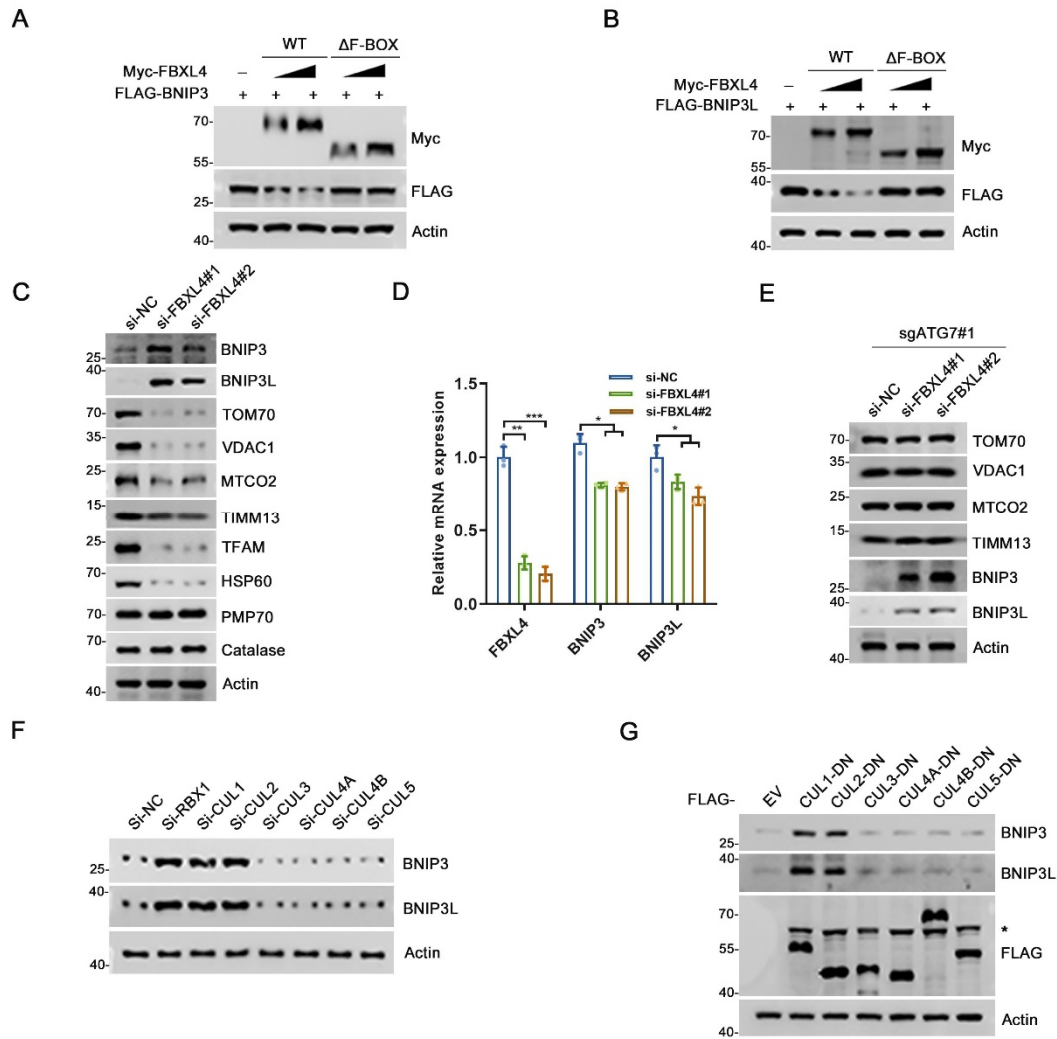


Supplementary Materials for

**FBXL4 Mutations Cause Excessive Mitophagy via BNIP3/BNIP3L
Accumulation Leading to Mitochondrial DNA Depletion Syndrome**



Supplementary Figure 1. FBXL4 mediates ubiquitination of BNIP3 and BNIP3L (related to Figure 2).

