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Supplementary Materials for

FOXO1 deficiency impairs proteostasis in aged T cells

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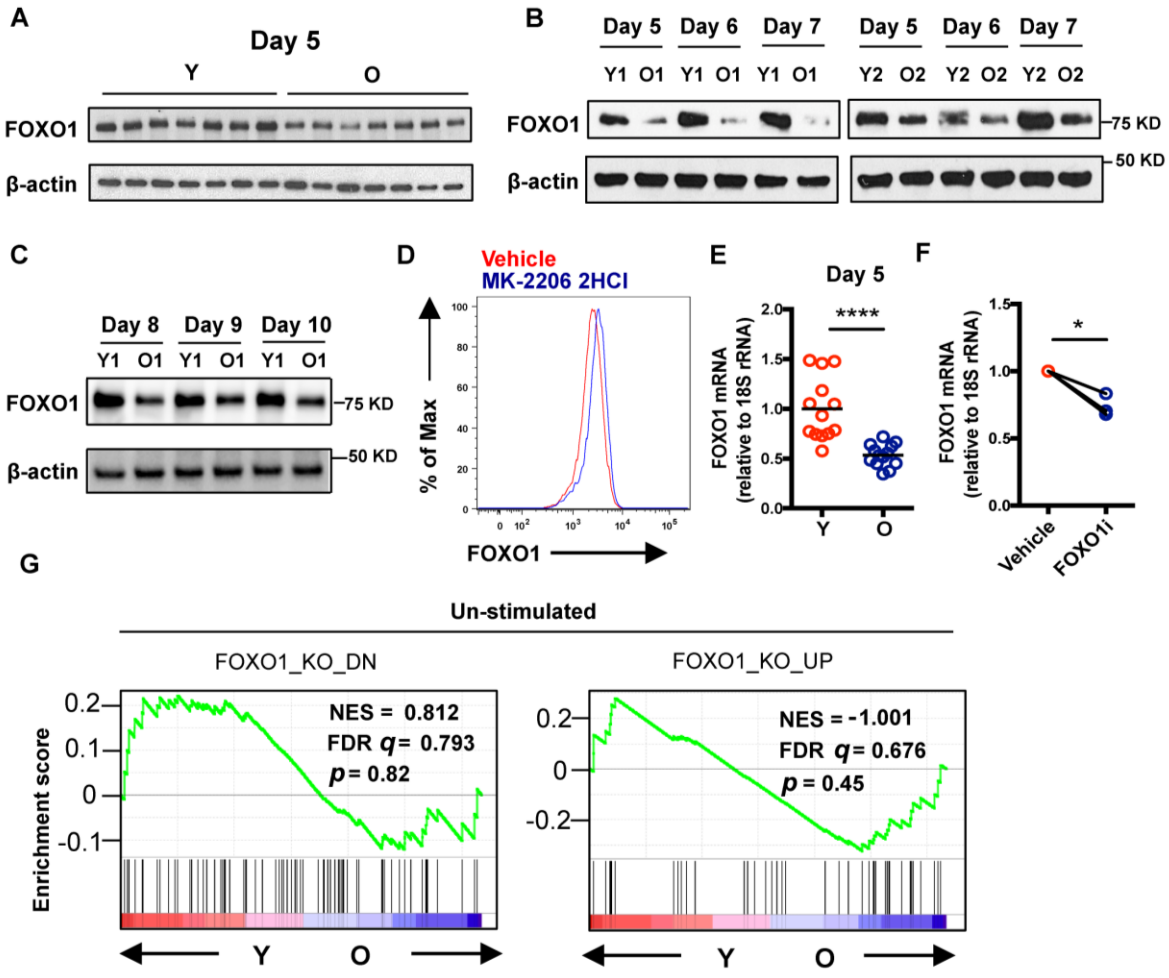

