



UNIVERSITAT DE VIC  
UNIVERSITAT CENTRAL  
DE CATALUNYA



## **FINAL THESIS**

### **Proteome and phospho-proteome study of Molm-13 cell line in Acute Myeloid Leukemia (AML)**

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Biotechnology Degree

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Vic, January 2016

## **Acknowledgments**

There are many people who have contributed to my final career thesis. First of all, I would like to thank my advisor at Vic University, Eliandre de Oliveira Cacheado. I could always mail her or go to her office to solve any doubt I had.

Thank you to Professor Frode Berven, the director of PROBE who trusted in me to let me carry my thesis in his laboratory. He gave me the chance to attend the Bioinformatics for proteomics course that PROBE organized where I have acquired a lot of bioinformatics knowledge and I started to use new programs. I am also thankful to my advisors during my stay in PROBE, Professor Frode Selheim and Maria Hernandez, thank you for all the time you put into teaching and helping me. I could always talk to them for anything that happened to me and I learned a lot from them and their work. I always received support to keep working and pushed myself to achieve better results in my experiments. I am thankful for the patience and the time Maria spent on teaching me new techniques and I can say that it is because of her dedication that I know how to properly work in a laboratory. Thank you to Olav Mjaavatten, an engineer in PROBE and the main responsible for the maintenance and running of the mass spectrometers. He always helped me when I had to inject my samples to the mass spectrometer and analyze the results. Thank you Nina, who is responsible of the cell culture laboratory and always willing to help me. I always had space available to carry my experiments.

I am grateful for the availability and kindness of my teammates in Norway. I had a positive experience and it had strongly enriched my studies and will be very useful to push further my work as a student and future professional.

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## **1. Abbreviations**

ACN – Acetonitrile

AML- Acute Myeloid Leukemia

APAF-1- Apoptosis Protease Activating Factor 1

BAK1- Bcl-2 antagonist/killer 1

BCA- Bicinchoninic Acid Assay

Bcl- B- Cell Lymphoma

CARD- Caspase Recruitment Domain

CI- Chemical Ionization

CID- Collision Induced Dissociation

CML- Chronic Myeloid Leukemia

Da- Dalton

DNA- Deoxyribonucleic Acid

EI- Electron Ionization

ER- Endoplasmatic Reticulum

ERK- Extracellular Signal- regulated Kinases

ESI- Electrospray Ionization

FA- Formic Acid

FAB- Fast Atom Bombardment

FASP-Filter Aided Sample Preparation

FASP-DD- Filter Aided Sample Preparation- Double Digestion

FLI1- Friend Leukemia Integration 1

FLT3- receptor Fms-Like Tyrosine kinase 3

FLT3-L- ligand Fms-Like Tyrosine kinase 3

GndCl- Guanidium chloride

IMAC- Immobilized Metal Affinity Chromatography

ISD- In Solution Digestion

ITD- Internal Tandem Duplication

Lys-C- endoproteinase Lysine C

MALDI- Matrix Assisted Laser Desorption Ionization

MAPK- Mitogen Activated Protein Kinase  
MS- Mass Spectrometry  
NK-Natural Killer  
NF- $\kappa$ B- Nuclear Factor kappaB  
OA- Okadaic Acid  
PCR- Polymerase Chain Reaction  
PI3K- Phosphoinositide 3-Kinase  
PP1- Protein Phosphatase 1  
PP2- Protein Phosphatase 2  
PTM- Post Translational Modification  
RF- Radio Frequency  
SAX- Strong Anion Exchange  
SCX- Strong Cation Exchange  
SDS- Sodium Dodecyl Sulfate  
SDS-RPS- Mix Moded  
STAT5- Signal Transducer and Activator of Transcription 5  
STK38- Serine/Threonine Kinase 38  
TCEP- Tris(2-Carboxyethyl)phosphine  
TFA- Trifluoroacetic Acid  
TiO<sub>2</sub>- Titanium Dioxide  
TNF- Tumor Necrosis Factor  
TNFAIP3- Tumor Necrosis Factor Alpha Induced Protein 3  
TNFAIP8- Tumor Necrosis Factor Alpha Induced Protein 8  
Trp- Endoproteinase Trypsin  
VRK3- Inactive Serine/Threonine protein kinase  
WT-Wild Type

## 2. Summary Final Thesis

### Biotechnology Degree

Title: Proteome and phospho-proteome study of Molm-13 cell line in Acute Myeloid Leukemia (AML)

Key words: Acute Myeloid Leukemia, FLT3 ligand, Okadaic Acid, FASP and  $TiO_2$

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Date: 13 January 2016

The main goal of this thesis was to study the changes in the proteome of Molm-13 cells from Acute Myeloid Leukemia when treated with a proliferation and cell differentiation inductor (FLT3 ligand) and with an apoptosis inductor (Okadaic Acid, OA, FLT3 ligand binds to FLT3 receptor which auto phosphorylates inducing cell proliferation and differentiation. FLT3 receptor is commonly mutated in AML, this mutation is named FLT3-ITD. Signaling pathways like RAS/MAP kinase, AKT/PI-3 kinase and JAK/STAT are activated depending on ITD localization.

Some other cells were treated with Okadaic Acid, a toxin and a potent inhibitor of the Serine/ Threonine (Ser/Thr) protein phosphatases 1 and 2, which blocks the activation of ERK5 a protein present in the MAP kinase pathway.

To perform proteomics analysis, protein content of each sample was digested by Filter Aided Sample Preparation Double Digestion method and a peptide aliquot was enriched by Titanium Dioxide to get phosphorylated peptides. Samples were analysed by mass spectrometry with the Orbitrap Elite.

More than 3000 proteins were identified in the different samples analysed. Bcl-6, Bcl-2, STAT and TNFAIP8 are important proteins which contribute to tumor cell progression and inhibit the apoptosis process by inhibiting caspases function. These mentioned proteins were found in Wild type (untreated cells) and FLT3- ligand induced cells. Whereas in Okadaic Acid treated cells different proteins were expressed as apoptotic inducer, these proteins are p53 and TNF along more proteins. FLT3- ITD mutation is probably localized in the Endoplasmatic Reticulum (ER).

### 3. Introduction

#### 3.1 Acute Myeloid Leukemia (AML)

Leukemias are cancers that start in cells that would normally develop into different types of blood cells. Acute Myeloid Leukemia (AML) is the most common type of leukemia and relatively rare disease occurring in approximately 10,500 cases per year in United States. Cells from relapsed patients of AML were used to carry out this project. AML starts in the bone marrow and it quickly moves into the blood. “Acute” means that this leukemia can progress quickly if not treated, and would probably be fatal in a few months. “Myeloid” refers to the type of cells this leukemia starts from.

Two types of mutations are needed to develop leukemia, process known as leukemogenesis: the mutations belonging to class I and class II. Class I mutations promote survival and proliferation of the cells, e.i FLT3 and c-KIT protein receptors. The second class of mutations impairs transcription factor that are essential for the regulation of normal myeloid development, e.i translocations of the genes targeting myeloid differentiation. Occurrence of class II mutations results in aberrant gene expression and abnormal development of hematopoietic cells. This is known as “two-hit” model with presence of both fusion genes and activating mutation of tyrosine kinases.

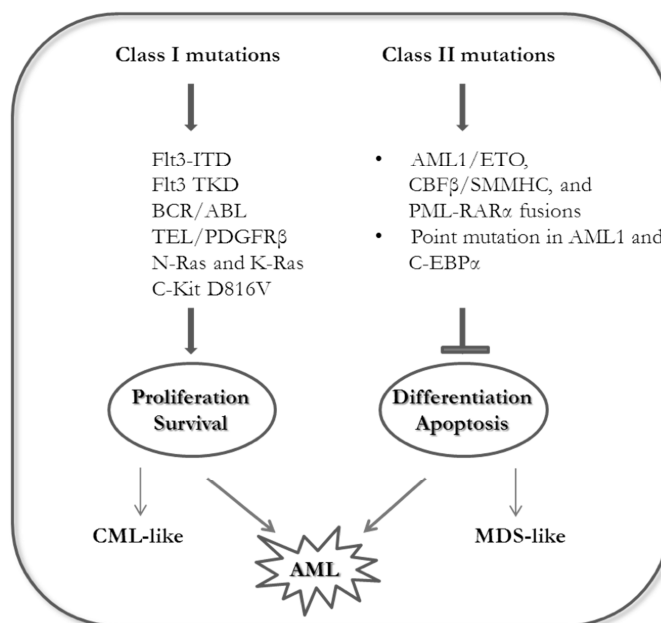


Figure 1: The two-hit-model of leukemogenesis.



Scientists are making progress in understanding how changes in the DNA in normal bone marrow cells can cause them to develop into leukemia cells. As researchers have found different mutations as chromosomal translocations or other changes that often occur in AML, it is becoming clear that there are many types of AML. This progress in understanding the DNA changes in AML cells has provided highly sensitive tests for detecting the smallest amount of leukemia cells left after treatment. The polymerase chain reaction (PCR) is a technique in molecular biology used to amplify a single copy of DNA generating thousands of copies of a particular DNA sequence. This test looks for certain changes in the structure or function of genes and can identify even very small numbers of AML cells after treatment in a sample based on their gene translocation. Although genetic analysis has made a lot of progress to understand AML, proteomics analysis has identified for the first time novel proteins that may either help to form a differential prognosis or be used as biological markers, providing potential new targets for therapies of AML.

### 3.2 FLT3 receptor, ligand and downstream pathways

FLT-3 gene is located in chromosome 13 and it encodes for a tyrosine kinase receptor class III. This receptor is composed of five extracellular immunoglobulin domains, a transmembrane domain, a juxtamembrane domain and a tyrosine-kinase domain consisting of two lobes that are connected by a tyrosine-kinase insert.

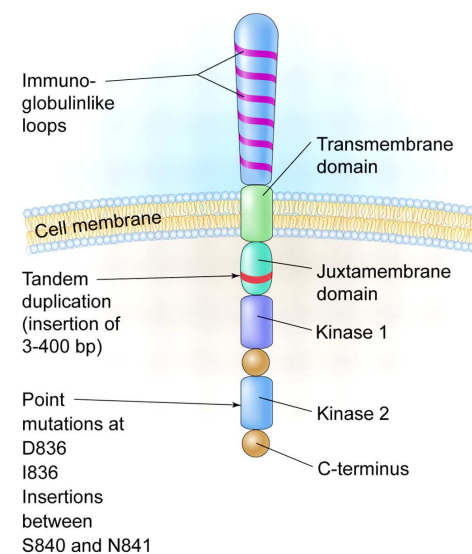


Figure 2: Structure of FLT3 receptor

FLT3 receptor is activated by FLT3 ligand (FL). The ligand binding leads to receptor dimerization and activation via auto-phosphorylation of multiple tyrosine kinases. The activated receptor recruits a number of protein, e.g Src, SHC and GRB2 to form a complex of protein-protein interaction, which lead to activation of downstream pathways including, PI3K, AKT, ERK 1/2 and ERK5.

When receptor gets activated it undergoes conformational changes leading to activation of its intrinsic kinase activity, which leads to phosphorylation of signaling molecules leading to different cell responses like survival, differentiation, proliferation and apoptosis. FLT3 is involved in several main signaling pathways like RAS/MAP kinase, AKT/PI-3 kinase and JAK/ STAT, resulting in various cellular responses such as cell growth, proliferation, differentiation and apoptosis.

