Granzyme B enters mitochondria in a Sam50, Tim22 and mtHsp70-dependent manner to induce apoptosis.

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Supplemental Material and Methods:

Mice, cell lines and growth conditions

The C57BL/6J wild-type female mice were obtained by the Animal Facility of the University of Geneva and used for liver mitochondrial purification in accordance with approved animal protocol by the cantonal veterinarian.

Hela cells were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate 6mM hepes free acid, 1.6 mM L-glutamine, and 50 µM β-mercaptoethanol. Hela-CD80 cells were grown in same medium plus puromycin. 721.221 cells overexpressing NDUFS1-V5-D255A were grown as in Jacquemin et al 2014[18]. HeLa TOM40 sh and SAM50 sh were grown as in Ott, C 2015[49]. Hela expressing doxycyclin (Dox)-inducible B2Δ19-HA or SU9-V5 were cultured in DMEM supplemented with Tet System Approved FBS (TAKARA Clontech). Hela cell and 721.221 were used as target cells for NK cells.

Parental YT-Indy were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate 6 mM hepes free acid, 1.6 mM L-glutamine, and 50 µM β-mercaptoethanol. YT-Indy silenced for endogenously expressed granzyme B, K and M (YT-shGzm) were grown in same medium as parental YT Indy plus puromycin. YT-shGzm re-expressing either wild type GB (YT-wtGB), GB KR243-244AA (YT-KR/AA), wild type GA (YT-wtGA), or GM (YT-wtGM) were grown in same medium as parental YT Indy cells plus puromycin and blasticidin.

Hela were from ATCC and 721.221 and YT-Indy was a kind gift of Pr. Lieberman. The cells were regularly tested for mycoplasma to unsure the usage of mycoplasma free cells.

YPH499 parental strain (MATa ade2-101 his3-Δ200 leu2-Δ1 trp1-Δ63 ura3=52 lys2-801)[54], YPH499 tom70::HIS3[37], YTJB4 MATa Leu2 His4 Lys2[32], YTJB64, tom20::LEU2[37], YPH499 ΔMas37/Tob37, YPH499gal/his8-Tob55[55], Δtom40+ pRS314-Tom40-2 KKY3.2 (TK3), Δtom40+ pRS314-Tom40-3 KKY3.3 (TK4), Δtom40+ pRS314-Tom40-4 KKY3.4 (TK5), thermosensitive strain for Tom40, and the wild type control Δtom40+ pRS314-Tom40wt KKY3.7 (TK8)[34] were kind gifts of Professor Doron Rapaport. PK80A parental strain (His4-713, Lys2, Ura3-52, ΔTrp1, Leu2-3,112 wild type SSC1), thermosensitive strains for SSC1 PK81 SSC1-2 (Ade2-101, Lys2, Ura3-52, Leu2-3.112, ΔTrp1 SSC1-2(LEU2) and PK83 SSC1-3 (Ade21.1, Lys2, Ura3-52, ΔTrp, SSCI-3(LEU2)) yeast strains were a kind gift of Professor Elizabeth Craig. The temperature-sensitive alleles Tim23-76 (YPH-BG-TIM23-76), tim22-14 (YPH499 22-M4) were kind gift of Professor Nikolaus Pfanner and Dr. Bernard Guiard. Yeast strain M22-2-1[40] was a kind gift of Professor Marco Colombini. Yeast cells were grown under aerobic conditions in YPD (1% bacto yeast extract, 2% bactopeptone, and 2% glucose) or YPGal medium (1% bacto yeast extract, 2% bactopeptone, and 2% galactose) depending on conditions. Single colony seed cultures were used to refresh larger cultures under shaking (~ 140 rpm). Mitochondrial purification was done when cultures reached OD_{600} 0.8.

Antibodies and reagents

Antibodies against V5 (V5-10) and FLAG (M2), Proteinase k (PK), PMSF, doxycycline and Laminin were from Sigma Aldrich; anti-GzmB (GB7), anti-PARP (H-250), anti-HSP60 (H-300), anti-tubulin (B-7), anti-Tom40 (H300), anti-SAMM50 (N-15), anti-HAX-1 (FL-279), anti-TIM44 (sc-21934), anti-yeast TIM23 yN-19 (sc-14048), donkey and-goat (sc-2020), and anti-NDUFS1 H-300 (sc-99232) were from Santa Cruz Biotechnology; anti-GzmA (GA6) from AbD Serotec; anti-GzmM (4D11) from Novus Biological; anti-HA (3F10) from Roche; anti-κ-light chain-HRP from Bethyl Laboratories; anti-Tom20 from BD Biosciences; anti-Caspase 3 (8G10) and anti-Histone H3 from Cell Signaling; anti-NDUFS1 from Epitomics; anti-GAPDH and anti-PORIN/VDAC1 (16G9E6BC4) from Abcam; Anti-mouse IgG-HRP, anti-rabbit IgG-HRP from GE Healthcare. Polyclonal anti-GB (PA5-17457), monoclonal anti-GB for immune fluorescence clone GB11 and Lipofectamine® 3000 were

from Thermo Fisher Scientific. Anti-Bid was from R&D system. Yeast anti-TOB55 and anti-TOM40 were kind gifts from Professor Doron Rapaport and anti-yeast TOM20 was a kind gift of Professor Nikolaus Pfanner. Valinomycin, zVAD-fmk and DEVD-fmk were from Calbiochem. All enzymes for molecular cloning were from New England BioLabs® Inc. Plasmids encoding Rev-caspase 3 and C/A Rev caspase 3 were kind gifts of Professor Alnemri.

In vitro Transcription/Translation.

The pGEM®-4Z plasmid (Promega) was used as cloning vector for the *in vitro* synthesis of Dihydrofolate reductase (DHFR) and fusion proteins used in import assay experiments. DHFR was amplified by PCR from pQE60-Su9 (1-69) DHFR (kind gift of Dr. Dejana Mokranjac), cut either with EcoRI/NotI or NotI/BamHI, and inserted into pGEM®-4Z plasmid. The presequence of subunit 9 of the *N. crassa* F1F0-ATPase was amplified by PCR from pQE60- Su9 (1-69) DHFR and fused to the EcoRI/NotI digested pGEM®-4Z-DHFR. N- and C-terminus of human granzyme B were obtained by primer annealing and fused to either EcoRI/NotI or NotI/BamHI digested pGEM®-4Z-DHFR. All constructs were sequence verified. ^{35}S-Met-radiolabeled proteins were obtained from the TnT® SP6 High-Yield Wheat Germ Protein Expression System (Promega) using 3 µg of DNA and 2 µl of ^{35}S-Met label (Hartmann analytic, IS-103).

Primers for DHFR amplification:

EcoRI-DHFR_Fw (AAAAAAGAATTCATGGTTCGACCATTGAACTGC) and DHFR-NotI_Rv (ATGATGATTGCGGCCGCGCCGTCAAACTTATACTTGATG) for DHFR at 5’.

NotI-DHFR_Fw (ACGACGGCGCGGCCGCAATCATGGTTCGACCATTG) and DHFR-BamHI_Rv (TTTTTTGGATCCTTATTAAAACTTATACTTGATGCC) for DHFR at 3’.

Primers for N- and C-terminal GzmB:

3’Ct_Fw
(GGCCGCGGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAAACGCTACTAATAAGATATCG),

3’Ct_Rv
(GATCCGATATCTTATTAGTAGCGTTTCATGGTTTTCTTTATCCAGTGTACAAAGCTTGAGACCGC) for DHFR-Ct construct;

5’Ct_Fw
(AATTCGATGGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAAACGCTACAGGCCTGC),

5’Ct_Rv
(GGCCGCAGGCCTGTAGCGTTTCATGGTTTTCTTTATCCAGTGTACAAAGCTTGAGACCATCG) for Ct-DHFR construct;

3’Nt_Fw
(GGCCGCGATCATCGGGGGACATGAGGCCAAGCCCCACTCCCGCCCCTACTAATAAAGGCCTG),

3’Nt_Rv
(GATCCAGGCCTTTATTAGTAGGGGCGGGAGTGGGGCTTGGCCTCATGTCCCCCGATGATCGC) for DHFR-Nt construct;

5’Nt_Fw
(AATTCGATGATCATCGGGGGACATGAGGCCAAGCCCCACTCCCGCCCCTACAGGCCTGC),

5’Nt_Rv

(GGCCGCAGGCCTGTAGGGGCGGGAGTGGGGCTTGGCCTCATGTCCCCCGATGATCATCG) for Nt-DHFR construct.

Isolation of yeast mitochondria.

Yeast mitochondria were isolated by differential centrifugation according to a published method[56] with some minor modifications: 1 mM sodium orthovanadate (Na_3VO_4); 0.2 μM okadaic acid ($C_{44}H_{68}O_{13}$) phosphatase inhibitors were added at the homogenization step and in the final resuspension of the mitochondrial pellet while BSA was omitted. Mitochondria concentration was measured by Pierce™BCA Protein Assay kit (Thermo Fisher). Yeast mitochondria were aliquoted and stored at -80^{o}C.

Isolation of mouse liver mitochondria.

Intact mouse liver mitochondria were purified according to a published method[57], resuspended in IBc buffer (10 mM Tris-MOPS, 1 mM EGTA/Tris, 0.2 M sucrose, pH 7.4) plus 1 mM sodium orthovanadate (Na_3VO_4), and 0.2 µM okadaic acid ($C_{44}H_{68}O_{13}$) phosphatase inhibitors, then used immediately.

Mitochondrial import assay.

