# PROFILING THE CHROMATOME TO SCOUT FOR CHROMATIN-ASSOCIATED METABOLIC VULNERABILITIES IN TRIPLE NEGATIVE BREAST CANCER

**Student:** Laura García López

**Academic tutor:** María del Carmen Portillo Guisado

**Professional tutor:** Sara Sdelci

---

**Bachelor's Degree Final Project**

Degree in Biotechnology

June 17th, 2019 (Tarragona)

# Acknowledgements

Firstly, I would like to thank **Sara**. For having trusted on me from the very beginning and for giving me this opportunity. Thank you for instil in me the keen to learn, to overcome myself and for always have time and advice for me. Thank you also to **Maria**, to always have a sincere smile prepared when I lost mine and for her infinite patience. And **Laura**, for her "*everything will be fine*", we are better together. If I had to choose one, it would be the coffee and chocolate moments, girls.

Thank you to **Carmen**, my academic tutor from the URV, for being always there for me and for her help while I was writing and organizing the thesis.

Thank you also to my **parents** and my **brother**, to be always a reference and unconditional support.

Today, I feel fully involved in the study of breast cancer and I would like to thank all the people who, during these months by chance, have made me believe that what we do make sense. And that behind all of this there are **women**, mothers, sisters and daughters who trust in what we do.

Primer de tot, gràcies a la **Sara**. Per haver confiat en mi des del principi i haverme regalat aquesta oportunitat. Gràcies, per contagiarme les ganes d'aprendre, de superar-me i per tenir sempre temps i consell per a mí. Gràcies també a la **Maria**, per tenir sempre un somriure sincer preparat quan jo perdía el meu i per la seva paciencia infinita. I a **Laura**, formem el millor equip, i pels seus "*everything will be fine*". Si hagués de triar-ne un, em quedaría amb els moments de café i xocolata, noies.

Gràcies a la **Carmen,** la meva tutora de la URV, per la seva ajuda durant tot el procès d'escriure i organitzar el treball.

Gràcies també als meus **pares** i al meu **germà**, per ser sempre referent i suport incondicional.

A dia d'avui, em sento plenament involucrada en l'estudi del càncer de mama i m'agradaria agraïr a totes les persones que, durant aquests mesos per casualitat o causalitat, m'he creuat i m'han fet creure que el que fem té sentit. Que darrere de tot hi ha **dones**, mares, germanes i filles que confíen en el que fem.

# Table of contents

# 1. Centre for Genomic Regulation

This bachelor thesis is based on the internship I performed in the group of Sara Sdelci (https://www.crg.eu/en/programmes-groups/sdelci-lab) at the Gene Regulation, Stem Cells and Cancer program at the Centre for Genomic Regulation (CRG, Barcelona). Results reported in this thesis were obtained in the period comprised between February and May 2019. Currently, I am continuing the internship and I will finish it in September 2019. The Centre for Genomic Regulation (https://www.crg.eu/) was founded in 2000 with the aim of creating an organization dedicated to the translation of basic biomedical research. Therefore, its mission is to discover and communicate scientific advancement for the benefit of society, public health and economic prosperity. For this purpose, the CRG aims to recruit the best researchers in the field of biology and biomedicine and provides them with the latest scientific and technological support.

The CRG is part of the Parc de Recerca Biomèdica de Barcelona (PRBB), which is located at 88 Doctor Aiguader Street (Barcelona), specifically in The Barceloneta district. The PRBB comprises six independent centers: IMIM, CEXS-UPF, CRG, EMBL, ISCGlobal and IBE (UPF-CSIC).

The CRG is organized into three main areas: scientific area, facilities, scientific-technical and administration. Four programs linked together compose the scientific core: Genomics and Bioinformatics; Cell and Developmental Biology; Gene Regulation, Stem Cells and Cancer; and Systems Biology.

The four research programmes are divided into 28 groups, besides the central facilities and technological platforms. In terms of central facilities, there are 8 different units: 1) Advanced optical microscopy, 2) Genomics, 3) Proteomics, 4) FACS, 5) Bioinformatics, 6) Biomolecular screening and protein technologies, 7) Tissue engineering, 8) histology unit.

# 2. Abstract

The treatment of breast cancer is still a worldwide health challenge, due to the high intrinsic heterogeneity of the disease. There are four fundamental types of breast cancer, which are characterized by the expression of ER, PrR and/or HER2, or by the absence of all those three receptors (triple negative breast cancer). The triple negative is the most aggressive one because no targeted therapy has been found until now, fact that limits the treatment to non-selective and aggressive chemotherapeutic agents, and strongly reduces the probability of patient survival.

In this project, we want to study the role of cancer metabolism in triple negative breast cancer with the final aim to identify a targeted therapy for this unmet medical need. The focus of our team is to study the role of cancer metabolism in epigenetic and transcriptional regulation. It has become evident during the last years that certain metabolic enzymes localize on chromatin despite their canonical cytoplasmic localization and function. We aim to study the role of metabolic enzymes specifically recruited on chromatin in breast cancer cells, to understand their contribution to tumour progression. For this, we set up a protocol for chromatin purification MS-coupled, which allows us to investigate the chromatome of selected breast cancer cell lines recapitulating the different breast cancer phenotypes. By studying the chromatome of those cell lines, we will select enzymes specifically recruited to chromatin in triple negative breast cancer, and investigate what is their function there. The identification of a metabolic enzyme/pathway whose chromatin localization is selectively required in triple negative breast cancer could open up new possibilities for the successful treatment of this aggressive tumour.

**Keywords**: breast cancer, triple negative breast cancer, chromatin, chromatome, Western Blot, mass spectrometry, metabolic enzymes, metabolic pathways.

# 3. Introduction

## 3.1 Breast cancer

Breast cancer is defined as the uncontrolled growth of breast cells that leads to a malignant tumour in the breast. The high heterogeneity of breast cancer is normally caused by the combination of genetic abnormalities and unknown epigenetic landscapes. Each year 1.5 million women are diagnosed with breast cancer in the world. For this reason, breast cancer is the most common type of malignant neoplasm worldwide among females (Sun et al. 2017; Dai et al. 2017; Kamińska et al. 2015).

Statistic indicate that breast cancer is the second cause of death in women. In Spain, only in 2018, 32.825 new cases of breast cancer have been diagnosed (Sociedad Española de Oncología Médica, https://seom.org/; Asociación Española Contra el Cancer, https://www.aecc.es/es). Therefore, in Spain breast cancer is the cancer type with the highest incidence among women, followed by colorectal and lung cancer. Although breast cancer usually affects women, there is a small percentage of men who also suffer from this neoplasm (>1%). However, this low penetrance in the male sex has seen an increase during the last three years (Sun et al 2017).

The incidence of breast cancer increases every year, since in 2012 there were less cases (25.215) which have been progressively incrementing (Sociedad Española de Oncología Médica, https://seom.org/; Asociación Española Contra el Cancer, https://www.aecc.es/es). This increased number is not just given by the higher number of people affected by the disease, but also it is due to the improvement of the detection methods and the increase in life expectancy because of the amelioration of the treatment (Sun et al. 2017).

Statistically speaking, the definition of prevalence when talking about tumour progression takes into account the number of patients diagnosed with a particular tumour who are still alive five years after the first diagnosis. In these terms, tumours with a higher survival rate carry a higher prevalence. Given that in 2017, it was estimated that breast cancer was the most prevalent type of tumour, we can understand that patient suffering from breast cancer have overall a good prognosis when compared to other cancer patients. However, given the high grade of heterogeneity of the disease, which includes both intra-patient and inter-patient heterogeneity, it is very difficult to establish a patron that would allow for an effective treatment of this malignance in every patient (Sociedad Española de Oncología Médica, https://seom.org/).

## 3.2 Histological subtypes

Breast cancer can originate in any of the cells of the mammary gland, which entails a wide range of morphological characteristics, immunohistochemical profiles and unique histopathological subtypes that make this disease to be very heterogenous. Carcinomas are the most common breast cancer lesion (>95%), followed by sarcomas and lymphomas (Makki, 2015). In histological terms, the specific type of affected cells is what determines the phenotype of the tumour and consequently gives the tumour name. Therefore, breast cancer called carcinoma originates from epithelial cells. More specifically, since the epithelial cells come from the mammary gland, breast carcinomas are defined as adenocarcinomas. Adenocarcinomas of the breast usually begin either in the milk lobules, which are the sites where milk is actually produced, or in the milk ducts, which is where the milk is secreted and collected (Barroso-Sousa and Metzger-Filho, 2016).

### *3.2.1 Breast lobular adenocarcinoma*

Lobular carcinoma accounts for less than 20% of breast cancer lesions, and originates in the lobes of the mammary gland which are responsible for milk production (Figure 1). In the lobular carcinoma in situ (LCIS), the cancer cells are restricted inside the lobe and do not migrate to other tissues, contrary to the invasive lobular carcinoma (ILC). ILCs generally display features associated with good prognosis, such epithelial-like morphology and oestrogen receptor positivity, which makes them sensitive to hormone receptor antagonist therapy (e.g. Tamoxifen). However, this type of tumour can also progress and become highly metastatic (Reed et al., 2015).

### *3.2.2 Breast ductal adenocarcinoma*

Breast ductal carcinoma begins in the ducts that conduct the milk to the nipple (Figure 1), and it represents between the 85-90% of all breast lesions. Breast ductal carcinoma is divided into ductal carcinoma in situ (DCIS), where cancer cells are limited to the primary lesion and do not migrate, and invasive ductal carcinoma (IDC), which is more aggressive, not circumscribed to the primary lesion and, therefore, able to metastasize (Makki, 2015). IDC is the most common type of breast cancer, and it fast spread to the lymph-nodes and neighbouring breast tissues (Breast Cancer Organization, https://www.breastcancer.org/). Because IDC is the most frequent and dangerous breast cancer, **we will focus on this particular type of breast cancer** for the development of this bachelor thesis.

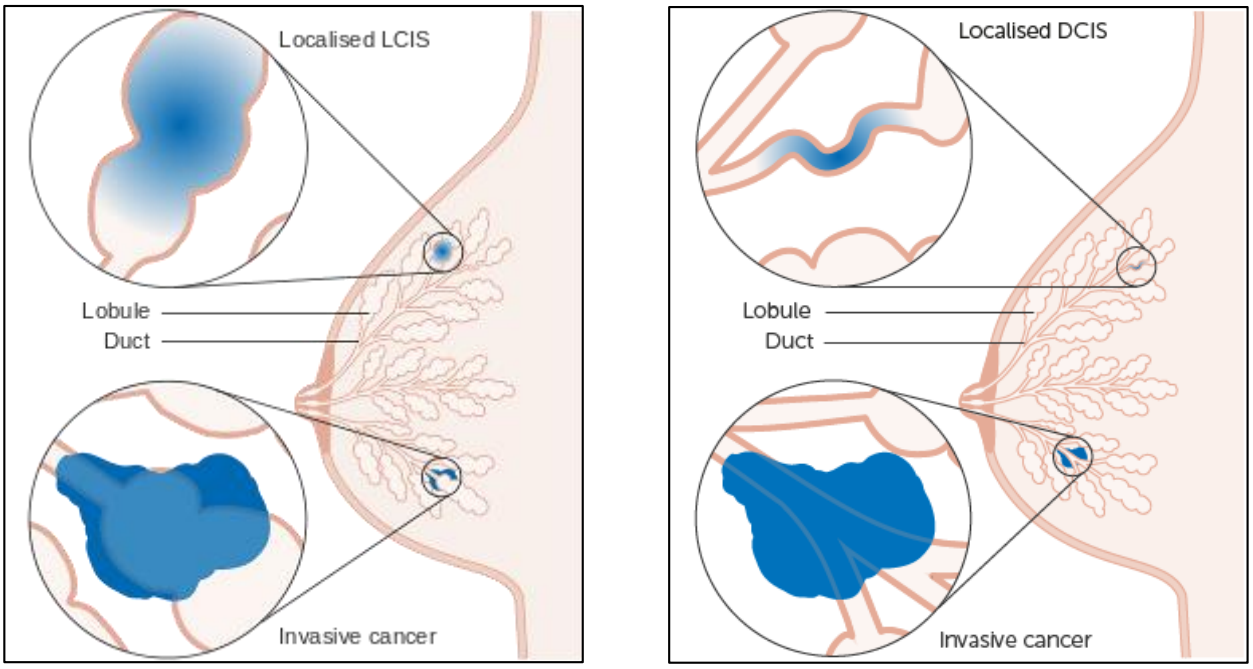



**Figure 1**. **Cartoon representing the origin of breast lobular and ductal carcinomas.** The two most widespread breast cancer subtypes are named according to the breast part in which they initiate (left, lobular; right, ductal). It is possible to differentiate the in situ carcinoma (upper part) from the invasive subtypes (lower part), which are able to invade the neighbouring breast tissues and give metastasis.

## 3.3 Molecular classification of ductal adenocarcinomas

Breast cancer has been classically classified based on the expression of oestrogen receptor (ER) and progesterone receptor (PrR), which have been used as diagnostic biomarkers to stratify breast cancer patients into good (ER+/PrR+) and bad (ER-/PrR-) prognosis (Figure 2). The same classification applies to IDCs, since they account for >85% of breast cancer lesions (Makki, 2015). The expression of ER and PrR usually indicates that breast cancer cells rely on hormone signalling for their proliferation. The receptors are usually localized in the cytoplasm of the cell and the binding with their ligands (oestrogen or progesterone) induces a conformational change, which allows the translocation of the complex to the nucleus. In the nucleus, ER and PrR act like transcription factors and activate the expression of target genes that are required for the proliferation of the hormone receptor-positive cancer cells. For this reason, the presence of ER/PrR makes the cancer responsive to compounds that mimics the hormone but have the ability to block the receptor in its original conformation (hormone receptor antagonist: e.g. Tamoxifen), therefore impeding the nuclear translocation and the activation of genes involved in cell proliferation. The combination of Tamoxifen and chemotherapy has given good results in the eradication of this breast cancer subtype (Ahmed et al., 2015).

The majority of breast cancers are ER+/PrR+ (75%), and about 50% of those are human epidermal growth factor receptor (HER2) +. Contrary to ER/PrR expression, the overexpression of HER2 indicates bad prognosis and ER+/PrR+/HER2+ cancer cells are usually more aggressive than ER+/PrR+/HER2- cancer cells. However, the development of a HER2-targeted therapy made possible to limit the proliferation of cancer cells belonging to this subgroup of tumours, considerably improving the outcome of the treatment (Ahmed et al., 2015).

Even though targeted HER2 and hormone therapies have been proved to be very effective, breast cancer is intrinsically very heterogeneous and tumour cells initially expressing ER, PrR and/or HER2 can evolve into the so-called triple negative phenotype (ER-/PrR-/HER2-). Triple negative phenotype lack the expression of every of those receptors and therefore, do not rely on their activity for the proliferation. Given the lack of specific biomarkers, this breast cancer subtype is particularly hard to target and, therefore, has the worst prognosis among all breast cancer subtypes (Bauer et al., 2007).

In addition to the receptors expression, breast cancer cells can be classified according to their epithelial-like or mesenchymal-like phenotype. Interestingly, breast cancer cells expressing ER and PrR usually maintain the epithelial phenotype, which associates with less invasion and metastasize capacity. In contrast, triple negative breast cancer cells are characterized by a mesenchymal-like phenotype, which correlates with their higher aggressiveness (Lehmann et al., 2011).

To recapitulate, breast cancer is typically classified as (Figure 2):

- **ER+/PrR+/HER2-:** The group includes those tumours that are positive for hormone receptors (ER and PrR) and rely on hormones for their growth. The expression of hormone receptor generally indicates good prognosis because it makes cancer cell sensitive to hormone receptor antagonist therapy, which blocks the hormone receptors signalling, therefore impairing cancer cell proliferation.

