

Supplemental Information

Mitochondrial Regulation of the 26S Proteasome

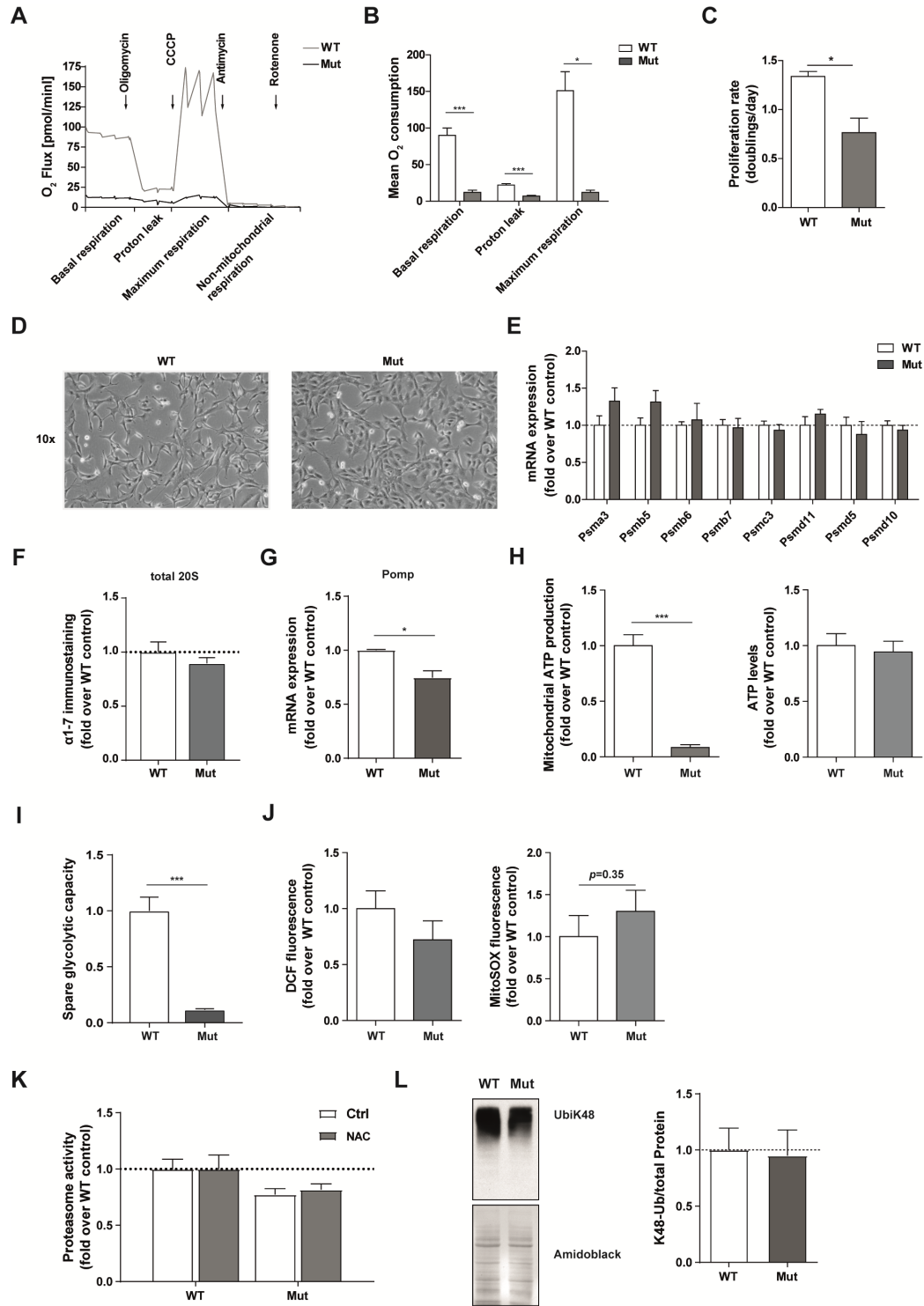
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Supplemental information

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Figure S1: related to Figure 1



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6 **Figure S1: related to Figure 1**