Import assays were performed in mouse liver mitochondria (1.6 mg/ml) or yeast mitochondria (2 mg/ml). 30µl of mitochondria were incubated with 5 µL of ^{35}S-Met-radiolabeled or 0.2 µM purified granzyme in import buffer (1.2 M sorbitol, 160 mM KCl, 20 mM Mg-acetate, 4 mM KH_2PO_4, 5 mM EDTA, 5 mM $MnCl_2$, 100 mM HEPES-KOH, pH 7.2 plus 2.5 mM ATP, 5 mM NADH, 0.25 µg/ml creatine phosphokinase, and 25 mM creatine phosphate) or SH buffer (0.6 M Sorbitol, 20 mM Hepes-KOH, pH 7.4) in the presence or absence of 4 µM valinomycin. Samples in SH+ valinomycin buffer were preincubated for 10 minutes at RT. All the samples were then incubated at 37°C (mouse mitochondria) or 25°C (yeast mitochondria) for 15 minutes or indicated time. After incubation, half of the sample was kept untreated (INPUT) and the rest treated with 50 µg/ml PK in a final volume of 1 ml SH buffer for 10 minutes on ice (IMPORTED). Then, PK was inhibited by adding 1 mM PMSF (phenylmethylsulfonyl fluoride) for 10 minutes on ice. Mitochondria were spun down at 12000 g for 15 minutes and resuspended in loading buffer to perform Western blot. The lanes -PK represent the total amount of GB bound (specifically or not) and unbound to the mitochondria. The % of import determined by densitometry and calculated as followed:

$$\%\ \text{of import} = 100 * \left(\frac{(+PK)}{(loading\ control)}\right) / \left(\frac{(-PK)}{(loading\ control)}\right)$$

Where the (+PK) and the (-PK) were always normalized with their respective loading control.

Submitochondrial localization of proteins.

Swelling experiments were performed from freshly isolated yeast mitochondria (0,8 mg/ml). Granzymes (0.2μM) were incubated with mitochondria in import buffer to a final volume of 30μl for 15 minutes at 25°C. The samples were spun down at 12'000 g, for 5 minutes at 4°C, resuspended in 400μl of isotonic SET buffer (250mM sucrose; 10mM Tris/HCl pH7.6; 1mM EDTA. ET buffer: 10mM Tris/HCl pH 7.6; 1mM EDTA) or hypotonic ET buffer (10 mM Tris/HCl pH 7.6 1 mM EDTA) for swelling and incubated for 30 minutes on ice. Swelled samples were occasionally vortexed to favor the generation of mitoplasts. The samples were the spun and the –PK resuspended in 5x loading buffer, while the +PK were resuspended in 100μl of SET buffer plus PK to a final concentration of 50μg/ml and incubated for 15 minutes on ice. PK was inhibited with 4mM PMSF for 10 minutes on ice. Samples were spun down and resuspended in 1x loading buffer. One sample was resuspended in 50μl of 1% Triton for 10 minutes on ice and then spun down at 14'000 rpm at 4°C. The supernatant was treated with PK and PMSF as before and the 5x sample buffer was added.

Mitochondrial $\Delta\Psi$m measurement.

To assess $\Delta\Psi$m, YPH499 and M22-2-1 mitochondria (1 mg/ml) were incubated with 1.5 μM of JC-1 to a final volume of 30 μl in import buffer (see mitochondrial import assay) for 15 minutes at RT. Mitochondria were washed two times with SH buffer. Mitochondria were then analyzed by flow cytometry with an AccuriC6 flow cytometer (BD Biosciences).

Blue Native gel.

30μl of yeast mitochondria at 2 mg/ml were incubated with 0.2 μM biotinylated granzyme in import buffer for the indicated time at 25^0C. The samples were spun at 12000 g and mitochondrial pullet resuspended in BN gel lysis buffer (20 mM Tris pH7.4, 0.1 mM EDTA,

50 mM NaCl, 10% glycerol and 1% digitonin freshly added. We added 1 µl of 0.2 M of PMSF to avoid protein degradation. After 30 minutes samples were spun at 12000 g and 40 µl of supernatant were combined with 6 µl of 50% glycerol and 5 µl of 5% coomassie blue. These samples were run directly on precast 3-12% gradient NativePAGE from Invitrogen Thermo Fisher. Gels were equilibrated for 30 minute with 1x transfer buffer without ethanol and blotted for 2 hours at 150 mA on PVDF membrane using a semi-dry transfer apparatus. Membranes were fixed for 20 minutes with 100% methanol before blocking in 5% non-fat milk and either incubated for 1 hour at room temperature with streptavidin HRP or probed with either anti-yeast Tob55 or yeast Tom40 primary antibody.

Lactococcus lactis cloning and import assay.

The NICE® lactococcus lactis expression strain NZ9100 (MoBiTec GmbH, Germany) was cultured at 30°C in M17 containing 0.5% Glucose, 20 mM MgCl2, and 2 mM CaCl2. Bacteria were electroporated according to the manufacturer's protocol with the expression plasmid pNZ8148 containing the coding sequence of yeast Tom40, Tob55 or Por1. The cDNA encoding for yeast Por1, Tom40 and Tob55/Sam50 were a kind gift of Professor Doron Rapaport.

Primers for cloning into pNZ8148:

Yeast Tom40 FW: AAAAAAAGCTTATAAAGGAGGTAAAAAATGTCTGCACCAACTCC

Yeast Tom40 RV: AAAAAAAGCTTTCACAATTGAGGAAGAG

Yeast Por1 (VDAC1) FW:

AAAAAGGTACCATAAAGGAGGTAAAAAATGTCTCCTCCAGTTTAC

Yeast Por1 (VDAC1) RV: AAAAAAAGCTTTCAAGCGTCGAAGGAC

Yeast Tob55 (Sam50) FW:

AAAAAGGTACCATAAAGGAGGTAAAAAATGACCTCATCATCTGG

Yeast Tob55 (Sam50) RV: AAAAAAAGCTTTTATAAAAATGCCAGACCAAGAC

After selection with 10 µg/ml chloramphenicol on M17agar plates, the clones expressing Tom40, Tob55 or Por1 were grown until O.D.600 = 0.5 before addition of 100 ng/ml Nisin. After overnight incubation at 30°C with vigorous shaking, the cell wall was removed with 1 mg/ml lysozyme for 5 minutes at 37°C, washed with PBS, and incubated with 400 nM GB for 15 minutes at 37°C. These two steps were done in presence of 1 mM sodium orthovanadate and 0.2 µM okadaic acid phosphatase inhibitors. Half of the sample was stored (INPUT), and the rest was treated with 50 µg/ml of proteinase K in a final volume of 1 ml PBS for10 minutes on ice. PK was then inhibited by adding 1 mM PMSF for 10 minutes on ice. The samples were spun and resuspended in loading buffer (IMPORTED). For Western blot, 5 µl of input and 20 µl of imported samples were loaded.

Confocal microscopy.

HeLa cells transfected with Mito-RFP (Clontech) and ZsGreen-Gzms were used. ZsGreen was amplified by PCR from pZsGreen 1-1 plasmid (Clontech) and cut with NotI and BamHI or PacI restriction enzymes. Gzms cDNA were amplified by PCR from Origene vectors (GzmB: RC206495; GzmA: RC204852; GzmH: SC126062; GzmK: RC207798; GzmM: RC206497) and digested with BamHI and EcoRI restriction enzymes or PacI and EcoRI in the case of GH. ZsGreen and Gzms were ligated together in pQCXIP plasmid (Clontech). HeLa cells were plated at a density of ~2 × 10^4 cells in 24-well plates on coverslips overnight. Cells were transfected in serum free medium with Lipofectamine® 2000 (Life Technologies), and after two hours, transfection medium was changed with complete medium. 24 hours later cells were fixed and coverslips mounted. Confocal microscopy images were obtained from a Zeiss LSM700 confocal microscopy with a 63x objective and analyzed by ImageJ software. ImageJ “Colocalization Threshold” plug-in was used to determine Mander’s coefficient.

Primers for ZsGreen-Gzm cloning:

ZsGreen_Fw: AAAAAAGCGGCCGCCGCCACCATGGCCCAGTCCAA

(BamHI) ZsGreen_Rv: AAAAAAGGATCC**ACCACCACCACCACC**GGGCAAGGCGGAG

or

(PacI) ZsGreen_Rv: AAAAAATTAATTAA**ACCACCACCACCACC**GGGCAAGGCGGAG

The linker between ZsGreen and Gzms is in bold.

Wild type GB_Fw: AAAAAAGGATCCATCATCGGGGGACATGAGGCC (common for wild type and mutant GzmB).

Wild type GB_Rv: AAAAAAGAATTCTTATTAGTAGCGTTTCATGGTTTTC

KR243/244AA GB_Rv: AAAAAAGAATTCTTATTAGTAAGCAGCCATGGTTTTC

KR243/244EE GB_Rv: AAAAAAGAATTCTTATTAGTATTCTTCCATGGTTTTC

GA_Fw: AAAAAAGGATCCATTATTGGAGGAAATGAAGT

GA_Rv: AAAAAAGAATTCTTATTAAACTGCTCCCTTGATAGTC

(PacI) GH_Fw: AAAAAATTAATTAACATCATCGGGGGCCATGAGG

GH_Rv: AAAAAAGAATTCTTATTAGAGGCGCTTCATTGTTCTC

GK_Fw: AAAAAAGGATCCATTATTGGAGGGAAAGAAG

GK_Rv: AAAAAAGAATTCTTATTAATTTGTATGAGGCGGGACAAG

GM_Fw: AAAAAAGGATCCATCATCGGGGGCCGGGAGG

GM_Rv: AAAAAAGAATTCTTATTAGGCCGATCGGCCGGTGACC

Effector-target killing assay.

