The role of ERAP1 and HLA-B27 in Ankylosing Spondylitis pathogenesis

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Abstract

HLA-B27 and ERAP1 are the two strongest predisposing genetic factors to Ankylosing Spondylitis (AS). As a key aminopeptidase in MHC class I presentation, ERAP1 potentially contributes to AS pathogenesis through altering HLA-B27 peptide presentation. In this thesis, I first studied the effect of AS-associated ERAP1 variation on its enzyme activity in vitro. Trimming of two N-terminally extended HLA-B27 epitopes was decreased by K528R mutation; the effect of R725Q was however substrate-dependent. I also investigated the effects of ERAP1 silencing on the repertoire of peptides bound to HLA-B27 and on peptide presentation to Cytotoxic T lymphocytes (CTLs). In both HeLa.B27 and C1R.B27 cells, the proportion of 9mer peptides, the canonical MHC class I peptide length, was decreased by ERAP1 silencing whereas the percentage of longer peptides (11-13mer) was increased. Surprisingly, following ERAP1 silencing, C-terminally extended versions of 9mer and 10mer peptides were readily identified. In both HeLa.B27 and mouse fibroblasts expressing HLA-B27, ERAP1 silencing/knockout reduced recognition by KK10-specific HLA-B27-restricted CTLs following recombinant vaccinia viral infection or transfection with minigenes expressing KK10 precursors. Interestingly, KK10 CTL recognition following extended KK10 minigene transfection was reduced in the presence of the AS protective variant, K528R-ERAP1 compared to wildtype ERAP1. The effects of ERAP1 inhibition (Leucinethiol), silencing (siRNA & shRNA) and introduction in ERAAP-/- cells on cell surface HLA-B27 expression were also studied. My finding validates the role of ERAP1 and HLA-B27 interaction in AS pathogenesis indicated by GWAS. ERAP1 inhibition could potentially be used for treatment in AS and other ERAP1-associated diseases.

Declaration

I declare that the work presented in this D.Phil thesis entitled "The role of ERAP1 and HLA-B27 in Ankylosing Spondylitis pathogenesis" is entirely my own work, except for where the contributions of my collaborators have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this University or elsewhere.

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Attributions

The work undertaken in this thesis is my own original work. Parts of the thesis contain work done in collaboration with others. The contribution of others is listed below and in some cases within the text.

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Abbreviations

AS - Ankylosing Spondylitis

HLA - Human Leukocyte Antigen

B27 - HLA-B27

β_2m - Beta-2 Microglobulin

CCMP - Centre for Cellular and Molecular Physiology

CTL - Cytotoxic T-Lymphocyte

ddNTPs - Dideoxynucleotide Triphosphate

dNDP - Deoxynucleotide Diphosphate

dNTP - Deoxynucleotide Triphosphate

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic Acid

ER - Endoplasmic Reticulum

ERAAP - Endoplasmic Reticulum Aminopeptidase associated with Antigen Processing

ERAP1 - Endoplasmic Reticulum Aminopeptidase 1

ERAP2 - Endoplasmic Reticulum Aminopeptidase 2

ERAD - ER-associated protein degradation

GWAS - Genome-wide Association Study

HCL - Hydrochloric Acid

HLA - Human Leukocyte Antigen

HT - Heterotrimer (Heavy chain, β_2m and peptide complex)

IFNγ - Interferon Gamma

KIR3DL2 - Killer Cell Immunoglobulin-like Receptor 3 DL2

LB - Lysogeny Broth

LCL - Lymphoblastoid Cell Line

Leu-AMC - L-Leucine-7-Amino-4-Methylcoumarin

LeuSH - Leucinethiol

LILRB2 - Leukocyte Ig-like Receptor B2

mAb - Monoclonal Antibody

mFib.B27 - ERAAP-/- Murine fibroblast transduced to express HLA-B27

MHC - Major Histocompatibility Complex

mRNA - Messenger Ribonucleic Acid

Mw - Molecular Weight

nsSNP - Non-synonymous Single Nucleotide Polymorphism

PBS - Phosphate Buffered Saline

PBMCs - Peripheral Blood Mononuclear Cells

PCR - Polymerase Chain Reaction

PLC - Peptide Loading Complex

SDS-PAGE - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

shRNA - small hairpin RNA

siRNA - small interfering RNA

SNP - Single Nucleotide Polymorphism

SpA - Spondyloarthropathy

TAP - Transporter Associated with Antigen Processing

TCR - T Cell Receptor

TGF-β - Transforming Growth Factor Beta

TNF - Tumour Necrosis Factor

TNFR1 - Tumour Necrosis Factor Receptor 1

TNFα - Tumour Necrosis Factor Alpha

UPR - Unfolded Protein Response

WIMM - Weatherall Institute of Molecular Medicine

WT - Wild-Type

WTCCC - Wellcome Trust Case-Control Consortium

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Chapter 1. Introduction

1.1 Ankylosing Spondylitis

Ankylosing Spondylitis (**AS**) is the prototypic form of the seronegative Spondyloarthropathies (SpA) which also includes reactive arthritis, arthritis related to inflammatory bowel disease, a subgroup of juvenile idiopathic arthritis, and some forms of psoriatic arthritis (Landewe and van Tubergen 2009). AS is a common chronic inflammatory arthritis and mainly affects joints in the spine and the sacroiliac joint in the pelvis, and can cause eventual fusion of the spine (Jimenez-Balderas and Mintz 1993). Extra-articular features include uveitis and lung fibrosis. AS is also found more commonly associated with inflammatory bowel disease (**IBD**) and psoriasis.

1.1.1 Spondyloarthropathies

SpAs predominately affect the axial skeleton (spinal, thoratic, and pelvic joints) while peripheral arthritis and peripheral enthesitis also exist in a large subgroup of patients. Inflammation may also cause extra articular symptoms (psoriasis, anterior uveitis, urethritis, and inflammatory bowel disease). These diseases are grouped because they are simultaneously or sequentially identified in the same patient or in a family member. Moreover, similar clinical characteristics such as eye involvement and enthesopathy are shared by these disorders (Moll JM 1974). The facts that HLA-B27

transgenic rats develop the various clinical features that are noted in human beings with spondyloarthritis also support these grouping (R. E. Hammer et al. 1990).

1.1.2 Genetics of AS

Familial aggregation studies have shown that genetic risk factors contribute to approximately 80-90% of the susceptibility to AS (Calin et al. 1984). In AS twins studies, concordance rate is significantly stronger in monozygotic (6 out of 8, 75%) compared to dizygotic (4 out of 32, 12.5%) twins, which confirms that this aggregation is due to genetic factors rather than environment (Brown et al. 1997).

1.1.2.1 HLA-B27

The association of HLA‑B27 with AS was first described in 1970s, supported by the finding that HLA-B27 is present in 88-96% of AS patients, compared with 4-9% of control individuals (Brewerton 1973; Schlosstein L 1973). HLA-B27 also increased the AS concordance rate of dizygotic twins from 12.5% (4 out of 32) to 27% (4 out of 15) (Brown et al. 1997). The role of HLA-B27 was further confirmed by the spontaneous spondyloarthritis-like disease in HLA-B27 transgenic rats with human β_2m and over-expressed HLA-B27 (R. E. Hammer et al. 1990). Indeed, the association of HLA-B27 with AS is the strongest genetic association known in a complex disease. However, only around 5% of Caucasians carrying HLA-B27 develop AS (Khan 1996). The finding of a greater prevalence of disease in B27 positive first-degree relatives of probands than in random B27 positive controls, suggests the existence of other genes for susceptibility (Calin et al. 1984; Woodrow et al. 1983).

1.1.2.1.1 HLA-B27 subtypes

The major histocompatibility complex (**MHC**) is a highly diverse genomic region containing more than 200 genes. As a MHC class I gene, HLA-B27 is also highly polymorphic with over 60 subtypes identified so far. These differ from each other at only a few amino acids, which are mainly within the alpha 1 and alpha 2 domains that form the peptide binding groove (Reveille and Maganti 2009). This could affect the peptides binding specificity, the rate of HLA-B27 folding and/or subsequent interaction with T or NK cells. The HLA-B*27:05 is found in all populations and thought to be the ancestor of other subtypes in evolution.

Not all HLA-B27 subtypes are AS-associated. Among the common subtypes (Table 1.1), HLA-B*27:05 (Caucasians), HLA-B*27:04 (Asians) and HLA-B*27:02 (Jews and Mediterranean populations) are strongly associated with AS, whereas HLA-B*27:06 (Indonesia, Malaysia and Thailand) and HLA-B*27:09 (Sardinia) do not seem to be associated with AS (D'Amato et al. 1995; Paladini et al. 2005).

HLA-B*27:09 differs from HLA-B*27:05 by only a single amino acid change at position 116. This residue, which resides within the F-pocket of the peptide binding-site, is responsible for anchoring the C-terminus of bound peptides and its substitution (Asp116His in HLA-B*27:09) results in a change in the peptide binding specificity (Ramos et al. 2002). Similarly HLA-B*27:06 differs from HLA-B*27:04 by two amino acids, at positions 114 and again at 116 which alters its peptide

specificity range by 10-12 percent (Sesma et al. 2002). This indicates that the peptide-binding specificity of HLA-B27 is possibly important in susceptibility to AS.

Populations	*02	*03	*04	*05	*06	*07	*09
Northern Europe	10			90			
Denmark	10			90			
Southern Europe	20			80			
Spain (Galicia)	18			80			
Northern Spain	7			91		1	
Azores		7		86		7	
Italy	38			50		4	7
Sardinia				75			25
Greece	34	8		50		8	
Cyprus (Greeks)	52			32		17	
Turkey	30			43		14	
Lebanon	24	12		35		30	
Jewish	48		3	38		13	
Siberia	14			84			
Northern India			33	61		6	
Western India			34	34		18	
Japan			82	18			
Chinese	2		66	31		2	
Singapore (Chinese)			89	2	9		
Taiwan	0.5		87	4	7	2	
Taiwan (Han-Chinese)			94	6			
Taiwan (Aborigines)			100				
Chinese Indonesian			38		62		
Native Indonesians			6	6	89		
Malays			19	6	72	3	
Thailand			42	5	53		
Maoris			36	64			
Brazil	10			80		3	
North Africa	50			50			
West African		32		68			

Table 1.1 Common HLA-B*27 subtype frequencies (%) in different world populations (adapted from (Akkoc and Khan 2005))

1.1.2.2 Other MHC genes

In humans, the MHC complex consists of more than 200 genes located close together on chromosome 6. Efforts have been made to elucidate the role of other genes in the MHC region in the pathogenesis of AS with HLA-Bw60 being the best example (W. Maksymowych 1999). It was first reported to be associated with AS in 1989 (Robinson et al. 1989), and this association has subsequently been confirmed in white European Britons (Brown et al. 1996) and in B27-negative Taiwanese AS cases (Wei et al. 2004). In the MHC region, MICA (Su et al. 2006), TNF (Sousa et al. 2009), HSP70 (Fabian et al. 2009) and HLA-class II region genes like DRB1 (Said-Nahal et al. 2002) and LMP2 (W. Maksymowych 1999) have been reported to be AS-associated. However, further confirmation of these genes has been confounded by the linkage disequilibrium, the non-random association of alleles at two or more loci, in this region. In the recent GWAS, few SNPs in the MHC region achieved genome-wide significance, except for the HLA-B locus, when controlling for the presence of HLA-B27 (Evans et al. 2011). This implies that other HLA-B alleles or genes nearby, such as MICA or HLA-C, could possibly be contributing to AS susceptibility.

1.1.2.3 Non-MHC genes

Genome-wide association studies (GWAS) have identified several of non-MHC genes (Table 1.2) (Reveille 2012). In this review, these genes are suggested in contribution of approximately 2.09% of the overall heritability of AS, with 23.3% provided by HLA‑B27 (Brown 2011).

Gene name or chromosomal region	Most highly associated SNP	Odds ratio	Overall contribution to AS heritability (%)[12]
HLA-B27	rs4349859	90.4	23.3
IL23R	rs11209026	1.90	0.31
LTBR-TNFRSF1A	rs11616188	1.38	0.075
2p15	rs10865331	1.36	0.54
ERAP1	rs30187	1.35	0.34
KIF21B	rs2297909	1.25	0.25
21q22	rs378108	1.25	0.035
TBKBP1	rs8070463	1.24	0.054
ANTXR2	rs4389526	1.21	0.054
PTGER4	rs10440635	1.20	0.052
RUNX3	rs11249215	1.19	0.12
IL12B	rs6556416	1.18	0.11
CARD9	rs10781500	1.18	0.034
IL1R2	rs2310173	1.18	0.12
Total	–	–	25.39

Abbreviations: AS, ankylosing spondylitis; SNP, single nucleotide polymorphism.

Table 1.2 Estimated contribution to heritability of AS of confirmed susceptibility genes (from (Reveille 2012))

1.1.2.3.1 ERAP1

The genetic association of endoplasmic reticulum aminopeptidase - 1 (ERAP1) and AS was first reported by the Wellcome Trust Case Control Consortium and the Australo-Anglo-American Spondylitis Consortium (WTCCC-TASC study) in 2007 (Burton et al. 2007). They scanned 14,500 nonsynonymous single-nucleotide polymorphisms (SNPs) and found that, in British white patients with AS, the major allele of the *ERAP1* SNPs *rs30187* and *rs27044* and minor allele of *rs10050860*, *rs17482078* and *rs2287987* was considerably less frequent. These five alleles are

therefore protective in AS. Interestingly, each of these alleles leads to a one amino acid change from wild type ERAP1 (WT-ERAP1), suggesting that WT-ERAP1 is pathogenic (Table 1.3). The association between ERAP1 and AS was then amply replicated in the UK and other populations, including Canadian, Han Chinese, Korean and Portuguese (Choi et al. 2009; Davidson et al. 2009; D. Harvey et al. 2009; W. P. Maksymowych et al. 2009; Pimentel-Santos et al. 2009).

In addition to SNPs, the association of ERAP1 haplotype with AS has also been shown in a study of approximately 1000 Canadian AS patients and 1500 controls. The rs27044-C/rs10050860-C/rs30187-T haplotype is strongly associated with AS (anti-sense strand: rs27044-C is an AS-protective variant; the other two are AS predisposing wild type variants), while the rs30187-C/rs26618-T/rs26653-G haplotype is protective (anti-sense: rs30187-C is an AS-protective variant; the others are wild type).

Strikingly, the predisposition to AS by ERAP1 was recently found to only exist in HLA‑B27 positive individuals (Evans et al. 2011), which indicates that HLA-B27 and ERAP1 have contributed jointly to disease susceptibility with mechanism to be revealed. Two protective ERAP1 alleles, rs30187 (Lys528Arg) and rs17482078 (Arg725Gln), were both reported to trim substrate peptides slower than wild-type ERAP1 and are in fact loss-of-function variants (Evans et al. 2011). Meanwhile, ERAP1 expression was elevated in the blood monocyte-derived dendritic cells (DC) of AS patients compared to healthy controls (Campbell et al. 2011). Together, they

suggest a possible role of hyper-functional ERAP1 in promoting ankylosing spondylitis.

ERAP1 SNP	Amino acid position	Sense strand Major allele (Anti-sense strand base pair subscript)	Minor allele	Base change + Amino acid change (major allele→ minor allele)	AS-predisposing allele
rs30187	528	G_C	A_T	AGG → AAG R(Arg) → K(Lys)	Minor allele (coding for Lys)
rs27044	730	G_C	C_G	GAA → CAA E(Glu) → Q(Gln)	Minor allele (coding for Gln)
rs17482078	725	G_C	A_T	CGA → CAA R(Arg) → Q(Gln)	Major allele (coding for Arg)
rs10050860	575	G_C	A_T	GAC → AAC D(Asp) → N(Asn)	Major allele (coding for Asp)
rs2287987	349	A_T	G_C	ATG → GTG M(Met) → V(Val)	Major allele (coding for Met)

Table 1.3 AS-associated ERAP1 SNPs (adapted from (Keidel et al. 2013)

1.1.2.3.2 IL23R

The association of IL23R with AS was first reported in GWAS reported by the Wellcome Trust Case Control Consortium (WTCCC) and the Australo-Anglo-American Spondylitis Consortium (TASC) (Burton et al. 2007). Ever since, several studies on different populations (Canadians, Portuguese and Spanish) have confirmed the initial finding (Karaderi et al. 2009; Rahman et al. 2008; Rueda et al. 2008). However, this association was not seen in patients from Korean and China (C. Chen et al. 2012; Sung et al. 2009), which was likely due to the possibility that the associated SNPs are not polymorphic in these cohorts.

IL23R is the receptor for IL23, it is embedded in cell membrane of several types of immune system cells, including T cells, natural killer (NK) cells, monocytes, and dendritic cells. The ligation of IL23 to IL23R activates the STAT3 pathway which promotes expansion and maintenance of Th17 cells (Layh-Schmitt and Colbert 2008). IL-17 (A and F) released by Th17 cells is highly pro-inflammatory and has been implicated in the pathogenesis of many chronic inflammatory disorders, such as spondyloarthritis, rheumatoid arthritis, psoriasis, asthma (Girolomoni et al. 2012; Irving 2010; Layh-Schmitt and Colbert 2008; Zhang et al. 2012).

Interestingly, the primary disease-associated SNP in IL23R, rs11209026, was found to be protective in AS, IBD and psoriasis (Karaderi et al. 2009; Pidasheva et al. 2011; Zhu et al. 2012). This SNP results in an R381Q substitution in IL23R which causes the loss of IL23R function in STAT3 phosphorylation when stimulated with IL-23 (Pidasheva et al. 2011). This provides an explanation for the protective role of R381Q

in AS and suggests a potential therapeutic application using IL-23 targeting neutralizing antibody or small molecular inhibitors. Several clinical trials are currently undergoing.

1.1.2.3.3 Other genes

In addition to HLA-B27, ERAP1 and IL23R, the gene deserts on chromosome 2p15 and 21q22 was also shown strongly associated with AS (Reveille et al. 2010). GWAS studies also reported several genes that have potential associations with AS, including tumour necrosis factor (TNF) receptor 1 (TNFSF1A), kinesin family member 21B (KIF21B), TBK1 binding protein 1 (TBKBP1), the vascular morphogenesis protein geneanthrax toxin receptor 2 (ANTXR2), prostaglandin E receptor 4 (PTGER4), runt-related transcription factor 3 (RUNX3), interleukin 12B (IL12B), interleukin 1 receptor 2(IL1R2), the innate immune receptor caspase recruitment domain family member 9 (CARD9) and so on (Burton et al. 2007; Evans et al. 2011; Pointon et al. ; Pointon et al. 2010; Reveille et al. 2010; Sims et al. 2008; Zinovieva et al. 2009).

1.1.3 Symptoms and diagnosis

AS affects men more than women by a ratio of about 3:1, the disease also usually takes a more severe course in men than women. The primary symptoms are typically chronic pain and stiffness in the middle part of the spine or sometimes the entire spine, often with pain referred to one or other buttock or the back of the thigh from the sacroiliac joint (van der Linden et al. 1984). Other common symptoms are generalized fatigue and sometimes nausea. In a proportion of cases, patients also develop extra-articular features include uveitis and subclinical bowel inflammation. Less

commonly, aortitis, apical lung fibrosis and ectasia of the sacral nerve root sheaths may occur. Pain is often severe at rest, but improves with physical activity. However, many experience inflammation and pain to varying degrees regardless of rest and movement.

Diagnosis of AS has been difficult. There are no specific symptoms and reliable disease markers. The main symptom, chronic back pain, is common present in patients suffering mechanical back pain. C-reactive protein (CRP) concentration and the erythrocyte sedimentation rate (ESR) have been measured clinically for diagnosis. However, both of them only increase during acute inflammatory periods. There are many with AS have normal level results, yet are experiencing a significant amount of inflammation in their bodies.

The modified New York criteria for AS have often been applied as a diagnostic instrument by clinicians. It covers both of clinical features and radiologic changes in sacroiliac joint (Table 1.4) (van der Linden et al. 1984). However, the structural changes identified on plain radiographs can often take up to 10 years to develop. It rises the problem of diagnostic delays (5 to 10 years) making this criteria not helpful in early stage of disease (Sieper et al. 2009).

New classification criteria for axial spondyloarthritis (SpA) have been developed and validated by the Assessment of SpondyloArthritis international Society Study group (ASAS) in order to facilitate better management of SpA patients (Table 1.5)

(Rudwaleit et al. 2009; Sieper et al. 2009). Magnetic resonance imaging (MRI) is included in the ASAS classification criteria as a new radiographic criterion for SpA due to its ability to detect sacroiliitis and spinal enthesitis in an early phase of the disease, when definite changes cannot be seen on X-ray. Apart from the diagnosis based on sacroiliitis on imaging, the new criteria also contain a clinical arm where a positive result for HLA B27 testing is the anchor criterion, which together with the presence of at least two specified clinical features allow axial SpA classification, when X-ray or MRI findings are absent or equivocal.

A. Diagnosis
1. Clinical criteria
   a) Low back pain and stiffness for more than 3 months which improves with exercise, but is not relieved by rest.
   b) Limitation of motion of the lumbar spine in both the sagittal and frontal planes.
   c) Limitation of chest expansion relative to normal values corrected for age and sex
2. Radiologic criterion
   Sacroiliitis grade ≥2 bilaterally or sacroiliitis grade 3–4 unilaterally.

B. Grading
1. Definite ankylosing spondylitis if the radiologic criterion is associated with at least 1 clinical criterion.
2. Probable ankylosing spondylitis if:
   a) Three clinical criteria are present.
   b) The radiologic criterion is present without any signs or symptoms satisfying the clinical criteria. (Other causes of sacroiliitis should be considered.)

Table 1.4 Modified New York criteria for AS (Adapted from (van der Linden et al. 1984))

Sacroiliitis on imaging*
plus
≥ 1 SpA-feature**

or

HLA-B27
plus
≥ 2 other SpA-feature**

** SpA features:

- Inflammatory back pain
- Arthritis
- Enthesitis (heel)
- Uveitis
- Dactylitis
- Psoriasis
- Crohn´s disease/ulceratice colitis
- Good response to NSAIDs
- Family history for SpA
- HLA-B27
- Elevated CRP

* Sacroiliitis on imaging:

- Active (acute) inflammation on MRI highly suggestive of sacroiliitis associated with SpA
  or
- Definite radiographic sacroiliitis according to mod. New York criteria

Table 1.5 ASAS-classification criteria for axial SpA (in patients with back pain ≥ 3 months and age at onset < 45 years) (Adapted from (Sieper et al. 2009))

1.1.4 Disease assessment and treatment

Disease activity in AS reflects the extent of inflammation and is assessed in daily clinical practice with the Bath ankylosing spondylitis disease activity index (BASDAI) (Garrett et al. 1994). The other two commonly used systems are the Bath ankylosing spondylitis functional index (BASFI) (Calin et al. 1994) and Bath ankylosing spondylitis metrology index (BASMI) (Jenkinson et al. 1994) for the function assessment and clinical measurements for AS assessment. More recently, The Ankylosing Spondylitis Disease Activity Score (ASDAS) was developed (Lukas et al. 2009), it combines level of acute phase reactants (C-reactive protein (CRP) and

erythrocyte sedimentation rate (ESR)) with four questions related to patient self-assessment (back pain, duration of morning stiffness, patient global and peripheral pain/swelling). Preliminary data suggest that the ASDAS is more discriminative than BASDAI when in assessment of TNF blockers (van der Heijde et al. 2009a). However, the usefulness of this new index needs further assess by clinicians in daily practice.

Various treatments have been developed to improve the condition of patient as well as prevent subsequent clinical deterioration. Non-steroidal anti-inflammatory drugs (NSAIDs) are the first-line treatment of AS which rapidly relieves pain and stiffness after 2-3 days. Apart from the dramatic symptomatic effect, NSAIDs have been reported to delay radiological progression of spine disease when taken by patients continuously on a daily basis over 2 years, compared with an on-demand treatment schedule (Wanders et al. 2005). However, the daily intake of NSAIDs is restricted by its gastrointestinal and cardiovascular side effects.

The antirheumatic drugs which have been shown to be effective in the treatment of rheumatic arthritis, however, have not shown efficacy for axial spondyloarthritis (SpA) and enthesitis (J. Chen and Liu 2005; J. Chen et al. 2006; van Denderen et al. 2005). Some efficacy was reported for peripheral arthritis and other extra-articular features such as psoriasis, uveitis and inflammatory bowel disease.

The success of TNF blockade in active and refractory disease has been the major therapeutic advance in spondyloarthritis treatment (Brandt et al. 2000; Braun et al. 2002; Van den Bosch et al. 2000). Studies on its efficacy in pre-radiographic axial spondyloarthritis and undifferentiated peripheral spondyloarthritis are ongoing. Despite its dramatic effectiveness in therapy for SpA, TNF blockade also has its limitations. Firstly, 20-40% patients do not respond well to treatment whereas no reliable prediction for that is available (Rudwaleit et al. 2004; Rudwaleit et al. 2008). Secondly, almost all patients relapse within 6-12 months after interruption of treatment, which means that longlasting remission cannot be achieved (Baraliakos et al. 2005). Thirdly, although the TNF blockade was reported able to stop joint destruction (van der Heijde et al. 2007), it fails to substantially slow new bone formation in SpA (van der Heijde et al. 2008b; van der Heijde et al. 2008a; van der Heijde et al. 2009b). It is still unclear whether this effect is because TNF blockade was started too late in the course of disease or due to the fact that new bone formation is not related to TNF-driven inflammation.

Therefore, despite the high efficacy of TNF blockade, there is still a strong need for both anti-inflammatory drugs and treatments aiming for disease remission and bone structure protection. Several other biological agents have been tested in small proof-of-concept trials in ankylosing spondylitis and psoriatic arthritis based on their efficacy in related diseases such as rheumatoid arthritis and psoriasis (anti-CD20 antibody rituximab, anti-CD11a antibody, anti-LFA3 antibody) (Nocturne et al. ; Song et al. 2010). According to the recent genetic studies, the blocking strategy was also applied on other cytokines, including the IL-1, IL-23, IL-12, IL-17 and IL-6.

Antagonists or neutralizing antibodies targeting these cytokines have been developed and testing trials are ongoing.

1.2 The MHC class I antigen presentation

MHC class I molecules are expressed by all nucleated cells, they present peptides derived from cytosolic and nuclear proteins at cell surface to CD8+ T cells for immune surveillance. The incorrectly folded and old self-proteins or foreign proteins are degraded by the proteasome, the resulting peptides are translocated from cytosol into endoplasmic reticulum (ER) by transporter associated with antigen presentation (TAP) (Ortmann et al. 1994) (Figure 1.1). In the ER, the peptides are further trimmed to optimal length (8-9 mer) by ER aminopeptidases such as ERAP1 before being assembled with the MHC class I heavy chain and light chain (beta-2 microglobulin). The nascent MHC class I molecule without peptides is stabilized by ER chaperones such as ERp57, calreticulin, protein disulfide isomerase (PDI) and tapasin (Kang et al. 2009; Sadasivan et al. 1996). Tapasin also interacts with TAP, providing a bridge for peptides delivery to MHC class I molecules (Suh et al. 1999). After loading of peptides, the chaperons are released and the peptide - MHC class I complex travels through the Golgi to cell surface. The un-associated MHC class I molecules are translocated into the cytoplasm and degraded by the proteasome through the ER-associated protein degradation (ERAD) system (Hughes et al. 1997)

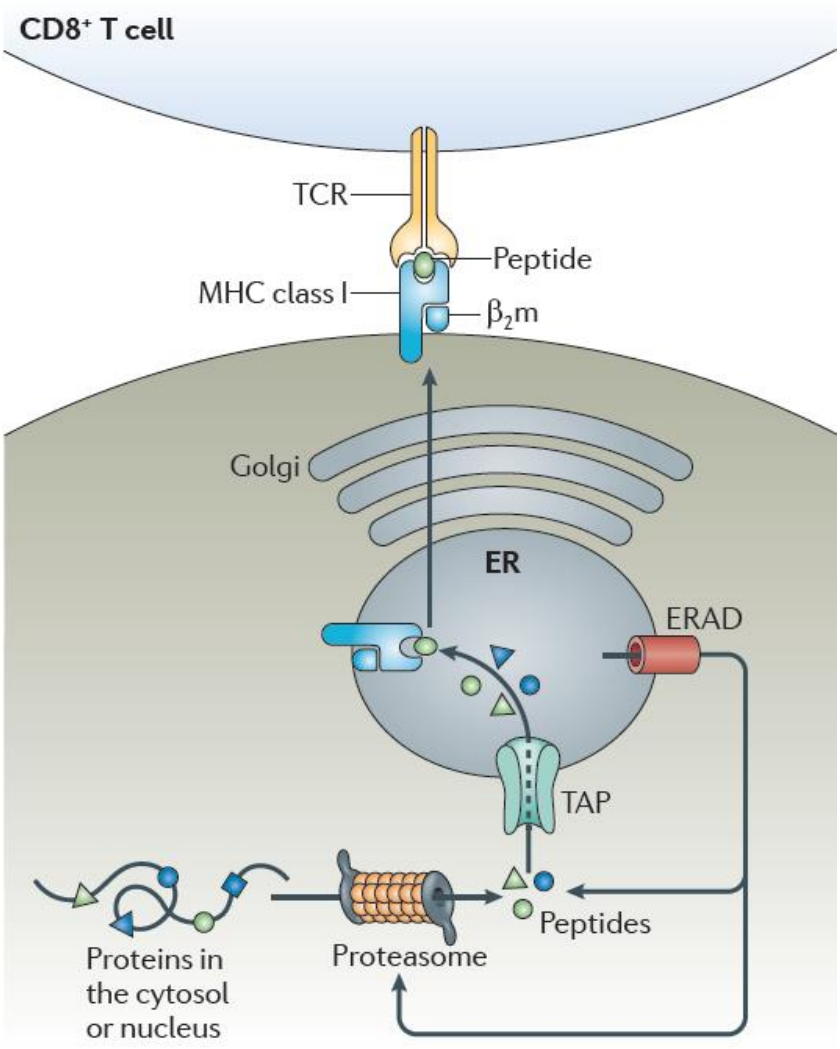


Figure 1.1 The MHC class I antigen presentation pathway (from (J. Neefjes et al. 2011)). The cytosol or nucleus-derived antigens are first degraded by the proteasome in the cytoplasm. The peptides generated are transported into the ER by the transporter associated with antigen presentation (TAP). The antigenic peptides are then loaded onto nascent MHC class I molecules. They are then transported though the Golgi and eventually expressed on the cell surface for CD8+ T cells recognition through T cell receptors (TCR). Incorrectly folded MHC class I proteins can be translocated into cytoplasm for proteasome degradation through the ER-associated protein degradation (ERAD) system.

1.2.1 Proteins degradation and the proteasome

Abnormal intracellular proteins are known to be the source of peptides for MHC class I complex, these proteins are thought to be digested by proteasome at the end of their functional lives. However, it has been found that around 30% to 70% of all proteins are rapidly degraded after synthesis before forming functional proteins (Schubert et al. 2000). These proteins, called defective ribosomal products (DRiPs), are normally the result of defective transcription or translation. DRiPs are immediately degraded for the prevention of protein aggregation, which would affect cell viability.

Unassociated MHC class I molecules are degraded through the ERAD system. In fact, a substantial fraction of MHC class I molecules never binds proper peptides according to pulse chase experiments (J. J. Neefjes and Ploegh 1988). These molecules could be degraded and presented to cell surface as antigens, such as the HLA-B27 peptide MRVTAPRTL derived from its own signal sequence (Purcell et al. 2001).

The proteasome is a ATP-dependent, multisubunit protease located in the nucleus and the cytoplasm in eukaryotes, its main functions are protein degradation and the production of MHC class I ligands (Peters et al. 1994). The 26S proteasome is composed of three subunits: one 20S core that is hollow and has protease activity and two 19S regulator subunits located at two ends of 20S core allowing the access of ubiquitinated and unfolded proteins (Figure 1.2). The proteasome determines the carboxyl terminus of its peptides products, which only need amino terminal trimming by aminopeptidases like ERAP1 before loading to MHC class I peptide binding groove (Cascio et al. 2001). In addition to the constitutive proteasome, the

immunoproteasome expressed by many immune cells and the thymus-specific proteasome expressed in thymic epithelial cells have been described (Sijts and Kloetzel 2011). Interestingly, immunoproteasomes were reported to be more active than constitutive proteasomes in contributing to protein homeostasis upon interferon-induced oxidative stress (Seifert et al. 2010). This indicates that immunoproteasomes may function to handle an expanded protein substrate pool rather than being more selective in making peptides for MHC class I presentation.

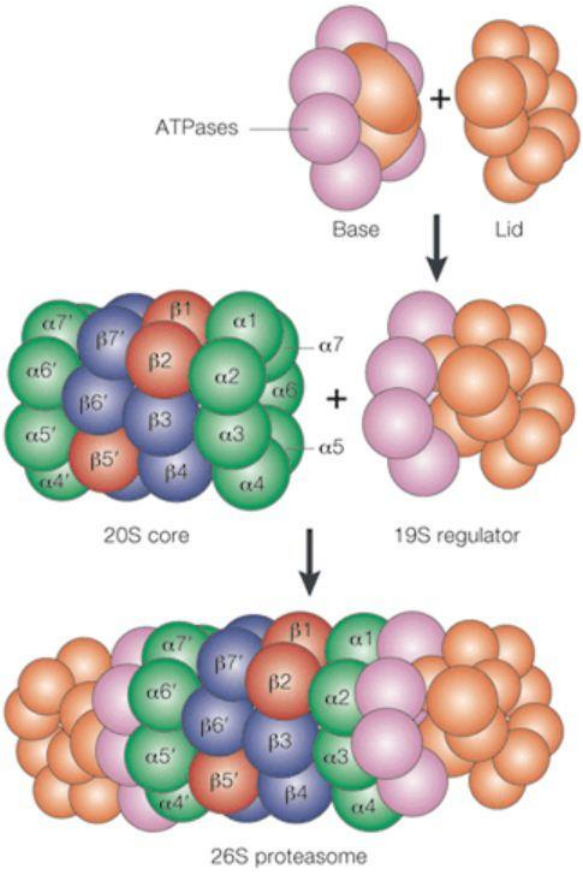


Figure 1.2 Proteasome structure (Adapted from (Kloetzel 2001). The 26S proteasome is constituted by one catalytic subunit (20S) and two regulatory subunits (19S). The 20S proteasome, the subunit has protease activity, consists of four heptameric staggered rings. The two outer rings are composed of α subunits (α1–α7, green), the two inner rings contain β- subunits (β1–β7, blue and red). β1, β2 and β5 subunits in red harbour the six active sites. The 19S subunit includes a base and a lid. The base is composed of six ATPase (purple) and two non-ATPase subunits (orange). The lid, composed by ten non-ATPase subunits (orange), is important for the binding of substrates.

1.2.2 Peptide loading complex (PLC)

The complex of TAP, tapasin, MHC class I, ERp57 and calreticulin is called the peptide loading complex (PLC). The transporter associated with antigen processing (TAP) is an ATP-dependent transporter located on the ER membrane, it binds 8 to 16-residue peptides with equal affinity, while translocation of peptides into the ER is most efficient for peptides that are 8 to 12 residues long (J. J. Neefjes et al. 1993). As TAP is not located at the nuclear face of the nucleus, nuclear peptides must first diffuse into the cytosol before their encounter with TAP (Reits et al. 2003).

In the ER, TAP binds to a key component of PLC, tapasin (Wearsch and Cresswell 2008). Tapasin recruits nascent MHC class I molecules to the TAP and enhances loading of MHC class I with high-affinity peptides. The nascent MHC class I complex without peptide is also stabilized by two additional chaperons, calreticulin and ERp57. Calreticulin is a lectin-like protein that binds N-glycosylated MHC class I molecules, which stabilize the interaction tapasin and MHC class I molecules. ERp57, along with calnexin (CNX), assists the folding of the MHC class I heavy chain (HC) and formation of disulfide bonds in the α2 and α3 domains. Overall, the HC/β2m dimer is stabilized by its association with TAP, tapasin, calreticulin and ERp57 in the PLC (Figure 1.3). Following binding to a peptide of sufficient affinity, MHC class I complex is disassociated with chaperons and passes the Golgi and eventually be expressed on cell surface.

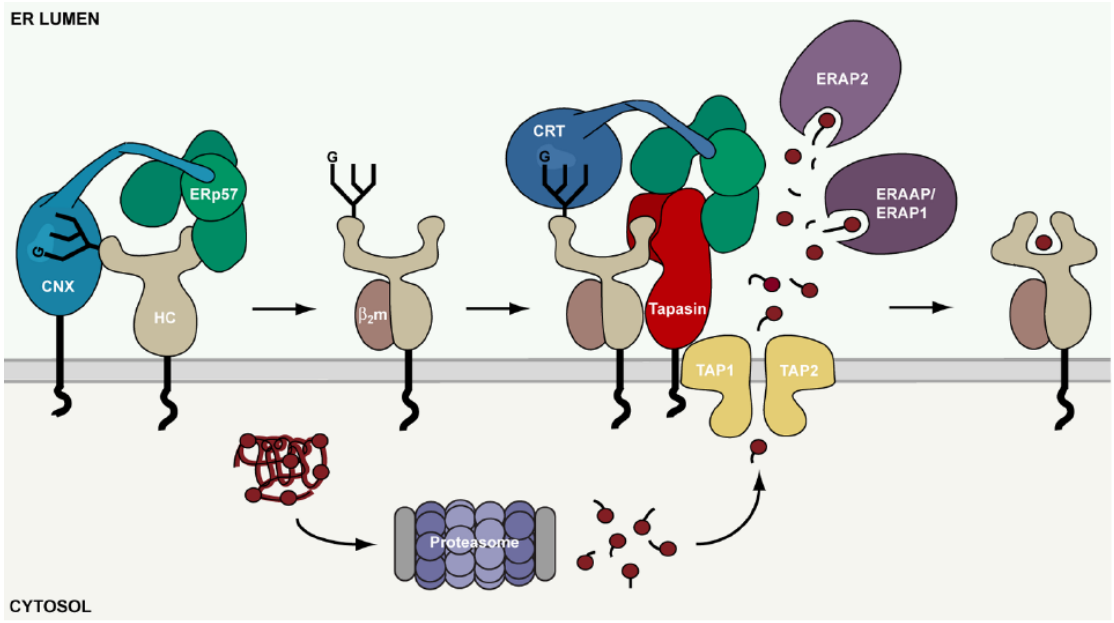


Figure 1.3 ER quality control and MHC class I assembly (adapted from (Wearsch and Cresswell 2008)). Calnexin (CNX) and ERp57 assist the folding of the MHC class I heavy chain (HC) and the formation of disulfide bonds in the α2 and α3 domains. The HC then assembles with β2m to generate an empty heterodimer which is stabilized by its association with other proteins in the peptide loading complex (PLC), tapasin, ERp57, calreticulin (CRT) and TAP. Tapasin bridges the nascent MHC class I to TAP to facilitate the efficient loading of peptides transported from the cytosol by TAP. These cytosolic protein-derived peptides are also further trimmed by ER aminopeptidases, ERAAP/ERAP1 (mice and humans) and ERAP2 (humans only). Optimal MHC peptides with appropriate length and affinity are loaded onto empty MHC class I molecule by the PLC. Following peptide binding, mature MHC class I dissociates from the PLC and traffics to the cell surface.

1.3 ERAP1 and AS

The association of ERAP1 polymorphism and AS was first reported in 2007 by genotyping 14,500 nonsynonymous single-nucleotide polymorphism (SNPs) (Burton et al. 2007). This association was then further confirmed in different populations in Europe and Asia (Choi et al. 2009; Davidson et al. 2009; D. Harvey et al. 2009; Pimentel-Santos et al. 2009; van der Heijde et al. 2009b). In addition to case-control studies, familial AS study also showed the association of AS with the ERAP1 SNP, rs30187 (Tsui et al. 2010).

ERAP1 is a key component in MHC class I presentation, it trims peptides to optimal length (8-9 residues) for their binding to MHC class I molecules (Saric et al. 2002; York et al. 2002). The finding that ERAP1 polymorphisms only affect AS risk in HLA-B27 positive individuals indicates the effects of ERAP1 on HLA-B27 presentation could be critical in AS pathogenesis (Evans et al. 2011). ERAP1 was also previously reported to cleave cell surface cytokines receptors: IL-1RII, IL-6Rα and TNFRI for downregulation of their proinflammatory signaling (X. e. a. Cui 2002; X. Cui et al. 2003b, 2003a). However, the level of soluble receptors (sTNFRI, sIL-1RII and sIL-6R) in AS patients was not affected by ERAP1 polymorphism (Haroon et al. 2010). Moreover, the levels of soluble TNFR and IL-6R in supernatant of cultured mouse spleen cells were similar between ERAAP -/- and control mouse (Evans et al. 2011). Therefore, the mechanism of ERAP1 polymorphism affecting AS susceptibility is more likely through HLA-B27 presentation rather than cytokine receptors cleavage.

1.3.1 ERAP1 and its functions

1.3.1.1 Identification of ERAP1

Proteolysis of abnormal proteins by proteasomes in the cytoplasm is the main source of peptides or peptide precursors presented by MHC class I molecules, which determines the C-terminal of the MHC class I peptide ligands (Cascio et al. 2001). In addition to cytoplasmic peptide trimming, a factor present in the endoplasmic reticulum (ER) was found to trim N-terminally extended MHC class I epitopes to the optimal length for presentation, which was completely inhibited by the metalloaminopeptidase inhibitor (Brouwenstijn et al. 2001; Komlosh et al. 2001). These finding suggest the existence of an ER-resident metalloaminopeptidase important for trimming of N-terminal extension of MHC class I-associated peptides. This unknown aminopeptidase was independently identified and termed ER-aminopeptidase 1 (ERAP1) (Saric et al. 2002) or ER aminopeptidase associated with antigen processing (ERAAP) (Serwold et al. 2002). I shall use ERAP1 hereafter.

1.3.1.2 ERAP1 peptide trimming properties

Following identification of ERAP1, this aminopeptidase's activity and substrate specificity was further characterized. Using in vitro peptide digestion, purified ERAP1 was found to trim peptides that were ten residues or longer and spared eight-residue peptides (York et al. 2002). Interestingly, some 9mer peptides were also digested, which indicates that ERAP1 both generates and degrades MHC class I peptides. Later, the "molecular ruler" mechanism of ERAP1 was suggested based on the finding that its substrates of preference are 9-16mer peptides, which are efficiently

transported into the ER by the transporter associated with antigen processing (TAP) (Chang et al. 2005). This hypothesis was also supported by the shared binding preference between ERAP1 and MHC class I molecules for peptides with hydrophobic C termini.

Trimming peptides from their N-termini, ERAP1's enzyme specificity should theoretically be affected by the N-terminal sequence of substrates. Indeed, in both of cell-free biochemical system and in intact cells, ERAP1 was found to chop off various amino acids on the N terminus of epitope precursors but at markedly different rates (Hearn et al. 2009). Among the twenty possible amino acids, L, M, and Y were cleaved off efficiently while D, E, K, P, R, V, and W were inefficiently trimmed, the rest were moderately cut off. These preferences were independent of the epitope core sequence. This finding was further supported by the abundance of amino acids found to be efficiently trimmed by ERAP1 in the N-terminal flanking sequences of known epitopes from database.

Another unique property of ERAP1 is that its substrate preference is affected by the peptide internal sequence (Evnouchidou et al. 2008). By in vitro digestion of a collection of peptides, ERAP1 was found to preferentially trim peptides with internal sequences containing positively charged or hydrophobic residues when N-terminal residue is identical. The trimming rate was increased by up to 100 fold for single residue substitutions and more than 40,000 fold for multiple residue substitutions. Moreover, another key characteristic of ERAP1 is its inability to cleave N-terminal residue when it is followed by a proline (Serwold et al. 2001; Serwold et al. 2002).

1.3.1.3 ERAP1 and MHC class I expression and peptide presentation

If and how does ERAP1 effect MHC class I expression and peptide presentation are important questions for better understanding of the role of ERAP1 in MHC class I pathway. To address this, mice lacking ERAAP were generated. Loss of ERAAP was found to disrupt the generation of natural MHC class I peptides, decrease the stability of MHC class I complex and reduce CD8(+) T cell responses (G. E. Hammer et al. 2006). In addition to causing the loss of antigenic peptides, the absence of ERAAP also produced many unstable and highly immunogenic peptides which elicited potent CD8+ T cell and B cell responses (G. E. Hammer et al. 2007).

In addition to natural MHC class I peptides, the presentation of viral peptides are also affected by the absence of ERAP1. In mice infected with vaccinia virus or lymphocytic choriomeningitis virus (LCMV), ERAP1-knockout altered the immune response to different viral peptides. The results were diverse, an increase, a decrease or no change (York et al. 2006). This study indicates that ERAP1 plays an important role in immunodominance by generating and destroying MHC class I epitopes.

The importance of ERAP1 in MHC class I presentation is also reflected by the recently reported viral evasion mechanism through microRNA targeting ERAP1 (Kim et al. 2011). Human cytomegalovirus microRNA, miR-US4-1, was found to block the presentation of viral antigenic peptides to CD8(+) T cell responses by down-regulating ERAP1 expression.

MHC class I molecules do not just passively receive peptides generated by ERAP1, its presence has been shown to be essential for generation of optimal epitopes. In the original study showing that ER cleaves off the N terminal flanking residues of the final antigenic peptide, this property was found only with the existence of appropriate MHC molecules (Brouwenstijn et al. 2001). Later, ERAAP was found to degrade the antigenic precursors in the ER in the absence of MHC I but needs MHC I molecules to generate the final peptides because of the protection of proteolytic intermediates through binding by MHC class I (Kanaseki et al. 2006). The protection of peptides by MHC class I against ERAP1 cleavage is supported by an in vitro ERAP1 trimming study showing that H-2L^{d} class I molecule protects an HIV N-extended epitope from digestion by ERAP1 (Infantes et al. 2010).

1.3.1.4 Crystal structures of ERAP1

Solving the crystal structure of ERAP1 would help for better understanding of the mechanism of ERAP1's unique catalytic properties, and provide a structural explanation for the reduced activity previously observed in an ERAP1 variant, K528R, which is the most confirmed AS-associated protective SNP (Evans et al. 2011; Kochan et al. 2011). In 2011, two groups independently determined the crystal structure of human wild-type ERAP1 (Kochan et al. 2011; Nguyen et al. 2011). One group co-crystallized the ERAP1 bound to bestatin, an aminopeptidase inhibitor at 3.0 Å and revealed the open conformation of ERAP1 (Nguyen et al. 2011). The other crystallized ERAP1 in two different space groups and determined two crystal forms indicting both of open and closed states of ERAP1 (Kochan et al. 2011) shown in

Figure 1.4. ERAP1 consists of four domains (I: green, II: orange, III: yellow, IV: teal), with domain II being the catalytic site and domain IV being the regulatory site. The regulatory domain IV shields the catalytic domain II in the closed state and moves away from the protease domain (II) for the access of substrate to the active site.

