
E.
HAP1 +/
HAP1 -/ IRE1GFP
2 nM
10 nM
50 nM
0 4 8 4 8 4 8 0 4 8 4 8 4 8 TG (hr)
500 bp
400 bp
*
u
s
F.
End.
Overexp.
0 4 8 0 4 8 TG (hr)
500 bp
400 bp
*
u
s

Suppl. Fig. 3


A.
100
80
60
40
20
0
% XBP1 splicing
0
2
4
6
8
Time (hrs)
WT
K907A
B.
K907A
WT
40
30
20
10
0
Clusters/Cell
0 2 4 6 8 0 2 4 6 8
C.
K907A
WT
100
80
60
40
20
0
Cluster Intensity (units x10-5)
0 2 4 6 8 0 2 4 6 8
hours

Suppl. Fig. 4


A.
SubAB 0.1µg/ml
SubAB 0.3µg/ml
UNT
2h
4h
6h
8h
2h
4h
6h
8h
75kD
αBiP
25kD
Full length
Cleaved
α14.3.3
B.
WT + Tm
WT + SubAB
K907A + Tm
Cluster Size (µm²)
20
15
10
5
0
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8
Time (hrs)
C.
Relative BiP expression
15
10
5
0
0
2
4
6
8
Time (hrs)
WT
WT + 4µ8c
K907A