Folding and Endocytosis of Anthrax Toxin Receptors

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Summary

Bacillus anthracis is the causative agent of the infectious disease anthrax. The disease is caused after taking up the spores by either inhalation, ingestion or through skin lesion. They then germinate inside the body and start secreting the tripartite anthrax toxin. This toxin can hijack the two anthrax toxin receptors, CMG2 (ANTXR2) or TEM8 (ANTXR1) to enter host cells. Once inside the host cell, the toxin will start to interfere with essential cellular pathways, ultimately leading to severe damage and death.

The two anthrax toxin receptors have been well described in the context of anthrax toxin pathogenesis. Their normal, physiological function however has been studied far less. Mutations in either one of the two proteins leads to a severe genetic disease, underlining the importance CMG2 and TEM8 normally have.

My thesis therefore aimed at better understanding the physiological function of the two receptors.

In a first part, we chose to study the impact of N-glycosylation on the folding, trafficking and, finally, the function of CMG2 and TEM8. N-glycosylation is a very common posttranslational modification and has been shown to play an important role in various cellular processes such as protein folding, stability, cell adhesion, endocytosis, cell-cell recognition and trafficking. We show that both receptors are glycosylated on the predicted sites, with varying impacts on different functions of the proteins. TEM8 crucially relies on the presence of the sugar sidechains, as a non-glycosylated mutant will fail to fold, will be retained in the ER and is non-functional. CMG2 is less sensitive to defects in glycosylation, even a non-glycosylated mutant will be able to reach its target destination and function correctly. However, in the simultaneous presence of mutations seen in Hyaline Fibromatis Syndrome, N-glycosylation seems to function as a genetic buffer, stabilizing the mutated protein. N-glycosylation helps to increase the folding capacity of both receptors and therefore impacts on their physiological function.

In a second part our objective was to find new interaction partners of CMG2 and study their effect on toxin endocytosis as a suitable functional output. We identified 4 new proteins that regulate anthrax toxin entry via CMG2: RNF41 and Cbl, both ubiquitin E3 ligases, MARK2, a serine-threonine kinase and USP8, a deubiquitinating enzyme. In the absence of any of these four proteins, toxin entry into cells was strongly delayed or blocked. Of these four new regulators, Cbl and MARK2 are conserved for toxin entry via TEM8. We found that both RNF41 and Cbl can ubiquitinate CMG2, but regulate different steps of toxin entry. We also established a flow cytometry-based endocytosis assay for CMG2 and TEM8, enabling us to look at endocytosis and internalization kinetics in the absence of the pathogenic ligand anthrax toxin. Both receptors continuously internalize from the plasma membrane an endocytic process, which is regulated by ligand-binding and the multimerization of the receptors. Interestingly, CMG2 and TEM8 show a striking difference in their endocytosis rate, suggesting an important functional role of this behavior as well.

Keywords: CMG2, N-glycosylation, anthrax toxin, endocytosis

Zusammenfassung

Die Infektionskrankheit Anthrax (Milzbrand) wird durch den bakteriellen Erreger *Bacillus anthracis* verursacht. Die Krankheit wird ausgelöst nach der Aufnahme der Sporen in den Körper des Wirts und der Keimung der Sporen. Dies führt zur Produktion und Sekretion des dreiteiligen Anthraxtoxins. Um in die Zelle zu gelangen, macht sich das Toxin die beiden zellulären Anthraxrezeptoren CMG2 (ANTXR2) und TEM8 (ANTXR1) zu Nutze. Sobald das Toxin in die Zelle gelangt ist, dereguliert es essentielle zelluläre Signalwege, die letzendlich zu grossem Schaden und Zelltod führen.

Obwohl beide Toxinrezeptoren im Kontext von Milzbrand gut erforscht sind, ist ihre physiologische Rolle bis jetzt unklar. Mutationen in jedem der beiden Rezeptoren führen zu einer schweren genetischen Erkrankung. Dies unterstreicht die wichtige Rolle, die CMG2 und TEM8 normalerweise im Körper spielen.

Meine Doktorarbeit hatte zum Ziel, die physiologische Funktion dieser beiden Rezeptoren besser zu verstehen.

In einem ersten Projekt haben wir die Auswirkungen von N-Glykosylierung auf Faltung, Transport und Funktion von CMG2 und TEM8 untersucht. N-Glykosylierung ist eine häufig vorkommende posttranslationale Modifikation, die eine wichtige Rolle in verschiedenen, essentiellen zellulären Prozessen spielt. Dazu gehören die Faltung und Stabilität von Proteinen, Zelladhäsion, Endozytose, Zell-Zell-Erkennung und Proteintransport. Wir konnten zeigen, dass beide Rezeptoren glykosyliert sind und dass dies verschiedene Auswirkungen auf die diversen Funktionen der Proteine hat. Für TEM8 ist die Glykosylierung äusserst wichtig. Ein nicht-glykosyliertes Protein kann sich nicht korrekt falten, wird im ER zurückbehalten und ist nicht funktionsfähig. CMG2 hingegen ist resistenter gegenüber einenm Verlust der Glykosylgruppen. Auch ein nicht-glykosyliertes Protein kann sein zelluläres Ziel erreichen und korrekt funktionieren. Interessanterweise scheint, bei gleichzeitigem Vorkommen von Mutationen des Hyalinen Fibromatosis Syndrom, Glykosylierung eine genetische Pufferrolle einzunehmen. Dies stabilisiert das mutierte Protein. N-Glykosylierung von CMG2 und TEM8 hilft den Proteinen, sich zu falten und hat folglich einen Einfluss auf deren physiologische Funktion.

Im zweiten Projekt war das Ziel, neue Interaktionspartner von CMG2 zu finden. Zudem wollten wir deren Effekt auf die Endozytose von Anthraxtoxin als geeigneten Funktionstest untersuchen. Wir haben vier neue Proteine gefunden, die die Endozytose von Anthraxtoxin via CMG2 regulieren: RNF41 und Cbl, beides E3-Ubiquitin-Ligasen, MARK2, eine Serin-Threonin-Kinase und USP8, ein deubiquitinierendes Enzym. Ohne jedes einzelne dieser vier Proteine war die Toxinendozytose stark verlangsamt oder blockiert. Von diesen vier Proteinen sind Cbl und MARK2 auch in der Endozytose von Anthraxtoxin via TEM8 involviert. RNF41 und Cbl regulieren beide die Ubiquitinierung von CMG2, sind aber an verschiedenen Etappen des Prozesses beteiligt. Wir haben des weiteren einen neuen, auf dem Prinzip der Durchflusszytometrie basierenden Test entwickelt, der es uns ermöglicht, die Endozytose von CMG2 und TEM8 und deren Kinetik in der Abwesenheit des pathogenen Ligandes Anthraxtoxin zu untersuchen. Beide Rezeptoren internalisieren kontinuierlich von der Zelloberfläche, ein Prozess, der von dem Binden eines Liganden und der Multimerisierung des Rezeptors abhängt. CMG2 und TEM8 internalisieren mit sehr unterschiedlichen Zeiten, was auf ein unterschiedliches physiologisches Verhalten hindeuten könnte.

Schlüsselwörter: CMG2, N-Glykosylierung, Anthraxtoxin, Endozytose

Abbreviations

AAA ATPase	ATPases Associated with diverse cellular Activities
ANTXR	Anthrax toxin receptor
Cbl	Casitas B-lineage lymphoma
CBD	Cytoskeleton binding domain
CIE	Clathrin independent endocytosis
CMG2	Capillary morphogenesis gene 2
CME	Clathrin mediated endocytosis
DUB	Deubiquitinating enzyme
ECM	Extracellular matrix
EE	Early endosome
EGFR	Epidermal growth factor receptor
EF	Edema factor
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ERQC	ER quality control
ESCRT	Endosomal sorting complex required for transport
FRAP	Fluorescence recovery after photobleaching
GAPO	Growth retardation, alopecia, pseudoanodontia and optic atrophy
GPCR	G-protein coupled receptor
HFS	Hyaline Fibromatosis Syndrome
ILV	Intralumenal vesicles
LE	Late endosome
LF	Lethal factor
MARK2	MAP/microtubule affinity regulating kinase 2
MIDAS	Metal ion dependent adhesion site
MVB	Multivesicular body
OST	Oligosaccharyl transferase
PA	Protective antigen
PI	Phosphatidylinositol
PM	Plasma membrane
RNF41	RING finger protein 41
RTK	Receptor tyrosine kinase
TEM8	Tumor endothelial marker 8
TM	Transmembrane
USP8	Ubiquitin specific peptidase 8
vWA	Von Willebrand Factor A

I. Introduction

Anthrax is an infectious disease caused by the Gram-positive bacterium *Bacillus anthracis*. While it is a well known old disease that often affects cattle or humans in contact with animals, it received public attention after the terrorist attacks of 9/11, when letters containing the infectious spores were sent to governmental institutions in the US, leading to 11 deaths. As many pathogens, *B. anthracis* has developed strategies to hijack the host cell machinery to colonize its host and spread. One of the major virulence factors is the anthrax toxin, the entry of which into cells of the host crucially relies on the presence of anthrax toxin receptors. In this thesis we have studied folding and endocytosis of the two anthrax toxin receptors, both in a physiological as well as in a pathological context.

1. The two anthrax toxin receptors

Both CMG2 and TEM8, also known as anthrax toxin receptors (ANTXR 2 and 1 respectively) were initially found in screens of physiological/pathological functions. Yet they have been more famously studied in their role as toxin receptors during infection. Here, I will talk first about their function in a physiological context and will discuss the toxin entry in a later chapter.

CMG2 protein

Capillary morphogenesis gene 2 (CMG2) was initially discovered in an *in vitro* capillary formation assay in endothelial cells, where it was strongly upregulated after 8h [1]. CMG2 is a ubiquitiously expressed protein, highly conserved but only present in vertebrates [2]. It is a 55kDa type I transmembrane (TM) protein, which contains an extracellular von Willebrand factor type A (vWA) domain, followed by an Ig-like domain, a TM span of 23 amino acids and a tail of 148 amino acids, which is predicted to be unfolded [2] (Fig.1A). There are four different isoforms [3] (Fig.1B), with isoform 4 being the one mainly studied in this work.
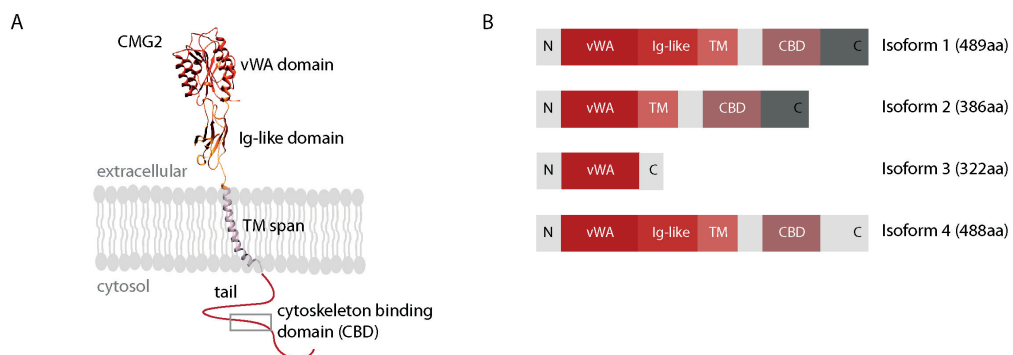
Fig.1: *Structure of CMG2 A)* *Schematic structure of CMG2 based on crystal structure of the vWA domain (1TZN) and modeling of the Ig-like domain [4].* **B)** *Depiction of the 4 isoforms of CMG2. All are TM proteins, except isoform 3 that is predicted to be secreted. The last 13 amino acids of isoform 4 differ from isoform 1 and 2.*

The vWA domain is a protein-protein interaction domain, which is found in several proteins that interact with proteins of the extracellular matrix (ECM). The most prominent examples of these vWA-containing proteins are integrins, where the domain is called I (inserted) domain [5]. The vWA domain of CMG2 contains a metal ion-dependent adhesion site (MIDAS), which is responsible for ligand binding [3].

The function of the Ig-like domain of CMG2 is so far still unknown. But it features two disulfide bonds, which are necessary for correct folding of the protein [4] and for proper insertion of the anthrax toxin into the endosomal membrane during anthrax toxin entry [6] (see in a later chapter). It also harbors two N-glycosylation sites, which are advantageous for protein folding, especially in the presence of other destabilizing mutations [7].

Directly after the TM span, there are two cysteines that can be palmitoylated, with a third site further downstream (S. Blaskovic, unpublished work). Palmitoylation is the reversible addition of a C16 fatty acid to cysteine residues of proteins, both soluble and TM [8]. For CMG2 this posttranslational modification affects protein stability at the cell surface (S. Blaskovic, unpublished work). The tail of CMG2 is predicted to be unstructured [9]. Disordered domains occur quite frequently in proteins and could be important for signaling and regulation [10]. The exact function of this domain in CMG2 remains to be determined.

TEM8 protein

The only other protein homologous to CMG2 is Tumor endothelial marker 8 (TEM8). It is also a type I TM protein and the two proteins share around 60% homology in the vWA domain and 68% identity in the first 145 residues of the cytoplasmic tail [11]. The tail of CMG2 and TEM8 contains a highly conserved cytoskeleton-binding domain (CBD), which was initially identified in TEM8 [12]. The vWA domain of TEM8 contains two N-glycosylation sites and one in the Ig-like domain, which is conserved between CMG2 and TEM8. The glycan sidechains are important for TEM8 folding and trafficking, as a glycosylation-deficient mutant cannot fold correctly, will be retained in the ER and degraded [7].

sp|Q9H6X2|ANTR1_HUMAN/1-564 1 MATAERRALGIGFQWLSLATLVLICAGQGG- RREDGGPACYGGFDLYFILDKS 52
sp|P58335|ANTR2_HUMAN/1-489 1 -MVAERSPARSPGSWLFPGLWLLVLSGPGGLLRAQEQPSCRRAFDLYFVLDKS 52

sp|Q9H6X2|ANTR1_HUMAN/1-564 53 GSVLHHWNEIYYFVEQLAHKFISPQLRMSFIVFSTRGTTLMKLTEDREQIRQG 105
sp|P58335|ANTR2_HUMAN/1-489 53 GSVANNWIEIYNFVQQLAERFVSPEMRLSFIVFSSQATIILPLTGDRGKISKG 105

sp|Q9H6X2|ANTR1_HUMAN/1-564 106 LEELQKVLPGGDTYMHEGFERASEQIYYENRQGYRTASVIIALTDGELHEDLF 158
sp|P58335|ANTR2_HUMAN/1-489 106 LEDLKRVSPVGETYIHEGLKLANEQI- -QKAGGLKTSSIIIALTDGKLDGLVP 156

sp|Q9H6X2|ANTR1_HUMAN/1-564 159 FYSEREANRSRDLGAIVYCVGVKDFNETQLARIADSKDHVFPVNDGFQALQGI 211
sp|P58335|ANTR2_HUMAN/1-489 157 SYAEKEAKISRSLGASVYCVGVLDFEQAQLERIADSKEQVFPVKGGFQALKGI 209

sp|Q9H6X2|ANTR1_HUMAN/1-564 212 IHSILKKSCIEILAAEPSTICAGESFQVVVRGNGFRHARNVDRVLCSFKINDS 264
sp|P58335|ANTR2_HUMAN/1-489 210 INSILAQSCTEILELQPSSVCVGEEFQIVLSGRGFMLGSRNGSVLCTYTVNET 262

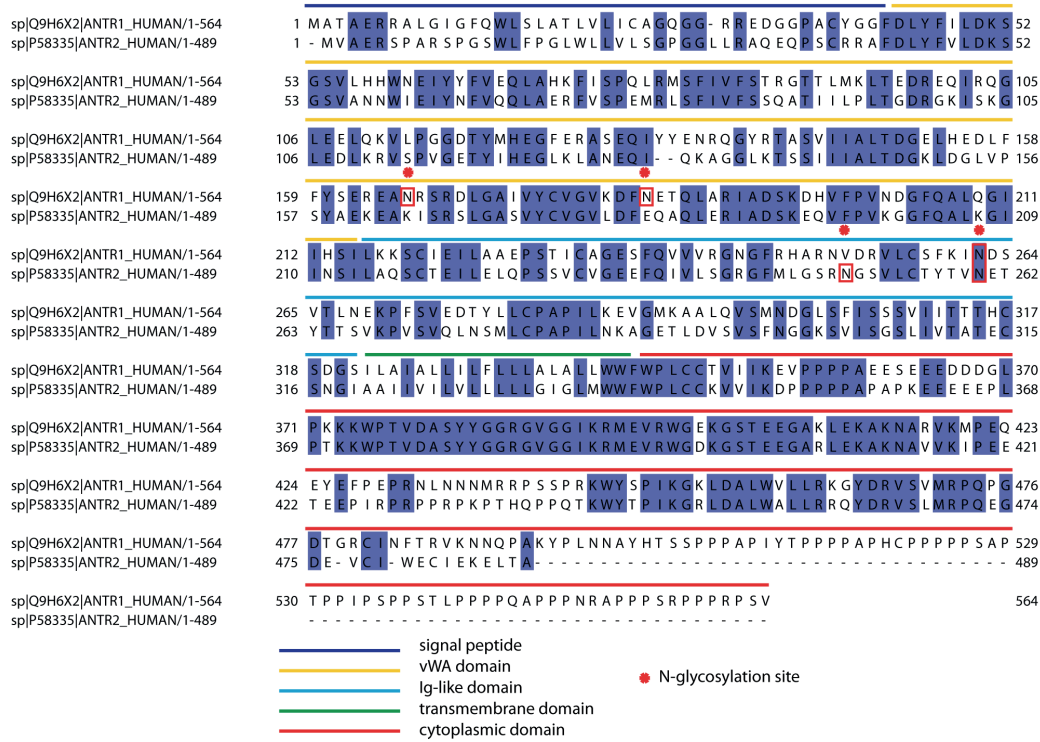sp|Q9H6X2|ANTR1_HUMAN/1-564 265 VTLNEKPFSVEDTYLLCPAPILKEVGMKAALQVSMNDGLSFISSSVIITTTHC 317
sp|P58335|ANTR2_HUMAN/1-489 263 YTTSVKPVSVQLNSMLCPAPILNKAGETLDVSVSFNGGKSVISGSLIVTATEC 315

sp|Q9H6X2|ANTR1_HUMAN/1-564 318 SDGSILAIALLILFLLLALALLWWFWPLCCTVIIKEVPPPPAEESEEEDDDGL 370
sp|P58335|ANTR2_HUMAN/1-489 316 SNGIAAIIVILVLLLLLGIGLMWWFWPLCCKVVIKDPPPPPAPAPKEEEEEPL 368

sp|Q9H6X2|ANTR1_HUMAN/1-564 371 PKKKWPTVDASYYGGRGVGGIKRMEVRWGEKGSTEEGAKLEKAKNARVKMPEQ 423
sp|P58335|ANTR2_HUMAN/1-489 369 PTKKKWPTVDASYYGGRGVGGIKRMEVRWGDKGSTEEGARLEKAKNAVVKIPEE 421

sp|Q9H6X2|ANTR1_HUMAN/1-564 424 EYEFPEPRNLNNNMRRPSSPRKWYSPIKGKLDALWVLLRKGYDRVSVMRPQPG 476
sp|P58335|ANTR2_HUMAN/1-489 422 TEEPIRPRPPRPKPTHQPPQTKWYTPIKGRLDALWALLRRQYDRVSLMRPQEG 474

sp|Q9H6X2|ANTR1_HUMAN/1-564 477 DTGRCINFTRVKNNQPAKYPLNNAYHTSSPPPAPIYTPPPPAPHCPPPPPSAP 529
sp|P58335|ANTR2_HUMAN/1-489 475 DE- VCI- WECIEKELTA- 489

sp|Q9H6X2|ANTR1_HUMAN/1-564 530 TPPIPSPPSTLPPPPQAPPPNRAPPPSRPPPRPSV 564
sp|P58335|ANTR2_HUMAN/1-489 -

signal peptide
vWA domain
Ig-like domain   ✱ N-glycosylation site
transmembrane domain
cytoplasmic domain

Fig.2: Sequence alignement between CMG2 and TEM8 *TEM8 (ANTXR1) and CMG2 (ANTXR2) were aligned and the protein domains are indicated [7].*

TEM8 is expressed in a variety of tissues, especially in epithelial cells [13], but was initially identified as upregulated during tumor angiogenesis [14].

Physiological role of CMG2 and TEM8

Almost 15 years after their discovery, the physiological role of CMG2 and TEM8 remains elusive. However, there are some promising leads for the two proteins, stemming from both *in vitro* and *in vivo* work.

The discovery of both proteins is linked to angiogenesis. CMG2 is expressed in the vascular endothelium of mice and in both endothelial cells of normal and cancer tissues [15]. In the same study, it was also found to be important for proliferation and capillary formation in Human Umbilical Vein Endothelial Cells (HUVECs) [15]. Therefore, efforts are undertaken to develop CMG2 as a potential therapeutic target in tumor angiogenesis [16].

TEM8, in addition to being highly expressed in tumor angiogenesis, was found to be upregulated in vasculature during the embryonic development in mice, yet undetectable in adult tissues [17]. Also, physiological angiogenesis is not impaired in TEM8 knockout mice [18]. It seems that vessel density and patterning during a specific phase of embryonic development are controlled by TEM8 in response to the canonical Wnt signaling pathway [19]. Missense mutations in TEM8 were also shown to be one of the causes of infantile hemangioma, which are regions of increased and disorganized angiogenesis [20]. This role in angiogenesis has also linked TEM8 increasingly to cancer

and its expression has been observed in a variety of cancers. Breast cancer cell lines that express TEM8 are more aggressive and invasive, also in an *in vivo* mouse model [21]. Also, TEM8 has been described as a marker for cancer stem cells [22] and cancer-specific circulating endothelial cells [23]. Therefore, TEM8 has increasingly become the focus of anti-cancer therapy, targeting cancer angiogenesis and thereby cutting off the supply line of the tumor [24, 25].

Another common theme of CMG2 and TEM8 is the link to the Wnt signaling pathway. Both receptors can interact with the Wnt coreceptor LRP5 and 6 [26, 27]. Also, TEM8 seems to function as a modulator/enhancer of the Wnt signaling pathway in developmental angiogenesis [19] and in cancer stem cells [22].

The link to Wnt seems evolutionary conserved. The closest homolog of CMG2 in zebrafish, Antxr2a, is responsible for polarization, again through interaction with the Wnt signaling pathway [28]. It is responsible for correct positioning of the mitotic spindle during zebrafish development. The Wnt pathway leads to a formation of a cortical actin cap in dividing epiblast cells. This will recruit Antxr2a and it will act as a RhoA effector, activating zDia2 that exerts a force on the spindle, ensuring correct orientation and subsequent cell division [28].

Both receptors can also bind actin in human cells by a domain of approximately 30 residues in the cytoplasmic tail [12 and J.Bürgi, unpublished work]. TEM8 was found to bind to actin, but this interaction was abolished when it bound to its surrogate ligand anthrax toxin on the extracellular part [29-31]. The same is true for CMG2 (J. Bürgi, unpublished work), but in contrast to TEM8, CMG2 in tissue culture cells it seems to be constantly occupied at steady state. Therefore, its ability to bind actin was only discovered recently with the help of a ligand-binding deficient mutant, D50A (J. Bürgi, unpublished work). Many of these studies rely on the binding of anthrax toxin as a surrogate ligand, as the true nature of the physiological binding partner of both receptors remains controversial.

In the first description of CMG2 in 2001, it was reported that the recombinant vWA of CMG2 can *in vitro* bind to the ECM proteins collagen IV and laminin [1]. This suggests a role in binding to or regulating the ECM. Strengthening this hypothesis, CMG2 knockout mice show an accumulation of collagen I, VI and fibronectin in the uterus [32, 33]. TEM8 as well was found to bind to ECM proteins, but in this case it was collagen I and VI [34, 35]. TEM8 knockout mice show also an accumulation of ECM in the uterus, but also additionally in the ovaries and the skin and enlarged teeth [36]. How this relates to angiogenesis is unclear, but the dysregulation of ECM is the link to the human diseases that are caused by mutations in CMG2 and TEM8: Hyaline Fibromatosis and GAPO syndrome.

Diseases associated with CMG2 and TEM8

Hyaline Fibromatosis Syndrome (HFS) is a rare autosomal, recessive disease caused by mutations in *cmg2* [37, 38]. The main disease symptom of HFS is the accumulation of ECM in organs and skin [39, 40]. These nodules seem to primarily form at sites of mechanical stress [41] and seem to mainly consist of collagen VI and glycosaminoglycans

[42, 43]. Other symptoms include failure to thrive, joint flexion contracture, recurrent infections and diarrhea and a higher susceptibility to bone lesions [2, 44]. Patients do not display mental retardation, consistent with the absence of CMG2 expression in brain [41]. For more severe cases, patients rarely survive their first two years. Mild cases are highly disfiguring and debilitating but patients can survive well into their adult years.

The mutations of CMG2 causing HFS characterized so far are all loss-of-function mutations and can generally be classified into 4 classes [2]. Class I consists of mutations in the vWA domain that do not affect folding or targeting to the plasma membrane but cannot bind the ligand, such as D50N. Class II are mutations in either vWA or Ig-like domain that affect folding of the protein, thereby leading to ER retention and degradation [45, 46]. Class III groups mutations that lead to mRNA degradation by the nonsense-mediated degradation (NMD) pathway due to the insertion of a premature stop codon [9]. Class IV mutations map to the cytosolic tail and are targeted properly to the PM. However, their function is impaired, through mechanisms that remain to be identified.

GAPO stands for growth retardation, alopecia, pseudoanodontia and optic atrophy and is caused by mutations in TEM8 [47]. Again, symptoms include accumulation of ECM but also dental abnormalities, which fits well with symptoms found in TEM8 knockout mice [18, 47, 48]. Patients display characteristic facial features, such as frontal bossing, broad forehead, widely spaced eyes, sparse facial hair including eyebrows and eyelashes and thickened lips, making the condition easily recognizable [47, 48].

So far only around 30 patients have been diagnosed with GAPO syndrome. The causative gene having been identified only very recently, no genotype-phenotype analyses have been reported yet.

2. Endocytosis and its regulation

Endocytosis in general

Endocytosis is a term that describes the general uptake for a plethora of substrates into the cell from the plasma membrane by vesicular traffic. Endocytic cargo can be liquid or solid, can be receptor-mediated or unspecific, includes components of the PM such as lipids or membrane proteins but also extracellular particles as diverse as ECM proteins, nutrients or pathogens, both viral and bacterial.
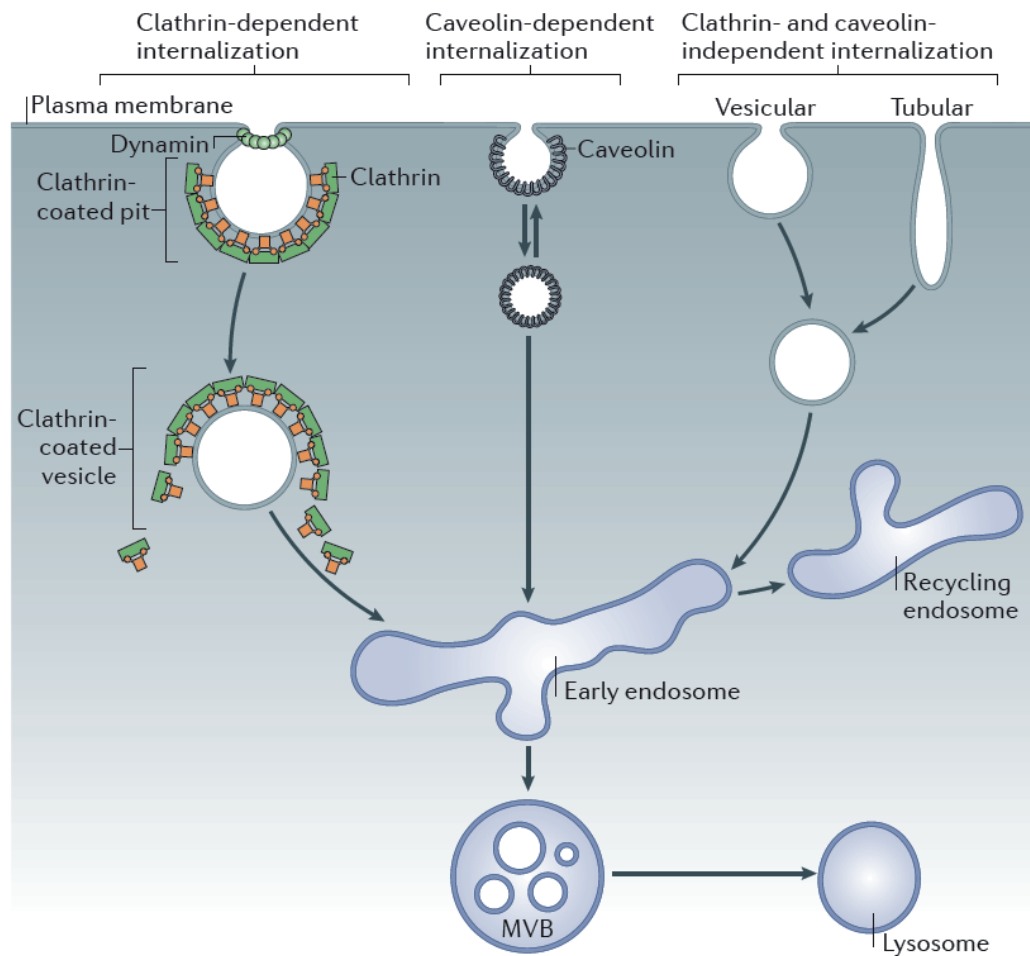
Fig.3: Schematic depiction of pathways into the cell There are several pathways into the cell. In general, they can be separated into clathrin-dependent, caveolin-dependent and other, clathrin- and caveolin-independent mechanisms. All pathways eventually converge at the level of early endosomes, the major hub for incoming cargo. Image from [49]

To get into a cell, endocytic cargo can take many routes. There is phagocytosis and macropinocytosis, both pathways that involve a large deformation of the membrane towards the outside. These entryways will not be discussed further here.

