Robust T cell activation requires an eIF3-driven burst in T cell

receptor translation

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ABSTRACT

Activation of T cells requires a rapid surge in cellular protein synthesis. However, the role

of translation initiation in the early induction of specific genes remains unclear. Here we

show human translation initiation factor eIF3 interacts with select immune system related

mRNAs including those encoding the T cell receptor (TCR) subunits TCRA and TCRB.

Binding of eIF3 to the *TCRA* and *TCRB* mRNA 3'-untranslated regions (3'-UTRs)

depends on CD28 coreceptor signaling and regulates a burst in TCR translation required

for robust T cell activation. Use of the *TCRA* or *TCRB* 3'-UTRs to control expression of

an anti-CD19 chimeric antigen receptor (CAR) improves the ability of CAR-T cells to kill

tumor cells *in vitro*. These results identify a new mechanism of eIF3-mediated translation

control that can aid T cell engineering for immunotherapy applications.

KEYWORDS

INTRODUCTION

Translation initiation serves as a key gatekeeper of protein synthesis in eukaryotes and requires the action of eukaryotic initiation factor 3 (eIF3) (Hernández et al., 2020; Pelletier and Sonenberg, 2019). In humans, eIF3 is a 13-subunit protein complex that coordinates the cellular machinery in positioning ribosomes at the mRNA start codon. Several lines of evidence indicate eIF3 also serves specialized roles in cellular translation, by recognizing specific RNA structures in the 5'-untranslated regions (5'-UTRs) of target mRNAs (Lee et al., 2015), binding the 7-methyl-guanosine (m^7G) cap (Lamper et al., 2020; Lee et al., 2016) or through interactions with *N*-6-methyl-adenosine (m^6A) post-transcriptional modifications in mRNAs (Meyer et al., 2015). Binding to these *cis*-regulatory elements in mRNA can lead to translation activation or repression, depending on the RNA sequence and structural context (Lee et al., 2016, 2015; Meyer et al., 2015; de la Parra et al., 2018). These noncanonical functions for eIF3 can aid cell proliferation (Lee et al., 2015), or allow cells to rapidly adapt to stresses such as heat shock (Meyer et al., 2015). T cell activation requires a rapid increase in protein synthesis within the first few hours that also involves eIF3 (Ahern et al., 1974; Kleijn and Proud, 2002; Miyamoto et al., 2005; Ricciardi et al., 2018). However, whether eIF3 serves a general or more specific role in T cell activation is unknown.

Translation in non-activated lymphocytes is limited by the availability of translation initiation factors (Ahern et al., 1974; Mao et al., 1992; Wolf et al., 2020). The mRNAs for several translation initiation factors, including those for many eIF3 subunits, are repressed in resting T cells and are rapidly translated within hours of activation (Wolf et al., 2020). Additionally, nearly-inactive eIF3 in quiescent T cells is activated to form translation

initiation complexes in the first few hours after stimulation (Miyamoto et al., 2005).

Activation of eIF3 coincides with the recruitment of subunit eIF3j to eIF3 and translation

initiation complexes (Miyamoto et al., 2005). Post-translational modifications are also

thought to contribute to the early increase in translation, for example activation of the

guanine nucleotide exchange factor eIF2B (Kleijn and Proud, 2002). By contrast, the level

of the canonical mRNA cap-binding complex eIF4F–composed of translation initiation

factors eIF4E, eIF4G and eIF4A– increases much later after T cell activation (Mao et al.,

1992).

Recent studies have identified translational control of specific transcripts required

for the rewiring of metabolism needed for effector T cell function. Translation of these

specific transcripts involved in glycolysis and fatty acid synthesis occurs within 1-3 days

of T cell activation (Ricciardi et al., 2018), and depends on the activity of eIF4F. Hundreds

of other mRNAs translationally repressed in resting T cells are rapidly translated within

hours after activation, including those encoding key transcription factors and ribosomal

proteins, in addition to the translation initiation factors noted above (Wolf et al., 2020).

Most of these mRNAs are sensitive to mTOR inhibition, highlighting the importance of

eIF4F for the translation of these specific transcripts. However, a small subset of mRNAs

evade mTOR inhibition by an unknown mechanism (Wolf et al., 2020). Whether eIF3

selectively regulates these or other mRNAs early in T cell activation is not known.

In the adaptive immune system, T cells are activated when a foreign antigen is

recognized by the T cell receptor (TCR). However, robust T cell activation requires a

second signal generated by interactions between the T cell and antigen presenting cell

mediated by the co-receptor CD28 (Esensten et al., 2016). This two-signal mechanism

enables T cells to adopt a fully active state and avoid becoming unresponsive (Chen and

Flies, 2013). The process of T cell activation can be mimicked *in vitro* using antibodies

targeting both the TCR and CD28 (anti-CD3 and anti-CD28 antibodies, respectively)

(Harding et al., 1992), which has greatly aided the dissection of molecular mechanisms

underlying T cell activation.

Insights from these studies have also inspired efforts to engineer T cells for

immunotherapy applications such as treating cancers (Chen and Flies, 2013). T cells can

be engineered to express chimeric antigen receptors (CARs) that specifically target

antigens on the surface of cancer cells, and signal through protein elements derived from

both the TCR and CD28 or other co-stimulatory receptors (Chen and Flies, 2013;

Globerson Levin et al., 2021). The most successful of these CAR T cells have been used

to treat CD19-positive B cell malignancies, with dramatic results (Friedman et al., 2018;

Kalos et al., 2011; Kochenderfer et al., 2013; Qin et al., 2020; Wang et al., 2020).

However, CAR T cells still have a number of drawbacks, including toxicity, CAR T cell

exhaustion, limited persistence and poor efficacy in solid tumors. These problems

highlight the need for a deeper understanding of T cell activation and how it can be

controlled in CAR T cells (Globerson Levin et al., 2021; Watanabe et al., 2018).

Here, we identified mRNAs that specifically bind eIF3 in activated T cells, including

many encoding proteins involved in immune cell function such as the TCR. We mapped

the eIF3-dependent *cis*-regulatory elements in the mRNAs encoding the TCR alpha and

beta subunits (TCRA and TCRB, respectively), finding the 3'-untranslated regions (3'-

UTRs) of these mRNAs control a rapid burst in TCRA and TCRB translation that depends

on CD28 coreceptor signaling. Finally, we use this information to engineer T cells

expressing chimeric antigen receptors to modulate the dynamics of CAR expression and

improve the ability of CAR T cells to kill tumor cells *in vitro*.

RESULTS

A specific suite of RNAs interact with eIF3 in activated Jurkat cells

To delineate how eIF3 contributes to T cell activation, we first identified RNAs that

directly interact with eIF3 in Jurkat cells activated for 5 hours with phorbol 12-myristate

13-acetate and ionomycin (PMA+I), using photoactivatable ribonucleoside-enhanced

crosslinking and immunoprecipitation (PAR-CLIP) (Hafner et al., 2010; Lee et al., 2015;

Mukherjee et al., 2019) (**Figure 1A**). In the Jurkat PAR-CLIP experiments, RNA

crosslinked to eight of the thirteen eIF3 subunits, as identified by mass spectrometry:

subunits EIF3A, EIF3B, EIF3D and EIF3G as previously seen in HEK293T cells (Lee et

al., 2015), as well as subunits EIF3C, EIF3E, EIF3F, and EIF3L (**Figure 1B, Figure 1–**

figure supplement 1A-1B, Supplementary File 1). Consistent with its role in T cell

activation, eIF3 crosslinked to a substantially larger number of mRNAs (~75x more) in

activated Jurkat cells compared to control non-activated cells (**Figure1–figure**

supplement 1C-1D and Supplementary Files 2 and 3). Notably, in activated Jurkat cells

eIF3 interacted with mRNAs enriched for those encoding proteins central to immune cell

function, in contrast to those previously identified in HEK293T cells (Lee et al., 2015)

(**Figure 1C and Figure 1–figure supplement 1E**). The extent of eIF3 crosslinking in

activated Jurkat cells does not correlate with mRNA abundance (**Figure 1–figure**

supplement 1F and Supplementary File 4), suggesting the enrichment of immune

system related mRNAs reflects the involvement of eIF3 in specific regulation of T cell

activation.

In activated Jurkat cells, eIF3 showed a multitude of crosslinking patterns on

different mRNAs (**Figure 1D-1E and Figure 1–figure supplement 1D, 1G and 1H**),

consistent with varied roles for eIF3 in T cell activation and function. Many of the mRNAs

have a single PAR-CLIP site in the 5'-UTR as observed in HEK293T cells (Lee et al.,

2015) (**Figure 1D, Figure 1–figure supplement 1D, Supplementary File 3**). However,

eIF3 crosslinked to some mRNAs across the entire length of the transcript, from the

beginning of the 5'-UTR through the 3'-UTR (**Figure 1E and Figure 1–figure**

supplement 1H). This "pan-mRNA" pattern of eIF3 crosslinking–which includes

polyadenylated mRNAs as well as histone mRNAs–has not been observed before.

Interestingly, a number of these mRNAs encode proteins important for T cell activation,

including both the alpha and beta subunits of the T cell receptor (TCR), subunits TCRA

and TCRB (**Figure 1E, Supplementary File 5**).

TCRA and *TCRB* **mRNAs form distinct puncta in activated Jurkat cells**

Crosslinking in PAR-CLIP experiments requires direct interaction between the

RNA and protein of interest (Ascano et al., 2012). Thus, the pan-mRNA pattern of

crosslinking between eIF3 and certain mRNAs suggests formation of ribonucleoprotein

complexes (RNPs) highly enriched in eIF3. Notably, the pan-mRNA crosslinking pattern

in the *TCRA* and *TCRB* mRNAs occurs in activated but not in non-activated Jurkat cells

(**Figure 1E**), suggesting eIF3 may contribute to increased translation of these mRNAs

rather than their repression. We therefore examined *TCRA* and *TCRB* mRNA localization

in activated Jurkat cells, to determine whether they colocalized with repressive

environments such as P bodies or stress granules (Tauber et al., 2020). Since Jurkat cells

have a defined TCR, we designed fluorescence in situ hybridization (FISH) probes across

the entire *TCRA* and *TCRB* transcripts to examine their localization. Interestingly, the

TCRA and *TCRB* mRNAs formed distinct puncta in Jurkat cells activated with anti-

CD3/anti-CD28 antibodies, which induce both TCR and CD28 coreceptor signaling

required for robust T cell activation (Harding et al., 1992). However, these mRNAs did not

co-localize with either P bodies or stress granules, or with each other (**Figure 1F, Figure**

1–figure supplement 2A and 2B). These results indicate that the *TCRA* and *TCRB*

mRNAs are not translationally repressed but are likely localized to translation "hot spots."

Pan-mRNAs remain bound to eIF3 in translating ribosomes

The pan-mRNA crosslinking pattern suggests that eIF3 remains bound to the

TCRA and *TCRB* mRNAs while they are actively translated. To capture *TCRA* and *TCRB*

mRNAs in translating ribosomes and examine their interactions with eIF3, we analyzed

polysomes in Jurkat cells activated with anti-CD3/anti-CD28 antibodies (**Figure 2A**)

(Harding et al., 1992). The cells were first treated with protein-protein crosslinker

dithiobis(succinimidyl propionate) (DSP) before isolating polysomes on sucrose gradients

(**Figure 2–figure supplement 1A**). We then incubated the cell lysates with RNase H and

DNA oligonucleotides designed to cleave the mRNAs specifically between the 5'-UTR,

coding sequence (CDS), and 3'-UTR (**Figure 2B, Supplementary File 6**). This protocol

efficiently cleaved the mRNAs and prevented the released 5'-UTR and 3'-UTR elements

from entering polysomes (**Figure2–figure supplement 1B-1E**). It also allowed us to

detect eIF3 interactions with the mRNA CDS regions independent of eIF3 interactions

with the UTR sequences identified in the PAR-CLIP analysis. We detected mRNAs

interacting with eIF3 in the polysomes by performing anti-EIF3B immunoprecipitations

followed by qRT-PCR (**Figure 2A**). We compared both *TCRA* and *TCRB* mRNAs to

another pan-crosslinked mRNA, *DUSP2*, and to two mRNAs that crosslinked to eIF3 only

through their 5'-UTRs (*EGR1*, *TRIM28*). Using primers to the CDS regions of the mRNAs,

we found that eIF3 only immunoprecipitated the pan-crosslinked mRNAs (*TCRA*, *TCRB*,

DUSP2) from polysomes, but not mRNAs that only crosslinked to eIF3 through their 5'-

UTRs (*EGR1*, *TRIM28*) (**Figure 2C and 2D**). Importantly, all of these mRNAs are present

in translating ribosomes and can be immunoprecipitated with eIF3 when the mRNAs are

left intact (RNase H treatment without DNA oligos) (**Figure 2E**).

We also tested whether these mRNAs interact with eIF3 similarly in primary human

T cells during activation. We could not examine *TCRA* and *TCRB* mRNAs in primary

human T cells, as these mRNAs do not have a unique sequence in the 5'-UTR or variable

region of the CDS to which we could design DNA oligonucleotides as described above.

Therefore, we tested the distribution of *DUSP2*, *EGR1* and *TRIM28* mRNAs in primary

human T cells activated with anti-CD3/anti-CD28 antibodies (Harding et al., 1992). As

observed in Jurkat cells, *DUSP2* mRNA remained bound to eIF3 through its CDS region

in polysomes whereas the *EGR1* and *TRIM28* mRNAs did not remain bound (**Figure 2F–**

2H). We also confirmed that the 5'-UTR and 3'-UTR elements of these mRNAs are

efficiently cleaved and did not enter polysomes (**Figure2–figure supplement 1F–1H**).

Taken together, these results indicate that, in activated T cells, eIF3 remains bound to

the coding sequences (CDS) of the pan-mRNAs *TCRA*, *TCRB* and *DUSP2* in polysomes

independent of their 5'-UTR and 3'-UTR elements. These results further support the

model that *TCRA* and *TCRB* mRNA puncta represent translation "hot spots," and the pan-

mRNA crosslinking pattern reflects eIF3 binding to these mRNAs during translation

elongation.

eIF3 interacts with the *TCRA* and *TCRB* mRNA 3'-UTRs and controls a burst in

translation during T cell activation

Although crosslinking of eIF3 to the CDS regions of the *TCRA* and *TCRB* mRNAs

(**Figure 1E**) indicates eIF3 remains bound to them during translation elongation (**Figure**

2 and Figure 2–figure supplement 1), we wondered whether the 5'-UTRs and 3'-UTRs

might play a role in recruiting these mRNAs to translating ribosomes. The *TCRA* and

TCRB genes encode a different, often short 5'-UTR for each variable region of the mature

locus (Omer et al., 2021; Scaviner and Lefranc, 2000), suggesting the 5'-UTR is unlikely

to harbor eIF3-dependent regulatory elements. We therefore focused on the roles of the

TCRA and *TCRB* 3'-UTRs. We constructed nanoluciferase reporters fused to the WT

TCRA or *TCRB* mRNA 3'-UTR sequence, to 3'-UTRs with the eIF3 PAR-CLIP site deleted

(*ΔPAR*) or to 3'-UTRs with the reversed sequence of the eIF3 PAR-CLIP site (R*PAR*,

i.e. 5'-3' sequence reversed to 3'-5' direction to maintain the length of the 3'-UTR) (**Figure**

3A). We then stably expressed these mRNAs in primary human T cells using lentiviral

transduction and activated these T cells using anti-CD3/anti-CD28 antibodies. T cells

expressing the reporters with the WT *TCRA* or *TCRB* mRNA 3'-UTR sequences produced

substantially higher luminescence that peaked 1 hour after activation, while cells

expressing nanoluciferase from reporters with a deletion or reversal of the eIF3 PAR-

CLIP site sequence showed no apparent burst in translation (**Figure 3B**). The *TCRA*

ΔPAR or R*PAR or *TCRB ΔPAR* or R*PAR mutations, however, did not cause significant

defects in the nanoluciferase mRNA levels when compared to reporters with the

corresponding WT 3'-UTR sequences (**Figure 3C**)**.** This suggests the burst in

nanoluciferase expression observed with the WT *TCRA* or *TCRB* mRNA 3'-UTR

sequences is regulated posttranscriptionally. Immunoprecipitation of eIF3 followed by

qRT-PCR quantification of nanoluciferase mRNA showed that less nanoluciferase mRNA

bound to eIF3 when the 3'-UTR PAR-CLIP site was either deleted or reversed, compared

to nanoluciferase mRNAs carrying the WT *TCRA* or *TCRB* 3'-UTR (**Figure 3D and 3E**).