ERAP1 polymorphisms reported associated with AS were labeled on the structure (Fig. 1.5A). The multiply confirmed mutation, Lys528Arg, is distant to the active site located on the surface of domain III. However, it forms polar interactions in the closed state with surrounding residues Asn414, Ser415, and Ser416 (Fig. 1.5B) from the catalytic domain which are further stabilized by Arg906, Gln910 and Glu913from domain IV. In the open conformation, Lys528 interacts only with Asn414 and Ser416 (Fig. 1.5C). These interactions of Lys528 with adjacent residues are possibly altered by substitution of Lys by Arg through formation of hydrogen bonds, polar changes or increased side-chain size. The Lys528Arg mutation could therefore result in defective transitions from the open to the closed form, thus affecting catalysis (Kochan et al. 2011).

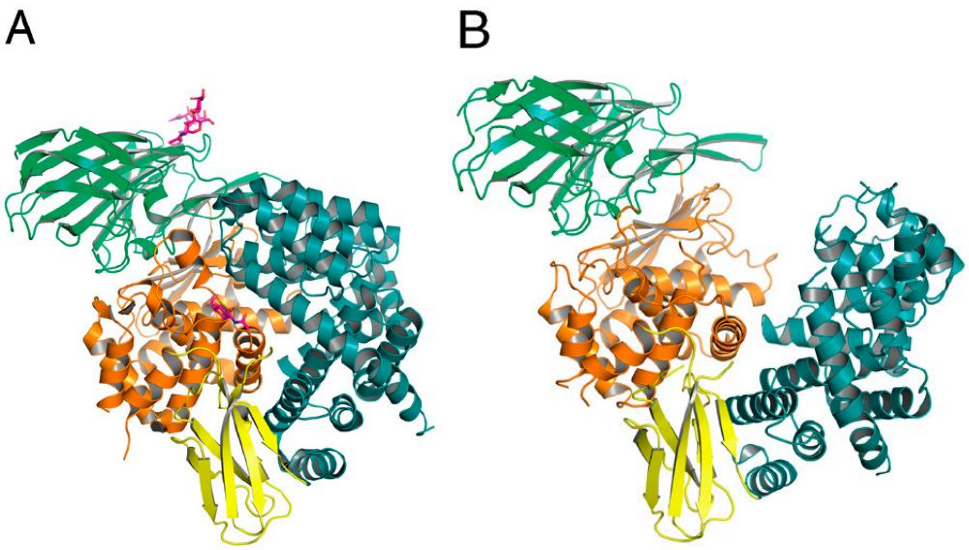


Figure 1.4 Crystal structure of human ERAP1. A. Closed form of ERAP1. ERAP1 consists of four domains (I: green, II: orange, III: yellow, IV: teal) N-glycosylation sites are colored in magenta. B Open form of ERAP1 (adapted from (Kochan et al. 2011).

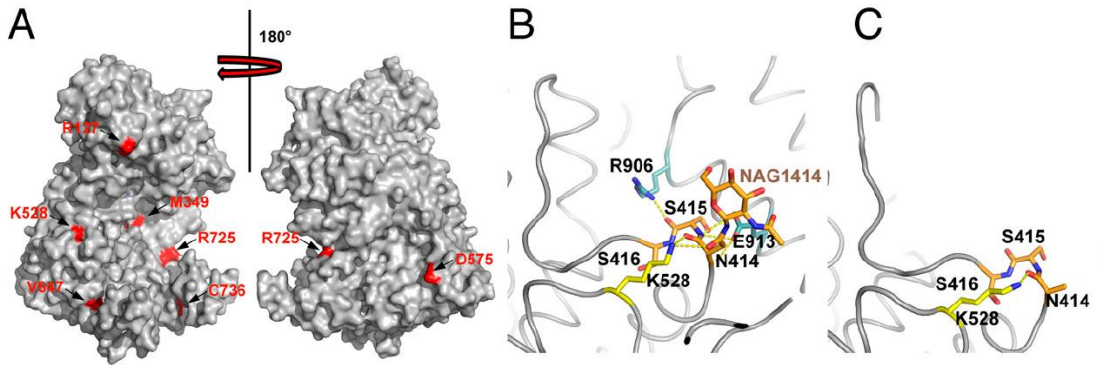


Figure 1.5 Polymorphism associated with ankylosing spondylitis. A. Surface representation of ERAP1 Mutations associated to AS is colored in red. B & C. Interactions of Lys528 with surrounding amino acids in the closed state (B) and the open state (C) (from (Kochan et al. 2011)).

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1.3.1.5 ERAP1 and cytokine receptor shedding

In 2002, one group reported the interaction of ERAP1 and TNFR1 in a yeast two-hybrid study and showed the co-localization of ERAP1 and TNFR1 on bronchial airway cells by confocal microscope (X. Cui et al. 2002). They also showed that the over-expression of ERAP1 decreased cell surface TNFR1 and increased the level of soluble TNFR1 in the culture supernatant. Later, ERAP1 was reported also to be required for shedding of interleukin-6 receptor (IL6-R) and the type II IL-1 decoy receptor (IL-1RII) (X. Cui et al. 2003b, 2003a). Therefore, the altered cytokine receptor shedding caused by ERAP1 polymorphisms could possibly change the

circulating cytokine receptors and subsequently affect cytokine activity and AS progress. However, a recent study using ELISA showed that none of the three circulating receptors, TNFR1, IL6-R and IL-1RII, correlate with ERAP1 polymorphisms (Haroon et al. 2010). TNFR1 was associated with the inflammation markers, C-reactive protein and the erythrocyte sedimentation rate, which suggested that TNFR1 cleavage might be a response rather than the cause of inflammation.

1.3.1.6 ERAP1 and ERAP2

Endoplasmic reticulum aminopeptidase 2 (ERAP2) is an ERAP1 homologue with different substrate preference, it has been showed to concertedly trim long peptides with ERAP1 by both substrate digestion in vitro and cellular antigen presentation in vivo (Saveanu et al. 2005). ERAP2 shares 49% amino acid sequence homology with ERAP1 and was found to have the crystal structure similar to that of ERAP1 in its closed conformation (Ascher et al. 2012; Birtley et al. 2012).

ERAP1 and ERAP2 were also found localized together in the ER by immunofluorescence staining and likely form heterodimeric complexes by immunoprecipitation (Saveanu et al. 2005). Although the genetic association of ERAP2 with AS was not found in case-control studies, a haplotype containing rs27044[G] rs30187[T] for ERAP1 and rs2549782[T] for ERAP2 was reported associated with familial ankylosing spondylitis (Tsui et al. 2010). Whether ERAP2 is involved in AS pathogenesis needs further investigation.

1.3.2 ERAP1 and HLA-B27 in AS pathogenesis

As mentioned above, ERAP1 only affects AS risk in population carrying HLA-B27 (Evans et al. 2011), which implies the importance of ERAP1 and HLA-B27 interaction in AS pathogenesis. Throughout years of intensive and extensive research on AS, three main theories have been proposed: "molecular mimicry", "cell surface B27 free heavy chain expression" and "unfolded protein response (UPR)". As the key enzyme producing peptide ligands for MHC class I molecules, ERAP1 could potentially contribute to AS development through all three theories.

1.3.2.1 ERAP1 and molecular mimicry

MHC class I molecules' classical function is the presentation of antigenic peptide to CD8+ T lymphocytes for immune surveillance. To prevent autoimmunity, the host is usually tolerant to peptides that are derived from self proteins. It is, however, possible that tolerance to a self peptide will be lost if an infectious pathogen activates the immune system through a pathogen-derived peptide that mimics the self peptide. This mechanism forms the foundation of the arthritogenic peptide hypothesis, also known as the molecular mimicry hypothesis (Lopez de Castro 2007). Occurrence of this mechanism *in vivo* is supported by the discovery of T lymphocytes that are responsive to peptides derived from self antigens, collagen and aggrecan from cartilage and vasoactive intestinal peptide receptor 1 (VIPR1) (Atagunduz et al. 2005; Fiorillo et al. 2000; Zou et al. 2005). Abnormal ERAP1 could possibly cause or enhance the generation of mimicry peptides and therefore could be involved in this hypothesis. However, this theory is confronted by the finding that the CD8 T cells are not essential to the pathogenesis of arthritis or colitis in HLA-B27 transgenic rats and arthritis or spondylitis in HLA–B27/human β2-microglobulin–transgenic rats (May et

al. 2003; Taurog et al. 2009).

1.3.2.2 ERAP1 and unfolded protein response

It was first raised in 1999 that misfolding of HLA-B27 due to its B pocket was possibly involved in Spondyloarthtitis susceptibility conferred by HLA-B27 (Mear et al. 1999). Later, the unpaired Cys67 and residues at the base of the B pocket were shown to dramatically impair HLA-B27 heavy chain folding in contribution to HLA-B27 misfolding (Dangoria et al. 2002). This theory is supported by the finding that the unfolded protein response (UPR) is activated in macrophages derived from the bone marrow of HLA-B27/human β_2m transgenic rats but not in HLA-B7/human β_2m transgenic or non-transgenic rats (Turner et al. 2005). The activation of the UPR in rat macrophages was later found to be caused by the accumulation of B27 misfolded heavy chains and correlated strongly with the degree of HLA-B27 up-regulation (Turner et al. 2007). In addition to the animal model, high UPR-marker expression in synovial biopsies of active AS patients and increased expressions of Bip, CHOP, and XBP-1 in monocytes/macrophages of patients with SpA also suggest the possible participation of UPR in the pathogenesis of AS (Dong et al. 2008; Feng et al. 2012; Gu et al. 2002).

UPR has been shown to be activated in AS, but how does it contribute to disease development and progress? In HLA-B27/human β_2m transgenic rats, HLA-B27 misfolding and UPR activation in macrophages was found to cause ER stress and enhance the production of IL-23, a cytokine up-regulate the IL-17. The strong activation of IL-23/IL-17 axis in the colon of B27/human β_2m -transgenic rats and the expansion of Th17 cells in SpA patients makes IL-23/IL-17 axis a potential link

between UPR and immune dysregulation in AS (DeLay et al. 2009; Jandus et al. 2008).

ERAP1 supplies ligands for HLA-B27 folding in the ER, its abnormality could therefore destroy too much or generate too little peptides to HLA-B27 which could both increase proportion of misfolded heavy chain and activate or enhance the UPR.

However, this theory was also challenged by other studies. Firstly, the induction of additional copies of human β_2m to HLA-B27-transgenic rats reduced UPR but did not ease arthritis and spondylitis (Tran et al. 2006). Secondly, in AS patients, IL-23 up-regulation by lipopolysaccharide (LPS) in macrophage was dissociated with the unfolded protein response (Zeng et al. 2011). Moreover, the confirmation of interleukin-17 overexpression in peripheral blood and gut of SpA patients has also encountered difficulties (Ciccia et al. 2009; Shen et al. 2009).

1.3.2.3 Possible influence of ERAP1 on cell surface B27 free heavy chains

I have already discussed how the intracellular misfolded B27 causes ER stress and activates the IL23/IL17 axis forming the cornerstone of UPR hypothesis. Alternatively, cell surface B27 heavy chains, including homodimers formed after dissociation of cell surface heterotrimers, are capable of interacting with immune regulatory receptors such as KIR3DL2. This may contribute to AS pathogenesis and is termed the “cell surface B27 free heavy chain” hypothesis.

The unusual ability of HLA-B27 to form β_{2m}-free heavy chain homodimers in vitro was first reported in 1999 (Allen et al. 1999). An unpaired Cys67 in the alpha1

domain was shown important for this dimerization through disulphide bonding. Later, it was reported that not only Cys67 but also Cys164 can be involved in homodimer formation in HLA-B27 expressing cells (the rat C58 thymoma cell line and human HeLa cells) (Antoniou et al. 2004). Populations of these B27 homodimers were reactive with the HC10 antibody (specific for HLA class I free heavy chains), the W6/32 antibody (specific for the α2 helix of native class I complexes) but not the ME1 antibody (recognizing the α1 helix of HLA-B27, -B7, -B42, -B67, -B73 and Bw22) (Allen et al. 1999; Kollnberger et al. 2002).

In addition to in vitro study, many studies have shown the presence of intracellular and/or cell surface B27 homodimer in cell lines and blood/joint samples from animal model and AS patient. It was first shown that the free heavy chains were found at the surface of HLA-B27-transfected T2 cells by surface labeling and W6/32 immunoprecipitation (Allen et al. 1999). Later, B27 homodimers were shown to be expressed by both C1R.B27 and PBMCs from an HLA-B27 positive AS patient by HC10 immunoprecipitation using 2-dimensional isoelectric focusing (2D-IEF) (Kollnberger et al. 2002). In HLA-B27 transgenic rats and strains of HLA-B27-transgenic β_2m-deficient mice, β_2m-free HLA-B27 heavy chain homodimers and multimers were also expressed both intracellularly and on the surface of leukocytes as detected by HC-10 (Kollnberger et al. 2004). Moreover, cell-surface HLA-B27 homodimers were also reported to be expressed on monocytes of SpA patients to a higher level compared to normal controls and patients with rheumatoid arthritis by HC10 staining (Kollnberger et al. 2002; Tsai et al. 2002). Infiltration of these free heavy chain-expressing monocytes was also found in the synovium of an hip joint of a AS patient by immunohistochemistry staining using

HC10 antibody (Tsai et al. 2002). More recently, the formation of both intracelluar and cell surface HLA-B27 homodimers was also reported in activated dendritic-like cell lines and LPS-stimulated human monocyte-derived dendritic cells (Santos et al. 2008).

HLA-B27 has been shown capable of forming β_2m-free disulfide-bonded homodimers in vitro, but what is the mechanism of HLA-B27 homodimer formation in vivo? Studies using lymphoblastoid cells suggests that HLA-B27 homodimers form in the ER but appear unable to egress to the cell surface in human cells (Bird et al. 2003). Cell surface HLA-B27 homodimers are likely formed from cell-surface heterodimers via an endosome-dependent recycling pathway.

How cell surface HLA-B27 free heavy chains are involved in AS pathogenesis is a key question to be answered in the cell surface B27 free heavy chain theory. Several immune regulatory receptors have been found bind to HLA-B27 homodimers including KIR3DL1, KIR3DL2, immunoglobulin-like transcript 4 (ILT4)/ leukocyte Ig-like receptor B2 (LILRB2) in humans and rodent paired immunoglobulin-like receptors (PIRs) by staining using B27 homodimer tetramers (Kollnberger et al. 2002; Kollnberger et al. 2004). Strikingly, KIR3DL2 only binds to B27 homodimers but not β_2m-associated B27 heterotrimers (Kollnberger and Bowness 2009). Additionally, the interaction of B27 homodimers with KIR3DL2 is independent of peptide used in refolding (Kollnberger et al. 2007). More recently, the leukocyte Ig-like Receptor B2 (LILRB2) was also shown to bind to HLA-B27 homodimers and free heavy chains more strongly than classical HLA-B27 heterotrimer (Giles et al. 2012). These data supports the hypothesis that B27 homodimers may interact either qualitatively or

quantitatively differently with immune receptors to promote AS.

In SpA patients, KIR3DL2 was found to be more commonly expressed by NK and CD4+ T cells from peripheral blood and synovial fluid compared to RA and healthy controls (Chan et al. 2005). Furthermore, KIR3DL2+ NK cells were reported to be protected from apoptosis by culture with a cell line expressing B27 homodimers. Additionally, KIR3DL2+ IL-17-producing CD4+ T cells were found to be increased in AS compared to healthy controls (Bowness et al. 2011). Moreover, APCs expressing B27 homodimers stimulated the survival, proliferation, and IL-17 production of KIR3DL2+ CD4 T cells.

To confirm B27 homodimer expression in SpA and its potential as a therapeutic target, a monoclonal antibody against B27 homodimer (HD6) has been generated (Payeli et al. 2012). HD6 also inhibited the survival and proliferation of KIR3DL2+ NK cells by competing with KIR3DL2 for binding to HLA-B27 homodimer. IL17 produced by peripheral blood mononuclear cells (PBMCs) from AS patients was also inhibited by this antibody. These results support the “cell surface B27 free heavy chain” theory and suggest the potential of the HD6 antibody in SpA research and treatment.

Since ERAP1 is the key aminopeptidase for generation of HLA-B27 peptides; alterations of its function could affect the quality of peptides provided to B27 within the ER. Cell surface B27 molecules folded with sub-optimal peptides might be less stable and lose their peptides. Consequently, expression of cell surface B27 free heavy chains are increased which contribute to AS pathogenesis through interaction with immune receptors like KIR3DL2 and LILRB2.

1.4 Project aims

Despite of decades of intensive research, the pathogenesis of Ankylosing spondylitis remains unclear. Recent breakthroughs in AS genetics have provided insight into of disease mechanism by identifying novel AS-associated genes. Among these genes, ERAP1 has been shown to have the strongest association with AS and, strikingly, only affects disease susceptibility in HLA-B27 + individuals. I hypothesized that, as a key aminopeptidase in MHC class I presentation, ERAP1 might be involved in AS through effecting the expression and peptide presentation of HLA-B27. In this thesis, I have aimed to answer following questions which will be addressed separately in chapter 3, 4, 5 and 6.

:

1. Does ERAP1 mutation change its enzyme activity towards B27 antigenic peptide precursors?
2. Does ERAP1 silencing change the repertoire of B27-bound peptides?
3. Does ERAP1 silencing affect the presentation of a defined B27 epitope to cytotoxic T lymphocytes?
4. Does ERAP1 silencing or inhibition affect the expression of cell surface B27 free heavy chains?

Chapter 2. Materials and methods

2.1 Cell lines

A MHC class I-low cell line, LCL 721.221 (hereafter termed 221), was transfected to express HLA-B*27:05 (221.B27) (Shimizu et al. 1988). In addition to HLA-B27, 221 cells were also transfected to express HLA-B35 and HLA-A3 as control cell lines. The second MHC class I-low cell lines, C1R.B27 cells were previously generated from C1R, an HLA-A, B negative mutant B lymphoblastoid cell line, by HLA-B*27:05 transfection (Bird et al. 2003).

HeLa cell, a cell line derived from cervical cancer cells, was transduced to stably express HLA-B*27:05 (HeLa.B27). ERAAP-/- mouse fibroblasts were transduced with lentivirus to stably express HLA-B*27:05 (mFib.B27) or HA-tagged HLA-B*B27:05 (mFib.B27HA). All three cell lines have been cultured in

C1R.B27 and HeLa.B27 were then treated with ERAP1-shRNA/ctr-shRNA lentivirus to stably silencing ERAP1 expression (C1R.B27-ERAP1-shRNA/ctr-shRNA and HeLa.B27-ERAP1-shRNA/ctr-shRNA).

2.2 Cell culture

C1R.B27 and 221.B27/B35/A3 cells were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 0.1 mg/ml of streptomycin, and 100 units/ml of penicillin (R10). R10 supplemented with G418 (500 μg/ml) was used for cell culture every one month to maintain the expression of HLA-B27/B35/A3.

HeLa.B27, mFib.B27 and mFib.B27HA cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 0.1 mg/ml of streptomycin, and 100 units/ml of penicillin (D10).

HeLa.B27 and C1R.B27 cells transduced with lentiviral shRNA were cultured in the same condition as HeLa.B27 and C1R.B27 cells.

2.3 Peptide trimming assay

The substrates peptides were chemically synthesized and purified with HPLC. Then 100 μM peptide substrates were incubated with recombinant ERAP1s in time course experiments at 37℃ (Up to 6h, 16 time points). At each time point, 50ul of reaction was taken out and mixed with 0.6% trifluoroacetic acid to stop the reaction. The peptide products were analyzed on a Q-TOF mass spectrometry. The signal intensity of all peptide products were added up, then the percentage of each product was graphed.

2.4 Synthetic peptides

Peptides were synthesized in the in-house facility by Dr Kati di Gleria using an automated peptide synthesizer (396 MPS, Advanced Chemtech) by conventional solid phase Fmoc chemistry, then analyzed for purity of >90% by reverse phase high performance liquid chromatography (HPLC). Peptides for in vitro peptide trimming assay were further purified by reverse HPLC. Peptides were dissolved in PBS or DMSO and stored at -20 ^{o}C.

2.5 Leu-AMC (L-Leucine-4-methylcoumaryl-7-amide) fluorescence activity assay

Reactions were performed in cuvettes using a SPECTRAmax M2 spectrofluorometer (Molecular Devices) with a final reaction volume of 200 µl. ERAP1 (250 ng/ml) was added to 500 µM Leu-AMC in 20mM Tris-HCL pH 7.5, NaCl 100mM and the fluorescent signal was observed over 2.5 min by monitoring excitation at 380 nm and emission at 460 nm. Activities of ERAP1 before and after heat incubation (6 hours at 37 degree) were compared, assuming un-heated ERAP1 to have 100 percent activity.

2.6 Expression and purification of recombinant ERAP1s

Wild type and mutant ERAP1 were cloned into pFastBac and transformed into competent DH10Bac Escherichia coli cells containing the baculovirus genome. The purified bacmid DNAs were used to transfect Sf9 (Spodoptera frugiperda) insect cells. Viruses were collected three days post infection and after amplification used for infection and protein production. For recombinant protein production, cell cultures

were collected 3 to 5 days post infection and ERAP1 purified from the culture medium by immobilized metal-ion affinity chromatography (**IMAC**).

2.7 Generation of lentivirus and lentiviral transduction of cell lines

All lentivirus-transduced cell lines generated in this thesis were produced using the supernatant from cultured HEK 293T cells that had been transiently transfected to produce lentivirus particles as described below. The HLA-B*27:05 and HA-tagged HLA-B*27:05 lentiviral plasmids were previously constructed in our lab using the pHR-SIN-BX-IRES-Em plasmid. The plenti4v5dest plasmid (V496–10, invitrogen) was used to generate ERAP1 constructs.

To make lentivirus particles, 293T cells in D10 media were co-transfected at a ratio of 1:1:1 with 3 different plasmids: 1) a lentiviral plasmid (pHR-SIN-BX-IRES-Em or); plenti4v5dest); 2) an HIV-based packaging plasmid (pCMVR8.91); 3) an HIV-based envelope plasmid (pMD-G). The pHR-SIN-BX-IRES-Em plasmid contains the transgene, which is flanked by the HIV-1 derived cis-acting sequences necessary for packaging, reverse transcription, and integration (a CMV promoter and the Woodchuck post-transcriptional regulatory element (WPRE)), and contains an eGFP gene. The pCMVR8.91 and pMD-G plasmids encode gag/pol (with *tat*, *rev*, and virulence genes deleted) and the G protein of VSV (VSV-G), respectively. The resulting disabled particles are able to infect most mammalian cells (Zufferey et al., 1997). 100 ul of DMEM with addition of 3 ul GeneJuice (70967, Merck Millipore) was vortexed and incubated at room temperature for 5 min before it is mixed with 1.5

ug of DNA (0.5 ug from each plasmids) and incubated at room temperature for another 15 minutes. After that, the 100µl mixture was added to 293T cells (30~50% confluent) in 6 well plates with 1ml D10 media and cells were incubated at 37 ℃ for 3 days. Supernatant was aspirated and centrifuged (3,000rpm, 10min) and filtered through 0.45 um filter disk. 250-500µl used to infect $2x10^5$ target cells in 24-well plates. After 5 days, successful lentiviral transduction was verified by either checking for GFP expression where appropriate or by staining with MHC class I-specific antibodies.

2.8 FACS staining and analysis

Cells were added to 96-well plates at 1-2 x 10^5/well and washed with cold PBS before addition of primary antibodies against surface markers at appropriate concentrations in 50µl R10. Cells were incubated on ice for 20 min, washed twice in FACS wash buffer (PBS with 1% FCS) and then, where necessary, incubated with secondary antibodies for a further 20 min on ice in dark. Cells were washed again twice in FACS wash buffer and then fixed by addition of 200µl/well fixing buffer (FACS wash buffer with 1.5% formaldehyde (47608, Sigma, UK)). To exclude dead cells, a violet LIVE/DEAD® stain was included by adding 4 ul/well of a 1:100 dilution of the violet fluorescent reactive dye reconstituted as per the manufacturer's instructions (L34955, Invitrogen, UK). Stained cells were read on a 9-colour CyAn ADP flow cytometer (Dako, Denmark) and analyzed using FlowJo software.

2.9 Cryopreservation of cells

Cells were centrifuged at 1500 rpm for 3 min and the pellet was resuspended in cold

freezing media (heat-inactivated FCS supplemented with 10% sterile dimethyl sulphoxide (D2650, Sigma-Aldrich, UK)). 1ml aliquots were transferred into chilled screw-cap freezing vials that were then placed into a cryo-container (5100, Nalgene, USA) and stored at -80 ℃ or in liquid nitrogen for long term storage. Frozen cells were removed from storage and immediately placed in a 37 ℃ water bath for thawing, washed once by centrifugation at 1500 rpm for 3 min in warm R10, then resuspended in appropriate media.

2.10 ERAP1 inhibition assay by Leucinethiol

Cells were split and then cultured for 24 hours in 6-well plates in R10 with or without 30uM of Leucinethiol ($4x10^5$ cells/well in 2ml of medium). Here, DTT was not used to activate Leucinethiol because its presence remarkably reduced HC-10 staining potentially through breaking down the disulfide bond that is crucial for HLA-B27 heavy chain homodimer formation (data not shown). HLA-B27 expression was detected by the staining with HC-10, ME1 and isotype control antibodies followed by flow cytometery analysis.

2.11 Construction of ERAP1 lentiviral plasmid

Firstly, primers were designed to produce blunt-end PCR products with CACC sequence at the 5′ end and Kozak consensus sequence (CACCATG). The PCR products were TOPO-cloned into the pENTR™ TOPO vector and then the recombinant vectors were transformed into One Shot competent *E. coli*. The TOPO vector with ERAP1 sequence was isolated from the positive transformant, and ligated

to Plenti4V5dest vector through LR recombination reaction. The reaction mix was then used to transform One Shot Stbl3™ Competent *E. coli*; and the ERAP1 lentiviral plasmid was isolated from positive transformant.

2.12 Stable ERAP1 silencing by lentiviral ERAP1-shRNA

Five ERAP1 siRNA candidates were screened using HeLa.B27 cells; the most effective one was chosen to make ERAP1-shRNA. A 60mer oligonucleotide was first designed to connect the sense and antisense sequence of selected siRNA with a 9mer oligonucleotide (TTCAAGAGA) (Figure 2.1). This 60-mer oligonucleotide was annealed with its antisense, and then cloned to a shRNA plasmid (Oligoengine pSUPER RNAi System). To stably express ERAP1-shRNA, H1 promoter and the ERAP1-shRNA was cut out from the shRNA plasmid and cloned to a lentiviral plasmid which contains the IRES-GFP. The ERAP1-shRNA was cloned into a lentiviral plasmid for stably transducing C1R.B27 and HeLa.B27 cells. A lentiviral control-shRNA was also constructed and applied in parallel. GFP, encoded by an IRES-GFP gene contained in the plasmid, was used to monitor the level of shRNA expression.

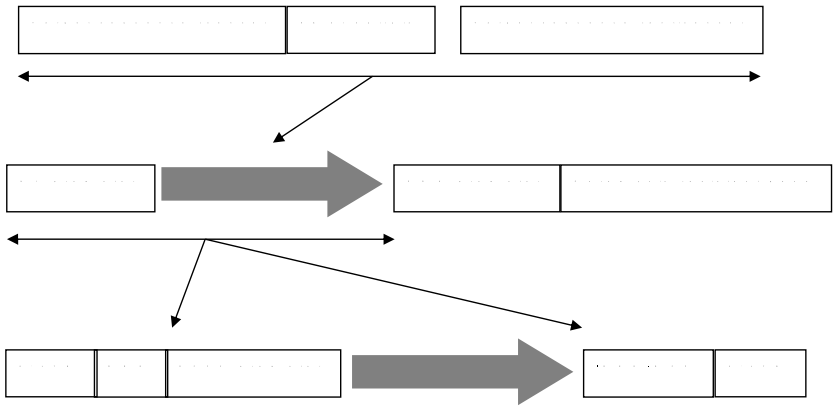


Figure 2.1 Construction of lentiviral ERAP1-shRNA.

2.13 Transfection of ERAP1 lentiviral plasmid into ERAAP-/- mFib.B27 cells

The ERAAP-/- mFib.B27 cells were plated onto 6-well plate the day before transfection to reach 50% confluency on the day of transfection. For each well, 1.5ug of DNA, 4.5ul of GeneJuice and 100ul of DMEM were used for transfection. Firstly, GeneJuice was added to DMEM dropwise, vortexed and incubated at room temperature for 5 minutes. Then the GeneJuice/DMEM mix was added to plasmid DNA, gently mixed and incubated at room temperature for 30 minutes. Finally, the mixture was added to cells evenly. The cells were analyzed 48 hours after transfection.

2.14 Peptide labelling using stable isotope labelling with amino acids in cell culture (SILAC)

For better comparison of B27-bound peptides from HeLa.B27 ctr-shRNA and HeLa.B27 ERAP1-shRNA, these two lines were labelled with normal ^{14}N4 L-Arginine (Arg-0) and 4 Da heavier isotope ^{15}N4 L-Arginine (Arg-4) separately. The cells were grown in the Arginine-depleted SILAC medium supplemented with Arg-0 or Arg-4 for 10 passages to achieve complete incorporation of the isotope-labelled amino acid before they were mixed before lysis and preparation for isolation of HLA-B27 bound peptides. The same approach was applied for C1R.B27 cells. The Arg-0 or Arg-4 labelled SILAC medium was supplemented with 10% dialyzed fetal bovine serum, 0.1 mg/ml of streptomycin, and 100 units/ml of penicillin.

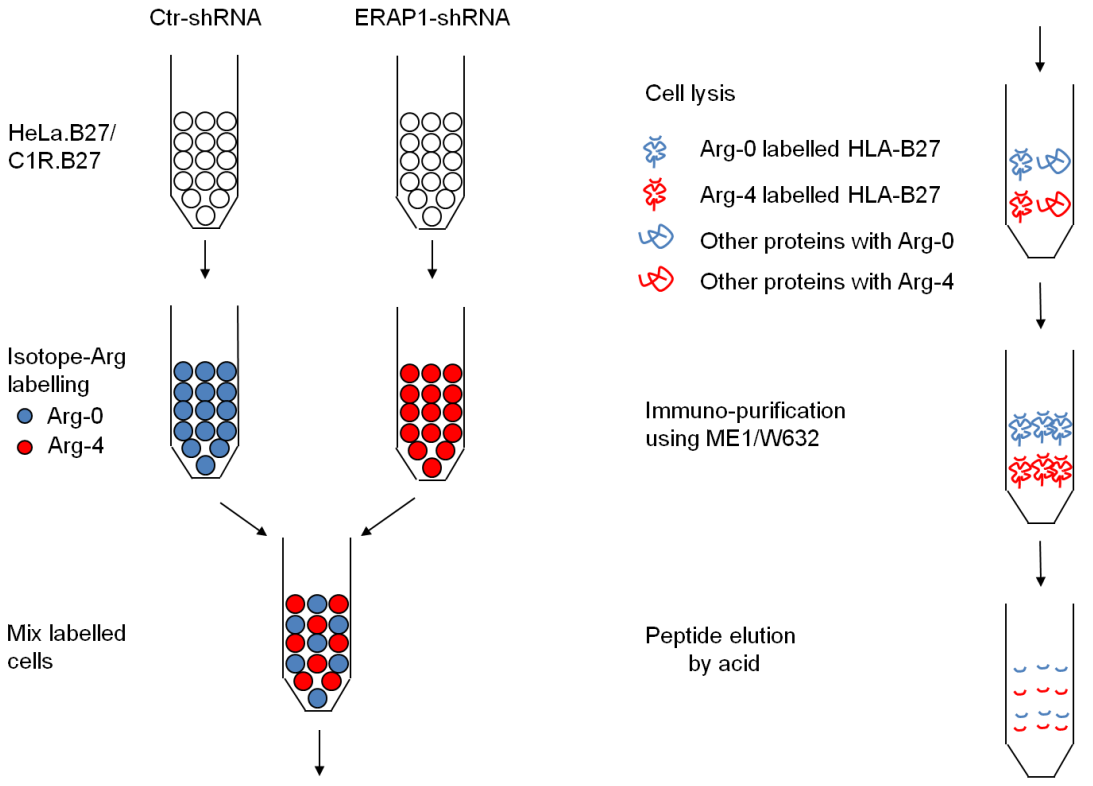


Figure 2.2 Peptide labelling using stable isotope labelling with amino acids in cell culture (SILAC)

2.15 Isolation of HLA-B27 bound peptides

1-5 x10^8 SILAC-labelled HeLa.B27 or C1R.B27 cells were lysed, the HLA-B27 complexes in the lysate were immunoprecipitated with ME-1 (specific for HLA-B27, B7, Bw22) or W632 (specific for a monomorphic HLA-A, B, C determinant) using protein G Dynabeads or protein G sepharose. The peptides bound to HLA-B27 were eluted by addition of 0.6% trifluoroacetic acid (TFA) or 10% acetic acid. The supernatant was centrifuged at 13,000 rpm at 4 degree for 30min to remove precipitated proteins. The final supernatant was kept at -80 ℃ until analysis.

2.16 Endoplasmic reticulum enrichment

In order to study the effect of ERAP1 silencing on peptides in the ER, ER was enriched through ultracentrifugation. 1-5 x10^8 SILAC-labelled HeLa.B27 or C1R.B27 cells were washed with 10x volume of PBS, then incubated in 3x volume of hypotonic buffer at 4 degree for 20min. Cells were pelleted and re-suspended in 2x volume of isotonic buffer, then passed though needle (25 gauge for C1R.B27, 30 gauge for HeLa.B27) 10 times, then stroked 10 times in a glass homogenizer (7ml, Dounce). The homogenate was then centrifuged at 1,000 x g for 10 minutes at 4 ℃ to remove nuclei and unlysed cells. The supernatant was transferred to a new tube and centrifuged at 12,000 x g for 15 minutes at 4 ℃. The resulting supernatant was then centrifuged at 100,000 x g for 1 hour at 4 ℃ for isolation of the crude microsomal fraction. The final pellet was lysed for western blot to confirm that ER was enriched and for mass spectrometry to analyze ER peptides.

2.17 Mass spectrometry and data analysis.

The isolated HLA-B27 peptides were further purified by HPLC (Ultimate 3000) on a ProSwift RP-1S 4,6 x 50 mm column (Thermo Scientific) essentially as described (Evnouchidou et al. 2011). Peptides were then analyzed by nano-LC-MS using a nAcquity LC (Waters, C18, 75 µm x 250 mm, 1.7 µm particle size) and a Thermo LTQ Orbitrap Velos (60,000 Resolution, Top 20, CID) workflow using a gradient of 1-40% Acetonitrile in 60 min at a flow rate of 250nl/min as described previously (Fischer et al. 2012). Peaklists were generated using MSConvert (Chambers et al. 2012)) and searched against the SwissProt database with the taxonomy restriction 'mouse' (09-2012, 16554 entries) or 'human' (09/2012, 20306 entries) or a custom made database comprising human and HIV-1 taxonomies (09/2012, 20290 entries) using Mascot v2.3.01 (http://www.matrixscience.com/), without enzymatic restriction and 10ppm / 0.5 Da mass deviations in MS / MS/MS. Oxidation of methionine, deamidation of asparagine and glutamine and SILAC labels R10 and R4 were used as variable modifications.

2.18 HIV gag-p24 Recombinant vaccinia virus (HIV gag rVV) and cell lines infection

HIV gag p24 rVV was from a co-operator Dr Tao Dong (Human immunology unit, Weatherall institute of molecular medicine, John Radcliffe hospital). Cells were washed using RPMI twice before incubated with rVV at 37^{o}C for 1 hour (5pfu/cell). Infected cells were then washed once in RPMI to remove rVV and cultured at 37^{o}C for another 1 hour before CTL activation assay.

2.19 Cytotoxic T cell activation assay using IFN-γ ELISPOT

A CTL clone specific for HIV-gag B27 epitope, KRWIILGLNK (KK10), was kindly made by Yanchun Peng from Dr Tao Dong's group. 400 CTLs and 8,000 B27 expressing cells infected by HIV-gag rVV or transfected by KK10 mini-genes were cultured in the 96-well ELISPOT plate pre-coated with captured IFN-γ antibody in complete RPMI overnight. After incubation, the plate was washed 6 times with 200μl of PBS per well then incubated with IFN-γ detection antibody for 2-4h at room temperature. The washing step was repeated and then 50 μl/well of diluted streptavidin (1 μg/ml) was added and the plates were incubated at room temperature for a further 1-2 h. The washing step was repeated for a final time and 50 μl of AP developing buffer (170-6432, Biorad, USA) was added per well, and the plate incubated for 5-15 min at room temperature until spots appeared. Plates were then washed with tap water and air-dried. Once dry, plates were read and analysed using AID ELISPOT Software.

2.20 KK10 mini-genes construction and transfection

Our co-operators, Dr Edd James and Dr Emma Reeves from Professor Tim Elliot's lab (Southampton General Hospital) have made several KK10 mini-genes. They encode ER-targeted KK10 and ER-targeted VK15 (N-terminal extended KK10, VGEIYKRWIILGLNK), KK10 and VK15 without ER signal. A negative control mini-gene, ER-targeted SHL8 encoding a H2-K^b epitope SIINFEHL, was also made.

The ER signal used was derive from adenovirus. Cells were plated one day before transfection using GeneJuice (Merck Millipore).

2.21 HLA-B27 thermo-stability assay

HeLa.B27 or mFib.B27HA cells were lysed by RIPA buffer (Cell signalling) supplement with complete protease inhibitor cocktail tablets (Roche). Equal amount of lysate were aliquoted to four 1.5 mL tubes and incubated for 1 hour at 4, 37, 50 and 60 ^{o}C respectively. The lysate from each tube was then immunoprecipitated respectively using ME1 (for HeLa.B27 cells) or W6/32 (for mFib.B27 cells) and blotted using HC-10 (for HeLa.B27 cells) or anti-HA antibody (for mFib.B27 cells) respectively. The HLA-B27 thermo-stability was compared between ctr-shRNA and ERAP1-shRNA transduced HeLa.B27 cells, or between ctr-plasmid and WT-ERAP1 transfected mFib.B27HA cells.

Chapter 3. Effects of AS-associated ERAP1 variations on enzyme activity in vitro peptide trimming

3.1 Introduction

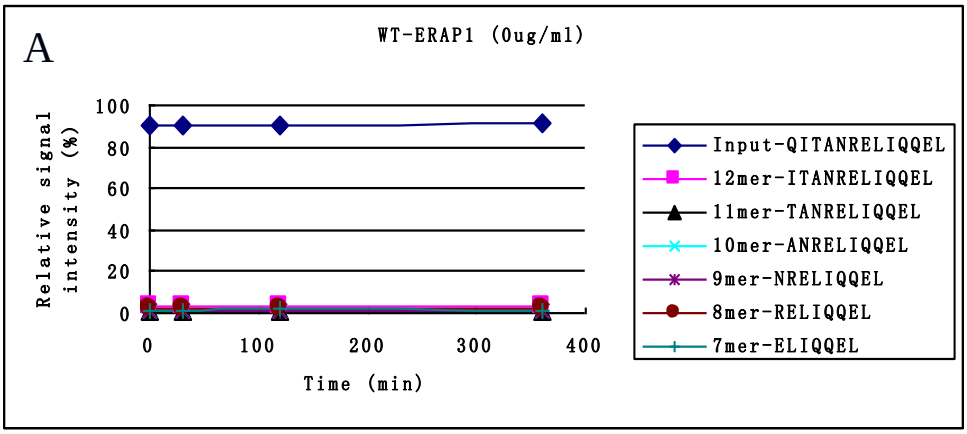
One major function of ERAP1 is the trimming of peptides in the ER to an optimal length for presentation by MHC class 1 molecules. Hence, the in vitro peptide trimming assay is a good tool for comparing peptide processing between WT-ERAP1 and ERAP1 variants. Being the most prevalent and likely ancestral subtype, HLA-B*27:05 has been the subtype selected for all studies in this thesis (hereafter termed HLA-B27). In order to study the effect of ERAP1 variation on the processing and generation of HLA-B27 epitopes, two N-terminally extended HLA-B27 antigenic peptides were used as substrates (QITANRELIQQEL derived from Chlamydia and ELRSRYWAIRTR from Influenza nucleoprotein 383-391, the underlined parts are the epitope peptides. Both peptides were synthesized and HPLC-purified by Katalin Di Gleria).

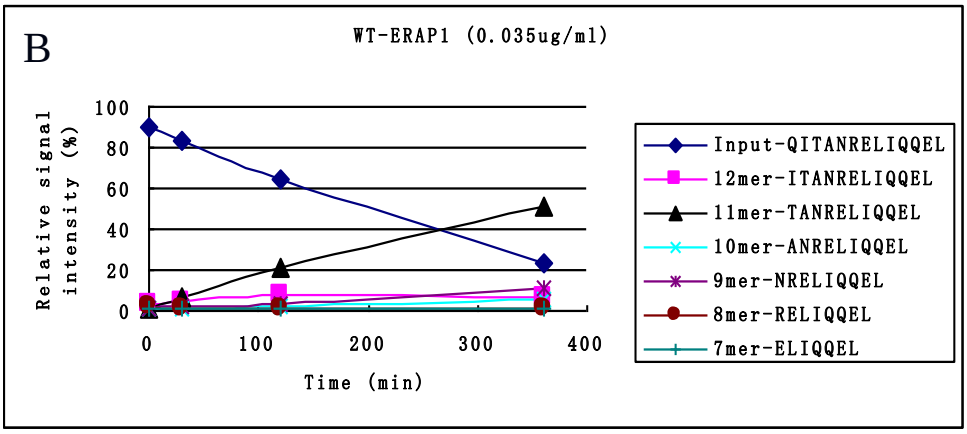
In this Chapter, two protective ERAP1 variants, K528R-ERAP1 and R725Q-ERAP1, were compared with WT-ERAP1 in their trimming of HLA-B27 epitope precursors, QITANRELIQQEL and ELRSRYWAIRTR. The degradation rate of input peptide and generation of peptide products affected by ERAP1 variation were studied by Mass Spectrometry.

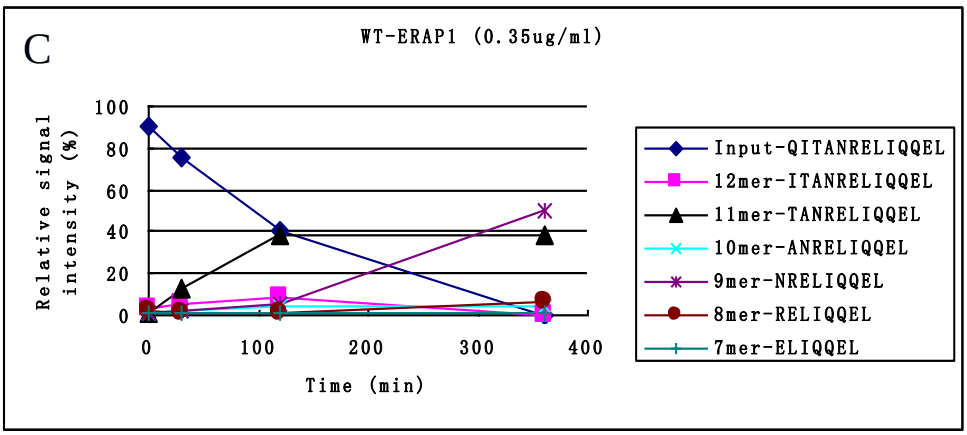
3.2 Results

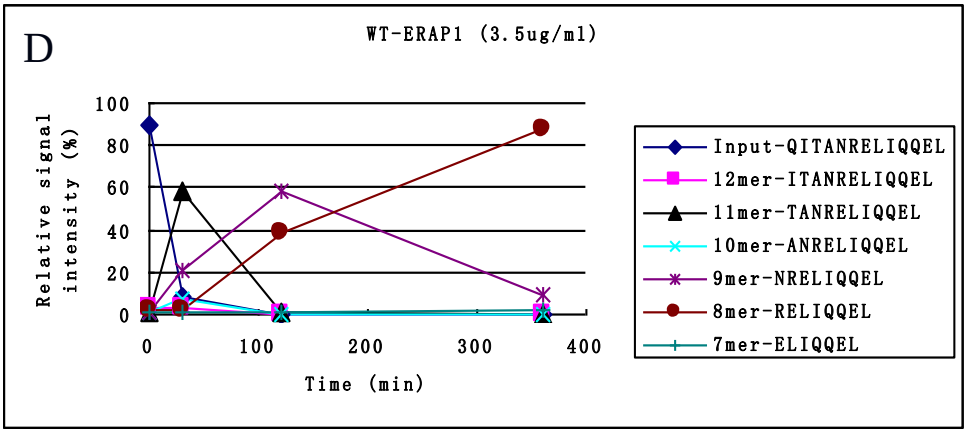
3.2.1 Titration of enzyme concentration using WT-ERAP1

In previous studies involved in peptide trimming by ERAP1, the concentration of 3.5 μg/ml was used (Chang et al. 2005; Saric et al. 2002). To look for suitable concentration of recombinant ERAP1 for in vitro peptide trimming studies, I first carried out a titration of WT-ERAP1 in vitro using QITANRELIQQEL as the substrate (Figure 3.1, recombinant WT-ERAP1 and ERAP1 variants used later were kindly made and provided by Dr David Harvey from Botnar Research Center). ERAP1 was able to trim the input 13mer peptide (QITANRELIQQEL) to N-terminally truncated peptides (12mer, 11mer, 10mer, 9mer and 8mer). The observation that, in the absence of ERAP1, input peptide was stable at 37 ℃ confirmed that these shorter peptides were indeed products of input peptide trimming by ERAP1 (Figure 3.1 A). The degradation rate of input 13mer peptide arose with the increase of ERAP1 concentration, from 0.035, 0.35, 3.5 to 10 μg/ml (Figure 3.1 B-E). The trimming by ERAP1 stopped at 8mer even when the highest concentration, 10 μg/ml were used. 3.5 μg/ml ERAP1 trimmed input 13mer peptide as efficient as 10 μg/ml and generated similar final peptide products at the latest time point. I therefore choose 3.5 μg/ml as the enzyme concentration for following experiments.