Fig. S1, related to Fig. 1. Age-associated failure to restore FOXO1 activity in naïve CD4$^+$ T cell responses. (A) Naïve CD4$^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days. FOXO1 protein expression was measured by Western blotting. Representative Western blots of cells from seven young and seven old individuals. (B) Kinetic analysis of FOXO1 protein expression on days 5, 6 and 7 after activation. Representative Western blots of cells from two young and two old individuals. (C) Kinetic analysis of FOXO1 protein expression on day 8, 9 and 10 after activation. Representative Western blots of cells from one young and one old individual. (D) Old naïve CD4$^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, the last 2 days in the presence of vehicle or an AKT inhibitor (MK-2206 2HCl). FOXO1 protein expression was determined by flow cytometry. The histogram shown is representative of 3 independent experiments. (E) *FOXO1* transcripts from naïve CD4$^+$ T cells on day 5 after activation as described in (A) were measured by RT-PCR. Results are shown relative to the mean result of cells from young individuals. The horizontal lines represent mean values; comparison by two-tailed unpaired t-test. (F) Day 5-stimulated naïve CD4$^+$ T cells were treated with FOXO1 inhibitor for 6 hours. *FOXO1* transcripts were measured by RT-PCR. Results are shown relative to vehicle cells. Comparison by two-tailed paired t-test). (G) GSEA analysis comparing fold transcript differences in unstimulated young compared to unstimulated old naïve CD4$^+$ T cells

with that of experimental datasets from mouse *Foxo1* knockout unstimulated T cells (*46*). *p < 0.05, ****p < 0.0001.