The 721.221 overexpressing NDUFS1-D255A-V5 ($0.1x10^6$) in 50 µl of medium (HBSS containing 1.55 mM CaCl2, 17.5 mM glucose, 10 mM Hepes, pH 7.4) were mixed with an equal volume of medium containing effector cells (YT-Indy) at the indicated effector:target (E:T) ratios and incubated at 37^0C for the indicated times. For caspase inhibition, target cells were pretreated for 30 minutes with 50 µM zVAD-fmk + 50 µM DEVD-fmk before incubation with effector cells. Then 5x sample buffer was added, and the sample was analyzed by Western blot.

For the calcein release assay, Hela expressing CD80, cells were preloaded with 1 µM Calcein AM (Life Technologies) for 10 minutes then washed 3 times with HBSS. Then $1x10^4$ target cells in 100 µl of HBSS containing 1.55 mM CaCl2, 17.5 mM glucose, 10 mM Hepes, pH 7.4 5% FCS were incubated with effector cells as done previously for the indicated time. Specific killing was determined by monitoring the calcein released in the supernatant.

$$\%\text{specific release} = 100\frac{release - spontaneous\ release}{\max release - spontaneous\ release}$$

Dox-inducible B2Δ19-HA and Su9-V5 HeLa cells were cultured for 96 hours ± 0.5 µg/mL Dox. At 72 hours, $5x10^3$ cells were plated in 96-well plate in 100 µl of medium (DMEM with Tet System Approved FBS). The next day, HeLa cells were mixed with an equal volume of medium containing effector cells (YT-Indy) at the indicated Effector:Target ratios and incubated overnight. Following this, effector cells were washed with HBSS, and methylene blue (0.2% methylene in 250ml methanol + 250 ml ddH2O) was added for 30 minutes at RT. The plates were washed in distilled tap water, and elution was performed with 0.1 M HCl. Absorbance was read at 630 nm.

HeLa Tom40 and Sam50 shRNA cells were cultured for three days ± 1 µg/ml Dox. At day 2, $3x10^3$ cells were plated in 96-well plate coated with 10 µg/ml laminin. The next day, HeLa cells were incubated eight hours with an equal volume of medium containing effector cells (YT-Indy, YT-wtGB, YT-GA, YT-GM) at the indicated Effector:Target ratios. Effector cells were then washed away with HBSS, and PrestoBlue™Cell Viability Reagent (Invitrogen) was added to cells in culture medium. Absorbance was read 16 hours later at 570 nm.

ROS measurement in target cells during killer cell attack

HeLa-CD80 target cells were stained with 1uM cellTrace CFSE in HBSS during 15 min, then washed three times in HBSS and resuspended at 100 000 cells/ml in assay buffer (HBSS, 10mM Hepes pH7.4, 17.5mM Glucose, 1.55mM CaCl2, 10% FBS). For each condition, 100 µl of CFSE-labelled target cells were incubated with 100 µl of assay buffer containing the appropriate number of YT Indy effector cells. After 2 hours at 37^0C, cells

were placed on ice and every single tube was incubated with 5 µM dihydroethidium (DHE) (ThermoFisher) for exactly 4 min at room temperature before analysis on a CyAn flow cytometer (Beckman Coulter). Dihydroethidium fluorescence signal intensity was analyzed after gating on CFSE-positive cells.

Cell death induction by UV or CH11

Hela Sam50sh or Hela Tom40 sh were induced for 4 days with 2µg/ml doxycycline (Dox) renewed every 2 days, then seeded on the 3rd day in a 96well-plate at 3000 cells/well in 100ul of DMEM. Dox was kept where required. Cell were either expose to 5 minutes UV and incubated for 16 hours. In some cases cells were treated with 2.5 µg/ml cycloheximide for 1h before adding 1 µg/ml of CH11 for 6 hours. Cell death were assessed by methylene blue staining as before.

In vivo experiments

U251 glioma cells constitutively expressing renilla luciferase were subcutaneously implanted to 4-6 weeks female hairless NOD.Cg-PrkdcscidHrhr/NCrHsd mice (Invigo) at $2x10^6$ cells/mice in 250µl of PBS. Three days post implantation, tumor size was analyzed by bioluminescence. At day 8 post implantation, some animals received 10^7 YT-shGzm, YT-wtGB or YT-KR/AA GB killer cells as indicated. Tumor size was followed at day 10 and day 17 post implantation. For bioluminescence analysis of the tumor size, mice were anesthetized by inhalation of 4% isoflurane and injected with 0.1 mg/g body weight of ViviRen *in vivo* renilla luciferase substrate (Promega). Mice were immediately placed in the IVIS Spectrum heated plate (PerkinElmer) and image acquire using *Living Image 4.5* acquisition software (PerkinElmer) with continuous isoflurane anesthesia (1,5 et 2,5% during all the acquisition time). Following acquisition mice were monitored until they recover from anesthesia before returning them to the housing facility. Quantification of the bioluminescence imaging (BLI) signal was performed with the same software. Mice were monitored daily for any sign of suffering in which case they were sacrificed. This study was carried out in strict accordance with the Swiss Regulation and the protocol was

approved by the Cantonal Commission for Animal Experiment of the Directorate General for Health of the Department of Employment, Social Affairs, and Health. The specific permit number for this study is: GE/142/15. The animals (typically 5 animals per cage; minimum floor space per animal 13 cm2) were kept under controlled environmental conditions in the specific pathogen free animal facility with a 12L:12D cycle. Mice were housed in enriched IVC cages containing autoclaved bedding, nesting material and a smart home. They were provided with irradiated food and 0.22 µm filtered tap water. Mice were under ocular inspection of their general health by animal facility dedicated staff and veterinary support is available if needed. At the end of the experiment the animals were humanely killed by lethal injection of 150 mg/kg of pentobarbital intraperitoneally. The lethal dose of pentobarbital prevent any suffering of the animal since with this procedure the animal die under profound anesthesia. n = 5mice/group.

Gram-staining

Gram staining was performed to determine the optimal condition of treatment with lysozyme for removing *L. lactis* cell wall before import assay. After treatment with lysozyme, bacteria were washed with PBS, transferred on a glass slide, air-dried and heat-fixed over a gentle flame. Fixed cells were stained with crystal violet for 1 min, gently washed with water and stained again with Gram's iodine for 3 min. After a gentle wash with water, bacteria were quickly decolorized with 95% ethanol and washed with water before counterstaining with safranin for 1 min. Cells were washed with water and air-dried before examination under the microscope.

Cell fractionation.

Cells ($0.5x10^6$) were washed in PBS and resuspended in 100 µl of cytosolic extraction buffer (70 mM KCl, 137 mM NaCl, 1.4 mM KH2PO4 pH 7.2, 4.3 mM Na2HPO4, 250 mM Sucrose, 400 µg/ml digitonin and protease inhibitors) on ice for 5 min, then centrifuged at 1000 g for 5 min. The supernatant was stored as the cytosolic fraction (C). The pellet was resuspended in 100 µl of mitochondrial lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 2

mM EDTA, 2 mM EGTA, 0.22% Triton X-100, 0.3% NP-40, protease inhibitors). After 10 min centrifugation at 10,000 g, the supernatant was kept as the mitochondrial-enriched fraction (M). The Nuclear fraction (N) was obtained after resuspension of the pellet in RIPA lysis buffer and sonication.

Recombinant protein purification.

C-DNA encoding for wild type GB, GB (HIS236A, LYS239/240A) and GB (KR243/244AA) with an N-terminal enterokinase site but without His tag, were cloned in pHLSeq vector (kind gift of Dr. Radu Aricescu) between AgeI and KpnI restriction sites with the following primer sets:

Common GB_Fw:

5'AAAAAAACCGGTGATGATGATGATAAAATCATCGGGGGACATGAGGCC3'

GB wild type_Rv: 5'TTATTAGGTACCTTATTAGTAGCGTTTCATGGTTTTCTTTATCC3'

GB (H236A-L239/240A)_Rv:

5'TTATTAGGTACCTTATTAGTAGCGTTTCATGGTAGCAGCTATCCAAGCTACAAAGC TTGAGACTTTGGTG3'

GB (KR243/244AA)_Rv:

5' TTATTAGGTACCTTATTAGTAAGCAGCCATGGTTTTCTTTATCCAGTG3'

The 293T cells were seeded 150 mm culture dishes to obtain 60-70% confluency the next day. One hour before transfection, medium was replaced with new medium without antibiotics containing 25 μM chloroquine. Cells were transfected with Calcium-Phosphate transfection method. For each dish, 50 μg plasmid DNA was mixed with 0.25M $CaCl_2$ and ddH2O to a final volume of 2.5 ml. 2.5 ml of 2x HBS (282 mM NaCl, 50 mM Hepes, 1.5 mM phosphate sodium; pH: 7.05) were added drop wise to the DNA solution and incubated for 2 to 5 minutes at RT. The solution was then sprinkled on the cells and incubated for 10 hours. Cells were then rinsed with pre-warmed PBS and serum-free medium (ExCell from Sigma) was added for 72 hours. The culture medium was harvested

and spun at 400 g to eliminate cells and then spun at 4000 g to eliminate cell debris. Granzymes were purified on heparin column according to the method of Thiery et al 2010[52]. The fractions were screened for granzymes by SDS-gel and coomassie staining. The granzyme positive fractions were combined with 1 U porcine enterokinase/20ml and dialyzed overnight in 4 liters of enterokinase buffer (150mM NaCl, 1mM CaI, 50mM Tris, PH 7.4). Enterokinase from porcine intestine was from Sigma. The next day, dialysis buffer was changed for Mono S column buffer A (150 mM NaCl, 50 mM bisTris, pH 5.8) for four hours. The solution was then filtered and run on Mono S column according to Thiery et al 2010[52].

