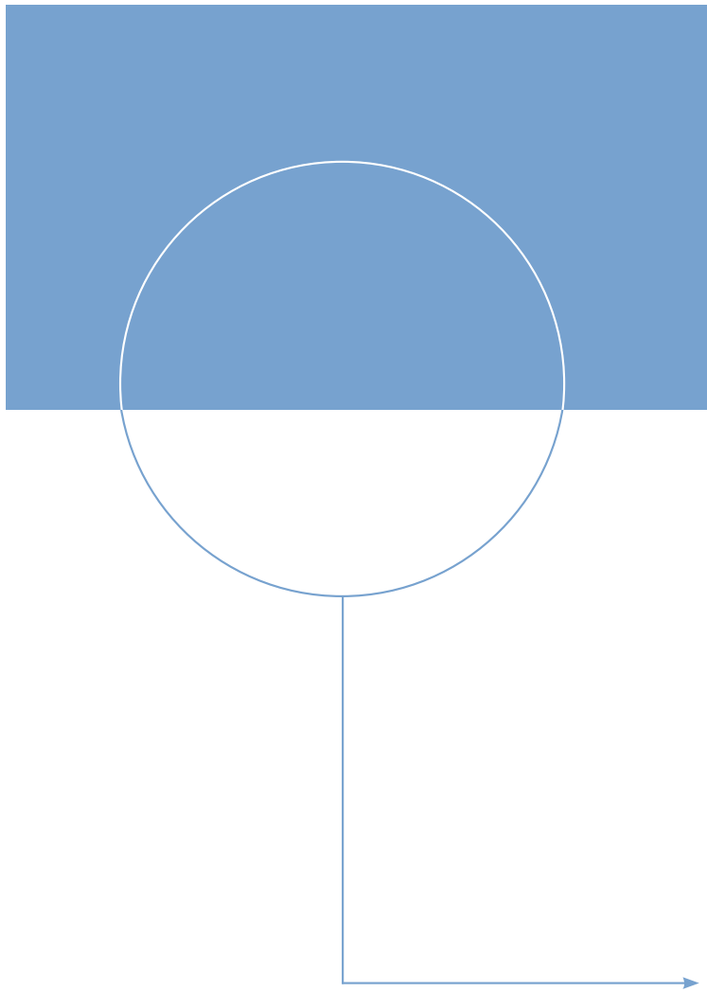

Master's thesis

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Cell and Molecular Biology
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Abstract

Multiple Myeloma (MM) is B-cell malignancy characterized by the accumulation and dissemination of antibody-secreting plasma cells throughout the bone marrow. MM is a disease with a high genomic heterogeneity with a slow progression. Despite the new advances and new treatments is still regarded as incurable, therefore, the finding of new targets and treatments are crucial. PRL-3 is a phosphatase that has been described to have an important role in migration in several malignancies. This role has been suggested to be important for malignant plasma cells migration. In addition, PRL-3 is activated by IL-6 stimulation, and important growth factor for MM cells.

In this thesis we analysed the alterations caused by PRL-3 in the phosphoproteome. This analysis revealed that PRL-3 under the stimulation of IL-6 can dysregulate the phosphorylation state of proteins related with several pathways such as, CD-28 stimulation, BCR signaling, cell cycle and interleukin signaling. In addition, the biological processes detected were related with protein post-translational modification, mRNA splicing, and cardiac development. After considering the study of the different pathways, we decided to focus on the B cell receptor (BCR) signaling, as it was an unexpected result, due to this pathway should not be relevant in differentiated plasma cells.

Our findings show that c-CBL is over-expressed and over-phosphorylated (y674) as a consequence of PRL-3 overexpression. Moreover, we confirmed a fact shown previously, that PRL-3 overexpression leads to the overactivation of LYN. We also suggest that this fact can protect PRL-3 cells against LYN inhibition with bafetinib. We also propose that PRL-3 overexpression can maintain the activation of this NF-κB pathway when is treated with a moderate inhibitor concentration (5µM). However, PRL-3 seems to not be related with other important tyrosine kinases for the BCR signaling, such as SYK or BTK. Our results indicate the PRL-3 is not related with the neither the overexpression nor the up-phosphorylation of BTK. Moreover, is possible that PRL-3 can downregulate BTK expression, and its phosphorylation on the y551 residue, the catalytic site of the protein. This differences in the expression and activity level, are not translated into any significant benefit, when BTK inhibition was tested. In addition, despite SYK inhibition can induce cell death in INA-6 cells, PRL-3 does not confer any advantage, against SYK inhibition.

In conclusion, after compiling all the results we cannot confirm that cells use PRL-3 to activate the BCR signaling to increase the cell proliferation and migration. However, we cannot discard the relevance of BCR signaling for MM cells´ development.