7 (A) Mitochondrial oxygen flux monitored over time at base line condition, after addition of
8 oligomycin (determining proton leak), CCCP (assessing maximal respiration), or antimycin A and
9 rotenone (assaying non-mitochondrial respiration) as analyzed by the Seahorse XF analyzer.
10 Curves show mean values from three independent wildtype (WT) and four independent
11 mutator (Mut) cell lines. (B) Mean oxygen consumption rates in WT (n=3) and mutator (n=4)
12 cells. Bar graphs show mean \pm SEM. Significance was determined using two-way ANOVA with
13 Bonferroni multiple comparison test. (C) Proliferation rates of WT (n=3) and mutator (n=4) cell
14 lines were determined by counting cells at day 1 and day 5 after seeding of the cells. Doublings
15 per day were then calculated as explained in the methods part. Bar graphs show mean \pm SEM.
16 Significance was determined using student's unpaired t-test. (D) Representative images show
17 cellular morphology of WT and mutator MEFs. Magnification: 10x. (E) RT-qPCR analysis of
18 proteasome subunit mRNA expression in WT (n=3) and mutator (n=4) cells. Data represent
19 mean \pm SEM relative to WT control. Statistical test: two-way ANOVA with Bonferroni multiple
20 comparison test. (F) Quantification of amounts of total 20S complexes in WT and mutator cells
21 as resolved by blotting of native gels and immunostaining for the 20S subunits α 1-7. Bar graph
22 shows combined signals for 30S, 26S and 20S related to WT controls. Significance was
23 determined using student's unpaired t-test. (G) RT-qPCR analysis of Pomp mRNA expression in
24 WT (n=3) and mutator (n=4) cells. Data represent mean \pm SEM relative to WT control. Statistical
25 test: unpaired t-test. (H) Relative mitochondrial ATP production (oligomycin sensitive
26 respiration) in WT (n=3) and mutator (n=4) cells as analyzed by the Seahorse XF analyzer.
27 Relative cellular ATP levels in WT (n=3) and mutator (n=4) cells as measured by a luciferase-

based assay. (I) Extracellular acidification rate (ECAR) was measured using the Seahorse XF analyzer to determine the spare glycolytic capacity in WT (n=3) and Mut MEFs (n=4). Values were normalized to the mean of WT controls. Significance was determined using student's unpaired t-test. (J) Cellular ROS was measured using H₂DCFDA staining and flow cytometry in WT (n=2) and mutator (n=2) cell lines. Flow cytometry analysis of WT (n=3) and mutator (n=4) cell lines stained with MitoSOX Red for detection of mitochondrial superoxides. Data represent mean±SEM relative to WT. Significance was determined using student's unpaired t-test. (K) CT-L proteasome activity in WT (n=3) and mtDNA mutator (n=4) MEFs after treatment with control or 5 mM NAC-containing medium for 2 h. Bars show mean±SEM. Values were displayed as fold change relative to WT. (L) Analysis of ubiquitinated proteins in WT and mutator cells. Representative Western blots of UbiK48-linked proteins. Bar graphs show Amido black normalized levels of UbiK48-linked proteins in WT (n=3) and mutator (Mut) cell lines (n=4) (mean±SEM). Significance was determined using student's unpaired t-test.

