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Generating genetically modified skin cell lines to investigate the function of LRIG3 and DIRAS1 in skin cancer

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Declaration of Authenticity

I herewith declare that I wrote this thesis on my own and did not use any unnamed sources or aid. Thus, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made by correct citation. This includes any thoughts taken over directly or indirectly from printed books and articles as well as all kinds of online material. It also includes my own translations from sources in a different language.
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Abstract

Skin cancer is the most prevalent form of cancer worldwide, with both melanoma and non-melanoma skin cancer cases increasing in recent years. GTP-binding protein Di-Ras 1 (*DIRAS1*) and leucine-rich repeats and immunoglobulin-like domains 3 (*LRIG3*) play major role in tumor development, but only little is known about functions of these proteins in skin cancer. Our previous investigations discovered that *DIRAS*1 and *LRIG3* were upregulated in the A431 (squamous cell carcinoma) and A375 (melanoma) cell lines. From these findings, we hypothesized that *DIRAS1* and *LRIG3* may have tumorigenic functions in skin cancer. To gain further insights into the role of *DIRAS*1 and *LRIG3* in skin cancer development, we planned to perform gene expression manipulation in three different skin cell lines. These included two skin cancer cell lines, A375 and A431, as well as HaCaT (immortalized human keratinocytes). To investigate the function of *LRIG3* and *DIRAS1* in these skin cell lines, we tried to overexpress *DIRAS1* and *LRIG3* genes in all three cell lines. In addition, we planned to delete *DIRAS1* and *LRIG3* genes by using clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology to generate knockout cell lines for both genes. However, we were not able to establish an overexpressing vector for *DIRAS1* due to the obstacle in amplifying *DIRAS1* cDNA. Moreover, in none of the cell lines, the *LRIG3* gene was efficiently deleted. We also faced a problem to obtain single cell colonies of *DIRAS1*-knockout (KO) HaCaT cells due to their inherent difficulty in transfection. However, we successfully generated *LRIG3*-overexpressing A375, A431, and HaCaT cells. Likewise, we accomplished to produce *DIRAS1*-KO A375 and A431 cell lines. The fundamental hallmarks of cancer such as proliferation, migration as well as invasion will be analyzed in the present genetically manipulated cell lines.

Abbreviations

BCC	Basal cell carcinoma
bp	Base pairs
CAS9	CRISPR-associated protein 9
cDNA	Cloned DNA
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DEMEM	Dulbecco´s Modified Eagle´s medium
DIRAS	Diverse rat sarcoma virus protein
E. coli	Escherichia coli
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
GDP	Guanosine triphosphate
GFP	Green florescent protein
GOI	Gene of Interest
gRNA	Guide RNA
GTPase	Enzymes that bind to the nucleotide guanosine triphosphate
GTP	Guanosine triphosphate
HaCaT	Cultured Human Keratinocyte
HRP	Horse-Radish-Peroxidase
INDEL	Insertion or deletion mutations
kDa	Kilodalton
KO	Knock out
LB-Medium	Luria-Bertani (LB) broth
LRIG	Leucine-rich repeats and immunoglobulin-like domains
MAP	Mitogen activated pathway

NC	Negative control
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PI3K-Akt	Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway
PVDF	Polyvinylidene fluoride
RAS	Rat sarcoma virus
rSAP	Shrimp alkaline phosphatase
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulfate
S.O.C.	Super Optimal broth with Catabolite repression
TBS	Tris Buffered Saline
TBST	Tris Buffered saline with Tween20

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1. Introduction

1.1 Skin Structure

The skin is the largest organ of the body and is accountable for a wide variety of functions, such as protection, tactile sensation, and thermoregulation. However, the skin also plays an important role in metabolic pathways and sexual signaling via the production and release of pheromones.(Mescher, Anthony L. 2018) To further understand the function of the skin, it is important to apprehend its structure and composition. The skin can be divided into three major layers: the epidermis, dermis and subcutis/hypodermis (Figure 1).(Welsch and Deller 2014) The following chapters aim to give a brief overview of the different skin layers.

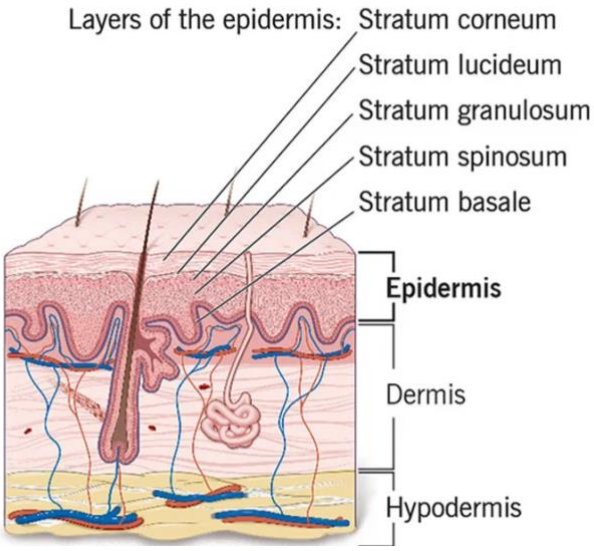


Figure 1: Skin Structure (Cleveland Clinic 2018)

1.1.1 Epidermis

The epidermis, the uppermost layer of the skin, can be further divided into the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. However, it is important to note that the stratum lucidum only exists in areas where the skin has a thick stratum corneum, such as the skin on the soles of the feet.

The stratum basale consists of a single layer of cubic keratinocytes with a high mitotic rate. Nevertheless, the ability of the cells to divide subsides when they are localized in the more superficial parts of the epidermis. In fact, the cells are only alive and capable of dividing themselves in the stratum basale, stratum spinosum, and in parts of the stratum granulosum, where the degeneration of the skin cells begins. Moreover, with the decrease of the cell's mitotic functions, the resilience against mechanical forces increases. This is due to the fact, that the cells in the upper layers of the skin produce a higher amount of a protein called keratin.

These keratin producing cells are known as keratinocytes, which make up the vast majority of cells in the epidermis. The uppermost layer of keratinocytes is especially rich in the protein and make up the entirety of the stratum corneum. These most superficial cells are

also known as squamous keratinocytes.(Mescher, Anthony L. 2018) Except for keratinocytes, some other cell types can be found in the epidermis: Melanocytes, which are responsible for the production of melanin resulting in the pigmentation of our skin. These cells originate from the neural crest and can be found in several different body parts, such as in the mesencephalon or the iris.(Yamaguchi and Hearing 2014) Antigen-presenting cells known as Langerhans cells can be found in the epidermis and, Merkel cells which are involved in the detection and neuronal processing of light touch stimuli. However, it is important to know that these three different cell types cannot be detected in the stratum corneum, stratum lucidum nor in the stratum granulosum, for they can only be found in the deeper levels of the epidermis where they are embedded between keratinocytes.(Welsch and Deller 2014)

1.1.2 Dermis

The main function of the dermis is to provide protection to the underlaying organs and nerves from damage due to physical force. In this context, collagen plays a vital role. Collagen is a protein that grants the skin elasticity and strength.(Welsch and Deller 2014) Additionally, the dermis also provides the upper skin layers with nutrients via numerous small capillaries. The fist layer of the dermis, inferior to the stratum basale of the epidermis, is called papillary dermis or layer and is especially rich in blood vessels. This layer consists of loose connective tissue in which several different cell types (mast cells, fibroblasts, dendritic cells, and migrated leukocytes) are enclosed by the elastic collagen fibers. The second layer of the dermis, or stratum reticularis, is much thicker and denser than the prior layer. The reticular layer is especially abundant in collagen type 3 a much stronger but less elastic subtype of collagen.(Mescher, Anthony L. 2018)

1.1.3 Hypodermis

The inmost layer of the skin, the hypodermis, subcutaneous tissue, also referred to as subcutis, consist of loose connective tissue. Additionally, to collagen fibers, a high number of adipocytes are located in this layer of the skin. This abundance of fat tissue is especially important for additional protection and for the body´s thermal insulation.(Mescher, Anthony L. 2018)

1.2 Skin cancer

Skin cancer is the most common type of cancer worldwide. Incidence of melanoma and non-melanoma skin cancer have increased tremendously in recent years. Skin cancer can be subdivided into two groups, depending on which cell type the tumor arose from. These sub-groups are called melanoma and non-melanoma skin cancer. The umbrella term non-melanoma skin cancer refers to all types of abnormal cell growth in the skin e.g., basal cell carcinoma or squamous cell carcinoma.(Mayo Clinic 2020) Causes for the development of skin cancer are multifactorial. Nonetheless, unprotected prolonged exposure to UV-radiation is considered the most relevant cause of skin cancer development. Still, a lot of genetic predisposition and pre-existing illnesses can influence the severity of skin cancer or the development itself. In recent years numerous genes could also be linked to the development of skin cancer and its heredity. Still, as there are many genes which are under suspicion to intervene with or even promote the development of cancerous growth in the skin, further research in this field has to be conducted.(PDQ Cancer Genetics Editorial Board 2002)

1.2.1 Non-melanoma skin cancer

In both, squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), keratinocytes are the affected cell types. These two types of skin cancer are therefore part of the non-melanoma skin cancer group. The distinction between the two sub-groups of non-melanoma skin cancers is important to state: In BCC only the keratinocytes of the stratum basale in the epidermis, are affected. On the other hand, abnormal cancerous growth of keratinocytes in the more upper layers of the epidermis are commonly referred to as SCC.(Huang and Balmain 2014)
Furthermore, it is important to note, that the survival rate of SCC compared to BCC is significantly lower. In a retrospective cohort study from 2015, the mortality risk of BCC and SCC was estimated separately when compared to a cohort of non-skin cancer related cancer patients (control group). The study found a 25% higher mortality risk within patients diagnosed with SCC when compared to the control group. However, patients with BCC only had a mortality risk increase at around 1%.(Rees et al. 2015) The reason behind the significant poorer survival rate within patients diagnosed with SCC is not quite clear yet and must be further studied. However, it has been stated that SCC tend to migrate and build metastasis much more easily than BCC which might be a contributing factor to the lower survival rate.(Huang and Balmain 2014)

1.2.2. Melanoma

Melanoma is accountable for the most deaths (roughly 60%) associated with skin cancer, even though it is not the most common type.(Hawryluk and Tsao 2014) If detected early, the survival rate over a 5-year period is 99%. However, if not treated properly or at all and the cancer is allowed to progress, metastasize, and infiltrate other organs, the survival rate decreases to 30%.(American Cancer Society 2016) In melanoma the melanocytes are affected, these cells are not only responsible for the color of the skin but also determine the

color of our hair and eyes. As mentioned previously, many genes are associated with the development of skin cancer, this is especially true for melanoma, as this sub-category of skin cancer is particularly heterogeneous on a molecular level. Unsurprisingly, many genes have been identified to play a significant role not only in the development but also the clinical symptoms and features that patients are confronted with.(Hawryluk and Tsao 2014) With the increasing relevance of personalized- and precision medicine, one can hope that even more relevant genes can be uncovered and used as tumor markers which can then be targeted for highly specific treatment purposes.

1.3 LRIG

There are three different leucine-rich repeats and immunoglobulin-like domains (*LRIG*) genes occurring in the human genome, namely *LRIG1, LRIG2* and *LRIG3*. *LRIG1* has first been successfully cloned from a mouse brain in 1996.(Suzuki et al. 1996) In 2002 a link between the expression of *LRIG1* and tumor growth in a multitude of different human tissue samples has been discovered. Additionally, a link between *LRIG´*s function and epidermal growth factor (EGF) receptor signaling could be made.(Hedman et al. 2002) Since then, the *LRIG´*s family involvement in different kind of cancers has been the subject of numerous studies. The mechanism of action as well as the structure of the transcribed proteins will be further discussed in the following chapters.