Table of contents

Abbreviations

7-AAD	7-amino actinomycin D
AKT/PKB	Protein kinase B
BCR	B-cell signaling
Biogrid	Biological general repository for interaction database
BLNK	B-cell linker
BM	Bone Marrow
BSA	Bovin serum albumin
BTK	Bruton´s Tyrosine Kinase
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CTG	CellTiter-Glo
CTLA	Cytotoxic T-Lymphocyte Antigen 4
CTR	Control
DEX	Differentially expressed
DMSO	Dimethyl Sulfoxide
DTT	dithiothreitol
DUSP	Dual-specificity phosphatases
ERK	Extracellular signal-regulated kinases
FCS	Fetal Calf Serum
FDR	False Discovery Rate
GO	gene ontology
HDAC	Mitogen-Activated Protein Kinases
HMCL	Human Myeloma Cell Lyne
HSP60	Hedgehog Protein 60
IC50	half inhibitory concentration
Ig	Immunoglobulin
IGF-1	Insulin-like-growth factor
IL	Interleukin
IL	Interleukin
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Loading Control
LMNA	Lamin A/C
MAPK	Mitogen-Activated Protein Kinases
MCODE	Molecular Complex Detection
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple Myeloma

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
ORA1	Olfactory receptor class A-like protein 1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Plasma cells
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kynase C
PLC	Phospholipase C
PRL	Phosphatase of regenerating liver
PS	phosphatidylserine
PTPs	protein tyrosine phosphatase
RLU	relative luciferase units
RPMI	Roswell Park Memorial Institute medium
Ser (s)	Serina
SHIP	Src homology 2 domain containing inositol polyphosphate
SIGNOR	SIGnaling Network Open Resource
SOCE	Store-operated calcium channel
SRRM	Serine/Arginine Repetitive Matrix
STAT	signal transducer and activator of transcription
STIM	Stromal interaction molecule
STRING	Search Tool for the Retrieval of Interacting Genes/Protein
SYK	Spleen tyrosine kinase
TGF	Transforming growth factor
Thr (T)	Threonine
TMPO	Thymopoietin
Tyr (y)	Tyrosine

1. Introduction

1.1. Multiple Myeloma

Multiple myeloma (MM) is a B-cell lineage malignancy characterized by the accumulation and dissemination of malignant plasma cells (PCs) throughout the bone marrow (BM). The symptoms are caused by the uncontrolled growth of plasma cells that leads to the overproduction of monoclonal immunoglobulins (or monoclonal immunoglobulin light chains), which is commonly referred to as the M component. Around 20% of patients have abnormal plasma cells secreting only monoclonal free light chains, and a small percentage of patients do not secret the M component. Common clinical features are usually referred as CRAB, hypercalcemia, renal failure, anaemia, and bone lesions (2, 3).

There is a set of diseases caused by the accumulation of the monoclonal protein M, named monoclonal gammopathies, of which the MM is part. The most common condition within this group is monoclonal gammopathy of undetermined significance (MGUS). This is an asymptomatic disease characterized by the infiltration of clonal plasma cells into the bone marrow, and the consequent accumulation of M protein. The appearance of this disorder usually precedes the development of MM. In some cases, an intermediate stage called smouldering myeloma can emerge. The progression through the different stages constitutes a slow process driven by the accumulation of genetic alterations (Figure 1) (2, 3).

These genetic alterations can occur during the process of generating the immunoglobulin repertoire of B-cells. This is due to the double-strand DNA breaks that happen in the immunoglobulin loci that are necessary for class-switch recombination and somatic hypermutation. These DNA breaks can generate aberrant fusions of DNA and chromosomal translocations, which normally are removed without consequences. Nevertheless, these abnormal DNA arrangements can include some oncogenes that give cells a growth advantage over the normal cells, that help them in the evolutive race within the bone marrow microenvironment (2). Therefore, translocations could be a possible initiating event that led to the emergence of MGUS and the subsequent stages that may eventually end in MM. In addition to translocations, aneuploidy could be an alternative or cooperating event that end in the appearance of MM cells (2, 4).

With regard to epidemiology MM, The American Cancer Society predicts that in 2021, in USA, almost 34,920 new cases will be diagnosed, which will lead to approximately 12,410 deaths (5). This makes MM the second most common hematologic malignancy after non- Hodgkin lymphoma, representing around a 10% of all hematological malignancies (2). Nowadays, despite the advances in understanding the disease, the most cutting-edge treatments they are only capable of prolonging the patient survival by 2 or 3 years, thus MM remains incurable with a high relapse frequency (2, 3, 6, 7). The aforementioned reasons illustrate the urgent need for further research which focusses on targeting the weak points of MM.

