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Knockdown of TRIM5 α or TRIM11 Increases Lentiviral Vector Transduction Efficiency of Human Muller Cells

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Abstract

The goal of this study was to determine the expression and distribution of the host restriction factors (RFs) TRIM5 α and TRIM11 in non-human primate (NHP) neural retina tissue and the human Muller cell line MIO-M1. In addition, experiments were performed to determine the effect of TRIM5 α and TRIM11 knockdown on FIVGFP transduction of MIO-M1 cells with the goal of devising strategies to increase the efficiency of lentiviral (LV) gene delivery vectors. Immunofluorescence (IF) studies indicated that TRIM5 α and TRIM11 were localized predominantly in nuclei within the outer nuclear layer (ONL) and inner nuclear layer (INL) of NHP retina tissue. Double label IF indicated that TRIM5 α and TRIM11 were localized to some of the retinal Muller cell nuclei. MIO-M1 cells expressed TRIM5 α predominantly in the nucleus and TRIM11 primarily in the cytosol. FIVGFP transduction efficiency was significantly increased, at 4 and 7 days post transduction, in TRIM5 α and TRIM11 knockdown clones (KD) compared to WT MIO-M1 cells. In addition, pretreatment with the proteasome inhibitor MG132 increased the transduction efficiency of FIVGFP in WT MIO-M1 cells. The nuclear translocation of NF- κ B (p65), at 72 hours post FIVGFP transduction, was enhanced in TRIM5 α and TRIM11 KD clones. The expression of TRIM5 α and TRIM11 in macaque neural retina tissue and MIO-M1 cells indicate the presence of these RFs in NHP retina and human Muller cells. Our data indicate that even partial knockdown of TRIM5 α or TRIM11, or a short proteasome inhibitor pretreatment, can increase the transduction efficiency of a LV vector.

Keywords

Retina; Muller cells; TRIM5 α ; TRIM11; Gene therapy; Viral vectors; Proteasome Inhibitor

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1.0 Introduction:

The retina is an ideal target organ for gene therapy for several reasons including the compartmentalization of intraocular tissues, accessibility for microsurgical delivery of viral vectors, and immune privilege due to the intraocular environment (Kumaran et al., 2018). Gene therapy holds great promise for the treatment of inherited retinal dystrophies (IRD), a group of clinically and genetically heterogeneous degenerative disorders (Bennett 2017, Kumaran et al., 2018, Ludwig et al., 2019, Ong et al., 2019, Trapani and Auricchio 2018, Ziccardi et al., 2019). Adeno-associated virus (AAV) has emerged as the vector of choice for many gene delivery studies, although its limited transgene capacity of approximately 4.8 kilobases (kb) may require dual vector strategies (Bennett 2017, Hori et al., 2019, Kumaran et al., 2018, Trapani et al., 2014). Recently, the FDA approved the first gene therapy for an IRD using an AAV Vector (Georgiadis et al., 2016). For larger transgenes, lentiviral vectors (LVs), including non-integrating variants, can be utilized (Annoni et al., 2019, Balaggan and Ali 2012). The desire to avoid HIV-based vectors has led to the use of non-primate vectors including equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV) for gene delivery (Balaggan et al., 2006, Cavalieri et al., 2018, Saenz et al., 2012). To date, sub-retinal administration has achieved high efficiency lentiviral transduction only in the retina pigment epithelium (RPE) (Balaggan and Ali 2012, Ziccardi et al., 2019), but EIAV may also transduce photoreceptor cells (Balaggan et al., 2006).

The efficacy of LV gene therapy can be influenced by factors including innate immune responses and cellular restriction factors (RFs), which block key steps in the viral life cycle, often in a species-specific manner (Annoni et al., 2019, Borsotti et al., 2016, Jia et al., 2015, Kajaste-Rudnitski and Naldini 2015, van Tol et al., 2017). RFs including APOBEC3G, tripartite motif (TRIM) proteins, tetherin, SAMHD1, and Mx2 can disrupt LV replication in mammalian cells (Borsotti et al., 2016, Kajaste-Rudnitski and Naldini 2015, Uchil et al., 2008, Yuan et al., 2016). The effect of these restriction factors must be considered when designing vectors for viral gene delivery, regardless of the intended cellular target.

The TRIM protein family is a group of type I interferon-inducible E3 ligases which regulate a variety of cellular functions including cell cycle progression, signal transduction, and transcription (van Gent et al., 2018). A large number of the approximately 80 human TRIM proteins are induced after viral infection or by interferon stimulation as part of the host antiviral response (Koepke et al., 2020). The antiviral activities of TRIM proteins include direct targeting of viral components, regulation of the innate immune response, and modulation of autophagy-mediated defenses (Koepke et al., 2020). A minimum of seven TRIM proteins, including TRIM5 α , TRIM11, TRIM19, TRIM22, TRIM33, TRIM34 and TRIM37 can restrict LV replication (Koepke et al., 2020, van Tol et al., 2017).

The best studied of the TRIM proteins, TRIM5 α , binds to and destabilizes the viral capsid via K63-linked ubiquitination (Imam et al., 2019), leading to premature disassembly and a reduction in reverse transcription (Stremlau et al., 2004). TRIM5 α also plays a role in the innate immune response by promoting AP-1 and NF- κ B signaling, through K63-ubiquitination of the TAK1 kinase complex, which leads to the transcription of inflammatory

cytokines (Grutter and Luban 2012, Hage and Rajsbaum 2019, Imam et al., 2019, Lascano et al., 2016, Pertel et al., 2011). Another TRIM protein, TRIM11, interacts with the incoming LV capsid to accelerate uncoating and block reverse transcription, as well as inhibiting the release of viral particles from cells (Koepke et al., 2020, Yuan et al., 2016).

The majority of TRIM proteins are expressed ubiquitously, but their subcellular localization can vary (Reymond et al., 2001). In HeLa and U2OS cells, TRIM5 α was located in cytoplasmic bodies while TRIM11 was distributed more evenly throughout the nucleus and cytoplasm (Reymond et al., 2001, Wu et al., 2006). TRIM function may be linked to intracellular distribution, as alteration of TRIM5 α localization by the proteasome inhibitor MG132 disrupted targeting of the HIV-1 reverse transcription complex to the proteasome (Danielson et al., 2012, Wu et al., 2006). Interestingly, MG132 treatment increased the transduction efficiency of FIVGFP in human trabecular meshwork cells and monkey organ-cultured anterior segments, indicating a role for the proteasome in retroviral restriction in these cells (Aktas et al., 2018). Targeted LNEIE mutations in the HIV-1 capsid protein were able to overcome rhesus TRIM5 α -mediated capsid degradation, which resulted in higher transduction efficiency in both rhesus and human primary T cells (He et al., 2017). These experiments indicate that reducing host cell TRIM restriction can significantly improve the efficiency of LV gene delivery vectors.

In this study we determined that the restriction factors TRIM5 α and TRIM11 are expressed in NHP retina Muller cell nuclei. TRIM5 α was predominantly expressed in MIO-M1 cell nuclei, while TRIM11 was mainly expressed in the cytosol of this cell type. We demonstrated that knockdown of TRIM5 α or TRIM11 in MIO-M1 cells, or pretreatment with a proteasome inhibitor, significantly increased the transduction efficiency of the LV vector FIVGFP.

