INVESTIGATING THE ROLE OF COILED-COIL DOMAIN CONTAINING 124 (CCDC124) IN INNATE ANTIVIRAL IMMUNE RESPONSE

by

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ABSTRACT

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The innate immune system acts as the first line of defense in a non-specific manner against infectious diseases as well as malignant transformation. Natural Killer (NK) cells are members of innate immune system which are particularly responsible for killing virus-infected cells and tumor cells. Distinct properties of NK cells are remarkable in terms of cancer immunotherapy. Among several approaches, genetic modification of NK cells to enhance their immune function is widely studied with promising results but *in vitro* gene delivery into NK cells is highly challenging. HIV-1 based lentiviral vector systems for stable gene transfer have been used in most of the studies that aim genetic modification of NK cells. However, viral resistance of NK cells causes low efficiency and reduced stability, but enhancement of gene delivery efficiency is possible to achieve with small-molecule kinase inhibitors, such as BX795. Stress granule assembly is known to be associated with antiviral responses. This study aims to study the effect of *CCDC124 gene* which may be associated with stress granule formation and antiviral response during lentiviral gene transfer to NK cells. To investigate the mechanism, CRISPR/Cas9 system

was used to knock out CCDC124 and other genes that may be involved in the intracellular response against lentiviral vectors in HCT116, NK-92 and YTS cell lines. We compared the responses of different cell lines to lentiviral transduction and observed significant change in transduction efficiencies. Additionally, stress granule formation in CCDC124 knockout NK-92 cells is examined. Our findings present novel insights into the resistance of NK cells to lentiviral gene delivery and provide useful tools to improve genetic modification of NK cells.

ÖZET

SARILI-SARMAL BÖLGE BULUNDURAN 124 (CCDC124) PROTEİNİNİN DOĞAL BAĞIŞIKLIK SİSTEMİNİN ANTİVİRAL YANITINDAKİ ROLÜ

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Anahtar Kelimeler: Doğal öldürücü hücreler, lentiviral vektör, CRISPR, viral transdüksiyon

Doğal bağışıklık sistemi, organizmaların enfeksiyonlara ve tümörlere karşı öncül bağışıklamaya ihtiyaç duymadan oluşturduğu ilk adım savunma sistemidir. Doğal Öldürücü (NK) hücreleri, doğal bağışıklık sisteminin bir elemanı olup özellikle virüsle enfekte olmuş hücreleri ve tümörleri hedef alır. NK hücrelerinin özgül yetenekleri kanser immünoterapsinde kullanılmak üzere gelecek vaad etmektedir. Pek çok immünoterapi yaklaşımı arasından, bağışıklık sistemi hücrelerinde genetik modifikasyon ile bu hücrelerin aktivitelerini artırma üzerine çalışmalar yapılmış ve başarılı sonuçlar alınmıştır ancak NK hücreleri üzerinde yapılan *in vitro* genetik modifikasyon denemelerinin başarı oranları düşüktür. Bu sebeple NK hücrelerinde stabil gen transferi çalışmaları HIV-1 bazlı lentiviral vektörler üzerinde yoğunlaşmıştır. NK hücrelerinin virüslere karşı olan dirençleri, instabilite ve verimliliğin düşmesine sebep olmaktadır. BX795 gibi küçük molekül kinaz inhibitörleri ile yapılan çalışmalarda daha verimli viral transdüksüyon sonuçları elde edilebilmiştir. Bu çalışmada hücre içi stres granülleri ile ilişkisi olduğu düşünülen *CCDC124* geni hedeflenmiş ve viral transdüksiyon sırasındaki antiviral rolü araştırılmıştır. Çalışmanın deneysel kısmında CRISPR/Cas9 sistemi kullanılarak

HCT116, NK-92 ve YTS hücrelerinde CCDC124 geni ve diğer aday genleri susturulmuştur. Geliştirilen hücrelerin lentiviral transdüksiyon sırasındaki davranışları incelenmiştir ve lentiviral gen transferi yüzdelerinde önemli değişimler gözlenmiştir. Ek olarak *CCDC124* geni susturulmuş olan NK-92 hücrelerinde stres granül oluşumuna bakılmıştır. Bu çalışmadaki bulgular NK hücrelerinde genetik modifikasyon yaklaşımlarının geliştirilmesine ve NK hücrelerinin virüslere karşı direnç mekanizmasının anlaşılmasına katkı sağlayacaktır.

To my lovely family…

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
ϵ	Epsilon
ζ	Zeta
µg	microgram
µl	Microliter
µM	Micromolar
ADCC	Antibody-dependent cellular cytotoxicity
ALR	AIM2-like receptor
APC	Allophycocyanin
$CaCl_2$	Calcium chloride
CAR	Chimeric antigen receptor
Cas9	CRISPR-associcated nuclease 9
CCD	coiled-coil domain
CCDC124	coiled-coil domain containing 124
CLL	Chronic lymphocytic leukemia
CLR	C-type lectin receptors
CMV	Cytomegalovirus
CO_2	Carbondioxide
CRISPR	Clustered Regularly Interspaced Short Palidromic Repeat
ddH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxade
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DSB	Double stranded break
dsDNA	Double stranded DNA
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic translation initiation factor
ER	Endoplasmic reticulum
FACS	Fluoroscence activated cell sorter
FBS	Fetal Bovine Serum
G3BP	GTPase-activating protein-binding protein
GAP	GTPase-activating proteins
GFP	Green fluorescent protein
GM	Genetically modified
GvH	Graft versus host
HBS	HEPES buffered saline

HBV	Hepatitis B virus
HDR	Homology directed repair
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSV	Herpes Simplex Virus
IDR	Intrinsically Disordered Region
IFN	Interferon
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IL-2	Interleukin-2
ILC	Innate lymphoid cell
IMiD	Immunomodulatory imide drug
iRFP	Near-infrared fluorescent protein
LB	Luria Broth
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAPKK	Mitogen-Activated Protein Kinase
MCMV	Murine cytomegalovirus
MDA5	Melanoma Differentiation-Associated protein 5
MEM	Minimum Essential Media
MHC	Major Histocompatibilty Complex
ml	Mililiter
mM	Milimolar
MOI	Multiplicity of infection
mRNP	Messenger ribonucleoprotein
NHEJ	Non-homologous end joining
NK	Natural Killer
NLR	Nucleotide-binding oligomerization domain-like receptors
OXO	(5Z)-7-Oxozeaenol
PAM	Protospacer adjacent motif
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PIPES	piperazine-N,N′-bis (2-ethanesulfonic acid)
PKR	protein kinase R
poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
qRT-PCR	quantitative Reverse-Transcription PCR
RasGEF1b	RasGEF Domain Family Member 1B
RBP	RNA-binding protein
RIG-I	Retinoic acid-inducible Gene-I
RLR	RIG-1 like receptors
RNA	Ribonucleic acid
rpm	Round per minute
RPMI	Roswell Park Memorial Institute
SG	Stress granule
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
ssRNA	Single stranded RNA
TBE	Tris Borate EDTA

TBK	TANK-binding kinase
TCR	T cell receptor
TIA	T cell restricted intracellular antigen
TIAR	TIA-1 related protein
TLR	Toll-like receptor
TLR3	Toll-like receptor 3
TME	Tumor microenvironment
Treg	Regulatory T cell
TRIM	Tripartite Motif Containing
VSV-G	Vesicular stomatitis virus G
WT	Wild type

1. INTRODUCTION

1.1. Natural Killer Cells

1.1.1. Innate Immune System Member: NK Cells

The human body continuously interacts with pathogens through air, food or direct contact. These harsh environmental conditions create a necessity for enduring defense mechanisms that can protect the body against invading pathogens. The first line of this defense is the skin and the mucus surrounding the respiratory system, both of which create physical barriers to stop entry of pathogens into the body. However, small pathogens such as viruses or microorganisms can find ways to infiltrate into the body. The immune system steps in at this point to prevent the host from invasion of pathogenic microorganisms. Traditionally, the immune system is studied under two categories: The Innate Immune System which acts rapidly and in a non-specific manner and the Adaptive Immune System which acts more slowly but has the characteristics of antigen-specificity and memory. Cellular components of adaptive immune system, T and B cells, are evolved to recognize the pathogen specifically and get activated through the recognition which results in proliferation and response against that specific pathogen. Moreover, T and B cells can develop immunological memory against pathogens which helps host to respond more quickly during a second infection by the same agent (Mulder et al. 2019). Natural killer (NK) cells, innate lymphoid cells (ILCs) and phagocytes constitute cellular components of the Innate Immune System.

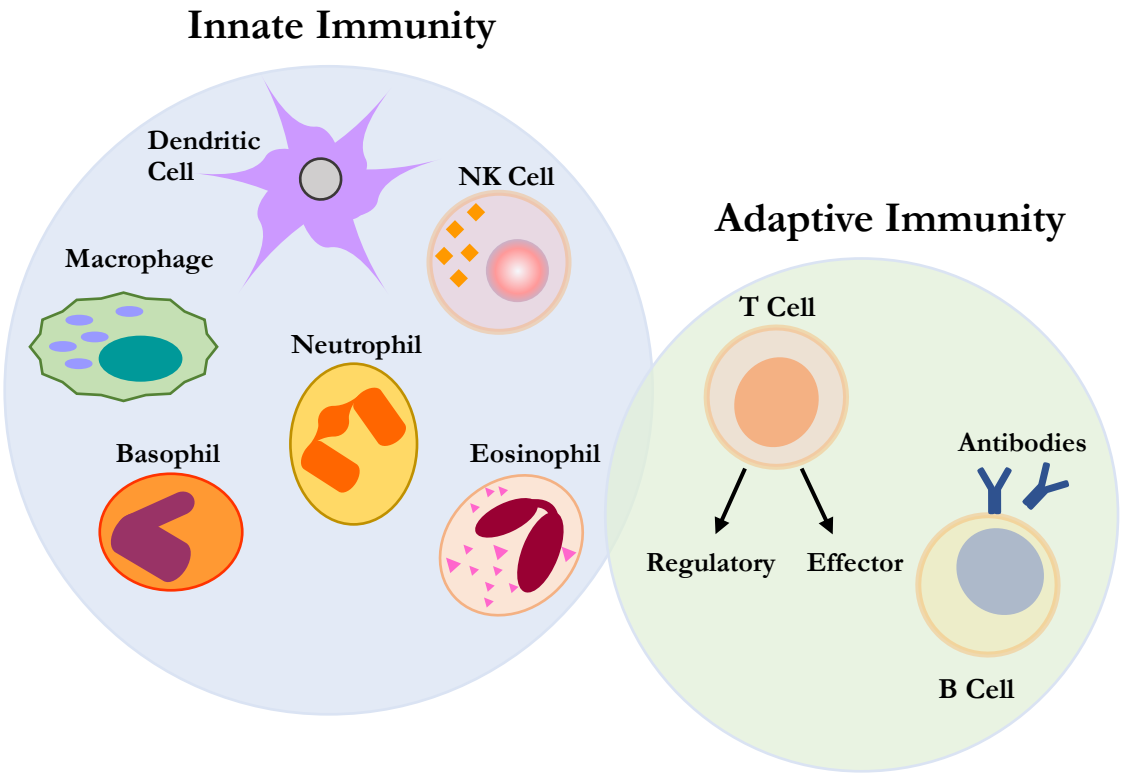


Figure 1. Cellular compartments of the immune system

Particularly, the innate immune system acts as the primary defense mechanism due to rapid response time and non-specific activity against a wide range of molecules that are common among different pathogens (Alberts et al. 2002). The innate immune system does not explicitly recognize the pathogen, but through its cells and receptors, recognizes molecular patterns common among pathogens to trigger activation and effector functions. Most cells in the immune system later contribute to this response by cytokine production, but dendritic cells, macrophages, and natural killer (NK) cells play an essential role as members of the innate immune system (Koenderman, Buurman, and Daha 2014) that also initiate activation of adaptive immunity.

1.1.2. Role of NK Cells in Innate Immunity

NK cells respond against transformed or virally infected cells by inducing target cells to undergo apoptosis. The response of NK cells is not antigen-specific, but NK cells track major histocompatibility complex class I (MHC-I) molecules on the host cell membrane. The MHC-I molecule plays a central role in recognition of target cells by cytotoxic cells of the immune system, that is T cells and NK cells. Both T cells and NK cells bind to MHC-I molecules, but the outcome of this binding differs dramatically in the two cell

types. T cells bind MHC-I via their T Cell Receptor (TCR) as a result of foreign peptide presented on MHC-I or foreign MHC molecule and get activated. Unlike T cells, NK cells scan self-MHC-I molecule to use the interaction as a regulator of activating and inhibitory mechanism. In this way, T cells are trained to recognize pathogens through tumor or virus-infected cell-specific antigens, but NK cells are specialized in killing cells that have impaired MHC-I molecule, or those have lost MHC-I expression (Sun and Lanier 2011). The phenomenon of recognition mechanism that enables NK cells to detect MHC-I non-expressing cells called missing-self recognition (Kärre 2008).

NK cell binding to MHC-I through inhibitory receptors implements self-recognition so that healthy cells can escape from cytotoxic activity of NK cells. Therefore, most vertebrate cells show high expression of MHC-I on their cell surface. Malignant transformation may inherently cause mutations which reduce MHC-I expression and enable immune escape from T cell-mediated lysis. Similarly, virus-infected cells may show low expression of MHC-I as several viruses have developed mechanisms of MHC-I downregulation. For example HIV encodes proteins that block MHC-I gene transcription, or herpes simplex virus blocks the translocation of the peptide that is required for MHC-I formation or cytomegalovirus drags MHC-I into proteasomes for degradation (Topham and Hewitt 2009). In these cases of MHC-I loss in transformed or virus-infected cells, NK cells step in to mediate target cell lysis by missing-self recognition.

1.2. NK-92 Cell Line

1.2.1. Characteristics of NK-92

NK-92 is a model NK cell line that was derived from a 50-year-old male non-Hodgkin's lymphoma patient in 1992. Proliferation and function of the NK-92 cell line depending on the presence of IL-2 in cell culture media and the cell line can survive barely up to 72 hours without IL-2 stimulation. The expression of CD56 on the cell surface is present however they are negative for CD16 expression which is distinct from primary NK cells. Detailed examination shows that NK-92 cell line displays functional characteristics of induced NK cells (Gong, Maki, and Klingemann 1994). The similarity of NK-92 functional responses to primary NK cells establishes a promising platform in understanding the biology of NK cells.

Main consideration in NK cell studies is the source of NK cells, where NK cells constitute only 10-15% of circulating blood cells which makes it inconvenient to isolate sufficient amount of NK cells. Additionally, *ex vivo* expansion of NK cells demands multiple cytokines which are sometimes supplemented via genetically modified feeder cell lines for cost-efficiency concerns, whereas the only requirement is for the NK-92 cell line is IL-2. More importantly, the unpredictable risk of graft-versus-host (GvH) reaction in allogenic NK transplantation may restrict the studies with primary NK cells whereas the more well-defined stable phenotype of NK-92 cells makes them more predictable and less susceptible to adverse effects (Klingemann, Boissel, and Toneguzzo 2016). All these circumstances put NK-92 cell line as a model in clinical research and clinical trials with the NK-92 cell line are ongoing (Hu et al. 2019).

1.3. Natural Killer Cell-based Cancer Immunotherapy Strategies

As mentioned above, NK cells are involved in the immune response during cancer and microbial infections. As a part of the innate immune system, these effector lymphocytes are responsible for restricting tumor growth and spread. NK cells are also able to provide indirect cytotoxic functions by cytokine production. While the endogenous NK cells of the body try to fight malignancies and infections, failure of these defense mechanisms due to the immunosuppressive effect of the tumor is a commonly observed phenomenon. In such cases, activation of endogenous NK cells or adoptive transfer of NK cells can be used as an approach to boost the anti-tumor NK cell activity. NK cell manipulation studies show higher efficiency in anti-tumor response, successful results in organ transplantation, and regulation of autoimmune diseases (Vivier et al. 2008). NK cells have been widely studied, and there are various approaches developed to induce NK cell function.

1.3.1. Enhancing Natural Killer Cell Activity with Cytokine Administration

Interleukins (ILs) are secreted cytokines which regulate immune response by mediating growth, differentiation, activation, proliferation, and survival of lymphocytes (T. Jiang, Zhou, and Ren 2016). Among these proteins, interleukin-2 (IL-2) has a fundamental role in NK cell biology in terms of proliferation and cytotoxicity. IL-2 is a small cytokine that is mainly produced by $CD4^+$ T cells. Additionally, $CD8^+$ T cells, NK cells and dendritic cells (Zelante et al. 2012) have the potential to secrete IL-2. Functional characteristics of IL-2 has significant impact on immune cells such as enhancing cytotoxicity of $CD8^+$ T

cells and NK cells, differentiation of T cells and proliferation of NK cells. IL-2 has high affinity against its own receptor, when it is formed by its three subunits IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132). Trimeric formation of IL-2R is found on limited group of cells such as activated T cells and Regulatory T cells (T_{reg}) because of IL-2Rα expression levels (Liao, Lin, and Leonard 2011). NK cells show high expression of β and γ subunits of IL-2 receptor, however extrinsic IL-2 stimulation can trigger α subunit expression (T. Jiang, Zhou, and Ren 2016). It is reported that IL-2Rα alone is inefficient to induce signal transduction which requires at least dimeric formation of β and γ subunits (Abbas et al. 2018; Hodge et al. 2000).