1.3.1 Structure of *LRIG* proteins

The *LRIG* family members are transmembrane proteins. The proteins have three subdomains: a cytosolic domain, a transmembrane domain, and an extracellular domain. Within the extracellular domain, the leucin rich repeats can be found. Leucin rich repeats are structural protein motives that consist of 20-30 amino acids. The occurrence of the amino acid leucin is unusually high in these sequences, hence the name. The function of these repeats is most likely mainly structural.(Kobe and Kajava 2001) However, some argue that leucin rich repeats might play a role in protein-protein interactions. This is due to the hydrophobic nature of leucin itself, which leads to a high affinity of binding other proteins, and therefore avoiding contact with water.(Kobe and Deisenhofer 1994) It is important to note, that *LRIG* proteins share a high similarity regarding the extracellular- and transmembrane domain, but differ widely when comparing the cytoplasmatic tail and cysteine N-flanking domain. As shown in Figure 2, each member of the *LRIG* protein family has a unique number of amino acids in the cytoplasmatic tail. Additionally, the differences in the cysteine N-flanking domain, a structure that is believed to provide structure in protein-protein interactions, is also notable.(Simion,Cedano-Prieto, and Sweeney 2014) Figure 2 demonstrates that *LRIG2* is the only member of the *LRIG* family that does not possess a cysteine N-flanking domain. This could be an explanation why the function of the three different *LRIG* genes cannot be considered homogenous.(Lindquist et al. 2014)

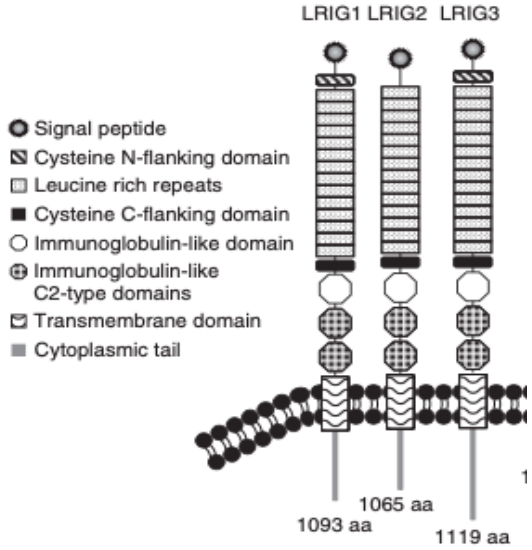


Figure 2: Structure of LRIG proteins (C.Simon et al. the lrig family))

1.3.2 Mechanism of action

LRIG proteins are involved in the regulation of receptor tyrosine kinase (RTK) signaling, which play a crucial role in cell proliferation, differentiation, and survival. The LRIG family consists of three members: *LRIG1, LRIG2*, and *LRIG3*, which are believed to have a similar mechanism of action.(Sigismund, Avanzato, and Lanzetti 2018) The primary mechanism of action of *LRIG1* is to negatively regulate RTK signaling. LRIG1 binds to and destabilizes RTKs, such as the epidermal growth factor receptor (EGFR), at the cell surface, preventing their internalization and degradation. This leads to a reduction in RTK signaling and activation of various signaling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. *LRIG1* achieves this negative regulation through several mechanisms. First, LRIG1 competes with RTK ligands, such as growth factors, for binding to the extracellular domain of the RTK. This reduces the activation of the RTK and the associated signaling pathways. Second, *LRIG1* can directly interact with and inhibit the activity of RTKs, such as EGFR. LRIG1 can bind to the kinase domain of EGFR and inhibit its activity, leading to reduced RTK signaling.(Hedman et al. 2002) While, *LRIG1* is considered to be a negative regulator for EGFR, meaning decreasing the activity of the receptor), a definitive statement for *LRIG2* and *LRIG3* cannot yet be made.(Hedman et al. 2002) However, a reduction of *LRIG3* expression in squamous cervix carcinoma cells has been demonstrated, while EGFR expression was increased. Meaning that *LRIG3* could also function as a negative regulator of EGFR while *LRIG2* is suspected to have an opposing effect.(Lindquist et al. 2014) (Guo et al. 2015) Also, important to note that there is a correlation between *LRIG1* and skin cancer.(Hoesl et al. 2021) Studies suggests that this protein may be a potential therapeutic target for the treatment of this disease, as *LRIG1* is frequently downregulated in skin cancer cells. This downregulation is associated with a more aggressive phenotype and poorer

patient outcomes.(Tanemura et al. 2005) Additionally, a study in 2008, suggested a link between the *LRIG* family and psoriasis. The *LRIG* protein levels of both healthy and psoriatic skin were compared. Interestingly, no significant difference in the expression level could be found. However, an altered expression pattern of the *LRIG* proteins could be detected in the psoriatic epidermis. Indicating a link between *LRIG* protein expression and skin pathology.(Karlsson et al. 2008) However, at this point these are just assumptions and little is known about the function of *LRIG3* in the skin. To be able to make a verified statement about the mechanism of action as well as to determine the safety and efficacy of *LRIG*-targeted therapies further research and studies are needed.

1.4 DIRAS

The *DIRAS* genes encode for small monomeric GTPases. There are three known functional *DIRAS* genes within the human genome: *DIRAS1*, *DIRAS2*, and *DIRAS3*, which belong to the RAS superfamily. The DIRAS protein is involved in a variety of cellular processes, including cell proliferation, differentiation, and survival. It has been shown to play a role in the development and progression of various types of cancer, and aberrant expression of the *DIRAS* genes have been associated with poor prognosis in cancer patients.(Li et al. 2019). The mechanism of action as well as the structure of the transcribed proteins will be further discussed in the following chapters.

1.4.1 Structure of DIRAS proteins

DIRAS proteins have a structure that is similar to other members of the RAS superfamily, with a conserved GTP-binding domain in the center of the protein that plays a critical role in protein activation and downstream signaling. The whole protein is relatively small as it consists of around 220 amino acids (exact number may vary depending on which *DIRAS* gene encodes for the protein). In addition to the conserved GTP-binding domain, *DIRAS* proteins have a unique C-terminal domain that is longer than that of other RAS superfamily members. The C-terminal cite is often described as a loop. This cite is important for the attachment of the protein to the cell membrane and interaction with downstream effectors.(Bourne, Sanders, and McCormick 1991)

1.4.2 Mechanism of action

The exact mechanism of action of *DIRAS* proteins is not fully understood, but it is known that they function as small GTPases, which means that they are involved in signaling pathways that regulate various cellular processes. In their active GTP-bound form, *DIRAS* proteins interact with downstream effectors to regulate cellular functions such as cell proliferation, differentiation, and survival. GTPases are small molecules that bind to guanosine triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP). This reaction is crucial for many molecular mechanisms like signal transduction of extracellular signals into the nuclei. GTPases are known as molecular switches as they are enzymatically inactive when bound to GDP and active when bound to GTP.(Li et al. 2019) *DIRAS* proteins have also been shown to interact with other proteins and signaling pathways,

including the PI3K-Akt pathway, which is involved in cell growth and survival, and the MAPK/ERK pathway, which is involved in cell proliferation and differentiation. The MAPK pathway includes a multitude of different proteins that are necessary for the transduction of extracellular signals to the nucleus of the cell, where the transcription of genes can be modulated. Therefore, it is an important signaling pathway in tumorigenesis.(Song et al. 2019). While there are a few publications on the role of *DIRAS1* in the context of cancer, the role of this gene in the development skin cancer is not explored yet.

2. Aim of the study

As *LRIG3* and *DIRAS1* are under suspicion to play role in the development of skin cancer, we aimed to generate 3 different skin cell lines in which the expression of *DIRAS1* and *LRIG3* is either knocked out or up-regulated. This should lead to a better understanding of human *LRIG3* and *DIRAS1* roles in tumor development.

3. Materials

3.1 Cells and plasmids

Cells / Plasmids	Brand	Location
A375 Cell Line	Eppendorf	Germany
A431 Cell Line	Eppendorf	Germany
HaCaT Cell Line	Eppendorf	Germany
pIRES2-AcGFP	Clontech	Saint-Germain-en-Laye, France
pSpCas9(BB)-2A-GFP (PX458)	Addgene	Teddington, UK
10-β- competent E.Coli	New England Biolabs	Frankfurt am Main, Germany

3.2 Equipment

Equipment Name	Brand	Location
Blotting membranes and filter paper	Thermo Fischer Scientific	Vienna, Austria
Cell culture plates (96-6 well plates)	Sarstedt	Vienna, Austria
Centrifuge, Hermle (Z.326K)/Eppendorf (5804R)	Gosheim/Eppendorf	Germany
ChemiDoc MP	Bio-Rad	Vienna, Austria
Clear non-binding, flat-bottom microplate	Greiner Bio-one	Kremsmünster, Austria
Cryovial, 1,5 ml	Thermo Fisher	Vienna, Austria
DS-11 FX spectrophotometer	Denovix-Biozym Biotech	Vienna, Austria
Eppendorf Tubes (1,5 ml; 2 ml)	Eppendorf	Hamburg, Germany
Falcon Tubes (15 ml and 50 ml)	Sarstedt	Hamburg, Germany
Fluorescence Microscope	Zeis	Vienna, Austria
Incubator	Binder	Tuttlingen, Germany
Inverted Microscope	Nikon	-
Lamina Flow	Thermo Fisher Science	Vienna, Austria
Micro Pipettes and Tips (filtered and non-filtered)	Rainin and Eppendorf	-
Microcentrifuge tube	Bio-Rad	Vienna, Austria
Nitril Examination Gloves	Hartmann	Vienna, Austria
Petri dishes (10 cm diameter)	Carl Roth, Falcon, Thermo Fisher	-
Pipetting aid	Eppendorf	Eppendorf, Germany
Scraper	Daigger Scientific-VWR International	Vienna, Austria
Serological Pipettes (5ml-50ml)	Sarstedt	Vienna, Austria
Thermocycler	Peqlab, VWR GmbH.	Vienna, Austria
Vortex	IKA	Staufen, Germany
Warterbath	Argo Lab	Carpi, Italy
XCell SureLock™ Mini-Cell	Thermo Fischer Scientfic	Vienna, Austria

3.3 Chemicals

Chemicals	Brand	Location
100 bp ladder	New England Bio Labs Inc	Frankfurt am Main, Germany
10x NEBuffer,	New England Biolabs Inc.	Frankfurt am Main, Germany
Agarose-Powder,	Sigma-Aldrich	Hoeilaart, Belgium
Anti-goat IgG, HRP-linked Antibody	Thermo Fisher Scientific	Vienna, Austria
Bovine serum albumin (BSA)	Sigma-Aldrich	Hoeilaart, Belgium
Clarity Western ECL Substrate	Bio-Rad	Vienna, Austria
dH2O	-	-
DMSO	Sigma-Aldrich	Hoeilaart, Belgium
DNeasy Blood & Tissue Kitt	QIAGEN	Hilden, Germany
DPBS (Dulbecco´s Phosphate Buffered Saline	Thermo Fisher Scientific	Vienna, Austria
Dulbecco's Balanced Salt Solution (DBSS)	Gibco-Thermo Fisher Scientific	Vienna, Austria
Ethanol (70%, 90%)	Roth	Krems, Austria
FCS	Sigma-Aldrich	Hoeilaart, Belgium
Gel 4-12%, Bis-Tris-gel, 10-well/12-well	Invitrogen	Darmstadt, Germany
Invitrogen™ Lipofectamine™ 3000 Transfektionsreagenz,	Invitrogen	Darmstadt, Germany
LRIG3 Goat	Biotechne	Prais, France
Luria Agar	Sigma-Aldrich	Hoeilaart, Belgium
Methanol	Merk	Vienna, Austria
Monarch DNA Gel Extraction Kit	New England Bio Labs Inc.	Frankfurt am Main, Germany
NaCl	Sigma-Adlrich	Hoeilaart, Belgium
NuPAGE Antioxidans	Thermo Fischer Scientific	Vienna, Austria
NuPAGE® LDS Sample Buffer (4X)	Thermo Fischer Scientific	Vienna, Austria
NuPAGE® Reducing Agent (10X)	Thermo Fischer Scientific	Vienna, Austria
20X NuPAGE® SDS Running Buffer (MOPS)	Thermo Fischer Scientific	Vienna, Austria
NuPAGE Transfer Buffer,	Thermo Fischer Scientific	Vienna, Austria
OneTaq Quick-Load 2X Master Mix with Standard Buffer	New England Biolabs Inc.	Frankfurt am Main, Germany
Opti-Mem	Gibco, Thermo Fisher Scientific	Vienna, Austria
Penstrep	Gibco, Thermo Fisher Scientific	Vienna, Austria
Pierce Detergent Compatible Bradford Assay Kit	Thermo Fisher Scientific	Vienna, Austria
Pierce RIPA-buffer	Thermo Fisher Scientific	Vienna, Austria

Protein ladder (PageRuler Prestained Protein ladder)	Thermo Fisher Scientific	Vienna, Austria
Qiagen MIDI-Prep Kitt	QIAGEN	Hilden, Germany
QIAquick PCR Purification Kit	QIAGEN	Hilden, Germany
Rabbit anti-human beta tubulin	Cell Signaling Technology	Vienna, Austria
Shrimp Alkaline Phosphatase (rSAP)	New England Biolabs Inc.	Frankfurt am Main, Germany
S.O.C Medium	Thermo Fisher Scientific	Vienna, Austria
Stable cell DMEM high glucose	Sigma-Aldrich	Hoeilaart, Belgium
T4-ligase buffer	New England Biolabs Inc.	Frankfurt am Main, Germany
T4-ligase	New England Biolabs Inc.	Frankfurt am Main, Germany
TRIS base	Sigma-Aldrich	Hoeilaart, Belgium
TRIS-HCL	Sigma-Aldrich	Hoeilaart, Belgium

3.4 Buffer recipes

Buffer	**Brand**	**Location**
TBS 10X (0.2M Tris-base + 15.5 M NaCl adjust pH to 7.4 using HCL	Components from Sigma-Aldrich	Hoeilaart, Belgium
TBST: 100 ml of TBS 10X + 500 µl of Tween 20 + 900 ml of deionized H2O	Components from Sigma-Aldrich	Hoeilaart, Belgium
Native buffer (0.025 M Tris-base, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4)	Components from Sigma-Aldrich	Hoeilaart, Belgium

3.5 Enzymes

Enzymes	Brand	Location
*Bgl*II	New England Biolabs Inc.	Frankfurt am Main, Germany
*Xcm*I	New England Biolabs Inc.	Frankfurt am Main, Germany
*EcoR*I	New England Biolabs Inc.	Frankfurt am Main, Germany
Trypsin (0,1% and 0,25% in DPBS without Ca and Mg),	Gibco-Thermo-Fisher Scientific	Vienna, Austria

3.6 Sequences of oligos and primers

hLRIG3	
Plasmid sequences (pSpCas9(BB)-2A-GFP (px458))	
Px458_h*LRIG3*_KO_F (5'->3')	CACCGAATCTGGGACCAGTCTCGGC
Px458_h*LRIG3*_KO_R (5'->3')	AAACGCCGAGACTGGTCCCAGATTC
Donor-ssDNA_h*LRIG3*_KO	CCTATGGAACTCCCACTCAAGGTTCCTGCAAACATACATGA GGAATTCTGCTGATATTCACTTACA AGGAGAGAAGTGTAATATTTGCCGAGACTGGTCCCAGAGG AATTCTTTGAAATGGTCTCCAATTCATTGTTGTTCAGTTTCC TACAAATGCCAAAAAAAAAAAGAAAAAGAAAAGCTGATT AGGTAATCTGTAA
Primers for genotyping	
h*LRIG3*-Gen-F (5'->3')	TGGTGTTCATAGAGATACTTC
h*LRIG3*-Gen-R (5'->3')	TCTCCACTAGGAACTACG

hDIRAS	
Plasmid sequence (pSpCas9(BB)-2A-GFP (px458))	
Px458_h*DIRAS1*_KO_F	CACCGCTGCAGATCACAGACACCAC
Px458_h*DIRAS1*_KO_R	AAACGTGGTGTCTGTGATCTGCAGC
Donor:ssDNA_ h*DIRAS1* _KO	TGACGGAGAACACCAGGATGAAGGCGTGGCCCTTG GAGATGGACAGGCGCTGCATGGCCGGGAACTGGTG GCTGTTTGTGGAATTCGGTGTCTGTGATCTGCAGCGT GCACACGCTCTTGTCGCAGCTGATCACCTGCCGGTAG GTGTCCTCGATGGTGGGGATGTAGGTGTCGCGGAACG TGCCCTTCACGAAGCGCAG
Primers for genotyping:	
h*DIRAS1*-Gen-F (5'->3')	TGGCCTTTGACGTGTGAC
h*DIRAS1*-Gen-R (5'->3')	TCATCTTGGCCGAGGTCTC

4. Methods

Generating genetically altered cell lines

To manipulate the expression of *LRIG3* and *1* in the skin cell lines, A375 (melanoma cell line),A431 (SCC cell line), and HaCaT (immortalized keratinocytes), we used an overexpressing vector (pIRES2-AcGFP1) or CRISPR-Cas9 approaches for generating knockout cell lines, respectively.

