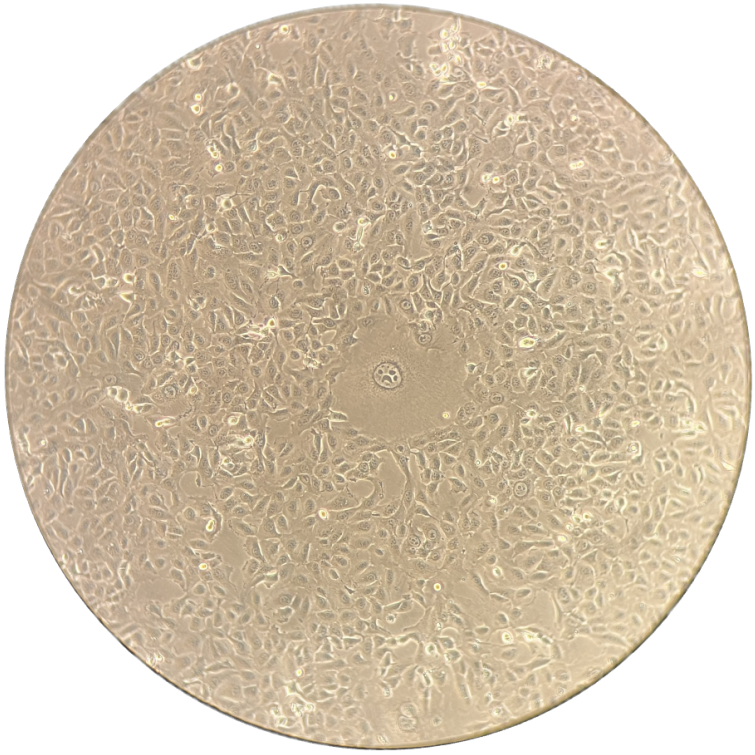
CHALMERS
UNIVERSITY OF TECHNOLOGY



Chromatin binding patterns of SWI/SNF chromatin remodeling complex components and interaction partners in FET sarcoma

Master s thesis in Biotechnology

KAJSA GRÖNQVIST

DEP RTMENT OF LIFE SCIENCES
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2024
www.chalmers.se

M ster's thesis 2024

Chromatin binding patterns of SWI/SNF chromatin remodeling complex components and interaction partners in FET sarcoma

Kajsa Grönqvist

CHALMERS
UNIVERSITY OF TECHNOLOGY

Department of Life Sciences
Division of Chemic l Biology
Ståhlberg group, Sahlgrenska Center for Cancer Research
Ch lmers University of Technology
Gothenburg, Sweden 2024

Sincere thanks to Malin for teaching and guiding me throughout the project.

Chromatin binding patterns of SWI/SNF chromatin remodeling complex components and interaction partners in FET sarcoma
K JS GRÖNQVIST

Supervisors: Malin Lindén & nders Ståhlberg, Sahlgrenska Center for Cancer Research
Examiner: Fredrik Westerlund, Department of Life Sciences, Chalmers Univeristy of Technology

Master's Thesis 2024
Department of Life Sciences
Division of Chemical Biology
Chalmers University of Technology
SE-412 96 Gothenburg

Cover: Picture of HT1080 FUS-DDIT3-EGFP cells in a microscope.

Typeset in LaTeX
Gothenburg, Sweden 2024

Abstract

Sarcoma is a group of cancers originating in supportive tissues in the body, such as muscle, bone, and fat. FET sarcoma, often found in young patients, is a subgroup of sarcoma. Treatment plans for certain types of FET sarcomas, can leave patients at a high risk of developing a secondary cancer later in life. FET sarcomas are characterized by the presence of fusion genes consisting of a member from the FET family partnered with a transcription factor for example *DDIT3*, and these fusion genes encode FET fusion oncoproteins, also called FET-FOPs. The FET fusion oncogenes are in several cases the only mutation present in FET sarcomas, indicating that the mutation can induce oncogenesis. FET-FOPs have been found to interact with the SWI/SNF chromatin remodeling complex, which is a complex responsible for remodeling nucleosomes to enable access to DNA for processes such as transcription.

This project aimed to determine the chromatin binding strength of SWI/SNF components and known interaction partners in the cell line HT1080 with stable expression of different FET fusion oncoproteins. This was done by using sequential salt extraction (SSE), a method used to solubilize nuclear proteins iteratively with increasing salinity. The salt concentration at which the proteins were extracted reflects their binding strength. The protocol for SSE was optimized to use on HT1080 as the nuclei in the cells were prone to rupturing, resulting in gel pellets that did not allow collection of the nuclear fractions. The change in the protocol that gave desirable results was to resuspend the cell pellet in a buffer with low salt concentration before adding a higher salt concentration buffer as this minimized the occurrence of local high salt concentration, which was believed to be the cause of the ruptured nuclei. Three antibodies targeting the SWI/SNF components BCL7A/B/C were also optimized to be used on Western blot to ensure that they targeted the correct proteins. The final extracts from SSE were then evaluated on Western blot with antibodies targeting SWI/SNF components and known interaction partners to assess if the expression of FET-FOPs alter the chromatin binding strength. SWI/SNF components displayed a unique salt extraction profile, different from most interaction partners but no patterns in regard to functional module or SWI/SNF subtype was observed. The results indicated that the expression of FET-FOPs could have an influence on the binding strength of SWI/SNF components and interaction partners as different expression profiles were detected for some proteins. However, more experiments are needed to confirm this conclusion. By discovering more about the cellular mechanisms between FET-FOPs and SWI/SNF, new targeted treatments for FET sarcomas may be explored.

Keywords: FET sarcoma, SWI/SNF (BAF) chromatin remodeling complex, sequential salt extraction, Western blot, binding profiles

Abbreviations

FET-FOP	FET fusion oncoprotein
SWI/SNF	SWItch/Sucrose Non-Fermentable
MLS	Myxoid Liposarcoma
DDIT3	*DNA damage-inducible transcript 3*
cBAF	Canonical BAF complex
PBAF	Polybromo-BAF complex
GBAF/ncBAF	GLTSCR1/1L-BAF or Non-canonical BAF
PRC2	Polycomb Repressive Complex 2
SSE	Sequential Salt Extraction
EB	Extraction Buffer

TABLE OF CONTENTS

1 Introduction

Cancer is one of the leading causes of death worldwide with nearly one in six deaths in 2020 [1]. Cancer is an umbrella term for abnormal cell growth caused by mutations. This means that most cancers have no common cause nor treatment, making research on the underlying mechanisms of different types of cancers essential in order to find effective treatments.

Sarcoma is a type of cancer that exists in the supporting tissues in the body, e.g. bones, cartilage and muscles [2]. Sarcomas are divided into two main types: bone and soft tissue. It is a very diverse cancer type, there are over 50 types and they all have different treatments and survival rates. Some sarcomas are caused by FET fusion oncoproteins (FET-FOPs) (Figure 1), also known as FET sarcomas. FET-FOPs can induce tumor development, often being the sole mutation present in tumor cells, and are thought to function as aberrant transcription factors. This project has studied the FET-FOPs characteristic for myxoid liposarcoma (MLS) and low-grade fibromyxoid sarcoma, namely FUS-DDIT3 and FUS-CREB3L2. FET proteins interact with the SWI/SNF chromatin remodeling complex, a large protein complex the cell uses to regulate gene expression. FET-FOPs have been shown to interact more with SWI/SNF than regular FET proteins, indicating that some type of dysregulated interaction occurs between SWI/SNF and FET-FOPs, which might contribute to the oncogenic mechanism in FET sarcoma [3]. Exploring the relationship between FET-FOPs and the SWI/SNF chromatin remodeling complex enables further research of new therapeutic targets for FET sarcomas.

1.1 Aim

The major aim of this project is to determine the chromatin binding strength of SWI/SNF components and other known interaction partners of FET oncoproteins in a model system: the HT1080 cell line with stable expression of different FET fusion oncoproteins. The project also has two minor aims which are to evaluate new antibodies on Western blot (SWI/SNF components BCL7A, BCL7B and BCL7C) and optimize sequential salt protein extraction in the mentioned model system.

2 Theory

The following section will cover important concepts such as FET oncoproteins and FET sarcoma, the SWI/SNF chromatin remodeling complex and the methods used to study these, including Western blot and sequential salt extraction.

2.1 FET proteins and FET fusion oncoproteins

The protein family FET consists of FUS, EWSR1 and TAF15, which are similar RNA- and DNA-binding proteins that have been found to be involved in several important cell processes [4]. These processes include RNA processing and transportation, and gene transcription, making FET proteins involved in gene regulation.

Fusion genes derive from chromosomal translocations and consist of a parent gene at the 5' end followed by a breakpoint where a 3' parent gene connects [5]. Transcription of the fusion gene is regulated mostly by the 5' end, but there are cases where sequences in the 3' gene influences expression of the gene. Fusion genes that lead to cancer are called fusion oncogenes, and the proteins they encode are called fusion oncoproteins.

FET fusion oncogenes consist of a gene from the FET family (*FUS, EWSR1* or *TAF15*) as the 5'-partner and an alternative DNA-binding transcription factor gene as 3'-partner (Figure 1). These fusion oncogenes encode FET fusion oncoproteins (FET-FOPs). Each variant of FET-FOPs is specific for one tumor type, with few exceptions. The FET N-terminal can in some cases be exchanged for one of the other FET proteins while still showcasing the same tumor type.

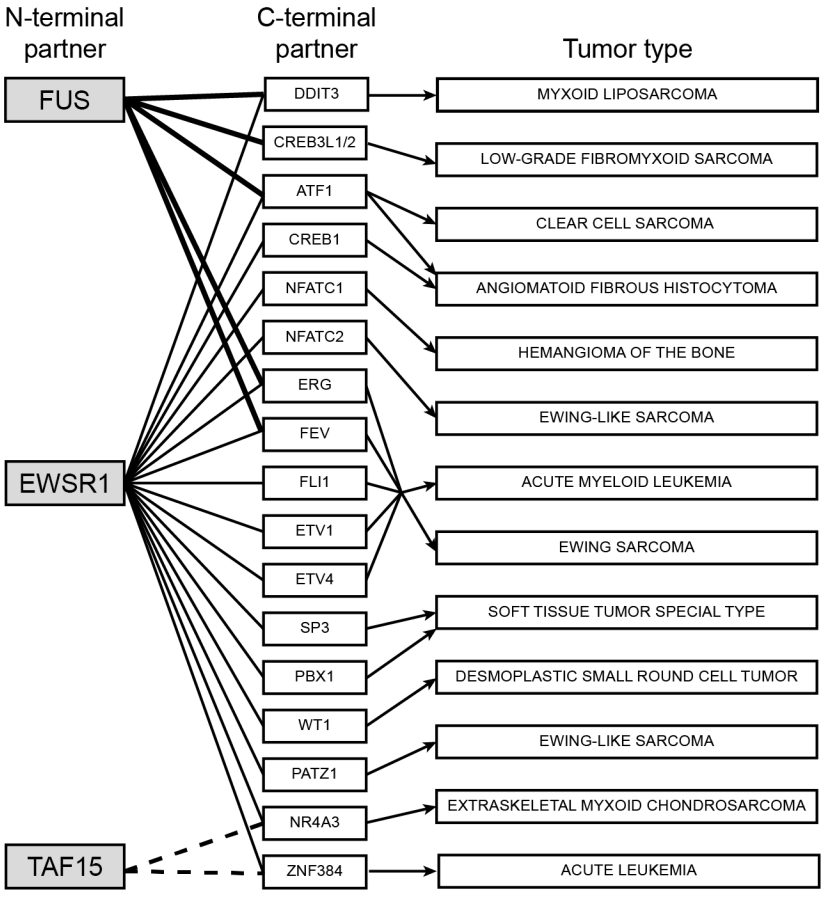


Figure 1. ***Combinations of FET fusion oncoproteins listing the N-terminal FET proteins together with their C-terminal partners and their associated tumor type. Adapted from*** [3]

2.2 FET sarcomas

The FET sarcoma Myxoid liposarcoma (MLS) accounts for about 20% of all liposarcomas and about 5% of adult soft tissue sarcomas [6]. The most common FET-FOP in MLS is a fusion of FUS as the N-terminal partner and the entire reading frame of DDIT3 as the C-terminal partner [7]. There are also few cases where EWSR1 is the N-terminal partner. In total, at least 12 different fusions of either FUS-DDIT3 or EWSR1-DDIT3 have been identified. FUS-CREB3L2 is a FET-FOP characteristic for low-grade fibromyxoid sarcoma, which is a rare type of sarcoma mostly affecting young adults [8].

One FET sarcoma whose FET-FOP was not studied in this project due to time restraints but shows the importance of studying the mechanisms behind FET sarcoma is Ewing sarcoma. It is the second most common type of bone cancer in children [9]. The most frequent FET-FOP is EWSR1-FLI1 [10]. Ewing sarcoma is an aggressive tumor type and the treatment is very intense. Research has shown that the majority of patients have chronic treatment-related complications and that there is an increased risk of a secondary cancer developing later in life. The intense treatment procedure and risk for long lasting complications further highlights the importance of developing new treatment plans.

2.3 The SWI/SNF chromatin remodeling complex

Cells use chromatin remodeling complexes to be able to regulate gene expression, repair DNA, and replicate DNA by reorganizing chromatin and nucleosomes [11]. This reorganization allows proteins to access the DNA, facilitating essential cellular processes. One of the chromatin remodeling complexes is the SWI/SNF (SWItch/Sucrose Non-Fermentable) complex, consisting of around 15 subunits that remodels nucleosomes via ATP-hydrolysis [12]. Studies have found 29 proteins that are a part of the SWI/SNF complex, and while there are many theoretical combinations of them, there are only three main subtypes described: canonical BAF complex (cBAF), polybromo-BAF complex (PBAF) and GLTSCR1/1L-BAF (GBAF/ncBAF) [13]. All SWI/SNF complexes are assembled by the formation of a core unit containing BAF170 and BAF155 and one of BAF60A, B and C. This core unit then creates an intermediate complex with subtype-specific proteins before finalizing the complex assembly with an ATPase module. All SWI/SNF components can be found in Table 1. Mutations of SWI/SNF genes are present in 20% of cancers, but alterations of the processes chromatin remodeling complexes facilitate are present in almost all human cancers [14].

2.3.1 The SWI/SNF component BCL7

One of the least studied components in SWI/SNF is the BCL7 (B-cell CLL/lymphoma 7) protein family which consists of three members: BCL7A, BCL7B and BCL7C. The cellular roles of the BCL7 family are largely unknown, but some type of BCL7 is present in all SWI/SNF complexes, giving BCL7 an involvement in chromatin remodeling [15]. It has also been reported that BCL7 is involved in negative regulation of DNA-templated transcription, the cell cycle and regulation of transcription by RNA polymerase II [16]. All BCL7 proteins have low tissue specificity and are located in the nucleus in most tissues.

Table 1. Summary of SWI/SNF components and corresponding subtype(s). Protein names in bold represent standardized nomenclature.

Protein	Alternative name	SWI/SNF subtype
BAF155	**SMARCC1**	All, core module
BAF60A	**SMARCD1**	
BAF60B	**SMARCD2**	
BAF60C	**SMARCD3**	
BAF170	**SMARCC2**	cBAF and PBAF, core module
BAF57	**SMARCE1**	
BAF47	**SMARCB1**	
BRG1	**SMARCA4**	All, ATPase
BRM	**SMARCA2**	
BAF53A	**ACTL6A**	All, ATPase module
BAF53B	**ACTL6B**	
β-ACTIN	**ACTB**	
BCL7A		
BCL7B		
BCL7C		
SS18	SSXT	cBAF and GBAF, ATPase module
SS18L1	CREST	
ARID1A	BAF250A	cBAF-specific
ARID1B	BAF250B	
BAF45B	**DPF1**	
BAF45C	**DPF3**	
BAF45D	**DPF2**	
ARID2	BAF200	PBAF-specific
PBRM1	BAF180	
BAF45A	**PHF10**	
BRD7		
BRD9		GBAF-specific
GLTSCR1	**BICRA**	
GLTSCR1L	**BICRAL**	

2.3.2 Interaction partners to the SWI/SNF complex

Both normal FET proteins and FET fusion oncoproteins bind to and interact with SWI/SNF complexes, giving FET-FOPs a direct link to chromatin reorganization and transcriptional regulation [3]. Driver mutations of SWI/SNF genes are however not present in FET sarcoma. Lindén *et al.,* (2019) found that FET-FOPs interact with SWI/SNF more than normal FET proteins, indicating that FET-FOPs have a dysregulated interaction with SWI/SNF which in turn can deregulate gene expression, leading to cancer [3].

