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Role of Tetraspanins in SARS-CoV-2 Fusion and Entry

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ROLE OF TETRASPANINS IN SARS-COV-2 FUSION AND ENTRY

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LIST OF ABBREVIATIONS

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2
S: Spike
E: Envelope
N: Nucleocapsid
M: Matrix
NSP: Non-structural proteins
hACE2: Human angiotensin-converting enzyme 2
RBD: Receptor binding domain
FP: Fusion peptide
ER: Endoplasmic reticulum
ERGIC: ER-to-Golgi intermediate compartment
EV: Extracellular vesicles
VOCs: Variants of concern
SEL: Small extracellular loop
LEL: Large extracellular loop
TEMs: Tetraspanins enriched microdomains
MCH I: Major histocompatibility complexes I
MCH II: Major histocompatibility complexes II
APC: Antigen presenting cell
IS: Immune synapse
LFA-1: Lymphocyte function associated antigen-1
TCR: T cell-receptor
MoDCs: Monocyte-derived dendritic cells
VCAM-1: Vascular cell adhesion molecule-1
ICAM-1: Intercellular adhesion molecule-1
JAM-1: Junctional adhesion molecule-1
BCR: B cell receptor
IL-4: Interleukin 4
MERS-CoV: Middle East Respiratory Syndrome-Coronavirus
229-E-CoV: Human Coronavirus 229-E
DPP4: Dipeptidyl peptidase-4
APN: Aminopeptidase N
IAV: Influenza A virus
HATs: Human airway trypsin-like proteases
HIV: Human immunodeficiency virus
DMEM: Dulbecco's modified Eagle medium

FBS: Fetal bovine serum
VLPs: Virus like particles
SFM: Serum-free media
HRP: Horseradish peroxidase
PBS: Phosphate-buffered saline
SEM: Standard error of the mean
RLU: Relative light unit
PLA: Proximity ligation assays
IF: Immunofluorescent

CHAPTER ONE

LITERATURE REVIEW

SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped positive-sense, single-stranded RNA virus, belonging to the family of coronaviruses. It was first documented in late 2019 in Wuhan, China and has since spread throughout the globe, leading to over 500 million cases and over 6 million deaths. Recent variants contain a 29.8-29.9 kb genome, encoding for 4 structural proteins: Spike (S), envelope (E), nucleocapsid (N), and matrix (M) proteins, as well as 16 non-structural proteins (nsp) and 11 accessory proteins (Redondo et al., 2021; Shang et al., 2021). There are many genetic differences between severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2. However, the most notable similarity is that they both target human angiotensin-converting enzyme 2 (hACE2) (Hoffmann et al., 2020; Starr et al., 2022; Telenti et al., 2022; Wells et al., 2021). Furthermore, there is sufficient homology between SARS-CoV and SARS-CoV-2 spike that anti-SARS-CoV antibodies can cross react with SARS-CoV-2 (Hoffmann et al., 2020).

Extensive studies have allowed an understanding of the SARS-2 infection process, but more specifically and importantly, SARS-CoV-2 fusion and entry into host cells requires 3 main components: The first component is the S glycoprotein, with at least one monomer with its receptor binding domain (RBD) in its “up” conformation, which mediates fusion and entry. Secondly, the virus requires its target receptor for the virus to bind target cells. When the virion’s S protein binds to ACE2, conformational changes occur within the spike’s structure. As S1 binds

to ACE2, S2 anchors the viral particle in the host cell membrane. Furthermore, ACE2 binding exposes the S2' site of the spike which can then be proteolytically cleaved by the third player in the fusion and entry process, host proteases, which for SARS-2, are found in the forms of either TMPRSS2 or Cathepsin-B/L.(Hoffmann et al., 2020) Which of either protease cleaves the S2' site is dependent on the entry pathway the virus utilizes to enter the cell.

There are 2 main pathways a coronavirus can undergo to enter the host cell and release its genome into the cytoplasm: "Early" entry via membrane fusion or "late" endocytic entry(Bayati et al., 2021; Jackson et al., 2022). When proteases on the surface are sufficiently present, in the case of SARS-2, TMPRSS2, S2' is proteolytically cleaved, the fusion peptide (FP) is released, allowing the formation of a fusion pore, allowing viral RNA to be released into the cytoplasm. Alternatively, through clathrin-mediated endocytosis, the bound virus-ACE2 complex is taken in an endosome. Within the endosome, S2' site is cleaved by cathepsin, allowing for the virus to fuse with the endosomal membrane and release its genetic contents into the cytoplasm. Once inside the cell, viral RNA is released, translated in the cytoplasm, and then assembled into maturation within the endoplasmic reticulum (ER), ER-to-Golgi intermediate compartment (ERGIC), and Golgi(Jackson et al., 2022; Klein et al., 2020; Scherer et al., 2022). After assembly and full maturation, there are two proposed pathways of egress for the virus, via a biosynthetic exocytosis or neutralized lysosomal trafficking towards the cell surface(Ghosh et al., 2020; V'Kovski et al., 2021), where they are then released into the extracellular environment and continue infection to nearby cells.

The spread of SARS-CoV-2 throughout the world immediately suggests that it is drastically more infective that SARS-CoV. While this was proven to be true, shortly after its original reports, the D614G mutant rapidly took the position of the predominant variant across the globe

(Korber et al., 2020). D164G did not grant increased immune evasion nor did it affect ACE2 affinity, however the mutations within this variant provide viral particles with increase spike density per particle and decreased S1 shedding (Zhang et al., 2020). This allowed for a virus with more readily stable and available spike, increasing overall infectivity. This increased infectivity was shown to be true not only on susceptible cells, but also on susceptible, ACE2 containing extracellular vesicles (EVs) (Qing et al., 2021). With such a significant mutation, this allowed the D614G mutant to be the predominant variant for the majority of 2020.

With no other major variants emerging during the majority of 2020, SARS-CoV-2 was thought to be a slow mutating virus. However, in late 2020, further variants of concern (VOCs) world begin to emerge and eventually overtake the D614G mutants as the predominant variant, due to immune pressure from current measures rising predominantly in highly populated areas. While there a mutation such as D614G increased infectivity by increasing the density and stability of the spike, other subsequent mutations have increased viral fitness through immune evasion, ACE2 affinity, changes in its preferred environment, and even crossing towards other species (Li et al., 2020; Lupala et al., 2022; Mohammadi et al., 2021; Ozono et al., 2021). These mutants include the Alpha variant, which emerged in the U.K, Beta, first emergent in South Africa, Gamma, first documented in Brazil, Delta, first documented in India and, Omicron, identified in South Africa in late 2021.

The Alpha variant was first discovered in November 2020 in the UK and was the variant to overtake the D614G variant as the prevalent variant present in infected cases, holding that position up until the emergence of Delta. Alpha features not only mutations within the spike, providing increased ACE2 binding affinity, but also changes outside the spike, providing Alpha avenues of immune evasion against host immune responses and decreased antibody

neutralization(Rees-Spear et al., 2021; Wang et al., 2021). Notably, the N501Y confers the spike a higher binding affinity to ACE2, increasing the infectivity of the virus(Ali et al., 2021). With increased infectivity and decreased susceptibility to measures present during that time, Alpha was a superior virus than the D614G mutant and spread throughout the globe.