4.1.1 Isolation of the h*LRI3*.1 and h*LRIG3*.2 genes

To clone the *LRIG3.1* and *LRIG3.2* cDNA into the pIRES2-AcGFP1 vector (Clontech, Saint-Germain-en-Laye, France) (Figure 4.1), we first cut out the GOI and opened the vector (StrataClone) bearing them using restriction enzyme *Eco*RI (New England Biolabs, Frankfurt am Main, Germany). To explain in more detail, 2 µg of plasmids StrataClone (SC)-*hLRIG3.1* or SC-*hLRIG3.2* or pIRES2-AcGFP1 vector was exposed to 20 unit (2 µL) of EcoRI and 6 µL of NEBuffer (rCutSmartTM) (New England Biolabs, Franfurt am Main Germany). Thereafter, the volume was adjusted to 60 µl with nuclease-free water. The reaction mixtures were incubated in a thermocycler at the following condition: a) 37 °C for 90 minutes b) 65 °C for 20 minutes followed by a 5-minute incubation on ice. In next step, in order to prevent religation of the linearized vector, we used Shrimp Alkaline Phosphatase (rSAP) (New England Biolabs, Frankfurt am Main, Germany) to dephosphorylate 5´end of the vector. To that end, 1 unit (1 µl) of rSAP was added to the pIRES2-AcGFP1 vector and was incubated at 37 °C for 10 minutes. Reaction was stopped by heat-inactivation at 75 °C for 5 minutes. To separate h*LRIG3.1* and h*LRIG3.2* DNA fragments as well as pIRES2-AcGFP1 vector based on their size and molecular weight, we performed electrophoresis on 1% w/v agarose gel. DNA fragments at the desired size (h*LRIG3.1*: 3221 bp, h*LRIG3.2*: 3581 bp and pIRES2-AcGFP1 vector: 5307 bp) were excised from the agarose gel and were weighed. The DNA fragments were extracted using monarch DNA gel extraction kit (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer's instructions. In brief, four volumes of gel dissolving buffer were added to each gel slice, followed by a 10-minute incubation at 50 °C while shaking. After loading the samples on the columns, they were centrifuged for 1 minute. Columns were washed twice and were then transferred into a new 1.5 ml microtube for elution with 10 µl of elution buffer. Denovix DS-11 FX spectrophotometer was used to determine DNA concentration.

4.1.2 Ligation of h*LRIG3*.1 and h*LRIG3*.2 genes into the pIRES-AcGFP1 vector

Ligation describes the process of inserting the isolated DNA (GOI) into a suitable vector (in this case pIRES2-AcGFP) using T4 DNA ligase enzyme. To describe this process shortly: following items were combined; a) the gel-purified pIRES2-AcGFP vector b) the gel-purified h*LRIG3*.1 or h*LRIG3*.2 DNA fragments c) T4 ligase buffer d) T4 ligase enzyme (both from New England Biolabs, Frankfurt am Main, Germany) e) dH2O to a

total of 20 µl volume. The mixtures were incubated at 16 °C overnight followed by a 3-hour incubation at room temperature (RT).

4.1.3 Transformation and analysis of bacterial transformants

Transformation describes the process of merging a recombinant plasmid with an appropriate bacterium (often E. coli is the bacterium of choice). The ligation mixtures containing the pIRES2-AcGFP1 vector with the h*LRIG3.1* or h*LRIG3.2* cDNA were added to the previously thawed 10-β competent E.coli bacteria. The probes underwent heat-shock treatment. This process includes several steps of warming and rapidly cooling the samples down (30-minute incubation on ice, incubation in 42 °C water bath for 45 seconds, followed by 2 minutes of incubation on ice). Afterwards 250 µl Super Optimal broth with Catabolite repression medium (S.O.C.) (Thermo Fisher Scientific, Vienna, Austria) was added. To be able to analyze the transformants, single bacterial colonies on kanamycin infused agar plates were generated. The bacteria which were used to create the transformants had an intentional resistance to kanamycin, resulting in the growth of only kanamycin-resistant bacterial colonies from 10-β E. coli. Subsequently, 10 single colonies from *hLRIG3.1*, and *hLRIG3.2* were chosen, respectively, to undergo DNA extraction. After successfully extracting the DNA from the bacterial transformants using Monarch Plasmid DNA Miniprep kit (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer's instruction, the DNA concertation was determined using Denovix DS-11 FX spectrophotometer. To determine if the gene was inserted into the vector in the right direction, an enzyme digestion had to be performed. In this instance, *Bgl*II enzyme (New England Biolabs, Frankfurt am Main, Germany) was used for the digestion. This enzyme cuts at two distinct sites (A/GATCT) in the pIRES2 AcGFP1 vector. Once in the sequence of the vector itself and once in the sequence of GOI. Figure 3 indicates the schematic plasmid maps and cutting sites in both cases of correct and incorrect insertions.
If the gene was in fact inserted in the right direction, following two bands (Table 1) can be detected through gel electrophoresis in a 1% agarose gel.

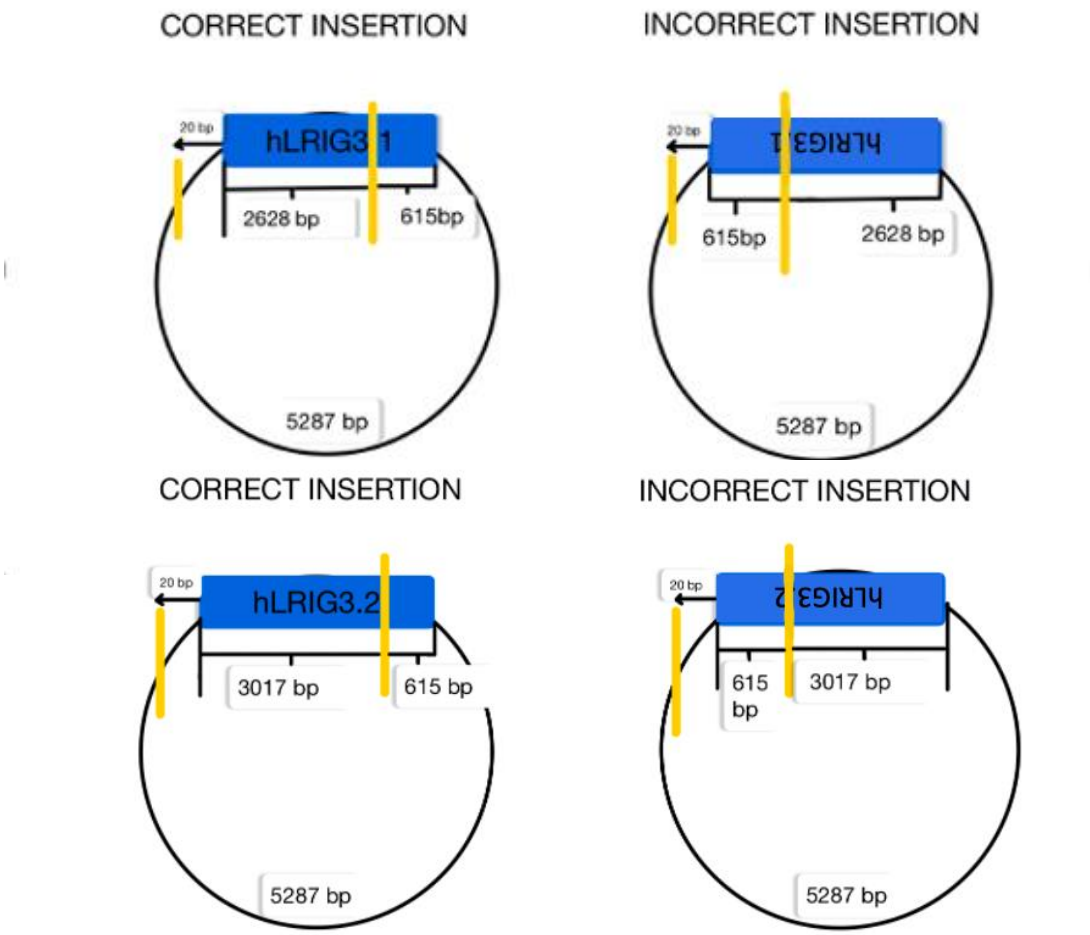
	*LRIG*3.1	*LRIG*3.2
bp of first band	5882 bp	5882 bp
bp of second band	2648 bp	3037 bp

Table 1: Expected bands if gene was inserted correctly

However, if the gel electrophoresis results in bands depicted in Table 2, the GOI was inserted in the wrong direction.

	*LRIG*3.1	*LRIG*3.2
bp of first band	7895 bp	8284 bp
bp of second band	635 bp	635 bp

Table 2: Expected bands if gene was inserted incorrectly



3: Schematic representation of vector digestion, On the top hLRIG3.1, on the bottom hLRIG3.2, yellow lines represent cutting Sites of EcoRI

After determining in which single colonies the gene was inserted correctly, plasmid DNA of two positive colonies one from h*LRIG*3.1 and one from h*LRIG*3.2, were sent for sanger sequencing using CMV, forward primer (see chapter material) The results of the sequencing were then analyzed using NCBI (The National Center for Biotechnology Information) and Nucleotide BLAST (The Basic Local Alignment SearchTool). We were able to confirm an alignment between our sequencing results and the human mRNA of *LRIG3* variants 1 and 2. Therefore, the plasmids were used for transfection into the cells. To purify plasmid DNA from the bacterial transformants for transfection-grade application, we utilized QIAfilter Plasmid Midi Kit (QIAGEN Haiden, Germany). The procedure was carried out as follows; First, we picked one single bacterial colony to start and let it dissolve in 3 ml of LB medium (Sigma, Hoeilaart, Belgium) in a 5-ml round-bottom tube. This starting culture was incubated at 37 °C for 8 hours with vigorous shaking. Afterwards, we diluted the starting culture with LB medium in a ratio of 1:1000. The diluted mixture was again incubated under the same conditions as before overnight. Then, the bacterial cells were harvested by centrifugation at 2992 xg for 20 minutes at 4 °C. Thereafter, plasmid purification was performed on the bacterial pellet using QIAfilter Plasmid Midi Kit (QIAGEN, Haiden, Germany) according to the manufacturer's instruction. It is a modified alkaline lysis procedure, followed by

binding of plasmid DNA to the QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. (Qiagen and Qiagen 2005)

