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Viral immune evasion strategies:

The human cytomegalovirus US2 immunoevasin and protein degradation from the endoplasmic reticulum

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Estratégias virais de evasão do sistema imunitário: a imunoevasina US2 do citomegalovírus humano e a degradação de proteínas do retículo endoplasmático

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Prefácio

Nesta dissertação apresentam-se os resultados do trabalho desenvolvido entre 2001 e 2006, no Departamento de Patologia da *Harvard Medical School* (Boston, Massachusetts, EUA) de Outubro de 2001 a Agosto de 2005, e no *Whitehead Institute for Biomedical Resarch* (Cambridge, Massachusetts, EUA), de Setembro de 2005 a Agosto de 2006 sob a orientação do Professor Doutor Hidde L. Ploegh (supervisor internacional), e tendo como supervisor nacional o Professor Doutor João Pedro Simas.

Este trabalho teve como principal objectivo o estudo de factores celulares envolvidos no mecanismo de retro-translocação do retículo endoplasmático que é usurpado pelas proteínas US2 e US11 do Citomegalovírus humano (CMVH) para induzir a degradação da cadeia pesada da classe I do complexo maior (principal) de histocompatibilidade (MHC) e com isso impedir a detecção do vírus pelo sistema imunitário.

A presente dissertação encontra-se dividida em 6 capítulos: *Introduction*, onde se encontram resumidos os conhecimentos até à data acerca do processo de retro-translocação do retículo endoplasmático catalizado pelas duas imunoevasinas US2 e US11 do CMVH. *Results*, onde são apresentados os resultados originais obtidos neste estudo, parte dos quais são apresentados na forma de artigos no último capítulo, e parte dos quais são resultados preliminares. *Discussion*, onde são discutidos detalhadamente os resultados obtidos e conclusões e perspectivas futuras são apresentadas. *Material and Methods*, com a descrição dos métodos utilizados durante a investigação levada a cabo neste estudo. *References,* onde são enumeradas as publicações consultadas durante a elaboração deste documento. Finalmente, *Publications*, onde são incluídos os artigos publicados em revistas científicas durante o período de execução da presente dissertação.

Como previsto no Artigo 40º do Regulamento de Estudos Pós-graduados da Universidade de Lisboa (Deliberação nº 961/2003), a presente dissertação encontra-se redigida em língua inglesa, contendo um resumo alargado (mais de 1200 palavras) em língua portuguesa.

Como previsto no Decreto de Lei 388/70, art. 8º, parágrafo 2, parte integral dos resultados apresentados encontra-se publicada nos seguintes manuscriptos:

Manuscripto 1. **Furman MH, Loureiro J, Ploegh HL, Tortorella D.** Ubiquitinylation of the cytosolic domain of a type I membrane protein is not required to initiate its dislocation from the endoplasmic reticulum. *J Biol Chem*. 2003 Sep 12; 278(37): 34804-11.

Manuscripto 2. **Loureiro J, Lilley BN, Spooner E, Noriega V, Tortorella D, Ploegh HL.** Signal peptide peptidase is required for dislocation from the endoplasmic reticulum. *Nature*. 2006 Jun 15; 441(7095): 894-7.

Manuscripto 3. **Loureiro J, Ploegh HL.** Antigen presentation and the ubiquitin-proteasome system in host-pathogen interactions. *Advances in Immunology* 2006; 92:225-305.

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Resumo

Genomas relativamente pequenos e elevadas taxas de replicação permitem a acumulação de mutações a virus e bactérias, que vão assim constituindo novos desafios para o sistema imunitário do hospedeiro. Por outro lado, a resposta imunitária de um hospedeiro progressivamente melhor adaptado, modificada ao longo de uma história de co-evolução, torna a vida do agente patogénico cada vez mais complicada. Não constitui, portanto, surpresa a constatação que micróbios patogénicos procurem evitar detecção ou modular a resposta imunitária do hospedeiro, muitas vezes re-dirigindo vias celulares normais para seu benefício. Os vírus persistentes da familia *Herpesviridae* como o Citomegalovírus humano (CMVH) são capazes de causar uma infecção vitalícia latente, com reactivação esporádica e controlada em face de um sistema imunitário completamente equipado do hospedeiro saudável. Esta estratégia viral de sobrevivência depende de uma forma crítica de proteínas com funções de imunoevasão ou "imunoevasinas", produtos de genes virais dedicados à subversão do sistema imunitário do hospedeiro para escapar eliminação por este último.

A via de apresentação de antigéneos pela classe I do complexo maior (ou principal) de histocompatibilidade (MHC) é utilizada pelo sistema imunitário para amostragem do ambiente intracelular. Péptidos antigénicos gerados pela actividade proteolítica do proteosoma citoplasmático são carregados em moléculas da classe I do complexo MHC acabadas de sintetizar no retículo endoplasmático (RE), após o que o complexo trimérico de MHC - composto pela cadeia pesada (CP) de MHC, cadeia leve de MHC e péptido antigénico - é transportado através da via secretora até à superfície da célula, onde é sujeito a escrutínio pelo sistema imunitário. Quando uma célula é infectada por um vírus, proteínas citoplasmáticas de origem viral são sujeitas a degradação pelo proteosoma tal como as proteínas de origem celular, resultando na produção de antigéneos virais que ganham acesso à via de apresentação de antigéneos pela classe I do MHC. Linfócitos T citotóxicos (CTLs) inspeccionam os produtos do MHC à superfície celular para identificação de péptidos antigénicos de origem viral, enquanto que as células *natural killer* (NK cells) procuram perturbações na expressão de moléculas do complexo MHC à superfície da célula infectada. Detecção quer de antigéneos víricos, quer de perturbações na expressão de produtos da classe I do MHC – ambos causados por infecções virais – pode conduzir à activação

do programa citolítico dos linfócitos T ou das células NK, e, por fim, à destruição da célula que serve de refúgio ao vírus. Porque funciona na amostragem do ambiente intracelular habitado pelos vírus, a via de apresentação de antigéneos pela classe I do MHC representa um desafio formidável para os patogéneos virais e é, não surpreendentemente, alvo de muitas estratégias virais de evasão do sistema imunitário, particularmente pelos vírus da família *Herpesviridae*. Um deles, o Citomegalovírus humano (CMVH), codifica múltiplas imunoevasinas que interferem com a via de apresentação de antigéneos pela classe I do MHC. Os genes US2 e US11, localizados na região *unique short* (US) do genoma do CMVH codificam duas glicoproteínas residentes na membrana do RE que catalizam o transporte (logo após a sua síntese) da cadeia pesada (CP) da classe I do MHC (uma glicoproteína transmembranar com topologia de tipo I) através da membrana do RE para o citoplasma, resultando na sua degradação pelo proteosoma uma vez no citoplasma. Este movimento de proteínas do retículo endoplasmático (RE) para o citoplasma, uma vez que ocorre no sentido contrário ao da sua translocação (*translocation*) aquando da síntese proteica, é designado retro-translocação ou *dislocation*.

O processo induzido pelas proteínas virais apresenta muitas similaridades com mecanismos de controlo de qualidade que operam ao nível do RE e que asseguram a fidelidade e a regulação da expressão proteica durante a vida e a diferenciação celulares. Após inserção na membrana do RE ou translocação para o interior do RE, as proteínas que não atingem os estágios finais de *folding* (estrutura secundária e terciária) ou *assembly* (estrutura quaternária, no caso de proteínas que fazem parte de complexos proteicos) são destruídas. Proteínas da membrana ou do lumen do RE que são reconhecidas como *misfolded* ou *misassembled,* ou que são reguladas por proteólise em resposta a factores ambientais, são retro-translocadas, atingindo assim o citoplasma onde têm lugar os processos de remoção das cadeias de açúcares (*deglycosylation*), ubiquitinação, e, por fim, proteólise, através do qual o proteosoma citoplasmático destrói os polipéptidos aberrantes. A maquinaria celular envolvida na extracção de proteínas *misfolded* ou *misassembled* encontra-se ainda pouco definida. A degradação das moléculas da cadeia pesada (CP) da classe I do MHC pelas proteínas US2 e US11 não só permite ao CMVH escapar detecção pelo sistema imunitário mas constitui um sistema privilegiado de estudo do processo de retro-translocação, um processo de importância mais generalizada em casos de doenças causadas por acumulação de proteínas *misfolded* ao longo da via secretora,

eliminação de proteínas com *folding* mais lento ou mais difícil, ou intoxicação celular por toxinas bacterianas ou vírus que têm de ser retro-translocados.

A retro-translocação induzida pelas imunoevasinas US2 e US11 é uma reacção (ou conjunto de reacções) única não só em termos da rapidez e da especificidade da degradação causada, mas também porque as moléculas da CP da classe I do MHC não se podem classificar como *misfolded* ou *misassembled* nem são degradadas em resposta a sinais normais de regulação celular, mas em resultado da expressão de quer uma quer outra proteína viral. Apesar destas características singulares, o estudo da reacção catalizada pelo virus permitiu a caracterização de importantes pormenores mecanísticos do processo de retro-translocação: o estudo da via de retro-translocação mediada pela imunoevasina US11, em particular, levou à descoberta de componentes deconhecidos da maquinaria celular, as proteínas da família DERLIN, cujo envolvimento mais generalizado na retro-translocação em células não infectadas por CMVH veio, entretanto, a ser demonstrado.

Embora tenham como alvo o mesmo substrato (CPs do MHC) e induzam o mesmo cenário (degradação das CPs do MHC), os pormenores mecanísticos das reacções catalizadas pelas duas imunoevasinas virais diferem, por exemplo, a nível da identidade dos alelos de MHC que são alvo de destruição ou dos requisitos em termos do comprimento e da sequência da cauda citoplasmática da CP evidenciados por cada uma das proteínas virais. Nesta tese nós mostramos que as imunoevasinas US2 e US11 diferem também na sua capacidade de usar como alvo de destruição estágios distintos de *folding* dos produtos do locus MHC. Nós produzimos uma molécula de CP *unfolded* através de *site-directed mutagenesis* das duas cisteínas que formam a ponte dissulfídica do domínio alpha 3 situado na região da CP mais próxima da membrana, e mostramos que apenas a imunoevasina US11 é capaz de induzir a degradação deste mutante. O facto de a proteína US11 utilizar como alvo moléculas de CP independentemente do seu estágio de *folding/assembly* ao passo que a proteína US2 utiliza como alvo apenas estruturas de classe I do MHC no seu estado *folded* sugere que as imunoevasinas US2 e US11 actuam em diferentes fases da via biossintética das moléculas da classe I do MHC.

As diferenças entre as reacções de retro-translocação catalizadas por US2 e US11 estendem-se à maquinaria celular utilizada por cada uma das imunoevasinas virais: a proteína US11 usa o seu domínio transmembranar para recrutar as CPs do MHC para um homólogo humano da proteína Der1p de levedura, e que é essencial para

a degradação de um subconjunto de proteínas *misfolded* do RE. Curiosamente, a proteína Der1-like (DERLIN-1) humana é crucial no caso do US11, mas não é necessária para o processo catalizado pelo US2. Na realidade, as proteínas celulares essenciais para a retro-translocação catalizada pela imunoevasina US2 não foram ainda caracterizadas. Nós realizamos um *screen* para a sua identificacão através da comparação entre parceiros de interacção de uma molécula de US2 *wild type* (activa) com os parceiros de interacção de uma molécula de US2 mutante (inactiva) que não é capaz de catalizar a retro-translocação das CPs da classe I do MHC devido à ausência dos aminoácidos que constituem a sua cauda citoplasmática. Nós identificamos assim a *signal peptide peptidase* (SPP) como um parceiro de associação específico para a forma activa da imunoevasina US2. Nós mostramos que a redução dos níveis de SPP através de *RNA interference* (RNAi) inibe a retro-translocação catalizada por US2, mas não por US11. Também demonstramos que a imunoevasina US11, capaz de recrutar a proteína DERLIN-1, não recruta a proteína SPP, e vice-versa. Em conjunto com os dados de RNAi, as nossas observações sugerem que as duas imunoevasinas virais recrutam componentes diferentes da maquinaria celular e possivelmente subvertem duas vias distintas de retro-translocação do RE.

A proteína *signal peptide peptidase* ou SPP é uma aspartase da família das presenilinas (envolvidas nos processos de *Notch signaling* e doença de Alzheimer's) que cataliza proteólise no plano intramembranar e que, como o próprio nome sugere, cliva os fragmentos dos péptidos sinal que são deixados na membrana do RE após clivagem pela *signal peptidase*. A primeira função atribuída à SPP foi a produção de epítopos (*ligands*) para a molécula de HLA-E, um tipo não clássico de MHC. Os epítopos apresentados pela molécula não canónica de HLA-E são fragmentos do péptido sinal de moléculas polimórficas da classe I do MHC como os alelos HLA-A e HLA-B, fragmentos esses gerados por proteólise intramembranar catalizada pela SPP. A apresentação de antigéneos via HLA-E é importante para a monitorização levada a cabo pelas células NK, uma vez que lhes permite a detecção de perturbações na síntese de produtos polimórficos da classe I do MHC. O facto de a redução dos níveis de SPP através de RNAi inibir a retro-translocação da CP catalizada por US2 implica a SPP na via US2 e sugere uma nova função para esta aspartase intramembranar no processo de retro-translocação do RE.

Nós também apresentamos evidência de uma papel para a proteína p97 na retro-translocação por US2. O complexo trimérico Cdc48p(p97)/NPL4/UFD1, formado pela

AAATPase p97 (Cdc48p em levedura) e pelos seus dois co-factores NPL4 e UFD1, é importante para o reconhecimento e/ou a extracção de substratos de degradação poliubiquitinados da membrana do RE, num passo que precede a sua degradação pelo proteosoma. Embora o envolvimento da proteína p97 tivesse já sido mostrado no caso do US11 em células semi-permeabilizadas, nós confirmamos que a proteína p97 é importante também em células US11 intactas: a redução dos níveis de p97 através de RNAi causaram retro-translocação deficiente da CP de MHC em células que expressam US11. O mesmo *knockdown* por RNAi da ATPase p97 em células US2 também compromete a retro-translocação da CP de MHC, constituindo a primeira evidência de um papel da p97 na via US2 de retro-translocação. A convergência das vias US2 e US11 de retro-translocação, presumivelmente num passo anterior à degradação pelo proteosoma catalizado pelo complexo p97/NPL4/UFD1, ocorre apesar da existência de vias distintas (dependentes ou de DERLIN-1 ou de SPP) que aparentemente não se interceptam a não ser aquando da extracção do substrato da membrana do RE, reforçando a noção de que existem múltiplas vias de retro-translocação.

Mais, nós mostramos que a cauda citoplasmática de US2 não só é necessária mas é também suficiente para recrutar a SPP. Nós construimos moléculas mutantes de US2 sem um ou mais elementos de sequência/estruturais de US2 para analizar a contribuição do lumen, do segmento transmembranar, e da cauda citoplasmática da proteína US2 para a associação com SPP; uma "mini-molécula" situada na membrana do RE e contendo da molécula de US2 apenas os aminoácidos que constituem a cauda citoplasmática é ainda capaz de recrutar SPP. Mais ainda, ao investigar a contribuição do segmento transmembranar (STM) de US2, descobrimos que o STM de US2 é dispensável para a associação com SPP mas imprescindível para a função de retro-translocação da imunoevasina US2: uma molécula quimérica de US2, em que o STM foi substituído pelo STM de uma outra proteína transmembranar de tipo I não aparentada com US2, não é activa na função de retro-translocação, apesar de manter a capacidade de se associar com o substrato, as CPs de MHC, e com a SPP. Estas observações possibilitaram-nos revisitar e refinar o modelo de como a imunoevasina US2 funciona: a retro-translocação da CP de MHC do RE depende não só do recrutamento da SPP (através da cauda citoplasmática de US2) mas também de interacções adicionais que se dão ao nível da membrana do RE (através do segmento intramembranar de US2). É possível que o segmento transmembranar de US2 esteja envolvido numa posterior interacção com a proteína SPP e/ou no recrutamento de/interacção com outra

proteína(s) envolvida(s) no processo de retro-translocação. Finalmente, nós levamos a cabo uma discussão das possíveis implicações dos nossos resultados no estudo da retro-translocação catalizada por proteínas virais para o controlo de qualidade no retículo endoplasmático num contexto celular mais generalizado.

Abstract

Relatively small genomes and high replication rates allow viruses and bacteria to accumulate mutations, continuously presenting the host immune system with new challenges. On the other side of the trenches, an increasingly well-adjusted host immune response, shaped by co-evolutionary history, makes a pathogen's life a rather complicated endeavor. It is, therefore, no surprise that pathogens either escape detection or modulate the host immune response, often by redirecting normal cellular pathways to their advantage [1]. Persistent viruses of the *Herpesviridae* family such as human cytomegalovirus (HCMV) can cause a life-long latent infection, with controlled sporadic reactivation in the face of a fully primed host immune system. This viral survival strategy is critically dependent on immune evasion proteins or "immunoevasins", viral gene products committed to subvert the host immune system to escape elimination [2-4].

The class I Major Histocompatibility Complex (MHC) antigen presentation pathway is utilized by the immune system for sampling of the intracellular environment [5]. Peptide antigens derived from cytosolic proteolysis are loaded on newly synthesized class I MHC molecules in the endoplasmic reticulum (ER), after which the trimeric class I MHC complex - composed of the type I membrane glycoprotein class I MHC heavy chain (HC), the soluble light chain, and a short antigenic peptide of 8 to 10 aminoacids – travels through the secretory pathway to the cell surface, where it is subjected to scrutiny by the immune system. When a cell is virally infected, cytosolic proteins of viral origin, like endogenous cellular proteins, are subjected to proteasomal degradation, and thus virus-derived peptides access the class I MHC antigen presentation pathway. Cytotoxic T lymphocytes (CTLs) inspect surface class I MHC products for the presence of virus-derived peptide antigens, whereas natural killer (NK) cells score for perturbations in expression of class I MHC molecules at the surface of the virus-infected cell. Detection of either – both of which can be caused by viruses - can lead to activation of the CTL and NK cell cytolytic program, ultimately resulting in destruction of the cell harboring the virus [6]. Because it is involved in sampling of the intracellular environment inhabited by viruses, the class I MHC antigen presentation pathway presents a formidable challenge for viral pathogens and is, not surprisingly, the target of many viral immune evasion strategies, particularly by herpesviruses [4,7-11].

HCMV alone encodes several immunoevasins that interfere with the class I MHC antigen presentation pathway [3]. The HCMV unique short region (US) genes US2 and US11 encode two ER-resident type I membrane glycoproteins capable of catalyzing the transport of newly synthesized class I MHC HC molecules through the ER membrane to the cytosol, leading to HC degradation by the cytosolic proteasome. This ER-to-cytosol transport, as it constitutes movement of proteins in the reverse direction of translocation of nascent polypeptide chains into the ER [12,13], is termed retro-translocation or dislocation. HCMV US2 and US11-induced HC dislocation from the ER membrane and subsequent HC degradation allows HCMV to avoid cell surface display of viral peptides and thus evade the host immune surveillance afforded by the class I MHC pathway.

The virus-induced dislocation process bears many similarities to cellular ER quality control mechanisms, which ensure the fidelity and regulation of protein expression during cell life and differentiation [14,15]. After insertion into the ER membrane or lumen, proteins that fail to fold or assemble correctly are destroyed. Misfolded or misassembled ER proteins, or ER proteins that are subject to degradation in response to environmental cues, are dislocated or retro-translocated into the cytosol, where deglycosylation, ubiquitination, and ultimately proteasomal proteolysis dispense with the defective polypeptides [15]. The machinery involved in the extraction of misfolded proteins from the ER is poorly defined. The degradation of class I MHC molecules not only endows HCMV with the capacity to evade the immune system [12,13] but also constitutes a model system for the study of dislocation as a cellular process of general importance, for example, in the context of diseases caused by accumulation of misfolded proteins in the secretory pathway or premature degradation of proteins with slow folding kinetics, or intoxication by some bacterial toxins and viruses [16-19].

US2- and US11-mediated dislocation of class I MHC heavy chains is unique in terms of the speed and specificity of degradation [4,12,13,20] and because HCs are neither misfolded nor degraded as a result of normal cellular signals like most known substrates, but as a result of the expression of either viral protein. Notwithstanding the unique nature of this virus-mediated process, the US2 and US11 systems have allowed the characterization of important mechanistic details of the dislocation reaction: the study of the US11 pathway, in particular, led to the discovery of previously unknown components of the cellular dislocation machinery, the DERLIN proteins, that have since been shown to be involved in ER dislocation more generally [21-26].

Although targeting the same substrate (HCs) and inducing the same outcome (HC degradation), the two viral immunoevasins have been shown to differ in many of the mechanistic details of the dislocation reaction, such as which class I MHC alleles are targeted by each viral product or what the HC cytoplasmic segment (cytoplasmic tail) length and sequence requirements each displays. Here we show that US2 and US11 also differ in their ability to target distinct folding stages of class I MHC products. We generated an unfolded HC molecule through site-directed mutagenesis of the cysteine residues that form the membrane-proximal alpha3 domain disulphide bond, and show that only US11 is able to catalyze its degradation [27]. The fact that US11 targets HC molecules independently of their folding/assembly status while US2 targets only folded class I MHC structures suggests that US11 and US2 act at different stages of the class I MHC biosynthetic pathway.

The differences between the US2- and US11-mediated dislocation processes extend to the cellular machinery that each utilizes: US11 uses its transmembrane domain to recruit HCs to a human homologue of yeast Der1p, Der1-like protein 1 or DERLIN-1, a protein essential for the degradation of a subset of misfolded ER proteins. Interestingly, DERLIN-1 is essential for the degradation of HC molecules induced by US11, but not by US2 [21]. In fact, proteins essential for US2-dependent HC dislocation have remained elusive. We conducted a screen for such proteins by comparing interacting partners of wild type (active) US2 with interacting partners of a dislocation-incompetent US2 mutant that lacks the US2 cytosolic tail. We identified signal peptide peptidase (SPP) as a specific partner for the active form of US2. We showed that reduction of SPP levels by RNA interference (RNAi) inhibits HC dislocation by US2, whereas the same knockdown of SPP has no effect on US11-mediated dislocation of HC. Moreover, we showed that while US2 binds SPP, it does not bind DERLIN-1; conversely, US11 binds DERLIN-1 but not SPP. Taken together, our data suggests that the two viral immunoevasins utilize distinct cellular machinery and possibly subvert two distinct pathways of dislocation from the mammalian ER.

SPP is an ER-resident presenilin-type intramembrane-cleaving aspartic protease which, as the name suggests, cleaves signal peptide remnants resulting from prior signal peptidase processing from the ER membrane [28]. The first role assigned to SPP was the generation of HLA-E epitopes, i.e., peptide fragments derived from intramembrane cleavage of the signal sequence of polymorphic class I MHC molecules such as HLA-A and HLA-B. HLA-E presentation is important for NK cell surveillance as

it allows NK cells to detect perturbations in synthesis of polymorphic class I MHC products [29]. The fact that reduction of SPP levels by RNAi inhibits HC dislocation by US2 implicates SPP in the US2 dislocation pathway and suggests a novel role for this intramembrane-cleaving aspartic protease in ER dislocation.

We also present evidence for a role of the p97 protein in HC dislocation by US2. The trimeric Cdc48p(p97)/NPL4/UFD1 complex, composed of the AAATPase p97 (Cdc48p in yeast) and its co-factors NPL4 and UFD1, is involved in recognition and/or extraction of polyubiquitinated ERAD substrates from the ER membrane, in a step that precedes their proteasomal degradation [30,31]. Although the involvement of p97 in US11-mediated dislocation had been shown previously in semi-permeabilized cells [32], we confirmed the p97 requirement in intact US11 cells: reduction of p97 levels by RNAi leads to impaired HC degradation in US11 cells. The same p97 knockdown also impairs US2-mediated HC dislocation, constituting the first evidence for a role of the p97/NPL4/UFD1 complex in the US2 pathway. This convergence of the US2 and US11 pathways, presumably at a pre-proteasomal step catalyzed by the p97/NPL4/UFD1 complex, occurs notwithstanding the seemingly non-overlapping SPP/DERLIN-1 routes of substrate recognition/targeting to dislocation prior to extraction from the ER membrane, reinforcing the notion of the existence of multiple ER dislocation pathways.

Furthermore, we show that the US2 cytosolic tail is not only necessary but also sufficient for recruitment of SPP. We generated mutant US2 molecules lacking one or more US2 sequence/structural elements to analyze the contribution of the US2 lumen, the US2 transmembrane domain (TMD) and the US2 tail to association with SPP; a "mini-molecule" targeted to the ER membrane and containing only the tail aminoacids of the US2 molecule is still capable of binding to SPP. More importantly, while investigating the contribution of the US2 TMD we found that the US2 TMD is dispensable for association with SPP, yet crucial for US2 function in HC dislocation: a chimeric US2 molecule in which the US2 TMD has been replaced with that of an unrelated type I membrane glycoprotein is inactive in HC dislocation, although retaining the ability to bind the HC substrate and SPP. This has allowed us to refine the model for US2's role in dislocation from the ER membrane: HC dislocation is dependent not just on US2 tail-mediated recruitment of SPP but also on additional US2 TMD-mediated interactions within the plane of the membrane. We hypothesize that the US2 TMD is involved in further engagement of SPP or in the recruitment or engagement

of other protein(s) involved in dislocation. Finally, we discuss the implications of our findings in viral protein-induced dislocation to cellular ER quality control.

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List of abbreviations

MHC, Major Histocompatibility Complex
HCMV, Human Cytomegalovirus
ER, Endoplasmic Reticulum
US2, HCMV unique short region gene/protein 2
US11, HCMV unique short region gene/protein 11
HC, class I MHC heavy chain
SPP, Signal peptide peptidase
HLA, Human leukocyte antigen
Ig, Immunoglobulin
NK, Natural killer
RNAi, RNA interference
eGFP, enhanced green fluorescent protein
TMD, Transmembrane domain
TCR, T cell receptor
pAPC, professional antigen-presenting cell
$beta_2m$, $beta_2$-microglobulin or class I MHC light chain
CXN, calnexin
CRT, calreticulin
TAP, transporter associated with antigen presentation
ERAAP, ER aminopeptidase associated with antigen processing
CTL, cytotoxic T lymphocyte (same as $CD8^+$ T cell)
DRIPs, defective ribosomal products
Ub, ubiquitin
DUB, deubiquitinating enzyme
E1, ubiquitin-activating enzyme
E2, ubiquitin-conjugating enzyme (same as UBC)
E3, ubiquitin-ligase enzyme
HECT, homologous to E6-AP carboxyl terminus
RING, really interesting new gene
SCF, Skp1-Cullin1-F-box
IFN, interferon
SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis
shRNA, short hairpin RNA
ctrl, control
U-box, UFD2-homology box
UBL, ubiquitin-like modifier
SUMO, small ubiquitin-like modifier
NEDD8, neuronal precursor cell-expressed developmentally down-regulated 8
ISG15, IFN-stimulated gene product of 15 kDa
UBC, ubiquitin-conjugating enzyme (same as E2)
CFTR, cystic fibrosis transmembrane conductance regulator
ERAD, endoplasmic reticulum-associated degradation
HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase
Hsp, heat shock protein
CHIP, C-terminus of Hsc70-interacting protein
UGGT, UDP-Glucose:glycoprotein glucosyltransferase
EDEM, ER degradation-enhancing alpha-mannosidase-like protein

Yos9p, yeast osteosarcoma 9 protein

PDI, protein disulphide isomerase

TPRs, tetratricopeptide repeats

Hrd, HMGR degradation

Doa10p, degradation of transcription factor Mat alpha2 protein 10

Der1p and Der3p, degradation from the endoplasmic reticulum protein 1 and 3

VCP, valosin-containing protein (same as Cdc48p or p97)

CPY, carboxypeptidase yscY

CPY*, misfolded variant of CPY

UPR, unfolded protein response

VIMP, VCP-interacting membrane protein

ZL_3VS, carboxyl benzyl-leucyl-leucyl-leucyl vinylsulfone

To Luís

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Chapter 1. Introduction

Host-virus interactions

The vertebrate body is equipped to deal with invading pathogens, whether by means of mechanical barriers such as the skin and other epithelial surfaces, or by means of the innate and adaptive branches of its immune system [33]. The highly sophisticated vertebrate immune system is thus the appropriate battleground for microbial pathogens, selecting for those that devise successful strategies to avoid detection and elimination [33,34]. Viruses employ two basic survival strategies: acute and persistent infection. Acute or “hit and run” viruses are mainly cytolytic, highly infectious, and propagate through escape to a new host before immunological resolution of the viral infection or death of the host occurs. Persistent or “hit and stay” viruses resist complete elimination by the immune system and persist in a latent state for the lifetime of the host; controlled sporadic reactivation ensures transmission to a new host and thus survival of both host and virus [34]. For example, human cytomegalovirus (HCMV) is ubiquitous in human populations worldwide and establishes a benign life-long infection, causing practically no disease symptoms in the immunocompetent host. Yet, perturbation of the delicate balance between the virus and the host immune system leads to life-threatening infections in immunocompromised individuals such as AIDS patients and organ transplant recipients, and to congenital defects if the mother undergoes primary infection during pregnancy [35,36]. In host-virus interactions, the game of viral persistence and elimination is critically dependent on viral manipulation of the host immune response, shaped by co-evolutionary history. Examination of viral gene products has revealed how highly successful viruses such as HCMV employ a multitude of immune evasion strategies to escape detection and control by innate and adaptive host mechanisms [2,4,33,37].

The host immune response against viruses

Innate immunity comprises the phagocytic and inflammatory systems, with phagocytes like macrophages and neutrophils, and soluble mediators such as cytokines and complement. Cellular mechanisms provide protection where phagocytosis and humoral defenses fail. Natural killer (NK) cells are the main cellular effector of the innate antiviral response [34,38,39] and are amongst the first lymphocytes to sense the release of inflammatory cytokines and

perturbations in expression of class I Major Histocompatibility Complex (MHC) molecules and other surface molecules which are triggered by viral infection. NK cells can not only destroy the virus-infected cell but also produce cytokines that stimulate an ensuing and more sophisticated adaptive response [39,40].

The adaptive immune system includes the thymus-derived T lymphocytes (or T cells), and the bone marrow-derived B lymphocytes (or B cells), dendritic cells and macrophages. The two subsets of T lymphocytes, $CD8^+$ and $CD4^+$ T cells, possess distinct T cell receptors (TCRs), CD8 and CD4, respectively, that interact with their co-receptors on the surface of the target cell, the polymorphic class I and class II MHC molecules [33]. Class I and class II MHC molecules provide the context in which antigenic peptide epitopes originated by intracellular proteolysis are displayed at the cell surface for recognition by T cells. In a simplified view of antigen presentation, the two pathways have evolved to sample different sources of antigen to which they have access: the class I MHC pathway, present in nearly all nucleated cells, usually deals with endogenously-generated proteins and is crucial for triggering activation of $CD8^+$ T cells; the class II MHC pathway is present only in so-called professional antigen-presenting cells (pAPCs), and deals with exogenous antigens and the activation of $CD4^+$ T cells [41-43].

Class I MHC molecules loaded with viral peptide epitopes on pAPCs are recognized by naïve T cells of complementary receptor specificity. A successful interaction, requiring the presence of the appropriate co-stimulatory molecules, leads to lymphocyte activation or "priming" [43]. Several days after exposure to the virus, primed CTLs that specifically recognize the viral epitopes are deployed from the lymphoid organs into the circulation and the intercellular fluids, ready to seek out and destroy somatic cells that displays signs of virus presence on their surface class I MHC molecules. Recognition via interactions of the antigen-specific TCR on the CTL surface with a virus peptide embedded in the peptide-binding pocket of a class I MHC complex at the surface of the target cell results in activation of CTL effector functions [44-46]. Activation of the cytolytic program of CTLs ultimately leads to lysis and destruction of the virus-infected cell [6].

There is an exception to the "rule" of class I MHC pathway devotion to display of peptide antigens from endogenously generated proteins. Professional APCs (pAPCs) such as dendritic cells and macrophages can acquire and process exogenous material and present it at the cell surface in class I MHC products, a process called cross-presentation [47]. Priming of virus antigen-specific CTLs, for example, requires viral peptides to be displayed on class I

MHC molecules at the surface of pAPCs. With few exceptions, however, viruses normally do not infect professional APCs and thus virus-derived proteins are not endogenously generated by the pAPC. Non-infected professional APCs, mostly dendritic cells, can “cross-prime” naïve T cells with virus-derived peptides only after acquiring virus-derived material, i.e, through phagocytosis of apoptotic virus-infected cells. Cross-presentation is therefore essential for development of virus-antigen-specific CTLs and immunity to viruses [48,49].

Detection and elimination of viral pathogens is a formidable challenge for all organisms, even those afforded with the sophisticated mammalian immune system, as viruses have evolved sophisticated mechanisms to subvert host processes to ensure their own replication and transmission [1,4,9,34,50]. Viruses such as HCMV encode several “immunoevasins”, viral gene products committed to subvert the host immune system to evade virus elimination [51,52]. Because of its involvement in sampling of the intracellular space inhabited by viruses, the class I MHC antigen presentation pathway provides numerous points of interference for viral pathogens and is targeted extensively by these immunoevasins, as we shall discuss.

Class I MHC antigen presentation

The trimeric class I MHC complex is composed of the 43 kDa class I MHC heavy chain, the 12 kDa light chain or beta$_2$-microglobulin, and an antigenic peptide typically 8-10 aminoacids long. The alpha 1 and alpha 2 domains of the class I MHC molecule form a cleft or groove that displays the antigenic peptide, while immunoglobulin (Ig)-like domains of the heavy chain (alpha 3 domain) and the light chain or beta$_2$-microglobulin (beta$_2$m) support the peptide-binding cleft [53] (**Fig.1**).

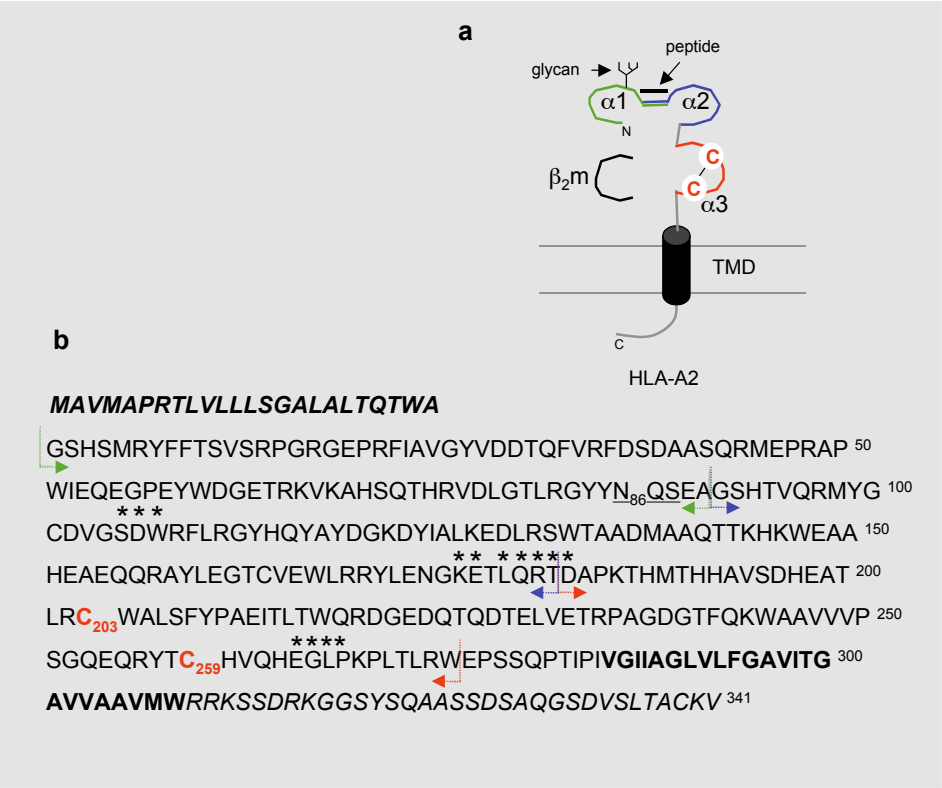

Figure 1. The class I MHC heavy chain (HC) molecule. **a.** Schematic representation of the topology of the HLA-A2 protein in the ER membrane, with the lumenal alpha 1, alpha 2 and alpha 3 domains colored in green, blue and red, respectively. The cysteine (C) residues that form the alpha 3 domain disulphide bond are indicated. **b.** HLA-A2 primary aminoacid sequence. The signal sequence is in bold italicized type, the *N*-glycan attachment sequence is underlined, the transmembrane domain is in bold type and the cytosolic tail residues are italicized. The sequences in between the colored arrows correspond to the alpha 1, alpha 2, and alpha 3 domains within the HC lumenal portion. The alpha 3 domain cysteine residues 203 and 259 are highlighted in red. The asterisks (*) indicate residues in HLA-A2 that contact US2.

The synthesis and assembly of class I MHC complexes takes place in the endoplasmic reticulum (ER) and consists of a multi-step process. In the course of synthesis, the class I

MHC heavy chain (HC) is inserted into the endoplasmic reticulum (ER) membrane and *N*-glycosylated, and binds to the transmembrane chaperone calnexin (CNX), at which point folding and intrachain disulfide bond formation take place. Once dissociated from CNX, the HC binds its soluble partner subunit, beta$_2$m, and enters the peptide-loading complex (PLC). The PLC is comprised of two MHC-encoded components, TAP and tapasin, and two "house-keeping" ER proteins, calreticulin (CRT) and ERp57. The transporter associated with antigen presentation (TAP) is an ATP-dependent pump with two subunits, TAP1 and TAP2, that transports peptides into the ER. Tapasin, a transmembrane glycoprotein, mediates the interaction between the TAP transporter and peptide-free HC/beta$_2$m dimers and is important for stability of class I MHC molecules and optimization of the peptide cargo presented to T cells [54-56]. Soluble CRT and ERp57, a glycoprotein chaperone and a thiol oxidoreductase, respectively, normally involved in folding of nascent glycoproteins, promote assembly of the class I MHC complex [57-59]. Just recently, protein disulphide isomerase (PDI), an enzyme involved in disulphide bond formation, has been proposed as a fifth component of the PLC, regulating the oxidation state of the disulphide bond in the peptide-binding groove to facilitate peptide optimization [60].

The peptide antigen cargo for class I MHC originates from proteasomal proteolysis in the cytosol. The array of proteasome-generated peptides is delivered to the ER lumen by the TAP transporter, often times after further trimming by cytosolic and/or ER-resident peptidases like the ER aminopeptidase associated with antigen processing (ERAAP) to guarantee a custom-fit of the peptide antigens into the peptide-binding groove on the HC/beta$_2$m dimer [61-64]. Empty HC molecules are detained in the ER by virtue of interaction with tapasin, until assembly with beta$_2$m and peptide takes place, at which point the trimeric HC/beta$_2$m/peptide complex is released from the PLC and allowed to exit the ER and enter the secretory pathway (**Fig.2**).

