Table S1. RNF126 siRNA duplexes used in this study.

Gene	No.	Sense sequence
Human RNF126 (ORF)	556	3’-GAGTCCTGCACTCAAACCCTATGGA-5’
Human RNF126 (ORF)	602	3’-CACTCAAACCCTATGGACTACGCCT-5’
Human RNF126 (3’-UTR)	1198	3’-CCTCTGTCTAACCTCACCCTCTAAA-5’
Human RNF126 (3’-UTR)	1219	3’-TAAACGTTCAGCGGTGGAAAGATTT-5’

ORF, open reading frame; UTR, untranslated region.

Table S2. Commercially available antibodies used in this study.

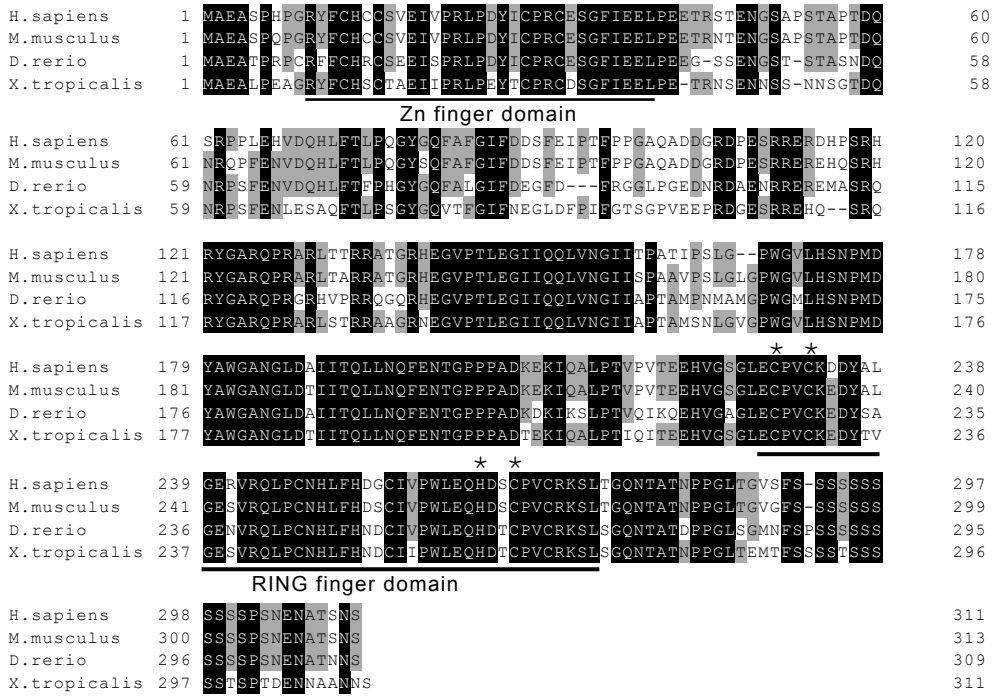
Antigen	Provider	Catalog number
DDB1	Zymed	37-6200
Phospho-H2A.X (Ser^{139})	Millipore	05-636
Histone H3	Abcam	ab1791
Hsc70	BD Biosciences	610607
I-SceI	Santa Cruz	sc-98269
Ku70	Santa Cruz	sc-1487
Ku80	Santa Cruz	sc-9034
Multi-ubiquitin (FK2)	MBL	D058-3
RNF8	Santa Cruz	sc-271462
α-Tubulin (B-5-1-2)	Sigma	T6074
FLAG (M2)	Sigma	F3165
Myc (9E10)	Santa Cruz	sc-40
GFP	MBL	598
MBP	Santa Cruz	sc-808
T7	Novagen	69552-3
6×His	Novagen	70796-3
CENP-F	Santa Cruz	sc-135865

Table S3. Identification of RNF126-associated proteins by proteomics analysis.

