Supplemental Materials

Figure S1. Colocalization of endogenous Nrf2 and endogenous Crif1. (A) Immunocytochemistry for Nrf2 and Crif1 localization before and after *t*-BHQ treatment. Cells (DU-145) treated with DMSO or *t*-BHQ (100 μM, for 30 min or 1 h) were stained with anti-Nrf2 or anti-Crif1 antibody as described in Experimental Procedures. (B-C) Whole cell lysates (B) and both cytosolic and nuclear fractions (C) of cells treated with 100 μM of *t*-BHQ for the indicated times were extracted and then analyzed on WBs with the designated antibodies. The anti-α/β-tubulin (cytosolic maker) and anti-Lamin B1 (nuclear maker) antibodies were used as loading controls and to monitor the quality of the cytosol/nuclear fractionation method.

Figure S2. Crif1 partially interacts with Neh1 (bZIP) domain (DNA binding domain) of Nrf2. (A) A schematic drawing of Nrf2 deletion mutants containing a bZIP domain used for interaction studies. (B) *In vitro* synthesized (IVT) ^{35}S-labeled FLAG-Crif1 and ^{35}S-labeled Nrf2 containing bZIP domains interact. ^{35}S-labeled proteins were mixed, immunoprecipitated with an anti-FLAG antibody, subjected to SDS-PAGE, and exposed to X-ray film. These experiments were repeated three times and yielded similar results; a representative result is shown. Control antibody (mouse IgG) was used as a control for IP.

Figure S3. Crif1 regulates Nrf2 protein half-life. (A) Over-expressed Crif1 reduces Nrf2 half-life. HEK293 cells co-transfected with FLAG-Crif1, FLAG-Nrf2 or pCDNA3 (empty vector) for 24 h were incubated with CHX (10 μg/ml) for the times indicated before WB analysis. Anti-FLAG antibody was used to detect both exogenously expressed Nrf2 (130 kDa) and Crif1 (30 kDa). The FLAG-Nrf2 band densities were quantified and then normalized to the total FLAG-Nrf2 protein level found at the zero time point (before adding CHX) in the pCDNA3-transfected group and demonstrated as a linear graph (see below WB image). (B) Down-regulated Crif1 effects on the half-life of endogenous Nrf2 protein in the presence of SFN. Total lysates of MCF-7 cells pre-treated with siRNA (control or Crif1-3 and -4) for 72 h, then incubated with SFN (5μM for 4h) and then treated with CHX (10 μg/ml) for the times indicated were analyzed on WBs with anti-Nrf2 and anti-Crif1 mouse monoclonal antibodies. The intensities of the Nrf2-containing bands in the presence of control-siRNA were normalized to the total endogenous Nrf2 protein in the presence of Crif1-siRNA (without CHX treatment) and demonstrated as a linear graph (below WB image).

Figure S4. Interactions between Crif1 and quadruple point mutants (lysine to arginine) of Nrf2. Wild-type or a quadruple point mutant protein fragment (from 4 lysine [K] residues to 4 arginine [R] residues) of (A) N-terminal or (B) C-terminal Nrf2 deletion mutant constructs (see Figure 4B for the diagram) co-transfected with either FLAG-Crif1 or FLAG-Keap1 in HEK293 cells were immunoprecipitated with anti-His (Nrf2) antibody and then subjected to WB analysis as indicated. One-tenth of the total cell lysates was also used for WB analysis to determine the amount of protein expressed.

Figure S5. Over-expression of Crif1 reduced SFN (or *t*-BHQ)-stimulated Nrf2 protein level. (A) Over-expression of Crif1 reduced endogenous Nrf2 protein levels after SFN treatment. Cells (MCF-7) infected with Ad-GFP or Ad-GFP-Crif1 were treated with SFN (5 μM) and harvested at the indicated times. (B) Over-expression of Crif1 reduced Nrf2 induction following *t*-BHQ (100 μM). Cells (MCF-7) infected overnight with Ad-GFP or Ad-GFP-Crif1 were treated with *t*-BHQ and harvested at 0, 8, 16 and 24 h. Anti-Nrf2 and anti-GFP antibodies were used to monitor the expression of Nrf2 and Crif1.