Once displayed at the cell surface, the antigen-loaded class I MHC complex is ready for inspection by the antigen-specific polymorphic T cell receptor (TCR) on circulating CTLs or by invariant receptors on NK cells [43,63,65-67].

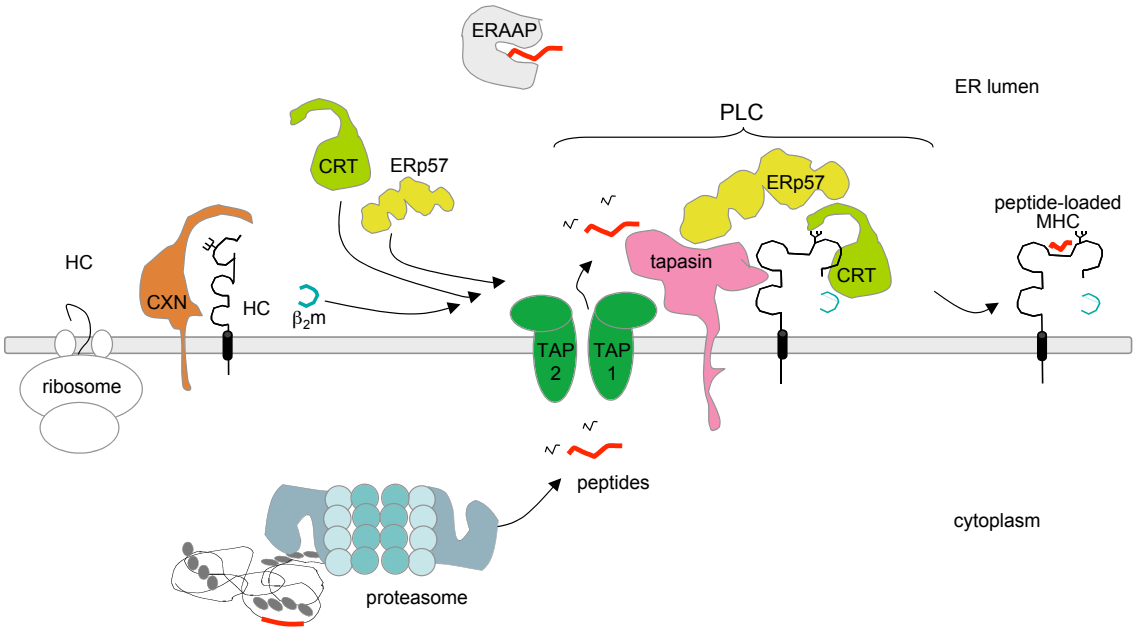

Figure 2. Schematic representation of the class I Major Histocompatibility Complex (MHC) assembly and loading pathway in the endoplasmic reticulum (ER). The newly synthesized class I MHC heavy chain (HC) initially associates with the chaperone calnexin (CNX), initiating folding and disulfide bond formation. HC is then incorporated, along with the class I MHC light chain or beta2-microglobulin (beta$_2$m), into the peptide-loading complex, which involves the transporter associated with antigen presentation (TAP), tapasin, calreticulin (CRT), and the thiol oxidoreductase ERp57. Peptides generated in the cytosol are TAP-translocated into the ER lumen where they may be trimmed by the ER-associated aminopeptidase ERAAP to the correct length of 8–10 amino acids. A peptide of the appropriate sequence binds to the HC/beta$_2$m dimer, initiating its dissociation from the peptide-loading complex.

Antigen recognition by CTLs and NK cells

Class I MHC products on most cells present exclusively “self” peptides, derived from the cell’s own proteins, the majority of which results from protein synthesis on free ribosomes in the cytoplasm. Because of the intrinsic error-prone nature of protein synthesis and folding, a sizable fraction of translation products (estimated at up to 30%) may never result in a finished product. These defective ribosomal products (DRIPs) are destroyed within 20-30 minutes of their synthesis by the cytosolic proteasomal pathway [68-71]. The apparent wastefulness of the protein synthesis process has a crucial immunological function, as this ongoing degradation of newly synthesized and nascent proteins provides a continuous supply of class I MHC ligands that can be readily detected by CTLs. In tumor cells or cells infected by a virus, mutated forms of endogenous proteins or viral proteins will compete with the host’s own proteins for presentation by class I MHC products. As “non-self” (tumor- or virus-derived) peptides displayed in the context of class I MHC products accumulate at the cell surface, their

chance of triggering activation of CTLs with a cognate receptor increases, ultimately leading to destruction of the tumor or virus-infected cell [72,73].

The selective pressure imposed by immune surveillance has made loss of class I MHC expression a hallmark of some tumors and virus-infected cells, as this allows them to be invisible to CTLs. There is, however, a back-up system for when lack of class I MHC expression impairs the CTL response: NK cells. NK cells display both activating and inhibitory receptors at their surface, which recognize different ligands at the surface of target cells. NK cell activity is ultimately determined by the integration of signals that are perceived by the NK cell surface receptors [67]. All NK cells express at least one inhibitory receptor, which engages class I MHC molecules on the surface of the target cell, resulting in down-regulation of NK cell effector functions. Low levels or absence of class I MHC products on the surface of the target cell relieve the inhibitory signals and lead to NK cell cytotoxicity, resulting in clearance of the virus-infected or tumor cells [67,74,75].

Viral interference with class I MHC antigen presentation

Antigen-presenting class I MHC molecules are crucial for detection and elimination of virus-infected cells by CTLs and NK cells [76-80]. This selective pressure leads viruses to keep evolving sophisticated immune evasion strategies targeting virtually every step of the class I MHC antigen presentation pathway. Viral immunoevasins can sequester class I MHC products intracellularly by inhibiting peptide loading (HSV ICP47, HCMV US6, Bovine Herpes-virus-1 UL49.5), by causing ER retention of heavy chain molecules (Adenovirus E3/19K and HCMV US3), by blocking exit of class I MHC complexes from the ER-to-Golgi complex (MCMV m152), by misdirecting MHC complexes to lysosomal compartments (MCMV m06 and HHV-7 U21), by internalizing MHC complexes from the cell surface (KSHV K3 and K5 and HIV Nef), or by inhibiting proteolysis and generation of the antigenic peptide (Epstein-Barr virus nuclear antigen-1 or EBNA-1, HCMV E protein pp65 and HIV Tat). Others can interfere with CTL and NK cell recognition at the cell surface by encoding class I MHC-like molecules and class I MHC ligands (HCMV UL18, UL40 and UL142 and MCMV m04), or can prevent cell surface display of class I MHC products by catalyzing degradation of class I MHC heavy chains by the Ub-proteasome system shortly after their biosynthesis (HCMV US2 and US11 and MHV-68 mK3) [4,76,79,81-84].

HCMV avoids control by conventional CTLs and NK cells

HCMV alone encodes several immunoevasins that frustrate recognition of virus-infected cells or activation of CTLs and NK cells by manipulating synthesis and trafficking of class I MHC locus products along the secretory pathway [85]. While many viruses target cellular factors in a number of subcellular compartments, the several HCMV-encoded immunoevasins confine their activity mostly to the endoplasmic reticulum to achieve downregulation of class I MHC products, as shown in **Figure 3**.

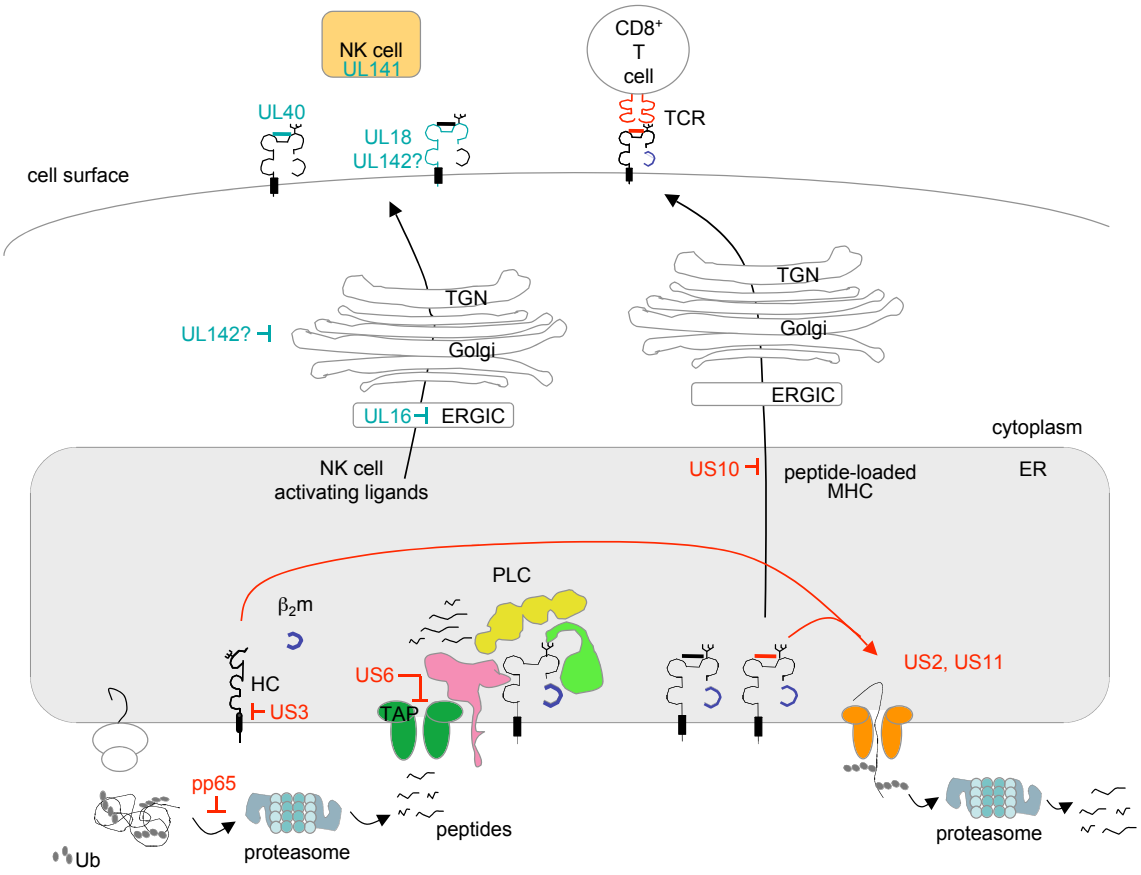

Figure 3. Schematic view of human cytomegalovirus (HCMV) immunoevasins that target the class I MHC antigen presentation pathway to avoid recognition and elimination of virus-infected cells. The HCMV immune evasion maneuvers aimed primarily at preventing recognition by $CD8^+$ T cells range from inhibition of proteolysis and generation of the antigenic peptide (pp65 protein) in the cytoplasm, to inhibition of peptide loading in the ER by blocking the TAP transporter (US6), to retention of class I MHC molecules in the ER (US3), to blocking exit of class I MHC complexes from the ER-to-Golgi complex (US10), and to catalyzing dislocation or extraction of class I MHC products from the ER membrane for subsequent degradation by the Ub-proteasome system (US2 and US11). Activation of NK cells is presumed to be prevented by HCMV immunoevasins expressed within the infected cell, that cause intracellular retention of activating receptor ligands (UL16 and perhaps UL142), encode class I MHC-like molecules that function as decoys (UL18 and perhaps UL142) or HLA-E ligands that maintain HLA-E expression, inhibiting NK cell activating receptors (UL40), or by HCMV proteins that act at the level of NK cell itself, causing down-regulation of NK cell activating receptors (UL141) or engaging the activating receptor in a way that disrupts the activating signaling pathway (HCMV pp65). Curiously, rather then inactivate NK cells, UL18 and UL40 may instead activate NK-T cells.

HCMV can downregulate cell surface expression of class I MHC molecules by the concerted action of a set of proteins encoded within the unique short (US) region of its genome [86]. The type I membrane glycoprotein US3, encoded by an immediate early US gene, is the first to be synthesized and binds to class I MHC heavy chains and retains them in the ER membrane [87,88]. The mechanism of HC retention by US3 is not entirely clear, and could be caused by the presence of an ER retention signal on the US3 lumenal domain [89] and/or to US3-mediated inhibition of tapasin and impairment of peptide optimization [90]. US6 inhibits peptide loading of the class I MHC molecules by blocking the TAP transporter [91-93]: the US6 type I ER-membrane glycoprotein has a bulky lumenal domain that binds to the TAP 1 and TAP 2 subunits of the TAP transporter from within the ER lumen, inhibiting ATP binding and thus TAP-mediated peptide translocation into the ER [94,95]. US10 delays trafficking of class I MHC HCs and stalls them in the ER through a poorly characterized mechanism [96]. US2 and US11 catalyze destruction of class I MHC HCs from the ER membrane by targeting them to the Ub-proteasome system [12,13], a process we discuss in detail in a later section.

Viral infection (as well as individual expression of each of the aforementioned immunoevasins in cell lines) abolishes cell-surface expression of class I MHC complexes [86,87]. This prevents antigen-specific recognition by CTLs, but invites activation of NK cells, as these lyse cells that lose class I MHC expression [67]. HCMV can, however, escape recognition by NK cells and NK cell-mediated lysis through expression of several immunoevasins, for the most part encoded within the unique long (UL) region of the HCMV genome [83,97-100]. The activating receptor NKG2D on the NK cell recognizes divergent families of class I MHC-related ligands, like the MIC and ULBP products, on the target cell. HCMV UL16 retains the ULBP-1, -2 and MIC-B NKG2D ligands in the ER-to-Golgi (ERGIC) compartment of the target cell, preventing NKG2D recognition and thus NK cell activation [101]. In addition to encoding a class I MHC-like decoy molecule, HCMV UL142 has been proposed to down-regulate the NKG2D ligand MIC-A. HCMV UL141 and pp65 act at the level of the NK effector cell rather than the antigen-presenting cell, also preventing NK cell activation. UL141 down-regulates the NK cell-activating receptors CD226 (DNAM-1) and CD96 (TACTILE) [102]. The pp65 tegument protein engages the activating receptor NKp30 and causes dissociation of a receptor-associated signaling module, disrupting the activating signaling pathway that would lead to NK cell activation [83,103].

NK cell responses also rely on receptors that recognize class I MHC locus products. The killer cell immunoglobulin-like receptor (*KIR*) genes encode a family of activating and

inhibitory receptors that recognize human leukocyte antigen (HLA)-A, -B and –C. The CD94/NKG2 receptors recognize the non-classical class I MHC molecule HLA-E, which presents fragments derived from the signal sequences of classical class I MHC molecules, delivering an inhibitory signal to CD94/NKG2 receptors. UL40 encodes a peptide whose sequence is exactly homologous to the HLA-E binding leader peptide from HLA-C locus products, thereby loading and maintaining HLA-E on infected cells [104,105] even when other class I MHC products are down-regulated. UL18 encodes a viral class I MHC homolog which function as a decoy for the class I MHC invariant inhibitory receptor CD85j/LIR-1/ILT-2, which recognizes class I MHC molecules. However, expression of the UL18 and UL40 does not prevent NK cell-mediated lysis [77,106].

Paradoxically, the HCMV UL18 and UL40 “immunoevasins” may instead activate a special subset of T cells that are crucial for control of viral infection [107-110]. The invariant (NK cell-like) inhibitory receptor CD85j is expressed not only by natural killer cells but also by some T-cell subsets, in which it specifically recognizes class I MHC molecules and down-regulates antigen-specific T cell functions [111,112]. Initially, UL18 was thought to engage CD85j on the NK cell, inhibiting NK cell effector functions [97,113], but, in fact, the UL18 interaction with CD85j at the T cell membrane leads to activation (not inhibition) of these non-MHC-restricted CTLs or NK-T cells [109 110]. The UL40-derived HLA-E peptide increases cell surface expression of HLA-E, which not only prevents activation by NK cells but is also important in activation of HLA-E-restricted CTLs which can kill HCMV-infected cells [114]. This HCMV-mediated NK-T cell activation is proposed to be a viral strategy of ensuring survival of the host by allowing some level of protection from the initial wave of NK cell-mediated antiviral response.

Promiscuous viruses and viral immunoevasins: the importance of multitasking

One emerging principle of viral immune evasion, particularly with herpesviruses, is that a single virus encodes multiple immunoevasins aimed at downregulation of class I MHC molecules. The multiple HCMV immunoevasins have marked allelic preferences [20,115-119], are proposed to allow HCMV to counter the high degree of class I polymorphism in the human population. Moreover, they may allow HCMV to control the cell surface repertoire of class I MHC molecules, as to preserve inhibitory signals to NK cells. Indeed, the known human NK

receptors for class I MHC focus primarily on the HLA-C and HLA-E locus products. By selectively downregulating the very potent antigen-presenting HLA-A and HLA-B locus products and allowing expression of mostly non-classical locus products (such as HLA-E, for example) that exhibit limited polymorphism, HCMV may diminish antigen presentation to CTLs while limiting NK cell activation [120]. Moreover, the multiple HCMV immunoevasins display distinct temporal and spatial patterns of expression [86,87] that are important in the context of infection. Murine cytomegalovirus (MCMV) also encodes multiple immunoevasins (unrelated to those of HCMV in terms of gene and protein sequence) that cause class I MHC downregulation from the cell surface [121]; the HCMV immunoevasins are likely to have synergistic and antagonistic interactions important for control of immune surveillance and host survival in the context of viral infection, as has been shown for MCMV [52,78,80,122].

Another emerging principle of viral immune evasion is that many viral immune evasion proteins bind multiple cellular targets, preventing recognition by different effectors of the immune response [79]. Several gamma-herpesviruses and poxviruses, for example, encode immunoevasins with E3 ligase activity, the K3 homologs, that include the Kaposi's-sarcoma-associated herpesvirus (KSHV) kK3 and kK5 (also called modulator of immune recognition MIR-1 and MIR-2, respectively), the murine gamma-herpesvirus 68 (MHV-68) mK3, and the rabbit myxoma virus M153R [123]. KSHV kK3 and kK5 and myxoma virus M153R catalyze ubiquitination of cell surface class I MHC heavy chains, thus providing the trigger for their internalization from the cell membrane and sorting for lysosomal degradation [124]. In addition to down-regulation of class I MHC molecules, some of these K3 homologs downregulate CD4, CD1d (a class I MHC-like molecule that presents lipids), and others [11,123,125-128], reducing recognition by $CD8^+$ T cells, $CD4^+$ T cells and CD1d-restricted NK-T cells, respectively. Similarly, HIV Nef prevents cell surface expression of class I and class II MHC molecules, CD4, CD28, and CD1a [129]. US2, for example, induces degradation of diverse class I MHC molecules (HLA-A, HLA-B, HLA-C and HLA-G), and is proposed to also cause the destruction of some class II MHC molecules and the hemochromatosis protein HFE [130]. How these viral proteins can act on molecules with such different primary structures is rather remarkable, as is the finding that herpesviruses such as HCMV, with over 200 genes, target multiple recognition pathways using a single viral protein. Remarkable, too, is the fact that HCMV has evolved two proteins, US2 and US11, that target the same substrate, class I MHC molecules, by a deceivingly similar dislocation reaction with very distinct mechanistic details, as we shall discuss.

HCMV US2 and US11 catalyze destruction of class I MHC heavy chains by subverting ER dislocation

The early stages of synthesis of class I MHC heavy chains (HCs) involve translocation of the nascent polypeptide chain into the ER through the Sec61 complex or translocon, insertion into the ER membrane and *N*-linked glycosylation. In HCMV-infected cells, expression of the early gene products US2 and US11 causes destruction of HCs very shortly after completion of their synthesis [87]. The half life of the HC protein is reduced from hours to a mere 2-5 minutes in HCMV-infected cells or in U373-MG astrocytoma cells stably transfected with either US2 or US11, with US11 displaying a slightly more robust degradation capacity [12]. The US2 and US11 glycoproteins are able to specifically associate with HC molecules and to catalyze the transport of newly synthesized, membrane-inserted HCs through the ER membrane into the cytosol, a process called retro-translocation or dislocation [12,13]. Upon dislocation from the ER membrane, removal of the HC *N*-linked glycan by the cytosolic peptide *N*-glycanase (PNGase) takes place, as well as ubiquitin conjugation and, ultimately, destruction by the cytosolic proteasome [12,13]. This US2- and US11-mediated ER-to-cytosol dislocation, also called retrograde-translocation or retro-translocation as it describes the movement of proteins in the reverse direction of the translocation process [18,131], shares numerous similarities with normal cellular ER quality control and degradation of misfolded or misassembled proteins [16,18,131,132]. No cellular homologs (or otherwise) or known sequence motifs exist in US2 or US11 that would suggest their function. The proposed *modus operandi* for the HCMV US2 and US11 immunoevasins is that they recognize and associate in a highly specific manner with class I MHC HCs and presumably deliver them to cellular dislocation machinery as substrates, subverting the cellular dislocation factors usually involved in removal of misfolded or misassembled ER proteins for destruction of class I MHC HCs (**Fig.4**).

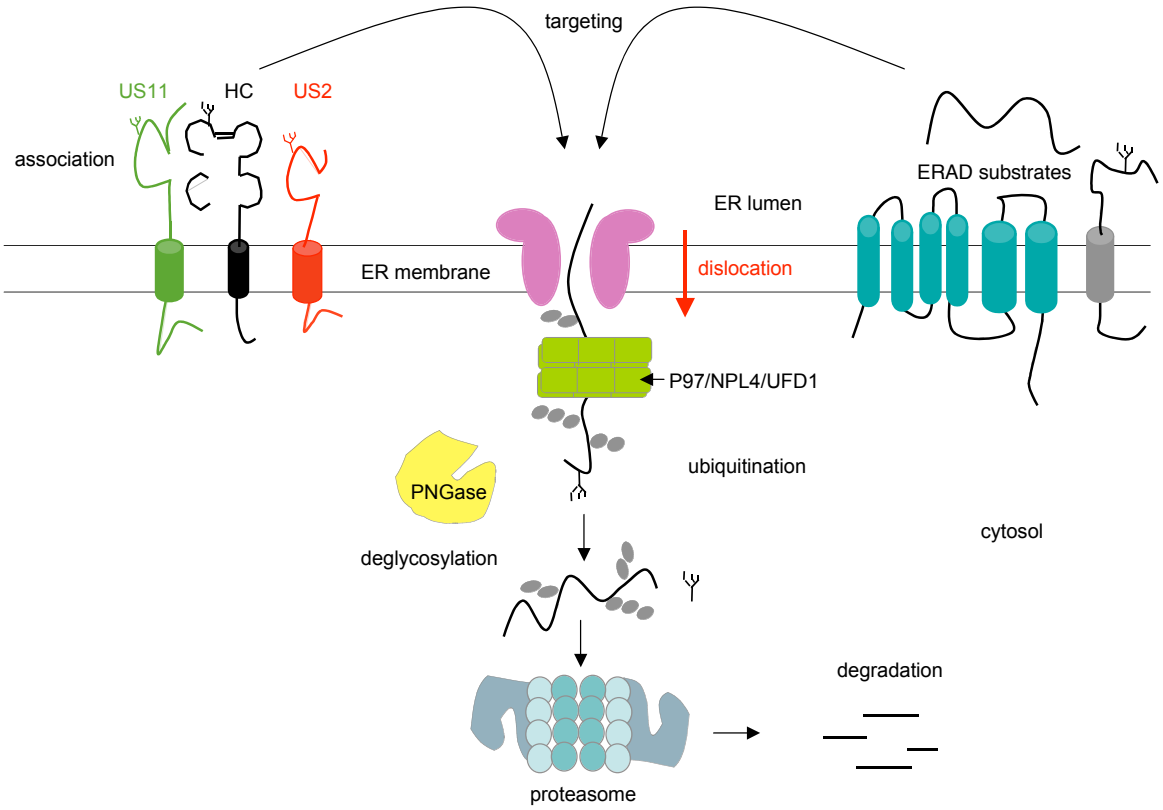

Figure 4. The HCMV US2- and US11-mediated dislocation of class I MHC heavy chains resembles normal cellular pathways of ER-associated degradation (ERAD). Class I MHC heavy chain (HC), US2 and US11 are type I membrane glycoproteins. US2 and US11 associate with HC soon after its synthesis and target HC for transport across the ER membrane into the cytosol, a process termed dislocation (or retrograde translocation). Once in the cytosol, HC is deglycosylated by the cytosolic peptide N-glycanase (PNGase), ubiquitinated, and degraded by the proteasome. This mechanism shares features of the normal cellular pathways of degradation of misfolded or misassembled ER proteins such as the cystic fibrosis conductance regulator (CFTR), the orfan T-cell receptor alpha (TCR alpha) subunit, tyrosinase and alpha 1-antitrypsin, or of regulated degradation of proteins like 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR).

ER protein quality control and ER-associated degradation

ER quality control is a homeostatic process that involves elaborate machinery that monitors the folding and assembly of newly synthesized proteins. Although tightly controlled, ER protein synthesis is not always successful. Proteins may sustain damage or fail to complete their synthesis early during biogenesis; they may instead be trapped in an

irreversible non-native conformation or a mutation may result in a structural alteration that leads to misfolding, as is the case for the cystic fibrosis conductance regulator (CFTR) [133,134], mutant plasma alpha1-antitrypsin [135] or tyrosinase [136]. Alternatively, proteins may be expressed in the absence of their cognate subunits, as is the case for unassembled subunits of TCR alpha [137,138]. In addition to its role in ER quality control, degradation from the ER can also be employed in the physiological regulated proteolysis of normal ER proteins whose degradation is subject to metabolic cues, such as that of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in response to sterol levels [14,139]. Accurate discrimination between proteins that are actively folding, fully folded/assembled, and misfolded/misassembled is required, as the cell must handle each of the three classes of ER quality control substrates differently. Actively folding proteins are retained in the ER and shielded from degradation, folded/assembled proteins destined for export are packaged into transport vesicles, and misfolded/misassembled proteins are retained and sorted for degradation in the cytosol by the Ub–proteasome system [140].

The selective protein export from the ER to the cytosol followed by proteasomal degradation is known as ER-associated degradation (ERAD) [17]. A feature of this ER-associated protein degradation is the spatial separation between targeting of substrates and their proteolysis, which requires substrate export from the ER lumen or membrane to the cytoplasm through a so far unknown channel in the ER membrane [131,141]. This complicated multi-step process involves substrate recognition in the ER lumen or membrane, targeting for dislocation, removal from the ER membrane, ubiquitination upon exposure to the cytosol, deglycosylation (in the case of glycoproteins) and finally destruction by the cytosolic proteasome [131,132,142].

The ubiquitin-proteasome system

All cellular proteins, regardless of their half-life, are subject to turnover. The main pathway for degradation of short-lived proteins in the cytoplasm of eukaryotic cells is the Ub-proteasome system, pivotal to numerous cellular processes such as cell cycle control, transcriptional regulation, signal transduction, membrane trafficking and many others [143,144] (**Fig.5**). Ubiquitin is a small 76 amino acid protein, synthesized as a precursor that is

processed by deubiquitinating enzymes (DUBs) to expose the glycine-glycine sequence at the Ub C-terminus. Glycine (G) 76 constitutes the site of Ub attachment to a lysine (K) residue on the target protein. In the Ub conjugation cascade, ATP-dependent Ub activation is catalyzed by the E1 (Ub-activating) enzyme, which adenylates the Ub C-terminus, allowing the subsequent formation of a high-energy thioester bond between Ub G76 and the cysteine residue on the E1 active site. Ub is then transferred from the E1 cysteinyl side-chain to a cysteinyl group on one of several E2 (Ub-conjugating) enzymes. Finally, one of hundreds of E3 (Ub-ligase) enzymes, binds the Ub-E2 complex and the substrate, thus facilitating the transfer of Ub to a lysine residue in the substrate via an amide (isopeptide) bond [143] (**Fig.5.a**).

Ub conjugation has much more wide-ranging effects than targeting of the Ub-conjugated substrate for proteasomal destruction: chain length, linkage type and type of Ub-like protein (UBL) attached also influence the outcome of the Ub-conjugated substrate. The multiple Ub moieties in a polyUb chain (chains of 4 or more Ub moieties) are linked to one another by an isopeptide bond between a lysine residue on one Ub molecule (usually on K48) and the C-terminal carboxyl group of the next Ub on the chain. Targeting of proteins for proteasomal proteolysis generally requires polyubiquitination in a K48-type linkage. By contrast to polyUb, substrate monoubiquitination or attachment of non-canonical Ub chains – Ub chains with non-K48 linkages such as K63 and K29 linkages – usually have non-proteolytic functions in DNA repair, endocytosis, signal transduction, transcriptional regulation and ribosomal function [145,146]. Monoubiquitination, which can occur on a single lysine residue or on several lysine residues in a substrate (multiubiquitination), for example, constitutes an extremely important sorting signal in the endocytic pathway [147,148]. Ub-like molecules or modifiers (UBLs), like SUMO, NEDD8 or ISG15, share structural homology with Ub and can also be conjugated onto protein substrates, mostly with outcomes other than proteasomal degradation [149,150] (**Fig.5.b**).

Deubiquitinating enzymes (DUBs) cleave isopeptide bonds to remove ubiquitin from the substrate or from polyubiquitin chains, and are a very diverse group of cysteine proteases and metalloproteases [151]. They have very diverse specificity properties in terms of the Ub or UBL moiety and target proteins from which they catalyze deubiquitination, and can be further regulated by subcellular localization or association with different binding partners, thus providing a mechanism for dynamic control of Ub chain-length and substrate fate (**Fig.5.a**) [152-155].

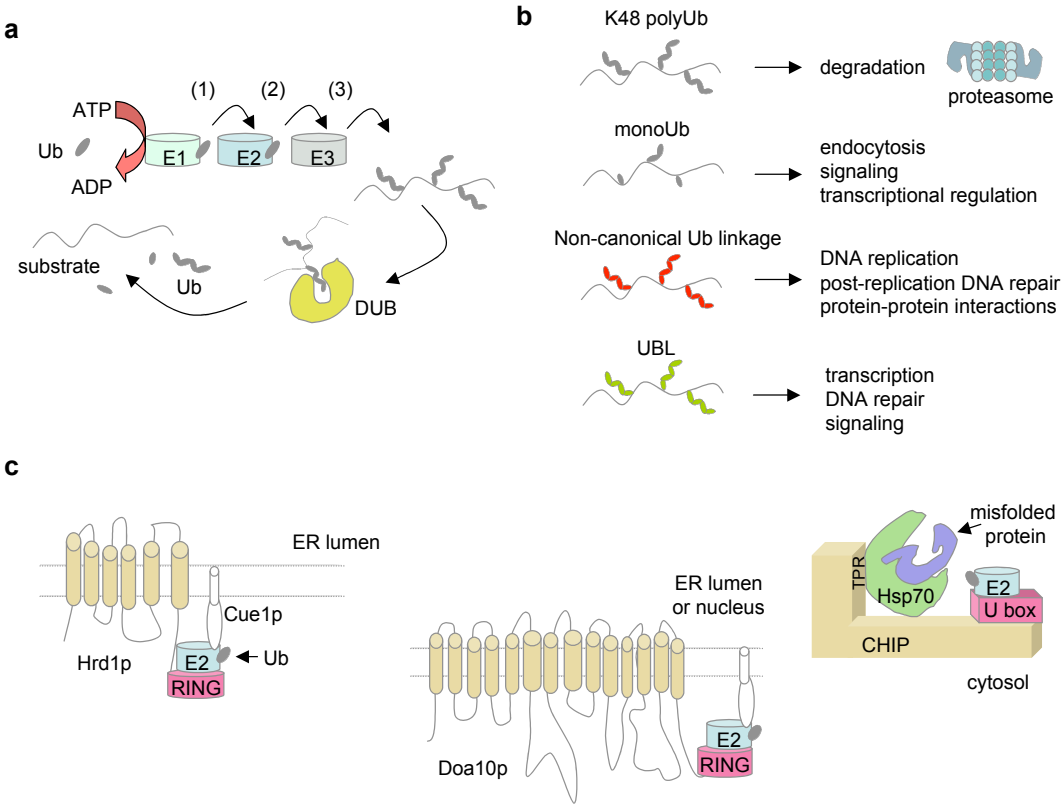

Figure 5. The ubiquitin–proteasome system. **a.** Simplified view of the ubiquitin (Ub) conjugation cascade: (1) Ub is activated by the E1 Ub-activating enzyme; (2) activated Ub forms a thiol ester with an E2 Ub-conjugating (UBC) enzyme; (c) activated Ub is transferred from the E2 to the substrate (or ubiquitin) lysine residue by an E3 Ub-ligase enzyme, in the case of RING domain E3s; for HECT E3s, the final step involves an E3-ubiquitin thiol ester. Deubiquitination - by deubiquitinating enzymes (DUBs) - can revert Ub conjugation and substrate fate. **b.** The type and length of the Ub chain as well as attachment of Ub-like modifiers (UBL) may influence the outcome of Ub conjugation. **c.** E3s play crucial roles in substrate selection and are regulated by localization, oligomerization, associated E2s, post-translational modifications and degradation. Hrd1p and Doa10p are yeast E3 ligases that are multi-spanning membrane proteins of the ER and, in the case of Doa10p, nuclear envelope. The C-terminus of Heat Shock Protein-Interacting Chaperone (CHIP) E3 ligase is cytosolic.

E3 ligases can be divided into two broad classes: the HECT (homologous to E6-AP carboxyl terminus)-domain ligases or the RING (really interesting new gene)-like domain ligases. In HECT E3s, ubiquitin is transferred from the E2 to a conserved cysteine residue in the HECT domain, followed by attack of this thioester by a lysine on the substrate [144]. The RING-CH domain is a ring finger motif with a cysteine residue in the fourth zinc-coordinating

position and a histidine residue in the fifth. RING-type E3s are more abundant and do not form an obligatory thioester intermediate with ubiquitin; rather they bring the Ub-loaded E2s and the substrate into proximity, thus facilitating the Ub transfer from the E2 to the substrate [144]. The functions of E3 ligases are also tightly regulated by means of signal-induced mechanisms such as localization, oligomerization, degradation and post-translational modifications, making E3s master orchestrators of specificity in the Ub conjugation cascade (**Fig.5.c**). This multi-step mechanism, much like phosphorylation, endows protein ubiquitination with a high degree of specificity and flexibility, which is paramount to its important biological functions [144,156-159].

The proteasome

The 26S proteasome, very abundant in the cytosol, is a multisubunit protease composed of the 20S and 19S proteasome complexes. The 20S proteasome (or central core particle) has the general architecture of a barrel, formed by 4 stacked rings of 7 subunits each, the outer two rings being composed of alpha subunits and the innermost two rings of beta subunits [160]. The beta subunits, which line the proteasome inner cavity, carry out the catalytic activity. For mammalian proteasomes, only three of the 7 beta subunits in each ring are catalytically active. Access to this cavity occurs through narrow pores (with a diameter on the order of 10-15 Å) at both ends of the barrel, so it is usually assumed that protein substrates must be unfolded prior to their delivery to the catalytic chamber [160-162]. Also at both ends of the core particle there is the 19S cap complex, whose functions range from recognition of poly-Ub chains on target proteins, to unfolding of the substrate to facilitate entry into the catalytic cavity and to deubiquitination activity [163-167]. Owing to the extreme topological diversity of ER proteins that must be examined by the ER quality control machinery, accurate substrate selection is of crucial importance. We shall now examine some of the common mechanistic themes in ERAD substrate selection.

Mechanisms of ERAD substrate recognition

Signature ERAD signals. The signature signals that allow factors that act in ER quality control to recognize and target ERAD substrates are presumably protein substructures such as hydrophobic patches, unpaired cysteine residues, and immature glycans. In the case of non-glycosylated substrates, Kar2p/BiP and other chaperones associate with hydrophobic patches exposed on the surface of misfolded proteins. ER-resident oxidoreductases like the PDI family members bind free thiols and catalyze disulfide bond formation and isomerization. Oxidoreductases may also play a role in ER quality control, for instance, by unfolding certain substrates prior to degradation [168,169] or by aiding in transfer of proteins with improper disulfide bonds to their cognate ERAD pathway [170,171]. For glycoprotein quality control, the calnexin/calreticulin cycle is of crucial importance.

The calnexin/calreticulin cycle. Immature glycoproteins are retained in the ER until productive folding takes place by the calnexin/calreticulin (CNX/CRT) lectin-type chaperones [140,172,173]. Terminally misfolded proteins, that is, proteins that after extensive CXN/CRT cycle folding attempts still fail to acquire their native conformation, must be removed from the CXN/CRT cycle, a step that is regulated by progressive trimming of sugar chains. *N*-linked glycoproteins are expressed in the ER and covalently co-translationally modified with pre-assembled core glycans ($Glucose_3$-$Mannose_9$-*N*-$Acetylglucosamine_2$), which are transferred by the oligosaccharide transferase (OST) complex from the intermediate lipid dolichol to an asparagine residue in Asparagine-X-Serine/Threonine motifs on the target protein. Cleavages and re-additions of the innermost glucose residue, by glucosidases I and II and UDP-Glucose:glycoprotein glucosyltransferase (UGGT), respectively, to the terminal mannose A residue prolong folding attempts in the CXN/CRT cycle. As a generally accepted concept, persistent ER retention leads to mannose trimming by the slow-acting ER mannosidase I [174], with the latter's slow reaction kinetics functioning as a timer for glycoprotein ERAD. Mannose trimming, either because glycans trimmed to $mannose_8$ (or lower) structures are sub-optimal glucosidase II and UGGT substrates or weaker ligands for CXN and CRT, acts as a symptom of aborted folding and facilitates extraction from the CXN/CRT cycle [175,176]. ER-resident mannose-binding lectins such as Htm1p/EDEMs and Yos9p/OS9 can also recognize misfolded glycoproteins and effectively decode the Man_8 degradation tag to interrupt futile CXN/CRT-chaperoned folding attempts of mannose-trimmed non-native glycoproteins [177-188].

N-linked glycans. *N*-linked glycans can serve as degradation tags not only in the CXN/CRT cycle but also after release of ERAD substrates into the cytosol [189,190]. The F-box sugar motif-containing proteins Fbs1 and Fbs2 bind high mannose *N*-linked glycoproteins [191]. When complexed with the Fbs1/Fbs2 substrate adaptor(s), the cytosolic SCF^{Fbs1} or SCF^{Fbs2} E3 ligases are rendered specific for glycoproteins that have been dislocated from the ER [192,193]. Interestingly, the C-terminus of Hsp70-interacting protein (CHIP) U-box E3 ligase, which usually serves as a co-chaperone for the heat shock protein Hsp70/Hsp90 chaperones, can be "manipulated" to function in ER glycoprotein turnover. The CHIP N-terminal tetratricopeptide repeat (TPR) motif traditionally mediates interactions with Hsps loaded with misfolded proteins, resulting in ubiquitination of misfolded proteins (**Fig.5.c**); however, its TPR motif can also bind to Fbs2 [190], resulting in ubiquitination of misfolded glycoproteins that have been dislocated.