A
WT-ERAP1 (0ug/ml)
Relative signal intensity (%)
100
80
60
40
20
0
0
100
200
300
400
Time (min)
Input-QITANRELIQQEL
12mer-ITANRELIQQEL
11mer-TANRELIQQEL
10mer-ANRELIQQEL
9mer-NRELIQQEL
8mer-RELIQQEL
7mer-ELIQQEL


B
WT-ERAP1 (0.035ug/ml)
Relative signal intensity (%)
100
80
60
40
20
0
0
100
200
300
400
Time (min)
Input-QITANRELIQQEL
12mer-ITANRELIQQEL
11mer-TANRELIQQEL
10mer-ANRELIQQEL
9mer-NRELIQQEL
8mer-RELIQQEL
7mer-ELIQQEL


C
WT-ERAP1 (0.35ug/ml)
Relative signal intensity (%)
100
80
60
40
20
0
0
100
200
300
400
Time (min)
Input-QITANRELIQQEL
12mer-ITANRELIQQEL
11mer-TANRELIQQEL
10mer-ANRELIQQEL
9mer-NRELIQQEL
8mer-RELIQQEL
7mer-ELIQQEL


D
WT-ERAP1 (3.5ug/ml)
Relative signal intensity (%)
100
80
60
40
20
0
0
100
200
300
400
Time (min)
Input-QITANRELIQQEL
12mer-ITANRELIQQEL
11mer-TANRELIQQEL
10mer-ANRELIQQEL
9mer-NRELIQQEL
8mer-RELIQQEL
7mer-ELIQQEL

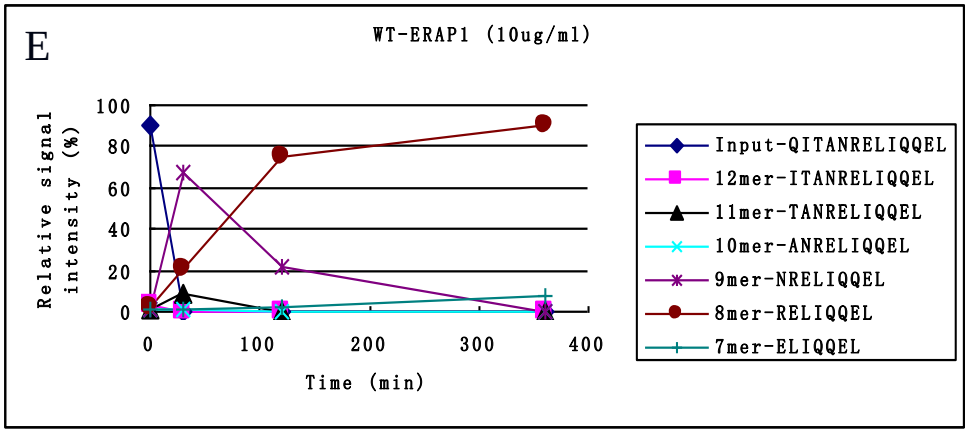


Figure 3.1 WT-ERAP1 titration on QITANRELIQQEL. The Chlamydia-derived substrate, QITANRELIQQEL, was incubated with different concentrations of recombinant WT-ERAP1 at 37 ℃ (0, 0.035, 0.35, 3.5 and 10 μg/ml), A-E respectively. Peptide trimming products were collected at four time points (0, 30, 120 and 360 minutes). For each time point, the input 13mer peptide and the peptides generated by ERAP1 trimming (12mer, 11mer, 10mer, 9mer, 8mer and 7mer) were analyzed on a Q-TOF mass analyzer. The signal intensity of all peptide products was added up; the percentage of each product was graphed. Experiment was carried out once.

3.2.2 ERAP1 is stable at 37℃

To confirm that the reason that ERAP1 cleavage stops at a 8mer in the titration experiments is not due to the loss of enzyme activity, I then tested the thermo-stability of ERAP1 with the Leucine-AMC assays (carried out by Dr David Harvey). I found that after 6 hours incubation at 37 degrees, all ERAP1 variants remained more than 80% of enzyme activity (Figure 3.2).

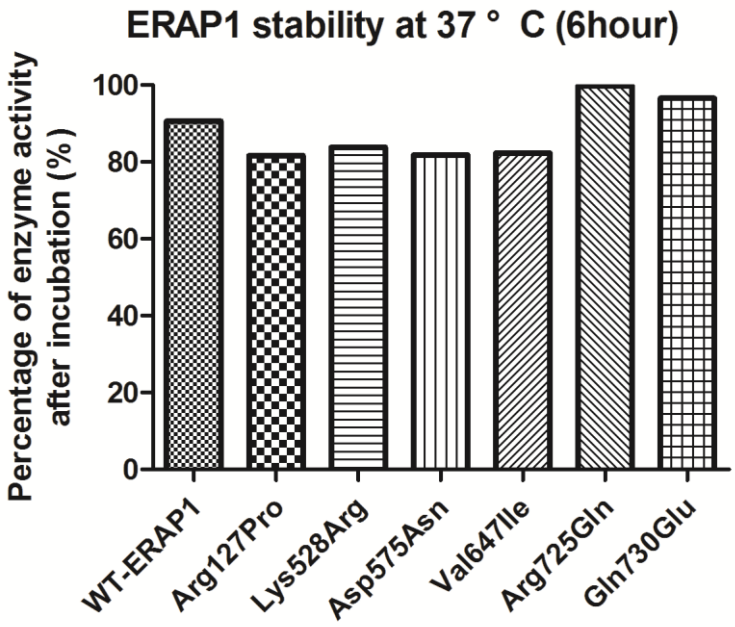


Figure 3.2 ERAP1 is stable at 37℃. Recombinant WT-ERAP1 and ERAP1 variants were incubated at 37℃ for 6 hours before determination of enzyme activity using the Leucine-AMC assay. The activities of ERAP1 before and after incubation were compared; the percentage of remained activity is shown. Experiment was carried out once.

3.2.3 Exclusion of contamination with other aminopeptidases

ERAP1 was previously reported to be unable to trim Proline residues and stop trimming at one amino acid before Proline (Serwold et al. 2001). To exclude possible contamination by other enzymes in peptide trimming assays, the Chlamydia-derived substrate with a residue change from Tyrosine to Proline at the third position (QI*P*ANRELIQQEL) was synthesize and compared with the unchanged substrate (QI*T*ANRELIQQEL) in the trimming (Figure 3.3). The presence of Proline at position 3 decelerated the trimming of 13mer input peptide and completely stopped the degradation of 12mer peptide which was a Pro2 peptide. This result agrees with previous study mentioned above and confirms the purity of recombinant ERAP1 and the absence of other aminopeptidases in this assay.

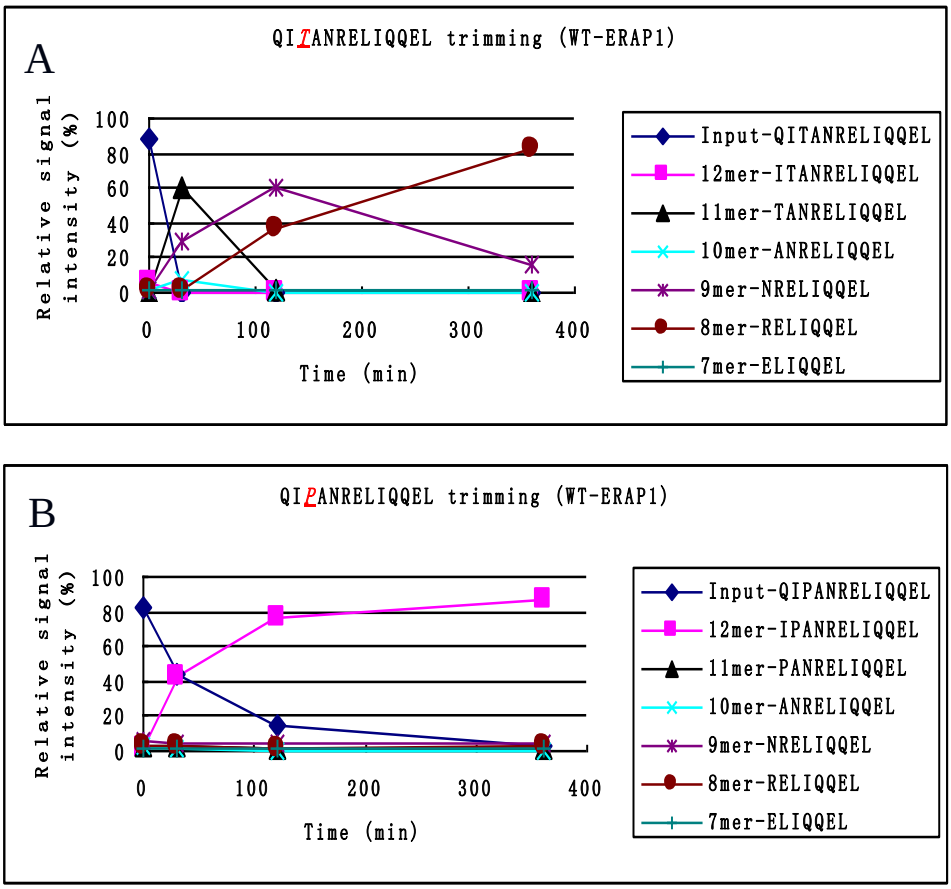


Figure 3.3 Exclusion of contamination by other aminopeptidases. WT-ERAP1 (3.5 μg/ml) was incubated with QI*T*ANRELIQQEL or QI*P*ANRELIQQEL at 37℃ for 30, 120 and 360 minutes respectively. Peptide trimming products were collected at four time points (0, 30, 120 and 360 minutes). For each time point, the input 13mer peptide and the peptides generated by ERAP1 trimming (12mer, 11mer, 10mer, 9mer, 8mer and 7mer) were analyzed on a Q-TOF mass analyzer. The signal intensity of all peptide products was added up; the percentage of each product was graphed. Experiment was carried out once.

3.2.4 K528R-ERAP1 trims peptides slower than WT-ERAP1

3.2.4.1 K528R-ERAP1 trims ELRSRYWAIRTR(Flu) slower than WT-ERAP1

I first compared K528R-ERAP1 and WT-ERAP1 in the trimming of Flu-derived substrate (ELRSRYWAIRTR). Figure 3.4 shows that at the latest time point (360 min), approximately 40% of input peptide is degraded by WT-ERAP1 compared to 10% in the trimming by K528R-ERAP1. WT-ERAP1 therefore trims ELRSRYWAIRTR more efficiently than K528R-ERAP1, although neither WT- nor 528-ERAP1 degrades the input peptide completely.

Looking at individual peptide intermediates, K528R-ERAP1 generates much less 11mer, 10mer, 9mer and 8mer than WT-ERAP1 (Figure 3.5). The 7mer only fluctuates below 4%, possibly due to the contamination from input peptide rather than the trimming product. Peptides shorter than 7mer were un-detectable.

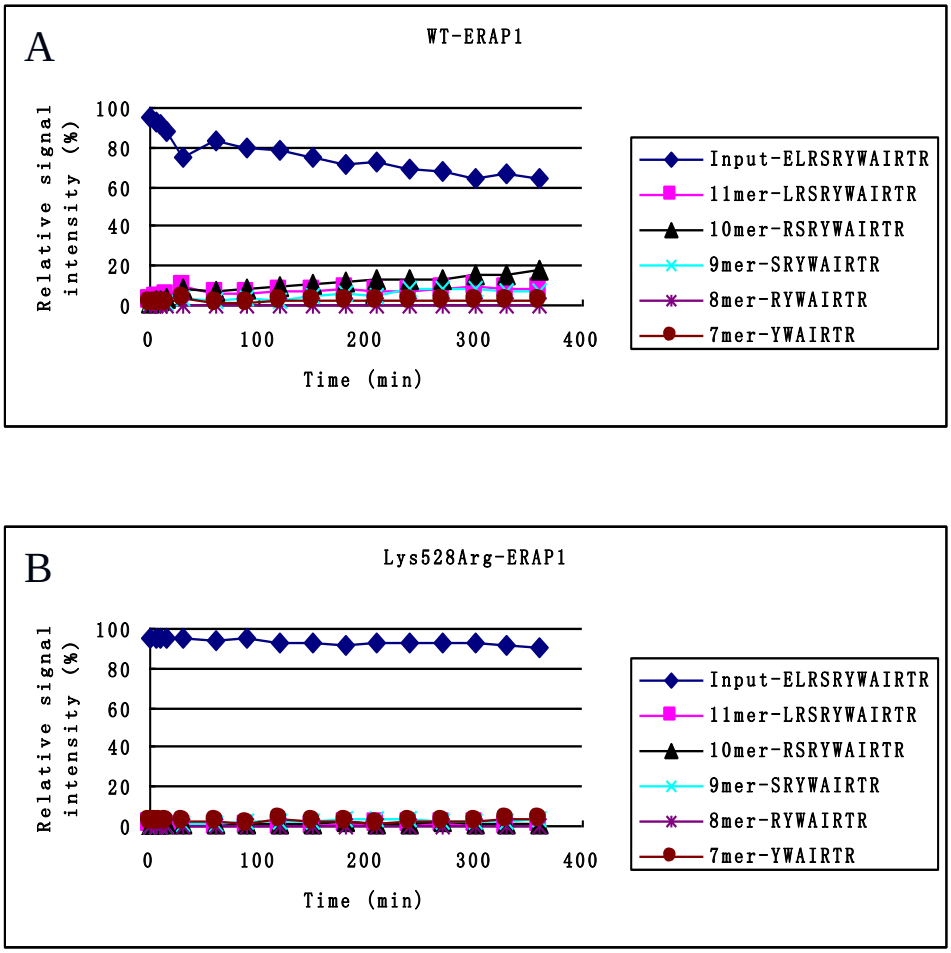


Figure 3.4 K528R-ERAP1 trims flu-derived substrate slower than WT-ERAP1. Recombinant WT- or K528R ERAP1 (3.5 μg/ml) was incubated with Flu-derived 12mer input peptide (ELRSRYWAIRTR) for 360 minutes. Peptide products were collected at 0, 5, 10, 30 minutes and then every 30 minutes till the end of assay. For each time point, the input 12mer peptide and the peptides generated by ERAP1 trimming (11mer, 10mer, 9mer, 8mer and 7mer) were analyzed on a Q-TOF mass analyzer. The signal intensity of all peptide products was added up; the percentage of each product was graphed. Experiment was carried out three times, representative result is shown here.

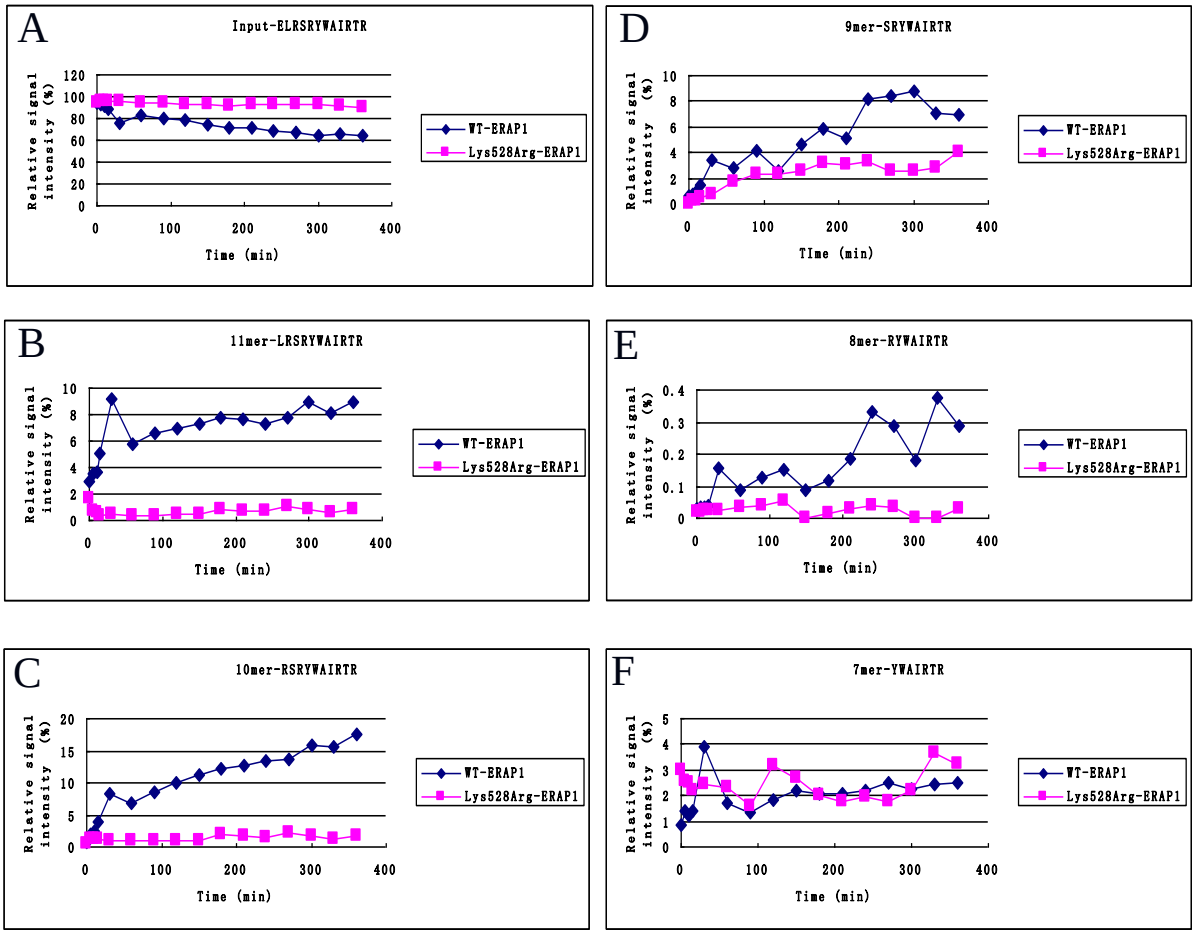


Figure 3.5 K528R-ERAP1 generates less 8mer/9mer than WT-ERAP1. The comparisons of input peptide and each peptide product between WT-ERAP1 and K528R-ERAP1 are shown separately in graph A-F. (Different presentation of same data shown in Figure 3.4)

3.2.4.2 K528R-ERAP1 trims QITANRELIQQEL (Chlamydia) slower than WT-ERAP1

In addition to Flu-derived peptide, a Chlamydia peptide (QITANRELIQQEL) was also used as the substrate for comparison of WT-ERAP1 and K528R-ERAP1 enzyme activity (Figure 3.6 and Figure 3.7). I found the input peptide was quickly degraded by WT-ERAP1 at the 30 minutes time point. WT-ERAP1 then efficiently degraded 12mer, 11mer and 10mer until it generated final products predominantly composed of 8mer and 9mer. In contrast, in the trimming by K528R-ERAP1, input peptide was not degraded until the 150 minutes time point. In addition to decreased input peptide trimming rate, K528R mutation also slowed down the trimming rate of 12mer peptide and made ERAP1 unable to trim 11mer peptides.

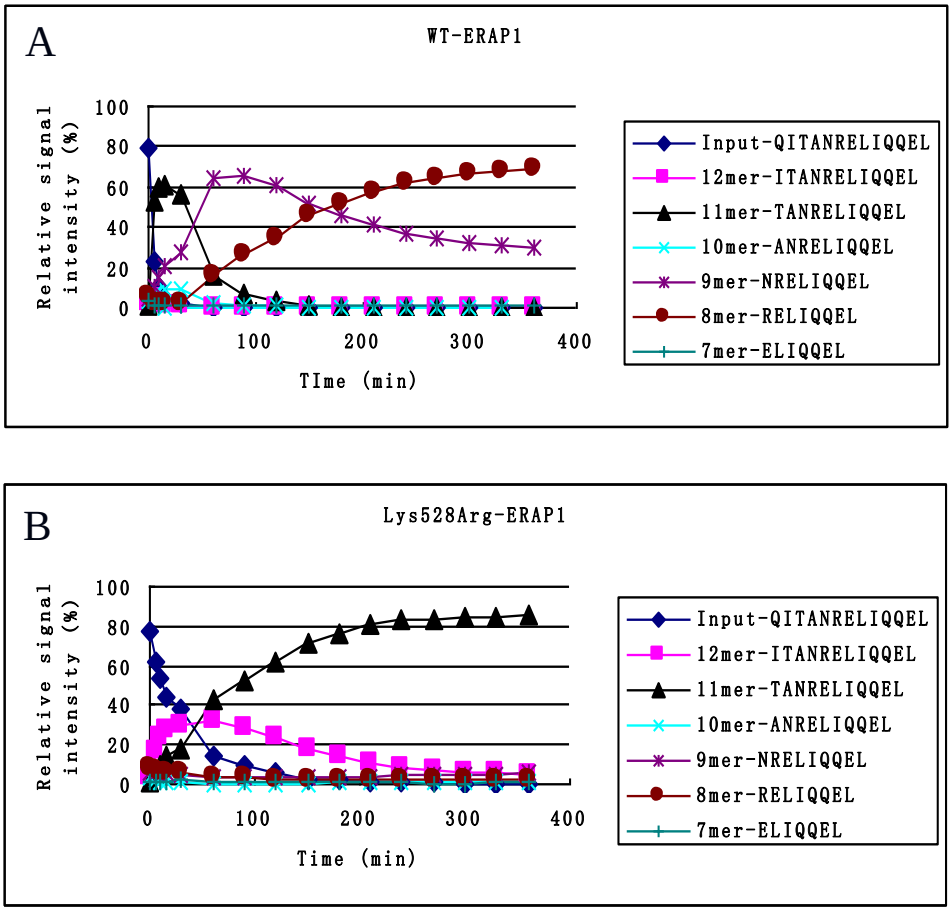


Figure 3.6 K528R-ERAP1 trims a Chlamydia-derived 13mer peptide slower than WT-ERAP1. Recombinant WT- or K528R ERAP1 (3.5 μg/ml) was incubated with Chlamydia-derived 13mer input peptide (QITANRELIQQEL) for 360 minutes. Peptide products were collected at 0, 5, 10, 30 minutes and then every 30 minutes till the end of assay. For each time point, the input 13mer peptide and the peptides generated by ERAP1 trimming (12mer, 11mer, 10mer, 9mer, 8mer and 7mer) were analyzed on a Q-TOF mass analyzer. The signal intensity of all peptide products was added up; the percentage of each product was graphed. Experiment was carried out three times, representative result is shown here.

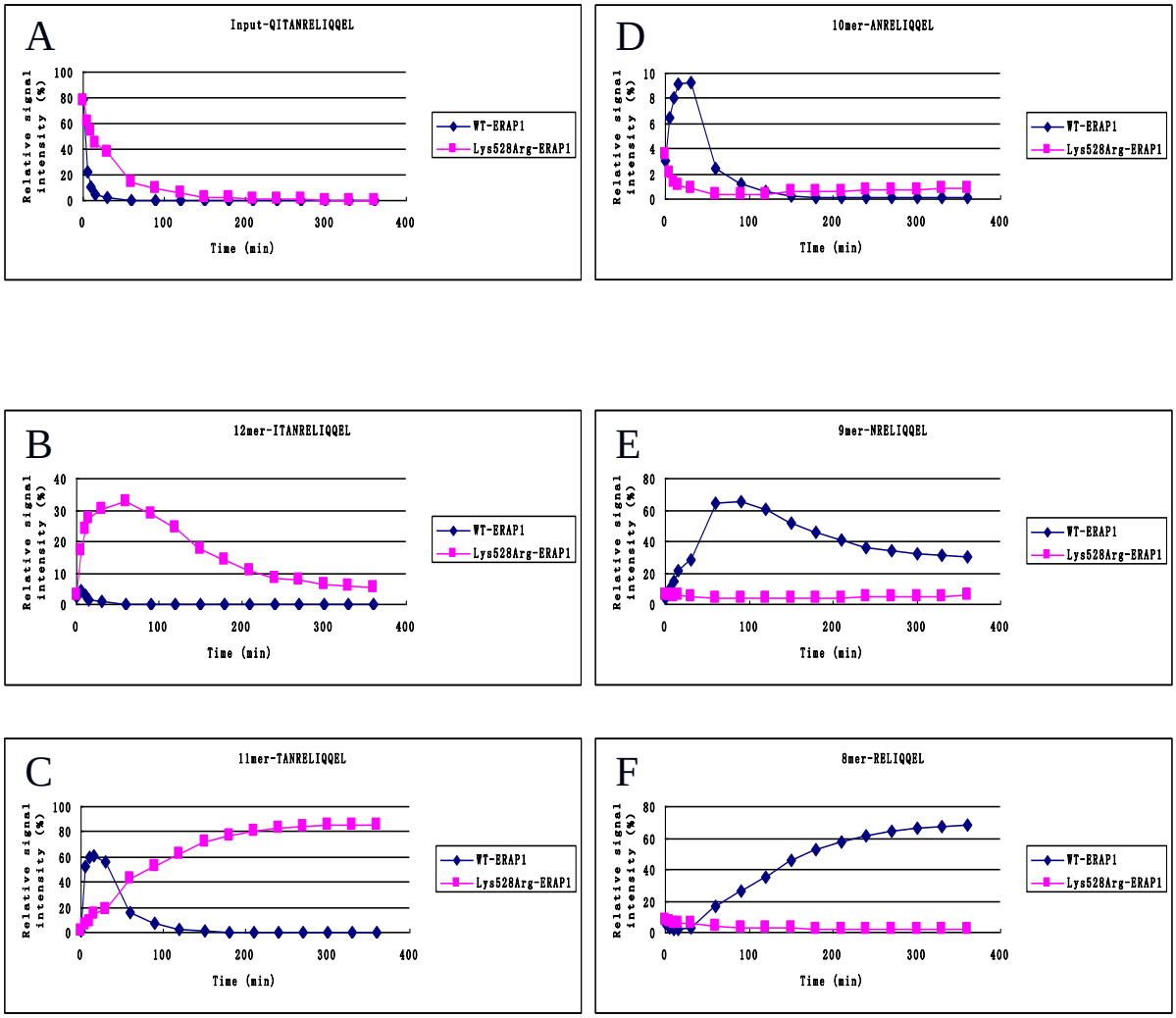


Figure 3.7 Trimming by K528R-ERAP1 of Chlamydia-derived 13mer stops at 11mer while the WT-ERAP1 stops at 8/9mer. The comparisons of input peptide and each peptide product between WT-ERAP1 and K528R-ERAP1 are shown separately in graph A-F. (Different presentation of same data shown in Figure 3.6)

3.2.5 R725Q variation affects ERAP1 peptide trimming in a substrate-dependent manner

3.2.5.1 R725Q-ERAP1 generates more 8/9mer than WT-ERAP1 from the Flu 13mer peptide

Figure 3.8 shows that degradation of 12mer peptide, ELRSRYWAIRTR, was similar between R725Q-ERAP1 and WT-ERAP1, both of which cleaved approximately 40% of input peptides at the end of the assay.

However, the composition of final peptide products was altered by the R725Q mutation (Figure 3.9). The 11mer and 10mer peptides were more abundant in the trimming by WT-ERAP1. In contrast, R725Q-ERAP1 generated more 8mer and 9mer peptides. These results suggests that, although not affecting the trimming of the 12mer input peptide, R725Q-ERAP1 is more efficient at the trimming of 11/10mer peptides than WT-ERAP1 and generates more 8/9mer peptides.

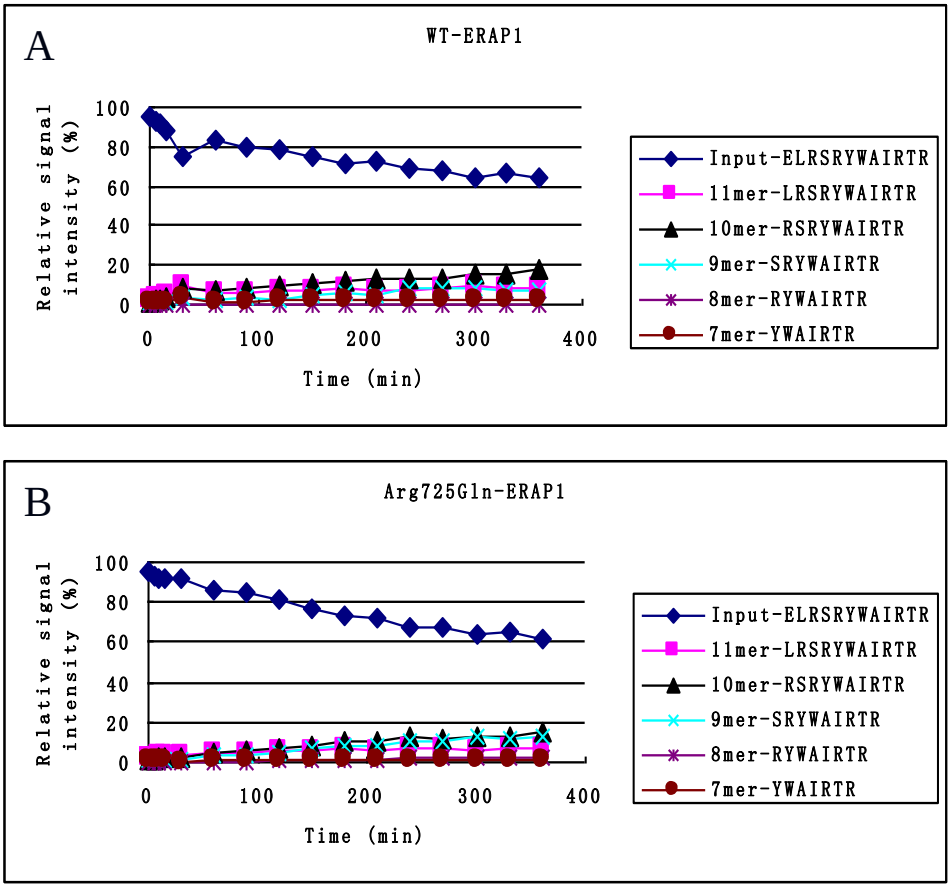


Figure 3.8 R725Q-ERAP1 and WT-ERAP1 process the ELRSRYWAIRTR in a similar rate. Recombinant WT- or R725Q ERAP1 (3.5 μg/ml) was incubated with Flu-derived 12mer input peptide (ELRSRYWAIRTR) for 360 minutes. Peptide products were collected at 0, 5, 10, 30 minutes and then every 30 minutes till the end of assay. For each time point, the input 12mer peptide and the peptides generated by ERAP1 trimming (11mer, 10mer, 9mer, 8mer and 7mer) were analyzed on a Q-TOF mass analyzer. The signal intensity of all peptide products was added up; the percentage of each product was graphed. Experiment was carried out three times, representative result is shown here.

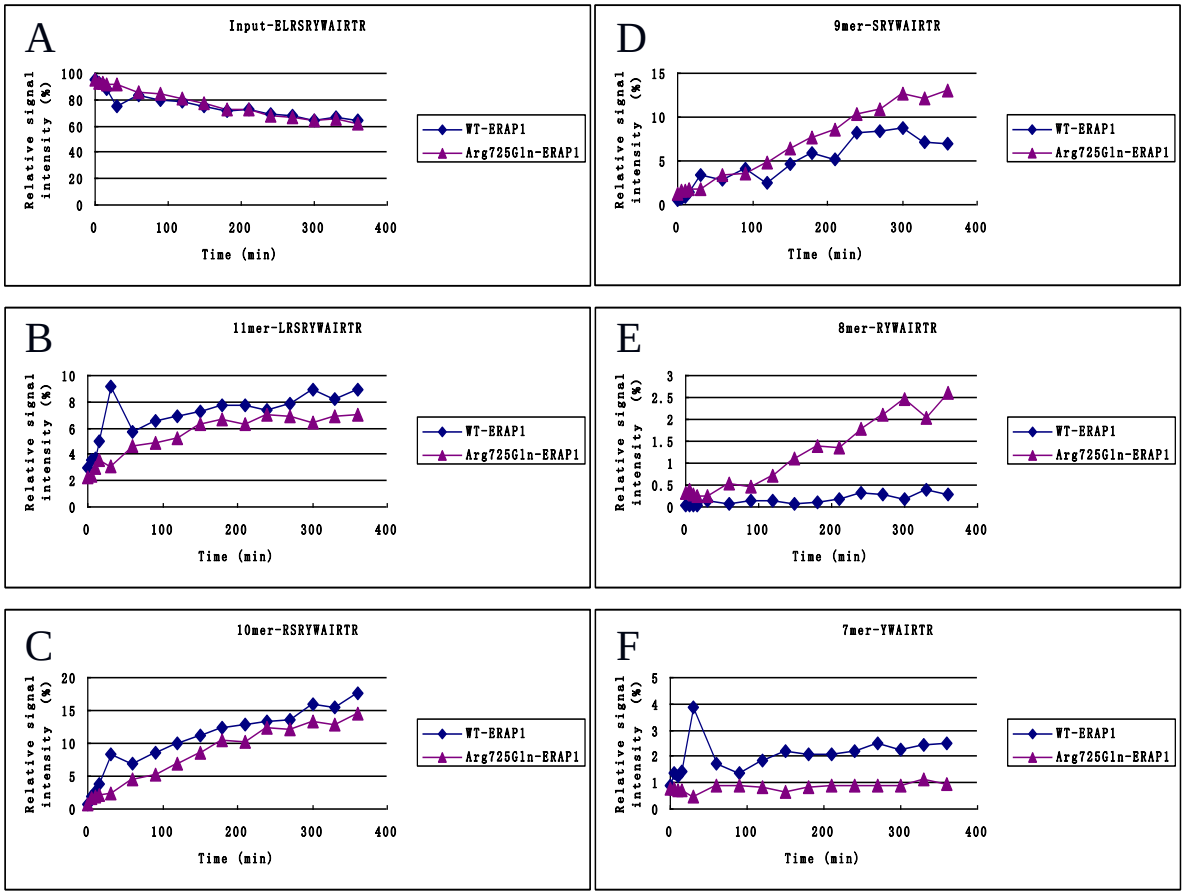


Figure 3.9 R725Q-ERAP1 generates more 8/9mer than WT-ERAP1 in the trimming of ELR<u>SRYWAIRTR</u>. (Different presentation of same data shown in Figure 3.8)

3.2.5.2 R725Q-ERAP1 trims QITANRELIQQEL peptide slower than WT-ERAP1

Different from ELRSRYWAIRTR peptide, trimming of QITANRELIQQEL was decelerated by R725Q mutation (Figure 3.10). The input 13mer was completely degraded by R725Q-ERAP1 after 60 minutes compared to 30 minutes in the trimming by WT-ERAP1.

Although both WT-ERAP1 and R725Q-ERAP1 completely trimmed 12mer, 11mer and 10mer at the end of assay, the variant was less efficient (Figure 3.11). R725Q-ERAP1 also trimmed 9mer peptide slower resulting in 60% of 9mer at 360 minutes time point compared to 30% in the trimming by WT-ERAP1.

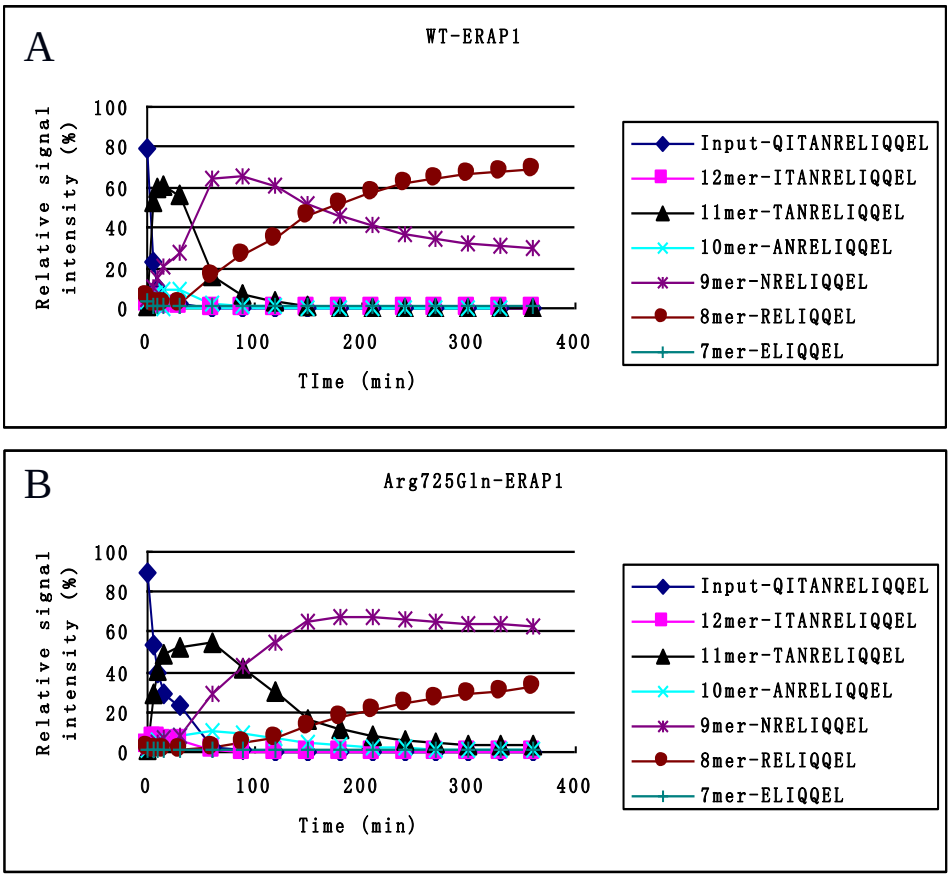


Figure 3.10 Trimming of QITANRELIQQEL by R725Q-ERAP1 and WT-ERAP1 Recombinant WT- or R725Q ERAP1 (3.5 μg/ml) was incubated with Chlamydia-derived 13mer input peptide (QITANRELIQQEL) for 360 minutes. Peptide products were collected at 0, 5, 10, 30 minutes and then every 30 minutes till the end of assay. For each time point, the input 13mer peptide and the peptides generated by ERAP1 trimming (12mer, 11mer, 10mer, 9mer, 8mer and 7mer) were analyzed on a Q-TOF mass analyzer. The signal intensity of all peptide products was added up; the percentage of each product was graphed. Experiment was carried out three times, representative result is shown here.

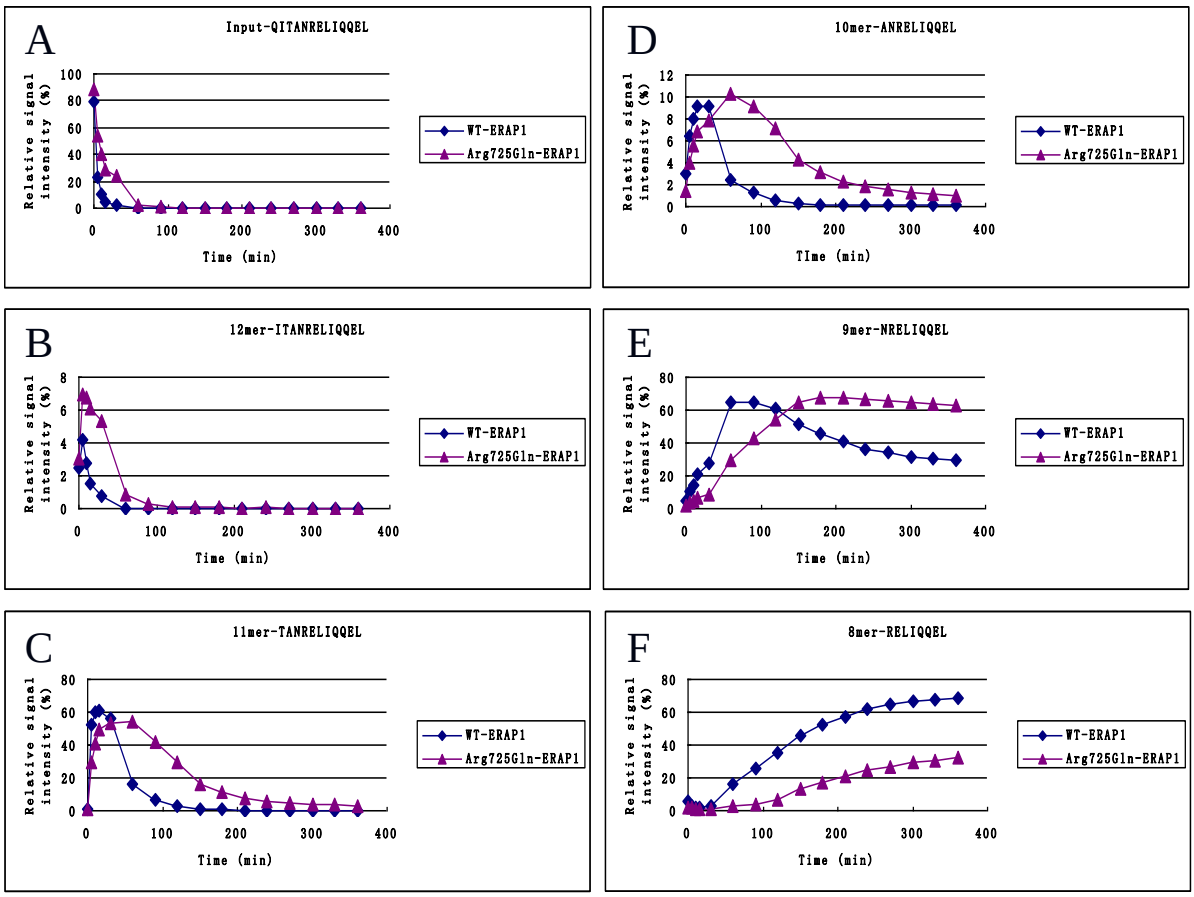


Figure 3.11 R725Q-ERAP1 trims the QITANRELIQQEL slower than WT-ERAP1 but generates similar amount of 8mer and 9mer. (Different presentation of same data shown in Figure 3.10.)

3.3 Discussion

My data show that mutation of ERAP1 changes the rate and nature of trimming of N-terminal extended HLA-B27 antigenic peptides in vitro. K528R-ERAP1 cleaves both flu-derived and Chlamydia-derived substrates less efficiently than WT-ERAP1. Moreover, K528R-ERAP1 trimming of QITANRELIQQEL stops at 11mer when WT-ERAP1 stops at 8/9mer. These results agree with previous finding that K528R-ERAP1 reduced enzyme activity in removing N–terminal Leucine/ Tryptophan (Evans et al. 2011; Goto et al. 2006).

The effect of R725Q mutation on ERAP1 function varies for different substrates: R725Q-ERAP1 trims ELRSRYWAIRTR at a rate similar to WT-ERAP1, but cleaves 10/11mer intermediate peptides more efficiently and generates more 8mer/9mer. However, QITANRELIQQEL and intermediate peptides (12mer-9mer) were trimmed by R725Q-ERAP1 at a lower rate than WT-ERAP1. Therefore, the effect of R725Q variation on ERAP1 enzyme activity is likely substrate-dependent.

It has been suggested that K528R substitution may result in defective transition from the open to the close state of ERAP1, thus affecting the enzyme activity (Kochan et al. 2011). How R725Q affects ERAP1 activity is unclear. R725Q-ERAP1 has been found to trim N-terminal Leucine residues less efficiently than WT-ERAP1 in the Leu-AMC assay (David Harvey 2010), its effect on cleavage of other residues is not known. One possible explanation for the R725Q-ERAP1's substrate-dependent effect observed here is that this variant reduces the trimming of some N-terminal residues (such as Glutamine from QITANRELIQQEL), does not affect some (such as Glutamic acid

from ELRSRYWAIRTR) and possibly enhances others. To confirm this assumption, a panel of peptides carrying different N-terminal residues, designed based on current substrates (XITANRELIQQEL and XLRSRYWAIRTR), could be used as the substrates to compare the enzyme activity between WT-ERAP1 and R725Q-ERAP1. Similarly, these peptides can also be used to study if the effect of K528R substitution on ERAP1 is independent of the P1 residues of its substrates.

Here, effect of ERAP1 mutation on its peptide trimming ability was examined in vitro using two HLA-B27 epitopes. Experiments using more HLA-B27 peptides would further confirm my results. Studies on the effect of ERAP1 on HLA-B27 expression and peptide presentation to CTLs will be shown and discussed in following chapters.

Chapter 4. ERAP1 determines the length and sequence of peptides bound to HLA-B27

4.1 Introduction

In all nucleated cells, MHC class I proteins bind peptides arising from degraded endogenous proteins and present them at the cell surface for immune surveillance. Antigens from self-proteins are usually tolerated; those from viral protein or abnormal protein in tumour cells are recognized by cytolytic T-lymphocytes (CTL) and NK cells resulting in the killing towards abnormal cells. To be properly folded and exported to the cell surface, class I molecules need to be loaded with optimal peptide ligands for the formation of a stable heterotrimer formed of heavy chain, β_2 microglobulin (β_2m) and peptide. Therefore, the peptide repertoire of a MHC class I protein is critical both immunologically and biochemically.

The association HLA-B27 with ankylosing spondylitis (AS) is among the strongest genetic associations with a common polygenic disease, although its mechanism remains unclear. Attempting to explain the possible role of HLA-B27 in AS pathogenesis, several theories have been suggested. As discussed before (**1.3.2**, **Chapter 1**), the three main hypotheses are the "arthritogenic peptide hypothesis", the "misfolding hypothesis" and the "cell surface HLA-B27 free heavy chain hypothesis" (Chatzikyriakidou et al. 2011). Abnormal HLA-B27 peptide repertoire caused by ERAP1 variation could be involved in all three theories. The decreased or enhanced

generation of mimicry peptides could be involved in the arthritogenic peptide hypothesis. A lower number of optimal peptides supplied to HLA-B27 might lead to a slower rate of HLA-B27 folding and increased misfolding, which can subsequently trigger an intracellular signaling response in the ER known as the Unfolded Protein Response (UPR) (Turner et al. 2007). Lastly, abnormal ERAP1 might produce sub-optimal peptide ligands for HLA-B27 and generate unstable HLA-B27 complexes which consequently increase the formation of cell surface B27 free heavy chain and HLA-B27 homodimers.

Deficiency of murine endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) has been shown to alter the repertoire of peptides bound to H-2D^b and H-2K^b and subsequent CTL recognition of cytomegalovirus epitope (Blanchard et al. 2010). I therefore set out to determine if ERAP1 silencing alters the repertoire of peptides bound to HLA-B27.

4.2 Results

4.2.1 Optimization of HLA-B27 peptide isolation

In previous studies of the HLA-B27 peptidome, protein A/G sepharose-based immunoprecipitation has been used for isolation of HLA-B27 complexes (Ben Dror et al. 2010; Lopez de Castro). Since ERAP1 has been shown to trim elongated peptides to 8mers or 9mers for their binding to MHC class I molecules (York et al. 2002), its silencing might result in binding of sub-optimal peptides to HLA-B27 and formation of less stable B27 hetero-trimers. Therefore, the conventional method is possibly too

long a process to identify peptides from unstable HLA-B27 complexes. To avoid losing sub-optimal peptides, I used a less time-consuming alternative to sepharose, protein G Dynabeads, for immunoprecipitation (which could be done in one hour). ME1, a mAb binding for HLA-B27, B7 and Bw22 complexes, was used to isolate HLA-B27 in immunoprecipitation. Table 5.1 A shows that 86 and 4 HLA-B27-bound peptides were identified in HeLa.B27 and C1R.B27 cells respectively. The numbers of peptides isolated were too low for comparison between ERAP1-competent and –deficient cells. Dr Roman Fisher and I found that one problem was contamination of polyethylene glycol (PEG) which shadowed peptide signals in mass spectrometry (data not shown).

I therefore decided to go back to Sepharose-based immunoprecipitation. Dr Nicola Ternette from Dr Benedikt Kessler's group kindly provided me with an MHC peptide isolation protocol and gave me a demonstration. Using this technique, I managed to indentify around 400 peptides from either HeLa.B27 or C1R.B27 cells (Table 5.1 B). Protein G Sepharose immunoprecipitation was therefore used in subsequent studies.

A ME1-Protein G Dynabeads

	Number of peptides
HeLa.B27	86
C1R.B27	4

B ME1-Protein G Sepharose

	Number of peptides
HeLa.B27	439
C1R.B27	362

Table 5.1 HLA-B27 peptide extraction using Protein G sepharose is more efficient than Protein G Dynabeads. ME1 immunoprecipitation using Protein G Dynabeads (A) or Protein G Sepharose (B) was carried out using HeLa.B27 or C1R.B27. Numbers of peptides isolated and sequenced by MS/MS using these two approaches are shown.

4.2.1 Both C and N-terminally extended peptides are bound to HLA-B27 following ERAP1 silencing

To examine the impact of ERAP1 on HLA-B27 peptide repertoire, I compared naturally processed peptides eluted from HLA-B27 expressed in ERAP1-sufficient and -silenced HeLa.B27 and C1R.B27 cells. In order to minimize the introduction of experimental or analytic error, I first labelled cells with different Arginine isotopes, and pooled the samples before HLA-B27-bound peptide enrichment and MS/MS analysis (details described in **2.14, Chapter 2**). Arginine was selected for SILAC isotopic labelling as it is a dominant "anchor" amino acid at position 2 of HLA-B27 peptide ligands (Guo et al. 1993; Lopez de Castro et al. 2004; Madden et al. 1992). Moreover, the Arg2 anchor is not an anchor residue for the other alleles expressed in C1R (HLA-Cw4 and HLA-B35).

