

**Nonsense Mediated Decay Associated Pioneer Round of  
Translation as Source for Peptides for Presentation  
by MHC Class I**

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## **Abstract**

### **Nonsense mediated decay associated pioneer round of translation as source for peptides for presentation by MHC class I**

Nonsense mediated decay (NMD) plays a critical role in the mRNA quality control mechanism protecting cells from aberrant mRNAs containing premature termination codons (PTC). Recognition of these PTCs requires a pioneer round of translation prior to the bulk translation of mRNAs for cellular protein synthesis, therefore producing a pool of immediate early peptides.

Effectiveness of the immune surveillance system is insured by the timely and complete MHC I mediated presentation of a pool of precisely cleaved peptides representing all cellular proteins. So far sources of antigenic peptides are known to include regular degraded proteins, defective ribosomal products (DRiPs) and cryptic peptides starting with a non-AUG start codon. Here the contribution of peptides originating from the pioneer translation to the pool of peptides presented by MHC I molecules is described.

Depletion of the essential NMD factor hUpf1 as well as the pioneer translation initiation complex factors CBP80 and CBP20 reveal impaired MHC I antigen presentation. In sharp contrast, targeted interference with bulk translation without effecting pioneer translation permits antigen presentation. Taken together these findings put products of the pioneer round of translation into an important position as novel source for antigenic peptides and reveal a new relationship between NMD and immune surveillance.

*The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.*

*Albert Einstein*

I dedicate this work to Chiaowen Deng

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# **1. Introduction**

The first part of the introduction starts with a bird's view of the immune system providing an overview of the recognition of pathogens and the different layers of defense against them. It will focus on the generation, processing and presentation of peptide sequences in adjunction to the Major Histocompatibility Complex. The second part will shine light on another surveillance mechanism of the cell, Nonsense Mediated Decay, insuring the integrity of the genetic information for protein translation.

This study in its entirety will then demonstrate the interrelatedness and synergies of these two biological systems.

## **1.1 The immune system**

Virtually all organisms have developed elaborate defense mechanisms to recognize and destroy pathogens. Simple unicellular organisms as well as complex eukaryotes were faced with the same central problem of immunology: the accurate, efficient and timely distinction between self and non-self structures. Within the evolution of vertebrates the immune recognition acquired additional levels of complexity, which can be divided into two major arms.

The innate immune system as the evolutionary oldest part consists of several mechanisms including humoral defense, mucosal barriers and inflammation. It further encompasses germ line encoded pattern recognition receptors with the ability to detect structures

common to multiple groups of pathogens. In mammals the Toll-like receptors (TLR), originally discovered in *Drosophila melanogaster* (Anderson, Bokla et al. 1985; Anderson, Jurgens et al. 1985), take a central role in detecting microbial molecules. This group of transmembrane receptors bind to highly conserved features of pathogens including double stranded RNA from viruses and lipopolysaccharid (LPS), lipoteichoic acid (LTA), flagellin and unmethylated CpG DNA from bacteria (Medzhitov and Janeway 1997). They represent the trigger of a signaling cascade activating multiple genes controlling the function of the complement system, the natural killer (NK) cells, cytokine secretion, apoptosis and phagocytosis. While the innate immune system can rapidly and successfully clear the organism from many infections, it is closely linked to the second line of defense – the adaptive immune system. Particularly NF- $\kappa$ B mediated activation of the interferon response serves as bridge between innate and adaptive defense (Barton and Medzhitov 2003). Interferon type I, consisting of INF  $\alpha$  and  $\beta$  and the less characterized  $\epsilon$ ,  $\kappa$ ,  $\omega$ ,  $\delta$  and  $\tau$ , is mainly expressed in virus infected cells. Interferon type II only comprises of one cytokine, INF  $\gamma$ . It takes a central role in activating the major histocompatibility class I and class II (MHC) pathway, the proteasome, apoptosis, natural killer cells and macrophages as well as inducing more than 200 other genes (Boehm, Klamp et al. 1997; Kloetzel 2004).

### **1.1.1 Adaptive immunity**

The adaptive immune system establishes immunological memory by recognizing previously encountered antigens and mounting a highly targeted immune reaction. It is composed of two arms, the humoral and cell mediated immune response. While both B-lymphocytes and T-lymphocytes have their origin in the hematopoietic stem cells of the bone marrow, their functions are highly specialized. The receptors expressed on the large and very diverse set of B-cells recognize pathogens in the extracellular milieu and induce defense through the secretion of antibodies. Pathogens that escape extracellular surveillance and that enter host cells become targets for cytotoxic CD8 T-lymphocyte detection and destruction. Host cells display antigens, short peptide sequences representing virtually all sources of protein originating from self or foreign structures inside the cell. Major histocompatibility complex class I (MHC I) molecules serve as crucial interaction partners ensuring the binding, conformation and integrity of the peptides for display to CD8 T-lymphocytes. In order for the antigen presentation pathway to create a complete and up to date “inventory of the cell” on its surface, multiple steps of a complex process have to be continuously and precisely orchestrated. Proteins and polypeptides are channeled through chaperones, cleaved by the proteasome and several proteases in the cytosol, transported by the transporter associated with antigen presentation (TAP), edited in the ER, loaded to MHC class I molecules and finally displayed on the cell surface for CD8 T-lymphocyte screening. These subsequent steps of the antigen presentation pathway will be discussed in detail.

### 1.1.2 The Major Histocompatibility Complex class I

One of the most diverse proteins, MHC class I molecules are encoded in a large genomic region of the human chromosome 6 (chromosome 17 in mice) together with about 140 other genes including MHC II, nonclassical MHCs and several other components of the immune system. Their discovery and initial description by George Davis Snell, Baruj Benacerraf and Jean Baptiste Dausset represents one of the hallmarks of modern immunology (Snell 1992). The biological significance was discovered by Zinkernagel and Doherty when observing that virus induced CD8 T-cells recognize virus infected cells in a MHC class I restricted way (Zinkernagel and Doherty 1974). MHC class I molecules consist of a membrane spanning 45 kDa  $\alpha$  chain and a non covalently associated invariant 12 kDa  $\beta_2m$  microglobulin chain. The alpha chain has three domains  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  with the peptide binding groove positioned between  $\alpha_1$  and  $\alpha_2$ . Each MHC class I molecule binds to a spectrum of different peptides consisting of 8 to 10 amino acids. The human leukocyte antigen (HLA) genes consist of HLA A, B and C as well as the non-polymorphic HLA E, HLA F, HLA G. Mouse MHC class I molecules have a distinct nomenclature with major antigens H-2K, D and L. A polymorphism with hundreds of alleles for each locus creates a remarkable diversity of expressed MHC class I molecules between individuals. Variations in HLA allele expression are a major determinant for susceptibility for many infections, tumor progression, auto immune diseases and transplantation tolerance (Nolan, Gaudieri et al. 2006). While MHC class I molecules are expressed in almost all nucleated cells in order to insure complete surveillance and protection from infections and potentially harmful mutations their

expression level varies considerably. Lymphocytes show relatively high expression levels of around  $5 \times 10^5$  MHC class I molecules per cell in comparison to fibroblast, liver and muscle cells with low expression. Some neuron and sperm cells have almost undetectable levels of MHC class I surface expression.

### **1.1.3 The MHC class I - peptide interaction: a perfect match**

Allelic variation creates hundreds of different MHC class I molecules but it is no match for the level of diversity added by the peptides. The interface between the MHC class I molecule and the peptide has to achieve an extraordinary task therein: binding to a huge array of different peptides from virtually all self and foreign protein sources while retaining the ligand on the cell surface and ensuring access for the T-cell receptor (TCR) for recognition. A first insight into the MHC class I peptide interface was gained when crystallographic structures of the MHC became available. The  $\alpha_1$  and  $\alpha_2$  chains each contribute four strands of an antiparallel  $\beta$ -sheet and a long interrupted helix to built an approximately 30 Å long and 12 Å wide peptide binding groove (Madden 1995; Rudolph, Stanfield et al. 2006). Particularly the residues pointing inside this groove comprise high variability enabling to interact with a multitude of ligands. In contrast to the MHC class II binding groove both ends are closed by a set of highly conserved residues limiting the maximum length of ligand chains. The MHC class I molecules are only stable with a bound peptide. Eluting peptides from MHC class I molecules and subsequent sequencing helped to define conserved and flexible residues of the peptide ligands and to create

valuable bioinformatics tools for antigen sequence analysis (Falk, Rotzschke et al. 1991). One or two positions p<sub>2</sub>, p<sub>3</sub> and the p<sub>9</sub> C-terminus are usually highly conserved anchor residues ensuring a stable interaction with the MHC class I molecule. This leaves high flexibility for the remaining six or seven residues creating diversity in the magnitude of 10<sup>8</sup> different peptides (Shastri, Schwab et al. 2002). While the vast majority of peptides are between 8 and 10 amino acids in length some exceptions due to bulging and extension over the peptide groove have been described (Fremont, Matsumura et al. 1992; Collins, Garboczi et al. 1994; Speir, Stevens et al. 2001). Finally it should be noted that MHC class I molecules are not just passive recipients of their ligands but rather actively involved in their generation. In what is called the “Rammensee-Paradox” two contrary ideas propose either the rapid generation and protection of peptides exclusively in the cytosol or the binding and subsequent trimming of peptides with MHC class I molecules acting as templates in the ER (Shastri, Schwab et al. 2002). Recent evidence points the way to common ground of these hypotheses with MHC class I molecules binding to suitable peptides and providing protection from further degradation together with additional trimming to finalize the peptide in the ER (Kanaseki, Blanchard et al. 2006).

## **1.1.4 Origins of antigenic peptides presented by MHC class I molecules**

### **1.1.4a Defective Ribosomal Products (DRiPs)**

The efficiency of the MHC class I immune surveillance depends on the complete, continuous and rapid presentation of virtually all self and foreign sources of peptides. For a long time antigenic peptides have been thought to predominantly originate in conventional cellular proteins being degraded according to their normal half live (Moore, Carbone et al. 1988). This concept was initially supported by findings correlating the protein stability and degradation to the rate of cell surface presentation for at least some particular proteins (Grant, Michalek et al. 1995; Sijts and Pamer 1997). Cellular proteins have a half -life ranging from minutes to several days with an estimated average life span of 10 hours before being degraded and becoming potential sources of antigen presentation. Within the last 10 years it became more and more clear that antigen presentation is often closely linked to new synthesis of polypeptides (Yewdell and Nicchitta 2006) . Evidence from several studies suggests that 30% or more of newly synthesized proteins are directly funneled into destruction through the interplay of the proteasome and several peptidases. Several hypotheses have been postulated to explain the fast degradation of these polypeptides with defective ribosomal products (DRiP's) being the most prominent concept. This idea suggests that many newly generated polypeptide chains cannot fold to their correct three dimensional confirmation within a certain time frame. The reason for the inability of these peptide chains to achieve their correct tertiary structure has been target of much speculation. Alternative reading frames

or problems in the mRNA generation, protein biosynthesis, translation termination, sub-cellular targeting or assembly with subunits and folding factors have been proposed. Further it has been observed that the generation of antigenic peptides is independent of the stability of endogenously synthesized peptides (Goth, Nguyen et al. 1996). Therefore not the normal protein turnover but a certain subset it is relevant for antigen presentation. Additionally a set of potent chemical inhibitors of various stages of protein biosynthesis played a crucial role in elucidating the direct relationship between translation and antigenic peptide generation. Several previous studies as well as experimental data herein narrowed down the time required for translation of many antigenic peptides to less than 10 minutes (Schubert, Anton et al. 2000). Particularly in the case of viral evasion of a cell, fast availability of antigens from immediate early gene products as well as of often long lived coat proteins becomes crucial for efficient host defense. Taken together monitoring protein synthesis as opposed to cellular protein turn over simply makes immunological sense.

#### **1.1.4b Rapidly degraded Products (RDPs)**

In contrast to DRiPs the term rapidly degraded products (RDPs) refers to the majority of otherwise normal, physiologically relevant nascent polypeptide chains in healthy cells that are degraded prior to reaching their destined functional conformation. (Qian, Princiotta et al. 2006) Cells maintain a fine balance between chaperone assisted folding and degradation through the ubiquitin or other pathways (Hershko, Ciechanover et al.

1979; Wilkinson 2005) It was shown that a large subset of nascent polypeptide chains, probably up to 70% are targeted for immediate degradation.

#### **1.1.4c Cryptic Translation**

For long translation was manifested as ribosomal recognition of an AUG start codon and generation of a continuous polypeptide chain subsided by folding to a predetermined protein conformation. Recent findings draw a different picture demonstrating several sources of unconventional or therefore cryptic translation products (Shastri, Schwab et al. 2002; Ho and Green 2006) Alternative mRNA splicing as well as frame shift events during transcription account for the generation of multiple antigenic peptides that efficiently initiate cytotoxic T-cell responses. Further examples include the recognition of alternative reading frames using downstream AUG codons for initiation as well as translation of conventionally untranslated regions in the 5'UTR and 3'UTR or intron sequences (Uenaka, Ono et al. 1994; Guilloux, Lucas et al. 1996). The now common practice of cell transfection of mini-genes encoding only short peptide sequences further illustrates the obsolescence of full length proteins for successful T-cell recognition. In stark contrast to a long prevailing dogma translation initiation can occur at codons other than AUG (Peabody 1989). At least six different initiation codons have been identified that result in peptide generation and constitutive display by MHC class I molecules. Especially for the case of CUG mediated initiation, consistent levels of CD8 T-cell activating antigens are observed in vitro using mini-genes as well as in a transgenic

mouse model (Malarkannan, Horng et al. 1999; Schwab, Li et al. 2003; Schwab, Shugart et al. 2004). Surprisingly initiation with CUG encodes a leucine rather than a “wobble” methionine. Current studies utilize primer extension inhibition assays, so called “toe-printing”, as means to monitor the precise binding region of the initiating ribosome. Several compounds have been identified that differentially impact CUG or AUG mediated initiation of translation (Green, Ow et al.; Kozak and Shatkin 1978). Especially with the discovery of internal ribosomal entry sites (IRES) it became clear, that alternative machineries of translation not only appear in mammalian cells but serve distinct functions far beyond their original viral role (Pfungsten and Kieft 2008). Different regulatory and refining mechanisms of translational control will be discussed later. So far these findings pave the road for future hypotheses about an alternative CUG based initiation mechanism that can generate antigenic peptides. The function of CUG start codons in the biosynthesis of full length proteins still remains to be elucidated.

#### **1.1.4d Antigens generated through peptide splicing**

Another pool of unanticipated antigens involving peptide splicing was discovered. Usually MHC class I presented peptides are expected to consist of a continuous sequence of 8 to 10 amino acids. Vigneron et al. demonstrated the antigenicity of two non-continuous and rejoined peptide fragments of melanocytic glycoprotein (gp100) in melanoma cells (Vigneron, Stroobant et al. 2004). An initial 13mer precursor peptide required proteasomal processing resulting in the excision of four amino acids and

subsequent trans-peptidation involving an acyl-enzyme intermediate in order to be recognized by human CD 8 T lymphocytes. Similarly Hanada et al. have shown the immune recognition of a fibroblast growth factor-5 (FGF-5) derived spliced nonamer peptid (Hanada, Yewdell et al. 2004). The diversity of unconventional peptide sources recently reached a highlight with the unanticipated example of a 10 residue peptide consisting of a tetra- and hexamer being rejoined in reverse order (Warren, Vigneron et al. 2006).

#### **1.1.4e Cross-presentation of peptides**

Cross-presentation denotes the ability of antigen presenting cells (APC's) to receive, process and present exogenous peptides via MHC class I molecules on their cell surface to trigger CD 8 T-cell activation. Cross-presentation considerably deviates from some characteristics of other antigenic sources described above as it is more prone to utilize full length protein. The ability to present exogenous peptide is mainly limited to dendritic cells (DC) with marked differences between distinct subsets of cells (Mellman and Steinman 2001; Guermonprez and Amigorena 2005). This process is particularly important for the immune surveillance of cells that do not actively undergo antigen presentation themselves. It enables dendritic cells to present viral or tumor antigens even in the case that they are not directly effected therefore closing an otherwise detrimental gap in the immune defense. While the phenomenon of cross-presentation is already known since 1976, several details of the pathway remain in the dark (Bevan 1976; Bevan

1976). Exogenous material is largely acquired by endocytosis of whole or partial cells leaving potential antigens in the phagosome. This opens the question of how peptides reach the cytosol and proteasome for further processing. “Leaky” passing through the phagosomal membrane as well as a specific transport mechanism involving Sec61 are current possibilities under investigation (Ackerman, Giodini et al. 2006). Alternatively fusion of phagosomes with the ER compartment and direct processing and loading of antigenic peptides to MHC class I molecules within the lumen of the phagosome has been proposed (Rock and Shen 2005). A remarkable extension of established cross-presentation pathways imposed the demonstration of active transfer of peptides between the cytosol of adherent cells through gap-junctions into antigen presenting cells (Neijssen, Pang et al. 2007). Additionally various approaches of cancer immunization using antigens, completely folded tumor proteins as well as whole cells prove the importance of cross-presentation for in vivo immune functions (Osanto 1997).

#### **1.1.4f Pioneer round of translation peptides**

Pioneer round of translation peptides are by definition the polypeptides generated within the first round of translation of each newly transcribed and spliced mRNA. They were discovered in the context of an mRNA surveillance mechanism called Nonsense mediated decay (NMD) and might combine several of the features of peptide sources described so far. At this point the subsequent steps of the generation and processing of antigens to their final presentation on the cell surface will be followed. The important

and novel role of pioneer peptides will be introduced within the context of NMD and described in detail in section 1.2.

### **1.1.5 Creating antigenic precursors through proteasomal degradation**

Despite the very different life time of structural proteins or unfolded polypeptide chains they eventually reach their fate of proteolysis through the proteasome and cytosolic proteases. The central function of the proteasome is the degradation of nuclear, cytosolic and transmembrane proteins that have reached their life span. While this process is crucial for the elimination of regulatory or infunctional proteins it also generates precursors of antigenic peptides of various length and stability for MHC class I mediated presentation. Crucial findings about the importance and direct involvement of the proteasome for antigen presentation came from studies with small molecule inhibitors. After the initial finding of reduced antigen presentation of a chicken ovalbumin derived peptide by MHC class I molecules various inhibitors like LLnL, MG115, MG132, lactacystin and to a lesser extent LLM became an important tool to study antigen presentation (Rock, Gramm et al. 1994; Anton, Bennink et al. 2001). The 26S proteasome consists of a barrel-shaped 20S catalytic core domain harboring all catalytically active residues and two 19S subunits that define substrate specificity. The proteasome recognizes polyubiquitin-labels and other degrons of proteins earmarked for destruction and degrades them in an ATP dependent process (Varshavsky 1997). Interestingly the two proteasomal subunits LMP2 and LMP7 are encoded in a INF  $\gamma$  controlled gene

region in direct proximity to the TAP-genes indicating their importance for the immune response. Upon INF  $\gamma$  induction these chains replace the constitutively expressed 20S proteasome subunits. This complex with altered proteolytic functions is called the immunoproteasome. The proteasomal activator PA28 has been described as an interaction partner, which induces small conformational changes of the 20S proteasome. These alterations potentially induce subtle changes in the orientation of some of the active residues of the 20S proteasome leading to the generation of slightly longer peptides. Accordingly it has been demonstrated that the expression level of PA28 alters the pool of recognized antigens on the cell surface (Sun, Sijts et al. 2002). With the discovery of the proteasome maturation protein (POMP) another factor entered the complex stage of proteasomal regulation. POMP serves as a chaperone tightly regulating the assembly and deterioration of the immunoproteasome. In response to viral induced cytokine release the immunoproteasome is rapidly up regulated while cells smoothly return to constitutive proteasome expression when an immune response becomes obsolete (Heink, Ludwig et al. 2005). Constitutive expression of the immunoproteasome in immune privileged brain and ocular tissue as well as indications of altered CD8 T cells gave rise to the idea of some involvement in alternative immunological processes beyond antigen presentation (Chen, Norbury et al. 2001; Singh, Awasthi et al. 2002). Other proteases like leucine aminopeptidase and tripeptidyl peptidase (TPP) seem to be involved in cytosolic processing even though their effect on the surface antigen repertoire remains to be demonstrated conclusively (Stoltze, Schirle et al. 2000; Reits, Neijssen et al. 2004).

### **1.1.6 Chaperone function and antigen processing in the cytosol**

The folding, assembly and eventually the degeneration of proteins is largely dependent on the interplay of chaperones, proteases and the proteasome. A role of chaperones in protecting them from their ultimate fate of degradation into single amino acids, in regulating subcellular peptide concentration and in guiding them to MHC class I molecules has been suspected for long (Li and Srivastava 1994). Among others the chaperones GRP94, HSP90, GRP78 and HSP70 have been associated with altered antigen presentation and CD8 T-cell recognition while their precise interaction mechanism with the proteolytic peptide intermediates remained unresolved (Gullo and Teoh 2004). A detailed analysis of HSP90 revealed its interaction with C-terminally extended peptide precursors taking advantage of the well characterized SIINFEKL system in conjunction with high performance liquid chromatography (HPLC) fractionation. Differential RNA interference mediated depletion demonstrated the crucial function of HSP90 $\alpha$  as well as the co-chaperonin CHIP in protecting and channeling C-terminally extended precursors for MHC class I loading (Kunisawa and Shastri 2006). In a previous study the selective role of group II chaperonin TRiC for the expression of peptide-loaded MHC class I molecules on the cell surface became evident (Kunisawa and Shastri 2003). Accordingly also chemical inhibition of HSP90 with radicicol geldanamycin , 17-DMAG and 17-AAG showed the generation of “empty” MHC class I molecules and reduced levels of T-cell activation. So far a predominantly post-proteasomal role for the interaction of HSP90 antigenic precursors is anticipated by the authors (Callahan, Garg et al. 2008). Very recently HSP90 has been also reported to

directly facilitate the assembly and function of the 26S proteasome therefore subsequently accounting for alterations in the surface presentation of peptides bound to MHC class I molecules (Yamano, Mizukami et al. 2008). Additional observations of HSP90 participation in DC mediated cross-presentation are a reminder that our understanding of the complete picture of chaperone function in antigen presentation still requires further elucidation (Giodini and Cresswell 2008).

### **1.1.7 From cytosol to ER: peptide transport via TAP**

The transporter associated with antigen processing (TAP) was almost simultaneously discovered by four laboratories in 1990. Initially two genes were found in direct proximity to the MHC locus with high sequence homology to ABC (ATP Binding Cassette) transporters. They encode a dimeric transmembrane complex named TAP1 and TAP2 that turned out to be responsible for the ER translocation of the vast majority of antigenic peptide precursors (Deverson, Gow et al. 1990; Monaco, Cho et al. 1990; Spies, Bresnahan et al. 1990; Trowsdale, Hanson et al. 1990). The TAP mutant human cell line LBL 721.134 (TAP1 mutant) (Cerundolo, Alexander et al. 1990) as well as the murine cell line RMA-S (TAP2 mutant) (Powis, Townsend et al. 1991) were crucial in establishing a functional link between wildtype TAP1 and TAP2 expression and MHC class I mediated CD8 T-cell stimulation. In both cell lines MHC class I peptide presentation could be reestablished by DNA transfection of TAP1 or 2 gene encoding DNA sequences. Severely reduced MHC class I surface expression as well as complete

absence of CD4 and CD8 T-cells was also observed in a TAP1 deficient mouse (Van Kaer, Ashton-Rickardt et al. 1992). Peptide translocation assays using N-glycosylated or respectively radiolabeled peptides in Streptolysin O permeabilized cells added the final evidence for the ATP-dependent ER translocation of antigenic peptides through TAP (Androlewicz, Anderson et al. 1993; Neefjes, Momburg et al. 1993). Androlewicz et al. further determined the preferred length of peptides transported by TAP as between 8 to 12 amino acids. Peptide chains in the length of 13-30 amino acids are transported to a much lesser extent while longer peptides are inevitably cleaved prior to transport. A clear correlation between the sequence requirements for TAP transport and the binding preferences of their respective MHC class I became evident. Human peptides allow a larger variety of peptide sequences both for TAP transport and MHC class I binding while mouse peptides are on the other side of the spectrum with more restrictive sequence specificity. The C-terminal flanking residues are a major determinant for TAP transport with basic and hydrophobic amino acids being more efficiently translocated in mouse, human and rat cells (Androlewicz and Cresswell 1996). While preferences for TAP transport generally reflect the requirements for MHC class I binding there are some marked exceptions. Four immunodominant viral peptides were found to be very inefficiently transported by TAP. A proline in position 3 was identified as a consensus amino acid preventing efficient TAP transport (Neisig, Roelse et al. 1995). A seminal study described the importance of new translation for TAP mediated transport of peptides emphasizing not only sequence features but also the origin of a peptide for efficient transport and presentation. In this case the authors used fluorescence recovery after photo bleaching (FRAP) with a TAP1 green fluorescence (GFP) fusion protein in order to

determine TAP1 mobility within the ER membrane as a measure for active peptide translocation (Reits, Vos et al. 2000). Different alleles encoding the TAP1 and 2 complex helped to gain further insight into the mechanism of TAP transport. The alternative rat allele RF1.Aa enabled to directly analyze the changes in MHC class I peptide surface presentation due to selectivity of the TAP transport (Powis, Young et al. 1996; Knittler, Gulow et al. 1998). Over the time of evolution viruses have developed evasion strategies against almost all major components of the immune system. TAP remains no exception. The Herpes simplex virus (HSV) expresses immediate early protein ICP47 which binds to TAP therefore preventing any further viral peptides from translocation and antigen presentation (Fruh, Ahn et al. 1995; Hill, Jugovic et al. 1995). Similarly proteins like human cytomegalovirus derived US6, the bovine herpesvirus type 1 encoded UL49.5 and the BNFL2a protein of Epstein Barr virus bind to TAP and introduce subtle conformational changes to arrest transport (Hislop, Rensing et al. 2007; Oosten, Koppers-Lalic et al. 2007). Finally there are some TAP independent pathways that lead to productive MHC class I loading and surface presentation of peptides. In the case of the completely TAP deficient human cell line T2 721.174 no surface expression of MHC class I HLA B and HLA C, but about 20-50 % of normal surface presentation of MHC class I HLA A2 can be detected. Here signal sequence derived peptides translocated into the ER by the signal recognition pore SRP account the for the supply of antigenic peptides (Wei and Cresswell 1992).

### 1.1.8 The ER based Peptide Loading Complex

MHC class I molecules mostly receive their peptides when associated to TAP as well as to the peptide loading complex (PLC). This multi protein mechanism consisting of calnexin, calreticulin, tapasin, ERp57 and several further factors coordinates peptide binding, quality control and editing in a precisely orchestrated process (Peaper and Cresswell 2008).

As a prerequisite of peptide binding calnexin facilitates correct assembly and folding of the microglobulin  $\beta_2m$  jointly with the heavy chain (HC). This 90 kDa transmembrane protein includes a globular domain mediating glycan binding as well as a P-domain important for ERp57 interaction. The ER resident chaperone calnexin prevents protein aggregation and helps to target misfolded protein for degradation (Molinari, Eriksson et al. 2004). Together with the homologous lectin calreticulin they both act in a calnexin-calreticulin cycle facilitating the folding of glycoproteins while exposing them to additional folding factors such as the below mentioned ERp57 (Hammond and Helenius 1993). Taken together MHC class I peptide assembly can be envisioned as a distinct process of protein folding with the involvement of multiple chaperones.

Within the PLC tapasin serves as an initial factor insuring the stability of the complex as well as the optimal loading of the MHC class I molecule. Tapasin is a 48 kDa transmembrane glycoprotein with an ER signal sequence encoded in the MHC locus of chromosome 6. It mediates the contact between TAP and the MHC class I molecule after assembly of the heavy chain and the microglobulin  $\beta_2m$  (Grande, Androlewicz et al.

1995; Ortmann, Copeman et al. 1997). Detailed analysis on the stoichiometric interaction demonstrated a 4:4:1 ratio of MHC class I, tapasin and TAP molecules (Bangia and Cresswell 2005). A recent study using blue native-PAGE introduced some controversy postulated two distinct oligomers of 350 and 450 kDa with the larger consisting of 2 tapasin molecules and each one MHC class I and calreticulin molecule (Rufer, Leonhardt et al. 2007). While the precise mechanism of tapasin remains unknown at least two distinct functions have been described. First tapasin increases the peptide-binding affinity of MHC class I peptide complexes as shown in a non-peptide-specific approach (Williams, Peh et al. 2002). Second it exerts a quality control function in the ER preventing the export of empty or low affinity MHC class I peptide complexes to the golgi network (Barnden, Purcell et al. 2000). Several additional functions have been suggested linking tapasin to TAP stability and transport efficiency, dissociation of peptides from MHC class I molecules and direct interaction with ERp57 among others. While tapasin is essential for the vast majority of peptides being loaded to MHC class I molecules there are reports of several cases with very low tapasin dependency (Peh, Burrows et al. 1998; Goodall, Ellis et al. 2006). Most striking is the case of HLA-B 4405 and HLA-B 4402 where a single amino acid exchange accounts for a large difference in tapasin requirement for folding, maturation and antigen presentation (Park, Lee et al. 2003).

Only recently the covalent association of tapasin with the thiol oxidoreductase ERp57 was discovered (Peaper, Wearsch et al. 2005). ERp57 belongs to the family of protein disulfide isomerases (PDI) functioning in oxidative glycoprotein folding. Beyond its involvement in the PLC it is widely distributed in the cell including the cell surface,

cytosol and nucleus (Turano, Coppari et al. 2002). A mouse model with B-cell specific ERp57 depletion leading to a strong reduction of MHC class I K<sup>b</sup> and D<sup>b</sup> surface presentation provides evidence for its important role in antigen presentation (Garbi, Tanaka et al. 2006). Further ERp57 increases the interaction time between tapasin, MHC class I potentially by inducing a conformational change within tapasin. Taken together ERp57 seems to be a central stabilizer of the PLC due to its covalent bond with tapasin and weak interactions with calreticulin.

### **1.1.9 The final cut: peptide editing in the ER**

In addition to the mere folding and binding events described in the previous paragraph peptides in the ER undergo a precise editing and trimming process to obtain the final peptides for MHC class I mediated antigen presentation. Proteolytic intermediates that reach the ER typically include N-terminal extensions imposing the requirement for further processing within the ER (Paz, Brouwenstijn et al. 1999; Rock, York et al. 2004; Kanaseki, Blanchard et al. 2006). The C-termini are often already cleaved at their final position within the cytosol. These crucial observations were initially made when analyzing the transport and processing of peptides including prolin residues. As mentioned above, an aminoterminal proline at the position p<sub>1</sub>, p<sub>2</sub> and p<sub>3</sub> (called X-P, X=any amino acid, P= prolin) makes notoriously bad substrates for TAP binding and transport (Neisig, Roelse et al. 1995; van Endert, Riganelli et al. 1995). But despite their poor translocation properties particularly a prolin at anchor position p<sub>2</sub> represents a very

frequent antigen sequence presented by MHC class I molecules (Rammensee, Bachmann et al. 1999). This conundrum was resolved with the discovery of the ER resident amino peptidase associated with antigen processing (ERAAP). N-terminally extended peptides ( $X_n\text{-P}$ ,  $n \geq 2$ ) with the proline “hidden” in the center of the peptide intermediate are efficiently transported by TAP and are precisely cleaved only upon arrival in the ER. ERAAP was initially purified from mouse microsomes. The interferon  $\gamma$  inducible protein is localized in the ER with a similar distribution as MHC class I molecules. Reduction of the aminopeptidase activity using the inhibitor leucinethiol as well as RNAi mediated depletion revealed the functional role of ERAAP in cleaving peptide N-termini to generate the final antigens (Serwold, Gonzalez et al. 2002). The analysis of mice deficient of ERAAP demonstrated changes of MHC class I surface expression (Hammer, Gonzalez et al. 2006; York, Brehm et al. 2006). These were not only manifested in the overall number of MHC class I molecules on the cell surface but rather in a difference of the diversity of antigens presented on the surface. While the surface expression of MHC class I molecules H-2K<sup>b</sup>, H2-K<sup>d</sup>, H2-D<sup>b</sup> and H2-D<sup>d</sup> only decreased about 20%, H-2L<sup>d</sup> was reduced 70% in ERAAP deficient mice. Interestingly only H-2 L<sup>d</sup> molecules include X-P peptides with a proline at position 2 in their repertoire. MHC class I peptide complexes in ERAAP deficient cells showed a reduced binding quality with a 10-20% higher dissociation rate. Most strikingly at least two peptides recognized by the T-cell hybridoma 30NXZ (H13-H-2D<sup>b</sup>) and 1AZ (H47-H-2D<sup>b</sup>) showed a significant increase in HPLC purified extracts of ERAAP -/- splenocytes (Hammer, Gonzalez et al. 2006). Detailed analysis of the antigen repertoire revealed not only a large group of peptides dependent on ERAAP expression but also a group of peptides that are presented on the

surface despite their incomplete trimming by ERAAP. These peptides uniquely expressed in ERAAP deficient cells induce strong immunogenicity in their wildtype counterparts (Hammer, Gonzalez et al. 2007). Additionally there is evidence that ERAAP acts in a synergetic cooperation jointly with the MHC molecule to generate the final MHC class I peptide complex (Paz, Brouwenstijn et al. 1999; Kanaseki, Blanchard et al. 2006). Taken together ERAAP is not only shaping the antigen repertoire but has been identified as the central ER resident aminopeptidase required for antigen trimming in mice. In human cells a homologous ERAAP (also termed ERAP1) interacts with another ER resident aminopeptidase L-RAP (also called ERAP 2) in order to generate the final antigens (Saric, Chang et al. 2002; Tanioka, Hattori et al. 2003; Saveanu, Carroll et al. 2005).