Beta was first reported May 2020 in South Africa. The Beta variant not only featured the N501Y mutation, which confers increased infectivity, but unlike Alpha, also contained mutations K417N and E484K, which play a large role immune evasion from neutralizing antibodies, and most notably, Beta shows much stronger resistance to antibodies than other variants due to its unique mutations (Reincke et al., 2022; Uwamino et al., 2022; Wang et al., 2021). Gamma, first identified November 2020 in Brazil, shared these key mutations with Beta, except for featuring a K417T substitution instead. The K417T mutation, however, was inferior in resisting antibody neutralization than K417N(Hoffmann et al., 2021; Lazarevic et al., 2021)

Delta, first identified October 2020 in India, was the next variant to ultimately overtake all others up until Omicron emerged. It bears key mutations D614G, L452R, P618R, T478K, and K417N, which have all been reported to enhance infectivity in varying degrees(Dhawan et al., 2022; Di Giacomo et al., 2021). Some strains were also reported to have an E484Q mutation, which provide immune evasiveness. However B.1.617.2, which became the predominant strain, did not have this change(Mohammadi et al., 2021). As previously mentioned K417N is well known to provide immune evasion. As with other mutations within the RBD, L452R has also been reported to decrease the effectiveness of neutralizing antibodies(Garcia-Beltran et al., 2021). The combination of spike mutations as well as novel mutations within the RBD would allow Delta to lead the third wave of the pandemic and would remain that way for nearly a year until the rise of Omicron.

Following Delta, Omicron has become the present variant of the virus circulating the population since emerging in November 2021. Omicron has shifted its niche to the upper respiratory tract, shows decreased pathogenicity and replication, and is less likely to enter the cell through membrane fusion, due to decreased interaction with TMPRSS2 (Armando et al., 2022; Meng et al., 2022; Shuai et al., 2022), clearly being unique to previous variants. Based on this, one could wonder how Omicron was able to lead the most recent wave of infection. Despite this change , Omicron still contains over 30 mutations in the spike, with 15 of them being in the RBD (Saxena et al., 2022). This high number of changes ultimately affects the efficacy of present vaccine efforts. Furthermore, other changes present in previous variants such as K417N, E484K, and N501Y are well known to be responsible for either increased ACE2 binding, therefore infectivity, and transmissibility(Ali et al., 2021; Poudel et al., 2022). Notably, the presence of "Deltacron" variants, which is an Omicron variants containing key changes found in Delta, suggests the possibility of recombination events between Omicron and other existing variants, which could lead higher variability that previously expected (Ou et al., 2022)

Omicron is a challenging obstacle as we continue through the pandemic. We now face a virus that as expected, with new changes, decreases current treatments, can infect more readily, and can transmit itself more easily due to its preference to the upper respiratory tract. It contains many of the optimal parts of previous variants and with its own changes, contains very few downsides in terms of infection. Lower interaction with TMPRSS2 also means that it has changed the way it interacts with target cells, opting to enter through the endocytic pathway(Meng et al., 2022; Shuai et al., 2022). With this change in entry pathway, understanding what other factors might influence viral infection become imperative as we face Omicron and potentially other future variants.

Tetraspanins: A Surface Overview

The cell membrane is home to an enormous ecosystem of proteins, all which communicate and interact with one another to maintain a viable environment for the cell. Among all these particles, there is a family of proteins known as tetraspanins. Tetraspanins are transmembrane proteins composed of 4 transmembrane domains, 3 short intracellular domains, a small extracellular loop (SEL) and a large extracellular loop (LEL). They support a wide array of functions, including roles in cell adhesion, signalling, motility, particle trafficking, and proliferation by interacting with nearby surface proteins on the membrane surface (Charrin et al., 2009; Rocha-Perugini et al., 2017; Termini & Gillette, 2017; Xing et al., 2020). These interactions between tetraspanins and other particles take place largely within tetraspanins enriched microdomains (TEMs), where the tetraspanins are most abundant are more readily available to interact with other nearby proteins (Yáñez-Mó et al., 2009).

As mentioned before, tetraspanins interact with a plethora of membrane proteins within TEMs to support or inhibit various cellular functions. CD9 for example, influences signaling in the immune system by regulation adhesion molecules. Antigens bound to major histocompatibility complexes I and II (MHC I and II) are recognized by T cells to trigger T cell-mediated immune responses. This is done in a region between the antigen presenting cell (APC) and the T cell called the immune synapse (IS) (Reyes et al., 2018). The IS is enriched with adhesion molecules to promote APC to T cell contact. CD9 has been shown to decrease the accumulation of lymphocyte function associated antigen-1 (LFA-1), whose co-stimulation with T cell receptor (TCR) facilitates T-cell mediated activation (Suzuki et al., 2007). CD9 is also known to play a role in the maturation of monocyte-derived dendritic cells (MoDCs) by stimulating MHC II egress as well as promoting the endocytosis and recycling of MHC II in mature MoDCs(Rocha-

Perugini et al., 2017). Additionally, CD9 can modulate the sheddase capability of a distintegrin and metalloproteinase-17 (ADAM-17)(Gutiérrez-López et al., 2011), subsequently affecting the shedding of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1)(Garton et al., 2003; Gooz, 2010), intercellular adhesion molecule-1 (ICAM-1)(Gooz, 2010; Tsakadze et al., 2006), and junctional adhesion molecule-1 (JAM-1)(Gooz, 2010; Koenen et al., 2009). This effect caused by CD9 proves very interesting, as both upregulation and downregulation of CD9 can lead to positive effects via ADAM17 modulation. HPV16 is dependent on ADAM17 activity as ADAM17-mediated ERK activation is required for HPV receptor to be assembled (Mikuličić et al., 2019) . Reports show that only specific concentration of CD9 is able to promote ADAM17 activity and as such, ERK activation (Mikuličić et al., 2020). Therefore, when outside this optimal range, ADAM17 and ERK are decreased, leading to an inhibition in HPV16 infection.

Like CD9, CD81 has near ubiquitous distributions amongst cells. CD9 and CD81 are considered partner proteins and are often thought to compensate in activity in the absence of the other. However, CD81 still affects many cellular functions and in similar fashion, plays roles that influence immune activity. For example, CD81 has been shown to be required for CD19 trafficking towards the cell surface(Shoham et al., 2006; Van Zelm et al., 2010). When CD81 forms complexes with CD19 and CD21 within TEMs, this complex can facilitate B cell activation when costimulated with B cell receptor (BCR) (Pieta et al., 2013). CD81 is present in the IS between B cells and T cells, contributing to the maturation of the IS when during antigen presentation between the two cell types (Rocha-Perugini et al., 2013) and more so, are important for both interleukin 4 (IL-4) production(Maecker et al., 1998) and downstream T-cell mediated signalling(Deng et al., 2002).