Other pathways include clathrin-dependent, caveolin-dependent and clathrin- and caveolin-independent mechanisms (Fig.3).

Clathrin-mediated endocytosis

Although by far not the only pathway for endocytic cargo to enter a cell, clathrin-mediated endocytosis (CME) is the most extensively described and studied endocytic pathway and appears in all eukaryotic cells. Knockout of clathrin or several other core proteins of CME are lethal in flies, worms and mice [50-53], underlining the importance of this process.

Fig.4: Five steps in clathrin-mediated endocytosis A) Depicted are the five steps of clathrin-mediated endocytosis (CME): nucleation, cargo selection, coat assembly, scission and uncoating with the key proteins of every step. B) Interactome of the proteins involved in CME, with the major hub proteins being AP2 and clathrin. Image from [49]
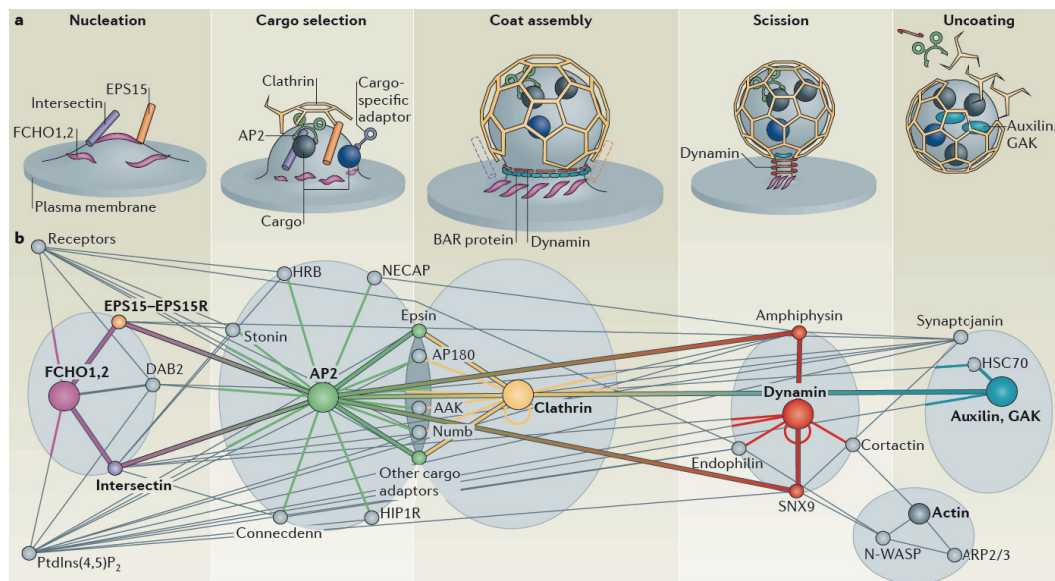
Endocytosis via clathrin can be separated into 5 different steps: initiation, cargo selection, coat assembly, scission and finally uncoating.

For the initiation, an invagination of the PM at the site of endocytosis will form. It seems that the definition of the site, where this invagination will occur, might be mediated by binding of FCHo1/2, EPS15 and intersectins [54, 55]. They preferentially bind to PI(4,5)P$_2$ at the PM and FCHo proteins have membrane-bending activity, thereby helping the formation of a stable invagination [54, 56].

After this, the endocytic cargo needs to be selected. This process is mediated by AP2, one of the core proteins of CME and other adaptor proteins, binding to AP2. AP2 can either bind receptors directly or it binds adaptor proteins, which in turn bind receptors at the PM [57-59]. The specificity of AP-2 acting preferentially at the PM stems from its ability to bind PI(4,5)P$_2$ [60, 61]. AP-2 not only helps in cargo selection but will also recruit clathrin from the cytosol to the nascent pits [62]. The clathrin triskelia will form a lattice around the curvature, thereby stabilizing the curvature. However, it has been observed that clathrin can also assemble on flat membranes for certain cell types and will form a stable lattice with a comparable low turnover rate [63], challenging the notion of clathrin only being assembled after membrane curvature has been established. Indeed, a recent finding shows that clathrin seems to be first present as a flat lattice and during the formation of a clathrin-coated pit undergoes continuous rearrangement as curvature increases [64].

Once the clathrin-coated vesicle has formed, it needs to pinch off from the PM to traffic towards the endocytic pathway. For this, the action of dynamin is needed [65, 66]. Dynamin is a GTPase and will form a ring-like helix structure around the "neck" of the budding vesicle [67]. The specific recruitement of dynamin to the strongly curved neck

of a clathrin coated pit (CCP) can either be mediated by curvature-sensing proteins with a BAR domain such as amphiphysin or endophilin [68, 69] or by curvature itself [70, 71]. It will then undergo a GTP dependent conformational change [67] leading to the constriction of the helix. After constriction, the vesicle needs to detach from the PM. How exactly this occurs remains a matter of debate, yet some general hypothesis about this process exist. One possibility is that fission is a spontaneous process, facilitated by constriction of dynamin and dependent on membrane tension [72, 73] and lipid composition [74].

The last step is the uncoating of the vesicle. This process is mediated by the ATPase HSC70 and its cofactor auxilin [75, 76]. It also seems that changes in the lipid composition of the vesicle are required for uncoating, as the lipid phosphatase synaptojanin is also essential in this process [77]. It will act preferentially on curved membranes [78], creating PI(4)P from PI(4,5)P$_2$. This might serve as a recruiting signal for auxilin, which binds to PI(4)P [79]and auxilin in turn will recruit HSC70 [80, 81]. With uncoating the clathrin coat becomes available again for other nascent pits while the new vesicle progresses through the endocytic pathway.

Clathrin-independent endocytosis

Apart from CME, a large number of different pathways into cells exist. Although very different from one another, they are grouped under the term clathrin-independent endocytosis (CIE).
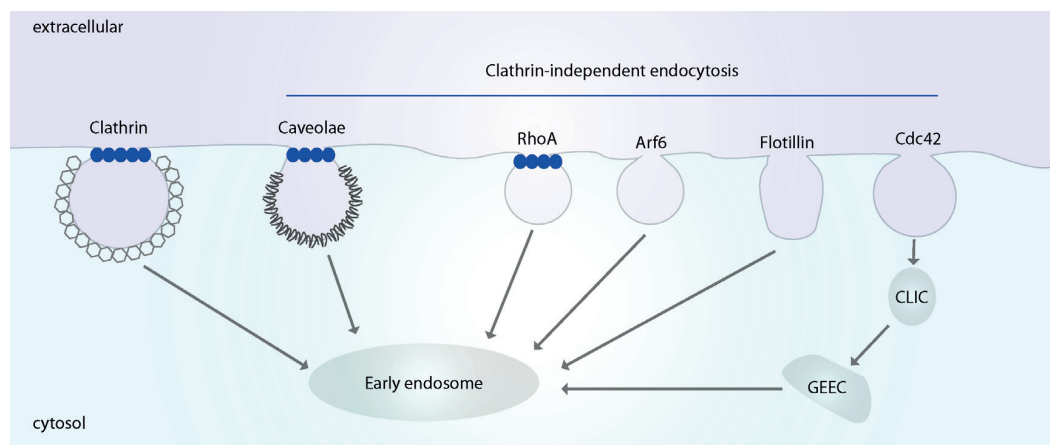
Fig.5: Pathways of clathrin-independent endocytosis *Clathrin-independent endocytosis can to date be classified into 5 different routes that are either dynamin-dependent (blue circles) or –independent. All pathways eventually converge in the EE, from where cargo is sorted to recycling or to degradation.*

For further specification, the pathways are classified as either dynamin-dependent or –independent and are generally divided into five routes: caveolae, RhoA-, Flotillin-, Cdc42- or Arf6-regulated [82] . This list is probably not exhaustive, as novel pathways continue to be described, such as fast endophilin-mediated endocytosis (FEME) [83].

In general the requirements for cargo selection for CIE is not well understood and so far, no specific adaptors, such as those found for CME, have been identified for CIE [84]. Additionally, it seems that these pathways are at least partly overlapping for some cargo proteins and might be highly cell-type and context-dependent [85, 86].

In the absence of the classical curvature-generating proteins from CME, it seems that CIE (except caveolar endocytocis) has established a general theme with actin as a core component for generating pits. Curvature generating proteins on the in- and outside could help with clustering receptors and applying bending force to generate stable pits without caveolar and clathrin coats [87].

Caveolae

Caveolae are stable invaginations of the PM containing caveolin, its accessory proteins called cavins and that are rich in sphingolipids and cholesterol [88]. Although normally stable at the PM [89], caveolae can become endocytosed vesicles when stimulated [90, 91]. The internalization process of caveolae is dynamin-dependent [92] and in contrast to CME, the coat structure is not disassembled after budding [90].

Caveolae, apart from being endocytic carriers, seem to be dominantly active in their "second life" and are important in PM architecture and signaling [88].

RhoA-dependent endocytosis

This pathway was initially discovered as the pathway to internalize interleukin 2 (IL-2) receptor [93]. It is now also clear that other receptors can make use of this pathway [84, 94]. As caveolae and CME, it depends on dynamin and also on the presence of lipid rafts [93].

Cdc42-dependent endocytosis (CLIC/GEEC pathway)

Cdc42, like RhoA, is a small GTPase that regulates this dynamin-independent pathway [95]. The most prominent cargoes of this pathway are GPI-anchored proteins [95, 96] and it seems to be the major fluid uptake route in cells [97]. The correct functioning of the pathway depends on cholesterol and actin [98]. The vesicle carriers of this pathway are called clathrin- and dynamin-independent carriers (CLICs) [99] and will mature into GPI-AP enriched early endosomal compartments (GEECs) [95] that will eventually fuse with early endosomes [100].

Arf6-dependent endocytosis

This pathway is probably the most cryptic CIE route to date. It is supposed to play a role in the internalization of e.g. MHC class I, β1 integrins and GPI-AP [84]. However, it might be that it is not necessarily endocytosis but rather recycling that is regulated by this small GTPase [101]. Further debate is on the subject if Arf6 depends on dynamin or not,

how cell-type variability can be accounted for [84, 85] and if Arf6 and Cdc42-dependent pathways are really distinct or might intertwine [82].

Flotillin-dependent endocytosis

Flotillin-dependent endocytosis has been described as an additional way for cargo to get into cells in a clathrin- and dynamin-independent manner [102]. Flotillins can induce curvature of the membrane, circumventing the need for other curvature-inducing and stabilizing proteins [103]. Although initially described as not dependent on dynamin, some studies show a dependency on dynamin [104, 105]. Potentially, flotillins recruit specific cargo and target them to other, dynamin-dependent or –independent mechanisms.

Endosomal pathway/endosome maturation
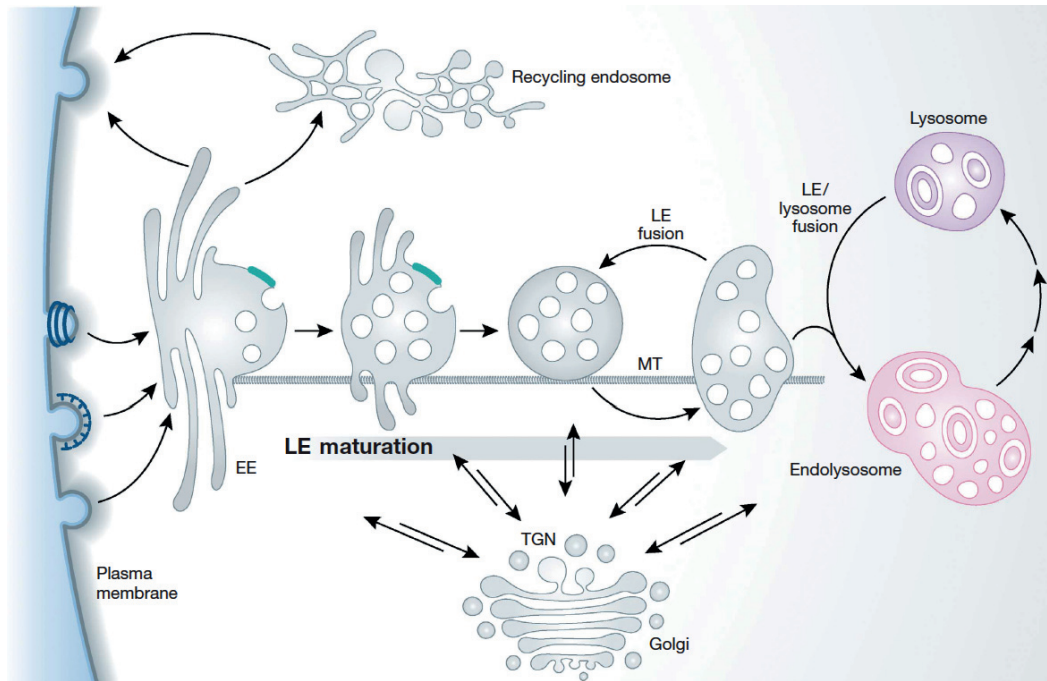
Fig.6: Schematic depiction of the general endocytic pathway *After being endocytosed, cargo is delivered to early endosomes (EE), from where it can either be recycled back to the PM or can continue by maturation on to late endosomes (LE). Finally, LE merge with lysosomes to endolysosomes, which will eventually mature to lysosomes. Figure from [106]*

Early endosomes (EE) are the first place cargo encounters after endocytosis and also the main sorting compartment of the endocytic pathway [106]. EE are vesicles loosely defined by several characteristics: they have a pH of 6.8-5.9, making them slightly acidic [107], the presence of Rab5, a small GTPase [108] and the phospholipid PI(3)P [106, 109] and their structure with both vacuolar and tubular domains. The tubular domains

can vary in their membrane composition and their function, linked to their function as different sorting regions/vesicle generation hubs [110-112]. The vacuolar part of EE seems to be the domain that matures into late endosomes (LE). Generally speaking, these LE are more acidic than EE, with a pH ranging from 6.0 to 4.9 [107], Rab5 is replaced by Rab7 [108, 113], PI(3)P is converted into PI(3,5)P$_2$ by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) [114-116] and the lumen of LE contains a large number of intraluminal vesicles (ILV). LE will fuse with lysosomes, creating endolysosomes, where most of the degradation processes take part.

But the default pathway for cargo arriving in EE is not degradation but rather recycling. In this case, receptors will traffic to EE and will be retargeted to the PM via recycling endosomes. To be selected for degradation, receptors seemingly need to display a characteristic signal, otherwise they will follow the bulk back to the surface [117].

The maturation from EE to LE can be described by different, intertwining and interdependent processes taking part. The first one is the so-called Rab switch, where different Rab proteins present on EE will be exchanged by others during maturation. Especially Rab5 and Rab7 have been described as master regulators of endosome maturation. On EE, Rab5 and many of its effector proteins are present, giving the compartment its identity [108, 109]. One of the effectors is the VPS34/p150 complex, which is a PI kinase complex and produces the EE signature lipid PI(3)P [118, 119]. To mature into LE, Rab5 is replaced by Rab7 [113, 120]. This switch also changes the recruitment of accessory proteins, modifying the identity of the vesicle and e.g. ensuring that the maturing endosomes will only fuse with downstream vesicles such as other LE or lysosomes, thereby securing the unidirectionality of the pathway [121].

Vesicles in the endocytic pathway also have their own specific lipid "fingerprint" which changes during maturation with PI(3)P being the prominent lipid for early and PI(3,5)P$_2$ for late endosomes. These lipids in turn recruit specific subsets of effector proteins that bind to their destination vesicle via PI-binding domains, such as FYVE, PH, PX, PHOX and BAR [122, 123]. Among these effector proteins are also PI kinases, thereby regulating local generation of the necessary PI species. An example is the generation of PI(3,5)P$_2$ by PIKfyve, which will bind to PI(3)P rich membranes, i.e. membranes of EE with its FYVE domain, generate PI(3,5)P$_2$ and thereby contribute to the maturation to LE [115, 116, 124] .

Other markers of endosome maturation include change in size and appearance, the ability to communicate with the PM via recycling is lost, change in intracellular localization, changes in ion composition and different associated proteins and the formation of ILV [106] . The presence of ILV characterizes the appearance of LE, which are therefore also termed multivesicular bodies (MVB).

ESCRT system and ubiquitin-dependent sorting

Formation of ILV can already appear in EE [125] and is mediated by the endosomal sorting complex required for transport (ESCRT) [126-130]. The ESCRT system consists of five complexes that take part in the different processes during cargo selection for degradation, bud formation and membrane scission [131]. The ESCRT complex plays

also a role in other cellular processes such as autophagy and cytokinesis [132], yet this will not be discussed here.

Table 1: List of protein components of the ESCRT complexes in both yeast and human from [133]

The complexes		Function	Necessary for MVB sorting?
Yeast	Human		
ESCRT-0		Binds ubiquitinated cargo and	
Vps27	Hrs	PtdIns(3)P	Yes
Hse1	STAM1/2		No
ESCRT-I		Binds ESCRT-0,-II, and	
Vps23	Tsg101	ubiquitinated cargo;	Yes
Vps28	Vps28	localizes to midbody during	Yes
Vps37	Vps37A-D	cell division	Yes
Mvb12	hMvb12A, B		Slight defect
—	UBAP1		Yes
ESCRT-II		Binds ESCRT-I and nucleates	
Vps36	EAP45	ESCRT-III; binds	Yes
Vps22	EAP30	ubiquitinated cargo and	Yes
Vps25	EAP20	PtdIns(3)P	Yes
ESCRT-III		Sculpts MVB vesicles and	
Vps20	CHMP6	drives cytokinesis	Yes
Snf7	CHMP4A-D		Yes
Vps24	CHMP3		Yes
Vps2	CHMP2A, B		Yes
Vps60	CHMP5		Yes
Bro1	Alix		Yes
Ist1	IST1		No
Did2	CHMP1		Yes
AAA ATPases		Drives ESCRT-III disassembly	
Vps4	Vps4A and B	severs MT during	Yes
—	Spastin	abscission	Yes

In early endosomes, cargo that has entered the endocytic pathway needs to be separated into a pool that will go back to its original localization via recycling and a pool that is destined for degradation. With recycling being the default pathway, sorting to the degradative system needs to be mediated by a signal. This signal is mostly the ubiquitination of target proteins [134].

Ubiquitination is a posttranslational modification that consists of the attachment of the 76 amino acid peptide ubiquitin to lysine residues of target proteins [135]. Ubiquitination controls a plethora of cellular processes, similar to phosphorylation, yet ubiquitination tops phosphorylation in terms of complexity as ubiquitin can itself be ubiquitinated, leading to complicated chains and linkages.
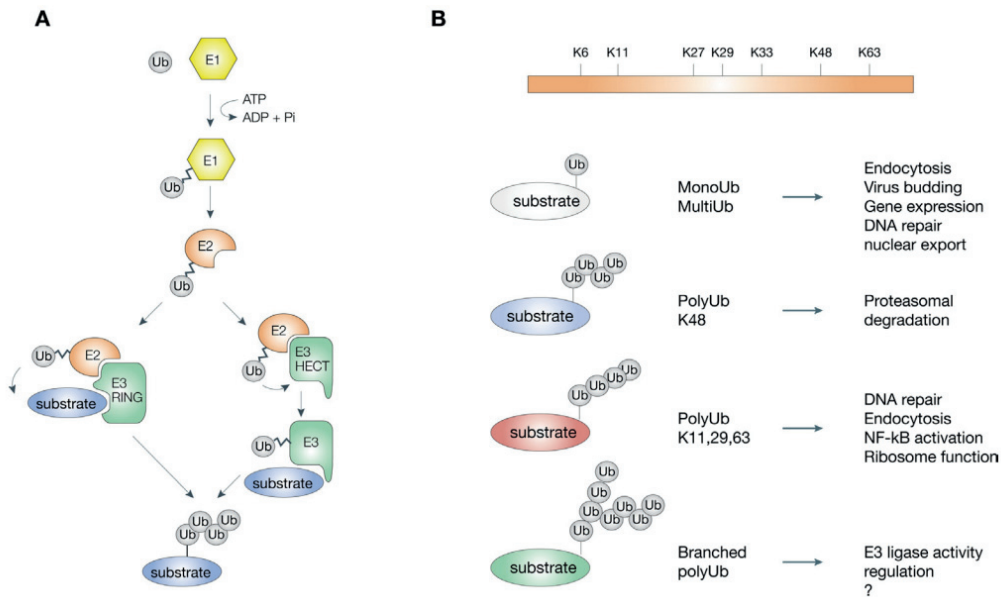
Fig.7: Enzymatic reaction of ubiquitination and different linkages A) Shown is the enzymatic, ATP-dependent cascasde, leading to the ubiquitination of the target protein. E1 ubiquitin-activating enzyme acquires an Ub moiety and passes it on to E2 ubiquitin-conjugating enzymes. From there it will go to members of either the RING or HECT family of E3 ligases, which will eventually pass it on to the substrate. B) Structure of the ubiquitin protein with its lysines and the consequences of the different linkages on the fate of the ubiquitinated protein. Image from [136]

The attachment of ubiquitin is an ATP-dependent three-step process, mediated by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases [137] (Fig.7A). There are only two E1 and 37 E2 enzymes but over 600 E3 ligases [135]. The ubiquitin is attached by its C-terminal glycine residue to lysines of the target protein by the E3 ligases. Ubiquitin itself contains 7 lysines (K6, K11, K27, K29, K33, K48 and K63) which themselves can be ubiquitinated. The linkage and length of the ubiquitin modification determine the fate of the modified protein. Additionally, it has been recently reported that ubiquitin can be phosphorylated on Ser65, adding an additional layer of regulation [138]. Ubiquitination is a reversible modification and the breakdown of ubiqutitin chains is mediated by enzymes called deubiquitinases (DUBs) [139].

Although it is not clear, what effect exactly every possible ubiquitination pattern will have, some "canonical" patterns have been studied quite extensively. The most famous is the K48 polyubiquitin chain, a signal for proteasomal degradation [140]. The ubiquitin signal for endocytosis was long believed to be only monoubiquitination, but also K63 linked chains have been reported to control this process [141-143]. Other processes that ubiquitin controls range from DNA repair, gene expression, to autophagy and virus budding (Fig.7B).

Proteins can be ubiquitinated in a variety of different patterns. The pattern associated with targeting to ILV seems to be primarily K63-linked ubiquitination, either in form of poly-, mono- or multimono-ubiquitin chains [134]. The ubiquitinated cargo is bound by

the ESCRT complexes, which contain ubiquitin-binding motifs and is then handed over to the next complex in line [131, 133, 134].
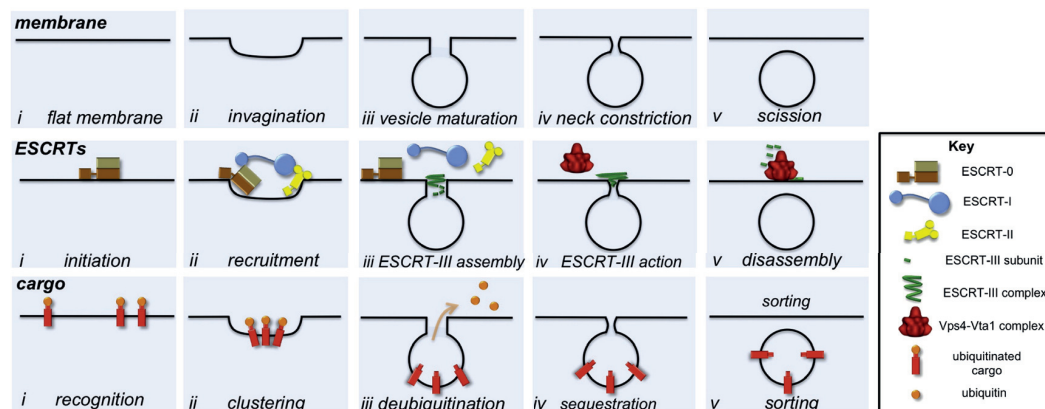

Fig.8: Depiction of the actions of ESCRT complexes Shown is the movement and deformation of the membrane, the different ESCRT complexes associated at each step and the consequences for the protein cargo. Image from [144]

The first to encounter ubiquitinated cargo is the ESCRT-0 complex. In mammalian cells, ESCRT-0 consists of Hrs and STAM1/2 [129, 130] and will, in addition to ubiquitin, also bind to PI(3)P via the FYVE domain of Hrs, thereby ensuring faithful localization to early endosomes [145]. STAM contains a SH3 domain, which will recruit and interact with two deubiquitinating enzymes: AMSH and USP8 (UBPY) [146, 147]. By modifying the ubiquitination of proteins, these two proteins can affect the fate of cargo that associates with ESCRT-0. Depending on the type of modification and also on the protein, cargo will either by sent to recycling or to degradation following deubiquitination by AMSH and USP8 [148]. USP8, in addition to modifying cargo, also seems to stabilize Hrs and STAM2, thereby contributing to normal functioning and morphology of endosomes [149, 150].

ESCRT-0 has now clustered cargo together and then mediates recruitment of ESCRT-I to endosomes [151, 152], thereby conveniently ensuring the handover to the next complex. ESCRT-I also binds to ESCRT-II, forming a supercomplex that is important for the budding of the membrane in ILV formation [153]. ESCRT-II will then recruit ESCRT-III, which will finally act on pinching off the membrane and thereby finalizing the process of a successful ILV formation [154]. For the disassembly of ESCRT-III, the last complex, Vps4-Vta1 is needed [155]. Vps4 is an AAA ATPase and associates with Vta, forming a complex that will render the membrane-bound ESCRT-III complex soluble again, ready for a new cycle [144].

Endocytosis and signaling

The link between endocytosis and signaling has usually been the notion that after endocytosis, the signal of e.g. receptor tyrosine kinases is stopped, given that they are sequestered from the PM and degraded. But more recently, increasing attention has been

given to the fact that these two processes seem to be intimately linked and that endocytosis can control signaling but also vice versa [156].

First, endocytosis can indeed attenuate signaling by changing the amount or the localization of receptors at the surface. Attenuation of the signal can also be achieved by physically separating the ligand from the receptor e.g. by a change of lipid species, which differs between PM and endosomes [157]. On the other hand, signaling can continue from endosomes e.g. for EGFR or GPCR [158, 159] or might exclusively be restricted to endosomes, such as in some signaling cascades of the TGFβ receptor [160]. Where signaling occurs might affect the outcome and change the proteins that are activated downstream [161] but also how receptors enter the cell influences their signaling [162].

Signaling can also have an impact on endocytosis. Studies on the EGFR show that activation of the receptor by its ligand can increase clathrin coated pit formation through the downstream activation Src kinase, thereby increasing its uptake [163, 164]. Other impacts are e.g. on the speed of endocytosis [165] or on the morphology of the pathway [166], thereby showing that cargo itself can tweak its entry route.

Pathogen entry

Many pathogenic agents, such as viruses, bacteria or toxins need to get into their host cell to replicate or to exert their function. Therefore, the easiest way in is to hijack already existing pathways and potentially even customize them for the respective needs.

Viruses and endocytosis

For viruses, entering cells by endocytosis is not only an easy way in but also comes with several other advantages. Endocytosis delivers viruses through the cytoplasm close to the nucleus, thereby avoiding the path through the cytoplasm. The identity of the maturing endocytic vesicles helps the virus to determine its localization in the cell and the change in pH can provide an important cue for uncoating and penetration [167, 168]. Other proteins, such as proteases present in endosomes can help activate viral particles [169, 170] and finally, entry through endosomes hides the virus as it leaves no trace at the PM, thereby probably helping immune avoidance [171, 172]. There are viruses such as HIV-1 that can establish a successful infection both by entering the cell via endocytosis and by fusion with the PM [173, 174], but the majority of viruses depend on at least a part of the endocytic machinery for an efficient entry [172]. Generally speaking, viruses can exploit any given endocytic pathway, from CME, caveolae and also CIE or even completely novel pathways [175, 176]. They might even manipulate the speed of uptake by invoking formation of CCP, such as influenza virus [177] or stimulate CME for increased cholesterol trafficking to create favorable replication conditions, such as enteroviruses [178]. All this has made viruses a favorite for deciphering intracellular trafficking pathways for decades and more recently, also a promising approach for intracellular delivery of drugs and proteins [179, 180].

Bacteria and endocytosis

Bacteria as an endocytic cargo are extremely large, ranging typically between 1-5 μm. This means that classical pathways, as described earlier cannot be applied here. In general, bacteria are taken up into cells by an-actin dependent reorganization of the PM, either by a so-called zipper or trigger mechanism [172]. For the zipper mechanism, some bacteria such as *Listeria monocytogenes* or uropathogenic *Escherichia coli* will bind to receptors at the PM, trigger recruitment of clathrin and potentially other factors and leads to actin rearrangement, ultimately engulfing the bacteria [181, 182]. It seems that clathrin serves as a cellular platform from which activation of internalization can be initiated instead of its canonical role.

The trigger mechanism is used e.g. by *Shigella* and *Salmonella* to enter cells. Here, the bacteria actively initiate uptake by injecting effector proteins into the cell, which will induce extensive rearrangements of the actin cytoskeleton and formation of ruffles which will eventually envelope the bacteria [172].

Once inside the cell, bacteria by all means need to avoid reaching the lysosome. The tactics to do this reach from perforating and destroying the endosomes by secreted toxins or by blocking maturation by different means [183].

