SynDIG1: A virion-incorporated reverse topology IFITM protein enhances lentiviral transduction

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Preface

This thesis conforms to the McGill University “Preparation of a Thesis” guidelines for thesis preparation and is written in the traditional monograph style and the works presented are not used for other theses. Chapter 1 provides a background, literature review, rationale and hypothesis. Chapter 2 outlines the methods and materials used in the experimental design. Chapter 3 presents the major findings and corresponding figures, and Chapter 4 discusses the findings and provides a critical review of the results and well as a future direction.

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Abstract:

Low lentiviral transduction efficiency of key cell types such as haematopoietic stem cells poses a significant challenge to efficient ex vivo gene therapy. A great deal of focus has been on the treatment of target cells with small molecules that transiently enhance lentiviral transduction such as rapamycin or cyclosporine H. However, these treatments are often cell type specific and may also impact the viability and pluripotency of the cell population. We present a proof of concept for a novel approach to enhance lentiviral transduction efficiency via the incorporation of a neuron-specific protein SynDIG1 on the viral envelope. This protein possesses the exact reverse topology as the antiviral interferon induced transmembrane protein 3 (IFITM3) and may induce negative membrane curvature of the virion envelope. In support of the hypothesis, virion incorporation of SynDIG1 led to modest increases in transduction efficiency independent of the target cell type. Rationale-based alterations to key residues led to further enhancement of transduction efficiency and transduction of mesenchymal stem cells (MSCs) using modified SynDIG1 was able to enhance transduction efficiency by 4-fold. Further modifications to the protein and refinement of the virus production protocol may continue to enhance the phenotype and lead to the adoption of the approach during commercial lentiviral particle manufacturing.

Résumé:

La faible efficacité de la transduction lentivirale de types de cellules fondamentales tels que la cellule souche hématopoïétique constitue un obstacle notable au succès de la thérapie génique ex vivo. Beaucoup d'emphase a été mise sur le traitement des cellules cibles à l'aide de petites molécules pouvant transitoirement améliorer le processus de transduction lentivirale, comme la rapamycine ou la cyclosporine H. Cependant, ces traitements sont souvent spécifiques à un type de cellule en particulier et peuvent influer sur la viabilité et la pluripotence de la population cellulaire visée. La présente est une démonstration de faisabilité pour une approche innovatrice ayant pour but d'améliorer l'efficacité de la transduction lentivirale via l'incorporation d'une protéine spécifique aux neurones, SynDIG1, au-dessus de l'enveloppe virale. Cette protéine possède une topologie inversée exacte à celle de l'IFITM3 (interferon induced transmembrane protein 3) antivirale et pourrait induire une courbure membranaire négative de l'enveloppe du virion. L'incorporation de la protéine SynDIG1 a généré de modestes améliorations au niveau de l'efficacité de la transduction, indépendamment du type de cellule cible. L'altération justifiée de résidus essentiels a aussi mené à de plus amples progrès au niveau de l'efficacité de la transduction, et la transduction de cellules souches mésenchymateuses (CSM) par l'entremise de SynDIG1 modifiée a permis de multiplier le taux d'efficacité de la transduction par quatre. Davantage de modifications à la protéine ainsi que le peaufinage du protocole de production des virus peuvent perpétuer l'amélioration du phénotype et mener à l'adoption de cette approche lors de la fabrication de particules lentivirales à des fins commerciales.

List of Abbreviations

AAV1	Adeno-associated virus type-1
ADA-SCID	Adenosine deaminase deficiency
ALL	Acute Lymphoblastic Leukemia
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP2	Adaptor Protein 2
BAR	Bin/Amphiphysin/Rvs
CAR-T	Chimeric antigen receptor T-Cells
CFDA	Chinese food and drug administration
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
CsA	Cyclosporine A
CsH	Cyclosporine H
DAG	Diacylglycerol
EPR	Paramagnetic resonance
ER	Endoplasmic reticulum
ESCRT	Endosomal complex required for transport
FDA	Food and drug administration
Gag	Group-associated antigen
SCID-X1	X-linked severe combined immunodeficiency
HEK	Human embryonic kidney
HIV-1	Human immunodeficiency virus type-1
HNSC	Head and neck squamous cell carcinoma

HSCs	Haematopoietic stem cells
HTLV-1	Human T lymphotropic virus type-1
IFITM3	Interferon inducible transmembrane protein 3
Ig	Immunoglobulin
ISG	Interferon stimulated gene
LDLR	Low density lipoprotein receptor
LPC	Lysophosphatidylcholine
LPLD	Lipoprotein lipase deficiency
LTR	Long-terminal repeat
MSCs	Mesenchymal stem cells
MLV	Murine leukemia virus
MVB	Multivesicular bodies
NMDA	N-methyl-D-aspartate (NMDA)
NMR	Nuclear magnetic resonance
OSBP	Oxysterol binding protein
PBST	Phosphate buffered saline, 0.1% Tween 20
PtdSer	Phosphatidylserine
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
RAFI	Amphipathic fusion inhibitors
RT	Reverse transcriptase
SAMHD1	SAM and HD domain containing deoxynucleoside
SD	Standard deviation
scFv	Short chain variable antibody fragment

SIRP- α	Signal-regulatory protein alpha
SIV	Simian immunodeficiency virus
SynDIG1	Synapse differentiation inducing gene 1
TSP-1	Thrombospondin 1
VAPA	Vesicle associated protein A

Chapter 1

Introduction

1.1 A brief account of gene therapy

In the past 20 years, gene therapy – the precise introduction of a transgene into a patient to combat disease – has emerged as a promising approach for the treatment and cure of previously incurable illnesses[1,2,3,4,5,6]. The applications of genetic precision medicine can be seen in the development of viral based gene therapies for the treatment of monogeneic diseases as well as in the design of targeted immunotherapies such as chimeric antigen receptor T cells (CAR-T) for the treatment of blood cancers[7]. As of February 2016, there have been over 2300 gene therapy clinical trials conducted worldwide[8]. An initial clinical trial using the gammaretroviral vector murine leukemia virus (MLV) to treat X-linked severe combined immune deficiency (SCID-X1) – where the γc gene encoding the receptor subunit for interleukin-2, -4, -7, -9, -15, -21 is non-functional – was deemed highly successful[9,10,11,12,13,14,15,16]. However, it soon became apparent that there were serious safety concerns regarding the potential of the inserted transgene to activate proto-oncogenes or inactivate tumor suppressor genes[17,18,19]. This genotoxic effect – termed insertional mutagenesis – drove the expansion of white blood cells and was responsible for inducing leukemia in 3 of 20 patients who received the treatment, resulting in one death[20,21]. However, the past three decades has seen numerous advances in the field that include the invention of the human immunodeficiency virus type-1 (HIV-1) derived lentiviral vectors that to date have not been shown to induce insertional mutagenesis[22,23,24,25,26,27,28]. Viral vector production has also been greatly improved to eliminate the possibility of viral reversion[29,30]. New gene editing platforms

such as the clustered regularly interspaced short palindromic repeats (CRISPR) have further added to the potential of pinpoint genetic alterations [31,32,33,34,35,36,37,38,39,40,41,42,43,44]. The Chinese food and drug administration (CFDA) was the first to approve a commercialized gene therapy Gendicine in 2003 for the treatment of head and neck squamous cell carcinoma (HNSCC)[45]. Using engineering adenoviruses that carry a wild-type p53 gene, transduction with this vector causes p53 deficient cancer cells to undergo apoptosis[46]. Gendicine can be used in combination with existing chemotherapy and radiotherapy approaches and has seen positive clinical outcomes. Since then a number of other gene therapies have also been approved, such as Alipogene tiparvovec (trade name Glybera) in the European Union in 2012 for the treatment of lipoprotein lipase deficiency (LPLD)[47,48, 49]. Glybera uses an adeno-associated virus 1 (AAV1) to introduce a functional copy of the lipoprotein lipase gene into the patient's cells[50]. In 2016, Strimvelis, another gene therapy using an *ex vivo* stem cell expansion for the treatment of adenosine deaminase deficiency (ADA-SCID) was approved by the European Commission[51,52]. With this approach, CD34+ haematopoietic stem cells are extracted from patients and transduced with gamma-retroviral particles that deliver a functional copy of the adenosine deaminase (ADA) gene[53]. Following transduction and expansion of the cellular population, the cells are transplanted back into the patient[54]. Clinical trials reported a 100% survival rate and 75% of treated individuals no longer require further treatment with enzyme replacement therapy[53]. Transgene insertion has also been adapted to insert an artificial protein, the chimeric antigen receptor into T cells for the treatment of B-cell acute lymphoblastic leukemia (ALL)[54,55,56,57,58,59]. This approach, named tisangenlecleucel (trade name Kymriah) fuses a short chain

variable antibody fragment (scFv) targeting the B-cell surface protein CD19 with a T-cell receptor[60,61]. This approach has been shown to be highly effective in a phase II clinical trial[62,63]. In 2018, the food and drug administration (FDA) approved Kymriah for treatment of late stage B-cell ALL[64].

1.2 Challenges facing Gene Therapy and Current Strategies

Although the field of gene therapy – from pre-clinical conceptualization to commercialization – has progressed rapidly, there remain a number of challenges to overcome in order to achieve cost-effective and accessible treatments[52,56]. One of the most important hurdles in virus based cell therapy is the low transduction efficiency of target cells[65,66]. Owing to intrinsic properties of different cell types, many clinically relevant cells such as haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and T-cells are difficult to transduce efficiently[65].

A number of strategies have been developed to enhance the efficiency of lentiviral transduction *in vitro*, ranging from physical and protocol-based approaches to the addition of small molecules[67,68,69]. There have been a number of reports that aim to optimize the transduction conditions of different cell types[70,71]. The addition of transduction promoting polycations such as polybrene reduces the negative-negative charge repulsion between the viral and cellular membrane[70]. However, polybrene can be toxic to certain cell types and has been shown to limit the proliferation of MSCs[72,73]. Other small molecules such as rapamycin, and cyclosporine A (CsA) has shown only modest effects on the order of ~2-3 fold enhancement[74,75]. However, rapamycin is a well-characterized inducer of autophagy and may not be the optimal

molecule to administer to stem cells[76]. And while the very recently reported cyclosporine H (CsH) was shown to enhance lentiviral transduction in HSCs by 10-fold, a very high concentration (8mM) of the molecule was used and raising CsH dose further led to cytotoxic effects[77]. There have also been reports of using microfluidics to bring virus particles and cells in close proximity, which has demonstrated modest success[69].

Other groups have focused on enhancing the production capabilities of virus producing cells using small molecules such as caffeine or sodium butyrate, which are important to alleviating the high cost of lentiviral particle production[67,23]. Siglec-9, a sialic acid binding immunoglobulin (Ig) superfamily lectin, has also been shown to enhance lentiviral particle production[68]. More recently, a trans-acting protein Tax from the human T-lymphotrophic virus type-1 (HTLV-1) can also increase lentiviral transduction efficiency by greatly enhancing transcription from cytomegalovirus (CMV) and HIV-1 long-terminal repeat (LTR) promoters[78]. Co-transfection of Tax encoding plasmid during lentiviral particle production increased virion production and release by 10-fold, albeit with concerns that Tax protein may be incorporated into the lentiviral particle and lead to adverse signal transduction in target cells[78].