Interestingly, although the *TCRA* and *TCRB* mRNA 3'-UTR elements that crosslinked to

eIF3 do not share any conserved sequences or RNA structural elements (Xu and

Mathews, 2016), the above results support the idea that eIF3 binding to specific

sequences or structures within the *TCRA* and *TCRB* 3'-UTRs controls a burst in

translation after T cell activation.

We next tested whether the *TCRA* and *TCRB* 3'-UTRs serve to initially recruit eIF3

to the *TCRA* and *TCRB* mRNAs, as eIF3 does not remain stably bound to these 3'-UTRs

in translating ribosomes (**Figure 1–figure supplement 2G**). We made nanoluciferase

reporters in which we replaced the eIF3 PAR-CLIP site in the *TCRA* 3'-UTR with

sequences from the hepatitis C viral internal ribosome entry site (HCV IRES domain

IIIabc) or the *JUN* mRNA 5'-UTR, each previously shown to bind directly to eIF3 (Kieft et

al., 2001; Lee et al., 2015) (**Figure 3F**). We stably transduced these constructs into

primary T cells and measured nanoluciferase activity after activating the cells with anti-

CD3/anti-CD28 antibodies. By contrast to the *TCRA ΔPAR* 3'-UTR, the HCV IRES IIIabc

and *JUN* sequences increased nanoluciferase translation (**Figure 3G**). This was true

despite the fact that there was less of an increase in mRNA abundance for the HCV IRES

IIIabc and *JUN* constructs compared to the *TCRA ΔPAR* 3'-UTR (**Figure 3H**). The HCV

IRES IIIabc and *JUN* sequences also rescued eIF3 binding to the reporter mRNAs

(**Figure 3I**) upon T cell activation. These results are consistent with the engineered 3'-

UTRs recruiting eIF3 to the mRNA. However, the dynamics of translation activation

induced by the 3'-UTRs harboring the HCV IRES or *JUN* sequences did not recapitulate

the effects of the WT *TCRA* 3'-UTR. Both engineered 3'-UTRs increased nanoluciferase

levels within 30 minutes of activation, in contrast to the WT *TCRA* 3'-UTR (**Figure 3G**).

Furthermore, neither engineered 3'-UTR led to a marked decrease of nanoluciferase

levels after the 1 hour peak in luminescence seen with the WT *TCRA* 3'-UTR (**Figure**

3G). Taken together, the nanoluciferase reporter experiments reveal the *TCRA* and

TCRB mRNA 3'-UTRs are necessary and sufficient to drive a burst in translation after T

cell activation. They also suggest these 3'-UTR elements recruit eIF3 to the *TCRA* and

TCRB mRNAs to drive a burst in translation of the TCR alpha and beta subunits.

The *TCRA* and *TCRB* mRNA 3'-UTRs control a burst in translation in a CD28-

dependent manner

Since T cell activation in some cases does not require the TCR (Siefken et al.,

1998), we also tested whether the burst in translation controlled by the *TCRA* and *TCRB*

mRNA 3'-UTRs could be induced by activation of either the TCR or CD28 individually

(**Figure 4**). We found anti-CD28 stimulation alone was sufficient to cause a transient burst

in translation in the nanoluciferase reporters with the WT *TCRA* or *TCRB* mRNA 3'-UTRs,

whereas anti-CD3 stimulation caused a continuous increase in reporter protein

expression (**Figure 4B–4D**). Interestingly, the burst in translation required the reporters

to be membrane tethered via a N-terminal transmembrane helix (from CD3zeta) that is

co-translationally inserted into the membrane (Call and Wucherpfennig, 2005) (**Figure**

4A–4D and Figure 4–figure supplement 1A–1C), consistent with the fact that CD28

signaling involves multiple membrane-associated events (Boomer and Green, 2010).

Moreover, the reporters with *TCRA ΔPAR* and *TCRB ΔPAR* 3'-UTRs failed to show a

burst in expression even when the reporter proteins were tethered to the membrane

(**Figure 4A–4D**), supporting the role for eIF3 in the translational burst. Taken together,

these results support the model that the CD28 costimulatory pathway drives an early burst

in TCR translation after T cell activation, mediated by eIF3 binding to the *TCRA* and *TCRB*

3'-UTRs.

We also tested the proximal signaling pathways downstream of CD28 required for

the dynamic regulation of reporter expression by using inhibitors of the kinases AKT (Choi

et al., 2016; Wu et al., 2020) and mTOR (Thoreen et al., 2009). In T cells expressing the

membrane-tethered nanoluciferase reporter mRNA with the WT *TCRA* 3'-UTR (**Figure**

4A), treatment with the mTOR inhibitor Torin 1 had no effect on the rapid increase in

reporter expression as seen with the DMSO control. However the burst in nanoluciferase

expression was blocked when AKT kinase activity was inhibited with AZD5363 (AZD)

(**Figure 4E–4F and Figure 4–figure supplement 1D-1E**). Altogether, these data indicate

that the transient burst in TCR expression likely requires specific interactions between

eIF3 and the *TCRA* and *TCRB* 3'-UTRs and also membrane-proximal CD28 signaling.

Furthermore, these results support a model in which T cell activation requires CD28

costimulation to elicit an initial positive signal involving AKT kinase activity, which is later

repressed by a negative feedback loop also mediated by CD28 signaling and cis-

regulatory elements in the *TCRA* and *TCRB* mRNA 3'-UTRs.

eIF3 interactions with the *TCRA* and *TCRB* mRNA 3'-UTRs regulate a burst in TCR

translation important for T cell activation

Given that the *TCRA* and *TCRB* mRNA 3'-UTRs are necessary and sufficient to

drive a burst in translation after T cell activation, we examined their effects on the

endogenous levels of the TCR. We analyzed the expression of TCR protein levels using

western blots probed with an anti-TCRA antibody, since the formation of an intact TCR is

required to stabilize both the TCRA and TCRB subunits (Koning et al., 1988; Ohashi et

al., 1985). As seen with the nanoluciferase reporters, TCRA levels rose and peaked

approximately one hour after T cell activation with anti-CD3/anti-CD28 antibodies (**Figure**

5A). Furthermore, the early burst in TCRA translation is dependent on CD28 but not on

TCR signaling (**Figure 5A**), also as observed with membrane-tethered reporters (**Figure**

4). To more directly assess the role of the *TCRA* and *TCRB* mRNA 3'-UTRs, we used

CRISPR-Cas9 genome editing to delete the eIF3 PAR-CLIP sites in either the *TCRA* or

TCRB mRNA 3'-UTRs in primary T cells from two healthy human donors (**Figure 5–figure**

supplement 1A and Supplementary File 6). PCR analysis showed successful deletion

of the eIF3 PAR-CLIP site in the *TCRA* 3'-UTR or in the *TCRB* 3'-UTR (*TCRA ΔPAR* or

TCRB ΔPAR, respectively) in 43% - 49% of the alleles (**Figure 5–figure supplement**

1B). A scrambled sgRNA (SC), which does not target any site in the human genome was

used as a control. We first measured the total endogenous TCRA protein levels by

western blot in *TCRA* or *TCRB* 3'-UTR edited cells versus control cells at different time

points after activating with anti-CD3/anti-CD28 antibodies (**Figure 5B**). The SC control

cells – which should behave as wild-type T cells – exhibited a substantial burst in TCRA

protein levels immediately after activation (~1 hr). By contrast, TCRA protein levels were

nearly absent or clearly reduced at early time points after activation in both *TCRA ΔPAR*

and *TCRB ΔPAR* cell populations (**Figure 5B**). Only at later time points did TCRA levels

in the *TCRA ΔPAR* and *TCRB ΔPAR* cell populations begin to increase. These results

share the same pattern of expression seen with the nanoluciferase reporters described

above (**Figure 3 and Figure 4**).

We next asked whether the burst in TCR expression driven by the *TCRA* and

TCRB 3'-UTRs affected downstream steps in T cell activation. During TCR-dependent T

cell activation, membrane proteins reorganize at the interface between the T cell and

antigen presenting cell (APC) to form an immunological synapse (Huppa and Davis,

2003). TCR cluster formation is a central aspect of immune synapse formation (Cochran

et al., 2001). Therefore we tested whether TCR cluster formation is affected by reduced

TCR protein levels in *TCRA ΔPAR* and *TCRB ΔPAR* T cells at early time points after

activation. We used the same cell populations as those used for the western blots above

(**Figure 5B**), to correlate the TCRA protein levels observed in the western blots with TCR

clustering. We performed immunofluorescence on SC, *TCRA ΔPAR* and *TCRB ΔPAR* T

cell populations using anti-TCRA and anti-TCRB antibodies to detect the TCR. Both

TCRA ΔPAR and *TCRB ΔPAR* cells had fewer cells forming TCR clusters, especially at

the early time point compared to SC control cells when activated with anti-CD3/anti-CD28

(**Figure 5C and Figure 5–figure supplement 1C**). Consistent with the TCRA expression

levels observed in western blots and the rate of TCR clustering (**Figure 5B and 5C**), both

TCRA ΔPAR and *TCRB ΔPAR* cells expressed lower amounts of cell surface TCR

compared to SC control cells or cells edited with a single gRNA when tested by flow

cytometric analysis after PMA+I activation (**Figure 5–figure supplement 1D and 1E**).

To test whether the defect in TCR clustering in the *TCRA ΔPAR* and *TCRB ΔPAR*

cell populations reflects a general deficiency in T cell activation, we measured the T cell

activation markers CD69 and CD25 (IL2RA) by flow cytometry (**Figure 5–figure**

supplement 1F and Figure 5–figure supplement 2A and 2B). Fewer cells in the *TCRA*

ΔPAR and *TCRB ΔPAR* CD8+ and CD4+ primary T cell populations expressed CD69 at

early time points after activation (5-8 hours) (**Figure 5–figure supplement 2C and 2D**)

and fewer expressed both CD69 and CD25 at later time points after activation, compared

to SC control cells (**Figure 5D–5E and Figure 5–figure supplement 2E and 2F**). We

also found the *TCRA ΔPAR* and *TCRB ΔPAR* T cell populations secreted lower amounts

of cytokines IL2 (**Figure 5F and Figure 5–figure supplement 2G)** and IFNγ (**Figure 5G**

and Figure 5–figure supplement 2H), compared to the SC control cells. Taken together,

the *TCRA ΔPAR* and *TCRB ΔPAR* primary T cell populations exhibited multiple early and

late T cell activation defects. These results support the model that after T cell activation,

eIF3 binding to the *TCRA* and *TCRB* mRNA 3'-UTRs drives an early burst in TCR

translation that is required for many subsequent steps in T cell activation.

To obtain additional mechanistic understanding of eIF3-mediated regulation of

TCR translation we generated clonal *TCRA ΔPAR* and *TCRB ΔPAR* Jurkat cells using

the CRISPR-Cas9 genome editing strategy developed for primary T cells (**Figure 5–**

figure supplement 1A, Supplementary File 6). We first measured total TCR levels

using western blots and an anti-TCRA antibody as described above (**Figure 5A**), at

different time points after activation with anti-CD3/anti-CD28 antibodies. WT Jurkat cells

showed a TCR translational burst that peaked 5-8 hours after activation (**Figure 5–figure**

supplement 3A). By contrast, both *TCRA ΔPAR* and *TCRB ΔPAR* Jurkat cell populations

expressed lower levels of the TCR proteins compared to WT cells, and failed to show a

burst in TCR expression at early time points after activation (**Figure 5–figure**

supplement 3A). Importantly, *TCRA* and *TCRB* mRNA levels were unaffected or even

increased in the *TCRA ΔPAR* and *TCRB ΔPAR* Jurkat cells (**Figure 5–figure**

supplement 3B and 3C), similar to our observations with nanoluciferase reporters in

primary T cells (**Figure 3C**). This is consistent with TCR expression levels being regulated

post-transcriptionally by eIF3 interactions with the *TCRA* or *TCRB* mRNA 3'-UTR

elements. We then tested whether deleting eIF3 binding sites in both *TCRA* and *TCRB*

mRNA 3'-UTRs affected their interaction with eIF3. In both *TCRA ΔPAR* and *TCRB ΔPAR*

cells, eIF3 bound to significantly lower amounts of the *TCRA* and *TCRB* mRNAs

compared to WT cells at both the 5 hour and 8 hour time points after anti-CD3/anti-CD28

activation (**Figure 5–figure supplement 3D and 3E**). This indicates deleting the eIF3

PAR-CLIP sites in the 3'-UTR disrupts eIF3 interactions with the *TCRA* and *TCRB*

mRNAs substantially. Together with the nanoluciferase reporter experiments in primary

T cells (**Figure 3**), these results support the model that eIF3 binding to the 3'-UTR

elements of the *TCRA* and *TCRB* mRNAs mediates the rapid burst in TCR translation

after T cell activation.

The *TCRA* and *TCRB* mRNA 3'-UTRs enhance anti-CD19 CAR T cell function

T cells engineered to express chimeric antigen receptors (CARs) for cancer

immunotherapy now in use clinically employ artificial 3'-UTRs in the CAR-encoding

mRNA, a woodchuck hepatitis viral posttranscriptional regulatory element (*WPRE*)

(Milone et al., 2009), or a retroviral 3'-long terminal repeat (*3'-LTR*) (Kochenderfer et al.,

2009). However, it is not known whether these 3'-UTR elements provide optimal CAR

expression or CAR T cell function. To test whether these 3'-UTRs induce a transient burst

in translation as observed with the WT *TCRA* or *TCRB* 3'-UTRs, we fused the *WPRE* and

3'-LTR 3'-UTR sequences to nanoluciferase reporters and expressed these in primary T

cells (**Figure 6A**). In contrast to the *TCRA* and *TCRB* 3'-UTRs (**Figure 3 and Figure 4**),

the *WPRE* and *3'-LTR* 3'-UTR elements failed to induce the early burst in nanoluciferase

expression (**Figure 6A**). These data suggest that fusing the *TCRA* or *TCRB* 3'-UTR

sequences to engineered CAR open reading frames could be used to obtain more

physiological expression dynamics seen for the endogenous TCR.

We therefore engineered primary human T cells to express an anti-CD19 CAR

currently in use clinically to treat B cell lymphomas (Kalos et al., 2011; Milone et al., 2009;

June et al., 2014). We used lentiviral transduction to express the anti-CD19 CAR from

mRNAs with either *WPRE*, *3'-LTR*, *TCRA* or *TCRB* 3'-UTRs to make anti-CD19 CAR T

cells (**Figure 6B and 6C**). We then stimulated these CAR T cells by incubating them with

CD19-expressing leukemia cells (NALM6) and measured the CAR expression by western

blot analysis at different time points. Interestingly, the *TCRA* and *TCRB* 3'-UTRs induced

a burst in anti-CD19 CAR protein levels within 5 hours of NALM6 cell addition, whereas

the *WPRE* and *3'-LTR* sequences delayed the burst in CAR expression to ~24 hrs (**Figure**

6D). In these CAR T cells, TCR expression also followed the burst in CAR expression,

and was dependent on the presence of the CAR in addition to the NALM6 cells (**Figure**

6–figure supplement 1A). Lastly, we tested whether the timing of CAR protein

expression correlates with the killing capacity of these CAR T cells. Using *in vitro*

cytotoxicity assays (**Figure 6E**), CAR T cells expressing the CAR using either the *TCRA*

or *TCRB* 3'-UTR showed more robust killing of two tumor cell lines compared to CARs

using either the *WPRE* or *3'-LTR* 3'-UTRs (**Figure 6F–6G and Figure 6–figure**

supplement 1B and 1C). This is consistent with the importance of an early burst of CAR

translation for optimal CAR T cell function. These results also support the idea that using

the native *TCRA* or *TCRB* 3'-UTRs for CAR expression can be used to improve CAR T

cell function.