IL-2 has been applied in the clinic to the patients diagnosed with metastatic renal cell carcinoma and metastatic melanoma as monotherapy with good clinical results and tumor regression. Promising results led IL-2 to be approved for metastatic renal cell carcinoma and metastatic melanoma treatment in 1992 and 1998, respectively. Even though IL-2 treatment demonstrated tumor regression, side effects such as cytokine storm related to the high dose administration of IL-2 and induction of immunosuppressive T_{regs} in low dose IL-2 treated patients diverted studies to combination of IL-2 with other cytokines such as IFN-α. Due to cytotoxicity of high dose IL-2, reduced dose IL-2 regimen was tested with substitute cytokine combinations, but it did not show significant difference. Taken into consideration, potential of IL-2 to trigger immune response would be better treatment when it is combined with cell-based therapies (T. Jiang, Zhou, and Ren 2016). Recombinant IL-2, known as Proleukin®, has been used in the clinic to boost immune system cells against metastatic renal cell carcinoma and metastatic melanoma (Childs and Carlsten 2015). Co-administration of *ex vivo* expanded T cells and IL-2, also shows a significant response, but low *in vivo* survival rates of expanded cells indicates the necessity of better *ex vivo* culture protocols.

In a similar manner, IL-15, which has therapeutic use in the clinic, plays crucial role in NK cell development, survival and activity. IL-15 binds to IL-15Rα with high affinity, besides that IL-15 can also bind IL-2Rβ and γ subunits. Due to shared receptor subunits, IL-15 and IL-2 show similar functional properties. Additionally, IL-15 has its own distinct immunoregulatory properties as well. IL-15 is a 15 kDa protein which is secreted by monocytes, macrophages, dendritic cells, fibroblasts, bone marrow stromal cells, and nerve cells constitutively (Waldmann and Tagaya 2002; Perera et al. 2012). IL-15 has a vital role in cytokine expression and cytotoxic activity of NK cells. IL-15-induced NK

cells show higher cytolytic activity via upregulation of activator receptor NKG2D (C. Zhang et al. 2008).

As a cytokine regulator, IL-15 induces expression of IFN-γ, TNF-α, and GM-CSF in NK cells when combined with IL-12. IL-15 dysfunction or failure in expression is associated with viral infection-related diseases which are directly related to NK cell participation in antiviral defense mechanism. A study on a patient who lacks NK cell activity shows that NK cell deficiency constitutes sensitivity against herpesvirus (Biron, Byron, and Sullivan 1989). Similarly, IL-15 expression is one of the targets of HIV-1 infection. After viral infection, inflammatory cytokine expression increases by various cell types such as NK cells, dendritic cells, and T cells. Upregulated cytokine levels increase $CD4^+$ T susceptibility to HIV (Manganaro et al. 2018) and disease progression causes $CD4^+$ T cell death and disorder in T cell, B cell, and NK cell function. Likewise, disrupted IL-15 expression leads to reduced NK cell development and proliferation. These findings suggest that IL-15 has a great potential to reconstitute NK cell activity during viral infection or cancer disease. On the other hand, IL-15 stimulation of HIV infected $CD4^+$ T cells would enhance viral replication and cause disease progression. Although the promising results of cytokine use as therapeutic agent, it has crucial restrictions and other approaches emerged for cancer immunotherapy (Perera et al. 2012).

1.3.2. IMiD-induced NK Cell Proliferation and Activation

A chemical compound, thalidomide, was discovered in the 1950s to cure nausea in pregnancy which was later used as an angiogenesis inhibitor. In the late 50s, severe birth defects were identified on the babies whose mothers used thalidomide treatment during the pregnancy. This is also known as Thalidomide Syndrome. These events lead researchers to study molecular mechanism of thalidomide and potential effect on angiogenesis (Vargesson 2013). Along with the effect on angiogenesis, research on Thalidomide revealed several immunomodulatory functions of Thalidomide, particularly in inducing cytokine production. Thalidomide and related immunomodulatory drugs (IMiDs), which are thalidomide derivatives, pomalidomide (Pomalyst/Imnovid®) and lenalidomide (Revlimid®), have been widely studied and demonstrated as indirect NK cell activity enhancers. The immunomodulatory mechanism of IMiDs is explained as a co-stimulatory signal to T cells to enhance proliferation and induce IL-2 and IFN-γ secretion (Anderson 2005; Davies et al. 2001; Haslett et al. 1998). Molecular mechanism

of thalidomide and derivatives are still being studied and not fully understood. Even so, successful results have been reported for both anti-angiogenic effect and immunomodulatory function on the patients who are diagnosed with multiple myeloma (Quach et al. 2010). However, preclinical outcomes are restricted in clinical practice because of the challenging characteristics of cancer disease and IMiDs still need to be studied in detail and improved.

1.3.3. Retargeting NK cells Against Tumors via Monoclonal Antibodies (mAbs)

The use of antibodies or engineered proteins in targeted cancer immunotherapy has been an emerging research topic for several years (Mayes, Hance, and Hoos 2018). Tumor-targeted monoclonal antibodies (mAbs) with higher affinity have increased the success rates of cancer treatment strategies (Adler and Dimitrov 2012). The primary role of mAbs is targeting the tumor directly to kill or indirectly to suppress tumor growth. mAb treatments enable antigen-specific interactions with host immune system components to induce or reactivate immune responses (Childs and Carlsten 2015). More specifically, antibody-coated target cells are destroyed in a process called Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) in which NK cells play a significant role.

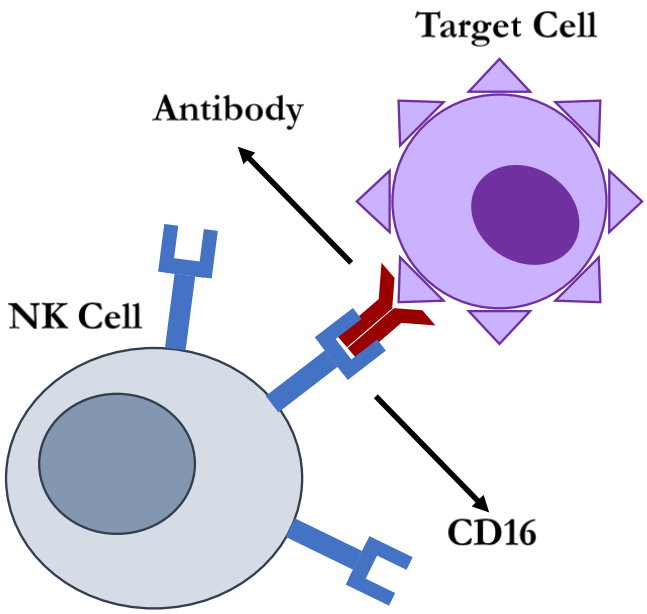


Figure 2. Antibody-Dependent Cell-mediated Cytotoxicity

Identification of ADCC was first given by Erna Möller in 1965 as Contact-induced Cytotoxicity by Lymphoid Cells (MOELLER 1965). The description demonstrates the effect of rabbit antiserum on lymphoid cells, which lead cells to accumulate around the

tumor. Further studies revealed that immunoglobulin content of the antiserum is the factor that activates immune cells and directs them to tumor region (MacLennan, Loewi, and Harding 1970). It is known that Fc receptors for immunoglobulin G (IgG) are presented on immune cells (Adler and Dimitrov 2012).

FcγR family is composed of four classes, and human NK cells express two among these four types. NK cells do not express the inhibitor FcγR so that the significance of NK cells in ADCC depends on the two forms, FcγRIIC (CD32c) and FcγRIIIA (CD16a) which have roles on starting signal transduction of NK cell-activating pathways upon binding Fc portions of the antibodies bound to the surface of the target cell. Current mAb therapies in clinical use mediate most of their ADCC effects through mainly NK cells and the other FcR expressing immune cells such as macrophages. Examples include Rituxan® (rituximab) and Erbitux® (cetuximab) which are targeted to CD20 and EGFR, respectively, as well as several other studies with other mAbs that demonstrate higher NK cell activity, such as Herceptin® (trastuzumab) (Alderson and Sondel 2011), GAZYVA® (obinutuzumab) and anti-GD2 mAb (Wang et al. 2015).

1.3.4. Genetic Manipulation of NK cells for Cancer Immunotherapy

NK cell cytotoxicity is mediated by activating and inhibitory receptors that are present on the membrane of NK cells without any prior stimulation (Pegram et al. 2011). In terms of cancer immunotherapy, NK cell function depends on the interaction between effector NK cells and tumor cells (Sun and Lanier 2011). However, during cancer development, impairments in the metabolism of the tumor microenvironment (TME) causes accumulation of immunosuppressive factors leading to inhibition of NK cells among other effector populations of the immune system. Emerging applications in NK cell genetic manipulation to let NK cells escape from immunosuppression consist of various approaches for enhancing persistence or cytotoxic activity (Chambers, Lupo, and Matosevic 2018). Early studies aiming to genetically modify NK cells are applied to enhance persistence via endogenous cytokine expression. As it is mentioned in previous part, IL-2 has a vital role in NK cell survival and proliferation. It is also demonstrated that systemic IL-2 administration may have adverse clinical side effects, for that matter stable endogenous gene expression gained importance in immunotherapeutic approaches. First endogenously IL-2 expressing NK cells are achieved by Miller et al. in 1997 by retroviral transduction. Despite the challenges in determining experimental procedures,

they optimized the retroviral transduction protocol to successfully obtain IL-2 expressing NK cells and reported proliferation for 7 days after IL-2 withdrawal (J. S. Miller et al. 1997). A similar study with different protocol on NK-92 and YT cell lines also indicates the potential of NK cell-based cytokine gene therapy with Nagashima et al. reporting exogenous IL-2 independent proliferation for more than 5 months and enhanced cytotoxic activity *in vivo* (Nagashima et al. 1998).

For non-viral genetic modification of NK cells, Grund et al. demonstrated DNA electroporation application on the NK-92 cell line. This study reports optimal conditions for NK cell modification via electroporation method (Grund and Muise-Helmericks 2005) for transfer of the EGFP gene. Further studies that are inspired by electroporation showed successful genetic manipulation of various NK cell lines. A study with NKL cell line shows IL-15 gene delivery with electroporation. Their findings suggest that transfected IL-15 gene is expressed stably and they observed improved proliferation and reduced apoptotic cells with enhanced *in vitro* cytotoxic activity against human hepatocellular carcinoma (W. Jiang, Zhang, and Tian 2008). Although the improvements on electroporation transfection in several approaches (Carlsten et al. 2014; Boissel et al. 2009) have been stated, the challenges of the technique restrict its clinical use. Most concerning limitation in transfection via electroporation is cell death during the primary electric pulse. Electroporation induced cell death decreases the efficiency or even leaves the method completely non-functional (Piñero et al. 1997). Likewise, different approaches such as nucleofection (D. Zhang et al. 2015), lipofection (Regis et al. 2017) and trogocytosis (Cho et al. 2014) have been examined to non-virally modify NK cell genome. However, standardized protocols needed for each technique remain as the main consideration (Matosevic 2018). Taking into account the gene delivery efficiency and clinical efficacy of genetically modified cells, the use of retroviral or lentiviral vectors for gene delivery are currently most common for genetic modification of NK cells. These will be covered in the next chapter of this thesis.

Recent studies with genetically modified (GM) NK cells are focused on chimeric antigen receptor (CAR) gene delivery to trigger recognition of target cell and redirect cytotoxic activity against a specific cell surface antigen found on the tumor cells. Müller et al. used NK-92 cell line to generate CD20 specific effector cells against B cell lymphomas and reported specificity cytotoxic activity of retrovirally transduced NK cells against only CD20 expressing cells (Müller et al. 2008). Another study with primary NK cells was

aimed to generate chronic lymphocytic leukemia (CLL) and Raji targeting NK cells via the use of a CD19-targeted CAR and reported high efficacy, both *in vitro* and *in vivo* of GM NK cells (Liu et al. 2018). Similar studies have reported promising results (Yvon et al. 2017; Velasquez et al. 2016), but limitations in retroviral gene delivery and development of lentiviral vector technologies have in the last two decades shifted the focus more on lentiviral gene delivery.

Similarly to retroviral studies, there are increasing numbers of studies with lentiviral vectors to develop CAR-expressing NK cells (Steinbach et al. 2014; Kobayashi et al. 2014). Our group and others have also recently used lentiviral vectors to express functional TCR complexes on NK cell lines and enable for the first time the targeting of intracellular antigens by NK cells (Mensali et al. 2019; Parlar et al. 2019). Although applications vary, and viral vectors seem to outperform non-viral approaches, the common denominator in genetic modification studies remains that the overall gene delivery in NK cells remains relatively low. A study with primary NK cells to set several lentiviral transduction parameters shows no relation between lentiviral transduction and functional properties of NK cells, though challenges remain problematic in lentiviral gene delivery (Carlsten and Childs 2015; Micucci et al. 2006).

1.4. Lentiviral Vectors

Lentiviruses are HIV-based viruses which are a subclass of the retroviridae family (Naldini, Blömer, et al. 1996). Lentiviral vectors derived from these viruses have become efficient tools in gene therapy. Recent challenges in gene therapy and other vector systems exhibit increasing demand for engineered lentiviral vectors. Especially, distinct properties of lentiviral systems such as the potential to transduce a large variety of dividing and non-dividing cells with stable transgene expression prove their importance.

1.4.1. Life Cycle of a Lentivirus

To better understand the underlying mechanism of lentiviral vector systems, an examination of the life cycle of a retrovirus is of paramount importance. Viral integration begins with attachment of the infectious particle to the target cell by connection between the envelope glycoprotein and cell surface receptors. When binding is achieved, viral envelope fuses with the target cell membrane which results in the release of the virion into the target cell cytoplasm. The capsid gets uncoated, and through reverse

transcription, single-stranded viral RNA (ssRNA) is converted into double-stranded DNA (dsDNA) and transported into the nucleus. The transport to the nucleus is maintained by a pre-integration complex that can facilitate active transport into the nucleus in lentiviruses such as HIV-1 while gammaretroviruses lack this active transport mechanism. This difference is critical since gammaretroviruses must wait for the cell cycle to proceed for access into the nucleus during prophase where the nuclear envelope breaks down. When viral DNA is integrated into cell genome, expression of viral genes begin. Viral ssRNA and proteins get enfolded and form virus particle proximal to the cell membrane where new viral particles bud off the infected cell (Buchschacher and Wong-Staal 2000; Escors and Breckpot 2010).

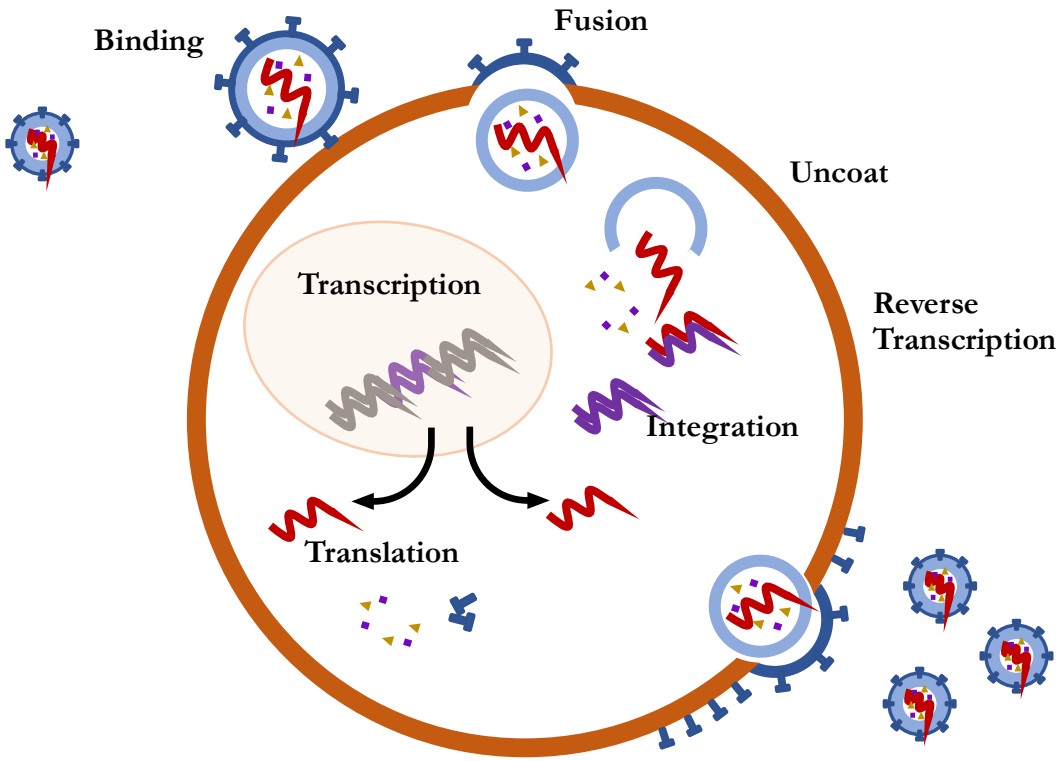


Figure 3. Life cycle of a retrovirus

1.4.2. Development of Lentiviral Vectors

Several restrictions and safety concerns discovered by the early adapters of retroviral systems lead the development of safer and more efficient lentiviral vectors based on HIV-1. Lentiviral vectors are significantly distinct from retroviral vectors in their ability to transduce non-dividing cells as they have the potential to actively transport into the nucleus. This leads to a relatively safer integration profile for lentiviral vectors (Milone and O'Doherty 2018; Cooray, Howe, and Thrasher 2012). Lentiviral vectors are

developed and explained in three generations. First-generation lentiviral vectors consist of two constructs; packaging plasmid and the vector containing gene of interest. Packaging plasmid in this system includes most of HIV genes (including the envelope gene) but lacks packaging signal (ψ). Therefore, this plasmid alone is deficient for viral particle production. Moreover, plasmid contains cytomegalovirus (CMV) promoter and polyadenylation site at 5' and 3' ends instead of long terminal repeats (LTR). For second-generation vectors, furface glycoprotein of vesicular stomatitis virus (VSV-G) is encoded on a separate plasmid as the envelope plasmid and third vector contains target gene with required genes for packaging and reverse transcription (Naldini, Blomer, et al. 1996).

