Journal Club

Mitochondrial translation is required for sustained killing by cytotoxic T cells

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Professor Gillian M. Griffiths

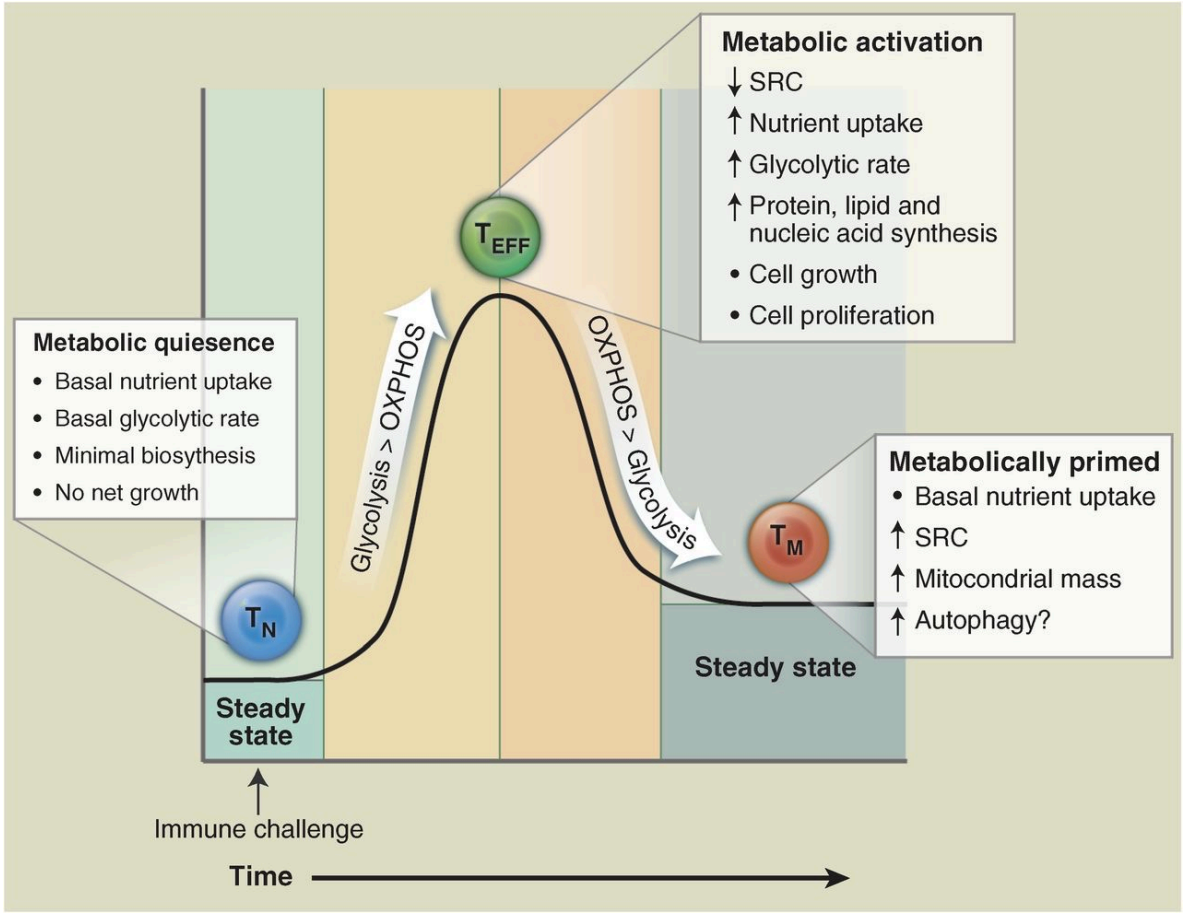
- Gillian Griffiths, is a British cell biologist and immunologist.
- Griffiths was one of the first to show that immune cells have specialised mechanisms of secretion, and identified proteins and mechanisms that control cytotoxic T lymphocyte secretion.
- Griffiths is Professor of Cell Biology and Immunology at the University of Cambridge and is the Director of the Cambridge Institute for Medical Research.

Cytotoxic T lymphocytes (CTLs)

Cytotoxic T cell’s serial killing

- $CD8^+$ cytotoxic T lymphocytes (CTLs) are key players of the adaptive immune response. Activation of T cell receptors (TCRs) on naïve $CD8^+$ T cells converts them into effector CTLs able to kill tumorigenic and virally infected cells.
- CTLs initiate cell death by secreting cytotoxic proteins, including perforin and granzymes, which selectively trigger caspase activation and subsequent apoptosis.
- One feature that makes CTLs particularly effective killers is their ability to carry out sustained, serial killing, with a single CTL attacking multiple targets, one after another.

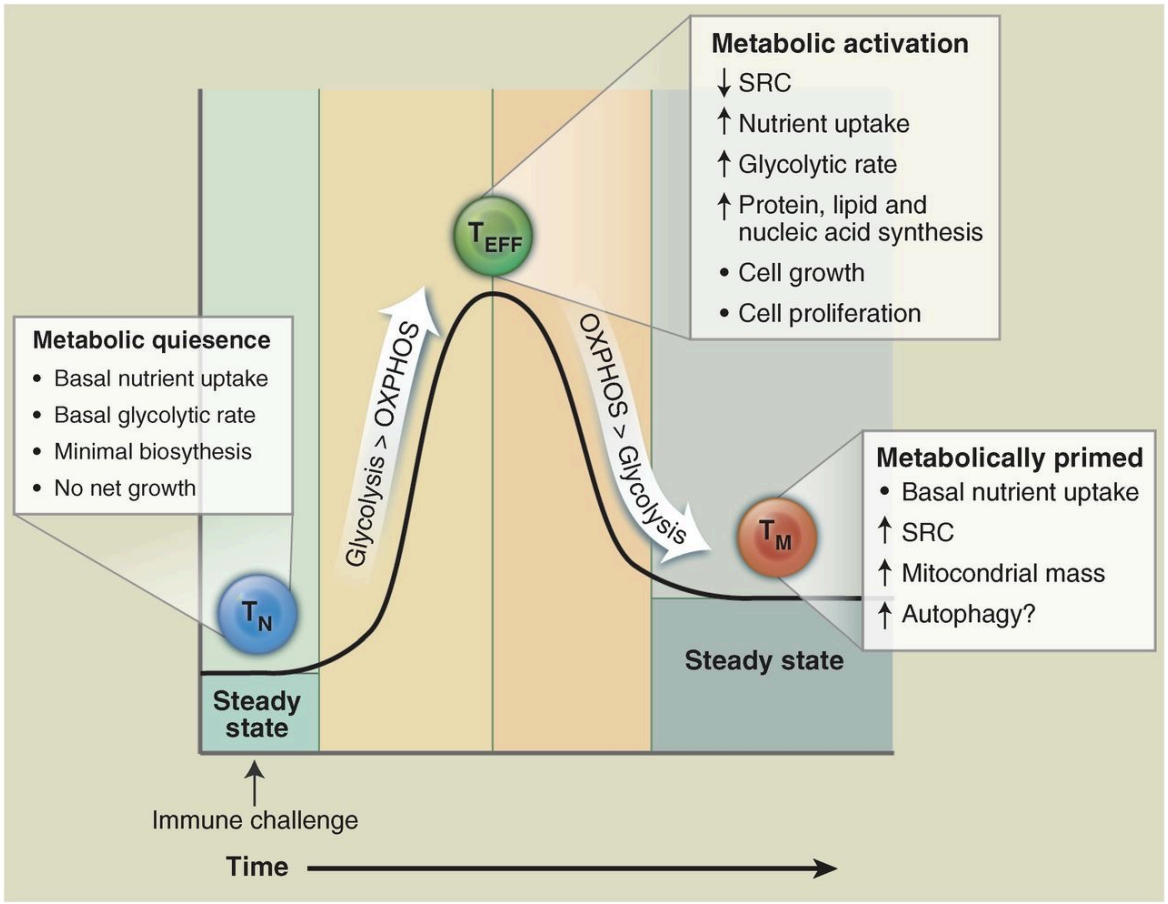
Mitochondria play crucial roles in the adaptive immune system, mediating the development, metabolism, and activation of T cells



- Mitochondria play crucial roles in the adaptive immune system, mediating the development, metabolism, and activation of T cells.
- There is an increased reliance on glycolysis (decreased OXPHOS) in activated effector T cells.

T_N, naïve T cell
T_{EFF}, Effective T cell

Mitochondria may play a previously unappreciated role in CTL biology

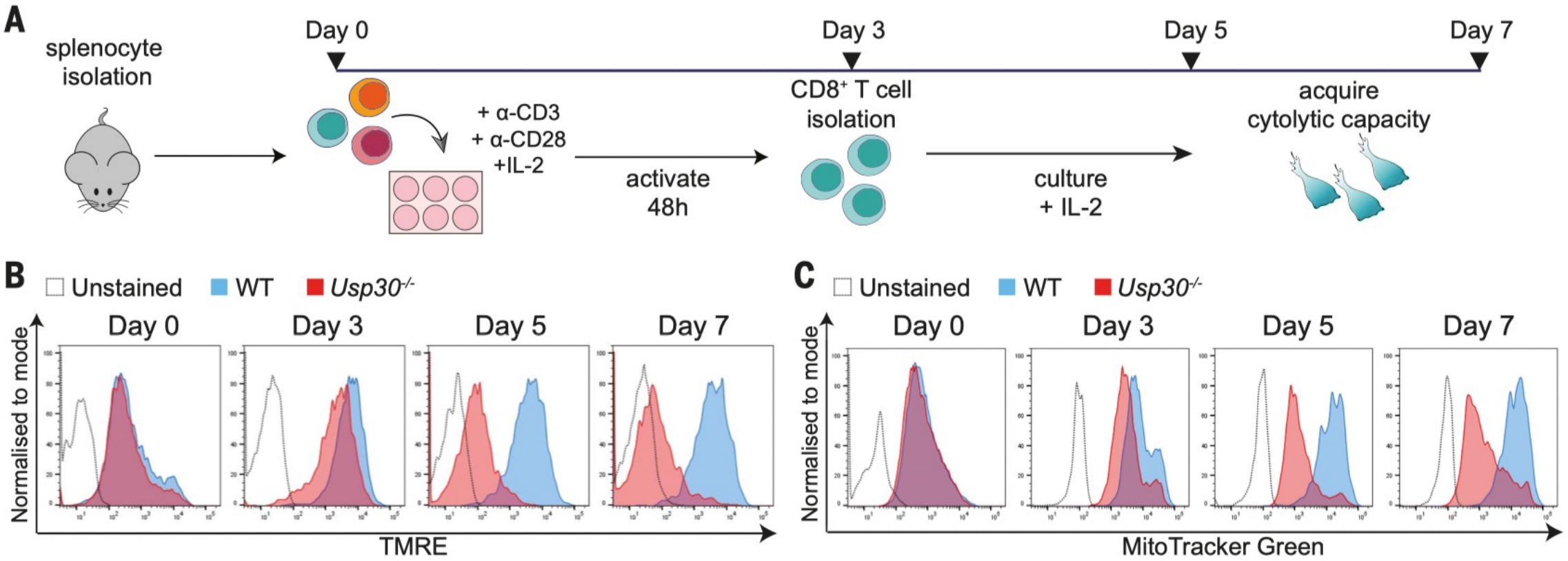


- The loss of USP30-mediated deubiquitination results in increased mitophagy, reducing cellular mitochondrial content.
- A large-scale screen of single-gene deletion mice for immunological function identified USP30 as a regulator of CTL killing. T cell development was normal in Usp30$^{-/-}$ (KO) mice, but CTLs generated after activation of naïve CD8^{+} T cells showed reduced killing of target cells.
- The role of USP30, and by inference mitochondria, in CTL function was intriguing because paradoxically there is an decreased reliance on OXPHOS in activated effector T cells.
- These observations raised the possibility that mitochondria play a previously unappreciated role in CTL biology and pointed to USP30 as a starting point for investigating this connection.