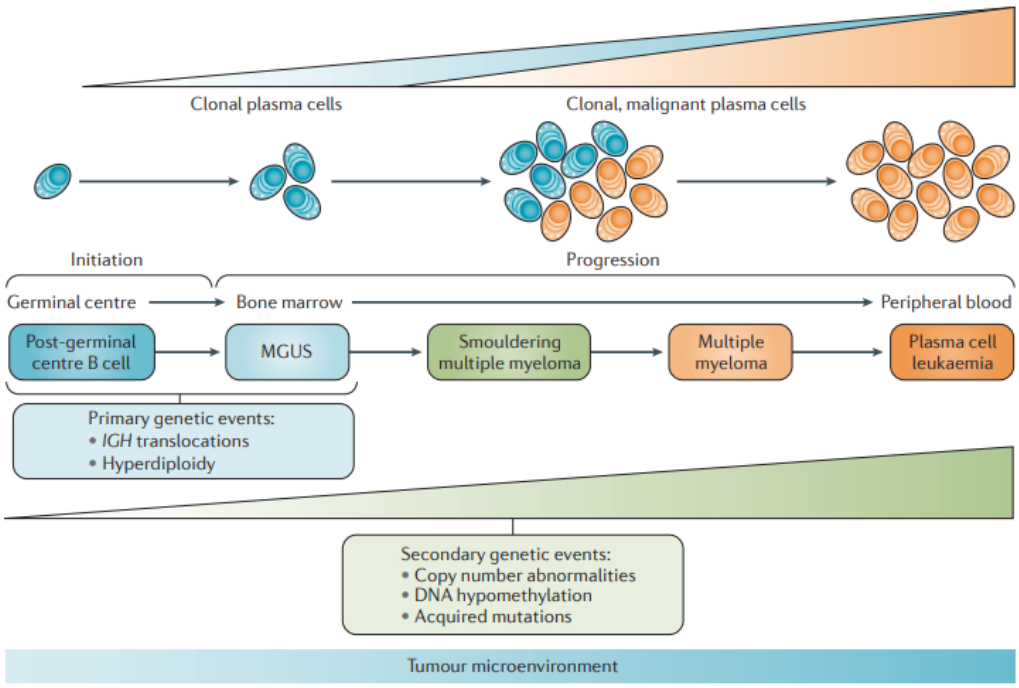


Figure 1. The development of monoclonal gammopathies. Multiple myeloma follows a slow multistep process which starts with the emergence of precursor asymptomatic disease stages, such as monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM). These stages are usually caused by primary genetic events, including translocations in the immunoglobulin heavy-chain gene (IGH), aneuploidy, and hyperdiploidy. The primary genetic events will predispose the cells to secondary genetic events, causing further accumulation of mutations and leading to MM. The disease can continue accumulating mutations and progress to bone marrow-independent diseases, such as extramedullary myeloma and plasma cell leukaemia. Reprinted by the permission of Spring Nature: Copyright © 2017, Macmillan Publishers Limited (2).

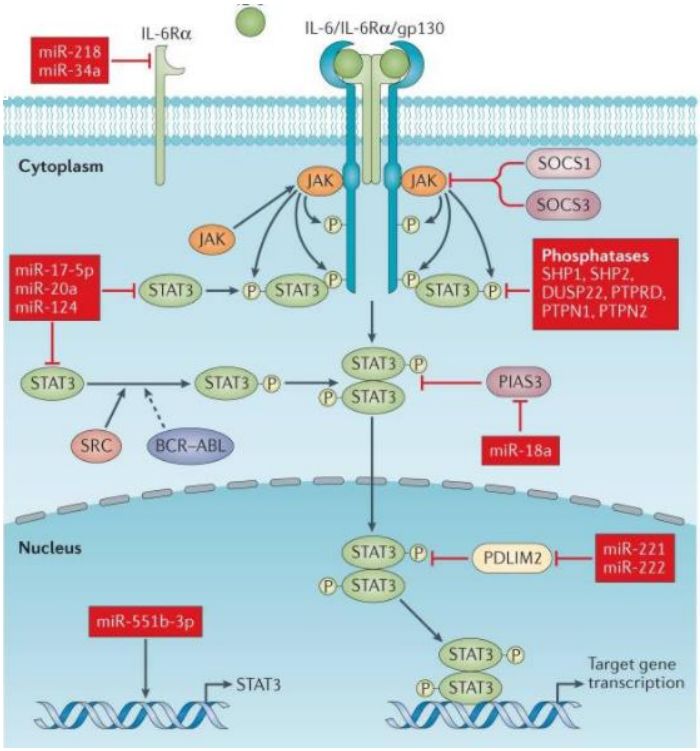
1.2. Growth factors

Myeloma cells require growth factors provided by BM microenvironment for proliferation and survival, many of these growth factors are cytokines(8, 9). Cytokines are small (15–20 kDa) and short-lived proteins important in autocrine and paracrine signalling. They are considered as key factors for the development and the activity of the immune system (9, 10). One of the most important cytokines for the malignant plasma cells survival in the BM is interleukin (IL)-6. When this cytokine binds to its receptor it activates MAPK and JAK/STAT3 signalling pathways leading to IL-6 increasing MM cell proliferation, survival, drug resistance and migration (Figure 2a) (11, 12).