#### **1.1.10 MHC class I recognition and CD8 T-cell response**

All T-cells originate as double negative hematopoietic stem cells of the bone marrow. As they progress in their development they migrate into the thymus and rearrange their TCR genes. Those with productive CD8 rearrangement subsequently pass through positive and negative selection leading to a population of lymphocytes that confer both MHC class I self restriction and self tolerance. This process leaves a diverse pool of mature immunocompetent CD8 T-cells that have a high reactivity to potentially foreign antigens. These naïve CD8 T-cells remain as resting cells in a G<sub>0</sub> stage of the cell cycle while continuously circulating between the blood and lymph system. Upon TCR recognition of an appropriate antigen presented by a MHC class I molecule, the T-cell mounts a primary

immune response and enters into cell cycle progression and upregulation of IL-2. While effector T-cells carry out multiple functions from cytokine secretion to cytotoxic killing another subset serves as memory T cells ensuring a secondary response if ever encountering the same antigen again.

Alterations within the pool of presented antigens often manifest themselves in a reduced number of CD8 T-cells. Depletion or mutations of many of the components of the MHC class I antigen presentation pathway discussed in the paragraphs above lead to a severely deficient CD8 T-cell repertoire. Lack of TAP, tapasin or  $\beta_2m$  leads to an almost total depletion of CD8 cells. Absence of ERAAP, LMP-7, LMP-2, LMP-10 and PA28 $\beta$  predominantly induce a reduction in the diversity while maintaining similar total numbers of CD8 T-cells.

#### **1.1.11 Antigen Presentation and Immunodominance – not just a numbers game**

Antigen presentation and CD8 T-cell recognition serve the ultimate goal to filter out those very few foreign antigens derived from viruses, bacteria or mutated proteins out of an ocean of peptides originating from host cell proteins. The complexity of this task and the sheer numbers of molecules involved are astonishing and deserve some further consideration.

Immunodominance is defined as the ability of a particular antigen to be generated, presented and recognized by CD8 T-cells to finally mount a strong, specific immune response. A step down the hierarchy are subdominant antigens which trigger weak to

barely detectable immune responses. Of all the peptides only an estimated 1% bind to MHC class I molecules with an minimum MHC class I affinity of about  $K_d > 500$  nM required for surface presentation (Yewdell 2006). Generation through translation, proteolytic liberation, efficient TAP transport, MHC class I allele expression and respective binding and finally the numbers of afferent APCs and their complementary T-cells all contribute to immunodominance. Moutaftsi et al. examined the immunodominance hierarchy for the example of the vaccinia virus WR strain (VACV-WR) (Moutaftsi, Peters et al. 2006). The following numbers represent a rough picture: From a theoretical pool of 175,000 potential poxvirus 8, 9 or 10mer antigens about 35,000 are proteolytically liberated, 30,000 are transported by TAP, 150 bound by MHC class I, 75 recognized by a TCR and finally 50 are actual antigens leading to a immune response. Many of the immunodominant viral antigens represent early gene products underlining the importance of a fast generation and presentation of MHC class I peptides (Yewdell 2006). The absolute number of a MHC class I peptide complex required to trigger a T-cell recognition is believed to be in the magnitude of about 10 molecules per cell surface. Understanding immunodominance mechanisms is particular important for vaccine generation, cancer treatment as well as gaining insight into autoimmunity.

## **1.2 Nonsense Mediated Decay**

### **1.2.1 Nonsense Mediated Decay and Antigen Presentation**

Previous sections stressed the varied origins of peptides that are utilized for antigen presentation. Within the vivid field of Nonsense Mediated Decay (NMD) a completely unanticipated source of translational products was brought to the light. This mRNA control mechanism screens for premature termination codons (PTCs) 55 or more nucleotides upstream of an exon exon junction. Since stop codons are only defined within an open reading frame by screening through a ribosome, this imposed the requirement of a pioneer round of translation of each mRNA prior to the onset of bulk translation. The very first round of translation is known to require a set of specific factors associated with the exon junction complex (EJC), which is removed by the initial passage of the first ribosome. These pioneer round of translation peptides represent the first translation products of an mRNA transcript prior to the production of functional proteins on a large scale. This makes them an ideal source of peptides for MHC class I mediated presentation without any “additional cost” to the cell demonstrating the synergy of two major surveillance mechanisms - “at the cross roads of NMD and MHC I”.

### **1.2.2 The Discovery of Nonsense Mediated Decay**

While the cellular machinery has multiple quality control mechanisms in place to insure the extraordinary high fidelity of the protein production machinery, mistakes do occur.

Especially truncated proteins, which are initially translated but lack a functional residue or domain are particularly harmful as they may act as dominant negative factors counteracting their original purpose in the cell. Examples for human diseases associated to partially translated proteins are abundant and well documented ranging from many forms of cystic fibrosis to  $\beta$ -thalassemia. An estimated 30% of all hereditary diseases are due to a frame shift or nonsense mutation resulting in a premature termination codon (Frischmeyer and Dietz 1999). In many cases a direct relationship between the severity of the phenotype and the position of the PTC could be identified (Hall and Thein 1994). But the earliest reports date back to as far as the 70s when the destabilizing effect of premature termination codons on mRNA transcripts was observed in yeast (Losson and Lacroute 1979). Soon the importance of intron positioning in relationship to the premature termination codon and splicing events was understood as well (Senapathy 1986). In the field of immunology VDJ rearrangement of the T-cell receptor and the immunoglobulins (Ig) come to mind. This particularly prominent mechanism of alternative splicing is prone to creating two thirds of unproductive rearrangements do to frame shifts and subsequent nonsense mutations. Cesar Milstein and colleagues observed the down regulation of mRNA transcripts that incorporated nonsense codons when studying the hypermutation mechanism for the antibody response (Lozano, Maertzdorf et al. 1994). Similar observations were made by the Koehler laboratory. The group reported that the extent of reduction in mutated IgM-mRNA levels directly depends on the position of the nonsense codon within the gene (Baumann, Potash et al. 1985). A quality control mechanism down regulating the expression of unproductive TCR mRNA transcripts was found thereafter (Li and Wilkinson 1998; Gudikote and Wilkinson 2002).

By today several seminal discoveries have been made to understand the mechanism and several sub-pathways to deal with premature termination codons in organisms as different as *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus* and *Homo sapiens* (Behm-Ansmant, Kashima et al. 2007). Early embryonic lethality and extinction of all hematopoietic stem cells in mouse models deficient of central factors of nonsense mediated decay leave no doubt of the essential importance of this process for life (Medghalchi, Frischmeyer et al. 2001; Weischenfeldt, Damgaard et al. 2008).

### **1.2.3 Nonsense Mediated Decay: The components**

#### **1.2.3a UPF proteins, central factors of NMD**

Within the last decade the picture of post transcriptional quality control and mRNA regulation has become much more complex. The lifetime of mRNAs is largely determined by their interaction with a plethora of host factors (Moore 2005). The central determinants of the NMD machinery are the up-frameshift proteins Upf1, Upf2 and the two alternative forms Upf3a and Upf3b (also termed Upf3 and Upf3X). Originally discovered in yeast, homologue proteins were so far described in all other eukaryotic organisms examined (Leeds, Wood et al. 1992; Cui, Hagan et al. 1995; Lee and Culbertson 1995). In mammalian cells Upf proteins are part of a large multi protein exon junction complex (EJC) consisting of at least 12 factors including Y14, eIF4AIII,

MAGOH and MLN51 (Le Hir, Izaurralde et al. 2000; Le Hir, Moore et al. 2000). Upf1, the by far best described component is Upf1, a 130 kDa group 1 ATP-dependent helicase with binding domains for Upf2, Upf3 and mRNA.

The localization of Upf1 as a shuttle protein both in the cytosol as well as in the nucleus reflects its separable functions both as final trigger for the mRNA decay cascade as well as in facilitating the early mRNA biogenesis (Wang, Czaplinski et al. 2001; Mendell, ap Rhys et al. 2002; Isken, Kim et al. 2008). The imminent role of Upf1 becomes evident in the embryonic lethality of Upf1 knock out mice and the failure to generate vital Upf1 negative embryonic fibroblast cell lines (Medghalchi, Frischmeyer et al. 2001; Weischenfeldt, Damgaard et al. 2008). The function of Upf1 is vigilantly regulated in a cycle of phosphorylation and dephosphorylation requiring at least four different factors. While the suppressor with morphogenetic effect on genitalia-1 (SMG-1) phosphorylates Upf1, SMG-5, 6 and 7 are dephosphorylating agents (Page, Carr et al. 1999; Grimson, O'Connor et al. 2004). A human Upf1 dominant negative protein with an arginine to cysteine exchange at residue 844 has been shown to reduce the targeted decay of mRNAs containing a PTC about two to threefold (Sun, Perlick et al. 1998). Fusion of Upf1 to the mRNA binding MS2 virus protein, resembling more of a gain-of-function approach, have led to a destabilization effect of mRNA transcripts when including a MS2 binding domain (Hosoda, Kim et al. 2005).

The 148 kDa adapter protein Upf2 includes binding domains for Upf1 as well as Upf3 which are both required for NMD in mammals. With a N-terminal nuclear localization domain it is present both in the nucleus as well as in the perinuclear cytosol. Its essential

role for NMD was recently demonstrated in a conditional Upf2 knock out mouse with severe defects of the hematopoietic stem cells system (Weischenfeldt, Damgaard et al. 2008).

So far there is no distinct function of the NMD pathway allocated to the Upf3 protein, but it might be involved in the second signal response. While Upf3 is predominantly nuclear it is also found as a shuttle protein in the cytoplasm. Beside its Upf2 interaction domain it binds directly to the EJC. Interestingly the genome of mammals contains two genes named Upf3a on chromosome 13 and Upf3b on the X-chromosome (or Upf3 and Upf3X) respectively (Lykke-Andersen, Shu et al. 2000; Serin, Gersappe et al. 2001). As mentioned above the Upf proteins are believed to be essential for NMD and vitality. Despite this fact there are reports for several family lines with defective truncated Upf3b genes that lead to a severe phenotype of mental retardation. It might be due to at least partial redundancy between Upf3a and Upf3b that NMD and vitality in these individuals can be maintained (Tarpey, Raymond et al. 2007).

### **1.2.3b Cap and PolyA binding proteins in NMD**

Another set of key components was found when examining the earliest events of the NMD pathway namely the pioneer round of translation. While the detailed mechanism of this very first event both in protein translation and NMD will be discussed below, the most important factors shall be introduced here. Freshly synthesized pre-mRNA binds

with its 5' prime to a cap binding complex (CBC) consisting the cap binding protein 80 (CBP80) and 20 (CBP20) with a rough molecular weight of 80 and 20 kDa respectively. Cristallography as well as extensive mutagenesis including alanine scanning contributed to the comprehensive picture of the CBP80/20 heterodimer.

CBP80, similarly to eIF4G, comprises of three major MIF4G domains, typical for RNA interacting proteins. CBP20 interacts with two of these domains while also binding RNA with a ribonucleoprotein domain (Mazza, Ohno et al. 2001; Marintchev and Wagner 2005). Four residues have been identified in establishing the interface of the heterodimer. Especially the Y43A mutation in CBP20 proved valuable as a dominant negative protein disrupting the binding to RNA and therefore disturbing the function as CBC (Cusack 2006). CBP80 proved to be essential for NMD by binding to Upf1 and promoting the interaction between Upf1 and Upf2 (Ishigaki, Li et al. 2001; Hosoda, Kim et al. 2005). The 3' prime polyadenylated tail of the pre-mRNA interacts with the polyA binding protein 1 (PABP1, also called PABPC1) and 2 (PABP2, also called PABPN1). PABP2 has been identified as the very first binding factor within the first translation round of newly transcribed mRNA and shows predominantly nuclear localization while PABP1 also facilitates multiple rounds of translation through the polyribosome (Behm-Ansmant, Gatfield et al. 2007). Additionally PABP2 was found to be associated with CBP80 but not with eIF4E associated mRNA (Wahle 1991; Ishigaki, Li et al. 2001).

#### **1.2.4 Destined for destruction: recognition of target mRNAs for NMD**

The mRNA regulation cycle of NMD is mostly described as a “downstream marker model” emphasizing the role of the stop codon positioning within the transcript in determining its fate (Shyu, Wilkinson et al. 2008). In mammalian cells mRNAs that include a stop codon >50-55 nucleotides upstream of an exon-exon junction generated through the splicing of an intron are destined for NMD. Accordingly premature termination codons appearing closer than 50-55 nucleotides upstream to an exon-exon junction site will fall through the screening of NMD and will not trigger a decaying process (Zhang, Sun et al. 1998). This model imposes a problem when considering the yeast genome or for example histone genes which rarely include introns at all but are both sensitive to Upf1 down regulation (Maquat and Li 2001; Kaygun and Marzluff 2005). Also in the case of TCR gene transcripts the NMD rules for intron positioning in relationship to a PTC are markedly relaxed (Carter, Li et al. 1996). Here an alternative second signal of undefined origin has yet to be detected in order to label a particular mRNA for destruction. This incident is usually described as the “aberrant termination model” (Shyu, Wilkinson et al. 2008). The development of the NMD pathway from a mere PTC recognition system to a general mRNA surveillance and regulation mechanism of the cell has been pointedly described as a versatile transition from “a vacuum cleaner to a swiss army knife” (Neu-Yilik, Gehring et al. 2004). By now NMD related pathways include Staufen1 Mediated Decay (SMD), alternative splicing, telomere maintenance, mTor signaling and regulation of genome stability (Le Hir and Seraphin 2008)(Chan, Huang et al. 2007; Kim, Furic et al. 2007; Isken and Maquat 2008).

Notably Upf1 seem to play a more promiscuous role being involved in almost all of the described pathways while Upf2, Upf3 and CBP80/20 are mainly limited to their role in PTC detection mechanisms.

### **1.2.5 The NMD mechanism: Pioneer round vs. bulk translation**

Despite the diversity of nonsense surveillance pathways they all share the requirement for initial translation in order to recognize a start codon and a subsequent in frame premature stop codon or aberrant 3'UTR. Newly synthesized and spliced mRNA is distinctly different from previously translated mRNA by still having the EJC deposited 20-24 nucleotides upstream of exon-exon junctions (Le Hir, Izaurralde et al. 2000; Le Hir, Moore et al. 2000). Additionally nuclear mRNA assembles several other factors including the heterodimer CBP80/20 at the 5'cap and PABP1 and PABP2 at the 3' terminus as well as eIF4G as core factor of the translation complex (Ishigaki, Li et al. 2001; Lejeune, Ranganathan et al. 2004). During this process the occurrence of a premature termination codon halts the EJC for a short period of time triggering the recruitment of Upf3 and subsequently Upf2 and Upf1 concealing the fate of the PTC containing mRNA for destruction. In the case of a functional mRNA transcript the first ribosome passing the complete gene displaces the EJC and triggers the exchange of CBP80/20 to elongation factor 4E (eIF4E) as well as the release of PABP2 (Chiu, Lejeune et al. 2004). This synthesis of the very first polypeptide from a given mRNA transcript was named "pioneer round of translation" by the Maquat lab. No matter

whether the mRNA is degraded later on or serves as template for multiple rounds of translation, exactly one polypeptide for every newly spliced mRNA in the cell is synthesized within the pioneer round.

The production of bulk quantities of protein facilitating polyribosomes is then carried out through a translation complex including eIF4E, eIF4G and PABP1 among many other factors. While eIF4G remains to take the center stage of the translation initiation complex, the heterodimer CBP80/20 is exchanged against eIF4E which is solely used for bulk translation. Similar to the role of CBP80/20 the factor eIF4E interacts with the m7GpppN 5' terminus of mRNA subsequently unwinding the 5'UTR to provide access for the 40S ribosomal subunit. In mammalian cells three isoforms of 4E- binding proteins (4E-BP1, 2 and 3) regulate eIF4E activity by binding and therefore preventing access to eIF4G. The factor 4E-BP1 itself contains six phosphorylation sites. Only dephosphorylated forms of 4E-BP1 can bind to eIF4E in order to down regulate bulk translation (Sonenberg 2008). It was demonstrated that phosphorylation sites threonine 37 and 46 are essential for subsequent phosphorylation of all six sites. The double mutant T37A and T46A effectively prevents phosphorylation of 4E-BP1 and constitutively binds to eIF4E (Burnett, Barrow et al. 1998) (Gingras, Gygi et al. 1999).

The 4E-BP1 T37A T46A double mutant therefore became an important tool to inhibit eIF4E dependent bulk translation while not interfering with CBP80/20 dependent pioneer translation.

Bulk translation takes place at the rough ER or in association with free ribosomes in the cytosol. But there is a yet unsettled debate about the location of the pioneer round of

translation (Wilkinson and Shyu 2002; Iborra, Escargueil et al. 2004). At least in yeast the pioneer round of translation does not appear to take place in the nucleus (Kuperwasser, Brogna et al. 2004). While there are several findings supporting the concept of a solely nuclear translation, many results are consistent with a pioneer round of translation at the nuclear pore during or right after mRNA export to the cytosol (Iborra, Jackson et al. 2004).

As mentioned above pioneer translation and therefore NMD require mRNA to bind the CBC consisting of CBP80 and CBP20. Therefore several studies addressed the question whether cap-independent mRNA translation initiated through an internal ribosome entry site (IRES) could be target for NMD. Many viruses use IRES sequences upstream of the start codon to attract the initial binding of a ribosome making cap-binding and polyA-binding factors obsolete (Hellen and Sarnow 2001; Vagner, Galy et al. 2001). But experiments have shown that IRES vary greatly in their requirement for translation factors. One of the first studies addressing the minimal requirements for pioneer translation and NMD used a IRES derived from encephalomyocarditis virus (EMCV) (Holbrook, Neu-Yilik et al. 2006). Their original finding that EMCV IRES initiated mRNAs can undergo NMD disputes the importance of the CBC for pioneer translation. But a later study establishes that EMCV IRES mRNA is indeed associated to CBP80 and CBP20 supporting the role of the CBC in facilitating pioneer translation (Woeller, Gaspari et al. 2008). Finally NMD fails to target nonsense-containing transcripts that initiate translation from the cricket paralysis virus (CrPV) IRES (Isken, Kim et al. 2008). IRES from dicistroviruses like CrPV do not require any translation factors or initiator

tRNA in order to assemble ribosomes and initiate productive translation (Schuler, Connell et al. 2006).

Taken together the CBP80/20 heterodimer proves to be the essential component of pioneer translation. Its counterpart during bulk translation is the 4E-BP1 regulated factor eIF4E.

### **1.2.6 Pioneer peptides as source for MHC class I mediated antigen presentation**

For a long time the pioneer round of translation has been merely seen as a prerequisite for PTC detection than as a distinct mechanism of translation. Several recent studies shifted the focus from the mRNA decay mechanism to understanding the occurrence and special features of the pioneer round of translation itself. Here NMD serves rather as an assay for pioneer translation than as the object of investigation. It has been determined that the pioneer round of translation and the biosynthesis of at least one polypeptide from each mRNA transcript can occur even under heat shock conditions that usually not permit translation (Marin-Vinader, van Genesen et al. 2006). Similarly pioneer translation continues under prolonged hypoxia as well as serum starvation (Oh, Kim et al. 2007; Oh, Kim et al. 2007). Another study positions the pioneer translation at a check point for the control of subsequent steady state translation under the control of the mTOR/S6K1 pathway (Ma, Yoon et al. 2008). These findings not only confirm that NMD appears even under conditions that abolish bulk translation but also opens additional possibilities for the cellular use of pioneer peptides. It has been shown by multiple studies as well as been

confirmed herein that MHC class I mediated antigen presentation directly depends on new translation. Since there is a continuous need for antigen presentation in the cell to adequately protect an organism from infection, pioneer peptides present a potential source of peptides for MHC class I presentation.

### **1.3 Aim of the thesis research**

MHC class I mediated antigen presentation and NMD are two central surveillance pathways in the cell. The goal is to determine if NMD is involved in producing or altering antigenic peptides presented by MHC class molecules. This could be the case through two mechanisms.

#### 1.) “Antigenic peptides as NMD targets”

In this scenario antigen encoding mRNA transcripts with a premature termination codon would be degraded through the NMD mechanism leading to a reduced level of surface presentation of these peptides via MHC class I molecules.

#### 2.) “Peptides of the Pioneer Round of Translation as Antigens”

Products of the pioneer round of translation itself are cleaved by the proteasome, transported by TAP, processed in the ER and presented by MHC class I on the cell surface.

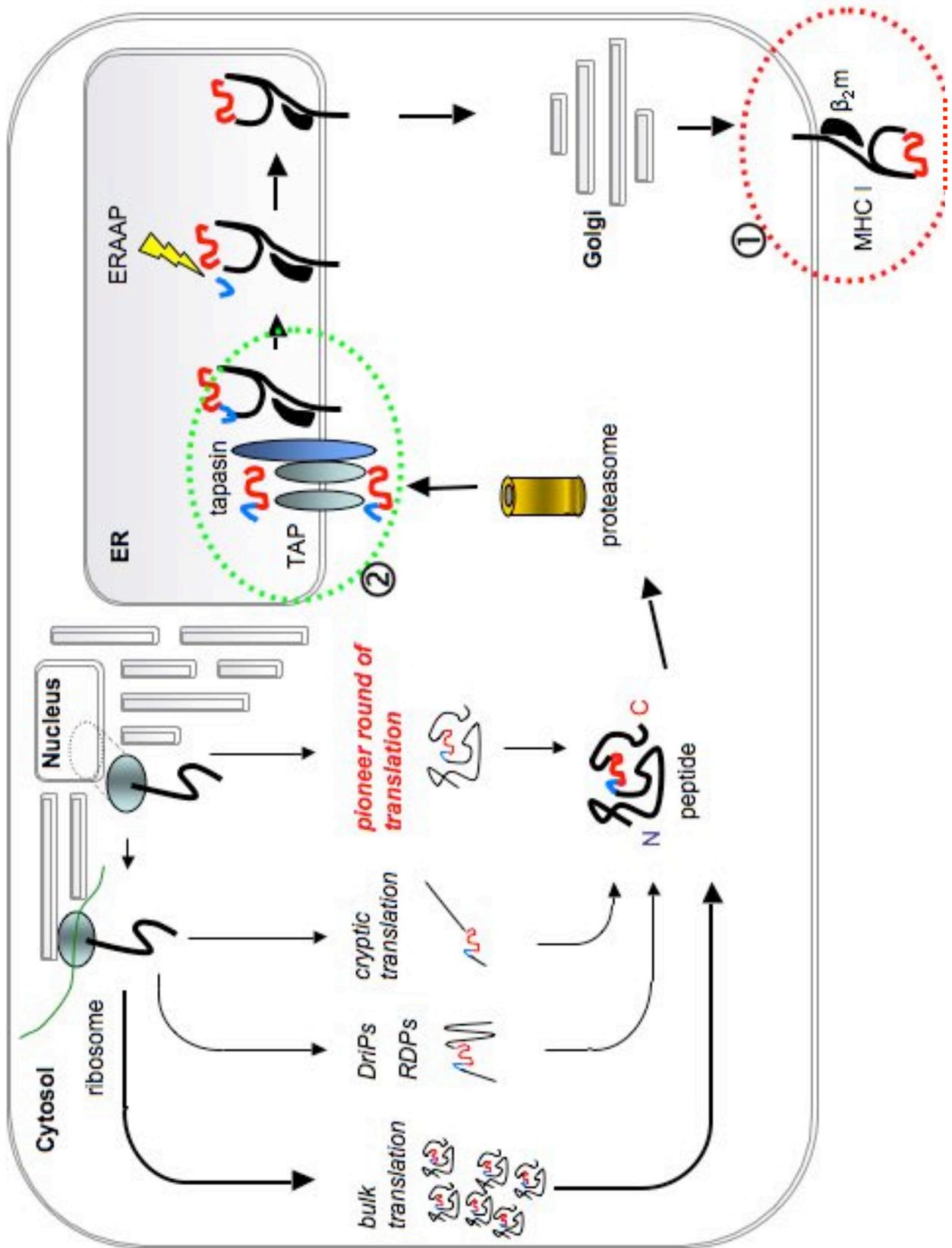
Many native proteins have a live cycle of many hours to several days before they start being degraded and become accessible for antigen presentation. For the effectiveness of the immune system it is important, that virtually all cellular proteins are presented on the

cell surface in a rapid and continuous process. Ideally, new peptides are sampled early after translation before the generation of bulk quantities of proteins. Especially in the case of viral infections as a major challenge for the antigen presentation pathway, speed of presentation is a key to preventing the spreading of infections. These theoretical considerations found it's match in recent experimental findings. It was shown that several unconventional sources of translation products are actual sources of antigenic peptides. These include cryptic translation products starting with non-AUG codons, peptides from usually untranslated regions and endocytosed or cross-presentated peptides as well as rejoined peptide chains. Within the last decade more and more evidence accumulated demonstrating the importance of freshly translated peptides as major source for antigenic peptides. Here the defective ribosomal products – hypothesis takes a center stage suggesting a subset of not completely folded proteins as immediate early antigenic peptides. Within the field of Nonsense Mediated Decay an unexpected source of translational products were discovered. This mRNA control mechanism screens for premature termination codons 55 or more nucleotides upstream of an exon exon junction. Since stop codons are only defined within an open reading frame by screening through a ribosome, this imposed the requirement of a pioneer round of translation of each mRNA prior to the onset of bulk translation. The very first round of translation is known to require a set of specific factors associated with the exon junction complex which are removed by the initial passage of the first ribosome. These pioneer round of translation peptides represent the first translation products of an mRNA transcript prior to the production of functional proteins on a large scale. This makes them an ideal source of peptides for MHC class I mediated presentation without any “additional cost” to the cell

demonstrating the synergy of two major surveillance mechanisms - “at the cross road of NMD and MHC I”.

This study aims to provide independent lines of evidence for the establishment of “Peptides of the Pioneer Round of Translation as Antigens” as stated in option 2). Direct involvement of pioneer peptides in antigen presentation would add a previously unknown source of antigenic peptides to the repertoire of peptides presented by MHC I molecules. It would not only show a combined starting step of two major surveillance pathways in the cell but also provide a novel mechanism for the generation of immediate early peptides. Furthermore an answer to these questions has the potential to integrate previous findings about DRiPs and RDPs with new results about pioneer peptides into a combined picture of the origin of early translation products for antigen presentation.

**Fig.1 Origins of peptide for antigen presentation.** Newly spliced mRNA assembles with the ribosomal subunits and factors of the pioneer round of translation before the onset of conventional translation for the biosynthesis of bulk quantities of peptide chains. New proteins are folded to their correct confirmation, serve a function as structural proteins or enzymes and eventually reach their live time for proteasomal degradation. Alternatively most defective ribosomal products (DRiPs) or cryptic translation products are funneled into proteasomal destruction after short time periods. Peptides from these different origins are transported by TAP to the ER, loaded to MHC class I molecules, edited by ERAAP and finally presented to T-cells at the cell surface. It has yet to be determined to what extent these different sources of translation contribute to the pool of antigenic peptides. Transport of peptides by TAP (green dotted line) and surface presentation (rod dotted line) are examined in this study.



## **2**    **Results**

### **2.1**    **Intron positioning and antigen presentation**

NMD has been described as a central mechanism insuring the fidelity of mRNA transcripts as well as regulating the expression of multiple genes. Two potential mechanisms for the interaction of NMD and MHC class I mediated antigen presentation have been proposed (see section 1.3: aim of this study):

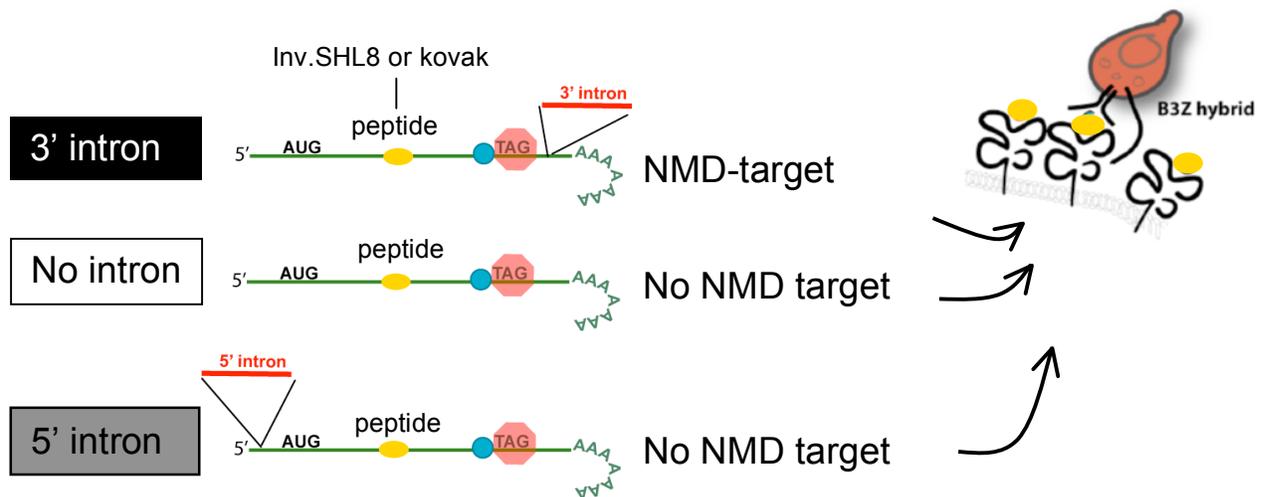
- 1.) “Antigenic peptides as NMD targets”
- 2.) “Peptides of the Pioneer Round of Translation as antigens”

The first hypothesis mainly suggests mRNA transcripts encoding for antigenic peptides as one more group of targets under the regulation of the already omnipresent NMD pathway. In sharp contrast the second hypothesis proposes a completely novel source of translation for the generation of antigenic peptides potentially explaining the importance of early translation for antigen presentation.

The emphasis of this section is on testing the initial hypothesis 1. In general two events have to prelude the activation of the NMD pathway and the decay of a specific mRNA for any given transcript. First the premature stop codon has to be detected within an open reading frame through the process of the pioneer round of translation. A second signal

has to be recognized distinguishing the premature termination codon from a conventionally occurring normal stop codon. While different second signal mechanisms evolved in organisms as distantly related as *A. thaliana* and *D. melanogaster* the case for mammalian NMD signaling is made by the positioning of the intron in context of the premature termination codon. E Nagy established the 50-55 base pair rule: “A PTC must be 50-55 base pairs upstream of an intron splicing site in order to elicit NMD.” (Nagy Maquat 1998) This second signal rule remained to be crucial for the vast majority of mammalian mRNA transcripts until today (Scofield, Hong et al. 2007). To address the validity of the first hypothesis several sets of expression vectors with an open reading frame with an antigen encoding sequence, a subsequent PTC and introns in different positions were generated (Fig.2). The first set of expression constructs are based on the invariant chain (Ii) followed by the antigen SIINFEHL (SHL8) in the vector pcDNA1. This construct was shown to liberate the antigenic peptide very efficiently leading to a particularly strong activation of B3Z T-cells upon presentation. An intron was positioned more than 55 base pairs down stream of the PTC (3'intron, black), completely removed (No intron, white) or inserted together with the splicing donor and splicing acceptor site upstream of start codon (5'intron, grey). An additional set of three expression vectors was created based on the ovalbumine residue 253-386 derived sequence kovak, including the K<sup>b</sup> restricted SIINFEHL (OVA<sub>258-265</sub>) peptide. (Note: The original SIINFEKL sequence of ovalbumine was changed to SIINFEHL in order to make the octapeptide refractory to trypsin digestion, both are recognized equally by B3Z T-cells (Kunisawa and Shastri 2003) (Paz, Brouwenstijn et al. 1999)).

In both cases the 3' intron constructs fulfill the requirements of a PTC 50-55 base pairs upstream of an intron and are therefore predicted targets of NMD. The constructs with no intron or an intron in 5' position are not expected to elicit NMD.



**Fig.2 Antigen encoding DNA constructs with different intron positioning.** Two sets of three pcDNA1 vector based DNA constructs were generated. The first set is based on a fusion protein encoding the invariant chain (Ii) and the ovalbumine derived antigen sequence SIINFEHL followed by a premature termination codon. The three transfection vectors contain an intron sequence in 3' position (black box), no intron (white box) or an intron in 5' position in front of the start codon (grey box). Only the 3' intron complies with the 55bp-rule making it a target for NMD. A second set of three constructs was designed in the same way based on the ovalbumine derived kovak sequence including the SIINFEHL peptide.

### 2.1.1 NMD dependent mRNA regulation does not correlate with T-cell activation

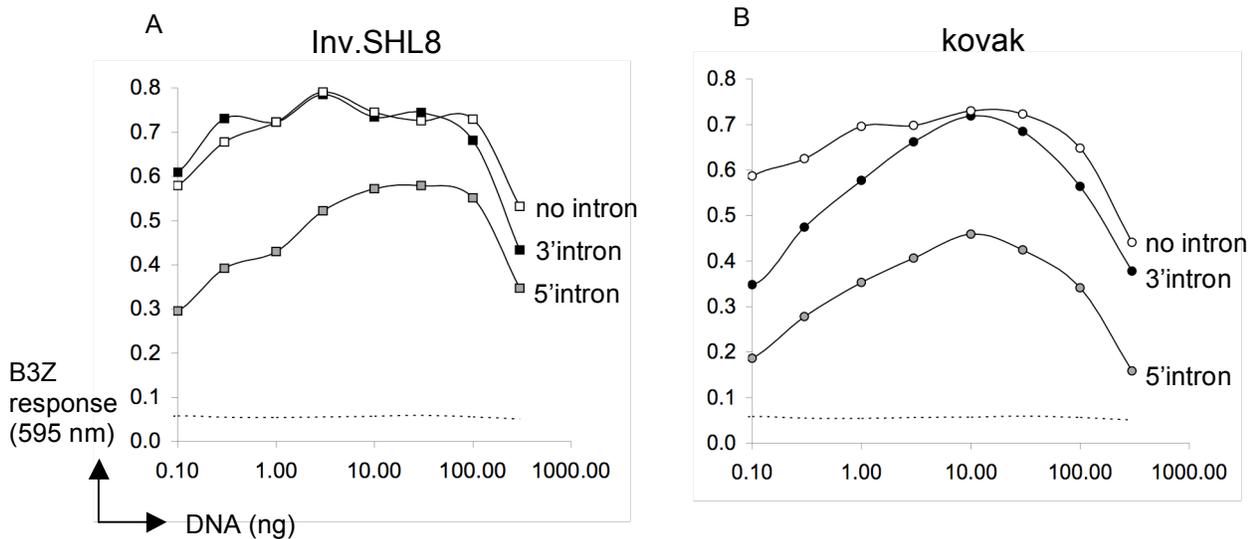
The above described set of three expression vectors on the basis of the kovak open reading frame with a 3' intron, No intron or a 5' intron were transiently transfected into HeLa K<sup>b</sup> cells. The expression of kovak mRNA was determined 48 hours after

transfection and relative amounts were calculated using the  $2^{-\Delta\Delta C}$  method normalized to actin mRNA (Fig.3A) (Livak and Schmittgen 2001). In compliance with the predictions of NMD the 3'intron demonstrates the lowest mRNA expression level. Both the construct with no intron as well as a 5'intron, which lack the second signal to be recognized for the decay mechanism, show a 8.2 or 7.6 fold higher mRNA expression level. Next it was tested if these mRNA levels are also reflected in similar intensities of T-cell activation (Fig.3B). Here the 3'intron as well as the no intron containing kovak expression vectors accounted for an almost 2 fold higher B3Z T-cell response than the 5'intron sequence. Taken together the mRNA expression level did not seem to correlate with the T-cell activation rejecting a mere dependence of antigen presentation on NMD associated mRNA regulation.