Figure S2: related to Figure 2

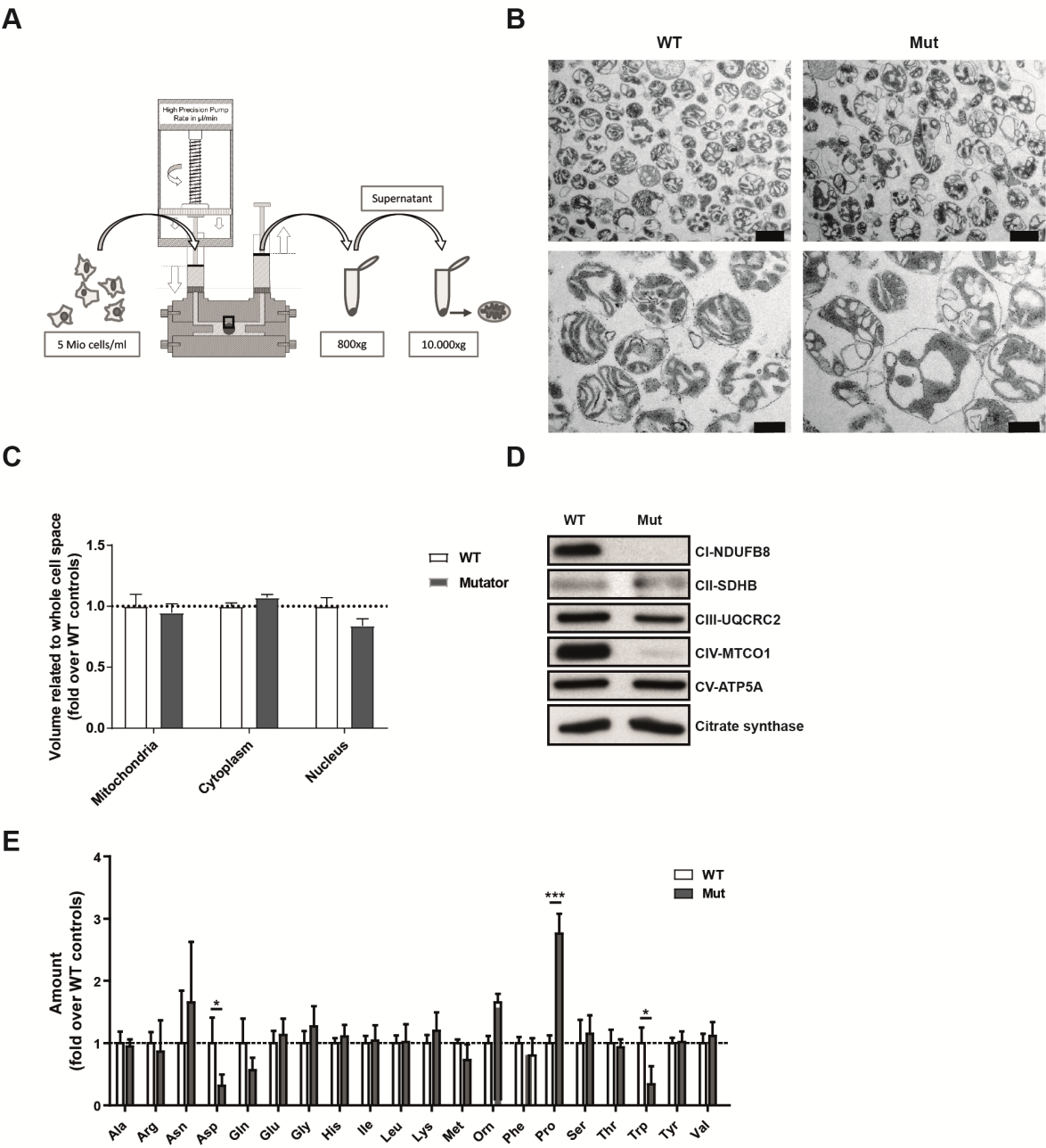


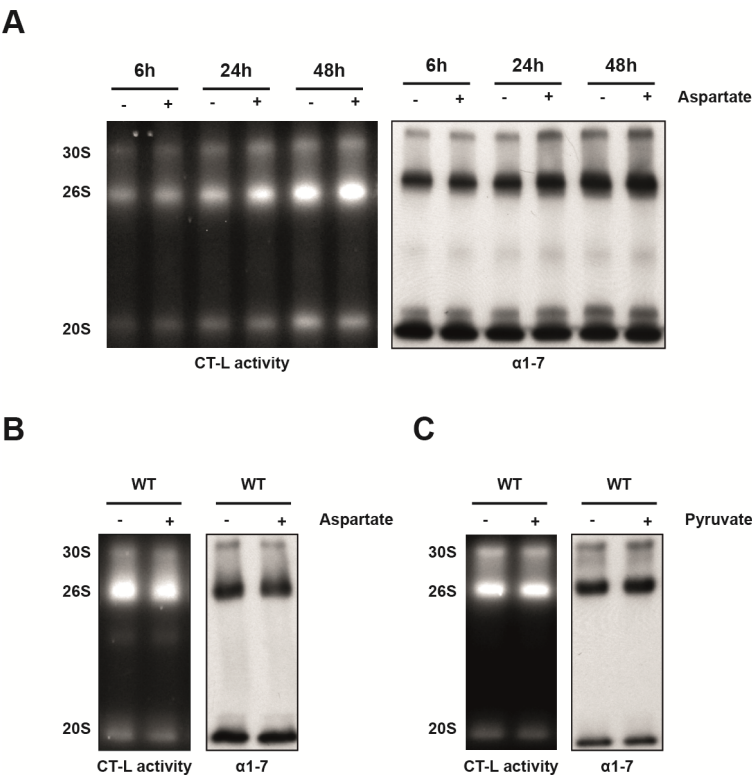
Figure S2: related to Figure 2

(A) Scheme of the mitochondrial isolation procedure. A high precision pump ensures, via gastight syringes, the continuous sample delivery in a constant rate to the “Balch-