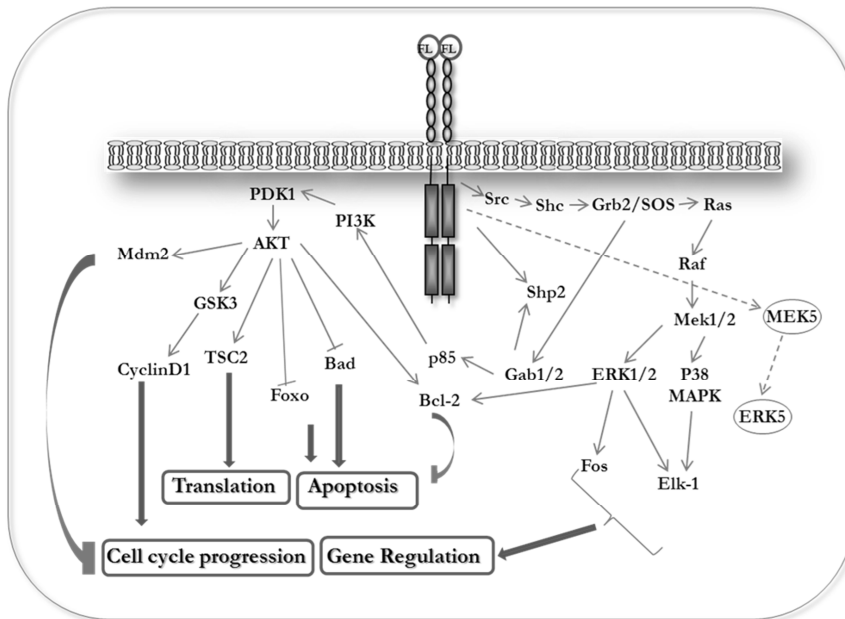


Figure 3: The main signal transduction pathways of wild-type FLTR3 receptor.

### 3.3 FLT3 and Acute Myeloid Leukemia

The FLT3 gene is one of the most commonly mutated genes in AML and the most common alteration in FLT3 is the internal tandem duplication (ITD). ITDs are in-frame mutations caused by duplication of various in length fragments encoding the JM domain of FLT3 receptor (encoded by exon 14 and 15). The length of ITD insert can influence the clinical outcome in AML patients.

Different size of ITDs leads to variation in the length duplications of specific amino acid residues, within JM domain, which are involved in receptor activation. The cause of ITD mutation is not known but the duplication can result from a failure in mismatch repair mechanism during the replication of DNA. FLT3-ITD mutation correlates with a poor prognosis and higher risk of relapse of AML patients.

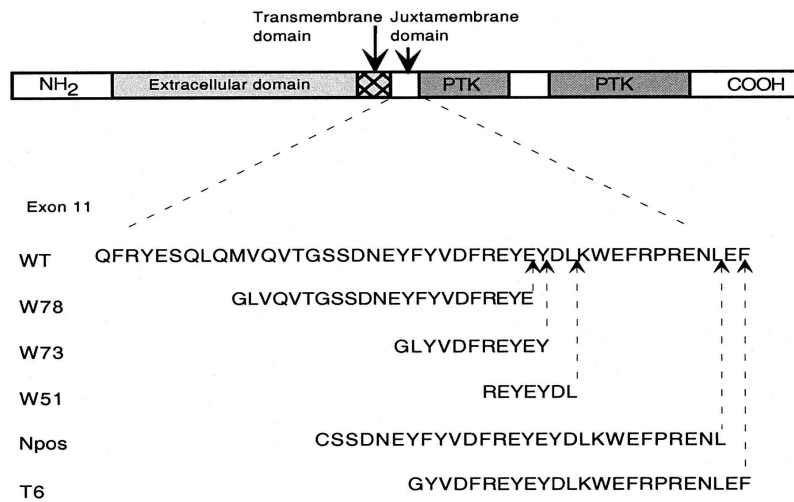


Figure 4: Schematic diagram of the FLT3 receptor tyrosine kinase.

In the figure 4 is represented the amino acid sequence of exon 11, which encodes the juxtamembrane sequence, is shown below. WT is the wild- type sequence and W78, W73, W51, Npos and T6 are the FLT3-ITD mutations where the duplicated residues are shown, together with an arrow indicating the duplication position with respect to wild-type exon 11.

Depending on ITD localization in the FLT3 receptor different signaling pathways can be activated. It has been shown that FLT3-ITD can activate STAT3 and STAT5 but not PIR3K and MAPK when localized at the endoplasmic reticulum (ER). On the contrary,

FLT3-ITD localized in the membrane has been found to strongly activate PI3K and MAPK pathways but fails to activate STAT5. Depending on ITD localization in the FLT3 receptor different signaling pathways can be activated. It has been shown that FLT3-ITD can activate STAT3 and STAT5 but not PI3K and MAPK when localized at the endoplasmic reticulum (ER). On the contrary, FLT3-ITD localized in the membrane has been found to strongly activate PI3K and MAPK pathways but fails to activate STAT5. The worse outcome of AML patients carrying FLT3-ITD expressing cells might be due to STAT5 activation that causes anti-apoptotic signaling in these cells. (Elena Razumovskaya 3)

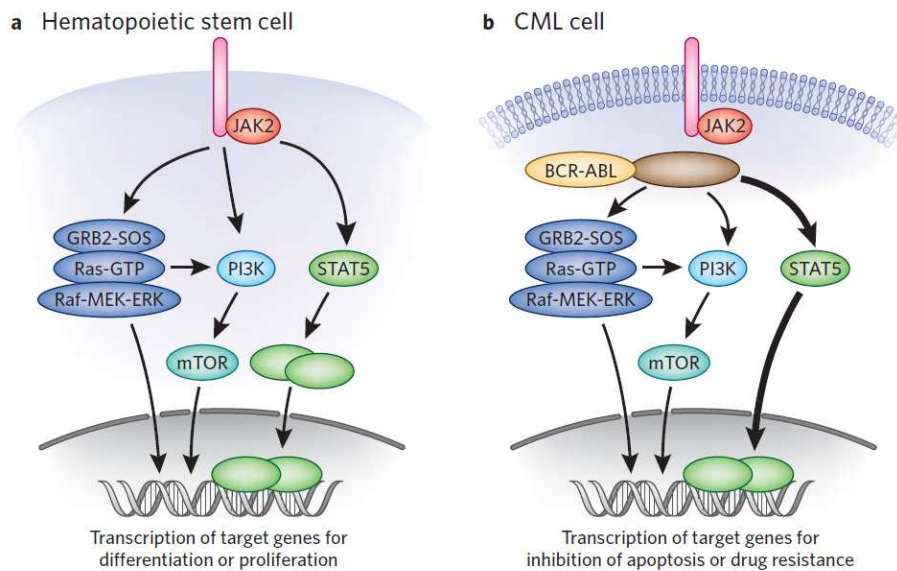


Figure 5: MAPK pathway in an Hematopoietic stem cell and in a Chronic Myeloid Leukemia cell (CML).

STAT5 protein is activated in CML cells causing the transcription of genes for inhibition of apoptosis. In the figure 5 is also exposed that in Hematopoietic cells STAT5 causes transcription of genes for differentiation or proliferation.

### 3.4 Okadaic acid (OA)

Okadaic acid,  $C_{44}H_{68}O_{13}$ , is a toxin produced by species of dinoflagellates. It is a potent inhibitor of the Serine/Threonine (Ser/Thr) protein phosphatases 1 and 2 (PP1 and PP2), which blocks the activation of extracellular-signal-regulated protein kinase 5 (ERK5), a member of the MAP kinase family activated by growth factors. Protein Ser/Thr phosphatase is a phosphoprotein phosphatase that acts upon the phosphate group found in serine or threonine residues of a wide range of phosphoproteins including enzyme that are phosphorylated by a kinase.

Reversible phosphorylation is a very important mechanism of signal transduction in eukaryotic cells and is mediated by a series of kinases and phosphatases. Disruption of the regulation of protein phosphorylation can lead to altered cellular behavior and result in diseases including cancer.

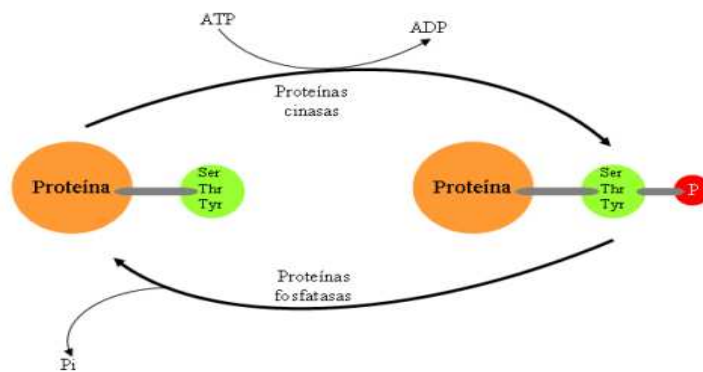


Figure 6: Reversible phosphorylation.

Extracellular signal- regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family and has the Thr-X-Tyr (TXY) activation motif. ERK5 is activated by growth factors and have an important role in the regulation of cell proliferation and differentiation.

## b ERK5 Structure

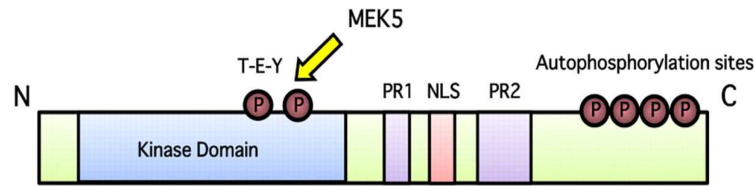


Figure 7: Schematic structure of Extracellular signal- regulated kinase 5 (ERK5).

### 3.5 Effect of OA in cells and apoptosis process

Cells react to OA treatment starting an apoptotic process. Apoptosis is the process of programmed cell death that occurs in multicellular organisms. Cells experiment morphology changes and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. These changes produce cell fragments called apoptotic bodies.

In addition to its importance as biological phenomenon, defective apoptotic processes have been implicated in a wide variety of diseases. Excessive apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer. The intracellular machinery responsible for apoptosis depends on a family of proteases that have a cysteine at their active site and cleave their target proteins at specific aspartic acids. They are therefore called caspases. They are synthesized in the cell as inactive precursors, which are usually activated by cleavage at aspartic acids, by other caspases. Once activated, caspases cleave, and thereby activate other procaspases resulting in amplifying proteolytic cascade.

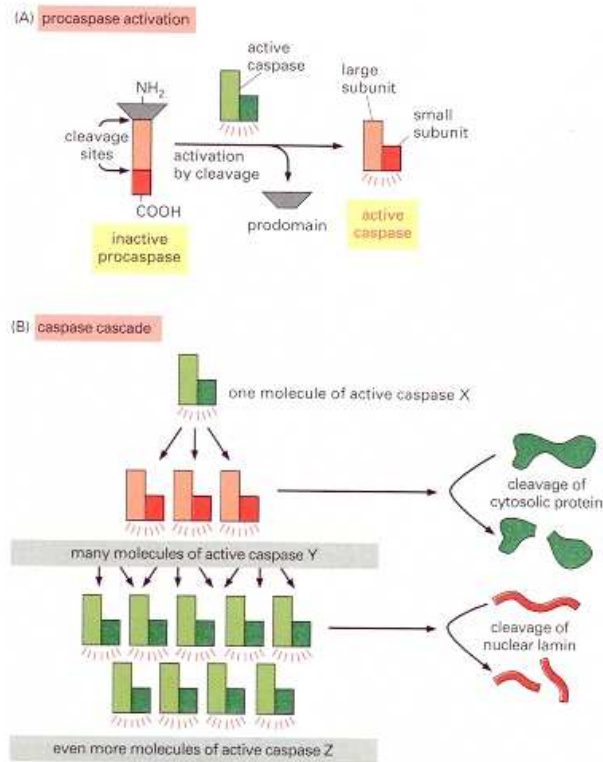


Figure 8: This illustration explains the process to activate a caspase and the cascade it begins.

### 3.6 Proteomics and AML

Proteomics is the large-scale study of proteins, particularly their structure and functions. Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. The proteome is the entire set of proteins produce by an organism.

This varies with time and distinct requirements, or stresses, that a cell or organism undergoes. Genes are first transcript to mRNA and then traduced to protein. Now, it is known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depend on the gene it is transcribed from an on the current physiological state of the cell. Proteomics confirms the presence of the protein and provides a direct measure of the quantity present. Many proteins are also subject to a wide variety of chemical modifications after translation. Many of these post-translational modifications (PTMs), such as phosphorylations, are critical to the protein's function.

To perform the proteomic approaches Molm-13 cell line established from the peripheral

blood of a patient at relapse of acute myeloid leukemia were used. Molm-13 and Molm-14 carry trisomy in chromosome 8, which is also present during myelodysplastic syndrome (MDS). MDS are a group of disorders caused by poorly formed or dysfunctional blood cells.

