UNIVERSITAT DE BARCELONA

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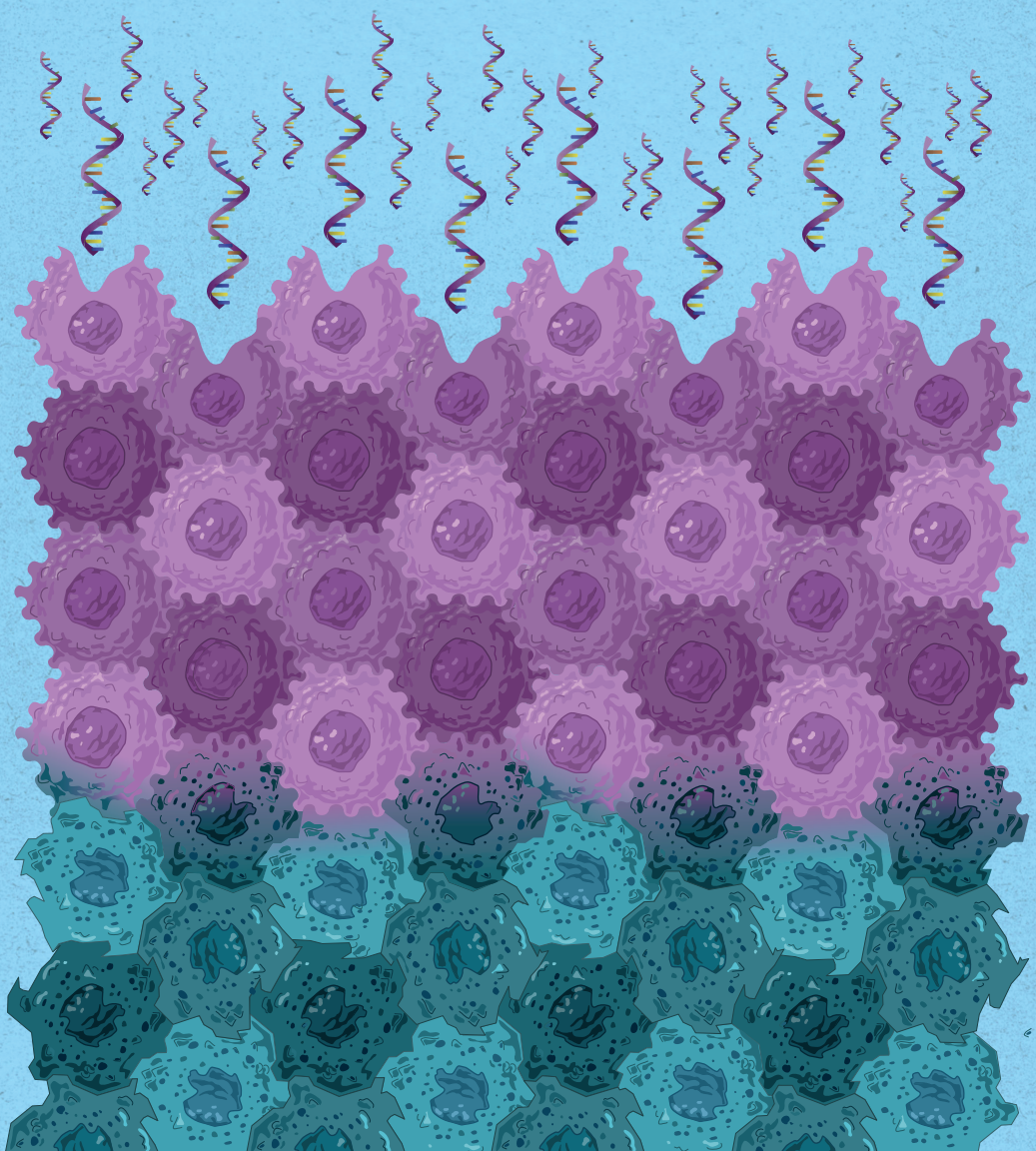

Universitat de Barcelona
Facultat de Biologia
IRB Barcelona
Programa de Doctorat en Biomedicina

Post-transcriptional control of anti-tumor immune responses

Memòria presentada per Marcos Fernández Alfara per optar al grau de doctor per la Universitat de Barcelona

Aquesta tesi ha estat realitzada sota la direcció del Dr. Raúl Méndez de la Iglesia a l'Institut de Recerca Biomèdica de Barcelona



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Barcelona, 2022

A Antonio, Patro, Luz y Pepe.

Al final del viaje está el horizonte.

Silvio Rodríguez

Contents

Abbreviations

4E-BPs	eIF4E-binding proteins
APA	Alternative polyadenylation
ARE	AU-rich-element
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BiP	Binding immunoglobulin protein
CAFs	Cancer-associated fibroblasts
CDK1	Cyclin-dependent kinase 1
CHOP	C/EBP homologous protein
CLP	Common lymphoid progenitor
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element binding protein
CPEB4-TKO	CPEB4 T cell-specificil Knock-Out
CTD	C-terminal domain
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte protein 4
DN	Double negative
DP	Double positive
eIF	eukaryotic Initiation Factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
ERK2	Extracellular signal-regulated kinase 2
GCN2	General control nonderepressible 2
GZMB	Ganzyme B
HRI	Heme-regulated inhibitor
HSC	Hematopoietic stem cell
HuR	Human antigen R

IFNγ	Interferon gamma
IP	Immunoprecipitation
IRE1α	Inositol-Requiring Enzyme 1 alpha
IRES	Internal ribosome entry site
ISR	Integrated stress response
KO	Knock-Out
m6A	N6-methyladenosine
MDSC	Myeloid-derived suppressor cells
MEF	Mouse embryonic fibroblast
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
miRNA	microRNA
mRMP	ribonucleoprotein particle
mRNA	messenger RNA
mTORC1	mammalian/mechanistic target of rapamycin complex 1
NK	Natural killer
NTD	N-terminal domain
OVA	Ovalbumin
P-bodies	Processing bodies
PAP	Poly(A) polymerase
PAS	Polyadenylation singal
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
PIC	Pre-initiation complex
PKR	Interferon-inducible RNA-dependent kinase
Pol II	RNA polymerase II
pre-mRNA	precursor mRNA
RBP	RNA bining protein

RIP-seq	RNA Immunoprecipitation and sequencing
ROS	Reactive oxygen species
RRM	RNA recognition motif
RT-qPCR	Real time quantitative polymerase chain reaction
snRNA	small nuclelar RNA
SP	Single positive
TC	Ternary complex
TCR	T Cell receptor
TEC	Thymic epithelial cells
Teff	Effector T cell
TIL	Tumor infiltrating lymphocyte
TKO	T cell-specific KO
TM	Memory T cells
TME	Tumor microenvironment
TN	Naïve T cell
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
TSP	Thymus-seeding progenitor
TTP	Tristetraprolin
uORF	Upstream open reading frame
UPR	Unfolded protein response
UTR	Untranslated region
WT	Wild type
XBP1	X-Box binding protein 1

Abstract

Translation of mRNAs into proteins is a highly regulated step of gene expression. In cancer, post-transcriptional and translational control have a critical effect on transformation, controlling proliferation, survival, stemness or metastatic capacity of tumor cells. However, cancer development not only depends on cell-intrinsic properties, but it is also influenced by the tumor microenvironment (TME). mRNA translation is pivotal in this communication, as tumor cytokine production, vascularization promotion or immune recognition are translationally regulated processes. However, the role of translational control in other components of the TME is largely unknown.

Cytoplasmic polyadenylation element binding proteins (CPEBs) are a family of four (CPEB1-4) RNA binding proteins that regulate mRNA translation and stability. In the context of cancer, CPEB function, and in particular CPEB4, has been mostly studied in tumor cells, where it mainly acts as a tumor promoter. However, its role in different cell types of the TME is completely unexplored.

In the present work, we characterized a novel role for CPEB4 in T cell mediated anti-tumor immunity. We observed that CPEB4 in T cells is required for an efficient anti-tumor effector response. CPEB4 is upregulated in activated and effector T cells by activation-induced endoplasmic reticulum (ER) stress and, in turn, it regulates mRNAs required for stress adaptation. Therefore, CPEB4-mediated gene expression control allows cellular adaptation to ER stress, improving T cell effector function and anti-tumor activity.

Introduction

1. Post-transcriptional control of gene expression

Gene expression is the process through which genetic information stored in the DNA is converted into functional gene products, namely RNA and proteins. In general terms, it encompasses two main steps: synthesis of an intermediary nucleotide sequence (messenger RNA, mRNA) from a DNA template and translation of mRNAs into proteins. This framework for genetic information transmission is known as the central dogma of molecular biology and was proposed by Francis Crick in 1957 (Crick, 1958).

Since the establishment of the central dogma, it became clear that not all genes are expressed at the same time and at similar levels in every cell. Gene expression regulation ensures that only those proteins required for specific functions are produced, providing cells with the adequate plasticity to respond and adapt to environmental changes (Kotliar et al., 2019; Strober et al., 2019). In pluricellular organisms, differential gene expression lies at the foundation of cell identity and fate, since although all cells present the same genome, each differentiated cell type specifically produces those proteins required for their specialized function (Kotliar et al., 2019; Strober et al., 2019).

Given that gene expression must be so tightly controlled, cells have evolved a complex, multilayered and interconnected network of regulatory mechanisms that sequentially modulates gene expression at each step. Due to historical and technical reasons, transcription has been the most studied process, leading to a comprehensive annotation of regulatory DNA elements, *trans*-acting factors, epigenetic marks and chromatin structures that influence mRNA synthesis, as well as their modulation by signaling pathways in health and disease. However, the technical developments in the last years have greatly expanded our knowledge of post-transcriptional gene expression control. It is now known that translation can be regulated at multiple levels, and that mRNAs, rather than being passively recognized by the ribosome, have different fates in the cytoplasm. In this context, a superfamily of proteins with RNA recognition activity, known as RNA-binding proteins (RBPs) have emerged

as master regulators of mRNA localization, stability and translation, with key functions in physiological and pathological conditions (Gebauer et al., 2020). In this section, we will review the basics concepts of mRNA processing and translation required to better understand the function of the CPEB family of RBPs.

1.1. From transcription to the cytoplasm: making a functional mRNA

In order to be functional, eukaryotic mRNAs undergo a co-transcriptional maturation process that involves an extensive remodeling of the mRNA molecule (Bentley, 2014). Pre-mRNA processing is not only spatiotemporally coupled with transcription, but both processes are also mechanistically interdependent. The C-terminal domain (CTD) of RNA polymerase II (Pol II) acts as a loading platform that carries mRNA processing machinery along the nascent mRNA molecule (Martinez-Rucobo et al., 2015). As the nascent mRNA exits Pol II, it is bound by a plethora of RBPs that catalyze different reactions to form a competent ribonucleoprotein particle (mRNP) that can be exported to the cytoplasm. In general terms, maturation of mRNA requires attachment of a cap to the 5' end, removal of introns by splicing, and formation of a 3' end by endonucleolytic cleavage and addition of a non-templated poly(A) tail (Figure 1a).

1.1.1. 5' end processing

The first step in mRNA processing, 5' end capping, is the attachment of a N7-methyl guanosine (m^7G) linked by a 5'-5' triphosphate chain to the first transcribed nucleotide (Topisirovic et al., 2011). Cap addition is obtained through 3 consecutive reactions that start as soon as the first 20-30 nucleotides of the nascent mRNA exit Pol II. Capping immediately shields mRNAs from 5' exonuclease-dependent degradation and is required for subsequent processing steps, export to the cytoplasm and translation initiation (Topisirovic et al., 2011). These functions are mediated by different cap-binding protein

complexes that constitute important signaling nodes of decisive influence on mRNA function and fate.

1.1.2. Splicing and alternative splicing

Splicing consists in the removal of introns from pre-mRNAs and ligation of adjacent exons in two sequential trans-esterification reactions (Wilkinson et al., 2020). Splicing is carried out by the spliceosome, a large molecular machine comprising several small-nuclear RNA (snRNA) and hundreds of proteins in humans. As a result of splicing, exons can be retained or removed in the final mRNA molecule, generating different protein isoforms (what is known

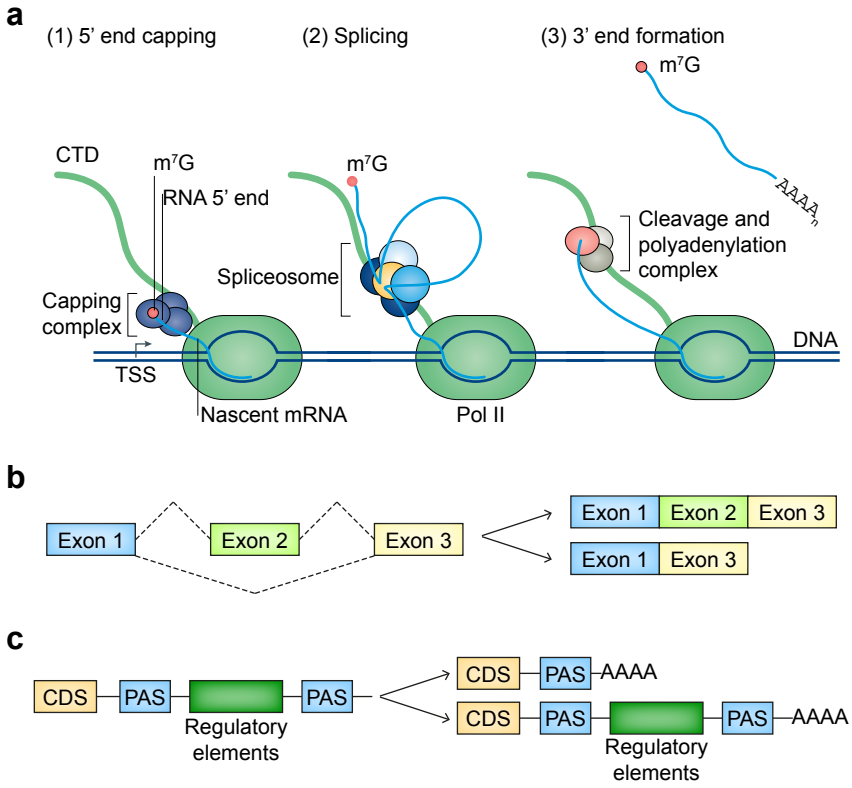

Figure 1. Pre-mRNA processing. a) When an mRNA is transcribed, it is modified in its 5' and 3' end by capping and cleavage and polyadenylation, respectively. As the mRNA molecule is being produced, introns are removed by splicing. Adapted from (Desterro et al., 2020). b) Alternative splicing generates different proteins products as exons can be removed from mRNAs. c) Alternative polyadenylation though differential PAS selection determines the presence or absence of regulatory elements in the 3' UTR of mRNAs.

as alternative splicing) and expanding the protein products that can be derived from a single gene (Figure 1b). The plasticity that alternative splicing confers to gene expression is cell type- and context-dependent, representing a rich and diverse layer of gene expression heterogeneity that cells modify to proliferate, differentiate or respond to any stimuli (Baralle and Giudice, 2017). In fact, 92-94% or human genes undergo alternative splicing, illustrating the importance of this mechanism of gene expression regulation (Wang et al., 2008).

1.1.3. Cleavage and polyadenylation

To become a fully mature mRNA, eukaryotic pre-mRNAs (except for replication-dependent histones) undergo cleavage and polyadenylation of their 3' end (Baralle and Giudice, 2017). This process is subdivided in 2 consecutive reactions: endonucleolytic cleavage of the nascent RNA followed by addition of a non-templated poly(A) tail at the 3' terminus of the cleaved product by a poly(A) polymerase (PAP). Cleavage is guided by several *cis* acting elements present in the sequence of the pre-mRNA that are recognized by *trans*-acting RBP complexes. The main RNA motifs are the polyadenylation signal (PAS), which consist of a degenerated A-rich hexamer A[A/U]UAAA, and the U/GU-rich element, both present in the 3' region of the mRNA. These motifs are bound respectively by the cleavage and polyadenylation specific factor (CPSF) and the cleavage stimulating factor (CsF). These factors, together with cleavage factor I and II, stimulate the cleavage. Then, CPSF recruits PAP, which will append a poly(A) tail, and poly(A) binding protein nuclear I (PABPNI), which binds the newly synthetized tail and prevents its degradation. The length of the tail is highly variable between species and, within the same species, between mRNA molecules, but it is pivotal for mRNA export, stability and translation (Proudfoot, 2011).

Interestingly, half of human mRNAs can be cleaved and polyadenylated in multiple sites, a process known as alternative polyadenylation (APA) (Figure 1c). APA generates a greater diversity of transcripts with shorter or longer 3' UTR, which results in the presence or absence of regulatory elements such

as microRNA or RBP binding sites (Baralle and Giudice, 2017). Importantly, presence or absence these motifs is crucial to determine the fate and localization of the mRNA and, consequently, of the final protein product (Berkovits and Mayr, 2015). On the contrary, PAS selection is also regulated by *cis*-regulatory elements such as RBP binding sites that, depending on the specific cellular context, modulate the usage of alternative PAS (Bava et al., 2013). As a result, APA plays a major role in tissue specification, homeostasis and disease (Gruber and Zavolan, 2019; Jia et al., 2017; Masamha and Wagner, 2018; Mayr and Bartel, 2009).

1.1.4. mRNA modifications

Finally, mRNAs can be enzymatically modified on specific nucleotides. More than 100 modifications have been described, but among all of them the reversible methylation of adenosine in position N6 (m^6A) has emerged as a dynamic, widespread and potent regulator of mRNA maturation, stability, translation and transport (Zhao et al., 2016). m^6A is generated by a *writer* complex, composed by methyltransferases METTL3 and METTL4. Conversely, m^6A is removed by *eraser* demethylases FTO and ALKBH5. Importantly, m6A can be recognized by different RBPs (*readers*) and, depending on the specific reader bound to the modification, it can either inhibit or activate translation. Physiologically, m6A and the proteins involved in its dynamics have been recently shown to play pivotal roles in cancer or immune system regulation (Barbieri and Kouzarides, 2020; Han et al., 2019; Shulman and Stern-Ginossar, 2020).

1.2. Modes of translational control

Once mRNA processing is finished, the mature mRNA molecule will be composed of a m^7G cap at the 5' end, 5' and 3' untranslated regions flanking the coding sequence, and a poly(A) tail closing the 3' end. Each region has specific regulatory properties that will determine the fate of the mRNA: if, how and where the mRNA will be translated (Figure 2).

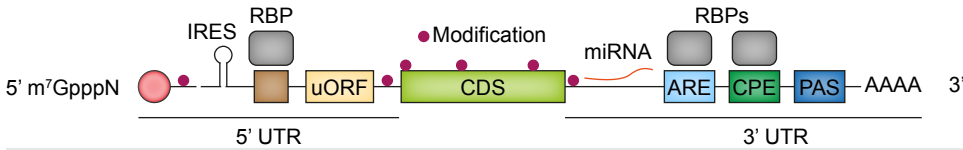

Figure 2. Structure of a mature mRNA and its *cis*-regulatory elements.

Translation is vital for cellular homeostasis as it connects genetic information to functional proteins. However, it is also the most expensive biosynthetic process, comprising the highest share of energy expenditure in the cell (Verduyn et al., 1991). Therefore, cells must carefully adapt their translation rates in response to stimuli such as environmental stresses (heat-shock, hypoxia), extracellular signaling (hormones, nutrients) or intracellular cues (energy status, cell function). Translational control induces both quantitative and qualitative changes in the translatome either by regulating general translation or by achieving a targeted, transcript-specific, motif-directed modulation, ensuring that the right proteins are made in the right time and place. The structural features that regulate mRNA translation are multiple and affect initiation (secondary structure, IRES, alternative initiation sites, upstream open reading frames, mRNA modifications, RBPs), elongation (codon usage) or mRNA stability (RBP and microRNA binding sites, mRNA modifications) (Figure 2). For the interest of this work, we will focus on mechanisms and pathways that regulate cap-dependent translation and RBPs. For a thorough description of other features refer to: Hinnebusch et al., 2016; Jonas and Izaurralde, 2015; Xu and Ruggero, 2020; Zhao et al., 2016.

1.2.1. mRNA translation

Protein synthesis is divided in three steps: initiation, elongation and termination, being initiation the rate-limiting and more complex phase of translation (Sonenberg and Hinnebusch, 2009). Engagement of the ribosomal machinery involves more than 25 proteins and is, if not the main, one of the most fundamental determinants of the translation efficiency of an mRNA. In most eukaryotic mRNAs, translation is initiated by cap-dependent recruitment of ribosomal machinery to the 5' UTR of mRNAs followed by ribosomal scanning towards the initiation codon (Figure 3).

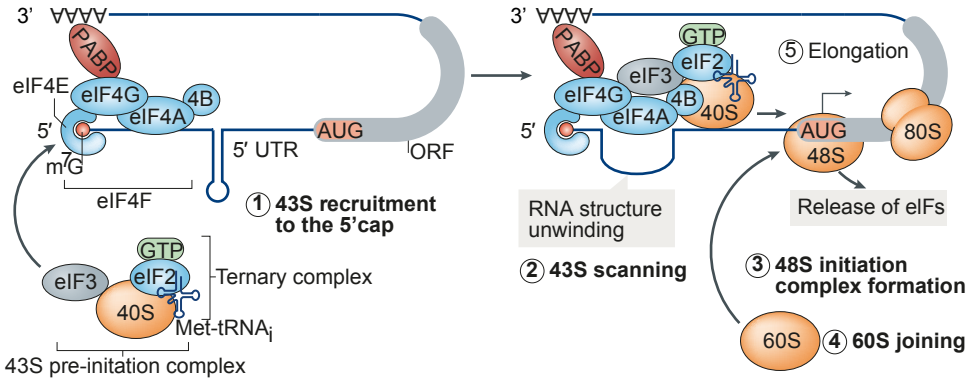

Figure 3. Overview of cap-dependent translation initiation. Cap-dependent translation requires the formation of the ternary complex and its recruitment to the 5'UTR cap of the mRNA to form 43S the pre-initiation complex (PIC) (step 1). Once bound, the PIC starts scanning the mRNA (step 2) until it reaches the initiation codon (step 3), where the 60S subunit of the ribosome joins (step 4) to form an elongation-competent ribosome (step 5). Adapted from (Leppek et al., 2017).

The first step in translation initiation is the formation of the ternary complex, which results from the binding of the initiator methionyl transfer RNA (Met-tRNAi) and GTP to the eIF2 complex (an heterotrimer comprising subunits α, β, and γ) (Hashem and Frank, 2018). The interaction of the ternary complex with the 40S small ribosomal subunit and a group of translation initiation factors (eIF1, eIF1A, eIF3, and eIF5) leads to the formation of the 43S pre-initiation complex (PIC). The PIC is recruited to the 5' end of the mRNA by the eIF4F complex, which is formed by the cap-binding subunit eIF4E, the RNA helicase eIF4A, the stabilization subunit eIF4B, and the scaffolding subunit eIF4G. eIF4G binds the polyadenylate-binding protein (PABP), therefore bringing the 5' and the 3' end together in a process of pseudocircularization known as the close loop model (Wells et al., 1998). The formation of the loop, albeit not strictly necessary for all steps of translation (Adivarahan et al., 2018), greatly increases ribosomal loading efficiency and recycling and, subsequently, protein production (Sonenberg and Hinnebusch, 2009).

Once PIC is bound to the mRNA, it scans the mRNA until an initiation codon in a specific sequence context is detected (Dever and Green, 2012). Then,

eIFs are released and the 60S large ribosomal subunit is recruited to form the fully-competent 80S ribosome that initiates translation elongation. During elongation, amino acids are added into the nascent protein chain by the 80S ribosome together with the translation elongation factors eEF1A and eEF2 and the pool of tRNAs. At the termination codon, peptide-chain releasing factors free the nascent protein and translation is terminated.

1.2.2. The 5′ cap as a hub of translation regulation

Given that initiation is the rate-limiting step in translation, it is controlled by multiple mechanisms (Jackson et al., 2010). The formation of the cap-binding protein complex is one of the main regulatory nodes of translation where different signaling pathways interact (Figure 4). eIF4E-eIF4G interaction is inhibited by the family of eIF4E-binding proteins (4E-BPs), which are directly regulated by mammalian target of rapamycin complex 1 (mTORC1) phosphorylation (Roux and Topisirovic, 2012). mTORC1 is a central hub in cellular signaling, where pathways such as Akt/PI3K, MAPK or AMPK converge, and therefore bridges cellular translation requirements with nutrient levels, energy levels, proliferation rate or metabolic status (Figure 4) (Saxton and Sabatini, 2017). In contexts where protein synthesis must be low, such as nutrient-limiting conditions, mTORC1 is inactive, and thus hypophosphorylated 4E-BPs are able to bind and sequester eIF4E, preventing translation initiation. However, when mTORC1 is active, as in growth-permitting conditions, 4E-BPs are hyperphosphorylated, releasing eIF4E and allowing translation (Gingras et al., 1999). Although *a priori* this mechanism should affect all cap-dependent mRNAs, it has been shown that only those that present a 5' terminal oligopyrimidine (TOP) motif are mTORC1-regulated (Thoreen et al., 2012).

Besides 4E-BPs, mTORC1 phosphorylates S6 kinases (S6K), that downstream phosphorylate ribosomal protein S6 (pS6) to promote translation initiation, and, in addition, inhibits EF2 kinase to stimulate translation elongation (Wang et al., 2001; Xu and Ruggero, 2020). Concurrently to mTORC1 activation, MAPK pathway also stimulates translation through MNK1/2-dependent

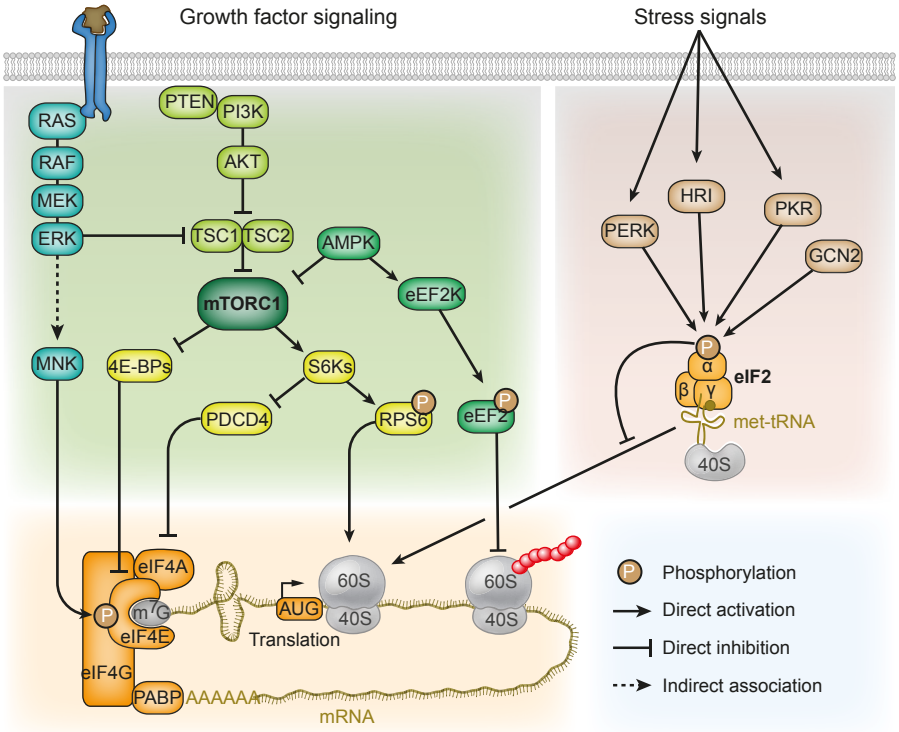

Figure 4. Signaling pathways regulating cap-dependent translation. In nutrient- or growth factor-rich conditions, Akt/PI3K and MAPK pathways converge on mTORC1 to activate translation. mTORC1 phosphorylates and inhibit 4E-BPs, allowing the formation of the eIF4F complex. mTORC1 also phosphorylates S6K, which in turn stimulates translation elongation. In parallel, MAPK pathway promotes translation initiation through MNK-dependent phosphorylation of eIF4E. In stress conditions, when translation must be stopped, AMPK is activated to repress mTORC1. Depending on the specific stressor, four different kinases (PERK, HRI, PKR and GCN2) phosphorylate eIF2α subunit to repress global protein synthesis while increasing translation of specific, stress-response mRNAs. Adapted from (Xu and Ruggero, 2020).

phosphorylation of eIF4E, although the underlying mechanism is still unknown (Joshi et al., 1995; Roux and Topisirovic, 2012)

Another major strategy of global translational control is targeting the formation of the ternary complex by phosphorylation of the eIF2 α subunit (Wek, 2018) (Figure 4). eIF2 is a G-protein that, in its active state, is bound to GTP. Upon recognition of the start codon, GTP is hydrolyzed and the inactive GDP-bound form of eIF2 is recycled back to its GTP-bound state by

the guanine nucleotide exchange factor eIF2B. Phosphorylation of the eIF2α subunit blocks eIF2 dissociation from eIF2B, preventing the reconstitution of a functional ternary complex and inhibiting general translation (Adomavicius et al., 2019). Phosphorylation of eIF2α is carried out in response to cellular stress by four different kinases from four different signaling pathways in what is collectively known as the integrated stress response (ISR) (Pakos-Zebrucka et al., 2016). These four kinases include: general control nonderepressible 2 (GCN2), activated by amino acid deprivation (Berlanga et al., 1999); interferon-inducible RNA-dependent kinase (PKR), activated in response to a viral infection (Proud, 1995); heme-regulated inhibitor (HRI), activated by heme deficiency (de Haro et al., 1996) and the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) (Harding et al., 1999), activated by accumulation of misfolded proteins in the ER.

Thereby, eIF2α acts as a signaling hub through which cells can stop translation when homeostasis is perturbed. The kinases and pathways highlighted above are interrelated and, in addition to halting translation, activate cellular responses to either overcome the initiating insult, adapt or eventually succumb to it. A trigger of stress responses of particular interest for this work is the accumulation of misfolded proteins in the ER, that will be covered in the following section.

1.2.3. Translational control during ER stress and the UPR

Approximately one third of all eukaryotic proteins are synthetized, modified and folded in the secretory pathway. Proteins enter the ER from the cytoplasm co-translationally in an unfolded state, and, as they complete their translation, undergo chaperone-assisted folding to become functional (Rapoport et al., 2017). This process is highly error-prone, and cells have acquired quality control mechanisms to maintain an optimal ER performance (Sun and Brodsky, 2019). However, under certain physiological or pathological circumstances, unfolded/misfolded proteins accumulate and ER homeostasis is disrupted, impacting not only on cellular protein production capacity, but also on metabolism, signaling

and ultimately survival. The condition where the ER presents an overload of unfolded proteins is generally known as ER stress, and can be triggered by a myriad of stimuli such as high protein production demand, misbalance in the redox status, defects in protein quality control mechanisms, lack of nutrients, viral infections, etc.

Given the constant challenges that the ER has to meet, cells have evolved a surveillance and response system to ensure a balance between protein synthesis demand and ER folding capacity: the unfolded protein response (UPR) (Hetz et al., 2020). The UPR is designed to sense unfolded protein overload and ignite a network of signaling pathways that reprogram transcription, translation and ER function to clear misfolded proteins and restore ER homeostasis. The UPR is composed of three distinct branches controlled by three transmembrane sensors localized at the ER: ATF6, IRE1α and PERK (Figure 5) (Cox et al., 1993; Harding et al., 1999; Morl et al., 1993; Yoshida et al., 1998). They are characterized by a luminal domain that is able to sense unfolded proteins through its interaction with the chaperone BiP (Bertolotti et al., 2000; Shen et al., 2002). BiP is a chaperone that assists protein folding; in homeostasis, BiP is present in excess in the ER lumen and binds the luminal domain of the three receptors, preventing their activation. However, during ER stress, BiP associates with misfolded proteins due to its higher affinity for them, thereby releasing ER stress sensors and priming them for activation.