As influential as they are in cellular functions, tetraspanins can also be linked to the pathogenesis of various diseases. Tetraspanins are frequently used as markers for cancer tissue as well as tumor metastasis and have been suggested as targets for cancer therapy(Malla et al., 2018; Xing et al., 2020). Tetraspanins play roles in EV formation, trafficking, and selection of cargo(Andreu & Yáñez-Mó, 2014; Gurung et al., 2021; Larios et al., 2020; Perez-Hernandez et al., 2013) and are therefore commonly used biomarkers used to indentify EVs. The presence of tetraspanins within EVs which can be used to track the invasion of cancer into neighboring tissue (Brzozowski et al., 2018) and as such for cancer prognosis(Hoshino et al., 2020; Malla et al., 2018).

Tetraspanins have also reported to promote pathogen entry, assembly, and egress(Earnest et al., 2017; Earnest et al., 2015; Hantak et al., 2019; Li et al., 2014). This is true for both bacteria and viruses as they interact with target receptors and other co-factors within TEMs. It has been reported in our lab that CD9 promotes Middle East Respiratory Syndrome-coronavirus (MERS-CoV) and human coronavirus 229-E (229-E-CoV) entry into host cells(Earnest et al., 2017; Earnest et al., 2015; Hantak et al., 2019). CD9 promotes the entry MERS-CoV and 229-E-CoV by directly recruiting their target receptors Dipeptidyl peptidase-4 (DPP4) and aminopeptidase N (APN), respectively into TEMs allowing for a high density of receptor at the cell surface accessible to virus. Additionally, CD9 is also shown to converge DDP4 with TMPRSS2 within TEMs, which as previously mentioned, promotes coronavirus membrane fusion. Notably, a significant portion of CoV receptors and associated proteases were also reported to be found in TEMs(Earnest et al., 2015).

Tetraspanins can influence viral infection and egress on viruses outside of the coronavirus family Influenza A virus (IAV) requires sialylated glycolipids or glycoproteins as target

receptors and human airway trypsin-like proteases (HATs) for its entry. HATs were shown to be abundant in TEMs (Earnest et al., 2015) and CD81 has been shown to play roles in both IAV uncoating and egress(He et al., 2013). When IAV is bound to the cell membrane CD81 facilitates entry through endocytosis. These CD81 positive endosomes also allows efficient uncoating. During virus budding, CD81 is recruited for scission, as virus buds within CD81 knockdown cells are shown to be elongated compared to WT and are much more likely to remain attached to the cell membrane.(He et al., 2013)

We discussed earlier that HPV16 required an optimal amount of CD9 to promote its entry(Mikuličić et al., 2019; Mikuličić et al., 2020). However, once HPV16 enters the host cell, CD63 recruits syntenin-1 to transfer the virus into multivesicular endosomes.(Gräßel et al., 2016; New et al., 2021), allowing HPV16 to uncoat and be transport itself into the nucleus.

Human immunodeficiency virus (HIV) can likely be affected by the largest variety of tetraspanins, as they influence various throughout binding, entry, assembly, and egress (Jolly & Sattentau, 2007; New et al., 2021). For example, CD81 and CD9 knockdown enhances HIV envelope (env)-mediated endocytosis, and their overexpression decreases env-mediated endocytosis (Gordón-Alonso et al., 2006). A separate report also showed that CD63 silencing, and CD63 deletion mutants resulted in diminished HIV entry (Li et al., 2014; Yoshida et al., 2008). Furthermore, downregulating CD63 seems to inhibit HIV viral replication amongst varying cell types (Chen et al., 2008; Fu et al., 2015; Li et al., 2014). Finally, HIV Env and Gag proteins associate with CD81 and CD63(Jolly & Sattentau, 2007). CD9, CD63, and CD81 TEMs to the virological synapse between infected and uninfected CD4 positive T cells (Jolly & Sattentau, 2007), indicating that tetraspanins are relevant to HIV secretion and egress.

Being present throughout most of HIV's replication cycle, as well key steps during the replication of other viruses this elucidates the importance of tetraspanins for viral entry and replication. Understanding tetraspanins at a larger scale becomes difficult due to the immense variety of interaction that take place between tetraspanins and other membrane proteins. However, as we progress our understanding of the role of tetraspanins in viral processes, perhaps we can obtain a better understanding of the intricacies that the varying viral species hold.

With extensive evidence that tetraspanins can influence the infection process of various viruses, including coronaviruses, it is only natural that we seek to uncover any inteactions between tetraspanins and SARS-CoV-2. While we have reports about other potential co-factors and alternative target receptors (Jackson et al., 2022; Trougakos et al., 2021), we have yet to see whether such interactions can occur between tetraspanins and SARS-CoV-2 ACE2**.** Further understanding underlying interactions between tetraspanins and host factors during SARS-CoV-2 infection becomes paramount as we continue through the present pandemic. In this thesis, we investigate the potential role tetraspanins CD9, CD63, CD81 play in SARS-CoV-2 fusion and entry. Our results demonstrate that overexpression of CD9 and CD63 inhibit SARS-CoV-2 VLP cell entry in HeLa cells while EVs overexpressing ACE2 and high quantities of either CD9 or CD81 inhibit VLP membrane fusion. However, neither CD9 or CD81 influenced overall transduction of Nluc in WT, CD9KO, or CD81KO HeLa cells. Furthermore, we also show that when co-overexpression of tetraspanin and ACE2-LgBit is present in EVs and HeLa cells, the quantity of ACE2 per is diminished. However, when we account this decrease of ACE2 to our transduction studies, we find that tetraspanins ultimately promote ACE2 efficient entry.

CHAPTER TWO

MATERIALS AND METHODS

Cells

HEK293T, HeLa (obtained from Ed Campbell, Loyola University Chicago) CD9 KO, and CD81 KO HeLa (generated as described below) cells were maintained in Dulbecco's modified Eagle medium (DMEM)-10% fetal bovine serum (FBS) (containing 10 mM HEPES, 100 nM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin G, and 100 μg/ml streptomycin and supplemented with 10% FBS; Atlanta Biologicals). All cell lines were cultured in a 5% CO_2 incubator at 37°C.

Plasmid Construction

Full-length SARS-CoV S (GenBank: AY278741.1) and SARS-CoV-2 S, E, M, and N (GenBank: NC_045512.2) genes were synthesized by Genscript, Inc. as human codon-optimized cDNAs, and inserted into pcDNA3.1 expression vectors. HiBiT-N was constructed by fusing HiBiT peptide (VSGWRLFKKIS) coding sequences with linker (GSSGGSSG) to the 5′ end of the N gene, as described in (Kumar et al., 2021; Qing et al., 2021). The pCMV-LgBiT expression plasmid was purchased from Promega. pcDNA3.1-hACE2-LgBiT was constructed by fusing the coding sequence of LgBiT to the 3′ end of hACE2 gene. PcDNA3.1-hCD9-FLAG and pcDNA3.1-hCD81-FLAg were constructed by fusing the coding sequence of the FLAG epitope tag at the 3' end of the CD9 or CD81 gene.

