Inducibilna i reverzibilna razgradnja proteinskog kompleksa za stvaranje mikroRNA u diferencirajućim ljudskim matičnim stanicama

Žužić, Marta

Master's thesis / Diplomski rad

2016

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Science / Sveučilište u Zagrebu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:217:257861

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Download date / Datum preuzimanja: **2024-03-28**



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University of Zagreb
Faculty of Science
Department of Biology

Marta Žužić

Inducible and reversible disruption of the microRNA processing machinery in differentiating human stem cells

Inducibilna i reverzibilna razgradnja proteinskog kompleksa za stvaranje mikroRNA u diferencirajućim ljudskim matičnim stanicama

Graduation Thesis

Zagreb, 2016.

This graduation thesis was conducted at the Center for Regenerative Therapies Dresden, Cluster of Excellence, TU Dresden, under supervision of Dr. Phil. Volker Busskamp. Cosupervision was done by Dr. Petra Korać, Asst. Prof. The thesis was handed for the evaluation to the Department of Biology at the Faculty of Science, University of Zagreb in order to obtain the title of master of molecular biology.

I would like to express my gratitude to my supervisor Dr. Phil. Volker Busskamp, for giving me the opportunity to conduct my thesis in his group at the Center for Regenerative Therapies Dresden and for the continuous support of my study, predoc Lisa K. Kutsche for introducing me to the topic and her advices which guided me through this study, and the entire Busskamp group for advising me and helping me on the way.

I would like to thank my cosupervisor Dr. Petra Korać, Asst. Prof., for useful comments and remarks.

Last but not the least, I would like to thank my family and friends for their encouragement and support throughout the entire process.

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek Diplomski rad

INDUCIBILNA I REVERZIBILNA RAZGRADNJA PROTEINSKOG KOMPLEKSA ZA STVARANJE mikroRNA U DIFERENCIRAJUĆIM LJUDSKIM MATIČNIM STANICAMA

Marta Žužić
Rooseveltov trg 6, 10000 Zagreb, Hrvatska

mikroRNA (miRNA) su razred malih, nekodirajućih RNA koje reguliraju gensku eskpresiju na posttranskripcijskoj razini. Poznato je da reguliraju mnogobrojne biološke procese i stanične funkcije. Mnoge miRNA su i same dinamično regulirane tijekom razvoja centralnog živčanog sustava te različito raspoređene u odraslom mozgu, što ukazuje na njihovu esencijalnu ulogu u razvoju i funkciji živčanog sustava. miRNA profil se mijenja i tijekom *in vitro* diferencijacije ljudskih induciranih pluripotentnih matičnih stanica u bipolarne neurone, no njihova funkcija tijekom tog procesa i dalje nije u potpunosti jasna. Za istraživanje utjecaja miRNA tijekom neurogeneze i vremenskog aspekta njihove regulacije, koristeći tehnologiju CRISPR/Cas9, uspostavljen je degradirajući sustav baziran na auksinu za inducibilni prekid puta obrade miRNA u stanicama iNGN.

(47 stranica, 28 slika, 6 tablica, 49 literaturnih navoda, jezik izvornika: engleski)

Rad je pohranjen u Središnjoj biološkoj knjižnici

Ključne riječi: mikroRNA, degradirajući sustav baziran na auksinu, DGCR8, stanice iNGN

Voditelj: Dr. Volker Busskamp

Ocjenitelj: Dr. sc. Petra Korać, doc., Dr. sc. Željka Vidaković-Cifrek, izv. prof., Dr. sc. Tomislav Ivanković, doc.

Rad prihvaćen: 1. 12. 2016.

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Science
Department of Biology Graduation Thesis

INDUCIBLE AND REVERSIBLE DISRUPTION OF THE microRNA PROCESSING MACHINERY IN DIFFERENTIATING HUMAN STEM CELLS

Marta Žužić
Rooseveltov trg, 10000 Zagreb, Croatia

microRNAs (miRNAs) are a class of small, noncoding RNAs that regulate gene expression at the post-transcriptional level. They have been demonstrated to regulate a lot of biological pathways and cellular functions. Many miRNAs are dynamically regulated during central nervous system development and are spatially expressed in adult brain indicating their essential roles in neural development and function. Furthermore, miRNA profile changes during *in vitro* differentiation of human induced pluripotent stem cells to bipolar neurons. However, their function during the process is not yet fully known. To study the influence of miRNAs during neurogenesis and the temporal aspect of this regulation, the auxin-based degron system for inducible disruption of the miRNA processing machinery was established in iNGN cells, using CRISPR/Cas9 technology.

(47 pages, 28 figures, 6 tables, 49 references, original in: English)

Thesis deposited in the Central Biological Library

Key words: microRNA, auxin-based degron system, DGCR8, iNGN cells

Supervisor: Dr. Volker Busskamp

Reviewers: Dr. Petra Korać, Asst. Prof., Dr. Željka Vidaković-Cifrek, Assoc. Prof., Dr. Tomislav Ivanković, Asst. Prof.

Thesis accepted: 1. 12. 2016.

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1. INTRODUCTION

1.1 Neurogenesis

Neurons are electrically excitable cells that process and transmit information through electrical and chemical signals. Neurons are, together with glial cells, the core components of the brain and spinal cord of the central nervous system. Both neurons and glial cells are generated from neural stem cells and progenitor cells in a process called neurogenesis that occurs during embryonic and perinatal stages as well as throughout adult life in restricted brain regions (Ming and Song, 2011). Neurogenesis is regulated by the dynamic interplay between transcription factors, epigenetic control, microRNA (miRNA) regulators, and cell-extrinsic signals from the "niche" where stem cells reside (Shi et al., 2008).

Early neurogenesis begins with segregation of the neural plate from the ectoderm of the trilaminar embryo by folding to form the neural groove, which then fuses to form the neural tube and the associated neural crest. Later neurogenesis in the central nervous system is the proliferation of ventricular neural stem cells, differentiation, migration and lamination of the developing neural system (Hill, 2016). Adult neurogenesis is spatially restricted to two "neurogenic" brain regions, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus where new dentate granule cells are generated, and the subventricular zone (SVZ) of the lateral ventricles where new neurons are generated and then migrate to the olfactory bulb to become interneurons (Gage, 2000).

Neurons are postmitotic cells, meaning that they are not capable of proliferation. Furthermore, given the fact that neurons are dying in neurodegenerative diseases, such as Alzheimer's, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), and that adult neurogenesis is restricted only to SGZ and SVZ, there is a big interest in differentiating induced pluripotent stem cells into different types of functional neurons *in vitro*, that could be used as a substitute for the degenerated ones as well as disease models to understand better the pathology.

1.2 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are a specific kind of pluripotent stem cells that can be generated from adult cells. The method to generate iPS cells from mouse fibroblasts was discovered by Shinya Yamanaka's group, when they showed that only four specific genes encoding transcription factors (Oct4, Sox2, Klf4 and c-Myc) can reprogram adult cells to a pluripotent stem cell state (Takahashi and Yamanaka, 2006.). The same was shown for human fibroblasts the year after (Takahashi et al., 2007). The generation of such iPS cells can be described in a few steps. First, fibroblasts are collected from the adult organisms. Then, four reprogramming transcription factors Oct4, Sox2, Klf4 and c-Myc are introduced into the cultured somatic cells. The cells are maintained under embryonic stem cell conditions and a small fraction becomes iPS cells after few weeks. The new generated iPS cells can then be extracted, expanded and finally differentiated into various cell types from all three germ layers, including neurons.

1.2.1 Inducible Neurogenin iPS cells

In vivo, neuronal differentiation is a complex mechanism involving many transcription factors and regulating cascades (He & Rosenfeld, 1991). Most protocols for the neuronal differentiation imitate developmental steps by applying stepwise environmental perturbations to cells and push them from one developmental step to the next. However, these differentiation protocols with multiple steps are never optimal and require months to be completed. Furthermore, they often show a high variability and low yields of generated neurons. To simplify the neuronal differentiation protocols and help to elucidate the gene regulatory mechanisms of stem cell-derived neurons, a special rapid and robust differentiation protocol was developed that leads to highly homogeneous neurons (Busskamp et al., 2014a). A bicistronic doxycycline-inducible Neurogenin1/2 expression cassette was delivered into human iPS cells by a lentiviral system and led to a stable and small molecule-inducible Neurogenin iPS cell line (iNGN cell line). The induction relies on a tet-on system: when doxycycline is added to the medium, Neurogenin-1 and Neurogenin-2 are expressed. This results in a homogeneous population of functional bipolar neurons within four days (Figure 1).

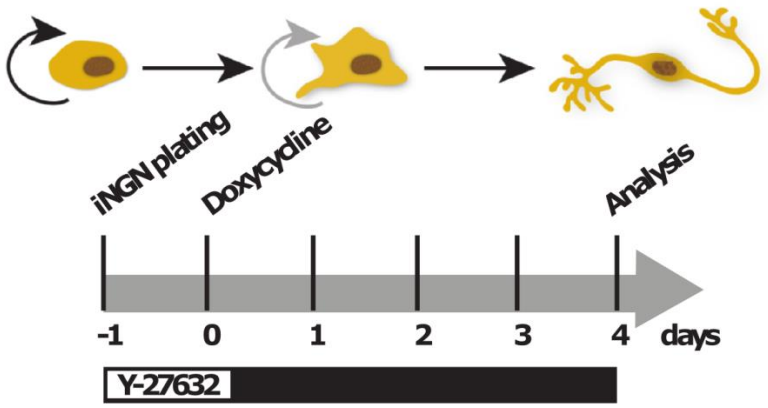


Figure 1. Doxycycline activates transcription of Neurogenin1/2 transcription factors which leads to the iNGN differentiation to bipolar neurons within 4 days. iNGN cells lose their self-renewing capacity 2 days after adding Doxycycline and become post-mitotic (Busskamp et al., 2014a).

Furthermore, it is known that networks of key transcription factors and miRNAs are involved in the neuronal differentiation of iNGN cells. During the differentiation of iNGN cells, their miRNA profile changes. On day 0, the uninduced iNGN samples have miRNA signatures of stem cells with the miR-302/367 cluster dominating their profile. In contrast, on day 4 a neuronal signature with miR-124, miR-96 and miR-9 being the most abundant ones is present (Figure 2). Those changes suggest that miRNAs are important during neurogenesis. However, their functions are not yet fully understood.

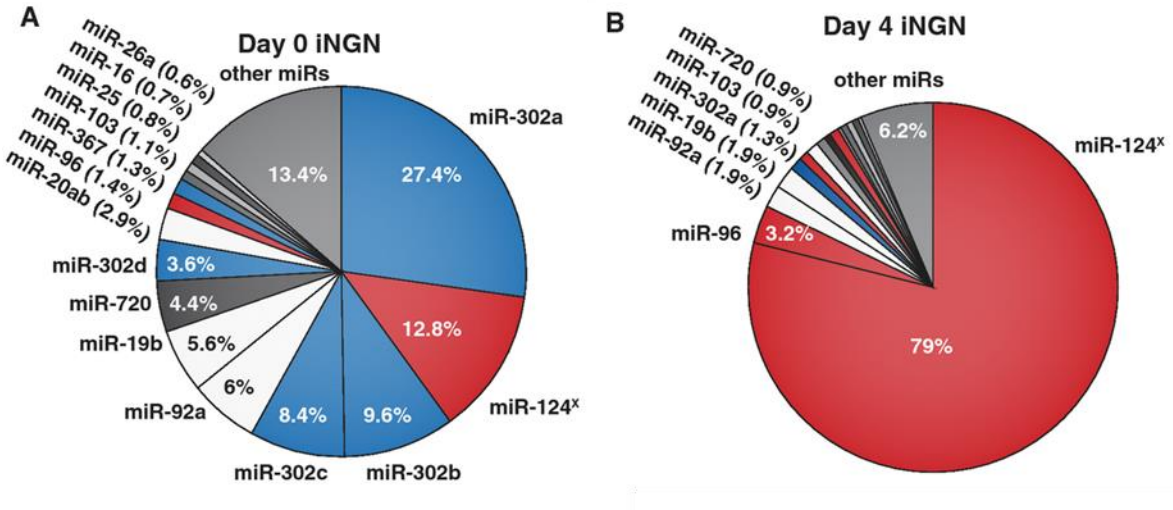


Figure 2. Relative abundance of the miRNA in A) uninduced and B) 4-days differentiated iNGN cells. The miRNAs associated with stem cell fate are indicated in blue and the neuronal miRNAs in red (Busskamp et al., 2014a).

1.3 microRNA

microRNAs (miRNAs) are short (20-23 nucleotides), non-coding, single-stranded RNA molecules that regulate gene expression at the post-transcriptional level (Bartel et al., 2004). miRNA biogenesis starts in the nucleus where the primary transcripts (pri-miRNAs) get transcribed from an intergenic or from an intronic region of the hosting gene by RNA polymerase II or III. The pri-miRNA is endonucleolytically cleaved by the nuclear micro-processor complex formed by the RNase III enzyme Drosha and the Di George critical region 8 (DGCR8) protein. The two RNase domains of Drosha cleave the pri-miRNA, whereas DGCR8 directly and stably interacts with the pri-miRNA and determines its precise cleavage site. The resulting precursor miRNA (pre-miRNA) is exported to the cytoplasm by the Exporting-5-RAN-GTP and cleaved to the mature miRNA duplex by a complex consisting of the ribonuclease Dicer and the transactivator RNA-binding protein (TRPB). The functional miRNA strand is embedded into Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC). Incorporated in RISC, the miRNA binds to the 3' untranslated region (UTR) of target mRNA in a sequence specific manner, resulting in repression of mRNA translation or destabilization of mRNA transcripts through cleavage or deadenylation (Figure 3) (Winter et al., 2009).

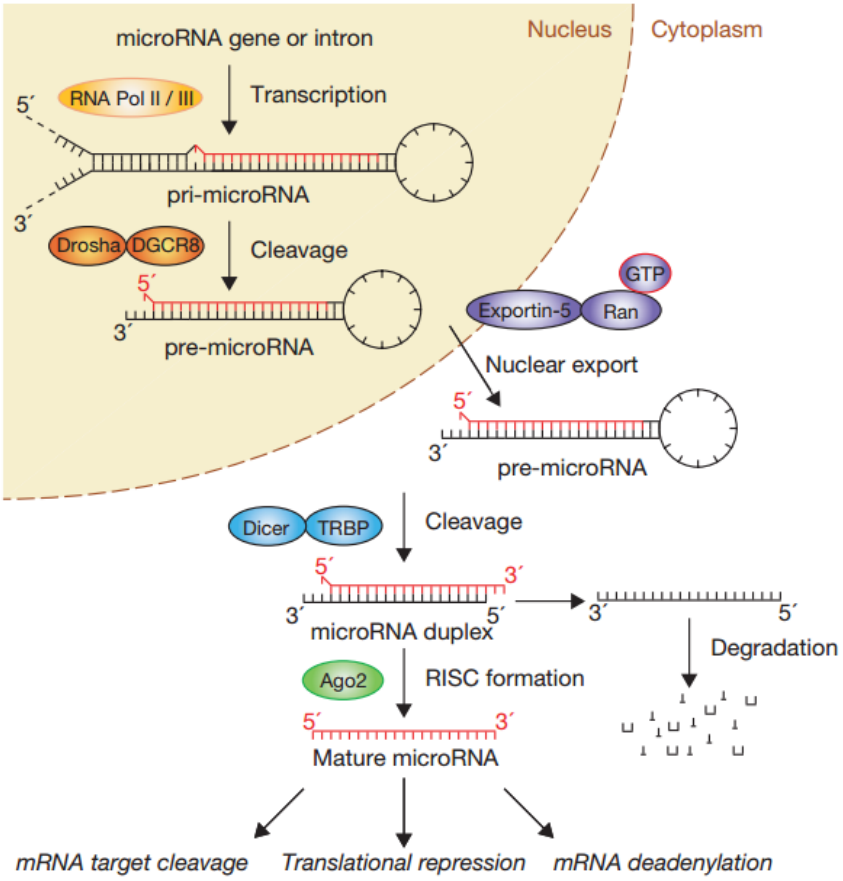


Figure 3. miRNA processing pathway. pri-miRNA is transcribed by RNA polymerase II or III and cleaved by the Drosha-DGCR8 complex in the nucleus. The resulting pre-miRNA is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, the RNase Dicer in complex with TRBP cleaves the pre-miRNA to its mature length. The mature miRNA is loaded together with AGO proteins into the RISC, where it guides the RISC to silence a target mRNAs through mRNA cleavage, translational repression or deadenylation (Winter et al., 2009).