(A) 293T cells were transfected with FLAG-BNIP3 and increasing amount of Myc-FBXL4 and Myc-FBXL4- Δ F-BOX mutant. The WCL were prepared for WB with the indicated antibodies.

(B) 293T cells were transfected with FLAG-BNIP3L and increasing amount of Myc-FBXL4 and Myc-FBXL4- Δ F-BOX. The WCL were prepared for WB with the indicated antibodies.

(C) WCL from H1299 cells transfected with FBXL4-specific siRNA or siNC were prepared for WB with the indicated antibodies. FBXL4 KO cell lines were generated through lentiCRISPRv2 methods. The WCL from parental and FBXL4 KO H1299 cells were prepared for WB with the indicated antibodies.

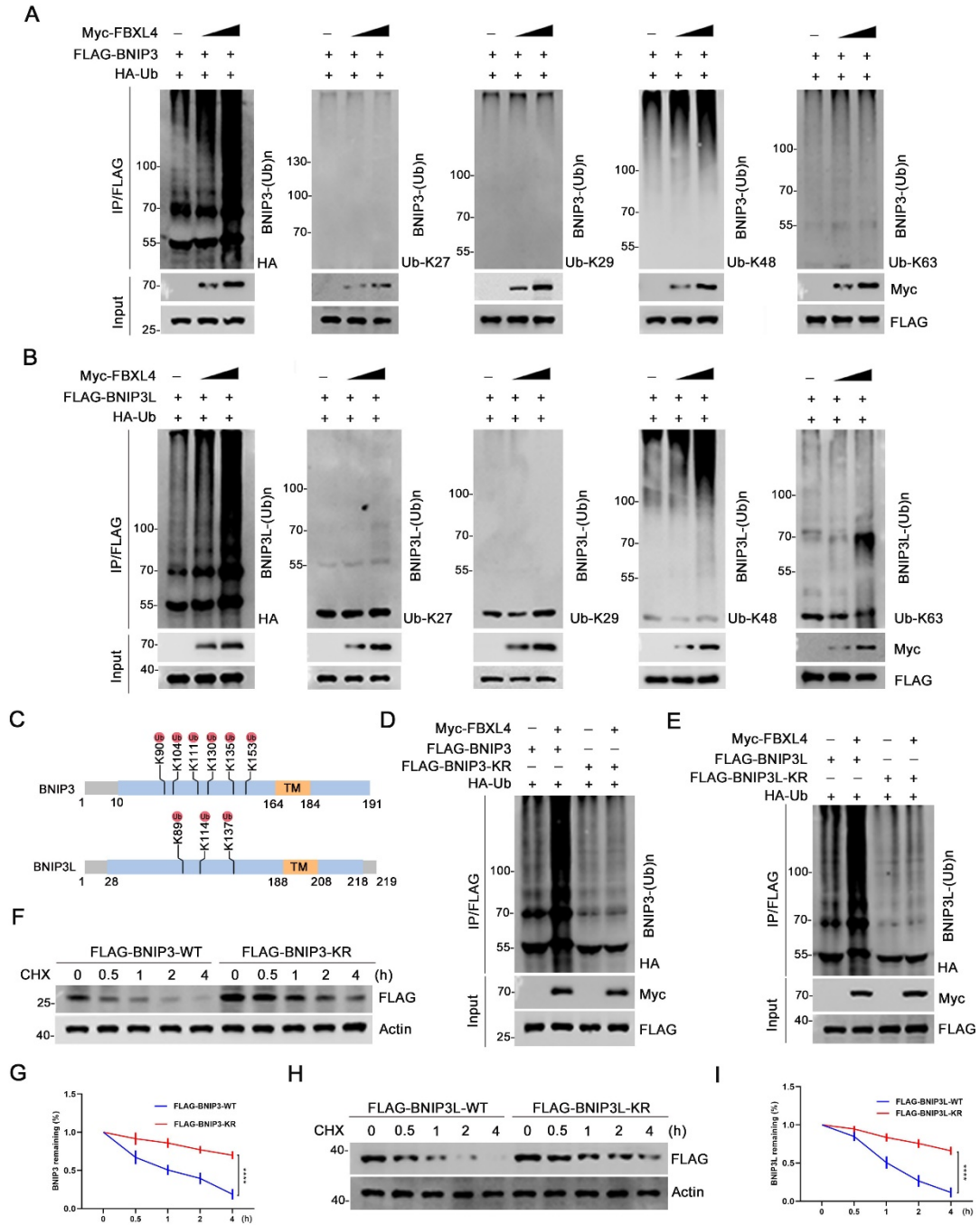
(D) RT-qPCR measurement of FBXL4, BNIP3 and BNIP3L mRNA expression in H1299 cells

