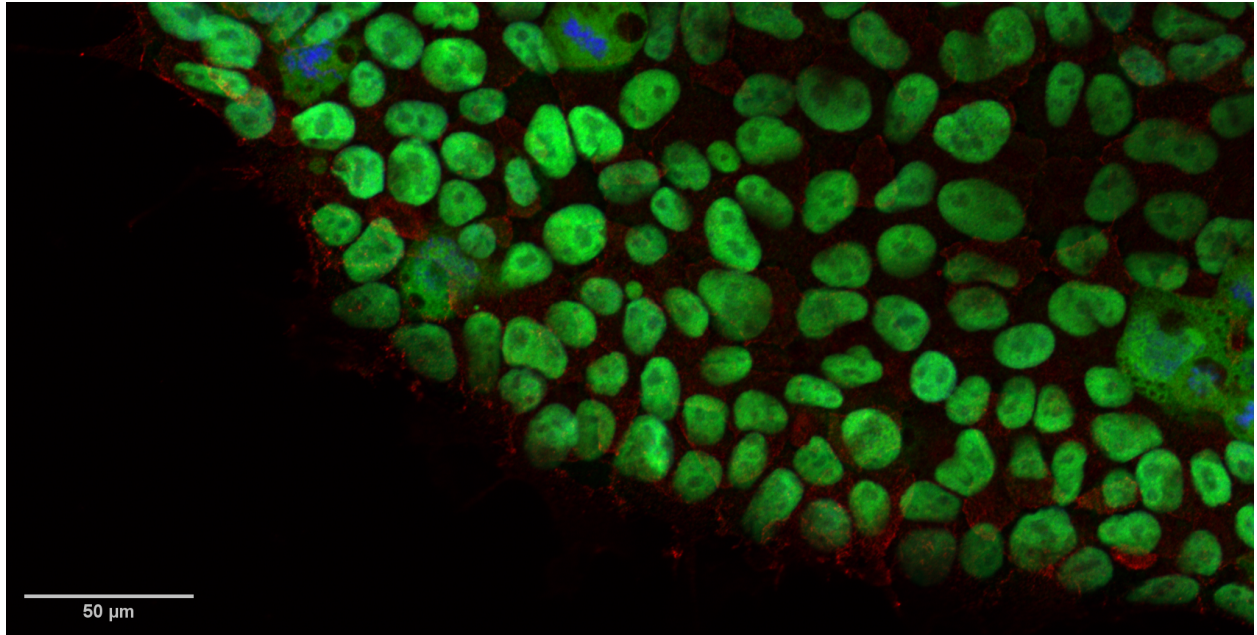




CHALMERS
UNIVERSITY OF TECHNOLOGY



Mitochondrial Implications Associated with 3q29 Schizophrenia Risk Copy Number Variant

Investigating phenotypic consequences of rare genomic alterations in embryonic stem cells and induced neurons

Master's thesis in Biotechnology

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DEPARTMENT OF PHYSICS AND ASTRONOMY

CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2026
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Cover: Confocal microscopy image of 3q29 edited human embryonic stem cells stained for pluripotency markers TRA160(red) and OCT4(green) together with nuclear dye DAPI(blue).

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Abstract

The 3q29 chromosomal region appear to have a significant role in normal neuronal development. The heterozygous deletion as well as the one copy duplication are linked to psychiatric conditions and neurodevelopmental disorders, respectively. Through genome wide studies, the 3q29 deletion has been identified as one of the greatest risk factors for Schizophrenia. The disorder is influenced by numerous genetic risk variants with unknown consequential mechanisms even after decades under scrutiny. Dysregulated mitochondria is hypothesized to play an upstream role in the pathogenesis, as this organelle generates the majority of cellular energy including that of synaptic formation and function. How 3q29 dosage alterations could implicate mitochondria is yet to be uncovered, hence the aim of this study.

Isogenic embryonic stem cell lines engineered using CRISPR technology were genotyped for 3q29 copy number. Seventy five clones were screened, resulting in three possible duplication clones and eleven deletion clones. Genotyped cell lines were expanded and characterized via staining of transcription factors indicating their pluripotency and undifferentiated state. Clones could thereafter be lysed in order to quantify protein subunits of electron transport chain complexes once the protocol was established. Several iterations of adjusting the Western blot methodology eventually resulted in detection of all subunits. Two batches of previously induced neurons were also investigated to bridge different time points during development. Preliminary results indicate that edited stem cells have a lower expression of mitochondrial protein complexes, with complex V appearing the most stable. The stability of complex V and the lower expression of protein SDHA in 3q29del cells are consistent with previous literature in mice. Neuronal protein complexes show an overall high variability except for the COX4 protein being more expressed in duplication clones.

To conclude, the identified stem cell lines together with the established Western blot protocol allow for further investigation targeting protein quantification of the transport chain complex subunits. The identified expression trends also motivate continuation of these experiments.

Keywords: 3q29, Neurodevelopment, Copy Number Variants, Genetics, Mitochondria, Schizophrenia, Embryonic Stem cells, induced Neurons

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Elin Esborn, Gothenburg, May 2026

Acronyms

The following acronyms have been used throughout the project, listed in alphabetical order.

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
B-Actin	Beta Actin
BSA	Bovine Serum Albumin
CNV	Copy Number Variant
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
D2	Dopaminergic subtype receptor 2
D	Day
Del	Deletion
DNA	Deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
Dup	Duplication
ETC	Electron transport chain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEK-293T	Human Embryonic Kidney cell line expressing SV40 large T antigen
hESC	Human Embryonic Stem Cell
iN	Induced Neuron
ICC	Immunocytochemistry
ID	Intellectual disability
iPSC	Induced pluripotent stem cell
kD	Kilodalton
MRI	Magnetic Resonance Imaging
Ngn2	Neurogenin-2
OxPhos	Oxidative phosphorylation
PFA	Paraformaldehyde
P	Passage
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
SCHEMA	Schizophrenia Exome Sequencing Meta-Analysis
SCZ	Schizophrenia
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween 20
VDAC	Voltage-Dependent Anion Channel
WT	Wildtype

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1

Introduction

Disorders with high heritability motivate mapping of the underlying genetic architecture. By association, rare genetic variants have been linked to disorders using genome-wide observational study designs [1]. Identification of these risk mutations provide clues about pathways and mechanisms worthy of scrutinization and consequential downstream processes. Such discoveries enable uncovering of common clinical phenotypes, an ultimate aim across studies in complex, heritable disorders [2]. By revealing vulnerabilities of distinct risk factors, these findings can allow for therapeutic targets and early intervention strategies.

One such identified region thought to require precise dosage for normal neural development and function is 3q29. Normally expressed in two alleles, a single copy deletion and duplication have associations with psychiatric conditions and neurodevelopmental disorders, respectively [3] [4]. The heterozygous 3q29 deletion has been identified as one of the strongest genetic risk factors for Schizophrenia, conferring a 40 fold increase in risk [5]. The 3q29 duplication, meaning three copies, has been associated with intellectual disability [3]. The cellular and molecular effects of 3q29 copy alterations are yet to be fully understood.

Schizophrenia affects as much as 1% of the world population and is considered chronic [6]. Typical onset occur in early adolescence with a preceding prodromal period with subtle changes in both behaviors and thinking [6]. The disorder is considered a major cause of disability worldwide, and life expectancy is reduced around 15 years [1]. The disorder is known to run in families, supported by twin studies reporting heritability estimates ranging between 60-65 percent [1]. Criteria in the Diagnostic and statistical manual of mental disorders (5th ed.) guide clinical assessment, where symptoms can be categorized in three domains ; positive-, negative- and cognitive symptoms [7]. Positive symptoms are those added to normal function, including hallucinations and delusions. Negative symptoms includes apathy and social withdrawal. Cognitive symptoms are implications related to thinking and attention, including retaining information in the working memory.

Three copies of 3q29, called a duplication syndrome, is associated with numerous abnormalities including intellectual disability (ID) [3]. ID involves deficits in functioning and adaptive skills including learning, problem solving, reasoning and abstract thinking [8]. A characteristic of ID is low IQ test scores of 70 at most, two standard deviations below the mean. Thus, the diagnosis is primary based on the implications of adaptive functioning and onset has to occur in childhood [9].

This thesis aims to address the gap between disorders associated with 3q29 dosage alterations and underlying biological consequences of such rare genetic polymorphisms. Disrupted pathways converging at the mitochondria could result in propagating downstream effects, but whether this organelle is implicated in development of 3q29 altered cells remain elusive. To enable the investigation, 3q29 edited human embryonic stem cell lines required genotyping to identify biological replicates of these model systems, followed by pluripotency characterization. Furthermore, a methodology had to be established to ultimately quantify mitochondrial proteins in stem cells together with differentiated neurons.

2

Background

2.1 Embryonic stem cell cultures

Human Embryonic Stem cells (hES) can be used as in vitro models to investigate early human development and differentiation [10]. Through in vitro fertilization, these cells are derived from the inner cell mass of blastocyst with indefinite proliferation capacity [11]. These cells are pluripotent, meaning they can differentiate into any cell type originating from the three embryonic germ layers: endo-, meso- or ectoderm. Cell line H1, one of the most extensively characterized cell lines in stem cell research, was used in this study [12]. Since derivation of these cells required destruction of a human blastocyst structure, this work comes with mild ethical concerns. Even though the H1 stem cell line is widely accepted, the crux surround the lack of consensus around what moment life beings [13].

It's important to acknowledge limitations of stem cells as model systems. First, the human brain is difficult to mimic due to it's high complexity, and the lack of physiological context such as vascularisation and immune function likely affect results. Second, the environmental and epigenetic influences are completely excluded in the closed systems of a dish. Especially in research of psychiatric disorders where the environmental influences have a significant role, these models do not reflect those contributions on neurobiology [7].

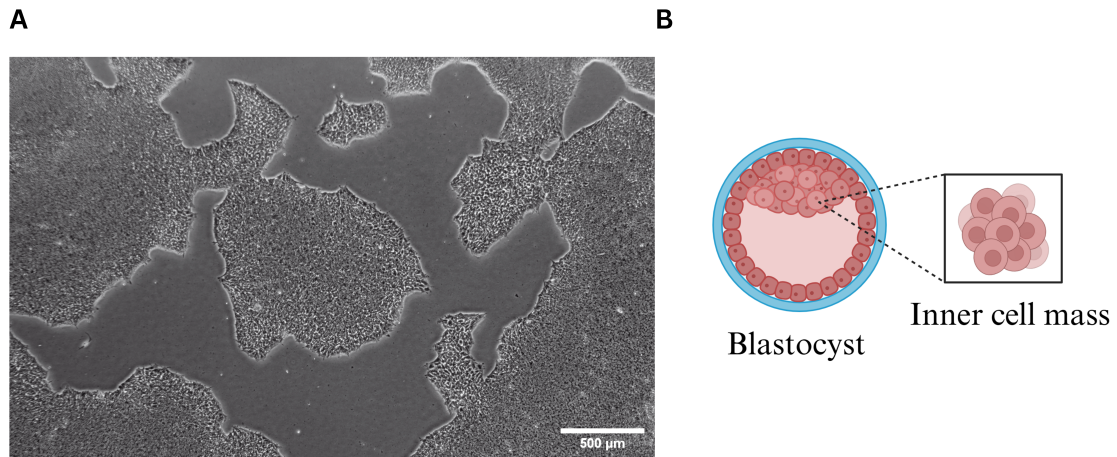


Figure 2.1: A) Morphology of human embryonic stem cells passage 44 cell line F65. Brightfield microscopy image taken under standard culture conditions. Scale bar showing 500 μm B) Schematic illustration of the human blastocyst structure including the inner cell mass, the trophectoderm and blastocyst cavity. Created in <https://BioRender.com>

2.2 Glutamatergic neurons induced through transcription factor expression

Differentiation of pluripotent stem cells to glutamatergic, excitatory neurons can be done using the Neurogenin-2-induction (Ngn2) method [14]. By forced overexpression of the transcription factor (TF) named Neurogenin 2, homogeneous cultures of neurons are generated fast and with high reproducibility [15]. The TF is delivered utilizing lentiviral vectors and placed under a doxycycline-responsive promoter, generating a Tet-ON system [15]. When the undifferentiated cells are exposed to the antibiotic Doxycycline, transcription of the Ngn2 gene is induced. By using this method, both the problem of brain tissue availability and ethical constraints are avoided. In this study, iNs differentiated from 3q29 edited hES cells were investigated for mitochondrial protein expression. These iNs are often co-cultured with glia cells as they provide structure and support for neuronal maturation [16]. Both cells from rodents and humans can be utilized for this purpose.

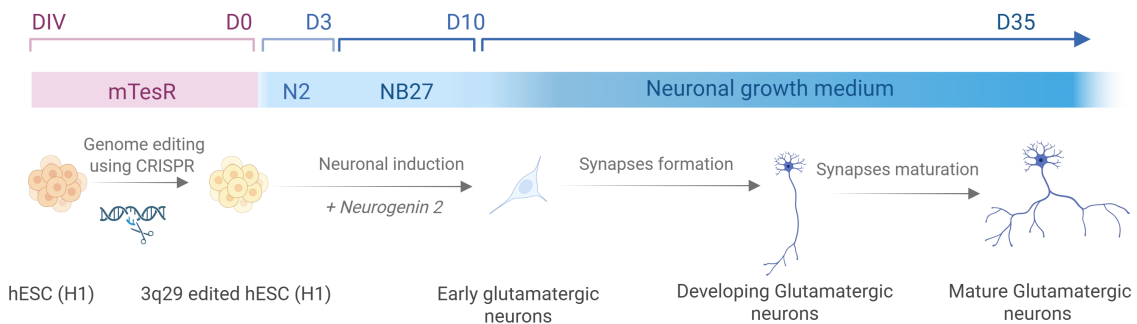


Figure 2.2: Induced glutamatergic neurons differentiated from 3q29 CRISPR edited hESCs from cell line H1. Through homologous recombination, alternative dosage of the target loci generate deletion, wildtype or duplication clones. The neuronal induction is induced by overexpression of the neurogenin-2 TF, and the timeline corresponds to stages of development. A defining hallmark is the formation of synapses as neurons evolve. Created in <https://BioRender.com>

2.3 Copy number variants and PAK2

Copy Number Variants (CNVs) are alterations of DNA segments ranging between kilo and megabases [17]. They have had an important evolutionary role in terms of introducing genetic variability in species [18]. When such polymorphisms occur in critical regions that require precise dosage, the consequences can have severe developmental implications. The alterations are often introduced during homologous recombination during meiosis, and more likely in regions flanked by low copy repeats with high genetic similarity [18].