Production of Knockout Cell Lines

CD9 and CD81 KO HeLa cell lines were produced as described in (Earnest et al., 2017). pSpCas9-BB-2A-puro was digested with Esp3I (Fermentas) for 4h at 37°C. The digested plasmid was purified and ligated with annealed guide DNAs specific for CD9 or CD81. Tetraspanin-specific pSpCas9-BB-2A-puro plasmids were transfected into HeLa cells. After 72h, cells were selected with 4 μg/ml puromycin for 96h. Selected cells were serially-diluted to isolate clonal populations and clones were selected by western blot.

Virus-like Particle (VLP) Production

HiBiT-N-tagged virus-like particles (VLPs) were produced as described previously (Kumar et al., 2021). Briefly, equimolar amounts of full-length CoV S, E (envelope), M (membrane), HiBiT-N-encoding, plasmids (total, 16 μg) were LipoD (SignaGen Laboratories) transfected into 10^7 HEK293T cells. To produce spikeless ("no-S") VLPs, the S expression plasmids were replaced with empty vector plasmids. At 6 h posttransfection, cells were replenished with fresh DMEM-10% FBS. HiBiT-N VLPs were collected in FBS-free DMEM (also referred as SFM) from 24 to 48 h posttransfection. FBS-free DMEM containing HiBiT-N VLPs were clarified by centrifugation (300 × *g*, 4°C, 10 min; 3,000 × *g*, 4°C, 10 min). Clarified samples were then pelleted through a 20% sucrose cushion in SFM (SW28, 7500rpm, 4°C, 24hr). Pellets were then resuspended using fresh FBS-free DMEM to 1/80 of the original medium volumes.

Conversely, clarified VLPs were concentrated 100-fold by ultrafiltration (Amicon; 100kDa). Concentrated samples were then overlayed by a 50% sucrose and 20% sucrose cushions and were banded (SW41, 39000rpm, 4°C, 2h). Fractions from the interface between both cushions was then collected and complemented with LgBit containing detergent and measured Nluc

quantities using a Veritas microplate luminometer. All collected VLP samples were stored at -80°C.

Western Blot and Antibodies

Samples in SDS solubilizer (0.0625 M Tris·HCl [pH 6.8], 10% glycerol, 0.01% bromophenol blue, and 2% [wt/vol] SDS with and without 2% 2-mercaptoethanol) were heated at 95°C for 5 min, electrophoresed through 10% (wt/vol) polyacrylamide-SDS gels, transferred to nitrocellulose membranes (Bio-Rad), and incubated with rabbit monoclonal anti-hACE2 (Invitrogen; catalog no. MA5-32307),,mouse anti-C9 (EMD Millipore; lot no. 2613109), mouse monoclonal anti CD-63 (BD Pharmingen; lot no. 6138688), mouse monoclonal anti CD81(BD Pharmingen; lot no. 6119813), mouse monoclonal anti FLAG (F1804; Sigma), goat polyclonal anti rabbit IgG (Perkin Elmer; lot no. 10473846), or goat polyclonal anti mouse IgG (Perkin Elmer; lot no. 10437708). After incubation with appropriate horseradish peroxidase (HRP)-tagged secondary antibodies and chemiluminescent substrate (Thermo Fisher), the blots were imaged and processed with a FluorChem E apparatus (Protein Simple).1

Cell-free Fusion Assay

Cell-free fusion assays required ACE2-LgBiT EVs. To obtain these EVs, HEK293T target cells were LipoD transfected with pcDNA3.1-hACE2-LgBiT. At 6 h posttransfection, transfection media were removed, rinsed, and replaced with FBS-free DMEM. Media were collected at 48 h post-transfection, clarified (300 × *g*, 4°C, 10 min; 3,000 × *g*, 4°C, 10 min), and concentrated 100-fold by ultrafiltration (Amicon; 100 kDa). EVs were then purified using SEC (qEV original; Izon, Inc.) using phosphate-buffered saline (PBS; pH 7.4) as the eluant. Peak EV fractions were identified by the addition of HiBiT-containing detergent and subsequent Nluc measurement by luminometry. EVs were stored at 4°C.

Cell-free fusion assays were performed by mixing HiBiT-N VLPs, each introduced at equivalent HiBiT concentrations, with hACE2-LgBiT EVs +/- the overexpression of hCD9-FLAG or hCD81-FLAG, the NanoLuc substrate (catalog no. N2420; Promega), and trypsin (Sigma; 50 ng/μl or as indicated) in 96-well multiwell plates. After 10 min at 4°C, sample plates were loaded into a GloMax luminometer maintained at 37°C. VLP-EV cell-free fusions were quantified as Nluc accumulations over time. For data presentation, the Nluc recordings from samples containing control "bald" (no-S) VLPs were normalized to values of 1.0, and the fold increases over levels for this control condition were calculated and plotted as fold fusion.

Cell Entry Assay

HeLa target cells were LipoD transfected with pcDNA3.1-hACE2-LgBiT and either pcDNA3.1-hCD9, pcDNA3.1-hCD63, pcDNA3.1-hCD81 or empty vector. At 2 days posttransfection, cells were incubated with a live-cell Nluc substrate (Nano-Glo Vivazine; Promega), and 1 h later, HiBiT-N VLPs were inoculated at equivalent HiBiT inputs +/- trypsin (25ng/μL). HiBiT-N VLPs lacking S proteins (no S) served as negative controls. In 20-minute intervals following VLP inoculation, Nluc levels were quantified using a Veritas microplate luminometer. For data presentation, the Nluc recordings in cultures inoculated with bald (no-S) VLPs were normalized to values of 1.0, and the fold increases over this control condition were calculated and plotted as fold entry.

Cell Complementation Assay

WT and either CD9 KO or CD81 KO HeLa target cells were LipoD transfected with pcDNA3.1-hACE2-LgBiT and 10-fold dilutions of either pcDNA3.1-hCD9-FLAG, pcDNA3.1-hCD81-FLAG or empty vector. At 2 days posttransfection, cells were complemented with HiBit containing detergent and Nluc quantities were measured in a Veritas microplate luminometer.

Cell Transduction Assay

WT and either CD9 KO or CD81 KO HeLa HeLa target cells were LipoD transfected with pcDNA3.1-hACE2-LgBiT and 10-fold dilutions of either pcDNA3.1-hCD9-FLAG, pcDNA3.1-hCD81-FLAG or empty vector. At 2 days posttransfection, were rinsed with pre-warmed SFM, inoculated with PS9-Nanoluc, N-HiBit containing VLPs under trypsin conditions and incubated for 2h at 37°C. Following 2h incubation, cells were added fresh FBS and incubated over 18 h. After 18 h, cells were lysed with detergent and Nluc quantities were measured in a Veritas microplate luminometer. For data presentation, the Nluc recordings in cultures inoculated with bald (no-S) VLPs were normalized to values of 1.0, and the fold increases over this control condition were calculated and plotted as fold transduction.