Interactions between miRNAs and mRNAs require sequence homology at the 5' end of the miRNA. However, variance in the degree of complementation in the remaining sequence allows a single miRNA to target a wide range of mRNAs (50 to >100 mRNAs), often regulating multiple genes within a common pathway. One of the pathways for which it is known that miRNAs are required is the neuronal development (Ivey et al., 2015). As the neurons mature, miRNAs regulate some of the key processes including developmental timing, neuronal cell proliferation, neuronal differentiation, cell fate determination, synaptogenesis and gliogenesis (Figure 4) (Nampoothiri et al., 2016). However, among the hundreds of miRNA identified thus far, only a limited number have been assigned target mRNAs and specific functions.

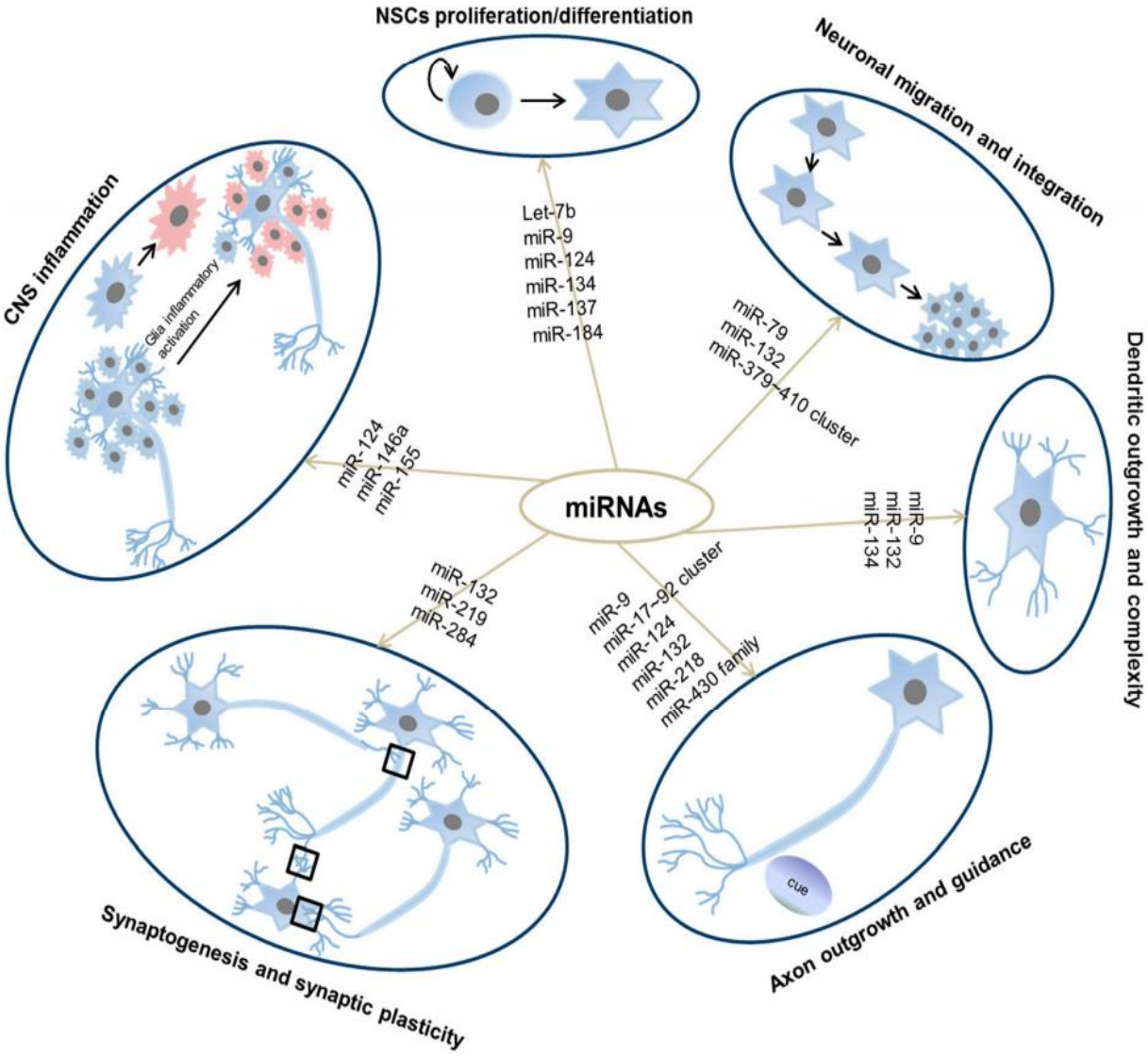


Figure 4. miRNAs roles during neuronal development. The same miRNAs are often regulating more than one step during neuronal development. Red indicates activated microglia and black squares indicate synapses (Cao et al., 2016).

1.4 Auxin-based degron system

In human cells, protein expression can be controlled in many different ways. When expression is controlled at the DNA (Sauer et al., 1988) or mRNA levels (Elbashir et al., 2001), protein depletion is indirect, which is a major limitation for stable proteins as DGCR8, as they would persist in cells for long time after disruption of its coding sequence or RNAi (RNA interference) mediated mRNA degradation (Busskamp et al., 2014b). Furthermore, gene deletion is not reversible and suppression of mRNA accumulation can be insufficient. Another way of controlling protein expression is at the protein level itself by using cell-permeable small molecules that induce posttranslational degradation (Nishimura et al., 2009).

Plants have evolved a system in which the plant hormone auxin directly induces degradation of the AUX/IAA family of transcription repressors by a specific form of the SCF E3-ubiquitin ligase (Grey et al. 2001). Auxins are a class of plant hormones that control gene expression during plant growth and development (Teale et al., 2006), with indole-3-acetic acid (IAA) being the most abundant one. It binds to the F-box transport inhibitor response 1 (Tir1), which is a part of the SCF (Skp, Cullin, F-box) ubiquitin ligase complex, and promotes its interaction with the AUX/IAA transcription repressors. SCF-Tir1 then recruits an E2 ubiquitin ligase that polyubiquitylates the repressor resulting in its degradation by the proteasome (Figure 5).

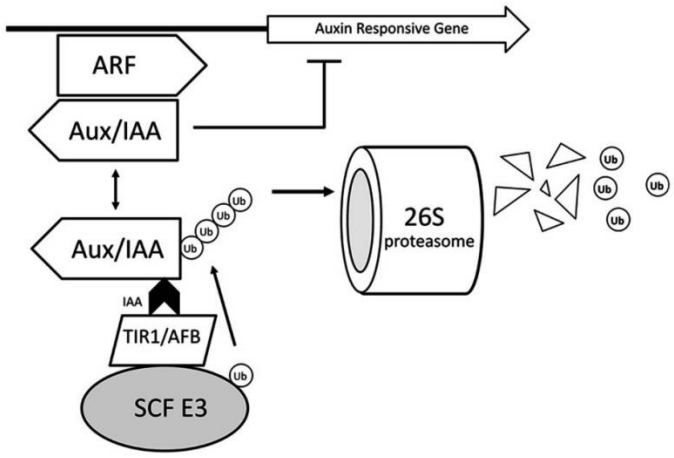


Figure 5. Auxin action mechanism based on Tir1. Upon binding of auxin to the Tir1 subunit of the SCF complex, their affinity toward the AUX/IAA proteins is enhanced, leading to ubiquitination and degradation of the latter in the proteasomes (Da Costa et al., 2013).

Human cells lack the auxin response but share the SCF degradation pathway, which is why it is possible to implement the system from the plants and use a small molecule auxin to conditionally control protein stability. Therefore, Tir1 protein has to be introduced and the protein of interest has to be tagged with the auxin responsive domain (so-called auxin inducible degron, AID) (Figure 6). The AID system has already been established and it has been demonstrated that it promotes degradation of proteins in a variety of human cell lines. This applies to degradation of stably expressed GFP in HeLa and HEK293 cells (Nishimura et al. 2009), polo-kinase 4 (Plk4), cyclin B1, histone H2B, centromere protein A (CENP-A), histone H3, telomeric repeat-binding factor 2 (TRF2) in colorectal cancer cell line DLD-1 (Holland et al., 2012) and RAD21 and DHC1 in colorectal cancer HCT116 cells (Natsume et al., 2016).

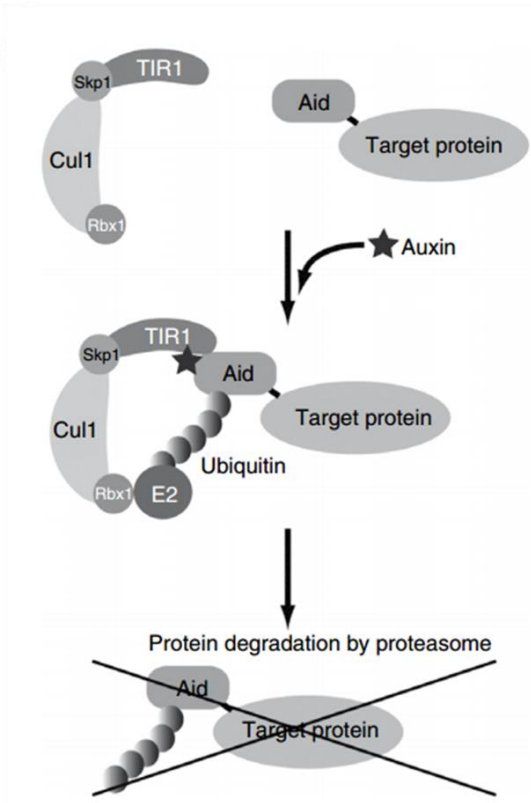


Figure 6. AID system used for degrading protein of interest in human cells. Auxin binding to Tir1 promotes the interaction between Tir1 and the AID degron of the target protein. SCF-Tir1 recruits E2 ligase resulting in ubiquitination of the AID degron. Finally, the target is degraded by the proteasome (Nishimura et al. 2009).

1.5 CRISPR/Cas9 technology

Clustered Regularly Interspaced Short Palindrome Repeats (CRISPR) and variable arrays of the CRISPR-associated (Cas) genes constitute archaeal and bacterial mechanism of defence against invading phages and plasmids that functions analogously to the eukaryotic RNA interference systems (Barrangou et al., 2007). In 2012, Charpentier and Doudna highlighted the potential to exploit this bacterial immune system for RNA-programmable genome editing. Since then, CRISPR/Cas9 technology has been widely used in biology, biotechnology and biomedicine.

When utilized for genome engineering, this system includes Cas9 endonuclease and single guide RNA (gRNA) along with an optional section of DNA repair template. gRNA binds to the complementary sequence in the genome and targets Cas9 endonuclease to introduce double strand break (DSB) next to the protospacer adjacent motif (PAM) sequence. Therefore, gRNAs are synthesized to recognize gene sequences having a PAM sequence at the 3'end (5'-NGG-3') (Cong et al., 2013; Mali et al., 2013) (Figure 7).

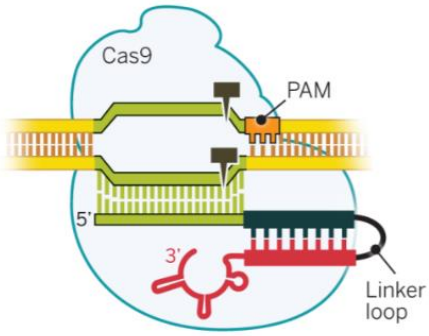


Figure 7. CRISPR/Cas9 mechanism of action. Cas9 enzyme (blue) generates breaks in double-stranded DNA by using its two catalytic centers to cleave each strand of a DNA target site next to a PAM sequence (orange) and matching the 20-nucleotide sequence of the single guide RNA (green) (Charpentier and Doudna, 2014).

The Cas9-introduced DSB can be repaired with non-homologous end joining (NHEJ) that is error prone and usually introduces insertion or deletion (indel) mutations often resulting in the knock-out of the targeted gene. If there is a DNA repair template present that carries enough homology, homologous recombination (HDR) will occur and the gene of interest will be knocked-in at the DSB (Figure 8).

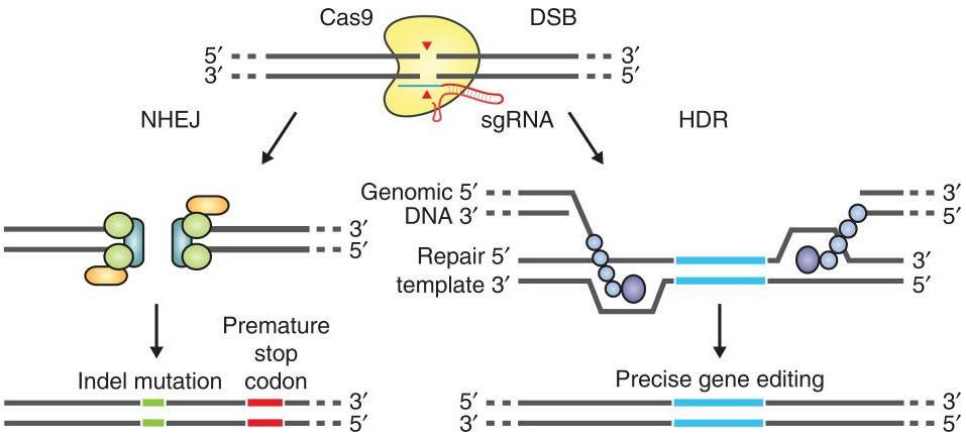


Figure 8. Gene editing after the DSB repair. DSBs induced by Cas9 can be repaired in one of two ways. In the error-prone NHEJ pathway, the ends of a DSB are processed by DNA repair machinery and rejoined, which can result in random indel mutations leading to frameshifts and creation of a premature stop codon. Alternatively, a repair template can be supplied to HDR, which allows precise editing (Ran et al., 2013).

1.6 Hypothesis

The hypothesis of the study is that depletion of the most active miRNAs during the four days differentiation of iNGN cells to neurons can reveal unknown miRNA functions during neurogenesis. Therefore, the aim is to establish the AID system in iNGN cells using CRISPR/Cas9 system, for the inducible disruption of the miRNA processing machinery by eliminating DGCR8 protein.

2. MATERIALS AND METHODS

2.1 MATERIALS

For this study, iNGN C4 cell line was used. iNGN is the Personal Genome Project iPS cell line, derived from Participant #1 (PGP1, hu43860C) (Coriel, USA) genetically modified by lentiviral gene transfer and genomic integration of the doxycycline-inducible Neurogenin and rTA3 vectors (Busskamp et al., 2014a). One Shot® Stbl3™ Chemically Competent *E. coli* (Thermo Fisher Scientific, USA) derived from the HB101 *E. coli* strain, was used for cloning. The transformation efficiency of the Stbl3 chemically competent cells is greater than 1×10^8 cfu/µg plasmid DNA. Validation of the CRISPR/Cas9 set-up was done in human embryonic kidney cells 293T (HEK293T) because of their high transfection efficiency.