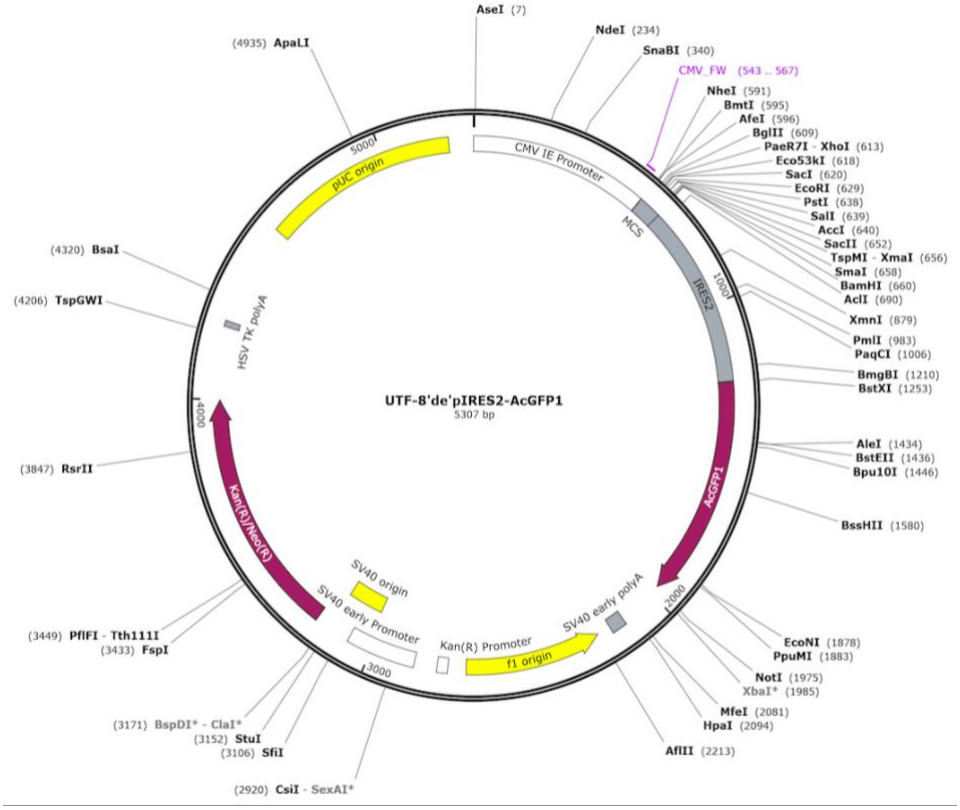


Figure 4: Map of Overexpressing Vector, pIRES2-AcGFP. Created by SnapGene

4.1.4 Transfection of cell lines with h*LRIG*3.1 and h*LRIG*3.2 overexpressing vectors

Transfection and transformation both describe the process of inserting a plasmid inside a specific cell with the intend to change its genomic code. Transformation is the term that is being used when procaryotic cells, like bacteria, are merged with a plasmid, while the term transfection is being reserved for eucaryotic cell lines.(Seeber 2000) For this method, the eucaryotic cells- HaCaT, A375, and A431 (Cell Lines Services, Eppelheim, Germany)- have been revived, cultured and split repeatedly until a confluence of 80% in a 6-well dish was achieved. All cells had been cultured in Dulbecco´s Modified Eagle´s medium (Thermo Fischer Scientific, Vienna, Austria) (DEMEM), containing phenol red as a pH-indicator. The medium was supplemented with 10% fetal calf serum (FCS) (Thermo Fischer Scientific, Vienna, Austria) and 1% penicillin/streptomycin. Before transfecting the cells with the generated plasmid, the cells were washed twice with 5 ml Phosphate Buffered Saline (PBS) (Thermo Fischer Scientific, Vienna, Austria). Thereupon the Transfection could be carried through. To describe this process in more detail: a cocktail comprising 7.5 µl Lipofectamine 3000 Reagent (Invitrogen, Darmstadt, Germany) and 125 µl Opti-MEM Medium (Gibco, Thermo Fischer Scientific, Vienna, Austria) was prepared. Subsequently, we prepared another cocktail consisting of DNA (4 µg of plasmid DNA) in 125 µl of Opti- MEM Medium and 8 µl of P3000™ Reagent (Invitrogen, Darmstadt, Germany). Afterwards 2 cocktails were mixed (1:1 ratio) dropwise and the mixture was incubated for 5 minutes at room temperature. Then the mixture was pipetted evenly to the cells. We let the cells incubate at 37 °C overnight.(Thermo Fisher Scientific 2016) The next day we analyzed the cells under the fluorescence microscope to determine if the cells glow green. We observed that the cells were emitting green fluorescence, therefore; overexpression had been successfully performed and the cells were harvested due to the fact, that our utilized approach was a transient transfection.

4.1.5 Protein isolation and quantification from h*LRIG*3.1 and h*LRIG*3.2 overexpressing cell lines

Before harvesting the overexpressing cell-lines, the cells were washed twice with 5 ml of cold DPBS (Gibco,Thermo Fischer Scientific, Vienna, Germany). The cells were harvested using a scraper. 500 µl of ice-cold native buffer (0.025 M Tris-base, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4) was added to the detached cells. The cells were pipetted up and down several times. We transferred the cell suspension into a 1,5 ml Eppendorf tube and vortexed it for 20 seconds. Next the samples were incubated on ice for 15 minutes. Afterwards we centrifuged the samples for 10 minutes with 17005 xg at 4 °C. Then we collected the supernatant and made aliquots. The samples were kept at -80 °C until they were further needed. It is important to note, that additionally to the overexpressing cells, non-transfected cells from each cell line were harvested for protein isolation as our negative control.

Before performing the western blot, the protein levels of the samples were determined via Bradford Essay. This was done to establish a suitable dilution ratio for the samples later in the western blot. To describe this process shortly: First we prepared our standards, a

dilution series containing bovine serum albumin (BSA) (Thermo Fischer Scientific, Vienna, Austria) in native buffer. The dilution series contained 9 dilution steps with a final BSA concentration of: 0, 25, 125 ,250 ,500, 750, 1000, 1500, and 2000 µg. We pipetted 10 µl of the samples and standards into a well of a microplate. Subsequently 300 µl of Pierce detergent compatible Bradford assay reagent (Thermofischer, Austria, Vienna) was added to each sample. The plate was incubated for 5 minutes at room temperature. Next the absorbance was measured at 595 nm with Tecan sunrise plate reader. A four-parameter logistic (4-PL) standard curve was created to calculate the concentrations of proteins isolated from each cell line using a Graph-Pad Prism software v9.

4.1.6 Confirmation of h*LRIG*3.1 and h*LRIG*3.2 overexpression in genetically modified cell lines using western blot

Western blot is a technique used to detect and analyze specific proteins in a sample by separating them by size through gel electrophoresis and transferring them onto a membrane, which is then probed with specific antibodies to identify the target protein.(Lee 2007) First, we diluted the samples with NuPAGE LDS Sample Buffer (4X) (Invitrogen, Darmstadt, Germany) accordingly to the outcome of the Bradford assay to reach a final concentration of 25 µg of protein in each sample. Next 1:10 dilution of NuPAGE Reducing Agent (10X) was added. The volume was adjusted to 15 µl with deionized water. After the sample preparation the preparation of the running chamber was started. First the 20X NuPAGE SDS Running Buffer MOPS (Invitrogen, Darmstadt, Germany) was diluted with deionized water to get 1l of 1x NuPAGE SDS Running Buffer. Thereupon 200 ml of 1X NuPAGE SDS Running Buffer was taken out and infused with 500 µl of NuPAGE Antioxidant (Invitrogen, Darmstadt, Germany) for use in the inner Buffer chamber of the XCell Sure Lock Mini-Cell chamber (Invitrogen, Darmstadt, Germany). The chamber was assembled with the NuPAGE gel (Invitrogen, Darmstadt, Germany) and filled with the prepared buffer solutions according to the companies' instructions. Next 15 µl of the samples and 5 µl of the PageRuler Prestained Protein ladder (Invitrogen, Darmstadt, Germany) were loaded on the gel. We let the gel run at 200 V for 50 minutes. Afterward, blotting pads and filter paper (Invitrogen, Darmstadt, Germany) were soaked in 1X NuPAGE Transfer Buffer. We pre-wetted the PVDF membrane for 30 seconds in methanol and rinsed it in deionized water, then the membrane was also places in 1X NuPAGE Transfer Buffer. After the gel electrophoresis, the gel was carefully removed from the chamber. The saturated blotting pads were placed into the blotting cathode core. Then the soaked filter paper followed by the gel was added to the cathode. The gel was then covered with the transfer membrane followed by a pre-soaked filter paper (Figure 5). Lastly, we added 2 pre-soaked blotting pads on top of the blotting sandwiches and placed the anode core on top of the pads.

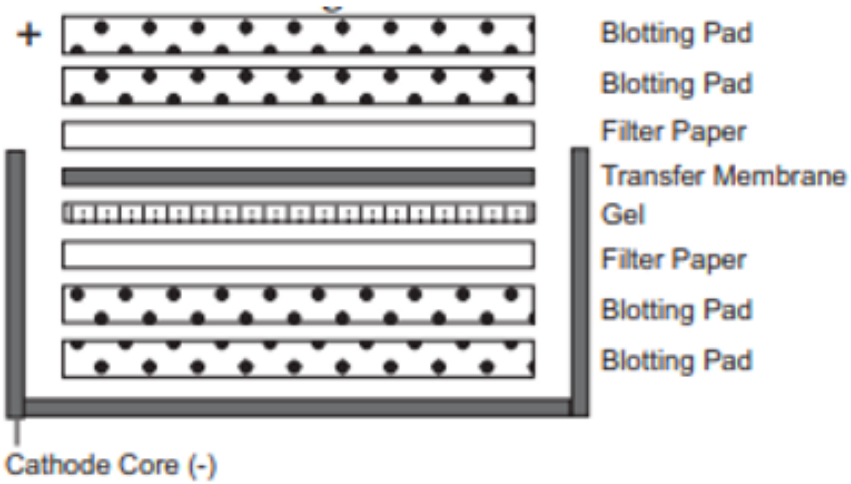


Figure 5: Assembled Blotting-Sandwich, Invitrogen

Subsequently the blot module is placed into the gel chamber. We filled the blot module with 1X NuPAGE Transfer Buffer until the blotting sandwich was completely covered. The lower buffer chamber was filled with deionized water. After the gel, membrane and filter papers were assembled, an electric current (30 V for 1h) is applied to the gel and the membrane which transfers the proteins onto the membrane accordingly to their molecular weight and charge. (Kurien and Scofield 2006) Afterwards the membrane was blocked in 15 ml of 5% skim milk in TBST (Thermo Fischer Scientific, Vienna, Austria) for one hour on a shaker at RT. This means a substance is utilized to bind to the non-occupied binding spots on the membrane. This step is vital as to avoid non-specific binding of the specialized antibody in the next steps. A higher background noise could be the result if mistakes occur in the immune detection. After blocking, the membrane was washed using 1X TBST 3 time for 5 minutes. Thereupon the membrane is placed into an incubation tube with 5 ml of goat anti-human LRIG3 (1:2000 dilution) (Biotechne, Paris, France) or rabbit anti-human beta tubulin (1:2000 dilution) (Cell Signaling Technology, Vienna, Austria) primary antibodies. We let the membranes incubate at 4 °C on a rotator overnight. The next day we washed the membranes 3 times for 10 minutes on shaker with 1X TBST. Next the membranes were supplied with 5 ml of the according secondary antibody, anti-goat IgG (1:2500) (Thermo Fischer Scientific, Vienna, Austria) or anti-rabbit IgG (1:2500) (Cell Signaling Technology, Vienna, Austria) HRP-linked Antibodies. The membranes were incubated for 1 hour at RT on a shaker. After, the membranes were washed 3 times with 1X TBST for 10 minutes at RT on a shaker. After the membranes were incubated in 4 ml Biorad´s Clarity Western ECL Substrate (Biorad, Vienna, Austria). We placed the membranes in a Western blot development device (ChemiDoc MP, Biorad, Vienna, Austria) and pictures of the membrane were taken. The outcome of the Western blot will be discussed in the chapter "results".

4.2 Generating h*DIRAS*1-and h*LRIG*3-knockout cell lines

In contrast to the process of overexpression, knock out is a method to annihilate the GOI in a cell's genome. This can be done through a multitude of methods, with the most popular and widely used being Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)- Crispr associated Protein 9 (Cas 9). This tool is used to modify the genome of cells permanently and was awarded with the Nobel prize for chemistry in 2020 for its groundbreaking contribution to the field of genome editing. CRISPR occurs naturally in the bacterial genome and plays a vital part in the bacteria´s defense against bacteriophages. Bacteriophages multiply by inserting their own genetic code into its host. Therefore CRISPR-Cas9 is responsible to find and cut out the foreign genetic regions, to allow the bacteria to stay uninfected. First, small RNA sequences are transcribed, that match the targeted bacteriophage´s DNA site. Those small sequences, also called CRISPR RNAs, can guide the CRISPR-Cas9 system to the foreign DNA. Subsequently, Cas9 the CRISPR associated protein, is responsible for cutting out the targeted DNA. Researchers have found that this technology can also be applied in eucaryotic cells. In fact, the CRISPR-Cas 9 system is not only capable of cutting out foreign genomic sequences but can also modify the cell´s own genetic code provided the CRISPR-Cas9 system is matched with a designed guide RNA (gRNA) to be able to detect and successfully attach to the GOI. A designed guide RNA (gRNA) in CRISPR-Cas9 system is able to detect and successfully attach to the GOI and induce an alteration in GOI.(Ran et al. 2013)
The gRNA that matched to the DNA of h*LRIG3* and h*DIRAS1* respectively was designed beforehand. These sequences were then inserted into two separate vectors. Vector contains sequences encoding CRISPR-Cas9 and the green florescent protein (GFP). In contrast to the process of establishing overexpressing cell lines, one vector-construct for hLRIG3 sufficed to be used to deactivate both isoforms. The applied CRISPR-Cas9 system is supposed to mutate h*LRIG3* gene, through induction of INDEL (a genetic mutation which occurred through insertion or deletion of a single nucleotide) in a region where the two isoforms of hLRIG3 are extremely homologous. Therefore, both hLRIG3.1 and hLRIG3.2 could be switched off by the same construct. This INDEL results in a frameshift of the translation machinery and subsequently should lead to a non- synonymous (a different protein than intended) or non-functioning mutation (the function of the synthesized protein is changed). Although the vector and gRNA have been designed and established beforehand, a short overview of the process will be given in the following chapters to grant a better understanding of the procedure.

