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Pioneer Round of Translation Supplies Peptides for Major Histocompatibility Complex Class I Presentation

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Pioneer Round of Translation Supplies Peptides for Major Histocompatibility Complex Class I Presentation

By

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Abstract

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Cytotoxic T cells monitor MHC class I complexes on antigen presenting cells for the potential presence of any non-self peptides that could derive from viral infection or cancerous cells. Effective immune surveillance requires that MHC class I molecules display a peptide repertoire on the surface representing all cellular proteins. This ensures that foreign antigens from all sources are presented. How the peptide repertoire can be comprehensive despite the large differences in abundance and stability of individual proteins is not known.

The pioneer round of translation is the first round of translation that occurs on newly spliced mRNA. It is associated with nonsense-mediated decay of mRNAs, allowing cells to detect and eliminate the aberrant mRNAs containing premature stop codons. We showed here that the peptide presentation by MHC I molecules was strongly influenced by the pioneer round of translation. Inhibition of the pioneer round of translation by knockdown of CBP80, a nuclear cap-binding protein, reduced peptide presentation by MHC class I molecules on the cell surface without affecting global protein synthesis. On the other hand, introduction of premature stop codons changed mRNA stability without affecting peptide presentation by MHC I on the cell surface. In addition, intronless mRNAs bypassing the pioneer round of translation resulted in reduced MHC I presentation on the cell surface. Furthermore, knockdown of CBP80 led to the deficiency of the cytosolic peptides delivered by the transporter associated with antigen processing (TAP) for the MHC class I pathway. Together, these findings highlight the importance of the pioneer round of translation in deriving peptides to generate a comprehensive display of virtually all endogenous proteins for the immune surveillance.

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Chapter 1: Introduction

Overview of Immune System

Innate Immune System

As the oldest part of immunity, innate defense mechanisms began with simple unicellular organisms and evolved into a more complex innate immune system in multicellular organisms. It is the first level of protective immune response, which generally involves phagocytic cells, basophils, mast cells, eosinophils, and natural killer cells. Unlike adaptive immunity, the innate immune response is an antigen-nonspecific defense mechanism and lacks immunologic memory.

The molecular components of innate responses include complement, acute-phase proteins, and cytokines such as the interferons. An innate immune response is initiated within several hours, by the host, after exposure to almost any microbe to prevent infection. The microbes are recognized by the germline-encoded pattern recognition receptors based on their structural motifs or the molecular patterns of common pathogens, which are known as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002; Akira et al. 2003; Underhill and Ozinsky 2002; Medzhitov et al. 2001; Greenberg and Grinstein 2002). PAMPs include components of microbial cell walls such as lipopolysaccharide and peptidoglycan, proteins, bacterial and viral nucleic acid.

Adaptive Immune System

In vertebrates, another strategy to provide immunity is through the adaptive immune system. This acts as a second line of defense and provides immunological memory. It is composed of two classes of responses, the antibody responses and T cell mediated immune responses (carried out by B cells and T cells respectively). The interaction between antigen presenting cells (APCs) and T lymphocytes is the keystone of adaptive immunity.

Induction of adaptive immunity not only depends on specific antigen recognition by the antigen receptors but also depends on signals delivered by activation of the innate immune response. While innate immune responses are generated mainly at sites of infection, adaptive immune responses are generated in secondary lymphoid tissues and organs such as the lymph nodes and the spleen.

In mammalian immunity, the adaptive immune system selectively activates and expands antigen-specific clones from a large pool of T and B lymphocytes that carry a diverse repertoire of antigen receptors. Antigen-binding regions of the T cell and B cell receptors (BCRs and TCRs) are generated through recombination of prototypic variable (*V*), diversity (*D*), and joining (*J*) gene segments (Hedrick et al. 1984; Tonegawa et al. 1983; Yanagi et al. 1984). V(D)J recombination or somatic recombination occurs in progenitors of B and T cells and is catalyzed by recombination-activating genes (RAG1/RAG2). RAG1/2 recognize recombination signal sequences (RSSs) that flank VDJ gene segments and induce DNA cleavage (Schatz et al. 1989). The generation of antigen occurs in cells where intracellular proteins are processed by proteolytic cleavage and subsequently presented by major-histocompatibility-complex (MHC)

molecules on the surface of antigen presenting cells. There are two classes of MHC molecules: MHC class I presents endogenously derived peptides and MHC class II presents exogenously derived peptides.

CD8 T cells

Thymocytes (T cells) are the key mediators in cell-mediated immune response. T cell progenitors originate from hematopoietic stem cells (HSCs) in the bone marrow (Orkin et al. 2008). They migrate to the thymus to learn how to distinguish self and non-self antigens (Miller et al. 1961). Supported by the unique microenvironment of the thymus, immature T cells undergo a series of well-defined and coordinated developmental stages including gene rearrangements at the TCR β, γ, and δ loci and cell-surface marker selection (Anderson et al. 1996; Petrie et al. 2002). The specificity and affinity of receptor-ligand pair interactions between T cells and self-peptide-MHC complexes play a key role in T cell survival and differentiation. The ultimate goal of thymic education is to generate self-tolerant CD4+ helper T cells and CD8+ cytotoxic T cells to establish a peripheral T cell repertoire.

Cytotoxic T lymphocytes (CTL) expressing the cell surface glycoprotein CD8 play an important role in adaptive immune system. They scan peptides that are presented by MHC class I molecules on the surface of cells. After recognizing foreign antigens from intracellular infections or mutant proteins as a result of tumorigenesis, CD8+ T cells exert their cytotoxic functions to destroy target cells (Cresswell et al. 2005). Granule-mediated or Fas-mediated cytotoxic pathways are two major death-inducing strategies used by CD8+ T cells for non-self antigen-bearing target cells (Liu et al. 1995). However, CTLs are also known to secrete several cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα), when target cells are in the vicinity (Murali-Krishna et al. 1998; Slifka et al. 2000).

Overview of MHC class I antigen presentation pathway

MHC I-associated peptides are typically of 8 to 11 residues in length and are generated from proteins endogenously synthesized by host cell ribosomes. Source proteins are degraded by cytosolic and nuclear proteasomes and trimmed by aminopeptidases in the cytosol or in the endoplasmic reticulum. Transporter associated with antigen processing (TAP) translocates peptides into the lumen of the endoplasmic reticulum (ER). In the ER, tapasin recruits MHC class I molecules to TAP, in association with the peptide-loading complex, to enhance peptide loading onto MHC class I molecules. The binding of peptides is required for the correct folding of MHC class I molecules and their release from the ER. MHC class I peptide complexes are then transported to the plasma membrane to be presented to CD8+ T cells.

MHC class I molecules

Major histocompatibility complex (MHC) class I molecules are expressed on the plasma membrane of almost all nucleated cells to present peptides derived from cytosolic proteins. These MHC class I molecules and peptides together form ligands for CD8+ T cell receptors and are important for immune development and immune surveillance.

Human class I molecules are encoded by three highly polymorphic genes called human leukocyte antigen or HLA genes: HLA-A, -B, and -C. The gene of MHC is located on the human chromosome 6. It is one of the most polymorphic genes and in humans it contains more than 200 alleles. Murine homologs of these MHC class I molecules are H-2D, H-2K and H-2L genes on the mouse chromosome 17. MHC class I molecules are capable of presenting a broad repertoire of peptides generated from intracellular proteins. Alternatively, MHC class I molecules may be loaded with peptides derived from extracellular proteins through cross presentation. The recognition of non-self peptides by CD8+ T cells results in a hierarchy of TCR responses in the order of cytotoxicity, cytokine expression, proliferation and differentiation.

The crystal structure of MHC class I molecules was first published in 1987 (Bjorkman et al. 1987). The MHC class I molecule is composed of two polypeptide chains: the transmembrane protein α-chain (heavy chain) and soluble protein β-microglobulin (β2m). Only the α-chain is polymorphic and encoded by a variant HLA gene. The α-chain is composed of three domains. The α-1 and α-2 domains form the peptide-binding groove (Falk et al. 1990), which consists of two long α-helices and an eight-stranded anti-parallel β-sheet. It is in the binding groove that a peptide of 8-10 amino acids in length binds to MHC class I molecules and there are 2-3 "anchor residues", one of which is always at the C terminal of peptides (Rammensee et al. 1995). The side chain of anchor residues are complementary to the surface of binding groove of MHC I molecules. Therefore, anchor residues can hold peptides in place by burying within the binding groove. It follows that the changes in the surface feature of binding groove and corresponding anchor residues permit a large diversity of MHC I peptides.

Other than classical MHC class I molecules, there are a group of non-classical class I molecules (or MHC class Ib molecules). They function in both innate and adaptive immunity at most levels of the immune response. These molecules are conserved among species and distinguished from classical MHC I by their limited polymorphism.

Antigen processing in cytosol

Peptides generated by cytosolic proteolytic systems are the precursors for the antigen processing pathway. The continual destruction of intracellular proteins serves important homeostatic functions like eliminating misfolded proteins or regulating signaling responses. This provides a large and dynamic population of peptides derived from almost all cellular proteins for antigen supply.

The ubiquitin-proteasome machinery performs protein degradation inside the cell. Ubiquitin is a small regulatory protein that is attached to the protein substrate and serves as a "tag" for subsequent signaling. The 26S proteasome recognizes the polyubiqutin chains that are conjugated to the protein substrate and consequently degrades the protein. The structure of 26S proteasome resembles a cylinder composed of a 20S proteasome core and a 19S complex of subunits. The 20S core functions as a protein cleavage site and the 19S complex functions as an access to the core to capture and unfold the ubiquitinated substrates (Kloetzel et al. 2004; Braun et al. 1999; Ferrell et al. 2000). The importance of the proteasome in the antigen-processing pathway is highlighted by its formidable task of digesting intracellular proteins continuously. In support of this, the use of several proteasome inhibitors reduces antigen production from cellular

proteins and the assembly of most class I molecules (Rock et al. 1994; Rock et al. 2002; Rock et al. 2004). The majority of peptides generated by the proteasome during breakdown of intracellular proteins is further digested into their constituent amino acids or are too short to bind MHC class I molecules (Saric et al. 2004; Tortorella et al. 2000; Kisselev et al. 1999; Totes et al. 2001). Only a small fraction of peptides are delivered into the ER where they bind to MHC I molecules and are transported to the cell surface for presentation to cytotoxic T cells.

In response to oxidative stress and proinflammatory cytokines, mainly IFN-γ, the cells of the immune system upregulate proteasomal subunits LMP2, MECL-1 and LMP7. These inducible catalytic subunits promote formation of a new proteasome - the immunopreoteasome by replacing standard catalytic subunits β1, β2, and β5 in nascent proteasome 20S core, respectively. Because of chymotrypsin-like activity in LMP2, the immunopreoteasome tends to generate peptides with hydrophobic amino acids at the C-terminus, which provides more peptides for MHC I binding.

The resulting polypeptides of proteasome degradation are approximately 7-24 amino acids long and can be trimmed by additional cytosolic proteases for further destruction. These proteases include leucine aminopeptidase, tripeptidyl peptidase II (TPPII), bleomycin hydrolase and puromycin-sensitive aminopeptidase. Their exo- or endo-proteolytic activities not only facilitate recycling of amino acids but also promote generation of MHC I peptides. Consistent with double roles of peptidases, overexpression of peptidases thimet oligopeptidase led to decreased MHC I surface expression (Reits et al 2003; Saric et al 2004; York et al 2003), suggesting the balance between destroying and generating epitopes is tightly regulated. Studies on the effect of individual peptidase deficiency on antigen presentation through in vitro siRNA knockdown or knockout mice (York et al. 2006; Kawahara et al. 2009; Firat et al. 2007; Kim et al. 2009; Towne et al. 2007; Towne et al. 2005) suggested limited roles of these peptidases. While these peptidases work in concert to generate epitopes from peptides, they are not essential for most antigen presentation.

Cytoplasmic chaperones

Cytoplasmic chaperones are actively involved in protein folding, assembly, translocation and degradation. They play a role in MHC I antigen presentation through protecting cytosolic peptides from degradation by cytoplasmic proteases. They also facilitate peptide folding and shuttle peptides to the TAP transporter. Among these chaperones, heat shock protein 90 (hsp90), hsp70, gp96 and tailless complex polypeptide-1 ring complex (TRiC) are intensively studied on their effect on antigen presentation.

A biochemical study revealed that α isoform of hsp90 is associated with C-terminal proteolytic intermediates (Kunisawa et al. 2006). Knockdown of hsp90α resulted in loss of these intermediates and consequently decreased pMHC I expression. In addition, treatment of cells with hsp90 inhibitor geldanamycin down-regulated surface MHC class I expression, as hsp90 may facilitate the assembly and function of the 26S proteasome (Yamano et al. 2008). Immunization of peptides in complex with hsp90 or hsp70 increased the specific cytotoxic T lymphocyte (CTL) responses in mice compared to immunization with peptides alone (Binder et al. 2001; MacAry et al. 2004). Introduction of these complexes with hsp70 into the mice also

converted tolerogenic response to the activation of autoimmunity in vivo (Millar et al. 2003). Group II chaperonine TRiC protected peptides with N-terminal flanking residues from degradation by directly binding to them. Inhibition of TRiC in cells using siRNA reduced expression of peptide-loaded MHC class I molecules (Kunisawa et al. 2003), suggesting that TRiC serves a function in protecting proteolytic intermediates for ER transport. Heat shock protein gp96 is an endoplasmic reticulum (ER)-resident stress protein. Like its cytosolic cognate hsp90, gp96 is associated with TAP-translocated peptides in the ER (Lammert et al. 1997). Immunization of mice with isolated gp96 induced protective immunity (Arnold et al. 1997). The data also showed that the antigens associated with gp96 could be TAP-dependent and TAP-independent, suggesting the ability of gp96 in inducing specific CTL responses to a wide range of intracellular antigens. However, absence of gp96 did not impair cell viability and had no detectable effect on the expression of the vast majority of cell surface receptors including CD44, CD29, CD54 and H2-K^b (Randow et al. 2001). This observation may be due to the redundant functions of chaperones. To understand the roles of chaperones in antigen presentation, further elucidation of individual contributions of each chaperone is still required. In addition, the participation of these immunochaperones in cross-presentation by carrying and introducing antigens into antigen presenting cells (Binder et al. 2000; Delneste et al. 2002; Berwin et al. 2003; Facciponte et al. 2007) established importance of the association between chaperones and peptide intermediates.

Peptide loading complex (PLC): Peptide loading and stabilization of MHC

The assembly of MHC class I molecule with peptides is a coordinated mechanism that requires multiple factors to work in a precisely orchestrated process (Peaper and Cresswell 2008). In addition to TAP heterodimer, the peptide loading complex (PLC) contains calnexin, calreticulin, tapasin, ERp57, β2m and MHC I heavy chain (Elliott and Williams 2005; Ortmann et al. 1997). This section details the role that calnexin, calreticulin, tapasin, ERp57 play to facilitate optimal peptide loading.

TAP transports peptides into the ER

Transporter associated with antigen processing (TAP) plays an essential role in MHC class I peptide presentation pathway. It transports peptides from the cytosol to the ER where MHC class I peptide complexes are assembled. TAP is an ATP-binding cassette (ABC) transporter that is localized in ER membrane (Neefjes et al. 1993). It is a heterodimer of TAP1 and TAP2 whose genes lie in the MHC class II region in chromosome 6. TAP proteins are composed of a hydrophobic domain and a hydrophilic nuclear binding domain (NBD), which permits ATP binding and hydrolysis. TAP translocates peptides from the cytoplasm into the ER by binding peptides and hydrolyzing two ATP molecules (van Endert et al. 2002): One ATP molecule to open its pore and another ATP to complete the translocation cycle.

TAP has some preference for peptide size and sequence. Most peptides transported by TAP are 9-12 amino acids in length although longer peptides may be transported. Human and mouse TAP transporters have similar preference on peptide sequence. They both translocate peptides with hydrophobic C-termini more efficiently, and do not favor peptides with a proline at the N-terminal positions (usually at positions 1-3) (van Endert et al. 1995; Momburg et al. 1994;

Burgevin et al. 2008). The peptides with prolines at the beginning of the sequence, particularly at position 2 (denoted as X-P-X_n motif where X=any amino acid, P= prolin) are poorly transported by TAP. Despite this, there are a number of MHC I haplotypes, including mouse L^d and human HLA-B7, that prefer peptides with X-P-X_n motif (Rammensee et al. 1995). Therefore, peptides with extended N-terminus are transported into the ER and subsequently trimmed by aminopeptidases like ERAAP.

TAP is not fully active all the time. Supported by a recent study on TAP activity using GFP-tagged TAP molecules, approximately only one third of TAP molecules are translocating peptides (Reits et al. 2000). TAP activity is associated with its lateral diffusion rate within the ER membrane. TAP diffusion rate increases when it is inactive and decreases when TAP is transporting peptides. One observation was that TAP mobility increases during viral infection, due to the rapid increase in the intracellular peptide pool (Reits et al. 2000). This suggested that TAP mobility is dependent on its substrates.

Most known antigenic peptides are TAP-dependent. In support of this, MHC class I expression in the mice drastically decreased and the presented peptide pattern altered as well in the absence of TAP1 (Van Kaer et al. 1992), while human cell line lacking TAP1 lost almost all MHC I expression (Spies et al. 1990; Spies et al. 1991). Despite their defects in class I antigen processing, TAP-deficient cells do express class I-bound peptides that are independent of TAP. Sequence analysis has revealed that these peptides are derived from protein signal sequences or other sources (Wei et al. 1992; Weinzierl et al. 2008). Consistent with this, impairment of TAP function allowed for MHC-I presentation of alternative peptides that are commonly barred by influx of TAP-transported peptides into the ER.

ERp57, calnexin, clareticulin and Tapasin

The thiol oxidoreductase ERp57 belongs to the family of protein disulfide isomerases (PDI). ERp57 functions in oxidative glycoprotein folding by mediating disulfide bond formation (Ellgaard and Frickel 2003). *In vitro* studies have revealed the activity of reductase, oxidase, and isomerase in ERp57, but its main activity *in vivo* remains unclear (Frickel et al. 2004). ERp57 interacts with ER lectin-like chaperone calnexin (CNX) and calreticulin (CRT) (Frickel et al. 2004; Pollock et al. 2004; Russel et al. 2004). To assess the role of ERp57 in MHC I presentation, ERp57 was specifically depleted in mouse B cells. In these cells, surface expression of murine MHC class I molecule K^b was decreased and the recruitment of K^b into the PLC was altered (Garbi et al. 2006).

Calnexin and calreticulin are two major chaperones involved in the folding and subunit assembly of MHC class I in the ER (Pamer et al. 1998). Calnexin is involved in the early stage of MHC I assembly. It recruits ERp57 to mediate disulfide bond formation in MHC I heavy chain while protecting class I heavy chain from degradation. Calreticulin has a critical role in the peptide loading of class I molecules (van Leeuwen et al. 1996; Sadasivan et al. 1996; Farmery et al. 2000).

Tapasin functions as a bridge between MHC class I/β2m complexes and TAP. The influence of tapasin on the stability of TAP transporter was demonstrated in the tapasin-deficient

mice, whose expression of TAP protein was reduced by 100-fold (Garbi et al. 2003). The absence of tapasin leads to loss of PLC (Ortmann et al. 1996; Dong et al. 2009; Peaper et al. 2005; Tan et al. 2002; Simone et al. 2012; Dick et al. 2002; Bangia et al. 1999). Furthermore, reduction in K^b and D^b surface expression and impairment of CD8 T cell responses were observed in tapasin-deficient mice. But the effect was not as severe as that seen in TAP-deficienct animals. These data indicate that tapasin is essential for presentation of T cell epitopes (Garbi et al. 2000, Grandea et al. 2000).

ER aminopeptidase associated with antigen processing

Proteolytic intermediates generated in cytosol are usually cleaved at their C-terminal positions while their extended N-termini require further processing typically within the ER (Paz et al. 1999; Rock et al. 2004; Kanaseki et al. 2006). These assumptions were initially made when peptides with X-P-X_n motif were identified presented by ~20% of MHC class I molecules. Peptides with proline at N-terminus, particularly at position 2, turned out to be the least efficiently translocated by TAP transporter and thus unlikely to enter the ER unless they were transported as longer precursor with N-terminal extension (Neisig et al. 1995; van Endert et al. 1995).

Accumulating evidence suggests that TAP-transported peptides are longer than the antigenic peptides presented on the cell surface (Androlowicz and Cresswell 1996; Yellen-Shaw, 1997). Further investigation on these additional trimming events in the ER indicates the existence of a specific protease capable of trimming N-terminal but not C-terminal residues on the antigen precursors, called ER resident aminopeptidase associated with antigen processing (ERAAP) (Serwold et al. 2001). It is suggested that ERAAP cleaves residues from the N-termini of longer peptide precursors and stalls at X-P sequence, resulting in the X-P-X_n motif.

In addition to the reduced surface pMHC I, altered expression of several endogenous peptides was observed in the cells knocked-down with ERAAP siRNA (Serwold et al. 2001). ERAAP deficient mice have reduced expression of MHC class I molecules but MHC II molecules are not affected (Hammer et al. 2006). In addition, MHC I molecules present more novel peptides and form unstable complexes in the ERAAP-deficient mice (Hammer et al. 2007). The ERAAP-deficiency prevents the generation of some epitopes and promotes the generation of other epitopes, while some peptide presentation is not affected, indicating that there are ERAAP-dependent and ERAAP-independent peptides (York et al. 2006; Firat et al. 2007). In addition to classical MHC I peptides, ERAAP-deficiency also has an effect on non-classical MHC class Ia and class Ib (Yan et al. 2006; Nagarajan et al. 2012).

Sources of MHC I antigenic peptides

MHC I provides ligands for CD8+ T cell TCR receptors to alert the immune system to the presence of intracellular pathogens or transformed cells. Most peptides presented by MHC class I are derived from cytosolic or nuclear proteins, which are degraded at different stages. It is the mission of the antigen processing pathway to create a diverse repertoire of peptides representative of self and potentially non-self proteins in cell such that immune system is able to capture any detrimental changes (Shastri et al. 2002).

Antigenic peptides derive from newly synthesized proteins

The primary source of MHC I antigens was thought to be the byproducts of mature intracellular protein turnover (Moore et al. 1988). In support of this, it was observed that antigenic peptides were derived from mature full-length proteins introduced into the cells (Moore et al. 1988). However, evidence rising from multiple studies implies other sources of class I antigens. It has been noticed that there is a rapid presentation of viral-derived peptides after viral infection despite the long half-life of viral proteins (Esquivel et al. 1992), which suggests an additional mechanism, other than normal protein turnover, that controls peptide generation. Peptides were also proposed to originate from newly synthesized proteins through rapid degradation (Schubert et al. 2000; Yewdell et al. 1996). All of these indicate that a great majority of peptides sampled by MHC class I molecules are generated from newly synthesized proteins, to ensure rapid presentation to the immune system.

Defective ribosomal products (DRiPs)

Mutations or errors occurring during mRNA splicing, transcription or translation, if not detected and corrected by system, would accumulate during protein production. It is proposed that the resulting defective proteins, called defective ribosomal products (DRiPs), are presumably targeted for rapid degradation (Yewdell et al. 1996), providing peptide substrates for MHC class I molecules. Accordingly, DRiPs is defined as "prematurely terminated polypeptides and misfolded polypeptides produced from translation of *bona fide* mRNAs in the proper reading frame" (Yewdell et al. 1996). The hypothesis is based on the finding that CD8+ T cells can recognize virus-infected cells instantly after viral penetration (less than 60 minutes), given the relatively long half-life of viral proteins (Esquivel et al. 1992).

Approximately 30% or more of newly synthesized polypeptides are directly signaled to destruction through proteasome and peptidases. DRiPs were proposed to explain the mechanism for rapid presentation of foreign peptides, especially antigens derived from highly stable viral proteins (Esquivel et al. 1992). Because it is conventionally thought that MHC I antigens are derived from normal protein turnover but further evidence reveals that the generation of antigenic peptides is independent of the stability of endogenous proteins (Goth et al. 1996). To distinguish DRiPs from peptides arising from turnover of normal proteins with either long or short half-lives (Qian et al. 2006), DRiPs definition has updated to include defective polypeptides originating from alternative/shifting reading frame and downstream initiation on *bona fide* mRNAs (Shastri et al. 1995; Wang et al. 1996; Bullock et al. 1996; Fetten et al. 1991; Berglund et al. 2007) and all other errors accumulated during processing of gene expression (Netzer et al. 2009), which are rapidly degraded and enter the class I pathway. In support of DRiPs hypothesis, multiple kinetic studies on antigen presentation have been done. For example, K^b-SIINFEKL presentation from recombinant vaccinia virus (VV) in the infected cells was quantitated (Princiotta et al. 2003). The results revealed a rapidly degraded SIINFEKL peptide (half-life in minutes) compared to a slow degradation rate of the fused virus protein (half-life in hours).

Cryptic translation: Non-AUG translation as a source of antigen peptides

Translation initiation from codons other than the canonical AUG codon occurs constitutively in many different organisms as well as in normal mammalian cells (Peabody 1989,

Schwab et al. 2003). It has been demonstrated that antigenic peptides can come from sources other than translation of the primary open reading frames (ORFs), such as alternative open reading frames (ARFs), intronic sequences, 5′ untranslated region or intron-exon junction of an mRNA (Uenaka et al. 1994; Guilloux et al. 1996). The fact that unconventional peptides can elicit cytotoxic T cell responses indicates that they may play an important role in immune surveillance. The information gained from non-coding region or non-canonical reading frame may expand the pool of antigenic peptides.

