Supporting information for

Centrioles control the capacity, but not the specificity, of cytotoxic T cell killing

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Contains SI materials and methods along with 7 SI figures

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SI Materials and methods

Constructs. pMSCV vectors containing a linked cassette comprising a puromycin resistance gene, an internal ribosome entry site, and either CFP or RFP were prepared by subcloning the CFP and RFP coding sequences into pMSCV PIG using the NcoI and SalI restriction sites. The coding sequence for Cre recombinase was inserted into the resulting vectors (pMSCV PIC and pMSCV PIR) and pMSCV PIG using the BglII and EcoRI restriction sites in a process that destroyed the BglII recognition sequence. The retroviral pHluorin-Lamp1 and Lifeact-GFP constructs have been described (1, 2).

Mice and cell culture. The animal protocols used for this study were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center. *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ mice on the FVB background were crossed with OT1 TCR transgenic mice on the C57BL/6 background to obtain OT1 *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and OT1 *Sas4*$^{+/fl}$*Trp53*$^{fl/fl}$ animals. RMA-s cells were maintained in RPMI containing 10% (vol/vol) FCS, and Phoenix E cells were maintained in DMEM medium containing 10% (vol/vol) FCS.

Cytotoxicity experiments. RMA-s target cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) or the membrane dye PKH26, loaded with different concentrations of OVA and mixed in a 96-well V-bottomed plate with CellTrace Violet (CTV) stained OT1 cells. To assess killing, cells were mixed at a 1:1 or 1:10 E:T ratio and incubated for 4-24 h at 37 °C. In certain experiments, CTLs were combined with both OVA-loaded targets and unloaded bystander RMA-s cells at a 1:1:1 ratio. At the assay endpoint, specific lysis of RMA-s cells was determined by flow cytometry as previously described (3). For degranulation experiments, the E:T ratio was 1:1 and cells were incubated at 37 °C for 90 min in the presence of eFluor 660-labeled Lamp1 Monoclonal Antibody (clone eBio1D4B, eBioscience). Lamp1 staining was then assessed by flow cytometry. In certain experiments, microtubules were depolymerized by preincubation for 10 min with 30 μM nocodazole, which was then maintained for the duration of the experiment. To measure conjugate formation, CTLs and targets were mixed 1:3, lightly centrifuged (100 × g) to encourage cell contact, and incubated for 20 min at 37 °C. Cells were then resuspended in the presence of 2% PFA, washed in FACS buffer (phosphate buffered saline (PBS) + 4% FCS), and analyzed by flow cytometry. Conjugate formation was quantified as (CFSE+ CTV+)/(CTV+).

Western blots. To assess TCR-induced signaling pathways, serum and IL2 starved OT1 CTLs were incubated with streptavidin polystyrene beads (Spherotech) coated with H2-K^b-OVA and ICAM1 at a 1:1 ratio for various times at 37 °C. To evaluate mTORC1 signaling, CTLs were starved in PBS and then stimulated for various times with RPMI containing 10% (vol/vol) FCS and IL2. Stimulated cells were lysed in 2 × cold lysis buffer (50 mM TrisHCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40 and 0.25% sodium deoxycholate) containing phosphatase inhibitors (1 mM NaF and 0.1 mM Na_3VO_4) and protease inhibitors (cOmplete mini cocktail, EDTA-free, Roche). Immunoblots were carried out using the following antibodies: pAkt (Phospho-Akt (Ser473) Ab; Cell Signaling Technology), pErk1/2 (Phospho-Thr202/Tyr204; clone D13.14.4E; Cell Signaling Technology), IκB (Cell Signaling Technology), pS6 Kinase (Phospho-Thr389; clone 108D2; Cell Signaling Technology), and p4EBP1 (Phospho-Thr389; clone 236B4; Cell Signaling Technology). Actin served as a loading control (clone AC-15, Sigma). Perforin levels were assessed by immunoblot using a polyclonal antibody (Cell Signaling Technology, #3693)

Functional assays. To assess IFNγ and GzmB secretion, OT1 CTLs were mixed 1:1 with RMA-s cells loaded with increasing concentrations of OVA and incubated for 4-6 hours at 37 °C. Secreted IFNγ and GzmB were detected by ELISA using an established anti-IFNγ antibody pair (clone AN-18 for capture, eBioscience, biotinylated clone XMG1.2 for detection, BD Biosciences) and a mouse GzmB ELISA kit (Invitrogen #88-8022), respectively. In certain experiments GolgiPlug (BD Biosciences, manufacturer recommended concentration) was included in the assay medium and IFNγ production measured by intracellular staining with fluorescently labeled anti-IFNγ antibodies (clone XMG1.2, TONBO). To quantify TCR-induced proliferation, OT1 CTLs were CTV-labeled and mixed with OVA-loaded, irradiated C57BL/6 splenocytes. CTV dilution was assessed on a daily basis by flow cytometry. Intracellular GzmB levels were measured by flow cytometry using Alexa 647 labeled anti-GzmB (clone GRB11, Biolegend). To assess lysosomal content, CTLs were incubated with 50 nM lysotracker red (Thermofisher) for 60 min at 37 °C and then analyzed by flow cytometry. To assess lysosomal degradation, CTLs were mixed with 10 μg/ml DQ-BSA and 10 μg/ml TMR-BSA (both from Thermofisher) for various times at 37 °C, after which their green and red fluorescence was determined by flow cytometry. To monitor TCR downregulation, OT1 CTLs were mixed 1:1 with H2-K^b-OVA- and ICAM1-coated beads at 37 °C for various times, after which TCR surface expression was assessed flow cytometrically using labeled antibodies against CD3ε (clone 500A2, eBioscience). Surface LFA1 was quantified using labeled antibodies against CD18 (clone M18/2, eBioscience).