Sequential checkpoints. Recognition of defective ER lumenal proteins may rely on lumenal quality control (QC) systems like the lumenal Hsp70 family chaperone Kar2p (BiP in mammals). For recognition of transmembrane ERAD substrates, a more elaborate QC setup with sequential quality-control checkpoints may be required: for example, the Hsp70/Hsp90 cytosolic chaperone complexes may first monitor the cytosolic domain of the transmembrane protein and, if the latter is deemed defective, facilitate ubiquitination of Hsp70/Hsp90-bound substrates by association with the CHIP E3 ligase. If, on the other hand, the cytoplasmic domain is inspected and deemed properly folded (or assembled) by the cytosolic checkpoint, the lumenal domain is then subjected to inspection by the lumenal checkpoint Kar2p/BiP system and delivered for dislocation from the ER in case of aberrant folding or assembly [131,194-196]. Sequential checkpoints are, for example, involved in triage of CFTRΔF508, a Cl^- ion channel with a single aminoacid deletion, the misfolding and premature degradation of which causes cystic fibrosis. These sequential checkpoints monitor the conformation of different regions of the nascent CFTR molecule, can independently target CFTRΔF508 for ERAD, and are staffed by distinct ER-membrane inserted or cytosolic E3 ligases [25,177,195,197-199].

ER-to-Golgi transport. Another possibility, at least in yeast, is that misfolded proteins escape to the Golgi where they become endowed with a signal for destruction (the identity of which remains elusive) and are then recycled to the ER [200,201]. This ER-Golgi shuttling model was proposed because mutations in several secretory pathway genes (like Ufe1p, Sec23p and Erv29p) compromise degradation of ERAD substrates [200,201], invoking a functional

secretory pathway for efficient degradation of misfolded proteins from the ER. Since the Ufe1p and Sec23p have since been shown to be required for maintenance of proper ER structure [202], the effects on protein degradation may also simply be pleiotropic consequences of perturbing the normal arquitecture of the ER [131,173].

ERAD E3 ligases. ERAD substrate selection can be performed by ERAD E3s, most of which are at the ER membrane - as is the case for the yeast Hrd1p/Der3p E3 complex [14] - or can be directed to ERAD – as is the case for the ER/nuclear envelope membrane Doa10p in yeast or the cytosolic CHIP complex in mammalian cells [131,132,142,203] (**Fig.5.c**). Yeast Hrd1p/Der3p is likely the best characterized example of an ERAD E3. It was first discovered in a genetic screen for genes involved in HMGR degradation (Hrd) [204]. Hrd1p (or Der3p) is an ER-resident RING-finger Ub ligase with 6 predicted transmembrane domains and the C-terminal RING-motif facing the cytosol. Hrd1p forms a stable complex with Hrd3p, a single-spanning ER membrane protein with a large ER luminal domain. Hrd3p can bind to ER chaperones loaded with misfolded proteins via TPRs in its lumenal domain, regulate Hrd1p ubiquitination activity, and recruit the Cdc48p(p97)/Npl4p/Ufd1p complex, which binds polyUb-conjugated proteins and delivers them to the proteasome. Hrd3p thus functions as a linchpin between substrate recognition, ubiquitination and delivery to the proteasome for degradation [205]. The Hrd1p/Hrd3p complex associates with the Ubc7p/Cue1p E2 dimer (the soluble Ubc7p protein becomes active only when tethered to the ER membrane by its membrane-anchoring co-factor Cue1p), and can recruit other components, such as Der1p, for degradation of some substrates [205]. Degradation from the ER 1 protein (Der1p) was initially discovered by Wolf and collaborators in a screen for yeast proteins involved in degradation of CPY*, the misfolded variant of the vacuolar soluble carboxypeptidase yscY or CPY [206]. Der1p and its mammalian orthologs, the Der-like proteins DERLIN-1, -2, and –3, span the ER membrane 4 times and are predicted to function as substrate adaptors for specific subsets of ERAD substrates, as membrane receptors for the Cdc48p(p97)/Npl4/Ufd1 complex, or even as a channel for ejection of degradation substrates from the ER membrane [21,22,26,205] (**Fig.6**).

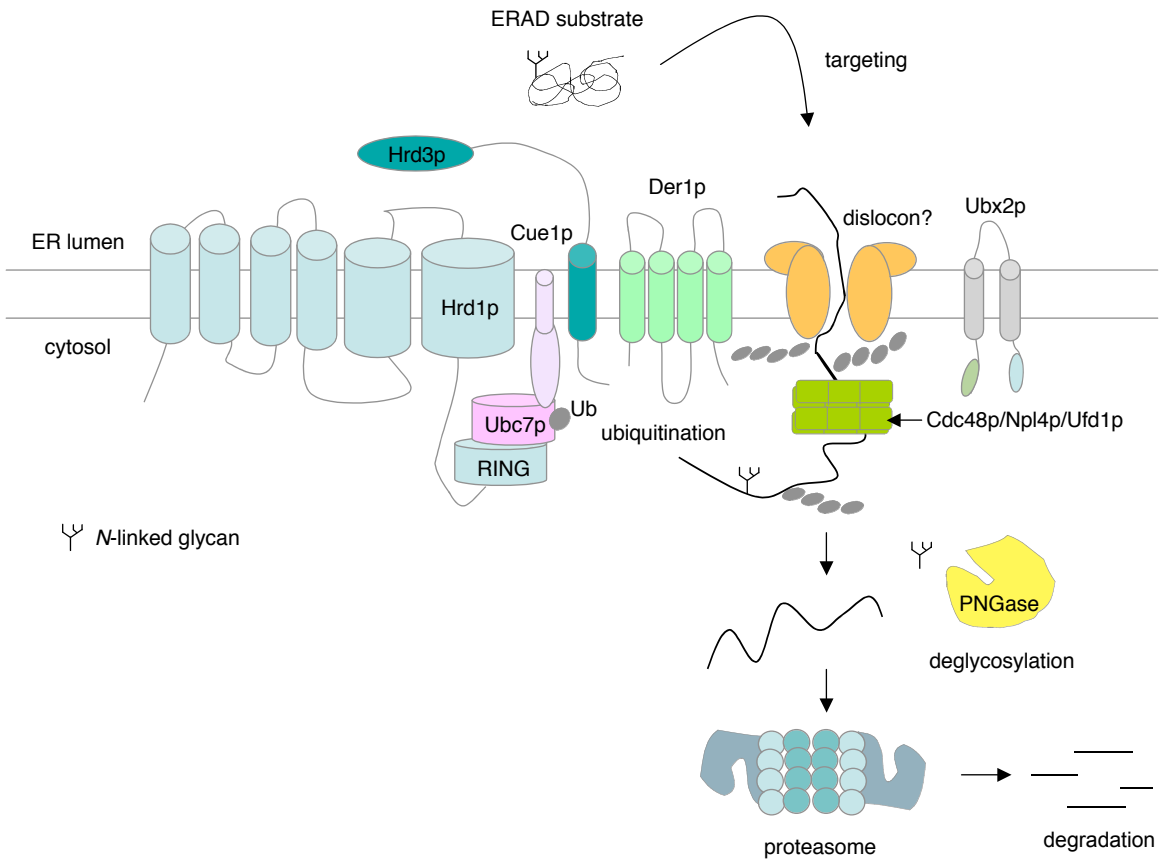

Figure 6. ER quality control and associated degradation (ERAD) is a multi-step process that can be coordinated by ERAD E3s. ER quality control includes substrate selection and targeting for dislocation, export through the ER membrane (dislocation), ubiquitination and, in the case of glycoproteins, deglycosylation, as well as delivery of Ub conjugates to the proteasome by the Cdc48p(p97)/Npl4/Ufd1 complex and/or other Ub escorting (shuttling) factors. In yeast, the Hrd1p/Der3p-Hrd3p E3 ligase participates in multiple steps of ER, from substrate selection in the ER lumen or membrane to ubiquitination in the cytosol. This organization scheme is likely to be fairly conserved in mammalian cells.

Another E3 ligase that participates in yeast ERAD is degradation of transcription factor Matalpha2–10 protein (Doa10p) [207], a multi-spanning transmembrane protein of the ER/nuclear envelope [208]. Both E3s can cooperate with distinct E2s to ubiquitinate distinct subsets of degradation substrates [14,207,209-211]. The Hrd1p and Doa10p E3 ligases, in fact, function in distinct ERAD pathways [212,213]. There are three proposed ERAD pathways, defined by distinct protein complexes assembled around particular E3 ligases, that handle degradation substrates based on the site of the misfolded lesion: membrane and soluble proteins with lesions in their lumenal domains are targeted to the ERAD-L pathway, membrane and soluble proteins with lesions in their cytoplasmic domains are targeted to the ERAD-C pathway and proteins with misfolded transmembrane domains are targeted to the

newly described ERAD-M pathway [212,213]. The ERAD-L, -C, and –M pathways assemble distinct multi-protein complexes that enable substrate selection in the ER lumen or membrane and delivery to downstream components such as a putative dislocon and downstream factors like the Cdc48p(p97)/Npl4/Ufd1 complex, ubiquitin shuttling factors and the proteasome [212,213].

The mammalian Hrd3p homolog SEL1L associates with HRD1 and the HRD1/SEL1L E3 complex seems to be organized similarly to its yeast homolog [22,214,215]. The HRD1/SEL1L complex is capable of recruiting other ER membrane and ER membrane-associated proteins like Cdc48p(p97)/NPL4/UFD1 and DERLINs, forming multisubunit complexes that coordinate dislocation steps from substrate selection to delivery to the proteasome, for at least a subset of ER degradation substrates [22,214,215]. The same seems to apply to a second predicted homolog of Hrd1p/Der3p, the gp78 E3 ligase [216]. gp78, identified as the tumor autocrine motility factor receptor (AMFR) [217] and later as an E3 ligase [218,219] has also been shown to be able to recruit and organize multiple ERAD factors [220-222].

The elusive dislocon

Export of proteins through the ER membrane most likely takes place via an aqueous channel that allows the passage of polypeptides through the highly hydrophobic ER membrane environment, while maintaining proper ionic balance between the ER and the cytoplasm. The Sec61 channel, the very same channel responsible for protein import into the ER, was initially thought to mediate transport in the reverse direction [12,223-225], with accessory factors regulating directionality and specificity of the channel. The extent to which Sec61 is involved in ER dislocation or the identity of the “dislocon” are not without controversy, and the search for a dislocation channel(s) is a subject of intense research [131,132].

Mammalian DERLIN-1 is involved in dislocation from the ER [21,26] and was proposed to constitute a channel for protein export from the ER membrane to the cytosol [26], as we mentioned above. The tetra-spanning ER membrane DERLINs -1, -2, and -3 can homo- and heteroligomerize, and could presumably form higher order structures with channel-like properties [21,26]. Conclusive evidence for a role of DERLIN-1 as a channel is still unavailable, and in any case, DERLIN-1 is unlikely to be the only channel, as turnover of some ERAD substrates does not rely on DERLIN-1 function [21,208]. DERLINs -2 and –3 are obvious

candidates that could function in place of DERLIN-1. Alternatively, Der1p/DERLINs may act to deliver a particular substrate to a channel/another adaptor in its cognate dislocation pathway, for instance through association with Hrd1p(Der3p)/Hrd3p (in yeast) or HRD1/(SEL1L or gp78) multiprotein complexes [22,215], which could integrate formation of a channel or dislocon, offering obvious advantages in terms of control of both specificity and directionality of the dislocation process.

The Cdc48p(p97)/NPL4/UFD1 complex

Yeast Cdc48p or mammalian p97 (also termed valosin-containing protein or VCP) is an essential protein of the AAA ATPase (ATPases associated with various cellular activities) family, conserved from archaea to mammals, whose functions include mitotic spindle disassembly, membrane traffic and fusion, nucleic acid repair and replication, and Ub-proteasome degradation [226]. Cdc48p/p97 is a motor protein that generates energy from ATP binding and hydrolysis; it forms a homohexameric barrel structure, with each subunit containing two AAA domains that contain the Walker motifs essential for ATPase activity; the 6 subunits are arranged in a ring with a pore in the center [227]. Cdc48p/p97 interacts with different adaptor proteins, which regulate its function [30]. Cdc48p/p97 itself can recognize denatured proteins non-specifically [228] and has an affinity for polyubiquitin chains [229]. When in complex with the nuclear protein localization 4 (Npl4p) and Ub fusion degradation 1 (Ufd1p) co-factors, both of which possess polyUb-binding domains, Cdc48p/p97 activity is directed to ER degradation [31,32,131].

In yeast and in mammals alike, the trimeric Cdc48p(p97)/NPL4/UFD1 complex is proposed to function in an essential post-ubiquitination, pre-proteasomal proteolysis step [230,231], in one of two ways: the ATP-hydrolytic activity of the AAA ATPase p97 may provide the driving force for dislocation, that is, to extract substrates through the ER membrane, or may simply be required to liberate already dislocated substrates from the cytosolic face of the ER membrane [132,142,171,209,232]. More recent evidence suggests that Cdc48p/p97 may be dispensable for extraction of (at least some) soluble substrates and rather function as a non-essential but important auxiliary factor that facilitates extraction of thermodynamically stable (transmembrane) domains or proteins from the ER membrane [198,233].

Driving dislocation and the long road to the proteasome

The 26S proteasome also possesses a AAA ATPase at the base of the 19S proteasome regulatory complex [234,235] which could provide the driving force for dislocation. In support of this suggestion, for some substrates such as tyrosinase [236], ribophorin 332 [237], TCRalpha [138] and CD3δ [238,239], dislocation and proteasomal degradation are tightly coupled, such that ER membrane extraction does not take place when proteasome activity is compromised. Furthermore, the proteasome interacts with the ER membrane [240], perhaps directly or through an ER-membrane docking receptor [241]. Indeed, both Cdc48p/p97 and the 19S regulatory complex AAA ATPases present the necessary attributes to provide the ATPase activity that drives export of substrates from the ER membrane, as they both associate with the ER membrane and the 20S proteasome, bind to polyubiquitinated substrates, exhibit unfolding/segregating activity, and facilitate degradation of ERAD substrates [230,242-245].

PolyUb-binding escort factors

ERAD substrates may be escorted or "guided" from a dislocation channel to the proteasome by a cascade of Ub-binding factors [188,244,246-251]. Cytosolic polyUb-binding factors like Rad23p (HR23B in mammals) and Dsk2p (hPLIC-1 and -2 in mammals) bind multiubiquitin chains through their Ub-associated (UBA) motif and bind the 19S proteasome through their ubiquitin-like (UBL) motif, and enhance degradation of model ERAD substrates [252-257]. This has led to the suggestion that they act as shuttles that escort ubiquitinated proteins to the proteasome, perhaps protecting Ub chains from DUB activity thus maintaining the substrate in a proteolysis-competent state [144]. They may also provide directionality and even dictate the pathway used for proteasomal targeting [132,244,248,258].

The polyUb-binding protein Cdc48p/p97 itself can influence substrate fate by simultaneously binding Ufd2p and one of two factors that can counteract its action: Otu1p, a deubiquitinating enzyme which can disassemble the polyUb chains, and Ufd3p protein, a protein of unknown function [259,260], which competes with Ufd2p for the same docking site on Cdc48p/p97 [261]. Selective recruitment of Ub-processing factors, which occurs also with mammalian p97 [262], could allow p97 to control the size of the polyUb chain avoiding formation of excessive polyUb chain sizes and, more importantly, tip the balance towards substrate degradation or release from the degradation cascade [244,261].

Deglycosylation

Peptide *N*-glycanase 1 (PNGase1) is a soluble cytosolic deglycosylating enzyme encoded by the *PNG1* gene that is expressed, albeit at low levels, in all eukaryotes, and that removes asparagine (*N*)-linked glycan chains from misfolded substrates prior to proteasomal degradation [263,264]. Deglycosylation is not essential for proteasomal degradation, as genetic ablation of *PNG1* [263] or chemical inhibition of PNGase by the Z-VAD-fmk inhibitor [265] cause no general degradation defects or simply a decreased degradation rate depending on the substrate, suggesting that PNGase merely facilitates more efficient proteasomal proteolysis of *N*-linked glycoproteins through glycan removal. Both in yeast and mammals, however, there is generally a physical association between PNGase and the proteasome [266] that suggests a tightly knit relationship between the two. It is possible that misfolded protein substrates may first be deglycosylated by ER-associated or free PNGase, then identified by Ub shuttling factors, and subsequently targeted to the nearby proteasome [266,267]. Mammalian PNGase has also been found to associate with ER membrane E3 ligases, such as gp78, the Cdc48p(p97)/NPL4/UFD1 complex and cytosolic Ub shuttling factors [268]. In this scenario, this complex, perhaps through the E3 ligase, would be able to couple the activities of dislocation, ubiquitination and deglycosylation, as well as escort of misfolded glycoproteins to the proteasome [222,267,268].

US2- and US11-mediated dislocation: mechanistic details

US11 structure/function

US11 is a type I membrane glycoprotein of 215 aminoacids with a single *N*-linked glycan and a molecular mass of 30 kDa [13]. Notwithstanding the lack of an ER-retention motif, US11 is an ER-resident protein. The US11 signal sequence directs the protein to the ER membrane despite its unusual delayed cleavage [269,270]. The lumenal domain of US11, predicted to constitute an immunoglobulin-like fold similar to that of US2 - for which structural information is available [271] - is responsible for interaction with HCs. Analysis of the structural requirements for US11 function reveals that the US11 transmembrane domain (TMD) is critical for US11 function (**Fig.7**).

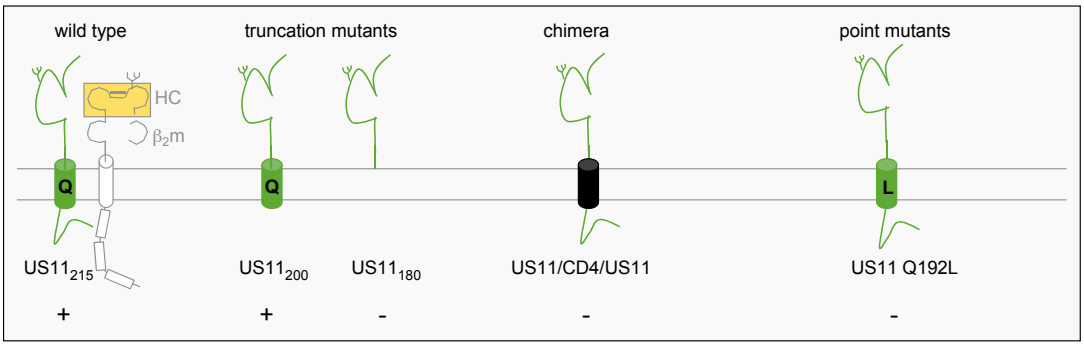

Figure 7. US11 function is dependent on its transmembrane domain. Schematic representation of US11 mutants used to assess the contribution of different domains or aminoacid residues of the viral protein to its function in HC dislocation. All mutants shown bind to HC within the alpha1/alpha2 region of the HC molecule (yellow box). Those that catalyze HC dislocation and degradation are identified by a (+) signal and those shown to be dislocation-incompetent are identified by a (-) signal.

The US11 cytosolic tail is dispensable for US11 function, as a cytosolic tail truncation mutant, $US11_{200}$, can still induce dislocation of HCs. In contrast, US11 TMD truncation and substitution results in US11 mutants, $US11_{180}$ and US11/CD4/US11, respectively, incapable of catalyzing dislocation, although maintaining the ability to bind HCs [270]. Furthermore, mutational analysis of the US11 TMD revealed that US11 function is critically dependent on the glutamine (Q) residue at position 192 within the US11 TMD. Mutation of the polar glutamine to a hydrophobic leucine (L) abolishes US11 function: although it is able to bind HCs, the US11 Q192L mutant is inactive in dislocation [21]. In fact, all non-functional US11

mutants - $US11_{180}$, US11/CD4/US11 and US11 Q192L - form a stable complex with HC which is retained in the ER through a poorly characterized mechanism [85]. The polar glutamine residue in the US11 TMD was initially predicted to form inter-helical hydrogen bonds within the lipid bilayer to allow a contact with a component of the dislocation machinery [270]. A functional screen for proteins that interact specifically with the active version of US11 and not with US11 Q192L then led to the identification of DERLIN-1 as a cellular factor required for HC dislocation mediated by US11 [21].

Substrate selection by US11

US11 is predicted to bind the class I MHC molecule within the peptide-binding alpha 1 and alpha 2 domains of HC [116] (**Fig.7,** yellow box). US11 is somewhat promiscuous in terms of substrate selection, as it can target a broad range of HLA locus products and even some murine class I MHC molecules [116,118]. Moreover, US11 is able to associate with HCs independently of their synthesis stage or the stage of assembly with $beta_2m$ [27,272].

Besides ER-lumenal regions, a strong determinant for class I MHC dislocation by US11 is the presence and length of the cytosolic (C-terminal) tail of the HC protein [117]. US11 dislocates only HCs that possess a cytosolic domain of approximately 30 aminoacids, albeit independently of its sequence: tailless HLA-A2 and even HLA-A2 Δ2, a mutant lacking only the last two aminoacids of its cytosolic tail, are not degraded by US11 [116]. HLA-E, for example, becomes sensitive to US11-mediated downregulation when its cytoplasmic tail of 29 aminoacids is extended by only 2 additional residues [117]. Not surprisingly, truncation of the C-terminal 10 residues of the cytosolic tail renders the HLA-A2 Δ10 mutant refractory to US11-mediated dislocation. However, substitution of the HLA-A2 C-terminal 10 aminoacids by the 10 aminoacid-long HA tag (HLA-A2 Δ10HA mutant) restores sensitivity to dislocation by US11 (Margo H. Furman, Mayra E. Lorenzo and Hidde L. Ploegh, unpublished observations). The fact that US11 substrate targeting involves measurement of HC tail length has led to the suggestion of a cytosolic “pocket”-shaped adaptor for the HC tail, but its identity is so far unknown.

Because a functional Ub-proteasome system and substrate ubiquitination are usually required for dislocation, we and others have analysed these requirements for US11. Although a functional ubiquitin system is required for HC dislocation by US11 [273,274], ubiquitination of

the substrate itself may not need to occur. Class I MHC HC molecules are ubiquitinated when they gain access to the cytosol, by a so far unknown E3 ligase. Most of the class I MHC HC molecules are soluble, while a small portion is membrane-associated [275]. Upon proteasomal inhibition, most cytosolic HCs lack any traces of Ub modification, perhaps due to the action of so far unknown DUB activity. When Ub conjugation is prevented by depletion of Ub in semi-permeabilized US11 cells or by incubation at the non-permissive temperature of a cell line with a temperature-sensitive E1 enzyme, class I MHC HC molecules are not extracted from the ER membrane and their ER lumenal domains are resistant to proteinase K [32,273,275], suggesting that ubiquitination is necessary for dislocation of HCs from the ER membrane. When all lysines in HLA-A2 were replaced and ubiquitination at its N-terminus was prevented, the HC molecule could still be dislocated by US11, albeit somewhat less efficiently [276], suggesting that ubiquitination of the HC itself need not take place. The overall requirement for ubiquitination, however, suggests that ubiquitin conjugation to other components is involved in the dislocation reaction.

US2 structure/function

US2 is a type I membrane glycoprotein of 199 amino acids with a single *N*-linked glycan and a molecular mass of 22kDa [12]. Despite lacking any obvious ER-retention motif, US2 is an ER-resident protein. The unusual non-cleavable signal sequence of US2 directs the protein to the ER, albeit inefficiently, and can yield ER insertion (also without signal peptide cleavage) upon transfer to other type I membrane proteins [277]. Despite a reduced sequence identity (12%) with molecules containing a similar tertiary structure, the US2 lumenal domain acquires an Ig-like fold of seven beta-strands (spanning residues 43 to 137), which mediates association with the lumenal domain of HC at the junction between the peptide-binding cleft and the alpha 3 domain [20]. Most of the polymorphic residues in the class I MHC HC are concentrated in the alpha 1 and alpha 2 domain regions that contact the antigenic peptide or the T cell receptor (TCR), and US2 does not compete with the TCR for association with HC (neither does US11) [20]. The rest of the US2 protein is composed of a transmembrane segment and a short cytosolic tail of 14 aminoacids (residues 186 to 199) (**Fig.8**).

a

MNNLWKAWVGLWTSMGPLIRLPDGITKAGEDALRPWKSTAKHPWFQIEDN 50

RCYIDNGKLFARGSIVGN$_{68}$MSRFVFDPKADYGGVGENLYVHADDVEFVPGE 100

SLKWNVRNLDVMPIFETLALRLVLQGDVIWLRCVPELRVDYTSSAYMWNM 150

QYGMVRKSYTH**VAWTIVFYSINITLLVLFIVYVTV***D*$_{186}$*CNLSMMWMRFFVC*$_{199}$

b

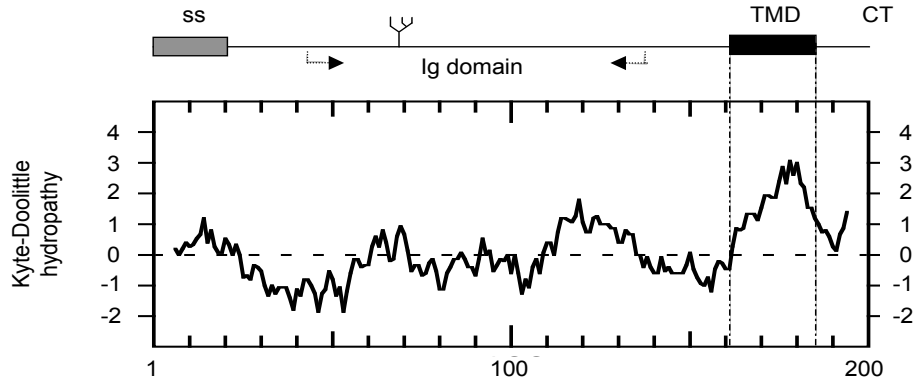

Figure 8. The US2 protein. **a.** Aminoacid sequence of the US2 type I membrane glycoprotein. The non-cleavable signal sequence is in subscript type, the asparagine 68 (N_{68}) *N*-linked glycosylation sequon is underlined, the transmembrane domain is in bold type and the cytosolic tail residues (residues 186 to 199) are italicized. Residues in red indicate tail aminoacids that abrogate US2 function when mutated. The sequence in between the two arrows corresponds to the residues within the US2 lumenal domain that form its immunoglobulin (Ig)-like domain with the two cysteines that form the disulphide bond within the Ig fold highlighted in blue. The aminoacids indicated by an asterisk (*) represent US2 residues that contact HLA-A2. **b.** US2 Kyte-Doolittle hydropathy plot. The US2 signal sequence (ss), lumenal domain, transmembrane domain (TMD) and cytosolic tail (CT) are indicated.

Unlike US11, US2 is an unstable protein: a significant portion of newly synthesized US2 constitutively fails to achieve insertion into the ER membrane presumably due to low efficiency of its signal sequence, therefore remaining non-glycosylated. This 20kDa non-glycosylated cytosolic US2 is very rapidly degraded by cytosolic proteasomes [12,277]. Only the 22kDa fraction of US2 that effectively becomes ER membrane-inserted and *N*-linked glycosylated catalyzes HC dislocation. Like HC, ER-inserted US2 is subjected to dislocation and degradation, albeit with distinct kinetics: US2 has a half-life of about 1 hr [12,277].

While association between US2 and class I MHC molecules is a pre-requisite for dislocation, the mere interaction between them is not sufficient. Analysis of the structural requirements for US2 function reveals that the US2 tail is critical for US2 function (**Fig.9**).

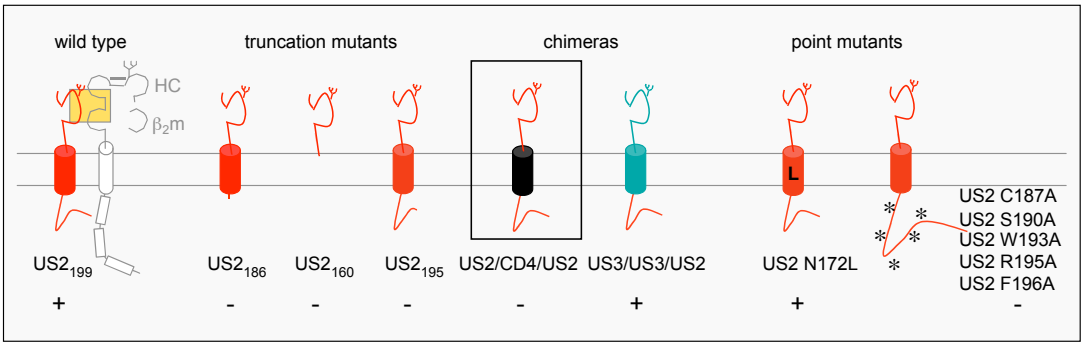

Figure 9. US2 function is dependent on its cytosolic tail (and transmembrane domain as reported in the results section). Schematic representation of US2 mutants used to assess the contribution of different US2 domains or aminoacid residues to its function in HC dislocation. All mutants shown bind to HC in the boundaries of the alpha2/alpha3 regions of the HC molecule (yellow box). Those that catalyze HC dislocation and degradation are identified by a (+) signal and those shown to be dislocation-incompetent are identified by a (-) signal.

While the US2 residues amino-terminal to the Ig-like fold can be replaced by an exogenous signal sequence without affecting US2 function [277], truncated US2 lacking its short cytosolic tail of only 14 aminoacids, $US2_{186}$, is incapable of catalyzing dislocation, albeit capable of binding to HC molecules [278]. Just recently, US2 function was shown to be abolished by either truncation of just 4 tail aminoacids or introduction of point mutations within the US2 tail ($US2_{195}$, and US2 C187A, S190A, W193A, R195A and F196A mutants), with no prejudice to HC binding [279]. Addition of the US2 tail sequence to the US3 immunoevasin, which has a similar type I membrane glycoprotein and Ig-like fold organization, presumably renders the US3lumen/US3TMD/US2tail (US3/US3/US2) chimeric protein able to catalyze HC degradation [280]. The cytosolic tail of US2 is, therefore, critical for US2 function in dislocation.

The US2 cytosolic tail mediates dislocation only when present in *cis* configuration, as chimeric molecules generated by swapping the cytosolic domain of US2 and HC with each other (US2/US2/HC and HC/HC/US2) or by swapping of the lumenal domains (US2/HC/HC and HC/US2/US2) abolish the dislocation reaction, despite stable association of the molecules *in vivo* [281]. In contrast with inactive mutants of US11, which cause HC retention in the ER, US2 mutants that do not dislocate HCs stall HC only briefly in the ER, despite a very stable interaction between US2 and class I MHC complexes [27,271], after which exit from the

ER and progression through the secretory pathway takes place. The reasons for this apparently more dynamic interaction of US2 with HC are so far unknown. Mutation of the polar asparagine (N) residue at position 172 within the US2 TMD (**Fig.8**) does not compromise US2 function (**Fig.9**): the US2 N172L mutant, unlike its US11 Q192L counterpart, maintains the ability to dislocate HC molecules [85], further illustrating the differences between the modes of action of the US2 and US11 immunoevasins.

Substrate selection by US2

The US2 ER-lumenal domain allows US2 to distinguish between class I MHC locus products, dictating a highly specific association. Allele specificity is largely determined by a small region of the alpha 2-alpha 3 junction of the HC (**Fig.9**, yellow box). The HLA-A2 residues that contact US2 (**Fig.2.b,** asterisks) are present in only certain groups of HLA locus products, notably HLA-A, certain HLA-B products and HLA-G [20,119]. This allele-specific association results in US2-mediated destabilization exclusively of class I MHC heavy chains that possess the consensus US2 contact residues such as most of HLA-A and –B alleles [117,119,282]. Alleles that do not conform to the consensus US2 binding site, such as HLA-B7 and HLA-Cw3, are spared from degradation [20,119,283,284]. Interestingly, the naturally resistant HLA-E molecules can be rendered susceptible to US2-mediated degradation by mutation of only 4 residues (LHLE to QRTD) to reconstitute the HLA-A2 consensus binding site for US2 (**Fig.2.b**) [117].

The class I MHC cytosolic tail may not be required for US2-mediated dislocation. Initial experiments from our lab performed in U373-MG human astrocytoma cells showed that over-expressed tailless HLA-A2 (corresponding to HLA-$A2_{312}$ or HLA Δ29), in contrast to over-expressed full-length HLA-A2, was not degraded [285]. The same was true for HLA-A2 Δ30 [286]. However, the HLA-A2 tail is not required for degradation by US2 in murine J26 cells, which, obviously, do not express human HCs [119]. This discrepancy can perhaps be explained by impaired degradation of tailless HLA-A2 due to an exceeding of the capacity of the US2 dislocation machinery with over-expressed HA-HLA-A2. Indeed, tailless HLA-A2 introduced by *in vitro* translation into microsomes from US2-expressing astrocytoma cells is degraded [287], presumably because microsomes have been depleted of endogenous class I MHC products due to US2 expression. Furthermore, US2 tolerates significant perturbations in the length and

sequence of the HC cytoplasmic tail, as it maintains the ability to dislocate class I MHC molecules C-terminally extended by fusion of GFP to the C-terminus [288].

In terms of assembly of the class I MHC complex, the US2 molecule forms a highly stable complex with the class I MHC trimeric complex of HC/beta$_2$m/peptide, as seen by analysis of the crystal structure of US2 bound to the class I MHC complex [271]. US2 binding does not alter the conformation of the folded class I MHC complex, and therefore its effect on class I MHC HC stability is not attributable to a direct disruption of the quaternary structure of the class I MHC complex. That US2 can bind the folded structure in such a stable fashion suggests that it may only recognize a folded form of class I MHC molecules. This is inferred also from the ready recovery of US2 in association with W6/32-reactive material from lysates of US2 cells [27]. The mouse monoclonal antibody W6/32 recognizes a monomorphic epitope on HLA heavy chains, irrespectively of the HLA allele but dependent on the presence of beta$_2$m [289,290]. W6/32-reactivity of HC molecules reactivity can be used to directly monitor assembly of the class I MHC complex [291]. More direct evidence comes from the fact that US2 is incapable of targeting HCs for degradation in astrocytoma cells with knockdown levels of the HC partner subunit beta$_2$m [292]. Unassembled class I MHC molecules produced in a beta$_2$m-defficient melanoma cell line have, however, been reported to be targeted by both US2 and US11 [272]. Experiments to directly test whether US2 requires a folded MHC class I complex for targeting to dislocation are described in the results chapter [27].

As for the ubiquitination requirements for US2, neither removal of the HC tail nor of the lysine residues within the HC cytosolic tail block dislocation by US2 [27,119]. However, a functional ubiquitin system [27,276] is required and mutation of all HLA-A2 lumenal lysines and blockage of N-terminal ubiquitin fusion abolishes dislocation by US2 [276]. This suggests that, unlike for US11, substrate ubiquitination is essential for US2-mediated dislocation and that HC ubiquitination must occur within the HC lumenal domain.

A summary of the substrate requirements for US2 and US11 is shown in **Figure 10**.

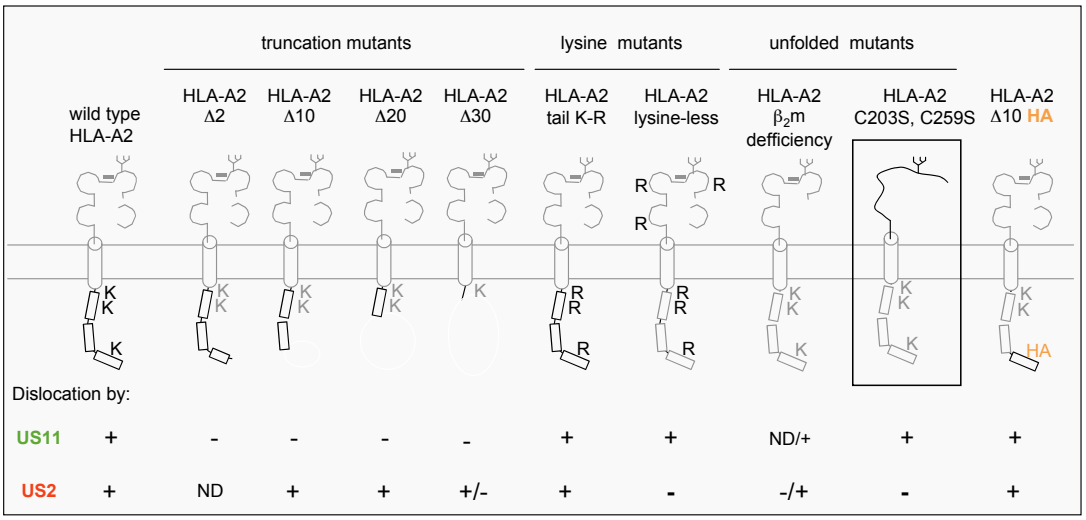

Figure 10. US2 and US11 have distinct substrate requirements. Schematic representation of the HLA-A2 mutants used to study US2 and US11 substrate preferences. The requirements analyzed included tail length and sequence, ubiquitination, and assembly and folding stage, for which the unfolded HLA-A2 C227S,C283S mutant (box) was used (described in the Results section). All mutants shown bind to US2 and US11. (+) and (-) indicate mutants that are or are not subject to dislocation by the viral protein; (ND), not determined. Mutants for which there are conflicting reports are identified by both (+) and (-). See text for details and references.

Triggering dislocation

The fact that HC ubiquitination in US2 cells occurs within the HC lumenal domain [276] raises the question of how ER-lumen located HC lysine residues (or the HC N-terminus) become accessible to the cytosolic ubiquitination machinery. Moreover, it suggests that while required for dislocation by US2, HC ubiquitination is not its trigger. US2 must, therefore, induce a “partial dislocation” or '”prolapse” of the lumenal domain of HC into the cytosol; as the HC lumenal domain begins to emerge in the cytosolic face of the ER, ubiquitination can take place [27,275]. This partial dislocation model may not be valid for US11, as the HC itself need not be a ubiquitin acceptor [276] and HC lumenal domains are not exposed to the cytoplasm when ubiquitination is blocked [274].

The instability and proteasomal degradation of US2 suggested that US2 bound to the folded class I MHC complex is dislocated from the ER membrane into the cytosol [12]. Export through the ER membrane of a folded structure such as the class I MHC complex, with bound US2, is a highly controversial idea, as it would require a channel of considerable size. However, HC molecules are exported from the ER membrane with their bulky glycan moieties

still attached [265,292], and HC molecules fused at their N-termini to folded domains such as enhanced green fluorescent protein (eGFP) and ligand-bound dihydrofolate reductase (DHFR) have been proposed to arrive in the cytoplasm after dislocation [293,294].