DISCUSSION

The eIF3 PAR-CLIP experiment we present here provides a snapshot of eIF3-RNA

interactions that occur at the time TCR translation is most sensitive to eIF3 regulation (5

hours in Jurkat cells, **Figure 5–figure supplement 3A**). At this point in time, eIF3

crosslinks to multiple mRNAs encoding proteins involved in immune cell function

(**Supplemental Files 2 and 3**). Interestingly, the patterns of eIF3 crosslinking, which for

a number of mRNAs include interactions with the protein coding sequence and 3'-UTR

(**Figure 1 and Figure 1–figure supplement 1G and 1H**), suggest an active role for eIF3

in promoting translation of these mRNAs. In support of this model, the two examples of

pan-mRNAs we examined here (*TCRA* and *TCRB*) reside in puncta distinct from P bodies

and stress granules (**Figure 1F**) and bind to eIF3 via the mRNA coding sequence on

translating ribosomes (**Figure 2**). However, this eIF3-mediated translation activation is

transient, lasting only 1-2 hours in primary T cells (**Figure 5A**). Importantly, the *TCRA* and

TCRB mRNA 3'-UTR elements are necessary and sufficient to control this burst in

translation (**Figure 3**). Recent evidence suggests that eIF3 can remain associated with

translating ribosomes (Bohlen et al., 2020; Lin et al., 2019; Wagner et al., 2020), a

phenomenon that seems to be enhanced for the pan-mRNAs identified here. Additional

layers of translation regulation also contribute to T cell function (Tan et al., 2017),

particularly with respect to mTOR signaling (Miyamoto et al., 2005; Myers et al., 2019)

and carbon metabolism (Manfrini et al., 2017; Ricciardi et al., 2018). The present PAR-

CLIP experiments should help to elucidate additional roles for eIF3-mediated translation

regulation and to map the system-wide role of translation regulation in T cell activation.

Recent experiments indicate that T cells must cross a threshold of T cell receptor

signaling to commit to activation and proliferation (Au-Yeung et al., 2014, 2017), setting

up a "digital" response to antigen recognition (Allison et al., 2016; Au-Yeung et al., 2014,

2017; Richard et al., 2018). The response threshold involves integration of intensity and

duration of TCR signaling (Au-Yeung et al., 2014, 2017; Richard et al., 2018), and spans

a wide range of TCR antigen affinity (Allison et al., 2016; Au-Yeung et al., 2014, 2017;

Richard et al., 2018). Notably, T cell commitment to clonal expansion and differentiation

can occur within as little as 1 to 2 hours of TCR stimulation for effector CD4+ and naive

CD8+ T cells (Iezzi et al., 1998; van Stipdonk et al., 2001). Remarkably, this time period

spans the burst in TCR protein synthesis mediated by eIF3 interactions with the *TCRA*

and *TCRB* mRNA 3'-UTR elements (**Figure 5A**). Naive CD4+ T cells require a longer

duration of TCR signaling of ~20 hours (Iezzi et al., 1998; Schrum et al., 2005). Although

we were not able to distinguish levels of TCR translation in isolated CD8+ and CD4+ T

cells (**Figure 5**), subsequent events in T cell activation including CD69 and CD25

expression, and IL2 and IFNγ secretion, were equally affected in CD8+ and CD4+ cells

in which the *TCRA* or *TCRB* 3'-UTR PAR-CLIP sites were deleted (**Figure 5C–5F**). In an

immune response, CD28 engagement serves as the second signal required for T cell

activation (Harding and Allison, 1993; Harding et al., 1992) and affects the first minutes

of TCR-mediated signaling (Green et al., 1994, 2000; Michel et al., 2001; Shahinian et

al., 1993; Tuosto and Acuto, 1998). Here we show CD28-mediated signaling is also

needed for the burst of TCR translation on the hour timescale in primary T cells (Buckler

et al., 2006) (**Figure 4**). Taken together, our results indicate that eIF3 controls *TCRA* and

TCRB mRNA translation during the first critical hours after antigen recognition that leads

to subsequent T cell commitment to proliferation and differentiation (**Figure 5**). The

requirement for CD28 in PD-1 mediated inhibition of T cell activation (Hui et al., 2017;

Kamphorst et al., 2017) also suggests eIF3-mediated control of TCR expression may

affect PD-1 checkpoint blockade-based cancer immunotherapy (Jiang et al., 2019).

Cell immunotherapies targeting various cancers have made great strides, due to

the engineering of chimeric antigen receptors that couple antigen recognition to

intracellular signaling domains that activate cytotoxic T cells. However, CAR T cells often

fail to eradicate cancers due to loss of activity over time, e.g. T cell exhaustion (Globerson

Levin et al., 2021; Watanabe et al., 2018). Our results using nanoluciferase reporters

indicated that eIF3-responsive mRNA 3'-UTR elements could be used to improve

chimeric antigen receptor expression and CAR T cell responsiveness (Eyquem et al.,

2017; Globerson Levin et al., 2021; Watanabe et al., 2018). We used this information to

improve the ability of CAR T cells to kill tumor cells *in vitro*. With a clinically validated anti-

CD19 CAR, we found that using the *TCRA* or *TCRB* mRNA 3'-UTRs dramatically

shortened the lag time before the burst in CAR protein expression upon exposure to tumor

cells (**Figure 6D**). This short lag time correlated with improved tumor cell killing in

cytotoxicity assays (**Figure 6E and 6F**). The burst in CAR protein expression was still not

as rapid as that of the endogenous TCR in T cells stimulated by anti-CD3/anti-CD28

antibodies, however (**Figure 5A**). This could be due to multiple factors, including CAR

affinity for the CD19 antigen or the activity of the 4-1BB costimulatory domain used in the

present CAR (Kalos et al., 2011; Milone et al., 2009; June et al., 2014) instead of the CD28

signaling domain (Kochenderfer et al., 2009). It is also possible that the 3'-UTRs of the

endogenous TCR subunits, which were left intact in the present cytotoxicity assays, and

which responded to CAR signaling (**Figure 6–figure supplement 1A**), titrated cellular

factors required for even shorter response times. T cell engineering to knock out the

endogenous TCR or improved CAR design may shorten the lag before the burst in CAR

expression and further improve CAR T cell function. Taken together, our experiments

delineate the central role of eIF3 in T cell activation and highlight the importance of

understanding translation regulation in immune cells to open new avenues for

engineering improved cell therapies (**Figure 7**).

References and Notes:

Ahern, T., Sampson, J., and Kay, J.E. (1974). Initiation of protein synthesis during

lymphocyte stimulation. Nature *248*, 519–521.

Allison, K.A., Sajti, E., Collier, J.G., Gosselin, D., Troutman, T.D., Stone, E.L., Hedrick,

S.M., and Glass, C.K. (2016). Affinity and dose of TCR engagement yield proportional

enhancer and gene activity in CD4+ T cells. Elife *5*.

Ascano, M., Hafner, M., Cekan, P., Gerstberger, S., and Tuschl, T. (2012). Identification

of RNA-protein interaction networks using PAR-CLIP. Wiley Interdiscip. Rev. RNA *3*,

159–177.

Au-Yeung, B.B., Zikherman, J., Mueller, J.L., Ashouri, J.F., Matloubian, M., Cheng,

D.A., Chen, Y., Shokat, K.M., and Weiss, A. (2014). A sharp T-cell antigen receptor

signaling threshold for T-cell proliferation. Proc. Natl. Acad. Sci. U. S. A. *111*, E3679–

E3688.

Au-Yeung, B.B., Smith, G.A., Mueller, J.L., Heyn, C.S., Jaszczak, R.G., Weiss, A., and

Zikherman, J. (2017). IL-2 Modulates the TCR Signaling Threshold for CD8 but Not

CD4 T Cell Proliferation on a Single-Cell Level. J. Immunol. *198*, 2445–2456.

Bohlen, J., Fenzl, K., Kramer, G., Bukau, B., and Teleman, A.A. (2020). Selective 40S

Footprinting Reveals Cap-Tethered Ribosome Scanning in Human Cells. Mol. Cell *79*,

561–574.e5.

Boomer, J.S., and Green, J.M. (2010). An enigmatic tail of CD28 signaling. Cold Spring

Harb. Perspect. Biol. *2*, a002436.

Buckler, J.L., Walsh, P.T., Porrett, P.M., Choi, Y., and Turka, L.A. (2006). Cutting edge:

T cell requirement for CD28 costimulation is due to negative regulation of TCR signals

by PTEN. J. Immunol. *177*, 4262–4266.

Call, M.E., and Wucherpfennig, K.W. (2005). The T cell receptor: critical role of the

membrane environment in receptor assembly and function. Annu. Rev. Immunol. *23*,

101–125.

Chen, L., and Flies, D.B. (2013). Molecular mechanisms of T cell co-stimulation and co-

inhibition. Nat. Rev. Immunol. *13*, 227–242.

Choi, A.-R., Kim, J.-H., Woo, Y.H., Cheon, J.H., Kim, H.S., and Yoon, S. (2016). Co-

treatment of LY294002 or MK-2206 with AZD5363 Attenuates AZD5363-induced

Increase in the Level of Phosphorylated AKT. Anticancer Res. *36*, 5849–5858.

Cochran, J.R., Aivazian, D., Cameron, T.O., and Stern, L.J. (2001). Receptor clustering

and transmembrane signaling in T cells. Trends Biochem. Sci. *26*, 304–310.

Dong, D., Zheng, L., Lin, J., Zhang, B., Zhu, Y., Li, N., Xie, S., Wang, Y., Gao, N., and

Huang, Z. (2019). Structural basis of assembly of the human T cell receptor-CD3

complex. Nature *573*, 546–552.

Esensten, J.H., Helou, Y.A., Chopra, G., Weiss, A., and Bluestone, J.A. (2016). CD28

Costimulation: From Mechanism to Therapy. Immunity *44*, 973–988.

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S.J.C., Hamieh, M.,

Cunanan, K.M., Odak, A., Gönen, M., and Sadelain, M. (2017). Targeting a CAR to the

TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature *543*, 113–117.

Friedman, K.M., Garrett, T.E., Evans, J.W., Horton, H.M., Latimer, H.J., Seidel, S.L.,

Horvath, C.J., and Morgan, R.A. (2018). Effective Targeting of Multiple B-Cell

Maturation Antigen-Expressing Hematological Malignances by Anti-B-Cell Maturation

Antigen Chimeric Antigen Receptor T Cells. Hum. Gene Ther. *29*, 585–601.

Globerson Levin, A., Rivière, I., Eshhar, Z., and Sadelain, M. (2021). CAR T cells:

Building on the CD19 paradigm. Eur. J. Immunol.

Green, J.M., Noel, P.J., Sperling, A.I., Walunas, T.L., Gray, G.S., Bluestone, J.A., and

Thompson, C.B. (1994). Absence of B7-dependent responses in CD28-deficient mice.

Immunity *1*, 501–508.

Green, J.M., Karpitskiy, V., Kimzey, S.L., and Shaw, A.S. (2000). Coordinate regulation

of T cell activation by CD2 and CD28. J. Immunol. *164*, 3591–3595.

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P.,

Rothballer, A., Ascano, M., Jr, Jungkamp, A.-C., Munschauer, M., et al. (2010).

Transcriptome-wide identification of RNA-binding protein and microRNA target sites by

PAR-CLIP. Cell *141*, 129–141.

Harding, F.A., and Allison, J.P. (1993). CD28-B7 interactions allow the induction of

CD8+ cytotoxic T lymphocytes in the absence of exogenous help. J. Exp. Med. *177*,

1791–1796.

Harding, F.A., McArthur, J.G., Gross, J.A., Raulet, D.H., and Allison, J.P. (1992). CD28-

mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-

cell clones. Nature *356*, 607–609.

Hernández, G., García, A., Sonenberg, N., and Lasko, P. (2020). Unorthodox

Mechanisms to Initiate Translation Open Novel Paths for Gene Expression. J. Mol. Biol.

432, 166702.

Hui, E., Cheung, J., Zhu, J., Su, X., Taylor, M.J., Wallweber, H.A., Sasmal, D.K., Huang,

J., Kim, J.M., Mellman, I., et al. (2017). T cell costimulatory receptor CD28 is a primary

target for PD-1-mediated inhibition. Science *355*, 1428–1433.

Huppa, J.B., and Davis, M.M. (2003). T-cell-antigen recognition and the immunological

synapse. Nat. Rev. Immunol. *3*, 973–983.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic

stimulation determines the fate of naive and effector T cells. Immunity *8*, 89–95.

Jiang, Y., Chen, M., Nie, H., and Yuan, Y. (2019). PD-1 and PD-L1 in cancer

immunotherapy: clinical implications and future considerations. Hum. Vaccin.

Immunother. *15*, 1111–1122.

June, C.H., Levine, B.L., Porter, D.L., Kalos, M.D., and Milone, M.C. (2014).

Compositions for treatment of cancer.

Kalos, M., Levine, B.L., Porter, D.L., Katz, S., Grupp, S.A., Bagg, A., and June, C.H.

(2011). T cells with chimeric antigen receptors have potent antitumor effects and can

establish memory in patients with advanced leukemia. Sci. Transl. Med. *3*, 95ra73.

Kamphorst, A.O., Wieland, A., Nasti, T., Yang, S., Zhang, R., Barber, D.L., Konieczny,

B.T., Daugherty, C.Z., Koenig, L., Yu, K., et al. (2017). Rescue of exhausted CD8 T

cells by PD-1-targeted therapies is CD28-dependent. Science *355*, 1423–1427.

Kieft, J.S., Zhou, K., Jubin, R., and Doudna, J.A. (2001). Mechanism of ribosome

recruitment by hepatitis C IRES RNA. RNA *7*, 194–206.

Kleijn, M., and Proud, C.G. (2002). The regulation of protein synthesis and translation

factors by CD3 and CD28 in human primary T lymphocytes. BMC Biochem. *3*, 11.

Kochenderfer, J.N., Feldman, S.A., Zhao, Y., Xu, H., Black, M.A., Morgan, R.A., Wilson,

W.H., and Rosenberg, S.A. (2009). Construction and preclinical evaluation of an anti-

CD19 chimeric antigen receptor. J. Immunother. *32*, 689–702.

Kochenderfer, J.N., Dudley, M.E., Carpenter, R.O., Kassim, S.H., Rose, J.J., Telford,

W.G., Hakim, F.T., Halverson, D.C., Fowler, D.H., Hardy, N.M., et al. (2013). Donor-

derived CD19-targeted T cells cause regression of malignancy persisting after

allogeneic hematopoietic stem cell transplantation. Blood *122*, 4129–4139.

Koning, F., Lew, A.M., Maloy, W.L., Valas, R., and Coligan, J.E. (1988). The

biosynthesis and assembly of T cell receptor alpha- and beta-chains with the CD3

complex. J. Immunol. *140*, 3126–3134.

Lamper, A.M., Fleming, R.H., Ladd, K.M., and Lee, A.S.Y. (2020). A phosphorylation-

regulated eIF3d translation switch mediates cellular adaptation to metabolic stress.

Science *370*, 853–856.

Lee, A.S., Kranzusch, P.J., Doudna, J.A., and Cate, J.H.D. (2016). eIF3d is an mRNA

cap-binding protein that is required for specialized translation initiation. Nature *536*, 96–

99.

Lee, A.S.Y., Kranzusch, P.J., and Cate, J.H.D. (2015). eIF3 targets cell-proliferation

messenger RNAs for translational activation or repression. Nature *522*, 111–114.

Lin, Y., Li, F., Huang, L., Duan, H., Fang, J., Sun, L., Xing, X., Tian, G., Cheng, Y.,

Yang, X., et al. (2019). eIF3 associates with 80S ribosomes to promote translation

elongation, mitochondrial homeostasis, and muscle health.

Manfrini, N., Ricciardi, S., Miluzio, A., Fedeli, M., Scagliola, A., Gallo, S., Brina, D.,

Adler, T., Busch, D.H., Gailus-Durner, V., et al. (2017). High levels of eukaryotic

Initiation Factor 6 (eIF6) are required for immune system homeostasis and for steering

the glycolytic flux of TCR-stimulated CD4+ T cells in both mice and humans. Dev.

Comp. Immunol. *77*, 69–76.

Mao, X., Green, J.M., Safer, B., Lindsten, T., Frederickson, R.M., Miyamoto, S.,

Sonenberg, N., and Thompson, C.B. (1992). Regulation of translation initiation factor

gene expression during human T cell activation. J. Biol. Chem. *267*, 20444–20450.

Meyer, K.D., Patil, D.P., Zhou, J., Zinoviev, A., Skabkin, M.A., Elemento, O., Pestova,

T.V., Qian, S.-B., and Jaffrey, S.R. (2015). 5' UTR m(6)A Promotes Cap-Independent

Translation. Cell *163*, 999–1010.

Michel, F., Attal-Bonnefoy, G., Mangino, G., Mise-Omata, S., and Acuto, O. (2001).

CD28 as a molecular amplifier extending TCR ligation and signaling capabilities.

Immunity *15*, 935–945.

Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D.,

Samanta, M., Lakhal, M., Gloss, B., Danet-Desnoyers, G., et al. (2009). Chimeric

receptors containing CD137 signal transduction domains mediate enhanced survival of

T cells and increased antileukemic efficacy in vivo. Mol. Ther. *17*, 1453–1464.