T_N, naïve T cell
T_{EFF}, Effective T cell

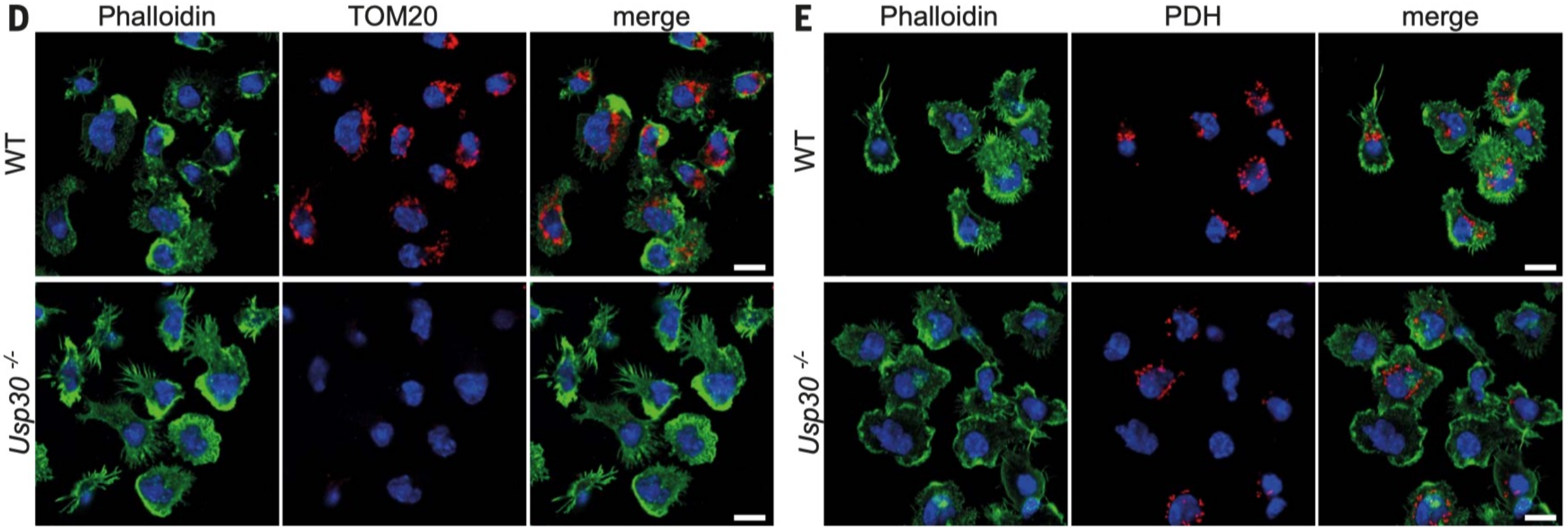
1. Usp30 deletion leads to mitochondrial depletion in effector $CD8^+$ T cells.
2. Mitochondrial depletion leads to oxidative phosphorylation-independent inhibition of killing.
3. Mitochondrial depletion leads to translation attenuation and reduced expression of key cytolytic proteins.
4. Mitochondrial translation is required for sustained T cell killing.

Isolation and stimulation of mouse CD8⁺ T cells



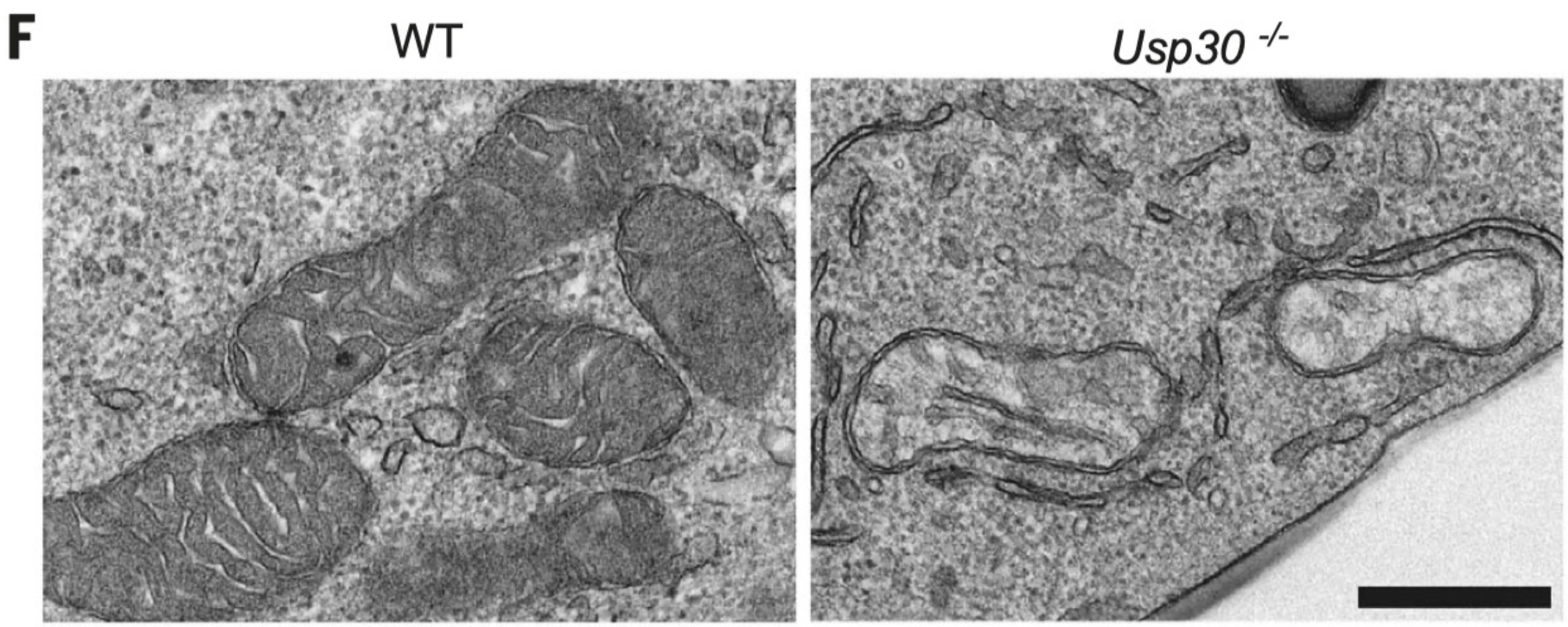
TMRE, labelling mitochondrial membrane potential
MitoTracker Green, labelling mitochondrial mass

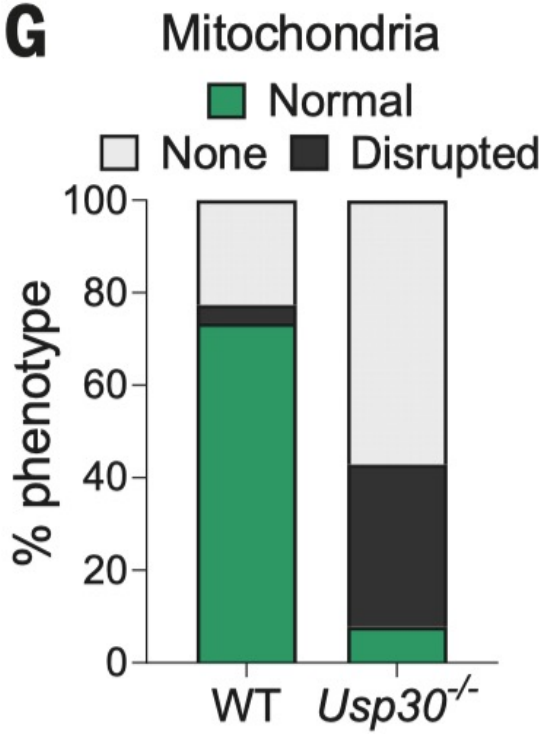
KO CTLs showed reduced levels of both the mitochondrial outer-membrane protein TOM20 as well as the mitochondrial matrix PDH



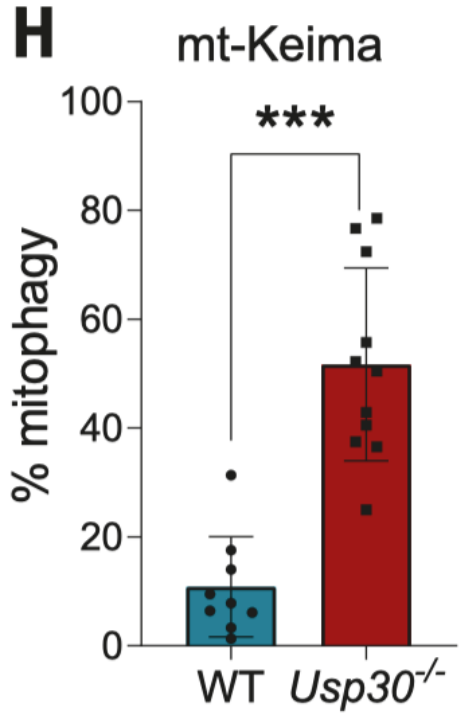
Phalloidin (actin filaments)
TOM20 (outer mitochondrial membrane)
PDH (mitochondrial matrix)

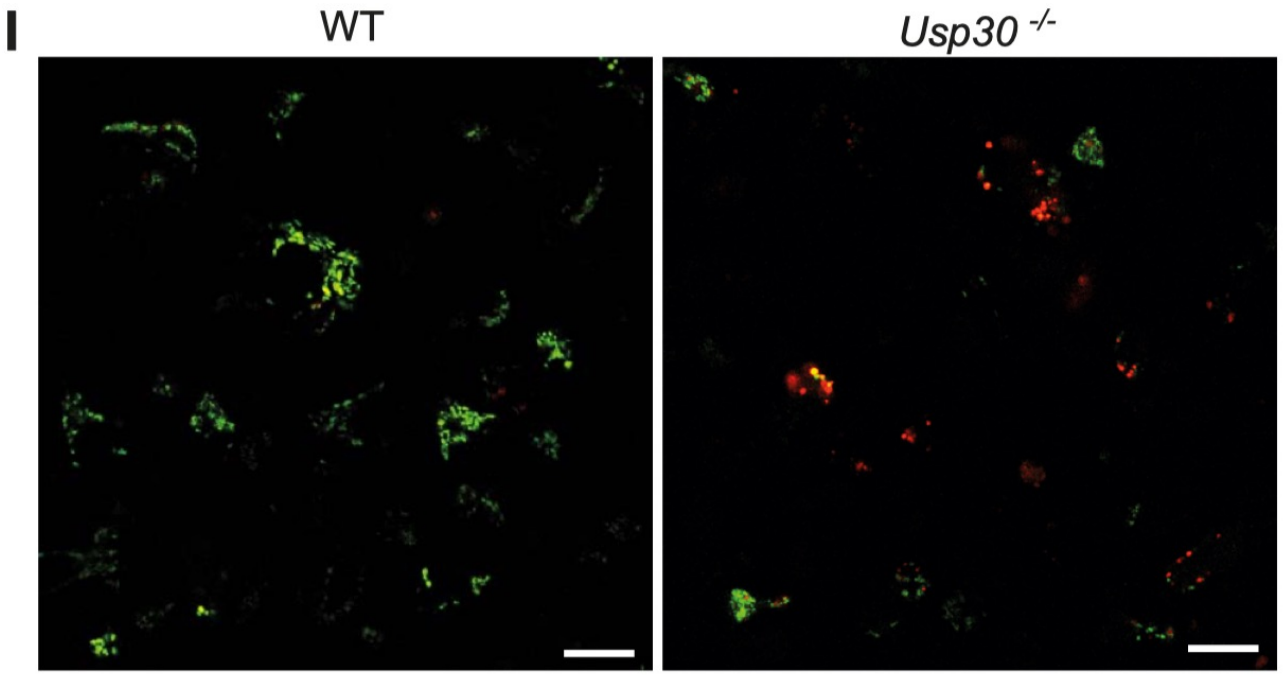
Using transmission electron microscopy (TEM), CTLs showed disrupted mitochondrial morphology