Quantitative RT-PCR. Total RNA from either control or DKO CTLs was extracted using the RNeasy Mini Kit (Qiagen), and RT-PCR was performed using the SuperScript™ III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed using the Fast SYBR™ Green Master Mix (Applied Biosystems). Samples were processed using the BioRad CFX96 Real-Time System, and melting curve analysis was performed using BioRad-CFX Manager software. Gene expression level was determined by normalizing transcripts levels of the gene of interest to the level of the GAPDH housekeeping gene. The fold gene expression of each transcript relative to the control was calculated using the cycle threshold (Ct) method.

Fixed imaging. Stimulatory glass surfaces and supported lipid bilayers bearing either H2-K^b-OVA and ICAM1 or ICAM1 alone were prepared as described previously (2). OT1 CTLs were incubated for 10 min at 37 °C on these surfaces and then fixed by adding 4% paraformaldehyde for 5 min followed by ice-cold methanol for 15 min. Samples were then blocked in PBS with 0.5% Triton X-100 solution supplemented with 2% goat serum for 1 h at room temperature and incubated overnight at 4 °C with mixtures of the following primary antibodies: anti-centrin (clone 20H5; Millipore), anti-pericentrin (ab4448; Abcam), anti-γTubulin (clone GTU-88; Sigma), anti-βTubulin (clone TUB 2.1; Sigma), anti GM130 (clone 35/GM130; BD Biosciences), anti-Lamp1 (clone 1D4B; eBioscience), anti-GzmB (Polyclonal; ThermoFisher Scientific), anti-Rab7 (clone D95F2; Cell Signaling), anti-EEA1 (clone F.43.1; ThermoFisher Scientific). For F-actin staining, the methanol fixation step was omitted and cells were stained using Alexa 594-labeled phalloidin (ThermoFisher) and anti-LFA1 (clone M14/7; eBioscience). After primary antibody staining and washing, samples were incubated with the appropriate secondary antibody for 2 h at room temperature, washed and then imaged. In certain experiments, DAPI or Hoechst was added just prior to imaging to visualize cell nuclei. Confocal microscopy was performed with a Leica SP8 laser scanning microscope fitted with a white light laser and a 40 × objective lens. TIRF imaging was carried out using an

Olympus OMAC system (IX-81 stage) outfitted with 561 nm and 488 nm lasers and a 60 × objective lens.

Live imaging. For Ca^{2+} imaging, OT1 CTLs were loaded with 5 μg/ml Fura2-AM (ThermoFisher Scientific), washed, and then imaged on stimulatory glass surfaces coated with H2-K^b-OVA and ICAM1. Images were acquired using 340 nm and 380 nm excitation every 30 seconds for 30 min with a 20 × objective lens (Olympus). For live imaging of degranulation, OT1 CTLs expressing pHluorin-Lamp1 and a fluorescent cellular label (typically RFP) were imaged on stimulatory glass surfaces coated with H2-K^b-OVA and ICAM1 using a Leica Sp5 laser scanning confocal microscope outfitted with 561 nm and 488 nm excitation lasers and a 40 × objective lens. For each imaging run, a 12 μm z-stack (0.5 μm intervals) was collected every 15 s. In certain experiments, microtubules were depolymerized by preincubation for 10 min with 30 μM nocodazole, which was then maintained in the imaging medium for the duration of the experiment. To visualize synaptic F-actin dynamics, OT1 CTLs expressing Lifeact-GFP were added to bilayers containing H2-K^b-OVA and ICAM1 and imaged every 5 s by TIRF microscopy for 30 min using a 60 × objective lens. For microwell cytotoxicity experiments, PDMS grids containing 50×50×25 μm wells were submerged in imaging medium and seeded with CTV-labeled OT1 CTLs and CFSE-labeled RMA-s cells that had been pulsed with 1 μM OVA. In general, individual wells contained between 1-3 CTLs and 1-3 RMA-s cells. 1 μM PI (Life Technologies) was included in the medium to enable real time visualization of dying cells. CTV-labeled CTLs were added and the cells imaged using a 20 × objective lens (Olympus) at 10 min intervals for 12 hr. Brightfield, CFSE, CTV, and PI images were collected at each time point. The number of target cell killing events (identified by PI influx) was scored for each well and sorted based on the initial number of CTLs and targets in the well. Wells with no IS formation or with initial IS formation after 10 hours were excluded from the analysis.