2.0 Methods:

2.1 Macaque retina tissue

Eyes from euthanized rhesus macaques (*Macaca mulatta*) were obtained as they became available from the Wisconsin National Primate Research Center of the University of Wisconsin-Madison. Animals were free of infectious agents at the time of sacrifice and no animals were deliberately sacrificed for these studies. Macaque eyes were kept on ice and dissected within one hour of sacrifice. Posterior eye cups were incubated with phosphate-buffered saline (PBS)/1 mM EDTA for 30 minutes at 37°C to loosen neural retina tissue and separate the retina from RPE cells. Neural retina tissue was rinsed in PBS before proceeding with further studies. All experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2 Cell Culture

The human Muller cell line MIO-M1 (Limb et al., 2002) and the human retinal pigment epithelial cell line ARPE-19 (ATCC CRL-2302) were maintained in DMEM/F12 (Cellgro/Mediatech, Manassas, VA, 10-090-CV)/10% FBS supplemented with a 1:100 dilution of L-glutamine-penicillin-streptomycin (SIGMA, St. Louis, MO, G1146) at 37°C in 5% CO₂.

HEK293 TN cells were maintained in DMEM /10% FBS supplemented with a 1:100 dilution of L-glutamine-penicillin-streptomycin at 37°C in 5% CO₂.

2.3 CRISPR knockdown

MIO-M1 cells were transfected with equal amounts of TRIM5 α CRISPR/Cas9 KO Plasmid (h) and TRIM5 α HDR Plasmid (h) (Santa Cruz Biotechnology (SCBT), Dallas, TX, sc-409348, sc-409348-HDR) or TRIM11 CRISPR/Cas9 KO Plasmid (h) and TRIM11 HDR Plasmid (h) (SCBT, sc-408290, sc-408290-HDR) following the Lipofectamine 3000 reagent protocol (Invitrogen, Carlsbad, CA, L300000). Cells were maintained in DMEM/F12/10% FBS with a 1:100 dilution of L-glutamine-penicillin-streptomycin. Puromycin (SCBT, sc-108071A, 1 μ g/ml) was added to the culture media at 3 days post transduction to select for transduced cells. Puromycin resistant cells were cloned by limiting dilution. Equal microgram amounts of wildtype (WT) Muller and TRIM5 α , or WT Muller and TRIM11 KD lysates, were screened for protein knockdown by immunoblotting with TRIM5 α or TRIM11 antibodies. Quantification of knockdown was performed on ECL images from three separate immunoblots utilizing ImageJ (<http://rsb.info.nih.gov/ij/>) and the mean percent WT expression was graphed.

2.4 shRNA knockdown

ARPE-19 cells were transduced with TRIM5 shRNA lentiviral particles (Santa Cruz Biotechnology (SCBT), sc-61718-V) or TRIM11 shRNA lentiviral particles (SCBT, sc-76734-V) at an MOI of 2 in the presence of 10 mg/ml Polybrene (SCBT, sc-134220). Cells were maintained in DMEM/F12/10% FBS with the addition of Primocin (InvivoGen, San Diego, CA, ant-pm-1, 1:500). Puromycin (SCBT, sc-108071A 1, μ g/ml) was added to the culture media at 3 days post transduction to select for transduced cells. Puromycin resistant cells were cloned by limiting dilution and screened for protein knockdown by immunoblotting with TRIM5 α or TRIM11 antibodies.

2.5 Immunoblotting

Lysates were prepared from macaque neural retina tissue immediately post-sacrifice (Gerhardinger et al., 2001). MIO-M1 cell lysates were prepared in Laemmli's sample buffer (BIORAD, Hercules, CA, 1610737) with added protease inhibitor (SIGMA P8340, 1:100) and phosphatase inhibitor (MilliporeSigma, Burlington, MA, 524633, 1:50) cocktails. Protein concentrations were determined by Pierce 660nm assay (Thermo Scientific, Rockford, IL, 2260). Lysates were electrophoresed on 4–15% Mini-PROTEAN TGX precast gels (BIORAD, 4568084S) with BenchMark Pre-stained Protein Standard (Invitrogen, Carlsbad, CA 10748–010) as a molecular weight marker (MWM). Proteins were electrophoretically transferred to nitrocellulose prior to blocking with 5% non-fat dry milk in Genius Buffer I (100mM maleic acid, 150mM NaCl, pH 7.5) containing 0.3% v/v Tween-20. The primary antibodies were diluted, as indicated, in 5% non-fat dry milk in Genius Buffer I, and incubated ON at 4°C followed by washing in Genius Buffer I containing 0.3% v/v Tween-20. HRP-conjugated secondary antibodies were diluted 1:5000 and applied for 1 hour at RT. After washing, the blots were developed using WesternSure PREMIUM chemilluminant Substrate (LI-COR, Lincoln, NE, 926–95000).

The following primary antibodies were used for immunoblotting: rabbit anti-TRIM5 α (Leinco Technologies, Fenton, MO, T331, 1:500), mouse anti-TRIM5 (SCBT, sc-373864), and rabbit anti-TRIM11 (Abcam, Cambridge, MA, ab111694, 1:1000). The secondary antibodies used for immunoblotting were mouse anti-rabbit HRP (SCBT, sc-2357, 1:5000) and goat anti-mouse HRP (Novus Biologicals, Centennial, CO, NBP1-75144, 1:5000).

2.6 Immunofluorescence

Neural retina tissue obtained from euthanized macaques was rinsed in PBS and fixed in 10% neutral buffered formalin (Thermo Fisher, Kalamazoo, MI, 305-510) for 24 hours before paraffin embedding and sectioning. Tissue sections were de-paraffinized and antigen retrieval was performed via heat treatment with pH 6 citrate buffer (10mM citric acid/ 0.05% Tween, pH 6, 20 minutes 92°C). Slides were blocked with 5% normal donkey serum (NDS) (ImmunoReagents Inc., Raleigh, NC, SP-072-020113) before incubation with the primary antibody diluted in 2.5% NDS/PBS overnight (ON) at 4°C.

MIO-M1 cells were plated on poly-L-lysine (SIGMA, P4707) coated chamber slides (Millipore, PEZGS0896). Cells were fixed in 4% paraformaldehyde/PBS and permeabilized for 10 minutes in 0.1% TritonX-100/PBS. Slides were then blocked with 5% NDS before incubation with the primary antibody diluted in 2.5% NDS/PBS for 1 hour at RT.

The secondary antibodies, diluted 1:400 in PBS containing 2.5% NDS, were applied for 1 hour at RT. Slides were stained with 1 μ g/ml Hoechst (Invitrogen, 33342) to visualize nuclei before mounting with Immu-mount (Thermo Scientific, 9990402). Fluorescence images (40x objective) were taken with a Zeiss AxioCam 702 mono camera on a Zeiss Axio Imager.Z2 microscope with Zeiss ZEN 2.3 pro software (Zeiss Microimaging, Oberkochen, Germany). Scale bars represent 20 micrometers (μ m).

The primary antibodies utilized for IF included: rabbit anti-TRIM5 α (Leinco Technologies, T331, 1:100), rabbit anti-TRIM11 (Abcam, ab111694, 1:100), mouse anti-vimentin (SCBT, sc-373717, 1:200), mouse anti-calretinin (SCBT, sc-365956, 1:50), mouse anti-PKC α (SCBT, sc-8393, 1:50), rabbit anti-FLAG Tag (SIGMA, SAB4301135, 1:140), rabbit anti-TurboGFP (Evrogen, Moscow, Russia, AB513, 1:1000), and goat anti-NF- κ B (p65) (SCBT, sc-372, 1:50). The secondary antibodies included: goat anti-mouse Alexa Fluor 594 (Life Technologies, Carlsbad, CA, A11032), goat anti-rabbit Alexa Fluor 488 (Life Technologies, A11008), and donkey anti-goat Alexa Fluor 594 (Invitrogen, A11058).