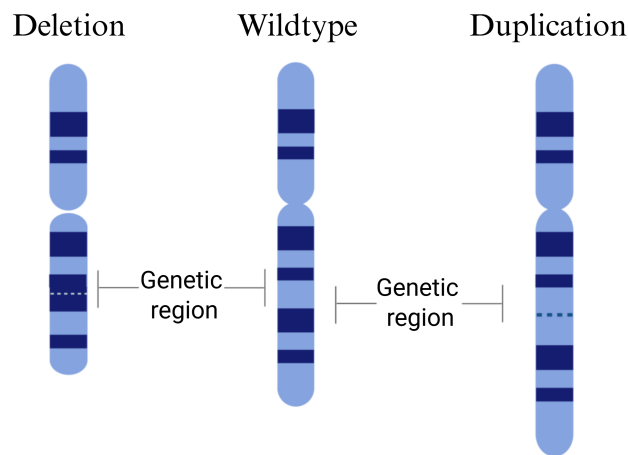


Figure 2.3: Modified schematic illustration of copy number variation Adapted from: [19] Created in <https://BioRender.com>

The 3q29 region is located at the long arm of human chromosome 3, spans about 1.6 megabases and includes 22 genes encoding proteins [5]. As mentioned, the heterozygous deletion and duplication of this loci have been associated with SCZ and ID, respectively [5].

The PAK2 gene is located within the 3q29 region encodes a multifunctional threonine kinase. [20]. In this study, PAK2 was during genotyping using a qPCR based method the target sequence expected to reflect the copy number of 3q29 dosage on a DNA level. This protein also served as a positive control during mitochondrial protein quantification in Western blot experiments. The assumption that PAK2 on a DNA level reflects transcriptional protein levels was based on evidence from single cell RNA sequencing results of human cortical organoids [5]. The study showed in several cell types, including neural progenitors and neurons, that 3q29 deletion clones produce less mRNA transcript than controls. PAK2 had one of the largest and most consistent transcriptional reductions across 3q29 protein encoding genes. Moreover, mRNA expression and protein levels are generally considered to be reasonably correlated [21]. The evidence from that study provide rationale for some choices in experiments for this study. Furthermore, illustration 2.4 show that PAK2 is located within the 3q29 region, while the gene TNK2 is found in the low copy repeat regions outside of 3q29.

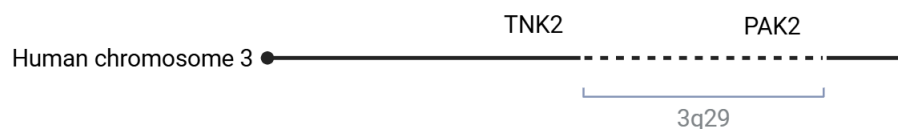


Figure 2.4: Schematic illustration of human chromosome 3 showing the PAK2 gene within the 1.6 megabase 3q29 region while TNK2 is located outside of it. Created in <https://BioRender.com>

2.4 Evolving understanding of Schizophrenia

Several influential theories attempt to explain the causes of Schizophrenia. One out of two longstanding, still relevant and evolving theories is the dopamine hypothesis [22]. In brief, excessive dopamine signaling is responsive for positive symptoms, such as hallucinations and delusions, and supported by symptom alleviation in patients using D2 receptor antagonists. Today's healthcare uses Dopamine receptor subtype 2 (D2) antagonists as the first line of pharmacological intervention for psychotic disorders [23]. The second persistent influential theory, called the neurodevelopmental model, proposes that the disorder arises from prenatal or early life events causing an abnormal trajectory of evolving brains [24]. The perturbation results in an increased susceptibility in individuals which likely onset in adolescence. Given this background, no single theory can fully explain the disorder and neither a single dysfunction. There are rather multiple interacting processes constituting Schizophrenia, classifying it as a complex disorder [25].

Synapses are sites of intercellular communication between neurons [26]. Dendritic spines are projections of neurons where excitatory synapses are located [26]. A growing and substantial body of literature show repeatedly reduced synaptic density and dendritic volume reductions in SCZ cases and models [27]. Several lines of evidence points in the direction; Post mortem-, patient derived induced Pluripotent Stem cells (iPSCs)-, PET- and MRI studies. Structurally, gray matter volume in cortical regions are predominantly attenuated in SCZ subjects [27]. These changes occur without reductions of neuronal or glia soma, but is attributed to the change in synaptic densities and dendritic volume reductions. The PET studies, allowing for investigation of synaptic densities in vivo, report less abundance reflected in synaptic markers in comparison to control [22]. Similar findings are found in iPSC studies, where reductions of both pre- and post synaptic markers and signaling alterations has been identified. This evidence also align with post mortem investigations, even though it's unclear whether antipsychotic medication is a confounder or even the driver of those findings [7]. Anyhow, compelling evidence from multiple approaches converge on the same observations, but causal mechanisms are insufficiently understood [1].

Studies in genomics have contributed with robust findings of risk factors, but also clues about biological pathways and cell types implicated in SCZ [10]. One such study published in 2022 by the consortium SCHEMA (Schizophrenia Exome Sequencing Meta-Analysis), was one of the largest exome sequencing analyses of SCZ associated genetic risk variants. In this meta analysis, high risk CNVs, protein truncating variants and common variants were compared in terms of risk for developing SCZ. The 3q29 deletion was reported to have the largest effect size of all risk alleles [4]. It is important to recognize that even the highest risk variant is considered an unfortunate risk that contribute to an individual's predisposition. Therefore, even rare, high risk variants like the 3q29 deletion are found in healthy individuals and only in a subgroup of patients [1].

2.5 The electron transport chain

The mitochondria is the primary energy generating organelle in eukaryotic cells, stored in bonds of Adenosine Triphosphate (ATP) [28]. Most ATP is generated through the oxidative phosphorylation (OxPhos) pathway where the electron transport chain (ETC) facilitates energy production. The ETC is located at the inner mitochondrial membrane and consists of five protein complexes. Electrons are shuttled from complex I-IV which drive proton pumping across the membrane. As protons flow down their gradient through the enzyme ATP Synthase, also referred to as complex V, phosphorylation of Adenosine Diphosphate (ADP) to ATP is facilitated.

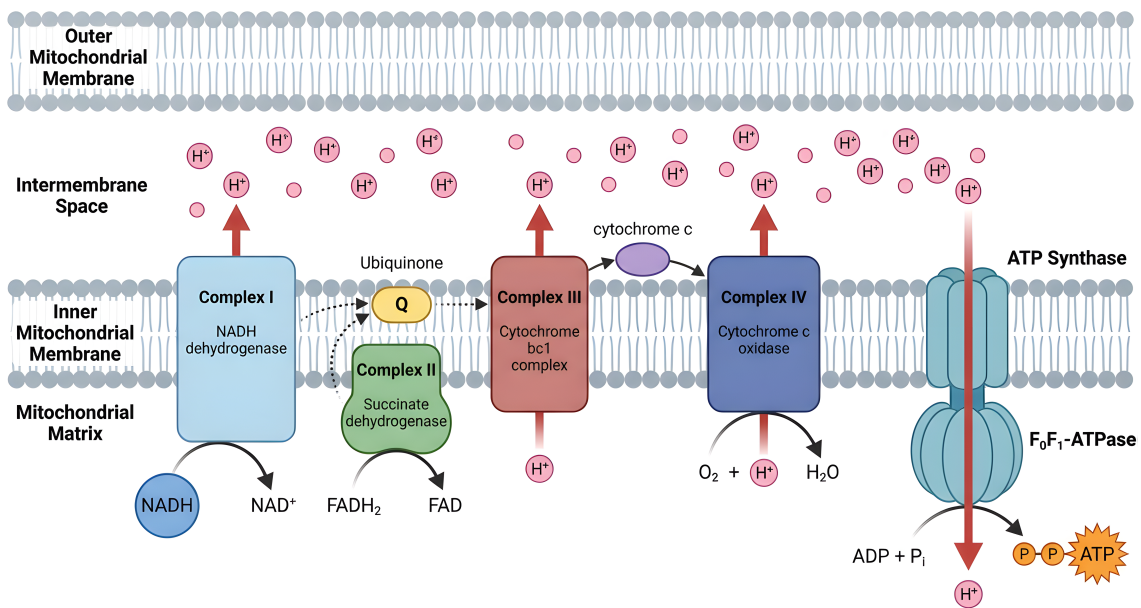


Figure 2.5: The electron transport chain located at the inner mitochondrial membrane facilitating ATP production through oxidative phosphorylation. Protein complexes consist of multiple subunits in which seven were quantified in this study. Created in <https://figurelabs.ai>

In this study, subunits of each protein complex have been identified and quantified using Western blot. These subunits include I-NDUF8B, II-SDHB, II-SDHA, III-UQCRC2, IV-COX2, IV-COX4, and V-ATP5A. These include both nuclear- and mitochondrial encoded proteins [29] [30].

2.6 Linking mitochondrial dysregulation to synaptic pathology

Returning to possible implications in neurodevelopmental disorders with a focus on SCZ, the mitochondria has been hypothesized to be implicated in the disorder [28]. Dysregulated mitochondria could be a possible contributor to the convergent findings of synaptic and dendritic spine reductions, the latter discussed in section 2.4 [28] [31]. Synaptic formation and function are energy intensive processes, where synaptic neurotransmission alone consume 80-90 percent of all cerebral ATP in developed neurons [28]. The dependence for sustained and high energy demand make these connections especially vulnerable to mitochondrial dysfunction.

In early development, neural progenitor cells shift from the energy system glycolysis to the more efficient oxidative phosphorylation to meet higher energy demands [5]. This stage is hypothesized to be a critical window underlying disruptions in neuronal signaling and metabolism [32]. The shift was mimicked in 3q29 deleted cells by Purcell and colleges by utilizing galactose medium followed by measurements of oxygen consumption indicative of energy system [5]. The authors found that 3q29 edited neural progenitor cells had a blunted response, indicating a worse adaptation to the intended energy system OxPhos. They additionally investigated the ETC in 3q29 deleted mice cortical cells, and found selective downregulation of OxPhos protein subunits II and IV. The structural finding could drive the impairment in adaptation of energy systems found in parallel, but this was not proved. The two downregulated subunits were II-SDHA and IV-COX4 which also are targets in this study. Here, the mice cell findings are aimed to be validated in human cell models (hESC and iNs).

A recent study by Robinette and colleagues modeled SCZ-risk variants in an in silico network with the intention of finding convergent consequential pathways [20]. Both the 3q29 deletion and 22q11 deletion had significant overlaps in pathways affecting mitochondrial translation. The 3q29 region was also identified to have the highest proportion of highly interactive proteins with the mitochondrial interactome, meaning mitochondrial encoded and associated proteins.

2.7 Fold Change Calculations of Gene Expression

Probe based quantitative Polymerase Chain Reaction (qPCR) is a specific and sensitive method to quantify nucleic acids [33]. The method utilizes florescent probes that specifically binds to the target sequence, and a signal proportional to the amount of DNA can be detected. Both the DNA template and the reference reactions are run in a thermocycler, including the steps of denaturation, annealing, and elongation of the DNA. For each cycle, the amount of DNA doubles, resulting in a signal increasing exponentially as described in equation 2.1.

$$N = n * 2^x \quad (2.1)$$

N =final number of DNA molecules, n =initial number of DNA molecules, x =number of PCR cycles.

Samples with more copies of the sequence in question have detectable signals at lower cycle numbers. For example, a single run for a heterozygous copy number deletion ($n=1$) lags one cycle behind the reference signal present in two copies ($n=2$). The difference in florescence between target gene and reference gene allows for relative quantitation. Cycle threshold, the C_t value, reflects the number of cycles until the florescent signal passes a set threshold.

$$\Delta C_t = C_{t,target} - C_{t,reference}$$

The ΔC_t value reflects how much target DNA there is relative to the reference gene in the same sample which corrects for technical variability.

The mean delta Ct of all technical replicates is thereafter found per sample.

$$\mu(\Delta C_t) = \frac{\sum_{w=1}^n (\Delta C_T)_w}{n}$$

The mean ΔC_t is also calculated for the calibrator sample which carries two copies of the target sequence. The amount of target sequence for each sample is therefore in relation to the calibrator as seen in the equation below.

$$\Delta \Delta C_t = \mu(\Delta C_{t,target}) - \mu(\Delta C_{t,calibrator})$$

The $\Delta \Delta C_t$ can furthermore be used to calculate the absolute copy number by using the logarithmic relationship between cycle threshold and DNA amounts described in equation 2.1.

2.8 Aims

Biological replicates of 3q29 altered clones were needed for this study. To identify replicates, seventy five CRISPR engineered isogenic lines of hESCs were genotyped using Taqman Copy Number Variation assay. Validated clones further intended for Western blot experiments require verification of pluripotency markers. This study used immunocytochemistry (ICC) to achieve this aim. To further quantify mitochondrial OxPhos proteins of hESCs, a Western blot methodology had to be established and optimized for the purpose. The time frame of this objective was undefined, but also an unavoidable step to progress to the final aim. That is, applying the established methodology to quantify OxPhos protein expression in the three genotypes of hESCs. The intention was also to extend the investigation to induced neurons to study several time points of development. Before proceeding to iNs, an additional experiment was required to investigate whether the glia cells, co-cultured with neurons, would contaminate results by also being targeted during immunoblotting. The experiment inform wether glia free neuronal cultures is a requirement for utilizing these cells in Western blots.