As an alternative to US2 accompanying the folded MHC complex out of the ER, one can invoke unfolding prior to or during export. Protein disulphide isomerase (PDI)-mediated unfolding of cholera toxin A subunit in the ER occurs prior to its dislocation [169] and reduction of disulphide bonds in the HC molecule occurs prior to deglycosylation [295], which does not preclude that unfolding could take place only shortly after release from the ER membrane. But whether there is a strict requirement for unfolding is not known and more experiments are necessary to address this issue.

The HCMV US2 and US11 immunoevasins subvert cellular dislocation pathways to degrade class I MHC heavy chains

Although both US2 and US11 catalyze destruction of the same substrate (HC) via a seemingly similar reaction (dislocation) with the same outcome, proteasomal degradation. The US2- and US11-mediated dislocation pathways differ substantially in terms of the mechanistic details of the dislocation reaction. US2 and US11 functional domains differ, with US11 relying mostly on TMD-mediated interactions and US2 relying mostly on its cytosolic tail (**Figs.7, 9**). The two immunoevasins display stringent and non-overlapping requirements in terms of which HLA alleles and assembly and ubiquitination status of the class I MHC HC either viral protein is able to target for dislocation and proteasomal destruction (**Fig.10**). This prompted us to analyze the influence of the class I MHC folding status on dislocation catalyzed by US2 and US11. Although constituting only indirect evidence, the distinct substrate requirements imposed by US2 and US11 argue strongly for differential engagement of dislocation factor(s) in the ER. The direct evidence came from the discovery of DERLIN-1 as a crucial cellular partner for US11, but not for US2 [21]. DERLIN-1 is a component of a large multiprotein complex at the ER membrane which is able to couple substrate selection (by means of US11), dislocation (through a dislocon or maybe even through the DERLIN-1 protein itself), ubiquitination (through the HRD1/SEL1L E3 complex), recruitment of the p97/NPL4/UFD1 complex, and proteasomal degradation of class I MHC HCs [22]. The fact that dislocation of the same substrate, class I MHC HC, by US2 is not dependent on DERLIN-1 [21] and that the

mammalian Hrd3p homolog, SEL1L, seems to be similarly involved in HC dislocation by US11 but not by US2 [214], again argues for the existence of very substantial mechanistic differences between US2 and US11 that must extend to recruitment of distinct ER dislocation machinery by US2, thus far completely unknown. This prompted us to investigate the ER dislocation machinery utilized by US2 and led us to the discovery of a new ER dislocation player.

Chapter 2. Results

Substrate requirements for HC dislocation: US2 *versus* US11

US2 and US11 target newly synthesized class I MHC heavy chains (HCs) for dislocation and proteasomal degradation by distinct strategies. Some indirect clues can be derived from the distinct functional domains of US2 and US11: US2 relies mostly on its cytosolic tail (**Fig.9**) and US11 relies mostly on its transmembrane domain (**Fig.7**). Moreover, the HC sequence and structure requirements for recognition and targeting for dislocation by US2 and US11 also differ: the US2 and US11 contact regions with HC are distinct (**Figs.7, 9**), and so are the requirements in terms of HC tail length and sequence, as well as the folding state and stage of assembly with beta$_2$m (**Fig.10**).

In order to assess the US2 and US11 requirements in terms of substrate conformation, we generated an unfolded class I MHC HC molecule by eliminating the disulfide bridge within its membrane-proximal immunoglobulin (Ig)-like alpha 3 domain (**Fig.11**).

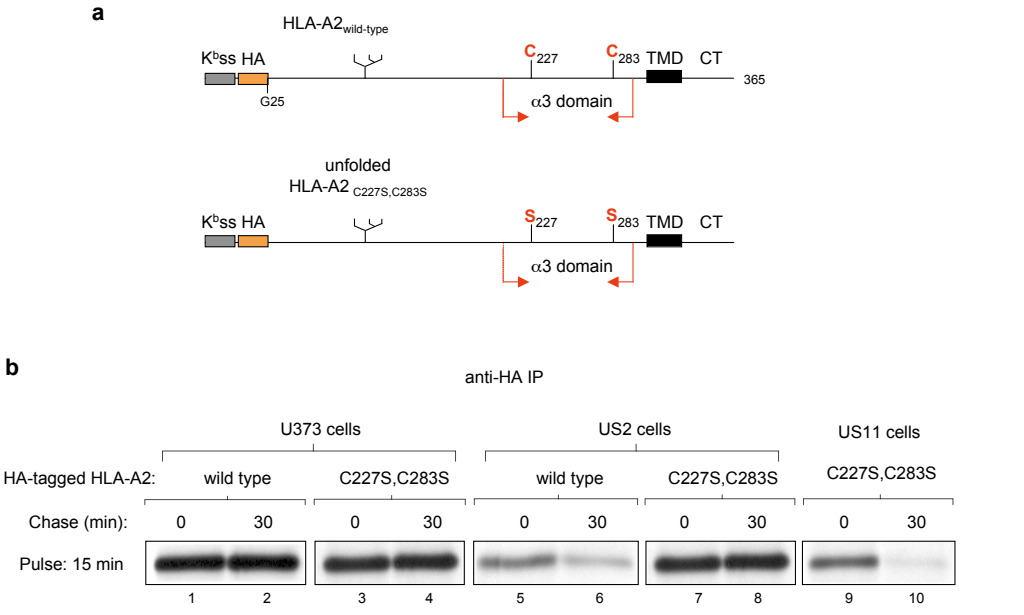

Figure 11. US2 targets only folded class I MHC molecules for degradation. **a.** Schematic representation of (folded) HA-HLA-A2$_{wild\ type}$ and (unfolded) HA-HLA-A2$_{C227S,C283S}$. The latter has the two cysteine (C) residues in the membrane-proximal alpha3 domain substituted by serine (S) residues. **b.** Partially folded class I MHC molecules are degraded by US11 but not by US2. Control U373 cells, US2 cells and US11 cells expressing HA-HLA-A2 $_{wild\ type}$ and HA-HLA-A2 $_{C227S,C283S}$ were metabolically labeled for 15 min and chased for up to 30 min. Lysates were prepared with 0.5% NP-40, HA-tagged HLA-A2 was recovered with 12CA5 anti-HA antibody, and the immunoprecipitates were analyzed by 12.5% SDS-PAGE, followed by fluorography.

As disulfide bridges help maintain the proper fold of class I MHC molecules, mutation of the cysteine residues that establish the disulphide bond within the alpha 3 domain should yield an incompletely folded class I MHC molecule – either completely unfolded or with a more flexible conformation (partially unfolded) than that of wild type HC. We replaced cysteine (C) residues 227 and 283 in HLA-A2 with serine (S) residues by means of site-directed mutagenesis (**Fig.11.a**). Note that we numbered the HC aminoacid residues from the initiator methionine (M) up to valine (V) 365, in which case cysteine residues 227 and 283 form the alpha 3 domain disulphide bond. When considering the HLA-A2 obtained after signal sequence cleavage, glycine (G) 25 is the first aminoacid and the alpha 3 cysteines are numbered 203 and 259 (**Fig.2.b**). We obtained astrocytoma (U373-MG) cells expressing K^bss-HA-tagged HLA-$A2_{wild\ type}$ and K^bss-HA-tagged HLA-$A2_{C227S,C283S}$ (**Fig.11.a**) by means of retroviral transduction (for details see material and methods chapter). The presence of an N-terminal HA-tag allowed us to distinguish the mutant from the endogenous heavy chains and the inclusion of an heterologous signal sequence, that of the mouse class I MHC molecule H2-K^b (K^bss), upstream of the HA tag assured targeting of these constructs to the ER membrane (data not shown), as described previously [296]. We expressed K^bss-HA-tagged HLA-$A2_{wild\ type}$ and K^bss-HA-tagged HLA-$A2_{C227S,C283S}$ in control astrocytoma cells (designated U373 cells or control cells, in which neither viral protein is expressed), and in stable cell lines expressing US2 (US2 cells) or US11 (US11 cells).

We assessed the stability of the HA-tagged HCs in the presence of US2 and US11 by pulse/chase analysis (**Fig.11.b**). Cells stably expressing HA-HLA-$A2_{wild\ type}$ and HA-HLA-$A2_{C227S,C283S}$ were metabolically labeled (using a mixture of radioactive aminoacids cysteine and methionine) for a short (15 minutes) period of time - the *pulse* – and then subjected to a *chase* (in the presence of a large excess of non-radioactive aminoacid mixture) of up to 30 minutes to evaluate the fate of the HC molecules synthesized during the pulse period. The HA-tagged molecules were specifically recovered from cell lysates with anti-HA antibody and the immunoprecipitates were analyzed by SDS–PAGE followed by fluorography. The difference between the amount of HC molecules synthesized during the pulse period and therefore radioactively labelled (seen as the amount of HC immunoprecipitate obtained from cell lysates obtained immediately after the pulse, i.e, at the 0 min time point) and the amount of HC molecules retrieved at the end of the chase period (at the 30 min time point) can be attributed to HC destabilization (degradation) by the cytosolic proteasome. Strikingly, although

the unfolded HA-HLA-$A2_{C227S,C283S}$ molecules are unstable in US11 cells, as assessed by the failure to recover radioactive HC molecules after 30 min of chase, they are completely stable in US2 cells (**Fig.11.b**, compare lanes 9 and 10 with lanes 7 and 8). The HA-HLA-$A2_{wild\ type}$ molecule, on the other hand, is unstable in the presence of both US2 (**Fig.11.b**, lanes 5 and 6) and US11 [285]. In control cells, both the HA-HLA-$A2_{wild\ type}$ and HA-HLA-$A2_{C227S,C283S}$ molecules are stable over the course of the chase (**Fig.11.b**, lanes 1 and 2 and lanes 3 and 4), suggesting that the observed destabilization (or lack thereof, in the case of US2) is specifically due to expression of the viral protein. It appears that class I MHC molecules must acquire a properly folded conformation to be targeted for degradation by US2, whereas US11-mediated degradation is not dependent on the tertiary structure of class I MHC molecules.

The cellular dislocation machineries of the US2 and US11 pathways differ: a functional screen for US2 interaction partners

US11 utilizes an ER dislocation pathway that is dependent on DERLIN-1 to cause dislocation and degradation of class I MHC heavy chains, while US2 operates through a DERLIN-1-independent mechanism [21]. In an attempt to find cellular proteins required for US2's function, we decided to use an affinity purification approach based on functional criteria, an approach that has been successfully applied to US11[21]. We hypothesized that proteins co-immunopurified only with a form of US2 that is active in HC dislocation and not with a mutant form of US2 that is inactive in dislocation might account for the functional properties of the active US2 molecule.

We inspected the structure/function evidence available for US2 in order to design such a functional screen. The US2 tail (residues 186 to 199), with the aminoacid sequence DCNLSMMWMRFFVC, is essential for dislocation: $US2_{186}$ or tailless US2, a tail deletion mutant of US2 (**Fig.8.a**) [containing only the most membrane-proximal aspartate (D) residue of the cytosolic tail to ensure proper ER membrane insertion of the US2 transmembrane domain (TMD) segment], is dislocation-incompetent [278]. Furthermore, a chimeric mutant of US3 with the US2 tail fused to the lumenal and transmembrane domains of US3 tail is rendered capable of catalyzing dislocation [280].

We used an affinity purification approach similar to that used for identification of US11-associated factors [21] for the selective retrieval of proteins that interact with US2 and tailless US2 (**Fig.12**).

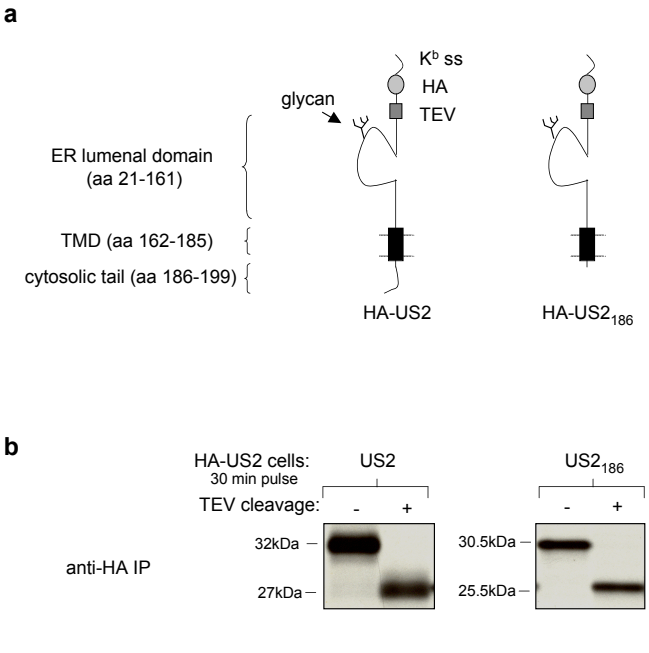

Figure 12. Tandem affinity purification constructs used for a functional screen for US2-associated proteins. **a.** Schematic representation of US2 constructs used for affinity purification. K^b ss, murine H2-K^b signal sequence; HA, influenza haemagglutinin (HA) epitope; TEV, tobacco etch virus protease cleavage site. **b.** Analysis of HA-US2 molecules by metabolic labeling. Astrocytoma cells expressing HA-US2 or $US2_{186}$ were pulsed for 30 min and lysed in 1%SDS. The lysates were immunoprecipitated with 12CA5 anti-HA antibody, treated with TEV protease where indicated (+) and subjected to 12.5% SDS-PAGE and fluorography.

We attached an amino-terminal affinity tag composed of the K^b signal sequence for effective targeting to the ER membrane, a haemagglutinin (HA) epitope followed by a tobacco etch virus (TEV) protease cleavage site and an additional spacer to the lumenal domain of wild type US2 (residues 21 to 199) or of the tailless US2 mutant $US2_{186}$ (residues 21 to 186) (**Fig.12.a**). The K^bss-HA-TEV-tag thus attached substituted the first 20 aminoacids of US2 corresponding to the US2 non-cleavable signal sequence [277] (**Fig.8.a**). We generated astrocytoma cells stably expressing K^bss-HA-TEV-tagged US2 and K^bss-HA-TEV-tagged $US2_{186}$ (HA-US2 and HA-$US2_{186}$ for simplicity) by retroviral transduction. We were able to retrieve the HA-US2 and HA-$US2_{186}$ molecules from lysates of metabolically labeled cells using an anti-HA antibody and were able to cleave them to completion with TEV protease (**Fig.12.b**). The HA-US2 and HA-$US2_{186}$ molecules migrated on SDS-PAGE at the expected molecular mass of glycosylated (ER-inserted) K^bss-HA-TEV-tagged HA-US2 and HA-$US2_{186}$, that is, 32 and 30.5 kDa, respectively. Both proteins were cleaved to completion by TEV protease, as assessed by the shift in their molecular masses to 27 and 25.5 kDa, respectively, upon incubation of the HA immunoprecipitates with TEV.

Indeed, the HA-US2 and HA-$US2_{186}$ constructs are correctly targeted to the ER

membrane and *N*-linked glycosylated (**Fig.13**).

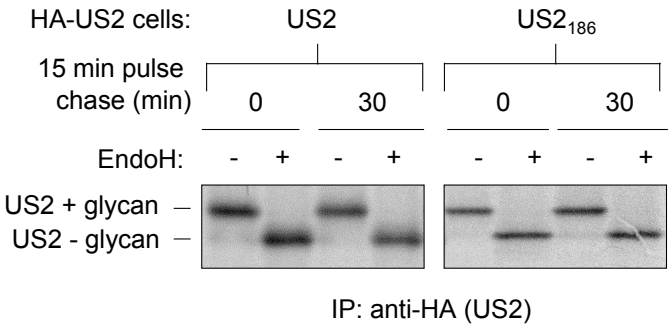

Figure 13. Both tagged US2 constructs are ER-resident glycoproteins. Astrocytoma cells were pulsed for 15 min and chased for up to 30 min in the presence of the proteasome inhibitor ZL_3VS and lysed in 1% SDS. Cell lysates were immunoprecipitated with 12CA5 anti-HA antibody followed by Endoglycosidase H (Endo H) digestion. Immunoprecipitates were then subjected to 12.5% SDS-PAGE and fluorography. The positions of glycosylated and deglycosylated HA-US2 are indicated.

HA-US2 and HA-$US2_{186}$ cells were metabolically labeled and lysates immunoprecipitated with anti-HA antibody followed by treatment with recombinant Endo-beta-N-acetylglucosaminidase H (Endo H). Endo H is an endoglycosidase secreted by *Streptomyces plicatus* which hydrolyzes the glycosidic bond between the core asparagine (*N*)-linked acetyglucosamine residues of *N*-linked high-mannose oligosaccharides. The high-mannose-type carbohydrate backbones recognized by Endo H are characteristic of glycosylated proteins resident in the proximal secretory pathway (ER to medial-Golgi). After exit from the ER, proteins proceed through the secretory pathway, and most glycoproteins acquire Endo H resistance in the medial-Golgi [297]. Therefore, sensitivity or insensitivity to Endo H can reflect the maturation state of glycoproteins. After a 15 min pulse, the HA-tagged US2 proteins were sensitive to Endo H treatment as seen by the shift in their molecular mass upon treatment with the recombinant endoglycosidase (+), showing that they had acquired their *N*-linked glycan upon ER membrane-insertion. Moreover, they were still in the proximal secretory pathway after 30 min as they remained completely sensitive to treatment with Endo H. Continued sensitivity to Endo H digestion therefore showed that both HA-US2 and HA-$US2_{186}$ are ER-resident glycoproteins.

Both HA-US2 and HA-$US2_{186}$ are capable of association with HC (**Fig. 14**).

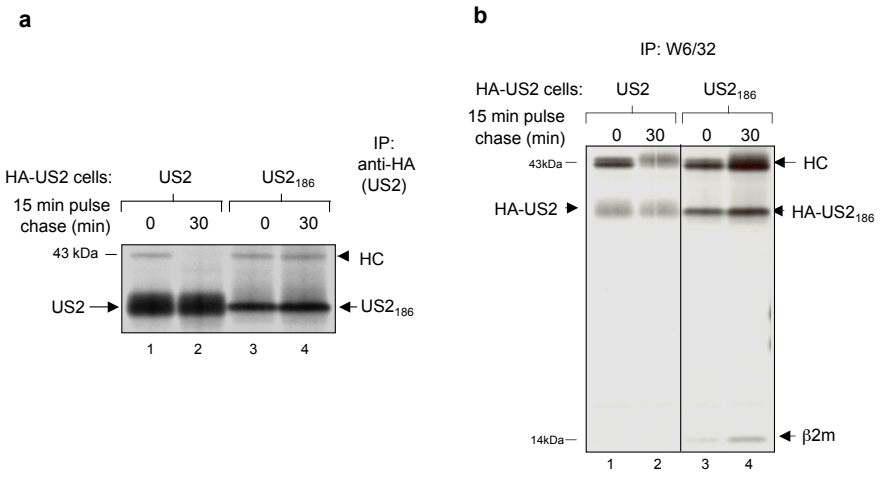

Figure 14. HA-US2$_{186}$, although dislocation-incompetent, retains the ability to associate with HC in the ER. **a.** Inspection of US2 immunoprecipitates reveals HC molecules associated with both HA-tagged US2 and US2$_{186}$. HA-US2 and HA-US2$_{186}$ cells were were pulsed for 15 min and chased for up to 30 min, in the presence of the proteasome inhibitor ZL_3VS. Anti-US2 (**a**.) and W6/32 (**b**.) immunoprecipitates were performed from 0.5% NP-40 lysates and subjected to 12.5% SDS-PAGE and fluorography. **b.** Inspection of W6/32 immunoprecipitates reveals the presence of both HA-US2 and HA-US2$_{186}$ cells in association with properly folded HCs.

Inspection of anti-US2 immunoprecipitates revealed the presence of associated HC molecules (**Fig.14.a**). At the onset of the chase (0 min time point), HC molecules could be detected in association with both HA-tagged US2 molecules (**Fig.14.a**, compare lanes 1 and 3), but after 30 min, we could no longer detect HC in association with HA-US2, although it was still seen in association with HA-US2$_{186}$ (**Fig.14.a**, compare lanes 2 and 4).

Inspection of W6/32 immunoprecipitated reveals the presence of associated HA-US2 and HA-US2$_{186}$ (**Fig.14.b**). As mentioned in the introduction, W6/32 recognizes an epitope on HLA molecules that is only displayed in presence of beta$_2$m [289,290]. We observe increased W6/32-reactivity in HA-US2$_{186}$ cells, as compared to HA-US2 cells, suggesting that a greater proportion of HC molecules assemble with beta2m and enter the secretory pathway in HA-US2$_{186}$ cells. Retrieval of both HA-US2 and HA-US2$_{186}$ from W6/32 immunoprecipitates (**Fig.14.b**) confirms that neither addition of the HA-TEV-tag nor removal of the US2 tail prevent association of US2 with its substrate, folded class I MHC complexes [27].

The more transient association of HC with HA-US2 (**Fig.14.a**) and the increased W6/32-reactivity in HA-US2$_{186}$ cells (**Fig.14.b**) are suggestive of HC degradation taking place

in HA-US2 cells and not in HA-US2$_{186}$ cells.

Indeed, although both HA-US2 and HA-US2$_{186}$ get targeted to the ER membrane and associate with their target molecule (HC), they display distinct functional properties: HA-US2 is functional in catalyzing HC dislocation, whereas the tailless HA-US2$_{186}$ mutant is inactive (**Fig.15**).

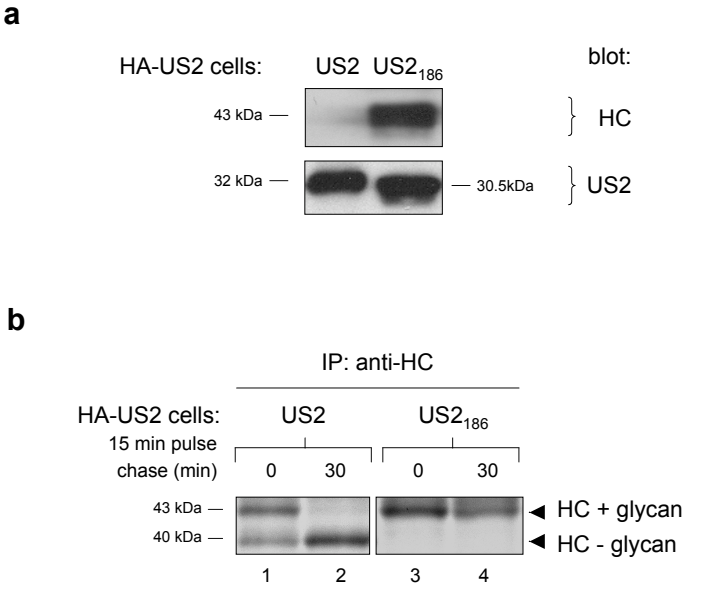

Figure 15. HA-US2$_{186}$ is dislocation-incompetent. **a.** HC stability in astrocytoma cells expressing the HA-tagged US2 molecules. Immunoblot analysis performed with the indicated antibodies. **b.** Only HA-US2 is capable of catalyzing dislocation. Astrocytoma cells expressing HA-US2 and HA-US2$_{186}$ were pulsed for 15 min and chased for up to 30 min in the presence of the proteasome inhibitor ZL$_3$VS. HC molecules were recovered from 0.5% NP-40 lysates with anti-HC (HC-70) antibody and the immunoprecipitates were then subjected to 12.5% SDS-PAGE and fluorography. The positions of the glycosylated and deglycosylated HC are indicated.

Analysis of the steady state levels of HC by immunoblot showed that HC molecules were almost undetectable in HA-US2 cells, but stable in HA-US2$_{186}$ cells (**Fig.15.a**). Moreover, analysis of HC stability in HA-US2 and HA-US2$_{186}$ cells by pulse/chase in the presence of proteasome inhibitor revealed the hallmark conversion of ER-resident glycosylated HC (HC+glycan) into cytosolic deglycosylated HC (HC-glycan) in HA-US2 cells (**Fig.15.b**, compare lanes 2 and 1), indicative of ER-to-cytosol transport [12]. In HA-US2$_{186}$ cells, however, the hallmark deglycosylated HC intermediate was not observed (**Fig.15.b**, lane 4), showing that the HA-US2$_{186}$ mutant is ineffective in dislocation. We conclude that, just like what was seen for their untagged counterparts [278], the N-terminally K^{b}ss-HA-TEV-tagged US2 molecules are targeted to the ER membrane, are *N*-glycosylated, associate with HC molecules shortly after synthesis, and catalyze dislocation in a US2 tail-dependent manner.

After validating the functional properties of the HA-US2 constructs, we performed a large-scale purification of US2 and US2-associated proteins from control U373-MG astrocytoma cells (expressing no US2) and from U373-MG cells that expressed either HA-US2 or HA-US2$_{186}$. Digitonin, a mild non-ionic detergent used to solubilize membrane-bound

proteins [275], was used to prepare lysates from control (-), HA-US2 and HA-US2$_{186}$ cells. These digitonin lysates were subjected to immunoprecipitation with anti-HA antibody-coupled agarose beads, and the bound material was then treated with TEV protease to release US2 and US2-associated proteins. The TEV eluates obtained were loaded on an 8% SDS-PAGE and subjected to silver staining. Bands revealed by silver staining were excised from the gel and tryptic digests of the polypeptides present were subsequently analyzed by tandem mass spectrometry (MS/MS), revealing the identity of proteins associated with HA-US2 and/or HA-US2$_{186}$ (**Fig.16**).

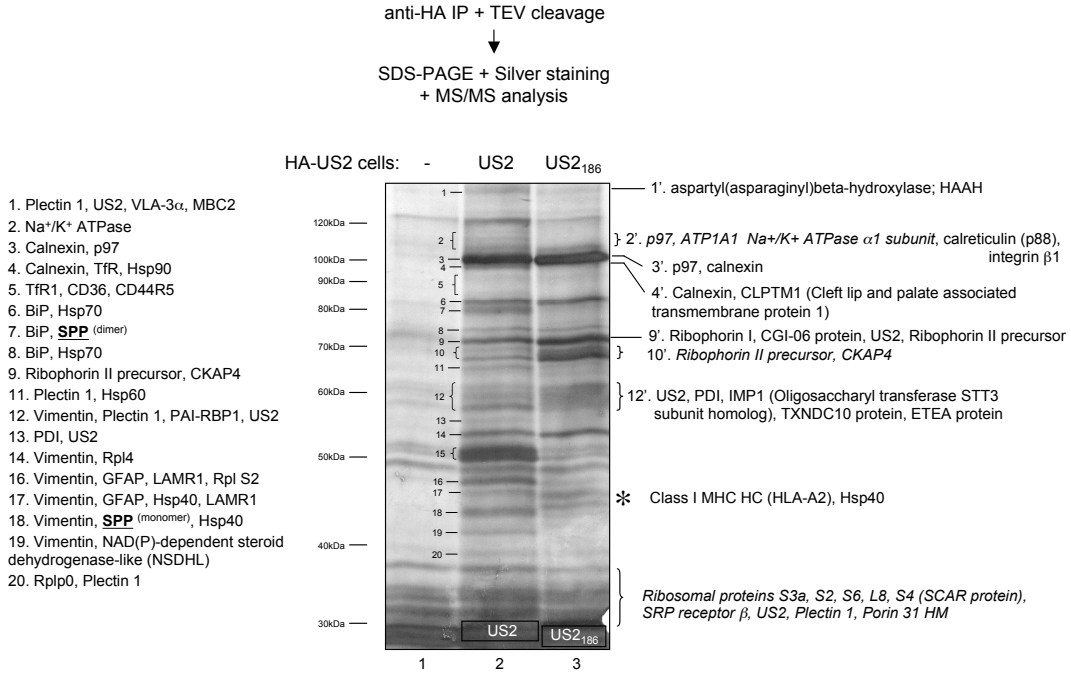

Figure 16. Large-scale affinity purification of US2 and US2-associated proteins from U373-MG control cells (-) or cells expressing HA-TEV-tagged US2 or US2$_{186}$. Lysates were immunoprecipitated with 3F10 anti-HA antibody and bound proteins were released by TEV cleavage, subjected to 8% SDS-PAGE and revealed by silver staining. The bands indicated were excised and subjected to mass spectrometry analysis and database searching to identify the polypeptides present. For each band (x) excised from lane 2, we excised a corresponding band (x') from lane 3. Shown are the most prominent proteins found in each band indicated: proteins retrieved by both US2 and US2$_{186}$ in corresponding bands are italicized. We have not obtained nor shown data for all peptides present in the bands excised and have for now focused mainly on SPP (bands 7 and 18). For most proteins other than US2 and SPP, validation awaits further experiments. The asterisk indicates class I MHC heavy chain. IP, immunoprecipitation.

The TEV protease and the two US2 constructs after release by TEV cleavage co-migrate in the 25-30 kDa range of the SDS-PAGE, in which we found a very high number of US2 peptides (**Fig.16**, boxed areas in lanes 2 and 3). Consistent with our previous findings

(**Fig.14**), we identified class I MHC HC molecules in association with HA-US2$_{186}$ (**Fig.16**, lane 3, asterisk).

MS/MS analysis of tryptic digests of the polypeptides retrieved from the bands identified in the gel revealed the presence of several proteins that co-purified with both HA-US2 and HA-US2$_{186}$, which we reasoned to be unlikely to account for the mutant phenotype of HA-US2$_{186}$. These proteins included the ER chaperones calnexin and BiP, the oxidoreductase PDI, subunits of the oligosaccharide transferase complex (ribophorin I and ribophorin II), the cytosolic chaperones Hsp90, Hsp60 and Hsp40, and the cytosolic AAA ATPase p97 (**Fig.16**). The possible significance of these interactions will be addressed briefly in the discussion.

Instead of assessing the possible functional implications of the interaction partners of both US2 molecules, we focused on polypeptides retrieved uniquely by HA–US2 and not by HA-US2$_{186}$. Amongst other proteins, we detected the prominent presence of signal peptide peptidase (SPP) as a specific association partner of dislocation-competent (active) HA-US2 (**Fig.17**). MS/MS analysis of tryptic digests obtained from bands 7 and 18 (**Fig.16**) yielded 8 peptides that were assigned to the 377 aminoacid protein SPP with an overall 22% sequence coverage (82/377 aminoacids) (**Fig.17.a**). SPP is an ER resident protein of 40-45kDa that is predicted to span the ER membrane 7 times, and belongs to the presenilin (PS)/SPP-Like (SPPL) superfamily of intramembrane-cleaving aspartic proteases [298]. PS/SPPL proteases are characterized by the ability to cleave substrate polypeptides within a transmembrane region, by possessing two active site aspartate (D) residues within the conserved motifs Y*D* and LGLG*D* in adjacent membrane-spanning regions [298-300], and by a C-terminal PAL motif that is required for normal active site conformation [300] (**Fig.17.b**).

a

MDSALSDPHNGSAEAGGPTNSTTRPPSTPEGIALAYGSLLMALLPIFFG 50
ALRSVRCAR**GKNASDMPETITSR**DAARFPIIASCTLLGLYLFFKIFSQEY 100
INLLLSMYFFVLGILALSHTISPFMNK**FFPASFPNRQYQLLFTQGSGENK** 150
EEIINYEFDTKDLVCLGLSSIVGVWYLLRKHWIANNLFGLAFSLNGVELL 200
HLNNVSTGCILLGGLFIYDVFWVFGTNVMVTVAKSFEAPIK**LVFPQDLLE** 250
KGLEANNFAMLGLG**D**VVIPGIFIALLLRFDISLKKNTHTYFYTSFAAYIF 300
GLGLTIFIMHIFKHAQPALLYLVPACIGFPVLVALAK**GEVTEMFSYEESN** 350
PKDPAAVTESKEGTEASASKGLE*KKEK* 377

b

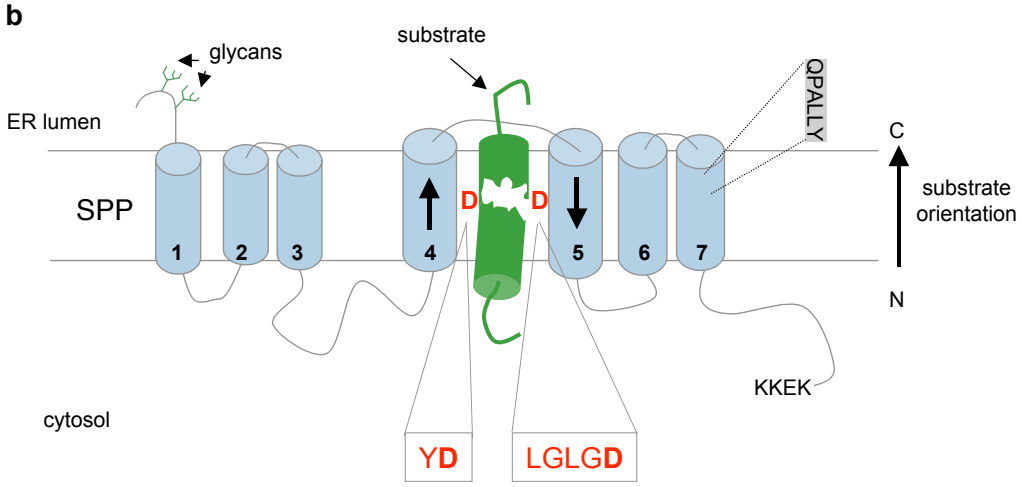

Figure 17. Signal peptide peptidase (SPP) is an intramembrane-cleaving aspartic protease of the presenilin (PS)/SPP-Like family. **a.** SPP amino acid sequence. The peptides that were identified by MS/MS are in boldface type. The conserved active site motifs characteristic of the PS/SPPL protease family are shown in red, with the catalytic aspartates in red boldface type. The conserved PAL motif, also characteristic of the PS/PSSL family is boxed in gray, and the KKEK ER retrieval signal at the SPP C-terminus is shown in italic. The putative transmembrane domain sequences are underlined. The two *N*-linked glycosylation sites at the N terminus of the SPP protein are shown in green. Residues shown in blue correspond to residues mutated in *Drosophila* SPP point mutants (L95F and P248L) that phenocopy SPP active site mutants. **b.** Predicted topology of the SPP molecule. Arrows indicate the orientation of the transmembrane regions containing the catalytic motifs YD and LGLGD. The type II orientation of the SPP substrate is also indicated, as well as its predicted localization within the SPP catalytic site relative to the catalytic dyad.

The common role of intramembrane-cleaving proteases is to liberate signaling molecules from membrane-bound precursors with consequent activation or repression of signaling cascades [299,301-305]. SPP (also called minor Histocompatibility antigen H13, IMP1 or hIMP1, IMPAS, MSTP086, Presenilin-like protein 3 or PSENL3 or PSL3 and dJ324O17.1) plays an important role in immune surveillance as it generates the epitopes for the non-classical HLA-E molecule [29]. SPP is also responsible for cleavage of the internal signal sequence of the hepatitis C virus (HCV) polyprotein which is required for maturation of the HCV core protein [306]. Additionally, SPP has been proposed to modulate the interaction of signal peptide remnants of HIV gp160 and preprolactin with calmodulin [307]. Of note, SPP has

been found in association with a truncated version of a polytopic ER protein in an *in vitro* system [308].

SPP has a type I signal anchor sequence for initiation of protein translocation and membrane insertion, two *N*-linked glycosylation sites near its N-terminus (localized in the ER lumen), and a C-terminus exposed towards the cytosol containing an ER-retention signal, KKEK [298,309] (**Fig.17.b**). SPP migrates as an 85 to 95 kDa SDS-resistant homodimer in SDS-PAGE; upon heating (or treatment with acid) the dimer dissociates and the SPP monomers (of 40 to 45 kDa) can be resolved [310], as observed upon analysis of anti-SPP immunoprecipitates heat-treated for the indicated times prior to SDS-PAGE (**Fig.18**, compare lanes 1 and 5).

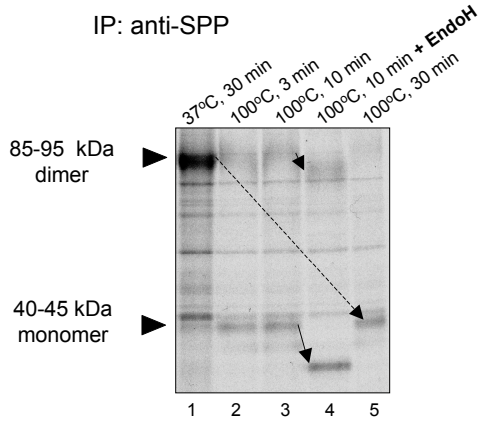

Figure 18. The ratio of SDS-stable SPP dimer/monomer is dependent on the treatment of samples before loading on the gel. SPP immunoprecipitates obtained from 1% SDS lysates of U373 cells radioactively labeled for 2 hr were split into 5 aliquots, ressuspended in reducing sample buffer, and treated as indicated before loading on a 12.5% SDS-PAGE. Endo H, Endoglycosidase H. IP, immunoprecipitation.

Treatment with endoglycosidase H results in removal of the two *N*-linked glycans from the SPP molecule, confirming it is an ER-resident glycoprotein (**Fig.18**, compare lanes 1 and 4).

To confirm the mass spectrometry data on the US2/SPP interaction, native (digitonin) lysates from HA-US2 or HA-US2$_{186}$ cells were subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-SPP antiserum (US2 IP/SPP blot) (**Fig.19**).

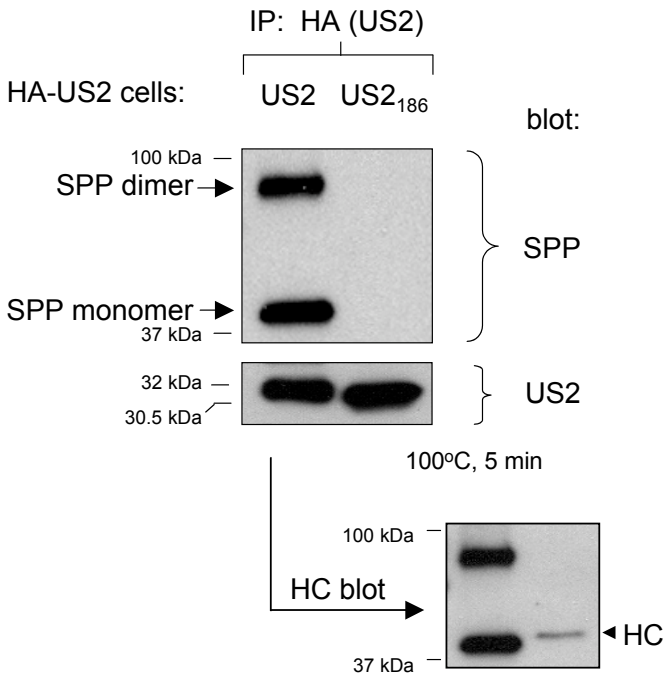

Figure 19. The US2 tail recruits signal peptide peptidase. Native (1% digitonin) lysates of astrocytoma cells expressing HA-US2 and HA-US2$_{186}$ were immunoprecipitated with 3F10 anti-HA antibody to retrieve US2 and US2-associated proteins. The immunoprecipitates were then subjected to 4-15% SDS-PAGE and immunoblot analysis with anti-SPP and anti-US2 antibodies. The blot was subsequently treated with anti-HC (HC-10) antibody (inset). As samples were boiled for only 3 min at 100^{0}C before loading on the gel, SPP is seen in its dimeric and monomeric forms.

