Expanded View Figures

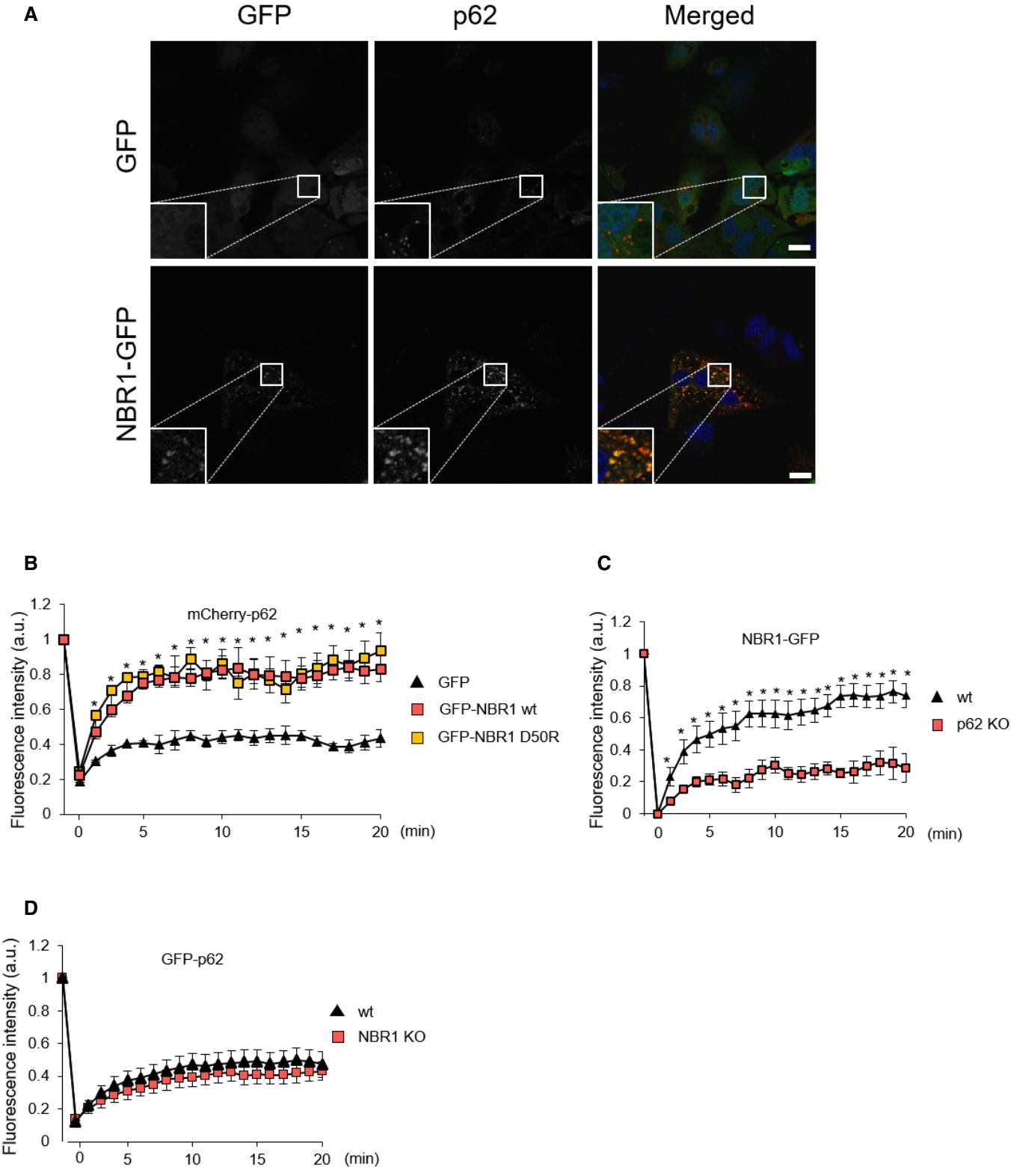

Figure EV1.

Figure EV1. Mobility analysis of structures positive for p62 and/or NBR1.