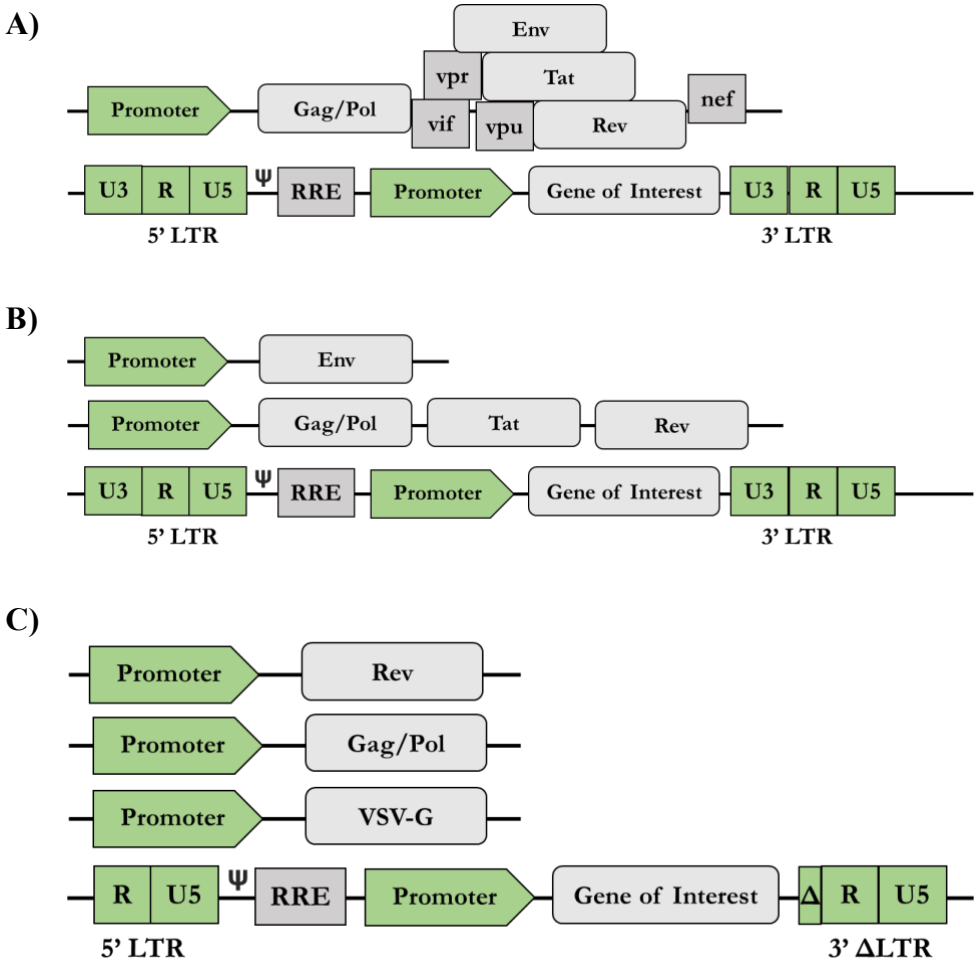


Figure 4. Development of lentiviral vectors. (A) First-generation lentiviral vectors, (B) Second generation lentiviral vectors, (C) Third generation lentiviral vectors

Further studies with HIV-based lentiviral vectors has revealed that elimination of accessory proteins does not interfere with transduction efficiency (Gruber et al. 2000). Later, it is understood that accessory genes have a role in survival *in vivo* but do not

participate in viral replication where regulatory genes fulfill the requirement (Milone and O'Doherty 2018). In accordance with these findings, second-generation lentiviral vectors are developed lacking *Vip, Vpr, Vpu* and *Nef* genes (Zufferey et al. 1997). For next version, safety concerns are prioritized for development of vectors. In third-generation lentiviral vectors, *gag/pol* and *rev* genes are encoded on separate plasmids which creates a requirement for three necessary constructs for packaging. Utilization of engineered LTRs in this version leaves *tat* gene dysfunctional so that third generation vectors do not include *tat* gene. Further safety improvements are applied on the 3'LTR by by disruption of the U3 region, which provides a self-inactivation function (Breckpot, Aerts, and Thielemans 2007; Milone and O'Doherty 2018).

1.5. Innate Antiviral Defense Mechanism

1.5.1. Innate Pattern Recognition System

Innate immune system members are evolved to generate rapid response against pathogens without antigen specificity. The interaction signals between host and pathogen are received by pathogen-recognition receptors (PRRs), which regulate recognition through pathogen-associated molecular patterns (PAMPs) (Kumar, Kawai, and Akira 2011a). Unlike the antigen-specific adaptive immune system components; conserved molecular patterns activate innate immune response via general carbohydrates, lipoproteins or nucleic acids (Kumar, Kawai, and Akira 2011a). PRR induced activation results in upregulated cytokine production, mainly interferons and inflammatory cytokines. Identified PRRs are divided into five groups which are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NOD-like/NLRs) and AIM2-like receptors (ALRs) (Brubaker et al. 2015). Some of these receptors are involved in viral component recognition to generate antiviral innate immunity.

Table 1. Recognition of viral RNA by PRRs

Pattern Recognition Receptors (PRRs) Involved in Viral Infections

Endosomal Recognition				Cytosolic Recognition		
Toll-Like Receptor Family (TLRs)				RIG-I Like Receptor Family (RLRs)		
TLR3	TLR7	TLR8	TLR9	DDX58 (RIG-I)	IFIH1 (MDA5)	LGP2
Viral dsRNA	Viral ssRNA		CpG DNA	Short dsRNA	Long dsRNA	Regulatory
Type 1 IFN and proinflammatory cytokine expression						

1.5.2. Antiviral Innate Immune Response via Stress Granule Formation

Among many PRRs, TLRs are widely studied receptors that have roles against several types of microorganisms. Human TLRs are divided into ten subclasses with different targets. TLR1, 2, 4, 5 and 6 are transmembrane proteins to recognize usually glycoprotein or lipid-based PAMPs, on the other hand, TLR3, 7, 8 and 9 are endosome located for nucleic acid targets (Kumar, Kawai, and Akira 2011b). TLR9 is responsible for sensing viral DNA that contains unmethylated CpG motifs which are typically found in herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) genome. The recognition of viral DNA by TLR9 leads to recruitment of the adaptor protein, MyD88, to induce downstream of its signaling pathway where NF-κB gets activated and upregulates Type I IFN and inflammatory cytokines such as TNF-α expression (Wagner 2009). TLR7/8 also trigger the same cascade of signaling pathway by sensing viral ssRNA of RNA viruses (Akira and Hemmi n.d.). TLR3 is assigned to recognize Polyinosinic:polycytidylic acid (poly I:C) which has similar structural properties with dsRNA also known as the synthetic analog of dsRNA. Binding of TLR3 to poly I:C leads to recruitment of TRIF adaptor protein that induces NF-κB activation and results in upregulation of Type I IFN and inflammatory cytokines such as TNF-α expression (Kumar, Kawai, and Akira 2009). Some intracellular PRRs have the same responsibility as endosomal TLRs, but they patrol the cytoplasm instead of the endosome. RLRs participate in cytoplasmic recognition of PAMPs. RLR family is composed of three identified proteins, RIG-I, IFIH1 and LGP2 (Bruns and Horvath 2014). Retinoic acid-inducible gene I (RIG-I) is able to recognize viral nucleic acids through the 5′-triphosphorylated uncapped viral ssRNA. Healthy host cells carry capped ssRNA so RIG-I can distinguish host and viral nucleic acids (Thompson et al. 2011). Even though MDA5 mechanism has not been identified, different

form RIG-I, MDA5 recognizes larger fragments of viral RNA with lower affinity (Bruns and Horvath 2014). LGP-2 is considered to be associated with MDA5 recognition to assist MDA5 binding (Rodriguez, Bruns, and Horvath 2014). As a result of RIG-I and viral RNA interaction, RIG-I recruits IPS-1 which is followed by induction of TBK1 that phosphorylates IRF-3 and IRF-7 transcription factors for the Type I IFN expression (Kato et al. 2006). The third component of the RLR family, LGP2 acts as a mediator of RLR related viral RNA recognition and antiviral response. Both inhibitory roles in knockout mice and activator roles synergic to MDA5 of LGP2 have been reported but need to be further investigated (Bruns and Horvath 2014).

Cellular restriction factors also participate in intracellular recognition of viral compounds. These factors are expressed constitutively in a number of cell types and contain recognition motifs against viral components (Blanco-Melo, Venkatesh, and Bieniasz 2012). Among cellular restriction factors, several members of the tripartite motif (TRIM) family carry out antiviral activity. TRIM protein activity is defined by structural features that follow N-terminal RING E3 ligase domain, one or two B-box domains, and a coiled-coil domain. Especially α isoform of *TRIM5* gene recognizes viral capsid proteins and acts as restrictor of viral replication or inhibitor of viral infection (Colomer-Lluch et al. 2018).

Detection of viral nucleic acids in the cytoplasm also induces several other pathways such as apoptosis (Danthi 2016) or stress granule formation (Onomoto et al. 2014). Stress granules, a type of membrane-less organelles, are dense cytoplasmic foci which are clustered untranslated messenger ribonucleoproteins (mRNPs). Stress granule formation takes place under stress conditions, for example during viral infections (Protter and Parker 2016b). Mass spectrometry analysis has revealed some components of stress granules that are mainly RNA-binding proteins (RBPs) (Jain et al. 2016). (Protter and Parker 2016c). Numerous viruses are reported as inducers of stress granule formation by activating RNA-dependent protein kinase (PKR) and eukaryotic initiation factor (eIF) kinases (Onomoto et al. 2014). PKR activation is demonstrated by the interaction between PKR and ssRNA (Mayo and Cole 2017) or dsRNA (Lemaire et al. 2008a). Additionally, PKR activation can be induced by IFN stimulation (Pindel and Sadler 2011) which is a result of upregulated expression levels of IFN during viral inflammation (Onomoto et al. 2012a). In consequence of viral infection, activated PKR leads to eIF2α phosphorylation

(Lemaire et al. 2008b) and inhibits viral translation inhibition via stress granule formation (Onomoto et al. 2012b).

1.5.3. Possible Role of CCDC124 in Antiviral Response Through Stress Granules

Non-RNA-binding proteins such as translation initiation factors and RNA-binding proteins constitute the stress granule assembly (C. L. Miller 2011). RasGAP-SH3-binding protein (G3BP) is identified as an RNA-binding protein with two isoforms G3BP1 and G3BP2 (Tourrière et al. 2003). Direct relation of G3BP1 in stress granule formation is reported in multiple studies and is used as a stress granule marker (McCormick and Khaperskyy 2017). Other RNA-binding proteins that are prominent in stress granules, T-cell intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR) together with G3BP have common features called intrinsically disordered regions (IDR) (Protter and Parker 2016a). According to structural analysis and classification of IDRs, some of the IDRs contain coiled-coil based complexes (van der Lee et al. 2014). The coiled-coil domains (CCDs) are motifs found in proteins that have a crucial role in cellular structure and signal transduction of eukaryotic cells (Li et al. 2016). One of the members of this family and recently characterized *Coiled-coil domain containing 124 (CCDC124)* gene is conserved in most species. Localization of CCDC124 in centrosome during cell division has been demonstrated without dependency to centrosome formation. However, absence of CCDC124 causes impaired cytokinesis which results with multinucleated cells. It has been reported that CCDC124 plays a role in cytokinesis by interacting with Ras-guanine-nucleotide exchange factors 1b (RasGEF1b) (Telkoparan et al. 2013). As mentioned previously, Toll-like receptor 3 (TLR3) is one of the TLR family members which is responsible for viral RNA recognition, mostly found in intracellular compartments, such as endoplasmic reticulum (ER), lysosomes or endosomes (Jensen and Thomsen 2012). TLR induced upregulation in expression of RasGEF1b and localization in early endosomes is demonstrated in murine macrophages (Andrade et al. 2010). Similar to RasGEF1b, it is also confirmed that TLR3 localizes to early endosomes (Funami et al. 2007). The interaction between TLR3 and RasGEF1b is still unidentified.

2. AIM OF THE STUDY

Natural killer cells are known to be resistant against lentiviral gene delivery. NK cells provide the first line of host protection mechanism, especially against viral threats. The recognition is not antigen-specific; instead, NK cells utilize specific intracellular patterns to detect the pathogen and respond to infection. This defense mechanism in primary NK cells is also seen in the NK-92 cell line which hinders the efficiency of gene delivery to NK cells using lentiviral vectors. To overcome this issue, small kinase inhibitors have been adapted to viral transduction methods to increase viral transduction efficiencies. The use of BX795, targeted to TBK1/IKKε pathway, during lentiviral transduction have been shown previously as transduction efficiency enhancer in NK-92 cell line and primary human NK cells (Sutlu et al. 2012). Our previous studies also confirm the enhancer effect of (5Z)-7-Oxozeaenol (OXO) which is targeted to MAPKK pathway. However, critical mediators of innate antiviral pathways have not been clarified yet.

Lentiviruses vary from retroviruses in their ability to integrate into non-dividing cells. However, intracellular dynamics during cytokinesis still have an impact on lentiviral gene delivery. Even though lentiviruses have been known to be able to deliver their gene into non-dividing cells, higher transduction efficiencies have been shown during G2 phase of the cell cycle (S. Zhang et al. 2006). These findings suggest that lentiviral transduction would be reduced in cells with impaired cytokinesis. CCDC124 protein has been identified as an agent that participates in cytokinesis by localizing in centrosomes. CCDC124 knockout cells have shown inability to divide and return to G1 phase. As a result of cytokinesis role, we considered that CCDC124 absence would have a proviral effect on the innate immune response.

On the other hand, stress granules have been reported as inhibitor of viral infections and viral replication. Our next consideration is the potential role of CCDC124 in stress granule formation because of its possible interaction with stress granule components. The aim of this study is to reveal the mechanism of CCDC124 in innate antiviral response by investigating lentiviral transduction efficiencies in CCDC124 knockout cell lines.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Table 2. List of chemicals

Chemicals and Media Components	Company
(5Z)-7-Oxozeaenol	Sigma, Germany
2-Mercaptoethanol	Sigma, Germany
Agar	Sigma, Germany
Agarose	Sigma, Germany
Ampicillin Sodium Salt	CellGro, USA
Boric Acid	Sigma, Germany
Bovine Serum Albumin	neoFroxx, Germany
BX795	Sigma, Germany
Chloroquine	Sigma, Germany
Distilled Water	Merck Millipore, USA
DMEM	GIBCO, USA
DMSO	Sigma, Germany
DNA Gel Loading Dye, 6X	NEB, USA
DPBS	Sigma, Germany
EDTA	Applichem, Germany
Ethanol	Sigma, Germany
Ethidium Bromide	Sigma, Germany
Fetal Bovine Serum	Thermo Fischer Scientific, USA
HEPES Solution, 1 M	Sigma, Germany
Hoechst 33342 Solution (20 mM)	Thermo Fischer Scientific, USA
Interleukin-2	Proleukin, Novartis
Isopropanol	Sigma, Germany
LB Broth	Sigma, Germany
L-glutamine, 200 mM	Thermo Fischer Scientific, USA
MEM Non-Essential Amino Acid Solution	Thermo Fischer Scientific, USA
MEM Vitamin Solution, 100X	Thermo Fischer Scientific, USA
Methanol	Sigma, Germany
Mowiol Mounting Medium	Sigma, Germany
NaCl	Sigma, Germany
RNAase A	Thermo Fischer Scientific, USA
PIPES	Sigma, Germany
Poly-L-lysine	Sigma, Germany

Protamine Sulfate	GIBCO, USA
RPMI 1640	GIBCO, USA
Triton X-100	Sigma, Germany
Sodium Pyruvate Solution, 100 mM	GIBCO, USA
Trypsin-EDTA	GIBCO, USA

3.1.2. Equipment

Table 3. List of equipment

Equipment	**Company**
Autoclave	Hirayama, HiClave HV-110, Japan
Balance	ISOLAB, 302.31.002, Germany
Centrifuge	Eppendorf, 5415D, Germany
	Eppendorf, 5702, Germany
	VWR, MegaStar 3.0R, USA
	Beckman Coulter, Allegra X-15R, USA
CO_2 Incubator	Thermo Fisher, Heracell Vios 160i, USA
	Binder, Germany
Deep Freezer	-80 ^oC, Forma, Thermo ElectronCorp., USA
	-20 ^oC, Bosch, Turkey
Electrophoresis Apparatus	Biorad Inc., USA
Filters (0.22 mm and 0.45mm)	Merck Millipore, USA
Flow cytometer	BD LSR Fortessa, USA
Freezing Container	Mr. Frosty, Thermo Fischer Scientific, USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Hemocytometer	ISOLAB, Neubauer, 075.03.001, Germany
Ice Machine	Scotsman Inc., AF20, USA
Laminar Flow	Heraeus, HeraSafe HS 12, Germany
	Heraeus, HeraSafe KS, Germany
LightCycler® 480	Roche, Switzerland
Liquid Nitrogen Tank	Taylor-Wharton, 300RS, USA
Magnetic Stirrer	VELP Scientifica, Italy
Microliter Pipettes	Gilson, Pipetman, France
	ISOLAB, Germany
	Thermo Fisher Scientific, USA
Microscope	Zeiss, Primo Vert, Germany
	Zeiss Observer Z1, Germany
	Zeiss Confocal LSM 880, Germany
Microwave Oven	Bosch, Turkey
pH Meter	Mettler Toledo, USA
Refrigerator	Bosch, Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	New Brunswick Sci., USA
	NanoDrop 2000, Thermo Fischer Scientific, USA
Thermocycler	C1000 Touch, Biorad, USA
	Eppendorf, Mastercycler, Germany
	PTC-200, MJ Research Inc., Canada

3.1.3. Buffers and Solutions

Agarose Gel: For 100 ml 1% w/v gel, 1 g of agarose powder was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.

