Freie Universität Berlin

Department of Biology, Chemistry and Pharmacy

Bachelor degree course: Biology (B.Sc.)

The Role of CD63-Large Extracellular Loop on HIV-1 entry and cell to cell transfer

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Carried out at Robert Koch-Institut – Division 18 – HIV and other Retroviruses

– Berlin, March 2014 –

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Acknowledgement

I would like to express my very great appreciation to Dr. Joachim Denner and Mr. Daniel Ivanusic for their valuable suggestions during the planning and development of this research work. Their willingness to give their time so generously has been very much appreciated.

I would also like to thank Dr. Kazimierz Madela and Mrs. Gudrun Holland for their help in doing the comparative microscopy and the orientation study.

My special thanks are extended to Mr. Daniel Smalley, for his encouragement and support throughout my study.

Finally I wish to thank Mrs. Valeska Esch and Mrs. Johanna Reich for their patient and useful critics and advice.

Abstract

Regarding to the World Health Organization’s 2011 published list of the top ten causes of death worldwide, HIV/ AIDS is still listed as the 6th most frequent reason for fatality. Previous analyses with a Yeast two-hybrid (Y2H) screen identified the tetraspanin CD63 as an interaction partner of the HIV-1 protein gp41 and unveiled some highly conserved cysteines in its Large Extracellular Loop as involved interaction sites between those two proteins. The aim of this study was to investigate the role of the CD63's Large extracellular loop on HIV-1 entry and cell-to-cell transfer. For this purpose several expression plasmids encoding for recombinant variants of the Large Extracellular Loop were generated. One did express the CD63 without the whole loop and four contained in each case a different mutation of one or two of the six cysteines.

Studies of the recombinant protein's expression with the confocal laser scanning microscope showed that only one cystein mutant was completely expressed like the wild type CD63. Two cysteine mutants were not expressed consistently throughout the HEK293T cells and one appeared to be not expressed on the surface of the plasma membrane. Surprisingly, recombinant protein without the Large Extracellular Loop was expressed predominantly on the surface of the plasma membrane. Subsequently some foreign protein sequences were introduced instead of this loop. It could be confirmed that these foreign proteins can be added without affecting the overall efficiency of the proteins integration into the cell membrane. Furthermore, introducing certain foreign proteins does neither affect the protein folding nor the protein orientation.

Once this membrane transport system is established, it could be used in the field of immunisation studies, antibody production or other immunological experiments, where the exposure of certain proteins on the cell surface of mammalian cells is of interest. Unfortunately, due to strict time constraints experiments investigating the recombinant protein's influence on HIV-1 entry and cell-to-cell transfer could not be performed.

The results gathered during the course of this research revealed some promise of usefulness. However, in regards to the CD63's overall influence on cell free virus infectivity and cell-to-cell transfer in T lymphocytes, it would appear the current understanding is that no significant influence can be shown. This would suggest that, here too, no significant results are to be expected.

List of Abbreviations

Abbreviation	Explanation
2YT-medium	2x Yeast extract and tryptone medium
A	Adenine
AIDS	Acquired Immune Deficiency Syndrome
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
CFP	Cyane Fluorescent Protein
CD63-ΔLEL	CD63 without the LEL-coding sequence
CPRG	Chlorophenolred-β-D-galactopyranoside
kDa	Kilo Dalton
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTPs	deoxycytidine Nucleosid triphosphates
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
Env	Envelope, glycoproteins
Gag	Group-specific antigen
GFP	Green Fluorescent Protein
HBS	HEPES-Buffered Saline
HCV	Hepatitis-C Virus
HEK293T	Human Embryonal Kidney cells

HeLa	Cervical cancer cell line taken from Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human Immunodeficiency Virus type 1
HTLV-1	Human T-cell leukaemia virus type 1
IS	Immunological Synapse
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LB-Medium	Lysogeny Broth Medium
LEL	Large Extracellular Loop
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
OD	Optical density
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
pCMV	Expression vector: Cytomegalovirus plasmid
PCR	Polymerase chain reaction
pfu	Plaque-forming units
Pol	Polymerase, enzymatic activities
RNA	Ribonucleic acid
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute Medium
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEL	Small Extracellular Loop
SEM-cLSM	Scanning Electron Microscope – confocal Laser Scanning Microscopy
SOC-Medium	Super Optimal Broth Medium + 20mM Glucose
TAE	Tris-acetate-EDTA buffer

TB-Medium	Terrific Broth Medium
TEM	Tetraspanin-enriched microdomain
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFB	Transfection buffer
TM	Transmembrane region
Tris	Tris (hydroxymethyl) aminomethane
VS	Virological synapse
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y2H	Yeast two-hybrid

1. Introduction

The *Acquired Immune Deficiency Syndrome* (AIDS) is a disease caused by the *Human Immunodeficiency Virus* (HIV). The World Health Organization (WHO)'s 2011 published list of the top ten causes of death worldwide, still lists HIV/ AIDS as the 6th most frequent reason for fatality. After more than 20 years of research and great advances in the antiretroviral therapy, it remains one of the world's major public health issues, one that has claimed more than 36 million lives to date [1]. The WHO further estimates around 35.2 million people living with HIV worldwide, while just one quarter of those are having access to the vital antiretroviral medication for therapy [2]. Although this therapy mostly decreases the virus load in the blood to under a detectable limit, the persistence of HIV in some special human immune cells remains a determining factor preventing HIV from being completely eradicated. Despite a consistently increasing knowledge of this lentivirus, mechanisms of the cell entry and that persistence are not yet fully understood. HIV-1 remains particularly prevalent within macrophages and T-lymphocytes, due to their comparably long life span of up to ten years.

Furthermore, it has been well documented that the virus can be transferred efficiently from cell to cell via structures called Virological Synapses (VS), which appear to be special adhesion sites between infected and uninfected cells [3]. Chen et al. could show that HIV-1 transferred that way is, in addition, neutralization-resistant [4]. Therefore, the understanding of this particular spreading mode becomes even more vital. Continuing studies showed that this transport system and the formation of the synapse is mainly driven by Env-CD4 interactions [5], [4], while others documented the involvement of tetraspanins [3], [6]. Earlier analyses with a Yeast two-hybrid (Y2H) screen identified the tetraspanin CD63 as an interaction partner of the HIV-1 protein gp41 and showed that some highly conserved cysteines in its Large Extracellular Loop (LEL) are important for the binding between those two proteins [7]. To analyse the role of the LEL in this interaction, several expression plasmids encoding for recombinant variants of the Large Extracellular Loop were generated. One did express the CD63 without this loop and four contained in each case a different mutation of one or two of the six cysteines. The purpose of my study was to investigate the influence of the CD63-LEL on HIV-1 entry and cell-to-cell transfer.

1.1. The Human Immunodeficiency Virus (HIV)

HIV is a retrovirus from the subgroup of lentiviruses, with a size of approximately 100 nm. The difference between the genomes of the AIDS causing subtype HIV-1 and the subtype HIV-2 is 40%. An envelope surrounds the capsid that is characteristical for the retrovirus and consists of the p24 capsid protein. Inside of the capsid there are two viral, single stranded RNA copies that are complexed with the p7-protein, but not covalently linked to each other. The viral glycoproteins, such as gp120 and gp41, part of the functionally active complex that is involved in the virus budding, is anchored as transmembrane protein within the envelope. The virus further contains the enzymes reverse transcriptase, integrase and protease. The around 7000 nucleotide long retrovirus genome encodes for group-specific antigen (gag), polymerase (Pol) and enzymatic activity and for envelope(Env) and glycoprotein gene products. It further contains additional protein encoding gene regions for regulatory and accessory proteins [8].

HIV can be transferred from one human to another, either via unprotected sexual intercourse, via transfusions of infected blood or while injecting drugs with used needles. Summed up, it can be transmitted via specific infectious body fluids. Hence, it can further be transmitted from a mother to her baby during the birth process or while breast feeding.

As the HIV genome itself only encodes 15 proteins, it must exploit multiple functions and proteins of the host cell for a productive infection. More than 250 host factors are required by HIV-1 [9]. The virus entry into its host cell usually consists of three steps: (i) the envelope gp120 protein of the virion attaches to the $CD4^+$ host cell surface, (ii) the now conformationally altered gp120 interplays with CCR5 and CXR4 co-receptors and, finally, (iii) the envelope gp41-protein mediates the fusion with the target cell [10].

Nevertheless, there has been evidence that the infection of certain cell-types occurs independently of these factors [8]. Once the RNA enters into the cell plasma of the host cell, the reverse transcriptase starts to transform it into a double stranded DNA molecule via several steps. Due to this enzyme's lack of a proof reading activity, the statistical mutation rate occurs to be very high. The following transport of this molecule into the nucleus is driven by further proteins and enzymes. Once the viral DNA has been inserted into the nucleus, the integrase incorporates it into the DNA genome of the host cell. This makes HIV the only known virus that is enabled to

infect cells in the stationary phase and not just during mitosis. Once this DNA starts getting expressed and an mRNA is built, the viral proteins get transcribed and a new viral RNA is built as well. These viral components then bind together and bud to a new virion with all its glycoproteins at the host cell membrane. After a process of maturation, the particles can infect a new cell and the replication cycle can start over again [8].

As HIV integrates into the host DNA, it can remain in a transcriptionally inactive or latent form for extended periods [11]. During infection, HIV remains and concentrates in some reservoirs in its host. Organelles within macrophages were described in an earlier study as preferential sites for virus accumulation in macrophages [12]. Current research provided that not just macrophages, but also T cells and dendritic cells are main reservoirs of HIV, even under retroviral therapy [13]. Due to the macrophages resistance to the process of apoptosis, they are persistent cellular reservoirs of HIV. Recent data showed that the human lung serves as an anatomical reservoir, as a result of the close proximity of a large number of immune cells, which provide ideal conditions for a cell-to-cell spread of HIV [14].

1.2. The Virological Synapse

The VS has not yet been investigated very well. First studies suggest that it shares some characteristics with the better known Immunological Synapse (IS), which forms between antigen presenting cells and T lymphocytes [15]. It is also comparable to the neural synapse, as in case of the VS the effector cell does not fuse with the target cell. This seems illogical in the beginning due to the fact that effector cells are enriched with Env and Gag at their surface while the target cells are enriched with CD4 and CXR4 co-receptors. The tetraspanin CD63 and HIV-1 have been co-localised at this structure during live cell imaging [7]. Further, the tetraspanins CD9 and CD81 seem to prevent the fusion process through their presence at the virological postsynapse and presynapse [16].

1.3. Structure of tetraspanins

Tetraspanins are a 33-member super family of four-span transmembrane glyco-proteins with a very characteristic structure, from which their name is derived. These proteins, also known as TM-4's, pass the cell membrane four times while building two typical extracellular regions, the Small Extracellular Loop (SEL) and the Large

Extracellular Loop (LEL). They further contain short cytoplasmic N- and C-termini as well as a shorter inner loop structure (Fig.1) that separates the SEL from the LEL [16]. An interspecies key feature that defines the tetraspanins, is at least four and up to six cysteines within the LEL, where one cysteine couple is part of a highly conserved -CCG- motif. Due to the typically formed disulphide bonds a sub-loop is created, which represents another defining feature. This explains the LEL organization in two structural subdomains. The first one appears to have a primarily structurally conserved fold. The second one begins after the -CCG- motif, and is extremely variable in its size, secondary fold, and its structure.