A Wild-type hepatocytes were infected with GFP or NBR1-GFP adenovirus for 48 h and then immunostained with anti-p62 antibody. Each inset is a magnified image. Bars: 20 μm.

B–D FRAP assay. *p62*-knockout MEFs were co-transfected mCherry-p62 together with GFP, GFP-NBR1, or GFP-NBR1 D50R. Forty-eight hours after the transfection, the signal recovery after photobleaching was measured and quantified (B). GFP-NBR1 was transfected into wild-type or *p62*-knockout MEFs. Forty-eight hours after the transfection, the signal recovery after photobleaching was measured and quantified (C). GFP-p62 was transfected into wild-type or *Nbr1*-knockout MEFs. Forty-eight hours after the transfection, the signal recovery after photobleaching was measured and quantified (D). Three independent biological replicates were performed on each experiment. Data are shown as means ± SE. $*P < 0.05$ as determined by Welch's *t*-test.

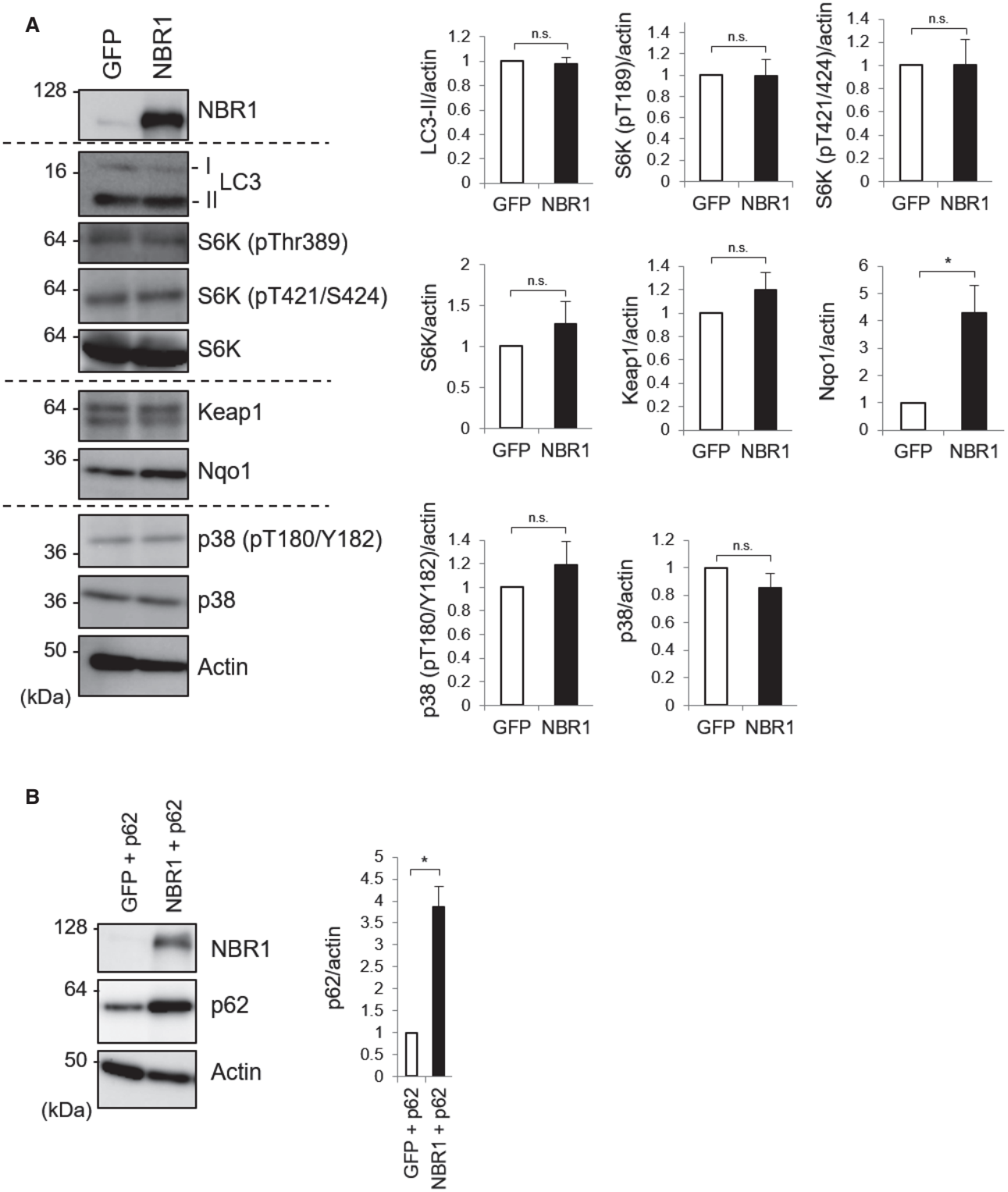
A
GFP
NBR1
128
NBR1
16
- I
- II
LC3
64
S6K (pThr389)
64
S6K (pT421/S424)
64
S6K
64
Keap1
36
Nqo1
36
p38 (pT180/Y182)
36
p38
50
Actin
(kDa)
LC3-II/actin
S6K (pT189)/actin
S6K (pT421/424)/actin
S6K/actin
Keap1/actin
Nqo1/actin
p38 (pT180/Y182)/actin
p38/actin
n.s.
*
GFP NBR1
B
GFP + p62
NBR1 + p62
128
NBR1
64
p62
50
Actin
(kDa)
p62/actin
*
GFP + p62
NBR1 + p62