**Fig.3 Quantification of mRNA expression and T-cell activation.** HeLa K<sup>b</sup> cells were transiently transfected with a pcDNA1 vector based kovak encoding DNA construct including either a 3'intron (black), no intron (white) or a 5' intron (grey). RNA was purified and kovak mRNA quantified by real time PCR (A). Relative kovak mRNA expression for each construct was determined by  $2^{-\Delta\Delta CT}$  method normalized to actin mRNA expression. Antigenic T-cell activation of each of the three constructs was measured in a B3Z T-cell assay (B).

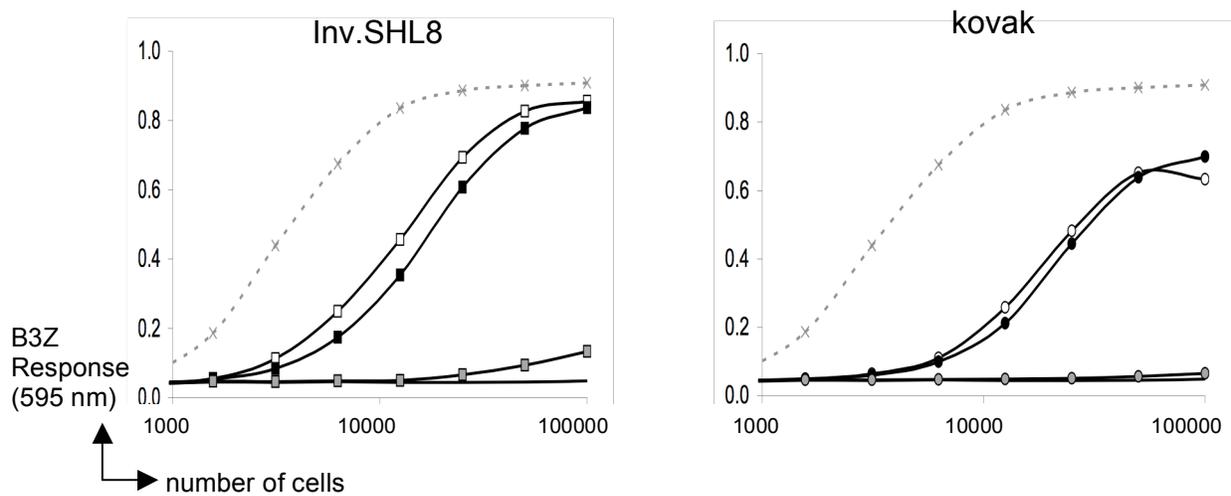
In order to quantify the T-cell activation based on the intron position of the Inv./SHL8 or kovak expression vectors two additional approaches were pursued. First the vector DNA was transiently transfected into HEK293T cells in a 1:3 dilution series (Fig.4A and B). Consistent with direct T-cell presentation assays as shown in Fig.3B the relative relationship between constructs with no intron, a 3' intron and a 5' intron remained the same. The independent sets of expression vectors based on Inv.SHL8 and kovak demonstrated a reduced B3Z T-cell activation for 5' intron antigenic sequences. The peak T-cell activation for kovak based constructs appeared at a DNA transfection concentration of 10 ng of DNA (Fig.4B).



**Fig.4 DNA titration reveals differences in T-cell activation.** Semiadhesive human HEK 293T cells were transfected with MHC K<sup>b</sup> and B7-2 in preparation for a B3Z T-cell assay. Additionally they were cotransfected with one of the three Inv.SHL8 (A) or three kovak (B) based antigen expression vectors with different intron positions in a 1:3 DNA dilution series. Transfection was carried out following the DEAE dextran/Chloroquine protocol. For both Inv.SHL8 (A) and kovak (B) based constructs 3'intron (black) and no intron (white) vectors show a higher T-cell activation than 5'intron (grey) containing vectors. Untransfected cells serve as negative control (dashed).

Finally peptide extraction enables the by far best quantitative assessment of antigenic peptides generated in the cells (Fig.5). Transiently transfected HeLa K<sup>b</sup> cells were boiled in 10% acetic acid for peptide extraction. After vacuum drying the purified peptides were added to murine K89 cells in a 1:3 titration for antigen presentation. The B3Z T-cell activation assay revealed the large disparity of antigen presentation between 3'intron and no intron containing expression constructs with markedly high T-cell response on the one side, and the 5'intron constructs with T-cell activation just above the detection level on the other side. Generally Inv.SHL8 constructs show a higher B3Z response as kovak constructs. This is consistent with prior observations due to a more efficient liberation of the SHL8 antigenic peptide in the Inv.SHL8 sequence.

In conclusion the antigen expression constructs show a very distinct T-cell activation ability dependent on their intron position. The mRNA level by itself does not predict the intensity of T-cell activation. This finding cannot be deduced from the predictions made by the current understanding of NMD dependent mRNA regulation.

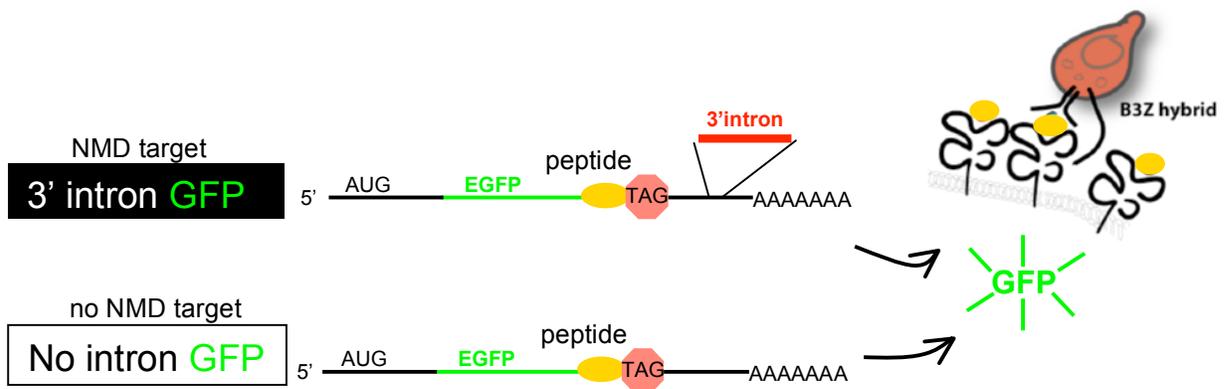


**Fig.5 Quantification of antigen presentation after peptide extraction.** For quantitative determination of T-cell activation both sets of Inv.SHL8 or kovak encoding vector constructs were transfected into HeLa K<sup>b</sup> cells. Peptides were extracted from 500.000 cells each and reincubated with 100.000 K89 cells for optimal antigen presentation to B3Z cells. Inv.SHL8 3'intron / No intron / 5'intron (black/white/grey square) in left graph and kovak 3'intron, No intron, 5' intron (black/white/grey circle) in right graph. Synthetic peptide SHL8 in a 1:3 titration starting at 1000 pM as positive control (dashed with cross), untransfected cells as negative control (black line).

### 2.1.2 GFP based NMD-reporter constructs for antigen presentation

A GFP based NMD-reporter system has been developed to monitor the activity of the NMD pathway in different cell lines and experimental approaches (Paillusson, Hirschi et al. 2005). Again the distinguishing feature between the two expression vectors is the presence of a 3'intron in accordance with the 50-55 base pair rule making only the first one a target for NMD. This approach presents several advantages in comparison to

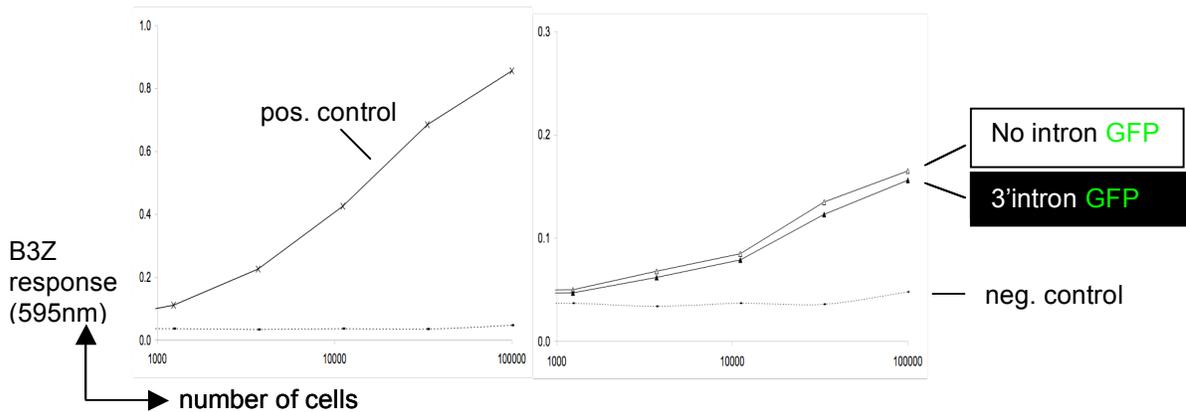
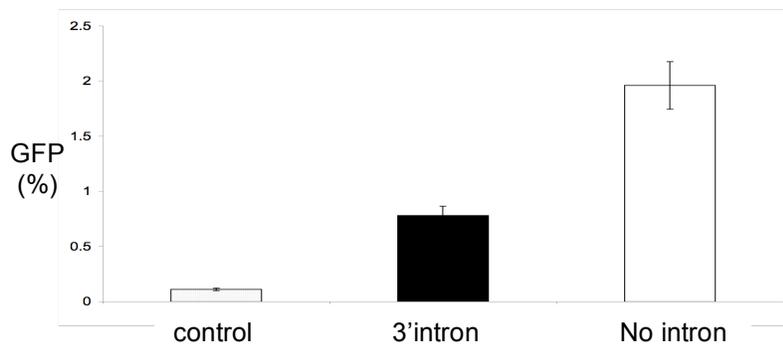
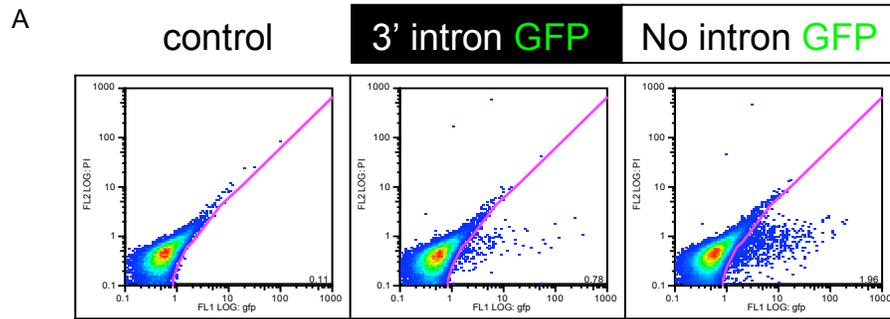
previously described methods measuring mRNA levels based on RT-PCR or northern blotting. Monitoring GFP levels with flow cytometry enables the fast and high throughput analysis of NMD activity. Additionally this assay is based on the actual protein level allowing more direct conclusions about the amount of peptides for antigen presentation. The previously described NMD-reporter constructs were modified for the purpose of examining antigen presentation by inserting the lysine flanked SIINFEHL encoding sequence at the C-terminus of the EGFP protein directly followed by a stop codon for efficient peptide liberation (Fig.6).



**Fig.6 GFP based NMD-reporter constructs modified for antigen presentation.** Both pCl vector constructs consist of a TCR $\beta$  leader - EGFP fusion sequence containing a PTC. The first contains a 3' intron making it a target for NMD (black) while the intron is deleted in the second construct rendering it unsusceptible to NMD (white). Comparison of GFP expression was used previously as measure for NMD activity. Here the sequence encoding the antigenic peptide SIINFEHL was inserted at the C-terminus of the EGFP in order to examine antigen presentation activity.

First the competence of HeLa K<sup>b</sup> cells to undergo NMD was demonstrated as described previously in the literature (Paillusson, Hirschi et al. 2005). 100 ng of DNA of the GFP based NMD reporter constructs was transiently transfected into  $2.5 \times 10^5$  cells and the GFP expression was determined by flow cytometry after 48 hours (Fig.7A). The total percentage of GFP expressing cells in the 3'intron construct accumulated to 0.78% while the no intron transfected cells showed 1.96% GFP positive cells. This demonstrates good correlation with the prediction of the 3'intron being a target of NMD. Then antigen presentation was determined as activation of B3Z T-cells (Fig.7B). Interestingly both NMD-reporter constructs demonstrated an equal level of B3Z activation independent of their susceptibility to NMD degradation. The T-cell response of both GFP based constructs was comparatively low as the control transfection with a kovak construct leads to a far higher B3Z activation. Low transfection efficiency of the backbone vectors together with a potentially less efficient peptide liberation might account for this generally low presentation activity. The equivocal T-cell activation of both vectors independent of their NMD dependent degradation underlines the findings of previous experiments with the Inv.SHL8 or kovak based expression vectors. As there are multiple steps influencing the efficiency of peptide presentation and immunodominance it can be difficult to pinpoint the factor responsible for the low antigenicity of the 5'intron constructs as well as the high antigenicity of the 3'intron construct despite the strong down regulation of it's mRNA. As described before it is often not possible to reliably predict the immunogenicity of a given peptide sequence. But as for this case, these experiments taken together serve the purpose of rejecting the first hypothesis of this study. The NMD pathway does not just alter the antigenicity of peptides by merely

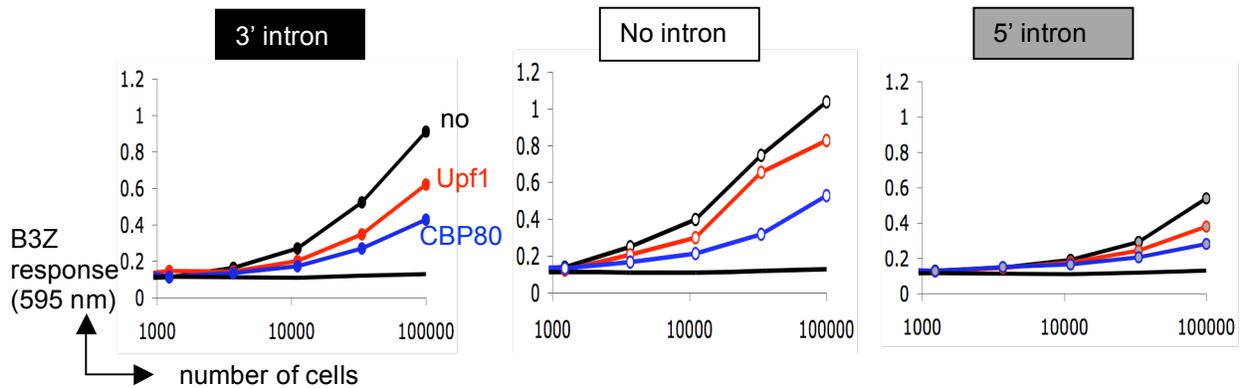
changing their mRNA or protein levels according to the targeting rules for mRNA decay. The answer may lie deeper within the mechanism of the NMD pathway as demonstrated by the following experiments.



**Fig.7 GFP expression and T-cell activation of NMD reporter constructs.** GFP expression of both NMD reporter constructs was determined in transiently transfected HeLa K<sup>b</sup> cells (A). The 3'intron construct, a target of NMD, showed GFP expression in 0.78 % of the cells (black). The NMD resistant construct lacking the intron demonstrated an increased level of 1.96 % GFP positive cells (white). As a negative control cells were transfected with a kovak encoding vector without GFP. Antigen presentation was accessed by B3Z T-cell assay (B). Both NMD reporter constructs showed very similar T-cell activation independent of intron presence (right panel, black and white triangles). A kovak encoding vector served as positive control (left panel, cross) for T-cell activation and untransfected cells as negative control (dashed).

### **2.1.3 Inhibition of pioneer translation reduces T-cell activation independent of intron position.**

Since the first hypothesis did not present a conclusive explanation of the varied antigenicity of different constructs, the following experiments focus on the pioneer round of translation by itself as an integral part of the NMD pathway and a potential source for the generation of antigenic peptides. The kovak based vectors with either the intron shuffled to the 3' or 5' position or the intron being deleted were transfected once more into HeLa K<sup>b</sup> cells (Fig.8). Here they were cotransfected with synthetic small interfering RNA molecules targeting either CBP80 or Upf1. Both factors have been shown to be essential for the NMD pathway. Depletion of these factors results in the inhibition of the NMD mechanism and therefore an upregulation of mRNAs previously suppressed by NMD. Surprisingly, depletion of these factors led to a reduction of antigen presentation in all three constructs. This reduction was more pronounced for CBP80 RNAi than for Upf1 RNAi but independent of the intron position.



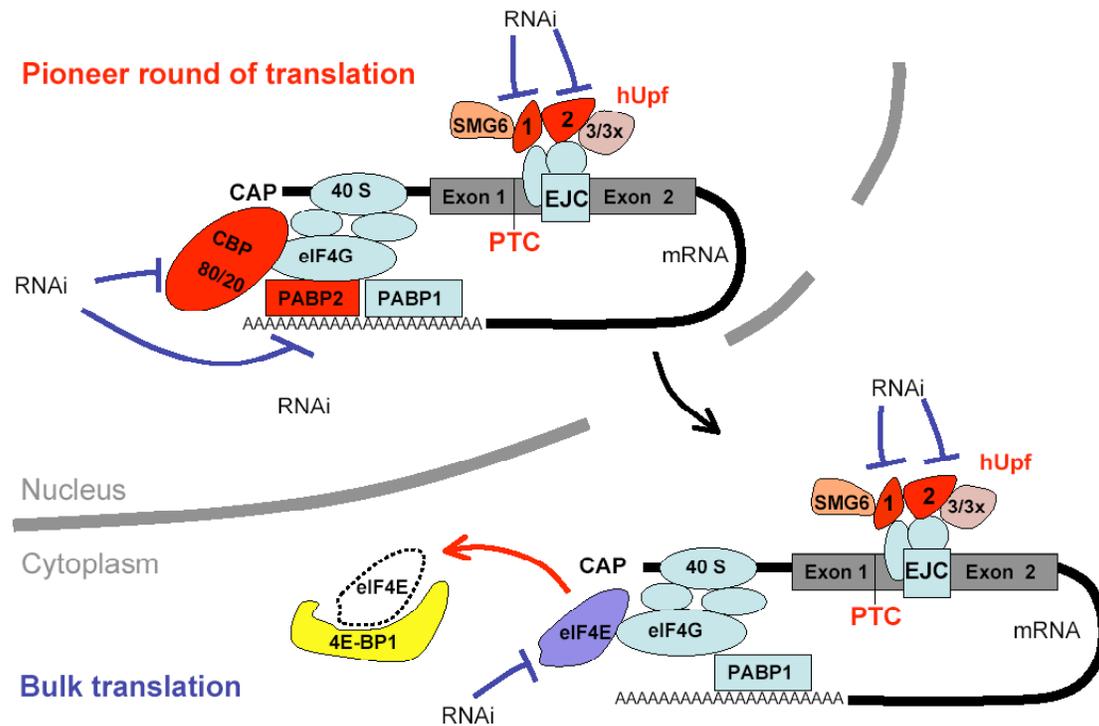
**Fig.8 Inhibition of pioneer translation reduces T-cell activation independent of intron position.** HeLa K<sup>b</sup> cells were cotransfected with one of the kovak based pcDNA1 vector constructs containing either a 3' intron (black), no intron (white) or a 5' intron (grey) and RNAi targeting either Upf1 (red line), CBP80 (blue line) or no RNAi (black line). Independent of intron position and susceptibility to NMD, Upf1 depleted cells showed an intermediate and CBP80 depleted cells a strong reduction in B3Z T-cell activation.

## **2.2 Effects of pioneer vs. cap dependent bulk translation on MHC class I expression**

Only with the discovery of nonsense mediated decay the requirement for a pioneer round of translation and therefore the possibility of a novel source of antigenic peptides became evident. This chapter aims to establish the pioneer round of translation as a separate mechanism of polypeptide generation as well as to demonstrate its effects on MHC class I mediated antigen presentation.

First an assay system to differentially interfere with either the pioneer round of translation or with the eIF4E and cap dependent bulk translation was developed (Fig.9). RNA interference sequences and dominant negative DNA constructs were generated targeting key elements of either the pioneer round or cap dependent bulk translation including CBP80, CBP20, PABP2, Upf1, Upf2, 4E-BP1 and eIF4E. The direct effect of target protein depletion as well as the inhibitory function on the subsets of translation were determined by ways of immunoblotting and radio labeling. Two different approaches were taken to precisely monitor antigen presentation in tissue culture cell lines. In order to access the overall surface expression of MHC class I molecules, flow cytometry in conjunction with antibodies binding to human and murine MHC class I complexes was used. To track the surface presentation of specific antigenic peptides, different T-cell clones were used in activation assays. A citric acid based protocol for the removal of initial MHC class I peptide complexes and subsequent surface reassembly was used to determine small differences in antigen presentation. To further characterize

the speed and accuracy of the peptide generation their interdependence with cell cycle progression, cytokine levels, chemical inhibitors and starvation conditions were addressed.



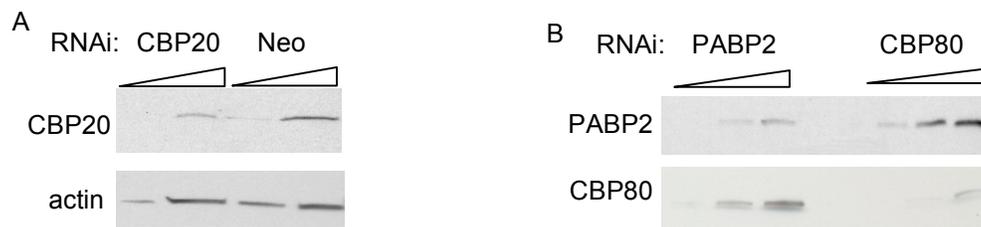
**Fig.9 Differential inhibition of pioneer round of translation and bulk translation.** Newly spliced mRNA assembles with the EJC, pioneer translation and NMD factors in the nucleus. The pioneer round of translation exclusively uses CBP80 and PABP2 (red) for initiation of translation. After the displacement of the EJC, eIF4E (dark blue) facilitates bulk translation. 4E-BP1 (yellow) competes with the translation initiation complex for binding to eIF4E. It therefore regulates protein biosynthesis depending on its phosphorylation stage. Factors like eIF4G that are jointly used for pioneer translation and bulk translation are depicted in light blue.

### **2.2.1 RNAi mediated depletion of the pioneer round of translation factors CBP80, CBP20, Upf1 and PABP2.**

As described in section 1.2.5 the pioneer round of translation includes several features distinguishing it from the bulk translation used for the generation of large quantities of cellular proteins. In order to examine the contribution of different sources of translation for antigen presentation, a method to exclusively target the pioneer round of translation was established. Translation of newly spliced mRNA transcripts is facilitated by the CBP80/20 heterodimer which is exchanged to eIF4E right after the first ribosome finishes the initial round of translation (Ishigaki, Li et al. 2001; Lejeune, Ishigaki et al. 2002; Chiu, Lejeune et al. 2004; Oh, Kim et al. 2007). Both proteins CBP80 and CBP20 are essential for this function. Additionally CBP80 promotes the interaction of Upf1 and Upf2. Upf1 was shown to be a factor of central importance being involved in early events of pioneer translation as well as being the final trigger for the mRNA decay (Hosoda, Kim et al. 2005). PABP2 is another protein exclusive for pioneer translation. Several other components like eIF4G are shared by both the pioneer as well as the bulk translation (Ishigaki, Li et al. 2001). Therefore CBP80, CBP20, Upf1 and PABP2 were targeted using interference RNA. Small interfering RNA oligonucleotides targeting the human sequences were designed newly or according to previously published data. Conditions for the transient transfection of the synthetic RNAi molecules and adequate down regulation of target proteins were optimized for different subclones of human HeLa cell cultures to develop efficient protocols. Small interfering RNA molecules targeting

neomycin phosphotransferase II, with a random sequence or only the lipid based transfection reagent without RNA molecules served as control for unspecific effects. The expression level of the targeted proteins was determined by immunoblot analysis. The protein bands were quantified in comparison to actin. The depicted immunoblots represent at least two independent experiments.

For the small interfering RNA targeting human CBP80 (Fig.10B) and human Upf1 (Fig.11D) a reduction of the protein level to about 25 % of the control treated cells was achieved. Similar depletion was achieved for CBP20 (Fig.10A) as well as PABP2 (Fig.10B).



**Fig.10 RNAi transfection leads to reduced protein level of CBP20, PABP2 and CBP80.** HeLa cells were transfected with small interfering RNA, cell homogenate was separated on 4-20% gradient SDS-PAGE gels and probed with antibodies raised against CBP20, PABP2 and CBP80 (A and B). Anti actin antibody served as loading control (B).

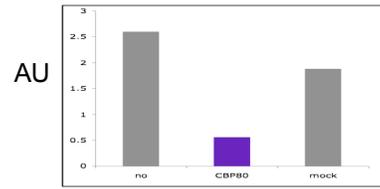
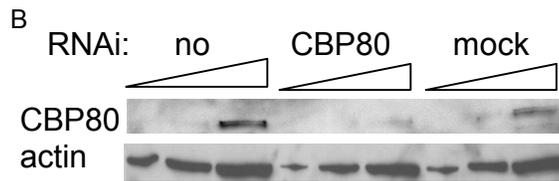
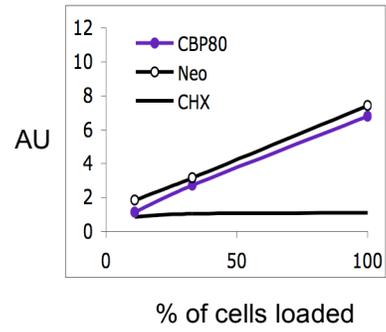
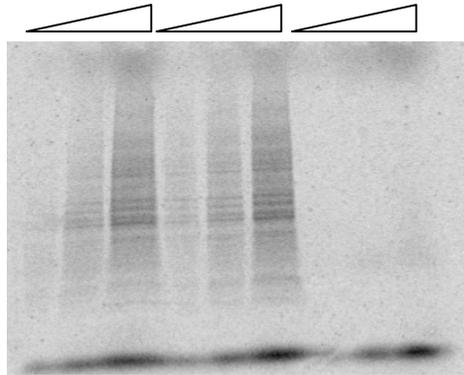
### **2.2.2 RNAi mediated inhibition of the pioneer round of translation does not effect the overall protein biosynthesis.**

Most mRNAs form polyribosome structures and undergo multiple rounds of translation before they reach their lifetime. Only PTC containing transcripts are limited to the pioneer round of translation before being degraded in a NMD dependent manner. Generally each newly synthesized mRNA will be translated into at least one pioneer round of translation product. To our knowledge there are no reports or even assays to determine the fate of pioneer round of translation products as unfolded polypeptide chains or proteins with a fully functional conformation. Taken together it can be assumed that the pioneer round of translation by itself only contributes very small and most likely undetectable quantities of protein to the overall pool of protein generated within the cell. In order to confirm that the RNAi mediated depletion of the pioneer round of translation factors CBP80 and Upf1 do not alter the overall synthesis of new polypeptides, radioactive incorporation assays were performed. Through radiolabeling any newly synthesized polypeptide chains are detected independent of length, conformation, reading frame or correct full-length translation. Since many of these alternative modes of translation might contribute to antigen generation, radiolabeling has an advantage over Western blotting of reference proteins that require full-length translation for antibody recognition. CBP80 and Upf1 RNAi depleted HeLa cells were incubated for various times in growth conditions including culture medium with S<sup>35</sup> radiolabeled cysteine and / or methionine. Afterwards excessive radioactivity was washed of, cells were homogenized and peptides were loaded on a reducing SDS-PAGE gel for separation. The

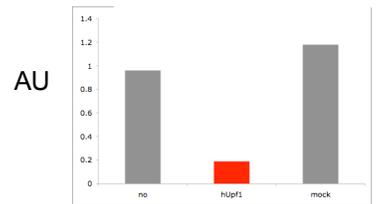
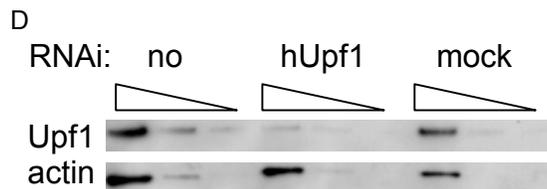
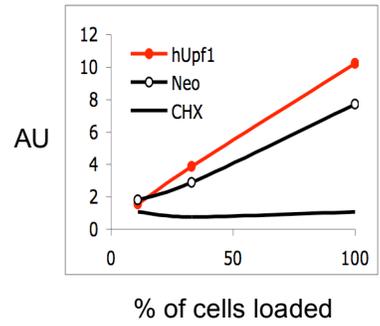
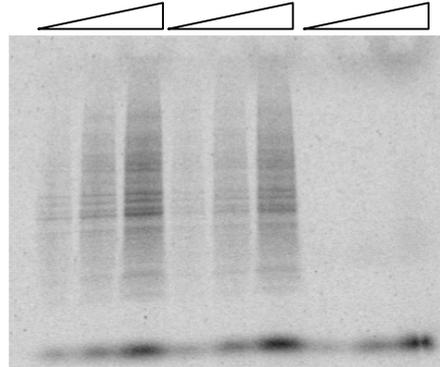
amounts of  $S^{35}$  incorporation was assessed by phosphoimaging and quantified. Cells transfected with unrelated RNAi served as a positive control, untransfected cells treated with the potent translation inhibitor cycloheximide at a concentration of 100  $\mu\text{g/ml}$  were used as a negative control.

Treatment of cycloheximide led to a reduction of  $S^{35}$  labeled amino acids below the detection level of the assay. CBP80 RNAi transfected HeLa cells showed a very slight reduction in  $S^{35}$  incorporation. Cells with a reduced level of Upf1 expression demonstrated an increase of translation activity of about 15% in comparison to mock treated cells. Several studies facilitating gene chip assays demonstrated the ability of Upf1 to alter the expression levels of multiple genes (Mendell, Sharifi et al. 2004; Chan, Huang et al. 2007). Out of 4000 genes examined after RNAi mediated inhibition of Upf1 in HeLa cells, at least 197 genes were shown to be consistently up regulated and 176 genes were down regulated by a factor of 1.9 or more. This effect might also account for the 15% increase in  $S^{35}$  incorporation detected here. The overall band pattern in the SDS-PAGE gel for mock, CBP80 or Upf1 RNAi transfected cells did not show any visible differences indicating similar sizes of the newly generated polypeptides. Changes in the size distribution could have been an indicator for different efficiency of initiation or elongation (Fig.11A and Fig.11C). Taken together no reduction of overall protein biosynthesis was detected after inhibition of the pioneer round of translation neither through CBP80 nor Upf1.

A RNAi: CBP80 mock CHX100



C RNAi: hUpf1 mock CHX100



**Fig.11 RNAi mediated depletion of the pioneer round of translation does not effect the overall protein biosynthesis.** Human HeLa cells were transiently transfected with short interfering RNA against CBP80, Upf1, neomycin phosphotransferase II (mock) or no RNAi. For  $S^{35}$  incorporation assays  $2.5 \times 10^5$  cells were cultured for 72 h and radiolabeled for 40 min with methionine and cysteine under growth conditions in RPMI medium. Cycloheximide was used at  $100 \mu\text{g/ml}$ . Cells were homogenized and the equivalent of 100,000 cells in a 1/3 dilution series was separated on a 4-20% gradient gel.  $S^{35}$  incorporation was quantified and depicted as arbitrary units (AU) to percent of cells. For protein detection the equivalent of 120,000 cells for CBP80 (80kDa) and 100,000 cells for Upf1 (137kDa) in a 1/3 dilution series were SDS-PAGE separated and immunoblotted. Bands were normalized to actin (43 kDa) and quantified. Data is representative of two or more experiments.