Figure S6. Crif1 reduces Nrf2 accumulation at ARE-containing enhancer regions of Nrf2 target gene (HO-1). MCF-7 cells were transfected for 24 h with expression vectors for FLAG-Crif1, His-V5-Nrf2 or both vectors and then fixed in formaldehyde and prepared for ChIP assays using mouse IgG (control), anti-His and anti-FLAG antibodies as described in Experimental Procedures. (A) A diagram of

ARE-containing and non-ARE-containing DNA sequence regions of the HO-1 enhancer region. (B-C) ARE-containing HO-1 enhancer genomic DNA sequence (B) and non-ARE-containing genomic DNA sequence regions (C) were PCR-amplified by using appropriate HO-1 primers. Mouse IgG was used as the negative, non-specific antibody control. DNA sequences, locations and primers are described in Experimental Procedures. (D) Quantitative real-time PCR confirms ChIP assay results of the ARE-containing HO-1 enhancer region (B).

Figure S1

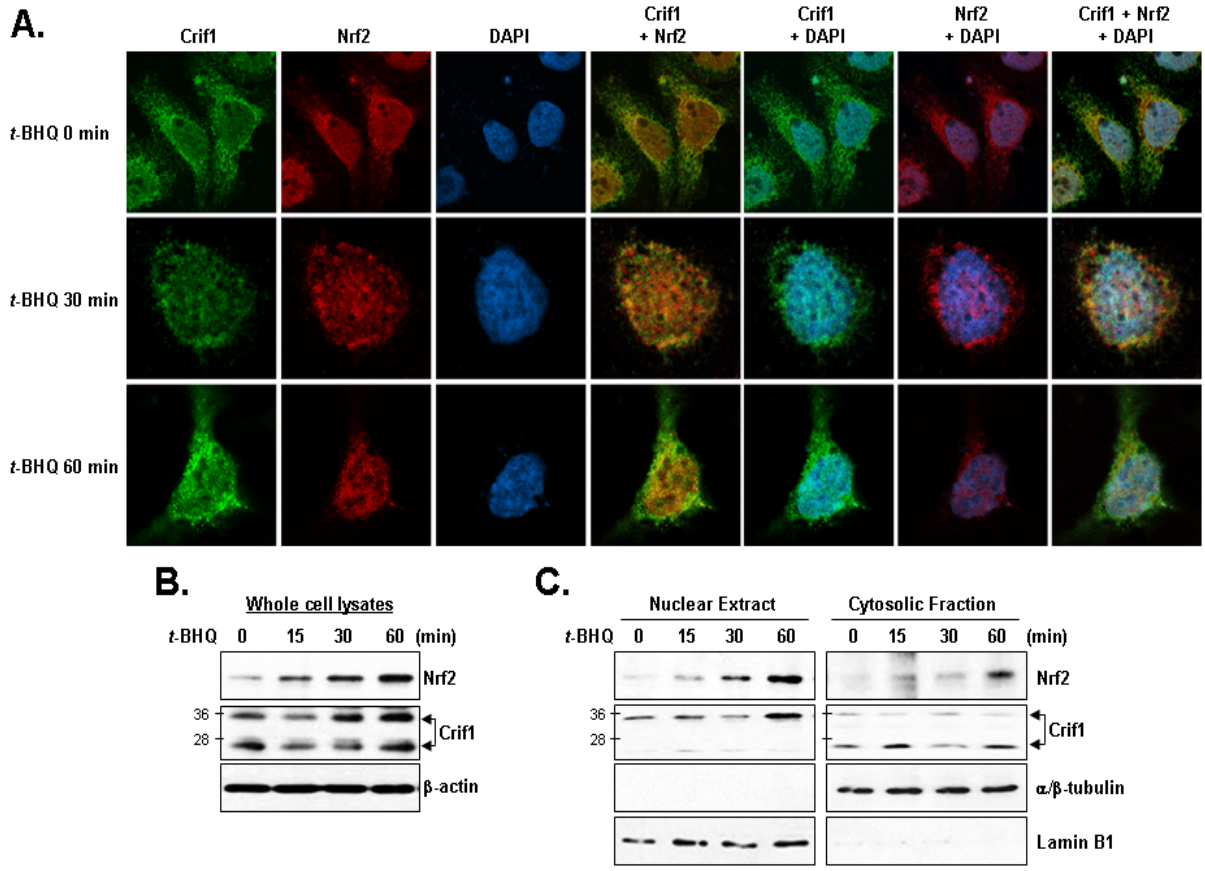

A.
Crif1
Nrf2
DAPI
Crif1 + Nrf2
Crif1 + DAPI
Nrf2 + DAPI
Crif1 + Nrf2 + DAPI
t-BHQ 0 min
t-BHQ 30 min
t-BHQ 60 min
B.
Whole cell lysates
t-BHQ 0 15 30 60 (min)
Nrf2
36
28
Crif1
β-actin
C.
Nuclear Extract
Cytosolic Fraction
t-BHQ 0 15 30 60 0 15 30 60 (min)
Nrf2
36
28
Crif1
α/β-tubulin
Lamin B1

Figure S2

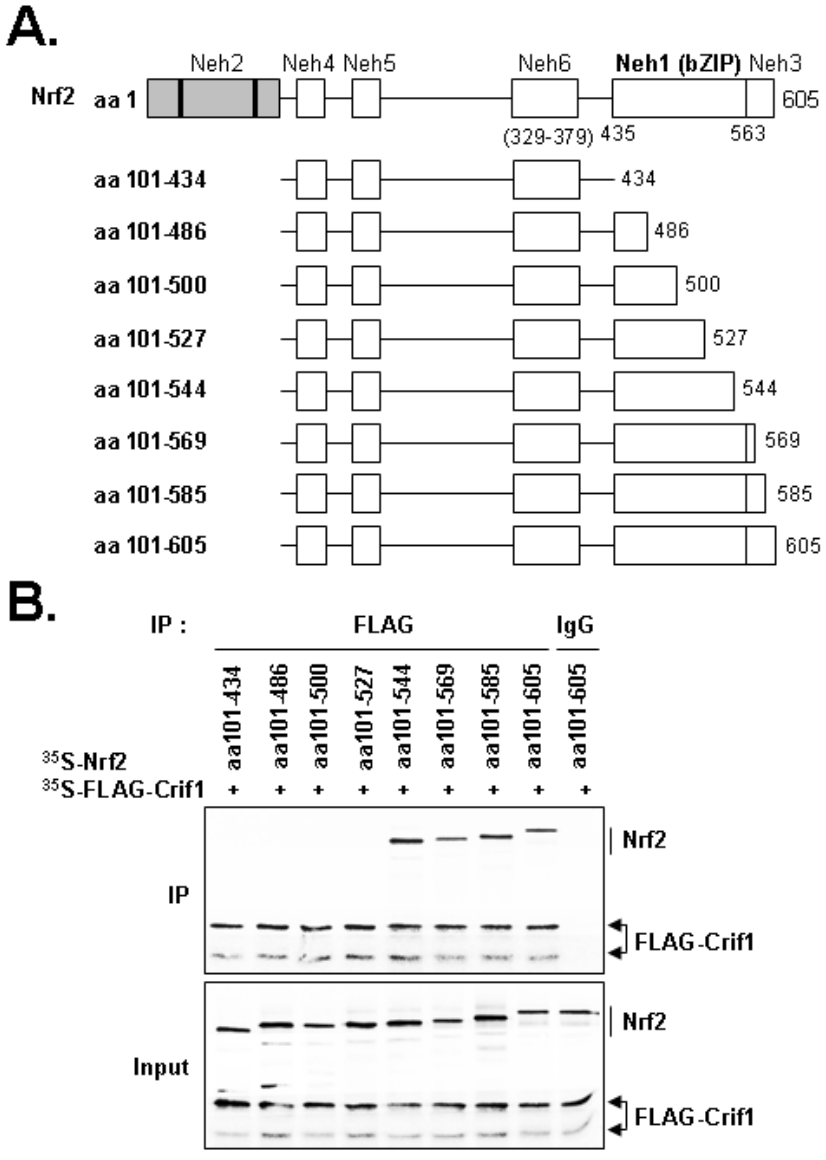

A.
Neh2
Neh4 Neh5
Neh6
Neh1 (bZIP) Neh3
Nrf2
aa 1
605
(329-379) 435
563
aa 101-434
434
aa 101-486
486
aa 101-500
500
aa 101-527
527
aa 101-544
544
aa 101-569
569
aa 101-585
585
aa 101-605
605
B.
IP :
FLAG
IgG
aa101-434
aa101-486
aa101-500
aa101-527
aa101-544
aa101-569
aa101-585
aa101-605
aa101-605
35S-Nrf2
35S-FLAG-Crif1
+ + + + + + + + +
IP
Nrf2
FLAG-Crif1
Input
Nrf2
FLAG-Crif1