SPP (both in its dimeric and monomeric form) was present in the HA-US2 immunoprecipitate while absent from that obtained from HA-US2$_{186}$ cells, confirming the specific interaction of SPP with wild type US2, but not with US2$_{186}$ (**Fig.19**, SPP blot), despite the preserved interaction of US2$_{186}$ with HC, as seen by immunoblotting with anti-HC antiserum (**Fig.19**, HC blot). The US2 levels were comparable as analyzed by immunoblot with anti-US2 antibody (**Fig.19**, US2 blot).

To further confirm the US2/SPP interaction, 5% of input material used for the large-scale purification experiment was analyzed by immunoblotting with anti-US2 and anti-SPP antibodies, revealing TEV-cleaved US2 (27 kDa) and US2$_{186}$ (25.5 kDa), as well as SPP in association only with full-length US2 (**Fig.20**).

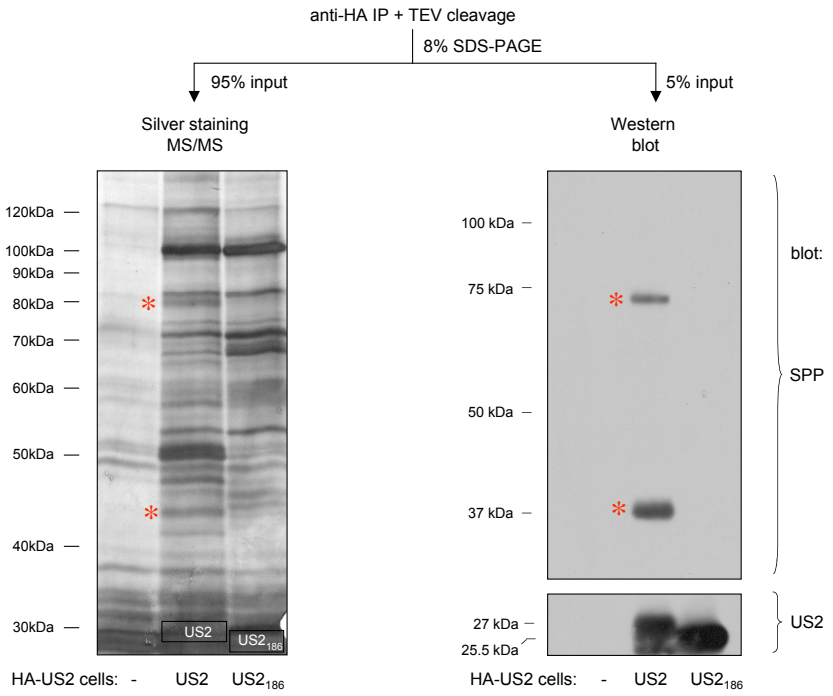

Figure 20. Immunoblot analysis of the samples used for the large-scale affinity purification of US2 and US2-associated proteins. A 5% aliquot of the samples that were subjected to MS/MS mass spectrometry analysis (left panel) was loaded on the same 8% SDS-PAGE and subjected to immunoblot analysis (right panel). Immunoblot analysis with anti-SPP and anti-US2 antibodies showed that the levels of HA-US2 and HA-US2$_{186}$ were comparable (right panel, US2 blot) and confirmed the MS/MS-indicated presence of signal peptide peptidase (asterisks) in HA-US2 immunoprecipitates. IP, immunoprecipitation.

Analysis of the large-scale affinity purification protocol, therefore, enabled identification of a *bona fide* cellular interaction partner for US2.

The immunoprecipitation of SPP from US2 and HA-US2 cells overexpressing tagged versions of SPP also allows recovery of US2 (**Fig.21**).

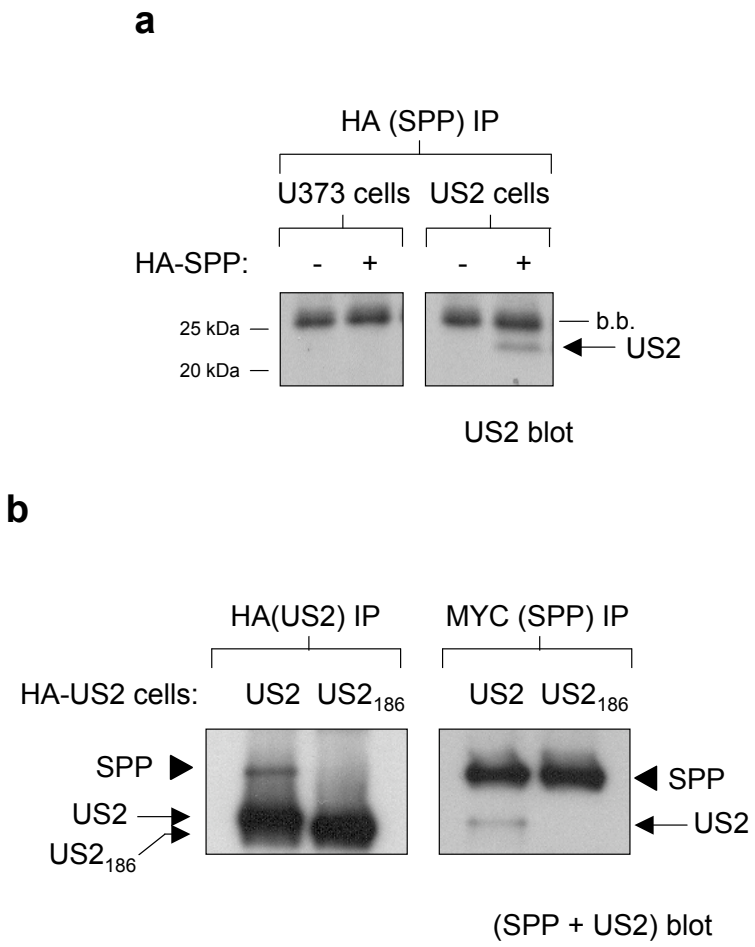

Figure 21. Signal peptide peptidase binds US2 in a US2 cytosolic tail-dependent manner. **a.** SPP binds a 22 kDa protein that is absent from control U373 cells and present in cells expressing US2. Immunoprecipitations from native (1% digitonin) lysates of U373 or US2 cells expressing an empty vector control (-) or HA-TEV-tagged SPP (HA-SPP) (+) were performed with anti-HA antibody, and subjected to 4-15% SDS-PAGE followed by immunoblot analysis with anti-US2 antibody. b.b., background band. **b.** Signal peptide peptidase does not bind the tailless $US2_{186}$. Digitonin lysates of cells expressing N-MYC-tagged SPP and HA-US2 or HA-$US2_{186}$ were immunoprecipitated with anti-HA and anti-MYC antibodies. The immunoprecipitates were then subjected to 4-15% SDS-PAGE and immunoblotted for SPP and US2. Samples were boiled for only 3 min at 100^0C. For clarity purposes, we show only the area of the gel where the SPP monomer migrates, but a similar association was also seen for the dimeric form of the SPP protein. IP, immunoprecipitation.

We generated U373 and US2 cells expressing N-terminally HA-TEV-tagged SPP (HA-SPP) and analyzed anti-HA (SPP) immunoprecipitates for the presence of US2. SPP bound a 22 kDa protein that was absent from control U373 cells and present in US2 cells (**Fig.21.a**), confirming SPP binding to US2. Moreover, we confirmed that SPP binds US2 in a US2 tail-dependent manner (**Fig.21.b**). We generated HA-US2 or HA-$US2_{186}$ cells expressing N-terminally MYC-TEV-tagged SPP (MYC-SPP) and analyzed anti-MYC immunoprecipitates by immunoblot with anti-US2 antibody. The anti-MYC (SPP) immunoprecipitates revealed the presence of HA-US2, but not HA-$US2_{186}$ (**Fig.21.b**), again confirming that SPP does not associate with $US2_{186}$. The same results were obtained for C-terminally MYC-tagged SPP expressed in HA-US2 cells (data not shown). We conclude that SPP interacts with US2, an interaction that requires an intact US2 cytoplasmic tail.

Although identification of SPP in association only with functional US2 suggests a role for SPP in HC dislocation, we sought to obtain direct functional evidence for SPP involvement through interference (RNAi). We used a retrovirus (pRETRO)-based system for transient and stable expression of short hairpin RNAs (shRNAs) in mammalian cells [311] to reduce SPP protein expression levels (**Fig.22.**).

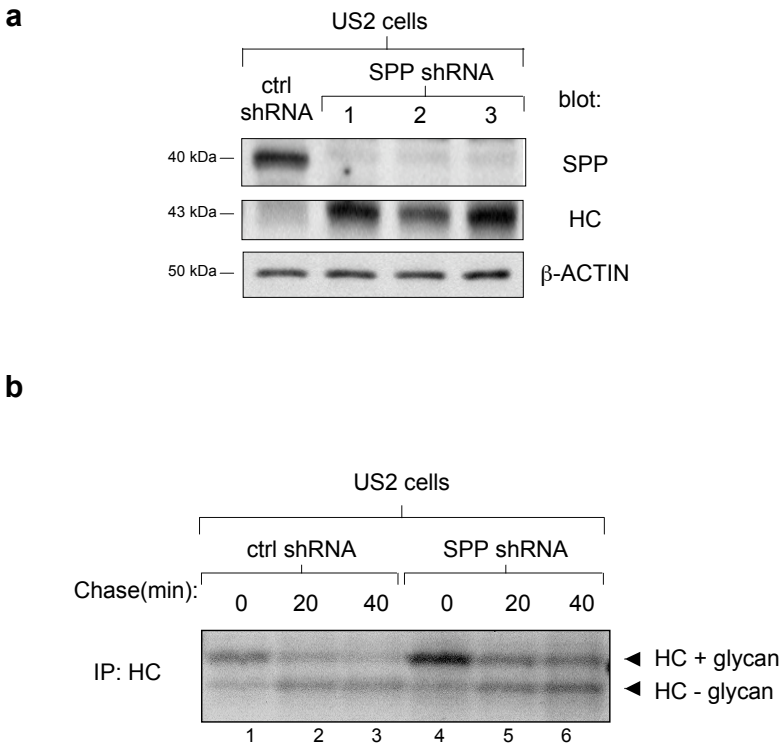

Figure 22. Signal peptide peptidase (SPP) is required for US2-mediated HC dislocation. **a.** Reduction of SPP protein levels by RNAi-mediated knockdown results in stabilization of HC. Immunoblot analysis of 0.5% NP-40 lysates of US2 cells expressing the indicated shRNA and subjected to 4-15% SDS-PAGE followed by immunoblot analysis with anti-SPP, anti-HC (HC-10) and anti-beta-Actin antibodies. For clarity purposes, we show only the area of the blot where the SPP monomer runs. Beta-Actin levels were used as a loading control. **b.** Knockdown of SPP results in impaired HC dislocation. US2 cells expressing a control shRNA (against eGFP) or 3 different shRNAs against SPP [SPP shRNAs (1+2+4)] were pulsed for 20 min and chased for up to 40 min in the presence of the proteasome inhibitor ZL_3VS. Lysates were obtained with 0.5% NP-40 and HC and US2 molecules were immunoprecipitated with anti-HC (HC-70) and anti-US2 antibodies and then subjected to 12.5% SDS-PAGE and fluorography. The positions of the glycosylated (+glycan) and deglycosylated (-glycan) HC and US2 are indicated. IP, immunoprecipitation.

A robust RNAi-mediated reduction of SPP protein levels (SPP knockdown) was achieved with three different SPP shRNA constructs targeting three different 21 nucleotide sequences within the SPP mRNA, SPP.1, SPP.2 and SPP.3, as seen by immunoblot analysis

(**Fig.22.a**, SPP blot, compare SPP levels in cells expressing SPP shRNAs or an shRNA directed against the unrelated control protein, eGFP). Whereas knockdown of SPP had no effect on HC levels in control U373-MG cells (data not shown), US2 cells that express each of the three SPP shRNAs had increased levels of HC at steady state (**Fig.22.a**, HC blot). If shRNAs are highly specific for their cognate target, then shRNAs designed against the same gene, but with different nucleotide sequences, should have similar effects [312]. We observed the same effect on HC levels (increase) with shRNAs SPP.1, SPP.2, and SPP.3 (**Fig.22.a**, HC blot), as well as with shRNAs SPP.4, SPP.5, SPP.6, SPP.7 and SPP.8 (data not shown), strongly implying that the observed effect on HC dislocation was specific.

Impairment of HC dislocation in US2 cells with an SPP knockdown could also be seen in pulse/chase experiments (**Fig.22.b**). US2 cells expressing the control shRNA or a mixture of SPP shRNAs 1, 2 and 4 were metabolically labeled in the presence of proteasome inhibitor and anti-HC immunoprecipitates were analyzed by SDS-PAGE. In US2 cells expressing the control shRNA, we observed loss of glycosylated HC (HC+glycan) over time and increased recovery of deglycosylated HC (HC-glycan) (**Fig.22.b,** compare lane 3 to lane 1). Conversion of glycosylated HC into cytosolic deglycosylated HC intermediate is a hallmark of dislocation, and at the end of the chase roughly 80% of the total HCs were deglycosylated (cytosolic) (**Fig.22.b**, lane 3). In contrast, in US2 cells expressing SPP knockdown constructs (US2/SPP knockdown cells), we observed a slower rate of dislocation: at the end of the chase, the ratio of deglycosylated to total HC was only about 50% (**Fig.22.b**, lane 6). The amount of remaining dislocated HCs (50%) detected probably reflected the failure of the SPP shRNA to completely eliminate the expression of SPP and hence the failure in completely blocking dislocation.

We sought to obtain further confirmation that the SPP knockdown obtained was a bona fide RNAi effect and not the result of off-target effects [313] (**Fig.23**). The reduction in SPP protein levels could also be observed through RT-PCR analysis of messenger RNA levels (**Fig.23.a**). Reduction of SPP expression at both the mRNA and protein level confirmed the observed effect as classical shRNA-related RNAi (and not microRNA-related) [313]. As an additional control for shRNA specificity, we assessed shRNA dose- and sequence-dependence (**Fig.23.b,c**).

For testing shRNA dose-dependence, US2 cells were infected with different titers of control (anti-eGFP) shRNA virus and SPP.1 shRNA virus and analyzed by immunoblot. Different titers consisted of 1:1, 1:2 and 1:3 dilutions of the virus supernatant with cell culture

medium. Titration of the shRNA by infection with different dilutions of shRNA-expressing retrovirus showed an SPP shRNA virus dose-dependence SPP knockdown and that accumulation of HC molecules is proportional to the SPP knockdown achieved (**Fig.23.b**). The same was true for experiments in which an increased level of SPP knockdown was obtained by performing multiple rounds of infection with the SPP shRNA virus (data not shown).

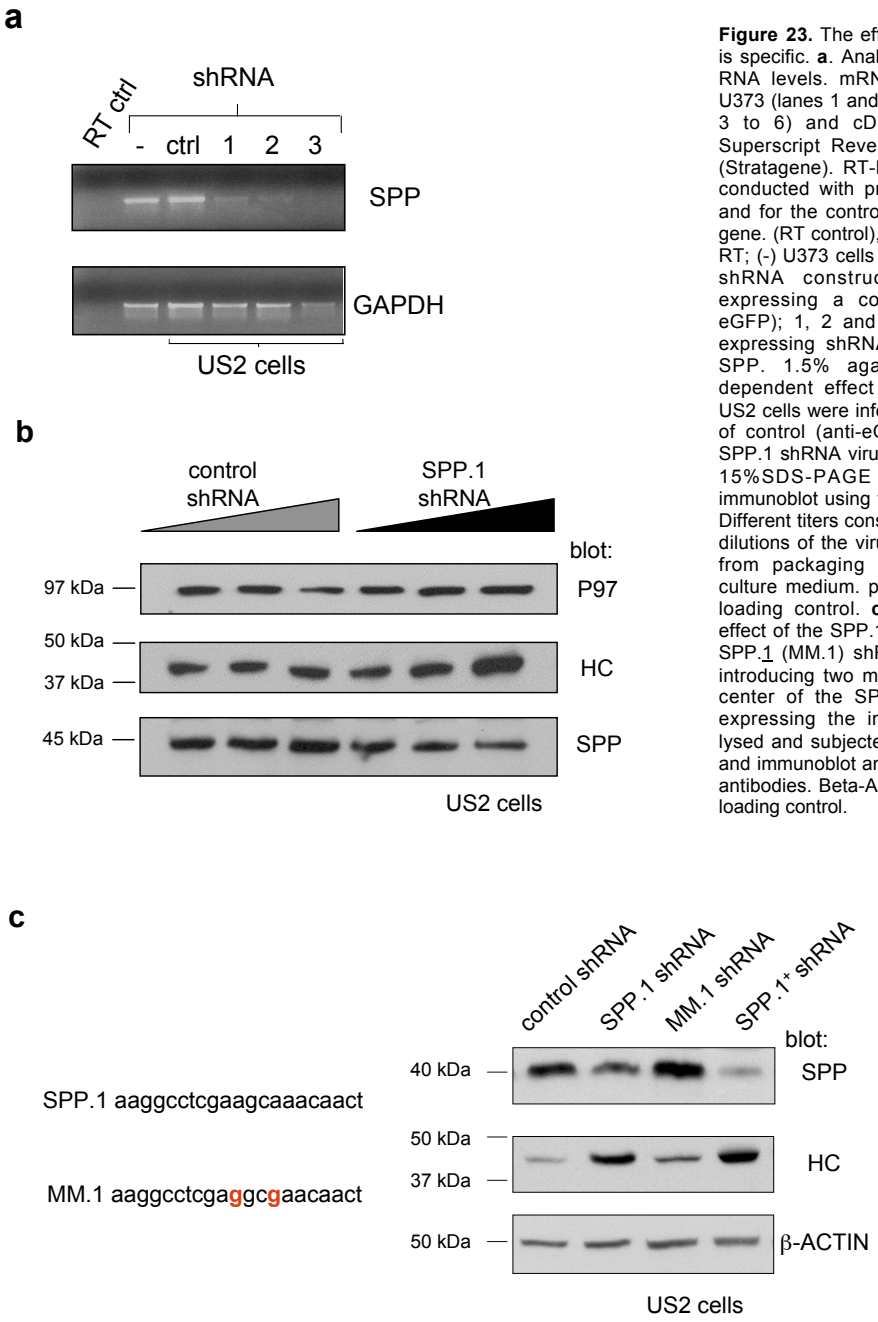

Figure 23. The effect of the SPP shRNAs is specific. **a**. Analysis of SPP messenger RNA levels. mRNA was extracted from U373 (lanes 1 and 2) and US2 cells (lanes 3 to 6) and cDNA was produced by Superscript Reverse Transcriptase (RT) (Stratagene). RT-PCR analysis was then conducted with primers specific for SPP and for the control GAPDH housekeeping gene. (RT control), sample not treated with RT; (-) U373 cells that do not express any shRNA construct; (ctrl), US2 cells expressing a control shRNA (against eGFP); 1, 2 and 3 refers to US2 cells expressing shRNAs 1, 2 and 4 against SPP. 1.5% agarose gel. **b.** Dose-dependent effect of the SPP.1 shRNA. US2 cells were infected with different titers of control (anti-eGFP) shRNA virus and SPP.1 shRNA virus, lysed, subjected to 4-15%SDS-PAGE and analyzed by immunoblot using the indicated antibodies. Different titers consisted of 1:1, 1:2 and 1:3 dilutions of the virus supernatant obtained from packaging cells with normal cell culture medium. p97 levels were used as loading control. **c.** Sequence-dependent effect of the SPP.1 shRNA. A mismatched SPP.1 (MM.1) shRNA was generated by introducing two mismatched bases in the center of the SPP.1 21mer. US2 cells expressing the indicated shRNAs were lysed and subjected to 4-15% SDS-PAGE and immunoblot analysis with the indicated antibodies. Beta-Actin levels were used as loading control.

For testing shRNA sequence-dependence, we tested the effects of a mismatched SPP.1 shRNA control (termed mismatched SPP.1 or MM.1), consisting of a two-base-pair change in the middle of the SPP.1 shRNA (**Fig.23.c**). We tested US2 cells expressing SPP.1 shRNA and MM.1 shRNA by immunoblot analysis and observed that mismatch of the SPP.1 shRNA sequence ablates its SPP knockdown effect and its effect on HC levels, confirming sequence-specificity (**Fig.23.c**). Taken together, our data support the notion that the interaction of SPP with US2 is essential for US2-mediated ER dislocation of class I MHC HC molecules.

US2 and US11 differ in the mechanistic details of dislocation and, of note, the analysis performed for US11 that led to identification of DERLIN-1 did not identify SPP [21]. Moreover, overexpression of a C-terminally eGFP-tagged version of DERLIN-1 blocks US11- but not US2-mediated HC dislocation [21]. It is, therefore, likely that the SPP-dependent pathway utilized by US2 is not co-opted by US11. To directly test this, we assessed the effects of an SPP knockdown in US11-mediated HC dislocation (**Fig.24**).

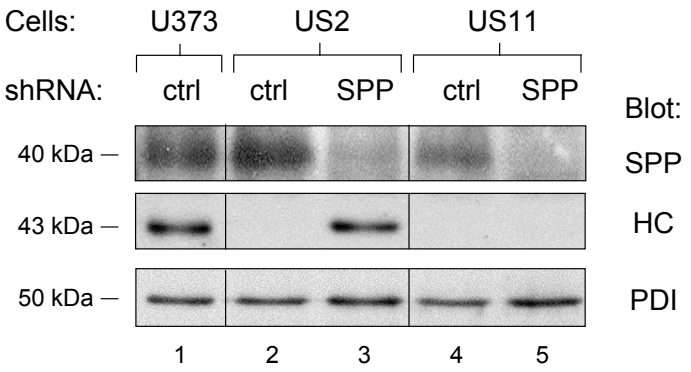

Figure 24. SPP knockdown impairs dislocation of HC by US2 but not US11. Lysates of US2 or US11 cells expressing a mixture of SPP shRNAs (1+2+4) (lanes 3 and 5), or a control shRNA against eGFP (ctrl) (lanes 2 and 4) were subjected to 4-15% SDS-PAGE and immunoblotted with the indicated antibodies. PDI levels were used as loading control. U373 cells were used as a control for HC levels in the absence of US2 and US11.

Lysates of US2 or US11 cells expressing SPP shRNAs or a control shRNA were subjected to immunoblot analysis. Whereas US2/SPP knockdown cells showed elevated HC levels (**Fig.24.** lane 3), the same decrease in SPP expression had no effect on the HC levels in US11 cells (**Fig.24**, lane 5). SPP is therefore specifically required for HC dislocation mediated by US2, but not by US11.

The association between the viral proteins and the cellular dislocation machinery that they recruit is highly specific (**Fig.25**).

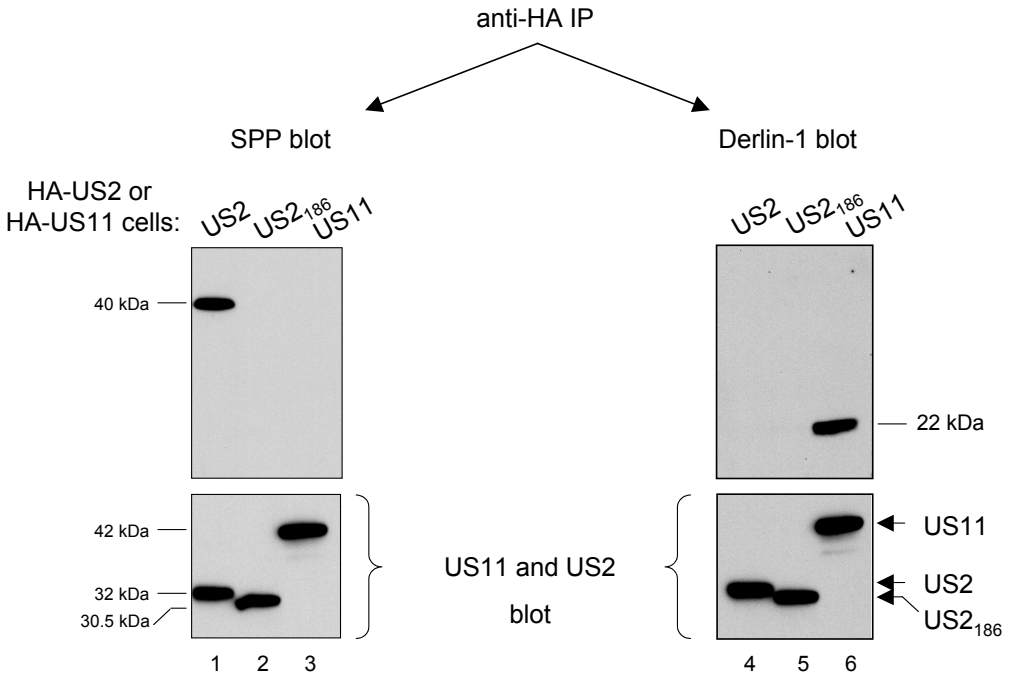

Figure 25. The SPP-dependent ER dislocation pathway co-opted by US2 is distinct from that used by US11, as US2 binds SPP and not DERLIN-1, while US11 binds DERLIN-1 and not SPP. Immunoprecipitations from native (1% digitonin) lysates of cells expressing HA-US2, HA-US2$_{186}$, and HA-US11 were performed with 3F10 anti-HA antibody to retrieve US2/US11 and US2- or US11-associated proteins. Anti-HA immunoprecipitates were subjected to 10% SDS-PAGE and immunoblot analysis was performed with the antibodies indicated.

We performed anti-HA immunoprecipitations from lysates of cells expressing HA-US2, HA-US2$_{186}$, and HA-US11 to retrieve US2/US11 and US2- or US11-associated proteins. As expected, we observed SPP in association with US2 (**Fig.25**, lane 1), and DERLIN-1 in association with US11 (**Fig.25**, lane 6). In contrast, we did not observe DERLIN-1 in anti-US2 immunoprecipitates (**Fig.25**, lane 4) nor SPP in anti-US11 immunoprecipitates (**Fig.25**, lane 3), albeit equal expression levels of the HA-tagged US2 and US11 proteins (**Fig.25**, bottom panels). Therefore, we conclude that US2 binds SPP and not DERLIN-1 and, conversely, that US11 binds DERLIN-1 and not SPP. Taken together, our observations and those described in the functional screen for US11 association partners [21] further support the notion that the ER degradation pathways co-opted by the HCMV immunoevasins US2 and US11 are distinct.

Amongst the many unanswered questions in HC dislocation by US2 is whether or not the Cdc48p(p97)/NPL4/UFD1 complex is required, as has been shown for US11. We found

the p97 AAA ATPase, a rather “usual suspect” in ER dislocation, in association with both HA-US2 and HA-US2$_{186}$ (**Fig.16**, bands 3 and 3’, respectively). While Elizabeth Klemm, an MIT graduate student, was doing a one-month long rotation in our lab, we decided to assess the functional relevance of the US2/p97 interaction for dislocation by analyzing the effects of a knockdown of p97 by RNAi (**Fig.26**).

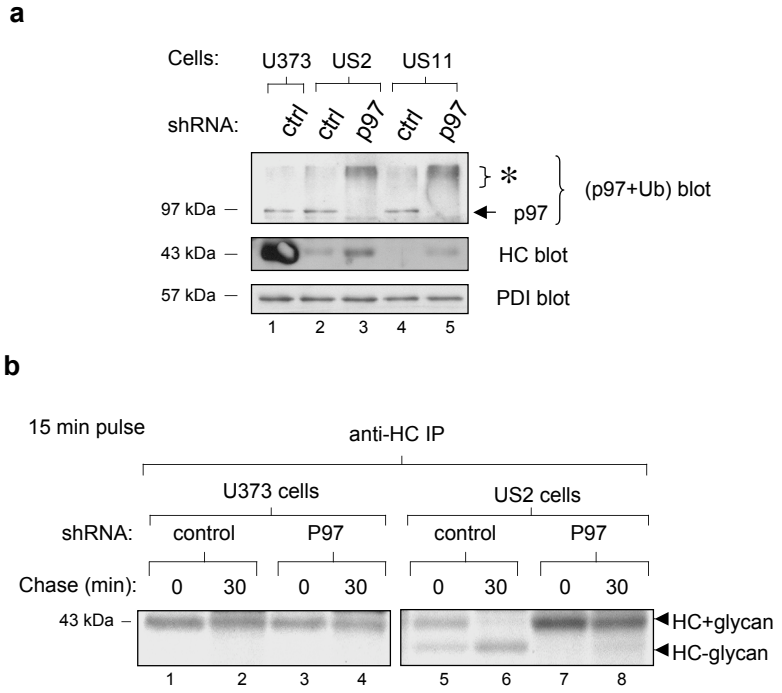

Figure 26. P97 is required for HC dislocation by both US2 and US11. **a.** U373, US2 and US11 cells expressing an shRNA against the AAA ATPase p97 or a control shRNA against eGFP (ctrl) were treated with 10mM NEM for 15 min and lysed with 0.5%NP-40. Lysates were subjected to 4-15% SDS-PAGE and immunoblot analysis with the indicated antibodies. The (p97+Ub) blot refers to an anti-p97 blot followed by a sequential anti-Ub blot to assess p97 knockdown levels as well as accumulation of polyUb conjugates (asterisk). PDI levels were used as a loading control. **b.** U373 and US2 cells were pulsed for 15 min and chased for up to 30 min, lysed in 0.5% NP-40 and subjected to anti-HC immunoprecipitation with HC-70 antibody, followed by 12.5% SDS-PAGE and fluourography. Experiment conducted by Elizabeth Klemm.

We assessed HC levels in U373, US2 and US11 cells expressing an shRNA against p97 or a control shRNA by immunoblot analysis (**Fig.26.a)**. Cells were treated with *N*-ethyl maleimide (NEM) prior to western blotting. NEM is a membrane-permeable thiol-blocking agent that irreversibly replaces the hydrogen atom in thiol (or sulfhydryl) (-SH) groups, present, for example, on cysteine proteases. Ubiquitin addition and removal reactions, by ubiquitin ligases and deubiquitinating enzymes, respectively, depend on cysteine residue on their active sites. NEM can therefore be employed to block these activities. NEM incubation prior to immunoblot analysis should allow us to prevent deubiquitination and enrich for ubiquitinated material in the cell lysates. Indeed, incubation with anti-p97 antibody followed by incubation with anti-Ub antibody revealed a robust knockdown of p97 both in US2 and US11 cells (**Fig.26.a**, p97+Ub blot, arrow), and a generalized accumulation of high molecular weight polyUb conjugates of cellular proteins (**Fig.26.a**, p97+Ub blot, asterisk). The p97 knockdown resulted in increased recovery of HC not only in US11 cells (**Fig.26.a**, HC blot,

compare lanes 5 and 4) as has been described in semi-permeabilized cells [32], but also in US2 cells (**Fig.26.a**, HC blot, compare lanes 3 and 2). We assessed HC stability in control U373 cells and US2 cells expressing the control shRNA or the shRNA against p97 by pulse/chase in the presence of proteasome inhibitor (**Fig.26.b**). After 30 min of chase, US2 cells expressing the control shRNA show almost complete conversion of glycosylated into deglycosylated HC (HC-glycan) (**Fig.26.b**, lane 6). By contrast, the cytosolic deglycosylated HC intermediate is practically absent from US2/p97 knockdown cells (**Fig.26.b**, lane 8). We conclude that HC dislocation by US2 requires p97, as has been shown for US11 [32]. It thus seems that the US2 and US11 pathways of dislocation of class I MHC HC molecules, although distinct at (presumably early) steps of dislocation that rely on ER membrane-resident proteins such as SPP and DERLIN-1, respectively, intercept at a (presumably posterior) p97-dependent step.

We are currently investigating the possible involvement of the catalytic activity of the SPP protease in US2-mediated dislocation (**Fig.27**).

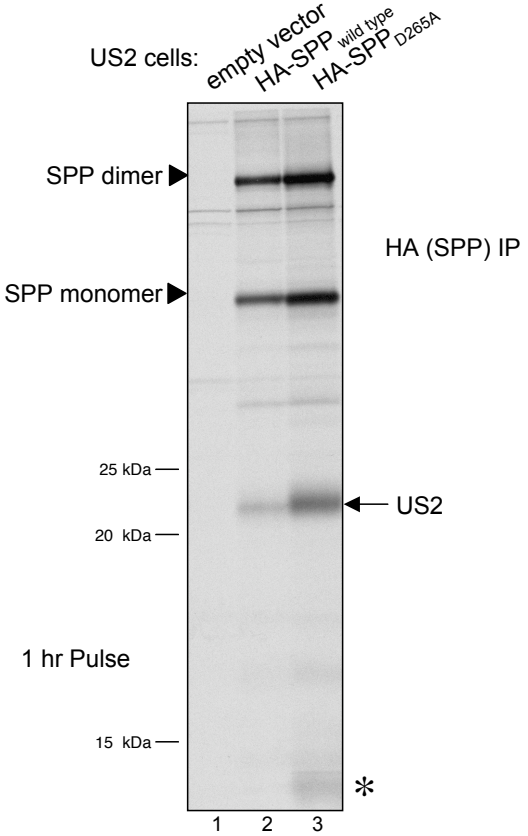

Figure 27. SPP associates with US2 irrespectively of its catalytic activity. US2 cells over-expressing HA-TEV-tagged SPP were metabolically labeled for 1 hour, lysed in 1% digitonin, immunoprecipitated with 12CA5 anti-HA antibody, and subjected to cleavage with TEV protease before loading on a 12.5% SDS-PAGE and fluorography. The radioactive labeling seen at the bottom of the gel labeled with an asterisk is of unknown origin.

Like all PS/SPPL proteases, SPP possesses two active site aspartate (D) residues within the conserved motifs Y*D* and LGLG*D* localized in adjacent membrane-spanning regions; the SPP catalytic dyad is formed by D218 and D265, presumably located in transmembrane domains (TMD) 4 and 5, respectively (**Fig.17**). Mutation of the conserved transmembrane aspartate D265 to an alanine (A) renders the SPP D265A mutant inactive in intramembrane proteolysis [298]. We generated an N-terminally HA-TEV-tagged SPP D265A mutant (HA-SPP_{D265A}) and obtained stable US2 cell lines expressing both HA-SPP_{wt} and HA-SPP_{D265A} by retroviral transduction.

We found that catalytically inactive SPP can still bind US2 (**Fig.27**). Lysates from metabolically labeled US2 cells expressing HA-SPP_{wt} or HA-SPP_{D265A} were immunoprecipitated with anti-HA antibody and subjected to cleavage with TEV protease. The anti-HA immunoprecipitates allowed the recovery of HA-SPP_{wt} and HA-SPP_{D265A} - seen in both dimeric and monomeric forms - as expected, as well as the recovery of US2 (all indicated by arrows in **Fig.27**). Most importantly, US2 is found in anti-HA immunoprecipitates from cells expressing both HA-SPP_{wt} and HA-SPP_{D265A} and, in fact, the amount of US2 recovered in association with catalytically inactive SPP is higher than that co-purified with wild type SPP (**Fig.27,** compare lane 3 with lane 2). The US2/SPP interaction may, therefore, not be dependent on SPP activity.

Interestingly, we found that SPP overexpression results in increased levels of the US2 protein (**Fig.28**).

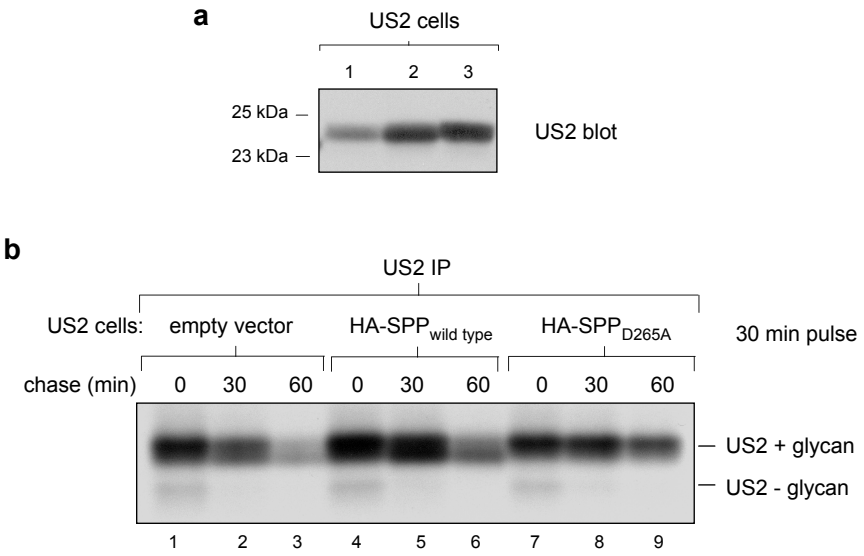

Figure 28. SPP overexpression results in increased stability of *N*-glycosylated US2 (US2+glycan). **a.** Steady state levels of the US2 protein are increased in cells overexpressing both wild type and catalytically mutant SPP. US2 cells expressing empty vector control (1), HA-$SPP_{wild\ type}$ (2) or HA-SPP_{D265A} (3) were lysed in 0.5% NP-40 and subjected to 15% SDS-PAGE and then immunoblot analysis with anti-US2 antibody. **c.** (US2+glycan) is stabilized in cells overexpressing HA-SPP, irrespectively of its catalytic activity. US2 cells expressing empty vector, HA-$SPP_{wild\ type}$ (2) or HA-SPP_{D265A} were pulsed for 30 min and chased for up to 60 min in the absence of proteasome inhibitor. Cells were then lysed with 0.5%NP-40, US2 molecules were immunoprecipitated with anti-US2 antibody and the immunoprecipitates subjected to 15% SDS-PAGE and fluorography. The positions of glycosylated (US2+glycan) and deglycosylated (US2-glycan) protein are indicated. IP, immunoprecipitation.

Anti-US2 immunoblot analysis of US2 cells expressing an empty vector control (1), HA-SPP_{wt} (2) or HA-SPP_{D265A} (3) reveals that the steady state levels of US2 are higher in cells expressing SPP, regardless of its activity (**Fig.28.a**, compare lanes 2 and 3 to control lane 1). As mentioned in the introduction, US2 is an unstable protein, subject to dislocation and proteasomal degradation [12,277]. The increased levels of US2 in cells expressing SPP therefore prompted us to investigate whether US2 dislocation and degradation was affected (**Fig.28.b**). US2 cells expressing an empty vector control, HA-SPP_{wt} and HA-SPP_{D265A} were metabolically labeled in the absence of proteasome inhibitor and lysates were immunoprecipitated with anti-US2 antibody (**Fig.28.b**). As expected, in US2 cells expressing the empty vector control, the (US2-glycan) protein disappeared very quickly during the course of the chase and (US2+glycan) was unstable, albeit to a smaller extent than (US2-glycan) (**Fig.28**, lanes 1-3). In cells that express HA-$SPP_{wild\ type}$ and HA-SPP_{D265A}, (US2+glycan) seemed to be stabilized (**Fig.28.b**, compare levels of US2+glycan present in lanes 6 and 9 to lane 3), suggesting that SPP overexpression results in impaired degradation and therefore stabilization of the (US2+glycan) protein. We are in the process of analyzing the effects of SPP overexpression on HC dislocation.