2.7 FIVGFP production

Packaging of the FIVGFP vector was described previously (Slauson et al., 2015). Briefly, the FIV-based vector pCDF1-MCS2-EF1 α -copGFP (1.74mg, System Biosciences, Palo Alto, CA, CD111B-1) was co-transfected into HEK293TN cells with pFIV-34-N (326 μ g) and pVSVG (408 μ g) in eight 500 cm² culture plates. Culture supernatants were collected after 48 hours and centrifuged at 24,000g for 30 minutes to pellet viral particles. The pellets were resuspended in Hanks balanced salt solution (HBSS) (Mediatech, 21-023-CM) and centrifuged through a 20% sucrose cushion in PBS. Viral pellets were resuspended in HBSS and stored at -80°C. Viral titers were determined by quantitation of fluorescent cells following serial dilution and transduction of Crandell-Rees feline kidney cells (CRFK).

2.8 FIVGFP Transduction

Wild type MIO-M1, TRIM5 α KD clones 13 and 14, and TRIM11 KD clones 5 and 6 were transduced ON at 37° with FIVGFP (Multiplicity of infection (MOI) of 95). The following day, cells were trypsinized and plated onto poly-L-lysine coated glass chamber slides. At four or seven days post transduction, cells were fixed, permeabilized, blocked, and stained with anti-TurboGFP and goat anti-rabbit AlexaFluor 488 as described above. Cells were also stained with 1 μ g/ml Hoechst to visualize nuclei. DAPI positive nuclei and GFP positive cells were counted in five fields for each cell type and data was expressed as mean percent GFP positive cells. *p* values were determined by two-sample t-test. Sample size (N)=5. Similar experiments were performed with the ARPE-19 TRIM5 α or TRIM11 KD clones.

2.9 MG132 assay

MG132 cytotoxicity was determined using a commercially available MTS assay (Cell Titer 96 Aqueous One Solution, Promega, Madison, WI, G3580). WT MIO-M1, TRIM5 α -13 KD, or TRIM11-6 KD cells were seeded in a collagen treated (Advanced BioMatrix, Carlsbad, CA, PureCol, 5005) 96-well plate at 6×10^4 cells/well and grown overnight in complete medium (DMEM/F12/10% FBS supplemented with a 1:100 dilution of L-glutamine-penicillin-streptomycin) at 37°C in 5% CO₂. The next day, the media was aspirated and replaced with 100 μ l of complete media containing increasing concentrations of MG132 (SIGMA, Z-Leu-Leu-Leu-al, C2211) with an adjusted final concentration of 0.05% DMSO (SIGMA, D2650). After a 1 hour incubation at 37°C, the media was replaced with 100 μ l complete media, and 20 μ l Cell Titer 96 Aqueous solution was added to each well. The plate was incubated at 37°C for 1 hour and the absorbance was measured at 490 nm using a Biotek Neo2 plate reader (Biotek, Winooski, VT). OD values were blanked using the absorbance from media only control wells. Samples were run in duplicate and the percent cell viability was calculated by setting the mean blanked absorbance value of the 0 μ M MG132 wells at 100 percent. The data was graphed with error bars representing SEM.

WT MIO-M1, TRIM5 α -13 KD, and TRIM11-6 KD cells were pretreated with media (DMEM/F12/10% FBS supplemented with a 1:100 dilution of L-glutamine-penicillin-streptomycin), media plus 0.05% DMSO, or media plus 20 μ M MG132/0.05% DMSO for 1 hour at 37°C. Cells were washed 3 times in PBS, media was added to wells, and cells were transduced ON at 37° with FIVGFP (MOI of 20). The following day, the cells were trypsinized and plated onto poly-L-lysine coated glass chamber slides. At six days post transduction, cells were fixed, permeabilized, blocked, and stained with anti-TurboGFP and goat anti-rabbit AlexaFLuor 488 as described above. Cells were also stained with 1 μ g/ml Hoechst to visualize nuclei. DAPI positive nuclei and GFP positive cells were counted in five fields for each cell type and data was expressed as mean percent GFP positive cells. *p* values were determined by two-sample t-test. Sample size (N)=5. The experiment was run in duplicate with similar results.

2.10 NF κ B (p65) Activation

MIO-M1 WT, TRIM5 α KD, and TRIM11 KD cells were plated in duplicate on poly-L-lysine coated chamber slides and transduced with FIVGFP (MOI of 12) at 37°C for 72 hours. Cells were fixed, permeabilized, and blocked as described above. Slides were stained

with goat anti-NF κ B(p65), followed by donkey anti-goat AlexaFluor 594. Nuclei were stained with Hoechst (1 μ g/ml) before mounting with Immu-mount. The total cell number (DAPI positive nuclei) and p65 positive nuclei were determined in five random fields per well and data was expressed as mean percent nuclear p65. *p* values were determined by two-sample t-test. Sample size (N)=10.

3.0 Results:

3.1 TRIM5 α and TRIM11 are expressed in NHP retina tissue

To determine whether the restriction factors TRIM5 α and TRIM11 were expressed in NHP neural retina tissue, immunoblots were performed. Blotting of retina tissue lysate with a rabbit anti-TRIM5 α antibody detected a doublet near the expected molecular weight (MW) of TRIM5 α (Figure 1A). A polyclonal antibody against TRIM11 recognized a single band near the expected MW of TRIM11 (Figure 1B) indicating that both TRIM5 α and TRIM11 proteins are produced in NHP retina tissue.

In HeLa cells, TRIM5 α proteins localize mainly to cytoplasmic bodies, whereas TRIM11 is expressed in a diffuse pattern in both the cytosol and nucleus (Reymond et al., 2001). To our knowledge, the expression pattern of these restriction factors has not been determined in macaque neural retina tissue. To examine the cellular distribution of TRIM5 α and TRIM11 within the neural retina, IF was performed on paraffin embedded macaque retina tissue. The TRIM5 α antibody detected protein expression predominantly in nuclei of the outer nuclear layer (ONL) and inner nuclear layer (INL), as well as some scattered nuclei in the ganglion cell layer (GCL) (Figure 2A). TRIM11 expression was mainly detected in nuclei of the INL, with fainter staining in ONL nuclei, and some scattered expression in GCL nuclei (Figure 2D).

To identify which retinal cells expressed TRIM5 α and TRIM11 in their nuclei, double label IF was performed with the TRIM5 α or TRIM11 antibody and cell type specific marker antibodies including vimentin (Muller), calretinin (amacrine), and PKC α (rod bipolar) (Sauter et al., 2018). Staining with the anti-vimentin antibody identified long, filamentous Muller cells which extended throughout the retina (Figure 2B, E). When the TRIM5 α or TRIM11 images were merged with the vimentin stains, it became apparent that some of the vimentin-labeled cells expressed TRIM5 α or TRIM11 in their nuclei (Figure 2C, F). These images indicate that Muller cells in the macaque retina express TRIM5 α and TRIM11 proteins in their nuclei. Double label IF with calretinin and PKC α antibodies did not reveal TRIM5 α or TRIM11 expression localizing to amacrine or rod bipolar cells in macaque neural retina tissue (data not shown).