Figure S3

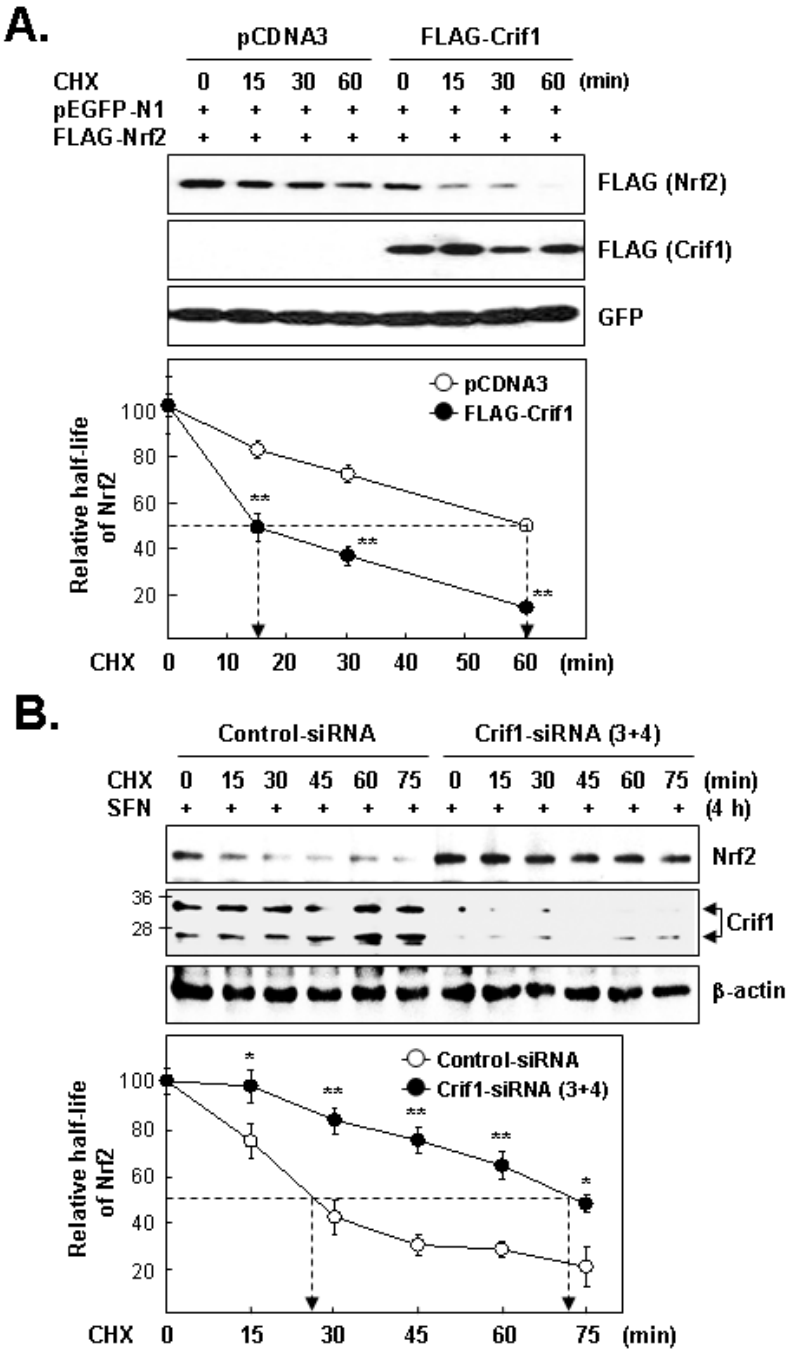

A.
pCDNA3
FLAG-Crif1
CHX 0 15 30 60 0 15 30 60 (min)
pEGFP-N1 + + + + + + + +
FLAG-Nrf2 + + + + + + + +
FLAG (Nrf2)
FLAG (Crif1)
GFP
pCDNA3
FLAG-Crif1
Relative half-life of Nrf2
100
80
60
40
20
**
**
**
CHX 0 10 20 30 40 50 60 (min)
B.
Control-siRNA
Crif1-siRNA (3+4)
CHX 0 15 30 45 60 75 0 15 30 45 60 75 (min)
SFN + + + + + + + + + + + + (4 h)
Nrf2
36
28
Crif1
β-actin
Control-siRNA
Crif1-siRNA (3+4)
Relative half-life of Nrf2
100
80
60
40
20
*
**
**
**
*
CHX 0 15 30 45 60 75 (min)