Since the bulk of the SPP protein is in the plane of the membrane and likely to be available for intramembrane contacts with the US2 transmembrane domain (US2 TMD), we decided to address whether or not the US2 TMD is also required for SPP interaction (**Fig.29**). We installed the K^bss-HA-TEV tag on a chimeric construct consisting of the US2 lumenal domain, the transmembrane segment of the unrelated type I membrane protein CD4 [314], and the US2 cytosolic tail, designated HA-US2/CD4/US2 for simplicity (**Fig.29.a**). Native (digitonin) lysates from HA-US2, HA-$US2_{186}$ or HA-US2/CD4/US2 cells were subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-SPP antiserum (US2 IP/SPP blot). Although HA-US2/CD4/US2 had a “wrong” transmembrane domain, SPP was still found in association with this US2 mutant (**Fig.29.b**). The expression levels of the HA-US2 molecules in the different cell lines were comparable as analyzed by

immunoblot with anti-US2 antibody (**Fig.29.b**, US2 blot). Because HA-US2/CD4/US2 is able to bind SPP, the US2 TMD must be dispensable for interaction with SPP.

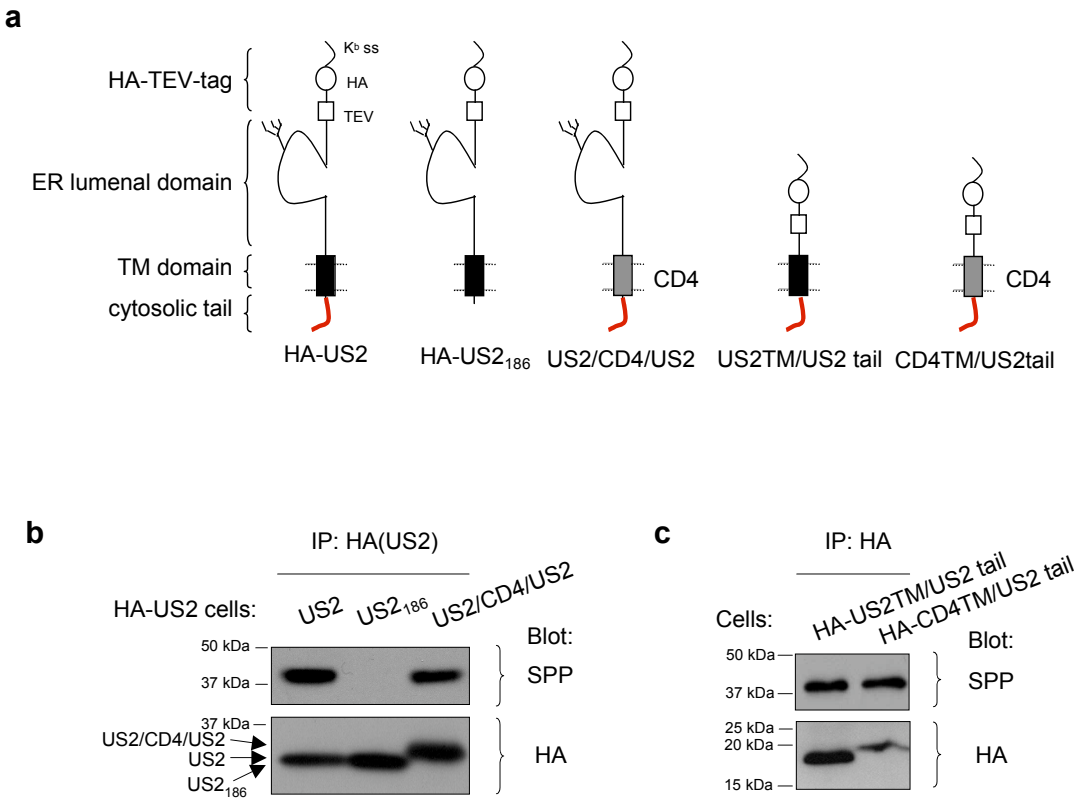

Figure 29. The US2 tail is both necessary and sufficient for recruitment of SPP. **a.** Schematic representation of K^bss-HA-TEV-tagged HA-US2, HA-$US2_{186}$, US2/CD4/US2, US2TM/US2tail and CD4TM/US2tail proteins. **b.** The transmembrane domain of US2 is not required for binding to SPP. Immunoprecipitations from native (1% digitonin) lysates of cells expressing the HA-US2 molecules indicated were performed with 3F10 anti-HA antibody to retrieve US2 and US2-associated proteins. Samples were subjected to 10% SDS-PAGE and immunoblot analysis with anti-SPP antibody and 12CA5 anti-HA antibody. **c.** The US2 tail is the sole requirement for binding to SPP. US2 cells expressing the HA-US2 "mini-proteins" were treated as described above. IP, immunoprecipitation.

We generated two molecules composed of the K^bss-HA-TEV tag, a transmembrane domain (the US2TMD or the CD4 TMD) and the US2 cytosolic tail, with the minimal sequence requirements to be targeted to the ER membrane but lacking the US2 lumenal domain (**Fig.29.a**). These "mini-constructs" were still capable of binding SPP, as seen by immunoblot analysis of anti-HA immunoprecipitates of cells expressing HA-US2TMD/US2tail and HA-CD4TM/US2 tail (**Fig.29.c**), showing that the US2 lumenal domain is not involved in binding to SPP. The Ig-like fold in the US2 lumenal domain is responsible for the allele-specific

association of US2 with HC [20], hence neither HA-US2TMD/US2tail nor HA-CD4TM-US2tail should be able to bind HC, implying that binding of US2 to SPP is not dependent on the presence of the substrate, HC. Moreover, because the HA-CD4TM-US2tail mini-protein (which contains no US2 sequence element other than the tail) is still able to recruit SPP (**Fig.29.c**), we conclude that the cytosolic tail of US2 is not only necessary but also sufficient for mediating association with SPP.

Curiously, the HA-US2/CD4/US2 molecule, although capable of recruiting SPP and of binding HC, is inactive in dislocation (**Fig.30**).

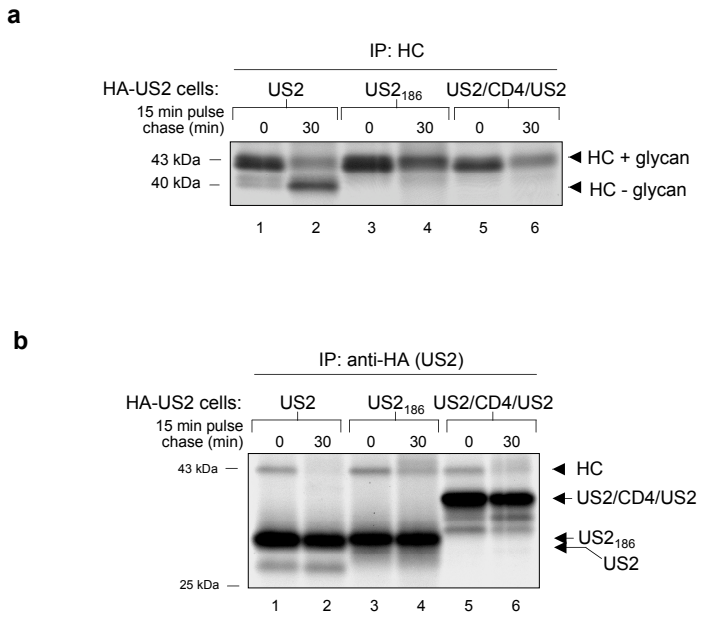

Figure 30. The US2 TMD is dispensable for association with SPP and HC but critical for dislocation. **a.** The HA-US2/CD4/US2 chimeric molecule is dislocation-incompetent. Astrocytoma cells expressing HA-US2, HA-US2$_{186}$, and HA-US2/CD4/US2 were pulsed for 15 min and chased for up to 30 min in the presence of the proteasome inhibitor ZL_3VS. HC molecules in 0.5%NP-40 lysates were recovered with anti-HC (HC-70) antibody and subjected to 12.5% SDS-PAGE and fluorography. The positions of the glycosylated and deglycosylated HC are indicated. **b.** The HA-US2/CD4/US2 chimeric molecule associates with HC. Astrocytoma cells expressing the HA-tagged US2 proteins were treated as described above, but immunoprecipitates were obtained with 12CA5 anti-HA antibody. The position of HC molecules associated with HA-tagged US2 is indicated.

We analyzed HC dislocation in cells expressing HA-US2, HA-US2$_{186}$, and HA-US2/CD4/US2 by pulse/chase in the presence of proteasome inhibitor. While HA-US2 could catalyze dislocation of HC as seen by the hallmark recovery of deglycosylated HC at the end of the chase (**Fig.30.a,** lane 2), no deglycosylated HC intermediate was detected in HA-US2/CD4/US2 cells (**Fig.30.a**, lane 6), much like what was seen for HA-US2$_{186}$ cells (**Fig.30.a**, lane 4). The HA-US2/CD4/US2 molecule contains the US2 lumenal domain, so it should retain the ability to bind HC. Indeed, the HA-US2/CD4/US2 mutant associates with HC (**Fig.30.b**). Anti-HA (US2) immunoprecipitates from cells expressing HA-US2/CD4/US2 revealed the presence of associated HC, readily visible at the onset of the chase (**Fig.30.b**,

lane 5), showing that the lack of dislocation activity of HA-US2/CD4/US2 is not due to lack of association with its substrate. The fact that a US2 construct lacking the US2 transmembrane domain (TMD) is not functional suggests that HC dislocation is dependent not just on (US2 tail-mediated) recruitment of SPP but also on additional (US2 TMD-mediated) interactions within the plane of the membrane.

US2 molecules may form oligomers that are dependent on the presence of the US2 transmembrane domain (**Fig.31**).

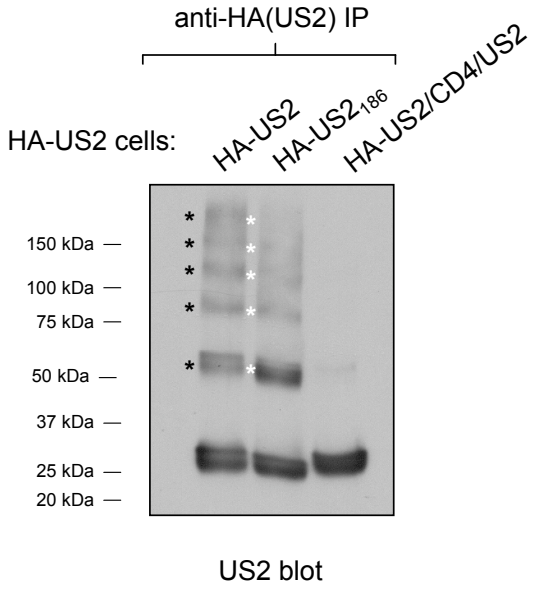

Figure 31. US2 molecules may form oligomers through interactions between the US2 transmembrane domains. Native (1% digitonin) lysates of astrocytoma cells that express HA-US2, HA-US2$_{186}$ and HA-US2/CD4/US2 were immunoprecipitated with 3F10 anti-HA antibody, subjected to 4-15% SDS-PAGE and immunoblotted with anti-US2 antibody. IP, immunoprecipitation.

We analyzed native lysates of cells expressing HA-US2, HA-US2$_{186}$ and HA-US2/CD4/US2 by immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-US2 antibody. Anti-HA immunoprecipitation from HA-US2 and HA-US2$_{186}$ cells contain monomeric HA-US2 and HA-US2$_{186}$ and higher order US2 antibody-reactive bands that are absent from HA-US2/CD4/US2 immunoprecipitates (**Fig.31**, asterisks). The molecular masses of the HA-US2 molecules (which both have a molecular mass of about 30kDa) and those of the anti-US2 antibody-reactive bands (estimated by visual inspection to be around 60, 90, 120kDa, and higher) are consistent with the possibility that they are dimeric, trimeric, and higher order oligomers of the HA-US2 and HA-US2$_{186}$ proteins (**Fig.28**). These anti-US2-reactive bands may simply represent US2 aggregates perhaps due to an overexpression

artifact. The fact that these anti-US2 antibody reactive-bands are absent from HA-US2/CD4/US2 immunoprecipitates, however, argues for specificity in formation of these structures and suggests that the US2 TMD may play a role in formation of oligomerized versions of US2.

We have started to investigate a possible role for a protein other than SPP that associates specifically with active US2, the NAD (P)-dependent steroid dehydrogenase-like protein (NSDHL) (**Fig.32**).

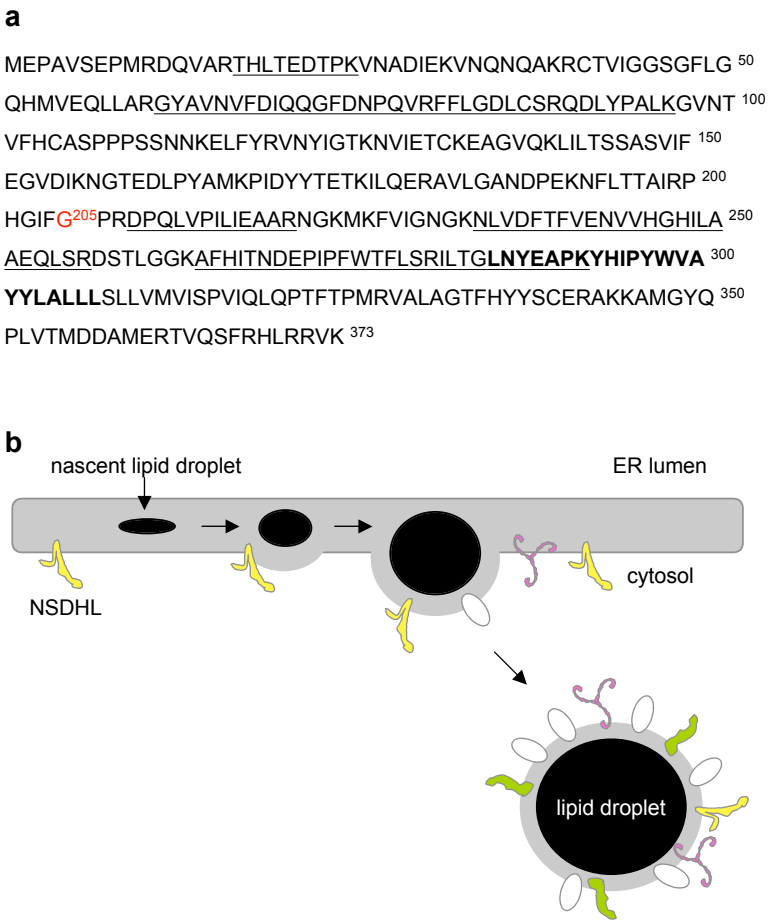

Figure 32. The NAD(P)-dependent steroid dehydrogenase-like (NSDHL) protein. **a.** NSDHL aminoacid sequence. The aminoacid sequences covered by the 8 peptides that were identified by MS/MS and attributed to NSDHL (see **Fig.16**, band 19) are underlined. The predicted transmembrane domain is shown in boldface type. Mutations in NSDHL, such as the glycine (G) 205 point mutant (in red), are commonly associated with CHILD syndrome. **b.** Schematic representation of the predicted topology of NSDHL and its trafficking from the ER membrane to lipid droplets. NSDHL is an enzyme involved in cholesterol biosynthesis, and is localized to the ER and to lipid droplet membranes. Lipid droplets are lipid storage structures, with an inner core composed of neutral lipids and a membrane that is decorated by proteins such as NSDHL and others. NSDHL is proposed to have both N- and C-termini in the cytosol.

Analysis by mass spectrometry of tryptic digests of the polypeptides in band 19 specific

for dislocation-competent HA-US2 (**Fig.16**) yielded 8 peptides that were assigned to the NSDHL protein with an overall 30% sequence coverage (111/373 aminoacids) (**Fig.32.a**). The NSDHL enzyme is involved in cholesterol biosynthesis: it functions as a C-3 sterol dehydrogenase (or decarboxylase) involved in the complex series of reactions that remove the two C4 methyl groups from the cholesterol precursor lanosterol [315-317]. Mutations in the NSDHL gene are associated with human congenital hemidysplasia with ichthyosiform nevus and limb defects (CHILD) syndrome, a very severe X-linked, male lethal embryonic developmental disorder, and mouse bare patches and striated mutations [315,318-321]. The 42 kDa NSDHL protein is expressed in all cells [322] and is mainly localized to the ER membrane and to lipid storage droplets [316,317]. The proposed topology of the NSDHL protein is one in which both the N- and C-terminal domains extrude from the ER membrane into the cytoplasm and flank a single transmembrane domain [317] (**Fig.32.b**). Lipid droplets are cytoplasmic structures with a single phospholipid bilayer presumably derived from the outer (cytoplasmic) ER membrane leaflet [323] surrounding a central neutral lipid core. NSDHL on the ER membrane is proposed to diffuse laterally to nascent lipid droplets [317], coating the surface of the lipid droplet while excluded from the lipid core, like other lipid droplet-associated proteins [324] (**Fig.32.b**).

NSDHL and US2 indeed associate in a US2 tail-dependent manner (**Fig.33**).

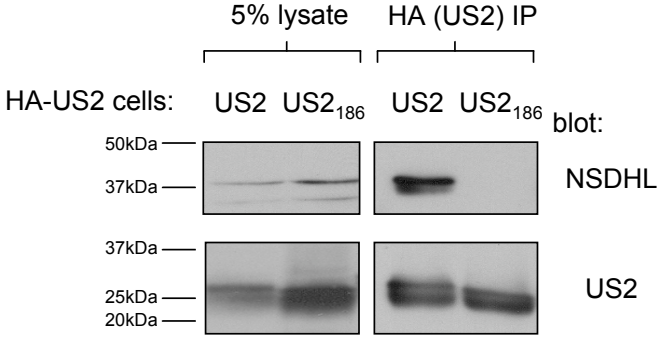

Figure 33. NSDHL associates with US2 in a US2 tail-dependent manner. Native (1% digitonin) lysates of astrocytoma cells expressing HA-US2 and HA-US2$_{186}$ were immunoprecipitated with 3F10 anti-HA antibody to retrieve US2 and US2-associated proteins. The immunoprecipitates were subjected to 4-15% SDS-PAGE and immunoblotted with anti-NSDHL and anti-US2 antibodies. IP, immunoprecipitation. Experiment conducted by Richard Wells.

We subjected digitonin (native) lysates from cells expressing HA-US2 or HA-US2$_{186}$ cells to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-NSDHL and anti-US2 antibody. We found NSDHL associated with HA-US2 while absent from

HA-US2$_{186}$ immunoprecipitates, confirming the specific interaction of NSDHL with wild type HA-US2 (**Fig.33**, NSDHL blot). The US2 levels were comparable as analyzed by immunoblot with anti-US2 antibody (**Fig.33**, US2 blot).

The US2 tail, in fact, is not only necessary but also sufficient for mediating interaction with NSDHL (**Fig.34**).

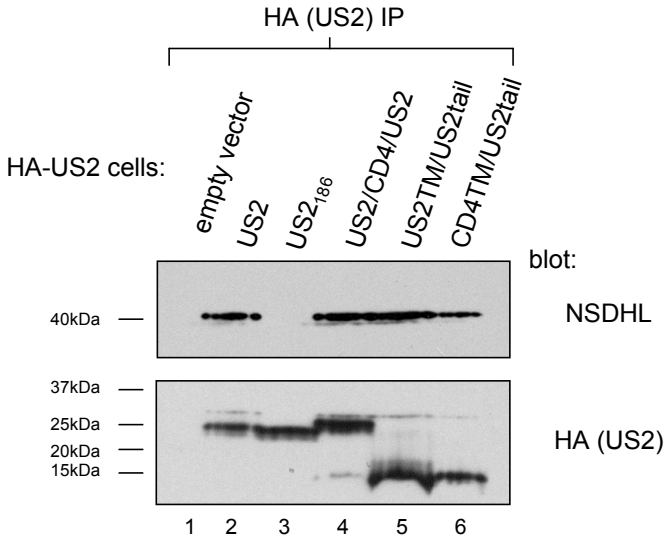

Figure 34. The US2 cytosolic tail is necessary and sufficient for recruitment of NSDHL. Native (1% digitonin) lysates of U373-MG astrocytoma cells expressing an empty vector control or the indicated HA-TEV-tagged constructs were immunoprecipitated with 3F10 anti-HA antibody. The immunoprecipitates were subjected to 4-15% SDS-PAGE and immunoblotted with anti-NSDHL and anti-HA (12CA5) antibodies. IP, immunoprecipitation. Experiment conducted by Richard Wells.

Digitonin lysates of U373 cells expressing an empty vector control or the several HA-TEV-tagged-US2 constructs used before (**Fig.26.a**), were immunoprecipitated with 3F10 anti-HA antibody and immunoblotted with anti-NSDHL antibody and anti-HA antibody (**Fig.34**). The HA-CD4TM/US2tail “mini-protein”, containing no US2 aminoacid sequence other than the US2 tail residues, still allowed recovery of NSDHL (**Fig.34**, lane 6), showing that the US2 tail is sufficient for recruitment of NSDHL and that the US2/NSDHL interaction is not dependent on the presence of HC molecules.

Interestingly, SPP and NSDHL do not seem to interact (**Fig.35**).

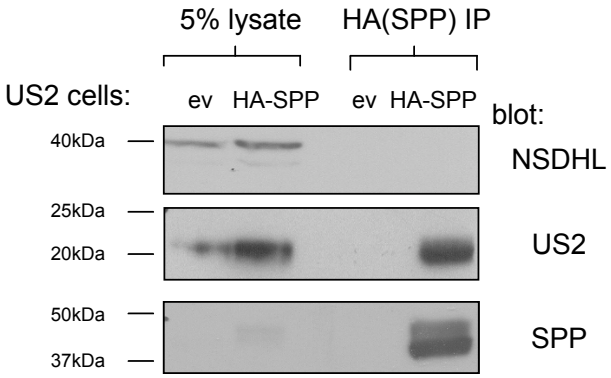

Figure 35. NSDHL and SPP do not seem to associate. Native (1% digitonin) lysates of U373-MG astrocytoma cells expressing an empty vector (ev) control or wild type HA-TEV-tagged SPP (HA-SPP) were immunoprecipitated with 3F10 anti-HA antibody. The immunoprecipitates were subjected to 4-15% SDS-PAGE and immunoblotted with anti-NSDHL, anti-US2 and anti-SPP antibodies. IP, immunoprecipitation. Experiment conducted by Richard Wells.

We analyzed anti-HA immunoprecipitates from digitonin (native) lysates of US2 cells that express an empty vector control or HA-tagged SPP by immunoblotting with anti-NSDHL, anti-US2 and anti-SPP antibodies. While US2 was readily recovered in association with HA-SPP (**Fig.34**, US2 blot), no NSDHL protein was seen in anti-SPP immunoprecipitates (**Fig.34,** NSDHL blot), suggesting that SPP and NSDHL do not associate, although both are recruited by means of the US2 tail, a stretch of only 14 aminoacid residues.

We are currently exploring NSDHL's contribution to US2-mediated dislocation by assessing the effects of RNAi-mediated NSDHL knockdown on HC levels. Our very preliminary results with expression in US2 cells of a mixture of two shRNAs against NSDHL, NSDHL.1 and NSDHL.2, by retroviral transduction, indicate that the stability of HC is increased in US2 cells in a manner that is proportional to the degree of NSDHL knockdown (data not shown). We are cloning more NSDHL shRNA-expressing retroviruses (NSDHL.3, NSDHL.4 and NSDHL.5) to further characterize this putative effect.

Chapter 3. Discussion

US2 and US11 act at different stages of the class I MHC biosynthetic pathway

US2 and US11 differ in their ability to target unfolded class I MHC molecules

The HCMV US2 and US11 immunoevasins target newly synthesized class I MHC heavy chains for dislocation and proteasomal degradation, causing downregulation of class I MHC complexes from the cell surface, thus allowing HCMV to remain undetected by the host immune system [4]. Although the substrate (HC molecules) and the outcome (proteasomal degradation) of the dislocation reaction catalyzed by US2 and US11 is the same, the two viral proteins display clear differences in the mechanistic details of one of the initial stages of the dislocation process, substrate recognition (**Figs.7,9,10**). To examine whether US2 and US11 target distinct folding states of the class I MHC complex, we mutated the two cysteine residues that form the disulfide bridge within the membrane-proximal Ig-like alpha 3 domain of the class I MHC heavy chain. This unfolded HC mutant is unstable in US11-expressing astrocytoma cells, but stable in US2 cells (**Fig.11**), which suggests that US11 can mediate dislocation and proteasomal degradation of HC molecules regardless of their tertiary structure or folding status, while US2 seems to require properly folded HCs as a substrate.

In support of this notion, US2 binds the properly folded and assembled trimeric class I MHC complex, as seen by analysis of the quaternary structure of the class I MHC HC/beta$_2$m/peptide complex [271]. Moreover, although both US2 and US11 can bind W6/32-reactive material, only US11 can be found in immunoprecipitates of free class I MHC heavy chains [20], and US2-mediated HC dislocation is impaired in astrocytoma cells with reduced levels of the beta$_2$m (or class I MHC light chain) subunit obtained by RNAi [292]. However, heavy chain molecules expressed in beta$_2$m-defficient human melanoma cells are presumably targeted by both US2 and US11 [272] (**Fig.10,** +/-). The reason for this discrepancy is presently unknown, but we hypothesize it may result from cell line-specific characteristics.

We conclude that US11 recognizes a motif that arises earlier in the class I MHC biosynthetic pathway, whereas US2 acts at a stage where folding and assembly of the class I MHC molecules has already taken place. It seems paradoxical that HCMV should encode two proteins, US2 and US11, with apparently redundant functions. As strong selection pressures constantly maintain compact virus genomes, it is a fair assumption that expression of both US2 and US11 benefits HCMV. Perhaps by focusing on different stages of the class I MHC

biosynthetic pathway, US2 and US11 safeguard against the escape of class I MHC molecules from the ER.

Signal peptide peptidase (SPP) is required for HC dislocation by US2

The US2 tail recruits SPP

Although US2 and US11 catalyze dislocation and degradation of the same substrate, HC molecules, the viral proteins do not utilize the same dislocation machinery [21]. US11 recruits DERLIN-1, a component of a multiprotein complex at the ER membrane which encompasses functions from substrate selection to dislocation, ubiquitination, ER membrane extraction and degradation in the cytoplasm [22,215]. Because the mechanistic details of the US2- and the US11-mediated dislocation reaction differ and because dislocation by US2 is not dependent on DERLIN-1 [21], we decided to investigate the identity of the cellular dislocation machinery hijacked by US2. We conducted a functional biochemical screen for proteins involved in US2-mediated dislocation, screening for proteins that were selectively co-immunopurified with an HA-TEV-tagged version of dislocation-efficient full length US2 and not with HA-TEV-tagged dislocation-ineffective $US2_{186}$, a cytosolic tail deletion mutant that is inactive in HC dislocation (**Fig.16**). We first showed that addition of the HA-TEV-tag containing an exogenous signal sequence (that of the K^b mouse class I MHC molecule) followed by an HA epitope and a TEV protease recognition sequence allowed recovery of the HA-US2 molecules by immunoprecipitation with anti-HA antibodies and elution of US2 and US2-associated proteins by TEV-protease-mediated cleavage, as well as targeting of the HA-US2 constructs to the ER membrane and association with the substrate molecules (class I MHC HC molecules) (**Figs.12,13,14**). We then demonstrated that, like untagged US2 and $US2_{186}$ molecules, HA-US2 and HA- $US2_{186}$ display distinct functional properties depending on the presence or absence of the US2 cytosolic tail (**Fig.15**). HA-US2 is active in catalyzing HC degradation by inducing its dislocation from the ER membrane with ensuing proteasomal degradation, as can be seen by the failure to detect HC levels at steady state in HA-US2 cells (**Fig.15.a**), or by the recovery of the deglycosylated HC intermediate from metabolically labelled HA-US2 cells treated with proteasome inhibitor (**Fig.15.b**). In contrast, HA-$US2_{186}$ is completely non-functional, as steady state levels of HC molecules similar to those present in control cells can be readily detected in HA-$US2_{186}$ cells (**Fig.15.a**), and no HC deglycosylated intermediate can be detected in metabolically labeled HA-$US2_{186}$ cells (**Fig.15.b**). Rather, HC

accumulates in a glycosylated (HC+glycan), presumably ER membrane-associated state that is protected from the deglycosylation activity of the cytosolic PNGase (**Fig.15.b**, lane 4). Furthermore, comparison of the mutually exclusive retrieval of free HCs with an anti-HC antibody [325] (**Fig.15.b**) and assembled HCs, with the W6/32 antibody (**Fig.14.b**), can establish what fraction of the HC molecules are assembled with beta$_2$m and therefore allowed to progress through the secretory pathway [12,27,326,327]. The amount of HCs that acquire W6/32-reactivity by the end of the chase period is increased in HA-US2$_{186}$ cells when compared to HA-US2 cells (**Fig.14.b**, compare lanes 2 and 4 for amount of HC and beta$_2$m recovered), suggesting increased assembly of the class I MHC complex, likely because HC molecules are not targeted for degradation (**Fig.15.b**). Therefore, and despite a very stable interaction between HA-US2$_{186}$ and class I MHC molecules (**Fig.14.a,b**), tailless HA-US2$_{186}$ only retains HCs in the ER very transiently: HC molecules initially associate with HA-US2$_{186}$ but are not targeted for dislocation from the ER membrane and are then allowed to exit the ER and progress through the secretory pathway (**Fig.14.b**). The US2 tail is, therefore, required for targeting of HCs for dislocation.

We hypothesized that proteins that associate only with active full length HA-US2 may constitute good candidates for cellular partners of US2 in its dislocation function. We identified the ER-resident intramembrane-cleaving aspartic protease signal peptide peptidase (SPP) as one of the specific interaction partners for HA-US2 (**Fig.16**) and showed that this highly specific interaction is critically dependent on the presence of the US2 tail (**Figs.19, 20, 21**).

SPP is required for US2-mediated HC dislocation

RNAi-mediated reduction of SPP protein levels in US2 cells results in impairment of HC degradation (**Fig.22**). HC molecules in US2 cells are stabilized by SPP knockdown, as can be seen by the elevated steady state levels of HC in SPP knockdown cells expressing three distinct shRNA constructs (**Fig.22.a**). In US2 cells expressing normal (control) levels of SPP, newly synthesized glycosylated HCs (HC+glycan) are converted almost to completion into a deglycosylated, cytosolic form (HC-glycan) (**Fig.22.b**, lanes 1 to 3). Total conversion of (HC+glycan) into (HC-glycan) would be visible at an additional 60 min chase point. Notwithstanding the incomplete dislocation observed in control cells, in cells with knockdown levels of SPP a smaller fraction of HC molecules are dislocated from the ER membrane into the cytosol, as can be seen by the reduced ratio of (HC-glycan) to (HC+glycan) (**Fig.22.b**,

lanes 4 to 6). We conclude that the US2 tail-recruited SPP is involved in HC dislocation mediated by US2.

SPP is a presenilin-type intramembrane-cleaving aspartic protease

The human genome encodes seven related members of the PS/SPPL superfamily of GXG*D* aspartic proteases, also referred to as PS homologues (PSHs) or intramembrane proteases (IMPAS): presenilin 1, presenilin 2, SPP and four SPP-like proteins, SPP2a, SPP2b, and SPP2c and SPP3 [299]. In humans, SPP is responsible for clearance of signal peptide remnants from the ER membrane: upon insertion of secretory proteins into the ER, their signal sequence is cleaved by the ER luminal protein signal peptidase, leaving the signal peptide anchored in the ER membrane. The ER membrane–anchored signal peptide is subsequently cleaved within the transmembrane region by the intramembrane-cleaving SPP, which is specific for type II-oriented membrane segments. The resulting signal peptide fragments are released into the cytosol (N-terminal portion) or into the ER lumen (C-terminal portion). The latter peptides may easily bind to class I MHC molecules in the ER lumen. The HLA-A2 molecule, for instance, is known to bind signal sequence-derived peptides [29].

Presenilin (PS) 1 and -2, on the other hand, are the catalytic components of gamma-secretase, a high molecular weight complex containing PS and three other subunits, nicastrin, APH-1 and beta-catenin [302]. Gamma-secretase plays a role in processing of the beta-amyloid precursor protein (APP) into $A_{beta}40$ and $A_{beta}42$, peptides that constitute the principal components of the beta–amyloid plaques in Alzheimer's disease (AD); gamma-secretase-mediated processing of the Notch receptor is required for development [328]. Presenilins also possess gamma-secretase-independent functions, functioning as ER Ca^{2+} leak channels [329] and in intracellular trafficking [330,331].

Although the sequence homology is limited, the protein sequences of PSs, SPP and SPP-like aspartic proteases can be aligned almost throughout the entire length [332] as they all have a similar multispanning membrane topology. Aspartic protease transition state analogues, in fact, target both PSs and SPP and inhibit both activities [333]. SPP and SPPLs share high homology particularly in the C-terminal half of the molecules, which contains the catalytic residues and the conserved sequence PAL motif and, therefore, their mechanism of action is presumed to be similar [299].

Substrate recruitment and subsequent cleavage by intramembrane proteases are separable events, as the substrate-docking site is external to the catalytic cavity and can be

blocked by substrate analogs without preventing catalytic activity [334-337]. Another common feature is that PS and SPP substrates must be generated by a prior cleavage event: SPP, for example, can only cleave signal sequence remnants in the ER membrane subsequently to signal sequence cleavage by signal peptidase [338].

Despite common structural features, there are major differences between PSs and SPP. The most striking difference is the opposite orientation of the catalytic domains within the plane of the membrane, with PSs cleaving type I-membrane substrates (such as the Notch receptor and APP) and SPP cleaving type II-oriented transmembrane peptides (such as the aforementioned signal sequences and hepatitis C virus core protein precursor). In terms of substrate recognition, SPP displays an important difference with respect to gamma-secretase: the apparent requirement for one or more helix-breaking and –bending residues in the transmembrane region of the target, possibly to add the necessary flexibility to its rigid helical structure as to expose the scissile peptide bond to proteolytic attack [338]. Furthermore, SPP-mediated cleavage is also dependent on the identity of residues flanking the membrane-spanning region, thought to affect the perpendicular flexibility of the transmembrane helix [338]. Moreover, PSs undergo endoproteolysis for activation and appear to act as the proteolytic subunits of gamma-secretase, whereas SPP activity requires neither endoproteolytic activation nor additional subunits, although it most likely requires homodimerization: the vast majority of SPP in mammalian cells presumably exists in the dimeric form and the SPP dimer is labeled by transition-state analog inhibitors of gamma-secretase that only react against the active protease [339], suggesting that the biologically active form of SPP is the dimer [310]. The function of the four SPP-like proteins is, at this point, unknown [28].

SPP plays a role in diverse cellular processes

SPP and generation of HLA-E epitopes. The first process to be discovered that depends on SPP was the generation of the peptide ligands for the non-classical class I MHC molecule HLA-E [29] (**Fig.36**).

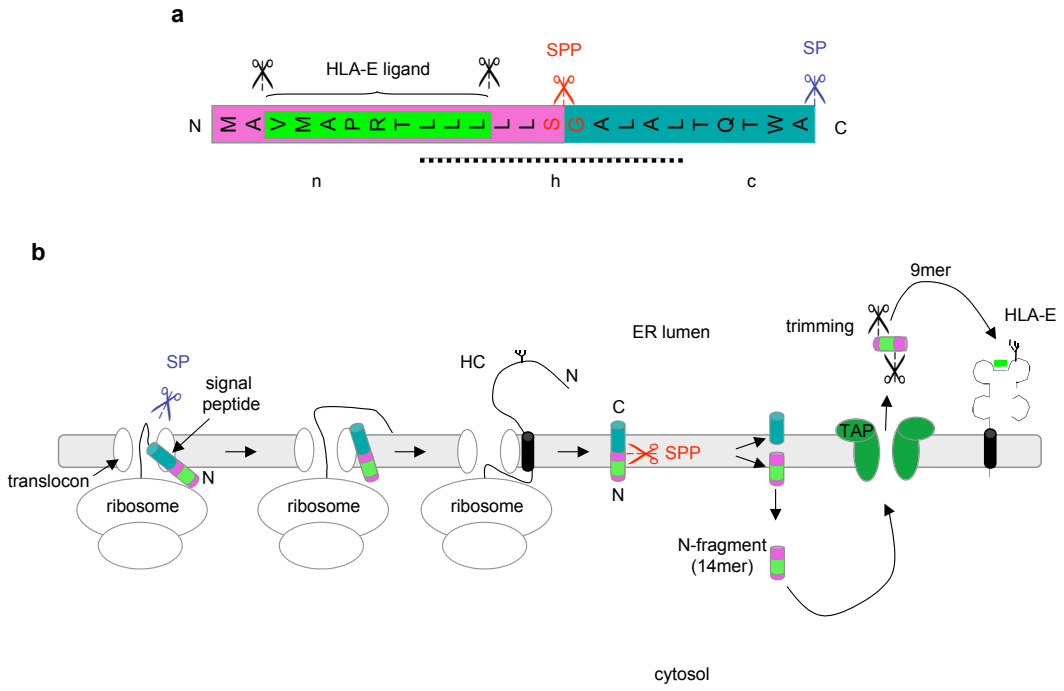

Figure 36. SPP and generation of HLA-E peptide ligands. **a.** Signal peptides from classical (polymorphic) class I MHC heavy chains contain a highly conserved stretch of nine aminoacids (9mer), the HLA-E epitope (shown in green). SPP cleaves at helix-breaking residues (shown in red) within the hydrophobic (h) region (underlined) of the signal peptide. **b.** During biosynthesis, the class I MHC HC signal peptide is first cleaved by signal peptidase (SP) and then processed by SPP. The HLA-E ligand-containing N-terminal fragment (14mer) is released into the cytosol and TAP-transported back into the ER lumen. Trimming of the N-terminal fragment in the cytosol and/or in the ER lumen generates the 9mer epitope that is loaded on the non-classical monomorphic class I MHC HLA-E molecule for presentation at the cell surface to natural killer cells.