4.2.1 Cloning of gRNA into pSpCas9(BB)-2A-GFP (PX458) backbone

The term "cloning gRNA" typically refers to the process of generating multiple copies of gRNA molecules, which are short RNA sequences used in the CRISPR-Cas9 gene editing system to guide the Cas9 enzyme to specific target sites in the genome and subsequent modification. Cloning gRNA involves inserting the gRNA sequence into a suitable plasmid, which serves as a carrier for replication of the gRNA sequence in bacteria. The cloned gRNA can then be amplified, purified, and used in the following steps.(Nageshwaran et al. 2018)

4.2.1.1 Annealing of gRNA oligos

The process of annealing the gRNA refers to bringing together two short single-stranded gRNA molecules or oligos, to form a double-stranded gRNA molecule. Typically, this was achieved by mixing two complementary gRNA oligos in a buffer solution (NEB 4 Buffer, New England Biolabs Inc., Frankfurt am Main, Germany) and then heating the mixture to 95°C for 5 minutes in MJ Research PTC-200 thermocycler, to denature the oligos. Then it was cooled down by 0.1 °C per second to 25 °C to anneal or reassociate into a stable double-stranded gRNA molecule.(Nageshwaran et al. 2018)

4.2.1.2 Digestion of the vector and ligation of annealed RNA into pSpCas9(BB)-2A-GFP (PX458) backbone

The process of ligating the annealed gRNA into a plasmid (in this case pSpCAS9(BB)-2A-GFP (PX458)) is similar to the ligation process for the overexpressing vector. In brief, the reaction consisted of 40 fmol of the vector (pSpCAS9(BB)-2A-GFP (PX458)), 120 fmol of the annealed diluted h*LRIG3* or h*DIRAS1* gRNA oligos (ratio 1:000), 30 U of T4 ligase and 1:10 dilution of of T4 ligase buffer (New England Biolabs Inc. Frankfurt am Main, Germany). In addition to the ligation, a digestion was performed. Therefore 1 µl of Fast Digest *Bpi*I (Thermo Fisher Scientific, Vienna, Austria) was added. *Bpi*I cut site is shown in Figure 4.5. Water was added to reach a final volume of 20 µl in the reaction. The reaction was incubated in MJ Research PTC-200 thermocycler (37 °C for 5 min followed by 20 °5 min for 12 cycles, 37 °C for 30 min, and 65 °C for 10 min.The DNA ligase enzyme facilitates the creation of covalent bonds between nucleotides, permanently connecting them together. Once the DNA insert is linked to the backbone, the entire plasmid can be introduced into bacterial cells for replication.(Green and Sambrook 2012)

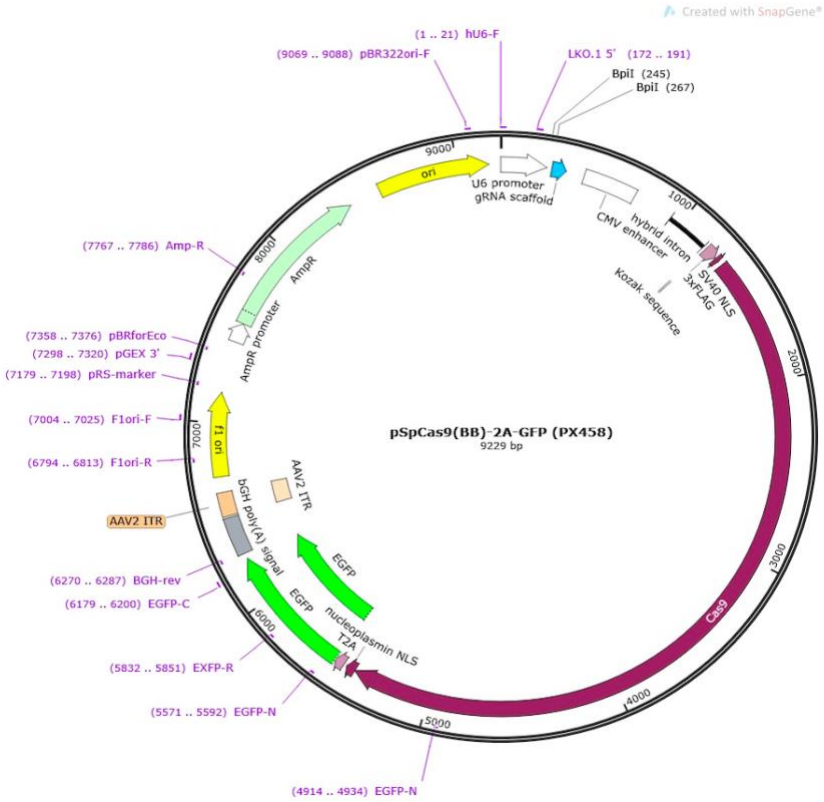


Figure 6: Map of pSpCas9(BB)-2A-GFP (PX458) Vector. Created by SnapGene

4.2.2 Transformation

For the transformation to 10-β E.coli bacteria , 5 µl of the ligation mixture was added. The bacteria containing the ligation mixture underwent heat shock treatment (30-minute incubation on ice, incubation in 42 °C water bath for 45 seconds, followed by 2 minutes of incubation on ice), 500 µl of S.O.C (Invitrogen, Darmstadt, Germany) was added. After an incubation time of 1 hour at 37 °C, 100 µl of bacteria was plated on LB-Agar-Ampicillin plates. After the transformation process (as described in greater detail for the overexpressing vectors) and generation of single bacterial colonies, the bacteria were analyzed to determine if the insertion of the plasmid was successful. Typically, 10 single bacterial colonies were picked to undergo DNA extraction using Monarch Plasmid DNA Miniprep kit (New England Biolabs, Frankfurt am Main, Germany). Next the extracted DNA was subjected to enzyme digestion using corresponding restriction enzymes. If the desired bands were observed, DNA plasmids were sent for Sanger. After confirmation by sequencing results for the correct insertion of the gRNA into the plasmid, a QIAfilter Plasmid Midi Kit (QIAGEN, Haiden, Germany) was carried out, to isolate a larger quantity of the ligated plasmids from the bacteria. This DNA can then be used for transfection.

4.2.3 Transfection of cell lines with hDIRAS1- & hLRIG3- pSpCas9(BB)-2A-GFP vector

The process of transfecting the eucaryotic cells with the vector carrying the knockout construct is similar to the transfection with the plasmid that carried the construct for the overexpressing cell lines. In both cases Lipofectamine 3000 and P3000 Reagent (both from Invitrogen, Darmstadt, Germany) were utilized. However, while the oligonucleotide cocktail for the overexpressing cell lines were supplied with 4 µg of the plasmid´s DNA (*hLRIG3.1* or *hLRIG3.2*), the oligonucleotide cocktail for the knockout transfection consisted of 2.5 µg of the plasmid´s DNA (px458-gRNA *hLRIG3* or *hDIRAS1*) and 1.5 µg of the donor oligo. The cells were transfected when they reached a confluency of 80% in a 6-well plate. After one day the cells could be checked under the microscope to determine if the cells glowed green and therefore if the first step of the transfection was successful. However, opposed to the overexpressing cell lines, the knockout construct is stable. Therefore, the cells could be further cultured and split. Precedingly, the cells were sorted via fluorescence-activated cell sorting (FACS).

4.2.4 Screening the transfected cells

Fluorescence Activated Cell Sorting (FACS) is a technique used to separate and analyze cells based on their fluorescent properties. For this method cells are either expressing a fluorescent protein e.g., green fluorescent protein (GFP) or labeled beforehand with a fluorescent-conjugated antibody. The cells are then passed through a flow cytometer, which uses lasers to detect and quantify the fluorescence emitted by the labeled cells. Based on their fluorescence intensity, cells can be sorted into different populations.(Basu et al. 2010) Here, transfected h*DIRAS*1- & h*LRIG*3- pSpCas9(BB)-2A-GFP cells were sorted based on the GFP expression. Before sorting the cells, they were washed with preheated DPBS and trypsinized (both Gibco-Thermo Fischer Scientific, Vienna, Austria). After the cells were detached, 3 ml of cell culture medium was added to neutralize the trypsin. The cell suspension was transferred to a 15 ml falcon tube and centrifuged at 300 g for 5 minutes. After discarding the supernatant, the cells were resuspended in 1 ml of plain medium (DMEM with 1% P/S without FCS). Then the cells were sorted via the BD FACSCantoII. Additionally, to determine which cells emit green light and therefore were successfully transfected, the BD FACS CantoII was able to generate single cell of the transfected cell lines. By the end of the process, we generated two 96-well plates per cell line- HaCAT, A431, A437.

4.2.5 Culturing of single-cell clones

The single-cell clones were further cultivated. When a confluency of 85-90% was achieved, the cells were detached from the well´s surface using trypsin (0.1% trypsin and 0.25% Trypsin were used for the tumor cell lines, and HaCaT cells, respectively) (Gibco-Thermo Fischer Scientific, Vienna Austria) and passaged into a bigger plate. This process was repeated until the cells reached a confluency of at least 85% in a 24-well plate. Then samples for DNA were taken and the remaining cells were frozen. To describe this process shortly: Firstly, the medium from the wells that should be frozen was aspirated. Then we washed the cells twice with preheated DPBS (Gibco-Thermo Fisher Scientific, Vienna, Austria). In between the liquid was aspirated again. Then 150 µl trypsin was added to the cells. The cells were incubated for 7-10 minutes at 37 °C, however it should be noted that the incubation time can vary. After the cells were detached, 550 µl of medium was added to the wells to stop the trypsinization. The cell suspension was transferred to a 1.5 ml Eppendorf tube and the wells were washed twice more with 550 µl of medium and transferred to the tube. From this suspension 250 µl were transferred into an Eppendorf tube for DNA isolation. The samples for DNA isolation were centrifuged at 3622 xg for 10 minutes at 4 °C. Afterwards the supernatant was discarded, and the cell pellet was stored at -20 °C until they were further needed for DNA isolation.
The rest of the cells were frozen. To be more precise: the rest of the cell suspension (1400 µl) was centrifuged at 300 xg for 5 minutes. Meanwhile, the cryovials (Thermo Fisher) were prepared by adding 80 µl of freezing medium containing 20% dimethyl sulfoxide in FCS to them (Thermo Fisher Scientific, Vienna, Austria). After centrifugation, the supernatant was aspirated, and the cells were resuspended in 80 µl of medium. Next, the cell suspension was added to the cryovial containing the freezing medium in a dropwise-manner. The cryovials were then kept at -80 °C in a freezer. After a maximum of 2 weeks the cryovials were transferred to a liquid nitrogen tank. If the desired genetic mutation could be verified via genotyping, the frozen cells could then be revived later in the project. The frozen cells were then thawed, cultured, and passaged until they reached 100% confluency in a 6-well plate. Then the cells could be harvested using the cell scrape (same procedure as with the overexpressing cell lines). From these samples enough protein could be isolated to confirm the genetic mutation on a protein level

4.2.6 Screening of h*DIRAS*1- and h*LRIG*3-KO single-cell clones

To confirm the cells knockout mutation, the acquired samples were genotyped via PCR.
If the PCR and the following gel electrophoresis verified the knockout, the samples were sent for sanger sequencing. If the sequencing results proved the desired mutation it was planned to proceed with western blot.

4.2.6.1 DNA isolation

To isolate DNA from the genetically altered knockout cells for the PCR, we used the frozen cell pellets. For this purpose, the Qiagen DNeasy Blood & Tissue Kit (QIAGEN, Haiden, Germany) was used. The procedure was followed as recommended by the company's instruction. The procedure briefly went as follows: First we resuspended the thawed cell pellet in 200 µl of DPBS (Gibco-Thermofisher, Vienna, Austria) inside Eppendorf tubes. Thereupon, we added Proteinase K (20 µl) and Buffer AL (200 µl) (both contained in the Qiagen kit) to each resuspended pellet. The mixture was then vortexed for a few seconds. Upon that, we let the samples incubate for 10 minutes at 57 °C. Afterwards 200 µl of 96% Ethanol was added to each sample and vortexed again. The cell-lysate mixture was pipetted into DNeasy spin columns and centrifuged for 1 minute at 7442 xg at room temperature. We discarded the flow through and inserted the spin column into a new collection tube. After 2 times of washing steps, DNA was eluted by centrifugation for one minute at 17532 xg. The amount of elution buffer (AE) was reduced to 35 µl yield a higher concentration of DNA in the final solution. Additionally, we reloaded the eluted DNA and let it centrifuge again. The final centrifugation was carried out at 7442 gx for 1 minute. (DNeasy ® Blood & Tissue Handbook 2020) Afterwards the DNA concentration was measured using Denovix DS-11 FX spectrophotometer.

4.2.6.2 Genotyping using PCR

Oligo donor of *hDIRAS1* has been designed in a way that EcoRI cut site was incorporated into the oligo; meaning that after PCR followed by a digestion with EcoRI, *hDIRAS1*-KO single-cell clones, would yield into 2 bands of 290 bp and 280 bp. While the wild-type sequence would show a single band at 563 bp. In oligo donor of hLRIG3, there is no cut site for any restriction enzyme, whereas there is a cut site for *Xcm*I enzyme in the wild-type sequence. Therefore, after PCR and digestion with *Xcm*I, *hLRIG3*-KO sequences would have a single band at 823 bp, while wild-type sequence would result in 2 bands at 402 bp and 420 bp. To screen *hDIRAS1*- and *hLRIG3*-KO single-cell clones, we conducted PCR using master mix OneTaq Quick Load 2X (New England Biolabs Inc., Frankfurt am Main, Germany), and 10 µM of each primers (see Material section) followed by a PCR condition for *hLRIG3* and *hDIRAS1*(table 3). When PCR was carried out in thermocycler peqSTAR, PCR products of the hLRIG3 and *hDIRAS1* single-cell clones were subjected to digestion by *Xcm*I and *EcoR*I enzymes (both from New England Biolabs Inc., Frankfurt am Main, Germany), respectively, at 37 °C for 1 hour. This was done to confirm that the GOI was in fact knocked out in the cell genome. Following incubation with enzymes, a gel electrophoresis was conducted using the 1% agarose gel.

Temperature (° C)	Time	Cycle
PCR-condition for h*LRIG*3		
94	30 s	1
94	15 s	35x
45	20 s	
68	45 s	
68	5 m	1
PCR-conditions for h*DIRAS*1		
94	30 s	1
94	15 s	35x
56	20 s	
68	45 s	
68	5 m	1

Table .3: PCR-conditions for hLRIG3 and hDIRAS1

If the gel electrophoresis confirmed a knockout mutation, the PCR was repeated with the positive clones. Afterwards the remaining PCR product was cleaned up using QIAquick PCR Purification Kit (QUIAGEN, Haiden, Germany) and sent for sanger sequencing using the forward primer. The results of the sequencing were then analyzed using ApE, a plasmid editor software.