In the case of ATF6, BiP dissociation allows its translocation towards the Golgi apparatus, where its cytosolic domain is proteolytically cleaved (Shen et al., 2002). The cytosolic fragment contains a transcription factor that translocate into the nucleus to activate transcription of ER chaperones, protein folding and maturation enzymes as well as misfolded protein clearance factors through the ER-associated degradation (ERAD) pathway (Haze et al., 1999; Wu et al., 2007). For IRE1α and PERK, BiP release induces their dimerization and autophosphorylation (Zhou et al., 2006). IRE1α phosphorylation activates its endoribonuclease activity, promoting the excision of a 26-nucleotide intron from the mRNA encoding for the transcription factor XBP1 (Calfon et al., 2002; Yoshida et al., 2001). This spliced version of XBP1, XBP1s, is an active

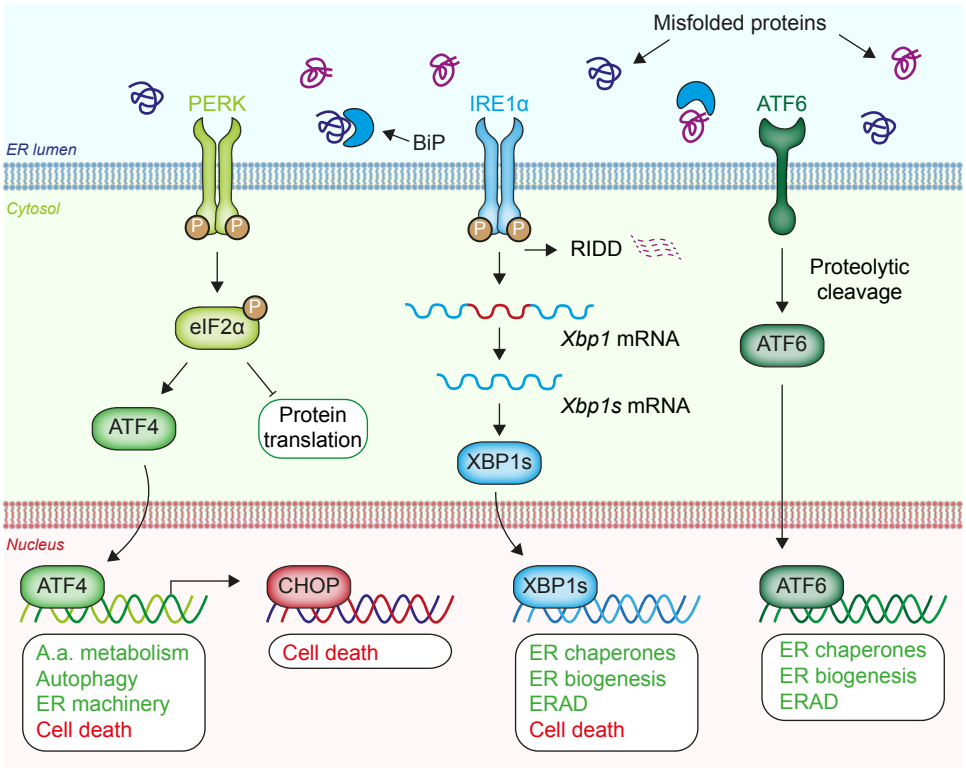

Figure 5. The UPR pathway. The UPR is composed of three parallel branches, each of them containing a different sensor: PERK, IRE1α and ATF6. These receptors are kept inactive by the chaperone BiP; however, when misfolded proteins accumulate in the ER, BiP is titrated away and the receptors become active. ATF6 is transported to the Golgi apparatus where it undergoes proteolytic cleavage to release a transcription factor. IRE1α promotes splicing of *Xbp1* mRNA to generate an active form, XBP1s, a transcription factor that controls UPR genes. IRE1α also claves a subset of mRNA to decrease protein translation at the ER in a process known as IRE1α -dependent decay (RIDD). Active PERK phosphorylates eIF2α in order to decrease global translation rates. However, a subset of uORF-containing mRNA such as *Atf4* increase their translation in this context. The three pathways cooperate to alleviate the accumulation of misfolded proteins and promote cell survival; however, if stress persist or cells cannot adapt to it, cell death promoters such as CHOP will be expressed and cells will undergo apoptosis. A.a: amino acid; ERAD: endoplasmic reticulum associated protein degradation.

form that, in parallel with ATF6, activates genes of ER chaperones, enzymes, ERAD components as well as ER and Golgi biosynthesis. IRE1α also cleaves and primes for degradation a subset of mRNAs and microRNAs in order to lower mRNA abundance and decrease ER translation in a process called regulated IRE1α -dependent decay (RIDD) (Hollien and Weissman, 2006).

On the other hand, as described above, active PERK phosphorylates eIF2α to transiently attenuate translation and alleviate the overwhelmed ER protein synthesis machinery (Harding et al., 1999). However, phosphorylation of eIF2α paradoxically results in the permissive translation of a subset of mRNAs that contain upstream open reading frames (uORFs) in their 5' UTR (Hinnebusch et al., 2016). In normal conditions, uORFs attenuate translation from the main ORF by sequestering highly abundant PIC before the scanning ribosome arrives at the main ORF. However, when eIF2α is phosphorylated and ternary complex are limited, PICs initiate scanning without the ternary complex attached, and therefore when they reach an uORFs it is bypassed. As scanning continues, ternary complex will eventually join the scanning, ternary complex-free PIC and, once they arrive at the main ORF, they will be fully competent to start translation. Among these uORF-containing mRNAs is ATF4, a transcription factor that thanks to this elegant translational control is upregulated during ER stress. Subsequently, ATF4 activates genes involved in ER protein folding, amino acid metabolism, redox signaling or autophagy, to complement ATF6 and XBP1s actions (B'chir et al., 2013; Harding et al., 1999, 2003).

In summary, the UPR activates a cellular response that increases ER capacity (protein translocation, folding and secretion), reduces the accumulation of misfolded proteins and attenuates protein influx into the ER. These responses are initially transient and communicate with other signaling and metabolic pathways in the cell to coordinately overcome, or adapt to, ER stress.

1.2.4. Terminal vs. adaptive responses in chronic ER stress

If ER stress persists in time, and the UPR is unable to sustain proteostasis, it will switch towards a pro-apoptotic program known as terminal UPR that will eventually cause cell death (Iurlaro and Muñoz-Pinedo, 2016). This maladaptation to ER stress is mediated by ATF4-induced transcription factor CHOP, which activates the mitochondrial apoptotic cascade (Puthalakath et al., 2007). The molecular mechanisms that govern the switch from adaptation

to cell death is yet unclear (Iurlaro and Muñoz-Pinedo, 2016). The final cellular fate is multifactorial and context dependent: it is influenced both by characteristics of the ER-stress inducer (type, duration and intensity) and of the specific cell type that is suffering it (basal ER translation capability, UPR strength, metabolic and proliferative status).

In fact, very little is known about the adaptive UPR program in chronic ER stress settings. It is worth noting that most of the mechanistic insights of the UPR have been drawn from systems in which ER stress is chemically induced *in vitro*. Although valuable, these conditions allow mostly the study of acute, strong UPR induction, which do not necessary represent physiological or pathological conditions, where ER stress is linked to the normal function of the cell and is chronically sustained without necessarily causing cell death. The mechanistic nuances of a sustained, mild chronic UPR are now starting to be revealed. For instance, it has been proposed that the m6A writer METTL14 suppresses CHOP-dependent apoptosis, promoting liver adaptation to chronic proteotoxicity and avoiding terminal apoptosis (Wei et al., 2021).

In the past years, UPR has been shown to play important roles in brain and neurodegeneration (Hetz and Saxena, 2017), cardiovascular system (Ren et al., 2021), immune system (Grootjans et al., 2016), liver function (Reibe and Febbraio, 2019) or cancer development (Wang and Kaufman, 2014). Understanding how UPR signaling is required for normal cell function, and, conversely, how it can contribute to pathology is an active area of research where exciting results are granted.

1.2.5. 3' UTRs: translational control by RBPs

The 3' UTR of mRNAs also plays a decisive regulatory role on mRNA fate. It acts as a bar code crowded with functional *cis* motifs where *trans*-acting factors such as RBPs bind (Mayr, 2019). Novel high-throughput interactome approaches have allowed the systematic identification of proteins bound to mRNAs, expanding the number of RBPs to nearly 2000 in mice, which has been collectively termed the RBPome (Hentze et al., 2018). The combination

of RBPs and other constituents on an mRNA molecule generates a unique mRNP particle that cells need to interpret to modulate gene expression (Gehring et al., 2017). This "mRNP code" controls protein output in a temporal- and spatial-specific manner, allowing for a rapid cellular adaptation to environmental changes without needing *de novo* transcription. For instance, in certain situations where translation cannot occur, mRNPs are sequestered into translationally silent, membrane-less organelles such as processing (P) bodies or stress granules, where mRNAs will be stored until needed or targeted for degradation (Decker and Parker, 2012). In addition, a given RBP can coregulate multiple mRNAs that encode for proteins of the same biological process (known as RNA regulons), allowing a coordinated expression of gene sets whose functions are interrelated (Keene, 2007).

Besides sorting mRNAs into specific cellular locations, RBPs control mRNA stability and translation efficiency, mostly through modulation of poly(A) tails. The first motifs that were discovered to impact poly(A) tail post-transcriptionally are AU-rich elements (AREs) (Meijlink et al., 1985). AREs are recognized by ARE-binding proteins that have dual roles on mRNAs. Some members of the family, such as Tristetraprolin (TTP), recruit a deadenylation complex that promotes mRNA decay and inhibits translation. However, this function is counteracted by proteins like human antigen R (HuR), which by competitively binding to the same AREs as TTP prevents mRNA deadenylation and therefore promotes its stabilization and translation (Otsuka et al., 2019). Since the discovery of AREs, more motifs and RBPs implicated in poly(A) tail dynamics have been characterized, adding plasticity to mRNA modulation and expanding the complexity of translational control at the 3' UTR (Weill et al., 2012).

1.3. Translational control by cytoplasmic polyadenylation

As explained above, poly(A) tails are vital for preventing mRNA degradation and stimulating translation efficiency. So far, we have seen that poly(A) tails, rather than being static, are dynamically modulated after mRNA processing

by deadenylation, with negative consequences for stability and translation. However, mRNAs can be re-anylated in the cytoplasm by the action of non-canonical PAPs, being GLD2 the most prominent. This allows the re-activation of previously deadenylated mRNAs, or the further lengthening of a poly(A) tail, with a concomitant positive effect in stability and translation efficiency. Cytoplasmic polyadenylation is a fast, transcript-specific and reversible mechanism of translational control, allowing rapid adaptation of cells to environmental changes (Weill et al., 2012). One of the main families of proteins that regulate gene expression through modulation of the poly(A) tail are the cytoplasmic polyadenylation element binding proteins (CPEBs).

1.3.1. Cytoplasmic polyadenylation element binding proteins

In vertebrates, the CPEB family of proteins is composed of 4 members, CPEB1-4 (Figure 6). CPEB2-4 are more closely related, being CPEB1 the most distant paralogue. CPEB orthologues have been identified in other species; for instance, *Drosophila* presents two (Orb1-2) and *C. elegans* four (cpe1-3 and fog1) (Fernández-Miranda and Méndez, 2012; Ivshina et al., 2014).

All four CPEBs share a common C-terminal domain with two RNA recognition motifs (RRMs) and two zinc-finger like domains (Hake et al., 1998). The RRMs are required for direct RNA binding, whereas the zinc-finger domains increase protein-protein and protein-RNA affinity (Afroz et al., 2014). On the contrary, the N-terminal domains greatly differ in length and amino acid composition between CPEBs. Interestingly, this region lacks any recognizable structure, being intrinsically disordered. In addition, most of the described post-translational modifications affecting CPEB1-4 occur in the N-terminal domain (Guillén-Boixet et al., 2016; Mendez et al., 2000a; Pavlopoulos et al., 2011; Setoyama et al., 2007, Duran-Arque, *in preparation*).

CPEBs recognize in their target mRNA a specific AU-rich motif termed cytoplasmic polyadenylation element (CPE). The consensus CPE sequences are UUUUAAU and UUUUAU although several non-consensus variants have been identified (McGrew and Richter, 1990; Piqué et al., 2008). However, for

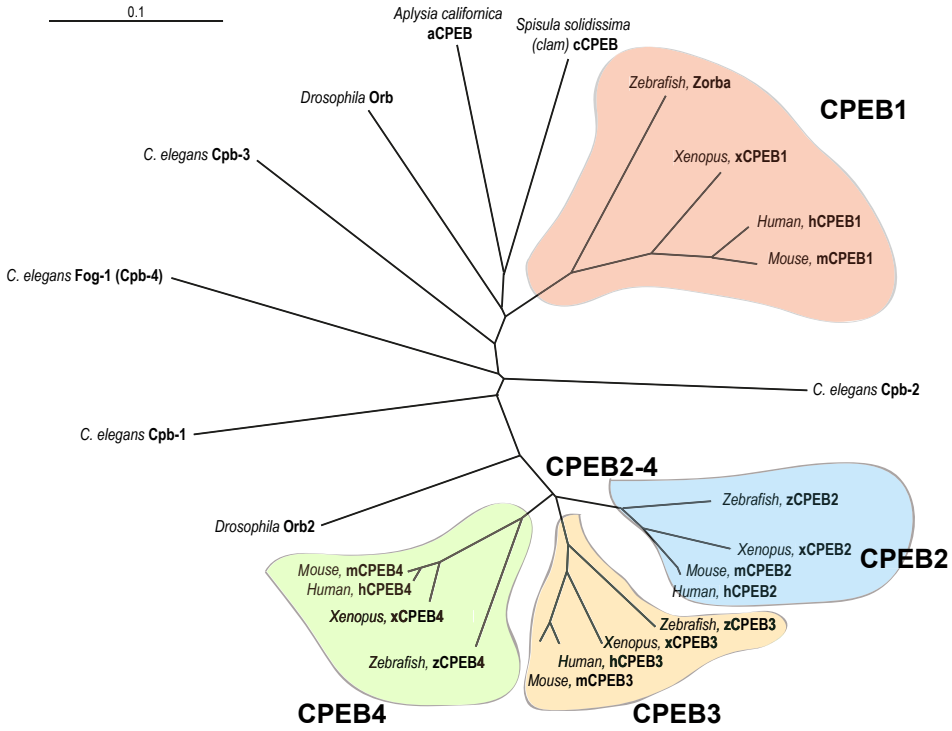

Figure 6. Phylogenetic analysis of CPEBs. Unrooted phylogenetic tree of CPEB proteins based on a multiple protein sequence alignment. As depicted, orthologs are closer than paralogues. CPEB1 vertebrate orthologues are the most distant members of the family. Adapted from (Fernández-Miranda and Méndez, 2012).

cytoplasmic polyadenylation to take place, CPEs must be surrounded by a specific sequence environment that allows CPEB function (Piqué et al., 2008). Genome-wide analysis of CPE presence in 3'UTRs suggested that up to 20% of human protein coding genes could be targeted by CPEBs, indicating that cytoplasmic polyadenylation is a widespread mechanism of translational control (Belloc and Méndez, 2008; Piqué et al., 2008). Given that the four CPEBs recognize the same sequence, it has been debated whether all four regulate the same targets. It has been proposed that CPEB1 presents higher affinity for canonical CPEs (Afroz et al., 2014), and *in vitro* experiments suggested that CPEB3-4 might need an extra U-loop motif for binding, not recognized by CPEB1 (Huang et al., 2006). However, subsequent studies showed that CPEB2-4 bind the same CPE as CPEB1 and that their targets at least partially overlap with CPEB1 (Igea et al., 2010; Novoa et al., 2010; Pavlopoulos et

al., 2011, Duran-Arque, *in preparation*). Although still an open question, the structural features of CPEBs imply that all of them can potentially recognize the same targets competing or acting coordinately on the same target mRNA. Nevertheless, since they are regulated by different pathways and mechanisms, they are involved in different cellular responses. In fact, gene expression analysis indicates that CPEBs expression pattern across tissue and cell types only partially overlaps and data from single CPEB knockout mouse models show that each of them has distinct functions (see below).

1.3.2. Mechanisms and regulation of CPEB function

Unlike most of the other poly(A) tail modulators, CPEBs exert bifunctional regulation on mRNA translation: depending on the context, CPEBs promote deadenylation to repress mRNA translation, or assemble a polyadenylation complex that elongates poly(A) tails and stimulates translation. Most of our knowledge about CPEB function has been acquired studying CPEB1 during *Xenopus laevis* oocyte meiotic maturation. *Xenopus* oocytes are arrested at prophase of meiosis I (PI), but upon progesterone stimulation they re-activate and progress with the meiotic cycle. In this specific context, CPEB1 is responsible for the translational repression of maternal mRNAs at PI, and their subsequent cytoplasmic polyadenylation and activation in response to progesterone (Mendez and Richter, 2001). CPEB1-mediated translational repression occurs upon CPEB1 dimerization and the recognition of at least two CPEs spaced by less than 50 nucleotides in its target mRNA (Piqué et al., 2008). Since CPEB1 is catalytically inactive, its main function is to recruit a repression complex that shortens poly(A) tails and prevents translation initiation. The complete picture of the repression complex remains elusive, and three different, mutually-exclusive models have been proposed to explain CPEB1-mediated translational repression. The first one proposes that CPEB1 directly recruits the deadenylase PARN, shortening the poly(A) tail and preventing the formation of the closed-loop (Kim and Richter, 2006); the second and third models contemplate the inhibition of translation initiation by preventing the formation of a translation-competent cap binding complex,

either by the recruitment of the eIF4E-inhibiting protein maskin (Stebbins-Boaz et al., 1999), or by recruiting an isoform of eIF4E, eIF4E-1b, that is unable to interact with eIF4G (Andrei et al., 2005; Minshall et al., 2007).

Upon progesterone stimulation, CPEB1 is phosphorylated by Aurora Kinase A at Ser174, leading to a rearrangement of its binding partners that activates CPEB1-mediated polyadenylation (Figure 7). The interaction of phosphorylated CPEB1 with CPSF recruits the PAP Gld2, which finally

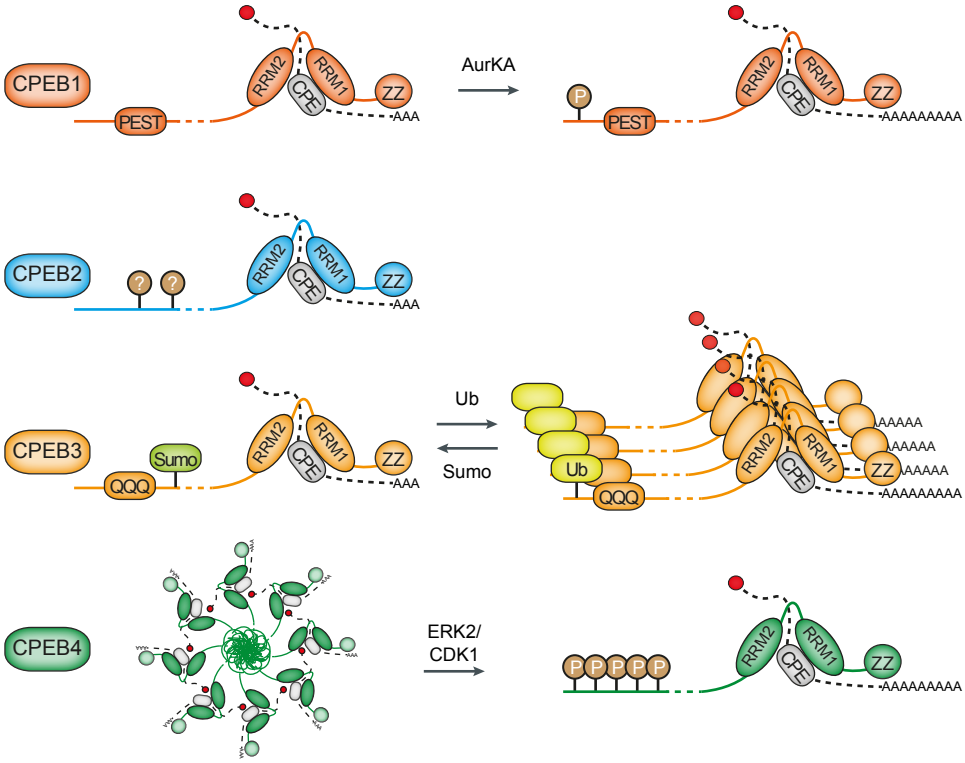

Figure 7. Regulation of CPEB function. CPEB1-4 share a common C-terminal domain but differ in their N-terminal domain, which provides them with specific modes of regulation. CPEB1 switch from repressor to activator upon a single phosphorylation by Aurora Kinase A (AurKA). No specific post-translational modification has been yet assigned to CPEB2. CPEB3 activity is determined by monoubiquitination or SUMOylation, which controls its fibrilization into a functional amyloid. If CPEB4 is not phosphorylated, it undergoes liquid-like phase separation and represses it target mRNA; however, upon hyperphosphorylation by ERK2 and CDK1, it solubilizes and activate mRNA polyadenylation. Ub: ubiquitin; SUMO: SUMOylation; P: phosphorylation; QQQ: polyglutamine stretch. The mRNA is depicted as a dashed line with a red cap at its 5'UTR and a poly(A) tail at its 3' end.

elongates poly(A) tails (Kim and Richter, 2006; Mendez et al., 2000b, 2000a). In human fibroblasts, CPEB1 has been shown to recruit a second PAP, GLD4, to p53 mRNA, although the mechanism that dictates the differential PAP recruitment is not known (Burns et al., 2011). CPEB1 is also phosphorylated by CDK1 and PLK1 in its PEST-box domain, targeting it for ubiquitin-mediated degradation (Mendez et al., 2002; Setoyama et al., 2007).

In addition to temporally regulating translation, CPEB1 is required for subcellular localization of mRNAs. For instance, it brings CPE-containing mRNAs to meiotic and mitotic spindles to enable cell cycle progression though localized translation (Eliscovich et al., 2008; Pascual et al., 2020a). In neurons, CPEB1 contributes to transport mRNAs towards dendrites, where they are translated upon synaptic stimulation (Huang et al., 2002). Therefore, CPEB1 not only controls when mRNAs are translated, but also where. Besides its role in the cytoplasm, CPEB1 also mediates alternative polyadenylation in the nucleus (Bava et al., 2013)

The mechanistic details explaining the function of the other members of the family are limited (Figure 7). CPEB2, probably the less known member of the four, was first reported to promote HIF1α translation upon insulin stimulation by cytoplasmic polyadenylation (Hägele et al., 2009). However, it was later reported that CPEB2 repressed *Hif1a* mRNA under normoxia by inhibiting translation elongation, and therefore translational activation resulted just from its dissociation from *Hif1a* mRNA (Chen and Huang, 2012). Recent reports have suggested its role as a translational activator in neurons (Lu et al., 2017), thermogenesis (Chen et al., 2018) and mammary gland development and tumorigenesis (Pascual et al., 2020b). However, the mechanism governing CPEB2 function and its potential switch from repressor to activator is still unknown. CPEB3 has been proposed to exert its activator function in neurons by forming a functional amyloid that is regulated by sumoylation and mono-ubiquitination (Drisaldi et al., 2015; Fioriti et al., 2015; Pavlopoulos et al., 2011) (Figure 7). In basal conditions, CPEB3 is SUMOylated and represses its target mRNAs, but upon synaptic stimulation it is ubiquitinated, triggering its amyloid fibrilization and activation. This process is key for learning and

long-term memory potentiation (Fioriti et al., 2015)

Regarding CPEB4, its role in translation activation through cytoplasmic polyadenylation has been shown in different scenarios such as meiosis maturation (Igea et al., 2010), cell cycle progression (Novoa et al., 2010) or angiogenesis (Calderone et al., 2016). Recent work has demonstrated that in proliferating cells CPEB4 switches from repressor to activator due to its phosphorylation status and aggregation capacity (Guillén-Boixet et al., 2016) (Figure 7). When CPEB4 is unphosphorylated, it phase-separates into inactive, liquid-like droplets through multiple inter-molecular interactions of its intrinsically-disordered N-terminal domain. However, upon hyperphosphorylation by ERK2 and CDK1, CPEB4 re-dissolves in the cytoplasm and promotes translational activation of its targets. Interestingly, CPEB4 (and CPEB2-3) mRNA harbors in its 3' UTR CPEs that are recognized by CPEB1 and CPEB4 itself, generating a positive feedback loop that further enhances CPEB4 expression when is upregulated (Igea et al., 2010). CPEB4 translation is also upregulated during ER stress due to the presence of uORFs in its 5'UTR. In fact, CPEB4 has been shown to be a fundamental player of the translational branch of the late, adaptive UPR (Maillo et al., 2017). In response to ER stress, CPEB4 coordinates the expression of CPE-containing mRNA related to ER homeostasis. CPEB4-mediated regulation occurs as a second wave of translational reprogramming following the first, uORF-mediated wave. Therefore, CPEB4 allows an extended, transcription-independent adaptation to ER stress that is vital for liver homeostasis.

2. Immunosurveillance of tumor growth

Cancer cells are highly influenced by their communication with the microenvironment. Tumor and stromal cells engage in a constant, bidirectional crosstalk that ultimately influences tumor initiation, progression, prognosis and therapy efficacy (Quail and Joyce, 2013). In response to cancer cell signals, the tumor microenvironment (TME) evolves as tumors grow, and, in turn, it communicates back to tumor cells modulating tumor progression.

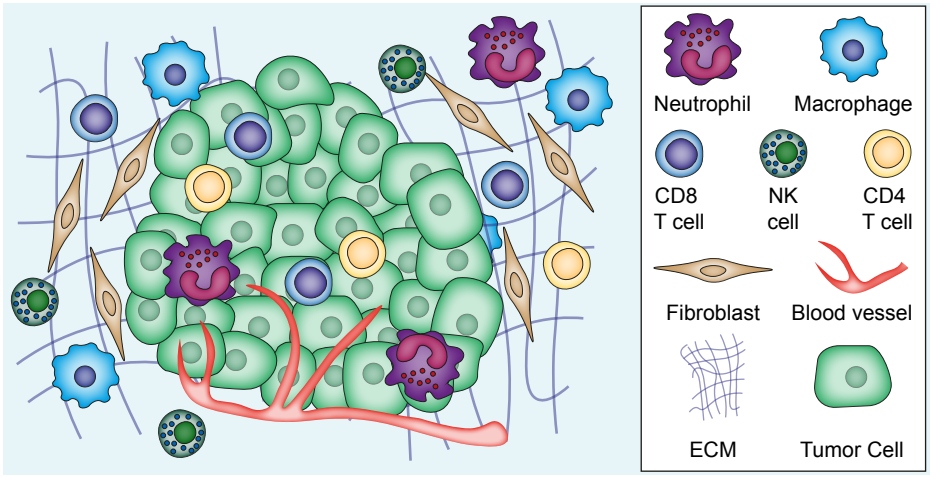

Figure 8. The tumor microenvironment (TME). Simplified cartoon of the components of the TME. Fibroblast produce extracellular matrix and signaling molecules required for tumor growth. Endothelial cells generate blood vessels to deliver nutrients and oxygen into the tumor. Immune cells such as macrophages, neutrophils, NK cells and CD8 and CD4 lymphocytes infiltrate tumors with either anti- or protumor effects. Inspired from (Quail and Joyce, 2013).

The TME is a heterogeneous mix of endothelial cells, fibroblasts and immune cells present in different proportions and activation states (Quail and Joyce, 2013) (Figure 8). In the recent years, the interaction between tumor and immune cells has become central in tumor biology. The first association between cancer and immune cells was reported by Rudolph Virchow in the 19th century when he observed leukocytes infiltrated in tumors (Balkwill and Mantovani, 2001). Inflammation and immune infiltration in the TME are intimately linked with the genetic background of each tumor, as oncogenic signaling dictates the production of chemokines and cytokines that recruit and activate immune cells (Binnewies et al., 2018). This results in a complex, tumor-specific, location-, time- and cell type-dependent function of immune cells within the TME, but that generally can be regarded as either anti- or pro- tumorigenic (Fearon, 2016; Greten and Grivennikov, 2019). For instance, macrophages, innate immune cells specialized in phagocytosis, present pro-tumor activity by generating an inflammatory milieu that promotes cancer cell survival and proliferation while concurrently suppresses anti-tumor immune cells (DeNardo and Ruffell, 2019). Similarly, neutrophils generally contribute

to cancer cell survival and proliferation though paracrine signaling, both by direct action on tumor cells or by inhibiting anti-tumor responses, although they have also been shown to present anti-tumorigenic activity (Hedrick and Malanchi, 2021).

As a consequence of their transformation process, tumor cells can be selectively recognized and killed by immune cells, mainly natural killer (NK) cells and T lymphocytes. NK cells present receptors that recognize damaged cells, and as cancer cells undergo extensive cellular damage, they can be targeted by NK cells (Huntington et al., 2020). Besides cellular damage, tumor cells progressively accumulate mutations in protein-coding genes that ultimately result in the production of tumor-specific neoantigens. T lymphocytes are able to selectively recognize these neoantigens and subsequently eliminate every tumor cell that expresses them (Waldman et al., 2020). T cells are therefore one of the most potent effectors of anti-tumor immune responses, and in fact their ability to detect and kill tumor cells has led to the recent development of immunotherapy, a novel therapeutic modality that harnesses the power of the immune system against tumors (Waldman et al., 2020).

However, T cells are not always able to control cancer development: tumors modify the TME to generate hostile conditions for T cell function, and cancer cells utilize numerous tricks to avoid immune surveillance. Moreover, T cells present intrinsic mechanisms that dampen their function, which are highjacked by tumor cells to prevent T cell recognition. Therefore, understanding the molecular mechanisms that control T cell activation and (dys)function in the context of the TME is pivotal to avoid tumor immune evasion. In this section, we will review the current knowledge of T cell differentiation, activation and function, with a special focus on mechanisms of translational control that govern the tumor-T cell crosstalk within the TME.

2.1. T cell function, activation and fate

2.1.1. T cell development and activation

The adaptive immune system has evolved to selectively recognize and eliminate external pathogens through the recognition of "non-self" antigens. It is comprised of two major subtypes of lymphocytes: B cells and T cells. B cells respond to circulating antigens by secreting protective antibodies (LeBien and Tedder, 2008). T cells recognize peptides derived from intracellularly processed antigens loaded onto major histocompatibility complex (MHC) molecules present in the extracellular membrane of antigen presenting cells (such as dendritic cells). There are two broad classes of T cells, based on the expression of the co-receptor CD4 or CD8. CD4 T cells, also called T helper cells, recognize antigens in the context of MHC class II and coordinate the adaptive immune system responses by secreting cytokines and chemokines with different pro- or anti- inflammatory properties (Borst et al., 2018). CD8 T cells recognize MHC class I-loaded antigens and present cytotoxic capacity to kill damaged, infected or neoplastic cells (Kumar et al., 2018). MCH-I carries antigens from intracellular origin, whereas MHC-II loads extracellular antigens; however, specialized antigen presenting cells like dendritic cells can load extracellular antigens into MHC-I molecules through a process called cross-presentation (Joffre et al., 2012)

Each T cell clone is characterized by expressing a unique T cell receptor (TCR), a glycoprotein complex that recognizes a specific antigen. The TCR is composed by a copy of the highly polymorphic α and a β chain, responsible of antigen recognition, and a group of signaling chains termed CD3 γ, δ, ε and ζ (Alcover et al., 2018). The acquisition of a functional TCR occurs during T cell fate acquisition in the thymus (Figure 9). Pre-thymic T cell precursors are generated in the bone marrow, where hematopoietic stem cells (HSC) differentiate into still multipotent but lineage-restricted progenitors such as common lymphoid progenitors (CLPs) (Krueger et al., 2017). These cells travel through the blood as thymus-seeding progenitors (TSPs) and colonize the thymus. Here, TSPs

undergo a series of differentiation steps that are identified by the progressive acquisition of T cell markers and loss of multilineage differentiation potential.

The earliest thymic progenitors are termed double negative (DN) due to the lack of CD4 and CD8 expression, and can be further subdivided into DN1-4 by the markers CD25 and CD44 (Koch and Radtke, 2011). DN1 ($CD44^+CD25^-$) receive Notch signals that prevent differentiation into other immune lineages (natural killer, macrophages or dendritic cells). DN2 ($CD44^+CD25^+$) rearrange the TCR β locus to generate a pre-TCR as they enter the DN3 ($CD44^-CD25^+$) stage. DN3 undergo a vital checkpoint known as β-selection in which only those cells that have successfully rearranged a functional pre-TCR will survive. Past DN3, all non-T cell differentiation options are lost. DN4 ($CD44^-CD25^-$) acquire CD4 and CD8 expression as they transit into double positive (DP, $CD4^+CD8^+$) thymocytes, where the α locus is rearranged to generate a

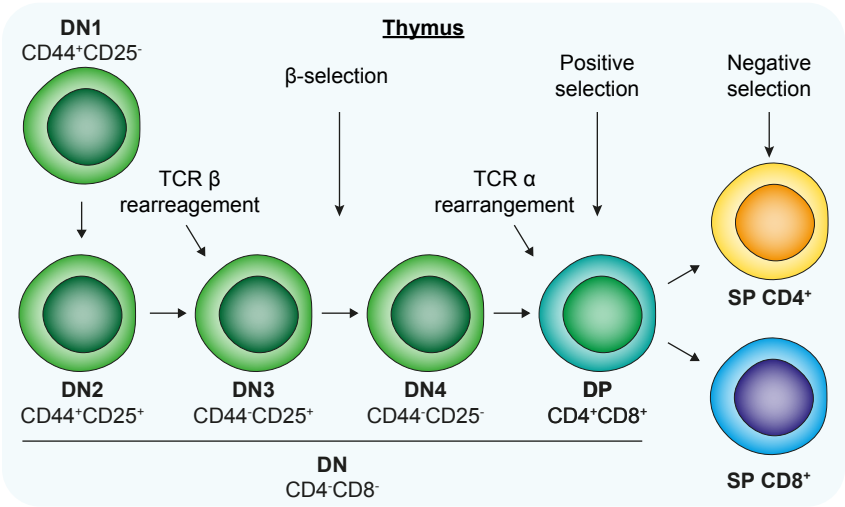

Figure 9. T cell development in the thymus. When thymic precursors enter the thymus, they start a step-wise differentiation process to generate mature, functional CD4 and CD8 T cells. Double negative (DN) cells restrict their differentiation potential and as they enter the DN3 stage they rearrange the TCR beta locus. Cells that express a proper TCRβ will pass though β-selection and enter the double positive (DP) stage, where they rearrange the α locus. DP cells with a fully functional TCR undergo positive selection and, depending on which MCH molecule can recognize, differentiate into single positive (SP) CD4 or CD8. SP cells will be subjected to negative selection, where only hose T cells with a self-tolerant TCR will survive and egress into the bloodstream. The receptors that mark each progenitor are depicted below the name.

fully-functional TCR (Klein et al., 2014). DP cells undergo another checkpoint known as positive selection, where only those DP cells that are able to recognize with intermediate avidity MHC molecules will survive. Depending on whether DP recognize MHC class I or II during positive selection, they will differentiate into CD8 or CD4 single positive (SP), respectively. Finally, negative selection will take place, where only SP clones with TCRs that do not recognize self-antigens will survive and exit into the periphery as naïve cells. This whole selection process is crucial to generate a tolerant immune system that will ignore self-tissues and only react against "non-self" antigens from external pathogens or neoantigens such as those present in cancer cells.