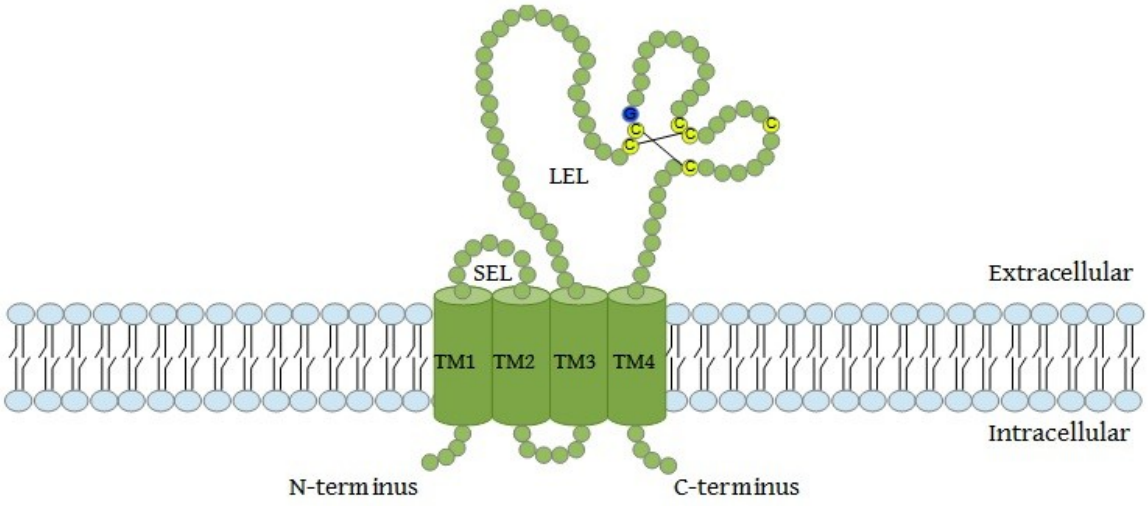

Fig.1: The tetraspanin model shows the typical protein structure. The four transmembrane regions (TM) are spanning the cell membrane, the Small Extracellular Loop (SEL), a small intracellular loop that separates the SEL from the LEL, and the Large Extracellular Loop (LEL) with six cysteines, as found in CD63, with bonding disulphide bridges . (own illustration)

However, the disulphide bridges remain the key organising factor [17]. Simultaneously this protein structure is hyper-variable within the tetraspanin family and homologous between different species [18]. Some tetraspanins contain an additional cysteine couple, such as the tetraspanin CD63. Therefore, they have altogether six cysteines within the LEL. Based on the amino acid sequence of the LEL, Seigneuret et al. subdivided the tetraspanin family into four subgroups. Over the past few years studies revealed the presence of tetraspanin-enriched microdomains (TEMs) within the lipid bilayer. These are specialised regions on the membrane surface with a higher density of tetraspanins. For example Nydegger et al. discovered CD9, CD63, CD81 and CD82 containing TEMs on the surface of fixed HeLa cells [19].

This phenomenon has also been shown in live cells, where these proteins have been found to locally concentrate and work as “interaction platforms” [20].

1.4. Functions of tetraspanins

Phylogenetic analysis, as done by Huang et al. in 2005, revealed that tetraspanins seem to play an important role in the transition from unicellular to multicellular organisms. It has been shown that these proteins have undergone an intense evolution and the different cysteine patterns of the LEL have been introduced as an important classification factor. Tetraspanins are not only expressed in metazoans but also in protozoan, amoebae, fungi and plants. *Drosophila melanogaster,* for example, has 36 tetraspanins, *Caenorhabditis elegans* on the other hand only has 20 [21]. However, Meacker et al. further documented that tetraspanins are distributed in almost all cell types and that each cell type expresses various and different kinds as well as different amounts of tetraspanins. Interestingly, some tetraspanins, such as CD9, CD63, CD81 and CD82, can be found in almost every tissue, whereas some others appear only in special tissues, like CD37 in B-cells [22]. Generally, it can be said that these molecules play a role in cell-cell and cell-matrix interactions, which include processes such as cell adhesion, cell migration, signal transduction, activation, proliferation and differentiation [23].

Each of these group members can bind with special protein partners such as integrins, co-receptor molecules, major histocompatibility complex antigens and cytoplasmic kinases [24] via the LEL as the main protein binding domain [21]. Based on the named subgroup system it has been found that members of the same subgroup and therefore with similar structural LEL characteristics can partially assume each other’s functions [16]. Therefore, just a few knock-out experiments lead to dramatic phenotypes, for example a CD9 knock-out in mice resulted in a loss of fertility caused by an inability of the oocytes to fuse with the sperm, or in the case of peripherin (CD22) knock-out mice, which resulted in a retinal degeneration [25]. Tetraspanins furthermore play regulative roles in cancer development. CD9 monoclonal antibodies, for example, can inhibit the transendothelial migration of melanoma cells [26]. They also have been found to both promote and suppress development of metastasis [27].

Last but not least, tetraspanins have been shown to be important interaction partners of different viruses. CD81 for instance is an essential binding partner for the HCV

(Hepatitis C virus) envelope protein E2 [28]. Another research [29] revealed that the CD82 interaction with the membrane glycoprotein of the Human T-cell Leukaemia Virus type 1 (HTLV-1) inhibits cell-to-cell fusion and virus transmission. And finally, tetraspanins influence the assembly, budding, entry and cell-to-cell transfer of the Human Immunodeficiency Virus type 1 (HIV-1).

1.5. CD63 and HIV-1

Some of the earlier studies that investigated the incorporation of proteins from host cells into HIV-1 particles revealed CD63 to be one of them [30, 31]. Soon after that it was established that HIV-1 exits at tetraspanin enriched segments of the cell membrane. Another early research found that the non-random process of CD63 incorporation into virions is specifically found when they are released from T lymphocytes [32]. Those previous findings were strengthened about ten years later, when several studies showed that HIV-1 exits on TEMs that are enriched with CD9, CD63, CD81 and CD82 in epithelial cells, T lymphocytes, macrophages, and dendritic cells [33-37].

It remained unclear, though, whether tetraspanins play a functional role in the budding process itself. Krementsov et al. found that although the treatment of virus producing HeLa cells with an anti-CD9 antibody reduced the virus release [38], the knockdown of CD9, CD63 and CD81 had no effect on the rate of HIV-1 release from these cells [3]. Their studies further revealed that tetraspanins have an impact on the infectivity of released virus particles and the cell-to-cell transmission. Interestingly, the overexpression of CD63 in co-transfected HeLa cells decreased the infectivity of cell-free virus dramatically and inhibited, just like the CD9, but not the CD81 overexpression, cell-to-cell transmission of HIV-1. The tetraspanin knockdown, contrary to expectations, had no effect on the cell-free infectivity and only the CD81 knockdown enhanced cell-to-cell transmission of HIV-1.

Further they provided evidence that HIV-1 can modulate the tetraspanin level in virus producing cells to maximize its efficiency. There was a downregulation of all tetraspanins in newly infected Jurkat T lymphocytes, whereas there was no such downregulation to be found in infected HeLa cells. Finally, despite this down-regulating effect, a tetraspanin concentration was still detectable at the Virological Synapse [3]. The scientists involved in this study therefore concluded: “Altogether,

currently available evidence suggests that tetraspanins do not generally act as budding co-factors for HIV-1” [16].

Therefore on the one hand, findings demonstrated that the treatment of macrophages and T lymphocytes with an anti-CD63 antibody inhibited the infection of these cells up to a level of 50%, but only if they were infected with a R5 tropic HIV-1 strain. No such effect was shown for cells infected with X4 tropic strains of HIV-1 [39]. On the other hand, it could be shown that recombinant CD63 LELs also have a specifically inhibitory effect on macrophage infection, but contrary to the antibody test, also on the X4-tropic virus [40]. The viral attachment and cell-cell fusion were not affected by CD63 silencing, but the CD63-depleted macrophages repeatedly showed an effect, this time a significant decrease in the initiation and completion of the reverse transcription of the virus [41]. This study also revealed that a down regulation of CD63 inhibits the p24 production, a late protein of the HIV replication cycle. Li et al.'s findings suggest an early post entry role for CD63, either prior to or at the step of reverse transcription.

1.6. The aim of this study

The aim of this study is to determine whether different mutations of the CD63-LEL have an effect on several steps of the CD63 development and HIV infection. First of all, it was of interest, whether a depletion of the complete LEL, or one of the point mutations of the LEL's cysteines would prevent the protein from being integrated into the cell membrane at all. As far as it is known, no research has unveiled this process to date. This research therefore started with the generation of recombinant expression plasmids. The used cDNA originates from a Y2H library. The pCMV expression vector was used to overexpress the recombinant proteins in eukaryotic cells. The second part of this research deals with the influence of these recombinant LELs on HIV-1 entry and the cell-to-cell transmission. The main focus was the LEL because of its importance as a protein binding region. Four different HIV-1 strains were used for the infection experiments. Three of them, as well as all constructs, were tagged with fluorescent proteins, which made it possible to easily localise them with the confocal laser scanning microscope.

2. Material and Methods

2.1. Material

2.1.1. PCR- Components

Table 1: All used PCR components and their manufacturers

PCR- Components	Manufacturer
10x *PfuUltra*™ II Reaction Buffer	Agilent Technologies
10x FastDigest Buffer	FD ThermoScientific
5 x T4 DNA Ligase Buffer	NewEngland BioLabs®
Abi BigDye 3.1.	Terminator Chemie
Deoxycytidine nucleoside triphosphates (dNTPs)	Agilent Technologies
NEBuffer 2,NEBuffer 3, NEBuffer 4	NewEngland BioLabs®
PfuUltra™ II Fusion HS DNA Polymerase	Agilent Technologies
Restriction enzyme ApaI	NewEngland BioLabs® + FD ThermoScientific
Restriction enzyme BamHI	NewEngland BioLabs® + FD ThermoScientific
Restriction enzyme EcoRI	NewEngland BioLabs® + FD ThermoScientific
Restriction enzyme EcoRV	NewEngland BioLabs® + FD ThermoScientific
Restriction enzyme HindIII	NewEngland BioLabs® + FD ThermoScientific
Restriction enzyme MfeI	FD ThermoScientific
Restriction enzyme PstI	NewEngland BioLabs® + FD ThermoScientific
Restriction enzyme XhoI	NewEngland BioLabs® + FD ThermoScientific
T4 DNA Ligase	NewEngland BioLabs®

2.1.2. Primer

Table 2: All primers used with their abbreviation and sequence, all primers were ordered from Sigma Aldrich