Toxins and endocytosis

Protein toxins, such as botulinum, tetanus, Shiga, anthrax or ricin toxins also make use of the various entry routes to gain access into their target cells. In fact, the notion of clathrin-independent endocytosis originally stems from the observation that ricin, a plant toxin, can enter cells even in the absence of clathrin [184]. The classical entry route of toxins is that they will bind to their receptor at the PM and will then enter the cell either via clathrin-dependent or –independent mechanisms. Sometimes this is not exclusive: some toxins have been reported to enter via several pathways, such as Shiga toxin, which can enter by several mechanisms, despite binding exclusively to one receptor [185]. The same is true for cholera toxin [186]. Toxins are also excellent in modifying their own uptake. Shiga toxin will reorganize lipids on the PM to maximize uptake [187] or will increase the formation of CCP via the tyrosine kinase Syk [188]. Like viruses, toxins have been used extensively in studying endocytosis and are a promising approach for intracellular targeting of drugs. There is even an approved immunotoxin consisting of a fusion of diphtheria toxin and IL2, which is used in T-cell lymphoma [185].

Anthrax toxin endocytosis

Anthrax is an infectious disease that is caused mainly by the exotoxins of the Gram-positive, spore-forming bacteria *Bacillus anthracis*. *B. anthracis* is a member of the *Bacillus* family and is commonly found in soil. The animals that are most commonly affected with anthrax are herbivores and infection can occur worldwide, although cases are rare and only sporadic [189, 190].

Anthrax has received a large amount of attention after the bioterrorism attacks of 2001 in the US. Weaponized, i.e. easily inhalable anthrax spore preparations led to 22 cases of anthrax of which 11 were fatal. In an earlier incident in 1979 in Russia where there was an unintentional release of anthrax spores, 77 cases were diagnosed of which 66 were fatal [190].

Anthrax is acquired by spores entering the body, either through a lesion, by inhalation or ingestion. Cutaneous anthrax, which is when anthrax spores enter the body through a skin lesion, is the most common but also the most treatable form of anthrax. If spores are inhaled or ingested, the disease is much more difficult to manage. For cutaneous anthrax, localized tissue edema and necrosis lead to a black eschar at the lesion site, a very distinct feature of anthrax. Gastrointestinal and inhalation anthrax present themselves with very non-specific symptoms and then in the acute phase will develop high fever, toxaemia and sepsis, which can be fatal [189, 191].

One of the main virulence factors of *B.anthracis* is the anthrax toxin. The anthrax toxin is a tripartite AB toxin with the three subunits Protective Antigen (PA), Edema Factor (EF) and Lethal Factor (LF). PA is the receptor-binding subunit, whereas EF and LF are the enzymatic subunits of the toxin. All the toxins are encoded on the virulence plasmid pXO1 [192].
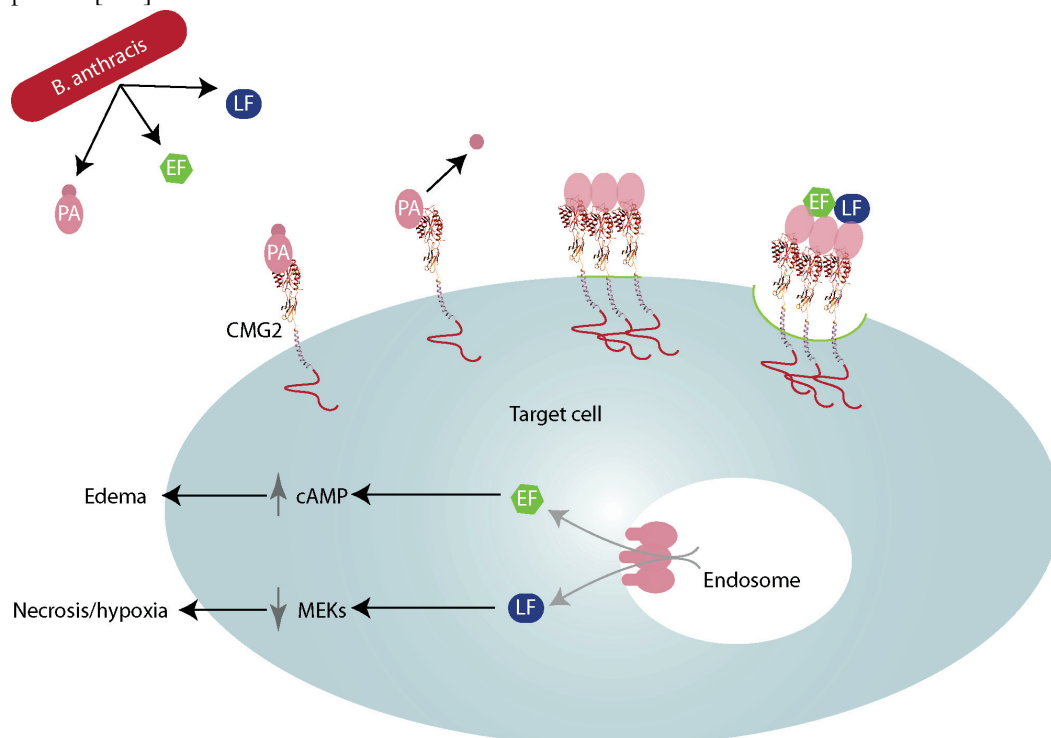
Fig.9: Entry of anthrax toxin into target cells. B.anthracis *secretes 3 subunits of the anthrax toxin: protective antigen (PA), edema factor (EF) and lethal factor (LF). PA will bind to either of the anthrax toxin receptors, CMG2 or TEM8, will be cleaved by furin and then heptamerize in lipid rafts. There it can bind the enzymatic subunits EF and LF and subsequently internalize. In the endosome, the heptamer will insert into the membrane, form a translocation pore and allow dislocation of EF and LF to the cytoplasm. EF elevates cytosolic cAMP levels and LF cleaves MEKs, thereby leading to edema and necrosis.*

All three toxin subunits are secreted by the bacteria and as a first step, PA^{83}, the 83kDa form of PA will bind to the vWA domain of one of the two anthrax toxin receptors on the target cell, CMG2 or TEM8. The main anthrax toxin receptor *in vivo* is CMG2 [193]. This might be partially due to a higher affinity of PA for CMG2, which ranges from 1000-fold for purified vWA domains [194] to only about 11-fold in cell culture experiments [193]. As PA^{83} is unable to interact with EF or LF, the toxin has to be modified. Therefore, after binding to the receptor, the toxin will be cleaved by furin, which will produce PA^{63} [195, 196]. The cleavage recognition site on PA is Arg-X-X-Arg, which when changed to Pro-Gly-Gly, cannot be cleaved, thereby rendering the toxin unable to proceed after binding to the receptor [197]. After this step the receptor-toxin complex multimerizes, either into heptamers or octamers [198, 199]. Up to 3 subunits of either LF or EF can then bind to the complex. These multimers then partition into specific nanodomains of the plasma membrane, so-called lipid rafts [200]. The binding and heptamerization leads to the activation of Src-like kinases, which can then phosphorylate the receptor on its cytoplasmic tail [201]. This phosphorylation is a prerequisite for ubiquitination, which is important for endocytosis [201, 202]. TEM8 is ubiquitinated by Cbl, the E3 ligase of CMG2 so far remains unknown [202]. Another protein that is involved at this phase is β -Arrestin, an adaptor protein in CME [29]. Anthrax toxin endocytosis is a clathrin-dependent process [29] although it might also be able to use alternative routes [203]. Actin also plays a role in the endocytosis of both receptors. It pre-organizes TEM8 in clusters at the PM and then is important in the internalization of both receptors in a toxin-bound state [29]. Once inside the endocytic pathway, the heptamer will at one point insert into the membrane and form a pore that will allow LF and EF to translocate to the other side. This translocation is proton-gradient driven [204] and follows the model of a Brownian-ratchet model [205]. To pass through the narrow PA channel, LF and EF partially unfold in the acidic environment of the endosome [206]. LF and EF can act on their targets in the cytoplasm. LF is a zinc-dependent metalloproteinase. It cleaves MEKs, which are MAP kinase kinases and have a vast inhibitory effect on diverse cellular pathways [207]. EF is a calmodulin-dependent adenylyl cyclase, which leads to the increase of cAMP, a second messenger impacting on a variety of cellular functions [191]. Whether this translocation occurs at the early or late endosome, depends on the receptor the toxin is bound to. TEM8-bound PA will insert already at early endosomes, CMG2-bound PA only at late endosomes [208]. Translocation of EF and LF seems to occur across the PA pore into the lumen of ILV. Release of the enzymatic subunit to the cytoplasm can then occur by backfusion of these ILVs with the limiting membrane [209]. The ILVs can however also eventually be released as exosomes and transmit the enzymatic toxin subunits to naïve cells, circumventing receptor usage, thereby acting as a long-term and long-range transmitter for anthrax toxin [210].

28

3. Partner proteins of CMG2

To study the physiological function of CMG2 more in detail, several potential partner proteins were identified by either Yeast 2 Hybrid screens or proteomic studies. Here, the four proteins studied during this thesis are rapidly introduced.

MARK2

The microtubule-affinity regulating kinases (MARK) or Par-1 kinases are part of the AMPK/Snf1 subfamily of the Ca^{2+}-calmodulin-dependent protein kinases (CaMK) [211]. In mammals we can find MARK1-4 (corresponding to Par1c, b, a, d) and each isoform can occur in different splice forms [212]. As the name implies, MARKs were initially identified as regulators of microtubule dynamics by means of phosphorylating microtubule-associated proteins (MAPs) [211]. Yet over the years it has become clear that they are also involved in several other key cellular processes such as establishment of cell polarity, neuronal differentiation, intracellular trafficking, cell migration and neurodegeneration [213].

MARK2 (Par1b) has been extensively described in the establishment of polarity in various mammalian cells. MARK2 is a downstream target of aPKC, another kinase master regulator of cell polarity and is involved in the establishment of asymmetric membrane domains [214, 215]. aPKC phosphorylates a threonine residue (T508 for isoform 3) which antagonizes MARK2 activity. The phosphorylation promotes binding to 14-3-3 proteins, sequesters MARK2 away from the basolateral membrane, where it is usually found and acts negatively on its kinase activity [214, 215].

MARK2 controls polarity by acting on the microtubule cytoskeleton [216] but also regulates the RhoA-dependent rearrangements of the actin cytoskeleton [217]. The effects of MARK2 on the cytoskeleton are also apparent in cells infected with *Helicobacter pylori*. CagA, a virulence factor of *H.pylori*, associates with and inhibits MARK2 [218]. CagA activity leads to cells with an elongated shape, causing the so-called hummingbird phenotype. This phenotype partly depends on stabilization of microtubules, breakdown of focal adhesions and activation of myosin II, all of which are regulated by MARK2. By inhibiting MARK2, CagA ensures an efficient breakdown in polarity and the establishment of the hummingbird phenotype [219].

Another way of establishing polarity for MARK2 is by communicating with the ECM [220, 221]. Rho kinase (ROCK) ensures basolateral positioning of MARK2 in mouse epithelium, which in turn controls the correct spatial deposition of basement membrane [221]. Almost the same is true in MDCK cells, where MARK2 regulates the localization of the dystroglycan complex to the basolateral side, which is a spatial cue for deposition of laminin, a member of the basement membrane [222]. Also in MDCK cells, MARK2 controls the actin organization as well as the cell-ECM matrix communications by regulating the scaffolding protein IRSp53 [220].

MARK2 contributes to the establishment of polarization by affecting polarized trafficking [223, 224]. It was also shown that MARK2 can interact with AP-2, thereby associating with CCVs [225].

Interestingly, as for CMG2, MARK2 has repeatedly been associated with the Wnt signaling pathway. In *Drosophila*, *Xenopus* and in mammalian cells MARK2 potentiates Wnt signaling and treating cells with Wnt vice versa increases MARK2 activity [226]. MARK2 phosphorylates Dishevelled, which leads to a downstream β-catenin stabilization in the canonical Wnt pathway [226]. This regulation is important during different steps of development and might be mediated by different isoforms [227]. MARK2 also seems to be a link between the TGFβ pathway and the non-canonical Wnt pathway. MARK2, Dishevelled and SMAD4 form a complex upon Wnt5a stimulation, mediating crosstalk between the two pathways [228].

RNF41

RNF41 (Nrdp1/FLRF) is an E3 ligase, which was first found to control trafficking of neuregulin receptors ErbB3 and ErbB4 [229]. It is ubiquitiously expressed with a higher signal in brain, heart and skeletal muscle cells [229] and also in prostate, ovary and testes [230]. RNF41 ubiquitinates ErbB3 and promotes its degradation, independent if ligand is bound or not [229, 230]. RNF41 also autoubiquitinates itself, which leads to its degradation by the proteasome, thereby making the protein in cells very unstable [229, 230]. This autoubiquitination requires oligomerization of RNF41, which is dispensable for ubiquitination of its substrates [231]. RNF41 can be stabilized by the DUB USP8, which removes the ubiquitin moiety from RNF41 [232]. Interestingly, it seems as if USP8 is stabilized in response to ligand binding of the ErbB receptors and this in turn stabilizes RNF41, ultimately leading to a downregulation of the pathway [233].

The regulation of the two proteins might be both ways, as RNF41 was also shown to ubiquitinate USP8, induce its degradation and thereby also affect stability of the ESCRT-0 complex [234].

RNF41 also regulates degradation of other receptors or proteins, such as parkin [235], cytokine receptors [236] and MyD88 [237]. For some proteins, interaction with RNF41 actually targets them away from degradation, potentially by rerouting them to other compartments [238].

In addition to a role in trafficking and degradation, RNF41 has also been linked to polarity. A proteomic study suggests that RNF41 might be the missing mammalian orthologue of Par2, a protein involved in polarity in *C.elegans* and *Drosophila* [239]. Par2 interacts with Par1, where the mammalian homologues are members of the MARK family, such as MARK2. In another study, RNF41 was found to be a novel MARK2 effector in establishing cell polarity in mammalian epithelia cells [240]. MARK2 phosphorylates RNF41 at S254 and this in turn is important for the correct positioning of laminin receptor to the basolateral side and subsequent correct deposition of the basement membrane protein laminin [240]. This study also considers the idea of RNF41 being functionally orthologous to Par2.

USP8

The deubiquitinating enzyme USP8 (UBPY) was first discovered to be associated with the ESCRT-0 complex [147]. Hrs and STAM, the two ESCRT-0 complex members are linked to two DUBs, namely AMSH and USP8. In the absence of USP8, ubiquitinated proteins accumulate on endosomes, including components of the ESCRT complex, and MVBs tend to accumulate [149, 241]. Depletion of USP8 *in vivo* is embryonic lethal and even a conditional knockout in adult mice results in liver failure [150]. Again, endosomes display an abnormal enlarged phenotype, which is accompanied by gradual loss of several growth factor receptors and the ESCRT-0 complex [150].

Several receptors or more specifically their trafficking has been shown to be regulated by USP8. CXCR4, a chemokine receptor is stabilized in the absence of USP8 and accumulates at the early endosome [242]. Activated receptor tyrosine kinases, such as EGFR and met, are also stabilized in the absence of USP8 [241]. But, especially for EGFR, there is conflicting data on the effect of USP8. USP8 was shown to drive EGFR degradation by promoting sorting into MVBs after being activated by Src [243-245]. Other studies claim that USP8 rescues EGFR from degradation by targeting it to recycling rather then to the degradative pathway [149, 246]. So far it is unclear, which scenario is correct or whether the observed effects are context-dependent.

As described earlier, USP8 and RNF41 seem to be in the same pathway. They cross-regulate each other, thereby impacting on the decision if a receptor is sent to recycling or degradation [234].

Very recently, mutations in USP8 were found to cause Cushing's disease [247-249], which is the overexpression of cortisol from the pituitary, most often as a result from a pituitary adenoma. The mutations that were identified are gain of function mutations located in exon 14, located around the 14-3-3 binding motif. USP8 can no longer bind to 14-3-3, thereby showing increased DUB activity. They target EGFR to recycling, prolonging its signaling after receptor activation [249].

Cbl

Cbl (Casitas B-lineage Lymphoma) is also an E3 ubiquitin ligase of a highly conserved family that has homologues in *Drosophila*, *C.elegans* and even *Dictyostelium discoideum*. Initially, Cbl was found as v-Cbl, what is now known to be a viral oncogene in murine leukemia virus [250]. The cellular homologue c-Cbl or Cbl is actually a longer form of this truncated version and is implicated in various cellular processes, in particular in the regulation of receptor tyrosine kinase (RTK) trafficking [251, 252].

The consensus is that ubiquitination of cell surface receptors ultimately promotes their lysosomal degradation [253]. However, at what step exactly Cbl acts on the receptor after ligand binding remains a controversial subject. Cbl can directly ubiquitinate receptors, which could mediate the first step of internalization [254]. This view has been challenged, suggesting that ubiquitination of EGFR is important for sorting to degradation but not for endocytosis [255]. Also, the role of Cbl in endocytosis might be independent from its role as an E3 ligase but might be more linked to its function as an adaptor protein. Cbl contains a Tyrosine Kinase Binding (TKB) domain, which can bind

to phosphorylated tyrosines in specific peptides of protein tyrosine kinases, such as EGFR, Src/Fyn or ZAP70. It also has a C-terminal proline rich region that can bind SH3-domain containing proteins such as CIN85, Grb2 or again Src/Fyn [253]. This makes it a perfect adaptor protein. After ligand binding to the EGFR, Cbl becomes activated and can recruit CIN85. This leads to the formation of a large complex of proteins required for endocytosis, such as curvature-inducing endophilins, synaptojanin, which acts on membrane lipid composition or ARAP3, a protein linked to cytoskeletal rearrangements [256-258]. Interestingly, ARAP3 has been shown to be important for anthrax toxin entry as well [259]. Therefore, Cbl might act directly and indirectly on endocytosis of cell surface receptors, determining their fate at different steps of the endocytosis process.

Note: This introduction contains parts of a review manuscript in preparation.

4. Glycosylation and ERQC/ERAD

Glycosylation

Posttranslational modifications of proteins are important for and modulate a variety of functions in proteins. Attaching e.g. phosphate groups, lipid moieties or glycan sidechains can change the properties of a protein and subsequently its functions and activity. Glycosylation is one of the most common protein modifications and bioinformatics estimate that more than 50% of eukaryotic proteins are glycosylated [260]. The process of glycosylation is conserved between eukaryotes and prokaryotes [261] and glycosylation is implicated in various cellular processes such as protein folding, stability, trafficking, localization, endocytosis, interactions and adhesion [262-264]. There are five types of glycosylation: N-, C-, O- and P-glycosylation as well as glypiation, with N-glycosylation being the most common one [265]. N-glycosylation consists of two steps: the attachment of a core oligosaccharide to the asparagine residue of the N-X-S/T consensus sequence (where X is any amino acid except proline) and initial trimming in the ER followed by additional trimming and extension of the glycan sidechains in the Golgi [266, 267]. The core oligosaccharide, which is co-translationally attached to the asparagine residue of transmembrane and secreted proteins by the oligosaccharyltransferase (OST) consists of $Glc_3Man_9GlcNAc_2$ (Glc: glucose, Man: mannose, GlcNAc: *N*-acetylglucosamine). The building blocks of the chains consist of ten monosaccharides: Glc, Man, GlcNAc, Fuc (fucose), Gal (galactose), GalNAc (*N*-galactosamine), GlcA (glucuronic acid), IdoA (iduronic acid), SA (sialic acid) and Xyl (xylose). The estimates of possible glycan sidechains in the human glycome come up with a number of around 7000, highlighting the diversity that glycosylation can confer to its protein substrate [268].

N-glycosylation and protein folding

Although N-glycosylation affects various processes, its best characterized role is in protein folding. Before being shuttled to their final destination in the cell and becoming active, proteins need to fold correctly. For membrane and secreted proteins, folding occurs in the ER and generally with the help of ER-resident chaperones. These proteins need to distinguish between newly synthesized and terminally misfolded proteins in the densely packed ER and therefore decide their fate. This is a tricky task and N-glycosylation has been proposed to be both advantageous for folding [269] and also to help the ER quality control machinery to distinguish these two protein groups [270].
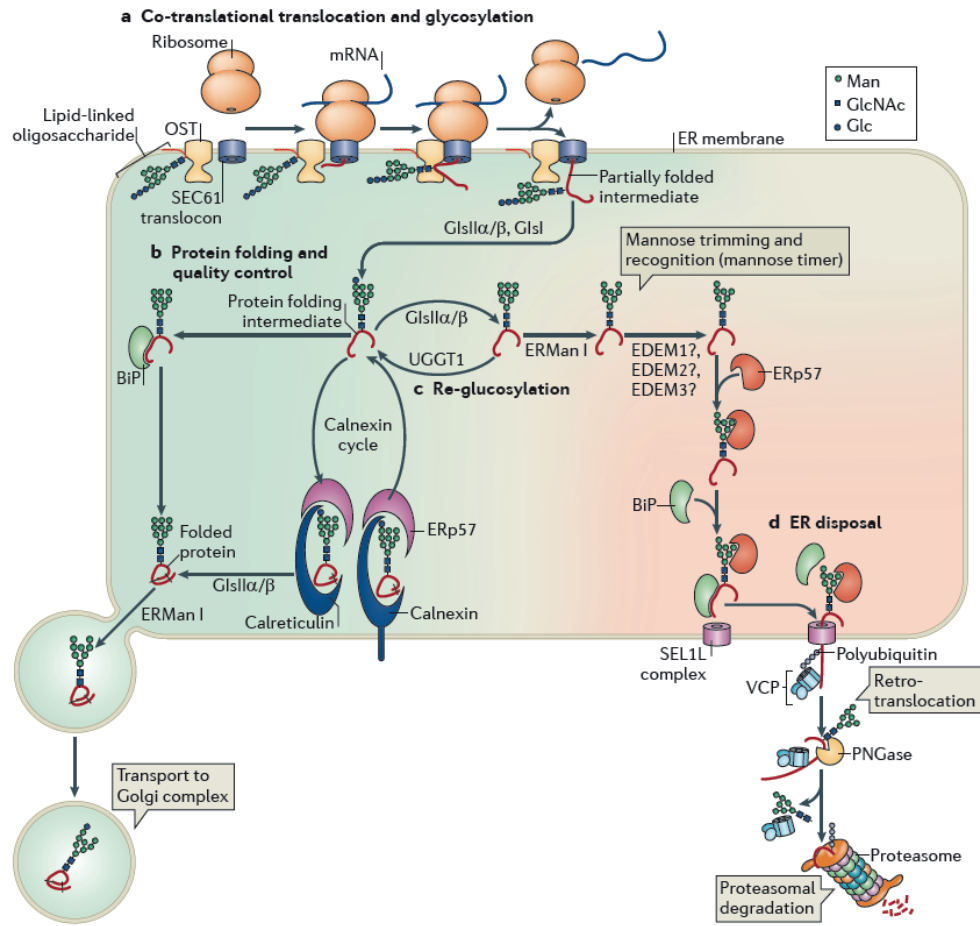
Fig.10: Scheme of N-glycosylation in the ER and its role in ER quality control and ER associated degradation *In short: proteins are co-translationally glycosylated with the core oligosaccharide, transferred to the acceptor sequence by the OST. After initial trimming of the two outermost glucose residues, the protein is prone to interact with the ER resident chaperones calnexin or calreticulin. If the protein folds correctly, the last glucose residue is trimmed and the protein is transported to its final destination via the Golgi complex. If folding is unsuccessful, the protein will be targeted to ER associated degradation (ERAD) by ER quality control (ERQC). The glycan sidechain is extensively trimmed to its mannose residues, which then acts as a targeting signal for ERAD. The terminally misfolded proteins are retrotranslocated out of the ER and degraded by the proteasome. Figure from [264]*

Proteins are co-translationally glycosylated during their entry into the ER by the oligosaccaryl transferase (OST) [267, 271] and the first glucose residue is immediately trimmed by α-glucosidase I. This di-glycosylated protein species was long believed to be only an intermediate. Recently, malectin, an ER-resident lectin specifically binding these proteins, was discovered [272]. It seems that already at this step, malectin can recognize misfolded proteins and target them for degradation [273-275]. The second trimming of a glucose residue, this time by α-glucosidase II, leads to the association of the protein with either calnexin or calreticulin. Calnexin and calreticulin are ER-resident lectins, sharing both structural as well as binding similarities: they both bind to monoglycosylated oligomannose glycans in a calcium-dependent manner [276]. Calnexin, itself a transmembrane protein, can bind both soluble as well as transmembrane proteins.

Calreticulin, which is soluble, can also bind both types but seems to prefer soluble substrates [270]. It was initially thought that both calnexin and calreticulin were specific for glycosylated proteins, but more recent works suggests that they can also assist the folding of non-glycosylated substrates by binding to peptide regions instead of the glycan sidechain [277, 278]. Nevertheless, both proteins are essential for the correct functioning of cells, as knockout mice for either calreticulin or calnexin either are embryonic lethal for calreticulin or are born with strong motor disorders for calnexin, with only very few mice surviving past 48h after birth [279, 280].

While interacting with either calnexin or calreticulin, proteins try to fold correctly. The third and last glucose residue can be removed by α-glucosidase II, which is the enzyme already responsible for the removal of the second glucose residue. In contrast to the first reaction, removal of the last glucose residue decreases the association of the protein with the chaperone, thereby releasing it. From here, there are two possible fates of the protein, depending on its folding state:

1) **The protein is correctly folded**

In this case, the protein exits the ER via COP II vesicles towards the Golgi apparatus [281]. Here, the glycan sidechain undergoes further trimming and extensions. These modifications are mediated by a plethora of glycosidases, glycosyltransferases and other glycan processing enzymes that are organized in the Golgi in a *cis*-to-*trans* fashion [282]. Through all these modifications, the proteins can achieve a high degree of complexity in their glycan structure. Additionally, several intrinsic characteristics of the Golgi contribute to the diversity. First, trafficking of the enzymes but also the substrates in the Golgi makes the glycan processing a dynamic and competitive process, with the result that even a single site on a protein might be modified differently. Second, the pH of the Golgi has been shown to modify efficiency of glycosylation reactions [283] and lastly, the presence and correct functioning of the conserved oligomeric Golgi (COG) complex is crucial for correct glycan modifications to occur [284, 285].

After traversing the Golgi and having gone through all the modification steps, the proteins will be routed to their final destination in or outside the cell.

2) **The protein is not correctly folded**

In this case, the protein is recognized and will be reglucosylated by UDP-Glc:glycoprotein glucosyltransferase (UGGT1), which in turn leads to re-association with calnexin or calreticulin [286, 287]. Subsequent cycles of α-glucosidase II and UGGT1 action give the misfolded protein several chances to finally achieve a native conformation. If this fails, the terminally misfolded protein needs to be removed from the ER, as accumulation of misfolded proteins in the ER would lead to ER stress and damage the cell. The misfolded protein will be recognized by ER quality control (ERQC) and will be then targeted to ER associated degradation (ERAD).
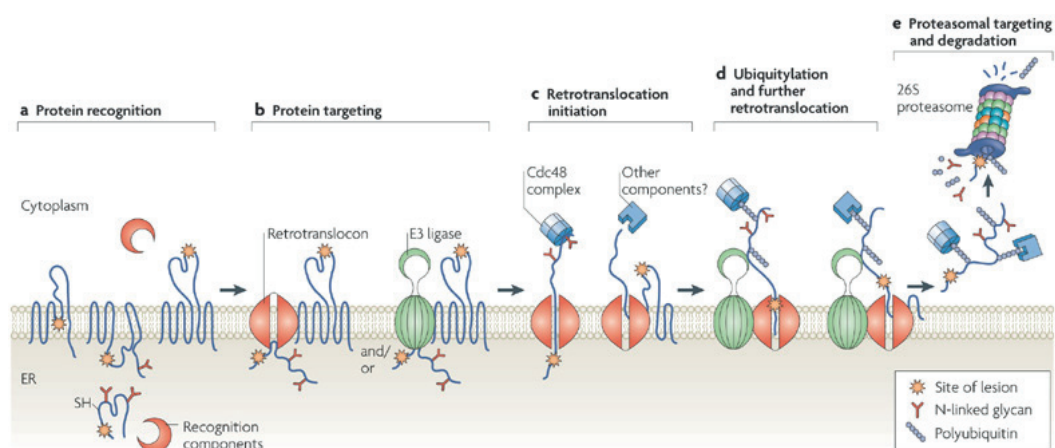
Fig.11: Scheme of the different steps of ER-associated degradation (ERAD) *Proteins that are terminally misfolded or not correctly assembled will be recognized by ERQC and targeted for degradation. After retrotranslocation to the cytoplasm through a still unknown channel, the protein will be sent to the proteasome and will be degraded. Figure from [288].*

After several futile folding attempts and thus a prolonged residence time in the ER, misfolded proteins are recognized and tagged for degradation. To do so, ER-resident mannosidases will extensively trim the glycan sidechain, generating a tag for ERAD [289-291]. The mannose residues are recognized by ER lectins OS-9 and XTP3-B [292-295]. For non-glycosylated proteins, ERAD targets also seem to be processed by the same proteins, although in this case they seem to be recognized by BiP and GRP94 [296, 297]. Degradation of ERAD substrates occurs in the cytosol by the proteasome. Therefore, a retrotranslocation step is required to deliver the proteins from the ER to the cytosol. Although extensive research in this field has been going on for years, the exact nature of the retrotranslocation channel is still elusive. Several candidates involve Sec61, several E3 ligases, members of the Derlin family and others, as reviewed in e.g. [288, 298, 299].
Retrotranslocation in most cases is an ubiquitination-dependent step. Substrates are ubiquitinated on the cytoplasmic side and are then extracted from the membrane. This action is either performed by the proteasome directly [300, 301] or by the Cdc48 complex [302]. In mammals, this complex consists of the AAA ATPase p97 and the two cofactors UFD1 and NPL4. After extracting the ERAD substrate to the cytosol, the Cdc48 complex hands it over for degradation to the proteasome, potentially with the help of additional proteins [303]. The glycan sidechains are removed by a glycanase [304-306] and the substrate might be deubiquitinated before entering the proteasome [288].