T cell activation begins when a naïve T cell recognizes its cognate antigen on an MHC molecule presented by an antigen presenting cell (Smith-Garvin et al., 2009) (Figure 10). However, if a T cell is stimulated only through its TCR, it will enter an unresponsive state known as anergy (Wells, 2009). For proper T cell activation, a second signal from the CD28 receptor (termed co-stimulation) is required (Hara et al., 1985). Antigen presenting cells express the CD28 ligands CD80 and CD86, and therefore are able to provide T cells with both signals simultaneously (Sharpe and Freeman, 2002). In these conditions, T cells initiate a signaling cascade that activate Akt/PI3K, RAS/ERK, JNK, p38 and calcium release (Gaud et al., 2018). This early signaling events induce anti-apoptotic proteins and IL-2 production, which in turn stimulates survival and clonal expansion. In addition, they rewire T cell metabolism to achieve a high anabolic and glycolytic rate in order to provide the building blocks required for proliferation and differentiation into effector (Teff) cells (Klein Geltink et al., 2018)

1.2.2. T cell subsets and fate

Once activation has started, T cells trigger multiple signaling, metabolic and transcriptional programs that will allow them to differentiate into functional Teff. In the case of CD4 cells, several subsets with distinct functional properties are generated by lineage-specifying cytokines and transcription factors (Saravia

et al., 2019). The first two subsets to be discovered were termed Th1 and Th2 (Mosmann et al., 1986). Th1 cells produce the proinflammatory cytokines IFNγ, TNFα and IL-2, and regulate anti-viral and anti-tumor immunity; Th2 cells are characterized by IL-4, IL-5, and IL-13 secretion and are vital against extracellular pathogens such as helminths. Th1 fate is promoted by IL-12 signaling and T-bet and Eomes transcription factors, whereas Th2 relies on IL-4 and GATA-3 (Hsieh et al., 1993; Ouyang et al., 1998; Szabo et al., 2000).

From this initial two, the collection of CD4 subsets has expanded. Regulatory T cells (Tregs), characterized by the expression of CD25 receptor and FOXP3 transcription factor, are required for suppressing T cell activity (Togashi et al., 2019). Th17 cells produce IL-17A, IL-17F, and IL-22 and are critical for gut microbiota regulation (Harrington et al., 2005; Park et al., 2005). Th17s are generated in the presence of IL-1β, IL-6, IL-21, IL-23, and TGF-β and are marked by the transcription factor RORγt (Bettelli et al., 2006; Ivanov et al., 2006; McGeachy et al., 2009; Veldhoen et al., 2006; Zhou et al., 2007; Zielinski et al., 2012). Another distinguished CD4 subset are T follicular helper (T_{FH}) cells, which promote B cell responses though IL-21 production (Bryant et al., 2007). Metabolism is also key to define T cell subset functionality: proinflammatory subsets such as Th1, Th2 or Th17 rely on glycolysis and oxidative phosphorylation, whereas Tregs suppress glycolysis and engage into fatty acid oxidation for their suppressive function (Klein Geltink et al., 2018). The CD4 subset repertoire is still expanding as new markers and functions allow a more in-depth characterization (Saravia et al., 2019).

As for CD8 T cells, their activation stimulates T-bet and Eomes-dependent differentiation into cytotoxic T lymphocytes (CTLs), that produce inflammatory cytokines such as IFNγ, TNFα and cytolytic proteins such as the protease granzyme B (GZMB) and the pore-forming protein perforin (Cui and Kaech, 2010) (Figure 10). CTLs are able to recognize exogenous or neo-antigens in infected or neoplastic cells and selectively kill them. CTLs also increase their metabolism and rely on high glycolysis and oxidative phosphorylation rates to sustain their effector function (Klein Geltink et al., 2018).

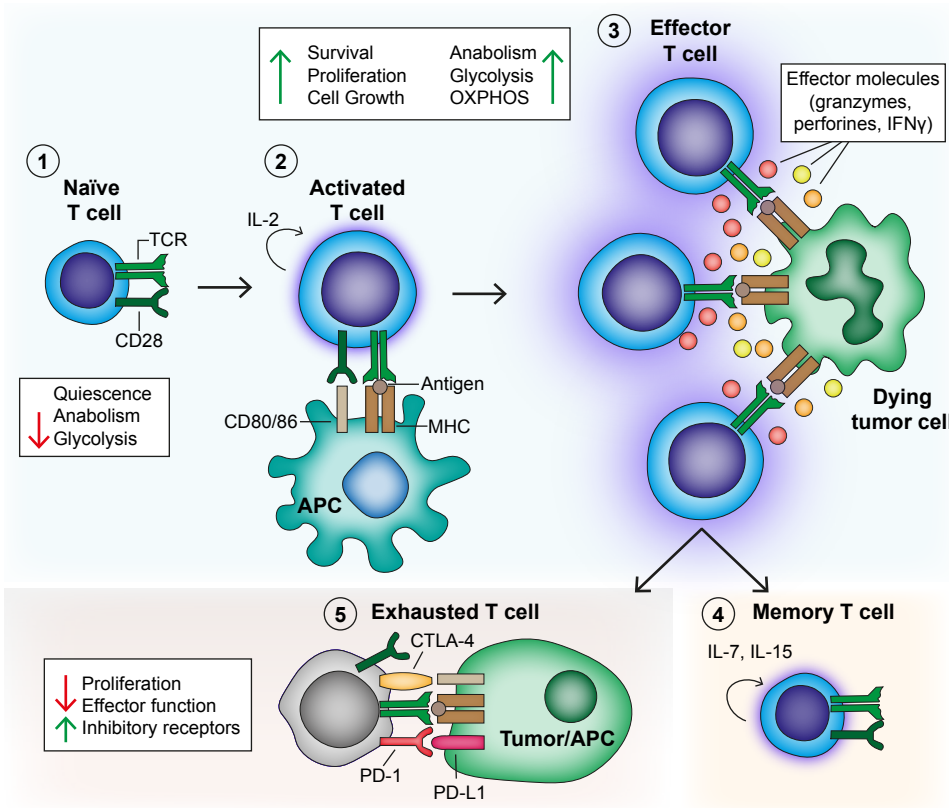

Figure 10. T cell activation and differentiation. Schematic depiction of CD8 lymphocyte activation and differentiation. Naïve CD8 cells are quiescent and present a low anabolic and glycolytic rate (1). Upon antigen recognition and CD28 co-stimulation by an antigen presenting cell (APC), CD8 cells start their activation process, which requires IL-2 dependent induction of proliferation, survival and cell growth (2). Activation induces the upregulation of anabolic pathways and produces a metabolic rewiring that increases glycolysis and oxidative phosphorylation (OXPHOS). Activation initiates the differentiation of naïve cells into effector cells, which are capable of recognizing target cells (such as tumor cells) and eliminate them though the secretion of effector molecules (granzymes, perforins, IFNγ) (3). In cases where the antigen is cleared, CD8 cells differentiate into memory cells, which are primed to respond faster in a second encounter with the antigen (4). Memory cells are long-lived and undergo a slow, stem cell-like IL-7 and IL-15-dependent homeostatic proliferation. If antigen clearance is not accomplished and T cells are exposed to it for a long period of time, they enter a dysfunctional state known as exhaustion (5). Exhausted T cells are characterized by the expression of inhibitory receptors such as PD-1 or CTLA-4 that inhibit TCR and CD28 signaling, impairing T cell proliferation and effector molecule production. Cancer cells induce T cell exhaustion by upregulating inhibitory receptor ligands such as PD-L1.

Following antigen clearance and resolution of inflammation, the expanded T cell clones will die due to cytokine withdrawal (Lenardo et al., 1999). However, a small subset of cells persists and differentiate into memory cells (T_M) (Figure 10). T_M are required for long-term immunological memory, as they are primed for a faster and stronger response to the same antigen on a second encounter (Mueller et al., 2013). T_M downregulate their effector program modulating the expression of counter-regulatory transcription factors such as T-bet, Blimp1 or Bcl6 (Kaech and Cui, 2012) and acquire stem-cell like survival properties, undergoing slow, IL-7 and IL-15-driven homeostatic self-renewal (Surh and Sprent, 2008). Metabolically, T_M decrease glycolysis to favor fatty acid oxidation and mitochondrial respiration (Pearce et al., 2009); in fact, they increase their mitochondrial spare respiratory capacity, which might be required for a rapid "recall" response in the event of a second antigen stimulation (van der Windt et al., 2012).

In pathological contexts where antigen stimulation persists for a long period of time, like chronic infections or cancer, T cells can become exhausted, a dysfunctional state characterized by a progressive loss of effector function sustained by the expression of multiple inhibitory receptors and transcription factors (McLane et al., 2019) (Figure 10). These inhibitory receptors, also known as checkpoint molecules, are a collection of negative regulators of T cell activation that have evolved to fine-tune T cell responses and avoid hyperactivation, being PD-1 and CTLA-4 the most prominent. Inhibitory receptors are normally upregulated during T cell activation and are vital to promote immune tolerance and prevent autoimmunity (Fife and Bluestone, 2008). However, their function is exacerbated in cancer and dampens anti-tumor responses. CTLA-4 prevents early T cell activation by sequestering CD28 ligands and preventing co-stimulation, whereas PD-1 recognizes its own ligands (mainly PD-L1) on target cells and inhibits TCR signaling (Buchbinder and Desai, 2016). Ultimately, the action of inhibitory receptors decreases T cell proliferation and cytokine production (Patsoukis et al., 2012; Zajac et al., 1998), suppresses glycolysis (Patsoukis et al., 2015), dysregulates mitochondrial dynamics (Bengsch et al., 2016; Scharping et al., 2016) and establishes a

transcriptional program that renders T cells unable to exert their function (Doering et al., 2012). In the last years, the number of checkpoint receptors has greatly increased, indicating the diversification of mechanisms to restrain T cell function (Lee et al., 2021).

2.2. Translational control of T cell activation and differentiation

As we have seen, T cells undergo massive changes during activation to proliferate, grow in size, and become fully functional. Gene expression must be tightly controlled to ensure that T cells transit smoothly through the different activation states and acquire the functional properties that each specific context demands. Although transcriptional control is pivotal to this process, recent reports have found little correlation between mRNA levels and protein levels in effector T cells, suggesting that translational control is also key to generate functional effector T cells (Howden et al., 2019). Translational control provides T cells with a fast and plastic mechanism of gene expression regulation that allows them to quickly adapt to different functional environments. Understanding how mRNAs are differentially translated and how this affects T cell function is nowadays a thriving and exciting area of exploration.

2.2.1. General translation during T cell activation and differentiation

During T cell activation, adaptation of global translation to the specific growth and proliferative rate of each differentiation step is pivotal (Araki et al., 2017). General translation is massively induced shortly after antigen recognition, and, in the case of CD8, activated cells present 3-4-fold higher total amount of protein than naïve lymphocytes, concomitant with their increase in size (Howden et al., 2019). Molecularly, two of the main signaling nodes responsible for these translational changes are MYC and mTORC1 (Huang et al., 2020; Man and Kallies, 2015).

MYC has pleiotropic effects in T cells, but one of the major characterized functions is to increase glucose and glutamine metabolism (Wang et al., 2011). Translation and metabolic reprogramming are intimately linked, since anabolic metabolism provides building blocks required for translation. MYC also upregulates amino acid transporters not only to support glutaminolysis but also to provide materials for protein synthesis (Marchingo et al., 2020). Similarly, MYC is known to upregulate ribosome biogenesis, therefore increasing the supply of translation machinery to maintain high translation rates (van Riggelen et al., 2010). mTORC1 signaling has a profound effect on T cell translation as well, controlling the translation of around 20% of total cell mass in cytotoxic T lymphocytes (Howden et al., 2019; Hukelmann et al., 2016). mTORC1 regulation of translation is crucial to dictate the fate of both CD4 and CD8 T cells, as differentiation into each effector subtype and memory cells has specific mTORC1 requirements (Araki et al., 2009; Delgoffe et al., 2009). For instance, high translation and mTORC1 levels are required for cytotoxic CD8 function and Treg expansion in tumors (de Ponte Conti et al., 2021). Downstream mTORC1, each signaling branch has distinct effects on T cell differentiation. S6K activity is dispensable for T cell proliferation and growth, but is required for differentiation into Th17 cells (Kurebayashi et al., 2012; So et al., 2016). On the other hand, the 4E-BP-eIF4E axis is indispensable for T cell activation (So et al., 2016), and has different effects on non-Treg and Treg $CD4^+$ T cells translatomes (Bjur et al., 2013).

Although upregulation of translation is a direct consequence of the activation-induced signaling and metabolic rewiring, recent reports indicate that translation can also act upstream of metabolic pathways. As previously discussed, T cells first re-direct their metabolism to achieve a high anabolic and glycolytic rate to differentiate into functional effector cells (Klein Geltink et al., 2018). In this context, translation of pre-formed glucose transporter *Glut1* and Acetyl-CoA carboxylase *Acc1* mRNAs after TCR activation is required to activate glycolytic and fatty acid synthesis metabolism respectively (Ricciardi et al., 2018). Therefore, blocking translation prevents metabolic reprogramming and T cell activation, even in the presence of proper TCR stimulation. In

addition, ribosome profiling data from $CD4^+$ Th1 cells has identified numerous metabolic enzymes under translational control, although the specific mRNA features that determine their translation efficiency remain unknown (Manfrini et al., 2020). These data indicate that translation is not just a passive process though which T cells build proteins, but is a highly regulated process that influences T cell metabolism, proliferation and fate.

The increase in translation, proliferation, cell size and secretory capacity concomitant to TCR signaling generates ER stress and activates the UPR (Cao et al., 2019; Pino et al., 2008; Takano et al., 2008). Although it might seem paradoxical, activation-induced UPR signaling is required for T cell differentiation and function (Kamimura and Bevan, 2008; Thaxton et al., 2017). However, given the dual function of the UPR, activation of the ER stress response can also be detrimental for T cell function. For instance, phosphorylation of eIF2α and stress granule formation precludes IL-4 secretion in re-stimulated Th2 cells, albeit it is required for proper differentiation (Scheu et al., 2006). Similarly, activation of PERK signaling upregulates CHOP, which in turn diminishes CD8 differentiation into effector cells (Cao et al., 2019).

2.2.2. RBPs in T cell activation and differentiation

RBPs have also been shown to play a role in T cell activation and differentiation (Turner and DÍaz-Muñoz, 2018) (Figure 11). ARE-mediated regulation by HuR and TTP-like proteins ZFP36L1/2 is critical for thymic development (Hodson et al., 2010; Papadaki et al., 2009; Vogel et al., 2016). In mature T cells, TTP restrains T cell activation and antiviral T cell function, whereas HuR stabilizes transcription factor mRNAs for adequate T cell differentiation (Moore et al., 2018; Ramgolam et al., 2010; Stellato et al., 2011). Of note, cytokine production is tightly post-transcriptionally regulated by ARE-binding proteins. For instance, in activated T cells, AREs decrease *Ifng* and *Tnfa* mRNA stability and translation efficiency (Moore et al., 2018), but in memory T cells, ZFP36L2 stores ready-to-use, pre-formed *Ifng* mRNA by blocking its translation until re-stimulation (Salerno et al., 2018). Deletion of *Ifng* AREs

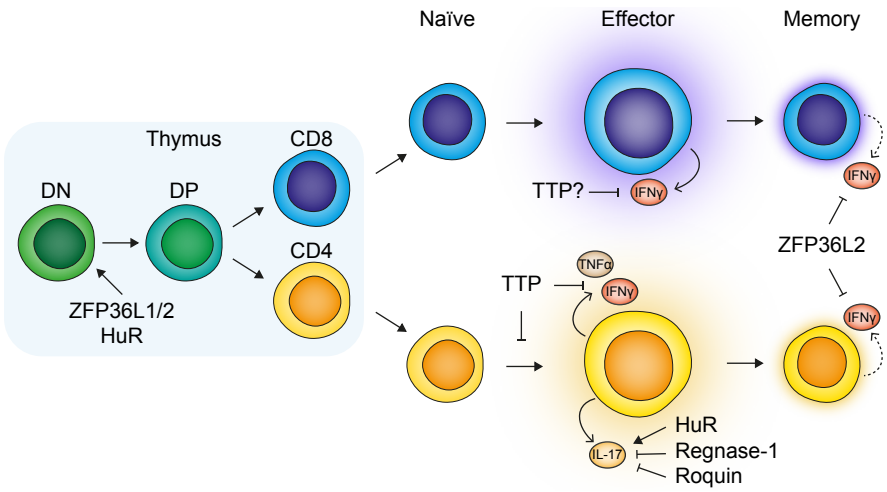

Figure 11. RBPs regulate T cell development and differentiation. Several RBPs have been shown to play a role in thymic T cell development and subsequent activation and differentiation. ARE-binding proteins HuR and ZFP36L1/2 regulate proliferation and TCR recombination in double negative (DN) thymic precursors. In mature cells, TTP block CD4 activation and production of Th1 cytokines TNFα and IFNγ. RBPs also control CD4 differentiation into Th17 cells: HuR stabilizes *Il17* mRNA, but regnase-1 and roquin block Th17 fate. In CD8, destabilizing AREs present in *Ifng* mRNA 3'UTR decreases anti-tumor immunity, although the specific ARE-binding protein that regulate IFNγ in this context is still unknown. In memory cells, TTP-like ZFP36L2 blocks *Ifng* mRNA translation, but this inihibition is lost upon re-activation, enabling memory cells to rapidly respond upon a new challenge.

increases IFNγ production and enhances T cell anti-tumor activity (Salerno et al., 2019). Interestingly, if glycolysis is blocked during activation, GAPDH directly binds *Ifng* AREs and inhibits its translation, providing a direct link between metabolism and T cell functionality (Chang et al., 2013). In models of autoimmune encephalomyelitis, HuR binds *Il17* mRNA and regulates CD4 Th17 response during inflammation (Chen et al., 2013).

Other RBPs have also been involved in T cell activation and differentiation. Conditional deletion of Roquin1 and 2, which promote the decay of target mRNAs via the recruitment of CCR4-NOT deadenylation complex, induces lupus-like autoimmune disease in mice (Vogel et al., 2013). In CD4 T cells, Regnase-1 downregulates the expression of cytokines and co-stimulatory molecules through cleavage of their 3'-UTRs, thereby inhibiting effector

T-cell activation (Uehata et al., 2013). In addition, Roquin and Regnase-1 act coordinately to negatively regulate Th17 fate and inflammation (Garg et al., 2015; Jeltsch et al., 2014). As our knowledge of RBPs function in T cell activation expands, recent efforts have been devoted to reveal the full identity of T cell RBPome (Perez-Perri et al., 2018). Although these datasets are valuable, more work is required to characterize the mRNAs bound by these RBPs, the sequences that determine their binding and their ultimate effect on protein production. Together, these data will allow us to establish post-transcriptional gene regulation networks that could explain the nuanced and intricated fates of mRNAs during T cell activation.

2.3. Translational control of T cell-mediated anti-tumor immunity

Translational control has been shown to be key for virtually all hallmarks of cancer, from promoting cell proliferation and growth autonomously to influencing metastatic capacity (extensively reviewed in Fabbri et al., 2021; Xu and Ruggero, 2020). In the last years, elegant reports have revealed that translational control modulates how tumor cells are eliminated by the immune system. The recognition and elimination of cancer cells requires a coordinated action of cell types that cooperate to mount a strong and durable immune response in a timely and localized manner. T cells recognize cancer cells in the context of the TME, which imposes a detrimental signaling and metabolic milieu that interferes with T cell function and enhances immune evasion. In addition, tumor cells can promote T cell exhaustion by upregulating immune checkpoint receptor ligands, "don't eat me" proteins that block T cell action. In this section, we will review the translational mechanisms that govern immune checkpoint ligand production in cancer cells, how altered translation can generate neoantigens in tumor cells and how T cell translation is adapted to the specific metabolic constrains of the TME.

2.3.1. Translational control of immune checkpoint proteins in tumor cells

Oncogenic pathways regulate immune checkpoint proteins at multiple levels, including their translation and mRNA stability (Spranger and Gajewski, 2018). The most studied checkpoint protein has been PD-1 ligand PD-L1, a transmembrane protein expressed by tumor and myeloid cells (Cha et al., 2019). The first evidence of translational control of PD-L1 expression was found in a model of glioma, where it was shown that activation of the PI3K pathway by PTEN loss resulted in increased PD-L1 (*Cd247*) mRNA recruitment to polysomes and translation (Parsa et al., 2007). Although at that time the features of PD-L1 mRNA that could mediate its differential polysome recruitment were not identified, recent work has revealed that PD-L1 mRNA contains in its 5'UTR inhibitory uORFs that prevent its translation in non-oncogenic conditions (Suresh et al., 2020; Xu et al., 2019). However, in tumor cells, the induction of the integrated stress response results in PD-L1 enhanced translation, suggesting that tumor evasion is a consequence of oncogenic stress. In liver cancer, MYC and RAS signaling pathways cooperate to allow the bypass of those inhibitory uORFs in an eIF4E dependent manner (Xu et al., 2019), whereas in lung cancer eIF5B stimulates PD-L1 translation in heme-deficient conditions (Suresh et al., 2020). PD-L1 expression is also upregulated by STAT1 in response to inflammatory signals such as IFNγ. Interestingly, STAT1 translation is regulated by eIF4F due to its highly structured 5'UTR. Inhibition of eIF4F resulted in decreased STAT1 translation and lack of PD-L1 induction in response to IFNγ (Cerezo et al., 2018). These advances have shown that preventing translation of PD-L1 or PD-L1-inducing proteins by inhibiting eiF4E or eIF4F holds great therapeutic potential as immunotherapy (Figure 12).

Immune checkpoint protein expression has also been shown to be regulated by mRNA stability. PD-L1 mRNA presents a complex 3'UTR containing several AREs (Coelho et al., 2017). In normal conditions, TTP and KSRP bind PD-L1 AREs and destabilize the mRNA; however, in response to oncogenic

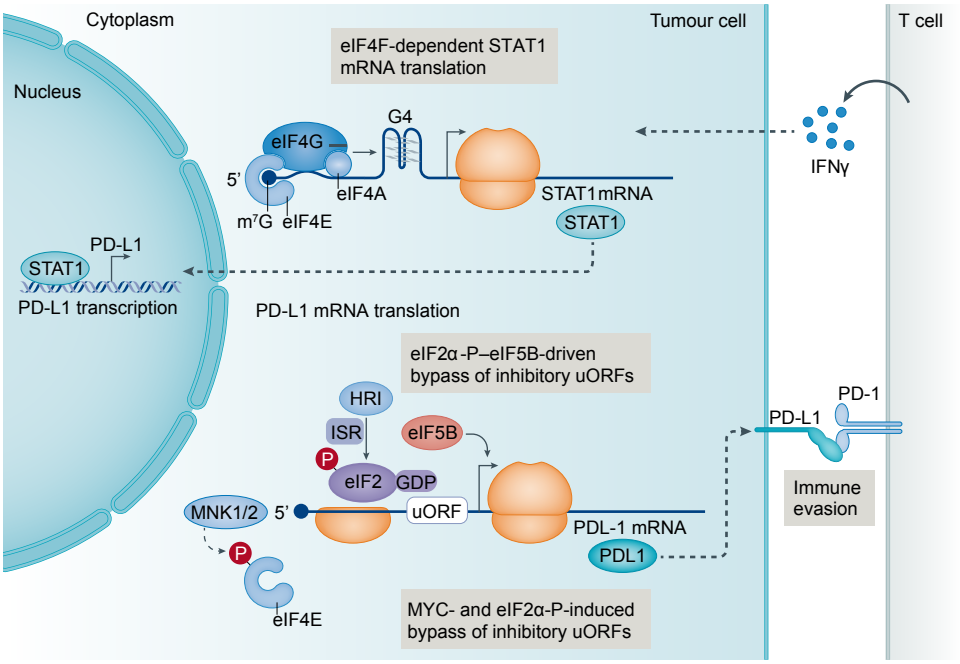

Figure 12. Translational control of PD-L1 expression. Top: IFNγ-induced PD-L1 expression is controlled by the eukaryotic initiation factor 4F (eIF4F) complex-dependent translation of signal transducer and activator of transcription 1 (STAT1) mRNA. STAT1 in turn stimulates PD-L1 transcription. The 5' UTR of *Stat1* contains G-quadruplex (G4) secondary RNA structure that requires eIF4A helicase activity to allow translation. Bottom: the activation of the integrated stress response (ISR) bypasses the inhibitory uORF present in PD-L1 mRNA 5' UTR. This bypass is accomplished in response to HRI activation due to heme deficiency in a phospho-eIF2α and eIF5B-dependent manner. Alternatively, oncogenic signals from Myc also leads to a phospho-eIF2α-dependent uORF bypass. Adapted from (Fabbri et al., 2021).

RAS-p38 signaling, TTP is downregulated and PD-L1 mRNA is stabilized. In addition, genetic alterations resulting in PD-L1 3'UTR shortening have been found in multiple human cancers (Kataoka et al., 2016). In this context, PD-L1 mRNA does not present destabilizing AREs (nor microRNA binding sites) and its expression is increased, promoting in immune evasion of tumor cells. Surprisingly, recent reports have established a new role for intracellular PD-L1 as an RBP regulating RNA stability, extending the list of proteins with moonlighting RBP activity (Tu et al., 2019).

2.3.2. Generation of translation-dependent neoantigens

The recognition of tumor cells by T lymphocytes is possible due to the presence of neoantigens for which central tolerance has not been established (Garcia-Garijo et al., 2019). Neoantigens usually arise from nonsynonymous mutations that create amino acid sequences specific to tumor cells. However, translation from "cryptic" regions of mRNAs that under homeostatic circumstances are non-translated can also produce peptides potentially recognized by the immune system (Starck et al., 2016). For instance, translation of uORFs generate peptides that can be loaded onto MCH class I molecules (Wang et al., 1996). Similarly, depletion of the ribosomal protein RPL28 in human melanoma cells promotes translation of uORF peptides that are loaded into HLA molecules and increase CD8 cytotoxic activity (Wei et al., 2019). In addition, tryptophan depletion in indoleamine 2,3-dyoxygenase (IDO1)-expressing melanoma cells causes frameshift events that generate aberrant peptides that are recognized by T cells (Bartok et al., 2020). In the next years, understanding globally how cellular stress rewires translation in cancer will allow us to identify the full repertoire of cancer-specific alternative ORF-encoded peptides. Expanding the list of known tumor neoantigens could open new therapeutic strategies to selectively target T cells towards cancer cells.

2.3.3. Stress in the TME causes T cell dysfunction

As tumors evolve, cancer cells shape a unique metabolic landscape in the TME. The high metabolic activity of tumor cells and lack of adequate blood flow renders the tumor hypoxic, acidic, nutrient deprived and accumulated with metabolic byproducts (Elia and Haigis, 2021). These metabolic constrains clash with the special requirements that T cells need to become functional, which is exploited by tumor cells *in vivo* to prevent immune recognition (Sugiura and Rathmell, 2018). For instance, the limited availability of glucose in the TME, coupled to the high demand from tumor cells, results in a metabolic competition between cell types that leads to T cell dysfunction (Chang et al., 2015; Ho et al., 2015). On top of withdrawing glucose from T cells, the high

glycolytic activity of tumor cells results in the accumulation of lactate, which inhibits T cell infiltration, proliferation and cytokine production (Brand et al., 2016; Fischer et al., 2007). A similar mechanism occurs with amino acids such as glutamine or tryptophan, essential for T cell function but scarce in the TME. In the case of tryptophan, tumor cell consumption of this amino acid not only depletes it from the microenvironment but also results in its conversion into kynurenine, a byproduct of tryptophan catabolism that promotes the generation of immunosuppressive Treg cells (Mezrich et al., 2010; Munn and Mellor, 2013). Lipid accumulation has also been shown to be detrimental for T cell function. Tumor infiltrating T lymphocytes (TILs) uptake oxidized LDL and long chain fatty acids that promote lipid peroxidation, causing T cell exhaustion or ferroptosis (Ma et al., 2021; Xu et al., 2021). In addition, T cell effector program can be inhibited by high extracellular concentration of potassium, which is released into the TME by necrotic cells (Eil et al., 2016; Vodnala et al., 2019)

In the recent years, it has been observed that the metabolic limitations suffered by T cells in the TME converge in a maladaptive UPR that reduces fitness and effector function (Cao et al., 2019; Ma et al., 2019; Song et al., 2018). Secreted factors from ovarian cancer cells suppress glucose import into T cells, which activates the IRE1α-XBP1 axis of UPR in T cells (Song et al., 2018). ER-stressed CD4 T cells have decreased mitochondrial respiration, IFNγ production and effector function. In melanoma, cholesterol accumulation promotes ER stress in CD8 TILs and drives their exhaustion in an XBP1s-dependent manner (Ma et al., 2019). Similarly, CHOP has been found to negatively regulate the effector phenotype in CD8 cells by repressing T-bet expression and reducing proliferation and metabolic fitness (Cao et al., 2019). These findings illustrate that activation of the terminal UPR causes maladaptive signaling that impairs T cell function. However, as explained above, the fact that UPR is also physiologically induced during T cell activation and is required for T cell differentiation suggests that UPR signaling has different consequences on T cells depending on the duration, intensity of the signaling and the context where activation occurs. Dissecting the specific mechanisms that promote adaptation

to stress and isolating them from those that lead to cellular dysfunction will allow us to improve T cell function in stressed conditions.

3. CPEBs in cancer: from cell-autonomous functions to TME modulation

As we have discussed, translational control is key for virtually every aspect of tumor biology. As key regulators of cell cycle and differentiation, CPEBs have been shown to be pivotal for tumor development. Among the four members of the family, CPEB1 and CPEB4 have been the most studied in the context of cancer, although recent reports have characterized a role for CPEB2 as a mediator of estrogen receptor-dependent tumor growth in breast cancer (Pascual et al., 2020b). CPEB1 is downregulated in several types of human tumors such as ovarian, gastric, breast, myeloma and colorectal cancer (Caldeira et al., 2012; Hansen et al., 2009; Heller et al., 2008), suggesting its role as a tumor suppressor. In line with these results, skin papilloma formation in carcinogen-treated animals is accelerated in CPEB1 KO mice (Burns and Richter, 2008) and human and mouse fibroblast that lack CPEB1 are able to bypass oncogene-induced senescence (Burns and Richter, 2008; Groisman et al., 2006; Groppo and Richter, 2011). CPEB4, in contrast, has been shown to have an opposite role in tumorigenesis, being a crucial tumor promoter in pancreatic ductal adenocarcinoma, glioma and melanoma (Boustani et al., 2016; Ortiz-Zapater et al., 2011; Pérez-Guijarro et al., 2016). In melanoma, CPEB4 activates the translation of lineage-specific melanoma drivers MITF and RAB27A required to sustain a high proliferative rate (Pérez-Guijarro et al., 2016). In pancreatic ductal adenocarcinoma, knock-down of CPEB4 does not affect cell division *in vitro* but decreases tumor growth and invasion *in vivo*, which correlates with lower tumor vascularization and proliferation (Ortiz-Zapater et al., 2011). Analysis of CPEB4 mRNA targets revealed that CPEB4 was required for production of tissue plasminogen activator, a key factor for tumor angiogenesis and invasion. These results suggested that in pancreatic cancer cells CPEB4 was controlling the architecture of the TME contributing

to the formation of a vascularized, tumor promoter microenvironment. Collectively, these findings indicate that CPEBs regulate tumor cell intrinsic processes such as proliferation or linage specification but also (at least for CPEB4) mediate the communication with its surroundings.