The project was carried out in the University of Bergen, Norway. The Proteomics Unit (PROBE) is a national core facility and performs large scale protein analysis. The proteomics research group is full equipped with a wide range of proteomics tools, from the identification of proteins present in a sample to comparison of protein expression profiles between different samples.

### 3.7 Mass Spectrometry

Mass spectrometry (MS) is an essential technique in proteomics due to its high capacity of analysis, sensitivity and precision in the determination of molecular masses. MS is an important analytical technique that helps identify the amount and type of chemicals present in a sample by measuring the mass to charge ratio. A mass spectrum is a plot of the ion signal as a function of the mass to charge ratio. The spectra are used to determine the masses of particles and molecules, and to elucidate the chemical structures of molecules. such as peptides and other chemical compounds.

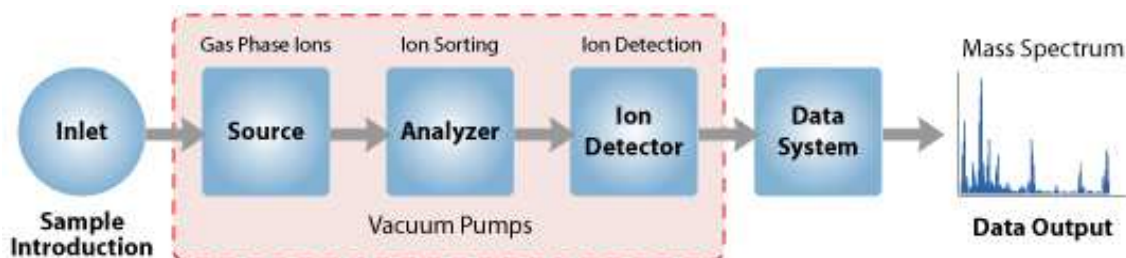


Figure 9: Scheme of a mass spectrometer.

The ion source is the part of the mass spectrometer that ionizes the analyte. A magnetic or electric field transport ions to the mass analyzer. There are different ion sources and the selection is based on the physic-chemical properties of the analyte and the energy transferred in the ionization process. Electron and chemical ionization are used for gases and vapor. In chemical ionization sources, the analyte is ionized by chemical ion-molecule reaction during collisions in the source. Two techniques usually used in proteome samples are electrospray ionization (ESI) and matrix- assisted laser ionization.



### 3.7.1 Ionization sources

Hard ionization techniques are processes that impart high quantities of residual energy in the subject molecule invoking large degrees of fragmentation. Resultant ions tend to have mass/charge ratio ( $m/z$ ) lower than the molecular mass. The most common example of hard ionization is electron ionization (EI).

Soft ionization refers to the processes that impart little residual energy onto the subject molecule and as such result in little fragmentation. Some examples are fast atom bombardment (FAB), chemical ionization (CI), electrospray ionization (ESI) and matrix-assisted laser ionization (MALDI).

Electrospray ionization is a technique used in mass spectrometry to produce ions using an electrospray in which a high voltage is applied to a liquid to create an aerosol. It is especially useful in producing ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionized. ESI is different than other atmospheric pressure ionization processes since it may produce multiply charged ions, extending the mass range of the analyzer. A capillary needle is the inlet into the apparatus for the liquid sample. Once in the capillary needle, the liquid sample is nebulized and charged. There is a large amount of pressure being applied to the capillary needle, which in effect nebulizes the liquid sample forming a mist. The positive applied voltage will place a charge on the droplets. Therefore, the mist that is ejected from the needle will be comprised of charged molecular ions.

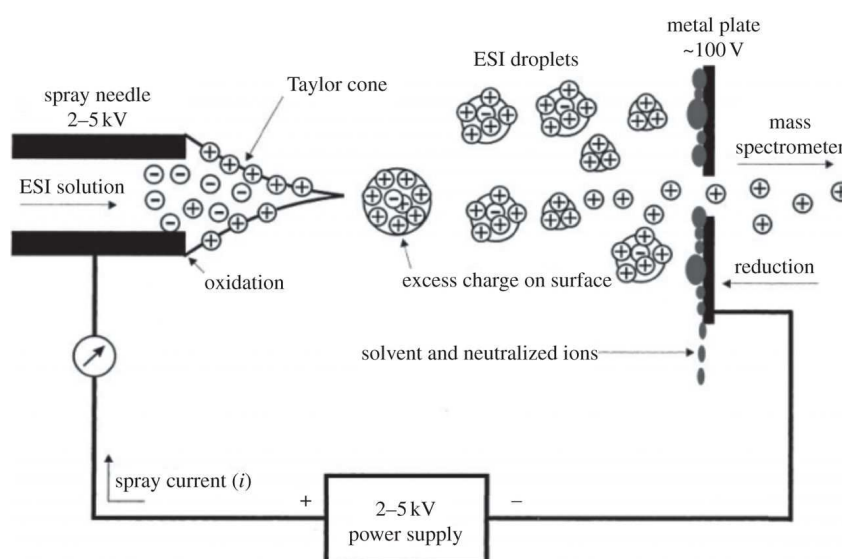


Figure 10: Scheme of Electrospray Ionization (ESI)

MALDI methodology is a two-step process. First, the peptides are dissolved in an acidic aqueous solution and mixed with an organic solution that absorbs laser irradiation, known as matrix. Matrix structure depends on its application.

After the vaporization of the solvents, the matrix is crystallized and the analyte is retained in the crystal. The result is a solid where the molecule of the analyte are isolated from each other and completely surrounded by molecules from the matrix. In the second step the solid is irradiated by intense pulses of laser, usually the laser is  $N_2$  and the wavelength is 337nm. The heat released causes the sublimation of the matrix molecules to vapor phase, which surrounds the analyte molecules. From this ionization technique mono-charged ions are obtained. The molecular mass observed corresponds to the molecular mass of the compound plus 1 Dalton (Da), in the case the ions are positively charged.

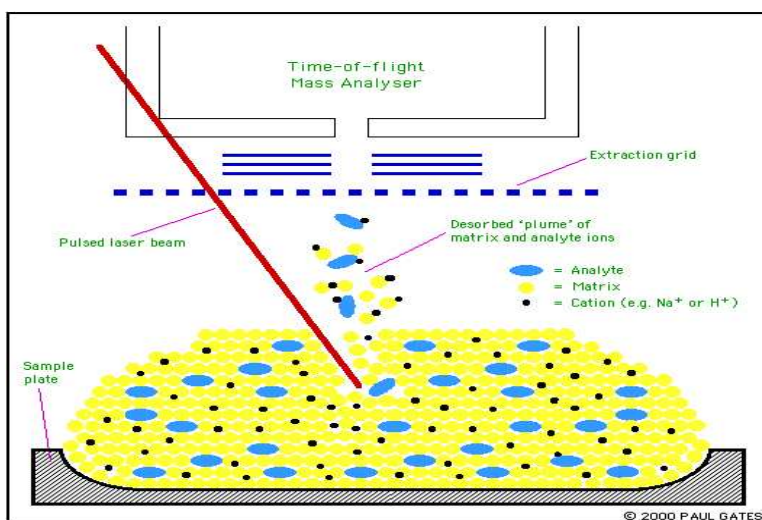


Figure 11: Structure of MALDI matrix

### 3.7.2 Mass analyzer

Once the ions are obtained, these are accelerated to the analyzer where are separated according their  $m/z$  ratio. Mass analyzer uses an electric and/or magnetic field to affect the path and/or velocity of the charged particles. There are different analyzers and the analyzer determines some important characteristics of the experiment as the resolution and the  $m/z$  range.

Quadrupole mass filter uses oscillating electrical fields to selectively stabilize or destabilize the paths of ions through a radio frequency (RF) quadrupole field created

between 4 parallel rods. Only the ions in a certain range of  $m/z$  ratio are passed through the system, but changes to the potentials on the rods allow a wide range of  $m/z$  values.

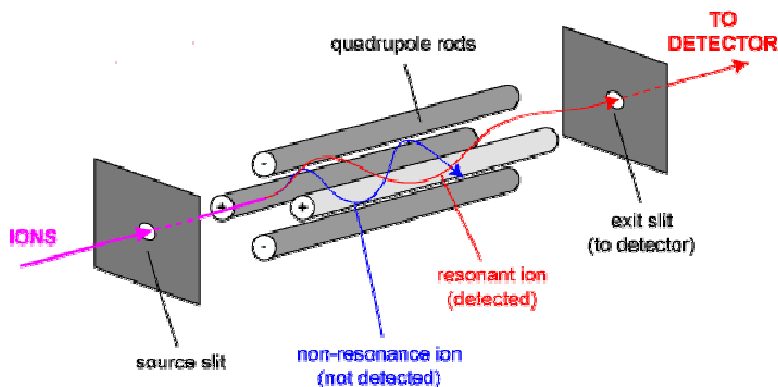


Figure 12: Quadrupole mass structure

#### Ion trap:

Ions generated in the ionization source are attracted to the capillary due to the application of an electrical potential and are quickly focused into the analyzer through the field created by two octapoles placed in tandem. The entrance of the ions is regulated by changes in the polarity of the entrance lens. This entrance of ions by pulse differ the ion trap from the quadrupole where ions continually enter. The time that the ions remained in the trap is known as ionization period. Ions overload induce distortions in the electric field resulting in an efficiency decrease.

As in the quadrupole, the ion trap is also an ion filter. The  $m/z$  ratio is selected and isolated through the application of different RF. The final element of the mass spectrometer is the detector. The detector records either the charge induced or the current produced when an ion passes by. In a scanning instrument, the signal produced in the detector will produce a mass spectrum, a record of ions as a function of  $m/z$ . The sensitivity, accuracy and response time are important criteria that distinguish different detection systems.

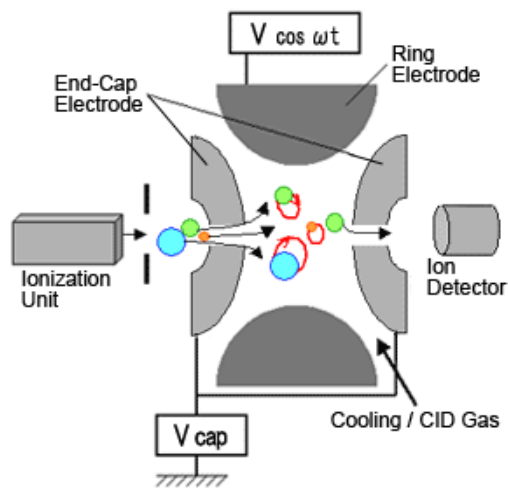


Figure 13: Scheme of an ion trap mass analyzer.

### 3.7.3 Data and analysis

The most common data representation is the mass spectrum. Since the precise peptide sequence is deciphered through the set of fragment masses, the interpretation of mass spectra requires combined use of various techniques. Usually the data obtained experimentally by mass spectrometry is compared to the theoretical mass data of public protein databases (i.e: SwissProt, NCBI).

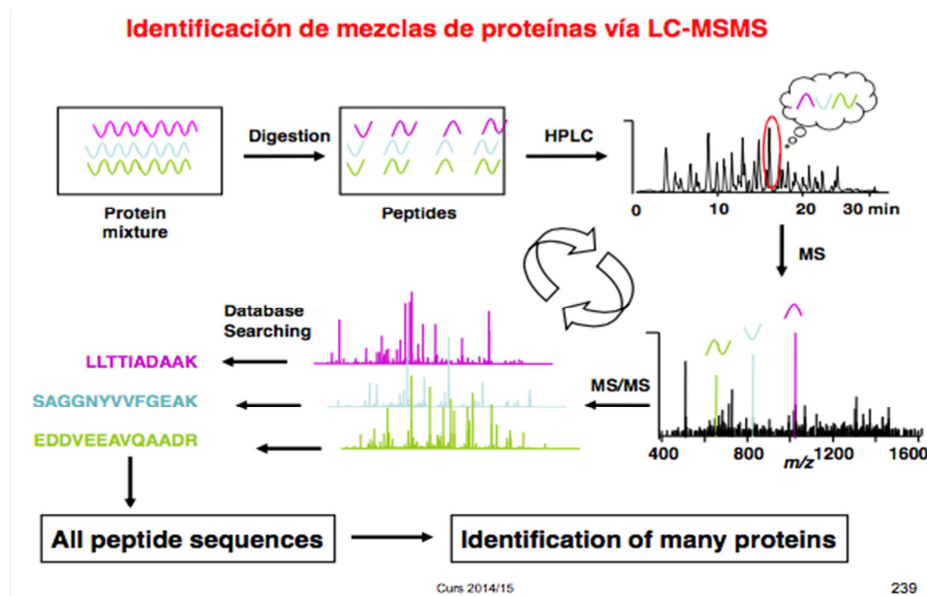


Figure 14: Diagram of the protein identification by mass spectrometry.