Figure S4

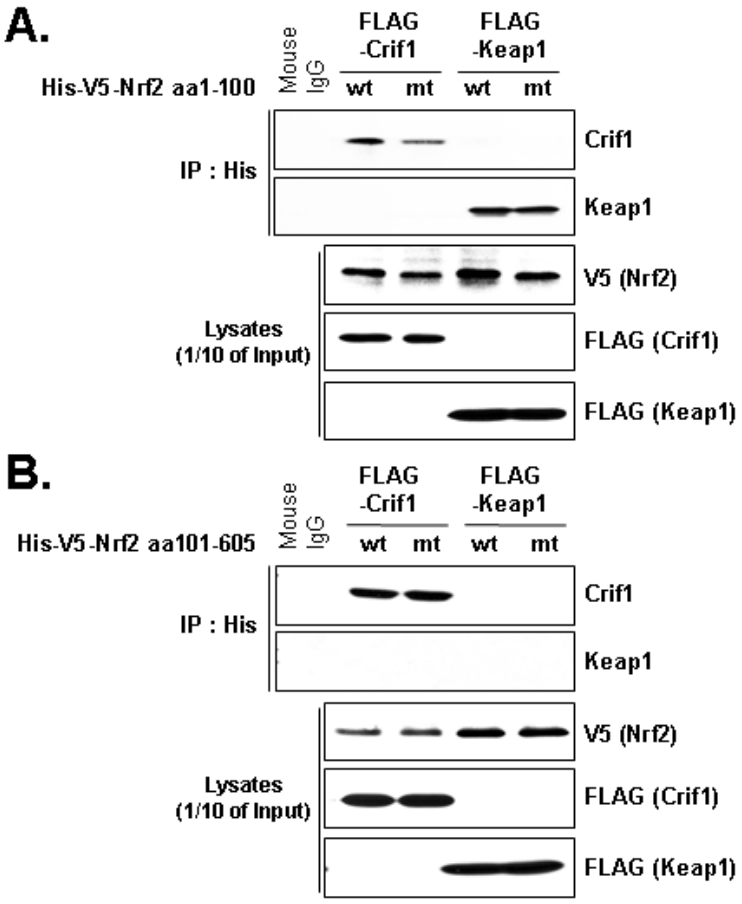

A.
His-V5-Nrf2 aa1-100
Mouse IgG
FLAG -Crif1
FLAG -Keap1
wt
mt
wt
mt
IP : His
Crif1
Keap1
Lysates (1/10 of Input)
V5 (Nrf2)
FLAG (Crif1)
FLAG (Keap1)
B.
His-V5-Nrf2 aa101-605
Mouse IgG
FLAG -Crif1
FLAG -Keap1
wt
mt
wt
mt
IP : His
Crif1
Keap1
Lysates (1/10 of Input)
V5 (Nrf2)
FLAG (Crif1)
FLAG (Keap1)