In addition to IL-6, BM cells secrete other growth factors such as insulin-like growth factor 1 (IGF-1) (13). This compound is a key anabolic growth factor that has been described as a proliferative and antiapoptotic factor in MM, that also plays an important role in the process of MM dissemination. Those characteristics make this compound as an important factor for the progression to extramedullary disease, as it is able to promote migration through the activation of the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathway in MM cells. This pathway drives the acquisition of epithelial to mesenchymal transition-like features, helping in the dissemination process (Figure 2b) (13, 14).

Considering the relevance of cytokines in MM, researchers have been focused on understanding their functions in order to develop new treatments. However, no cytokine inhibiting drug has succeeded in clinical trials yet. This is probably due cytokine redundancy, meaning that several cytokines can activate the same pro-survival signalling pathways. Thus, targeting intracellular signalling protein downstream of multiple cytokines holds greater promise (11). The phosphatase of regenerating liver-3 (PRL-3) has been previously identified as one of the molecules that were up-regulated in the cytokine-dependent myeloma cell lines after stimulation with various growth factors, including IL-6 and IGF-1(15).

A)



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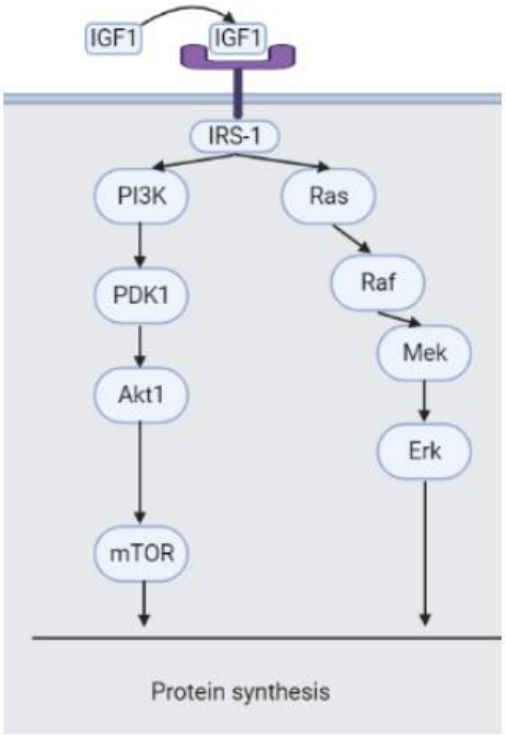


Figure 2. Cytokines can activate multiple pathways.

A) Pathway responsible for the protein synthesis by the stimulation of IGF-1.

B) Pathway responsible for the increase of MM cell proliferation, survival, drug resistance and migration, triggered by IL6.

1.3. Protein phosphorylation

Protein phosphorylation is one of the most common post-translational modifications which regulates the biological processes within a cell, thus alterations in the phosphorylation pattern can promote the emergence of malignancies (16-18). When a phosphate group is either added or removed, the protein´s physical characteristics are altered, which in turn changes the respective activation state (19).

The enzymes in charge of phosphorylation and dephosphorylation are kinases and phosphatases, respectively (20). Aberrations in the regulation of the activity of these enzymes can provoke alterations in signalling pathways that lead to the emergence of malignancies. By analysing the phosphoproteome it is possible to investigate aberrantly activated signalling pathways and identify therapeutic targets in cancers (16, 21).

1.4. PRL family

The phosphatase of regenerating liver (PRL) family is part of a bigger family of enzymes called protein tyrosine phosphatases (PTPs). These phosphatases have crucial roles in the regulation of essential signalling pathways involved in the control of cell proliferation, adhesion, migration, differentiation, and survival/apoptosis, and when these pathways are altered (22, 23), they contribute to some of the hallmarks of cancer (24). For this reason, the aberrant tyrosine phosphorylation caused by the altered PTP activity or expression has been implicated in the progression of some malignancies.

The PRL family is a well conserved group of phosphatases formed by three members, PRL-1, PRL-2 and PRL-3. In the past few years PRL enzymes have emerged as important proteins involved in the metastatic process in many cancer types, as elevated PRL expression, especially PRL-3, was found in those aggressive tumors (22, 25, 26).