Miyamoto, S., Patel, P., and Hershey, J.W.B. (2005). Changes in ribosomal binding

activity of eIF3 correlate with increased translation rates during activation of T

lymphocytes. J. Biol. Chem. *280*, 28251–28264.

Mukherjee, N., Wessels, H.-H., Lebedeva, S., Sajek, M., Ghanbari, M., Garzia, A.,

Munteanu, A., Yusuf, D., Farazi, T., Hoell, J.I., et al. (2019). Deciphering human

ribonucleoprotein regulatory networks. Nucleic Acids Res. *47*, 570–581.

Myers, D.R., Wheeler, B., and Roose, J.P. (2019). mTOR and other effector kinase

signals that impact T cell function and activity. Immunol. Rev. *291*, 134–153.

Ohashi, P.S., Mak, T.W., Van den Elsen, P., Yanagi, Y., Yoshikai, Y., Calman, A.F.,

Terhorst, C., Stobo, J.D., and Weiss, A. (1985). Reconstitution of an active surface

T3/T-cell antigen receptor by DNA transfer. Nature *316*, 606–609.

Omer, A., Peres, A., Rodriguez, O.L., Watson, C.T., Lees, W., Polak, P., Collins, A.M.,

and Yaari, G. (2021). T Cell Receptor Beta (TRB) Germline Variability is Revealed by

Inference From Repertoire Data.

Park, J.H., Rivière, I., Gonen, M., Wang, X., Sénéchal, B., Curran, K.J., Sauter, C.,

Wang, Y., Santomasso, B., Mead, E., et al. (2018). Long-Term Follow-up of CD19 CAR

Therapy in Acute Lymphoblastic Leukemia. N. Engl. J. Med. *378*, 449–459.

de la Parra, C., Ernlund, A., Alard, A., Ruggles, K., Ueberheide, B., and Schneider, R.J.

(2018). A widespread alternate form of cap-dependent mRNA translation initiation. Nat.

Commun. *9*, 3068.

Pelletier, J., and Sonenberg, N. (2019). The Organizing Principles of Eukaryotic

Ribosome Recruitment. Annu. Rev. Biochem. *88*, 307–335.

Qin, J.S., Johnstone, T.G., Baturevych, A., Hause, R.J., Ragan, S.P., Clouser, C.R.,

Jones, J.C., Ponce, R., Krejsa, C.M., Salmon, R.A., et al. (2020). Antitumor Potency of

an Anti-CD19 Chimeric Antigen Receptor T-Cell Therapy, Lisocabtagene Maraleucel in

Combination With Ibrutinib or Acalabrutinib. J. Immunother. *43*, 107–120.

Ricciardi, S., Manfrini, N., Alfieri, R., Calamita, P., Crosti, M.C., Gallo, S., Müller, R.,

Pagani, M., Abrignani, S., and Biffo, S. (2018). The Translational Machinery of Human

CD4+ T Cells Is Poised for Activation and Controls the Switch from Quiescence to

Metabolic Remodeling. Cell Metab. *28*, 895–906.e5.

Richard, A.C., Lun, A.T.L., Lau, W.W.Y., Göttgens, B., Marioni, J.C., and Griffiths, G.M.

(2018). T cell cytolytic capacity is independent of initial stimulation strength. Nat.

Immunol. *19*, 849–858.

Scaviner, D., and Lefranc, M.P. (2000). The human T cell receptor alpha variable

(TRAV) genes. Exp. Clin. Immunogenet. *17*, 83–96.

Schrum, A.G., Palmer, E., and Turka, L.A. (2005). Distinct temporal programming of

naive CD4+ T cells for cell division versus TCR-dependent death susceptibility by

antigen-presenting macrophages. Eur. J. Immunol. *35*, 449–459.

Shahinian, A., Pfeffer, K., Lee, K.P., Kündig, T.M., Kishihara, K., Wakeham, A., Kawai,

K., Ohashi, P.S., Thompson, C.B., and Mak, T.W. (1993). Differential T cell

costimulatory requirements in CD28-deficient mice. Science *261*, 609–612.

Siefken, R., Klein-Hessling, S., Serfling, E., Kurrle, R., and Schwinzer, R. (1998). A

CD28-associated signaling pathway leading to cytokine gene transcription and T cell

proliferation without TCR engagement. J. Immunol. *161*, 1645–1651.

van Stipdonk, M.J., Lemmens, E.E., and Schoenberger, S.P. (2001). Naïve CTLs

require a single brief period of antigenic stimulation for clonal expansion and

differentiation. Nat. Immunol. *2*, 423–429.

Tan, H., Yang, K., Li, Y., Shaw, T.I., Wang, Y., Blanco, D.B., Wang, X., Cho, J.-H.,

Wang, H., Rankin, S., et al. (2017). Integrative Proteomics and Phosphoproteomics

Profiling Reveals Dynamic Signaling Networks and Bioenergetics Pathways Underlying

T Cell Activation. Immunity *46*, 488–503.

Tauber, D., Tauber, G., and Parker, R. (2020). Mechanisms and Regulation of RNA

Condensation in RNP Granule Formation. Trends Biochem. Sci. *45*, 764–778.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J.,

Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mammalian target

of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J. Biol. Chem.

284, 8023–8032.

Tuosto, L., and Acuto, O. (1998). CD28 affects the earliest signaling events generated

by TCR engagement. Eur. J. Immunol. *28*, 2131–2142.

Wagner, S., Herrmannová, A., Hronová, V., Gunišová, S., Sen, N.D., Hannan, R.D.,

Hinnebusch, A.G., Shirokikh, N.E., Preiss, T., and Valášek, L.S. (2020). Selective

Translation Complex Profiling Reveals Staged Initiation and Co-translational Assembly

of Initiation Factor Complexes. Mol. Cell *79*, 546–560.e7.

Wang, M., Munoz, J., Goy, A., Locke, F.L., Jacobson, C.A., Hill, B.T., Timmerman, J.M.,

Holmes, H., Jaglowski, S., Flinn, I.W., et al. (2020). KTE-X19 CAR T-Cell Therapy in

Relapsed or Refractory Mantle-Cell Lymphoma. N. Engl. J. Med. *382*, 1331–1342.

Watanabe, K., Kuramitsu, S., Posey, A.D., Jr, and June, C.H. (2018). Expanding the

Therapeutic Window for CAR T Cell Therapy in Solid Tumors: The Knowns and

Unknowns of CAR T Cell Biology. Front. Immunol. *9*, 2486.

Wolf, T., Jin, W., Zoppi, G., Vogel, I.A., Akhmedov, M., Bleck, C.K.E., Beltraminelli, T.,

Rieckmann, J.C., Ramirez, N.J., Benevento, M., et al. (2020). Dynamics in protein

translation sustaining T cell preparedness. Nat. Immunol. *21*, 927–937.

Wu, Y.-H., Huang, Y.-F., Chen, C.-C., Huang, C.-Y., and Chou, C.-Y. (2020).

Comparing PI3K/Akt Inhibitors Used in Ovarian Cancer Treatment. Front. Pharmacol.

11, 206.

Xu, Z.Z., and Mathews, D.H. (2016). Prediction of Secondary Structures Conserved in

Multiple RNA Sequences. Methods Mol. Biol. *1490*, 35–50.

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Project and experiments were conceived and designed by D.D.S. and J.H.D.C. D.D.S.

carried out all experiments, with assistance from B.E.S. and G.H.C. for image

acquisition and analysis, from T.R., R.A.A., F.B. and A.M. for primary T cell genome

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Figure 1. eIF3 interacts with specific mRNAs related to immune function in activated Jurkat cells. (**A**) Schematic of the PAR-CLIP experiment in Jurkat cells, showing steps through cell harvesting. (**B**) Phosphorimage of SDS polyacrylamide gel resolving 5′ ^{32}P-labeled RNAs crosslinked to eIF3 subunits in activated and non-activated Jurkat cells, from one of two biological replicates. (**C**) Venn diagram of genes identified by eIF3 PAR-CLIP in activated Jurkat cells from two biological replicates, compared with

the eIF3 PAR-CLIP mRNAs previously identified in HEK293T cells. The same number of

mRNAs from the Jurkat cell PAR-CLIP libraries, ranked by total reads mapped to a given

gene, are compared to the number of mRNAs identified in the HEK293T PAR-CLIP

experiments. (**D**) Varied mRNA crosslinking patterns to eIF3 in activated Jurkat cells.

Cumulative plot showing mRNA crosslinking in sample EIF3A/C/B to predominantly the

5'-UTR (n = 396, 414 mRNAs in replicates 1 and 2, respectively), and across the entire

length of some mRNAs ("pan-mRNAs", n = 634, 621 mRNAs). The 5'-UTR, CDS, and 3'-

UTR regions are shown normalized by length. (**E**) Crosslinking of the eIF3 subunits as

indicated across the entire *TCRA* and *TCRB* mRNAs, in activated and non-activated

Jurkat cells. 5'-UTR, coding sequence (CDS), and 3'-UTR elements (below) along with

the variable (V), joining (J) and constant (C) regions (above) for the mapped TCR genes

in Jurkat cells are shown. The blue and red vertical lines in the plotted reads indicate the

amount of T-C transitions vs other mutations, respectively for a particular nucleotide. The

TCRA and *TCRB* mRNAs are present in both non-activated and activated Jurkat cells

(**Supplementary File 4**). (**F**) FISH analysis of *TCRA* and *TCRB* mRNAs (yellow and

magenta, respectively) and P bodies (top) marked by the location of DCP1 (cyan) and

stress granules (bottom) marked by the location of G3BP1 (cyan), in activated Jurkat

cells. Graphs to the right of the images indicate Pearson's correlation coefficients (PCCs)

of *TCRA* and *TCRB* mRNAs localizing with each other or with P bodies or stress granules.

TetraSpeck^{TM} microsphere beads were used as a positive control for colocalization.

Labels on the x axis are, B: TetraSpeck^{TM} microsphere beads, C: activated Jurkat cells.

(*n* = 5, P <0.008, for PCC values of cells relative to bead colocalization, across all the

channels tested, using the Wilcoxon rank-sum test). Images are representative of one

experiment of the five independent experiments in the graphs.

Figure 2. eIF3 remains bound to the coding sequences (CDS) of pan-mRNAs

independent of their 5'-UTR and 3'-UTR elements in actively translating ribosomes.

(**A**) Schematic outlining the RNase H-based assay of eIF3 interactions with mRNAs in

polysomes. DSP refers to the dithiobis (succinimidyl propionate) crosslinking agent.

Oligos, DNA oligos designed for RNase H-mediated targeting and cleavage of specific

mRNAs. (**B**) Strategy for detecting mRNA fragments released by RNase H digestion. Red

arrows denote DNA oligos for RNase H-mediated targeting of mRNAs. RT-qPCR primers

(black) were used to detect the CDS regions of the mRNAs. (**C**) Amount of eIF3-bound

mRNA co-immunoprecipitated by an anti-EIF3B antibody (Lee et al., 2015), from

polysome fractions of Jurkat cells treated with RNase H and oligos targeting the CDS-

UTR junctions (red arrows diagrammed in panel **B**). (**D**) Amount of eIF3-bound mRNA

co-immunoprecipitated with IgG beads, from polysome fractions of Jurkat cell lysate

treated with RNase H and oligos targeting the CDS-UTR junctions. (**E**) Amount of eIF3-

bound mRNA co-immunoprecipitated by the anti-EIF3B antibody, from polysome

fractions of Jurkat cell lysate treated only with RNase H. (**F**) Amount of eIF3-bound mRNA

co-immunoprecipitated by an anti-EIF3B antibody, from polysome fractions of primary

human T cells treated with RNase H and oligos targeting the CDS-UTR junctions (red

arrows diagrammed in panel **B**). (**G**) Amount of eIF3-bound mRNA co-

immunoprecipitated with IgG beads, from polysome fractions of primary human T cell

lysate treated with RNase H and oligos targeting the CDS-UTR junctions. (**H**) Amount of

eIF3-bound mRNA co-immunoprecipitated by the anti-EIF3B antibody, from polysome

fractions of primary human T cell lysate treated only with RNase H. In panels **C**–**H**, the

percentage is relative to the amount of total mRNA present in the polysome fraction prior

to immunoprecipitation. All the immunoprecipitation experiments in panels **C**–**H** were

carried out in biological duplicate with one technical triplicate shown (n = 3, with mean

and standard deviations shown). The primary human T cell experiment was done using

2 donors.

Figure 3. Interaction of eIF3 with *TCRA* and *TCRB* mRNA 3'-UTR elements mediates a burst in TCR translation in primary human T cells. (**A**) Schematic of nanoluciferase reporters stably expressed in primary human T cells. The reporters carry the *HBB* 5'-UTR and WT, *ΔPAR* or R**PAR* 3'-UTRs. WT, intact 3'-UTR from either *TCRA* or *TCRB* mRNA; *ΔPAR*, 3'-UTR of *TCRA* or *TCRB* with the eIF3 PAR-CLIP site deleted; R**PAR*, reversed

PAR-CLIP sequence in the 3'-UTR of *TCRA* or *TCRB* mRNA. (**B**) Luciferase activity in

anti-CD3/anti-CD28 activated T cells stably expressing nanoluciferase constructs

described in **A**, relative to non-activated controls (ACT/NoACT). (**C**) Changes in

nanoluciferase mRNA levels in **B**, as determined by qRT-PCR. (**D**) Schematic of

immunoprecipitation of eIF3 using an anti-EIF3B antibody (Lee et al., 2015), followed by

qRT-PCR to quantify the amount of nanoluciferase mRNA bound to eIF3. (**E**)

Immunoprecipitation as shown in **D** showing the amount of nanoluciferase mRNA bound

to eIF3 in T cells stably transduced with either *TCRA* (left) or *TCRB* (right) WT, *ΔPAR* or

R**PAR* 3'-UTRs after activation with anti-CD3/anti-CD28 antibodies for 1 hour. The

percent mRNA bound to anti-EIF3B beads is calculated relative to total mRNA isolated

from the cells. (**F**) Schematic of nanoluciferase reporters stably expressed in primary

human T cells. Nanoluciferase reporters carry the *HBB* 5'-UTR and WT, *ΔPAR,* R**PAR*

of the *TCRA* 3'-UTR, or *ΔPAR* of the *TCRA* 3'-UTR replaced with either a *JUN* 5'-UTR

hairpin or hepatitis C viral (HCV) internal ribosome entry site (domain IIIabc). (**G**)

Luciferase activity in anti-CD3/anti-CD28 activated T cells stably expressing

nanoluciferase constructs described in **F**, relative to non-activated controls. (**H**) Changes

in nanoluciferase mRNA levels in **G**, as determined by qRT-PCR. (**I**) Immunoprecipitation

as shown in **D** to quantify the amount of nanoluciferase mRNA bound to eIF3 in T cells

stably expressing nanoluciferase constructs in **F** after activation with anti-CD3/anti-CD28

antibodies for 0.5, 1, 3 and 5 hours. The percent mRNA bound to anti-EIF3B beads is

calculated relative to total mRNA isolated from the cells. All experiments were carried out

in triplicate (3 separate wells per condition), with mean and standard deviations shown.

All the primary human T cell experiments were performed using two donors and data from

one representative donor is shown.

Figure 4. The burst in nanoluciferase reporter translation requires membrane-proximal CD28 signaling. (**A**) Schematic of the membrane-tethered nanoluciferase reporters stably expressed in primary human T cells. (Top) Wild-type CD3-zeta protein with asterisk indicating two amino acids mutated in the transmembrane region to prevent association with the TCR (Dong et al., 2019) (EX: extracellular, TM: transmembrane). (Bottom) Schematic of the nanoluciferase reporters. Nanoluciferase is fused C-terminal to the extracellular and transmembrane segments of CD3-zeta, mutated to prevent TCR association. The reporters carry the *HBB* 5'-UTR and *TCRA, TCRB*, *TCRA ΔPAR,* or

TCRB ΔPAR 3'-UTR. (**B**) Luciferase activity in primary human T cells stably expressing

membrane-tethered reporters described in **A** stimulated with anti-CD3/anti-CD28

antibodies, relative to non-activated controls (ACT/NoACT). (**C**) Luciferase activity in

primary human T cells stably expressing membrane-tethered reporters described in **A**,

and activated only with anti-CD28 antibodies, relative to non-activated controls. (**D**)

Luciferase activity in primary human T cells stably expressing membrane-tethered

reporters described in **A**, and activated only with anti-CD3 antibodies, relative to non-

activated controls. (**E**) Luciferase activity in primary human T cells stably expressing

membrane-tethered reporter with WT *TCRA* 3'-UTR described in **A** inhibited with either

AZD5363(AZD) to inhibit AKT activity or Torin 1 to inhibit mTOR before activating with

anti-CD3/anti-CD28 antibodies, relative to non-activated controls. In panels **A**–**E**, all

experiments were carried out in triplicate (3 separate wells per condition), with mean and

standard deviations shown. (**F**) Western blot carried out to measure AKT activity in the

presence of AZD5363 (AZD) using an anti-Phospho-GSK-3β antibody or mTOR activity

in the presence of Torin 1 using an anti-Phospho-4EBP1 antibody, for the samples in **E**.