CHAPTER THREE

RESULTS

CD9 and CD63 Inhibit SARS-2 Entry

Tetraspanins are well known to promote viral entry. This can be done either by promoting the accumulation of target receptor, host proteases, or promoting other pathways such as endocytosis. As such, we sought to test whether such an effect would be present in the case of SARS-CoV-2. To perform our cell entry assay, we transfected HeLa cells with ACE2-LgBit + and either pcDNA3.1-hCD9, pcDNA3.1-hCD63, pcDNA3.1-hCD81, or empty plasmid. We had attempted to generate ACE2-LgBit+TMPRSS2 overexpressing Hela cells, however, the cells had very little survival rate, and were thus deemed unfit for the assay. 2 days posttransfection, we inoculated the cells with Bald (no S) or D614G spike VLPs, then read for luminescence every 20 minutes for 2 hours. We do find that while CD81 overexpressing cells lead to a modest decrease in VLP entry (less than 2-fold), CD9 and CD63 overexpression result in up to 5-fold decrease in entry when compared to our ACE2 control during this time lapse (Fig 1a). While we have yet to understand the interactions occurring to cause this inhibition, we find that the presence of CD9 and CD63 inhibit SARS-CoV-2 entry.

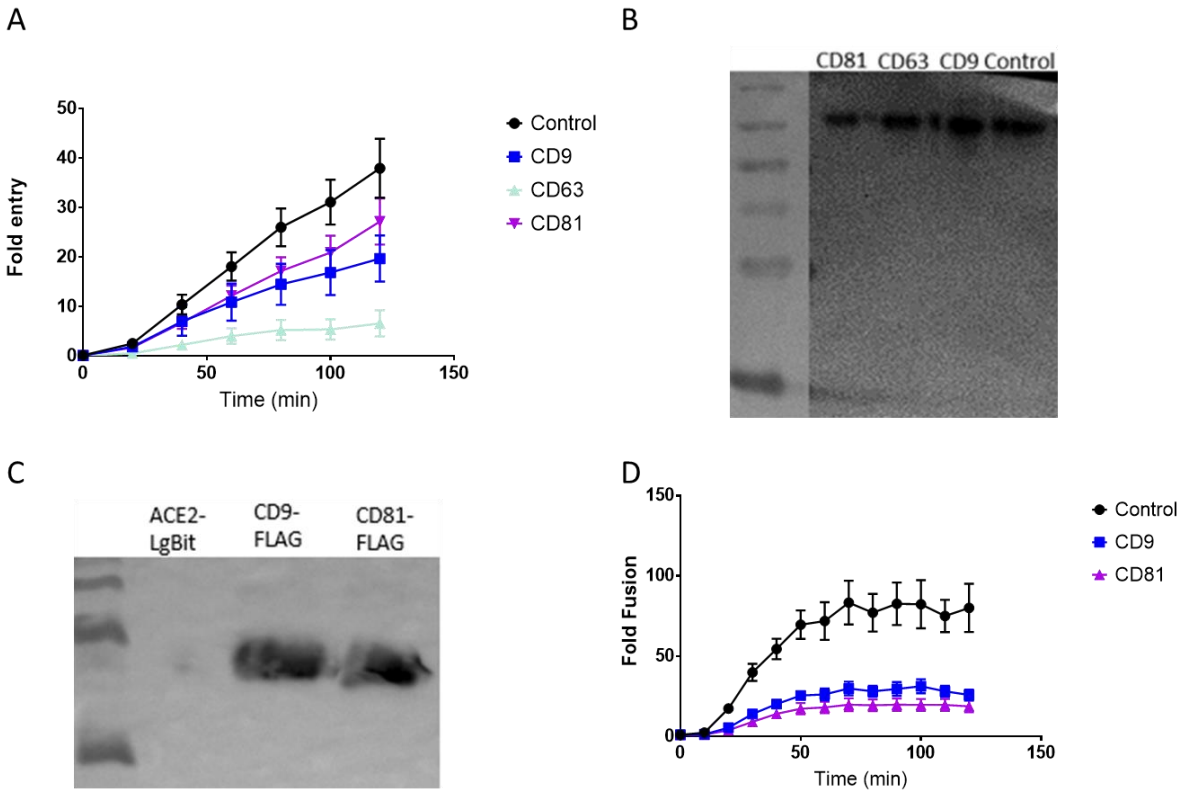

Figure 1. Tetraspanins Inhibit SARS-2 Fusion and Entry. HeLa cells overexpressing ACE2-LgBit and either hCD9, hCD63, hCD81, or no tetraspanin were inoculated with N-HiBit VLPs under trypsin conditions (A). Fold entry is calculated as spike-mediated entry over background (no S) signal. Values and standard error of the mean (SEM) are representative of n=3 experiments. EVs expressing ACE2-LgBit and either hCD9, hCD63, hCD81, or no tetraspanin were subjected to WB analysis to detect the presence of ACE2 (B). EVs expressing ACE2-LgBit and either hCD9-FLAG, hCD81-FLAG, or no tetraspanin were subjected in equimolar volumes of Lg-Bit to WB analysis to detect the presence of FLAG (C). EVs expressing ACE2-LgBit and either hCD9-FLAG, hCD81-FLAG, or no tetraspanin were inoculated with N-HiBit containing VLPs under trypsin conditions (D). Fold fusion is calculated as spike-mediated fusion over background (no S) signal Values and standard error of the mean (SEM) are representative of n=3 experiments.

Tetraspanin Levels are Proportional to ACE2-LgBit Quantities in Overexpressing EVs

Before we can assess the role of tetraspanins in the SARS-CoV-2 fusion, we needed to create environment where could reduce the entry process just to the fusion step. It's been shown that ACE2 containing EVs can be targeted by SARS-CoV-2 (Cocozza et al., 2020), but we first

needed to determine whether we could generate functional EVs overexpressing both ACE2 and tetraspanins. For this, we transfected HEK 293T cells with equimolar amounts of ACE2-LgBit and either hCD9, hCD63, or hCD81 encoding plasmids, with conditions lacking tetraspanin instead being co-transfected with empty vector plasmid. Following 48 hours post-transfection, media was collected, clarified through ultracentrifugation, purified using SEC, and then quantified via complementation using excess extraneous HiBit and HiBit extracellular substrate. Volumes containing equivalent luminescent values were then added to an SDS solubilizer solution (without 2-mercaptoethanol for tetraspanin containing EVs), heated at 95°C, electrophoresed in 10% polyacrylamide gel, transferred into nitrocellulose membrane, and then probed for ACE2 (Fig 1b). We were able to find that ACE2 quantities were proportional to the amounts of LgBit present. The ability to accurately normalize ACE2 input allows us to remove ACE2 variation in future EV based assays. However, we acknowledge using different antibodies, each unique to each tetraspanin candidate, could ultimately lead to variation in results, and therefore, provide inaccuracies when comparing tetraspanin variations. To account for this, we generated EVs CD9 and CD81 overexpressing EVs, with each tetraspanin now linked to a FLAG epitope tag. By probing for FLAG rather than individual tetraspanin, we both eliminate the need for multiple antibodies and allows for more reliable comparison when we observe tetraspanin expression. As such, we prepared ACE-2 LgBit and ACE-2LgBit + CD9/81 conditions were prepared as previously described (Fig 1c). We then found that FLAG quantities were equivalent between both our CD9-FLAG and CD81-FLAG conditions, suggesting that tetraspanin expression within our EVs amount could also be normalized based on luminescent values. Here we find that we can easily generate quantifiable and comparable conditions of different EVs, that we can use for future cell-free assays.