The PRC2 polycomb repressor complex is another interaction partner to SWI/SNF [12]. The complex represses heterochromatin mainly through the catalytic enzyme EZH2, keeping DNA tightly packed and transcriptionally inactive. SWI/SNF and PRC2 work together in opposing roles to maintain a balanced regulation of chromatin structure and gene expression, where SWI/SNF enable open chromatin structures while PRC2 promotes a closed, repressive state. A

dysregulation between this relationship can lead to disease, such as cancer. Another protein that has been found to interact with SWI/SNF is BRD4, a bromodomain protein that has an involvement in several cellular processes, including transcriptional control and DNA damage repair [17, 18]. BRD4 was also reported to interact with FET-FOPs, contributing to oncogenic gene expression in MLS [19].

2.4 Method theory

This chapter will explain the theory behind the most important methods used during the project.

2.4.1 Cell culture

Cancer cell cultures are grown in flasks in a 2D model system to study cell mechanisms and epigenetics. Cell cultures are often used as the first model system during biological studies as it is relatively easy and cheap compared to whole tumors, for example, of which material is very limited. Cell culturing is also suited in this project as the utilized methods require many cells during several months. This project used the fibrosarcoma cell line HT1080, which are adherent cells derived from the connective tissues of a male patient with fibrosarcoma [20]. A vector containing a FET fusion oncogene, FUS-DDIT3 and FUS-CREB3L2, have been inserted into the cells to study the impact and differences a FET fusion gene has in the cells. The vector also contains EGFP (enhanced green fluorescent protein), which makes the cells visible in a fluorescence microscope, and an antibiotic resistance gene as a selection marker [21].

2.4.2 Sequential salt extraction

Sequential salt extraction (SSE) is an extended version of nuclear protein extraction where the cell pellet is resuspended in several concentrations of salt instead of only one. The aim of the method is to extract nuclear proteins with the help of an extraction buffer containing salt in increasing concentration. Salt, in this project KCl, dissociates into ions (K^+ and Cl^-) when it is dissolved in an aqueous solution. These ions affect and interfere with the ionic bonds between proteins and DNA and other nuclear proteins [22]. By resuspending the cell pellet in increasing salt concentrations, it is possible to approximate the protein binding strength to chromatin in the nucleus. This is because the salt breaks the chromatin-protein bonds, and the released proteins are transported out of the nucleus and can be collected. A higher salt concentration corresponds to a higher binding strength.

Cells can be detached from the plate surface either through scraping or by using 0.25% Trypsin, but Trypsin cleaves proteins and nucleic acids and can therefore affect downstream results, so scraping was used during SSE in this project. The cells are then allowed to swell in a hypotonic lysis buffer to facilitate easier cell lysis. The disruption of the outer cell membrane is performed with a syringe and a small gauge needle. However, successful cell lysis needs to be verified with the help of Trypan blue, which is a dye normally used to determine cell viability [23]. Cells with intact membranes will not take up any dye and will remain uncolored but dead cells with disrupted membranes will turn blue as the dye binds to intracellular proteins within the cell [24]. Benzonase is added to the cells before the membrane disruption to help protein disassociation from the nuclei and to cleave released DNA and RNA, to ensure that DNA does not increase the viscosity of the cell suspension [25]. The supernatant (cytoplasmic fraction) is carefully removed after centrifugation, and the pellet is then resuspended and incubated in extraction buffer (EB) three times with increasing salt concentrations. The extraction buffer has a higher salt concentration to compensate for the dilution from the cells' inherent liquid volume,

resulting in a final concentration of 250 mM, 500 mM and 1000 mM KCl. The supernatant is removed after centrifugation for all salt concentrations and saved as nuclear fractions. The pellet was avoided during the collection of supernatant in order to not contaminate any samples, and if there was a risk of touching the pellet during the removal, the affected sample was discarded and the volume registered. To make the nuclear fractions usable in Western blot, they are diluted to 150 mM as a high salt concentration in Western blot affects how the samples travel through the gel and therefore the results. This dilution factor was accounted for during the calculations of loading volume and dilution for Western blot.

A protein concentration measurement kit from BioRad was used in order to know how many µg of protein was loaded in Western blot. The kit is a calorimetric assay and is based on the Lowry assay [26]. The proteins in the sample react with copper from one of the reagents, these complexes then reduce a Folin reagent and the solution develops a blue color. The absorbance is analyzed in a spectrophotometer at 690 nm and compared to standards based on bovine serum albumin to estimate the protein concentration of the samples.

2.4.3 Western blot

Western blot is a common method used to analyze proteins in different samples [27]. It uses gel electrophoresis to separate denatured proteins by molecular weight and the separated proteins are then transferred to a membrane. The membrane is blocked with either 5% milk or 5% Bovine albumin serum in Tris-Buffered Saline Tween-20 (TBS-T) to prevent unspecific binding of antibodies to the membrane. Proteins are labeled with primary antibodies that are corresponding to the proteins of interest which are detected with the help of horseradish peroxidase (HRP)-conjugated secondary antibodies with the same species as the primary antibody. To detect the proteins, equal volumes of a stable peroxide and a luminol-based enhanced substrate solution are added to the membrane which produces chemiluminescent signals as luminol oxidizes and comes back from an excited state [28]. These signals are captured with charge-coupled device (CCD) cameras.

Primary antibodies that are used in Western blot need to be optimized in regard to the blocking buffer used and dilution of the antibody in order to get ideal results. The desired results are separated, clear bands that are neither weak nor too strong to be able to analyze and compare results between protein samples. The choice of antibody is very important for Western blot as the results are dependent on the antibodies detecting the correct proteins by binding to them. Antibodies are either monoclonal or polyclonal, depending on how they are produced. Monoclonal antibodies are produced by immune cells that are clones of a unique parent cell, leading to the antibody only detecting a single specific epitope [29]. Polyclonal antibodies are produced by different immune cells and can therefore detect several epitopes of an antigen. Western blot is favorable to use in this project as it allows for detection of specific proteins in a complex mixture containing many other proteins. The method can also be used semi-quantitively, which allows for numeric comparison between samples, which is one of the aims in this thesis.

3 Methods

Several methods have been used in this project and the following section will describe how to perform them.

3.1 Cell culture

The fibrosarcoma cell line HT1080 (ATCC, CCL-121) was cultured to be used in experiments. The culture mainly focused on HT1080 wild type cells and stable clones expressing FUS-DDIT3-EGFP, FUS-CREB3L2-EGFP and EGFP. Cell lines containing FUS-DDIT3 and EGFP had previously been established by Ståhlberg group and FUS-CREB3L2 was purchased from (and cloned by) VectorBuilder. The cells were cultured in T25 flasks with RPMI 1640 GlutaMAX medium (Gibco, 61870036) with addition of 5% FBS (Gibco, 10270106) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, 15140122), and incubated at 37°C with 5% CO_2. The cell lines with a vector received 500 µg/ml addition of Geniticin (Gibco, 10131035), with the exception of the cell line with FUS-CREB3L2 vector which received 1.5 µg/ml Puromycin (Gibco, A1113803). The cells were split 1:8-1:12 approximately two times a week and discarded after 15-20 passages. MLS cell lines (MLS Avory (2645-92), MLS 402 (402-91) and MLS 1765 (1765-94), established by Ståhlberg group) were used in whole cell extraction and Western blot for antibody optimization experiments. They were treated in the same manner as HT1080 WT and were cultured by a colleague until the extractions.

3.2 Sequential salt extraction

The protocol was adapted from [30]. HT1080 cells were expanded to T175 flasks 5 days before the extraction and passaged onto 1-5 plates with the same area as T175 flasks 2 days before the extraction. The number of plates depended on the confluency and state of the cells and their rate of proliferation. To ensure high cell confluency and thriving cells, the cells were fed the day before extraction by removing approximately half of the medium and replacing it with fresh medium.

The cells were scraped from the plates with ice cold DPBS (Gibco, 14190-094) and centrifuged at 4°C for 10 min at 450 rcf. All solutions, tubes and cells were kept on ice during the whole extraction procedure. The packed cell volume (PCV) was estimated, and the supernatant was discarded. The pellet was resuspended in 5xPCV of hypotonic lysis buffer (HLB) [10mM KCl, 10 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 1 mM DTT, 1X Protease inhibitor cocktail (DTT and Protease inhibitor added fresh) and MilliQ] and incubated for 15 minutes on ice to let the cells swell. The cell suspension was centrifuged at 4°C for 5 minutes at 400 rcf. The supernatant was discarded and the pellet was gently resuspended in 2xPCV of HLB. Benzonase dilution was prepared by adding Benzonase (Merck, 101654) to Benzonase dilution buffer [50 mM Tris pH 8.0, 20 mM NaCl, 2 mM $MgCl_2$ and MilliQ] to get a final concentration of 250 U/ml. The

Benzonase dilution was added to the cell suspension, (3xPCV)/50. The cell membranes were then disrupted by using a nr. 27 hypodermic needle and syringe. The syringe was first filled with HLB and ejected in a single steady stroke to minimize the amount of air incorporated into the sample. The syringe was then filled with the cell suspension and ejected with a single steady stroke five times to ensure sufficient cell lysis. The cell suspension was incubated at 4°C for 15 minutes on an end-over-end mixer and then centrifuged at 4°C for 20 minutes at 10 000 rcf. The supernatant was transferred to a new tube and the collected volume was estimated. This was the cytoplasmic fraction. Aliquots of the cytoplasmic fraction were taken to be used in protein concentration measurement and Western blot. LDS sample buffer (Invitrogen, NP0007) was added to the Western blot aliquot in a 13:5 ratio, where 13 is the sample and 5 is the LDS sample buffer.

The pellet was carefully resuspended in 2/3xPCV of 250 mM extraction buffer [0.42 M KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 10% Glycerol, 1X Protease inhibitor cocktail (added fresh) and MilliQ] and incubated on ice on a shake table for 30 minutes. It was then centrifuged at 4°C for 5 minutes at 20 000 rcf. The supernatant (nuclear fraction) was transferred to a new tube. The pellet was resuspended in 500 mM and then 1000 mM extraction buffer [0.6/1.2 M KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 10% Glycerol, 1X Protease inhibitor cocktail (added fresh) and MilliQ] with the same steps as for the first nuclear fraction. The collected volume of all fractions was recorded, also keeping record of how many µl that was discarded in case of pellet contamination. The nuclear fractions were diluted to 150 mM with dilution buffer [10 mM Tris pH 7.5, 0.1 mM EDTA, 10% Glycerol, 1X Protease inhibitor cocktail (added fresh) and MilliQ]. Aliquots were taken to be used in protein concentration measurement and Western blot. LDS sample buffer was added to the Western blot aliquots. The aliquots were stored in -20°C and the cytoplasmic and nuclear fractions were snap-frozen with dry ice and stored in -80°C. The protein amount in the fractions was measured with a protein concentration measurement kit (Bio-Rad, 5000112) and a spectrophotometer. The 250 mM and 500 mM extracts were diluted further (1:5 and 1:2, respectively) during the protein concentration measurement to ensure that the absorbance was within the range of optimal detection.

To verify disruption of the outer cell membrane, 5 µl samples were taken during SSE before swelling, after swelling, after 2 strokes and after 5 strokes. 5 µl Trypan blue (Invitrogen, T10282) was added to the samples and loaded onto a C-Chip (Digital Bio, DHC-B02). The ratio of cells with disrupted cell membrane (blue) compared to whole cells (white) was estimated in a microscope.

3.3 Whole cell extraction

HT1080 and MLS cells were cultured in T25 flasks. Flasks with a confluent monolayer of cells were put on ice and the medium was removed. The cells were scraped from the flask in PBS and centrifuged at 4°C for 6 minutes at 450 rcf. The supernatant was discarded and the pellet was resuspended in RIPA lysis buffer (Thermo Fisher, 89900) [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% sodium deoxycholate, 0.1% SDS] with addition of 1X EDTA (Thermo Fisher, 1861274) and 1X Protease and Phosphatase inhibitors (Thermo Scientific, 1861281). The cell lysate solution was incubated for 10 minutes on ice, and the solution was mixed gently with a pipette after 5 and 10 minutes of incubation. The cell solution was divided into two tubes and one of the tubes was centrifuged at 4°C for 10 minutes at 14 000 rcf to remove cell debris. The supernatant was transferred to a new tube avoiding the pellet in order not to contaminate the sample. Aliquots of both centrifuged and non-centrifuged solutions were taken to be used in protein concentration measurements and Western blot as described

above. For some cell lines, specifically uncentrifuged samples of HT1080, the sample turned into a gel when LDS sample buffer was added, most likely because of released DNA. In an effort to reduce the viscosity, the affected samples were sonicated in cycles of 30 sec on/30 sec off at 4°C with the Bioruptor Pico from Diagenode.

3.4 Western blot

Protein extracts from sequential salt extraction and whole cell extraction were size separated through gel electrophoresis using the NuPAGE system for Western blot (Thermo Fisher). Each load (well) of whole cell extracts was calculated and diluted to be 10 µg using the information received from the protein concentration measurements. The dilution buffer for whole cell extracts contained RIPA lysis buffer and LDS sample buffer, with a 13:5 ratio as mentioned before. For the extracts from SSE, their dilution factor and total volume including the discarded (contaminated) volumes were taken into account to load an equal percentage of each fraction across the salt concentrations within one cell line. Thus, maximum volume of the 1000 mM fraction was loaded on the gel and the 250 mM and 500 mM fractions were diluted with IP wash buffer [150 mM KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 10% Glycerol, MilliQ] and LDS sample buffer in a 13:5 ratio. All extracts were mixed with 10% sample reducing agent (Invitrogen, NP0004), resulting in a final ratio of 13:5:2 in the samples, and denatured at 70°C for 10 minutes (90°C if histones were evaluated) before loading 12.5 µl sample and 10 µl SHARP ladder (Invitrogen, 57318) on a 15-well 4-12% Bis-Tris gel (Invitrogen, NP0323BOX). The gel was run for 1 hour (50 min for histones to ensure that they do not travel too far down on the gel) at 150 V for the first 10 min and 200 V for the remaining time. The proteins were then transferred to membranes with 0.45 µm pore size (Invitrogen, LC2005) by laying the membrane on the gel and putting transfer buffer [20X Transfer buffer (Invitrogen, NP0006), 10 % EtOH, 1000X Antioxidant (Invitrogen, NP0005), MilliQ]-dipped filter papers to protect them, and then surrounding the gel-membrane sandwich with transfer buffer-soaked sponges to keep it from drying out. The gel-membrane sandwich and the sponges were put into a container for the NuPAGE system and were run for 1 hour and 15 min at 30 V. Cold water was put outside of the container but within the system to minimize heating and risk of drying the membrane. After the transfer, the membranes were cut to be able to visualize several primary antibodies per membrane while maintaining optimum space around each band and then blocked in either 5% skim milk powder or 5% bovine albumin serum mixed with TBS-T [50 mM Tris-HCl pH 6.8, 50 mM NaCl, 0.1% Tween 20] in room temperature for at least 30 minutes. The primary antibodies were added in the block buffer (Appendix A), and the membranes were incubated at 4°C overnight on a shaker. The membranes were washed in TBS-T for 3x10 min to remove the excess primary antibody before incubation with anti-rabbit or anti-mouse HRP-conjugated secondary antibody (Thermo Scientific, #32460 (anti-rabbit), #32430 (anti-mouse)) at 1:1000 dilution in block buffer for 1 hour in room temperature. The membranes were washed again in TBS-T before incubation with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, 34076). The proteins in the membranes were then detected via chemiluminescent signals in ImageQuant Amersham 800 (GE Healthcare). The Western blot signals were quantified in ImageJ's gel analyzer tool by marking a small rectangular area around each signal with the same box size for each antibody. ImageJ then created a plot of the signal, the background was removed manually by drawing a line through the base of the signals and the remaining area was calculated and used as the signal strength.