While the aforementioned studies address numerous important issues surrounding the production and transduction efficiency of lentiviral particles, no studies so far have attempted to alter the nature of the lentiviral particle itself. There have been reports of incorporating the simian immunodeficiency virus (SIV) accessory protein Vpx into lentivial particles to degrade SAM and HD domain containing

deoxynucleoside triphosphate triphosphohydrolase 1 (SAMHD1) in target cells – a critical restriction factor of the reverse transcription step of retroviral gene transfer[79,80,81]. But this avenue addresses a blockade of gene transfer rather than enhancing the intrinsic efficiency of gene transfer and is again somewhat dependent on cell type. An approach that can be ubiquitously applied to any target cell type and that is not based on increasing the titres of virus particle production may be a more appealing means of addressing the transduction efficiency bottleneck in gene therapy.

1.3 Virus Fusion and Membrane Curvature

An enduring concept in biochemistry is the existence of a rate-limiting step in any biosynthesis reaction[82]. These steps are virtually always at the beginning, since it would be frustrating and energetically nonsensical to be in equilibrium with a product until significant resources have been expended[83]. Since the first step of a productive infection/transduction must necessarily be virus fusion, methods to enhance the basal efficiency of membrane fusion at the level of the individual viral particle may be an effective means of enhancing transduction efficiency.

The properties of lipid bilayers and its impact on membrane fusion have been investigated since the early 70s[84,85,86,87]. Since then a number of groups have found that a critical determinant of membrane fusion potential is the curvature of the lipid bilayer[88,89,90,91,92,93]. By convention, positive membrane curvature is defined as curvature towards the center of the enclosure (either towards the cytoplasm or in the case of the viral envelope towards the virion core). Negative membrane curvature is

then curvature away from the cytoplasm or virion core. Since all free virus particles characterized thus far are metabolically inactive, the entirety of the required energy to overcome the unfavourable conformation of membrane hemifusion and fusion pore formation is then fully reliant on the energy of viral fusion peptides[94,95]. One of the initial steps of membrane fusion is to bring the virus and cellular membranes together, the negative-negative charge repulsions of the phospholipid head groups is a critical barrier to successful fusion[96]. Positive membrane curvature leads to a "flatter" membrane and will thus require a higher overall surface area to come in close contact with the opposing membrane[97]. This increase in the required potential energy may result a majority of virions being unable to perform successful viral fusion[98]. On the other hand, negative membrane curvature leads to the opposite effect and lowers there required energy for successful fusion of viruses[90]. Therefore, a simple and reproducible method to induce negative membrane curvature on the viral envelope may be an effective means to increase lentiviral transduction efficiency.

1.4 Factors influencing Membrane Curvature

Lipid composition at the local membrane is important, where both the size of the lipid head group and the length of the acyl chain can contribute to the local membrane curvature (Figure 1). Of the common phospholipids, phosphatidylserine (PtdSer) and phosphatidylcholine (PtdCho) do not directly contribute to membrane curvature since the width of the phosphate head group and acyl fatty acid chain are more or less the same[99]. Phosphatidylethanolamine (PtdEtn) and diacylglcerol (DAG) possess larger acyl groups than phosphate head groups and so would contribute to curvature that

results in a squeezing of the head group (Figure 1)[100]. Finally, phospholipids with larger head groups than acyl groups, such as the phosphatidylinositol phosphates (PtdIns) and lysophosphatidylcholine (LPC) bend towards the acyl chains (Figure 1)[100]. In the absence of protein, phospholipid species composition on a lipid bilayer is responsible for its basal curvature (Figure 1)[100].

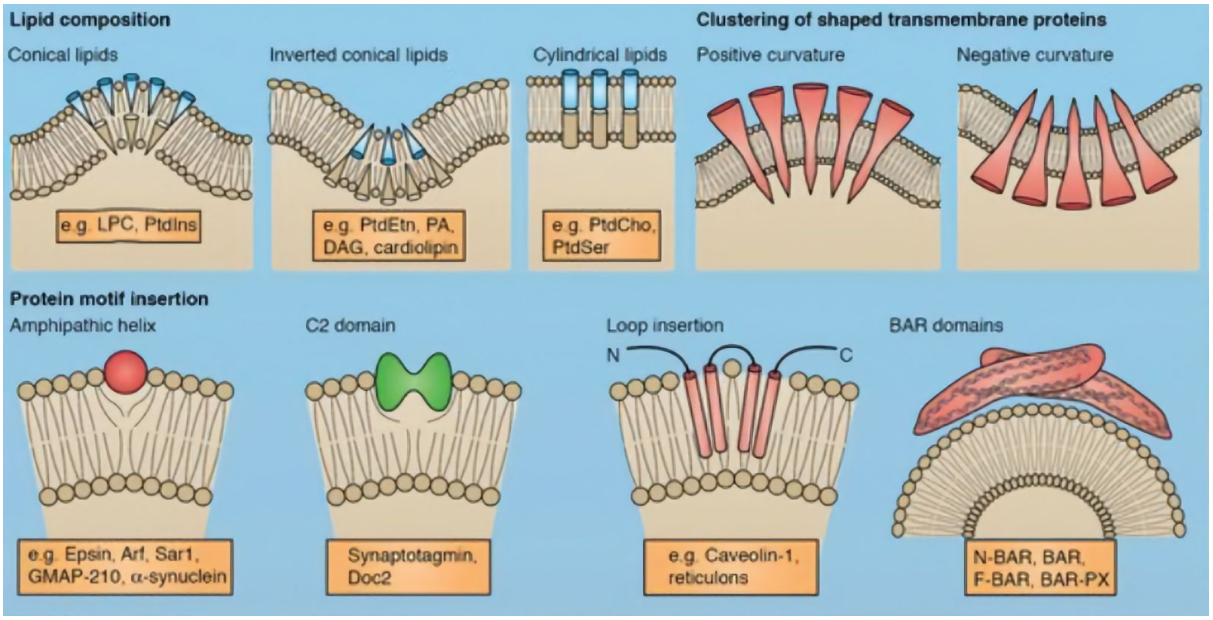

Figure 1. Phospholipids and Proteins that Affect Membrane Curvature. Depending on the size of the phosphate head groups compared to the fatty acid chains, different phospholipids exert either negative or positive membrane inducing effects on the lipid bilayer. Clustering of transmembrane proteins that are either outside-heavy or inside-heavy would lead to spontaneous membrane curvature generation. Amphipathic helices, C2 domains, loop insertions and BAR domains have all been shown to alter local membrane curvature based on the side of the lipid bilayer of the insertion. Adapted from: McMahon, Harvey T., and Emmanuel Boucrot. "Membrane Curvature at a Glance." *Journal of Cell Science* 128.6 (2015): 1065. Print.

Lipid composition on the lipid bilayer can be actively altered by lipid flippases and floppases and unequal distributions of one particular species of phospholipid would

result in curvature[101,102]. PtdSer is actively maintained on the inner leaflet and its exposure on the other leaflet is usually indicative of apoptosis[103,104]. Integral membrane proteins as well as membrane-associated cytoplasmic proteins have been shown to exert moderate to significant influence on membrane curvature[100]. For example, receptors or transporters that are asymmetric (ie. a protein that is larger on one side of the membrane than it is on the other) can alter membrane curvature by exerting physical pressure on the lipid bilayer, such as the clustering of transferrin and low density lipoprotein receptors (LDLR) and subsequent pit formation during clathrin-mediated endocytosis[105]. Integral membrane proteins can also form scaffolds via multimerization that may force the lipid bilayer to adopt a particular curvature[106]. Integral membrane proteins that contain amphipathic helices or other intramembrane domains as part of its structure can very effectively alter membrane curvature via their hydrophobic residues[107]. These proteins force an imbalance on the lipid bilayer and drive a wedge into the membrane, resulting in curvature of the lipid bilayer that can be either positive or negative depending on the location of the amphipathic helix or intramembrane domain[107]. Examples include epsins, endophilins, α-synuclein, annexin B12 and the endosomal complex required for transport (ESCRT)-III subunit CHMP-4B. Bin/Amphiphysin/Rvs (BAR) domains on proteins such as amphiphysin form dimers that interact with lipid bilayer via electrostatic interactions between the positively charged amino acids such as lysine and arginine and negatively charged phospholipids, leading to the adoption of the intrinsic curvature of the proteins on the membrane[100].

The cytoskeletal network actively affects membrane curvature, a phenomenon that can be readily observed in structures such as the endoplasmic reticulum (ER), Golgi, filopodia and lamellipodia[108]. The cytoskeleton also maintains the shape of cellular membranes by providing structural support for certain morphologies[108]. However, these phenomena cannot be applied to the engineering of lentiviral particles due to the absence of metabolic activity of viruses as well as the macroscopic nature of cytoskeletal alterations.

Of the various methods to alter membrane curvature in the cell, both alterations to lipid composition and protein-based approaches may be translated onto the lentiviral particle to induce negative membrane curvature. However, a protein-based approach may be more advantageous due to the comparative ease with which protein expression can be manipulated in the current laboratory molecular protocols.

There has been a number of precedents for utilizing protein incorporation on to the lentiviral envelope as a means of altering the properties of the produced virion. Following the production of structural proteins and viral genomic RNA, lentiviral particle release is mediated by the group-associated antigen (Gag) polyprotein, which self-assembles at the plasma membrane following its translation and contains a number of motifs that are required for the recruitment of the ESCRT pathway proteins[109,110,111,112,113]. ESCRT is the collective term for a set of three protein complexes that are responsible for reverse membrane scission (ie. budding or fission of a membrane away from the cytoplasm)[114,115]. ESCRT has been shown to function in budding of HIV-1 and lentiviral particles from the plasma membrane, the

generation of multivesicular bodies (MVB) and cleavage of daughter cells following cytokinesis[109,114, 116]. The p6 region of Gag contains the P(T/S)AP motif, which directly binds the ESCRT-I complex protein TSG101 to recruit the recruitment of the complex to the plasma membrane[117,118]. The YPXL domain (where X can be any amino acid) on the p9 region of Gag can bind and recruit ALIX, a cellular protein that initiates ESCRT protein assembly[119,120,121]. A further as yet uncharacterized motif is responsible for the recruitment of NEDD4L, an E3 ubiquitin ligase that is also required for virion release and other ESCRT complex functions, although the mechanism and specific protein-protein interactions are not well-understood[122,123,124].

Since HIV-1 -- and by extension lentiviral particles -- bud from the plasma membrane, highly expressed integral membrane proteins that have significant subcellular localization at the cellular surface may be readily incorporated on the lentiviral envelope[125]. Indeed, a number of groups have utilized this concept to incorporate proteins of interest by expressing the proteins in virus producing cells during virus production. *Rodriquez-Frade et al.* explored the possibility of incorporating FasL, a membrane bound inducer of apoptosis, as a means of maintaining T-cell homeostasis[126]. The approach demonstrated efficacy in a murine model of arthritis, where local administration of FasL incorporated lentiviral particles led to appreciable reductions of inflammation and anti-collagen II IgG. More recently, virion incorporation of CD-47 -- a “don’t eat me” signal protein that binds thrombospondin-1(TSP-1) and signal-regulatory protein alpha (SIRP-α) -- reduced virion uptake by professional phagocytes and resulted in an increase of *in vivo* lentiviral particle gene delivery to the liver in a non-human primate model[127,128].