Blocking Solution: For 50 mL solution, 1 g BSA was dissolved in 50 mL PBS-T.

Calcium Chloride ($CaCl_2$) Solution: 60 mM $CaCl_2$ (diluted from 1 M stock), 15% Glycerol, 10 mM PIPES (pH 7.00) were mixed and sterilized by autoclaving at 121°C for 15 minutes and stored at 4°C.

DAPI Solution: For DAPI solution, 1:100.000 dilution of DAPI dye was prepared in blocking solution

HBS Solution (2X): 280 mM NaCl, 50 mM HEPES and 1.5 mM Na_2HPO_4 were mixed and pH was adjusted to 7.1 with 10 M NaOH and sterilized by filtering with 0.22 µm filter and stored at -20°C

Phosphate-buffered saline (PBS): For 1000 ml 1X solution, 100 ml 10X DPBS was added to 900 ml ddH_2O and the solution was filter-sterilized.

PBS-T: For 50 mL solution, 50 µL of Triton X-100 was filled up to 50 mL with 1X PBS.

PI Solution: 0.5 µg of PI stain was dissolved in 100µl in PBS.

Tris-Borate-EDTA (TBE) Buffer: For 1 L 5X stock solution, 54 g Tris-base, 27.5 g boric acid, and 20 ml 0.5M EDTA (pH 8.00) were dissolved in 1 L of ddH_2O. The solution is stored at room temperature (RT) and diluted 1 to 10 with ddH_2O for working solution of 0.5X TBE.

3.1.4. Growth Media

Luria Broth (LB): For 1 L 1X LB media, 20 g LB powder was dissolved in 1 L ddH2O and then autoclaved at 121°C for 15 minutes. For selection, kanamycin at a final concentration of 50 µg/ml or ampicillin at a final concentration of 100 µg/ml was added to liquid medium just before use.

LB-Agar: For 1X agar medium in 1L, 20 g LB powder and 15 g bacterial agar powder were dissolved in 1 L ddH_2O and then autoclaved at 121°C for 15 minutes. Then, autoclaved LB agar is mixed with antibiotic of interest at the desired ratio. Kanamycin at a final concentration of 50 µg/ml or ampicillin at a final concentration of 100 µg/ml was

added to prepared medium just before pouring into sterile Petri dishes. Sterile agar plates were kept at 4°C.

DMEM: 293T, 293FT, and HCT116 cells were maintained in culture in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-Glutamine, 1mM Sodium Pyruvate, 0.1mM MEM Non-essential amino acid solution, and 25mM HEPES solution.

RPMI: NK-92 and YTS cell lines were maintained in culture in RPMI1640 supplemented with 20% heat-inactivated fetal bovine serum, 25 mM HEPES, 2 mM L-Glutamine, 1X MEM vitamins, 0.1 mM MEM Non-essential amino acid solution, 1 mM Sodium Pyruvate, and 0.1 mM 2-mercaptoethanol. For the NK-92 cell line, 1000 U/ml Interleukin-2 was added to culture every 48 hours.

Freezing medium: All the cell lines were frozen in heat-inactivated fetal bovine serum containing 6% DMSO (v/v).

3.1.5. Commercial Kits

Table 4. Commercial kits

Commercial Kit	Company
LightCycler® 480 SYBR Green I Master Kit	Fermentas, USA
Nucleo Spin® Plasmid Midiprep Kit	Macherey-Nagel, USA
RNA isolation kit	Zymo Research, USA
RvertAid First Strand cDNA Synthesis Kit	Thermo Fisher, USA
Taq DNA Polymerase with Standard *Taq* (Mg-free) Buffer Kit	NEB, USA

3.1.6. Enzymes

All the restriction enzymes, polymerases and PCR reaction supplements are obtained from either Fermentas or New England Biolabs.

3.1.7. Antibodies

Table 5. List of antibodies

Antibody	**Company**
Anti-G3BP1 (ab56574)	Abcam, UK
Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor ® 594 Conjugate)	CST, The Netherlands
Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate)	CST, The Netherlands
CCDC124 Antibody, A301-835A	Bethyl Lab, USA
Mouse APC anti-CD56 (NCAM 16.2)	BD Biosciences, USA

3.1.8. Bacterial Strains

Top10 strain is used for lentiviral construct amplifications.

3.1.9. Mammalian Cell Lines

HEK293FT: Human embryonic kidney 293 (HEK293) cell line derivative that stably expresses the large T antigen of SV40 virus and has fast-growing specificity (Invitrogen R70007).

HEK293T: Human embryonic kidney 293 (HEK293) cell line derivative that stably expresses the large T antigen of SV40 virus

HCT116: Human colorectal carcinoma (HCT116) cell line that was derived from adult male, are positive for transforming growth factor-beta 1 (TGF beta 1) and beta 2 (TGF beta 2) expression (ATCC® CCL-247™)

NK-92: IL-2 dependent human natural killer cell line, derived from 50 years old male malignant non-Hodgkin's lymphoma patient (ATCC® CRL 2407™).

YTS: Derivative of YT cell line that was originally from a 15-year old male with acute lymphoblastic leukemia (ALL) were TCR-negative cells with NK cell activity (DSMZ ACC 434).

3.1.10. Plasmids and CRISPR Constructs

The CRISPR constructs and plasmid those were used in this study are listed below.

Table 6. List of CRISPR Constructs. All CRISPR constructs were cloned into LeGO-iG2p backbone

Target Gene		sgRNA Sequence
AAVS1	Top	CACC**TAGGACAGGGATCACCGGGG**
	Bottom	AAAC**CCCCGGTGATCCCTGTCCTA**
CCDC124	Top	CACC**GGCGCAGCGTGTCCTCGATC**
	Bottom	AAAC**GATCGAGGACACGCTGCGCC**
DDX58	Top	CACC**GGGGTCTTCCGGATATAATCC**
	Bottom	AAAC**GGATTATATCCGGAAGACCCC**
IFIH1	Top	CACC**GCGAATTCCCGAGTCCAACCA**
	Bottom	AAAC**TGGTTGGACTCGGGAATTCGC**
PATZ1	Top	CACC**TGGCTGCTACACATACC**
	Bottom	AAAC**GGTATGTGTAGCAGCCA**
TLR3	Top	CACC**GTTCGGAGCATCAGTCGTTGA**
	Bottom	AAAC**TCAACGACTGATGCTCCGAAC**

Table 7. List of plasmids

Plasmid Name	Purpose of Use	Source
pMDLg/pRRE	Virus production/packaging plasmid (*Gag/Pol*)	Addgene (#12251)
pRSV-REV	Virus production/packaging plasmid (*Rev*)	Addgene (#12253)
pCMV-VSV-g	Virus production/packaging plasmid (*Env*)	Addgene (#8454)
LeGO-G2	Lentiviral construct for GFP expression	Kind gift from Prof. Boris Fehse of University Medical Center Hamburg-Eppendorf, Hamburg, Germany
LeGO-iG2-Puro	Lentiviral construct for GFP expression with Puromycin resistance gene for selection	Kind gift from Prof. Boris Fehse of University Medical Center Hamburg-Eppendorf, Hamburg, Germany
LeGO-iRFP670	Lentiviral construct for iRFP expression	Kind gift from Adil Doğanay Duru of Nova Southeastern University, Florida, USA

3.1.11. Software, Computer-based Programs and Websites

Table 8. List of used software, computer-based programs and websites.

Software, Program and Website	Company/Web Address	Purpose of Use
Addgene	https://www.addgene.org/	Plasmid map and sequence information
BD FACSDiva	BD Biosciences	Flow cytometry control software
CLC Main Workbench v7.7	CLC bio	Constructing vector maps, restriction analysis, DNA sequencing analysis, DNA alignments, etc
FlowJo v10	Tree Star Inc.	Analyzing raw flow cytometry data
LightCycler 480 SW 1.5	ROCHE	Analyzing qPCR results
Office 365	Microsoft	Analytical calculations
Origin 9.0	OriginLab Corp.	Drawing graphs and plots

3.2. Methods

3.2.1. Bacterial Culture

E. coli cells were cultured in LB media with ampicillin and grown at 37^{o}C with 220 rpm shaking. For single colony picking, cells were spread on Petri dishes which had been prepared with ampicillin. Cell spread applied by glass beads and plates were placed into 37^{o}C incubator for overnight incubation. For long term storage of bacteria, single colonies grown overnight in liquid culture were further diluted 1:3 and were grown for another 3 hours at 37^{o}C with 221 rpm shaking. Bacteria were taken at log phase of growth and mixed with glycerol in 1 ml at final 10% (w/v) and preserved in cryotubes at -80^{o}C. Macherey-Nagel Midiprep Kits were applied for DNA isolation according to manufacturer's protocols. The final DNA concentration and purity were measured by a NanoDrop spectrophotometer.

3.2.2. Mammalian Cell Culture

Cell Thaw: Cells that are preserved in liquid nitrogen in cryotubes were taken on ice and slowly brought to RT. 15 ml tubes were prepared for each cell with 5 ml FBS. When the cell suspension was at RT, 1 ml frozen sample was pipetted very carefully into FBS, taking 2-3 minutes in total to avoid harming cells and dilute remnants of DMSO. The cells were then centrifuged at 300g for 5 minutes, and the supernatant was discarded. The

cell pellet was resuspended with complete media to reach 500,000-700,000 cells/ml concentration, and the cells were followed every day after thaw.

Maintenance of Cell Lines: 293T, 293FT, and HCT116 cells were maintained in complete DMEM medium in sterile tissue culture flasks with filtered caps at an incubator set to 37°C with 5% CO2. Cells were split when maximum 90% confluency was reached. The supernatant was discarded, and cells were washed with DPBS and trypsin was added to cell culture flasks and incubated in 37°C incubator with 5% CO_2 for 5 minutes. Then the cells were resuspended in complete DMEM and split at 1:3 to 1:10 ratio and split every two days, never letting them reach full confluency.

NK-92 and YTS cells were maintained in complete RPMI medium in sterile tissue culture flasks with filtered caps at an incubator set to 37°C with 5% CO_2. Cells were kept at a density between 300,000 cells/ml to 1,000,000 cells/ml. 1000 U/ml human Interleukin-2 (IL-2) was added every 48 hours for NK-92 cells.

Cryopreservation: All types of cell lines were split one day before freezing to a concentration of 500,000 cells/ml for suspension cells and to a confluency of 30-40% for adherent cells. The next day, cells to be frozen were counted and at least $3x10^6$ cells were frozen per vial. For each vial, cells were centrifuged at 300g for 5 minutes where supernatant was discarded, and the pellet was resuspended in 0.5ml FBS and incubated on ice for 15-20 minutes. In the meantime, 0.5 ml FBS with 12% DMSO was prepared fresh and incubated on ice. When the incubation was over, 0.5 ml cell suspension was mixed with 0.5 ml freezing medium to reach 6% DMSO in 1 ml. Cells were stored in cryotubes in -80°C for at least 24 hours, then in liquid nitrogen for long term storage.

3.2.3. DNA Ladder

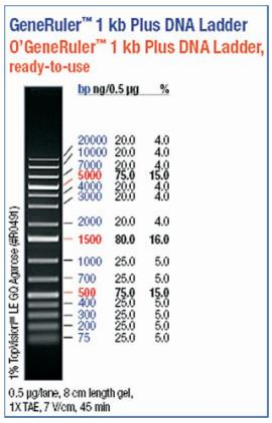


Figure 5. DNA ladder that was used in electroporation experiments

3.2.4. Lentiviral Vector Production

293FT cells were used as a transfection host in lentiviral vector production. 100 mm cell culture dishes were prepared by coating with 2 ml of filtered poly-L-lysine solution (0.1% w/v in H_2O). After 8 min of incubation, leftover poly-L-lysine solution was removed, and dishes were rinsed with sterile ddH_2O to dispose any residual of poly-L-lysine solution. When dishes get dried, $5x10^6$ 293FT cells were seeded to each dish and placed into incubator to be cultured overnight. Next morning, cells were transfected via calcium phosphate transfection method. For each dish, 7.5 µg of plasmid containing gene of interest, 3.75 µg of pMDLg/pRRE, 2.25 µg of pRSV-REV and 1.5 µg of phCMV-VSV-G plasmids were combined and completed to 450 µL of mixture with sterile ddH_2O. 50 µL of 2.5 M $CaCl_2$ was added to mixture slowly, and whole mixture was dissolved in 2X HBS buffer drop by drop while HBS buffer was being bubbled. During 15 minutes incubation of plasmids at room temperature, medium of the transfection dishes was replaced with fresh, 25 µM chloroquine containing DMEM-Glutamax. At the end of incubation of plasmids, plasmid mixture was distributed to transfection dishes dropwise, and dishes were placed to incubator. No later than 8-10 hours of incubation, chloroquine containing medium was discarded and fresh DMEM-Glutamax was given to the cells. Virus containing supernatants were collected in following 24 and 36 hours. The collected supernatant was filtered with 0.45 µm filters and stored in -80°C. 50 µL of aliquots from each gene of interest containing virus stored separately to determine virus titer by the transduction of 293FT cells.

3.2.5. Virus Titration

293FT cells were seeded in 24-well-plate with the concentration of $0.5x10^5$ cells/well and plates were placed into the incubator to let cells to adhere onto the well bottom in 4-5 hours. Then, serial concentrations of viral supernatant were given to cells in the presence of 8 µg/ml protamine sulfate. Titration plate was cultured for 16 hours, and supernatant was replaced with fresh DMEM. 48 hours after medium change, transduction efficiencies of different concentrations were obtained with flow cytometry. Depending on the fluorescent protein-expressing population percentage, multiplicity of infection (MOI) was calculated.

3.2.6. Lentiviral Transduction

Suspension Cells: For each lentiviral transduction to generate knockout cells, 10^6 NK-92 or YTS cells per T25 flask were seeded with an appropriate amount of virus supernatant in the presence of 8 μg/ml of protamine sulfate and 1.5 μM OXO. Cells were incubated at 37°C, 5% CO_2 overnight and next day in the morning, cells were taken to sterile tubes and centrifuged at 300g for 5 minutes at room temperature to replace viral supernatant with fresh growth medium.

For each lentiviral transduction to determine transduction efficiencies in different conditions, $2.5x10^5$ NK-92 and YTS cells per well were seeded in 24-well-plates at specified MOI in the presence of 8 μg/ml Protamine Sulfate and 1000 U/ml IL-2 (only for NK-92 cells) for 6 hours. Depending on the experimental setup 3 μM BX795 or 1.5 μM OXO was involved in lentiviral transduction. First 1 hour of 6 hours incubation took place in centrifuge at 1000g and 37°C, afterward plates were placed into incubators for the rest of the transduction period. After culturing cells in virus-containing media for the given time, plates were centrifuged for 45 minutes at 1000g with acceleration 9 and deceleration 4 for transductions done in 24-well-plates. Virus containing supernatant was completely removed and cells were cultured in their regular growth media for 72 hours before flow cytometry analysis.

Adherent Cells: For each lentiviral transduction, $0.5x10^5$ HCT116 and HEK293T cells per well were seeded in 12-well-plates for overnight culture. Next day in the morning, virus soup at specified MOI in the presence of 8 μg/ml Protamine Sulfate was introduced to the cells for 6 hours. Depending on the experimental setup 3 μM BX795 and/or 1.5 μM OXO was involved in lentiviral transduction. After culturing cells in virus-containing media for the given time, virus-containing supernatant was completely removed and cells were cultured in their regular growth media for 72 hours before flow cytometry analysis.

3.2.7. Flow Cytometry

For determining transgene expression, adherent cells were trypsinized and collected in PBS containing 0.1% FBS. Supernatants were discarded by centrifuge and cells carried on to analysis in PBS.

For surface staining, NK-92 cells were washed once with PBS and stained with an appropriate amount of anti-CD56-APC on ice and in dark for 20 minutes. Cells were washed once more and carried on to analysis in PBS.

For cell-cycle analysis, suspension cells were set to 500.000 cell/ml a day ahead then 10^6 cells of all cell types were taken for PI staining. Cell suspensions were centrifuged at 300g for 5 minutes, and supernatants were discarded. Cells washed with PBS, and ice-cold 70% ethanol (prepared previously and placed in -20°C) was added dropwise to fix cells for 30 minutes at +4°C. Cell suspensions were then centrifuged at 300g for 5 minutes, and supernatants were discarded then washed with PBS and centrifugation applied again. Pellets were resuspended in RNAase A containing PBS and placed into incubator for 15 minutes incubation at 37°C. In the meantime, PI solution was prepared in PBS. At the end of incubation, cell suspensions were centrifuged at 300g for 5 minutes, and supernatants were discarded. PI solution was added onto pellets and tubes were incubated at room temperature for 15 minutes. After the incubation, more PBS was added to each tube and cells were carried on to flow cytometry analysis.