3

Methods

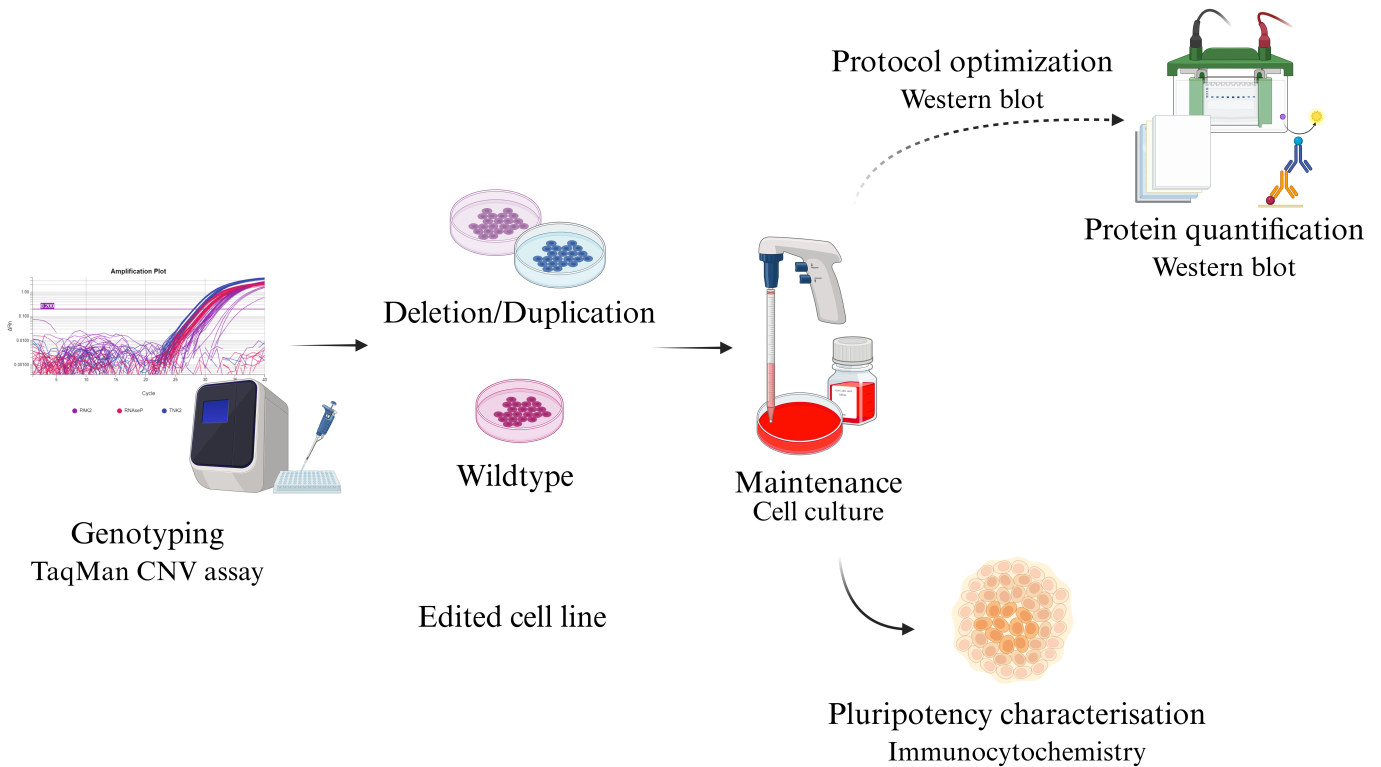


Figure 3.1: Overview of experimental workflow. Cells were characterized through genotyping by identifying their copy number of the 3q29 region. Several lines were cultured before characterization of pluripotency markers using immunocytochemistry (ICC). A western blot workflow was in parallel established, and mitochondrial proteins from stem cells in culture could be collected and quantified using the established methodology. Two batches of induced neurons were additionally investigated, but the induction itself was not included in this study. Illustration created in <https://BioRender.com>

3.1 TaqMan Copy Number Variation assay

Materials

Samples used for genotyping were wet genomic DNA from hES cells that previously had been picked using both Fluorescence Activated Cell Sorting (FACS) and Man-

ually (M). The DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, 69504) before frozen down and used in the Copy Number Variation Assay.

The TaqMan Copy Number Assay consisted of two primers and a FAM dye-labeled MGB probe. These were designed to target the PAK2 gene within the 3q29 region. The positive control was chosen to be TNK2, a gene located in the low copy repeat region outside of 3q29 (Illustration in section 2.3 figure 2.4). The TaqMan Copy Number Reference Assay contained two primers and a VIC dye-labeled probe. These were designed to detect RNaseP, a gene constitutively expressed and present in two copies in human diploid cells. Samples previously established to carry the 3q29 deletion (F13), duplication (F1) and Wildtype (F6) H1 cells through whole genome sequencing was used for comparison. Lastly, to mitigate contamination risk, a no template control was run simultaneously.

Method

DNA samples were extracted using DNeasy Blood and Tissue Kit (Qiagen, 69504) according to manufacturers instructions. Several samples were previously extracted using the same method and therefore directly thawed. In the final step of DNA extraction, 200 μ l AE elution buffer was added to samples before centrifugation 1 min at 6000g. To determine the concentration and purity of DNA, samples were analyzed using a spectrometer (Thermo scientific Nanodrop Spectrophotometer 1000). An absorbance ratio above 1.7 (280/260 nm) were considered pure and contained sufficient genetic material. These samples were diluted to 5 ng/ml in ddH₂O, while a few samples containing less genetic material (<1.7) was used up completely for the experiment. The PCR reaction mix was prepared using TaqMan Genotyping Mastermix(2x), PAK2 (20x) or TNK2 (20x), Taqman Copy Number Reference Assay (20X), and nuclease free water to a total volume of 11 μ l per well including 10 overage. 8 μ l of mastermix was added to a 96 well plate to each well, and samples were added in volumes of 2 μ l. The plate was sealed using an optical adhesive film before put into the thermocycler (Qunantstudio 3, Bio-Rad) with the cycling conditions listed in table 3.1.

Phase	ΔT	Stable temperature(°C)	Time (min)
Initial Denaturation	1.6*	95*	10
Denaturation	constant	96*	15
Annealing / Extension	-1.6*	60*	1

Table 3.1: Run Method for a total of 40 cycles

Data analysis in Copy Caller Software and Excel

Design and analysis 2.8.0 software (Applied Biosystems) was used for plate setup and run method. Applied Biosystems Copy Caller Software version 2.1 was thereafter the platform used to perform relative quantitation analysis. The software pre-process data to reduce noise and remove poor quality signals. The ΔC_t threshold were set to 5.5, meaning the lowest allowed amplification and therefore the filter value. The

relative amount of target gene to the reference were calculated, and the remaining $\Delta\Delta C_t$ and copy number calculations was carried out in excel. Full derivation of calculations are found in section 2.7.

3.2 Cell culture

H1 hES cells were obtained from WiCell (H1 RB18522 p33), HEK293T cells were obtained from ATCC (CRL-11268 lot 63696280), and glia cells were prepared in house. The ethical permits for mouse glia were the following ethical permit numbers: ID: 6419/24, Diarienummer: Dnr 5.8.18-13036/2024 (eligible until 25/09/28).

hES cells were stored in the -80°C freezer until cultured. Samples were thawed and cultured using dishes coated with Matrigel[®] and in mTesR plus medium. Every other day the medium was changed and passaged when confluency reached between 50-70%. All cells were maintained in a 37°C incubator with 5 % CO_2 . Stem cells prepared for immunofluorescent staining were passaged into Ibidi[®] plates and cultured until fixation. Cells used for western blots were collected in ependorf tubes and frozen down in -80°C until experiment initiation. Figure 2.1 A) is taken with brightfield microscope (ECLISPE TS2, NIKON) of hES cells in culture.

3.3 Immunocytochemistry

Cells were fixed using Paraformaldehyde(PFA) 4% with 5% sucrose for 15-20 minutes inside of the chemical hood at RT. The PFA was discarded and cells were washed three times with Phosphate Buffered Saline (PBS) with 5 minutes in between washes. Cells were blocked using 2% Bovine Serum Albumin (BSA) in PBS to prevent unspecific binding 1 hour at RT. The species specific primary antibody Mouse-anti-TRA160 (1:1000, channel 46465, Cell Signaling) was diluted in blocking buffer 2% BSA in PBS before added to wells and incubated overnight at RT. Secondary antibody (Goat-anti-Mouse, 700-channel, 1:1000, 925-68070, LICOR) was prepared together with nuclear staining dye DAPI (1:1000) in blocking buffer. Cells were incubated in solution 1 hour at RT.

To stain the nuclear proteins SOX2, OCT4 and NANOG, cells had to first be permeabilized using Triton-X100 0.3% in PBS for 10 minutes at RT. Species specific primary antibodies were prepared in blocking buffer using 2% BSA in PBS. The primary antibodies rabbit-anti-Sox2 (1:400, 3579, Cell signaling), Rb-NANOG (1:200, D73G4, Cell signaling), Rb-OCT4 (1:200, 27505, Cell signaling) were added to wells and incubated overnight. Species specific secondary antibodies were prepared in 2% BSA in PBS (Goat-anti-Rabbit, 1:1000, 800-channel, 925-32211, LICOR) and incubated one hour at RT. Plates were stored in -4 degrees until imaging using a Widefield microscope (ECLISPE TS2, Nikon)

3.4 Western blot

Protein samples were extracted from hESCs, iNs, mouse glia cells and human Embryonic Kidney cells carrying the SV40 Large T antigen (HEK-293T) in separate experiments. HEK-293T cells were utilized during the phase of protocol optimization presented in subsequent section 4.3.2 in order to save sample until the methodology was established. Evaluation of four parameters were targeted during the phase of protocol optimization; cell lysis methods, transfer conditions, blocking buffers, and primary antibodies. Once the methodology was functional it was applied on edited hES samples as well as the iNs. The materials and method presented in this section is that of the final protocol. In figure 3.2, the seven mitochondrial protein complex subunits that were targeted in this study are displayed. These are referred to as OxPhos proteins as they are components in the process of oxidative phosphorylation.

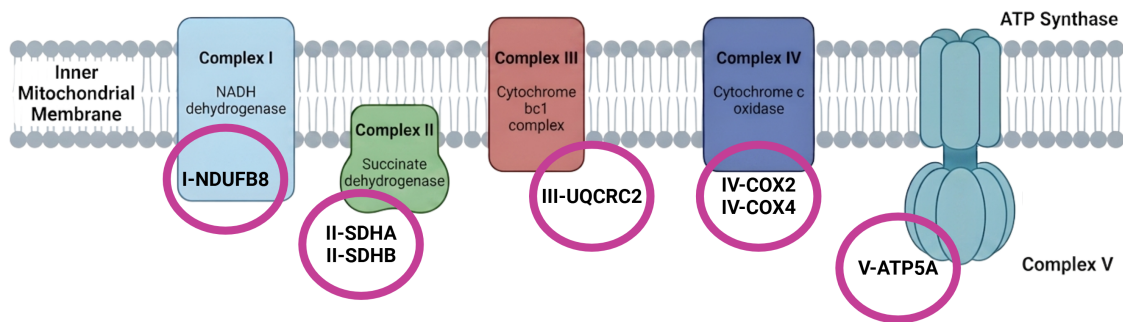


Figure 3.2: Electron transport chain with protein complexes consisting of several protein subunits. Purple circles indicate which complex (I-V) each subunit belongs too. The seven subunits in these circles were targeted in this study and referred to as OxPhos proteins.

Created in <https://BioRender.com>.

Materials and method

Cells were stored in the -80°C freezer before thawed on ice for 30 minutes. 100 ml RIPA solution (50x Roche, 100x PSMF) were added per sample. Cell lysis method included sonification on ice for 5 minutes and centrifugation at 1500g for 8 minutes at 4 degrees. The supernatant containing solubilized proteins were collected and pellet discarded.

A Bicinchonic acid (BCA) curve using Pierce BCA Protein Assay Kit was used to determine the concentration of samples. This was not a requirement for HEK-293T samples since the total protein amount was used and where therefore simply aliquoted in PCR tubes ($15\ \mu\text{l}$) before adding $3,75\ \mu\text{l}$ amount of 5x Laemmli buffer with DTT 100mM. The hES and iN samples were diluted to $1\ \mu\text{g}/\text{ml}$ i RIPA based on BCA curve before mixing with 5x Laemmli buffer with DTT 100mM.

Proteins were separated in a 4% sodium dodecyl sulfate - polyackrylamide gel elec-

trophoresis in precast tris-glycine TGX gels. 12.5 microliters of sample were loaded per well and 2 microliters of the BioRad Precision Plus Protein Dual Color marker (BIO-RAD). The gels were run at 10 min 80V and 60 minutes 160V.

The wet transfer from gel to nitrocellulose membrane was done in Towbin buffer 10x (25mM tris, 0,2M glycine and 20% methanol), soaking the transfer sponges, filter papers, membranes and gel. The transfer was ran in cold room (4°C) at varying conditions. 400 MA with constant up to 100Volts for 35 minutes. Transfer efficiency were checked by Ponceau staining before proceeding.

Several primary antibodies were used during immunoblotting to target the protein of interest listed in table 4.5. The primary antibodies for OxPhos proteins (Rb-Anti-Total OXPHOS Antibody Cocktail antibody, 1:1000, Ab317270, Abcam), individual protein OxPhos complexes SDHA (Rb-SDHA Polyclonal Antibody, 1:1000 PA5-66693, Abcam) and COX4 (Rb-Cox4 Monoclonal Antibody, 1:1000, MA5-15078, Abcam) as well as PAK2 (Rb-Pak2 Monoclonal Antibody, 1:2500, ab76293, Invitrogen) were incubated overnight in 4 °C after application. The species specific secondary antibodies (Goat-anti-Mouse, 1:10 000, 800-channel, 925-32210, LICOR) was applied and incubated 1 hour at RT for all mentioned proteins. Lastly, the loading controls, GAPDH (mono-Mouse, 1:5000, ab8245, Abcam), and B-actin (mono-Mouse, 1:1000, 3700, Cell Signaling Technology), were targeted with species specific secondary antibodies in the 700-channel (Goat-anti-Mouse, 700-channel, 1:1000, 925-68070, LICOR). Acquisition of images of membranes was done using Odyssey CLx imager (LI-COR).

Data analysis in Image Studio

Image studio (LI-COR) version 6.1 was the software used for Western blot quantification. Pixel intensities are proportional to protein amount within the linear detection range. Bands are restricted in both channels by hand drawn squares. The median background noise were subtracted from each square before normalizing each band to the assigned protein in the 700 channel. Normalization was done by first finding a normalization factor based on the well with the highest response. The remaining singals was divided by the factor to get normalization factors. The corresponding lanes were in turn divided by this factor to finally adjust for loading variability.

3.5 Usage of AI

The AI tools used during this thesis has been, for educational purposes, ChatGPT to discuss articles with to deepen the understanding about related topics. It also assisted in generating LATEX code and was used for sorting the list of Acronyms in alphabetical order. AI tools in Biorender and Figurelabs were utilized when creating figure 2.5 and 3.2 of the electron transport chain.

4

Results

4.1 Identification of genotypes in stemcells

Out of 75 screened hES cell lines, several were identified as possible duplication or deletion clones. Figure 4.1 illustrates the mean of the two technical replicates for calculated copy numbers.

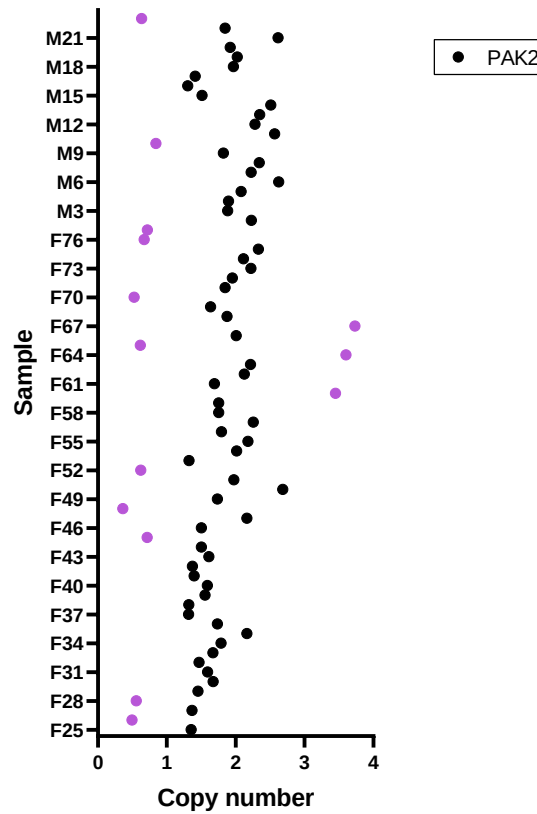


Figure 4.1: Genotyping for 3q29 copy number variants. Plot displays mean of calculated copy number for two technical replicates per sample. Purple datapoints indicates candidate deletion- or duplication clones, while black indicates wildtype. Every third name of samples are displayed on the y-axis where F: FACs sorted clones; M: Manually picked clones

A total of three duplication clones and eleven deletion clones were identified, but copy numbers were interpreted at a continuum. The most promising 3q29 alterations

where repeated in the assay. The second time around, an additional two clones were included for a total of 4 technical replicates per sample. Control protein TNK2 located in the low copy repeat region outside of 3q29 was additionally included to ensure specific CRISPR editing, also displayed in figure 2.4. Table 4.1 presents an overview of cell lines included in either every round of experimentation or where they served a purpose for comparison with the newly identified clones. The high confidence in copy number of these lines is due to validation using Whole Genome Sequencing (WGS).