3.2 Expression patterns of TRIM5 α and TRIM11 in a human Muller cell line

Since macaque Muller cell nuclei were shown to express both TRIM5 α and TRIM11, we wanted to determine the cellular distribution pattern of these restriction factors in the human Muller cell line MIO-M1. MIO-M1 cells stained with the TRIM5 α antibody demonstrated bright nuclear staining, and faint cytoplasmic staining (Figure 3C) compared to a no primary antibody control (Figure 3A) or a nonspecific rabbit polyclonal antibody control (Figure

3B). TRIM11 staining was cytosolic and diffuse, although some MIO-M1 cells also appeared to express TRIM11 in their nuclei (Figure 3D). These images indicate that TRIM5 α protein was primarily expressed in the nuclei, while TRIM11 expression appeared to be mainly cytoplasmic, in MIO-M1 cells.

3.3 Knockdown of TRIM5 α or TRIM11 increased FIVGFP transduction

CRISPR technology was utilized to knock down TRIM5 α or TRIM11 proteins in MIO-M1 cells and puromycin resistant clones were screened by immunoblotting with TRIM5 α and TRIM11 antibodies. We obtained two clones with decreased levels of TRIM5 α (Figure 4A) and two clones with decreased levels of TRIM11 (Figure 4B). ImageJ analysis of three separate immunoblots indicated that TRIM5 α KD clones 13 and 14 had a mean of 39.6% or 49.2%, respectively, of WT levels of TRIM5 α (Figure 4C). Analysis of TRIM11 clones 5 and 6 indicated that they expressed a mean of 39.9% or 42.7%, respectively, of WT levels of TRIM11 (Figure 4C). Total knockdown was not achieved in MIO-M1 cells for either TRIM5 α or TRIM11, suggesting that these proteins are necessary for cell survival. We were also not able to generate a viable double KD of TRIM5 α and TRIM11 in this cell type.

To determine if partial knockdown of TRIM5 α or TRIM11 affected the transduction efficiency of a non-human lentiviral gene delivery vector in human cells, we exposed WT MIO-M1 cells, TRIM5 α KD clones, and TRIM11 KD clones to FIVGFP. At 4 or 7 days post transduction, cells were fixed and stained with an anti-TurboGFP antibody to visualize GFP positive cells. The transduction efficiency of FIVGFP was very low in WT MIO-M1 cells, as expected for a feline retrovirus in human cells, with only 16.7% of cells expressing GFP at seven days post transduction (Figure 5B). In contrast, there was a roughly two-fold increase in the number of GFP positive cells in the two TRIM5 α KD clones and the two TRIM11 KD clones, compared to WT MIO-M1 cells, at four days post transduction (Figure 5A). By seven days post transduction, the overall number of GFP positive cells had increased, and TRIM5 α -13 KD and TRIM11-6 KD clones still had a significantly higher transduction efficiency compared to WT MIO-M1 cells (Figure 5B). These results, which were consistent in four replicate experiments, indicate that a decrease in the amount of TRIM5 α or TRIM11 protein allowed for enhanced FIVGFP transduction of the human Muller cell line MIO-M1.

To study the effect of TRIM5 α or TRIM11 knockdown in a different retinal cell line, we utilized shRNA technology to isolate stable knockdown clones in the human retinal pigment epithelial cell line ARPE-19. As with the MIO-M1 cells, we were not able to obtain total knockout of either TRIM5 α or TRIM11 in ARPE-19 cells, suggesting that these proteins are vital for cellular function. FIVGFP transduction efficiency experiments with the ARPE-19 TRIM5 α or TRIM11 KD clones yielded inconsistent results for reasons that are not clear at this time (data not shown).

3.4 Proteasome inhibition increased transduction of MIO-M1 cells

Prior to testing the effect of the proteasome inhibitor MG132 on transduction efficiency in MIO-M1 cells, the toxicity of the DMSO solvent and MG132 was determined. WT MIO-M1, TRIM5 α -13 KD, or TRIM11-6 KD cells were exposed to increasing concentrations of

MG132 in 0.05% DMSO for 1 hour at 37°C. A MTS assay indicated that no toxicity was observed for either 0.05% DMSO alone or concentrations of up to 20 µM MG132 for any of the Muller cells tested (Fig 6A).

To determine the effect of proteasome inhibitor pretreatment on FIVGFP transduction efficiency, we incubated WT MIO-M1 cells, TRIM5α-13 KD, and TRIM11-6 KD cells with 20 µM MG132 for 1 hour at 37°C. After washing with PBS, the cells were challenged with FIVGFP (MOI 20) for 6 days at 37°C. MG132 pretreatment had a significant effect on the transduction efficiency of FIVGFP in WT MIO-M1 cells with GFP positive cells increasing from 9.5% in media treated cells to 16% in MG132 treated cells (Fig 6B). DMSO alone did not have a significant effect on transduction efficiency (Fig 6B) and these results were consistent in duplicate experiments. We did not observe an increase in FIVGFP transduction efficiency after MG132 pretreatment in either TRIM5α-13 KD or TRIM11-6 KD cells (data not shown).

3.5 NF-κB (p65) activation is increased in transduced TRIM5α and TRIM11 knockdown cells

To determine whether TRIM5α or TRIM11 knockdown affect NF-κB activation, WT MIO-M1, TRIM5α KD, or TRIM11 KD cells were challenged with FIVGFP. Seventy-two hours post transduction, cells were fixed and stained with an antibody to NF-κB (p65). The percent nuclear p65 was calculated to serve as an indicator of NF-κB (p65) activation. An average of 4.4% of WT MIO-M1 cells exhibited p65 staining in their nuclei after a 72-hour FIVGFP challenge (Figure 7). In contrast, TRIM5α KD cells exhibited an approximate three-fold increase (13.1%) in the percent of cells with nuclear p65 staining following FIVGFP exposure (Fig 7). TRIM11 KD cells had an even greater increase in nuclear p65 staining (17.2%), compared to WT MIO-M1 cells, following FIVGFP challenge (Fig 7). These experiments indicate that KD of the restriction factors TRIM5α or TRIM11 in the human Muller cell line MIO-M1 resulted in significantly higher levels of NFκB (p65) activation at later time points post FIVGFP transduction.

4.0 Discussion:

Although the expression of some TRIM proteins, including TRIM11, have been characterized in the developing mouse retina (Chowdhury et al., 2018), researchers have not yet explored the distribution of TRIM proteins in the primate retina. Given our interest in factors that reduce the efficiency of lentiviral gene delivery vectors, we focused on TRIM5α and TRIM11 in this study. Our data show that both proteins are expressed in NHP retina and that TRIM5α and TRIM11 were localized to some Muller cell nuclei. Reports of TRIM localization in HeLa and U2OS cells indicated cytoplasmic localization of TRIM5α and diffuse localization of TRIM11, suggesting that localization may be cell-type dependent (Reymond et. al., 2001; Wu et. al., 2006). TRIM5α is also expressed in neuroblastoma cell lines but the localization was not reported (Singh et. al., 2014). The nuclear distribution of TRIM11 in NHP retina was consistent with reports of TRIM11 localization in multiple cell types (Darshit and Ramanathan, 2016; Jabbari et.al., 2018; Rathinam and Fitzgerald, 2011; Reymond et. al., 2001).

Analysis of the human Muller cell line MIO-M1 indicated that TRIM5 α was expressed predominantly in the nuclei while TRIM11 was primarily cytoplasmic. Expression of both proteins in Muller cells would be consistent with the role of Muller cells in responses to microbial infection and innate immune response signaling (Kumar et al., 2013, Kumar and Shamsuddin 2012). The differences in localization of these proteins in NHP retina and the MIO-M1 cells could be due to several factors including species-specific differences or differences due to tissue and cell processing. Finally, the differences may be related to the fact that we were comparing cells *in situ* with immortalized cells in tissue culture. Environmental factors are known to regulate expression and localization of TRIM proteins (Singh et al., 2014) and the tissue and cell culture environments are quite different. The NHP tissue was obtained from pathogen-free animals and the MIO-M1 cells were not exposed to virus prior to analysis so infection related differences should not be relevant. It will be interesting to examine the localization of TRIM proteins in the context of an infection.