Electron microscopy. OT1 CTL populations were pelleted and resuspended in fresh, growth medium at 1 × 10^6 cells/ml. Each CTL population was mixed 1:1 with EL4 targets pre-incubated for 40-60 min with 1 μM OVA, left at room temperature for 5 min and incubated for 30-35 min at 37 °C to form conjugates. Conjugated suspensions were fixed by adding an equal volume of 5% gluteraldehyde/ 4% paraformaldehyde in PBS to give a final concentration of 2.5% gluteraldehyde/ 2% paraformaldehyde and left for 10 min. Then, the fixed cells were pelleted and fresh 1 × fixative in 0.1M Sodium Cacodylate added over the pellets. Pellets were subsequently processed for osmium fixation, urynal acetate staining en bloc and embedding in EPON as previously described for cells adhered on dishes (4, 5). 70-80 nm serial sections of CTLs conjugated to targets were collected on film-coated slot grids, stained with lead citrate, and the area of interest in each cell (identified by looking for the Golgi complex) followed over a depth of ~1-2 μm using an FEI Tecnai G2 Spirit BioTWIN TEM (Eindhoven, Netherlands). Images were captured using a Gatan US1000 CCD camera and FEI TIA software.

Image analysis. Imaging data were analyzed using SlideBook (3I), Imaris (Bitplane), Excel (Microsoft), Prism (GraphPad), and Matlab (MathWorks). Ca^{2+} responses were quantified by first normalizing the ratiometric Fura2 response of each individual cell to the last time point before the initial influx of Ca^{2+}, and then by aligning and averaging all responses in the data set based on this initial time of influx. $Centrin^+$ puncta (Fig. 1B, S1B) were quantified by first establishing a high intensity threshold for all images of DKO and wild type control CTLs taken on the same day and then counting the number of fluorescent objects in each cell above this threshold. Radial analysis of microtubule and

Golgi staining (Fig. 1C, S1D-E) was performed using a custom Matlab script. First, 180 linescans were taken through each cell at 1° increments and then averaged and amplitude-normalized to yield an intensity profile for that cell. Then, multiple intensity profiles (normalized for cell size) were averaged to generate the graphs shown in Fig. 1C and S1E. Distances between degranulation events and the stimulatory surface (Fig. 3C, 3E) were determined with the Imaris Measurement Toolkit using yz or xz projections of confocal stacks. Pearson's Correlation Coefficients (Fig. 5D, S7C) were calculated in Imaris for each image (10-20 cells per image). To determine the size distribution of intracellular compartments (Fig. 5E, S7D), 3-dimensional surfaces encompassing the compartments were first established in Imaris by intensity thresholding. Then, the volumes of all particles were output as an Excel file. For each compartment in question, identical intensity thresholds were applied to images of DKO and wild type control CTLs taken on the same day.