Primer	Sequence in 5'- 3' direction
C145A,C146A for	GCAGGCAGATTTTAAGGCTGCTGGGGCTGCTAACTACACAGATTG
C145A,C146A rev	CAATCTGTGTAGTTAGCAGCCCCAGCAGCCTTAAAATCTGCCTGC
C169A,C170Afor	GAACCGAGTCCCCGACTCCGCTGCTTGATGTTACTGTGGG
C169A,C170A rev	CCCACAGTAACATCAATAGCAGCGTCGGGGACTCGGTTC
C177A for	GCATTGATGTTACTGTGGGCGCTGGGATTAATTTCAACG
C177A rev	CGTTGAAATTAATCCCAGCGCCCACAGTAACATCAATGC
C191A for	GGCGATCCATAAGGAGGGCGCTGTGGAGAAGATTGGGGG
C191A rev	CCCCCAATCTTCTCCACAGCGCCCTCCTTATGGATCGCC
4A3/TM1-3 for	TTTTTTGGATCCATGGCGGTGGAAGGAGGAATG
4A3/TM1-3 rev	TTTTTCTGCAGCTTATCTCTAAACACA
4A3/TM4 for	TTTTTGATATCGGAGGAGGAGGAAA AAATGTGCTGG
4A3/TM4 rev	TTTTTTAAGCTTCATCACCTC GTAGCC ACTTCT
4A3/gp41 for	TTTTTGAATTCCTGACGGTACAGGCCAGAC
4A3/gp41 rev	TTTTTGATATCCTGCCTAACTCTATTCACT
4A3/YFP for	TTTTTGAATTCATGGTGAGCAAGGGCGAGGA
4A3/YFP rev	TTTTTGATATCCTTTCTGAGTCCGGACTTGT

2.1.3. Buffer and Media

Table 3: All used Buffer stocks and media with their ingredients

Buffer/ Media	Ingredients
1 x HBS- Buffer	5 mM KCl; 137 M NaCl; 0,7 mM Na_2HPO_4; 7,5 mM D-glucose; 21 mM HEPES
2YT- Medium	1,6% Bacto tryptone; 1% Bacto yeast extract; 0,5% NaCl
50x TAE - Buffer	2 M Tris, 1 M glacial acetic acid; 100 ml 0,5 M EDTA
DMEM-complete	500 ml DMEM, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg /ml streptomycin, 10% BSA
DMEM-serum free	500 ml DMEM, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg /ml streptomycin
LB- Medium	1% Bacto tryptone; 0,5% Bacto yeast extract; 1% NaCl
PBS- Buffer	2,7 mM KCl; 137 M NaCl; 1,5 mM KH_2PO_4; 8,1 mM Na_2HPO_4
RPMI- complete	500 ml RPMI, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg /ml streptomycin, 10% BSA
RPMI-serum free	500 ml RPMI, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg /ml streptomycin
Running buffer (SDS-PAGE)	25 mM Tris, 192 mM glycine, 0,1% (w/v) SDS (pH 8,3)
Laemli buffer 2x	50 mM Tris-HCl pH 6,8; 2% SDS; 10% glycerol; 1% β-mercaptoethanole; 12,5 mM EDTA; 0,02% bromophenol blue
SOC- Medium	2% Bacto tryptone; 0,5% Bacto yeast extract; 0,05% NaCl; 2,5 mM Kcl; 10 mM $MgCl_2$; 20 mM Glucose
TB- Medium	1,2% Bacto tryptone; 2,4% Bacto yeast extract; 0,5% Glycerol; 1M $KHPO_4$
TFB I	dH_2O depending on volume; 5 % (v/v) Glycerine; 100 mM RbCl ; 30 mM Kaliumacetate; 10 mM $CaCl_2$; pH 5,8; 50 mM $MnCl_2$
TFB II	dH_2O depending on volume; 15 % Glycerine; 10 mM RbCl; 10 mM MOPS; 75 mM $CaCl_2$; pH 6,8;

2.1.4. Other chemicals

Table 4: All other chemicals that were used including their respective manufacturers

Name	Manufacturer
6x Orange DNA Loading Dye	Thermo Fisher Scientific Inc.
Agarose	Carl Roth GmbH + Co. KG
Ampicillin sodium salt	Carl Roth GmbH + Co. KG
Benzonase® Nuclease	Sigma-Aldrich® Chemie GmbH
Bovine Serum Albumine (BSA)	NewEngland BioLabs® GmbH
CPRG	Sigma-Aldrich® Chemie GmbH
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich® Chemie GmbH
Ethidium bromide	Sigma-Aldrich® Chemie GmbH
Kanamycin sodium salt	Carl Roth GmbH + Co. KG
Metafectene®PRO	Biontex Laboratories GmbH
O´GeneRuler™ DNA Ladder Mix	Thermo Fisher Scientific Inc.
Penicillin/Streptomycin	Biochrom AG Berlin
Pierce ECL Western blotting substrate	Thermo Fisher Scientific Inc.
SDS	Sigma-Aldrich® Chemie GmbH
Triton X-100	Carl Roth GmbH + Co. KG -
Trypsine-EDTA	Biochrom AG Berlin
β-Mercaptoethanol	Sigma-Aldrich® Chemie GmbH

2.1.5. Biological material

Table 5: All biological components used; all strains were already existing at the laboratory

Abbreviation	Description/ manufacturer
ER 9295	Transgene *E.coli* for transfection that is dam^-/dcm^- (Nous Bio)
g-anti-FLAG	goat anti-FLAG antibody (first antibody) - (Dako)
g-anti-HRP	goat-anti-HRP konjugate (second antibody) - (Dako)
HEK293T	Human Embryonic Kidney 293T cells (National Institute of Health)
HIV-iGFP	Vector carrying HIV proviral genome with an internal Green Fluorescent Protein (GFP); R5 tropic (Chen)
HIV-IRES R5	Vector carrying HIV proviral genome with an external GFP; R5 tropic (Kirchhoff, Ulm)
HIV-IRES X4	Vector carrying HIV proviral genome with an external GFP; X4 tropic (Kirchhoff, Ulm)
Jurkat cells	Human T-lymphocyte cells - acute T-cell leukaemia (S. Norley)
m-anti-mCherry	mouse-anti-mCherry antibody (first antibody) - (Abcum)
m-anti-HRP	Mouse-anti-HRP antibody (second antibody) - (Abcum)
pCMV/CD63	Expression vector of the Cytomegalie virus where the CD63 sequence is introduced at the multiple cloning site (Ivanusic)
pNL4-3	Vector carrying HIV proviral genome; X4 tropic (National Institute of Health)
r-anti-GFP	rabbit-anti-GFP antibody (Abcam, ab6556)
anti-human-immunogold	Anti-human antibody with 10 nm gold particles (Dako)
anti-rabbit-immunogold	Anti-rabbit antibody with 10 nm gold particles (Dako)
r-anti-HRP	rabbit-anti-HRP conjugate (Dako) (second antibody)
TZM-bl	Transgene version of Human cervical cancer cell line taken from Henrietta Lacks (HeLa cells) – having CD4 and chemokine receptors
XL-1 blue	Transgene *Escherichia coli (E.coli)* strain for transfection (Stratagene)
XL-10 gold	Transgene *E.coli* strain for transfection (Stratagene)

2.1.6. Kits

Table 6: All kits used and their respective manufacturers

Name of the kit	Manufacturer
EndoFree® Plasmid Maxi Kit	QIAGEN N.V.
Plasmid Midi Kit	QIAGEN N.V.
Spin DNA Extraction Kit Invisorb®	Stratec molecular GmbH
Spin Plasmid Mini *Two* Kits Invisorb®	Stratec molecular GmbH

2.1.7. Laboratory apparatus

Table 7: All laboratory apparatus used and their respective manufacturers

Apparatus name	Manufacturer
BioPhotometer 8,5 mm	Eppendorf AG Hamburg, Germany
Centrifuge 5804R	Eppendorf AG Hamburg, Germany
Centrifuge 5810 R	Eppendorf AG Hamburg, Germany
ChemoCam Imager 3.2	Intas Science Imaging Instruments GmbH, Germany
Classic Series C24 Incubator shaker	Eppendorf New Brunswick Scientific AG Hamburg, Germany
Confocal laser scanning microscope Zeiss LSM 780	Carl Zeiss AG Germany
Ecotron shaker	INFORS HAT Germany
Megafuge 1.0R	Hereaus Holding GmbH, Germany
Mini Spin Centrifuge	Eppendorf AG Hamburg, Germany
Spektrophotometer ND-1000	NanoDrop® ThermoScientific Germany
Thermocycler vapo.protect Mastercycler pros	Eppendorf AG Hamburg, Germany

2.2. Methods

2.2.1. Preparation of antibiotic containing agar plates

For the preparation of the plates, 7 g of agar were dissolved in 500 ml LB-Medium. The mixture was warmed until it started boiling and the agar was melted completely. After cooling the solution down to a temperature of 60°C, either 100 µg/ml of Ampicillin or 40 µg/ml of Kanamycin, was added and everything was then mixed carefully and thoroughly. Under sterile conditions, the LB-agar was poured into the petri dishes. Until usage, the dishes were stored at 4°C.

2.2.2. Preparation of 1% agarose gels

The gel electrophoresis required agarose gels, which were mixed from 50 ml 1x TAE-buffer and 0,5 g of agarose. The mixture was heated in the microwave until it started boiling and the solution became clear. After allowing the solution to cool down to 40°C 5 µl of Ethidium bromide (10 mg/ml) were added. Everything was mixed thoroughly and then poured into the casting tray with a comb for the wells. The hardened gel was then placed into a gel electrophoresis apparatus that was filled with 1x TAE-buffer.

2.2.3. Preparation and transformation of competent bacteria cells

2.2.3.1. Preparation of chemically competent cells

Cells were grown in each 50 ml of Terrific-Broth Medium (TB-Medium), which had been inoculated with 100 µl of XL-1 blue or XL-10 gold *E.coli* strains, which was stored at -80°C. The mixture was incubated at 37°C overnight. The next day 200 ml Lysogeny-Broth (LB) Medium was inoculated with 1 ml of these overnight bacteria cultures. The bacteria were growing up to an OD_{600} of 0,4 – 0,7 at 37°C and 200 rounds per minute (rpm) in the incubator shaker. Afterwards the cells were centrifuged (4°C; 3500 rpm; 5 minutes) and resuspended in 60 ml TFB I, which was cooled down on ice. The suspension was left on ice for 90 minutes and centrifuged again (4°C; 3500 rpm; 5 minutes) after which, the pellet was resuspended in 8 ml TFB II solution. 100 µl aliquots were prepared from the resulting bacteria mixture and flash frozen in liquid nitrogen before they were stored at -80°C until usage.

2.2.3.2. Transformation of chemically competent cells

The chemically competent *E. coli* aliquots were slowly defrosted on ice and then mixed with 1 to 5 ng of plasmid-DNA. The tubes were continuously stored on ice and incubated for 30 minutes, before the bacteria plasmid mixture was heat shocked for 90 seconds at 42°C and once again incubated on ice for 5 minutes. After adding 150 µl of Terrific Broth (TB) Medium the suspension was stored at 37°C for another 30 minutes and then plated on Kanamycin containing agar plates. These petri dishes were left in the incubator (37°C) overnight until single colonies became visible.

2.2.4. Plasmid amplification

After successful transformation, the single colonies were transferred under sterile conditions into a 100 ml Erlenmeyer flask filled with 20 ml Kanamycin containing TB-Medium (40 µg/ml). The mixture was incubated in a shaker for at least six hours at 37°C and 200 rpm. There were at least two mutants picked and amplified from each successful transformation cycle. Once a good number of bacteria had grown, they were miniprepped to harvest the plasmid-DNA. The DNA concentration was measured using a Nanodrop spectro-photometer at a wave-length of 260 nm.