TRIM5 α and TRIM11 bind to HIV-1 capsid complexes and accelerate uncoating (Yuan et al., 2016) despite low sequence homology (Li et al., 2007). Our results indicate that knockdown of TRIM5 α in MIO-M1 cells resulted in a significant increase in transduction efficiency of the FIVGFP vector, suggesting that TRIM5 α is involved in FIV restriction. Previously a negative correlation between TRIM5 α expression and HIV-1 vector transduction efficiency in human and rhesus CD34+ cells was reported (Evans et al., 2014). Transient knockdown of TRIM5 α in CD4+ lymphocytes or a human neuroblastoma cell line (SK-N-AS) also increased HIV-1 infection (Singh et al., 2014). These results indicate that even a partial decrease in TRIM5 α protein can enhance lentiviral transduction in multiple cell types.

Our data show that knockdown of TRIM11 in MIO-M1 cells also increased FIVGFP transduction efficiency. TRIM11 was initially identified as a RF that inhibited HIV entry (Uchil et al., 2008). Yuan et al. (2016) found that the inhibition involved direct binding of TRIM11 to the capsid resulting in accelerated uncoating, decreased capsid stability, and inhibition of early stages of infection. TRIM11 also disrupts nuclear import of viral DNA (Yuan et al., 2014; Yuan et al., 2016), activates the proteasome (Chen et al., 2018), and suppresses AIM2 inflammasomes (Liu et al., 2016). This study supports a role for TRIM11 in restriction of lentiviral gds in human Muller cells and demonstrates that transduction efficiency can be increased by partial knockdown of TRIM11.

Other methods to overcome TRIM mediated restriction may include alteration of viral capsid proteins (He et al., 2017), the use of linkage-specific deubiquitinating enzymes (Imam et al., 2019), or proteasome inhibitor treatment (Aktas et al., 2018, Danielson et al., 2012, Wu et al., 2006). Our data indicate that pretreatment with the proteasome inhibitor MG132 significantly increased the transduction efficiency of a lentiviral vector in human Muller cells. These results are consistent with our previous studies on human trabecular meshwork cells and support a role for the use of proteasome inhibitors prior to ocular gene therapy (Aktas et al., 2018). Previous studies have linked the association of rhesus TRIM5 α and proteasomes to restriction of HIV-1 in HeLa cells (Wu et al., 2006, Danielson et al., 2012). Our experiments reinforce the importance of proteasome function in lentiviral

restriction and demonstrate this link for the first time in a human Muller cell line. Interestingly, TRIM 11 restriction on HIV-1 was not sensitive to proteasome inhibitors in HEK293 cells, revealing that although there is some functional overlap in terms of capsid binding, there are differences in effector functions of the TRIM5 α and TRIM11 proteins (Tareen et al., 2011, Yuan et al., 2016). We did not observe an increase in FIVGFP transduction efficiency in MG132 treated TRIM5 α or TRIM11 knockdown clones and future studies will be needed to explain this finding.

The early induction of NF- κ B is essential for efficient lentiviral gene expression, yet it also triggers an antiviral innate immune response (Heusinger and Kirchhoff 2017). Lentiviruses cope with this by enhancing NF- κ B activity early in infection and suppressing it at later stages. Our data show that the increase in FIVGFP transduction efficiency in TRIM5 α or TRIM11 KD MIO-M1 cells was accompanied by increased nuclear translocation of NF- κ B (p65) at 3 days post transduction. Since the FIVGFP gdv used in our studies is not replication competent, later stages of the lentiviral life cycle, in which NF- κ B is usually suppressed, are not present in our system. This observation provides another way to quantitate transduction efficiency in cell types that are difficult to transduce, and suggests a role for NF- κ B activation in efficient gene delivery by lentiviral vectors.

In summary, we have identified TRIM5 α and TRIM11 expression in Muller cell nuclei of NHP retina, suggesting that these restriction factors may play a role in reducing the transduction efficiency of lentiviral gene delivery vectors in this tissue. We have also shown in a cultured Muller cell line that knockdown of either TRIM5 α or TRIM11, or pretreatment with a proteasome inhibitor, increased the transduction efficiency of a lentiviral vector. These results suggest that transient inhibition of host restriction factors, just prior to vector delivery, could improve transduction efficiency of retinal cells and result in vector sparing which is especially important in ocular gene delivery due to the small volume of vector that can be administered (Aktas et al., 2018). Future experiments will determine if transient knockdown of TRIM5 α or TRIM11 in primary NHP or human Muller cells increases transduction efficiency of lentiviral vectors.

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Highlights

1. NHP retina tissue expresses TRIM5 α and TRIM11 in Muller cell nuclei
2. Cultured human Muller cells express TRIM5 α in nuclei and TRIM11 in the cytosol
3. Knockdown of TRIM5 α or TRIM11 increased transduction efficiency of FIVGFP
4. Proteasome inhibitor pretreatment increased transduction efficiency of FIVGFP
5. NF- κ B (p65) activation was increased in TRIM5 α and TRIM11 knockdown clones

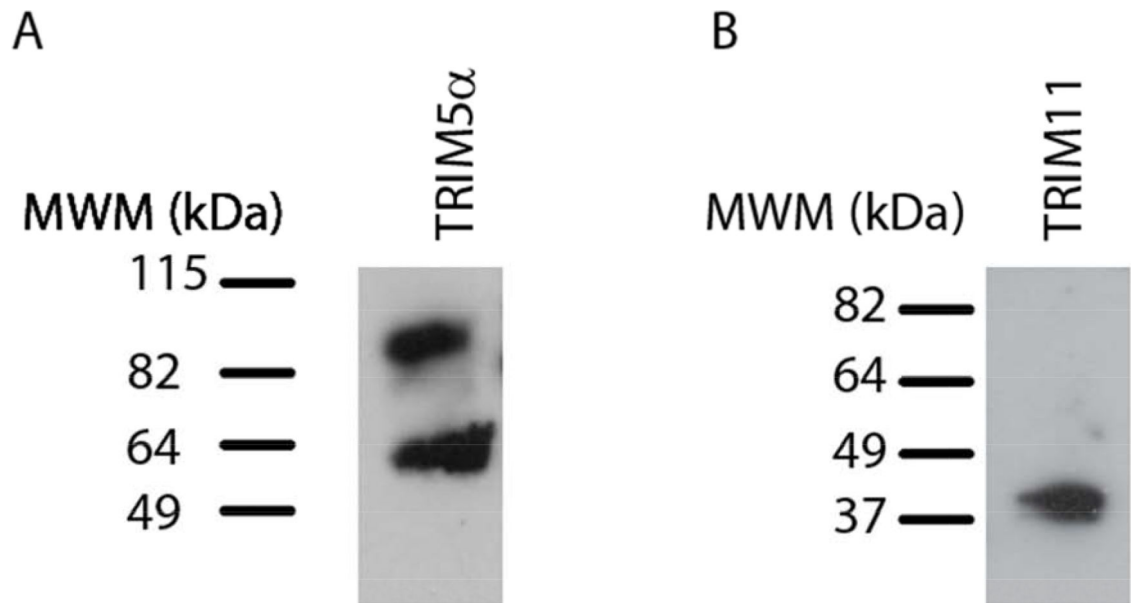


Figure 1: Expression of TRIM5 α and TRIM11 proteins in NHP retina tissue. Immunoblotting of macaque retina tissue lysates was performed with A) rabbit anti-TRIM5 α or B) rabbit anti-TRIM11 primary antibodies. Goat anti-rabbit HRP-conjugated secondary antibodies were applied prior to ECL.