homogenizer". Cell breakage occurs upon passage through a defined clearance (square), which is adjusted by selecting tungsten carbide balls of different diameters. The cell homogenate is collected in a second syringe and transferred to a 2ml Eppendorf cup for differential centrifugation. (B) Representative electron microscopy images of mitochondria isolated from one WT and mutator cell line. Scale bar: upper panel 1 μm , lower panel 500 nm. (C) Proportion of mitochondrial volume was quantified as described by Hacker and Lucocq (Hacker and Lucocq, 2014). In total, 30 electron micrographs (1000x magnification) from three wildtype clones (two technical replicates, each) and 43 electron micrographs (1000x magnification) from four mutator clones (two technical each) were used for quantification. Data represent mean \pm SEM relative to WT controls. Statistical test: student's unpaired t-test. (D) Representative Western blot analysis of single respiratory chain complex subunits in mitochondria isolated from one WT and mutator cell line. Four independent isolations were used for Western blots. Citrate synthase served as a loading control. (E) Quantification of the amount of 20 amino acids in WT (n=3) and mutator (n=4) cells. 6 replicates were measured for each cell line and the respective values were normalized to the cell number of each cell line. Data represent mean \pm SEM relative to WT controls. Statistical test: student's unpaired t-test.

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67 **Figure S3: related to Figure 3**



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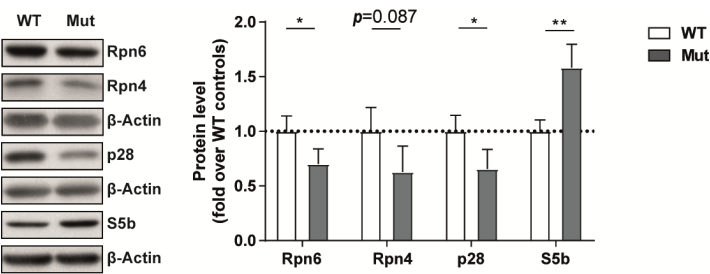
69 **Figure S3: related to Figure 3**

70 (A) Representative native gel analysis of active proteasome complexes in cell lysates from one
71 mutator cell line treated with 10 mM aspartate for 6 h, 24 h and 48 h. Chymotrypsin-like (CT-L)
72 substrate overlay assay and immunoblotting for 20S α 1-7 subunits is shown. (B+C) Native gel of
73 active proteasome complexes in cell lysates from WT cells (n=3) treated with 10 mM aspartate
74 or 1 mM Pyruvate for 72 h. Chymotrypsin-like (CT-L) substrate overlay assay and
75 immunoblotting for 20S α 1-7 subunits is shown.

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78 **Figure S4: related to Figure 4**



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83 Western blot analysis of 26S proteasome assembly factor expression in WT and mutator cells.

84 β -Actin was used as a loading control. Densitometric analysis shows mean \pm SEM from three WT