However, as we have seen, the communication between tumor cells and its environment is bidirectional, and other cell types present in the TME such as immune cells influence tumor growth. Although in the context of cancer most of the work performed to date with CPEBs have been focused on tumor cells, data from non-tumor settings have shown that CPEBs are required for inflammation and immune responses. CPEB1, for instance, regulates IL-6 production and protects mice from endotoxic shock (Ivshina et al., 2015). Similarly, CPEB4 is required in macrophages to regulate inflammation resolution and its absence exacerbates response to lipopolysaccharide both *in vitro* and *in vivo* (Suñer et al., 2021). In addition, CPEB4 positively regulates the inflammatory profile of adipocytes, activating the recruitment of macrophages to visceral fat and promoting obesity (Pell et al., 2021). Finally, in CD4 lymphocytes and innate lymphoid cells, CPEB4 contributes to intestinal homeostasis and inflammatory disease by translationally activating the protective cytokine IL-22 (Sibilio et al., *accepted*). This shows that CPEBs regulate intercellular communication and contribute to orchestrate complex physiological and pathological responses where multiple cell types must interact coordinately. However, how CPEBs influence the recruitment, activation, differentiation or functionality of immune cells within the TME is still unexplored. For this reason, the study of CPEBs, and in particular CPEB4, in the TME might shed light on how translational control in non-transformed cells is required for tumor progression.

Objectives

The main goal of this work is to characterize the role of CPEB-mediated post-transcriptional control of gene expression in non-transformed cells of the TME. In particular, we focus our study in the contribution of CPEB4 to the pro- or anti-tumor function of immune cells.

Overall, our specific goals are:

1. Exploration of the potential impact of modulating CPEB4 in the TME on cancer development.

2. Generation and phenotypic characterization of mouse models of CPEB4 loss-of-function in cell types of the TME.

3. Analysis of CPEB4 expression and regulation in specific cell types and subsets of the TME.

4. Identification of mRNA targets and pathways regulated by CPEB4, with an emphasis in the molecular mechanisms required for cellular function in the TME.

Materials & Methods

Mice

Ubiquitous, constitutive CPEB4 KO mouse (Cpeb4$^{-/-}$) and Cpeb4$^{lox/lox}$ mouse were previously described (Calderone et al., 2016; Maillo et al., 2017). T cell-specific CPEB4 knockout mice (CPEB4-TKO) were obtained by crossing Cpeb4$^{lox/lox}$ mice with CD4-Cre (Lee et al., 2001) transgenic animals from Jackson and were maintained in a pure C57BL6/J background. Cpeb4$^{-/-}$ mice were backcrossed for seven generations onto the C57BL/6J background. OT-I mice were obtained from Jackson. To generate Cpeb4-TKO/OT-I mice, OT-I mice were crossed with Cpeb4-TKO mice. Mice were maintained in a specific-pathogen-free (SPF) facility with a 12-h light–dark cycle and given *ad libitum* access to standard diet and water. Animals were used at 7-12 weeks of age with sex- and age-matched controls.

Cell lines

B16F10 murine melanoma cell line was obtained from ATCC. B16F10 cells expressing OVA-GFP were provided by D. Sancho and had been described previously (Sancho et al., 2008). B16F10 TGL cells were generated by infecting B16F10 cells with retrovirus containing the TGL (Thymidine kinase-GFP-Luciferase, provide by R. Gomis) plasmid. Briefly, HEK–293 T cells were transfected with TGL vector and plasmids encoding retroviral particles using standard methods. B16F10 were subjected to two consecutive rounds of infection and expanded for 7 days without selection. At day 7, GFP+ cells were isolated by fluorescence-activated cell sorter (FACS) and expanded. Cells were cultured in DMEM d-glucose medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2mM L-glutamine (Gibco). Mouse colorectal tumor organoids (MTOs) carrying patient-specific oncogenic mutations and expressing luciferase have been previously described (Tauriello et al., 2018).

In vivo tumor studies

For subcutaneous tumor growth, $1x10^5$ B16F10 or $2.5x10^5$ B16F10 OVA-GFP cells were injected in the mouse flank. Tumors were allowed to grow for 14 days except in experiments were survival curves were calculated. Survival time was defined as the time required for a tumor to reach a volume of 500 mm^3. Tumor dimensions were measured 2-3 times a week with a digital caliper and tumor volume was calculated by applying the following formula: $Volume = length \times width^2 / 2$. For melanoma experimental metastasis assays, $4x10^5$ B16F10 TGL cells were injected in 100 µl of PBS into lateral tail veins and were allowed to grow for 14 days. For liver colonization experiments, intrasplenic injections of MTOs were performed as previously described (Tauriello et al., 2018). Briefly, MTOs cultured in standard conditions were dissociated with trypsin into a single cell suspension and injected at a ratio of $5x10^5$ cells per animal. Growth kinetics of luciferase-expressing melanoma or colorectal cancer organoids were monitored with *in vivo* bioluminescence using an IVIS-Spectrum (Perkin-Elmer). Mice were anesthetized before receiving a retroorbital injection of 50 µl D-luciferin at 15 mg/ml (Resem BV). Total photon flux measurements were normalized per mouse to day 0 post-injection values. The number of metastatic foci at the endpoint of the experiment was manually counted. For melanoma lung metastasis, the area of each nodule was measure on H&E sections with QPath 0.2.3.

Tumor, spleen, lymph node, thymus and blood processing

Tumors were minced in RPMI containing 0.5 mg/ml collagenase I (Sigma Aldrich) and 20 µg/ml DNase I (Roche) and incubated in a gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec) using the program 37C_m_TDK_1. Samples were then filtered through a 70 µm cell strainer and washed with 2% FBS HBSS (FACS buffer) to obtain a single cell suspension. Spleen, thymus and lymph nodes were mechanically processed into a single cell suspension. Blood was collected and supplemented with 50mM EDTA to prevent coagulation. Erythrocytes were lysed using isotonic ammonium chloride solution before

performing antibody staining.

Flow cytometry analysis

For cell surface staining, cells were incubated for 20 minutes on ice with the following antibodies: anti-CD45 BV605 (clone 30-F11, 1:400, Biolegend), anti-CD3ε PerCPCy5.5 (clone 145-2C11, 1:100, Biolegend), anti-CD4 APC-eFluor780/FITC (clone GK1.5, 1:200, eBiosciences), anti-CD8 BV786/FITC (clone 53-6.7, 1:400, BD Biosciences), anti-CD44 PE/APC (clone IM7, 1:200, Biolegend), anti-CD11b FITC (clone M1/70, 1:200 BD Biosciences), anti-CD69 FITC (clone H1.2F3, 1:200, Biolegend), anti-CD25 PE-Cy7/APC (clone PC61.5, 1:300, eBiosciences), anti-CD62L PE-Cy7 (clone MEL-14, 1:200, eBiosciences), anti-Tim3 PE (clone RMT3-23, 1:100, Biolegend), anti-Lag-3 APC (clone C9B7W, 1:100, Biolegend), anti-PD-1 PE (clone 29F.1A12, 1:200, Biolegend).

For CPEB4 intracellular staining, T cells were enriched with density gradient centrifugation (800g, 30 min) at 25 °C with 40% and 80% percoll (GE Healthcare). Cells were labeled with surface antibodies followed by fixation/permeabilization with Cytofix/Cytoperm kit (BD Biosciences). Cells were then blocked with permeabilization buffer containing 10% Donkey serum (Merk) and 2% BSA for 30 minutes on ice. Cells were then incubated with anti-CPEB4 (Mouse Monoclonal ERE149C, 1:100, homemade) diluted in permeabilization buffer with 2% donkey serum 2% BSA for 30 minutes at room temperature. Cells were subsequently incubated with donkey anti-mouse AF647 (1:1000, Invitrogen) for 30 minutes on ice. The specificity of the antibody was confirmed by performing the staining with Cpeb4$^{-/-}$ cells.

For cytokine intracellular staining, cells were re-stimulated *in vitro* with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma), 1 μg/ml ionomycin (Sigma) and Golgiplug inhibitor (1:1000, BD Biosciences) for 3 hours. Surface antigens were labeled before fixation/permeabilization with Cytofix/Cytoperm kit (BD Biosciences), followed by incubation with anti-IFNγ APC (clone XMG1.2, 1:200, Biolegend) and anti-TNFα PE (clone MP6-XT22,

1:200, Biolegend). GZMB staining was performed in the same conditions but without re-stimulation using an anti-GZMB PE antibody (clone NGZB, 1:200, Ebiosciences). For transcription factor staining, fixation/permeabilization was performed with FOXP3 / Transcription Factor Staining Buffer Set (eBiosciences) after surface staining. Anti-FOXP3 PE (clone FJK-16s, 1:100, eBiosciences), anti-Tbet APC (clone 4B10, 1:100, Biolegend) and anti-Eomes PE (clone Dan11mag, eBiosciences, 1:100) were used. For apoptotic cell analysis, cells were staining with AnnexinV-Cy5 (BD. Biosciences) according to the manufacturer instructions.

FACS analyses were performed using a FACS Aria Fusion (BD Biosciences) with BD FACSDiva software (v.8.0.1). Data were analyzed using FlowJo. DAPI or LIVE/DEAD™ Fixable Violet/Yellow Dead Cell Stain Kit (ThermoFisher) was used to exclude dead cells in non-fixed/fixed cells respectively. Complete gating strategies are found in appendix 1.

Immunohistochemistry and immunofluorescence

Tumors and lungs were fixed in 10% neutral buffered formalin solution and embedded in paraffin. 3 μm sections were air dried and further dried overnight at 60 ºC. Antigen retrieval was performed with Tris-EDTA buffer (pH 9) using a PT Link (Dako). Endogenous peroxidase was quenched by 10-min incubation with peroxidase blocking solution (Dako REAL, S2023). For CD3 IHC, anti-CD3 antibody (1:100, IS50330, Dako) was incubated for 2 hours at room temperature. A biotin-free, ready-to-use BrightVision poly–horseradish peroxidase (HRP)–anti-rabbit immunoglobulin G (Immunologic, DPVR-110HRP) was used as secondary antibody. Sections were counterstained with hematoxylin (Dako, S202084) and mounted with toluene-free mounting medium (Dako, CS705). For immunofluorescence, anti-CD8 (1:1000, ab217344, Abacam), anti-CD31 (1:300, ab28364, Abcam) or anti-CD140a (1:100, AF1062, R&D) were incubated over-night at 4° and Alexa secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) were used. Images were acquired with a NanoZoomer-2.0 HT C9600 scanner (Hamamatsu). Image

analysis was performed using QPath 0.2.3.

Mouse T cell culture and *ex vivo* activation

Spleen and lymph nodes from WT, Cpeb4$^{-/-}$ and CPEB4-TKO were mechanically processed into single cell suspension. CD8 or CD4 T cells were purified using Dynabeads™ FlowComp™ Mouse CD8 Kit (ThemoFisher) or Dynabeads™ FlowComp™ Mouse CD4 Kit (ThemoFisher) respectively. Isolated cell purity was confirmed by flow cytometry (>95%). T cells were cultured in RPMI medium (Gibco) supplemented with 10% Fetal Bovine Serum, 1% penicillin/streptomycin, 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco) and 55 μM β-mercaptoethanol at 37ºC, 5% CO2 and 5% O_2. Purified T cells were activated with 1 μg/ml anti-CD3 antibody (clone 145-2C11, MA5-17655, ThermoFisher), 1 μg/ml anti-CD28 antibody (clone CD28.6, 16-0288-85, eBiosciences) and 20 ng/ml IL-2 (212-12, Peprotech) for the indicated periods. OT-I WT and CPEB4-TKO splenocytes were processed into single cell suspension and plated into T cell media supplemented as described above. For OT-I activation, 1 μg/ml $OVA_{257-264}$ peptide (vac-sin, InvivoGen) and 20 ng/ml IL-2 were added to the media. Activated OT-I CD8 cells were purified at the indicated timepoints for downstream analysis by Dynabeads™ FlowComp™ Mouse CD8 Kit.

Immunoblotting

Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with phosphatase and protease inhibitors and protein concentration was determined by DC Protein assay (Bio-Rad). Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Sigma) for 1h at 400 mA. Membranes were blocked in 5% milk and incubated with antibodies against CPEB4 (Mouse Monoclonal ERE149C, 1:500, homemade), β-Actin-HRP (1:15000, ab49900, Abcam), DDIT4 (1:1000, 10638-1-AP, Proteintech), HERPUD2 (1:500, sc-398583, Santa Cruz) and Phospho-eiF2α (s51) (1:1000,

9721S, Cell Signaling).

Lambda protein phosphatase assay (l-PPase).

Primary CD8 were isolated and activated as explained above. 48h post-activation, cells were lysed with l-PPase reaction buffer (New England BioLabs, Ipswich, MA) supplemented with 0.4% NP-40 and EDTA-free protease inhibitors (Sigma-Aldrich). Phosphatase reaction was performed according to the manufacturer's instructions.

Proliferation analysis by CFSE staining

CFSE (Invitrogen) staining was performed following the manufacturer's instructions. Briefly, $1x10^6$ purified $CD8^+$ T cells were resuspended in 1 of PBS containing 1 μM CFSE (Thermo Fisher). Cells were incubated for 20 min at 37 °C, and staining was stopped by adding five volumes of cell culture medium. Cells were washed and activated as described above. After 3 days, cells were analyzed by flow cytometry.

Ex vivo cytotoxicity assay

Wild type and TKO OT-I CD8 T cells were activated as described above, and 48 hours after activation T cells were purified by percoll density gradient. OT-I cells were subsequently co-culture with a mix of 50% B16F10 OVA-GFP target cells labelled with 5 μM CFSE and 50% B16F10, non-target cells labelled with 1 μM CellTrace Far Red (ThermoFisher) at different effector:target ratios (0.5:1, 1:1, 2:1, 4:1) maintaining tumor cell number constant. After 16h, tumor cell viability was examined by flow cytometry comparing the % of CFSE+ and far red+ cells. Cell survival percentage was calculated as % survival $= 100 \times$ (sample % target cell $\div$ sample % non-target cell) $\div$ (control % target $\div$ control % non-target cell). Tumor cells cultured without OT-I cells were used as control.

Seahorse analysis

OCR and ECAR were measured using a XFe24 extracellular flux analyzer (Seahorse Bioscience). CD8 T cells were activated with CD3/CD28/IL2 for 48h, washed and plated onto poly-L-lysine (0.5 μg/ml, Sigma Aldrich) coated XFe24 plates ($3x10^5$ cells/well). Cells were subjected to a Seahorse XF Cell Mito Stress Test using non-buffer XF RPMI medium supplemented with 10 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate (all from Agilent technologies). OCR was measured in basal conditions and after sequential addition of 1 μM oligomycin, 1 carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and 100 nM rotenone plus 1 μM antimycin A (Rot + AA). Basal and maximal respiration were calculated by subtracting non-mitochondrial respiration (Rot + AA values) from basal or FCCP-mediated respiration respectively. Maximal respiration values were normalized to basal respiration. ECAR was measured in basal conditions.

ELISA

IFNγ concentration in T cell culture supernatants was determined by enzyme-linking immunosorbent assay ELISA) using the Mouse IFN-gamma DuoSet ELISA kit (R&D systems) following the manufacturer's instructions.

Tauroursodeoxycholic acid (TUDCA) treatments

For ER-mediated CPEB4 protein upregulation experiments, TUDCA 250 μM or vehicle was added 2 hours after activation to CD8. Cells were harvested 24 hours after activation. For rescue experiments, TUDCA 250 μM was added at the last 24h of a 48 hours activation period.

RT-qPCR analysis

Total RNA was extracted by TRIzol reagent (Invitrogen), followed by DNAse treatment (Ambion). 500-100 ng of RNA were then retrotranscribed

into cDNA using random hexamers with SuperScript IV (ThermoFisher). Quantitative real-time PCR was performed in a QuantStudio 6 Flex (Applied Biosystems) using PowerUp SYBR Green Master Mix (ThermoFisher). RNA quantifications were normalized to *Tbp* as endogenous control. Primers are listed in Table 1.

RNA-seq

Resting or 48h-activated Cpeb4$^{+/+}$ and Cpeb4$^{-/-}$ primary CD8 were harvested, washed twice with PBS and RNA was extracted as explained above. Samples were processed at the IRB Functional Genomics Facility following standard procedures and libraries were sequenced by Illiumina 50-bp single-end. Reads were aligned against the UCSC mm10 genome with STAR 2.7.0a (Dobin et al., 2013) and default options. Counts at gene level (Ensembl GRC. m38.07) were obtained using featureCounts with default options. DESeq2 1.22 was used to detect differentially expressed genes between groups of interest using Benjamini-Hochberg adjusted pvalues (Love et al., 2014). For gene set enrichment analysis, analysis was performed using regularized log transformation (rlog) applied to the count data using the DESeq2 R package 1.22, with the Roast (Wu et al., 2010) method using the MaxMean statistic. Hallmark gene set was obtained from the Broad Institute MSigDB website (Liberzon et al., 2015) and mapped from human to mouse genes using homology information from Ensembl biomart archive July 2016.

RNA-immunoprecipitation-sequencing analysis

30-40 million Cpeb4$^{+/+}$ and Cpeb4$^{-/-}$ primary CD8 (pooled from 3-4 animals) were isolated and activated as described above for 48 hours in biological triplicates. Activated CD8 were harvested, washed twice with 15 ml of cold PBS and incubated in 15 ml of FBS-free, 0,5% formaldehyde DMEM for 5 min at room temperature with soft agitation to allow crosslinking of RNA-binding proteins to target RNAs. Crosslinking was quenched by adding 5 ml of 1M glycine for 5 min. Cells were washed twice with cold PBS, lysed in 1 ml of RIPA

buffer (25 mM Tris-Cl pH7.6, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 100 mM EDTA, 150 mM NaCl) containing protease inhibitors and RNase inhibitors and sonicated for 5 min at low intensity with a Standard Bioruptor Diagenode. Extracts were precleared and immunoprecipitated (overnight, 4ºC on rotation) with 30 µg of anti-CPEB4 antibody (Mouse Monoclonal ERE149C, homemade) bound to 150 µl of Dynabeads Protein G (Invitrogen). Beads were washed 5 times with cold RIPA buffer supplemented with Protease inhibitors. For protein extraction, beads were resuspended in 100 µl Proteinase-K buffer with 70 µg of Proteinase-K (Roche) and incubated 60 min at 65ºC. RNA was extracted by standard phenol-chloroform, followed by Turbo DNA-free Kit (Ambion) treatment. Samples were processed at the IRB Functional Genomics Facility following standard procedures and libraries were sequenced by Illiumina 50-bp single-end. Reads from $Cpeb4^{+/+}$ and $Cpeb4^{-/-}$ CD8 inputs and IPs in biological triplicates were aligned against UCSC mm10 rRNA genome (October 2016) to identify rRNA contaminated reads with Bowtie1 using default options (Langmead et al., 2009). Non-rRNA reads (Bowtie1 unmapped) were then aligned against the UCSC mm10 genome with Bowtie 2 using default options (Langmead and Salzberg, 2012). TDF files for visual inspection were generated with IGVTools 2 (Thorvaldsdóttir et al., 2013). 3' UTR coordinates for the mm10 genome were obtained from Ensembl Biomart (March 2017). 3' UTR counts were generated with R 3.5.1 and featureCounts from RSubread v1.32.4 (Liao et al., 2019) with options allowMultiOverlap=TRUE, countMultiMappingReads=FALSE, minMQS=1 over mm10 3' UTR regions. CPEB4 targets were defined by an interaction analysis of $Cpeb4^{+/+}$ and $Cpeb4^{-/-}$ RIP samples and their respective input controls ($Cpeb4^{+/+}$IP/ $Cpeb4^{+/+}$ Input versus $Cpeb4^{-/-}$IP/$Cpeb4^{-/-}$Input) using DESeq2 (Love et al., 2014), with raw pvalue <0.05 and fold change >1.5 as thresholds. Gene set enrichment for selected targets was performed using the online Enrichr tool (November 5th 2020) (Kuleshov et al., 2016). For the analysis of CPE-A containing mRNAs, the script developed by Pique et al. (Piqué et al., 2008) was run over mm10 3UTR reference sequences (Biomart Ensembl archive february 2014).

Statistics and reproducibility

Data are represented as mean ± SD and statistics were analyzed with GraphPad Prism software. For two group comparison, Mann-Whitney test was performed; for multiple comparison, one- or two-way ANOVA with Sidak correction; for survival, Log-rank (Mantel-Cox) test was used. Linear model with random effects was used for *in vivo* growth of colorectal cancer liver metastasis. P values < 0.05 were considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. For animal studies, aged- and sex-matched animals were used. The experiment was blinded before experimental analysis. Littermates were used whenever possible, and animals from different groups were kept in the same cage. Experiments were repeated independently with similar results as indicated in the figure legend.

Results

1. Depletion of CPEB4 in the whole tumor microenvironment increases tumor growth and reduces T cell infiltration

In order to address the potential contribution of CPEB4 in the TME, we subcutaneously engrafted B16F10 melanoma cells into syngeneic mice either WT (Cpeb4$^{+/+}$) or knock-out for CPEB4 (Cpeb4$^{-/-}$) and monitored tumor growth (Figure 13a). Melanoma tumors from KO hosts developed more rapidly, suggesting an anti-tumor role for CPEB4 in the TME (Figure 13b). Comparative analysis of the TME cellular composition revealed a reduced infiltration of CD8 lymphocytes in tumors from KO animals (Figure 13c), but similar proportions of CD45^{+} immune cells (Figure 13d), CD4 lymphocytes (Figure 13e), CD11b^{+} myeloid cells (Figure 13f) and CD11b^{+}F4/80^{+} macrophages (Figure 13g). Additionally, tumor vascularization and presence of CD140a^{+}cancer-associated fibroblast were similar between tumors grown in animals from either genotype (Figure 13h, i).

To extend these findings into a metastatic setting, we performed an experimental metastasis assay injecting intravenously luciferase-expressing B16F10 cells (B16F10 TGL) into WT and CPEB4 KO hosts. Analysis of luciferase levels up to 14 days post-injection showed an increase in luciferase signal in KO hosts (Figure 14a). The augmented metastatic burden observed in KO animals originated from the development of larger metastatic foci, but not from an increased number of foci (Figure 14b, c), suggesting an effect in metastatic growth rather than homing or implantation. In line with these observations, KO lungs were less infiltrated with CD3^{+} lymphocytes (Figure 14d). Altogether, these results indicate that CPEB4 depletion in the TME favors tumor growth and suggest that this effect is T cell mediated.

2. T cell-intrinsic CPEB4 is dispensable for late T cell development and T cell homeostasis

Given that phenotypes observed in CPEB4 full-body knockout could stem from developmental defects or combination of multiple actions in

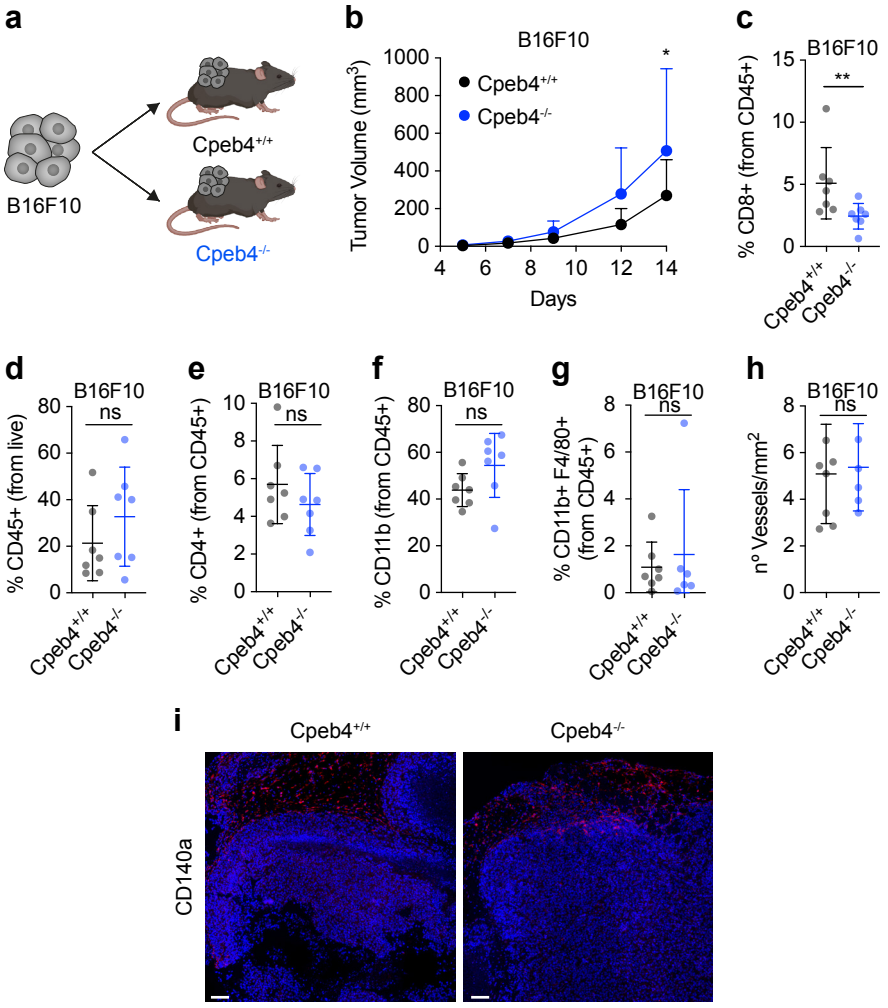

Figure 13. CPEB4 depletion in the whole tumor microenvironment impairs anti-tumor immunity. a) Schematic representation of experimental design. b) Tumor growth of B16F10 cells injected subcutaneously into Cpeb4$^{+/+}$ (n = 8) or Cpeb4$^{-/-}$ mice (n = 9). c-g) Flow cytometry analysis of CD8^{+} T cell, CD45^{+} total immune cell, CD4^{+} T cell, CD11b^{+} myeloid cell and CD11b^{+}F4/80^{+} macrophage infiltration in B16F10 tumors from Cpeb4$^{+/+}$ or Cpeb4$^{-/-}$ mice 14 days post-injection (n = 7). h) IF quantification of CD31^{+} blood vessels in B16F10 tumors from Cpeb4$^{+/+}$ or Cpeb4$^{-/-}$ mice 14 days post-injection (n = 7). i) Representative images of CD140a immunofluorescence marking fibroblasts in B16F10 tumors from Cpeb4$^{+/+}$ or Cpeb4$^{-/-}$ mice 14 days post-injection. Scale bar 100 µm. Data are represented as mean ± s.d. Statistical analysis was performed by 2-way ANOVA with Sidak correction (b) and Mann-Whitney test (c-h). ns = not significant, *p < 0.05, **p < 0.01. Data are representative of 2-3 independent experiments (b-i).

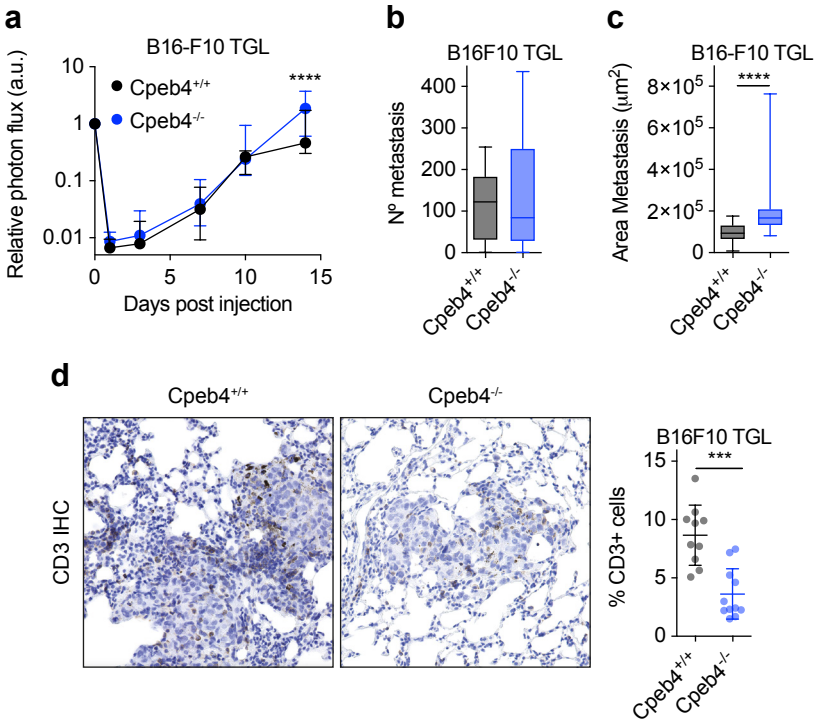

Figure 14. CPEB4 depletion in the metastatic niche promotes metastatic growth. a) Quantification of luminescence signal of lung metastasis from Cpeb4$^{+/+}$ (n = 20) or Cpeb4$^{-/-}$ (n = 21) mice injected via tail vein with B16F10 TGL cells. b, c) Lung metastasis foci number (b) and area (c) from Cpeb4$^{+/+}$ (n = 20) or Cpeb4$^{-/-}$ (n = 21) mice injected via tail vein with B16F10 TGL cells 14 days post-injection. d) Representative images (left panel) and quantification (right panel) of CD3 immunohistochemistry of lungs from Cpeb4$^{+/+}$ (n = 10)) or Cpeb4$^{-/-}$ (n = 11) mice injected via tail vein with B16F10 TGL cells 14 days post-injection. Data are represented as mean ± s.d, except in (a) where median + 95% confidence interval is represented. Statistical analysis was performed by 2-way ANOVA with Sidak correction (a) and Mann-Whitney test (b-d). ns = not significant, ***p < 0.001, ****p < 0.0001. Data are pooled from 2 independent experiments (a-d).

different cell types, we sought to determine whether the antitumoral effect of CPEB4 in the TME was due to its intrinsic function in T cells by specifically deleting it in CD4 and CD8 T cells. To this end, we crossed a CD4-Cre mouse line (Lee et al., 2001) with animals carrying a floxed allele of CPEB4 (CPEB4-TKO). CD4-Cre is first expressed in double positive thymocytes, and therefore in this model both CD4 and CD8 cells are knockout for CPEB4, even though mature CD8 cells lose CD4 expression (Figure 15a).

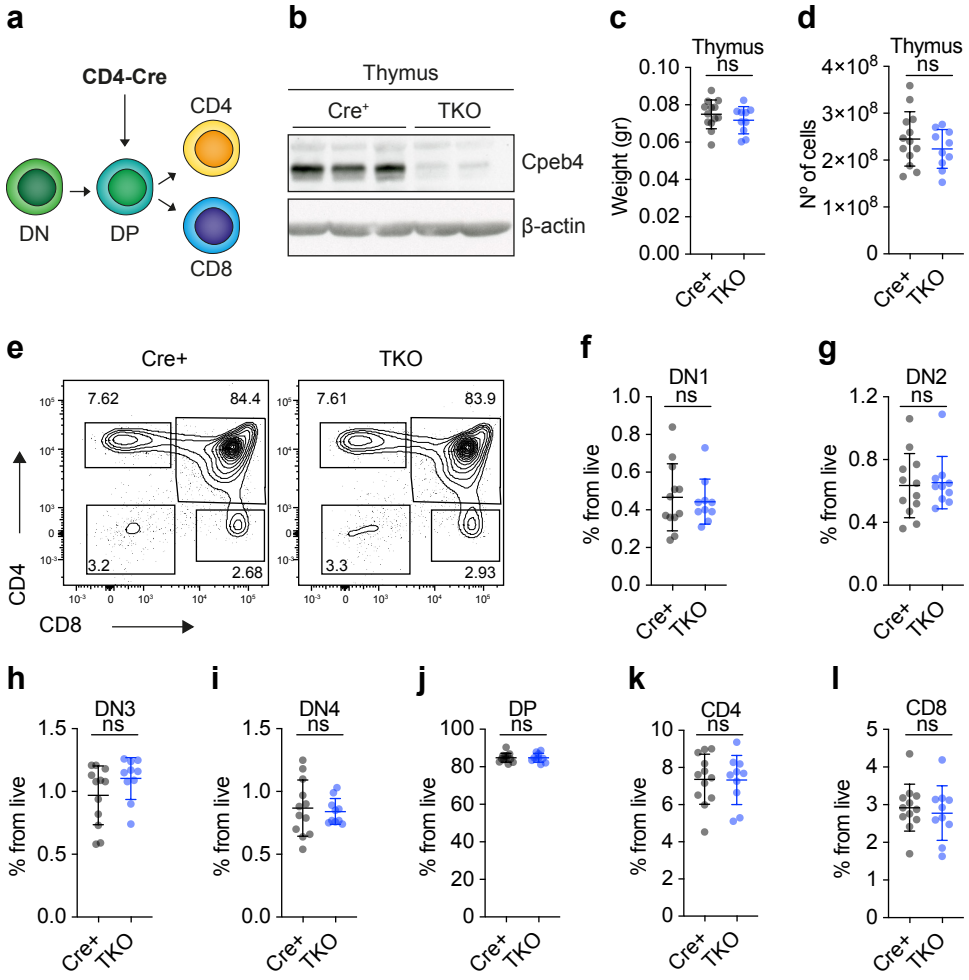

Figure 15. T-cell specific CPEB4 depletion does not affect thymic development. a) Schematic representation of CD4-cre cassette expression during thymic development. b) Representative western blot of CPEB4 protein levels in thymi from Cre+ and TKO; β-actin is used as loading control. c, d) Thymus weight (c) and cellularity (d) of Cre+ (n = 13) and TKO (n = 10) animals. e-l) Representative plots (e) and quantification (f-l) of flow cytometry analysis of thymic T cell precursor DN1 (f), DN2 (g), DN3 (h), DN4 (i), DP (j), CD4 k) and CD8 (l) frequencies from Cre+ (n = 12) and TKO (n = 10) animals. Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test (c, d, f-l). ns = not significant. Data are pooled from 3-4 independent experiments.