### 2.2.3 Targeting of eIF4E and 4E-BP1 reduces overall protein biosynthesis

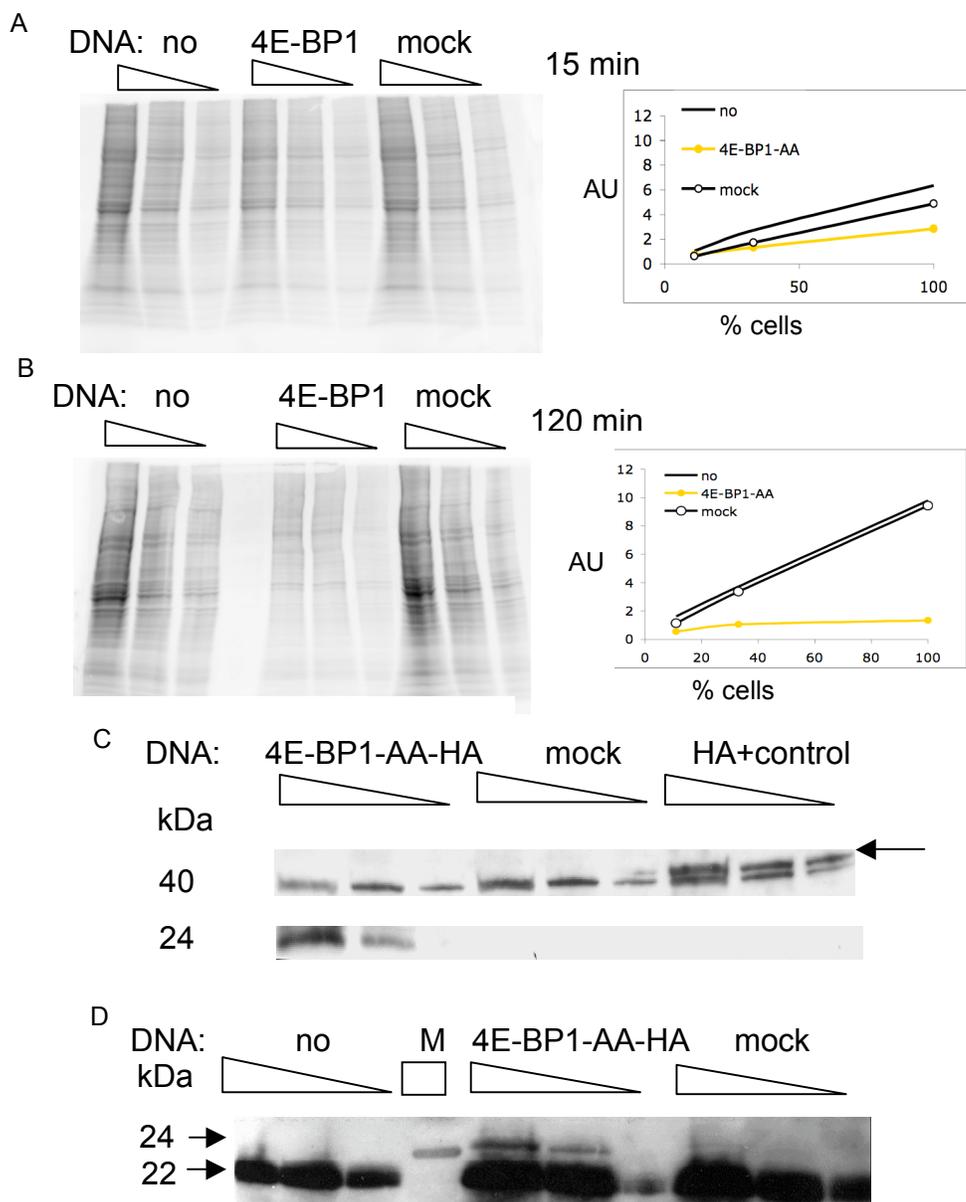
As mentioned above the shift from pioneer round of translation to bulk translation requires the transition from CBP80 to eIF4E. Within the steady state generation of protein biosynthesis eIF4E is regulated by 4E-BP1 factors. Overexpression of 4E-BP1 has been shown to stall translation. The dominant negative mutant 4E-BP1-AA lacks the two residues Thr37 and Thr46 (Gingras, Raught et al. 2001). Therefore it remains unphosphorylated and constitutively inhibits eIF4E function even more efficiently. Transient transfection with the dominant negative 4E-BP1-AA expression vector leads to a reduced incorporation of  $S^{35}$  methionine in comparison to cells transfected with an empty vector or no DNA. This inhibition was detectable after 15 min of  $S^{35}$  methionine incorporation (Fig.12A) and was even more pronounced after 120 min of translation (Fig.12B). Expression of the dominant negative 4E-BP1-AA vector was confirmed using immunoblotting. The first blot was probed with an antibody recognizing the HA-Tag of the 4E-BP1-AA vector. A band at 24kDa confirmed the expression of the dominant

negative protein. The band at 40kDa is unrelated to the experiment, the band at 43kDa a control transfection with the HA-tagged protein Trim21 (arrow) (Fig.12C). For an additional blot an antibody directly against 4E-BP1 is used. It recognized the wildtype protein of constitutively expressed 4E-BP1 at 22 kDa as well as the slightly larger transiently transfected 4E-BP1-AA at 24 kDa (Fig.12D).

Alternatively eIF4E can be directly targeted with small interfering RNA in order to achieve bulk translation inhibition. Transfection with eIF4E RNAi led to a reduction of  $S^{35}$  methionine and cysteine incorporation both after 30 and 75 min of translation. The translation rate of cells with depleted eIF4E was about 60 % of mock treated cells (Fig. 13A and B). In order to determine the effectiveness of the small interfering RNA eIF4E depletion was determined by immunoblotting. eIF4E bands were quantified and normalized to actin bands demonstrating a reduction to 30 % of protein level (Fig.13C).

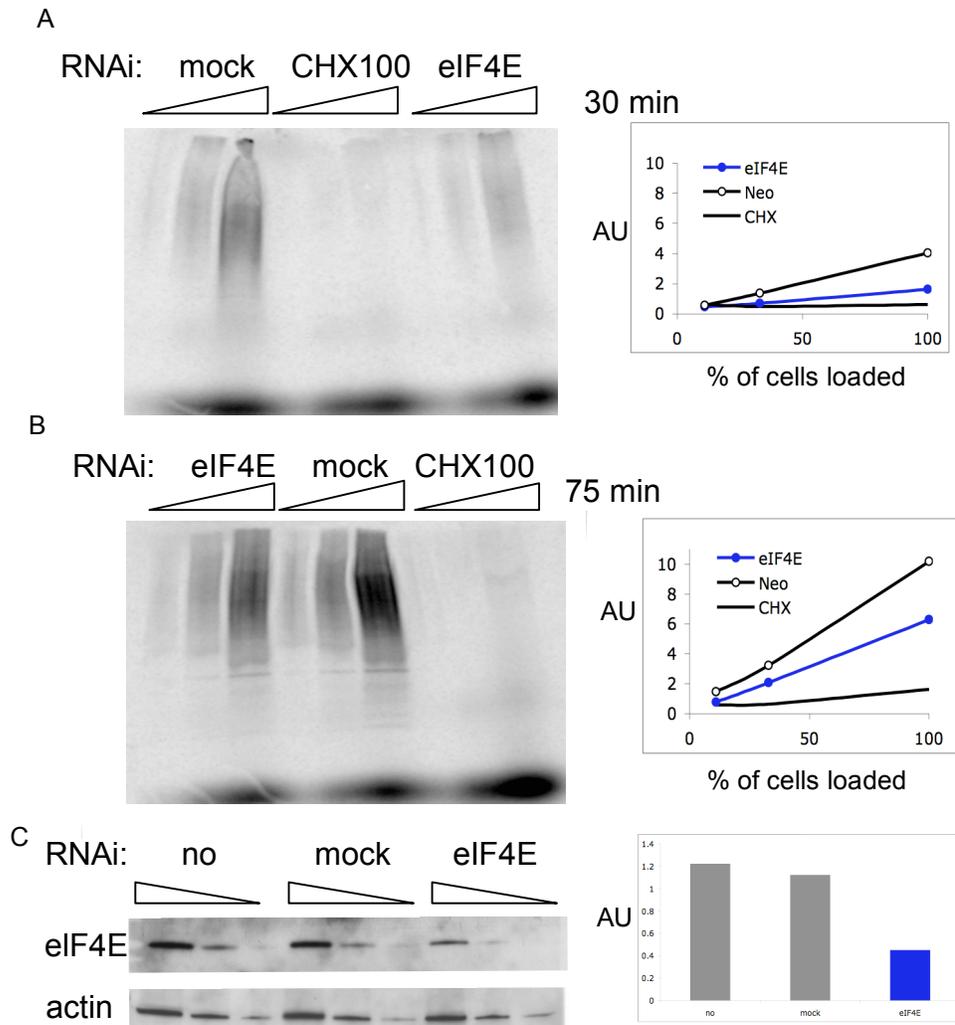
Taken together both DNA overexpression of dominant negative 4E-BP1-AA and depletion of eIF4E reduced bulk translation and generation of new polypeptides.

Treatment of cells with cycloheximide at a concentration of 100  $\mu$ g/ml stalled translation completely.



**Fig.12 Overexpression of dominant negative 4E-BP1-AA reduces translation**

HeLa cells were transfected with a vector encoding the dominant negative 4E-BP1-AA sequence followed by a HA-tag or empty vector. For  $S^{35}$  incorporation assays cells were cultured for 72 h, radiolabeled for 15 min (A) or 120 min (B) with  $S^{35}$  methionine under growth conditions in RPMI medium. Cycloheximide was used at 100 $\mu$ g/ml. Cells were homogenized and the equivalent of 300,000 cells in a 1/3 dilution series was separated on a 4-20% gradient gel.  $S^{35}$  incorporation was quantified and depicted as arbitrary units (AU) to percent of cells. For immunoblotting 400,000 cells were homogenized and loaded for separation onto a 4-20% SDS-PAGE gel in a 1/3 dilution series. The blot was probed with antibody raised against the HA-tag of the 4E-BP1-AA protein. 4E-BP1-AA was detected at 24 kDa, a control transfected Trim21 protein at 43kDa (C). Additionally constitutive wt 4E-BP1 as well as transiently transfected 4E-BP1-AA was detected directly (D).



**Fig.13 RNAi mediated depletion of translation factor eIF4E reduces protein biosynthesis.** HeLa cells were transfected with RNAi against eIF4E of random sequence RNAi. For  $S^{35}$  incorporation assays cells were cultured for 72 h, radiolabeled for 30 min (A) or 75 min (B) with  $S^{35}$  methionine under growth conditions in RPMI medium. Cycloheximide was used at  $100\mu\text{g}/\text{ml}$ . Cells were homogenized and the equivalent of 150,000 cells in a 1/3 dilution series was separated on a 4-20% gradient gel.  $S^{35}$  incorporation was quantified and depicted as arbitrary units (AU) to percent of cells (A and B). For immunoblotting 200,000 cells were homogenized and loaded for separation onto a 4-20% SDS-PAGE gel in a 1/3 dilution series. The blot was probed with antibody against eIF4E (25kDa) and actin (43 kDa) for normalization (C).

#### **2.2.4 Depletion of Upf1 reduces surface presentation of MHC class I**

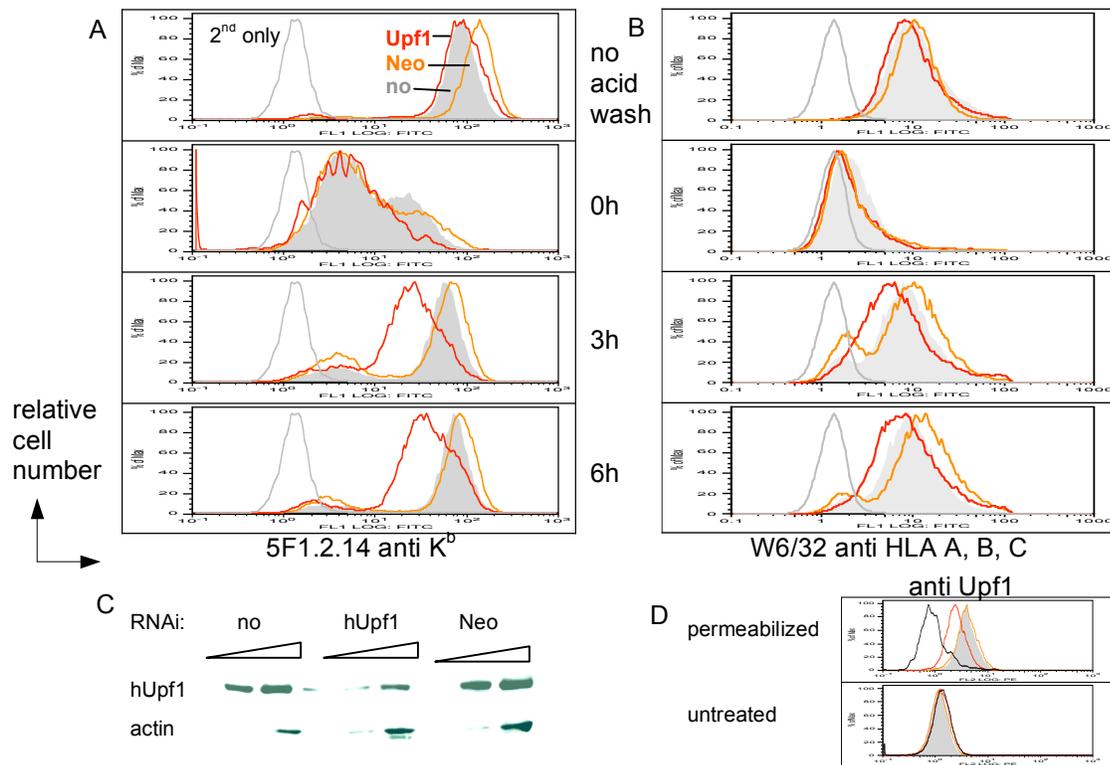
In the previous paragraphs a differential strategy to inhibit either the pioneer round of translation or the bulk synthesis of polypeptides was established. It was demonstrated that pioneer translation does not have a detectable effect on the overall generation of polypeptides. Several known sources of translation including cryptic translation and DRiPs are not contributing to the general synthesis of proteins while they play an important role in producing antigens for MHC class I mediated presentation (Shastri, Schwab et al. 2002). Here it shall be tested, if depletion of factors essential for the pioneer round of translation have any effect on the cell surface presentation of MHC class I. When MHC class I molecules that are loaded with their matching antigenic peptides finally reach the cell surface, they went through a cascade of rigorous steps optimizing the quality and binding properties of the peptides. They usually have a  $K_D$  of more than 500 nM and stably remain on the cell surface for hours or even days (Yewdell 2006). Therefore changes in the supply or processing of peptides often do not directly result in alterations of the MHC class I levels on the surface. Previously a method was established to extract the peptides from the binding cleft of the MHC class I molecule on the cell surface (Kunisawa and Shastri 2003). This is achieved with an acid wash procedure with PBS buffered citric acid at pH 3.1 for 1 to 5 minutes immediately followed by several steps of PBS rinsing. Conditions were adapted for several cell lines as well as murine primary cells. Adhesive cells remain attached to the culture dish during the acid wash treatment allowing for good conditions for continuous growth and culture of the cells. Removal of the peptide leads to the reinternalization and recycling of the heavy chain.

The  $\beta_2m$  chain, which is only non-covalently attached to the heavy chain, is permanently removed through the washing procedure. Within a time course of several hours newly generated MHC class I molecules are loaded and presented at the cell surface. The time of surface recovery is highly dependent of the cell line as well as the specific MHC class I peptide complex and has to be adjusted for the experimental conditions. Significant differences in the recovery times even for different subclones of the same cell line were observed. Generally the time for complete recovery ranges between 4 and 10 hours with murine MHC D<sup>b</sup> being relatively fast and MHC K<sup>b</sup> being slightly slower in achieving full recovery. In addition to respective primary and secondary antibodies, cells were stained with propidium iodine to select only living cells for analysis. Propidium iodine positive cells, generally a population of less than 0.5% of the cells that remain after citric acid washing, were excluded from the analysis.

Here human HeLa cells stably transfected for the murine MHC class I molecule K<sup>b</sup> as well as for the ovalbumine derived antigen precursor Kovak were used. They allow monitoring of the human HLA A, B and C as well as the murine K<sup>b</sup> MHC class I molecules during the acid wash recovery experiments. Cells were depleted for Upf1 using small interfering RNA, peptides were removed through acid wash treatment, and cells were placed under growth conditions for various times from 0 to 6 hours (Fig. 14A and B). After both 3 and 6 hours a significantly reduced level of K<sup>b</sup> surface expression was detected (Fig. 14A). This observation was also consistent with a lower level of surface HLA A, B and C (Fig. 14B). This observation was consistent in more than 10 experiments. General RNAi mediated depletion of Upf1 was confirmed with immunoblotting (Fig. 14C). Since western blotting can only demonstrate an overall

reduction of Upf1 in a pool of homogenized cells, Upf1 protein levels were additionally accessed on a per cell basis using flow cytometry. Cellular Upf1 protein levels were reduced in all permeabilized cells as demonstrated by the shift of the graph for Upf1 RNAi treated cells to the left (Fig. 14D, red line). Unpermeabilized cells served as a control for Upf1 specificity.

Together these experiments indicate a loss of MHC class I surface presentation in Upf1 depleted cells.



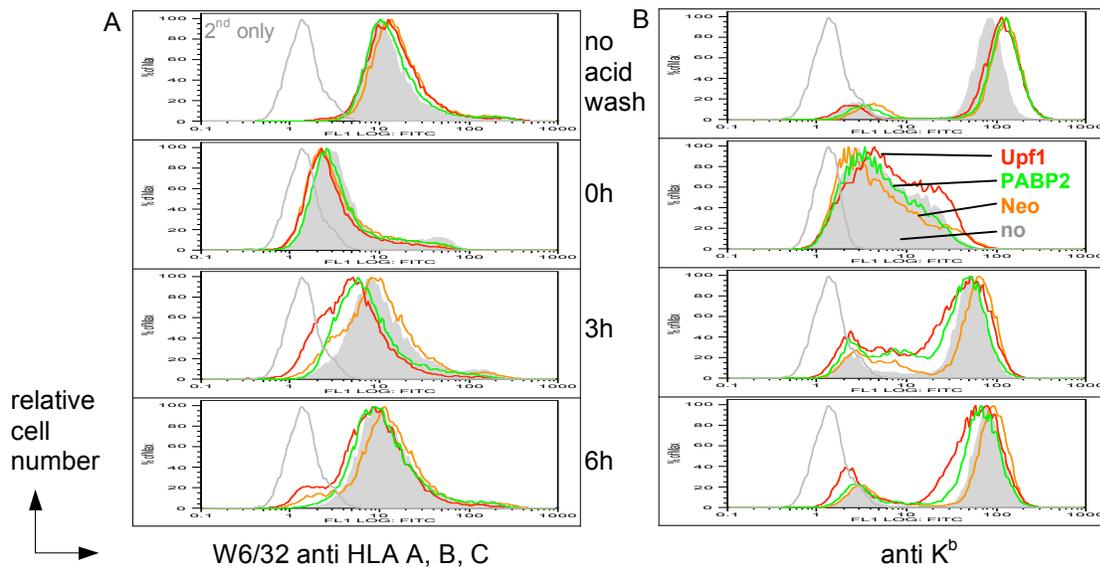
**Fig.14 Transient depletion of Upf1 reduces the surface presentation of MHC class I.**

HeLa Kovak/K<sup>b</sup> cells were transiently transfected with RNAi targeting Upf1 (red), neomycin phosphotransferase II (orange) or no RNAi (grey shadow). Cells were untreated (no acid wash) or acid washed and placed under culture conditions for various times 90, 3, 6h). MHC surface levels were monitored by flow cytometry using primary antibody 5F1.2.14 against K<sup>b</sup> or W6/32 (A) against HLA A, B, C (b). RNAi mediated depletion of Upf1 was confirmed by immunoblotting (C) and flow cytometry (D). Cells were permeabilized with -20°C prechilled EtOH and stained for internal Upf1 protein levels.

### **2.2.5 Reduction of PABP2 expression leads to intermediate MHC class I surface recovery**

PABP1 and PABP2 have been described as protein factors present at the translation initiation complex of newly spliced mRNA (Ishigaki, Li et al. 2001). After the pioneer round of translation PABP2 is removed from the assembly, while PABP1 remains bound to the Poly-A tail of the mRNA facilitating further rounds of translation. Here the effect of PABP2 depletion on MHC class I surface expression was directly compared to the previously demonstrated Upf1 mediated MHC class I surface reduction (Fig.15). As described in the experiments above HeLa Kovak/K<sup>b</sup> were transfected with small interfering RNA molecules, treated with citric acid solution for the removal of initial MHC class I surface molecules and recovery was monitored over a 6 hour time interval. Flow cytometric determination of the MHC class I HLA A, B, and C surface expression levels showed a clear difference in recovery between Upf1 depleted cells on the one hand and irrelevant neomycin phosphotransferase II RNAi or untransfected cells after 3 hours. Absolute numbers of mean fluorescence for the 3h time point were 24.8 for Upf1, 29.4 for PABP2, 42.4 for Neo and 41 for no RNAi. Therefore cells with a reduced PABP2 level showed an intermediate phenotype with HLA A, B and C levels lower than control transfected cells but slightly higher than Upf1 diminished cells. After 6 hours the absolute differences were less pronounced while the same relative differences between Upf1, PABP2, Neo and untransfected cells remained (Fig.15A). Analysis of K<sup>b</sup> surface expression presented similar findings at the 3 and 6 hour time points with more pronounced MHC class I reduction for Upf1 and intermediate MHC class I reduction for

PABP2 depleted cells. While this pattern was consistent in at least two independent experiments there remain several explanations for an intermediate effect of PABP2 depletion on MHC class I surface recovery. These include more technical findings like a less pronounced efficiency of the small interfering RNA transfection in reducing the PABP2 target protein level or more functional explanations like a less stringent requirement for PABP2 or a redundancy between PABP1 and PABP2 in facilitating the pioneer round of translation mechanism. Since it might be difficult to conclusively distinguish between these possibilities, further experiments focused on additional factors of the pioneer round of translation with more pronounced effects. In summary PABP2 reduction shows intermediate inhibition of MHC class I surface expression.



**Fig.15 Transient depletion of PABP2 reduces the surface presentation of MHC class I to a lesser extent than Upf1.** HeLa Kovak/K<sup>b</sup> cells were transiently transfected with RNAi targeting PABP2 (green), Upf1 (red), neomycin phosphotransferase II (orange) or no RNAi (grey shadow). Cells were acid treated as described above. MHC surface levels were monitored using primary antibody or W6/32 (A) or 5F1.2.14 (B). Depletion of PABP2 reduced the recovery of both HLA A, B and C as well as K<sup>b</sup> but to a lesser extent than Upf1 depletion.

### **2.2.6 Depletion of the pioneer round of translation factor CBP80 reduces surface presentation of MHC class I**

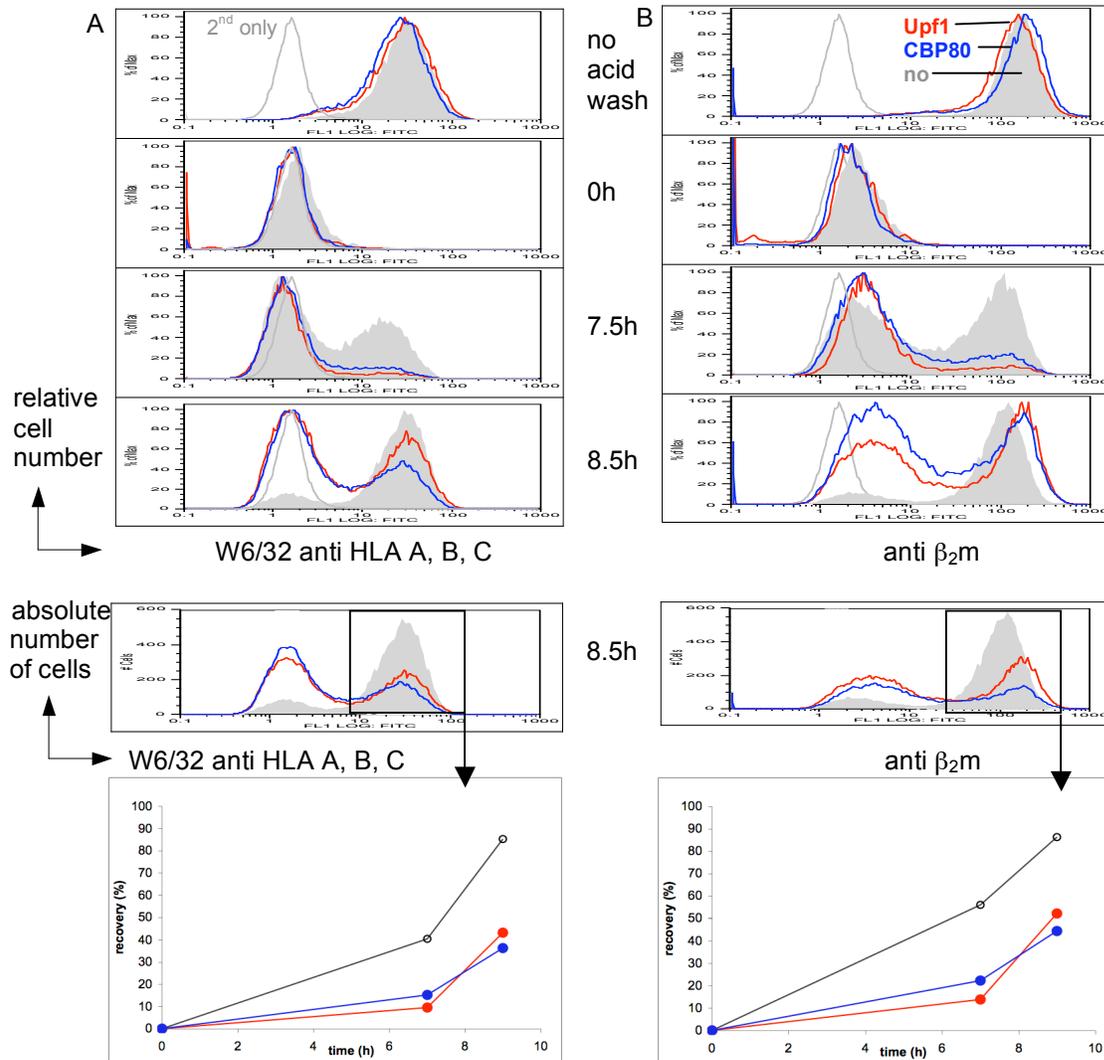
CBP80 has been described as the defining protein for the pioneer round of translation. Jointly with CBP20 it interacts with the cap structure of the newly spliced mRNA as well as eIF4G (Marintchev and Wagner 2005). Since it is removed from the translation initiation complex right after the first passage of the ribosome it is the ideal target to demonstrate the involvement of the pioneer round of translation in antigen presentation (Lejeune, Ishigaki et al. 2002; Chiu, Lejeune et al. 2004). This represents an advantage over experiments focusing solely on Upf1, which has been demonstrated to be part of the pioneer translation but also takes part in final processes of mRNA decay as well as general gene regulation (Mendell, Sharifi et al. 2004; Chan, Huang et al. 2007).

Additionally, a subclone of HeLa cells (termed AS1) were used which showed a particularly slow MHC class I surface recovery pattern. After surface removal these cells only regain their prior level of MHC class I expression after about 8.5 hours. This helps to examine any differences of MHC class I recovery times of cells deficient for the pioneer round of translation. Cells were transfected with small interfering RNA molecules, washed with citric acid buffer and placed under culture conditions as described above. After initial optimization experiments flow cytometry analysis was performed in a time course with measuring intervals prior to acid wash, right after acid wash and at 7.5 and 8.5 hours of culture. CBP80 as well as Upf1 depleted cells showed a very similar or slightly reduced overall MHC class I level as assessed with antibody

W6/32 (Fig.16A). Acid wash completely removed MHC class I complexes reducing the mean fluorescence in all cases to the level of only secondary antibody staining. After 7.5 hours 42 % of untransfected cells recover to their level of MHC class I surface expression prior to acid wash. At the same time only 9-11% of the Upf1 or CBP80 depleted cells show full MHC class I expression. This disparity remains at the 8.5 hour time point with 85 % of control cells fully recovering their HLA A, B and C expression. In comparison only 38 and 40 % of CBP80 and Upf1 depleted cells recover at this time interval. The separate panel line shows the recovery pattern at the 8.5 hour time point with absolute numbers of cells on the y-axis as well as the gate (box with arrow) used for the determination of the percentage of fully recovered cells.

Assembly of functional MHC class I molecules on the surface requires the noncovalent binding of  $\beta_2m$  with the heavy chain as well as association with an appropriate peptide. Therefore the overall surface expression of MHC class I complexes can be determined by flow cytometric measurement of  $\beta_2m$  (Fig.16B). Also here the recovery follows a similar pattern with only 13 and 16 % of Upf1 and CBP80 depleted cells in comparison to 55% of untreated cells with full  $\beta_2m$  surface expression after 7.5 hours. After 8.5 hours the difference between 43 and 41 % of Upf1 and CBP80 transfected cells to 83% of control cells regaining their prior  $\beta_2m$  levels remains large. Targeting of CBP20 results in a comparable delay of MHC class I expression after acid wash confirming above findings and ruling out unspecific or off target effects of the small interfering RNA molecules. While the underlying reasons for the bimodal MHC class I recovery pattern were addressed in additional experiments, CBP80 expression and the pioneer round of

translation can be postulated as an essential requirement for timely MHC class I surface presentation.



**Fig.16 Bimodal distribution of MHC class I surface expression in Upf1 and CBP80 depleted HeLa cells.** MHC class I surface recovery is cell line dependent. A subclone of HeLa cells were transiently transfected with RNAi targeting Upf1 (red), CBP80 (blue) or no RNAi (grey shadow). MHC class I surface recovery was accessed after 0, 7.5 and 8.5 h using antibody against HLA A, B and C (A) or beta2m (B). MHC class I surface level is depicted in relative and for the 8.5h time point additionally in absolute cell numbers. A gate was chosen for cells with fully recovered MHC levels (square box with arrow) and the percentage of cells recovered at each time point were depicted in separate diagrams (bottom).

### **2.2.7 RNAi mediated depletion of CBP80, CBP20 and Upf1 decreases activation of three different T-cell lines.**

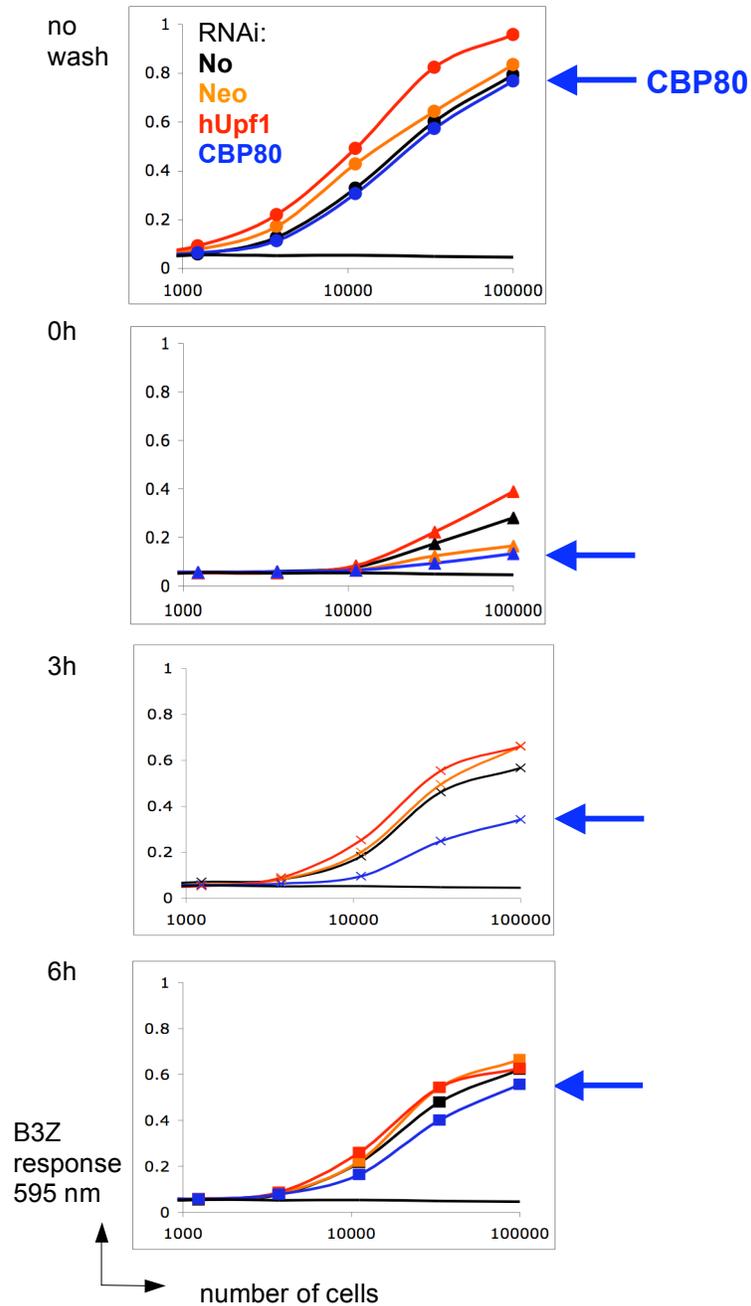
The previous sections postulate a reduced number of MHC class I molecules in cells transiently depleted of the pioneer round or translation factors CBP80 and Upf1. MHC class I levels were assessed as total amount of K<sup>b</sup> or HLA A, B and C molecules on the surface recognized by specific antibodies. In order to determine the effect of a diminished pioneer round of translation on the generation and surface presentation of specific antigenic peptides acid wash recovery experiments were performed with subsequent T-cell activation assays (Fig.17). HeLa Kovak/K<sup>b</sup> cells were RNAi transfected and citric acid treated for MHC class I surface removal as described before. Afterwards cells were placed under culture conditions for recovery of MHC class I surface expression for 0, 3 and 6 hours. Peptides were extracted from 500,000 cells of each condition resuspended and titrated in a 1 to 3 dilution series (see methods 4.9). Peptides were incubated with 100,000 HeLa K<sup>b</sup> cells as antigen presenting cells and 100,000 B3Z T-cells. B3Z cells recognize the K<sup>b</sup> restricted ovalbumine derived antigen SIINFEHL. After 16 hours T-cell activation was measured in a CPRG assay (Fig .17).