Purification of native wild type and KR243/244AA GB.

Native wild type and KR243/244AA GB were purified according to the method of Thiery et al 2010[52] from YT-wtGB and YT-KR243/244AA GB cells, respectively.

Granzyme B activity assay.

The activity of purified granzyme B was measured by using the chromogenic substrate N-Acetyl-Ile-Glu-Pro-Asp-p-nitroanilide (Sigma). Granzyme B was diluted at 200 nM in HBSS, 10 mM Hepes, pH 7.4 in the presence of 160 µM of substrate in a final volume of 100 µl, and the absorbance was measured at 405 nm every minute during 45 minutes in 96-well plates with the SpectraMax Paradigm plate reader (Molecular Devices).

GzmB staining for confocal microscopy

HeLa-CD80 cells were seeded on laminin-coated coverslips 24 hours before addition of YT effector cells at a 2:1 E:T ratio. After 30 min of incubation, cells were fixed with 2% PFA for 20 min at room temperature, and for 20 min in the presence of 50 mM NH4Cl. Cells were washed with PBS and permeabilized in PBS 0.2% Triton X-100 during 5 min. After washes in PBS, cells were blocked for 30 min in PBS 10% FBS, washed again in PBS and incubated for 1 hour at room temperature with anti-Granzyme B antibody (1/100

in PBS 0.05% Triton X-100) (Invitrogen, clone GB11). Coverslips were washed 3 times with PBS and incubated 1 hour at 4°C in PBS 0.05% Triton X-100 containing 1/250 Anti-mouse IgG (H+L) Alexa Fluor 488 secondary antibody (Molecular Probes). After 3 washes in PBS 0.05% Triton X-100 and 2 washed in PBS, coverslips were mounted on glass slides with mounting medium containing Dapi. Z-stack images were collected using an LSM 700 confocal laser scanning microscope (Zeiss).

Biotinylation of GB and GA.

GA and GB 10 µg each were oxidized by 30 minutes incubation in cold 20 mM of freshly dissolved sodium Meta-periodate in 100 mM of sodium acetate pH5.5. Excess periodate were removed on 7k MWCO Zeba spin desalting column equilibrated with 100 mM sodium phosphate buffer pH 7.2. Desalted oxidized granzyme are incubated for two hours at room temperature with 5 mM of biotin hydrazide before a last step of desalting to remove excess of biotin hydrazide. Final material is concentrated and used in import assay.

Expression of GB-cytoFRET and GB-mitoFRET reporter.

A human codon optimized synthetic gene encoding ECFP linked to EYFP by the VGPDFGR29 GB cleavable linker was cloned between AgeI and PacI restriction sites in pQCXIP (Clontech) for GB-cytoFRET reporter. For GB-MitoFRET reporter, three mitochondrial targeting sequences of Cox8a were added in frame between NotI and AgeI.

Sequence of GB-cytoFRET reporter:

GACC**ACCGGT**AACGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGT
GGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTG
TCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCT
GCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCT
GGGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTT
CAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC
GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG

AACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGC
ACAAGCTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAG
AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCG
TGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCT
GCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAAC
GAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTC
TCGGCATGGACGAGCTGTACAAGTCCGGACTCAGATCTAGCGGCGTGGGCCCCGA
CTTCGGCCGCGTGTACGGCAGCGGCTCCACCATGGTGAGCAAGGGCGAGGAGCT
GTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGAC
CCTGAAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG
ACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGC
AGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCAT
CTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGC
GACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACC
GCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCG
AGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCG
ACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAG
CAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCC
GCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAATAATTAATTAAC**GGATC**
CGGAATTCCG.

(AgeI and PacI sites, respectively, are in bold).

Sequence of 3xCox8a:

AG**GCGGCCGC**ATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGCTC
GGCCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGGGGGATCTGTC
CGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGCTCGGCCCGGCGGCTCCC
AGTGCCGCGCGCCAAGATCCATTCGTTGGGGGAtctgTCCGTCCTGACGCCGCTGC
TGCTGCGGGGCTTGACAGGCTCGGCCCGGCGGCTCCCAGTGCCGCGCGCCAAGA
TCCATTCGTTGGGGGATctgGATCCC**ACCGGT**AACGC.

(NotI and AgeI sites, respectively, are in bold).

Expression of GA, GB and GM in YT Indy.

The endogenous granzymes expressed in YT Indy were silenced by transducing the cells with lentiviruses generated in 293T cells transfected with the pLVX-shRNA1 vector containing GM, GH or GB shRNA together with the lenti-X HTX packaging mix (Clontech). Cells were selected with puromycin, and the clones showing the best silencing of all granzymes were selected by limiting dilution. For reconstitution of granzyme expression in the YT shRNA clones, cells were transduced with lentiviral particles generated from pLVX-EF1α expressing GA, GB wt, or GB mutant and selected with blasticidin. The pLVX-EF1α-blasticidin were generated by swapping puromycin with blasticidin from pLVX-EF1 EF1α puromycin (Takara).

Granzyme 3' UTR target sequences:

(GB) TATTTATTCAGTTGCTGGC;

(GM) GGACCAATAAATCATAATG;

(GH) TATTACAGTCCTGCAACCC.

Primers for expression of reconstituted GA, GB (wild type and mutants) and GM:

GA_Fw: AAAAAGCGGCCGCGCCGCCACCATGAGGAACTCCTATAG

GA_Rv: AAAAAGGATCCTTAAACTGCTCCCTTGATAGTC

GB_Fw: AAAAAAGCGGCCGCGCCGCCACCATGCAACCAATCCTGCTTC

Wild type GB_Rv: AAAAAGGATCCTTAGTAGCGTTTCATGGTTTTC

KR243/244EE GB_Rv: AAAAAGGATCCTTAGTAGGCTGCCATGGTTTTC

GM_Fw: AAAAAGCGGCCGCGCCGCCACCATGGAGGCCTGCGTGTC

GM_Rv: AAAAAGGATCCTTAGGCCGATCGGCCGGTGAC

B2Δ19-HA and SU9_V5 overexpressing HeLa.

The pET21+ Cytb2 (1-167) Δ19 DHFR, and pQE60-Su9 (1-69) DHFR, a kind gift of Dr. Dejana Mokranjac, were used as template to subclone B2Δ19-HA and Su9-V5 between the NotI and EcoRI restriction sites of pLVX TRE3G (Takara Clontech) with the following set of primers.

pLVX-TRE3G B2Δ19 Fw :

GGCCCGCGGCCGCAATGCTAAAATACAAACCTTTACTAAAAATCTCG

pLVX-TRE3G B2Δ19 Rv :

CCCGGTAGAATTCTCATCAAGCGTAATCTGGAACATCGTATGGGTAAAACTTatACtT GATGCCTTTTTCC

pLVX-TRE3G SU9Fw :

ATCCGGGCCCGCGGCCGCAATGGCCTCCACTCGTGTCCTCGC

pLVX-TRE3G SU9Rv :

CCCGGTAGAATTCTTATTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGG CTTACCAAACTTatACtTGATGCC

Lentivirus was produced, and HeLa tet cells were transduced following Lenti-X™ Tet-ON® 3G Inducible Expression System protocol. Dox (1 µg/ml) was added to cell medium for B2Δ19-HA and SU9-V5 overexpression.

Immunoprecipitation.

C-terminal GzmA residues 232-SKKHLNWIIMTIKGAV-246 or GzmB residues 231-VSSFVHWIKKTMKRY-245 were fused in C-terminus of two B domains of protein A, BB-CtA and BB-CtB, respectively. As a control, two BB domains were fused in the C-terminus of full length GB to generate GB-BB fusion protein.

Human codon optimized gblocks for BB domain of protein A were used.

5'-B/B

AAAAAA**ACCGGT**ATGGCAGATAATAAGTTTAACAAAGAGCAGCAGAACGCCTTCTA
CGAAATCTTGCACTTGCCTAACCTGAACGAAGAACAGAGAAATGGCTTTATTCAAAG
CCTTAAGGATGATCCGAGCCAGAGCGCTAACCTCCTGGCTGAGGCTAAAAAACTCA
ACGACGCCCAGGCACCTAAAGCTGACAACAAATTTAATAAAGAGCAGCAGAACGCC
TTCTACGAGATACTGCACCTTCCTAATCTCAATGAAGAGCAAAGGAATGGCTTTATT
CAATCTCTGAAGGACGATCCTAGTCAGTCCGCAAACCTGCTGGCCGAAGCGAAGA
AGCTGAACGATGCCCAGGCTCCTAAGGAAAATCTCTATTTCCAAGGGGACGGC**GC
GGCCGC**GTAATAA

AgeI and NotI sites are in bold.

3’-B/B

AAAAAA**GCGGCCGC**AGCAGATAACAAGTTCAACAAAGAGCAGCAGAACGCCTTCTA
CGAGATTCTGCATCTGCCGAACCTGAATGAGGAACAGAGAAACGGCTTCATCCAAT
CACTCAAGGATGATCCATCTCAATCCGCAAATCTTCTGGCAGAGGCCAAGAAACTC
AACGATGCGCAGGCCCCCAAGGCGGATAACAAGTTCAACAAAGAGCAGCAGAACG
CCTTCTACGAGATCCTGCACCTTCCCAATCTGAATGAGGAGCAGCGCAACGGTTTC
ATCCAGTCTCTCAAGGACGACCCCAGCCAATCAGCCAACCTCCTCGCAGAAGCTAA
AAAACTTAATGACGCCCAGGCTCCTAAA**GGTACC**TAATAA

NotI and KpnI sites are in bold.