HSP90 was used as a loading control. All the primary human T cell experiments were

performed using two donors and data from one representative donor is shown.

Figure 5. eIF3 binding to the *TCRA* and *TCRB* mRNA 3'-UTR elements is required

for a rapid burst in TCR translation and robust activation of primary human T cells.

(**A**) Western blots of TCRA protein levels in T cells as a function of time after different

modes of activation. HSP90 was used as a loading control. (**B**) Western blots measuring

TCRA protein levels as a function of time after anti-CD3/anti-CD28 activation. Cell lines

used are labeled on the left: *TCRA ΔPAR*, *TCRB ΔPAR*, and SC (scrambled gRNA).

HSP90 was used as a loading control. Schematics of *TCRA* and *TCRB* mRNAs with and

without eIF3 PAR-CLIP sites are shown above. SC control cells have the WT 3'-UTRs for

TCRA and *TCRB* mRNAs. (**C**) The number of T cells with one or more TCR clusters

measured by anti-TCRA/anti-TCRB protein staining and epifluorescence microscopy as

a function of time after anti-CD3/anti-CD28 activation. A total of 100 cells from each donor

were imaged for *TCRA ΔPAR* (*n* = 2 donors, stained with anti-TCRA antibody), *TCRB*

ΔPAR (*n* = 2 donors, stained with anti-TCRB antibody), and SC cell lines (*n* = 2 donors,

each stained separately with anti-TCRA and anti-TCRB antibodies). Values are mean ±

standard deviation. (**D**) Flow cytometric analysis measuring T cell activation markers

CD69 (early activation marker) and CD25 (mid-activation marker), quantifying the mean

percent of CD4+ T cells that are CD69+ CD25+. (**E**) Flow cytometric analysis of CD8+ T

cells, quantifying the mean percent of CD8+ T cells that are CD69+ CD25+. Cells sorted

as shown in **Figure 5–figure supplement 2**. (**F**) Quantification of IL2 secreted from SC,

TCRA ΔPAR, TCRB ΔPAR and *ΔTCR* cell populations at different time points after

stimulation with anti-CD3/anti-CD28 antibodies, as determined by ELISA. (**G**)

Quantification IFNγ secreted from the cells in **F**, as determined by ELISA. In panels **D**–**G**,

all experiments were carried out in triplicate (3 separate wells per condition), with mean

and standard deviations shown. Representative results from 1 donor are shown.

Figure 6. The *TCRA* and *TCRB* mRNA 3'-UTR elements enhance CAR T cell

function. (**A**) (Top) Schematic of membrane-tethered nanoluciferase reporters stably

expressed in primary human T cells. The reporters have the *HBB* 5'-UTR and either the

TCRA 3'-UTR, the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

(*WPRE*) or the gammaretroviral 3'-Long terminal repeat (*3'-LTR*) as 3'-UTR. (Bottom)

Luciferase activity in primary human T cells stably expressing membrane-tethered

nanoluciferase reporters described above and activated with anti-CD3/anti-CD28

antibodies, relative to non-activated cells. Representative results from one of two donors

are shown (n = 3 separate wells, with mean and standard deviations shown). (**B**)

Schematics of FMC63-BBZ CAR cDNA sequence fused 5' of the *WPRE, 3'-LTR, TCRA*

3'-UTR or *TCRB* 3'-UTR*.* The membrane-tethered nanoluciferase reporter with *TCRA* 3'-

UTR was used as a control for the effects of lentiviral expression. (**C**) Timeline of CAR T

cell generation from primary human T cells and the experiments performed. (**D**) Western

blots measuring FMC63-BBZ CAR protein levels as a function of time after incubation

with NALM6 cells. CAR T cells expressing the constructs in **B** are labeled: Control*,*

WPRE, 3'-LTR, TCRA and *TCRB* 3'-UTR. HSP90 was used as a loading control (n = 2

donors). (**E**) Schematic describing the cytotoxicity assay used to detect live tumor cells

after incubation with FMC63-BBZ CAR cells using flow cytometric analysis. (**F**) Cytotoxic

activity of FMC63-BBZ CARs fused to various 3'-UTRs described in **B** after incubating

with NALM6 cells for 24 and 48 hours. (**G**) Cytotoxic activity of FMC63-BBZ CARs fused

to various 3'-UTRs described in **B** after incubating with Jeko 1 cells for 24 and 48 hours.

In panels **F** and **G**, representative results for one of two donors are shown (n = 3 separate

wells, with mean and standard deviations shown).

Figure 7. Model for robust T cell activation and improved CAR T cell function mediated by eIF3 interactions with the *TCRA* and *TCRB* mRNAs. Left, a T cell activated upon antigen recognition by the TCR and CD28 costimulatory signal leads to recruitment of the *TCRA* and *TCRB* mRNAs to translation "hot-spots" due to eIF3 binding to the mRNA 3'-UTRs. This results in a short burst in TCRA and TCRB translation followed by a robust increase in T cell function as measured by CD69 and CD25 expression and cytokine release. Right, activation of CAR T cells expressing CAR mRNAs with the *TCRA* or *TCRB* 3'-UTRs leads to a burst in CAR translation and improved tumor cell killing.

Supplementary Information for

Robust T cell activation requires a burst in T cell receptor translation

mediated by eIF3

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This PDF file includes:

Materials and Methods

Figure supplements

Captions to Supplemental Files 1-6

Other Supplemental Information for this manuscript include the following:

Supplemental Files 1-6

Materials and Methods

Jurkat cell culture

Human Jurkat, Clone E6-1 (ATCC TIB-152) was purchased from American Type

Culture Collection (ATCC) and was maintained in RPMI 1640 Medium (ATCC

modification) with 10% FBS (VWR Life Science Seradigm) and 0.01% Penicillin-

Streptomycin (10,000 U/mL) (ThermoFisher). The cells were maintained between 1

million to 8 million cells/mL. When cells were stimulated they were always maintained at

8 million cells/mL.

Jurkat cell stimulation

The Jurkat cells used for the PAR-CLIP experiment were stimulated with 1X Cell

Stimulation Cocktail, containing phorbol 12-myristate 13-acetate (PMA) and ionomycin

(Thermofisher, Cat. #: 00-4970-93) to ensure a large proportion of the cells were

activated. Unless otherwise stated, all other experiments involving activated Jurkat cells

used anti-CD3/anti-CD28 antibodies (Tonbo) for stimulation. Flat bottom plates were

coated with anti-CD3 antibody at a 10 µg/mL concentration, and anti-CD28 antibody was

added to the cell culture media at a concentration of 5 µg/mL.

Isolation of human primary T cells

Primary human T cells were isolated from healthy human donors from

leukoreduction chambers after Trima Apheresis (Vitalant, formerly Blood Centers of the

Pacific). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood

samples by Ficoll centrifugation using SepMate tubes (STEMCELL) per manufacturer's

instructions. T cells were isolated from PBMCs from all cell sources by magnetic negative

selection using an EasySep Human T Cell Isolation Kit (STEMCELL), per manufacturer's

instructions. Unless otherwise noted, isolated T cells were stimulated with anti-CD3/anti-

CD28 antibodies (Tonbo) as described above and used immediately.

<u>Primary human T cell culture</u>

Bulk T cells were cultured in XVivo15 medium (Lonza) with 5% fetal bovine serum

(FBS), 50 µM 2-mercaptoethanol (Sigma), and 10 µM *N*-acetyl-cystine (Sigma) or

ImmunoCult™-XF T Cell Expansion Medium (StemCell). Immediately after isolation, T

cells were either frozen or stimulated for 2 days with anti-human CD3/CD28 magnetic

Dynabeads (ThermoFisher) at a bead to cell concentration of 1:1, along with cytokine IL-

2 (UCSF Pharmacy). For T cells cultured after electroporation, the media was

supplemented with IL-2 at 500 U/mL. Throughout the culture period T cells were

maintained at an approximate density of 1 million cells per mL of media. Every 2–3 days

after electroporation, additional media was added, along with fresh IL-2 at 500 U/mL

concentration, and the cells were transferred to larger culture flasks as necessary to

maintain a density of 1 million cells per mL. For all the other times T cells were maintained

at 50U/mL IL-2 with the addition of fresh media every 2 days.

<u>Primary human T cell stimulation</u>

For edited primary human T cells, the cells were transferred to fresh media lacking

IL-2 after 9 days of culturing. The T cells were then stimulated with anti-CD3/anti-CD28

antibodies using flat bottom plates coated with anti-CD3 antibody (Tonbo) at a 10 µg/mL

concentration, and anti-CD28 antibody (Tonbo) added to the cell culture media at a

concentration of 5 µg/mL. In all other primary human T cell stimulation experiments the

cells were stimulated with anti-CD3/anti-CD28 antibodies as mentioned above along with

50 U/mL IL-2.

<u>4-thiouridine optimization for PAR-CLIP experiments</u>

We used Jurkat cells as a model for T cells, as PAR-CLIP experiments require a

large number of cells labeled with 4-thiouridine (Sigma) at a non-toxic concentration

(Ascano et al., 2012). Jurkat cells also have a defined T cell receptor and transcriptome,

avoiding the donor-to-donor variability of primary T cells. Jurkat cells were seeded, so

that they reached 8×10^5 cells ml^{-1} seeding density on the day of the experiment. Varying

concentrations of 4-thiouridine (s4U) were added to the cells (50 µM, 75 µM, 100 µM, or

none as a negative control). The cells were then incubated for different time points: 8

hours, 10 hours, 12 hours, or 16 hours. After each incubation time cell viability was

determined using the CellTiter-Glo assay (Promega), according to the manufacturer's

instructions. Concentrations at which the relative luminescence in the presence of s^{4}U

(luminescence of the s^{4}U treated cells/luminescence of the untreated cells) exceeded

95% were considered non-toxic. Based on these measurements, we used 50 µM of 4-

thiouridine for PAR-CLIP experiments.

PAR-CLIP

Two biological replicates were used to perform PAR-CLIP analysis as described

in (Lee et al., 2015), with modifications for Jurkat cells. 50 µM of 4-thiouridine was

determined as non-toxic to Jurkat cells over the time course of the PAR-CLIP experiments

(Ascano et al., 2012). A total of 55 million Jurkat cells seeded at 8 million cells ml^{-1} was

treated with 50 µM of 4-thiouridine for 7 hours, then stimulated with 1X Cell Stimulation

Cocktail for 5 hours in media containing 50 µM of 4-thiouridine (**Figure 1A**). The same

number of cells were treated with 50 µM of 4-thiouridine for 12 hours without stimulation

as a non-activated control. The cells were then crosslinked on ice with 365 nm UV

irradiation at an energy dose of 0.2 J cm^{-2}. The cells were pelleted by centrifugation at

100 x g for 15 min at 4 °C, and the pellet was resuspended in three volumes of NP40 lysis

buffer (50 mM HEPES-KOH pH 7.5, 150 mM KCl, 2 mM EDTA, 0.5% Nonidet P-40

alternative, 0.5 mM dithiothreitol (DTT), 1 Complete Mini EDTA-free Protease Inhibitor

Cocktail tablet (Roche)). The cell suspension was then incubated on ice for 10 min,

passed through an 18G needle five times, and centrifuged at 13,000 x g for 15 min at 4

°C and RNAs were lightly digested by treatment with MNase (Thermo Scientific) at a final

concentration of 0.05 U µl^{-1} for 20 min at 16 °C. For each PAR-CLIP assay 1000 µl of

Dynabeads (Invitrogen) and 800 µl of anti-EIF3B antibody (Bethyl A301-761A) were used.

The remaining steps of the PAR-CLIP analysis were performed exactly as described in

(Danan et al., 2016; Lee et al., 2015) with the exception of using MNase at 5 U µl^{-1} for the

on-bead digestion step.

Mass spectrometry

To identify eIF3 subunits that crosslinked with RNAs in the PAR-CLIP experiments,

a portion of eIF3 immunoprecipitated using Dynabeads as described above were treated

with nonradioactive ATP (NEB) during the T4 polynucleotide kinase labeling step. The

nonradioactive samples were then run on the same gel next to the radiolabeled PAR-

CLIP samples, Coomassie stained (Pierce) and the bands that matched with the

phosphorimager printout were excised from the gel and submitted for identification using

one-dimensional LC-MS/MS (**Supplementary File 1**).

<u>PAR-CLIP computational analysis</u>

PAR-CLIP cDNA libraries were sequenced on an Illumina HiSeq 2500 platform.

To eliminate potential PCR biases during PAR-CLIP library preparation, a random bar

code was introduced into the 3' adapter and all the reads that matched the random

barcode were collapsed into single reads. Clusters of overlapping sequence reads

mapped against the human genome version hg38 were generated using the PARalyzer

software (Corcoran et al., 2011) incorporated into the PARpipe pipeline

(https://ohlerlab.mdc-berlin.de/software/PARpipe_119/, (Mukherjee et al., 2019) with the

settings below. Binding sites were categorized using the Gencode GRCh38.p12 GTF

annotations (gencode.v21.annotation.gtf), https://www.gencodegenes.org/human/.

The PARpipe settings used were:

Conversion = T>C

Minimum read count per group = 5

Minimum read count per cluster = 7

Minimum read count for kde = 3

Minimum cluster size = 11

Minimum conversion locations for cluster = 2

Minimum conversion count for cluster = 2

Minimum read count for cluster inclusion = 1

Minimum read length = 20

Maximum number of non conversion mismatches = 1

Comparison of eIF3 PAR-CLIP results in Jurkat and HEK293T cells

To compare RNAs in activated Jurkat cells crosslinked to eIF3 with those

crosslinked to eIF3 in HEK293T cells (Lee et al., 2015), the gene cluster lists

(*.gene_cl.csv) from APA_REP1, APA_REP2, APB_REP1, APB_REP2, APD_REP1,

and APD_REP2 were used (see **Supplementary File 3**). The genes were first sorted by

total read counts ("ReadCountSum") from high to low for each library to obtain the top

candidate genes. Then, the same number of top candidate genes from these sorted lists

as the number of genes identified in HEK293T cells, for eIF3 subunit EIF3A, EIF3B, and

EIF3D crosslinked to RNA (Lee et al., 2015), were chosen for comparison.

PAR-CLIP Pathway analysis

To determine biological pathways enriched in the set of mRNAs that crosslinked

to eIF3 in activated Jurkat cells, genes with at least 100 total aligned reads were used,

as determined in the PARpipe analysis described above (Mukherjee et al., 2019), from

the EIF3A/C/B samples. Since PAR-CLIP reads are short, it is not possible to determine

with certainty which mRNA transcript isoform cross-linked with eIF3. Therefore the most

abundant mRNA transcript isoform for each gene was chosen, as determined by

transcriptome profiling using kallisto (protein_coding category) (Bray et al., 2016), as

described in the Transcriptome Profiling section. Even with this choice, eIF3 crosslinks to

mRNAs do not correlate with mRNA abundance (**Figure 1–figure supplement 1F**).

Human genome GRCh38.p13 annotation was used to extract mRNA lengths by 5'-UTR,

coding region and 3'-UTR (Ensembl Biomart) (Cunningham et al., 2019). These genes

were then sorted by the density of PAR-CLIP reads in the mRNA 5'-UTR region, prior to

mapping pathways of transcripts that crosslinked to eIF3. Due to the complexity of *TCRA*

and *TCRB* transcript annotation, these transcripts were excluded from the normalization

calculation, but included in the pathway analysis. The top 500 genes from the resulting

EIF3A/C/B PAR-CLIP gene lists were used, sorted as described above, to analyze gene

enrichment profiles in the STRING database (Szklarczyk et al., 2019). The top tissue-

specific categories involve the immune system and T cell function (**Supplementary File**

5). Note that the STRING database categories are not mutually exclusive gene lists, and

do not include TCR subunits in its analysis.