Cell line	3q29 Genotype	Role
H1	Wildtype	Calibrator sample
F1	Deletion	Comparative sample
F6	Wildtype	Comparative sample
F13	Duplication	Comparative sample

Table 4.1: Cell lines included in either every Taqman copy number analysis experiment or when serving the purpose of comparison. All data is expressed relative to H1(WT), and all comparative samples have been validated through whole genome sequencing

Results for the mean calculated copy numbers are displayed in figure 4.2, each datapoint representing a single well. Again, PAK2 expression reflects 3q29 copy number, while TNK2 is the positive control expected in two copies.

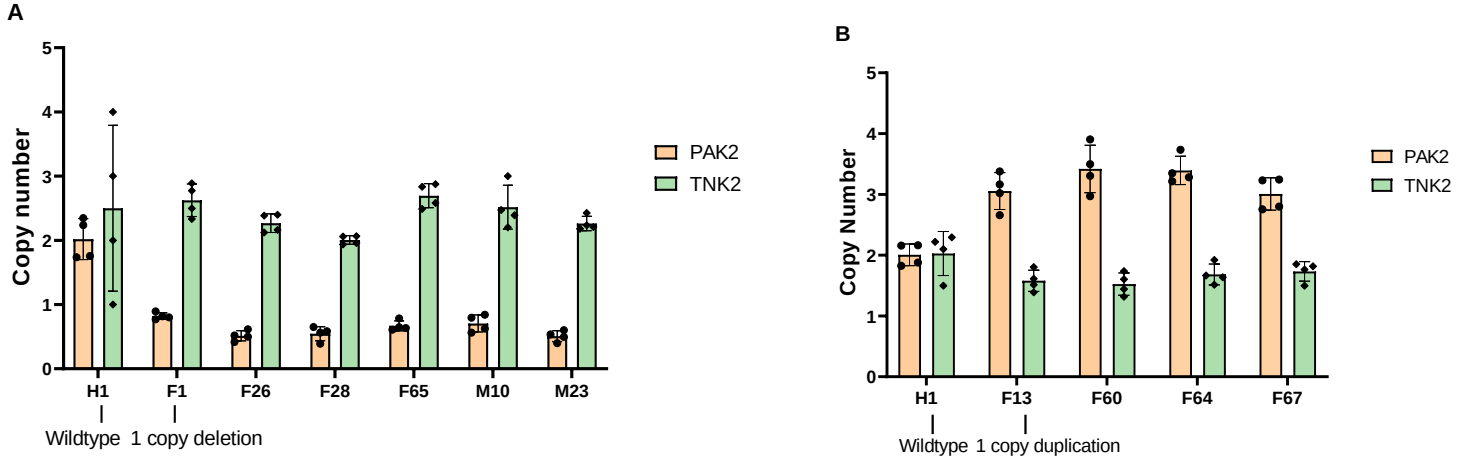


Figure 4.2: Mean and standard deviation of 3q29 copy number for four technical replicates. **A)** Relative PAK2 abundance, reflecting copy number of 3q29, in candidate deletion clones together with positive control TNK2 expected in two copies. **B)** Relative PAK2 abundance, reflecting 3q29 copy number in candidate duplication clones together with positive control TNK2 expected in two copies

Candidate Del/Dup clones all displayed expression of PAK2 close to corresponding expected copy number. Expectations were based on data in figure 4.1 displaying the first experimental run including these clones. TNK2 also appeared consistent across samples, but with some uncertainty in the calibrator sample H1(WT) showing high

variability in plot A of figure 4.2. One cell line per genotype were picked for proliferation in culture based on this experiment. Since all screened clones looked promising based on data in figure and 4.2 A) and B), the choice of clones could have been any of these. The picked clones were decided to be F65(Deletion), F60(Duplication) and F62(Wildtype). The WT clone was based on data in Figure 4.1 as this genotype was the most probable out of all CRISPR edited clones and therefore did not need an additional assay.

The clones picked for proliferation in culture required an additional assay run with calculated copy numbers displayed in figure 4.3. The reason of the additional experiment was due to a questionable result in a future Western blot experiment when quantifying PAK2 protein expression in F60(Dup) appearing close to that of the Wildtype clone F62. The assumed correlation between genetic makeup and protein expression was discussed in background section 2.3.

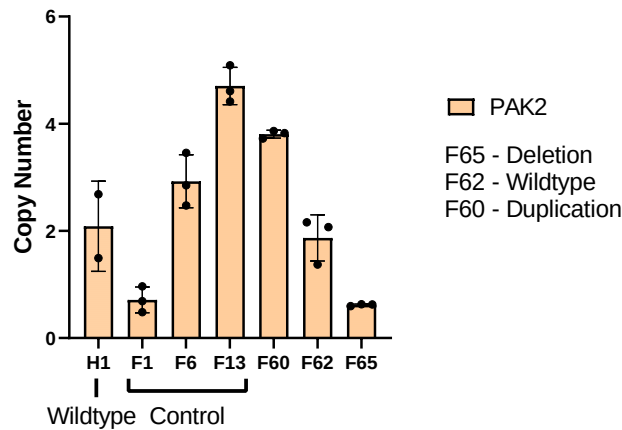


Figure 4.3: Mean and standard deviation of copy number for three technical replicates. The experiment was conducted to verify genotypes of F60, F62 and F60 due to conflicting results in a future experiment. H1: Wildtype and calibrator sample; Comparative samples: F1: 1 copy deletion; F6: Wildtype F13: 1 copy duplication

Noteworthy of figure 4.3 is that the calibrator sample H1(WT) appears in two replicates due to the third having an undetectable signal from PCR amplification. Clones included for comparison (F1, F6, F13) and the newly identified clones (F65, F62, F60) are all displayed relative to the calibrator sample H1(WT). Only having two replicates that in turn appear variable likely shift the results of all copy numbers. Therefore, the main takeaway from this experiment were that clones are likely aligned with their expected copy numbers of 3q29, but with some uncertainty of the F60(Dup) clone as this bar appear in between F13(Dup) and F6(WT).

4.2 SOX2, NANOG, OCT4, and TRA160 expressed in hES cell lines

To ensure that picked stem cells are pluripotent and undifferentiated, stem cell markers SOX2, NANOG, OCT4 and TRA160 were stained for. Stem cell properties were identified for all included cell lines displayed in figure 4.4. The quality of cells was therefore sufficient to continue culturing these lines intended for Western blot experiments. Additionally, nuclear dye DAPI(blue) was included to ensure individual cells would be visible.

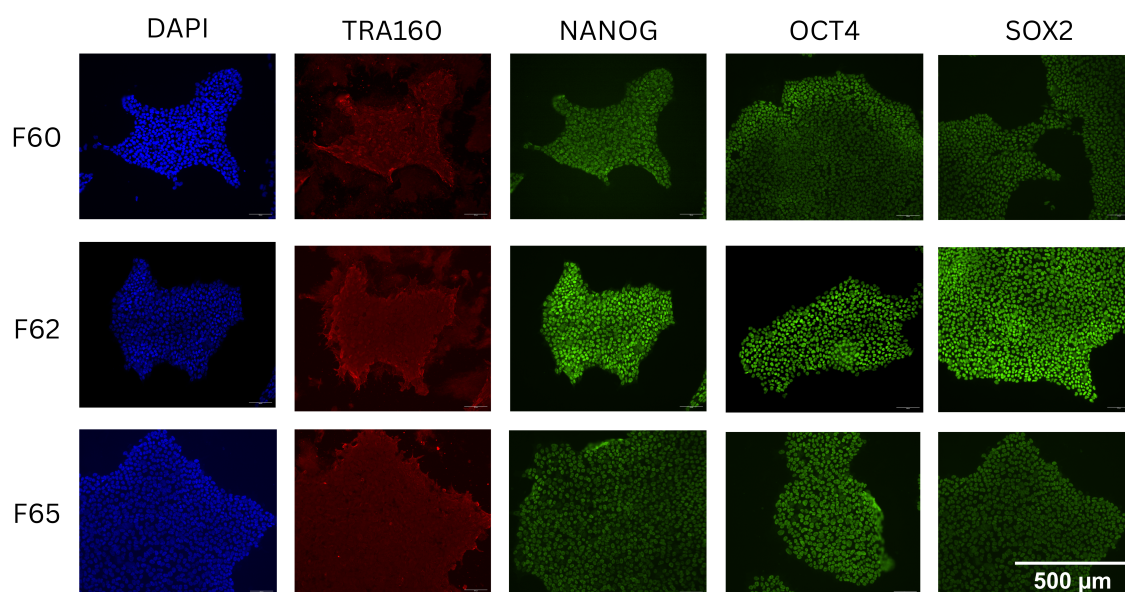


Figure 4.4: Immunocytochemistry using fluorescent markers of embryonic stem cell lines F60(Dup), F62(Wt) and F65(Del) expressing pluripotency markers. Nuclear markers include SOX2, NANOG, OCT4, while TRA160 expressed at the cellular surface. Nuclear Dye DAPI was included to ensure visualization of all cells.

4.3 Quantification of mitochondrial protein subunits using Western blot

4.3.1 Initial targeting of OxPhos proteins in edited hES cells

Experiment 1: testing the method

The first Western blot analysis was conducted using 3q29 edited Del/WT/Dup p48 hES cells. The target of quantification were five mitochondrial protein subunits (OxPhos proteins) and the positive control was PAK2 expected to be less abundant in 3q29 deletion clones and in higher quantity for duplication clones. OxPhos proteins were normalized against Glyceraldehyde -3 Phosphate Dehydrogenase (GAPDH), and PAK2 against Beta-Actin (B-actin) as these proteins are constitutively expressed in human cells.

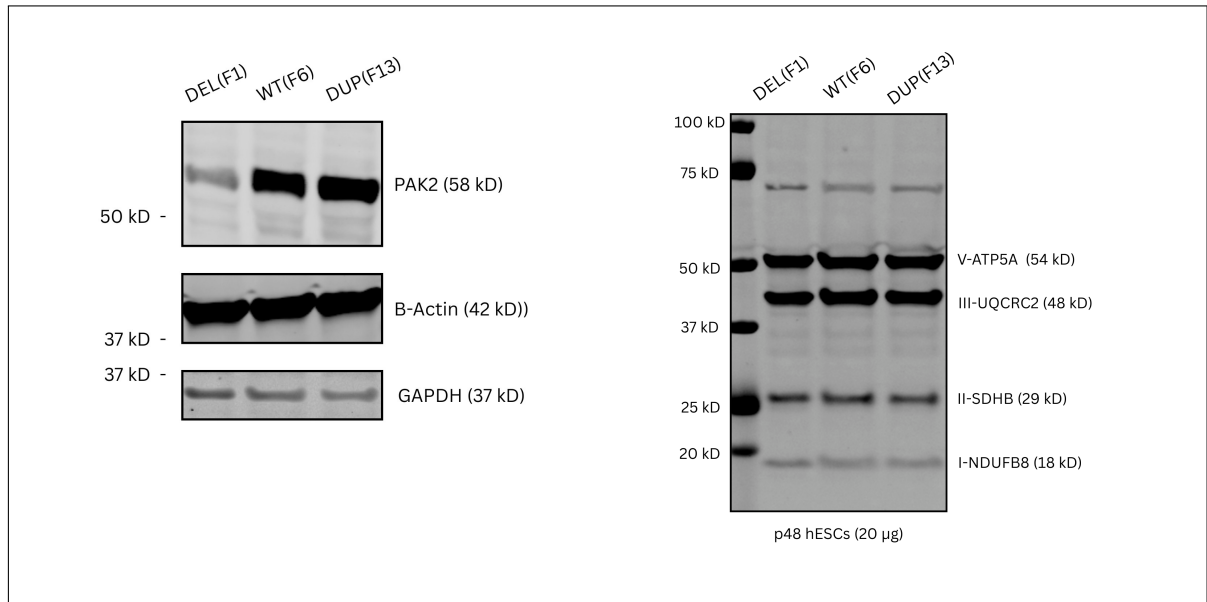


Figure 4.5: Positive control PAK2 with reference proteins GAPDH and B-Actin. Four out of five mitochondrial protein subunits (OxPhos proteins) was identified in P48 hES cells.

As expected for a heterozygous deletion, PAK2 appeared visually weaker in sample F1(Del) relative to WT(F6) and DUP(F13). As the OxPhos membrane in Figure 4.3 displays, OxPhos protein detection was incomplete. Complex IV-COX2 expected at 22 kiloDalton (kD) did not appear on the membrane. The band detected at 70 kD is considered unspecific binding of antibodies.

4.3.2 Protocol optimization

In order to continue the investigation of 3q29 dosage effects on mitochondrial protein expression, all OxPhos proteins had to be detectable. To achieve this the methodology required modifications. In section 4.3.2, all adjusted parameters during the phase of protocol optimization are presented with corresponding tables. Each experiment is then outlined in chronological order with reasoning behind adjustments. All experiments in section 4.3.2 was done in Human Embryonic Kidney (HEK-293T) cells in order to save sample.

Cell lysis

The initial step of the Western blot assay is protein extraction and separation from cells. The treatments were evaluated subjectively with the aspiration of getting low viscosity samples to decrease loading variability in gel and high protein yield on the final membranes while minimizing risk of protein degradation. All mechanical and thermal stressors that were applied at different time points are listed in table 4.2.

Treatment	Ultrasound (min)	Centrifuge 1500 rcf (min/°C)	Metal bath (°C)	Metal bath (min)
1	–	–	–	–
2	5	–	–	–
3	–	–	37	30
4	–	–	70	80
5	10	8/4	–	–
6	5	8/4	–	–
7	5	8/4	37	30
8	5	8/4	70	8

Table 4.2: Treatment options for cell lysis

Transfer conditions

During transfer, negatively charged proteins migrate from gel to membrane due to the application of an electrical field. Efficiency is impacted by transfer time and applied current, and these parameters in should be adapted to the molecular weights of proteins. For example, a lighter protein transfers quick but is also at a higher risk of passing through the membrane. Table 4.3 lists the tested currents, voltages and transfer times while figure 4.6 illustrates the setup across experiments.