In the absence of SIINFEHL antigen, HeLa cells are not capable of triggering any B3Z specific response serving as a negative control for the assay (black line at bottom). Upf1 depleted cells (red) show as similar or slightly increased rate of T-cell activation in comparison to untransfected (black) or neomycin phosphotransferase II (orange) control transfected cells at all time points. In sharp contrast to that, the initial absorbance of CBP80 (blue) depleted cells of 0.8 prior to acid wash was reduced to 0.12 right after acid

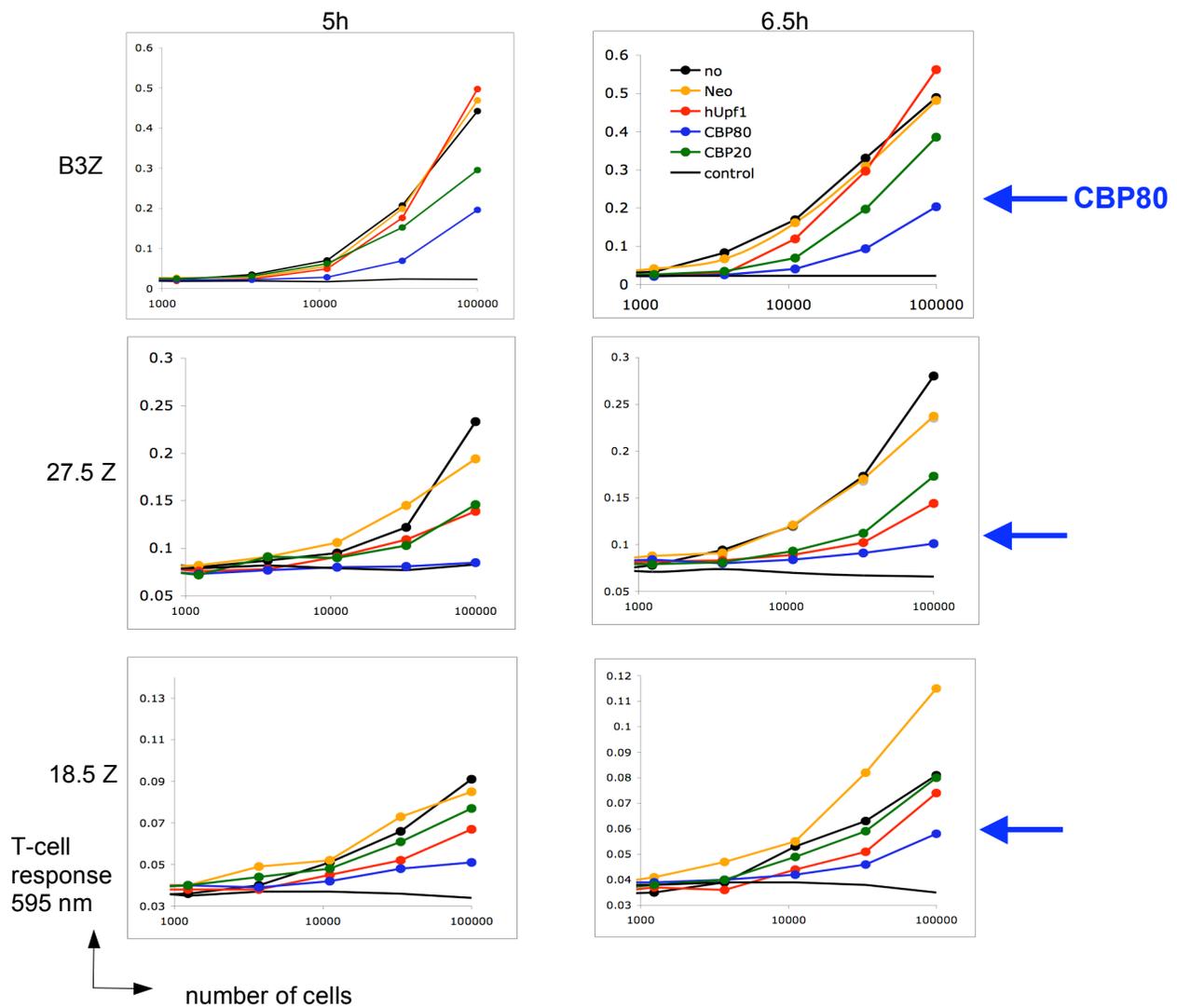
treatment and only recovers slowly to 0.37 after 3 hours and 0.58 after 6 hours. Therefore antigen presenting HeLa cells are only capable of triggering a B3Z T-cell response of about half the intensity of control-transfected cells. This finding goes along with previous observations of a reduced MHC class I surface expression in CBP80 deficient cells as detected by flow cytometry. It underlines the importance of pioneer factors for efficient antigen generation and surface presentation. The unaltered or even slightly increased T-cell activation of Upf1 depleted cells might find its explanation in the multiple roles of Upf1 in altering gene expression of many genes. In conclusion this experiment has demonstrated the dependency of MHC class I surface presentation on CBP80 pioneer translation for at least one specific antigen. Additionally it should be underlined that with this experimental design extracting antigenic peptides and presenting them separately through different cells, unspecific effects of the RNAi treatment on MHC class I maintenance or regulation can be ruled out.

Two additional T-cell lines were used in a similar experimental setup in order to substantiate these results. Small interfering RNA transfection for CBP80 (blue), CBP20 (green), Upf1 (red), neomycin phosphotransferase II (orange) and no RNA as well as acid wash were performed as described above (Fig.17). Here 100,000 HeLa Kovak/K<sup>b</sup> cells were used in a direct presentation assay. Brefeldin A was added in the concentration of 5µg/ml in order to prevent any further Golgi transport of MHC class I peptide complexes during the incubation with the T-cells. For activation assays the T-cell lines 18.5Z and 27.5Z were used in addition to B3Z. Both recognize a specific antigen of unknown sequence. In accordance with the previous experiment (Fig.17) Upf1, Neo and no RNAi

transfected cells show similar B3Z T-cell activation both after 5 and 6.5 hours. CBP20 RNAi transfected cell demonstrate an intermediate T-cell response in the CPRG assay while CBP80 RNAi transfected cells lead to a markedly reduced B3Z activation. In a similar way CBP80 depletion leads to a severely reduced 27.5 T-cell activation. Here Upf1 as well as CBP20 reduction goes along with a T-cell response less than half of the control samples. This is consistent with 18.5 T-cell activation with the lowest activation of CBP80 RNAi treated cells, slightly increased responses of Upf1 and CBP20 RNAi treated cells. Consistent with all experiments CBP80 deficient antigen presenting cells demonstrate a markedly reduced T-cell recognition further confirming measurements of overall MCH class I surface levels as determined by antibodies and flow cytometry. CBP20 and Upf1 RNAi transfection seem to have a lesser effect on antigen presentation. Interestingly Upf1 reduction altered the 18.5Z and 27.5Z peptides to a larger extent than the B3Z recognized SIINFEHL peptide.



**Fig.17 Peptides extracted from CBP80 depleted cells show reduced B3Z T-cell activation.** HeLa Kovak/ $K^b$  cells were transfected with RNAi targeting CBP80 (blue), Upf1 (red), Neo (orange) or no RNA (black). After citric acid wash cells were placed under culture conditions for 0, 3 or 6 h. Peptides were extracted from 500,000 cells and presented by HeLa  $K^b$  cells to B3Z T-cells for CPRG assay measurement. CBP80 depleted cells demonstrate lower B3Z T-cell activation after 3 h.

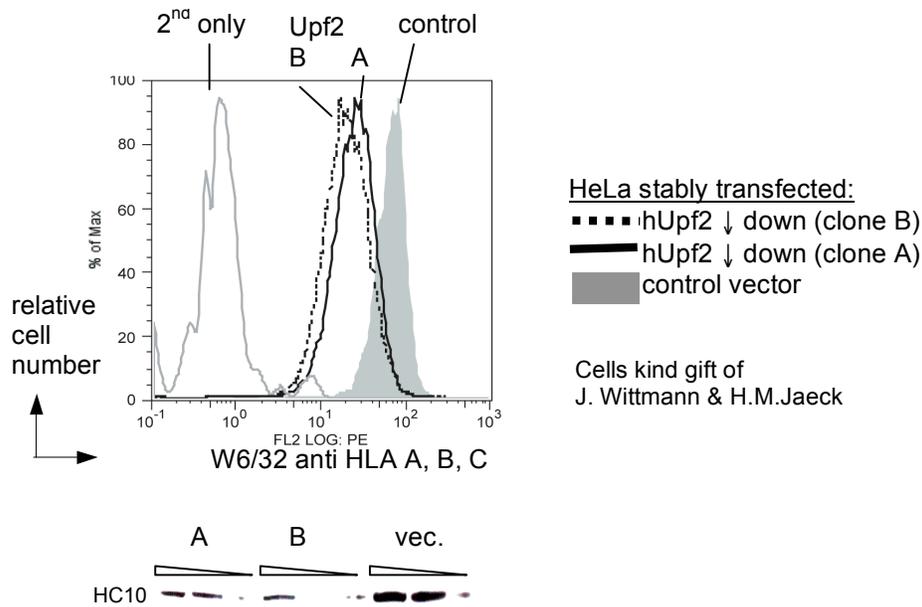


**Fig.18 CBP80, CBP20 and Upf1 depleted cells show reduced activation of three different T-cell lines.** HeLa Kovak/ $K^b$  cells were transfected with RNAi targeting CBP80 (blue), CBP20 (green), Upf1 (red), Neo (orange) or no RNA (black), washed with citric acid buffer and cultured for 5 or 6.5 h for MHC class I surface recovery. 100,000 cells each were used in CPRG T-cell assays with the cell lines B3Z, 27.5Z or 18.5Z in the presence of brefeldin A. T-cell activation was determined as absorbance at 595nm. The T-cell response for all three cell lines is strongly diminished in CBP80 depleted cells and to some extent reduced in CBP20 depleted cells. Upf1 depleted cells show only a reduced T-cell activation for 18.5Z and 27.5Z while leaving B3Z recognition unaffected.

### 2.2.8 Permanent Upf2 reduction lowers MHC class I expression

Nonsense mediated decay is required for viability at an organismal as well a cellular level. Attempts to generate an Upf1 knock out mouse model were doomed by the early embryonic lethality of Upf1 deficient cells (Medghalchi, Frischmeyer et al. 2001). Here the requirement of Upf1 even prevented the generation of cell lines with permanently diminished factor expression. Cell lines stably transfected with Upf1 targeting small hairpin RNA sequences incorporated within an puromycin selectable expression vector did not generate any stable cell lines viable after 10 days (Wittmann, Hol et al. 2006). In contrast two functional and partially redundant Upf3 homologues are encoded in mammals. In humans dysfunctionality in one of these genes is tolerated even though it is manifested in a severe disease phenotype (Tarpey, Raymond et al. 2007). This leaves Upf2 as potential target for stable down regulation via vector encoded small hairpin RNA (Wittmann, Hol et al. 2006). HeLa cells were selected for stable incorporation of the Puro-Upf2-shRNA construct by culturing in the presence of 10 µg/ml puromycin and reduced Upf2 protein expression was confirmed by immunoblotting. The two HeLa cell line subclones shown below were provided by Juergen Wittmann and Hans-Martin Jaeck (Fig.19). Both independent clones show permanently about 5 fold reduced MHC class I levels as recognized by staining with antibody W6/32. The total combined cellular and surface amount of MHC heavy chain in both Upf2 diminished subclones as well as control cells was additionally assessed by immunoblotting using antibody HC10. The Western blot confirmed the overall reduction of heavy chain. Since the MHC class I molecule is only stable in the confirmation bound to an antigenic peptide these findings

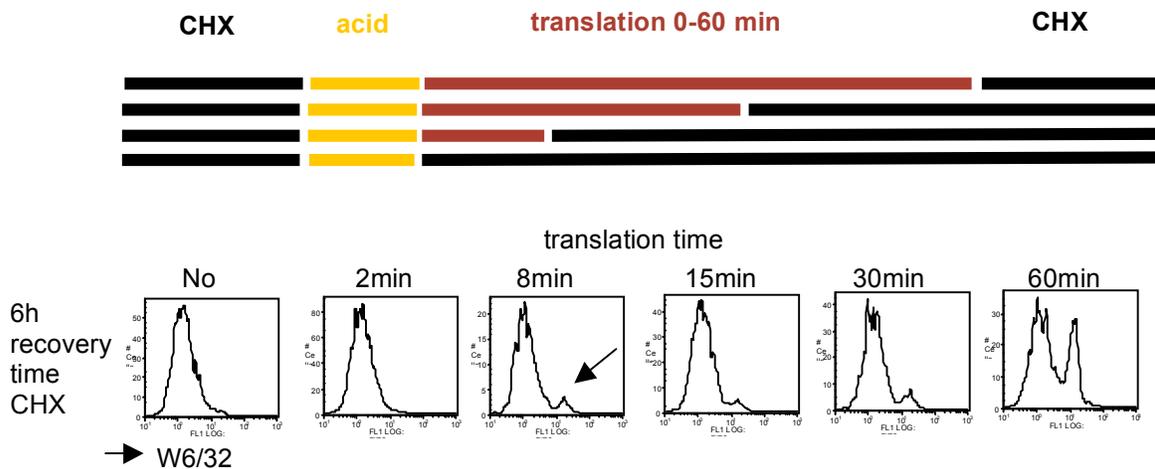
are another indicator for the lack of sufficient antigen generation in the absence of Upf2. While this is so far the only example of a viable mammalian cell line with permanently reduced expression of a factor of the pioneer round of translation and NMD pathway, there will likely be more cell lines and primary cells originating from a recently established Upf2 conditional knock out mouse.



**Fig.19 Stable depletion of Upf2 reduces both MHC class I surface and internal HC levels.** HeLa cells were transfected with a shRNA vector targeting Upf2. Subclones with stable down regulation of Upf2 were selected for further analysis. MHC class I surface expression was determined by flow using antibody W6/32. Total heavy chain expression was measured using immunoblotting with antibody HC10.

### **2.2.9 Short time intervals of new translation are sufficient for antigen presentation**

It was demonstrated that ongoing translation is a requirement for antigenic peptide generation and presentation (Qian, Reits et al. 2006). In order to examine the minimum requirements of translation for antigen production cycloheximide was used in a time course experiment (Fig.20). HeLa cells were treated for 2 hours prior to the acid wash removal to ensure that recently translated polypeptides are already processed and transported to the surface. MHC class I surface molecules were removed with citric acid and cells were permitted to recover for various time intervals from 0 to 60 min to progress with translation. After that translation was again interrupted by addition of cycloheximide and cells were cultured for a total of 6 hours followed by flow cytometry analysis using antibody W6/32. As expected cells that were continuously exposed to 100  $\mu\text{g/ml}$  cycloheximide were not able to translate peptides for presentation. While 2 minutes were not sufficient, 8 or more minutes of translation generated peptide MHC class one complexes detectable on the surface. This confirms the dependency of antigenic peptide generation on new translation. Further it demonstrates that the very first peptides generated within several minutes already contribute to antigen presentation.



**Fig.20 Minimum time requirement for peptide generation.** HeLa cells were pretreated with 100  $\mu\text{g/ml}$  CHX for 2 h prior to acid wash. Cells were placed in recovery conditions for time intervals of 0-60 min before interruption of translation with CHX. MHC class I surface recovery was determined 6 h after citric acid treatment by flow cytometry using antibody W6/32. Permission of 8 min of translation is sufficient for the generation of peptides presented on the cell surface.

### 2.2.10 Inhibition of bulk translation does not effect antigen presentation

Multiple rounds of eIF4E-dependent bulk translation of mRNA transcripts account for the generation of the waste majority of proteins in a cell. Here it shall be examined if a reduction of this steady state translation pathway has any measurable effect on antigen generation and MHC class I mediated surface presentation. In the first experiment inhibition of bulk translation has been achieved as described above. HeLa Kovak/ $K^b$  cells have been transfected with the dominant negative 4E-BP1-AA vector construct (red), the wildtype 4E-BP1 DNA (orange), an empty vector (yellow) or no DNA (grey shadow

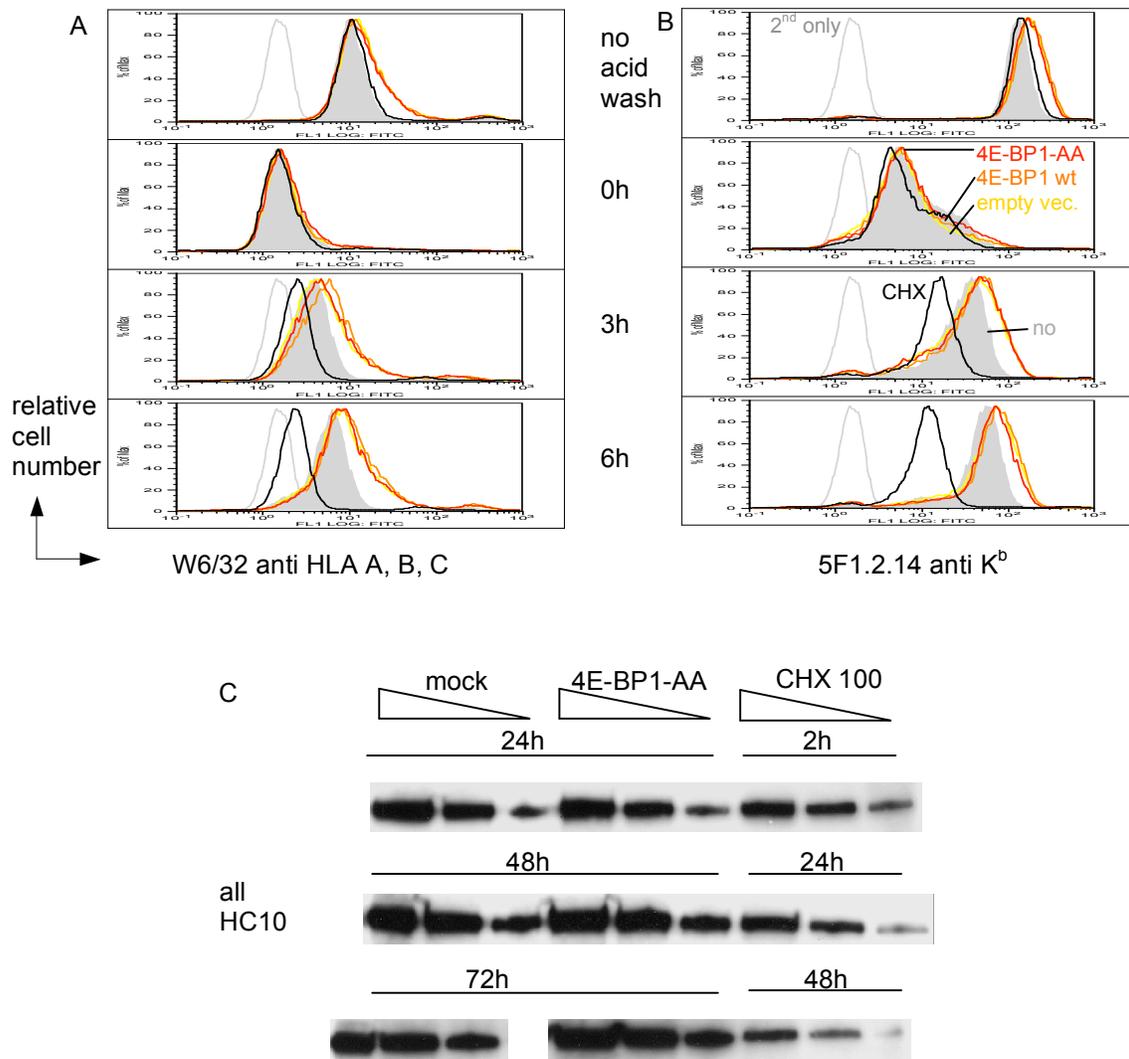
(Fig.21 A and B). Additionally cells were incubated with cycloheximide at the concentration of 100 µg/ml (black). Cycloheximide interferes with the translocation therefore abolishing all translation elongation. MHC class I surface expression was monitored by flow cytometry analysis using antibodies W6/32 and 5F1.2.14. All cells transfected with any of the vector constructs showed some MHC class I upregulation. This is an unspecific effect apparently related to transfection itself. No differences in MHC class I surface expression between the 4E-BP1-AA, 4E-BP1 or empty vector constructs were detected after 3 and 6 hours. Even though 4E-BP1-AA transfection has been demonstrated to significantly reduce the amount of newly generated polypeptide chains it did not impose any detectable effect of MHC class I mediated antigen presentation. In stark contrast cells with cycloheximide regained only initial surface expression at the 3 hour time point. No further MHC class I reappearance was measured after 6 hours. The initial recovery might be due to the time required for cycloheximide to enter the cell, bind to the ribosomes and stall new translation initiation. During this time period recently loaded MHC-peptide complexes can continue to progress on their passage from the ER through the Golgi to the cell surface independent of cycloheximide. So inhibition of overall translation proved to be a very effective measure to abolish peptide generation and presentation. This finding is supported by the strict requirement of ongoing translation for antigen generation (Qian, Reits et al. 2006).

Additionally it was demonstrated that the MHC class I heavy chain is abundantly present during the course of the experiment (Fig.21 C). With a relatively long protein half-life the heavy chain is continuously present during 4E-BP1-AA expression. Cycloheximide

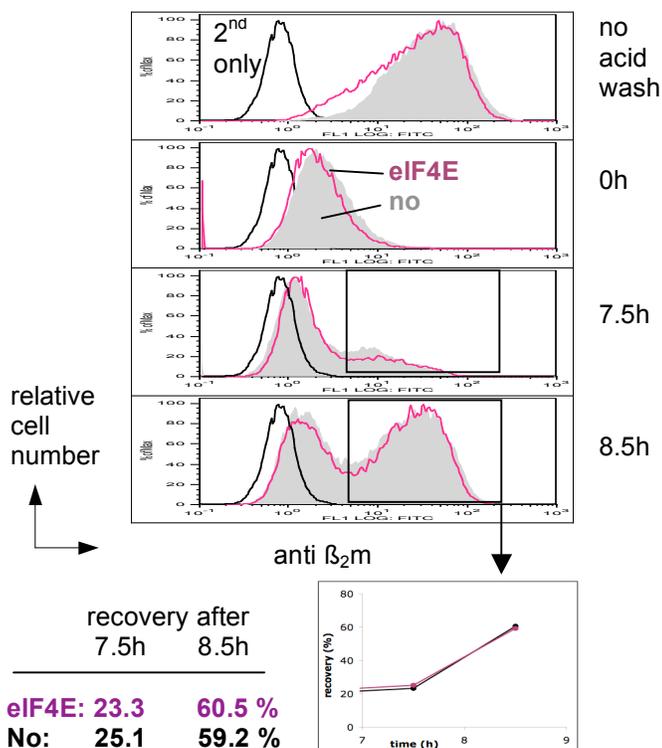
incubation only gradually decreases the overall pool of heavy chain after 2 hours. Even after 1 and 2 days of cycloheximide treatment at 100 µg/ml substantial amounts of heavy chain can be detected by immunoblotting. Therefore the availability of heavy chain does not seem to be the reason for stalled MHC class I surface presentation but rather the interference with the generation of new peptides.

4E-BP1-AA binds to eIF4E therefore preventing it from functioning in bulk translation. Alternatively small interfering RNA against eIF4E can prevent the assembly of the initiation complex for bulk translation. HeLa AS1 cells with depleted eIF4E were shown to recover their MHC class I surface level in the same pattern as untransfected cells. Both eIF4E depleted and control cells recover to 23.3 and 25.1 % after 7.5 hours and to 60.5 and 59.2 % respectively after 8.5 hours. No significant effect on MHC class I surface presentation could be detected upon reduced eIF4E expression and bulk translation.

Taken all these findings together, antigen presentation remains to be dependent on the active and ongoing translation of RNAs. Alterations of the eIF4E dependent bulk translation do not change antigenic peptide generation. It has to be considered that bulk translation usually produces large quantities of polypeptides and that even a very effective RNAi targeting strategy is unlikely to completely abrogate bulk translation, particularly the relatively small copy numbers needed for the MHC class I mediated antigen presentation. But on the other hand these finding continue to underline the importance of the pioneer round of translation for antigen generation, which might just generate minuscule amounts of peptides while altering antigen surface levels severely.



**Fig.21 Inhibition of 4E-BP1 regulated bulk translation does not alter MHC class I expression.** HeLa Kovak/K<sup>b</sup> cells were transiently transfected with no DNA (grey shadow) or DNA constructs encoding dominant negative 4E-BP1-AA (red), wildtype 4E-BP1 (orange) or empty vector (yellow). 100 µg/ml cycloheximide was added in the recovery medium to inhibit overall translation (black line). After acid wash flow cytometric analysis was performed using antibody W6/32 (A) and 5F1.2.14 (B). No apparent effect of 4E-BP1AA or 4E-BP1 wt on MHC class I recovery was detected. Additionally cells were transfected with 4E-BP1-AA or incubated with cycloheximide for various times between 2 and 72 hours as indicated. The equivalent of 100.000 cells or a subsequent dilution 1/3 was loaded on a 4-20% SDS-PAGE gradient gel and probed for MHC class I heavy chain expression.



**Fig.22 Inhibition of eIF4E dependent bulk translation does not alter MHC class I expression.** HeLa AS1 cells were transfected with eIF4E (purple) or no (grey shadow) RNAi, acid treated and cultured for 0, 7.5 and 8.5 hours before staining with antibody against  $\beta_2m$  to access total MHC class I surface levels. MHC class I expression is depicted as percentage of cells with completely regained surface expression (box, table & graph). Both eIF4E and control cells recover MHC class I levels to equal extent after 7.5 and 8.5 hours.

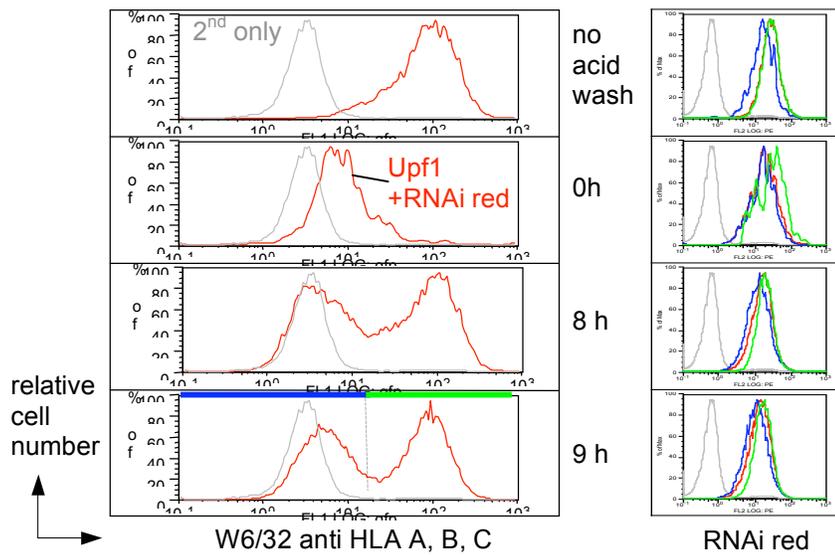
### 2.2.11 Bimodal distribution of MHC class I recovery in HeLa AS1 cells

In multiple experiments a bimodal recovery pattern as depicted in Fig.22 was observed. Two distinguishable peaks became particularly visible with a large difference between the maximum fluorescence labeling before acid wash and the minimum right after acid wash. Cells like HeLa AS1 with a relatively slow surface recovery of MHC class I molecules show very distinctive peaks. Since these experiments take an important

position within this study the recovery pattern of subpopulations was studied in more detail. Three factors were identified that could potentially account for differences in MHC class I expression between these subpopulations.

- 1.) Transfection efficiency of small interfering RNA is different for MHC class I high and low expressing cells
- 2.) RNAi mediated down regulation of target proteins differs in subpopulations
- 3.) Differences in the stage of the cell cycle account for bimodal MHC class I surface reappearance.

First it was confirmed that small interfering RNA transfection was equally efficient for the different subsets of cells. HeLa AS1 cells were cotransfected with Upf1 RNAi and an equal amount random sequence RNA of the same length with a red fluorescence tag. The cells were then used for an acid wash recovery experiment and analyzed by flow cytometry for the surface expression of HLA A, B and C as well as the uptake of red fluorescence from the control RNA. The red fluorescence was determined for each time point before acid treatment and at 0, 8 and 9 hours of culture for the entire peak (red) as well as separately for the 50% cells with lower (blue) and higher (green) MHC class I expression. All cells transfected with RNAi<sub>red</sub> showed high level of red fluorescence confirming the RNAi uptake of almost 100% of the cells. RNAi<sub>red</sub> transfection levels were comparable between MHC class I low (blue) and high (green) expressing cells within a significant increased above control cells (grey) in mean red fluorescence.

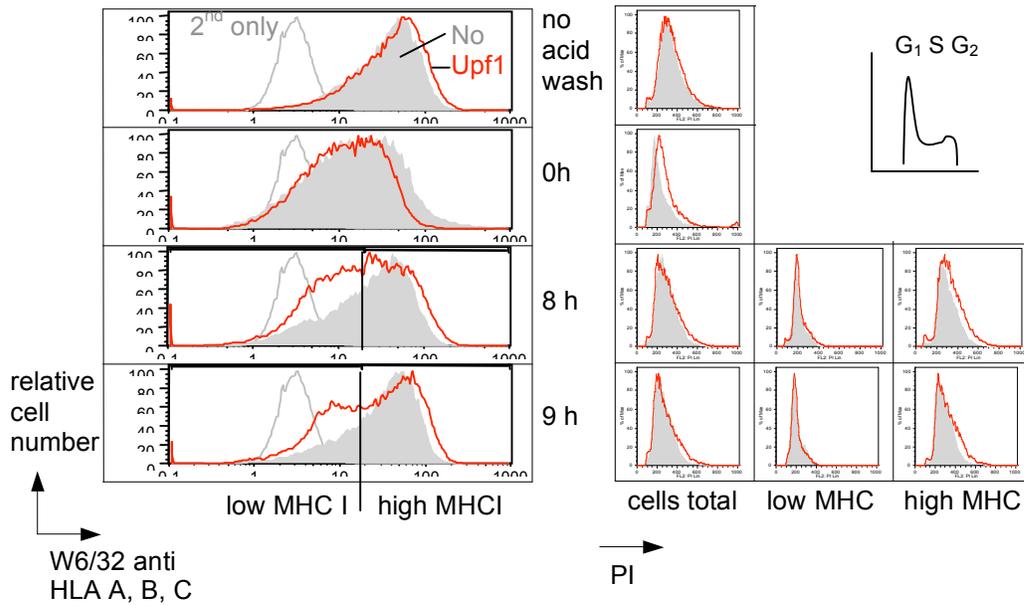


**Fig.23 RNAi transfection efficiency consistent in subsets of recovering cells.** HeLa AS1 cells were cotransfected with equal amounts of Upf1 and red fluorescence labeled random sequence RNAi, washed with citric acid and cultured for 0, 8 or 9 hours. MHC class I expression was probed with W6/32 staining (left). RNA<sub>red</sub> uptake was analyzed for the total amount of cells (red), 50 % cells with lowest MHC class I expression (blue) and 50 % with highest MHC class I expression (green) (right). All subsets of cells demonstrated RNA<sub>red</sub> uptake.

Further the interdependence of MHC class I surface expression and the cell cycle was addressed. As noted before new generation of MHC class I peptide complexes requires ongoing protein translation. Within the progression of the cell through the cell cycle different subsets of translation are more or less active. During cell division global cap-dependent protein translation is slowed down by stalling the ribosomes in the elongation step while IRES dependent translation of many cytokines and mitosis factors is up regulated (Sivan, Kedersha et al. 2007) (Pyronnet, Pradayrol et al. 2000). Due to the

differential regulation of these modes of translation, a potential correlation between MHC class I surface recovery and the cell cycle progression was examined. HeLa AS1 cells were transfected with Upf1 (red) or no (grey shadow) small interfering RNA and acid treated (Fig.24). Cells were stained at different time points during their recovery with antibody W6/32 (left panel). The cell permeable propidium iodine was added as DNA marker to determine the cell cycle stage (right panel). The DNA content is depicted as linear propidium iodine staining with the G<sub>1</sub> phase corresponding to 2 n, the G<sub>2</sub> phase with 4 n and S phase with an intermediate set of chromosomes between 2 and 4 n. The propidium iodine stained DNA content was determined for the total of recovering cells as well as for the 50 % cells with lower or respectively higher MHC class I surface expression. Cells with low levels of surface MHC class I were predominantly in the G<sub>1</sub> phase while cells with higher MHC class I expression progressed to a larger extent from G<sub>1</sub> through S to G<sub>2</sub>. This effect has been consistent in three independent experiments in HeLa cell as well as murine K89 cells. It is consistent with additional experiments using the DNA staining reagent Hoechst 33342 in fixed cells in order to measure cell cycle progression. No significant difference between Upf1 RNAi or untransfected cells could be detected in this experimental approach. Human Upf1 has been recently described to interact with DNA synthesis and DNA damage response during S-phase progression (Azzalin and Lingner 2006). Since this could not be confirmed for Upf2 or in non human cell lines it might be an effect depending rather on some Upf1 specific gene regulation than on NMD or pioneer round of translation in general. While the correlation of cell cycle stages and MHC class I surface expression might be interesting for further

experimentation it does not explain reduced antigen presentation upon pioneer round of translation inhibition.



**Fig.24 MHC class I surface recovery correlates with cell cycle progression**

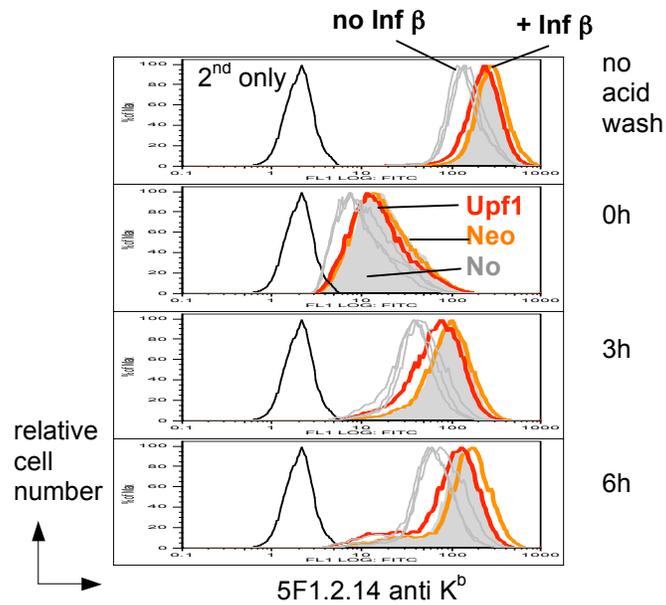
HeLa AS1 cells were transfected with Upf1 (red) or no RNAi (grey shadow), washed with citric acid, cultured for 0, 8 or 9 hours and analyzed with antibody W6/32 staining (left). Staining with propidium iodide for DNA content was used to determine cell cycle stage for the total of cells as well as the 50 % cells with lowest and highest MHC class I expression (right). Lower MHC class I correlates with predominantly G<sub>1</sub>, higher MHC class I with progression from G<sub>1</sub> through S to G<sub>2</sub>. No significantly different cell cycle stage could be detected for Upf1 or no RNAi transfected cells.