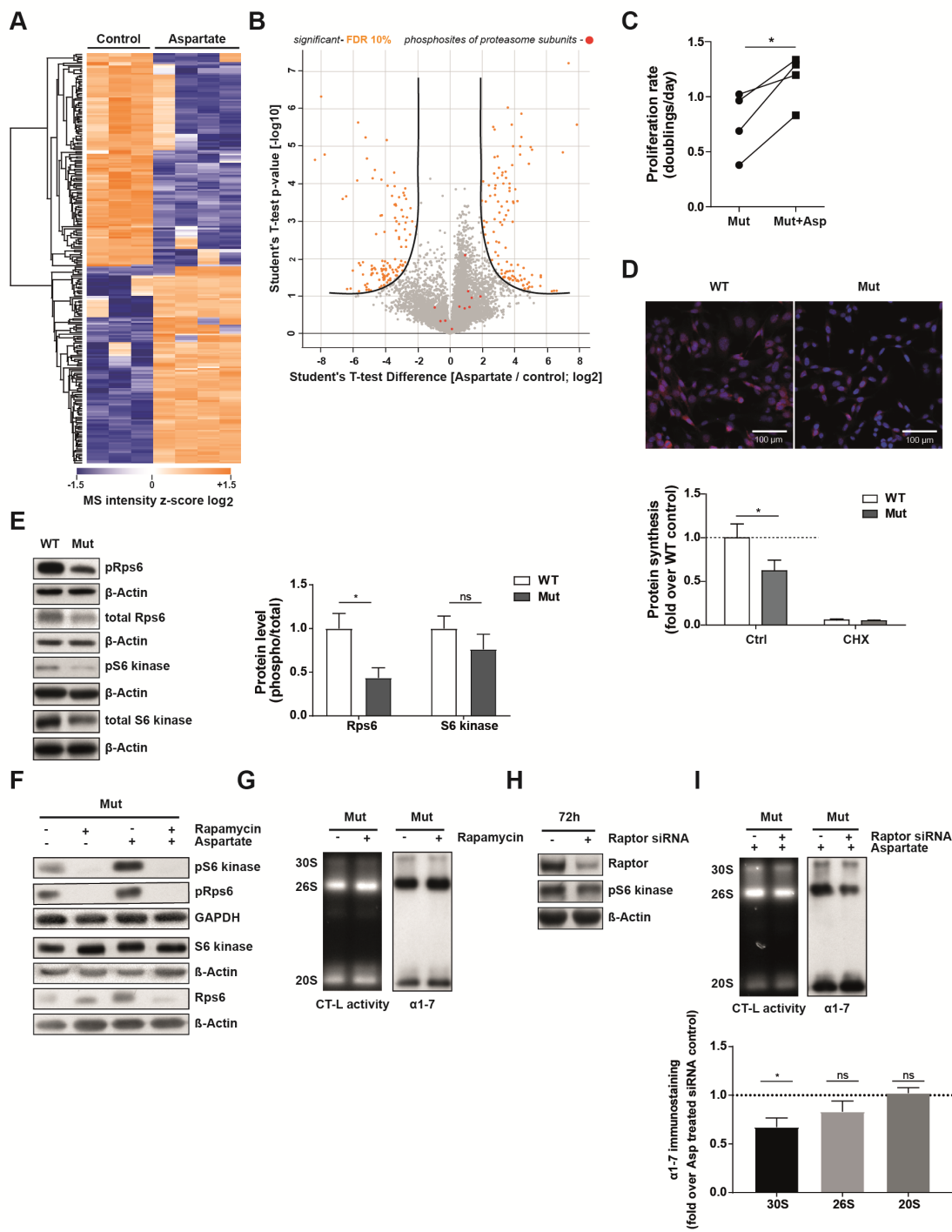
85 and four mutator cell lines. Significance was determined using student's unpaired t-test.

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89 **Figure S5: related to Figure 5**



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91 **Figure S5: related to Figure 5**

92 (A) Heatmap of 233 phosphosites significantly regulated by aspartate treatment compared to
93 non-treated controls. Each row corresponds to a single distinct phosphosite. Rows are ordered
94 according to unsupervised hierarchical clustering (Pearson correlation of z-score), after
95 imputation to replace missing values. (B) The depicted volcano plot shows significantly altered
96 phosphorylation sites relative to controls with a 10 % FDR. Phosphosites of proteasome
97 subunits are shown in red. (C) Proliferation rates of mutator (n=4) cell lines treated with 10 mM
98 aspartate were determined by counting cells at day 1 and day 5 after seeding of the cells.
99 Doublings per day were then calculated as explained in the methods part. Graph shows
100 increase for each mutator cell line after aspartate supplementation. Significance was
101 determined using student's paired t-test. (D) Representative fluorescence images showing
102 nascent protein synthesis (red signal) and cell nuclei (blue signal) in WT (n=3) and mutator (n=4)
103 cells. (Cycloheximide CHX) treatment with 100 μ M for 4 h served as a negative control. Scale
104 bar: 100 μ m. Graph shows mean \pm SEM relative to WT for Ctrl and CHX treated samples.
105 Statistical test: Two-way ANOVA with Bonferroni multiple comparison test. (E) Analysis of
106 mTOR signaling in WT (n=3) mutator (n=4) cells. Representative Western blots of total and
107 phosphorylated levels of p70 S6 kinase and S6 ribosomal protein (Rps6). Bar graphs show β -
108 Actin normalized phospho-protein levels related to total levels of the respective protein in WT
109 and mutator cells (Mean \pm SEM). Significance was determined using student's unpaired t-test. (F)
110 Analysis of mTOR signaling upon treatment with 0.5 nM rapamycin and 10 mM aspartate for 72
111 h in one mutator cell line. Representative Western blots of total and phosphorylated levels of
112 p70 S6 kinase, S6 ribosomal protein (Rps6). GAPDH and β -Actin was used as a loading control.
113 (G) Representative native gel analysis of active proteasome complexes in cell lysates from one