Time (min)	Ampere (A)	Volt (V)
40	0.4	100
≈42	0.4	100
30	0.5	75–100

Table 4.3: Conditions during transfer

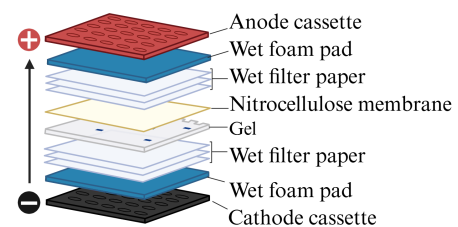


Figure 4.6: Transfer setup. Negatively charged proteins migrate towards the anode by applying an electric field

Blocking buffer

Detection of proteins during immunoblotting can be disrupted by excessive blocking, leading to weaker bands disappearance as the imaging process depends on florescent signals. Bovine Serum Albumin (Sigma Aldrich) in TBS-T were the weaker option to Intercept blocking buffer (LICOR-BIO) during this study.

Blocking buffer
Intercept blocking buffer (LICOR-BIO)
3% Bovine Serum Albumin (Sigma Aldrich) in TBS-T

Table 4.4: Blocking buffers to prevent unspecific binding of primary antibodies

Primary antibody

Primary antibodies are used to target the protein of interest during immunoblotting by binding. The florescent secondary antibodies do in turn bind the primary, enabling protein identification during imaging. In this study, multiple batches of the same antibody were included to exclude batch variation. The first four in the list of antibodies in table 4.5 are cocktails, meaning multiple antibodies are included in a single vial.

Primary antibody	Dilution	Catalog #	Lot #
OxPhos Human WB antibody	1:300	Invitrogen 45-8199	AC4650214
OxPhos Human WB antibody	1:300	Invitrogen 45-8199	79626621
OxPhos Human WB antibody	1:300	Invitrogen 45-8199	80055753A
Rb-Anti-Total OXPHOS Antibody Cocktail antibody	1:1000	Ab317270	1088747-40
Rb-SDHA Polyclonal Antibody	1:1000	PA5-66693	R90361
Rb-Cox4 Monoclonal Antibody	1:1000	MA5-15078	79983358
Rb-Pak2 Monoclonal Antibody	1:2500	AB76293	1026246-27
GAPDH mono mouse	1:5000	ab8245	-
Actin- β (8H10D10) mono Mouse	1:1000	3700	-

Table 4.5: Primary antibodies used for immunoblotting

Experiment 2: cell lysis methods

The target of modification for the first experiment in HEK-293T cells was cell lysis methods. The viscosity of samples were considerably implicated by treatment choice. Samples exposed to ultrasound and centrifugation had a less viscous character and were therefore easier to load in the gel used during electrophoresis. The resulting membrane displayed in Figure 4.7 were still lacking OxPhos protein COX2, so the conclusion could be drawn that the problem was not to be solved by different lysis methods. Thus, loading of gel using less viscous samples seemed beneficial to minimize variability. The ultrasound and centrifugation was therefore brought into future experiments.

All figures of HEK293T cells have corresponding lysis methods above individual lanes of the membrane. These methods are additionally listed in table 4.2. Each table below membranes additionally contains a hyperlink by clicking "(Table 4.2)" to the different cell lysis treatments.

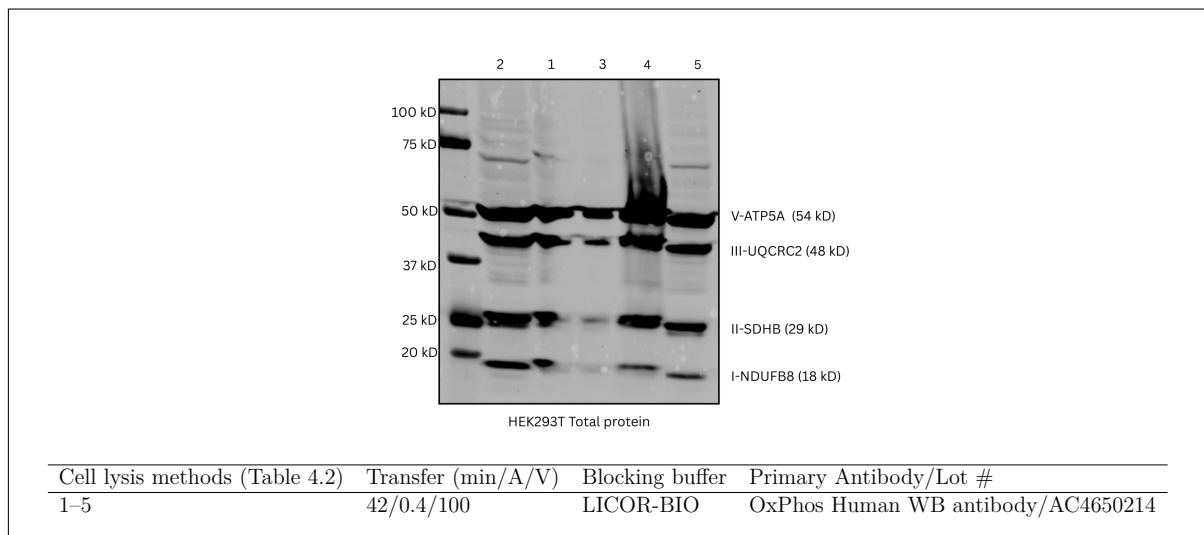


Figure 4.7: HEK-293T cells with cell lysis methods as target modification

Experiment 3: new primary antibody batch and reduced transfer time

A new batch of the OxPhos Human WB antibody (Invitrogen) was applied in the immunoblotting. The antibody batch from Experiment 2 was again applied on a separate membrane to ensure that no other influences affected the outcome. All cell lysis methods included ultrasound, and all but one centrifugation. The resulting membranes were still lacking OxPhos protein COX2 of complex IV.

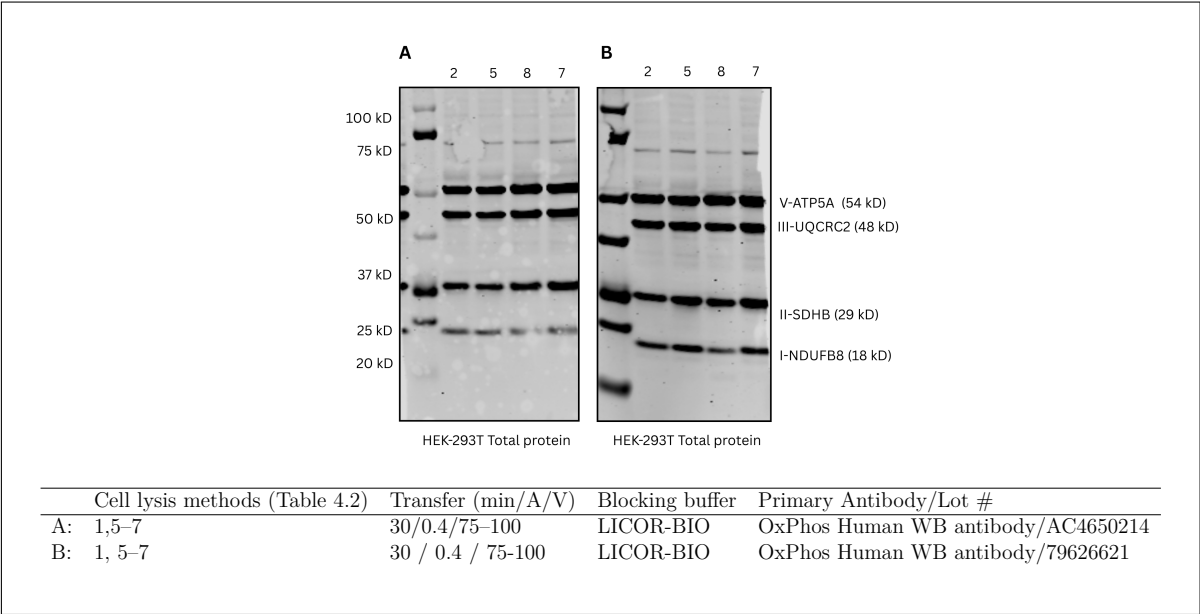


Figure 4.8: Test of new OxPhos antibody batch. **A)** Old lot AC4650214 **B)** New lot 9626621

Experiment 4: targeting individual protein subunits SDHA and COX4

The following experiment was conducted with the purpose to target only two subunits of the OxPhos proteins. These subunits, SDHA and COX4, belong to the same are simply different subunits than previous experiments but belong to the same complexes II and IV (explained in section 3.4). Other experimental conditions were kept the same as in the previous experiment 4.8. Both proteins, Figure 4.8 display successful detection of both SDHA and COX4.

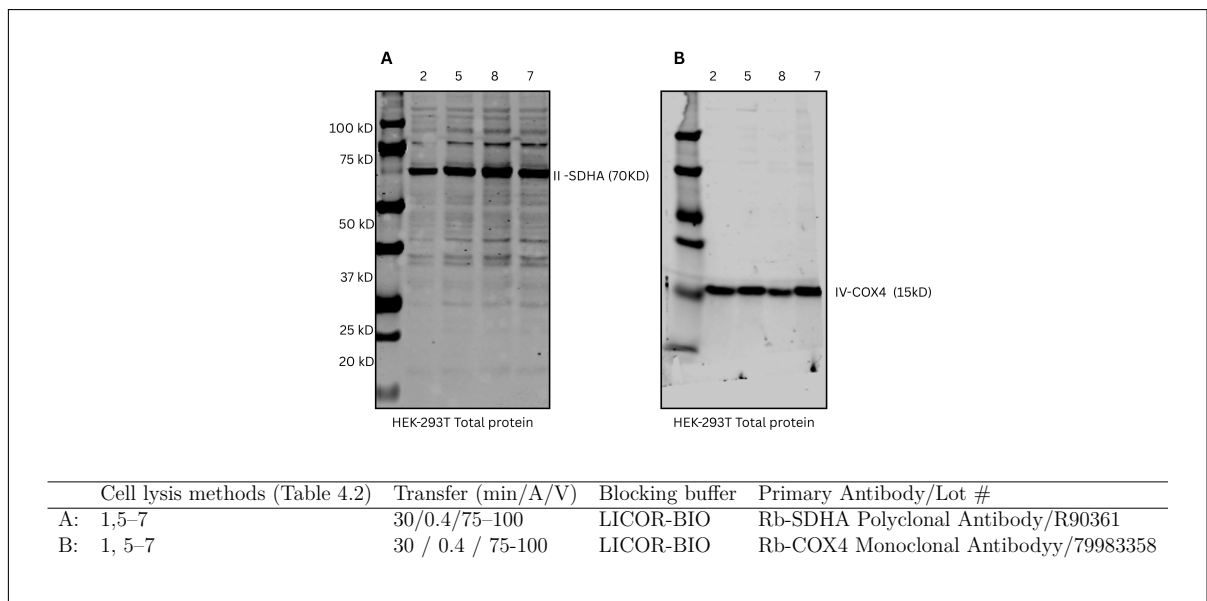


Figure 4.9: Targeting of two OxPhos proteins with independent antibody batches Rb-SDHA Polyclonal Antibody and Rb-COX4 Monoclonal Antibody **A)** Detected II-SDHA at 70 kD. **B)** Detected IV-COX4 at 15 kD

Experiment 5: blocking with alternative buffer

After consultation with other researchers, it was hypothesized that two factors where making complex COX2 disappear when utilizing the antibody cocktail. First, excessive blocking could be hiding the smaller protein band. Second, some proteins are heat sensitive and can therefore not be boiled during cell lysis. Therefore, experiment 5 excluded all metal baths during lysis, and switched to BSA 3% in TBS-T as an alternative blocking buffer. Resulting membrane displayed in figure 4.10 appeared to have a faint band at 22 kD, the height of COX2, but was too blurry and weak for quantification. The conclusion was drawn that a slightly stronger COX2 protein appeared using the previous blocking buffer from LICOR-BIO and was therefore kept in subsequent experiments.

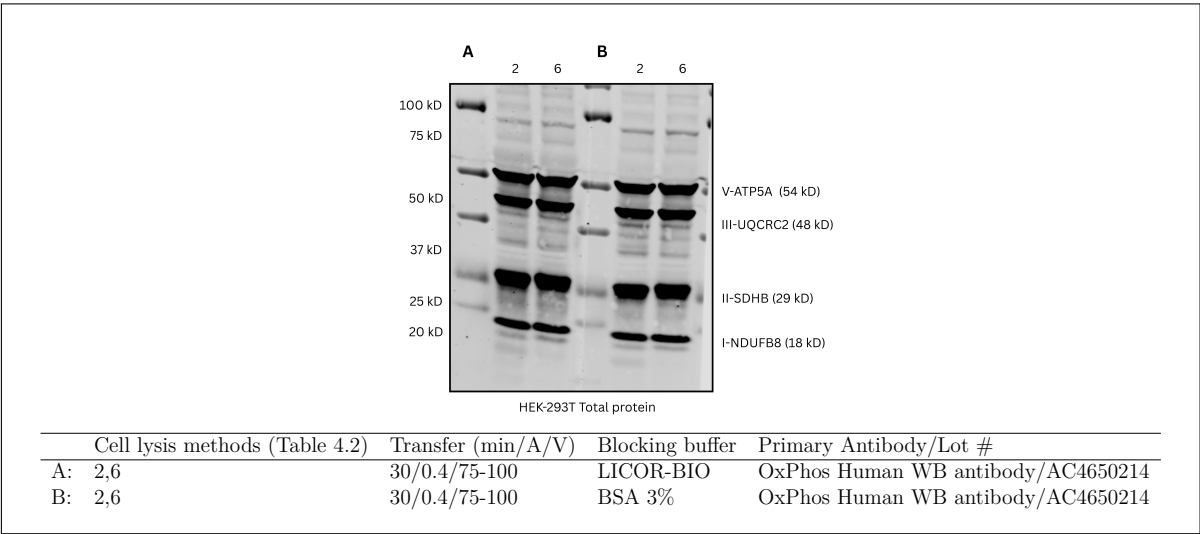


Figure 4.10: Target adjustment was reducing blocking, used to prevent unspecific binding during immunoblotting, to allow weaker florescent signals appear during imaging. **A)** Blocking buffer from LICOR BIO **B)** Weaker alternative, BSA 3% in TBS-T

Experiment 6: new primary antibody batch

When discovered that the previous two batches of OxPhos Human WB antibody (Invitrogen) came from the same production batch, a third was delivered from the antibody supplier. The purpose of experiment 6 was to test this third new batch. The resulting membrane was still lacking COX2.