Figure S5

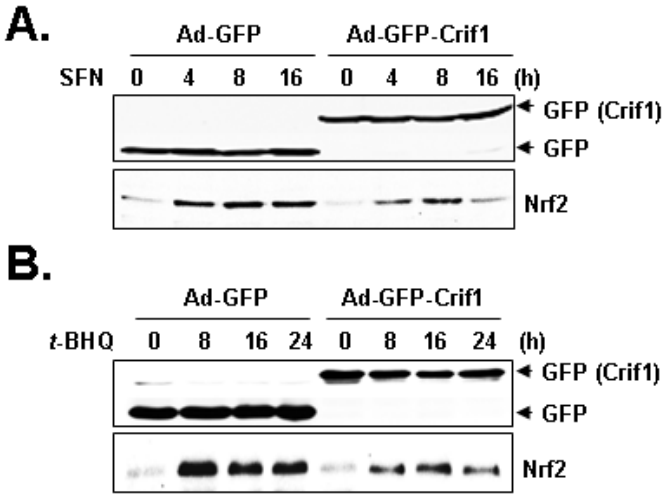
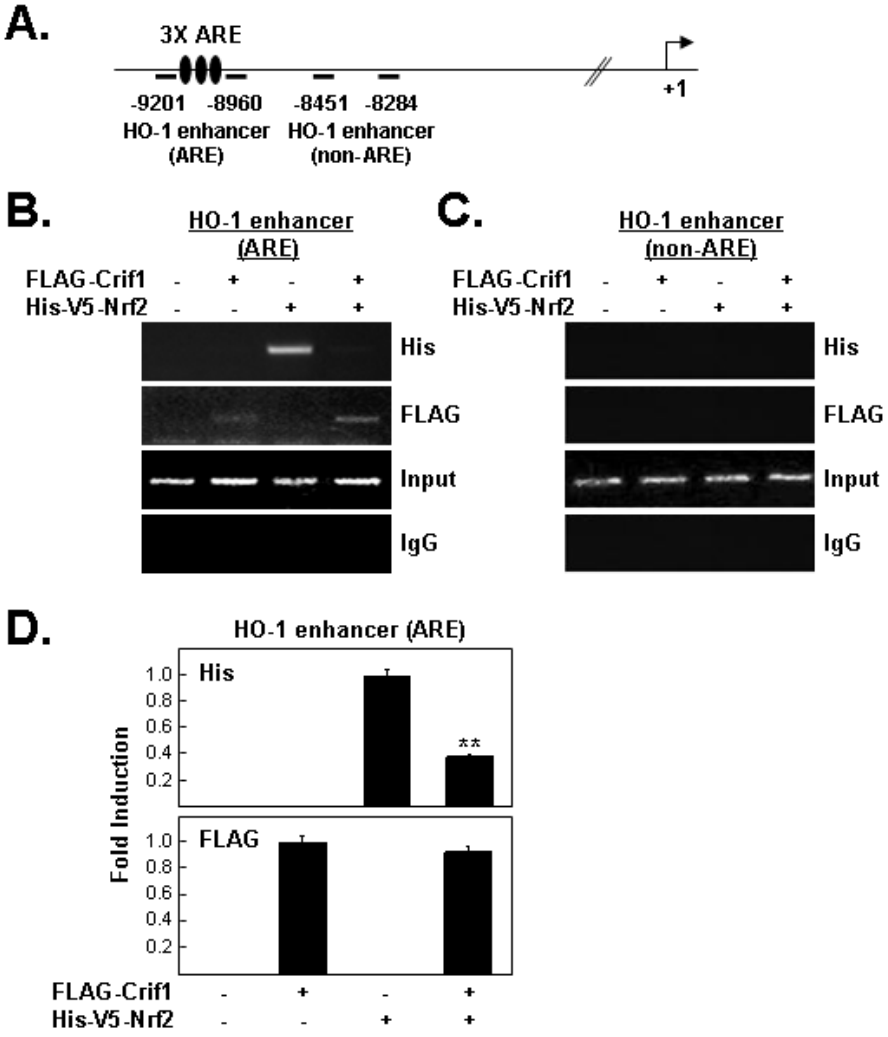

A.
Ad-GFP
Ad-GFP-Crif1
SFN 0 4 8 16 0 4 8 16 (h)
GFP (Crif1)
GFP
Nrf2
B.
Ad-GFP
Ad-GFP-Crif1
t-BHQ 0 8 16 24 0 8 16 24 (h)
GFP (Crif1)
GFP
Nrf2

Figure S6


A.
3X ARE
-9201
-8960
-8451
-8284
+1
HO-1 enhancer (ARE)
HO-1 enhancer (non-ARE)
B.
HO-1 enhancer (ARE)
FLAG-Crif1 - + - +
His-V5-Nrf2 - - + +
His
FLAG
Input
IgG
C.
HO-1 enhancer (non-ARE)
FLAG-Crif1 - + - +
His-V5-Nrf2 - - + +
His
FLAG
Input
IgG
D.
HO-1 enhancer (ARE)
Fold Induction
His
FLAG
1.0
0.8
0.6
0.4
0.2
**
FLAG-Crif1 - + - +
His-V5-Nrf2 - - + +