Figure EV2.

Figure EV2. NBR1 influences Nrf2 activation but not other p62-related pathways.

A The primary hepatocytes prepared from wild-type mice and infected with adenovirus GFP or NBR1 for 48 h employed in Fig 1A were subjected to immunoblot analysis with the indicated antibodies. Data shown are representative of three separate experiments. Bar graphs indicate the quantitative densitometric analysis of the indicated proteins relative to actin. Data are shown as means ± SE. *$P < 0.05$ as determined by Welch's *t*-test.

B Primary hepatocytes prepared from *p62*^{Flf}; Alb-*Cre* mice were infected with adenovirus expressing GFP or NBR1 in combination with p62 for 48 h. Cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. Data shown are representative of three separate experiments. Bar graphs indicate the quantitative densitometric analysis of p62 relative to actin. Data are shown as means ± SE. *$P < 0.05$ as determined by Welch's *t*-test.

Source data are available online for this figure.

Figure EV3. Generation of *Nbr1*-conditional knockout mice.

A Schematic representation of the targeting vector and the targeted allele of the *Nbr1* gene. The coding exons numbered in accordance with the initiation site as exon 1 are depicted by gray boxes. Exon 21 was fused to a cDNA fragment encoded by exons 22, 23, and 24 (aa 2,523–2,964), and GFP and polyA signal sequence was added. Neo-resistant gene cassette (neo) with FLP sequences (representing semi-ellipsoid) was ligated behind the polyA signal sequence. The black triangles indicate the loxP sequence. The probe for Southern blot analysis is shown as a gray ellipse.

B Southern blot analysis of genomic DNA extracted from mice tails. Wild-type and Flox alleles are indicated.

C Expression of the *Nbr1* transcript in MEFs. Transcripts from the indicated genotypes were detected by real-time PCR analysis. Data are shown as means ± SE calculated from three independent biological replicates.

D MEFs from the indicated genotypes were subjected to immunoblot analysis with the anti-NBR1 and anti-actin antibodies. Data shown are representative of three separate experiments.

E Immunofluorescence microscopy. Primary hepatocytes from the indicated genotypes were isolated, cultured in full media (Fed) or amino acid-deprived (Stv) for 12 h, and then subjected to immunostaining with an anti-LC3 antibody. Bars: 20 μm.

F Hepatocytes from wild-type, *p62*-, and *Nbr1*-knockout mice were isolated and labeled with [^{14}C] leucine for 24 h, and degradation of long-lived protein in deprived (Stv) or non-deprived (—) condition was measured. E64d and pepstatin (EP) were added as indicated. Data are shown as means ± SE. *$P < 0.05$, **$P < 0.01$ as determined by Welch's *t*-test derived from three independent biological replicates.