Analysis of CPEB4 protein levels confirmed CPEB4 depletion in CPEB4-TKO thymi (Figure 15b). Ablation of CPEB4 in DP thymocytes did not affect thymic development, as thymus weight (Figure 15c), cellularity (Figure 15d) and precursor composition (Figure 15e-l) was comparable between

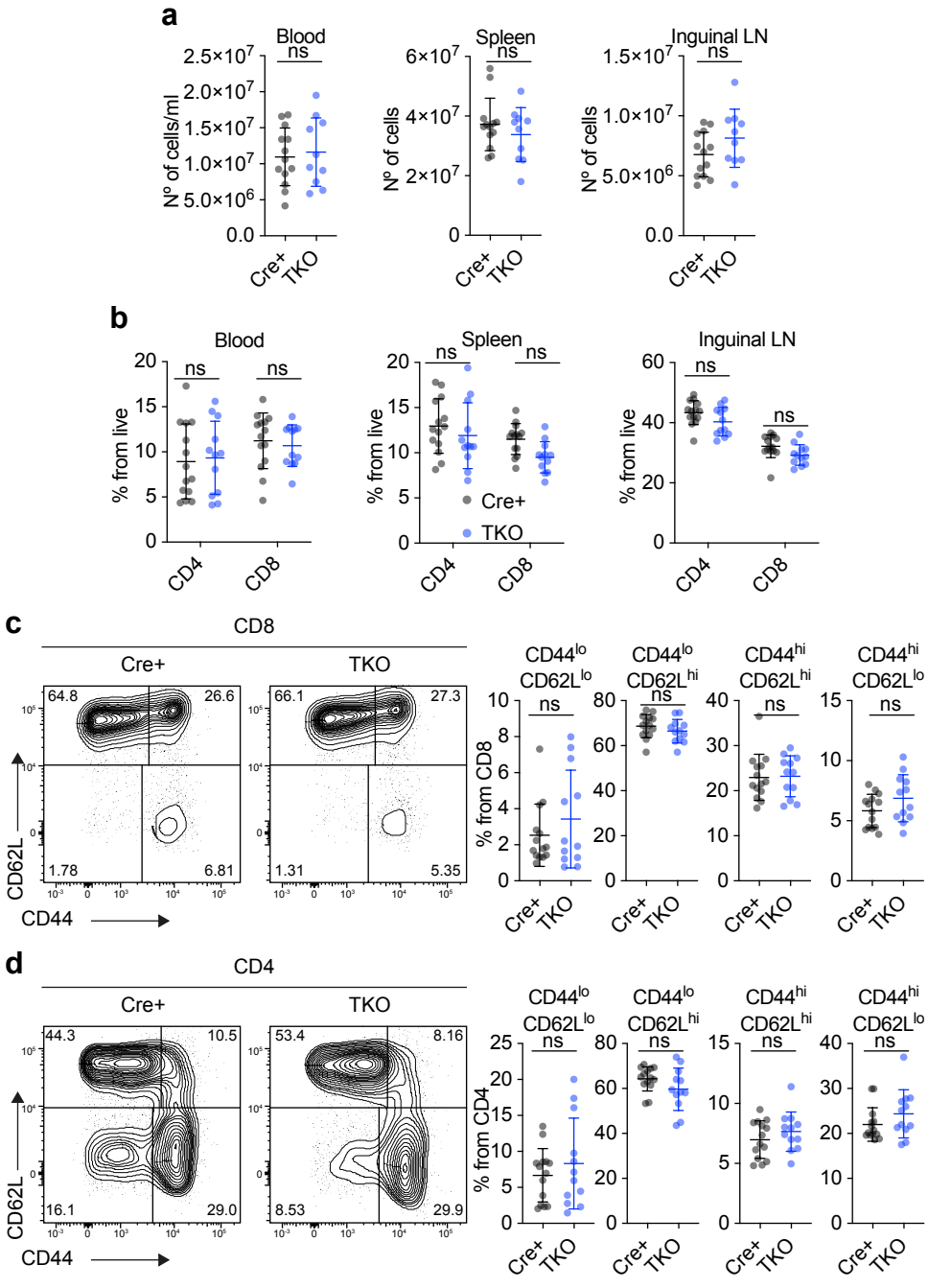

Figure 16. T-cell specific CPEB4 depletion does not affect T cell homeostasis.
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◄— **Figure 16. T-cell specific CPEB4 depletion does not affect T cell homeostasis.** a) Nº of cells per ml in blood (left panel) and cellularity of spleen (middle panel) and inguinal lymph node (right panel) of Cre+ (n = 13) and TKO (n = 10) animals b) Frequencies of CD8 and CD4 T cells in blood (left panel), spleen (middle panel) and inguinal lymph node (right panel) from Cre+ (n = 14) and TKO (n = 12) animals. c, d) Representative plots (left panels) and quantification (right panels) of stem-cell like memory ($CD44^{lo}CD62L^{lo}$), naïve ($CD44^{lo}CD62L^{hi}$), central memory ($CD44^{hi}CD62L^{hi}$) and effector memory ($CD44^{hi}CD62L^{lo}$) frequencies in CD8 (c) and CD4 (d) splenocytes from Cre+ (n = 14) and TKO (n = 12) animals. Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test (a,c,d) or by one-way ANOVA with Sidak correction (b). ns = not significant. Data are pooled from 3-4 independent experiments.

CPEB4-TKO and WT (Cre+) animals. Moreover, no changes in number nor frequency of T cells in blood, spleen or lymph nodes associated to CPEB4 depletion were detected (Figure 16a, b). Homeostatic differentiation of naïve ($CD44^{lo}CD62L^{hi}$) cells into effector memory ($CD44^{hi}CD62L^{lo}$) and central memory $CD44^{hi}CD62L^{hi}$) was also unaffected in both CD8 and CD4 lymphocytes (Figure 16c, d). These results indicate that CPEB4 is dispensable for thymic development from the DP stage and for T cell homeostasis in unchallenged animals.

3. CPEB4 positively regulates T cell mediated anti-tumor immunity

To test whether T-cell mediated anti-tumor activity required CPEB4, control and CPEB4-TKO mice were subcutaneously engrafted with B16F10 melanoma cells expressing the ovalbumin antigen (B16F10 OVA) (Sancho et al., 2008). The expression of OVA renders these cells more immunogenic and make them a better model to study tumor immunity, as B16F10 are poorly immunogenic (Wang et al., 1998). As observed in full-body CPEB4-KO, loss of CPEB4 in T cells increased tumor growth (Figure 17a), resulting in a decreased survival span (Figure 17b). To test if this effect was specific for the melanoma model, we also performed intra-splenic injection of colorectal cancer organoids expressing luciferase (Tauriello et al., 2018) and monitored liver metastasis growth. As for the melanoma cells, metastatic burden was

increased in CPEB4-TKO mice (Figure 17c), indicating that CPEB4 acts as a positive mediator of T cell anti-tumor immunity across tumor types.

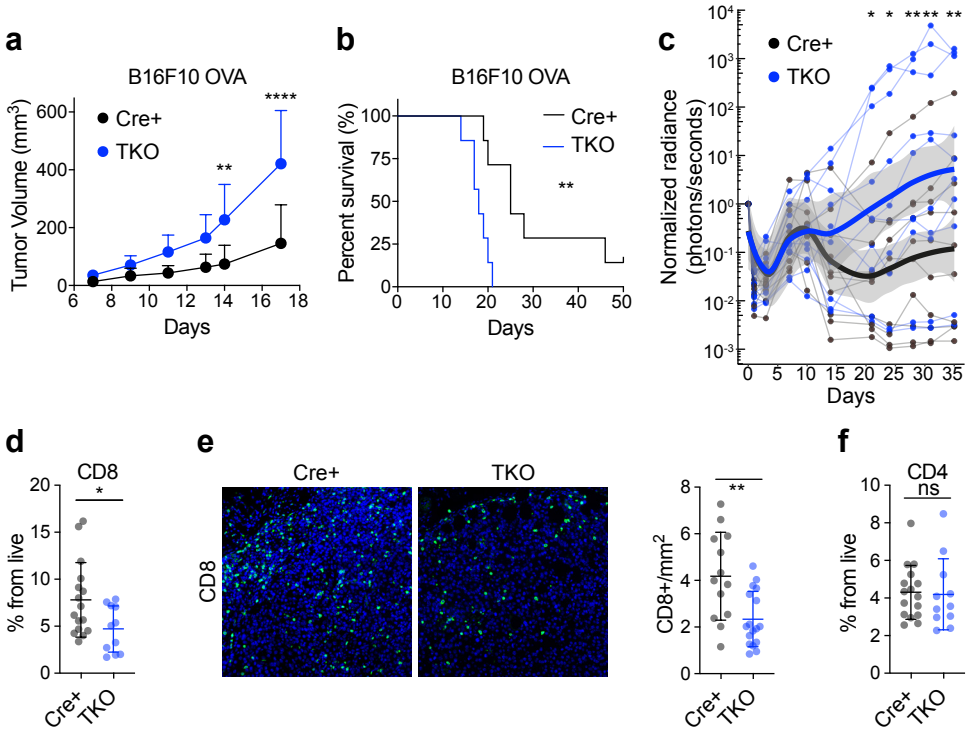

Figure 17. T-cell specific CPEB4 depletion impairs anti-tumor immunity. a, b) Tumor growth (a) and survival curves (b) of Cre+ or TKO mice injected subcutaneously with B16F10 OVA cells (n = 7). c) Quantification of bioluminescence signal of liver metastasis from Cre+ or TKO mice injected intra-spleen with colorectal cancer organoids expressing luciferase (n = 10). d) Flow cytometry analysis of CD8 T cell infiltration in B16F10 OVA tumors from Cre+ (n = 16) or TKO (n = 11) mice 14 days post-injection. e) Representative images (left panels) and quantification (right panel) of CD8 IF from Cre+ (n = 13) and TKO (n = 17) B16F10 OVA tumors. f) Flow cytometry analysis of CD4 T cell infiltration in B16F10 OVA tumors from Cre+ (n = 16) or TKO (n = 11) mice 14 days post-injection. Data are represented as mean ± s.d, except in (c) where points and lines represent individual mice, trend lines (bold) show a LOESS model with 95% confidence interval (grey band). Statistical analysis was performed by 2-way ANOVA with Sidak correction (a), Mann-Whitney test (d-f), linear model with random effects (c) and Mantel-Cox test (b); ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are representative of 2-5 independent experiments (a-c) or pooled from 2-3 independent experiments (d-f).

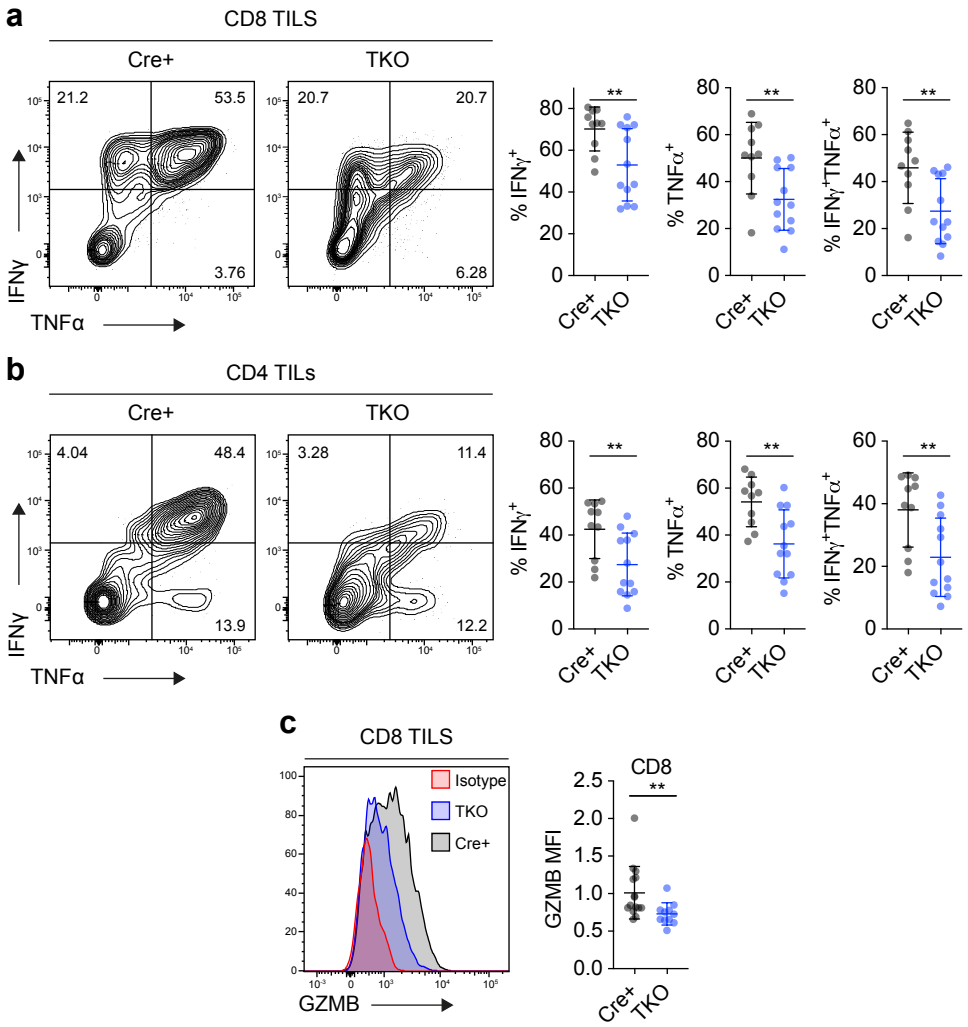

Figure 18. T-cell specific CPEB4 depletion decreases tumor-infiltrating lymphocyte effector function. a, b) Representative plots (left panels) and quantification (right panels) of IFNγ and TNFα-producing CD8 (a) or CD4 (b) cells infiltrated in B16F10 OVA tumors from Cre+ (n = 10) and TKO (n = 12) animals. c) Representative plot (left panel) and quantification (right panel) of GZMB MFI of CD8 TILs in Cre+ (n = 15) and TKO (n = 11) injected s.c. with B16F10 OVA cells. MFI values are normalized to Cre+ mice. Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test (a-c); **p < 0.01. Data are pooled from 2 independent experiments (a-c).

Since all our previous observations pointed out to a functional defect in T cells, we immunophenotyped TILs of control and TKO animals. In line with results from full-body KO, CD8 infiltration was reduced in B16F10 OVA tumors from CPEB4-TKO mice, measured by FACS (Figure 17d) and immunofluorescence

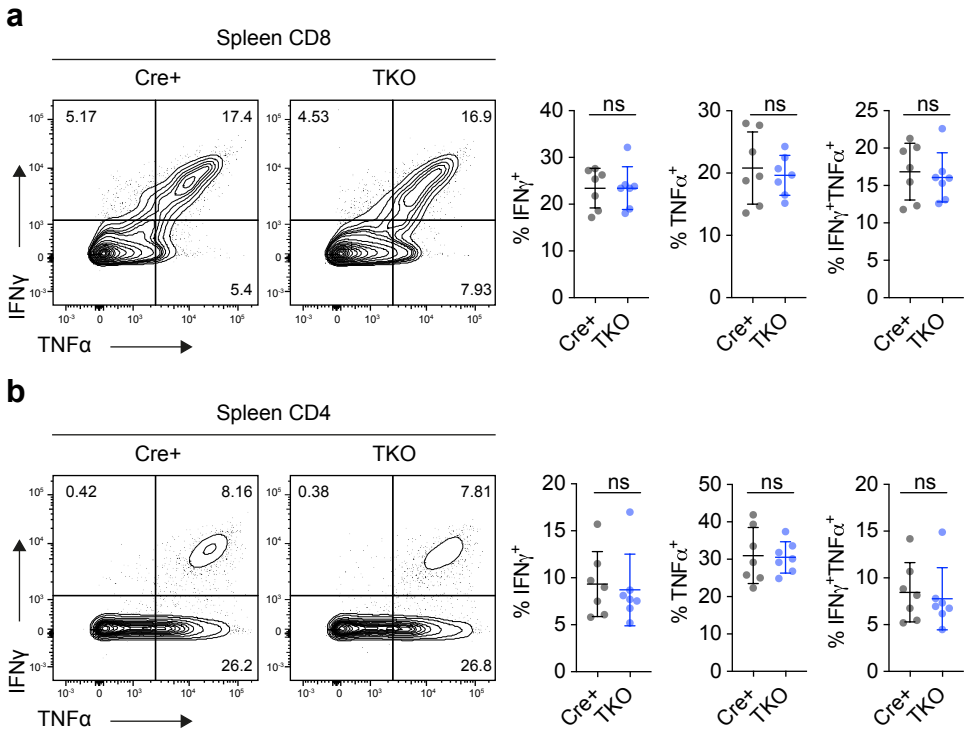

Figure 19. Effector function defects in CPEB4-TKO are specific of tumor infiltrating lymphocytes. a, b) Representative plots (left panels) and quantification (right panels) of IFNγ and TNFα-producing CD8 (a) or CD4 (b) splenocytes of B16F10 OVA tumor-bearing Cre+ (n = 7) and TKO (n = 7) animals. Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test (a,b). n.s. = not significant. The data are representative of 2 independent experiments.

(Figure 17e), whereas CD4 TILs levels were comparable to control animals (Figure 17f). Moreover, CPEB4-TKO TILs presented a defect in production of effector T cell mediators, as observed by the decrease in IFNγ$^+$, TNFα$^+$ and IFNγ$^+$TNFα$^+$ CD8 and CD4 TILs (Figure 18a, b) and GZMB expression in CD8 TILs (Figure 18c). Importantly, these differences were not observed in splenic lymphocytes from tumor-bearing animals (Figure 19a, b), suggesting that CPEB4 was required for effector T cell function specifically in active, intra-tumor lymphocytes.

Of note, this phenotype does not seem to originate from an exacerbated exhaustion, as typical exhaustion markers such as Tim3, Lag3 and PD-1 were largely unaffected (Figure 20a, b). Defects on effector T cell can also stem

from increased immunosuppressive signals from Tregs: however, we did not observe changes in the proportions of FOXP3+ Tregs between control and CPEB4-TKO mice (Figure 20c). Together, these observations indicate that T cells require CPEB4 to acquire or sustain their effector function and control tumor growth, in a mechanism independent of exhaustion or Treg differentiation. Depletion of CPEB4 in T cells therefore causes a defective anti-tumor response leading to an increase in tumor growth. On the contrary, T cell development and homeostasis do not appear to require CPEB4.

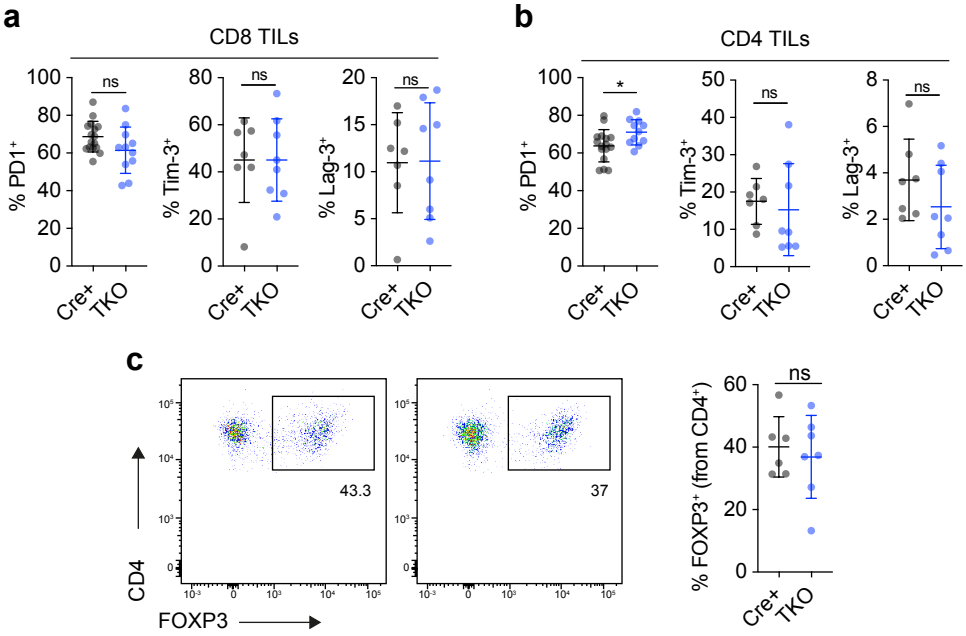

Figure 20. Effector function defects are not caused by increased exhaustion of Treg infiltration. a, b) Frequencies of PD1+ (left panel), Tim3+ (middle panel) and Lag3+ (right panel) CD8 (a) and CD4 (b) infiltrated in B16F10 OVA tumors from Cre+ (n = 7, except for PD1+ n = 16) and TKO (n = 8 except for PD1+ n = 11) animals. c) Representative flow cytometry plots (left panels) and quantification of FOXP3+ CD4 cells infiltrated in B16F10 OVA tumors from Cre+ (n = 6) and TKO (n = 7) animals. Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test (a-c); ns = not significant, *p < 0.05. Data are representative of 2-3 independent experiments (a-c).

4. CPEB4 is upregulated in effector CD8 TILs and activated T cells

To further define the role of CPEB4 in T cell function we tested whether CPEB4 was differentially expressed among T cell subsets. First, we analyzed CPEB4 protein levels in B16F10 TILs versus spleen or draining lymph nodes (dLN) of tumor-bearing animals by flow cytometry using a CPEB4-specific antibody (Figure 21a). CPEB4 was strongly upregulated in CD8 TILs

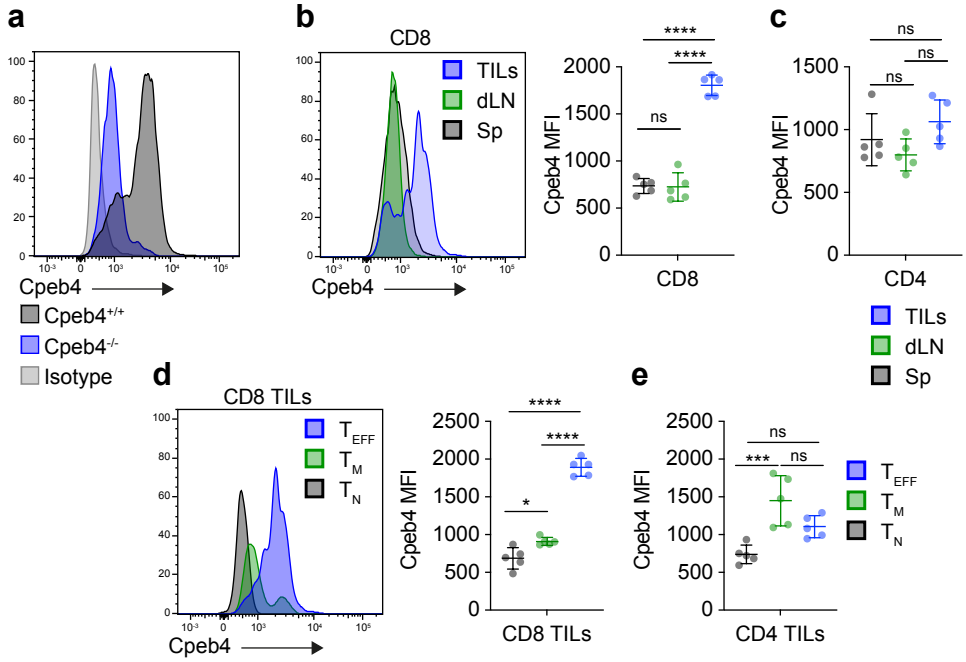

Figure 21. CPEB4 protein levels are upregulated in effector CD8 TILs. a) Flow cytometry histogram of CPEB4 intracellular staining of CD8 infiltrated in B16F10 tumors from Cpeb4$^{+/+}$ or Cpeb4$^{-/-}$ host showing specificity of anti-CPEB4 antibody. b) Histogram (left panel) and quantification (right panel) of flow-cytometric analysis of CPEB4 expression in CD8 cells from spleen, draining lymph node and B16F10 tumors of tumor-bearing mice (n = 5). c) Flow cytometry analysis of CPEB4 MFI in CD4 from B16F10 tumors, spleen and draining lymph node of tumor-bearing mice (n = 5). d) Histogram (left panel) and quantification (right panel) of CPEB4 MFI in T_N, T_{eff} and T_M CD8 TILs from B16F10 tumors (n = 5). e) Quantification of CPEB4 MFI in T_N, T_{eff} and T_M CD4 TILs from B16F10 tumors. Data are represented as mean ± s.d. Statistical analysis was performed by 1-way ANOVA with Tukey correction (b-e). ns. = not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are representative of 2-3 independent experiments (b-e).

compared to splenic or lymph node CD8 cells (Figure 21b), whereas CPEB4 levels in CD4 from the three different sources were comparable (Figure 21c). A deeper analysis of CPEB4 expression revealed that CPEB4 was strongly upregulated in effector CD8 TILs, while memory CD8 cells only presented a modest increase compared to naïve cells (Figure 21d). In contrast, CPEB4 levels in CD4 TILs were increased in memory cells compared to effector or naïve cells (Figure 21e). This suggests that CPEB4 is heavily upregulated in effector CD8 lymphocytes, whereas its levels in CD4 lymphocytes increase in memory cells.

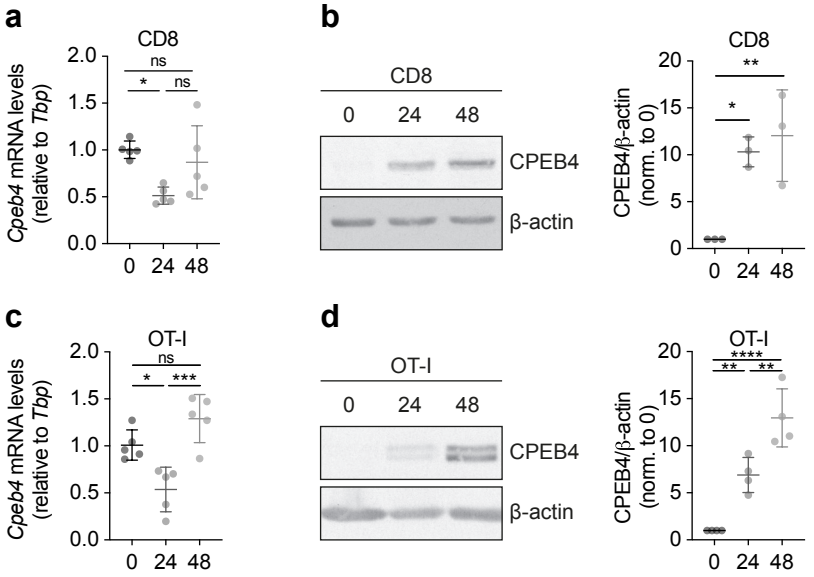

Figure 22. CPEB4 protein, but not mRNA, is upregulated during CD8 T cell activation *ex vivo*. a) qPCR of *Cpeb4* mRNA in CD8 resting or activated *ex vivo* with CD3/CD28/IL-2 at the indicated time-points; *Tbp* is used as endogenous control (n = 5). b) Western blot (left panel) and quantification (right panel) of CPEB4 protein expression in CD8 resting or activated *ex vivo* as in a at the indicated time-points; β-actin is used as loading control (n = 3). c) qPCR of *Cpeb4* mRNA in OT-I cells resting or *ex vivo* activated with OVA/IL-2 at the indicated time-points; *Tbp* is used as endogenous control (n = 5). d) Western blot (left panel) and quantification (right panel) of CPEB4 protein expression in OT-I cells resting or *ex vivo* activated as in c at the indicated time-points; β-actin is used as loading control (n = 4). Data are represented as mean ± s.d. Statistical analysis was performed by one-way ANOVA with Tukey correction (a-d). ns. = not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data are representative of 2-3 independent experiments (A-F).

Next, we monitored CPEB4 protein and mRNA levels during T cell activation *ex vivo*. Strikingly, *Cpeb4* mRNA was reduced upon activation (Figure 22a), while CPEB4 protein levels started from virtually undetectable in unstimulated CD8 cells to became strongly upregulated upon activation (Figure 22b). Albeit changes in protein stability cannot be ruled out, this apparent contradiction between mRNA and protein levels is frequently observed for mRNAs under strong translational control, such as *Cpeb4* mRNA (Igea et al., 2010; Maillo et al., 2017) Similar results were obtained when CD8 cells from OT-I animals were activated *ex vivo* with OVA/IL-2 (Figure 22c, d). In CD4 T cells *Cpeb4* mRNA levels remained constant (Figure 23a) and, as observed *in vivo*, CPEB4 protein upregulation was much more modest compared to CD8 cells (Figure 23b). Together, these results indicate that CPEB4 protein is accumulated in effector CD8 cells, and this increase is induced upon T cell activation, potentially in a post-transcriptional manner. This regulation, or the extent of it, is different between CD8 and CD4 T cells.

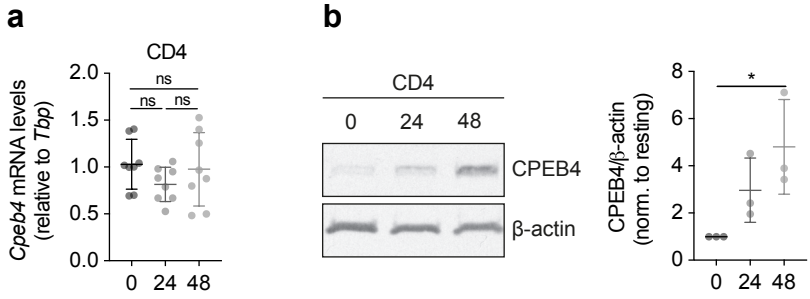

Figure 23. CPEB4 protein, but not mRNA, is upregulated during CD4 T cell activation *ex vivo*. a) qPCR of *Cpeb4* mRNA in CD4 resting or activated *ex vivo* with CD3/CD28/IL-2 at the indicated time-points; *Tbp* is used as endogenous control (n = 8). b) Western blot (left panel) and quantification (right panel) of CPEB4 protein expression in CD4 resting or activated *ex vivo* as in a at the indicated time-points; β-actin is used as loading control (n = 3). Data are represented as mean ± s.d. Statistical analysis was performed by 1-way ANOVA with Tukey correction (a, b). Data are representative of 2 independent experiments (b) or pooled from 3 independent experiments (a).

5. CPEB4 sustains effector fuction and metabolic fitness, but not early activation or differentiation induction

Given the correlation between CPEB4 protein expression pattern in CD8 lymphocytes and the effector phenotype defects observed *in vivo* in CPEB4-KO models, we decided to further investigate the role of CPEB4 in CD8 effector function. T cell activation induces a cascade of signaling pathways, metabolic adaptations and transcription factor regulation that results in clonal expansion and effector fate acquisition. Then, effector function must be sustained until antigens are cleared. Defects in effector phenotype can therefore arise either from problems in the differentiation cascade or from subsequent difficulties in sustaining functionality.

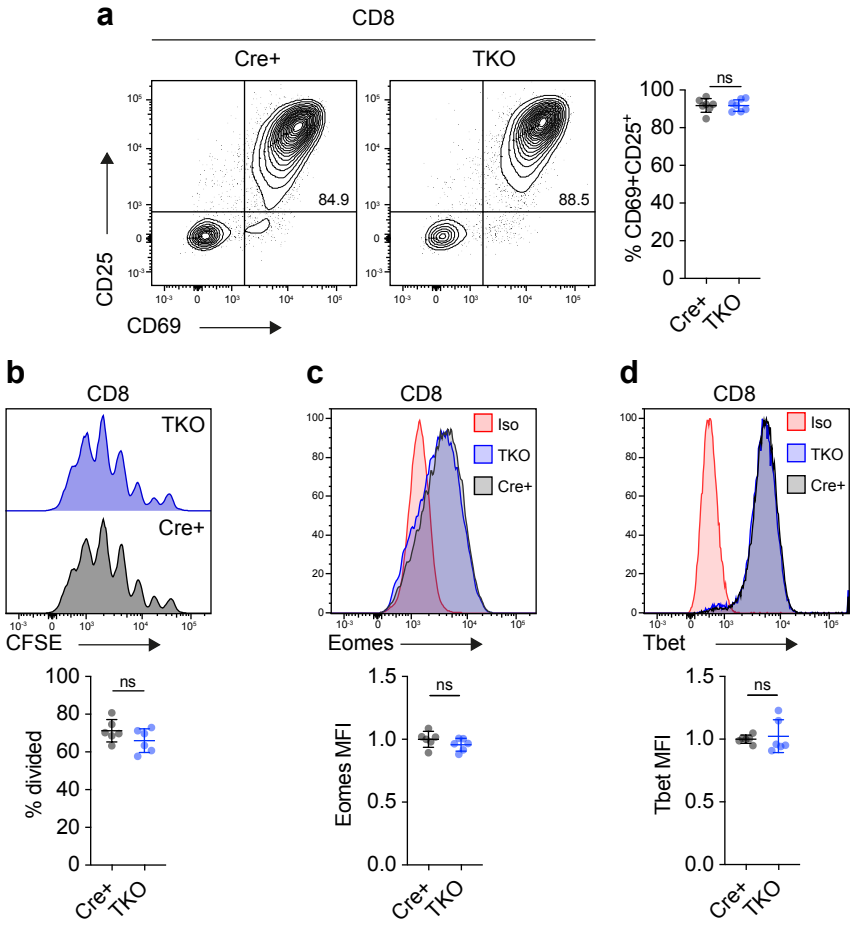

Figure 24. CPEB4 is dispensable for CD8 early activation, proliferation and induction of differentiation *ex vivo*. *(figure legend on next page)* ⟶

◄— Figure 24. CPEB4 is dispensable for CD8 early activation, proliferation and induction of differentiation ***ex vivo*****.** a) Representative flow cytometry plots (left panels) and quantification (right panel) of early activation markers CD25 and CD69 in CD8 cells activated ex vivo with CD3/CD28/IL-2 for 24h (n = 7). b) Proliferation assay of carboxyfluorescein succinimidyl ester (CFSE)-labelled CD8 cells activated ex vivo with CD3/CD28/IL-2 for 72h. Left: representative histogram of CD8 T cell proliferation (n = 6); right: quantification of the percentage of divided cells (n = 6). c, d) Representative histograms (left panels) and quantification (right panels) of Eomes (c) and T-bet (d) median fluorescent intensity from Cre+ and TKO CD8 activated with CD3/CD28/IL-2 for 48h (n = 6). Values are normalized to Cre+. Data are represented as mean ± s.d. Statistical analysis was performed Mann-Whitney test (a-d). ns = not significant. Data are representative of 2 independent experiments (c, d) or pooled from 2 independent experiments (a, b).