Supplemental Figures

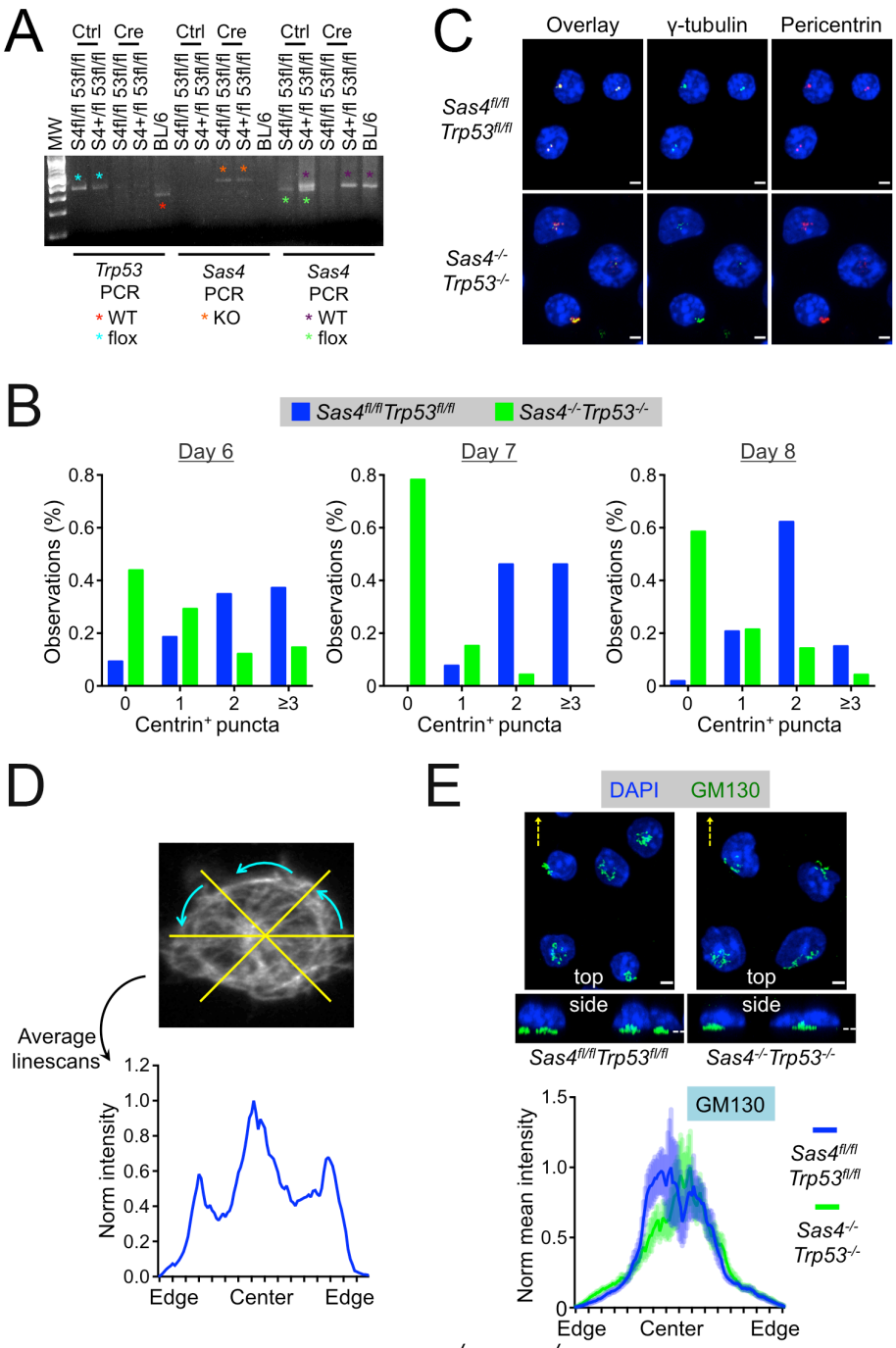

Figure S1. Architectural analysis of *Sas4*$^{-/-}$*Trp53*$^{-/-}$ CTLs. (A) PCR genotyping of the *Sas4* and *Trp53* deletions. T cells from OT1 *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ (S4fl/fl 53fl/fl) and OT1 *Sas4*$^{+/fl}$*Trp53*$^{fl/fl}$ (S4+/fl 53fl/fl) mice were stimulated with antigen and transduced with either control (Ctrl) or Cre-expressing retroviruses. After additional culturing and FACS purification, DNA extracts were PCR genotyped using primer mixtures for wild type and floxed *Trp53* (left), the *Sas4* knockout allele (middle), and wild type and floxed *Sas4* (right). Diagnostic bands are indicated with colored asterisks. Tail DNA from C57BL/6

mice (BL/6) was included as a wild type control. MW = molecular weight ladder. (B) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ naïve OT1 T cells were stimulated with OVA-loaded splenocytes and then transduced with Cre expressing or control retrovirus after 48 hours. Subsequently, CTLs were stained with antibodies against centrin and the number of centrin^{+} puncta in each cell quantified at the indicated time points, which denote days after initial antigen stimulation. For each graph, N ≥ 39 *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ cells and N ≥ 41 *Sas4*$^{-/-}$*Trp53*$^{-/-}$ cells. (C) Representative confocal images of *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs stained with antibodies against the indicated centrosomal proteins. Scale bars = 3 μm. (D) Schematic diagram of the radial image analysis protocol. Multiple linescans (yellow lines on image to the left) were generated by rotating an initial horizontal line by 1° increments over 180°. These linescans were then averaged to yield an intensity profile for the entire cell (below), which was then averaged with other cell profiles to generate the curves shown in Figure 1C and Figure S1E. (E) Above, representative confocal images of *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs stained with antibodies against GM130. Top view (z-projection) images are shown above with corresponding side views (y-projections) below. Dotted white lines indicate the plane of the IS in the side views. The axis of rotation used to generate the side view is indicated in yellow in the top views. Scale bars = 2 μm. Below, normalized GM130 fluorescence intensity within radial domains between the center and the edge of the cell (N ≥ 12 for each cell type). Error bars denote standard error of the mean (SEM).