4 Results

This section includes results from the optimization of antibodies on Western blot, optimization of sequential salt extraction, and the results and quantification from Western blot with sequential salt extracts from HT1080 WT, EGFP, FUS-DDIT3-EGFP and FUS-CREB3L2-EGFP.

4.1 Optimization of antibodies on Western blot

Optimization of four antibodies (Table 2) from the BCL7 protein family was made using Western blot: BCL7A from Invitrogen (PA5-27123), BCL7B from Santa Cruz (sc-134278), BCL7B from Abcam (ab172358) and BCL7C from Novus Biologicals (NBP2-15559). The antibodies were tested with dilutions ranging from 1:100 to 1:3000, depending on the recommended dilution from the company, with BSA and milk as blocking buffer. Whole cell extracts of HT1080 and MLS cell lines were used as samples during the testing, both uncentrifuged and centrifuged samples. The goal of the optimization is to evaluate if the antibody is suitable to use to detect the target proteins and decide what dilution and blocking buffer to use in further experiments. A good result is clear, strong and separated bands at expected sizes with minimal background noise and unspecific binding. The recommendations for further use of the antibodies can be seen in Appendix A.

Table 2. The tested antibodies: size, expected band size, recommended dilution, clonality and species.

Antibody	**Protein size from NeXtProt (kDa)**	**Expected band size from company (kDa)**	**Recommended dilution**	**Clonality and species**
BCL7A (Invitrogen, PA5-27123)	22.8 & 25	40	1:500-1:3000	Polyclonal, rabbit
BCL7B (Santa Cruz, sc-134278)	15.5, 16, 17.9 & 22.2	32	1:100-1:1000	Monoclonal, mouse
BCL7B (Abcam, ab172358)	15.5, 16, 17.9 & 22.2	27	1:1000	Polyclonal, mouse
BCL7C (Novus Biologicals, NBP2-15559)	23.4 & 26.4	40	1:500-1:3000	Polyclonal, rabbit

4.1.1 BCL7A

The evaluated BCL7A antibody came from Invitrogen and was tested on Western blot with MLS 402, HT1080 WT and FUS-DDIT3-EGFP samples from whole cell extraction as input (Figure 2). The dilutions of the antibody used were 1:500-1:3000, based on recommendations from the company. BSA and milk were tested as blocking buffers.

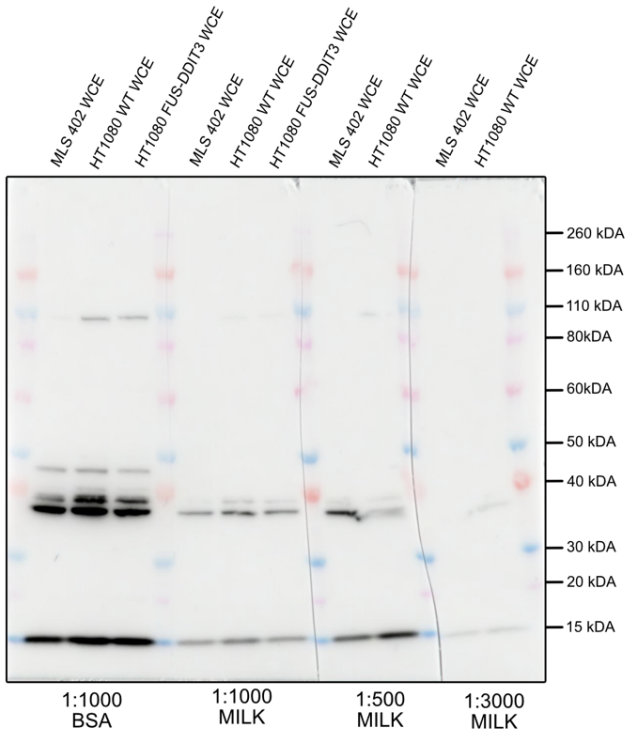


Figure 2. ***Evaluation of BCL7A antibody (PA5-27123) on Western blot with whole cell extracts (WCE, not centrifuged) of MLS 402, HT1080 WT and HT1080 FUS-DDIT3-EGFP. Blocking buffer and the dilutions of the antibody are indicated in the figure. The exposure time was 4.1 seconds using automatic exposure based on the strongest signal.***

The expected band size is just under 40 kDa according to the company. The strongest band (double band) for all dilutions were at the expected size and can therefore be concluded to be BCL7A. Clear and strong bands were observed for all dilutions and blocking buffers except 1:3000 milk. The bands at 1:3000 were weak due to the fact that less antibody was used, creating less signals. There was also a disturbance in the bands from HT1080 WT in 1:500 and MLS 402 in 1:3000. The absence of bands in this area is most likely because of an air bubble between the gel and membrane during the transfer, resulting in a non-ideal transfer and a lack of proteins in the affected area. Another set of strong bands were observed at 15 kDa, and these are most likely due to unspecific binding to smaller proteins. This is however not a problem as the bands are far away from the expected band size and can therefore be excluded. In the membrane blocked with BSA some weaker bands were seen up towards 50 kDa and 110 kDa and these are probably due to unspecific binding and insufficient blocking. The recommended dilution and blocking buffer for further use is 1:1000 milk.

4.1.2 BCL7B

The first BCL7B antibody that was optimized was one from Santa Cruz (Figure 3A). It was tested with the recommended dilutions with BSA and milk using the same samples as previously. The results did not show any band at the expected 32 kDa band size, but instead showed strong bands at 160 kDa for HT1080 samples, and very weak bands for MLS, visible only in the membrane blocked with BSA. There were weak bands a little bit above expected band size on the BSA membrane, but as they are not visible at all in the milk membranes, it cannot be concluded to be BCL7B. The antibody was tested again at a later date with 1:500 dilution and BSA (Figure 3B) to see if it was human error behind the first test, for example if the wrong antibody was used (since such a strong band was seen at a completely different size). The results of the second Western blot (**Fel! Hittar inte referenskälla.**) were very similar to the first, but stronger bands at the expected band size were observed. Because of this, a final Western blot was performed with one MLS cell line and HT1080 WT and FUS-DDIT3-EGFP

cell lines, centrifuged and non-centrifuged samples, with 1:500 dilution and both BSA and milk (Figure 4). The strongest signals were again at 160 kDa, except for MLS which showed no strong signals. There were a few signals that stood out from the background noise at around the expected band size in the BSA-membrane, but these were not as strong as the previous experiment and were not visible at all in the milk-membrane even when it was detected in the machine by itself, as seen in Figure 4. All of the testing concludes that the received antibody from the company does not accurately detect BCL7B and should not be used further.

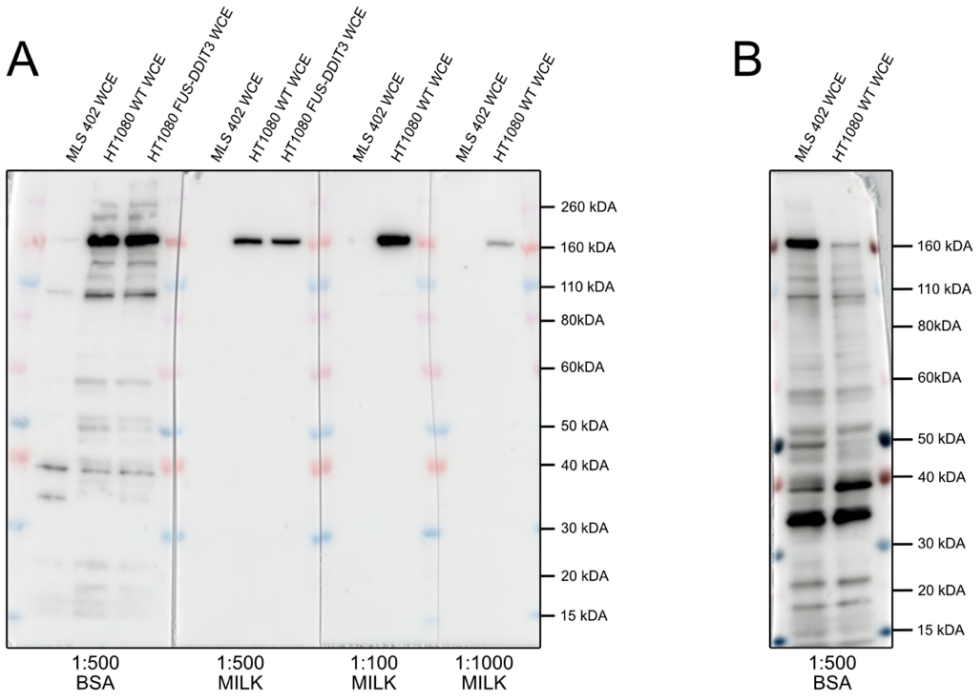


Figure 3. ***Evaluation of BCL7B antibody from Santa Cruz*** **(sc-134278)** ***on Western blot with whole cell extracts (WCE, not centrifuged) of MLS402, HT1080 WT and FUS-DDIT3-EGFP as input. Blocking buffer and the dilutions of the antibody are indicated in the figure. A) First evaluation of the antibody. B) Second evaluation of the antibody. The exposure time was 14.6 seconds and 24.3 seconds, respectively, using automatic exposure based on the strongest signal.***

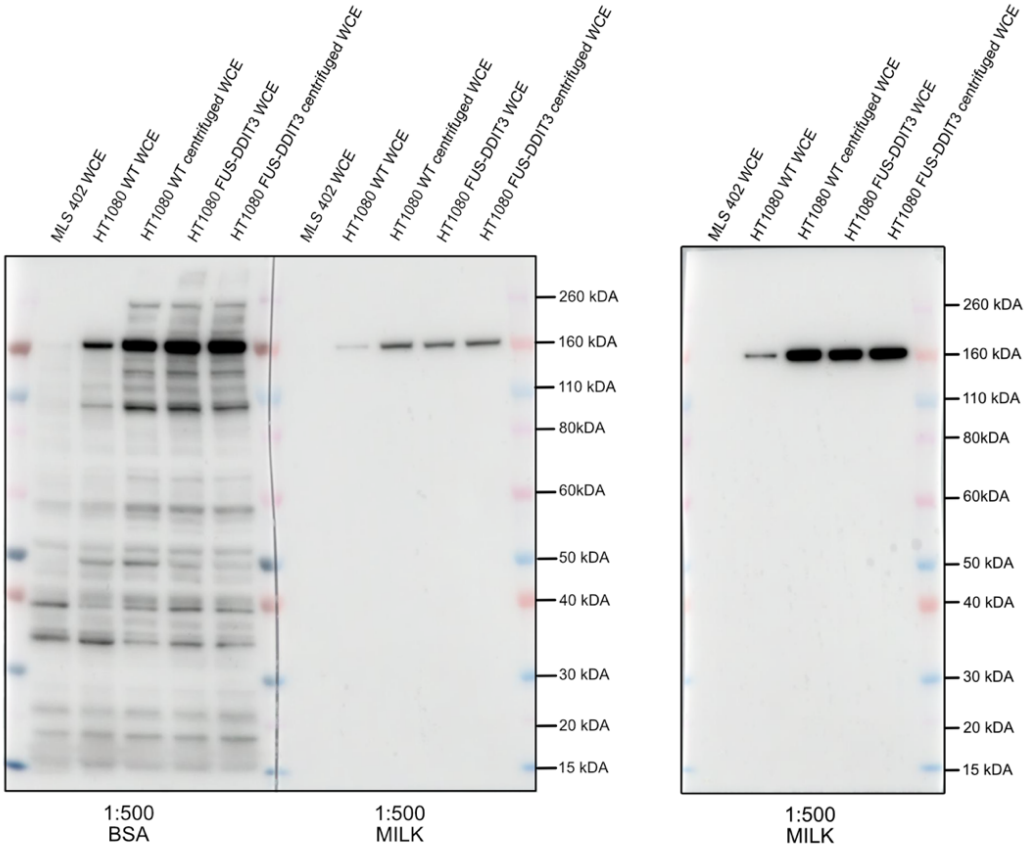


Figure 4. ***Final evaluation of BCL7B antibody from Santa Cruz*** **(sc-134278)** ***on Western blot with whole cell extracts (WCE) of MLS 402, HT1080 WT and FUS-DDIT3- EGFP (non-centrifuged and centrifuged) as input. Blocking buffer and the dilution of the antibody are indicated in the figure. The right part of the membrane (milk block buffer) was also detected separately. The exposure time was 13.3 seconds and 59.6 seconds, respectively, using automatic exposure based on the strongest signal.***

Because the first BCL7B antibody did not work, a new antibody was ordered from Abcam. They only recommended one dilution, 1:1000, but to test the antibody, a range of 1:500-1:2000 was used. The expected band size from the company was 27 kDa and the observed bands were slightly above 30 kDa, seen in Figure 5, and can be assumed to be the correct protein, as proteins travel differently on various gels. The BSA-membrane has a lot of background which makes it hard to distinguish the correct bands and BSA should therefore not be used as blocking buffer with this antibody. There was a bit of unspecific binding on the 1:500 membrane with bands at 15, 40, 60 and 110 kDa. A few of these bands can also faintly be seen in 1:1000 milk, but not at all in 1:2000 which solidifies the theory that it is unspecific binding, perhaps from excessive amounts of antibody. There were also a few vertical bands in 1:500 and it is unclear why they appeared. One reason could be damage to the membrane, allowing either primary or secondary antibodies to get stuck which will then produce a chemiluminescent signal during detection. The exposure time for the membranes blocked with milk was quite long compared to for example BCL7A, which had an exposure time of 4.1 seconds while BCL7B (Abcam) had 2 minutes 18 seconds. Longer exposure and detection times indicate lower amount of protein or a suboptimal antibody. In order to have detection times that are not too dissimilar to other antibodies, the recommendation for further use is 1:500 milk even though there is some unspecific binding, as this does not interfere with the correct band at 32 kDa.