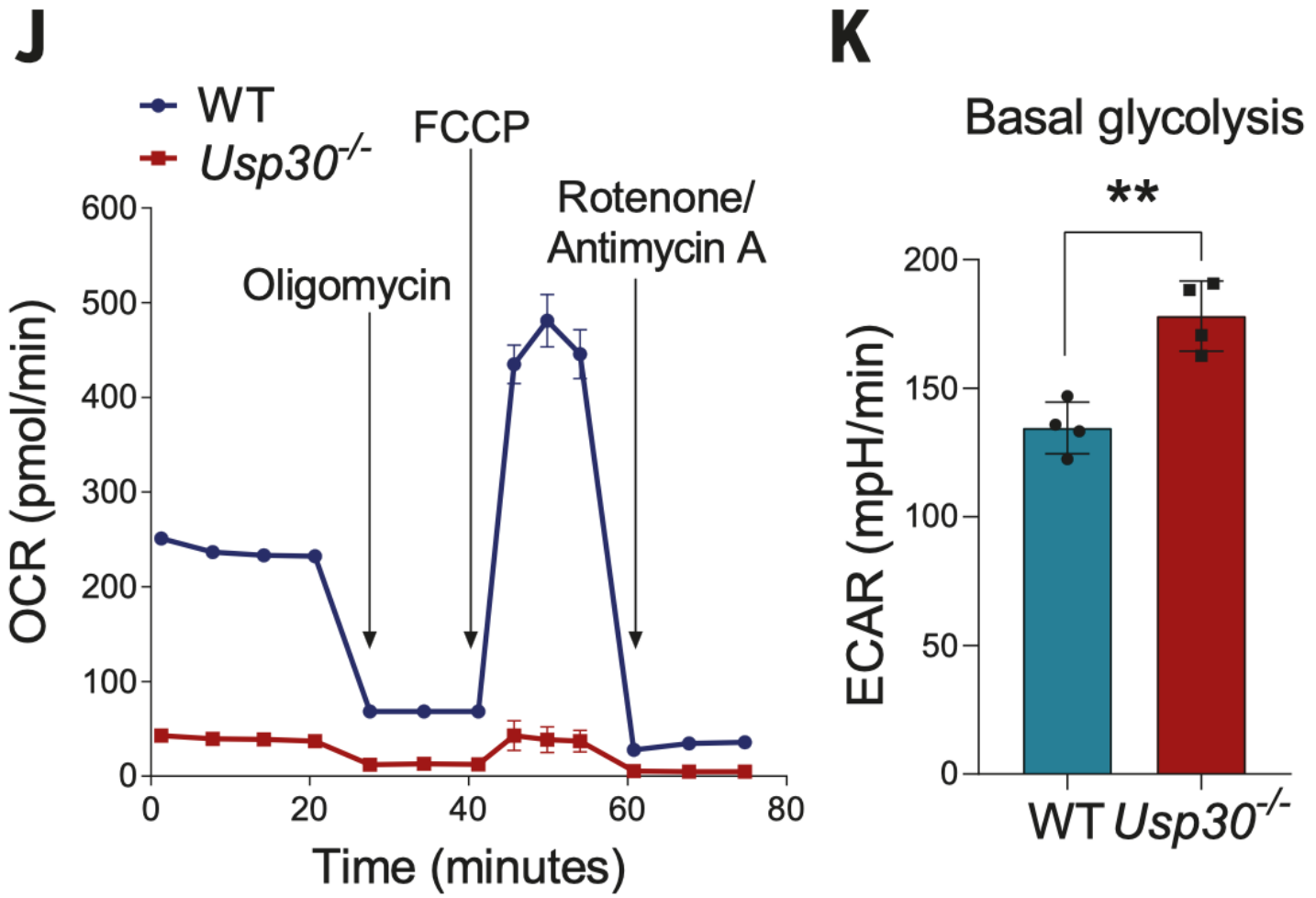
An increased mitophagic signal (red) in KO CTLs





mt-Keima, mitophagy reporter (red)

The decrease in oxygen consumption rate (OCR) was accompanied by an increase in extracellular acidification rate (ECAR), suggesting an even greater reliance on glycolysis in KO compared with that of WT CTLs



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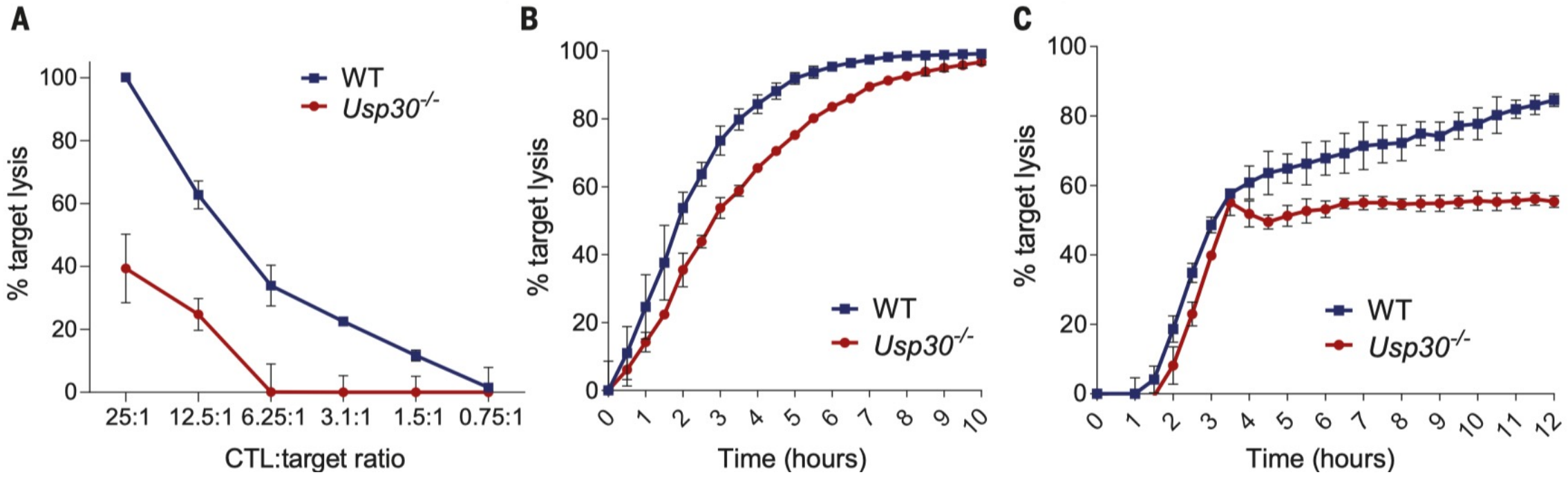
Day 5 (activated) KO CTLs exhibited a reduced killing capacity, particularly when required to kill for extended periods or when the number of CTLs was limiting



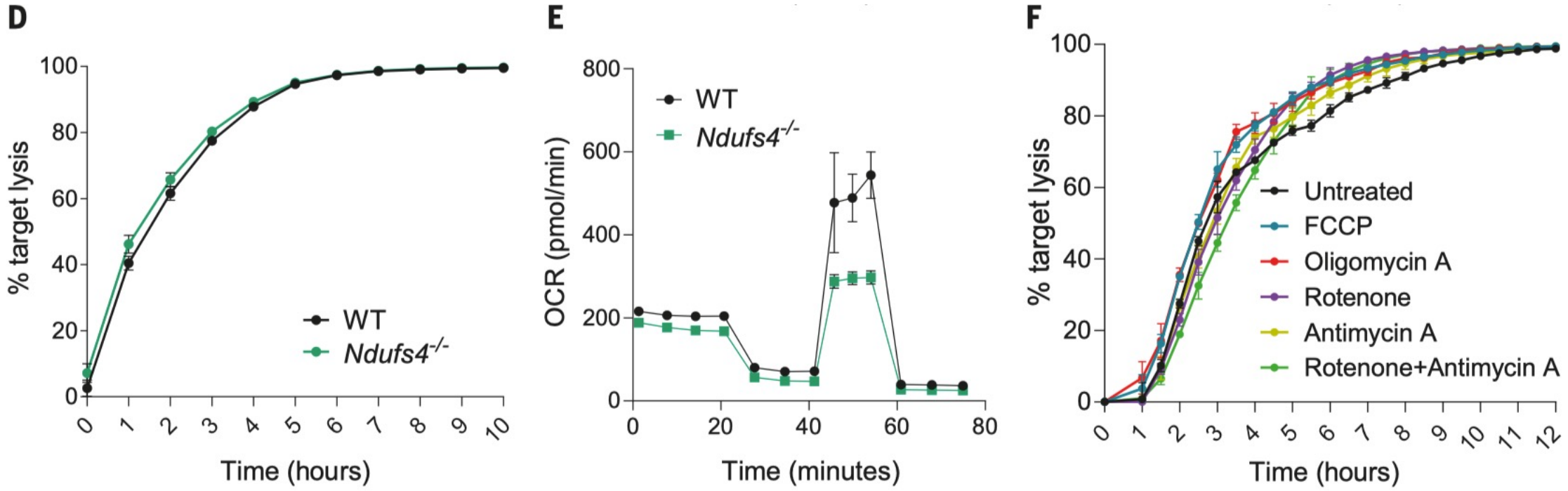
CTL-to-target ratio, 10:1

CTL-to-target ratio, 1:1

In a short-term (2.5 hours) killing assay, KO CTLs failed to kill targets until the ratio of CTL to targets was >7 and only killed 40% of target cells when CTLs outnumbered targets by 25:1.