3.2.8. PCR

NEB *Taq* DNA Polymerase with Standard *Taq* (Mg-free) Buffer Kit was applied according to the manufacturer's protocol.

3.2.9. qRT-PCR

RNA isolation: $3x10^6$ cells were seeded for each type of cells, one day before the RNA isolation and the next day, all cells were lysed. Zymo Research RNA isolation Kits were applied according to manufacturer's protocols. The final RNA concentration and purity were measured by NanoDrop spectrophotometer.

cDNA Synthesis: RevertAid First Strand cDNA Synthesis Kit was applied according to manufacturer's protocols.

qPCR: LightCycler® 480 SYBR Green I Master Kit was applied according to manufacturer's protocols and analysis was done according to $2^{-\Delta\Delta Ct}$ method.

3.2.10. Immunofluorescence Microscopy

For microscopy experiments, coverslips were coated with a poly-L-lysine solution (0.1% w/v in H_2O) for an hour at 37°C in 6-well-plates. The leftover poly-L-lysine solution was removed, and wells were rinsed with sterile ddH_2O to dispose any residual of poly-L-lysine solution. $2x10^6$ NK-92 cells were seeded to each well then 6-well-plates were centrifuged at 900 rpm for 3 minutes and placed into 37°C and 44°C separately to cultured for an extra hour. At the end of incubation, plates were centrifuged again with same

conditions. After centrifugation, supernatant and unattached cells were removed, and wells were rinsed twice with PBS at room temperature. Ice-cold methanol (-20°C) was given to each well to fix cells and plates were incubated at +4°C for 10 minutes. When fixation was done, methanol was discarded, and wells were washed twice with PBS-T. Blocking solution was added to the wells to block and permeabilize the cells for 1 hour at room temperature on shaker. At the end of blocking, coverslips were stained primary antibody for 1 hour at room temperature or overnight at +4°C. Coverslips were washed with PBS-T three times for 5 minutes while gently shaking. Secondary antibody staining took place for 1 hour in the dark and coverslips were washed with PBS-T three times for 5 minutes while gently shaking. DAPI staining solution was added onto the coverslips and incubated for 10 minutes then washed with ddH_2O for 15 minutes. Coverslips were mounted to the slides with one drop of Mowiol and brought to confocal microscopy.

4. RESULTS

4.1. The Use of Small Molecule Kinase Inhibitors in Lentiviral Gene Delivery

The use of BX795 and OXO have been shown to reveal their role in multiple viral transductions. It has been reported that measles virus-mediated immunosuppression during viral infection can cause higher susceptibility of cells to secondary infections. Likewise, other virus types have shown side reactions as viral immunosuppression (Naniche and Oldstone 2000). To reveal the interference of this mechanism, we suppressed immunological pathways with small kinase inhibitors to compare immunosuppression effect on secondary viral transduction (Figure 6).

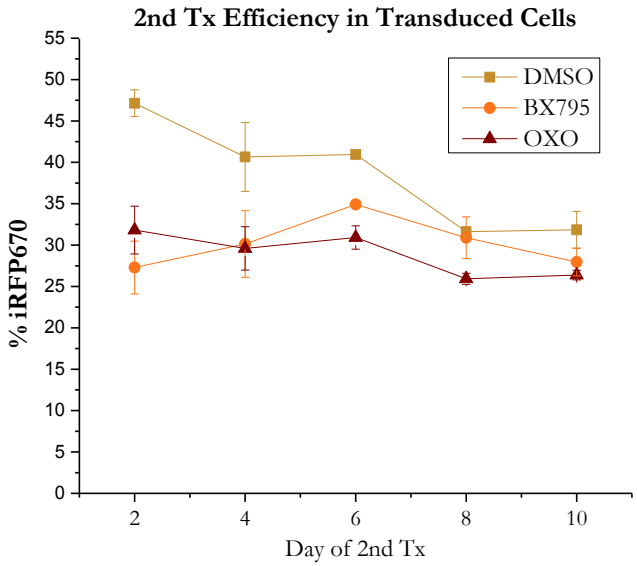


Figure 6. The effect of kinase inhibitors on gene delivery tendency. NK-92 cells were transduced with LeGO-G2 for 6 hr in the presence of DMSO, BX795 (3 µM) and OXO (1.5 µM) on day 0. Cells were introduced to LeGO-iRFP670 as the secondary transduction on 2nd, 4th, 6th, 8th and 10th day after first transduction. Flow cytometry analysis was done on 3rd day after each transduction event.

In order to determine the effect of kinase inhibitors on further transductions, the proportion of double-positive cells to only GFP expressing cells were calculated. The cells that have been transduced with LeGO-G2 in the presence of inhibitors have shown similar efficiencies for 10 days period. On the other hand, absence of inhibitor in first transduction affects the antiviral behavior of transduced cells and decrease in gene delivery rates on these cells were observed. Our results indicate that if small molecule kinase inhibitors are not used in the first transduction, a subpopulation of cells that are more permissive to viral vector entry are targeted, and this reflects as increased transduction efficiency during second transduction, an effect that lasts for at least a week after the first transduction. On the other hand, the use of inhibitors during the first transduction removes this bias from the experiment. We concluded that utilization of kinase inhibitors during the first transduction is critical for lentiviral CRISPR/Cas9-based knockout of candidate genes in order to circumvent this bias.

4.2. CCDC124 Expression in Studied Cell Lines

CCDC124 expression was confirmed in all targeted cell lines via RT-PCR method (Figure 7). HeLa Fucci cells were included in PCR but excluded in further experiments.

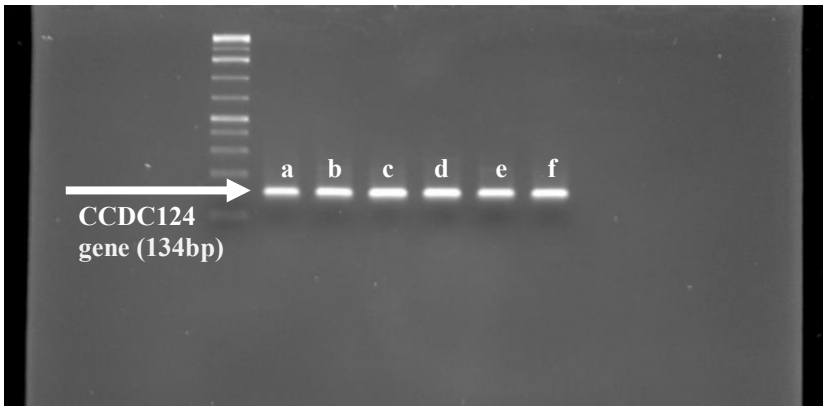


Figure 7. *CCDC124* gene expression. **a.** HCT116 WT **b.** HCT116 p53-/- **c.** 293T **d.** HeLa Fucci **e.** NK-92 **f.** YTS

4.3. Generation of Knockout Cell Lines via the Lentiviral CRISPR/Cas9 System

4.3.1. CRISPR/Cas9-mediated Gene Knock-out

Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR, represents a highly effective and widely used RNA-guided genomic tool (Nair et al. 2019). Experimental CRISPR/Cas9 (CRISPR-associated nuclease 9) system can be designed to create double-

stranded breaks (DSBs) on DNA at specified point that is orchestrated by single-guide RNA (sgRNA) (Shalem et al. 2014). After the binding event between spacer sequence of sgRNA and target sequence of host DNA, Cas protein recognizes protospacer adjacent motif (PAM), which is next to target sequence and cleaves the target DNA (Pickar-Oliver and Gersbach 2019). Subsequent DSB repair mechanisms can follow two separate mechanisms, non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Wyman and Kanaar 2006). Gene knockout application of CRISPR/Cas9 system relies on the error-prone NHEJ which usually ends up in insertions or deletions at the cleavage site (Khan, Yuen, and Luo 2019) that causes heterogeneity in NHEJ products (Waters et al. 2014). The error in repair mechanism may lead loss of function in target protein or induces protein degradation because of damaged protein structure (Sherman and Goldberg 2001). Recent improvements on CRISPR/Cas9 system provide stable expression of sgRNA and Cas9 protein via using lentiviral vectors (Khan, Yuen, and Luo 2019). Development of lentiviral CRISPR vectors facilitates knockout of specific target studies and promotes the future of CRISPR-based tool usage in clinic.

4.3.2. Knockout Cell Line Generation

The lentiCRISPR viruses of target genes were introduced to 10^6 cells in a T25 flask and cultured overnight in the presence of OXO and protamine sulfate. In the morning, virus-containing supernatant was discarded and replaced with fresh medium. After 24 hours, Puromycin selection was started to obtain an enriched population of Cas9 expressing cells. Since lentiCRISPR constructs do not contain any fluorescent protein, Puromycin selection was maintained until knockout cells get synchronized with wild type cells. Besides that, as control, LeGO-iG2p virus with low MOI was also introduced to cell cells to follow Puromycin selection with GFP expression. When GFP expressing cells reach >90%, we accepted lentiCRISPR transduced cells as selected. Further experiments were conducted to confirm gene silencing.

4.4. Characterization of Knockout Cell Lines

4.4.1. Analysis of Target Gene Expression Levels

CCDC124 mRNA levels of target cell lines were determined by qRT-PCR and normalized to GAPDH levels of each group. Our results show that following CRISPR/Cas9 modification, CCDC124 is significantly downregulated (Figures 8, 9, 10).

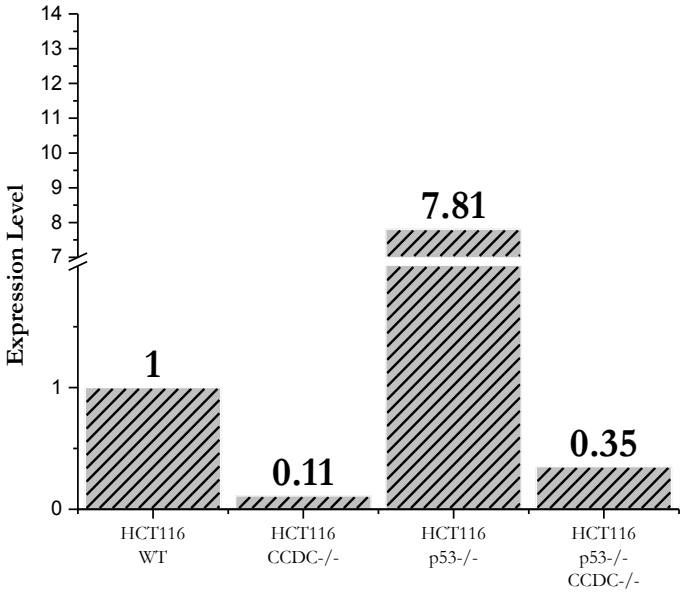


Figure 8. *CCDC124* gene mRNA expression levels in HCT116 cell lines

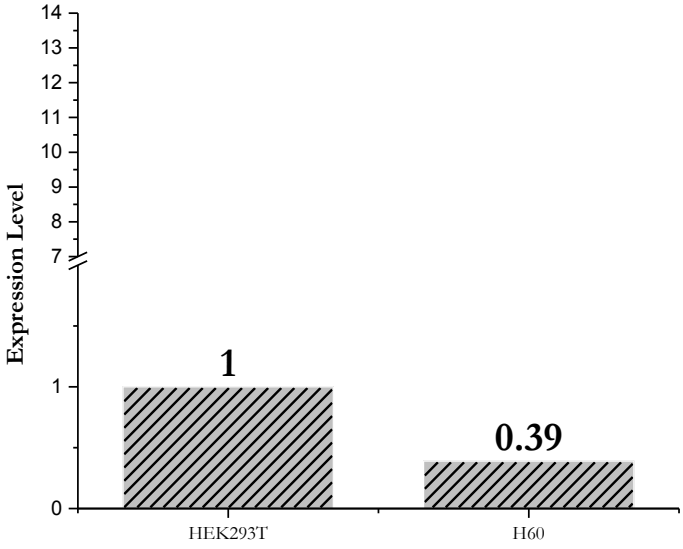


Figure 9. *CCDC124* gene mRNA expression levels in 293T cell lines

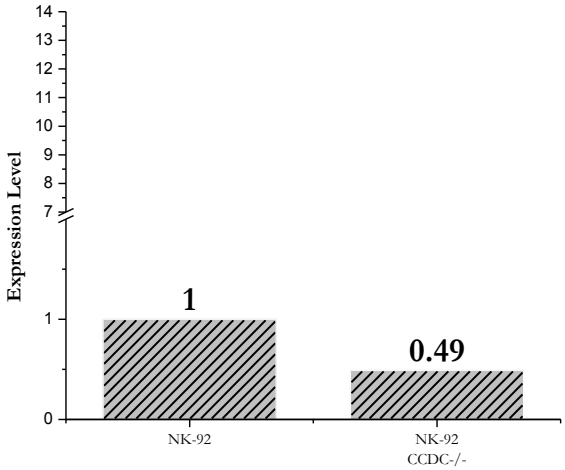
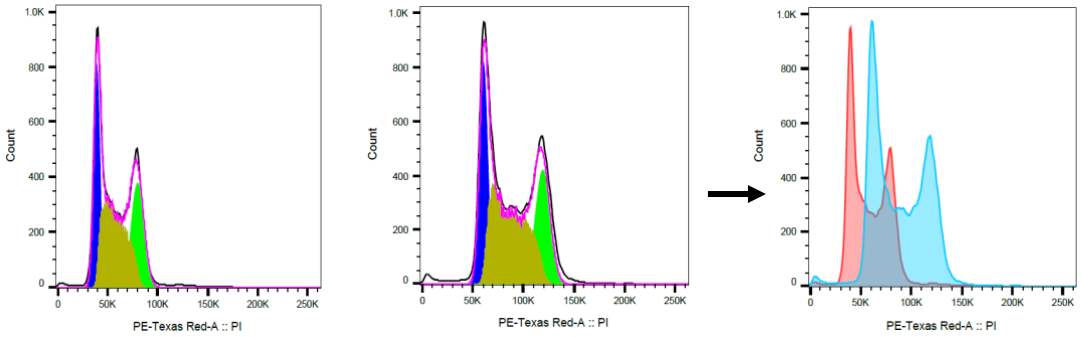


Figure 10. *CCDC124* gene mRNA expression levels in NK-92 cell line

4.4.2. Cell-Cycle Analysis of Knockout Cell Lines

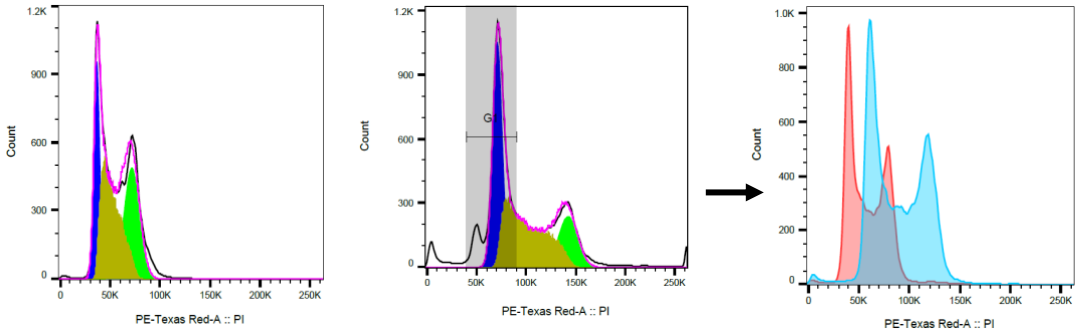
Cell-cycle progression of each cell group was analyzed by PI staining in flow cytometry.



%G1 : 28.2
%S : 42.2
%G2 : 26.2

%G1 : 26.5
%S : 44.0
%G2 : 23.5

Figure 11. DNA contents of HCT116 WT and CCDC124-/- cells

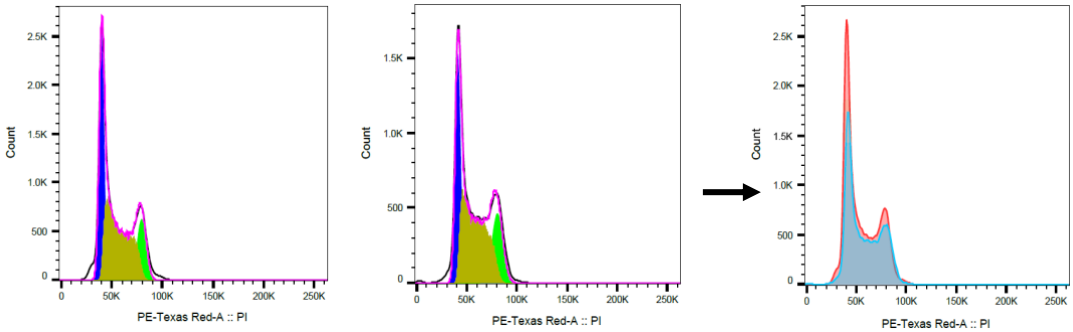


%G1 : 24.7
%S : 40.8
%G2 : 32.4

%G1 : 34.3
%S : 36.2
%G2 : 15.3

Figure 12. DNA contents of HCT116 p53- and p53-/- CCDC124-/- cells

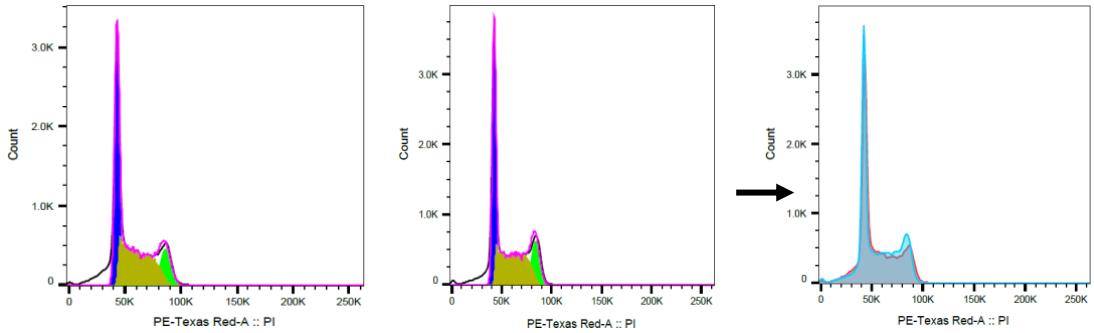
Cell-cycle analysis revealed no significant differences between CCDC124-/- and WT cells, but both CCDC124-/- groups showed right-shift on the flow cytometer plot according to their control groups (Figures 11 and 12). We conclude that while cell-cycle is not affected, the total DNA content of the cells is increased possibly due to defects in cytokinesis and formation of multi-nucleated cells.