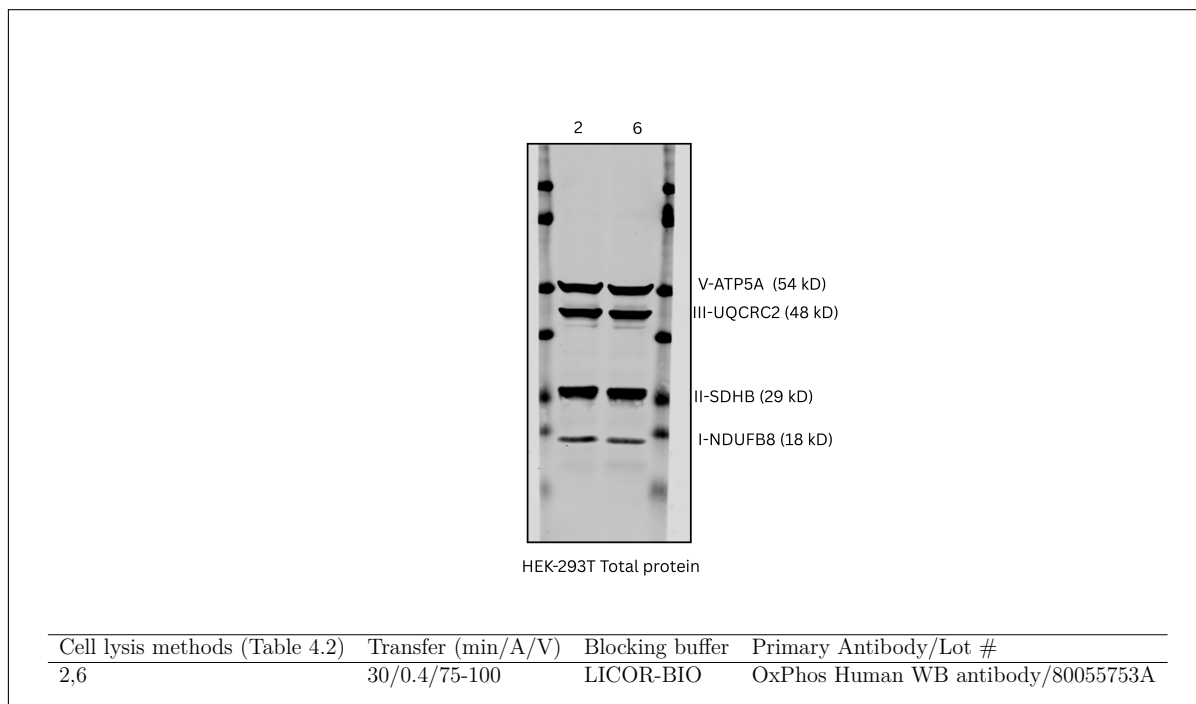


Figure 4.11: Target adjustment: Test of a new Invitrogen antibody produced in a separate batch

Experiment 7: primary antibody cocktail from Abcam

A new primary antibody cocktail (Rb-Anti-Total OXPHOS Antibody Cocktail antibody, 1:1000, Ab317270, Abcam) was tested. Lysis method, Transfer time, blocking buffer were kept same as the previous experiment 6. All OxPhos proteins were detected and a conclusive protocol was finalized.

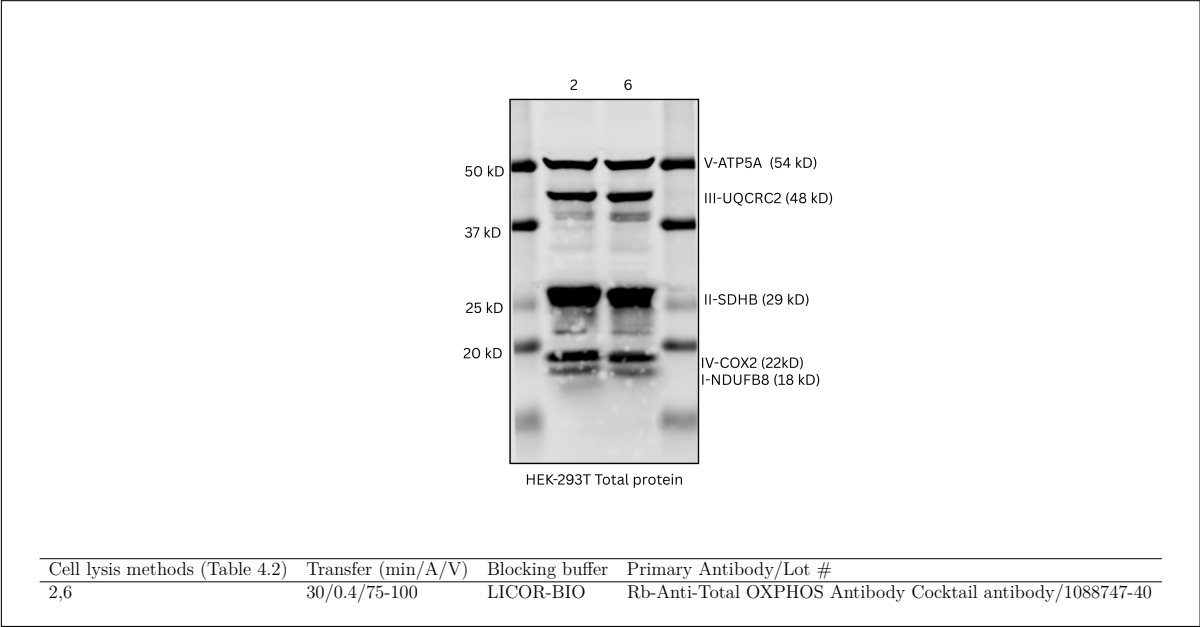


Figure 4.12: Target adjustment: Immunoblotting with Anti-Total OXPHOS Antibody Cocktail antibody. All five OxPhos proteins were successfully targeted.

4.3.3 Final OxPhos protein quantification in edited hES cells

With the established protocol, quantification of OxPhos proteins in hESCs could be initiated. Experiments included clones two cell lines per genotype listed in table 4.6.

Cell line	3q29 Genotype	Number of experiments
F1	Deletion	2
F6	Wildtype	2
F13	Duplication	2
F65	Deletion	1
F62	Wildtype	1
F60	Duplication	1

Table 4.6: Summary of all hES cell lines in which OxPhos proteins were quantified using the established Western blot methodology

With a total of three experiments in stem cells from two independent cell lines per genotype, the presented data simply display trends without the inclusion of statistical tests. That is to avoid drawing population based conclusions from an unrepresentative sample. When more than one experiment was conducted for a single cell line, this is shown as the mean expression in a single datapoint. All OxPhos expression is presented relative to Wildtype clones ($y=1$).

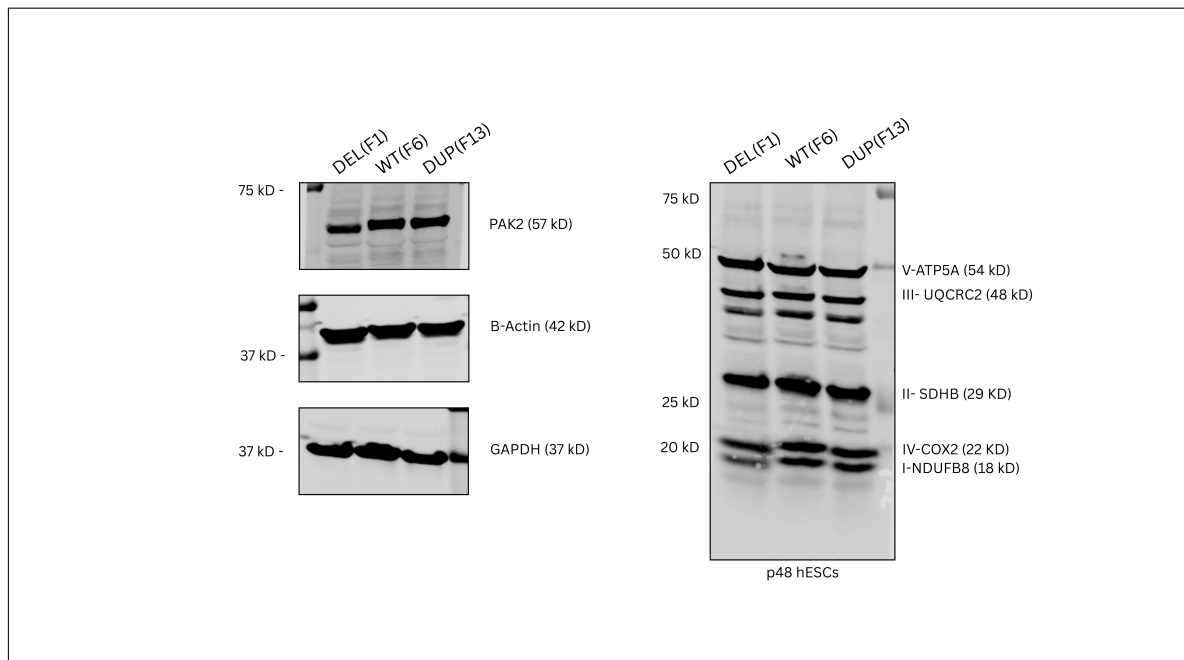


Figure 4.13: Representative membranes out of three in stem cells. Included positive control was PAK2 normalized to B-Actin. GAPDH was used for normalization of OxPhos proteins collected from P48 hES cells

Assessment of assay reproducibility in hESCs

Figure 4.14 shows the variability of the assay itself by comparing two independent experiments using clones from the same cell line for each genotype. Since these are clones originating from the same lines F1, F6 and F13, low biological difference is expected and is why they are suitable to evaluate the reproducibility of the assay itself. Data reveal high variability for most OxPhos complexes with more stable expression in duplication clones in complexes III-V.

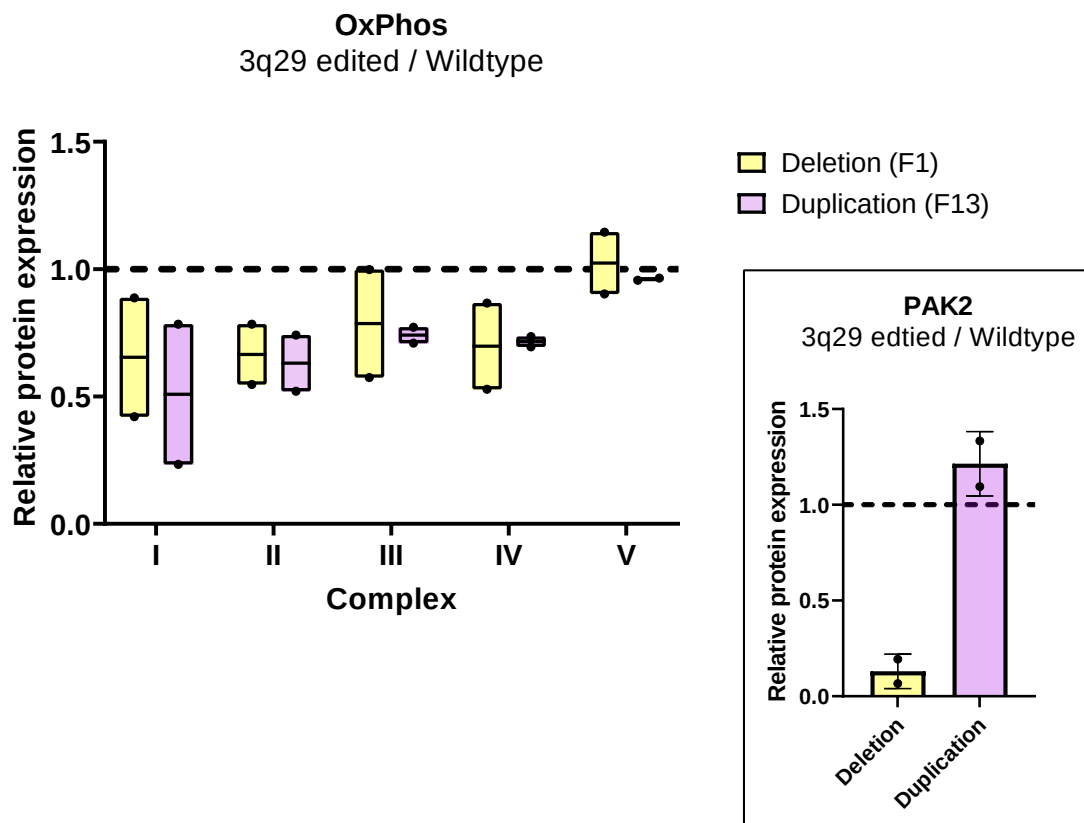


Figure 4.14: Mean and standard deviation of OxPhos proteins from two independent experiments using one biological replicate per genotype; Deletion: F1, Wildtype: F6; Duplication: F13. Data is relative to wildtype, and PAK2 expression is the positive control

OxPhos protein expression in hESCs

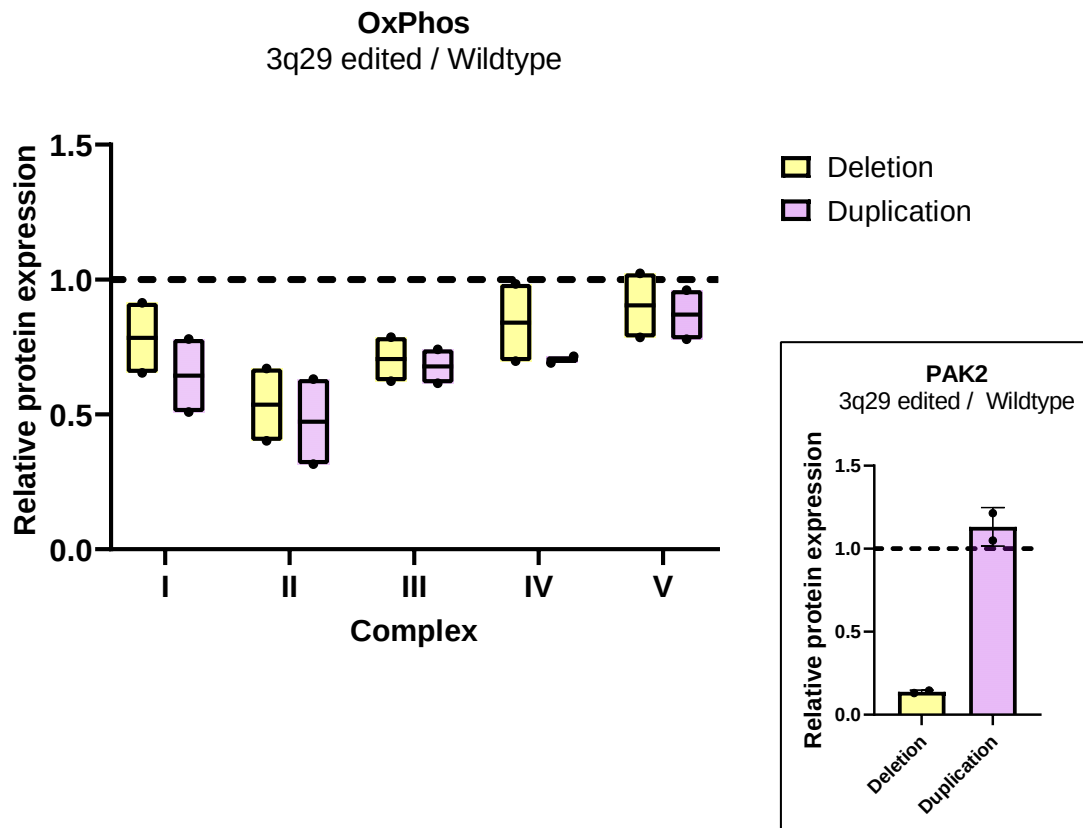


Figure 4.15: Mean and standard deviation from three independent experiments with clones from two biological replicates per genotype; Deletion: F1, F65 Duplication: F13 ; Wildtype: F6. Data is relative to wildtype, and PAK2 expression is the positive control

Figure 4.15 instead displays trends of OxPhos expression including two biological replicates from three experiments. The mean expression for F1, F6, F13 is represented in a single datapoint. Complex I-IV show trends of less protein expression for both genotypes, while complex V appears relatively stable. Variability is overall high with exception for subunits of complex III in deletion clones, and III, IV and V in duplication clones. The PAK2 expression appeared close to that of wildtype's in the single experiment including the F60(Dup) cell line. That experiment caused the concern about F60's true copy number and motivated the additional run of the Taqman copy number variation assay previously presented in section 4.1 figure 4.3

The overall pattern points towards reduced expression of mitochondrial protein subunits for either CNV risk variant, with exception for the more stable complex V. The trend clearly points towards a phenotypic difference in mitochondrial protein expression compared to wildtype cells.