A longer-term assay with 10 CTLs per target showed that KO CTLs took almost twice as long as WT CTLs to kill all targets

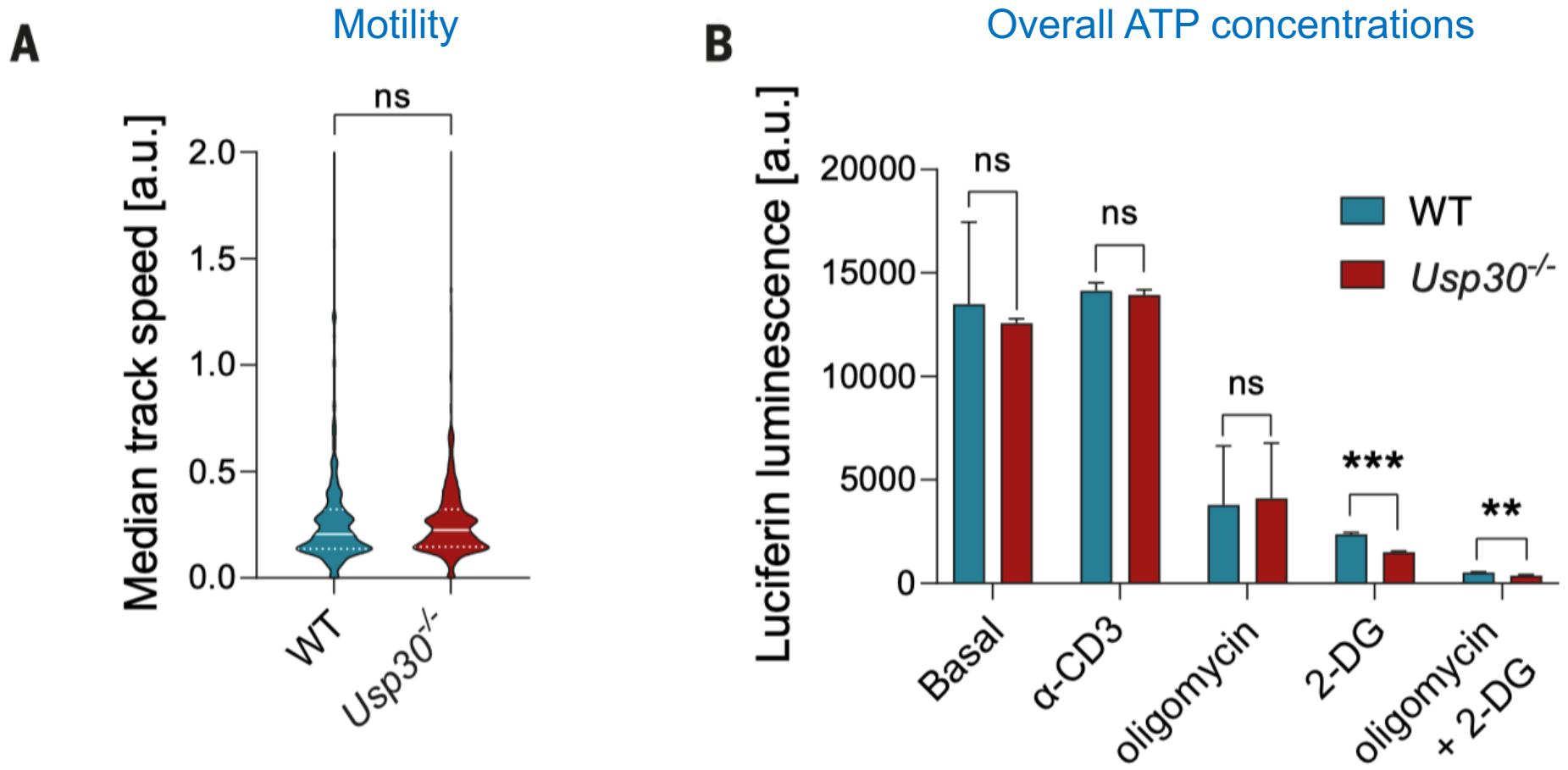
Mitochondrial oxidative phosphorylation don’t contribute to CTL killing



$Ndufs4^{-/-}$, Complex I was disrupted
FCCP, interfering with the proton gradient
Oligomycin A, an inhibitor of Complex V
Rotenone, an inhibitor of Complex I
Antimycin A, an inhibitor of Complex III

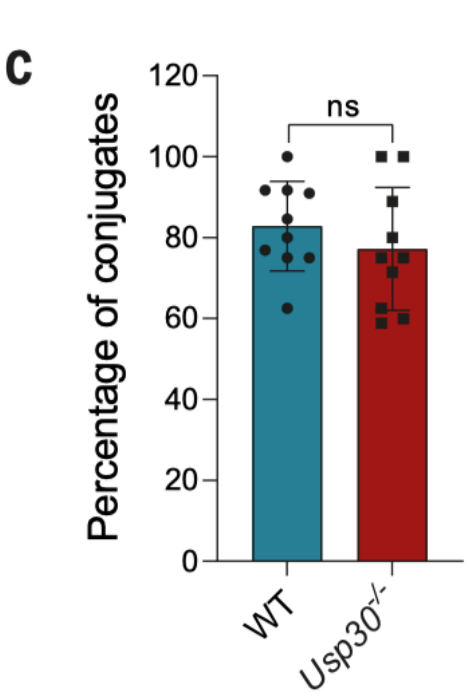
Because mitochondrial adenosine 5′-triphosphate (ATP) has been shown to play a role in lymphocyte migration, they asked whether motility or ATP levels were affected in day 5 KO CTLs.

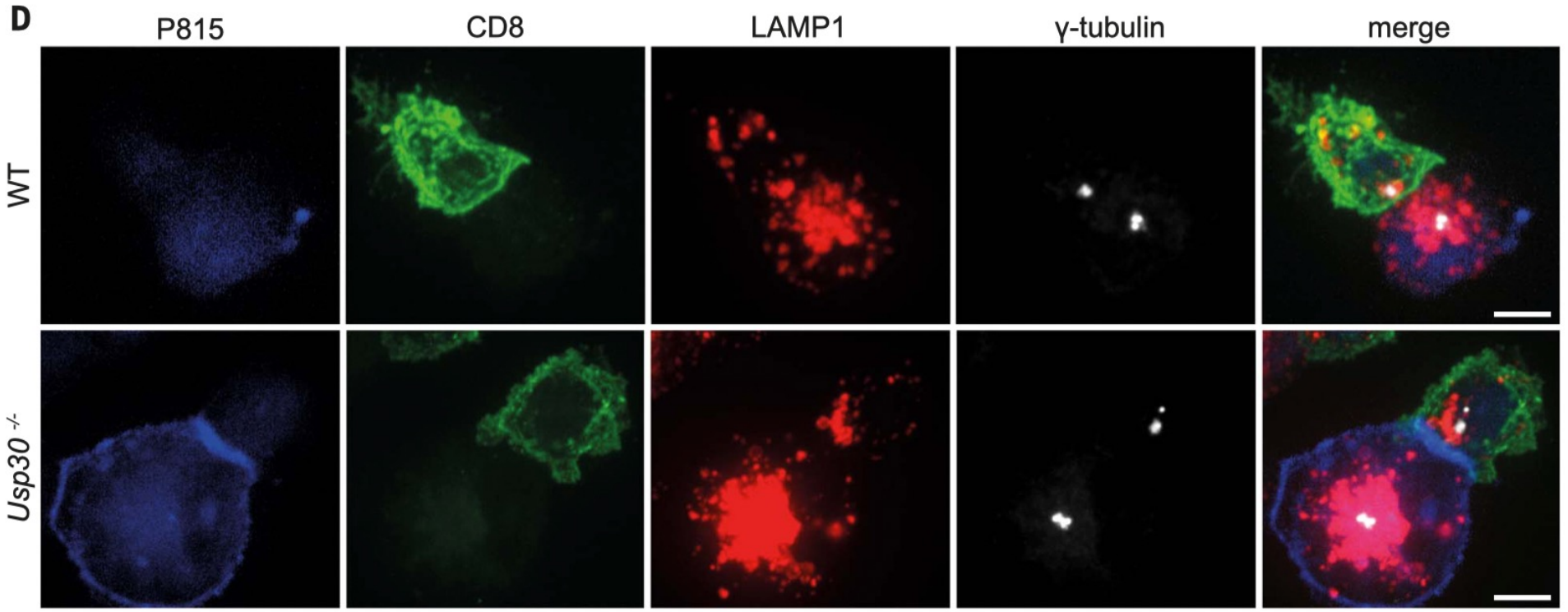
Loss of USP30 does not impair motility or overall ATP concentrations



- anti-CD3, mimicking target encounter by cross-linking TCR
- Oligomycin, inhibitor of oxidative phosphorylation
- 2-DG, inhibitor of glycolysis

No defect in conjugate formation between day 5 KO CTLs and target cells or in polarization of the centrosome or cytolytic granules to the synapse formed between CTLs and targets

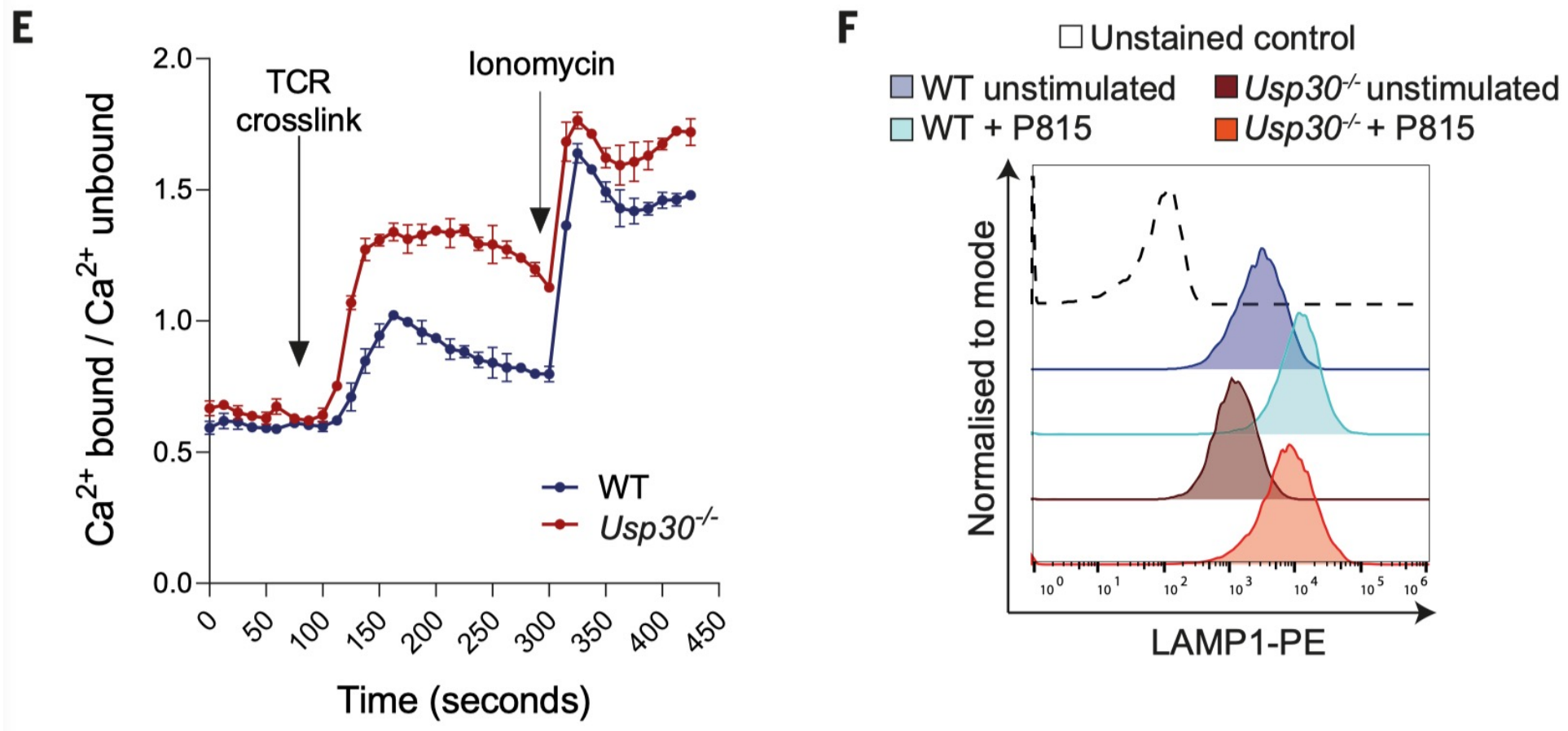




Quantitation of conjugate formation

Conjugates formed by day 5 WT or KO CTLs (green) with P815 targets (blue) showing polarization of cytolytic granules (red) and centrosome (white)