Cryptic translation products or cryptic pMHC I are the unconventional peptides encoded by alternative reading frames resulting from alternative mRNA splicing as well as frame shift events (Shastri et al. 2002; Ho and Green 2006). Cryptic translation is initiated with or without AUG initiation codons. At least 6 non-AUG codons that are capable of initiating translation have been identified: CUG, ACG, GUG, AUU, AUC and UUG (Shastri et al. 1995, Malarkannan et al. 1999). Research on alternative translation suggests that an initiator methionine tRNA (Met-tRNAiMet) could mediate the initiation of non-AUG start codon through 'wobble' interactions. In addition, recent studies show an initiator tRNA charged with leucine rather than a canonical methionine initiates at CUG start codon for cryptic translation (Starck et al. 2008; Starck et al. 2012).

Unlike peptides generated by conventional AUG initiated open reading frames, cryptic peptides are expressed at low levels. It has been shown that cryptic translation is relatively enhanced when conventional translation is inhibited by cellular stresses such as nutrient deprivation and oxidative stress. Although the mechanism of cryptic translation still remains to be elucidated, several studies on protein products from alternative translation show that this translational process is crucial for cellular function and is probably finely tuned (Arnaud et al. 1999; Bruening and Pelletier, 1996; Short and Pfarr, 2002; Tee and Jaffe, 2001; Touriol et al. 2003; Zhao et al., 2006). These findings suggest that cryptic translation may provide alternative MHC I peptides under cellular stress or add diversity to antigenic peptide pool.

The Pioneer round of translation

The fundamental question of immune surveillance is discrimination between self and non-self peptides. To do this, cells present a diverse library of peptides from all gene products that can be sampled by MHC class I molecules. Whether antigens come from a functional protein or a truncated polypeptide is irrelevant. Unconventional sources such as cryptic translation provides cellular information that is not contained in the primary open reading frame; DRiPs proposes rapid presentation from rapid degradation of defective polypeptides. Several correlation studies on the time course of peptide generation and precursor processing revealed that antigen presentation is related with precursor synthesis rather than protein degradation (Townsend et al. 1988; Gueguen et al. 1996; Goth et al. 1996). These findings suggested a link between new protein synthesis and antigen presentation.

An overview on nonsense-mediated mRNA decay

Eukaryotic cells regulate their gene expression at multiple levels, including transcription, mRNA splicing, mRNA export, translation and mRNA degradation. These mechanisms act in

concert to ensure the efficiency and fidelity of gene expression, however mutations do occur at different steps and cause subsequent consequences. For instance, frameshift or nonsense mutations in mRNAs can result in premature termination codons (PTCs) and lead to generation of truncated proteins with deleterious dominant-negative effect or gain-of-function activity. Failure to detect and eliminate these abnormal transcripts may result in genetic disorders. Examples of genetic disorders in which nonsense mutations are known to be among the causes include cystic fibrosis, Duchenne muscular dystrophy, Hurler syndrome and β-thalassemia. It has been estimated that 30% of inherited diseases and many of cancers are caused by PTCs (Frischmeyer et al. 1999). Several mRNA surveillance mechanisms are currently known to function in cells to ensure the quality of transcripts.

Nonsense-mediated mRNA decay (NMD) is a post-transcriptional surveillance mechanism evolutionarily conserved in eukaryotes. It controls the quality of gene expression by degrading the mRNAs of mutant genes that contain premature stop codons (Behm-Ansmant et al. 2007; Chang et al. 2007; Isken and Maquat, 2007). In addition, NMD controls proper expression of natural genes by downregulating selected normal transcripts, such as mRNAs important for amino acid transport and metabolism (Mendell et al. 2004; Wittmann et al. 2006; Lareau et al. 2007; Ni et al. 2007). For example, gene expression profiling has revealed that NMD regulates ~10-20% of transcriptome in yeast, Drosophila or human cells (Mendell et al. 2004; He et al. 2003; Lelivelt et al. 1999).

The basic mechanism by which a nonsense mutation signals mRNA for decay is not fully understood. Indeed, how a PTC is defined in *S. cerevisiae* and *Drosophila* are different from humans even though several NMD factors are conserved in these organisms (Gatfield et al. 2003). In mammals, the recognition of PTC often requires the participation of exon-exon junctions. While in *S. cerevisiae* and *Drosophila*, the PTC-containing mRNAs derived from intronless genes are also subjected to NMD, suggesting that NMD is independent of exon-exon junctions (Baker et al. 2004; Maquat 2004; Gatfield et al. 2003).

Mechanism of nonsense-mediated mRNA decay

Here we focus on the current most prevalent mammalian model of NMD. After mRNA splicing, an exon junction complex (EJC) is deposited approximately 20 nucleotides upstream of an exon-exon junction, serving as a position-specific marker and binding platform for factors involved in mRNA pathways. Whether a nonsense transcript is recognized and targeted for degradation depends on the relative position of its nonsense codons to the downstream sequence and associated translational complex. In general, PTCs that reside at least 50-55 nucleotides upstream of the next exon-exon junction are NMD-activating nonsense mutations, the most common feature that distinguishes mRNAs degraded by NMD from normal mRNAs. In contrast, the nonsense mutations within the last exon do not activate NMD as there are no more exon junction complex (EJC) downstream, and thus yield a stable mRNA that directs the synthesis of C-terminally truncated polypeptides (Nagy and Maquat 1998; Thermann et al. 1998). For example, the nonsense codon in the last exon of human β-globin gene is resistant to NMD decay, which results in accumulated truncated β-globin proteins and dominantly inherited thalassemia as a consequence (Thein et al. 1990; Kugler et al. 1995).

NMD is associated with mRNA translation. According to the current model, a translating ribosome removes EJCs and associated NMD factors Up-frameshift 2 (Upf2) and Upf3, which allows the mRNA to enter the phase of bulk translation. If the ribosome stops at a PTC, translation is terminated and NMD factors such as Upf1 protein are recruited to elicit mRNA degradation. The nucleases and co-activators that mediate general mRNA decay pathway are also involved in NMD.

Essential factors for NMD

The key components for NMD activity were first discovered in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Hodgkin et al. 1989; Leeds et al. 1991; Cui et al. 1995; He et al. 1997). UPF1-3 (identified in yeast and known as SMG2-4 in C.elegans) proteins comprise the core complex in NMD pathway and their function is conserved in eukaryotes (Page et al. 1999; Serin et al 2001; Gatfield et al. 2003). Inhibition of the UPF1 and UPF2 results in the stabilization of PTC-containing mRNAs in *S. cerevisiae*, *C. elegans*, *Drosophila* and human cells (Hodgkin et al. 1989; Leeds et al. 1991; Cui et al. 1995; He et al. 1997; Lykke-Andersen et al. 2000; Mendell et al. 2002). UPF3 is also critical for NMD but its function in human cells is difficult to assess, as there are two paralogs hUPF3a and hUPF3b. It has been reported that hUpf3a is much less efficient than hUpf3b in triggering NMD (Kunz 2006).

Upf3 is concentrated in the nucleus but shuttles to the cytoplasm and is thought to recruit Upf2. Upf2 is located in the perinuclear area and may associate with mRNAs via Upf3 and the EJC (Kadlec et al. 2004; Lykke-Andersen et al. 2000; Serinet et al. 2001). The RNA helicase Upf1, mostly in cytoplasm, plays a key role in the NMD by setting up a connection between translational machinery and decay pathway (Leeds et al. 1991; He et al. 1997; Page et al. 1999; Lykke-Andersen et al. 2000; Mendell et al. 2002; Czaplinski 1998). Upf1 is thought to be associated transiently with EJC and recruited to NMD core complex via Upf2 when NMD is activated. The importance of Upf1 to NMD is supported by the report that mouse embryos lacking Upf1 resorb shortly after implantation because of NMD failure (Medghalchi et al. 2001). In addition, the UPF1-deficient blastocysts obtained 3.5 days post-coitum undergo apoptosis in culture after a short period of cell expansion (Medghalchi et al. 2001). As NMD functions in both regulating normal gene expression and eliminating aberrant transcripts, it is not surprising that the unviability of embryos and cells is due to failure of NMD. The alternative functions of Upf1 in other pathways may also contribute to the apoptosis.

Pioneer round of translation supports NMD

In mammals, NMD of mRNA generally occurs as a consequence of a pioneer round of translation when a ribosome stalls at a nonsense codon (Ishigaki et al. 2001; Sato et al. 2008). The pioneer round of translation is the first round of translation that every mRNA undergoes after being newly synthesized and spliced. Precursor mRNA (pre-mRNA) is bound by nuclear cap-binding protein (CBP) heterodimer CBP80/20 at 5'-cap and the major nuclear poly(A)-binding protein PABPN1 at 3'-end (Chiu et al. 2004; Ishigaki et al. 2001). Mature mRNA generated from pre-mRNA after splicing is bound by CBP80/20, PABPN1, the major cytoplasmic poly(A)-binding protein PABPC and exon junction complex (EJC) (Chiu et al. 2004; Ishigaki et al. 2001; Lejeune et al. 2004). The EJC also associates with NMD factors, including

Upf3a/Upf3b, Upf2 and presumably Upf1 (Kim et al. 2004; Lykke-Andersen et al. 2000; Lykke-Andersen et al. 2001; Mendell et al. 2000; Ohnishi et al. 2003; Serin et al. 2001). The resulting mRNP, or pioneer translation complex, initiates a first round of translation, which is thought to link to NMD for mRNA surveillance. Previous studies suggest that pioneer round of translation occurs in nuclei when mRNAs are degraded via nucleus-associated NMD, and in the cytosol when mRNAs are degraded via cytoplasmic NMD (Dahlberg et al. 2003; Maquat 2002; Dreumont et al. 2004; Moriarty et al. 1998). On the other hand, more recent evidence suggests that the majority of HeLa cell NMD occurs in the cytoplasm () and NMD factors and substrates are observed in the cytoplasmic mRNP granules called processing bodies ().

As described above, upon the recognition of the premature termination codon, NMD is triggered. The pioneer round of translation complex helps recruit a series of NMD factors to form an mRNA surveillance complex, which targets the mRNA for degradation. The decay of NMD substrates was thought to occur at both end of mRNA, which thus involves decapping and 5'-to-3' exonucleolytic mechanism as well as deadenylation followed by 3'-to-5' exosomal activities (Lejeune et al. 2003; Chen and Shyu 2003; Couttet and Grange 2004). However, degradation mechanisms of NMD have been suggested to differ among species. Recent evidence suggests that endonucleolytic cleavage is the degradation mechanisms of NMD in metazoan and the metazoan-specific NMD factor SMG6 is the responsible endonuclease (Eberle et al. 2009).

CBP80/20 resides mostly in the nucleus but also shuttles to cytoplasm. NMD does not target mRNA when an mRNP is remodeled after pioneer round of translation, where eIF4E replaces CBP80/20 for cap binding, PABPC replaces PAPBN1 for poly-A tail, and EJCs are removed from mRNA (Ishigaki et al. 2001; Chiu et al. 2004; Hosoda et al. 2005). This CBP80/20-restriction mechanism is due to the functions of CBP80 and EJCs in NMD and can be explained by the absence of CBP80 and EJCs in steady-state translation. CBP80 promotes NMD by strengthening the interaction of Upf2 with Upf1 (Hosoda et al. 2005). Being associated with CBP80, Upf1 is the last of the Upf proteins that get recruited to trigger NMD (Lykke-Andersen et al. 2001; Hosoda et al. 2005; Kim et al. 2005; Singh et al. 2007). This suggested that CBP80 is no longer required for NMD once Upf1 interacts with Upf2. EJCs are also removed by translating ribosomes during pioneer round of translation (Dostie & Dreyfuss, 2002).

Mechanism of pioneer round of translation

As described above, the initiation complexes of pioneer round of translation are different from steady-state translation initiation complexes. The most distinguishing features are cap-binding proteins and poly(A)-binding proteins (PABPs). Additionally, pioneer translation initiation complexes contain exon junction proteins, which are removed during translation. These specialized components determine the resulting distinguishing functions of each translation initiation complex. It naturally follows that the pioneer round of translation and steady-state translation responses differently to cellular stimuli. For example, steady-state translation is inactivated during prolonged cell stress such as hypoxia or serum starvation mainly due to the activation of 4E-BP1, which specifically binds and inhibits steady-state cap-binding protein eIF4E (Koritzinsky et al. 2006). Whereas the pioneer round of translation remains efficient under these circumstances to maintain the surveillance of newly synthesized mRNAs, which are

presumably ready for bulk translation of cellular proteins once stress is gone (Chiu et al. 2004; Matsuda et al. 2007; Woeller et al. 2008; Oh et al. 2007a,b).

Meanwhile, the pioneer round of translation and steady-state translation share many common initiation factors, as eIF2, eIF3, eIF4AI, eIF4G, PABPC1, eRF1 and eRF3 are co-immunopurified not only with eIF4E but also with CBP80 (McKendrick et al. 2001; Chiu et al. 2004; Lejeune et al. 2004; Hosoda et al. 2006; Kashima et al. 2006; Isken et al. 2008). Furthermore, both CBP80/20 complex (CBC)-bound mRNAs and eIF4E-bound mRNAs are detected on polysomes (Stephenson and Maquat 1996; Chiu et al. 2004; Sato et al. 2008). Further evidences showed that polysomes associated with CBC-bound mRNAs are smaller than the polysomes associated with eIF4E-bound mRNAs. This is consistent with the finding that CBC-bound mRNAs are exported to the cytoplasm, where protein translation occurs (Visa et al. 1996; Chiu et al. 2004; Cheng et al. 2006). Like steady-state translation, the pioneer round of translation is carried out by more than one ribosome, determined by the initiation efficiency and the length of open reading frame (Isken and Maquat, 2008; Isken et al. 2008).

Cap-binding protein CBP80/20

Nuclear cap-binding complex is a heterodimer that comprises a 20-kDa CBP20 and an 80-kDa regulatory protein CBP80. Signaling from growth factors and Rho-GTPase Cdc42 can stimulate CBC binding to the cap structure of mRNAs (Chou and Blenis, 1996; Wilson and Cerione, 2000). CBC facilitates various RNA processing events including pre-mRNA splicing, mRNA export, translation, nonsense mediated mRNA decay and microRNA (miRNA) regulated gene silencing. For example, PHAX bridges CBC to CRM1 (also known as exportin-1) for the nuclear export of U snRNAs (Ohno et al. 2000; Segref et al. 2001). The binding of CstF to CBC mediates the cleavage in 3'-end formation (Flaherty et al. 1997). The association between CBP80 and the poly(A)-specific RNase (PARN) abrogates mRNA deadenylation (Balatsos et al. 2006).

As mRNA cap-binding proteins, CBC and eIF4E have no sequence homology (Marcotrigiano et al. 1997). Mazza et al. looked at the 2Å resolution crystal structure of human CBC (Mazza et al. 2001). Human CBP80 is 790 amino acids in length, and CBP20 is 156 amino acids in length. CBP20 contains a classical RNA binding motif (Izaurralde et al. 1995) and is highly conserved from yeast to human. Unlike CBP20, human CBP80 showed three helical domains with highly ordered structure, each consisting of successive antiparallel pairs of helices. The three domains are connected with two linkers and all of them are structurally similar to a middle domain of eIF4G, called MIF4G domain (Ponting 2000; Marcotrigiano et al. 2001). The second and third domains of CBP80 are associated with CBP20.

How mRNAs undergo steady-state translation after the pioneer round of translation remains obscure, and little is known about how CBC is replaced by eIF4E at the 5'-cap of mRNAs. It seems the process of the pioneer round of translation itself may enhance the conversion of initiation complexes, suggested by the evidences that both removal of EJCs and replacement of PABPN1 by PABPC1 require occurrence of the pioneer round of translation. However, translation alone does not augment the replacement of CBC by eIF4E.

Importin α (IMPα) is a member of the importin/karyopherin family of nucleocytoplasmic transport proteins. It generally binds the nuclear localization signal (NLS) of cytoplasmic cargo, such as the N-terminal end of CBP80 (Goldfarb et al. 2009). The direct interaction of nuclear transport receptor importin β (IMPβ) with IMPα in the cytoplasm promotes the nuclear import of IMPα–NLS complex. The association between IMPβ and IMPα-cargo is regulated by the GTPase Ran (Lee et al. 2005; Cook et al. 2007), as the binding of IMPβ to GTP-Ran dissociates IMPβ from IMPα and substrate. It has been shown that the binding of IMPβ to IMPα-CBC complex in the cytoplasm promotes the replacement of CBC by eIF4E at the 5'-cap of mRNAs (Sato and Maquat, 2009). This suggested that the dissociation of CBC from the mRNAs promotes the binding of eIF4E to unoccupied 5' caps of mRNAs, which is independent of translation.

Translation initiation factor CTIF

CBP80/20-dependent translation initiation factor (CTIF) has been identified as a component of pioneer translation initiation complex (Kim et al. 2009). It directly interacts with CBP80 and is not involved in steady-state translation. Consistent with this, depletion of CTIF led to selective inhibition of the CBP80/20-bound mRNA translation *in vitro* and consequently the decreased level of CBP80-associated polysome fractions (Kim et al. 2009; Choe et al. 2012), which can be restored by addition of exogenous CTIF. But the relative distribution of eIF4E in the polysome fractions or eIF4E-bound mRNA translation was not affected. In addition, down-regulation of CTIF blocked nonsense codon mediated decay of mRNAs (Kim et al. 2009). CTIF contains the middle domain of eIF4GI (MIF4G) and is linked with eIF3 and eIF4AIII. While the molecular mechanism for mRNAs remains unclear, CTIF is found to interact with eIF3g, a component in the eIF3 complex (Kim et al. 2009). eIF3 complex is involved in ribosome recruitment. It is apparent that CTIF functions as an adaptor protein to assist recruitment of ribosome to CBP80/20-bound mRNAs via eIF3 complex.

Thesis Research

Cytotoxic T cells monitor MHC class I complexes on antigen presenting cells (APCs) for the potential presence of any non-self peptides that could derive from viral infection or cancerous cells. APCs present antigens to CD8+ T cells in a "blind" manner, without knowing which antigenic peptides are self or foreign. Therefore the key for immune surveillance is to sample antigens from a wide range of precursors. This ensures that foreign antigens from all sources are presented. In addition, immune evasion mechanisms tend to prevent steady-state translation (Janeway et al. 2001). Therefore antigen derived from alternative methods of translation could be highly significant for immune surveillance. One interesting question is what sampling strategies the cell takes to maximize immune surveillance.

One finding on the comprehensive list of MHC I peptides suggests an impressive diversity of antigen precursors with a wide range in cellular functions, locations and abundance (Rammensee et al. 1995). This is consistent with the immune surveillance principal that antigen processing pathway aims to act on most, if not all, proteins available in the cell. Another important study was done to assess correlation between transcriptome (cellular mRNA levels) and immunopeptidome (surface MHC-peptide density) with combined microarrays and mass

spectrometry in the EBV-transformed B-cell lines (Granados et al. 2012). The data indicates that density of MHC I peptides derived from transcripts bearing miRNA response elements is not correlated with their mRNA levels, as about half of identified peptides are derived from low-abundance mRNAs. These results raise the question on the competition among peptides, especially the peptides derived from low abundant or even a single copy of translation product. Involvement of the pioneer round of translation in MHC I peptide synthesis may explain why every endogenous peptide species can be equally sampled by MHC I molecules. One advantage of the pioneer round of translation is that it generates a diverse spectrum of polypeptides with identical quantity, provided that it takes place only once on every mRNA. Furthermore, the possible rapid degradation of pioneer translational products, which are presumably have no function in the cell, could provide for rapid presentation of antigens and thus early detection of virus- or tumor-specific peptides. The following chapters focus on studies on whether the pioneer round of translation is involved in MHC I antigen presentation.

Chapter 2: CBP80 deficiency decreases surface MHC class I presentation

Summary

It has been known that MHC class I molecules mainly sample peptides from newly synthesized ribosomal products, of conventional or unconventional sources, including cryptic translation and DRiPs (Shastri et al. 2002; Yewdell et al. 1996). The pioneer round of translation generates polypeptides from almost all new mRNAs; we reasoned that inhibition of pioneer round of translation would have effect on antigen generation. After MHC class I complexes are transported onto cell surface, they usually remain stable on the cell surface for hours and even days (Yewdell, 2006). Therefore the overall level of MHC I surface expression may not be altered instantly upon the changes in cellular peptide quantity. To assess the generation of antigenic peptides in living cells, we employed an "acid wash" protocol (Storkus et al. 1993) that allows us to track the newly assembled MHC I peptide complex on the cell surface. The acid wash irreversibly removes existing MHC I peptide complex on cell surface with a pH3.1 citric acid buffer, while the cell membrane remains intact. The bound peptides will be dissociated from MHC I molecules and β2-microglobulin will be released from class I heavy chain. Cells are continued to culture in growth medium, during which MHC I molecules in the ER are loaded with new peptides and exported to the cell surface. The assembly rate of new pMHC-I reflects the supply of peptides inside cell. Nuclear cap binding protein CBP80 is crucial for the initiation of the pioneer round of translation. We found that absence of CBP80 decreased surface MHC class I presentation, which suggests that pioneer round of translation is indispensable for intact MHC class I presentation.

Results

Loss of CBP80 does not affect the overall protein biosynthesis

In general, newly synthesized and spliced mRNA will be translated by a pioneer round of translation. mRNAs containing PTCs will be degraded by NMD after being recognized during the pioneer round of translation. Normal transcripts undergo multiple rounds of steady-state translation before the end of their life span. Therefore it can be assumed that the pioneer round of translation only contributes a very small fraction of protein to the overall intracellular protein pool. To confirm whether the down-regulation of CBP80 alters the overall synthesis of polypeptides, we assessed the new protein synthesis using radioactive S^{35} incorporation assays.

CBP80 siRNA transfected HeLa-K^b (HeLa cells stably expressing murine MHC class I molecule K^b) cells were incubated in DMEM medium free of methionine/cysteine for 30 minutes after which S^{35} radiolabeled cysteine/methionine was added into the culture. 15 minutes later, excessive radioactivity was washed off, cells were lysed and lysates were loaded on a reducing SDS-PAGE gel for separation. The amounts of S^{35} incorporation was measured by phosphoimaging and quantified. Cells transfected with scrambled siRNA served as a positive control and cells treated with cycloheximide were used as a negative control. Pre-incubation with 100 μg/ml translation inhibitor cycloheximide abolished generation of new polypeptides in the cells (**Fig 1**). Down-regulation of CBP80 led to an undetectable decrease in S^{35} labeled proteins,

compared to mock transfected cells (**Fig 1b-c**). The depletion of CBP80 was confirmed with western blotting (**Fig 1d**).

Targeting of elF4E and 4E-BP1 reduces overall protein biosynthesis

Eukaryotic translation initiation factor 4E (eIF4E) is a limiting component of conventional translation initiation. eIF4E binding protein 1 (4E-BP1) directly interacts with eIF4E. Association between hypophosphorylated 4E-BP1 and eIF4E inhibits assembly of initiation complex and represses cap-dependent translation. 4E-BP1 is inactive when phosphorylated by kinases such as insulin stimulated MAP-kinase or mTORC1 in response to various cellular signals. Phosphorylated 4E-BP1 dissociates from eIF4E and consequently activates mRNA translation. 4E-BP1-AA is a dominant negative mutant in which alanine replaces two serine residues Thr-37/46 (Gingras et al. 2001). Therefore it remains unphosphorylated and constitutively binds eIF4E.

We suspect block of steady translation using eIF4E RNAi impairs overall protein synthesis and consequently cellular processing. To test our hypothesis, HeLa-K^b cells transfected with eIF4E siRNA or 4E-BP1-AA DNA plasmid were subject to radioactive S^{35} incorporation assay. 4E-BP1-AA led to significant reduction in translation, as reflected by the diminished incorporation of S^{35} in comparison to cells transfected with scrambled siRNA (**Fig 2**). Expression of 4E-BP1-AA protein in the transfected cells was confirmed by western blotting probed with an antibody recognizing HA-Tag fused to 4E-BP1 (**Fig 2d**). The addition of eIF4E siRNA almost completely stalled translation of new proteins, as observed cycloheximide (**Fig 2b-c**). The results suggested that blocking steady-state translation by expressing either dominant negative 4E-BP1-AA or eIF4E siRNA reduced generation of new polypeptides.