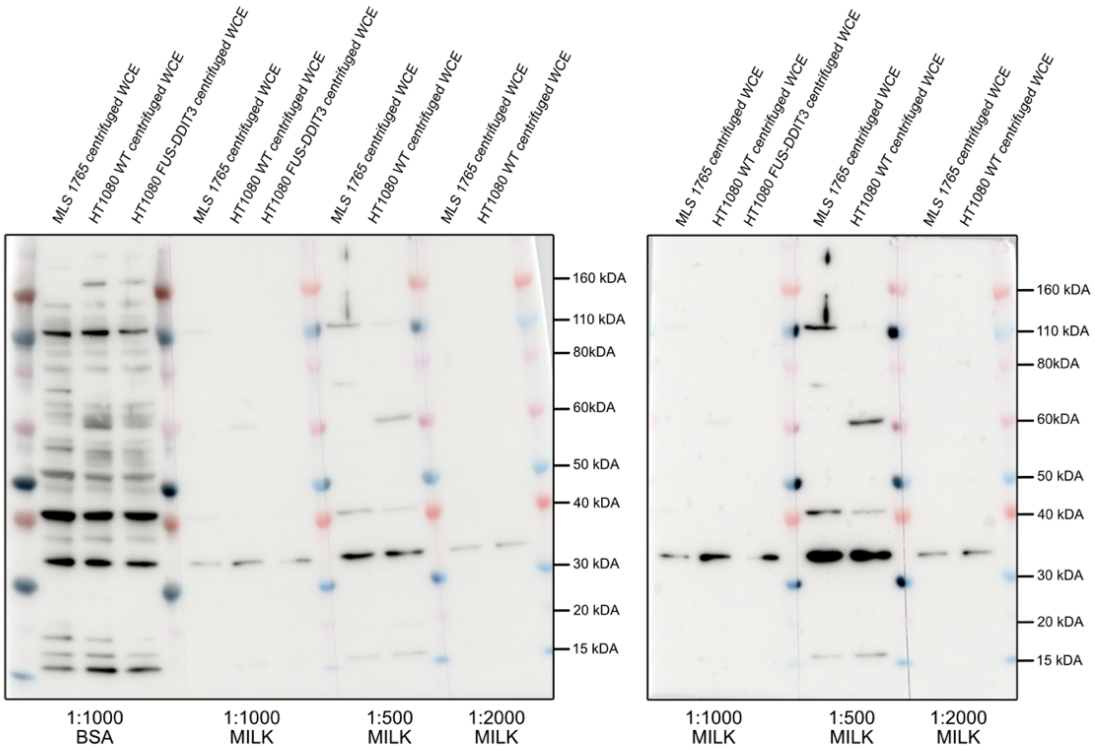


Figure 5. ***Evaluation of BCL7B antibody from Abcam*** **(ab172358)** ***on Western blot with whole cell extracts (WCE) of MLS 1765, HT1080 WT and FUS-DDIT3-GFP (all centrifuged) as input. Blocking buffer and the dilutions of the antibody are indicated in the figure. The right part of the membrane (milk block buffer) was also detected separately. The exposure time was 1 minute 0.4 seconds and 2 minutes 18 seconds, respectively, using automatic exposure based on the strongest signal.***

4.1.3 BCL7C

A BCL7C antibody from Novus Biologicals was tested and optimized. As with the other BCL7-antibodies, samples from whole cell extractions were used as input and both BSA and milk was used as a blocking buffer, with a dilution range of 1:500-1:3000, based on the suggestion from Abcam.

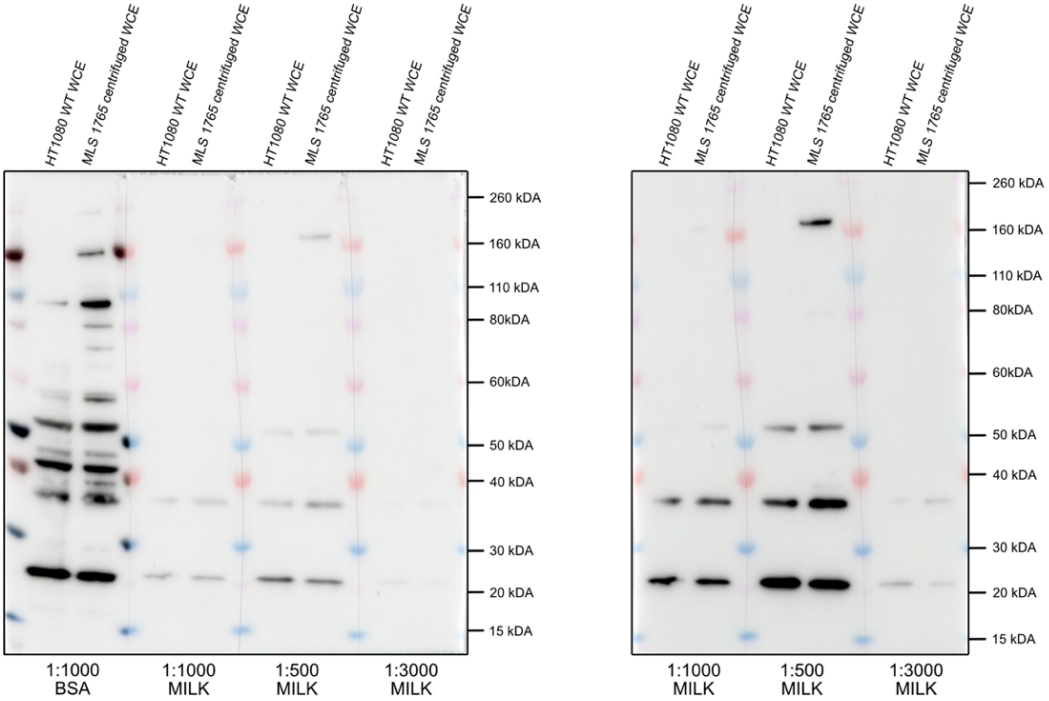


Figure 6. ***Evaluation of BCL7C antibody*** **(NBP2-15559)** ***on Western blot with whole cell extracts (WCE) samples as input (not centrifuged or centrifuged). Blocking buffer and the dilution of the antibody are indicated in the figure. The right part of the membrane (milk block buffer) was also detected separately. The exposure time was 16.9 seconds and 1 minute 6.4 seconds, respectively, using automatic exposure based on the strongest signal.***

Clear bands across all cell lines were observed at 20 and ~37 kDa. The band at 37 kDa is very close to 40 kDa and can be presumed to be BCL7C. The band at 20kDa is probably unspecific binding as all isoforms of BCL7C are bigger than 20 kDa. The background noise in the BSA-membrane is too high and the correct bands are therefore hard to distinguish. One of the antibodies or the developing liquid seems to have bound into the ladder in the BSA membrane as well which can skew the machine's detection time for the correct bands which is not preferable. The milk membranes by themselves have a detection time of about 1 minute and it is the 1:500 membrane that has the strongest signal. The recommendation for future experiments is to use 1:500 dilution with milk as the blocking buffer to get the strongest signal while having minimal background.

4.2 Optimization of sequential salt extraction

The major part of this project was spent on optimizing sequential salt extraction (SSE) for HT1080 cells. Lindén *et al.* 2022, used the same method for MLS cell lines [30] but initial tests found that the pellet from HT1080 cells became gel-like and could not be resuspended at high salt concentrations (not published). The first trial in this project followed the original protocol

and faced the same problem, verifying that the method needed to be modified. The optimization was based on the method described in 3.2 and several different approaches were used in an effort to minimize the occurrence of gel in the pellet to be able to collect the entire nuclear salt fractions, these are summarized in (Table 3).

Table 3. The attempts and modification of the protocol of SSE. *EB = extraction buffer

Attempt	HT1080 cell line	Packed cell volume (PCV)	Change in protocol	Comments
1	WT FUS-DDIT3-EGFP	450-500 300	None	Gel-pellet at 1000 mM EB
2a	WT	250	Double volume of Benzonase	Worked well, no gel formation
2b	WT	240	Slow addition of EB* in 4 parts while pipetting the pellet gently	Worked well, no gel formation
3	FUS-DDIT3-EGFP	300	Double volume of Benzonase + slow addition of EB while pipetting the pellet gently	Turned to gel at the addition of 1000 mM EB, showed signs of gel formation at 500 mM
4	FUS-DDIT3-EGFP	150	Double volume of Benzonase + resuspension of the pellet in a low concentration EB before adding a high concentration EB to get a final concentration of 500 mM and 1000 mM	Worked well, no gel formation

The very first SSE was done on HT1080 WT and FUS-DDIT3-EGFP cells by following the original protocol. Cells from both cell lines probably exploded during the addition of 1000 mM extraction buffer (EB) and released DNA, resulting in a gel-pellet that could not be resuspended nor properly centrifuged. Centrifuging a gel-pellet results in a pellet that does not stay at the bottom of the tube, making it difficult to extract the supernatant without contamination. There is also a high risk of not being able to collect all of the supernatant or being able to collect the supernatant at all since it is trapped inside the gel. The first approach to optimize the method was to double the amount of Benzonase added, as Benzonase breaks down DNA and RNA and reduces the viscosity thereby, in theory, hopefully avoiding the formation of a gel-pellet. The first attempt went well and there was no gel formation. The manufacturer of Benzonase declares, however, that the enzyme activity is inhibited by monovalent cations concentrations over 300 mM, such as K^+ and Cl^-. The current method therefore adds Benzonase when the cells are suspended in hypotonic lysis buffer, allowing Benzonase to break down DNA and RNA before the nuclear extraction steps which quickly reach concentrations over 300 mM KCl, and

high salt concentrations are necessary for the purpose of the lab. Because of this, doubling the amount of Benzonase is not a method change that will always ensure that there is no gel formation. At the same time, another change was evaluated in another batch of cells where EB was added slowly in 4 parts while simultaneously pipetting the pellet to resuspend it in order to minimize isolated areas of high concentrations of salt that disrupt the cells. This went well for all concentrations of salt and there was no gel formation but pipetting the pellet without much liquid in the tube was tricky as it was quite firm, and there was some bubble formation. Thus, both first attempts seemed to be successful. For the next attempt, both of the previous measures of optimization were combined, comprised of double amount of Benzonase and slow addition of EB while pipetting the pellet, this time extracting HT1080 cells with FUS-DDIT3-EGFP suspected to be even more prone to gel formation. Instead of adding the EB in 4 parts, a bigger pipette was used that could hold all of the liquid that was going to be added and the liquid was slowly added while using a second pipette for resuspension, which was easier than stopping and having to refill the pipette tip several times. This attempt did not work, and the pellet turned to gel during the addition of 1000 mM EB and showed signs of gel during the addition of 500 mM EB, as the pellet did not resuspend as easily as it had done before. During the whole attempt, it was still hard to break up the pellet during pipetting when there was very little liquid in the tube and bubbles formed as a result, even at 250 mM EB. The bubbles did not disappear after the incubation and centrifugation which made it hard to remove all of the supernatant and to get a correct volume estimate of the supernatant, which is important for calculations of protein quantity later on. The stiffness of the pellet could be the result of a higher PCV, i.e. more cells, causing further difficulties in breaking up the pellet. Later experiments were often designed to have a lower PCV, at 150-200 µl, to reduce this problem. Overall, the attempted changes were not sufficient to perform SSE on transfected HT1080 cells.

We believed that one cause of the formation of a gel-pellet could be that small areas of the pellet are exposed to a high concentration of salt during the resuspension when the pellet is firm and hard to pipette. This disrupts the nuclei in that area, causing an increase in viscosity which in turn causes difficulty in resuspending the pellet and other areas will have the same problem with high concentrations of salt and disrupted nuclei. This led to the next attempt, which was to have double the amount of Benzonase and to resuspend the pellet in a lower concentration of EB first and then adding an EB with a high concentration to get a final concentration of 500 mM and 1000 mM. Because there had never been any problem of resuspending the pellet in 250 mM EB, it was added as described in 3.2 which was 2/3xPCV. For 500 mM, 1/3xPCV of 250 mM EB and 1/3xPCV of 750 mM EB was added instead, and for 1000 mM, 1/3xPCV of 500 mM EB and 1/3xPCV of 1500 mM EB was added. This worked very well, and because the pellet was already resuspended with a low concentration for 500 mM and 1000 mM, the high concentration EB could be incorporated into the suspension easily, avoiding gel formation. This modification in method was used during the rest of the project with four cell lines: HT1080 WT, EGFP, FUS-DDIT3-EGFP and FUS-CREB3L2-EGFP. Gel formations occurred during one extraction of FUS-DDIT3-EGFP and EGFP cells with the modified method, and this was likely because of the usage of a week old Benzonase solution instead of a freshly made one and/or a PCV that was too high (400 µl).

The final modified and optimized method follows all the same steps as described in 3.2, except that the volume of Benzonase is (3xPCV)/25, and the Benzonase solution should be made fresh to ensure maximum enzyme activity. Another difference to the protocol is the EB, which now consists of 4 concentrations of KCl: 250, 500, 750 and 1500 mM. The modified extraction buffer is [0.25/0.5/0.75/1.5 M KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 10% Glycerol, 1X

Protease inhibitor cocktail (added fresh) and MilliQ]. These buffers do not take any dilution from the liquid in the cells into consideration, which the original buffer does.

4.2.1 Evaluation of cell disruption

Aliquots of cell suspension were taken during the SSE to evaluate how efficient the syringe was at disrupting the outer cell membranes. After cell disruption, cell lysis should be at least 80% to ensure sufficient collection of the cytoplasmic fraction and creation of a nuclear pellet from where nuclear proteins can be extracted. The aliquots were mixed with Trypan blue, which is a dye that stains disrupted cells, turning them blue, while leaving undisrupted cells white. Thus, almost all cells should be undisrupted before using the syringe and almost all cells should be disrupted by the syringe and needle. This is to ensure that the fraction collected after the disruption contains cytoplasmic proteins and that these are not lost in earlier steps. As a control, trypsinated cells were stained with Trypan blue. Trypsin should in theory not break down the outer cell membrane, only the proteins that the cells use to adhere to a surface, and therefore the cells should remain white after Trypan blue is added. The trypsinated cells can be seen in Figure 7, where almost all cells are white, showing very few signs of disruption.

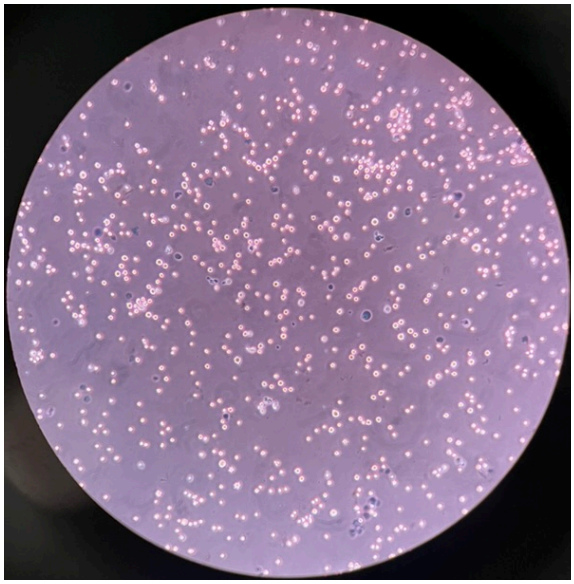


Figure 7. ***Picture of trypsinated cells, stained with Trypan blue.***

HT1080 cells had previously been evaluated to ensure sufficient cell disruption after 5 strokes, similar to MLS cell lines while EWS cell lines only required 2 strokes. However, although HT1080 with all the different vectors had not been evaluated, small differences between these cell lines were expected. Here, the cell disruption was evaluated for all cell lines used in SSE. As mentioned earlier, the SSE protocol is based on the premise that the cells have intact membranes before the syringe and a needle is used to disrupt them. This was however not the case with the HT1080 cells that were used during the project, as seen in Figure 8A. This picture was taken after the cells were allowed to swell, but before any disruption with the syringe. Most cells are clearly blue, and therefore have a disrupted outer membrane. Because the cells did not show signs of disruption when they were trypsinated, and they were not damaged in the cell swelling step or by resuspension in a small 10 µl pipette (not shown), they were most likely damaged by the scraping that detaches the cells, a somewhat unexpected result. This was however not a problem for the results in this project as we were only interested in the proteins located in the nucleus, but it can be a problem if the cytoplasmic proteins are the proteins of interest as many proteins are lost after the first rounds of centrifugation, where the supernatant is not saved. Figure 8B and 9C show the cells after 2 and 5 strokes, respectively, and while the result is not optimal as there were still many white cells after 5 strokes, the syringe seems to have had an effect on disrupting the cells as the ratio between blue and white cells is bigger in Figure 8C compared to Figure 8A. There is an area in Figure 8C that has a bigger blue spot,

indicated by the arrow, that can be caused by several factors such as aggregation of cells or disrupted nuclei. Because it is only a small area and some white cells are left, the recommendation for further experiments is to perform 5 strokes for HT1080 WT. HT0180 FUS-DDIT3-EGFP looked very similar to WT and have the same recommendation.