5’ or 3’ BB domains were cloned in pHLsec vector.

GB cDNA was amplified by PCR and cloned between AgeI and KpnI in pHLsec vector at 5’ of BB domain of protein A, using the following primers:

GB-BB AgeI Fw;

aaaaaaaccggtgatgatgataagATCATCGGGGGACATGAGGCC

GB-BB Rv:

GATTGCGGCCGCGCCGTCGTAGCGTTTCATGGTTTTCTTTATCC

C-terminus of GA and GB were cloned between NotI and KpnI.

CtermA_Fw:

AAAAAAGCGGCCGCGTCAAAGAAACACCTCAACTGGATAATTATGACTATCAAGGG AGCAGTTTAATAAGGTACC AAAAAA

CtermA_Rv:

TTTTTTGGTACCTTATTAAACTGCTCCCTTGATAGTCATAATTATCCAGTTGAGGTGT TTCTTTGACGCGGCCGCTTTTTT

CtermB_Fw:

AAAAAAGCGGCCGCGGTCTCAAGCTTTGTACACTGGATAAAGAAAACCATGAAACG CTACTAATAA GGTACC AAAAAA

CtermB_Rv:

TTTTTTGGTACCTTATTAGTAGCGTTTCATGGTTTTCTTTATCCAGTGTACAAAGCTT GAGACCGCGGCCGCTTTTTT

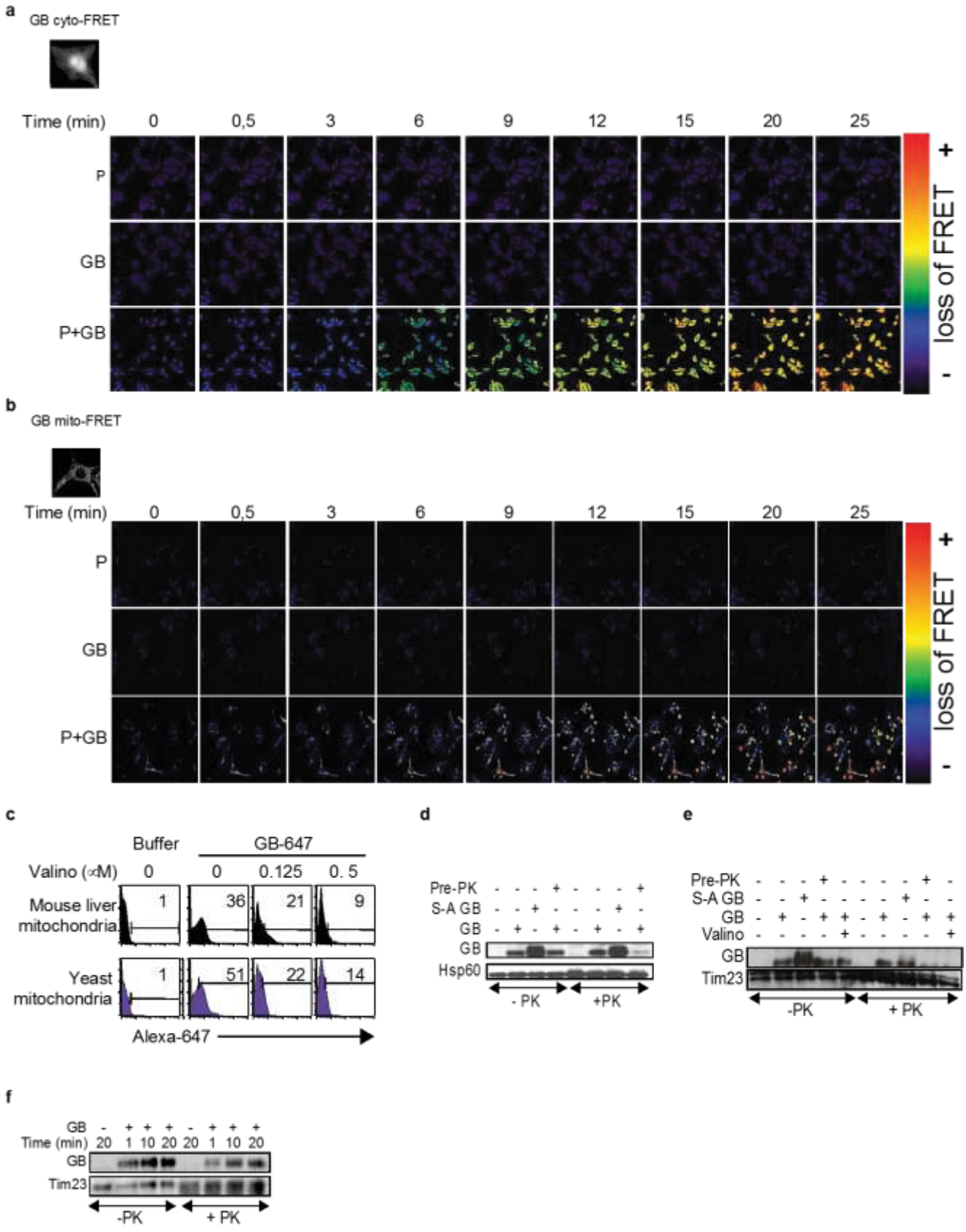
Purified mouse liver mitochondria (100 µg), were incubated with 2 µg of BB-fused proteins in SH buffer (see import assay) for 15 minutes on ice and washed in 1 ml of SH buffer. The samples were spun 10 minutes at 12000 g, and the pellet was resuspended in 100 µl of SH. The DSP cross-linker (Thermo Fisher) was added at a final concentration of 1 mM for 30 minutes at RT and then inactivated by adding 5 µl of 1.5 M Tris, pH 8.0 for 15 minutes at RT. The samples were spun as before, and the pellet resuspended in 100 µl of lysis buffer (50 mM potassium acetate; 10 mM MOPS, 20% glycerol) plus 1% digitonin and 10 mM PMSF for 45 minutes at 4°C. 500 µl of lysis buffer was added and the samples were spun as previously. The supernatants were incubated O/N at 4°C with Pierce™ Streptavidin Magnetic Beads and 15 µl of biotinylated-Fc protein (EZ-link™Sulfo-NHS-SS-Biotin, Thermo scientific). The next day, beads were washed 2 to 3 times with lysis buffer plus 0.2% digitonin, and elution was performed in 100 µl of Glycine-HCl, pH 2.5. Samples were loaded on NuPAGE®Novex® 12% Bis-Tris native gel (Life Technologies). Gels were silver stained using SilverQuest™ staining kit (Life Technologies), and bands of interest were analyzed by mass-spectrometry.

Statistical Analysis

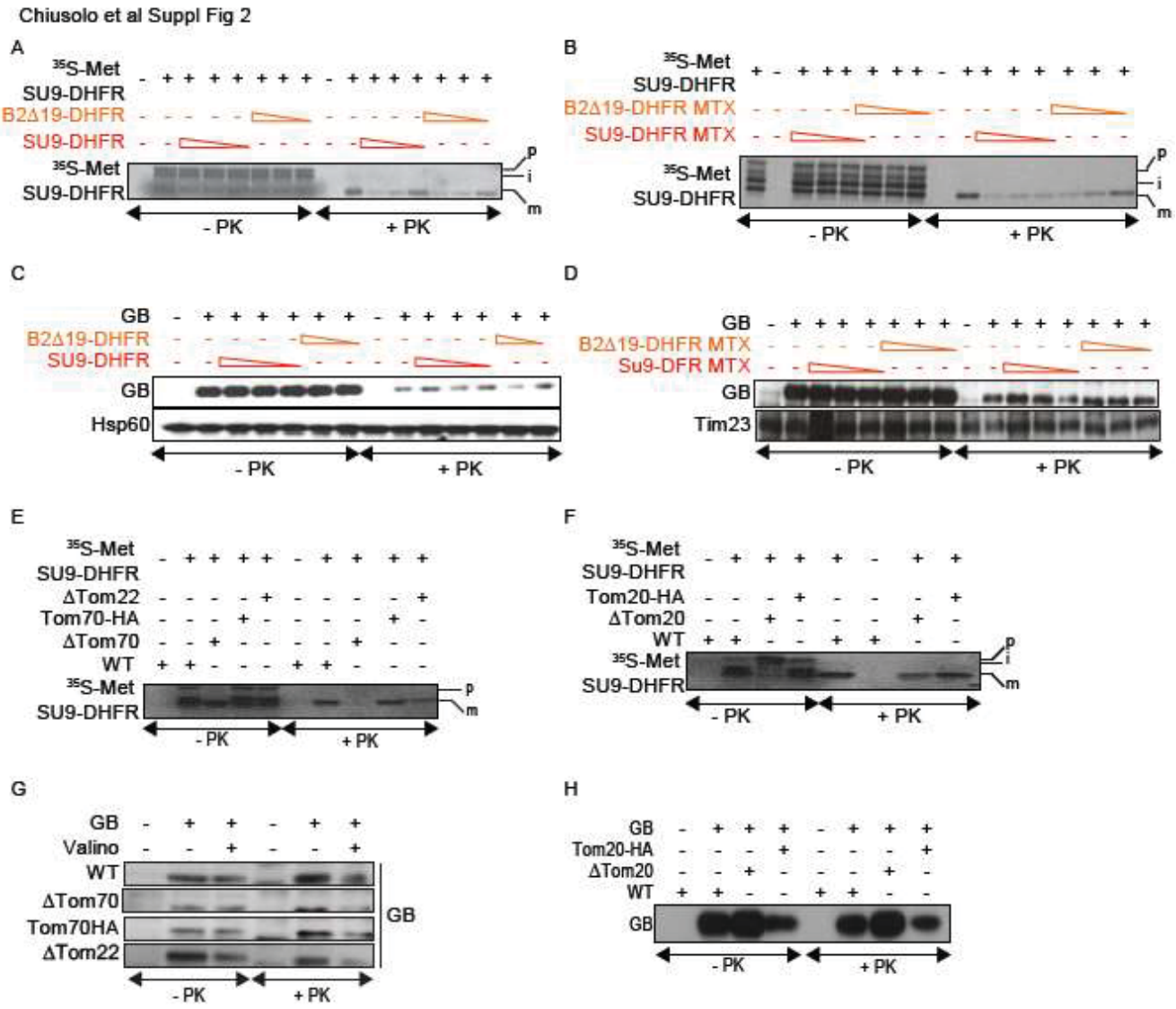
Data represent mean +/- SEM or mean +/- SD of indicated number of independent experiments. Statistical significance has been calculated by a two-tailed or one-tailed Student t-test between samples and P values are indicated in the legends.