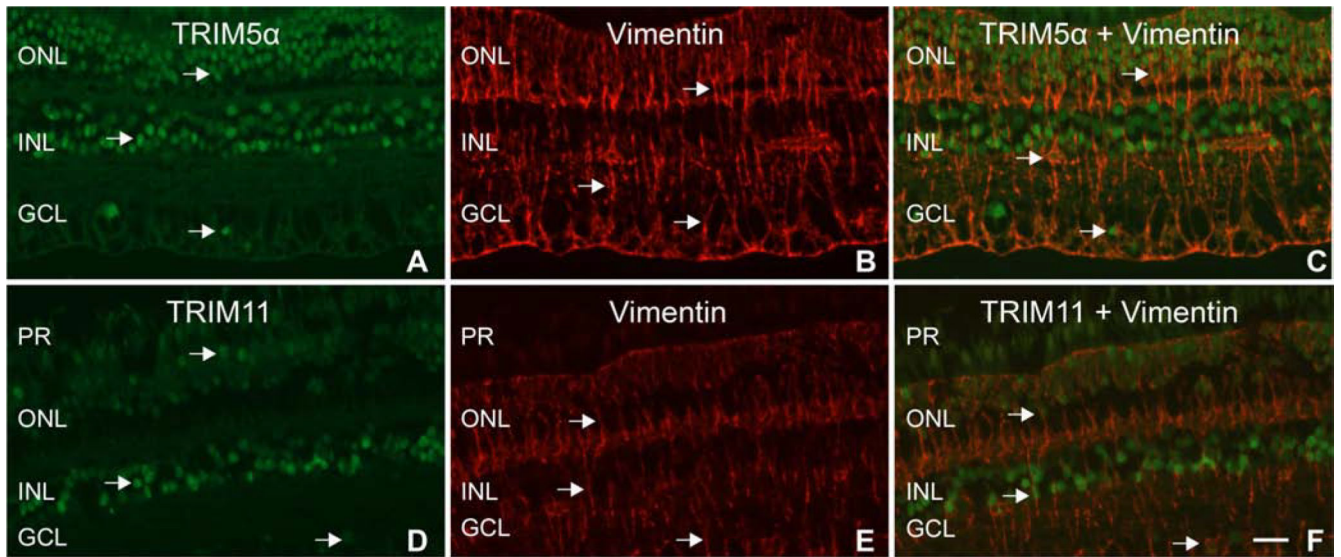


Figure 2: Expression patterns of TRIM5 α and TRIM11 in macaque neural retina tissue:

A) Arrows indicate TRIM5 α staining. B and E) Arrows indicate vimentin staining of Muller cells. C) Merged image with arrows indicating localization of TRIM5 α staining to Muller cell nuclei. D) Arrows indicate TRIM11 staining. F) Merged image indicating localization of TRIM11 staining to Muller cell nuclei. PR= photoreceptor layer, ONL= outer nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer. Scale bar= 20 μ m.

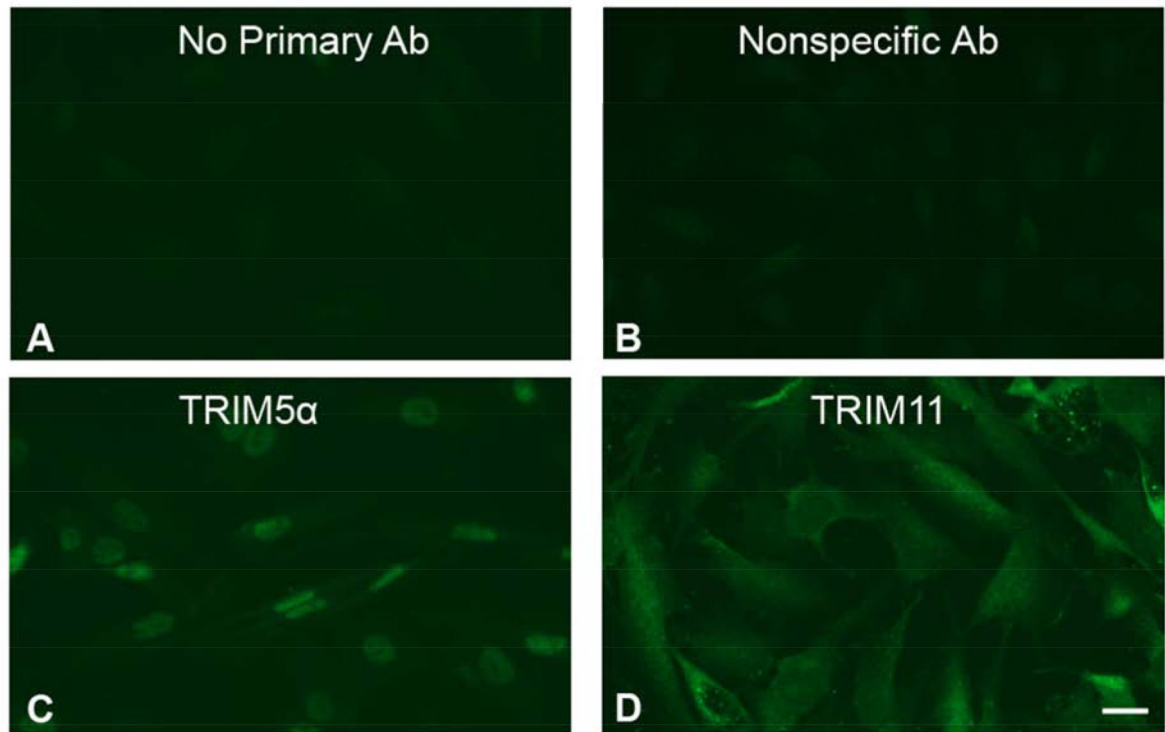


Figure 3: Expression patterns of TRIM5 α and TRIM11 in MIO-M1 cells.

MIO-M1 cells were fixed, permeabilized, and stained with A) no primary antibody, B) a nonspecific rabbit anti-FLAG Tag antibody, C) rabbit anti-TRIM5 α , or D) rabbit anti-TRIM11 followed by a goat anti-rabbit Alexa Fluor 488 secondary antibody. All polyclonal antibodies were diluted to a final concentration of 10 $\mu\text{g/ml}$.