5. Results

5.1 Generation of h*LRIG*3.1- and h*LRIG*3.2-overexpressing cell lines

5.1.1 Establishing h*LRIG*3.1- and h*LRIG*3.2-pIRES2-AcGFP1 overexpressing vectors

As described in section 4.1.3, the clones were analyzed by using restriction enzyme digestion with *EcoR*I (Figure 7). The precise expected bands in the gel electrophoresis are mentioned and explained in detail in Table 1. One positive clone each from *hLRIG3.1* and *hLRIG3.2* were selected for further analysis. Plasmid DNA from two positive colonies (hLRIG3.1 and hLRIG3.2) were sequenced using CMV and a forward primer. The sequencing results were analyzed using NCBI and Nucleotide BLAST, confirming alignment with human LRIG3 mRNA variants 1 and 2. These plasmids were subsequently transfected into cells after successful confirmation

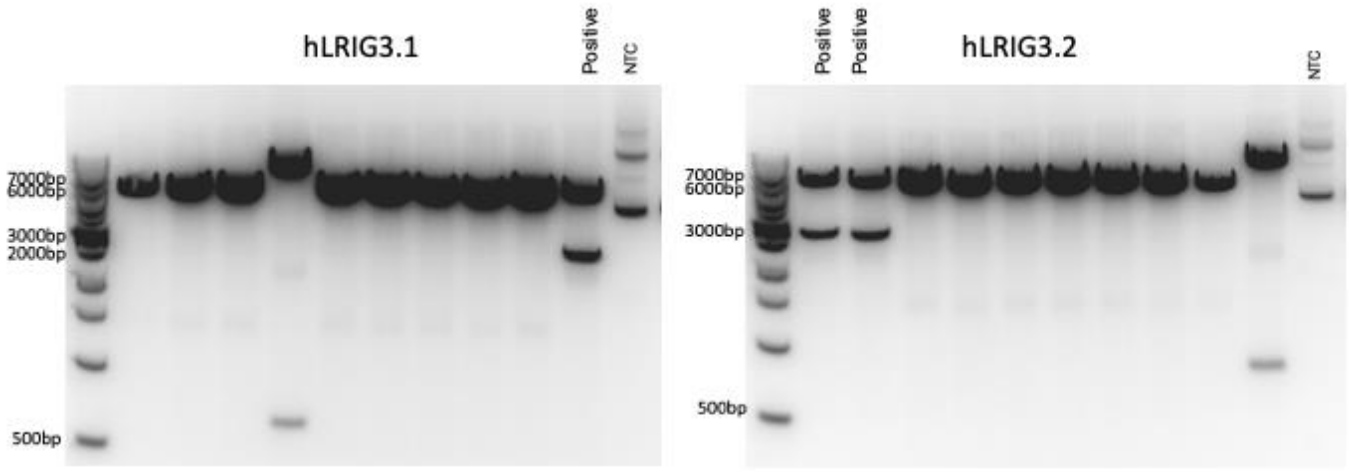


Figure 7: Correct insertion of cDNA into pIRES2-AcGFP1, for hLRIG3.1: in one clone the correct insertion was verified (lane 11); for hLRIG3.2: in two clones the correct insertion was verified (lane 2 and 3).

5.1.2 Successful transfection of hLRIG3.1- and hLRIG3.2-pIRES2-AcGFP1 vectors into cell lines

The fluorescence microscopy confirmed that we successfully transfected the *hLRIG3.1*- and *hLRIG3.2*-pIRES2-AcGFP1 vectors were successfully transfected into skin cell lines A375, A431, and HaCaT (Figure 8). No GFP-expressing cells can be seen in the negative control (NC) cell's, which were transfected with empty vector (Figure 8). Important to mention is the absence of *hDIRAS1* overexpressing cells. We were not able to establish a suitable overexpressing vector for *hDIRAS1*. Further research and trails are needed for generation of the *hDRIAS1* overexpressing cell lines.

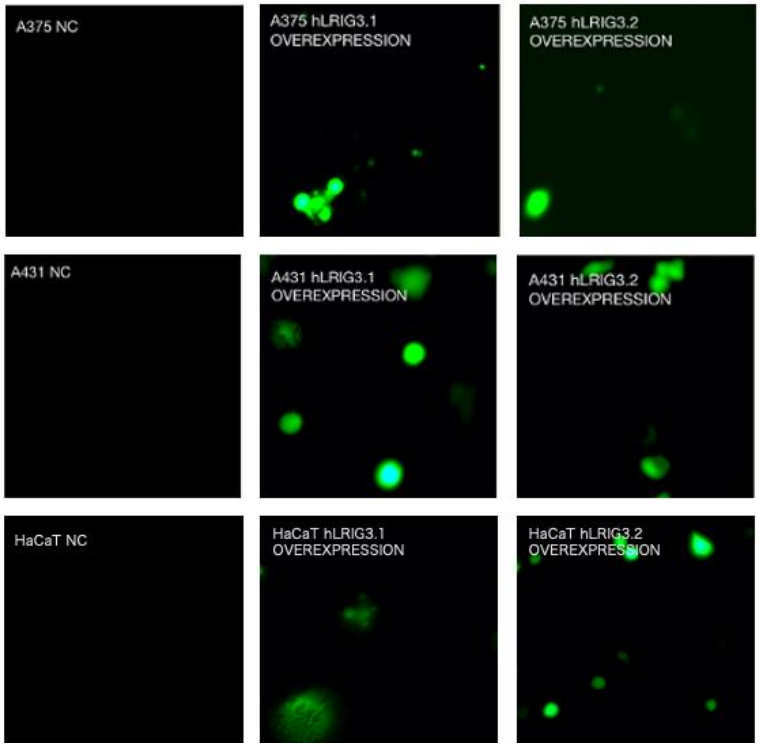


Figure 8: Immunofluorescence pictures of GFP-hLRIG3.1- and hLRIG3.2-overexpressing cell lines and negative control, fluorescence microscopy.

5.1.3 Overexpression of hLRIG3 in cell lines

To provide evidence that the above-mentioned transfected cell lines are overexpressing *hLRIG3*, we carried out a western blot assay. In addition to the detection of the *hLRIG3* protein, beta-tubulin, a reference protein, was used as an internal control. Beta-tubulin has a molecular weight of 55 kDa and the molecular weight of hLRIG3 is approximately 140 kDa. We confirmed the overexpression of *hLRIG3* in all cell lines (A375, A431 and HaCaT). We observed a second band in some cell lines such as HaCaT-hLRIG3.2, A375-*hLRIG3.1* and -*hLRIG3.2* cells (Figure 9A). We assumed that this band might be a soluble *hLRIG3* at around 110 kDa. (Cheng et al. 2019)The overexpression of hLRIG3.1 and *hLRIG3.2* were verified in the cell lines A375 and HaCaT (Figure 9A). While in A431 cell line, we only identified overexpression of *hLRIG3.2* and could not decipher a higher expression level in *hLRIG3.1* (Figure 9B).

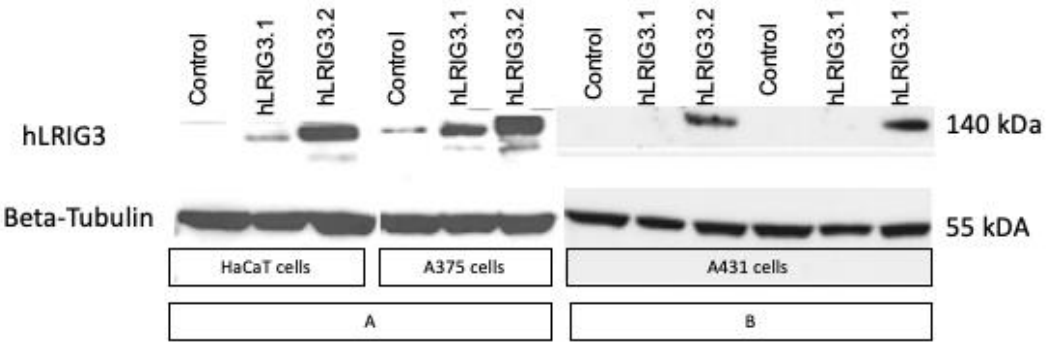


Figure 9.: Western blot results, hLRIG3.1-and hLRIG3.2-overexpressing cells, panel A: HaCaT and A375, panel B: A431 cells.

5.2 Generation of h*LRIG*3- and h*DIRAS*1-KO cell-lines

5.2.1 Successful transfection of px458-h*LRIG3*gRNA and px458-h*DIRAS*1gRNA plasmids into cell lines

The florescence microscopy corroborated that we accomplished to transfect the px458-*hLRIG3*gRNA and px458-h*DIRAS*1gRNA plasmids into the skin cell lines A375, A431 and HaCaT (Figure 10). Cells transfected with empty vector were used as negative control cells. In these cells we could not detect any green fluorescence emission.

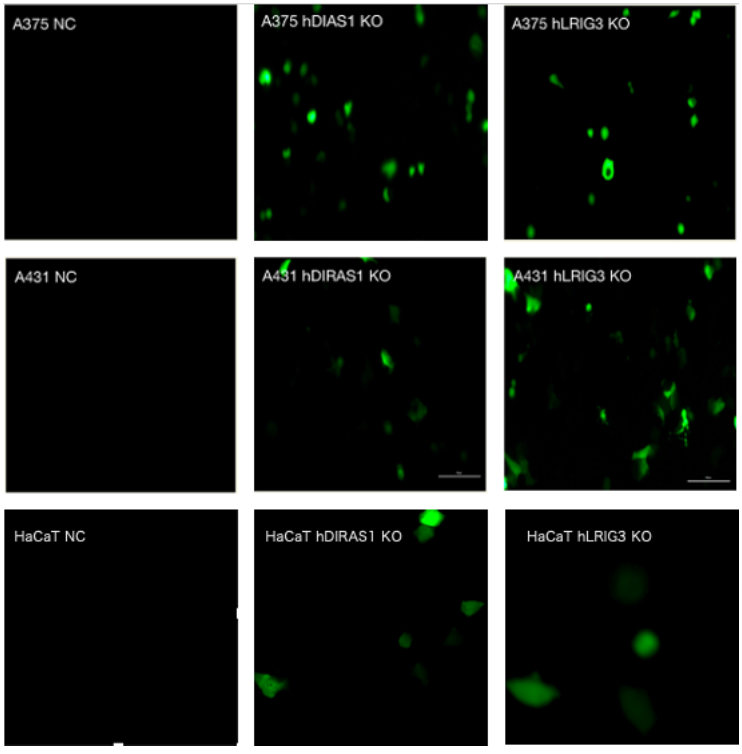


Figure 10: Immunofluorescence pictures of GFP-hLRIG3- and hDIRAS1-knockout cell lines and negative control.

5.2.2 Genotyping of single cell clones

It is important to note, that the sufficient cultivation of HaCaT cell line after the transfection was not successful to a large extent. We were only able to generate and subsequently take samples from 11 single cell colonies. We did proceed to isolate DNA from the samples and genotype them. Unfortunately, all samples were tested negative. Due to that reason, in the following chapter only the results from cell line A375 and A431 will be mentioned.

5.2.2.1 Genotyping of h*LRIG*3 single cell clones

We collected 64 single cell clones from px458-*hLRIG3*gRNA-transfected A375 cell lines. Individual clones were then screened for hLRIG3 knockout by PCR. PCR and succeeding gel-electrophoresis showed that none of the clones possessed the desired knockout mutation, as the bands could be seen around the 400 bp mark (positive result bands should be at 823 bp meaning that they should not be cut by *Xcm*I) (Figure 11).

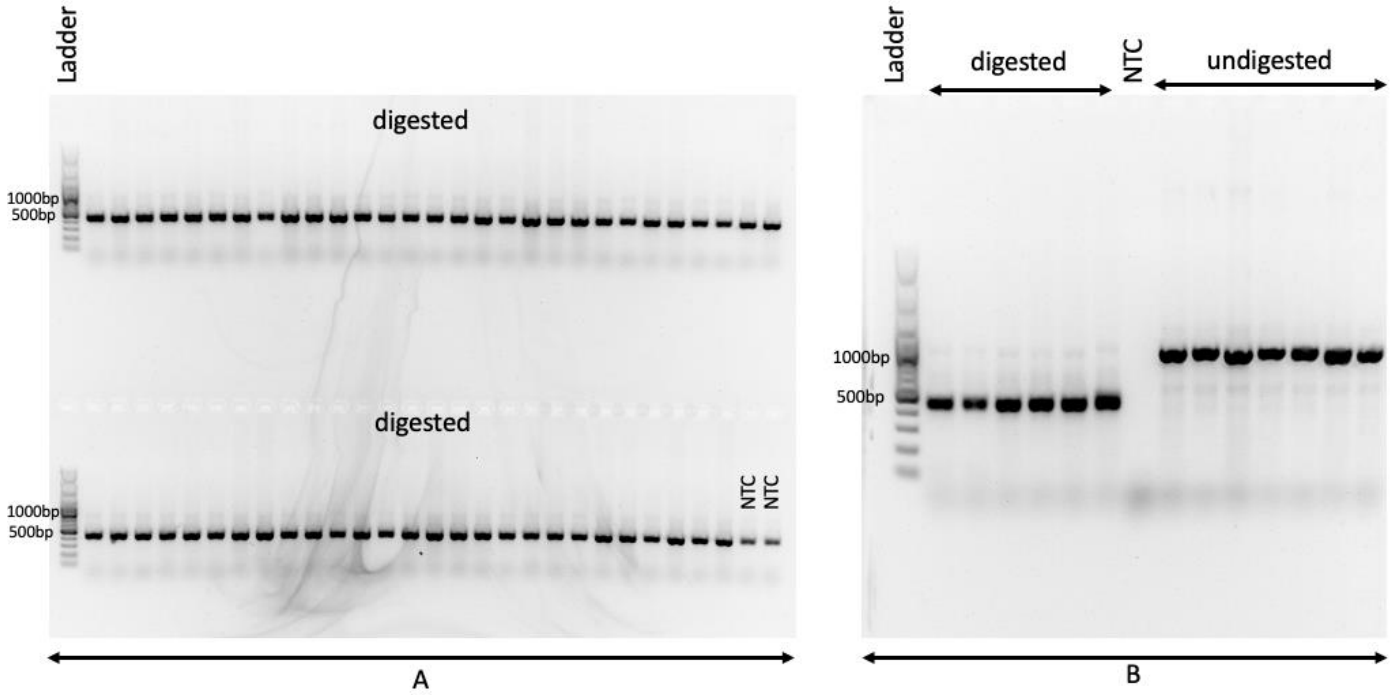


Figure 11: Gel-electrophoresis of A375 hLRIG3 knock-out PCR results with 100bp ladder. Panel A: 58 digested negative clones (around 400bp) with two NTCs, panel B: PCR on remaining single clones: On the left, the first 6 samples are A375 hLRIG3 digested clones, all of which lacked the intended knockout mutation. After the six lanes one non-template control (NTC) can be seen. On the right. 7 hLRIG3 undigested clones were added as an additional control.