The amount of misfolded proteins varies greatly and depends on factors such as folding rate, enzyme availability and metabolic state of the cell but also differs for any given protein. This means that even under ideal conditions, certain proteins will be degraded and the efficiency of protein can be rather low [307, 308]. For notoriously bad folder such as CFTR, only around 25% of even wildtype proteins under the best conditions will make it past the ER [309], whereas other proteins such as α1AT can attain efficiencies up to 90% [293]. This means that ERQC and ERAD are processes that have to be

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extremely well tuned. Too efficient or too weak ERQC will lead to pathological phenotypes, either by degrading proteins too fast or by an accumulation of un-/misfolded proteins. Therefore, their activity is regulated by ERAD tuning. This model is based on the segregation and selective degradation of ERAD factors, thereby modulating the efficiency of the whole process [310].

Also, ERQC factors need a reliable way to recognize terminally misfolded proteins and to distinguish them from folding intermediates. Both protein species share similarities by exposing hydrophobic patches, which in a correctly folded protein would not be accessible [288, 299]. For unassembled subunits of oligomeric complexes, which are also considered ERAD substrates, it seems that the exposure of unshielded, charged residues in the transmembrane domain serve as a degron sequence [311-313].

As most of the conserved cellular pathways, the ERAD pathway can and will be hijacked by pathogenic agents. Bacterial toxins such as cholera toxin, Shiga and Shiga-like toxin but also the plant toxin ricin utilize ERAD to get from the ER to the cytosol [314]. Hijacking ERQC and ERAD was also shown for Simian Virus 40 (SV40) and mouse polyomavirus, both trafficking from endosomes to the ER and from there to the cytosol and eventually the nucleus [315, 316]. Other viruses modify ERAD for their advantage. Human cytomegalovirus infection leads to the degradation of MHC class I and II receptors with the help of ERAD factors, thereby dampening the immune response [317, 318]. HIV can downregulate the expression of its cell surface receptor CD4 by inducing the proteasome-mediated degradation of CD4 [319]. Hepatitis virus B and C in contrast downregulate ERAD function, thereby helping to establish a persistent infection [320, 321].

II. Results: Folding of anthrax toxin receptors

Differential Dependence on N-Glycosylation of Anthrax Toxin Receptors CMG2 and TEM8

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Abstract

ANTXR 1 and 2, also known as TEM8 and CMG2, are two type I membrane proteins, which have been extensively studied for their role as anthrax toxin receptors, but with a still elusive physiological function. Here we have analyzed the importance of N-glycosylation on folding, trafficking and ligand binding of these closely related proteins. We find that TEM8 has a stringent dependence on N-glycosylation. The presence of at least one glycan on each of its two extracellular domains, the vWA and Ig-like domains, is indeed necessary for efficient trafficking to the cell surface. In the absence of any N-linked glycans, TEM8 fails to fold correctly and is recognized by the ER quality control machinery. Expression of N-glycosylation mutants reveals that CMG2 is less vulnerable to sugar loss. The absence of N-linked glycans in one of the extracellular domains indeed has little impact on folding, trafficking or receptor function of the wild type protein expressed in tissue culture cells. N-glycans do, however, seem required in primary fibroblasts from human patients. Here, the presence of N-linked sugars increases the tolerance to mutations in *cmg2* causing the rare genetic disease Hyaline Fibromatosis Syndrome. It thus appears that CMG2 glycosylation provides a buffer towards genetic variation by promoting folding of the protein in the ER lumen.

Introduction

N-Glycosylation is one of the most prevalent protein modifications and is largely conserved between eukaryotes and prokaryotes [1]. Based on bio-informatics analyses, it has been estimated that more than 50% of all eukaryotic proteins are glycosylated [2] underlining the importance of these modifications in diverse cellular processes including protein folding, stability, trafficking, endocytosis, cell adhesion and cellular recognition events [3].

N-linked protein glycosylation can be viewed as a two-step process with a first step in the endoplasmic reticulum (ER) and the second in the Golgi. In the ER, a core oligosaccharide consisting of $Glc_3Man_9GlcNac_2$ (Glc: glucose, Man: mannose, GlcNAc: *N*-acetylglucosamine) is attached to asparagine residues within the N-X-S/T consensus sequence and undergoes initial trimming. When the protein is transported to the Golgi, the oligosaccharide undergoes further modifications, in particular the addition of complex sugars [4, 5].

N-glycosylation plays an important role in protein folding in the ER. The core oligosaccharide is attached to the polypeptide chain as it is translocated into the ER lumen across the translocon pore. The two outer glucoses are immediately removed by glucosidases I and II. This enables association of the nascent polypeptide chain with calnexin or calreticulin, chaperones involved in ER folding and quality control [6, 7]. These two ER-resident lectins specifically bind monoglucosylated oligomannose glycans [8]. If folding and, when relevant, oligomerization are successful, the newly synthesized protein exits the ER via COPII coated vesicles and is subsequently routed to its final destination [9].

If folding is aberrantly delayed or unsuccessful, the protein can be targeted to ER-associated degradation (ERAD) [10]. Prolonged presence in the ER leads to extensive mannose trimming of the oligosaccharide, which acts as a targeting signal for ERAD [11–13]. Exposed mannoses are recognized by the ERAD lectins OS-9 and XTP3-B [14–16]. Since degradation is proteasome mediated, ERAD substrates must be retrotranslocated to the cytoplasm through a ubiquitination-mediated process [17]. Once in the cytosol, the remaining glycans are removed by a glycanase, before the protein is handed over to the proteasome for degradation [18, 19]. Thus N-linked sugars play crucial roles first in promoting protein folding via glucose residues and then targeting the protein to degradation via the mannose residues.

We here investigated the importance of N-glycosylation in the trafficking and function of two surface receptors probably involved in the homeostasis of the extracellular matrix, namely TEM8 (tumor endothelial marker 8) and CMG2 (capillary morphogenesis gene 2). TEM8 and CMG2 are two highly homologous type I membrane proteins, composed of an extracellular von Willebrandt A domain (60% identity between the two proteins), an Ig-like domain, a transmembrane domain and a cytosolic tail that differs in size between the two proteins but is initiated by a 68% identical domain (S1 Fig.).

TEM8 was discovered as an upregulated gene in human tumor endothelium [20]. It was subsequently found to serve as a receptor for anthrax toxin, hence the name anthrax toxin receptor 1 (ANTXR1) [21]. Recent studies describe TEM8 as a cancer [22–24] and cancer stem cell [25] marker due to its upregulation in tumor, but not physiological, vasculature. Consistently, interfering with TEM8 leads to a decrease in tumor angiogenesis [26–28]. Mutations in TEM8 can lead to GAPO syndrome, a rare, complex and severe autosomal-recessive disorder [29, 30].

CMG2 was found as the second most upregulated gene in a 3D culture of endothelial cells [31]. Its vWA domain was found to bind *in vitro* to the ECM proteins laminin and collagen type IV [31]. CMG2 knockout mice display an accumulation of ECM in different organs [32, 33], indicating a role for CMG2 in the homeostasis of ECM. This notion is strengthened by the pathology of patients afflicted with Hyaline Fibromatosis Syndrome, a disease caused by mutations in *cmg2*. Patients suffer from the accumulation of a hyaline material in skin and other organs which can be life threatening or highly debilitating [34]. Most reported mutations in the vWA and the Ig-like domain of CMG2 lead to misfolding of the protein, provoking its retention in the ER and its degradation by ERAD, resulting in loss of protein function [35]. The drastic effect of these point mutations suggests a sensitive folding landscape for CMG2.

Both TEM8 and CMG2 contain potential N-glycosylation sites: N166 and N184 in the vWA and N262 in the Ig-like domain of TEM8, and N250 and N260 in the Ig-like domain of CMG2. In this study, we show that glycosylation can occur at all potential sites and that glycosylation is necessary for TEM8 to fold, exit the ER and reach the plasma membrane. We found that CMG2 is less dependent on glycosylation for folding and thus its N-glycosylation-independent exit from the ER depends on the folding capacity/ER quality control stringency of the cell. Importantly, the dependence on glycosylation becomes apparent when genetic mutations such as

those found in Hyaline Fibromatosis Syndrome patients, decrease folding efficiency of the extracellular domains.

Materials and Methods

Cells and reagents

HeLa cells were grown in Modified Eagle's medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamine, non-essential amino acids, penicillin and streptomycin. RpeI cells, patient fibroblasts and Flp-In T-REx 293 CMG2 cells (Invitrogen, Carlsbad, CA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM penicillin and streptomycin. For induction of CMG2, Flp-In T-REx 293 CMG2 cells were treated for 24h with 0.1 μg/ml doxycycline.

The human CMG2 (isoform 4, Uniprot P58335–4) gene, with a V5 epitope at the C-terminus was cloned in the pcDNA3.1/V5-HIS-TOPO expression vector (Invitrogen, Carlsbad, CA) and was provided by J. Martignetti (Mount Sinai School of Medicine, New York, NY; Dowling et al., 2003). For stable cell lines, CMG2 was cloned into the pcDNA5/FRT/TO vector (Invitrogen, Carlsbad, CA) and transfected cells were selected according to the manufacturer's protocol. The human TEM8 (isoform 1, Uniprot Q9H6X2–1) gene with an HA epitope was cloned in the pIREShyg2 vector (Liu and Leppla, 2003). Mutations were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). All plasmids were transfected into cells using Fugene according to the manufacturer's protocol (Promega, Madison, WI).

Anthrax toxin was purified as described before [36]. Polyclonal goat antibody (#771B) against Protective Antigen (PA) was from List Biological Laboratories (Campbell, CA) and used at 1/2000 dilution, monoclonal mouse V5 antibody (#R960–25) was from Invitrogen (Carlsbad, CA) and used at 1/2000 dilution; monoclonal rat HA-HRP antibody (#12 013 819 001) was from Roche (Basel, Switzerland) and used at 1/2000 dilution; monoclonal mouse Ubiquitin antibody (#sc-8017) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used at 1/500 dilution; polyclonal rabbit calnexin antibody was produced by Eurogentec for our lab [37] and used at 1/2000 dilution; polyclonal rabbit BiP antibody (#ab21685) from Abcam (Cambridge, UK) used at 1/1000 dilution; polyclonal rabbit antibody against TEM8 was generated in our lab and used at 1/2000 and polyclonal goat CMG2 antibody (#AF2940) was from R&D Systems (Minneapolis, MN) and used at 1/2000 dilution.

HRP-conjugated secondary antibodies were from Pierce Chemical Co. (Rockford, IL) and Alexa-conjugated secondary antibodies from Molecular Probes (Invitrogen, Carlsbad, CA). Streptavidin-agarose conjugated beads were from Sigma-Aldrich (St. Louis, MO), protein G beads were from GE Healthcare (Uppsala, Sweden) and HA-beads from Roche (Basel, Switzerland);

Treatments with N-glycosidase and Endoglycosidase H (New England Biolabs, Ipswich, MA) were performed as previously described [38].

Immunofluorescence

Transiently transfected HeLa cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X100, and stained with antibodies against the V5 or HA tag and the ER marker BiP followed by an appropriate secondary antibody. Images were acquired using a Plan-Apochromat 63x/1.4 oil objective on a Zeiss LSM 710 (Carl Zeiss Microimaging, Thornwood, NY), equipped with an Axiocam MRm camera using the Zen 2009 acquisition software. FIJI and Adobe Illustrator software were used to prepare the figures.

Surface biotinylation and immunoprecipitation

For immunoprecipitation, confluent cells were washed three times with PBS. Cells were lysed by incubation for 30 min at 4°C with 0.5% NP-40, 500 mM Tris-HCl, pH 7.4, 20 mM EDTA, 10 mM NaF, 30 mM sodium pyrophosphate decahydrate, 2 mM benzamidine, 1 mM PMSF, 1 mM NEM and a cocktail of protease inhibitors (Roche, Basel, Switzerland). Cells lysates were incubated overnight at 4°C with anti-V5 antibody and protein G sepharose beads for CMG2, or HA-beads for TEM8. For the ubiquitination assay, cells were treated for 4h with 10 μM MG132 or 0.1 μM Bafilomycin A1 (Sigma) before cell lysis.

For surface biotinylation, confluent cells were incubated with 0.2 mg/ml NHS-SS-biotin (Pierce) in PBS for 30 min at 4°C and washed 3x 10 min with sterile PBS containing 100 mM NH_4Cl. Cells were lysed and the lysate was immunoprecipitated with streptavidin-coated aga-rose beads. After SDS-PAGE and Western blotting against the V5 or HA antibody, biotinylated CMG2 or TEM8 proteins and expression level were quantified with Image Quant TL 2005 / Typhoon software (GE Healthcare) or BioID software (Fusion). Biotinylated proteins values were normalized to expression level values. Expression of mutants at the cell surface was nor-malized to that of the WT protein. To assess surface expression of non-glycosylated endoge-nous protein, cells were treated with 5 μg/ml tunicamycin (Sigma) for 16h and then labeled as described above.

For *in vivo* anthrax protective antigen (PA) binding, transiently transfected HeLa cells were incubated for 1h at 4°C with 500 ng/ml PA[83] in internalization medium (IM medium), (Glas-gow minimal essential medium, Invitrogen, 10mM HEPES, pH 7.4). Cells were washed twice with warm IM medium to remove excess toxin and incubated for 10 min at 37°C to induce cleavage and heptamerization. Cells were then lysed and immunoprecipitated against TEM8-HA or CMG2-V5. For *in vitro* PA binding, transiently transfected HeLa cells were lysed and the lysate was incubated for 1h at 4°C with 1μg/ml PA[83]. Lysates were immunoprecipitated against TEM8-HA or CMG2-V5.

Graphics

Molecular graphics and analyses were performed with the UCSF Chimera package [39]. For TEM8, the graphic is based on structure 3N2N from the Protein Data Bank (http://www.rcsb.org/pdb/). For CMG2, the graphic is based on structure 1TZN from PDB and on modeling from [40].

Ethical Statement

Primary Human fibroblasts from a control and a Hyaline Fibromatosis syndrome patient were obtained with patient consent, research using these cells was approved by the "Commission Cantonale d'éthique de la recherche sur l'être humain" and are registered under the approval No A070055 of the Swiss Federal Office of the Environment. Patients provided written consent that patient derived cells could be used in any studied aimed at a better understanding of Hya-line Fibromatosis syndrome and the gene involved. The patient signed a standard consent form approved by the ethics committee.

Results

TEM8 and CMG2 can undergo N-glycosylation on all predicted sites

TEM8 and CMG2 have been shown to be glycosylated, yet the location and number of sites have not been determined [38]. They respectively contain 3 and 2 predicted N-glycosylation sites, of which only one is conserved: N262 in TEM8 corresponding to N260 in CMG2 within
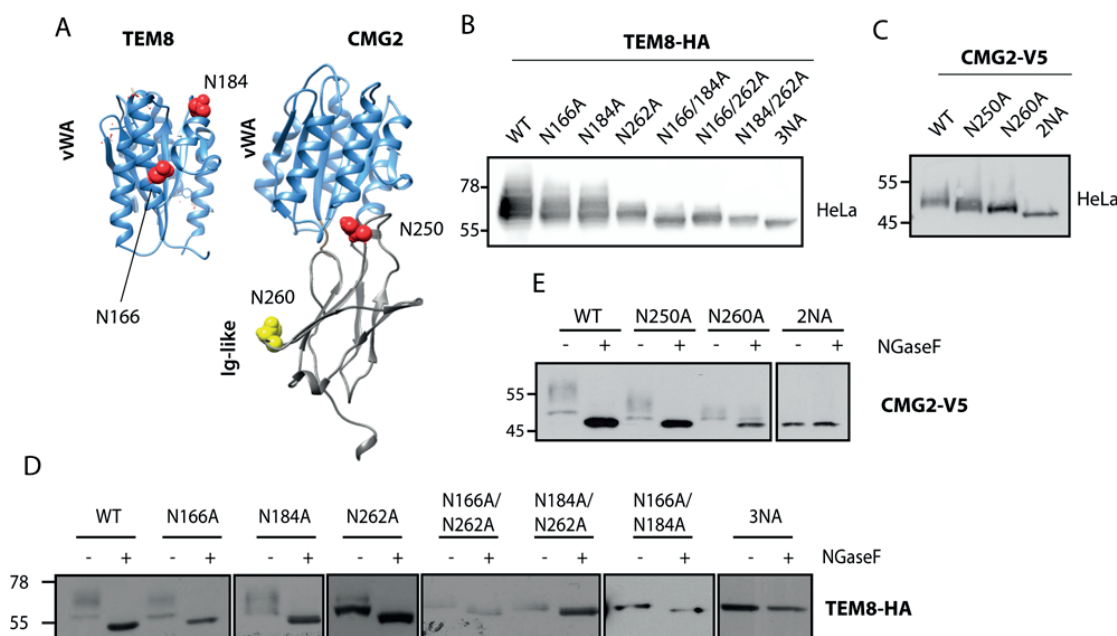
Fig 1. CMG2 and TEM8 can undergo N-glycosylation on all predicted sites. A) Graphics depicting glycosylation sites on TEM8 and CMG2. Sites in red are unique to the respective proteins, N260 in CMG2 (yellow) corresponds to N262 in TEM8. **B)** Expression of TEM8 glycosylation mutants in HeLa cells. Cells were transfected for 48h with the respective cDNAs. Expression was analyzed by SDS-PAGE and Western Blotting. **C)** Expression of all CMG2 glycosylation mutants in HeLa cells. Cells were transfected for 48h with the respective cDNAs. Expression was analyzed by SDS-PAGE and Western Blotting. **D)** Endoglycosidase F (NGaseF) treatment on TEM8 glycosylation mutants. 40 μg of cell extracts were treated or not with NGaseF and analyzed by SDS-PAGE and Western Blotting. **E)** Endoglycosidase F (NGaseF) treatment on CMG2 glycosylation mutants. 40 μg of cell extracts were treated or not with NGaseF and analyzed by SDS-PAGE and Western Blotting.

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the Ig-like domain (Fig. 1A). To test whether these sites can be modified, we generated single, double and triple asparagine mutants. Expression of the mutants was analyzed by transient transfection of HeLa cells (Fig. 1BC). For both TEM8 and CMG2, mutation of a single asparagine residue, at whatever position, was sufficient to induce a change in electrophoretic mobility. The most dramatic change was observed when mutating the conserved site in the Ig-like domain (N262/N260, Fig. 1BC). Mobility increased as more asparagines were mutated. Also, TEM8 and CMG2, which migrate as smears when WT, migrated as a well-defined single band as glycosylation sites were mutated (Fig. 1BC). All together this analysis indicates that all sites can be modified in WT TEM8 and CMG2.

To confirm that all predicted sites can indeed be glycosylated, Total Cell Extracts (TCE) of cells expressing the mutants were treated with N-Glycosidase F (NGaseF), an enzyme that removes all glycan side chains of a protein, regardless of their modification or localization [41]. For both TEM8 and CMG2, NGaseF-treatment led to a change in electrophoretic mobility of WT and all mutants, with the exception of the mutants with all potential sites mutated (Fig. 1DE), indicating that all sites can be modified *in vivo*. Note that all sites might not be modified simultaneously, and thus cells might express differentially glycosylated species.

The number of glycan sidechains and their localization determine trafficking efficiency of the protein

The first hurdle for any protein is to fold. Because glycosylation has been shown to promote folding of certain proteins such as the multi-pass membrane protein cystic fibrosis transmembrane conductance regulator CFTR [42] or human immunodeficiency virus-1 (HIV-1) protein gp120 [43], we investigated whether mutating glycosylation sites in CMG2 and TEM8 affects their folding. As a readout, we utilized the modification of N-linked sugars by Golgi enzymes since proper folding is a prerequisite for ER exit and trafficking to the Golgi apparatus. Modification of N-linked sugars by Golgi enzymes renders them insensitive to the enzyme Endoglycosidase H (EndoH). Transiently expressed WT TEM8 or CMG2 migrate as a smear, corresponding to the Golgi modified form, and a lower molecular weight band, corresponding to the immature ER form, which is EndoH sensitive [35, 38]. All single CMG2 and TEM8 glycosylation mutants migrated as an EndoH insensitive smear and an EndoH sensitive band, indicating that a fraction of all mutants is able to fold correctly, exit the ER and reach the Golgi (Fig. 2A). Fully glycosylation deficient mutants cannot be analyzed using this assay.

Following sugar modification in the Golgi, CMG2 and TEM8 are transported to the plasma membrane where they exert anthrax toxin receptor and presumably physiological functions. To assess whether glycosylation influences surface targeting, we performed a surface biotinylation assay. This assay allows the affinity isolation of the surface population and comparison to the overall expression levels. Mutation of either of the two vWA domain glycosylation sites in TEM8, N166 and N184, did not affect surface expression when compared to WT, but simultaneous mutation of both led to a 50% decrease in surface expression (Fig. 2BC). A similar drop in surface expression was observed when mutating N262 in the Ig-like domain (Fig. 2BC). A pronounced drop in surface expression was observed when all three sites where modified (Fig. 2BC). Thus TEM8 requires two N-linked glycans for efficient trafficking to the cell surface, of which one site must be N262.

For CMG2, loss of either of the two glycosylation sites within the Ig-like domain lead to a 30% drop in plasma membrane targeting (Fig. 2DE). Surface targeting was also impaired when both sites were absent (Fig. 2DE), yet at least 50% was still properly addressed to the plasma membrane.

Thus plasma membrane targeting of both TEM8 and CMG2 is enhanced by the presence of two N-linked sugars, one of which must reside in the Ig-like domain.

TEM8 glycosylation promotes ER exit

The surface biotinylation analysis indicates that fully glycosylation-deficient TEM8 and CMG2 do not or only partially reach the plasma membrane. We analyzed their localization by immunofluorescence microscopy. As expected, the WT form of TEM8 and CMG2 could be detected at the plasma membrane as well as intracellular structures (Fig. 3AB and S2 Fig.). Plasma membrane staining was also observed for CMG2 2NA (Fig. 3B and S2 Fig.). Note that immunofluorescence does not allow a quantitative analysis of surface expression as does surface biotinylation (Fig. 2). In contrast, the triple 3NA TEM8 mutant was absent from the plasma membrane and showed clear ER staining, co-localizing with the ER chaperone BiP (Fig. 3A).

Non-glycosylated TEM8 is an ER quality control and ERAD substrate

The above observations indicate that glycosylation promotes ER exit, raising the possibility that in the full absence of glycosylation, folding in the ER is impaired, leading to recognition of the protein by the ER quality control and possible targeting to ERAD. To address this issue, we
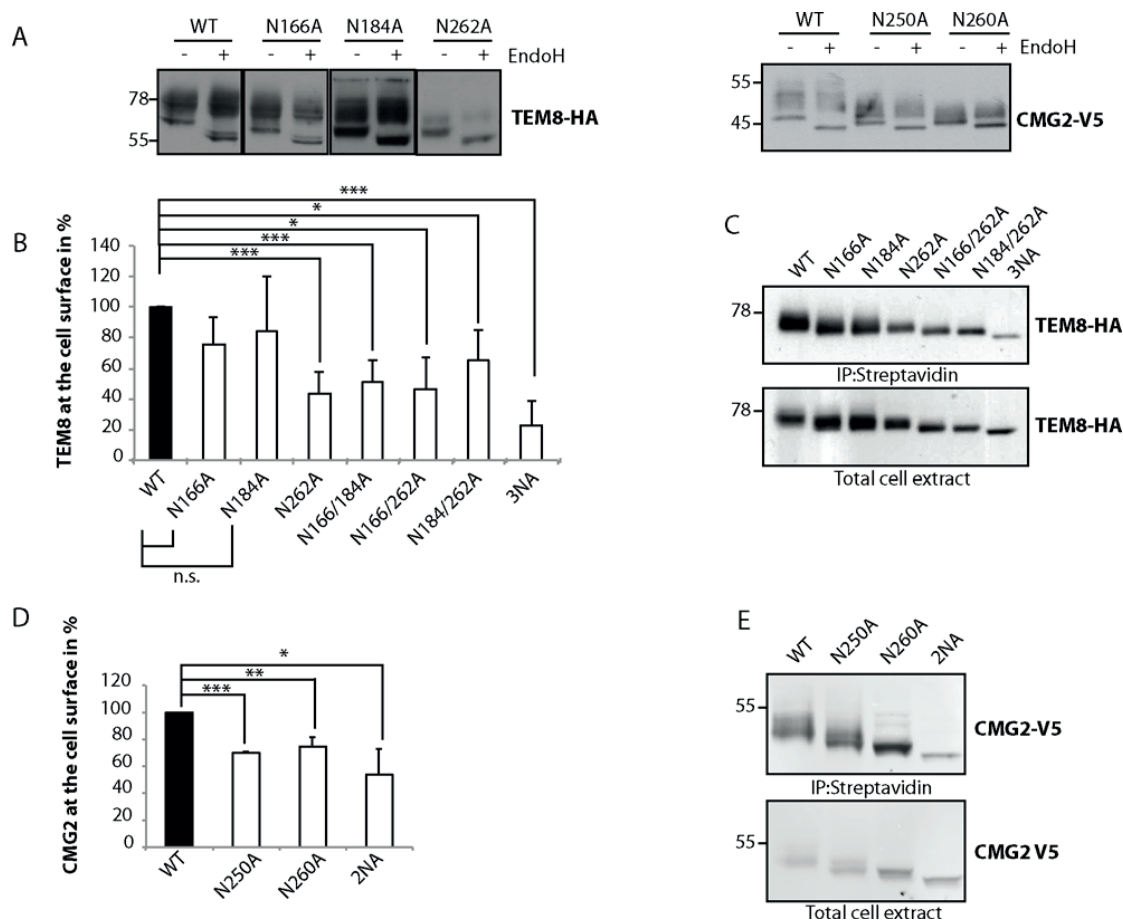
Fig 2. Number and localization of glycan sidechains determine trafficking efficiency of TEM8 and CMG2. A) Endoglycosidase H (EndoH) treatment on TEM8 and CMG2 single mutants. HeLa cells were transfected for 48h with the respective cDNAs. 40 μg of cell extracts were treated or not with EndoH as described before. Samples were analyzed by SDS-PAGE and Western Blotting. B) Quantification of surface biotinylation experiments to determine amount of TEM8 at the cell surface. All mutants were corrected for their expression levels and then normalized to WT, which was set at 100%. Errors represent standard deviation. Statistics were calculated using an unpaired t-test. n ≥ 3. * p≤0.05, ** p≤0.01, *** p≤0.001 C) Representative Western Blots of surface biotinylation. HeLa cells were transfected 48h with the respective cDNAs. Proteins at the cell surface were labeled with biotin, immunoprecipitated with streptavidin beads and blotted against TEM8-HA. D) Quantification of surface biotinylation experiments to determine amount of CMG2 at the cell surface. All mutants were corrected for their expression levels and then normalized to WT, which was set at 100%. Errors represent standard deviation. Statistics were calculated using an unpaired t-test. n ≥ 3. * p≤0.05, ** p≤0.01, *** p≤0.001 E) Representative Western Blots of surface biotinylation. HeLa cells were transfected 48h with the respective cDNAs. Proteins at the cell surface were labeled with biotin, immunoprecipitated with streptavidin beads and blotted against CMG2-V5.

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monitored the ubiquitination status of TEM8 and CMG2, since this post-translation modification is required for targeting the protein to the proteasome. Since ubiquitination might lead to rapid degradation, rendering the species undetectable, we treated cells with the proteasome inhibitor MG132. We also monitored the effect of Bafilomycin A1, which inhibits acidification of the endosome, thereby preventing lysosomal protein degradation, since this is the second major protein degradation route in the cell. Inhibitors were used only for a few hours to minimize the potential secondary effects on protein translation for example. For both WT and the N262A mutant of TEM8, a limited smear of ubiquitination was observed, which was enhanced
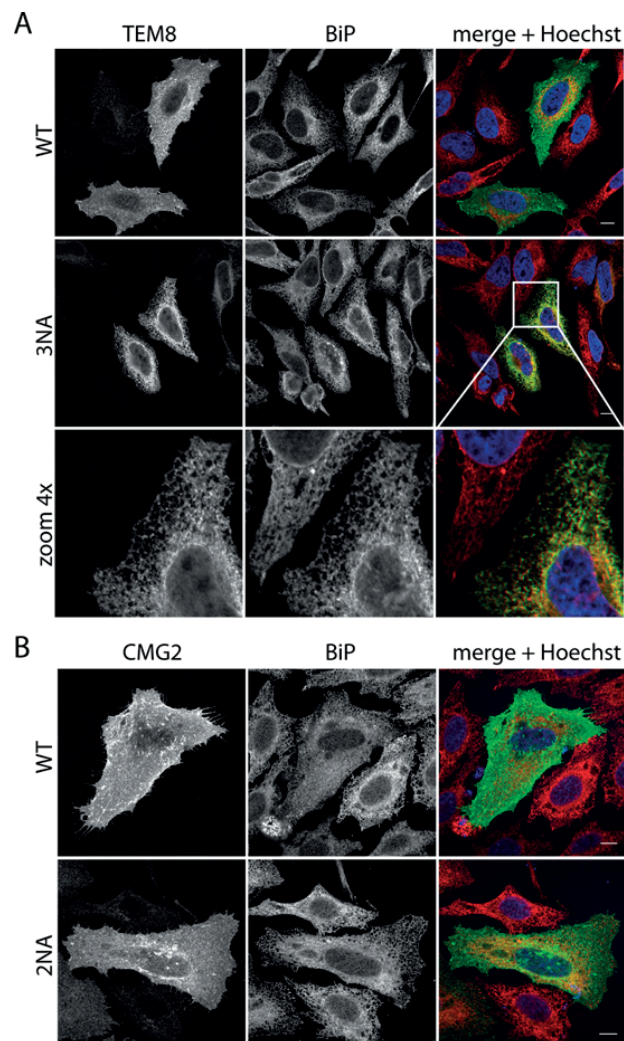
Fig 3. Localization of TEM8 and CMG2 glycosylation mutants. A) Immunofluorescence of transiently transfected HeLa cells. Cells were transfected for 48h with the respective cDNAs. Cells were fixed, permeabilized and stained for TEM8-HA, endogenous BiP and Hoechst. Scalebars represent 10 μm. **B)** Immunofluorescence of transiently transfected HeLa cells. Cells were transfected for 48h with the respective cDNAs. Cells were fixed, permeabilized and stained for CMG2-V5, endogenous BiP and Hoechst. Scalebars represent 10 μm.