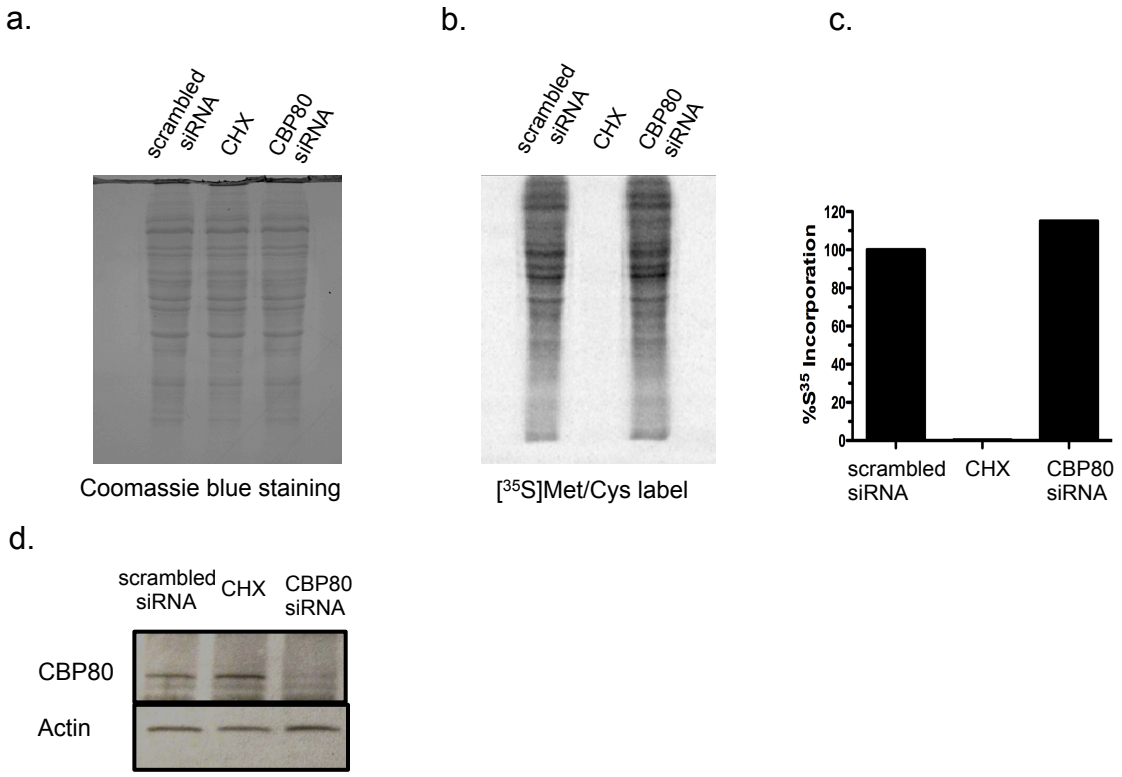
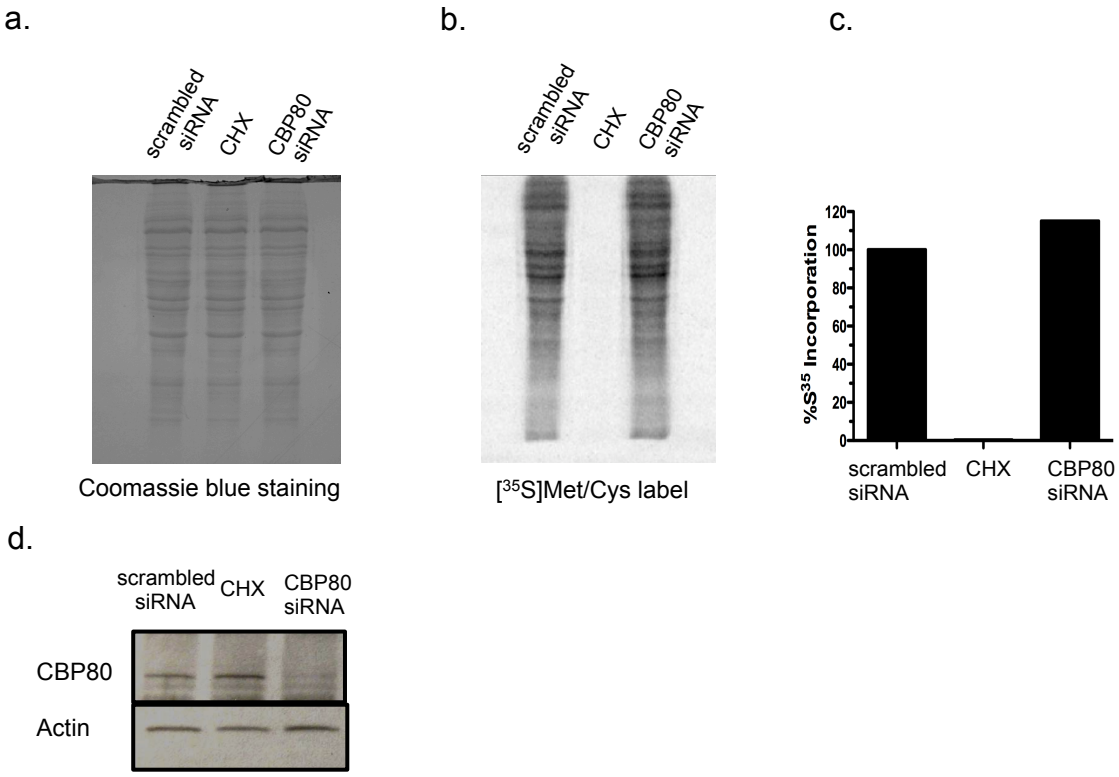


Figure 1. Inhibition of CBP80 does not affect overall protein synthesis. Radioactive S^{35} incorporation assay measured newly synthesized proteins in HeLa-K^b cells incubated with cycloheximide (100 µg/ml, 2 hours) or transiently transfected with scrambled or CBP80 siRNA (48 hours). The treated cells were incubated with S^{35} [Met/Cys] in Met/ Cys-free medium for 15 minutes and subjected to western blotting. (a) Coomassie blue stained SDS-PAGE gel. (b) Phosphor-imaging of dried SDS-PAGE gel. (c) The amount of S^{35} labeled newly synthesized proteins was quantified by setting the scrambled siRNA sample to 100 percent and the rest relative to the control. (d) Western blotting of the samples in (a-c).



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Targeting of eIF4E reduces K^b protein level

The results of S^{35} incorporation assay indicated that the absence of eIF4E in the cell decreased the cellular production of new proteins, whereas the absence of CBP80 did not (**Fig 3**). To test if the absence of eIF4E or CBP80 reduces the K^b expression level and in turn impairs MHC I antigen processing, mouse MHC class I H-$2K^b$ expression level was examined by western blotting upon knockdown of CBP80 or eIF4E (**Fig 4b**). In line with the results of S^{35} incorporation experiment, a reduced level of K^b expression was observed in the cells treated with eIF4E siRNA but no detectable difference in the cells treated with control or CBP80 siRNA.

Endoglycosidase H (Endo H) will only remove simple N-linked glycans which are added to the proteins in the endoplasmic reticulum (ER), while complex N-glycosidic linkage found in the Golgi are Endo H-resistant and can be cleaved by PNGase F (Comunale et al. 2004; Dwek et al. 1993; Dwek et al. 2002) (**Fig 4a**). Endo H and PNGase F assay is often used to assess the distribution of secreted proteins in the ER and Golgi. MHC class I complex are retained in the ER until they are loaded with peptide substrates and transported onto the cell surface. HeLa-K^b cells treated with or without CBP80 or eIF4E siRNA were lysed and subjected to Endo H or PNGase F enzymatic reaction. The K^b expression level in the digested cell lysates were resolved by western blotting (**Fig 4c**). Endo H treatment revealed two bands at around 48kDa and 40kDa, presumably derived from the high-molecular-weight K^b form in the untreated sample. The upper band represents the K^b molecules in the post-ER compartments while the lower band represents K^b retained in the ER. Glycosidase PNGase F removes both simple and complex N-linked oligosaccharides. A single band of K^b protein at around 40 kDa was observed after pretreatment with the PNGase F. There was less amount of K^b in the cells treated with eIF4E siRNA compared to the cells treated scrambled or CBP80 siRNA (**Fig 4c**). Taken together, these results suggest that knockdown CBP80 does not reduce MHC class I molecule expression level whereas knockdown of eIF4E does. Therefore, I will focus on the effect of CBP80-downregulation on the MHC I peptide presentation in the following experiments because knockdown of eIF4E largely reduced new protein synthesis and K^b protein expression, which could in turn interfere with the MHC antigen processing pathway.

Inhibition of pioneer translation decreases surface MHC class I presentation

Human HeLa cells and HeLa-K^b stable cell lines were used to examine the expression of human HLA A/B/C as well as mouse K^b peptides during acid washing experiments. To block the pioneer round of translation and bulk translation, cells were depleted for CBP80 using small interfering RNA (siRNA), or scrambled siRNA for nonspecific effects of siRNA. To better estimate the cumulative effects of translational inhibition, cells were also pretreated for 2 hours with 100ug/ml of protein synthesis inhibitor cycloheximide to stop overall production of new protein. After a 2 minute acid wash, the acid pH was neutralized with PBS and the cells were allowed to recover in normal culture medium during which MHC class I complex continued to form in the ER. The newly formed pMHC I can be monitored, immediately after the acid wash and 3 or 6 hours later, by flow cytometry using peptide dependent anti-class I antibodies such as W6/32 for human HLA and 5F1 for the transfected mouse K^b. In addition, cells were stained with TORPO-3 to exclude the dead cells from analysis.

The flow cytometry data showed gradual increase of surface peptide-K^b from 3 hours to 6 hours after acid wash. The results indicated surface expression of K^b significantly decreased in

the cells treated with CBP80 siRNA (**Fig 5**). As expected, the surface expression was even lower in the cells incubated with cycloheximide, which should block both pioneer and bulk translation (**Fig 5**). For example, at 3-hour time point, the number of cells with recovered surface peptide-K^b reproducibly diminished by 45% in CBP80-depleted population and 88% in cycloheximide treated population (**Fig 5**). The observation was consistent with HLA expression in HeLa cells (**Fig 6**). Therefore, it appears that loss of CBP80 leads to reduction in pMHC I expression. In addition, it was noted that there was a slight recovery of pMHC I in the cells incubated with cycloheximide. This is not surprising as recently loaded MHC I complexes in the ER and loading of peptides from the degraded proteins before addition of cycloheximide would progressively travel to the cell surface.

CBP80/20-dependent translation initiation factor (CTIF) has been shown to be involved in initiation of the pioneer round of translation as an adaptor protein linking CBP80/20 and the eIF3 complex (Kim, 2009; Choe 2012). Therefore, CTIF supports pioneer translation initiation by promoting recruitment of ribosome. For example, depletion of CTIF selectively blocks translation of mRNAs bound by CBP80 (Kim et al. 2009). CTIF expression in HeLa-K^b cells was down-regulated using specific siRNA against CTIF. The cells were subject to acid wash recovery assay as described. Consistent with the strong surface pMHC I reduction seen in the previous experiments (**Fig 7**), CTIF cells showed a remarkable decrease similar in magnitude to CBP80 down-regulated cells (**Fig 7**). Whether these decreases are a result of pMHC I reduction due to the reduction in the peptide supply or whether there is impaired MHC I processing pathway inside cell because of protein synthesis shutdown remains to be determined.

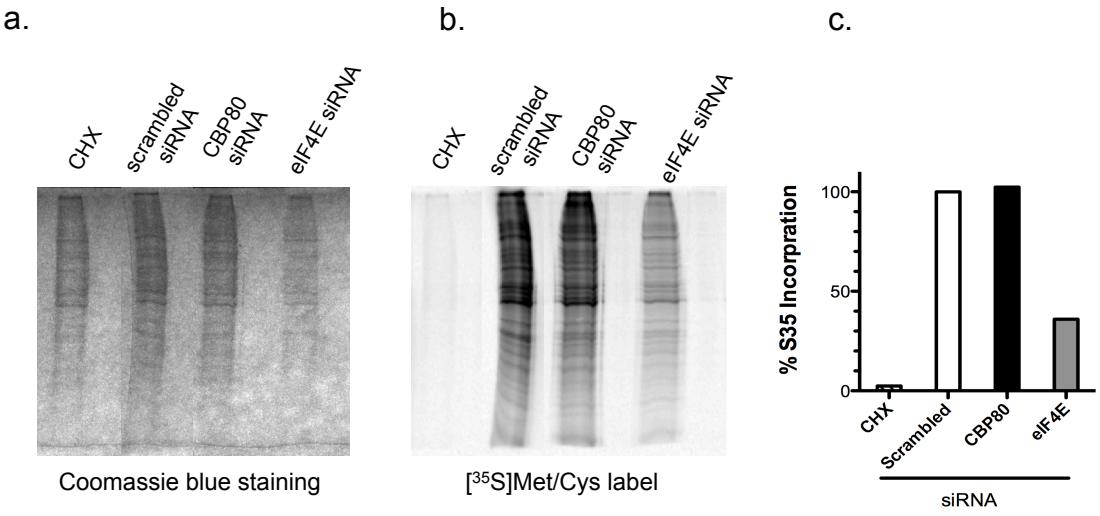


Figure 3. Inhibition of pioneer translation does not affect overall protein synthesis. Radioactive S^{35} incorporation assay measured newly synthesized proteins in HeLa-K^b cells incubated with cycloheximide (100 µg/ml, 2 hours) or transiently transfected with scrambled, CBP80 or eIF4E siRNA (48 hours). The treated cells were incubated with S^{35} [Met/Cys] in Met/Cys-free medium for 15 minutes and subjected to western blot. (a) Coomassie blue stained SDS-PAGE gel. (b) Phosphor-imaging of dried SDS-PAGE gel. (c) The amount of S^{35} labeled newly synthesized proteins was quantified by setting the scrambled siRNA sample to 100 percent and the rest relative to the control.

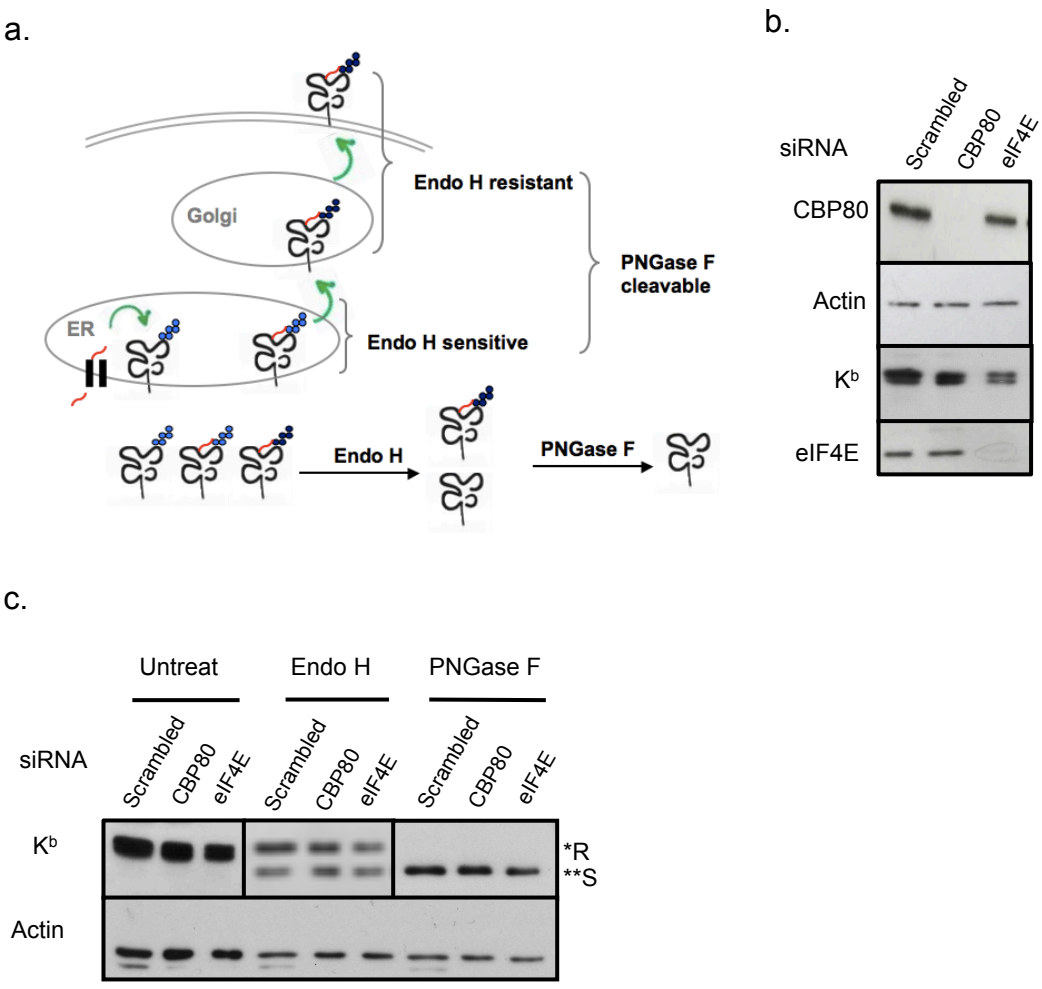


Figure 4. Down-regulation of CBP80 does not decrease K^b protein expression level. (a) Schematics of Endo H and PNGase F cleavage assay. (b) HeLa-K^b cells were transiently transfected with scrambled, CBP80 or eIF4E siRNA (48 hours). Cells were subjected to western blotting with the indicated antibodies. (c) The cell lysate of each sample in (b) was equally divided into three groups and treated with mock, Endo H or PNGase F enzymes for 2 hours. The treated lysates were then subjected to western blotting with the indicated antibodies. *R represents the K^b resistant to Endo H cleavage and **S represents the K^b sensitive to Endo H cleavage.

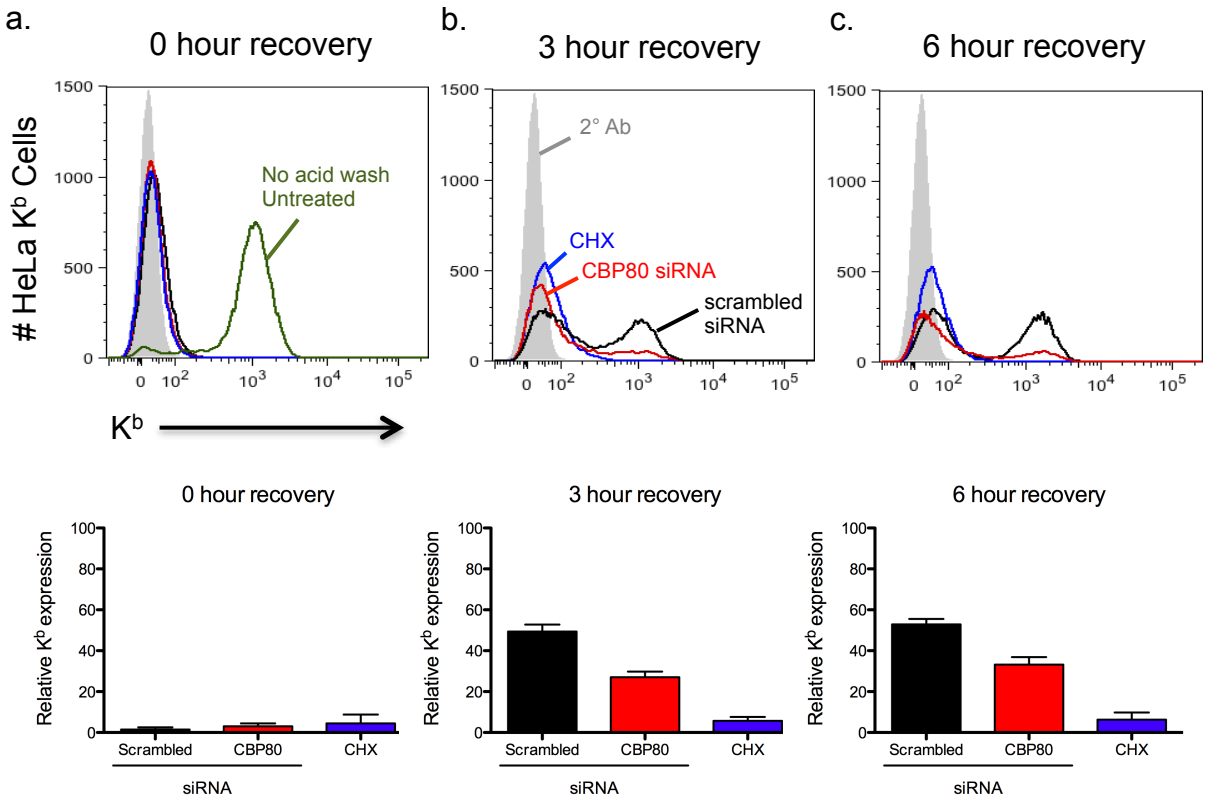


Figure 5. Down-regulation of pioneer translation reduces surface K^b expression. (a-c) Surface expression of H-2K^b on the HeLa-K^b cells that were pre-incubated with cycloheximide (100 µg/ml, 1 hour) or transiently transfected with scrambled or CBP80 siRNA (72 hours) during acid wash assay. Cells were co-stained with 5F1 and To-Pro-3 viability dye and then analyzed by flow cytometry (a) immediately after acid wash, or after cultured in growth medium for (b) 3 hours and (c) 6 hours. Data is representative of 5 independent experiments. Bar graphs summarize percentage of living cells with K^b expression in 3 independent experiments.

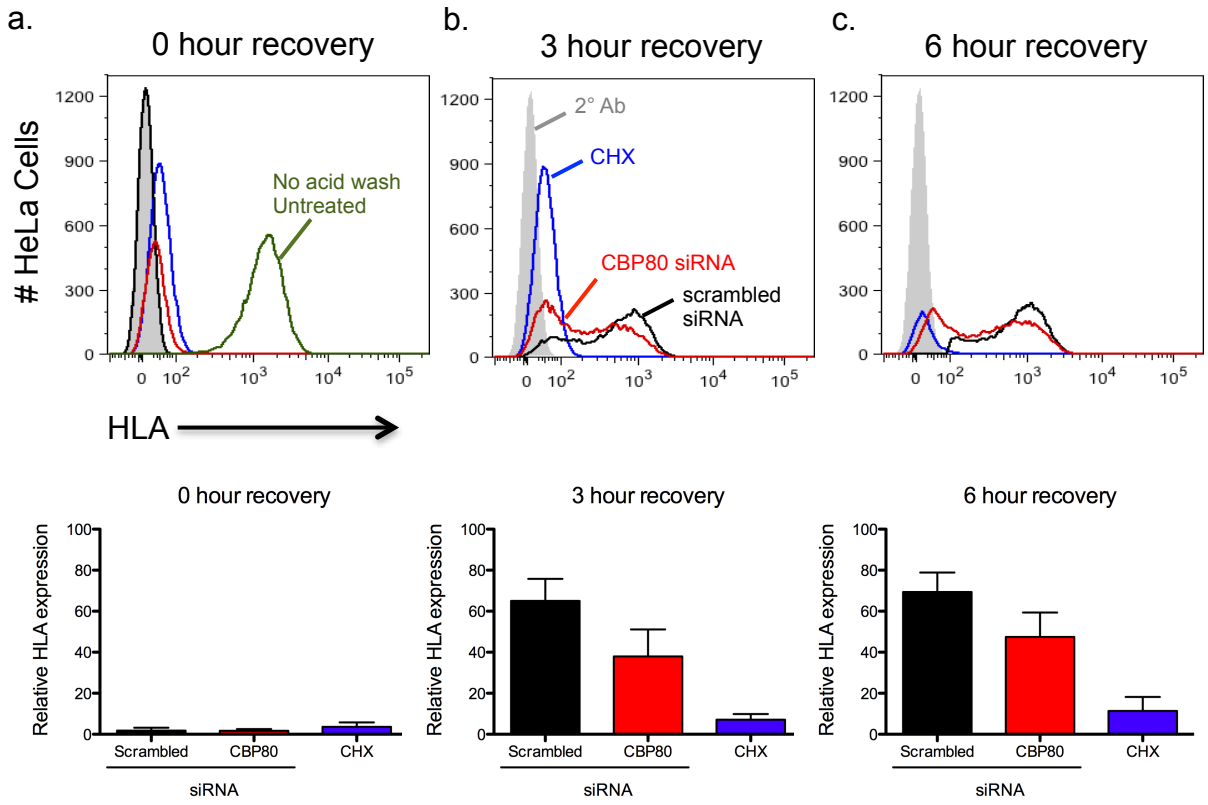


Figure 6. Down-regulation of pioneer translation reduces surface human pMHC I expression. (a-c) Surface expression of HLA-A,B,C on HeLa cells that were pre-incubated with cycloheximide (100 µg/ml, 1 hour) or transiently transfected with scrambled or CBP80 siRNA (72 hours) during acid wash assay. Untreated and not acid-washed cells were used as a negative control. Cells were co-stained with W6/32 and To-Pro-3 viability dye and then analyzed by flow cytometry (a) immediately after acid wash, or after cultured in growth medium for (b) 3 hours and (c) 6 hours. Data is representative of 5 independent experiments. Bar graphs summarize percentage of living cells with HLA expression in 3 independent experiments.