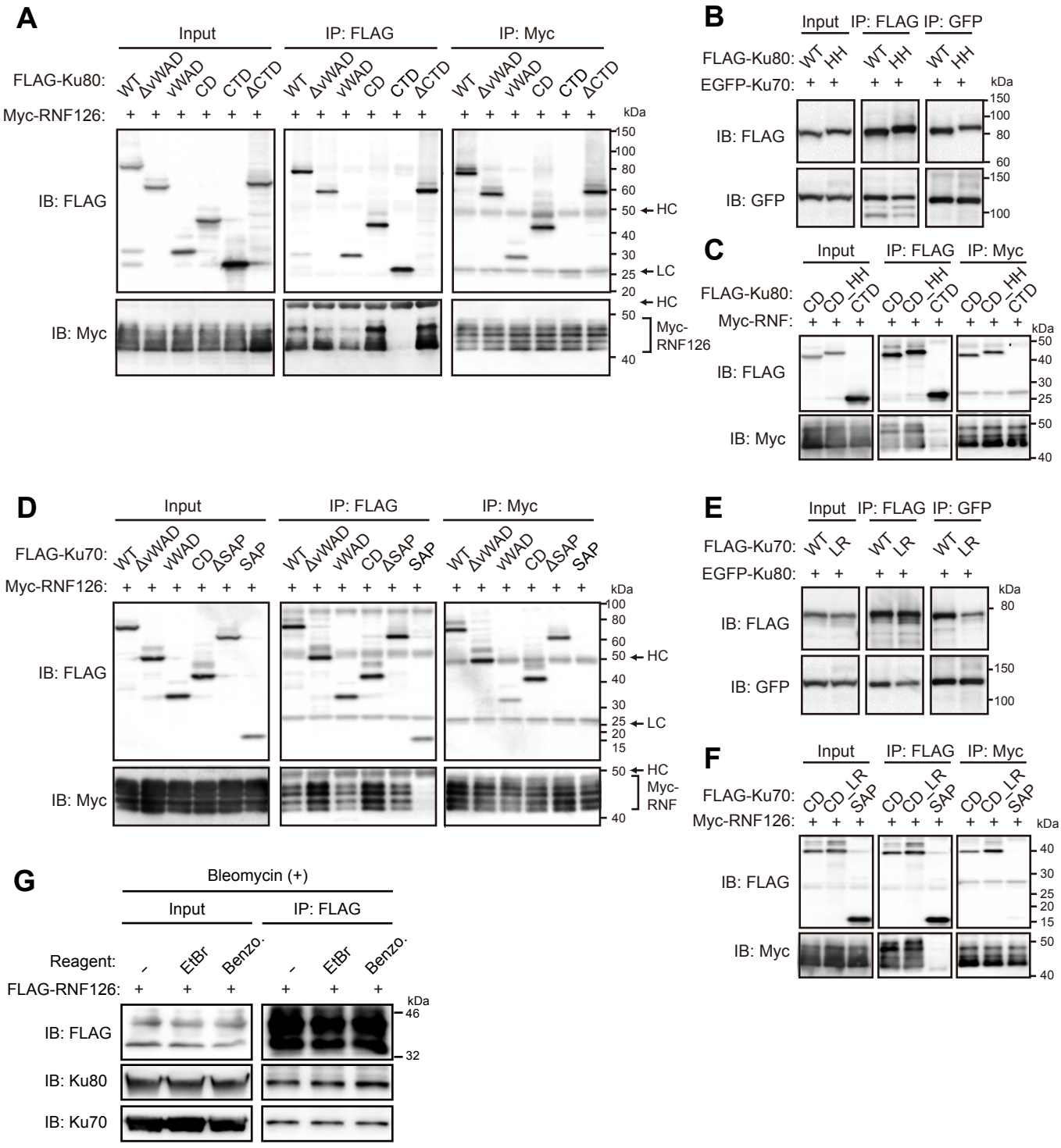
Gene symbol	Protein name
ACATE2	likely ortholog of mouse acyl-coenzyme A thioesterase 2, mitochondrial
ALDH3A2	aldehyde dehydrogenase 3 family, member A2
ASNA1	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)
ATP5C1	ATP synthase, H^+ transporting, mitochondrial F1 complex, gamma polypeptide 1
BAT3	HLA-B associated transcript 3; isoform unknown
BCAP31	B-cell receptor-associated protein 31
C7orf20	chromosome 7 open reading frame 20
CANX	calnexin
DDB1	damage-specific DNA binding protein 1, 127 kDa
GCS1	glucosidase I
GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12
IRS4	insulin receptor substrate 4
MAGED1	melanoma antigen, family D, 1
MAGED2	melanoma antigen, family D, 2
MLSTD2	male sterility domain containing 2
NAP1L1	nucleosome assembly protein 1-like 1
OCIA	ovarian carcinoma immunoreactive antigen
PRO1855	hypothetical protein PRO1855
PSMA4	proteasome (prosome, macropain) subunit, alpha type, 4
SKP1A	S-phase kinase-associated protein 1A (p19A)
SLC25A5	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5
SLC27A4	solute carrier family 27 (fatty acid transporter), member 4
TIMM13	translocase of inner mitochondrial membrane 13 homolog (yeast)
TIMM8A	translocase of inner mitochondrial membrane 8 homolog A (yeast)
TIMM8B	translocase of inner mitochondrial membrane 8 homolog B (yeast)
UBE2D3	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)
UBL4	ubiquitin-like 4
XRCC5	x-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80 kDa)
YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase

HEK293T cells transiently expressing FLAG-tagged human RNF126 were subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates were subjected

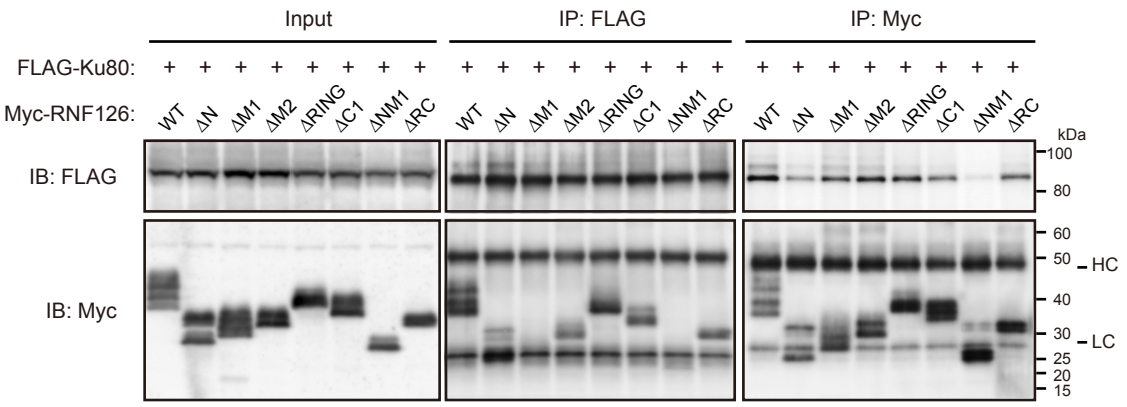
to LC–MS/MS analysis. Proteins reproducibly detected in at least three out of four independent experiments are listed.