Source data are available online for this figure.

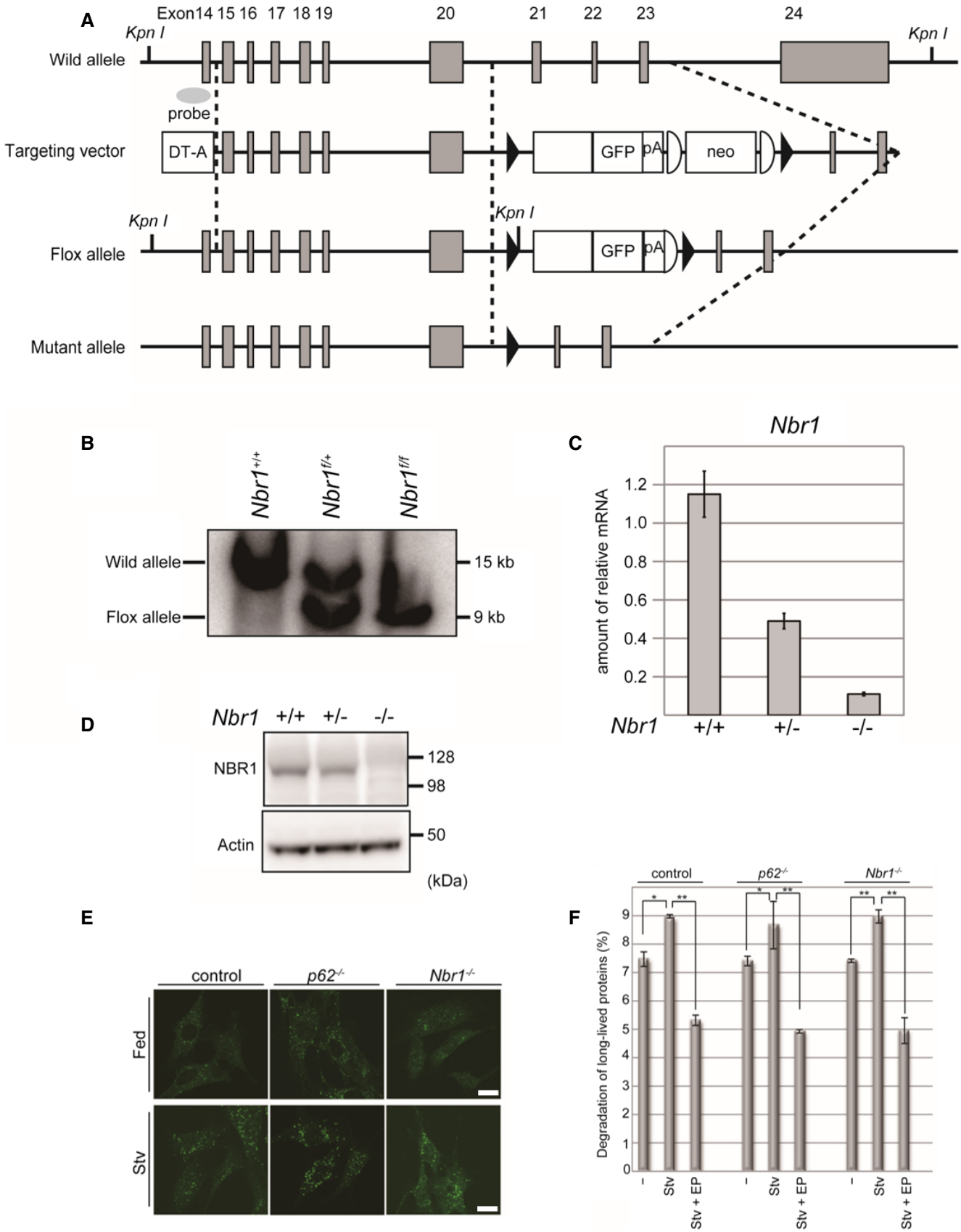
A
Exon14 15 16 17 18 19 20 21 22 23 24
Kpn I
Kpn I
Wild allele
probe
Targeting vector
DT-A
GFP
pA
neo
Kpn I
Kpn I
Flox allele
GFP
pA
Mutant allele
B
Nbr1+/+
Nbr1f/+
Nbr1f/f
Wild allele
Flox allele
15 kb
9 kb
C
Nbr1
amount of relative mRNA
1.2
1.0
0.8
0.6
0.4
0.2
Nbr1 +/+ +/- -/-
D
Nbr1 +/+ +/- -/-
NBR1
128
98
Actin
50
(kDa)
E
control
p62-/-
Nbr1-/-
Fed
Stv
F
control
p62-/-
Nbr1-/-
Degradation of long-lived proteins (%)
9
8
7
6
5
4
3
2
1
0
-
Stv
Stv + EP
-
Stv
Stv + EP
-
Stv
Stv + EP