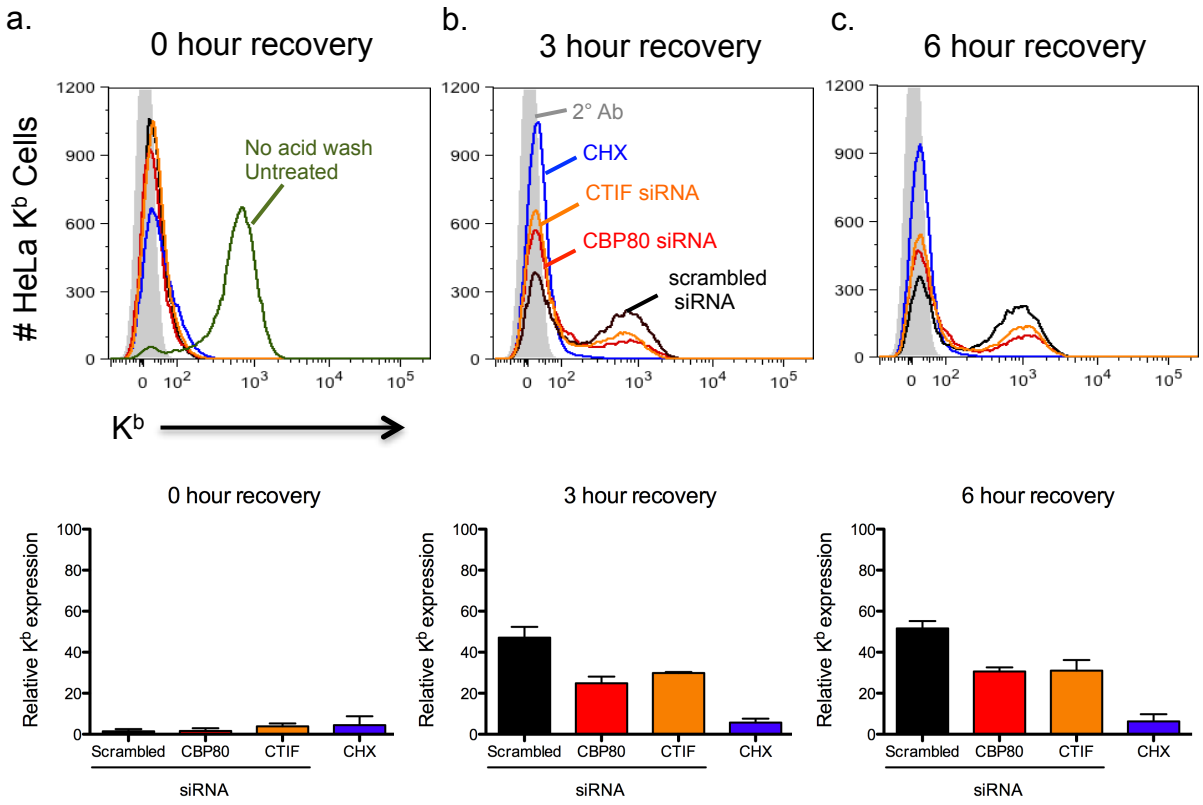


Figure 7. Inhibition of CTIF reduces surface pMHC I expression. (a-c) Surface expression of H2-K^b on HeLa-K^b cells that were pre-incubated with cycloheximide (100 µg/ml, 1 hour) or transiently transfected with scrambled, CBP80 or CTIF siRNA (72 hours) during acid wash assay. Cells were co-stained with 5F1 and To-Pro-3 viability dye and analyzed by flow cytometry (a) immediately after acid wash, or after cultured in growth medium for (b) 3 hours and (c) 6 hours. Data is representative of 3 independent experiments. Bar graphs summarize percentage of living cells with K^b expression in 3 independent experiments.

Enhancement of pioneer translation accelerates MHC I complex recovery

mRNAs bound by CBP80/20 cap-binding protein complex (CBC) undergo a pioneer round of translation that is important for mRNA quality control. mRNA containing premature stop codons will be recognized during the pioneer round of translation and destroyed by the associated NMD machinery. Normal mRNA passes this quality control and is, thereafter, taken over by the eIF4E cap-binding protein in the subsequent rounds of steady-state translation. However, it is not clear how the pioneer round of translation complex is remolded to eIF4E-dependent translation initiation complex. The pioneer round of translation alone did not affect replacement of CBP80/20 by eIF4E in cytosol, although it enhanced the replacement of poly-(A) binding protein PABPN1 (Sato and Maquat, 2009).

Importin β (IMPβ) was identified to play a role in the remodeling of the pioneer round of translation complex (Sato and Maquat, 2009). The binding of IMPβ to IMPα in the cytosol mediates the import of IMPα-substrate complex into the nucleus. Subsequently, the binding of IMPβ to the CBC-IMPα at the cap of newly synthesized mRNA promotes the import of CBC into the nucleus and thus allows the binding of eIF4E to the unoccupied 5'-cap of mRNA. The co-immunoprecipitation experiment showed that the association between mRNA and CBP80 was decreased by the increased interaction of IMPα with IMPβ and vice versa (Sato and Maquat, 2009). Based on these studies, we hypothesized that replacement of CBC by eIF4E would be delayed if association of IMPβ with IMPα was inhibited, which could subsequently lead to an increase in MHC I antigen presentation.

The binding of nuclear RAS-related small guanine nucleotide-binding protein RAN with IMPβ releases IMPα-substrate complex into the nucleoplasm, after which the complex of RAN-GTP-IMPβ is transported into the cytoplasm where the hydrolysis of GTP in RAN-GTP dissociates IMPβ from RAN-GTP (Cook et al. 2007). RanQ69L is a GTPase deficient variant of RAN that binds but cannot hydrolyze GTP (Bischoff et al. 1994; Klebe et al. 1995). Therefore, RanQ69L inhibits import of CBC into nuclei by sequestering IMPβ from IMPα. In addition, the binding of IMPβ to IMPα can be interfered with using the IBB (IMPβ-binding) domain of IMPα (Sato H and Maquat L, 2009), a 41-amino-acid in length region of IMPα that binds directly to IMPβ (Görlich et al. 1996; Palmeri and Malim 1999), functioning as a dominant negative protein. To test our hypothesis, a DNA plasmid expressing RANQ69L or IBB was transiently transfected into HeLa-K^b cells. Surface expression of K^b peptide complex was measured by flow cytometry after acid wash recovery (**Fig 8**). The recovery rate of K^b complexes was enhanced on the cells expressing RANQ69L or IBB, compared to mock transfected cells. The data showed that surface K^b pMHC I expression increased when the association between CBC and 5'-cap of mRNA was enhanced. This is in line with our previous finding that CBC-dependent translation contributes to the pool of MHC class I peptides.

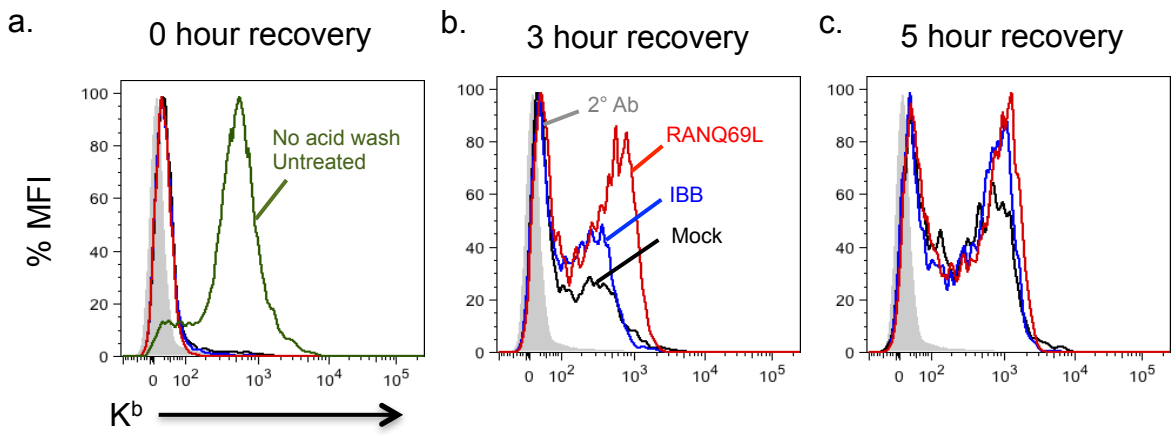


Figure 8. Enhancement of pioneer translation increases surface pMHC I expression. (a-c) Surface expression of H2-K^b on HeLa-K^b cells that were transiently transfected with pcDNA-V5-RAN(Q69L) or pEYFP-IBB construct for 48 hours, during acid wash assay. Cells were co-stained with 5F1 anti-K^b and To-Pro-3 viability dye, and then analyzed by flow cytometry (a) immediately after acid wash, or after cultured in growth medium for (b) 3 hours and (c) 5 hours. Data is representative of 2 independent experiments.

Discussion

Taken together, inhibition of the pioneer round of translation decreased surface MHC class I HLA and K^b expression, without detectable reduction in overall protein biosynthesis or MHC I molecular level (**Fig 4b-c**).

In the parallel experiments of acid wash recovery, inhibition of steady-state translation also led to a slower recovery rate of new MHC class I HLA and K^b complexes on the cell surface. Upon closer examination, down-regulation of eIF4E reduced pMHC I expression slightly more in HeLa-K^b cells and slightly less in HeLa cells relative to CPB80. It might be due to the different specificity for peptides by the endogenous HLA MHC I molecules of HeLa cells. For example, HLA-C molecules are more selectively in peptide binding than the HLA-A and HLA-B molecules. This limiting step results in a slow transportation of HLA-C peptide complex to the cell surface. Therefore, we suspected that deficiency in peptide supply resulting from blocking steady-state translation was not a limiting step for HLA-C expression. Another concern of prolonged down-regulation of eIF4E is that the absence of eIF4E inhibits the overall protein synthesis, it may cause downstream negative effects on multiple cellular signaling, MHC class I levels in the ER and other processes that contribute to antigen processing.

Based on our hypothesis that depletion of CBP80 decreases the amount of peptides available for MHC I molecules, the amount of empty K^b in the absence of CBP80 was likely to be higher than that in the untreated cells. However, we did not observe a significantly increased level of empty K^b retained in the ER compared to the peptide bound K^b molecules in post-ER compartments, with knockdown of CBP80. Because the majority of K^b complexes are expressed on the cell surface, it is possible that K^b complexes form before the detection of transient siRNA depletion by the antibody as K^b in post-ER compartments. To bypass the limitation of siRNA depletion and immunoblotting, S^{35} pulse-chase combined with immunoprecipitation of K^b can be performed to visualize the newly formed empty K^b in the ER from newly exported K^b molecules.

Because RanQ69L and IBB block the association of IMPβ with IMPα-CBC, CBC cannot be promptly replaced by eIF4E in the cells over-expressing RanQ69L and IBB. This could lead to an increase of the pioneer round of translation and inhibition of steady-state translation. Interestingly, an increased recovery rate of surface K^b pMHC I expression was observed in the cells expressing RANQ69L or IBB compared to the untreated cells. This raised the possibility that the pioneer translational products are rapidly degraded and enter the MHC I antigen pathway after synthesis. To test the possibility, kinetics assay on the presentation of MHC I antigens can be performed with or without the inhibition of IMPβ and IMPα-CBC binding complex.

Chapter 3: Pioneer translation products can be presented by MHC class I

Summary

Although the pioneer round of translation and steady-state translation are functionally distinct and use different cap-binding proteins, they share many common initiation factors and use the common pool of ribosomes and amino acids to generate polypeptides from same mRNA templates. Due to the technical difficulty in separating translational products of the pioneer round of translation from those of steady-state translation *in vivo*, it is necessary to build a cell-based system that allows us to measure presentation of peptides specifically from the pioneer translation substrates. mRNAs containing premature termination codons (PTCs) are presumably degraded by NMD after undergoing the pioneer round of translation. The constraint of these mRNAs by NMD makes them the best candidates for a reporter of the pioneer round of translation.

We generated SIINFEKL (SL8) epitope reporter constructs by fusing the SL8 gene to wild-type human hemoglobin β gene or a mutant variant that contains PTCs. The mRNA level of SL8 in the mutant reporter was substantially lower than the wild-type because of the degradation of the mutant hemoglobin β mRNA. But both reporters generated identical amount of SL8 peptides. This suggests that the mutant reporter may be more efficient at generating peptides. In the cells with knockdown of CBP80, both reporters generated significantly less amount of SL8 peptides. These results indicated that the pioneer round of translation was involved in production of SL8 peptide in cell.

Results

Generation of NMD-reporter constructs for antigen presentation

Human hemoglobin β gene has a naturally occurring mutant form that contains one nonsense mutation at codon 39 (CAG -> TAG) (Baserga et al. 1988). This nonsense mutation results in the premature termination of its mRNA translation. It has been reported that the degradation of β-globin mutant mRNA is due to nonsense-mediated mRNA decay and the levels of the mutant mRNA were around 5% to 15% of normal levels (Boelz et al. 2006). We took advantage of this mutation to design a model for the study of polypeptide products of the pioneer round of translation. Luciferase based NMD-reporter assays using hemoglobin β gene were previously reported by Boelz and his colleagues (Boelz et al. 2006). They fused a Renilla-luciferase to either wild-type (WT) β-globin or mutant β-globin with a PTC at position 39 (NS39) and found that the protein and mRNA level.of NS39 was around 9% and 15%, respectively, of the wildtype expression.

We constructed a pair of DNA reporters with SIINFEKL minigene fused into either WT or NS39 luciferase-β-globin constructs. The SIINFEKL gene was inserted right after the start codon of β-globin using PCR (**Fig 9a**). As previously reported (Boelz et al. 2006), the relative mRNA level of the NS39 reporter was remarkably lower than that of the wild-type reporter in the real-time PCR experiment (**Fig 9b**). To directly quantify the amount of proteins actually translated from the reporters, a dual luciferase assay was performed on the transfected cells to measure the levels of luciferase activities corresponding to WT and NS39 β-globin (**Fig 9c**). Consistent with the real-time PCR results, the luciferase activity in the cells transfected with

NS39 reporter was only around 15% of WT reporter-transfected cells (**Fig 9c**). This indicated that the NS39 PTC resulted in the destruction of the reporter mRNA. However, the surface expression of SL8 peptide was low with both WT and NS39 reporters.

As antigen processing of an epitope can also be affected by the location of the epitope in the protein precursor, it is possible that the Renilla-luciferase, which is on the N-terminus of SL8, interfered with the cleavage and transport of the SL8 precursor peptide. Therefore, the luciferase gene was removed from the original reporter while the rest of the construct sequence including SL8-βglobin remained the same (**Fig 10a**).

PTC and WT reporters stimulate T cells equally

To measure the mRNA levels of the new SL8-βglobin reporters, we extracted the total RNA from the HeLa-K^b cells transfected with WT or PTC SL8-βglobin constructs. Semi-quantitative PCR experiments were performed on the cDNAs, that were *in vitro*-transcribed from the RNAs, using two sets of primers against the regions before and after the SL8 minigene as indicated (**Fig 10a**). The PCR data demonstrated that the relative mRNA level of the PTC reporter was 8 folds lower than that of the wild-type reporter (**Fig 10b**). It implied that the mutant mRNA was mostly degraded by NMD in cells.

To assess the peptide presentation by the HeLa-K^b cells transfected with two different reporters, B3Z T cell assay was performed. The responses of B3Z T hybridomas to the two sets of transfected cells were comparable (**Fig 10c**), given the dramatic difference in mRNA level of SL8. The results implied that MHC class I K^b molecules presented SL8 peptides generated from the pioneer round of translation, on the cell surface. However, from this experiment we could not rule out the possibility that SL8 peptides were generated from any remaining PTC mRNA in steady-state translation.

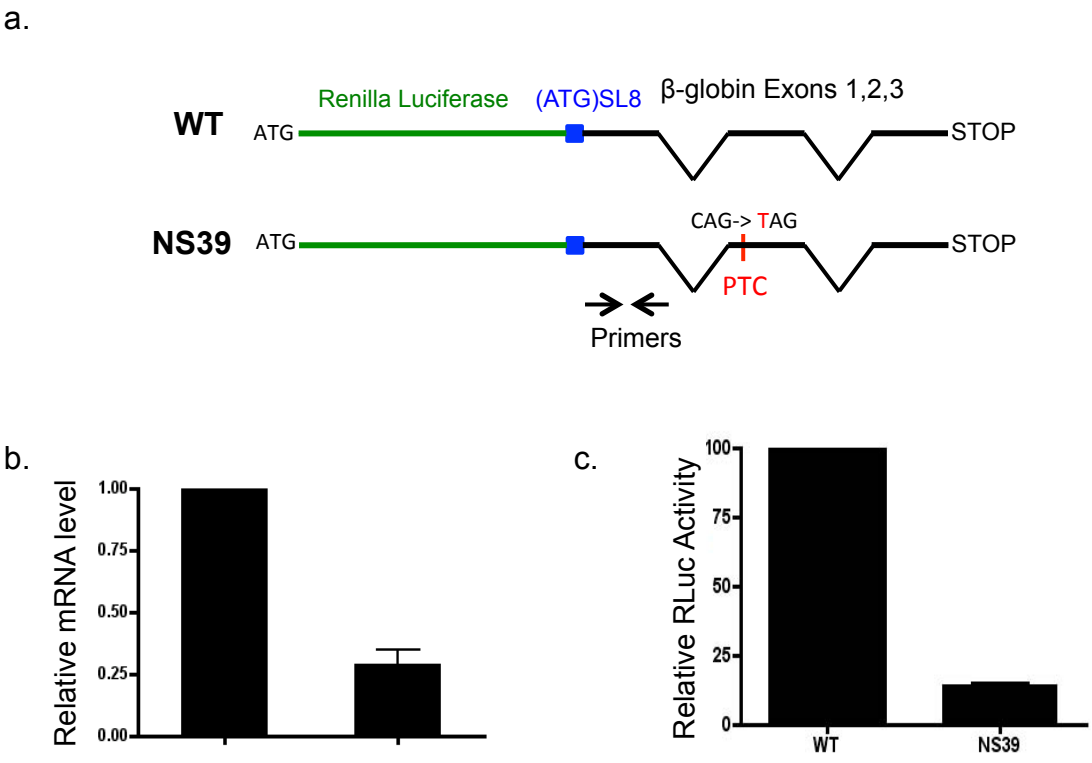


Figure 9. Generation of luciferase SIINFEKL(SL8)-β-globin reporter. (a) Schematic of wild type (WT) renilla-luciferase-SL8-βglobin and premature (NS39) renilla-luciferase-SL8-βglobin genes. The SL8 epitope, globin gene, premature stop codon (PTC) and normal stop codon are indicated. The binding positions of PCR primers for RT-PCR in (b) are labeled. **(**b) WT and NS39 construct were transiently transfected into the HeLa-K^b cells. The mRNA levels of SL8-βglobin in two cell samples were measured by reverse quantitative PCR and normalized with internal GAPDH mRNA level. (c) Firefly-luciferase construct and WT or NS39 construct were transiently transfected into the HeLa-K^b cells. The Renilla-luciferase activity of each sample was measured and normalized with internal firefly luciferase activity. Above data are average of three independent experiments.

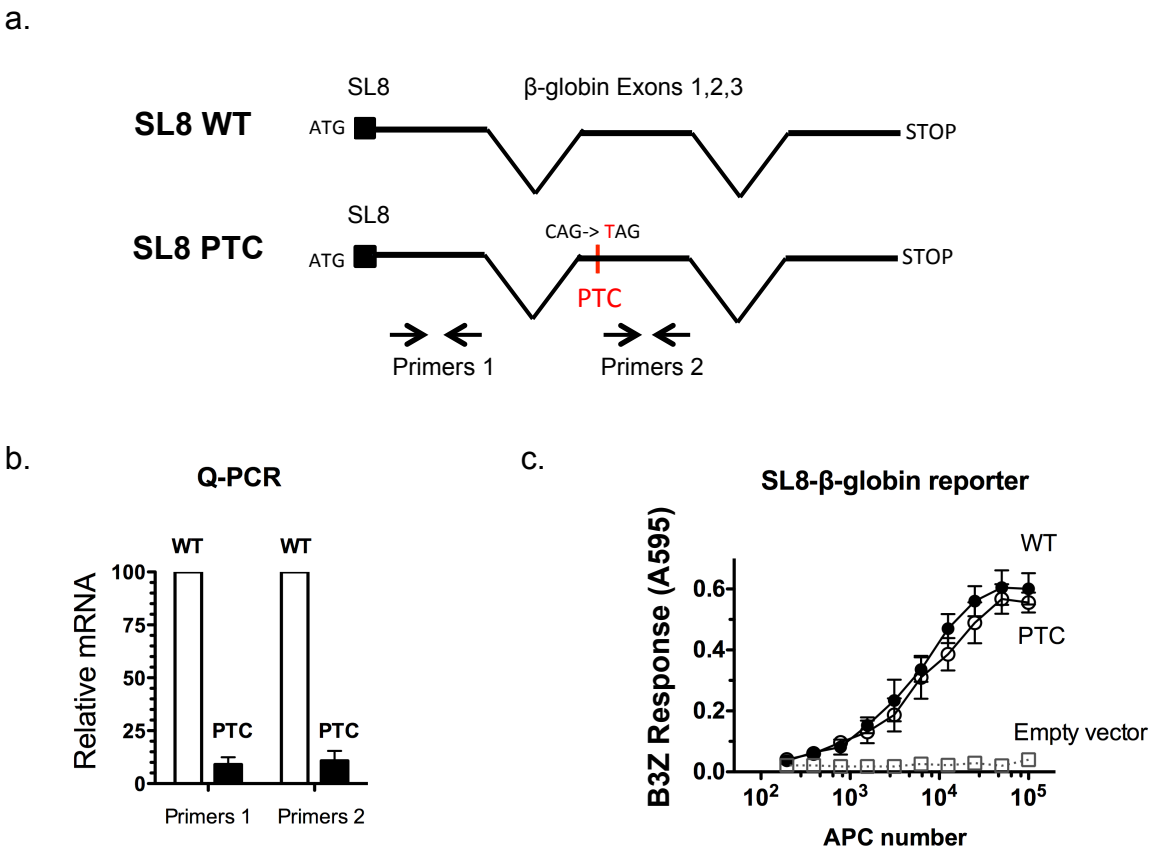


Figure 10. Generation of SL8-β-globin reporter. (a) Schematic of wild type (WT) SL8-βglobin and premature (PTC) SL8-βglobin genes. The SL8 epitope, beta-globin gene, premature stop codon (PTC) and normal stop codon are indicated. The binding positions of PCR primers for RT-PCR in (b) are labeled. (b) HeLa-K^b cells were transfected with WT or PTC SL8 constructs as indicated (a). Total RNA was extracted from cells to make first strand cDNAs for real time PCR. Two sets of primers as indicated in (a) were used for validation. The relative mRNA levels were normalized using internal GAPDH mRNA. (c) HeLa-K^b presentation of WT or PTC SL8 peptide to the B3Z T cell hybridoma. Data in (b) and (c) are summary of 3 independent experiments.

CBP80 deficiency disrupted MHC I presentation of SL8-reporter

To validate the effect of the pioneer round of translation on antigen presentation, we further down-regulated CBP80 in cells using siRNA. CBP80 is a key component for the pioneer translation initiation and recruitment of NMD factors. If the SL8 peptides originated from the pioneer translation products, depletion of CBP80 would lead to a diminished B3Z response to the cells transfected with either WT or PTC SL8 reporter.

To determine the effect of pioneer translation on MHC I presentation, we quantified surface expression of SL8 peptide on HeLa-K^b cells in the presence or absence of CBP80. HeLa-K^b cells were transiently transfected with either CBP80 or scrambled siRNA. After 2 days, cells were transfected with the WT or PTC SL8-βglobin plasmid. The protein expression levels of WT and PTC SL8-βglobin in the transfected cells were examined by western blotting (**Fig 11a**). The data showed that WT SL8-βglobin protein level was identical in the cells with or without CBP80, but PTC SL8-βglobin protein was barely detected. To avoid saturation of K^b-SIINFEKL on the cell surface, it is necessary to treat the cell with citric acid to remove existing pMHC I complexes from the cell surface. Following 4 hours culture, cells were incubated with B3Z T cell hybridomas and K^b-SIINFEKL recovery was measured (**Fig 11b**). Interestingly, cells without down-regulation of CBP80 showed identical K^b-SIINFEKL presentation regardless of mRNA stability or protein expression of SL8-βglobin, whereas depletion of CBP80 led to a reduction of K^b-SIINFEKL presentation in both WT and PTC SL8 reporter-transfected cells. This is consistent with our hypothesis and previous observation of slow recovery of overall MHC class I expression.

To accurately measure peptides generated by the pioneer round of translation and by steady-state translation, a similar experiment was performed. Instead of incubating transfected HeLa-K^b cells with B3Z T cells directly, the total peptide mixtures were extracted from HeLa-K^b cells after acid wash recovery. Peptides were then loaded on antigen presenting cells, K^b expressing L-fibroblasts (K89), and then cultured with the B3Z hybridoma to quantify the intracellular production of SL8 peptides (**Fig 11c**). Consistent with the cell surface presentation assay, peptides extracted from both cells stimulated B3Z equally and a reduction was observed only when CBP80 was down-regulated.

To compare this to steady-state translation, we did the same experiment with the cells depleted of eIF4E and observed even more pronounced reduction in SL8 presentation. Due to the down-regulation effect of eIF4E-deficiency on both global protein synthesis and K^b protein level, we cannot safely conclude that MHC class I molecules mainly sample peptides from steady-state translational products. On the other hand, SL8 reporter transfected cells after CBP80 depletion were still able to stimulate B3Z T cells. One explanation could be incomplete inhibition of CBP80. Another possibility that we cannot exclude is that new proteins from steady-state translation constitute a fraction of peptide pool. At this stage, it is fair to say that the pioneer round of translation and steady-state translation are both involved in generation of MHC I peptide precursors.