The PRL members are expressed in different healthy tissues. PRL-1 and PRL-2 are almost ubiquitously expressed, while PRL-3 have a more restricted expression pattern (22, 27), this fact makes this member a more promising target to prevent cancer development.

1.3. PRL-3

PRL-3 is encoded by the gene PTP4A3. In healthy individuals, it is expressed during earlier developmental stages, in tissues related to the vascular system, such as fetal heart, developing blood vessels, and developing erythrocytes, but not in their mature forms (22, 25, 28). This expression pattern places PRL-3 as a possible key protein in the regulation of angiogenesis observed in the tumors. Therefore PRL-3 expression has been described ectopically in many cancers (29) . Taking these facts into account, PRL-3 has been proposed to be an important protein involved in the initiation of metastasis, as it has been observed to be deregulated early in the metastatic process, it could be a key metastatic driver that helps progression of malignant cells from primary to other tissues in the body (12, 25) .

In relation to MM, it has been associated with poor prognosis in MM patients as it can protect cells against apoptosis, and it constitutes an important factor involved in the acquisition of metabolic adaptations in MM (30, 31) .

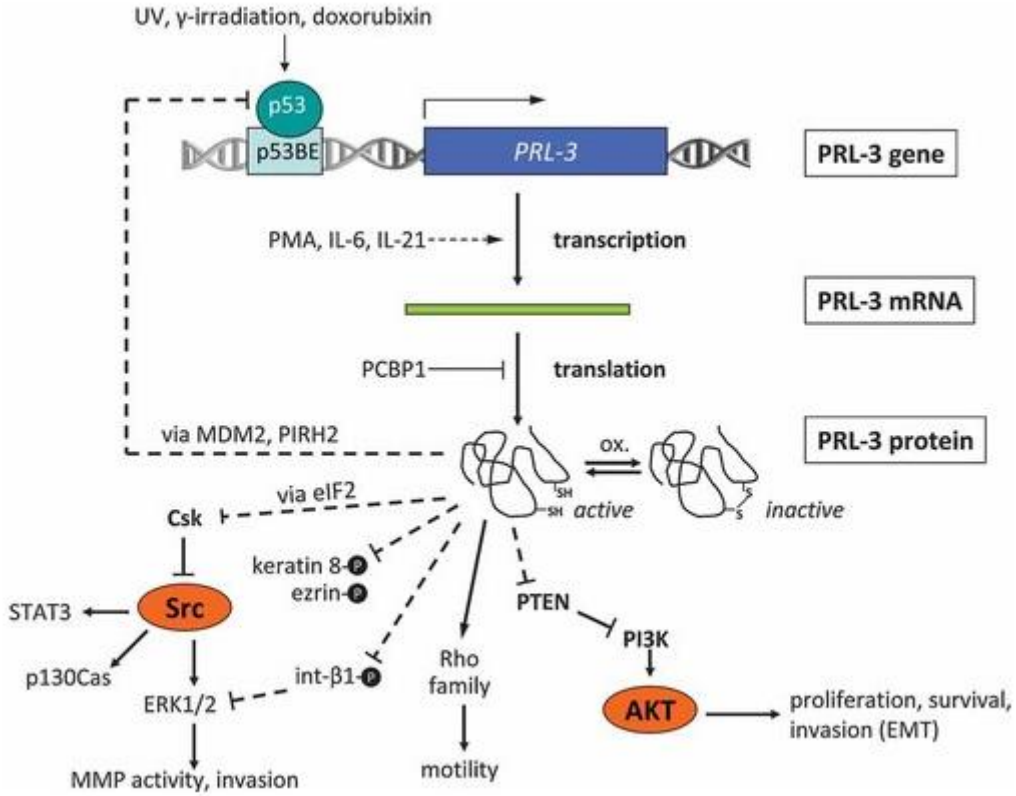


Figure 3. Previously described pathways trough which PRL-3 can induce metastasis.PRL-3 can inhibit some of the negative regulators that are able to control proliferation, survival, and invasion. It can inhibit phosphatase and tensin homologue deleted on chromosome 10 (PTEN) which is the key negative regulator of the Akt pathway leading to an uncontrolled proliferation and invasion. PRL-3 is also able to downregulate c-Src tyrosine kinase (Csk) expression so it can not regulate the activity of Src culminating in the excessive activation of some oncogenic pathways including ERK1/2, STAT3, and p130Cas, producing an increase in the cell invasion ability. PRL-3 is also involved in the downregulation of p53, an important transcription factor considered as the guardian of the genome due to its capacity to inhibit the proliferation of cells with chromosomic aberrations. Reprinted by the permission of John Wiley and Sons,