The ME1 monoclonal antibody was first used to immunoprecipitate HLA-B27 complexes from HeLa.B27 and C1R.B27 cells. In order to exclude possible bias caused by the ME1 antibody, I also used W6/32 to immunoprecipitate HLA-B27 from C1R.B27 cells, which express relatively little class I before transfection. Figure 4.1 shows that ME1 only stains HLA-B27-transduced HeLa and C1R, W632 stains HeLa and C1R but to a lesser extent compared to cells expressing B27.

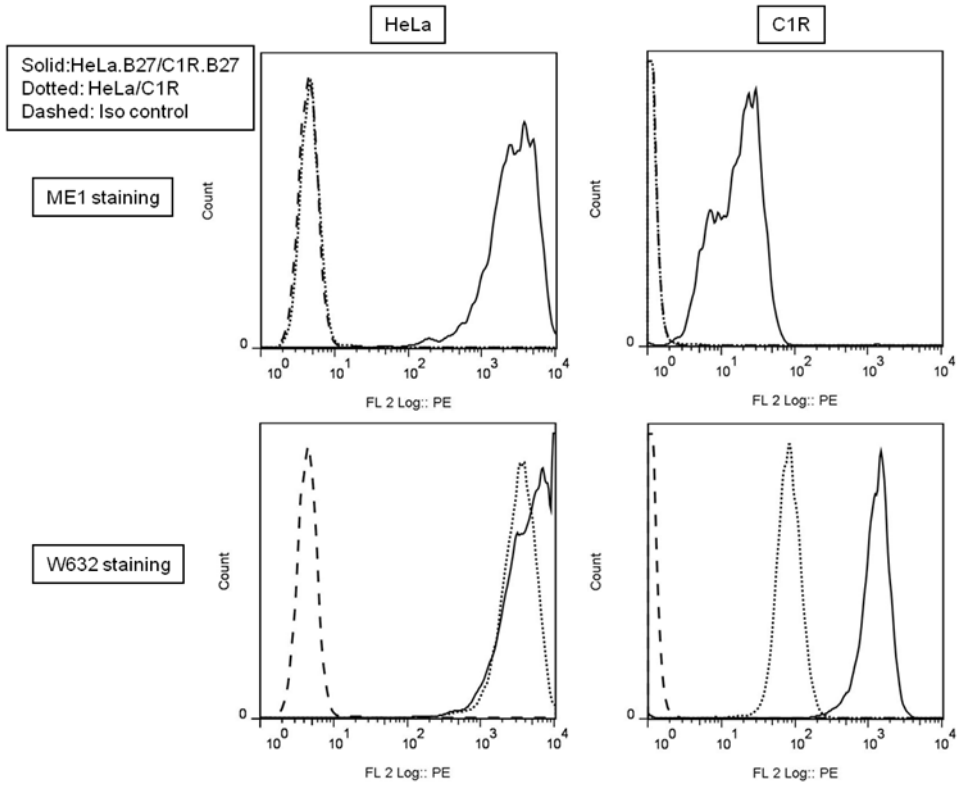


Figure 4.1 ME1 stains only HeLa.B27 and C1R.B27 cells, W6/32 stains HeLa and C1R but to a lesser extent compared to cells expressing B27. ME1 and W632 were used to stain HeLa.B27/C1R.B27 and HeLa/C1R cells. Expression of HLA-B27 was either achieved through lentiviral transduction (HeLa.B27) or transfection (C1R.B27). Results are representative of n=3 experiments.

The immunoprecipitated HLA-B27 complexes were then treated with 10% acetic acid to elute peptides. Proteins, mainly HLA-B27 heavy chain and β_2m, were also released due to the breakdown of HLA-B27 complexes. These proteins need to be removed because their presence affects identification of peptides in MS/MS. Extracted HLA-B27-bound peptides were therefore further purified by HPLC. Figure 4.2 shows separation of peptides from proteins using C1R.B27-immunoprecipitated by ME1 as an example. Peptides were eluted mainly between 5 minutes and 11 minutes, these fractions were therefore used for sequencing by tandem mass spectrometry (MS/MS). Fractions containing β_2m and heavy chain, which were eluted after 12 minutes, were discarded.

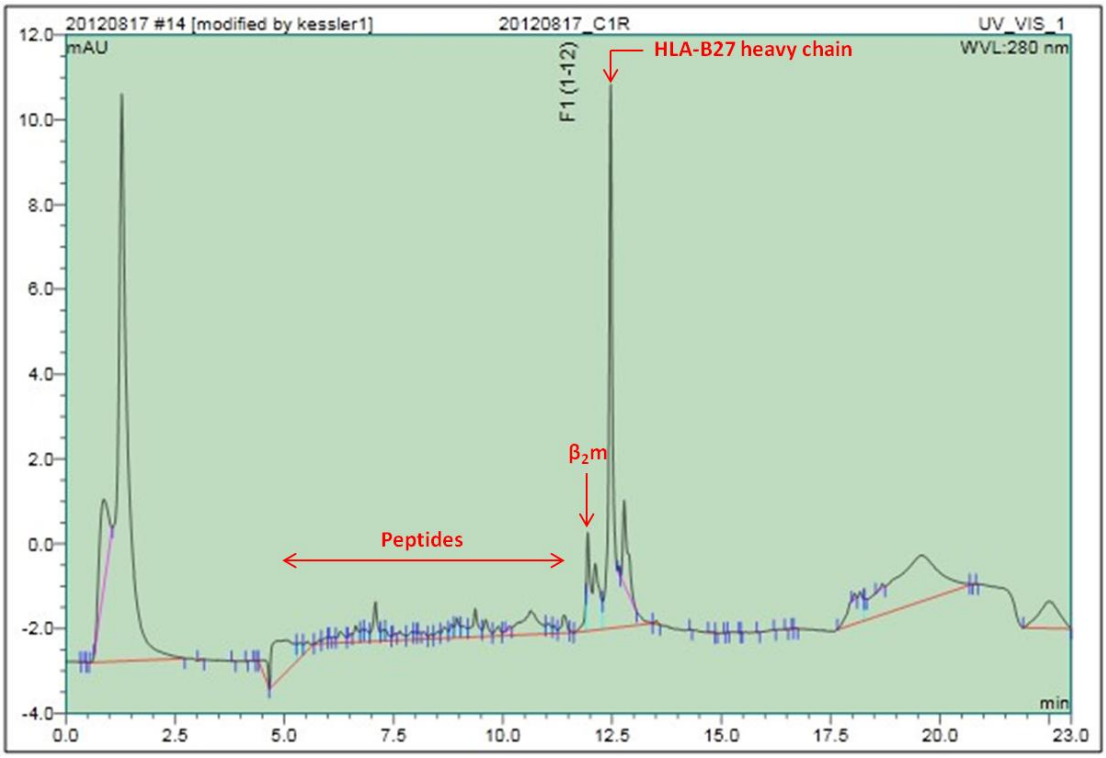


Figure 4.2 HPLC purification of extracted HLA-B27-bound peptides. Peptides isolated from C1R.B27 by ME1 immunoprecipitation were fractionated by HPLC (details shown in **2.17**, **Chapter 2**). Peptides were first eluted followed by B2m and HLA-B27 heavy chain. Area marked "peptides" was collected for mass spectrometry analysis.

HLA-B27-bound peptides were then compared between ctr-shRNA and ERAP1-shRNA transduced HeLa.B27 cells (ME1 immunoprecipitation) or C1R.B27 cells (ME1/W632 immunoprecipitation). Figure 4.3 shows that expression of endogenous ERAP1 was remarkably reduced by ERAP1-shRNA but not ctr-shRNA. The screening of ERAP1 siRNA candidates and construction of lentiviral ERAP1-shRNA based on the sequence of ERAP1 siRNA selected will be described in **Chapter 6**.

I first looked for evidence of extended versions of peptides identified in either ctr-shRNA- or ERAP1-shRNA-transduced cells. Table 5.2 shows that 30 extended peptides were identified in ERAP1-silenced cells compared to 3 in ERAP1-competant cells. 25 out of the 31 peptides carrying "core" sequence have been previously identified in contrast to only 16 out of 33 extended peptides (Ben Dror et al. 2010; Fiorillo et al. 1997; Jardetzky et al. 1991; Lopez de Castro et al. 2004). Surprisingly, 24 of the 30 extended peptides identified in ERAP1-silenced cells were C-terminally extended. This likely reflects the inability of HLA-B27 to bind N-terminally extended peptides. 29 of these 30 extended peptides carried Arginine at position 2; also confirming the critical anchor role of Arg2.

In addition to this qualitative study, I also carried out quantitative analysis on selected peptides using extracted ion chromatography. Figure 4.4 shows that the abundance of three canonical 9mer peptides was either reduced (GRLPSSWAL) or unaffected (VMAPRTLIL and LRNQSVFNF) by ERAP1 silencing, whereas the extended peptides (GRLPSSWALFSP, TRVMAPRTLIL and SRLRNQSVFNF) were constantly more abundant in ERAP1-silenced cells.

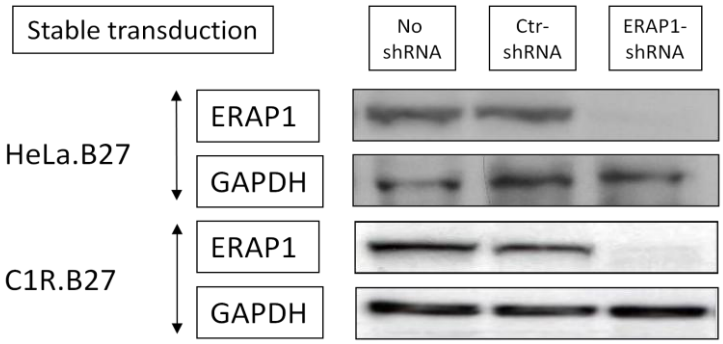


Figure 4.3. Stable silencing of endogenous ERAP1 by Lentiviral shRNA. ERAP1 expression by untransduced and ctr-shRNA/ERAP1-shRNA transduced HeLa.B27/C1R.B27 cells was examined using western blotting. GAPDH was used as protein loading control.

A **HeLa.B27 ME1** - Ctr-shRNA vs **ERAP1-shRNA**

Peptide sequence	Reference	Protein	Accession Number
Extended peptides identified in ERAP1-silenced cells			
K R L E G L S K *K K R L E G L S K	This study This study	Ceramide synthase 5 (CERS5)	Q8N5B7
G R I D K P I L K G R I D K P I L K A	(ref 1-3) (ref 2)	60S ribosomal protein L8 (RPL8)	P62917
F R V P F I Y G H F R V P F I Y G H K Y	(ref 2) This study	Synaptic glycoprotein SC2 (TECR)	Q9NZ01
G R L P S S W A L G R L P S S W A L F S P	This study This study	T-cell immunomodulatory protein (ITFG1)	Q8TB96
A R L D H K F D L A R L D H K F D L M	(ref 2) (ref 2)	Tubulin alpha-1A chain (TUBA1A)	Q9BQE3
L R F P G Q L N A L R F P G Q L N A D L	(ref 2) This study	Tubulin beta chain (TUBB)	P07437
A R F S P D G Q Y L A R F S P D G Q Y L V	(ref 1 & 2) (ref 2)	WD40 repeat-containing protein SMU1	Q2TAY7
G R I L S G V V T K G R I L S G V V T K M	(ref 1, 2, 4) (ref 2)	40S ribosomal protein S11 (RPS11)	P62280
A R L D H K F D L M A R L D H K F D L M Y A R L D H K F D L M Y A	(ref 2) (ref 2) This study	Tubulin alpha-1A chain (TUBA1A)	Q9BQE3
G R W G P T F P S S Y G R W G P T F P S S A G F	(ref 2) This study	Coiled-coil domain-containing protein 34 (CCDC34)	Q96HJ3
Extended peptides identified in non-silenced cells			
S R V N I P K V L R S R V N I P K V L	(ref 1 & 2) (ref 2)	Clathrin heavy chain 1	Q00610
S R F D A S F F G V H P K S R F D A S F F G V	(ref 2) (ref 2)	Fatty acid synthase	P49327

B **C1R.B27 ME1** - Ctr-shRNA vs **ERAP1-shRNA**

Peptide sequence	Reference	Protein	Accession number
Extended peptides identified in ERAP1-silenced cells			
L R V T P F I L K L R V T P F I L K K	(ref 2) This study	Mitochondrial import receptor subunit TOM5 homolog (TOMM5)	Q8N4H5
G R L A A I V A K G R L A A I V A K Q V	(ref 1 & 2) This study	60S ribosomal protein L13a (RPL13A)	P40429
V R L L L P G E L A K V R L L L P G E L A K H	(ref 1 & 2) (ref 2)	Histone H2B type 2-F (HIST2H2BF)	Q5QNW6
Extended peptides identified in non-silenced cells			
F R Y N G L I H R K F R Y N G L I H R	This study (ref 1, 2, 3)	60S ribosomal protein L28(RPL28)	P46779

C **C1R.B27 W632 -** Ctr-shRNA vs **ERAP1-shRNA**

Peptide sequence	Reference	Protein	Accession number
Extended peptides identified in ERAP1-silenced cells			
A H E V G W K Y *G R L **A H E V G W K Y**	This study (ref 2)	60S ribosomal protein L13a (RPL13A)	Q8N4H5
Q I E D F E A R *R R L **Q I E D F E A R**	This study (ref 2)	NADH dehydrogenase 1 alpha subcomplex subunit 13	Q9P0J0
L R N Q S V F N F *S R **L R N Q S V F N F**	(ref 1 & 2) This study	Farnesyl-diphosphate farnesyltransferase 1 (FDFT1)	P37268
V M A P R T L I L *T R **V M A P R T L I L** *M R **V M A P R T L I L**	This study This study This study	HLA class I histocompatibility antigen (HLA-C)	P30504
L R V T P F I L K **L R V T P F I L K K**	(ref 2) This study	Mitochondrial import receptor subunit TOM5 homolog	Q8N4H5
G R L A A I V A K **G R L A A I V A K Q V**	(ref 1 & 2) This study	60S ribosomal protein L13a (RPL13A)	P40429
G R W P G S S L Y **G R W P G S S L Y Y**	(ref 1) (ref 1 & 4)	Lamin-B receptor (LBR)	Q14739
L R Y P M A V G L **L R Y P M A V G L N K**	(ref 1 & 2)) This study	60S ribosomal protein L36 (RPL36)	Q9Y3U8
S R I H P V S T M **S R I H P V S T M V K**	(ref 1 & 2) (ref 2)	L-lactate dehydrogenase B chain (LDHB)	P07195
A R A T L S S I R H **A R A T L S S I R H M I R**	This study This study	60S ribosomal protein L28 (RPL28)	P46779
A R F S P D G Q Y L **A R F S P D G Q Y L V**	(ref 1 & 2) (ref 2)	WD40 repeat-containing protein SMU1(SMU1)	Q2TAY7
G R I L S G V V T K **G R I L S G V V T K M**	(ref 1, 2, 4) (ref 2)	40S ribosomal protein S11 (RPS11)	P62280
G R W P K K S A E F **G R W P K K S A E F L L**	This study This study	60S ribosomal protein L17 (RPL17)	P18621
A R L H P R A V N C **A R L H P R A V N C R**	This study This study	Ubiquitin-60S ribosomal protein L40 (UBA52)	P62986
V R L L L P G E L A K **V R L L L P G E L A K H**	(ref 1 & 2) (ref 2)	Histone H2B (HIST1H2B)	Q5QNW6
Extended peptides identified in non-silenced cells			
None			

Table 5.2 Extended peptides eluted from HLA-B27 following ERAP1-shRNA silencing in A) HeLa.B27 and B) C1R.B27 immunoprecipitated with ME1 antibody and C) C1R.B27 immunoprecipitated with W6/32. Datasets of peptides identified from control (upper of each pair) and ERAP1-shRNA silenced cells (**lower in bold**) were cross searched for extended or truncated forms. The 'core' sequences of the extended peptides are underlined. 30 extended peptides were identified from

ERAP1-silenced cells and 3 (marked *) from control shRNA-transduced cells. Peptides previously identified are labeled with ref 1 (Lopez de Castro et al. 2004), ref 2 (Ben Dror et al. 2010), ref 3 (Jardetzky et al. 1991) and ref 4 (Fiorillo et al. 1997). Peptide-associated proteins and their accession numbers are also shown.

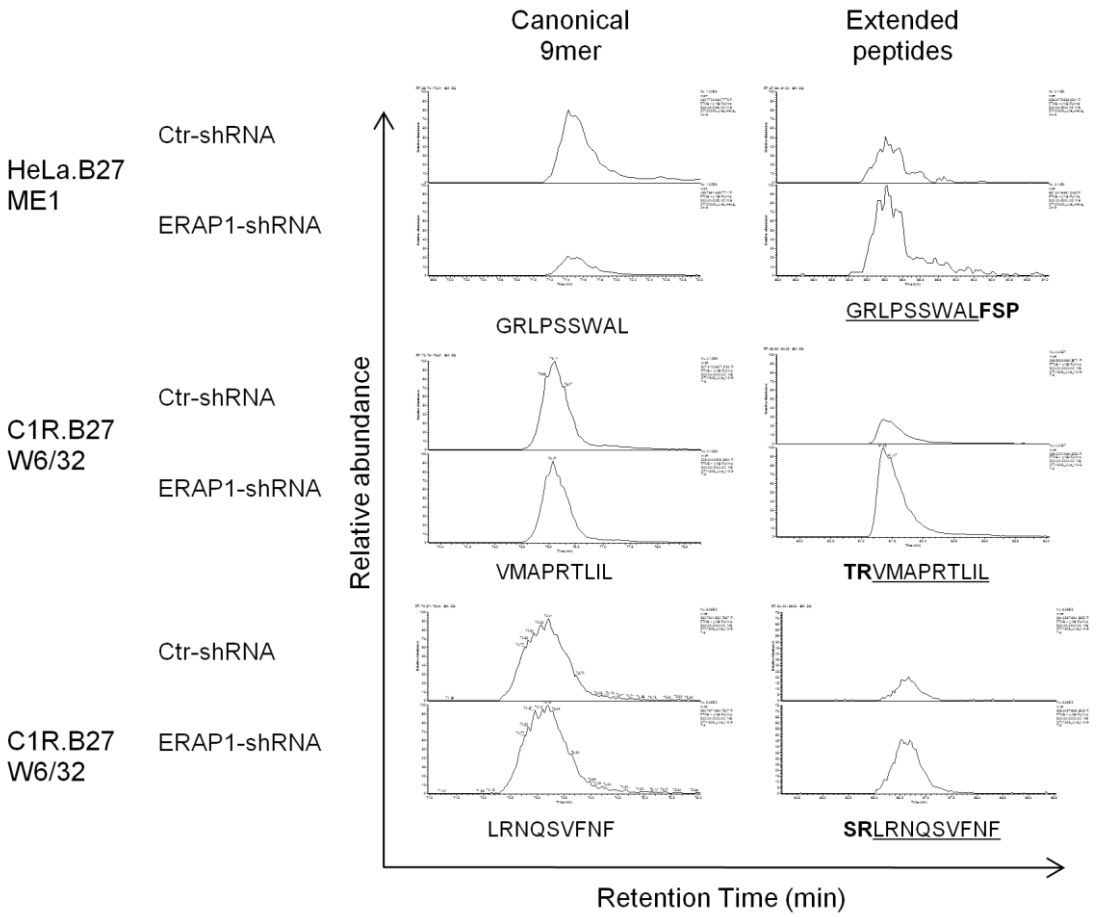


Figure 4.4 Increased quantities of extended peptides identified following ERAP1-silencing. Relative abundance of the peptides identified were compared between ERAP1-silenced and -competent cells. The 'core' sequences of the extended peptides are underlined. GRLPSSWALFSP, TRVMAPRTLIL and SRLRNQSVFNF are derived from ITFG1 (Q8TB96), HLA-C (P30504) and FDFT1 (P37268) respectively.

4.2.2 ERAP1 silencing increases the quantities of long peptides bound to HLA-B27

I next asked if silencing of ERAP1 affects the length of peptides bound to HLA-B27. To minimize any possible contamination, I specifically enumerated all Arg2-containing peptides eluted from HeLa.B27 and C1R.B27. A total of 600 peptide sequences were obtained from the 3 control-shRNA, and 604 from the 3 ERAP1-shRNA-treated cells. Figure 4.5 shows the effects of ERAP1-silencing on the percentage of peptides of different lengths (9mer, 10mer, 11mer, 12mer, 13mer and 14mer) eluted. In HeLa.B27 cells, ERAP1 silencing decreased the proportion of 9mer peptides, from 45.4% to 17.6%, (Figure 4.5 A). By contrast, percentages of 10mer, 11mer and 12mer peptides were increased. Similarly, in C1R.B27 (using either ME1 or W6/32 for immuno-precipitation) the percentage of 9mer peptides was decreased by ERAP1 silencing whereas proportions of 11mer, 12mer and 13mer were increased (Figure 4.5 B and C). These results show that, at least in HeLa.B27 and C1R.B27 cells, ERAP1 determines repertoire of HLA-B27-bound peptides in a non-redundant manner.

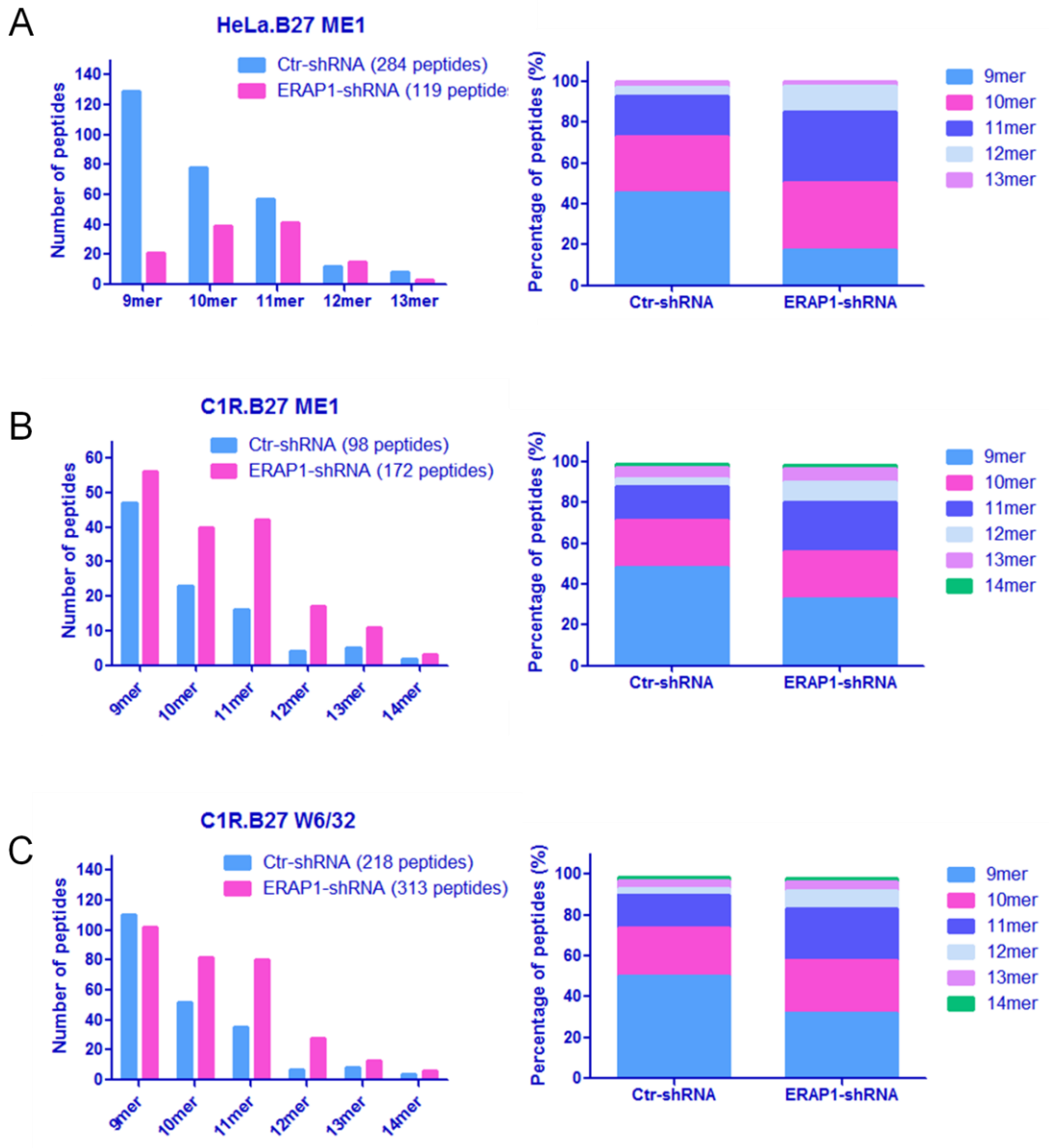


Figure 4.5 The percentage of long HLA-B27-bound peptides, 11mer-13mer, was increased when ERAP1 is silenced. Number (left-hand panels) and percentage (right-hand panels) of HLA-B27-bound peptides of different lengths (9mer, 10mer, 11mer, 12mer, 13mer and 14mer) were compared between cells with ctr-shRNA and ERAP1-shRNA. A. HLA-B27 peptides isolated from HeLa.B27 cells using ME1. B. HLA-B27 peptides isolated from C1R.B27 cells using ME1. C. HLA-B27 peptides isolated from C1R.B27 cells using W6/32. Number of experiment (n=1) has been limited by time and expense of SILAC.

4.2.3 ERAP1 affects the nature of the P1 amino acid of peptides bound to HLA-B27

ERAP1 has been reported to differentially trim different N-terminal amino acid residues (Hearn et al. 2009). Therefore, I asked if silencing of ERAP1 altered the amino acid (AA) usage of P1 residues in peptides eluted from HLA-B27. The frequencies of P1 AA of HLA-B27-bound peptides were compared between cells with ctr-shRNA and ERAP1-shRNA (Figure 4.6). For HLA-B27 peptides isolated from HeLa.B27 cells, uncharged polar AA (S, Y and T) were more abundant at P1 in ERAP1-silenced cells whereas the percentage of charged polar AA (E and R) was higher in ERAP1-competent cells (Figure 4.6 A). In C1R.B27 cells, ERAP1 silencing increased the frequency at P1 of uncharged polar AA (Y and S) and hydrophobic AA (K and M), and decreased charged polar AA (R and E) (Figure 4.6 B and C). Difference in AA usage at other positions, P3-P5 and C-terminal P1-P5, was not obvious (Figure 4.7). Some changes were seen at P5 and C-terminal P1-P4, but residues affected were mostly in low abundance (Figure 4.7 C-G). P2 residue usage was not studied as only Arg2 peptides were selected for analysis.

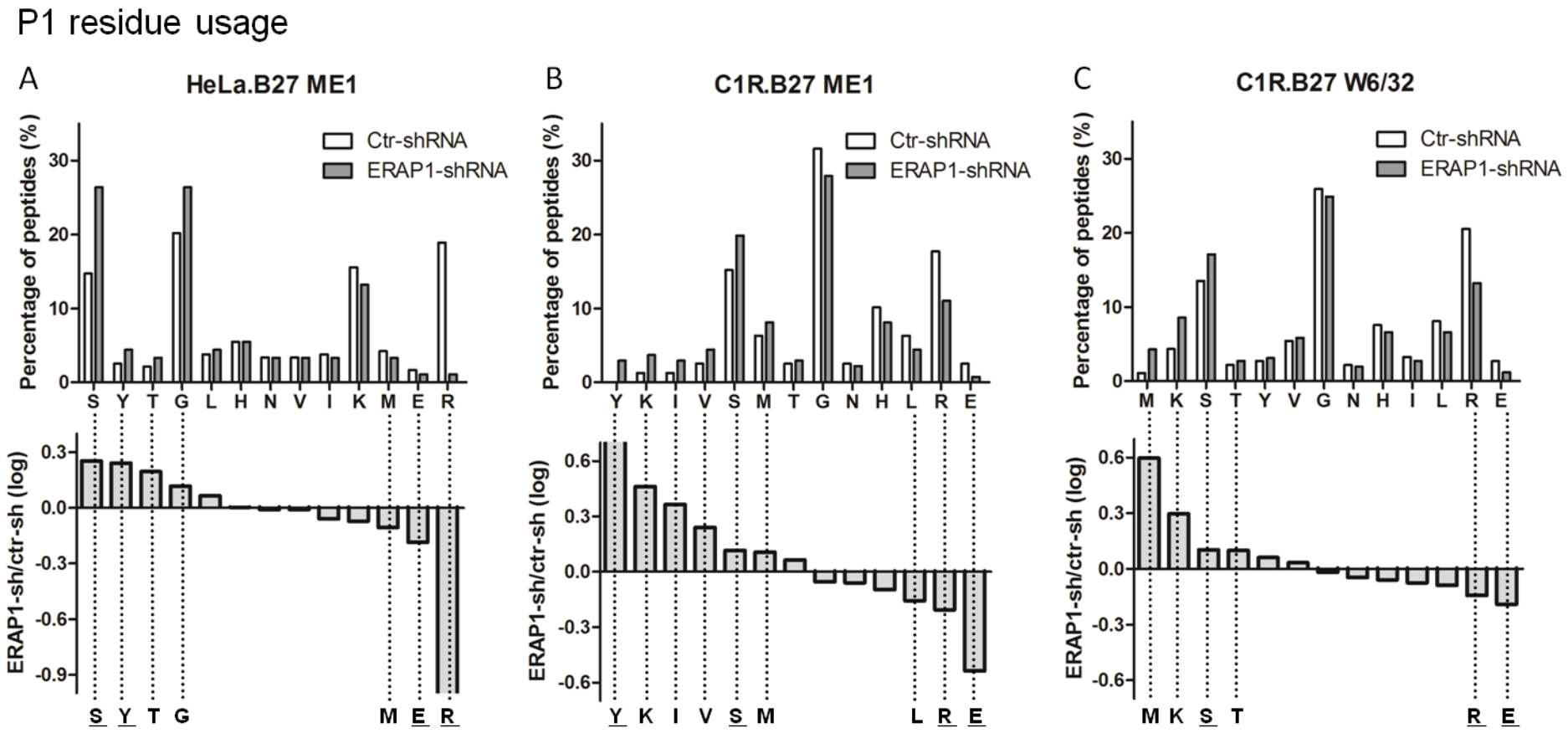
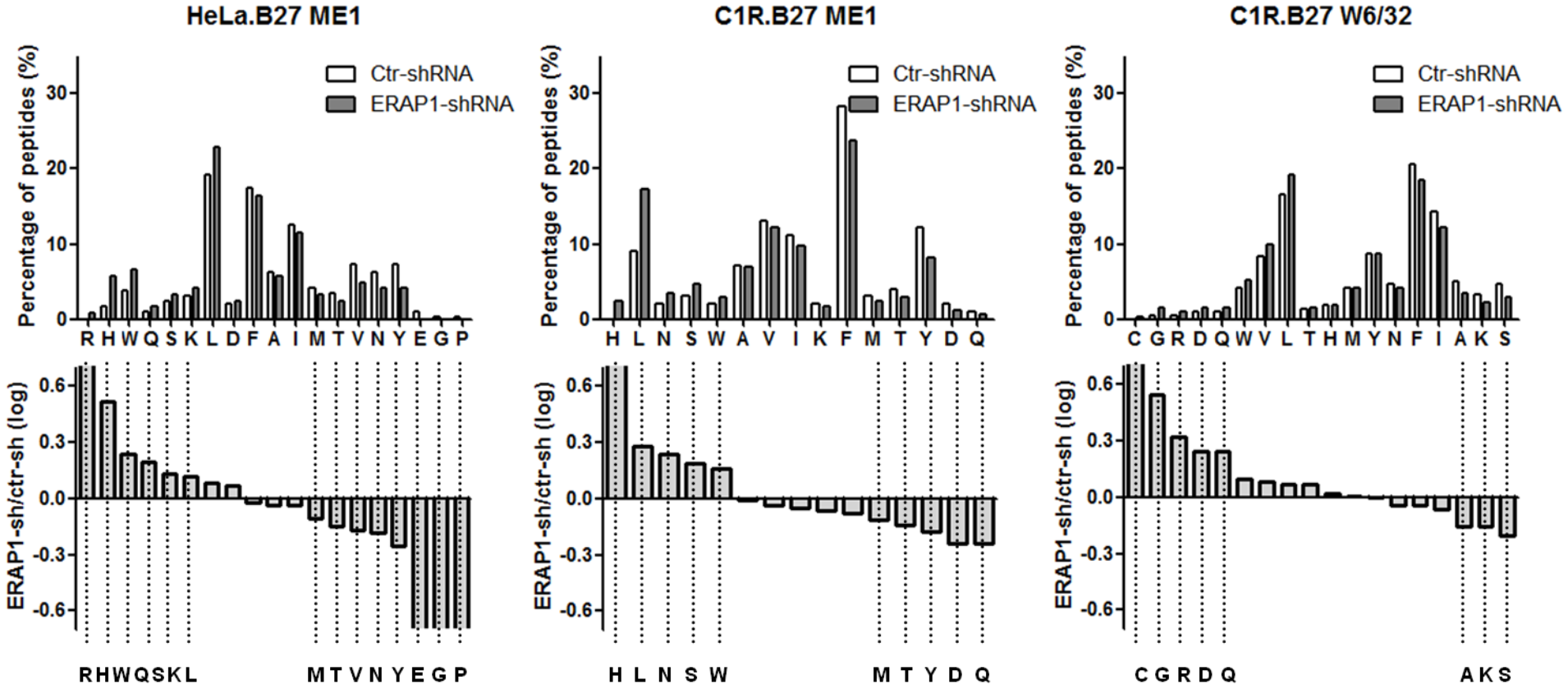
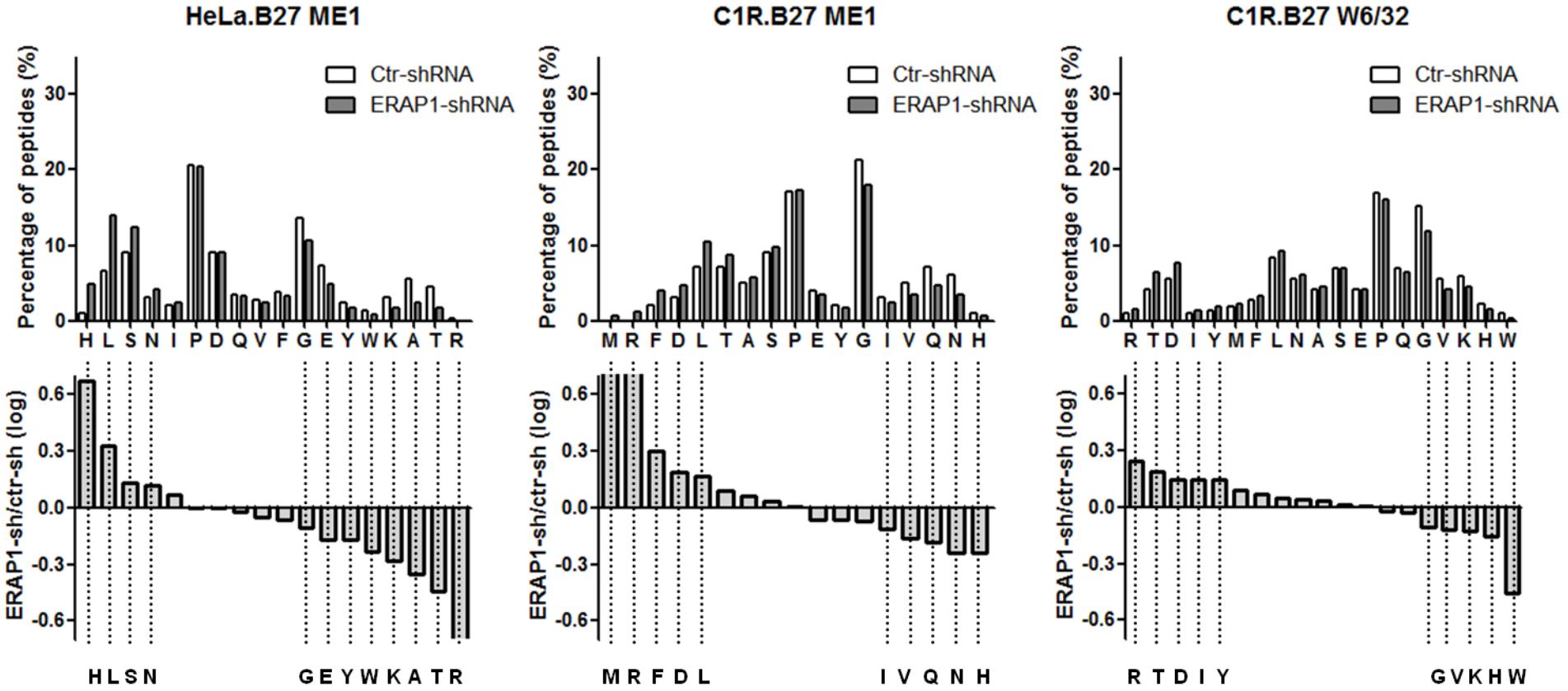


Figure 4.6 ERAP1 affects the nature of the P1 amino acid of peptides bound to HLA-B27. The amino acid usage of HLA-B27-bound peptides at position 1 was compared between cells with ctr-shRNA and ERAP1-shRNA. The order of residues was arranged based on the log ratio of peptide percentages between ERAP1-shRNA and ctr-shRNA (ERAP1-sh/ctr-sh (log)). Residues with the ERAP1-sh/ctr-sh log ratio >0.1 or <-0.1 were highlighted with dashed line above. P, D and W were not included because of their low abundance. A. HLA-B27 peptides isolated from HeLa.B27 cells using ME1. B. HLA-B27 peptides isolated from C1R.B27 cells using ME1. C. HLA-B27 peptides isolated from C1R.B27 cells using W6/32. Number of experiment (n=1) has been limited by time and expense of SILAC.