A characteristic feature of a signal sequence is its tripartite structure: a polar N-terminal n-region, a hydrophobic core (h-region) of 7–15 residues, within which the SPP cleavage site is located, and a polar C-terminal c-region that contains the consensus sequence for signal peptide cleavage [340]. During biosynthesis of polymorphic class I MHC molecules, their signal sequences are first cleaved by signal peptidase, liberating a membrane-spanning peptide target for SPP. Signal sequences from polymorphic class I MHC heavy chains such as HLA-A

and HLA-B contain a highly conserved stretch of nine aminoacids that constitutes the HLA-E epitope. SPP cleaves at helix-breaking residues within the hydrophobic (h) region of the signal peptide in the center of the membrane-spanning helix. The HLA-E epitope-containing N-terminal cleavage product is released into the cytosol and then TAP-transported into the ER lumen. Trimming of the N-terminal fragment to a nine-residue-long peptide then allows loading on the non-classical class I MHC molecule HLA-E [28]. By presenting fragments derived from their signal sequences, HLA-E reports proper biosynthesis of polymorphic class I MHC molecules, delivering an inhibitory signal to NK cells that usually prevents activation of the NK cell cytotoxic program [67]. Therefore, in humans, SPP performs an important immunological surveillance function.

SPP and processing of the hepatitis C virus core protein. SPP is involved in processing of the hepatitis C virus (HCV) core protein [306] (**Fig.37**).

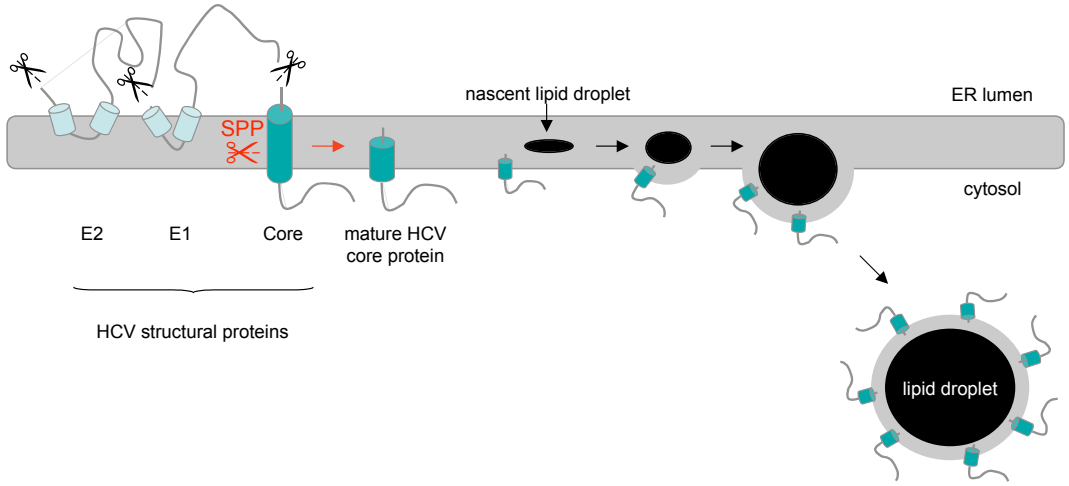

Figure 37. SPP and maturation of the hepatitis C virus (HCV) core protein. The HCV core protein is the most N-terminal component of the viral polyprotein, followed by the membrane glycoproteins E1 and E2. The E1, E2 and core proteins constitute the HCV structural proteins. Targeting of the nascent HCV polyprotein to the ER membrane is directed by an internal signal sequence located between the core protein and E1. Core protein maturation comprises two cleavage events, signal peptidase (SP)-mediated processing of the signal sequence, and SPP-mediated cleavage within the HCV core membrane-spanning region. The latter cleavage event allows release of the mature core protein from the ER membrane and incorporation into budding lipid droplets. Lipid droplets are lipid storage structures, with an inner core composed of neutral lipids and a protein-decorated membrane.

HCV is a single-stranded RNA virus with a single open reading frame encoding a large polyprotein. The N-terminal portion of the HCV polyprotein encodes the structural components of the HCV virion, the core protein (thought to constitute the virion capsid) and the E1 and E2 envelope glycoproteins. The mature structural components of the HCV virion are produced through a series of cleavage events catalyzed by cellular proteases [299]. The core protein is the

most N-terminal portion of the polyprotein and is followed by the signal sequence of the E1 envelope glycoprotein. The E1 signal sequence targets the polyprotein to the ER membrane and induces translocation of E1 into the ER lumen. Cleavage by signal peptidase liberates the N-terminal end of E1, leaving the core protein anchored (by the E1 signal peptide) in the ER membrane. SPP-mediated intramembrane proteolysis of the E1 signal sequence then results in release of the HCV core protein from the ER membrane [306], thus freeing the mature core protein for incorporation into lipid droplets [299]. SPP-mediated HCV core protein maturation and trafficking to lipid droplets and the outer mitochondrial membrane may be critical for viral assembly and life cycle [341] and may also affect cellular lipid metabolism and apoptosis [342-348]. It is possible that SPP serves as a modulator of these important cellular functions of HCV.

SPP and calmodulin signaling. SPP may regulate the interaction of signal peptide remnants of HIV gp160 envelope protein and preprolactin (p-Prl) with calmodulin. While the n-region of most signal sequences comprises only a few residues, some signal sequences have extended n-regions, of up to 150 residues. The function of such long n-regions is not known. Both the p-Prl and the gp160 signal sequence have an extended basic n-region that can potentially form a basic amphiphilic alpha-helix, a feature of CaM-binding domains [349], not found in the majority of signal sequences. SPP-mediated cleavage releases this CaM-binding domain on the N-terminal fragment of the p-Prl and gp160 signal peptides into the cytosol. The functional and physiological significance of an interaction between the p-Prl and p-gp160 signal peptide fragments that are released into the cytosol and calmodulin (CaM) could be due to a regulatory function of the signal peptide fragments. CaM-dependent processes could be enhanced or inhibited depending on the amounts of CaM-binding signal peptide fragments generated and released into the cytosol [307].

SPP and development. The SPP orthologues in *Drosophila melanogaster, Spp,* and in *Caenorhabditis elegans*, *Imp-2*, play an essential role in development: SPP protease activity seems to be essential for larval development both in the fly and in the nematode [350,351]. Although the mechanism by which SPP mutations impairs developmental processes in the fly is currently unknown, in *C. elegans* the molting defect induced by *imp-2* deficiency was mimicked by cholesterol depletion and by deficiency in *lrp-1*, a homolog of mammalian lipoprotein receptor-related protein (LRP) receptors, suggesting a role for SPP in cholesterol and lipid metabolism [351].

A role for SPP in ER quality control and degradation

A role for SPP in ER quality control was first proposed by Stephen High and colleagues, who reported an association between SPP and a truncated version of a polytopic ER protein (opsin) in an *in vitro* system, and proposed SPP to be implicated in the recognition of misassembled transmembrane domains during membrane protein quality control at the ER [308]. US2-mediated dislocation of class I MHC HC molecules provides the first functional evidence of such a role for the intramembrane-cleaving protease SPP [352]. The precise mechanism/function of SPP in ER dislocation is presently unknown.

We showed that the US2 tail is not only necessary but also sufficient to recruit SPP (**Fig.29**). One of the unanswered questions is the location of the US2 tail-binding site on the SPP molecule. Direct or indirect binding of the US2 tail to SPP may occur within the cytosol-exposed surface of the molecule, or perhaps within the SPP substrate-docking site or the SPP catalytic site. In theory, the US2 tail, due to its unusually hydrophobic character considering its cytosolic disposition (**Fig.8.b**) could fold back into the membrane and access SPP even within the substrate-docking or proteolytic core surfaces. Any of the proposed direct interaction modes with the US2 tail could modulate SPP structure and/or enzymatic activity during dislocation.

An obvious possibility for how SPP contributes to ER dislocation is through its catalytic activity. Assuming the occurrence of a cleavage event during dislocation, which at this point has not been shown, it may occur within the transmembrane domain (TMD) of HC, US2 or another unidentified protein. Cleavage may be necessary for dislocation by contributing to extraction of HC or of US2 from the ER membrane, for example. SPP-mediated intramembrane cleavage within the HC TMD is, however, inconsistent with the observed recovery of full length HC in the dislocation reaction [12,13,265,292]. Instead, US2 could be subject to intramembrane cleavage by SPP as to “mature” US2 into a dislocation-effective form, in a manner analogous to the SPP requirement for maturation of the HCV core protein [306], or as a requirement for dislocation of US2 itself [12]. SPP-mediated intramembrane proteolysis requires that its hydrophobic membrane-spanning substrate has access to the SPP catalytic core in a type II orientation [299,338]. US2 (like HC) is a type I membrane glycoprotein, so the topology of its transmembrane domain is opposite to that of predicted SPP substrates. Intramembrane cleavage of the US2 TMD by SPP in this inverted orientation would presumably not occur, as

seen for other proteases [353,354]. Occupancy of the SPP catalytic site by an uncleavable "substrate" due to its wrong membrane orientation, as might be the case for the US2 TMD, could block SPP activity and thus affect HC dislocation. As an additional alternative, SPP may cleave a protein X in *trans* that influences export of HC or US2 from the membrane, either due to HC or US2 extraction along with protein X or due to release of a fragment of protein X with a post-cleavage regulatory role in the dislocation process.

The involvement of SPP need not be related to its catalytic activity. The cytosolic US2 tail may bind to the SPP molecule and modulate the structural properties of SPP in a way that favors HC dislocation. Proteolytic activity-dependent and proteolytic activity–independent functions of presenilins (PSs) have been reported [329,331,355] and likewise, SPP may be crucial for HC and/or US2 dislocation and degradation irrespectively of its catalytic properties. For example, PSs function as Ca^{2+} leak channels at the membrane, with important consequences for Ca^{2+} signaling and neuronal development [329]. Additionally, gamma-secretase-mediated cleavage of a large number of type I transmembrane proteins releases their C-terminal fragments (CTFs) [303,304]. PS1 knockout mice show delayed turnover of these gamma-secretase substrates, which accumulate as full-length proteins [331,356,357], leading to the proposal that the gamma-secretase activity of presenilins is required for degradation of these proteins. However, treatment with gamma-secretase inhibitors does not phenocopy PS deficiency [331], suggesting rather a gamma-secretase activity-independent effect. The US2 tail-recruited SPP may, for example, function as an adaptor protein for a dislocation complex, as a scaffold for recruitment of other dislocation factors, or even as a channel for dislocation.

We have no evidence to support this notion, but it is conceivable that SPP could form a dislocon: monomeric or dimeric SPP could form a channel in the ER membrane with 7 to 14 transmembrane domains through which dislocation substrates could be extracted. Should SPP form a dislocation channel in the ER membrane, binding of the US2 tail could cause changes in SPP structure that would trigger opening of the channel and HC dislocation from the ER membrane. This proposition is, however, entirely speculative. In the case of DERLINs, which span the membrane 4 times and are capable of oligomerization, a possible function as a channel was proposed [26], which, although supported by some experimental evidence [21,26], is still extremely controversial.

A less contentious hypothesis would be that US2 tail-recruited SPP is a component of a multiprotein dislocation complex that is specific for US2 in terms of the constituting subunits, yet perhaps similar in organization to that described for US11 [22,215]. The HA-CD4TM/US2tail

mini-construct (HA-CD4/US2) does not contain any portion of the US2 lumenal region, which possesses the class I MHC contact motifs that determine substrate selection (**Fig.8**) [271], and therefore cannot bind or degrade HC. Yet, HA-CD4/US2 is still capable of binding SPP (**Fig.29**), implying that binding to the substrate (HC) is not a pre-requisite for the association of US2 with SPP. In support of this, at steady state the US2/SPP association is readily observed despite almost undetectable levels of HC molecules (**Figs.15, 19**). US2 may therefore function as an adaptor molecule or a bridge that brings HC to a putative SPP-containing multiprotein dislocation complex [22] (**Fig.38**).

These multiprotein complexes are usually organized by an E3 ligase (Hrd1p/Der3p and Doa10p in yeast, perhaps HRD1/SEL1L or gp78 in mammals) catalyzing not only ubiquitination of the substrate but also substrate selection, dislocation, extraction from the membrane and proteasomal degradation through assembly with individual subunits capable of the various required activities [22,212,213,215,221,222]. SPP may function as a substrate adaptor, as has been proposed for DERLIN-1, in a dislocation pathway for a subset of ER degradation substrates that may include misfolded transmembrane proteins such as truncated opsin [308] and that is subverted by US2 to dislocate HC molecules. SPP may also function as a scaffolding protein, as its proposed multispanning topology would allow it to function in recruitment/assembly of proteins located in the ER lumen, the ER membrane or the cytosol to this putative complex. We do not know the identity of the E3 ligase or E2 enzymes involved in US2-mediated HC dislocation, as we did not retrieve any of these in our pull down (**Fig.16**), but identification of SPP association partners in US2 cells could help clarify this hypothesis.

SPP catalytic activity and HC dislocation

We have initiated a collaboration with Andrew C. Nyborg, a post-doctoral fellow in the laboratory of Todd E. Golde (Department of Neuroscience, Mayo Clinic Jacksonville, Florida, USA) that has developed a luciferase-based assay for monitoring SPP activity in mammalian cells [358]. Our very preliminary data suggest that expression of HA-US2, but not HA-US2$_{186}$ or HA-US11, results in down-modulation of SPP activity (Andrew Nyborg and Todd E. Golde, unpublished observations). Down-modulation of SPP activity should result in perturbations of HLA-E expression at the cell surface and, consequently, NK cell-mediated lysis of US2-expressing cells, which is in disagreement with previous reports. It would be interesting, however, to investigate whether or not the functions of the HCMV US2 immunoevasin extend beyond downregulation of classical class I MHC locus products from the cell surface (through

SPP-dependent ER dislocation) to avoid $CD8^+$ T cell recognition, to modulation of HLA-E expression (again through interaction with SPP) and avoidance of NK cell surveillance.

In the case of presenilins, for example, the C-terminal PAL motif has been shown to be important for correct orientation of the catalytic site [300], illustrating how these intramembrane-cleaving proteases are sensitive to structural perturbations. We predict that US2 binding to SPP could perturb SPP activity through disruption or occupancy of protein-protein interaction domains or through occupancy/blockage of the substrate-docking site or catalytic cavity, thus affecting access of true SPP substrates to the SPP catalytic core. We are in the process of determining whether the downmodulatory effect on SPP-mediated proteolysis seen for US2 is dependent on the US2 tail - by making use of the different HA-US2 mutants (Fig.21) – and whether or not there is a correlation between SPP activity and HC dislocation.

We have started to characterize the role of SPP activity through over-expression studies. Over-expression of a catalytic mutant of SPP with a mutation in one of the aspartate (D) residues in the catalytic dyad, SPP D265A. US2 can be found in association with catalytically inactive SPP (**Fig.27.a**). Obviously, the over-expressed mutant SPP molecules may form heterodimers with endogenous (untagged), catalytically active SPP, in which the latter alone may account for the presence of US2 molecules. We observe higher levels of US2 in association with catalytically inactive SPP when compared to equally over-expressed catalytically active SPP (**Fig.27.b**). It may be that the US2 tail can access the catalytic cavity of SPP independently of SPP catalytic activity but requires catalytic activity for release from the catalytic cavity, explaining the increased recovery of US2 in association with mutant SPP. Intriguingly, US2 is stabilized, i.e., less subject to dislocation in cells over-expressing SPP irrespectively of its catalytic activity (**Fig.28**) as well as in cells with knockdown levels of SPP (data not shown), arguing for a proteolytic cleavage-independent role of SPP in US2 dislocation. We are conducting experiments to address this and to assess the effects of SPP overexpression in HC dislocation by US2. Our rather preliminary results suggest that over-expressed SPP impairs HC irrespectively of its catalytic properties (data not shown). US2 is proposed to be dislocated along with class I MHC complexes [12], in which case degradation of US2 would be dependent on HC degradation. Indeed, in melanoma cells that do not express HC, US2 is not degraded [359]. The fact that SPP overexpression as well as SPP knockdown may affect US2 dislocation and HC dislocation supports the aforementioned hypothesis that US2 and HC are dislocated as a whole structure. It would also support the recovery of

ubiquitinated W6-32-reactive/folded class I MHC molecules from US2 cells [27]. HC and US2 degradation, however, differ in terms of DERLIN-1 and SEL1L requirements [21,214]. Further experiments are necessary, therefore, not only with the aforementioned SPP activity assay and catalytic mutants of SPP but also with SPP inhibitors, to allow a conclusive assessment of the contribution of the proteolytic properties of SPP to US2 and HC dislocation.

A more generalized role for intramembrane proteolysis in ER dislocation?

Intramembrane-cleaving proteases comprise three mechanistic classes: metalloproteases, the rhomboid serine proteases, and the PS/SPPL aspartic proteases [360]. Curiously, the rhomboid serine proteases, share a highly evolutionarily conserved homology domain of unknown function with the DERLINs [336]. This motif consists of a tryptophan (W) and arginine (R) motif and a closely located histidine residue. In rhomboids, mutations in the WR motif at least partially inhibit enzyme activity. The WR motif is situated in the center of a well-conserved loop between the two first transmembrane domains that folds back within the membrane in a potentially unstable conformation, where it is proposed to form a substrate gating mechanism [337]. It is tempting to speculate that our observations extend the connection from regulated intramembrane proteolysis to a direct involvement in ER dislocation, and that perhaps this motif in DERLIN proteins is required for their role in ER dislocation. PSs and the other SPP-like members of the PS/SPPL superfamily of intramembrane-cleaving aspartic proteases, so far of unknown function [299], and some of which may not reside in the ER [361], may likewise be involved in disposal of different degradation substrates. HCMV might just be exploiting a cellular degradation pathway that involves intramembrane-cleaving proteases for disposal of class I MHC heavy chains.

Other roles for SPP in vivo?

Although we have no evidence to support this hypothesis, SPP's role in dislocation of proteins from the ER to the cytosol could, in principle, be of importance in contexts such as cross-presentation and exit of bacterial toxins and viruses from the ER. As mentioned in the introduction, professional APCs and in particular dendritic cells are able to cross-prime naïve $CD8^+$ T cells for initiation of a T cell response to a foreign antigen [48]. There is considerable controversy as to the subsets of APCs endowed with this property, the exact nature of the antigen acquired, modes of antigen acquisition, and the intracellular mechanisms leading to

cross-presentation [43,47,362-364]. Nevertheless, this exogenous material is somehow processed, loaded and presented by class I MHC molecules, which implies that these antigens must seemingly cross the membrane of the phagocytic/endocytic compartment and gain access to cytosolic proteasomes [365]. How these exogenous antigens reach the cytosol is rather controversial. One of the possible mechanisms is fusion of the ER membrane with phagosomes [366,367], which would allow the ER dislocation machinery to have access to the antigenic proteins. The first reports of a role for proteins involved in degradation of misfolded ER proteins, such as p97 and the CHIP E3 ligase, in cross-presentation have recently surfaced [368,369]. It would be interesting to examine whether cross-presentation of exogenous antigens involves SPP.

A number of important bacterial and plant toxins traffic from the cell surface to the ER, and cause intoxication by modifying proteins in the cytosol [370]. Viruses such as SV40 and Polyoma also traffic from the cell surface to the ER [371,372], as part of their infectious life cycle. Such pathogenic material must cross the ER membrane in order to access their targets. Several of these toxins and viruses may utilize the ER-to-cytosol dislocation pathway as a means of accessing the cytosol [373-376]. Recent evidence suggests that the ricin toxin [377], the Cholera toxin A subunit [378], and Polyoma virus [19], may utilize proteins like EDEM, p97 and DERLIN-2, respectively, for exit from the ER. As SPP is involved in ER-to-cytosol dislocation, it may also participate in intoxication of mammalian cells by bacterial toxins and viruses.

HC dislocation: more than a tale of the US2 tail

Dislocation of class I MHC heavy chain molecules by US2 is a multi-step process (**Fig.38**). One of the initial steps is substrate recognition, in which association of US2 with the folded class I MHC complex takes place (**Fig.38**, arrow 1). Yet, association is not enough, as seen by the many HC and US2 mutant molecules that are capable of associating without ensuing dislocation (**Figs.9, 10**). The US2 cytosolic tail must be present for HC dislocation to take place, as the US2 tail is required for SPP recruitment, and SPP is necessary for dislocation (**Fig.38**, arrow 2).

The US2 transmembrane domain is also crucial for dislocation

We found that the US2 transmembrane domain (TMD), although dispensable for SPP recruitment, is critical for dislocation, as a chimeric mutant of US2 with the native US2 TMD replaced with that of the unrelated CD4 molecule is still able to associate with SPP but is inactive in dislocation (**Figs.29, 30**). HC dislocation is therefore dependent not just on (US2 tail-mediated) recruitment of SPP but also on additional (US2 TMD-mediated) interactions within the plane of the membrane (**Fig.38**, arrows 3 and 4). Interactions between TMDs are important for the assembly and function of membrane protein complexes [379], as has been elegantly shown for US11 [21,270]. The US2 TMD may be involved in the recruitment or engagement of other protein(s) involved in dislocation (**Fig.38**, arrow 3) or even further engagement of SPP (SPP-mediated cleavage or otherwise) (**Fig.38**, arrow 4). Additionally, SPP involvement may extend to direct interactions (SPP-mediated cleavage or otherwise) with other protein(s) involved in dislocation (**Fig.38**, arrow 5).

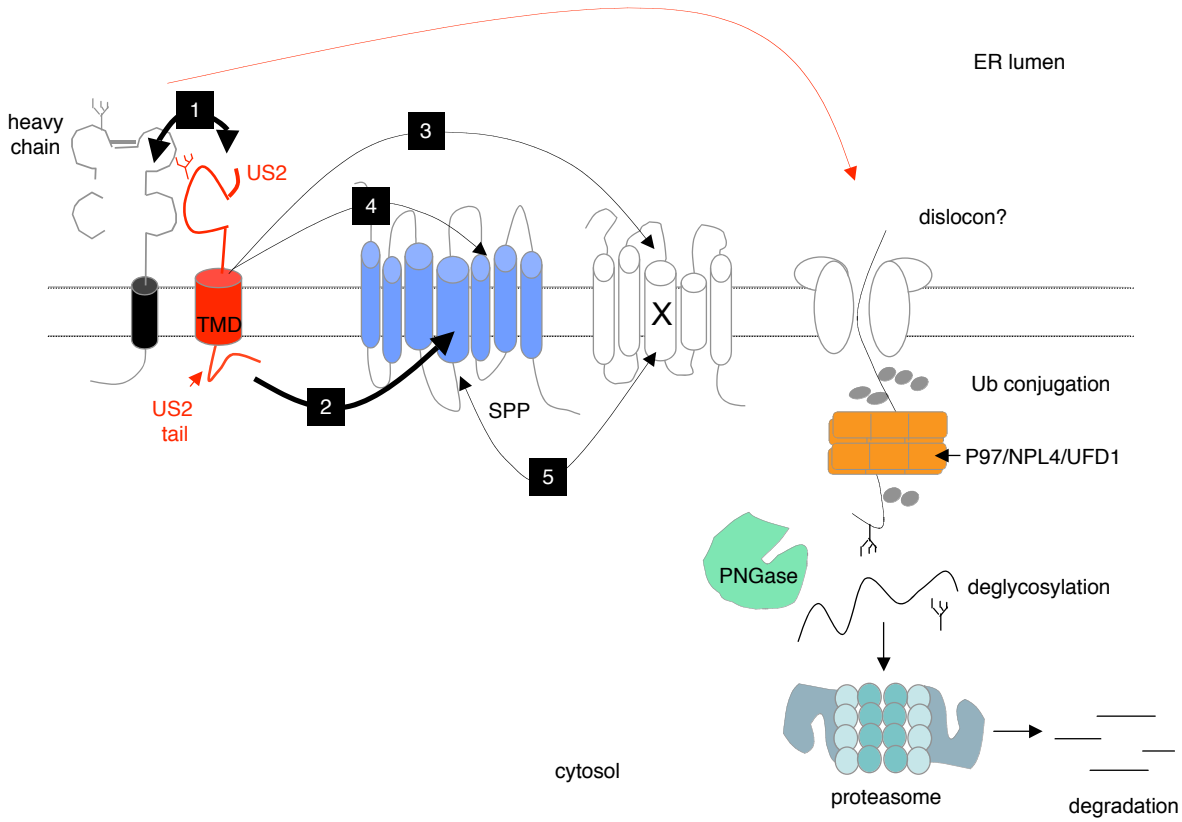

Figure 38. US2-mediated HC dislocation: a tale of the US2 tail, the US2 TMD and many other players. Thick arrows and colored structures indicate known US2 dislocation pathway components or interactions. Thin arrows and white structures indicate unknown/unverified components or interactions. Association of US2 with HC is mediated by the US2 lumenal domain (1). The US2 cytosolic tail binds SPP, one of the US2 interaction partners required for dislocation (2). The US2 transmembrane domain (US2 TMD) is responsible for an additional step in dislocation, perhaps through recruitment of so-far unidentified protein(s) (3) necessary for dislocation and/or due to an additional interaction with SPP, which may establish intramembrane contacts with US2 (4). SPP itself may be responsible for interactions that are important for dislocation (5) and which may or may not rely on its catalytic activity. The unidentified protein(s) marked with an X represents a putative interacting partner of US2 in the ER lumen, membrane or in the cytosol, and which may be recruited directly or otherwise.

We detected anti-US2 antibody-reactive bands on SDS-PAGE that are consistent with the possibility that they represent oligomeric forms of US2 (**Fig.31**). They can only be retrieved by anti-HA antibody and not by anti-US2 antibody (data not shown) and could simply constitute an artifact due to the presence of the HA tag or to HA-US2 over-expression. Because these anti-US2-reactive, higher molecular mass bands seem to require the presence of the US2TMD, as they do not appear in samples obtained from HA-US2/CD4/US2 cells (at least not to a great extent), we would argue that there is specificity in their formation and that the US2 TMD may contribute to US2 oligomerization. So far, however, we have no indication that oligomerization indeed occurs *in vivo* nor that it might contribute to US2 function. Nevertheless, these findings warrant further investigation.

The US2 lumenal domain

The lumenal domain of US2 may also have an important role in dislocation, which may extend beyond the mere recognition of the class I MHC heavy chain. A number of ER proteins associate with both versions of US2: BiP, calnexin, PDI, subunits of the oligosaccharide transferase complex and others, indicative of an interaction with the US2 lumenal domain. Because the US2 proteins were expressed at high levels, the observed associations may reflect mere chaperone-client interactions due to incomplete folding of US2. It is also possible, however, that US2 may use BiP and/or CNX and/or PDI to alter the structure of the lumenal domain of the class I MHC heavy chain prior to dislocation. As in the case of yeast substrates [380], US2 may employ BiP to help to maintain the class I MHC heavy chain in a conformation that can be dislocated readily. BiP may also disassemble the US2-class I MHC heavy chain complex prior to dislocation. PDI, for instance, could be responsible for unfolding of the complex between US2 and the folded HC/beta$_2$m/peptide complex [27,271], as is the case for bacterial toxins that must be dislocated [168,169,381]. US2 association with BiP may instead help US2 bridge HC onto BiP promoting interactions with other ER dislocation machinery components [359]. Identification of proteins (for example, by affinity purification) that do not associate with US2 lumenal mutants that bind HC but are inactive in dislocation [382], could reveal important clues regarding events that take place in the lumen prior to and during dislocation.

A HC dislocation story with many players: SPP and beyond

We have yet to explore many of the proteins other than SPP that are differentially associated with functional and non-functional US2 (**Fig.16**), although at least some of these may play a role in dislocation. We have shown some preliminary data that we have obtained for one of these proteins, NSDHL. Much like what happens with SPP, NSDHL associates specifically with dislocation-active HA-US2, and in fact the US2 tail is both necessary and sufficient to recruit NSDHL (**Figs.33, 34**). Curiously, although both SPP and NSDL are

recruited by motifs present in the 14 aminoacid US2 tail, they do not seem to associate (**Fig.35**), suggesting that if a connection exists between SPP and NSDHL, it does not involve physical association between the two proteins.

We are in the process of addressing whether the US2/NSDHL association is relevant for US2-mediated dislocation. Although so far we have no data to support this, our working hypothesis is that NSDHL, cholesterol metabolism and lipid droplets may be connected to US2's function in dislocation. Interestingly, as mentioned earlier, the HCV core protein, a known SPP substrate, is incorporated (like NSDHL) into lipid droplets upon SPP-mediated cleavage. Moreover, the molting defect induced by deficiency in SPP protease activity of the *Caenorhabditis elegans* SPP orthologue, *Imp-2,* is mimicked by cholesterol depletion and by deficiency in *Irp-1*, involved in lipid metabolism [351]. Some of the experiments in our "Future perspectives" should allows us to find if there is indeed a role for NSDHL and cholesterol in ER dislocation.

ER dislocation by viral immunoevasins: different pathways to a common fate

US2 and US11: two means to the same end

One theme that arises from the characterization of this process over the 10 years that have passed since its discovery is that HC dislocation is unique in many respects: HC molecules do not meet the requirement of being either misfolded or misassembled, yet their dislocation takes place by virtue of the presence of US2 or US11. Moreover, the speed of HC degradation is unrivaled by that of any other ER-associated degradation substrates [131,132,383].

Another "common theme" is that the viral proteins *differ* substantially in terms of the mechanistic details of the dislocation reaction. We have found yet another mechanistic difference between US2 and US11 at the substrate recognition stage, with US11 recognizing HCs earlier in their biosynthetic pathway and US2 targeting only properly folded HC molecules for dislocation and degradation. They also differ in terms of the cellular dislocation machinery that each subverts for dislocation of class I MHC molecules [21 352]. The knowledge that the transmembrane domain of US11 is essential for its function [270], led to the discovery of

DERLINs [21] and of DERLIN-containing multiprotein complexes at the ER membrane that function in US11-mediated HC dislocation and US11-independent dislocation of a subset of ER degradation substrates [19,22-26,197,214,215,384], providing the first direct evidence that the US11 immunoevasin subverts a normal cellular dislocation pathway for degradation of class I MHC HCs.

The knowledge that dislocation by US2 is not dependent on DERLIN-1 [21] and that the US2 tail is essential for its function [27], led to the discovery of SPP [352]. Although removal of signal peptide remnants from the ER membrane, assigned to SPP in animals and plants, is not a function exclusive to higher eukaryotes, a gene that encodes an orthologue of this enzyme is absent from the yeast genome [28,298]. Acquisition of the SPP gene by higher eukaryotes could have occurred, for instance, due to evolution of an immune system, as SPP is required for HLA-E antigen presentation [28], and its role may not be limited to signal peptide processing but extend to processes such as protein dislocation from the ER.

The US2 and US11 pathways intercept at a p97-dependent step

A knockdown of SPP impairs class I MHC HC dislocation by US2 but not US11 (**Fig.24**), and, in agreement with this, US2 does not bind DERLIN-1 and US11 does not bind SPP (**Fig.25**), further confirming that these two pathways are independent. However, the cytosolic p97/NPL4/UFD1 complex is found in association with US2 (**Fig.16**) and is necessary for HC dislocation mediated by the two proteins (**Fig.26**). Although p97 had been shown to be involved in US11-induced dislocation in a semi-permeabilized cell system - we have shown it for the first time in intact US11 cells (**Fig.26.a**) -, the involvement of p97 in US2 cells had not been shown. Hence, it seems that although the early ER-membrane events (targeting of substrate to the dislocation machinery and perhaps dislocation through the ER membrane) of the US11- and US2-mediated ER degradation pathways have distinct (DERLIN-1 and SPP) requirements, these routes of protein disposal from the ER membrane intersect at a pre-proteasomal step involving p97. This is reminiscent of the ERAD-L, -M, and –C pathways in yeast, where it has been shown that substrates with lesions in their lumenal, membrane, and cytosolic domains, respectively, are handled by distinct E3 ligase-assembled membrane components of distinct dislocation complexes, but that ultimately all substrates are dependent on the Cdc48p(p97)/NPL4/Ufd1 complex for extraction from the ER membrane and/or delivery

to the proteasome [212,385].

Convergence of the US2 and US11 dislocation pathways at the step of ER membrane extraction in terms of the Cdc48p(p97)/NPL4/UFD1 requirement suggests that a common cytosolic degradation fate may be brought about by different membrane events.

Additionally, the combined data on p97 and SPP is further validation of the affinity purification protocol used (**Fig.16**), as it allowed retrieval of SPP, an ER-membrane component, and also of p97, a cytosolic constituent of the US2 dislocation machinery. Because both US2 and tailless $US2_{186}$ recruit p97, the interaction between US2 and p97 is likely not mediated by the US2 cytosolic tail, although this is in disagreement with a report that substitution of the HCMV US3 cytosolic tail by that of US2 is sufficient for recruitment of p97 [280]. Rather, p97 recruitment to the ER membrane could be dependent on the cytosolic tail of the HC molecule: p97 has been suggested to bind to the HC cytosolic tail in US11 cells [116,229,274,296], but the HC tail may not be necessary for dislocation by US2 [119]. Cdc48p/p97 has been shown to be recruited to the ER membrane by E3 ligases (like Hrd1p and Doa10p in yeast or HRD1 and gp78 in mammals), by DERLIN-1, by VCP-interacting membrane protein (VIMP) or by ER membrane Ub regulatory X (Ubx) domain proteins such as yeast Ubx2p/Sel1p or mammalian UBXD2/erasin [22,26,386-389]. Likewise, US2 interaction with p97 may be indirect and occur as the result of the assembly of such a multiprotein complex, as seen for US11 [22].

Herpesviruses and ER dislocation

Like HCMV US2 and US11, the MHV-68 mK3 immunoevasin targets newly synthesized murine class I MHC heavy chains for dislocation from the ER membrane and proteasomal degradation [10,390-392]. It does so, however, by yet another distinct mechanism (**Fig.39**).

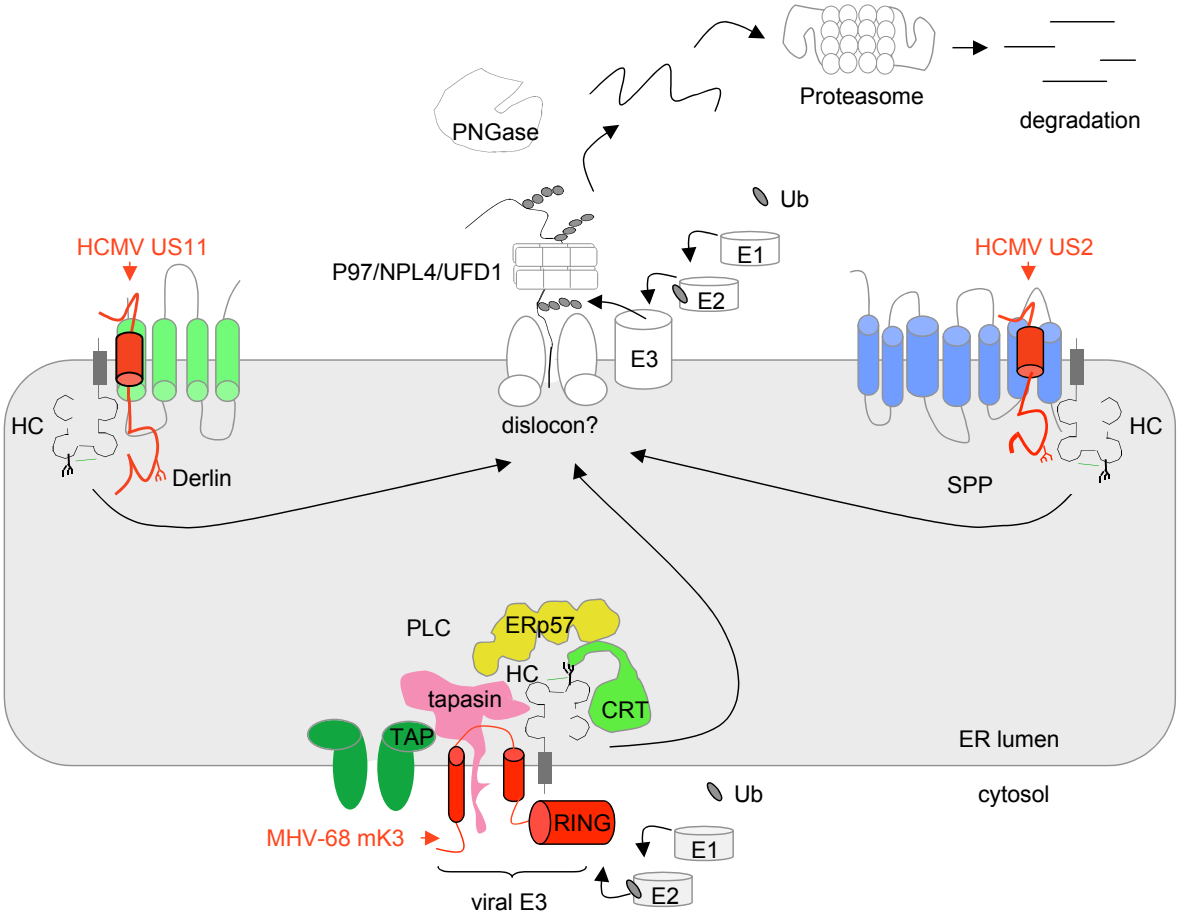

Figure 39. Schematic overview of the three endoplasmic reticulum (ER) dislocation pathways that are subverted by viral immunoevasins for dislocation of class I MHC heavy chain (HC) molecules. HCMV US2 and US11 proteins target human HC while MHV-68 mK3 targets murine HC for dislocation. The three viral proteins hijack distinct ER dislocation pathways. The MHV-68 mK3 protein itself has E3 ligase activity and uses the peptide loading complex (PLC) to target HC molecules while in the PLC; moreover, mK3 uses its E3 ligase activity (conferred by a cytosolic RING-finger-like domain) for HC ubiquitination, ultimately resulting in dislocation from the ER membrane. The HCMV US2 and US11 immunoevasins most likely function as adaptors that bind HC molecules and one or more component(s) of a cellular ER dislocation pathway, thus committing HCs for disposal from the ER. US11 binds DERLIN-1, an ER-membrane component of a putative multiprotein complex, which, in turn, is capable of coordinating substrate selection, dislocation, ubiquitination, deglycosylation and proteasomal degradation. Conversely, US2 binds signal peptide peptidase (SPP), proposed to be an ER-membrane component of a distinct multiprotein complex that functions in a non-overlapping dislocation pathway.

The mK3 immunoevasin is an E3 ligase of the aforementioned family of K3 homologs encoded by several herpes and poxviruses, of which several mammalian homologs, the MARCH proteins, have recently been found [11]. MHV-68 mK3 is a type III ER membrane protein, with E3 ligase activity conferred by its RING-related domain which faces the cytosol [391,393]. The mK3 E3 ligase uses the peptide loading complex (PLC) as a platform to impose the necessary proximity an orientation of its RING domain as to specifically ubiquitinate incompletely assembled heavy chains as they enter the PLC [10,392,394]. The main difference, therefore, resides in the fact that mK3 encompasses ubiquitination activity (by means of its RING domain), substrate selection (by means of its association with the peptide loading complex) and recruitment of dislocation machinery (like DERLIN-1 and p97) all in one polypeptide [24]. It is nothing short of remarkable how sequence and structurally unrelated herpesvirus proteins have converged into (although only superficially) similar mechanisms to dislocate newly synthesized class I MHC molecules from the ER membrane. The study of the mechanistic details of HC dislocation catalyzed by the MHV-68 mK3 and the HCMV US2 and US11 immunoevasins will most certainly keep providing new insights into ER dislocation more generally.