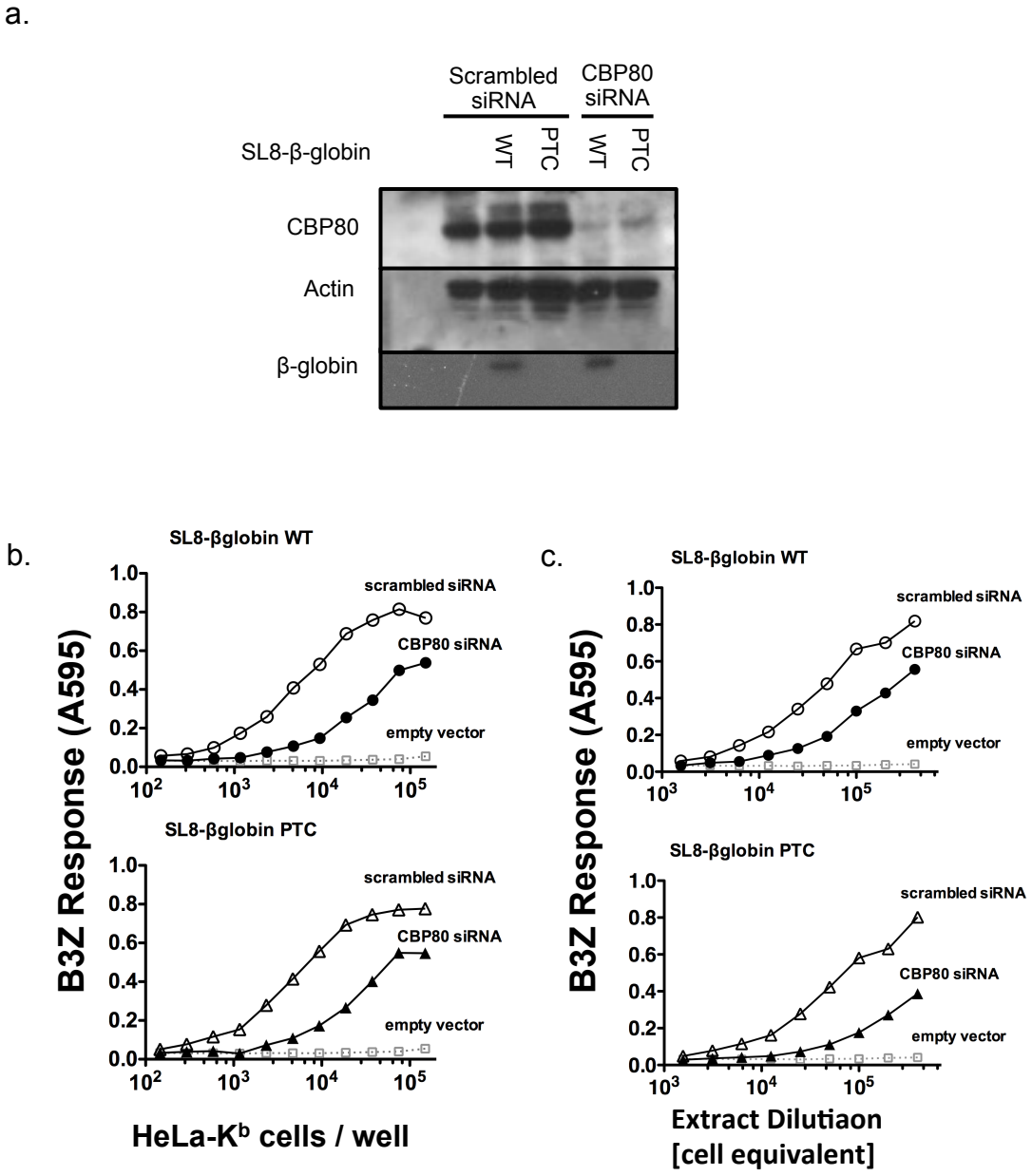


Figure 11. **MHC class I presentation of SL8 peptides in HeLa-K^b cells in the presence or absence of CBP80.** (a) HeLa-K^b cells were transfected with scrambled or CBP80 siRNA for 48 hours, and then with WT or PTC SL8-βglobin reporters or empty vector for 24 hours. Expression levels of the indicated proteins were examined by Western blotting. (b) 4 hours after mild acid washing, the cells or (c) exacted cellular peptides were presented to the B3Z T cell hybridoma. Data are representative of 3 independent experiments.

CBP80 depletion does not block nuclear mRNA export

CBP80 binds to pre-mRNAs in the nucleus. CBP80/20 complex facilitates various RNA processing events including pre-mRNA splicing, mRNA export, translation, nonsense mediated mRNA decay and microRNA (miRNA) regulated gene silencing. We have shown in the radioactive S^{35} incorporation experiments that inhibition of CBP80 did not impair the overall protein synthesis, which implied that loss of CBP80 did not compromise the mRNA export process. To test if down-regulation of CBP80 inhibits mRNA export from the nucleus to the cytosol, and specifically whether SL8 presentation was hindered by inefficient nuclear export and mRNA processing due to CBP80 depletion, we measured SL8-βglobin mRNA level in nuclear and cytosolic fractions. HeLa-K^b cells were transfected with WT or PTC SL8-βglobin constructs after depletion with scrambled or CBP80 siRNA for 48 hours. Cells were fractionated into cytosolic and nuclear fractions. Total RNAs were extracted and subjected to RT-PCR to quantify the SL8-βglobin mRNA level in the cytosol and nucleus, respectively. To confirm the fractionation, the cell fractions were tested by western blotting, using the antibodies against cytosolic protein marker α-tubulin and nuclear protein marker lamin-A (**Fig 12a**). RT-PCR results showed that SL8-βglobin mRNA level is comparable in cytosolic and nuclear fraction with or without CBP80 presence (**Fig 12b**). To test the SL8 peptide presentation in the presence or absence of CBP80, B3Z T cell response assay was performed (**Fig 12c**). Taken together, these data suggest that the reduction in SL8 peptide presentation, in the absence of CBP80, was not due to the decreased cytosolic SL8-βglobin mRNA level.

mRNAs bypassing pioneer translation generate lower presentation of peptides

NMD does not target mRNA that bound by eIF4E or the intronless mRNAs that do not contain EJCs (Ishigaki et al. 2001; Chiu et al. 2004; Hosoda et al. 2005). We have seen that loss of the pioneer round of translation reduced MHC class I presentation of SL8 peptides. Therefore, we can infer that if a transcript does not undergo the pioneer round of translation there would be fewer peptides generated. In nature, it is unknown whether such transcript exists. However we can generate mature mRNA *in vitro* by reverse transcription. The *in vitro* synthesized mRNAs have both a 5'-cap and a poly-A tail but do not contain introns or EJCs. Therefore they are not subjected to NMD degradation even if they contain a nonsense codon. The mRNAs, directly transfected into the cytosol, will be predominantly bound by eIF4E because CBP80/20 complexes are mostly located in the nucleus for new precursor mRNAs. This results in a type of mRNA that bypasses the pioneer round of translation.

To test our hypothesis that mRNA bypassing the pioneer round of translation results in peptide presentation, we generated SL8 mRNA *in vitro* by reverse transcription using WT or PTC SL8-βglobin reporter as templates. We then quantified mRNA transcripts and transfected equal amount of WT and PTC into CBP80-deficient HeLa-K^b cells. We found that both WT and PTC mRNA transfected cells stimulated B3Z hybridomas equally (**Fig 13**). In contrast to DNA transfected cells, mRNA transfected cells had the same ability in stimulating B3Z cells, with or without CPB80. The T cell responses were also dependent on the level of mRNAs transfected, suggesting that peptides generated from these mRNAs did not saturate the system. Taken together, we conclude that peptides generated during CBP80-initiated pioneer translation are presented by the MHC class I pathway.

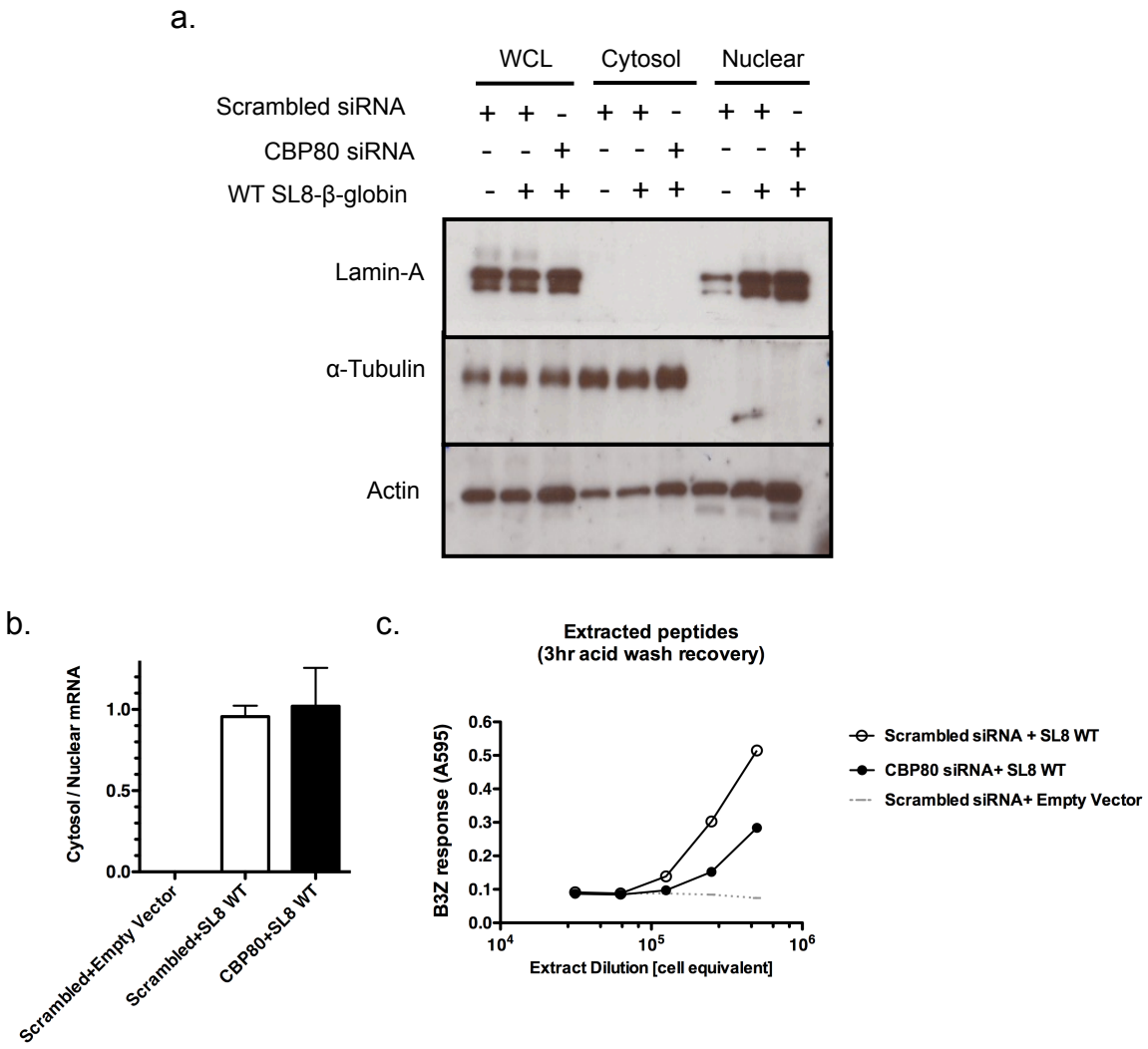


Figure 12. Depleting CBP80 does not inhibit SL8-βglobin mRNA exporting from cell nucleus. HeLa-K^b cells were treated with scrambled or CBP80 siRNA for 72 hours. 48 hours post-transfection, the cells were transfected with 2ug of wild type SL8-βglobin DNA plasmid for 24 hours before cell fractionation. The cells were divided into three groups for the experiments in (a-c). (a) Western blotting analysis of HeLa-K^b whole cell lysate (WCL), cytosol and nucleus confirmed fractionation. Actin served as loading control. (b) Quantitative RT-PCR measured the ratio of SL8-βglobin mRNA in the cytosolic fraction over the nuclear fraction after the mRNA level was normalized to the internal GAPDH mRNA level in the HeLa-K^b cell samples as in (a). The SL8-βglobin mRNA level in the HeLa-K^b cells transfected with empty vector was undetectable. (c) After mild acid washing, the cells were cultured in the growth medium for 3 hours. The peptides were then extracted from the cells. HeLa-K^b cells presented the extracted peptides to the B3Z T cell hybridoma.

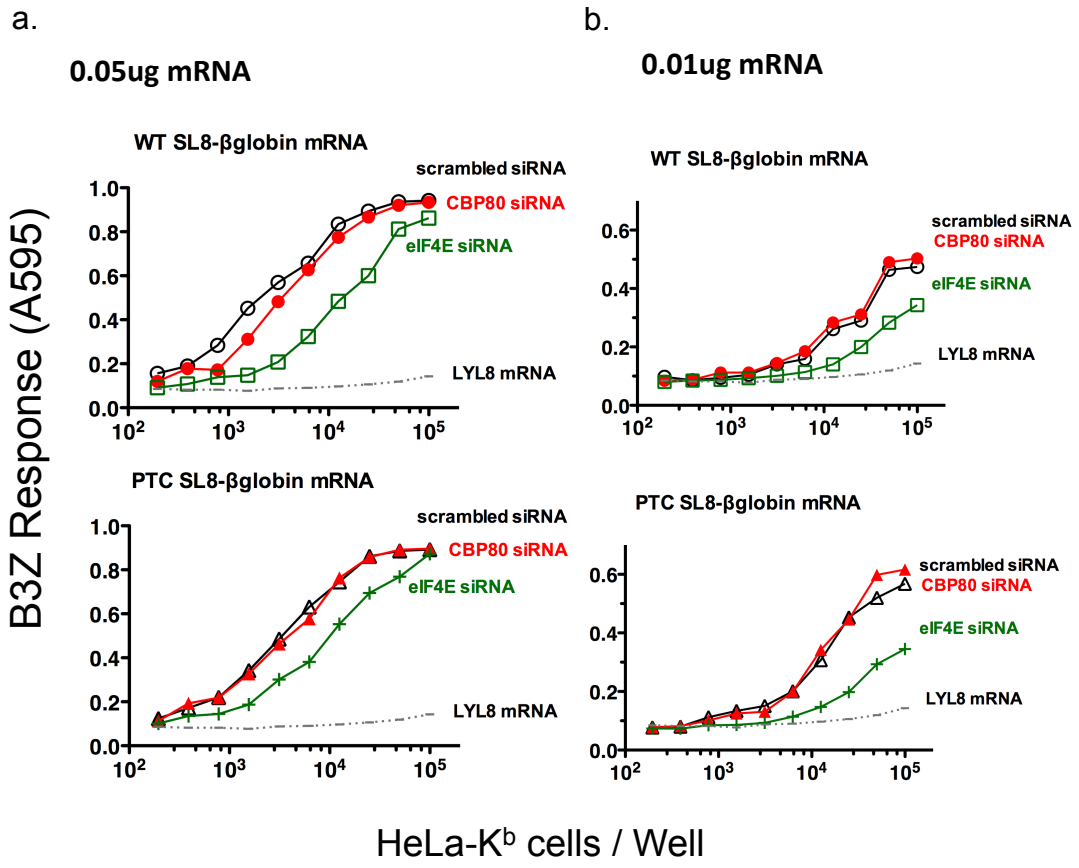


Figure 13. SL8-βglobin mRNAs bypassing pioneer translation generate lower MHC I presentation of SL8 peptides. WT or PTC SL8-βglobin mature mRNA was *in vitro* synthesized using purified RNA extracted from WT or PTC SL8-βglobin transfected HeLa-K^b cells. HeLa-K^b cells were transfected with scrambled (filled circle or triangle), CBP80 (circle or triangle) or eIF4E (cross or line) siRNA for 48 hours. The cells were then counted and re-plated with the same number of cells. (a) 0.05 ug or (b) 0.01 ug mRNAs were transfected into HeLa-K^b cells. YL8-epitope mRNA (gray dotted line) was transfected into HeLa-K^b cells as a negative control. 4 hours post-transfection, the cells were harvested and subjected to B3Z T cell response measurement.

Discussion

We established a reporter system to study the association between products of pioneer translation and MHC class I antigen presentation. K^b presentation of SL8 peptide was decreased by 30-50% when the pioneer round of translation was inhibited. The remaining surface expression of K^b-SL8 after CBP80 knockdown could be derived from other sources such as the SL8 precursor generated during eIF4E-dependent translation or due to incomplete CBP80 knockdown.

The pioneer round of translation appears to be beneficial for immune surveillance. First, almost every mature mRNA, if not all, undergoes CBP80/20 cap-binding protein complex (CBC)-mediated pioneer round of translation. It is even possible that non-canonical translation occurred on the alternative reading frame, such as the intronic sequence of mRNA, because pre-mRNAs before splicing are bound by CBC. The large diversity in the substrates of the pioneer round of translation, consequently, increases the diversity in the pool of MHC I peptides. Second, the amount of polypeptides generated during the pioneer round of translation is remarkably lower compared to the polypeptides generated during steady-state translation on each mRNA. The ability of pioneer translational polypeptides to enter MHC I antigen processing pathway explains how the immune system is able to sample peptides from relatively small pool of foreign proteins. Furthermore, if the polypeptides generated in the pioneer round of translation do not participate in cellular processing as normal proteins, they may get degraded rapidly and serve as peptide precursors timely. For example, virally infected cells and cancerous cells can be detected and eliminated before they spread.

Chapter 4: Assessing peptide supply using Fluorescence Recovery after Photo Bleaching

Summary

We want to further understand the molecular basis for the pioneer round of translation-dependent MHC class I expression. MHC class I molecules sample peptides in the ER transported through TAP. The peptide translocation causes conformational changes in TAP heterodimer complex. TAP molecules transport peptides through opened pore and return to closed conformation when inactive. This conformational change is associated with lateral mobility of TAP. Active TAP diffuses in the ER membrane at a slow rate whereas inactive TAP molecule diffuses more rapidly (Reits et al. 2000). Therefore, the TAP mobility is inversely proportional to the TAP activity. It has been shown that blocking the generation of cytosolic peptides leads to enhanced lateral diffusion rate of TAP molecules (Reits et al. 2000).

We hypothesized that if the products of the pioneer round of translation were indeed delivered by TAP into the ER we would observe that the TAP diffusion rate enhances under the inhibition of the pioneer round of translation. To examine the association between the pioneer round of translation and the intracellular peptide supply, we analyzed quantitative changes in the intracellular peptide pool by a TAP mobility assay, using human melanoma cell line Mel JuSo cells stably transfected with TAP1-GFP as described (Reits et al. 2000). The lateral mobility of the TAP in a single living cell can be tracked dynamically using a time-lapse protocol visualizing fluorescence recovery after photobleaching (FRAP). The diffusion rate of TAP in the presence or absence of the pioneer round of translation is then calculated. Inhibition of CBP80 resulted in enhanced TAP lateral mobility, which was restored by introduction of exogenous peptides. These results suggest that loss of CBP80 affects the cytosolic peptide supply to the TAP transporter in the MHC class I antigen processing pathway.

Mel Juso Cells and FRAP

Mel JuSo cells stably expressing TAP-GFP were a gift from the Neefjes lab (Reits et al. 2000). The transfected human TAP1 subunit was fused with green fluorescent protein (GFP) at its cytosolic carboxyl terminus. As described, these cells were selected to ensure correct assembly of transfected TAP1-GFP with the endogenous TAP2 because the functional dimerization of two subunits is essential for peptide translocation. Confocal microscopy showed typical meshwork staining of the endoplasmic reticulum in the cytosol and the nuclear envelope of these cells (**Fig 14a**).

To measure the amount of peptides transported into ER, Mel JuSo cells were cultured at 37°C under standard conditions as described (Wubbolts et al. 1996). During FRAP experiments, a circular spot was defined within the ER region close to the nuclear envelope of a living Mel Juso cell (**Fig 14a**). The spot was bleached and the fluorescence intensity within the spot before and after the bleaching was recorded every second for 25 seconds (**Fig 14b**). Another spot in another region within the same cell was monitored at the same time for fading of flueresence due to imaging (**Fig 14a**). The fluoresence intensity within the bleached spot recovered along the time course due to the diffusion of surrounding TAP-GFP molecules. The half-time of recovery

was calculated after correction with fluorescence of reference spot (**Fig 14b**). Figure 15 shows an example of how to compute diffusion constant D of TAP-GFP molecule in live cell samples manually. Assuming a Gaussian profile for the bleaching beam, the diffusion constant D can be simply calculated as $D = w^2/(4\tau_{1/2})$, where $\tau_{1/2}$ is the diffusion timescale for a bleached spot of radius w (**Fig 15**).

CBP80 deficiency enhanced TAP mobility

Previous studies characterized the influence of intracellular peptide content on mobility of TAP and found that TAP activity is dependent on new protein translation (Reits 2000, Yewdell et al. 1996). The peptide supply to the ER can be reduced using inhibitors targeting either translational procedures or proteasomal degradation, or ATP availability that is required for TAP function. Our own examination of TAP mobility replicated these findings. When dynamics of TAP-GFP were examined in control Mel Juso cells, the bleached area recovered fluorescence intensity slowly (**Fig 16a**). In contrast, recovery of TAP-GFP was enhanced in the cells depleted of ATP (**Fig 16a**) or treated with translation inhibitor cycloheximide (**Fig 16b**) .

To examine the effects of loss of CBP80 and hence the suppression of the pioneer round of translation on peptide generation, Mel Juso cells were transiently transfected with CBP80 siRNA and analyzed by FRAP. These results were compared with scrambled siRNA transfected cells, or cells incubated with proteasome inhibitor lactacystin, to better estimate the effects of CBP80 deficiency (**Fig 16c**). As expected, a substantial increase in TAP mobility was observed when cells were incubated with lactacystin. Knockdown of CBP80 resulted in an increase in TAP diffusion with same magnitude as reducing cytosolic peptide supply by lactacystin. This indicated the presence of a substantial mobile fraction of TAP molecules. Furthermore, the TAP diffusion rate was reciprocally dependent on the cellular protein level of CBP80 (**Fig 17**). Therefore, it appears that loss of CBP80 leads to reduced cytosol peptide supply to TAP transporter.

Peptide supply restored TAP mobility

An increase in TAP mobility could be the result of a non-specific effect of CBP80 siRNA; therefore, we tested whether the alteration of TAP mobility was due to peptide deficiency, by introducing peptides in control cells or cells transfected with CBP80 siRNA. TAT is an 11-mer peptide derived from HIV-1 trans-activating regulatory protein (Tat) domain. It can be efficiently taken up from the surrounding medium by numerous cell types in culture and it has a preference to localize in the cytoplasm. Therefore, as a biological tool, the cell membrane penetrating property of TAT may give cells an opportunity to acquire exogenous peptides from the cytoplasm. Considerably, the TAP mobility was restored in the cells with CBP80 knockdown after incubation with TAT for 30 minutes (**Fig 18a**). No substantial difference in the TAP mobility was observed in control cells. To independently validate the effect of permeable peptides, cells were also incubated with non-permeable conventional peptides SIINFEKL. By contrast, restoration was undetectable in both control cells and CBP80 cells (**Fig 18b**). The data suggested that the effect of CBP80 depletion on TAP mobility was mainly due to the absence of peptides synthesized from the pioneer round of translation.

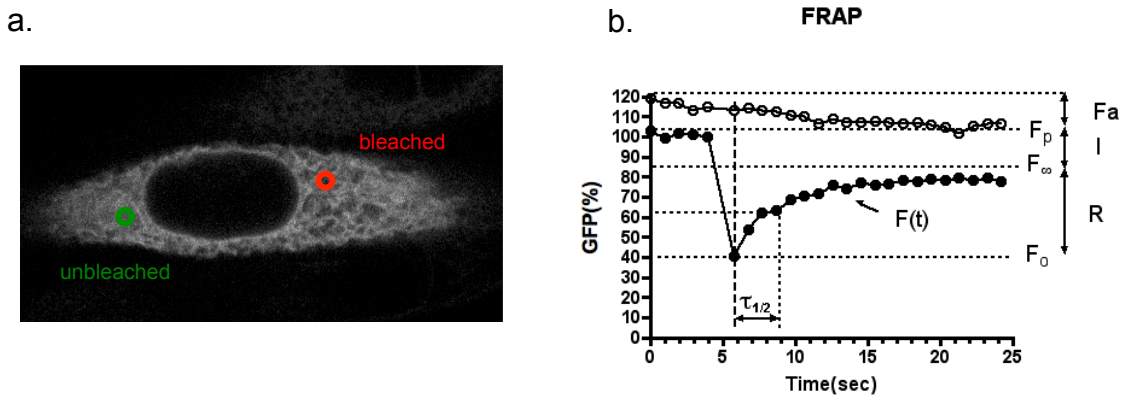
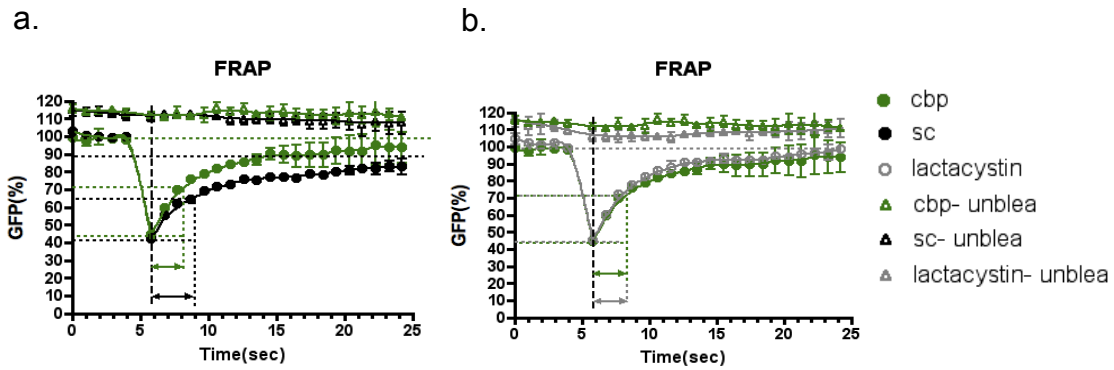


Figure 14. Measuring TAP mobility with fluorescence recovery after photobleaching (FRAP). (a) TAP1-GFP distribution in living Mel Juso cells. A defined circular spot (red) with radius 0.82 µm was bleached at 100% laser intensity with an argon / krypton 488 nm laser and fluorescence recovery through lateral diffusion of TAP1-GFP was monitored at 1 second time intervals. An unbleached spot (green) on the cell was used as a reference for fading fluorescence due to imaging. (b) Graphical presentation of the data collected during a FRAP experiment. A baseline of GFP fluorescence is collected before the photobleaching occurs (where the fluorescence significantly drops). Over the time course, the amount of fluorescence in the photobleached area (solid circle) increases and the fluorescence in the unbleached area (circle) fades due to imaging. Later, there is a stabilization of the amount of fluorescence recovery and a flat line is obtained. Fp: fluorescence intensity of the bleached region before bleaching. F∞ : upper bound of fluorescence recoverable after bleaching. Fo: Fluorescence of the region when photo-bleaching occurs. F(t): recovery curve as a function of time. Fa: fading of fluorescence. τ1/2:half time of recovery. I: immobile fraction. R: recovery, mobile fraction.



c.

$$\text{Diffusion}_{TAP\ I} = \frac{\omega^2}{4\,\tau_{1/2}}$$

	scramble siRNA	CBP80 siRNA	Lactacystin
$\tau_{1/2}$ (s)	3.174	2.337	2.301
D ($10^{-10}cm^2s^-$)	3.819	6.327	6.424

Figure 15. Calculation of half-time for fluorescence recovery and TAP diffusion rate. MelJuSo cells were transfected with scrambled or CBP80 siRNA. 48 hours post-transfection, the cells were incubated with or without lactacystin (10μM) for 30 minutes before subjected to FRAP assay. (a-b) Fluorescence data was collected during a FRAP experiment. Each data point was an average of the fluorescence data collected from three independent cells. (c) The lateral mobility of TAP1-GFP is calculated as diffusion coefficient D. ω: radius of bleached region (0.82 μm). $\tau_{1/2}$: half-time of recovery. D: diffusion coefficient for lateral motion.