2.2.5. Site-directed mutagenesis

A two primer mutagenesis-PCR was used to introduce the mutations. Using an already existing pCMV/CD63 vector, altogether four cystein mutations were generated, two double cystein mutations (C145A,C146A; C169A,C170A) and two single cystein mutations (C177A; C191A). Primers were used as shown in table 2 together with the *PfuUltra*™ II Fusion HS DNA Polymerase in a ratio of 1:25, *PfuUltra*™ II Fusion HS DNA Reaction Buffer and 40 mM dNTPs. The total reaction volume was 20 µl for each mutation. The initial solutions were then running the following temperature programme in a thermocycler:

-94°C 2 min
-94°C 45 s
-52°C 45 s
-72°C 3 min } 25 cycles
-72°C 5 min
-4°C ∞

From each batch 10 µl were used for the transformation of the chemically competent cells. Each mutagenesis was checked by Sanger sequencing. When the mutation was confirmed, a new overnight culture was grown in 200 ml of Kanamycin containing TB-Medium (INFORS HAT Ecotron shaker, 37°C, 200 rpm) by inoculating it with 10 µl of a glycerine culture (stored at -20°C). The following day the plasmid-DNA was extracted by using the Qiagen Plasmid Midi Kit, which was used according to the manufacturer's specifications. Then once again, the mutations were checked by Sanger Sequencing (Abi BigDye).

2.2.6. Cloning

To prepare the CD63-ΔLEL (CD63 without the LEL) cloning the modified primers 4A3/TM1-3 for, 4A3/TM1-3 rev, 4A3/TM4 for, 4A3/TM4 rev (table 2) and the pCMV/CD63 vector as the template were used to amplify the fragments that were introduced later. A mixture consisting of the vector, primer, dNTPs, *PfuUltra*™ II Fusion HS DNA Polymerase and *PfuUltra*™ II Fusion HS DNA Reaction buffer ran through the following temperature programme in the thermocycler:

-95°C 5 min
-94°C 30 s
-55°C 30 s
-72°C 1 min } 35 cycles
-72°C 5 min
-4°C ∞

For checking purposes of the vector and the amplified fragments an agarose gel electrophoresis was used. To that end a 1% agarose gel was loaded with O´GeneRuler™ DNA Ladder Mix and the samples were mixed with 6x Orange DNA Loading Dye. The gel was then placed into a gel electrophoresis apparatus that was filled with 1x TAE-buffer, and was then run for 30 minutes at a voltage of 90 V. The bands were visualized with UV light in the ChemoCam and then cut out with a scalpel. The fragment containing gel was cleaned up with the Spin DNA extraction kit that was used according to the manufacturer's specifications.

After a renewed measure of the DNA concentration through spectro-photometer, the vector and the fragments were digested with restriction enzymes. First the TM1-3

fragment and the empty pCMV vector were digested with BamHI and PstI restriction enzymes (NewEngland BioLabs®) for at least 5 hours. Then once again the DNA was run in an agarose gel electrophoresis, followed by another cleaning up and concentration measurement of the vector and the fragments.

The restricted and purified pCMV vectors were mixed with the TM1-3 fragments (1:5 ratio), 5x T4 DNA ligase buffer, and T4 DNA ligase. The solution was ligated for 30 minutes at 22°C, 30 minutes at 20°C and 60 minutes at 16°C. Following this, the ligated plasmids were transformed into the chemically competent cells as described and then amplified. The extracted DNA ran through another cycle of restriction digest with the same restriction enzymes. For this control digest, the DNA was restricted with FastDigest restriction enzymes from ThermoScientific for 30 minutes. This control was resolved by agarose gel electrophoresis as before, observed for characteristic restriction fragment pattern under UV transillumination and then compared with the undigested control. If a 5000 bp fragment and a 335 bp fragment became visible, the cloning was successful.

The pCMV/TM1-3 plasmid, as well as the TM4 insert, was digested with the restriction enzymes EcoRV and HindIII (NewEngland BioLabs®) for at least 5 hours and then, as described, resolved by agarose gel electrophoresis. The DNA was once again extracted from the gel and ligated with a 1:5 plasmid-insert-ratio. Once the transformation was successful, the clones were amplified and digested again with EcoRV and HindIII (ThermoScientific) to control the ligation. If the following gel electrophoresis showed a 5000 bp and a 130 bp band, the cloning of the second insert was successful.

Subsequent to the cloning and the mutagenesis, all five constructs pCMV/CD63-C145A,C146A, pCMV/CD63-C169A,C170A, pCMV/CD63-C177A, pCMV/CD63-C191A and pCMV/CD63-ΔLEL were tagged with the sequence for one fluorescent protein each, either with mCherry or YFP (Yellow fluorescent protein). Therefore the five constructs, the already existing pCMV/CD63-YFP, and the pCMV/CD63-mCherry vectors were transformed into ER 9295. The process was the same like the one with the chemically competent cells, except for not plating the cells but directly culturing them in a 50 ml Kanamycin containing TB-medium as overnight culture. The following day the cultures were miniprepped to extract the plasmids and the DNA concentration was measured. All constructs were then digested with the restriction enzymes ApaI and XhoI (NewEngland BioLabs®) for 6 hours at 25°C and 6 hours at

37°C. The restricted DNA was then resolved by agarose gel electrophoresis. The recombinant plasmids and the YFP and mCherry inserts were cleaned up with a gel extraction kit. Overall ten ligations were prepared. Each construct was once ligated with YFP, once with mCherry and then transformed into chemically competent cells. Subsequent to the amplification, the plasmids were extracted from the bacteria and then once again restricted by a control digest with HindIII and MfeI (ThermoScientific; 60 minutes, 37°C). The cloning was successful if the following gel electrophoresis showed a 5000 bp and a 1090 bp band.

The recombinant pCMV/CD63-ΔLEL-mCherry was further used as template for a series of experiments, generating CD63 hybrid molecules. Instead of just 4 glycines, as in the case of the LEL deletion, either a part of the HIV gp41, the gp 120 V3 or the fluorescent protein YFP were additionally inserted. The primers 4A3/gp41 for, 4A3/gp41 rev, 4A3/V3gp120 for and 4A3/V3gp120 rev (table 2) were used together with the HIV-iGFP as template. The primers 4A3/YFP for and 4A3/YFP rev were used with pCMV/CD63-YFP as template. The amplification was done as described before and the fragments, as well as the vector pCMV/CD63-ΔLEL-mCherry were restricted with EcoRI and EcoRV at 37°C for 5 hours. The fragments and the vector were then ligated.

2.2.7. Transfection

2.2.7.1. Calcium phosphate transfection

HEK293T or TZM-bl cells growing in culture bottles were split in the stage of growth. For this purpose the cells were washed twice with PBS and then trypsinated through incubation with trypsin at 37°C and 5% CO_2 atmosphere. After 10 minutes the cell suspension was buffered with DMEM-complete and then centrifuged (4 minutes, 800 rpm). The supernatant was discarded, the cells resuspended and then diluted with DMEM-complete in a 1:10 ratio. Afterwards the cells were cultured in either 6 well plates, ibidi 8 well plates, 24 well plates or 96 well plates (TZM-bl). Each well of the 6 well plates was filled with 3 ml of the prepared cell suspension, the 24 well plates with 500 ml each and the 96 well plates with 170 µl each. The plates were then stored in the incubator (37°C; 5% CO_2) for at least 24 hours, and grown until they were of sufficient size. The transfection media with the plasmid-DNA was then mixed as shown in table 8. The medium was carefully removed and replaced with fresh

DMEM-serum free, before the transfection medium was added to the cells dropwise. In case of co-transfection the amount of DNA was doubled due to the input of two different plasmids.

Table 8: Calcium phosphate transfection, amounts of buffer, $CaCl_2$ and DNA for different surface areas

Format	Culture media	Transfection media	1x HBS	2,5 M $CaCl_2$	Total DNA
6 well	2 ml	1,4 ml	86 µl	5,1 µl	5,1 µg
8 well Ibidi	100 µl	50 µl	25 µl	3 µl	1 µg
24 well	500 µl	280 µl	18 µl	1,1 µl	1,1 µg
96 well	100 µl	70 µl	4,5 µl	0,3 µl	0,3 µg

2.2.7.2. Transiently transfection of HEK293T cells

Metafectene®PRO transfection was used for cell transfection in ibidi 8 wells. For this method the cells were prepared as described for the calcium phosphate transfection. For each transfection two Eppendorf tubes with 50 ml DMEM-serum free were prepared. One was mixed with 1 µg of plasmid DNA and the second one was mixed with 2 µl Metafectene®PRO. Afterwards both mixtures were put together, carefully pipetted up and down and incubated at room temperature for 20 minutes. The mixture was then added to the cells dropwise.

2.2.7.3. Nucleofection

The nucleofection was used to transfect plasmids into Jurkat T cells. When in the stage of growth, the cells were centrifuged (4 minutes, 800 rpm), resuspended in RPMI-complete and the cell number was counted. For each 6 well $5x10^6$ cells were pelleted and resuspended with 82 µl of the manufacturer's solution 1, 18 µl Amaxa™ transfection reagent and 3 µl plasmid DNA. The solution was mixed thoroughly, transfected in the nucleofector and fresh RPMI-serum-free was added immediately. After that the T cells were transferred into 6 wells and the transfection was checked after 24 hours in the incubator (37°C; 5% CO_2).

2.2.8. SDS-Polyacrylamide gel electrophoresis and Western blot

To check the expression of all recombinant proteins, the transfected cells were lysed 48 hours post transfection. Once completed, the medium was carefully removed and the cells of each 6 well were lysed in 100 µl of Laemli buffer, using a sterile pipette tip to mix it thoroughly. In the next step 250 U of Benzonase® Nuclease was added to reduce the viscosity of the cell lysate and remove nucleic acids. The lysated protein samples were loaded on a 10% TGX gel (BioRad) and separated by SDS-PAGE in an electrophoresis chamber, which was filled with running buffer (10 minutes 100V; 40 minutes 200V).

The separated Protein lysates on the SDS-PAGE were transferred onto a Immobilon-P PVDF membrane (Milipore). All sample proteins were tagged with 0.5 µg/ml of a primary antibody (m-anti-mCherry or g-anti-FLAG) and the primary antibodies were detected with 0.125 µg/ml HRP-conjugated secondary antibodies (m-anti-HRP, r-anti-HRP or g-anti-HRP). The proteins were then visualized with the Pierce ECL Western blotting substrate, according to the manufacturers instruction. Western blot images were acquired using the ChemoCam device with implemented contrast correction.

2.2.9. Infection assay

The first step of the infection assay was to control if the provirus containing expression plasmids could be transfected successfully. For this purpose, HEK293T cells were transfected with either HIV-iGFP in each case with the calcium phosphate method. The transfections was checked with the fluorescent microscope for any visible GFP. To make sure that the fluorescent protein was attached to the actual virus, a correlative microscopy study was performed. Single transfected and therefore green fluorescent cells, were first searched with the confocal laser microscope and then co-localised with a scanning electron microscope. This study was performed by Dr. Kazimierz Madela (Robert Koch-Institut Berlin, Germany).