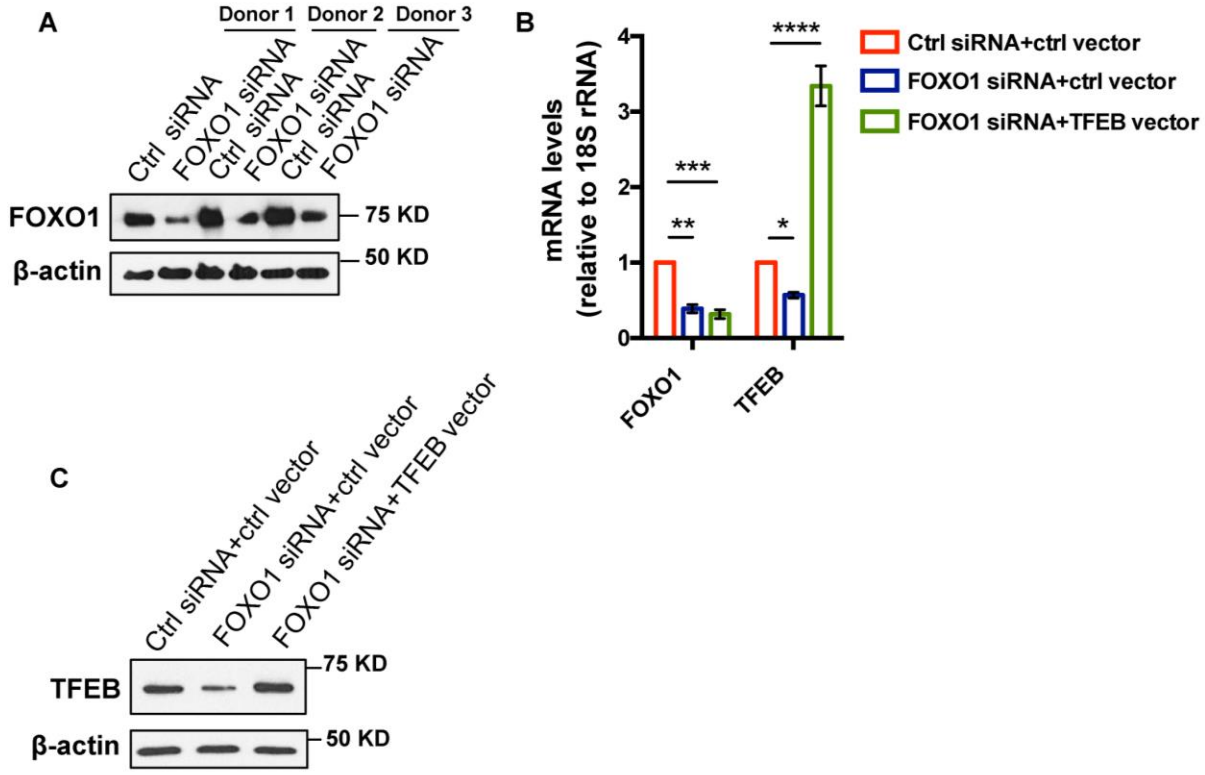

Fig. S2, related to Fig. 1. FOXO1 regulates TFEB expression in naïve CD4$^+$ T cells. (A) Naïve CD4$^+$ T cells from young healthy adults were activated with anti-CD3/anti-CD28 beads, transfected with FOXO1 or control siRNA on day 2 and then cultured on plates coated with 5 µg/ml anti-CD3 and 5 µg/ml anti-CD28. FOXO1 silencing was confirmed by Western blotting. (B-C) Naïve CD4$^+$ T cells were activated as described in (A) and transfected with indicated siRNA/plasmid. *FOXO1* and *TFEB* gene expression were quantified by RT-PCR (B). Results are normalized to control samples; mean ± SEM of 4-6 experiments, comparison by two-way ANOVA followed by Tukey's multiple comparison test. TFEB protein expression was determined by Western blotting (C). Western blots are representative of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

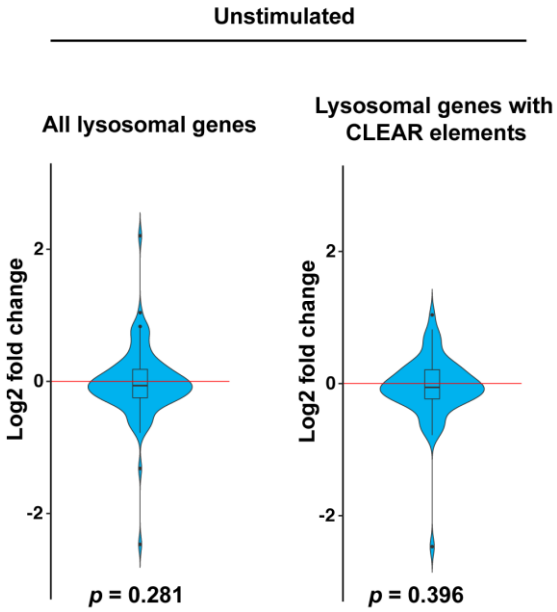

Fig. S3, related to Fig. 1. Age-related failure in re-expressing lysosomal genes. Transcriptome data from naïve $CD4^+$ T cells from 3 young and 3 old healthy individuals before stimulation, contrasting the results in Fig. 1G for stimulated cells. Violin plots and boxplots showing the fold gene expression differences of lysosomal genes, comparing the transcriptome of old to young T cells. Lysosomal gene sets were drawn from published data in human-derived cell lines (*14, 18*). Statistical analysis by Wilcoxon rank sum tests.

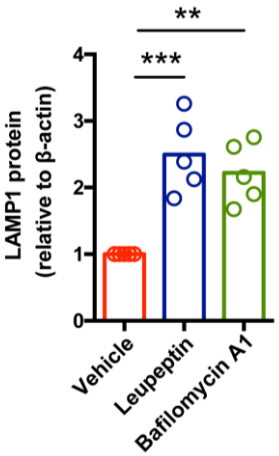

Fig. S4, related to Fig. 2. Lysosome inhibition increases LAMP1 protein expression. Naïve $CD4^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, with the last 2 days in the presence of vehicle or a lysosome inhibitor. LAMP1 protein expression was determined by Western blotting. Results are normalized to control samples; bars represent means of 5 experiments, comparison by one-way ANOVA followed by Tukey's multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$.