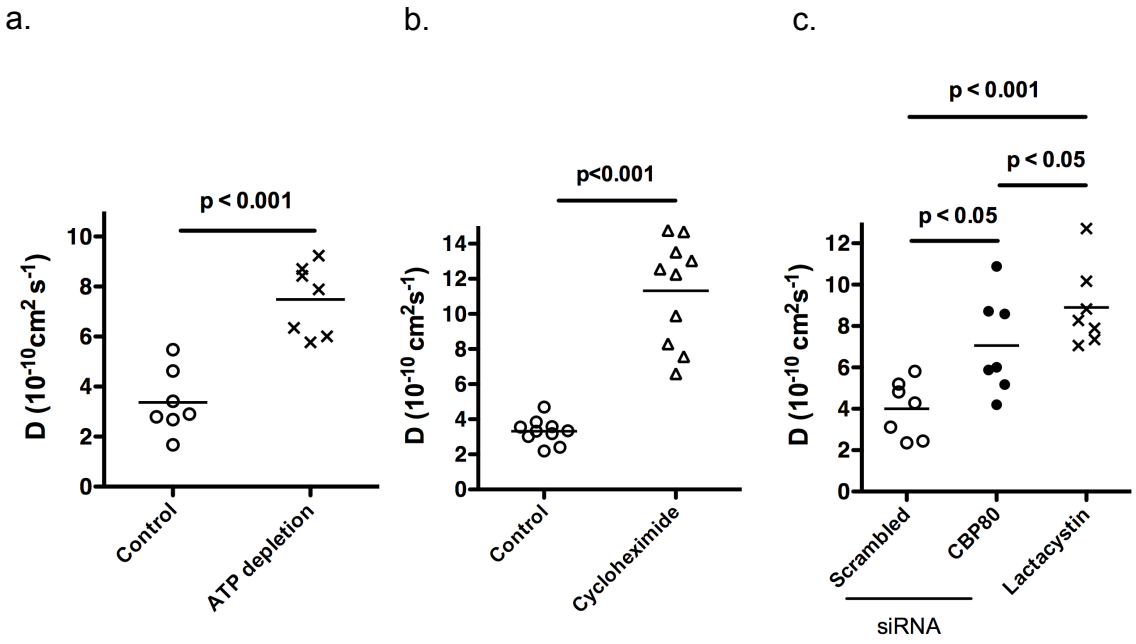


Figure 16. TAP lateral mobility in cells decreases without CBP80. Lateral mobility of TAP in Mel Juso cells in response to ATP depletion (a) or CHX (b), and CBP80 knockdown and lactacystin (c). The scrambled or CBP80 siRNA was transfected into Mel Juso cells for 72 hours before the assay. The cells were incubate with the drugs for 30 minutes before the assay. For each experiment, one dot represents one cell. Data are representative of 3 independent experiments.

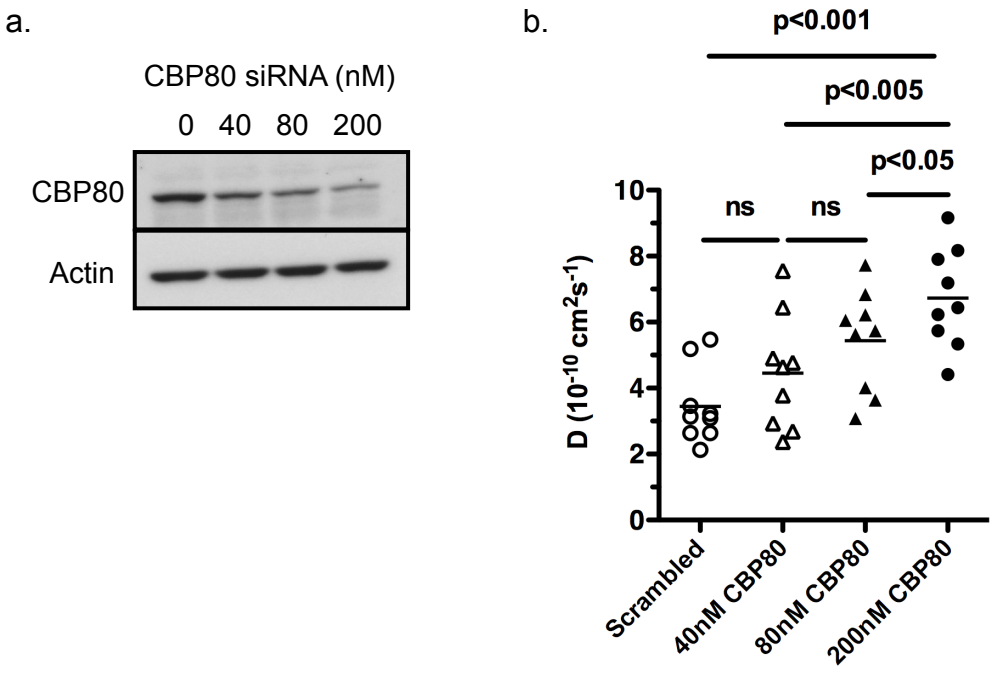


Figure 17. CBP80 depletion enhances TAP mobility. Lateral mobility of TAP in Mel JuSo cells transfected with scrambled siRNA or various concentrations of CBP80 siRNA for 72 hours. (a) Western blotting analysis confirmed the CBP80 protein level under the treatment of different concentrations of siRNA. (b) Lateral mobility of TAP in Mel JuSo cells. For each experiment, one dot represents one cell. Data are representative of 3 independent experiments.

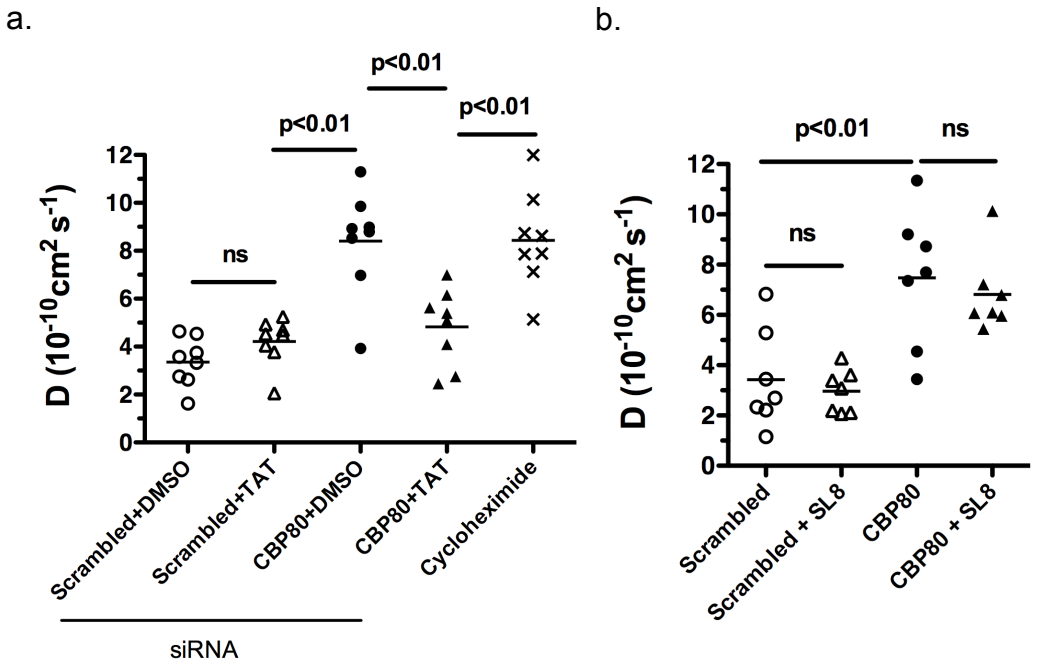


Figure 18. Supply of exogenous peptides restores TAP mobility. Lateral mobility of TAP in Mel JuSo cells transfected with scrambled or CBP80 siRNA for 72 hours. Mel JuSo cells were incubated with (a) the membrane penetrating TAT peptide 5uM or (b) non-penetrating SL8 synthetic peptide 5uM for 30 min. For each experiment, one dot represents one cell. Data are representative of at least two independent experiments.

Discussion

The TAP-FRAP experiments were aiming to measure the contribution of the pioneer round of translation to the peptide supply in an early antigen processing pathway. FRAP was originally established as a biophysical method quantifying the movement of molecules that diffuse freely or within the lipid bilayer. To track the TAP diffusion rate in living cells, the experimental setup was optimized to minimize the background variations. The confocal microscope was connected to an "incubator" chamber which contained a heater sample frame and CO_2 generator. Cell culture was transported into the chamber after the temperature was set to 37°C and the CO_2 concentration to 5%. Because the FRAP assay is based on single cell measurement, statistical significance of the results was achieved by gathering and averaging the data for at least 8 cells in each sample as described in the literature (Reits et al. 2000).

The findings of the FRAP experiment established a link between the pioneer round of translation and TAP-translocated peptides. Treatment of CBP80 siRNA directly increased TAP diffusion rate. The effect of CBP80 siRNA was not as great as cycloheximide or lactacystin. As cycloheximide interferes with translation elongation, it is able to block both the pioneer round of translation and steady-state translation. Lactacystin binds and inhibits catalytic subunits of the proteasome and thus the treatment of lactacystin downregulates the overall amount of cytosolic peptides available to TAP transporter. Consistent with previous experiments, these results suggest the pioneer round of translation is not the only source of MHC I peptides.

To achieve sufficient cytosolic peptide supply, a high concentration of external peptides as TAP substrate was added into the culture media. Alternatively, this can be achieved by microinjection of antigens to the cytosol of a single cell, to directly supply peptides. The advantage of microinjection is that we can track the TAP mobility in a single cell with or without extra peptide supply. However, this technique requires the change of current microscopy equipment.

Chapter 5: Antigen presentation in CBP80 deficient mice

Summary

Previous chapters focused primarily on analyzing the association between the pioneer round of translation and MHC class I antigen presentation in tissue culture cells. Notably, when the pioneer round of translation was inhibited by "knockdown" with small interfering RNA (siRNA) in cells, expression of pMHC I was reduced. Approaches using siRNA can be limited by transfection efficiencies, incomplete down regulation of target proteins or RNA mediated off target effects. In addition, the nature of the peptide pool generated by the pioneer round of translation in normal antigen presenting cells and the function of the pioneer round of translation in shaping the T cell repertoire for immune surveillance is not known. To assess the function of the pioneer round of translation *in vivo*, we want to generate mice lacking CBP80 and analyze the influence of CBP80-dependent pioneer translation on the generation of MHC class I peptide repertoire.

Results

Generation of chimera mice

In the mouse genome database, the structure of the gene encoding CBP80 showed that it contained 23 exons distributed over 33 kilobases (kb). Because of its large size, eliminating the entire locus was impractical. Gene trap strategy is a high-throughput knockout approach that is used to introduce insertional mutations in the genome. A gene trap vector contains a promoterless reporter gene and/or selectable genetic marker flanked by an upstream 3' splice site acceptor (SA) and a downstream polyadenylation sequence. The approach inserts reporter vectors randomly into the mammalian genome. When the vector is inserted into an intron of an expressed gene, the resulting insertional mutation creates a fusion transcript containing sequences from exons upstream to the insertion joined to the reporter marker. Gene trap therefore simultaneously inactivates the expression of the trapped gene at the insertion site.

We obtained from BayGenomics an embryonic stem cell line with a gene trap vector pGT1lxf inserted into the gene encoding CBP80 (**Fig 19a-b**). pGT1lxf contains a splice-acceptor sequence upstream of a reporter gene called β-geo. β-geo is a fusion of β-galactosidase and neomycin phosphotransferase II. The parental ES cell line was E14Tg2a.4 derived from mouse strain 129P2/OlaHsd. BayGenomics determined the identity of the trapped gene (CBP80) by 5' rapid amplification of cDNA ends (5' RACE) followed by automated DNA sequencing. To knockout the target gene using gene trap strategy, generally we need to select a clone that traps the furthest upstream of the target gene. We therefore chose the XG182 ES cell line after confirming by blasting the sequence tag of 5'RACE that the location of the gene trap in the 5' N-terminus (between the region of exon1 to exon2) of CBP80 gene. Immuno-blotting analysis of XG182 ES cells showed that in contrast to wild-type parental ES cell E14, CBP80 expression was decreased in XG182 with heterozygous CBP80 deficiency (**Fig 19c**).

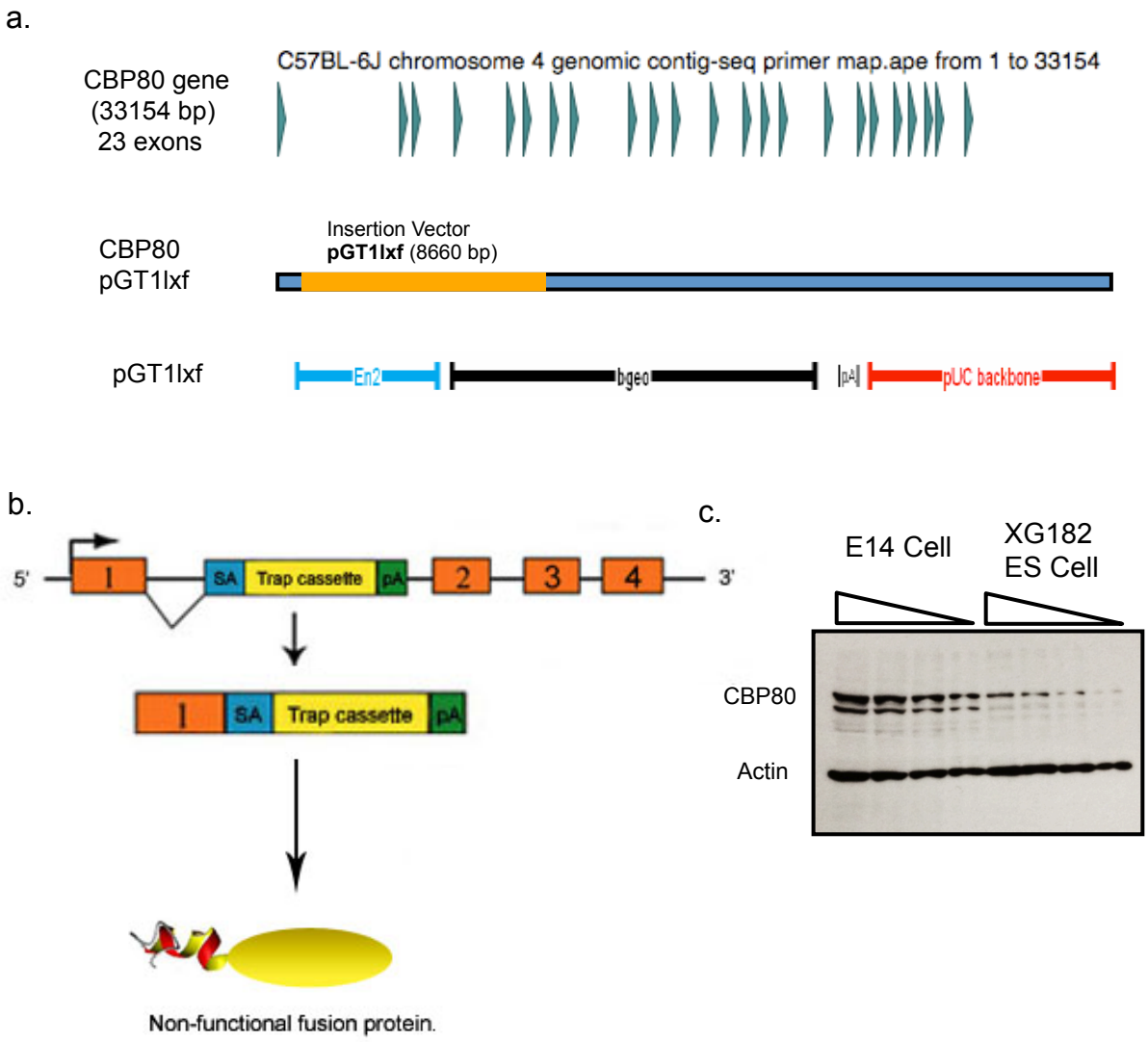


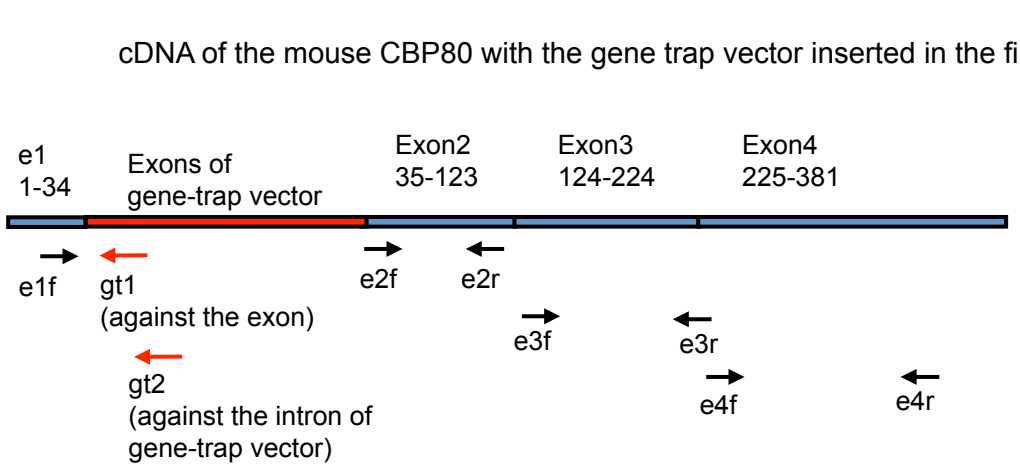
Figure 19. Gene-trap results in decreased CBP80 protein expression in XG182 ES cell line. (a) Schematic of mouse CBP80 genomic DNA and CBP80 gene with the gene trap vector pGT1lxf inserted in the first intron. (b) Schematic of gene trap strategy. Gene trap cassette with splicing site acceptor (SA) and translation termination sequence (poly(A)-tail sequence; pA) is inserted in the first intron of the target gene. After transcription and intron splicing, the first exon of the trapped gene abuts the splice acceptor sequence Engrailed-2 (En2) exon, which is part of the pGT1lxf vector. No vector intron sequence should be present. Translation of the insertion mutated gene creates a truncated non-functional fusion protein. (c) Western blotting of wild-type parental ES cell line E14 and mutated ES cell line XG182 with CBP80 gene-trapped in one allele. Loading of cell lysate is diluted in series. Actin serves as a loading control.

Because the first intron of CBP80 is 5.9 kb in length and the exact insertion position of the gene trap vector (8.6 kb) in the intron is not known, it is impractical to perform a PCR across the entire intron to verify the insertion. Therefore, reverse quantitative PCR was performed on the cDNA sequence transcribed *in vitro* from mutated CBP80 gene, to further verify the insertion of gene trap vector pGT1lxf (**Fig 20a**). Assuming the gene trap vector was inserted into the first intron of CBP80 gene on one allele, the fusion sequence composed of CBP80 exon 1 and the gene trap vector exon would be detectable by designing a forward primer in the exon1 and a reverse primer in the gene trap exon. The mRNA level of CBP80 gene was quantified relative to the mRNA level of internal GAPDH (**Fig 20b**). The results showed that RT-PCR detected the sequence of CBP80 exon1 fused to gene trap vector with the specific primers (e1f - gt1, **Fig 20a-b**). RT-PCR with primers e2f - gt2, which targets the potential sequence that is composed of CBP80 exon2 fused to the gene trap vector, did not show any detectable mRNA level (**Fig 20a-b**). Primer gt2 targets the intron sequence in the gene trap vector and thus serves as a negative control. Taken together, these results indicate that XG182 ES cell line contained a heterozygous CBP80 deficiency allele with the gene trap vector inserted in the first intron of CBP80.

We obtained one male chimera mouse with one allele deficient in CBP80, using the XG182 ES cell line. Germline offspring mice were obtained by breeding the XG182 chimera mouse with C57BL/6 females. The mutant allele will be detectable by designing a pair of primers in the exon of gene trap vector. Such PCR reactions should yield a single band, exclusively in samples from mutant cells or mouse tissues (**Fig 21a**). To genotype the mice, PCR was performed on the digested tail of each mouse. In the pups generated by the mating of two mice heterozygous for the mutated allele, none of them carried homozygous mutation, as determined by PCR. Figure 21-b shows an example of genotyping PCR data and the immunoblotting in Figure 21-c shows the expression of CBP80 protein in the corresponding mice.

So far the generation of a mouse line silenced for the pioneer round of translation is not known. The attempt to generate a mouse line depleted for NMD factor Upf1 was not successful due to the embryonic lethality at the blastocyst stage after only 3.5 days (Medghalchi et al. 2001). This finding not only states the importance of NMD during organism development but also suggests the mouse line depleted for the pioneer round of translation may not be successful because initiation of NMD is dependent on the pioneer round of translation. Therefore we tried to create a Cre-loxP based CBP80 conditional knockout mouse. We obtained an ES cell line with a Cre-loxP based conditional gene trap insertion in CBP80 to generate chimeric mice. However, the male chimera mouse obtained was not germline transmitted because none of the offspring mice (35 mice) contained the mutated allele.

a.



b.

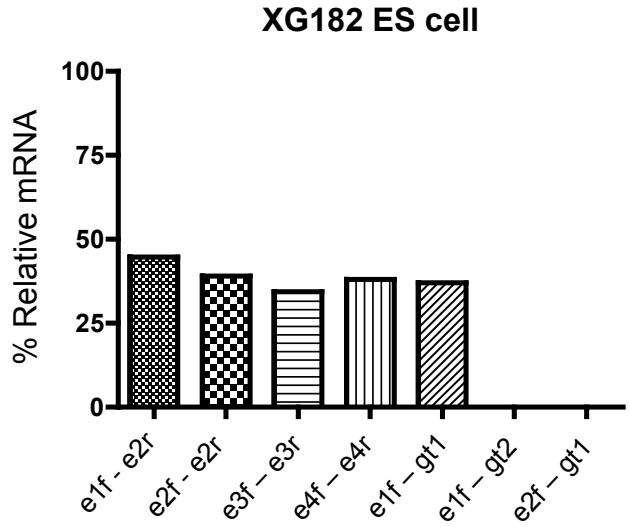


Figure 20. PCR of the ES cell line XG182. (a) Schematic of mouse CBP80 mRNA with the gene trap vector pGT1lxf inserted in the first intron. Primers for quantitative RT-PCR are indicated according to their targeting sequences. (b) Relative mRNA level of CBP80 was measured by reverse quantitative PCR and normalized with the internal GAPDH mRNA level.