These findings demonstrate the feasibility of utilizing virion incorporation engineering as a means to alter membrane curvature.

1.5 IFITM3: a Positive Membrane Curvature Inducing Protein

One of the key restriction factors of enveloped virus fusion is the interferon inducible transmembrane protein 3 (IFITM3) — one the only cellular factors identified thus far that blocks the entry step of lentiviruses. IFITM3 belongs to a family of small, single pass transmembrane proteins that block the critical fusion step of various enveloped viruses such as dengue virus, West Nile virus, Ebola virus, influenza A virus, vesicular stomatitis virus, and human immunodeficiency virus (HIV)[130,132,133,134,135,136,137]. Since VSV-G pseudotyped lentiviral particles are derived from HIV-1, IFITM3 has been shown to be one of the major restriction factors that limit lentiviral transduction efficiency[65]. What is more, it has recently been reported that a subset of interferon stimulated genes (ISGs) are intrinsically expressed in a number of stem cell lineages in the absence of interferon stimulation[138].

IFITM3 ranks consistently among the highest expressed genes and may be one of the key limiting factors of transduction efficiency in CD34+ haematopoietic stem cells, a key cell type in ex vivo stem cell therapy[77,138]. The precise mechanism of IFITM3 restriction has been the subject of intense investigation. One report suggests IFITM3 enhances cholesterol accumulation in the lipid bilayer of late endosomes by interacting with vesicle associated protein A (VAPA), which in turn disrupts VAPA association with oxysterol binding protein (OSBP)[137]. Accumulation of cholesterol in lipid bilayers leads to membrane rigidity that would in turn increase the energy

required to execute membrane fusion[137]. However, a number of other studies have failed to reproduce the findings, and instead show that membrane curvature alterations may be one of the key mechanisms to inhibit virus entry[139]. Palmitoylation has also been implicated in IFITM3 restriction of viral entry, as this reversible post-translational modification enhances the association of proteins with the lipid bilayer[95,140]. IFITM3 -- along with its other family members IFITM1, IFITM2, IFITM5 and IFITM10 -- belong to the dispanin family of integral membrane proteins, so named via bioinformatics prediction of two putative transmembrane regions in their protein coding sequence[141]. However, subsequent analysis have demonstrated that while the second predicted hydrophobic segment is indeed a full-pass transmembrane domain, the first hydrophobic segment is rather an intramembrane domain that spans the inner leaflet of the lipid bilayer (Figure 2)[142]. The specific protein topology of IFITM3 -- resembling and “L” shape -- may be critical for the membrane curvature alterations of this protein.

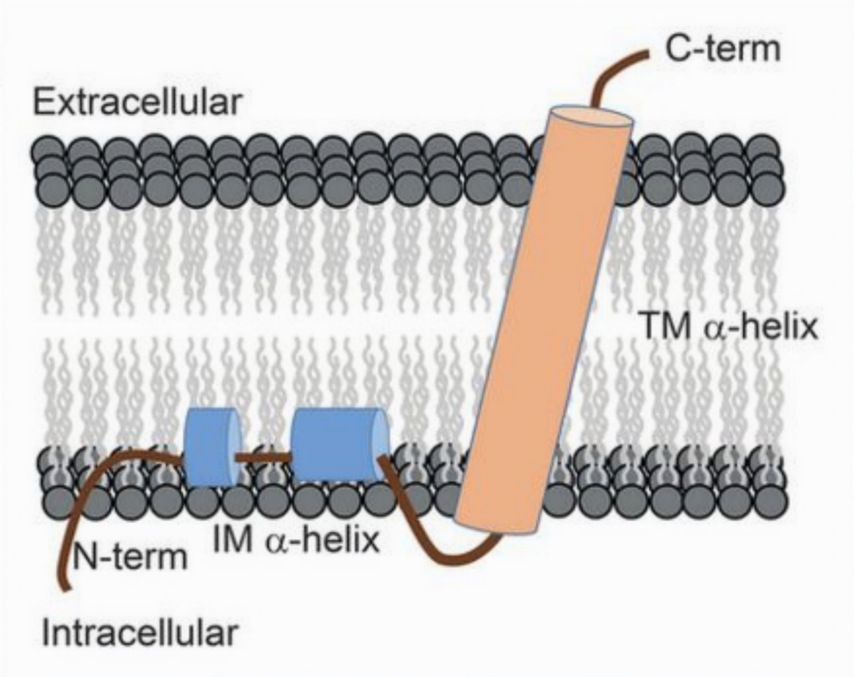

Figure 2. Schematic of IFITM3 membrane topology. Combined nuclear magnetic resonance (NMR) and paramagnetic resonance (EPR) studies have confirmed that the topology of IFITM3 consists of an N-terminus intramembrane segment followed by an intracellular loop and a full-pass transmembrane domain towards the C-terminus. The overall topology of IFITM3 can be conceived of as an "L" shaped integral membrane protein with an intramembrane domain embedded in the inner leaflet. Adapted from: Ling, Shenglong, et al. "Combined Approaches of Epr and Nmr Illustrate Only One Transmembrane Helix in the Human Ifitm3." *Scientific Reports* 6 (2016): 24029. Print.

1.6 IFITM3 Incorporation on the Virus Envelope

Immunohistochemistry has shown the subcellular localization of IFITM3 to be both at the late endosome as well as the plasma membrane. Interestingly, IFITM3 can be readily incorporated onto the envelope of HIV-1 virions (Figure 3)[143]. High expression of IFITM3 in virus producing cells would lead to accumulation of IFITM3 on the plasma membrane and subsequent incorporation during virus budding. It therefore follows that a hypothetical protein whose subcellular localization is also largely on the plasma membrane but whose topology is the exact opposite as IFITM3 would induce negative membrane curvature. Furthermore, if this protein were to be incorporated onto the envelope of VSV-G pseudotyped lentiviral particles, the intrinsic infectivity of these virions may be elevated.

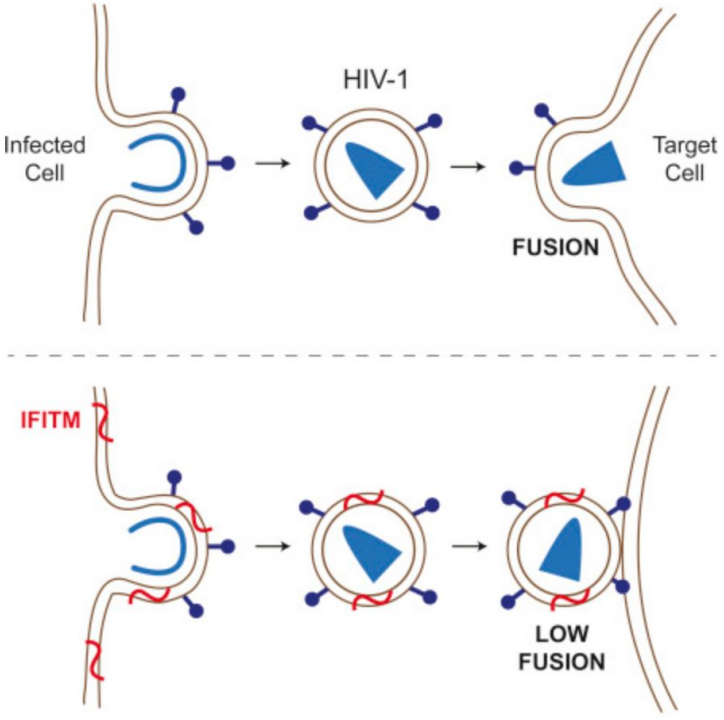

Figure 3. IFITM3 incorporated HIV-1 Virions Demonstrate Decreased Fusion of Target Cells. A high level of IFITM3 expression in the virus producing/infected cell would lead the protein's incorporation on the virus envelope, increased lipid order and increased positive membrane curvature, resulting in decreased viral fusion with the target cell. Adapted from: Compton, Alex A, et al. "Ifitm Proteins Incorporated into Hiv-1

Virions Impair Viral Fusion and Spread." *Cell Host & Microbe* 16.6 (2014): 736-47. Print.

1.7 SynDIG1: a Reverse Topology IFITM3 Protein

After a search of the relevant literature, we stumbled upon one elegant review paper of IFITM3 localization, topology and trafficking. *Chesarino et al.* highlighted the existence of a protein that does indeed appear to possess the exact opposite topology as IFITM3 (Figure 4)[144]. This protein — termed synapse differentiation inducing gene 1 (SynDIG1) — is a neuron specific protein whose only reported physiological function thus far is to recruit glutamatergic a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to developing dendrites[145]. However, the mechanism of this recruitment is unknown and recent reports suggests that SynDIG1 may also recruit the glutamatergic N-methyl-D-aspartate (NMDA) receptors by a general, as yet uncharacterized mechanism[146]. However, SynDIG1 topology and localization has been extensively characterized, possessing a type II transmembrane topology with a full pass transmembrane domain followed by an intramembrane domain towards the C-terminus[145]. Immunohistochemistry in human embryonic kidney (HEK)293T cells further indicated a subcellular localization of the early endosome and plasma membrane[145]. Palmitoylation at cysteine residues 191 and 192 has been implicated in activity dependent trafficking of SynDIG1, and may function in a similar fashion as the same modifications on IFITM3 and enhance lipid associations (Figure 4)[147].