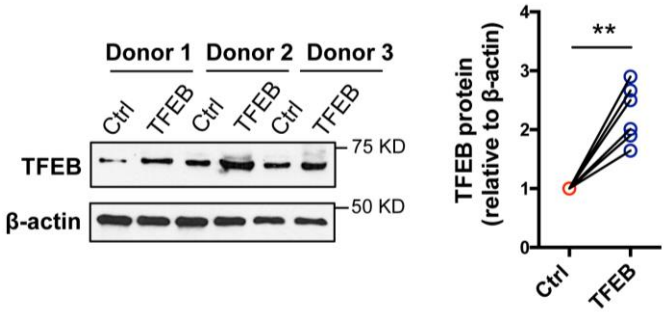

Fig. S5, related to Fig. 2. Overexpression of TFEB in activated naïve CD4$^+$ T cells. Resting naïve CD4$^+$ T cells from an old healthy individual were transfected with a TFEB expression vector or a control vector. Cells were then stimulated with anti-CD3/anti-CD28 beads for 5 days. TFEB protein expression was determined by Western blotting. Representative blots of cells from three old individual (left) and results normalized to control vector-transfected cells (right) (n = 6, two-tailed paired t-test). $^{**}p < 0.01$.

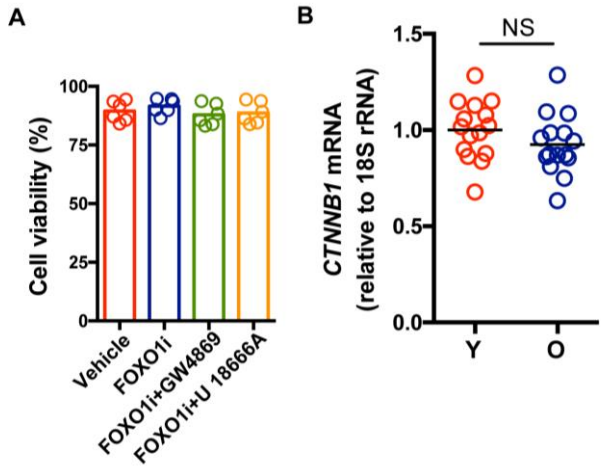

Fig. S6, related to Fig. 3. CTNNB1 gene expression does not change with aging in naïve CD4$^+$ T cells, in contrast to β-catenin protein expression. (A) Naïve CD4$^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, with the last 2 days in the presence of vehicle or indicated inhibitor. Cell viability was determined as the percentage of Annexin V negative population. (B) *CTNNB1* gene expression in day 5 stimulated CD4$^+$ naïve T cells was assessed by RT-PCR. Results from 15 young and 15 old healthy individuals (two-tailed unpaired t-test). The horizontal lines represent mean values. NS, not significant.