Once it was confirmed that the transfected cells expressed the different HIV-strains, HEK293T cells were cultured in 6 wells as described and then transfected using the calcium phosphate transfection method. The transfection was controlled with the fluorescent microscope 24 hours later. After 48 hours the medium was carefully removed with a pipette and then stored at -80°C until usage. The cells were covered with 2 ml of new DMEM-serum-free and restored to the CO_2 incubator (37°C, 5%

CO_2). The virus containing supernatants were collected 2, 5 and 7 days post transfection.

All supernatants were tested for actual virus and the amount of produced HIV-1 using a β-galactosidase assay (CPRG), as the provirus contains a β-galactosidase reporter gene. TZM-bl cells were prepared as described in 96 wells. The cells were then infected with the HIV-1 containing supernatants 24 hours later. Per well 100 µl of PBS mixed with 0,5% Triton X-100 were added, after the medium was removed and then incubated for 20 minutes at room temperature. Subsequent to that, 100 µl of a CPRG solution (4 mg/ml in PBS) was added and then incubated for at 37°C until the colour changed from yellow to dark red.

2.2.10. Control of the protein expression

All recombinant plasmids were transfected in 8 wells as described and ,after 48 hours, fixed for 20 min in 2% paraformaldehyde (PFA) which was solved in PBS. The five mCherry tagged constructs pCMV/CD63-C145A,C146A-mCherry, pCMV/CD63-C169A,C170A-mCherry, pCMV/CD63-C177A-mCherry,pCMV/CD63-C191A-mCherry and pCMV/CD63-ΔLEL-mCherry were each co-transfected with pCMV/CD63-YFP as control.

The YFP tagged pCMV/CD63-C145A,C146A-YFP, pCMV/CD63-C169A,C170A-YFP, pCMV/CD63-C177A-YFP, pCMV/CD63-C191A-YFP and pCMV/CD63-ΔLEL-YFP constructs were each co-transfected with pCMV/CD63-mCherry. As controls pCMV/mCherry or pCMV/YFP were used. The hybrid molecules pCMV/CD63-ΔLEL-YFP-mCherry, pCMV/CD63-ΔLEL-gp41-mCherry and pCMV/CD63-ΔLEL-gp120V3-mCherry were co-transfected with pCMV/CD63-YFP. The samples were then investigated with the confocal laser scanning microscope.

To confirm the correct orientation of the CD63 hybrid proteins, a special study was performed together with Mrs. Gudrun Holland (Robert Koch-Institut Berlin, Germany). Using 2F5 antibodies, anti-human-immunogold, r-anti-GFP and anti-rabbit-immunogold (10 nm gold particles) the plasma membrane surface of transfected HEK293T cells was investigated with the scanning electron microscope.

3. Results

3.1. Site-directed mutagenesis

The mutations used for the experiment were generated in the LEL of the tetraspanin CD63 as illustrated in table 10. Altogether four recombinant CD63-LEL expression plasmids were produced, each carrying either a single cysteine mutation, C177A; C191A; or a double cysteine mutation, C145A,C146A and C169A,C170A. Each cysteine was replaced with an alanin to prevent a frame shift. The resulting constructs are pCMV/CD63-C145A,C146A, pCMV/CD63-C169A,C170A, pCMV/CD63-C177A and pCMV/CD63-C191A.

Table 9: Site-directed mutagenesis of the CD63-LEL cysteines

C177A: 5´…GTTACTGTGGGC**TGT**GGG…3´ ↓ 5´…GTTACTGTGGGC**GCT**GGG…3´ C → A	**C191A:** 5´…CATAAGGAGGGC**TGT**GTG…3´ ↓ 5´…CATAAGGAGGGC**GCT**GTG…3´ C → A
C145A,C146A: 5´…GATTTTAAG**TGCTGT**GGG…3´ ↓ 5´…GATTTTAAG**GCTGCT**GGG…3´ CC → AA	**C169A,C170A:** 5´…CCCGACTCC**TGCTGC**ATT…3´ ↓ 5´…CCCGACTCC**GCTGCT**ATT…3´ CC → AA

3.2. Cloning

By cloning the two CD63 domains TM1-3, the section from the first to the third transmembrane region and TM4, the fourth transmembrane region encoding sequence, a recombinant CD63 without the LEL was generated. The results are illustrated in figure 2. Each protein includes the sequence for one fluorescent protein, either YFP or mCherry, which was proven by the control restriction digest with HindIII and MfeI. The resulting constructs are the expression plasmids pCMV/CD63-ΔLEL-mCherry and pCMV/CD63-ΔLEL-YFP.

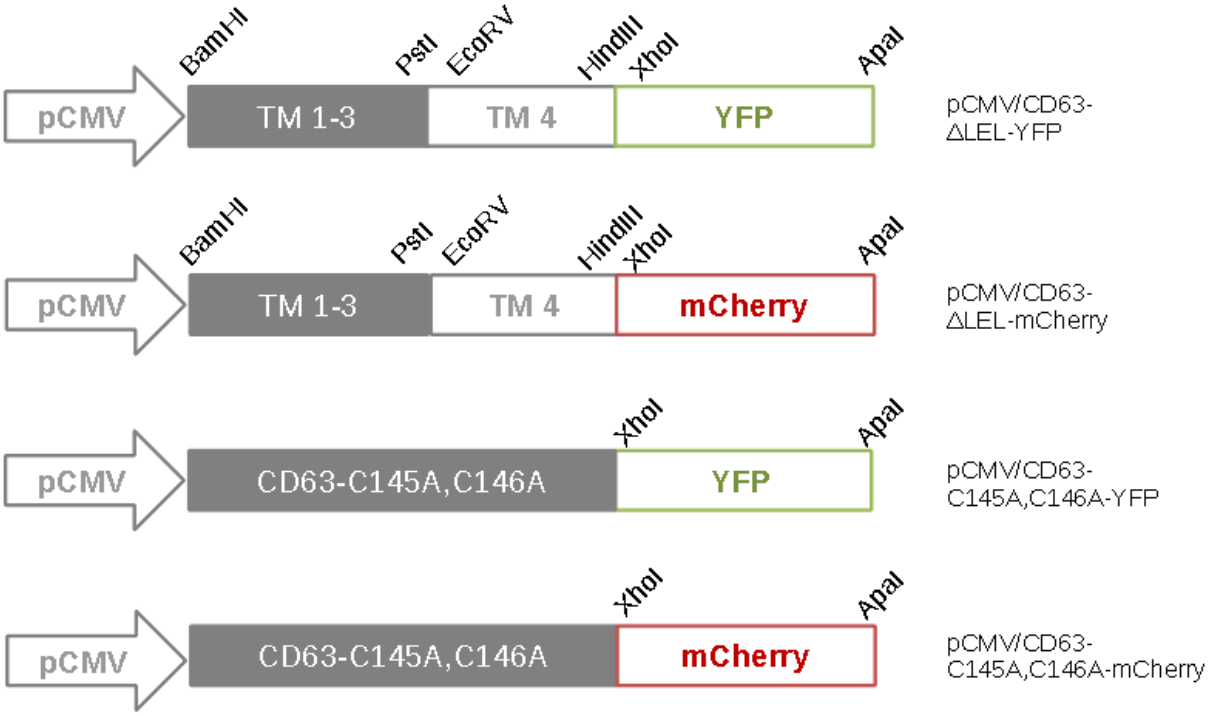

Fig.2: Schematic figure of the cloning and the used restriction enzymes; pCMV/CD63-ΔLEL-mCherry and pCMV/CD63-ΔLEL-YFP: into a pCMV vector the TM1-3, then the TM4 fragment and the mCherry/ YFP encoding sequences were introduced; pCMV/CD63-C145A,C146A-mCherry and pCMV/CD63-C145A,C146A-YFP: after point mutations of the cysteines, sequences encoding for YFP or mCherry were introduced, the same procedure was used for the other three point mutated molecules

All point mutated constructs were also tagged with the fluorescent proteins for further experiments (see fig. 2) Either a YFP or a mCherry encoding DNA sequence was introduced to each recombinant CD63 construct. Hence, altogether ten recombinant expression plasmids were produced. Each of them carries the sequence for a recombinant CD63-LEL and the sequence for a fluorescent protein that made it detectable with a fluorescent microscope. The resulting recombinant plasmids were provided a consistent nomenclature, which is illustrated in table 9.

Table 10: Nomenclature of the generated recombinant CD63-LEL constructs, starting with the name of the vector followed by the name of the protein, the introduced mutation and the fluorescent protein

mCherry tagged constructs	**YFP tagged constructs**
pCMV/CD63-C145A,C146A-mCherry	pCMV/CD63-C145A,C146A-YFP
pCMV/CD63-C169A,C170A-mCherry	pCMV/CD63-C169A,C170A-YFP
pCMV/CD63-C177A-mCherry	pCMV/CD63-C177A-YFP
pCMV/CD63-C191A-mCherry	pCMV/CD63-C191A-YFP
pCMV/CD63-ΔLEL-mCherry	pCMV/CD63-ΔLEL-YFP

3.3. Expression of CD63-LEL with introduced point mutations

The CD63 wild type is expressed predominantly on the surface of the plasma membrane of cells. Therefore the recombinant CD63 proteins were first checked for their expression on the plasma membrane. The pCMV/CD63-C177A-mCherry is expressed on the surface of the cell membrane of the HEK293T cells. This follows a similar pattern to the wild type CD63 (Fig. 3, pCMV/CD63-C177A-mCherry). The mutants pCMV/CD63-C191A-mCherry and pCMV/CD63-C169A,C170A-mCherry (Fig. 3) also showed this pattern of expression, however just in a part of the investigated cells. In the other part of the HEK293T cells, the CD63 appeared to be expressed differently from the wild type protein.