1.5. BCR signalling

In normal B-cells BCR signalling leads to the activation of B-cells in response to antigen binding to the BCR. The receptor is formed by a membrane-bound immunoglobulin (mIg) non-covalently associated with a heterodimer of CD79a (Igα) and CD79b (Igβ). When an antigen binds to the BCR, it triggers the activation of Src family kinases such as LYN and FYN that are responsible of the phosphorylation of the immunoglobulins that form the BCR (Igα and Igβ). This results in the phosphorylation of immunoreceptor tyrosine-based activation motives (ITAMs) in the BCR that lead to the recruitment of SYK kinase and its binding results in the activation and phosphorylation of Syk. The activation of SYK causes the phosphorylation of BLNK, which is an important protein that serves as a scaffold for the assembly of other proteins involved in the propagation of the signal, such as BTK and PLCγ2. This succession of phosphorylation events ends in the activation of the Ras pathway, PKC pathway, AKT/PI3K and calcium influx, that promote the activation of transcription factors such as NF-κB, Erk or JNK (32).

As the activation of BCR by an antigen provokes the activation of different transcription factors that induce cell differentiation and proliferation, so it is necessary to have a way of regulating this process to avoid an excessive proliferation. This is achieved

by negative signals that limit B cell activation and therefore preventing the appearance of malignancies. These negative signals are produced by the LYN kinase that is able to phosphorylate immunoreceptor tyrosine-based inhibition motifs (ITIM) of membrane receptors including CD22 (33, 34). Also, CBL is an important ubiquitin ligase responsible of the homeostasis of the BCR signalling targeting the ubiquitination of LYN and SYK (35). After the activation of the B-cell into a normal plasma cell, the BCR signalling is downregulated by those negative signals.

Therefore, BCR signalling pathway is essential for cell survival in normal B cells, as it activates several transcription factors that regulate cell fate decisions such as migration, proliferation, or apoptosis (36). It has been shown that this signalling pathway is altered in several B-cell malignancies, such as chronic lymphocytic leukaemia (CLL) and B cell lymphomas where the dysregulation of the pathway contributes to cell survival, proliferation, and resistance to apoptosis (36).

Consequently, BCR signalling pathway components have been investigated as therapeutic targets for some of these malignancies. However, little is known about the role of this cascade in MM, as plasma cells lose the BCR receptor complex, and the proteins in charge of activating the cascade in the healthy cell, appear downregulated once these cells are differentiated. Thus, plasma cells cannot recognize antigens anymore, although they still produce immunoglobulins in their secreted form.

In this way normal plasma cells can avoid an excessive activation and proliferation. This is the reason why the BCR signaling has been a target in some therapies for B-cells malignancies as they express the BCR complex, but in the case of MM this pathway has gone more unnoticed for researchers when they try to find new targets (37).

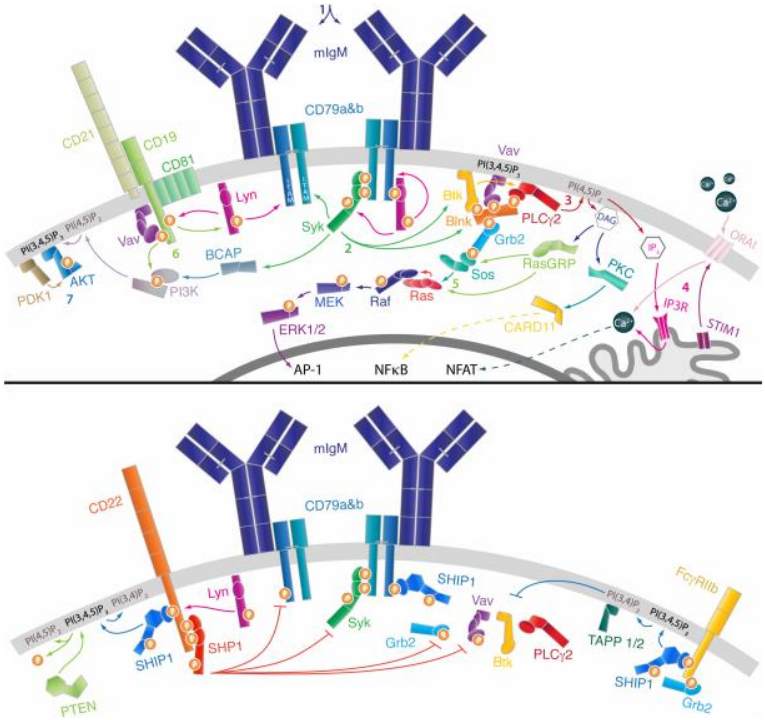


Figure 4. BCR signalling. A) Activation of the B-cell. B) Deactivation of the B-cell. Image extracted from (34). This image permits unrestricted use, distribution, and reproduction. Copyright © 2013 Faculty of 1000 Ltd.