## 4. Goals

The project “ Proteome and phosphor-proteome study of Molm-13 cell line in Acute Myeloid Leukemia (AML)” has a main objective, to study and understand the cell behavior and how the proteome changes when Okadaic Acid and FLT3-L promote apoptosis and proliferation of the tumor cells respectively.

Early apoptotic and proliferation inducers would be identified and later studied to understand their effect on the Molm-13 cells and the AML disease. The study of these proteins might help to keep studying AML and find new paths of study in order to have a therapeutic advance in this common disease. Also would be important to know how the phosphorylation PTM is implicated in cancer progression.

Another goal of this project is to optimize enzymatic digestions and phosphorylated peptides enrichment protocols for further experiments with Molm-13 cell line.

## 5. Methods and materials

### 5.1 Cell growth and sample preparation

Molm-13 cells were grown in RPMI1640 medium from Sigma supplemented 10% of fetal bovine serum (FBS) from Gibco. They need to be grown in a suspension culture at 36°C and 6%  $CO_2$  until the confluence is approximately 85%. Different conditions were used to grow these cells to later compare the proteome. In order to have enough amount of protein for the proteomic and phosphor-proteomic approaches I needed to have in each flask of cells 600  $\mu g$  of protein. I had two flasks with Molm-13 unstimulated cells as a control of the experiment. Two other flasks were induced with FLT3-ligand provided by Øystein Bruserud at section for Hematology, Department of Clinical Science, University of Bergen, Norway. The concentration used was 100 ng/ml. After 15 minutes induction was stopped by adding 1 ml of phosphate buffered saline (PBS) and centrifuged it for 4 minutes at 900 rpm. Last two flasks were induced with Okadaic Acid (OA) and order from Sigma.

Concentration used was 100nm and also stopped after 15 minute of induction. Once the samples are centrifuged I added the lysis buffer (4% SDS 50mM Tris-HCl pH 7.6) and heated it for 10 minutes 95°C. Next step is sonication of the sample for 2 minutes with pulses of 1 second on and 1 second off at 50% amplitude. Sonication is the act of applying sound energy to agitate particles presents in the sample, with that process what we want is to disrupt the cells and make the proteins accessible.

### 5.2 Protein Quantification

Protein quantification is an essential step to know how much protein our sample has and to decide how much enzyme we will need to digest the proteins.

We used the Bicinchoninic Acid Assay (BCA assay) in 96 well plates to perform the protein quantification. The BCA react consists on 10 ml react A plus 200  $\mu l$  react B. From this mixture, 200  $\mu l$  were added to each well and then mixed 30 min at 37 °C. The wavelength was 565 nm.

### Standard curve

Water	10 $\mu$ l	8 $\mu$ l	6 $\mu$ l	4 $\mu$ l	2 $\mu$ l	0 $\mu$ l
Bovine IgG	0 $\mu$ l	2 $\mu$ l	4 $\mu$ l	6 $\mu$ l	8 $\mu$ l	10 $\mu$ l
Lysis buffer	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l

### 5.3 Filter Aided Sample Preparation- Double Digestion (FASP-DD)

Once the protein concentration of the sample is known

- Add 0.1M DTT as a reducing agent in the sample and incubate it 5 min at 95 °C.
- Add 0.1M Tris /HCL pH 8.5, 8M Urea to reduce SDS concentration from 4% to 0.5%.
- Display the filter in the collection tube and fill it with the sample.
- Centrifugation for 15 min at 1200 rpm
- 200  $\mu$ l of 0.1M Tris/HCL pH 8.5, 8M Urea
- Centrifugation for 25 min at 1200 rpm and discard the flow through. Do it twice.
- Add 100  $\mu$ l Chloroacetamide and mix 1 min at 650 rpm. Incubate for 20 min in dark conditions without mixing. After these 20 minutes centrifuge the filters 15 min at 1200 rpm and discard flow through.
- Add 100  $\mu$ l 0.1M Tris/HCL pH 8.5, 8M Urea and centrifuge 15 minutes at 1200 rpm. Do it three times.
- Add 100  $\mu$ l 50mM Tris/HCL pH 8.5 and centrifuge 10 min at 1200 rpm. Discard flow through and do it three times.
- The digestion ratio is 1  $\mu$ g of enzyme: 100  $\mu$ g of protein (1:100; w/w). The concentration of Lys-C was 0.2  $\mu$ g/ $\mu$ l. Add the  $\mu$ l needed of Lys-C with 75  $\mu$ l of 50mM Tris/HCL pH 8.5 overnight at 37 °C
- Take the collection tube and centrifuge it for 10 min at 1200 rpm. Display a new collection tube to the same unit filter.

- Elute two times with 80  $\mu\text{l}$  50mM Tris/HCL pH 8,5. Tranfer the Lys-C elute to a low binding tube.
- The digestion ratio is 1  $\mu\text{g}$  of enzyme: 100  $\mu\text{g}$  of protein (1:100; w/w). The concentration of Trypsin (Trp) was 0.2  $\mu\text{g}/\mu\text{l}$ . Add the  $\mu\text{l}$  needed of Trp with 75  $\mu\text{l}$  of 50mM Tris/HCL pH 8.5 for two hours at 37 °C.
- Elute two times with 80  $\mu\text{l}$  50mM Tris/HCL pH 8.5.
- Add 50  $\mu\text{l}$  of 0.5M NaCl and centrifuge. Transfer the Trp elute to a low binding tube.
- The peptide content was estimated by UV light spectral density at 280nm using Nanodrop spectrophotometer ND 1000.

□



Figure 15: Microcon centrifugal filters used in the FASP protocol.

#### 5.4 Desalting

Desalting is the process to clean up the sample and remove the salts present in it. Cleaning up the sample it is an important step to remove salts presents in our sample that can interfere in the results obtained by mass spectrometry because it is very sensitive to small amounts of contaminants, detergents, buffers or salts used in the sample preparation. TFA is added to the sample before to start the desalting process. TFA does what it is called ion pair effect, the negative charge of this acid interacts with positive charged peptide residues and due to the hydrophobicity of the anion there is an increase of the affinity between peptides and the stationary phase of the reverse phase chromatography (RP-HPLC), this increment of the affinity means an increase in the



retention time and the peptide separation it is better, there is an improvement of the peaks width in the chromatogram.

The protocol of desalting is the following:

- Acidification of the sample to a final concentration of 1% of Trifluoroacetic acid (TFA) to inactivate endonuclease activity.
- Centrifuge it 5 min at 5000 rpm.

- Sample with at least 100  $\mu\text{g}$  of peptides use this column: Oasis HLB 96-well  $\mu\text{Elution}$  Plate, 2 mg Sorbent per Well, 30  $\mu\text{m}$  Particle Size

- Add 500  $\mu\text{l}$  80% Acetonitrile (ACN) 0.1% Acid Formic (FA) \*1
- Add 500  $\mu\text{l}$  0.1% FA \*1
- Add 500  $\mu\text{l}$  0.1% FA \*1
- Addition of the sample \*2
- Add 500  $\mu\text{l}$  0.1% FA \*1
- Add 500  $\mu\text{l}$  0.1% FA \*1
- Add 500  $\mu\text{l}$  0.1% FA \*1
- Change the washing plate for the elution plate. Elution with 100  $\mu\text{l}$  80% ACN 0.1% FA \*1
- Elution with 100  $\mu\text{l}$  80% ACN 0.1% FA \*1
  - \*: 1 min centrifugation at 4 °C and 200x g
  - \*2: 3 min centrifugation at 4 °C and 150x g

- Sample with more than least 100  $\mu\text{g}$  of peptides use this column: Oasis HLB 1 cc Vac Cartridge, 10 mg Sorbent per Cartridge, 30  $\mu\text{m}$  Particle Size.

- Display a 2ml low binding tube with the column
- Add 1 ml 0.1% FA
- Add 1 ml 80% ACN 0.1% FA
- Split it in 4 low binding tubes, 250  $\mu\text{l}$  per each one

## 5.5 Phosphopeptide enrichment with titanium dioxide

- Add 75  $\mu\text{l}$  of binding buffer (80% ACN, 5% TFA, 1M glycolic acid) and centrifuge it for 3 min at 3000 rpm. Discard flow through.
- Add 50% titanium beads and 50% binding buffer in a tube. Cut the tip to take the beads to avoid breaking them.
- Concentration of the beads was 100  $\mu\text{g}/\mu\text{l}$  and the ratio was 5 beads: 1 peptide.
- Add the  $\mu\text{l}$  of beads to the samples and rotate it for 30 minutes.
- After rotation, centrifuge it 5 min 3000 rpm.
- Take flow through and add to a low binding tube. Add half of the  $\mu\text{l}$  of the beads added previously.
- 30 min of rotation and centrifugation of 5 min at 3000 rpm
- Display a stage tip with one  $C_8$  in a 2ml standard eppendorf and add the sample. Centrifuge it 2 min 2000 rpm.
- Wash 1: 150  $\mu\text{l}$  of 80% ACN, 5% TFA, 1M glycolic acid + centrifugation 2 min 2000 rpm
- Wash 2: 150  $\mu\text{l}$  of 80% ACN, 0.2% TFA + centrifugation 2 min 2000 rpm
- Wash 3: 150  $\mu\text{l}$  of 20% ACN + centrifugation 2 min 2000 rpm
- Take stage tip and display it in a 1.5 ml low binding eppendorf.
- Elution: 50  $\mu\text{l}$  of 5% Ammonia, 60% ACN (3 times)
- Lyophilized the samples before refrigeration.

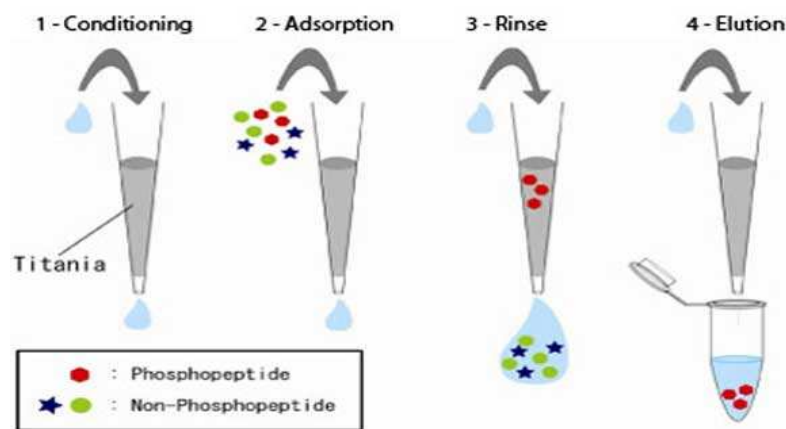


Figure 16: Schematic diagram of phospho-enrichment with titanium dioxide

## 5.6 Samples analyzed by Mass Spectrometry

After FASP and desalting process and before phosphor-peptide enrichment I got an aliquot of the samples to perform qualitative analysis by MS. The proteome samples and its peptide contents measured by the Nanodrop spectrophotometer ND 1000 were the following ones:

WT Lys-C	WT Trp	FLT3 Lys-C 1	FLT3 Trp 1	FLT3 Lys-C 2	FLT3 Trp 2	OA Lys-C 1	OA Trp 1	OA Lys-C 2	OA Trp 2
32.4 $\mu g$	15.51 $\mu g$	43.24 $\mu g$	19.6 $\mu g$	12.42 $\mu g$	2.61 $\mu g$	36 $\mu g$	7 $\mu g$	25.2 $\mu g$	31.2 $\mu g$

The rest of the samples were proceed with phospho-enrichment protocol explained above. After the phospho-enrichment I obtained the samples named as phospho samples to be analyzed by MS.