We were able to culture 27 single cell clones from px458-*hLRIG3*gRNA-transfected A431 cell lines for genotyping. As seen in figure 12 no *hLRIG3*-knockout mutations were confirmed in A431 cell lines.

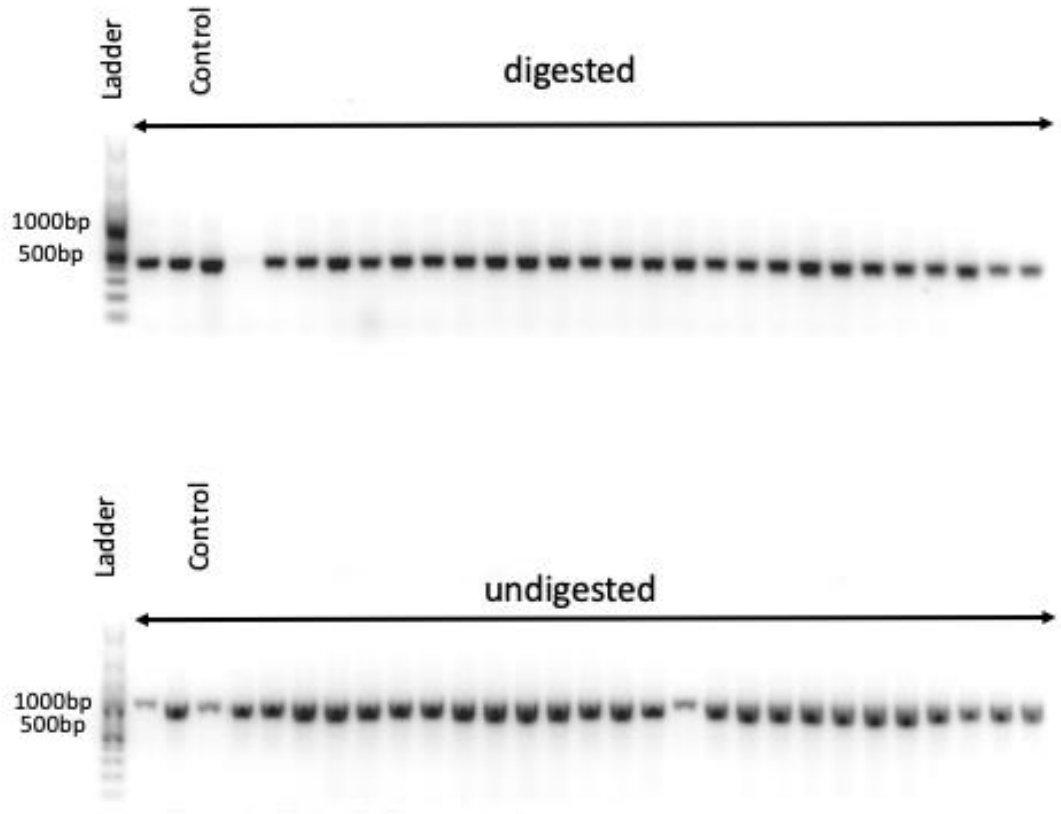


Figure 12: Gel-electrophoresis of A431 hLRIG3 knock-out PCR results with 100bp ladder. PCR and gel-electrophoresis was performed in duplicates. Once the samples were loaded onto the gel in their undigested form (lower row) and once the digestion had been performed (upper row).

5.2.2.2 Genotyping of h*DIRAS*1 single cell clones

We successfully cultured 38 single cell clones from px458-*hDIRAS1*gRNA-transfected A375 cell lines for genotyping. To ensure accuracy and reliability, we repeated the PCR several times. Based on positive results from the previous round of genotyping, we suspected that three clones (D1, D4, and F8 from plate 1) might carry the desired knockout mutation. However, none of the clones from plate 2 showed positive results. For the final run (as shown in figure 13), we performed PCR and gel electrophoresis in duplicate. We loaded the samples onto the gel in their undigested form (Rows: 2,4,6) and after digestion (Rows: 3,5,7), in addition to a no template control (NTC) (Row 8). Some primer dimers were detectable in the NTC. The results of the final round of PCR and gel electrophoresis confirmed the knockout mutation, as the bands of the digested clones were visible at around the 300 bp mark. As previously mentioned, the positive mutants should have bands at 280 bp and 290 bp.

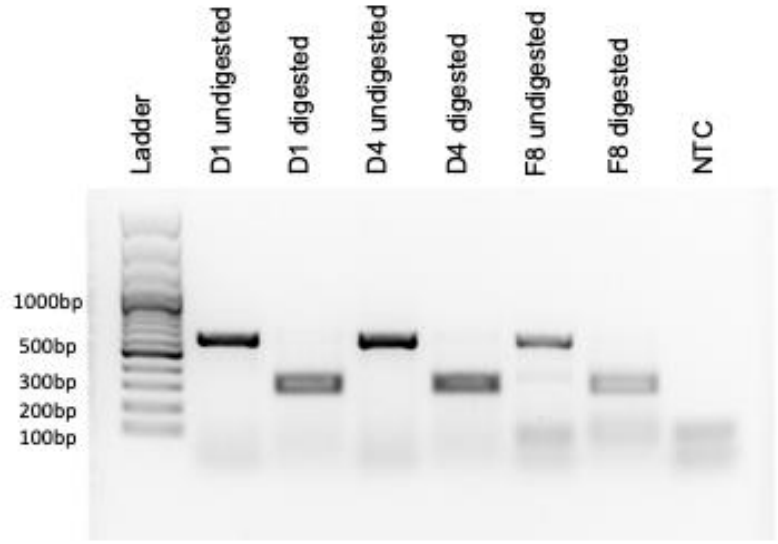


Figure 13: Confirmatory PCR for positive A375 hDIRAS1 knockout clones, digested (around 300 bp) and undigested (around 600 bp).

We collected 36 single cell clones from px458-*hDIRAS*1gRNA-transfected A431 cell lines for genotyping. Multiple rounds of PCR and gel-electrophoresis were performed. 5 different clones showed promising results and led us to believe that they might carry the favored knockout mutation. The promising clones were as follows: From plate 1: B6; From plate 2: B4, C5, D1, and G6. The bands from row 2-6 all lie at around the 300 bp band-mark and therefore carry the desired mutation.

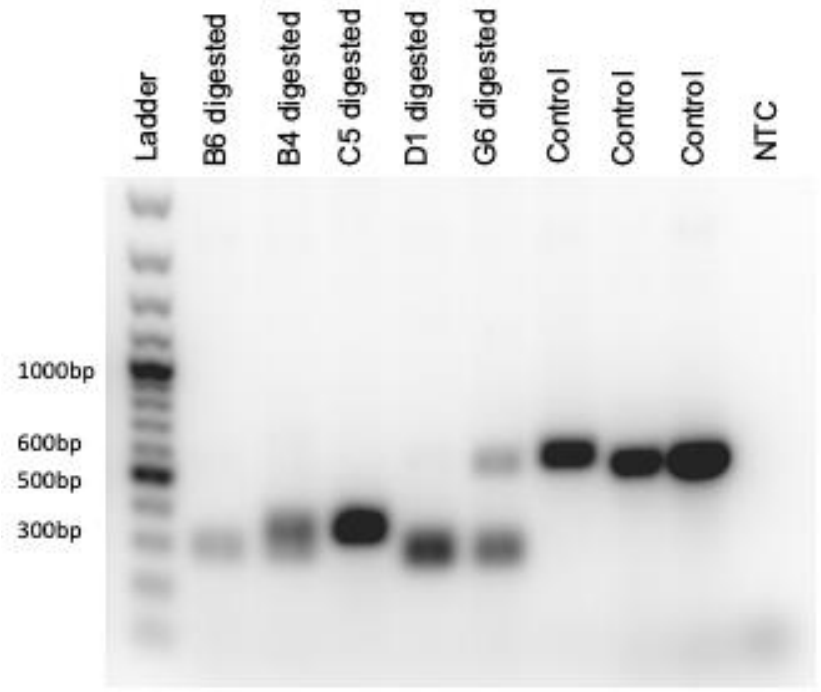


Figure 14: Confirmatory PCR for positive A431 hDIRAS1 knockout clones, digested and undigested sequences. In the final round of PCR, the five putative positive samples (rows 2-6) were loaded onto the gel, along with three undigested samples (rows 7-9) and a negative control (row 10).

5.3 Sequencing of the positive h*DIRAS*1-KO single cell clones

The three positive clones from *hDIRAS1*-KO A375 cell line were sent for sanger sequencing using the forward primer for genotyping (see Material section). The sequencing results were then analyzed by ApE, a plasmid editor software. We were able to confirm an alignment between our designed oligo donor sequence and the sequencing results. We detected the oligo donor with *EcoR*I cutting site insertion into the single cell clones' sequences (Figure 15). The knockout vector was found (as intended) to affect an insertion of a singular cytosine nucleotide in the second exon. Additionally, after we received the sanger sequencing outcomes, protein translation prediction was conducted via the use of the ApE software. Beforehand, the start- and stop-codons were determined using Ensembl. This procedure validated our hypothesis that the mutated gene translates to a non-sense from of the protein ordinarily found in wild-type cells resulting in nonsense-mediated decay. As such, we concluded that clones P.1-D.1, P.1-D.4, and P.1-F.8 from A375 cell lines are carrying the h*DIRAS1* gene mutation.

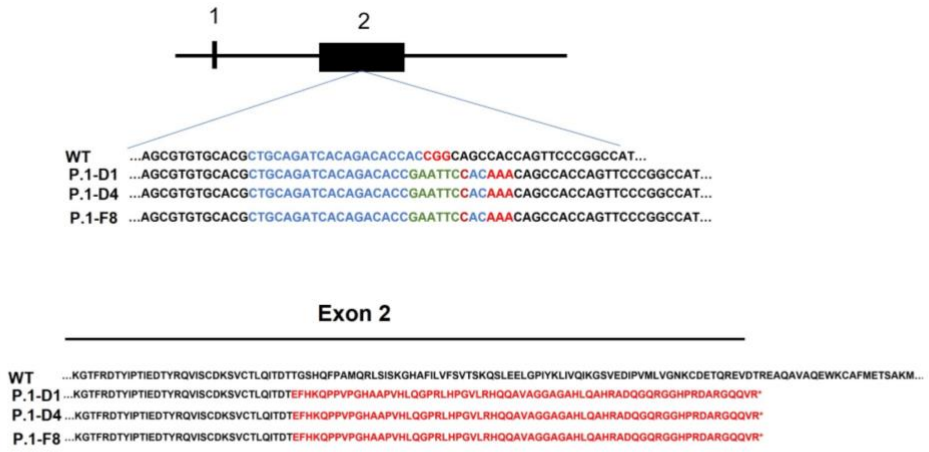
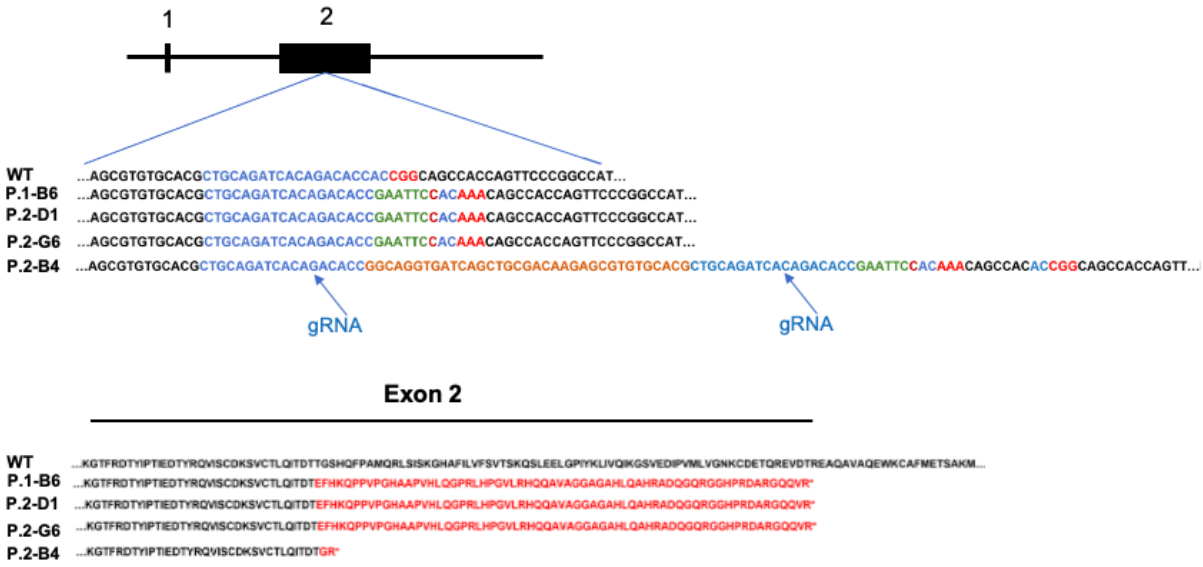


Figure 15: Schematic represetation of sanger sequencing results and predicted translated proteins of A375 hDIRAS1 knock-out cells.