### 2.2.12 Effect of Interferon $\beta$ on MHC class I surface expression

The cellular response to viral infection and the recognition of foreign RNA often includes an induction of interferon type I as well as a subsequent upregulation of several immune

functions including MHC class I expression and antigen presentation. Transfection with small interfering RNA has been suspected by several authors to include off-target effects including unspecific gene regulation and activation of an interferon response (Tschaharganeh, Ehemann et al. 2007; Stewart, Li et al. 2008). Therefore especially RNAi strategies targeting immune functions under the control of interferon have to be well controlled demonstrating not only depletion of the target protein but also excluding any additional effects due to cellular RNA recognition. Transfection with different RNA sequences against the same factor, random sequence RNAi constructs, constructs targeting irrelevant neomycin phosphotransferase, synthetic RNA molecules or vector encoded small hairpin RNA as well as different transfection reagents have been included in previous experiments as lines of precaution.

Here the dependency of murine K<sup>b</sup> MHC class I surface recovery on pioneer round of translation factor Upf1 was examined in the presence of interferon  $\beta$  (Fig.25). HeLa Kovak/K<sup>b</sup> cells were transfected with no, Neo or Upf1 small interfering RNA and treated with citric acid for MHC class I surface recovery analysis. Cell culture medium included 500u/ml of human interferon  $\beta$ . During a fully activated interferon  $\beta$  response cells recovered as observed before with Upf1 depleted cells (red) showing a reduced MHC class I surface reappearance after acid wash. In comparison cells transfected with the different synthetic RNAi molecules but without the addition of interferon  $\beta$  generally expressed lower levels of MHC class I while recovering in the same pattern (thin gray lines). In conclusion interferon  $\beta$  stimulation does not change the requirement of pioneer translation for fast MHC class I surface expression.



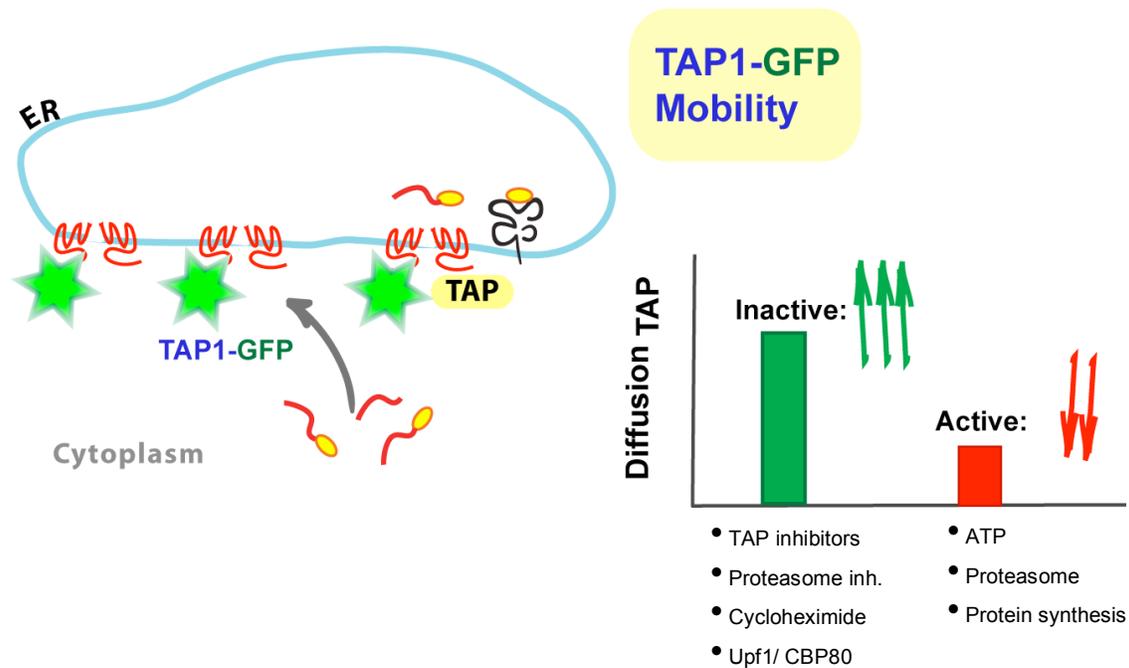
**Fig.25 Effect of interferon  $\beta$  on MHC class I expression.** Comparison of murine MHC class I K<sup>b</sup> surface expression in the presence or absence (thin grey lines) of 500u/ml of interferon  $\beta$  in HeLa Kovak/ K<sup>b</sup> cells transfected with no (grey shadow), Neo (orange) or Upf1 (red) RNAi in an acid wash recovery experiment. MHC class I levels are generally up regulated upon interferon  $\beta$  addition while slower MHC class I recovery in Upf1 depleted cells persists.

## **2.3 Direct assessment of peptide supply using Fluorescence**

### **Recovery after Photo Bleaching (FRAP)**

So far T-cell assays as well as antibody measurements of pMHC class I on the cell surface helped to reveal the relationship between factors of the NMD associated pioneer round of translation and MHC class I mediated antigen presentation. While translation and the generation of polypeptides are the very first events within the antigen presentation pathway, the final presentation at the cell surface represents the very end of the process. Here an additional technique is introduced in order to monitor the peptide transport across the ER membrane as an intermediate and essential step for efficient antigen presentation. TAP1 and TAP2 form a heterodimer upon recognition of suitable peptide targets for transport (see introduction “From cytosol to ER: peptide transport via TAP) (Androlewicz, Anderson et al. 1993). Next the assembly of the peptide loading complex including the TAP heterodimer, MHC class I, ERp57, calnexin, calreticulin, tapasin and further factors drastically increases the molecular size of the multiprotein complex (Peaper and Cresswell 2008). Indeed the radius of the complete TAP-PLC complex has been estimated to be ~600-1.000 Å (Marguet, Spiliotis et al. 1999). Formation of the TAP-PLC complex decreases the lateral mobility of TAP1 within the ER membrane. Eric Reitz and Jaque Neefjes were the first to take advantage of this change in mobility as a direct measure for ongoing peptide transport (Reits, Vos et al. 2000). Based on fluorescence recovery after photo bleaching (FRAP), a microscopy technique established already 30 years ago when fluorescent proteins became available,

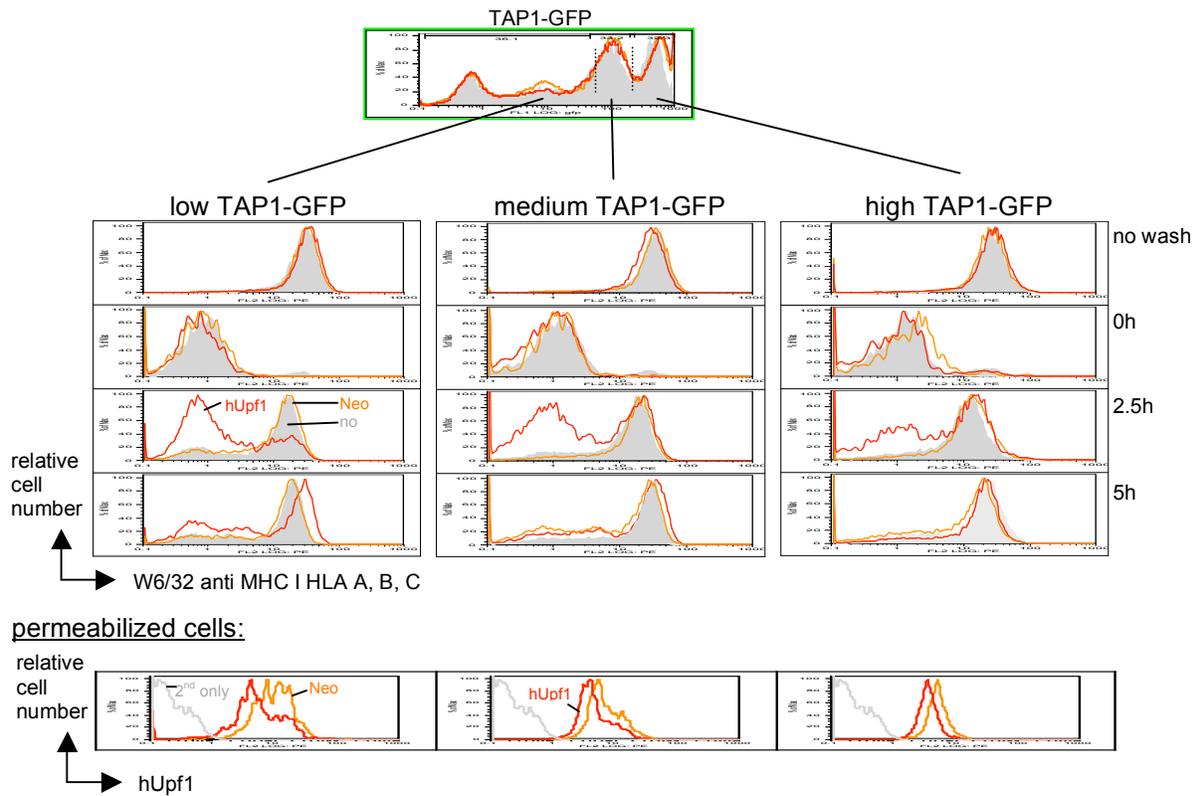
this method enables to monitor peptide transport in single living cells (Fig.26). In subsequent experiments the peptide supply was either reduced by using inhibitors targeting translation proteasomal degradation or peptide binding to TAP or allowing for optimal peptide generation under conditions of ongoing translation and proteasome function in the presence of ATP. The ultimate goal of these experiments was to determine the involvement of the pioneer round of translation associated with NMD in the generation of peptides for TAP transport.



**Fig.26 Lateral mobility of TAP directly depends on peptide supply.** The active transport and MHC class I binding of peptides leads to the formation of a TAP1 / TAP2 heterodimer as well as the assembly of a multi protein peptide loading complex (PLC). The increase in molecular weight and size of the protein complex reduces its lateral mobility within the ER membrane. This information is used for a direct assay of peptide transport to the ER in human MeJuSo cells by tracking the movement of an eGFP-TAP1 fusion protein with a fluorescence recovery after photo bleaching (FRAP) technique. Inhibitors of TAP, proteasome or translation increase TAP1-GFP mobility while conditions of active peptide generation decrease TAP1-GFP mobility.

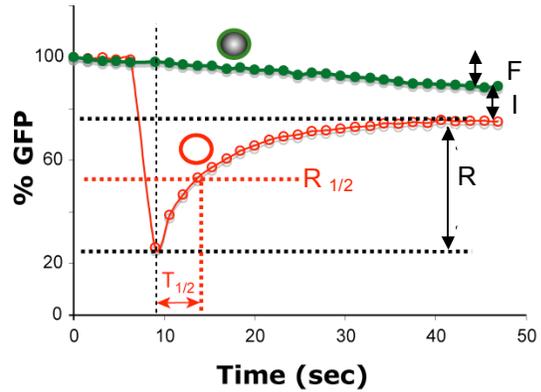
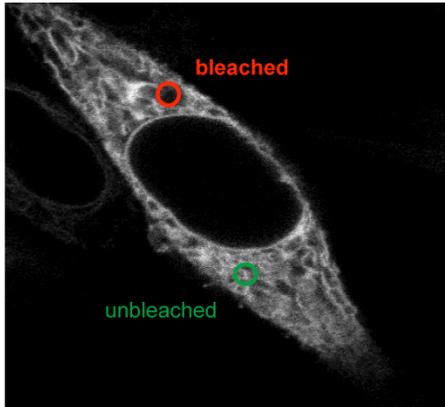
Human MelJuSo cells expressing a TAP1 protein with a C-terminal eGFP fusion were generously provided by the Neefjes laboratory. These cells have been described earlier as suitable targets for FRAP measurements (Reits, Vos et al. 2000). The correct assembly of the transfected TAP1-GFP with the endogenous TAP2 is essential for dimerization and peptide transport requiring an optimal ratio of about 1:1. Flow cytometric analysis of the TAP1-GFP expression revealed a multimodal pattern with subpopulations of low, medium and high TAP1-GFP expressing cells (Fig.27 top panel). Even after differential cell sorting of these subpopulations and sustained culturing of the cells the same multimodal pattern of TAP1-GFP expression was observed consistently. The MelJuSo cells were transiently transfected with siRNAi targeting either Upf1, neo or no RNA as described above. Initial TAP1-GFP as well as MHC class I expression was not effected by siRNA transfection. The cells were washed with citric acid for the removal of MHC class I surface complexes and placed under culture conditions for 2.5 or 5 hours prior to W6/32 antibody staining. Cells were analyzed for MHC class I surface expression as well as TAP1-GFP content. MelJuSo cells with depleted Upf1 protein demonstrated a delayed MHC class I surface recovery particularly after 2.5 hours. This effect was more pronounced for low than for high TAP1-GFP expressing cells. Only 29.5 % of low TAP1-GFP, but 45.4 % of medium and 57.4% of high TAP1-GFP expressing cells regained their full MHC class I surface level after 2.5 hours. This might be due to a generally higher protein biosynthesis in the subpopulation expressing high levels of TAP1-GFP. Therefore these cells might have a lower susceptibility for siRNA depletion. After 5 hours the majority of cells regained complete MHC class I surface expression independent of their TAP1-GFP expression or Upf1 depletion. Additionally cells were

permeabilized with Ethanol at  $-20^{\circ}\text{C}$  and stained for intracellular Upf1 expression (Fig. 27 bottom panel). This approach confirmed the down regulation of Upf1 protein expression upon siRNA transfection in low, medium and high TAP1-GFP expressing cells. In conclusion MelJuSo cells demonstrate Upf1 dependent MHC class I surface expression consistent with findings from experiments in HeLa cells. Cells with medium levels of TAP1-GFP expression were chosen for all following FRAP experiments since they combine both sufficient fluorescence for FRAP analysis as well as susceptibility for Upf1 dependent MHC expression. As recommended by Reits et al. they might also support a more efficient formation of the TAP heterodimer due to a more balanced ratio of TAP1-GFP to endogenous TAP2 proteins (Reits, Vos et al. 2000).



**Fig.27 TAP1-GFP expressing MelJuSo cells demonstrate reduced MHC class I surface recovery after RNAi mediated Upf1 depletion.** MelJuSo cells show a different intensity of TAP1-GFP expression (top panel). Cells with low, medium and high TAP1-GFP expression were monitored for MHC class I surface expression in an acid wash recovery time course experiment before treatment and at 0, 2.5 and 5h. Cells demonstrated delayed MHC class expression after 2.5 h after Upf1 (red) depletion (middle panels, neo orange, no RNAi grey shadow). Down regulation of Upf1 protein following RNAi transfection was demonstrated by detection of Upf1 protein levels in permeabilized cells with anti Upf1 antibody (bottom panel).

MelJuSo TAP1-GFP cells show a typical staining of the ER and nuclear envelope (Fig.28). For FRAP measurements a region  $\omega$  with a radius of 84 was monitored for 5 time intervals of 1.6 seconds, fluorophores were bleached with a pulse at 100% laser intensity of a 488 nm Argon laser and monitored for fluorescence reassembly through lateral TAP1-GFP motion for another 32 seconds (ROI red). A general fading of about 3-5 % of the initial fluorescence due to the detection laser was observed (ROI green). As depicted in the graph, an immobile fraction (I) as well as an actively recovering mobile fraction (R) can be distinguished. Excluding microdomains, alterations of membrane shape and other obstacles the movement within the membrane can be described as planar Brownian motion. Different mathematical models have been used to generate a diffusion constant  $D_{FRAP}$ . Here the equation for unrestricted two dimensional diffusion in a circular area without any recovery from outside the focal plane has been used (Axelrod, Koppel et al. 1976; Reits and Neefjes 2001; Chen, Lagerholm et al. 2006). This strategy has been successfully applied to determine diffusion constants for other membrane bound components including GFP-tagged MHC class I L<sup>d</sup> molecules (Marguet, Spiliotis et al. 1999).



$$\text{Diffusion}_{\text{TAP1}} = \frac{\omega^2 \times \gamma}{4T_{1/2}}$$

$\omega$ : radius of bleached region  
 $\gamma$ : correction factor  
 $T_{1/2}$ : half time of recovery diffusion  
 $D_{\text{FRAP}}$ : diffusion coefficient for lateral motion

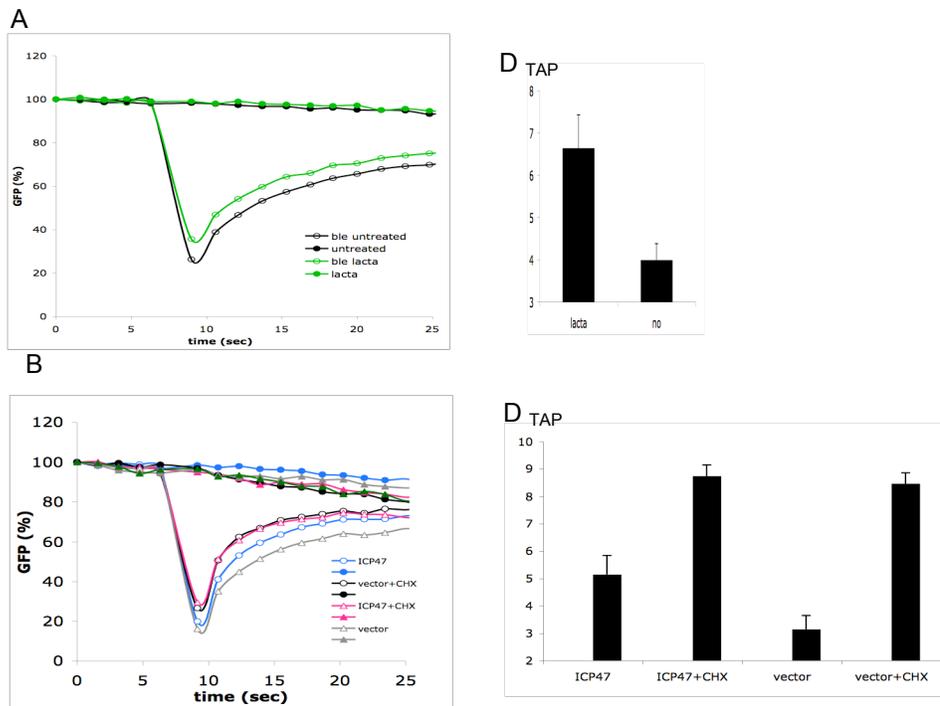
R: recovery rate, mobile fraction  
 I: immobile fraction,  
 F: fading of fluorescence

**Fig.28 Measuring Peptide transport with Fluorescence Recovery after Photo-Bleaching (FRAP).** TAP1-GFP was expressed in MelJuSO cells. A circular region was bleached at 100% laser intensity with an argon / krypton 488 nm laser and fluorescence recovery through lateral movement of TAP1-GFP was monitored at 1.6 second time intervals. A region on the opposing side of the cell was chosen as a reference for fluorescence due to fading. The lateral mobility of TAP1-GFP is calculated as  $D_{\text{FRAP}}$  Diffusion coefficient.

### 2.3.1 Inhibition of peptide supply increases TAP1-GFP membrane mobility

Reits et al. postulated a direct correlation between cytosolic peptide supply and lateral TAP1-GFP mobility in the membrane (Reits, Vos et al. 2000). In order to establish an experimental set up to measure the transport of antigenic peptide, several of their experiments were recapitulated. First TAP1-GFP expressing MelJuSo cells grown on a

microscopy cover slip were incubated for 30 minutes at 37°C with medium containing 200 µg/ml of the proteasome inhibitor lactacystin or medium alone. 10 cells of each condition expressing intermediate TAP1-GFP fluorescence were selected for FRAP analysis. The combined data for the cells with (green) or without lactacystin (black) are depicted in Fig. 29A. Incubation with the proteasomal inhibitor led to an accelerated fluorescence recovery indicating a higher lateral mobility of the TAP1-GFP molecule in the ER membrane. The TAP diffusion coefficient  $D_{TAP}$  was determined as  $6.63 \times 10^{-10}$  ( $\text{cm}^2 \text{s}^{-1}$ ) for lactacystin treated cells and as comparison to  $3.98 \times 10^{-10}$  ( $\text{cm}^2 \text{s}^{-1}$ ) for control cells. The effect of translational inhibition on TAP1-GFP mobility was tested using cycloheximide as an inhibitor of protein biosynthesis. Cells were incubated for 30 minutes with cycloheximide at a concentration of 100 µg/ml (Fig.29B). A further approach included the direct interference with the TAP1/TAP2 heterodimer to interact with peptide for transport (Fig.29B). The Herpes simplex virus (HSV) derived immediate early protein ICP47 forms a stable complex between its N-terminal residues and the TAP1/TAP2 heterodimer therefore abolishing further peptide transport (Galocha, Hill et al. 1997). Cells treated with cycloheximide and control vector or jointly together with ICP47 encoding vector demonstrated a high  $D_{TAP}$  of  $8.47 \times 10^{-10}$  ( $\text{cm}^2 \text{s}^{-1}$ ) and  $8.75 \times 10^{-10}$  ( $\text{cm}^2 \text{s}^{-1}$ ) respectively, indicating the abrogation of peptide transport. In comparison ICP47 vector alone had a  $D_{TAP}$  of  $5.15 \times 10^{-10}$  ( $\text{cm}^2 \text{s}^{-1}$ ) demonstrating a intermediate increase in TAP mobility in comparison to control transfected cells with a  $D_{TAP}$  of  $3.15 \times 10^{-10}$  ( $\text{cm}^2 \text{s}^{-1}$ ). Taken together inhibition of translation, proteasomal degradation and peptide transport all reduce lateral TAP mobility. This establishes TAP mobility as a good measure for peptide supply.

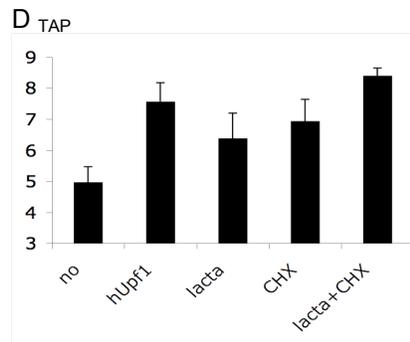
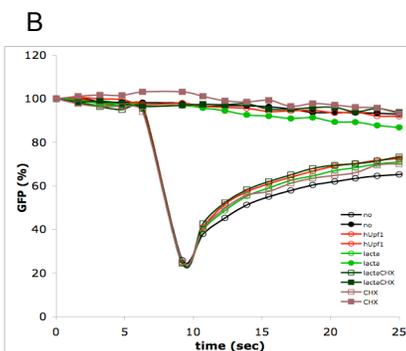
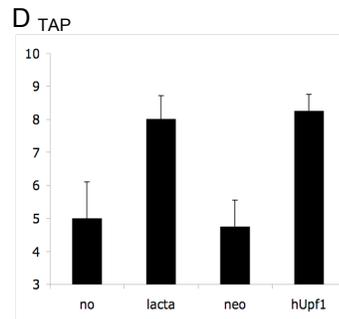
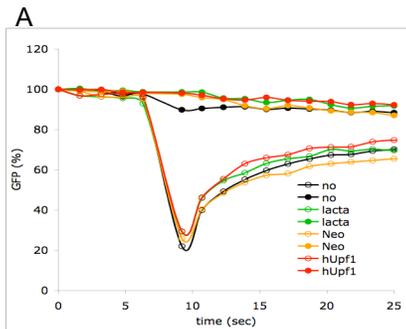


**Fig.29 Inhibition of peptide supply increases TAP1-GFP membrane mobility.** Protein degradation was inhibited by addition of 200  $\mu\text{g/ml}$  proteasome inhibitor lactacystin (green) leading to accelerated fluorescence recovery with a  $D_{\text{TAP}}$  of 6.63 in comparison to  $3.98 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$  for contral cells (A). Alternatively protein biosynthesis was inhibited with cycloheximide at a concentration of 100  $\mu\text{g/ml}$  or TAP transport was reduced by vector transfection of the herpes simplex derived ICP47 (B). Both cycloheximide and ICP47 treated cells demonstrate a  $D_{\text{TAP}}$  increase in comparison to control transfected cells indicating reduced peptide transport trough TAP.

### 2.3.2 Upf1 depletion increases TAP1 mobility

It was shown in previous experiments that siRNA induced depletion of Upf1 delays the recovery of surface MHC class I expression. Here we examined if Upf1 depletion also reduces the supply of peptides as measured by TAP1-GFP FRAP. MeJuSo cells were transfected with siRNA targeting Upf1 (red), neo (orange) or no RNA (black) (Fig.30A). As a control cells were incubated for 30 minutes with lactacystin (green). Upf1 siRNA

transfected and lactacystin incubated cells both had a comparatively high  $D_{TAP}$  of  $8.26 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$  and  $8.02 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$ . The  $D_{TAP}$  of Neo siRNA or control cells remained similarly low at  $4.75 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$  and  $5.01 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$ . The difference in TAP mobility between Upf1 siRNA and untransfected cells is also reflected in Fig 30B. Treatment with lactacystin, cycloheximide or both chemical inhibitors together demonstrated an increasing  $D_{TAP}$  value of  $6.39 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$ ,  $6.94 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$  and  $8.40 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$ . This implies a potentially synergetic effect of the inhibitors in decreasing peptide supply. In conclusion Upf1 depletion reduces peptide supply available for TAP mediated transport to a comparable extend as chemical inhibitors of translation or proteasomal degradation.



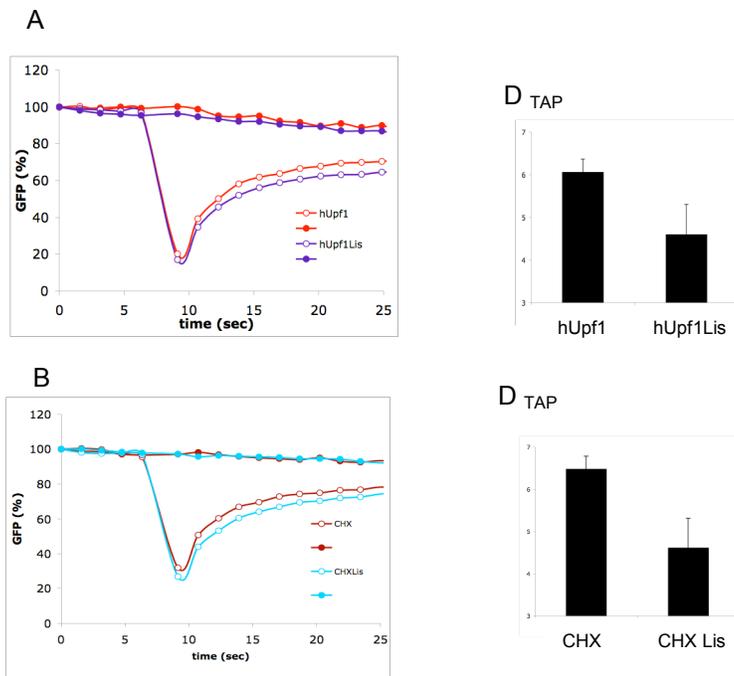
**Fig.30 hUpf1 depletion increases TAP mobility.** MelJuSo cells were transfected with siRNA targeting hUpf1 (red), neo (orange) or no RNA (black). Additionally cells were treated with lactacystin (green) for proteasomal inhibition (A). TAP mobility was increased for both lactacystin and hUpf1 RNAi. In (B) cells were again transfected with hUpf1 (red) or no (black) siRNA or chemical inhibitors lactacystin (green), cycloheximide (brown) or both (dark green). Lactacystin and cycloheximide showed a cumulative effect of  $D_{TAP}$  increase.

### 2.3.3 Exogenous peptide supply partially reverses high TAP mobility

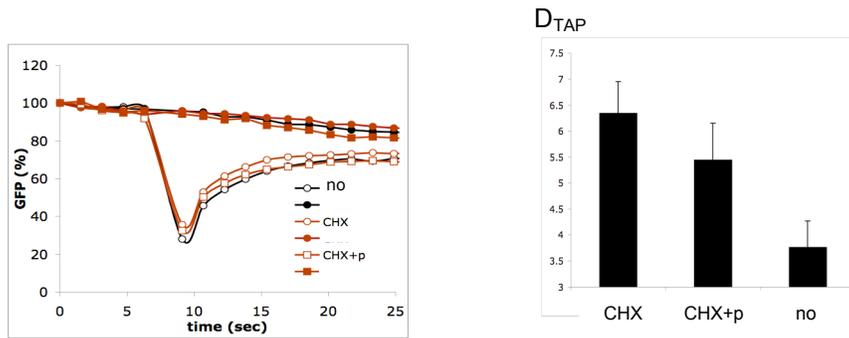
The chemical inhibitors cycloheximidine and lactacystin as well as siRNA targeting Upf1 increase TAP mobility. In order to verify that the higher TAP mobility is directly due to a lack of peptides for TAP association and transport, peptides of exogenous origin were supplied to the cells. *Listeria monocytogenes* infection was used as way to deliver peptides to the cytosol of the cells. The *Listeria monocytogenes* strain (acta<sup>-</sup>, LLO-kovak) expresses a LLO-kovak fusion protein in addition to its endogeneous genes. Further it should be noted that prokaryotic translation is not receptive to cycloheximide inhibition due to different ribosomal subunits. For the infection, cells were incubated for 1 hour with a MOI of 1:20. MelJuSo cells were transiently transfected with siRNA against Upf1 prior to addition of *Listeria monocytogenes* (Fig.31A). *Listeria* infection reduced the TAP mobility of hUpf1 depleted cells from  $6.06 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$  to  $4.60 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$ . *Listeria monocytogenes* induced a similar reduction in TAP mobility when added to cycloheximide treated cells bringing the  $D_{TAP}$  from  $6.49 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$  down to  $4.62 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$ .

Alternatively cycloheximide treated cells were incubated with a synthetic peptide in the culture medium at a concentration of 50  $\mu\text{M}$  (light blue) leading to a decrease in TAP

mobility (Fig.32). TAP mobility demonstrates a modest drop from  $6.35 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$  to  $5.45 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$  upon peptide addition. Control cells had a  $D_{\text{TAP}}$  of  $3.77 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1})$ . Taken together this indicates that uptake of exogenous peptide from the culture medium or through infection from *Listeria monocytogenes* can reverse a high TAP mobility due to a drained peptide supply.

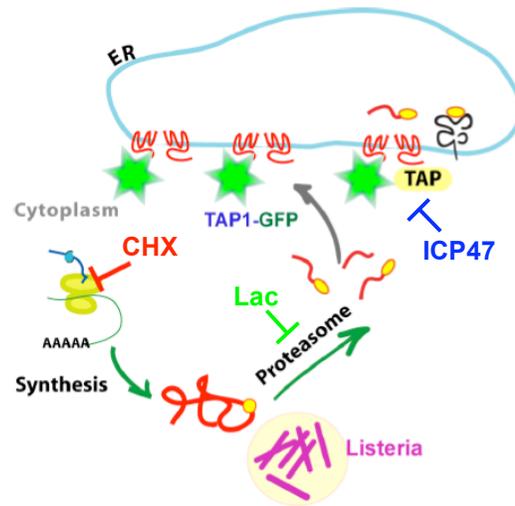


**Fig.31 *Listeria monocytogenes* peptide partially reverses high TAP mobility.** MelJuSo cells were transiently transfected with hUpf1 (red) and infected with a kovak expressing *Listeria monocytogenes* (purple) strain for 1 hour at a MOI of 1:20. Infection and therefore exogenous peptide supply led to reduced TAP mobility. Similarly addition of *Listeria monocytogenes* (light blue) reduced the  $D_{\text{TAP}}$  of cycloheximide (brown) treated cells (B).



**Fig.32 Exogenous peptide supply partially reverses high TAP mobility.** Synthetic peptide was added to the culture medium at a concentration of  $50\mu\text{M}$  together with the translation inhibitor cycloheximide 1 hour prior to FRAP analysis. TAP mobility demonstrates a modest drop from  $6.35 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$  to  $5.45 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$  upon peptide addition. Control cells had a  $D_{TAP}$  of  $3.77 \times 10^{-10} \text{ (cm}^2 \text{ s}^{-1}\text{)}$ .

In conclusion it was demonstrated that lateral mobility of the TAP1-GFP fusion protein in MelJuSO cells can be used to measure the active transport of peptide from the cytosol to the ER (Fig.34). The pool of peptides for TAP dependent transport depends on ongoing translation and proteasomal degradation as demonstrated with the chemical inhibitors cycloheximide and lactacystin. Depletion of hUpf1 and therefore inhibition of the pioneer round of translation reduces the amount of peptides available for TAP mediated transport and therefore increases  $D_{TAP}$ . A higher TAP mobility is the direct result of a diminished pool of peptides for TAP interaction and transport as it can be reversed by additional supply of exogenous peptide sources.



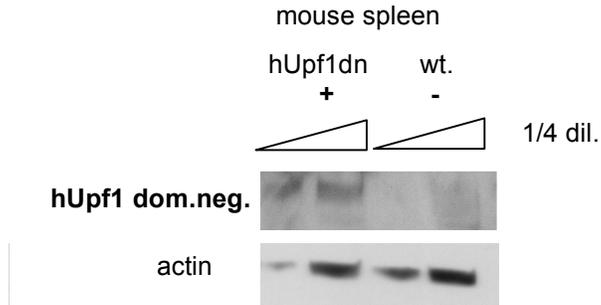
**Fig.33 Generation of peptides for TAP mediated transport.** The generation of peptides for TAP transport from the cytosol to the ER requires ongoing translation and proteasomal degradation as inhibition with cycloheximide and lactacystin reduces TAP transport. Depletion of hUpf1 reduces TAP transport. Supply of exogenous peptide through *Listeria monocytogenes* or high concentrations of synthetic peptide can reverse this effect. The HSV derived protein ICP47 can bind to TAP inhibiting further transport.