114 mutator cell line upon rapamycin treatment (0.5 nM) for 72 h. Chymotrypsin-like (CT-L)
115 substrate overlay assay and immunoblotting for 20S α 1-7 subunits is shown (H) Protein levels
116 of Raptor and phospho S6 kinase after aspartate supplementation and siRNA mediated Raptor
117 silencing in one mutator cell line (n=4 independent experiments). (I) Representative native gel
118 analysis of active proteasome complexes in cell lysates from one mutator cell line upon 72 h
119 treatment with 10 mM aspartate and siRNA mediated Raptor silencing. Chymotrypsin-like (CT-
120 L) substrate overlay assay and immunoblotting for 20S α 1-7 subunits is shown. Densitometric
121 analysis shows mean \pm SEM of fold change over control from four independent experiments.
122 Significance was determined using the one sample t-test.

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Figure S6: related to Figure 6

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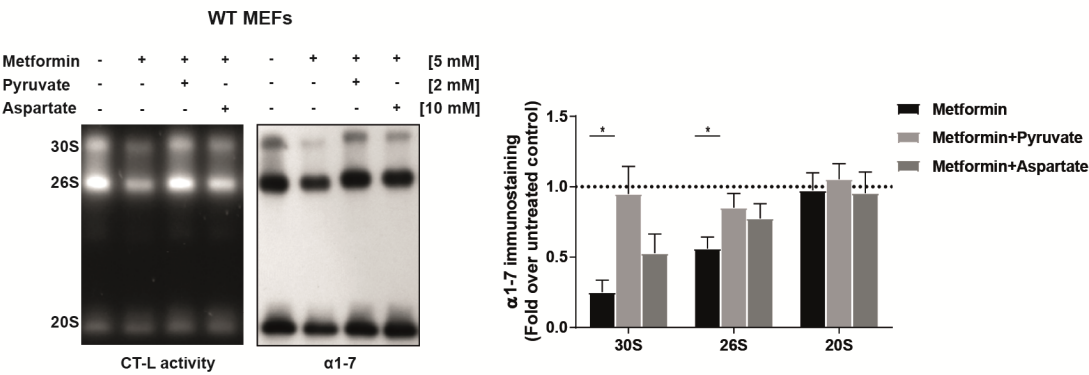


Figure S6: related to Figure 6

Representative native gel analysis of active proteasome complexes in cell lysates from one WT MEF cell line cotreated with 10 mM aspartate or 1 mM pyruvate together with 5 mM metformin for 72 h. Chymotrypsin-like (CT-L) substrate overlay assay and immunoblotting for 20S $\alpha 1-7$ subunits is shown. Bar graphs represent mean \pm SEM relative to respective untreated WT MEF cell line (n=4 independent experiments). Significance was determined using one sample t-test.