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by Bafilomycin A1 treatment, consistent with an ubiquitination-mediated targeting of TEM8 from the plasma membrane to lysosomes [38]. MG132 treatment led, as expected, to the appearance of a long smear, a hallmark of polyubiquitination, the targeting signal for the proteasome (Fig. 4A). These observations are consistent with the fact that upon transient expression of WT TEM8, the majority is targeted to the cell surface, from where degradation occurs in lysosomes, while a smaller a fraction remains in the ER and is degraded by the proteasome (Figs. 1 and 2). When a similar analysis was performed for the 3NA TEM8 mutant, no effect was
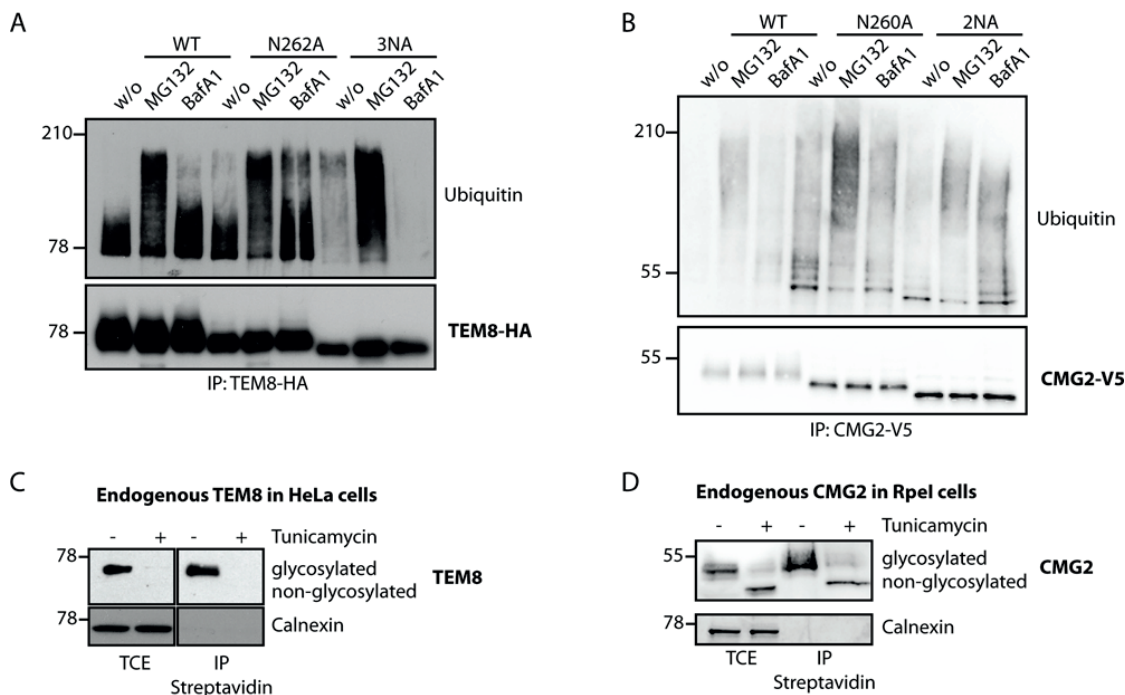
Fig 4. Non-glycosylated TEM8 is an ER quality control and ERAD substrate. A) HeLa cells were transfected for 48h with the respective cDNAs. Cells were treated or not with MG132, an inhibitor of the proteasome or Bafilomycin A1, a drug preventing endosomal acidification and thus lysosomal degradation. Immunoprecipitates against TEM8-HA were analyzed by SDS-PAGE and Western Blotting against Ubiquitin and TEM8-HA. **B)** HEK cells stably expressing CMG2 under the control of a tetracycline inducible promotor were induced for 24h with 0.1µg/ml doxycycline. Cells were treated or not with MG132 or Bafilomycin A1. Immunoprecipitates against CMG2-V5 were analyzed by SDS-PAGE and Western Blotting against Ubiquitin and CMG2-V5. **C)** HeLa cells were treated or not with tunicamycin, an antibiotic blocking the co-translational transfer of glycan sidechains in the ER by blocking the oligosaccharyltransferase (OST) for 16h. Surface proteins were labeled with biotin and immunoprecipitates against streptavidin were analysed for TEM8 or Calnexin as a negative control. **D)** RpeI cells were treated or not with tunicamycin for 16h. Surface proteins were labeled with biotin and immunoprecipitates against streptavidin were analysed for CMG2 or Calnexin as a negative control.

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observed upon Bafilomycin A1 treatment, consistent with the absence of TEM8 3NA at the plasma membrane. However a long smear, revealing polyubiquitination, was observed under control conditions and strongly enhanced upon MG132 treatment (Fig. 4A, lane 8). All together this analysis indicates that the 3NA TEM8 mutant is retained in the ER and targeted to the ERAD pathway. Note that while the majority of WT TEM8 folds and exists the ER, targeting to ERAD is also observed indicating that the efficiency of TEM8 folding is not 100%.

For CMG2, the ubiquitination pattern of WT and mutants was qualitatively similar, in all cases sensitive to both drugs, with a higher molecular weight smear upon MG132 treatment and a lower molecular weight smear upon neutralization of lysosomes (Fig. 4B). This behavior is consistent with our finding that a significant percentage (50%, Fig. 2D) of even the 2NA mutant reaches the plasma membrane.

The above experiments indicate that non-glycosylated TEM8 is retained in the ER and potentially an ERAD substrate, while CMG2, under these experimental conditions, is less dependent on glycosylation for its proper expression.

The above experiments were performed in HeLa or HEK cells transiently expressing TEM8 or CMG2 WT and glycosylation mutants and allowed us to reveal a differential sensitivity to glycosylation. Different cell types however have different capacities of protein folding and ER

quality control [44]. Also overexpression might saturate the ER capacity to promote folding of the overexpressed protein of interest. We therefore next monitored endogenously expressed proteins, TEM8 in HeLa cells (which do not express CMG2) and CMG2 in Rpe1 cells [45, 46]. N-glycosylation was prevented by treating cells for 16h with tunicamycin, a drug inhibiting the transfer of the core oligosaccharide to the nascent polypeptide chain by oligosaccharyltransferase (OST). Tunicamycin treatment led to a complete loss of TEM8 protein (Fig. 4C). Even after surface biotinylation and enrichment of surface proteins with streptavidin beads, TEM8 was undetectable. In contrast, expression of CMG2 was not significantly modified by tunicamycin (Fig. 4D). The CMG2 band migrated at a lower molecular weight as expected from the absence of glycosylation. Importantly, non-glycosylated CMG2 was transported to the cell surface as revealed by surface biotinylation. Since tunicamycin disrupts N-glycosylation of all proteins in the cell, the loss of TEM8 could potentially be due to gross secondary effects. This is however unlikely given the fact that these results are fully consistent with the surface biotinylation experiments performed on the glycosylation mutants and the lack of effect of tunicamycin on CMG2, which is highly homologous to TEM8. These experiments confirm that endogenous TEM8 is highly dependent on glycosylation for its expression, while in Rpe1 cells, endogenous CMG2 does not significantly rely on glycosylation for its plasma membrane targeting.

Loss of glycosylation affects binding of Anthrax toxin to TEM8 but not to CMG2

Since glycosylation is not required for targeting of CMG2 to the plasma membrane, we next investigated whether it is required for its ability to bind its ligand. Parallel experiments were performed for TEM8. We used protective antigen (PA), the receptor binding subunit of the anthrax toxin, as a surrogate ligand. As a negative control, we used the D50A mutants of both receptors, which harbors a mutation in the ligand-binding domain and therefore cannot bind PA [47, 48].

We monitored binding of full-length PA (PA^{83}) to the receptor, the processing to its 63 kDa form (PA^{63}) and the conversion of the 63 kDa form into an SDS-resistant oligomeric form (PA^{7mer}), using SDS-PAGE and Western Blotting. The presence of oligomers indicates that PA has been transported to endosomes, where the conversion to the membrane inserted SDS-resistant form occurs [49]. Upon addition of PA to cells expressing any of the single TEM8 mutants, we observed a decrease in PA binding, cleavage, and heptamer formation, as compared to WT. Only monomers were observed in immunoprecipitates of the N262A mutant (Fig. 5A). This could be due either due lower binding, impaired oligomerization or enhanced release of the oligomer from the receptors [50]. The triple, fully glycosylation-deficient TEM8 mutant failed to bind PA (Fig. 5A), not surprising given its ER localization. Since differences observed in this assay might, at least partly, be due to a decrease in surface expression of the mutants, we next monitored PA binding in vitro. In this assay, PA is added to cell lysates, bypassing the localization issue [35]. Of the single TEM8 mutants, only N262A showed a severe decrease in binding when compared to the WT. This was also true for double mutants and PA binding became essentially undetectable for the triple asparagine TEM8 mutant (Fig. 5B). Thus, glycosylation of TEM8, in particular at position 262, is required for efficient PA binding. Recombinant, bacterial expressed, TEM8 vWA domain is fully competent for PA binding [51], thus glycosylation per se is not required for ligand binding. Therefore the absence of PA binding to the 3NA mutant is likely due to a defect in folding in the cellular context in the absence of glycosylation, which would be consistent with the full ER retention of TEM8 3NA. In contrast, for CMG2, both in vivo and in vitro binding assays showed no significant difference in binding, cleavage, or heptamer formation between WT and mutant proteins partially or fully deficient in glycosylation (Fig. 5CD).
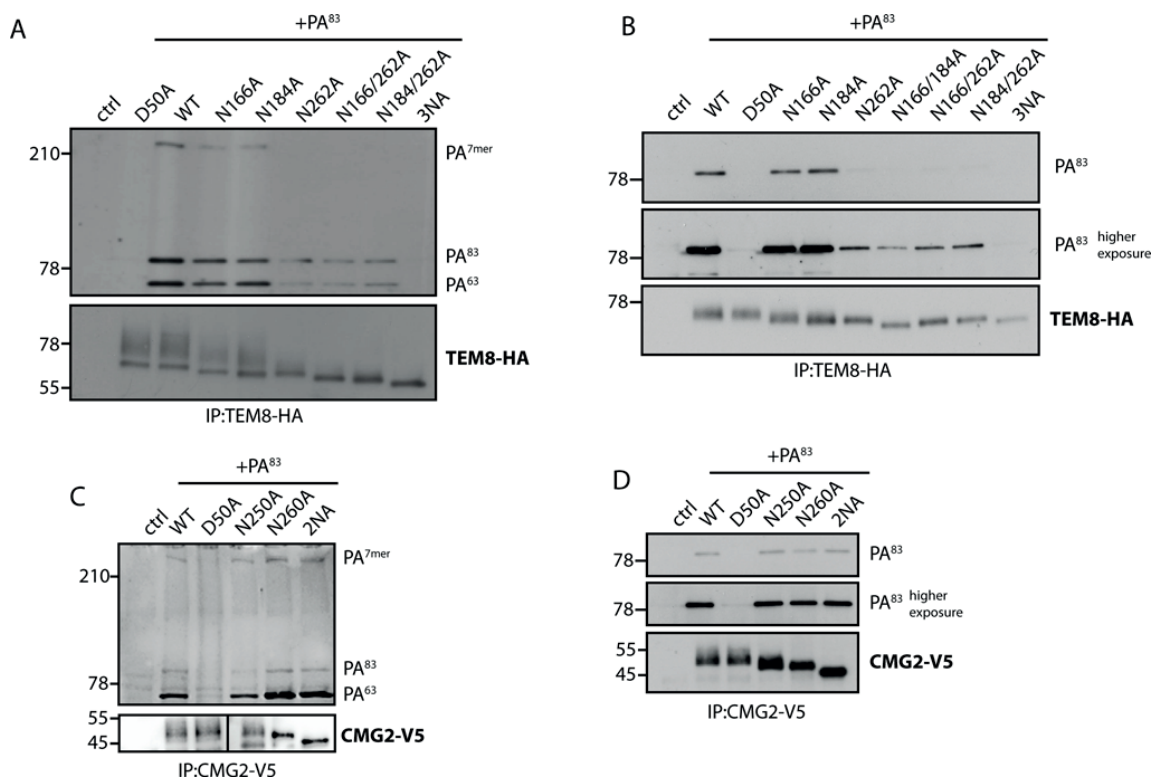
Fig 5. Loss of glycosylation affects binding of Anthrax toxin to TEM8 but not to CMG2. A and C) HeLa cells were transfected for 48h with the respective cDNAs. Cells were treated for 1h at 4°C with 500 ng/ml PA^{83} and shifted to 37°C for 10 min to induce cleavage and heptamerization. Immunoprecipitates against TEM8-HA/CMG2-V5 were analyzed by SDS-PAGE and Western Blotting against PA and TEM8-HA/CMG2-V5. Control cells are non-transfected. D50A is a binding deficient mutant that serves as a negative control. **B and D)** HeLa cells were transfected for 48h with the respective cDNAs. Cells were lysed and incubated for 1h at 4°C with 1 µg/ml PA^{83}. Immunoprecipitates against TEM8-HA/CMG2-V5 were analyzed by SDS-PAGE and Western Blotting against PA and TEM8-HA/CMG2-V5. Control cells are non-transfected. D50A is a binding deficient mutant that serves as a negative control.

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Glycosylation acts as a buffer for CMG2 ectodomain mutations

Our study reveals that in Rpe1 cells or upon transfection into HeLa cells, CMG2 is only mildly sensitive to the loss of N-glycosylation. Our analyses of Hyaline Fibromatosis Syndrome (HFS) mutations have however revealed that CMG2 is rather sensitive to ectodomain mutations in terms of folding [35, 40]. Indeed most ectodomain missense mutations were found to lead to defects in ER folding and targeting to ERAD [35, 40]. We therefore hypothesized that N-glycosylation of CMG2 might favor folding in the presence of protein destabilizing mutations and thus serve as a "buffer" for genetic variation. To test this hypothesis, we searched for a HFS inducing mutation that localizes to the ectodomain of CMG2 but does not affect plasma membrane targeting. This is a rare situation since we have found that most ectodomain mutations lead to CMG2 degradation by ERAD [35, 40]. We found a homozygous patient carrying the c.652T>C mutation which leads to p.C218R. Modification of Cys-218 leads to the disruption of the disulfide bond that links the N and C-termini of the vWA domain (Fig. 6A). We have previously shown that upon transient overexpression in tissue culture cells, the C218R mutant has a partial folding defect, leading to a 60% drop in surface expression yet its ability to bind

Fig 6. Glycosylation acts as a buffer for CMG2 ectodomain mutations. A) Graphic showing the disulfide bridge C39-C218 (blue) present in CMG2 WT B) Fibroblast cells were treated or not with tunicamycin for 16h and TCE were analyzed by SDS-PAGE and Western Blot. Representative Western Blot with control
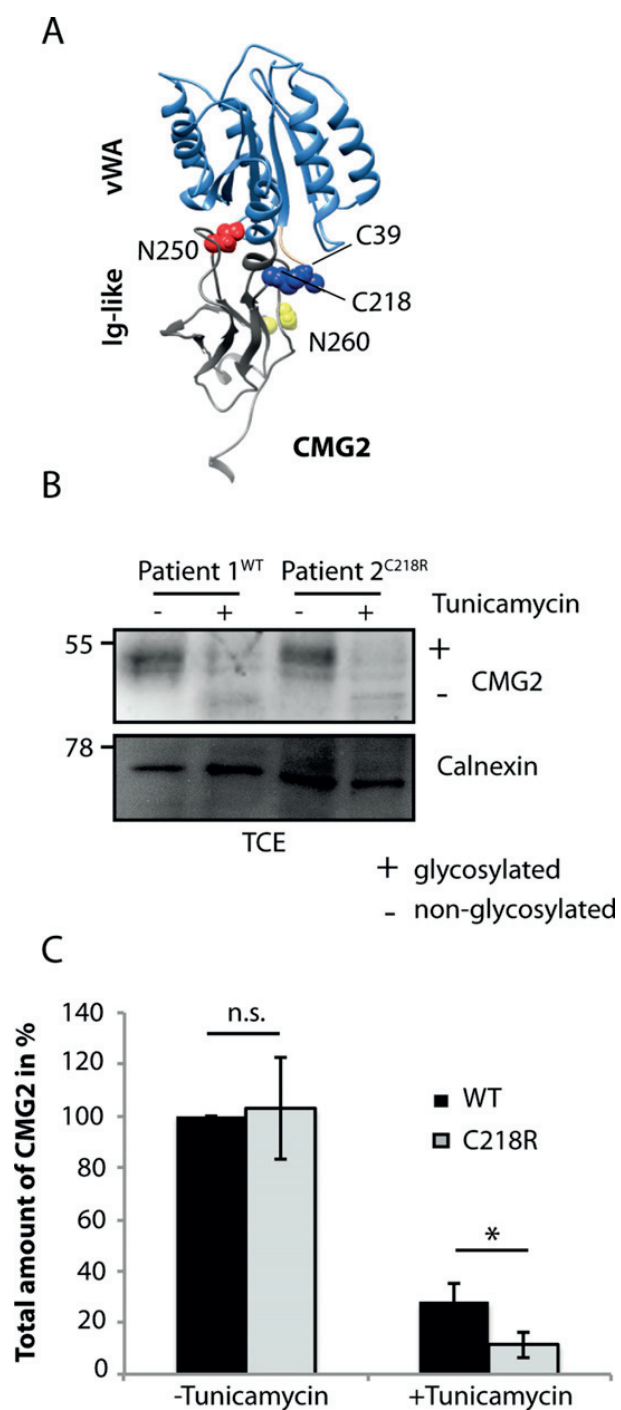
fibroblasts and patient fibroblasts. Calnexin serves as a loading control. **C)** Quantification of total protein levels. CMG2 levels are normalized to WT protein level without tunicamycin treatment, which was set to 100%. Statistics were calculated using an unpaired t-test. Errors represent standard deviation. $n \geq 3$.
* $p \leq 0.05$

PA is not impaired [35, 40]. Instead of transient expression in tissue culture cells, we could analyze patient cells. As shown in Fig. 6, CMG2 is expressed at the same level in cells from the C218R expressing patient as in cells from a control patient. Also the migration pattern of C218R CMG2 was similar to that of WT CMG2, indicating that sugar modifications and in particular addition of complex sugars in the Golgi had occurred. To investigate the importance of CMG2 glycosylation in patient-derived fibroblasts, cells were treated with tunicamycin. In marked contrast to what we observed in the tissue culture cell line Rpe1, expression of WT CMG2 dropped by 70% upon tunicamycin treatment indicating that in these primary human fibroblasts, glycosylation was required for efficient expression of WT CMG2. Consistently, tunicamycin also led to a drastic drop of the C218R CMG2 HFS variant (Fig. 6BC). Interestingly, the comparison of WT to C218R CMG2 expression under conditions of tunicamycin treatment showed that C218R expression was almost three fold lower than that of WT. Thus C218R is in fact a protein destabilizing mutation, but this is no longer apparent in the presence of glycosylation, a situation in which its expression is similar to that of WT. Thus consistent with our hypothesis, glycosylation appears to overcome the folding defect generated by the C218R mutation. As opposed to most other ectodomain mutations, C218R does not lead to a major loss of protein expression at the cell surface but to a loss of protein function, which we are currently addressing.

Discussion

TEM8 and CMG2 are two transmembrane surface glycoproteins involved in homeostasis of the extracellular matrix but can also act as anthrax toxin receptors. Mutations in the *tem8* or *cmg2* genes may lead to two severe genetic diseases, GAPO syndrome [29, 30] and Hyaline Fibromatosis syndrome, respectively [34]. While the genetic origin of GAPO syndrome has only recently been identified and thus no genotype-phenotype studies have yet been reported, our studies on the HFS mutations indicate that the disease is due to a loss of protein function, often as a consequence of the loss of protein expression [40]. Depending on the specific patient mutations, the defects of CMG2 expression were attributed to premature mRNA degradation or impaired folding in the ER. This led us to investigate the potential role of N-glycosylation on the folding and subsequent trafficking of TEM8 and CMG2.

We show that both proteins can undergo N-glycosylation on all of their predicted sites, three in TEM8 and two in CMG2, of which only one is conserved and localizes to the Ig-like domain. Transient overexpression experiments in tissue culture cells reveal that optimal expression is achieved when all sites are available to be modified. The requirement for glycosylation was however less stringent for CMG2 since a significant fraction of the protein was found to exit the ER, indicating proper folding, reach the plasma membrane and bind its ligand in the absence of N-linked sugars. In contrast, fully glycosylation-deficient TEM8 was retained in the ER and failed to bind anthrax PA toxin, together suggesting that folding was impaired. TEM8 was in fact affected by the loss of any of its glycosylation sites, and losses were to a large extent additive.

That sugars promote protein folding, trafficking and function has been reported for a variety of proteins, as reviewed in [3, 52]. Proteins that depend on glycosylation for folding and other functions are diverse and range from mammalian to viral and bacterial proteins [53, 54].

Depending on where the glycosylation sites are localized in proteins, sugars can have a local or a global impact on the protein, i.e. by influencing the secondary structure in the vicinity of the modified residue [55, 56]. The presence of sugars affects the hydrophilicity of the protein but also provides the opportunity to interact with lectin chaperones such as calnexin, further increasing the positive effect on folding. The present work leads to the unexpected finding that two proteins that are very closely related and show a high degree of sequence identity/similarity (S1 Fig.), differ significantly in their dependence towards glycosylation.

The tolerance of CMG2 toward the lack of glycosylation was however only apparent in certain cell types (HeLa and Rpe1 tested here). In primary human fibroblasts however, glycosylation of CMG2 appeared essential for proper expression, since tunicamycin led to a 70% drop in expression. This differential behavior likely reflects the difference in expression of the folding and quality control arsenal expressed by these cells. It has indeed been reported that the expression of chaperone and folding enzymes greatly differs between cells and tissues [44]. It could thus be that primary cells have a poorer folding capacity, a more stringent ER quality control and/or a more potent ERAD pathway, and that under these conditions optimal folding of CMG2 can only be achieved in the presence of N-linked glycans.

Moreover, glycosylation appears to provide a buffer towards genetic variation. Glycosylation would render CMG2 more tolerant to mutations that fail to fold without the assistance of lectin chaperones. Consistent with this hypothesis, we found that in the absence of glycosylation, C218R CMG2 was expressed at 30% of WT, whereas under normal glycosylation conditions, expression was similar to WT.

That chaperones can act as buffers for genetic variations has previously been shown. This might not only help proteins that have acquired a mutation to fold, but could also be a driver for genetic variation and evolution [57–59]. Our results now suggest a variation on this theme: by being modified with glycan side chains, a protein is more prone to interact longer or more often with lectin chaperones. This in turn would help the protein to fold correctly even if it harbors a destabilizing mutation. The glycosylated form of the protein would be more tolerant to mutations while not affecting or even promoting function. One could even envision that some mutants have a beneficial increased occupancy of the glycosylation sites. It was indeed observed that excessive N-glycosylation of Aquaporin 2 mutants increased their stability and promoted folding, thereby partly preventing their degradation [60]. The importance of N-glycosylation in protecting TEM8 and CMG2 will be further tested, as mutations in the encoding genes identified in GAPO and HSF patients will be reported.

Supporting Information

S1 Fig. Alignment of human TEM8 and CMG2. Sequence alignment using Jalview [61] for human TEM8 (ANTR1) isoform 1 and CMG2 (ANTR2) isoform 4. Indicated are the domains of the proteins as well as the glycosylation sites (asterisks).
(TIF)

S2 Fig. Immunofluorescence of TEM8 WT and CMG2 WT and N260A. Images shown in Fig. 3 of TEM8 WT, CMG2 WT and N260A with an additional zoomed image.
(TIF)

S3 Fig. Original blots of Figs. 1, 2 and 4. Uncropped blots shown in Figs. 1,2 and 4. Indicated is the area that was used for the figures.
(TIF)

S4 Fig. Original blots of Figs. 5 and 6. Uncropped blots shown in Figs. 5 and 6. Indicated is the area that was used for the figures.
(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: SF JD GVDG. Performed the experiments: SF JD. Analyzed the data: SF JD GVDG. Contributed reagents/materials/analysis tools: SF JD. Wrote the paper: SF GVDG.
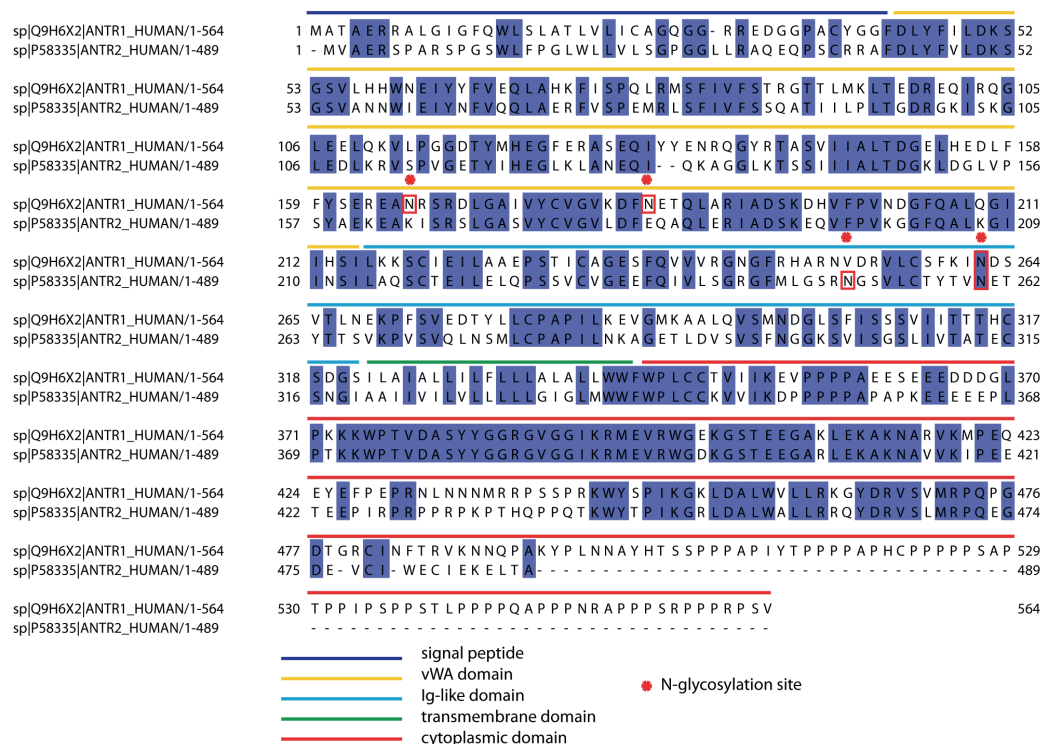
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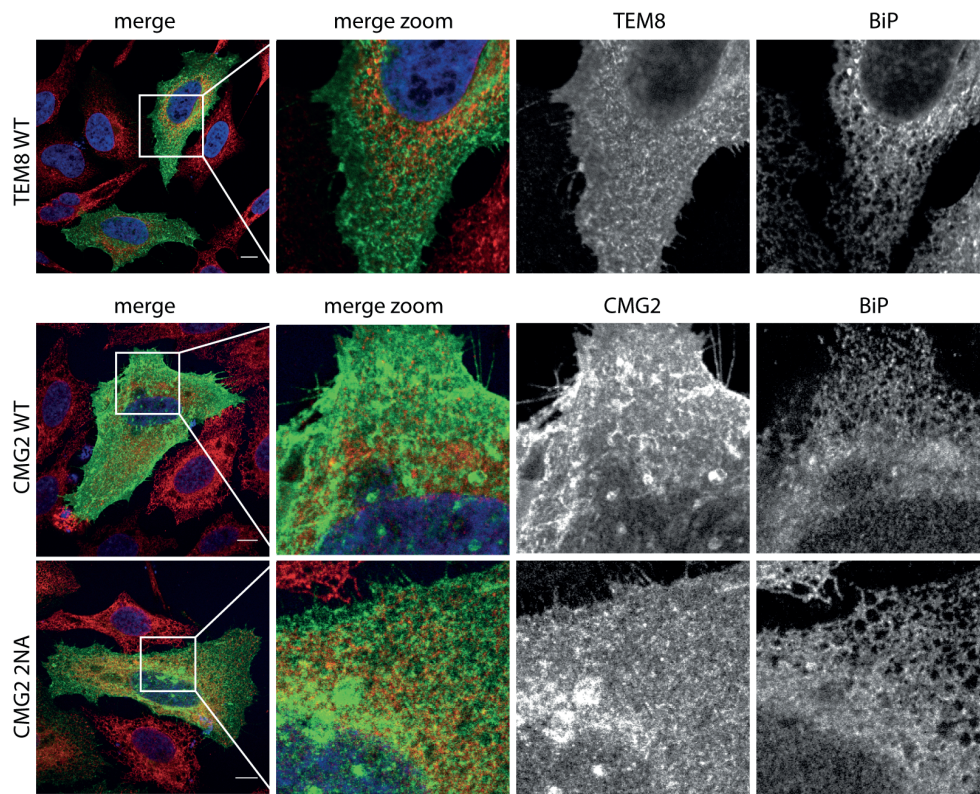
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sp|Q9H6X2|ANTR1_HUMAN/1-564
sp|P58335|ANTR2_HUMAN/1-489