%G1 : 34.0
%S : 45.1
%G2 : 15.2

%G1 : 30.4
%S : 46.6
%G2 : 18.4

Figure 13. DNA contents of HEK293T WT and H60 mutant cells



%G1 : 39.0
%S : 38.5
%G2 : 13.1

%G1 : 37.8
%S : 38.5
%G2 : 15.2

Figure 14. DNA contents of NK-92 WT and CCDC124-/- cells

For HEK293T (Figure 13) and NK-92 (Figure 14) cell lines, neither cell-cycle progression nor total cellular DNA content showed any difference between CCDC124-/- and control groups, indicating that CCDC124 does not cause a cytokinesis deficiency in these cell lines.

4.4.3. Cell Size Analysis on Knockout Cell Lines

Relative sizes of studied cells were observed by flow-cytometric light scattering measurements. Populations were first gated on FSC-A vs SSC-A plot, and single cells were gated FSC-A vs FSC-H plot. From single cells, FSC-A and SSC-A means of

populations were calculated by FlowJo software. Calculations showed that CCDC124-/- in HCT116, HEK293T, and NK-92 cells, increased the light scattering for both channels.

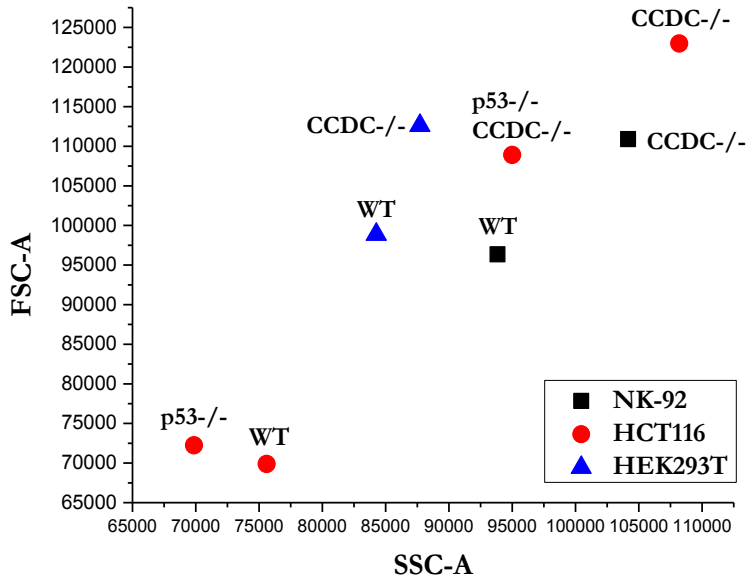


Figure 15. Cell Size Analysis

For all cell types, CCDC124-/- groups showed an increase in their cell sizes compared to the control groups. While this effect is most pronounced in HCT116 cells, HEK293T and NK-92 cells also showed some increase in cell size (Figure 15).

4.1. Antiviral Response of *CCDC124-/-* Cell Lines

4.1.1. Combined Transduction with Kinase Inhibitors

To estimate the antiviral behavior of cell lines and how it is affected by CCDC124, we introduced lentiviral vectors to these cells in three different MOIs, in the presence of small molecule kinase inhibitors. Unlike NK-92 cells, HCT116 cells responded to BX795 negatively in terms of transduction efficiency while OXO showed similar enhancer effect. In a recent study, the off-target effect of BX795 during herpes simplex virus infection has been reported (Jaishankar et al. 2018). Inhibition of Akt pathway by BX795 blocks viral protein translation. In this specific cell line, HCT116, BX795 presence during viral transduction restricts transgene integration.

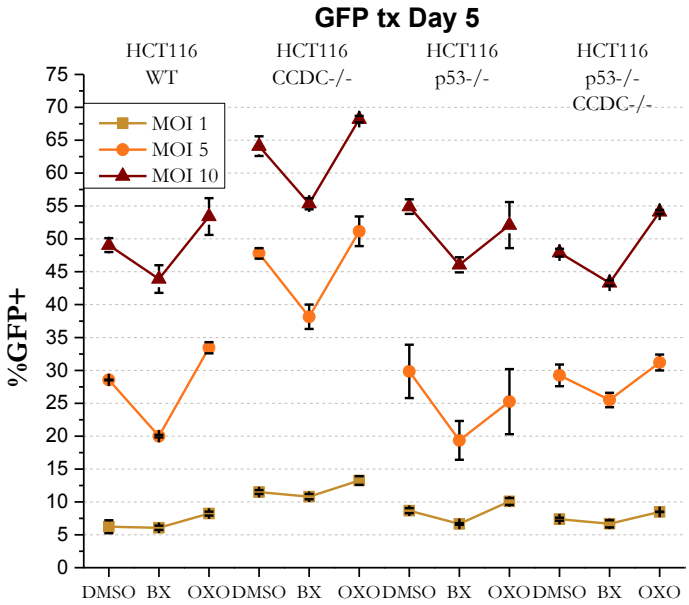


Figure 16. The efficiency of LeGO-G2 transduction to HCT116 cell lines in the presence of small kinase inhibitors

When transduction efficiency of CCDC124-/- cells is compared to WT cells, a significant increase was observed in CCDC124-/- cells in all three MOIs whereas p53-/- cells did not show any significant difference compared to WT cells (Figure 16). Similar efficiencies were observed in double knockout cells, p53-/- and CCDC124-/- as it was in WT and single p53-/- cells. These results indicate that CCDC124 is involved in the antiviral response in HCT116 cells, but this effect is neutralized by p53 knockout.

293T and H60 mutant responses against lentiviral vectors differ from the HCT116 cell line (Figure 17). Presence of BX795 did not show a significant difference when it is compared to DMSO presence during lentiviral transduction. However, OXO had negative effect on cells in terms of transduction efficiencies. Also, silencing *CCDC124* gene in 293T cells decreased the transduction efficiencies.

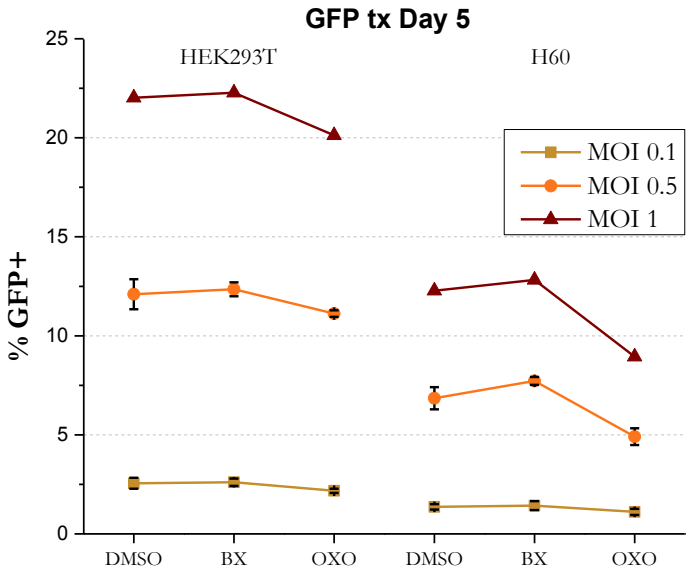


Figure 17. The efficiency of LeGO-G2 transduction to 293T cell lines in the presence of small kinase inhibitors

In NK-92 cells, it is observed that *CCDC124* gene had an extensive impact on gene delivery during lentiviral transduction (Figure 18). Flow cytometry results confirmed stable GFP expression for 14 days with high levels of GFP expressing population. Knocking out CCDC124 folded up transduction efficiencies ~40 times higher without any inhibitor even in low MOI. Small molecule kinase inhibitors enhanced the transduction efficiency up to 90%.

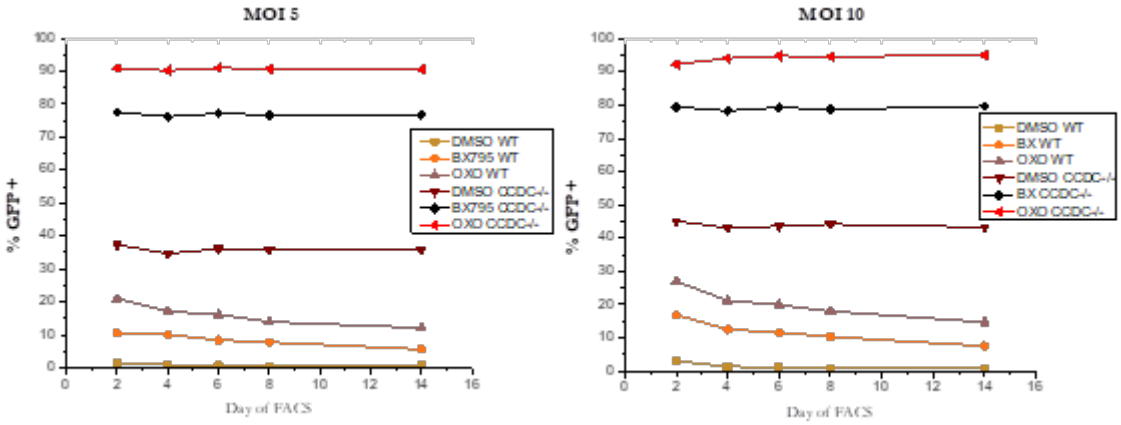


Figure 18. The efficiency of LeGO-G2 transduction to NK-92 cell lines in the presence of small kinase inhibitors

4.1.2. Effect of Target Gene Knockout on Lentiviral Gene Delivery

Further experiments conducted with CCDC124 overexpression plasmids and as a control iG2p since CCDC124 was cloned in iG2p backbone. Moreover, small molecule kinase inhibitors were excluded to investigate the role of *CCDC124* only on lentiviral transduction.

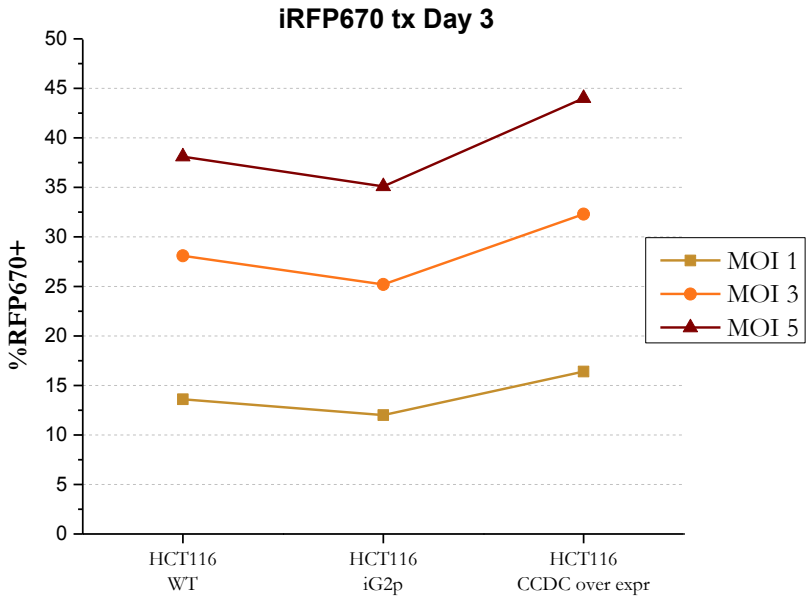


Figure 19. The efficiency of LeGO-iRFP670 transduction to HCT116 cell lines

Flow cytometry results show that prior introduction of lentiviral vector to HCT116 cells decrease efficiency in the second introduction of lentiviral vectors (Figure 19) even though small molecule kinase inhibitors had used in first transduction which is opposite effect that had been explained in section 4.1. Thus, CCDC124 overexpression had enhancing effect on transduction efficiency.

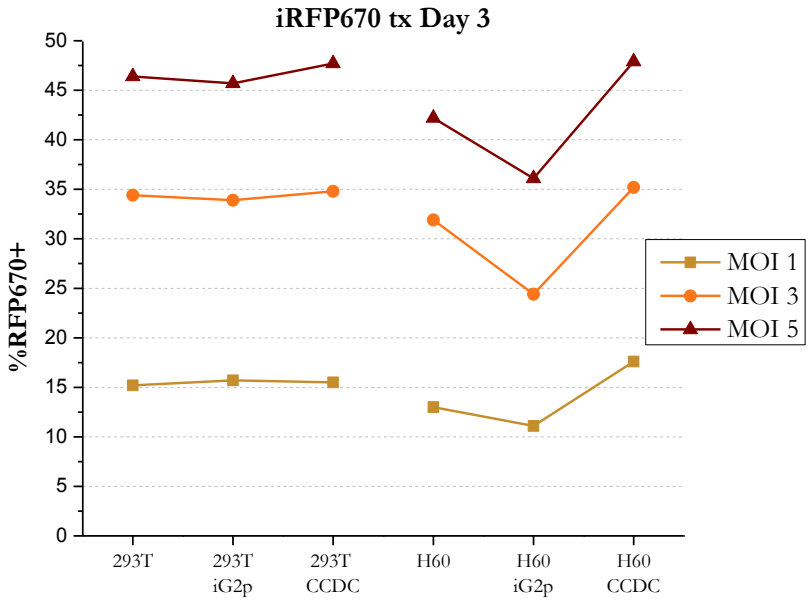


Figure 20. The efficiency of LeGO-iRFP670 transduction to 293T cell lines

WT 293T cells did not show a significant difference in lentiviral transduction efficiencies when transduced second time (Figure 20). Additionally, CCDC124 overexpression did not change efficiencies as well in low MOIs however slight increase observed in MOI 5. H60 mutant cells responded to secondary viral transduction similar to HCT116 cells with reduced transduction efficiency, but CCDC124 overexpression in mutant cells recovered the transduction efficiencies and carried up to the levels as WT cells.

YTS cell line experiments were conducted with iG2p, CCDC124-/-, and CCDC overexpressing cells against WT cells. CCDC124 expression of YTS cell lines was examined beforehand (Figure 21). Knockout and overexpression was confirmed and proceeded to further steps. Secondary viral transduction showed decrease in transduction efficiency (Figure 22). CCDC124-/- cells showed higher transduced population when it is compared to both WT and iG2p cells. For CCDC124 overexpressing cells, transduction values were lower than both WT and iG2p cells.

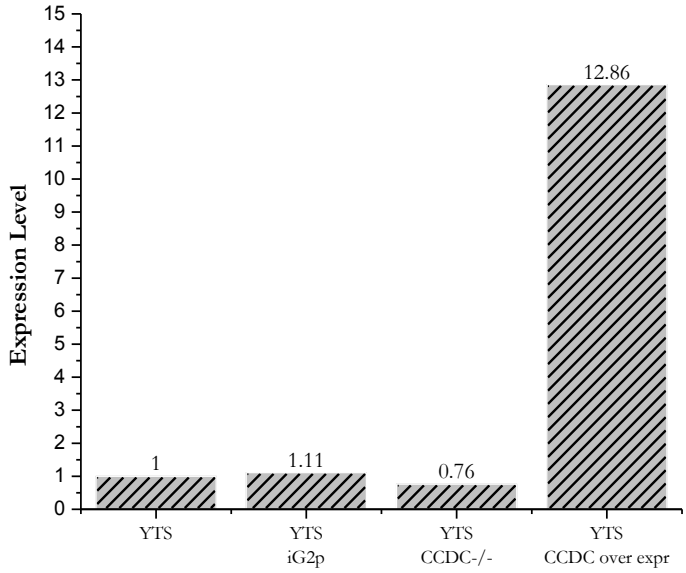


Figure 21. *CCDC124* gene mRNA levels in studied YTS cells

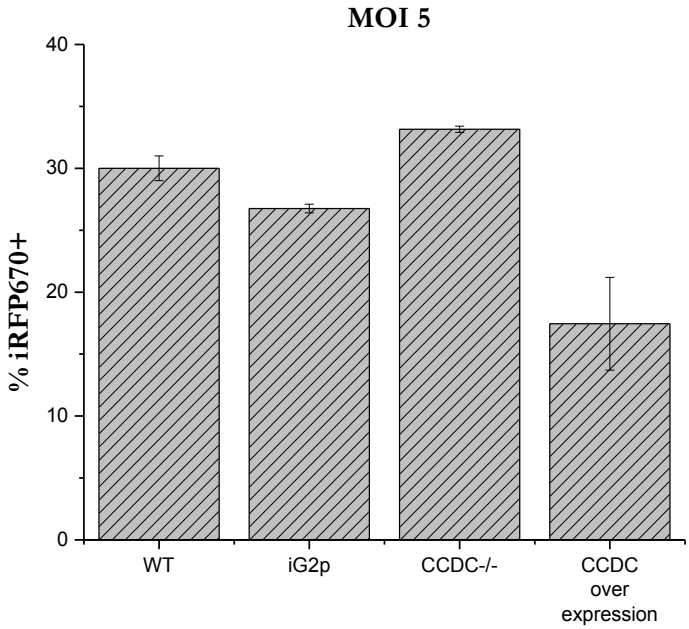


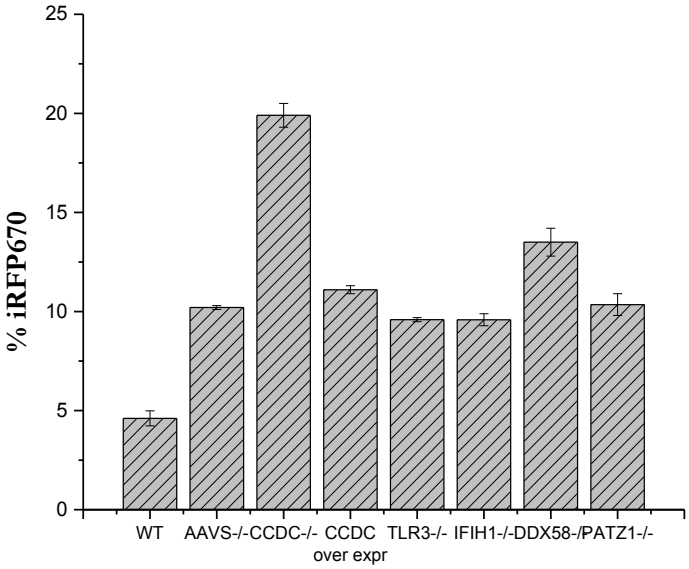
Figure 22. The efficiency of LeGO-iRFP670 transduction to YTS cell lines

4.1.3. The Role of CCDC124 Gene in Innate Antiviral Response of NK Cells

Viral RNA sensing pathways and pattern recognition receptors have been explained previously. Among these, we targeted *DDX58*, *IFIH1*, and *TLR3* to compare the antiviral response of *CCDC124* in the absence of these genes separately. All knockout genes were

confirmed with qRT-PCR (Figure 23b). Lentiviral transduction of LeGO-iRFP670 plasmid without any inhibitors has demonstrated the high impact of *CCDC124* gene in antiviral defense mechanism of NK-92 cells (Figure 23a).