In order to determine whether CPEB4 played a role in differentiation or maintenance of the effector phenotype, we monitored several parameters of T cell activation and differentiation *ex vivo*. First, we asked whether CPEB4 was required during the initial steps of activation by analysing the expression of the early activation markers CD25 and CD69 (Reddy et al., 2004). *Ex vivo* activated control or CPEB4-TKO CD8 presented similar proportions of $CD25^{+}CD69^{+}$ cells 24 hours post-activation (Figure 24a). Second, we measured the induction of proliferation, observing that both control and CPEB4-TKO CD8 expand at comparable levels upon activation (Figure 24b). Third, we analysed the expression levels of T-bet and Eomes, the main transcription factors required for effector phenotype induction (Pearce et al., 2003; Sullivan et al., 2003). Protein levels of both T-bet and Eomes were similar between control and CPEB4-TKO CD8s (Figure 24c, d). These results suggest that early activation, clonal expansion and transcriptional induction of differentiation were not affected in the absence of CPEB4.

However, in agreement with *in vivo* observations, *ex vivo* activated CPEB4-depleted CD8s presented defects in the production of effector molecules, as the proportion of $IFN\gamma^{+}$ cells (Figure 25a) and the secretion of IFNγ (Figure 25b) were diminished in in cells lacking CPEB4. In addition, absence of CPEB4 reduced metabolic fitness, illustrated by a decreased mitochondrial respiration, bot basal and maximal (Figure 25c), and lowered glycolysis (Basal ECAR,

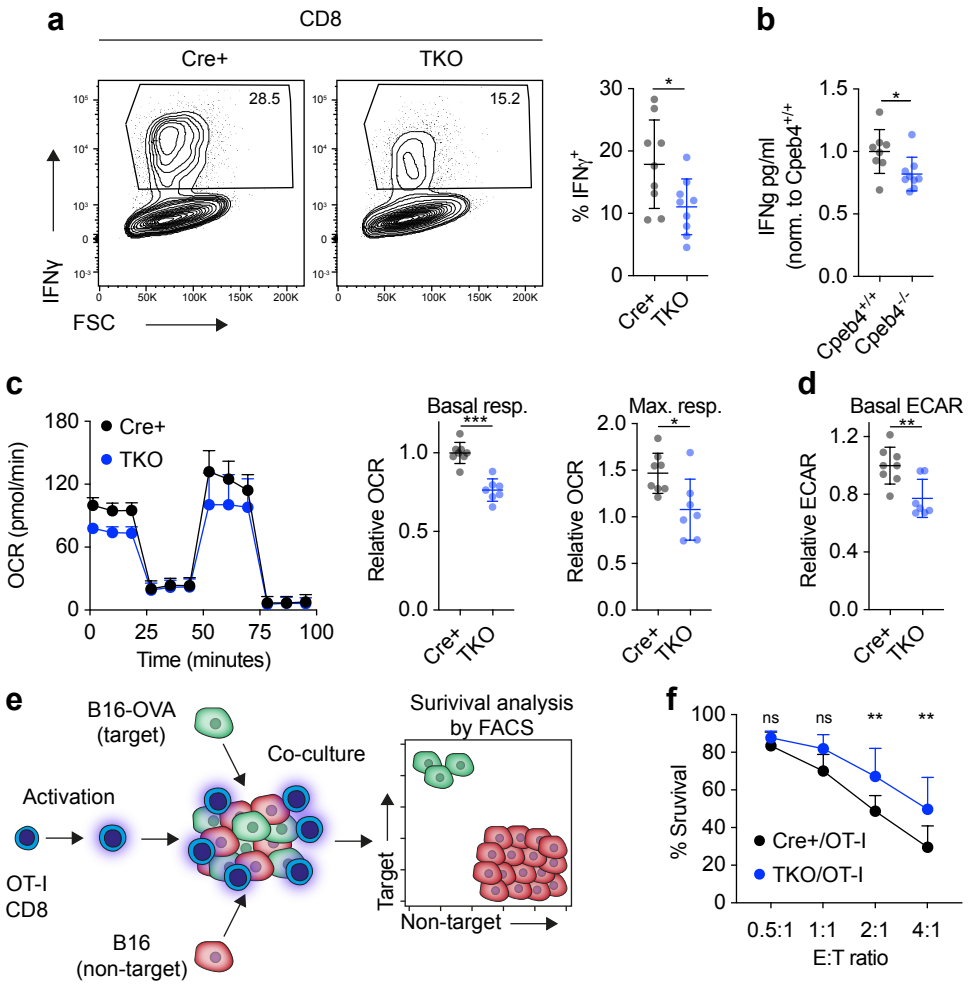

Figure 25. CPEB4 is required for CD8 effector function *ex vivo*. a) Flow cytometry plots (left panels) and quantification (right panel) of IFNγ$^+$ CD8 cells activated *ex vivo* with CD3/CD28/IL-2 for 24h (n = 9). b) Concentration of IFNγ in supernatants of cultured Cpeb4$^{+/+}$ (n = 8) and Cpeb4$^{-/-}$ (n = 9) CD8 cells activated as in a. c) Oxygen consumption rate of Cre+ (n = 8) or TKO (n = 7) CD8 activated *ex vivo* as in a after mitochondrial stress test analysis. Values are normalized to basal Cre+ measurements. d) Extracellular acidification rate (ECAR) of Cre+ (n = 8) or TKO (n = 7) CD8 activated *ex vivo* as in a measured in basal conditions normalized to Cre+. e) Schematic view of killing assay (see methods). f) Survival percentage of B16F10 OVA melanoma cells co-cultured with pre-activated Cre+/OT-I or TKO/OT-I cells at increasing effector:target cell rations. Survival was measured by flow cytometry 16h after co-culture (n = 4 for 0.5:1, n = 8 for 1:1, 2:1 and 4:1). Data are represented as mean ± s.d. Statistical analysis was performed by 2-way ANOVA with Sidak correction (f) and Mann-Whitney test (a-d). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001. Data are from 2-4 independent experiments (a-f).

Figure 25d). To derive a functional consequence of this defect in effector capacity and fitness, we developed a killing assay to monitor the cytotoxic activity of CPEB4 depleted CD8. With this aim, we crossed our CPEB4-TKO mouse with OT-I animals, which clonally express a TCR that recognizes the OVA antigen (Hogquist et al., 1994). We then isolated and activated *ex vivo* either control (Cre+) or CPEB4-TKO OT-I CD8 and co-cultured them with target B16F10 OVA cells to measure melanoma cell survival, using non-target B16F10 cells for normalization (Figure 25e, see Materials and Methods for details). In line with previous results, cytotoxic activity of CPEB4-TKO CD8 cells was reduced compared to control cells (Figure 25f). Together, these findings indicate that CPEB4 is required for maintaining CD8 effector function, and suggest that mechanistically CPEB4 function is downstream of early activation or differentiation processes.

6. CPEB4 deletion exacerbates activation-induced ER stress

To better understand the mechanistic basis for the observed phenotypes in T cell effector function, we compared the transcriptomes of CPEB4 WT or

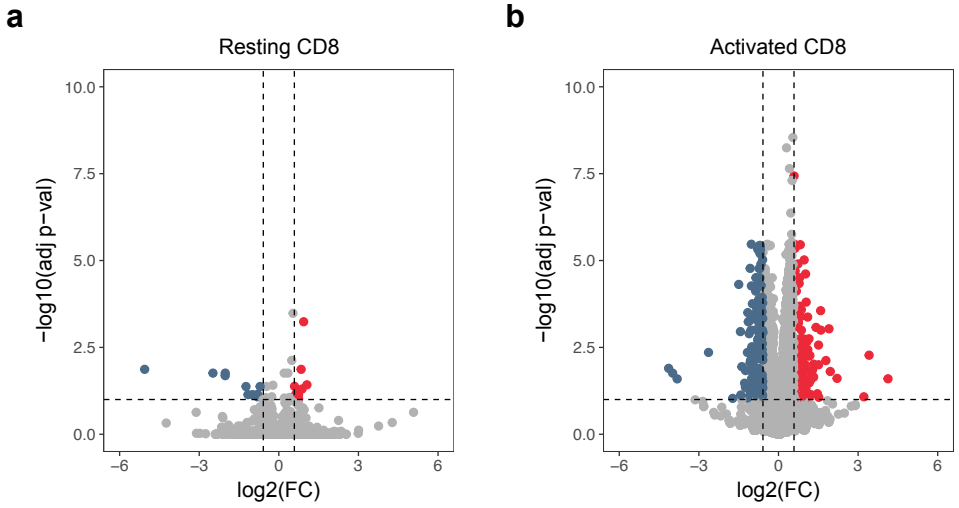

Figure 26. CPEB4 depletion does not affect gene expression in resting CD8. a, b) Volcano plot showing gene expression changes measured by RNA-seq in resting (a) or 48h CD3/CD28/IL-2 activated (b) Cpeb4$^{-/-}$ vs Cpeb4$^{+/+}$ CD8 cells. Genes with a fold change > log2(1.5) and an adjust p.val < 0.1 are colored in red; genes with a fold change < -log2(1.5) and an adjust p.val < 0.1 are colored in blue.

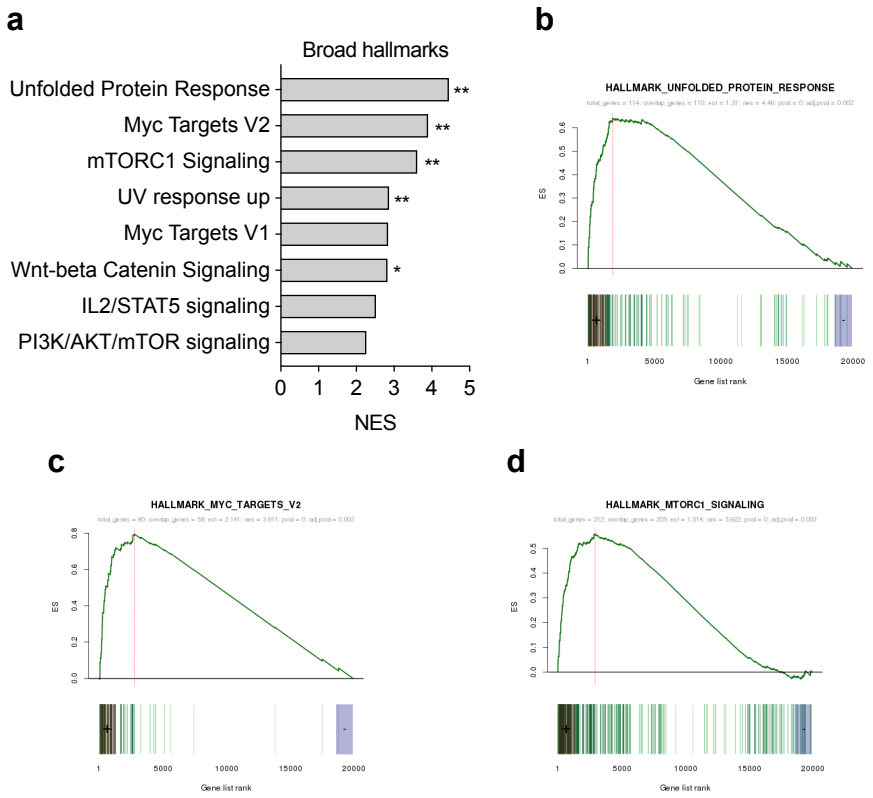

Figure 27. CPEB4 depletion exacerbates the unfolded protein response and anabolic pathways. a) Pathway enrichment analysis in Cpeb4$^{-/-}$ (n = 3) vs Cpeb4$^{+/+}$ (n = 3) CD8 cells activated *ex vivo* with CD3/CD28/IL-2 for 48h. Top pathways enriched from the Molecular Signatures Database Hallmarks collection are shown. b-d) Enrichment plot of unfolded protein response (b), Myc targets version 1 (c) and mTORC1 signaling (d) gene sets from the Molecular Signatures Database Hallmarks collection.

KO CD8 lymphocytes in resting or 48 hours *ex vivo* activated conditions. In agreement with the negligible levels of CPEB4 in resting CD8 cells, non-activated WT or CPEB4 KO lymphocytes displayed almost no differences in their transcriptome (Figure 26a). On the contrary, we detected differential expression of 301 mRNAs from lymphocytes activated in the presence or absence of CPEB4 (Figure 26b). Pathway enrichment analysis showed that the top differential categories upregulated in CPEB4 KO activated CD8 cells corresponded to UPR (Figure 27a, b) and anabolic pathways (mTORC1, MYC, Figure 27a, c, d). Among these top enriched gene sets, we were specially intrigued by the increase in UPR pathway.

The UPR is physiologically induced upon T cell activation as a result of the ER stress generated by the increase in T cell size, proliferation and secretory capacity, and is necessary for differentiation into effector cells (Cao et al., 2019; Kamimura and Bevan, 2008; Pino et al., 2008; Takano et al., 2008; Thaxton et al., 2017). However, in contexts where UPR signaling is exacerbated, it can lead to T cell dysfunction (Cao et al., 2019; Ma et al., 2019; Song et al., 2018). Given that previous results from our laboratory showed that CPEB4 is required for liver adaptation to pathological ER stress (Maillo et al., 2017), we hypothesized that CPEB4 could mediate CD8 cell adaptation to activation-induced, functional ER stress, and therefore its depletion causes a maladaptive, terminal UPR that causes cellular dysfunction.

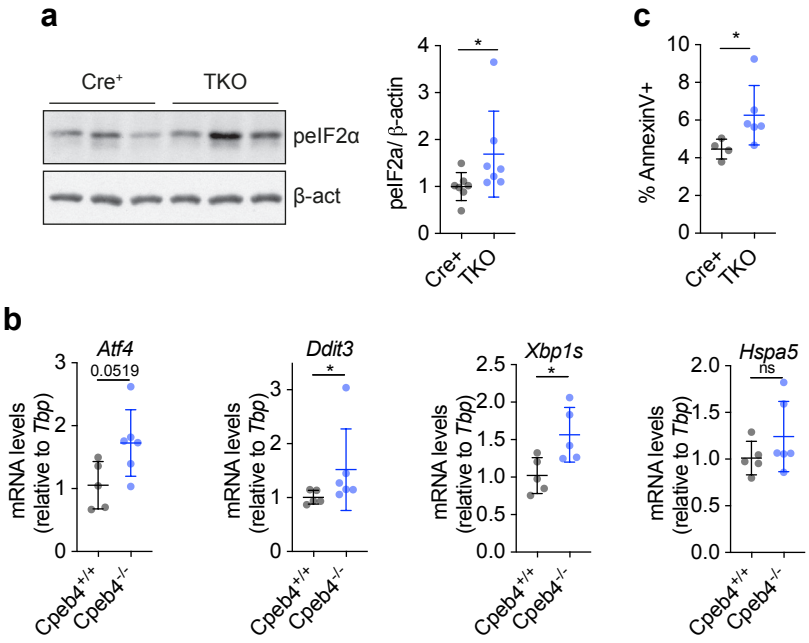

Figure 28. The UPR is exacerbated in activated, CPEB4 depleted CD8 lymphocytes. a) Western blot analysis (left panel) and quantification (right panel) of eIF2α phosphorylation in CD8 activated *ex vivo* with CD3/CD28/IL-2 for 48h; β-actin is used as loading control (n = 7). b) qPCR analysis of *Atf4*, *Ddit3*, *Xbp1s* and *Hspa5* mRNA levels in Cpeb4$^{+/+}$ (n = 5) and Cpeb4$^{-/-}$ (n = 6) CD8 cells activated as in a; *Tbp* is used as endogenous control (n = 5). c) Flow cytometry analysis of apoptotic AnnexinV+ Cre+ (n = 4) or TKO (n = 6) CD8 cells activated as in a. Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test a-c); ns = not significant, *p < 0.05. The data are representative of 2 independent experiments (c) or pooled from 2-3 independent experiments (a, b).

To functionally validate the increased UPR signalling in CPEB4-depleted cells, we measured markers of ER stress. As expected, eIF2α phosphorylation was increased in *ex vivo* activated CPEB4 KO CD8 (Figure 28a), together with an upregulation of *Atf4*, *Ddit3* (CHOP), and *Xbp1s* mRNA levels (Figure 28b). This upregulation of ER stress markers was concomitant with an increase in cell death (Figure 28c), suggesting that CPEB4 depletion caused a maladaptive terminal UPR upon activation.

7. CPEB4 regulates the expression of mRNAs required for adaptation to ER stress

Since our transcriptomic changes could originate from indirect mechanisms subsequent to CPEB4 depletion, we sought to determine whether CPEB4 was directly regulating adaptation to functional ER stress in T cells. Given that CPEB4 is an RBP that regulates its target mRNAs post-transcriptionally, we performed CPEB4-RNA immunoprecipitation and sequencing (RIP-seq) in activated CD8 cells to identify CPEB4-bound mRNAs, using CPEB4-KO cells as a background corrector. Western blot analysis of input and immunoprecipitated fractions showed a specific immunoprecipitation of CPEB4 only in wild-type conditions (Figure 29a). Sequencing of CPEB4 co-precipitated mRNAs identified 241 specific targets (fold change > 1.5, pval < 0.05, Table 2). RIP targets were enriched in genes that contained CPE regulatory motifs in their 3' UTR compared to the whole transcriptome (Figure 29b), indicating the specificity of the IP.

Pathway analysis of CPEB4 targets showed enrichment of anabolic (MYC, mTORC1), stress (UPR) and inflammation (NF-kB) signalling pathways (Figure 29c). Notably, the top enriched pathways among targets were the same top gene sets upregulated in our transcriptomic data (UPR, mTORC1 and MYC, Figure 27), suggesting that CPEB4 directly controls stress responses to ER stress and anabolic pathways in KO cells. Comparison of upregulated or downregulated mRNAs in KO CD8s and CPEB4 targets revealed that only 2 CPEB4 binders presented altered mRNA levels in KO CD8 cells (Figure

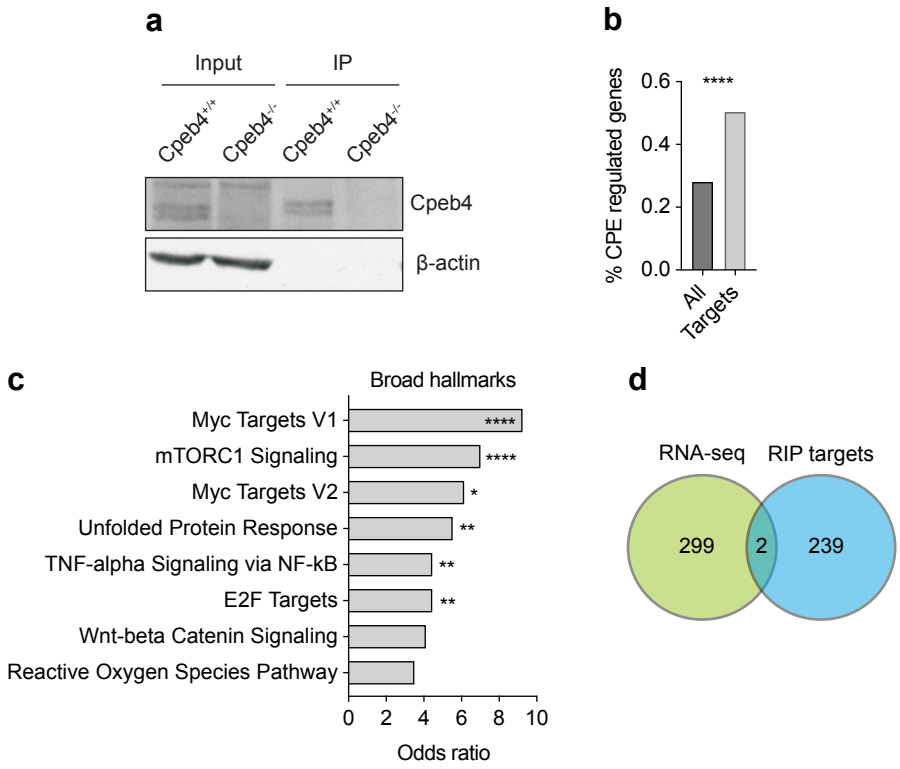

Figure 29. CPEB4-bound mRNAs are involved in the unfolded protein response and anabolic pathways. a) CPEB4 levels in input and immunoprecipitated fractions using anti-CPEB4 antibody of Cpeb4$^{+/+}$ and Cpeb4$^{-/-}$ CD8 cells activated *ex vivo* with CD3/CD28/IL-2 for 48h; β-actin is used as control (n = 3). b) Comparison of percentage of genes containing CPE element in their 3'UTR in the whole mouse transcriptome versus RIP targets. c) Pathway enrichment analysis in CPEB4 RIP targets. Top pathways enriched from the Molecular Signatures Database Hallmarks collection databased are shown, ordered by odds ratio. Statistical analysis was performed by Fisher's exact test (b).

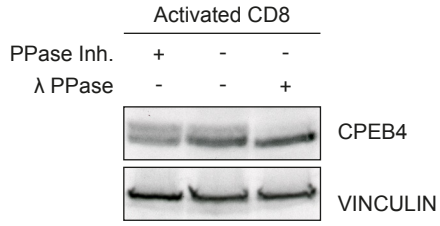

Figure 30. CPEB4 is phosphorylated in *ex vivo* activated CD8 cells. Determination of CPEB4 phosphorylation status in CD8 cells activated ex vivo with CD3/CD28/IL-2 for 48h by lambda phosphatase assay; VINCULIN is used as loading control

29d), suggesting that CPEB4 do not regulate mRNA levels or stability of its targets in this context. In fact, phosphatase assay indicated that CPEB4 is phosphorylated in CD8 (Figure 30), which has been previously described to promote CPEB4 function as a translational activator (Guillén-Boixet et al., 2016).

Among the 241 defined targets, CPEB4-bound mRNAs specifically included genes required to suppress anabolism in response to stress (*Ddit4*), heat-shock proteins (*Hspe1, Hspd1, Hsp90ab1, Hspa9*), ubiquitination and protein degradation machinery (*Herpud2, Psma7, Psma4, Vcp, Vimp*), ER translation (*Tap1, Ssr2, Ssr3, Erap1, Spcs2*) and ER protein folding (*Ppib, Mogs, Ostc, Pdia4, Rpn1, Erp44, Ero1l, Hyou1*) (Figure 31 and Table 2), further indicating the direct involvement of CPEB4 in ER stress responses. For DDIT4 and HERPUD2, we validated that their protein levels were reduced in the absence of CPEB4, without parallel changes in their respective mRNAs (Figure 32a-d). DDIT4 is negative regulator of mTORC1 signalling in response to cellular stresses such as hypoxia, heat shock or ER stress (Brugarolas et al., 2004; Wang et al., 2003; Whitney et al., 2009). HERPUD2 is involved in endoplasmic reticulum associated protein degradation (Huang et al., 2014). These examples suggest that CPEB4 coordinately increases ER capacity and diminishes anabolic pathways to allow adaptation to stress.

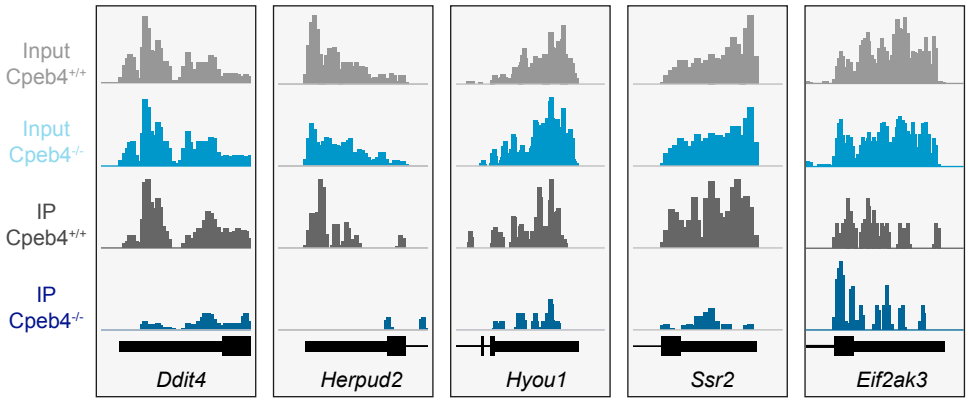

Figure 31. CPEB4 binds mRNAs related to adaptation to UPR, but does not regulate directly UPR sensors. 3'UTR RIP-seq reads depicting normalized RIP-seq coverage for inputs (light grey and blue) and IP (dark grey and blue) from Cpeb4$^{+/+}$ and Cpeb4$^{-/-}$ CD8. Image obtained using the integrated genome viewer (IGV).

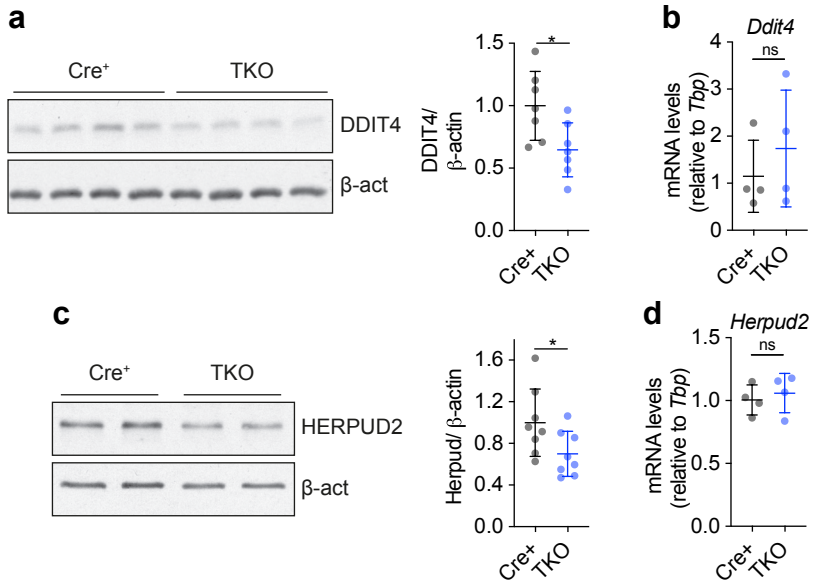

Figure 32. CPEB4 regulates DDIT4 and HERPUD2 protein levels. a) Western blot (left panel) and quantification (right panel) of DDIT4 protein levels in Cre+ and TKO CD8 cells activated *ex vivo* with CD3/CD28/IL-2 for 48h; β-actin is used as loading control (n = 7). b) qPCR of *Ddit4* mRNA levels in Cre+ and TKO CD8 cells activated as in a (n = 4). c) Western blot (left panel) and quantification (right panel) of HERPUD2 protein levels in Cre+ and TKO CD8 cells activated as in a; β-actin is used as loading control (n = 8). d) qPCR of *Herpud2* mRNA levels in Cre+ and TKO CD8 cells activated as in a (n = 4). Data are represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test; n.s. = not significant, *p < 0.05. Data are representative of 2 independent experiments (b, d) or pooled from 2-4 independent experiments (a, c).

It should be noted that neither mRNAs encoding the main UPR sensors (PERK, IRE1α, ATF6) nor mediators of the acute (ATF4, XBP1) or terminal (CHOP) responses were identified in the RIP (Figure 31 and Table 2). We also ruled out a direct regulation of CPEB4 on effector response mediators, since mRNAs encoding for effector molecules (IFNγ, TNFα, GZMB) or Teff transcription factors (T-bet, Eomes) were not bound by CPEB4 (Figure 33 and Table 2). Collectively, these results indicate that CPEB4 directly regulates adaptation to activation-induced ER stress, presumably by acting as a translational activator of genes required to suppress anabolism and augment ER translation and folding capacity. In contrast, CPEB4 does not regulate the expression of ER stress sensors and early mediators, nor the levels of effector molecules or transcription factors required to stablish the effector phenotype.

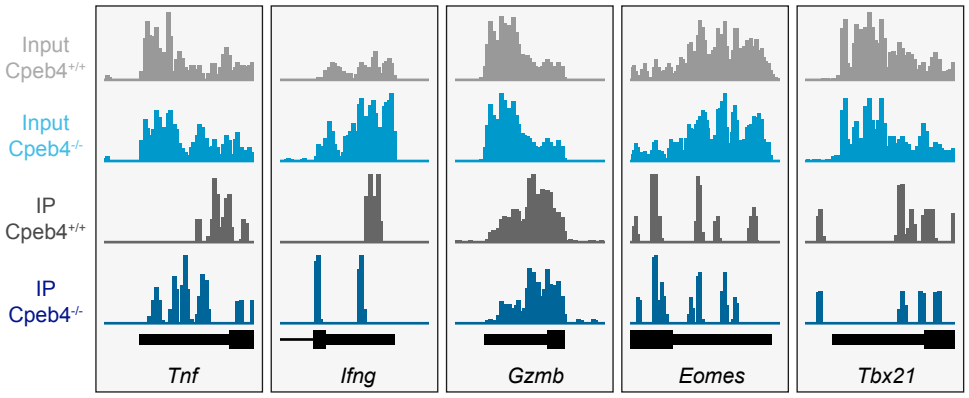

Figure 33. CPEB4 does not bind mRNAs related to CD8 effector function. 3'UTR RIP-seq reads of effector T cell genes depicting normalized RIP-seq coverage for inputs (light grey and blue) and IP (dark grey and blue) from Cpeb4$^{+/+}$ and Cpeb4$^{-/-}$ CD8. Image obtained using the integrated genome viewer (IGV).

8. Maladaptation to ER stress causes effector function defects in CPEB4-depleted lymphocytes

Our results so far placed CPEB4 as a downstream effector UPR singling required for T cell adaptation to activation-induced ER stress. Therefore, in the absence of CPEB4, CD8 cells present a maladaptation to exacerbated ER stress concomitant with a decrease in effector function. To mechanistically link the maladaptation to ER stress with the defects in effector phenotypes, we activated control or CPEB4-TKO CD8 cells in the presence or absence of tauroursodeoxycholic acid (TUDCA, T), a chemical chaperone that attenuates ER stress (Keestra-Gounder et al., 2016), and measured metabolic fitness and IFNγ production (Figure 34a). In order to allow UPR induction and CPEB4 upregulation in wild type cells, we added TUDCA only for the last 24 hours of stimulation. Strikingly, TUDCA treatment rescued metabolic fitness of CPEB4-TKO cells, as mitochondrial respiration (Figure 34b, c) and glycolysis (Figure 34d) were restored to control levels. Similarly, IFNγ production in CPEB4 depleted cells was partially rescued by TUDCA (Figure 34e). Altogether, these results confirm that maladaptation to ER stress in the absence of CPEB4 causes effector T cell dysfunction.