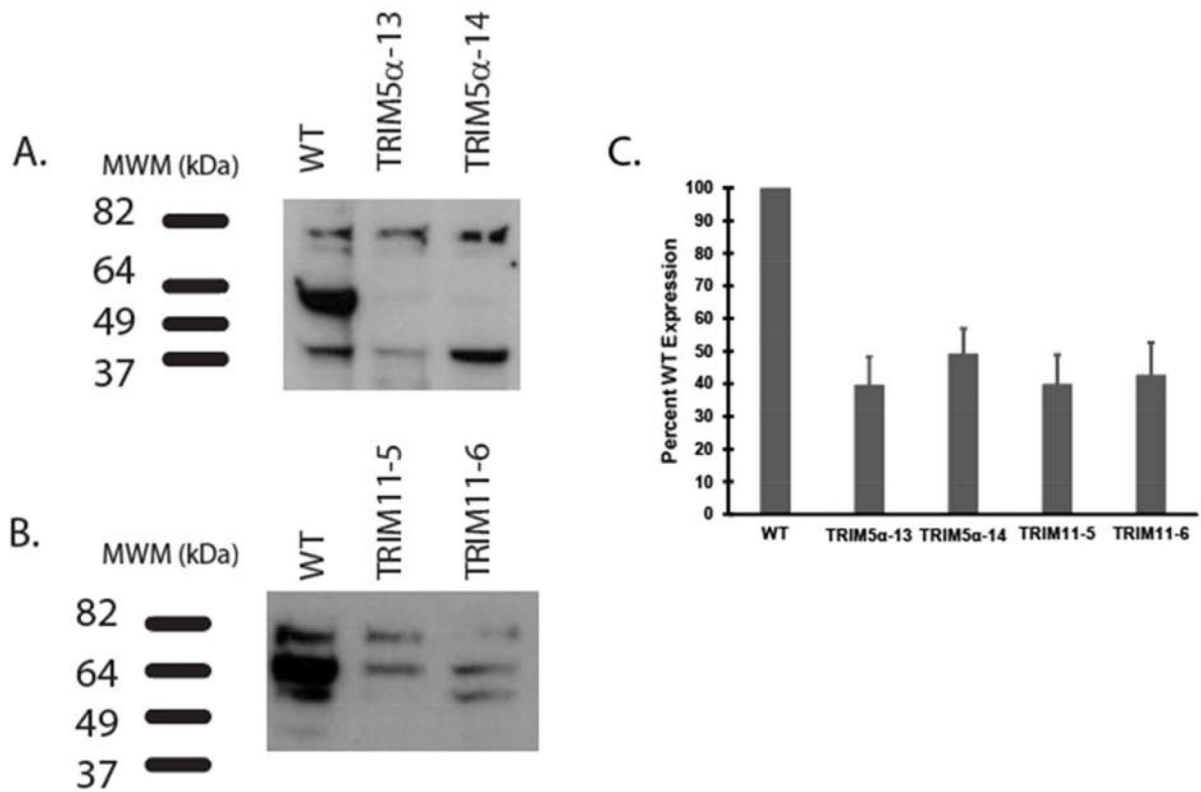


Figure 4: Immunoblots of TRIM5α and TRIM11 knockdown clones.

Equal micrograms of MIO-M1 WT and TRIM5α, or MIO-M1 WT and TRIM11 knockdown cell lysates, were electrophoresed and blotted with: A) mouse anti-TRIM5 or B) rabbit anti-TRIM11 antibodies. HRP-conjugated secondary antibodies were applied prior to ECL. C) ImageJ analysis was performed on three separate blots for each antibody and the mean percent WT expression was graphed with error bars representing standard error of the mean (SEM).

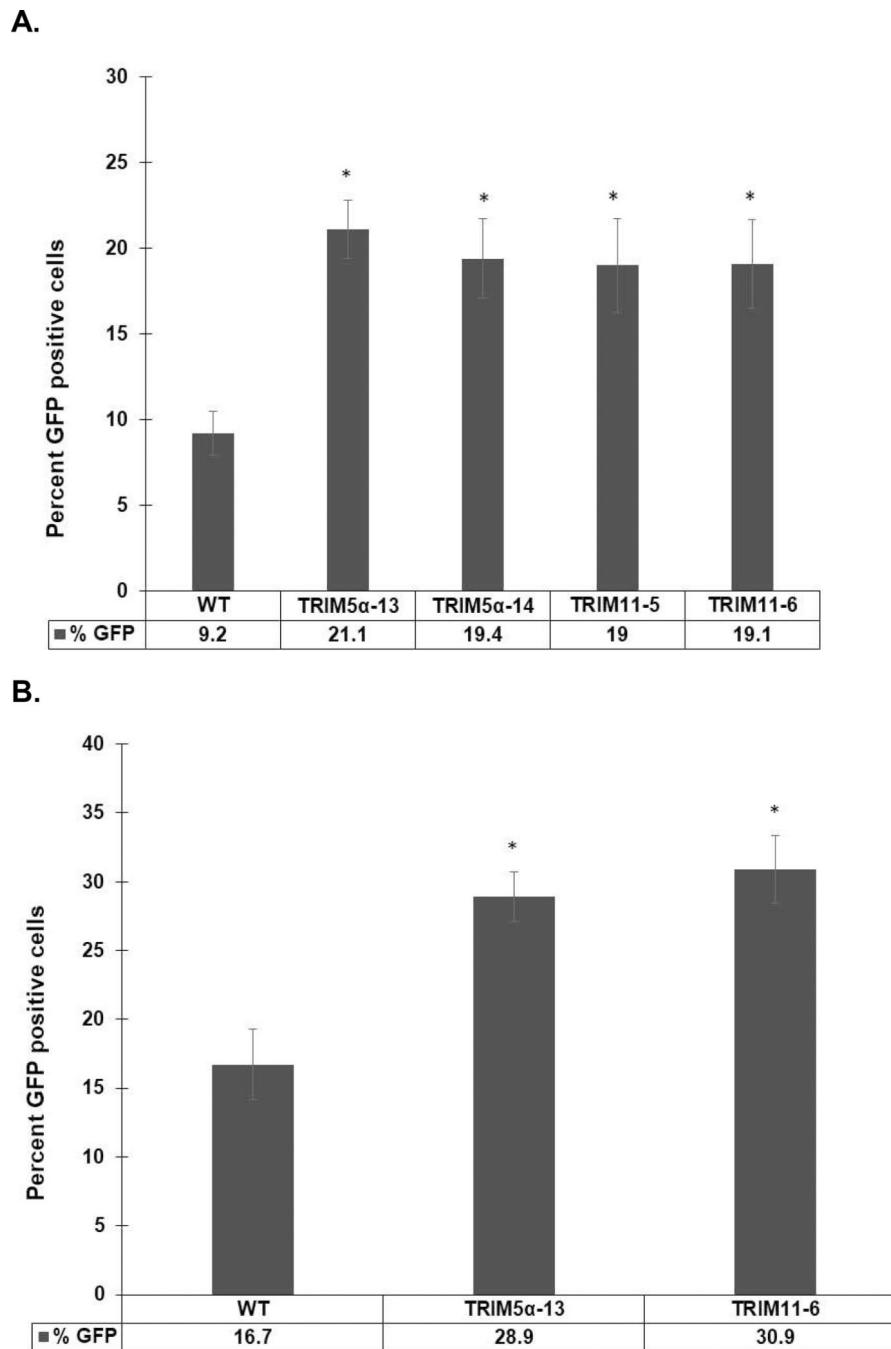


Figure 5: TRIM5 α or TRIM11 knockdown increased FIVGFP transduction efficiency in a human Muller cell line.

WT MIO-M1, TRIM5 α clones 13 and 14, and TRIM11 clones 5 and 6 were challenged with FIVGFP. Four days (A) or seven days (B) post transduction, cells were fixed and stained with an anti-TurboGFP antibody followed by goat anti-rabbit Alexa Fluor 488. Cells were stained with Hoechst to visualize nuclei. The percent GFP positive cells were determined in five fields for each cell type. Error bars represent SEM. p values were determined by two-sample t-test. Sample size (N) = 5. (*): $p < 0.02$.