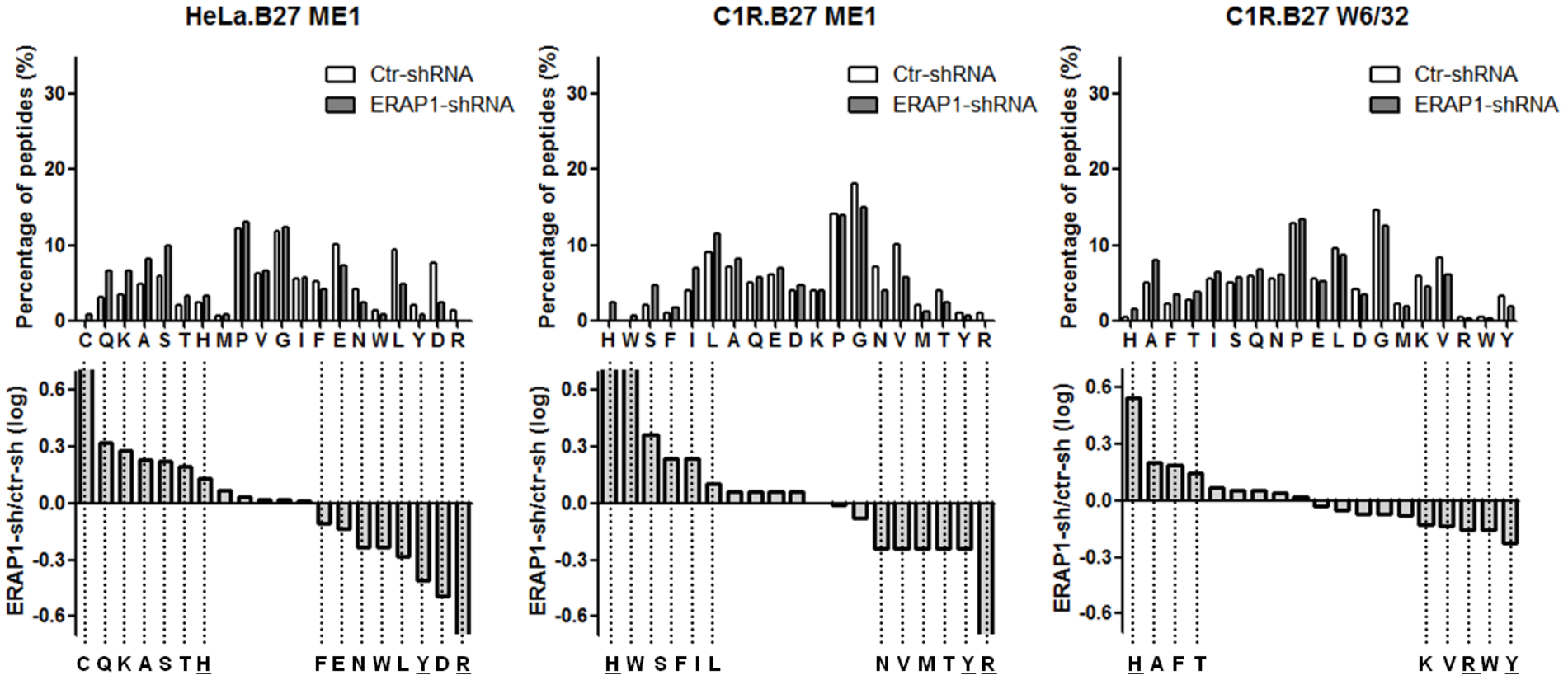
A. P3 residue usage



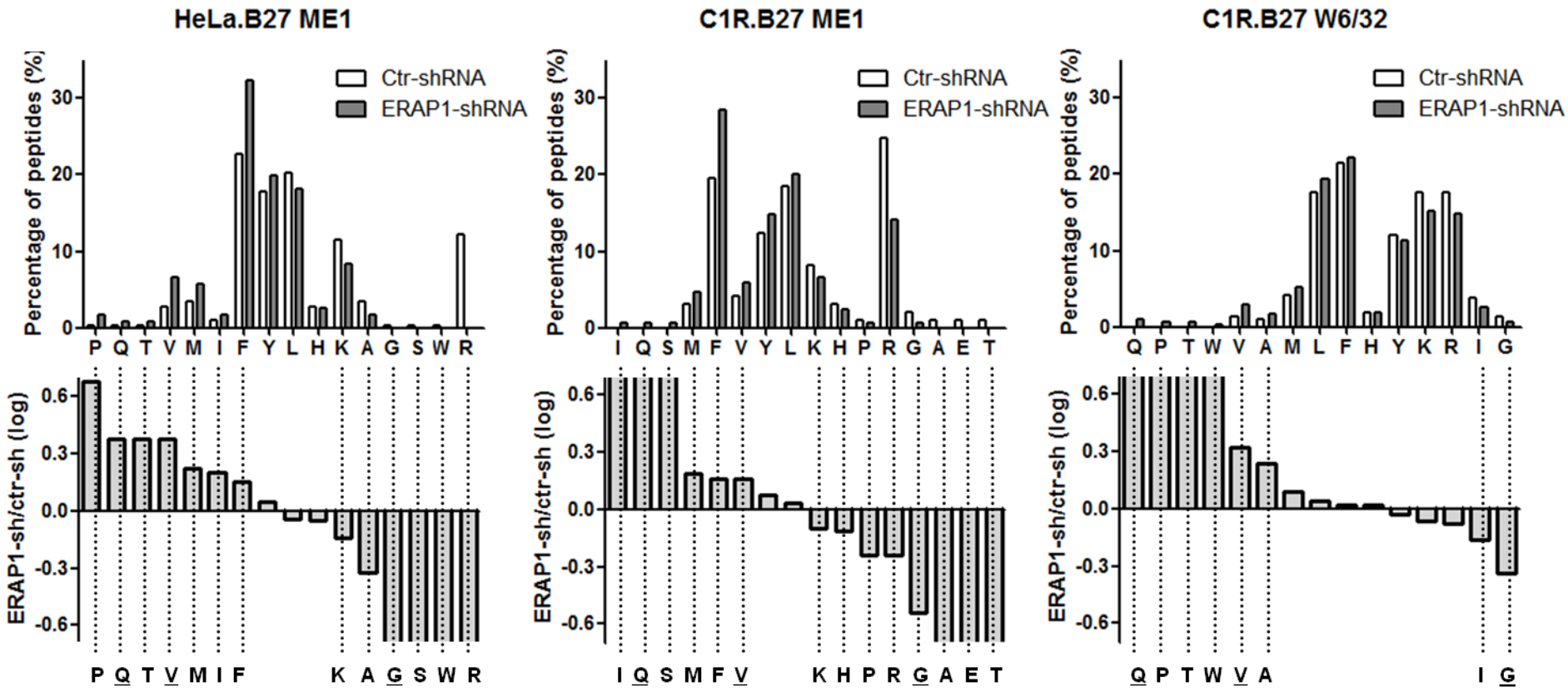
B. P4 residue usage



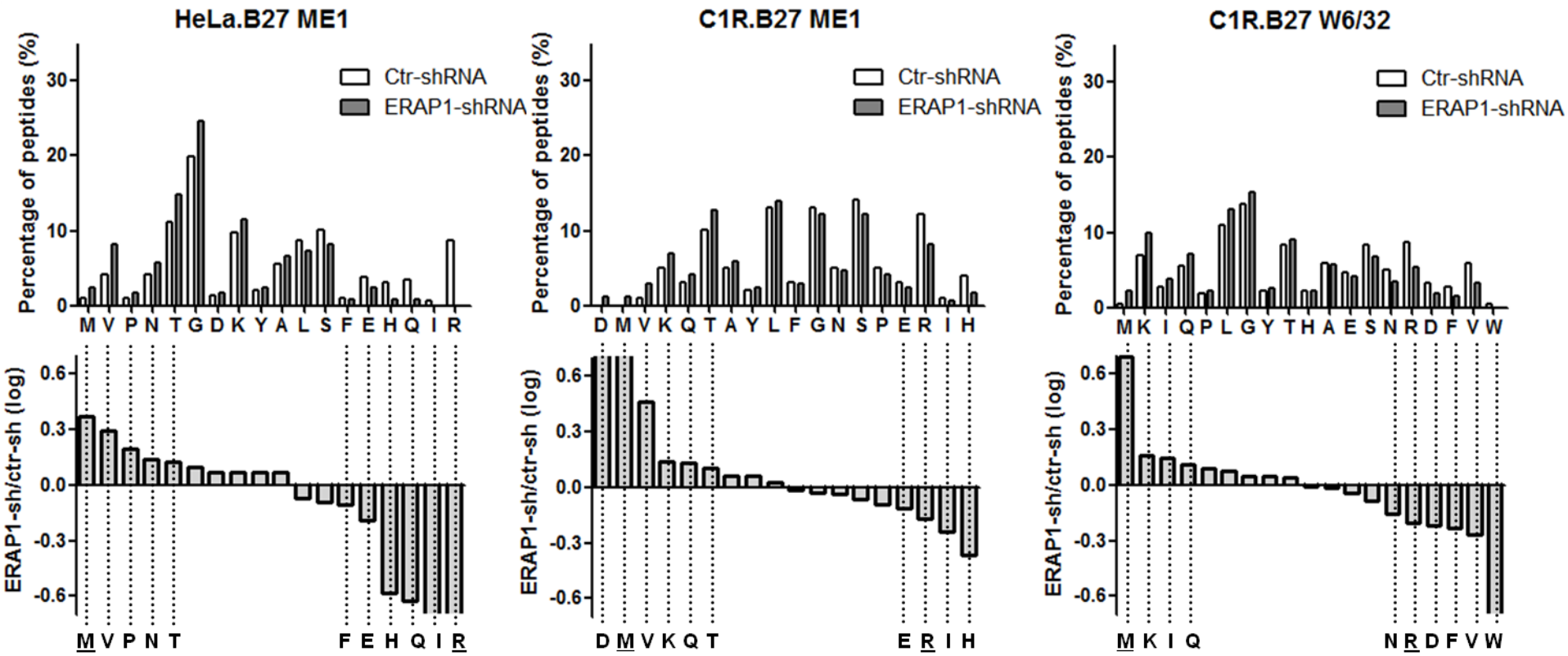
C. P5 residue usage



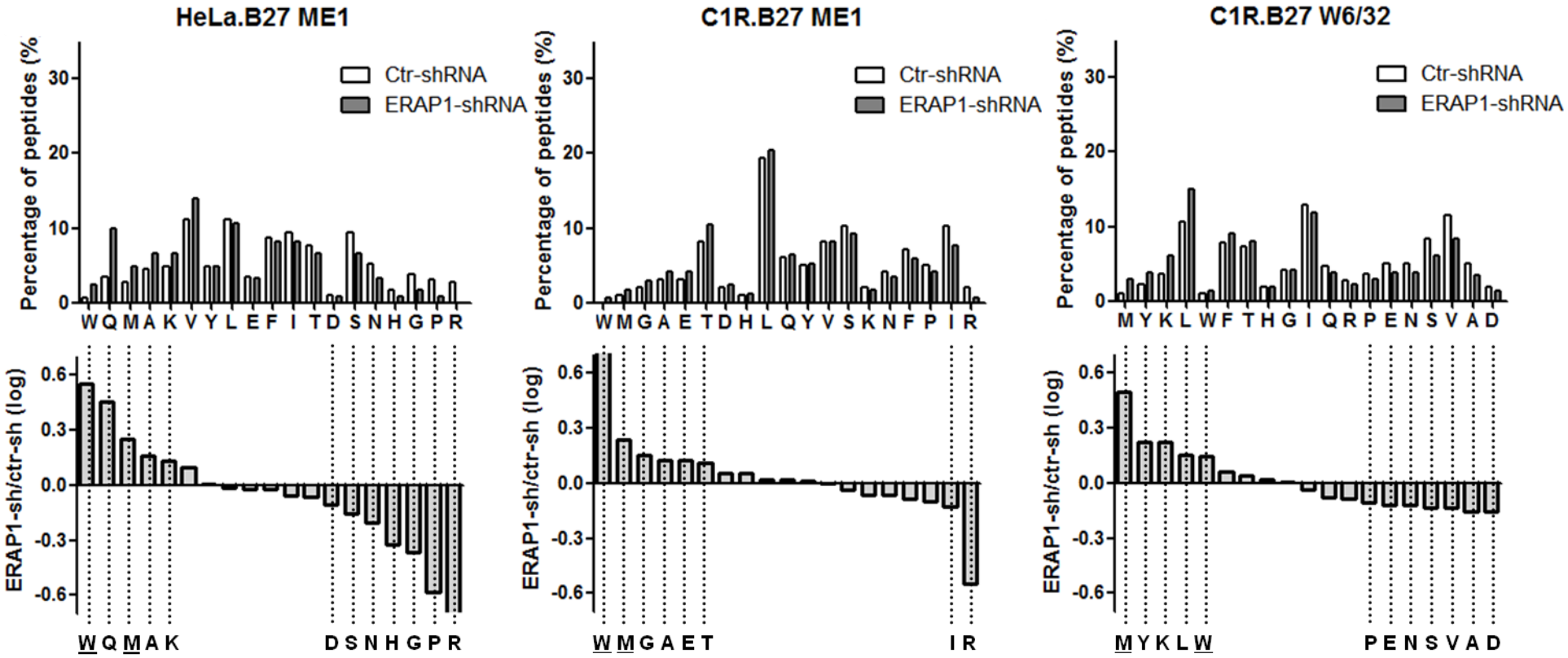
D. P [C-1] residue usage



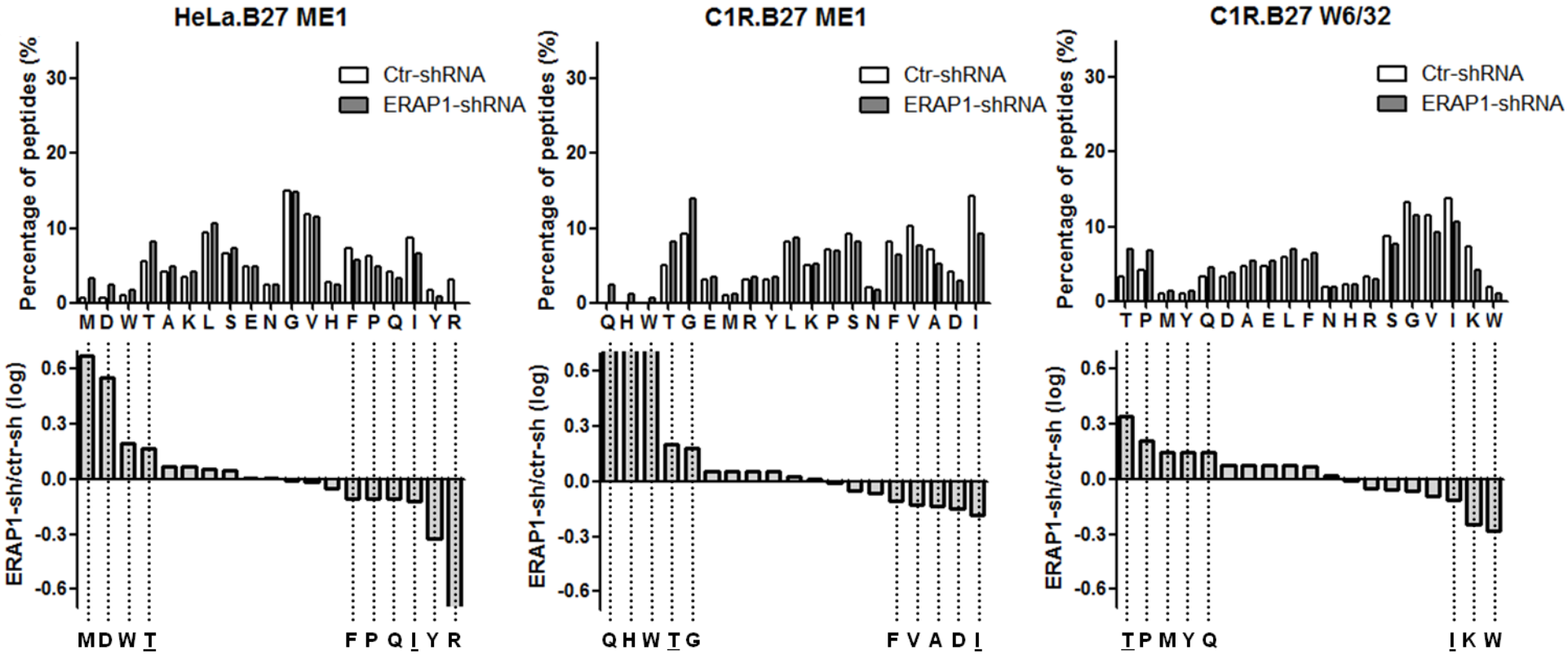
E. P [C-2] residue usage



F. P [C-3] residue usage



G. P [C-4] residue usage



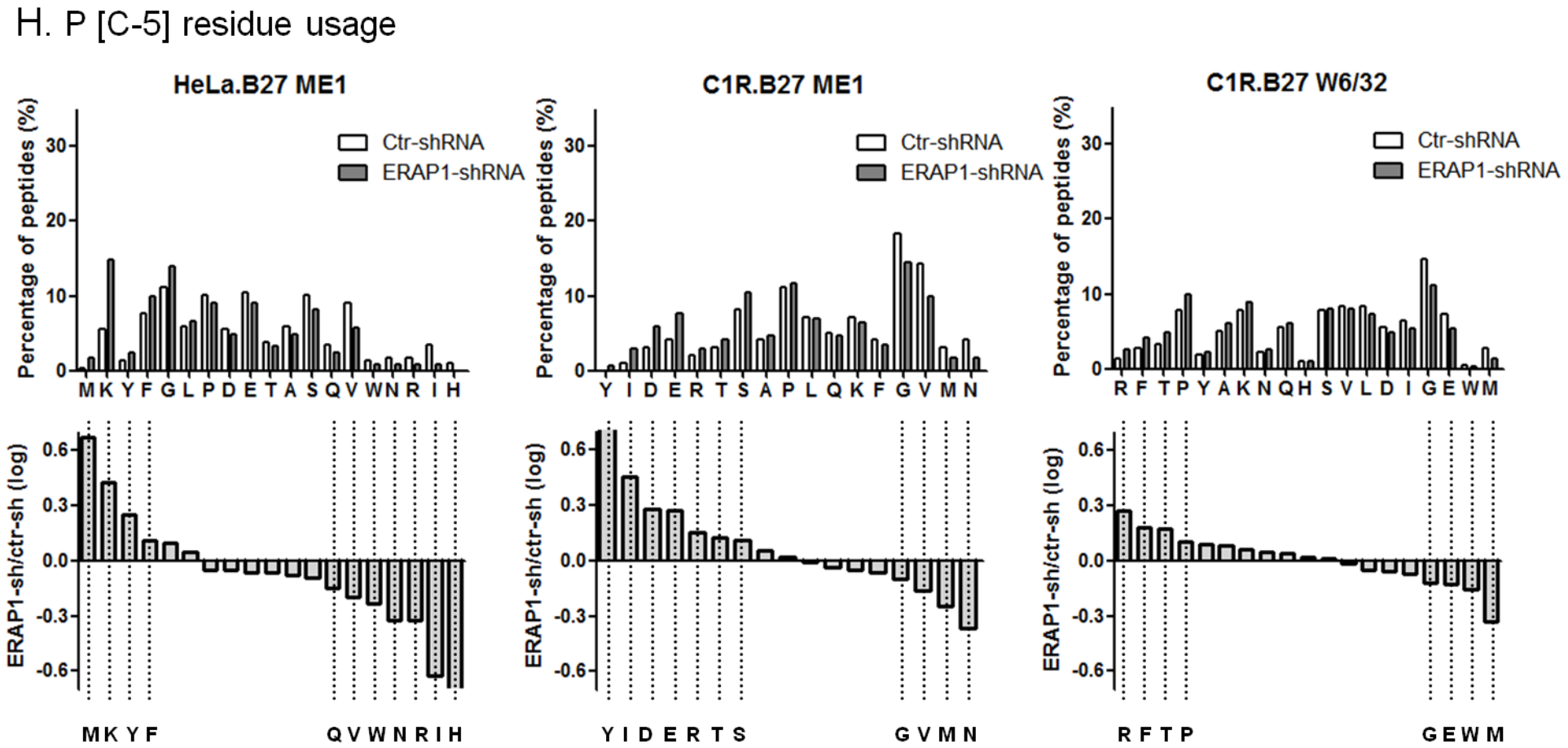


Figure 4.7 Effects of ERAP1 silencing on amino acid usage at P3-P5 and C-terminal P1-P5 of peptides bound to HLA-B27. The amino acid usage of HLA-B27-bound peptides at different positions was compared between cells with ctr-shRNA and ERAP1-shRNA. The order of residues was arranged based on the log ratio of peptide percentages between ERAP1-shRNA and ctr-shRNA (ERAP1-sh/ctr-sh (log)). Residues with the ERAP1-sh/ctr-sh log ratio >0.1 or <-0.1 were highlighted with dashed line above. Residues in low abundance were not included. A-H shows effect of ERAP1 silencing on residues usage at P3, P4, P5, P [C-1], P [C-2], P [C-3], P [C-4] and P [C-5] respectively. Number of experiment (n=1) has been limited by time and expense of SILAC.

4.2.4 The Effects of ERAP1 silencing on peptides present in the endoplasmic reticulum

ERAP1 is an aminopeptidase that trims its substrates from the N-terminus (York et al. 2002), it is therefore expected that silencing of this enzyme should lead to N-terminal extension of HLA-B27 epitopes. However, the long peptides identified in MS/MS were mainly C-terminally extended. This is possibly due to the strong conservation of Arg at position 2 in HLA-B27 ligands. Indeed, 5 out 6 N-terminally extended peptides identified in ERAP1-silenced cells were Arg2 peptides (Table 5.2). If this assumption is correct, N-terminally extended peptides caused by ERAP1 silencing should be present in the ER. I therefore studied the overall repertoire of peptides within the ER to look for evidence. For this purpose, isotope Arg-labelled ERAP1-silenced and -competent C1R.B27 cells were mixed and mechanically lysed before sequential centrifugation for ER enrichment (1,000 x g for 10 minutes, 12,000 x g for 15 minutes and 100,000 x g for 1 hour) (details described in **2.16, Chapter 2**). The pellet of each centrifugation and final supernatant were lysed by detergent and blotted for nuclear and ER markers (Figure 4.8 A). SP-1, a nuclear protein, was only found in 1K pellet suggesting the nuclei and intact cells were successfully removed by 1,000 x g centrifugation. In the 100K pellet, all four ER marker proteins (ERAP1, ERp57, Tapasin and calreticulin) were increased compared to 1K pellet, indicating that ER has been enriched. Moreover, the higher molecular weight band in the 100K pellet of ERp57 and calreticulin blots was removed by the 100,000 x g centrifugation. This suggests that the Golgi have been removed by 12,000 x g centrifugation (because protein glycosylation is one of the Golgi's main functions). Figure 4.8 B shows that similar amount of protein from each pellet and final supernatant has been loaded for western blotting. A coomassie blue-stained protein gel is used rather than a western

blot of housekeeping proteins (because they locate at certain cellular compartment and cannot be used as the loading control here, e.g. GAPDH in the cytosol, beta-actin in cytoskeleton).

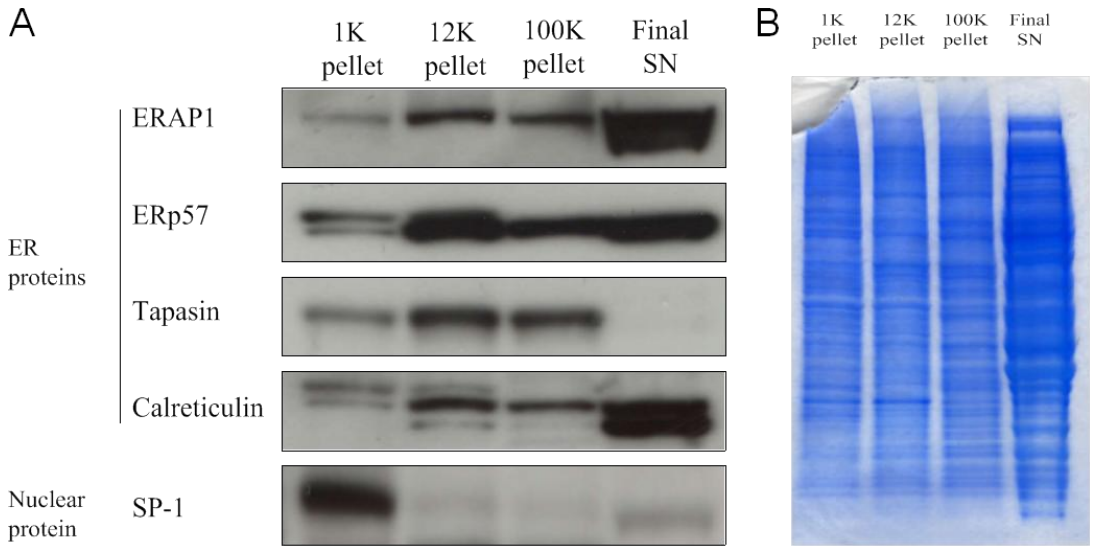


Figure 4.8 ER enrichment by sequential centrifugation. Isotope Arginine-labelled ERAP1-silenced and -competent C1R.B27 cells were mixed and mechanically lysed (5 x 10^8 cells from each). ER was then enriched by sequential centrifugation (1,000 x g for 10 minutes, 12,000 x g for 15 minutes and 100,000 x g for 1 hour). A. 1,000 x g pellet, 12,000 x g pellet, 100,000 x g pellet and final supernatant were lysed and blotted for ER proteins (ERAP1, ERp57, Tapasin and calreticulin) and nuclear protein (SP-1). B. Coomassie blue-staining of gel in A showing similar amount of proteins have been loaded for each lane.

The ER-enriched 100K pellet was lysed and treated with 0.1% TFA to precipitate proteins. The resulting supernatant containing peptides was purified by HPLC (data not shown) and analysed by MS/MS. 181 peptides were identified from C1R.B27 with ctr-shRNA, and 38 from the ERAP1-shRNA treated cells. Table 5.2 shows that 3 extended peptides were obtained from ERAP1-silenced cells compared to 1 in ERAP1-competent cells. Interestingly, all 4 peptides were C-terminally extended. Only 1 peptide had a P2 Arginine in either form.

ERAP1 has been reported to strongly prefer peptide substrates 9 -16 amino acids long (Chang et al. 2005). Silencing of ERAP1 could potentially increase the percentage of longer peptides resident in the ER. To test this hypothesis, I compared the percentage of 9mer, 10mer, 11mer, 12mer, 13mer, 14mer, 15mer and 16mer ER-resident peptides between ctr-shRNA and ERAP1-shRNA treated C1R.B27 cells (Figure 4.9). In agreement with the assumption, the percentages of long peptides, 12mer and 14mer, appeared to be increased by ERAP1 silencing. However, surprisingly, ERAP1 silencing increased the percentage of 9mer and decreased 10mer, 11mer and 16mer. The percentage of 13mer and 15mer was similar between ERAP1-competant and -deficient cells.

C1R.B27 **ER enrichment**	
Ctr-shRNA	**ERAP1-shRNA**
APGAEFAPNK	APGAEFAPNKR
APIDRVGQTIE	APIDRVGQTIER
NRAAPGAEFAPNK	NRAAPGAEFAPNKR
*RSAPGGGSKVPQK	*RSAPGGGSKVP

Table 5.2 Extended peptides from enriched-ER following ERAP1-shRNA silencing in C1R.B27 cells. Datasets of peptides identified from control and ERAP1-shRNA silenced cells were cross searched for extended or truncated forms. The 'core' sequences of the extended peptides are underlined. 3 extended peptides were identified from ERAP1-silenced cells and 1 (marked *) from control shRNA-transduced cells. Number of experiment (n=1) has been limited by time and expense of SILAC.

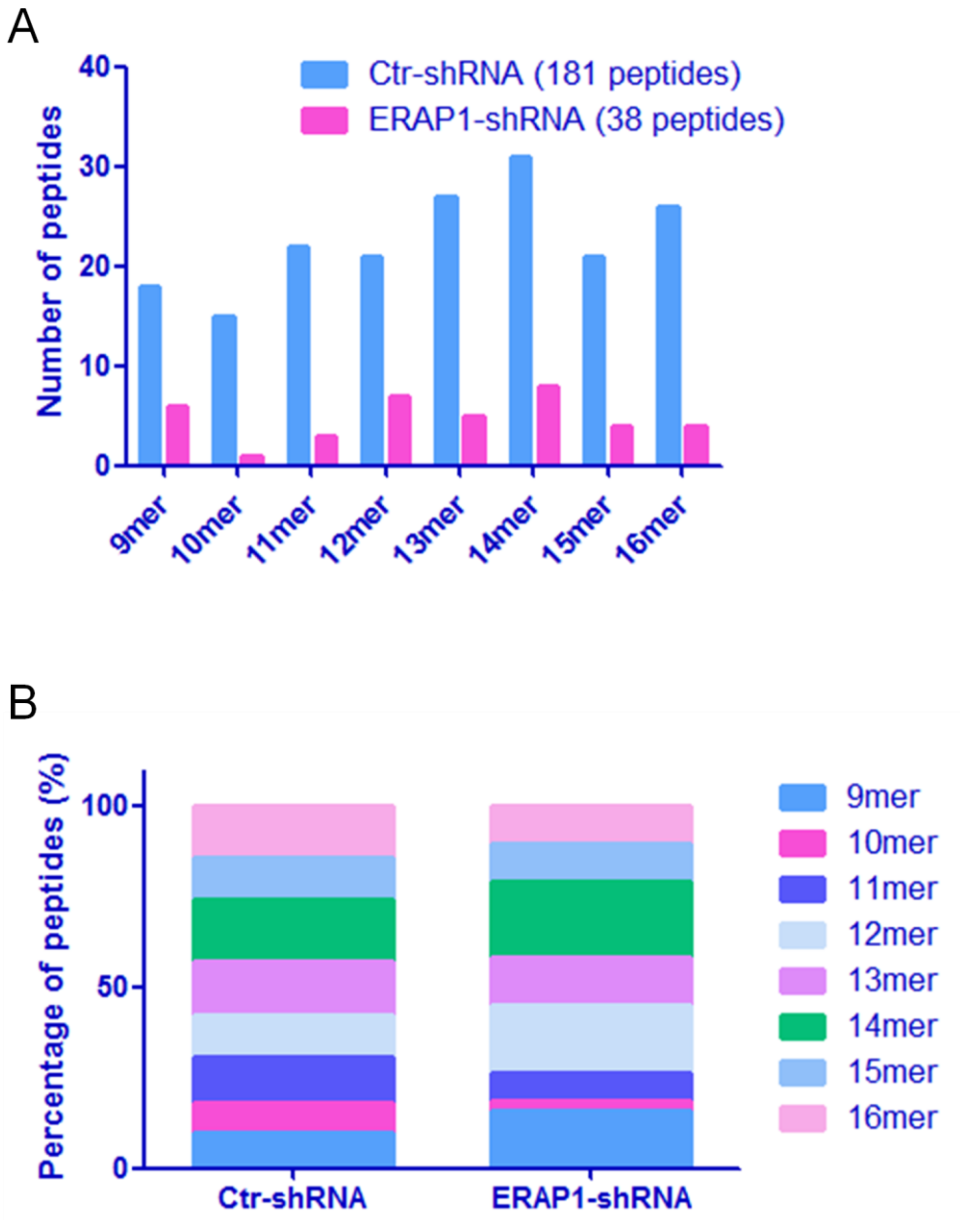


Figure 4.9 Effects of ERAP1 silencing on ER-resident peptides in C1R.B27 cells. ER-resident peptides of different length, 9mer, 10mer, 11mer, 12mer, 13mer, 14mer, 15mer and 16 mer were compared between control-shRNA and ERAP1-shRNA. A. Number of peptides of different length. B. Percentage of peptides of different length. Number of experiment (n=1) has been limited by time and expense of SILAC.

4.3 Discussion

My data show that ERAP1 plays a non-redundant role in shaping the repertoire of peptides bound to the AS-associated HLA-B27. Silencing of ERAP1 led to both N-terminal and C-terminal extensions of HLA-B27 epitopes, at least in 2 HLA-B27 transfected/transduced human cell lines. Interestingly, although ERAP1 trims peptides from the N-termini, these longer peptides were predominantly C-terminally extended. In contrast, extended MHC class I peptides caused by ERAAP knockout in mice were mainly N-terminally extended (Blanchard et al. 2010). The likely explanation is that N-terminally extended epitopes bind so poorly to HLA-B27 that they are rarely detected. This is supported by the finding that 5 out of 6 N-terminally extended peptides identified contained Arginine at P2 and could thus potentially bind in a shifted register. A model illustrating this is shown in Figure 4.10. Other possible explanations include a peptide editing function of ERAP1 or off target inhibition of the proteasome or of alternative peptidases by ERAP1-shRNA.

I also found that ERAP1 silencing increased the proportion of longer peptides (11 to 13 mer) bound to HLA-B27. This finding agrees with previous data on murine MHC-I showing that ERAAP-/- increases the length of peptides bound to H-2D^b and H-2K^b (Blanchard et al. 2010). I also found that ERAP-silencing changed the nature of P1 amino acid of peptides bound to HLA-B27, increasing uncharged polar residues (S, Y and T) whilst reducing the prevalence of E and R. This observation is in accordance with a previous study showing that charged N-terminal residues were relatively protected from ERAP1 degradation (Hearn et al. 2009).

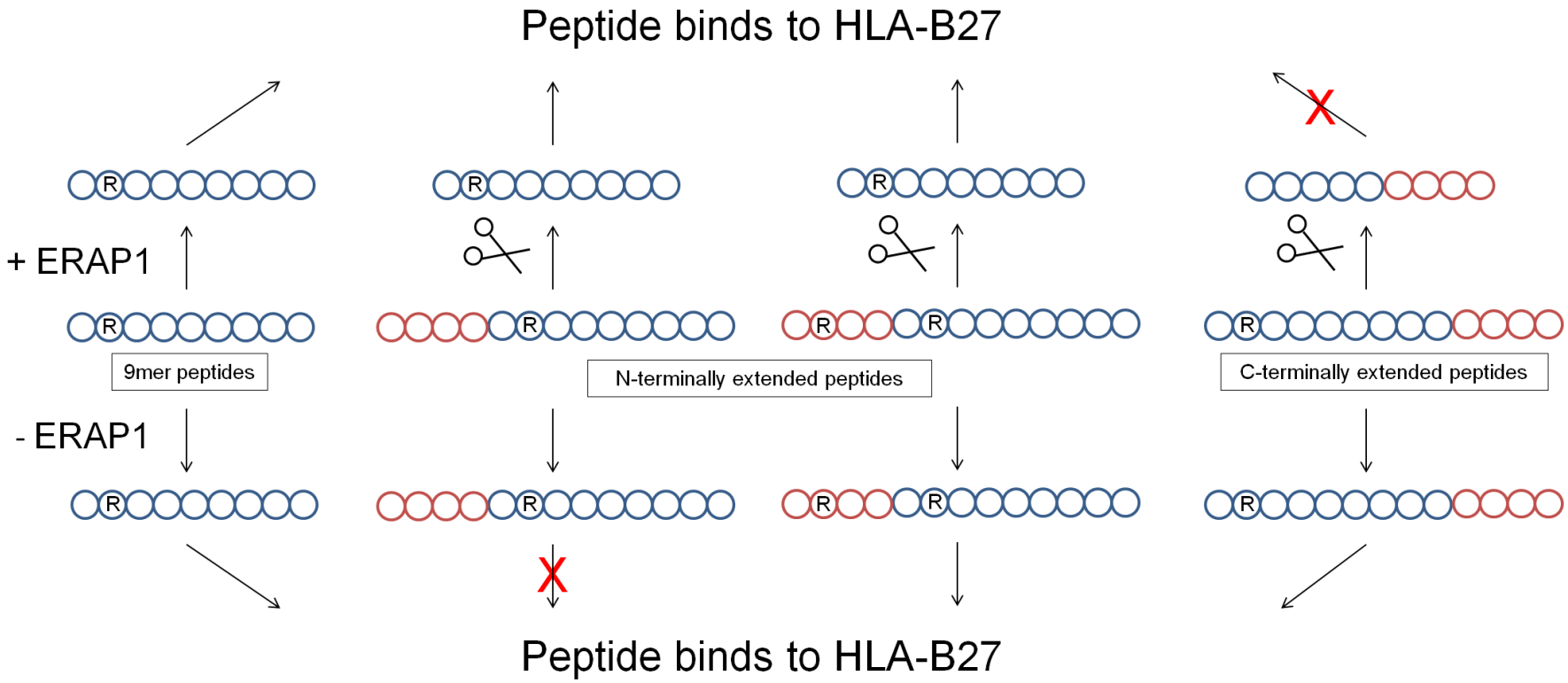


Figure 4.10 Model explaining the effect of ERAP1 silencing on the nature of peptides bound to HLA-B27 in the ER. 9mer Arg2-peptides are optimal ligands for HLA-B27.regardless of ERAP1 status. In absence of ERAP1, N-terminally extended peptides which do not have Arg2 are not trimmed and fail to bind to HLA-B27; N-terminally extended peptides carrying Arg2 and the C-terminally extended peptides are also not trimmed but may bind to HLA-B27.

It should be pointed out that the HLA-B27-bound peptides studied here were isolated from whole cell lysate. These peptides were therefore derived from both cell surface and intracellular HLA-B27 complexes. For better dissection of the role of ERAP1 in HLA-B27 peptide binding, HLA-B27 expressed on the cell surface and located intracellularly need be isolated separately. Papain can be used to solubilise surface MHC class I in prior to HLA-B27 immunoprecipitation (Jardetzky et al. 1991). Intracellular HLA-B27 complexes can then be isolated from papain-stripped cells.

If most N-terminally extended peptides caused by ERAP1 silencing were not eluted from HLA-B27 because of their poor binding in absence of Arg2, these peptides should be present in the ER. I therefore studied effect of ERAP1 silencing on peptides in enriched ER. Similar to HLA-B27-bound peptides isolated from whole cell lysate, absence of ERAP1 generated extended ER-resident peptides, which unexpectedly were all C-terminal extensions. This raises the possibility that these peptides with C-terminal extension are preferably destroyed by ERAP1. It is surprising that no N-terminally peptides were identified, given that N-terminally extended peptides, although in low numbers, were found in HLA-B27-bound peptides. The possible explanation is that the N-terminal peptide extensions in the ER were trimmed by other aminopeptidases (such as ERAP2). Although reliability of ER enrichment and the low number of peptides identified in ERAP1-silenced cells need to be considered as sources of potential bias.

The percentages of both peptides of optimal length (9mer) and long peptides (12mer and 14mer) in the ER were increased by ERAP1 silencing, suggesting that ERAP1 can destroy epitope in addition to epitope generation through trimming of long

peptides. Moreover, I also found that 10mer and 11mer peptides were decreased in ERAP1-silenced cells. This might reflect that the presence of ERAP1 can potentially protect some sub-optimal peptides from trimming by other ER aminopeptidases. Alternatively, this finding is possibly caused by decreased trimming of longer peptides, such as 12mer and 14mer, in the absence of ERAP1.

Only a minor proportion of ER-resident peptides carry Arginine at P2 (4 out of 38 in ERAP1-silenced cells, 6 out of 181 in ERAP1-competent cells). This might indicates that, in the ER, peptides optimal for HLA-B27 binding have been selected out by the expression of HLA-B27 in C1R cells. Therefore, ER enrichment using untransduced C1R cells will help for better understanding of the effect of ERAP1 silencing on generation of peptides optimal for HLA-B27 binding in the ER.

Overall, ERAP1 silencing affects the length and P1 residues usage of peptide bound to HLA-B27 and the peptides present in the ER. How ERAP1 affects the generation of HLA-B27-bound peptides in the ER needs further investigation.

Chapter 5. The effects of ERAP1 silencing and of allelic differences on peptide presentation by HLA-B27 to cytotoxic T lymphocyte

5.1 Introduction

The classic function of MHC class I molecules is presentation of antigen to $CD8^+$ T cells for immune surveillance. In the mouse, absence of ERAAP (ER aminopeptidase associated with antigen processing) has been shown to alter $CD8^+$ T cell responses to epitopes presented by MHC class I proteins. Loss of ERAAP reduces $CD8^+$ T cell responses by decreasing presentation of natural antigenic peptides (G. E. Hammer et al. 2006) and alters the immune response to viral peptides in mice infected with vaccinia virus or lymphocytic choriomeningitis virus (LCMV) (York et al. 2006).

I have shown in the previous chapter that ERAP1 silencing alters the length and P1 residue usage of the peptides bound to HLA-B27. It has been shown previously that P1 residue changes in an HLA-B27-bound epitope were able to influence recognition by the T cell receptor of CTL (Bowness et al. 1994). In order to study if the effect of ERAP1 silencing is biologically meaningful, the effect of ERAP1 silencing on presentation of HLA-B27 epitope to $CD8^+$ T cells was examined. An HLA-B27-restricted HIV-gag epitope (KK10)-specific cytotoxic T lymphocyte (CTL) clone (named 011-B5) was made and maintained by Dr Yanchun Peng from Dr Tao Dong's lab. The CTLs were co-cultured with HLA-B27-expressing cells infected with

HIV-gag recombinant vaccinia virus or transfected with minigene encoding HIV-gag-derived KK10 peptide precursors. As IFN-γ secretion is a marker of CTL activation, IFN-γ ELISPOT was used as readout. If and how ERAP1 silencing affects presentation of the KK10 epitope by HLA-B27 to a CTL clone were studied. The effect of induction of wild type and different ERAP1 alleles was also examined using ERAAP-/- murine fibroblasts.

5.2 Results

5.2.1 ERAP1 silencing reduces presentation to a KK10-specific HLA-B27-restricted CTL clone by vaccinia-infected HeLa.B27 cells

To optimise the conditions for HIV-Gag recombinant vaccinia virus (HIV-Gag rVV) infection, I first infected HeLa.B27 with increasing amount of rVV (0, 5, 10 and 20 pfu/cell) (Figure 5.1 A). 5 pfu/cell was able to provoke CTL activation as effectively as KK10 peptide pulsing, higher virus load did not further increase the activation of CTL. 5 pfu/cell was therefore enough to maximize the infection rate and chosen for later experiments. Notably, neither rVV infection nor KK10 pulsing of HeLa.B27 cells led to activation of a control CTL clone (Flu-A2). Figure 5.1 B compares CTL activation by co-culture of target cells (HeLa.B27) and effector cells (CTL) at different target to effector (T:E) ratios (80:1, 40:1, 20:1, 2:1). 20:1 was chosen for later experiments because any ratio higher did not significantly change the number of CTLs activated. I also compared the efficacy of HeLa.B27 cells rested for 1 hour and 3 hours post-infection for CTL activation and found no difference (Figure 5.1 C). 1 hour was therefore selected for later experiments.

To study the effect of ERAP1 on presentation of KK10 to CTLs, HeLa.B27 cells transduced with ctr-shRNA or ERAP1-shRNA were first infected with HIV-Gag rVV, and then co-cultured with HLA-B27-restricted KK10-specific CTLs. Figure 5.2 A shows that silencing of ERAP1 reduces CTL recognition by approximately 50% (P =0.001). ME1 staining showed that HLA-B27 expression levels were not significantly altered by ctr-shRNA or ERAP1-shRNA (Figure 5.2 B). To support the contention that this was indeed due to reduced presentation of KK10 on the cell surface, and to confirm the specificity of KK10-CTLs, I studied the activation of KK10-CTLs by a panel of NH2 or COOH-terminally extended KK10 peptides based on the Gag-P24 protein sequence (Figure 5.2 C). The canonical KK10 10mer was indeed found to activate notably more CTLs compared to other peptides (Figure 5.2 C) (albeit noting that KK10 was used to restimulate these CTL ex vivo). These data show for the first time that ERAP1 can affect presentation by HLA-B27 of a naturally occurring viral epitope to CTLs.

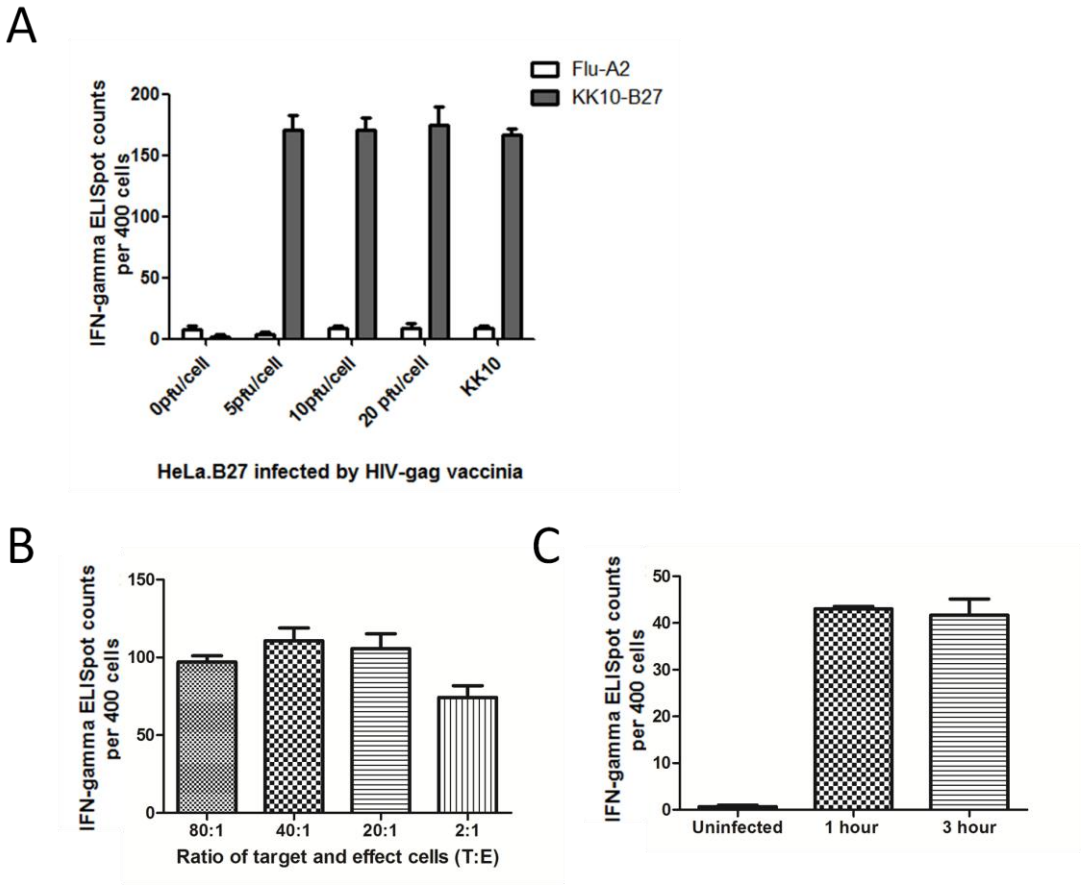


Figure 5.1 Optimization of HeLa.B27 infection using HIV-Gag recombinant vaccinia virus. Vaccinia-infected HeLa.B27 cells (8000 cells per well) were co-cultured with a CTL clone raised against KK10 (400 cells per well) for 16 hours. Measurement of IFN-gamma release using IFN-gamma ELISPOT was used to identify activated CTLs. A. Titration of amount of virus used for each cell (0, 5, 10, 20 pfu/cell). Flu-A2 CTL clone was used as negative control. KK10-B27 clone pulsed with KK10 peptide (10 nM, 1 hour incubation) was used as positive control. B. Effect of different target to effector ratio (80:1, 40:1, 20:1 and 2:1) on activation of KK10 CTLs. C. Effect of different resting time post-infection (1 and 3 hour) on KK10 CTLs activation. All experiments were repeated twice, representative experiments are shown.

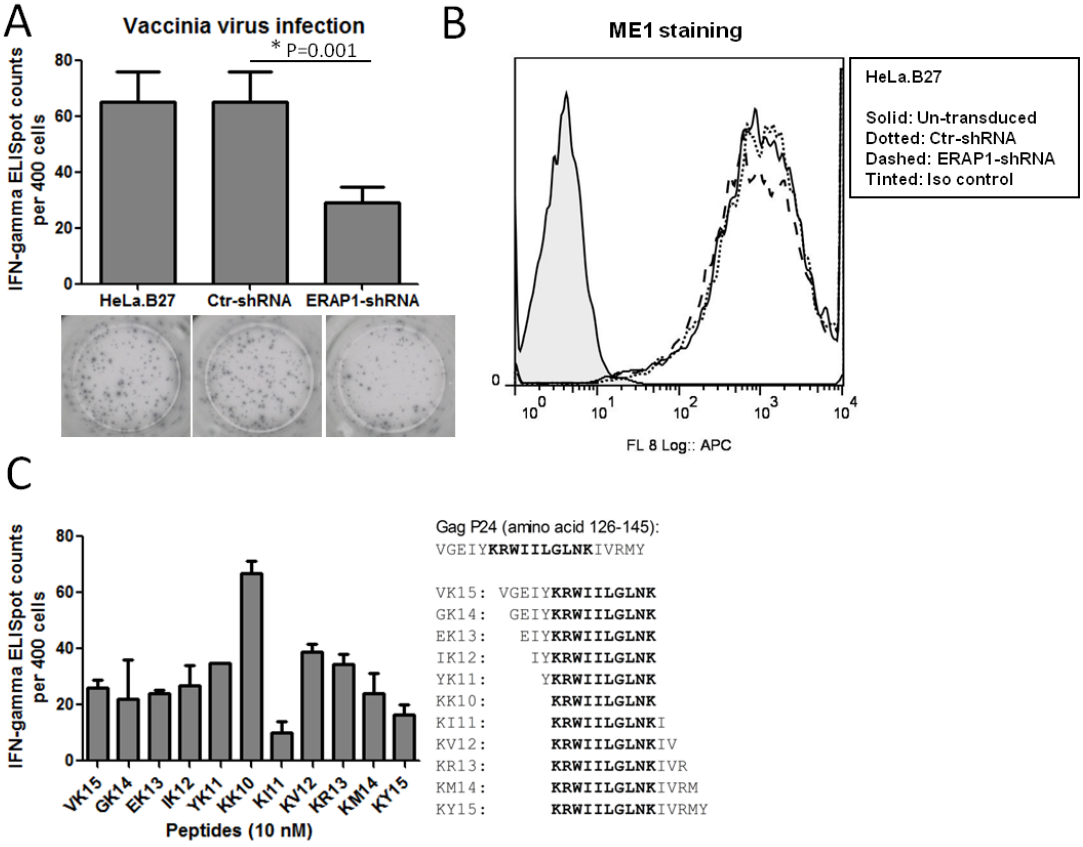


Figure 5.2 ERAP1 silencing reduces presentation by vaccinia-infected HeLa.B27 cells to a KK10-specific HLA-B27-restricted CTL clone. A. Un-transduced HeLa.B27 and HeLa.B27 with ctr-shRNA or ERAP1-shRNA were infected with recombinant HIV-Gag vaccinia virus and then co-cultured with CTLs (400 cells per well) for 16 hours. B. ERAP1-shRNA or ctr-shRNA did not affect expression of cell surface HLA-B2705 expression by HeLa.B27 cells. C. Specificity of CTL clone was examined by stimulating CTLs with a panel of extended KK10 peptides based on the Gag P24 protein sequence. Peptides were made by Dr Katalin Di Gleria. Experiment shown in A and B was repeated 3 times in triplicate, representative experiments are shown. Standard deviation and student's unpaired two-tailed t-test was used for statistics. Experiment shown in C was carried out once in triplicate.

5.2.2 ERAP1 silencing/knockout reduces presentation to a KK10-specific HLA-B27-restricted CTL clone by minigene-transfected HeLa.B27/Fib.B27

To further define the role of ERAP1 in presentation of KK10 to CTLs by HLA-B27, I used an N-terminally extended KK10 precursor VGEIYKRWIILGLNK (hereafter termed X5-KK10) whose expression was targeted to either the endoplasmic reticulum through an ER signal (ES) peptide (ES-X5-KK10) or the cytoplasm (X5-KK10) using minigene. Minigenes targeting expression of the KK10 10mer to ER (ES-KK10) or cytoplasm (KK10) were also used. Table 5.1 lists minigenes used for transfection. Dr Emma Reeves and Dr Edward James (University of Southampton) designed and made all minigene constructs and helped with the transfection. Figure 5.3 shows that ERAP1 silencing significantly reduces stimulation of KK10-specfic CTLs by HeLa.B27 when X5-KK10 is expressed in either the ER or the cytoplasm ($P<0.005$). Expression of a mouse Kb epitope directed into the ER (ES-ctr peptide) did not provoke any CTL activation.

In addition to silencing of ERAP1, I also introduced wild type ERAP1 (WT-ERAP1) into murine ERAAP-/- fibroblasts expressing HLA-B27 (hereafter termed ERAAP-/-mFib.B27). However, when using optimized T:E ratio (20:1), only around 5 CTLs were activated (Figure 5.4 A). This is possibly due to lower HLA-B27 expression in mFib.B27 cells compared to HeLa.B27 (data not shown) which results in fewer HLA-B27 molecules on the cell surface for interaction with T cell receptors from

CTLs. Indeed, higher T:E ratio (40:1, 80:1, 120:1) increased the number of activated CTLs (Figure 5.4 A, 80:1 selected for later experiments).

When ES-X5-KK10 was transfected into ERAAP-/- mFib.B27, CTL recognition was significantly increased by co-transfection with a plasmid encoding WT-ERAP1 ($P<0.001$, Figure 5.4 B). Interestingly, regardless of the presence of WT-ERAP1 or not, mFib.B27 cells transfected with X5-KK10 or KK10 minigenes without an ER targeting signal were unable to provoke a potent CTL response. Notably the presence of ERAP1 reduced the number of activated CTLs in mFib.B27 cells when KK10 was expressed in the ER (Figure 5.4 B), suggesting that ERAP1 can destroy HLA-B27 epitopes in addition to generating them. A slight decrease in cell surface HLA-B27 expression following WT-ERAP1 reconstitution was also observed and could have contributed (Figure 5.5 B). Taken together, these data show that ERAP1 significantly influences cell surface presentation of a naturally occurring HLA-B27 viral epitope.

Minigenes	Sequence	Expression location
X5-KK10	VGEIYKRWIILGLNK	Cytoplasm
KK10	KRWIILGLNK	Cytoplasm
ES-X5-KK10	VGEIYKRWIILGLNK	ER
ES-KK10	KRWIILGLNK	ER
ES-ctr peptide	SIINFEHL	ER

Table 5.1 Minigenes used for transfection. N-terminally extended KK10 precursor VGEIYKRWIILGLNK (X5-KK10) was targeted to either the endoplasmic reticulum through an ER signal (ES) peptide (ES-X5-KK10) or the cytoplasm (X5-KK10) using minigene. Minigenes targeting expression of the KK10 to ER (ES-KK10) or cytoplasm (KK10) were also used. A mouse Kb epitope, SIINFEHL, targeted to ER (ES-ctr peptide) was used as the negative control. All minigenes were designed and made by Dr Emma Reeves and Edward James (University of Southampton).

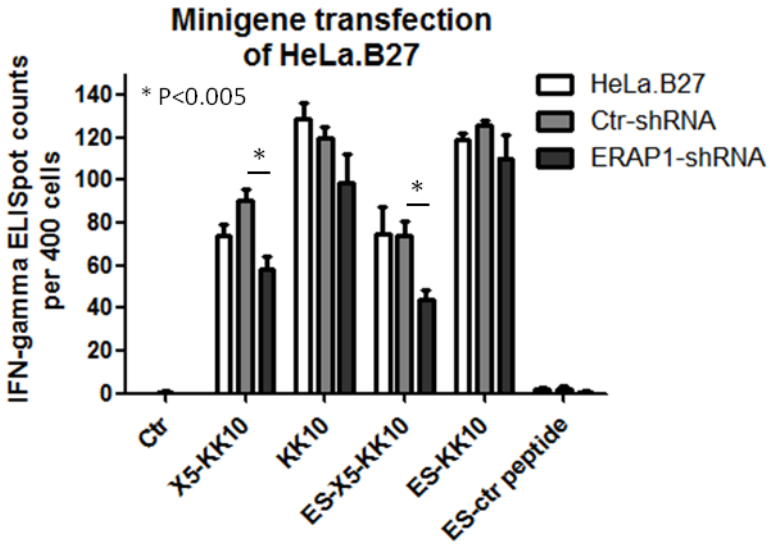


Figure 5.3 ERAP1 silencing reduces presentation to a KK10-specific HLA-B27-restricted CTL clone by minigene-transfected HeLa.B27. Un-transduced HeLa.B27 and HeLa.B27 with ctr-shRNA or ERAP1-shRNA were transfected with minigenes to express N-terminally extended KK10 15mer in the cytosol (X5-KK10) or in the ER (ES-X5-KK10) before co-culture with CTLs (400 cells per well). KK10 and a mouse Kb epitope were used as positive and negative control (KK10, ES-KK10 and ES-ctr peptide). Experiment was repeated 3 times in triplicate, representative experiments are shown. Standard deviation and student's unpaired two-tailed t-test was used for statistics.