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sp|Q9H6X2|ANTR1_HUMAN/1-564  530 TPPIPSPPSTLPPPPQAPPPNRAPPPSRPPPRPSV                    564
sp|P58335|ANTR2_HUMAN/1-489      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
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signal peptide
vWA domain
Ig-like domain   ※ N-glycosylation site
transmembrane domain
cytoplasmic domain

S1 Fig. Alignment of human TEM8 and CMG2 *Sequence alignment using Jalview for human TEM8 (ANTR1) isoform 1 and CMG2 (ANTR2) isoform 4. Indicated are the domains of the proteins as well as the glycosylation sites (asterisks).*

S2 Fig. Immunofluorescence of TEM8 WT and CMG2 WT and N260A *Images shown in Figure 3 of TEM8 WT, CMG2 WT and N260A with an additional zoomed image.*

Figure 1B

Figure 1C

Figure 1D

Figure 1D

Figure 1D

Figure 1D

Figure 1E

Figure 1E

Figure 2A

Figure 2A

Figure 2A

Figure 2A

Figure 2C

Figure 2C

Figure 2E

Figure 4A

Figure 4A

Figure 4B

Figure 4B

Figure 4C

Figure 4C

Figure 4C

Figure 4D

Figure 4D

S3 Fig. Original blots of Figures 1, 2 and 4 Uncropped blots shown in Figure 1,2 and 4. Indicated is the area that was used for the figures

Figure 5A   Figure 5A   Figure 5B   Figure 5B

Figure 5B   Figure 5C   Figure 5C   Figure 5C   Figure 5D

Figure 5D   Figure 5D   Figure 6B   Figure 6B
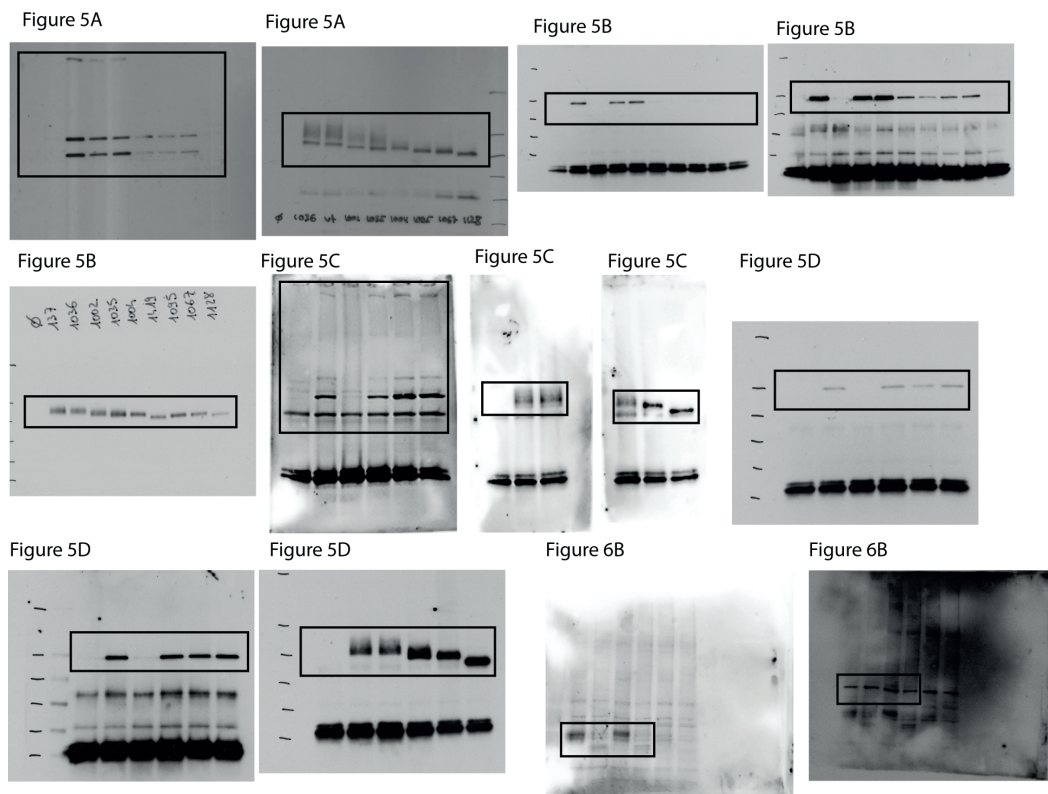
S4 Fig. Original blots of Figures 5 and 6 Uncropped blots shown in Figure 5 and 6. Indicated is the area that was used for the figures

II. Results: Endocytosis of anthrax toxin receptors

Pathological and physiological endocytosis of anthrax toxin receptors CMG2 and TEM8

Manuscript in preparation

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Abstract

CMG2 and TEM8 are two vertebrate-specific membrane proteins, identified as receptors of the anthrax toxin, a major virulence factor of the pathogenic bacterium *Bacillus anthracis*. These proteins are essential for the toxin to access target cells. Their physiological roles have however remained largely elusive, but their importance is highlighted by the fact that loss of function mutations in either protein leads to rare genetic diseases: Hyaline Fibromatosis Syndrome for CMG2, and GAPO syndrome for TEM8.

To better understand both the physiological and pathological roles of CMG2, we have searched for partner proteins using yeast-two hybrid screening. We identified and validated 4 new partners. We found that the E3 ligase Cbl and the serine-threonine kinase MARK2 are involved in regulated endocytosis of the anthrax toxin via both the CMG2 and TEM8 mediated pathways. Interestingly we found two proteins specifically affecting CMG2-mediated toxin endocytosis, the E3 ligase RNF41 and the deubiquitinating enzyme USP8.

We also assessed physiological endocytosis of the receptors and found that both CMG2 and TEM8 internalize even in the absence of their pathological ligand. This endocytosis is triggered by binding of a physiological ligand to CMG2 and subsequent clustering of the receptor. TEM8 undergoes constitutive endocytosis, which is enhanced upon ligand binding. Our work provides important insights into both pathological as well as physiological endocytosis of the two anthrax toxin receptors.

Introduction

Anthrax is an infectious disease, which is largely caused by the exotoxins of *B. anthracis*. Anthrax toxin is a tripartite toxin and consists of protective antigen (PA), the receptor binding subunit, as well as edema factor (EF) and lethal factor (LF), the two enzymatic subunits of the toxin. PA will bind to one of the two anthrax toxin receptors, TEM8 or CMG2 on the cell surface [1, 2]. The full length form is 83 kDa (PA^{83}), which will be cleaved by furin to the smaller 63 kDa form (PA^{63}) [3]. The receptor-toxin complexes will then multimerize either into heptamers (PA^{7mer}) or octamers [4, 5] and partition into specific microdomains of the plasma membrane [6]. The enzymatic subunits EF and LF can then bind to the multimer and the whole complex is internalized via clathrin-mediated endocytosis [7]. This process is regulated by phosphorylation and ubiquitination [8, 9]. Once in the endocytic pathway and therefore exposed to a lower pH, the PA multimer inserts into the membrane and allows for translocation of the enzymatic subunits across the membrane into the lumen of intraluminal vesicles (ILV) [10]. Both enzymatic subunits need to eventually reach the cytosol to function but can also be kept in ILVs for long-term storage and also release by exosomes as an efficient long-term and long-distance transmitter mechanism [11].

The anthrax toxin receptor responsible for lethality in mice is CMG2 [12], which was discovered as anthrax toxin receptor 2 (ANTXR2) in 2003 [1]. It is a type I transmembrane protein, with an extracellular von Willebrand A and an Ig-like domain [13]. The vWA domain is involved in binding PA and presumably the physiological ligand as well, as mutations in the ligand-binding site lead to the genetic disease Hyaline Fibromatosis Syndrome. The Ig-like domain, with so far uncharacterized function, contains two glycosylation sites. Glycosylation at these sites promotes folding of CMG2 in the ER, providing a buffering capacity for this mutation-prone protein [14]. After the single transmembrane span, CMG2 has a 148 amino acid long tail, which is predicted to be intrinsically unstructured and contains a conserved cytoskeleton-binding domain [13].

CMG2 was initially identified as the second most upregulated gene in *in vitro* capillary morphogenesis, hence the name Capillary Morphogenesis Gene 2 [15]. Mutations in *cmg2* were then found to cause the rare but severe genetic disease Hyaline Fibromatosis Syndrome (HFS) [16, 17]. The most prevalent symptom of this disease is the accumulation of hyaline material in the skin and organs of the patients [18], creating nodules mostly at sites of mechanic stress [19]. CMG2 knockout mice display a marked

fibrosis of the uterus, with an accumulation of fibronectin and collagens I and VI [20, 21]. *In vitro* studies indicate that CMG2 binds proteins of the extracellular matrix such as laminin and collagen IV [15]. Thus, although the exact role remains elusive, CMG2 appears to be involved in the homeostasis of the extracellular matrix.

The only protein in the genome that shows high homology to CMG2 is TEM8 [13], also called anthrax toxin receptor 1 (ANTXR1) [2], since it was the first protein identified as an anthrax toxin receptor. Mutations in *tem8* lead to another severe human genetic disease, GAPO syndrome [22]. While the apparent symptoms of Hyaline Fibromatosis and GAPO syndromes are very different, they share the accumulation of extracellular matrix proteins in different tissues. How the loss of CMG2 and TEM8 function leads to the accumulation of extracellular matrix is completely unclear. Given the opportunistic nature of bacterial pathogens, it is tempting to speculate that PA has hijacked the ability of CMG2 and TEM8 to bind and internalize an endogenous ligand.

Using a FACS-based assay we have analyzed endocytosis of CMG2 and TEM8 in the absence and presence of endogenous ligand and upon binding of the anthrax protective antigen. By studying the physiological entry of the two anthrax toxin receptors in the absence of their pathological ligand anthrax, we could show that both receptors internalize constantly from the cell surface. This internalization is dependent on ligand-binding and multimerization of the receptor.

Yeast-Two-Hybrid screens led us to investigate the role of four proteins in CMG2 and TEM8-mediated anthrax toxin entry: RNF41, an E3 ubiquitin ligase, MARK2, a serine/threonine kinase, Cbl, the E3 ligase for TEM8, and USP8, a deubiquitinating enzyme. We found that MARK2, Cbl and USP8 regulate either the surface expression or endocytosis of both receptors, while RNF41 specifically affects CMG2 uptake.

Material and Methods

Cells and reagents

RpeI cells, RpeI shTEM8 or shCMG2 and patient fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM penicillin and streptomycin.

The human CMG2 (isoform 4, Uniprot P58335-4) gene, tagged with a V5 epitope at the C-terminus, was cloned in the pcDNA3.1/V5-HIS-TOPO expression vector (Invitrogen, Carlsbad, CA) and provided by J. Martignetti (Mount Sinai School of Medicine, New York, NY; [16]). The ΔCBD mutant, missing residues 368-418, tagged with a V5 epitope at the C-terminus was cloned in the pIRES vector (Clontech, Japan).
The human RNF41 (Uniprot Q9H4P4), both full length and the dominant-negative carboxy terminus, tagged with a Flag epitope at the C-terminus were cloned in the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) and provided by K. Carraway III (UC Davis, Sacramento, CA; [23]).
All plasmids were transfected into cells using Fugene (Roche, Basel, Switzerland).

For knockdown experiments, cells were treated with 100 nmol siRNA/6cm dish for 72 h prior to experiments. siRNA against RNF41 (SI04267655), MARK2 (SI00288288) and USP8 (SI00073017) was from Qiagen (Venlo, The Netherlands). siRNA against c-Cbl (sc-29242) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). As a control, non-targeting siRNA (ATTGAACAAACGAAACAAGGA) was used. Transfection was done with Interferrin from Polyplus Transfections (Illkirch, France) according to the manufacturer's protocol.

Anthrax toxin was purified as described [24] or produced in our lab. For toxin experiments, cells were treated for 1 h at 4°C with 500 ng/ml PA[83] in internalization medium (IM medium), (Glasgow minimal essential medium, Invitrogen, 10mM HEPES, pH 7.4). Cells were washed twice with warm IM medium to remove excess toxin and incubated for different timepoints at 37°C to induce cleavage and heptamerization. To reveal surface heptamers, total cell extracts were treated for 10 min at RT with 145 mM NaCl and 20 mM MES-Tris, pH 4.5. Protein quantifications were done with the BC Assay Protein Quantification Kit from Interchim Uptima (Montluçon, France), according to the manufacturer's protocol.

68

Antibody against PA was from List Biological Laboratories (Campbell, CA), anti-V5 antibody was from Invitrogen (Carlsbad, CA); anti-actin antibody from Merck Millipore (Darmstadt, Germany); anti-Ub antibody (sc-8017) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti MARK2 from abnova (Taipei, Taiwan); anti RNF41 was from Bethyl Laboratories (Montgomery, TX); anti USP8 was from Cell Signaling (Danvers, MA). HRP-conjugated secondary antibodies were from Pierce Chemical Co. (Rockford, IL) and Alexa-conjugated secondary antibodies from Molecular Probes (Invitrogen, Carlsbad, CA). hCMG2 and hTEM8 hybridomas were generated in our lab. Streptavidin-agarose conjugated beads were from Sigma-Aldrich (St. Louis, MO), protein G beads were from GE Healthcare (Uppsala, Sweden) and HA-beads from Roche (Basel, Switzerland);

Immunoprecipitation

For immunoprecipitation, confluent cells were washed three times with PBS. Cells were lysed by incubation for 30 min at 4°C with 0.5% NP-40, 500 mM Tris-HCl, pH 7.4, 20 mM EDTA, 10 mM NaF, 30 mM sodium pyrophosphate decahydrate, 2 mM benzamidine, 1 mM PMSF, 1 mM NEM and a cocktail of protease inhibitors (Roche, Basel, Switzerland). For ubiquitination, 1 mM NEM was added to the buffer. Cell lysates were incubated over night at 4°C with antibody and protein G sepharose beads.

Co-immunoprecipitation

For immunoprecipitation, confluent cells were washed three times with PBS. Cells were lysed by incubation for 10 min at 4°C with 0.25% NP-40, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 20 mM sodium molybdate, 0.1 mM DTT and a cocktail of protease inhibitors (Roche, Basel, Switzerland). Cells were sonicated to complete lysis. Cells lysates were incubated over night at 4°C with antibody and the next day for 4 h with protein G sepharose beads.

Flow cytometry

Cells were labeled for 1h at 4°C or for 20 min at 37°C with antibody against the extracellular domain of CMG2 (rat 2F6, produced in our lab) or TEM8 (rat 9D1, produced in our lab), both diluted 1:2 in IM. Antibody was chased for 2 h at 37°C in complete medium. For assays with toxin, cells were starved for 16 h in medium without FCS and then treated for 1 h at 4°C with 500 ng/ml PA[83] (WT or cleavage-deficient U7

mutant) in the antibody/IM mix. Antibody was chased in medium without FCS containing 500 ng/ml PA[83] (WT or U7). Cells were stained for viability with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (lifetechnologies, Waltham, MA) according to the manufacturer's protocol. Cells were then fixed with BDFix solution (BD, Franklin Lakes, NJ) and stained with secondary antibody αrat 647 (Jackson ImmunoResearch, West Grove, PA) at 4°C for 30 min. Fluoresence was measured on a Gallios™ Flow Cytometer (Beckman Coulter) and data was analyzed with FlowJo (Ashland, OR).

Ethical Statement

Primary human fibroblasts from a control and a Hyaline Fibromatosis Syndrome patient were obtained with patient consent, research using these cells was approved by the "Commission Cantonale d'éthique de la recherche sur l'être humain" and are registered under the approval No A070055 of the Swiss Federal Office of the Environment. Patients provided written consent that patient derived cells could be used in any studied aimed at a better understanding of Hyaline Fibromatosis Syndrome and the gene involved. The patient signed a standard consent form approved by the ethics committee.

Results

Internalization of CMG2 is ligand-dependent and requires its multimerization

The prevailing view has been that CMG2 and TEM8 remain at the cell surface, unless the toxin binds, leading to rapid internalization. This has however not directly been addressed before. We set up a flow cytometry-based internalization assay, where disappearance of the receptor from the PM is measured over time using a specific anti-receptor antibody.
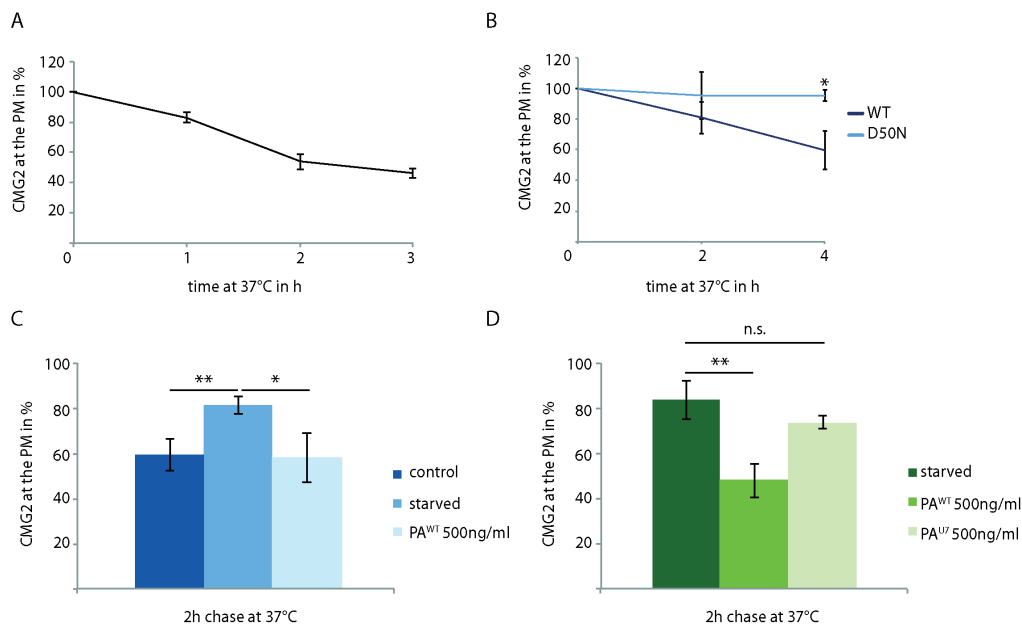
Figure 1: CMG2 internalization depends on presence of ligand and its multimerization

A) RpeI cells were labeled for 1 h at 4°C with antibody against the extracellular domain of CMG2. Antibody was chased for indicated times at 37°C in complete medium. Cells were stained for viability, fixed and stained with secondary antibody at 4°C for 30 min. Fluoresence was measured on a Gallios™ Flow Cytometer (Beckman Coulter). **B)** Patient fibroblasts (CMG2 WT or D50N) were labeled and analyzed as described above. Fluorescence at timepoint t=0 was set to 100% for WT and D50N. **C)** RpeI cells were cultivated in complete medium (control), starved for 16h in serum-free medium or starved for 16 h and recomplemented with 500 ng/ml PA^{WT} during labeling and chase. Cells were labeled and analyzed as described above. Fluorescence at timepoint t=0 was set to 100% for all conditions and levels after 2h were normalized to the respective levels at t=0. **D)** RpeI cells were either starved for 16 h in serum-free medium (starved) or starved for 16 h and recomplemented with 500 ng/ml PA^{WT} or 500 ng/ml PA^{U7} (non-cleavable toxin mutant) during labeling and chase. Cells were labeled and analyzed as described above. Fluorescence at timepoint t=0 was set to 100% for all conditions and levels after 2 h were normalized to the respective levels at t=0.

Statistics were calculated using an unpaired t-test. Errors represent standard deviation. n ≥ 3. * p≤0.05, ** p≤0.01

When following CMG2, it was apparent that the protein is lost from the cell surface as a function of time, with about 50% internalized after 3 h (Fig.1A). Recent studies have revealed that in tissue culture conditions, CMG2 is occupied by a ligand that is either present in the serum or synthesized by the cell (Bürgi et al., in preparation). To assess the impact of the physiological ligand on the observed internalization of CMG2, we used primary fibroblast obtained from a control patient or a patient suffering from HFS and harboring the D50N mutation [13]. The aspartic acid at position 50 is part of the Metal Ion Dependent Adhesion site (MIDAS) present in the vWA domain, and its mutation abolishes the ligand binding capacity. The FACS-based assay was used to compare the CMG2 internalization kinetics for both cell lines. While WT CMG2 was internalized as observed for Rpe1 cells, CMG2 D50N essentially remained at the surface over a timecourse of 4h (Fig.1B). Note this observation also excludes the possibility that the observed endocytosis of CMG2 is an artifact of antibody binding.

We observed that starvation of cells in a serum-free medium liberates CMG2 from its physiological ligand. When cells were thus starved for 16 hours in serum-free medium, internalization of the WT protein was markedly delayed compared to control conditions (Fig. 1C). To exclude that this was an indirect effect of starvation, endocytosis was monitored upon addition of PA^{WT} to starved cells. The toxin triggered CMG2 uptake, indicating that endocytosis could occur under starvation conditions and thus the lack of receptor entry was due to the absence of ligand (Fig.1C).

Upon binding to the receptor at the cell surface, PA^{WT} undergoes cleavage by furin followed by multimerization. The multimer is the pre-pore state and is required for pore formation in endosomal membranes and translocation of the enzymatic subunits to the cytoplasm. To test whether multimerization of the ligand affects CMG2 uptake, starved cells were treated with PA^{U7}, a mutant toxin deficient in the furin cleavage site and therefore multimerization-incompetent [25]. This mutant did not lead to significant uptake (Fig.1D). Therefore, endocytosis of CMG2 is dependent on the presence and binding of ligand as well as the formation of receptor multimers.

TEM8 undergoes constitutive endocytosis

For TEM8, the kinetics of internalization at steady state differs from CMG2. TEM8 rapidly internalizes roughly 50% of the labeled receptor population in the first hour, with a slow internalization rate in the two following hours (Fig.2A).
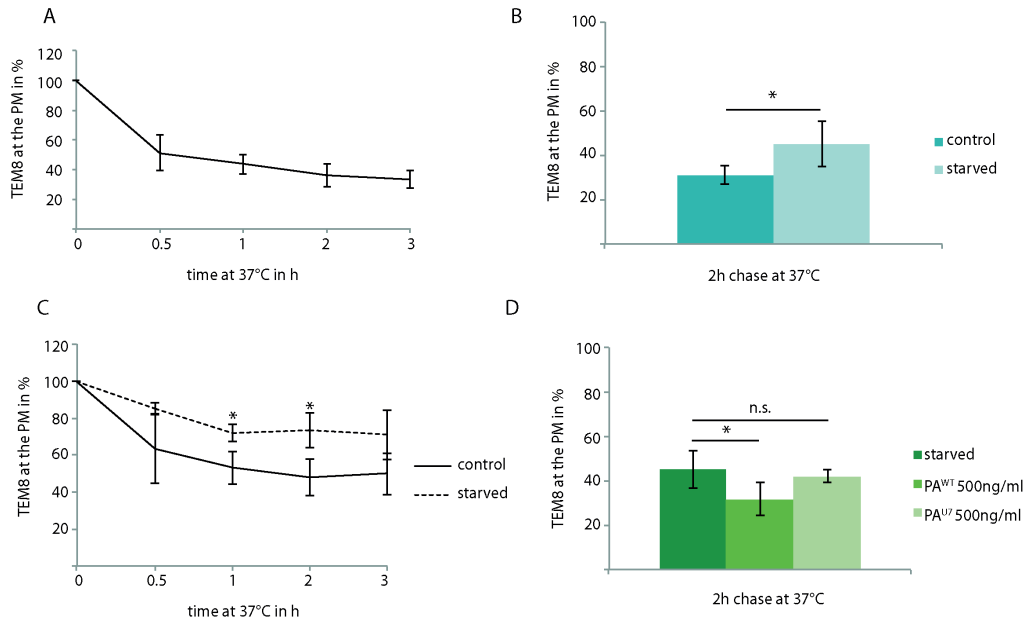
Figure 2: TEM8 undergoes constitutive endocytosis, which is enhanced upon ligand binding

A) RpeI cells were labeled for 1 h at 4°C with antibody against the extracellular domain of TEM8. Antibody was chased for indicated times at 37°C in complete medium. Cells were stained for viability, fixed and stained with secondary antibody at 4°C for 30 min. Fluoresence was measured on a Gallios™ Flow Cytometer (Beckman Coulter). **B)** RpeI cells were either starved for 16 h in serum-free medium or not. Cells were labeled and analyzed as described above. Fluorescence at timepoint t=0 was set to 100% for both conditions and levels after 2h were normalized to the respective levels at t=0. **C)** RpeI cells were starved for 16 h in serum-free medium or not and were then analyzed as in A). **D)** RpeI cells were either starved for 16h in serum-free medium (starved) or starved for 16 h and recomplemented with 500 ng/ml PA^{WT} or 500 ng/ml PA^{U7} (non-cleavable toxin mutant) during labeling and chase. Cells were labeled and analyzed as described above. Fluorescence at timepoint t=0 was set to 100% for all conditions and levels after 2h were normalized to the respective levels at t=0.

Statistics were calculated using an unpaired t-test. Errors represent standard deviation. n ≥ 3. * p≤0.05, ** p≤0.01

This was not behavior induced by the temperature switch from labeling at 4°C and chasing at 37°C, as receptors labeled for 20 min at 37°C showed the same kinetics (Fig. S1). When cells were serum-starved, endocytosis was attenuated compared to control conditions (Fig.2B) but still occurred to a significant extent. Therefore, TEM8 undergoes

constitutive endocytosis in starved cells but endocytosis is significantly enhanced by the presence of ligand (Fig. 2C). Again, only PA^{WT} but not PA^{U7} accelerated endocytosis in starved cells (Fig.2D).

Thus TEM8, in contrast to CMG2, undergoes ligand-independent, constitutive endocytosis. However, endocytosis is enhanced by the binding of the ligand and clustering of the receptor.

Identification of novel anthrax toxin receptor interacting proteins

To find new interaction partners for CMG2, several Yeast Two Hybrid (Y2H) screens were performed. The bait used in these screens was either the full-length protein or only its cytoskeleton-binding domain (CBD), which is conserved between TEM8 and CMG2 (Fig.3A) and was shown to interact with the actin cytoskeleton for TEM8 [26].
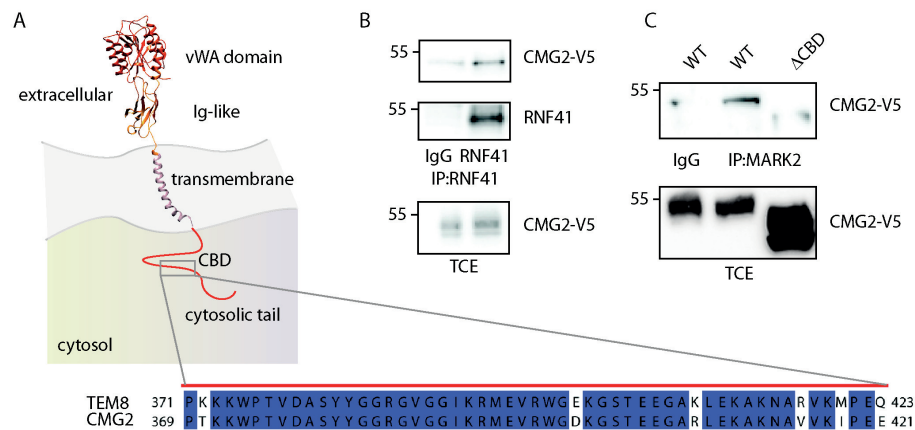
Figure 3: RNF41 and MARK2 are new interactors of CMG2

A) Scheme depicting the general structure of CMG2. CBD is the cytoskeleton-binding domain, conserved between TEM8 and CMG2 **B)** RpeI cells were transfected for 48 h with CMG2-V5 WT. Immunoprecipitates against RNF41 or as control unspecific IgGs were analyzed by SDS-PAGE and Western Blotting against CMG2-V5 and RNF41. Total cell extracts (TCE) were analyzed for expression levels of CMG2-V5. **C)** RpeI cells were transfected for 48 h with CMG2-V5 WT or ΔCBD. Immunoprecipitates against MARK2 or as control unspecific IgGs were analyzed by SDS-PAGE and Western Blotting against CMG2-V5. Total cell extracts (TCE) were analyzed for expression levels of CMG2-V5.

The first hit from a membrane Y2H with the full length CMG2 as bait, was the E3 ubiquitin ligase RNF41 (Nrdp1), an enzyme involved in the trafficking and downregulation of cell surface receptors. RNF41 could be co-immunoprecipitated with CMG2 WT (Fig.3B). From a Y2H screen in which the CBD was used as bait, the only hit was the serine/threonine kinase MARK2 (Par1b). This kinase is involved in regulating polarity [27] but was more recently also shown to phosphorylate and activate

RNF41 [28]. Again, MARK2 could be co-immunoprecipitated with CMG2 WT, but this time, as expected, not with the mutant lacking the CBD domain (Fig.3C). Thus, both RNF41 and MARK2 appear to be novel interactors of CMG2.