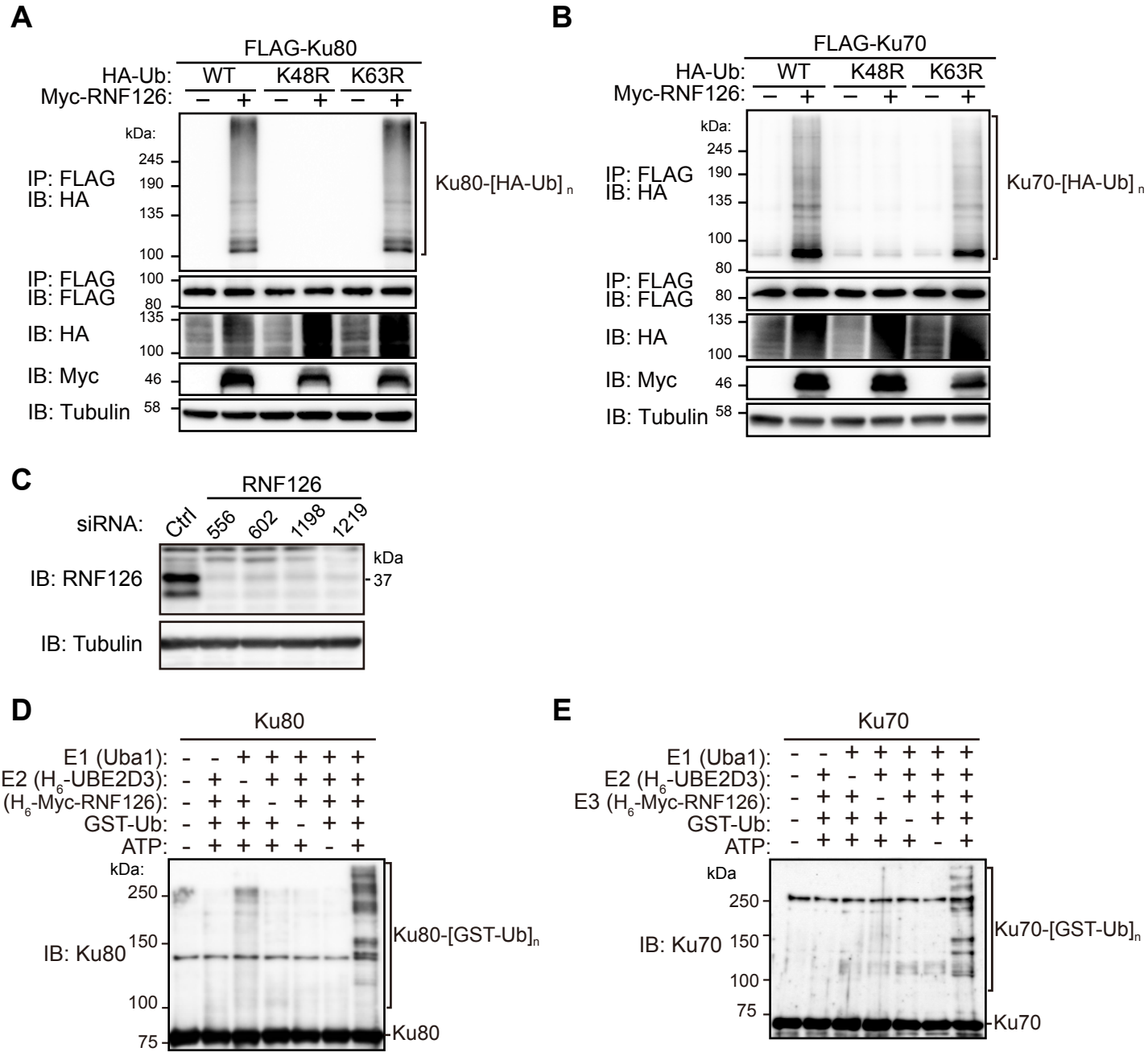
Supplementary Figure S1



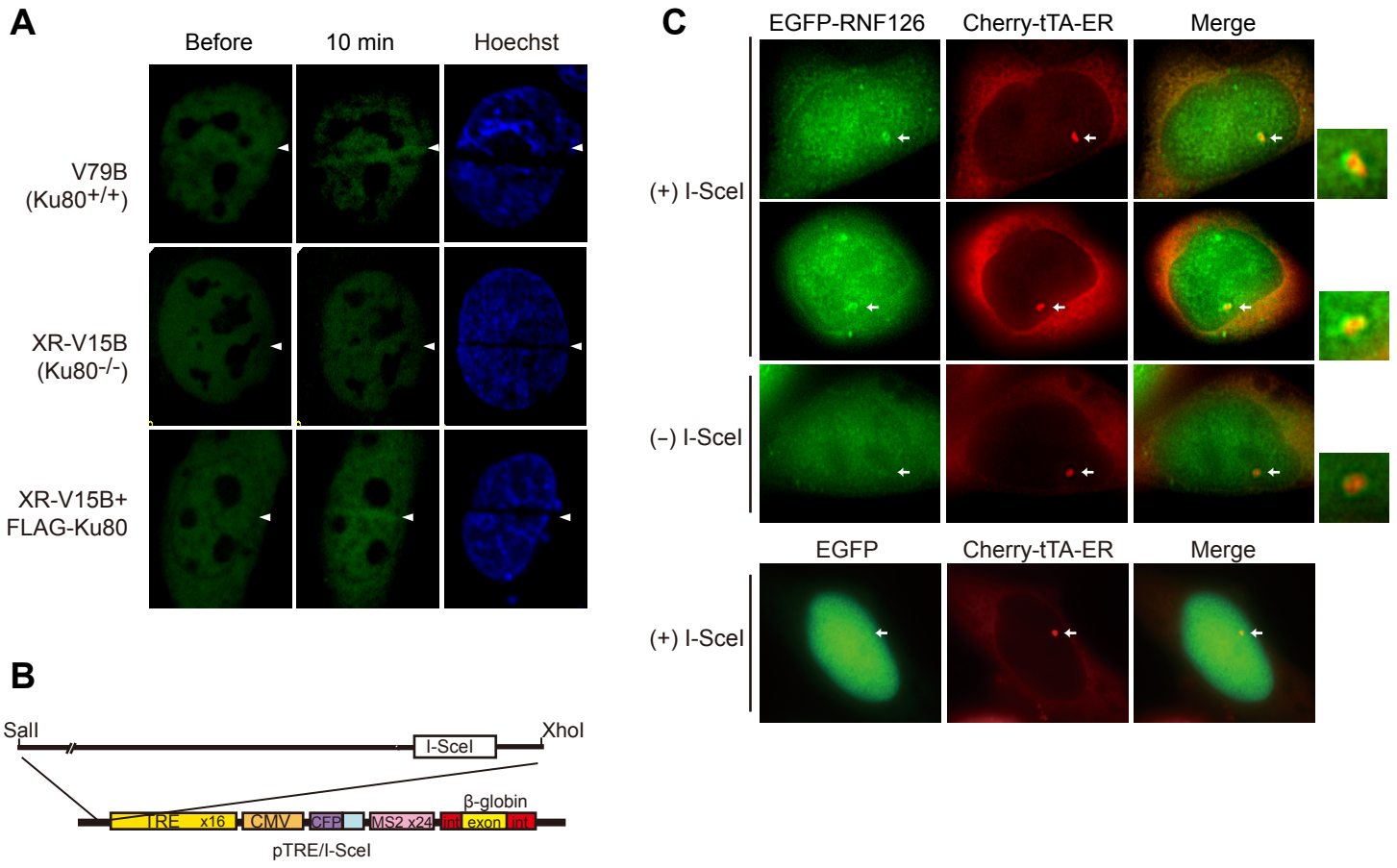
Supplementary Figure S2