Supplemental Figures:

Chiusolo et al Suppl. Fig 1

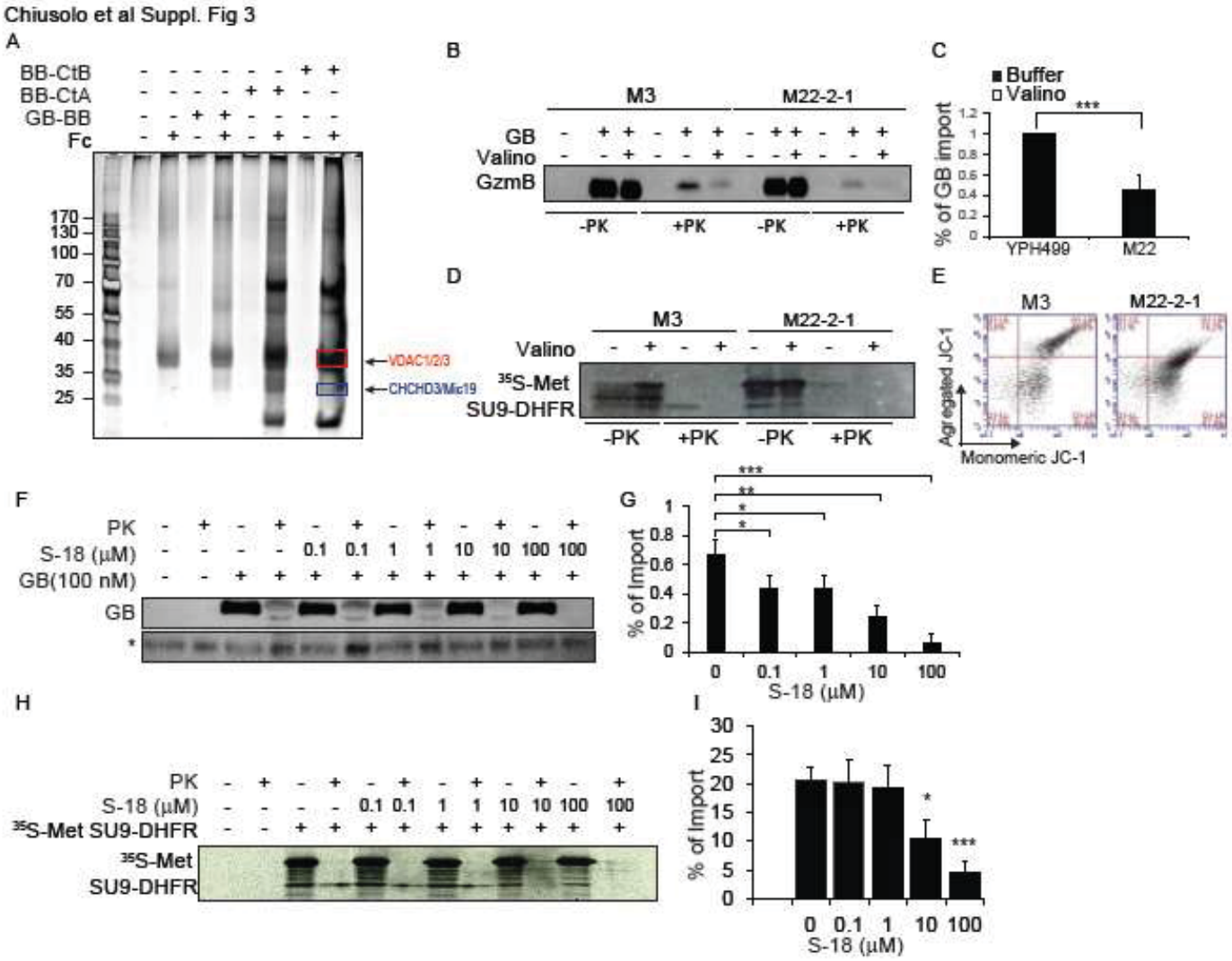


Suppl. Figure 1: GB enters Hela cell mitochondria. Hela cells overexpressing cytosolic (a) or mitochondrial targeted (b) GB-FRET reporter were treated with a sublytic dose of perforin alone, 0.4 μM of GB alone, or perforin plus 0.4 μM GB. GB activity was monitored by the loss of FRET signal by microscopy. Images are representative of three independent experiments. (c) GB entered both mouse liver (top raw) and yeast (bottom raw) mitochondria. Mitochondria, pretreated or not with valinomycin to disrupt the membrane potential, were incubated with Alexa 647-GB before removing the externally bound GB with a gentle proteinase K treatment. GB import was analyzed by FACS. FACS histograms are representative of three independent experiments. GB enters mouse liver (d) and yeast (e) mitochondria in a facilitated manner. Intact or proteinase K pretreated mitochondria were incubated with active GB or catalytically inactive s-aGB in import assay. Both active and inactive GB only entered intact mitochondria. Western blots are representative of three independent experiments. (f) GB enter yeast mitochondrial in a time dependent manner. Freshly purified yeast mitochondria were incubated with GB in import assay for the indicated time.



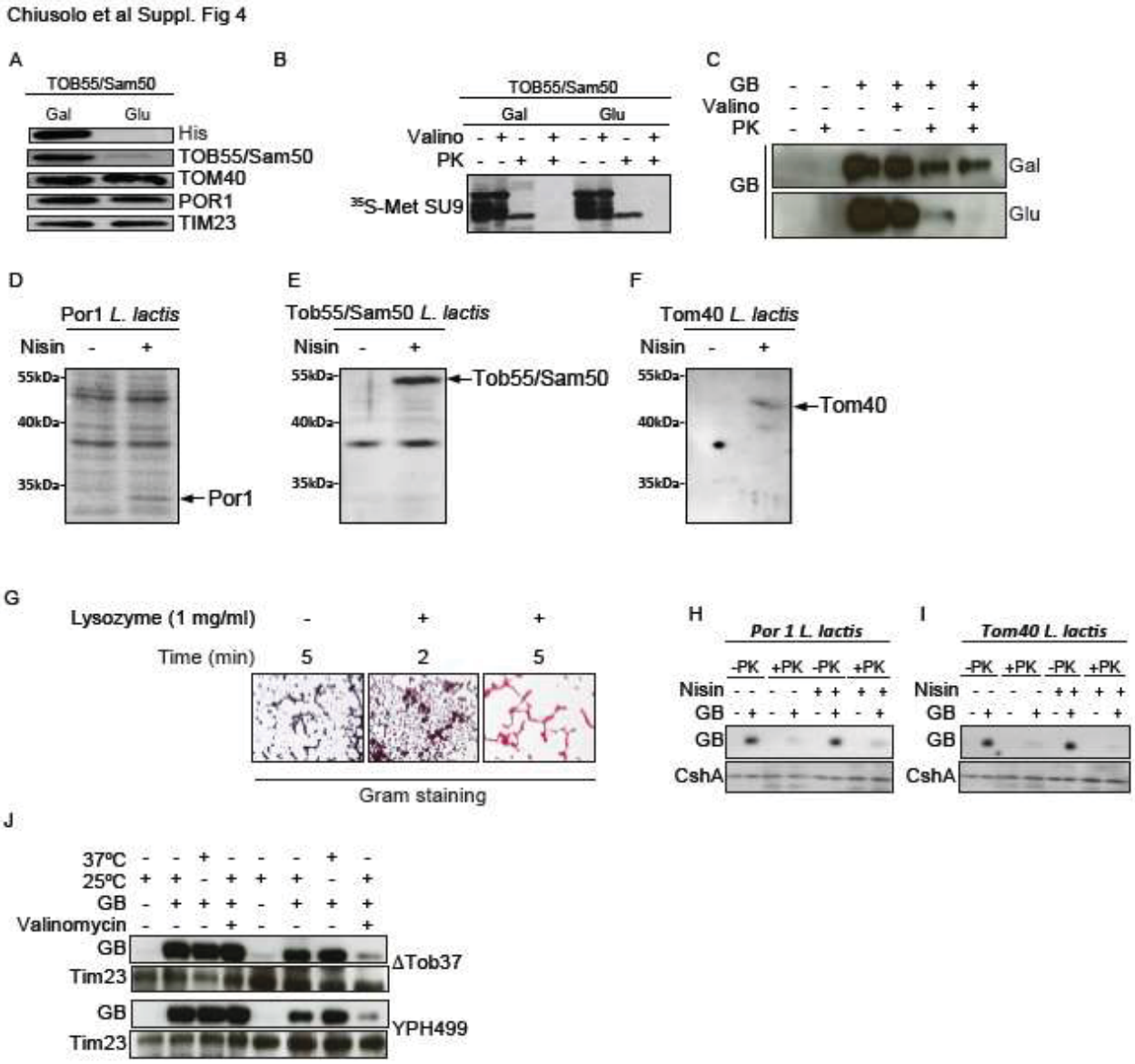
Suppl. Figure 2: TOM receptors are not required for GB mitochondrial import. (a-d) Non-labelled B2Δ19-DHFR and SU9-DHFR, in the absence (a and c) or presence of preincubation with methotrexate (b and d) were used to saturate mouse liver mitochondrial import machinery before testing the import of ^{35}S-Met labelled SU9-DHFR as a positive control (a-b) or GB (0.2 µM) import (c-d). While B2Δ19-DHFR and SU9-DHFR prevent the import of ^{35}S-Met SU9-DHFR, only B2Δ19-DHFR at the highest dose has an effect on GB import. This effect was no longer perceptible in the presence of methotrexate (MTX). (e) Mitochondria purified from wild type yeast, or yeast deficient for Tom70 (ΔTom70), overexpressing Tom70 (Tom70-HA), or deficient for Tom22 in a Tom70 over-expressing background (ΔTom22), were used in import assay for ^{35}S-Met SU9-DHFR. (f) Same as in