WT Lys-C	WT Trp	FLT3 Lys-C 1	FLT3 Trp 1	FLT3 Lys-C 2	FLT3 Trp 2	OA Lys-C 1	OA Trp 1	OA Lys-C 2	OA Trp 2
75,6 $\mu g$	18 $\mu g$	108 $\mu g$	260 $\mu g$	36.3 $\mu g$	66.6 $\mu g$	36 $\mu g$	6.05 $\mu g$	98.27 $\mu g$	34.18 $\mu g$

I had only one wild type replicate because I got less protein amount than expected from these samples. So, I decided to pool the cells from flask 1 and flask 2.

## 5.7 Mass spectrometry and chromatographic settings

About 0.5ug protein as tryptic peptides dissolved in 2% acetonitrile (ACN), 1% formic acid (FA), were injected into an Ultimate 3000 RSLC system (Thermo Scientific, Sunnyvale, California, USA) connected online to a linear quadrupole ion trap-orbitrap (LTQ-Orbitrap Elite) mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanospray Flex ion source (Thermo Scientific).

The sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2cm x 75µm i.d. nanoViper column, packed with 3µm C18 beads) at a flow rate of 5µl/min for 5 min with 0.1% trifluoroacetic acid (TFA).

Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 270 nl /min) on a 50 cm analytical column (Acclaim PepMap 100, 50cm x 75µm i.d. nanoViper column, packed with 3µm C18 beads). Solvent A and B was 0.1% TFA (vol/vol) in water and 100% ACN respectively.

The gradient composition was different depending on the sample analyzed. The gradients used for each sample are mentioned below.

- Molm-13 proteome samples enriched with Titanium Dioxide and flow through analyzed during 90 min (WT-Trp-FT, WT-LysC-FT OA-Trp-FT, OA-LysC-FT, FLT3-Trp-FT, FLT3-LysC-FT): The gradient for these samples was 5%B during 5 min (trapping) followed by 5-8%B over 0.5min, 8-37%B for the next 45 min, 37-90% over 5 min. The elution of hydrophobic peptides and the wash of the column were performed during 15 min with 90%B and 30 min with 5%B.

- Molm-13 proteome samples enriched with Titanium Dioxide analyzed during 140 min (WT-Trp, WT-LysC, OA-Trp, OA-LysC, FLT3-Trp, FLT3-LysC): In this case the gradient composition was 5 min of trapping with 5%B followed by 5-7%B over 0.5 min, 7-32% for the next 80 min, 32-40%B over 5 min, 40-90%B for 5 min. Elution for 30 min with 90%B and conditioning during 20 min with 5%B.

- Molm-13 proteome samples analyzed during 195 min (WT-Trp, WT-LysC, OA-Trp, OA-LysC, FLT3-Trp, FLT3-LysC): The gradient composition was 5%B during

trapping (5min) followed by 5-7%B over 0.5min, 7-32%B for the next 129min, 32-40%B over 10 min, and 40-90%B over 5min. Elution of very hydrophobic peptides and conditioning of the column were performed during 20 minutes isocratic elution with 90%B and 20 minutes isocratic elution with 5%B respectively.

The type of sample to be analyzed determined the method used. TOP12CID method and TOP15CID-MSA were used respectively to analyze the global proteome and the phosphor proteome.

TOP12CID method (global proteome):

The eluting peptides from the LC-column were ionized in the electrospray and analyzed by the LTQ-Orbitrap Elite. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.7.0 and Xcalibur 2.2.

Survey full scan MS spectra (from m/z 300 to 2,000) were acquired in the Orbitrap with resolution  $R = 240\ 000$  at m/z 400 (after accumulation to a target value of  $1e6$  in the linear ion trap with maximum allowed ion accumulation time of 300ms). The 12 most intense eluting peptides above a ion threshold value of 3000 counts, and charge states 2 or higher (determined from a preview orbitrap scan of 60 000 resolution), were sequentially isolated to a target value of  $1e4$  and fragmented in the high-pressure linear ion trap by low-energy CID (collision-induced-dissociation) with normalized collision energy of 35% and wideband-activation enabled. The maximum allowed accumulation time for CID was 150 ms, the isolation with maintained at 2 Da, activation  $q = 0.25$ , and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with two electron multiplier detectors. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 40s. Lock-mass internal calibration was not enabled.

TOP15CID-MSA method (phospho proteome):

Survey full scan MS spectra (from m/z 300 to 2,000) were acquired in the Orbitrap with resolution  $R = 60\ 000$  (140 min LC-run) or  $240\ 000$  (90 min LC-run) at m/z 400 (after

accumulation to a target value of  $1e6$  in the linear ion trap with maximum allowed ion accumulation time of 300ms). The 15 most intense eluting peptides above a ion threshold value of 5000 counts, and charge states 2 or higher, were sequentially isolated to a target value of  $1e4$  and fragmented in the high-pressure linear ion trap by low-energy CID (collision-induced-dissociation) with normalized collision energy of 35% and wideband-activation enabled. Multistage activation enabled pseudo MS3 of the 3 most intense neutral loss peaks from a list of 6 masses corresponding to single and multiple losses of  $H_3PO_4$  ( $m/z$  32, 49, 65.3, 73.5, 98, 147).

The maximum allowed accumulation time for CID was 150 ms, the isolation with maintained at 2 Da, activation  $q = 0.25$ , and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at rapid scan rate, and recorded with two electron multipliers. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 60s. Lock-mass internal calibration was not enabled.

Ion source parameter:

The spray and ion-source parameters were as follows. Ion spray voltage = 1800V, no sheath and auxiliary gas flow, and capillary temperature = 260 °C.

## 5.8 Data analysis with MaxQuant

MaxQuant was the software use to analyze the raw files. After downloaded the software I add the fasta file of Homo sapiens. Go to *Andromeda configuration*, click Sequences databases → add → fasta file and select your file. For label free experiments go to *General* and set *Multiplicity* “1”. *Label-free quantification* change the tab from “None” to “LFQ”. In the *Group-specific tab* and I set the digestion mode as trypsin or lysine C depending on the sample. I added oxidation of the methionine as a variable modification and as fixed modification I added carbamidomethylation. Enable *Match between runs* in order to transfer a protein identification to a non-identified MS feature in other LC-MS runs. In the *Advanced* tab change number of threads to the number of cores on your computer, I used 8 number of threads.

To search for post-translational modifications (PTMs) add also as a *Variable modification* the PTM you are interested to identify, in my case I set “Phospho (STY)”.

Results will end up in a combined folder and the PTM data will appear in this folder as a (Phospho (STY)Sites.txt) file.

### 5.9 Data analysis with Proteome Discoverer

When I had raw files of my final experiment from Bergen I did my searches with Proteome Discoverer 1.4. I had one fraction digested by Lys-c and one by Trp from every sample but later I joined this two fractions to have the completed proteome of the sample, the name of the samples changed, e.g from WT-L and WT-T to WT-LT. The processing node was Sequest HT and the database was SPH 150313 from Swiss Prot. I set up the Precursor Mass Tolerance as 10 and the Fragment Mass Tolerance as 0.6 Dalton (Da). The Max.Missed Cleavage Sites I set up it as 3.

I had two types of peptide modification. Static modifications was the alquilation of Cysteine amino acid known as carbamidomethylation and as a dynamic modification I had the Oxidation of the Methionin.

## 6. Results and Discussion

First of doing my final experiment which consists on treating Molm-13 cells with two different treatments (OA and FLT3-L), digestion of the cells by FASP-DD, desalting them and finally the phospho-enrichment of some aliquots I did some optimization on the protocols in order to decide which suits better for my experiment and to get a mass spectrum of quality to later analyze.

### 6.1 Cell growth

As mentioned before, Molm-13 cells were treated with Okadaic Acid and FLT3-L and the proteome was analyzed to determine differences induced by the two different treatments. Firstly, I performed optimization on the concentrations and incubation times to improve cells treatment conditions for both reagents. In the first experiment I used Okadaic Acid. I took 4 ml of cells and centrifugated 4 min at 900 rpm. I discard the flow through and I add 3 ml of RPMI1640 medium from Sigma supplemented 10% of fetal bovine serum (FBS) from Gibco and homogenized it. I counted the cells with the Neubauer Chamber. The cells counted were 111 cells what correspond to  $7.3 \times 10^5$  cells/ml. For this assay I needed 40000 cells for each well (400  $\mu$ l). I used three different concentrations of Okadaic Acid, 100, 300 and 500 nM. The incubation times, used were 30 min, 1h, 3h, 16h and 36h.

I did a 1:10 and 1:100 dilution of OA. In the wells with OA concentration 100 nM I added 4  $\mu$ l of OA dilution 1:100. In the wells with 300 nm I added 1.2  $\mu$ l of OA 1:10 and the last one was 0.2  $\mu$ l of 1.10 dilution. The induction with the different concentrations of OA was done in parallel, at the same time. Once the incubation time for each well was over, cells were transferred and fixed in another plate to be later analyzed in a fluorescent microscope.



## EXPERIMENT 1 OA

### **Incubation plate**

Control	100 nM OA	300 nM OA	500 nM OA
1.1	2.1	3.1	4.1
1.2	2.1	3.2	4.2

### **Fixation plate**

30 min	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2
1h	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2
3h	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2
16h	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2
36h	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2

The procedure used to perform the second experiment with OA was similar as explained before. Nevertheless, only the concentration 100 nM was applied because a higher concentration showed to be too harmful for the cells, much more cells were dead and the apoptosis stage was too much develop. The interesting aspect to recognize in the cells was an earlier stage of apoptosis, if the cells were treated longer with OA the apoptotic process will had gone to far and cells would been in the end-phase. The results would be the same but with different apoptosis inducers with the difference that the molecules involved in the induction phase will not be present and therefore not possible to study them. Same for the phosphorylation, the early phosphorylation patterns leads to the final results, the fate of the cell-proliferation and apoptosis. The times of incubation in the second experiment were also slightly different, 15 min, 30 min, 1h, 3h and 23h.

In the first experiment, number of death cells were counted in two different times to do a comparison between control cells and Okadaic acid induced cells.