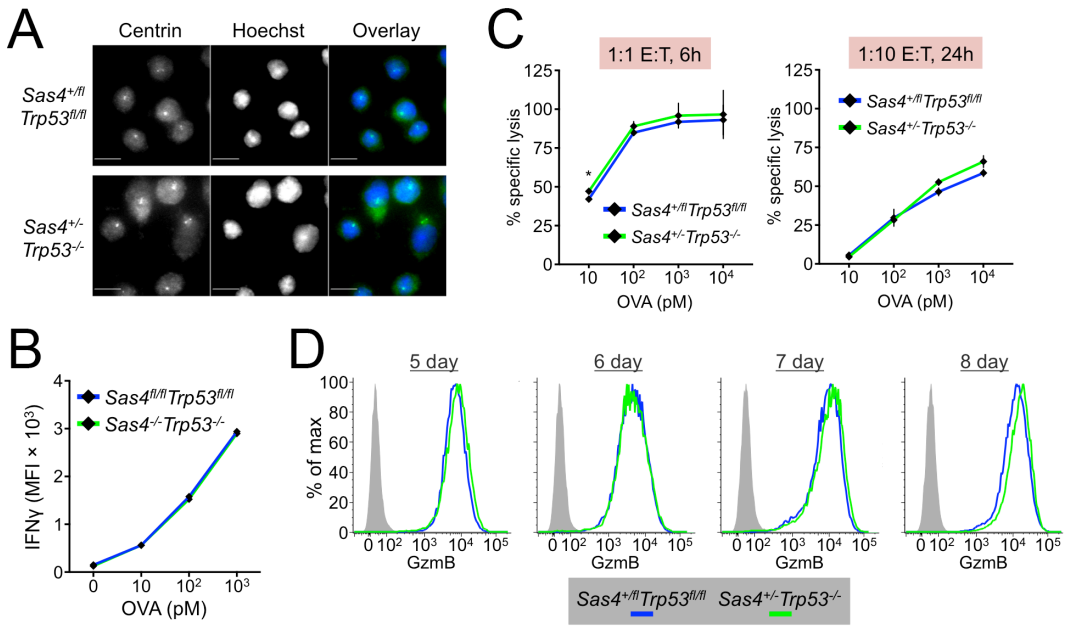

Figure S2. Architectural and functional analysis of *Sas4*$^{+/-}$*Trp53*$^{-/-}$ CTLs. (A) Representative images of *Sas4*$^{+/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{+/-}$*Trp53*$^{-/-}$ OT1 CTLs stained with antibodies against centrin. Scale bars = 10 μm. (B) *Sas4*$^{+/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were mixed with RMA-s cells pulsed with the indicated concentrations of OVA, and antigen-induced IFNγ production assessed by intracellular staining. (C) RMA-s target cells were loaded with increasing concentrations of OVA and mixed with *Sas4*$^{+/fl}$*Trp53*$^{fl/fl}$ or *Sas4*$^{+/-}$*Trp53*$^{-/-}$ OT1 CTLs. Specific lysis of RMA-s cells was assessed

at the indicated E:T ratios and times. (D) *Sas4*$^{+/fl}$*Trp53*$^{fl/fl}$ naïve OT1 T cells were stimulated with OVA-loaded splenocytes and then transduced with Cre expressing or control retrovirus after 48 hours. Subsequently, GzmB expression levels were assessed by flow cytometry in the developing CTLs at the indicated time points, which denote the number of days after initial antigen stimulation.

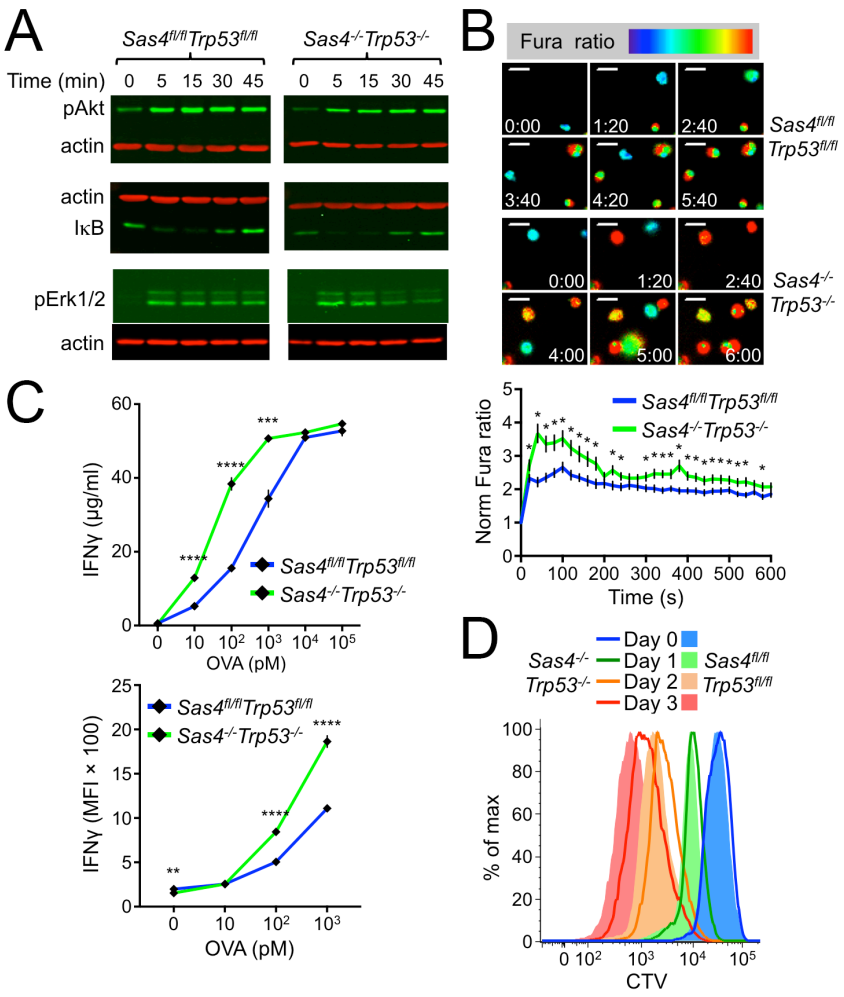

Figure S3. *Sas4*$^{-/-}$*Trp53*$^{-/-}$ CTLs respond to TCR stimulation. (A) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were mixed with beads coated with H2-K^{b}-OVA and ICAM1. At the indicated times, pAKT, IκB, and pErk1/2 were assessed by immunoblot, using actin as a loading control. (B) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were loaded with Fura2-AM and imaged on glass surfaces coated with H2-K^{b}-OVA and ICAM1. Above, representative time-lapse montages showing Fura ratio in pseudocolor, with warmer colors indicating higher intracellular Ca^{2+} concentrations. Time in M:SS is shown in each image. Scale bars = 10 μm. Below, mean normalized Fura ratio was graphed against time. N ≥ 29 for each cell type. P values (* indicates $P < 0.05$) were calculated by two-tailed Student's T-test. (C) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were mixed with RMA-s cells pulsed with the indicated concentrations of OVA. Antigen-induced IFNγ production was assessed by ELISA (above) and intracellular staining (below). P values (**, ***, and **** indicate $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively) were calculated by two-tailed Student's T-test. (D) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1