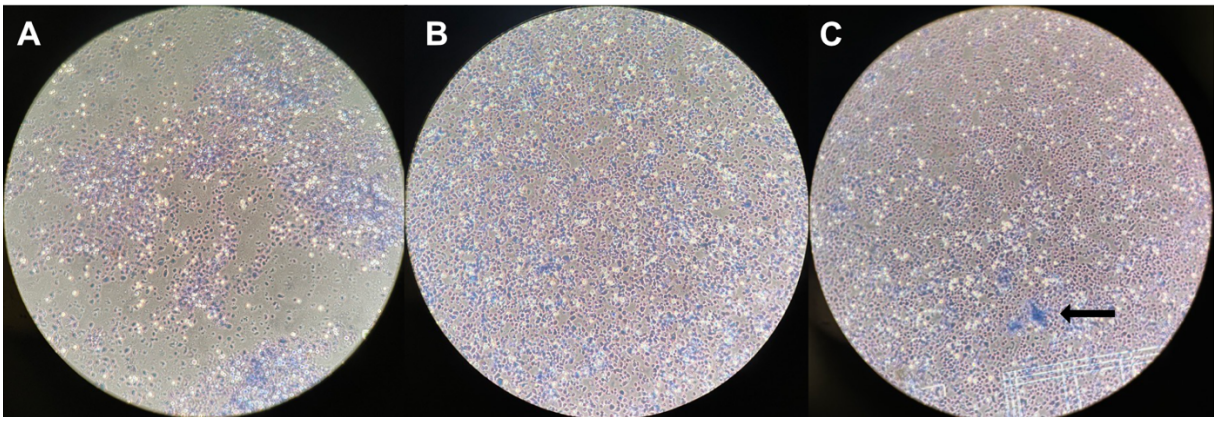


Figure 8. ***Aliquots of HT1080 WT cells during cell disruption, stained with Trypan blue. The pictures are representative of several trials. A) Cells before any strokes B) Cells after 2 strokes C) Cells after 5 strokes.***

The evaluation of cell disruption in HT1080 EGFP is pictured in Figure 9 and illustrates that some cells showed signs of disrupted nuclei even before any strokes had been performed as there are some spots that are a darker blue than the rest and there was obvious aggregation of cells on the chip. If some nuclei have burst, the sample becomes viscous and difficult to load onto the chip, which in turn makes it hard to analyze the sample properly. However, a conclusion can be drawn that there is too much nucleic disruption than what is desired. The disrupted nuclei in EGFP increased with the number of strokes and several spots of a darker blue with undefined borders were seen after 5 strokes (Figure 9C), leaning towards a recommendation that only 2 strokes should be performed in HT1080 EGFP, but further evaluation is needed.

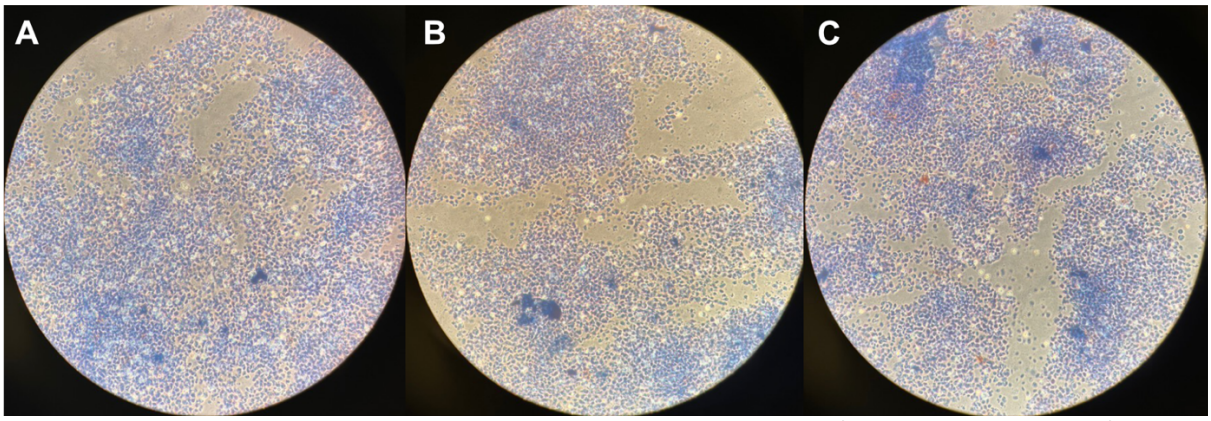


Figure 9. ***Pictures of HT0180 EGFP during cell disruption, stained with Trypan blue. A) Cells before any strokes B) Cells after 2 strokes C) Cells after 5 strokes.***

Sequential salt extraction of HT1080 FUS-CREB3L2-EGFP were performed for the first time, making these quality controls extra important. Of note, HT1080 FUS-CREB3L2-EGFP were not as damaged by the scraping as the other cell lines, see Figure 10. There was a clear majority of white cells before the strokes, and while the syringe had an effect on disrupting the outer membrane, there were still too many white cells after 5 strokes to get an efficient extraction

which was surprising. When there are many undisrupted cells, the extraction buffer most likely cannot solubilize the nuclear protein which leads to lower protein concentration in the extracts, as seen in Appendix B. Next time, a more thorough evaluation is needed where the cell disruption is continuously evaluated when performing the strokes. The most probable reason for the higher durability in FUS-CREB3L2 compared to the other cell lines is that they do not have the same HT1080 cells as a host or an impact of the fusion on the cell. EGFP and FUS-DDIT3-EGFP have been transfected into HT1080 WT cells by the lab group, while FUS-CREB3L2-EGFP were obtained as already transfected cells, with a different batch of HT1080 WT cells as a host. Even though all HT1080 cells originate from the same tissue sample, small changes within the cell can occur after years of cell culture at alternative locations, and impact, e.g., the durability of the outer membrane.

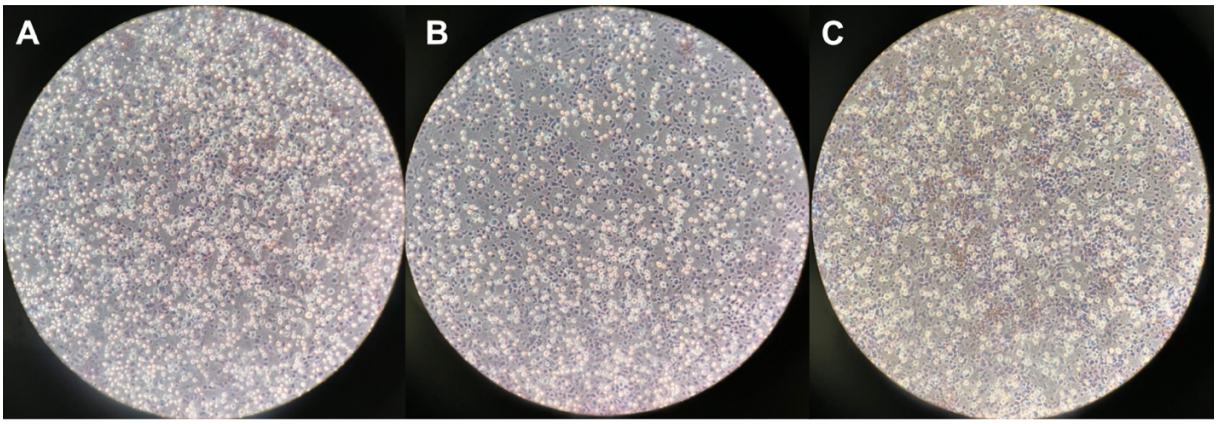


Figure 10. ***Pictures of HT1080 FUS-CREB3L2-EGFP during cell disruption, stained with Trypan blue. A) Cells before any strokes B) Cells after 2 strokes C) Cells after 5 strokes.***

4.3 Evaluation of chromatin binding strength in sequential salt extracts on Western blot

Successful sequential salt extraction of HT1080 WT, HT1080 EGFP and HT1080 with stable expression of FET fusions FUS-DDIT3-EGFP and FUS-CREB3L2-EGFP were performed. In order to evaluate if the proteins of interest were present in the SSE extracts and if so, when they were released from chromatin, Western blots were performed with antibodies targeting SWI/SNF components as well as some interaction partners to SWI/SNF. Appendix A lists all antibodies used, with respective dilution and blocking buffer. The signal strength of the bands from Western blots were quantified through ImageJ's gel analyzer tool and the relative protein amount was calculated with respect to the signal strengths within each cell line for every antibody. The extracts from WT and EGFP should in theory have very similar extraction profiles as the only difference between them is that EGFP cells is transfected with a vector that only expresses the EGFP protein which should not affect proteins binding to chromatin. This section will present the most interesting results from the evaluation, but pictures and plots of all of the proteins evaluated can be seen in Appendix C and D.

To ensure that the fusions were expressed in the cells, an EGFP antibody was used on Western blot. In theory, WT should have no expression of EGFP, the EGFP cell line should have an expression of EGFP at 30 kDa and the fusion cell lines should have expressions of EGFP at 80 kDa. The difference in size between the EGFP cell line and the fusion cell lines is because in

the fusions, the EGFP is expressed together with the length of the FET-FOP, creating a bigger protein. The result of the evaluation showed that the EGFP cell line correctly expressed EGFP at 30 kDa and that FUS-DDIT3-EGFP expressed EGFP with a size of 80 kDa (Figure 11). Of note, FUS-CREB3L2-EGFP did not show any expression of EGFP in this evaluation. The fusion was however evaluated by a colleague in whole-cell extracts collected a week earlier and expressed EGFP at 80 kDa during the evaluation. This gives reason to believe that the cells correctly expressed FUS-CREB3L2-EGFP even though it was not visible during the Western blot with SSE extracts, as it is highly improbable that the cells have lost the transfected vector in the matter of one week. An explanation to the loss could be that most of the EGFP is in the cytoplasmic fraction or that the incomplete cell disruption prevents sufficient collection of protein, but more experiments are needed to verify the expression of the fusion protein in sequential salt extracts.

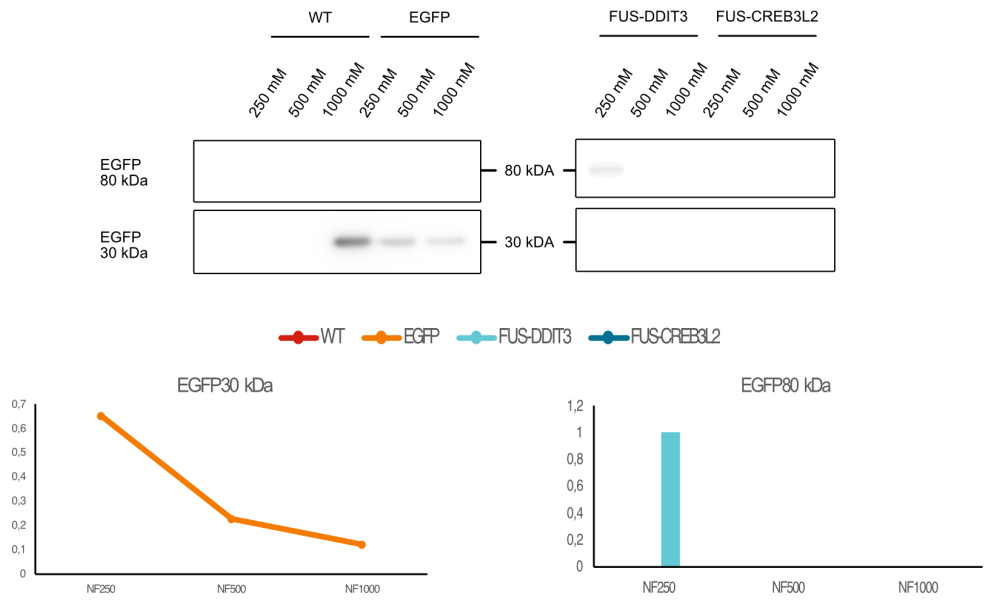


Figure 11. ***The expression of EGFP in the evaluated cell lines.***

Then, the binding strength of 20 different SWI/SNF components were evaluated: the core components BAF155, BAF60A, BAF170, BAF57, and BAF47, the ATPase components BRG1, BRM, BAF53A, BCL7A/B/C, and SS18, and the subtype-specific components ARID1A, ARID1B, ARID2, PBRM1, BRD7, BRD9, GLTSCR1, and GLTSCR1L (Figure 12 and Appendix C and D). The results showed that SWI/SNF components had similar extraction profiles in all HT1080 cell lines, with most of the proteins disassociating at 500 mM, as seen in Figure 12A-B where BRD7 represent the extraction profile of most SWI/SNF components. Thus, the extraction profiles did not differ for SWI/SNF components from different subtypes or functional modules. For several SWI/SNF components, three out of four cell lines had this distinctive triangle profile while one cell line showed protein dissociation at other salt concentrations. An example of this is BRM, a protein in the ATPase module in all SWI/SNF subtypes, which disassociated mostly in 1000 mM in FUS-CREB3L2-EGFP, whereas it released mostly in 500 mM in the other cell lines (Figure 12). FUS-CREB3L2-EGFP also had a different extraction profile for ARID2 and BRG1, where ARID2 was released at 500 mM and 1000 mM instead of mostly at 500 mM, and BRG1 was released 30-40% at all concentrations. BRG1 disassociated mostly at 500 mM in the other cell lines, but with some differences in the relative protein amount at each concentration. These results show that the fusionFUS-CREB3L2-EGFP might have an impact on the binding affinities of these proteins to chromatin.

Several proteins, namely BAF53A, GLTSCR1L, and BAF170, were extracted at a higher percentage in 250 mM and a lower percentage in 500 mM for FUS-DDIT3-EGFP compared to the other cell lines (Figure 12D). They still had the triangle shape seen in other cell lines and SWI/SNF components, but with a slightly changed relative protein amount from NF250 and NF500. This indicates that the fusion of FUS and DDIT3 might alter the epigenetic landscape to a certain extent. There was one protein in the SWI/SNF complex that did not follow the pattern of disassociation as the other SWI/SNF components, namely BCL7B. For all cell lines, BCL7B released at 250 mM while the BCL7A and BCL7C had a similar extraction profile as described earlier (Figure 13). None of the fusions had a divergent extraction profile for any of the BCL7 components.