Day 5 KO CTLs showed no defects in either TCR-stimulated calcium fluxes nor the release of granules in response to TCR activation



Changes of intracellular calcium concentration

Signal of granules release

- TCR crosslink, activating CTL
- Ionomycin, raising the intracellular calcium level
- P815, CTL target cell

- Mitochondrial depletion leads to oxidative phosphorylation-independent inhibition of killing.
- Loss of USP30 does not impair CTL ATP production, migration, target recognition or secretion.
- Given that all steps to secretion appeared to be intact, they asked whether the secretory granules that store the cytolytic proteins required for killing were affected in day 5 KO CTLs.

1. Usp30 deletion leads to mitochondrial depletion in effector $CD8^+$ T cells.
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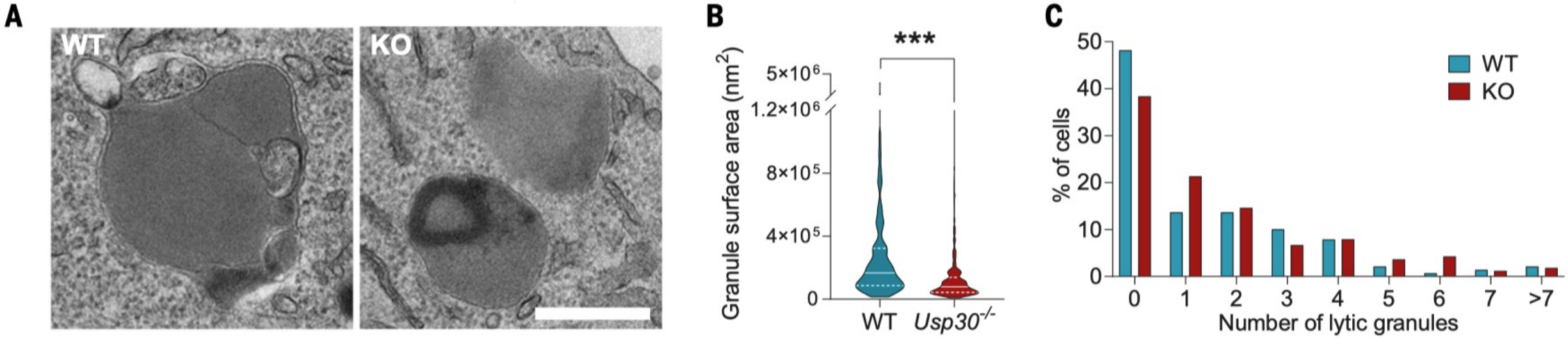
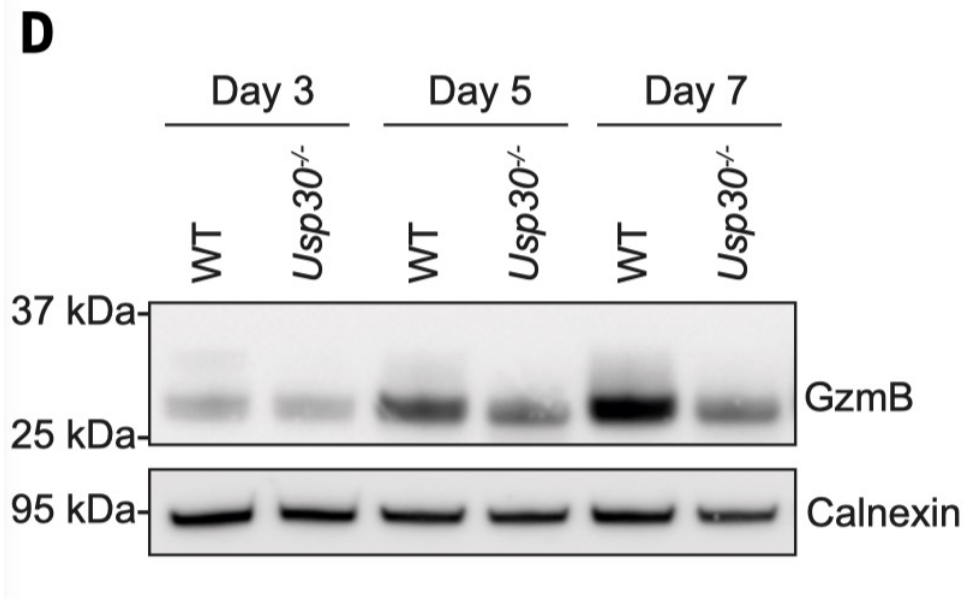
Although similar in number, the granules were smaller (quantitated by reduced surface area) in KO CTLs



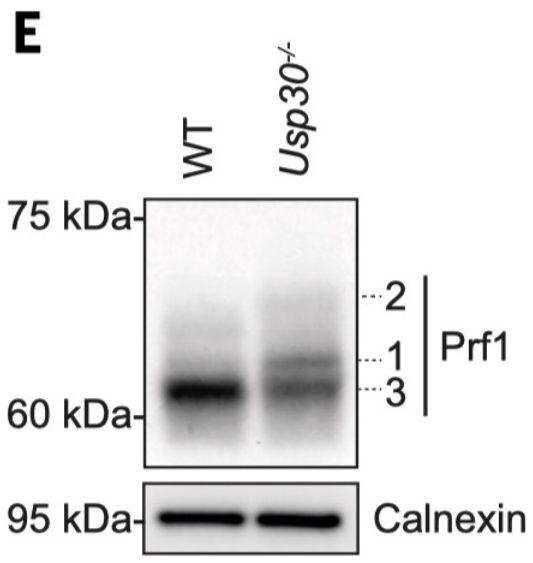
Scale bar, 500 nm

Lytic granules

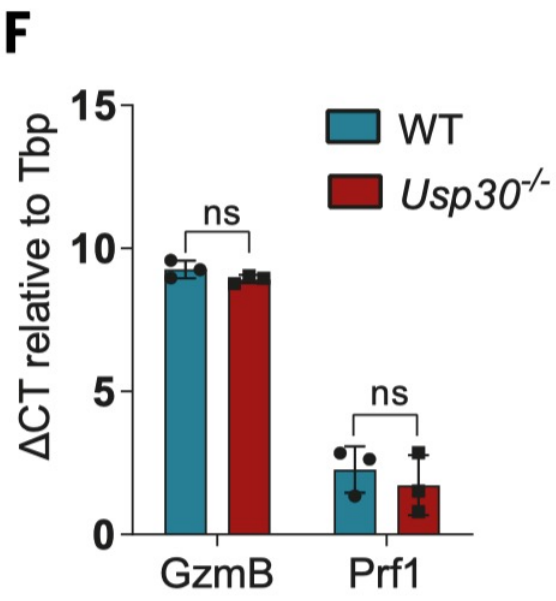
Two of the key cytolytic proteins in KO CTLs was reduced relative to that in WT CTLs after activation



Two of the key cytolytic proteins stored in granules are granzyme B (GzmB) and the pore-forming protein perforin (Prf1)

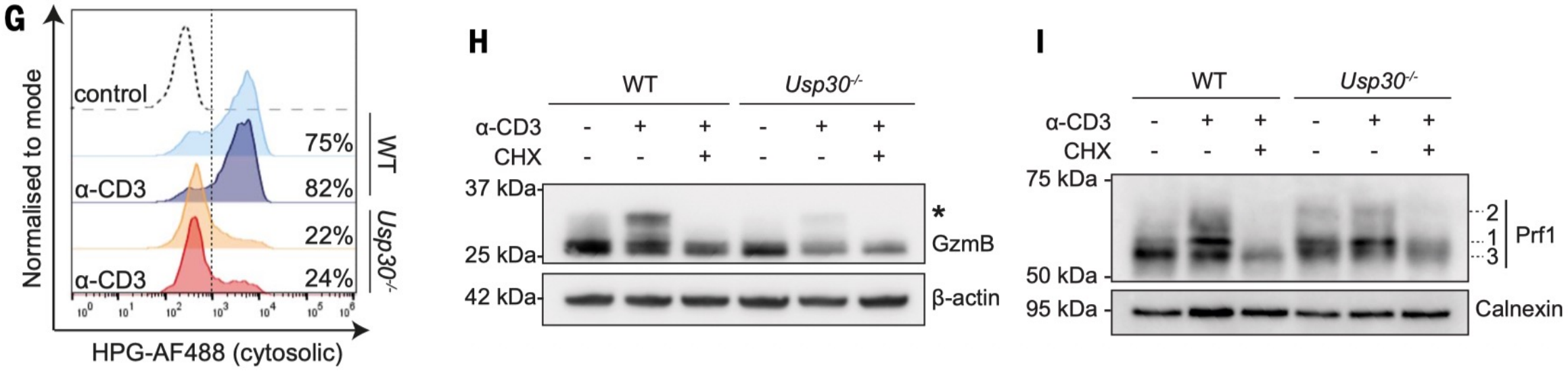


Prf1 forms:
Immature (1)
intermediate (2)
mature (3)



Unchanged mRNA

New synthesis was much reduced in the KO, suggesting that a defect in mRNA translation of cytolytic proteins might cause the loss of killing observed in KO CTLs