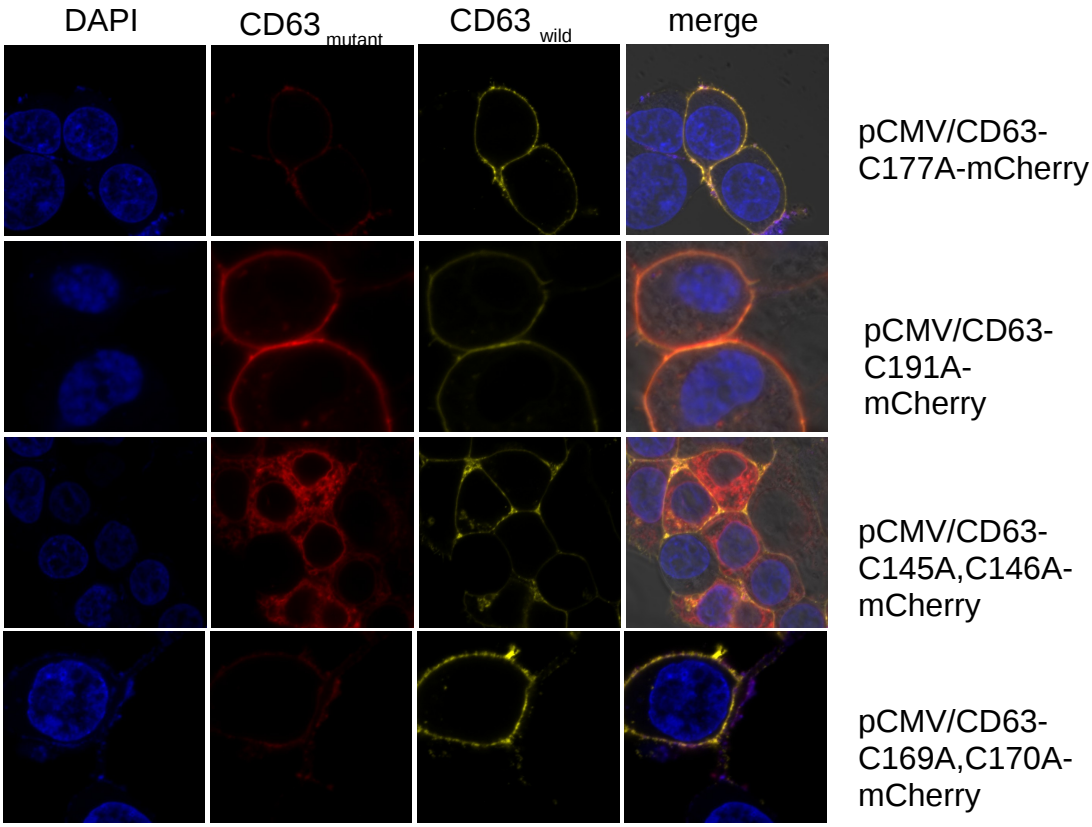

Fig. 3: Images were taken with the laser scanning microscope. HEK293T cells were co-transfected with Metafectene®PRO. Each row shows the co-transfected recombinant expression plasmid. Every mutant was co-transfected with pCMV/CD63-YFP as wild type. Cells were fixed with 2% PFA 48 hours post transfection and incubated with DAPI, to stain the cell nuclei, for 20 minutes.

This leaves pCMV/CD63-C177A as the only recombinant protein that was completely expressed like the wild type. The pCMV/CD63-C191A and the pCMV/CD63-C169A,C170A were not expressed consistently throughout the HEK293T cells. Figure 4 illustrates that these recombinant proteins were inconsistently expressed on the

cell membrane,with a number remaining inside of some cells, which appeared to be the main phenotype of the pCMV/CD63-C145A,C146A mutants.

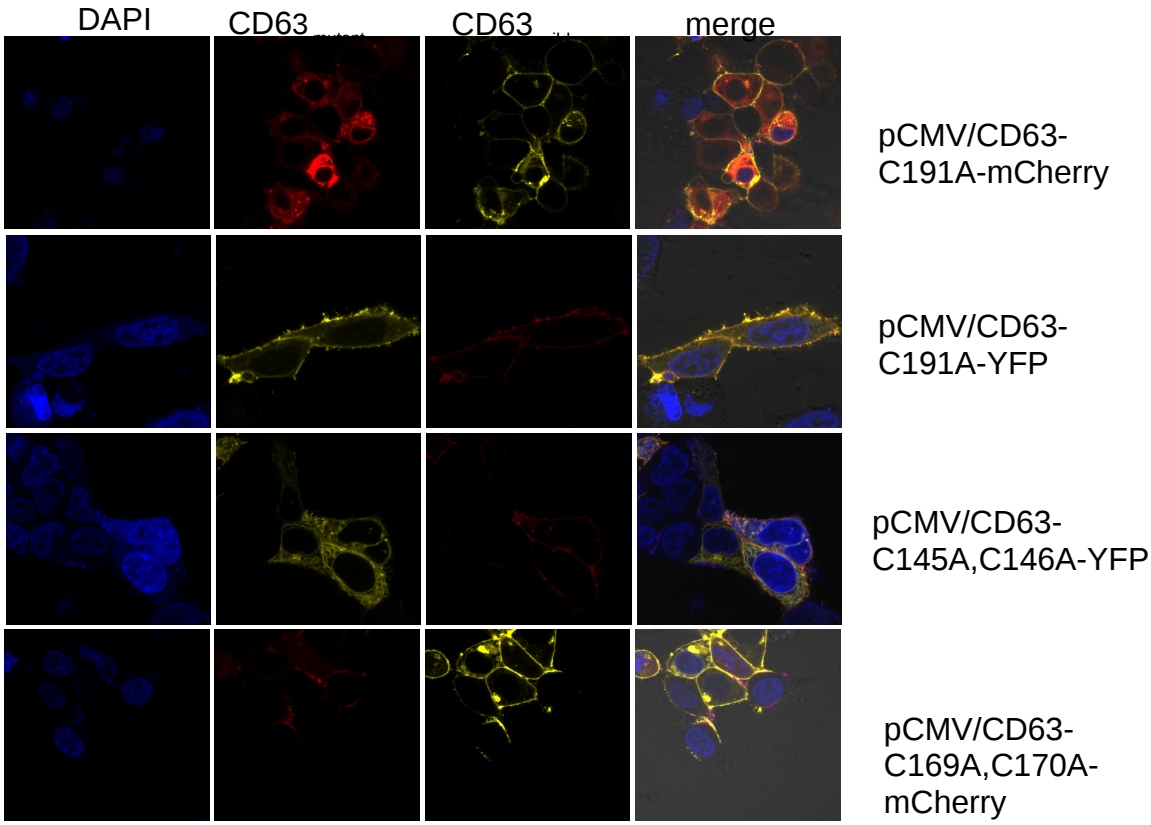

Fig. 4: Images were taken with the laser scanning microscope. HEK293T cells were co-transfected with Metafectene®PRO. Each row shows the co-transfected recombinant expression plasmid. When a YFP tagged construct was used, the mCherry tagged wildtype was co-transfected. When a mCherry tagged construct was used, the YFP tagged wildtype was co-transfected Cells were fixed with 2% PFA 48 hours post transfection and incubated with DAPI, to stain the cell nuclei, for 20 minutes.

The protein expression was further investigated by using a western blot analysis. This analysis showed that the recombinant proteins were found to be expressed (see Fig. 5). The western blot shows a general expression of the recombinant CD63 proteins. In Fig. 5, the pCMV/CD63-C191A transfection was not successful, therefore the expression of the protein can not be shown.

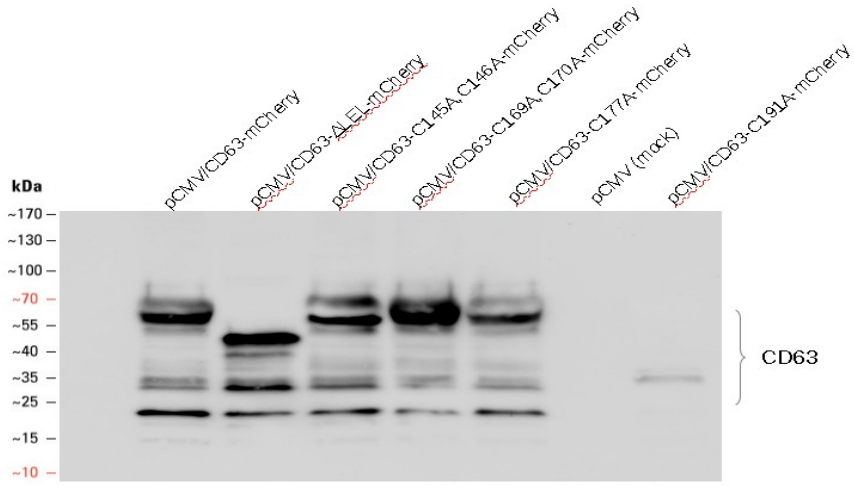

Fig. 5: Western blot analysis of the recombinant CD63 constructs with m-anti-mCherry and m-anti-HRP antibodies. The native pCMV vector was used as negative control, and pCMV/CD63-mCherry wild type as positive control.

3.4. Expression of CD63-ΔLEL and its hybrid molecules

As the CD63-LEL had been expected to play an important role during the process of integrating the tetraspanin into the membrane, it seemed surprising when the CD63-ΔLEL was expressed on the surface of the plasma membrane (Fig. 6). There were no differences seen between the mCherry tagged wild type CD63 and the ΔLEL variant of this protein. The western blot analysis confirmed the finding with showing that the CD63-ΔLEL was expressed (Fig. 7, pCMV/CD63-YFP + pCMV/CD63-ΔLEL-mCherry). Furthermore, this tetraspanin was expressed very efficiently, despite the fact that its largest extracellular region was deleted. These results inspired the idea of my supervisor D. Ivanusic to use the CD63-ΔLEL as a transport machine to express certain proteins on the plasma membrane surface. The cloning of some foreign proteins was done as illustrated in Fig. 8. Analysing the CD63- ΔLEL hybrid co-transfected HEK293T cells with the laser scanning microscope showed that even the recombinant hybrid molecules were expressed efficiently on the plasma membrane, just as the wild type control (Fig. 6, pCMV/CD63-YFP + pCMV/CD63-ΔLEL-gp41-mCherry).

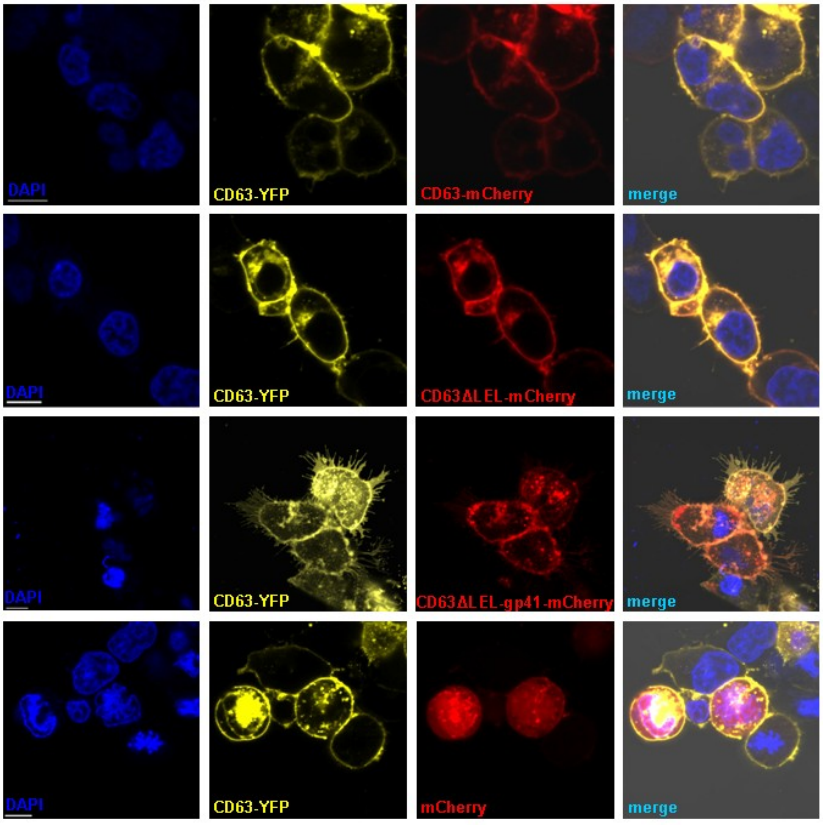

Fig. 6: Images were taken with the laser scanning microscope. HEK293T cells were co-transfected with Metafectene®PRO. Each row shows the co-transfected as described on the right side. Controls in the first row, both wild types were co-transfected; fourth row: the CD63-YFP wild type was co-transfected with a pCMV/mCherry expression vector. Cells were fixed with 2% PFA 48 hours post transfection and incubated with DAPI, to stain the cell nuclei, for 20 minutes.