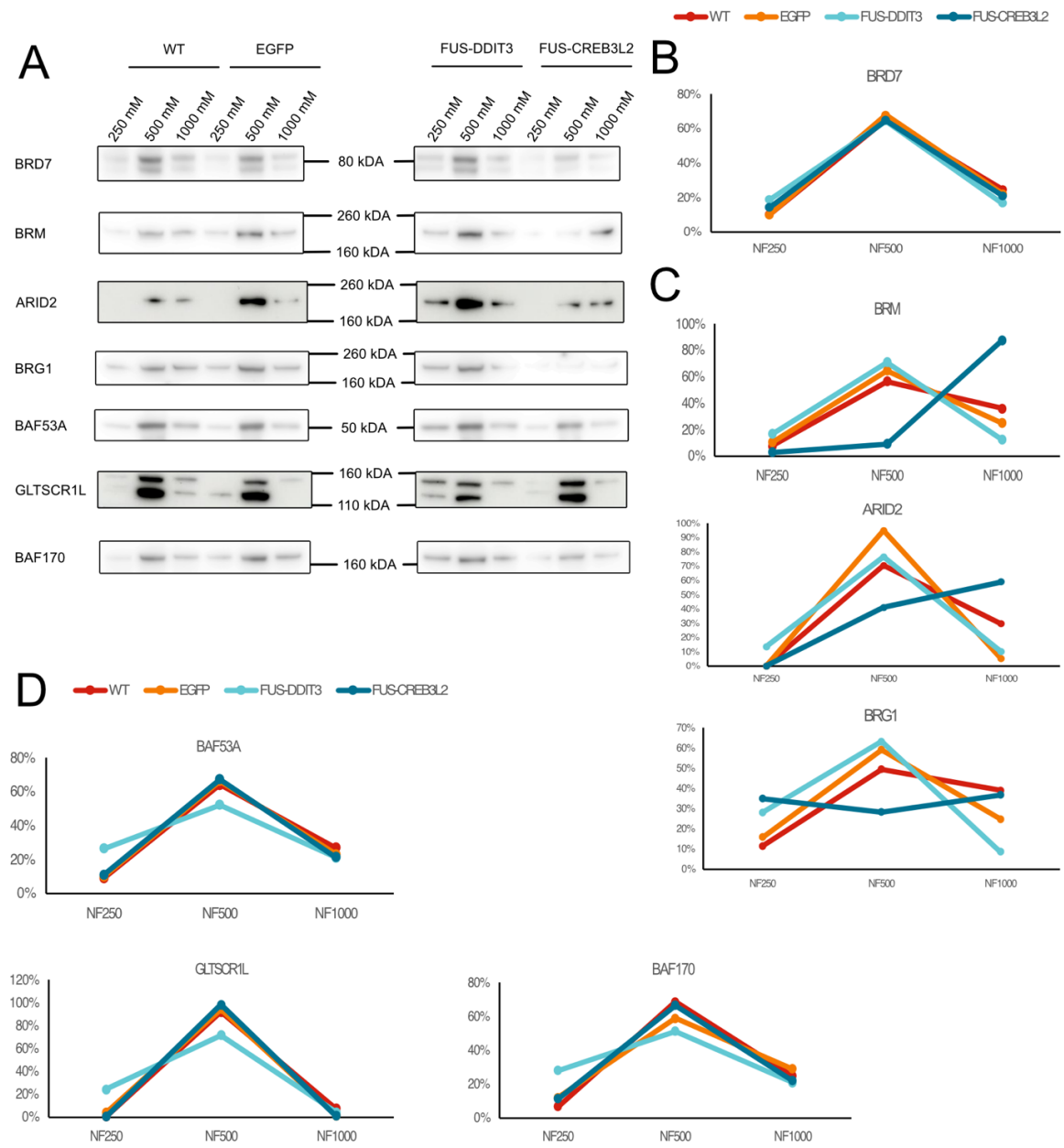


Figure 12. ***Analysis of chromatin binding strength for SWI/SNF components. A) Western blot analysis of SSE fractions 250, 500 and 1000 mM salt for SWI/SNF components BRD7, BRM, ARID2, BRG1, BAF53A, GLTSCR1L, BAF170 B) Representative graph of the extraction profile of SWI/SNF components, visualizing BRD7 C) Extraction profiles of SWI/SNF components where FUS-CREB3L2-EGPF have a different extraction profile D) Extraction profiles of SWI/SNF components where FUS-DDIT3-EGPF have a different extraction profile.***

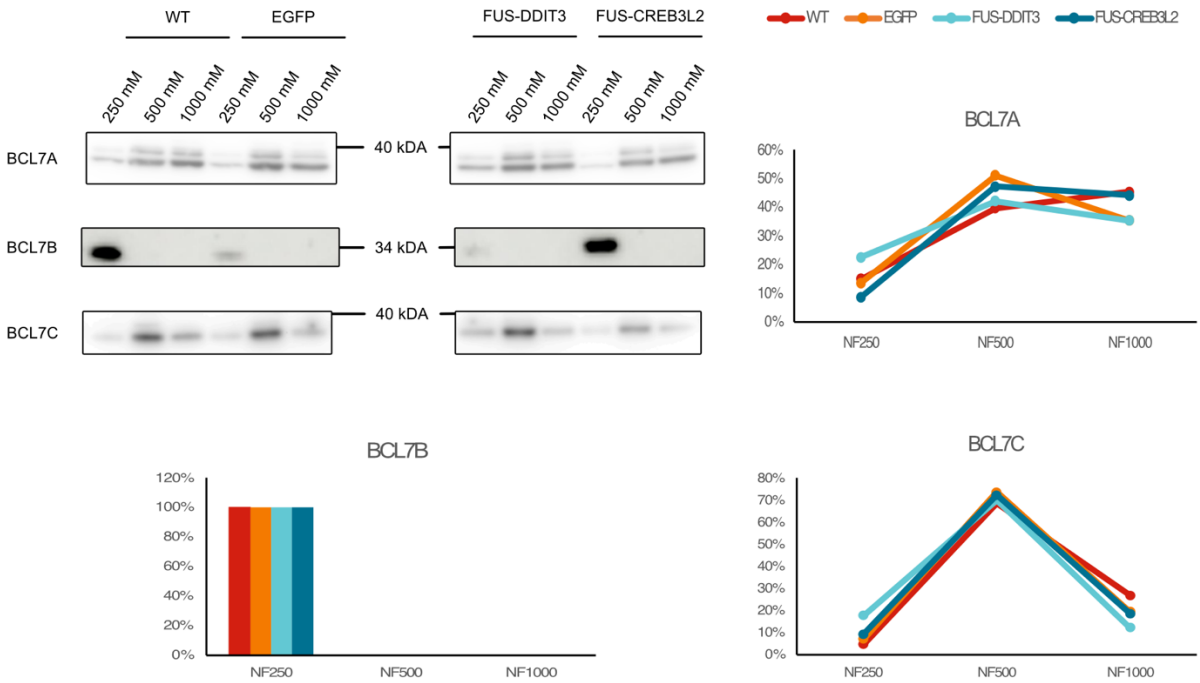


Figure 13. ***Western blot analysis and extraction profiles of BCL7A, B, and C, based on Western blot of SSE fractions 250, 500 and 1000 mM salt from HT1080 WT, EGFP, FUS-DDIT3-EGFP and FUS-CREB3L2-EGFP.***

Several known interaction partners of FET oncoproteins were also evaluated: the normal FET protein EWS and FUS, the PRC2-component EZH2, BRD4, and the histone component H4 (Figure 14 and Appendix C and D). Interestingly, the evaluated interaction partners did not showcase the same extraction profile as components of SWI/SNF, as seen in Figure 14. EWS, for example, had an almost linear extraction profile with the highest disassociation at 250 mM, but no big difference between the cell lines, indicating that the fusions do not have any impact on chromatin binding of EWS. FUS-CREB3L2-EGFP seems to have an impact on the binding strength of EZH2 as most of the proteins disassociated at 250 mM, whereas the proteins released quite evenly through all concentrations with the other cell lines. Two isoforms of BRD4 were detected during the Western blot evaluation. The band at 110 kDa showed a slight difference in disassociation between WT/EGFP and the fusions, where the fusions released a bit more at 250 mM than WT and EGFP. This indicates that the fusions might have a minor impact on this isoform of BRD4. The band at 160 kDa showed very different extraction profiles for all cell lines, and because WT and EGFP did not show any similarities, it is very hard to draw any conclusions regarding the impact FET-FOPs have on this protein isoform. One protein that the fusions might have an impact on is the H4 histone, where WT and EGFP had the same extraction profile, with most proteins released in 1000 mM while both fusions differ from the WT/EGFP profile and instead had a similar shape as the SWI/SNF components.

Interestingly, there were differences in extraction profiles with the fusions for both SWI/SNF components and interaction partners. But, there was no common trend observed between the different fusions and their divergent extraction profiles of SWI/SNF components, indicating that different FET fusions have distinct effects on chromatin binding perhaps by interacting with SWI/SNF complex with diverse compositions, however the observed variations were independent of the SWI/SNF subtype or function.

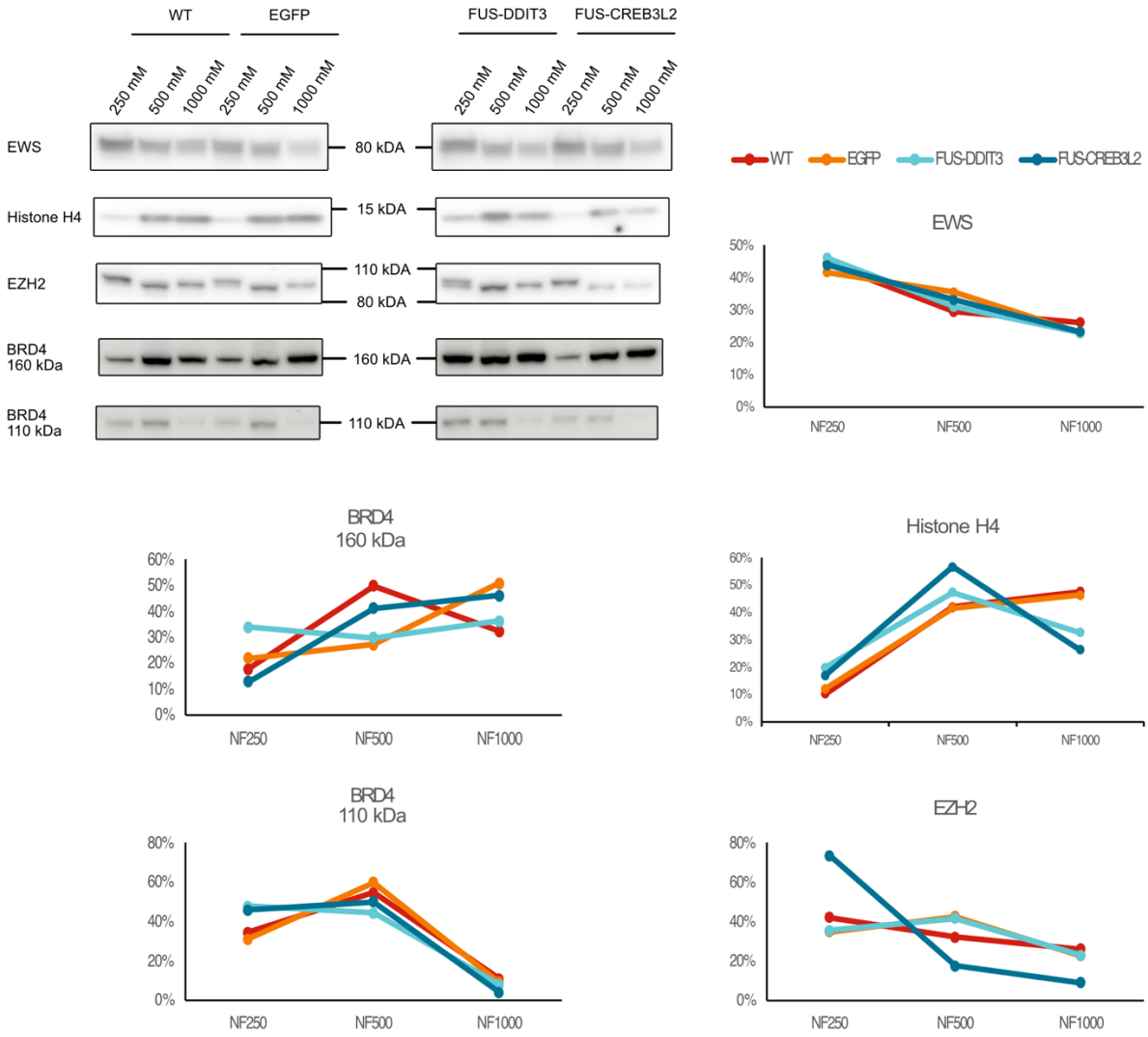


Figure 14. ***Analysis of chromatin binding strength for SWI/SNF components. Western blot analysis and extraction profiles of SSE fractions 250, 500 and 1000 mM salt for SWI/SNF interaction partners EWS, BRD4, EZH2 and the histone component H4.***

5 Discussion

This section will discuss methods used during the project, as well as the results and future prospects.

5.1 Optimization of BCL7 antibodies

The optimization of BCL7 antibodies went well and showed the importance of knowing what bands to look for and what blocking buffer and dilutions should be used when trying new antibodies. The purchased antibodies had been tested with knockouts by the companies to ensure its specificity, but none of the antibodies had citations in published articles, and very few articles have used BCL7 antibodies overall, making it hard to conclude that the visible bands were the correct ones. The known isoforms were studied through databases and published articles and the bands visible in the Western blots of this project were compared with the articles that have used BCL7 antibodies, all to ensure that the visible bands were correct. Knowing the protein size was especially important during the evaluation of BCL7B from Santa Cruz, which showed bands at 160 kDa. By knowing that the band should be around 40 kDa, it was fairly simple to determine that the antibody did not target the correct epitopes. The optimization also showed the importance of trying different blocking buffers and dilutions of the antibodies to get optimal signals with as little background noise as possible. Throughout all evaluations, the membranes blocked with BSA showed significant background, causing difficulties in determining the correct bands. This is often a problem with BSA as it does not block the membrane as efficiently as milk, and since BSA is also more expensive, milk is often a more suitable blocking buffer to use. The optimal dilution of the antibody during Western blot is based on the concentration and efficiency of the antibody, but also on the amount of protein in the sample. This is why testing different dilutions and measuring the protein concentration of samples are important, as it is then possible to modify the dilution to get a stronger signal while also being mindful of not wasting antibodies as they are often expensive.

5.2 Optimization of sequential salt extraction

The protocol for SSE is quite long with many steps, in comparison to whole cell extraction for example, so it was difficult to know where to modify the method in order to optimize it, i.e. stopping the occurrence of gel pellets. Because the gel comes from nuclei rupturing and releasing long strands of DNA, a natural start to the optimization was to double the amount of Benzonase, which is supposed to break down DNA, as well as adding EB slowly to minimize the stress and shock to the cells. These attempts were done separately on WT cells and worked well, and in the next attempt, the changes were combined during extraction of FUS-DDIT3-EGFP but failed, as described in 4.2. FUS-DDIT3-EGFP had in previous projects been more prone to gel formation than WT, the reason for this is unknown but could be because of the expressed FET-FOP as the EGFP cell line does not cause as much difficulty. A question also

arose regarding the efficiency of Benzonase treatment and if it helps in breaking down nucleic acids. It is unclear from the producer how much time Benzonase needs to work, or if the Benzonase even reaches inside the nuclei in the current protocol, but evaluating the need for Benzonase was considered to be too time consuming for this project. Benzonase is however used in SSE in published articles and have been seen to produce a better yield during former experiments within Ståhlberg group. The change of the protocol that had the best result was to first resuspend the pellet in a low salt concentration and then adding a high salt concentration to get a final salt concentration of 500 mM and 1000 mM. This gave the cells the least shock and difference in osmotic pressure, making the nuclei less likely to burst. The extraction buffers in the original protocol accounted for the dilution by the cells' liquid volume, resulting in final concentrations of 250, 500, and 1000 mM. The new buffers did not do this and had instead a concentration of 250, 500, 750 and 1500 mM before they were added to the cell pellet which makes the final concentrations somewhat lower than 250, 500 and 1000 mM. Other SSE protocols do not account for dilution, and it would be interesting to analyze the differences in extraction profiles when using buffers that consider cell dilution in future experiments. The reason for not taking the cells' inherent liquid into consideration when making the new buffers was because of time constraints. Another important aspect of SSE is how the cells are behaving during the expansion before the extraction. For the extraction to give valuable results, the cells cannot behave strangely as it indicates that the cells are under distress and exhibit altered protein expression profiles. Overall, SSE is a relatively simple method to use in order to get information about the epigenetic landscape on a global scale, and the method can be used to elucidate how changes in the cell, for example vector expression or cell treatments, can change the epigenetic landscape. This has also been shown by Porter, Connelly and Dykhuizen, 2017, where the extraction profiles of ARID1A, PBRM1 and BRD4 changed depending on the presence of writer and reader inhibitors [31].

5.3 Evaluation of chromatin binding strength in sequential salt extracts on Western blot

The quantification of SSE fractions is relative and done within each cell line. There are many opinions in the scientific community on how to quantify Western blots correctly, for example which software that should be used, how the background should be excluded, how much space to include in the quantified area etc. Most methods of quantification are therefore subjective and comparison between articles should be done carefully. Because the same method of quantification and calculations prior to the Western blots were done for all antibodies and cell lines during this project, comparisons can be made with the relative protein amounts from each cell line for every antibody.