transfected with FBXL4-specific siRNAs, Data are shown as means \pm SD (n = 3). *P* values are calculated by the Two-way ANOVA test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

(E) ATG7 KO HeLa cells were generated through CRISPR/Cas9 methods. The WCLs from ATG7 KO HeLa cells transfected with FBXL4-specific siRNAs or siNC were prepared for WB with the indicated antibodies.

(F) WB analysis of the indicated proteins in the WCL from HeLa cells transfected with indicated siRNAs.

(G) WB analysis of the indicated proteins in the WCL from 293T cells transfected with the indicated plasmids.



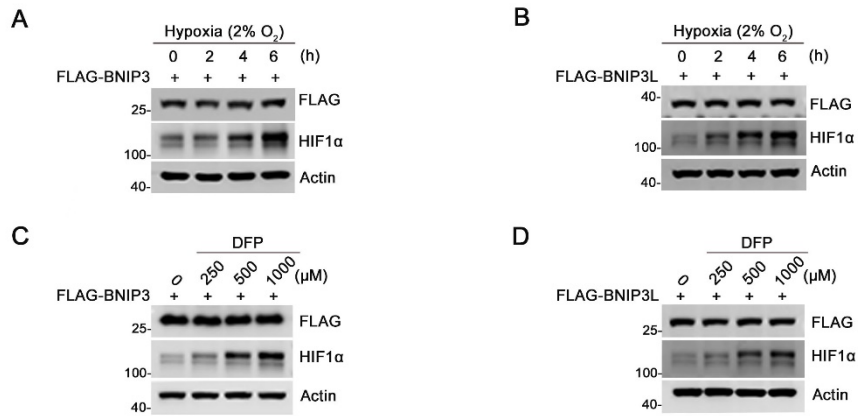
Supplementary Figure 2. FBXL4 promotes degradative ubiquitination of BNIP3/BNIP3L (related to Figure 2).

(A, B) WB analysis of the indicated proteins *in vivo* ubiquitination assays performed products and WCL from 293 T cells transfected with the indicated plasmids.

(C) The distribution of the ubiquitin attachment sites of BNIP3 and BNIP3L.

(D, E) WB analysis of the indicated proteins *in vivo* ubiquitination assays performed products and WCL from 293 T cells transfected with the indicated plasmids.