CTLs were incubated with OVA-loaded C57BL/6 splenocytes, and proliferation assessed by CTV dilution at the indicated time points. In B and C, error bars denote SEM.

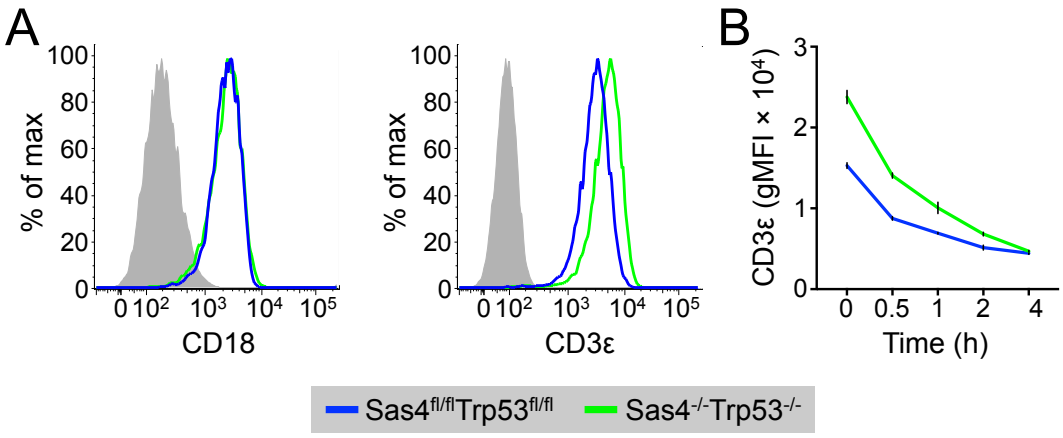

Figure S4. TCR and LFA1 expression of *Sas4*$^{-/-}$*Trp53*$^{-/-}$ CTLs. (A) Flow cytometric analysis of CD18 (LFA1 β chain) and CD3ε on *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs. (B) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were mixed with OVA-loaded RMA-s target cells and surface expression of CD3ε assessed by flow cytometry at the indicated times. Error bars denote SEM.

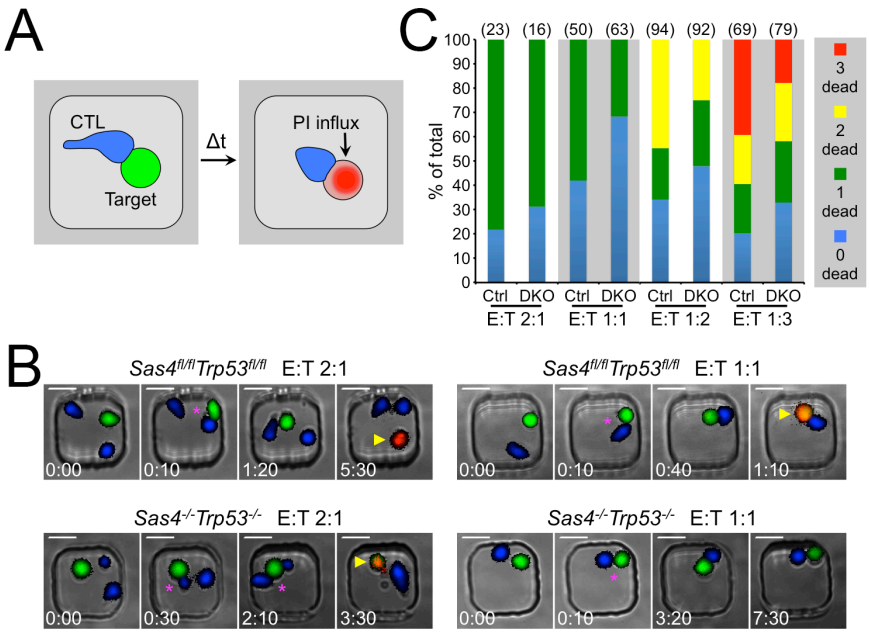

Figure S5. Imaging analysis of *Sas4*$^{-/-}$*Trp53*$^{-/-}$ CTL killing. *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were mixed with OVA-loaded RMA-s target cells and loaded into 50 μm × 50 μm microwells to facilitate imaging of many CTL-target cell conjugates for 10-12 hours. (A) Schematic diagram showing detection of target cell death by PI influx. (B) Representative time-lapse montages of microwells containing one CFSE labeled target cell (green) and either one (right, E:T 1:1) or two (left, E:T 2:1) CTV-labeled CTLs (blue) of the indicated genotype. Magenta asterisks denote conjugate formation and yellow

arrowheads the influx of PI. Time in H:MM is shown at the bottom left corner of each image. Scale bars = 10 μm. (C) Cumulative bar graph showing the distribution of outcomes in microwells containing the indicated E:T ratios. For each condition, the total number of wells analyzed is shown in parentheses above the bar. Ctrl = *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$, DKO = *Sas4*$^{-/-}$*Trp53*$^{-/-}$.