The result in this project showed a distinctive extraction profile for SWI/SNF components, where most of the disassociation occurred at 500 mM KCl. There is some indication that the fusion cell lines might have an impact on the chromatin binding strength of SWI/SNF components as the fusions exhibited a different extraction profile for a few of the components. The interaction partners of SWI/SNF did not have a common extraction profile, but the fusions seem to have some kind of impact on the chromatin binding strength. Interestingly, the expression profiles in the cell lines used in this project and the MLS cell lines used in Lindén *et al*, 2022 were very different even though MLS express FUS-DDIT3 [30]. The SWI/SNF components in MLS disassociated mostly at 250 mM instead of 500 mM, as they did during this project. The reason for this is hard to determine and can be due to several factors. One reason could be the difference in final concentration of the buffers in the extraction, as discussed in 5.2. When the cell's liquid volume is not considered, the 250 mM EB might be diluted to the

point where few proteins disassociate, and the proteins extracted at 250 mM in MLS in Lindén *et* al, 2019 will instead release at 500 mM during this project [3]. The difference in expression profiles could also be due to that HT1080 is a model system transfected with fusion genes while MLS cell lines are established from tumors inherently expressing FET-FOPs. Because HT1080 is a model system, it is not fully representative of cellular mechanisms that occur in cells with FET-FOPs in their native environment as they may arise from different cell-of-origins and contain different epigenetic landscapes. The use of SSE also gives information about the binding strengths on a global scale, but it is not possible to determine where the proteins of interest bind to chromatin. A higher binding strength can indicate that the proteins are bound to a more compact form of chromatin. Other methods, such as ChIP-seq, need to be performed in order to conclude where in the chromatin our proteins of interest bind to, and to identify changes in binding at individual locations. In conclusion, further experiments and replicates are required to make definitive conclusions about the chromatin binding strength of SWI/SNF components in HT1080 with stable expression of different FET-FOPs.

5.4 Final conclusions and future prospects

The project succeeded in its minor aims of optimizing BCL7 antibodies for Western blot and optimizing sequential salt extraction in HT1080 as a model system. The evaluation of BCL7 antibodies highlighted the importance of investigating several blocking buffers and antibody dilution to obtain optimal results. The importance of knowing the expected band size and size of the protein was also demonstrated, particularly during the trials with BCL7B from Santa Cruz where the antibody did not target the correct protein. The optimization of SSE demonstrated how minor changes can make major differences in successful retrieval of sequential salt extracts, as well as the importance of being consistent in your performance in order to evaluate where the method can be improved. There might be other parts of the protocol that can be improved to minimize the occurrence of gel formation and to maximize the yield, for example the Benzonase treatment, the buffers used and evaluating the disruption of the cell membrane more thoroughly. In future experiments with FUS-CREB3L2-EGFP, this evaluation is important to perform as the results in this project displayed that the disruption was not sufficient. It is crucial to evaluate the cell disruption in future experiments if new cell lines are introduced.

The major aim of determining the chromatin binding strength of SWI/SNF components and known interaction partners has been evaluated with cell lines expressing two different FET-FOPs. The quantifications from Western blot indicated that the FET-FOPs might influence the binding strength and the epigenetic landscape. However, the quantification has been performed without replicates and can therefore not be used to draw definitive conclusions. More experiments need to be performed to evaluate whether these results are valid. It would also be interesting to evaluate other FET-FOPs to make comparisons at a greater scale and to assess similarities and differences between them. It is important to note, however, that HT1080 is a model system and the results gained from experiments cannot be used to conclude that the observed cellular mechanisms occur in all cells with FET fusion oncoproteins. Hopefully, the experiments can still give us insight into the cellular mechanisms between FET-FOPs and SWI/SNF, and lead to the discovery of new targeted therapies in the future.

6 References

[1] J. Ferlay *et al.*, “Cancer statistics for the year 2020: An overview,” *Int J Cancer*, vol. 149, no. 4, pp. 778–789, Aug. 2021, doi: 10.1002/ijc.33588.

[2] “Sarkom,” 1177. Accessed: Feb. 29, 2024. [Online]. Available: https://www.1177.se/sjukdomar--besvar/cancer/cancerformer/sarkom/

[3] M. Lindén *et al.*, “ FET family fusion oncoproteins target the SWI / SNF chromatin remodeling complex ,” *EMBO Rep*, vol. 20, no. 5, May 2019, doi: 10.15252/embr.201845766.

[4] J. C. Schwartz, T. R. Cech, and R. R. Parker, “Biochemical properties and biological functions of FET proteins,” *Annual Review of Biochemistry*, vol. 84. Annual Reviews Inc., pp. 355–379, Jun. 02, 2015. doi: 10.1146/annurev-biochem-060614-034325.

[5] P. Åman, “Fusion genes in solid tumors,” *Cancer Biology*, vol. 9, pp. 303–318, 1999.

[6] C. D. M. Fletcher, J. A. Bridge, P. C. W. Hoogendorn, and F. Mertens, “WHO Classification of Tumors of Soft Tissue and Bone,” *Lyon: IARC Press*, vol. 4 ed, 2013, [Online]. Available: https://www.researchgate.net/publication/302956200

[7] S. Abaricia and A. C. Hirbe, “Diagnosis and Treatment of Myxoid Liposarcomas: Histology Matters,” *Current Treatment Options in Oncology*, vol. 19, no. 12. Springer New York LLC, Dec. 01, 2018. doi: 10.1007/s11864-018-0590-5.

[8] F. Chamberlain *et al.*, “Low-grade Fibromyxoid Sarcoma: Treatment outcomes and efficacy of chemotherapy,” *In Vivo (Brooklyn)*, vol. 34, no. 1, pp. 239–245, 2020, doi: 10.21873/invivo.11766.

[9] S. N. Hamilton, R. Carlson, H. Hasan, S. R. Rassekh, and K. Goddard, “Long-term Outcomes and Complications in Pediatric Ewing Sarcoma,” *American Journal of Clinical Oncology: Cancer Clinical Trials*, vol. 40, no. 4, pp. 423–428, 2017, doi: 10.1097/COC.0000000000000176.

[10] O. Delattre *et al.*, “Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours,” *Nature*, vol. 359, pp. 162–165, 1992, doi: https://doi.org/10.1038/359162a0.

[11] C. R. Clapier, J. Iwasa, B. R. Cairns, and C. L. Peterson, “Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes,” *Nature Reviews Molecular Cell Biology*, vol. 18, no. 7. Nature Publishing Group, pp. 407–422, Jul. 01, 2017. doi: 10.1038/nrm.2017.26.

[12] C. Kadoch, R. A. Copeland, and H. Keilhack, “PRC2 and SWI/SNF Chromatin Remodeling Complexes in Health and Disease,” *Biochemistry*, vol. 55, no. 11, pp. 1600–1614, Mar. 2016, doi: 10.1021/acs.biochem.5b01191.

[13] N. Mashtalir *et al.*, “Modular Organization and Assembly of SWI/SNF Family Chromatin Remodeling Complexes,” *Cell*, vol. 175, no. 5, pp. 1272-1288.e20, Nov. 2018, doi: 10.1016/j.cell.2018.09.032.

[14] R. M. Chabanon, D. Morel, and S. Postel-Vinay, “Exploiting epigenetic vulnerabilities in solid tumors: Novel therapeutic opportunities in the treatment of SWI/SNF-defective

cancers," *Seminars in Cancer Biology*, vol. 61. Academic Press, pp. 180–198, Apr. 01, 2020. doi: 10.1016/j.semcancer.2019.09.018.
[15] K. Taniuchi, M. Furihata, S. Naganuma, K. Dabanaka, K. Hanazaki, and T. Saibara, "Original Article BCL7B, a predictor of poor prognosis of pancreatic cancers, promotes cell motility and invasion by influencing CREB signaling," 2018. [Online]. Available: www.ajcr.us/
[16] "BCL7A - Function," NeXtProt. Accessed: Mar. 06, 2024. [Online]. Available: https://www.nextprot.org/entry/NX_Q4VC05/
[17] R. J. Conrad *et al.*, "The Short Isoform of BRD4 Promotes HIV-1 Latency by Engaging Repressive SWI/SNF Chromatin-Remodeling Complexes," *Mol Cell*, vol. 67, no. 6, pp. 1001-1012.e6, Sep. 2017, doi: 10.1016/j.molcel.2017.07.025.
[18] B. Donati, E. Lorenzini, and A. Ciarrocchi, "BRD4 and Cancer: Going beyond transcriptional regulation," *Molecular Cancer*, vol. 17, no. 1. BioMed Central Ltd., Nov. 22, 2018. doi: 10.1186/s12943-018-0915-9.
[19] Y. Chen *et al.*, "Bromodomain and extraterminal proteins foster the core transcriptional regulatory programs and confer vulnerability in liposarcoma," *Nat Commun*, vol. 10, no. 1, Dec. 2019, doi: 10.1038/s41467-019-09257-z.
[20] "HT-1080 [HT1080]," ATCC. Accessed: Feb. 29, 2024. [Online]. Available: https://www.atcc.org/products/ccl-121
[21] "G418 (Geneticin)," InvivoGen. Accessed: Feb. 29, 2024. [Online]. Available: https://www.invivogen.com/g418
[22] L. Grossmann and D. J. McClements, "Current insights into protein solubility: A review of its importance for alternative proteins," *Food Hydrocolloids*, vol. 137. Elsevier B.V., Apr. 01, 2023. doi: 10.1016/j.foodhyd.2022.108416.
[23] W. Strober, "Trypan Blue Exclusion Test of Cell Viability," *Curr Protoc Immunol*, vol. 111, no. 1, p. A3.B.1-A3.B.3, Nov. 2015, doi: 10.1002/0471142735.ima03bs111.
[24] "How does trypan blue work?," AAT Bioquest. Accessed: May 16, 2024. [Online]. Available: https://www.aatbio.com/resources/faq-frequently-asked-questions/How-does-trypan-blue-work
[25] "Benzonase® Endonuclease," Merck. Accessed: May 16, 2024. [Online]. Available: https://www.merckmillipore.com/SE/en/products/biopharmaceutical-manufacturing/harvest/cleavage-lysis-purification-harvest/benzonase-endonuclease/bnCb.qB.NXkAAAFDei54saSR,nav?ReferrerURL=https%3A%2F%2Fduckduckgo.com%2F
[26] Bio-Rad Laboratories, "DC Protein Assay Instruction Manual." 1979.
[27] T. Mahmood and P. C. Yang, "Western blot: Technique, theory, and trouble shooting," *N Am J Med Sci*, vol. 4, no. 9, pp. 429–434, Sep. 2012, doi: 10.4103/1947-2714.100998.
[28] ThermoFisher, "Chemiluminescence western blotting technical guide and protocols." Accessed: May 26, 2024. [Online]. Available: https://assets.thermofisher.com/TFS-Assets/BID/Application-Notes/TR0067-Chemi-Western-guide.pdf
[29] N. S. Lipman, L. R. Jackson, L. J. Trudel, and F. Weis-Garcia, "Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources." [Online]. Available: https://academic.oup.com/ilarjournal/article/46/3/258/738903
[30] M. Lindén *et al.*, "FET fusion oncoproteins interact with BRD4 and SWI/SNF chromatin remodelling complex subtypes in sarcoma," *Mol Oncol*, vol. 16, no. 13, pp. 2470–2495, Jul. 2022, doi: 10.1002/1878-0261.13195.

[31] E. G. Porter, K. E. Connelly, and E. C. Dykhuizen, “Sequential salt extractions for the analysis of bulk chromatin binding properties of chromatin modifying complexes,” *Journal of Visualized Experiments*, vol. 2017, no. 128, Oct. 2017, doi: 10.3791/55369.

Appendix A

List of all antibodies used during SSE evaluation on Western blot

Antibody	**Company**	**Expected size (kDa)**	**Dilution + blocking buffer**
ARID1A	Atlas Antibodies, HPA005456	250	1:1000 milk
ARID1B	Bethyl, A301-047A	250	1:2000 milk
ARID2	Santa Cruz, sc-166117	250	1:200 BSA
BAF170	Santa Cruz, sc-17838	170	1:400 BSA
BAF155	Santa Cruz, sc-32763	155	1:200 BSA
BAF60A	Santa Cruz, sc-135843	60	1:1000 milk
BAF57	Abcam, ab131328	57	1:1000 milk
BAF53A	Abcam, ab131272	53	1:1000 milk
BAF47	Santa Cruz, sc-166165	47	1:1000 BSA
BCL7A	Invitrogen, PA5-27123	40	1:1000 milk
BCL7B	Abcam, ab137245	40	1:500 milk
BCL7C	NovusBio, NBP2-15559	40	1:500 milk
BRD7	Santa Cruz, sc-376180	80	1:200 BSA
BRD9	Abcam, ab137245	80	1:5000 BSA
BRG1	Santa Cruz, sc-17796	190	1:1000 BSA
BRM	Abcam, ab15597	190	1:500 milk
GLTCR1	Santa Cruz, sc-515086	230	1:200 BSA
GLTCR1L	Atlas Antibodies, HPA029391	130	1:400 milk
PBRM1	Bethyl, A301-591A	190	1:5000 milk
SS18	Santa Cruz, sc-28698	50	1:200 milk
Histone H4	Millipore, #04-858	12	1:15000 milk
EZH2	Millipore, #07-689	90	1:1000 milk
BRD4	Abcam, ab128874	250, 160, 110, 80	1:500 BSA
EGFP	Clontech, 632381	80, 30	1:1000 milk
EWS	Santa Cruz, sc-28327	80	1:1000 milk
FUS	Santa Cruz, sc-47711	60	1:1000 milk

Appendix B

Summary of sequential salt extraction samples used in Western blot.