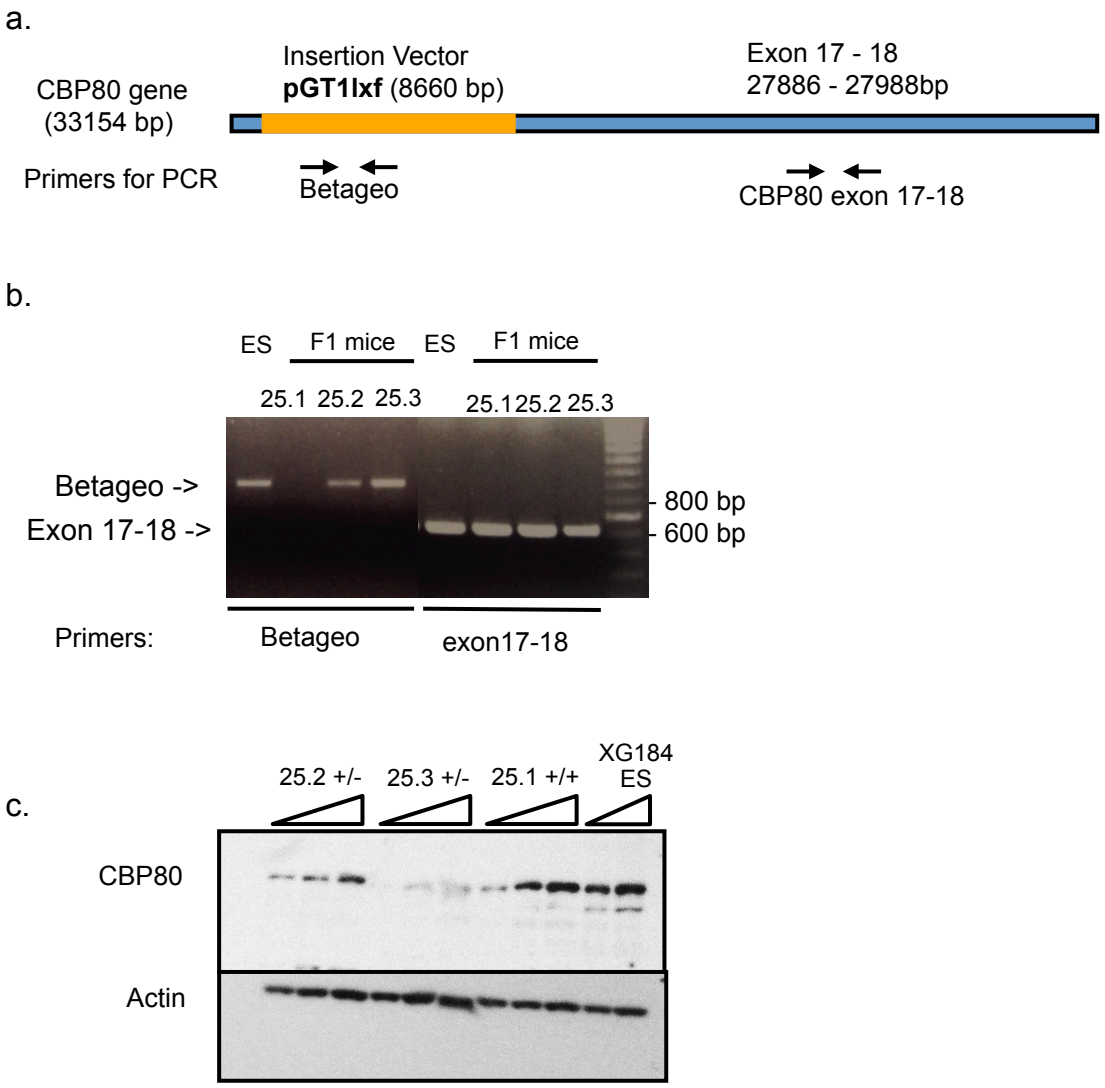


Figure 21. Genotyping of F1 mice. (a) Schematics of CBP80 gene trapped with pGT1lxf vector and the primers for genotyping of the mouse deficient in CBP80. The gene trap vector in the mutant allele is detectable by the primers Betageo. CBP80 exon is detectable by the primers exon17-18. (b) Genotyping of XG182 ES cells and the F1 mice using the primers indicated in (a). (c) Western blotting of ES cell line XG182 and the spleen cells of the F1 mice with wild-type alleles or with heterozygous CBP80 deficiency in one allele. Loading of cell lysate is diluted in series. Actin serves as a loading control.

Analysis of CBP80 deficient cells

The hallmark of disruption of MHC I antigen generation pathway is decreased expression of MHC I molecules on the cell surface. To assess the expression of different MHC class I molecules in mice with CBP80-deficiency in one allele, splenocytes were isolated from C57BL/6 mice and mice with wild-type (cbp80 +/+) or heterozygous (cbp80 +/-) alleles. After blocking of the Fc-receptors, cells were stained for surface MHC class I expression with antibodies against H-2K^b, H-2D^b, CD8+ and B-lymphocyte surface marker B220. After testing of several mice, total number of T lymphocytes was found to be similar in the spleen of wild-type and heterozygous mice. Although the population of cells expressing H-2K^b or H-2D^b was lower in the heterozygous mice (**Fig 22a-f**), no apparent differences were observed in expression of H-2K^b, H-2D^b molecules assessed by mean fluorescence intensity (**Fig 22g-h**). Because the loss of CBP80 is not complete in the heterozygous mice (**Fig 21c**), it is possible that functional CBP80 protein from the remaining wild-type allele is capable of initiating the pioneer round of translation and complements the loss of one allele. A greater decrease in expression of different MHC class I molecules is observed in cultured cells, because the siRNA knockdown of CBP80 in cultured cells is a potent approach that has depleted the majority, if not all, CBP80 protein in the cells.

Taken together there are no obvious indicators for an altered MHC class I expression in heterozygous mice. Noted that CBP80 is lost on only one allele in these mice, the unclear effect of the CBP80 deficiency on the peptide repertoire suggests that the heterozygous mouse is an insufficient model system. Future direction will be discussed in detail.

Analysis of pMHC I repertoire and T cell repertoire in heterozygous mice

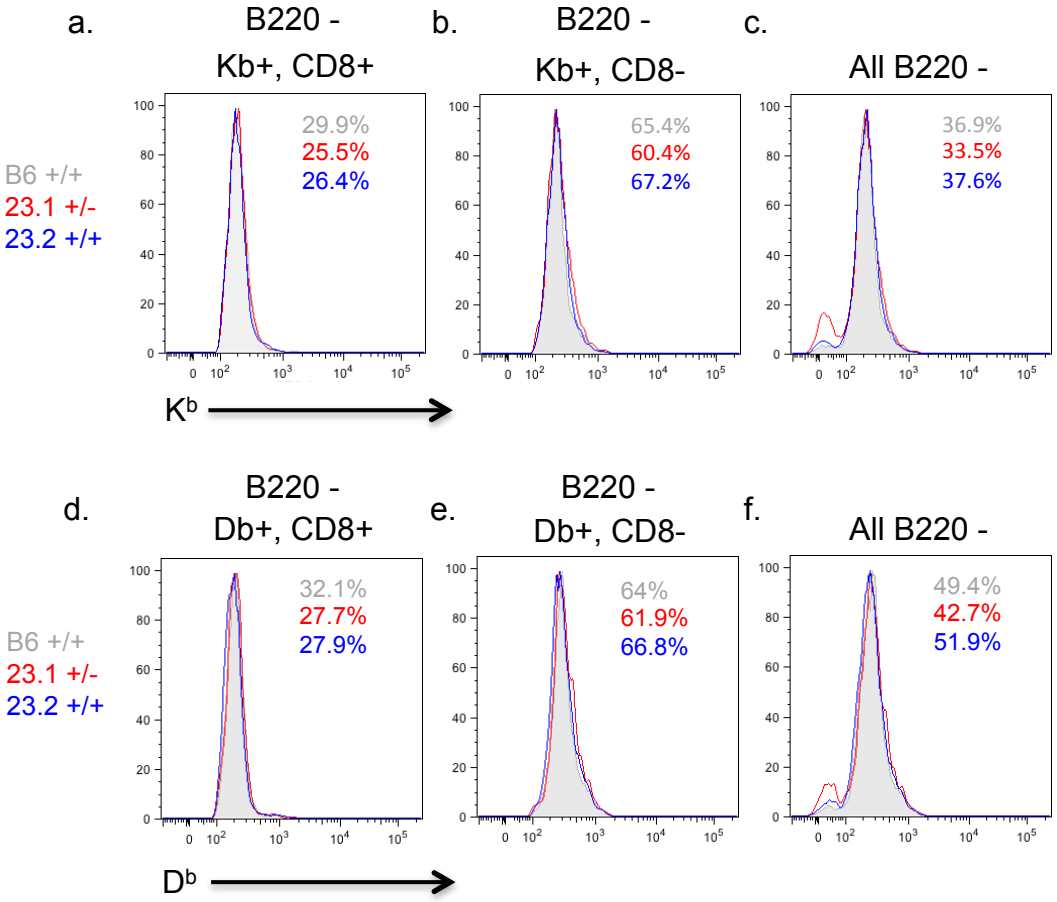
Immune surveillance by CD8+ T cells requires a diverse spectrum of peptides presented by MHC I molecules on the cell surface. When endogenous peptide supply is decreased by inhibition of the pioneer round of translation, MHC I molecules fail to assemble with peptides in the ER, resulting in altered quantity and quality of MHC I complexes on the cell surface.

Flow cytometry analysis on MHC class I molecules displays a general picture of MHC class I expression but it cannot reflect the diversity of peptides presented on cell surface. T cell activation assay is a more sensitive approach, which also assesses the potential ability of specific MHC class I complex in triggering a T cell mediated immune response. To assess the influence of CBP80-dependent pioneer translation on the generation of specific peptides bound to MHC I, we measured the presentation of several endogenous peptides on the surface of spleen cells from ERAAP-deficient (ERAAP), WT B6, CBP80 heterozygous (+/-) and WT littermate (+/+) (**Fig 23**). 1AZ T cell hybridoma recognizes a H-2D^b restricted peptide SCILLYIVI (SII9) peptide encoded by murine H47^a gene (Mendoza et al. 2001). 30NXZ T cell hybridomas are activated through a H-2D^b restricted peptide SSVVGVWYL (SVL9) derived from the H13^a histocompatibility gene (Hammer et al. 2006). 27.5Z T cell hybrid recognizes a H-2K^b restricted peptide of unknown sequence. LpAZ T cell hybridoma recognizes a H-2D^b restricted antigen SASPCNSTVL derived from H3^a gene (Serwold et al. 2001). pMHC I ligands recognized by 1AZ and 30NXZ hybridomas was enhanced on ERAAP-deficient cells as previously reported. Although variation in T cell responses was observed in independent experiments, these results did not show a clear distinction of heterozygous and wild-type counterparts in generation of

these specific peptides. This analysis is limited because it is unable to probe for the entire peptide species presented by MHC I molecules.

The development and maintenance of T cells relies on the expression of pMHC I *in vivo*. To test whether the T cell repertoire were changed in CBP80 deficient cells and to determine the overall extent to which the pMHC I repertoire was disrupted in CBP80 deficient mice, we used F1 heterozygous splenocytes as APCs to immunize their wild-type littermates. The F1 heterozygous and WT counterparts were obtained by mating the chimeric male with B6 females. Every offspring carries one set of alleles from each parent with the only difference being in the mutated CBP80 gene on one allele. If inhibition of the pioneer round of translation results in production of less stable peptide complexes or novel peptide species, WT T lymphocytes will respond with exquisite specificity to foreign peptides in heterozygous mice. Alternatively, WT T lymphocytes are tolerant to the normal pMHC I from both parents but heterozygous T lymphocytes may respond to potential "non-self" pMHC I generated in WT mice, due to the loss of CBP80 in one allele.

To test our hypothesis, we started by immunizing WT mice with heterozygous APCs. Splenocytes from male heterozygous mouse were injected intraperitoneally into two female WT littermates. As background control, splenocytes from male WT littermate were injected into two female WT counterparts. After 10 days, the host mice were immunized again to boost immune response. 10 days post-immunization, spleen cells from immunized mice were restimulated *in vitro* for a week by co-culture with irradiated spleen cells of female heterozygous mouse or female WT littermates. CD8+ T cell responsiveness was measured by incubating restimulated T cells with female heterozygous or WT APCs (spleen cells) and subsequently staining for IFN-γ. The WT anti-Het CD8+ T cells responded strongly to Het APCs but only slightly to self APCs (**Fig 24**). The WT anti-WT CD8+ T cells responded neither to Het APCs or self APCs (**Fig 25**). This implied that WT mice perceived pMHC I in heterozygous mice as non-self. However, after the second round of restimulation, although WT anti-Het CD8+ T cell population increased, they did not respond to Het APCs or self APCs (**Fig 26**), compared to the WT anti-WT CD8+ T cell controls (**Fig 27**). This suggests that the production of IFN-γ observed in the first restimulation was due to non-specific T cell response and therefore diminished during *in vitro* culture. Taken together, we think WT CD8+ T cells may be tolerant to pMHC I in heterozygous mice and we should assess the heterozygous CD8+ T cell response to WT APCs.



g.

H2-K^b	MFI K^b+CD8+	MFI K^b+CD8-	MFI All B220-
B6 +/+	191	224	210
23.1+/-	193	237	207
23.2+/+	188	232	213

h.

H2-D^b	MFI D^b+CD8+	MFI D^b+CD8-	MFI All B220-
B6 +/+	205	305	264
23.1+/-	204	315	260
23.2+/+	190	291	249

Figure 22. Analysis of MHC I expression on the CBP80-heterozygous cells. Surface expression of H-2K^b (a-c) or H-2D^b (d-e) on the spleen cells from a C57BL/6 mouse, a F1 mouse with CBP80-heterozygous (+/-) alleles and a CBP80 wild-type (+/+) littermate. Cells were Fc-receptor blocked and stained for MHC class I K^b, D^b and B220, CD8. B cells were excluded (B220-) and the cell population was then gated on CD8 positive (a and d) or CD8 negative (b and e) staining. (g-h) Mean fluorescence intensity (MFI) of cells in (a-c) and (d-e). The data is a representative of 3 independent experiments.

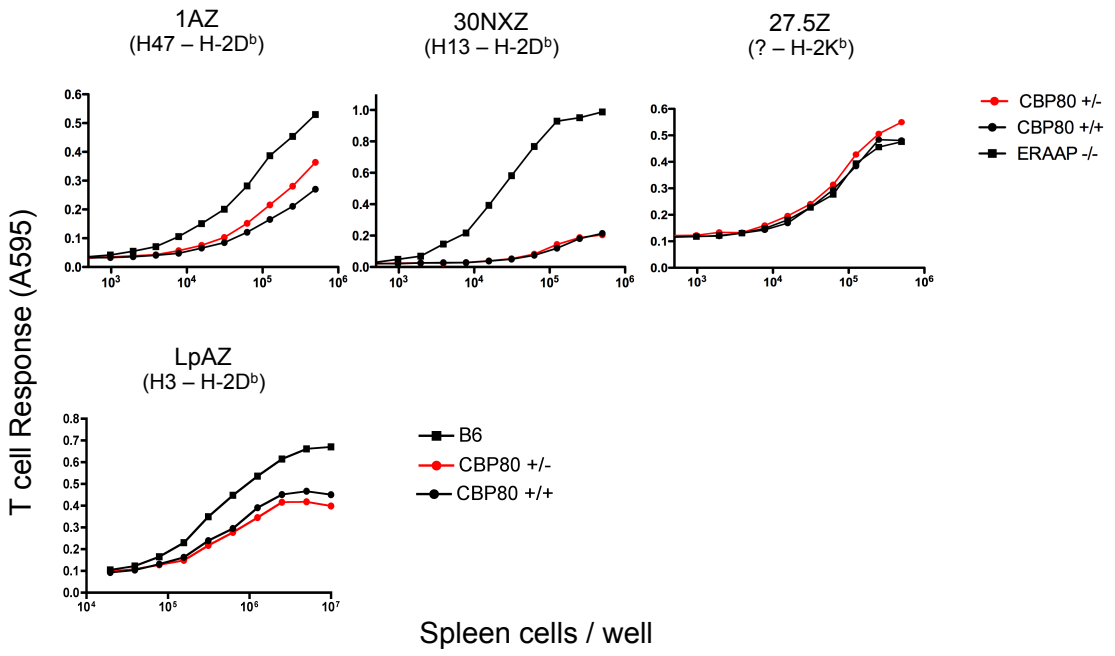


Figure 23. T cell activation of spleen cells in the CBP80-heterozygous mice. Spleen cells from the indicated male mice were stimulated with 200 ng/ml LPS overnight before incubation with CD8+ T cell hybridomas 1AZ, 30NXZ, 27.5Z or LpAZ. Hybridomas specifically recognize peptides encoded by the indicated genes and presented on H-2D^b or H-2K^b. Hybridoma response is determined by assessing conversion of the colorimetric substrate chlorophenol red-b-D-galactopyranoside to chlorophenol red measured at absorbance at 595 nm (A595). Data are representative of three independent experiments.

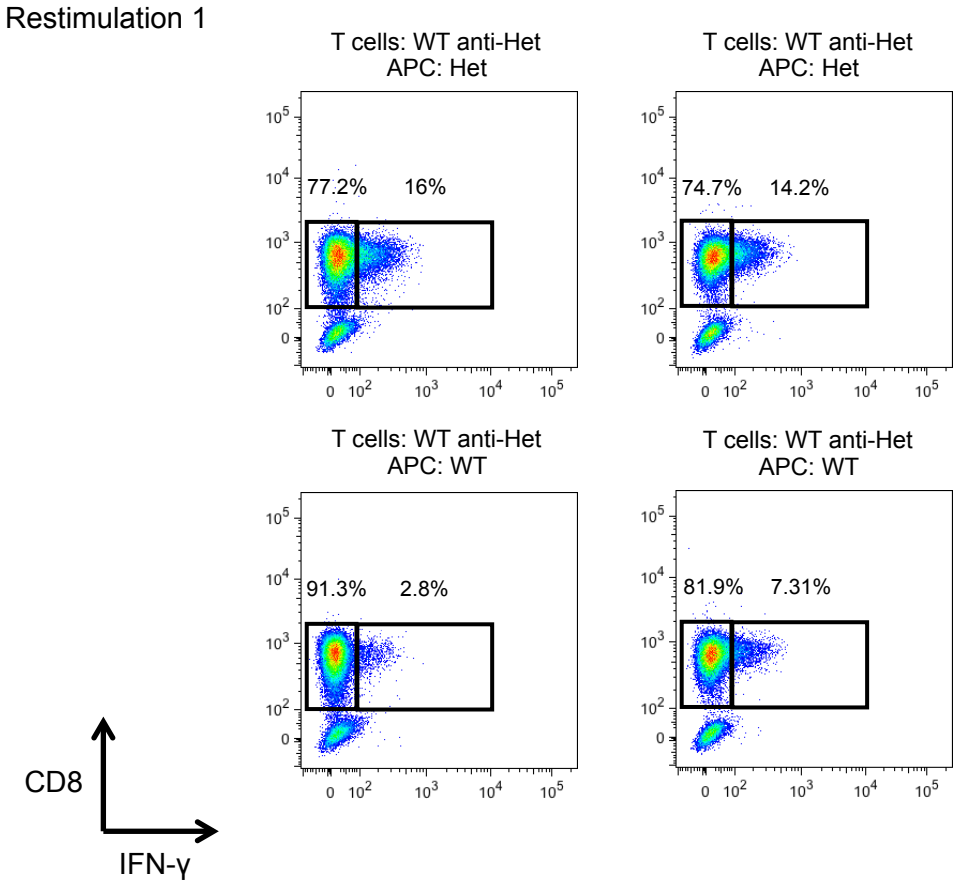


Figure 24. T cell response of CBP80-WT mice to CBP-hetetrozygous (Het) APCs after the first restimulation. Intracellular IFN-γ produced by WT anti-Het CD8+ T cells in response to WT or Het APCs. Cells displayed are restimulated spleen cells with positive TCR, CD4 and CD8 staining.

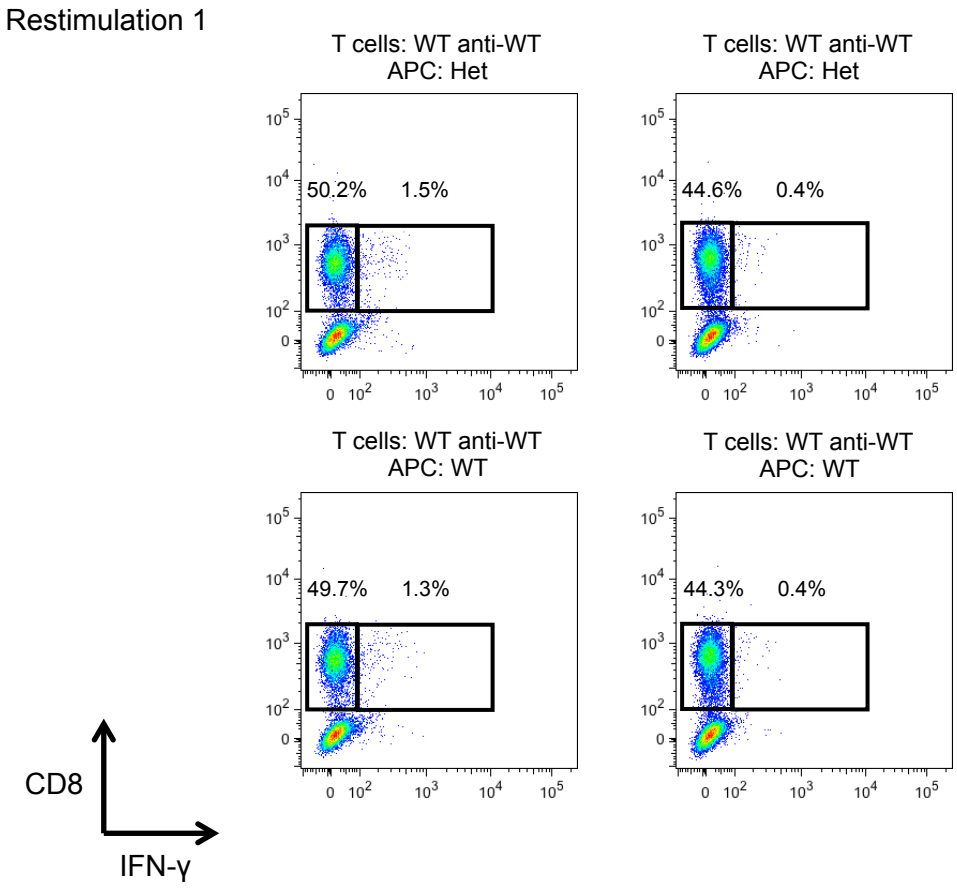


Figure 25. T cell response of CBP80-WT mice to WT APCs after the first restimulation. Intracellular IFN-γ produced by WT anti-WT CD8+ T cells in response to WT or Het APCs. Cells displayed are restimulated spleen cells with positive TCR, CD4 and CD8 staining.

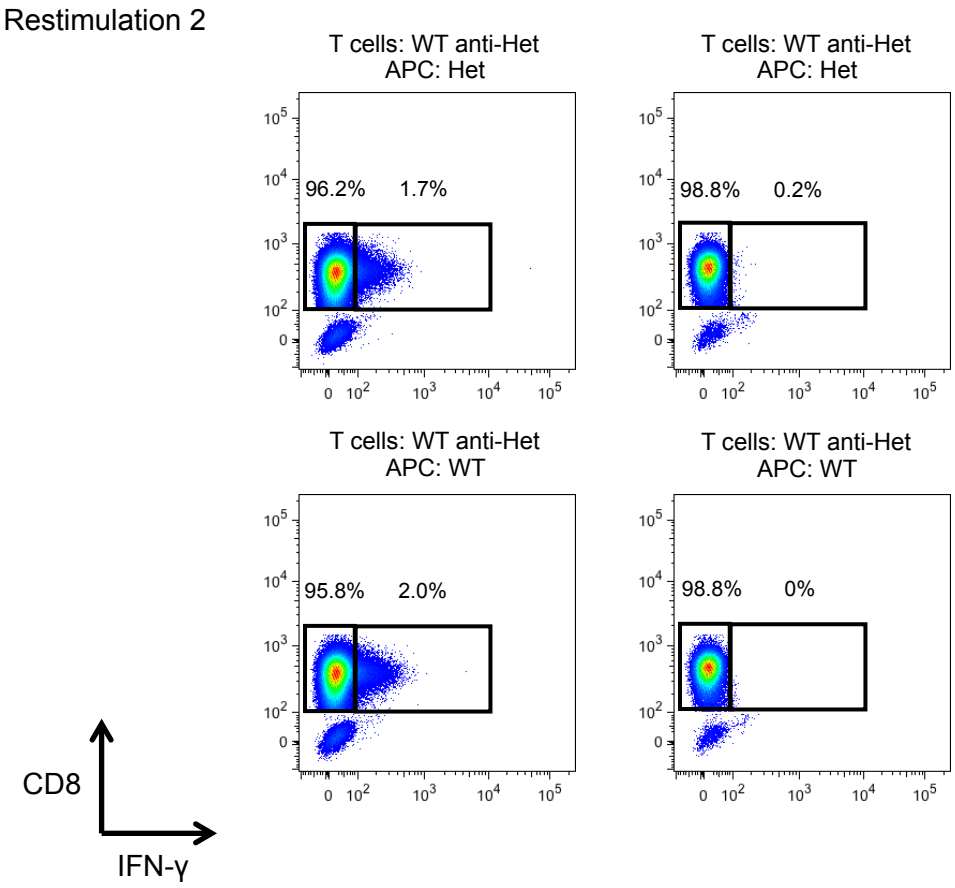


Figure 26. T cell response of CBP80-WT mice to CBP-hetetrozygous (Het) APCs after the second restimulation. Intracellular IFN-γ produced by WT anti-Het CD8+ T cells in response to WT or Het APCs. Cells displayed are restimulated spleen cells with positive TCR, CD4 and CD8 staining.