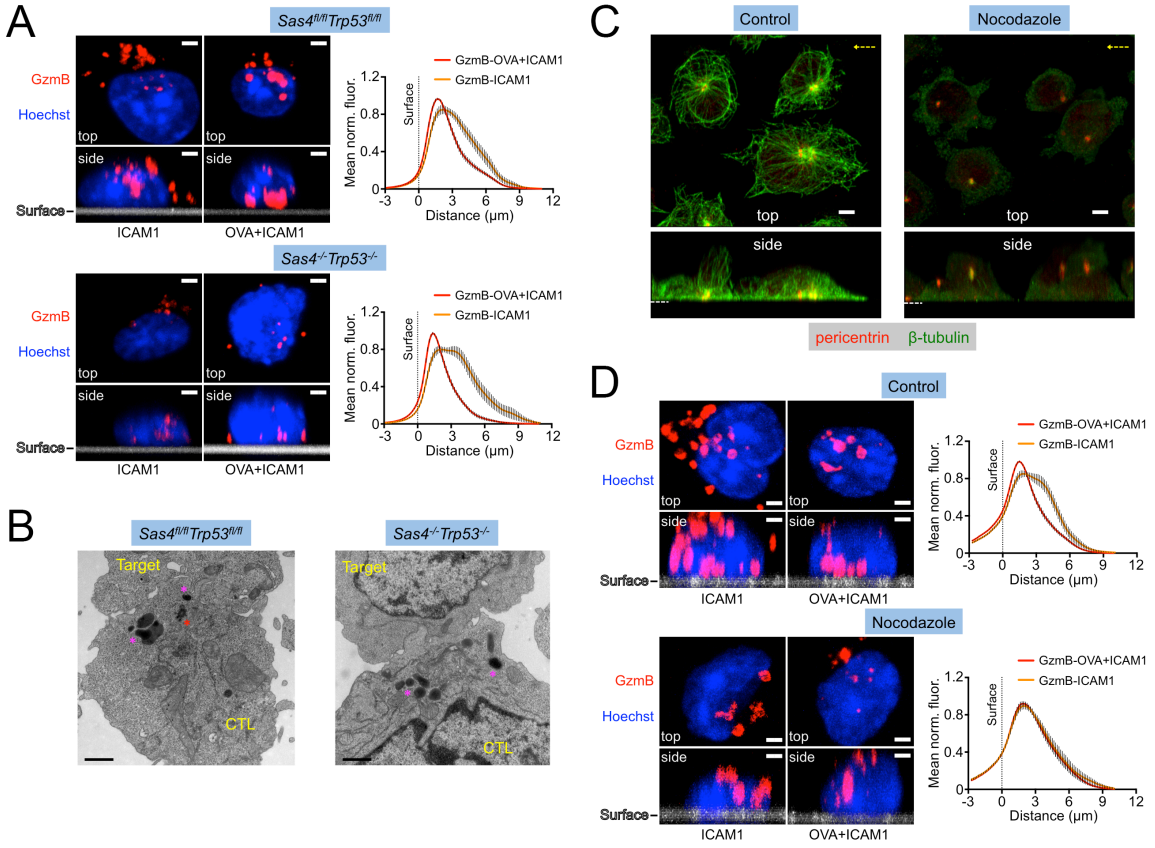

Figure S6. Centriole loss does not disrupt lytic granule polarization. (A) *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs were applied to glass surfaces coated with the indicated molecules (OVA = H2-K^{b}-OVA) and then fixed and stained with antibodies against GzmB. Scale bars = 3 μm. Left, confocal images of representative CTLs shown from the top and from the side, with nuclear Hoechst staining shown in blue. The stimulatory surface is indicated in side view images. Right, the average normalized fluorescence intensity distribution of the GzmB signal under each condition graphed as a function of distance from the stimulatory surface. (B) TEM images of conjugates formed between *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ or *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs and OVA-loaded EL4 target cells. Magenta asterisks indicate lytic granules, and a red asterisk denotes the centriole in the *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ CTL. Scale bars = 1 μm. (C) Representative confocal images of control and 30 μM nocodazole-treated OT1 CTLs stained with antibodies against pericentrin and β-tubulin. Top view (z-projection) images are shown above with corresponding side views (x-projections) below. Dotted white lines indicate the plane of the IS in the side views. The axis of rotation used to generate the side view is indicated in yellow in the top views. Scale bars = 3 μm. (D) OT1 CTLs were pretreated with 30 μM nocodazole or vehicle control (DMSO), applied to glass surfaces coated with the indicated molecules (OVA = H2-K^{b}-OVA), and then fixed and stained with antibodies against GzmB. Left,

confocal images of representative CTLs shown from the top and from the side, with nuclear Hoechst staining shown in blue. The stimulatory surface is indicated in side view images. Scale bars = 2 μm. Right, the average normalized fluorescence intensity distribution of the GzmB signal under each condition graphed as a function of distance from the stimulatory surface. All error bars denote SEM.