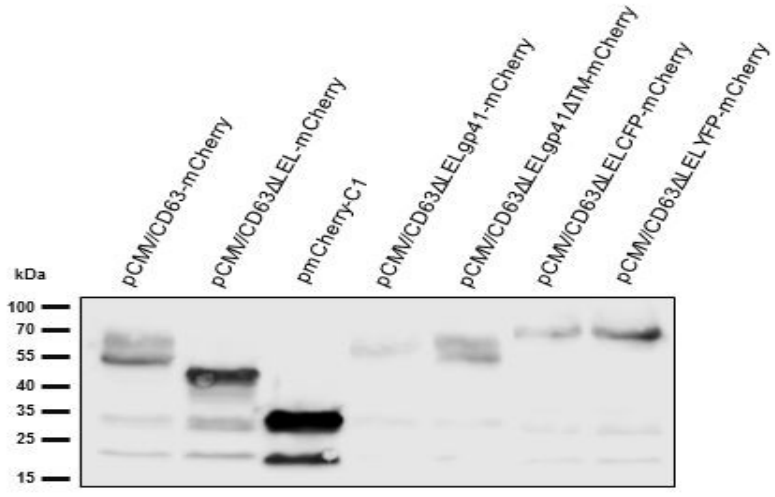

Fig. 7: Western blot analysis of the recombinant CD63-ΔLEL and the CD63-ΔLEL hybrid constructs with m-anti-mCherry and m-anti-HRP antibodies.

The insertion of the foreign proteins was confirmed via gel electrophoresis with the control digestion as described within the methods. To make sure that the protein was being expressed on the cell membrane, co-transfected HEK293T cells were investigated with the laser scanning microscope. But even then it remained unclear, if the protein folding was correct. To clarify the results, the fluorescent proteins YFP and CFP were introduced as illustrated in Fig. 8 and once again analysed with the laser scanning microscope (data not shown). These fluorescent proteins were used, because of their sensitivity to mis-foldings. Once the spatial confirmation of these proteins has changed, even on the minutest scale, they lose their fluorescent characteristics. However, as fluorescent proteins were detected, the right folding of introduced foreign proteins could be confirmed.

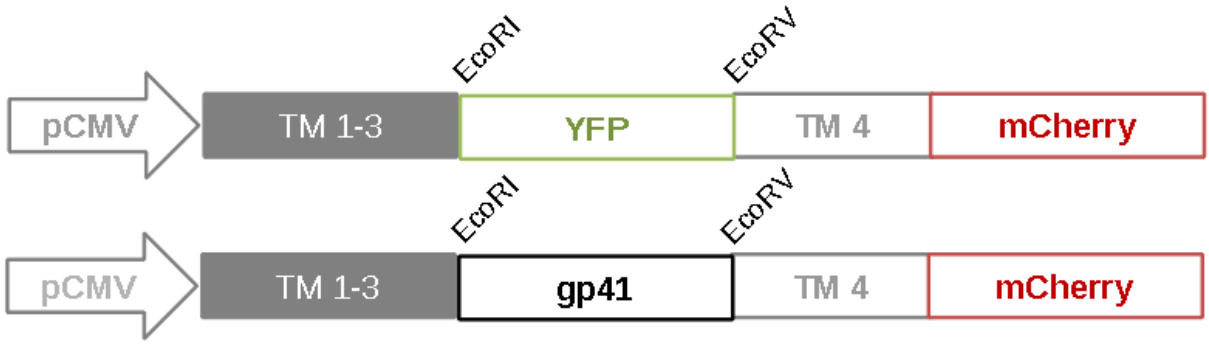

Fig. 8: Schematic figure of the cloning and the restriction enzymes used for the pCMV/CD63-ΔLEL-mCherry hybrids ; pCMV/CD63-ΔLEL-mCherry was used as vector. The fragments were introduced between the TM3 and the TM4, right where the LEL is supposed to be.

As it was still a possibility that the orientation of the protein within the membrane was not correct, therefore an orientation study as mentioned in the methods was performed together with Mrs. Gudrun Holland (Robert Koch-Institut Berlin, Germany). The foreign proteins that were introduced between TM3 and TM4 were tagged with a first antibody that was detected with a gold particles carrying second antibody. Afterwards the cell surface was investigated for these gold particles with the SEM. As the gold was detected on the cell membrane surface, the right orientation of the hybrid molecules could be confirmed. The results (Fig. 9) show that the protein is integrated into the plasma membrane in the correct orientation and therefore confirms that introducing foreign proteins into the CD63-ΔLEL does neither affect the protein folding nor the protein orientation.

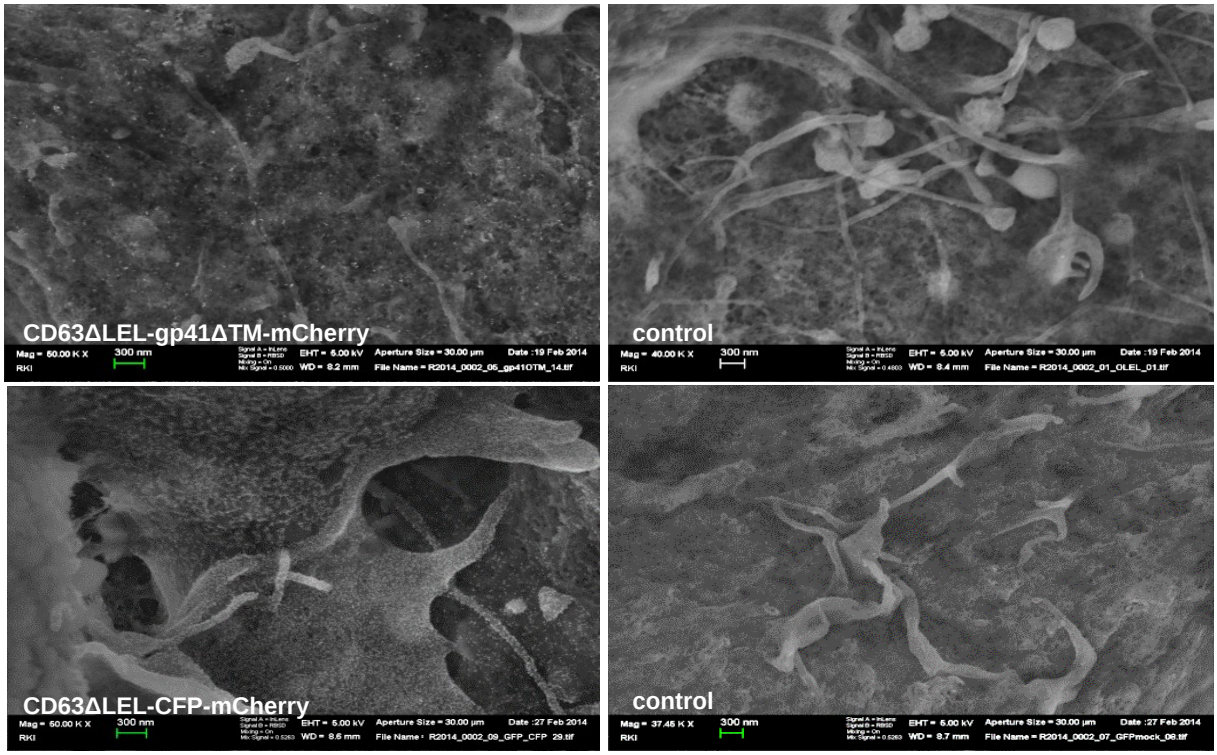

Fig. 9: Images were taken with the electron scanning microscope by Mrs. Gudrun Holland. As antiboies were used h-2F5 and anti-human-immunogold, r-anti-GFP and anti-rabbit-immunogold. The little dots on the surface of the HEK293T cells are 10 nm god particles that tag the CD63-ΔLEL hybrid molecules.

3.5. Infection assay

To confirm that the provirus containing expression plasmids were not just expressing the GFP but the actual virus, a comparative scanning electron microscope – confocal laser scanning microscope (SEM-cLSM) study was performed together with Dr. Kazimierz Madela (Robert Koch-Institut Berlin, Germany). For this purpose, HEK293T cells were transfected with either HIV-iGFP and then investigated with the cLSM (except for the pNL4-3) for any visible GFP. Single transfected, therefore green fluorescent cells, were first searched for with the confocal laser microscope and then correlatively localised with SEM (Fig. 10). The HIV1-iGFP provirus carrying expression plasmids were found to express successfully HIV-1, visualized in Figure 10 and 11, where the virus is visible during the budding on the cell surface of the transfected HEK293T cells.

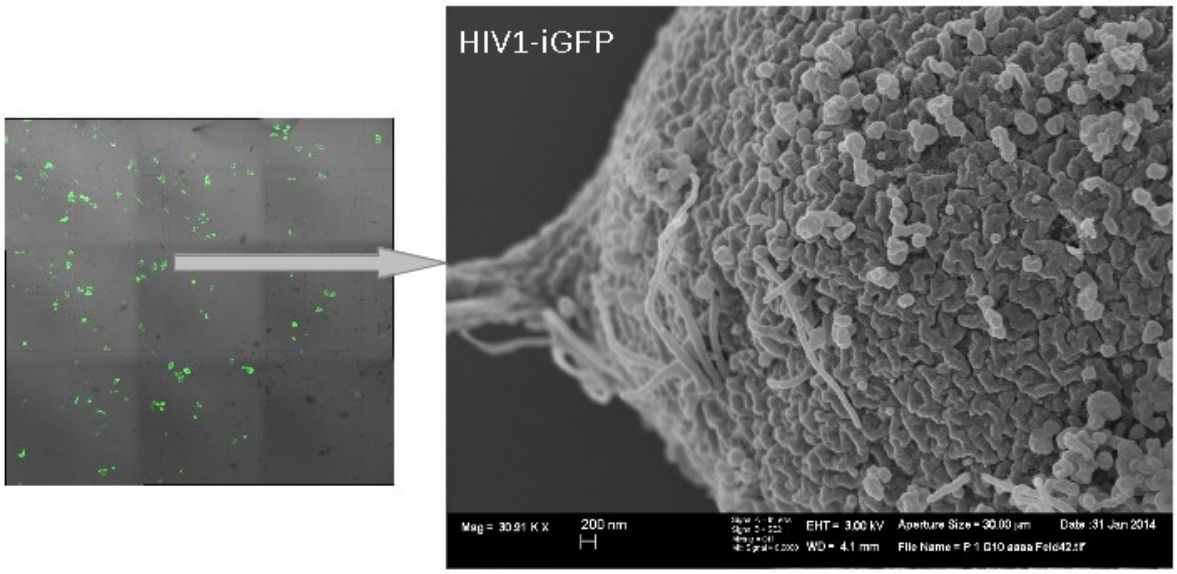

Fig. 10: Comparative SEM-cLSM study: images were taken by Dr. Kazimierz Madela (Robert Koch-Institut Berlin, Germany). After localising the HIV1-expressing cells via GFP expression with the cLSM, the exact same cells were then investigated with the SEM.