<u>Metagene analysis</u>

The PAR-CLIP genes sorted as described above in the "PAR-CLIP pathway

analysis" were used and mapped against the most abundant mRNA transcript isoforms

to generate cumulative plots of the reads. Reads for the *TCRA* and *TCRB* mRNAs were

manually extracted from the Bowtie version 1.0.0 Hg38 alignment of the eIF3 PAR-CLIP

reads. We did not identify reads mapped to the D segment of *TCRB* (e.g. to *TRBD2*) due

to its short length of 16 nucleotides. These reads were combined with the mapped reads

in the *.read.csv files generated by Parpipe. The combined reads were then sorted to

extract only reads annotated as 5'-UTR, start codon, coding, stop codon, and 3'-UTR.

The most abundant transcript isoform, as identified in the Transcriptome Profiling section

using kallisto (described below) was used. Reads mapped to the 5'-UTR and start codon

were normalized by the length of the 5'-UTR. Reads mapped to the coding region and

stop codon were normalized by the length of the coding region. Finally, reads mapped to

the 3'-UTR were normalized to the length of the 3'-UTR. Relative positions of the mapped

reads along a metagene were computed based on the locations each mapped read fell

within its respective feature. Relative positions were from -1 to 0 for the 5'-UTR, 0 to 1 for

the coding region, and 1 to 2 for the 3'-UTR. 5'-UTR values were computed by multiplying

the relative position by -1, whereas the 3'-UTR values were computed by adding 1 to the

relative position. Coding region relative positions were unchanged.

The empirical cumulative distribution frequency function from R package ggplot2

(Wickham et al., 2016) was used to build the metagene plots from the vector of relative

positions for the reads which mapped to a given set of reads. We defined mRNAs having

a ratio of normalized 5'-UTR reads divided by 3'-UTR reads of 20 or more as "5'-UTR

enriched" with respect to eIF3 crosslinking. All others were categorized as "pan-mRNAs,"

with eIF3 crosslinking across the entire length of the mRNA. The cut-off value 20 is not a

sharp boundary between the two categories of mRNA (**Figure 1–figure supplement 1G**).

Transcriptome Profiling

RNA samples were extracted from non-activated Jurkat cells or Jurkat cells activated for

5 hr with I+PMA, using the Direct-zol RNA Miniprep kit (Zymoresearch). The libraries were

prepared using TruSeq Stranded RNA LT Ribo-Zero Gold kit (Illumina) following the

manufacturer's instructions, with two biological replicates. Cutadapt (version 2.6) (Martin,

2011) with a minimum read length of 20, 5' end with a cutoff of 15 and the 3' end with a

cutoff of 10 in paired-end mode was used to remove adapters. RNA-seq reads were

pseudoaligned using kallisto v.0.46.0 run in quant mode with default parameters to

estimate transcript abundance (transcripts per million, TPM) (Bray et al., 2016). The

transcript index for kallisto was made with default parameters and GENCODE Release

32 (GRCh38.p13) FASTA file (Frankish et al., 2019).

RNA-FISH and immunofluorescence

Jurkat cells were washed with PBS, fixed with 3.7% (vol./vol.) paraformaldehyde

(VWR) for 10 min at room temperature and washed three times with PBS. PBS was

discarded and 1 ml 70% ethanol was added. The cells were incubated at 4°C for 16 hours.

The 70% ethanol was aspirated and the cells were washed once with 0.5 ml Stellaris RNA

wash buffer A (Biosearch technologies). The cells were then incubated with 100 µl

Stellaris hybridization buffer (Biosearch Technologies) containing Stellaris RNA FISH

probes (Biosearch Technologies) at a final concentration of 125 nM (**Supplementary File**

6) and with the relevant antibody (**Supplementary File 6**) for 16 hours at 28 °C. The cells

were then washed twice with 0.5 ml Stellaris RNA wash buffer A containing secondary

antibody conjugated with a fluorophore for 30 minutes at 37 °C in the dark. The second

Stellaris RNA wash buffer A contained DAPI in addition to the secondary antibody. Finally

the cells were washed once with 0.5 mL Stellaris RNA wash buffer B and mounted with

mounting solution (Invitrogen). All high resolution images were taken using confocal

ZEISS LSM 880 in Airyscan super-resolution mode, equipped withA Plan-Apochromat

63x/1.4 Oil objective (Zeiss). To measure colocalization of *TCRA* and *TCRB* mRNAs with

each other or with P bodies (using DCP1 antibody, **Supplementary File 6**) or stress

granules (G3BP1 antibody, **Supplementary File 6**) the cells were mounted along with

0.1 µm TetraSpeck™ microspheres (ThermoFisher) adhered to the slide according to

manufacturer's instructions, to be able to account for the chromatic shift during image

acquisition.

<u>Colocalization analysis</u>

To measure colocalization of *TCRA* and *TCRB* mRNAs with each other or with P

bodies (using DCP1 antibody) or stress granules (G3BP1 antibody), immunofluorescently

labelled cells (see above) were mounted along with 0.1 µm TetraSpeck™ microspheres

(ThermoFisher) adhered to the slide according to manufacturer's instructions. The

microspheres allowed for the correction of lateral and axial chromatic aberrations during

image acquisition.

Z-stacks were acquired with 35 nm x 35 nm x 190 nm voxels on a ZEISS LSM 880

in Airyscan super-resolution mode, equipped with a Plan-Apochromat 63x/1.4 Oil

objective (Zeiss). The images were then deconvolved to a lateral resolution of ≈150 nm

and an axial resolution of ≈500 nm (as confirmed by observing the discrete Fourier

transform of the z-stacks). After imaging a single cell, beads that were on the slide axial

to the cell were imaged to measure the corresponding lateral and axial chromatic

aberrations.

To quantify the relative colocalization, we developed an automated processing and

analysis   pipeline   in   ImageJ   1.52p   available   on   github:

https://github.com/Llamero/TCR_colocalization_analysis-macro.   Specifically,   the

chromatic aberrations in the z-stacks were compensated for by registering the channels

of the bead z-stack to one another, and then applying the same registration vectors to the

corresponding channels in the cell z-stack (Parslow et al., 2014). Each channel of a z-

stack was then thresholded to remove background in the image, and then the

colocalization between each pair of images was measured using the Pearson's

correlation coefficient. Samples in which any pair of channels in the bead z-stack had a

correlation of less than 0.45 were removed from final analysis, as this suggested that the

images had insufficient dynamic range in at least one of the channels for an accurate

deconvolution.

Polysome analysis of eIF3-associated mRNAs

To isolate polysomes from Jurkat cells, the cells were seeded to reach 8×10^5

cells/mL on the day of harvest and then stimulated with anti-CD3/anti-CD28 antibodies as

described above for 5 hours. To isolate polysomes from primary human T cells, the cells

were seeded to reach 1 x 10^6 cells/mL on the day of harvest and then stimulated with anti-

CD3 and anti-CD28 antibodies as described above for 1 hour. Both Jurkat and primary

human T cells were treated with 100 µg ml^{-1} cycloheximide (VWR) 5 minutes before

harvesting. Cells were then collected into a 50 ml falcon tube and rinsed once with ice

cold PBS (ThermoFisher) supplemented with 100 µg ml^{-1} cycloheximide. The cells were

then incubated with 0.5 mM of the crosslinking reagent dithiobis (succinimidyl propionate)

(DSP, Thermofisher, Cat. #: PG82081) and 100 µg ml^{-1} cycloheximide in PBS at room

temperature for 15 minutes, with rocking. The crosslinking reagent was then removed and

the cells were incubated with quenching reagent (PBS, 100 µg ml^{-1} cycloheximide and

300 mM Glycine(Sigma)) for 5 minutes on ice. The cells were then rinsed again with ice

cold PBS and flash frozen in liquid nitrogen.

A total of 4 x 10^8 cells were lysed with 400 µl hypotonic lysis buffer (10 mM Hepes

pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 1% Triton, 100 µg ml^{-1} cycloheximide,

and one Complete EDTA-free Proteinase Inhibitor Cocktail tablet (Roche)). The cells

were incubated for 10 min on ice and then passed through an 18G needle five times, and

centrifuged at 13,000 × g for 15 min at 4 °C. The 400 µl supernatant was then transferred

to a fresh eppendorf tube and subjected to RNase H digestion by adding the following

reagents: 3 mM MgCl$_2$, 10 mM DTT, 200 units RNase H (NEB) and 20 µl of SUPERasIN

(ThermoFisher), with a total of 4 µM DNA oligos (IDT), as indicated in the figure legends.

The mixture was then incubated at 37 °C for 20 minutes. After incubation 10 µl of the

RNase H treated lysate mixture was isolated to test the efficiency of the RNase H

digestion using qRT-PCR (**Figure 2–figure supplement 1B, 1D, and 1G**) and the rest of

the lysate was layered onto a 12 ml 10%-50% sucrose gradient, made with gradient buffer

consisting of: 10% sucrose (w/v) or 50% sucrose (w/v), 100 mM KCl, 20 mM Hepes pH

7.6, 5 mM $MgCl_2$, 1 mM DTT and 100 μg ml^{-1} cycloheximide. The gradient was centrifuged

at 36,000 rpm (222k x g) for 2 hours at 4 °C in a SW-41 rotor. The gradient was then

fractionated using the Brandel gradient fractionator and ISCO UA-6 UV detector and all

the polysome fractions (~ 5 ml) were collected into a fresh 15 ml falcon tube. 100 μl from

each of the polysome fractions was kept aside to measure the input RNA amounts, and

the rest of each polysome fraction was incubated with 100 μl of Dynabeads (Invitrogen)

conjugated with 40 μl of anti-EIF3B antibody (Bethyl A301-761A) for 16 hours, rotating at

4 °C. After incubation, the beads were rinsed three times with 1000 μl room temperature

NP40 lysis buffer (defined in the PAR-CLIP section), rotating for 5 minutes for each wash.

After the final wash the beads were resuspended in 400 μl of Trizol (Thermofisher), the

RNA was extracted and qRT-PCR was performed as described above.

<u>Western Blot</u>

Western blot analysis was performed using the antibodies listed in

Supplementary File 6.

<u>Total mRNA isolation and quantitative RT-PCR analysis</u>

Total RNA was isolated from whole cells for qRT-PCR using Quick RNA miniprep

plus kit from Zymo Research following the manufacturer's instructions. Quantitative RT-

PCR analysis was performed using the Power SYBR Green RNA-to-Ct 1-Step kit (Applied

Biosystems) according to the manufacturer's instructions, and the QuantStudio™ 3 Real-

Time PCR System (ThermoFisher). Each target mRNA was quantified in three biological

replicates, with each biological replicate having three technical replicates.

<u>Plasmids</u>

Nanoluciferase reporters (Hall et al., 2012) were constructed using the 5'-UTR of

the human beta globin mRNA (*HBB*) and a PEST destabilization domain. The PEST

domain reduces protein half-life (Voon et al., 2005) and was used to provide better time

resolution of nanoluciferase expression after T cell activation. The *TCRA* 3'-UTR and

TCRB 3'-UTR sequences were amplified from Jurkat genomic DNA. The nanoluciferease

sequence fused to a PEST domain was amplified from pNL1.2[*NlucP*] Vector Sequence

(Promega) and was cloned into a modified CD813A vector (System Biosciences) using

the In-Fusion® HD Cloning Kit (Takara). The subsequent mutations in the *TCRA* and

TCRB 3'-UTRs were generated using these initial constructs. For *TCRA ΔPAR*

constructs, nucleotides 102-338 in the 3'-UTR of *TCRA* mRNA were deleted. For *TCRB*

ΔPAR constructs, nucleotides 16-161 in the 3'-UTR of *TCRB* mRNA were deleted.

TCRA/TCRB ΔPAR, *TCRA/TCRB R*PAR, 3'-LTR* (3'-Long Terminal Repeat), *JUN* 5'-

UTR hairpin (**Supplementary File 6**) and HCV IRES domain III (**Supplementary File 6**)

sequences were purchased as gblocks from IDT and were cloned into this plasmid

backbone. The *WPRE* (Woodchuck Hepatitis Virus Posttranscriptional Regulatory

Element) 3'-UTR sequence was amplified from the CD813A-1 (System Biosciences)

vector.

For nanoluciferase reporters designed to be membrane-tethered, we fused the N-

terminal sequence of CD3-zeta spanning the transmembrane helix (amino acids 1-60)

ordered as a gblocks from IDT to the nanoluciferase sequence above. To prevent

interaction of the CD3-zeta-nanoluciferase fusion protein with the TCR, we made

mutations in the CD3-zeta derived transmembrane helix that would disrupt interactions

with the TCR, based on the cryo-EM structure of the complex (Dong et al., 2019) (PDB

entry 6JXR) and consistent with earlier biochemical results (Call et al., 2002). The two

mutations, L35F and D36V, are predicted to introduce a steric clash and disrupt an intra-

membrane salt bridge, respectively, with other subunits in the TCR holo-complex. These

CD3-zeta-nanoluciferase chimeras were cloned into the modified CD813A plasmids

described above.

Generation of primary human T cells stably expressing nanoluciferase reporters

For lentiviral production, HEK293T cells were plated at a density of 80% in T-75

flasks the night before transfection. The cells were then transfected with plasmids:

expressing the nanoluciferase, PsPAX2 and pCMV-VSV-G using the Lipofectamine 2000

reagent (ThermoFisher) following the manufacturer's instructions. Forty-eight hours after

transfection, the viral supernatant was collected, filtered and concentrated using PEG-it

Virus Precipitation Solution (System Biosciences) following the manufacturer's

instructions. The virus pellets were then resuspended in ImmunoCult™-XF T Cell

Expansion media and stored in -80 °C.

The primary human T cell transductions were done with multiple viral titers using

TransDux™ MAX Lentivirus Transduction Reagent (System Biosciences) following the

manufacturer's instructions. To test the viral transduction efficiency of the cells, forty-eight

hours after viral transduction the percent of cells expressing GFP was measured by FACS

analysis and cells expressing less than 30% GFP were treated with 1 µg ml^{-1} puromycin

(ThermoFisher) for 4 days or until ~90% of the cells are GFP positive.

<u>Luciferase reporter assays</u>

The stable cell lines expressing the Nanoluciferase reporters were stimulated with

anti-CD3/anti-CD28 antibodies with 50 U/mL IL-2 and the nanoluciferase activity was

assayed after 30 min, 1 hr, 3 hr, and 5 hr after stimulation using Nano-Glo® Luciferase

Assay System (Promega). For each time point 200,000 cells were tested in triplicate for

each cell line.

For assays of TCR and CD28 signaling requirements, the stable cell lines were

stimulated with anti-CD3 or anti-CD28 antibodies individually in the presence of 50 U/mL

IL-2, and assayed as described above. To identify signaling pathways downstream of

CD28, cells were incubated with AKT inhibitor AZD5363 at 1 µM (Cayman Chemical),

mTOR inhibitor Torin1 250 nM (Cayman Chemical) or DMSO for 3 hours prior to T cell

activation with anti-CD3/anti-CD28 antibodies (Tonbo) as indicated above. The cells were

assayed as described above, and the extent of AKT and mTOR inhibition were

determined by western blot analysis of phosphorylation of their substrates GSK-3β and

4E-BP1, respectively.

<u>RNA immunoprecipitation and qPCR</u>

The EIF3B-RNA immunoprecipitations were carried out following the exact same

conditions used for the PAR-CLIP analysis with the following changes. For each

immunoprecipitation, cell lysates were prepared in NP40 lysis buffer (defined in the PAR-

CLIP section) with 4 million cells. The lysates were then incubated with 50 µl Protein G

Dynabeads conjugated with 20 µl of anti-EIF3B antibody (Bethyl A301-761A) for two

hours at 4 °C. After incubation, the flow through was removed and the beads were washed

three times with 1 ml NP40 lysis buffer for each wash. The beads were then resuspended

in 400 µl TRIzol reagent (ThermoFisher) and vortexed for 1 minute. The RNA was

extracted following the manufacturer's instructions and qPCR was performed as

described above using primers listed in **Supplementary File 6**.

<u>sgRNA/Cas9 RNP production</u>

The sgRNA/Cas9 RNPs used to edit Jurkat cells were produced by complexing

sgRNA (Synthego) to Cas9 as described (Schumann et al., 2015) while RNPs to edit

Primary Human T cells were produced by complexing a two-component gRNA (crRNA

and tracrRNA, Dharmacon) to Cas9 as described in (Roth et al., 2018). The targeting

sequences for the sgRNAs and crRNA are given in **Supplementary File 6**. Recombinant

Cas9-NLS was obtained from MacroLab in the California Institute for Quantitative

Biosciences.