1.7. Objectives

In this thesis we tried to confirm the results provided by the *in-silico* assays with in vitro assays. Therefore, the first goal was to identify the overrepresented pathways found in a phosphoproteomic list that shows the differences in the phosphorylation state of several proteins upon PRL-3 overexpression.

After using systems biology and bioinformatic tools to create a network and find the overrepresented terms, we hypothesized that MM cells can dysregulate the BCR pathway and take advantage of the pre-existing interactions to activate the pathways that lead to B-cell proliferation and survival. Therefore, we investigated the most important proteins implied in the BCR signalling and how PRL-3 affect their phosphorylation state. Thus, by targeting those proteins we can possibly mitigate the advantages that PRL-3 confers to malignant cell for their survival.

2. Material and methods

2.1. Cell lines and culture conditions

In this thesis all experiments were done using human myeloma cell lines (HMCLs). These cell lines are derived from MM patients with an advanced stage of the disease or from extramedullary sites. HMCLs are able to proliferate continuously when they are cultured in the appropriate growth medium. This characteristic makes it easy to work with - as there is almost always a high number of cells available to do experiments. One problem comes when the *in-vitro* assays are wanted to be extrapolated to the situation found in the original tumor (38). This is complicated as the BM microenvironment has a big influence on the behaviour of the MM cells (8).

HMCLs used in this thesis were cultured in a humid atmosphere consisting of 5% CO2 at 37°C with different mediums depending on the cell line. The HMCLs are from different resources:

- JJN-3. Kind gift from Dr. I. M. Franklin, University of Birmingham, UK (39) .
- INA-6. Gentle gift from Dr. M Gramatzki, Erlangen, Germany (40) .
- ANBL-6. Kind gift from Dr. Jelinek, Mayo Clinic, Rochester, MN, USA (41) .
- U266. From America Type Culture Collection (ATCC), Rockville, MD, USA (42)

Table 1. The growth medium for the different HMCLs.

HMCLs	Growth medium
JJN-3	10% FCS in RPMI
INA-6	10% FCS in RPMI + IL6 (1ng/mL)
ANBL-6	10% FCS in RPMI + IL6 (1ng/mL)
U266	10% FCS in RPMI

Abbreviations and sources:

FCS: Fetal calf serum (gibco ® by life technologies)

RPMI: RPMI 1640 supplemented with 100 mg/mL of L-glutamine (both from Sigma-Aldrich, USA)

IL-6 gibco ® by life technologies

2.2. Bioinformatics

In the last years bioinformatics has emerged as an approach to increase the efficiency of the drug discovery process as it allows us to use large datasets acquired from the basic science at the same time(43). The wide diversity that we found in the genetics of cancer cells makes it difficult to study the genetic variations that lead to the appearance of malignancies. In this context, a bioinformatic approach can be used to identify the key altered pathways which the oncogenic properties rely on (43, 44).

Phospho-proteomics data provided by S. Elsaadi *et. al* was analysed using different databases that allow us to have a system approach to how PRL-3 influences the phosphorylation of the different proteins.

The data show how PRL3 overexpression can influence the serine/threonine and tyrosine phosphorylation of different proteins. This data includes the phosphorylation state of several proteins obtained from modified INA-6 cells (overexpressing PRL3 and control MOCK) in different conditions. In this thesis we only analysed the list of

differentially expressed (DEX) phospho-proteins that show the differences in phosphorylation of PRL3 against MOCK in presence of IL-6.

In order to analyse the data and know in which process are the proteins involved we decided to use a systems biology approach.

Systems biology can be defined as the science that "studies biological functions that emerges from interactions at all levels of the biological hierarchy" or, in other words, systems biology studies how the systems govern life (45).

Nowadays we can take advantage of the advances in the sequencing methods that allow us to get a vast amount of data with a relative low cost. This improvement in the data obtaining has been proportional to the development of new bioinformatics tools to analyse these data (46, 47). Using these tools, we are able to analyse a lot of data in an easier and faster way, allowing us to have a more comprehensive view of the interactions that take place in a cell and how those interactions can be affected by a disease or by external conditions. These advances together have allowed molecular biology to have a system approach. If we apply the study of the system properties to molecular biology, we can decipher the complexity and variety of interactions that happen inside a live cell. By employing the fundamentals bases of Systems biology, we can create detailed models of cell regulation, focusing on the signal-transduction cascades, thus providing a new point of view that can complement the classical methods used in drug discovery(48). Using these models, it is possible to make predictions that help to identify new targets for drugs, saving time and money compared to trial-and-error experiments. Furthermore, models can be used to find hidden mechanisms that help to alleviate and predict the side effects of drugs (45, 47, 48).