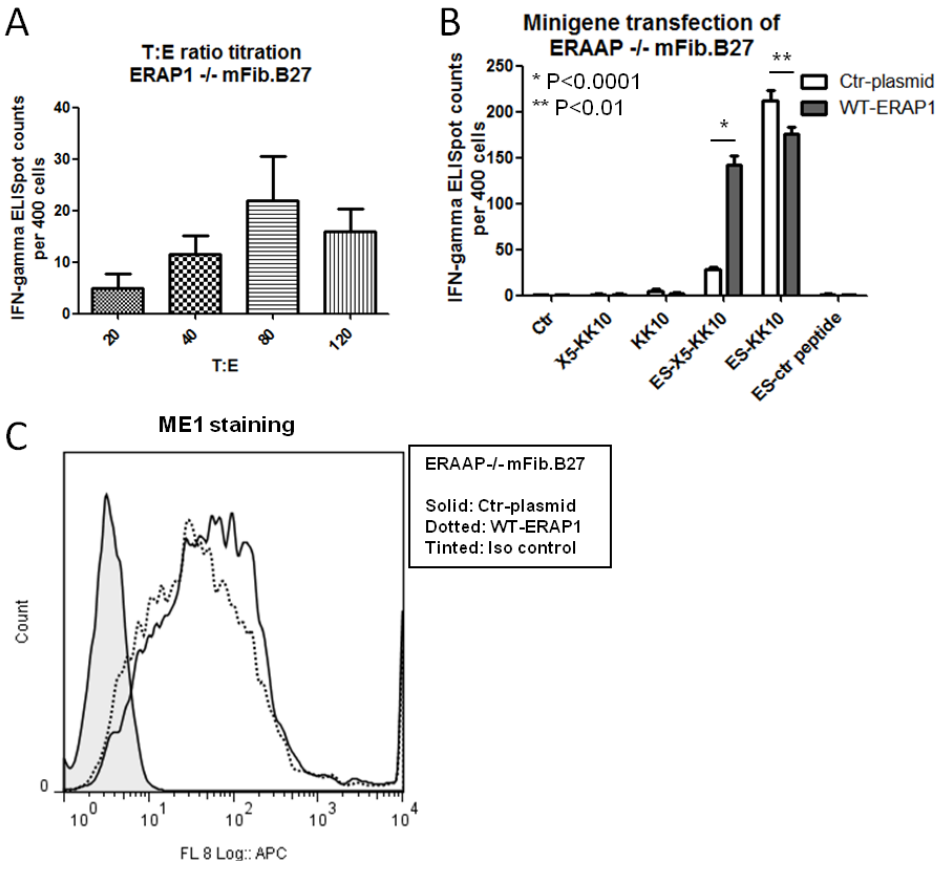
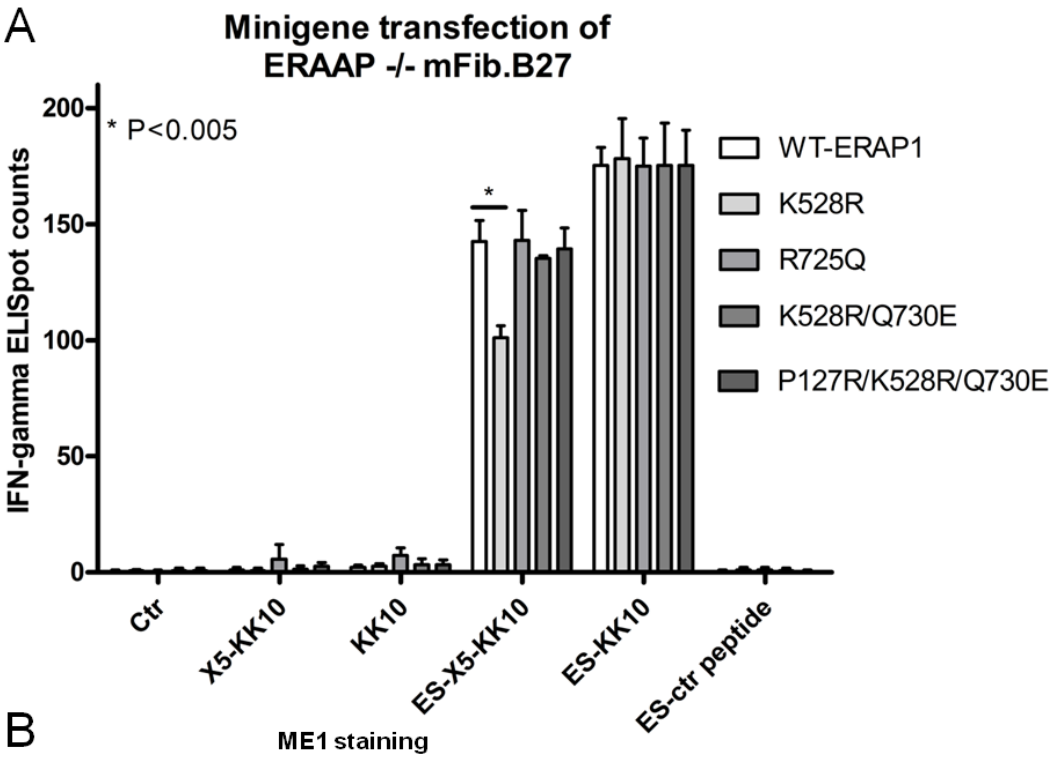
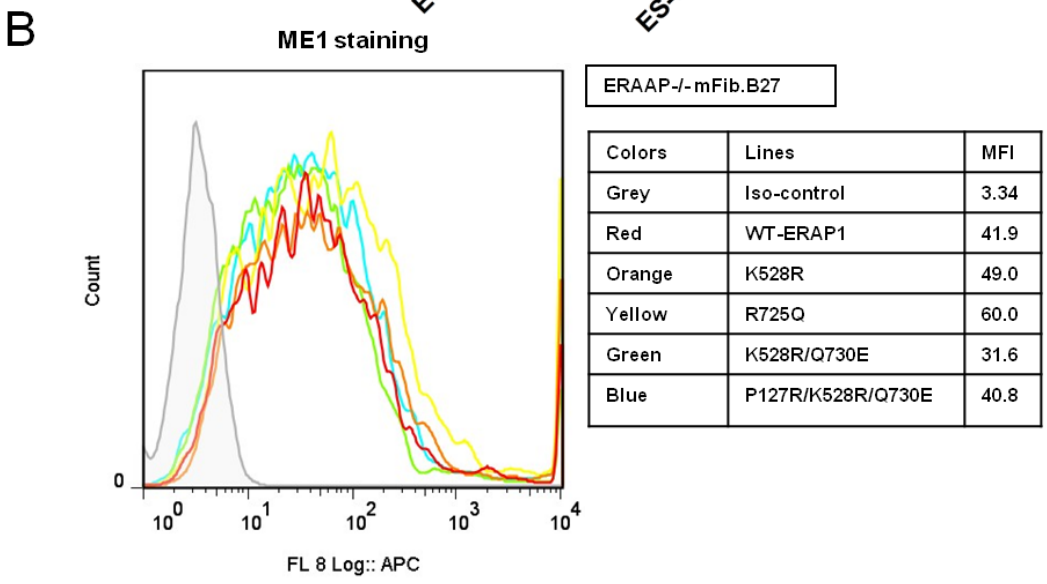


Figure 5.4 Introduction of ERAP1 increases presentation to a KK10-specific HLA-B27-restricted CTL clone by minigene-transfected ERAAP-/- mFib.B27. A. ERAAP-/- mFib.B27 cells were incubated with 10nM KK10 peptide for 1 hour in RPMI before co-culture with CTLs (400 cells per well) at different T:E ratios (20:1, 40:1, 80:1 and 120:1). B. Minigene-transfected ERAAP-/- mFib.B27 cells were co-transfected with WT-ERAP1 or ctr-plasmid. 48 hours later, cells were co-cultured with CTLs (400 cells per well). A minigene expressing an irrelevant peptide (a mouse Kb epitope, SL8) into the ER (ES-ctr peptide) was used as control for minigene transfection. C. Expression of cell surface HLA-B2705 by ERAP1-/- mFib.B27 was modestly decreased by introduction of WT-ERAP1 compared to ctr-plasmid. Experiment shown in A was carried out twice in triplicate; experiments shown in B and C were repeated 3 times in triplicate. Representative experiments are shown. Standard deviation and student's unpaired two-tailed t-test was used for statistics.

5.2.3 K528R-ERAP1, a protective variant in AS, reduces presentation of the HLA-B27-restricted KK10 epitope to CTLs.

We and others have shown that K528R-ERAP1 has a slower substrate trimming rate compared to WT-ERAP1 (Evans et al. 2011; Goto et al. 2006; Kochan et al. 2011). I therefore asked if cells with K528R-ERAP1 present endogenously-expressed KK10 peptide differently from those with WT-ERAP1. ERAAP-/- mFib.B27 cells were co-transfected with KK10 minigenes and plasmids encoding WT-ERAP1, K528R-ERAP1, R725Q-ERAP1, K528R/Q730E-ERAP1 or P127R/K528R/Q730E-ERAP1 (the latter two are common haplotypes of ERAP1 variants). ERAAP-/- mFib.B27 expressing K528R-ERAP1 were less well recognized by KK10 CTLs compared to WT-ERAP1 (Figure 5.5 A). Similar levels of ME1 staining between mFib.B27 transfected with WT-ERAP1 and K528R-ERAP1 confirms that this effect was not caused by altered HLA-B27 expression (Figure 5.5 B). Interestingly, neither K528R/Q730E-ERAP1 nor P127R/K528R/Q730E-ERAP1 reduced the activation of KK10 CTLs compared to WT-ERAP1. This result strongly suggests that Q730E can rescue the effect of the K528R mutation. Figure 5.5 C shows that transfected ERAP1 variants were expressed at similar level in ERAAP-/- mFib.B27 cells by western blotting.





ERAAP-/- mFib.B27

Colors	Lines	MFI
Grey	Iso-control	3.34
Red	WT-ERAP1	41.9
Orange	K528R	49.0
Yellow	R725Q	60.0
Green	K528R/Q730E	31.6
Blue	P127R/K528R/Q730E	40.8

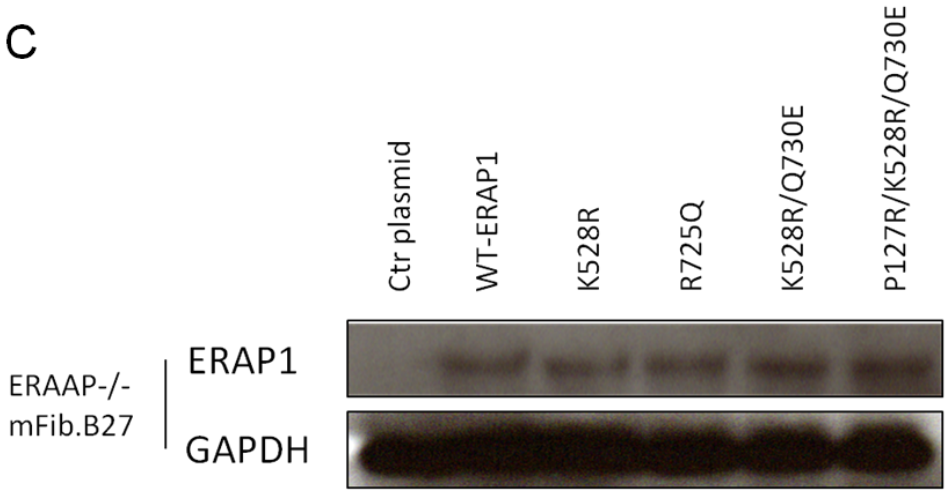


Figure 5.5 The AS-protective variant, K528R-ERAP1, decreases presentation of KK10 to CTLs. A. Activation of KK10 CTLs (ELISPOT) by ERAAP-/- mFib.B27 co-transfected with minigenes and ERAP1 variants (WT-ERAP1, K528R-ERAP1, R725Q-ERAP1, K528R/Q730E-ERAP1 and P127R/K528R/Q730E-ERAP1). B. Cell surface HLA-B27 expression of ERAAP-/- mFib.B27 transfected with ERAP1 variants. ME1 antibody was used to stain cell surface HLA-B27 of ERAAP-/- mFib.B27 transfected to express ERAP1 variants (WT-ERAP1, K528R-ERAP1, R725Q-ERAP1, K528R/Q730E-ERAP1 and P127R/K528R/Q730E-ERAP1). C. Western blotting showing expression of ERAP1 variants in transfected ERAAP-/- mFib.B27 cells. GAPDH was used as loading control.

5.3 Discussion

My data show for the first time that silencing of ERAP1 reduces presentation of a defined immunodominant viral epitope, KK10, to an HLA-B27-restricted CTL clone. Moreover, the CTL recognition was significantly increased by expression of ERAP1 in ERAAP-/- murine fibroblasts expressing HLA-B27. These finding agree with a previous study showing that loss of ERAAP diminished CD8+ T cell responses in mouse (G. E. Hammer et al. 2006). It should be pointed out that we have only studied a single 10mer viral HLA-B27 epitope, and that ERAP1 likely act differently on different epitopes. Indeed, a mouse study has shown that the effect of ERAAP knockout on the immune response to viral peptides is epitope-dependent (York et al. 2006). Whether ERAP1 silencing deduces presentation of other HLA-B27 epitopes needs further investigation.

I also found that X5-KK10 and KK10 minigene transfection provoked potent CTL responses only in HeLa.B27 cells but not mFib.B27 cells (Figure 5.3 and Figure 5.4). This difference between human and mouse might be due to the difference in substrate affinity of transporter associated with antigen processing (TAP). This assumption is supported by a previous study showing that murine TAP, not human TAP, exhibits low affinity in binding to peptides with C-terminal Lysine (Burgevin et al. 2008), such as X5-KK10 and KK10 peptides. Therefore, both of them were likely poorly transported into ER by mouse TAP. Another possible explanation is the difference of cytoplasmic aminopeptidase constitution between mouse and human. X5-KK10 and KK10 peptides were possibly degraded by aminopeptidases in the mouse cytoplasm before being transported into ER by TAP.

K528R-ERAP1, an ERAP1 variant that is protective in AS, also decreased KK10 presentation compared to WT-ERAP1. In chapter 3, I have shown that K528R-ERAP1 trims HLA-B27 epitope precursors much slower than WT-ERAP1. It was also reported previously by others that this ERAP1 variant has a slower substrate trimming rate compared to WT-ERAP1 (Evans et al. 2011; Goto et al. 2006). Therefore, similar to ERAP1 silencing, decreased ERAP1 activity associated with the K528R-mutation also reduced the presentation of a defined HLA-B27 epitope to CTLs. Interestingly, the presence of Q730E was able to rescue the effect of the K528R mutation in the assay I used. This needs further investigation both immunologically and genetically.

Moreover, in experiment shown in Figure 5.4, when KK10 was expressed in the ER, introduction of ERAP1 in ERAAP-/- mFib.B27 cells reduced presentation of KK10 to CTLs. Thus, the presence of ERAP1 can also probably destroy B27 epitopes. However, contribution by the slight decrease of cell surface HLA-B27 expression caused by ERAP1 silencing could not be excluded.

ERAAP-/- mFib.B27 has been used to study the effect of ERAP1 variation on KK10 presentation because of its complete absence of endogenous ERAP1/ERAAP. However, a potential limitation of this cell line is the difference between mouse and human β2m which will affect the folding and stability of HLA-B27 molecules. Only 68% protein homology is shared by human and mouse β2m. In HLA-B27-transgenic

mice, human β2m has been found to promote the surface expression of folded HLA–B27 (Kievits et al. 1989), raising the possibility that HLA-B27 assembly with mouse β2m is less efficient than with human β2m.

It should be pointed out that only one HLA-B27 epitope, KK10, was studied here. More epitopes need to be further tested to confirm my finding. Moreover, although IFN-γ ELISPOT has been commonly used as readout for CTL recognition (Humphreys et al. 2012; Rajapaksa et al. 2012; Ranasinghe et al. 2011), it is only one assay. Ideally, other methods such as Cr^{51} release and CD107 assay should be used to confirm my result.

Overall, both ERAP1 silencing/knockout and introduction of a protective ERAP1 allele (K528R-ERAP1) reduced presentation of a naturally occurring HLA-B27 epitope. Therefore, ERAP1 inhibition or silencing could potentially be used for therapy in AS and possibly other ERAP1-associated diseases.

Chapter 6. Effects of ERAP1 on cell surface expression of HLA-B27

6.1 Introduction

This chapter investigates the role of ERAP1 on cell surface expression of HLA-B27 in different forms. Peptides generated by the proteasome in the cytosol are transported by the Transporter associated with Antigen Processing (TAP) into the ER (J. J. Neefjes et al. 1993; York et al. 2002)), where precursors are further trimmed by ERAP1 to optimal length (8/9mer) to bind to MHC class I molecules, like HLA-B27 (J. J. Neefjes et al. 1993; York et al. 2002). ERAP1 therefore influences the quality of peptides provided to HLA-B27 and potentially affects the folding and expression of HLA-B27.

In addition to the classical heterotrimer formed by heavy chain, β_2m and peptide, HLA-B27 is able to form β_2m-free heavy chains. HLA-B27 heavy chain homodimers have been identified both in vitro and in vivo (cell lines, rodent AS models and AS patients) (Allen et al. 1999; Kollnberger et al. 2002; Kollnberger et al. 2004; Santos et al. 2008). Cell surface β_2m-free HLA-B27 heavy chains including HLA-B27 homodimers/multimers interact with immune regulatory receptors, such as KIR3DL2 and LILRB2 and could thus contribute to AS pathogenesis (Bowness et al. 2011; Giles et al. 2012; Kollnberger et al. 2002).

To investigate the role of ERAP1 in AS pathogenesis, I asked if cell surface

expression of HLA-B27 in different forms could be affected by the inhibition/silencing/reconstitution of ERAP1. The expression of folded and unfolded HLA-B27 on cell surface was examined by ME1 and HC10 antibodies respectively using flow cytometry. The effect of ERAP1 silencing on cell surface HLA-B27 restoration and the complex stability were also examined. HLA-B*27:05 (hereafter termed HLA-B27), the most prevalent and probably ancestral subtype, has been used for study.

6.2 Results

6.2.1 Inhibition of ERAP1 with Leucinethiol increases HC10 staining on the cell surface

I first studied the effect of ERAP1 inhibition on HLA-B27 expression by Leucinethiol (LeuSH), a potent inhibitor of aminopeptidases, including ERAAP (Kanaseki et al. 2006; Serwold et al. 2001). Figure 6.1 A shows that ME1 (a mAb specific for HLA-B27, B7, Bw22) only stains 221.B27, HC10 (recognizing MHC class I free heavy chain) also stains un-transfected 221 but to a much less extent compared to 221.B27. Following LeuSH treatment, both HC10 and ME1 staining were significantly increased by 1.26 and 1.47 fold respectively (Figure 6.1 B and C).

In order to exclude possible bias from 221.B27, I also used HeLa.B27 to study the effect of ERAP1 inhibition on HLA-B27 cell surface expression. ME1 did not stain un-transduced cells. HC10 exhibited only low level staining of HeLa cells but strongly stained HeLa.B27 (Figure 6.2A). Similar to 221.B27 cells, inhibition of

ERAP1 by LeuSH increased both cell surface HC10 and ME1 staining by 1.63 and 1.30 fold respectively.

In summary, these results suggest that treatment with Leucinethiol, a known ERAAP inhibitor, increases HC10 and ME1 staining on both 221.B27 and HeLa.B27 cells.

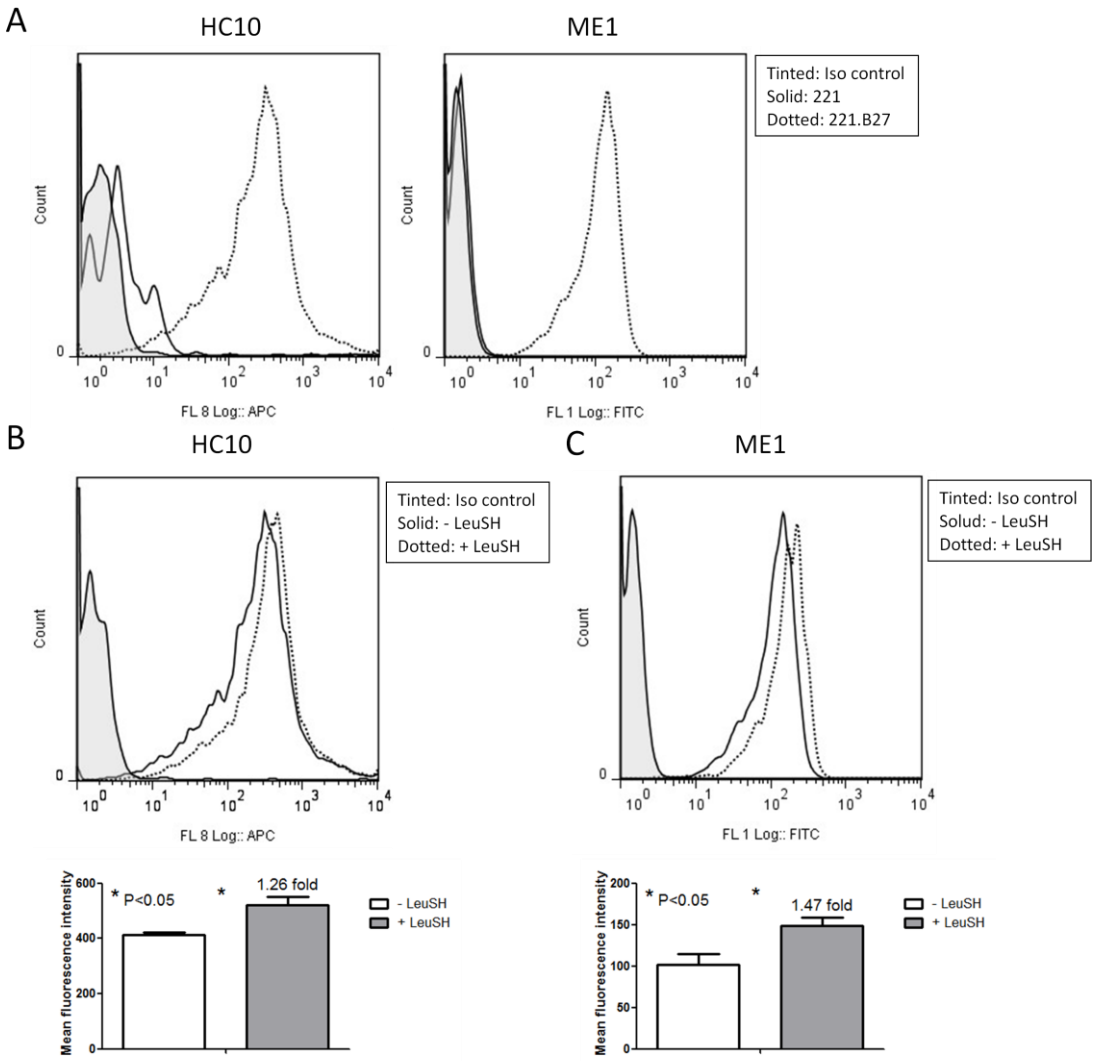


Figure 6.1 Treatment with the ERAP1 inhibitor, Leucinethiol, increases surface HC-10 and ME1 staining of 221.B27 cells. A. ME1 and HC10 staining of B27-transfected 221 compared to un-transfected cells. B. HC10 staining of 221.B27 cells was increased by LeuSH by 1.26 fold. C. ME1 staining of 221.B27 cells was increased by LeuSH by 1.47 fold. This experiment was repeated 3 times, representative graphs are shown here. Mean and standard deviation of mean fluorescence intensity (MFI) for one experiment are shown in the bar graphs below. Student's unpaired two-tailed t-test was used for statistics.

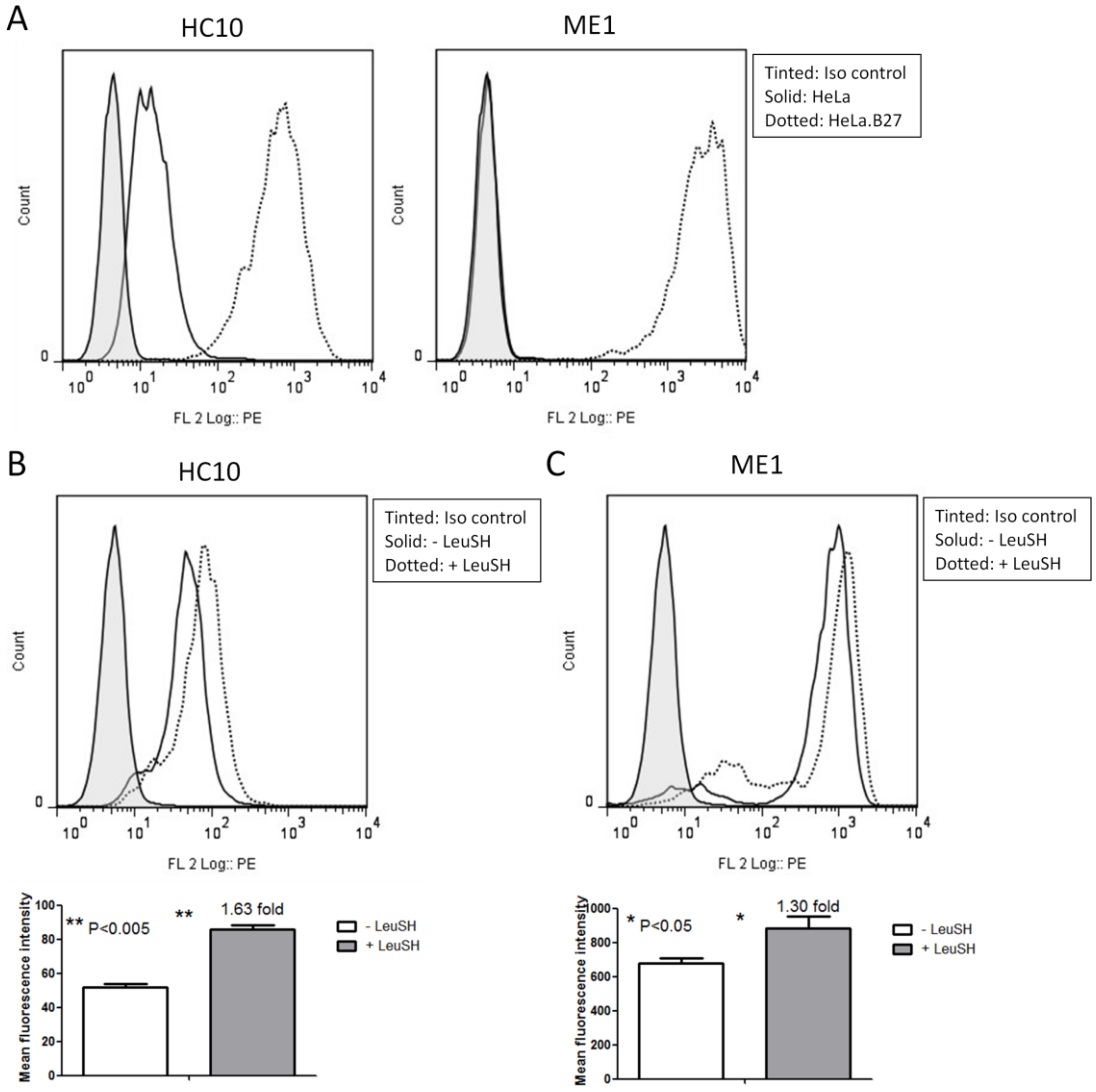


Figure 6.2 Treatment with the ERAP1 inhibitor, Leucinethiol, increases surface HC-10 and ME1 staining of HeLa.B27 cells. A. ME1 and HC10 staining of HeLa.B27 cells compared to HeLa cells. B. HC10 staining of HeLa.B27 cells was increased by LeuSH by 1.63 fold. C. ME1 staining of HeLa.B27 cells was increased by LeuSH by 1.30 fold. This experiment was repeated 3 times, representative graphs are shown here. Mean and standard deviation of MFI for one experiment are shown in the bar graphs below. Student's unpaired two-tailed t-test was used for statistics.

6.2.2 Effect of transient ERAP1 silencing or reconstitution on HLA-B27 expression

To exclude the possible off-target inhibition of alternative aminopeptidases by LeuSH, I attempted to specifically silence endogenous ERAP1 expression through siRNA. Five ERAP1 siRNA candidates targeting different ERAP1 coding areas were tested by transient transfection using HeLa.B27 cells (Figure 6.3 A). SiRNA-1 substantially reduced ERAP1 expression and was therefore selected for time course experiments examining the effect of ERAP1-siRNA or ctr-siRNA on ERAP1 expression 3 and 5 days post-transfection (Figure 6.3 B). I found, at both time points, ERAP1 expression was greatly decreased by ERAP1-siRNA but not ctr-siRNA. SiRNA, especially used in higher dose, can lead to cell toxicity and death. I therefore measured the viability of siRNA-transfected HeLa.B27 cells and found neither ERAP1-siRNA nor ctr-siRNA decreased percentage of viable cells (Figure 6.3 C).

I then studied the effect of transient ERAP1 siRNA silencing on HLA-B27 cell surface expression (Figure 6.4). Cell surface HC10 staining was decreased by ERAP1 siRNA to 0.72 fold compared to ctr-siRNA. Similarly, ME1 staining was also decreased by ERAP1 silencing; the level was 0.85 fold of cells treated with ctr-siRNA. ERAP1 silencing by siRNA therefore decreased both folded and HLA-B27 free heavy chains on cell surface.

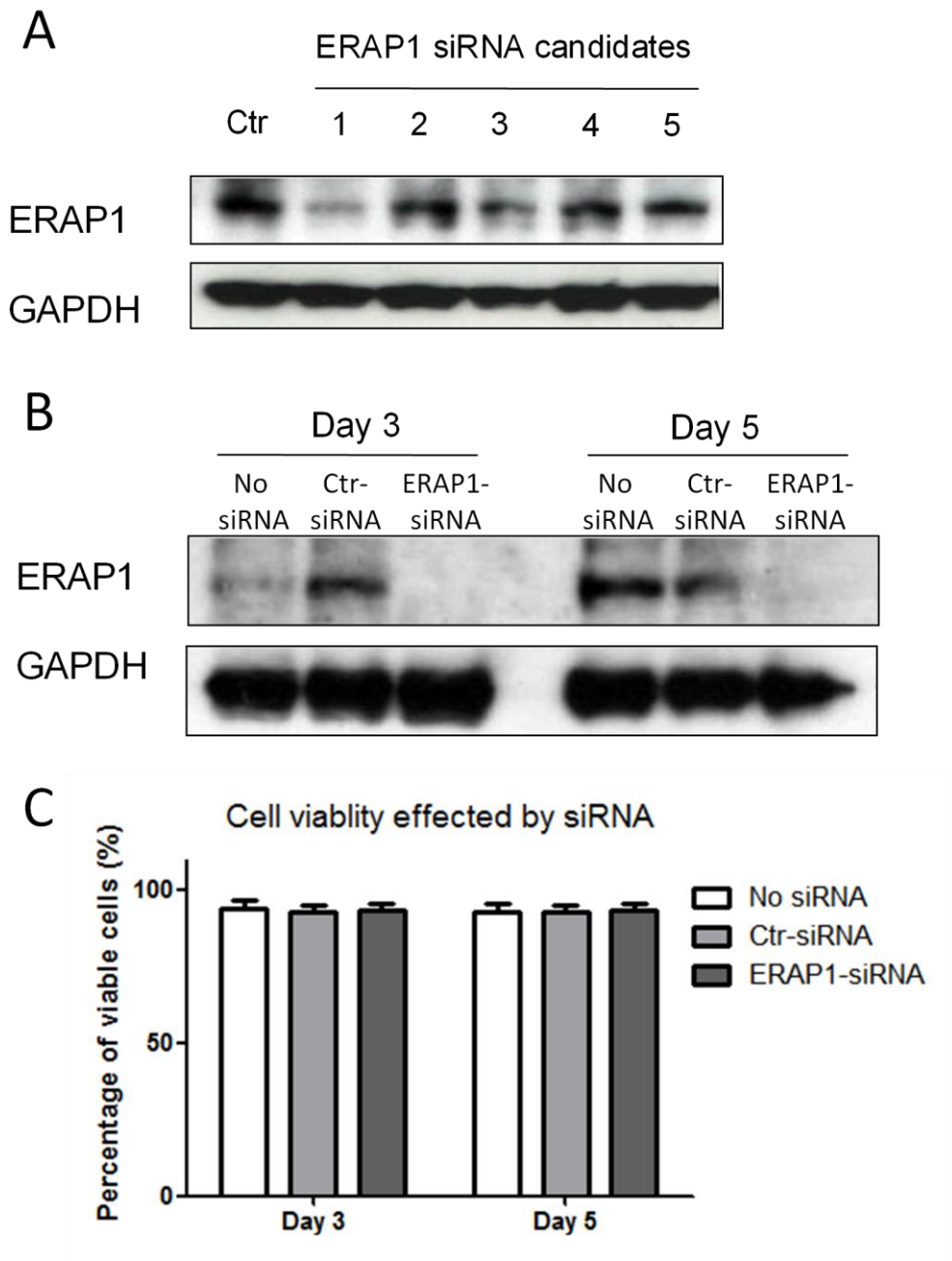


Figure 6.3 Screening of ERAP1 siRNA candidates. A. Effect of siRNA candidates on HeLa.B27 ERAP1 expression was measured by Western blot. Cells were harvested 3 days post-transfection. GAPDH was used as loading control. B and C. Effect of ERAP1-siRNA1, ctr-siRNA or no siRNA on ERAP1 expression and HeLa.B27 cell viability. Cells were harvested either 3 or 5 days after siRNA transfection. Trypan blue was used to examine cell viability.

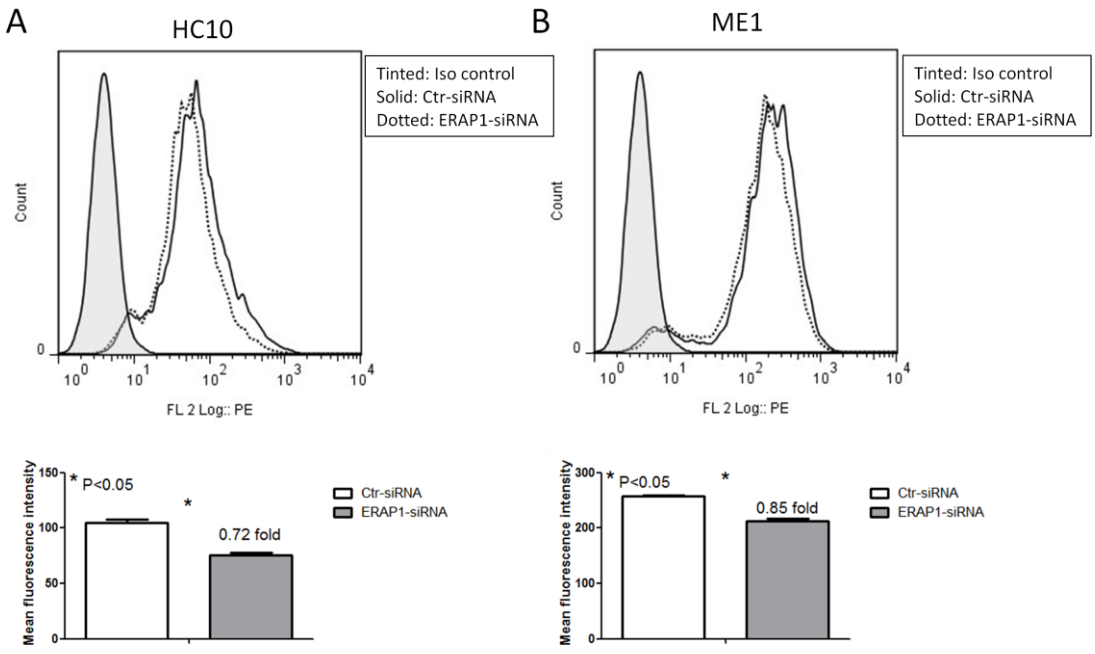
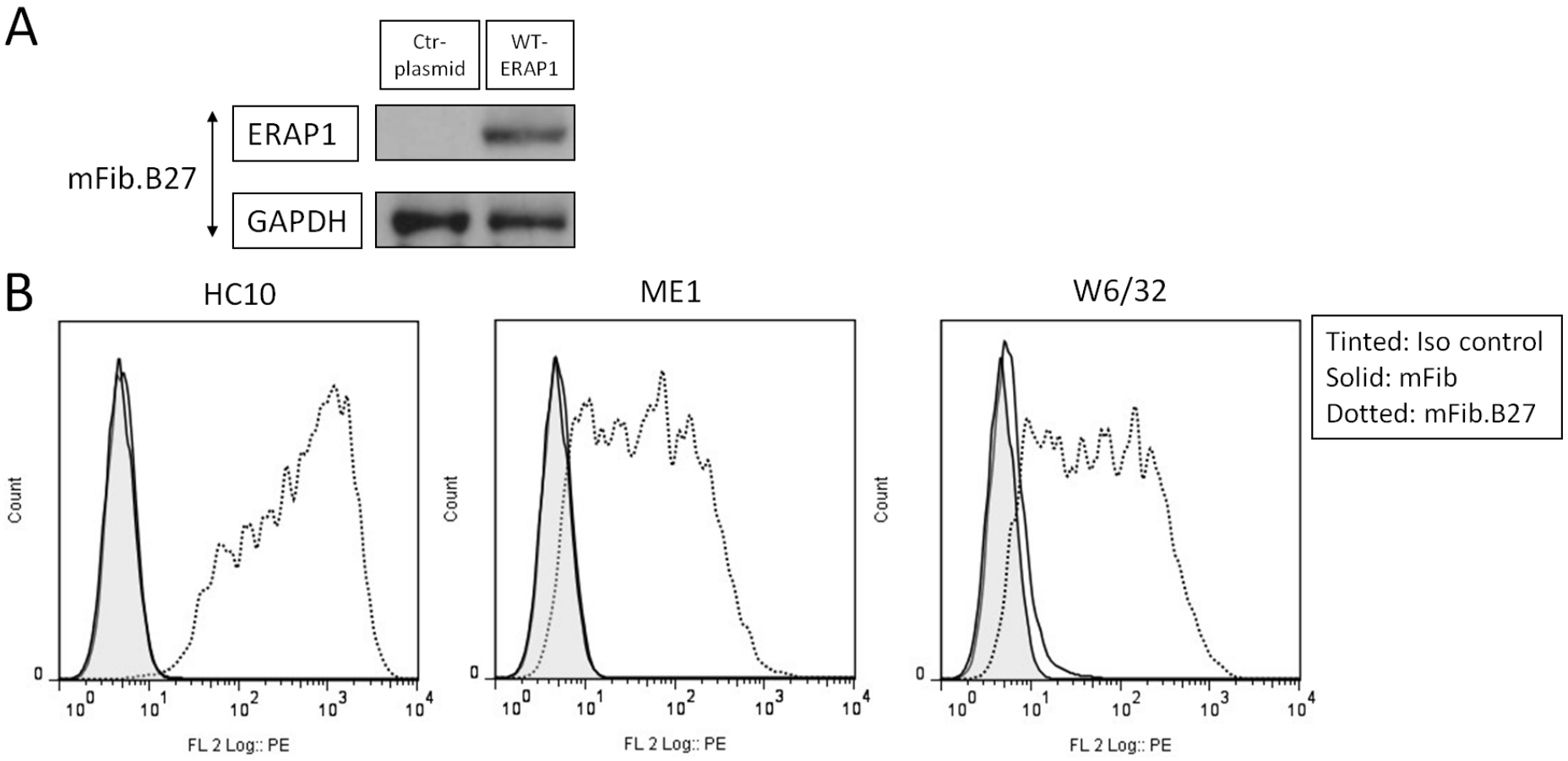


Figure 6.4 ERAP1 siRNA decreased both HC10 and ME1 staining of HeLa.B27 cells. A. Effect of ERAP1 siRNA transfection on HC10 staining of HeLa.B27. B. Effect of ERAP1 siRNA transfection HeLa.B27 cells ME1. This experiment was repeated 3 times, representative graphs are shown here. Mean and standard deviation of mean fluorescence intensity (MFI) for one experiment are shown in the bar graphs below. Student's unpaired two-tailed t-test was used for statistics.

In addition to silencing of ERAP1, I also re-constituted wild type ERAP1 (WT-ERAP1) in ERAAP-deficient cells (Figure 6.5 A). ERAAP -/- mouse fibroblasts, obtained from Prof. Nilabh Shastri, University of Berkeley, were transduced to stably express HLA-B27 (mFib.B27). In addition to HC10 and ME1, W6/32 was also used to exclude possible bias from ME1. Figure 6.5 B shows that HC10, ME1 and W6/32 only stain HLA-B27-transduced mFib, not un-transduced cells. In ERAP1-deficient mFib.B27 cells, cell surface HC10 staining was only 0.67 fold of cells with WT-ERAP1 (Figure 6.5 C). In contrast, ME1 and W6/32 staining were increased by 1.32 and 1.31 fold respectively in cells transfected with ctr-plasmid compared to those expressing WT-ERAP1 (Figure 6.5 D & E). Overall, induction of WT-ERAP1 in mFib.B27 cells increased misfolded HLA-B27 and decreased folded ones.

In summary, ERAP1 siRNA silencing/knockout alters both misfolded and folded HLA-B27 on the cell surface. Absence of ERAP1 reduced HC10 staining in both HeLa.B27 and mFib.B27 cells, but affected ME1 staining differently (a decrease in HeLa.B27 cells and an increase in mFib.B27). However, in both HeLa.B27 and mFib.B27 cells, ERAP1 silencing/knockout decreased the HC10/ME1 ratio, by 15% and 49% respectively (calculated from HC10 and ME1 fold changes presented in Figure 6.4 and 6.5).


A
Ctr-plasmid
WT-ERAP1
mFib.B27
ERAP1
GAPDH
B
HC10
ME1
W6/32
Count
FL 2 Log:: PE
Tinted: Iso control
Solid: mFib
Dotted: mFib.B27

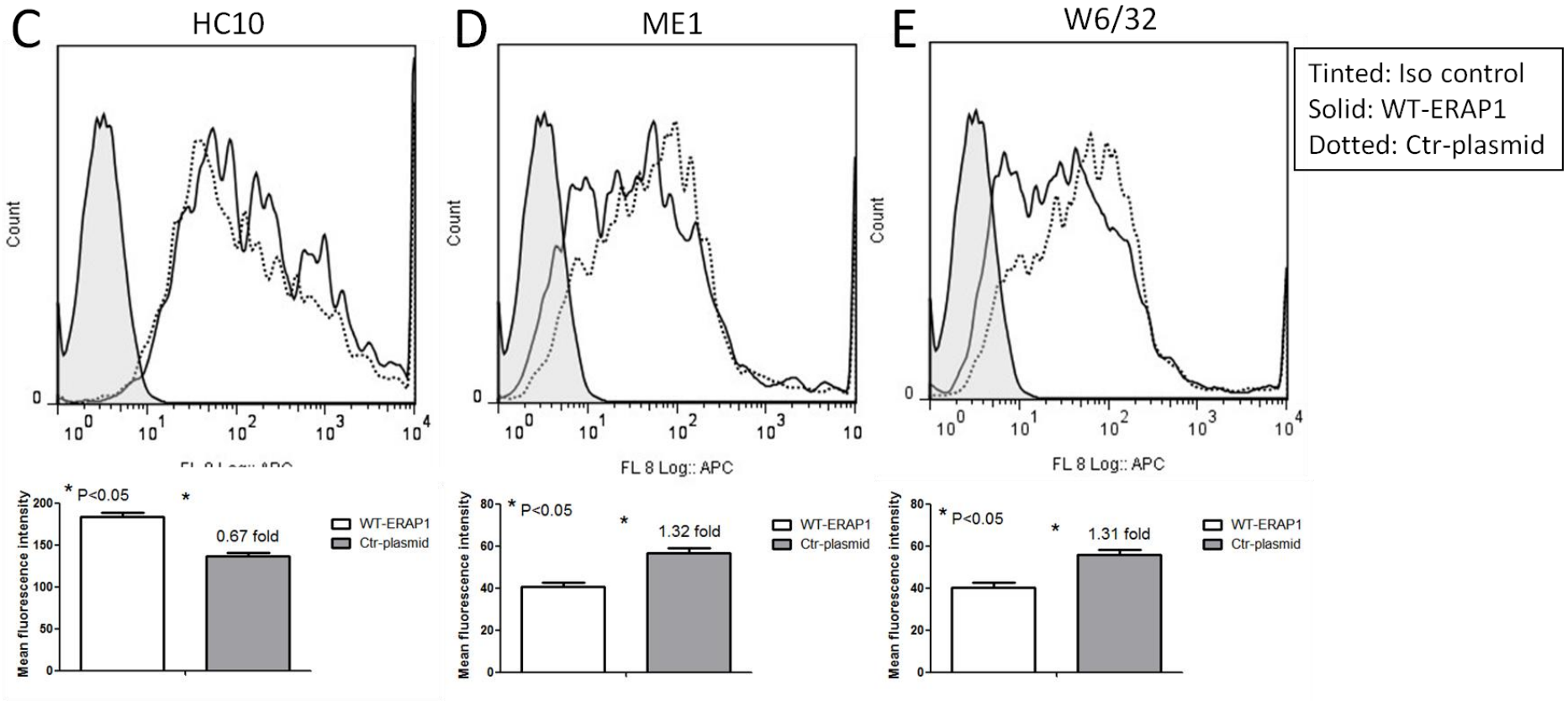


Figure 6.5 Introduction of WT-ERAP1 in ERAAP-/- mFib.B27 alters HLA-B27 cell surface expression. A. WT-ERAP1 was expressed in ERAAP-/- mFib.B27 cells by transient transfection. B. ME1 and HC10 staining of mFib.B27 cells compared to un-transduced mFib cells. C-E. HC10, ME1 and W6/32 staining of mFib.B27 cells with or without WT-ERAP1. Lower panel in C-E shows MFI of HC10, ME1 or W6/32 staining affected by absence of ERAP1. This experiment was repeated 3 times, representative graphs are shown here. Mean and standard deviation of MFI for one experiment are shown in the bar graphs below. Student's unpaired two-tailed t-test was used for statistics.