2.2 METHODS

2.2.1 Experimental set-up

To establish the AID system in iNGN cells for the inducible degradation of the DGCR8 protein, cells have to be genetically modified with Tir1 using PiggyBac transposon system and the DGCR8 has to be tagged with AID tag together with mKate2 as a fluorescent marker using CRISPR-Cas9 technology (Figure 9).

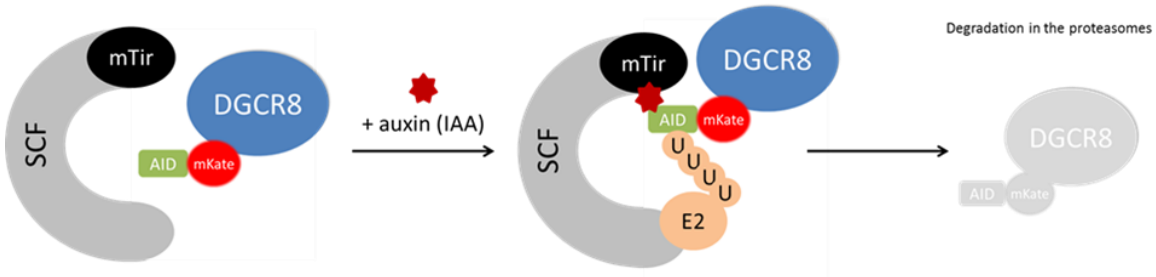


Figure 9. Auxin-based degron system mechanism of action in human cells. In the presence of auxin, mTir1 binds to the AID tagged protein of interest and initiates its polyubiqutination by the E2 ubiquitin ligase leading to degradation in the proteasomes.

For a knock-in of the AID domain from the system at the 3' end of the *DGCR8* gene, the gRNA has to target the Cas9 endonuclease around the *DGCR8* stop codon. The AID has to be supplied as a repair template surrounded by homologous regions to serve as substrate for the HDR pathway (Figure 10).

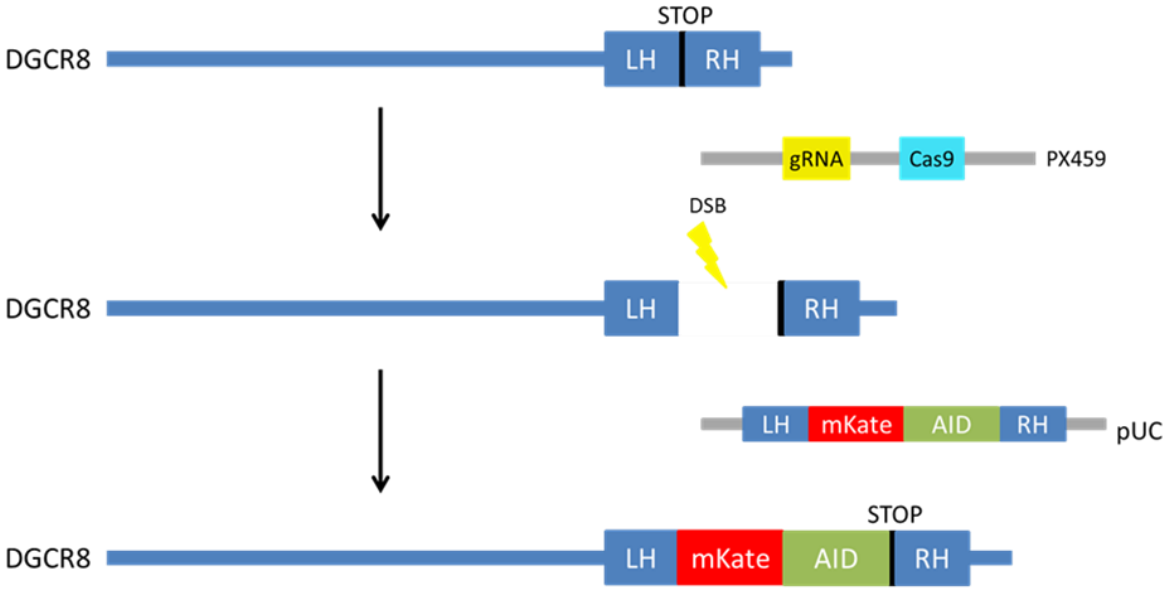


Figure 10. Establishment of an AID system for DGCR8 degradation using CRISPR/Cas9 technology. The Cas9-gRNA complex introduces DSB at the 3' end of the *DGCR8* gene. The DSB will be repaired with HDR triggered by long left and right homology arms (LH, RH) of the repair template resulting in gene knock-in.

2.2.2 PCR reactions

In all PCR reactions the KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA) and the ProFlex PCR System (Thermo Fisher Scientific, USA) were used. The standard PCR mix for the KAPA and the standard PCR conditions for the ProFlex system are shown in Table 1 and Table 2. A gradient PCR with annealing temperatures from T_m=58-68 °C was performed for each primer pair.

Table 1. Standard PCR mix for amplifying DNA.

Component	Volume	Final concentration
2X KAPA HiFi HotStart ReadyMix	25 µL	1 X
10µM forward primer	1.5 µL	0,3 µM
10µM reverse primer	1.5 µL	0,3 µM
100 ng template DNA	As required	2 ng/µL
dH_2O	Up to 50 µL	/

Table 2. Standard PCR conditions for amplifying DNA.

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	3 min	1
Denaturation	98 °C	20 sec	
Annealing	60-75 °C	15 sec	35
Extension	72 °C	15-60 sec/kb	
Final extension	72 °C	1 min/kb	1

2.2.3 Gel electrophoresis

The gel electrophoreses were performed with the pre-casted E-GEL EX 1% (Invitrogen, USA) and Bolt 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific, USA) or with an agarose gel made with UltraPure agarose. The UV-light chamber was used for imaging.

2.2.4 Transformation of DNA in competent bacteria

One Shot Stbl3 Chemically Competent *E. coli* (Invitrogen, USA) were thawed on ice for 15 min and 25 ng DNA was added by stirring with the pipette. After adding the DNA, the bacteria suspension was incubated on ice for 10 min; heat shocked at 42 °C for 45 sec and put on ice again for 5 min. For the bacteria to recover from the heat shock, 300 µl of SOC media were added and incubated for 1 h at 37 °C and shook 300 rpm. Finally, the suspension was plated on a pre-warmed LB-Antibiotic plate and incubated overnight at 37 °C.

2.2.5 Cloning

2.2.5.1 Gibson assembly

Gibson assembly (GA) was used for assembling multiple overlapping DNA molecules by the concerted action of a 5’ exonuclease, a DNA polymerase and a DNA ligase (GA reaction mix) (Gibson et al., 2009). GA primers that contain a 5’ end that is identical to an adjacent segment and a 3’ end that anneals to the target sequence were designed using Serial Cloner 2.6.1 Software (Serial Basics) (Figure 11).

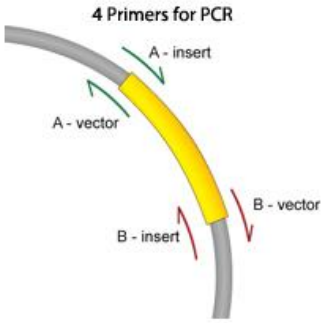


Figure 11. Gibson assembly primers design. Primers containing 5' end identical to the vector (grey) and 3' end to the insert (yellow) were designed (www.addgene.com).

PCR was performed with specific GA primers to amplify desired fragments from the plasmids (Table 3). PCR products were loaded on an E-GEL EX 1% agarose. After the electrophoresis, the right bands were cut from the gel, purified with the QIAquick Gel Extraction Kit (Qiagen, Germany) and the DNA concentrations were measured with a Nanophotometer P330 (Implen, Germany).

Table 3. GA primers for the amplification of selected fragments from different vectors and lengths of the amplified PCR products.

Primer	PCR template	PCR product	Sequence	Lenght
PB09-EF1a_F	III-80 – PB09-TRE-EF1a-TetO	EF1a	CGGGGAAAAGGCCTCCACGGCCAACTAGTGGATCTGCGATCGCTCCGGTG	625
EF1a-mTir_R			CCAGCACCTTTCTCTTCTTCTTGGGGGCGGCGGGGCCCATGGCGGC	
EF1a-mTir_F	IV-05-pCAGGS-NLS-mTIR-HA-p2A-mTIR-Puro	NLS-mTir-HA-p2A	AGAGCCGCCATGGGCCCCGCCGCCCCCAAGAAGAAGAGAAAGGTG	1798
mTir-P2A-Neo_R			CGTGCAATCCATCTTGTTCAATCATAGGTCCAGGGTTCTCCTCCAC	
mTir-P2A-Neo_F			AGACGTGGAGGAGAACCCTGGACCTATGATTGAACAAGATGGATTG	
Neo-PB09_R	II-53-hCas9	NeoR	ATTTTGTAATCCAGAGGTTGATTGTCGACTCAGAAGAACTCGTCAAGAAG	852
pUC -	iNGN C4 cDNA	Left	CGGCCAGTGAATTCGAGCTCGGTA	1377

hom1_F		homology arm	CCAGTCAGCATGCAGTTATGTTGCC	1343
hom1 - mKate2_R			TCACCATGGTGGCGACCGGTGGAT CCACGTCCACGGTGCACAG	
hom1 - mKate2_F		Right homology arm	TGAGCCCCTGTGCACCGTGGACGTG GATCCACCGGTCGCCACCATG	
mKate2 - AID_R			CTTTGGGACAGGCACCTCCGGATCC TCTGTGCCCCAGTTTGCTAGG	
mKate2 - AID_F	II-65-pmKate2-H2B	mKate	CTCCCTAGCAAACTGGGGCACAGA GGATCCGGAGGTGCCTGTCCC	696
AID - hom2_R			GCCCTGGCCCGTGCCACCTCCCTCA CTTATACATTCTCAGGTCAAT	
AID - hom2_F	IV-06-pcDNA5FRTTO_3xHA-Venus-3xminiAID	miniAID	AAAGATTGACCTGAGAATGTATAA GTGAGGGAGGTGGCACGGGC	204
hom2 - pUC_R			GCATGCCTGCAGGTCGACTCTAGA GTTGGTCACACAGGTAGTGAGGGA AG	

Vector III-80-PB09 was digested with SpeI and SalI restriction enzymes (NEB, USA). For this reaction, 4 µg of the vector DNA was mixed with 2 µL SpeI and 2 µL SalI in 2 µL 10 x Cutsmart buffer and 10 µL H_2O. Reaction mix was then incubated at 37 °C for 90 min. The same protocol was applied for the digestion of the II-60-pUC19 with KpnI and BamHI (NEB, USA). Digested vectors were loaded on the E-GEL EX 1% agarose and purified with QIAquick Gel Extraction Kit.

For the production of the IV-37-PB09_EF1a_mTir1_HA_p2A_Neo vector (Figure 12), EF1a, NLS-mTir-HA-p2A and NeoR fragments were mixed with 100 ng III-80-PB09 vector backbone previously cut with SpeI/SalI at equal molecular ratio. The same procedure was applied for left homology arm, mKate, miniAID and right homology arm mixed with II-60-pUC19 backbone previously cut with KpnI/BamHI for the assemble of the IV-52-pUC19_DGCR8-3'mKate2_AID (Figure 13).

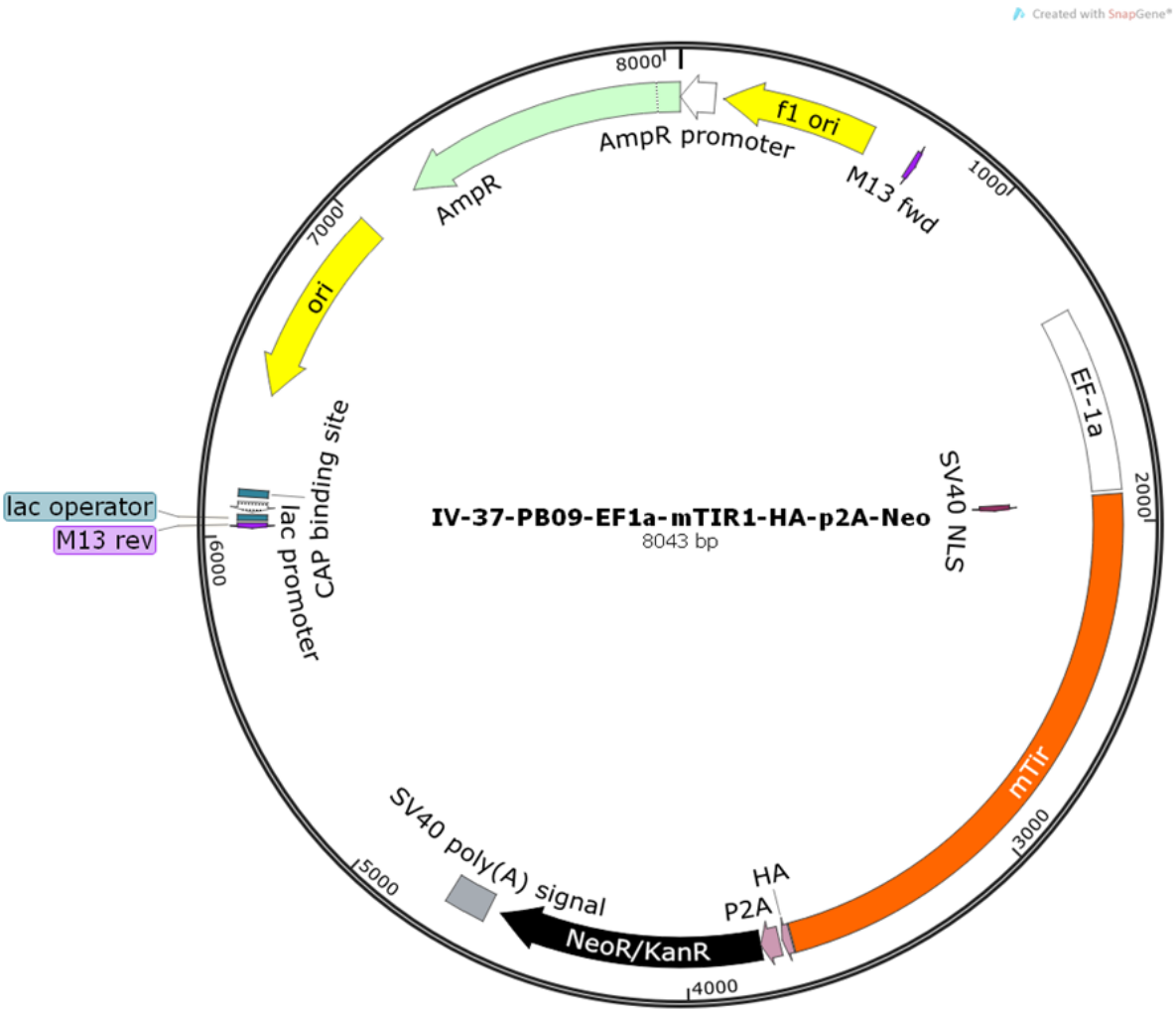


Figure 12. The IV-37 vector important for the introduction of the mTir1 protein into the iNGN cells using piggybac transposon system; carrying the mTir1 gene (brown) linked with the P2A sequence to the Neomycin resistance gene (orange).