## EXPERIMENT 2 OA

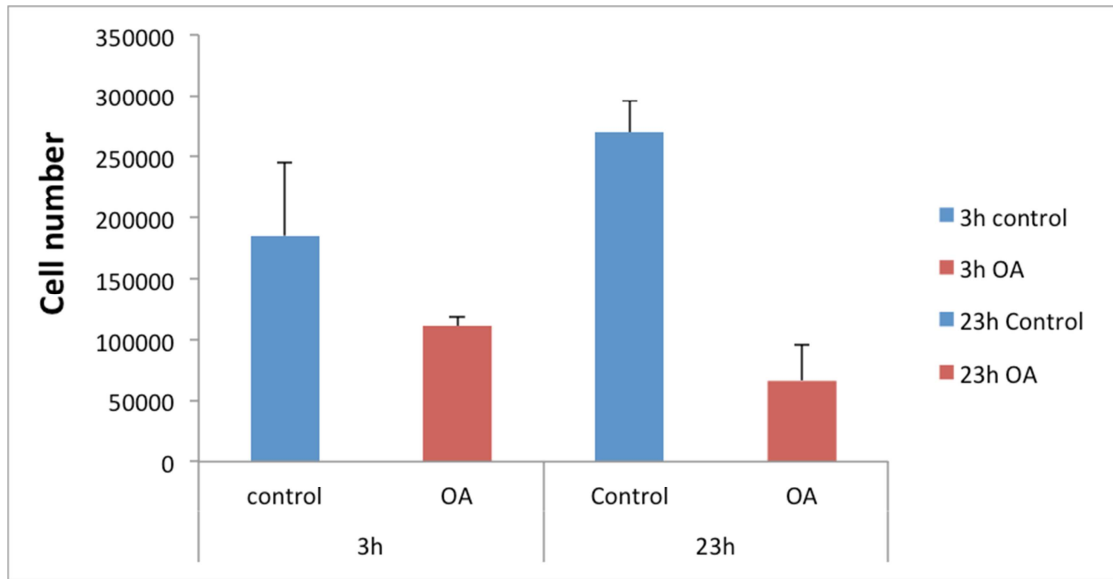
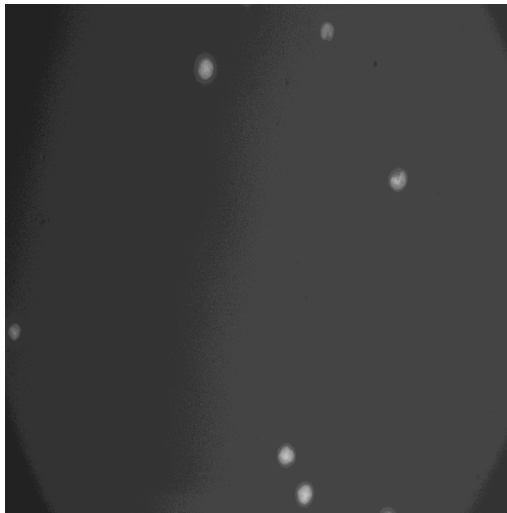
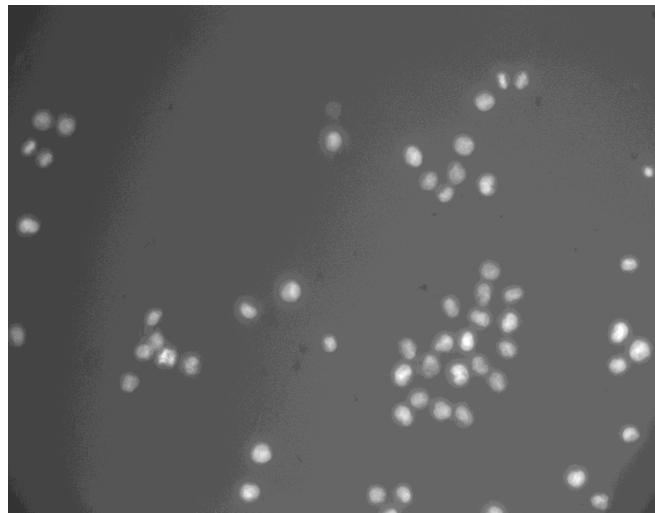


Figure 17: Comparative relation of living cells between the control and cells treated with Okadaic acid in two different times.

As observed in the graph 17 the number of dead cells increased with the time in the well incubated with Okadaic Acid. In contrast the number of living cells increased in the control wells of the experiment.



Picture A



Picture B

The first picture A shows Molm-13 cells after being treated for 15 minutes with OA. The other image b represents same cells after 30 minutes of incubation with OA. We can see in the image B how cells are in a high stage of the apoptotic process. Cells are

clearly more fragmented after 30 minutes of treatment than 15 minutes. After the exposition to OA, cells were treated with FLT3-L to see how they behave to this compound. The concentration of the cells were  $7.7 \times 10^5 \text{ cells/ml}$ . The starting concentration of the FLT3-L was  $1 \mu\text{g} / \text{ml}$  and the concentration needed was 100 ng/ml. Cells were stopped and counted at 16h, 24h, 39h and 48h.

CONTROL	100 ng FLT3-L
1.1	2.1
1.2	2.2

EXPERIMENT 1 FLT3-L

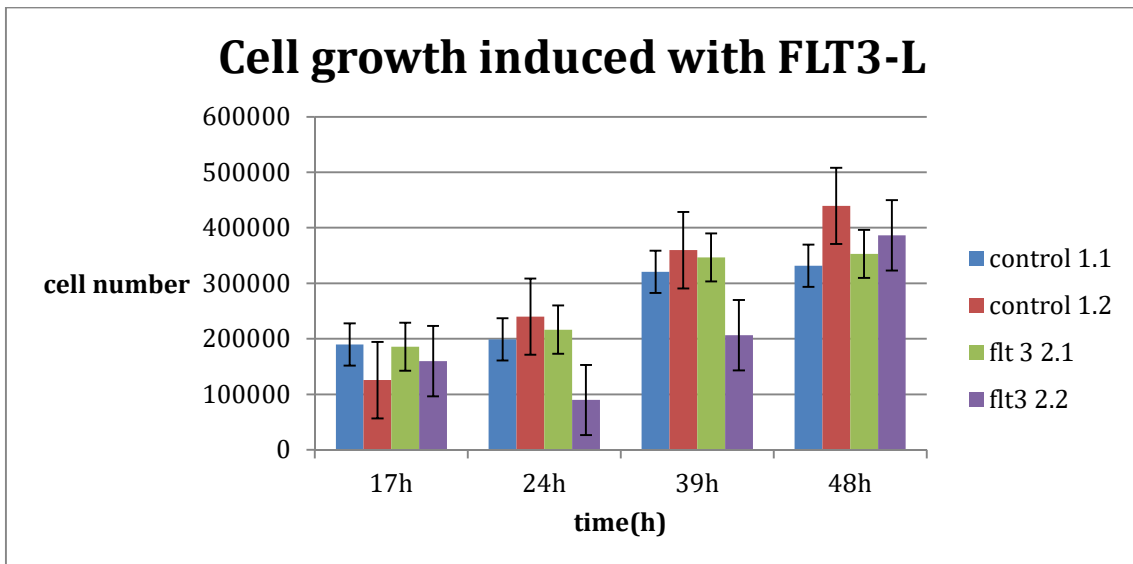


Figure 18: Number of the cells counted at 17,24, 39 and 48 hours of induction with FLT3-L.

As expected, in both Control wells and FLT3 wells we observed an increase in the number of cells, once, FLT3 ligand attached to its receptor causes proliferation of the

## 6.2 Protein quantification

Protein quantification is an essential step to know the amount of total proteins in the samples in order to calculate the amount of endonuclease needed to digest the proteins. The Bicinchoninic Acid Assay (BCA assay) was the method of choice due to the samples contained Sodium Dodecyl Sulfate (SDS) and it is incompatible with other protein quantification assays as Bradford.

BCA Protein Assay Kit is a two-component detergent compatible reagent set to measure total protein concentration compared to a protein standard. The BCA assay combines the reduction of  $Cu^{2+}$  to  $Cu^{+1}$ , this is a temperature dependent reaction (incubation at 37 °C). The amount reduced is proportional to the amount of protein present in solution. Next, two molecules of bicinchoninic acid (weak acid composed of two carboxylated quinoline rings) chelate with each  $Cu^{+1}$  ion and form a purple-colored product that absorbs light at a wavelength of 562nm. The bicinchoninic acid  $Cu^{+1}$  complex is influenced in protein samples by the presence of cysteine, tyrosine and tryptophan side chains. The absorbance values obtained by using a Biochrom Asys UVM 340 Microplate reader allow me to plot a standard curve. Absorbance values of unknown samples are then interpolated onto the plot or formula for the standard curve to determine their concentrations.

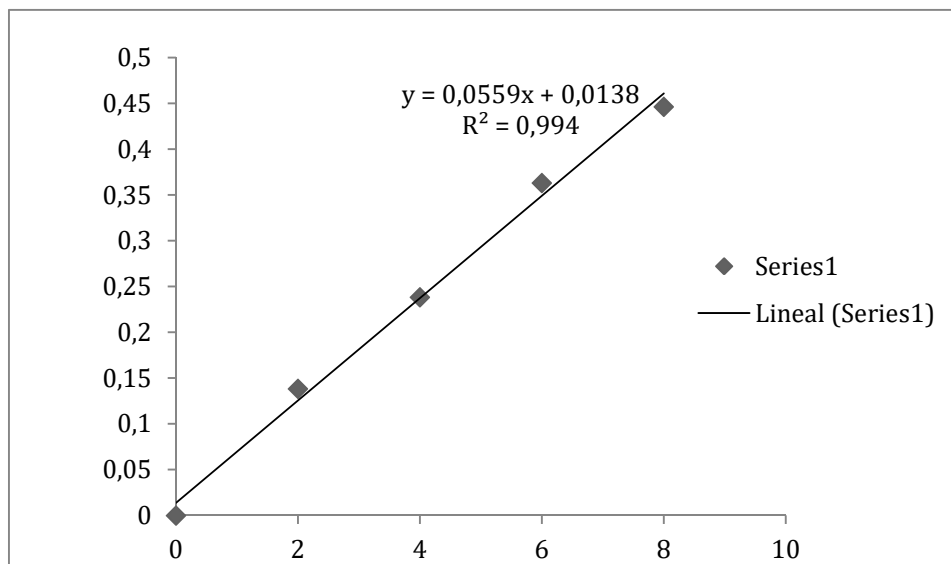


Figure 19: Standard curve used to determine the concentration of the unknown proteins present in the sample.

The initial protein concentration of the samples were:

WT 1	WT 2	FLT3 1	FLT3 2	OA 1	OA 2
248,01 $\mu g$	316,50 $\mu g$	643,96 $\mu g$	548,07 $\mu g$	422,17 $\mu g$	696,80 $\mu g$

### 6.3 Digestion and phosphopeptide enrichment protocol optimization

#### 6.3.1 Digestion method (ISD vs FASP)

Two enzymatic digestion approaches were compared. The first one was In Solution Digestion (ISD). The lysis buffer was composed by: 6M Guanidium hydrochloride (GndCl), 5mM tris(2-carboxyethyl) phosphine (TCEP), 10mM Cloroacetamide, 10 mM Tris/HCl pH 8.5. The advantage of this buffer is that in one step proteins are denatured because the ability of GndCl to break hydrogen bonds between amino acid residues. Disulfide bonds are reduced by TCEP and the thiol groups are alkylated with Cloroacetamide. GndCl is much more stable at high temperatures than Urea used in FASP method. Sonication is also needed in this method to make sure that proteins are accessible. After the cell lysis, protein quantification was carried by Bradford assay and the wavelength is at 595 nm. The digestion of the proteins was with lysC 0.2  $\mu g / \mu l$  1h at 37°C. After this incubation I added trypsin at the same concentration overnight at 37°C, the ratio was 1:100, enzyme/protein.

In contrast, FASP method combines the advantages of in-gel and in-solution digestion for mass spectrometry- based proteomics. This method consists on solubilized the proteome in SDS, which will be exchanged by urea on a standard filtration device (Microcon centrifugal filters from Milipore). SDS is the reagent of choice for total solubilization of cells and denaturation of the proteins due its capacity to bound the apolar part of the poli-peptide. But detergents, even in a small concentration, can preclude enzymatic digestion and dominate mass spectra owing to their ready ionizability and their great abundance compared to individual peptides. For that reason,

depletion of SDS is a prerequisite for efficient mass spectrometric analysis. Because in solution removal of SDS it is impossible, other methods such as FASP have been developed for analyzing membrane proteomes. Membrane proteins can be fully depleted from detergents by filtration in 8M urea. FASP combines strong detergents for universal solubilization before digestion and obtained purified peptides after digestion while avoiding the disadvantages of the gel format. The filter device used in this method acts as a proteomic reactor for detergent removal, buffer exchange, chemical modification and protein digestion. The steps of the FASP method are: depletion of low molecular weight components in urea buffer, carboamidomethylation of thiol group, digestion of proteins and elution of peptides.