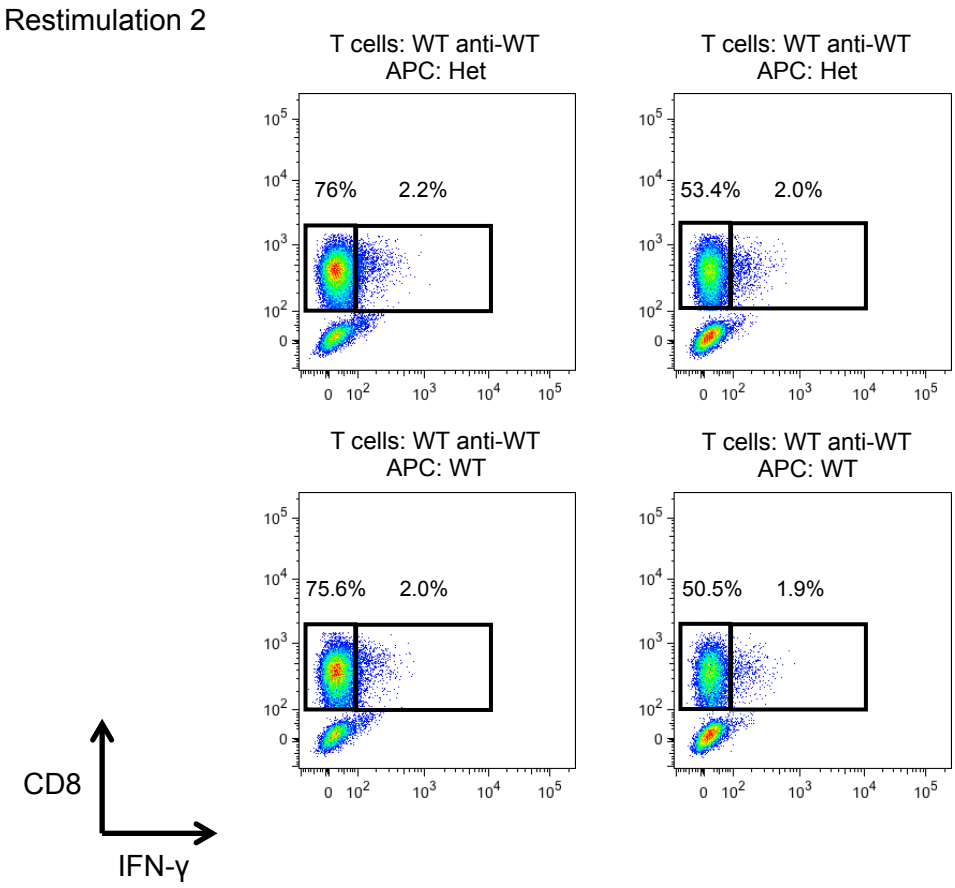


Figure 27. T cell response of CBP80-WT mice to WT APCs after the second restimulation. Intracellular IFN-γ produced by WT anti-WT CD8+ T cells in response to WT or Het APCs. Cells displayed are restimulated spleen cells with positive TCR, CD4 and CD8 staining.

Discussion

At this stage no mouse model for the pioneer round of translation pathway is available. We have established *in vitro* that knockdown of CBP80 decreased expression of pMHC I in tissue culture cells. However, it is unknown whether block of the pioneer round of translation only decreases the amount of endogenous peptides for presentation by MHC I molecules or alters the diversity of peptides at the same time. Since every newly spliced mRNA undergoes the pioneer round of translation and only the mRNAs that pass NMD surveillance pathway are translated by steady-state translation, it is presumable that the polypeptides of the pioneer round of translation are more diverse than those of steady-state translation. Therefore CBP80-knockout mice may lack tolerance to WT mice. We considered another possibility that inhibition of the pioneer round of translation also changes the quality of peptides on cell surface because loss of pioneer translation-specific peptides could enhance the presentation of peptides from normal mRNAs and therefore alters the T cell repertoire. Based on the preliminary results of immunization, we rule out the latter possibility. To test the former hypothesis, we will immunize the CBP80-knockout mice with WT APCs and measure the intracellular production of IFN-γ by heterozygous CD8+ T cells to WT APCs.

Since we have observed absorbed embryos in the female heterozygous mice mated with heterozygous males and no CBP80-/- homozygous mice have been acquired yet, it is very likely that a complete depletion of CBP80 is embryonically lethal. Besides the conditional knockout strategy, an alternative approach of studying CBP80-knockout cells is to culture CBP80-/- embryonic fibroblast cells, which will be further discussed in the next chapter.

Chapter 6: Future Directions

We have shown that the pioneer round of translation is required for MHC class I antigen presentation, using biochemical and immunological approaches. Our data suggests that the knockdown of CBP80 with siRNA reduced the surface expression of pMHC I in cultured cells. The pioneer round of translation is likely to provide a subset of constitutively presented peptides that are efficiently targeted to TAP.

However, the nature of peptides derived from pioneer round of translation is unknown. One challenge preventing the establishment of a model to study peptide precursors generated by pioneer round of translation is that it is difficult to distinguish between peptides yielded from pioneer round of translation versus those from steady-state translation. Although multiple factors in the initiation complex of pioneer translation are distinct from those in the initiation complex of steady-state translation, both translation machinery share common ribosomes, tRNAs and amino acids. The translated products from the mRNAs targeted by NMD serve as potential models for the study of the pioneer round of translation, but still it is not guaranteed that these products are merely from the pioneer round of translation rather than a mixture of products contributed by both the pioneer round of translation and steady-state translation.

This leads to another question that how fast and how efficiently the pioneer round of translation products are degraded and targeted to the TAP for MHC class I presentation. Could the pioneer round of translation be the earliest event for the generation of MHC I peptide precursors? While DRiPs has been proposed to explain for the rapid detection of virus-infected cells (Croft et al. 2013) by CD8+ T cells, the nature of DRiPs remains a major question. It appears that a fraction of rapidly degraded proteins is utilized for generation of antigenic peptides (Qian et al. 2006). There is no direct evidence that the rapid degradation of proteins is correlated with the abundance of the gene transcripts. As DRiPs are referred to the defective ribosomal products that fail to form a native, stable structure and degrade rapidly, it is possible that DRiPs are generated from the mutated mRNAs during the pioneer round of translation. Kinetics studies on the translated gene products from the pioneer round of translation, such as the presentation rate of epitopes in an mRNA degraded by NMD, may shed some light on this question.

What is also unknown is the subcellular location of the pioneer round of translation. Although it is likely that the pioneer round of translation of our reporter gene occurs in cytosol, our experiments using cell fractionation and RAN69QL did not imply the exact subcellular location of the pioneer round of translation. It has been showed that NMD of mRNAs occurs in both nuclear and cytosolic compartments (Dahlberg et al. 2003; Maquat, 2002; Dreumont et al. 2004; Moriarty et al. 1998). Several evidences on nuclear translation of mRNAs have been proposed (Allfrey et al. 1954; Goidl et al. 1975; Bohnsack et al. 2002; Iborra et al., 2001). These evidences include incorporation of amino acids or tRNA into the nuclear proteins, isolation of polyribosomes from the nucleus, and visualization of translation factors in the nucleus. Recently, nuclear translation on pre-spliced mRNAs was visualized (Apcher et al, 2013) using ribopuromycylation technique (David et al. 2012). Treatment of translation elongation inhibitors freezes polyribosomes with associated peptide chains in living cells. Puromycin, which is a Tyr-

tRNA mimetic, is then added to the living cells and subsequently enters nascent chains through ribosome-catalyzed incorporation. Therefore translation is visualized with antibodies against puromycin, peptides and nuclei marker proteins on the fixed and permeabilized cells. The combination of ribopuromycylation technique and polysome profiling may help to identify the subcellular location of pioneer translation start sites as well as the distribution of the pioneer translation initiation complexes.

One key thing for the immune surveillance is how the limited number of MHC class I molecules are not occupied by the redundant peptides from the most abundant protein products. Before antigenic peptide is loaded onto MHC class I molecules, there are several bottlenecks including proteasomal degradation, peptidase trimming and TAP transportation that the peptide needs to pass by competition with the overwhelming cytosolic peptides. Selective degradation of certain proteins by proteasomes (Qian et al. 2006) and compartmentalized antigen processing (Lev et al. 2010) has been suggested as strategies that the immune system take to prevent competition between peptides. If the pioneer round of translation provides a possible answer for the question that how the extremely low abundant gene products are presented by MHC I molecules, it may help develop approaches to the early identification of newly transformed cells with low mutation rates.

The questions discussed above would be extensively addressed with a pioneer round of translation-deficient mouse model. We are currently generating CBP80-knockout mouse embryonic fibroblasts (MEFs) from an early stage of embryos, by crossing between CBP80 heterozygous F1 mice. The embryo resorption was observed after day E9.5 and the resorption rate was high after day E11.5. Western blotting detected expression of full-length CBP80 in the MEFs derived from E11.5-12.5 embryos. These indicate that knockout of CBP80 could be embryonically lethal. Therefore, we will grow MEFs from the embryos at E7.5 to E8.5 and verify their genotypes by PCR and Southern blotting. Besides making CBP80-conditional knockout mice, another approach is to make bone marrow-chimeric mice by transplanting CBP80-deficient bone marrow cells with lenti-virus based transduction. The influence of the pioneer round of translation on the expression of MHC I molecules *in vivo* is unclear. To investigate this question, we can assess the number of lymphocyte subpopulation and MHC class I surface expression of the spleen cells in the CBP80-deficient mice, as well as the specific responses of CD8+ T cells in CBP80-deficient mice to wild-type mice.

Chapter 7: Materials and Methods

Plasmids

To generate pcDNA1-luciferase-SIINFEHL-betaglobin (WT and PTC), pCI-neo-luciferase-betaglobin (WT and PTC) previously described (Boelz et al. 2006) was used as template. A final ~1460 bp fragment of ATG-SIINFEKL-betaglobin flanked by XhoI and NotI sites was amplified by three rounds of PCR and ligated into pCI-neo vector using T4-DNA ligase (Promega). The PCR primers used for each PCR step in order are each of the forward primers
5'-ACTTTGAAAAGCTTGTGCATCTGACTCCTGAGGAGAAGT-3',
5'-ATGAGTATAATCAACTTTGAAAAGCTTGTGCATCTGACTC-3',
5'-AGTCCTCGAGACACCATGAGTATAATCAACTTTGAAAAGCTTGTGCA-3'
and reverse primer 5'-AGTCGCGGCCGC TTAGTGATACTTGTGGGCCAGGGCA-3'.

To generate pcDNA1-SIINFEHL-betaglobin (WT and PTC), a similar PCR method was used with different forward primers. An ~1450 bp fragment of ATG-SIINFEKL-betaglobin flanked by EcoRV and NotI sites was amplified and ligated into pcDNA1 vector. The PCR primers used in order are each of the forward primers
5'-ACTTTGAAAAGCTTGTGCATCTGACTCCTGAGGAGAAGT-3',
5'-ATG AGTATAATCAAC TTT GAA AAG CTT GTGCATCTGACTCC-3',
5'- AGTCGATATC ATGAGTATAATCAACTTTGAACACCTTGTGCA-3' and reverse primer 5'-AGTCGCGGCCGC TTAGTGATACTTGTGGGCCAGGGCA-3'.
pKW1608-IBB was kindly provided by Dr. Karsten Weis, pcDNA-V5-RAN(Q69L) by Dr. Richard Cerione, and pACTAG2/HA-AA-4EBP1 by Dr. Nahum Sonenberg.

Cell culture and transfection

HeLa and HeLa-K^b were cultured in RMPI 1640 (Hyclone) containing 10% fetal bovine serum (Hyclone), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 0.3 mg/ml glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mel Juso cells stably expressing TAP1-GFP were described (Reits et al., 2000) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (normal medium). Cells (2×10^5 cells/well for 6-well plates) were transiently transfected with 200nM (or as indicated) of *in vitro*-synthesized nonspecific siRNA, CBP80 siRNA or eIF4E siRNA (Thermo) using oligofectamine (Invitrogen). For expression of SL8 peptides, two days after siRNA transfection HeLa-K^b cells were transfected with1ug of indicated plasmids using lipofectamine (Invitrogen). Cellular CBP80, eIF4E and CTIF were down-regluated using,
5'-r(GCUGAUCUUCCUAACUACA)d(TT)-3' (Woeller et al., 2008)
5'-r(GGAUGGUAUUGAGCCUAUG)d(TT)-3' and
5'-r(GCAUCAACCUGAAUGACAU)d(TT)-3' (Kim et al., 2009), respectively.

The beta-galactosidase (lacZ)-inducible T cell hybridomas B3Z, 1AZ, 30NXZ, 27.5Z and LPAZ have been described (Hammer et al. 2006; Nagarajan et al. 2012).

Antibodies for flow cytometry

The following antibodies were purchased from BD Biosciences: anti-CD8α (H53-6.7), anti-CD4

(RM4-5), anti-TCRβ (H57-597), anti-CD19 (eBio1D3), anti-CD45R/B220 (RA3-6B2), antibody CD16/32 (Fc block, clone 93) and anti-IFNγ (XMG1.2). Antibodies purchased from BioLegend were anti-H-2K^b (AF6-88.5) and anti-H-2D^b (KH95).
PE anti-mouse IgG secondary antibody was from Invitrogen. Anti-HLA-A/B/C (W6/32) was from Santa Cruz Biotechnologies and anti-H-2K^b (5F1.5) was culture supernatant.

Acid wash recovery and T cell assay
For acid wash experiments, cells were treated with acid wash buffer (0.131 M citric acid, 0.066 M NaH2PO4 pH 3.1) for 2 min, washed twice with PBS and cultured in normal growth media for indicated time (Serwold et al. 2001).

To extract intracellular peptides, cells were lysed in 10% acetic acid and boiled for 10 minutes. After cell debris being removed by centrifugation, the samples were dried using vaccum centrifugation overnight. Dried extraction was resuspended in 25 μg/mL phenol red/PBS to adjust the pH to 7 using 0.1 N NaOH.

For T cell assay, neutralized peptide extraction was titrated in 96-wellplates and incubated with Lac Z-inducible B3Z T hybridomas (1×10^5 cells) and APCs (K89 or HeLa-K^b cells, 5×10^4 cells) for 16-22 h. Alternatively, transfected HeLa-K^b APCs were titrated and incubated with B3Z T hybridomas (1×10^5 cells) in 96-well plates. The T cell response was measured with the substrate chlorophenol red-β-D-galactopyranoside (CPRG). The product was measured with a 96-well plate reader at 595 nm and 655 nm as the reference wavelength.

To measure the cell surface expression of MHC class I complexes after acid wash recovery, HeLa or HeLa-K^b cells were stained for 20 minutes on ice with anti-HLA-A/B/C (W6/32) or anti-H-2K^b (5F1.5), respectively, before staining with anti-mouse secondary antibody (Invitrogen). Stained cells were washed and resuspended in FACS buffer (PBS+2.5% FBS) with cell viability indicator TO-PRO-3 (Life Technologies). Cells were analyzed by flow cytometry on an LSRII (Becton Dickinson, San Jose, CA) cytometer and the data was analyzed using the FlowJo software (TreeStar, Ashland, OR).

^{35}S labeling
To measure new protein synthesis using radioactive ^{35}S labeling, HeLa-K^b cells were transfected with siRNA as indicated. After two days, cells were incubated in methionine/cysteine free DMEM media for 30 minutes, followed by a 15-minute incubation with ^{35}S-methionine and 5% FBS in methionine/cysteine free DMEM media. Cells were then washed twice with PBS and lysed in lysis buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1mM DTT) including protease inhibitors (0.5 mM PMSF, 10 μM MG132, 2 μg/ml Leupeptin, 2 μg/ml Aprotonin and 10 μM Pepstatin A) for 30 minutes on ice. Cell debris was removed by centrifugation at 4°C for 20 minutes. The sample supernatant was mixed with 1×SDS loading buffer and boiled for 10 minutes. The protein extracts were separated by electrophoresis in SDS-polyacrylamide gels. Gels were stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories, Inc) for 1 hour and washed with distilled H_2O for at least 2 hours. Gel was then dried for 2 hours using BioRAD gel dryer model 583 (Bio-Rad Laboratories, Inc). Dried gel was exposed to a PhosphorImager Screen and imaged using Typhoon scanner.

Cell fractionation and Western blots
Cell cytosol and nuclear fractions were prepared using REAP method as described (Suzuki et al., 2010). Cell lysate sample was equally divided into three parts, followed by enzymatic reaction with Endo H (New England Biolabs), PNGase F (New England Biolabs) or mock, respectively, according to the manufacturer's instructions. The digested lysate sample was mixed with 1×SDS loading buffer and boiled for 10 minutes.

To detect protein levels, protein extracts were prepared as previously described and subsequently separated by electrophoresis in SDS-polyacrylamide gels, transferred to nylon polyvinylidene difluoride (PVDF) membranes (Hybond P; GE Healthcare, Piscataway, NJ), and probed with specific antibody. The following antibodies were used: anti-Actin (C-1) (Santa Cruz Biotechnologies), anti-eIF4E (Cell Signaling Technology), anti-NCBP1 (Proteintech Group and Abcam), anti-H-2K^b (Ab194), anti-HA (12CA5 (Covance)), anti-Lamin A (Abcam), anti anti-α-tubulin (Abcam). Horseradish peroxidase (HRP)-conjugated secondary anti-goat, anti-rabbit, and anti-mouse antibodies were purchased from Santa Cruz Biotechnology, Inc.

mRNA transcription and transfection
The DNA template plasmids pcDNA1-SIINFEHL-betaglobin (WT and PTC) were linearized with HpaI and purified using electrophoresis. The *in vitro* transcription was performed with mMEssage mMACHINE T7 Ultra kit (Invitrogen) and mRNAs were subsequently purified with MicroSpin G-25 column (GE Healthcare), according to the manufacturer's instructions. HeLa-K^b cells were first transfected with scrambled or CBP80 siRNA as described above. The cells were plated the night before at 5×10^5 cells/well for 6-well plates. The next day, mRNA were transfected at 0.05 μg per well for 4 hours using TransMessenger Transfection Reagent (Qiagen) according to the manufacturer's instructions.

qRT-PCR:
Total cell RNA was isolated using Trizol reagent (Invitrogen) followed by DNase digest (Promega), according to the manufacturer's instructions. RNA quality was analyzed using the Spectrophotometer NanoDrop 2000 (Thermo Scientific). 1 μg of quantified RNA was converted to cDNA by random priming using OligodT_{12-18} Primer (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. qPCR was performed with SYBR Green PCR Core Reagents (Applied Biosystems) on an 7300 Real Time PCR System machine (Applied Biosystems) with Sequence Detection Software (Applied Biosystems), according to the manufacturer's instructions.
cDNA of SL8 betaglobin was amplified using the primer pair 5'-CCTGAGGAGAAGTCTGCCGTTA-3' and 5'- CAAGGGTAGACCACCAGCAG-3', or 5'-GAAGGCTCATGGCAAGAAAG-3' and 5'-GCTCACTCAGTGTGGCAAAG-3'. cDNA of human GAPDH was amplified using the primer pair 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-GAGGCAGGGATGATGTTCTG-3'.

cDNA of XG182 ES cells was amplified using the following primer as indicated:

e1f (5’- GCACAGCTACGAGAACGATG-3’),
e2r (5’-AACAGGCACTCTTTTCCCCTA-3’),
gt1 (5’-GTTTTCCCAGTCACGACGTT-3’),
gt2 (5’-GAGGAGAAAGGGCAGAGGTT-3’),
e2f (5’-TGGACAACCTCACAAAAGGA-3’),
e3f (5’-TGGCTGGTGTTTTAGAAGCTG-3’),
e3r (5’-GGCATTCAGCAGTCCAACTAA-3’),
e4f (5’-GCACGTCTGTTACCTGAGAAGC-3’),
e4r (5’-AGCTTCTACAAATTCTCCTCCAAA-3’).

The simultaneous analysis of serial dilutions of RNA ensured that qRT-PCR analyses were quantitative. All samples were analyzed in triplicate and relative mRNA levels were determined from CT values according to the ΔΔCT method (Applied Biosystems) after normalization to GAPDH as a reference gene.

Fluorescence recovery after bleach (FRAP)
Confocal analysis of living Mel Juso cells was performed with a Zeiss LSM 710 AxioObserver Inverted confocal microscope system containing incubation chamber. The incubation chamber was set to a condition of 37°C and 5% CO_2. To inhibit proteasomes, cells were incubated with 10 μM lactacystin for 30 minutes. To deplete ATP, cells were incubated with 0.05% sodium azide and 50 μM 2-deoxyglucose for 30 minutes. To block translation, cells were incubated at 37°C in the presence of 200 μM cycloheximide for 30 min. For CBP80 down-regulation experiments, cells were transiently transfected with CBP80 siRNA as described for 72 hours. To measure TAP diffusion rate, one circular spot with a radius of 0.82 mm in the endoplasmic reticulum was bleached at full intensity. To monitor the fluorescence, a time-lapse photography was used. Another spot with the same size in the endoplasmic reticulum within the same cell was monitored at the same time during FRAP. The diffusion coefficient, D, is described by the formula $D = w^2/\tau$, where w is the radius of the circular bleaching spot (in our experiments was 0.82 mm), and τ is dependent on the laser intensity. The data was analyzed with EMBL Frap-analysis software (using Soumpasis Diffusion model). The fluorescence intensity of unbleached spot was used as baseline in analysis to correct the attenuation of fluorescence due to imaging. D was determined using 8 cells per experiment and depicted as means ± s.d.

Mice
BayGenomics ES cell line XG182 (Stock Number: 008178-UCD) containing gene trap vector (pGT1lxf) was purchased from MMRRC. The parental cell line E14Tg2a.4 (MMRRC Stock Number 015890-UCD) was derived from mouse strain 129P2/OlaHsd.
BayGenomics ES cell line XG182 passed pathogen screening and sequence confirmation performed by MMRRC. The sequence from the MMRRC lab is:
CGTATATGTATGCTATACGAAGTTATCGATCTGCGATCTGCGTTCTTCTTCTTTGGTT
TTCGGGACCTGGGACCATCGTTCTCGTAGCTGTGCCGCCTCCGCGACATGCTGCTTG
CTGGTGCGCAGCCGATCTGAGTTGCTAGCCGCCTGCAACCCCAGAACCGTCGGGCTG
CCGGCCGGCCGGTGCGCGGTCAGCGGCGAGGCGCGCGGACTCCCCCCCCCCCCCCMW
WMARWGKGGRRRAAAAAAAKAAAAAAARGWCGARACGAWCTGAATTGCTGSCCG
CCTGCWACCCCCAGAMCSGTCGGGCTGCCGGCSGGCGGTGCKCGGKCAGCGGCGAG

GCGSGCGMCCTCCCCCCCCCCCSCMWWTRWMSAAAAAAAAAAAAAARRGKT. The sequence BLASTs to Chr 4, Ncbp1, exon 1.

Chimera mice were generated by Gene Targeting Facility of University of California at Berkeley. Only one chimera mouse (male) from XG182 ES cell line was obtained. XG182 chimera mouse was crossed with C57BL/6J female mice to generate XG182 F1 mice. C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor ME). Potentially CBP80-deficient offspring was analyzed by genotyping-PCR of tail biopsies. A 751 bp fragment was detected with primers 5'-ATGTTGATGAAAGCTGG CTAC AGGAAGG -3' (forward) and 5'-AGCAGGATATCCTGCACCATC GTC T -3' (reverse) for the mutant allele with gene trap vector. A 526 bp fragment between CBP80 exon 17 and exon 18 was detected with primers 5'-AGTCCTTCAGTCACTCCTTCAGTGCT-3' (forward) and 5'-CTCCGCCAGAGTTTTGAAGACTTCATG-3' (reverse). Use of mice was under the approval of the Animal Care and Use Committee of the University of California at Berkeley.

Immunization

To generate WT anti-heterogyzous CBP80 T cells, female F1 wild-type mice were immunized intraperitoneally with $2x10^7$ spleen cells from male F1 heterozygous or wild-type mice, twice with a ten-day gap. Ten days after immunization, spleen cells were restimulated *in vitro* with irradiated spleen cells from female mice of the same genotype used for immunization. 20 U/ml of hIL-2 (BD Biosciences) was added into cell culture. The second restimulation was done 7 days later.

Measurement of surface Kb and Db

Spleen cells of the XG182 F1 mice were blasted overnight with 200 ng/ml LPS. Cells were first stained with Fc block (1:100 dilution in FACS buffer) followed by staining with surface marker antibodies (1:100 dilution in FACS buffer) including anti-CD8α, anti-B220, anti-H-$2K^b$ and anti-H-$2D^b$ for 15 minutes on ice. Cells were analyzed by flow cytometry and with FlowJo software.

Measurement of intracellular IFN-γ production

The CD8+ T cell responses of the immunized mice were assessed by measurement of intracellular IFN-γ production. Spleen cell APCs were depleted with CD4+ and CD8+ cells using magnetic beads (Dynal Biotech, Invitrogen) before incubated with restimulated CD8+ T cells. The mixture of APCs and CD8+ T cells were incubated for 5 hours with 1 μl/ml brefeldin A/GolgiPlug (BD Biosciences).

For staining, cells were first stained with Fc block (1:100 dilution in FACS buffer) followed by staining with surface marker antibodies (1:100 dilution in FACS buffer) including anti-CD8α, anti-CD4, anti-TCR and anti-CD19 for 15 minutes on ice. Cells were fixed using cytofix/cytoperm (BD Biosciences) for 20 minutes on ice or overnight at 4°C. After being washed in perm/wash buffer (BD Biosciences), cells were stained with anti-IFN-γ (1:100 dilution in perm/wash buffer) for 20 minutes on ice. Cells were analyzed by flow cytometry and with FlowJo softwared.

Statistical analysis

Analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance (p-value ≤ 0.05) was determined using the two-tailed unpaired t test.

Chapter 8: References

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