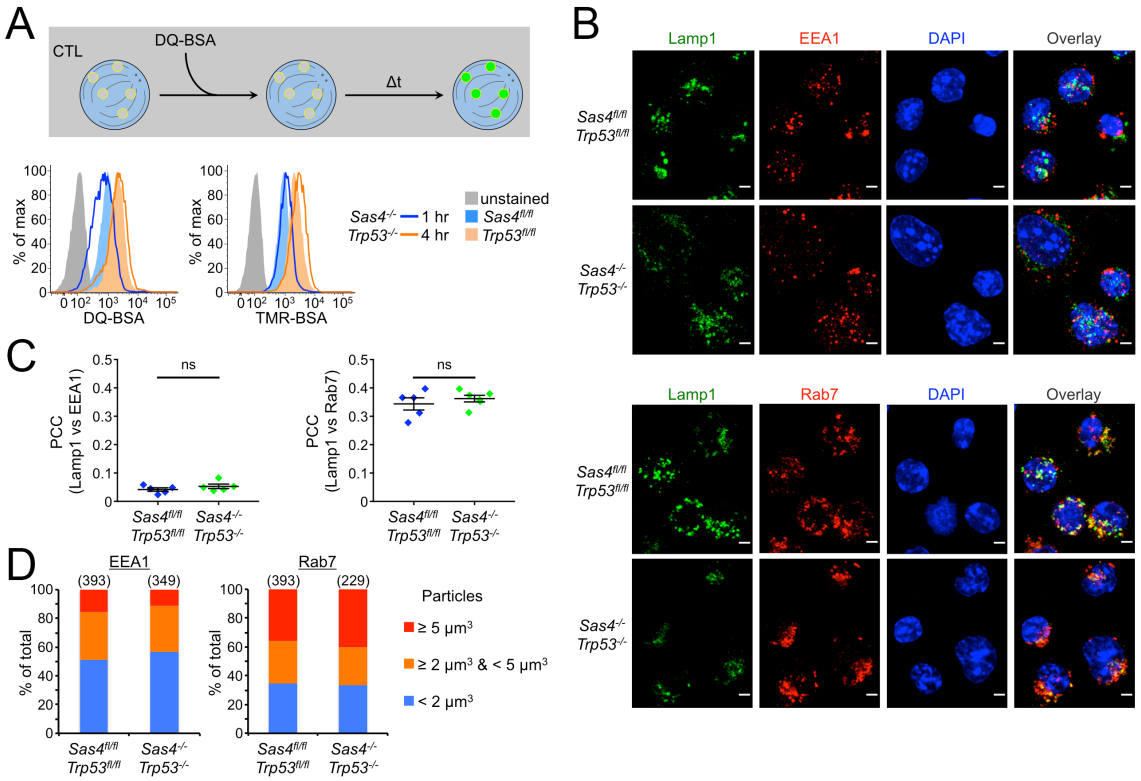

Figure S7. Endocytic and degradative capacity of *Sas4*$^{-/-}$*Trp53*$^{-/-}$ CTLs. (A) Above, schematic diagram of DQ-BSA uptake assay. Lysosomal degradation dequenches probe fluorescence. Below, flow cytometric analysis of DQ-BSA (left) and TMR-BSA (right) fluorescence at the indicated time points. (B) Representative confocal images of *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs stained with antibodies against the EEA1 and Lamp1 (above) and Rab7 and Lamp1 (below). Nuclear DAPI staining is shown in blue. Scale bars = 2 μm. (C) PCC between Lamp1 and EEA1 fluorescence (left) and between Lamp1 and Rab7 fluorescence (right) was determined for each image of *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs (N = 5). Each image contained 10-20 CTLs. P value was calculated by two-tailed Student's T-test. (D) Graph showing the distribution of EEA1^{+} (left) and Rab7^{+} (right) particle size (see Materials and methods) in *Sas4*$^{fl/fl}$*Trp53*$^{fl/fl}$ and *Sas4*$^{-/-}$*Trp53*$^{-/-}$ OT1 CTLs. The number of particles analyzed is indicated in parentheses above each bar.

SI References

1. Rak GD, Mace EM, Banerjee PP, Svitkina T, & Orange JS (2011) Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* 9(9):e1001151.
2. Le Floc'h A*, et al.* (2013) Annular PIP3 accumulation controls actin architecture and modulates cytotoxicity at the immunological synapse. *J Exp Med* 210(12):2721-2737.
3. Purbhoo MA, Irvine DJ, Huppa JB, & Davis MM (2004) T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol* 5(5):524-530.
4. Jenkins MR*, et al.* (2014) Distinct structural and catalytic roles for Zap70 in formation of the immunological synapse in CTL. *Elife* 3:e01310.
5. Tsun A*, et al.* (2011) Centrosome docking at the immunological synapse is controlled by Lck signaling. *J Cell Biol* 192(4):663-674.