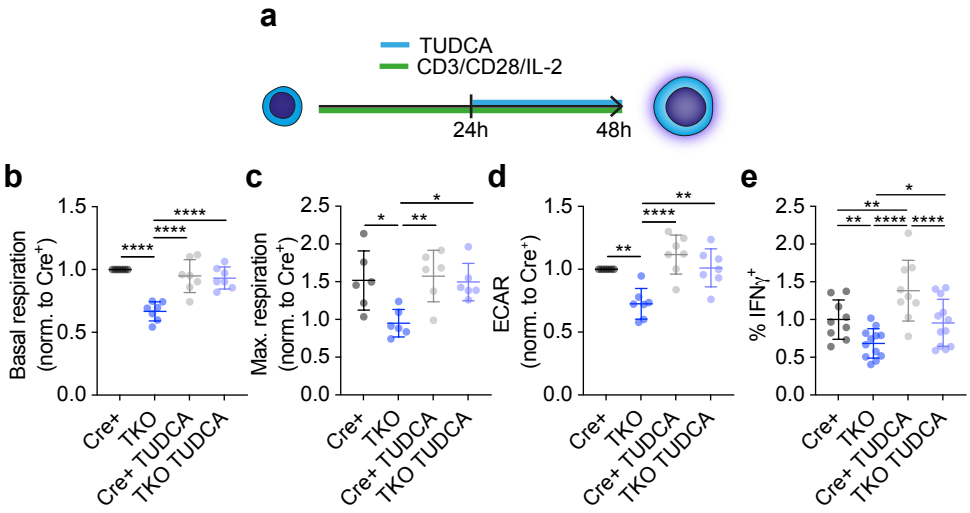

Figure 34. Alleviation of ER stress rescues effector phenotype defects in CPEB4 depleted cells. a) Schematic representation of experimental design. b, c) Basal oxygen consumption rate (b) and maximal oxygen consumption rate (c) after mitochondrial stress test analysis. Cre+ and TKO CD8 cells were activated *ex vivo* with CD3/CD28/IL-2 for 48h, and were treated with either Tauroursodeoxycholic Acid (TUDCA) 250 μM or vehicle for the last 24h. Values are normalized to basal Cre+ measurements. n = 7 for all conditions in basal respiration; n = 6 for all conditions in maximal respiration. d) Extracellular acidification rate (ECAR) in basal conditions for Cre+ and TKO CD8 cells activated and treated *ex vivo* as in b (n = 7). Values are normalized to basal Cre+ measurements. e) Quantification of IFNγ$^+$ CD8 cells activated and treated as in b (n = 9 for Cre+ and n = 12 for TKO in both conditions). Statistical analysis was performed by one-way ANOVA with Tukey correction (b-e). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, **** p <0.0001. The data are pooled from 3-4 independent experiments (b-e).

9. Activation-induced ER stress mediates CPEB4 translational upregulation in CD8 cells

Previous results from our laboratory showed that CPEB4 is translationally upregulated by the UPR due to the bypass of inhibitory uORFs present in its 5' UTR (Maillo et al., 2017). Therefore, knowing that CPEB4 was primarily mediating adaptation to ER stress, we asked whether the apparent discrepancy between CPEB4 protein upregulation and mRNA downregulation during T cell activation (Figure 22) was a consequence of UPR signaling upon T cell stimulation. In order to test this hypothesis, we activated CD8 cells in the presence of TUDCA and measured CPEB4 protein levels (Figure 35a).

As expected, CPEB4 protein was upregulated in activated, vehicle-treated T cells, but this upregulation was significantly decreased in TUDCA-treated cells (Figure 35b), without differences in *Cpeb4* mRNA levels (Figure 35c). In addition, no differences in general T cell activation using CD25 and CD69 induction as a proxy were observed between vehicle- and TUDCA-treated cells (Figure 35d), indicating that the lack of proper CPEB4 upregulation was not due to a general problem in T cell activation. Overall, these results suggest that CPEB4 protein levels in activated CD8 cells are partially controlled by the UPR, presumably through a translational bypass of its inhibitory uORFs (Maillo et al., 2017). This indicates that CPEB4 is an integral part of the UPR in

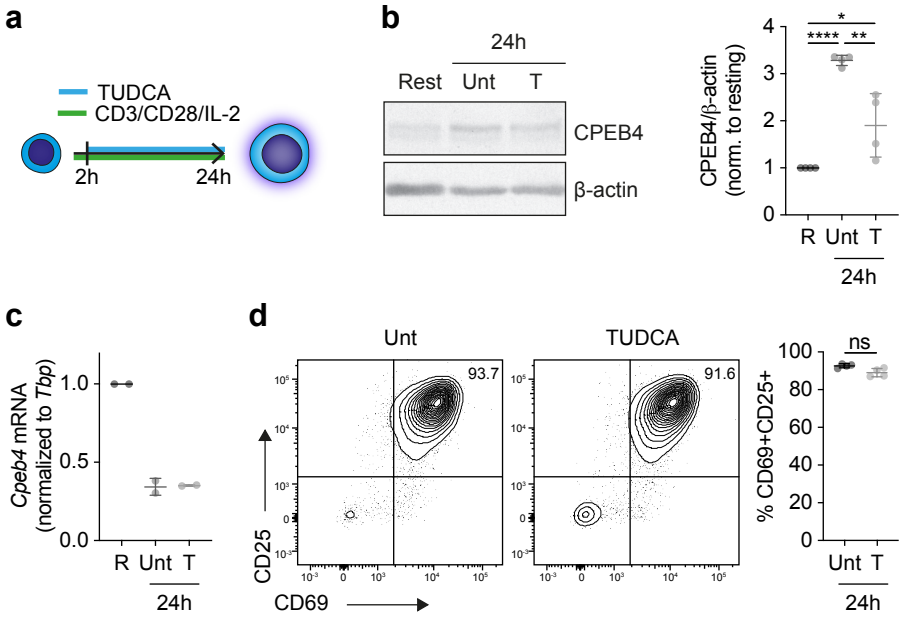

Figure 35. CPEB4 protein levels are translationally upregulated by the UPR. a) Schematic representation of experimental design. b) Western blot (left panel) and quantification (right panel) of CPEB4 protein levels in CD8 resting or activated with CD3/CD28/IL-2 for 24h in the presence or absence of Tauroursodeoxycholic Acid (TUDCA, T) 250 μM (n = 4) from 2 hours after plating. c) qPCR analysis of *Cpeb4* mRNA levels in CD8 resting or activated as in b (n = 2). d) Representative flow cytometry plots (left panel) and quantification (right panel) of $CD25^{+}CD69^{+}$ CD8 cells activated as in b (n = 4). Data are represented as mean ± s.d. Statistical analysis was performed by one-way ANOVA with Tukey correction (b) or Mann-Whitney test (d); n.s. = not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p <0.0001. The data are pooled from 2-3 independent experiments (b, d) or representative of one experiment (c).

T cells and that it is subsequently upregulated upon UPR induction to mediate cellular adaptation to activation-induced ER stress.

Discussion

In this work, we have unveiled a previously unappreciated role for CPEB4 in lymphocytes (Figure 36). CPEB4 is a downstream effector of UPR signaling in CD8 cells, as it is translationally regulated by activation-induced ER stress. It is required to promote adaptation to that stress and mitigate the potential damage of an exacerbated UPR. Mechanistically, CPEB4 regulates the translation of mRNAs encoding for stress proteins, ER translation machinery and protein folding and degradation machinery. Therefore, in the absence of CPEB4, uncontrolled ER stress leads to maladaptive, terminal UPR signaling that thwarts CD8 cell effector function, impairing cytokine production, metabolic fitness, survival and, ultimately, cytotoxic capacity. *In vivo*, this is translated into defective anti-tumor responses and increased tumor growth, placing CPEB4 as a critical mediator of effector T cell fitness that could be therapeutically harnessed to improve anti-tumor immunity. CPEB4-mediated adaptation to functional ER stress is thus required to allow proper maintenance of CD8 T cell effector phenotype.

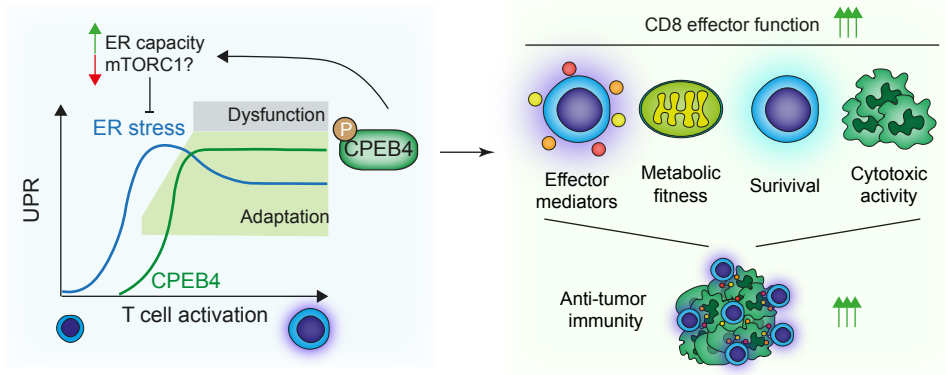

Figure 36. CPEB4-mediated adaptation to activation-induced ER stress promotes CD8 anti-tumor effector function. CPEB4, as a downstream effector of the UPR, is upregulated by activation-induced ER stress. In turn, CPEB4 mediates the expression of mRNAs that increase ER capacity and potentially diminish mTORC1 signaling, promoting functional adaptation to stress by keeping it within functional levels. This is required for CD8 effector function, particularly effector molecule production, metabolic fitness, survival and cytotoxic activity. *In vivo*, this results in effective anti-tumor responses.

In the following section, we will integrate the results presented in this thesis into the current literature, and discuss the potential implications for the fields of CPEB translational control, ER stress, T cell biology and tumor biology. In addition, we will highlight, when applicable, the technical or conceptual limitations of our work, and the future directions to either strengthen or expand our findings.

1. CPEB4 mediates adaptation to chronic, functional ER stress in CD8 T cells

Our previous work established CPEB4 as a novel mediator of late UPR singling (Maillo et al., 2017). CPEB4 was shown to be translationally upregulated in response to chemically-induced ER stress by PERK-dependent phosphorylation of eIF2α and bypass of its 5'UTR inhibitory uORFs. In turn, CPEB4 promoted the expression of CPE-containing mRNAs encoding for ER machinery required for ER stress adaptation and resolution, contributing *in vivo* to rectify high-fat diet-induced liver ER stress. Here, we have expanded CPEB4-mediated function during ER stress into a different scenario in which cells, in this case CD8 T cells, suffer a physiological, chronic ER stress to which they have to adapt in order to be functional.

In response to acute ER stress, UPR signaling is primarily devoted to adapt and resolve (if possible) the stress in order to return to homeostasis. In contrast, activation-induced ER stress in T lymphocytes is physiologically and inherently coupled to T cell activation and activity, and therefore it cannot be fully resolved, as it is derived from the normal function of the cell. The increase in protein synthesis required to support T cell size growth, proliferation and secretion in response to TCR signaling concomitantly generates ER stress that, in fact, is required for differentiation into effector cells (Kamimura and Bevan, 2008; Pino et al., 2008; Takano et al., 2008; Thaxton et al., 2017). This is not unique to T lymphocytes, as specialized secretory cells that present an intense ER protein production (like plasma cells or pancreatic beta cells) have a constant, adaptive UPR signaling to maintain ER homeostasis (Reimold et

al., 2001; Scheuner et al., 2001; Sharma et al., 2021; Tellier et al., 2016).

This illustrates that in physiological conditions in which the ER is submitted to an intense pressure, unavoidable chronic ER stress occurs and cells, rather than aiming to fully resolve it, try to adapt by maintaining ER stress within manageable levels that do not compromise functionality. The results presented in this work suggest that CPEB4 is central for this process. Since T cells suffer an inherent, chronic ER stress derived from their activation and effector function, CPEB4-mediated adaptive response increases ER capacity to keep stress levels within limits compatible with cytotoxic activity (Figure 36).

2. Adaptive vs terminal UPR in chronically stressed T cells

Maintaining a manageable level of ER stress is a complicated task, as perturbations in the intensity, duration or type of stress can switch the UPR into its terminal response. When T cells have to perform their function for an extended time (as in conditions of chronic antigen exposure such as cancer) or in suboptimal situations that trigger a higher ER stress (such as those observed in the TME) terminal UPR signaling can be activated, leading to cellular dysfunction (Cao et al., 2019; Ma et al., 2019; Song et al., 2018). Whereas at first these opposite contributions of UPR signaling might seem contradictory, it reflects the dual nature of UPR signaling pathways, as mediators such as ATF4 and XBP1s are responsible of activating both adaptive and terminal responses. In T cells, IRE1α/XBP1s signaling is necessary for cytotoxic T cell differentiation during acute viral infections (Kamimura and Bevan, 2008), but, in the context of cancer, it causes CD4 cell mitochondrial dysfunction in ovarian carcinoma and cholesterol-driven CD8 exhaustion in melanoma (Ma et al., 2019; Song et al., 2018). In these cases, the beneficial effects of XBP1s signaling in an acute infection setting are overridden in a chronic situation (Figure 37).

As for the PERK-dependent translational branch of the UPR, where CPEB4 plays a role, the situation is more nuanced depending on the member of the pathway. The classical view of the PERK/eIF2α/ATF4 axis stablishes that

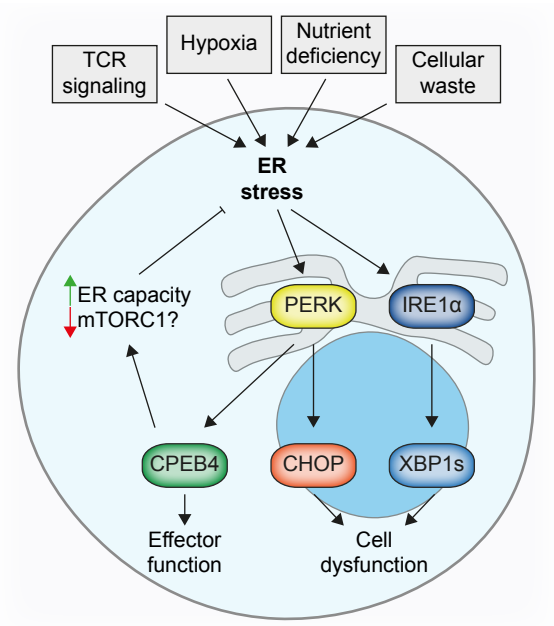

Figure 37. Adaptive vs terminal UPR in chronically stressed T cells. TCR signaling induces ER stress that activates PERK and IRE1α. In the TME, this is amplified by hypoxia, lack of nutrients or accumulation of cellular waste. This situation activates CHOP and XBP1s-dependent terminal UPR, causing T cell dysfunction. However, parallel upregulation of CPEB4 by PERK activates CPEB4-mediated adaptive response. CPEB4 in turn increases ER capacity and potentially inhibits mTORC1 signaling to reduce ER stress, allowing proper effector function.

if the initial ATF4 signaling does not resolve ER stress, it will activate the expression of the maladaptive, terminal UPR effector CHOP. In line with this view, recent publications have shown that PERK-dependent upregulation of CHOP diminished T cell effector function, mainly by repressing the expression of the master effector transcription factor T-bet (Cao et al., 2019). Therefore, CHOP transcriptionally blocks the effector fate, decreasing proliferation, effector molecule production and metabolic fitness (Figure 37).

Nevertheless, the results obtained in this work indicate that CPEB4 does not regulate directly effector fate or apoptosis. In addition, we have not identified targets of a terminal, maladaptive UPR in the present or previous work: CPEB4 seems to just regulate adaptive UPR signaling and not the terminal response. Although preliminary, as we cannot rule out technical problems due to insufficient resolution of our RIPs, these results prompt us to hypothesize

that CPEB4 differs from other UPR mediators such as ATF4 or XBP1s in its inability to switch from adaptive to terminal UPR. Whereas ATF4 or XBP1s present this dual role, CPEB4 seems to be an "adaptive-only" effector of the UPR, where its action does not switch into terminal signaling, potentially even in a setting of high, chronic ER stress such as the TME (Figure 37).

Still, UPR-mediated activation of CPEB4 does not occur isolated, but happens in the context of PERK signaling, where the other axes are also induced in parallel. Intriguingly, blocking PERK improves T cell effector function and anti-tumor immunity (Cao et al., 2019; Hurst et al., 2019), suggesting that in TILs the terminal PERK/eIF2α/ATF4/CHOP axis wins over the adaptive PERK/eIF2α/CPEB4 branch. A missing piece of the puzzle, general to ER stress research, is therefore what is the tipping point between adaptation vs terminal UPR. It is possible that if chronic stress is too high, CPEB4 adaptive function is not enough to prevent dysfunction. Since our data suggest that CPEB4 does not directly block terminal UPR, it is plausible to think that CPEB4 needs to interact with other adaptive mediators to shut down terminal UPR. In this line, recent reports have shown that m6A writers block CHOP signaling in chronic stress situations (Wei et al., 2021), suggesting that indeed a direct inhibition of terminal UPR might be needed in parallel to stress adaptation. Although is still unknown whether this also occurs in T cells, all the available observations indicate that during chronic stress multiple mechanisms should be deployed to allow cell function.

Adaptation to harsh conditions is key for T cell function. Besides ER stress, T cells have developed tools to functionally adapt to unfavorable situations such as high ROS (Yue et al., 2021), amino acid limitation (van de Velde et al., 2016) or fatty acid accumulation (Zhang et al., 2017) that can be therapeutically harnessed. We believe that CPEB4 is an integral part of T cell's adaptation toolkit and that its function becomes even more important in settings where multiple stresses converge, as happens in the TME. It has been hypothesized that blocking UPR signaling can boost immune responses against tumors, as it will prevent the detrimental effects of terminal UPR in effector T cells. However, inhibiting UPR pathways at the sensor level will also impair adaptive

responses, which, as we have seen in this work, are beneficial for T cell function. Therefore, finding mechanisms that only block terminal UPR mediators, while maintaining the adaptive UPR, or boost adaptive UPR to outcompete detrimental signaling could be of even greater therapeutic interest. Whether CPEB4 could be one of these targeted mechanisms remain to be explored.

3. CPEB4 as an anabolic rheostat: crosstalk with MYC, mTORC1 and ISR during chronic stress.

Besides UPR, two other major pathways appear to be directly regulated by CPEB4 in activated CD8: MYC, and mTORC1. These two anabolic pathways are critical for T cell activation and differentiation, and several lines of evidence suggest that CPEB4 communicates with them.

Myc, as a highly regulated mRNA, presents several CPEs in its 3'UTR and is translationally inhibited by CPEB1 in MEFs (Groisman et al., 2006). However, we have not identified MYC as a target of CPEB4 in CD8 cells, and in fact its protein levels are not altered in CPEB4-depleted cells (Figure 38). Even if CPEB4 does not regulate MYC protein itself, it might contribute to amplify the expression of a subset of MYC target genes. However, the fact that CPEB4 depletion does not affect proliferation in this context, suggests that if CPEB4 could modulate MYC signaling, it would not be through its action on cell cycle, as previously suggested (Groisman et al., 2006). This could be a novel setting to study CPEB4-MYC coregulation of other cellular functions.

In the case of mTORC1, our results show that CPEB4 translationally activates DDIT4 (an ER stress-induced negative regulator of mTORC1 (Whitney

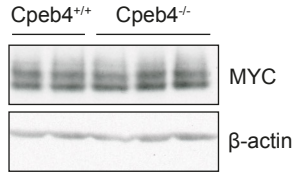

Figure 38. MYC protein levels do not change in CPEB4-depleted, activated CD8. Western blot of MYC protein levels in WT or CPEB4-KO CD8 activated with CD3/CD28/IL-2 for 48h. β-actin is used as a loading control.

et al., 2009)) and that, at least at transcriptomic level, CPEB4 depletion upregulates mTORC1 signaling. Thus, it can be hypothesized that as part of the adaptation process to chronic ER stress, CPEB4 upregulates DDIT4 to diminish mTORC1-depedent translation and reduce protein input into the ER (Figure 37). Indeed, it is known that inhibition of mTORC1 is a parallel mechanism to eIF2α phosphorylation that cells activate to decrease translation during ER stress (Appenzeller-Herzog and Hall, 2012; Ozcan et al., 2008). Nonetheless, this initial protein synthesis shut down is rapidly reverted in a negative feedback loop by ATF4 and CHOP. ATF4 upregulates amino acid transporters, increasing amino acid uptake and re-activating mTORC1 (Torrence et al., 2021). Similarly, ATF4 and CHOP transcriptionally upregulate phosphatases that de-phosphorylate eIF2α, which also re-activate protein translation (Han et al., 2013). While this feedback is of great importance during acute ER stress to return to homeostasis, if this increase in protein synthesis occurs when ER stress is still high, it causes cell death, showing that ATF4- dependent transcriptional activation of translation is detrimental for the cell (Han et al., 2013).

In our view, CPEB4 action on mTORC1 might provide cells with a complementary temporal mechanism to maintain protein synthesis low once ATF4 has re-ignited protein synthesis. In this model, if ER stress is not yet resolved, CPEB4 might still restrain mTOCR1 levels via DDIT4 (or other uncharacterized mechanisms) to prevent proteotoxic cell death. This mechanism can be specifically relevant for adaptation to chronic ER stress, where cells have to balance normal protein synthesis for their function while undergoing ER stress. Whether our phenotypes in CPEB4-null cells are also driven by mTORC1 hyperactivation in a context of high ER stress is still unknown. However, reports have shown that hyperactivation of mTORC1 by TSC1 abrogation resulted in T cell death and impaired antibacterial immune responses, although the mechanism was not characterized (Yang et al., 2011). Despite more work is required to actually prove CPEB4-mediated regulation of mTORC1, exploring this mechanism could allow us to find novel ways of understanding cellular adaptation to chronic ER stress and preventing

UPR-induced cell dysfunction and death.

The fact that CPEB4 is regulated by eIF2α makes it a potential downstream effector not only of UPR itself but of the whole ISR (Pakos-Zebrucka et al., 2016). Since, as explained at the introduction, eIF2α can be phosphorylated by four different kinases, heme deficiency, amino acid starvation and viral infection could, on top of ER stress, induce CPEB4 upregulation. However, CPEB4 has so far never been linked to any of these processes, although CPEB1 (which is not regulated by eIF2α) has been shown to be required for host and viral mRNA translation during viral infections (Batra et al., 2016).

Hence, it is plausible, for instance, to hypothesize that CPEB4 is induced in response to amino acid starvation through GCN2/eIF2α and mediates mTORC1 inhibition. In line with the model proposed for ER stress, CPEB4 would also adjust here the levels of mTORC1 signaling when amino acids are scarce. In this collective view, CPEB4 could generally act as an "anabolic rheostat" that fine-tunes anabolic signaling pathways in response to chronic stresses, preventing potentially toxic signaling without compromising cellular

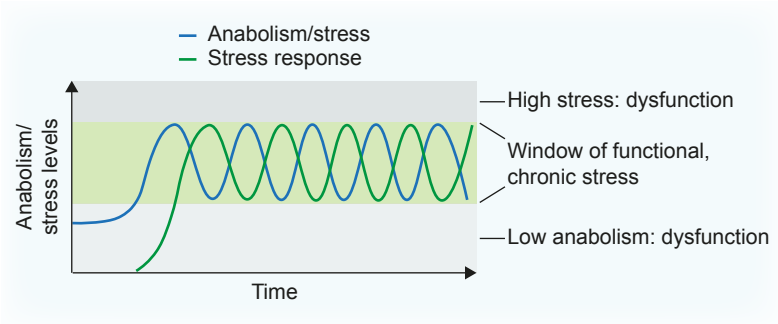

Figure 39. Hypothetical model for molecular oscillator-driven adaptation to anabolism-induced chronic ER stress. In contexts where stress (such as ER stress) is derived from normal cellular anabolic activity, cells need to adapt to that stress but cannot afford to shut down anabolism as it would compromise their activity. Therefore, a potential solution is to stablish stress responses that generate molecular oscillators that alternate stress high/low and anabolic high/low situations within a window compatible with cellular activity. As anabolic activity accumulates and generates stress, stress responses will ignite adaptation programs that will reduce anabolism. This reduces the stress, and concomitantly diminishes the stress response, resulting in a re-upregulation of anabolic pathways that will re-start the cycle again.

function. Adaptation to chronic stress could therefore be accomplished by oscillatory increases/decreases in stress/anabolism that maintain a window of sufficient anabolic signaling for the cell to be functional (for instance mTORC1-dirven protein production in T cells) while preventing the detrimental effects of that signaling (following the same example, accumulation of misfolded proteins in the ER) (Figure 39). Whether this mechanism occurs during CPEB4-driven adaptation to ER stress, and whether this is a general common nexus of the ISR and anabolism, are key questions yet to be answered.

4. CPEB4 as a translational activator during ER stress

As we have seen, CPEB4 is translationally regulated by activation-induced ER stress (Figure 35). Although, as explained above, CPEB4 was shown to increase upon chemically-induced ER stress, this is, to our knowledge, the first evidence of CPEB4 expression regulation by physiologically-induced UPR. Our experimental approach involved the use of the molecular chaperone TUDCA, which broadly decreases ER stress levels. However, this setting did not allow us to define the specific UPR pathway mediating CPEB4 upregulation in our context. Even though CPEB4 is clearly upregulated by PERK in MEFs (Maillo et al., 2017), it would be interesting to check by chemical or genetic ablation of PERK signaling whether this pathway also controls CPEB4 in activated lymphocytes. However, it is worth noting that TUDCA only partially prevented CPEB4 protein upregulation. Given that TCR signaling massively upregulates protein synthesis (Araki et al., 2017; Howden et al., 2019; de Ponte Conti et al., 2021), *Cpeb4* mRNA may also benefit from this general translation upregulation.

Nevertheless, it is known that CPEB4 binds and promotes translation of its own mRNA (Igea et al., 2010). Our analysis of CPEB4 phosphorylation status and expression of selected targets suggest that CPEB4 works as a translational activator in activated CD8 cells, as it has been widely shown in the literature (Calderone et al., 2016; Igea et al., 2010; Maillo et al., 2017; Novoa et al., 2010). This implies that CPEB4 induces poly(A) tail elongation of its target mRNAs

(potentially including itself) to increase their translation efficiency. Our model therefore suggests that poly(A) elongation is required during T cell activation to increase translation efficiency. In the translation-heavy context of T cell activation, transcripts might compete for translation machinery. On top of this, ER-stress dependent phosphorylation of eIF2α diminishes the pool of ternary complexes, aggravating the translational competition between mRNAs. In this situation, having a longer poly(A) tail can boost ribosomal recruitment and subsequently increase translation efficiency

Therefore, it is possible that once CPEB4 protein starts to be accumulated, it generates a positive feedback loop that further increases its own (and its targets') protein levels. In fact, when we treat CD8 cells with TUDCA for the last 24h of a 48h activation period, CPEB4 levels remain unchanged (Figure 40). However, if we perform the same treatment regime with cordycepin, an inhibitor of polyadenylation (Kondrashov et al., 2012), CPEB4 protein levels drop (Figure 40). Although these observations cannot be fully ascribed to CPEB4 itself, they have several implications. First, it shows that ER stress only promotes CPEB4 translation at early time points, when CPEB4 levels are very low, suggesting that once CPEB4 is upregulated its translation become uncoupled from ER stress. Second, that cytoplasmic polyadenylation might sustain CPEB4 (and other target mRNAs) protein levels after its initial UPR-driven induction. This preliminary observation goes in line with previous results from our lab that showed that CPE-containing transcripts increase their translation at later timepoints during the UPR compared to uORF-containing

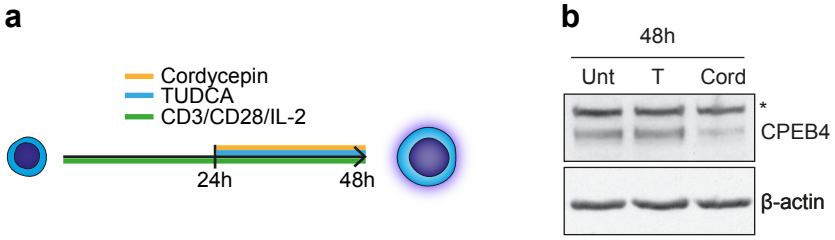

Figure 40. Polyadenylation increases CPEB4 protein levels after UPR-mediated upregulation. a) Schematic representation of experimental design. b) Western blot of CPEB4 protein levels in CD8 activated in the presence or absence of Tauroursodeoxycholic Acid (TUDCA, T) 250 μM or Cordycepin (Cord) 50 μM during the last 24h of activation (n = 1). β-actin is used as a loading control.

transcripts (Maillo et al., 2017). Whether, in the case of T cells, this regulatory switch occurs by cellular adaptation to ER stress and de-phosphorylation of eIF2α, or by a stronger positive effect on translation by poly(A) tail length than the inhibitory uORFs remains to be studied.

It should be noted that our data supporting CPEB4 positive regulation of its targets is only based on few selected candidates. To fully define CPEB4 effect on mRNA translation and final protein output, it would be of outmost interest to perform ribosome profiling in WT and CPEB4-depleted CD8. In addition, a transcriptome-wide analysis of mRNA poly(A) tail length in those conditions will provide us with a direct measurement of CPEB4 polyadenylation activity on transcripts. Integrating these 2 datasets with our RIP will allow us to fully determine CPEB4 contribution to gene expression in CD8 cells.

5. Integrating CPEB4 circuits with other RBPs in T cells

In the present work we have studied CPEB4 regulatory function isolated from other proteins. However, as explained in the introduction, RBPs, including CPEB4, do not generally work alone on its target mRNAs, but are part of a complex network of multiple *trans* acting factors that simultaneously influence mRNA fate. Therefore, although our work has laid the foundations of CPEB4 function in T cells, future experiments might be directed towards understanding the interplay between CPEB4 and other RBPs.

CPEB4 has been shown to interact and/or compete with CPEB1-3 and thus its action might be conditioned by the presence of the other members of the family. However, gene expression analysis has shown that *Cpeb1* mRNA is not detected in T cells, and *Cpeb2/3* are heavily downregulated upon T cell activation (Figure 41). We acknowledge that this behavior is, at least at 24h post-activation, similar to CPEB4, but given that CPEB2/3 are not regulated by ER stress (Maillo et al., 2017), and we failed to detect their protein expression with validated antibodies (data not shown), we think that CPEB2/3 expression is very low or negligible in this context. Therefore, we assume that most if not all of CPEB-mediated regulation in CD8 cells is performed by CPEB4.

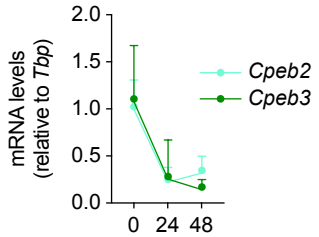

Figure 41. ***Cpeb2*** **and** ***Cpeb3*** **mRNAs are downregulated in activated CD8 cells.** qPCR of *Cpeb2* and *Cpeb3* mRNA in CD8 resting or activated *ex vivo* with CD3/CD28/IL-2 at the indicated time-points; *Tbp* is used as endogenous control (n = 5).

Another important RBP that crosstalks with CPEB4 is the ARE-binding protein TTP (Belloc and Méndez, 2008; Suñer et al., 2021). These two proteins exert opposite functions in mRNAs, as TTP induces poly(A) tail trimming while CPEB4 (when phosphorylated) promotes their elongation. These inverse functions have led to the proposition of an ARE/CPE score for mRNAs, which states that CPEB/CPE action of a mRNA is influenced by the presence of ARE elements in the 3'UTR and the expression of TTP (Suñer et al., 2021). On top of it, TTP destabilizes *Cpeb4* mRNA and has been shown to be active shortly after T cell activation (Moore et al., 2018), potentially explaining *Cpeb4* mRNA downregulation 24h after activation. Given that TTP negatively regulates T cell activation, albeit in a mechanism independent of ER stress, it would be interesting to check whether ARE/CPE score influences CPEB4 targets, or if CPEB4 regulation of CD8 effector function is independent of its crosstalk with TTP.

6. CPEB4 beyond ER stress and CD8 effector function: CD4 and memory cells

Our mechanistic work has focused mostly on CD8 cells. However, we also analyzed CPEB4 protein dynamics and *in vivo* loss-of-function phenotypes in CD4 lymphocytes. Whereas our *in vivo* data shows that CD4 Th1 effector molecule production is impaired in the absence of CPEB4 (as observed in CD8 cells), CPEB4 expression dynamics differ between CD4 and CD8 cells. In particular, CPEB4 protein upregulation in CD4 lymphocytes *ex vivo* was milder compared to CD8 cells, and *in vivo* CPEB4 highest protein levels were

found in memory cells. With our data, it is not possible to determine whether these divergences simply mirror the underlying differences between CD8 and CD4 during activation and differentiation, or if they are due to independent roles of CPEB4 in each T cell type.

Our model of CPEB4-mediated adaptation to activation-induced ER stress could also be applied to Th1 effector function, as maladaptation to ER stress also causes CD4 dysfunction (Song et al., 2018). In this framework, the intensity of ER stress could explain the differences in CPEB4 protein dynamics between CD8 and CD4 cells. Activated CD8 cells grow bigger in size and present higher protein content than CD4 (Howden et al., 2019), which can potentially generate a stronger ER stress and would suggest a stronger need for CPEB4 in CD8 cells. Nevertheless, recent data from our laboratory has characterized other functions of CPEB4 in CD4 effector cells. While our results indicate that CPEB4 does not directly regulate cytokine production in CD8, it controls IL-22 translation in CD4 Th22 cells (Sibilio et al., *accepted*). Whether this represents context-dependent mechanistic variations regarding which targets are bound and how are they influenced by CPEB4 is still an open question.

The upregulation of CPEB4 in CD4 memory cells is also intriguing. Memory cells downregulate effector programs and return to a stem-cell like state. However, they are primed for a faster response upon re-encounter with their antigen. Very elegant work has shown that pre-formed *Ifng* mRNA is stored by TTP-like proteins in order to prevent activation of memory cells in the absence of re-stimulation (Salerno et al., 2018). Given that memory cells stop proliferation and TCR signaling, shutting down CDK1 and ERK2 activity, it is fair to assume that CPEB4 will return to a translational repressor state. Therefore, CPEB4 could potentially sequester its target mRNAs into translationally-inactive granules in a similar fashion to TTP-like proteins, subsequently inducing differentiation into memory cells and/or sustaining preparedness for reactivation. In this model, adaptation to ER stress might no longer be needed and CPEB4 targets could potentially be different that those characterized in this thesis. Although heavy experimental work is required to

test this hypothesis, it could be of great interest to increase our understanding on translational regulation of stem cell-like states in adult tissues.