6.2.3 Effect of stable ERAP1 silencing on cell surface HLA.B27 expression

ERAP1 siRNA only delivers a transient silencing of protein expression and is therefore unable to be used to study the effect of long-term ERAP1 deficiency on HLA-B27 expression. Moreover, siRNA transfection into suspension cell lines, such as 221.B27, is much less efficient than adherent cells. I therefore generated a lentiviral shRNA based on the ERAP1 siRNA sequence. The ability of shRNA to silence ERAP1 expression was first confirmed by transient transfection using HeLa.B27 cells (Figure 6.6 A). ERAP1 shRNA lentivirus was then produced and applied on HeLa.B27, 221.B27 and C1R.B27 cells. Figure 6.6 B shows that endogenous ERAP1 expression by all three cell lines is largely down-regulated by ERAP1-shRNA, but not changed by ctr-shRNA. Figure 6.7 shows that ME1 stains C1R.B27 but not C1R cells, HC10 stains un-transfected cells but to a much less extent compared to transfected ones.

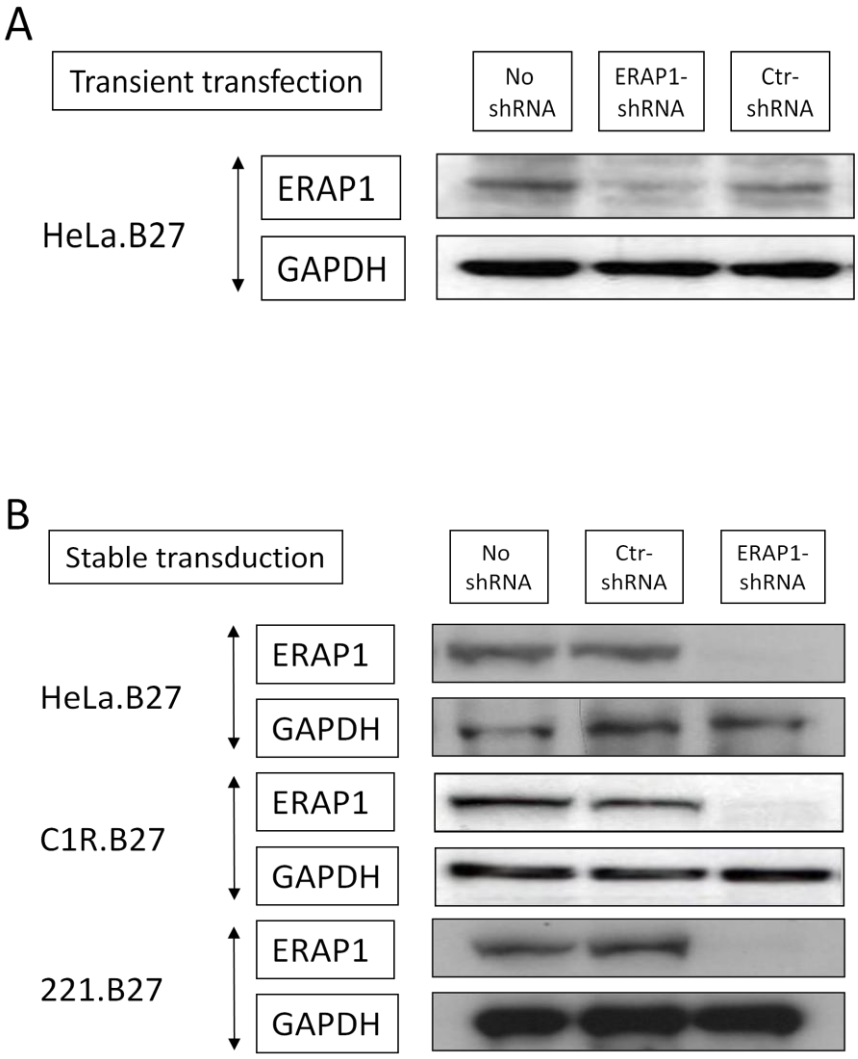


Figure 6.6 Stable silencing of ERAP1 expression using lentiviral shRNA. A. ERAP1-shRNA was tested by transient transfection using HeLa.B27 cells. B. Stable silencing of ERAP1 expression by HeLa.B27, C1R.B27 and 221.B27 cells using lentiviral shRNA. GAPDH was used as protein loading control.

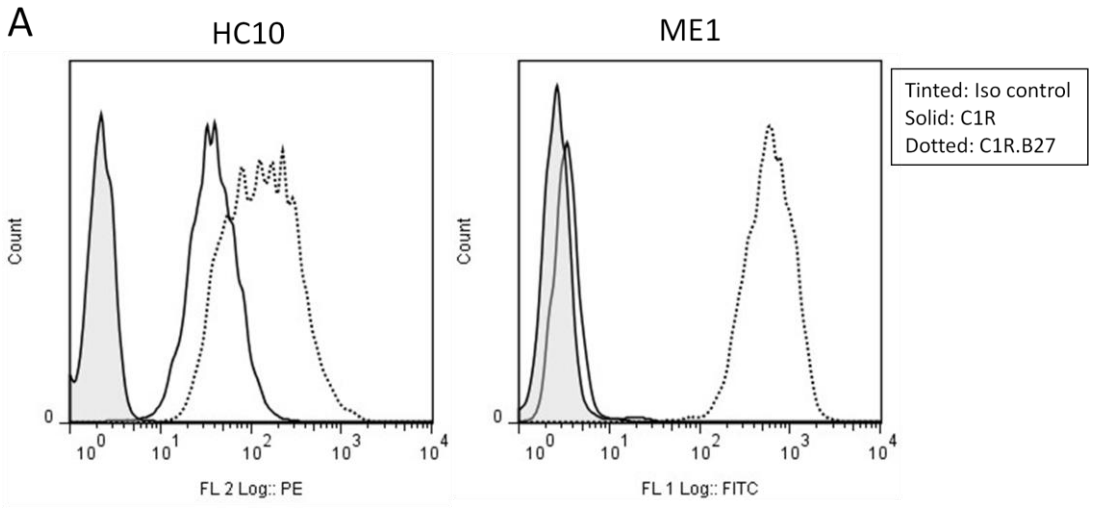


Figure 6.7 HC10 stains C1R cells but to a much less extent compared to HLA-B27-transfected cells, ME1 stains only C1R.B27 cells.

I then compared cell surface HLA-B27 expression between cells transduced with ctr-shRNA and ERAP1-shRNA (Figure 6.8). In HeLa.B27 and C1R.B27 cells, neither HC10 nor ME1 staining was significantly affected by stable ERAP1 silencing (Figure 6.8 A and B). Interestingly, in 221.B27 cells, ERAP1-shRNA remarkably increased HC10 staining and strongly decreased ME1 staining (Figure 6.8 C). However, expression of shRNA in 221.B27 cells, not in HeLa.B27 and C1R.B27 cells, led to some cytotoxic effects, such as decreased cell proliferation and increased cell size (data not shown). Moreover, expression ERAP1-shRNA in 221.B27 was much lower than that of ctr-shRNA, indicated by IRES-GFP (Figure 6.9 C), which may contributed to alteration of HLA-B27 expression. In contrast, IRES-GFP expression in HeLa.B27/C1R.B27 cells transduced with ctr-shRNA and ERAP1-shRNA was at similar level (Figure 6.9 A and B). Therefore, changes seen in 221.B27 cells need to be considered with great caution.

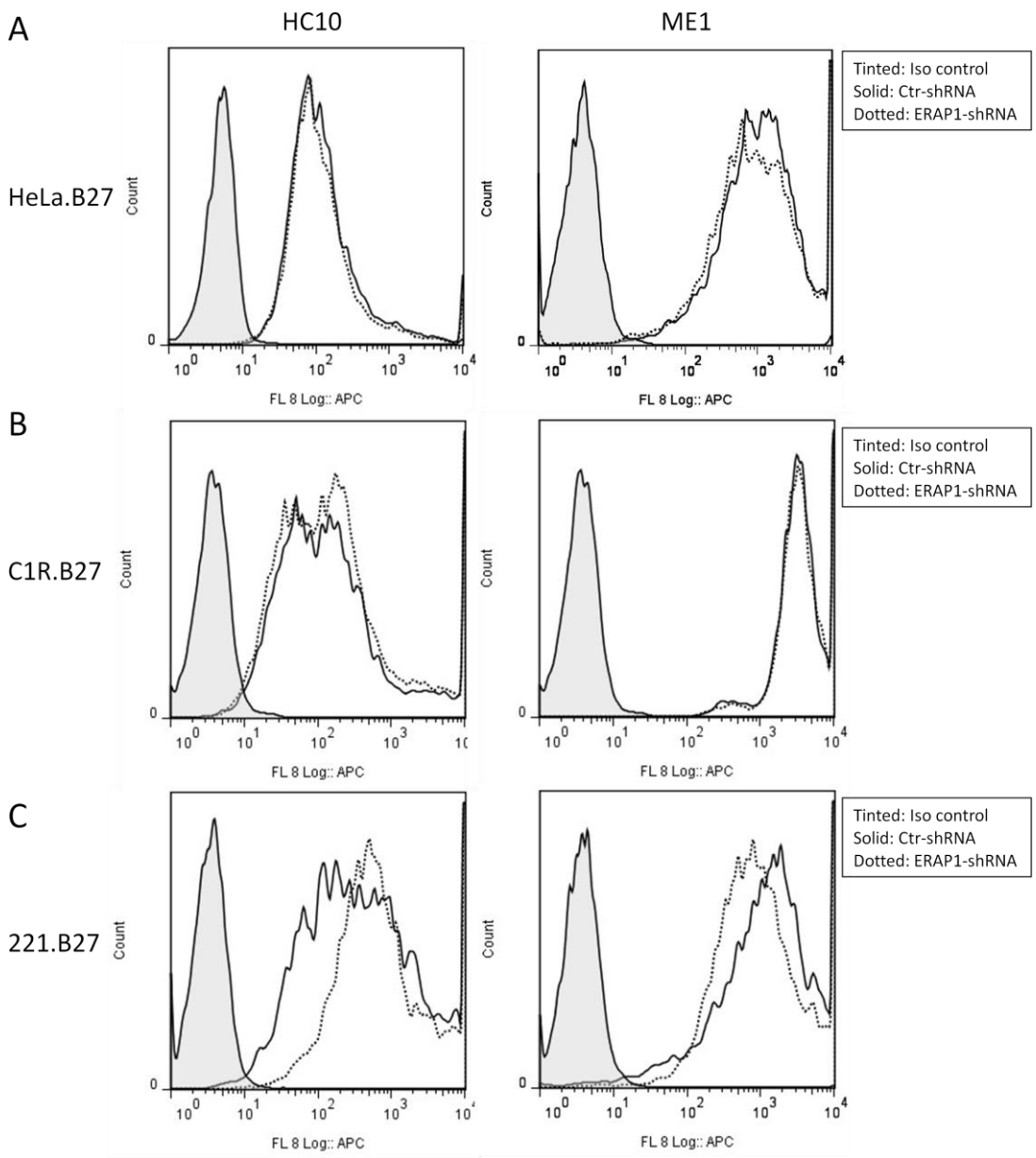


Figure 6.8 Effect of stable ERAP1 silencing on HLA-B27 cell surface expression by HeLa.B27, C1R.B27 and 221.B27 cells. HC10 and ME1 antibodies were used to stain misfolded and folded HLA-B27. All experiments were repeated at least 3 times, representative graphs are shown here.

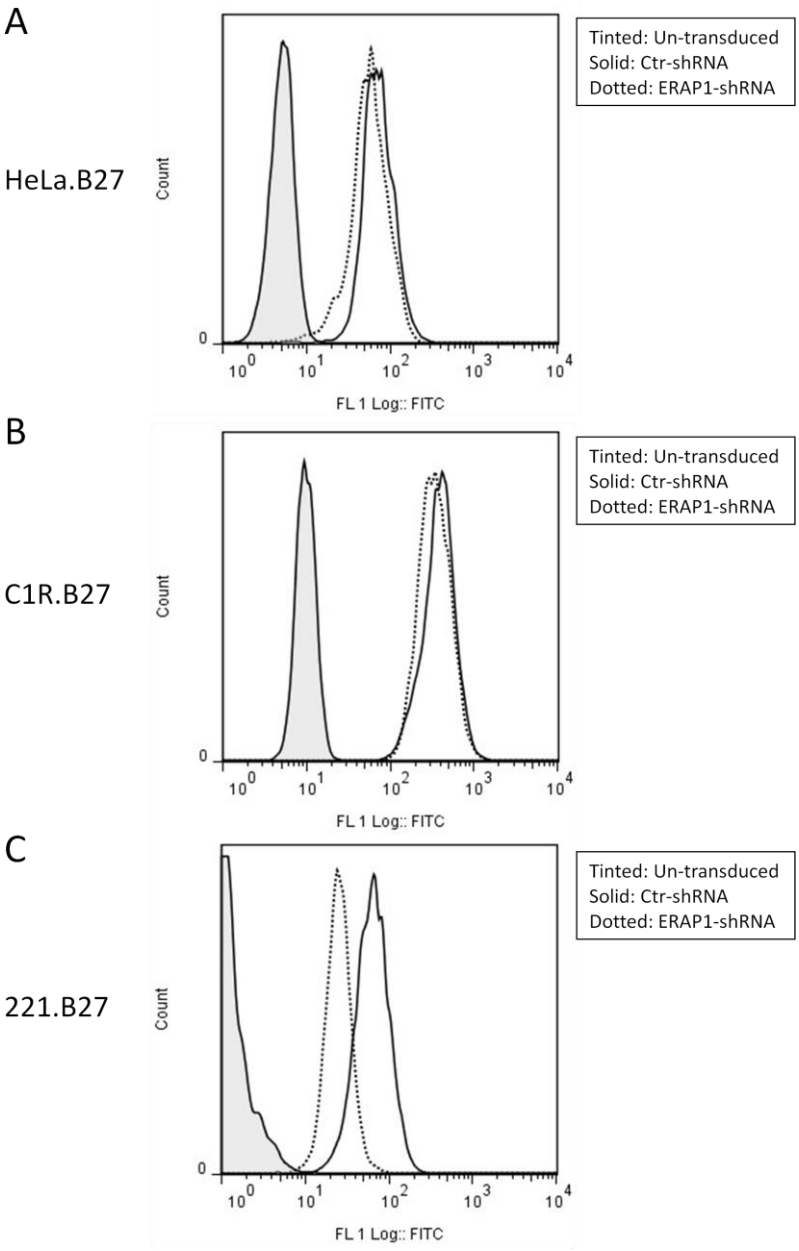


Figure 6.9 Expression of ctr-shRNA and ERAP1-shRNA is similar in HeLa.B27 and C1R.B27, but not in 221.B27 cells. The lentiviral shRNA plasmid used contains "IRES-GFP", intensity of GFP was therefore used as an indicator for expression of ctr-shRNA and ERAP1-shRNA. All experiments were repeated at least 3 times, representative graphs are shown here.

I then ask whether cell surface expression of other HLA alleles is affected by stable ERAP1 silencing. Two cell lines previously made in our group, 221.B35 and 221.A3, were treated with lentiviral ERAP1 shRNA. Similar to HLA-B27, HC10 was used for detection of misfolded HLA alleles. ME1 was replaced by W6/32, an antibody recognizing folded MHC class I molecules. Figure 6.10 shows that ERAP1 silencing led to a slight decrease (10-20%) in HC10 and W632 cell surface staining of HLA-B35/HLA-A3-transfected cells.

In summary, at least in HeLa.B27 and C1R.B27 cells, stable ERAP1 silencing has little effect on HLA-B27 expressed on cell surface. Expression of other HLA alleles, such as HLA-B35 and HLA-A3, is moderately decreased in the absence of ERAP1.

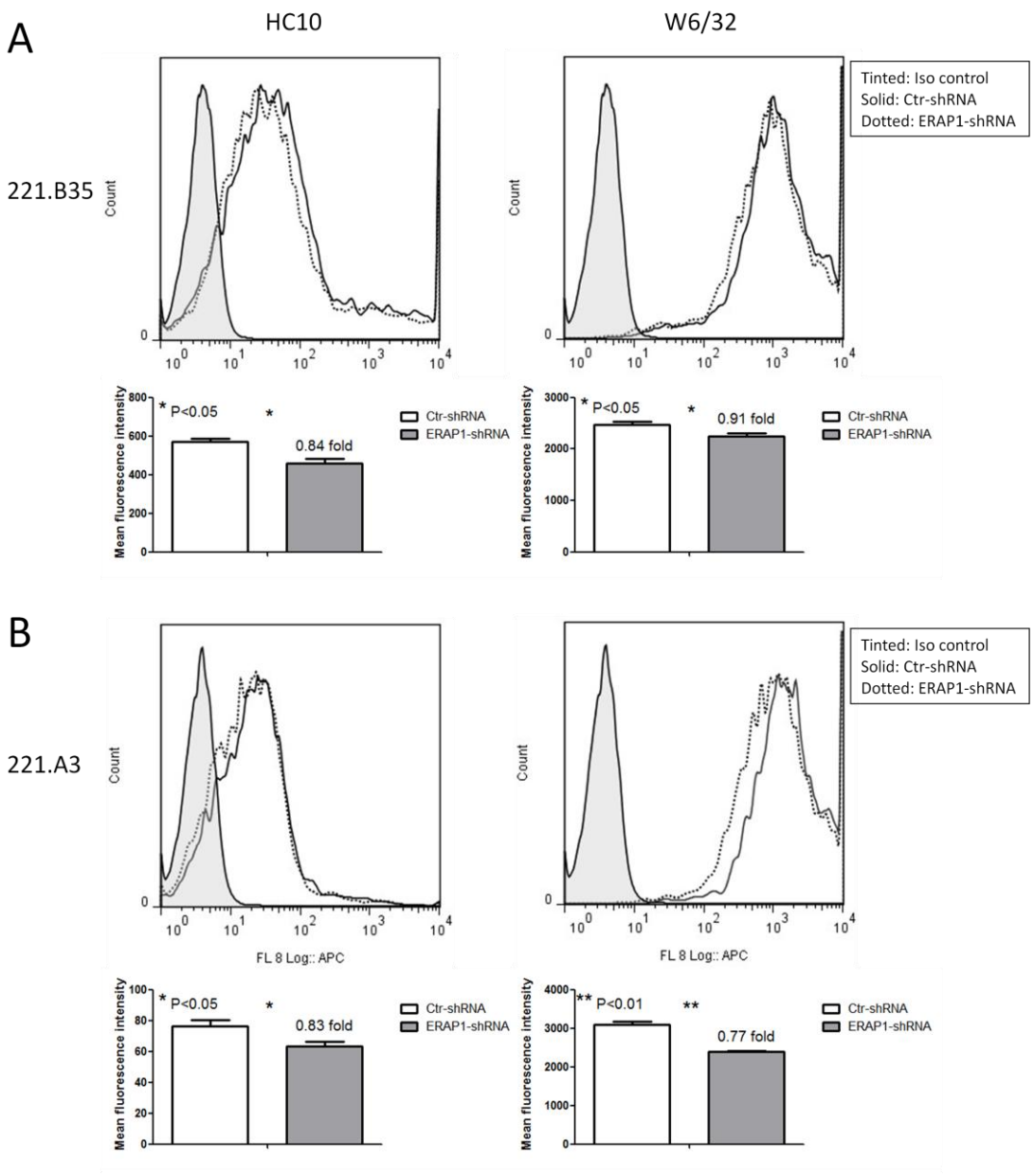


Figure 6.10 Stable ERAP1 silencing decreases cell surface HLA-B35 and HLA-A3 expression. 221 cells were transfected previously in the lab to express HLA-B35 (221.B35) or HLA-A3 (221.A3). Misfolded and folded MHC class I molecules on cell surface were stained by HC10 and W6/32 antibody respectively. Both experiments were repeated 3 times, representative graphs are shown here. Mean and standard deviation of MFI for one experiment are shown in the bar graphs below. Student's unpaired two-tailed t-test was used for statistics.

6.2.4 Effect of stable ERAP1 silencing on restoration of cell surface HLA-B27 expression following surface stripping

Cell surface HLA-B27 staining by ME1/HC10 only reflects the steady state. I next asked if silencing of ERAP1 changes the recovery rate of cell surface HLA-B27. Cells were first treated with activated papain (A-papain) to remove readily expressed HLA-B27 on cell surface and then incubated for 30, 60 and 180 minutes. In both HeLa.B27 and C1R.B27 cells, the restoration rate of neither HC10-reactive HLA-B27 (Figure 6.11 A and C) nor ME1-stained folded HLA-B27 (Figure 6.11 B and D) on cell surface was affected by the absence of ERAP1.

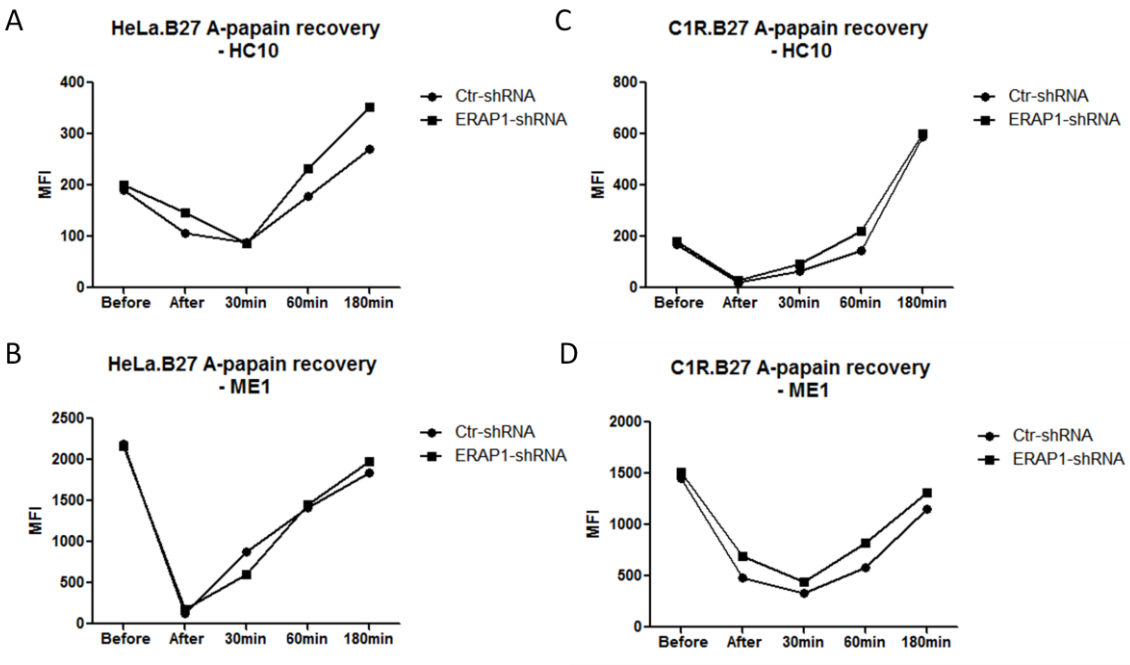


Figure 6.11 ERAP1 silencing does not affect restoration of HLA-B27 on cell surface. HeLa.B27 or C1R.B27 cells were treated with activated-papain (A-papain) to remove cell surface HLA-B27. Cells were harvested for HC10 and ME1 staining before A-papain treatment and 0, 30, 60 and 180 minutes after removing cell surface HLA-B27. Recovery rate of misfolded (HC10) and folded HLA-B27 (ME1) between ERAP1-competent and -silenced in HeLa.B27 (A and B) and C1R.B27 (C and D) were compared. Both experiments were repeated three times, representative graphs are shown here.

6.2.5 Effect of ERAP1 silencing/knockout on HLA-B27 stability

ERAP1 is a key aminopeptidase for generation of MHC class I peptides (Saric et al. 2002; Serwold et al. 2002; York et al. 2002). Silencing of ERAP1 could possibly alter repertoire of peptides loaded to HLA-B27 and affect stability of the complex. To test

this hypothesis, I compared the thermo-stability of ME1-isolated HLA-B27 between ERAP1-silenced and ERAP1-competent HeLa.B27 cells (Figure 6.12 A). Surprisingly, incubated at increasing temperature, folded HLA-B27 (45 kDa) showed similar degrading pattern between ERAP1-deficient and -competent cells (Figure 6.12 C). The thermo-stability of the 90 kDa band, likely HLA-B27 homodimer, was also not affected by ERAP1 silencing (Figure 6.12 B).

In addition to ERAP1 silencing, the effect of ERAP1 introduction into ERAAP-/- mFib.B27HA cells on HLA-B27 stability was also examined (Figure 6.13 A). Figure 6.13 B and C show that degradation of HLA-B27 caused by increased temperature was similar between cells transduced with ctr-shRNA and ERAP1-shRNA. Overall, ERAP1 silencing or knockout do not affect HLA-B27 stability.

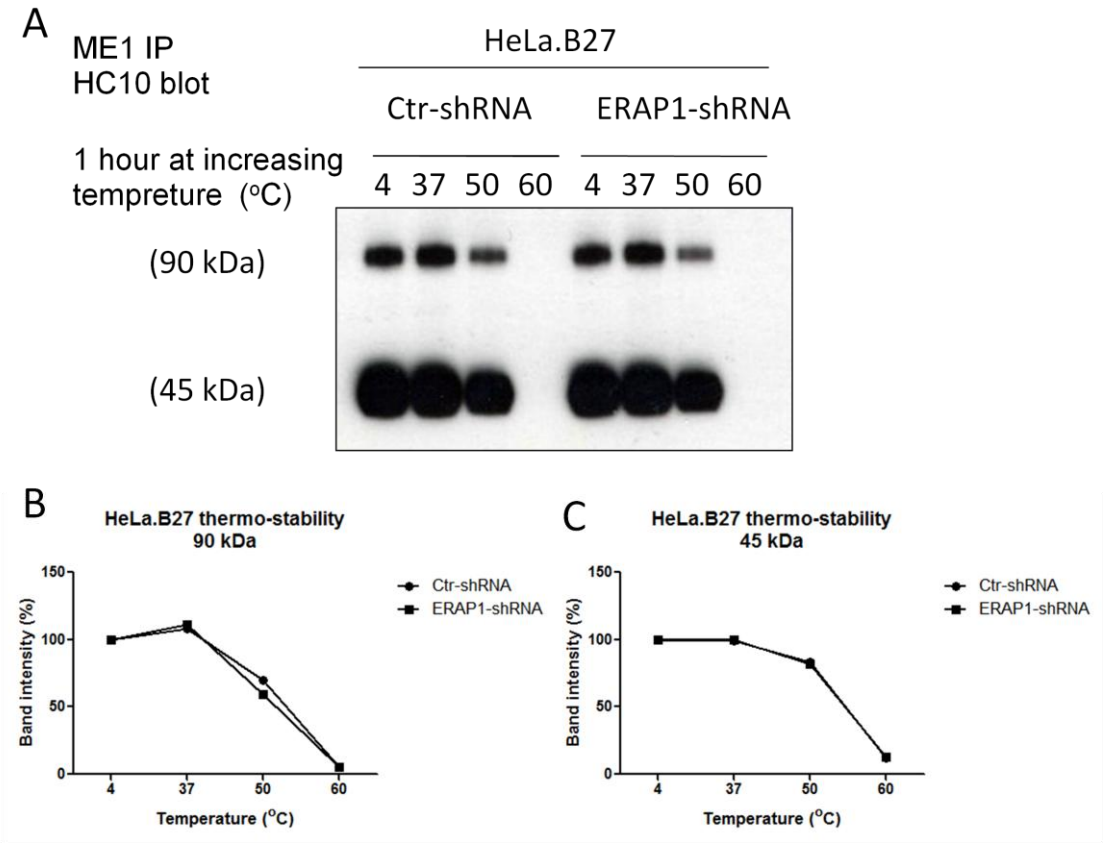


Figure 6.12 HLA-B27 thermo-stability is not affected by ERAP1 silencing. HeLa.B27 cells transduced with ctr-shRNA or ERAP1-shRNA were lysed and incubated at increasing temperature, 4, 37, 50 and 60 ^{o}C for one hour. Cell lysate was then immunoprecipitated using ME1 for isolation of HLA-B27 and run on a SDS-PAGE gel. HC10-HRP was used to probe HLA-B27 heavy chain. A. 45 kDa band represents folded HLA-B27, 90 kDa band is likely HLA-B27 heavy chain homodimer. Changes of 45 kDa band (B) and 90 kDa band (C) intensity by increase of temperature are shown. Intensity of protein bands were measured by ImageJ. Experiment was repeated twice, representative blot and graphs are shown here.

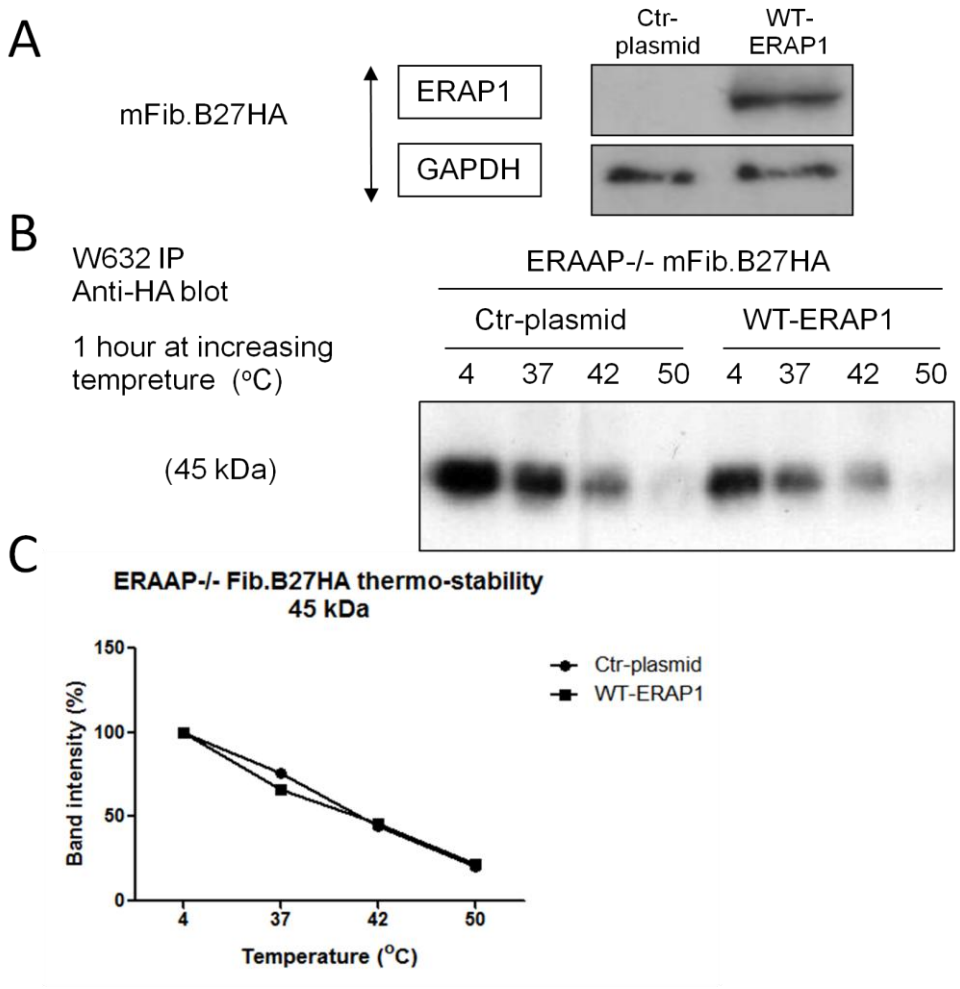


Figure 6.13 HLA-B27 thermo-stability is not affected by introduction of WT-ERAP1 into ERAAP-/- mouse fibroblast expressing HA-tagged HLA-B27. ERAAP-/- mFib cells were transduced to express HLA-B27 with HA tag (ERAAP-/- mFib.B27HA). Cells were then transfected with either WT-ERAP1 or control plasmid (A). 48 hours after transfection, cells were lysed and incubated at 4, 37, 42 and 50 ^{o}C for 1 hour. B. The lysate was then immunoprecipitated using W6/32 and run on a SDS-PAGE gel. Anti-HA antibody was used to probe HA-tagged HLA-B27 heavy chain. 45 kDa band represents folded HLA-B27. C. Intensity changes of the 45 kDa band with increasing temperature are shown. Intensity of protein bands were measured by ImageJ. The experiment was repeated twice, representative blot and graph are shown here.

6.3 Discussion

In this chapter, to study effect of ERAP1 on cell surface HLA-B27 expression, I have used a variety of approaches, including ERAP1 inhibition, siRNA/shRNA silencing and ERAP1 introduction. However, the results generated are not conclusive as these treatments do not always lead to the same changes (Table 6.1).

Treatment of 221.B27 and HeLa.B27 cells using LeuSH significantly increased both HC10 and ME1 staining of HLA-B27 on cell surface. However, the possibility of LeuSH inhibition of other aminopeptidases could not to be excluded. This result should therefore be interpreted with great caution.

Specific ERAP1 silencing through siRNA led to a decrease of both HC10 and ME1 staining in HeLa.B27 cells. This finding agrees with previous mouse studies showing that either ERAAP siRNA or knockout decreases folded MHC class I expression (they did not look at the unfolded MHC molecules) (G. E. Hammer et al. 2006; Serwold et al. 2002). ERAP1 siRNA has been reported have no effect on either folded or unfolded cell surface HLA-B27 in C1R.B27 cells (Haroon et al. 2012). Their result was possibly due to low siRNA transfection efficiency in suspension cells. Indeed, only approximately 70% ERAP1 expression was suppressed in this study. It should be pointed out that HeLa cells are not professional antigen presenting cells (APCs). ERAP1 siRNA silencing using dendritic cells (DC) or macrophages derived from HLA-B27 positive AS patients or HLA-B27-transfected/transduced DC/macrophage-like cell lines needs to be done to confirm my result.

Similar to ERAP1 siRNA, the absence of ERAP1 in mFib.B27 cells decreased cell surface HC10 staining. However, folded HLA-B27 on cell surface was increased in mFib.B27 cells, although the ratio of HC10/ME1 was decreased in both cells (Table 6.1). This difference might be due to differences between human and mouse in ER molecules constitution.

It is interesting that the effect of LeuSH and ERAP1 siRNA on cell surface HLA-B27 expression is different, or even contradictory. If the inhibition by LeuSH is indeed ERAP1-specific, the question is why inhibition of ERAP1 enzyme activity increases cell surface unfolded HLA-B27 but silencing of endogenous ERAP1 expression acts oppositely. One possible explanation is that, in addition to its role as a peptidase, ERAP1 might have a chaperone-like function which helps HLA-B27 molecules, especially those loaded with sub-optimal peptides, egress to cell surface. It is possible that ERAP1 siRNA increases unfolded HLA-B27 in the ER, which however could not egress to cell surface without ERAP1 as the chaperone. LeuSH only inhibits peptidase role of ERAP1, therefore increases unfolded HLA-B27 on the cell surface. This assumption is supported by previous study showing the presence of ERAP1 on cell surface by confocal microscopy (X. Cui et al. 2002). It could be tested by examining the presence of ERAP1 in HLA-B27-immunoprecipitated proteins, and vice versa.

Interestingly, stable ERAP1 silencing by lentivial shRNA had little effect on cell surface HLA-B27 expression. Even for other HLA alleles (HLA-B35 and HLA-A3), only a slight decrease was caused by ERAP1 shRNA. The different effect between ERAP1 shRNA and siRNA on HLA-B27 cell surface expression is presumably due to self-compensation mechanisms (e.g., induction of alternative aminopeptidase).

Consistent with this assumption, I found ERAP1 shRNA only transiently decreased folded and unfolded HLA-B27 on cell surface of HeLa.B27 post-transduction (data not shown).

Cell lines	221.B27			HeLa.B27			mFib.B27			C1R.B27		
MFI fold changes	HC10	ME1	HC10:ME1	HC10	ME1	HC10:ME1	HC10	ME1 W6/32	HC10:ME1 HC10:W6/32	HC10	ME1	HC10/ ME1
LeuSH	1.26	1.47	0.86	1.63	1.3	1.25						
ERAP1 siRNA/ ERAAP -/-				0.72	0.85	0.85	0.67	1.32 1.31	0.51 0.51			
ERAP1-shRNA	Results excluded			Unchanged						Unchanged		

Table 6.1 Summary of studies on the effect of LeuSH, ERAP1 siRNA, ERAAP -/-, ERAP1-shRNA on cell surface HLA-B27 expression.

The figures shown are fold changes of HC10/ME1 MFI caused by the inhibition/absence of ERAP1. The fold change bigger than 1, indicating an increase was highlighted in red. The numbers smaller than 1 were labelled in green. Boxes that are crossed off mean that no experiments were carried out using these combinations of cell lines (top row) and treatment (left column). The experiment studying effect of ERAP1-shRNA on HLA-B27 expression in 221.B27 cells was excluded due to cytotoxic effect and unpaired shRNA expression, labelled in yellow.

I also showed that, in HeLa.B27 and C1R.B27 cells, restoration of neither unfolded nor folded HLA-B27 on cell surface following activated-papain treatment was altered by ERAP1 shRNA silencing. In addition to activated-papain, other approach such as acid treatment could be used to remove cell surface MHC class I molecules to further confirm this result.

Moreover, in either HeLa.B27 or mFib.B27 cells, the absence of ERAP1 did not affect thermo-stability of HLA-B27 complexes. It should be pointed out that HLA-B27 has been isolated from whole cell lysate. Cell surface HLA-B27 at steady state may possibly dilute out changes contributed by intracellular unfolded HLA-B27. The ^{35}S Methionine pulse-chase labels newly generated proteins, and therefore could be used in prior to immunoprecipitation of HLA-B27 to confirm this result.

Overall, ERAP1 probably affects cell surface expression of HLA-B27. Whether the restoration and stability of HLA-B27 complex is affected by ERAP1 needs further investigation.

Chapter 7. Discussions and future work

The central aim of this thesis was to investigate the role of the interaction between ERAP1 and HLA-B27 in AS pathogenesis. In addressing this, I first showed, in vitro, that two protective AS-associated ERAP1 variants exhibited different substrate trimming efficiency from WT-ERAP1. K528R-ERAP1 trimmed both Flu-derived and Chlamydia-derived N-terminally extended HLA-B27 epitopes less efficiently than WT-ERAP1. The R725Q variation, however, affected ERAP1 activity in a substrate-dependent manner, increasing the trimming of Flu-derived substrate whereas decreasing the Chlamydia-derived peptide. In addition to in-vitro studies, I also showed in vivo that ERAP1 silencing increased the length of HLA-B27-bound peptides, which were predominantly C-terminally extended. The P1 residue of peptides bound to HLA-B27 was also affected by the absence of ERAP1. Furthermore, I have demonstrated that ERAP1 affects the presentation of an immunodominant viral HLA-B27 epitope (KK10) to CTLs. Both silencing in HeLa.B27 cells and replacement of WT-ERAP1 by K528R-variant in ERAAP-deficient mouse fibroblasts reduced the activation of HLA-B27-restricted KK10-specific CTLs. Finally, my data using cell lines suggest that ERAP1 probably affects cell surface expression of HLA-B27. The ratio of HC10 to ME1 staining (HC10/ME1) was decreased by the absence of ERAP1 in both siRNA-treated HeLa.B27 and ERAAP-/- mouse fibroblasts expressing HLA-B27. Collectively, these findings are consistent with a model in which ERAP1 affects disease through altered peptide repertoire, but also raise several questions relating to the role of ERAP1 and HLA-B27 in AS pathogenesis which require further investigation in future studies.

The first and key question is whether and how my data fit with the main AS pathogenesis theories: “cell surface HLA-B27 free heavy chain expression”, “molecular mimicry” and “unfolded protein response (UPR)”. With ERAP1 being a pathogenic gene increasing the susceptibility to AS, my finding that ERAP1 siRNA or absence of ERAP1 decreases HC10/ME1 suggests that ERAP1 possibly contribute to AS through increasing HLA-B27 free heavy chain expressed on cell surface. The molecular mimicry hypothesis is supported by in vitro peptide trimming and CTL activation assays. The protective AS-associated variant, K528R-ERAP1, trims N-terminally extended B27 epitopes slower in vitro than WT-ERAP1 and reduces activation of HLA-B27-restricted KK10-specific CTLs. These results would support the notion that ERAP1 increase susceptibility to AS by its key role in generation and presentation of “arthritogenic peptide”. The only hypothesis that my data does not support is the UPR hypothesis. HLA-B27 complex loaded with long peptides is known to be less stable compared to those carrying peptide of optimal length. Therefore, my finding that silencing of ERAP1 increased the length of HLA-B27-bound peptides indicates that presence of ERAP1 stabilizes HLA-B27 and should consequently alleviate UPR. The effect of ERAP1 silencing or variation on UPR could be further studied by examining UPR markers using ERAP1-silenced HeLa.B27/C1R.B27 cells and ERAAP-/- mFib.B27 cells transfected with ERAP1 variants. The 78-kDa glucose-regulated protein (GRP78) and C/EBP homology protein (CHOP) expression could be assessed both at the protein level using western blot or flow cytometry and at the RNA level using quantitative PCR. Slicing of X-box binding protein 1 (XBP1) could be also analyzed by quantitative PCR.

Considering the effect of ERAP1 on cell surface HLA-B27 and peptides bound to HLA-B27 together, one interesting question is that why ERAP1 silencing increases the length of peptides bound to HLA-B27 but decreases the ratio of HC10 and ME1 staining on the cell surface. This is contradictory because HLA-B27 carrying long peptides should be less stable and more likely to form HLA-B27 free heavy chains. One possible explanation is that these ERAP1-silencing-induced unstable HLA-B27 complexes are unable to be transported to cell surface. This assumption is supported by the previous finding from our group that HLA-B27 homodimers formed in the ER are unable to egress to the cell surface (Bird et al. 2003). Moreover, with the whole cell lysate being used for isolation of HLA-B27-bound peptides, longer peptides increased by ERAP1 silencing are likely contributed by intracellular HLA-B27 complexes. Comparing intracellular HC10/ME1 staining between ERAP1-deficient/silenced and ERAP1-competent cells will confirm this hypothesis. Additionally, the effect of ERAP1 silencing on peptides bound to intracellular HLA-B27 using papain/acid-stripped cells could be studied.

The free heavy chain theory proposes that cell surface B27 heavy chains are capable of interacting with immune regulatory receptors such as KIR3DL2 and LILRB2. Cells expressing HLA-B27 free heavy chains have been found to stimulate the survival and proliferation of KIR3DL2+ CD4 T cells and NK cells. Silencing of ERAP1, which led to decreased HC10/ME1 in FACS staining, is therefore likely to decrease the interaction of HLA-B27-expressing cells with KIR3DL2 and LILRB2. These could be tested by cell surface staining using KIR3DL2 and LILRB2 Fc proteins and by measuring proliferation of KIR3DL2+ NK/T cells in co-culture experiments.

It should be pointed out that, in my work, only two HLA-B27 epitopes (derived from Flu and Chlamydia) were used in in vitro peptide trimming assay, and one HLA-B27 epitope from HIV-Gag in CTL activation assay. My finding therefore does not necessarily reflect the effect of ERAP1 on all HLA-B27 epitopes. In order to further confirm the necessity of WT-ERAP1 in generation of HLA-B27 epitopes and perhaps further support the arthritogenic peptide theory, more epitopes need to be tested using these two experimental approaches. Moreover, the epitopes used in vitro can be further tested using CTL activation assay (for example, an NRELIQQEL-specific CTL clone could be generated to examine the effect of ERAP1 on peptide presentation by Chlamydia-infected or QITANRELIQQEL minigene-transfected cells). Vice versa, the N-terminally extended KK10 peptide, VK15, can be synthesized and used as a substrate in in vitro peptide trimming assays.

In this thesis, the effect of ERAP1 on peptides bound to HLA-B27 has been studied by comparing ERAP1-silenced and -competent cells. I have tried to compare WT-ERAP1 and K528R-ERAP1 expressed by ERAAP -/- mouse fibroblasts in the repertoire of peptides bound to HLA-B27. However, the number of peptides isolated was too low to analyse due to relatively poor HLA-B27 expression in mouse fibroblast. A possible solution is to use immortalized B cells of AS patients naturally carrying disease-associated ERAP1 variants. I have established a few B cell lines from patient PBMC using Epstein–Barr virus supernatant and planed to study the effect of ERAP1 polymorphism on HLA-B27-bound peptides.

I have shown in CTL activation assays that the Q730E-ERAP1 mutation rescued the reduction of KK10-specific CTLs response caused by K528R. Q730E has been previously shown to increase or decrease the peptide trimming efficiency, depending on the substrate (Evnouchidou et al. 2011). It is therefore possible that, with N-terminally extended KK10 (VK15) being the substrate, the Q730E mutation increases the enzyme activity which compensates the reduction by K528R. This assumption could be tested by in vitro peptide trimming assay, comparing the trimming of VK15 by WT-, K528R-, Q730E- and K528RQ730E-ERAP1.

In a previous study, ERAP1 expression in blood monocyte-derived dendritic cells (DCs) from AS patients was found to be higher than healthy controls (Campbell et al. 2011). My in vitro peptide trimming experiment shows that the enzyme efficiency of WT-ERAP1 was increased with increasing concentration, at least in a certain range (0 to 10 μg/ml) (Figure 3.1, **Chapter 3**). Therefore, over-expressed ERAP1 could potentially alter peptides supplied and bound to HLA-B27 and contribute to AS pathogenesis. This hypothesis can be tested using the methods described in this thesis.

In conclusion, the work presented in this thesis validates the role of ERAP1 and HLA-B27 interaction in AS pathogenesis indicated by GWAS. ERAP1 affects the length and sequence of peptides bound and presented by the AS-associated HLA-B27. Given that ERAP1 variation affects enzyme activity in vitro, it is thus likely that AS-associated ERAP1 alleles alter length and composition of HLA-B27-bound peptides in vivo. Moreover, I have shown that both ERAP1 silencing and introduction of a protective ERAP1 allele (K528R-ERAP1) reduced functional presentation of a

naturally occurring HLA-B27 epitope to CD8+ T cells. Therefore, ERAP1 inhibition or silencing could potentially be used for therapy in AS and other ERAP1-associated diseases.