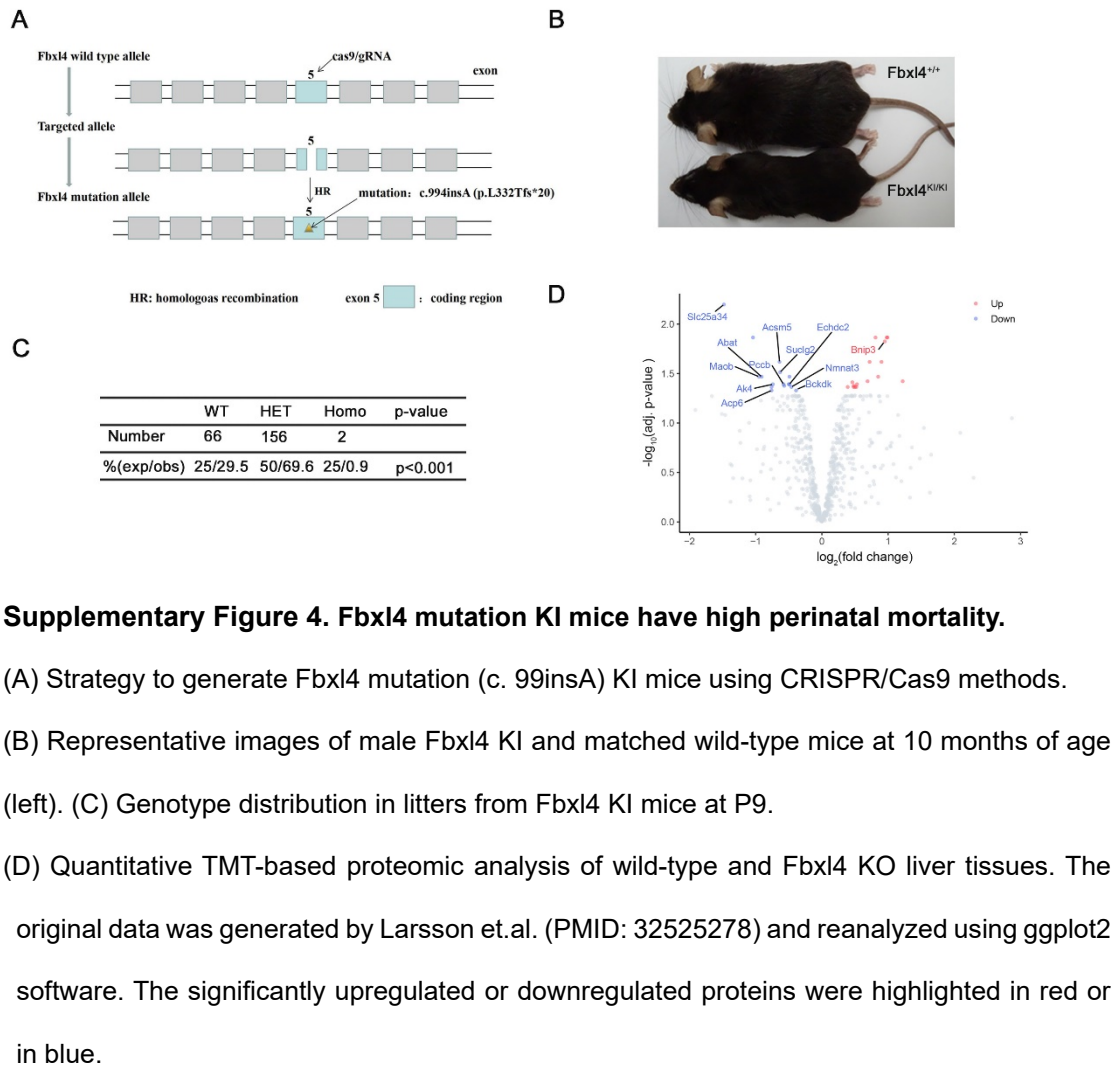
(F-I) WB analysis of the indicated proteins in the WCL of cells transfected with indicated plasmids and treated with cycloheximide (CHX, 50 μ g/ml) and harvested at different time points. At each time point, the intensity of BNIP3 (G) and BNIP3L (I) was normalized to the intensity of Actin and then to the value at 0 h. *P* values are calculated by the Two-way ANOVA test. *****p* <0.0001.