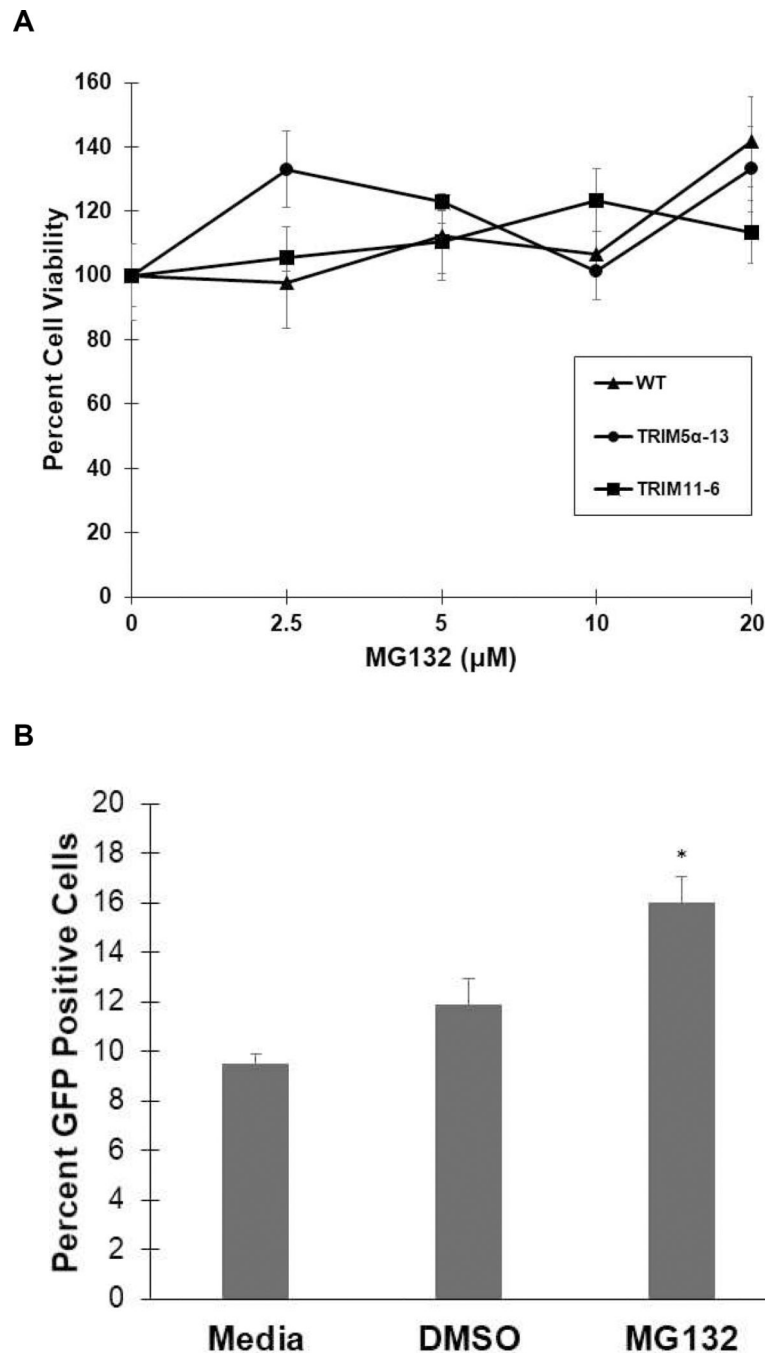


Figure 6: Proteasome inhibitor pretreatment increased FIVGFP transduction efficiency in MIO-M1 cells.

A) WT MIO-M1, TRIM5α-13 KD cells, and TRIM11-6 KD cells were incubated with increasing concentrations of MG132 for 1 hour at 37°C, followed by MTS assay. The mean percent viability was calculated and graphed with error bars representing the SEM. B) WT MIO-M1 cells were pretreated with media, 0.05% DMSO, or 20 μM MG132/0.05% DMSO for 1 hour at 37°C prior to FIVGFP transduction (MOI 20). Six days post transduction, the cells were fixed and stained with an anti-TurboGFP antibody followed by goat anti-rabbit AlexaFluor 488. Cells were stained with Hoechst to visualize nuclei. The percent GFP

positive cells were determined in five fields for each condition and the mean percent was graphed with error bars representing the SEM. p values were determined by two-sample t -test. Sample size (N)= 5. (*): $p=0.002$.

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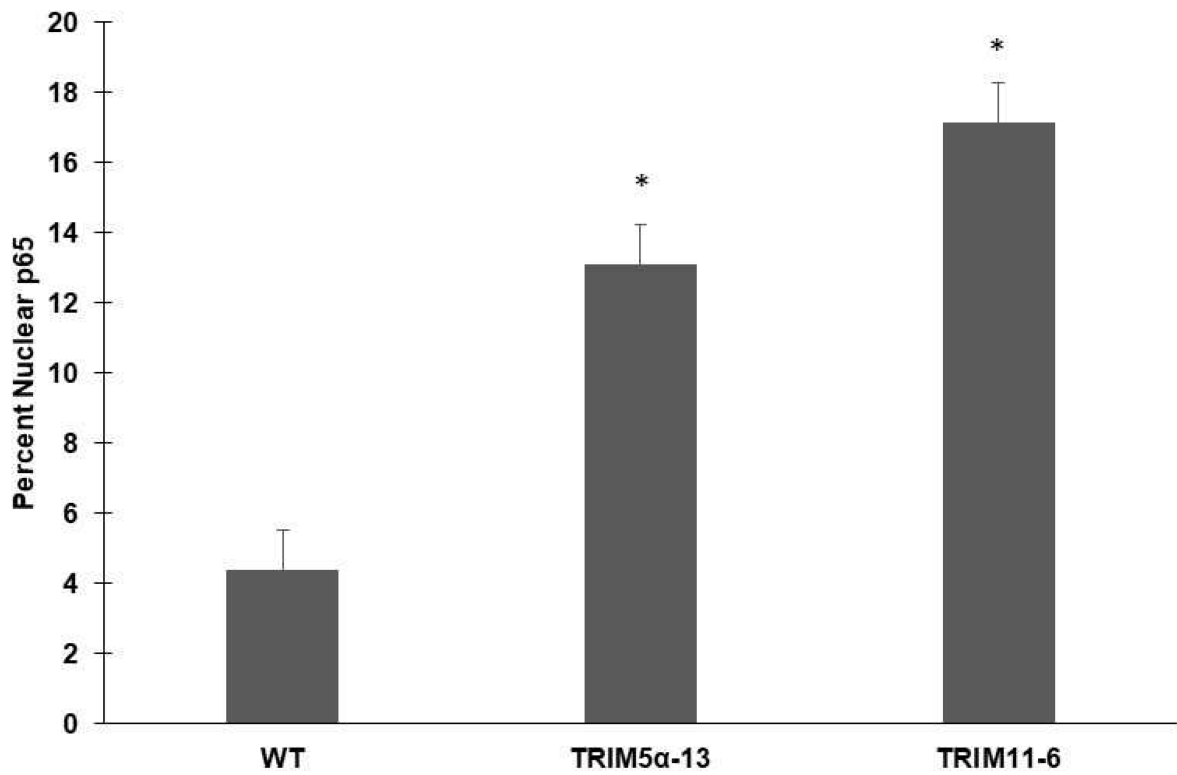


Figure 7: NF- κ B (p65) activation following FIVGFP transduction

Duplicate wells of WT, TRIM5 α -13 KD, and TRIM11-6 KD MIO-M1 cells were challenged with FIVGFP for 72 hours. Cells were fixed, permeabilized, and stained with an anti-NF- κ B (p65) antibody followed by donkey anti-goat Alexa Fluor 594. Cells were stained with Hoechst to visualize nuclei. The total number of cells and p65 positive nuclei were determined in five random fields per well and data was graphed as mean percent nuclear p65. Error bars represent SEM. *p* values were determined by two-sample t-test. (*): $p < 0.001$. Sample size (N)=10