<u>Primary T cell and Jurkat genome editing</u>

Jurkat cells used for electroporation were collected at passage 5 or lower and were

maintained at a seeding density of 8 million cells/mL or lower. Primary T cells were

isolated as described above. Prior to electroporation the Primary T cells were stimulated

with magnetic anti-CD3/anti-CD28 Dynabeads (ThermoFisher) for 48 hours. After 48

hours these beads were removed from the cells by placing cells on an EasySep cell

separation magnet for 2 min before electroporation. One million Jurkat (not activated) or

primary T cells cells (activated with anti-CD3/anti-CD28 Dynabeads for 48 hours) were

rinsed with PBS and then resuspended in 20 µl of Lonza electroporation buffer P3. The

cells were then mixed with 2.5 µl Cas9 RNPs (50 pmol total) along with 2 µl of a 127-

nucleotide non-specific single-stranded DNA oligonucleotide at 2 µg µl^{-1} (4 µg ssDNA

oligonucleotide total). The cells were then electroporated per well using a Lonza 4D 96-

well electroporation system with pulse code DN100 for Jurkat cells and EH115 for primary

human T cells. Immediately after electroporation, 80 µl of pre-warmed media (without

cytokines) was added to each well, and the cells were allowed to rest for 15 min at 37 °C

in a cell culture incubator while remaining in electroporation cuvettes. After 15 min, cells

were moved to final culture flasks. Jurkat cells were clonally selected by single cell sorting

into U-bottomed 96 well plates and by testing each clone using PCR primers flanking the

editing site (**Figure 5–figure supplement 1A**). The clones producing a single PCR band

of 1283 bp and 1022 bp were selected as clonal populations for *TCRA ΔPAR* and *TCRB*

ΔPAR respectively.

Genome edited populations of primary T cells with the *TCRA ΔPAR* and *TCRB*

ΔPAR mutations were determined by measuring the density of the PCR bands described

above resulting from the edited cell population compared to the PCR band from non

edited cells, using ImageJ. To compare with the *TCRA ΔPAR* or *TCRB ΔPAR* primary T

cell populations, we edited cells from both donors using each gRNA targeting the *TCRA*

3'-UTR and *TCRB* 3'-UTR region separately (single gRNA experiments), a gRNA

targeting the coding sequence (CDS) of *TCRA* which knocks out TCR expression with

high efficiency (Δ*TCR*) (Roth et al., 2018), a scrambled gRNA (SC) which does not target

any site in the human genome, and cells mixed with the gRNA/Cas9 RNPs but not

nucleofected.

<u>Analysis of TCR cluster formation</u>

WT, *TCRA ΔPAR* or *TCRB ΔPAR* T cells were activated with anti-CD3/anti-CD28

antibodies for 1, 3 or 5 hours, as described above. Cells were collected and stained with

anti-TCRA antibodies, followed by a secondary antibody Alexa Fluor 488 goat anti-mouse

IgG (Invitrogen). For the counting of cells containing TCR clusters, immunofluorescent

imaging was performed on a Revolve Epi-Fluorescence microscope (Echo), equipped

with an A Plan-Apochromat 40x objective (Olympus). Cells with substantial puncta

(arrows in **Figure 5–figure supplement 1C**) were scored as having TCR cluster

formation (**Figure 5C**).

<u>Flow cytometry and cell sorting</u>

Flow cytometric analysis was performed on an Attune NxT Acoustic Focusing

Cytometer (ThermoFisher). Surface staining for flow cytometry and cell sorting was

performed by pelleting cells and resuspending in 50 µl of FACS buffer (2% FBS in PBS)

with antibodies at a 1:100 concentration (**Supplementary File 4**) for 20 min at 4 °C in

the dark. Cells were washed twice in FACS buffer before resuspension and analysis.

<u>ELISA</u>

The cell culture supernatants were collected after each time point of activation with

anti-CD3/anti-CD28 antibodies for WT, *TCRA ΔPAR* or *TCRB ΔPAR* cells. For each

timepoint the same number of cells were used from each strain to be able to compare

across strains and time points. The amount of secreted IL-2 in the cell suspensions after

activation anti-CD3/anti-CD28 antibodies for WT, *TCRA ΔPAR* or *TCRB ΔPAR* cells were

measured by ELISA MAX™ Deluxe Set Human IL-2 (BioLegend) according to the

manufacturer's instructions.

<u>Chimeric antigen receptor (CAR) construct sequences</u>

The CDS region of the Juno anti-CD19 chimeric antigen receptor presently used

in the clinic (June et al., 2014; Kalos et al., 2011) was cloned into CD813A lentiviral

vectors (Systems Biosciences) with a common core EF1alpha promoter and 5'-UTR

with an inserted intron. The CDS sequence was followed by various 3'-UTRs. Two of

the 3'-UTRs are presently used in clinical CAR T cells, the Woodchuck Hepatitis Virus

Posttranscriptional Regulatory Element (*WPRE*) (June et al., 2014; Milone et al., 2009),

or the murine stem cell virus (MSCV) retroviral 3'-long terminal repeat (*3'-LTR*)

(Kochenderfer et al., 2009). We also cloned the full *TCRA* or *TCRB* 3'-UTR sequences

including the polyadenylation sites after the anti-CD19 CAR CDS sequence.

<u>Production of CAR T cells</u>

Production of the CAR-expressing lentiviruses was carried out as described

above using HEK293T cells. The viruses were then concentrated using PEG-it Virus

Precipitation Solution (System Biosciences) following the manufacturer's instructions.

The virus pellets were then resuspended in ImmunoCult™-XF T Cell Expansion media

and stored in -80 °C. Frozen primary human T cell pellets were thawed and then

stimulated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (Stemcell) for 48

hours and then transduced with various CAR viruses using TransDux™ MAX Lentivirus

Transduction Reagent (System Biosciences) as described above. Two days after

transduction the percent of cells expressing GFP was measured by FACS analysis and

cells expressing less than 30% GFP were treated with 1 μg mL^{-1} puromycin

(ThermoFisher) for 2 days or until ~90% of the cells are GFP positive. After removal of

puromycin the cells were seeded at 1x10^6 cells/mL upto 9 days from the day the cells

were transduced by adding fresh ImmunoCult™-XF T Cell Expansion Medium

(StemCell) and 50 U/mL IL-2 every other day. After 9 days the CAR T cells were used

for various assays.

<u>CAR expression dynamics</u>

To measure CAR expression dynamics by western blot analysis, CAR T cells

were incubated with NALM6 tumor cells at a ratio of 1:2 CAR T cell:NALM6 cells, in

ImmunoCult™-XF T Cell Expansion Medium (StemCell) and 50 U/mL IL-2. Cells were

collected at the indicated time points and processed for western blot analysis as

described above. The total protein expression level of the anti-CD19 CAR was detected

with an anti-CD3z antibody (Santa Cruz Biotechnology).

<u>CAR T-cell cytotoxicity assays</u>

CAR T cell cytotoxicity was determined using a FACS based assay. First,

NALM6 or Jeko 1 tumor cells were stained with CTV (CellTrace™ Violet, thermofisher).

Cells were resuspended in 10 mL PBS (5 x 10^6 cells), 5 µl CTV was added, and the

cells were incubated for 20 minutes at 37 °C. After incubation, 30 mL of RPMI media

was added and the cells incubated for an additional 5 minutes at 37 °C. The cells were

then gently pelleted, rinsed and resuspended in fresh ImmunoCult™-XF T Cell

Expansion Medium (StemCell) and 50 U/mL IL-2. Then in a round bottom 96-well plate,

20,000 stained tumor cells were cocultured with CAR T cells at different effector-target

ratios in a total volume of 200 µL for 24 hours, 48 hours and 72 hours. T cells

transduced with a membrane-tethered nanoluciferase reporter containing the *TCRA* 3'-

UTR (**Figure 4B**) were used as a negative control. The CAR T cell killing capacity was

measured by Flow Cytometry as diagrammed in **Figure 6–figure supplement 1B**.

Briefly, the 96-well plates were incubated at 4 °C for 30 minutes to terminate the killing

by CAR T cells. Then 2 µL of propidium iodide (1000x stock, Thermofisher) was added

to each column in the plate one by one and the living tumor cells were measured as

shown in **Figure 6–figure supplement 1B**.

Figure 1–figure supplement 1. eIF3 PAR-CLIP experiments in activated and non-

activated Jurkat cells. (**A**) Composition of eIF3 in I+PMA activated and non-activated

Jurkat cells after anti-EIF3B immunoprecipitation (IP), identified by mass spectrometry.

Shown is an SDS polyacrylamide gel stained with Coomassie Brilliant Blue. (**B**)

Composition of eIF3 in activated Jurkat cells determined by western blot after anti-EIF3B

IP. IN: input; FT: flow-through from anti-EIF3B IP beads; ELU: elution of eIF3 from anti-

EIF3B IP beads. Anti-EIF4G1 and anti-RPS19 western blots confirm the stringency of

bead wash steps (Lee et al., 2015). (**C**) (Top) The length distribution of PAR-CLIP clusters

mapped to RNAs crosslinked to eIF3 in the EIF3A/C/B samples, from activated (red) and

non-activated (grey) Jurkat cells. (Bottom) The frequency distribution of the number of

eIF3 PAR-CLIP clusters mapped to RNAs in the EIF3A/C/B samples, from activated (red)

and non-activated (grey) Jurkat cells. In all panels, the distributions are the average of

both biological replicates. RNA PAR-CLIP properties observed in the EIF3A/C/B sample

are representative of those seen in PAR-CLIP sequence reads of RNAs crosslinked to

other eIF3 subunits. (**D**) Categories of RNA crosslinked to eIF3, defined by clusters and

divided into RNA categories, in I+PMA activated Jurkat cells and non-activated Jurkat

cells (grey). In all panels, RNA categories include: 5'-UTR, 5'-UTR of mRNA; 3'-UTR, 3'-

UTR of mRNA; CDS, protein coding region of mRNA; Start codon, beginning of mRNA

CDS; Stop codon, stop codon region of mRNA; Intron, regions of pre-mRNA; Other, other

classes of RNA; No annotation, reads that map to the human genome but that have no

annotation assigned. A given mRNA may give rise to clusters in different mRNA

categories. (**E**) Pathway enrichment categories determined using the STRING Database

for both biological replicates of the EIF3A/C/B PAR-CLIP libraries (mRNAs with >= 100

reads). Number of genes in each pathway whose mRNAs crosslinked to eIF3 is shown

next to each bar. Note that the categories reported by the STRING Database are not

disjoint sets. Panels **C** through **E** show representative results from one of two biological

replicates. (**F**) Scatterplot of TPM of mRNAs expressed in activated Jurkat cells (most

abundant isoform, see Methods) versus mRNAs identified by PAR-CLIP as crosslinked

to eIF3 in the EIF3A/C/B samples, with ≥ 100 total read counts. n=1,029 and 1,035 for

PAR-CLIP hit genes plotted for replicates 1 and 2, respectively. The R-squared goodness

of fit to a linear equation is shown. (**G**) mRNAs sorted by increasing value of the ratio of

normalized 5'-UTR reads to normalized 3'-UTR reads in the EIF3A/C/B samples. Values

of the ratio of 5'-UTR/3'-UTR normalized reads > 200 have been truncated to a value of

200 in the plot (asterisk). The arrow indicates the threshold used to create cumulative

plots shown in **Figure 1D**. (**H**) Examples of eIF3 crosslinking to histone mRNAs

HIST1H2AI and *HIST1H3H* in activated Jurkat cells are shown. Transcription start sites

were determined from the FANTOM5 Database (Noguchi et al., 2017).

Figure 1–figure supplement 2. The *TCRA* and *TCRB* mRNAs form localized puncta

in activated T cells. (**A**) (right panel) Single optical section of TetraSpeck microspheres

(0.1 µm) adjacent to a Jurkat cell probed for P bodies. Scatter plots of pixel intensities in

different emission channels are shown: channel 1, 670 nm; channel 2, 570 nm; channel

3, 488 nm. (left panel) Single optical section of a Jurkat cell probed for *TCRA* mRNA

(channel 1), *TCRB* mRNA (channel 2), and DCP1 to mark P bodies (channel 3). (middle

panels) Scatter plots showing : channel 1 vs. channel 2, channel 1 vs. channel 3, and

channel 2 vs. channel 3. (**B**) (right panel) Single optical section and scatter plots for

TetraSpeck microspheres as in **A**, but adjacent to a Jurkat cell probed for stress granules.

(left panel) Single plane image of a Jurkat cell probed for *TCRA* mRNA (channel 1), *TCRB*

mRNA (channel 2), and G3BP1 to mark stress granules (channel 3). (middle panels)

Scatter plots as in **A**. Images of each cell are representative of those used in the analysis

in **Figure 1F**.

Figure 2–figure supplement 1. The *TCRA* and *TCRB* mRNAs remain bound to

elongating ribosomes via eIF3 in activated T cells. (**A**) Sucrose gradient

fractionation of polysomes from crosslinked Jurkat cells. Cell lysates treated with RNase

H, as indicated, were fractionated on 10%-50% sucrose gradients. Shown is the

absorbance at 254 nm, for one of two biological replicates. (**B**) Strategy for detecting

mRNA cleavage by RNase H digestion. RT-qPCR primers were designed to span the

mRNA digestion sites. (**C**) Strategy for detecting 5'-UTR or 3'-UTR mRNA fragments

released by RNase H digestion. RT-qPCR primers were designed within the 5'-UTR or

3'-UTR regions. (**D**) Fraction of intact mRNA segments remaining after RNase H

treatment of DSP-crosslinked cell lysates from Jurkat cells, in the presence or absence

of mRNA-specific DNA oligos for the indicated mRNAs. The mRNAs were detected

using RT-qPCR oligos as illustrated in panel **B**. (**E**) Amount of eIF3-bound 5'-UTR and

3'-UTR regions of the mRNA co-immunoprecipitated by anti-EIF3B antibody, from

polysome fractions of lysate from Jurkat cells treated with RNase H, either in the

absence (left) or presence (right) of mRNA-targeting DNA oligos. Percentage is relative

to the amount of total mRNA present in the polysome fraction prior to

immunoprecipitation. Primers to the mRNA 5'-UTR and 3'-UTR regions, as indicated in

panel **C**, were used for quantification**.** (**F**) Sucrose gradient fractionation of polysomes

from crosslinked primary human T cells. (**G**) Fraction of intact mRNA segments

remaining after RNase H treatment of DSP-crosslinked cell lysates from primary T cells,

in the presence or absence of mRNA-specific DNA oligos for the indicated mRNAs. The

mRNAs were detected using RT-qPCR oligos as illustrated in panel **B**. (**H**) Amount of

eIF3-bound 5'-UTR and 3'-UTR regions of the mRNA co-immunoprecipitated by anti-

EIF3B antibody, from polysome fractions of lysate from primary human T cells treated

with RNase H, either in the absence (left) or presence (right) of mRNA-targeting DNA

oligos. Percentage is relative to mRNA present in the polysome fraction prior to

immunoprecipitation. Primers to the mRNA 5'-UTR and 3'-UTR regions, as indicated in

panel **C**, were used for quantification. All experiments were carried out in biological

duplicate with one technical triplicate shown (*n* = 3, with mean and standard deviations

shown). All the primary human T cell experiments were performed using samples from

two donors and data from one representative donor are shown.

Figure 4–figure supplement 1. Nanoluciferase reporter expression in activated T cells. (**A**) Schematic of nanoluciferase reporters without a transmembrane helix (not membrane tethered) stably expressed in primary human T cells. The reporters carry the *HBB* 5'-UTR and *TCRA, TCRB, TCRA ΔPAR,* or *TCRB ΔPAR* 3'-UTR. (**B**) Luciferase activity in T cells stably expressing reporters described in **A** stimulated only with anti-CD28 antibodies, relative to non-activated controls (*n* = 3, with mean and standard deviations shown). (**C**) Luciferase activity in primary human T cells stably expressing reporters described in **A** stimulated only with anti-CD3 antibodies, relative to non-activated controls (*n* = 3, with mean and standard deviations shown). (**D**) Luciferase activity in primary human T cells stably expressing membrane-tethered reporter (described in the schematic above) with WT *TCRA* 3'-UTR inhibited with either

AZD5363(AZD) to inhibit AKT activity or Torin 1 to inhibit mTOR before activating with

anti-CD3/anti-CD28 antibodies, relative to non-activated controls. In panels **B**–**D**, all

experiments were carried out in triplicate (3 separate wells per condition), with mean and

standard deviations shown. (**E**) Western blot carried out to measure AKT activity in the

presence of AZD5363 (AZD) using an anti-Phospho-GSK-3β antibody or mTOR activity

in the presence of Torin 1 using an anti-Phospho-4EBP1 antibody for the samples in **D**.

HSP90 was used as a loading control. All the primary human T cell experiments were

performed using samples from two donors and data from one representative donor are

shown here. The data from the other donor are presented in **Figure 4F**.