In fact, our general knowledge of cytoplasmic granule dynamics and function in T cell biology is very limited. Understanding broadly how P bodies, stress granules or RBPs contribute to the establishment of memory could be of tremendous value to, for instance, improve responses to secondary infections.

7. CPEB4 in T cell development

Our results show that T cell-intrinsic CPEB4 is dispensable for T cell development from DP precursors and mature T cell homeostasis. However, recent data from our laboratory showed that full body CPEB4 KO have multiple defects in T cell homeostasis such as smaller lymph nodes and intestinal

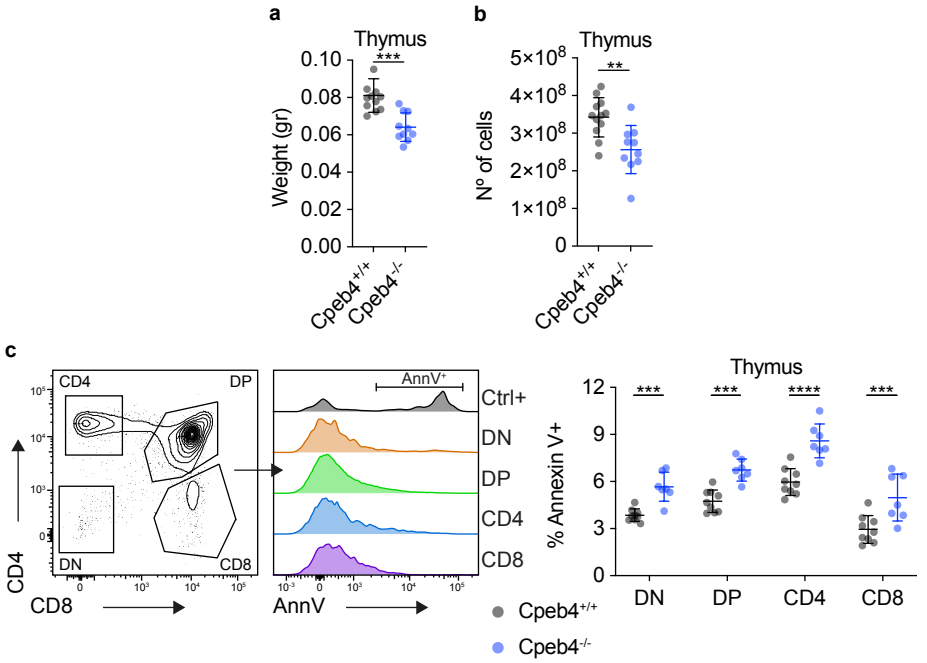

Figure 42. Cpeb4 full body KO animals present smaller thymi concomitant with increased cell death in all stages. a, b) Thymus weight (a) and cellularity (b) of Cpeb4$^{+/+}$ (n = 12) and CPEB4$^{-/-}$ (n = 10) animas. c) Gating strategy (left panel) and quantification (right panel) of Annexin V+ DN, DP, CD4 and CD8 thymocytes in Cpeb4$^{+/+}$ (n = 9) and Cpeb4$^{-/-}$ (n = 7) animals. Data represented as mean ± s.d. Statistical analysis was performed by Mann-Whitney test (a, b) or two-way ANOVA with Sidak correction (c). **p < 0.01, ***p < 0.001, **** p< 0.0001.

Peyer's patches, and decreased frequencies of T cells in blood, spleen and lymph nodes (Sibilio et al, *accepted*). Further analysis of T cell development has revealed that CPEB4 full-body KO animals present smaller thymi, with lower number of cells, concomitant with an increase in cell death in all precursor stages (Figure 42).

The lack of phenotype in mature T cell structures in CPEB4-TKO animals indicates that the problems observed in full KO animals cannot stem from cell-intrinsic defects in homeostasis. In addition, CPEB4 do not regulate thymic development from the DP stage, as CPEB4-TKO animals do not present thymic alterations. Then, which is the origin of T cell phenotypes observed in full body KO? We hypothesize that there are two main possibilities: that CPEB4 is required for much earlier, pre-DP T cell development, or that CPEB4 plays a role in other non-T cell types that contribute to T cell generation.

A preliminary analysis of *Cpeb4* mRNA expression along hematopoietic and T cell precursors from publicly available datasets (Heng et al., 2008) showed that *Cpeb4* becomes upregulated in DP thymocytes (Figure 43). However, its expression levels skyrockets when measured in thymic epithelial cells (TECs) (Figure 43). Although, as we have learnt from this work, mRNA levels do not always correlate with protein levels, this result might suggest that CPEB4 has a role in the thymic stroma rather than in T cell precursors. Among many different functions, TECs are responsible of producing IL-7, which is required

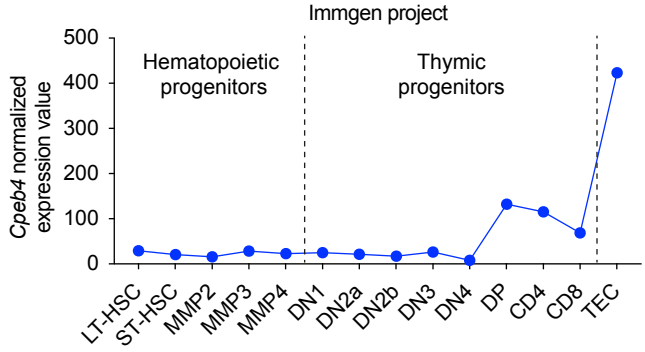

Figure 43. ***Cpeb4*** **mRNA is highly expressed in thymic epithelial cells compared to hematopoietic of thymic progenitors.** *Cpeb4* expression in hematopoietic progenitors and thymic progenitors and epithelial cells (TECs) by RNA-seq from the Immunological Genome Project (https://www.immgen.org/).

for thymocyte survival (Hong et al., 2012). Therefore, it can be speculated that CPEB4 positively regulates IL-7 production in TECs, and therefore its depletion causes the thymic phenotypes observed in CPEB4 full-body KO. In order to test this hypothesis, it would be interesting first to perform a bone marrow transplant to check if CPEB4-KO HSC can fully reconstitute the T cell lineage when the thymic stroma is WT for CPEB4. Additionally, it would be necessary to measure IL-7 levels in CPEB4-KO thymus. These analyses could shed light on how translational control contributes to intercellular communication during T cell development.

8. CPEB4 in other cell types of the TME

Our initial approach attempted to broadly assess CPEB4 function in the whole TME. Although we focused on T cells, we do not exclude possible roles for CPEB4 in other cell types of the TME that could have been missed in the models used in this work. For instance, UPR signaling has been shown to be critical for the immunomodulatory function of myeloid-derived suppressor cells (MDSCs) or the anti-tumor and anti-viral activity of NK cells (Dong et al., 2019; Mohamed et al., 2020; Wang et al., 2018). It is thus fair to hypothesize that CPEB4, as part of the UPR, also regulates MDSC and NK function.

Similarly, cancer assocaited fibroblasts (CAFs) or endothelial cells could also be influenced by CPEB4. Although in our melanoma model we did not find differences in vascularization, CPEB4 is known to regulate VEGFA expression during pathological angiogenesis in chronic liver disease (Calderone et al., 2016). Whether this function does not occur in tumors, or whether it could be tumor-type specific, remains unexplored. B16F10 cells are not a good model to study CAFs since, at least in our hands, B16 tumors are almost devoided of them. Given that CPEBs regulate the secretion of pro-inflammatory cytokines in MEFs (Groisman et al., 2006; Ivshina et al., 2015), it could be interesting to investigate the role of CPEB4 in CAFs using models where fibroblasts are known to be critical for tumor development, such as colorectal cancer (Calon et al., 2015). Beyond CPEBs, our understanding of translational control in the

TME is only starting to emerge. We hope that future research will uncover new translational mechanisms in the TME that can improve therapy and disease outcome.

In conclusion, in this thesis we have uncovered a novel mechanism of T cell adaptation to activation-induced ER stress (Figure 36). CPEB4-mediated adaptation to ER stress is required for T cell effector fitness and antitumor-immunity.

Conclusions

The present study provides new insights into CPEB4 function in T lymphocytes. We describe that CPEB4 is required for adaptation to activation-induced ER stress. As a result, CPEB4 positively regulates T cell effector phenotype, maintaining effector molecule production, metabolic fitness, survival and cytotoxic activity. Finally, we show that CPEB4 is required for efficient anti-tumor T cell responses *in vivo*.

The main conclusions of this work are the following:

1. CPEB4 regulates T cell-mediated anti-tumor immunity in vivo.

2. CPEB4 is dispensable for post-DP T cell development and homeostasis.

3. CPEB4 protein levels are post-transcriptionally upregulated in effector CD8 TILs and activated T cells.

4. CPEB4 sustains T-cell effector phenotype *in vivo* and *in vitro*, downstream of early activation or transcriptional induction or differentiation.

5. CPEB4 diminishes and promotes adaptation to activation-induced ER stress in CD8 cells.

6. CPEB4 preferentially binds and translationally upregulates UPR genes that increase ER protein translation, folding and degradation capacity together with stress genes that likely decrease anabolic pathways.

7. CPEB4 does neither bind mRNAs encoding for UPR sensors, early or terminal effectors, nor directly regulates transcript encoding for effector T cell transcription factors or mediators.

8. Effector T cell fitness and function requires CPEB4-mediated adaptation to activation-induced ER stress.

9. Activation-induced ER stress promotes CPEB4 translational upregulation.

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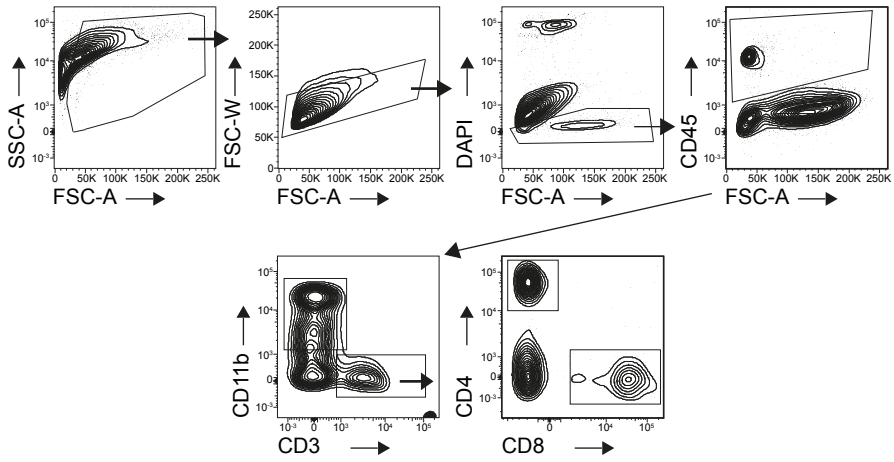
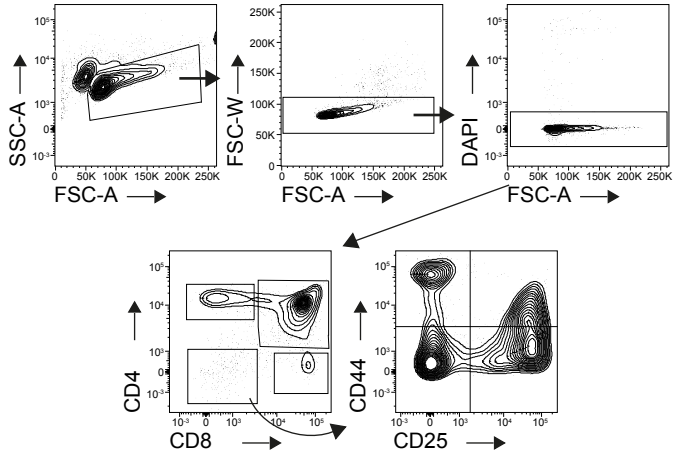
Appendix

Appendix 1. Gating Strategies

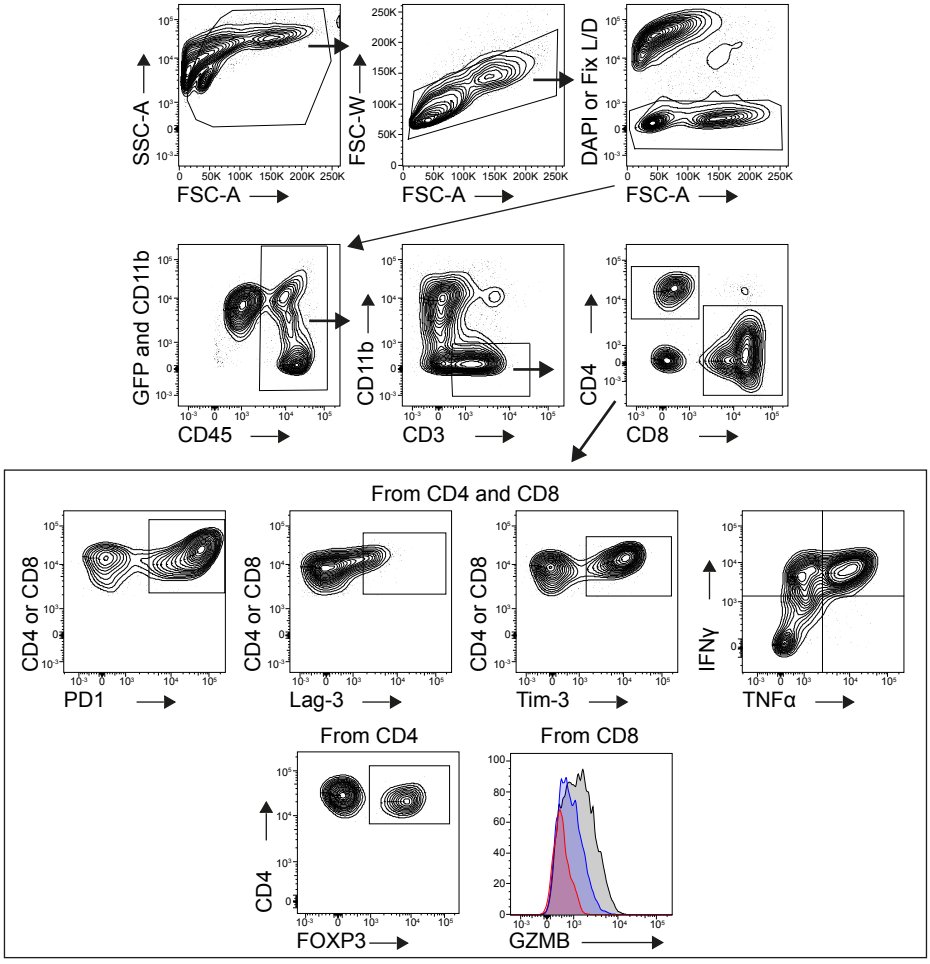
Gating strategy 1. Immune cells in B16F10 tumors (related to figure 13).



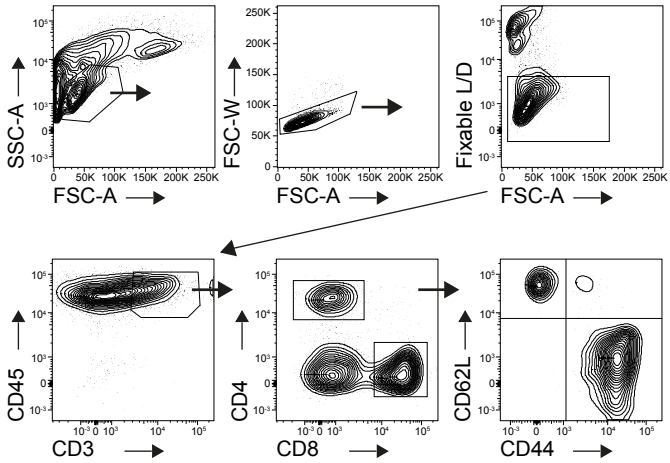
Gating strategy 2. Thymocyte subpopulations (related to figure 15).



Gating Strategy 3. TILs immunophenotyping (related to figures 17-20).



Gating strategy 4. CPEB4 levels in TILs/spleen/dLN (related to figure 21)



Appendix 2. Tables

Table 1: qPCR primers.

Gene	Forward	Reverse
m*Tbp*	AGAACAATCCAGACTAGCAGCA	GGGAACTTCACATCACAGCTC
m*Cpeb4*	CCAGAATGGGGAGAGAGTGG	CGGAAACTAGCTGTGATCTCATCT
m*Atf4*	ATGGCCGGCTATGGATGAT	CGAAGTCAAACTCTTTCAGATCCATT
m*Ddit3*	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
m*Xbp1s*	CTGAGTCCGCAGCAGGTG	GACCTCTGGGAGTTCCTCCA
m*Hspa5*	ACTTGGGGACCACCTATTCCT	ATCGCCAATCAGACGCTCC
m*Ddit4*	TCTTGTCCGCAATCTTCGCT	GGAGGACGAGAAACGATCCC
m*Herpud2*	ATGGACCAAAGTGGGATGGAG	TCAATGGTTTGCTAGGGTACAC

Table 2. RIP-seq targets

0610007P14Rik
1110059E24Rik
2410016O06Rik
2510039O18Rik
2700029M09Rik
2700060E02Rik
2810417H13Rik
Aaed1
Abcg1
Acd
Actr2
Actr3
Adprhl2
Aebp2
Ahi1
Akna
Anp32a
Arf5
Armc10
Arnt
Asf1b
Asrgl1
Atf1
Batf
Bccip
Bcl3
Bfar
Brat1
Brd3
Bud13
Bzw1
Bzw2
Cbx3
Ccdc38
Cd48
Cdc40
Chsy1
Ckap5
Cldnd1
Clk3
Clta
Cmip
Copb1
Cpsf4
Creb1
Csnk1g2
Dcp1a
Ddit4
Ddx47
Desi2
Dhx8
Dusp7
E2f4
Eed
Eif2a
Erh
Ero1l
Erp29
Esco1
Esyt1
Exosc9
F2r
Fam122b
Fam49b
Foxn2
Fzd7
G3bp1
Glul
Gm10263
Gm10335
Gmeb1
Golga3
Gsr
Gtf2h3
H1f0
H2afv
Hdac7
Hdgf
Herpud2
Hmgb1
Hmgb3
Hnrnpc
Hnrnpk
Hspa9
Hspe1
Hyou1
Id2
Ier3ip1
Ift52
Ints9
Irf2
Isg20l2
Itch
Kdm6b
Khdrbs1
Klf2
Kras
Lef1
Litaf
Llgl1
Lmnb1
Lsm14a
Lsm3
M6pr
Mad2l1
Magt1
Map3k12
Mcm3
Mcm4
Med11
Metap2
Mettl14
Mex3c
Mff
Mogs
Morf4l2
Msl2
Mta1
Mtmr9
Mtpn
Myl12a
Nde1
Ndufa7
Nedd1
Nelfa
Nelfb
Nfkbia
Npm1
Nudcd2
Ostc
Oxct1
Oxsm
P4ha1
Pabpc1
Pcbp2
Pdcd7
Pdia4
Plek
Poc5
Polr2d
Polr2h
Ppib
Ppp2r5e
Ppp6c
Prkar2b
Prkch
Prkd2
Prps2
Psma4
Psma7
Ptges3
Ptms

Table 2. RIP-seq targets *(continued)*

Pym1
Rab18
Ranbp1
Rap2c
Rbbp4
Rbm10
Rbm17
Rbm22
Rbm8a
Rhoa
Rpgrip1
Rpia
Rpl23a-ps3
Rpn1
Rps27rt
Rsl24d1
S100a10
Sacm1l
Samd1
Samsn1
Sc5d
Scaf4
Scpep1
Sec11a
Sec13
Set
Sf3b6
Sipa1l1
Slc16a1
Slc16a10
Slc25a24
Slc2a1
Smo
Sms
Snrpa
Snrpf
Snrpg
Snx18
Snx22
Snx3
Sox12
Sp1
Spcs2
Spint2
Spop
Srd5a3
Ssr2
Stk26
Stx4a
Supt16
Taf1
Taf3
Taf5
Tagln2
Taok3
Tap1
Tceal1
Tcf12
Tcf3
Tcf7
Telo2
Tfap4
Thg1l
Tiparp
Tipin
Tjap1
Tmod3
Tnfaip8
Tomm22
Tomm70a
Top1
Tpm1
Tpm4
Trp53
Tshz1
Txn1
Ubald2
Ugdh
Urb1
Usp1
Vasp
Vprbp
Wbp4
Yipf4
Zbtb22
Zfp219
Zfp36l1
Zfyve1
Znrd1

Appendix 3. Publications

Nuria Pell, Ester Garcia-Pras, Javier Gallego, Salvador Naranjo-Suarez, Alexandra Balvey, Clara Suñer, **Marcos Fernandez-Alfara**, Veronica Chanes, Julia Carbo, Marta Ramirez-Pedraza, Oscar Reina, Louise Thingholm, Corinna Bang, Malte Rühlemann, Andre Franke, Robert Schierwagen, Karl P. Rheinwalt, Jonel Trebicka, Raul Mendez, Mercedes Fernandez (2021). Targeting the cytoplasmic polyadenylation element-binding protein CPEB4 protects against diet-induced obesity and microbiome dysbiosis. *Molecular Metabolism*. Volume 54, 101388

Annarita Sibilio, Clara Suñer, **Marcos Fernandez-Alfara**, Judit Martin, Antonio Berenguer, Alexandre Calon, Veronica Chanes, Alba Millanes-Romero, Gonzalo Fernandez-Miranda, Eduard Batlle, Mercedes Fernandez, Raul Mendez (2022). Immune translational control by CPEB4 regulates intestinal inflammation resolution and colorectal cancer development. *iScience*. Accepted, pending publication.

Marcos Fernández-Alfara, Annarita Sibilio, Judit Martin, Victor Alcalde, Veronica Chanes, Adrià Cañellas, Eduard Batlle and Raúl Méndez. CPEB4-mediated adaptation to ER stess regulates anti-tumor immunity. Manuscript in preparation.

Appendix 4. Contributions

Marcos Fernández Alfara performed all the studies and contributed to experimental design, data analysis and interpretation, figure preparation, thesis writing and editing. Raúl Méndez conceived, directed the study and provided feedback during writing. Annarita Sibilio contributed to *in vivo* mouse experiments and data interpretation. Judit Martin and Victor Alcalde contributed to *in vivo* mouse experiments. Veronica Chanes performed tissue immunohistochemistry and immunofluorescence. Adrià Cañellas performed colorectal cancer organoid culture and intra-splenic injections supervised by Eduard Batlle. Joel Paz and Marta Lovera designed the cover.

Acknowledgements

El doctorado es una época emocionante e intensa, donde se suceden altibajos, momentos divertidos y otros frustrantes. A menudo parece que uno está solo, armado con una pipeta en un mar de falcons. Pero en cuanto se levanta la cabeza de la gradilla se ve a toda la gente que está detrás, o bueno, en cualquier dirección, apoyando y ayudando. Sin vosotros esta tesis no habría pasado del primer TAC. Así que gracias.

Gracias Raúl por darme la oportunidad de trabajar en tu laboratorio, por tu confianza desde el primer momento y por tener siempre la puerta abierta. Gracias a todo el laboratorio, por generar un ambiente en el que da gusto trabajar. Gracias Judit, no ha sido fácil, pero creo que he conseguido tenerte 5 años frita sin parar, ni las mejores patatas oye. Gracias por tu trabajo concienzudo, por tu motivación para hacer cosas nuevas, y por tantas horas codo con codo (literalmente) haciendo guarradas (quiero decir, machacando tumores, sacando linfonodos, preparando sumaje…). Esta tesis es inconcebible sin ti. Gracias Víctor, por acoplarte a mi otro codo con tus ganas de aprender y una personalidad inmensa. Eres in duda el hater más querible de Barcelona. Gracias a los dos por cuidar de los ratones; y gracias a los ratones, si las CPEBs ayudan a alguien algún día, el mérito es vuestro.

Gracias Annarita por hacerme hablar más despacio (mis padres te lo agradecen también) y por ayudarme a navegar por el sistema inmune. Gracias a Chiara, Eulalia y Vero por enseñarme vuestro compromiso y eficiencia, tanto en el laboratorio como fuera de él. Gracias Alba por tu perseverancia y tu disposición a ayudar. Gracias Gonzalo por tu generosidad infinita y ser mitad del principal activo para paliar el envejecimiento poblacional de España.

Y como no, gracias a las pinchafas, las que ya no están y las que quedan. A las honorables fundadoras del club de las bufandas enormes, Rosa, Clara e Irene. Gracias por vuestra vitalidad y entusiasmo, por acogerme en vuestra vorágine de planes y mostrarme el camino en los primeros años de tesis. Clara, gracias además por alargar tu estancia en el laboratorio y no irte al culo del mundo después, y así guiarme durante mis crisis científicas. Gracias Manu (Ministro, Ministrol, Don Manuel, NeoManuel o simplemente Manuel cuando la cosas se

pone seria), por darme tanto. No se si eres pinchafo de los que se fueron o de los que quedan. Por que, aunque no estés, no te has terminado de ir. Conocerte me ha ayudado a conocerme. Estos años el espejo has sido tú, pero me lo tenía callado.

Gracias Berta por llevarme siempre un poco más allá para aprender que, en efecto, un poco más allá es mejor. Gracias AnnaB por tu naturalidad. Y por cuidar mi salud coronaria. Si con tus "uuuuuuuuuuuuuu" agudos no me ha dado un infarto, ya nada lo hará. Y AnnaF, por ser cómplice de excusiones y sobre todo de reflexiones. Pocas veces aprendo tanto como cuando hablo contigo (y mira que hablo…). Te iba a escribir la dedicatoria en catalán, así como colofón del entrenamiento, pero me lo reservo para la intimitat, *¿val?* Y a las nuevas PhDs, Marina y Camilla, que vais con paso fuerte y decididas. No se que se hablará en unos años en el labo, si italiano, inglés, español o catalán, pero si no os explota la cabeza os habréis pasado el juego de la poliglotía.

Pasado el umbral del laboratorio, el IRB me ha brindado una colección de personas maravillosas. Gracias Pancho, por ser compañero de vida. Y porque tengo claro que esta etapa no va a ser la última juntos. Omega, oh, ooh, ooooooohh, tus rarezas me dan la vida. Y tu motivación, tesón y resiliencia, en la ciencia y, sobre todo, más allá, las admiro profundamente. Y bueno, claro, que voy a decir, es que estás MUY fuerte, eso es lo más importante. Joel y Marta, compañeros de salir a las 6 (bueno, ejem, o más tarde) y de aventuras de escalada. Gracias por vuestra vitalidad y ganas de aventura. Ah, y por la portada, que las contribuciones materiales no son menores que las espirituales. Gracias a Ele Mele, por todas las risas (sin querer) que me has dado, y hacerme sentir importante dándote la chapa.

Gracias a los compañeros del Student Council, Paloma (aka Palama, Pamana, Palomar, gracias por tener un nombre que da -sorprendentemente- tanto juego), Enric, Diego y compañía, por transformar la organización de eventos y el politiqueo en algo divertido y memorable. Gracias Isita por tu buen humor y tu socarronería. Hacer los seahorses sin ti (ocupando tu poyata) hubiera sido un dolor. A los mayores del IRB, Ernest (tú ya no se donde deberías ir, lo cual

es bueno), Craig, Ricardo, Leyre, Cris, Laura y alguno más que seguramente me deje (si leéis esto alguna vez, por favor, daos por aludidos) por las esquiadas, cool-offs y hacer del IRB un sitio donde ir por el pasillo sea una actividad que merezca la pena. Gracias a Sara y Maria, compis del Immunology club, por tanta ayuda mutua. Sin vosotras y los cruasanes el proyecto no hubiera salido. Gracias a mis queridos Jaume y Sonia de citometría, pasar tubos con vosotros es una fiesta. Sonia, algún día acabaremos a la hora, te lo prometo.

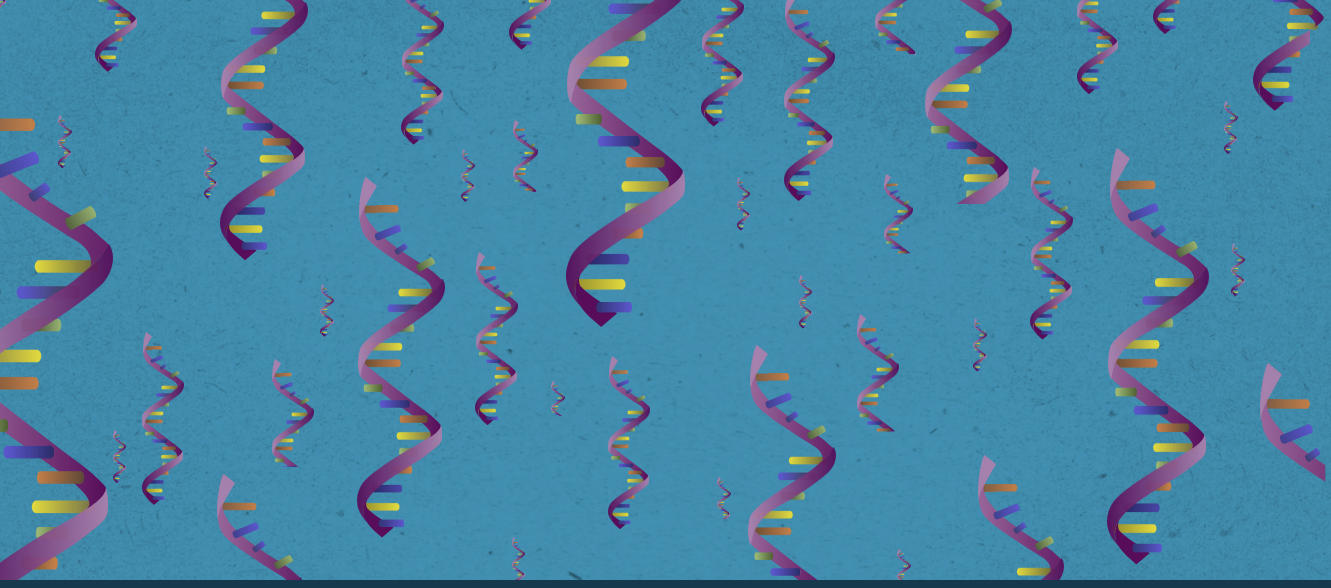
Ya fuera del IRB, quiero agradecer a todas las personas que me han acompañado en este viaje. A los amigos de Barcelona, ElenaF, ElenaM, Marc, Almu (Almö, Almonda, Almuerzo, otra que tal baila), Javi, María, Sofía, y los que, aunque no estén ya en Barcelona es como si siguieran aquí, Barri, Enrique y Jacobo. Gracias por estar siempre disponibles con una cerveza preparada y ganas de diversión. Gracias a Rodrigo y Luis, que ya venimos de más atrás, por convertir en suelo fértil todo lo que pisáis. Entre todos, habéis hecho que Barcelona sea mi casa, y convertido los momentos buenos en mejores, y los malos en… mejores también. Gracias a Bece, Mario, Fausto, Pinchi, Javi y Silvi por crecer juntos pasándolo bien, con buenas dosis de intensidad emocional, y querernos más allá de donde estemos. Pinchi, tu ya desde el instituto macho… las cosas bonitas te las digo en persona que es más divertido ver cómo te pones nervioso.

Thanks to my dear Penguins, María (you could have appeared multiple times), Dan, Vero, Anna Peanuts and those that have already been mentioned for making a dark and windy place feel like home. It's amazing how we've grown after Utrecht, and how it still feels like home whenever and wherever we meet. Gracias a los rajjcaifols, o como se escriba, Miguel Moisés y Vallejo, por acogerme como si fuera de toda la vida. Después de una tesis, sigo sin entender que tiene Manuel Becerra para que os mole tanto, pero, en fin, hay misterios inescrutables. Gracias a Carlos y Marina, por ser nuevos compañeros de aventuras en tierra, mar y, desde hace poco, también hielo.

Gracias a toda mi familia por haberme llenado de cariño en la distancia y, cuando ha sido posible, en la proximidad. Gracias a mis tíos y mis primas, Antonio, Mariajo, Rosa, Irene y Marina. Gracias a mis abuelos y abuelas. Esta

tesis es por vosotros. Gracias a mi hermana, por ponerme en mi sitio y su apoyo continuo. Y gracias a mis padres, que siempre están ahí empujando y me han dado todo el cariño, amor, confianza y libertad que se pueda imaginar. Gracias por estar siempre conmigo. Soy quien soy por vosotros.

Y muchas gracias Clara por esta maravillosa parte de nuestro viaje juntos. Gracias por ayudarme en mis altibajos y hacerme crecer. Gracias por cofundar los Pitechos. Gracias por tu transparencia disimulada. Gracias por compartir tu vida conmigo.



UNIVERSITAT DE BARCELONA

PhD Thesis
2022

INSTITUTE FOR RESEARCH IN BIOMEDICINE
IRB BARCELONA