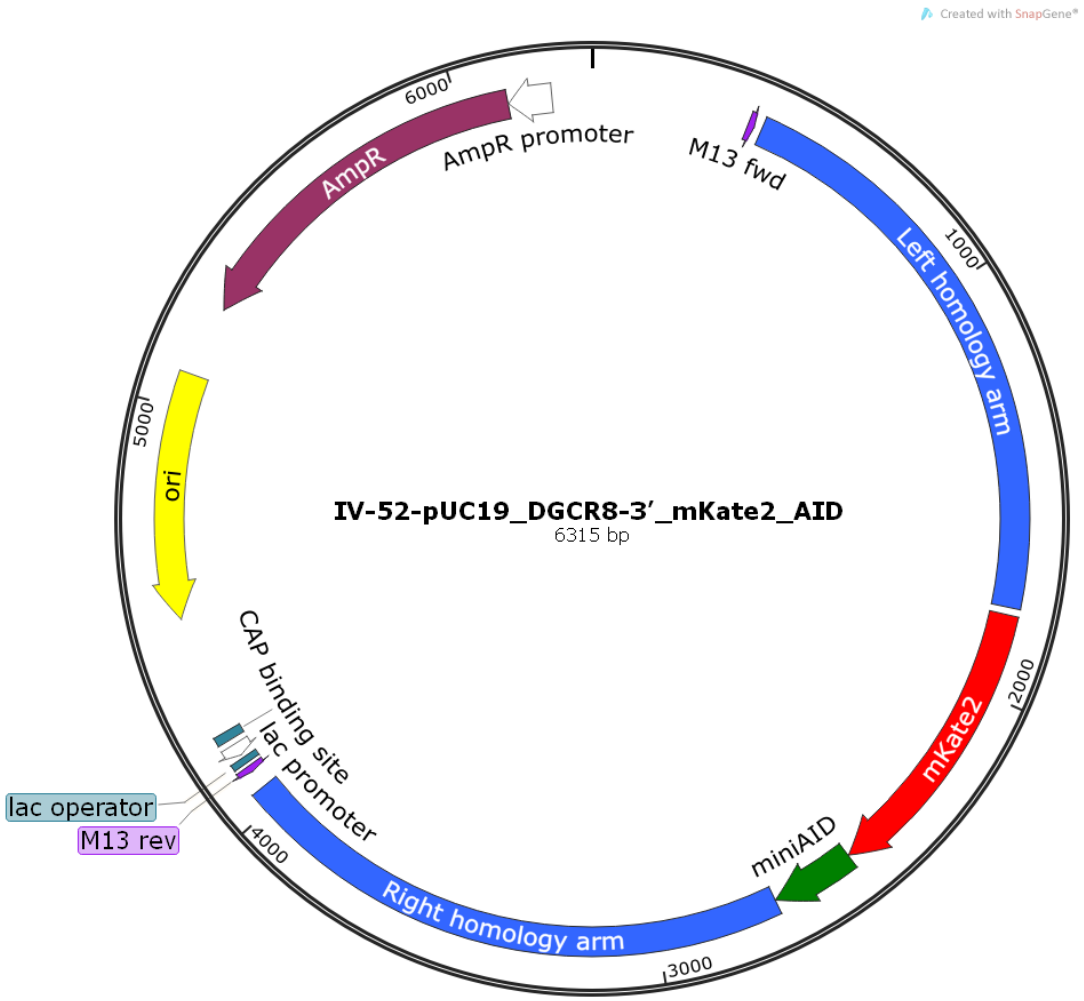


Figure 13. The IV-52 assembled for the CRISPR/Cas9 genome engineering; containing mKate2 (red) –AID (green) fragment flanked by ~ 1,5 kbp left and right homology arms (blue) important for homologous recombination of the tag sequence at the DSB introduced by Cas9 endonuclease

Afterwards, 5 µl of the combined fragments were added to 15 µl GA reaction mix (exonuclease, DNA polymerase and ligase) and incubated at 50 °C for 45 min. 4 µl of assemblies were transformed to Stbl3 and plated to LB-Antibiotic plates. On the next day four colonies of each GA were picked and separately transferred into a shake tube with 5 ml LB-medium with 10 µl Ampicillin (Amp). After overnight incubation, the bacteria suspension was spun down at 15000 x g for 20 min and the DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) in the QiaCube (Qiagen, Germany). To detect the clone with correctly ligated fragments and the fewest point mutations the whole inserted sequence of the different clones was analysed. The most appropriate clone was transferred to a flask with 50 ml LB-medium and 100 µl Ampicillin and used the next day to perform a larger, midi DNA preparation using the Qiagen Plasmid plus Midi Kit (Qiagen, Germany).

2.2.5.2 Cloning gRNAs into the PX459 vector

A gRNA was designed using the crispr.mit.edu online platform (Hsu et al., 2013). The gRNA with the least number of intragenic off targets and a specific bindings close to the DGCR8 stop codon was chosen. For the annealing, 2 µl of the top oligo (CACCGTGTGCACCGTGGACGTGTGA) and 2 µl of the bottom oligo (AAACTCACACGTCCACGGTGCACAC) were mixed together with 2 µl 10 x T4 ligation buffer (Thermo Fisher Scientific, USA), 1 µL T4 polynucleotide kinase (Thermo Fisher Scientific, USA) and 13 µL dH_2O. Annealing mix was transferred to the ProFlex PCR System (Table 4).

Table 4. PCR conditions for annealing top and bottom gRNA oligo.

Temperature	Ramp rate	Time
37 °C		30 min
95 °C		5 min
95 °C-25 °C	-5 °C/min	
4 °C		hold

For the digestion of the III-34-pSpCas9(BB)-2A-Puro (PX459) vector, 3 µg of the vector was mixed with 2 µl BbsI (NEB, USA) (10U/µL), 5 µl Buffer G (Thermo Fisher Scientific, USA) and filled with dH_2O up to 50 µl in total. The digestion mix was incubated at 37 °C for an hour. After digestion, the vector ends were dephosphorylated by adding 0.5 µL CIP alkaline phospatase and incubating at 37 °C for an hour. The digested and dephosphorylated vector backbone was loaded on the E-GEL EX 1% agarose and purified with QIAquick Gel Extraction Kit.

For the production of the IV-39-PX459_DGCR8-3'_gRNA vector (Figure 14), 100 ng purified vector backbone were mixed with 2 µl gRNA oligo hybrid (1:250 diluted) and 5 µl 2 x Mighty Mix (Takara Bio, USA) and incubated at 16 °C for 30 min. Next, 5 µl of the ligation reaction was used for transformation into Stbl3 cells. On the next day, four colonies were picked and transferred to 5 mL LB medium with Ampicilin. After overnight incubation, bacteria were spun down, and DNA was extracted using the QIAprep Spin Miniprep Kit. The DNA concentration was measured and the clones were sent for sequencing. Clone with correct gRNA was used for a midi DNA preparation.

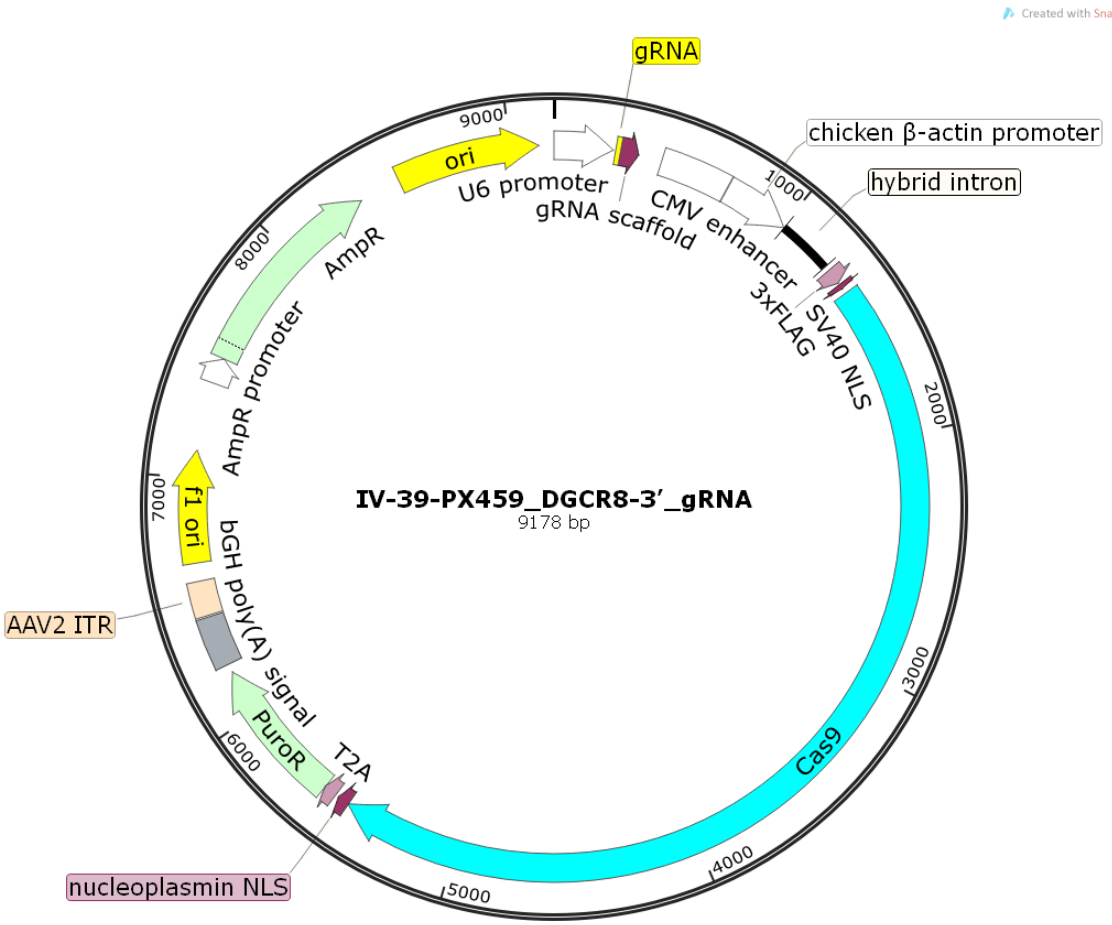


Figure 14. The IV-39 vector assembled for the CRISPR/Cas9 genome engineering; coding for the gRNA (yellow) that guides Cas9 protein (turquoise) to introduce the DSB at the 3' end of the DGCR8 gene.

2.2.6 Sequencing analysis

To ensure that the cloning reactions or CRISPR/Cas9 knock-in had been successful, sequencing samples were prepared using 100 ng/µl plasmid DNA (Mini preparations) or PCR product in 15 µl dH_2O, mixed with 2 µl sequencing primer previously designed with Primer3. Primers Piggybac_for (CAAAACTTTTATGAGGGACAGC), Piggybac_rev (AAGAACCCAGTTTGGGAACA) and mTir-P2A-Neo_R were used to sequence the EF1a-NLS-mTir-p2A-Neo fragment incorporated in III-80-PB09 vector, and primers M13_for, M13_rev (standard primers), mKate2_AID_F and hom2_pUC_R for sequencing the LH-mKate2-AID-RH fragment incorporated in II-60-pUC plasmid. For checking the mKate2-AID tag knock-in in the genome, primers Seq_mK_AID_for (CAGCAGGTGCAGGACATAGTG) and Seq_mK_AID_rev (CTTTTCAAAGAAGGGGAAACACAGA) were used. DNA solutions were sent to Eurofins for sequencing. Serial cloner was used for analysing and comparing the sequences.

2.2.7 Cell culture

2.2.7.1 Coating of the cell culture well plates

The attachment and proliferation of iPS cells depends on the cell culture plate surface. Therefore, before plating stem cells the plates were coated with Matrigel (Corning, USA). One aliquot of Matrigel (approx. 120 µl) was resuspended in 12 ml cold coating medium (DMEM + 1% Pen/Strep). After resuspending, 1 ml Matrigel solution was transferred to a 6-well plate, and distributed equally to guarantee that the complete surface is covered. For 12-well plate, 0.5 ml of the Matrigel solution was used, for 24-well plate 250 µl, etc. The coated plates were incubated at RT for at least 45 min before seeding cells.

2.2.7.2 Thawing iPS cells

iNGNs were taken from liquid nitrogen tank and kept on dry ice before thawing in a bead bath. Thawed cells were transferred to a 15 ml falcon tube and 6 ml PBS was added drop-wise. After spinning down the cell suspension at 1400 rpm for 4 min, the supernatant was aspired. The cell pellet was re-suspended in 2 ml mTeSR medium (StemCell Technologies, Canada) with Rho kinase inhibitor (ROCKi) (StemCell Technologies, Canada) and transferred to a 6-well plate. The plate was placed in the incubator at 37 °C and 5% CO_2. ROCKi (Y-27632) is a factor that enhances iPSCs survival upon single cell dissociation (Watanabe et al. 2007), but has to be removed from the mTeSR medium after 24 h.

2.2.7.3 Culturing iPS cells

iNGN cells were cultured on Matrigel-coated well plates in mTeSR medium and kept inside the incubator at 37 °C and 5% CO_2. When changing the media, mTeSR was aspirated, cells were washed once with PBS, and the new mTeSR was added (2 ml for 6-well plate, 1 ml for 12-well plate, etc.) using serological pipettes.

Cells were passaged when they reached 70-80% confluence. After washing with PBS, the cells were detached using TrypLE Express (Thermo Fisher Scientific, USA) (0.5 ml for 6-well plate, 250 µl for 12-well plate, etc.) for 5 min at 37 °C. PBS was used for collecting the detached cells to a falcon tube. The cell suspension was centrifuged at 1400 rpm for 4 min, the supernatant was removed and the pelleted cells were resuspended in 1 ml mTeSR + ROCKi for counting. Next, 10 µl of the cell suspension was mixed with 10 µl trypan blue and 10 µl of the mixture were transferred on a Countess cell counting chamber slide (Thermo Fisher Scientific, USA). Slide was inserted in the Countess II FL cell counter and the amount

of cells was calculated. Approx. 35000 cells/cm^3 were used for passaging. After 24 h cells were washed with PBS and the fresh medium without ROCKi was added.

2.2.7.4 Freezing iPS cells

iNGN cells were washed with PBS, detached using TripLE, washed with PBS again and transferred to a falcon tube. Cell suspension was spun down and the supernatant was aspirated. Cell pellets were resuspended in mFReSR (StemCell Technologies, Canada) (500 µl / 1 x 10^6 cells) and 500 µl were aliquoted in cryotubes that were labelled, put in a freezing container at -80 °C for 24 h and transferred to a liquid nitrogen tank afterwards.

2.2.8 PEI transfection

HEK293T cells were thawed following the protocol for thawing iNGN cells and plated to cell culture flask (75 cm^2) in 10 ml media (DMEM + 10% FBS + 1% Pen/Strep). After 4 days of propagation, they were detached with 2 ml trypsin and 1.5 x 10^6 cells were passaged to the 6-well plate in 1.5 ml DMEM + 10% FBS. The next day, old DMEM + 10% FBS was replaced with 1 mL fresh one. The transfection mix was prepared by mixing 200 µl PEI solution (9 µl PEI + 191 µl DMEM) with 200 µl DNA solution (3 µg PX459-DGCR8-3'gRNA vector DNA in 200 µl DMEM). After 30 min incubation, the transfection mix was added drop wise to 293T cells. Cells were selected using 3 µg/µl Puromycin and collected the next day.