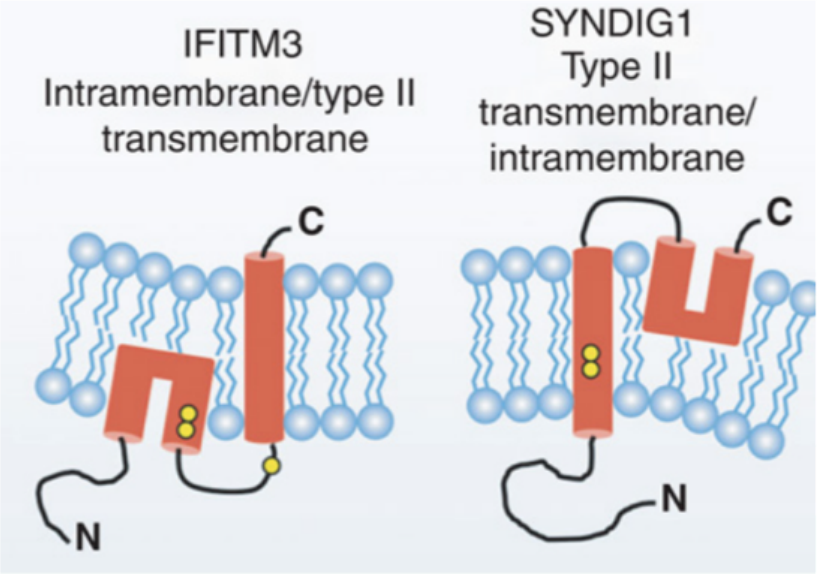

Figure 4. Comparing IFITM3 topology against SynDIG1 topology. The topology of IFITM3 can be conceived of as an "L" shaped integral membrane protein with an intramembrane segment on the inner leaflet. SynDIG1 is then a reverse "L" shaped protein with an intramembrane segment embedded on the outer leaflet. Yellow circles represent cysteines corresponding to residues 71,72, and 105 on IFITM3 and residues 191 and 192 on SynDIG1. These residues have been shown to be palmitoylated to enhance their associations with the lipid bilayer. Adapted from: Chesarino, Nicholas M et al. "Regulation of the trafficking and antiviral activity of IFITM3 by post-translational modifications." *Future microbiology* vol. 9,10 (2014): 1151-63. doi:10.2217/fmb.14.65

1.8 Rationale and Hypothesis

The low lentiviral transduction efficiency of certain cell types such as CD34+ hematopoietic stem cells and human primary macrophages have led to numerous lines of development in methods to overcome this bottleneck. While numerous methods have been explored ranging from chemical to physical to protein transduction, there have been no published attempts to alter the intrinsic transduction efficiency of the lentiviral particles at the level of the virion. Since IFITM3 incorporation has been characterized to exert a restrictive effect on the side of the virus envelope, a similar but opposite phenomenon may be achieved via the incorporation of SynDIG1, which is localized in part on the plasma membrane. Therefore, SynDIG1 overexpression in HEK293T cells during virus production may lead to its incorporation on the envelope of VSV-G pseudotyped lentiviral particles. Since the topology of these proteins is the exact reverse of each other, SynDIG1 incorporation may lead to an enhancement in lentiviral transduction efficiency that is based on enhancing the intrinsic infectivity of these lentiviral particles. Furthermore, since the manipulation is solely based upon alterations to lentiviral particles, a potential enhancement in lentiviral transduction may also be cell type independent and may provide a general mechanism to enhance lentiviral transduction.

Chapter 2

Materials and Methods

2.1 Antibodies

The primary antibodies used for immunoblotting are: mouse monoclonal anti-b-Tubulin IgG (Santa Cruz Biotechnology) (1:10000), rabbit polyclonal anti-SynDIG1 IgG (Alomone Labs) (1:1000), and mouse monoclonal anti-HIV-1 p24 (Abcam) (1:10000) and rabbit monoclonal anti-IFITM3 IgG (Cell Signalling) (1:10000). The secondary antibodies used for immunoblotting are: horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare) (1:10000) and horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare) (1:100000).

2.2 Cell Lines

Human embryonic kidney (HEK293T) cells and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% Fetal Bovine Serum (FBS) (Termo Fisher) and 1% Penicillin/Streptomycin (P/S) (Thermo Fisher).

SupT1 cells, MT4-QCXIP cells and MT4-IFITM3 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS (Termo Fisher), 1% Glutamine (Termo Fisher) and 1% P/S (Thermo Fisher).

2.3 Primary Cells

Human mesenchymal stem cells (MSCs) were maintained in Advanced Minimum Essential Medium (Alpha-MEM) (Thermo Fisher) supplemented with 20% FBS (Thermo Fisher), 1% Glutamine (Thermo Fisher) and 1% P/S (Thermo Fisher). MSCs were a gift from Dr. Nicoletta Eliopoulos.

2.4 Plasmids and Transfection

Mouse SynDIG1 (mSynDIG1) (NCBI Accession NM_001363096) was obtained from the laboratory of Dr. Elva Diaz (University of California, Davis School of Medicine) in the pHM6 vector (AddGene) and subsequently cloned into pcDNA3.1+ vector (Thermo Fisher) using the following primers:

pcDNA3.1-SD1-BamHI-For	5'-cataatggatcctacccatacgacgtcccagac-3'
pcDNA3.1-SD1-EcoRI-Rev	5'-cgggcggaatcctcacaggtggttgtttttg-3'

Human IFITM3 (NCBI Accession JQ610621) was cloned into pcDNA3.1+ vector using the following primers:

pcDNA3.1-TM3-BamHI-For	5'-cagtatggatccatggattacaaggatg-3'
pcDNA3.1-TM3-EcoRI-Rev	5'-cagagtgaattcctatccataggcctggaa-3'

Human SynDIG1 (hSynDIG1) was obtained from Genscript (NCBI Accession NM_024893.3) (Clone ID OHu04268) in pcDNA3.1+/C-(K)DYK vector and modified

into two additional constructs (G237I, T238V-hSynDIG1 and YEML-SynDIG1) using the following primers

hSD1-G237I,T238V	cctggcagtgctgtccatcaccattatcgtcggcgtctatgtgggcgtggccgtgg
hSD1-YEML-Rev	gaatatggatccgccaccatgtatgagatgctcgatggcatcattgaacagaag
hSD1-Rev	gatatagaattcttatcacttatcgtcgtcatccttgtaatccaggtggttgttcttggagaggtaggcgatg

Plasmids used to produce lentiviral particles were psPAX2 (Addgene), pLVX-TetOn-Luc (Takara Bio) and pCMV-VSV-G (Addgene).

Viruses were produced by transient transfection into HEK293T cells using polyethylenimine(PEI) as per the manufacturer's protocols.
Briefly, HEK293T cells were seeded in 35x10mm dish 20-24 hours before transfection. Cells were then transfected with psPAX2, pLVX-TetOn-Luc and pCMV-VSV-G in ration of 10:10:1 as well as pcDNA3.1+ mSynDIG1 or pcDNA3.1+ IFITM3 or pcDNA3.1+ vector and a DNA:PEI ratio of 6:1 mixed in 500µl OptiModified Eagle Medium (opti-MEM) (Invitrogen). For BlaM-Vpr incorporated lentiviral particles, HEK293T cells were transfected with psPAX2, pLVX-TetOn-Luc, pCMV-VSV-G, and pCMV4-Blam-Vpr (Addgene) at a ratio of 10:10:1:3 as well as cDNA3.1+ mSynDIG1 or pcDNA3.1+ IFITM3 or pcDNA3.1+ vector and a DNA:PEI ratio of 6:1 mixed in 500µl Opti-MEM. The mixture was incubated at room temperature for 15 minutes and slowly introduced each dish. Transfection media was replaced with fresh DMEM 6 hours post transfection. The supernatant was harvested at 48 hours post

transfection, filtered with a 2 micron syringe filter (Thermo Fisher), aliquoted and stored at -80°C.

2.5 Transduction

Adherent cells were seeded in 12 well plates 24 hours before transduction. Suspension cells were seeded in 24 well plates immediately prior to transduction. Cells were transduced with reverse transcriptase (RT) normalized lentiviral particles for 14-16 hours. Cells were then washed once in phosphate-buffered saline (PBS) (Thermofisher) and replaced with fresh media supplemented with 500ng/ml doxycycline. 48 hours post transduction, wells were washed once with PBS, lysed and luciferase measurements analyzed by a luminometer (Promega).

2.6 Western Blot

HEK293T cells were harvested and washed once in cold PBS and were lysed for 30 minutes in radioimmunoprecipitation assay (RIPA) buffer on ice supplemented with complete protease inhibitor. Cell lysates were purified by centrifuging the lysed cells at 32000rpm, 4°C for 20 minutes. 150µl of the purified cell lysates were mixed with 50µl 4Xloading buffer. 4ml of virus supernatant was ultracentrifuged under a 20% sucrose gradient at 35,000 rpm for 70 minutes. The pellet was resuspended in 60ul 1X loading buffer. Samples were loaded and run on SDS-polyacrylamide gels (SDS-PAGE). Gels were then transferred to PVDF membranes (Roche) and blocked in 5% non-fat milk dissolved in phosphate buffered saline, 0.1% Tween 20 (PBST) at room temperature for 1 hour or at 4°C overnight. The membranes were then incubated with the primary antibody at RT for 2 hours at

room temperature or at 4°C overnight, washed 3 times with PBST solution and then incubated with secondary antibody conjugated to horseradish peroxidase for 45 minutes. Membranes washed 3 times with PBST and were then blotted via chemiluminescence (ECL) substrate (PerkinElmer) as per the manufacturer's instructions.

2.7 HIV-1 Entry Assay

1*10^6 SupT1 cells were seeded in 24-well plates immediately prior to transduction. RT normalized lentiviral particles were used to transduce target cells supplemented with 5ug/ml polybrene, spin infected at 1800rpm for 45min, and incubated at 37°C in a 5% CO2 incubator for 2 hours. Cells were then washed in CO2 independent media (Thermo Fisher) and incubated for 1 hour in the dark with CCF2-AM loading solution[148]. This was followed by a single wash with development media (10ul probenecid, 100ul FBS, 1ml CO2 independent medium) and incubation overnight with development media in the dark. Samples were then collected by centrifugation at 1500rpm and washed twice with PBS supplemented with 2% FBS and finally fixed in PBS 4% paraformaldehyde (PFA), 1% FBS.

Chapter 3 Results

3.1 Mouse SynDIG1 co-transfection enhances lentiviral transduction efficiency in both adherent and suspension cell lines

The concept of SynDIG1 incorporation on lentiviral particles is illustrated in Figure 5. As had previously been established, IFITM3 overexpression in virus producing cells would lead to its incorporation onto the envelope of lentiviral particles, increase positive membrane curvature, and decrease fusion and by extension transduction efficiency.