(e) using mitochondria purified from wild type, Tom20 overexpressing (Tom20-HA), or deficient (ΔTom20) yeast strains. (g) Same as in (e) for GB import. Loss of Tom70 and Tom22 had no effect on GB import. (h) Same as in (f) for GB import. Loss of Tom20 had no effect on GB import. Blots and autoradiographies are all representative of three independent experiments.



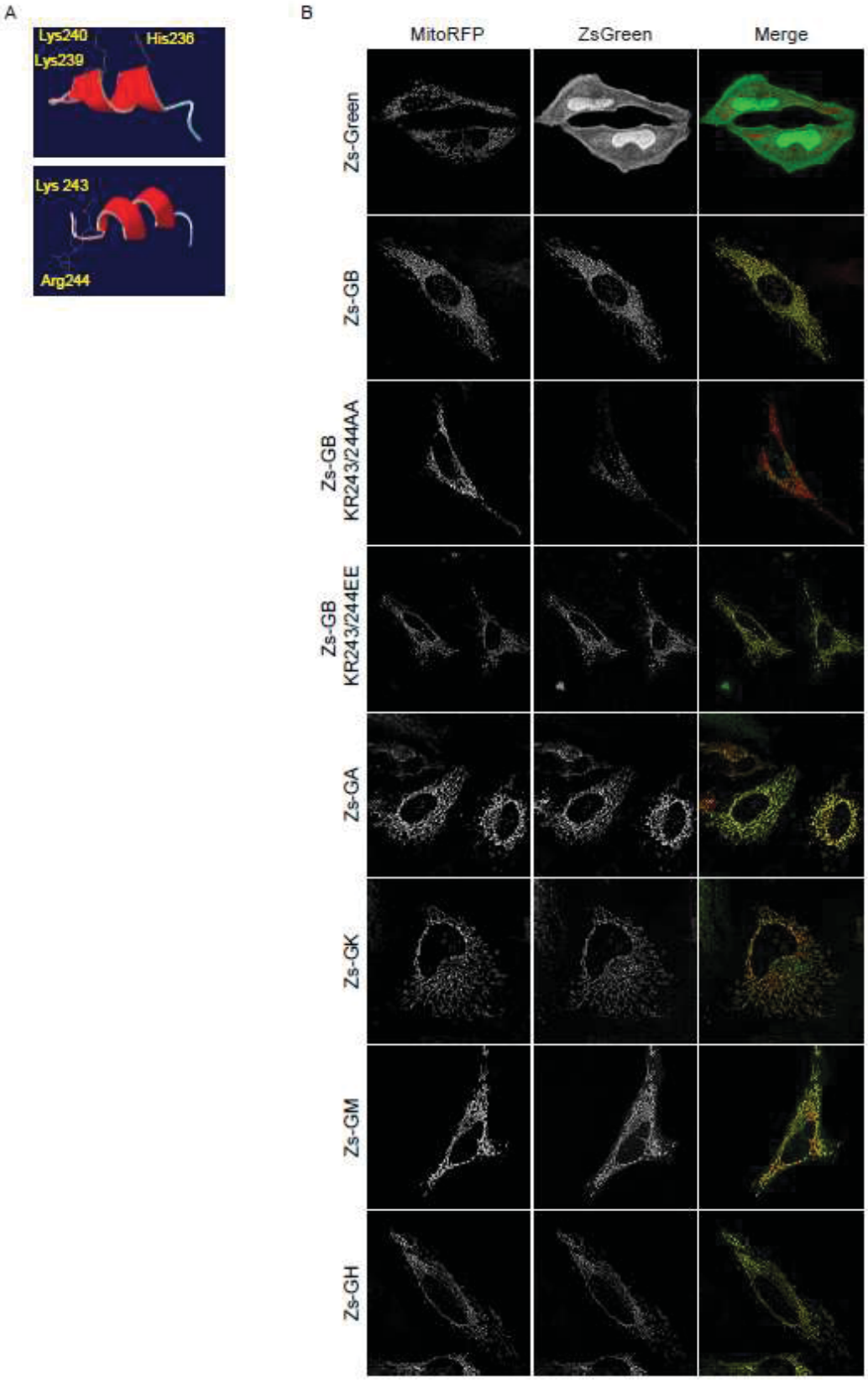
Suppl. Figure 3: Por1/VDAC deletion affect both SU9 and GB import. (a) C-terminal portion of GB or GA were fused to the C-terminus of two B domains of protein A to generate the fusion constructs BB-CtB and BB-CtA respectively that were used to carry affinity purification. GB with C-terminus blocked with two B domains of protein A (GB-BB) was used as negative control. Boxes highlight bands were characterized by mass spectrometry. (b) Mitochondria purified from YPH499 wild type or M22-2-1 yeast deficient

for Por1 and 2 were used in import of GB (0.2 µM). (c) Same as in (b) mean +/- SD of four independent experiments. (d) YPH499 wild type and M22-2-1 yeast mitochondria were used in ^{35}S-Met SU9 import. (e) Purified mitochondria from YPH499 an M22-2-1 yeast strains were analyzed by FACS for their membrane potential following JC-1 staining. (f) Mouse liver mitochondria preincubated or not with VDAC1 inhibitor S-18 were used in GB import. (g) Same as in (f), mean +/- SD of three independent experiments. (h) Same as in (f) using ^{35}S-Met SU9. (i) Same as in (h), mean +/- SD of three independent experiments. P value * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (two-sided t-test). Blots and autoradiographies are all representative of at least 3 independent experiments.