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9. Appendices

9.1 Lists of HLA-B*27:05-bound peptides from ERAP1-deficient and -competent cells

9.1.1 HeLa.B27 ME1

Ctr-shRNA	Length	ERAP1-shRNA	Length
GRIPGIYGR	9	EISDRLIGI	9
GRAPGGLSL	9	LPLPLKKDR	9
FVNDIFERI	9	QNLARTKGI	9
TRLPLISGF	9	KIRVSVIGL	9
KQWTEILHF	9	GRLNEPIKV	9
QRSSGIIRL	9	ARTPVMSTY	9
KSKDFVQVM	9	GRLVKDMKI	9
GRMENLASY	9	DTGRAAKLI	9
YRDWFSSQL	9	ELWELRRQQ	9
GRFGSGMNM	9	ARAGTFQAF	9
GRFLEIGKF	9	TRLPLISGF	9
MLMAGFIKI	9	NMVRVGTLI	9
LRYGEPTLR	9	SRTPVLMNF	9
QLMQQMIQL	9	YRKPKWNHL	9
ARIDEVVSR	9	GRNFWVSGL	9
TRYPDVQTR	9	AQGPTPRYF	9
ARFLFTTGF	9	KKIRNNPAL	9

SRFPEALRL	9	GRFGSGMNM	9
QVPHILQGL	9	ARFLFTTGF	9
HRAPPIIGY	9	SRVNIPKVL	9
SRFSGGFGA	9	RKNNRLLEV	9
ARLPLRLFL	9	GRFPQVQAF	9
VRLPYPLQM	9	NRYPGIVKK	9
LRNPLIAGK	9	SRLPQLVGV	9
YRKPKWNHL	9	SRLQEVFGH	9
GRIGVITNR	9	SRVLKVLSV	9
RNTLEKFTA	9	IRAAPPPLF	9
NRIKFVIKR	9	PGRRNHFAY	9
GRNFWVSGL	9	DRIHGSVAL	9
TAPRTLLLL	9	ARTPVTSTY	9
GRHGVFLEL	9	KAHSQTNRA	9
HRLWSHRSY	9	LRVPEKVPF	9
ARLGLGLLL	9	RVQNNNTLR	9
VRTPVLFGY	9	GRLTKHTKF	9
RRYYKPGLL	9	SRLPSLGAGF	10
GQYGNPLNK	9	GRWPGSSLYY	10
EPAQLLLLL	9	GRWPKKSAEF	10
VLEEQVLAL	9	SRLSIPTYGL	10
GQHTVLLVK	9	GRISITGVGF	10
LNIPKVLLP	9	QRYLDELMKL	10
DNYTITFLI	9	IRAFPNKQGY	10

IRLPSQYNF	9	MMRRSLRAAG	10
LTPLIKKYD	9	RALLGLSLLF	10
VRLPPITKF	9	SRLQAEIEGL	10
RNVSTPSAF	9	NRMEPLWTGF	10
YGFKVSIQN	9	KRLSSQLILL	10
GRIDKPILK	9	SRIHVPASGY	10
SRIHPVSTM	9	SRFDASFFGV	10
GRLVKDMKI	9	GRFHGEEVAV	10
ARLGVISKV	9	KKRLEGLSKQ	10
IRMPSLPSY	9	VNRGGGGFGA	10
ARLPWAGQL	9	NRFAGFGIGL	10
VCKLSSSVK	9	HRFEQAFYTY	10
SRFPYYNSL	9	SRLWPKIQGL	10
FRVPFIYGH	9	SRAGITMESY	10
ARIFQFQNF	9	GRISDFHETY	10
NPISQIGII	9	GIIRSPKSAL	10
KRMPWGWDY	9	ARFKGDGVKY	10
SRVNVSVGY	9	TRISIFGLGY	10
KRKDFLIMF	9	ARNPAMMQEM	10
YRFPSVSKL	9	SRLESGMQNM	10
AQGPTPRYF	9	YRKDAKENLH	10
LDIVKLLLP	9	LPRRSSRLFT	10
ARLDLERKV	9	RVLGSVGGQL	10
GRLPSSWAL	9	VRSSKELLLQ	10

QRGPVRVGF	9	SRHSGPFFTF	10
LELNKVQLL	9	GRFPDGTNGL	10
SRKTFSHEL	9	ARLDHKFDLM	10
ARTPVMSTY	9	MRLPSGKVGF	10
ARAGTFQAF	9	FRQYAKDIGF	10
GRFQKIKSM	9	GRIDKPILKA	10
KRLEEIPLI	9	SVSPVVRVAV	10
SRNAQSLGF	9	ARHKAEEVFY	10
LHRDDVEFI	9	SRVFVEEQVY	10
MVNLTQPAL	9	YRSPPLATGY	10
KEPLRSVLQ	9	GRMHAPGKGL	10
RRLPPIKEA	9	HRAPGFGFGI	10
GRYPGVSNY	9	GRILSGVVTK	10
ARLGITMKA	9	SRMLSGGFGY	10
LRVTPFILK	9	QRHDGKLWNL	10
SRTPVLMNF	9	SRDPSGTKGF	10
GRNSFEVRV	9	AQNGQLFGRF	10
GRTYPVQEY	9	KRLLPVFAQTF	11
GRVADVYRY	9	YRVTLNPPGTF	11
QRKERLYPL	9	GRFHGEEVAVK	11
KRLGLLAGF	9	NGGKEKRVAHF	11
RRLERLDHL	9	FRALCTGEKGF	11
SRLKASESF	9	MRLPGGPTLTF	11
HRQGFSYDY	9	MRLSQLKQLLK	11

ARTYLERHM	9	LRHSEPSKLTF	11
NRLPLVVSF	9	KRIGKDLSNTF	11
RRVPLPLLL	9	QRKDPSAFFSF	11
ARNLIYFGF	9	SRNSNTVVFVK	11
LFYFIVIII	9	SRSVALAVLAL	11
KKKIDILLK	9	SRIHPVSTMVK	11
GRWPWQGSL	9	KDTARGQNLLQ	11
SDDKSEYLF	9	FRVPFIYGHKY	11
ARFKERVGY	9	KRQGVVLGQNL	11
GRFDREFLF	9	SRHNIEGIFTF	11
QRFGPPVSR	9	KRISEQFTAMF	11
KRFSGTVRL	9	ERHGYIKGIVK	11
ARLDHKFDL	9	ARLDPKFLVNL	11
HRLPPVTSF	9	PNRLTCTLKLL	11
SRVNIPKVL	9	WQWNPSDRPSF	11
SRSGIRTGY	9	GRWGPTFPSSY	11
RREPGGNRL	9	GRAVEIGVKKF	11
ARSGEKITV	9	AARQFLLKWSF	11
HRLEELYTK	9	QRFQLPGNIGF	11
MTDLSKGVI	9	GRWQGNDIVVK	11
SRLATLNEK	9	ARKLPEKTQTF	11
RRIEYIEAR	9	ARLDTYMGKVF	11
NIEFKPESR	9	ARWLPEPGLGL	11
HKVMEFKSL	9	KRNIGVVVVGF	11

RRTSINVVR	9	ARLDELGGVYL	11
SAYGSVKAY	9	LRFPGQLNADL	11
QRQDIAFAY	9	ARDEFNLQKMM	11
KRLPPFETF	9	GRLAHEVGWKY	11
LELGLVEAI	9	ARLDHKFDLMY	11
NRYPGIVKK	9	RQYAKDIGFIK	11
ARIYHTIAY	9	HRLEELYTKKL	11
SRDPVQLNL	9	LRWIGDKEATY	11
SRLPQLVGV	9	SRFGQGSIIGV	11
ERLEKFFHR	9	SRYLNEKAVSY	11
KRFKGTESI	9	ARHLLEGLLQK	11
ARNIFPSNL	9	KRLSTPSASTY	11
DDITTSLRG	9	GRILSGVVTKM	11
RRDYLHYIR	9	ARFSPDGQYLV	11
GRYGGETKV	9	PGVSNTSRKTT	11
ARNPPGFAF	9	RGDLIKLFCII	11
SRLQEVFGH	9	ARNPSLKQQLF	11
EQLQEENLK	9	QRDPNSPLYSV	11
ARVTKVLGR	9	ARIGQTGTKSVF	12
GRYGAISGF	9	HRYPTSIASLAF	12
GDTLRLSAI	9	KRLNQVIFPVSY	12
RAIARTAVN	9	HRFIIGKKGQNL	12
NRTIVITSH	9	GRYSSESDVWSF	12
GRYTDVSTR	9	RLGLLMMILGLI	12

KAFQKIVVL	9	KRFGFPEGSVEL	12
KRLASSVLR	9	RTLGRSKGRLRL	12
SRNEGVATY	9	KRMNPNSPSITY	12
ASVAWLSGF	9	DFRSILPSNLLT	12
KRADLQSTF	9	SRFLQDPYSTTF	12
ARAPPPPRP	9	GRLPSSWALFSP	12
SRNPTFMGL	9	IRIGANVSNFTF	12
HRLPVEVAY	9	TRFLAEEGFYKF	12
IRAAPPPLF	9	QTWGGRGSQSTV	12
QRVPSFESF	9	VRLLLPGELAKH	12
FRYNGLIHR	9	ARLDHKFDLMYA	12
GRFSEPHAR	9	ARILLDNDHYAM	12
KRWEGGYER	9	ARSLPPPKLTDP	12
RRFPDLNRL	9	VRWYVDGPASTAF	13
RRVDAENRL	9	KRFGFPEGSVELY	13
SRFGNAFHL	9	FERIAGEASRLPH	13
IRHPNIITL	9	KRFSMVVQDGIVK	13
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KRISEQFTA	9		
SRNAQTFGF	9		
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TRIPAVKEK	9		
KRLGSLNKF	9		
ERSNVVITR	9		

NRIQEHFNK	9		
ARTPVTSTY	9		
RVNVVAPGF	9		
VRNEPTVSR	9		
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IRIQLPVVSK	10		
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FRFGESTTGF	10		

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SRIDLDELMK	10		
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KHINELTMKL	10		
ATALLASRGF	10		
TRISIFGLGY	10		
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MRLPGGPTLTF	11		
GRWQGNDIVVK	11		
SRAGPLSGKKF	11		
MRLSQLKQLLK	11		
WRKKQSDVMRF	11		
GRYGAISGFGL	11		
ARFPQLDSTSF	11		
GRFHGEEVAVK	11		
KRYDPDILLGY	11		
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ERHGYIKGIVK	11		
SRIHPVSTMVK	11		
KRLLPVFAQTF	11		
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FEPVVPLPDKI	11		
SRFGNAFLNRF	11		
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RRFVGHTKDVL	11		
WQWNPSDRPSF	11		

ILALHQLDLLQ	11		
MRAAVAASVGL	11		
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KRYDREFLLGF	11		
AFKAIKTEKLE	11		
GRSTGEAFVQF	11		
RRNLDIERPTY	11		
MRISPDQGQQF	11		
VHAPQVADAIL	11		
RQYAKDIGFIK	11		
VANVSLLALYK	11		
RRIEYIEARVT	11		
KRFDHSEIYTY	11		
LRWIGDKEATY	11		
EGGGLKIALAV	11		
NANPSPPPSPS	11		
RRMGPPVGGHR	11		
NRFSTPEQAAK	11		
RRLDSIVGPQL	11		
KRLSTPSASTY	11		
KRYEDQELTGK	11		
KRDESTGVIYR	11		
RRPIATAGLHR	11		
PKFEVIEKPQA	11		

RRLAEDEAFQR	11		
RRLLLEDLVKK	11		
LALDIEIATYR	11		
TDNTANGIALL	11		
RREPPPYGYRK	11		
RRFVEVGRVAY	11		
GGAKYAQNGQL	11		
EKTVQDLLNLM	11		
RRISESGIKKM	11		
FDTFCPLGPAL	11		
WYTSAVCPLGV	11		
QLQDGCEYVFI	11		
FRKDPNAEGRY	11		
MRVTAPRTLLLL	12		
EVTQPRTNTPVK	12		
KRFLGSGGFIGY	12		
QTWGGRGSQSTV	12		
VRLLLPGELAKH	12		
RRYLENGKETLQ	12		
GRFNDIHVPVRL	12		
LAFPLSPDWQVD	12		
KPMVVLGSSALQ	12		
NMEESLLSKLFP	12		
TRYNHPKPNLLY	12		

TEALPVGLIGMN	12		
RRMGESDDSILR	12		
MNPASKVIALKA	12		
GREFPEGEGRSK	12		
PHYTPEISTKLT	12		
ARARIVAEEFLK	12		
KRFADEGTVVKR	12		
IGQASGDLLRQI	12		
RRFVNVVPTFGK	12		
GRNSNSKRLLGY	12		
LKLTAPAVEAPV	12		
AQYKKDGADFAK	12		
DQVASLAGPGGQ	12		
AGEASRLAHYNKR	13		
KRFGFPEGSVELY	13		
GGEIFDHLVAHGR	13		
SRFDASFFGVHPK	13		
NGLGGPNGFPKPT	13		
GRIFLDHIGGTRL	13		
VDVSFVKGDSVRA	13		
RRLQIEDFEARIA	13		
RRFVGHTKDVLSV	13		
LEAPAARVGGAKV	13		
RRMGESDDSILRL	13		

KRFPGYDSESKEF	13		
KRFSMVVQDGIVK	13		
AGGHKLGLGLEFQA	14		
FVTPLTSMVVTKPE	14		
GTGGIQMGVTGGQL	14		
GIGSFTIIDGNQVS	14		
DASKKSDSNPLTEI	14		
PASPGAGNPPGTPK	14		
AGGHKVGLALELEA	14		
LYTLVTYVPVTTFK	14		

9.1.2 C1R.B27 ME1

Ctr-shRNA	Length	ERAP1-shRNA	Length
MLLAQLNAF	9	ARVTKVLGR	9
QIQQFHSQI	9	ARVPGPPAR	9
EIQKSALQI	9	GRIGVITNR	9
QQAELMLKM	9	SRSGIRTGY	9
LKKSNAPLV	9	ERSNVVITR	9
KQDTLLKLL	9	TRLFAVLRF	9
LEQSKALEL	9	GRFSGLLGR	9
IQWNGNYKL	9	GRYTDVSTR	9
TSTPTQLVQ	9	VRNEPTVSR	9
ELIQVKSEL	9	FRYNGLIHR	9
HRAQVIYTR	9	MRDPIRILV	9
HRAQVKRFT	9	GRDIPIVHR	9
QMLAVSATY	9	GRIGQAIAR	9
MGQFVLSIK	9	GRIPGIYGR	9
LVMLLQQPD	9	HRITIKKRF	9
TKTNRMPRW	9	MRLLPRLLL	9
KPSLMIQKA	9	HRAQVIYTR	9
EKVMEAFNI	9	ARLQTALLV	9
SRFIKFQEM	9	HRIGQQNEV	9
SRIHPVSTM	9	GGCRAFLIR	9
GRFGSGMNM	9	GRAALITSF	9
NMLQFNLQI	9	GRTLSDYNI	9

MRLLPRLLL	9	FRIEGLAPK	9
HRFYMIQSF	9	LRVTPFILK	9
EVVGRELLL	9	SRKESYSVY	9
GRAALITSF	9	ARSGEKITV	9
EKPGKAPNL	9	GRLEGWEVF	9
VVHVTIRGL	9	GRFLEIGKF	9
GQYGNPLNK	9	QRFPLSFGF	9
FRIEGLAPK	9	GRWPGSSLY	9
LRVTPFILK	9	GRFFLSSGL	9
ARVTKVLGR	9	FVQIGRFFL	9
ARVPGPPAR	9	GRLAAIVAK	9
ARLQTALLV	9	VRVPVTSNL	9
RRISGVDRY	9	LRYPMAVGL	9
GRIGVITNR	9	GRLTKHTKF	9
SLTAIQIGL	9	GRYPGVSNY	9
VESLKGILA	9	NRTIVITSH	9
GRFLEIGKF	9	SRFGNAFHL	9
SKTLSRFGI	9	GRLGSTVFV	9
KMVLLLNLQ	9	HRLPPVTSF	9
SGELKTLAL	9	SRFLKSDLF	9
QRFPLSFGF	9	ARIALLPLL	9
ERSNVVITR	9	ARLGVISKV	9
RRFTTSVVR	9	GRYGGETKV	9
KLSELHAYI	9	ARNPPGFAF	9

LRVDPVNFK	9	IRMPSLPSY	9
ERLEKFFHR	9	SRAGLQFPV	9
TRLFAVLRF	9	IRNDEELNK	9
GRFSGLLGR	9	LRVPEKVPF	9
EARELLPLL	9	RPDPKIPGL	9
GRFFLSSGL	9	NRMLKQMLF	9
VRNEPTVSR	9	IERVLGATL	9
FRYNGLIHR	9	ARTPVMSTY	9
GRLAAIVAK	9	SRAVQEFGL	9
LRYPMAVGL	9	QRYPNPYSK	9
GRYPGVSNY	9	PKTGMVSSR	9
ISEKELKLL	9	SRFIKFQEM	9
AFDLSQVLF	9	SRIHPVSTM	9
GRKGVAINF	9	GRFGSGMNM	9
MRDPIRILV	9	HRFYMIQSF	9
NRTIVITSH	9	QYNMAGVRTE	10
SRFGNAFHL	9	RRFVNVVPTF	10
GRDIPIVHR	9	ARTLLAILRL	10
HRLPPVTSF	9	SRVNIPKVLR	10
MPIKLKIGL	9	ARATLSSIRH	10
SRFNFDNKY	9	GRNQGGYDRY	10
LDQLSRLNI	9	SRFPNQNQTR	10
GRIGQAIAR	9	ARIPPVAVRL	10
KESALLNKL	9	YRFATDRNDF	10

GRIPGIYGR	9	SRIAIGPLRL	10
ARIALLPLL	9	GRVGDVYIPR	10
VVLFEGILV	9	RVRNATSPLT	10
ARLGVISKV	9	DSTISRRNAI	10
IFLDQLARL	9	MRVTAPRTLL	10
SRFSGGFGA	9	ARLSLTYERL	10
GRYGGETKV	9	RRISGVDRYY	10
IRMPSLPSY	9	GRIGQAIARR	10
IQKPQGLDP	9	DRALVDTLKF	10
LDIEKTKLL	9	HRYGDGGSTF	10
RLLPRLLLL	9	LRVTPFILKK	10
FRYPQDYQF	9	GRFPDGTNGL	10
ALPRTTPSV	9	GRFNGQFKTY	10
ARTPVMSTY	9	NRFAGFGIGL	10
SRAVQEFGL	9	LRIADLISSF	10
QRYPNPYSK	9	HRFEQAFYTY	10
RGLKAINVD	9	SRLPSLGAGF	10
NASIVTVIQL	10	SRHSGPFFTF	10
AGNVNAVDKL	10	SRLSIPTYGL	10
MILVNSFQLL	10	GRFLTGPLNL	10
ETGQVQKIVL	10	GRWPGSSLYY	10
LQSLVSIKQE	10	FRFGESTTGF	10
KQNLLRPMQL	10	IRKFLEGNSM	10
HNTLSFQTLR	10	MRLPSGKVGF	10

ESKQSAELNK	10	GRAPISNPGM	10
GVAKSQLLSY	10	ARLDHKFDLM	10
KQDLLAEKVL	10	KRFEELGVKF	10
VAINLIVQHI	10	HRYGDGGSSF	10
VSQLLRQAMH	10	HRFYGKNSSY	10
AIAQAVRGLI	10	ARVTGVPLSY	10
LKAENDRLKV	10	VRWSPKGTYL	10
PVTNSPQNLN	10	SRAGITMESY	10
YLSMGMVVLL	10	IRNDEELNKL	10
HRYGDGGSTF	10	TRLPPTPASK	10
SCCVLVFLLF	10	RRFVNVVPTFG	11
GQYGNPLNKY	10	GRLSKEDIERM	11
RRFVNVVPTF	10	GRFSHRIYVAY	11
GRFPDGTNGL	10	SRSQTSSFFTR	11
GRFNGQFKTY	10	SRFGNAFLNRF	11
NRFAGFGIGL	10	SRAGLQFPVGR	11
LPLRLRLLLL	10	ARFSPDDKYSR	11
RRISGVDRYY	10	GRFTGLESIFR	11
MLQMSILGSA	10	MRVTAPRTLLL	11
DPAPHKKVGF	10	RRYLENGKETL	11
RALSEQINIF	10	RRTGPPMGSRF	11
GRFLTGPLNL	10	RRIGLENFLLR	11
GRWPGSSLYY	10	RRNLDIERPTY	11
SVTGIFLPIL	10	RRLQIEDFEAR	11

ARTLLAILRL	10	QRYLSLVGQVY	11
KIYIDSNNNP	10	MRVPAQLLGLL	11
SRVNIPKVLR	10	GRSTGEAFVQF	11
ARATLSSIRH	10	FRLFPVPGSGL	11
FRYNGLIHRK	10	MRLPGGPTLTF	11
DMSVPAVAKL	10	VRLLLPGELAK	11
GRNQGGYDRY	10	SRIPFNQALVF	11
SRFPNQNQTR	10	GRKQDKGGFGF	11
KRFEELGVKF	10	RVLGQQGQELK	11
GRIGQAIARR	10	YRVTLNPPGTF	11
KDIMISIVGL	10	GRFGYLEGQEY	11
HRYGDGGSSF	10	ARFPQLDSTSF	11
RSERGGPFLF	10	GRSDLIPTIKF	11
HRFYGKNSSY	10	VYATDRGVVPL	11
LDNLVAILDI	10	HRVGPIEATSF	11
LHVAVIHKDV	10	SRSVALAVLAL	11
SRIAIGPLRL	10	MRISPDQGQQF	11
DHPYREIMKL	10	GRLAAIVAKQV	11
GRVGDVYIPR	10	GRLAHEVGWKY	11
QDIANKVLLA	10	LRWIGDKEATY	11
SASLPVTSLL	10	ARLDHKFDLMY	11
SSPLPVTSLI	10	GRSSQSSSQQF	11
MRVTAPRTLL	10	SRLNITYPMLF	11
GRWPKKSAEF	10	TRLPPTPLLLF	11

QRKSRGSESE	10	KRAFIITGQTY	11
QYTPPVVACV	10	KRLLPVFAQTF	11
DIGIVTSIEI	10	SRHVILGTQQF	11
DEQLPKQIPF	10	GRYDPSVKPPF	11
DTVAAMMNIKF	11	ARAALQELLSK	11
REIAVQIHSVI	11	SPSEPKEANRK	11
MGLGPGGQPID	11	MRVMAPRTLIL	11
ADIQQLYGGLL	11	RRFVNVVPTFGK	12
MTFKYNRQSMT	11	GEASRLAHYNKR	12
GIALIMLMAGF	11	ARFSPDDKYSRH	12
HLPPLGSTIVM	11	MRVTAPRTLLLL	12
TSLFTSIMMKA	11	RRYLENGKETLQ	12
MRVTAPRTLLL	11	RRMGESDDSILR	12
YCSSTVFLDTM	11	SRFSGSGSGTNF	12
KEIKILNIFGV	11	RTKGGNSAILNR	12
FIVPLTDLRIP	11	SRFSGSGSGTDF	12
RRFVNVVPTFG	11	MRVPAQLLGLLL	12
VRLLLPGELAK	11	FRLFPVPGSGLV	12
RRTGPPMGSRF	11	VRLLLPGELAKH	12
LEKYLSLQKIH	11	KRFGFPEGSVEL	12
FLGGCAGGLTL	11	YRVTLNPPGTFL	12
GRFGYLEGQEY	11	ARFQQGKDLQQY	12
YTLVGSLPLLI	11	TRFLAEEGFYKF	12
KLINGGIAGLI	11	YRFDPLGIVDGF	12

LRVDPVNFKLL	11	RVLSQQAASVVK	12
HRVGPIEATSF	11	ARHGLPLLPLLS	12
PKFEVIEKPQA	11	RGLGYTSLGIFL	12
SRSQTSSFFTR	11	ARILLDNDHYAM	12
RRIGLENFLLR	11	AGEASRLAHYNKR	13
LKLSCVQLLLQ	11	ARVPGPPARLGLP	13
GRSSQSSSQQF	11	RRFSPIPFPPLSY	13
SKDQVQLKDLL	11	RRMGESDDSILRL	13
AGQTTPVEPNI	11	RRYLENGKETLQR	13
NYKSPPANRCL	11	RRYNQSEAGSHTL	13
SRFGNAFLNRF	11	FRLFPVPGSGLVL	13
RRLQIEDFEAR	11	KRFGFPEGSVELY	13
KTVYALPTIAF	11	SRLSWLDGSGLGF	13
SRAGLQFPVGR	11	SRFDASFFGVHPK	13
ARFSPDDKYSR	11	VRWYVDGPASTAF	13
GRYDPSVKPPF	11	ARHGLPLLPLLSL	13
TSIVNLGTSGI	11	ARVPGPPARLGLPL	14
KDSSAMAVDAI	11	SRFSGSGSGTDFTL	14
RRYLENGKETL	11	GRFGADPEGSDLSF	14
MITLSTDGVII	11	DIVMLEKKIRTSSM	14
KTSCIVTAGQL	11		
RFPSNLSGAVG	11		
YQFDKVGILTL	11		
QSVIQSAGVSVL	12		

DGHNNNLLPQII	12		
RRFVNVVPTFGK	12		
LDTANGTPKVYK	12		
LSVLPQIKSTSF	12		
EQKAITAPVSLF	12		
QSSPSPSQLSHL	12		
SNITLLLTGLNM	12		
KNSTGVEIKRIM	12		
KEIKILNIFGVI	12		
EIKILNIFGVIK	12		
TIKLLNENSYVP	12		
DFIVPLTDLRIP	12		
MATPLLMQALPM	12		
KVDIVAINDPFI	12		
IFVKTLTGKTIT	12		
VNLNTIKRCLLI	12		
GEASRLAHYNKR	12		
RRMGESDDSILR	12		
LGLVLLRLGLHG	12		
GPISSILVNKYG	12		
TVKKIESFGSPK	12		
ARFSPDDKYSRH	12		
KGSSYKLLVEQA	12		
MRVTAPRTLLLL	12		

SGKVDIVAINDPF	13		
MATPLLMQALPMG	13		
LRAQVMEVKALPG	13		
VPPEVTVLTNSPV	13		
EIKILNIFGVIKG	13		
KEIKILNIFGVIK	13		
TIKLLNENSYVPR	13		
LDFFGNGPPVNYK	13		
DFIVPLTDLRIPS	13		
KVDIVAINDPFID	13		
IFVKTLTGKTITL	13		
LANIAILNNNLNT	13		
TNVVDIILAHHFG	13		
KAIFSPELYENIA	13		
AGEASRLAHYNKR	13		
RRMGESDDSILRL	13		
IAELDTLSEESYK	13		
ARVPGPPARLGLP	13		
VPDHVVWSLFNTL	13		
GGPISSILVNKYG	13		
RRFSPIPFPPLSY	13		
LNKLAFLNQFHFG	13		
NSAFGAQAGQYTY	13		
QAGCLGDDSPITL	13		

NSKTVYALPTIAF	13		
RRYLENGKETLQR	13		
GALKPGRAVEGPS	13		
QLLLQNIRHHSHFH	14		
YPHNGMIDLKYFPY	14		
LSAIIDLVCSTLGM	14		
VPPEVTVLTNSPVE	14		
EPSTVQILHSPAVE	14		
KEIKILNIFGVIKG	14		
TPDFIVPLTDLRIP	14		
NGKLVINGNPITIF	14		
GKVDIVAINDPFID	14		
KVDIVAINDPFIDL	14		
IFVKTLTGKTITLE	14		
GLANIAILNNNLNT	14		
LANIAILNNNLNTL	14		
LPLVIQALTNIEDK	14		
ARVPGPPARLGLPL	14		
AALREGGFLLLHTL	14		
LQQLKALLNHLDVG	14		
GRFGADPEGSDLSF	14		
IEKVEHSDLSFSKD	14		

9.1.3 C1R.B27 W6/32

Ctr-shRNA	Length	ERAP1-shRNA	Length
AASLGMILL	9	AAVEEGIVL	9
APALNALLV	9	ATAGEVGQF	9
ARAKIPLGF	9	ESDDSILRL	9
ASGLFIQAI	9	GVVFVTKKR	9
CITIKSTAI	9	IHRGGGQII	9
DLVCFVSGL	9	KVRAVGQRL	9
ESNYELEGK	9	LARGQPLPE	9
ETEQVNGLF	9	PRELAESVI	9
FDMGAEVGF	9	PSPVQLKAI	9
FDPSLLHLL	9	QLTLRKLQE	9
FDTAIAHLF	9	RAFPNKQGY	9
FGAITLNSM	9	RATLSSIRH	9
GFGGRGGGF	9	RDGYMGIGL	9
GHTKDVLSV	9	RFAGFGIGL	9
GIETELRSL	9	RFAPQFSGY	9
IIGASGLHL	9	RFATDRNDF	9
IQKDPVTPS	9	RFDASFFGV	9
IYIDSNNNP	9	RFDDPGLML	9
KAYLGSAIQ	9	RFEELGVKF	9
LAHVDLIEK	9	RFEQAFYTY	9
LDSGKELKI	9	RFEQLISRR	9
LGELVRLIL	9	RFGEMQLDF	9

LIHINLQFW	9	RFGESTTGF	9
LNNIPYHEG	9	RFGQDFSTF	9
LNSRLASYL	9	RFNGQFKTY	9
LRSPPTATI	9	RFNVLKVTK	9
LVSRLTGEA	9	RFPDGTNGL	9
MPVDKAKAP	9	RFPNQNQTR	9
NADLFRGTL	9	RFSPDGQYL	9
NELLSPLKD	9	RFTNGGKEK	9
NGDSYLVLI	9	RFYGKNSSY	9
NVVPTFGKK	9	RGRAAMIQV	9
PDNFQSRRP	9	RHMGIGKRK	9
PISDHEATL	9	RHSGPFFTF	9
QAAGQVLQF	9	RIDKPILKA	9
QHVHKSAVN	9	RIHGVGFKK	9
QYGNPLNKY	9	RILMEHIHK	9
RATLSSIRH	9	RILSGVVTK	9
RDGYMGIGL	9	RINVRIEHI	9
RFAGFGIGL	9	RIPSTPDSF	9
RFAPQFSGY	9	RIQQIIEGK	9
RFATDRNDF	9	RIRDIDLKR	9
RFEELGVKF	9	RISGVDRYY	9
RFEQLISRR	9	RKDAKSVKI	9
RFGESTTGF	9	RKFLEGNSM	9
RFGHGSAKL	9	RKNLLRTHT	9

RFGQDFSTF	9	RKTGQAPGY	9
RFNGQFKTY	9	RLDHKFDLM	9
RFNVLKVTK	9	RLDPNFLLK	9
RFPDGTNGL	9	RLEGIVNKM	9
RFPNQNQTR	9	RLEVKPHAL	9
RFSPDGQYL	9	RLKYPPFAK	9
RFTNGGKEK	9	RLNITYPML	9
RFVNVVPTF	9	RLPSGKVGF	9
RFYGKNSSY	9	RLPSLGAGF	9
RHMGIGKRK	9	RLQIEDFEA	9
RIHGVGFKK	9	RLQQVLTGL	9
RIKVNGIDY	9	RLSIPTYGL	9
RILMEHIHK	9	RLVKELEAK	9
RILSGVVTK	9	RMEPLWTGF	9
RINVRIEHI	9	RMPPFTQAF	9
RIQQIIEGK	9	RNKEIVHTF	9
RIRDIDLKR	9	RNKFGQLGL	9
RISGVDRYY	9	RNPGVKEGY	9
RIVAEEFLK	9	RQGGGRKGL	9
RKDAKSVKI	9	RQYAKDIGF	9
RLGGIAVAY	9	RSDVEAIAK	9
RLHPRAVNC	9	RSGMKIGRI	9
RLPSGKVGF	9	RTALIFGGF	9
RLPYLRTWF	9	RTLLAILRL	9

RMLLAHVDL	9	RTPVLMNFM	9
RNEKEKVLI	9	RVGDVYIPR	9
RNPGVKEGY	9	RVGIFPISY	9
RSDVEAIAK	9	RVLFPATGY	9
RSGMKIGRI	9	RVMAPRTLI	9
RSQVSTPRF	9	RVNIPKVLR	9
RSSLLMPYY	9	RVPAQLLGL	9
RTALIFGGF	9	RVTPFILKK	9
RVGDVYIPR	9	RWLPAGDAL	9
RVLFPATGY	9	RWLPLGLGL	9
RVNIPKVLR	9	RWPGSSLYY	9
RWLPAGDAL	9	RWPKKSAEF	9
RWPKKSAEF	9	RWQAIYKQF	9
RYEPPHIKK	9	RWQVAKELY	9
RYGDGGSTF	9	RWSPKGTYL	9
RYGNTPFLF	9	RYDMAQLRF	9
RYNGLIHRK	9	RYEPPHIKK	9
RYQDAIRVF	9	RYGNTPFLF	9
RYTPKEQLK	9	RYLDELMKL	9
SLSQSTSLF	9	RYNGLIHRK	9
STLPGVQIS	9	RYTPKEQLK	9
TFSKIPIGF	9	SKPKGPIRF	9
TKFILDTEM	9	SSAQPRLGR	9
TNVPLLVLI	9	VLGSVGGQL	9

TYFAQADAL	9	VRVLRLCTV	9
YLNPGSIRL	9	ADINGLRRVL	10
ALQQAHLLKN	10	DADKRITPLK	10
DGLITPTIAP	10	DKTILRALNQ	10
EDSQKLDNFL	10	FEILAPSSLR	10
ENMAVLTISS	10	LLQDSVDFSL	10
EPTSPAALVM	10	QYAKDIGFIK	10
GAMVEAGGLL	10	RAFIITGQTY	10
GFGGGSFRGS	10	RAGLQFPVGR	10
GLGSRARKWR	10	RAGPLSGKKF	10
GVPAVVVTII	10	RDEIEAMIKR	10
IALIMLMAGF	10	RDVVAKLISQ	10
IGNVKILRHK	10	RFDSKDEILL	10
IVPLTDLRIP	10	RFGPNGGLVF	10
KFEVIEKPQA	10	RFKSNVTKTM	10
LDSPGKVLLQ	10	RFLGKGGFAK	10
LEKYLGGLFV	10	RFLPQKIIYL	10
LGQIVSQRKL	10	RFNDLRFVGR	10
LHLLLVDCQA	10	RFNPNASSSF	10
LPLRLLCESM	10	RFSPDDKYSR	10
MINKQKGGLK	10	RFSPDGQYLV	10
PATASALLLL	10	RFSPKVSLPK	10
PGKVVVVTGA	10	RFVNVVPTFG	10
QILPKEQWTK	10	RGGTLAGHTQ	10

QYAKDIGFIK	10	RGRGGGGGAL	10
RAALQELLSK	10	RHVILGTQQF	10
RAGLQFPVGR	10	RIDPFVRTFL	10
RAGPLSGKKF	10	RIGVITNRER	10
RFGPNGGLVF	10	RIHPVSTMVK	10
RFGYLEGQEY	10	RILSGVVTKM	10
RFLGKGGFAK	10	RINVRIEHIK	10
RFNPNASSSF	10	RIPFNQALVF	10
RFSPDDKYSR	10	RISEQFTAMF	10
RFSPKVSLPK	10	RISLADIAQK	10
RFVGHTKDVL	10	RISPDQGQQF	10
RFVNVVPTFG	10	RKDAKSVKIK	10
RIDPFVRTFL	10	RKKQSDVMRF	10
RIGVITNRER	10	RKQDKGGFGF	10
RIKLEGIITR	10	RLAAIVAKQV	10
RINVRIEHIK	10	RLAHEVGWKY	10
RIPAEGRVAL	10	RLAVLPYELR	10
RISLADIAQK	10	RLDHKFDLMY	10
RKDAKSVKIK	10	RLDSIVGPQL	10
RKKQSDVMRF	10	RLEELYTKKL	10
RLAVLPYELR	10	RLFPVPGSGL	10
RLDSIVGPQL	10	RLGVAGQWRF	10
RLGVAGQWRF	10	RLHPRAVNCR	10
RLLLPGELAK	10	RLLKPGGMML	10

RLPSDVVTGY	10	RLLLPGELAK	10
RLQIEDFEAR	10	RLNITYPMLF	10
RLVPDSDVRF	10	RLPGGPTLTF	10
RMNVLADALK	10	RLPQGIVREL	10
RNPDTQWITK	10	RLPSDVVTGY	10
RSQTSSFFTR	10	RLQIEDFEAR	10
RTGPPMGSRF	10	RLRNQSVFNF	10
RVLQALEGLK	10	RLSNISHLNY	10
RVSTSHQPLR	10	RLVPDSDVRF	10
RWQGNDIVVK	10	RLVQSPNSYF	10
RYGAISGFGL	10	RMKLPKPPKP	10
RYLENGKETL	10	RMNVLADALK	10
SHCVVQAGLK	10	RMPEKVTWMR	10
SSVREVKSQM	10	RNPDTQWITK	10
TKAQIPILRM	10	RNPSLKQQLF	10
TQAPTFTQPL	10	RNSNTVVFVK	10
TTSIASVAGL	10	RSDLIPTIKF	10
VGPADRAAVL	10	RSQTSSFFTR	10
VSIGPVGKSK	10	RSTGEAFVQF	10
YCLTALPDEF	10	RTTSAGTGGM	10
YEAVAAVFIA	10	RVGPIEATSF	10
YKSPPANRCL	10	RVITEEEKNF	10
AGKIVPKSLLL	11	RVLQALEGLK	10
ALLSPSLRGTH	11	RVMAPRTLIL	10

DGQPVGAELGL	11	RVPFIYGHKY	10
DLDPETEQVNG	11	RVSTSHQPLR	10
EEMSEKPKKKK	11	RVTAPRTLLL	10
FVKTLTGKTIT	11	RVTLNPPGTF	10
GIPVVVAVNAF	11	RWDETPASQM	10
IILGSPSSGVT	11	RWGPTFPSSY	10
IVPLTDLRIPS	11	RWIGDKEATY	10
MALLASNSCFI	11	RWQGNDIVVK	10
MTWLPPLSAIQ	11	RYGAISGFGL	10
PEQKGVPTGFI	11	RYLENGKETL	10
PISSILVNKYG	11	RYLNEKAVSY	10
RARIVAEEFLK	11	RYPMAVGLNK	10
RFDDAVVQSDM	11	RYSQEPIKTF	10
RFVNVVPTFGK	11	RYTNKYSSQF	10
RGYLENGKETL	11	TFALLFGRRF	10
RKSSGGKGGSY	11	TTVARLIAIF	10
RMGESDDSILR	11	VTKRVELGIK	10
RYQKSTELLIR	11	YKRKSGSPSV	10
SLLGMRRGLNS	11	ASAALAGPASR	11
SPFLEGTVSLA	11	NSLAHLRAAPC	11
TPIIATLSSGA	11	RAGLKQVIIPR	11
VAVCDIPPRGL	11	RFDAGELITQR	11
EIKILNIFGVIK	12	RFDDAVVQSDM	11
FVKTLTGKTITL	12	RFDQVMKFIEK	11

GGGYGGDRGGGY	12	RFGFPEGSVEL	11
NGKLGIIARGDE	12	RFNDIHVPVRL	11
PLMRASGPGTMS	12	RFPYVLKEITK	11
RFVNVVPTFGKK	12	RFQQGKDLQQY	11
RLQIEDFEARIA	12	RFSGSGSGTDF	11
RMGESDDSILRL	12	RFVNVVPTFGK	11
RMLLAHVDLIEK	12	RGSAVIKFCLF	11
RMWPLAFGSGMG	12	RIGANVSNFTF	11
RVDPVNFKLLSH	12	RILLDNDHYAM	11
RWDEANAEKQRL	12	RLDHKFDLMYA	11
RYLENGKETLQR	12	RLLLPGELAKH	11
SSSPGPTGQPRR	12	RLNLNNTVLSK	11
VDIVAINDPFID	12	RMGESDDSILR	11
DLDPETEQVNGLF	13	RNDGVGQISGF	11
GFGGRGGGFGGGS	13	RQITTAQSVIL	11
KVDIVAINDPFID	13	RVPAQLLGLLL	11
PDFIVPLTDLRIP	13	RVQPGLLQWVK	11
PPEVTVLTNSPVE	13	RVTAPRTLLLL	11
RFGADPEGSDLSF	13	RVTIMPKDIQL	11
RFLNPDEVHAILR	13	RWPKKSAEFLL	11
RIDVVVNNAGILR	13	RYDPSISFSGM	11
RVPGPPARLGLPL	13	RYFNSKVPITR	11
DRIAGQVAAANKKH	14	RYLENGKETLQ	11
EPFAGLASSALSVF	14	RYQKSTELLIR	11

GFGGGSFRGSYGSS	14	VDVSILQRSLA	11
GGPGTASRPSSSRS	14	ETERFLSQVLGR	12
GTIKLLNENSYVPR	14	RATLSSIRHMIR	12
PGSVTSRLGSVFPF	14	RFGFPEGSVELY	12
PLLMQALPMGALPQ	14	RFPGYDSESKEF	12
		RFQVEQIGENKY	12
		RFVNVVPTFGKK	12
		RHGLPLLPLLSL	12
		RLQIEDFEARIA	12
		RMGESDDSILRL	12
		RMLLAHVDLIEK	12
		RMWPLAFGSGMG	12
		RWYVDGPASTAF	12
		RYFPGADEPLQY	12
		RYLENGKETLQR	12
		VRLSCAASGFDF	12
		RFGADPEGSDLSF	13
		RFLNPDEVHAILR	13
		RFSGSGSGTDFTL	13
		RIDVVVNNAGILR	13
		RVPGPPARLGLPL	13
		RVTAINARGSGKP	13
		ARTVASGGRELIEK	14
		GGPGTASRPSSSRS	14

		GTIKLLNENSYVPR	14
		NRFGIGPPVETIQR	14
		PGSVTSRLGSVFPF	14
		RFPGYDSESKEFNA	14

9.2 Lists of ER-resident peptides from ERAP1-deficient and -competent cells

9.2.1 C1R.B27

Ctr-shRNA	Length	ERAP1-shRNA	Length
AKELATKLG	9	IGSGVERMG	9
AVTVAPPGA	9	KRFEGLTQR	9
FPSVSAPSK	9	RKVGLIAAR	9
GPLGLDHMA	9	RVTVPLVRR	9
GRIDKPILK	9	VGSEIERMG	9
IGSGVERMG	9	MGPAIERMG	9
IRDTPAKNA	9	NWRIIPRNNP	10
LKGAEGQLQ	9	APGAEFAPNKR	11
LPPGLAVKE	9	RSAPGGGSKVP	11
LTHSPPDEL	9	APIDRVGQTIE	11
MGPAIERMG	9	AVTVAPPGARQG	12
PGAEFAPNK	9	GPALGAGIERMG	12
RKVGLIAAR	9	RAVPAEGVRTAE	12
SDQTVTVKE	9	RGVQIIDDEPQA	12
TPTPGHIMS	9	LAGPVRGVGGPS	12
VGSEIERMG	9	AAPIDRVGQTIE	12
VKAPLVLKD	9	APIDRVGQTIER	12
VKTNVPVKL	9	VAQKKASFLRAQE	13
APGAEFAPNK	10	NRAAPGAEFAPNK	13

APSETQFSHL	10	ERMVPAGMGAGLE	13
APVNVTTEVK	10	RSDLSVIQRNPKR	13
IEKFDKSKLK	10	LAGPVRGVGGPSQ	13
INEILSNALK	10	RMAAPIDRVGQTIE	14
IVKPVKVSAP	10	NRAAPGAEFAPNKR	14
KFFTTPSKSV	10	ATAPVNIAGSRTAA	14
LERMGANSLE	10	PGPRGLLGPKGPPG	14
LISGMEKPLP	10	RHTAAPTDPADGPV	14
NSDQTVTVKE	10	RVETGVLKPGMVVT	14
PEPAKSAPAP	10	VPAGMGAGLERMGP	14
SPEAQPKTLP	10	APIDRVGQTIERMG	14
STLLPKKPTA	10	KSKNGLYEIENGRPR	15
TPAPVEKSPA	10	TGTIKLLNENSYVPR	15
VEDIKAKMQA	10	VGVVLCNSLRYKIYL	15
AAPGAEFAPNK	11	ISGKRSAPGGGSKVP	15
APIDRVGQTIE	11	LERMAAPIDRVGQTIE	16
APVNVTTEVKS	11	IGRIEDVTPIPSDSTR	16
ETIMNQEKLAK	11	YGRGREEYEGPNKKPR	16
FIDKKPTSAKP	11	SISGKRSAPGGGSKVP	16
KIVKPVKVSAP	11		
KPAPPKPEPKP	11		
KTETQEKNPLP	11		
LIDRPAPHFER	11		
LKKKPTPVLLP	11		

LPPGLAVKELK	11
LVAKKPLTTTP	11
LVITDKEKAEK	11
RSAPGGGSKVP	11
SLGPSLATDKS	11
SNVKIQSTPVK	11
STAPEIKAPEP	11
TGVLKPGMVVT	11
VETGVLKPGMV	11
VTKTAGPIASA	11
YKEETIEKMQE	11
YPRSGTGQTIK	11
AAPIDRVGQTIE	12
AKPAPPKPEPKP	12
APAAAAAPAKVE	12
APVPTAPAAGAP	12
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GPVMDRMATGLE	12
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KPQKVAGGAAPS	12

KWPKKPKVPQST	12
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RAVPAEGVRTAE	12
RGVQIIDDEPQA	12
SPKAEDGATPSP	12
STAVTTSSAKIK	12
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AAAPASVPAQAPK	13
APVNVTTEVKSVE	13
APVPPVNEPETLK	13
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GPLGLDHMASSIE	13
ITSKPATLTTTSA	13
KPKAAKPKVVKPK	13
LAGPVRGVGGPSQ	13
LKKTETQEKNPLP	13
LNASKQDGPMPKP	13
LPPPPIPPPPPLS	13
LVITDKEKAEKLK	13
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PVATSQHHPGPQS	13
RSAPGGGSKVPQK	13
RSDLSVIQRNPKR	13
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SSAAQNAFKGNQI	13
TATPAPVEKSPAK	13
TISTSDPAEVLVK	13
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VAQKKASFLRAQE	13
VETGVLKPGMVVT	13
APAANSDQTVTVKE	14
APIDRVGQTIERMG	14
APVNVTTEVKSVEM	14
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AVQTASAHLPAPAA	14
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IKTLPPATSTEPSV	14
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LSEEPELSGGPPHI	14
NAPVPPVNEPETLK	14
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QAAAPASVPAQAPK	14
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RMAAPIDRVGQTIE	14
RVETGVLKPGMVVT	14
SIGGSAAPHTQSMQ	14
SITSKPATLTTTSA	14
SIVMETGNTKNSAL	14
SKETIEQEKQAGES	14
TATANQIPIISAEH	14
VPAGMGAGLERMGP	14
VPLDKQITIIDSPS	14
AAGVRNPQQHLNAQP	15
AVQKELSDMDYLKSK	15
DLQQWQGTRQKAENE	15
EKVEKPDTKEKKPEA	15
IIKNMPQMANFVYNM	15

ISGKRSAPGGGSKVP	15
KTETQEKNPLPSKET	15
LAAAEPTANGGLALA	15
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LTADSHPSYHTDGFN	15
NRAAPGAEFAPNKRR	15
QNDTPKGPQPPTVSP	15
QPPPLLPPSATGPDA	15
SKFPSSGPVTPQPTA	15
TPKKWPKKPKVPQST	15
TVGGPAPTPLLPPSA	15
VEAPEYIPISDDPKA	15
VVPTFGKKKGPNANS	15
AAPKQKIVKPVKVSAP	16
APAAGEEPSKEEGEPK	16
APEPSTVQILHSPAVE	16
APGPAAGGEAPKAAEA	16
GAAKEAAGKSSGPTSL	16
GEKVEKPDTKEKKPEA	16
HQQQQPPPPPQDSSKP	16
IGRIEDVTPIPSDSTR	16

ISTSDPAEVLVKNSQP	16
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KGGKPEPPAMPQPVPT	16
LERMAAPIDRVGQTIE	16
LHSSGINVNAAPFQSM	16
LKGVHSHKKKKIRTSP	16
LSSWDQAETPGHTPSL	16
REEASTSLPTKPTQGA	16
RGPLPAAPPVAPERQP	16
SISGKRSAPGGGSKVP	16
SKTPEGGPAPPPYSEV	16
SSTSSPKAEDGATPSP	16
SWDPSPVSSTVPAPSP	16
VEAPAANSDQTVTVKE	16
VGLNKGHKVTKNVSKP	16
VGVKKQKKPLVGKKAA	16
VIAMEEPAVPAPLPKK	16
YGRGREEYEGPNKKPR	16

10. Publications

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