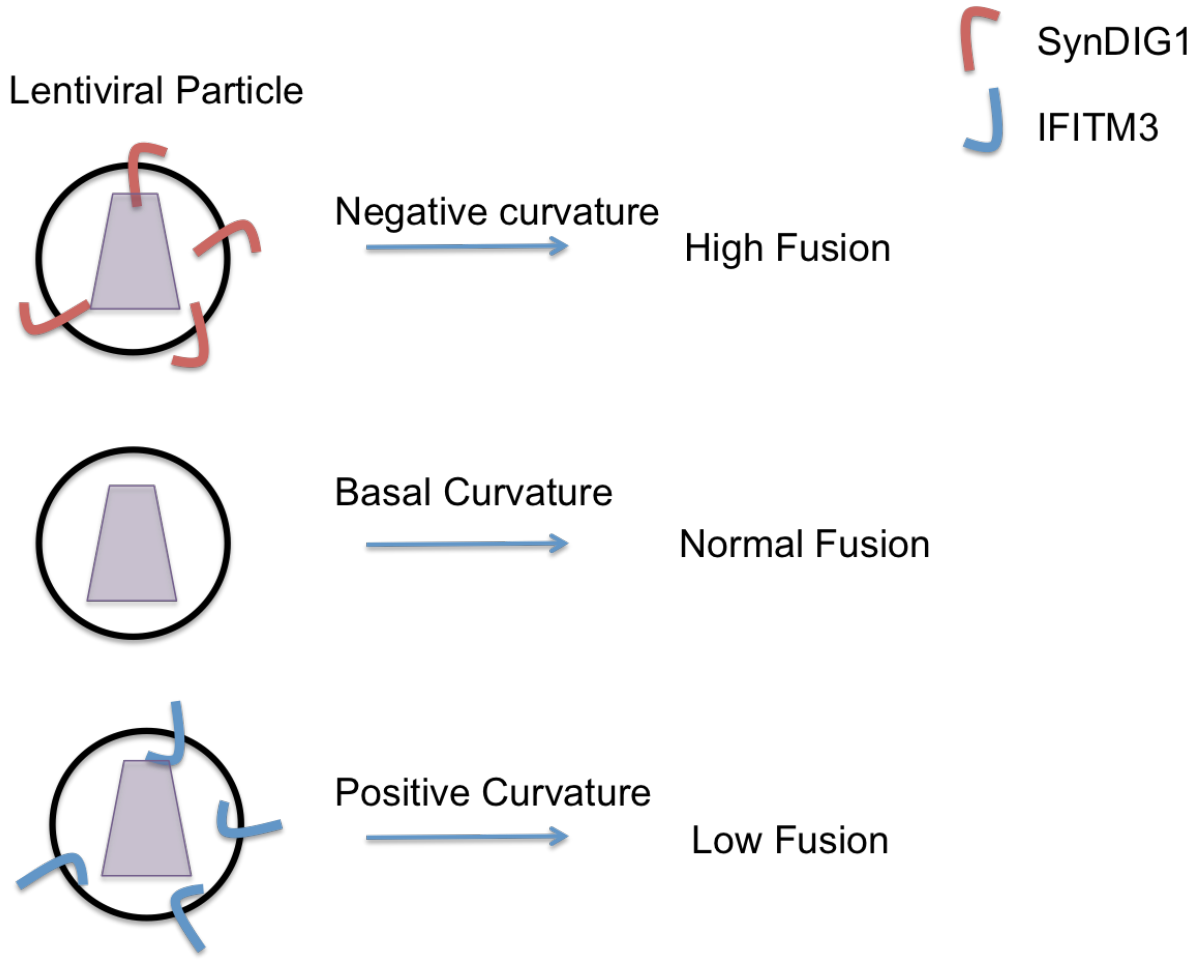

Figure 5. Schematic illustrating the effect of SynDIG1 incorporation, no incorporation, and IFITM3 incorporation on virus fusion efficiencies. SynDIG1 incorporation during lentiviral production in virus producing cells may lead to increased positive membrane curvature and enhanced lentiviral transduction efficiency. In the absence of protein incorporation (pcDNA3.1 vector control) the membrane curvature of the lentiviral envelope should be at its basal state. IFITM3 incorporation has been shown to decrease fusion efficiency of HIV-1 viruses and is included here as a positive control.

We surmised that overexpression of SynDIG1 may also lead to its incorporation on lentiviral particles, increase negative membrane curvature, and enhance membrane fusion and transduction efficiency. To test our hypothesis, we co-transfected mSynDIG1 with plasmids encoding the major components of lentiviruses to produce VSV-G pseudotyped lentiviral particles and harvested the supernatant 48 hours post transfection. The transfer plasmid of these viral vectors (pLVX-TetOn-Luc) contains a luciferase reporter gene under the control of a doxycycline inducible promotor. Using the reverse transcriptase (RT) assay, we normalized the amount of lentiviruses produced and using RT-normalized virus levels transduced HEK293T, HeLa, and SupT1 cells (Figure 6) and measured luciferase values following transduction by VSV-G pseudotyped lentiviral particles and induction by doxycycline. Both hSynDIG1 and IFITM3 slightly impacted reverse transcriptase activity compared to cDNA control, which may be the result of increased demand for the translation machinery in these co-transfected cells.

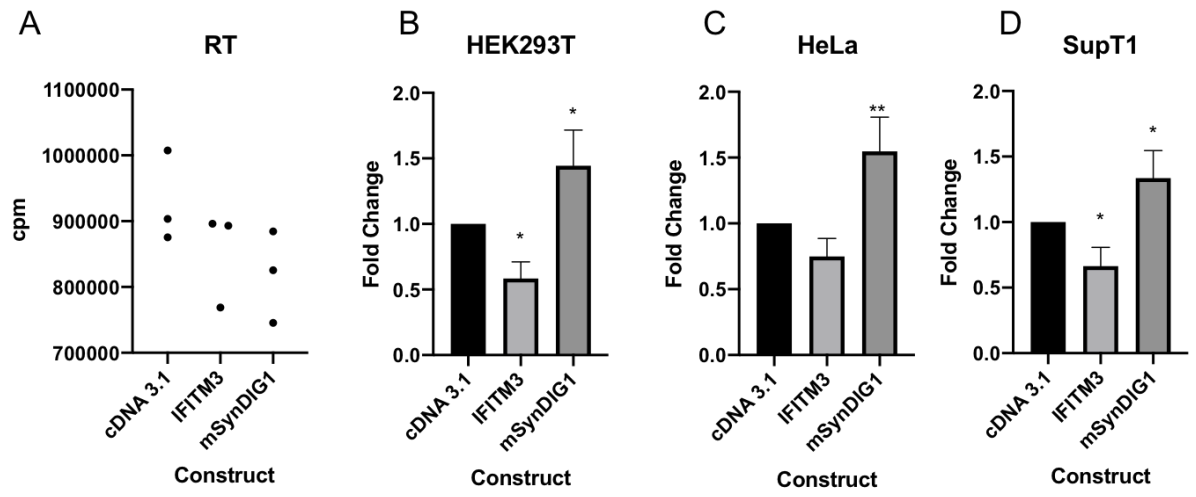

Figure 6. Mouse SynDIG1 co-transfection enhances transduction efficiency. Virus producing HEK293T cells were transduced with pVSV-G, psPAX2 and pLVX-TetOn-Luc and either pcDNA3.1, pcDNA3.1-SynDIG1, or pcDNA3.1-IFITM3 and tested for transduction efficiency by luciferase gene incorporation and activation in target cells. A) Reverse transcriptase (RT) values of the supernatant of virus producing cells harvested 48 hours post co-transfection. B/C) RT-normalized viral titres from pcDNA3.1, pcDNA3.1-SynDIG1 and pcDNA3.1-IFITM3 were used to transduced HEK293T cells. (B), HeLa cells (C), and SupT1 cells (D). Cells were stimulated with 500ng/ml Doxycycline 16 hours post transduction and harvested 48 hours post transduction for luciferase readings. Values represent mean ± standard deviation (SD) of a summary of 3 independent experiments. * $p < 0.033$, ** $p < 0.002$ by one-way ANOVA.

Compared to vector control, mSynDIG1 co-transfected lentiviral particles moderately enhanced transduction efficiency in all cell types analyzed by luciferase measurements (Figure 6B-D). In the same assay, IFITM3 co-transfected lentiviral particles decreased lentiviral transduction. Therefore, mSynDIG1 appeared to provide a virus intrinsic enhancement of lentiviral transduction efficiency.

3.2 Mouse SynDIG1 co-transfected lentiviral particles enhance transduction independent of IFITM3 expression in the target cell

To further determine whether target cell properties impact mSynDIG1 enhancement of lentiviral transduction, we transduced in parallel MT4-QCXIP and MT4-IFITM3 — both CD4+ T-cell lines — with protein incorporated lentiviral particles. As expected, the overall transduction efficiency of MT4-QCXIP was 4-5 fold higher than that of MT4-IFITM3 (Figure 7C). Nonetheless, mSynDIG1 co-transfected lentiviral particles continued to demonstrate a ~1.5 fold change in both cell lines, further indicating that the enhancement was independent of cell type or of IFITM3 levels in the target cell (Figure 7A,B).

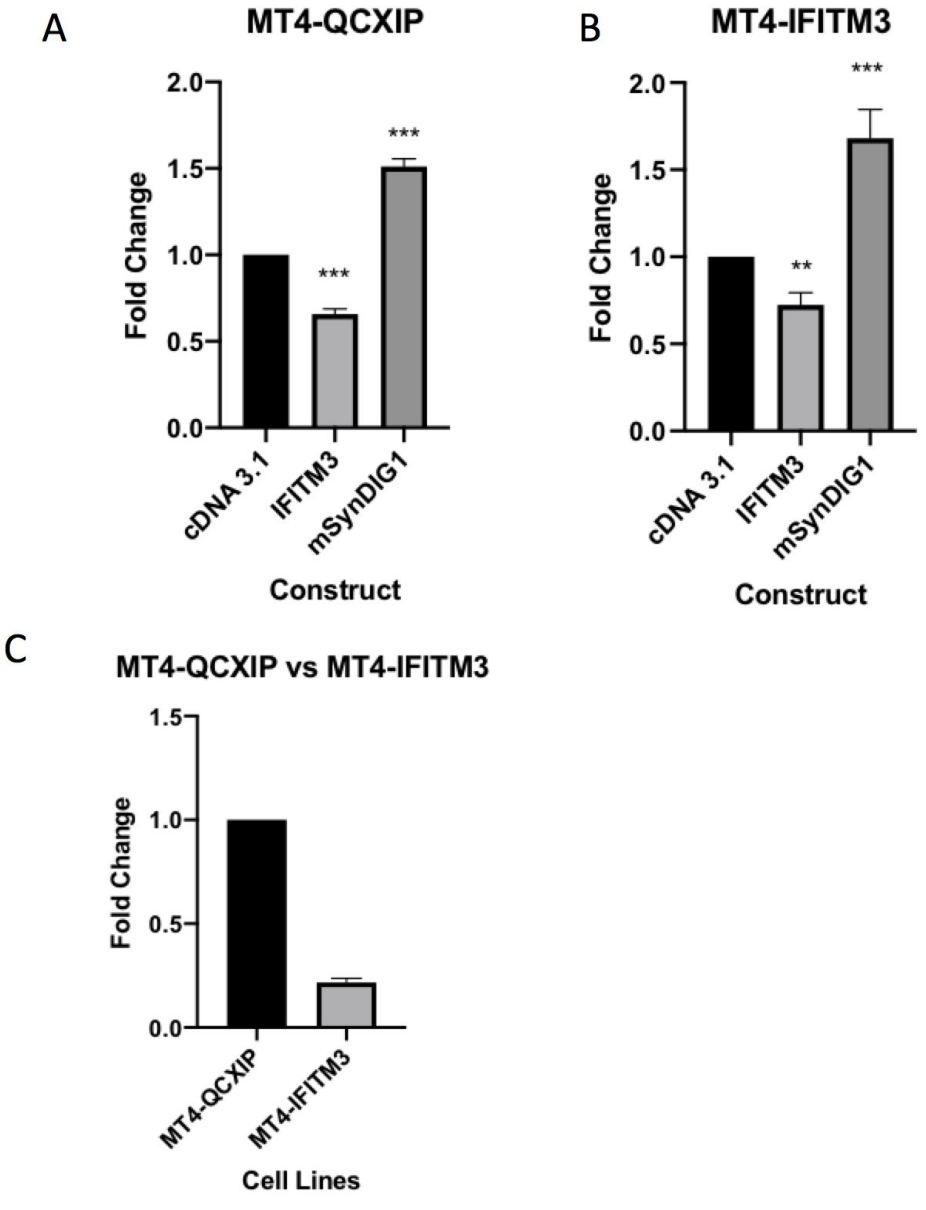

Figure 7. SynDIG1 co-transfected lentiviral particles enhance transduction efficiency independent of IFITM3 expression in the target cell. MT4-QCXIP pr MT4-IFITM3 cells lines were transduced in parallel to determine whether IFITM3 expression in the target cell affects the fold change of SynDIG1 virion incorporation. Cells were stimulated with 500ng/ml Doxycycline 16 hours post transduction and harvested 48 hours post transduction for luciferase measurements. A) Fold change in lentiviral transduction efficiency of MT4-QCXIP cells using RT-normalized lentiviral particles produced from virus producing cells transduced with pcDNA3.1, pcDNA3.1- SynDIG1 or pcDNA3.1-IFITM3. B) Fold change in lentiviral transduction efficiency of MT4-IFITM3 cells. C) Target cell expression of IFITM3 directly impact the overall infectivity of lentiviral particles. Values represent mean ± standard deviation (SD) of a summary of 3 independent experiments.
* $p < 0.033$, ** $p < 0.002$, *** $p < 0.001$ by one-way ANOVA.