Figure EV3.

Figure EV4. Phenotypic analysis of the *Nbr1*$^{f/f}$; Alb-*Cre* mice.

A Immunoblot analysis. Total homogenates were prepared from the livers of 6-week-old *Nbr1*$^{f/f}$ (n = 3, biological replicates) and *Nbr1*$^{f/f}$; Alb-*Cre* mice (n = 3, biological replicates). These were subjected to immunoblotting using the indicated antibodies. Bar graphs show the amounts of the indicated proteins measured by densitometry relative to actin. Data are shown as means ± SE determined by Welch's t-test.

B Gene expression of Nrf2 targets in *Nbr1*-deficient livers. Total RNA was prepared from the livers of 6-week-old *Nbr1*$^{f/f}$ (n = 3) and *Nbr1*$^{f/f}$; Alb-*Cre* mice (n = 3). Values were normalized against the amount of mRNA in the livers of *Nbr1*$^{f/f}$ mice. The experiments were performed three times. Data are shown as means ± SE determined by Welch's t-test.

C Liver weights (% per body weight) of mice described in (A). Data are shown as means ± SE determined by Welch's t-test from three independent biological replicates.

D Liver function tests of the mice used in (A). The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured. IU/l, international units/liter. Data are shown as means ± SE determined by Welch's t-test from three independent biological replicates.

E Hematoxylin and eosin staining. Liver sections from 6-week-old *Nbr1*$^{f/f}$ and *Nbr1*$^{f/f}$; Alb-*Cre* mice were prepared and stained with H&E. Data are representative of three separate experiments. Bar: 50 μm.

Source data are available online for this figure.