CD9 and CD81 Inhibit SARS-2 Membrane Fusion

CD9 and CD81 are well documented to promote viral entry, particularly CD9 promoting MERS coronavirus by presenting target receptor DPP4 and protease TMPRSS2 which are the key factors needed for MERS membrane fusion. Earlier we showed that tetraspanins can inhibit VLP entry. Therefore, we explored whether tetraspanins could a similar role on SARS-2 fusion. We generated EV conditions overexpressing ACE2-LgBit and either hCD9-FLAG, or hCD81-FLAG or no tetraspanin, to observe cell-free fusion. Since tetraspanins are known to interact with coronavirus target receptors, perhaps we can identify if the inhibition of entry is found during membrane fusion. EV conditions were normalized by RLU values, and therefore ACE2 quantities, were then added to 96-well plates wells and were then inoculated with equimolar amounts of Bald (No S) VLP or D614G spike VLPs. Inoculated wells were then incubated for 10 minutes at 4°C to allow for binding occur. Following incubation, a 30µL mixture of PBS, HiBit Extracellular substrate +/- trypsin (50ng/µL, used to replace TMPRSS2) was added to the wells and NLuc signal was then measure every 10 minutes for 2 hours (Fig 2). Our results show that when targeting EVs overexpressing ACE-LgBit as well as CD9 or CD81, D614G VLP membrane fusion is inhibited roughly 4 to 5-fold in relation to EVs overexpressing ACE-2 LgBit only. However, despite the inhibition, the tetraspanin containing conditions still show luminescent values 20 to 25-fold higher than the background, meaning that fusion is still occurring at a relatively high rate. This, however, allows to conclude that tetraspanins inhibit, but not completely surpress SARS-CoV-2 membrane fusion.

Tetraspanins Inhibit SARS-2 Entry by Reducing Overall ACE2 levels

We have previously shown that tetraspanins inhibit both fusion and entry. We now seek to understand through what mechanisms or interactions this inhibition takes place through. WT and

either CD9 or CD81 HeLa cells were transfected with pcDNA 3.1 ACE2-LgBit + 10-fold dilutions of either pcDNA3.1 hCD9-FLAG or pcDNA3.1 hCD81, with a “no tetraspanin” condition as a control. 2 days post transfection, the cells were lysed, supplemented excess HiBit and analyzed for luminescent activity (Fig. 3). Our results show that in the presence of excess tetraspanin, we observed a 8 to 10-fold decrease ACE2 levels in our 1:1 ACE2:tetraspanin input when compared to our ACE2 control . ACE2 levels then reflect no tetraspanin levels upon reaching a 1:100 dilution onwards. We recall that our previous cell entry assay was performed with 1:1 input ratio of ACE2 to tetraspanin. Thus, we find that the excess presence of tetraspanin decreased the overall ACE2 levels within these HeLa and furthermore, this decrease in the target receptor naturally leads to a decrease in SARS-CoV-2 entry.

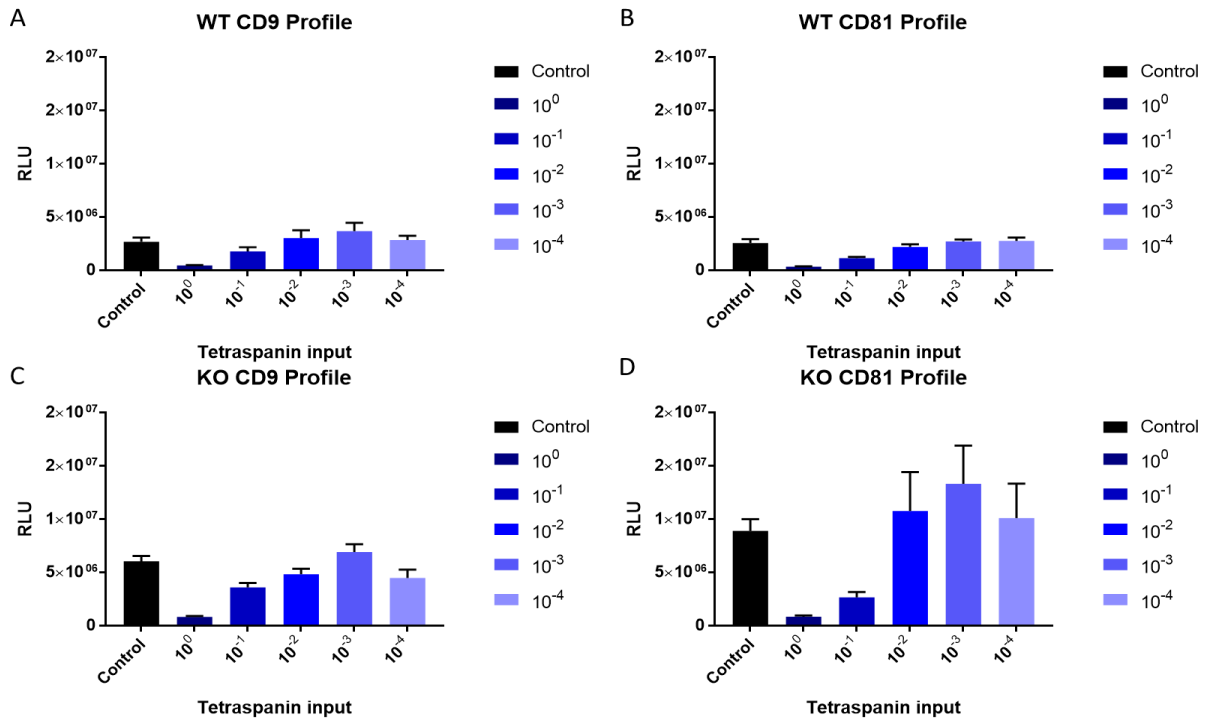

Figure 2. Excess Tetraspanins Reduces ACE2 Levels in HeLa Cells. WT (A&B) and either CD9 KO (C) or CD81 KO (D) were complemented with HiBit containing detergent and then analyzed for total Nluc signal. Values and SEM are representative of n=3 experiments.

Tetraspanins Promote Efficient SARS-2 Entry

We have tested and observed the different effects that tetraspanins have had on SARS-CoV-2 fusion and entry. However, we do not know whether this would also impact the replication of viral RNA from the particles successfully entering the cell. To understand whether our VLPs remain viable upon entry, we generated Bald and D614G VLPs with both N-HiBit as well as PS9-Nanoluciferase to be used for our cell transduction. Our hypothesis here is two-fold. First, we believe that if NLuc RNA is taken in and translated, then our VLPs are still functional following cell entry. Furthermore, we believed that should our VLPs be viable, then we should observe decreased amounts of NLuc, based on our previous findings. Cells from the same line used in Fig. 3 were inoculated with N-HiBit + PS9-NLuc VLPs and incubated as previously described. Following lysing, we then observed for total NLuc translated and expressed based on luminescent units over background (Fig 4). We observe comparable amounts of luminescence between both WT and KO cell lines. Furthermore, we observe no difference between our “no tetraspanin” and our tetraspanin expressing cells.