- **ER+/PrR+/HER2+:** Cells in this subtype expresses ER and/or PrR and HER2. Usually this type of cancer cells represents a transition between the ER/PrR-only positive subtype and the HER2-only positive subtype.

- **ER-/PrR-/HER2+:** The HER2 tumour subtype express HER2, without over-expressing the hormone receptors (ER and PrR). Usually, for this subtype of breast cancer, cytotoxic therapy

is combined with Trastuzumab, a specific monoclonal antibody that binds to HER2, blocking the signalling cascade that HER2 activates to support cancer cell proliferation.

- **ER-/PrR-/HER2-:** This phenotype corresponds to the triple negative breast cancer subtype, and cancer cells do not express ER, PrR or HER2, therefore their grow do not depend on the signalling cascades triggered by the activation of those receptors.

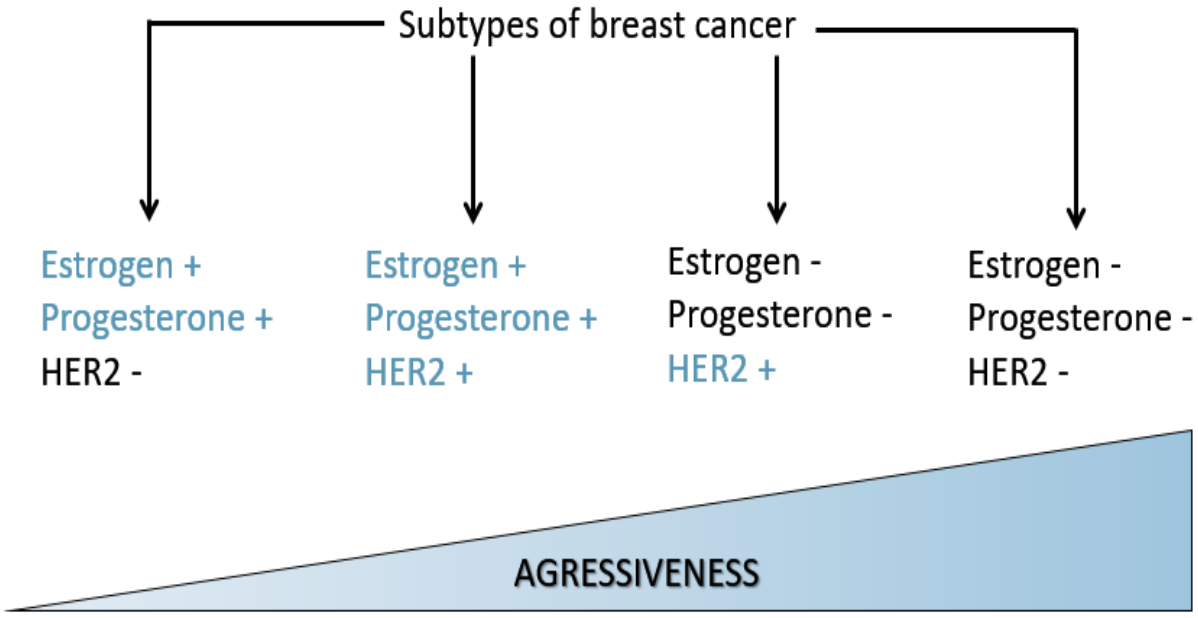



**Figure 2. Molecular subtypes of breast cancer according to the cellular receptors expressed**. The four different breast cancer subtypes phenotypes based on the receptors patterns, which is also associate to the aggressiveness of each breast cancer cell subtype. Subtype cells that do not express any of the receptors, such as triple negative subtype (MDA-MB-231), are considered the most aggressive ones because they are hard to target and, therefore, they are hard to find a treatment. There are also some breast cancer cell lines as examples representing every phenotype.

## 3.4 Triple negative breast cancer

Triple negative breast cancer (TNBC) subtype refers to the breast cancer cells that do not expresses the hormone receptors (ER and PrR) or HER2. As mentioned above, the lack of these receptors makes the cell hard to target in order to develop an efficient anticancer treatment. For this reason, this type of breast cancer is the most aggressive subtype, and it has the poorest outcome compared to other subtypes (Chavez et al. 2012).

TNBC represents approximately 10-15% of all breast cancers (Chavez et al. 2012) and patients suffering from it are treated with unspecific chemotherapeutic agents (specifically small molecules that block the synthesis of nucleic acids or microtubule dynamics). However, it is very common that TNBC cells develop overtime a mechanism to evolve and evade chemotherapy, which allow them to become chemotherapy resistant, proliferate and metastasize, condemning

the patient to death. That is why TNBC is an unmet medical need, and it is urgent to develop a targeted and efficient therapy for its eradication (Toft and Cryns, 2011).

## 3.5 The link between epigenetics and metabolism to target TNBC

Global changes in the epigenetic landscape and metabolism are both hallmarks of cancer (Flavahan et al., 2017; DeBerardinis and Chandel, 2016; Pavlova and Thompson, 2016). However, the direct interconnection between those two areas has remained elusive. During cancer, a deep and complex metabolic rewiring occurs to sustain tumour development and support the metastatic process. Although cancer has been traditionally described as a genetic disease, disruption of epigenetic regulation can lead to altered gene function and malignant cellular transformation (Hatziapostolou and Iliopoulos, 2011; Sharma et al., 2010). The connection between metabolism and epigenetics, however, remains indirect and relies on the evidence that the metabolic rewiring happening in cancer can mediate epigenetic changes by limiting or enhancing the availability of metabolic substrates (Reid et al., 2018; Wong et al., 2017; Kinnaird et al., 2016). Recently, the hypothesis that metabolic enzymes and their metabolites can directly impact on epigenetics and gene control has been proposed. Supporting this hypothesis, it has been shown that the pool of Acetyl-CoA produced in the nucleus has a direct impact on histone acetylation (Pietrocola et al., 2015), and that nuclear ATP production in breast cancer cells is essential to guarantee enough energy needed for chromatin remodeling in response to oestrogens (Wright et al., 2016). However, the majority of the molecular mechanisms linking cancer metabolism to epigenetic regulation are still far from understood.

During her postdoctoral research, Sara Sdelci (my supervisor for this bachelor thesis) investigated the role of metabolism influencing epigenetic regulation and gene expression. She discovered that MTHFD1, a crucial enzyme of the folate metabolism needed for the de novo synthesis of nucleotides, is a chromatin partner of BRD4, an epigenetic reader that promotes cancer progression via the transcriptional activation of several oncogenes, including Myc (Lovén et al., 2013; Zuber et al., 2011; Alekseyenko et al., 2015). She described that the interaction between BRD4 and MTHFD1 on chromatin is needed to maintain a certain transcriptional state in cancer cells, due to the production of folate metabolites in loco, which can immediately be used for the synthesis of nucleotides. Interestingly, the growth of aggressive xenograft tumours was arrested combining the inhibition of BRD4 and MTHFD1, while single treatments did not show any effect. This discovery opened up a novel cancer biology scenario in which metabolic

enzyme can directly influence the epigenetic and chromatin state of the cancer cells by making contact with chromatin.

The recent discoveries that metabolic enzymes can directly control epigenetics and transcription made us consider the existence of a specific chromatin metabolism able to influence cancer epigenetics and gene control. Therefore, within this project we propose to identify metabolic enzymes whose chromatin localization associates with the loss of epithelial-like morphology and Er/PrR expression and in TNBC. The identification of such scenario will give us the chance to target chromatin-bound metabolic enzymes to undo the TNBC phenotype, finally re-sensitizing TNBC cells to the hormone therapy. A change from a positive to negative status represent the classical evolution of hormone sensitive epithelial-like breast cancer into aggressive mesenchymal-like TNBC. The mechanisms responsible for repressing hormone receptors expression in TNBC are not clearly understood, but several possibilities have been proposed, including intra-tumour heterogeneity, clonal selection caused by chemotherapy, or a switch in tumour biology during cancer evolution (e.g. metabolic rewiring). From a clinical perspective, this last scenario is the most exciting one, because it encompasses the possibility to find the molecular and cellular mechanisms driving the "switch", and target them to restore ER and PrR expression in TNBC, which is rarely compromised by specific genetic mutations, thus indicating that an aberrant epigenetic regulation can drive breast cancer evolution.

# 4. Hypothesis and aim

Sara Sdelci has recently discovered that enzymes of the cytoplasmic folate pathway are recruited to chromatin in cancer cells where, together with epigenetic regulators, to control gene expression. This finding describes for the first time that metabolism just not happen in the cytoplasm of the cell (canonical metabolism), but can be translated to chromatin in order to support chromatin functions. In this contest, we speculate that the different metabolic needs of cancer cells can boost a complex metabolic reprograming which can comprise the re-localization of metabolic enzymes and pathways in non-canonical cellular compartments (e.g. chromatin). We furthermore speculate that the re-localization of metabolic entities can be tumour-dependent and tumour type and subtype-specific.

Given those hypothesis, the final aim of the project will be to identify chromatin-associated metabolic entities of each specific breast cancer phenotypes, with the scope (long-term) to discover novel chromatin-associated metabolic vulnerabilities of the TNBC subtype.

Therefore, this bachelor thesis focused on the performance and study of the breast cancer chromatome, which we define as the proteins that strictly associate with chromatin, with particular interest on the fraction of metabolic enzymes identified in each of the breast cancer subtypes.

# 5. Materials and methods

## 5.1 Cell lines and culture methods

| Cell line | Phenotype | Tissue | Disease | Reference |
|---|---|---|---|---|
| **T47D** | ER+/PrR+/HER2- | Mammary gland/breast. Derived from a metastatic site | Ductal carcinoma | ATCC® HTB-133™ |
| **MCF7** | ER+/PrR-/HER2- | Mammary gland/breast. Derived from metastatic site | Adenocarcinoma | ATCC® HTB-22™ |
| **BT-474** | ER+/PrR+/HER2+ | Mammary gland/breast; breast/duct | Ductal carcinoma | ATCC® HTB-20™ |
| **SK-BR-3** | ER-/PrR-/HER2+ | Mammary gland/breast. Derived from metastatic site | Adenocarcinoma | ATCC® HTB-30™ |
| **MDA-MB-231** | ER-/PrR-/HER2- | Mammary gland/breast. Derived from metastatic site | Adenocarcinoma | ATCC® HTB-26™ |

**Table 1**. **Human breast cancer cell lines used for the project**. Breast cancer cells lines that recapitulate each breast cancer phenotype we aimed to study. The reference is provided for the American Type Culture Collection (ATCC).

**T47D**

The T-47D cell line derives from the pleural effusion of an invasive ductal adenocarcinoma of a 54-years-old Caucasian female (Table 1). The cell line shows an epithelial-like morphology and overexpresses ER and PrR receptors (Figure 3). Differently from the MCF7 cell line, the T47D cell line markedly susceptible to progesterone and it is oestrogen insensitive when progesterone is present. For those characteristics, the T47D cell line represent an ideal experimental model to study the molecular basis of progesterone-dependent breast cancer (Yu et al., 2017).

**MCF-7**

The MCF-7 cell line was established from the pleural effusion of an invasive ductal carcinoma of a 69 years-old Caucasian female at metastatic stage (Table 1). The cell line shows epithelial-like morphology and expresses ER and PrR receptors but not HER2 receptor (Figure 3). Cell grow is oestrogen dependent making the MCF7 cells sensitive to ER antagonist treatment. For those characteristics this cell line is an excellent model to study the molecular basis of oestrogen-dependent breast cancer.

**BT-474**

The BT-474 cell line originates from an invasive ductal adenocarcinoma, which was isolated in 1978 from the primary tumour of a 60-year-old Caucasian female (Table 1). The cell line has an epithelial-like morphology and it is characterised by a high expression of ER, PrR and HER2 receptors (Figure 3). Even though the cells express ER and their grow increases when they are treated with oestrogens (e.g. oestradiol), they are not sensitive to the treatment with ER antagonist (e.g. Tamoxifen). For those characteristics, the cell line BT-474 is used as a model to study the molecular basis of cancer progression from its first stage (usually ER/PP positive and tamoxifen sensitive) to HER2 positive and tamoxifen insensitive (still retaining the expression of ER and PrR).

**SK-BR-3**

The SK-BR-3 cell line was established in the Memorial Sloan–Kettering Cancer Center (MSKCC) in 1970. Initially cells were isolated from a metastatic site of an invasive ductal adenocarcinoma of a 43-year-old Caucasian woman (Table 1). The SK-BR-3 cell line possess an epithelial-like morphology and express high levels of the HER2, which make them be classified as HER2 positive subtype. ER and PrR expression are not present in this cell line (Figure 3). For those characteristics, the SK-BR-3 cell line is a very good model for the study of the molecular basis of the HER2 breast cancer subtype.

**MDA-MB-231**

The MDA-MB-231 cell line is one of the most commonly used for the experimental and in vitro study of breast cancer. This cell line originates from metastatic ductal adenocarcinoma and was originally isolated in 1973 from the pleural effusion of a 51-year old Caucasian female (Table 1). The MDA-MB-231 cell line belongs to the TNBC, since it does not express oestrogen receptor (ER), progesterone receptor (PrR) and HER2 (Figure 3). Additionally, even though the tissue of origin is epithelial, this cell line shows a fibroblastic-like morphology, similar to the loss of the epithelial morphology usually observed during breast cancer progression, which correlates with high aggressiveness. Those characteristics make the MDA-MB-231 the perfect cellular model to study the molecular basis of TNBC.

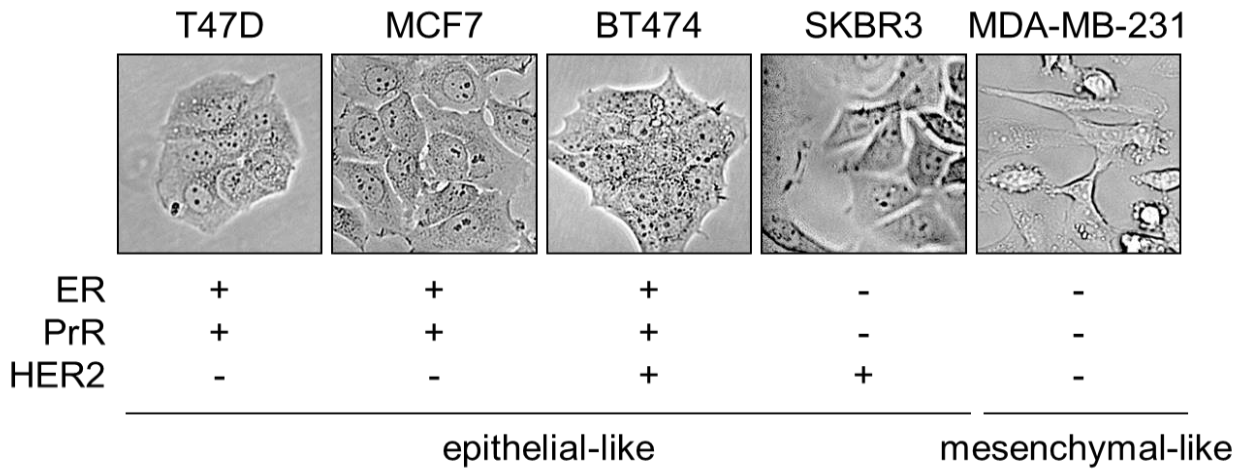



**Figure 3. Images of breast cancer subtypes.** The images of the cell lines, taken with the EVOS Cell Imaging System, are showing the phenotype of each breast cancer subtype.

**Culture methods**

Each cell line was cultured with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (Gibco; Thermo Fisher Scientific) and 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific) in a 5% CO2 atmosphere at 37˚C.

All the cell lines were provided by the CRG cell lines collection.