Conclusions and Future Perspectives

The HCMV immunoevasin US2 and US11 induce the proteasomal degradation of class I MHC heavy chains shortly after synthesis by catalyzing their dislocation from the ER membrane, a process normally used by cells to rid their ER of misfolded and misassembled proteins. Class I MHC molecules are not misfolded nor misassembled, yet the viral proteins are able to associate very stably with class I MHC molecules and deliver them to normal ER dislocation machinery components, with ensuing degradation. Although the end result is similar, the ER dislocation pathways subverted by US2 and US11 have long been thought to be distinct, for example, at the substrate recognition stage. In fact, we have obtained evidence that US2 and US11 act at different stages of the class I MHC biosynthetic pathway, as US11 targets unfolded and unassembled class I MHC HCs, whereas US2 targets only properly folded/assembled class I MHC heavy complexes fro degradation [27]. Direct evidence came recently through the identification of US2- and US11-specific cellular dislocation machinery [21,352] : US11 delivers class I MHC HCs to DERLIN-1, a component of a pathway that US2 does not utilize, in a process that is critically dependent on the US11 transmembrane domain

[21]. We have shown that the HCMV immunoevasin US2 uses its cytosolic tail to recruit signal peptide peptidase, an intramembrane-cleaving aspartic protease of the presenilin family, which is required for HC dislocation by US2 and not US11 [352]. The US2 and US11 pathways, although distinct at the level of the events taking place at the ER membrane intersect at a pre-proteasomal step, as both viral immunoevasins require the Cdc48p(p97)/NPL4/UFD1 complex for HC dislocation. We have also found that the US2 transmembrane domain is involved in an additional step (so far unknown) that is required for HC dislocation to take place [352]. The mechanism by which US2 operates will hopefully be clarified as we continue to characterize its structural domains and individual contributions to dislocation and host dislocation machinery involved in the US2 pathway.

The SPP catalytic activity and ER dislocation. We would like to continue our efforts to determine the contribution of the catalytic activity of the SPP protease to HC dislocation and US2 dislocation, by making use of over-expressed versions of SPP, in its wild type form and in the form of the D265A catalytic mutant as well as the L86F and P239L mutants. The *spp*^{5} and *spp*^{7} *Drosophila* mutants, with point mutations in the L95 and P248 positions of the *Drosophila* SPP gene (L86 and P239 in human SPP) phenocopy SPP active site mutants [350]. As L95 and P248 are evolutionarily conserved in the PS/SPPL superfamily and in the SPP family, respectively, they may also be important for human SPP and could be useful in further characterization of the role of SPP (**Fig.41**).

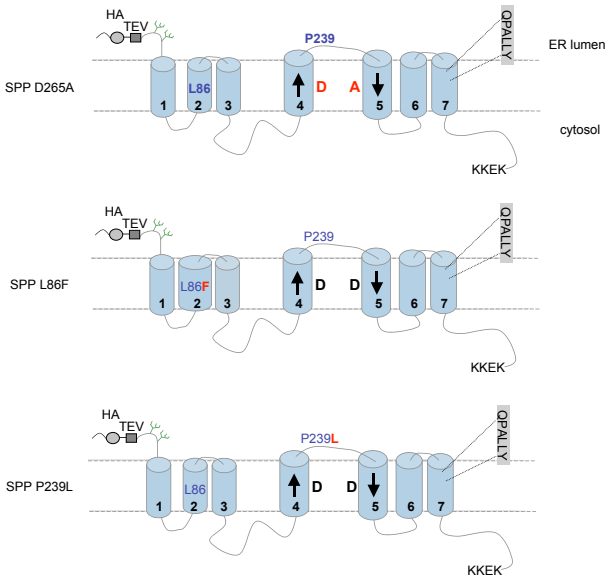

Figure 41. Schematic representation of the HA-TEV-tagged versions of SPP. SPP D265A mutant has a mutation in one of the aspartate (D) residues in the catalytic dyad, whereas L86F and P239L mutants have mutations that should phenocopy active site mutants, as they have been shown to do in *Drosophila*.

We have generated an N-terminally MYC-tagged version of the HCV core protein, which is cleaved by SPP, as a positive control for catalytic activity. We would also like to test SPP inhibitors. There are no cell-permeable inhibitors of SPP but there are cell-permeable inhibitors of gamma-secretase that also target SPP such as L-685, 458 (SIGMA). By comparing its effects to those of a cell-permeable gamma-secretase-specific inhibitor such as DAPT (SIGMA), one could assess the effect of blocking SPP activity on HC dislocation by US2. We would also like to expand our studies with the SPP activity assay in collaboration with Dr. Andrew Nyborg in the lab of Dr. Todd Golde. Moreover, we would like to determine the US2 tail interaction regions on the SPP molecule by expression of SPP truncation mutants in astrocytoma cells. In the event that these truncation mutants can not be expressed in mammalian cells, one could instead examine potential interactions of the US2 TMD with individual SPP TMDs using a bacterial system that measures interactions between heterologous membrane helices [270,395]. Identifying the precise SPP TMDs and/or amino acid contacts that form the interface between the US2 TMD and SPP would provide important information not only for the mechanism of dislocation but also for the mechanism of catalysis by the SPP protease.

The Cdc48p(P97)/NPL4/UFD1 complex in US2-mediated dislocation. To clarify the role of the p97/NPL4/UFD1 complex we are targeting NPL4 and UFD1 by RNAi using a variety of different shRNA constructs, as we have so far obtained only NPL4 and UFD1 knockdown levels that are either rather low and thus show no effect on HC dislocation or so high that massive cell death occurs due to the essential nature of these proteins. At the same time, we have generated mutant forms of Cdc48/p97: the p97 QQ mutant, with impaired ATP hydrolysis and the p97 ΔN mutant, lacking the N-terminal tail which mediates p97 binding to the ER membrane. The complex of the AAA ATPase Cdc48/p97 with Ufd1 and Npl4 is postulated to function in extraction of HCs from the ER membrane by providing the ATP-hydrolysis driving force for dislocation of ER degradation substrates to the cytosol or by acting as polyUb recognition module that can recognize substrates marked for destruction and shuttle them to the proteasome. The ATPase activity and Ub binding mutants should help shed some light on this issue. We have also generated retroviruses encoding a dominant negative form of Npl4 lacking the novel zinc-finger (NZF) domain (NPL4 ΔNZF) putatively involved in the recognition/binding of ubiquitin, and a dominant negative form of Ufd1, the UT6

domain, which possesses the p97 and Npl4 binding motifs and presumably capable of titrating p97 and Npl4 (**Fig.42**).

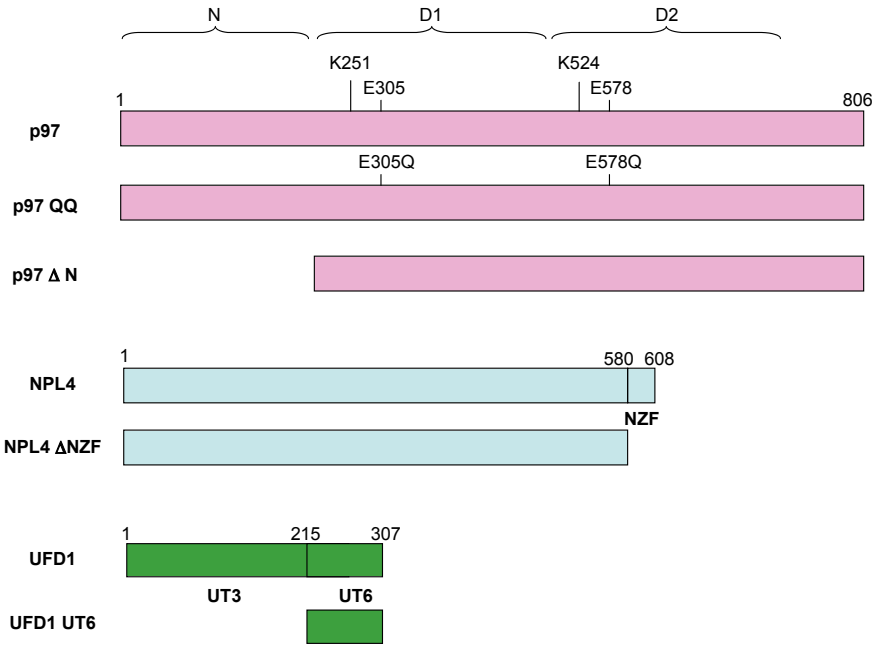

Figure 42. Schematic representation of p97, NPL4 and UFD1 constructs. N, N-terminal domain; D1 and D2, first and second ATPase domain, respectively. For the wild-type p97 protein, the lysine (K) residue in the Walker A motif and the glutamate (E) in the Walker B motif are indicated. For the mutants, they have been changed to glutamine (Q), as shown. The N terminal domain, responsible for p97 membrane attachment, is absent from the p97ΔN mutant. For NPL4, the NPL4 zinc finger (NZF) domain is shown, which is deleted in the NPL4 ΔNZF mutant. For UFD1, the UT3 and UT6 domains are indicated.

We are in the process of subcloning these cDNAs into retroviral expression vectors and establishing the conditions for testing the effect of the expression of these dominant negative forms of the components of the Cdc48p(p97)/NPL4/UFD1 complex in HC dislocation by US2.

The US2 transmembrane domain. We are presently conducting a functional screen for identification of US2 TMD-dependent cellular factors that aid in US2-mediated dislocation by analyzing interaction partners for the HA-US2/CD4/US2 mutant and to evaluate their contribution to dislocation. Moreover, identification of the aminoacid residues within the TMD that are important for dislocation could reveal important details in terms of ER membrane events during dislocation. We would also like to further characterize the putative contribution of US2 oligomerization to US2's function in dislocation, perhaps by analyzing these US2 TMD mutants for their ability to oligomerize. The HCMV US3 immunoevasin causes downregulation of class I MHC complexes from the cell surface by causing HC retention in the ER [87]. US3 and US2 molecules have a significant degree of homology (55% aminoacid similarity, 25% aminoacid identity), mostly within the lumenal domains [382]. A construct composed of the US3

lumenal and transmembrane domains (which bind HC) fused to the US2 tail (US3/US3/US2) is presumably capable of HC degradation, suggesting that the US2 cytosolic tail confers upon this chimeric molecule the ability to catalyze HC dislocation; the US2 tail on this chimera is presumably also sufficient for recruitment of p97, suggesting it contains a minimal determinant for recruitment of ER dislocation machinery [280]. Should a US2/US3/US2 chimera be active in dislocation, unlike US2/CD4/US2, comparison of the TMD sequences of US2, US3 and CD4 might provide clues as to why the US3 TMD but not the CD4 TMD should functionally replace that of US2 and have some predictive value when designing US2 TMD mutants.

NSDHL and US2. We would also like to pursue our investigation of the effects of NSDHL knockdown and overexpression on HC dislocation and on US2 dislocation. For this purpose we have generated 5 shRNA constructs against NSDHL, as well as N-terminally and C-terminally GFP-tagged versions of NSDHL and a CHILD syndrome-associated point mutant of NSDHL (G205S) [316] (**Fig.43**).

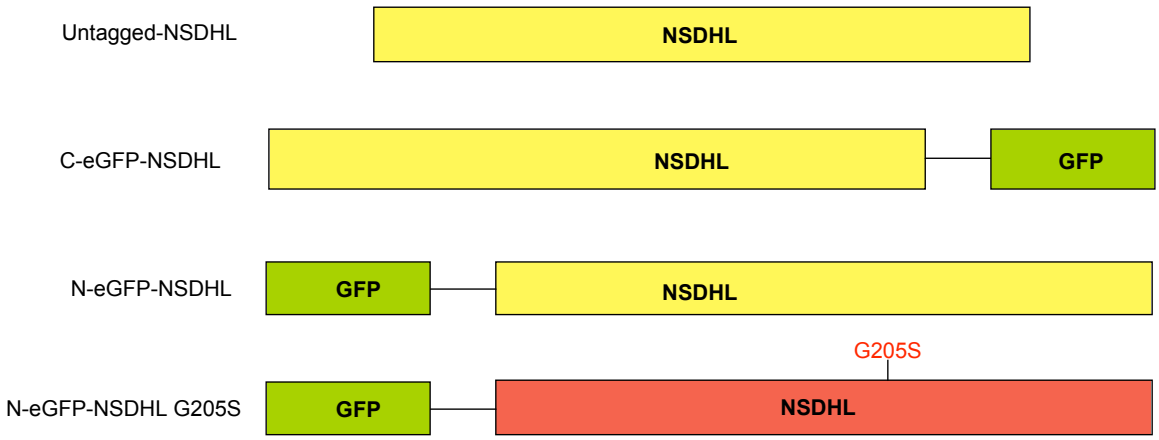

Figure 43. Schematic representation of NSDHL constructs in preparation. The wild-type NSDHL aminoacid sequence is shown in yellow, the CHILD syndrome-associated NSDHL G205S point mutant is shown in red, and the eGFP moiety attached at the amino (N) or carboxyl (C) terminus of the molecule is shown in green.

Our very preliminary results with NSDHL shRNAs indicate that the stability of HC is increased in US2/NSDHL knockdown cells (data not shown) and we are currently exploring these findings. With overexpression of NSDHL (both in untagged and tagged forms) we have

not observed any defects in HC dislocation (data not shown). However, human NSDHL with the CHILD syndrome-associated G205S missense mutation fails to localize to lipid droplets and fails to restore growth of a cholesterol auxotroph cell line in cholesterol-deficient medium [316], suggesting a functional significance of the localization of NSDHL on lipid droplets. We would like to assess whether expression of the NSDHL G205S mutant in US2 cells, and consequently mislocalization of NSDHL, might affect US2 localization and/or its function in class I MHC HC dislocation. We also plan to conduct experiments in which we prevent the formation or increase the accumulation of lipid droplets (through overexpression of the HCV core protein, for example), or in which we perturb cholesterol metabolism (through the use of statins, for example) and assess the possible contribution of lipid droplets and cholesterol metabolism to HC dislocation by the HCMV US2 immunovevasin.

Chapter 4. Material and methods

Cell culture.

U373-MG astrocytoma/glioblastoma cells and the human embryonic kidney cell line (HEK-293T) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum. U373-MG cells stably expressing HA-US2, HA-US2$_{186}$, HA-US2/CD4/US2, HA-US2TM/US2tail and HA-CD4TM/US2 tail were obtained by retroviral transduction (see below) with retroviral vectors constructed using the **pLNCX** (Clontech) backbone and selected and maintained in DMEM containing 0.5 mg/ml of geneticin (Invitrogen). US2, US11 and HA-US11 (neomycin-resistant) cells have been described [21,292]. HA-TEV and MYC-tagged SPP-expressing cells were obtained by retroviral transduction with SPP in **pLHCX** (Clontech) and selected and maintained in DMEM containing 0.250 mg/ml of hygromycin B (Roche).

cDNA Constructs.

The HA-HLA-A2$_{C227S,C283S}$ molecule was obtained by site-directed mutagenesis from the parental HA-HLA-A2 molecule in pcDNA3.1(+) (Invitrogen), which has been described [296]. Site-directed mutagenesis was performed using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers used introduced a (tgc) to (**a**gc) point mutation in the HA-HLA-A2 cDNA that results in a cysteine to serine substitution in the mutated protein, and were as follows: C227S FWD primer: gccaccctgagg**a**gctgggccctg and C227S REV primer: acgggcccagctcctcagggtggc. C283S FWD primer: gagcagagataca**c**cc**a**gccatgtgcagcatgag and C283S REV primer: ctcatgctgcacatggctggtgtaactctgctc. The HA-HLA-A2$_{C227S,C283S}$ cDNA was then subcloned into the retroviral expression vector. Brendan Lilley, a former graduate student in the lab, added a tandem affinity purification tag which he had previously used for US11 [21] to US2. This N-terminal tag consisted of a K^{b}ss-HA-TEV tag followed by a small spacer and was added to US2 residues 21-199 (US2) or 21-186 (US2$_{186}$) in frame by Brendan Lilley using a US2 construct in pcDNA3.1(+) [277]. From Brendan I obtained supernatants from HEK293T cells containing viruses expressing the HA-US2 and US2$_{186}$ constructs. Human SPP cDNA was previously described [298] and was a generous gift from Dr. Bruno Martoglio (ETH, Zurich, now at Novartis), PCR-amplified, N- and C-terminally MYC-tagged and subcloned into the **pLHCX** retroviral vector (Clontech). I added the K^{b}ss-HA-TEV-tag to the newly generated US2TM/US2 tail and CD4TM/US2 tail cDNAs in pcDNA3.1(+) and to the US2/CD4/US2 cDNA

[also in pcDNA3.1(+)] that I obtained from Vanessa Noriega and Domenico Tortorella (Mount Sinai School of Medicine, NY,USA) (**Fig.44**).

US2 sequence
MNNLWKAWVGLWTSMGPLIR
LPDGITKAGEDALRPWKSTAKHPWFQIEDNRCYIDNGKLFARGSIVGNMSRFVFDPKADYGG
VGENLYVHADDVEFVPGESLKWNVRNLDVMPIFETLALRLVLQGDVIWLRCVPELRVDYTSS
AYMWNMQYGMVRKSYTHVAWTIVFYSINITLLVLFIVYVTV**DCNLSMMWMRFFVC**

HA-US2 sequence
K^{b}ss-HA-TEV-spacer-tag-
LPDGITKAGEDALRPWKSTAKHPWFQIEDNRCYIDNGKLFARGSIVGNMSRFVFDPKADYGG
VGENLYVHADDVEFVPGESLKWNVRNLDVMPIFETLALRLVLQGDVIWLRCVPELRVDYTSS
AYMWNMQYGMVRKSYTHVAWTIVFYSINITLLVLFIVYVTV**DCNLSMMWMRFFVC**

HA-US2$_{186}$ sequence
K^{b}ss-HA-TEV-spacer-tag-
LPDGITKAGEDALRPWKSTAKHPWFQIEDNRCYIDNGKLFARGSIVGNMSRFVFDPKADYGG
VGENLYVHADDVEFVPGESLKWNVRNLDVMPIFETLALRLVLQGDVIWLRCVPELRVDYTSS
AYMWNMQYGMVRKSYTHVAWTIVFYSINITLLVLFIVYVTV**D**

HA-US2/CD4/US2 sequence
K^{b}ss-HA-TEV-spacer-tag-
LPDGITKAGEDALRPWKSTAKHPWFQIEDNRCYIDNGKLFARGSIVGNMSRFVFDPKADYGG
VGENLYVHADDVEFVPGESLKWNVRNLDVMPIFETLALRLVLQGDVIWLRCVPELRVDYTSS
AYMWNMQYGMVRKSYTQPMALIVLGGVAGLLLFIGLGIFF**DCNLSMMWMRFFVC**

HA-US2TM/US2tail sequence
K^{b}ss-HA-TEV-spacer-tag-VAWTIVFYSINITLLVLFIVYVTV**DCNLSMMWMRFFVC**

HA-CD4TM/US2tail sequence
K^{b}ss-HA-TEV-spacer-tag-QPMALIVLGGVAGLLLFIGLGIFF**DCNLSMMWMRFFVC**

Figure 44. The aminoacid sequences of wild type untagged US2 and all HA-tagged US2 constructs used in these studies. The US2 signal sequence, which has been replaced with the K^{b}ss-HA-TEV-spacer-tag is shown in subscript italic. The transmembrane domain aminoacid residues are shown in black (US2 TMD) or gray (CD4) boldface type and those of the cytosolic tail, when present, are shown in red.

RNAi constructs.

SPP shRNA 21 nucleotide-long (21mer) target sequences [unique 19mer sequences within the gene of interest and whenever possible preceded by two adenine (aa) bases] were chosen using an algorithm available at http://jura.wi.mit.edu/siRNAext/ and were cloned into

the **pRETRO-SUPER** expression system [311] using the *Bgl*II and *Hind*III sites on the vector. The 21mer sequences used were the following: SPP.1, (aa)ggcctcgaagcaaacaact, SPP.2, (aa)gggagaagtgacagagatg, SPP.3, (aa)gaatgcttcagacatgcct, and SPP.4, (aa)gaatgcttcagacatgcct [396]. As a control, we used a 20mer shRNA against the enhanced green fluorescent protein (eGFP), with the following sequence: gcaagctgaccctgaagttc [22]. In addition to the unrelated eGFP shRNA control, we made an additional RNAi mismatched control by making a two-base-pair change in the middle of the SPP.1 shRNA (mismatched SPP.1 or MM.1: (aa)ggcctcga**g**g c**g**aacaact). The p97 shRNA 21mer sequence, (aa)gtagggtatgatgacattg, has been previously described [397]. The NSDHL shRNA 21mers we designed were NSDHL.1, (aa)taacatcctttgaatgagt, NSDHL.2, (aa)ataacatcctttgaatgag, NSDHL.3, (aa)cgatcctgagaagaatttc, NSDHL.4, (aa)tgggaagaacttggtggac and NSDHL.5, (aa)gaacttggtggacttcacc. Briefly, two primers - a forward or sense primer, and a reverse or antisense primer - were synthesized per targeting sequence. The shRNA primers had the following sequences: shRNA sense primer, GATCCCC(19mer-sense)TTCAAGAGA(19mer-antisense)TTTTTGGAAA, and antisense primer, AGCTTTTCCAAAAA(19mer-sense)TCTCTTGAA(19mer-antisense)GGG. Each of the individual primers was 5' phosphorylated and PAGE-purified. The two primers were annealed and ligated into the *Bgl*II and *Hind*III sites on the pRETRO backbone. All retroviral constructs were sequence-verified and used to produce shRNA-expressing viruses. The shRNA-expressing target U373-MG human astrocytoma cells were obtained by retroviral transduction with pRETRO viruses [311] packaged in human embryonic kidney (HEK) 293T cells (as described below). The shRNA-expressing astrocytoma cells were typically assayed 48-96 hours after retroviral infection, as in most cases the knockdowns of the proteins led to decreased cell proliferation and survival.

Retrovirus packaging by HEK 293T cells

We always used freshly thawed, low-passage HEK293T cells (no more than 2-3 weeks old). The day prior to transfection, we plated HEK 293T cells at a density of $4x10^6$ cells/10 cm dish. The day of transfection, we mixed 4μg of viral plasmid, 3μg of VSV-G (envelope), and 3μg of gag-pol (structural proteins and RNA polimerase), re-precipitated the DNAs by adding 1/10 volume of 3M sodium acetate and 3 volumes of 95% ethanol (cold), which we then incubated at -80^0C for 30 min, spun down for 15 min at 4^0C at 14K rpm, washed with 70% ethanol, re-spun, and allowed to dry in the hood before ressuspending in 15 μl of sterile PBS. While the DNA was drying, we set up the transfection reagents by mixing, in a 1.5mL eppendorf tube,

200μl of warm Opti-MEM and 30 μl of Transi-T reagent (liposomes) (NOTE: the Transi-T must be added by drops of 10μl, making sure that it does not come in contact with the side of the tube). The tube was tapped briefly and left to sit for 10-15 min at RT. Afterwards, the DNA was added to the liposomes and left to sit at RT for 15 min. In the meantime, the HEK293T cell medium was replaced by 6 ml of OPTI-MEM. The DNA/liposomes mix was then added DROP-WISE. The plate was then rocked very gently and transferred to the viral incubator. The medium was replaced after 6-8 hours with complete DMEM. 48hrs post-transfection, we collected the viral supernatant, added polybrene (hexadimethrine bromide or 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide) (SIGMA) to a final concentration of 4 μg/ml and filtered the virus supernatant through a 0.45μm filter. We then either infected the target cells immediately or aliquoted and stored the virus supernatant at -80^0C.

Retroviral infection of target U373-MG astrocytoma cells.

The day before infection, we split U373 cells (target cells) into 6-well plates at a density of 300,000 cells/well. On the day of the infection, the virus supernatant (obtained from HEK293T cells) was applied to the target cells (U373 cells). Target cells were then spun at 2200 rpm, 2hrs, at RT. The virus supernatant was removed after centrifugation and replaced with fresh DMEM medium, after which cells were returned to the incubator. Antibiotic selection was initiated only after cells had gone through one round of cell division (typically 24hrs) to enrich for cells that had been retrovirally-transduced with the gene of interest. Cells were typically assayed 48-72 hrs post-infection.

RNA Purification and RT-PCR analysis.

The SPP RT-PCR primers used (SPP RT-PCR forward primer, 5'-TCCTGCACCTCAACAATGTC-3'; SPP RT-PCR reverse primer, 5'-AGGACAGGAAAACCGATGC-3') were previously described [396]. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for cDNA production, and the following GAPDH-specific primers (GAPDH RT-PCR forward primer, 5'-CCACCCATGGCAAATTCCATGGCA-3'; GAPDH RT-PCR reverse primer, 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. RT-PCR with these SPP- and GAPDH-specific primers should yield PCR products of 400bp and 600bp, respectively. Total cytoplasmic RNA from U373-MG astrocytoma cells was isolated using the RNeasy kit (Qiagen) and was

analyzed by RT-PCR. Typically, 5 µg of total messenger RNA were reverse-transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Briefly, the RNA was incubated with an oligo$(dT)_{12-18}$ primer and a dNTP mix for 5 min at 65°C and quickly chilled on ice. First-Strand RT buffer, $MgCl_2$, DTT and RNAse OUT reagent were added, mixed gently, and incubated for 2 min at 42°C, before addition of 50 units of SuperScript™ II Reverse Transcriptase and incubation for 50 min at 42°C. Reverse transcription was terminated by heat inactivation at 70°C for 15 min. 50 U of Rnase H were then added for 20 min at 37°C.

For PCR amplification of the target cDNAs thus obtained corresponding to the SPP and GAPDH messenger RNAs, we used the Platinum PCR Supermix (Invitrogen) with an initial step of 2 min at 94°C and 30 cycles of 15s at 94°C, 30s at 60°C and 30s at 72°C. The PCR products were analyzed on 2.0% agarose gels and visualized them by ethidium bromide staining. Control experiments indicated that amplification under these conditions was semi-quantitative, with increases in amplification products proportional to the amount of RNA utilized for RT-PCR. The products were not present in the absence of reverse transcription.

Metabolic labeling of cells and pulse–chase analysis

Cells were detached by trypsin treatment, followed by starvation in methionine/cysteine-free DMEM for 1hr at 37°C. Cells were metabolically labeled with 500µCi/ml of [^{35}S] methionine/cysteine (Perkin Elmer NEG-072 EXPRESS [^{35}S]Protein Labeling Mix, with 73% L-[^{35}S]methionine and 22% L-[^{35}S]cysteine) at 37°C for the times indicated, with the cells being collected immediately at the end of the pulse period and then lysed in 1% digitonin, 1% SDS or 0.5% NP-40. For pulse–chase experiments, at the end of the pulse, medium containing a large excess of non-radioactive aminoacids (the chase solution) was added and an aliquot of cells was immediately collected, the 0 minute (min) time point, while the remaining cells were chased (left in the chase solution) for the remainder of the chase period always at 37°C, with aliquots being collected at the indicated chase points. Cells were lysed in 1% SDS or 0.5% NP-40 and lysates were then equalized for incorporation of radioactive material using trichloroacetic acid precipitation (TCA) followed by scintillation counts. The desired molecules were then recovered from the lysates with an antibody against the molecule of interest or its tag, and the immunoprecipitates were analyzed by SDS–PAGE (typically 10% or 12.5% SDS-PAGR) followed by fluorography. TEV protease (Invitrogen) and endoglycosidase H (New England BioLabs) digestions were performed, where indicated,

according to the manufacturer's instructions. For some pulse/chase experiments, different pulse and chase periods were used as indicated in figures and figure legends.

Immunoprecipitations

Immunoprecipitations were performed as previously described [12,13,27]. Typically, lysates obtained from radioactively labeled cells were equalized for incorporation of radioactive material using trichloroacetic acid precipitation (TCA) followed by scintillation counts. Lysates obtained from non-radioactively labeled cells were equalized for total cellular protein content using the bicinchoninic acid (BCA) assay (Pierce). The desired molecules were then recovered from the lysates by incubation at 4^0C for 1 hr (with constant rocking) with an antibody against the molecule of interest or its tag. Protein A or Protein G-coupled agarose beads pre-equilibrated with lysis solution were then incubated with the lysates at 4^0C for 45min-1hr (with constant rocking). The immunoprecipitates were collected by centrifugation to pellet the beads (bound to the antibody, in turn binding the molecule of interest) and washed three times in wash buffer containing the same detergent used during the immunoprecipitation. After washing, the immunoprecipitates were boiled in 1x reducing sample buffer typically for 3-5 min, before loading on the gel.

Western Blots.

Cells were collected by incubation with 0.05% trypsin for 2-5 min, washed twice with PBS, and lysed in 0.5% NP-40. For p97 knockdown experiment, cells were pre-incubated with 10 mM N-ethyl maleimide (NEM) (SIGMA) for 15 min prior to trypsinization. Total protein content was normalized with the BCA assay (Pierce). Typically, 10-20 μg of total cellular protein was loaded per lane of a 4-15% SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted with the antibodies indicated. Immunoblot procedure: the PVDF membrane was blocked in PBS/0.2%Tween/5% milk, 1hr at RT (ON at 4C), and then rinsed briefly with PBS/0.2%Tween/2% milk (or PBS/T/2%). The primary antibody was added in PBS/T/2% at an appropriate dilution. The membrane was rocked in this solution at RT for 1 hr/ON at 4C, and washed 3x 5min with PBS/T/2%. The secondary antibody was then added: HRP-conjugated Goat anti-Mouse and anti-rabbit IgG (H+L) chain (Southern Biotechnologies) were used at a 1:20000 and 1:50000 dilution in PBS/T/2%, respectively. The membrane was again rocked at RT for 45', washed 3x 15min with PBS/T/2%, and finally developed by addition of the ECL reagents. Exposure times were typically 1-3 minutes, depending on amount of protein/lane

and level of protein expression in cells. The films used were typically Kodak X-OMAT Blue films.

IP/Westerns.

1% digitonin lysates were normalized for total protein content with the BCA assay (Pierce). Typically, 150-250 μg of total cellular protein was used for immunoprecipitation with anti-HA or anti-MYC antibody, run on a 4-15% or 10% SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted with the antibodies indicated as described above.

Antibodies.

Rabbit polyclonal anti-human HC, anti-US2 and anti-human PDI antibodies and mouse monoclonal W6/32 antibody have been described previously [277,289,290,295,327]. Mouse monoclonal anti-MYC, rabbit polyclonal anti-SPP, rat monoclonal 3F10 anti-HA and mouse monoclonal anti-beta-ACTIN antibodies were from Invitrogen, AbCam, Roche and Sigma, respectively. The mouse monoclonal 12CA5 hybridoma, producing an antibody against the epitope YPYDVPDYA of the haemagglutinin (HA) protein of influenza virus, has been described [398]. The rabbit polyclonal anti-NSDHL antibody was a kind gift from Dr. Masato Ohashi (Okazaki, Japan).

Large-scale affinity purification and mass-spectrometry.

The procedure was adapted from one described previously and developed in its initial form by Brendan Lilley [21]. Briefly, astrocytoma cells (10^8) stably expressing HA-US2, HA-US2$_{186}$, or no tagged protein (control cells), were lysed in 30 ml of ice-cold lysis buffer containing 1% digitonin ($C_{56}H_{92}O_{29}$, M.W. 1229.3) in 100mM Tris-HCl pH 7.4, 20mM NaCl, 70mM KCl, 12mM $MgCl_2$ with 1mM PMSF and complete, EDTA-free Protease inhibitor Cocktail tablets (Roche), by rocking at 4^0C for 1 hr. The lysate was cleared of debris by centrifugation at 20,000 g for 15min. US2 and associated proteins were retrieved by immunoprecipitation with 250 μl of compacted anti-HA 3F10 antibody beads (Roche) from 10 mg of cleared lysate. After 2hrs of incubation, beads were extensively washed in wash buffer (composition as lysis buffer, except with 0.2% digitonin and without protease inhibitors). Bound material was eluted by treatment with 50 units of TEV protease (Invitrogen) at 30^0C for 2hrs in TEV buffer with 1mM DTT and 0.2% digitonin. The eluate was boiled in reducing conditions for 3 min, and the resulting

polypeptides separated by 8% SDS–PAGE and revealed by silver staining. The bands of interest were excised, subjected to trypsinolysis, separated by liquid chromatography, analyzed by MS/MS and database-searched by Eric Spooner as described [21]. A schematic representation of the affinity purification protocol is shown in **Fig.47.**

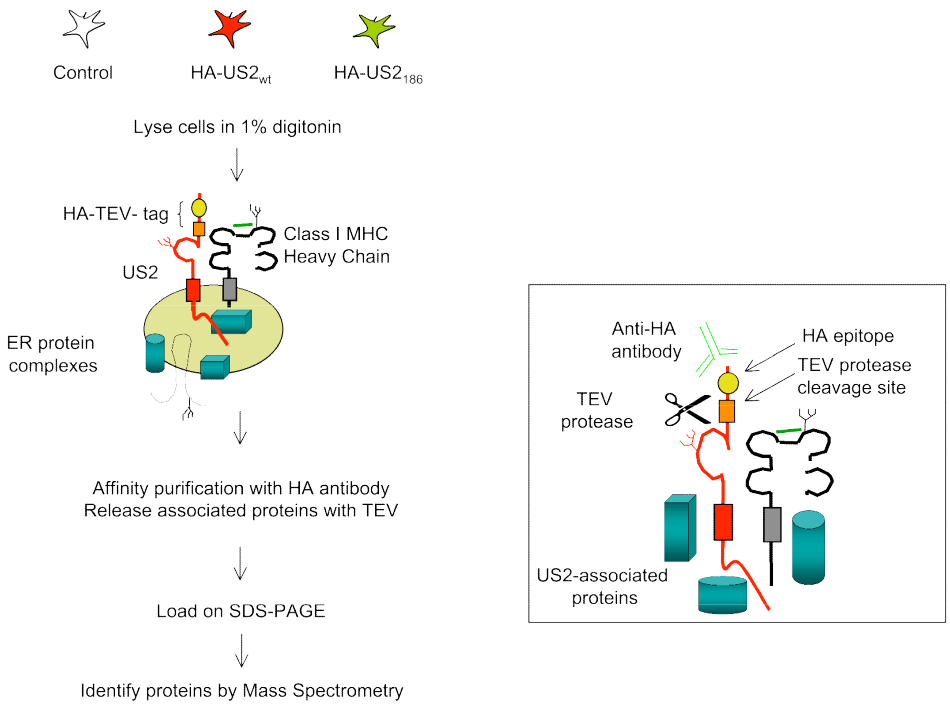

Figure 47. Affinity purification protocol used for retrieval of US2-associated proteins.

The data obtained from mass spectrometry analysis for comparison of proteins co-purified with HA-US2 and HA-US2$_{186}$ is summarized in **Fig.16.** The peptides obtained for SPP were: LVFPQDLLEK, NASDMPETITSRGEVTEMFSYEESNPK, GEVTEMFSYEESNPK, DPAAVTESK, FFPASFPNRQYQLLFTQGSGENK, LVFPQDLLEK, GKNASDMPETITSR and EEIINYEFDTK. The peptides obtained for NSDHL were: THLTEDTPK, GYAVNVFDIQQGFDNPQVR, FFLGDLCSR, AFHITNDEPIPFWTFLSR, DPQLVPILIEAAR, NLVDFTFVENVVHGHILA, QDLYPALK and ILTGLNYEAPK.

Mass-spec-compatible silver staining

The procedure was adapted from Blum et al, Electrophoresis, 8, pp93-99, 1987 and Schevchenko, A. et al. (1996) Anal. Chem. 68, 850-858. We fixed the gel in a mixture of 40%

ethanol and 10% acetic acid for 1 hr, after which we washed the gel in 30% ethanol, 2x20 min, followed by H_2O for 20 min. We then bathed the gel in freshly prepared sensitizing solution (0.02% $Na_2S_2O_3$) for 1 min, quickly washed the gel in H_2O, 3x20 sec, and then incubated it in freshly prepared cold 0.2% $AgNO_3$ solution, for 20 min at 4^oC.The gel was then washed in H_2O, 3x20 sec, after which we changed the gel chamber and washed the gel in H_2O for 1 min. Then we added half of the developing solution (3% Na_2CO_3, 0.05% formalin) and changed it when the developer turned yellow. After a quick wash in H_2O for 20 sec, the staining was then terminated in 5% Acetic Acid. Afterwards, the gel was washed in H_2O, 3x 10 min and left at 4^0C in 1% acetic acid before mass spectrometry analysis.

Gel fluorography

Fluorography was used to amplify the somewhat weak radiation signals from ^{35}S Meth/Cys-labeled proteins in SDS-PAGE gels, for which we used the 2,5-diphenyloxazole (PPO) fluorophore. Briefly, the gel was fixed in DMSO for 30 minutes (2x), followed by incubation with 22% 2,5-diphenyloxazole (PPO) in DMSO for 1hour. After addition of PPO to the gel, the gel was washed in water for 3x15 min to remove excess PPO in solution. Then, the gel was dried for about 2 hrs at 80C, placed inside a cassette containing a film of the appropriate size, and placed at –80C (at least) overnight. Radiation from molecules labeled with beta-emitting isotopes like ^{35}S excites the PPO fluor added to the gel, allowing detection of the radioactively labeled molecules retrieved by immunoprecipitation with specific antibodies by autoradiography, typically using Kodak BioMax MR-1 films.

NCBI accession numbers, tags and viral proteins used.

SPP: NM_030789, Gene ID 81502; CD4: NM_000616 Gene ID 920; NSDHL: NM_015922 Gene ID 50814; P97: NM_007126, Gene ID 7415; UFD1L: NM_005659, Gene ID: 7353; NPL4: NM_017921, Gene ID: 55666; eGFP: CVU55762;
HA tag: tacccatacgacgtaccagactacgca (YPYDVPDYA);
MYC tag: GAACAAAAACTCATCTCAGAAGAGGATCTG (EQKLISEEDL);
HA-TEV-SBP:
MVPCTLLLLLAAALAPTQTRAGTYPYDVPDYADYDIPTTASENLYFQGELKTAALAQHDEAEF SFEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPLE;
HCV core protein:
MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRG RRQPIPKARRPEGRTWAQPGYPWPLYGNEGCGWAGWLLSPRGSRPSWGPTDPRRRSRN LGKVIDTLTCGFADLMGYIPLVGAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLAL LSCLTVPASA

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Chapter 6. Publications

Manuscript 1

Manuscript 2

Manuscript 3