Figure S7: related to Figure 7

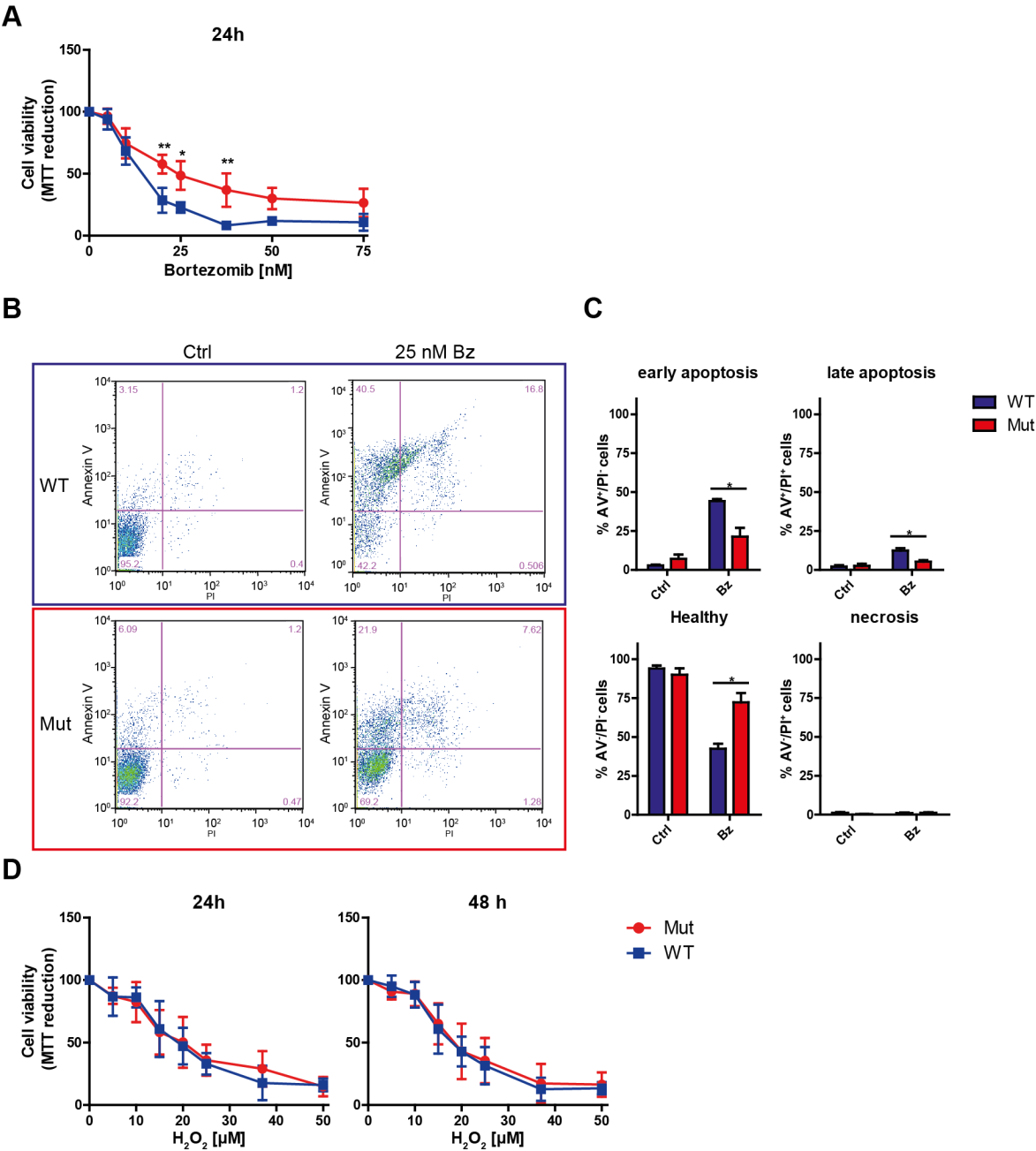


Figure S7: related to Figure 7

(A) MTT cellular viability assay of WT (n=3) and mutator (n=4) MEFs treated with increasing doses of the proteasome inhibitor Bortezomib for 24 h. Values were normalized to the solvent-treated control (0 nM Bortezomib) and are displayed as mean+SEM. Significance was determined using two-way ANOVA with Bonferroni multiple comparison test. *:p< 0.05, **:p< 0.01, ***:p< 0.001. (B) Representative flow cytometry analysis of Annexin V and PI staining of WT and mutator MEFs treated with either solvent or 25 nM Bortezomib (Bz) for 24 h. (C) Quantification of Annexin V/PI staining in WT (n=3) and Mut (n=4) cells. Cells were classified as healthy (Annexin V⁻/PI⁻), early apoptotic (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺), or necrotic (Annexin V⁻/PI⁺). Bar graphs show mean+SEM. Significance was determined using student's t-test. *:p< 0.05. (D) Cellular viability of WT (n=3) and mutator (n=4) MEFs treated with increasing concentrations of H₂O₂ for 24 or 48h. Values were normalized to the 0 μM H₂O₂-treated group and are displayed as mean+SEM.