## 5.2 Chromatin extraction

Chromatin was extracted from samples of 40 million cells from all the cell lines used on this study.

### *5.2.1 Crosslinking condition*

Cells were crosslinked in plate by adding formaldehyde at a final concentration of 1% (Pierce™ 16% Formaldehyde (w/v), Thermo-Fisher, 28908) for 10min. Fixation was quenched through the addition of Glycine 0.25M. Cells were washed with cold PBS twice and collected by scraping in falcon tubes (Falcon® Conical Tubes 50 mL or 15 mL). Finally, the cell suspension was centrifuged at 600 g for 7min at 4 ºC to isolate the cellular pellet. Cells were immediately processed for chromatin extraction or snap frozen in liquid nitrogen and stored at -80 ºC until further use.

### *5.2.2 Chromatome extraction*

Cellular pellet from crosslinked and non-crosslinked cells was resuspended in 1 mL of SB buffer (HEPES 10mM, KCl 10mM, MgCl2 1.5mM, sucrose 0.1% and proteinase inhibitor cocktail (Sigma 4693159001)). After a 30min incubation at 4ºC, the tubes were centrifuged for 3min at 2000g at 4ºC. Supernatant was stored for further analysis as it comprises the cytoplasmic fraction of cells and was used as control. The pellet containing the nuclei of the cells was resuspended in 0.5mL of SB buffer and then ultracentrifuged on a sucrose gradient over SC buffer (HEPES 10mM, KCL 10mM, MgCl2 1.5mM, Sucrose 2.1M and Proteinase Inhibitors. Ultracentrifugation was performed at 53000rpm for 3h at 4ºC with a reduced acceleration and deacceleration of 5 out of 10 in a TLA100.3 rotor and a Optima TableTop Ultracentrifuge (Beckman Coulter). After the ultracentrifugation, the supernatant containing the nuclear proteins was discarded while the pellet containing the chromatin and its associated proteins was resuspended on 0.5mL of buffer SB and later centrifuged at 20000g for 45min at 4ºC to remove the rest of the sucrose gradient. Two washing steps were then performed by using a buffer composed by HEPES 10mMM and proteinase inhibitors (Sigma 4693159001). The pellet contains the chromatin and its associated proteins.

To release the chromatin-associated proteins, a sonication procedure was performed followed by a DNA digestion step. For the sonication, the chromatin pellet was resuspended in 0.25mL of Benzonaze digestion buffer (HEPES 15mM, EDTA 1mM, EGTA 1mM, 5mg/mL TPCK, 1% of NP40

and Proteinase inhibitors (Sigma 4693159001)). We used a BioRuptor Pico sonication device from Diagenode, especially designed for chromatin, DNA and RNA shearing. Chromatin was sheared through 15 cycles of 30seg of sonication followed by 45seg of pause. DNA was then degraded through the enzymatic action of 1uL of Benzonaze (Millipore 70664) and also RNA was removed with the addition of the RNAse A at a 1:1000 dilution (Thermo EN0531). DNA and RNA digestions were performed during 30-40min incubation at 4ºC in a rotatory wheel. At this point, proteins associated to chromatin were released to solution.

Final chromatome sample from 40 million cells (253.5uL) was then split in three equal samples. Sample 1 was kept in its native state and immediately stored at -80ºC for further use. Samples 2 and 3 were treated to denature proteins either with 6M urea or with SDS 2%. Both samples were also stored at -80 ºC until used (See full protocol on Extended Data 1).

## 5.3 SDS-PAGE and Western Blot

For the initial characterization of the purified protein sample associated to the chromatin, we performed an SDS-PAGE followed by a Western Blot (WB). With the first one, we aimed to separate proteins according to their molecular weight and visualize the protein pattern of the samples. With the WB we aimed to identify specific proteins through antibody binding to validate the quality of the sample and the efficacy and efficiency of the purification process. Paired whole cell lysates and the cytoplasmic fractions were used as control samples.

SDS-PAGE was performed in a 12% acrylamide/bis-acrilamyde gel (Acrylamide 30%, Tris-HCL 1.5M, SDS 10%, APS 10%, TEMED 0.1%). Electrophoresis was performed at 100v for 1.5h. The PageRuler Plus Prestained Protein ladder (Thermo 26619) was used as protein marker. Gel was stained with BlueSafe (NYZ Biotech MB15201) for visualization (See full protocol on Extended Data 2).

For the WB, proteins in the acrylamide gel were transferred to a nitrocellulose membrane at 100v for 1h in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol). The membrane was stained with Ponceau S reagent (Sigma P7170-1L) to visualize the transferred proteins. After the transference, the membrane was blocked with 5% skimmed milk in TBS-T 1% buffer for 1h at room temperature and continuous shaking. Three to five washes were performed with TBT-T 1% followed by an overnight incubation at 4 ºC with the primary antibodies described in Table 2. Primary antibodies were diluted in the same blocking solution. After washing on the same

conditions as above, the membrane was incubated with the corresponding secondary antibodies dilutions (Table 2) in blocking solution for 1h at room temperature and continuous shaking. Three to five washing steps were performed as above (See full protocol on Extended Data 3). For the development of the chemiluminescent reaction we used the Western Lightining Plus-ECL reagent (PerkinElmer NEL105001EA). The reaction was visualized on an Amersham Imager 600 equipment. The time of exposure was adjusted depending on the intensity of the signal for each antibody and reaction.

| Primary Antibody | Protein recognized (MW, cellular localization) | Source | Product reference | Dilution | Secondary antibody |
|---|---|---|---|---|---|
| α-Vinculin | Vinculin (120 kDa, cytoplasm) | Mouse | Santa Cruz Biotechnology sc-25336 | 1:500 | Anti-mouse-HRP 1:1000 (ECL™, NA931V) |
| α-RCC1F-2 | RCC1F-2 (45 kDa. nucleus) | Rabbit | Santa Cruz Biotechnology sc-376049 | 1:500 | Anti-mouse-HRP 1:1000 (ECL™, NA931V) |
| α-VDAC1 | VDAC (35 kDa, cytosol) | Rabbit | Santa Cruz Biotechnology sc-390996 | 1:500 | Anti-mouse-HRP 1:1000 (ECL™, NA931V) |
| α-Actin | Actin (42 kDa, cytoplasm) | Rabbit | Sigma-Aldrich A2066 | 1:500 | Anti-rabbit-HRP 1:50.000 (ECL™, NA934V) |
| α-Histone-3 | Histone-3 (15 kDa, nucleus) | Mouse | Sigma-Aldrich H0164-200UL | 1:8000 | Anti-rabbit-HRP 1:50.000 (ECL™, NA934V) |

**Table 2.** Primary and secondary antibodies.

## 5.4 Mass spectrometry

### *5.4.1 Sample preparation*

Samples (20 µg) were reduced with dithiothreitol (100mM, 37 ºC, 60min) and alkylated in the dark with iodoacetamide (5µmol, 25 ºC, 20min). The resulting protein extract was wash with 2M

urea with 100 mM TRIS-HCL and then with 50mM ammonium bicarbonate for digestion with endoproteinase LysC (1:10 w:w, 37 ºC, o/n, Wako, cat #129-02541) and then for trypsin digestion (1:10 w:w, 37 ºC, 8h, Promega cat #V5113) following Wiśniewski et al. FASP procedure.

After digestion, peptide mix was acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc) prior to LC-MS/MS analysis.

### *5.4.2 Chromatographic and mass spectrometric analysis*

Samples were analysed using a LTQ-Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an EASY-nLC 1000 (Thermo Fisher Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column and were separated by reversed-phase chromatography using a 50-cm column with an inner diameter of 75µm, packed with 2µm C18 particles spectrometer (Thermo Scientific, San Jose, CA, USA).

Chromatographic gradients started at 95% buffer A and 5% buffer B with a flow rate of 300nl/min for 5min and gradually increased to 22% buffer B and 78% A in 79 min and then to 35% buffer B and 65% A in 11 min. After each analysis, the column was washed for 10 min with 10% buffer A and 90% buffer B. Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile.

The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.4kV and source temperature at 275 °C. Ultramark 1621 for the was used for external calibration of the FT mass analyser prior the analyses, and an internal calibration was performed using the background polysiloxane ion signal at m/z 445.1200. The acquisition was performed in data-dependent acquisition (DDA) mode and full MS scans with 1 micro scans at resolution of 120,000 were used over a mass range of m/z 350-1500 with detection in the Orbitrap mass analyser. Auto gain control (AGC) was set to 1E5 and charge state filtering disqualifying singly charged peptides was activated. In each cycle of data-dependent acquisition analysis, following each survey scan, the most intense ions above a threshold ion count of 10000 were selected for fragmentation. The number of selected precursor ions for fragmentation was determined by the "Top Speed" acquisition algorithm and a dynamic exclusion of 60seg. Fragment ion spectra were produced via high-energy collision dissociation (HCD) at normalized collision energy of 28% and they were acquired in the ion trap mass analyser. AGC was set to 1E4, and an isolation window of 1.6 m/z and a maximum injection time of 200ms were used. All data were acquired with Xcalibur software v4.1.31.9.

Digested bovine serum albumin (New England Biolabs cat #P8108S) was analysed between each sample to avoid sample carryover and to assure stability of the instrument and QCloud has been used to control instrument longitudinal performance during the project.

### *5.4.3 Data Analysis*

Acquired spectra were analysed using the Proteome Discoverer software suite (v2.0, Thermo Fisher Scientific) and the Mascot search engine (v2.6, Matrix Science). The data were searched against a Swiss-Prot human database (as in April 2019 entries) plus a list of common contaminants and all the corresponding decoy entries. For peptide identification a precursor ion mass tolerance of 7ppm was used for MS1 level, trypsin was chosen as enzyme, and up to three missed cleavages were allowed. The fragment ion mass tolerance was set to 0.5Da for MS2 spectra. Oxidation of methionine and N-terminal protein acetylation were used as variable modifications whereas carbamidomethylation on cysteines was set as a fixed modification. False discovery rate (FDR) in peptide identification was set to a maximum of 5%.

Protein abundance was estimated with Proteome Discoverer (v2.3) using the TOP 3 most abundant peptides per protein.

### *5.4.4 Bioinformatic analysis*

For the comparison of the protein lists retrieved by mass spectrometry among the samples we used the Panther Classification System (http://pantherdb.org/) following the protocol described in Thomas el. Al 2019. Protein lists were classified according to their molecular function, biological process and metabolic pathways among other criteria. Classifications were visualized by pie charts generated also through the Panther Classification System.

# 6. Results

In order to validate the goodness of the chromatome purification prior submitting the pool of sample (5 cell lines, three replicates per cell line) to mass spectrometry we have performed three quality checks with one replicate of the MCF7 cell line.

## 6.1 BlueSafe staining

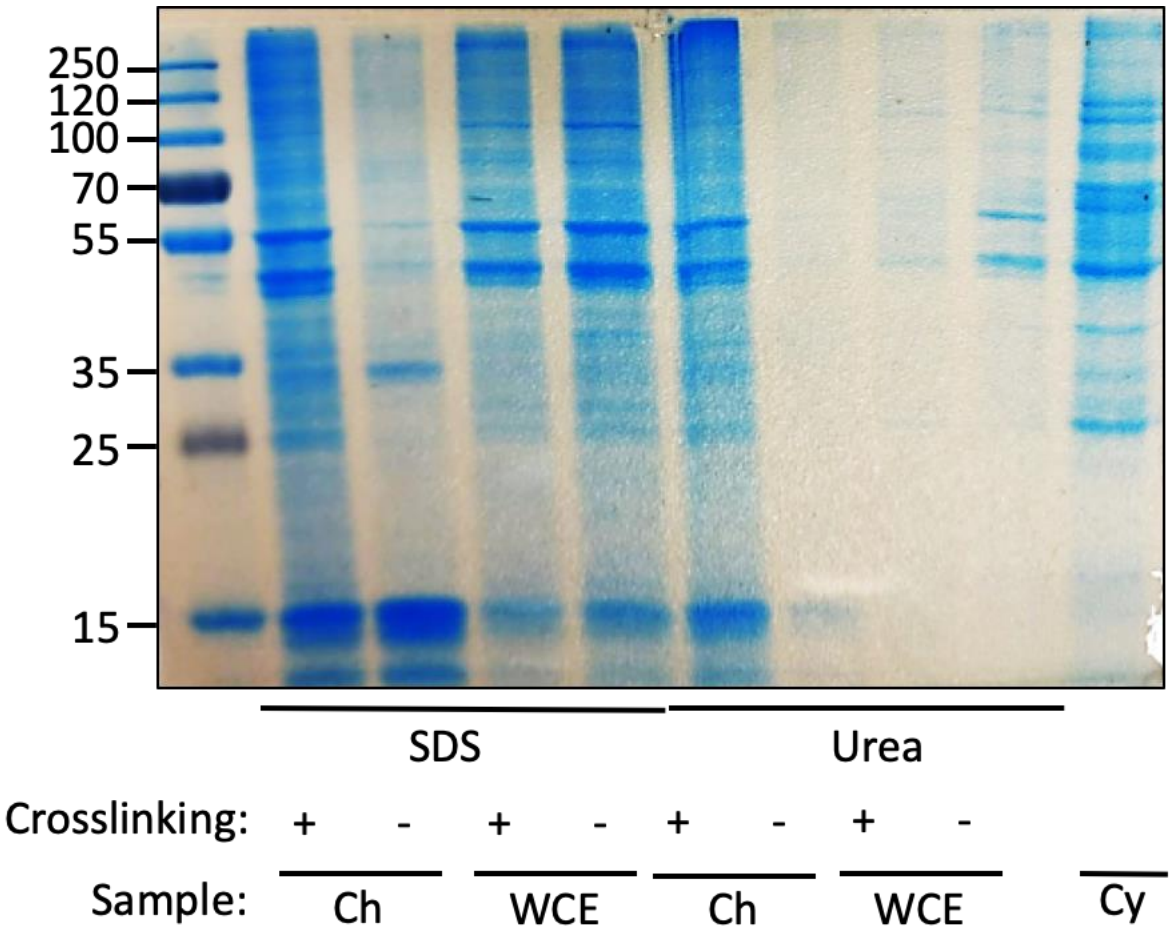



**Figure 4. BlueSafe staining of MCF7 SDS-gel**. Chromatome fraction (Ch), whole cell extract (WCE) and cytoplasm fraction (Cy).

The first quality check consisted in the preparation of Western Blot samples for the BlueSafe staining and the visualization of the protein bands directly on the acrylamide gel. With this experiment, we wanted to validate the presence of different protein patterns in the three-isolated fractions, which were the cytosol, the chromatome and the whole cell extract, as well as verify that the extraction of the sample worked in every used conditions (cell crosslink *vs* not-crosslink; pellet SDS *vs* urea denaturation).

As shown in Figure 4, all the urea-denaturised sample showed a weaker BlueSafe staining than SDS-denaturised samples, indicative of low protein amount, which suggested that the used urea treatment is not sufficient for the complete denaturation of the proteins. Additionally, we observed that the SDS-denaturised chromatome-crosslinked sample showed a very similar protein pattern respect to the cytoplasm and whole cell extract samples, either crosslinked or non-crosslinked. This similarity suggested us that most probably the chromatome extraction did not work properly possibly due to the over-fixation of the sample. Finally, the SDS-denaturised non-crosslinked chromatome sample showed a very different protein pattern respect to the cytoplasm and whole cell extract samples, indicative that the chromatin extraction may have worked properly for this condition. In this regard, in the non-crosslinked SDS-denaturised chromatome sample we observed a strong protein band at low molecular weight (around 15KDa) which may correspond to the histones proteins, which are known for being chromatin-associated proteins.