3.3 Rationale-driven alterations to human SynDIG1 further enhance transduction efficiency

Although the observed phenotype of mSynDIG1 enhancement was apparent in a variety of cell lines, the effect was very moderate, increasing transduction efficiency by only ~1.5 fold. As a result, we next investigated whether human SynDIG1 (hSynDIG1) protein can exert a similar function. In an attempt to further enhance the observed transduction efficiency, we devised two structural alterations to the residues of hSynDIG1. Since IFITM3 has been shown to be targeted to the late endosomes by a YXXΦ motif (where Y is tyrosine, X can be any amino acid, and Φ any hydrophobic residue), a tyrosine-based endosomal sorting motif that has been shown to engage and associate directly with the adaptor protein 2 (AP2), a critical component of the cellular endocytic machinery, we wondered whether the addition of this motif may enhance SynDIG1 activity[149]. To this end, we added a 4 residue YEML motif to the intracellular N-terminus of hSynDIG1 (see Materials and Methods). Additionally, studies have reported that amphipathic helices and intramembrane domains have the properties of wedges on the lipid bilayer[100]. That is, the more

significant the association of the intramembrane domain with the lipid bilayer, the stronger the ability of the intramembrane -- as a single entity -- to affect membrane curvature. Residues 229-249 on hSynDIG1 corresponds to the outer leaflet intramembrane domain. We hypothesized that point mutations that alter the residues at the center of the amphipathic helix to ones that are more hydrophobic may enhance the ability of hSynDIG1 to associated with the outer leaflet, thereby increasing the membrane curvature generation properties of hSynDIG1 per unit protein and by extension enhance membrane curvature (Figure 8A). Therefore, we generated a point mutant G237I, T238V where the relatively hydrophilic amino acids glycine and threonine have been replaced by isoleucine and valine respectively (Figure 8B).

Compared to mSynDIG1, hSynDIG1 demonstrated the same enhancement of lentiviral transduction efficiency in both HEK293T as well as SupT1 cells (Figure 8C,D). However, YEML-hSynDIG1 reduced transduction levels to that of the pcDNA3.1 vector control, which may be a result of decreased plasma membrane expression in the virus producing and less protein incorporation onto the lentiviral envelope (Figure 8C,D). G237I, T238V increased transduction efficiency over pcDNA3.1 vector control at ~2.0-2.5 fold in both HEK293T and SupT1 cells, further enhancing transduction efficiency compared to unaltered hSynDIG1 (Figure 8C,D). As expected, IFITM3 co-transfection decreased transduction efficiency (Figure 8C,D).

A

Residue	Hydrophobicity Index
Glycine	-0.4
Threonine	-0.7
Isoleucine	4.5
Valine	4.2

B

Wild Type: 229-LAVLSITIGTGVYVGVAVALI-249
Mutant: 229-LAVLSITIIVGVYVGVAVALI-249

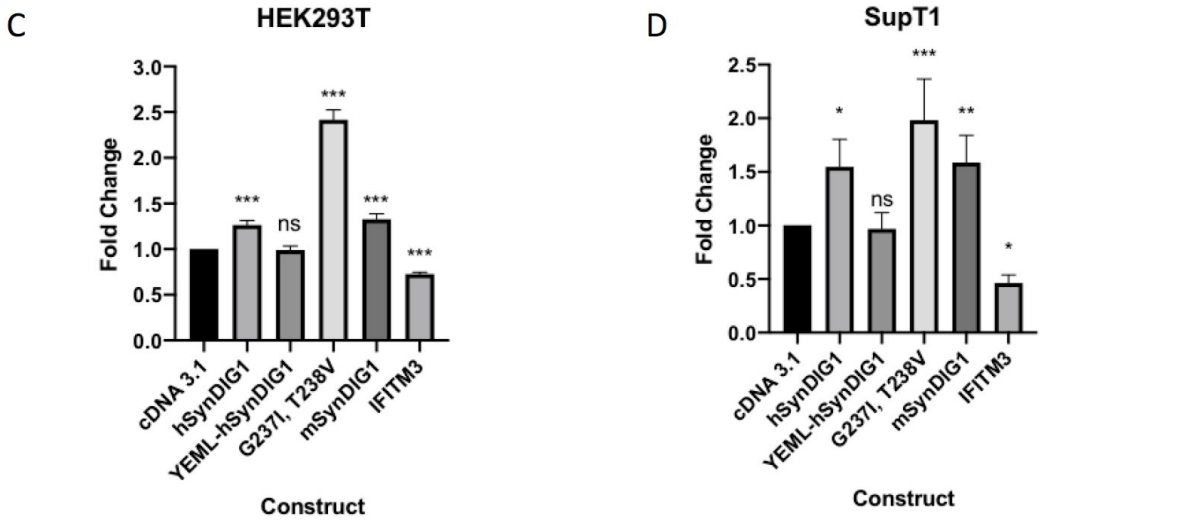

Figure 8. Transduction efficiency can be further enhanced by point mutations to the hSynDIG1 intramembrane segment. In order to further enhance transduction efficiency by hSynDIG1, two mutants were generated. A) Table of wild type amino acids and mutated residue along with their respective hydrophobic indexes. B) Human SynDIG1 transmembrane segment (residues 229-249). Red amino acids indicate altered amino acids from 237G, 238T on wild type SynDIG1 and 237I, 238V on mutant SynDIG1. C) Fold change by luciferase measurements in lentiviral transduction efficiency of HEK293T cells using various construct co-transfected RT-normalized lentiviral particles. D) Fold change in lentiviral transduction efficiency of SupT1 cells. Values represent mean ± standard deviation (SD) of a summary of 3 independent experiments.
ns = non-significant, * $p < 0.033$, ** $p < 0.002$, *** $p < 0.001$ by one-way ANOVA.

3.4 hSynDIG1 G237I, T238V co-transfection enhances transduction efficiency in human mesenchymal stem cells

While both mSynDIG1 and hSynDIG1 enhances transduction efficiency in a number of cell lines, for the approach to be feasible in a clinical setting, demonstrating of the

effect in primary cells is required. We therefore investigated the possibility that SynDIG1 co-transfected lentiviral particles enhances transduction efficiency in human mesenchymal stem cells (MSCs), which are of significant interest in gene therapy owing to their ability for self-renewal and capacity to differentiate into a number of different cell types such as osteoblasts, neurons, and muscle cells[150]. Co-transfection of mSynDIG1 and hSynDIG1 led to moderate increases in transduction efficiency in MSCs, as had been observed previously in cell lines (Figure 9). IFITM3 co-transfection led to a decrease in transduction efficiency. YEML-hSynDIG1 again was unable to enhance transduction efficiency. Human SynDIG1 mutant G237I, T238V led to a significant increase in transduction efficiency by ~4 fold, further demonstrating that this alteration may be an exciting avenue for further characterization and investigation (Figure 8).

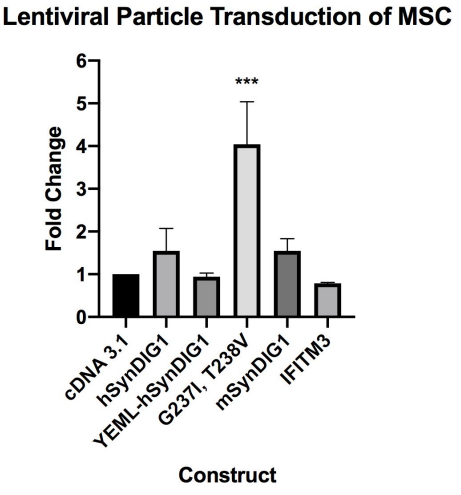

Figure 9. G237I, T238V-hSynDIG1 significantly enhances lentiviral transduction efficiency of MSCs. RT-normalized lentiviral particles produced with co-transfections from various pcDNA3.1-SynDIG1 constructs, pcDNA3.1 and pcDNA3.1-IFITM3. Fold change in lentiviral transduction efficiency by luciferase measurements of MSCs using various construct co-transfected RT-normalized lentiviral particles by luciferase readings. *** $p < 0.001$ by one-way ANOVA.

3.5 SynDIG1 protein is expressed in virus producing cells and lentiviral particles

To demonstrate that mSynDIG1 and hSynDIG1 protein is well-expressed, we probed for SynDIG1 expression by immunoblotting (Figure 10). In virus producing cells, hSynDIG1 and G237I, T238V are highly expressed. G237I, T238V appear to possess a lower kDa band that is largely absent in the other constructs, which may be indicative of a subspecies of a truncated version of the protein. YEML-hSynDIG1 and mSynDIG1 are not as well-expressed as other variants and may be due to