Figure 5–figure supplement 1. Generation and analysis of *TCRA ΔPAR* and *TCRB*

***ΔPAR* primary human T cells.** (**A**) CRISPR/Cas9 RNP mediated genome editing at the

TCRA and *TCRB* genomic loci in primary human T cells. Location of gRNA sites targeting

the *TCRA* 3'-UTR, to generate *TCRA ΔPAR* cells is highlighted in red. In *TCRA*, gRNA1

and gRNA2 target hg38 genomic locations chr14:22,551,700 and chr14:22,552,073,

respectively. Location of gRNA sites targeting the *TCRB* 3'-UTR, to generate *TCRB*

ΔPAR cells is highlighted in blue. In *TCRB*, gRNA1 and gRNA2 target hg38 genomic

locations chr7:142,802,561 and chr7:142,802,695, respectively. (**B**) Top gel: Analysis of

TCRA 3'-UTR PAR-CLIP site deletion efficiency. Total genomic DNA extracted from WT,

TCRA ΔPAR, TCRB ΔPAR (here serving as a negative control), scrambled gRNA

nucleofected (S) and non-nucleofected (N) cells was analyzed by PCR to measure the

editing efficiency. *TCRA ΔPAR* cells produced a 1283 bp PCR product compared to 1676

bp in S, N or *TCRB ΔPAR* cells (See panel **A**). Bottom gel: Analysis of *TCRB* 3'-UTR

PAR-CLIP site deletion efficiency. Total genomic DNA extracted from WT, *TCRA ΔPAR*

(here serving as a negative control)*, TCRB ΔPAR*, scrambled gRNA nucleofected (S) and

non-nucleofected (N) cells were analyzed by PCR to measure the editing efficiency.

TCRB ΔPAR cells produced a 1022 bp PCR product compared to 1190 bp in S, N or

TCRA ΔPAR cells (See panel **A**). The percentage of alleles with PAR-CLIP site deletion,

quantified by imageJ. (**C**) Representative immunofluorescence images used to count

TCR clusters in WT, *TCRA ΔPAR* and *TCRB ΔPAR* primary human T cells. The white

arrows indicate the cells that were scored as having TCR clusters. (**D**) Percentage of cells

expressing TCR on the cell surface of activated T cells measured by flow cytometric

analysis. TCR expressing cells in the cell populations tested: *N*, Non-nucleofected cells;

SC, Scrambled gRNA; *1gA*, *TCRA* gRNA 1; *2gA*, *TCRA* gRNA 2; *1+2 gA*, *TCRA* gRNA

1+2 (i.e. *TCRA ΔPAR*); *1gB*, *TCRB* gRNA 1; *2gB*, *TCRB* gRNA 2; *1+2 gB*, *TCRB* gRNA

1+2 (i.e. *TCRB ΔPAR*); *ΔTCR*, TCR gRNA targeting the CDS of *TCRA*. (n = 3 replicates

from three separate wells, with mean and standard deviation shown.) (**E**) Representative

density plots showing the percentage of TCR on the cell surface in activated T cells. The

cell lines shown are: SC, *TCRA ΔPAR*, *TCRB ΔPAR*, *ΔTCR* (negative control). In panels

D and **E**, representative results from one of two donors are shown. (**F**) Gating strategy for

flow cytometric analysis of CD69 and CD25 levels. Primary human T cells were gated to

isolate lymphocytes, followed by isolation of single cells, then live cells. Next, cells were

gated to separate CD4+ and CD8+ cells expressing T cell activation markers CD69 and

CD25. Shown is the workflow of the FACS gating, with an example of T cells activated

with anti-CD3/anti-CD28 antibodies for 8 hours.

Figure 5–figure supplement 2. Effects of *TCRA ΔPAR* and *TCRB ΔPAR* mutations

on different steps of T cell activation. (A and **B)** Representative density plots showing

the percentage of, **A** CD8+ T cells and **B**, CD4+ T cells expressing CD69 (early activation

marker), CD25 (mid-activation marker) or CD69 and CD25 after activation with anti-

CD3/anti-CD28 antibodies at different time points, analyzed by flow cytometry. The plots

represent one of the two donors. The cell lines shown are SC, *TCRA ΔPAR*, *TCRB ΔPAR*

and Δ*TCR* (negative control). **(C** and **D)** Flow cytometric analysis quantifying the mean

percent of **C,** CD8+ T and **D**, CD4+ T cells that are only CD69+. **(E** and **F)** Flow cytometric

analysis quantifying the mean percent of **E,** CD8+ T and **F**, CD4+ T cells that are

CD69+CD25+. (**G** and **H**) Quantification of **G**, secreted IL-2 and **H**, secreted IFNγ, for all

cell populations tested, as determined by ELISA. In panels **C**–**H** cell populations tested:

N, Non-nucleofected cells; SC, Scrambled gRNA; *1gA*, *TCRA* gRNA 1; *2gA*, *TCRA* gRNA

2; *1+2 gA*, *TCRA* gRNA 1+2 (i.e. *TCRA ΔPAR*); *1gB*, *TCRB* gRNA 1; *2gB*, *TCRB* gRNA

2; *1+2 gB*, *TCRB* gRNA 1+2 (i.e. *TCRB ΔPAR*); *ΔTCR* gRNA targeting the CDS of *TCRA*.

In panels **C** through **H**, all experiments were carried out in triplicate (3 separate wells per

condition), with mean and standard deviations shown. Representative results from one

donor are shown.

Figure 5–figure supplement 3. eIF3 binding to the *TCRA* and *TCRB* mRNA 3'-UTR

elements in Jurkat cells. (**A**) Western blots of TCRA protein levels as a function of time

after anti-CD3/anti-CD28 activation. Cell lines used: WT Jurkat cells, *TCRA ΔPAR* and

TCRB ΔPAR, Jurkat cells in which the eIF3 PAR-CLIP sites in the 3'-UTRs of the *TCRA*

and *TCRB* mRNAs, respectively, have been deleted. Schematics of *TCRA* and *TCRB*

mRNAs with and without eIF3 PAR-CLIP sites are shown above. HSP90 was used as a

loading control. Western blots are representative of two biological replicates. (**B**) *TCRA*

and *TCRB* mRNA levels in WT Jurkat cells, or Jurkat cells with the *TCRA* eIF3 3'-UTR

PAR-CLIP site deleted, as determined by qRT-PCR at different time points after anti-

CD3/anti-CD28 activation. (**C**) *TCRA* and *TCRB* mRNA levels in WT Jurkat cells, or Jurkat

cells with the *TCRB* eIF3 3'-UTR PAR-CLIP site deleted, as determined by qRT-PCR at

different time points after anti-CD3/anti-CD28 activation. (**D**) Schematic of

immunoprecipitation of eIF3 using an anti-EIF3B antibody, followed by qRT-PCR to

quantify the amount of nanoluciferase mRNA bound to eIF3. (**E**) Immunoprecipitation of

eIF3 as shown in **D** to quantify the amount of *TCRA* or *TCRB* mRNA bound to eIF3 in

Jurkat cells after activation with anti-CD3/anti-CD28 antibodies for 5 hours (left) and 8

hours (right). The percent mRNA bound to anti-EIF3B beads is calculated relative to total

mRNA isolated from the cells. In panels **B**–**E**, all experiments were carried out in triplicate

(3 separate wells per condition), with mean and standard deviations shown.

Representative results from one of two biological replicates are shown.

Figure 6–figure supplement 1. Effects of the *TCRA* and *TCRB* mRNA 3'-UTR

elements on CAR T cell function. (**A**) Western blots measuring TCRA protein levels as

a function of time in FMC63-BBZ CAR T cells after incubation with NALM6 tumor cells.

Cell lines used are labeled: Control*, WPRE, 3'-LTR, TCRA* and *TCRB* 3'-UTR

diagrammed in **Figure 6B**. HSP90 was used as a loading control. (**B**) Gating strategy for

flow cytometric analysis to detect live tumor cells after incubation with CAR T cells. This

includes: (1) both CAR T and tumor cell gating, (2) isolating single cells, (3) isolating live

cells based on propidium iodide staining and (4) separating cell trace violet (CTV) stained

vs non-stained cells to isolate live tumor cells. (**C**) Cytotoxic activity of FMC63-BBZ CARs

721   fused to various 3'-UTRs described in **Figure 6B** after incubating with NALM6 or Jeko1

722   tumor cell lines for 72 hours. Representative results for one of two donors are shown (*n*

723   = 3 separate wells, with mean and standard deviations shown).

Supplementary Files

Supplementary File 1. (Separate file) Subunits in eIF3 crosslinked to RNA in

activated Jurkat cells. Lists the eIF3 subunits, percent sequence coverage and number

of identified peptides.

Supplementary File 2. (Separate file) PARpipe statistics for eIF3 PAR-CLIP

samples. Samples are indexed in the first tab, including both biological replicates for

activated and non-activated Jurkat cells. Statistics are given for each library at the read,

cluster and group level.

Supplementary File 3. (Separate file) PAR-CLIP mapping to individual genes for

each eIF3 PAR-CLIP sample. Samples are indexed in the first tab, including both

biological replicates for activated and non-activated Jurkat cells. First lists the gene name

and number of clusters identified. The statistics also include: Sum, sum of that statistic

over all sites for that gene; Med, median of that statistic for all sites for that gene;

ReadCount, total reads mapping to the gene; T2C fraction, number of reads with T-to-C

conversions / number of reads; ConversionSpecificity, log (number of reads with T-to-C

conversions / number of reads with other conversions); UniqueReads, reads collapsed to

single copies. Also included: 5'utr/Intron/Exon/3'utr/Start_codon/Stop_codon, number of

sites mapping to that annotation category; Junction, number of sites mapping to a junction

between categories (coding-intron, coding-3'utr, etc.); GeneType, as described in the

gene_type category for this gene in the .gtf file used.

Supplementary File 4. (Separate file) Transcriptome analysis of non-activated or

activated Jurkat cells. Each tab lists transcript name and version, gene name, type of

transcript, length of transcript, and mean transcripts per million, calculated from two

biological replicates.

Supplementary File 5. (Separate file) Pathway enrichment analysis. Lists for both

biological replicates of the EIF3A/C/B PAR-CLIP libraries are included (genes with >= 100

reads), along with associated transcript names, lengths in nts of the 5'-UTR, coding

region, and 3'-UTR, and reads normalized to the lengths of the transcript regions. Tabs

also include the top tissue-specific pathway enrichment categories determined using the

STRING Database. These list: the Gene Ontology (GO) number, GO description,

observed gene count, background gene count, false discovery rate, and matching

proteins in the network by Ensembl protein ID, and by gene name.

Supplementary File 6. (Separate file) Reagent information for experiments. Lists

include antibodies used, PCR primers, qPCR primers, gRNA targeting sequences, and

FISH probes, and DNA oligos for RNase H experiments.

References

Ascano, M., Hafner, M., Cekan, P., Gerstberger, S., and Tuschl, T. (2012). Identification

of RNA-protein interaction networks using PAR-CLIP. Wiley Interdiscip. Rev. RNA *3*,

159–177.

Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic

RNA-seq quantification. Nat. Biotechnol. *34*, 525–527.

Call, M.E., Pyrdol, J., Wiedmann, M., and Wucherpfennig, K.W. (2002). The organizing

principle in the formation of the T cell receptor-CD3 complex. Cell.

Corcoran, D.L., Georgiev, S., Mukherjee, N., Gottwein, E., Skalsky, R.L., Keene, J.D.,

and Ohler, U. (2011). PARalyzer: definition of RNA binding sites from PAR-CLIP short-

read sequence data. Genome Biol. *12*, R79.

Cunningham, F., Achuthan, P., Akanni, W., Allen, J., Amode, M.R., Armean, I.M.,

Bennett, R., Bhai, J., Billis, K., Boddu, S., et al. (2019). Ensembl 2019. Nucleic Acids

Res. *47*, D745–D751.

Danan, C., Manickavel, S., and Hafner, M. (2016). PAR-CLIP: A Method for

Transcriptome-Wide Identification of RNA Binding Protein Interaction Sites. Methods

Mol. Biol. *1358*, 153–173.

Dong, D., Zheng, L., Lin, J., Zhang, B., Zhu, Y., Li, N., Xie, S., Wang, Y., Gao, N., and

Huang, Z. (2019). Structural basis of assembly of the human T cell receptor-CD3

complex. Nature *573*, 546–552.

Frankish, A., Diekhans, M., Ferreira, A.-M., Johnson, R., Jungreis, I., Loveland, J.,

Mudge, J.M., Sisu, C., Wright, J., Armstrong, J., et al. (2019). GENCODE reference

annotation for the human and mouse genomes. Nucleic Acids Res. *47*, D766–D773.

Hall, M.P., Unch, J., Binkowski, B.F., Valley, M.P., Butler, B.L., Wood, M.G., Otto, P.,

Zimmerman, K., Vidugiris, G., Machleidt, T., et al. (2012). Engineered luciferase

reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS

Chem. Biol. *7*, 1848–1857.

June, C.H., Levine, B.L., Porter, D.L., Kalos, M.D., and Milone, M.C. (2014).

Compositions for treatment of cancer.

Kalos, M., Levine, B.L., Porter, D.L., Katz, S., Grupp, S.A., Bagg, A., and June, C.H.

(2011). T cells with chimeric antigen receptors have potent antitumor effects and can

establish memory in patients with advanced leukemia. Sci. Transl. Med. *3*, 95ra73.

Kochenderfer, J.N., Feldman, S.A., Zhao, Y., Xu, H., Black, M.A., Morgan, R.A., Wilson,

W.H., and Rosenberg, S.A. (2009). Construction and preclinical evaluation of an anti-

CD19 chimeric antigen receptor. J. Immunother. *32*, 689–702.

Lee, A.S.Y., Kranzusch, P.J., and Cate, J.H.D. (2015). eIF3 targets cell-proliferation

messenger RNAs for translational activation or repression. Nature *522*, 111–114.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput

sequencing reads. EMBnet.journal *17*, 10–12.

Milone, M.C., Fish, J.D., Carpenito, C., Carroll, R.G., Binder, G.K., Teachey, D.,

Samanta, M., Lakhal, M., Gloss, B., Danet-Desnoyers, G., et al. (2009). Chimeric

receptors containing CD137 signal transduction domains mediate enhanced survival of

T cells and increased antileukemic efficacy in vivo. Mol. Ther. *17*, 1453–1464.

Mukherjee, N., Wessels, H.-H., Lebedeva, S., Sajek, M., Ghanbari, M., Garzia, A.,

Munteanu, A., Yusuf, D., Farazi, T., Hoell, J.I., et al. (2019). Deciphering human

ribonucleoprotein regulatory networks. Nucleic Acids Res. *47*, 570–581.

Noguchi, S., Arakawa, T., Fukuda, S., Furuno, M., Hasegawa, A., Hori, F., Ishikawa-

Kato, S., Kaida, K., Kaiho, A., Kanamori-Katayama, M., et al. (2017). FANTOM5 CAGE

profiles of human and mouse samples. Sci Data *4*, 170112.

Parslow, A., Cardona, A., and Bryson-Richardson, R.J. (2014). Sample drift correction

following 4D confocal time-lapse imaging. J. Vis. Exp.

Roth, T.L., Puig-Saus, C., Yu, R., Shifrut, E., Carnevale, J., Li, P.J., Hiatt, J., Saco, J.,

Krystofinski, P., Li, H., et al. (2018). Reprogramming human T cell function and

specificity with non-viral genome targeting. Nature *559*, 405–409.

Schumann, K., Lin, S., Boyer, E., Simeonov, D.R., Subramaniam, M., Gate, R.E.,

Haliburton, G.E., Ye, C.J., Bluestone, J.A., Doudna, J.A., et al. (2015). Generation of

knock-in primary human T cells using Cas9 ribonucleoproteins. Proc. Natl. Acad. Sci. U.

S. A. *112*, 10437–10442.

Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J.,

Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., et al. (2019). STRING v11:

protein-protein association networks with increased coverage, supporting functional

discovery in genome-wide experimental datasets. Nucleic Acids Res. *47*, D607–D613.

Voon, D.C., Subrata, L.S., Baltic, S., Leu, M.P., Whiteway, J.M., Wong, A., Knight, S.A.,

Christiansen, F.T., and Daly, J.M. (2005). Use of mRNA- and protein-destabilizing

elements to develop a highly responsive reporter system. Nucleic Acids Res. *33*, e27.

Wickham, H., Chang, W., and Others (2016). ggplot2: Create elegant data

visualisations using the grammar of graphics. R Package Version *2*.