## 6.2 Western Blot

To further validate the chromatome extraction in every used conditions we performed a WB (Figure 5) by transferring on a nitrocellulose membrane a sister acrylamide gel of the one stained with the BlueSafe and already presented above. By using antibodies recognizing protein commonly localizing either in the nucleus/chromatin (RCC1F-2 and Histone-3) or in the cytoplasm (Vinculin), here we aimed at identifying the best chromatome condition in term of purity (lack of contamination from the cytoplasm fraction).

The WB confirmed the near-absence of proteins in the urea denaturised samples. Additionally, for the crosslinked and SDS-denaturised chromatome sample we observed the presence of Vinculin, which indicated a clear cytoplasmic contamination. Finally, and as expected from the BlueSafe staining presented above, the non-crosslinked SDS-denaturised chromatome sample showed the absence of Vinculin and a strong signal for Histone 3 and RCC1F-2, indicative of the lack of cytoplasm contamination and a proper chromatin protein enrichment.

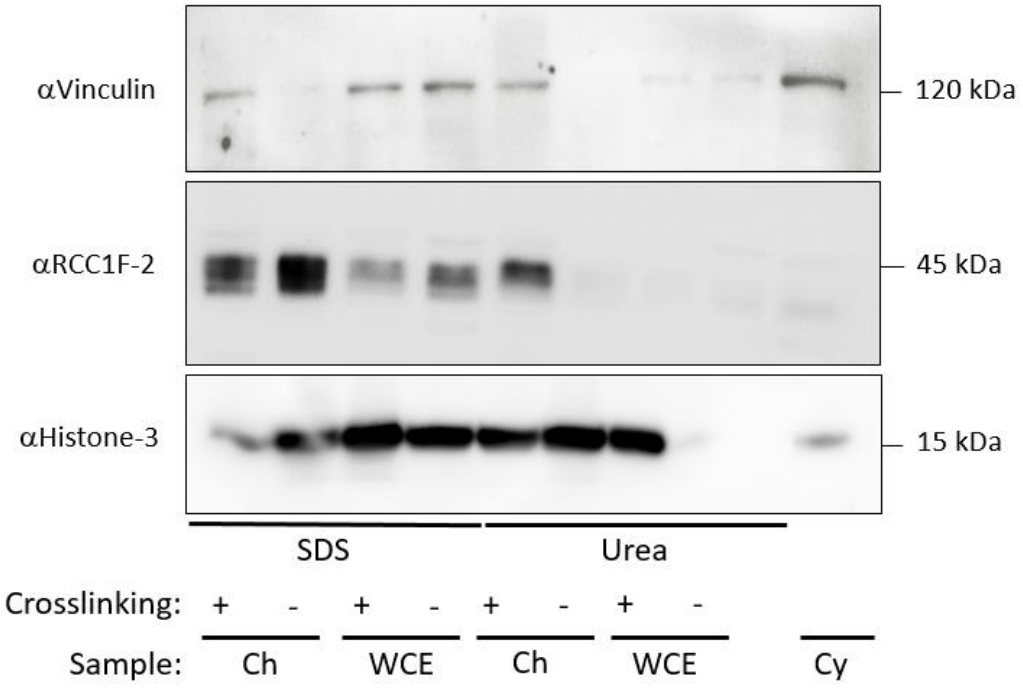



**Figure 5. MCF7 Western Blot**. Western Blot showing the different protein amount in every condition tested. Chromatome fraction (Ch), whole cell extract (WCE) and cytoplasm fraction (Cy). On the left side of the picture, the primary antibodies recognizing specific cytoplasm and nuclear/chromatin proteins are indicated.

## 6.3 Mass spectrometry

Finally, as third-layer of sample quality check we decided perform mass spectrometry with the SDS-denaturised, both crosslinked and non-crosslinked, samples (chromatome, cytoplasm and whole cell extract). This comparative analysis had the aim to identify the overlap between the different fractions and understand whether a consistent difference could be found between the chromatome samples and the cytoplasm/whole cell extract samples.

For the crosslinked samples, we retrieved 3.629 proteins in the chromatome sample, 2.345 proteins in the cytoplasmic sample and 3.688 proteins in the whole cell extract sample (Figure 6). The lower number of proteins observed in the cytoplasm sample is surprising because we were expecting to identify more proteins in this cellular fraction than on chromatin. Given that the BlueSafe staining and the Western Blot indicated that the crosslinked condition might not be the best one, we decided to perform a simple comparative analysis to check the protein overlap of the different samples. The comparative analysis indicated that the chromatome sample almost completely overlap with the whole cell extract sample, confirming that the chromatin-cytoplasm separation failed in the crosslinked condition.

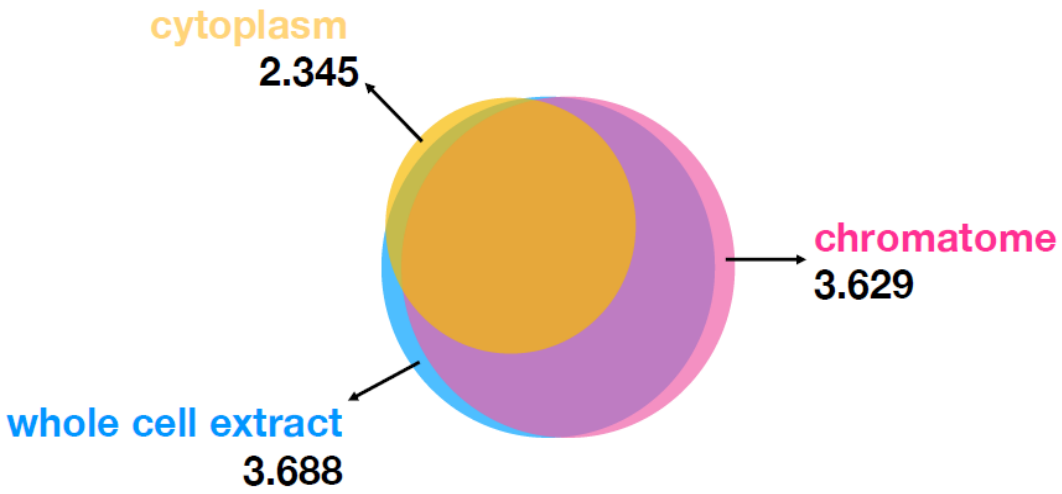



**Figure 6. Crosslinked samples.** Venn-diagram showing the overlap of the crosslinked and SDS-denaturised sample (whole cell extract, cytoplasm and chromatome).

For the non-crosslinked samples, we retrieved 1.346 proteins in the chromatome sample, 3661 proteins in the cytoplasmic sample and 3814 proteins in the whole cell extract sample (Figure 7). Therefore, in this condition it is clear that the chromatome sample is the one with the lowest amount of proteins, which is what we initially expected. When doing a comparative analysis, we could see that in the non-crosslinked condition the overlap between the chromatome sample and the whole cell extract decreased consistently respect to the crosslinked condition. Conversely, now the cytoplasm sample appears to be much more similar to the whole cell extract sample, as it would be logically expected. When comparing the chromatome with the cytoplasm samples (Figure 8), we can observe that of the 1.346 chromatome proteins, only 165 are exclusively found in the chromatome while the rest (1.181) is shared with the cytoplasm. The reason of this big overlap can be due to the fact that the protein translation happens in the cytoplasm even for those factors that have not a cytoplasmic function. The overlap between the chromatome and the cytoplasm fraction is actually the protein pool that interests us the most because we aim to find on chromatin metabolic enzyme usually localizing in the cytoplasm compartment.

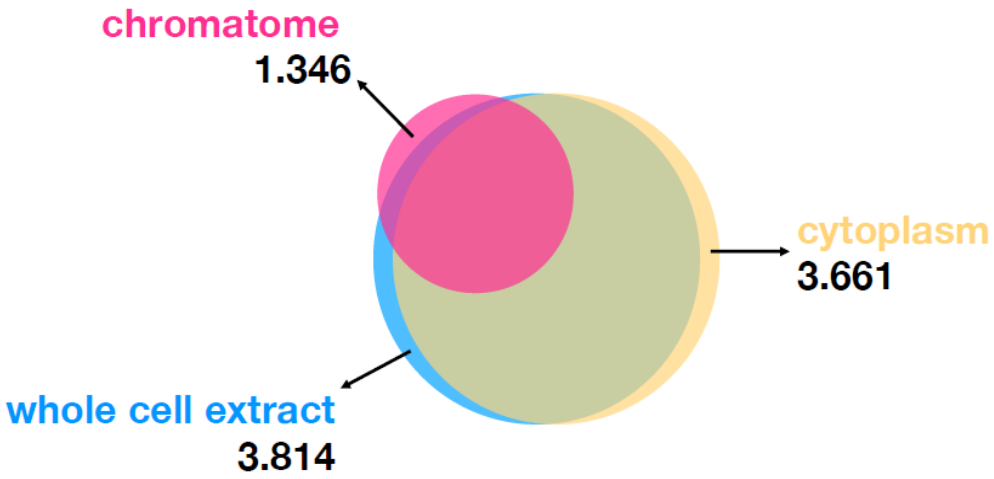



**Figure 7. Non-crosslinked samples.** Venn diagram showing the overlap of the non-crosslinked and SDS-denaturised samples (whole cell extract, cytoplasm and chromatome).

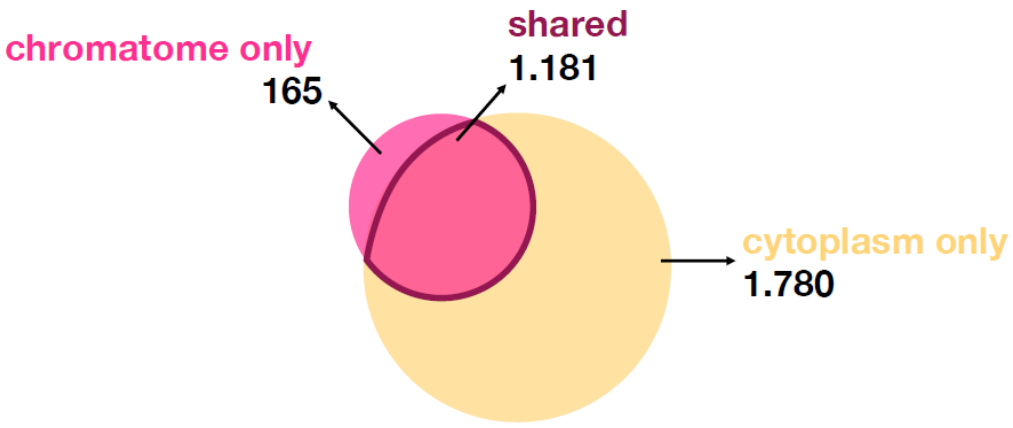



**Figure 8. Non-crosslinked cytoplasm and chromatome samples.** Venn diagram showing the overlap of the SDS-denaturised non-crosslinked cytoplasm and chromatome samples.

### *6.3.1 Panther analysis*

To verify the presence of metabolic enzymes on chromatin we did a simple bioinformatic analysis by using the online software PANTHER (Protein Analysis Through Evolutionary Relationships; http://pantherdb.org/). With this analysis we wanted to identify possible cellular process, in particular metabolic, that are occurring on chromatin. For this, we investigated which biological processes where enriched in the chromatome compartment by entering in PANTHER the 1.346 chromatome proteins. Interesting, the most represented biological process associated with the chromatome was the metabolic process (29,7%), giving a clear evidence that metabolic enzymes are associated with chromatin at least in the analysed breast cancer cell line (MCF7) (Figure 9). A deeper look into the metabolic process-associated proteins retrieved in the chromatome revealed that several enzymes of the canonical metabolism associate with chromatin, for example enzymes of the oxidative phosphorylation, the glycolysis, the de novo synthesis of nucleotides and the Krebs cycle (Figure 9).

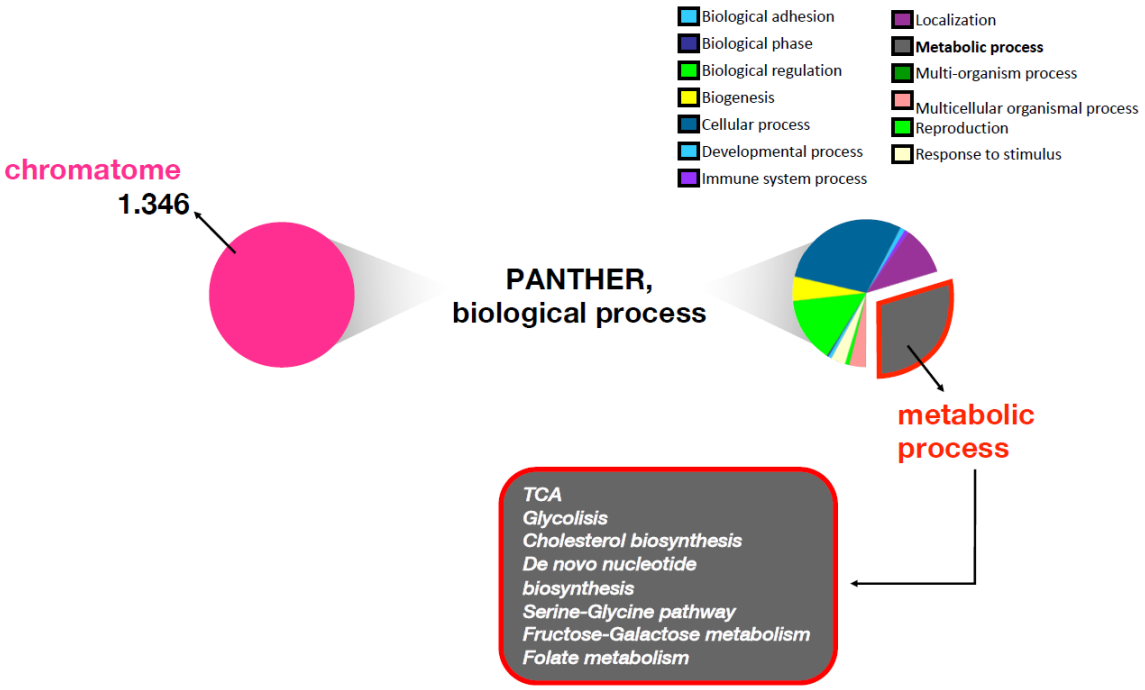



**Figure 3. PANTHER bioinformatics analysis**. Representation of the metabolic pathways identified associated with chromatin in MCF7

## 6.4 Identification of chromatome samples for mass spectrometry

Given the promising results of this first mass spectrometry experiment, which was done purely to perform a quality check of the chromatome extraction, we decided to perform mass spectrometry of the non-crosslinked and SDS-denaturised samples of all the cell lines. For a solid statistical analysis, at least 3 biological replicates are required. Therefore, the last part of the internship was devoted to the identification of good chromatome extractions among the ones performed for each cell line by WB assay. The results section that follows describes these data.