SDHA and COX4 expression in hESCs

SDHA and COX4, the OxPhos proteins targeted independently shown in section 4.3.2 Figure 4.9, was also an attempt of validating previous finding in mice by Purcell and colleges (Introduced in second paragraph of section 2.6) [5].

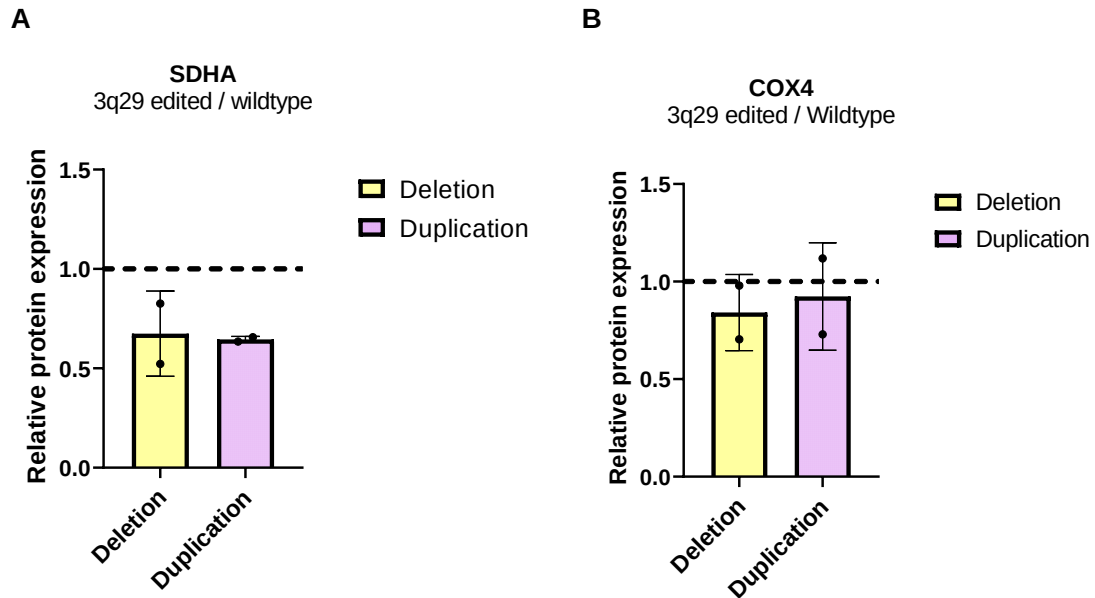


Figure 4.16: Expression of independently stained subunits displayed relative to wildtype. **A)** Mean and standard deviation of SDHA from three independent experiments using two biological replicates. **B)** Mean and standard deviation of COX4 with clones from two biological replicates Deletion: F1, F65; Duplication: F13, F60; Wildtype: F6, F62

The trend of SDHA show less expression relative to wildtype for either genotype. This finding, if replicated, would be consistent with literature in mice. The COX4 protein is seemingly less affected, but the high expression variability makes conclusions difficult to draw.

4.3.4 OxPhos protein quantification in induced neurons and glia cells

Name (including cell line)	3q29 Genotype	Day of collection
iN F1	Deletion	D3
iN F6	Wildtype	D3
iN F13	Duplication	D3
iN F1	Deletion	D4
iN F6	Wildtype	D4
iN F13	Duplication	D4
iN F1 w/ mGlia	Deletion	D35
iN F6 w/ mGlia	Wildtype	D35
iN F13 w/ mGlia	Duplication	D35

Table 4.7: Summary table of all induced neurons included in this study. All iNs were differentiated from the same cell line per genotype but collected at different timepoints after seeding and/or cultured with or without mouse glia.

Glia immunoblotting and OxPhos complex expression in iNs

Glutamatergic neurons differentiated using NGN2 induction method (see background section 2.2) were investigated for a single replicate per genotype. Figure 4.17 illustrates one out of three membranes which included stem cells and induced neurons collected day 4 after seeding.

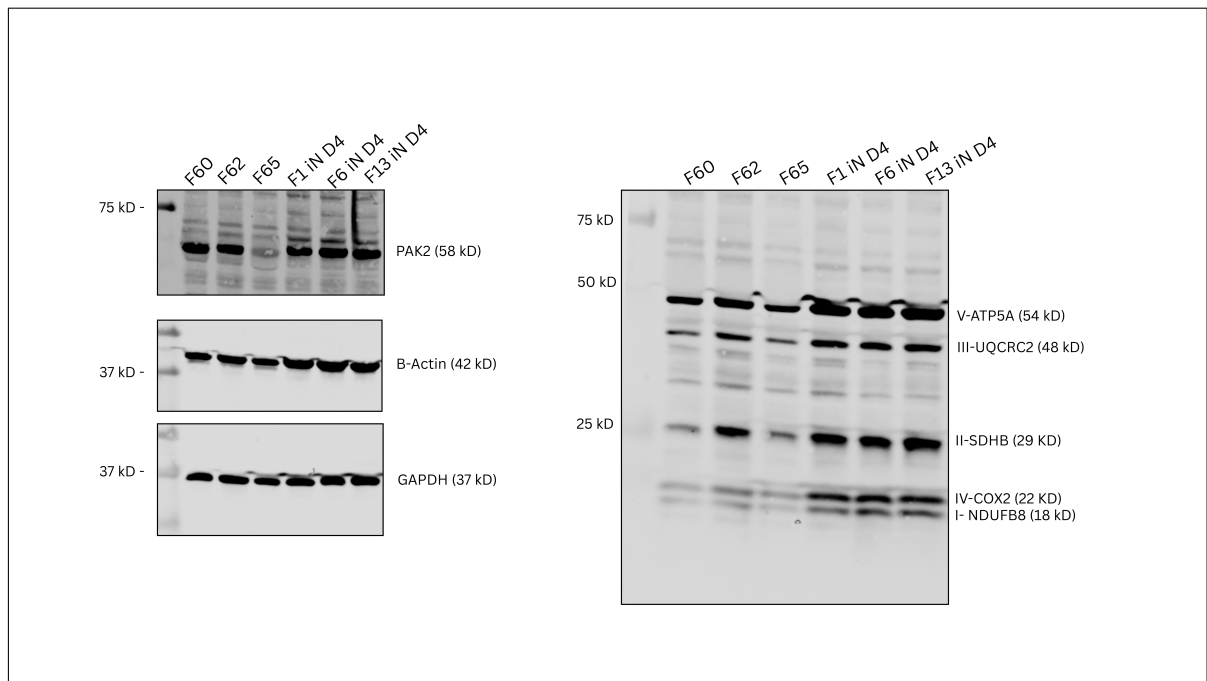


Figure 4.17: OxPhos proteins in hESCs(P44) and iNs collected day 4 after seeding cultured without glia cells. Left membranes show positive control PAK2 with loading controls GAPDH and B-Actin. F60: Duplication; F62: Wildtype; F65: Deletion; F1 iN D4: Deletion; F6 iN D4: Wildtype; F13 iN D4: Duplication

4. Results

Neurons were initially co-cultured with mouse glia cells for support and structure. Because of uncertainty whether the primary antibody could distinguish between mitochondrial proteins from glia and neurons, the question had to be resolved before proceeding to quantification. Figure 4.18 displays neurons collected at day 35 (D35) after seeding and co-cultured with mouse glia, but also lanes with only mouse glia. A complete discrimination between celltypes would be necessary to keep the two celltypes in co-culture as glia otherwise would contaminate results.

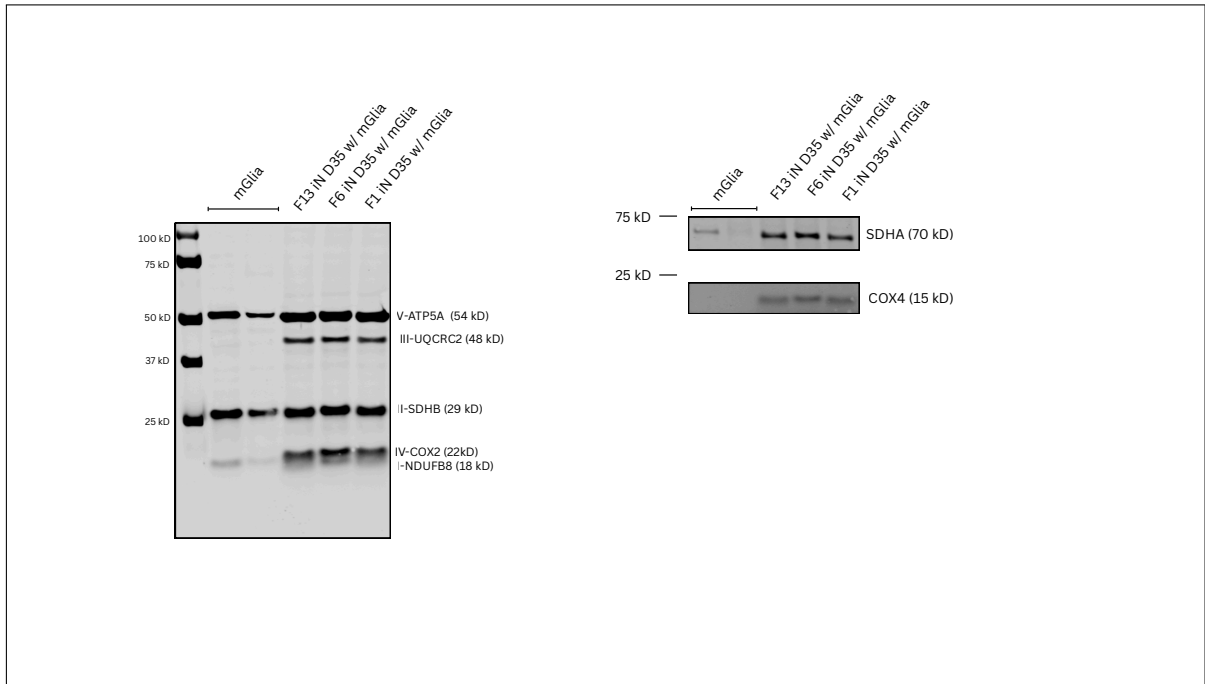


Figure 4.18: OxPhos complexes are selectively stained for in mouse glia, leading to contamination of protein quantification in iNs. SDHA and COX4 were to a lesser degree detected in mouse glia (right membranes) in comparison to remaining OxPhos complexes (left membrane)

The mouse glia did produce a signal for several OxPhos proteins. The primary antibody could therefore only to some extent differentiate between proteins from neurons and glia. Complete discrimination between the two celltypes would be sufficient to continue culturing neurons and glia together as results otherwise would be contaminated by glia proteins. The subsequent results for OxPhos proteins for iNs with glia is only included for SDHA and COX4 because, as seen in figure 4.18, these two proteins were remarkably less stained than OxPhos proteins using the antibody cocktail (left membrane).

iN cultures were from this point only conducted with neurons differentiated without glia cells, even though it compromises their viability. A representative membrane of these cultures are seen in previous figure 4.17.

Figure 4.19 and 4.20 show OxPhos protein expression from two iN batches collected one day apart after seeding.

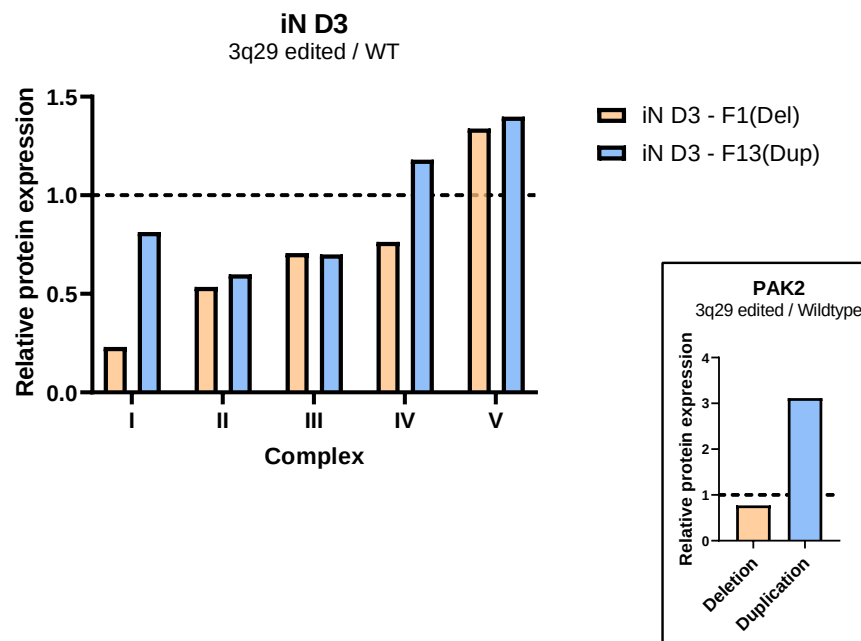


Figure 4.19: Expression of edited clones relative to wildtype from one experiment with neurons differentiated from F1, F6 and F13 cell lines collected three days (D3) after seeding. PAK2 expression is the positive control validating genotypes.