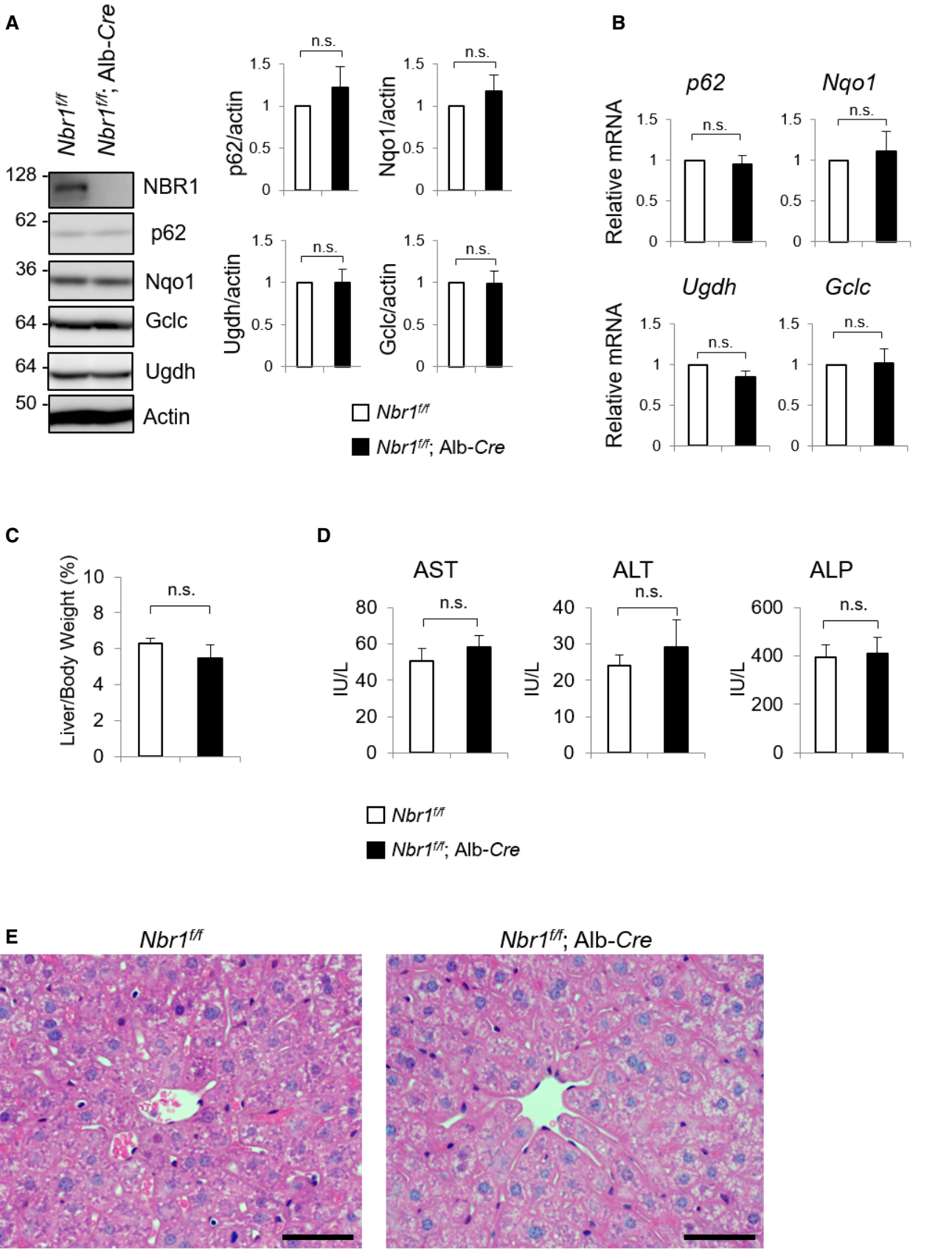
A
Nbr1f/f
Nbr1f/f; Alb-Cre
128
62
36
64
64
50
NBR1
p62
Nqo1
Gclc
Ugdh
Actin
p62/actin
Nqo1/actin
Ugdh/actin
Gclc/actin
n.s.
Nbr1f/f
Nbr1f/f; Alb-Cre
B
p62
Nqo1
Ugdh
Gclc
Relative mRNA
C
Liver/Body Weight (%)
D
AST
ALT
ALP
IU/L
E
Nbr1f/f
Nbr1f/f; Alb-Cre

Figure EV4.