2.2.9 T7 endonuclease I assay

DNA was isolated from the transfected HEK293T cells using Qiagen DNA Blood and Tissue Kit (Qiagen, USA) using QiaCube. Primers Gen_for (CACTCTGGTTTCACTCCTTGATGA) and Gen_rev (TAGCAATTTGAGTTGAGGGTTTCTT), previously designed with Primer3 software, were used to amplify a 2945 bp long fragment from the isolated DNA. The desired fragment was extracted from the gel after the electrophoresis and purified using Qiagen MinElute PCR Purification Kit (Qiagen, USA). The DNA concentration was measured using a Nanophotometer P330. Next, 2 µl NEB Buffer 2 (NEB, USA) was added to 200 ng DNA and filled with dH_2O up to 19 µl in a PCR tube. A hybridisation reaction was run in the ProFlex PCR System (Table 5).

Table 5. PCR conditions for the annealing of the PCR products.

Step	Temperature	Ramp rate	Time
Initial denaturation	95 °C		5 min
Annealing	95 °C – 85 °C	-2 °C /s	
	85 °C – 25 °C	-0.1 °C /s	
Hold	4 °C		hold

For the endonuclease digest, 1 µl of T7 endonuclease I was added to the annealed PCR products for 15 min at 37 °C. T7 endonuclease recognizes and cleaves non-perfectly matched DNA (Gushin et al., 2010). The reaction was stopped by adding 1 µl of 0.5 mM EDTA and the samples were analysed on a gel.

2.2.10 Nucleofection

Cells were detached with TripLE, spun down and resuspended in mTeSR + ROCKi for counting. 800000 cells were resuspended in 100 µL P3 supplement mix, previously prepared by mixing 82 µL of P3 Primary Cell 4D Nucleofector X Solution with 18 µL Supplement from the P1 Primary Cell 4D-Nucleofector Kit (Lonza, Switzerland). For piggyback transformation of iNGN cells with IV-37 (mTir-Neo), the cell suspension was transferred to a tube containing 10 µg of IV-37 and 2.5 µg of III-25, coding for transposase. On the other hand, for introducing CRISPR/Cas9 system in the iNGN cells, the cell suspension was mixed with 8 µg of the IV-39 (gRNA/Cas9) and 1.5 µg of the linear donor harbouring long homology arms, PCR amplified from the vector IV-52 (AID-mKate2). Samples were transferred to the cuvette and nuclefected with Lonza 4D nucleofector using pulse CB-156. After two days of propagation, cells nucleofected with IV-37 were selected with 100 µg/ml G418 for a few days, and cells carrying IV-39 with 0.5 µg/ml Puromycin for 12-16 h.

2.2.11 Fluorescence activated cell sorting (FACS)

Cells previously nucleofected with IV-39 (gRNA/Cas9) and linear donor (AID-mKate) were trypsinised, washed once in PBS and washed again in FACS buffer (PBS + 10% FBS + 1 mM EDTA). After centrifugation at 1400 rpm for 4 min, cells were resuspended in 500 µl FACS buffer for sorting, transferred to FACS tubes and put on ice. Before putting them in the BD Aria II cell sorter (BD Biosciences, USA), cells were filtered

to remove cell clumps. Gating was done in BD FACSDiva 8.0.1 software. For discriminating cell debris from iNGN cells, FSC-A vs. SSC-a plot was used, and for excluding doublets and clumps FSC-A vs. FSC-H and SSC-H vs. SSC-W plots. Nucleofected cells positive for mKate2 were sorted into the 96-well plate.

2.2.12 Cell colony picking and genotyping

To obtain monoclonal lines carrying the mKate2-AID tag, 500 and 1000 FACS sorted cells were seeded to a 6-well plate. After 8 days of propagation, single cells formed colonies that were big enough for picking under the microscope. The colonies were divided into half using a pipette tip. One half was transferred to the new 48-well plate for expansion, and the other half to the PCR tubes with 100 µl PBS. The 48-well plate with picked colonies was quickly transferred back to the incubator. PCR tubes were centrifuged for 2 min using a table top centrifuge and PBS was aspirated.

Cells from the PCR tubes were lysed, their proteins denatured and the remaining DNA was used as a template for the genotyping. For lysing the cells, 25 µl H_2O was added and the cell suspension was incubated in a PCR cycler at 95 °C for 10 min. 5 µl of protease K (Qiagen, USA) was added to the lysed cells for protein degradation and incubated at 50 °C for 60 min, followed by an incubation step at 75 °C for 15 min.

The cell lysate was used for genotyping with primers Gen_for and Gen_rev that were designed to bind outside of the left and right homology arms (Figure 15). Using these primers the monoclonal lines that are homozygous for the mKate2-AID tag can be distinguished from the heterozygous clones on the gel. After the PCR reaction, the products were analysed on a 1% agarose gel followed by electrophoresis.

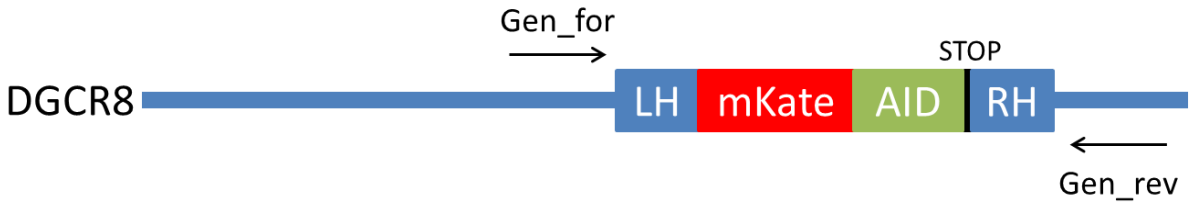


Figure 15. Primers Gen_for and Gen_rev used for genotyping were designed so that they bind outside of the homology arms.

2.2.13 Fluorescence microscopy

Cells were plated on µ-dishes with high glass bottom (Ibidi, USA) and analysed for fluorescence under the total internal reflection microscope two days after. Pictures taken under the transmission light and in red channel were overlayed and processed using Fiji (Schindelin et al., 2012).

2.2.14 Auxin preparation

0.1 g IAA was filled up with dH_2O to a volume of 1.144 ml in total. IAA solution was kept at 14 °C in dark box because it is light sensitive. 1:1000 (500 mM) was used in the media.

2.2.15 Protein isolation

Cells were scraped with a cold scraper in 300 µl ice-cold RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, USA), in 10 ml of which one protease inhibitor tablet (Roche, Switzerland) was added in advance, and transferred from the well to an ice-cold tube. After 30 min of constant agitation at 4 °C samples were centrifuged at 4 °C for 20 min at 12000 rpm. Supernatant containing proteins was kept and pellet with cell debris was discarded. Protein concentration was measured with BCA protein assay.

2.2.16 BCA protein assay

Samples were prepared using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) following manufacturer's protocol. 200 µl of working reagent, prepared using a 50:1 ratio of the kit reagents A:B, was mixed with 10 µl of pre-diluted BSA standards and protein samples and incubated at 60 °C for 5 min. Protein concentration was measured with a Nanodrop after generating the standard curve.

2.2.17 Western blot

Protein samples were thawed on ice and mixed with 10 µl LDS Sample Buffer (4x), 4 µl Reducing Agent (10x) (Novex, USA) and filled up to 40 µl with dH_2O. Samples were heated at 95 °C for 5 min and loaded on the Bolt 4-12% Bis-Tris Plus gel, together with 3 µl SeeBlue marker. Electrophoresis was run for 25 min on 200 V.

Blotting was done with an iBlot device (Thermo Fisher Scientific, USA), following the manufacturer's protocol. The pre-casted gel was wetted and placed on the transfer

membrane on the bottom stack followed by a water-wet filter paper, top stack and the absorbent pad. Program p0 (7 min) was run.

To prevent unspecific binding, the membrane was incubated in blocking milk (5% milk in PBS + 0.1% Tween20) for one hour in a falcon on a rotary module. 1:1000 (3 µl) anti-DGCR8 (Abcam, GB) and 1:5000 (0.6 µl) anti-GAPDH (Cell Signaling Technology, USA) antibodies were added to 3 ml milk (1% milk in PBS + 0.1% Tween20). After an overnight incubation in antibody solution on a rotary module at 4 °C, membrane was washed three times for 10 min in PBST (PBS + 0.1%Tween). The secondary antibody solution was prepared by adding 1:10000 (0.3 µl) of dye-labeled anti-rabbit secondary antibody (Li-Cor, USA) in 3 ml milk (1% milk in PBST). After an hour of incubation in secondary antibody solution in the dark, the membrane was washed 5 times for 10 min in PBST. The Odyssey imaging system (Li-Cor, USA) was used for protein detection.

2.2.18 RNA isolation

Cells were detached using TripLE and spun down at 1500 x g for 5 min. Pipettes and work space were cleaned with RNAzap. Pellets were resuspended in 500 µl Qiazol (Qiagen, Thermo Fisher Scientific, USA) and mixed well for homogenisation by pipetting. After 5 min of incubation, cell homogenates were added to phase lock tubes previously spun down at 1500 x g for 30 s. Next, 100 µl chloroform was added and the two phases were mixed by shaking for 15 s. The tubes were centrifuged at 10000 x g for 10 min at 4 °C. Subsequently, the upper aqueous phases were transferred to the new 2 ml tube. Samples were put into the QiaCube according to the protocol sheet, together with the reagents and columns for RNA purification from the miRNeasy Mini Kit (Qiagen, USA). The respective program for RNA isolation with Dnase digestion was started.

2.2.19 cDNA synthesis and mRNA RT-qPCR

Previously frozen RNA samples were thawed on ice. 1 µg of isolated RNA was filled up with RNA-free H_2O to 10 µl and mixed with 2 µl 10x RT buffer, 0.8 µL 25x dNTPs, 2 µL 10x random primers, 1 µL reverse transcriptase and 4.2 µL H_2O from the AB High Capacity cDNA RT Kit (Thermo Fisher Scientific, USA). Instead of 10 µl reaction mix, 10 µl H_2O was added to the no-RT control. For the reverse transcriptase to transcribe mRNA to cDNA, all samples were incubated in the PCR cycler at 25 °C for 10 min, 37 °C for 2 h, 85 °C for 5 min and 4 °C on hold.

After the incubation, cDNA samples were diluted 1:20 and 2 µl of the diluted samples were mixed with 6.25 µl Power SYBR green master mix (Applied Biosystems, USA), 0,375 µl forward and reverse primer and 3.5 µl dH_2O in 96-well plate. DGCR8_F (GCAGAGGTAATGGACGTTGG) and DGCR8_R (AGAGAAGCTCCGTAGAAGTTGAA) primers were used to check the amount of DGCR8 mRNA in the cells and ß-actin (ACTB), with ACTB_F (CCTCGCCTTTGCCGATCC) and ACTB_R (CGCGGCGATATCATCATCC) primers, was used as a reference. The StepOne Real-Time PCR machine (Thermo Fisher Scientific, USA) was run under conditions shown in Table 6 and and the StepOne software 2.2.2 was used for analysing the data.

Table 6. qPCR conditions from comparative qPCR program.

Cycle	Temperature [°C]	Time [min : s]
1	95	10
[2	95	0:10
3] x40	60	1

2.2.20 Exiqon cDNA synthesis and Exiqon miRNA RT-qPCR

First, dilutions were made from the thawed RNA samples so that the RNA concentration in each sample is 5 ng/µl. 1 µl of 5 ng/µl RNA sample was then mixed with reagents from the Universal cDNA synthesis kit (Exiqon, USA): 1 µl of 5x reaction buffer, 0.5 µl of enzyme mix and 2 µl RNA-free H_2O. In no-RT control samples, 1 µl of RNA was mixed with 4 µl RNA-free water. 5 µl in total were incubated at 42 °C for 60 min, 95 °C for 5 min and at 4 °C on hold.

After the incubation, 4 µl of 1:80 diluted cDNA samples were mixed with 5 µl of Power SYBR green master mix and 1 µl of Exiqon primer mix in 96-well plate. Exiqon miR-302a, Exiqon miR-103 and Exiqon 5S primer mix were used to check the expression of miRNA-302a, miRNA-103 and 5S as the reference. Afterwards, the plate was spun down, placed in the StepOne Real-Time PCR machine and the StepOne software was run (Table 18).

3. RESULTS

3.1 Analysis of the transfected HEK293T cells

To get a first estimate whether the targeting of the 3' end of the *DGCR8* gene with the designed gRNA is successful, a T7 endonuclease assay was performed. For this assay, HEK293T cells were transfected with the PX459-DGCR8-3'gRNA vector and the 3 kb DNA around the expected DSB was PCR amplified. After the digestion of the hybridized PCR products with the T7 endonuclease I, 3 bands were visible on the gel (Figure 16). The upper, bigger band (≈ 3 kb), that matches the length of the expected PCR product (2945 bp), and two smaller bands with less intensity (≈ 1.5 kb) are detectable. A forward primer was designed to bind 1552 bp upstream and a reverse primer to bind 1393 bp downstream of the desired DSB. Therefore, the upper from the two smaller bands corresponds to the fragment upstream of the cut introduced by the T7 endonuclease and the lower one to the downstream fragment. This shows that the targeting strategy is working in human cells and the cut can be produced close to *DGCR8*'s stop codon.

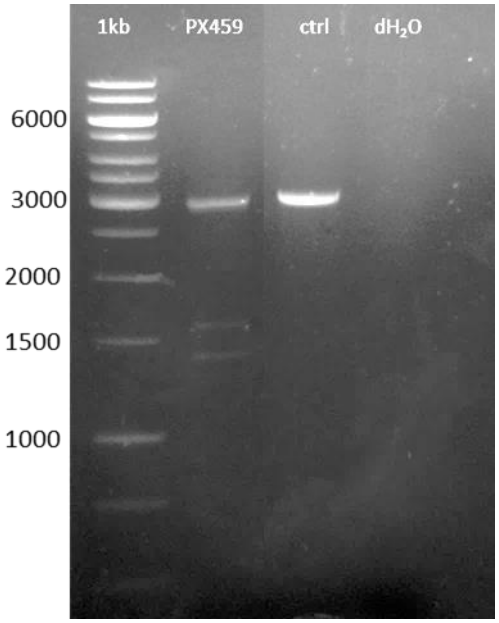
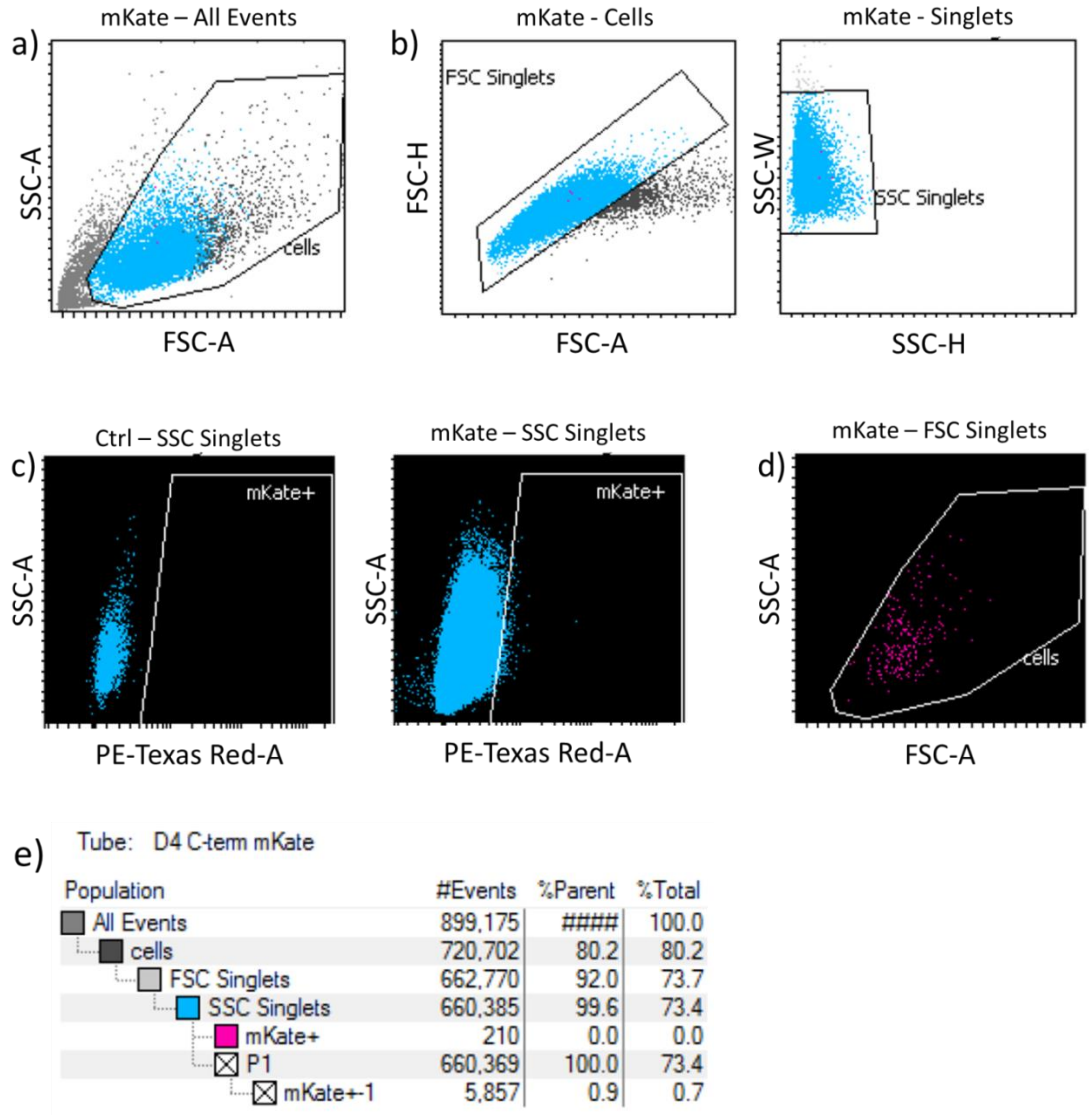