- **MCF7**

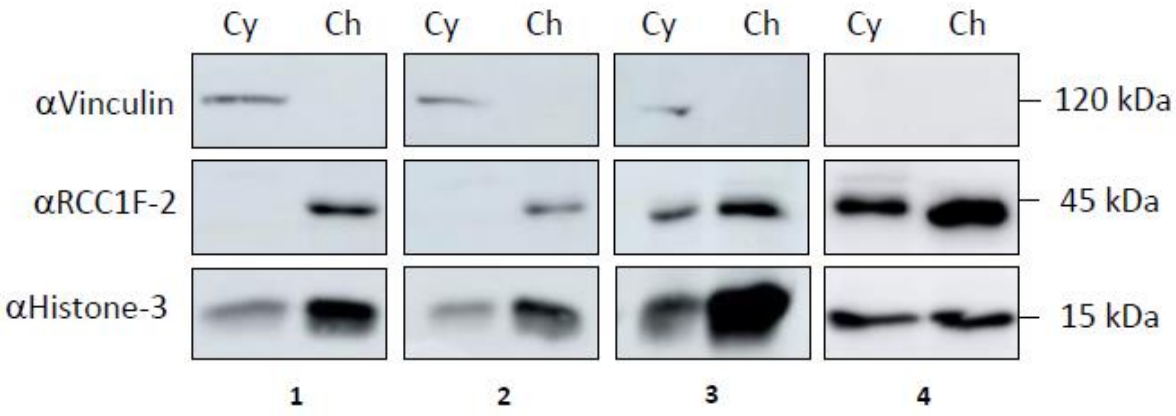



**Figure 4. Western Blot of MCF7**. For each lane 20µg of protein were load. Cy = cytoplasm; Ch =Chromatome.

Four replicates were prepared for the MCF7 cell line (Figure 10). Any replicate showed Vinculin presence in the chromatome fraction while at least in the replicates 1, and 4 Vinculin was present in the cytoplasm fraction. Additionally, Histone-3 and partially RCC1F-2 (for replicates 1 and 3) were considerably enriched in the chromatome fraction than in the cytoplasm fraction of those replicates, indicating that the chromatome isolation worked properly. Due to the fact that no RCC1F-2 or Histone-3 enrichment was observed in the chromatome fraction of replicate 2, we discarded this sample for mass spectrometry analysis.

- **T47D**

For the cell line T47D four replicates were prepared (Figure 11). Any replicate showed vinculin presence in the chromatome fraction while at least in the replicates 1, 2 and 3 Vinculin is present in the cytoplasm fraction. Additionally, RCC1F-2 and Histone-3 were considerably enriched in the

chromatome fraction than the cytoplasm fraction of those replicates, indicating that the chromatome isolation worked properly. Due to the fact that no RCC1F-2 or Histone-3 enrichment was observed in the chromatome fraction of replicate 4, we discarded this sample for mass spectrometry analysis.

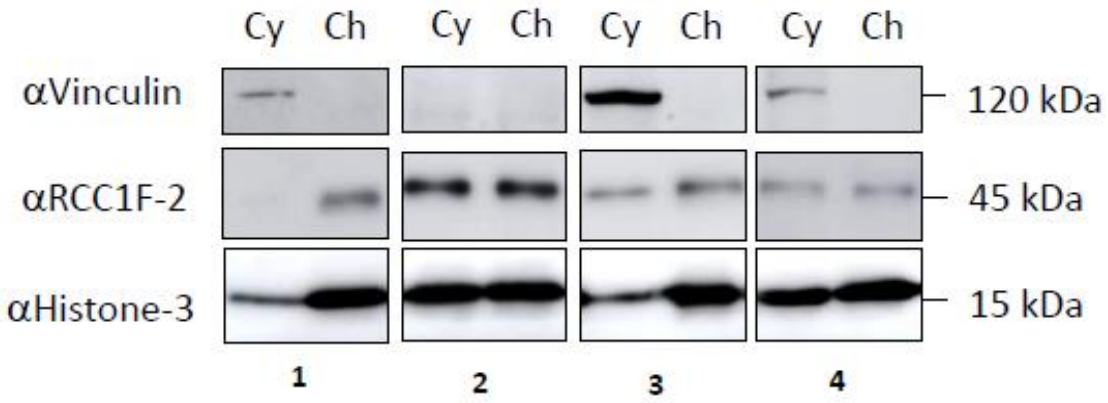



**Figure 5. Western Blot of T47D**. For each lane 20µg of protein were load. Cy = cytoplasm; Ch =Chromatome

- **BT-474**

For the cell line BT-474 six replicates were prepared (Figure 12). Any replicate showed Vinculin presence in the chromatome fraction while at least in the replicates 1, 2, 3 and 4 Vinculin was present in the cytoplasm fraction. Additionally, RCC1F-2 and Histone-3 were considerably enriched in the chromatome fraction than the cytoplasm fraction of those replicates, indicating that the chromatome isolation worked properly. Due to the fact that RCC1F-2 and Histone-3 enrichment was observed in the cytoplasm fraction of replicates 5 and 6, we concluded that the chromatome isolation did not work and discarded those sample for mass spectrometry analysis.

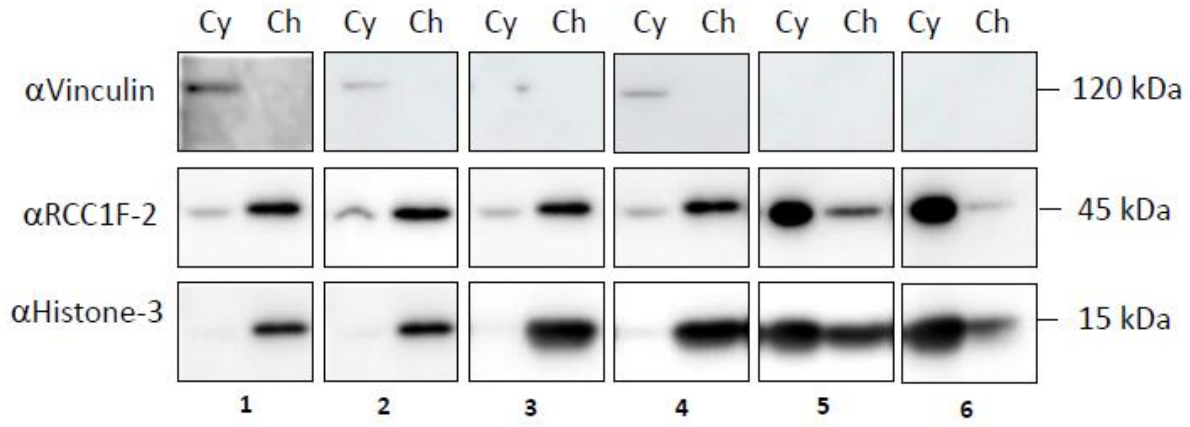



**Figure 6. Western Blot of BT-474**. For each lane 20µg of protein were load. Cy = cytoplasm; Ch = Chromatome

- **SK-BR-3**

For the cell line SK-BR-3 five replicates were prepared (Figure 13). Any replicate showed Vinculin presence in the chromatome fraction while was present in every cytoplasm fractions. Additionally, Histone-3 and partially RCC1F-2 (for replicates 2 and 4) were considerably enriched in the chromatome fractions than the cytoplasm fractions, indicating that the chromatome isolation worked. We selected replicates 1, 2 and 4 for mass spectrometry, however each of them was equally good a part from replicate 4 where the chromatome extraction worked optimally.

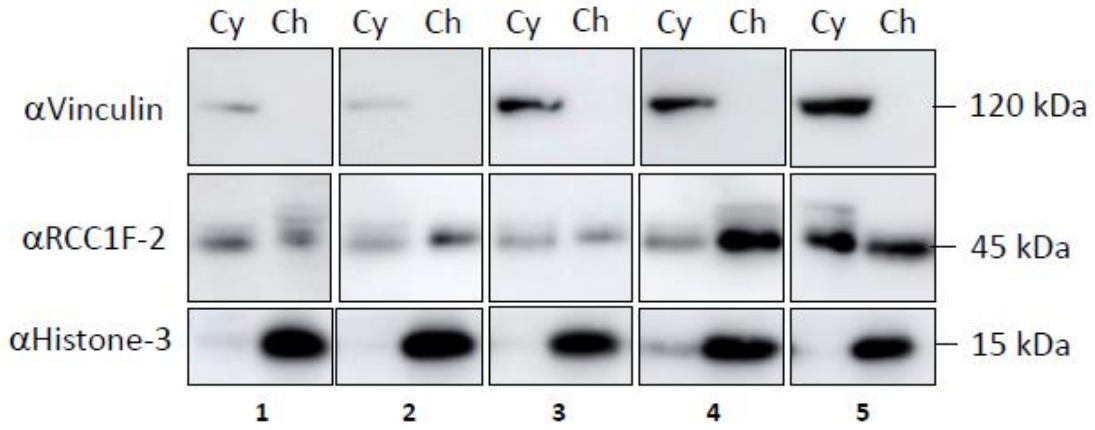



**Figure 7. Western Blot of Sk-BR-3**. For each lane 20 µg of protein were load. Cy = cytoplasm; Ch =Chromatome

- **MDA-MB-231**

For the cell line MDA-MB-231 seven replicates were prepared (Figure 14). Any replicate showed Vinculin or Actin (also a cytoplasm-associated protein) presence in the chromatome sample which were observed only in two of the cytoplasm extracts (replicates 5 and 6). Additionally, in replicates 5 and 6, RCC1F-2 and Histone-3 were considerably enriched in the chromatome fraction than in the cytoplasm fraction, indicating that the chromatome isolation worked for those samples. For the other replicates an inverted patron was observed, which RCC1F-2 and Histon-3 enrichment in the rather in the cytoplasm fraction than in the chromatome fraction, reason why those samples where discarded for mass spectrometry. Given that out of seven replicates only two were suitable for mass spectrometry analysis, more samples must be prepared for this cell line.

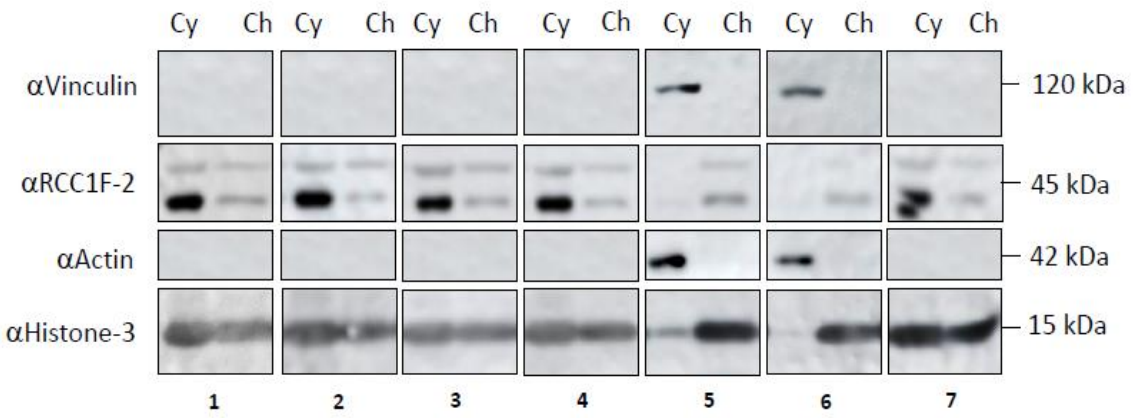



**Figure 8. Western Blot of MDA-MB-231**. For each lane 20 µg of protein were load. Cy = cytoplasm; Ch =Chromatome

# 7. Discussion

Breast cancer is the most prevalent one among women, with huge impact on the personal life, but also at the level of public health and society. Among breast cancer subtypes, the TNBC is still considered as an unmet medical need, due to the lack of an effective treatment capable to stop cancer progression, then leading to increased metastases incidence and death. The current treatment for TNBC is mainly focused on unspecific chemotherapy, which is often not effective, induces chemotherapy resistance in the cancer cells and is toxic for the proliferating non-cancer cells. That is why new and targeted strategies to address this particularly aggressive breast cancer subtype are needed (Bauer et al., 2007; Lehman et al., 2011; Sun, 2017)

It is known that cancer cells show a very distinct metabolic behaviour compared to normal cells. It has been proposed that a metabolic rewiring happens in cancer cells during tumour progression, leading to an increased cell proliferation, survival and long-term maintenance. Among all the described metabolic alterations, the increased glucose uptake and the fermentation of this glucose to lactate happen even in the presence of a fully functional mitochondria capable to perform cellular respiration. This characteristic metabolic profile it is known as Warburg effect, and despite been known for more than 10 years, its molecular mechanism it is still not fully understood. Additionally, it has been proven that the cancer metabolic rewiring influences gene expression, supporting transcriptional patterns that lead to an increased and abnormal cellular proliferation (Liu et al., 2010).

The project that is under development in the laboratory of Sara Sdelci aims to unveil the relationship between metabolism, epigenetics and cancer development and progression in order to find out metabolic vulnerabilities of cancer cells that could be valuable for the identification of new targeted treatments.

As for this bachelor thesis, we focused on breast cancer because of the amount of information available about it, such as the accurate description of the different subtypes naturally occurring, the existence of a non-targetable subtype (TNBC) and the availability of well-established cell lines recapitulating every breast cancer subtype.

We wanted to identify if there are metabolic enzymes associated to the chromatin on breast cancer cells, and whether they are chromatin-bound subtype-specific, and in which metabolic pathways they are involved.

The cell lines used for the development of this bachelor project recapitulate each breast cancer phenotype. Chromatin was extracted for MCF7 (ER+/PrR-/HER2-), T47D (ER+/PrR+/HER2-), BT-474 (ER+/PrR+/HER2+), SK-BR-3 (ER-/PrR-/HER2+) and MDA-MB-231 (ER-/PrR-/HER2-) cell lines representing the four subtypes of breast cancer (mainly ductal carcinoma). For all the cell lines, several biological replicates were prepared in order to validate them through WB before submitting the samples to mass spectrometry. For the sample validation, we analysed the presence of cytoplasmic proteins (Vinculin or Actin) on the chromatome fraction and the enrichment on Histone-3 and RCC1F-2 proteins, which are known to be chromatin-bound. In general, the absence of Vinculin or Actin signal and a clear enrichment of RCC1F-2 and Histone-3 in the chromatome fraction indicated that the chromatome extraction worked properly and that there was no cytoplasm contamination. Therefore, samples showing these two conditions were considered as optimal replicates and they will be used for the mass spectrometry analysis. Samples were cytoplasmic proteins were detected in the chromatome fraction were discarded as a clear signal of cytoplasmic contamination.

We tested different desaturating agents for the final chromatome sample: SDS and Urea. Sodium dodecyl sulfate (SDS) is an anionic detergent capable of denaturing secondary structures as well as tertiary structures not linked by disulfide bonds by binding to the protein chain exposing normally buried regions. The addition of SDS to a protein sample also leads to an increased negative charge of the protein directly related to it molecular weight (Schmid et al., 2016). On the other hand, urea denaturizes proteins through direct binding and changing the electrical charge of the hydrogen bonds, thus weakening the protein secondary and tertiary structure, and also by changing the properties of the solvent were the protein is (Schmid et al., 2016). In our hands, when using urea 6M as denaturing agent, we could appreciate a dramatic reduction on protein content of the sample as seen through SDS-PAGE (Figure 4), and also a lack of specific signal on the WB (Figure 5), indicating that there was a reduced amount of protein on those samples. Probably proteins precipitated when adding urea to the sample, and therefore those were discarded after the last centrifugation step. Those samples treated with SDS, behave as we expected: the proteins were denatured and remained in the supernatant thus being recovered and nicely appreciated on the SDS-PAGE and the WB (Figure 4 and 5). Therefore, we decided to discard the urea treated samples for further assays.

When analysing protein interactions there is always the risk of losing partners of the interacting complexes during the purification processes. To try to avoid this issue, the crosslink with formaldehyde 1% is very commonly used (Hoffman et al., 2015). With the crosslink reaction the protein interactions are chemically preserved and thus the non-covalent links could be also further identified. In our particular case, we tested the 1% formaldehyde crosslink to try to retrieve chromatin-bound protein complexes or metabolic pathways in their entirety, which otherwise could be only partially recovered in the non-crosslinked conditions. To test whether our crosslinked condition was more efficient on retrieving chromatin associated proteins than the non-crosslinked one, we compared the protein profile recovered from the chromatome of MCF7 cell line under crosslinked and non-crosslinked conditions (Figure 5). On the crosslinked condition, we observed a clear cytoplasmic contamination due to the presence of Vinculin in the crosslinked chromatome SDS denaturised, meaning that the chromatin isolation process was impaired by the crosslinking reaction. Specifically, when crosslinking, the chromatin isolation process is less effective on the elimination of non-chromatin related proteins. This was also evident when comparing the mass spectrometry results, as the sample purified from chromatin of crosslinked cells is almost identical to the whole cell extract sample, meaning that there was no selective purification (Figure 6).