A)



B)

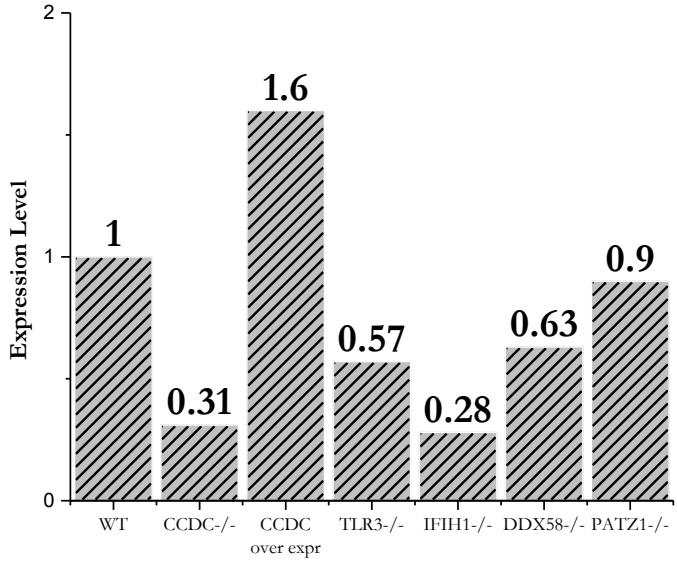


Figure 23. a) Lentiviral transduction efficiencies in knockout cells **b)** mRNA expression levels of separate genes.

4.1.4. CCDC124 and Stress Granules (SGs) in NK Cells

Stress granule formation was examined in temperature-related stress conditions in WT, CCDC124-/- and CCDC124 overexpressing NK-92 cells (Figures 24, 25 and 26). Even though CCDC124 is significantly silenced, its expression still exists in CCDC124-/- populations and low CCDC124 expression is visible in CCDC124-/- cells while overexpressing cells show higher brightness with anti-CCDC124 staining. Stress granule formation is observed in all cell types that have been cultured under stress conditions but and overlaps with CCDC124 staining, indicating that CCDC124 is present in the stress granules of NK-92 cells. The cells that have been maintained in normal conditions did not show stress granule formation.

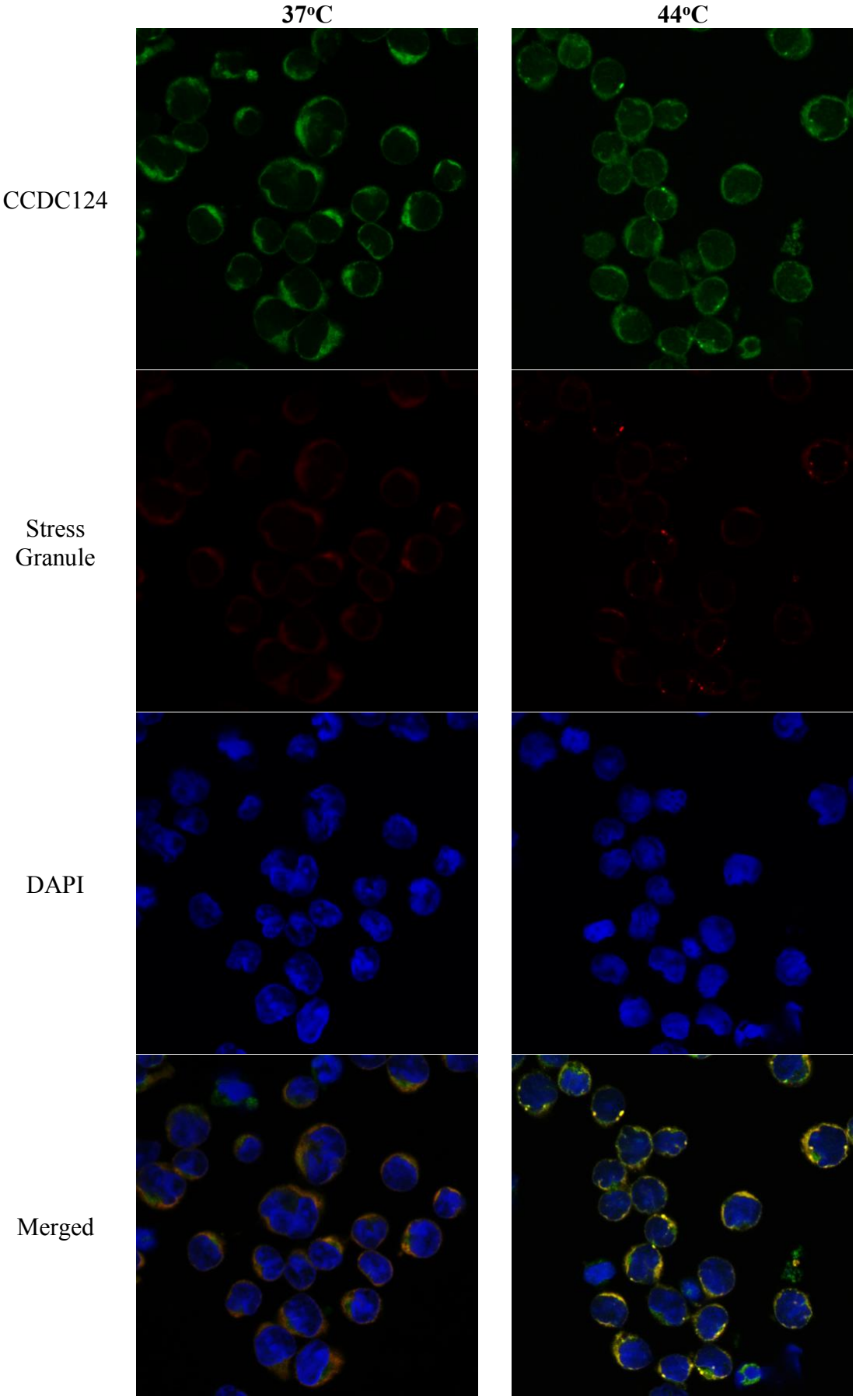


Figure 24. Stress granule formation in WT NK-92 cells

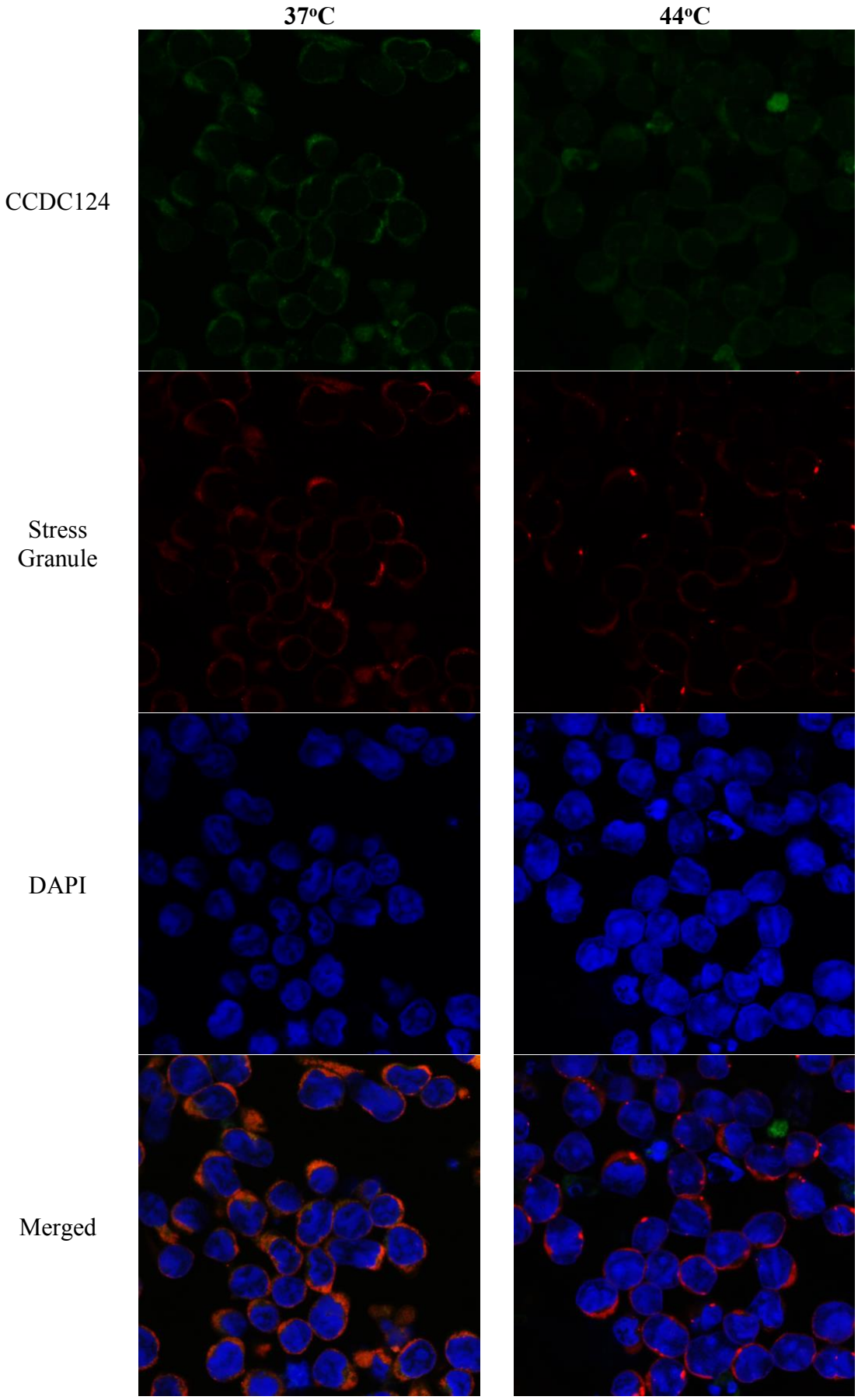


Figure 25. Stress granule formation in CCDC124-/- NK-92 cells

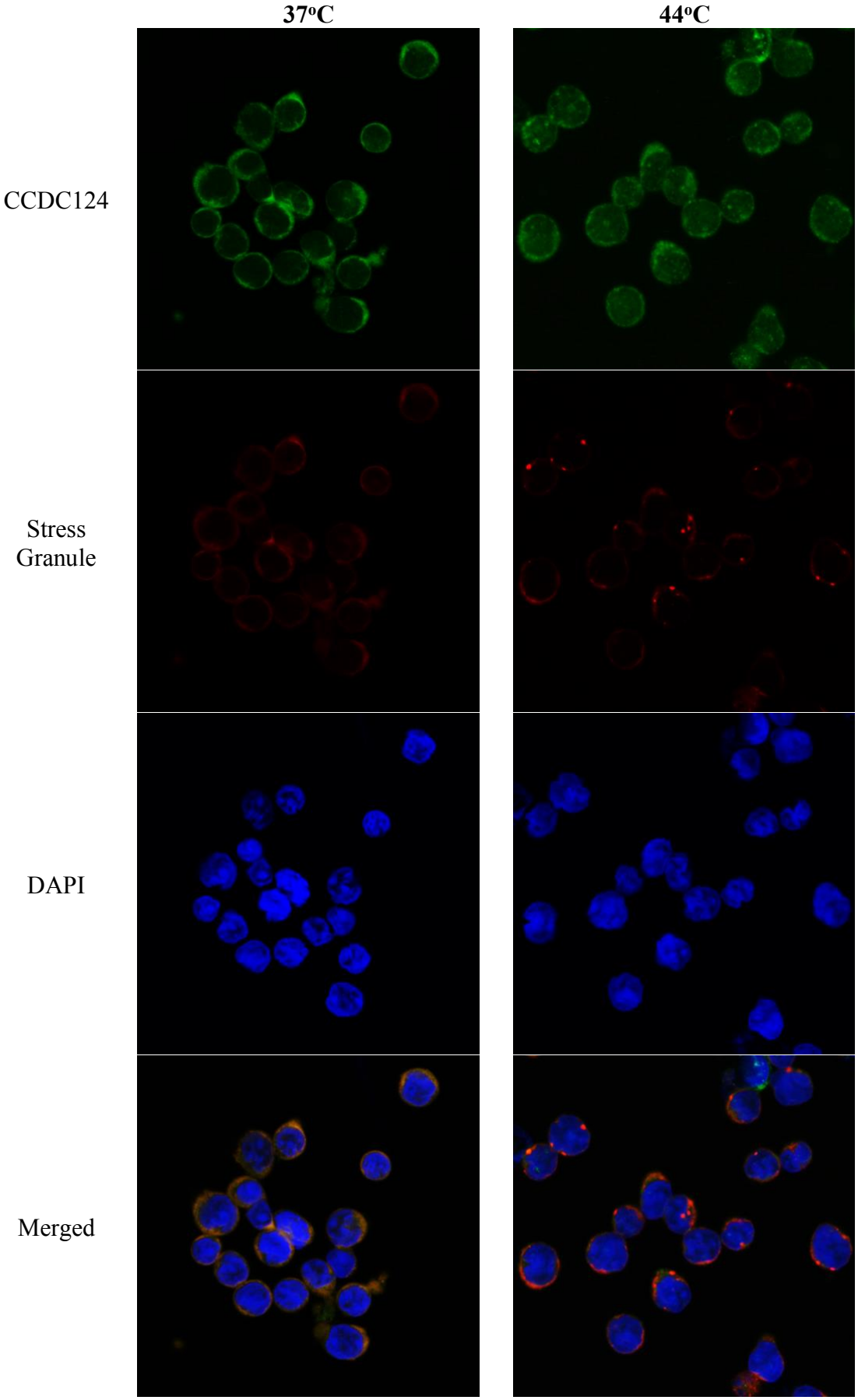


Figure 26. SG formation in CCDC124 over expressing NK-92 cells

5. DISCUSSION

Viruses are pathogens that manipulate intracellular machinery of target cells and disrupt hosts antiviral defense mechanism (García-Sastre 2017). Retroviruses are RNA containing virions that evolved various mechanisms to counteract and subvert the immune system (Johnson 2019). One of the strategies to overcome immune effector mechanism that HIV-1 follows is hiding its genetic material in the viral capsid so that HIV-1 can escape from intracellular defense system until it integrates its viral RNA (Guha and Ayyavoo 2013). However, recognition of viral products after integration may activate or disrupt several antiviral pathways. To investigate the effect of this activation for secondary virus introduction, we set consecutive transduction experiments in the presence and absence of small molecule inhibitors. Inhibition of antiviral pathways with inhibitor administration during lentiviral transduction may catalyze the integration of viral genome without disturbing those pathways. As a result, we observed in the secondary lentiviral transductions that, once cells get activated by viral vectors, they acquire higher susceptibility to viral insertion, possibly due to a selection bias during the first transduction. Our results suggest that when virus disrupts antiviral pathways, 7-10 days are required for cell to recover intracellular mechanisms and behave like uninfected cells. On the contrary, inhibition of these pathways keeps intracellular mechanism to work correctly for further lentiviral transductions. According to these findings, we used the small molecule inhibitor, OXO, for knockout cell line generation in order to decrease lentiviral transduction efficiency bias for secondary lentiviral transductions.