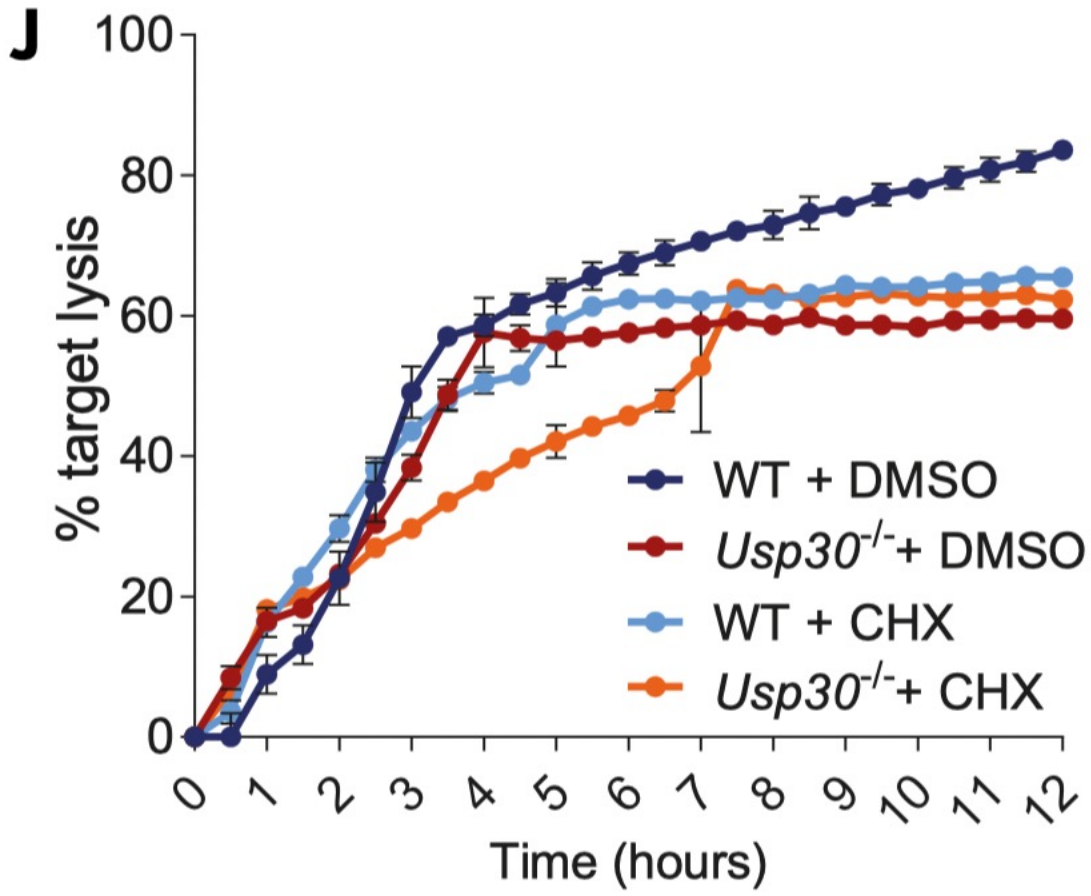


- HPG, methionine analog (monitoring protein synthesis)
- anti-CD3, mimicking target encounter by cross-linking TCR

- CHX, cytosolic protein synthesis inhibitor
- *, newly synthesized GzmB

Prf1 forms:
Immature (1)
intermediate (2)
mature (3)

WT CTLs killed for the first 4 hours, after which CHX treatment reduced cytotoxicity, mirroring the pattern of killing by KO CTLs



CHX, cytosolic protein synthesis inhibitor

- Mitochondrial depletion leads to translation attenuation and reduced expression of key cytolytic proteins.
- De novo protein synthesis is required for sustained CTL killing and is impaired in KO CTLs.
- To determine whether the translational defect affected all proteins equally, they used mass spectrometry to compare the proteomes of day 5 WT and KO CTLs before and after TCR activation.

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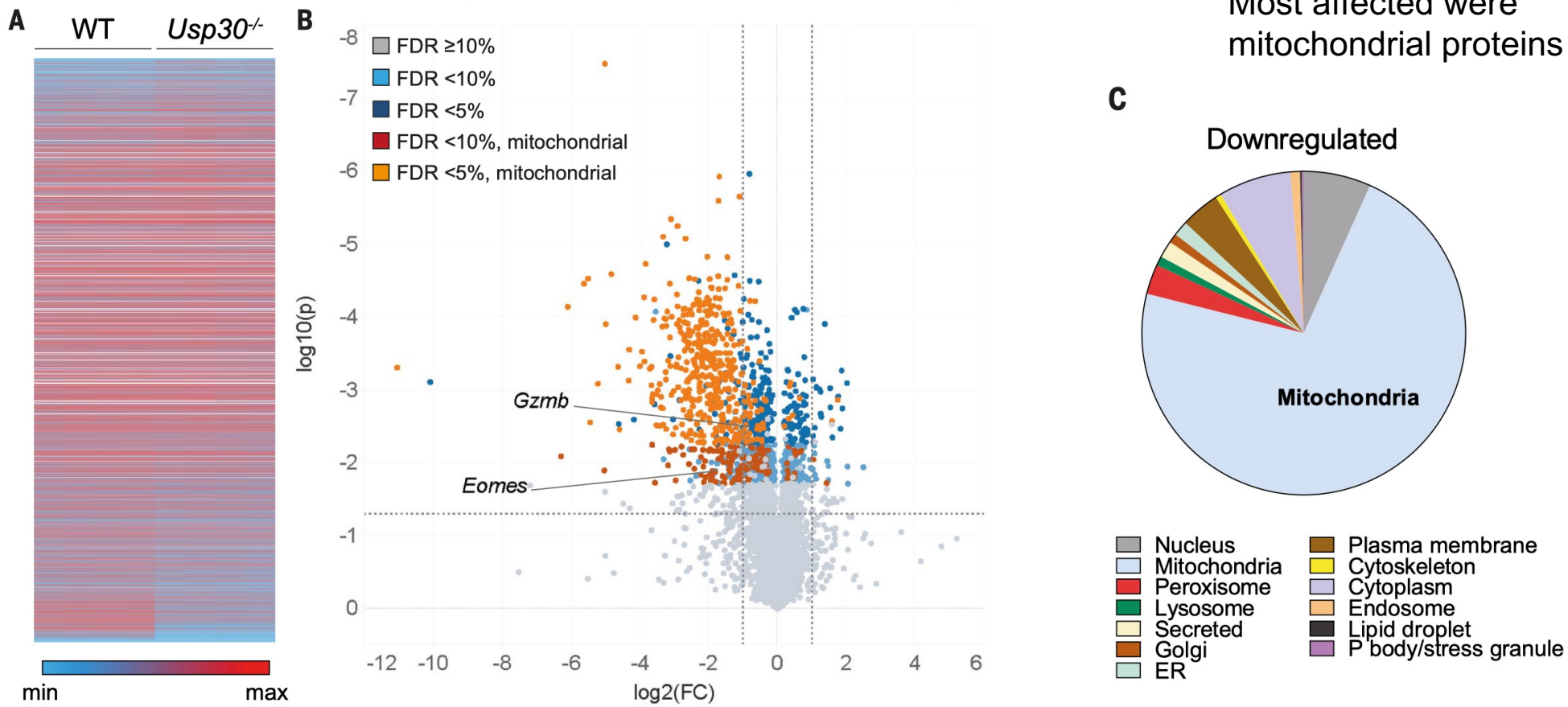
Only a subset of proteins (557) was found to have a significantly lower expression in KO CTLs [fold change > 2, false discovery rate (FDR) < 10%]

Day 5 CTL



Most affected were mitochondrial proteins (72%)

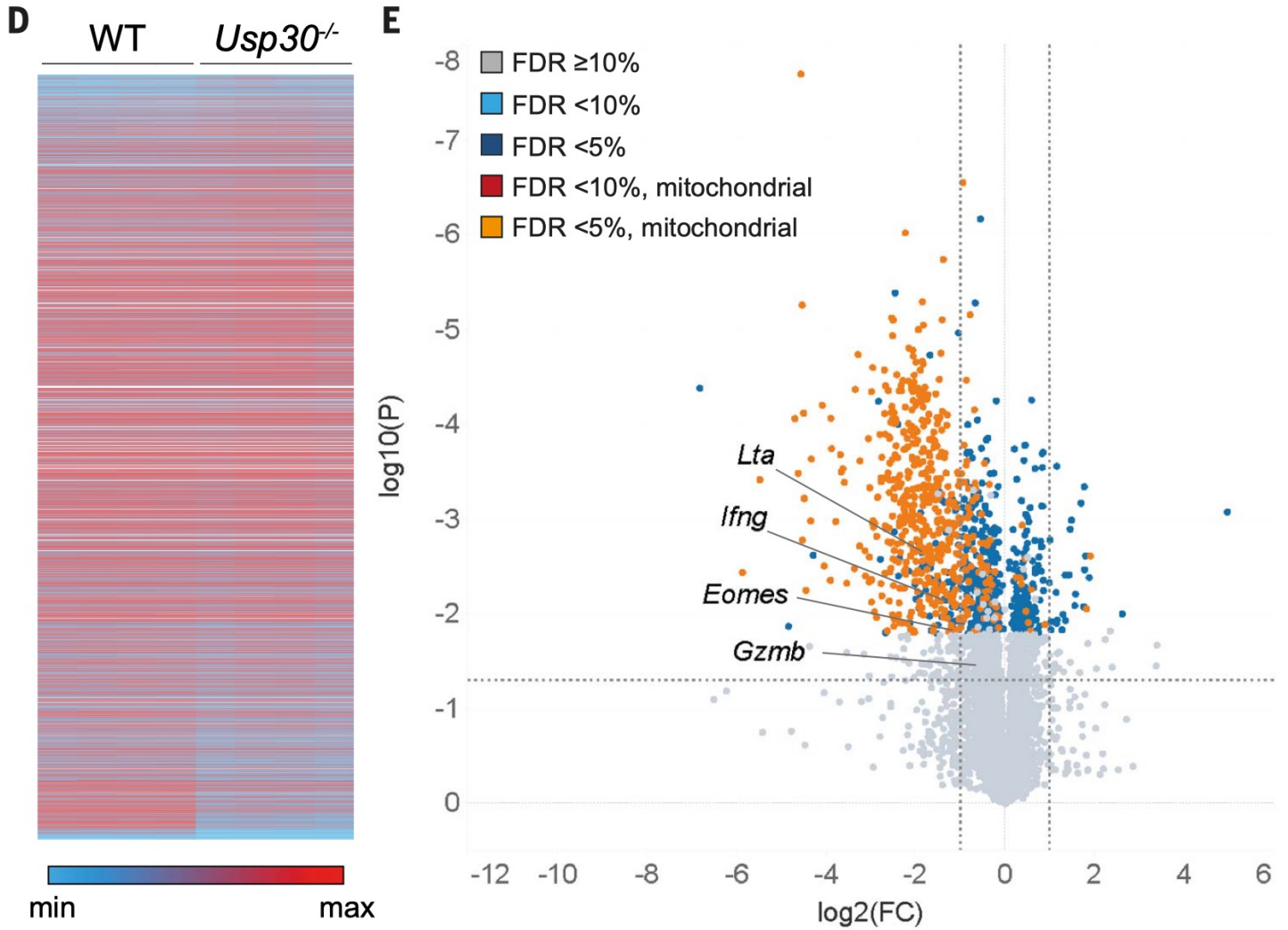
Highly downregulated proteins included GZMB and EOMES, a transcription factor that regulates expression of several cytotoxic proteins.

Cellular localization of down-regulated proteins in day 5 KO CTLs (5% FDR).