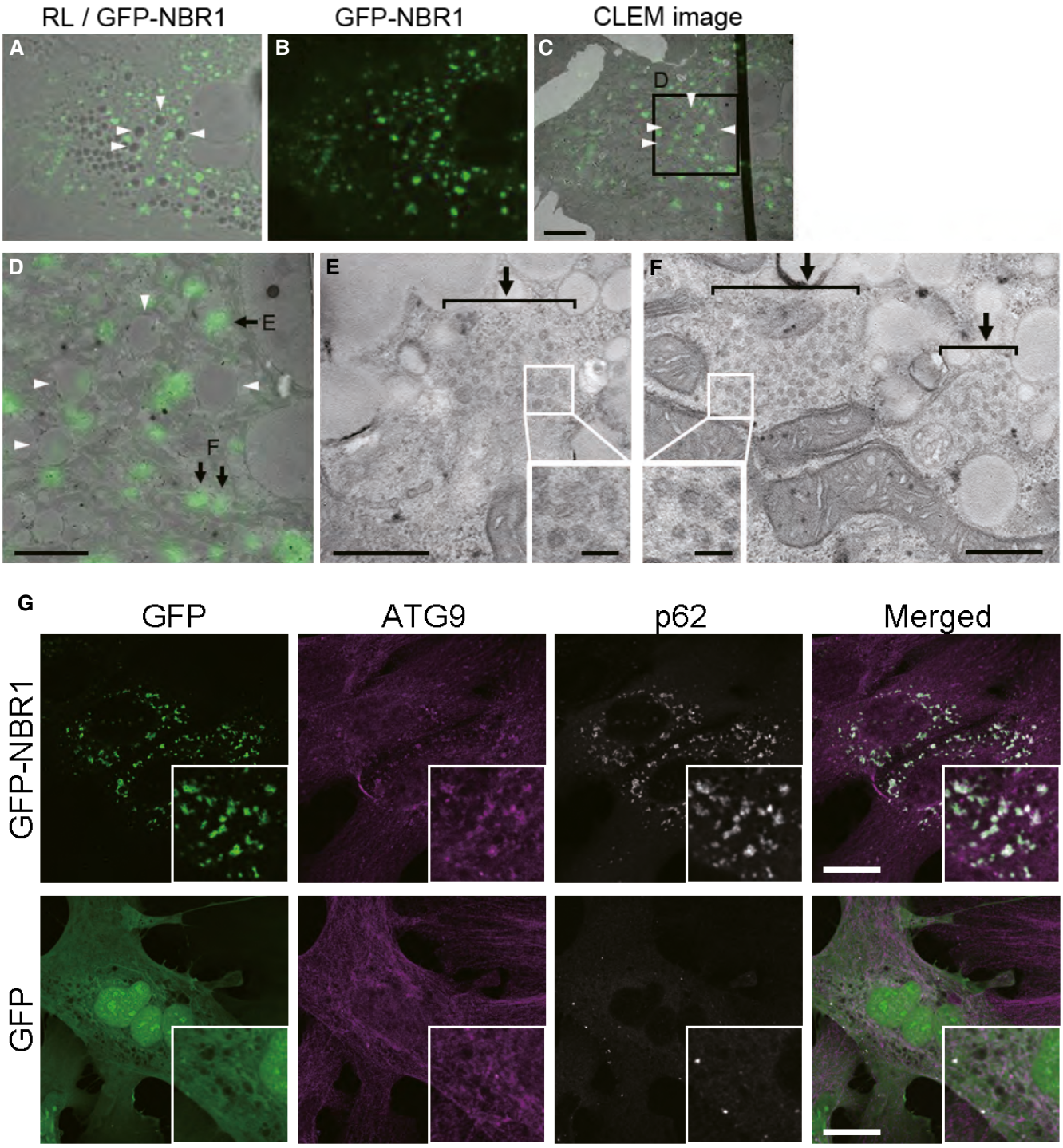

Figure EV5. Assembly of ATG9-vesicles onto p62 droplets by NBR1 overexpression.

A–F Correlative light-electron microscopy (CLEM). Wild-type hepatocytes were infected with GFP-NBR1 adenovirus for 48 h, and then, NBR1-positive structures (arrows) were identified by CLEM. The reflected light image (A) was aligned with the EM image (C) using 4 lipid droplets (arrowheads). The boxed region in C is enlarged and shown in D. Note that numerous small vesicles (approximately 40–50 nm in diameter; insets in E and F) are present in the GFP-NBR1-positive structures (arrows in E and F). Bars: 10 μm (C), 5 μm (D), 500 nm (E and F), and 100 nm (insets).

G Immunofluorescence microscopy. Wild-type hepatocytes were infected with GFP or GFP-NBR1 adenovirus for 48 h and then immunostained with anti-ATG9 antibody. Each inset is a magnified image. Bars: 20 μm. The overexpression of GFP-NBR1, but not GFP, induced the translocation of Atg9 onto the GFP-NBR1-positive structures.