As the 1% formaldehyde crosslink is a very broadly used technique which often gives good results and improves protein interaction studies, we are now optimizing this step for further chromatome purifications. We are reducing the time of crosslink, but also the amount of formaldehyde could be optimized. We are also testing the crosslink directly on the isolated nuclei (instead than intact cells), with the aim of reducing cytoplasm contamination. For this condition, the time and the amount of formaldehyde should be also optimized.

The non-crosslinked samples showed no cytoplasm contamination, and an overall good enrichment of RCC1F-2 and Histone-3 signal for every cell line. With those results in our hands, we decided to send the non-crosslinked samples to mass spectrometry as they were not contaminated and more enriched on chromatin-associated proteins.

Finally, another critical step during the chromatome purification is the initial cellular lysis. The initial cellular lysis is meant to release the cytoplasm to retrieve intact nuclei. An excess of time or strength of the lysis could lead to a partially undesired nuclei lysis, and thus the release of the nuclear proteins in the cytoplasm fraction with the corresponding loss of protein representation

on the chromatome fraction. We observed that for some of the cell lines (Figures 10-13 and 14), where we could detect a small amount of Histone-3 on the cytoplasmic fraction, meaning that the first cellular lysis was not optimal. Time and strength of the initial cellular lysis should be also optimized for each cell line and also for the number of starting cells.

We received a protein list from each sample sent to mass spectrometry, showing those identified proteins attached to chromatin according to the different sample preparation conditions. To analyse those protein lists we performed a comparative analysis by using Panther Classification System based on gene ontology. On the non-crosslinked sample, 165 proteins were identified only in the chromatome fraction, all from DNA-related processes as replication and transcription. Additionally, those proteins related to metabolism should be present on the overlapping protein list between the cytoplasmic and the chromatome fractions, as metabolic enzymes are supposed to perform their function in the cytoplasm. therefore, we performed a comparative analysis taking into account the represented biological processes by analysing the chromatome retrieved proteins. Metabolic processes were highly represented among the chromatin associated proteins, reinforcing the idea of their influence on gene expression and cancer progression (Figure 9).

The fact that metabolic enzymes (like those involved in glycolysis or in the tricarboxylic acid cycle) are associated with chromatin, makes us hypothesize that there are regions on chromatin where energy in form of ATP and nucleotides are needed for the proliferation and division of the tumours. On the other hand, the metabolic pathways involved in the biosynthesis of nucleotides invite to think that the *in loco* availability of nucleotides at active transcription sites can facilitate the transcription process of certain genes associated with cancer proliferation.

After validating the chromatome extraction protocol, each biological replicate prepared for every cell line has been tested by WB and three biological replicates of each of the breast cancer cell lines were selected and will be soon submitted to mass spectrometry analysis. From the mass spectrometry results we aim to be able to compare not only the specific chromatome core for each cell line, but to identify the common features among those from the same cancer subtype. Finally, and probably more important, we want to identify (if any) those metabolic enzymes or pathways associated to chromatin only in TNBC, in order to investigate specific pathways related to the development/progression of this untreatable cancer subtype and further use this information to propose new targets for TNBC treatment.

# 8. Conclusion

Inside all the deep knowledge about epigenetics, cancer and metabolism currently available, our work is aimed to solve basic biological questions (still not answered) related to the influence of metabolic enzymes on gene expression, specifically in the context of cancer. The project that is ongoing in the lab of Sara Sdelci will unveil the relationship between the already known Warburg effect (characteristic metabolic rewiring process of cancer cells) and its translation to the chromatin environment, with the consequent changes on gene expression and epigenetic regulation that can support cancer progression.

In this context, this bachelor thesis has contributed as follows:

1. We were able to stablish a protocol for the isolation of proteins associated to chromatin in cancer cell lines. This protocol should also work in any kind of mammalian cell with slight opportune optimization.
2. We demonstrated that the use of urea as denaturing agent is not recommended, as it seems to provoke inefficient protein denaturation and consequent protein precipitation.
3. We demonstrated that the cellular crosslink with formaldehyde 1% for 10min is inappropriate for the correct isolation of chromatin-bound proteins, as it provokes the contamination of the sample with cytoplasm-associated proteins
4. We performed WB analysis for sample validation based on the identification of proteins of the cytoplasm and chromatin fractions.
5. We were able to identify metabolic enzymes associated to chromatin in MCF7 breast cancer cell line through mass spectrometry of the chromatome.

# 9. Future perspectives

As the logical continuity of the research line, we will send for analysis through mass spectrometry a set of three biological replicates for all the breast cancer cell lines, in order to identify common and differential metabolic enzymes subsets among all of them. With this information we will be able to design genetic systems to perturb the cells and asses the influence of those metabolic enzymes on the proliferation of the cancerous cells and also evaluate them as possible therapeutic targets to prevent cancer development and progression. The main effort will be devoted to unveil this information in the TNBC cell line, in order to identify effective and specific cancer targets for this unmet medical need.

# 10. Self-evaluation

Thinking of what to say to assess this internship, I have realized that I do not have more than words of gratitude. It has been a period of deep learning above all. I have been able to learn and improve several techniques, such as the WB or the chromatin extraction, which I had just studied on paper before and now each detail makes much more sense in my mind.

When I arrived to the Sdelci Lab, I was the only student since the lab set up only 1-month prior my arrival, but from the first day I have always been treated as a fellow and Sara and Maria (the lab manager of the group) always have been supportive and respectful. Working in an environment in which people enjoy and believe in what they are doing made me grasp the essence of science and acquire confidence in myself. Additionally, I cannot even describe the satisfaction when I saw that my work was giving nice results.

From the first moment, the communication between all members of the group has been exceptional, which made easier the learning process. During the weekly lab meetings, we were discussing results and planning new experiments, carefully controlling the progress of the project. Additionally, we regularly organized Journal Clubs and, where I had the opportunity to present the following articles:

1) LLGL2 rescues nutrient stress by promoting leucine uptake
2) Interactome Rewiring Following Pharmacological Targeting of BET Bromodomains

Moreover, during my time in the CRG, I had the possibility to attend talks given by excellent scientists, such as Darío García-Lupiáñez or Arkaitz Carracedo, in addition to the weekly data seminars of the program "Gene regulation, stem cells and cancer". IU was invited to participate in the Gene regulation, stem cells and cancer retreat, which was organized in February and was an amazing experience. On the 22nd of March with Sara and Maria we attended the "IX Jornada de cromatina I epigenètica" in Barcelona (Sala Prat de la Riba, Seu de l'institut d'estudis Catalans), organized by the Societat Catalana de Biologia. For the occasion, I had the opportunity to present a poster entitled "Does chromatin drive breast cancer evolution?" and to listened to presentations of great scientists like Ferran Azorín, Manel Esteller or Sandra Peiró.

In less words, I could not have had a better start into the scientific world.

# 11. Bibliography

Sun, Y. S., Zhao, Z., Yang, Z. N., Xu, F., Lu, H. J., Zhu, Z. Y., … Zhu, H. P. (2017). Risk Factors and Preventions of Breast Cancer. *International journal of biological sciences*, *13*(11), 1387–1397.

Dai, X., Cheng, H., Bai, Z., & Li, J. (2017). Breast Cancer Cell Line Classification and Its Relevance with Breast Tumor Subtyping. *Journal of Cancer*, *8*(16), 3131–3141.

Kamińska, M., Ciszewski, T., Łopacka-Szatan, K., Miotła, P., & Starosławska, E. (2015). Breast cancer risk factors. *Przeglad Menopauzalny*, *14*(3), 196–202.

Makki, J. (2015). Diversity of breast carcinoma: Histological subtypes and clinical relevance. *Clinical Medicine Insights: Pathology*, *8*(1), 23–31.

Barroso-Sousa, R., & Metzger-Filho, O. (2016). Differences between invasive lobular and invasive ductal carcinoma of the breast: results and therapeutic implications. *Therapeutic advances in medical oncology*, *8*(4), 261–266.

Reed, M. E. M. C., Kutasovic, J. R., Lakhani, S. R., & Simpson, P. T. (2015). Invasive lobular carcinoma of the breast: Morphology, biomarkers and 'omics. *Breast Cancer Research*, *17*(1), 1–11.

Ahmed, S., Sami, A., & Xiang, J. (2015). HER2-directed therapy: current treatment options for HER2-positive breast cancer. *Breast Cancer*, *22*(2), 101–116.

Bauer, K. R., Brown, M., Cress, R. D., Parise, C. A., & Caggiano, V. (2007). Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: A population-based study from the California Cancer Registry. *Cancer*, *109*(9), 1721–1728.

Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of clinical investigation*, *121*(7), 2750–2767.

Chavez, K. J., Garimella, S. V., & Lipkowitz, S. (2012). Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. *Breast Disease*, *32 (1)*, 35–48.

Lee, S. M., Kang, E. J., Kim, J. H., Yun, J. M., Sun, D. S., Ko, Y. H., & Won, H. S. (2015). Triple-negative breast cancer that progressed as estrogen receptor-positive skin metastases. *Korean Journal of Internal Medicine*, *30*(3), 411–414.

Toft, D. J., & Cryns, V. L. (2011). Minireview: Basal-like breast cancer: from molecular profiles to targeted therapies. *Molecular endocrinology (Baltimore, Md.)*, *25*(2), 199–211.

Flavahan, W. A., Gaskell, E., & Bernstein, B. E. (2017). Epigenetic plasticity and the hallmarks of cancer. *Science*, *357*(6348).

DeBerardinis, R. J., & Chandel, N. S. (2016). Fundamentals of cancer metabolism. *Science Advances*, *2*(5).

Pavlova, N. N., & Thompson, C. B. (2016). The Emerging Hallmarks of Cancer Metabolism. *Cell metabolism*, *23*(1), 27–47.

Hatziapostolou, M., & Iliopoulos, D. (2011). Epigenetic aberrations during oncogenesis. *Cell and molecular life sciences, 68*(10), 1681–1702.

Sharma, S., Kelly, T. K., & Jones, P. A. (2009). Epigenetics in cancer. *Carcinogenesis*, *31*(1), 27–36.

Reid, M. A., Dai, Z., & Locasale, J. W. (2018). The impact of cellular metabolism on chromatin dynamics and epigenetics. *Nature Cell Biology, 19*(11), 1298–1306.

Wong, C. C., Qian, Y., & Yu, J. (2017). Interplay between epigenetics and metabolism in oncogenesis: mechanisms and therapeutic approaches. *Oncogene, 15(*24*),* 3359–3374.

Kinnaird, A., Zhao, S., Wellen, K. E., & Michelakis, E. D. (2016). Metabolic control of epigenetics. *Nature*. 16 (11), 694-707.

Pietrocola, F., Galluzzi, L., Bravo-San Pedro, J.M., Madeo, F. & Kroemer, G. (2015). Acetyl Coenzyme A: A central metabolite and second messenger. *Cell Metabolism*, 21(6), 805-21.

Wright, R. H. G., Lioutas, A., Dily, F. Le, Soronellas, D., Pohl, A., Bonet, J., … Beato, M. (2016). ADP-ribose – derived nuclear ATP synthesis by NUDIX5 is required for chromatin remodeling. *Science*, *352*(6290), 1221–1226.

Sdelci, S., Rendeiro, A.F, Rathert, P., You, W., Lin J.M.G, Ringler, A., …. Kubicek, S. (2019). MTHFD1 interaction with BRD4 links folate metabolism to transcriptional regulation. *Nature Genetics,* 51(6), 990-998.

Lovén, J., Hoke, H. A., Lin, C. Y., Lau, A., Orlando, D. A., Vakoc, C. R., … Young, R. A. (2013). Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell*, *153*(2), 320–334.

Zuber, J., Shi, J., Wang, E., Rappaport, A. R., Herrmann, H., Sison, E. A., … Vakoc, C. R. (2011). acute myeloid leukaemia. *Nature*, *478*(7370), 524–528.

Alekseyenko, A. A., Walsh, E. M., Wang, X., Grayson, A. R., Hsi, P. T., Kharchenko, P. V., … French, C. A. (2015). The oncogenic BRD4-NUT chromatin regulator drives aberrant transcription within large topological domains. *Genes and Development*, *29*(14), 1507–1523.

Yu, S., Kim, T., Yoo, K. H., & Kang, K. (2017). The T47D cell line is an ideal experimental model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. *Biochemical and Biophysical Research Communications*, *486*(3), 752–758.

Lacroix, M., & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Research and Treatment*, *83*(3), 249–289.

Wiśniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, *6*(5), 359–362.

Liu, X., Wang, X., Zhang, J., Lam, E. K. Y., Shin, V. Y., Cheng, A. S. L., … Jin, H. C. (2010). Warburg effect revisited: An epigenetic link between glycolysis and gastric carcinogenesis. *Oncogene*, *29*(3), 442–450.

Schmid, M., Prinz, T. K., Stäbler, A., & Sängerlaub, S. (2017). Effect of sodium sulfite, sodium dodecyl sulfate, and urea on the molecular interactions and properties of whey protein isolate-based films. *Frontiers in chemistry*, *4*, 49.

Hoffman, E. A., Frey, B. L., Smith, L. M., & Auble, D. T. (2015). Formaldehyde crosslinking: A tool for the study of chromatin complexes. *Journal of Biological Chemistry*, *290*(44), 26404–26411.

.