Upon TCR activation of CTLs, additional cytolytic proteins were down-regulated in KO CTLs, including IFNG, TNF-a and TNF-b

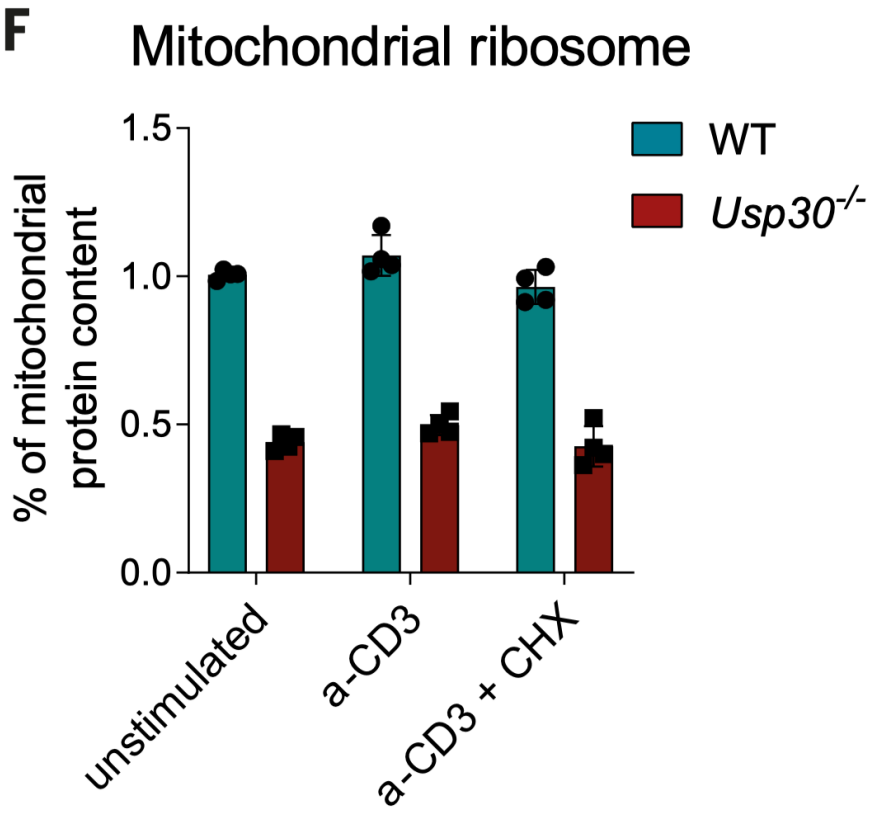
Day 5 CTL + anti-CD3

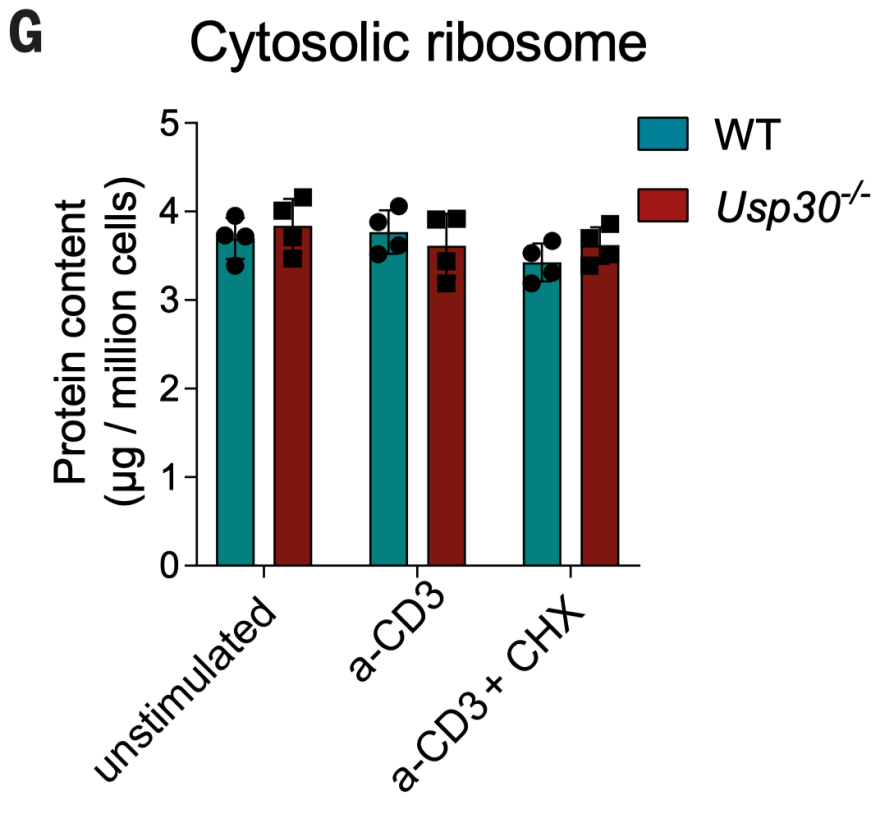
anti-CD3, mimicking target encounter by cross-linking TCR



Mitochondrial ribosomal proteins represented one of the most down-regulated subsets within the mitochondrial compartment both before and after TCR stimulation

Cytosolic ribosomes were unaffected, indicating that the translation attenuation observed in the KO CTLs was not caused by a defect in cytosolic ribosomal content



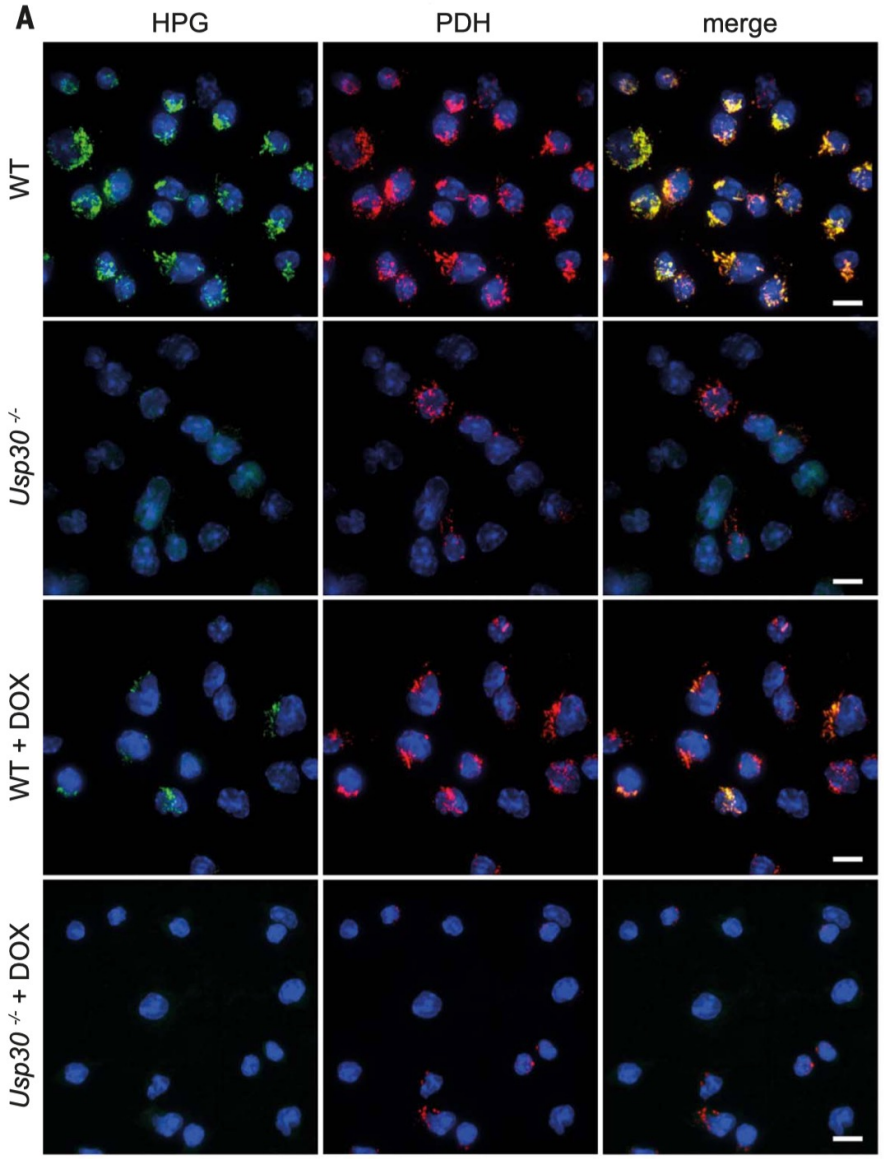


Percentage of mitochondrial ribosomal protein out of total mitochondrial peptides

Mitochondrial and cytosolic protein synthesis

It is now well established that mitochondrial translation can regulate the synthesis of cytosolic proteins (27, 28). In addition, recent in vivo studies suggest roles for mitochondrial translation in T cell function (29, 30). They therefore asked whether mitochondrial protein synthesis was disrupted in day 5 KO CTLs by examining HPG incorporation when cytosolic translation was inhibited by CHX.

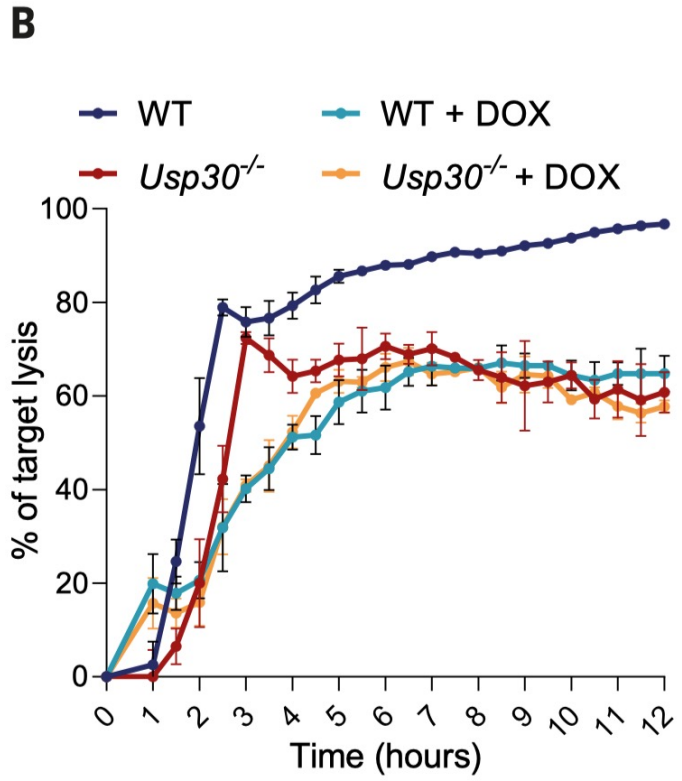
Mitochondrial translation was evident in WT CTLs, with HPG colocalizing with the mitochondrial marker, PDH. By contrast, there was little HPG incorporation in KO CTLs

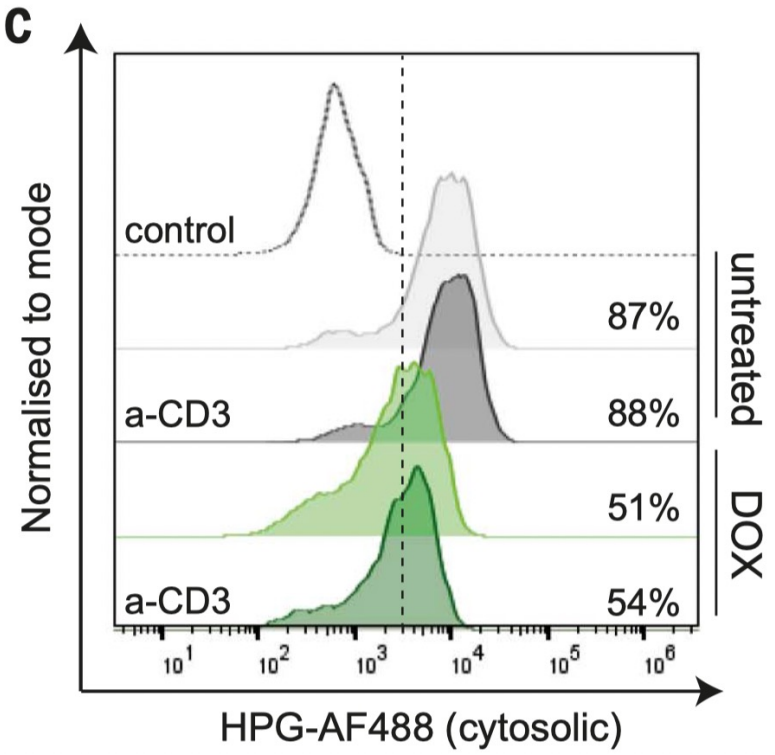


To directly address the role of mitochondrial protein translation in CTL killing, they disrupted mitochondrial protein translation pharmacologically using doxycycline (DOX).