The first step of endocytosis of CMG2 is regulated by RNF41

To test for functional interaction of these novel interactors with CMG2 and TEM8, expression of the receptor at the surface as well as endocytosis rates were measured in the presence or absence of the proteins. Here we also included Cbl, the previously identified E3 ligase of TEM8 [9] and USP8, the deubiquitinating enzyme related to stability and function of RNF41 [29, 30] in our analysis.
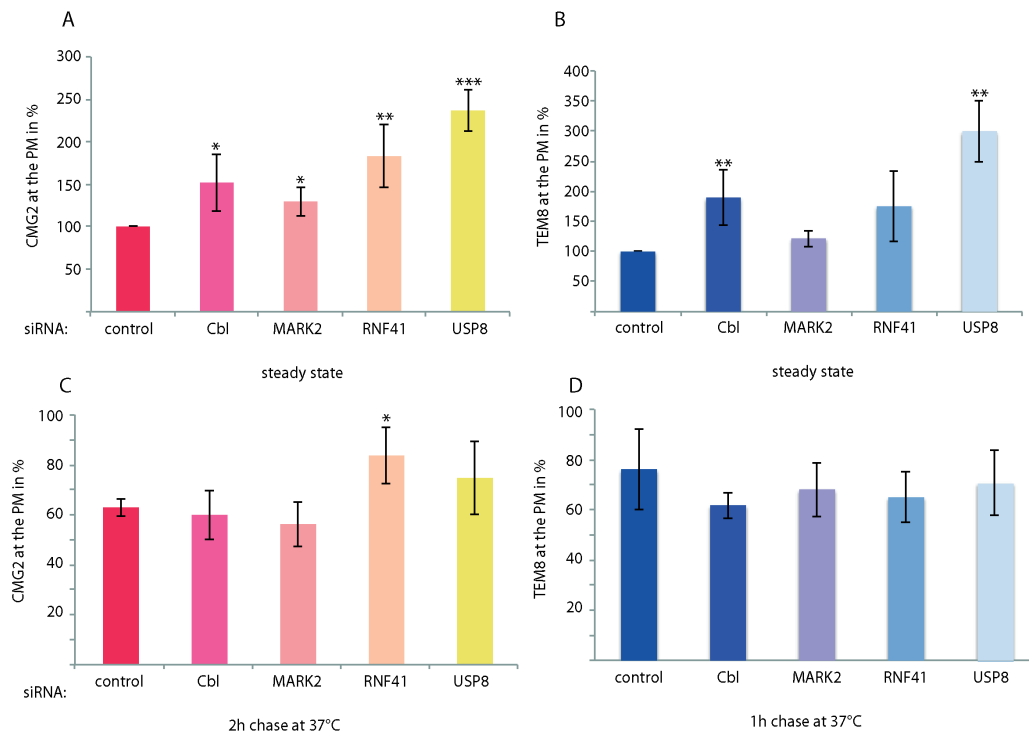
Figure 4: RNF41 mediates early steps of CMG2 endocytosis

A) RpeI cells were transfected for 72 h with control siRNA (control) or with siRNA against Cbl, MARK2, RNF41 or USP8. Cells were labeled for 1 h at 4°C with antibody against the extracellular domain of CMG2 and steady state PM expression of CMG2 was assessed directly without chase. Cells were stained for viability, fixed and stained with secondary antibody at 4°C for 30 min. Fluoresence was measured on a Gallios™ Flow Cytometer (Beckman Coulter). Fluorescence at timepoint t=0 was set to 100% for WT and levels for all other conditions were normalized to WT. **B)** RpeI cells were transfected for 72 h with control siRNA (control) or with siRNA against Cbl, MARK2, RNF41 or USP8. Cells were labeled for 1 h at 4°C with antibody against the extracellular domain of TEM8. Cells were treated and analyzed as in A) **C)** RpeI cells were transfected for 72 h with control siRNA (control) or with siRNA against Cbl, MARK2, RNF41 or USP8. Cells were labeled for 1 h at 4°C with antibody against the extracellular domain of CMG2.

Antibody was chased for 2 h at 37°C in complete medium. Cells were stained for viability, fixed and stained with secondary antibody at 4°C for 30 min. Fluoresence was measured on a Gallios™ Flow Cytometer (Beckman Coulter). Fluorescence at timepoint t=0 was set to 100% for all conditions and levels after 2 h were normalized to the respective levels at t=0. **D)** RpeI cells were transfected for 72 h with control siRNA (control) or with siRNA against Cbl, MARK2, RNF41 or USP8. Cells were labeled for 1 h at 4°C with antibody against the extracellular domain of TEM8. Cells were treated and analyzed as in C). Statistics were calculated using an unpaired t-test. Errors represent standard deviation. n ≥ 3. * p≤0.05, ** p≤0.01, *** p≤0.001

Knockdown efficiencies for all proteins were either confirmed by Western Blot or qPCR (Fig. S2A-C) or had been established previously for Cbl [9]. Silencing of any of the four proteins led to an increase of the steady state level of CMG2 at the cell surface (Fig.4A). For TEM8 however, only loss of Cbl and USP8 led to an elevated surface expression compared to control cells (Fig.4B).

An elevation in surface levels could be caused by a defect in endocytosis. However, CMG2 was more stable at the cell surface over time only when cells were depleted of RNF41 (Fig.4C). Endocytosis of TEM8 was not affected by the absence of all four proteins (Fig.4D). Therefore, only RNF41 controls physiological endocytosis of CMG2. The increase in surface expression for the other proteins might be explained by a defect in later steps of endocytosis, a long-term accumulation not measurable in the timecourse assessed here or a difference in recycling.

RNF41, MARK2, Cbl and USP8 affect anthrax toxin entry via CMG2

To assess endocytosis with the pathological ligand PA, we turned to a well-established assay for toxin binding and entry into cells [6, 7]. Three parameters can be monitored: 1) the binding of PA and its conversion for the 83 (PA^{83}) to the 63 kDa (PA^{63}) form, 2) the appearance of the SDS-resistant multimeric PA, which only forms once the PA multimer has undergone the conformational change leading to membrane insertion (Inserted PA^{7mer}) and 3) the total PA multimer (Total PA^{7mer}), which can be visualized by acidifying cell extracts in order to induce the conversion to the SDS-resistant for all PA multimers (note that PA multimerizes at the cell surface but that this multimer is SDS-sensitive and thus migrates as a monomer). Cells expressing only the main anthrax receptor CMG2 (RpeI shTEM8) were treated for different timepoints with toxin, either in the presence or absence of our protein of interest (Fig.5A and B).
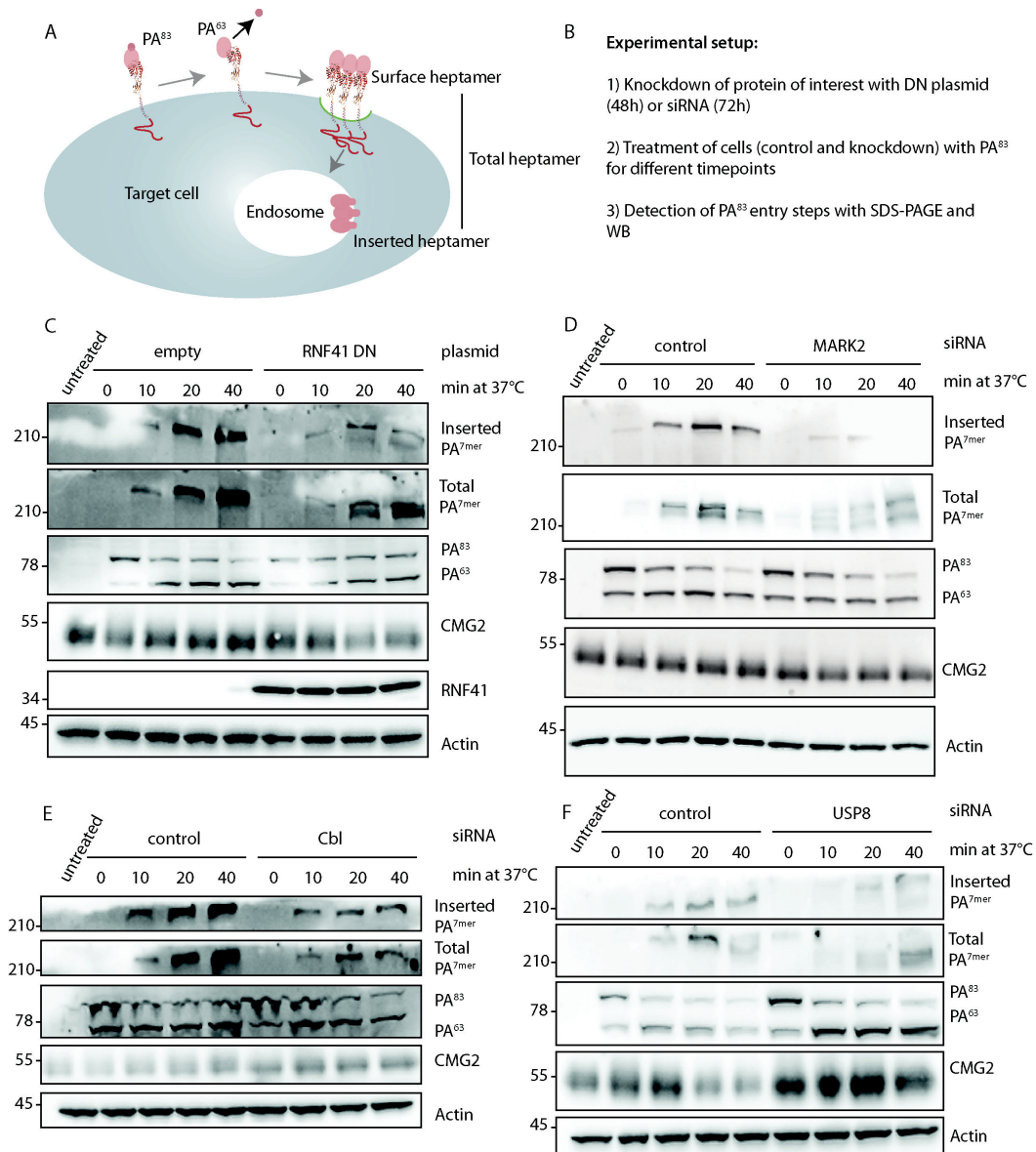
Figure 5: RNF41, MARK2, Cbl and USP8 affect anthrax toxin entry via CMG2

A) Scheme of toxin entry indicating the different steps that can be monitored in this assay **B)** Experimental setup for the toxin entry assay **C)** RpeI shTEM8 cells were either transfected for 48h with an control plasmid without insert (empty) or with a dominant-negative form of RNF41 (RNF41 DN) before toxin entry. Knockdowns for RNF41 DN has been established previously [23]. Cells were treated for 1 h at 4°C with 500 ng/ml PA[83] and shifted to 37°C for indicated times to induce cleavage and heptamerization. Proteins were quantified and 40 μg were loaded on 4-20% gradient SDS-PAGE gels and blotted against PA, CMG2 and actin (equal loading control). To visualize SDS-sensitive heptamers (surface heptamers), samples were treated with 145 mM NaCl and 20 mM MES-Tris, pH 4.5 for 10 min at RT before loading on the gel.

D)-F) RpeI shTEM8 cells were transfected for 72 h with control siRNA (control) or with siRNA against MARK2, Cbl or USP8 before toxin entry. Cells were treated as described for C).

For cells transfected with a dominant-negative form of RNF41, both endogenous levels of CMG2 as well as binding and cleavage efficiency of PA were comparable to the control cells. Heptamer insertion in endosomes was impaired, but not formation at the surface (Fig.5C). Absence of MARK2 results in a slight defect in surface formation and a strong inhibition of internalization for CMG2 (Fig.5D). Cells depleted of Cbl showed a decrease in both surface as well as inserted heptamers. This suggests that formation of the heptamer was inhibited, yet whatever heptamer was formed could efficiently enter the cells (Fig.5E). For cells lacking USP8, a striking increase in total protein concentration of CMG2 was observed. This increase was also reflected in the amount of PA bound to the receptor, as PA^{83} and PA^{63} were elevated. Yet both inserted and total heptamer were decreased compared to control cells (Fig.5F). Altogether these observations indicate that Cbl influences the surface distribution of CMG2, and thereby multimerization kinetics of PA, while RNF41 and MARK2 regulate PA-induced CMG2 endocytosis. USP8 has a more general effect, affecting surface expression and distribution and possibly also endocytosis.

MARK2 acts on both receptors to mediate internalization

To test the involvement of MARK2 and USP8 in toxin entry via TEM8, the same experiments were performed on RpeI shCMG2, cells that only express TEM8.
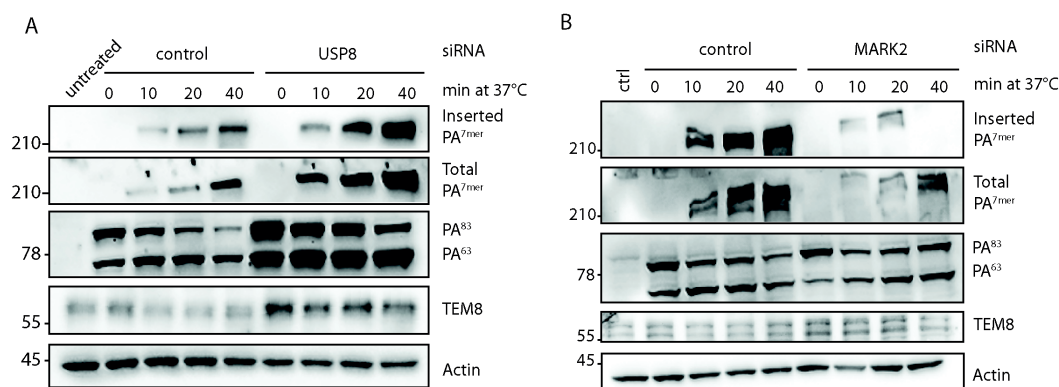
Figure 6: MARK2 also affects entry of anthrax toxin via TEM8

A) RpeI shCMG2 cells were transfected for 72 h with control siRNA (control) or with siRNA against USP8 before toxin entry. Cells were treated for 1 h at 4°C with 500 ng/ml PA^{83} and shifted to 37°C for indicated times to induce ubiquitination. Proteins were quantified and 40 µg were loaded on 4-20% gradient SDS-PAGE gels and blotted against PA, CMG2 and actin (equal loading control). To visualize SDS-sensitive heptamers (surface heptamers), samples were treated 145 mM NaCl and 20 mM MES-Tris, pH 4.5 for 10 min at RT before loading on the gel. **B)** RpeI shCMG2 cells were transfected for 72 h with control siRNA (control) or with siRNA against MARK2 before toxin entry. Cells were treated for 1 h at

4°C with 500 ng/ml PA[83] and shifted to 37°C for indicated times to induce ubiquitination. Immunoprecipitates against TEM8 were analyzed by SDS-PAGE and Western Blotting against ubiquitin, PA and actin (equal loading control).

As for CMG2, silencing of USP8 led to a strong increase in TEM8 protein levels, and a consistent increase in PA (Fig.6A). In contrast, multimerization (Total PA^{7mer}) and endocytosis (Inserted PA^{7mer}) were not inhibited but appeared increased, consistent with the increased toxin binding (Fig.6A).

In cells depleted of MARK2, levels of TEM8, as well as PA binding and cleavage were not changed. However, there was a decrease in heptamer formation and endocytosis (Fig.6B), comparable to the results of CMG2 (Fig.5D). MARK2 seems to be a conserved regulator of toxin entry, whereas USP8 impacts only on toxin entry via CMG2.

Differential E3 ligase requirement of CMG2 and TEM8

Cbl has been established as the E3 ligase for TEM8 and mediates entry by ubiquitination of the receptor after toxin binding [7, 9]. The involvement of Cbl in CMG2-mediated endocytosis has not been addressed and the present identification of RNF41 as a protein interacting with CMG2 raises the interesting possibility that despite their high level of sequence similarity, TEM8 and CMG2 have different requirements in terms of E3 ligases. To test this possibility, PA induced receptor ubiquitination was tested for both CMG2 and TEM8 in cells that express either one or the other receptor.
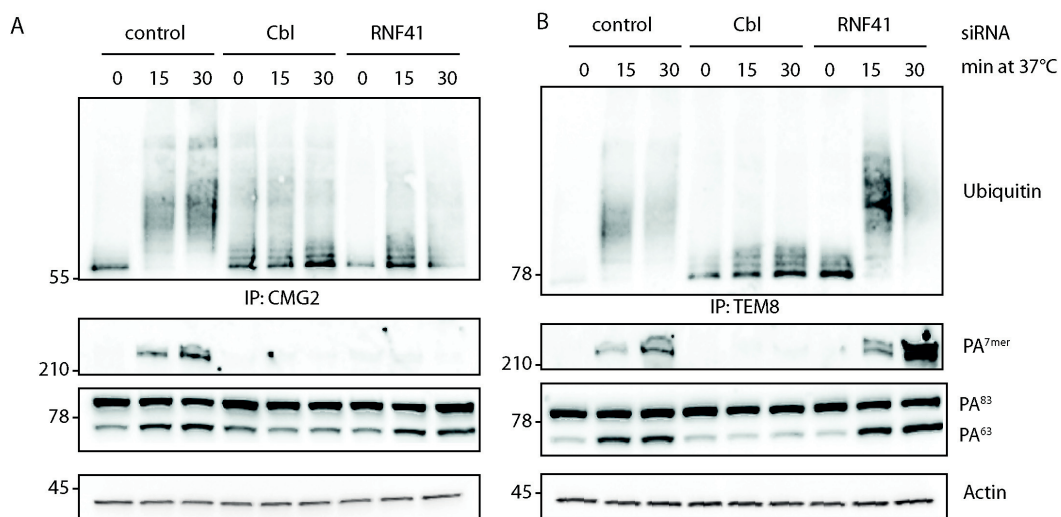
Figure 7: RNF41 is specific for CMG2 but not for TEM8

A) RpeI shTEM8 cells were transfected for 72h with control siRNA (control) or with siRNA against Cbl or RNF41 before toxin entry. Cells were treated for 1h at 4°C with 500 ng/ml PA[83] and shifted to 37°C

for indicated times to induce ubiquitination. Immunoprecipitates against CMG2 were analyzed by SDS-PAGE and Western Blotting against ubiquitin, PA and actin (equal loading control). **B)** RpeI shCMG2 cells were transfected for 72h with control siRNA (control) or with siRNA against Cbl or RNF41 before toxin entry. Cells were treated for 1h at 4°C with 500 ng/ml PA[83] and shifted to 37°C for indicated times to induce ubiquitination. Immunoprecipitates against TEM8 were analyzed by SDS-PAGE and Western Blotting against ubiquitin, PA and actin (equal loading control).

After toxin treatment under control conditions, over time both CMG2 and TEM8 will undergo PA-induced ubiquitination, which is absent at steady state (Fig.7A and B). In the absence of Cbl or RNF41, this ubiquitination is abolished after toxin binding for CMG2 (Fig. 7A). Additionally, a marked decrease in inserted heptamers can be observed, consistent with previous results (Fig.5C and E).

Silencing of Cbl strongly affects the ubiquitination of TEM8 and the formation of the SDS-resistance PA heptamer, as expected, while silencing of RNF41 has no effect (Fig. 7B). Thus, Cbl can modify the ubiquitination of both receptors, whereas RNF41 is specific for CMG2.

Discussion

Both CMG2 and TEM8, apart from their role as anthrax toxin receptors, serve important roles in their physiological context. This is evident from the two severe genetic diseases that are linked to mutations in these proteins, either HFS for CMG2 or GAPO syndrome for TEM8 [22, 31]. Here we apply new methods to investigate endocytosis, steady state kinetics and physiological behavior of these two interesting proteins and we identify new potential interactors of these receptors.

Physiological endocytosis of CMG2 and TEM8

We show, for the first time, that both receptors also internalize without their pathogenic ligand, although at very different rates. In the case of CMG2, this internalization is completely dependent on the presence and binding of a physiological ligand. Additionally, it seems that internalization only occurs if the ligand induces the multimerization of the receptor. In the absence of ligand or if this ligand fails to cluster the receptor, CMG2 remains at the cell surface. This is a concept that was already shown for the Transferrin receptor, where clustering promotes endocytosis [32]. Interestingly, the internalization kinetics of TEM8 differs drastically from those of CMG2. It appears that although the same concepts as described for CMG2 also apply for half of the TEM8 population, the other half internalizes constitutively. This means that potentially only 50% of the receptor present at the cell surface actually can engage in binding to a ligand whereas the other half cannot. It has been suggested before that TEM8 exists in two forms at the PM, in an open or closed conformation, similar to integrins [33]. We already know that in the absence of ligand binding, both CMG2 and TEM8 can bind actin [7 and L.Abrami, personal communication]. Binding of ligand will release this interaction [34]. In cells endogenously expressing CMG2 and TEM8, only TEM8 shows interaction with actin, suggesting that a majority of CMG2 is always bound to ligand whereas TEM8 is not. Our biphasic internalization curve could reflect this distribution. CMG2, on the other hand, is constantly internalized but at a slower rate, suggesting a more homogeneous population of receptor at the cell surface.

The internalization cue so far is not understood. If indeed CMG2 binds to collagen VI, as studies from our lab and others suggest, the question arises as to why and when the receptor internalizes and if the ligand internalizes at the same time. If CMG2, like integrins, coordinates adhesion, a possibility could be that endocytosis is triggered dependent on adhesion strength. For E-cadherin it was shown that large clusters of the

protein are targeted preferentially for endocytosis, thereby keeping adhesion strength equilibrated over time [35].

Novel regulators of anthrax toxin entry

In this study four new proteins acting on toxin entry via CMG2 were identified, of which two are shared with the pathway via TEM8. We have started to understand their mode of action on the intricate and highly regulated entry of anthrax toxin.

Both RNF41 and MARK2 affect internalization of the PA-CMG2 heptamer after toxin binding but only RNF41 also affects physiological endocytosis of CMG2. A recent paper suggests a model in which RNF41 activity depends on phosphorylation by MARK2 [36]. We show that internalization of toxin via TEM8 is also controlled by MARK2 but is completely independent of RNF41. This argues that apart from acting on RNF41, in our case MARK2 must have an additional, potentially direct effect on our receptors. This is strengthened by the fact that MARK2 was found in a Yeast-2-Hybrid screen with the CBD, a conserved cytosolic domain of CMG2 and TEM8. We have so far not been able to identify if MARK2 phosphorylates CMG2 and TEM8 directly. It could be that although the physical interaction is direct, the functional interaction between MARK2 and the receptors might not. Two studies have already placed MARK2 in the context of clathrin-mediated endocytosis. It can be co-purified with clathrin-coated vesicles and might facilitate their assembly [37] and seems to promote vesicle transport along microtubules [38]. One could imagine both possibilities for our case, which will be addressed in the future.

Cbl was already identified as the E3 ubiquitin ligase of TEM8 mediating endocytosis in an earlier study from our lab [9]. We now show that Cbl, together with RNF41 is also involved in the pathway of toxin uptake via CMG2. Interestingly, Cbl seems to have a different effect on CMG2 than TEM8. Whereas ubiquitination of TEM8 by Cbl regulates the internalization step [7], it mediates heptamer formation for CMG2. The internalization process of CMG2 in turn is regulated by RNF41, a protein, which is completely dispensable for toxin entry via TEM8. CMG2 therefore needs ubiquitination by two different E3 ligases to successfully enter after toxin binding. The two enzymes might act in concert, with Cbl starting to ubiquitinate the receptor, which promotes its heptamerization. Then, RNF41 could add on to the existing ubiquitin chain, either just extending it or by adding a differently linked chain. It could be that the extension of the

chain reaches a threshold required for endocytosis or that the signal consists of a differently linked ubiquitin chain. It is also interesting to note that TEM8 only seems to need one single E3 ligase, although we cannot completely exclude the possibility of another enzyme.

The last protein that was identified was USP8, a deubiquitinating enzyme. Two studies link USP8 to RNF41. The proteins mediate each other's stability and thus regulate receptor trafficking [29, 30]. But more famously, USP8 is known as a DUB associated with the ESCRT-0 complex at endosomes. There it deubiquitinates cargo and components of ESCRT-0, which can lead to degradation or recycling of this cargo and stabilization of the ESCRT complex, thereby maintaining normal endosome morphology and function [39-43]. Here a loss of USP8 only has an impact on toxin entry via CMG2 but not TEM8. If indeed USP8 and RNF41 are in the same pathway, this would be consistent with RNF41 only acting on CMG2 but not TEM8. Another possibility is that USP8's effect on early endosome dynamics might play a role in toxin entry as well. This could explain why the effect is limited to CMG2. To form a pore, PA needs to undergo a conformational change linked to the change in pH in endosomes. When PA is bound to TEM8, the pH requirements are already met in early endosomes, whereas if it is bound to CMG2, it needs to progress to later endosomes and their lower pH [44]. So even though here we show that overall receptor levels of CMG2 and TEM8 are elevated after knockdown of USP8, arguing for a misbalance of receptor turnover at the level of early endosomes during siRNA treatment, pore formation is only inhibited for CMG2.

This work sheds light on the endocytosis of the two anthrax toxin receptors, both in the presence and absence of its surrogate ligand anthrax toxin. For the first time, steady state behavior of CMG2 and TEM8 has been assessed and novel regulators of both pathological and physiological endocytosis of the two receptors were identified. Our results add crucial information to the research on the physiological function of CMG2 and TEM8.

Supplementary Figures
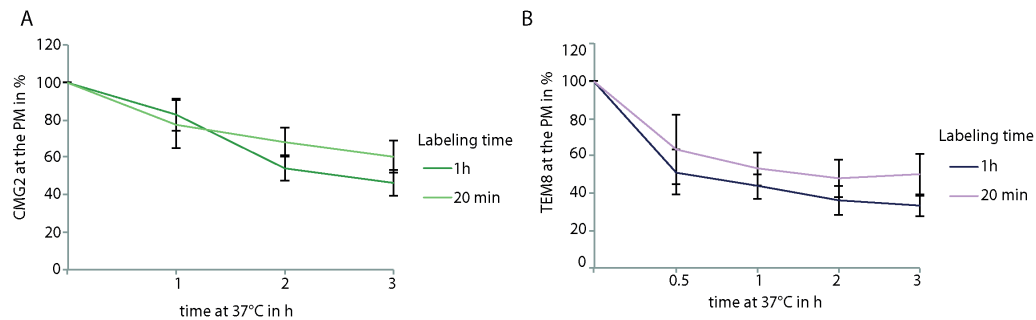
Figure S1: Internalization kinetics with different labeling times

A) RpeI cells were labeled for either 1 h at 4°C or for 20 min at 37°C with antibody against the extracellular domain of CMG2. Antibody was chased for indicated times at 37°C in complete medium. Cells were stained for viability, fixed and stained with secondary antibody at 4°C for 30 min. Fluoresence was measured on a Gallios™ Flow Cytometer (Beckman Coulter). **B)** RpeI cells were labeled for either 1 h at 4°C or for 20 min at 37°C with antibody against the extracellular domain of TEM8 and were labeled and analyzed as described above.
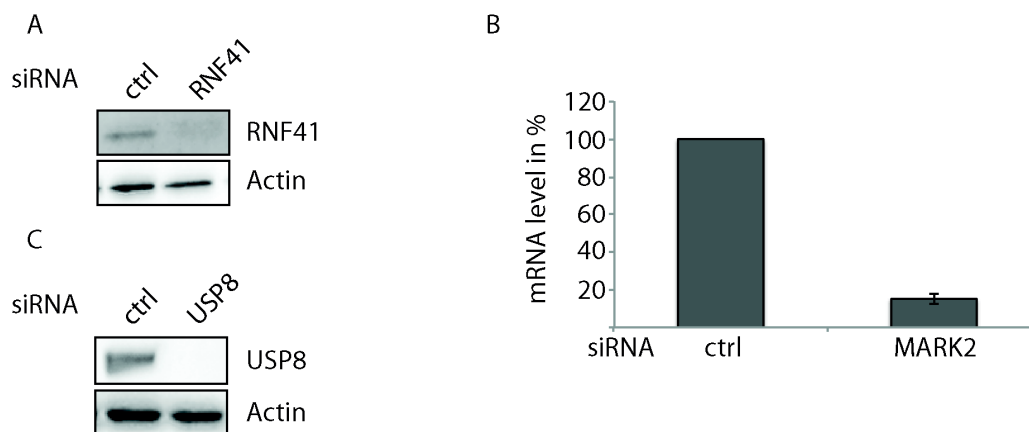
Figure S2: Control of knockdown for RNF41, USP8 and MARK2

RpeI cells were transfected for 72 h with control siRNA (ctrl) or with siRNA against MARK2, RNF41 or USP8 before toxin entry. **A)** Proteins were quantified and 40 μg were loaded on 4-20% gradient SDS-PAGE gels and blotted against RNF41 and actin (equal loading control). **B)** qPCR analysis was performed with specific primers against MARK2 and housekeeping genes as a control. n=3 **C)** Proteins were quantified and 40 μg were loaded on 4-20% gradient SDS-PAGE gels and blotted against USP8 and actin (equal loading control).

Author contributions

Conceived and designed the experiments: SF GVDG. Performed the experiments: SF (Fig.1-5, 6A and S2) LA (Fig.7) NP (Fig.6B and S1). Analyzed the data: SF LA GVDG. Contributed reagents/materials/analysis tools: SF LA NP. Wrote the paper: SF GVDG

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III. Conclusions and perspectives

In this work, we have investigated folding and endocytosis of the two anthrax toxin receptors CMG2 and TEM8. Both receptors, apart from their crucial role in anthrax pathogenesis, are also indispensable in a physiological context. Mutations in *cmg2* lead to Hyaline Fibromatosis Syndrome, whereas mutations in *tem8* are associated with GAPO syndrome. The physiological role of the two receptors to date remains elusive and knockout mice for either CMG2 or TEM8 have so far only been able to recapitulate limited aspects of the respective disease. Therefore, it is imperative to investigate the role of these two important proteins in more detail. In this thesis I presented two research projects and will integrate the findings into the big picture of CMG2 and TEM8 research.