# Extended Data 1. Chromatin extraction protocol

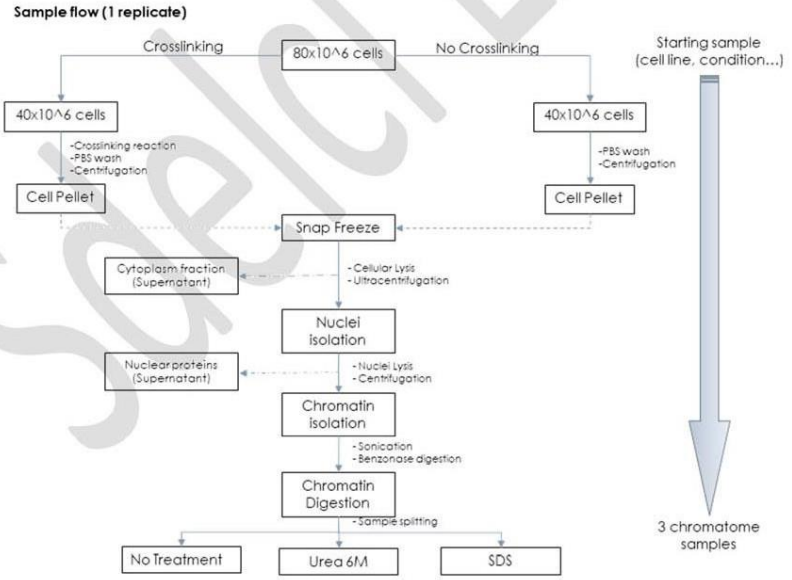
*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

| PROTOCOL | CHROMATOME ISOLATION |
|---|---|
| BASED ON | CeMM protocol. Ref J Proteomics. 2012 Sep 18;75(17):5493-506 |
| STANDARDIZED (Y/N) | Y |

**STEPS**

1. Culture cells
2. Preparation of working solutions
3. Crosslinking and cell harvesting
4. Cellular lysis
5. Nuclei isolation
6. Chromatin isolation
7. Sonication and Benzonase digestion
8. Sample splitting
9. Protein isolation
10. Protein quantification

**Graphical flow:**



*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

**DAY 0**

**1.- Culture cells**

- Culture cells on their appropriate media and amplify them as required to finally get 1-2x$10^7$ cells per sample
- Pellet the cells at 400g during 5 minutes
- Discard the media
- Wash the pellet one with PBS
- Snap freeze the pellet in liquid nitrogen

**DAY 1**

**2.- Preparation of working solutions:**

- Prepare the following buffers:

| Buffer | Volume | Composition |
|---|---|---|
| SB (for 10mL) | 10 mL | -100uL of 1M HEPES (pH 7.5) to reach 10mM<br>-100uL of 1M KCL to reach 10mM<br>-15uL of MgCL2 to reach 1.5mM<br>-1,3mL of 2,5M sucrose to reach 0.1%<br>-200uL of Proteinase inhibitor cocktail (50X)<br>-7.38mL of H2O<br>*(NOTE: for hard to lyse cells use 0.2% Triton in the buffer) |
| SC (for 10mL) | 10 mL | -100uL of 1M HEPES (pH 7.5) to reach 10mM<br>-100uL of 1M KCL to reach 10mM<br>-15uL of MgCL2 to reach 1.5mM<br>-8,3mL of 2,5M sucrose to reach 2.1M<br>-200uL of Proteinase inhibitor cocktail (50X)<br>-1.5mL of H2O |
| Washing buffer (for 10mL) | 10 mL | -100uL of 1M HEPES (pH 7.5) to reach 10mM<br>-200uL of Proteinase inhibitor cocktail (50X)<br>-9.7mL H2O |
| Sucrose 2.5M | 50mL | -42.7g sucrose<br>-50mL H2O |
| Benzonase digest buffer (for 5mL) | 5 mL | -100uL of 750mM HEPES to reach 15mM<br>-10uL of 0.5M EDTA to reach 1mM<br>-10uL of 0.5M EGTA to reach 1mM<br>-5uL TPCK of a 5mg/mL TPCK<br>-100uL of Proteinase inhibitor cocktail (50X)<br>-50uL of 10% NP40 to reach 0.1%<br>-4725uL H2O |

| | | |
|---|---|---|
| 2X SDS Lysis buffer (for 5mL) | 5 mL | -660uL of 750mM HEPES to reach 100mM<br>-2000 of 10% SDS to reach 4%<br>-10uL of 100mM PSMF to reach 2mM<br>-50uL of Proteinase inhibitor cocktail (50X)<br>-2270uL H2O |
| Urea 12M | 50 mL | -36g Urea<br>-50mL H2O |

**3.- Crosslinking and cell harvesting:**

* Crosslinking reaction is performed in order to "secure" the protein-DNA and protein-protein interactions. The objective is to try to recover protein complexes attached to the chromatin that otherwise would be dissociated. Note that non-crosslinked cells are used as control (same number of cells).

- Extract 1mL of the culture media from the plates

- Add 933mL of 16% Formaldehyde (w/v) methanol-free (Pierce 28908)

- Incubate 10min at room temperature

- Add 750uL Glycine 2.5M to quench the formaldehyde

- Incubate 5 min on ice

- Wash twice with PBS (***Note**: For non-crosslinked plates start from here to collect the cells)

- Scrape the cells with PBS and collect all the cells into a falcon tube

- Centrifuge at 600g for 7 minutes at 4ªC

- Snap freeze the cellular pellet

**DAY 2**

**4.- Cellular lysis:**

- Resuspend the cellular pellet (40x$10^6$ cells per sample) in 1mL of buffer SB (0.5mL for 20x$10^6$ cells)

- Incubate 30min in a rotating wheel at 4ºC (*Note: for hard to lyse cells use 45min of incubation)

- Spin the cells 3min at 2000g at 4ºC (Nuclei fraction is the pellet – Save the supernatant for cytoplasm)

- Extract the supernatant and store it for further analysis if required

*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

**5.- Nuclei isolation**

- Dissolve the pellet in 0.5mL of buffer SB

- For hard to lyse cells incubate for extra 30min more on ice

- Prepare the sucrose gradient in the ultracentrifuge tube (Beckman Ref. 349622). Add 2,5mL of buffer SC and the sample on top of it

- Be sure to equilibrate the tubes before ultracentrifugation on a balance. ***Note:** Please note that for the mentioned tubes the maximum volume for centrifugation is 3mL

- Centrifuge samples at 53000 rpm for 3h at 4ºC (Ultra table Top, Rotor TLA100.3). Acceleration and Deacceleration to the half (5).

- The nuclei content is on the pellet. Supernatant contains the nuclear proteins

**6.- Chromatin isolation**

- Resuspend the pellet in 0.5mL of buffer SB

- Spin cells for 45min at 4ºC at 20000g

- Pellet contains the chromatin

- Wash the pellet twice with 0.5mL of washing buffer

- Centrifuge 15min at 20000rpm 4ºC

- Discard the supernatant and either snap freeze the pellet on liquid nitrogen or move directly to the Benzonase digestion step

**DAY 3**

**7. Sonication and Benzonase digestion**

- Cool down the Diagenode Sonicator bath to 4ºC

- Dilute the benzonase stock (250U/mL) 1:10

- Add 250uL of lysis buffer to the cell pellet (for $40x10^6$ cells pellet)

- Transfer the sample to the diagenode tube

- Sonicate samples 30seg ON, 45seg OFF, 15 cycles

- Add 1uL of the Benzonase 1:10 dilution

- Add 2.5uL of the 1:1000 RNAse dilution

- Digest samples for 30-40min on a rotatory wheel at 4ºC

**8. Sample splitting**

- Split samples on 3 tubes (estimate the total sample volume)

a.- Snap freeze as control

b.- Add equal amount of Urea 12M solution to reach Urea 6M final concentration. Snap freeze for further analysis

c.- Continue to the SDS Lysis

**8. Protein isolation**

- Add equal amount of SDS Lysis buffer 2X to the sample (ratio 1:1)

- Incubate 10min at room temperature

- Heat at 99ºC for 5 minutes in a safe locked eppendorff tube

- Centrifuge at 16000g for 10 minutes at 20ºC

- Transfer supernatant to new tubes

**9. Protein quantification**

- Measure protein concentration of a sample dilution 1:10 by using BCA (using detergent compatible reagents) or directly to the nanodrop

**10.- Continue to Mass Spectrometry and/or Western Blot**

***Note** (Mass Espectrometry requirements):

- 3 biological replicates per cell line/condition

- 10ug total protein extract

- 1ug/uL ideal concentration

# Extended Data 2. SDS-PAGE protocol

*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

| PROTOCOL | SDS-PAGE |
|---|---|
| BASED ON | Cold Spring Harbor Protocols |
| STANDARDIZED (Y/N) | Y |

**Separation gel:**

- Prepare the separation gel mixing the reagents following the order of the table below, as you might need.

- Pour the gel mixture living around 2cm below the bottom of the comb for the stacking gel. Make sure to remove any bubble.

- Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will keep the polymerized gel from drying out.

- In ~30 min, the gel should be completely polymerized.

- Remove the isopropanol when the gel is polymerized. Wash with miliQ water and dry with a piece of paper.

**Stacking gel :**

- Pour stacking gel on top of the separation gel.

- Add combs to make wells. In around 30min the stacking gel should become completely polymerized

- Clamp gel into apparatus and fill both buffer chambers with gel running buffer according to the instructions for the specific apparatus

- Load samples and molecular mass protein markers into wells for separation by electrophoresis

*.- Choosing the appropriate Acrylamide % depending on the sample: The smaller the size of the protein of interest, the higher the percentage of acrylamide/bis. The bigger the size of the protein of interest, the lower the percentage of acrylamide/bis.



Pag.1 *Sdelci Lab. CRG, Barcelona*

*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

| **Buffer B1 4X** | 260mL |
|---|---|
| 1.5M Tris-HCL pH 8.8 | 250 mL |
| SDS 10% | 10 mL |

| **Buffer B2 4X** | 260mL |
|---|---|
| 1.5M Tris-HCL pH 6.8 | 250 mL |
| SDS 10% | 10 mL |

| **Stacking mix** | 100mL |
|---|---|
| Acrylamide 30% (29:1) | 16.7 mL |
| Buffer 2 4X | 25 mL |
| H2O mQ (mL) | 58.3 mL |

| **7 % Gels →** Recommended for separating proteins between 25-200 KDa | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total volume (mL) | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| Acrylamide 30% (29:1) (mL) | 1,17 | 2,34 | 3,51 | 4,68 | 5,85 | 7,02 | 8,19 | 9,36 | 10,53 | 11,7 |
| B1 4X (mL) | 1,25 | 2,5 | 3,75 | 5 | 6,25 | 7,5 | 8,75 | 10 | 11,25 | 12,5 |
| H2O (mL) | 2,58 | 5,16 | 7,74 | 10,32 | 12,9 | 15,48 | 18,06 | 20,64 | 23,22 | 25,8 |
| 10% APS (uL) | 50,00 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 450 | 500 |
| TEMED (uL) | 5,00 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| #Gels (BioRad) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

| **10 % Gels →** Recommended for separating proteins between 20-100 KDa | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total volume (mL) | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| Acrylamide 30% (29:1) (mL) | 1.67 | 3,34 | 5,01 | 6,68 | 8,35 | 10,02 | 11,69 | 13,36 | 15,03 | 16,7 |
| B1 4X (mL) | 1.25 | 2,5 | 3,75 | 5 | 6,25 | 7,5 | 8,75 | 10 | 11,25 | 12,5 |
| H2O (mL) | 2.08 | 4,16 | 6,24 | 8,32 | 10,4 | 12,48 | 14,56 | 16,64 | 18,72 | 20,8 |
| 10% APS (uL) | 50,00 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 450 | 500 |
| TEMED (uL) | 5,00 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| #Gels (BioRad) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

| **12 % Gels →** Recommended for separating proteins between 10-70 KDa | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total volume (mL) | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| Acrylamide 30% (29:1) (mL) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| B1 4X (mL) | 1.25 | 2,5 | 3,75 | 5 | 6,25 | 7,5 | 8,75 | 10 | 11,25 | 12,5 |
| H2O (mL) | 1.75 | 3,5 | 5,25 | 7 | 8,75 | 10,5 | 12,25 | 14 | 15,75 | 17,5 |
| 10% APS (uL) | 50,00 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 450 | 500 |
| TEMED (uL) | 5,00 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| #Gels (BioRad) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

| **15 % Gels →** Recommended for separating proteins between 12-45 KDa | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total volume (mL) | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| Acrylamide 30% (29:1) (mL) | 2.50 | 5 | 7,5 | 10 | 12,5 | 15 | 17,5 | 20 | 22,5 | 25 |
| B1 4X (mL) | 1.25 | 2,5 | 3,75 | 5 | 6,25 | 7,5 | 8,75 | 10 | 11,25 | 12,5 |
| H2O (mL) | 1.25 | 2,5 | 3,75 | 5 | 6,25 | 7,5 | 8,75 | 10 | 11,25 | 12,5 |
| 10% APS (uL) | 50,00 | 100 | 150 | 200 | 250 | 300 | 350 | 400 | 450 | 500 |
| TEMED (uL) | 5,00 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 |
| #Gels (BioRad) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

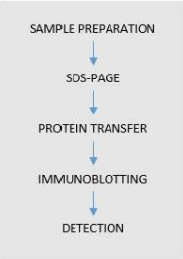
| **STACKING GEL** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total volume (mL) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| Stacking mix (mL) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 10% APS (uL) | 20 | 40 | 60 | 80 | 100 | 120 | 140 | 160 | 180 | 200 |
| TEMED (uL) | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 |
| #Gels (BioRad) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

# Extended Data 3. Western Blot protocol

*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

| PROTOCOL | Western-Blot |
|---|---|
| BASED ON | Novus Biologicals WB Handbook |
| STANDARDIZED (Y/N) | Y |

**WORKFLOW:**




**SOLUTIONS:**

| Name | Composition | Notes |
|---|---|---|
| Transfer buffer 1X (wet) | 25 mM Tris base<br>192 mM glycine<br>20% methanol<br>Adjust to pH to 8.3 | Available in the kitchen in 10X format. Methanol should be added after diluting to 1X. Recommended for small proteins |
| Transfer buffer 1X (semidry) | 48 mM Tris base<br>39 mM glycine<br>20% methanol<br>Adjust pH to 8.3 | Recommended for proteins above 100KDa |
| TBST 1X | 20 mM Tris base<br>150 mM NaCl<br>0.1% Tween 20 | TBS is available in the kitchen in 10X format. Tween should be added after diluting to 1X |
| Blocking solution | 5% Skim milk<br>TBST | Do not store the blocking solution for long to avoid contamination |
| Stripping solution 1 | 100 mM 2-mercaptoethanol<br>2% SDS<br>62.5 mM Tris-HCl<br>Adjust pH to 6.7 | For stripping by using the method with heat and detergent |
| Stripping solution 2 | 25 mM glycine-HCl<br>1% SDS<br>Adjust pH to 2 | For stripping with acid pH |

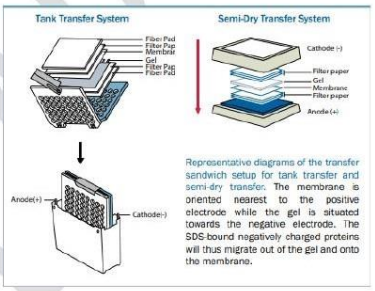
*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

**PROTEIN TRANSFER:**

1- Prepare PVDF membrane by wetting it in methanol for 30 seconds and then soaking it briefly in distilled water followed by 1X transfer buffer. Handle the membrane carefully, ideally with rounded tweezers to avoid scratching or puncturing the surface. Note: Do NOT wet nitrocellulose membranes with methanol or the membrane will dissolve.

2- Soak filter papers and sponges in the transfer buffer for 10 minutes prior to assembly of the transfer "sandwich"

3- Soak filter papers and sponges in the transfer buffer for 10 minutes prior to assembly of the transfer "sandwich". After electrophoresis, remove the gel from the electrophoresis apparatus and equilibrate it by soaking in transfer buffer for 10 minutes.



Representative diagrams of the transfer sandwich setup for tank transfer and semi-dry transfer. The membrane is oriented nearest to the positive electrode while the gel is situated towards the negative electrode. The SDS-bound negatively charged proteins will thus migrate out of the gel and onto the membrane.

4- Prepare the sandwich according to the illustration. Sequentially assemble the layers of the sandwich. Gently remove any air bubbles with a roller or pipette. Bubbles between the gel and the membrane will inhibit the transfer of proteins to the membrane.

5- Place the sandwich into a transfer cassette and perform semi-dry or wet transfer according to the manufacturer's instructions of the blotting apparatus. **Semi-dry transfer:** generally faster, better suited for larger proteins >100 kDa. Commonly used transfer time: 60 minutes at a constant current (1.25 mA/cm2) **Wet transfer:** recommended for smaller proteins. Commonly used transfer time: 60 minutes at a constant current (1.25 mA/cm2). **Tip:** Transfer time/voltage may require optimization. Over-transferring (or pulling protein all the way through the membrane) can occur and thus caution must be taken, especially for small proteins.

**IMMUNOBLOTTING:**

1- After transfer, rinse the membrane briefly in distilled water or 1X TBST.

2- *Ponceau staining:* If desired, stain the membrane with Ponceau red (a reversible protein stain) for 30 seconds to visualize protein bands to confirm that protein transfer was successful. Rinsing the membrane briefly with distilled water after Ponceau staining will reveal protein bands. Wash away Ponceau red with several washes in 1X TBST until membrane is clear. Additionally, Coomassie staining of the gel after transfer can help assure that proteins were completely transferred from the gel to the

membrane (minimal or no protein staining should be visible on a coomassie-stained gel after successful complete transfer).

3- Incubate membrane in blocking solution for 1 hour at room temperature or overnight at 4˚C with constant rocking.