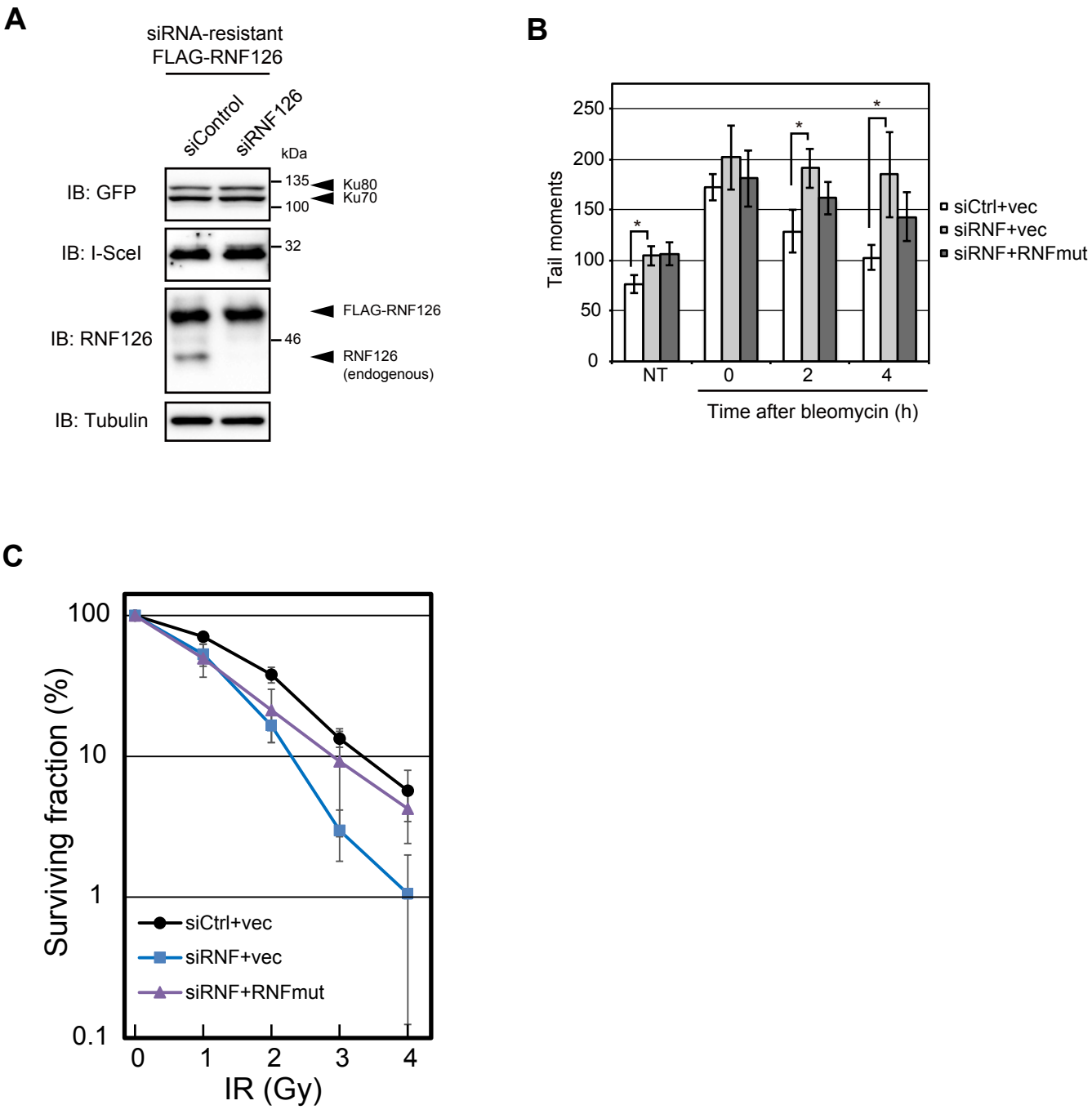
Supplementary Figure S3



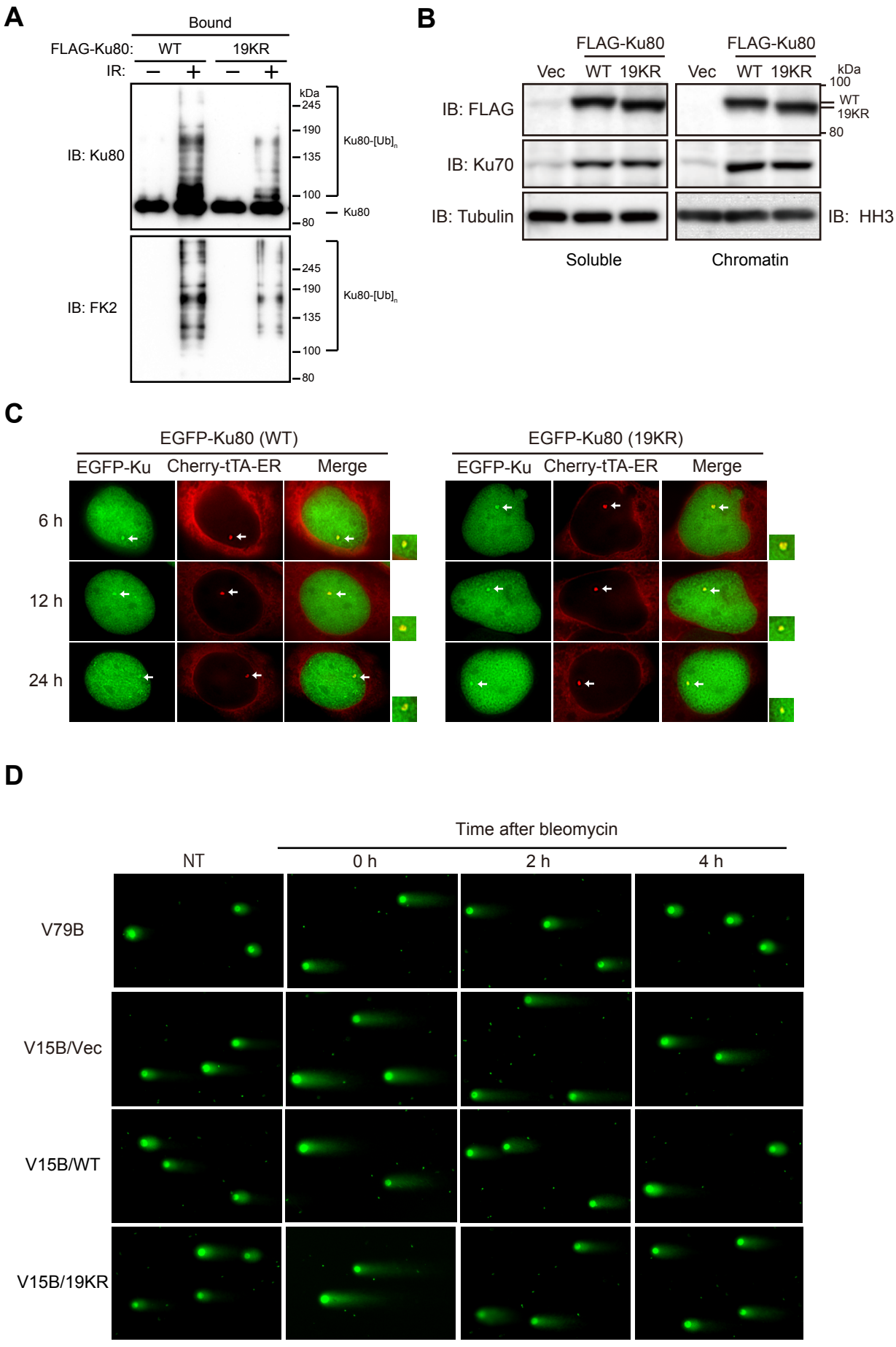
Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6



Supplementary Figure S7

Supplemental Figure Legends

Figure S1. Conserved domain structure of RNF126 in vertebrates.