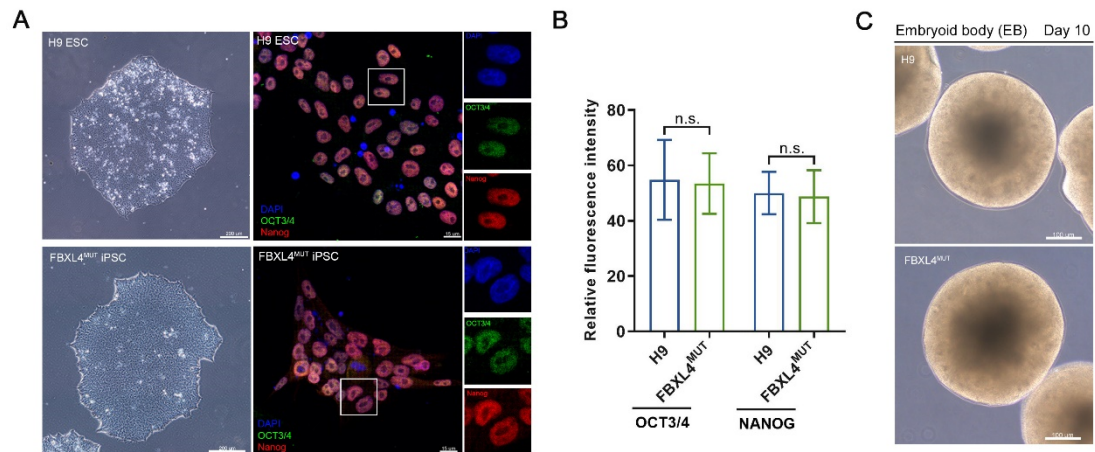


Supplementary Figure 3. Iron loss or hypoxia did not affect the protein stability of ectopically overexpressed BNIP3/3L (related to Figure 2).

(A, B) WB analysis of the indicated proteins in the WCL of cells transfected with indicated plasmids and exposed to hypoxia (2% O₂) for the indicated times.

(C, D) WB analysis of the indicated proteins in the WCL of cells transfected with indicated plasmids and treated with DFP (0, 250, 500, 1000 μM) for 24 h.