To confirm the silencing of *CCDC124* gene, we checked mRNA levels of *CCDC124* gene in each group of cell lines. All types of cell lines were confirmed in terms of silencing to varying extents. Interestingly, we observed increased CCDC124 mRNA levels in p53 knockout HCT116 cell line. This consequence might be related to their common interacting genes such as *APEX1* (Seemann and Hainaut 2005; Kristensen, Gsponer, and Foster 2012), *VCP* (Jethwa et al. 2018; Hülsmann et al. 2018b) and *XPO1* (Kırlı et al. 2015b; Santiago et al. 2013). All these three proteins are known to be related

to cell-cycle regulation, though their additional roles in intracellular mechanisms appear to be interfering with antiviral response and stress granule formation. The effect of the possible interaction between CCDC124 and p53 is also observed in lentiviral transduction results of HCT116 cell lines. Our first observation is increased transduction efficiency when *CCDC124* is knocked out alone, however double knockout HCT116 cells, *CCDC124-/-* and *p53-/-*, show similar transduction efficiency with CCDC124 expressing cell lines. *XPO1* is reported as the inhibitor of nuclear export of HIV-1 component Rev protein (Kırlı et al. 2015a). Another related gene, *VCP*, is identified as regulator of protein homeostasis and participator of intracellular membrane fusion under stress conditions (Hülsmann et al. 2018a). According to these findings, change in antiviral responses depending on the changing expression levels of *CCDC124* and *p53* genes may be related to interaction between CCDC124 and p53.

Higher lentiviral transduction efficiencies are expected to be observed in the presence of small molecule inhibitors. In accordance with this, OXO showed a slight increase, but presence of BX795 have shown opposite effect. As it is discussed before, a recently published study (Jaishankar et al. 2018) reported the unexpected effect of BX795 in HSV-1 infection. Jaishankar et al. suggested that inhibition of Akt pathway with BX795 also inhibits synthesis of viral proteins. This might be the reason for reduced levels of transduction efficiency in BX795 containing transduction events.

Our further considerations about CCDC124 lacking cells are related to the DNA content of the cells and cell sizes. Dysfunctional cytokinesis has been reported as a result of CCDC124 knockout, which leads the formation of bi- and multinucleated cells (Telkoparan et al. 2013). Our results with HCT116 cell lines also confirms cytokinetic consequences of CCDC124 protein.

Contrary to HCT116 lentiviral transduction results, CCDC124 knockout HEK293T cells have shown a decrease in transduction efficiency levels. Suppressor activity of transforming growth factor β-activated kinase 1 (TAK1) against transcription of hepatitis B virus in hepatoma cell lines have been reported previously (Pang et al. 2017). The same report demonstrated upregulated transcription levels of HBV during silenced or inhibited endogenous TAK1 expression. For further investigations, we recovered the expression levels of CCDC124 with CCDC124 overexpressing plasmid in H60 mutant cells and examined the transduction efficiencies compared to 293T and H60 mutant cells. Transduction levels showed that rescued CCDC124 raises the lentiviral transduction efficiency up to the same levels with control group. Characterization of HEK293T cell

lines showed that CCDC124 lacking cells are more abundant than control group without any increase in DNA content.

Regarding these findings, we applied the same procedures to natural killer cell lines, NK-92 and YTS. YTS cell lines showed expected results in lentiviral transduction efficiency, where CCDC124-/- YTS cells have higher viral genome integration and CCDC124 overexpressing cells less integration compared to WT YTS cells. A drastic effect of CCDC124 knockout was observed in the NK-92 cell line. Lentiviral transduction efficiencies in CCDC124-/- NK-92 cells have shown significantly higher transduction levels compared to control group, while the addition of small molecule inhibitors has shown even higher transduction efficiencies. Characterization experiments did not show difference in DNA content between CCDC124-/- and control groups; however, CCDC124-/- NK-92 cells are slightly larger than WT NK-92 cells as in other cell lines. To compare the antiviral effect of CCDC124 protein in antiviral response, related proteins such as RIG-I, MDA5, and TLR3, were also knocked-out and lentiviral transduction efficiencies were investigated. Among all knockout cell lines CCDC124-/- group have shown highest transduction efficiencies compared to control groups which are wild type NK-92 cells and AAVS-/- cells.

As it is mentioned previously, viral integration favors the dividing cells even if the lentiviral vectors can transduce both dividing and non-dividing cells. Therefore, it would be possible to expect the effect of CCDC124 on cytokinesis would decrease transduction efficiencies in knockout cells however these observations showed enhanced lentiviral transduction efficiencies which lead us to investigate the role of CCDC124 in stress granule related innate antiviral response. Confocal microscopy images of WT, CCDC124-/- and CCDC124 overexpressing NK-92 cells revealed that stress granule formation occurs under stress conditions but not in healthy maintained cells. Granular bodies are clearly visible in the cell groups that have been cultured under stress conditions. Merged images of different antibodies exhibit the co-localization of CCDC124 protein and stress granule marker G3BP1 only under stress conditions. However, stress granules were observed in all cell types that have expose to 44^{o}C which means CCDC124 interacts with stress granules, but its role in stress granule formation is not clear yet. Additionally, further investigations with virus-related stress conditions and stress granule formation should be examined to understand CCDC124 role in innate antiviral response.

6. CONCLUSION

This study indicated that CCDC124 protein is engaged in innate antiviral response. The use of small-molecule inhibitors in lentiviral transductions on NK-92 cell line showed significantly enhanced transduction values. On the other hand, off-target effect of these inhibitors has been demonstrated in different cancer cell lines which should be investigated in detail. Lentiviral transduction results in HCT116 cell lines showed the potential connection between CCDC124 and p53 proteins. CCDC124 relation in antiviral response was confirmed in HCT116 and NK cell lines. Consistent data in two natural killer cell lines, NK-92 and YTS, emphasizes the antiviral role of CCDC124 in innate immunity. Confocal microscopy results state that there is an interaction between CCDC124 and stress granules definitely however we need to further confirm its role in lentiviral stress granule formation.

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APPENDIX

APPENDIX A: Plasmid Maps

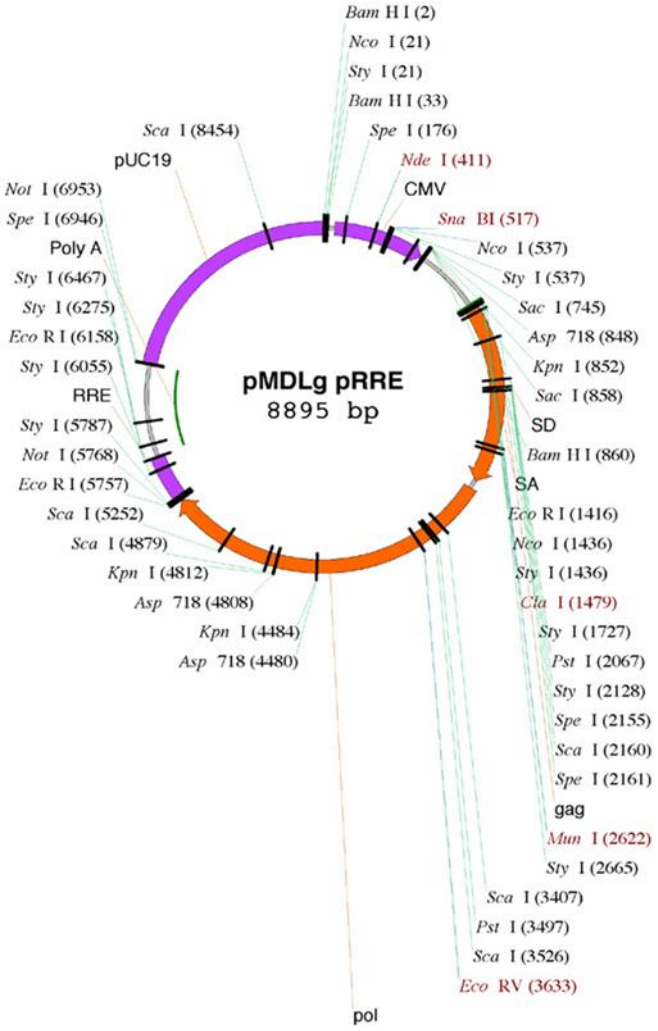

Figure 27. The vector map of pMDLg/pRRE

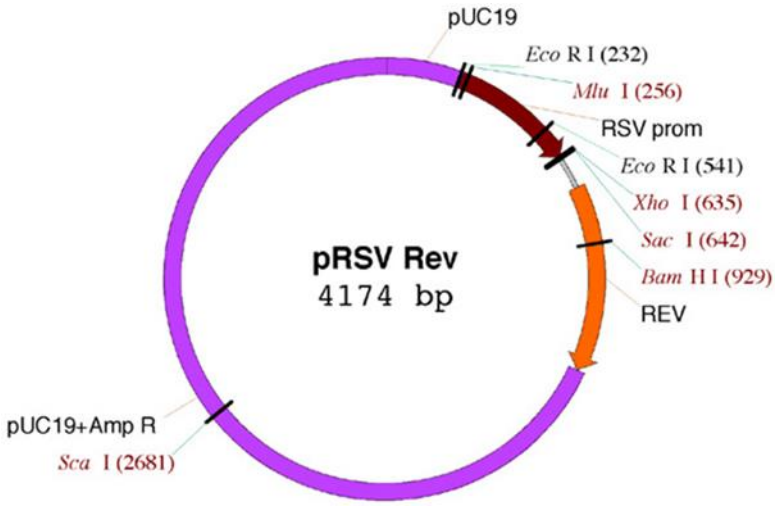

Figure 28. The vector map of pRSV-REV

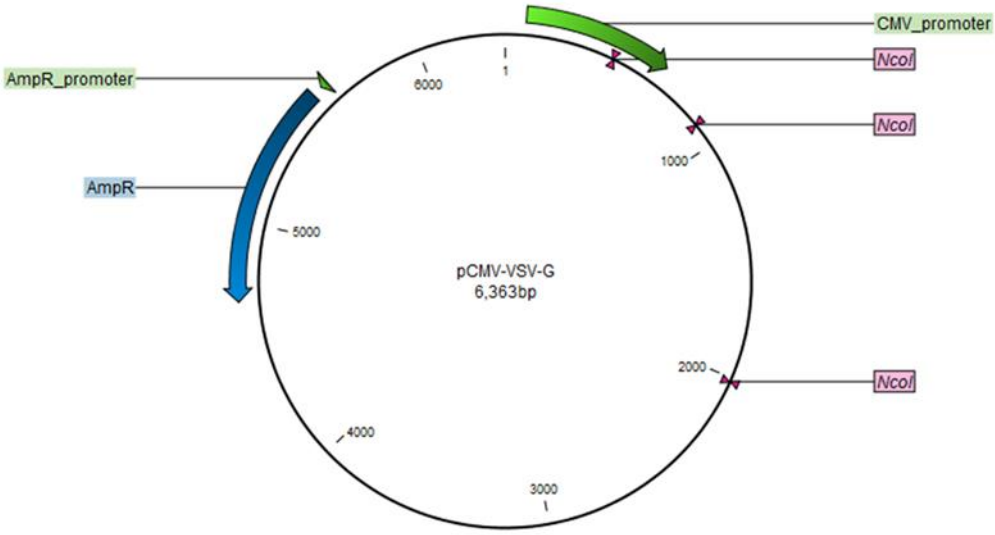

Figure 29. The vector map of pCMV-VSV-g

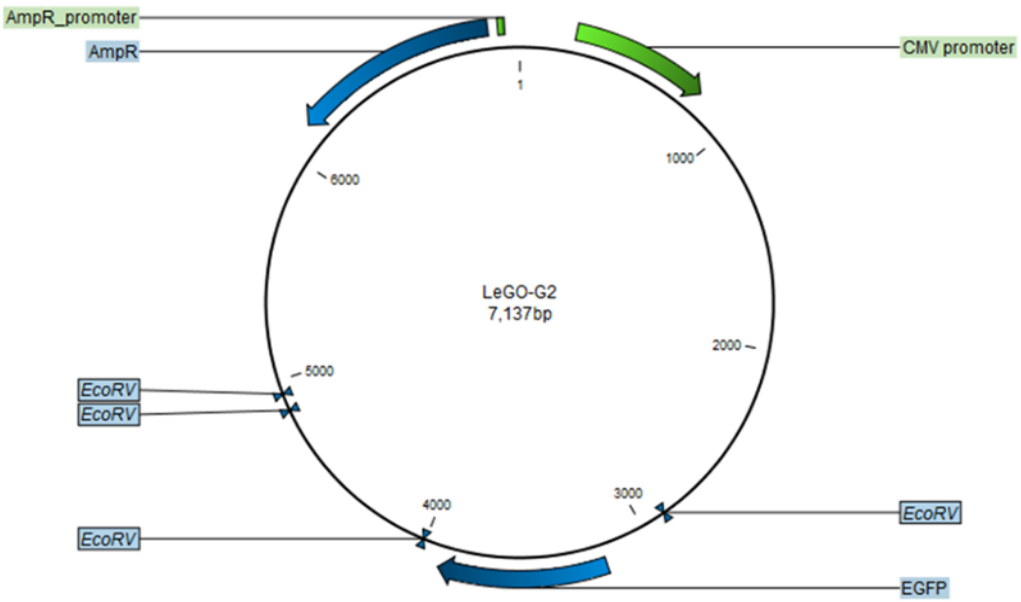

Figure 30. The vector map of LeGO-G2

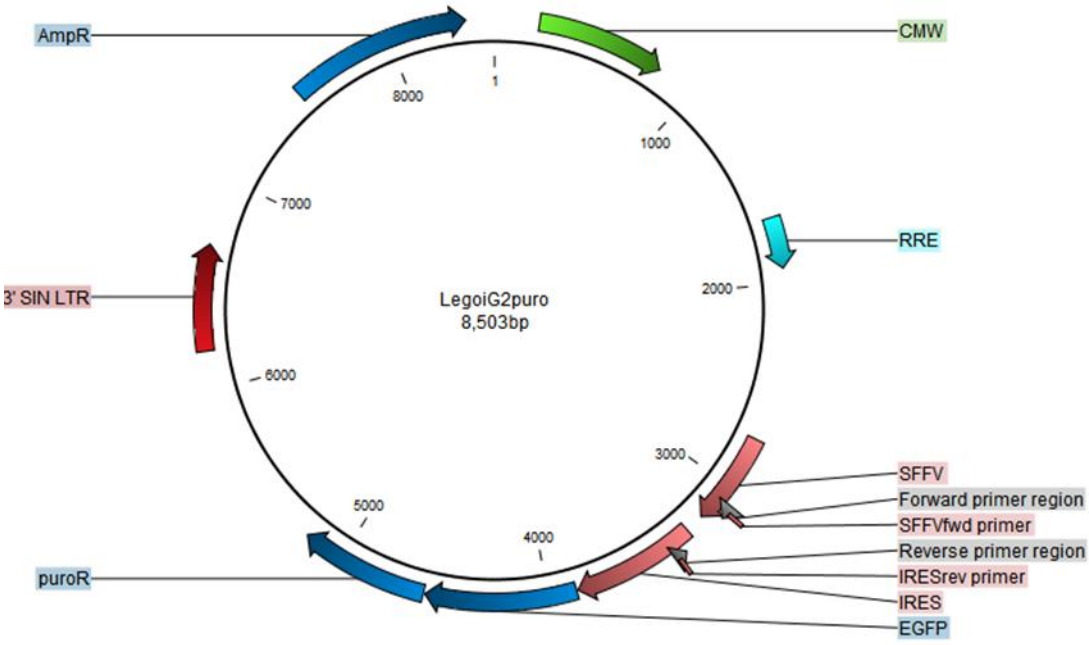

Figure 31. The vector map of LeGO-iG2puro

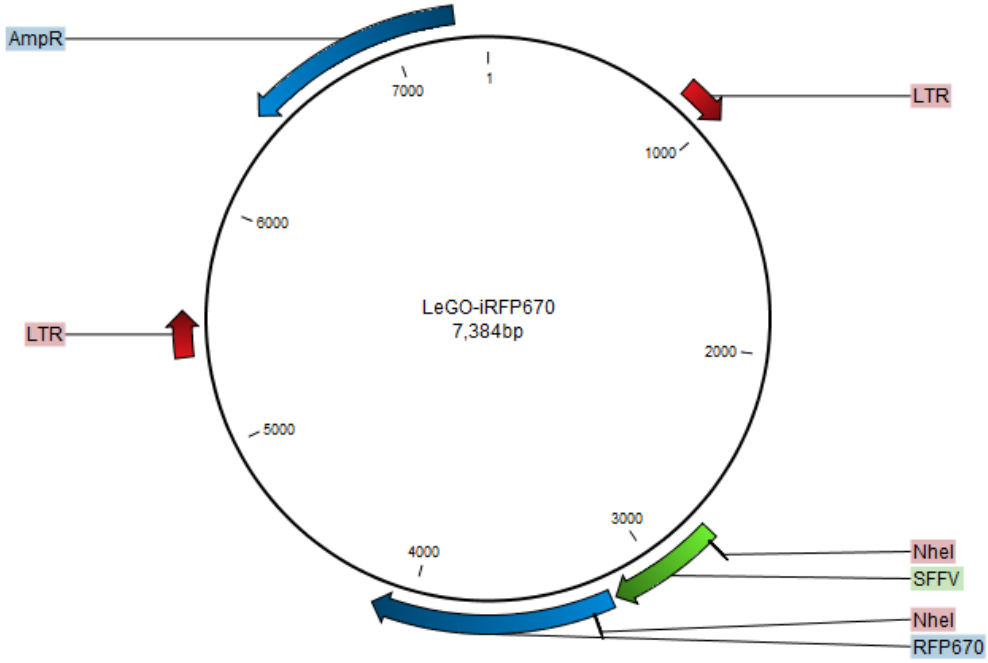

Figure 32. The vector map of iRFP670