The predicted amino acid sequences of human (*Homo sapiens*), mouse (*Mus musculus*), zebrafish (*Danio rerio*), and frog (*Xenopus tropicalis*) RNF126 are compared. Conserved residues are shaded in black (100% conservation) or gray (75% conservation). The zinc finger and RING finger domains are indicated. Asterisks denote conserved cysteine and histidine residues critical for ubiquitin ligase activity in the RING domain.

Figure S2. Delineation of the RNF126 binding regions of Ku70 and Ku80.

A RNF126 binding region of Ku80. HEK293T cells transiently expressing the indicated FLAG-tagged mutants of human Ku80 and Myc epitope–tagged mouse RNF126 were subjected to immunoprecipitation with antibodies to FLAG or to Myc, and the resulting precipitates (as well as 2.5% of the input cell lysates) were subjected to immunoblot analysis with the same antibodies. HC and LC, heavy and light chains of immunoglobulin.

B Effect of the HH (A453H/V454H) mutation of Ku80 on interaction with Ku70. HEK293T cells expressing EGFP-tagged human Ku70 and FLAG-tagged WT or HH mutant forms of

human Ku80 were subjected to immunoprecipitation with antibodies to FLAG or to GFP followed by immunoblot analysis with the same antibodies.

C Interaction of the CD_HH mutant of Ku80 with RNF126. HEK293T cells expressing Myc epitope–tagged mouse RNF126 and FLAG-tagged CD, CD_HH, or CTD mutant forms of human Ku80 were subjected to immunoprecipitation with antibodies to FLAG or to Myc followed by immunoblot analysis with the same antibodies.

D RNF126 binding region of human Ku70 examined as in **A**.

E Effect of the LR (L413R) mutation of Ku70 on interaction with Ku80 examined as in **B**.

F Interaction of the CD_LR mutant of Ku70 with RNF126 examined as in **C**.

G HEK293T cells transfected with an expression vector for FLAG-RNF126 were treated with bleomycin (5 μg/ml) for 1 h, and lysates prepared from the cells were then incubated with or without ethidium bromide (EtBr, 10 μg/ml) or Benzonase (Benzo., 5000 U/ml) for 15 min before immunoprecipitation with antibodies to FLAG. The resulting precipitates as well as the original cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.

Figure S3. Delineation of the Ku80 binding region of RNF126.

HEK293T cells transiently expressing the indicated Myc epitope–tagged mutants of mouse RNF126 as well as FLAG-tagged human Ku80 were subjected to immunoprecipitation with antibodies to FLAG or to Myc, and the resulting precipitates (as well as 2.5% of the input cell lysates) were subjected to immunoblot analysis with the same antibodies.

Figure S4. In vivo and in vitro ubiquitylation of Ku70 and Ku80 by RNF126.

A and **B** HEK293T cells transfected with expression vectors for FLAG-Ku80 (**A**) or FLAG-Ku70 (**B**), Myc-RNF126, and HA-tagged WT or K48R or K63R mutant forms of ubiquitin (Ub) were incubated with the proteasome inhibitor MG132 (10 μM) for 5 h, lysed, and subjected to immunoprecipitation with antibodies to FLAG under denaturing conditions (0.1% SDS). The resulting precipitates as well as the original cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.

C Efficiency of RNF126 knockdown by RNAi. U2OS cells were transfected for 48 h with one of four different RNF126 siRNAs or a control (Ctrl) siRNA and then subjected to immunoblot analysis with antibodies to RNF126. Unless indicated otherwise, RNF126 siRNA 602 was used in experiments for this study.

D and **E** Polyubiquitylation of Ku80 (**D**) and Ku70 (**E**) by recombinant RNF126 in vitro. Recombinant His_6-Myc-RNF126 as well as E1 (Uba1), E2 (His_6-UBE2D3), GST-ubiquitin, ATP, and recombinant Ku80 or Ku70 were incubated in the indicated combinations for 30 min at 26°C, after which ubiquitylated forms of Ku80 or Ku70 were detected by immunoblot analysis with corresponding antibodies.