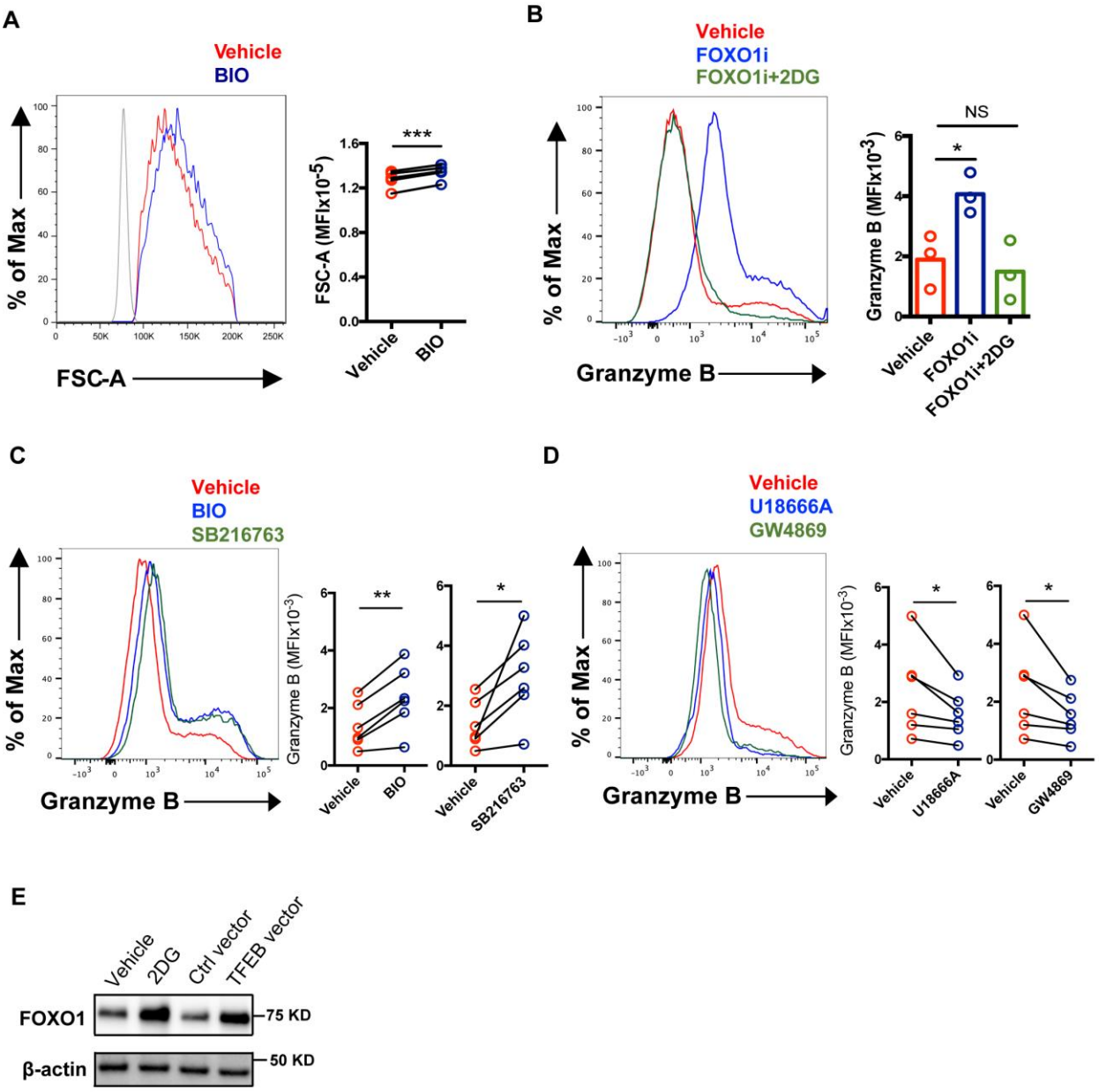

Fig. S7, related to Figs. 3 and 5. GSK3 regulates cell size and effector functions. (A) Naïve $CD4^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, with the last 24 hours in the presence of vehicle or a GSK3 inhibitor (BIO). FSC-A was determined by flow cytometry. Representative histograms (left) and results of samples from 5 young healthy individuals (right). Comparison by two-tailed paired t-test. (B) Naïve $CD4^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, with the last 2 days in the presence of vehicle, FOXO1 inhibitor or the combination of FOXO1 inhibitor and the glucose analog 2-deoxyglucose (2DG). Representative histograms of intracellular granzyme B after re-stimulation with PMA and

ionomycin (left) and cumulative results normalized to control cells (right). Comparison by two-tailed paired t-test. (C-D) Three days after anti-CD3/CD28 bead stimulation, naïve $CD4^+$ T cells from a young individual were cultured for 48 hours with control vehicle or a GSK3β inhibitor (C), whereas those from an old individual were cultured with an MVB/exosome inhibitor (D). Representative histograms of intracellular granzyme B after re-stimulation with PMA and ionomycin (left) and cumulative results normalized to control cells (right). Comparison by two-tailed paired t-test. (E) Naïve $CD4^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, with the last 2 days in the presence of vehicle or the glucose analog 2DG. Alternatively, cells were transfected with a TFEB expression vector or a control vector as described in fig. S5. FOXO1 protein expression was measured by Western blotting. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