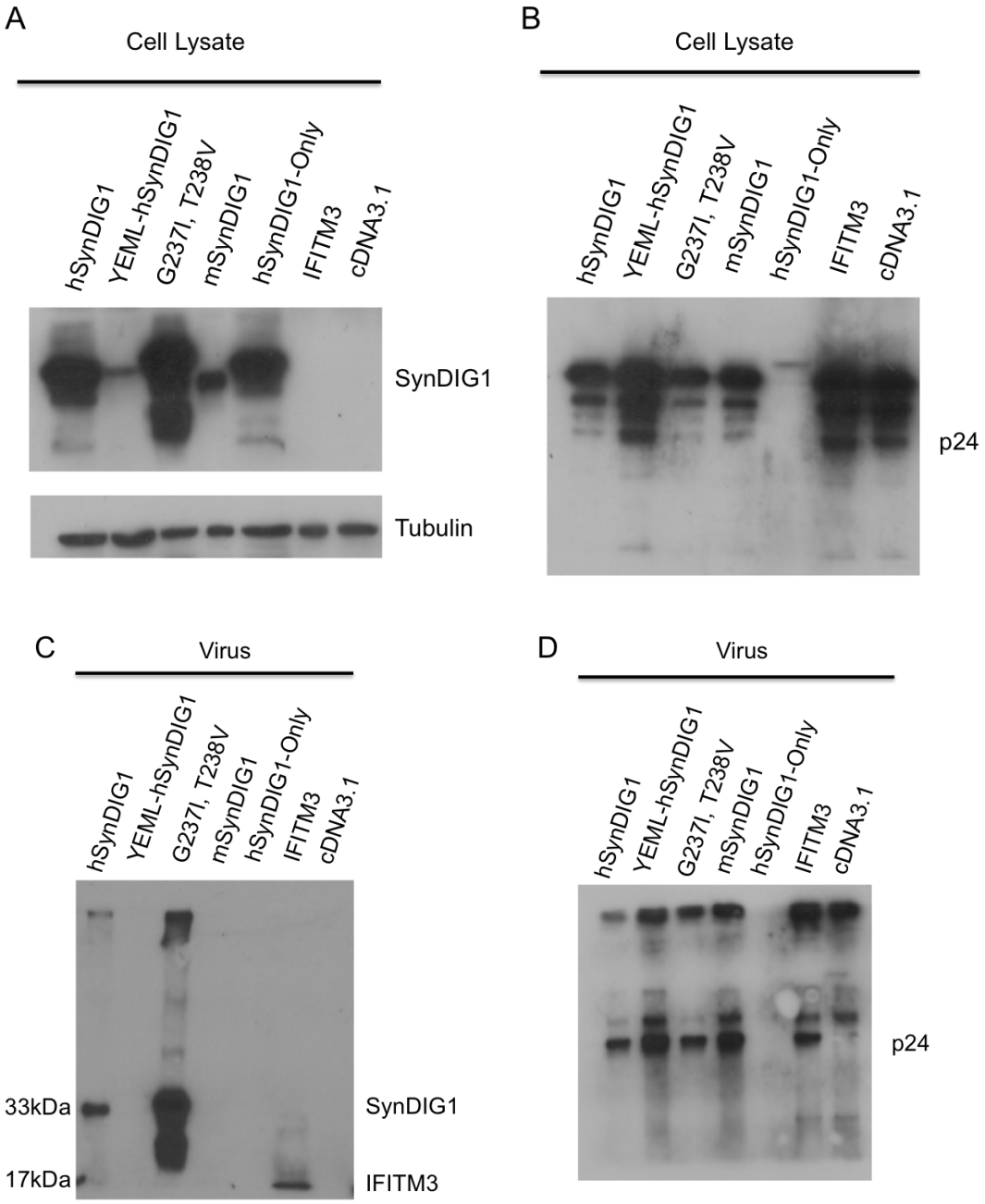

Figure 10. Western blot of cell lysates and viruses. A) hSynDIG1 and G237I, T238V is well-expressed in cell lysates. However, decreased expression is observed in YEML-hSynDIG1 and mSynDIG1. B) HIV-1 p24 is expressed in all samples save for hSynDIG1 only transfection. C) SynDIG1 protein expression is detected in hSynDIG lentiviral particles and G237I,T238V-hSynDIG1 but not in mSynDIG1, YEML or hSynDIG1 only samples. IFITM3 expression in the lentiviral particles is also detected. D) HIV-1 p24 in all virus producing samples.. SynDIG1-only transfection, where the virus assembly plasmids are not expressed, does not express p24 or SynDIG1, indicating that SynDIG1 does not spontaneously incorporate into exosomes.

the xenotypic variations inherent in altering subcellular localization and expressing a mouse protein in human cells. The p24 expression in virus producing cells is robust and indicates positive expression of lentiviral structural proteins. In ultracentrifuged lentiviral particles, only hSynDIG1, IFITM3, and G237I, T238V could be detected by immunoblotting, with G237I, T238V demonstrating the highest levels of protein incorporation, hinting at the potential reason for the higher levels of transduction efficiency when producing lentiviral particles with this construct. In human SynDIG1 only samples, hSynDIG1 is strongly expressed in cell lysates but not in ultracentrifuged samples, suggesting that hSynDIG1 does not readily incorporate into exosomes and that the observed hSynDIG1 expression is mainly due to its incorporation onto the lentiviral envelope. The p24 levels in the virus supernatant is relatively constant across the samples, indicating that none of the various constructs pose a significant impact on virus production. Immunoblotting for IFITM3 in the virus samples demonstrate that IFITM3 is incorporated into lentiviral particles, and confirm the effect of IFITM3 on the viral envelope.

3.6 BlaM-Vpr assay does not corroborate observed hSynDIG1 enhancemnet of lentiviral transduction

In order to demonstrate that hSynDIG1 directly enhances viral fusion, we employed a well established entry assay — the BlaM-Vpr — to assess the impact of protein incorporation onto lentiviral particles[148]. BlaM-Vpr is based on the incorporation of the BlaM-Vpr chimeric protein consisting of a beta-lactamase protein fused with the HIV-1 accessory protein Vpr. HIV-1 Vpr associates with HIV-1 Gag during virus

assembly at the plasma membrane and is readily incorporated into the target cell. The chimeric BlaM-Vpr would also incorporate into lentiviral particles. Target cells loaded with a fluorescent dye termed CCF2-AM may be cleaved by BlaM-Vpr in the cytoplasm, the amount of which is directly proportional to the number of virions that have fused with the target cell during transduction. CCF2-AM has an excitation/emission spectra of 408nm/530nm. Following cleavage by BlaM-Vpr, CCF2-AM is converted to CCF2, which now has an excitation/emission spectra of 408nm/460nm, corresponding to a green to blue transition that can be readily detected by flow cytometry.

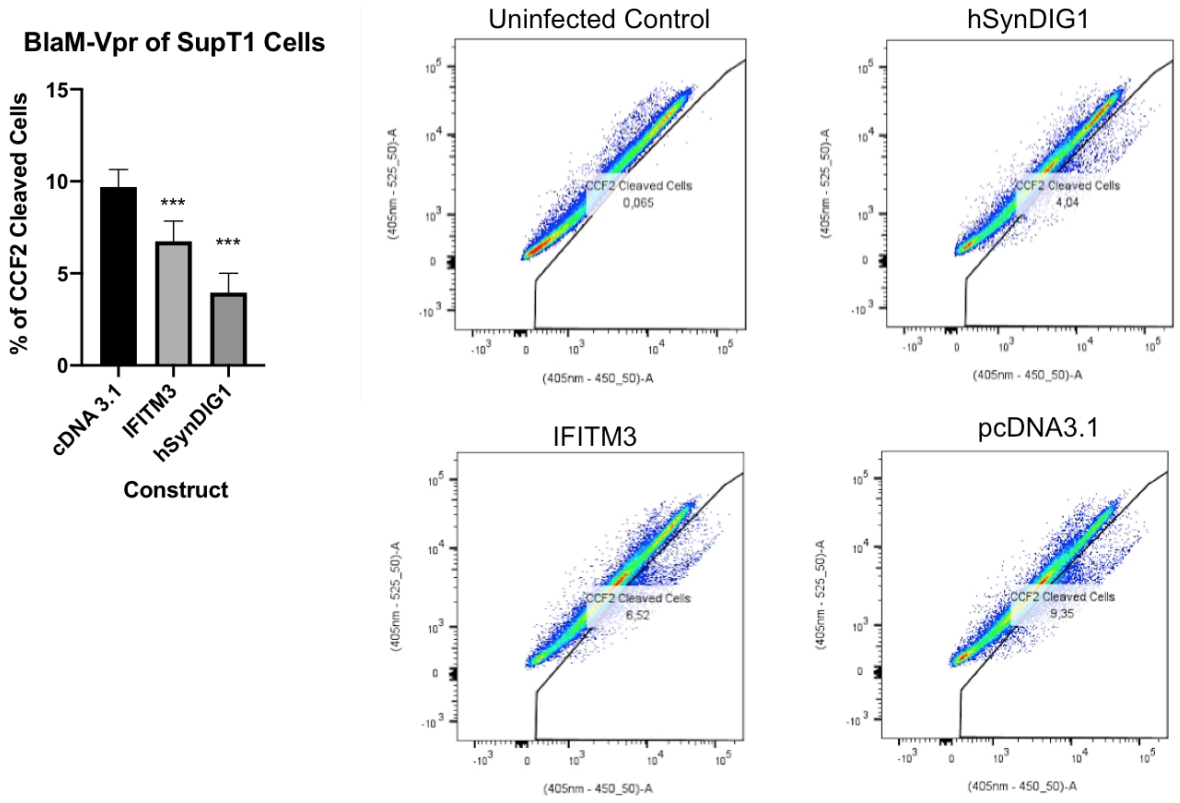

Figure 11. BlaM-Vpr assay of SupT1 cells was unable to corroborate the transduction results. RT-normalized hSynDIG1, IFITM3, or pcDNA3.1 incorporated lentiviral particles also incorporateing BlaM-Vpr chimeric fusion protein were transduced in SupT1 cells. BlaM-Vpr incorporated lentiviral particle transduction of SupT1 cells indicate that hSynDIG1 decreases viral entry by a greater amount than IFITM3 according to the percentage of CCF2-cleaved cells, which is known to inhibit viral entry. Sample flow cytometry data are shown. Data are an average of 4 independent experiments. *** $p < 0.001$ by one-way ANOVA.

RT normalized BlaM-Vpr incorporated lentiviral particles co-transfected with hSynDIG1, IFITM3, or pcDNA3.1 vector were used to transduce SupT1 cells to measure viral entry (Figure 11). IFITM3 incorporated lentiviral particles decreased lentiviral transduction, as had been established from previous reports. Unexpectedly, SynDIG1 incorporated lentiviral particles decreased lentiviral transduction efficiency more significantly than IFITM3 incorporation, in stark contrast to the results obtained from the transduction experiments (Figure 11). Since BlaM-Vpr is a direct assay of viral entry, hSynDIG1 may exert its transduction enhancing effects on another step of the lentiviral life cycle. Luciferase measurements are a readout of the overall steps of lentiviral transduction, including reverse transcription, nuclear import and integration. Alternatively, hSynDIG1 expression may decrease the ability of BlaM-Vpr to incorporate into lentiviral particles, either via direct competition for transcription and translation machinery, or by physical means. The N-terminus of hSynDIG1 consists of a large intracellular domain of 181 residues, which may interfere with BlaM-Vpr association with the Gag polyprotein since this large cytoplasmic domain is effectively intraviral following hSynDIG1 incorporation onto the lentiviral envelope (Figure 12)[145].