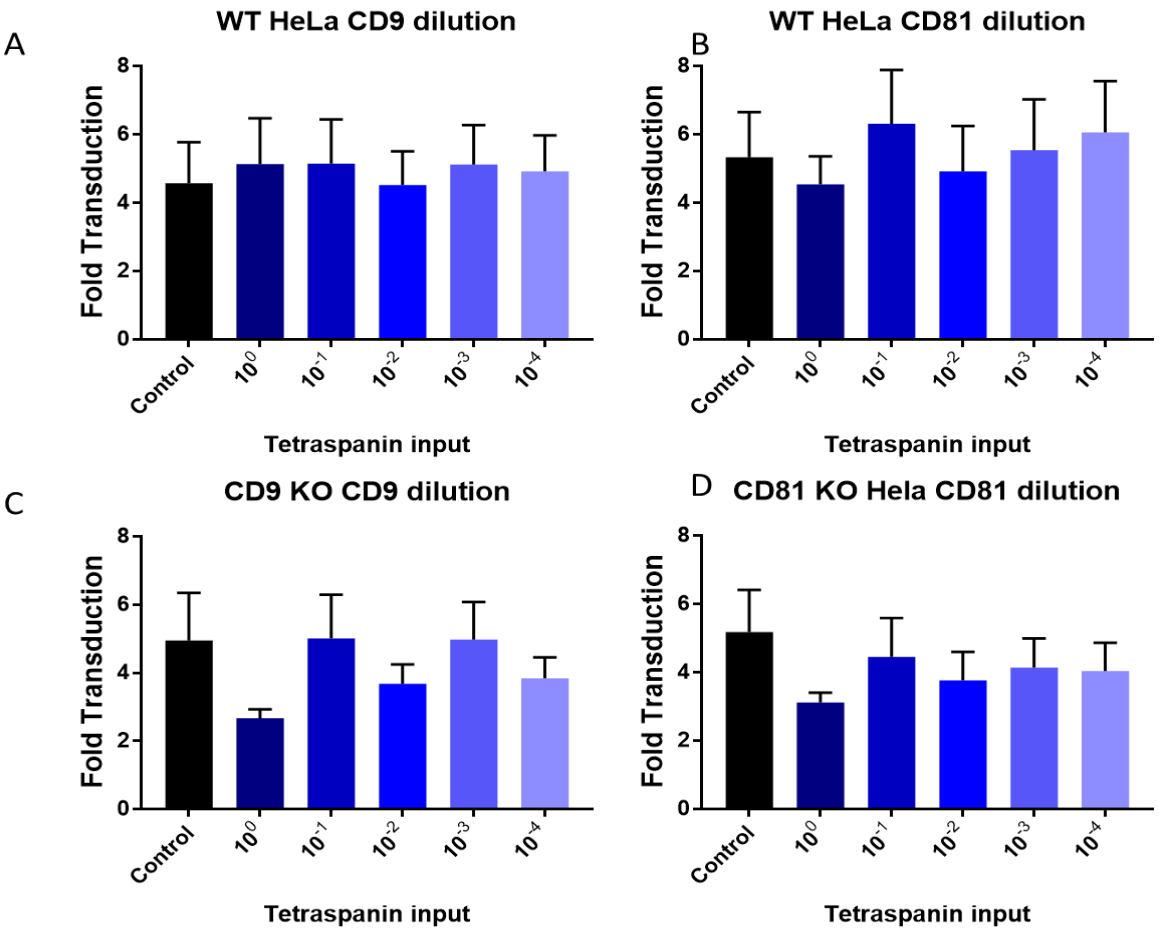

Figure 3. Tetraspanins Have No Effect on Transduction. WT (A&B) and either CD9 KO (C) or CD81 KO (D) were inoculated with PS9-Nanoluc, N-HiBit containing VLPs under trypsin containing conditions. Cells were then rinsed, incubated for 18 h, then lysed and analyzed for total Nluc signal. Values and SEM are representative of n=3 experiments.

However, we recall that higher concentrations of tetraspanins lead to a decrease in ACE-LgBit levels within these cells. To attribute for the difference in ACE2 between conditions we normalized the levels of transduction by comparing the amount of fold transduction per unit of ACE2 compared to the WT ACE2 only control from each tetraspanin group (Fig 5). From this comparison, we find that due to the decrease in ACE2 levels, high concentration of both CD9 and CD81 result in a much higher degree of transduction per available receptor, and thus allowing for a more efficient entry process for SARS-2.