Extraction date	PCV	Cell line	SSE fraction	Concentration [mg/ml]	Amount NF [µl]	Final dilution factor	Load Western blot [µl]	Load Western blot [µg]	Load Western blot [%]
240424	150	FUS-DDIT3-EGFP	NF250	1,80	197,2	1,00	3,97	4,30	1,5%
			NF500	2,04	330	1,67	6,65	8,16	1,5%
			NF1000	0,46	670	3,40	13,5	3,78	1,5%
240424	200	EGFP	NF250	1,24	273,7	1,00	4,34	3,23	1,1%
			NF500	1,67	442,2	1,62	7,02	7,06	1,1%
			NF1000	0,39	850,9	3,11	13,5	3,15	1,1%
240430	150	FUS-CREB3L2-EGFP	NF250	1,53	224,4	1,00	4,61	4,26	1,5%
			NF500	1,01	339,9	1,51	6,99	4,25	1,5%
			NF1000	0,24	656,6	2,93	13,5	1,94	1,5%
240506	150	WT	NF250	2,167	209,1	1,00	4,39	5,72	1,5%
			NF500	1,88	323,4	1,55	6,79	7,68	1,5%
			NF1000	0,59	643,2	3,08	13,5	4,79	1,5%

Calculation of final dilution factor

For 250 mM: Amount NF250 [µl]/Amount NF250 [µl]

For 500 mM: Amount NF500 [µl]/Amount NF250 [µl]

For 1000 mM: Amount NF1000 [µl]/Amount NF250 [µl]

Example (FUS-DDIT3-EGFP):
197,2/197,2 = 1,00
330/197,2 = 1,67
670/197,2 = 3,40

Calculation of load Western blot [µl]

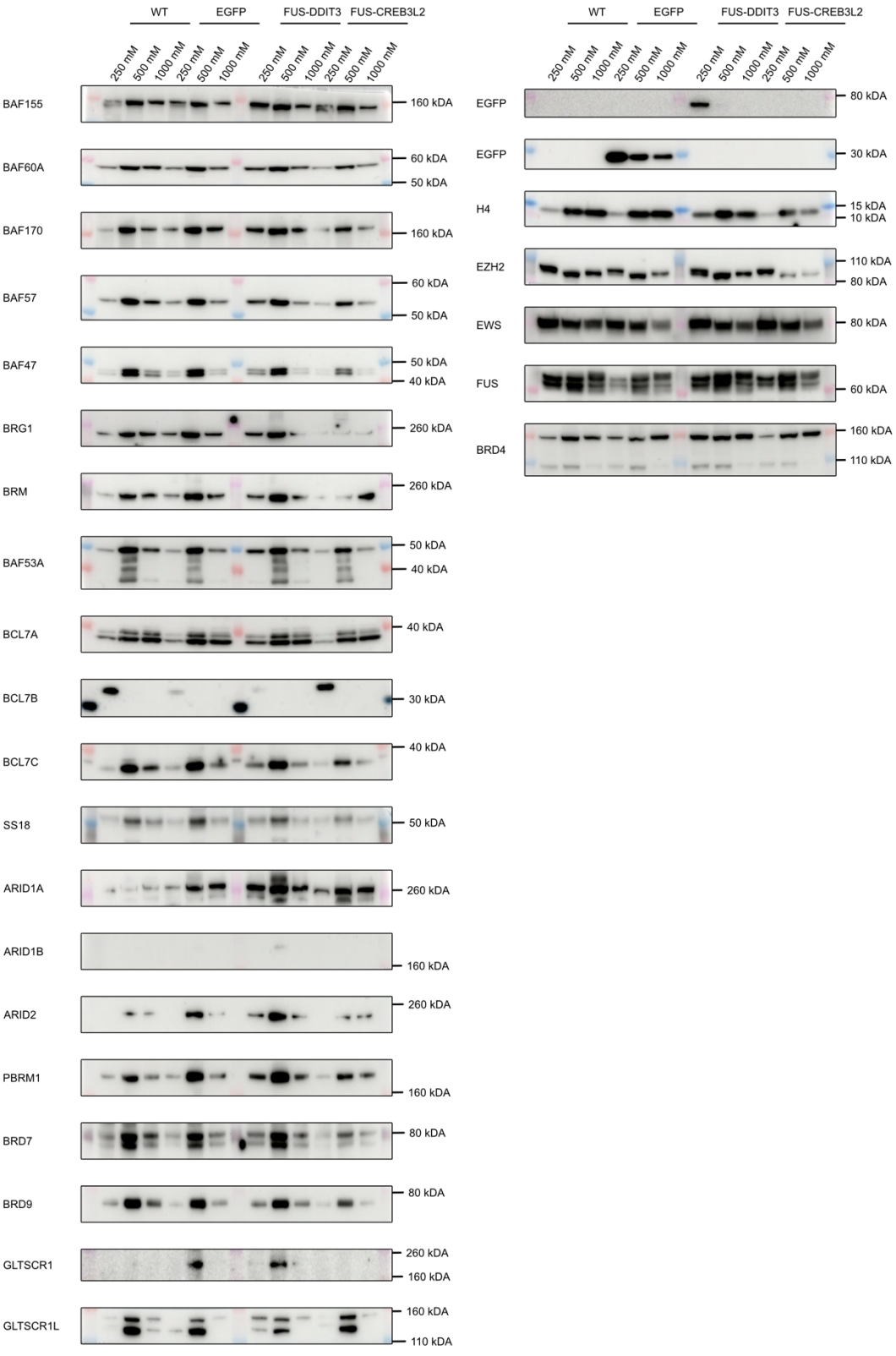
The load of NF1000 was maximized to 13,5 µl as this fraction had been diluted the most. The load of NF250 and NF500 were based on the final dilution factor and the load of NF1000.

For 250 mM: 13,5 (load NF1000) [µl]/Final dilution factor NF1000

For 500 mM: Load NF250 [µl]/Final dilution factor NF500

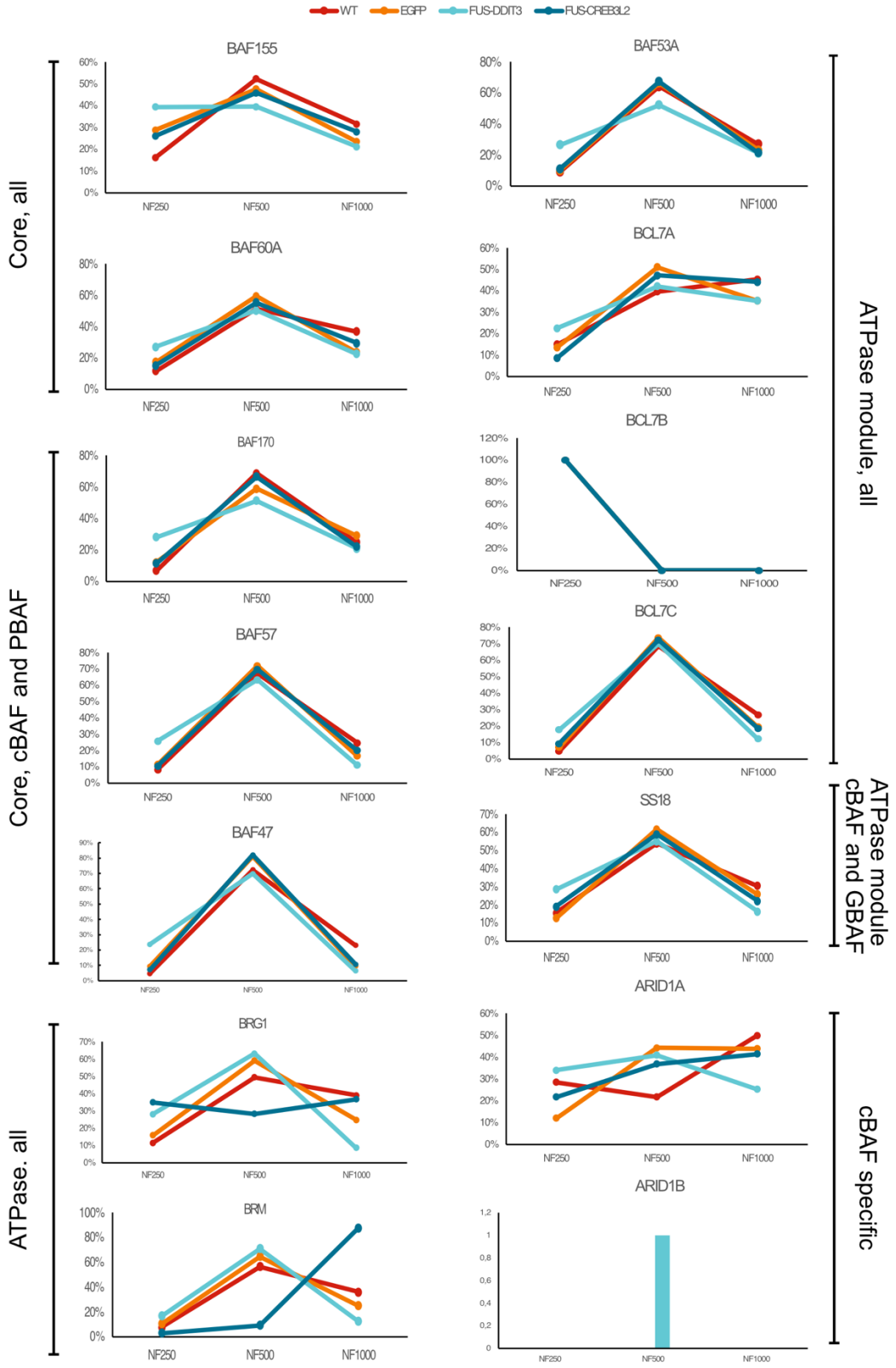
Appendix C

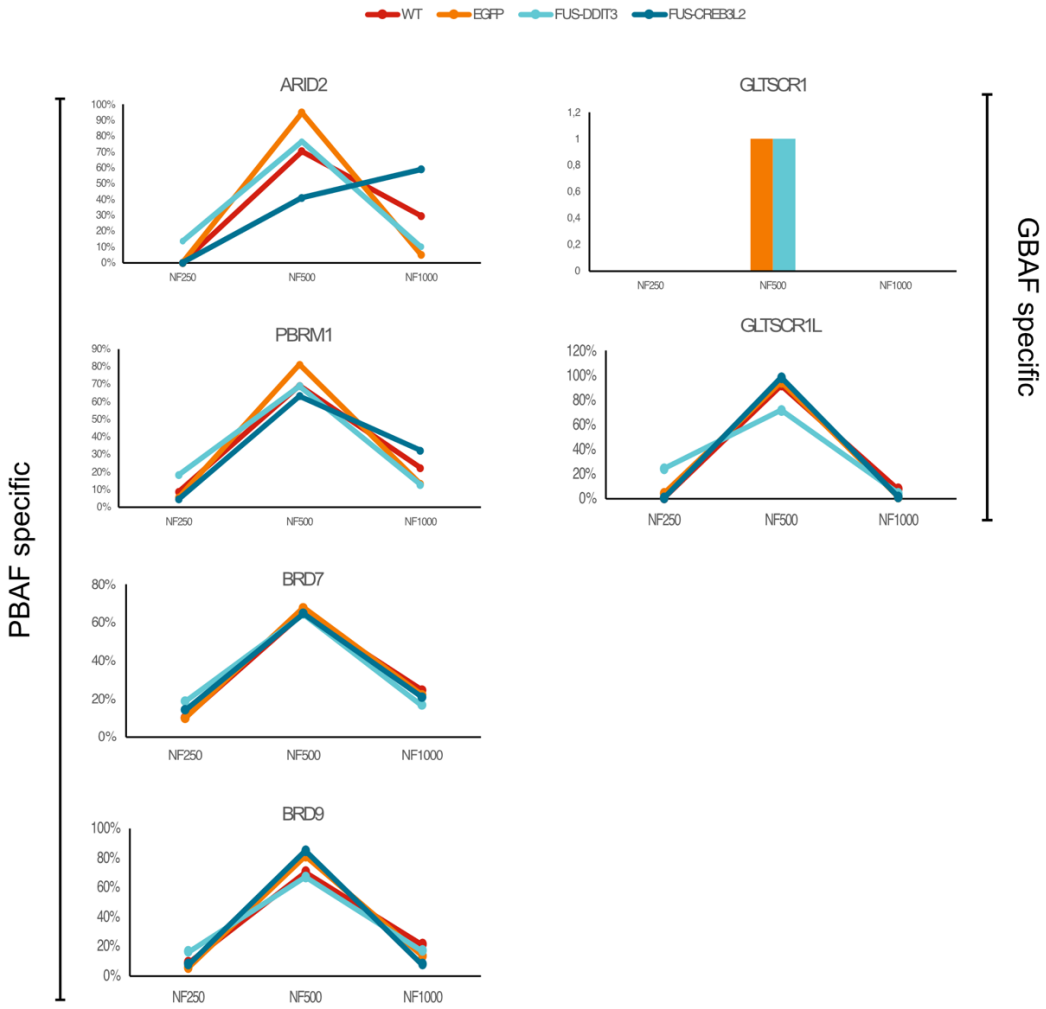
Western blot pictures of all samples and antibodies.

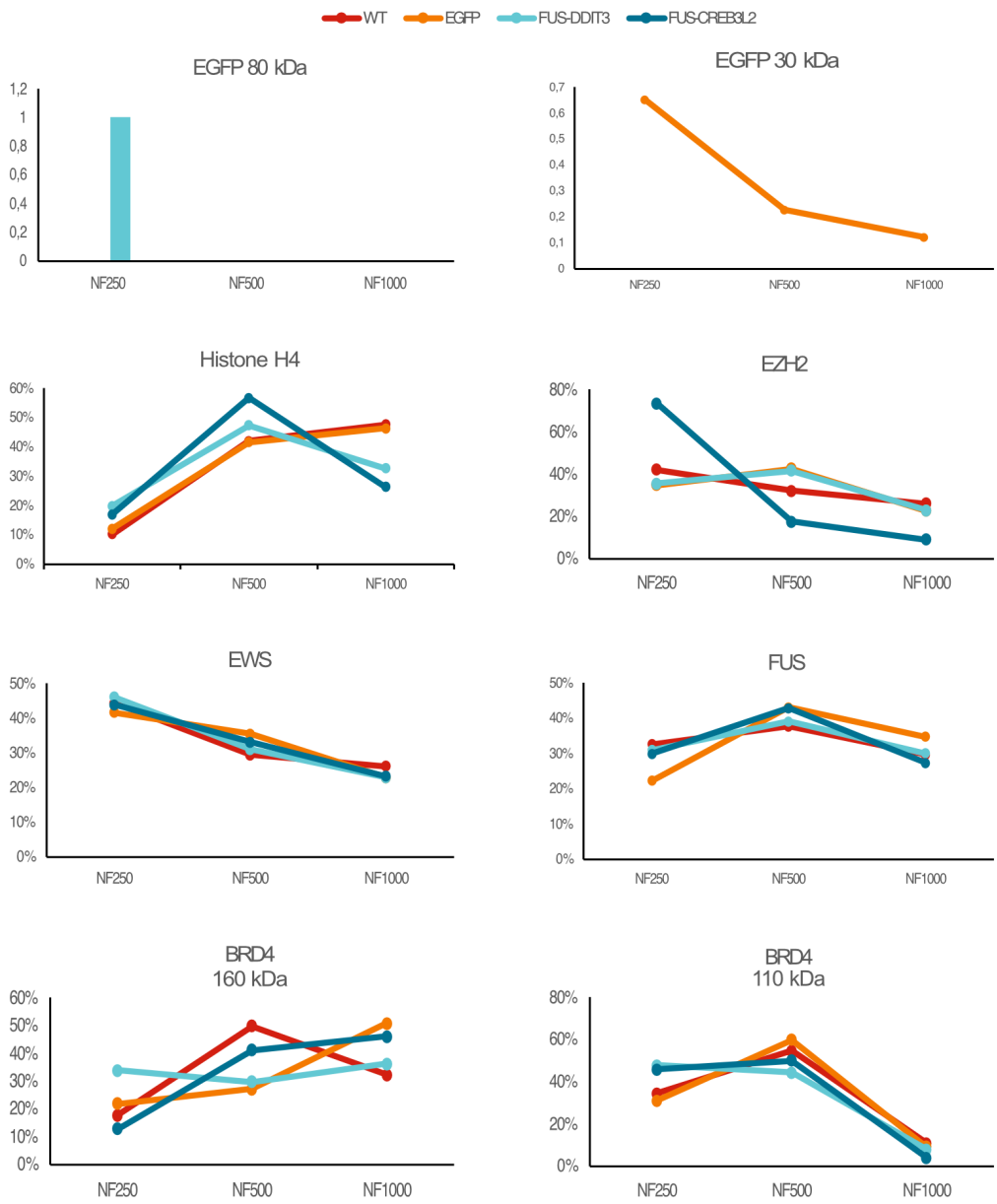


Appendix D

Plots from Western blot quantification.




WT
EGFP
FUS-DDIT3
FUS-CREB3L2
ARID2
GLTSCR1
PBRM1
GLTSCR1L
BRD7
BRD9
NF250
NF500
NF1000
PBAF specific
GBAF specific


WT
EGFP
FUS-DDIT3
FUS-CREB3L2
EGFP 80 kDa
1,2
1
0,8
0,6
0,4
0,2
0
NF250
NF500
NF1000
EGFP 30 kDa
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
NF250
NF500
NF1000
Histone H4
60%
50%
40%
30%
20%
10%
0%
NF250
NF500
NF1000
EZH2
80%
60%
40%
20%
0%
NF250
NF500
NF1000
EWS
50%
40%
30%
20%
10%
0%
NF250
NF500
NF1000
FUS
50%
40%
30%
20%
10%
0%
NF250
NF500
NF1000
BRD4
160 kDa
60%
50%
40%
30%
20%
10%
0%
NF250
NF500
NF1000
BRD4
110 kDa
80%
60%
40%
20%
0%
NF250
NF500
NF1000