HPG, methionine analog
PDH, labeling mitochondrial matrix
CHX, cytosolic protein synthesis inhibitor
DOX, disrupting mitochondrial protein translation

DOX treatment inhibited CTL cytotoxicity after 4 hours, which replicated the phenotype of KO CTL

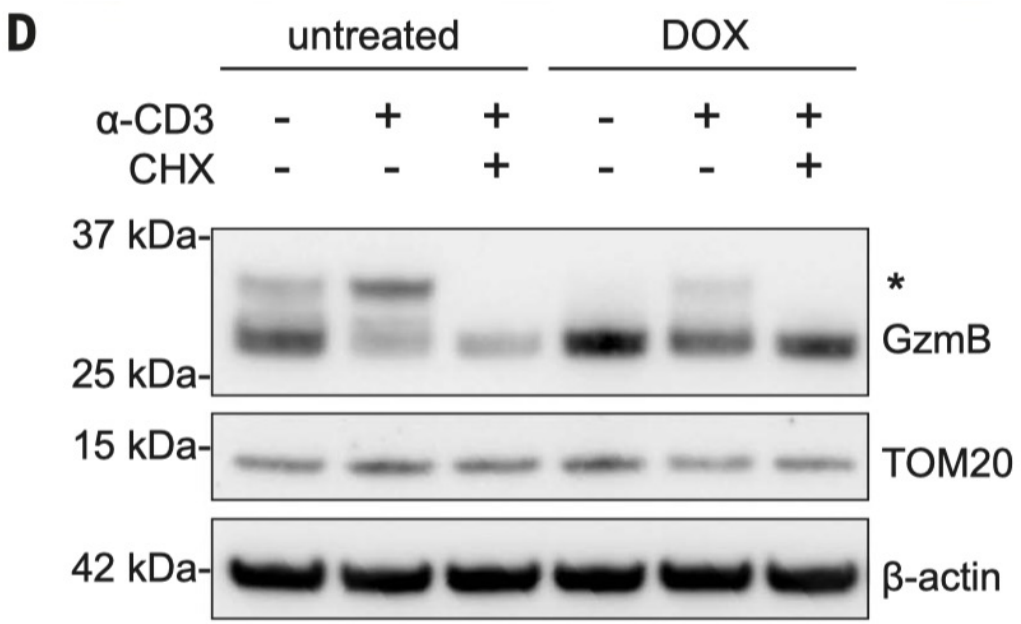


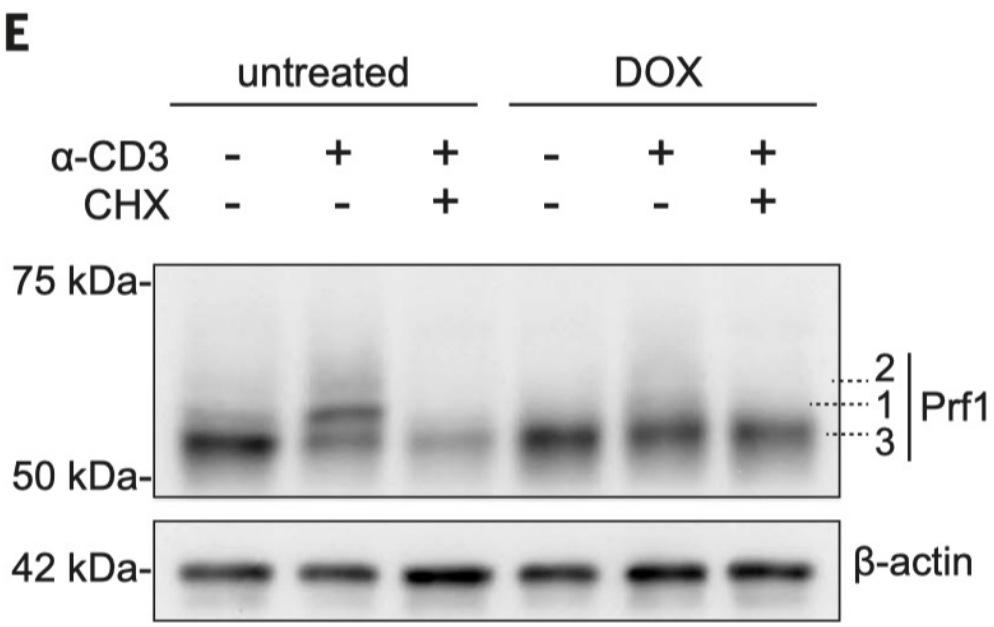


DOX, disrupting mitochondrial protein translation

Furthermore, cytosolic protein translation was inhibited after 1 hour of DOX treatment

De novo synthesis of granzyme B and perforin after TCR activation (4 hours) was also reduced





*, newly synthesized GzmB
TOM20, outer mitochondrial membrane

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Immature (1)
intermediate (2)
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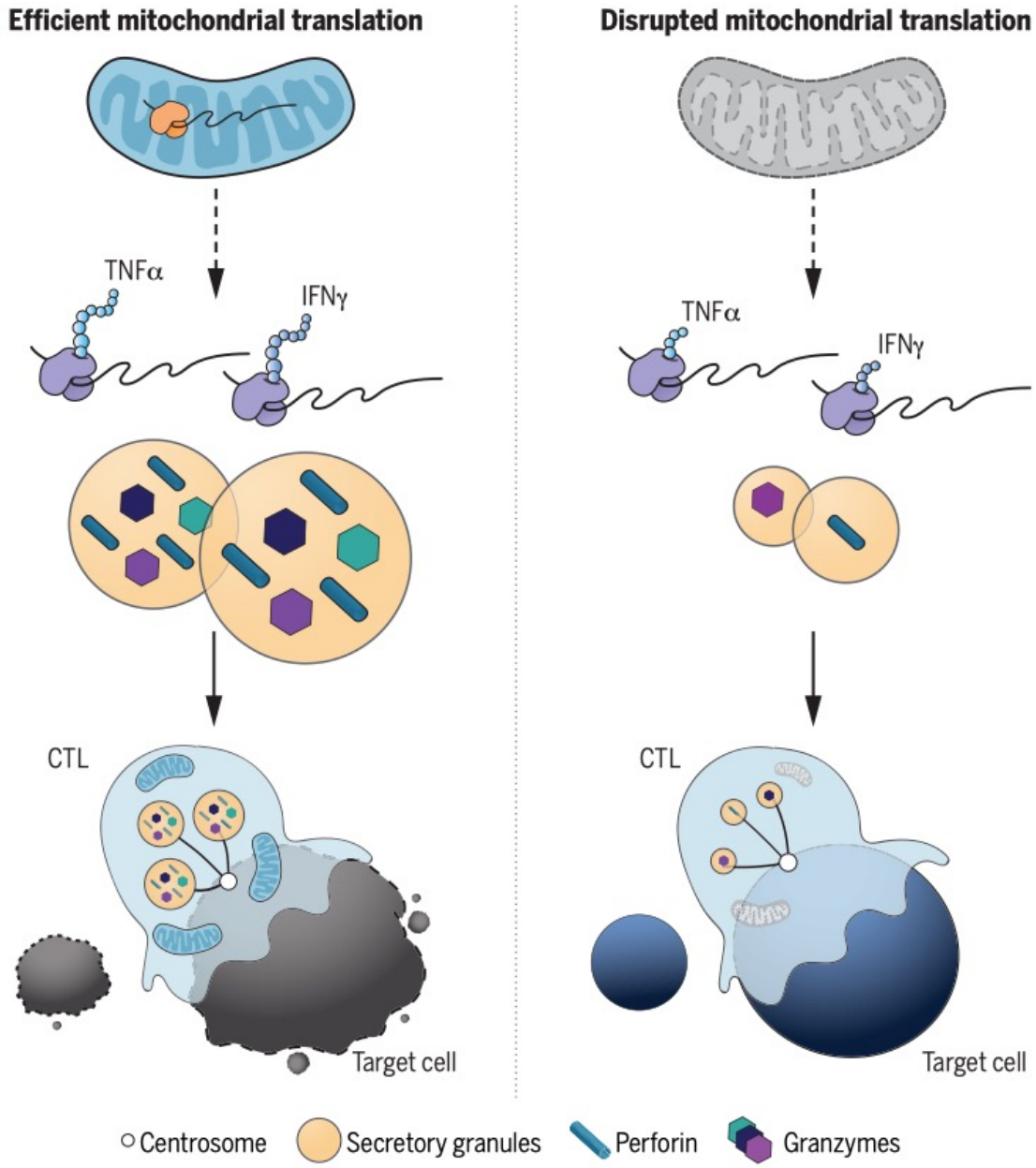

Summary

Mitochondria regulate sustained killing by CTLs.

Sustained killing of targets by CTLs requires replenishment of cytolytic proteins, including perforin and granzymes, in secretory granules and cytokines including TNF-a and IFN-g. This is controlled through mitochondrial translation, selective disruption of which prevents refueling, leaving CTLs unable to sustain killing.

Discussion

- Inhibition of mitochondrial translation is known to affect cytosolic translation by way of several pathways, including mechanistic target of rapamycin (mTOR) and the integrated stress response (ISR). However, these pathways do not appear to be causative in the mitochondrial regulation of CTL killing. The mTOR target was phosphorylated in both KO and WT CTLs (fig. S4). In addition, although they identified up-regulation of some ISR proteins in KO CTLs, the ISR inhibitor (ISRIB) only partially restored protein synthesis and was unable to restore killing in KO CTLs (fig. S7).

- Two distinctive features of the KO CTLs were decreased oxidative phosphorylation (and increased reliance on glycolysis) (Fig. 1, J and K) together with selectivity in the down-regulation of cytosolic protein synthesis (Fig. 5). Their proteomic analyses suggested an alternative mechanism that may contribute to the loss of killing when mitochondrial disruption triggers changes in metabolic pathways in the cell.

- Gene ontology analysis shows that 68% of commonly down-regulated proteins in KO and DOX-treated CTLs were involved in cellular metabolism. Many metabolic enzymes can “moonlight” (兼职) as RNA-binding proteins (RBPs), which regulate the translation of selected mRNAs. The moonlighting functions of RBP metabolic enzymes can be induced by changes in CTL metabolism and can regulate expression of cytolytic proteins, including granzyme B, TNF-a, and IFN-g. They propose that this metabolic rewiring leads to the selective decrease in proteins required for sustained killing when mitochondrial translation is impaired.

Thanks for your attention!