Figure 16. T7 endonuclease I essay. HEK293T cells were transfected with gRNA targeting *DGCR8*. Genomic PCRs were prepared and tested for CRISPR/Cas9 induced mutations by T7 endonuclease I assay. HEK293T cells transfected with PX459 vector show mismatches at the target sites.

3.2 Obtaining a homozygous cell line carrying the mKate2-AID tag

3.2.1 Fluorescence activated cells sorting analysis

iNGN cells nucleofected with the vector IV-39 (gRNA/Cas9) and the linear donor harbouring mKate2-AID tag flanked by homology arms were FACS sorted for the mKate2 fluorescent tag (Figure 17). Out of 660385 single iNGN cells, 210 were sorted as fluorescently labelled, meaning that CRISPR-Cas9 knock-in of the mKate2-AID tag was successful. It corresponds to 0.00032% CRISPR-Cas9 knock-in efficiency.



e)

Tube: D4 C-term mKate

Population	#Events	%Parent	%Total
All Events	899,175	#####	100.0
cells	720,702	80.2	80.2
FSC Singlets	662,770	92.0	73.7
SSC Singlets	660,385	99.6	73.4
mKate+	210	0.0	0.0
P1	660,369	100.0	73.4
mKate+-1	5,857	0.9	0.7

Figure 17. FACS sorted mKate2+ cells after the CRISPR-Cas9 knock-in of the AID-mKate2 tag at the 3' end of the DGCR8 gene. A) FSC-A vs. SSC-A profile. The "cells" gate corresponds to iNGN cells discriminated from the cell debris. B) FSC-A vs. FSC-H (left) and SSC-H vs. SSC-W (right) plot with light blue iNGN singlets and excluded black cell doublets and clumps. C) SSC-A vs. PE-Texas Red-A plot showing mKate2+ cells among all single iNGN cells in control sample (left) and nucleofected sample (right). D) mKate2+ cells displayed on a SSC-A vs. FSC-A plot. E) Numbers of events in different cell populations.

3.2.2 Genotyping of the obtained fluorescent clones

FACS sorted mKate2+ clones were isolated and the insertion of the mKate2-AID tag was checked by genomic PCR with primers that bind outside of left and right homology arms. The predicted length of the PCR fragment with mKate2-AID tag was 3848 bp, and without 2945 bp. Only the lower bands (≈ 3 kbp) of clones 3, 4, 6, 8, 11, 13, 14, 16, 20, 23 and 24, were visible, corresponding to the length of the left and right homology arms (Figure 18). Clones 9, 12, 15, 17 and 22 showed two bands, in which the upper one (≈ 4 kbp) matched the length of both homology arms and the mKate2-AID tag and the lower one the unaltered locus. Hence, these clones were heterozygous for the tag, meaning that CRISPR/Cas9 knock-in occurred at only one of the two *DGCR8* alleles present in human cells. Clones where only the upper band was present (2, 7, 10, 18 and 19), were presumably homozygous clones for the mKate2-AID tag and were further analysed by sequencing.

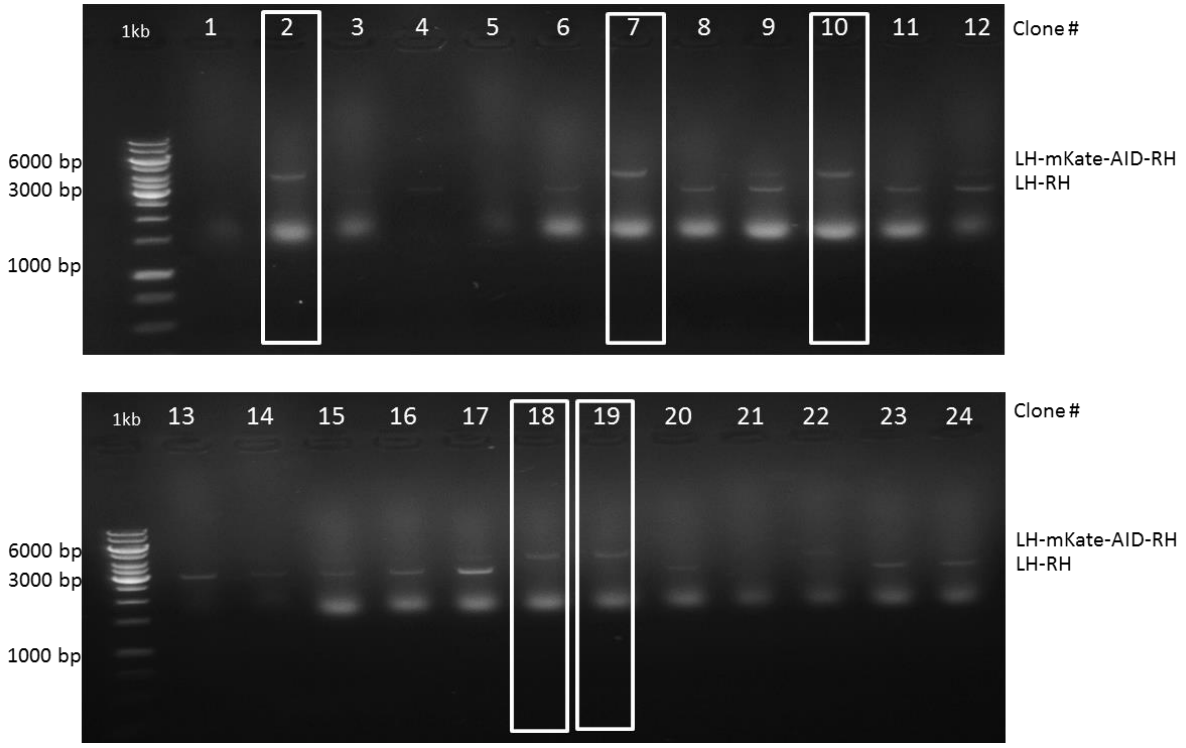


Figure 18. The genotyping of mKate2+ clones. Genomic PCRs were performed with primers designed to bind outside of LH and RH on 24 picked clones. Clones that show only one band are homozygous for mKate2-AID tag or for no tag, and clones with two bands are heterozygous. Clones 2, 7, 10, 18 and 19 seemed to be homozygous for the mKate2-AID tag.

3.2.3 Sequencing analysis of the genotyped fluorescent clones

The sequencing of the clones confirmed the genotyping results that the mKate2-AID fragment is successfully knocked-in before the stop codon at the both alleles. Experiments were proceeded with clone 19, therefore only results for clone 19 are shown (Figure 19 and 20). It was possible to distinguish homozygous iNGN clones from the heterozygous by comparing sequencing electropherograms (Figure 20 and 21). Furthermore, the sequencing indicated the presence of the correct reading frame, i.e. neither frameshift mutations leading to a truncated protein, nor any missense mutations were introduced.

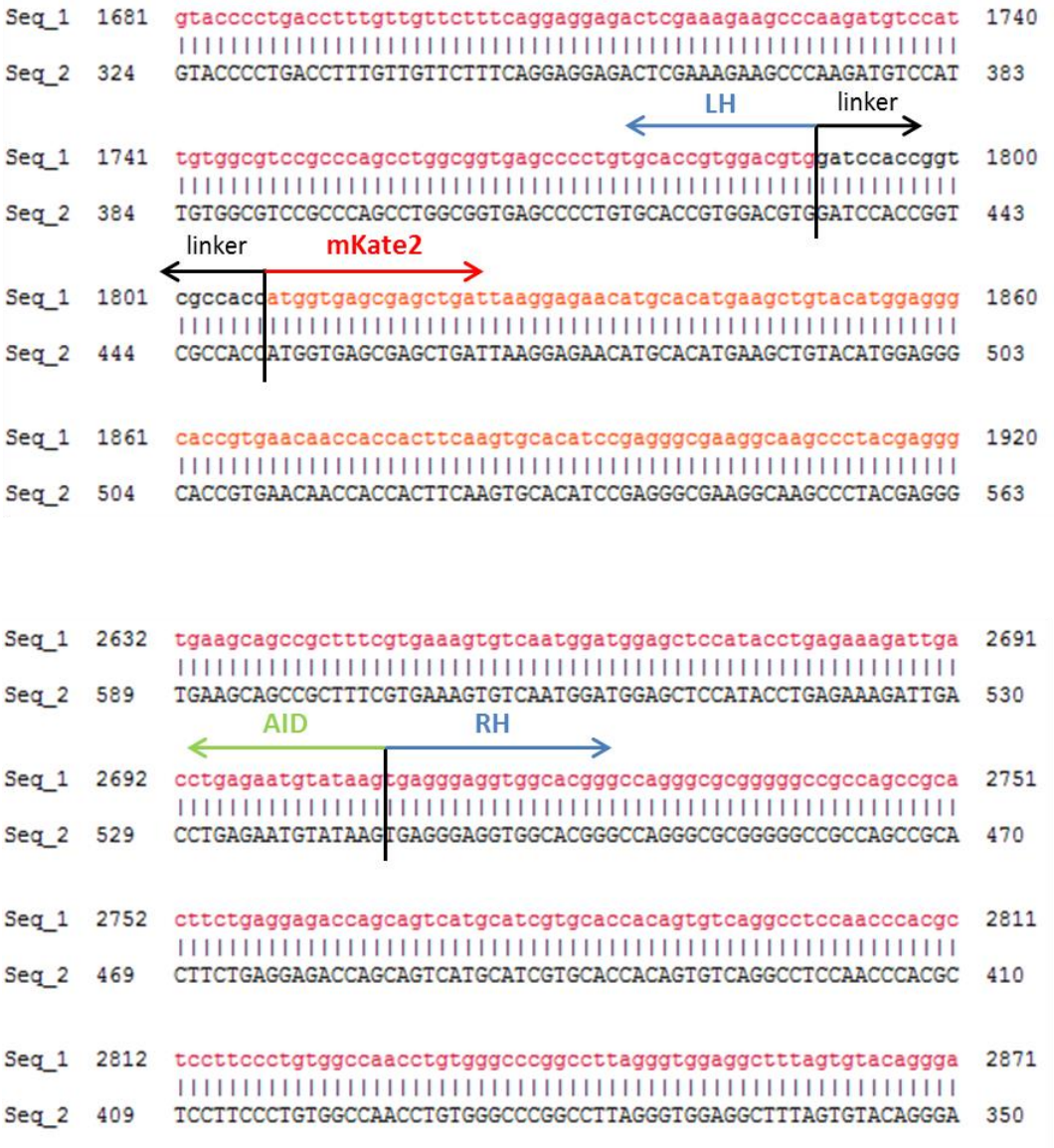


Figure 19. The sequencing analysis of the iNGN clone 19. IV-52 donor vector (Seq_1) and sequenced iNGN clone 19 (Seq_2) alignment in Serial cloner showed that mKate2-AID tag is present in iNGN cells.

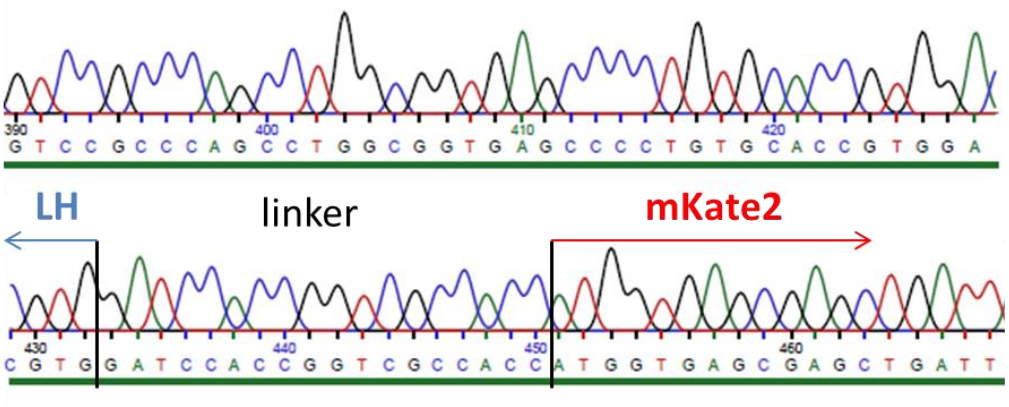


Figure 20. The sequencing electropherogram of the iNGN clone 19. Only one signal for A, G, C or T was present at each position of the sequenced DNA.

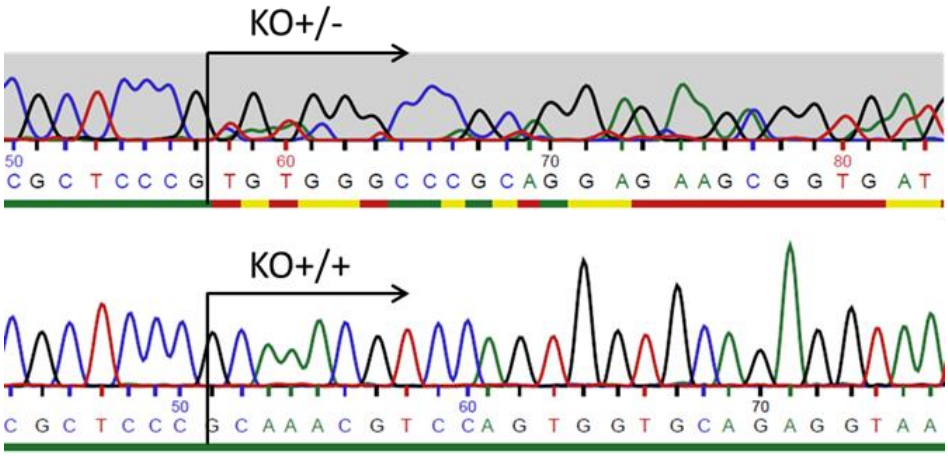


Figure 21. Difference in the electropherograms between heterozygote and homozygote for the DGCR8 knock-out. Two peaks are visible for each base position in the DNA in the heterozygote and only one peak in the homozygote.