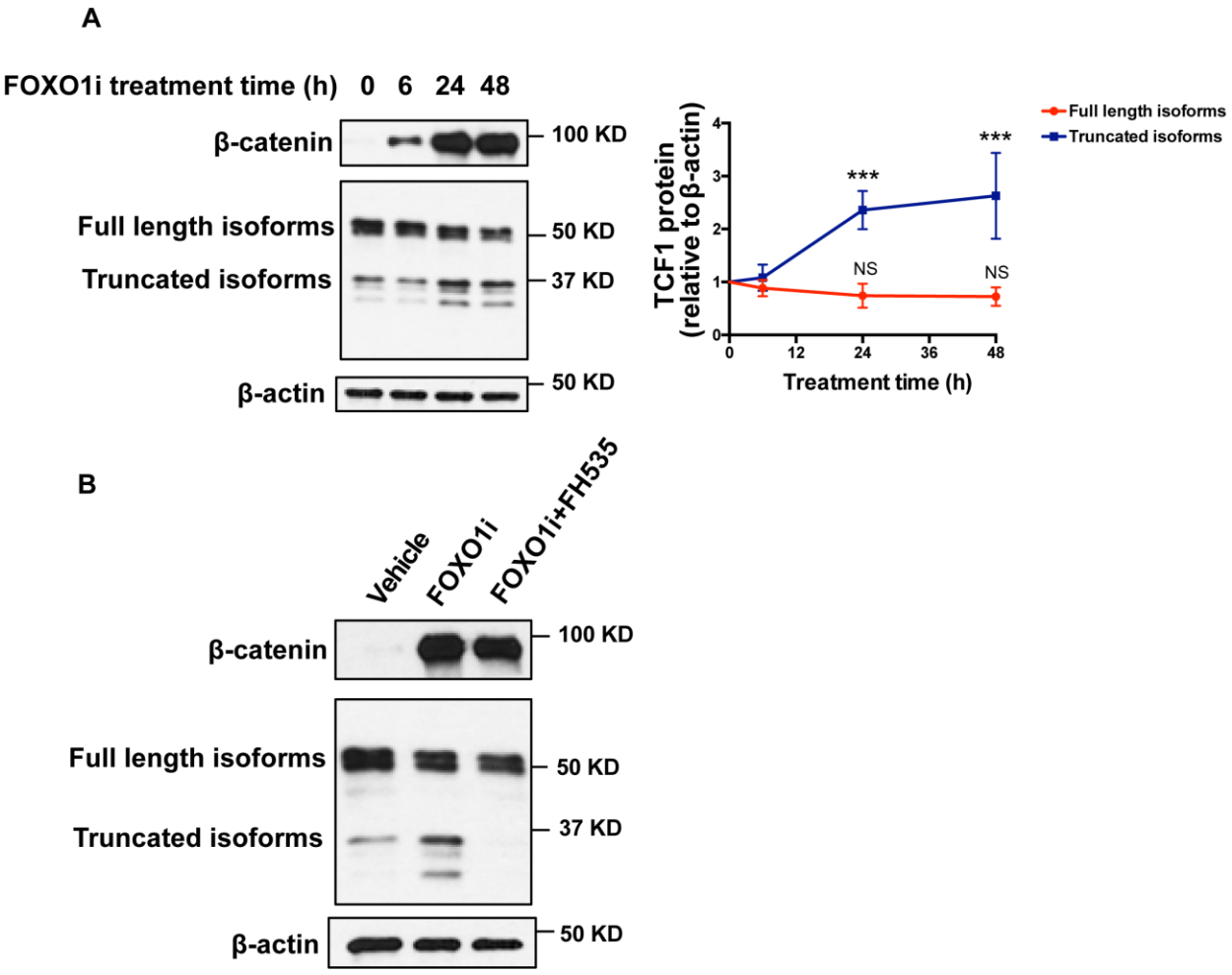

Fig. S8, related to Fig. 3 and 5. FOXO1 inhibition induces β-catenin, but only expression of truncated TCF1 protein. (A) Naïve $CD4^+$ T cells were activated with anti-CD3/anti-CD28 beads for 5 days, in the presence of vehicle or FOXO1 inhibitor during the last culture time. The full length and truncated isoforms of TCF1 were examined by Western blotting. Representative Western blots (left) and results normalized to untreated samples (right); mean ± SD of 3 experiments, comparison by two-way ANOVA followed by Tukey's multiple comparison test. (B) To prove that the increase of truncated isoforms following FOXO1 inhibition is β-catenin-dependent, naïve $CD4^+$ T cells were treated as in (A), with the last 24 hours in the presence of FOXO1 inhibitor alone or together with a β-catenin inhibitor (FH535). The full-length isoforms and truncated isoforms of TCF1 were examined by Western blotting. Western blots representative of 3 experiments. ***$p < 0.001$, NS. not significant.

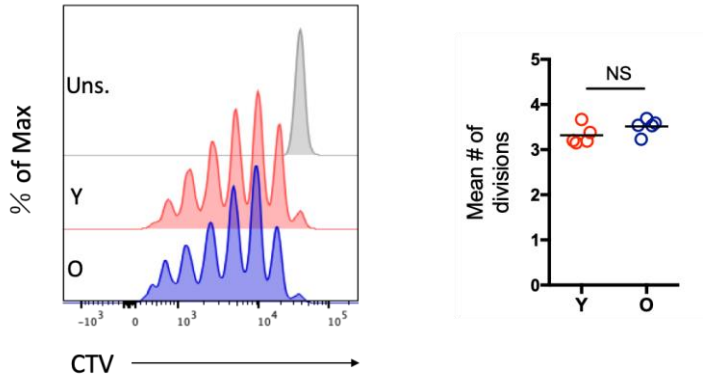