FASP method includes two protocols, single and double digestion. FASP- single digestion protocol means that the protein sample is once digested by Trypsin. In opposite, double digestion implies the consecutive digestion of the cell lysates with Lys-C and trypsin that allow the generation of two population of peptides. Proteins are firstly digested overnight by Lys-C and after the isolation of the peptides, material remaining on the filter was digested with trypsin only 2h. This consecutive proteolytic digestion enables identification up to 40% more proteins and phosphorylation sited in comparison to the commonly used one- step tryptic digestion (Wiśniewski JR, Mann M. “Consecutive Proteolytic Digestion in an Enzyme Reactor Increases Depth of Proteomic and Phosphoproteomic Analysis. *ACS Publications (2012): 2631-2637*”)

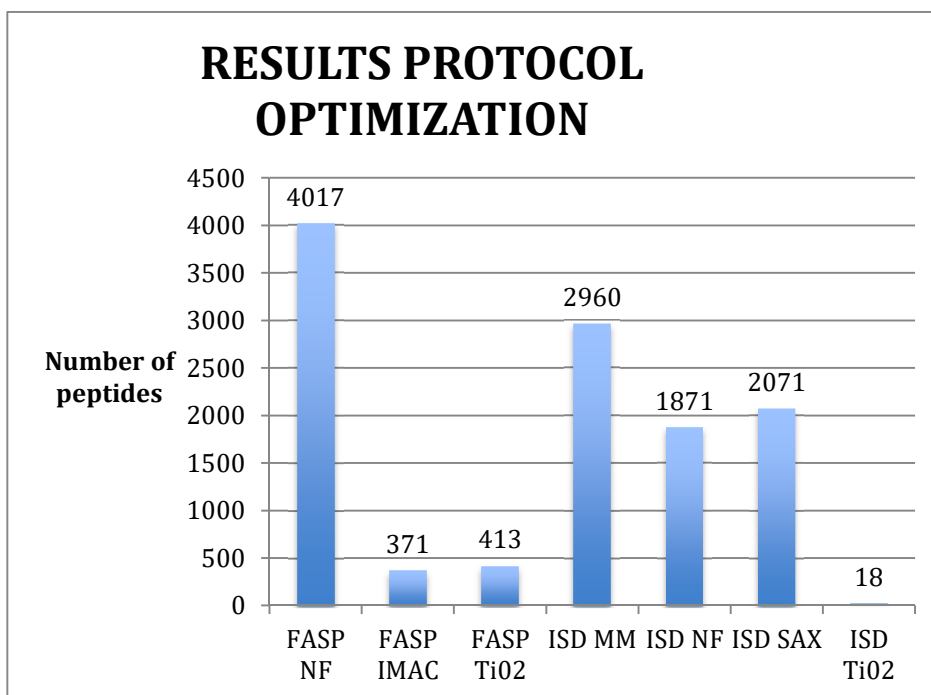


Figure 20: Comparison between the two digestion methods (FASP and ISD) and the phosphor-enrichment (IMAC and TiO<sub>2</sub>)

In the graph 20 are shown the number of peptides once the first samples were analyzed. FASP NF is a sample that corresponds to the cells digested by FASP method and were not fractionated. As seen in the graph this was the sample that had more number of peptides identified (4017). In contrast, there is a sample named ISD NF. The sample was digested by ISD method and also non-fractionated. The result indicates that 1871 peptides were identified. Therefore, FASP digestion method was the best method to digest Molm-13 cells in order to obtain a higher number of identified peptides.

### 6.3.2 Peptide fractionation

Once the samples were cleaned up of contaminants, detergents and salts by using the specific desalting protocol for each sample depending on the peptide amount, a peptide fractionation was done in the proteome samples.

By proceeding my samples to peptide fractionation I had different fractions to avoid interference between peptides and improve the proteome coverage. This peptide fractionation can be divided into three simple components: the column characteristics,

the mobile phase and the peptide properties (charge, polarity, hydrophobicity and size). Because I had 4 samples I decided to use three different fractions and the last sample was not fractionated. Strong anion exchange (SAX) was used in one of my peptide samples. The resin was positively charged and the negatively charged ions (anions) were linked. Strong cation exchange (SCX) the ions bind were positively charged (cations). Last fractionation was done by Mix mode (SDS-RPS), it is a poly(styrene-divinylbenzene) copolymer that has been modified by sulfonic acid groups to make it hydrophilic. Peptides are separated by its polar and non-polar characteristic. It consists on fractionating peptides by using a strong cation exchange matrix as a mobile phase and a hydrophobic interaction chromatography (HILIC)

### 6.3.3 Phosphopeptide enrichment (IMAC vs TiO<sub>2</sub>)

After desalting the samples higher amount of the peptides were taken in order to perform a phospho-enrichment. The characterization of phosphorylated proteins is a challenging analytical task since many of the proteins targeted for phosphorylation are low in. Highly efficient enrichment procedures are therefore required. Two samples were enriched using two different methods, one by the method Titanium Dioxide (TiO<sub>2</sub>) and the other one by IMAC enrichment.

IMAC uses metal cations as  $Fe^{+3}$  or  $Ga^{+3}$  that bind negatively charged group phosphate, the ratio has to be 50  $\mu g$ : 120  $\mu g$  (beads/peptides). The other method for selective phosphopeptide enrichment is TiO<sub>2</sub>. This highly efficient method for purification of phosphopeptides is well suited for the characterization of phosphoproteins from both *in vitro* and *in vivo* studies in combination with mass spectrometry (MS). The protocol consists in using titanium beads that bind phosphopeptides usually mono phosphopeptide.

For this enrichment is needed to know the exact amount of peptides in the sample have to use the correct volume of titanium beads in order to achieve the ratio 1:5 (beads/peptides). Samples were all injected and separated in a nano-liquid chromatography system coupled to mass spectrometry. The achieved results are explained in the following graphic.

As shown in graph 20, there were two samples digested with FASP enriched by TiO<sub>2</sub> or IMAC. 317 peptides were distinguished in the sample enriched with IMAC and 42 more

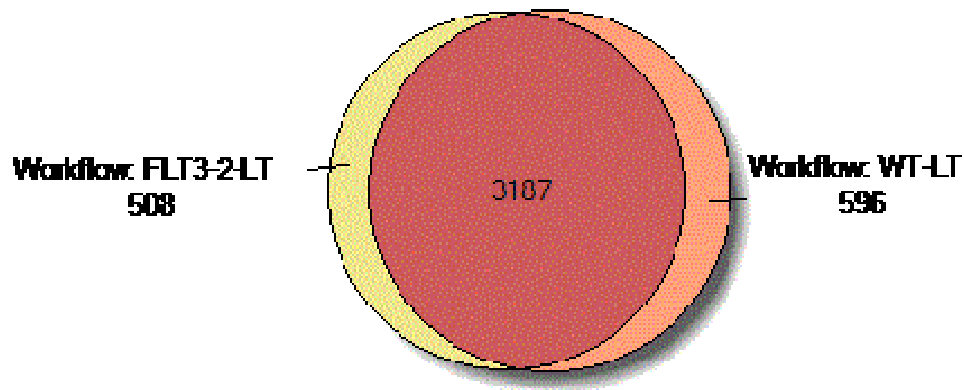


peptides were characterized by  $TiO_2$ . At this point, the phosphor enrichment method chosen to carry out the experiments was the Titanium Dioxide enrichment ( $TiO_2$ ).

#### 6.4 Comparison between Molm-13 samples untreated, OA and FLT3-L

In the present work Molm-13 cells were submitted to two different treatments in order to analyze the molecular mechanisms involved in the Acute Myeloid Leukemia: FLT3 and OA, respectively promoter of proliferation/survival and apoptosis.

- FLT3-2-LT and WT-LT



The comparison of the protein profile between Molm 13 treated with FLT3 and the control (cells not treated) showed that the samples share most of the proteins identified (3187 from 4291). Both samples have unique proteins that are expressed differently because one of them was submitted to the treatment. In the FLT3-2-LT sample some growth factors were found to be upregulated in comparison with the WT-LT. These growth factor proteins combined with the FLT3 ligand stimulated the proliferation of the Molm-13 cells as observed during the cells growing.

Other interesting protein identified in FLT3-2-LT was a signal transducer and activator of transcription 5B coded by the gene STAT5B (Uniprot accession P51692). The protein STAT5B is a member of the STAT family of transcription factors. In

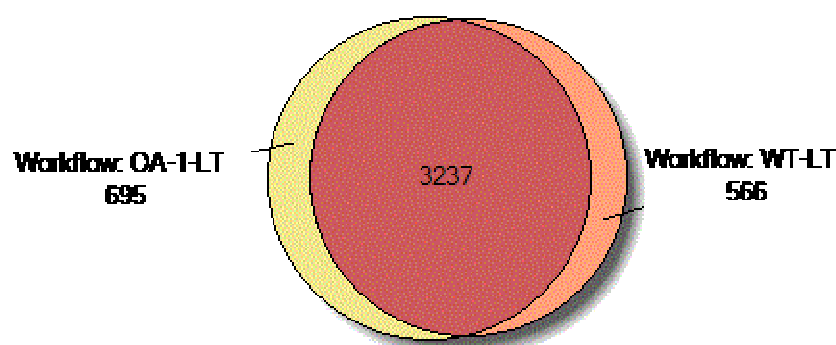
response to cytokines and growth factors, STAT family members are phosphorylated by the kinases associated to the receptor.

According to Bańkiewicz-Masiuk M, Machaliński B (2004) “STAT5 proteins are activated by many hematological cytokines and growth factors. Because STAT5s are constitutively activated in certain hematologic diseases, they are suggested to play an important role in leukemogenesis”. STAT1 and STAT3 proteins were also identified in the sample.

As states in part 3.3 and the results in the sample by Proteome Discoverer 1.4 shown and activation of STAT5 and STAT3 is probably that the ITD mutation in the FLT3 receptor is localized in the ER.

Bashman, Sathe, Grein, McClanahan, D’Andrea, Lees, Rascle studies confirmed that the STAT family comprises seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) which are essential mediators of cytokine, growth factor and hormone responses. STAT proteins play important function in a variety of pathways, from innate and acquired immunity to cell proliferation, differentiation and survival. Accordingly, inappropriate activation of STATs, in particular of STAT1, STAT3 and STAT5, is associated with a wide variety of human cancers and diseases and constitutively activated STAT signaling directly contributes to oncogenesis.

- WT-LT and OA1-LT

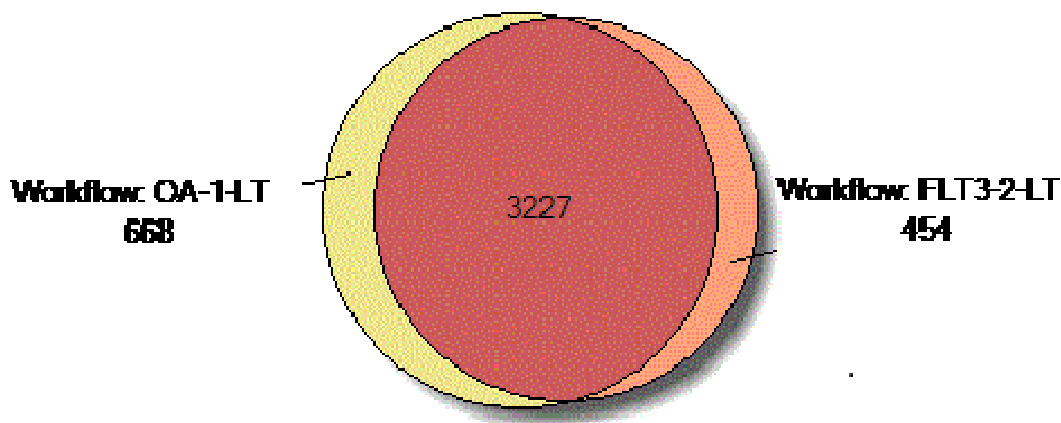


The same protein profile was observed when Molm-13 cells were treated with OA. Most of the proteins identified were common for both treated and non-treated cells.

The 695 proteins unique for the sample OA1-LT are expressed by the effect of the toxin to the cells. Some interesting proteins were identified exclusively in this sample:

a protein named Programmed cell death protein 2 (Q16342) that plays an important role in cell death and/or in regulation of cell proliferation. There is also a DNA repair protein probably because the Okadaic Acid has damaged the DNA and the cell expressed this protein in order to fix the DNA. The tumor suppressor p53 protein was also present in OA-LT. TP53 gene encodes proteins that bind to DNA and regulate the gene expression to prevent mutations of the genome, is a gene that plays a crucial role in preventing cancer formation. The protein p53 has many mechanisms of anticancer function and plays role in apoptosis and genomic stability. Other tumor suppressors were found in this sample.