3.2.4 Cell analysis under the fluorescence microscope

The previously sequenced clone 19 was analysed under the fluorescence microscope to localise the tagged DGCR8 protein. The fluorescence was observed, which is another confirmation of the successful CRISPR/Cas9 tagging (Figure 22). Furthermore, the fluorescence was observed only in the nucleus suggesting that mKate2-AID tag did not interfere with DGCR8 nuclear localization signal.

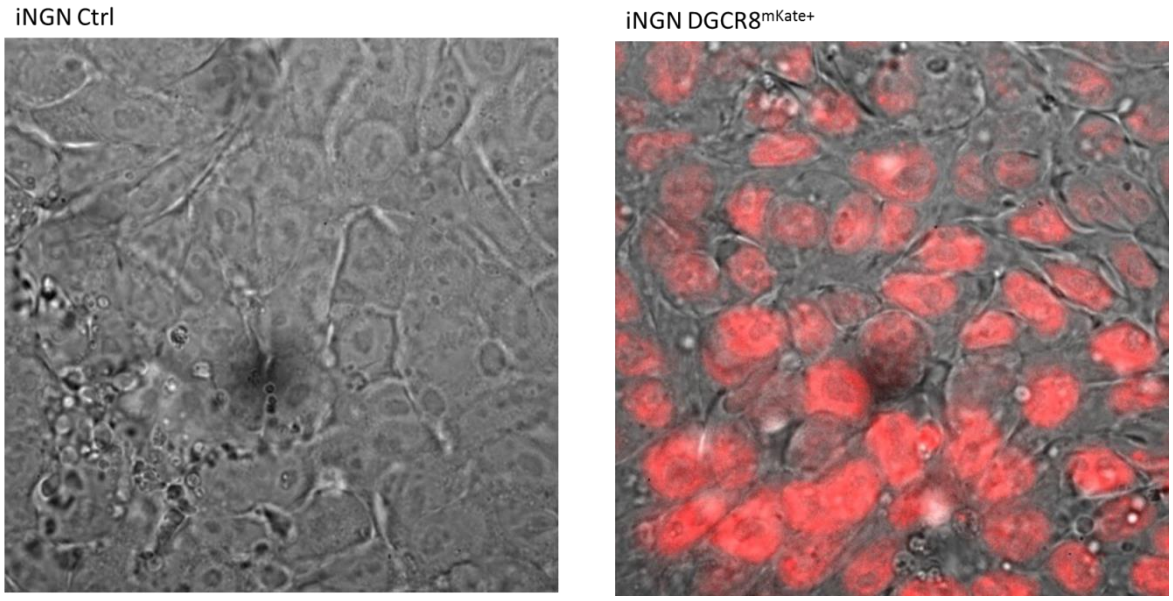


Figure 22. The fluorescence microscopy analysis of the iNGN cells. The clone 19 was positive for the mKate2-AID tag (right) and showed fluorescent signal localized in the nucleus.

3.3 Testing the auxin-based degron system in the iNGN cells

After nucleofection of the homozygous cell line carrying mKate2-AID tag with the PB09-mTir-Neo, three iNGN cell lines in total called mTirDGCR8, aidDGCR8 and degDGCR8 were produced. mTirDGCR8 expresses the Tir1 protein which is a part of the SCF ubiquitin ligase, aidDGCR8 expresses the mKate-AID tagged DGCR8 and degDGCR8 expresses both Tir1 and AID-tagged DGCR8 (Figure 23).

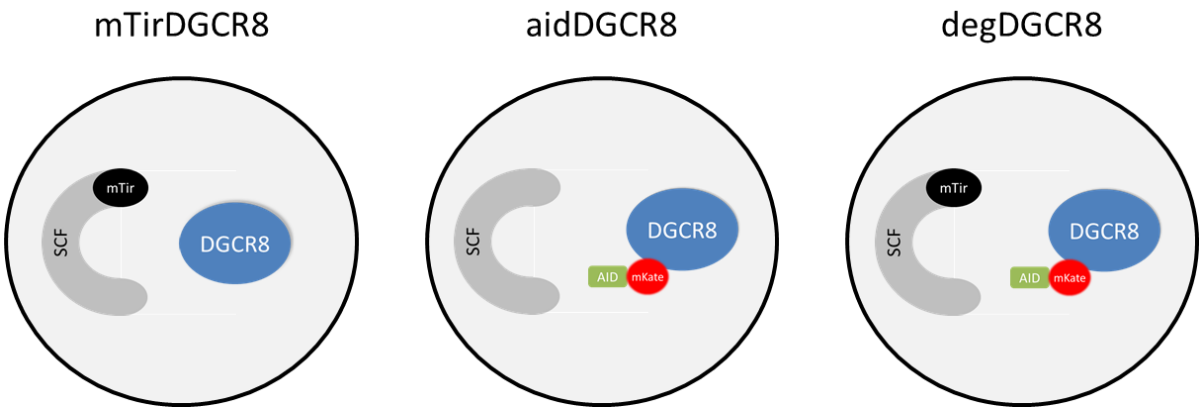


Figure 23. Produced iNGN cell lines. mTirDGCR8 expressing the mTir1 protein and the non-tagged DGCR8, aidDGCR8 expressing tagged DGCR8 and degDGCR8 expressing mTir and AID-tagged DGCR8 important for auxin-based degron system.

3.3.1 Survival of the cells treated with auxin

The mTirDGCR8, aidDGCR8 and degDGCR8 iNGN cell lines were treated with different IAA concentrations for 24 h (Figure 24). Immediately after adding IAA to the cell culture, colour of the cell media changed gradually to more pink with higher IAA concentrations because of the pH change. The same IAA concentrations showed no differences in the cell death frequency among all three cell lines. Furthermore, the same incidence in cell death occured in the un-treated cells and cells treated with 500 mM IAA. However, when cells were treated with IAA concentrations higher than 500 mM more apoptotic and necrotic (2000 mM IAA and higher) cell death was observed. Therefore, 500 mM IAA was used for the AID system activation.

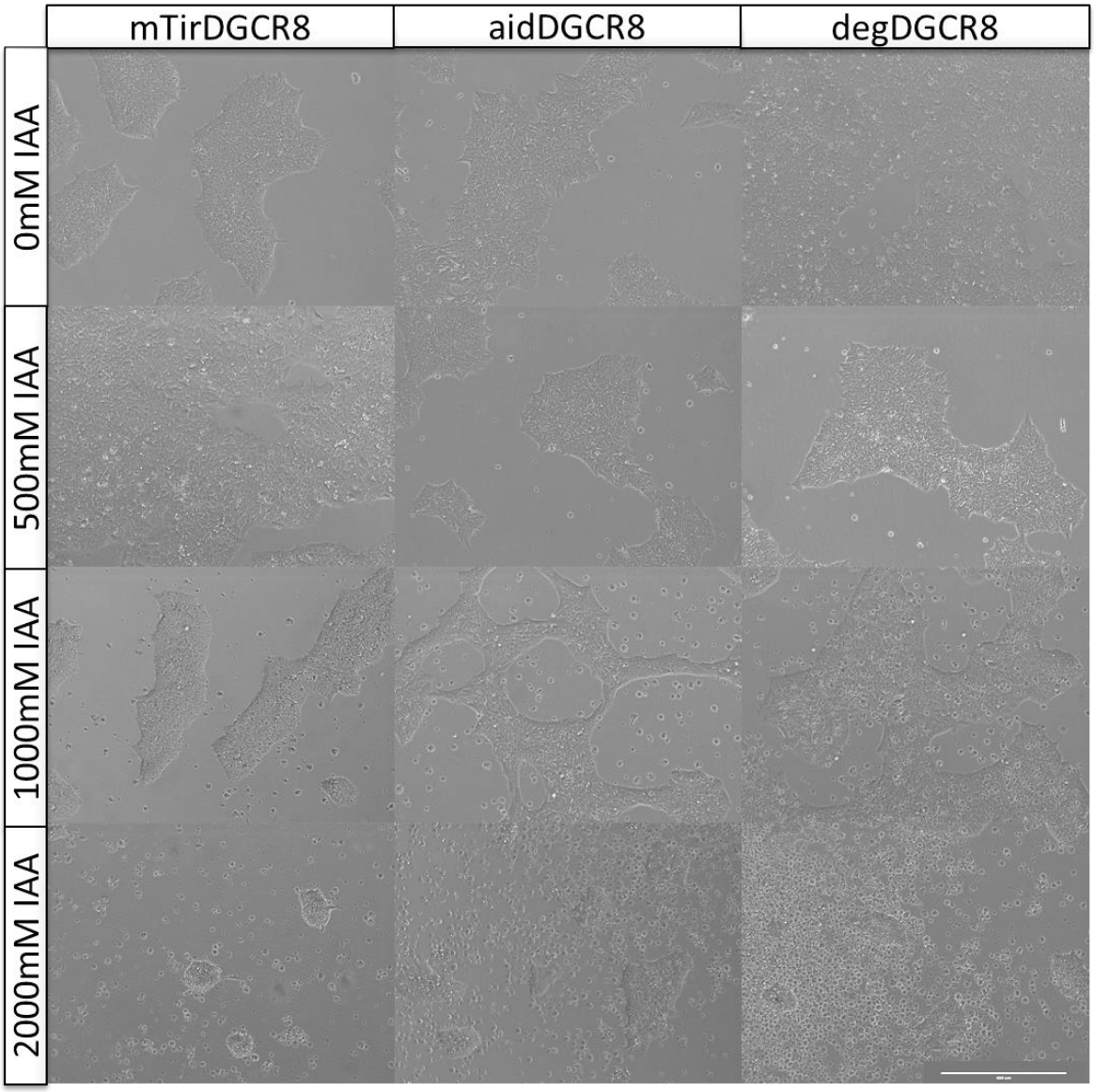


Figure 24. The mTirDGCR8, aidDGCR8 and degDGCR8 cell lines treated with 500 mM, 1000 mM and 2000 mM IAA for 24h. No difference in cell death was observed between controls and cells treated with 500 mM IAA, where many more cells died when treated with higher IAA concentrations. Scale bar 400 μm.

3.3.2 DGCR8 detection

The cell lines, mTirDGCR8, aidDGCR8 and degDGCR8 were treated with 500 mM IAA for 6h and 24h. DGCR8 from the mTirDGCR8 cells and DGCR8-mKate-AID from the aidDGCR8 and degDGCR8 were detected by immunoblotting using anti-DGCR8 antibodies (Figure 25). DGCR8 as expected appeared lower on the gel (86 kDa) and tagged DGCR8 higher (119 kDa) because of the AID-mKate2 knock-in, showing that the translation of the tagged protein was successful. 500 mM IAA had no impact on the protein abundance in the mTirDGCR8 and aidDGCR8 cells after 6h or 24h treatment. DGCR8 was still present in the degDGCR8 iNGNs that were not treated with IAA and after 6h treatment. However, degradation occurred 24h after adding IAA and less protein was detected. Not expectedly, two additional unspecific bands or DGCR8 isoforms appeared in aidDGCR8 and degDGCR8 iNGNs.

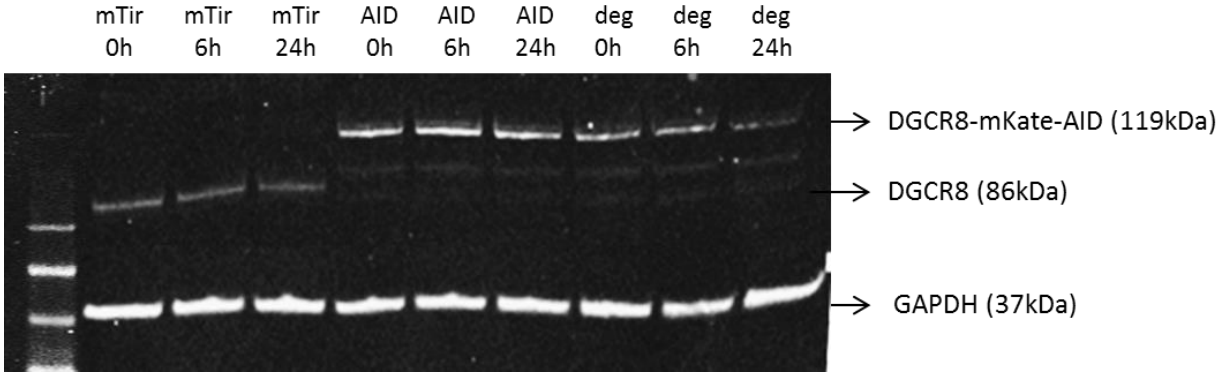


Figure 25. Nuclear DGCR8 fused with the AID degron controlled by the AID system in iNGN cells. DGCR8 and DGCR8-mKate-AID detected by immunoblotting using anti-DGCR8 antibodies and antiGAPDH as a loading control. Degradation of the AID-tagged DGCR8 observed after 24 h incubation in 500 mM IAA.

3.3.3 *DGCR8* expression analysis

The mRNA RT-qPCR was performed to test for any changes in the *DGCR8* expression levels after auxin treatment among the produced cell lines (Figure 26). The mTirDGCR8 and aidDGCR8 were treated with 500 mM IAA for 72 h and degDGCR8 were treated for 24, 48 and 72 h. The experiment was performed in triplicates and results were normalized to the no IAA sample. In mTirDGCR8 and degDGCR8, the DGCR8 expression did not significantly change among IAA-induced and control samples. For unknown reasons, there was a significant increase in 72 h IAA-induced mTirDGCR8 cells. Nevertheless, *DGCR8* expression levels in the degDGCR8 was not affected by IAA treatment suggesting that the degradation occurs only at the protein level.

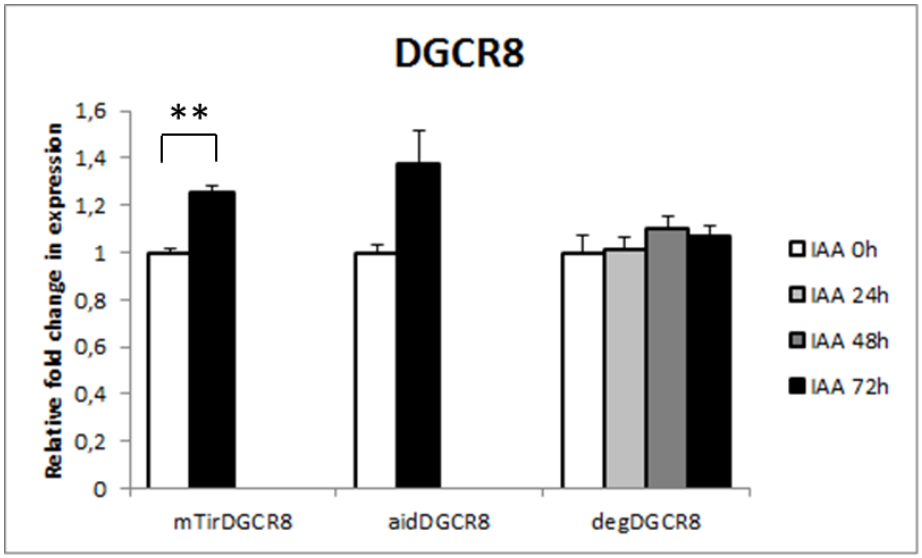


Figure 26. Relative fold change in *DGCR8* expression in mTirDGCR8 and aidDGCR8 treated with 500 mM IAA for 72 h and degDGCR8 treated for 24, 48 and 72 h. Significant change was observed in 72 h treated mTirDGCR8. IAA did not affect *DGCR8* expression in aidDGCR8 or degDGCR8 samples. ($p<0.01$ is shown by **)

3.3.4 miRNA expression analysis

To investigate the functionality of DGCR8 degradation, the levels of some candidate miRNA were assessed by RT-qPCR with and without IAA treatment. It was known that miRNA 302-a and miRNA-103 are expressed in iNGN cells. The same cell samples as for mRNA RT-qPCR (Figure 26) were used for Exiqon miRNA RT-qPCR (Figure 27 and 28). Tested miRNAs were still present in the cells after adding 500 mM. miRNA-103 did not show any significant change in expression and miRNA-302a expression was significantly

lowered in treated degDGCR8 cells after 24, 48 and 72 h indicating the reduced DGCR8 function after IAA treatment. However, the AID system failed to ablate all mature miRNAs.