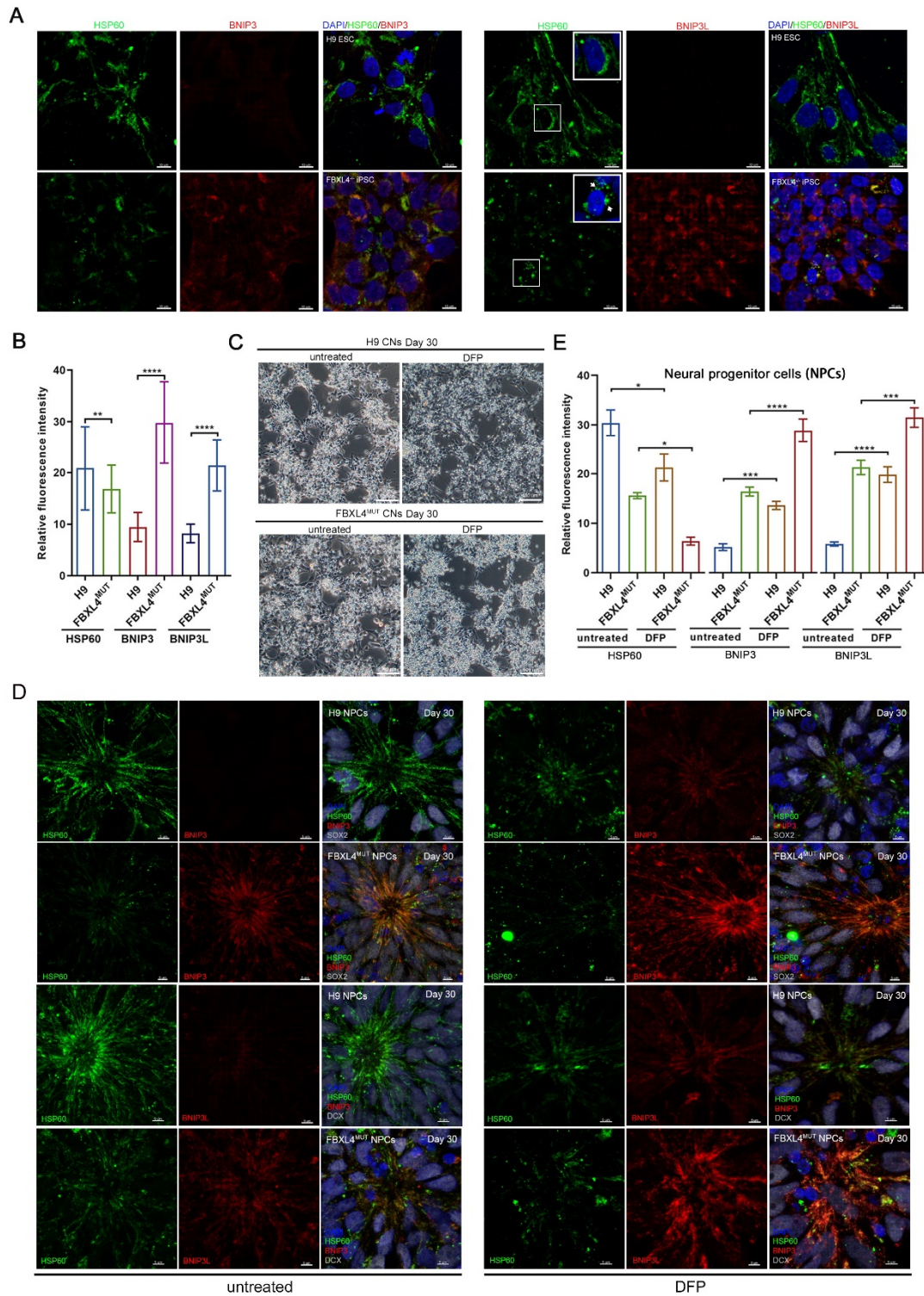


Supplementary Figure 5. Stemness markers and embryoid body formation are unaltered in MTDPS13 patient hiPSCs (related to Figure 6).

(A) Representative bright-field images (left) of H9 ESC and FBXL4^{MUT} iPSCs and images (right) of OCT3/4 and Nanog antibody immunostaining with a high-magnification view of typical stem cells. Scar bar, 15 and 200 μ m.

(B) Relative intensity mean of OCT3/4 and Nanog signals in H9 ESC and FBXL4^{MUT} iPSC. Data were shown as means \pm SD (n = 50). *P* values are calculated by the Two-way ANOVA test. n.s: no significant.

(C) Representative bright-field images of EB derived from H9 ESC and FBXL4^{MUT} iPSCs on day 10. EB: embryoid body. Scar bar, 100 μ m.



Supplementary Figure 6. Mitophagy is abnormally activated in cortical neurons induced from MTDPS13 patient hiPSCs (related to Figure 6).

(A) Representative images of HSP60, BNIP3 and BNIP3L antibody immunostaining in H9 ESCs and FBXL4^{MUT} iPSCs. White arrowhead: spot-like HSP60 structure. Scar bar, 10 μ m.

(B) Comparison of the HSP60, BNIP3 and BNIP3L immunostaining intensity in PSCs. Data

were shown as means \pm SD (n = 50). *P* values are calculated by the Two-way ANOVA test. ***P* < 0.005; *****P* < 0.0001.

(C) Representative bright-field images of DFP (500 μ M)-treated H9/FBXL4^{MUT} CNs (right) with untreated H9/FBXL4^{MUT} CNs on day 30. Scar bar, 100 μ m.

(D-E) Representative images (D) of HSP60, BNIP3 (or BNIP3L) antibody immunostaining in DFP (500 μ M)-treated H9/FBXL4^{MUT} NPCs (right) with untreated H9/FBXL4^{MUT} NPCs (left) of neural tube. Scar bar, 5 μ m. Comparison of the HSP60, BNIP3 and BNIP3L immunostaining intensity in NPCs. Data were shown as means \pm SD (n = 50). *P* values are calculated by the Two-way ANOVA test. **P* < 0.05; ****P* < 0.0005; *****P* < 0.0001.