- OA-1-LT and FLT3-2-LT



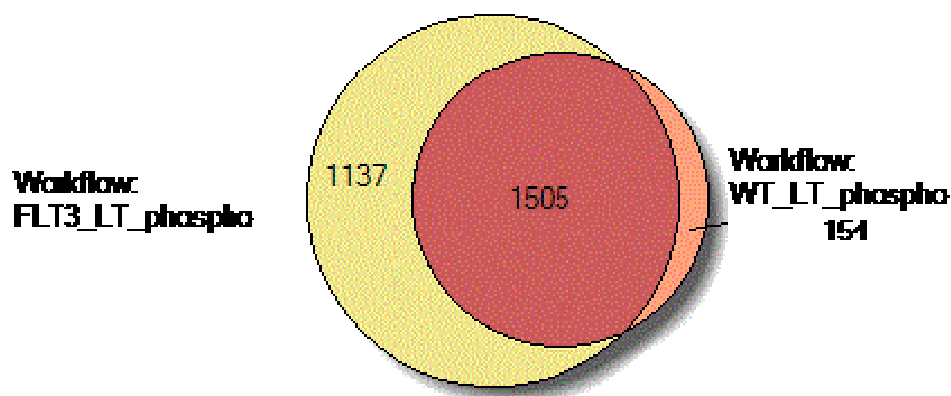
We also performed the comparison of the protein profile between the Molm-13 cells treated with OA and FLT3. The Venns diagram above shows that most of the proteins identified are common for both samples, nevertheless there are many unique proteins identified in each of the treatment. 668 proteins were exclusive for OA-1-LT samples. Some of the remarkable proteins identified in this sample are: protein Q6P589 known as tumor necrosis factor alpha-induced protein 8-like protein 2. The function of this protein is to act as a negative regulator of innate and adaptive immunity by maintaining immune homeostasis. Prevents hyperresponsiveness of the immune system and promotes Fas-induced apoptosis.

TNF is a cytokine able to induce fever, apoptotic cell death and to inhibit tumorigenesis. Dysregulation of TNF production is implicated in a variety of human diseases such as cancer. Other tumor necrosis factor as Q15628 is found in this sample because an overexpression of TRADD (encoded gene) leads to apoptosis.

Both samples content Ser/Thr protein phosphatases and kinases, but they are different proteins in each sample. This indicates that the cell responses differently to these two treatments used in the experiments.

### 6.5 Analysis of the phosphor-proteome of Molm-13 cells untreated, treated with OA and FLT3.

- FLT3-2-LT and WT-LT



In this case, after digestion samples were enriched in phosphopeptides by using TiO<sub>2</sub>. As can be seen in the Venns diagram above, the sample non treated (WT-LT-phosphor) just has 154 unique proteins. One of the proteins present in this samples is named Friend Leukemia integration 1 transcription factor, the gene that encodes the protein is FLI1. This gene is a proto-oncogene. When it is mutated or expressed at high levels it has the potential to cause cancer. B-cell lymphoma 2 and 6 other interesting proteins are also identified in WT-LT phospho sample. These are proteins that regulate cell death or apoptosis. Bcl-2 is specifically considered an important anti-apoptotic protein and is thus classified as an oncogene

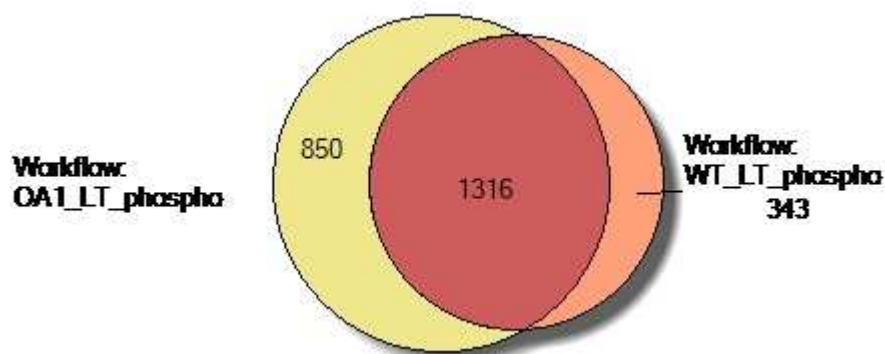
137 unique proteins were seen in the FLT3-LT phospho sample. One of the relevant proteins found in this samples was Tumor Necrosis factor alpha induced protein 8 (O95379). TNFAIP8 acts as a negative mediator of apoptosis and may play a role in tumor progression, suppresses the TNF-mediated apoptosis by inhibiting caspase-8 activity but not the processing of procaspase-8.

Rho guanine nucleotide exchange factor 2 is also distinguished in the sample and activates Rho-Gtpases by promoting the exchange of GDP to GTP. This protein is involved in leukemic cell differentiation, cell cycle regulation, innate immune response and cancer. The length of this protein is 986 amino acids and a phosphate group modifies 20 of them.

852 peptides from the 3259 peptides identified in the sample WT-LT were phosphorylated. In the case of the FLT3-LT phosphor sample 6481 peptides were identified and 1455 of them has at least one phosphate group.

This great difference in number of unique proteins in each sample evidences that cells are sensitive to the treatment used and that many pathways were activated to act against the stress the cells were experiencing.

- WT-LT and OA1-LT



2509 protein were identified in the comparison of the phosphoproteome of non treated Molm 13 cells with the cells treated with OA.. 1316 proteins were common in both of them. The sample OA1-LT phospho contains 850 unique proteins, 507 more proteins than WT-LT phospho.

OA1-LT- phospho contains proteins that are characteristic of the apoptotic process the cell experienced when the induction with Okadaic acid was done. One of these important proteins is Bcl-2 homologous antagonist/killer (BAK1). Bcl-2 protein was identified in WT-LT phospho samples and it is an important anti-apoptotic protein.

BAK1 protein function is that in the presence of an appropriate stimulus, in this case would be the induction with the toxin OA, accelerates programmed cell death by binding to and antagonizing the anti-apoptotic action of Bcl-2 protein. BAK1 is not phosphorylated but it has two methionine amino acids with an oxidation.

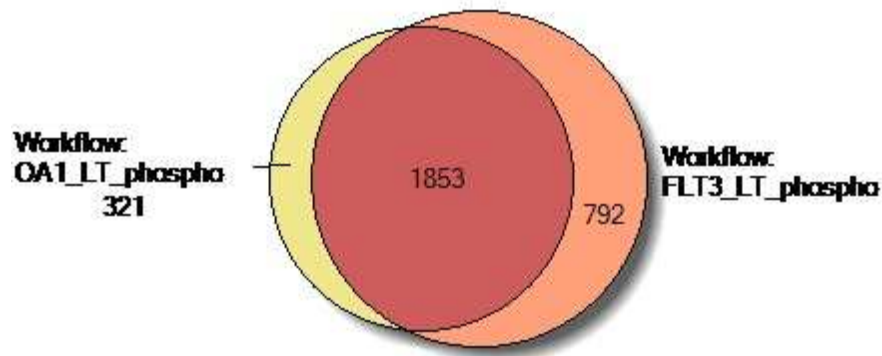
RAF proto-oncogene serine/threonine protein kinase (P04049) was also identified in this sample. Serine/Threonine protein kinase acts as a regulatory link between Ras GTPases and the MAP/ERK cascade, and this critical regulatory link functions as a determining cell fate decision including proliferation, differentiation, apoptosis, survival and oncogenic transformation. RAF-1 activation initiates a mitogen-activated protein kinase (MAPK) cascade that comprises a sequential phosphorylation of MAPK kinases. Can protect cells from apoptosis also by translocating to the mitochondria where it binds Bcl-2 and displaces Bcl-2 antagonist of cell death.

The caspases identified in this sample were Caspase-3 and Caspase-4, both involved in the activation cascade of caspases responsible for apoptosis execution.

A protein named Apoptosis-inducing factor 1, mitochondrial (O95831) was identified in the WT-LT phospho sample. . This is a protein that has two different functions, can act as NADH oxidoreductase and as a regulator of apoptosis. In response to apoptotic stimuli (in this sample was not any stimulus of apoptosis, because the sample was untreated) it is released from the mitochondria intermembrane space into the cytosol and to the nucleus, where it function as a pro-apoptotic factor in a caspase-independent pathway. In contrast, functions as an anti-apoptotic factor in normal mitochondria via its NADH oxidoreductase activity.

Both flow through of these samples, OA-1-FT-LT phospho and WT-FT-LT phospho, have the protein TNFAIP8 that is involved in tumor progression.

- OA-1-LT- and FLT3-2-LT



From the comparison of the phosphoproteome of Molm 13 cells treated with OA and FLT3 we observed that the sample induced with OA has 321 unique proteins. In contrast the sample induced with the FLT3 ligand has 792 exclusive proteins.

The OA1-LT phospho sample has some proteins that are important in the apoptotic process the cells experienced when were induced with Okadaic Acid. Inactive serine/threonine protein kinase VRK3 (Q8IV63) was identified in this sample. This is an inactive kinase that suppresses extracellular signal regulated kinases (ERK) activity by promoting phosphatase activity of Dual specificity protein phosphatase 3 that specifically dephosphorylates and inactivates ERK in the nucleus.

Another protein found was MOB kinase activity 2 that stimulates auto-phosphorylation and kinase activity of Serine/Threonine protein kinase 38 (STK38). STK38 is a negative regulator of MAP3K1/2 signaling. Converts MAP3K2 from its phosphorylated form to its not phosphorylated form and inhibits auto-phosphorylation of MAP3K2. The de-phosphorylation of ERK and MAP3K2 implies the cell death and the tumor do not progress.

NK tumor recognition protein was observed in OA1-LT phospho sample and it is involved in the function of Natural Killers (NK) cells. These cells are type of cytotoxic lymphocyte and responds to tumor formation by inducing the death of tumor cells.

Proteins that stimulate cell Molm-13 proliferation were found in the sample FLT3-LT phospho. B-cell lymphoma/leukemia 10 (O95999) is a protein that contains a caspase recruitment domain (CARD) and has been to induce apoptosis and to activate NF-kappaB. This protein is found to form a complex with the paracaspase MALT1.

MALT1 and their protein synergize in the activation of NF-kappaB, and the deregulation of either of them may contribute to the same pathogenetic process that leads to the malignancy. NF-kappaB is used as a regulator of genes that control cell proliferation and cell survival. Many different types of human tumors have NF-kB active. Active NF-kB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis. NF-kappaB was not identified in this sample because TNFAIP3 interacting proteins 1 is identified and acts as an inhibitor of NF-kappa-B activation. The inactivation of NF-kappa B leads to cell death what it is rare because the activation of FLT3 receptor implies cell proliferation and tumor progression. Otherwise a protein that acts as a negative mediator of apoptosis and plays a role in tumor progression was distinguished, this protein is named Tumor necrosis factor alpha induced protein 8 (O95379). This protein suppresses the TNF-mediated apoptosis by inhibiting caspase 8 activity.

In the flow through of this sample (FLT3-FT-LT phospho sample) were identified B-cell CLL/ lymphoma 7 protein family member C that plays an anti-apoptotic role and Apoptosis regulator Bcl-2 that suppresses apoptosis by controlling the mitochondrial membrane permeability. Inhibits caspase activity either by preventing the release of cytochrome c (transmembrane protein) and/or by binding to the apoptosis-activating factor (APAF-1). Both, Cytochrome c and APAF-1 were also identified in this sample.



## 7. Conclusions

Although replicates of the proteome and phosphor-proteome of Molm 13 cells have to be performed in order to take conclusions related to the expression of proteins related with cells proliferation and death, after analyzing the results of the protocols optimization and being studying the function's of some proteins that were identified in each sample, the conclusions are:

- FASP digestion method allows the identification of higher number of proteins than ISD method.
- Titanium Dioxide phospho enrichment presented better yield than IMAC in terms of phosphorylated peptides.
- Protein found to be present in FLT3-LT sample, indicate that FLT3-ITD mutant could be located in the ER.
- Proteins responsible to inhibit apoptosis as Bcl-2 and Bcl-6 were identified in the non-treated Molm 13 sample, which is in accordance to the behavior of tumor cell samples.
- Okadaic Acid induced cells have many tumor suppressors as p53 and TNF proteins to promote the cell death. (la apoptosis es un proceso normal de muerte celular)
- FLT3 samples have TNFAIP8 protein that inhibits caspase 8, responsible to mediate apoptotic process.
- Non-treated and FLT3 treated samples have similar proteins that promote cell proliferation and inhibit apoptosis.

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