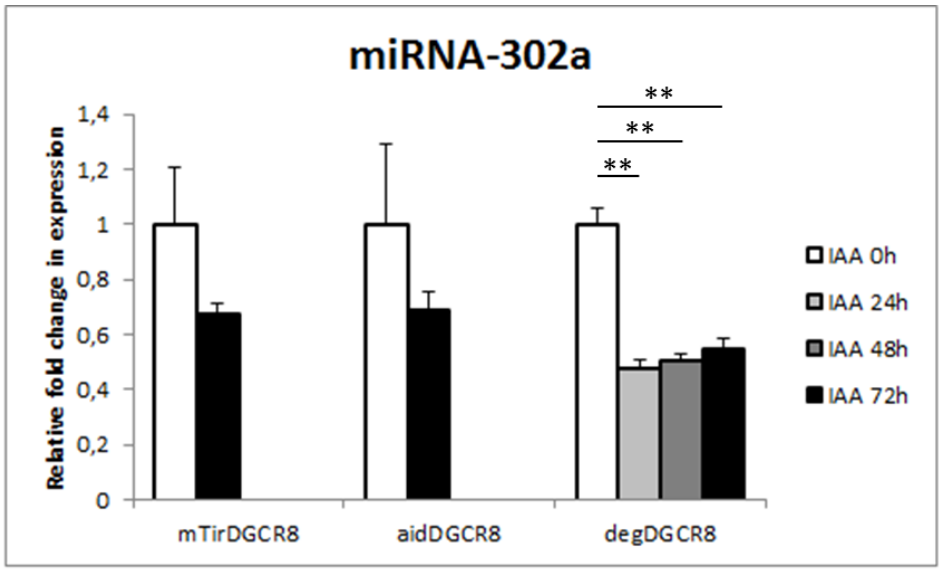


Figure 27. Relative fold change in miRNA-302a expression in mTirDGCR8 and aidDGCR8 treated with 500 mM IAA for 72h and degDGCR8 treated for 24, 48 and 72 h. degDGCR8 treated samples showed 50 % reduction in miRNA-302a expression. IAA did not significantly affect mTirDGCR8 and aidDGCR8 treated samples ($p<0.01$ is shown by **).

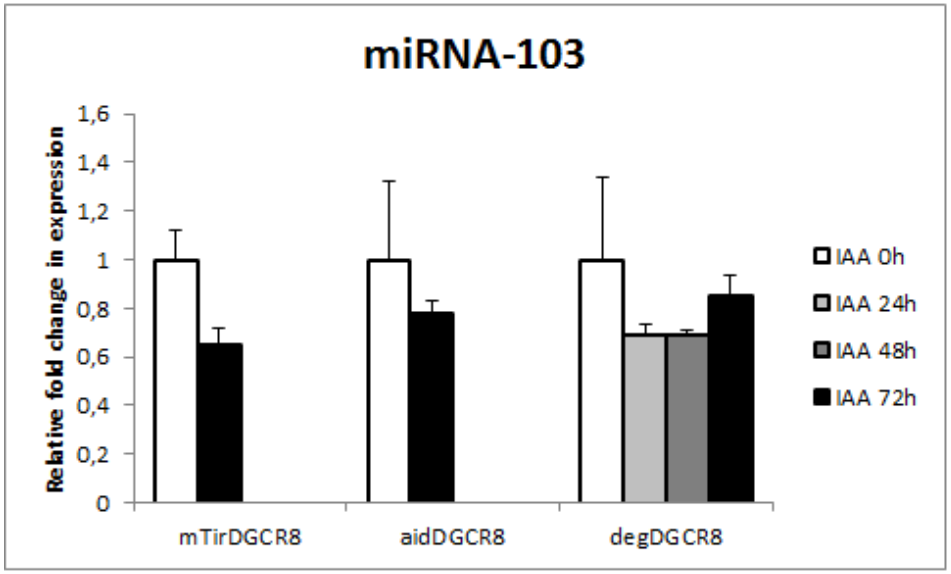


Figure 28. Relative fold change in miRNA-103 expression in mTirDGCR8 and aidDGCR8 treated with 500 mM IAA for 72 h and degDGCR8 treated for 24, 48 and 72 h. IAA did not significantly effect expression in any of the treated samples.

4. DISCUSSION

The aim of the study was to establish an AID system in iNGN cells for the inducible degradation of the DGCR8 protein that would lead to miRNA depletion in these cells. The construction of conditional AID mutants is dependent upon the introduction of two components into cells. The first component is the AID tag that is fused to the DGCR8 at the endogenous locus and the second component is the auxin responsive protein, mTir. In this experiment, I introduced the AID tag at the C-terminus of the DGCR8 protein using CRISPR/Cas9 technology and the mTir protein using piggybac transposon system and tested the efficacy of the introduced system in iNGN cells.

4.1 Obtaining a homozygous cell line using CRISPR/Cas9 technology

For the CRISPR/Cas9 technology, the linear donor DNA coding for mKate2-AID tag flanked by approx. 1.5 kbp homology arms was introduced in the iNGN cells. The knock-in efficiency was 0.00032%, which is lower than the reported 0,2% HDR efficiency in iPS cells (Miyaoka et al., 2016). The reason for very low CRISPR/Cas9 efficiency could be the instability of the PCR amplified linear donor in cells because of degradation by exonucleases. To improve donor stability, the PCR product could be cloned into a plasmid and used as a donor for HDR. Nevertheless, genotyping, sequencing and fluorescence microscopy showed that 1,5 kbp homology initiated HDR at the DSB leading to mKate2-AID knock-in at the *DGCR8* 3' end. Synthetic short left and right homology arms can be used instead of long sequences, which would lower the possibility of introducing mutations during the cloning and lower the number of sequencing reactions needed to verify the bacterial clone without mutations. In a recent paper published by Natsume and Kanemaki (2016), more than ten endogenous proteins have been successfully tagged with AID using only 175-220 bp homology arms. However, they were only used in human cancer cell lines and in mouse embryonic stem cells indicating the need to test whether the short-arm donors are efficient for CRISPR/Cas9-mediated HDR in human iPS cell lines.

A diploid human genome contains two alleles of each gene making a homozygous knock-in a time consuming process that usually requires more than one transfection. In this experiment, one transfection was enough to initiate HDR at both alleles simultaneously. Homozygous insertion was found in 6 out of 24 (25%) FACS sorted clones. Using fluorescent tag as a marker was useful for localizing tagged DGCR8 but required time for cells to propagate before and after FACS sorting. Instead of a fluorescent tag, antibiotic

resistance markers can be used. Two donor vectors containing different antibiotic resistance markers can be co-transfected, in order to tag both alleles simultaneously by selecting cells in the antibiotic presence. In this way, it would be possible to generate aidDGCR8 cell line by a single transfection and subsequent propagation.

Some of the FACS sorted clones were homozygous for the wild type suggesting that FACS also captured false-positive cells due to autofluorescence. However, it was possible to isolate iNGN cell clones that were homozygous for the tagged DGCR8 as tested by sequencing the genomic loci. The expression of the DGCR8-mKate2-AID fusion protein was confirmed further by immunoblotting. These data highlights successful application of the CRISPR/Cas9 technology for generation of a homozygous knock-in, thus adding the auxin degron to endogenous DGCR8.

4.2 The auxin-based degron system in iNGN cells

After obtaining a homozygous cell line, the mTir gene was introduced with the piggybac system and three cell lines in total were produced, mTirDGCR8, aidDGCR8 and degDGCR8. The western blot showed partial degradation of the DGCR8 in degDGCR8 cells after 24h treatment in 500 mM IAA suggesting that the introduced auxin-based system in iNGN cells is to some extend functional. Therefore, it is likely that mTir binds to AID and initiates its ubiquitination and the degradation inside the proteasomes. It is known from previous studies that *DGCR8* knock-out stem cells retain the phenotype but proliferate slowly and accumulate in G1 phase of the cell cycle (Wang et al., 2008). The stagnation in proliferation was not detectable, possibly due to the only partial DGCR8 degradation.

As shown by the transcription analysis of *DGCR8*, auxin itself did not have any impact on DGCR8's mRNA levels, which was expected because auxin, as a plant hormone, should not affect any cellular gene expression process in humans. However, it seemed that the AID system in iNGN cells was not efficient enough to degrade DGCR8 after 24 h IAA treatment, as shown in previous studies where other proteins were degraded within only 30 min (Nishimura et al., 2009). DGCR8 was still present in degDGCR8 that was not treated with the IAA meaning that AID system was not activated without adding IAA. Two additional DGCR8 isoforms visible on the blot could indicate changes in posttranslational modifications of the DGCR8-mKate2-AID protein, which could lower the affinity of the AID for mTir after IAA binding. Moreover, it could be that one allele carries a mutation that results in premature stop codon and truncated mKate2-AID tag but was not detected by sequencing.

In line that DGCR8 showed partial degradation after 24 h, two typical stem cell miRNAs miRNA-302a and miRNA-103 were still expressed although miRNA-302a significantly reduced, indicating that DGCR8 function was likely reduced in an IAA-dependent manner but incomplete. Exiqon primers are optimised to detect mature miRNA forms and hence it is unlikely that pri-miRNA species before DGCR8 processing were detected. It is possible that insufficient auxin amounts were added to the cell media. To be able to add higher concentrations, the pH of the auxin solution or cell medium has to be additionally buffered that it does not result in increased cell death. In addition, the mKate2-AID tag might change protein conformation leading to a lowered affinity to mTir in the presence of auxin. The mKate2 fluorescence intensity was not very high at FACS sorting suggesting low expression levels or an altered protein structure resulting in lower fluorescence. To avoid potential changes in conformation, a longer linker sequence (George and Heringa, 2002) can be added between *DGCR8*'s C-terminus and the mKate2-AID tag or alternatively, the mKate2-AID tag must be fused to the N-terminus. Drosha binding domain is localized at the C-terminus of the DGCR8 (Lee et al., 2006), which could be the reason for the altered protein structure of the tag.

In conclusion, AID system is established but its function is incomplete and has to be optimized. Several other conditional degron technologies, which allow for controlling the stability of degron-fused proteins by use of a small molecule, have been established (Banaszynski et al., 2006; Bonger et al., 2011; Chung et al., 2015; Neklesa et al., 2011) and could potentially be used for the DGCR8 degradation instead of the AID system.

4.3 Assessing miRNA roles during *in vitro* neurogenesis

iNGN cells are offering an effective model for studying the functions and mechanistic roles of miRNAs in human because their miRNA expression profiles during differentiation resemble those in neurogenesis *in vivo*. Moreover, they are differentiating to neurons through the neural progenitor state (Busskamp et al., 2014a). Many different miRNAs are known to contribute to neuronal subtype specification *in vivo* from neural progenitors (rewied by Stappert et al., 2015). Therefore, degradation of DGCR8 can be initiated by adding auxin before and after iNGN cells reach the progenitor state to see the differences between the overall impacts of miRNAs at those two time points during iNGN differentiation to neurons.

Furthermore, the AID system can be used for studying different miRNAs functions during iNGN neurogenesis. miRNAs known to be upregulated during iNGN differentiation

can be spiked in the cell environment where there is none present because of the abolished processing pathway to rescue the phenotype and to assess their individual functions Additionally, different miRNAs can be spiked in at the same time for studying their possible interconnections. For example, miRNA-124 and miRNA-9 can be introduced. These miRNAs are already known to be neurogenic and can induce direct conversion of fibroblasts into neuronal-like cells (Yoo et al., 2011; Ambasudhan et al., 2011; Xue et al., 2013). Upon neuronal differentiation, BAF53a (subunit composition of ATP dependent BAF chromatin remodeling complex) is replaced by its homolog BAF53b. Ectopic expression of miR-124 and miR-9 induces the down-regulation of BAF53a, allowing the incorporation of BAF53b and neurogenesis (Yoo et al., 2009). Therefore, it would be interesting to see if miRNA-124 and miRNA-9 would converse the conditional DGCR8 mutant phenotype to the wild type, and at what time point during iNGN neurogenesis. A possibility for further analyses could be to look for target genes for the different miRNAs that prove to have an impact on neurogenesis. Target genes can be found in miRNA databases (Chou, 2016) and their expression analysed by a quantitative qRT-PCR reaction.

4.4 Using miRNAs as tools to modulate neuronal cell fate *in vitro*

Considering that miRNAs are emerging as important players during *in vivo* neuronal subtype specification, they could be exploited as additional tools to modulate neuronal cell fate decisions *in vitro*. miRNA-133b and miRNA-132 were shown to have a negative impact on the generation of dopaminergic neurons from mouse embryonic stem cells (Kim et al., 2007; Yang et al., 2012). On the other hand, miRNA-181a and miRNA-125b were found to promote the generation of dopaminergic neurons in human neuroepithelial-like stem cells (Stappert et al., 2013). A subset of midbrain dopaminergic neurons degenerate in Parkinson's disease and are therefore of particular interest for neuro-regenerative stem cell research. Therefore, the miRNAs that could possibly be found to have an impact on iNGN neurogenesis can be envisioned as tools to direct the differentiation of pluripotent stem cells, as iNGN cells, and derived neural progenitor cells towards medically relevant neuronal subtypes.

5. CONCLUSION

The auxin-based degron system for the inducible disruption of the miRNA processing machinery showed reduced DGCR8 function in IAA dependent manner. Such results suggest that the auxin-based degron system is established in iNGN cells, but nevertheless this system has to be further optimized.

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CURRICULUM VITAE

I was born on 19th of January, 1993, in Zagreb. In 2011, I enrolled Undergraduate school of Molecular biology at the Faculty of Science, University of Zagreb. During that time I participated in "Night of biology" and "6th opened doors of Department of chemistry at the Faculty of science", for which I received two Special Rector's awards in 2012 and 2013. On the third year of my undergraduate studies I had a laboratory practice in the field of plant proteomics under the supervision of Prof. dr. sc. Biljana Balen. In 2014, I enrolled Graduate school of Molecular biology. My second laboratory practice was under the supervision of Doc. dr. sc. Inga Marijanović, in the field of stem cell biology. During the winter semester of the academic year 2015/2016, I studied Regenerative Biology and Medicine at the TU Dresden, Germany, as an Erasmus+ exchange student where I had a laboratory practice in the field of stem cell biology and neuroscience under the supervision of Dr. phil. Volker Busskamp. From April to September 2016, I participated in Erasmus+ Internship programme as an intern in Center for Regenerative Therapies Dresden, where I conducted my graduation thesis.