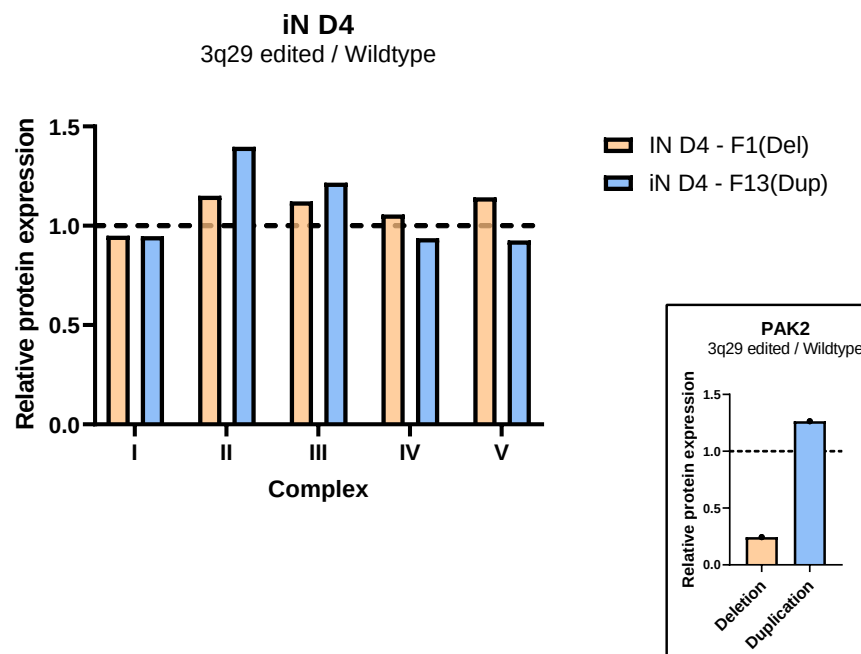


Figure 4.20: Expression of edited clones relative to wildtype from one experiment with neurons differentiated from F1, F6 and F13 cell lines collected four days (D4) after seeding. PAK2 expression is the positive control validating genotypes.

4. Results

iN D3 seemingly express a stronger phenotype than iN D4. This result should not be interpreted as a biological difference as these are batches from the same cell line with only one day apart. The results rather points towards a reproducibility issue of the experiment itself.

SDHA and COX4 expression in induced neurons

Proteins targeted individually, SDHA and COX4, show trends also indicating high variability. The most consistent trend was that of COX4 in F13(DUP) clones showing consistently higher expression across experiments.

To conclude, the derived data from all seven OxPhos proteins in iNs appear highly variable. The one trend appearing with high consistency is that of COX4 duplication clones showing overexpression compared to wildtype across experiments.

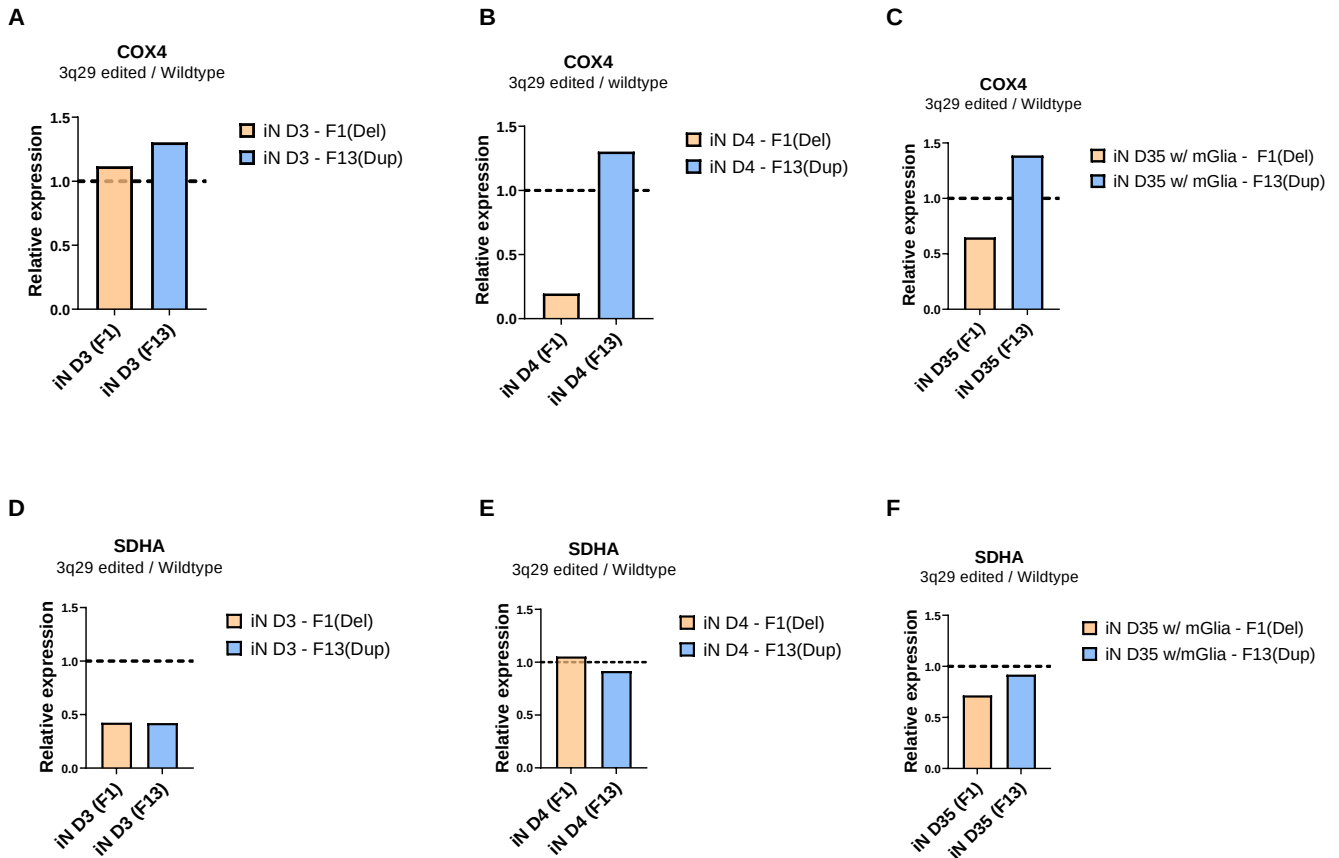


Figure 4.21: Expression relative to wildtype from one experiment with induced neurons differentiated from F1, F6 and F13 cell lines. Y axis show relative protein expression. **A)** COX4 expression in iNs collected 3 days after seeding **B)** COX4 expression in iNs collected 4 days after seeding **C)** COX4 expression in iNs cocultured with glia cells collected 35 days after seeding **D)** SDHA expression in iNs collected 3 days after seeding **E)** SDHA expression in iNs collected 4 days after seeding **F)** COX4 expression in iNs co-cultured with glia cells collected 35 days after seeding.

5

Discussion

Bridging the knowledge gap between genetic risk variants and the resulting phenotypes is crucial to understand neurodevelopmental disorders. In practice, this can guide therapeutic targets and allow for early intervention strategies. In this study, identification of biological replicates in 3q29 edited stem cell lines was an essential first step to enable investigation of phenotypic effects of the risk variants. Screening of clones using the Taqman CNV assay is considered a robust technique for validation [34]. Even so, cell line F60(Dup) had conflicting outputs when validated on a DNA level (Taqman CNV assay) versus on a protein level (positive control in Western blot). To resolve the uncertainty, the cell line can be sent for WGS.

Pluripotency and undifferentiated state of stemcells was validated in three out of the identified clones using Immunocytochemistry. These properties are requirements for homogeneous cultures in both the stem cell state and neuronal cultures differentiated from those.

A Western blot protocol was established to identify mitochondrial subunits in ETC-complexes in HEK-293T cells. After multiple iterations, the major identified issue was the primary antibody from Invitrogen, even though the fabricators themselves have a dataset of eighteen publications in which the antibody has been successfully utilized [35]. Why this antibody did not successfully target the five OxPhos proteins in this study remain unresolved. Instead, the Rb-Anti-Total OXPHOS Antibody Cocktail was included in the final protocol, together with the most effective lysis methods, transfer time and currency, and blocking buffer.

The established Western blot methodology could be applied to quantify expression in two embryonic stem cell lines and induced neurons. Observed expression patterns in OxPhos proteins for hES cells was an overall reduction with ATP5A of complex five being the least affected. Both the stability of this complex V subunit and reduced expression of COX4 in hES deletion clones are consistent with previous literature in mice [5]. Anyhow, more biological replicates are essential to draw empirical conclusions.

Glia mitochondria was confirmed to contaminate results in iNs, resulting in glia free neuronal cultures for subsequent experiments. Since these supporter cells are essential for neuronal viability, these neurons are likely to benefit from being collected early for experiments as done in this study [36]. OxPhos protein expressions in these iN clones were inconsistent. Overall, the most unambiguous trend was observed for

individual complex COX4 in F13(Dup) clones. The biological interpretation of expression trends in iNs and hESCs, if statistically significant after replication, will be discussed in section 5.0.2 discussing future directions.

5.0.1 Study limitations

High variability was expected due to the small sample size in this study for both quantified cell types, but results were more inconsistent than initially thought. A possible inference is the seemingly low assay reproducibility, motivated by data in technical replicates of hESCs from figure 4.14. Another interpretation is simply that OxPhos proteins are sensitive to extraneous variables. Neither of these two explanations are mutually exclusive. Several possible contributing factors should be considered. First, the lack of consistency in the pre-experimental phase in terms of storage and collection. For example, the D3 neurons was collected from culture, while D4 neurons was stored in -80°C and thawed before lysed the following day. The sample handling could therefore be more similar across experiments. Second, the *in silico* restriction of the bands' areas using Image Studio become difficult when membranes or images appear blurry. With more experiments, having more stringent data acceptance could mean excluding membranes with lower quality.

Arguably the greatest source of variability is the utilization of GAPDH as the only reference gene for OxPhos proteins in this study. GAPDH is a common loading control in Western blots for total cellular protein, but does not account for mitochondrial abundance [37]. In order to account for different mitochondrial densities across samples, a control protein expressed within the mitochondria itself must be included. As a suggestion, the Voltage dependent anion channel (VDAC) expressed at the outer mitochondrial membrane has been used as an indicator of mitochondrial abundance [38]. Protein amount would in this case be normalized per mitochondria and better represent expression differences across genotypes. Together with GAPDH, both total protein loading and mitochondrial abundance would be accounted for and better reflect electron transport chain dysregulations. Furthermore, the mentioned study by Purcell and colleges, finding selective downregulation of the OxPhos proteins in mice, utilized the most stable expression in complex V for normalization purposes [5].

5.0.2 Suggested methods for further investigation

This study aimed to identify a structural deviation in the ETC as a downstream consequence of 3q29 dosage alterations. Assuming OxPhos protein expression is significantly altered in edited cell lines after replicated Western blot experiments, a next step could be to investigate whether the structural impairment translates to a functional consequence. Such finding would in turn motivate the importance of precise protein expression in the transport chain. Previous genetic risk variant studies have identified reduced ATP levels in neurons secondary to selective downregulation of mitochondrial protein complexes [39] [5]. ATP levels is a desirable target of

measurement as reduced ATP output negatively can affect synaptic formation and function as discussed in background section 2.4. Thus, reduced ATP levels alone does not prove causation for synaptic impairment, but rather evidence supporting the hypothesis. Several assays can be used for the purpose of ATP measurements, suggestively a mitochondrial stress test using a Seahorse analyzer or a bioluminescence assay. The former measures oxygen consumption as an indirect measure of ATP production [5]. In the bioluminescence assay, the activity of OxPhos complexes is instead reflected by fluorescence as luciferase enzymes catalyses a light producing reaction in the presence of ATP [39].

Before allocating resources towards the functional assays, neartime efforts should continue Western blot experiments using remaining identified cell lines. The current results in protein expression are limited by both a small sample size and lack of biological replicates. This lead to the lack of statistical tests since the sample set is an insufficient representation for population level conclusions. Anyhow, the identified trends motivate continuation of these experiments provided the undeniable differences in protein expression across Deletion, Wildtype and Duplication clones.

A final consideration is conducting so called rescue experiments using CRISPR interference/activation using dCas9. The technology could be applied to identify individual driver genes within the 3q29 region. By modifying gene expression using RNA guided dCAS9 fused with transcriptional regulators, genes can be either repressed or activated[40]. The general objective of these rescue experiments is to determine whether a phenotype is restored followed by the intervention. In the context of this study, the electron transport chain is the phenotype of interest, and candidate genes are those located within 3q29. For example, overexpressing the PAK2 gene in the remaining allele of 3q29 deleted clones using CRISPR activation, and thereafter evaluate mitochondrial OxPhos protein expression.

6

Conclusion

Dysfunctional mitochondria, already hypothesised to play a role in SCZ pathology, was hypothesised to have altered protein expression due to 3q29 alterations. To enable the investigation of mitochondrial phenotypes as a consequence of the genetic risk variants, biological replicates of 3q29 edited Del/WT/Dup stem cells was identified. Genotyping was conducted to CRISPR engineered isogenic hES cell lines using a quantitative PCR based method. Out of seventyfive clones, three duplication and eleven deletion clones were recognized which allow for multiple biological replicates to be included in future experiments. Out of these cell lines, one per genotype (Del/WT/Dup) was characterised by utilizing immunocytochemistry targeting pluripotency markers SOX2, NANOG, OCT4 and TRA160. These cell lines, together with one already validated replicate per genotype, were used in Western blot experiments to quantify OxPhos protein expression. Before initiation a methodology had to first be established using HEK293T cells. The optimization period, including modification of cell lysis methods, transfer conditions, blocking buffers and primary antibodies, resulted in a final protocol that successfully targeted all OxPhos proteins. hESCs showed trends of less expression of edited cell lines, with complex V appearing the most stable. Expression data in induced neurons, after showing that glia mitochondria contaminated results leading to exclusion from neuronal cultures, appeared highly variable. The one consistent trend were that of IV-COX4 proteins being overexpressed in duplication clones. This study was limited by small sample sizes leading to trend assesment rather than statistical tests. Also, factors likely introducing variability should be corrected for before continuation of these experiments. However, the identified trends, particularly those in 3q29 edited hESCs, support further investigation of mitochondrial implications as a consequence of the genetic risk variants.

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Appendix 1

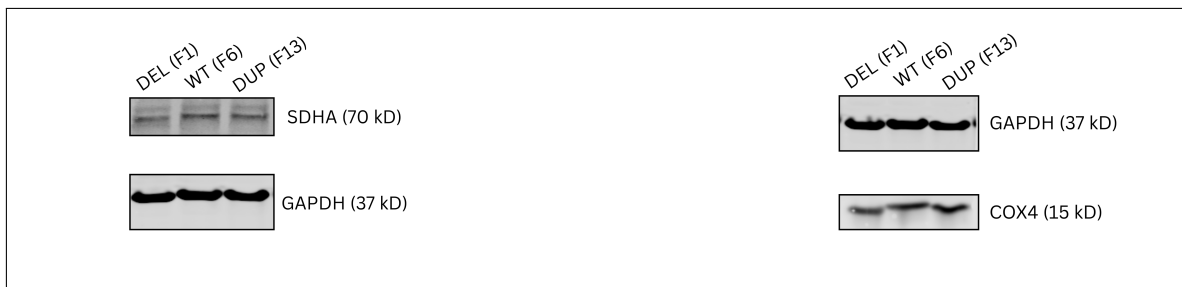


Figure A.1: Representative membrane of experiments for SDHA and COX4 quantification in hESCs. GAPDH included as a loading control.



Figure A.2: Representative membrane of experiments for SDHA and COX4 quantification in iNs. GAPDH included as a loading control. iN D3: Collected 3 days after seeding; iN D4: collected 4 days after seeding

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