4- Rinse the membrane for 5 mins in 1X TBST

5- Dilute the primary antibody to working concentration in 1X TBST with 5% milk or BSA (Whatever was chosen for blocking). **Note:** Typical working antibody dilutions range from 1:500 to 1:5000 or greater. Antibody specification sheets often contain suggested dilutions, but optimal dilutions may need to be determined experimentally. **Optional:** If using blue molecular weight markers, add 1 µg/mL Blue Marker Antibody to the primary antibody solution to visualize the molecular weight markers. This antibody does not cross react with protein lysates and will bind specifically to the blue dye of each molecular weight marker.

6- Incubate the membrane in primary antibody solution for 2-3 hours at room temperature or overnight at 4˚C with gentle rocking. Incubation time may require optimization. See antibody datasheet for recommended dilutions.

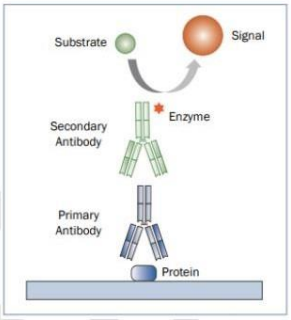
7- Wash the membrane with 1X TBST three times for 10 minutes each with gentle rocking. **Tip:** Increase the number of washes to 5-6 if high background occurs.

8- Incubate the membrane in the appropriate diluted secondary antibody (in 1X TBST and may include 1-5% milk or BSA) for 1 hour at room temperature with gentle rocking. **Note:** See primary antibody information sheet for proper secondary antibody selection- must recognize host species of the primary antibody. Secondary antibody concentration guidelines are listed in the product information sheet.

9- Wash the membrane in 1X TBST three times for 10 minutes each with gentle rocking. **Tip:** Do not let the membrane dry at any point during the blotting process.

**DETECTION:**

The method of detection is dependent on which enzyme is conjugated to the secondary antibody (or in some cases the primary antibody). The most commonly used enzyme is HRP. Once substrate has been added, it reacts with HRP and emits light. The emitted light can be detected using autoradiography film or a chemiluminescence imaging system. Fluorescence can be also used as detection method for those secondary antibodies conjugated to fluorescent dyes.




*Chemiluminiscence:*

1- Prepare the ECL substrate just prior to use according to the manufacturer's instructions.
2- Incubate the membrane in the substrate according to manufacturer's directions. Typical incubation times are 1-5 minutes. **Tip:** More sensitive substrates may require shorter incubation times or even dilution to achieve optimal signal and avoid overexposure.
3- Carefully remove the membrane from the detection reagent and sandwich it between layers of plastic (e.g. a sheet protector or plastic wrap).
4- Expose the membrane to autoradiography film in a dark room or image with a chemiluminescent imaging system. **Tips:** Clip the top right corner of your film as a guide for film orientation in a dark room. Use multiple exposure lengths to identify the most optimal exposure time.

**STRIPPING/RE-PROBING:**

A single blot can be analyzed sequentially with multiple antibodies by stripping one antibody from the blot and subsequently incubating with an additional antibody. This practice may be useful when sample is limited. Two methods are outlined below. The first method uses heat and detergent to release antibodies and the second uses low pH to inactivate the antigen binding site of the antibody.

| Method 1: Heat and detergent | Method 2: Acid pH |
|---|---|
| 1- In a fume hood, agitate the blot in stripping solution #1 for 30 minutes at 50˚C | 1- Agitate the blot in stripping solution #2 for 30 minutes at room temperature. |
| 2- Agitate the blot in 1X PBS for 10 minutes at room temperature. Repeat with fresh buffer. | 2- Agitate the blot in 1X PBS for 10 minutes at room temperature. Repeat with fresh buffer. |
| 3- Proceed to the blocking step of the immunoblotting protocol to re-probe the blot with a second antibody. | 3- Proceed to the blocking step of the immunoblotting protocol to re-probe the blot with a second antibody. |

*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

**BLOT STORAGE:**

PVDF is a chemically resistant polymer that has excellent long term stability. Sometimes it might be desirable to store a blot for future use, such as for stripping and re-probing.

1- Sandwich the dry PVDF blot between two clean sheets of Whatman 3MM paper
2- Place the sandwich between two sheets of card stock or thin cardboard.
3- Use paperclips to clip the stack together on the edges.
4- Place the stack in a plastic bag and seal the plastic bag closed
5- Store the blot at 4˚C for up to 2 weeks, -20˚C for up to 2 months, or -70˚C for more than 2 months.

Alternatively, the membrane could be stored in sealed plastic bags in dark conditions.

**Tip:** Thaw frozen blots to room temperature before removal from the plastic bag because frozen blots are prone to breakage. **Tip:** Blots can also be stored wet at 4˚C, but sodium azide should be added to prevent bacterial growth. As sodium azide inhibits HRP activity, it should be thoroughly washed out of a blot prior to use.

**NOTES ABOUT PROPER CONTROLS:**

| CONTROL SAMPLE | WHAT IS?/WHAT IS FOR?... | COMMONLY USED SAMPLES |
|---|---|---|
| Positive control | A positive control lysate is from a cell line or tissue sample that is known to express the protein of interest. This control will yield a positive band on the western blot, even if the test samples are negative for the protein of interest. This control is important to ensure that there were no issues in the western blotting protocol. It will also verify that any negative results are indeed negative. If the positive control lysate does not result in a positive signal, the western blotting protocol requires optimization. | - Samples from cells exhibiting overexpression of target protein<br>- Cell line/tissue/experimental condition with proven positive signal<br>- Purified recombinant protein |
| Negative control | A sample known to not contain the target protein. This control is important for determining whether non-specific binding (false positive result) has occurred in the western blotting procedure. | - Samples from knockdown or knockout tissue/cell lines<br>- Samples from RNA interference targeted lines<br>- Cell line/tissue/experimental condition with proven negative signal |
| Endogenous control | If testing a sample of recombinant protein, a positive endogenous control lysate known to express the target of interest is recommended. This control will help ensure that the western blotting protocol is working and indicate whether there might be an issue with the recombinant protein. | A cell lysate from a cell line/tissue with known expression of the target protein |

| | | |
|---|---|---|
| Loading control | Required for the semi-quantification of protein levels between wells. Expression of loading control protein must be equal across all wells to confirm that observed changes in target protein expression are true. Equal expression of loading controls confirms the samples have been equally loaded and protein has been evenly transferred from gel to membrane.<br>Considerations for choosing a loading control:<br>- **Detection size:** Choose a loading control that can be distinguished in MW from the target protein of interest.<br>- **Expression level:** Choose a loading control that is highly expressed in your sample. Common loading controls are highly expressed genes required for basic cellular processes and vitality, also known as housekeeping genes.<br>- **Expression consistency:** Choose a loading control that is ubiquitously and constitutively expressed. The expression should be unchanged throughout an experiment, regardless of experimental treatment, cell type, tissue type, etc. | - Depending on the kind of sample, cellular compartment etc…see table below |

| Common Loading Controls | Target | MW (kDa) |
|---|---|---|
| Cytoplasm/Whole Cell | Alpha-tubulin | 55 |
| | Beta-actin | 43 |
| | GAPDH | 37 |
| | Cyclophilin B | 21 |
| Mitochondria | HSP60 | 60 |
| | COX IV | 17 |
| Nucleus | Lamin B1 | 66 |
| | HDAC | 60 |
| | PCNA | 29 |
| | Histone H3 | 17 |
| Serum | Transferrin | 77 |

| Target Protein | Notes |
|---|---|
| Beta-actin | Not suitable for nuclear extract as beta-actin is a component of chromatin remodeling complexes. May not be suitable for studies involving subjects with a large age difference. |
| GAPDH | Not suitable for oxygen-related studies as hypoxia can upregulate GAPDH expression. May not be suitable for studies involving subjects with a large age difference. |
| Alpha-tubulin | May not be suitable for studies involving subjects with a large age difference. Tubulin expression can be affected by anti-cancer and anti-fungal drugs. |
| Lamin B1 | Not suitable for embryonic stem cells. |
| PCNA | Not suitable for serving as a loading control for non-proliferating cells. |
| COX IV | Many proteins run at around 15~17 kDa; hence, it may be necessary to consider an alternative control antibody if your protein of interest is similar in size to COX IV. |
| Transferrin | Transferrin levels can be influenced by some disease states and treatments such as retinoic acid. |
| Histone H3 | Many proteins run at around 15~17 kDa; hence, it may be necessary to consider an alternative control antibody if your protein of interest is similar in size to Histone H3. |

*SDELCI LAB´S PROTOCOL. CRG, Barcelona*

## PROTEIN EXTRACTION TIPS:

Recommended lysis buffers based on cellular location of protein of interest

| Subcellular Location | Recommended Buffer |
|---|---|
| Whole Cell Lysate | NP-40 |
| Nucleus | RIPA or nuclear fractionation for increased protein of interest concentration |
| Mitochondria | RIPA or mitochondrial fractionation for increased protein of interest concentration |
| Cytoplasm | Tris-HCl |
| Membrane-Bound Proteins | RIPA (SDS is generally consicered harsh and thus is often well-suited for difficult to solubilize proteins) |

Lysis buffer recipes

| NP-40 | RIPA | Tris-HCl |
|---|---|---|
| 150 mM NaCl<br>1% NP-40 or Triton X-100<br>50 mM Tris pH 8.0 | 150 mM NaCl<br>1% NP-40 or Triton X-100<br>0.5% sodium deoxycholate<br>0.1% SDS<br>50 mM Tris, pH 8.0 | 20 mM Tris-Hcl, pH 7.5 |

Common protease and phosphatase inhibitors

| Inhibitor | Target | Final Concentration |
|---|---|---|
| Aprotinin | Trypsin, chymotrypsin, plasmin | 2 µg/mL |
| Leupeptin | Lysosomal, tripsin, papain | 1-10 µg/mL |
| Pepstatin A | Aspartic proteases | 1 µg/mL |
| PMSF | Serine and cysteine proteases | 1 mM |
| EDTA | $Mg^{2+}$ and $Mn^{2+}$ metalloproteases | 1-5 mM |
| EGTA | $Ca^{2+}$ metalloproteases | 1 mM |
| Sodium fluoride | Serine & threonine phosphatases | 5-10 mM |
| Orthovanadate | Tyrosine phosphatases | 1 mM |
| Pyrophosphate | Serine & threonine phosphatases | 1-2 mM |
| B-glycerophosphate | Serine & threonine phosphatases | 1-2 mM |

## TROUBLESHOOTING:

| Detected problem | Possible causes | Solutions |
|---|---|---|
| No Signal or Weak Signal | Primary antibody concentration is too low | .- Increase the concentration of the primary antibody<br>.- Increase the incubation time to 4°C overnight<br>.- If re-used too many times, the effective antibody concentration may be too low; use fresh antibody to improve signal |
| | Target protein concentration is too low | .- Load more protein per well<br>.- Use a positive control control lysate known to express the target protein, an overexpression lysate, or a recombinant protein<br>.- Ensure lysis buffer is optimal for localization of target protein<br>.- Use immunoprecipitation or fractionation (i.e. nuclear fractionation) if necessary to increase the concentration of a non-abundant protein<br>.- Include protease inhibitors in the lysis buffer<br>.- Ensure the sample has not degraded |
| | Protein transfer from gel to membrane was unsuccessful | .- Confirm that proteins were successfully transferred to the membrane by Ponceau S staining of the membrane<br>.- Confirm that proteins were completely transferred by coomassie staining of the gel<br>.- Confirm equal transfer by analyzing loading control expression |
| | Primary and secondary antibody are not compatible | .- Ensure that secondary antibody was raised against the species in which the primary was raised (e.g. if primary was raised in mouse, use an anti-mouse secondary) |
| | Membrane choice was not ideal | .- Check the hydrophobicity/hydrophilicity of the antigen sequence<br>- PVDF membrane may work better for hydrophilic/polar/charged antigens<br>- Nitrocellulose may work better for hydrophobic/non-polar antigens |

| | | |
|---|---|---|
| | There are issues with blocking | .- Blocking for too long can mask certain epitopes and inhibit antibody binding<br>.- Reduce blocking time<br>.- Reduce concentration of blocking solution<br>.- Reduce percentage or remove blocking reagent from antibody incubation buffers<br>.- Switch to a different blocking solution |
| | Excessive washing of membrane | .- Detection reagent issues<br>.- Detection reagents can become inactive over time-<br>- Ensure reagents are fresh - Test by dot blotting secondary onto membrane and incubating with detection reagent<br>.- Use more sensitive reagents when working with low abundance proteins |
| | Image exposure was too short | Increase exposure time (check several times to achieve optimal exposure time) |
| | Antibody only recognizes native proteins | Do not use reduced, denatured proteins if working with an antibody that only recognizes native proteins |
| | Targets are low molecular weight | Reduce transfer time to prevent over transfer<br>- Use membranes with smaller pore size (0.2 µm vs. 0.45 µm)<br>- Wet transfer is recommended for small proteins |
| | Sodium azide contamination has occurred | Sodium azide (often used to store primary antibodies) inhibits HRP activity<br>- Ensure sufficient washing to remove presence of sodium azide<br>- Use sodium azide-free buffers |
| **High Uniform Background** | Insufficient blocking | - Increase blocking time and/or temperature<br>- Increase the concentration of blocking reagent (try up to 10%)<br>- Consider changing the blocking agent (milk vs. BSA)<br>-Include the optional blocking agents in antibody buffers (can also increase %) |
| | Blocking not compatible | - For phosphorylated protein detection, milk should not be used (milk and casein are phospho-protein rich)<br>- If your secondary is anti-bovine, anti-goat, or anti-sheep, use 5% serum from the host species of the secondary antibody as a blocking agent |
| | Non-specific binding due to high antibody concentration | - Lower concentration of primary or secondary (titrations may be helpful)<br>- Include blocking agents in antibody buffers<br>- Confirm the secondary is specific by omitting the primary and performing a secondary antibody only control blot |
| | Insufficient washing of unbound antibodies | Increase the number and/or time of washes |
| | Dry membrane | Make sure the membrane never becomes dry during the western blotting protocol |
| **Non-specific bands/wrong size or multiple bands** | Target protein is less abundant than the threshold of non-specific binding | - Load more protein in the SDS-PAGE gel<br>- Enrich low-abundance proteins by immunoprecipitation or fractionation |
| | Sample degradation | - Use fresh lysates<br>- Keep sample on ice until just before sample buffer addition and boiling<br>- Always include protease inhibitors and phosphatase inhibitors if detecting phosphorylated target |
| | Other protein isoforms may be present | - Alternative splicing, multimer formation, etc.<br>- May need an isoform-specific antibody |
| | Post-translational modifications may be present | - Predicted molecular weight can be influenced by many factors such as glycosylation, phosphorylation, protein processing (cleavage from a pro-form to a mature form)<br>- To confirm specificity, perform positive and negative controls such as recombinant protein or overexpression lysate, downregulated knockdown/knockout lysate |

| | | |
|---|---|---|
| Speckled or Swirled Background | Membrane mishandling | Minimize contact with membrane. Use clean tools to handle the membrane |
| | Air bubbles | Roll out any bubbles between the gel and membrane before transfer |
| | Insufficient washing | - Increase the volume of the washing buffer<br>- Increase the number and/or duration of the washes |
| Other Issues | White/hollow bands | Decrease the concentration of primary/secondary antibody or use less protein |
| | Smeared bands/lanes (sample overloading) | Load less protein into each lane |
| | "Smiling" bands | - Migration was too fast; decrease the voltage while running the gel<br>- Migration was too hot; run the gel in the cold room |
| | Molecular weight marker lane is black | - The antibody may react with the molecular weight marker<br>- Add a blank lane between the molecular weight marker and the first sample lane |