## 2.4 Antigen presentation in the hUpf1 transgenic mouse

Previous chapters focused primarily on analyzing the relationship between antigen presentation and the pioneer round of translation of the NMD pathway in cultured cells. Approaches using RNA interference can be limited by transfection efficiencies, incomplete or only transient down regulation of target proteins or RNA mediated off target effects. Particularly when focusing on the immune system certain limitations of cell lines were apparent. Immunization studies in mice would represent the par excellence standard to provide evidence for pioneer peptides as source for antigen presentation and in vivo T-cell recognition. So far the attempt to generate a mouse line depleted for the Upf1 gene was not successful as loss of Upf1 expression was embryonic lethal at the blastocyst stage after only 3.5 days (Medghalchi, Frischmeyer et al. 2001). This finding not only speaks to the importance of NMD at the organismal level but also blocks the road to the generation of Upf1 knock out embryonic stem cells. Dietz et al. tried to create a Cre-loxP based Upf1 conditional knockout mouse. Analysis of the resulting mice revealed only an incomplete depletion of the Upf1 gene (Dietz 2007).

Therefore the potential of a transgenic Upf1 dominant negative mouse in altering NMD and the pioneer round of translation was evaluated. The human Upf1 dominant negative construct was described before in competing with the wildtype protein and reversing NMD facilitated mRNA downregulation (Sun, Perlick et al. 1998). Here the human Upf1 R844C construct was used for the creating of a transgenic mouse by H. Dietz and colleagues and kindly provided to us. Verification of the successful integration of the hUpf1 R844C transgene was initially achieved by Southern blotting. Then a PCR based

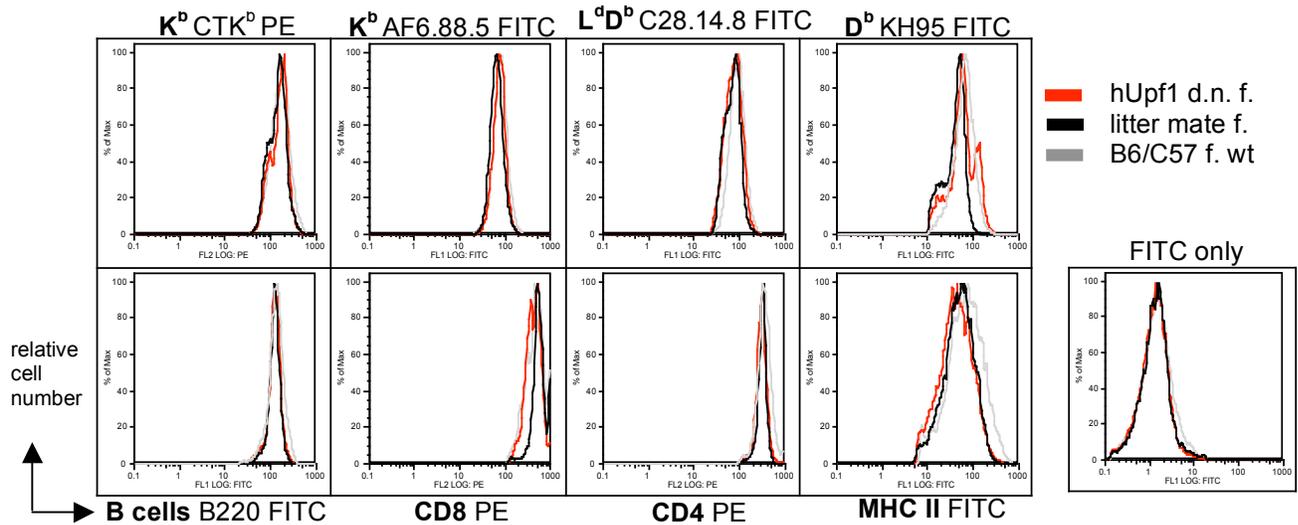




**Fig.35 Expression of the hUpf1 dominant negative protein.** Spleenocytes were isolated, homogenized and separated on a 4-20 % SDS PAGE gradient gel with a 1:4 dilution. Immunoblotting with a mouse polyclonal antibody recognizing only the human protein was used to very expression of the 137 kDa dominant negative Upf1 protein.

#### 2.4.1 Analysis of spleenocytes from hUpf1 dominant negative transgenic mice

Spleenocytes were isolated from hUpf1 dominant negative transgenic or wildtype mice (Fig.36). After blocking of the Fc-receptors, cells were stained for surface MHC class I expression using antibodies CTK<sup>b</sup> α K<sup>b</sup>, AF6.88.5 α K<sup>b</sup>, C28.14.8 α D<sup>b</sup> and KH95 α D<sup>b</sup>. Additionally cells were characterized for the surface markers B220 for B-lymphocytes, CD8, CD4 and MHC class II. After testing of several mice no apparent differences were noted in the expression of any of these surface markers in hUpf1 dominant negative transgenic or wildtype mice.



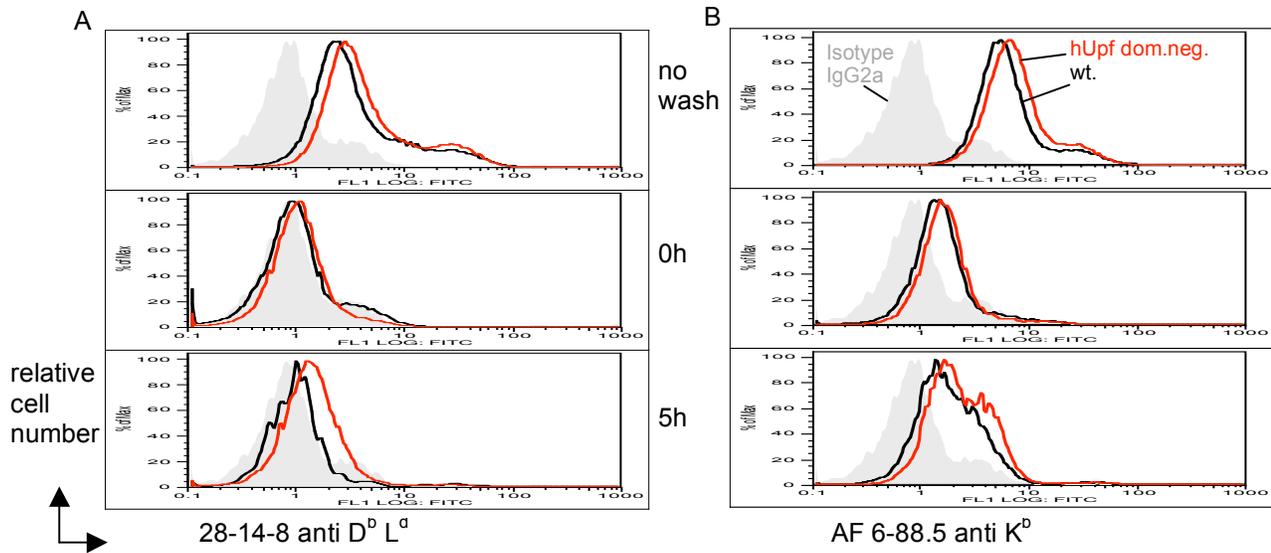
**Fig.36 Analysis of splenocytes derived from hUpf1 dominant negative transgenic mice.** Splenocytes from each a transgenic hUpf1 d.n., a litter mate and a B6/C57 wt. 4 month female mouse were extracted, Fc-receptor blocked and stained for MHC class I  $K^b$  and  $D^b$  expression (top row) and B220, CD8, CD4 and MHC class II (bottom row). Surface expression of these markers did not reveal significant differences.

#### 2.4.2 MHC class I surface recovery of hUpf1 dominant negative bone marrow derived dendritic cells

The steady state cell surface expression of MHC class I molecules often does not appropriately reflect the actual generation of MHC class I molecules and their peptide ligands within the cell. MHC class I complexes can be stable over longer times in association with their bound peptide. As for CBP80 or Upf1 RNAi depleted HeLa cells, the differences in peptide generation only became obvious when monitoring the assembly and presentation of new MHC class I peptide complexes using the acid wash and recovery approach described in chapter 2.2. Similarly the large differences in the peptide

repertoire of ERAAP deficient cells in comparison to wildtype antigen presenting cells did not become obvious by steady state detection of MHC class I surface expression but was discovered by rather detailed analysis of specific antigenic peptides by immunization studies (Hammer, Gonzalez et al. 2006).

Therefore bone marrow derived dendritic cells were generated for acid wash MHC class I surface recovery experiments (Fig.37). The total cells from transgenic and wildtype mice were extracted from the murine femur and cultured for 5 days in the presence of GM-CSF. Non-adhesive cells were treated with citric acid for 1 minute and placed under cell culture conditions for MHC class I recovery. For flow cytometry analysis Fc-receptors were blocked and MHC class I were stained with antibody 28.14.8 anti D<sup>b</sup>L<sup>d</sup> and AF 6-88.5 anti K<sup>b</sup> or with a IgG2a isotope control. For both antibodies hUpf1 dominant negative transgenic splenocytes showed a slightly higher expression than the wildtype cells. This remained true throughout the experiments at 0 hours directly after the acid treatment as well as after 5 hours of recovery. The recovery was incomplete after 5 hours as the cells take a longer time recovery to the original level. While primary cells derived from bone marrow represent a more heterogeneous cell population than cancer cell lines in tissue culture, the hUpf1 transgenic cells showed in multiple experiments a tendency to recover their MHC class I molecules faster than their wildtype counterparts. While this finding was unexpected at the beginning it might reflect a difference in the experimental approach between RNAi mediated depletion as described in previous chapters and dominant negative protein overexpression of Upf1. This will be further discussed below in section 3.4.

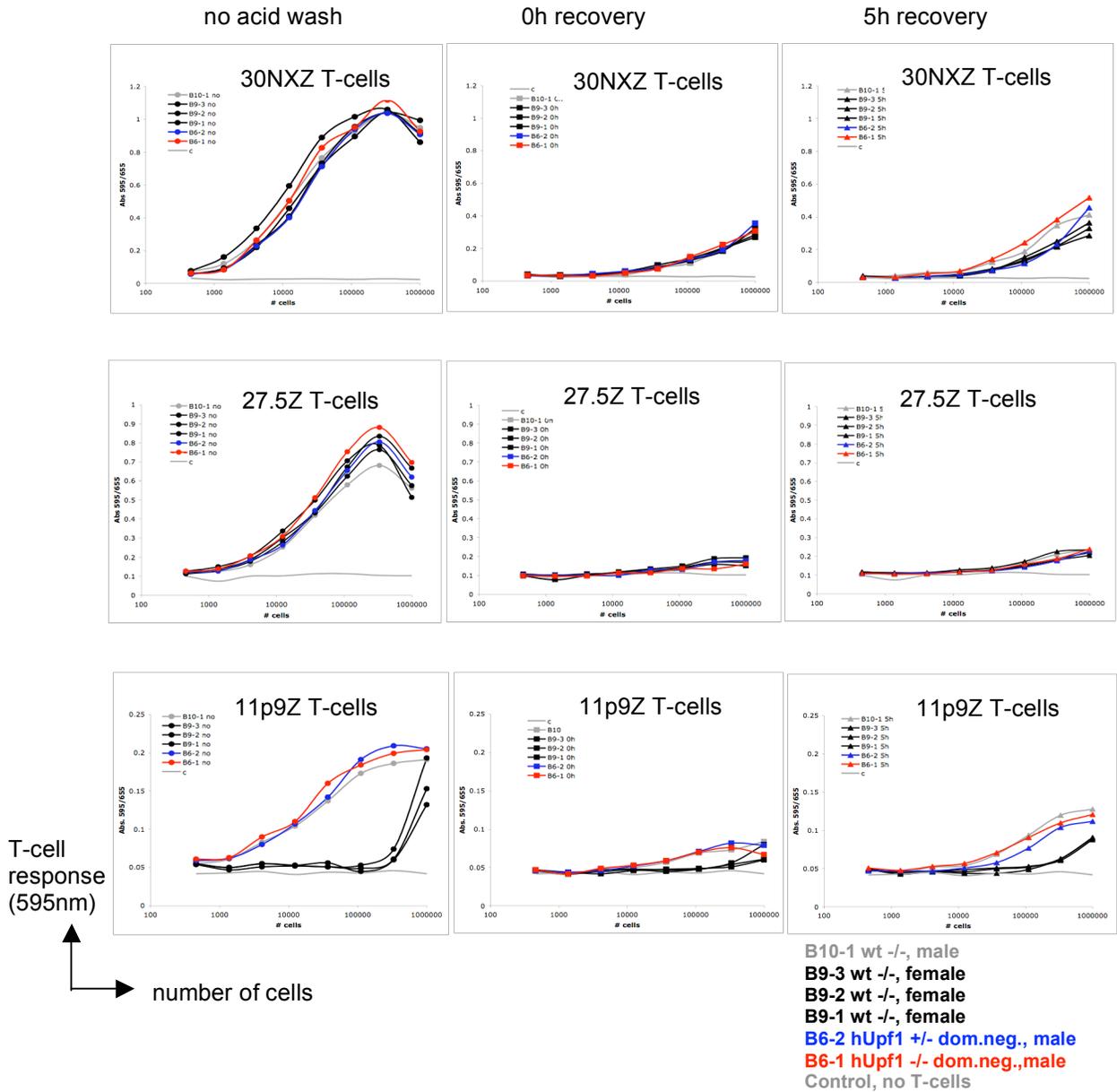


**Fig.37 MHC class I surface recovery of hUpf1 dominant negative bone marrow derived dendritic cells.** Bone marrow cells were extracted from femur bones of a transgenic hUpf1 dominant negative mouse (red) or a wildtype littermate (black). After 5 days of GM-CSF culture non-adhesive cells were harvested, red blood cells lysed and surface MHC class I molecules removed using the acetic acid wash procedure. Fc-receptors were blocked with CD 16-32 and cells were stained for D<sup>b</sup> and L<sup>d</sup> (A) or with a IgG2a isotope control (grey shadow). hUpf1 dominant negative transgenic cells show slightly higher MHC class I levels in comparison to wt cells.

### 2.4.3 Analysis of T-cell activation of hUpf1 dominant negative cells

Flow cytometry based staining of MHC class I molecules gives a general picture of different MHC class I subgroups. T-cell activation represents a far more specific and sensitive assay but also takes the actual potential of MHC class I peptide complexes in triggering a T-cell mediated immune response into account. In the next approach six mice were analyzed for MHC class I antigen binding (Fig.37) and also for T-cell activation assays (Fig.38). The acid wash recovery time course was described above. Then  $1 \times 10^6$  murine antigen presenting dendritic cells from each time point were titrated in a 1:3

dilution series and incubated overnight with  $10^5$  T-cells in the presence of  $5 \mu\text{g/ml}$  brefeldin A. Afterwards a CPRG assay was performed. 30NXZ T-cells recognize the  $D^b$  restricted peptide SSVVGVWYL (SVL9) derived from the H13a histocompatibility gene (Hammer, Gonzalez et al. 2006). The 30NXZ T-cell responses are initially high before acid treatment at an absorbance between 1.0 and 1.1. Citric acid is efficient in removing SVL9 peptide from the cell surface as the 30NXZ T-cell response goes down to 0.3 at 0 hours. After 5 hours an increased 30NXZ T-cell response was detected. This was particularly evident for the male hUpf1 dominant negative transgenic mouse shown by the red line. The 27.5Z T-cells recognize a  $K^b$  restricted peptide of unknown sequence. In comparison to 30NXZ T-cells their activation of a T-cell response remains very low after 5 hours with only a very small increase between 0 hours and 5 hours. The response does not allow for a clear distinction of intensities of transgenic or wildtype cells. Finally 11p9Z T-cells are activated through the  $D^b$  restricted antigen WMHHNMDLI (WI9) derived from the Y-chromosome encoded Uty gene (Greenfield, Scott et al. 1996). Accordingly only cells from male mice present this  $D^b$  restricted peptide. Wells with a high density of  $1 \times 10^6$  cells show an nonspecific response. Within the male mice there are no significant differences in the recovery. Taken together there are no obvious indicators for an altered MHC class I recovery pattern as detected by the T-cell lines 30NXZ, 27.5 and 11p9Z. This reflects also additional experiments performed in repeat of this analysis. No clear effect of the hUpf1 dominant negative protein expression on T-cell activation reflects possibly mainly on this transgenic mouse being an insufficient experimental system as it will be reflected in further detail in the discussion.



**Fig.38 T-cell activation of transgenic hUpf1 dominant negative mouse bone marrow derived dendritic cells.** Bone marrow derived dendritic cells were cultured as described in Fig.37.  $1 \times 10^6$  cells were diluted 1:3 in 96-well plates and incubated with  $1 \times 10^5$  cells of three different hybridoma T-cell lines: 30NXZ D<sup>b</sup> SVL9 H13a, 27.5Z K<sup>b</sup> and 11p9Z Db WI9 Uty. T-cell activity of antigen presenting cells was assessed by CPRG assay.

### **3 Discussion**

Previous chapters examined several aspects of the relationship between the pioneer round of translation and MHC class I mediated antigen presentation. Experimental findings were presented for intron positioning (2.1), pioneer vs. cap-dependent translation (2.2), TAP mobility as a measure for peptide supply (2.3) and a transgenic mouse model. Here the technical approach, the validity of each of the experimental results and the implications for the MHC class I presentation pathway are discussed. Further these findings are put into a context for a general model of pioneer round of translation peptides as source for antigen presentation. Finally an outlook defines yet to be answered questions that open future perspectives.

#### **3.1 Pioneer peptides itself rather than the NMD mechanism are important for antigen presentation**

The first set of experiments (2.1) aimed to clarify if NMD alters the translation and presentation of antigenic peptides. A first hypothesis focused on mRNAs encoding an antigenic peptide as potential target for NMD. Two sets of three DNA constructs were created based on either the Invariant chain / and SIINFEHL or the ovalbumine derived kovak sequence including SIINFEHL. An intron was shuffled to the 3' or 5' position or completely deleted.

The intron at the 5' UTR was mainly introduced in order to create a counterpart of the 3' construct with the exact same size of the vector to avoid potential changes in transfection efficiency. At the same time alterations in the 5' UTR might introduce a multitude of alternative initiation codons, sequences functioning as IRES, or with other cryptic or translation modifying elements that are difficult to account for (Komarova, Brocard et al. 2006; Graber and Holcik 2007). Therefore the central focus of these experiments lies on the comparison between the 3' and no intron constructs. This parallels the approach of many studies that helped to discover and examine the NMD pathway.

A sharp contrast of the mRNA levels between the 3' and no intron constructs was observed while there were only minor differences in antigen presentation. Since both 3' and no intron constructs elicit a strong B3Z T-cell response in a direct presentation assay as shown in Fig.3, two additional approaches were chosen. DNA titration of the vectors (Fig.4) as well as extraction of the peptides and presentation by K89 cells (Fig.5) helped to quantify the B3Z T-cell response. While the no intron construct accounts for a slightly stronger T-cell activation than the 3'intron construct, this does not reflect the magnitude of difference in the mRNA levels as determined by RT-PCR. Since this pattern was detected for both the invariant chain / SIINFEHL and the kovak vector set these findings seem to outline a more general rule.

At the same time two similar NMD-reporter vectors were published that included the advantage of eGFP expression for straightforward analysis of protein levels and decay (Paillusson, Hirschi et al. 2005). They were modified for the purpose of antigen

presentation assays by introducing the antigenic SIINFEHL sequence to the C-terminus of the protein. As already described by the authors in their publication the vectors achieved only modest transfection efficiencies and GFP expression levels. Nevertheless the system confirmed the 3' intron construct to be a target of NMD by showing lower GFP expression than the no intron construct (Fig.7). Despite the different GFP levels the B3Z T-cell activation of both constructs remained almost identical. This strengthened the prior results based on the invariant chain / SIINFEHL and kovak constructs. The mRNA and protein level and therefore NMD activity does not directly account for the antigen presentation intensity.

A recent study presented improved NMD-reporter constructs using a luciferase based system (Boelz, Neu-Yilik et al. 2006). This more robust NMD detection assay jointly with the described SIINFEHL insertion for B3Z recognition is currently under investigation.

A crucial finding for the direction of this study was the discovery of reduced antigenicity upon Upf1 and CBP80 depletion. HeLa K<sup>b</sup> cells depleted for these essential factors of the NMD pathway and the pioneer round of translation reduced the response of B3Z T-cells independent of the intron position. For a mere NMD effect the 3'intron would be expected to increase in expression and peptide presentation. A plethora of this kind of examples can be found in the literature (Zhang, Sun et al. 1998; Sun and Maquat 2000). But in this case quite the opposite was observed. The lack of Upf1 and even more so CBP80 reduced the generation and presentation of peptides for antigen presentation.

In conclusion these findings not only demonstrate that there is no straight forward relationship between the extent of protein expression and antigen presentation, but point out the potential role of the pioneer round of translation itself in generating antigens for MHC class I mediated presentation.

### **3.2 Time course experiments reveal the role of the pioneer round of translation for antigen presentation**

The results of the previous intron shuffling experiments altered the focus of this study. They indicated a direct role of the pioneer round of translation rather than the decay mechanism of NMD in antigen presentation. In section 2.2 first an experimental approach to distinguish between pioneer and bulk translation was developed. CBP80 and Upf1 were identified as key components that built a stable complex exclusive for the pioneer round of translation (Hosoda, Kim et al. 2005). Transient siRNA mediated depletion was chosen as a method that depleted the target protein levels to ~20% as shown by immunoblotting while it did not effect viability of the cells during the course of the experiments (Fig.11). S<sup>35</sup> methionin and cysteine incorporation assays demonstrated no reduction of protein biosynthesis in Upf1 or CBP80 depleted cells. Quite the opposite Upf1 inhibition rather increased the overall translation of about 15 %. As mentioned this finding is consistent with prior reports of Upf1 being involved in the upregulation of at least 197 genes by the factor of 1.9 or more (Mendell, Sharifi et al. 2004; Chan, Huang et al. 2007). Apart from that it should be noted that Upf1 might be involved in other

nonsense associated mechanisms. Target mRNAs of these pathways might be upregulated upon Upf1 reduction accounting for the 15% increase in translation. Generally the use of S<sup>35</sup> incorporation includes the advantage over alternative antibody based techniques, that it enables the detection of any kind of polypeptide independent of functionality, conformation or truncation with very high sensitivity. In addition, this experiment served the purpose to verify the complete inhibition of translation through cycloheximide treatment at a concentration of 100 µg/ml for 1 hour at 37°C. These conditions were used in several subsequent experiments.

Inhibition of cap dependent bulk translation by 4E-BP1 vector DNA and eIF4E siRNA transfection represented two independent approaches. Despite the far larger molecular weight and potentially lower transfection efficiency of the ~5600 base pair 4E-BP1 DNA construct, it demonstrated a stronger inhibition of translation than the 21 base pair eIF4E siRNA molecule. These data are in good correlation with previously published studies by other groups (Gingras, Gygi et al. 1999).

In conclusion these results confirmed the validity of the experimental design to separately interfere with pioneer or bulk translation and to jointly inhibit overall translation with cycloheximide.

The role of pioneer vs. bulk translation in antigen presentation was then put to the test in flow cytometry experiments using antibody staining against different MHC class I molecules. Monitoring the recovery of MHC class I molecules rather than the steady state surface expression was chosen as an experimental approach due to three earlier observations. First the MHC class I steady state expression of ERAAP deficient mice

revealed only minor changes on the surface while more elaborate assays elucidated significant differences in the antigen repertoire (Hammer, Gonzalez et al. 2006). Secondly the importance of new synthesis of peptides has already been described in the context of DRiPs as crucial for antigen presentation (Qian, Reits et al. 2006). And third the transient siRNA depletion might only effect newly generated peptides that were not assembled prior to the transfection.

While immunoblotting confirmed the down regulation of Upf1, PABP2, CBP80 and CBP20 in a pooled cell homogenate, flow cytometry based detection in permeabilized cells helped to confirm the depletion on a per cell basis (Fig.14D). Acid wash recovery experiments revealed a significant delay of MHC class I surface recovery in Upf1, PABP2 and CBP80 depleted cells (Fig.14, Fig.15, Fig.16). Initially HeLa cells stably expressing kovak and K<sup>b</sup> were used in these acid wash recovery studies since they allow for the detection of human MHC class I HLA A, B, C as well as murine K<sup>b</sup> molecules (Fig.14). It was later noticed that expression of the murine K<sup>b</sup> molecule in human HeLa cells competes and consistently reduces the surface level of human MHC class I expression (data not shown). A subclone of HeLa cells lacking the additional K<sup>b</sup> molecule (termed AS1) revealed an even larger delay in the recovery pattern of Upf1 and CBP80 deficient and control transfected cells (Fig.16).

Antibody staining in conjunction with flow cytometry offers the opportunity to monitor overall changes in MHC class I molecules. T-cell activation assays enable to track the MHC class I mediated presentation of defined peptide sequences with exceptionally high

sensitivity. Three different T-cell lines B3Z, 18.5Z and 27.5Z were used that recognize either the stably transfected kovak derived SIINFEHL or two unknown human endogenous peptide sequences. Depletion of CBP80 reduced the T-cell response of recovering APCs in all cases (Fig.18). Also siRNA mediated inhibition of CBP20 reduced the T-cell response to an intermediate level. CBP80 and CBP20 are equally important for the pioneer round of translation since they form a heterodimer that recognizes the newly spliced mRNA and facilitates the pioneer translation. Differences in the intensity of the T-cell response between CBP80 and CBP20 might be simply due to variations in the effectiveness of the siRNA in down regulating its target protein. The case might be different for Upf1 inhibition as it demonstrated a reduction in the T-cell response of 18.5Z and 27.5Z T-cells but no distinguishable effect on the B3Z response (Fig.17 and Fig.18). While no final explanations can be offered for this finding, at least two aspects should be pointed out. First the stably transfected kovak sequence might be integrated into the genome of the HeLa cells at different positions, which might alter gene regulation while the 18.5Z and 27.5Z recognized peptides are part of endogenously encoded proteins. Secondly, in contrast to CBP 80 and CBP 20, additional functions of Upf1 have been described by now that might alter the expression of different genes (Neu-Yilik and Kulozik 2008). Furthermore peptide extraction from siRNA depleted cells and presentation by untreated APCs as described in Fig.17 represents the advantage of directly quantifying antigenic peptide. It helps to rule out potential effects of siRNA transfection or unspecific targeting of factors involved in MHC class I synthesis or regulation. Additional evidence for the involvement of the pioneer round of translation in antigen presentation comes from inhibition of hUpf2 through HeLa cells stably

expressing a small hairpin RNA (Fig.19). Two separate clones demonstrate a consistent five fold down regulation of MHC class I surface expression.

So far several lines of evidence have been presented to demonstrate the requirement of ongoing pioneer translation for MHC class I mediated antigen presentation. The following experiments aimed to distinguish the effects of pioneer translation from cap dependent bulk translation as well as to further characterize how early events in translation control the final outcome of antigen presentation on the surface.

An essential discovery was made with the identification of newly translated polypeptides as major source for antigen presentation. While the original finding of DRiPs has been strengthened substantially within the last decade through several studies, an exhaustive answer to why new translation products govern antigen presentation has not been offered yet. The experiment in Fig.20 recapitulated the importance of immediate early translation for antigenic peptide generation. A short time interval of 8 minutes of translation is sufficient to synthesize peptides that can be detected with the antibody W6/32 on the cell surface. Inhibition of translation with cycloheximide completely abrogates antigen presentation despite the presence of large pools of previously generated proteins present in the cell. The pretreatment of cells with cycloheximide 2 hours prior to the acid wash and short time intervals of translation of 0, 2, 8, 15, 30 and 60 minutes aimed to exclude peptides that were currently in the process of transfer from the ER through the Golgi network to the surface.

The experimental approach in Fig.21 further confirms the notion of new translation products being essential for MHC class I mediated antigen presentation. Addition of cycloheximide (black line) abrogates the surface recovery. The initial increase of detected MHC class I molecules between the 0 hour and 3 hour time point is likely to be due to peptides that were generated right before cycloheximide incubation and were already on their way through the ER trimming and Golgi transport to the surface. The inhibition of antigen surface recovery of cycloheximide treated cells is not simply due to a lack of MHC class I synthesis as there is an abundant and stable pool of heavy chain present in the cell. A substantial amount of heavy chain could be detected even after 2, 24 and 48 hours of continuous translation inhibition (Fig.21C). The previously established system to exclusively target the cap dependent bulk translation provided particularly important information. Transfection with the 4E-BP1-AA dominant negative vector as well as targeting of eIF4E directly with siRNA both did not interfere with MHC class I surface recovery (Fig.21 and Fig.22). While both 4E-BP1-AA dominant negative vector or eIF4E siRNA transfection not completely abolish all bulk translation they did effect it substantially (Fig.12 and Fig.13). Therefore it remains remarkable that the reduction of bulk translation has no detectable effect on antigen presentation while inhibition of pioneer translation severely interferes with efficient MHC class I surface expression.

The following experiments in Fig.23, Fig.24 and Fig.25 serve as important controls for the so far established results. Fig.23 revisited the bimodal recovery pattern of HeLa AS1 cells after acid treatment. Co-transfection with fluorescence labeled siRNA demonstrated the equal transfection efficiency of both slower and faster recovering subpopulations of

cells. This helps to rule out a differential recovery due to different efficiency of siRNA mediated inhibition. Further, Fig.24 demonstrates that the extent of recovery correlates with PI staining for DNA with faster recovering cells showing also a faster progression through the cell cycle. Importantly hUpf1 siRNA did not directly effect the cell cycle progression within the course of the experiment as shown in the right panel of Fig.24. Findings have been confirmed with Hoechst 33342 staining in K89 cells. A recent report connected hUpf1 to the DNA damage response and repair (Azzalin and Lingner 2006). As the authors noted this is rather an effect of hUpf1 specific gene regulation than related to NMD or the pioneer round of translation in general. It has been stated before, that stable transfection with short hairpin RNA targeting Upf1 over a prolonged time period is lethal even in tissue culture cell lines (Wittmann, Hol et al. 2006).

Lastly the effect of interferon  $\beta$  induction of MHC class I molecules during the acid wash recovery time course with hUpf1 depleted cells has been examined (Fig.25). This was mainly done to control for any potential effects of RNAi transfection in triggering an interferon response and potential alterations in MHC class I expression. Generally interferon  $\beta$  incubation up regulated the MHC class I surface expression at all stages of the experiment while leaving the relative difference between Upf1 depleted and control transfected cells unaltered.

In conclusion chapter 2.2 established the direct importance of pioneer translation for MHC class I mediated antigen presentation. Furthermore it reaffirmed the requirement of immediate early translation for antigen synthesis and discounted the contribution of eIF4E dependent bulk translation for the generation of antigenic peptides.

### **3.3 Antigen supply can be quantified during TAP transport**

The TAP1-GFP FRAP experiments were aiming to quantify the effect of pioneer peptides not only at the very end of the antigen presentation pathway at cell surface but at an intermediate step of the process. First previous findings of delayed MHC class I surface recovery upon hUpf1 depletion were successfully confirmed in MelJuSo cells (Fig.27). Due to the differential TAP1-GFP expression pattern this was also established for low, medium and high GFP expressing cells. As the FRAP assay is based on single cell measurements these aspects become more important. Statistical significance of the results was achieved by gathering and averaging the data for at least 10 cells for each graph as described in the literature (Reits, Vos et al. 2000; Reits and Neefjes 2001). Still this certainly showed a limitation of the statistical accuracy of this method in comparison to measurements using flow cytometry with results based on 20.000 or more cells per sample. Special attention was given to the experimental setup, timing and temperature adjustments. After removal from the cell culture incubator cells were transported in a 37°C water bath and analyzed with a microscope with a preheated sample frame in order to limit FRAP variations due to temperature inconsistencies. FRAP was originally established as a biophysical method quantifying the movement of freely diffusing or bi-lipid membrane integrated molecules. Cellular measurements face additional challenges as the ER membrane is not a planar structure but heavily folded with inconsistencies including membrane rafts and intracellular movements. Taking all these effects into consideration the TAP1-GFP FRAP method established by Neefjes et al. represents an elegant method to measure peptide supply early on in the antigen presentation pathway.

Addition of small chemical inhibitors like lacacystin for the proteasomal degradation and cycloheximide for translation provide means to equally inhibit antigen presentation functions on cells attached to a microscope cover slip. Both show a significantly increased lateral TAP1-GFP mobility (Fig.29). This is in good correlation to their expected and previously published effect in decreasing the pool of peptides for TAP transport (Reits, Vos et al. 2000; Qian, Reits et al. 2006). Transfection with an ICP47 encoding vector serves as a further control, as the ICP47 protein directly interacts with TAP1 therefore reducing its peptide transport and complex assembly. Next hUpf1 siRNA transfection was used to establish a connection between pioneer peptide translation and TAP1-GFP mobility (Fig.30). As demonstrated before siRNA transfection has a high transfection efficiency reducing the target protein expression in the whole transfected cell population. This is important since the number of cells for FRAP measurements is limited to about 10 cells per condition due to technical considerations. hUpf1 depleted cells showed a consistently increased TAP1-GFP mobility indicating a reduced peptide supply for TAP transport. Furthermore positive controls reversing the effect of peptide pool reduction by adding exogenous peptides were conducted.

The addition of peptides through pathogen infection faces several challenges. First *Listeria monocytogens* are only infecting a subset of the given cells under the time course and conditions of the experiment (Fig.31). Since it would be an important improvement to the experimental approach to unequivocally distinguish highly infected from potentially uninfected cells for  $D_{TAP}$  measurements, a GFP as well as a RFP expressing *Listeria* strains have been recently obtained and will be used for future experiments. They

will provide the additional opportunity to interfere with prokaryotic translation using specific inhibitors like tetracycline or eukaryotic translation using cycloheximide.

The addition of synthetic peptide has been useful as another method of supplying peptide to the cell. This method requires high concentrations of external peptide as TAP substrate to achieve significant cellular uptake. As shown in Fig.32 it has the potential to moderately decrease TAP1-GFP mobility. Streptolysin O, a bacterial membrane pore forming toxin, has been used to increase the uptake of exogenous peptides, but this approach faces the additional challenge of adding an additional compound that potentially alters membrane features for FRAP measurements.

Finally microinjection represents an additional possibility to directly supply large quantities of peptide inside the cytosol of single cells cell. While this technique has already been used to reverse a high  $D_{TAP}$  in MelJuSo cells, it would require to change form the current setup of an upright microscope to an inverted microscope to enable the use of micromanipulators for injection.