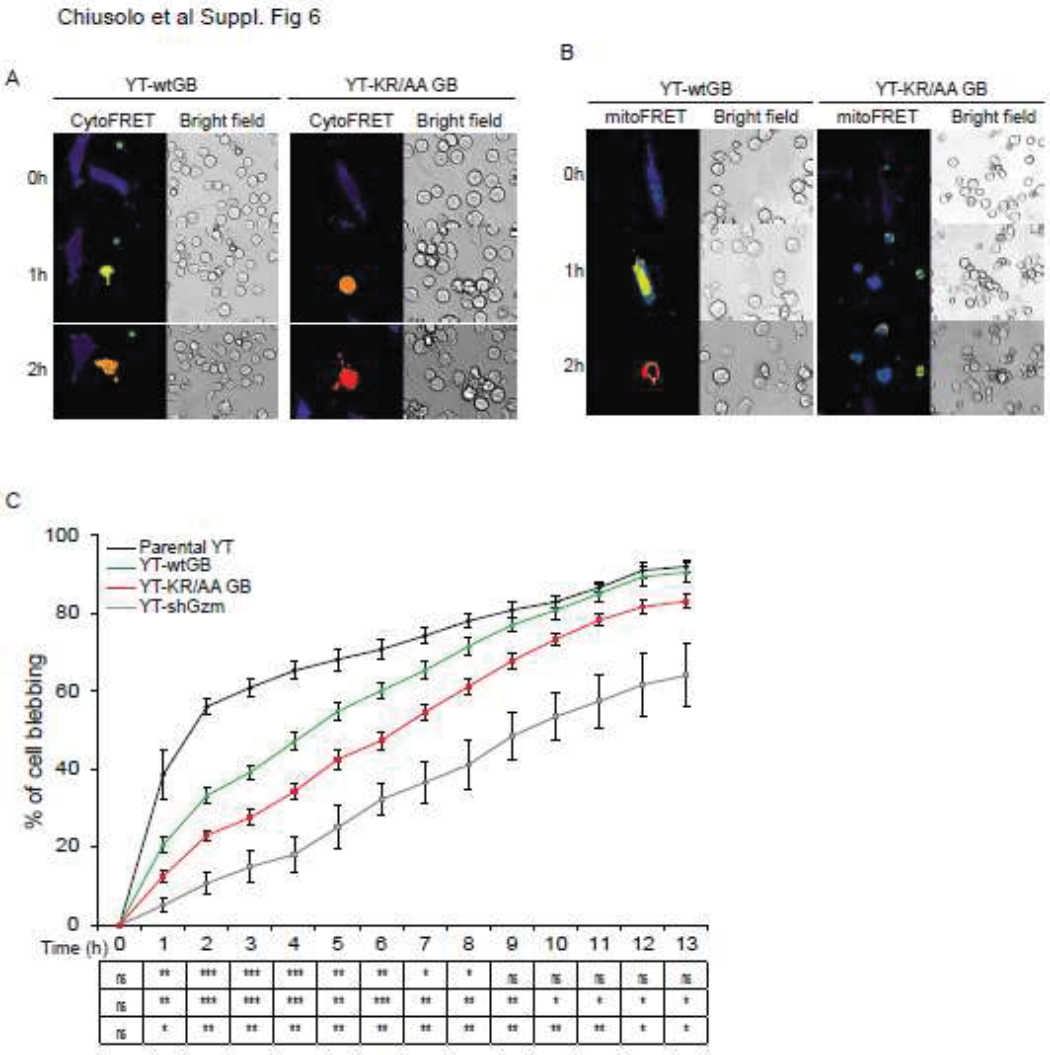


Suppl. Figure 4: Tob55/Sam50 is required for GB mitochondrial entry. (a) Mitochondria from yeast expressing Tob55/Sam50 under the control of promotor of galactose operon grown in galactose or glucose medium were analyzed for Tob55/Sam50, Tom40, Por1 and TIM23 expression by Western blot. (b and c). Mitochondrial purified as in (a) were used to import ^{35}S-Met SU9 (b) or GB (0.2 µM) (c). Loss of Tob55/Sam50 expression has no effect on ^{35}S-Met SU9-DHFR import; however, it significantly compromised GB mitochondrial entry. Lactococcus lactis (*L. lactis*) electroporated with construct encoding for Por1 (d), Tob55/Sam50 (e) or Tom40 (f) were induced or not with nisin, and the expression of Por 1, Tob55/Sam50, and Tom40 tested by Western blot or coomassie blue. (g) L. lactis were treated or not with lysozyme, and the integrity of their cell wall tested by Gram staining. *L. lactis* overexpressing Por1 (h) or TOM40 (i) were used in import assay for GB. (j) Mitochondria purified from YPH499 wild type yeast or deficient for Tob37 were used for the import of GB (0.2 µM). GB entered as efficiently both types of mitochondria. Blots are all representative of three independent experiments.

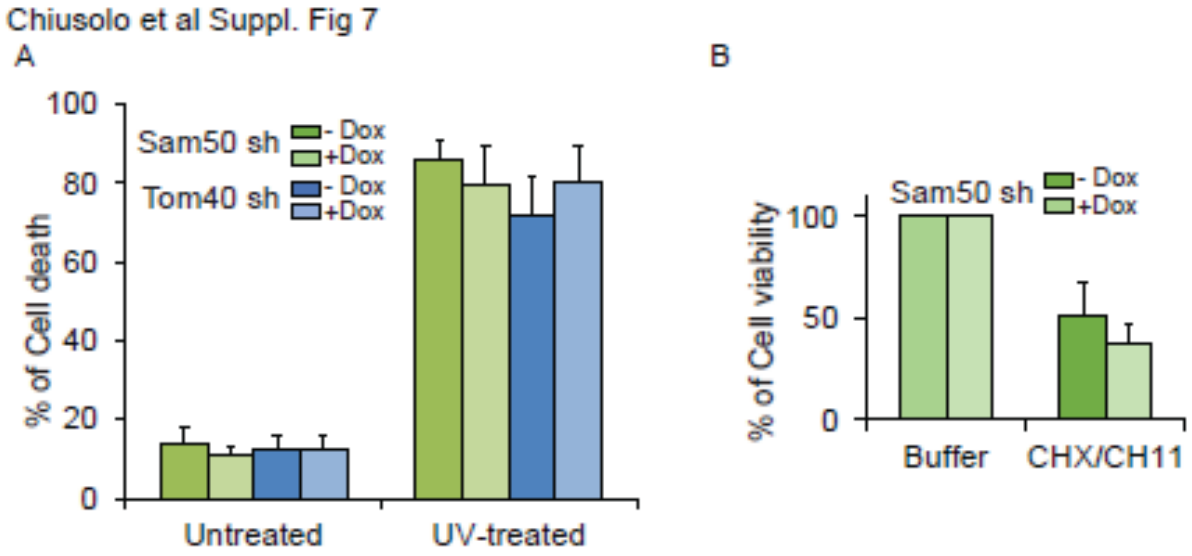
SupplChiusolo et al Fig 5



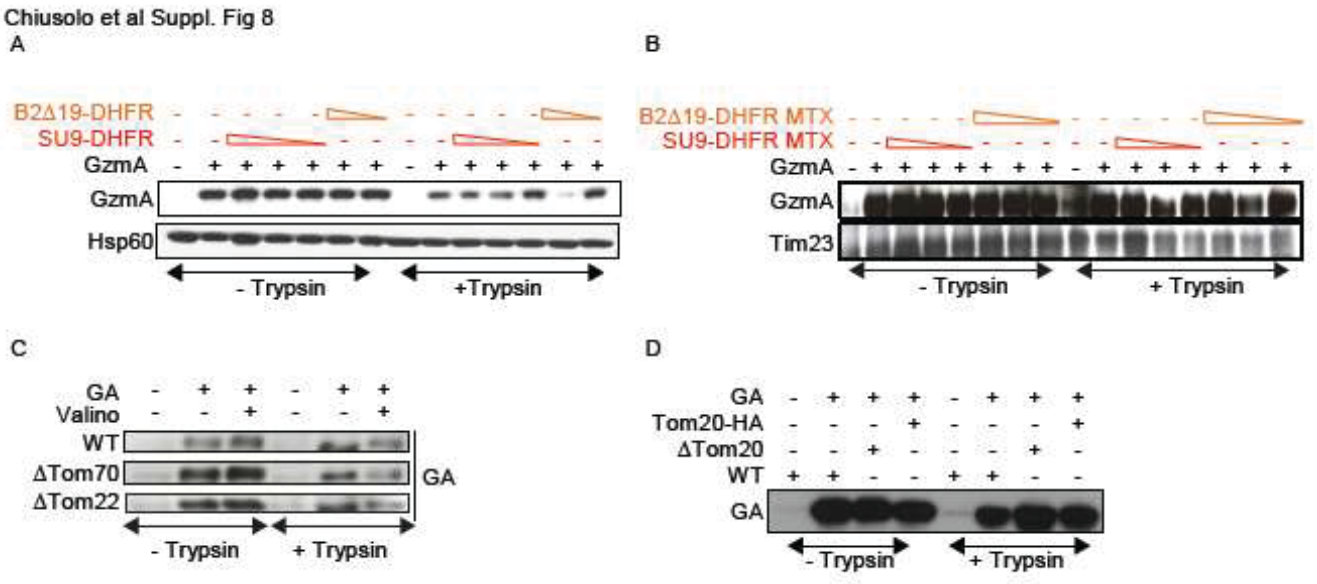
Suppl. Figure 5: (a) 3D structure of GB C-terminal tail. Highlighted are residues histidine 236 and lysines 239-240 (top panel) and residues lysine and arginine 243-244 (bottom panel). (b) The five human granzymes enter mitochondria. Hela cells overexpressing mitoRFP were co-transfected with either ZsGreen or ZsGreen fused in N-terminus of wild type GB, KR243/244AA GB, KR243/244EE GB, wild type GA, GK, GM or GH, and the subcellular localization of the granzymes was assessed by confocal microscopy. Images are representative of three independent experiments. The wild-type granzymes localized to the mitochondria while GB KR243/244AA did not.



Suppl. Figure 6: (a) YT-wtGB and YT-KR/AA GB were used to attack Hela CD80$^+$ cells expressing either cytosolic (a) or mitochondrial (b) GB-FRET reporter. Images are representative of three independent experiments. (c) Parental Y-Indy, YT-shGzm, YT-wtGB and YT-KR/AAGB were used to kill Hela CD80$^+$ cells. Cell death was assess by microscopy. Data represent mean +/- SD of at least four independent experiments. P value * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-sided t-test).



Suppl. Figure 7: Sam50 silence cells are still sensitive to cell death. (a) Hela Sam50sh and Hela Tom40sh were Dox-induced or not before 5 minutes exposure to UV irradiation. Cell death were assessed 16 hours later by methylene blue staining. (b) Hela Sam50sh were Dox-induced or not before treatment with 1 µg/ml of anti-Fas CH11 for 8 hours before cell death assessment by methylene blue staining. Data are representative of 3 independent experiments.



Suppl. Figure 8: TOM receptors are not required for GA mitochondrial import. Non-labelled B2Δ19-DHFR and SU9-DHFR in the absence (a) or presence (b) of preincubation with methotrexate were used to saturate mouse liver mitochondria import machinery before testing the import of GA (0.2 μM). Only B2Δ19-DHFR at the highest dose had an effect on GA import. This effect was no longer perceptible in the presence of methotrexate. (c) Mitochondria deficient for Tom70 (ΔTom70) overexpressing Tom70 (Tom70-HA) or deficient for Tom22 in a Tom70 overexpressing background (ΔTom22) were used in import assay for GA (0.2 μM). (d) Same as in (c) using mitochondria isolated from wild type, Tom20 overexpressing (Tom20-HA) or deficient (ΔTom20) yeast strains. Lake of Tom20 had no effect on GA import. Blots are all representative of three independent experiments.