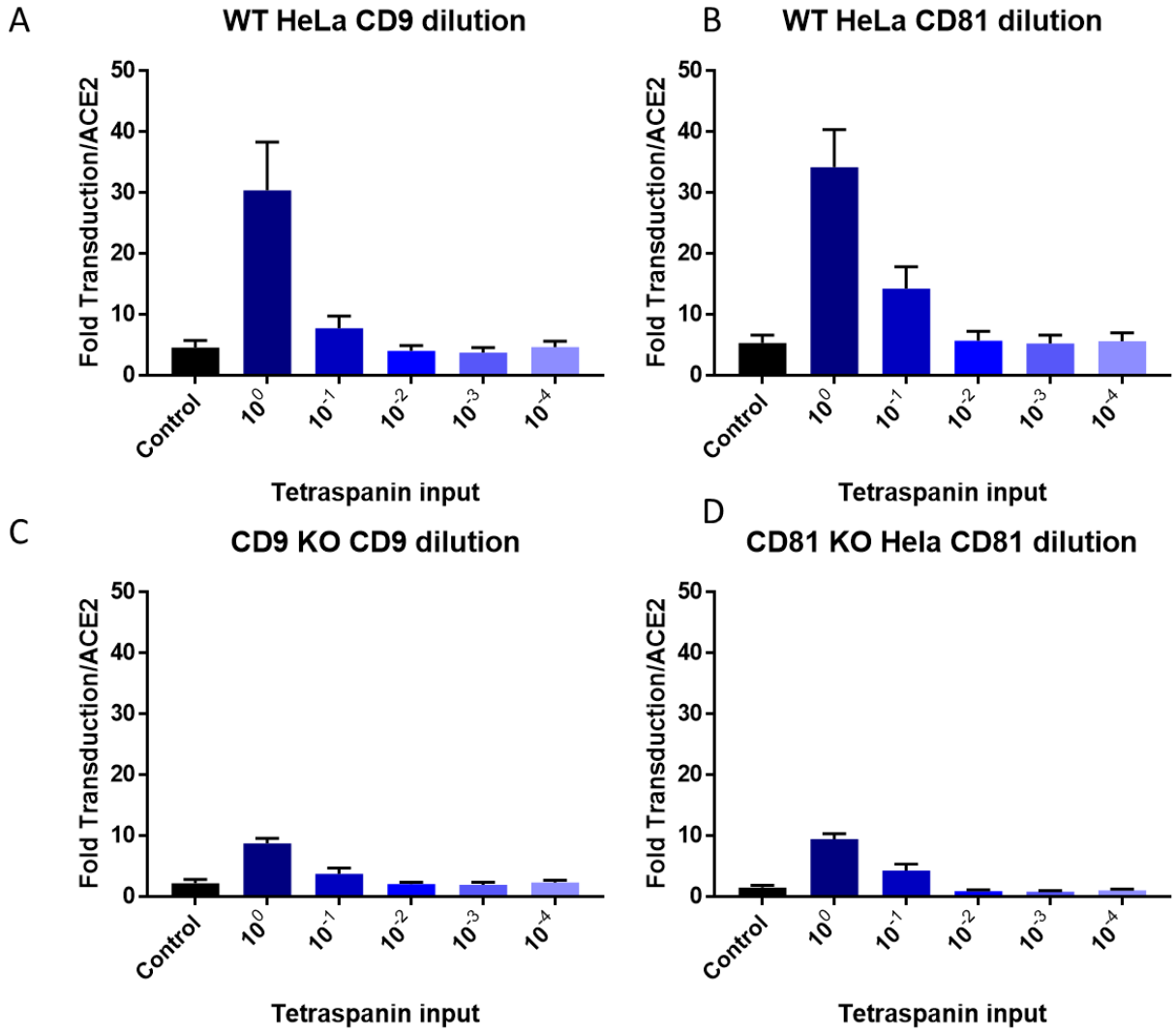

Figure 4: Tetraspanins Promote Receptor Efficient Entry. Ratios were generated from Figure 2, where the ACE2 only control from each WT set (A&B) was classified as "1.0" and was used to normalize all other conditions within the other WT (A&B) or KO cell line conditions (C&D) for each respective tetraspanin dilution set. Ratios were then applied to the values from Figure 3 to observe fold transduction per unit of ACE2.

CHAPTER FOUR

DISCUSSION

Tetraspanins are influential for both cellular processes and viral infection, including that of coronavirus, interacting with many other proteins on the membranes at the cell surface and vesicles alike. With the impact of SARS-CoV-2 has had on the world in recent years, learning more about potential host-factors and their entry processes remains paramount. Throughout this manuscript we showed that excess tetraspanins in both EVs and cells lead to diminished SARS-2 fusion and entry, respectively (Fig 2). We have also shown here that despite decreased entry, the level of transduced RNA remains unchanged (Fig 4). Furthermore, we also demonstrated that the decreased cell entry was due to an overall decrease in overexpressed ACE2 (Fig 3) and therefore, what we can conclude instead is that tetraspanins lead to higher efficiency in SARS-CoV-2 entry into host cells.

Despite these findings, there many gaps present within our knowledge. First of these notable gaps is our understanding of the interactions between tetraspanins is limited. We have shown that increased tetraspanins decrease the quantity of ACE2 within cells (Fig 3). Whether this is caused by an interaction between ACE2 and tetraspanins remains to be seen. We believe that the decrease in target receptor imply that ACE2 is present within TEMs, where tetraspanins are in abundance and in proximity with other interacting surface proteins. To confirm an interaction between ACE2 and nearby tetraspanins, perhaps we could use proximity ligation assays (PLA) or immunofluorescent (IF) microscopy to test this hypothesis. We also do not understand this efficient entry that we found during our transduction assays. One possibility is

that while tetraspanins are limiting entry through membrane fusion, they could instead be promoting entry through the endocytic pathway. CD81 is known to be endocytosed for degradation (Hosokawa et al., 2020). It is plausible that if a viral particle bound to ACE2 is near tetraspanins within TEMs, then they could be endocytosed alongside the tetraspanin and be able to enter the host cell. Additionally, while tetraspanins can recruit TMPRSS2, which is the protease utilized by both MERS-CoV and SARS-CoV-2 to undergo membrane fusion, we were unable to implement this protease due the poor conditions of cells and EVs alike. It is notable that while the D614G and other previous variants prefer membrane fusion as its method of entry, Omicron, the latest predominant variant , has been shown to favor endocytic entry(Meng et al., 2022; Shuai et al., 2022), which could become more relevant as we shift more towards understanding Omicron and future emerging variants. Finally, while our finding gives us some insight into fusion and egress, we believe that these studies could also be applied to better understand the assembly and egress of SARS-CoV-2. Tetraspanins are important markers for the sorting, trafficking, and secretion of cargo (Berditchevski & Odintsova, 2007; Perez-Hernandez et al., 2013). SARS-CoV-2 structural proteins are assembled between the ER, ER-to-Golgi intermediate compartment ERGIC and Golgi(Cortese et al., 2020; Scherer et al., 2022; V'Kovski et al., 2021). One future direction for this would be observe the assembly process and through IF microscopy or other methodologies, observe whether SARS-CoV-2 and tetraspanins co-exists within these areas of viral assembly.

However, we acknowledge that there are some limitations that need to be considered with these results. Firstly, as alluded to preciously, we recognize that these overexpression experiments do not necessarily reflect the environment found in vivo. Furthermore we also understand that while there are cell lines much more innately susceptible to SARS-CoV-2 than

HeLa cells such as Vero-E6 (Yeung et al., 2021), Vero cells are less susceptible to transfection, which was required for us to increase the expression of tetraspanins. Additionally, it was previously noted that CD9 KO HeLa lines were less adhesive than their WT counterparts (Earnest et al., 2017), which was observed during our experiments but we also saw this on CD81 KO HeLa cells. This effect is justified as tetraspanins play roles in cell adhesion (Charrin et al., 2014; Charrin et al., 2009; Reyes et al., 2018; Yáñez-Mó et al., 2009). This led us to decrease the amounts of SFM washes that were performed during the assay performed with the tetraspanin dilution input experiments, so that we would minimize the amount of cell loss from the KO groups. However, the lack of rinses means that there was a higher likelihood of extraneous LgBit or HiBit to remain within the wells during these experiments. In a more ideal scenario, more rinses would be performed to minimize the background signal caused by extracellular reporters. It is important to note that these are not points to invalidate these findings but rather provide better insight for the future investigations within this field. These should serve as a word of caution for those continue this research, so that future experiments can be performed with greater efficiency and accuracy.

Ultimately, there is much work to do as we continue to better understand SARS-CoV-2 its co-factors, and how they interact with one another during viral entry into the cell. However, as we've previously mentioned, these findings provide us with direction of where we can venture next with our research. Since the emergence of SARS-CoV-2 in late 2019, variants have been appearing faster than we can investigate, learn, and divulge our understanding of them. However, understanding both viral entry processes at a broader scale and tetraspanins, who are revealed to have more and more functions as years of research pass, perhaps this can give us the opportunity to become proactive in our research. With the virus constantly evolving and no mutating to evade

our current measures to combat it, such a position would allow the community to be prepared for the possible variants that could arise in the future.

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