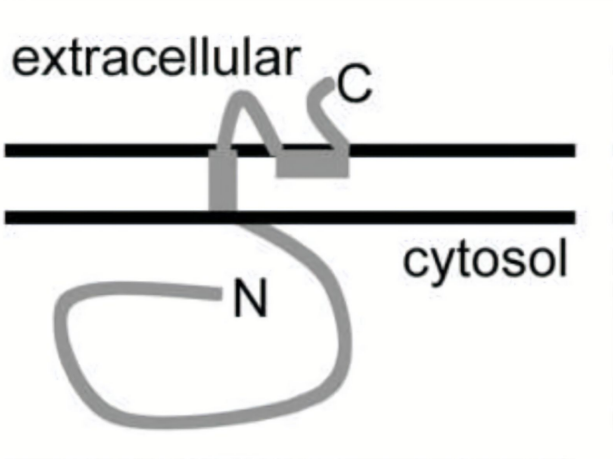

Figure 12. Schematic of SynDIG1 topology. A large intracellular domain comprising of more than half the total length of the protein may lead to decreased BlaM-Vpr chimeric protein incorporation in lentiviral particles via a competitive mechanism. Adapted from: Kalashnikova, Evgenia et al. “SynDIG1: an activity-regulated, AMPA- receptor-interacting transmembrane protein that regulates excitatory synapse development.” Neuron vol. 65,1 (2010): 80-93. doi:10.1016/j.neuron.2009.12.021

Chapter 4

Discussion and Conclusion

4.1 SynDIG1 incorporation enhances lentiviral transduction in a cell-type independent manner

A variety of strategies have been developed with regard to enhancing lentiviral transduction efficiency of target cells in *ex vivo* gene therapy ranging from small molecules to physical interventions. However, a majority of these approaches are cell-type specific and may cause undue alterations to the target cells that could jeopardize their pluripotency and viability. A virus-based approach would minimize any additional alterations to the target cell past the intended genetic insertions. Virus envelope engineering has seen rapid development in recent years, with examples the reader may refer to in Chapter 1. However, a majority of focus has been on receptor and avenues to utilize the viral envelope for signal transduction. These studies make use of the virus envelope as a means of carrying a certain protein to its destination. This thesis may be the first example of conceptualizing of the virus envelope — or the membrane of the virus envelope — as a direct entity that can be manipulated and engineering with the incorporated proteins.

Virus fusion is an energetically demanding process that has been shown to be inhibited by the proteins on the viral envelope such as IFITM3. We hypothesized that a protein with the exact opposite topology as IFITM3 would be able to induce negative membrane curvature on the membrane and enhance virus fusion if said

protein were to be able to be incorporated onto the lentiviral envelope. The SynDIG1 protein had previously been characterized and was demonstrated to be of the exact opposite topology as IFITM3. Since SynDIG1 is a neuron specific protein that does has not been shown to be critical for key cellular functions overexpression of SynDIG1 should not induce cytotoxicity. Furthermore, since this protein is expressed on the plasma membrane and early endosomes it may be easily incorporated onto the lentivirus envelope. Incorporation of this protein was confirmed by Western Blot for hSynDIG1 and G237I, T238V. However, although YEML-SynDIG1 and mSynDIG1 were detected in the cell lysates, we were unable to detect their expression on the viral envelope. This may be due to their lower expression levels in the cell lysate. Alternatively, the anti-SynDIG1 antibody is a monoclonal antibody that can detect mouse, rat and human SynDIG1. The antibody's polyclonal nature may have been a reason for the inability to detect mSynDIG1 in the virus supernatant. Mouse SynDIG1 co-transfected lentiviral particles resulted in a moderate increase in transduction efficiency, prompting us to investigate human SynDIG1 and interrogate its ability to enhance transduction efficiency. Furthermore, since the basal level of mSynDIG1 enhancement was quite moderate, we designed two rational changes to the protein, consisting of the addition of a YEML tyrosine-based late endosomal sorting motif and a two residue mutation in the middle of the intramembrane domain. Although we hypothesized that YEML-hSynDIG1 would further enhance lentiviral transduction efficiency, YEML-hSynDIG1 co-transfected lentiviral particles were not able to enhance transduction at all compared to the vector control. This may be due to an increase of YEML-hSynDIG1 sequestration in the late endosomes and consequently a decrease of YEML-hSynDIG1 expression on the plasma membrane

of the virus producing cell. However, Western Blot analysis of YEML-hSynDIG1 showed that the protein is not well expressed in cell lysates, and may be due to the inability of the protein to withstand the low pH conditions of the late endosome. The G237I, T238V construct exhibited a strong enhancement of lentiviral transduction, further enhancing the observed phenotype over unaltered hSynDIG1. We initially hypothesized that by altering the hydrophobicity at the center of the intramembrane domain, we are able to increase the association of this segment with the acyl fatty acid chains of the lipid bilayer. This may very well be the case. However, additional experiments may have to be conducted in order to ascertain this possibility. Alternatively, based on immunoblotting G237I, T238V appears to be more readily incorporated onto the lentiviral envelope and may be the mechanism for its increased enhancement.

4.2 SynDIG1 incorporation enhances lentiviral transduction in a cell type independent manner

The initial hypothesis and development of SynDIG1 incorporation was an attempt to develop a method of enhancing lentiviral transduction efficiency based on altering the intrinsic properties of virus-cell membrane fusion. We have demonstrated that SynDIG1 incorporation on the lentiviral envelope is indeed cell type specific, and have further demonstrated that this enhancement can be translated to stem cells, as is the case for MSCs. Exploiting the general properties of membrane fusion has been the impetus for the development of broad spectrum antivirals such as rigid amphipathic fusion inhibitors (RAFI), small molecules that generate increased membrane curvature on the viral envelope, thus eliminating the possibility of viral

fusion[151]. An opposite effect using small molecules may be achieved with negative membrane curvature inducing lipids such as oleic acid, which is non-toxic even at high doses and has even been shown to reduce inflammation[90,152]. Thus, pre-treating or treating virus producing cells with oleic acid may be an additional avenue to explore in a quest to generate negative membrane curvature on the lentiviral envelope. In summary, the concept of virus envelope engineering may provide an "all-round" approach to enhancing lentiviral transduction.

4.3 BlaM-Vpr indicates SynDIG1 inhibits viral entry

Although the transduction experiments demonstrated a robust phenotype of SynDIG1 incorporated lentiviral particles, we were unable to corroborate the phenotype by the BlaM-Vpr assay (Figure 11). This may hint at an alternative mechanism for SynDIG1-mediated enhancement of lentiviral transduction efficiency. However, we posit that a more likely explanation may be that SynDIG1 incorporation decreases the ability of lentiviral particles to incorporate the BlaM-Vpr chimeric protein. As previously mentioned, SynDIG1 contains a large intracellular domain of 181 residues that may impact the ability of viral accessory proteins to incorporate inside the virus particle. This hypothesis may be tested by producing SynDIG1 truncation constructs that curtail the large intracellular/intraviral segment. What is more, since SynDIG1 likely enhances lentiviral transduction via the C-terminus intramembrane domain, truncations to the cytosolic segment should not affect the ability of SynDIG1 to enhance transduction and may even enhance the observed effect since a smaller protein would provide more room to unfurl and package onto the viral envelope. BlaM-Vpr analysis using truncated hSynDIG1 constructs would be

able to accurately determine the validity of this hypothesis. Alternatively, an entry assay that is independent of BlaM-Vpr incorporation may also be a worthwhile pursuit. For example, the recently developed entry/uncoating assay based on RNA availability (EURT) may be able to determine whether the mechanism of SynDIG1 enhancement of lentiviral transduction is due to viral entry[153]. As its name suggests, EURT is based upon the availability of mRNA coding for the firefly luciferase gene and is independent of BlaM-Vpr incorporation into the virion.

4.4 Future directions and perspectives

Significant additional studies have to be performed to definitively demonstrate the feasibility of SynDIG1 incorporation. For example, it is unknown whether SynDIG1 incorporation would enhance the transduction of clinical grade lentiviral particles that must undergo numerous additional purification steps. Furthermore, CD34+ hematopoietic stem cells — an extremely significant cell type for *ex vivo* gene therapy — has not been tested. If these hurdles are overcome, potential side effects regarding SynDIG1 incorporation must also be addressed. However, we expect this approach to minimally affect target cells since SynDIG1 has not been shown to play a role in any critical signal transduction pathways. Furthermore, a protein incorporation-based approach would drastically limit the exposure of target cells to SynDIG1, and any residual proteins that have entered the target cell following absorption of the virus envelope following viral fusion would be degraded based on the half-life of SynDIG1.

Further alterations to the hSynDIG1 protein may also continue to enhance transduction efficiency. For example, residues 239 and 241 — also located at the center of the intramembrane domain — are composed of glycine and tyrosine, respectively, which are also relatively hydrophilic amino acids. Point mutations to these residues to more hydrophobic amino acids may further enhance either SynDIG1 negative membrane curvature generation or incorporation onto the viral envelope. However, these changes may affect the topology of SynDIG1 and convert the intramembrane domain to full pass transmembrane segment, which may completely ablate SynDIG1's capacity to generate negative membrane curvature.

Since membrane curvature is known to affect the hemifusion step of viral fusion, additional "boosts" that serve to enhance the ability for viral fusion proteins such as VSV-G to from a fusion pore may also enhance transduction efficiency. We propose that p15, fusion-associated small transmembrane (FAST) protein from the baboon reovirus p15 or its sub-components may also be able to enhance lentiviral transduction efficiency[154]. The p15 protein is a small, single pass transmembrane protein of 140 residues. Specifically, a study has shown that a specific ~20 residue motif — termed the hydrophobic patch (HP) — found on the intracellular side of p15 enhances fusion pore formation during cell-cell fusion, leading to syncytia formation and enhancing reovirus transmission and infectivity[154]. Furthermore, the mechanism behind this observation has been exquisitely defined. This segment appears to be a sensor of increasing positive membrane curvature, which occurs during the formation of the hemifusion intermediate, forcing the HP motif to embed into the inner leaflet, and push the inner leaflet towards its opposing member and enhance

fusion pore formation. Theoretically, SynDIG1 possesses a long intracellular domain that may be substituted for the reovirus p15 intracellular segment. If this augmented SynDIG1-p15 hybrid protein is incorporated onto the lentiviral envelope, negative membrane curvature may be generated via the C-terminus intramembrane domain of SynDIG1, leading to an enhancement of the formation of the hemifusion intermediate. Once this intermediate has been established, increasing pressure on the inner leaflet of the viral envelope may result in the insertion of the p15 HP into the phospholipids of the inner viral envelope and drive formation of the fusion pore. Furthermore, the intramembrane segment of SynDIG1 may be reiterated, and if the cellular machinery cooperates, a SynDIG1 protein with multiple intramembrane domains embedded in the outer leaflet may be created. This heavily altered SynDIG1 may provide a drastic increase in lentiviral transduction and not only overcome intrinsic IFITM3 mediated restriction but also membrane fusion in general.

4.5 Conclusion

We have demonstrated a proof of concept in utilizing protein incorporation to alter the intrinsic properties of lentiviral particles. Both native mouse and human SynDIG1 moderately enhance lentiviral transduction. Further alteration of the intramembrane segment led to a significant improvement in lentiviral transduction efficiency and we have demonstrated SynDIG1 incorporation to enhance transduction efficiency in MSCs, a pertinent cell type for gene therapy. Although this research is still in its infancy, we hypothesize that further alterations to SynDIG1, oleic acid incorporation during virus production, and reovirus FAST protein-SynDIG1 chimeric constructs would further enhance the observed effect. However, more work is required to

ascertain whether SynDIG1 incorporation is feasible on clinical grade lentiviral particles. Furthermore, the safety of SynDIG1 incorporation has not yet been evaluated *in vivo*. But I sincerely hope that the reader may find the research of this thesis suitable for further investment and continuation.

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