We also sent the five clones for sanger sequencing with the forward h*DIRAS1* primer for genotyping. Analysis of the sequencing results was done as explained above. As seen in the schematic representation of the sanger sequencing results (Figure 16) the knockout construct was integrated successfully in the 4 tested single cell colonies. However, an additional insertion occurred in the P.2-B.6 clone. We suspect that the additional random insertion happened during the double strand break repair. However, the underlying reason for the gRNA integration during the cellular repair mechanism remains is unclear. Nevertheless, this insertion also leads to the knockout of the gene and therefore to a non-sense translated protein, and further nonsense-mediated decay. In general, we found 4 positive single cell clones of *hDIRAS1*-KO A431 cell lines.



gRNA, CGG: PAM, GAATTC: ECORI restriction site, C: arbitrarily inserted nucleotide, AAA: arbitrary sequence that replaced PAM, Random insertion which has been introduced most likely due to the double-strand break (DSB) repair.

Figure 16.: Schematic representation of sanger sequencing results and predicted translated proteins of A431 hDIRAS1 knockout cells.

6. Discussion

6.1 Establishment of h*DIRAS*1 overexpressing vector

As was mentioned in the previous chapters, we encountered difficulties in establishing a suitable overexpressing vector for h*DIRAS*1, due to obstacles we faced in the amplification of *hDIRAS1* complimentary DNA (cDNA). In the following section the most common obstacles as well as possible solutions for *cDNA* cloning will be revisited and discussed.

6.1.1 Cloning of cDNA

cDNA is a kind of DNA, which is complimentary to a specific RNA sequence. cDNA is synthesized via reverse transcription. During this process, a single-stranded DNA molecule is synthesized from an RNA template using the enzyme reverse transcriptase. Thereupon a double stranded cDNA can be generated via DNA-polymerase and in the presence of dNTPs (deoxyribonucleotide triphosphate). The resulting cDNA can be used in a diverse range of molecular biology applications, such as molecular cloning.(Pelley 2012) During the generation of cDNA, a multitude of obstacles can arise. First of all, it is important to synthesize the cDNA from a high-quality RNA to guarantee optimal conditions for the reverse transcriptase to work. Furthermore, the amplification process can be challenging as well. Factors such as low concentration of the template cDNA, suboptimal primer design or mutations can affect the resulting double stranded cDNA greatly in a negative way.(Harbers 2008) One option to eliminate these factors could be to purchase a plasmid containing the cDNA insert from a commercial supplier. However, this option is undoubtedly paired to an increase of the projects cost and one should still evaluate the quality of the bought cDNA, as there is no guarantee for the sequences' quality. The cDNA quality can be tested via PCR followed by sanger sequencing to ensure the best possible outcome for the following procedures.(Pelley 2012) For this project we first tried to amplify *hDIRAS1* sequence from the available cDNA, but we were not successful to do so. It might be due to the factors mentioned above. Consequently, we adopted a strategy similar to the one used for *hLRIG3* by acquiring a commercially available cDNA vector construct. We carried out a PCR using our designed primers for amplification of h*DIRAS*1 cDNA and obtained an unexpected band after performance of gel electrophoresis. Subsequently, we sent the obtained sequence for sanger sequencing for quality control. Unfortunately, the commercially acquired cDNA did not correspond with the cDNA for h*DIRAS*1, rendering it unsuitable for our project. Furthermore, due to time restriction issues we were not able to undergo a second trial for the amplification of h*DIRAS*1 cDNA. Therefore, our efforts were limited to the overexpression of *hLRIG3.1* and *hLRIG3.2*.
Overall, the process of cloning cDNA can be very time intensive and error prone. Due to a strict time plan we were not able to identify and eliminate possible sources of error in our cloning process. However, with enough time and recourses the cloning and subsequent amplification of cDNA should be possible.

6.2. Overexpression of h*LRIG*3.1 in cell line A431

As it was mentioned in the section 5.1.2 of chapter "Results", the overexpression of *hLRIG3.1* in the cell line A431 proved to be unsuccessful. In this section possible reasons for the inability of establishing *hLRIG3.1* A431 overexpressing cells will be discussed. Firstly, factors such as a prolonged period between the transfection and the harvest of the cells could have led to the lack of the overexpression. This is due to the fact, that the vector construct cannot integrate into the cells' genome. Resulting in a non-permanent transient transfection. In transient transfection, plasmid DNA is expressed in the cytoplasm for a short period of time and decays over few days. The reason behind the instability of the construct lies in the inability for the cloning vector to integrate into the chromosome of the host cell. Therefore, the vector carrying the genetic information will not be replicated inside the cell. The more cells divide, the less emission of fluorescence would be. Additionally, the vector itself will be degenerated inside the cells cytoplasm, therefore making the overexpression only last for a few days.(Moo-Young 2019) Therefore, the transfected cells had to be harvested shortly after transfection was carried out.

Contradicting this theory would be the fact, that all the cell lines had been transfected and harvested at the same time. Nonetheless, it cannot be ruled out that the A431 cells in the specific well used for the overexpression of *hLRIG3.1* had an increased metabolic rate, allowing them to degenerate the overexpressing vector in a more time effective manner. It might be possible that the cells that were subjected to the hLRIG3.1 overexpressing vector had an increased amount of substrate or FCS provided in the cell culture medium before or after the transfection. This could potentially lead to an increased cellular metabolic rate. Other factors that might inflate the cells' metabolism include higher oxygen levels or the cells density in the plating.(Zhu and Thompson 2019) However, it is unlikely that these factors had a significant effect during this project, as all the cell samples were treated with the identical cell medium and were cultured under uniform condition. Furthermore, the possibility of vector degradation to the extent that overexpression was entirely absent in the western blot within the time frame between transfection and harvesting is not sufficiently studied yet to be able to make a definitive statement. Another theory would be that the cells were still in contact with a residual amount of antibiotics that the regular cell culture medium was supplied with. As was mentioned before, one should strive to transfect the cells without the presence of any antibiotic, as it can influence the transfection rate in a negative manner.(Gibco 2018) Though, the cells were washed twice with DPBS to ensure the absence of the antibiotic, a mistake could have occurred, leading to the transfection being carried out in the presence of the antibiotic. Nonetheless, the extent to which this potential error could have contributed to the overall failure of the overexpression remains uncertain. Additionally, another possible hypothesis would be that an error occurred during the transfection process, possibly during the preparation of the transfection dilutants containing P3000 and Lipofectamine. These substances are designed to induce cell membrane destabilization and promote successful transfection.(Thermo Fisher Scientific 2016) Even though this process was conducted with great care, under sterile conditions and under supervision, a minor pipetting error could have occurred leading to an unsuccessful transfection and subsequent overexpression. Another possibility to consider is the large size of the *hLRIG3.1*-pIRES-AcGFP vector (~14 kb) which makes its transfection

notoriously difficult and causes high cell death.(Søndergaard et al. 2020) Nevertheless, is worth noting that the cells were examined under a fluorescence microscope, and they emitted green light, indicating that the vector was most likely successfully integrated into the cells. At this point in time one can only speculate about the reasons for the failed overexpression of *hLRIG3.1* in A431 cell lines. In general, to obtain the highest transfection efficiency of A431 with hLRIG3.1-pIRES-AcGFP vector, optimization of the transfection conditions such as varying transfection reagent or ratio of plasmid DNA are required.

6.3 Alterations for cultivating HaCaT knockout cell-lines

As discussed in the preceding chapters, our efforts to generate HaCaT single cell clones proved to be challenging. We faced difficulties in obtaining a sufficient number of cells with suitable confluency within the 12- or 24-well plates, which hindered our ability to genotype them adequately. As a result, we were only able to obtain a small number of samples from single cell colonies that could be genotyped, all of which tested negative. In this chapter, we will investigate the potential factors that contributed to our difficulties in cultivating HaCaT cells and propose potential solutions for improving the cultivation of these cells.

6.3.1 Conditioned medium

Firstly, the importance of a suitable medium in cell culture should be discussed. The purpose of a cell culture medium is to provide a nutrient-rich environment for the growth and proliferation of cells in vitro. The medium typically contains a variety of essential nutrients, such as amino acids, vitamins, and minerals, as well as growth factors and other supplements to support cell growth and maintenance. The nutrient composition of cell culture medium plays a crucial role in the maintenance of single cell cultures. In vivo, cells rarely grow in isolation, and the process of single cell sorting and cultivation can compromise the viability of cultured cells. However, the use of conditioned medium can enhance the survival and growth rates of single cell colonies by mimicking the in vivo cellular microenvironment. Conditioned medium is a type of cell culture medium that has been exposed to cells or cellular components. Within secreted factors, such as growth factors, cytokines, and extracellular matrix proteins, are accumulated. These factors can modulate the behavior of cells cultured in conditioned media, altering their growth, or functional properties.(Dowling and Clynes 2011) We therefore propose the usage of conditioned medium in future experiments when single cell colonies should be cultivated.

6.3.2 Alternatives for FACS

FACS machines are capable of analyzing thousands of cells per second and can sort cells based on a wide range of parameters. However, the physical stress of the sorting process can affect cell viability, which can be a significant drawback in some experiments. Other methods for dividing cells based on their genotype and subsequently cultivating monoclonal cell lines, might be more suitable for sensitive cell types (like HaCaT). One alternative method might be limiting dilution cloning, a process that involves diluting a sample to isolate individual cells in each well, allowing for their quantification and analysis. (Ye et al. 2021) Despite the ability of the limiting dilution method to generate clonal populations,

its extensive time and labor requirements make it less appealing than alternative techniques such as fluorescence-activated cell sorting (FACS) for the generation of monoclonal single-cell colonies. It is important to note that with the limiting dilution method, the generation of a single-cell colony in a well is of statistical nature and, moreover, the probability of success is typically quite low. Despite its limitations in terms of precision and verification of single-cell presence compared to more advanced methodologies like FACS, the limiting dilution approach continues to represent a simple, gentle, and cost-effective option for obtaining single cells with moderate throughput.(Gross et al. 2015)An alternative to FACS for selecting transfected cells is neomycin selection, which involves introducing a recombinant plasmid containing the gene of interest (GOI) and a neomycin resistance gene into the target cells. Following transfection, the cells are exposed to a selective agent containing the antibiotic G418, which kills cells that have not taken up the resistance gene. The surviving cells are considered to be stably transfected.(Southern and Berg 1982) It is important to that there are various selection methods available for genetically modified cell lines. When choosing an appropriate approach, factors such as the cell type should be taken into consideration. Different cell types may have different requirements or preferences for certain selection methods, and this should be taken into account to ensure the most effective and reliable results.

6.4 Generation of knock out h*LRIG3* cell lines

As previously stated, we were unable to generate any hLRIG3 knockout cell clones in both A375 and A431 cells. This section will outline and propose some potential explanations for the outcome of the experiment. One possible factor contributing to the lack of successful knockout clones is the statistical likelihood of successful transfection and subsequent cell sorting via FACS, as well as additional cell culture treatments. It is plausible that we did generate some knockout clones, but they may not have survived until we could collect samples. In that sense, it might be possible that the knockout mutation may have interfered with the growth and survival of the cells, but currently there is no published research or literature to support this hypothesis in the case of *hLRIG3*. It is also noteworthy to mention a study conducted in 2021 that found a possible link between FACS cell sorting and an alteration of the MAPK p38 pathway in human primary T cells, which resulted in minor physiological changes in the cell line.(Andrä et al. 2020) Given the findings of this study, it is possible to hypothesize that FACS sorting may have had a notable impact on the skin cell lines used in our experiment, which could have influenced the metabolism of the skin cells or the knockout construct for *hLRIG3* itself. Further research is needed to investigate the potential effects of FACS sorting on skin cell lines and their subsequent ability to integrate knockout constructs.
However, the most plausible explanation for the low success rate would be an error in the construct design. Possible sources of error include but are not limited to: Incorrect selection of targeted gene sequence or the failure to properly screen for off-target effects.(Donnelly 2021) In order to make a definitive statement about the failure for the generation of the hLRIG3 knockout clones in A431 and A375, the experiment should be repeated.

7. Conclusion

Unfortunately, we were unable to establish an overexpressing vector for *DIRAS1* due to the obstacle of amplifying *hDIRAS1* cDNA. Additionally, we faced challenges in effectively deleting the *hLRIG3* gene in any of the cell lines. Obtaining single cell colonies of knockout (KO) HaCaT cells proved to be difficult due to their inherent difficulty in transfection. Despite facing challenges, we have successfully generated A375, A431, and HaCaT cells that overexpress *LRIG3.2*, as well as A375 and HaCaT cells that overexpress *hLRIG3.2.* Furthermore, we accomplished the production of *DIRAS1*-KO-A375 and *DIRAS1*-KO-A431.

In future projects, the aim is to analyze fundamental cancer hallmarks such as proliferation, migration, and invasion in these genetically manipulated cell lines to shed light on the role of *DIRAS1* and *LRIG3* in cancer development and progression. Despite the setbacks, we believe that our findings will contribute to a better understanding for the establishment of genetically altered skin cell lines within the genes *hLRIG3* and *hDIRAS1*.

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9. Figures

10. Tables