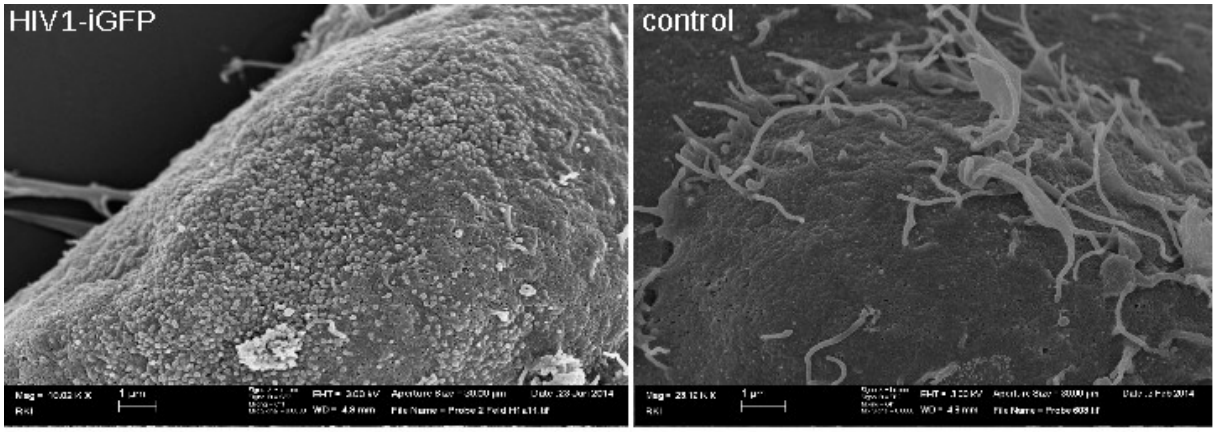

Fig. 11: Comparative SEM-cLSM study: images were taken by Dr. Kazimierz Madela (Robert Koch-Institut Berlin, Germany). In the left image HIV-1 budding structures are visualized on the surface of HEK293T cells.

The control of the virus amount in the supernatants was performed as described and the samples that turned dark red in the CPRG were picked. Afterwards is was planned to transfect TZM-bl cells with the four point mutated constructs and the pCMV/CD63- ΔLEL. Following this, it was supposed to infect the transfected cells with the produced and for infectivity tested virus strains to analyse the impact of the introduced mutations on HIV-1 entry and cell-to-cell transfer. Further it had been planned to reproduce this experiment with Jurkat T cells instead of HEK293T cells. Unfortunately, due to strict time constraints these experiments could not be performed.

4. Discussion

The purpose of this study was, to investigate whether a deletion of the complete LEL, or one of the point mutations of the LEL´s cysteines would prevent the protein from being integrated into the cell membrane, as this process has not yet been unveiled. It was hypothesised that the CD63-LEL plays an important role during this process and deleting it therefore seemed to be the first logical step in the study. Furthermore, it was known that changing a protein this way can itself prevent the protein expression. Hence, it was surprising to discover that the deletion of the complete LEL did not affect this process of integration into the plasma membrane, whereas the cysteine mutations did.

It could be shown that the exchange of disulphide bridge building cysteines inhibited the protein integration in three out of four cases. This seems plausible, as the C145,C146, C169,C170 and C191, but not the C177 are building the stabilising disulphide bonds (Fig. 1). The C145A,C146A mutation appeared to have the strongest effect on inhibiting the CD63 integration. One possible explanation for this phenomenon is, as both cysteines are involved in forming disulphide bridges none of them can be build without these particular amino acids. Due to the disulphide bonds role in folding and stabilising the tertiary structure of proteins [42], their removal most likely causes a wrong folding of the LEL and following this a mis-folded or missing subloop. This steric change in the protein structure might be a bigger handicap during membrane integration than deleting a whole domain (Fig. 12b). The C169,C170 and C191 mutations on the other hand, are each part of one disulphide bridge (Fig. 12a and Fig. 12b). Therefore, their deletion caused a partial change in the protein folding, which could explain that the inhibition of membrane integration was just partially found. The C177A mutation finally, did not cause a problem, as it is not involved in the tertiary structure stabilising disulphide bonds (Fig. 12a).

Further analysis of the constructs influence is still needed, to ascertain statistical data for the C191A and C169A,C170A mutation. Therefore it was planned to repeat this experiment more than once and to assess the number of cells with and without a surface expression. Unfortunately, due to strict time constraints these experiments could not be performed. But this is necessary to finally evaluate the inhibition in these cases. Altogether it can be said that the LEL is not the responsible CD63-domain that integrates the protein into the membrane. Nevertheless, a destabilisation of the LEL-subloop causes steric problems that inhibit the protein integration.

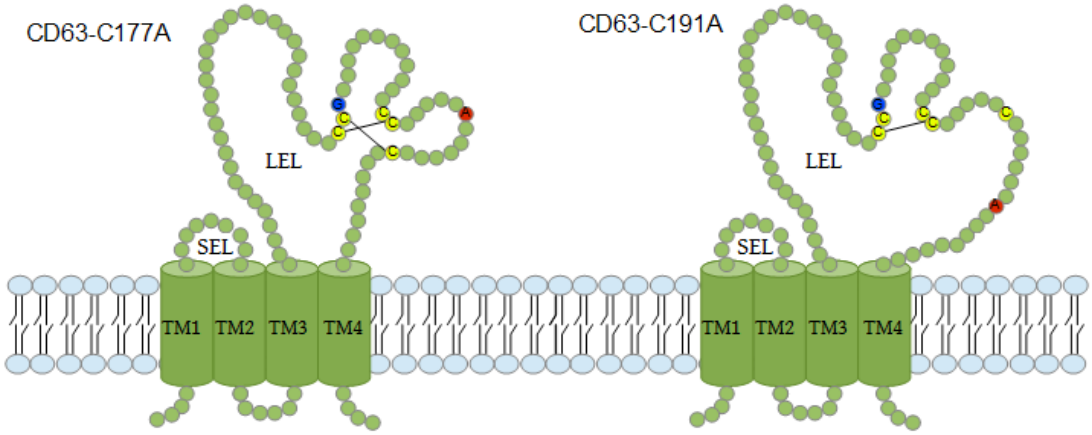

Fig. 12a: Schematic model of the changed protein folding after the introduced point mutations at the single cysteines (own illustration)

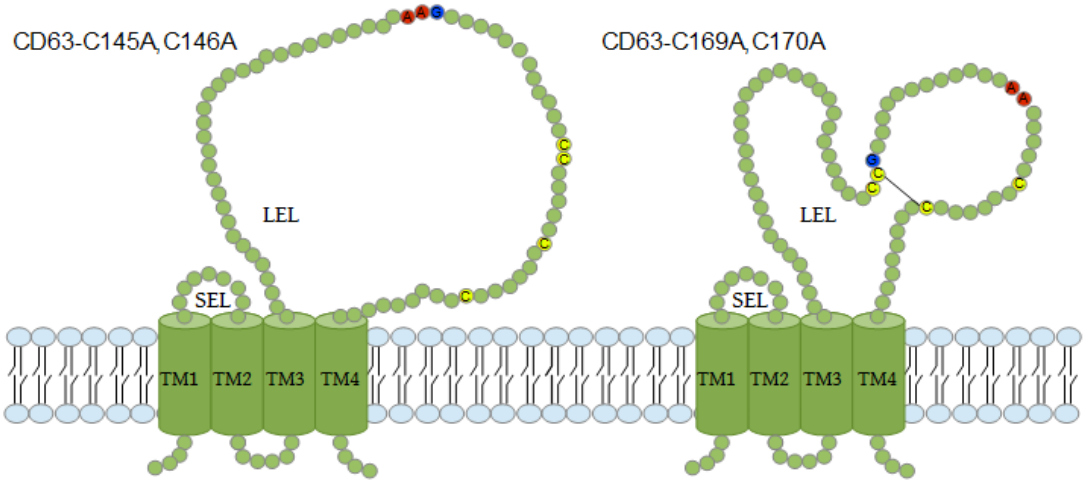

Fig. 12b: Schematic model of the changed protein folding after the introduced point mutations at the double cysteines (own illustration)

As a correct expression of the generated hybrid molecules was confirmed, the CD63-ΔLEL construct appears to be a sufficient transport machine to the cell membrane surface. Overall, it has very efficient expression, even after introducing foreign proteins, which could be used in the field of immunisation studies, antibody production or other immunological experiments, where the exposure of certain proteins on the cell surface is of interest. Another benefit of this system is that it can express proteins on the surface of mammalian cells, which would be a different starting point for immunisations for instance.

However, this study represents only the beginning in establishing this system. Although the correct folding and orientation of the hybrid proteins could be confirmed, it still needs to be investigated, what proteins can be successfully introduced.

Regarding to the previous described findings, the size of the foreign protein might be a limiting factor, as some proteins could cause the same steric problems as the mis-folded LEL. As the pCMV/CD63-ΔLEL-gp120-mCherry expression for example appeared to be lethal for the transfected HEK293T cells, although the hybrid protein had been expressed, the ideal conditions for this system to work are important for successful experiments. Further, it would be needed to test this protein expression in other mammalian systems, such as Jurkat T cells or HeLa cells.

Further analysis could start with a co-transfection of the generated constructs with HIV1-iGFP provirus carrying expression plasmids to examine the influence of the LEL-mutations on the infectivity of released cell-free virus. This is conceivable as Krementsov et al. confirmed an inhibited cell-to-cell transmission and decreased infectivity of cell free virus that was released from cells with a CD63 overexpression [3]. In general, all experiments were planned to be replicated in Jurkat T cells, to establish the system in immune cells that have the CD4 receptor and due to the different natural amount of CD63 in different cell types. In the case of earlier studies that worked with the overexpression of CD63, different results in different cell lines were already revealed [3]. Infection assays with the virus containing supernatants should be performed in Jurkat T cells to examine the influence of the expressed recombinant proteins on HIV-1 entry, as the process of CD63 incorporation into virions is specifically found when they are released from T lymphocytes [32]. It was further planned to co-culture R5 tropic infected Jurkat T cells with uninfected T lymphocytes. Uninfected Jurkat cells could be tagged with a cell tracker and the infection monitored 4, 8 and 24 hours post infection via flow cytometry. As an R5 tropic strain is not able to infect Jurkat T cells, but to be transferred from cell to cell, it can be used to evaluate the rate of cell-to-cell transmission for the purpose of investigating the influence of the CD63-LEL mutations. The CD63-ΔLEL protein is of great interest due to its role as protein binding region. However, although the CD63 tetraspanin was found to be interacting with gp41 [7], Thali et al. concluded that tetraspanins do not generally act as HIV-1 budding co-factors [16]. Although the results gathered during the course of this research project have showed some promise of usefulness in the scientific field, in regards to the CD63's overall influence on cell free virus infectivity and cell-to-cell transfer in T lymphocytes, it would appear the current understanding is that no significant influence can be shown [3]. This would suggest that, here too, no significant results are to be expected.

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