Figure S5. Accumulation of RNF126 at DSB sites is dependent on Ku80.

A V79B ($Ku80^{+/+}$) and XR-V15B ($Ku80^{-/-}$) cells expressing EGFP-tagged RNF126 were monitored for EGFP fluorescence before and 10 min after laser microirradiation. EGFP-RNF126 accumulated at laser-irradiated sites in V79B cells but not in XR-V15B cells. Accumulation of EGFP-RNF126 at such sites was observed, however, in XR-V15B cells expressing FLAG-Ku80. Arrowheads indicate path of irradiation.

B Schematic representation of the pTRE/I-SceI plasmid integrated into U2OS/TRE/I-SceI-19 cells.

C U2OS/TRE/I-SceI-19 cells were electroporated with expression vectors for Cherry-tTA-ER, I-SceI, and either EGFP-tagged RNF126 or EGFP (as indicated), exposed to tamoxifen for 6 h, and then examined for EGFP and Cherry fluorescence. Arrows indicate sites of Cherry-tTA-ER

accumulation, higher magnification views of which are shown in the insets for the merged images.

Figure S6. Attenuation of RNF126 siRNA–induced defects in DSB repair by expression of an siRNA-resistant form of RNF126.

A Immunoblot analysis of U2OS/TRE/I-SceI-19 cells that had been infected with a lentivirus encoding a FLAG-tagged siRNA-resistant form of RNF126, transfected with RNF126 or control siRNAs, and then subjected to electroporation with expression vectors for EGFP-tagged Ku70 and Ku80, I-SceI, and Cherry-tTA-ER. The nucleotide sequence targeted by the RNF126 siRNA was 5'-GAGTCCTGCACTCAAACCCTATGGA-3', whereas the corresponding siRNA-resistant sequence contained two mutations (5'-GAGTCCTGCACTCAAATCCCATGGA-3').

B U2OS/TRE/I-SceI-19 cells were infected with a lentivirus encoding an siRNA-resistant form of RNF126 (RNFmut) or the corresponding empty virus (vec), transfected with RNF126 or control siRNAs, treated (or not, NT) with bleomycin (0.1 μg/ml), incubated for the indicated times, and then subjected to a single-cell neutral gel electrophoresis (comet) assay of DSBs as in Figure 5A. Data are means ± SEM for tail moments in 750 cells from three independent experiments. *$P < 0.05$ (Student's *t* test).

C U2OS/TRE/I-SceI-19 cells infected with a lentivirus encoding an siRNA-resistant form of RNF126 (or with the corresponding empty virus) and transfected with RNF126 or control siRNAs were exposed to IR before determination of colony-forming ability as in Figure 5D. Data are means ± SEM from three independent experiments.

Figure S7. Ubiquitylation of Ku80 by RNF126 is required for Ku removal from DSB sites.

A XR-V15B cells stably expressing FLAG-tagged Ku80(WT) or Ku80(19KR) were exposed (or not) to IR (2 Gy), incubated with 10 μM MG132 for 5 h, and then lysed for consecutive precipitation of ubiquitylated proteins with the TUBE system and immunoprecipitation with antibodies to Ku80. The final precipitates were subjected to immunoblot analysis with antibodies to the indicated proteins.

B Immunoblot analysis of soluble and chromatin fractions prepared from XR-V15B cells stably expressing FLAG-tagged Ku80(WT) or Ku80(19KR).

C U2OS/TRE/I-SceI-19 cells electroporated with expression vectors for EGFP-Ku70, Cherry-tTA-ER, I-SceI, and EGFP-tagged Ku80(WT) or Ku80(19KR) were treated with tamoxifen and monitored for EGFP and Cherry fluorescence at 6, 12, and 24 h after electroporation. Arrows

indicate DSBs marked by Cherry-tTA-ER, higher magnification views of which are shown in the insets for the merged images.

D XR-V15B cells stably expressing FLAG-tagged Ku80(WT) or Ku80(19KR) were treated (or not, NT) with bleomycin (0.1 μg/ml) and then incubated for the indicated times, after which tail moments were examined by single-cell neutral gel electrophoresis (comet assay) as in Figure 6E. V79B ($Ku80^{+/+}$) cells and XR-V15B cells transfected with an empty vector (Vec) were analyzed as controls.