N-glycosylation of anthrax toxin receptors CMG2 and TEM8

N-glycosylation is a common posttranslational modification, which has been shown to be important for protein folding for a wide variety of substrates. We found that both CMG2 and TEM8 are glycosylated on their predicted sites and this is advantageous for folding and function of the proteins.

The role of glycosylation for CMG2 is especially intriguing, as it only seems to become important when other protein-destabilizing mutants are present. In this case, glycosylation acts as a buffer and increases folding efficiency for the mutants. It would be interesting to know if these mutants have an increased occupancy in glycosylation or are differentially glycosylated compared to the WT protein.

Another interesting aspect to understand would be why two proteins that are so closely related as CMG2 and TEM8 vary so substantially in their dependence on N-glycosylation. Even the glycosylation site in the Ig-like domain of the two proteins is conserved, but loss of this site affects CMG2 and TEM8 very differently.

We have not been able to assess the variation in folding efficiency of CMG2 and TEM8. Despite the high sequence similarity it seems that TEM8 presents crucial sequence alterations responsible for its higher tendency to misfold. So far, we have always considered CMG2, even WT, to be a protein that frequently fails to fold. This stems from our observations that a high number of known HFS mutations leads to misfolding of the protein and subsequent loss-of-function [2]. The genotype-phenotype correlation for GAPO syndrome is far less developed. It will therefore be interesting to see if mutations seen in GAPO syndrome are also highly correlated with the misfolding of TEM8 and which domains of the protein are most mutation-prone.

This work sheds light on the folding of the two anthrax toxin receptors and reveals unexpected, yet intriguing, differences between these two closely related receptors.

Behavior and distribution at the plasma membrane

After being synthesized, CMG2 rapidly travels to the plasma membrane. There, it encounters its ligand and will bind to it. This binding leads to a signal transduction that ultimately leads to the release of the actin cytoskeleton from the cytosolic tail (J. Bürgi, unpublished work).
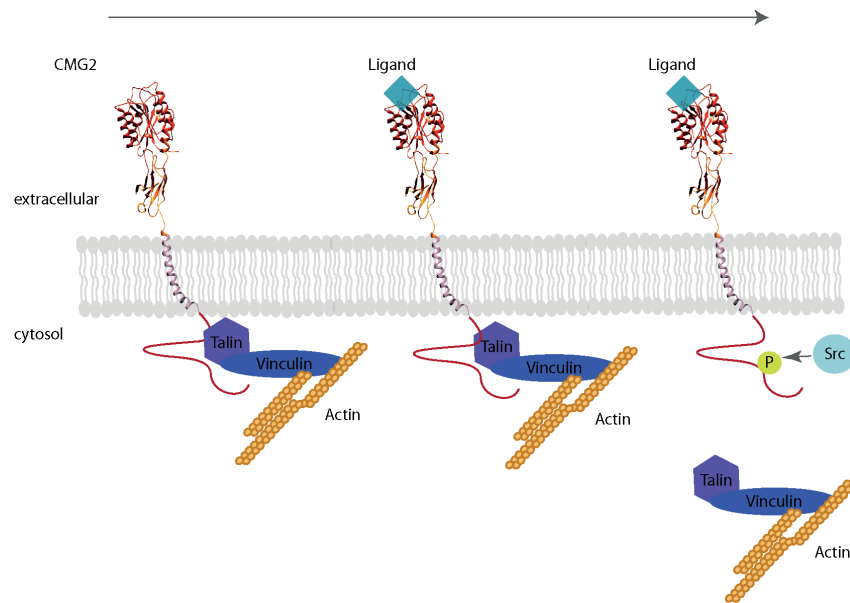
Fig.12 Graphical summary of actin binding and release CMG2 binds actin via talin and vinculin with its cytosolic tail at the plasma membrane if not bound to a ligand. Binding of a ligand to the extracellular domain of CMG2 leads to a release of actin, talin and vinculin. This action is mediated by Src phosphorylating CMG2.

Binding of the ligand to CMG2 will lead to a constant internalization of the receptor. This is dependent on the clustering of the receptor into multimers, as binding alone cannot restart endocytosis (Fig. 1D of the draft manuscript). This is interesting, as it implies that either CMG2 will actively segregate into clusters after binding or that the ligand can mediate this aggregation. It was shown that CMG2 can form homodimers and an extended FRAP method in collaboration with the lab of Gerhard Schütz (TU Vienna) showed that around 30% of CMG2 is present as a dimer at the cell surface (S. Blaskovic, unpublished results). It is possible that CMG2 is preorganized in dimers at the cell surface and that binding of a ligand leads to the formation of higher order clusters, which are then endocytosis-competent. Interestingly, TEM8 can self-associate though its TM domain and mutations in this domain block interaction and slow down heptamerization after toxin binding [322], nicely backing our hypothesis.

For the binding of PA, we already know that endocytosis requires the toxin-receptor complex to be heptameric, as single receptors, even when bound to the toxin, will not internalize. This preorganization and the fact that clustered receptors internalize preferentially, was already shown for the EGFR [323]. As CMG2 is a receptor without enzymatic activity, the question as to how signal transduction is achieved remains puzzling. The cytoplasmic tail of CMG2 could be a mediator in this process as it has

been described as a highly disordered domain [9]. After binding of a ligand to the extracellular domain, we could envision a conformational change of the tail, which would allow for binding of a different set of proteins. This creates two sets of "signaling hubs" for either a ligand-free or ligand-bound receptor, a concept that has been described for numerous proteins with disordered domains [324-326]. Another option is that the conformation of the tail could be involved in the organization of CMG2 at the PM, either confining it to monomers or mediating association into dimers or oligomers. A similar concept was shown for STIM1 in response to intracellular Ca^{2+} levels [327].

Interestingly, the steady-state endocytosis of CMG2 is a rather slow process (Fig. 1A of the draft manuscript) compared to endocytosis triggered by PA. Yet we know that a majority of the receptor is occupied with ligand at steady state (J. Bürgi, unpublished results). Potentially, clustering of CMG2 with the physiological ligand into multimers that can internalize correctly is a slow process that reflects the slow kinetics of endocytosis at steady state. PA, which has a higher affinity to CMG2 than the physiological ligand, might be able to speed up this process, thereby leading to a faster endocytosis rate. The concept that ligand can modulate entry speed has been recently proposed for the cannabinoid receptor 1, a GPCR [328].

For TEM8, we see a much faster initial drop in surface fluorescence than for CMG2 (Fig.2A of the draft manuscript). After this, the population at the surface seems to be declining much slower. This suggests the presence of two populations: one that can bind ligand and is regulated by this, similar to CMG2, and one that cannot bind ligand and therefore traffics ligand-independently. This might depend on our experimental setup. CMG2 and TEM8 not necessarily bind to the same ligand and it is unclear, to what the receptors are bound in cell-culture conditions. It could be interesting to test different conditions to find out more about the heterogeneity of TEM8 at the cell surface.

Recent experiments from our lab suggest that CMG2, when bound to ligand also binds to RhoA with its cytoplasmic tail (J. Bürgi, unpublished results). This indicates a competitive binding between either actin via talin and vinculin for "non-activated" e.g. ligand-free receptor and RhoA for "activated", e.g. ligand-bound receptor. CMG2 could be pre-organized by the actin cytoskeleton and could be re-arranged into spatially and functionally different clusters by RhoA, once activated by a ligand. This link with RhoA is evolutionary conserved. In zebrafish, binding of Antxr2a, the CMG2 homologue, to actin and RhoA mediates the mitotic spindle positioning [28]. For the moment it is unclear how this function in zebrafish could be translated to humans. A separate analysis of these two populations is challenging. By a long-term perturbation of RhoA, i.e. by siRNA treatment, the entity of the actin cytoskeleton would be affected, making it difficult to differentiate between the effects caused by actin loss or RhoA loss. One possible experiment to circumvent this would be a very short-term perturbation of RhoA with the "knock-sideways" technique [329], where the protein of interest is segregated away from its normal localization.

Regulation of endocytosis of CMG2 and TEM8

Once inside the endosomal pathway, the default destination for receptors is recycling back to the plasma membrane. The decision between recycling and degradation can be dependent on the concentration of the ligand as for EGFR [330] but might also be determined by affinity of the ligand.
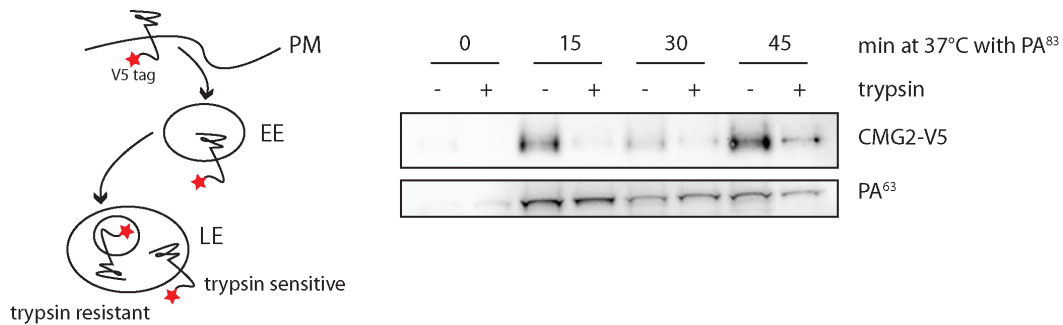
Fig. 13 Description and representative result of a protease-protection assay CMG2 with a V5 tag at its C-terminus (red star) is expressed in cells. The tag is sensitive to trypsin digestion after fractionation and enrichment of endosomes, when present in early endosomes (EE). It becomes protected from trypsin by inclusion into intraluminal vesicles in late endosomes (LE). This can be assessed by Western Blot. PA^{63} is always inside the endosomes, where it is continuously protected from trypsin treatment.

After the addition of toxin to the cells, we can see CMG2 in multivesicular endosomes already after a short time. A trypsin-resistant band appears in a protease protection assay (see above) and our toxin assays show similar results. For toxin bound to CMG2 this ensures that the receptor faithfully transports its pathological cargo to late endosomes where pore formation can occur. It would be interesting to test this also without toxin. PA could lead to a different or at least faster trafficking route of the receptor. A modification of the pathway would not be necessary for TEM8, as toxin associated to this receptor can already insert into the membrane of early endosomes.

A possible way of changing the fate of the receptor after internalization is a change in post-translational modifications. This might depend on which ligand is bound. When we look at ubiquitination after adding PA to the cells, we see the apparition of a long smear, consistent with the receptor being rapidly polyubiquitinated after toxin binding (Fig.7 of the draft manuscript). However, at steady state, this ubiquitination is mostly absent. This suggests that physiological endocytosis is regulated differently or with a lower level of polyubiquitination that we are unable to detect.
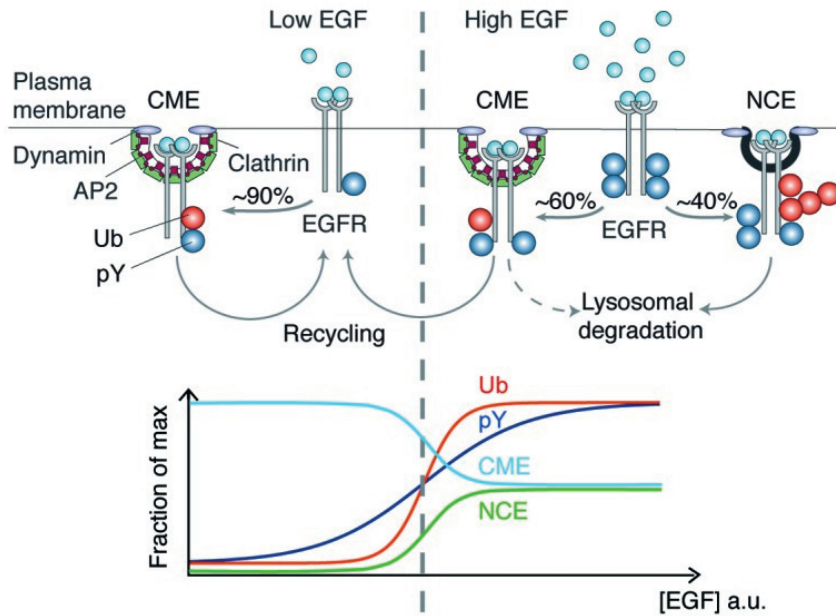
Fig. 14 Schematic of the threshold-mediated model for EGFR endocytosis Depending on the concentration of the ligand, EGFR will be either routed to CME and recycling, or mostly to CIE (or here NCE) and degradation. This depends on the level of phosphorylation, which controls the levels of ubiquitination. Higher levels of phosphorylation allow stable binding of Cbl and this in turn controls the levels of ubiquitination. Figure from [331]

This threshold model has already been proposed for the EGFR, where also the level of ubiquitination depends on the concentration of the ligand and this in turn regulates receptor fate [332]. Interestingly, this difference in ubiquitination also depends on the level of phosphorylation, a modification which also occurs in CMG2 and TEM8 after toxin binding [201].

Events after endocytosis

In the case of PA, the pathological ligand stays attached to the receptor and hitchhikes into the cell. We do not know if this is also the case for the physiological ligand. If the ligand is indeed, as shown from ours and other labs, collagen VI, it would be important to determine how this could be achieved. Collagen VI is a large protein, a fibrillar collagen, for which very little is known about its turnover. One report states that it is taken up by phagocytosis, followed by degradation in lysosomes [333]. Other collagens, such as collagen I, II, IV and V, can be internalized by the endocytic collagen receptor uPARAP/Endo180 and are degraded in the lysosome [334, 335]. Therefore, a scenario, in which collagen VI, or fragments of it are endocytosed with CMG2 and subsequently degraded, thereby regulating its turnover, can be envisioned. The accumulation of collagen VI in HFS patients and also in CMG2 knockout mice also strongly argues for a dysregulation of collagen VI turnover. We would need to test if indeed collagen VI can be taken up into cells and is capable of restarting endocytosis of CMG2 in starved cells, thereby acting as a *bona fide* ligand.

Another question we have started to address is the signal transduction after binding and internalization of the receptor. Even though CMG2 and TEM8 are cell surface receptors without intrinsic enzymatic activity, they might be able to indirectly convey signals to a downstream signaling pathway. We therefore used a commercially available phosphokinase assay to look at potential upregulation after ligand binding.
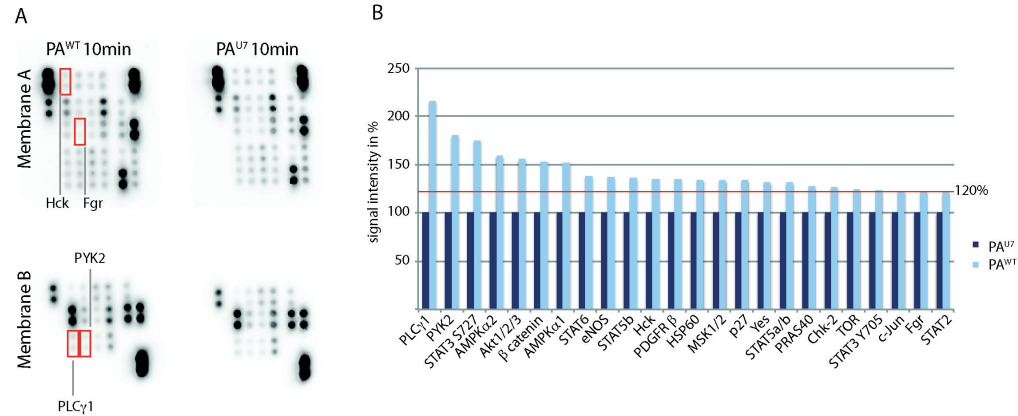
Fig. 15 PA binding and endocytosis activates proteins involved in actin rearrangement A) Fibroblasts *were starved for 16h in serum-free medium and were treated for 1h at 4°C with 500ng/ml PA^{WT} or 500ng/ml PA^{U7} (non-cleavable toxin mutant). Cells were shifted to 37°C for 10 min to induce cleavage and heptamerization. Proteins were quantified and 350 μg were used for each membrane set. In red are proteins that came up several times **B)** Quantification of control fibroblasts. As a cutoff, 120% in signal increase was used.*

When we measured the phosphorylation of the 43 substrates detectable in the kit and compared levels between PA^{WT}- and PA^{U7}-treated cells, we indeed found some interesting hits (see above). A target that was significantly upregulated in all three assays was the non-receptor tyrosine kinase Fgr, a member of the Src subfamily. It has been implicated in transmitting signals downstream of plasma membrane proteins lacking kinase activity, such as β2 integrin signaling [336]. This in turn affects cytoskeletal structure and rearrangements, both of the actin cytoskeleton in general and in focal adhesions [337]. Another hit was PLCγ1, which was only present in two out of three assays, but was the most highly elevated one in cells with both receptors. This enzyme is a PI(4,5)P$_2$ diesterase, producing diacylglycerol and IP$_3$ and has again been implicated in actin reorganization [338]. Among the upregulated targets found in one or two assays also functionally linked to the actin cytoskeleton were PYK2 (focal adhesion kinase 2) and Hck, also a non-receptor tyrosine kinase.

CMG2 was found in a proteomics study to be present in focal adhesions [339] and its functional relationship with proteins such as talin and vinculin (J.Bürgi, unpublished results) also places it in this cellular environment. It would therefore be intriguing to see if the dynamics of focal adhesions are altered in response to toxin entry.

Role of partner proteins in endocytosis of CMG2 and TEM8

In this work, we identified four new proteins that mediate entry of anthrax toxin into cells, namely RNF41, MARK2, Cbl and USP8. Cbl and MARK2 also mediate entry via TEM8. The model we propose is the following:
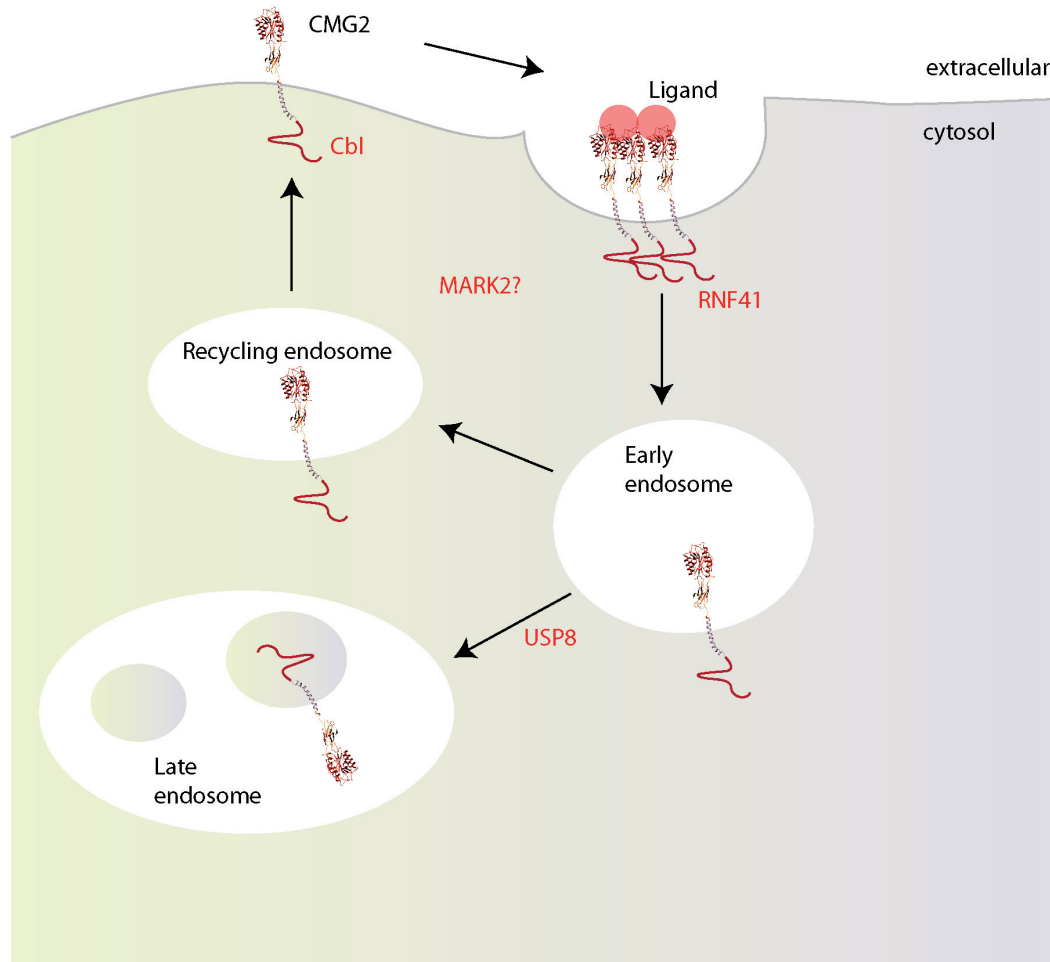
Fig. 16 Graphical abstract of partner proteins regulating the lifecycle of CMG2 CMG2 will be ubiquitinated upon ligand binding by Cbl, promoting heptamerization. Clustered CMG2 will be further ubiquitinated by RNF41, leading to endocytosis. At the early endosome, the receptor fate is decided. De-ubiquitination by USP8 leads to CMG2 being routed to late endosomes, where degradation of the receptor can occur. The exact role of MARK2 in this process remains unclear.

CMG2 is ubiquitinated by Cbl to promote heptamerization after ligand binding. Further ubiquitination by RNF41 mediates endocytosis of the multimeric ligand-receptor complex. At the endosome, deubiquitination of CMG2 could mediate either targeting to the recycling pathway or to late endosomes. This could be done by USP8, although this is a possibility that we still are adressing. We have not been able to pinpoint the exact step of endocytosis that MARK2 controls.

It is interesting to note that CMG2 seems to need two E3 ligases for an efficient entry after toxin binding. Cbl has been shown to mediate both mono- and polyubiquitination of receptors [340]. Our experiments show the apparition of a smear of ubiquitinated

CMG2 after toxin binding (Fig.7 of the draft manuscript), more consistent with polyubiquitination. RNF41 seems to lead to the polyubiquitination of its substrates, including itself [231, 341]. In our case, we could envision a scenario in which Cbl ubiquitinates CMG2 after toxin binding, promoting heptamerization. After this, RNF41 adds on to the ubiquitination on K350, reaching a threshold, which allows endocytosis and efficient targeting to the late endosomes. In the absence of toxin, only RNF41 seems to be required for endocytosis of CMG2. Potentially, this leads to a different ubiquitination pattern, also changing the fate of the receptor and promoting recycling instead of degradation. Although K350 seems to be the main ubiquitination site of CMG2 [202], we cannot exclude the possibility of other sites being ubiquitinated. This other ubiquitination might be too low for our methods to detect or is transient and therefore hard to capture. The conserved site in TEM8, K352, is the main, but not the only site of ubiquitination [202].

So far, we have not been able to show direct interaction between CMG2 and USP8. Nevertheless, it is possible that CMG2 is deubiquitinated by USP8 and this can promote the decision of recycling or degradation. Such a mechanism has been shown for USP8 and EGFR, where deubiquitination of EGFR by USP8 facilitates the sorting of the receptor to MVB with the help of the ESCRT complexes [241, 243, 245]. On the other hand, it is also possible for our effect being based on generally changed endosome dynamics, which have been observed for cells deficient in USP8 [149].

So far it is unclear, how MARK2 affects the endocytosis of anthrax toxin both via CMG2 and TEM8. The fact that MARK2 was identified in a Yeast Two Hybrid screen with a domain conserved between CMG2 and TEM8 argues for a direct interaction. However, we have not been able to show that phosphorylation of a serine or threonine residue in the cytosolic domain of the two receptors is involved in toxin entry. Therefore, it is possible that the effect of MARK2 is indirect and via a different interaction partner.

Integration of our data into a general model for function of CMG2

Research on CMG2 has led to a basic understanding of the function of this protein. However, more information is needed to enhance our knowledge about this important receptor.

The two main aspects of CMG2 that come up repeatedly are angiogenesis and binding to the extracellular matrix. How these two relate to each other is unclear. CMG2 was found in an *in vitro* screen for capillary formation. It was among the most upregulated genes in early capillary formation, i.e. during a specific point in capillary development [1]. The same is true for TEM8, which seems to be needed only during developmental or pathological angiogenesis [17]. To the best of our knowledge, there are no defects in angiogenesis in HFS patients. Data from a collaboration with the lab of Tatiana Petrova (Unil, Lausanne) however suggest a defect in correct lymph vessel formation in CMG2 knockout mice.

For TEM8, the link between vascular and fibrotic phenotypes is easier to understand. A recent paper links both vascular and connective tissue homeostasis to regulation by TEM8 [342]. Also, TEM8 was shown to interact with VEGFR2 in infantile hemangiomas [343] and finally, GAPO patients show both an accumulation of ECM

components as well as venous malformations and vascular abnormities [47, 344]. It seems that TGFβ1 signaling contributes to the profibrotic phenotype in TEM8 knockout mice [342]. Data from our lab also suggests that TGFβ signaling is linked to CMG2 (J.Bürgi, unpublished results). Although all this points in the same direction, further studies are needed to decipher the exact pathway at the molecular level.

In the context of binding to the extracellular matrix, our work adds important information to this process. We now know that both receptors internalize constantly. CMG2 internalizes rather slowly, with a homogenous population distribution. TEM8 however seems to have two distinct populations, that show distinct kinetics. Endocytosis is ligand-binding and multimerization dependent, two features that are most probably also true in the physiological context. It will now be crucial to assess the behavior of the potential physiological ligand(s) during endocytosis. When we compare our results to some key concepts of integrin endocytosis, we find some interesting similarities. Integrins bind to members of the ECM and seem to be important for remodeling of the ECM, especially of fibronectin via binding to α5β1 integrin [345]. Integrins recycle back to the PM after internalization with a half-time of either 3 min or 10 min, depending on the pathway. Estimations consider that the population of integrins at the PM will traffic through the recycling pathway at least once every 30 min [346]. Our data suggests a slower turnover for CMG2, although we would need to address the recycling times more accurately.

Our work places CMG2 into a context comparable with integrins, yet with a less dynamic turnover. This could be important in tissues that do not constantly undergo remodeling or that would need to be stabilized more than others. Regions with mechanical stress would need such a stabile reinforcement together with a tight control of ECM homeostasis. This could explain why HFS patients display nodules preferentially at sites of mechanical stress, sites that are particularly sensitive to loss of CMG2 function. This work significantly contributes to a more general understanding of CMG2's physiological function, by providing important insight into receptor trafficking, its regulation and by identifying key proteins functionally interacting with CMG2. It therefore provides us with a solid groundwork for further research on this important protein.

IV. References

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Institute of Biochemistry, ETH, Zurich, Switzerland   09/10 – 06/11
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        Studying the role of ERAD factor Bap31 in virus entry
Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland   03/10 – 06/10
    Laboratory of Brigitte Berger-Bächi, *research project*
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        Studying folding of anthrax toxin receptor 1 and 2

PUBLICATIONS

Friebe S, Deuquet J, van der Goot FG. Differential Dependence on N-Glycosylation of Anthrax Toxin Receptors CMG2 and TEM8. PloS one. 2015;10(3):e0119864. doi: 10.1371/journal.pone.0119864

Dengler V, Meier PS, Heusser R, Kupferschmied P, Fazekas J, **Friebe S**, Staufer SB, Majcherczyk PA, Moreillon P, Berger-Bächi B, McCallum N.
Deletion of hypothetical wall teichoic acid ligases in Staphylococcus aureus activates the cell wall stress response. FEMS Microbiol Lett. 2012 Aug;333(2):109-20. doi: 10.1111/j.1574-6968.2012.02603.x. Epub 2012 Jun 18.

Geiger R, Andritschke D, **Friebe S**, Herzog F, Luisoni S, Heger T, Helenius A.
BAP31 and BiP are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol. Nat Cell Biol. 2011 Sep 25;13(11):1305-14. doi: 10.1038/ncb2339.

PRESENTATIONS

Poster: **Friebe S,** Abrami L, van der Goot FG. "Unraveling the physiological role of anthrax toxin receptor 2." *EMBO Endocytosis Meeting 2015.* Mandelieu-la-Napoule, France. September 2015

Poster: **Friebe S,** Abrami L, Blaskovic S, van der Goot FG. "Novel mechanistic insights into anthrax toxin endocytosis." *LS2 Annual Meeting 2015.* Zurich, Switzerland. January 2015

Poster: **Friebe S,** Abrami L, van der Goot FG. "Novel mechanistic insights into anthrax toxin endocytosis." *ASCB Annual Meeting 2014.* Philadelphia, PA. December 2014

Poster: **Friebe S**, Abrami L, van der Goot FG. "The Fabulous Four of anthrax toxin endocytosis." *Global Health Institute EPFL Retreat 2014.* Les Diablerets, Switzerland. June 2014

Poster: **Friebe S,** van der Goot FG. "Anthrax toxin receptor 2 and MARK2- it takes two to get in." *LS2 Annual Meeting 2014.* Lausanne, Switzerland. February 2014

Poster: **Friebe S,** van der Goot FG. "Anthrax toxin receptor 2 and MARK2- it takes two to get in." *Labex Inform Workshop "Quantitative Biology of Signalling".* Cargèse, France. October 2013

Poster: **Friebe S,** Deuquet J, van der Goot FG. "Being sweet pays off: N-Glycosylation of anthrax toxin receptor 1." *Global Health Institute EPFL Retreat 2012.* Les Diablerets, Switzerland. June 2012

TEACHING EXPERIENCE

Supervisor for Master Project, EPFL, Lausanne, Switzerland   Spring 2015
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HONORS AND AWARDS

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Best Poster Award at the GHI Retreat	2014
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SKILLS

LANGUAGES
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PROFESSIONAL
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