Fig. S9, related to Fig. 6. Comparable proliferation rates in young and old naïve CD4$^+$ T cells after activation. Naïve CD4$^+$ T cells from five young and five older individuals were activated with anti-CD3/anti-CD28 beads for 5 days. Cell proliferation was monitored by Cell Trace Violet (CTV). Representative CTV histograms (left) and cumulative data of mean number of divisions for young and old cells (right). NS, not significant.

Table S1. Oligonucleotide primer sets used in this study.

Gene	Forward	Reverse
FOXO1	TCGTCATAATCTGTCCCTACACA	CGGCTTCGGCTCTTAGCAAA
TFEB	GACCCAGAAGCGAGAGCTCACA	TGTGATTGTCTTTCTTCTGCCG
CTSA	GTGCCCAGCCATTTTAGGTA	CTTCCGCACGTACGGGTTGT
CTSB	CCAGGGAGCAAGACAGAGAC	GAGACTGGCGTTCTCCAAAG
CTSD	GACACAGGCACTTCCCTCAT	CTCTGGGGACAGCTTGTAGC
CTSH	ACGGAGGAGTACCACCACAG	GCAATTCTGAGGCTCTGACC
CTSO	AGTGGGACAAACTCCAGCAC	CCTCTTTGATCCCACCTGAA
CTSS	TCTCTCAGTGCCCAGAACCT	GCCACAGCTTCTTTCAGGAC
CTSW	CCACCCCAAGAAGTACCAGA	GTGGCCTTGATCACACCTTT
GZMB	TCTCGACCCTACATGGCCTTA	TCCTGTTCTTTGATGTTGTGGG
PRF1	CTGCCACTCGGTCAGAATG	CGGAGGGTAGTCACATCCAT
PRDM1	TTCTCTTGGAAAAACGTGTGGG	GGAGCCGGAGCTAGACTTG
RUNX3	CAGGTTCAACGACCTTCGATT	GTGGTAGGTAGCCACTTGGG
CTNNB1	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATACTGTCCAT
18S rRNA	CGCCGCTAGAGGTGAAATTCT	CGAACCTCCGACTTTCGTTCT