Beside these future perspectives for the elaboration of the technique, the current results already provide a direct link between Upf1 mediated pioneer translation and the peptide supply for TAP transport. These findings further underline the importance of the pioneer round of translation for peptide generation.

### 3.4 Developing a mouse model for the pioneer round of translation

At this stage only very limited mouse models for the NMD pathway are available. Since a complete depletion of Upf1 was embryonic lethal and a conditional knock out strategy did not lead to a complete excision of all functional exons of Upf1, an alternative approach was pursued (Medghalchi, Frischmeyer et al. 2001). A transgenic mouse based on the R844C dominant negative human Upf1 construct was created by H. Dietz and colleagues. In contrast to stable transfection of hUpf1 shRNA into HeLa cells, which is lethal after about 10 days of tissue culture, overexpression of the R844C mutant hUpf1 protein in HeLa and COS cells is tolerated (Sun, Perlick et al. 1998; Wittmann, Hol et al. 2006). Expression of the R844C dominant negative hUpf1 vector in tissue culture cells increased the expression of otherwise decayed PTC containing mRNA about three fold. Taken together the dominant negative hUpf1 R844C transgenic mouse represented the best available model for the examination of NMD and pioneer round of translation associated effects on the immune system.

Integration of the transgene into the mouse genome was confirmed by Southern blotting (Dietz 2007) and PCR (Fig.34). Expression was verified by immunoblotting with an antibody specific for the human protein sequence of the transgene. No distinctive phenotype of the transgenic mouse could be detected for the surface expression of MHC class I, MHC class II, CD4, CD8 or B220 (Fig.36). T-cell responses after acid wash and recovery did not reveal consistent differences in activation of 30NXZ, 27.5Z or 11p9Z T-cells (Fig.38). In several cases increased expression of D<sup>b</sup>, L<sup>d</sup> and K<sup>b</sup> was detected throughout acid wash recovery time course experiments (Fig.37). So far the Dietz lab did

not report any additional phenotype that might assist to determine the functionality of the hUpf1 transgenic mouse model. That led us to revisit several of the original Upf1 mutagenesis studies in yeast as well as the current understanding of the domain structure of the mammalian Upf1 proteins (Czaplinski, Weng et al. 1995; Weng, Czaplinski et al. 1996; Sun, Perlick et al. 1998). By now there is abundant evidence that hUpf1 is a protein with multivalent functions including early nuclear interactions with CBP80 and hUpf2, mRNA binding and translation termination as well as facilitating the final decay of target mRNAs in the cytosol. This leaves room to speculate about the exact function of the hUpf1 R844C mutation in interfering with NMD. While the R844C mutation has been shown to interfere with the mRNA decay mechanism, the mutated protein might still be able to support other functions including mRNA binding and pioneer round of translation. Indeed the overexpression of mouse and human Upf1 might even enhance some functions in the transgenic mouse therefore accounting for the slight increase of MHC class I expression observed in acid wash recovery experiments (Fig.37). The complex phosphorylation cycle of hUpf1 has been described in several studies but its role in NMD and the pioneer round of translation still remains indefinite (Conti and Izaurralde 2005; Isken, Kim et al. 2008). An approach introducing mutations deleting the phosphorylation sites of Upf1 might be of promise. Additional experiments focusing on the less versatile proteins CBP80 and CBP20 of the pioneer round of translation are already under investigation. In this context several mutations were proposed that might interfere with the CBP80 / CBP20 heterodimer interface therefore disabling pioneer translation (Cusack 2006). Additionally lenti-virus based transduction or “nucleofection” (Amaxa Biosystems, Cologne Germany) of RNAi into mouse embryonic cells are

alternative approaches to advance the experiments from immortalized tissue culture cells to mouse primary cells. And finally the most promising opportunities to examine the role of the pioneer round of translation for antigen presentation at an organismal level opened up with the recent advent of the hUpf2 dominant negative mouse model (Weischenfeldt, Damgaard et al. 2008).

### 3.5 Final conclusions

- MHC class I mediated antigen presentation depends on very **early translation**.
- Specific inhibition of cap-dependent **bulk translation** does not significantly alter MHC class I dependent antigen presentation.
- Interference with the **pioneer round of translation** reduces the pool of peptides transported by TAP and presented by MHC class I on the cell surface.

Taken together these findings put products of the pioneer round of translation at an important position as peptides for MHC class I mediated antigen presentation.

## **4 Materials and Methods**

### **4.1 Small interfering RNA oligomer design and transfection**

Synthetic small interfering RNA molecules targeting mRNAs were designed using the publicly available bioinformatics algorithm from the Whitehead Institute (Tuschl 2004) and generated by Dharmacon (Dharmacon, Chicago IL). Generally siRNA sequences consisted of double stranded 19-23 base pair oligomers with 3' dTdT extensions. When indicated the design of previously validated and published siRNA molecules was used for oligomer synthesis. In the case of eIF4E and eIF2B siRNA oligomers with a proprietary sequence were purchased.

For transient depletion of the target mRNA 1 to  $2.5 \times 10^5$  cells were plated per well of a 6-well plate and incubated under growth conditions over night. Then medium was exchanged to 800  $\mu$ l of serum free medium. siRNA oligomers were incubated for 20 minutes with 4  $\mu$ l of oligofectamine reagent (Invitrogen, Carlsbad CA) in 200  $\mu$ l of serum without medium and added to the cells. After 3 hours 500  $\mu$ l of medium containing 3x serum was added. Cells were assayed for targeted mRNA or protein depletion after 48 or 72 hours.

## 4.2 List of small hairpin RNA oligomers:

- hUPF1**  
Definition: *homo sapiens* up frame shift 1  
Alternative Names: rent1 regulator of nonsense transcripts  
Accession Number: NM\_002911  
siRNA: 5' GAUGCAGUCCGCUCCAUdTdT 3'  
3' dTdTTCUACGUCAAGGCGAGGUA 5'  
(Mendell, ap Rhys et al. 2002)
- mUPF1**  
Definition: *mus musculus* up frame shift 1  
Alternative Names: rent1 regulator of nonsense transcripts  
Accession Number: AY597038  
siRNA: 5' GUGCCAGCUGCCAAAGCGCdTdT 3'  
3' dTdTTCACGGTCGACGGTTTCGCG 5'
- CBP80**  
Definition: *homo sapiens* nuclear cap binding protein subunit 1, 80 kDa  
Alternative Names: NCBP1  
Accession Number: NM\_002486  
siRNA: 5' GCUGAUCUCCUAACUACAdTdT 3'  
3' dTdTTCGACTAGAAGGATTGATGT 5'
- CBP20**  
Definition: *homo sapiens* nuclear cap binding protein subunit 2, 20 kDa  
Alternative Names: NCBP2  
Accession Number: NM\_007362  
siRNA: 5' CAGGUGUUUGACAAUUGAAUdTdT 3'  
3' dTdTGTCCACAACTGTTA ACTTAA 5'
- PABP2**  
Definition: *homo sapiens* poly A binding protein, nuclear 1  
Alternative Names: PABPN1  
Accession Number: NM\_004643  
siRNA: 5' AGAGUCAGUGAGGACUCCdTdT 3'  
3' dTdTTCCTCAGTCACTCCTGAAGG 5'

Neo

Definition: neomycin phosphotransferase II

Alternative Names:

Accession Number:

siRNA: 5' AAUGAACUGCAGGACGAGGCAdTdT 3'  
3' dTdTUUACUUGACGUCCUGCUCCGU 5'  
(Kunisawa and Shastri 2003)

eIF4E

Definition: homo sapiens eukaryotic translation initiation factor 4E

Alternative Names:

Accession Number: NM\_001968

siRNA: proprietary (Cell Signaling Technology, Danvers MA)

eIF2B

Definition: homo sapiens elongation factor 2B

Alternative Names:

Accession Number:

siRNA: proprietary (Cell Signaling Technology, Danvers MA)

In addition to synthetic small hairpin RNA molecules, several vector based RNA constructs were used. These mRNA hairpins require further processing by dicer and drosher within the cell to trigger degradation of their mRNA targets. In these cases cell lines were transfected as described and maintained under growth conditions including 1-5 µg/ml puromycin as selective reagent.

#### **4.3 Vector based DNA and small hairpin RNA constructs**

The GFP-based NMD reporter constructs have been described in detail for the analysis of NMD in eukaryotic cells (Paillusson, Hirschi et al. 2005). The SIINFEHL encoding

sequence directly followed by a stop codon was introduced into the GFP-based NMD reporter genes by integration of a DNA fragment at the BsrGI restriction site. The DNA fragment was generated by synthesizing both strands 5'-TTAACCTGTACAAAAGTATAATCAACTTTGAAAACTCTGATTGTACATTAACC-3' and 5'-GGTTAATGTACAATCAGAGTTTTTCAAAGTTGATTATACTTTTGTACAGGTAA-3', annealing in a temperature gradient and BsrGI digestion.

The small hairpin RNA constructs targeting hUpf1, hUpf2 and hSMG6 in the vector pSuper vector were kindly provided by Juergen Wittmann and Hans-Martin-Jaeck (University of Nuremberg-Erlangen, Germany) (Wittmann, Hol et al. 2006). The wildtype 4E-BP1 expression construct as well as the dominant negative (HA)-AA-4E-BP1 (Thr37/46Ala) protein in the pACTAG2 eukaryotic expression vector and backbone vector without insertion were provided by Nahum Sonenberg (McGill University, Vancouver, Canada) and Diane Fingar (University of Michigan, Ann Arbor MI).

The invariant chain (Ii) fused to Ova<sub>247-265</sub> containing the SIINFEHL sequence were integrated into the pcDNA1 vector (Invitrogen, Carlsbad CA) using the EcoRI and XbaI site. The 3' intron removal was achieved using two DraI sites. For 5' intron reintegration PCR based cloning using the forward primer 5'-CCCCAAGCTTTTAGAGGATCTTTGTGAAGGAACCTTACT-3' and the backward primer 5'-CCCCGGATCCGCATT CATTATGTTTCAGGTCAGG-3' and the cloning sites HindIII and BamHI was used. For the generation of three kovak based expression constructs with shuffled intron positions, the OVA<sub>138-386</sub> sequence was transferred into the pcDNA1 backbone vectors with the 3', 5' or no intron using the restriction sites EcoRI and XbaI.

The dominant negative human Upf1 expression construct with the arginine to cysteine mutation at residue 844 in the vector pCI was kindly provided by Harry Dietz (Johns Hopkins University, MD). CBP80 and CBP20 expression vectors for the generation of dominant negative interface mutations were shared by Ian Mattaj and Steven Cusack and have been mentioned in the literature before (Mazza, Ohno et al. 2001). A luciferase dependent chemiluminescence NMD reporter system has been described recently (Boelz, Neu-Yilik et al. 2006). The pCi-neo expression vectors for the *renilla* luciferase  $\beta$ -globin fusion protein with or without a PTC as well as a *firefly* luciferase control vector have been provided by Andreas Kulozik (EMBL / University of Heidelberg).

#### **4.4 Cell lines and cell culture reagents**

HeLa (human), 293 (human), 293T (human), HFF (human) and COS-7 (green monkey) are well characterized cell lines that were acquired from the American Tissue Culture Collection (ATCC, Manassas VA). HeLa cells stably transfected for K<sup>b</sup> or both K<sup>b</sup> and the ovalbumin derived KOVAK 138-386 precursor and K89 K<sup>b</sup>-L-cells (mouse) were described before (Kunisawa and Shastri 2003). MelJuSo (human) cells stably expressing a GFP-TAP I fusion protein were a generous gift from Jaque Neefjes and colleagues (Reits, Vos et al. 2000). The HeLa cell lines E1105 and E 1106 stably expressing hUpf2 shRNA and the HeLa cell line E1086 stably expressing empty pSuper vector were kindly provided by Juergen Wittmann and Hans-Martin Jaeck (Wittmann, Hol et al. 2006). They were maintained in RPMI complete medium containing 1  $\mu$ g/ml puromycin as selection

reagent.  $\beta$ -galactosidase (lacZ)-inducible 27.5Z, 18.5Z, 30NXZ, 11p9Z and B3Z T-cell hybridomas have been described earlier (Serwold, Gonzalez et al. 2002). Cell lines were maintained under growth conditions of 5 % CO<sub>2</sub> and humidified air at 37°C. DMEM (Invitrogen, Carlsbad CA) complete medium was used for culturing 293, 293T and MelJuSo cells. All other cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad CA) complete medium containing additional 2 mM glutamine, 1 mM sodium pyruvate, 50  $\mu$ M beta-mercaptoethanol, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all: Invitrogen, Carlsbad CA) and 10 % fetal bovine serum (Hyclone, Logan UT).

#### **4.5 Mice and primary cells**

Transgenic mice expressing the human Upf1 dominant negative R844C protein on the B6 background were received from the Dietz laboratory (Johns Hopkins University, Baltimore MD). Wildtype C57BL/6 (B6) mice were obtained from the Jackson Laboratory Bar Harbor, ME). For genotyping, DNA was purified from tail tissue using either the DNAeasy Tissue Kit (Qiagen, Valencia CA) or by phenol-chloroform extraction. The human Upf2 dominant negative DNA sequence was amplified by PCR using the forward primer 5'-GTCACAGCCCTTCTCTCAGG-3' and the backward primer 5'-CCTGGTA CGTGGAGTCCTGT-3'. PCR amplification of TAP1 with the forward primer 5'-ATTGAAGTTCCTGCGCCTCC-3' and the backward primer 5'-AGGCTCAGCG TGCCACTAAT was used as a positive control. Expression of the dominant negative human Upf1 R844C protein was verified by immunoblotting with an

antibody recognizing only the human protein. Bone marrow dendritic cell cultures were used for characterizing Upf2 dominant negative transgenic cells including the analysis of MHC class I surface expression in acid wash recovery experiments. Murine cells were collected from the femur, treated with RBC lysis buffer for 1 minute and cultured in uncoated cell culture plates. GM-CSF was added in the concentration of 10 ng/ml at day 3 and day 5 (Lutz, Kukutsch et al. 1999). Spleen cell cultures were generated accordingly. Non-adhesive cells were collected at day 5 to 7 for analysis. Cells were blocked with anti-Fc receptor antibody (2.4G2) prior to antibody staining for flow cytometry assessment. Additionally B lymphocyte depletion was achieved using sheep anti mouse IgG Dynabeads according to the manufacturers instruction (Invitrogen, Carlsbad CA).

#### **4.6 Transient transfection of cell lines**

For presentation assays, 1 to  $2.5 \times 10^5$  cells were plated in a single well of a 6-well plate, transfected with 1  $\mu$ g of plasmid DNA after 24 hours of initial culture and assayed for gene expression after 48-72 hours. 3  $\mu$ l of Fugene 6 (Roche, Indianapolis IN) was used as transfection reagent for each 1  $\mu$ g of vector DNA. For cotransfection of plasmid DNA and RNAi, Oligofectamine reagent (Invitrogen, Carlsbad CA) was used as described above. As controls DNA encoding the appropriate empty vector only or GFP were used. Alternatively purified vectors were transfected with 100  $\mu$ g/ml of DEAE-dextran and 100  $\mu$ M chloroquin in RPMI supplemented with 10 % Nu-serum as described before (Schenborn and Goiffon 2000).

#### **4.7 Acid wash MHC class I surface recovery assay**

Adhesive cells were grown in 6-well plates washed with PBS and incubated for 3 minutes with acid solution containing 0.13 M citric acid and 0.066 M monobasic sodium phosphate at pH 3.1. These conditions remove bound peptides and  $\beta_2m$  while leading to internalization of empty MHC class I molecules. Then the acid solution was aspirated, cells washed twice with 10 ml of PBS and placed again under growth conditions for various time points of MHC class I surface recovery for up to 20 hours. Semi-adhesive or suspension cells were lifted, transferred to 50 ml conical tubes, incubated with 2 ml of acid solution for 1-3 minutes, washed twice with 50 ml of PBS and placed under growth conditions for recovery. For complete inhibition of translation of new peptides, cycloheximide (Sigma, St. Louis MO) was added to a final concentration of 100  $\mu\text{g/ml}$  prior to acid wash. After the acid treatment cycloheximide in the same concentration was added in the recovery medium. MHC class I surface expression was consecutively assessed by flow cytometry with a FacScan (Coulter, Hialeah FL) and FlowJo software (Treestar, Ashland OR) and T-cell assay.

#### **4.8 Fluorescence recovery after photo bleaching**

Transport of peptides through TAP requires dimerization of TAP1 and TAP2 and is subsided by formation of the peptide loading complex (PLC) for transfer of peptides to the MHC class I complex. The size of the TAP dimer together with the PLC alters the

mobility within the ER membrane. In order to visualize the lateral mobility, TAP1 was fused to GFP and expressed in human MelJuSo cells. A region ROI<sub>1</sub> with a diameter of 0.84  $\mu\text{m}$  was bleached by 20 iterations of 488nm Argon / Krypton laser pulse at 100 % intensity. GFP intensity in a reference region ROI<sub>2</sub> at the opposing side of the cell was monitored in order to assess background photobleaching. Fluorescence was determined in a 20 cycle time course at 1.6 sec intervals at 25 % laser intensity by time lapse photography. For all microscope imaging a Zeiss 510 Axioplan META MaiTai at the Cancer Research Laboratory Molecular Imaging Center, UC Berkeley was used. Detailed FRAP protocols and studies were published before (Qian, Reits et al. 2006)(Reits and Neefjes 2001)(Reits, Vos et al. 2000).

#### **4.9 Peptide extraction**

For peptide extraction cell pellets were resuspended in 500  $\mu\text{l}$  of 10 % acetic acid in water at a pH of  $\sim 2$  and boiled for 10 minutes. After 15 minutes of centrifugation at 10,000 rpm supernatant was collected and liquid evaporated through vacuum centrifugation. Remaining peptides were resuspended in 50  $\mu\text{l}$  PBS with 25 ng/ml phenol red. After completed resuspension for 30 minutes on ice, pH was balanced to  $\sim 7$  using 0.1 M NaOH. Remaining cell debris was pelleted through short centrifugation and supernatant was titrated in a 96 well plate 1:2 or 1:3 in a volume of 50  $\mu\text{l}$ .  $1 \times 10^5$  antigen presenting cells K89 and  $1 \times 10^5$  hybridoma T-cells are added to each well.

#### **4.10 T-cell activation assays**

Upon recognition of the K<sup>b</sup> restricted SIINFEHL antigen, B3Z hybridoma T-cells express IL-2 promotor controlled  $\beta$ -galactosidase (LacZ) (Karttunen and Shastri 1991).

Enzymatic LacZ activity was measured by chromogenic chlorophenol red  $\beta$ -D-galactopyranoside (CPRG, 0.15 mM) (Roche, Indianapolis IN) catalysis to chlorophenol red in Z buffer (9 mM MgCl<sub>2</sub>, 0.125 % NP-40 alternative in PBS). Reaction product accumulation was read as 595 nm absorbance with a 635 nm reference wavelength using a Biorad Microplate Reader (Biorad, Hercules CA). Activation of 18.5Z, 27.5Z, 30NXZ and 11p9Z hybridoma T-cells was determined accordingly.

For MHC class I presentation assays  $1 \times 10^5$  T-cells were added in 96 well plates to serial dilutions of antigen presenting cells and cocultured overnight. Cells were spun down for 2 minutes at 2,000 rpm, culture medium was removed and 100  $\mu$ l of CPRG was added into each well. Plates were read after different incubation times.

#### **4.11 Western blotting**

Cells were lysed for 30 min. at 4°C with homogenization buffer (80  $\mu$ l 1 M Tris-HCl pH 7.6, 40  $\mu$ l NP40 alternative, 20  $\mu$ l 200 mM PMSF, 4 ml of H<sub>2</sub>O) containing a cocktail of protease inhibitors (either 4  $\mu$ l 1mg/ml leupeptin, 2  $\mu$ l 25  $\mu$ M pepstasion or one mini-tablet of inhibitors (Roche, Indianapolis) and spun down at 10,000 rpm, 4°C for 30 min.

The protein supernatant excluding the pelleted nuclei was combined with the same volume of sample buffer and loaded on a 10 % or 4-20 % gradient SDS polyacrylamide gel (Biorad, Hercules, CA). Separated, denatured proteins were transferred onto a nitrocellulose membrane and transferred using a Bio-Rad trans blot apparatus with 25 mM Tris, 0.192 M glycine and 20 % methanol as transfer buffer. After blocking with 1% dry skim milk in PBS, membranes were probed with various primary antibodies and secondary horseradish peroxidase conjugated antibodies in the listed dilutions. Protein bands were visualized using the ECL western blotting detection kit (Amersham, Buckinghamshire UK).

#### 4.12 Primary Antibodies

antibody	species	application	source
W6/32 $\alpha$ HLA A,B,C	mouse $\alpha$ human monoclonal	FC 1:500	ascites
W6/32 $\alpha$ HLA A,B,C FITC conjugated	mouse $\alpha$ human monoclonal	FC 1:500	Sigma, St.Louis MO F-5662
5F1.2.14 $\alpha$ K <sup>b</sup>	mouse monoclonal	FC 1:500	ascites
5F1.2.14 $\alpha$ K <sup>b</sup>	mouse monoclonal	FC 1:500	supernatant
$\alpha$ $\beta_2$ m	mouse $\alpha$ human monoclonal	FC 1:50 – 1:100	US Biological, Swampscott, MA M3890-5
AF6-88.5 $\alpha$ K <sup>b</sup> FITC conjugated		FC 1:50	BD Bioscience, San Jose CA
KH95 $\alpha$ D <sup>b</sup> FITC conjugated		FC 1:50	BD Bioscience, San Jose CA
C28.14.8 $\alpha$ D <sup>b</sup> FITC conjugated		FC 1:50	BD Bioscience, San Jose CA
CTK <sup>b</sup> $\alpha$ K <sup>b</sup> PE conjugated		FC 1:50	BD Bioscience, San Jose CA
Y3 $\alpha$ K <sup>b</sup> K <sup>k</sup>	mouse	FC 1:500	ascites
HC-10 $\alpha$ MHC I heavy chain	mouse $\alpha$ human	WB 1:100 FC 1:50	supernatant Helge Plough, MIT, MA
$\alpha$ hUpf1 / rent1	mouse $\alpha$ human polyclonal	WB 1:1000	Abcam, Cambridge, MA Ab43408
$\alpha$ hUpf1 / rent1	goat $\alpha$ human polyclonal	WB 1:1,000 – 1:2,500 FC 1:100	Abcam, Cambridge, MA Ab10510 & Ab10534

$\alpha$ hUpf2	rabbit $\alpha$ human polyclonal	WB 1:100	Hans-Martin Jaeck Universitate Nueremberg- Erlangen, Germany
$\alpha$ CBP80	rabbit $\alpha$ human polyclonal	FC 1:50	Abcam, Cambridge, MA
$\alpha$ CBP80	rabbit $\alpha$ human polyclonal	WB 1:500	Elisa Izaurrealde, EMBL Heidelberg, Germany
$\alpha$ CBP20	rabbit $\alpha$ human polyclonal	FC 1:50	Abgent, San Diego, CA AP1946b
$\alpha$ eIF4E	rabbit $\alpha$ human polyclonal	WB 1:1,000	Cell Signaling, Boston, MA 6310
$\alpha$ eIF4B	rabbit $\alpha$ human polyclonal	WB 1:500 - 1:1,000	Cell Signaling, Boston, MA 6310
$\alpha$ PABP2	rabbit $\alpha$ human polyclonal	WB 1:1,000	Elmar Wahle, Universitaet Halle, Germany
$\alpha$ 4E-BP1	rabbit $\alpha$ human polyclonal	WB 1:1,000	Cell Signaling, Boston, MA 9452
$\alpha$ actin	goat $\alpha$ human	WB 1:2,000	SC-1615
$\alpha$ neomycin phosphotransferase II	rabbit $\alpha$ human polyclonal	WB 1:1,000	Upstate, Lake Placid, NY
$\alpha$ neomycin phosphotransferase II	rabbit $\alpha$ human polyclonal	WB 1:1,000	5Prime-3Prime
$\alpha$ In-1	rat $\alpha$ human	WB 1:500	subernatant
$\alpha$ HA-Tag	rabbit $\alpha$ human polyclonal	WB 1:4,000	Abcam, Cambridge, MA Ab9110
ME1 $\alpha$ HLA B0702	mouse $\alpha$ human	FC 1:50	Abcam, Cambridge, MA

### 4.13 Secondary Antibodies

antibody	application	source
goat $\alpha$ mouse IgG FITC conjugated	FC 1:250	Cappel, Solon, OH
sheep $\alpha$ mouse HRP conjugated	FC 1:10,000 - 1:100,000	
goat $\alpha$ mouse IgG PE conjugated	FC 1:200	
rabbit $\alpha$ goat IgG HRP conjugated	WB 1:10,000 – 1:100,000	Zymax, San Luis Obispo, CA
donkey $\alpha$ rabbit HRP conjugated	WB 1:10,000 – 1:50,000	Amersham, Piscataway, NJ
rabbit $\alpha$ rat HRP conjugated	FC 1:10,000 – 1:100,00	Stressgen, Ann Arbor, MI SAB-200
swine $\alpha$ goat PE conjugated	FC 1:200	Invitrogen, Carlsbad, CA 1366799

#### 4.14 Definitions

**Pioneer Translation:** First round of translation of a mRNA after splicing in association with the EJC, CBP80, CBP20, Upf1 and PABP2. Might be located in close association with the nucleus or nuclear pore.

**Bulk translation:** Exclusively eIF4E dependent translation of bulk quantities of polypeptides and proteins in the cell. Might be termed steady state translation or conventional translation by some authors.

**Overall translation:** All translational pathways that lead to the generation of polypeptides through ribosomal activity including bulk translation, pioneer translation, IRES dependent translation, cryptic translation, DRiP and RDP generation etc.

#### 4.15 Abbreviations

17-AAG	HSP90 inhibitor
17-DMAG	HSP90 inhibitor
4E-BP1	elongation factor 4E binding protein 1
$\alpha$	anti
APC	antigen presenting cell
CBP20	also NCBP2 cap binding protein 20 kDa
CBP80	also NCBP1 cap binding protein 80 kDa
CPRG	chlorophenol red $\beta$ -D-galactopyranoside
DMSO	dimethyl sulfoxide
EJC	exon junction complex
eIF-2B	elongation factor 2B
eIF-4B	elongation factor 4B
eIF-4E	elongation factor 4E (only cap-dependent bulk translation)
eIF-4G	elongation factor 4G (both cap dependent bulk and pioneer translation)
EMCV	encephalomyocarditis virus
ERAAP	ER resident amino peptidase associated with antigen presentation

FC	flow cytometry
FGF-5	fibroblast growth factor
FITC	fluorescein resorcinolphthalein
FRAP	fluorescence recovery after photo bleaching
GFP	green fluorescent protein
GM-CSF	granulocyte monocyte colony stimulating factor
GRP94	glucose-regulated peptide ~ 94 kDa, ER resident chaperone, =gp96
gp96	see GRP94
gp100	melanocytic growth protein 100
h	human, <i>homo sapiens</i>
HC	heavy chain of major histocompatibility complex class I
HLA A/B/C	human MHC class human lymphocyte antigen A, B, C
Hoechst33342	fluorescent dye, excitation nm, emission nm
HPLC	High Performance Liquid Chromatography
HRP	horseradish peroxidase
HSP70 / 90	heat shock protein ~ 70 / 90 kDa, cytosolic
HSV	Herpes simplex virus
IRES	internal ribosome entry site
ICP-47	infected cell protein 47, herpes simplex virus encoded TAP inhibitor
Ii	invariant chain associated to major histocompatibility complex class II
INF	interferon
K <sub>d</sub>	dissociation constant
KOVAK	ovalbumine derived peptide sequence of amino acids 138-386
lac	lactacystin, organic compound, naturally synthesized by bacteria <i>Streptomyces</i> , potent proteasome inhibitor
LacZ	β-galactosidase
LB	Luria Bertini bacterial growth medium
LLM	N-acetyl-L-leuciny-L-leuciny-methional, weak 20S proteasome inhibitor
LLnL	N-acetyl-L-leuciny-L-leucinal-L-norleucinal, 20S proteasome inhibitor
LPS	lipopolysaccharide
LTA	lipoteichoic acid
m	mouse, <i>mus musculus</i>
MG115	N-carbobenzoxyl-L-leuciny-L-leuciny-L-norvalinal, potent proteasome inhibitor
MG132	N-carbobenzoyl-L-leuciny-L-leuciny-L-leucinal, potent proteasome inhibitor
MHC I	major histocompatibility complex class I
MOI	multiplicity of infection
OVA	chicken ovalbumin
PA28	proteasome activator
PABP2	poly adenin binding protein 2, also PABPN1
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PE	R-phycoerythrin
PLC	peptide loading complex

PMSF	phenylmethanesulphonylfluoride
POMP	proteasomal maturation protein
PTC	premature termination codon
RBC	red blood cell lysis buffer
rent1	regulator of upframe shift, also Upf1
RF1.Aa	<i>rattus norvegicus</i> TAP allele
rmp	rounds per minute
siRNA	small interfering RNA
shRNA	small hairpin RNA
SDS	sodium dodecyl sulfat
SHL8	ovalbumine amino acids 257-264 SIINFE <u>HL</u> , K to H substitution
SL8	ovalbumine derived peptide sequence of amino acids 257-264 SIINFEKL
SMG-1 to 7	suppressor with morphogenic effect on genitalia-1 to 7
SRP	signal recognition pore
TAP	transporter associated with antigen processing
TLR	toll-like receptor
TPP	tripeptidyl peptidase
TRiC	TCP-1 containing ring complex, group II chaperonin
UL49.5	<i>bovine herpes virus</i> type 1 encoded TAP inhibitor
Upf1	upframe shift protein 1, also rent1
Upf2	upframe shift protein 2
US6	<i>human cytomegalovirus</i> encoded TAP inhibitor
UTR	untranslated region of a gene in 3' or 5' position
VDJ	gene rearrangement of immunoglobulin und TCR genes
WB	western blotting
X-P	petide with X= any amino acid and P=prolin

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## **6 Appendices**

## 6.1 Lebenslauf Andreas Schmidt

Abitur am Rudolph-Brandes Gymnasium Bad Salzuflen	Mai 1997
Zivildienst in der Biologischen Station Ravensberg e.V., Herford	Aug.1997 - Sept.1998
Studium der Biologie an der Universität zu Köln Vordiplom in Biologie	Okt.1998 - Sept.2000 Sept.2000
Studium an der University of Oregon, Eugene USA Botschafterstipendium der Rotary-Stiftung USable Preis der Körber-Stiftung	Okt.2000 - Sept.2001
Studentische Hilfskraft Praktikum Biologie für Mediziner Universität zu Köln	Nov.2001 - Nov.2002
Chinesisch Sprachstudium Taipei, Taiwan R.O.C.	Juli.2002 - Okt.2002
Studium der Biologie an der Universität zu Köln Genetik, Biochemie und Pharmakologie Diplomarbeit am Institut für Genetik, Universität zu Köln Arbeitsgruppe Prof. Dr. Jonathan C. Howard Grundstipendium der Friedrich-Ebert Stiftung Diplom in Biologie	Okt.2001 - Mai 2004      Mai 2004
Wissenschaftlicher Mitarbeiter bei Amaxa Biosystems Köln	Mai 2004 - Aug.2004
Promotion in Immunologie Wissenschaftlicher Mitarbeiter im Department of Molecular and Cell Biology, University of California Berkeley, Arbeitsgruppe Prof. Dr. Nilabh Shastri Universität zu Köln, Institut für Genetik Betreuer Prof. Jonathan C. Howard Graduiertenstipendium der Friedrich-Ebert-Stiftung	Aug.2004 - Feb.2009
Management of Technology China Fellow Haas School of Business, University of California Berkeley	seit Jan. 2006
Mitglied im Beirat des German Academic International Network GAIN der Deutschen Forschungsgemeinschaft, des Deutschen Akademischen Austauschdienstes und der Alexander von Humboldt Stiftung	seit Aug. 2008

## **6.2 Erklärung nach §4, Absatz 9**

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Jonathan C. Howard Institut für Genetik der Universität zu Köln betreut worden.

Alle experimentellen Arbeiten im Rahmen dieser Dissertation sind entsprechend §6, Absatz 1 an der University of California Berkeley, Department of Molecular and Cell Biology, Division of Immunology unter Anleitung von Herrn Prof. Dr. Nilabh Shastri, durchgeführt worden.

## 6.3 Zusammenfassung

### **MHC I vermittelte Antigenpräsentation von Peptiden der “nonsense mediated decay” assoziierten Pionier-Runde der Translation.**

“Nonsense mediated decay” (NMD) spielt eine entscheidende Rolle in dem Qualitätskontroll-Mechanismus, der Zellen vor Stopp-Mutationen, so genannten “premature termination codons” (PTC) schützt. Erkennung dieser PTCs erfordert eine Pionier-Runde der Translation noch vor Beginn der konventionellen Translation von mRNA. Diese führt zur Synthese sehr früher Peptide.

Die Effektivität der Immunüberwachung wird in entscheidendem Masse von der schnellen und vollständigen MHC I vermittelten Präsentation von exakt prozessierten Peptiden, die alle zellulären Proteine repräsentieren, gewährleistet. Bisher ist bekannt, dass diese Peptide konventionell prozessierte Proteine, defekte ribosomale Produkte (DRiPs), und kryptische Peptide, deren Translation nicht mit einem AUG-Codon beginnt, umfassen. Hier wurde untersucht, welchen Beitrag Peptide aus der Pionier-Runde der Translation zu der MHC I vermittelten Antigenpräsentation leisten. Reduktion der für die Pionier-Runde der Translation essentiellen Faktoren hUpf1 und CBP80 führt zu verminderter Antigenpräsentation. Im Gegensatz dazu führt eine Reduktion der konventionellen Translation ohne Einschränkung der Pionier-Translation zu keiner Veränderung der MHC I vermittelten Antigenpräsentation. Aus diesen Ergebnissen geht die entscheidende Bedeutung der Peptide der Pionier-Runde der Translation für die Antigenpräsentation hervor und stellt eine neue Verbindung zwischen NMD und dem Immunsystem dar.