To that end, we used phospho-proteome data obtained from MM cells that overexpress PRL-3 to create system of interacting molecules. This type of modelling based on system biology can provide the basis to reach a better biological understanding about disease progression.

To create a network based on system biology we used Cytoscape. This software allows the creation of integrated models of biomolecular interaction networks. It is frequently used in science as it has the possibility to add multiple applications that makes easier to create a network with nodes and edges that connect them. All nodes that we add were from Homo sapiens genes, so all analysis we did were based on Homo sapiens biology(49).

To create the network, we imported validated protein-protein interactions from high throughput studies from Biogrid. This is the Biological General Repository for Interaction Database, which is an open access database dedicated to manually curated interactions extracted from publications and archives of protein, genetic and chemical interactions for all major model organism species and humans(50).

In order to confirm some interactions, we used SIGNOR 2.0 and String. Signor is a database that provides experimentally validated causal interactions between biological entities. SIGNOR acts both as a source of signalling information and a support for data analysis, modelling, and prediction. It works in a similar way as other databases already mentioned because it shows any protein-protein interactions in network. It is extremely

useful since it shows the mechanism behind each interaction and how it affects the proteins involved, as it illustrates whether the interactions cause either up or down-regulations in the target entity(51). To continue adding interactions and confirm the ones we had already added we used STRING (Search Tool for the Retrieval of Interacting Genes/Protein). This is an association database that collects, scores and integrates all knowledge published of protein–protein interaction information. It generates protein-protein interaction maps combining direct experimental evidence with computational predictions, including direct (physical) as well as indirect (functional) interactions, it also gives a general overview of the overexpressed pathways in the network and is also able to group the genes in clusters (52, 53).

Therefore, we ended up with three different networks that we merged into a final network. Although most interactions come from the Biogrid network, as interactions from high throughput experiments were manually added. String completed the network with experimental validated interactions from the primary interaction databases organized in the IMEx consortium, and from also interactions from Biogrid(52) that maybe were unvoluntary omitted. To ensure that the interactions added from String were reliable, we added only those ones that were confirmed by experiments and collected in the manually curated pathway databases that are integrated in the String software. As Signor only takes into account experimental data and is a manually curated database, all interactions shown by this tool were considered reliable. In this way it was possible to cover almost all the relevant interactions that connect the entities obtained from the phosphoproteomic set. The number of nodes and edges and how many of them were added from each network are shown in the Figure 5, and they are further explained in the Results section.

After we imported all the interactions that connect the proteins within our dataset, we use Reactome and BiNGO to figure out the pathways and GO terms appear overrepresented.

BiNGO is an opensource Java tool that can determine which Gene Ontology (GO) terms are significantly overrepresented in a set of genes. BiNGO takes advantage of Cytoscape´s interface to generate a hierarchical tree of overrepresented GO terms within an input network.

On the other hand, Reactome Knowledgebase was used to make an overrepresentation analysis and compare it with what we obtained in the BiNGO analysis. Reactome gives an overrepresentation of the

Figure 5.Diagram that shows the network creation process. The blue rectangles represent the databases used to import the protein-protein interactions that build the network, the yellow rectangles represent the tools used to look for the overrepresented ontologies or pathways, finally in orange rectangels represent the tools used to create clusters in order to have a better understand of the network organization.

pathways, while BiNGO gives an overrepresentation of GO terms, so by comparing them we can get a good view of the processes that our network is involved in.

These characteristics make Reactome a useful tool to understand different biological processes already known as well as a tool for discovering unexpected functional relationships(54). For doing the Reactome analysis the protein HLA-A was omitted as it has been named with several protein IDs, hence generating a lot of noise when searching for the overrepresented pathways.

BiNGO and Reactome make a hypergeometric test that gives a *P*-value. This P-value answers the question: "when sampling *X* genes (test set) out of *N* genes (reference set, either a graph or an annotation), what is the probability that *x* or more of these genes belong to a functional category or a pathway *C* shared by *n* of the *N* genes in the reference set?" (55). BiNGO and Reactome test the significance of all GO terms that are present in the tested set of genes. To perform this task, they make several statistical analyses at the same time, that can create false positives. In order to control this, both tools make a statistical validation of these false matches. As there is overlap between true and false positives, they can make a statistical estimation of the false positives present in the data processed. This estimation is called False discovery rate (FDR), and represents the corrected over-representation probability. FDR is the metric for global confidence assessment of a large-scale proteomics dataset (54, 56). This number is what we are using to select the most relevant and significant pathways and biological processes.

After using all these curated databases, we finished the network building, so the next step was to analyse it. With this purpose we made clusters, highly interconnected regions, to see how the network is organized. To perform this task, we used MCODE (Molecular Complex Detection) and jActiveModules. Both apps make clusters, but they use different algorithms. jActiveModules searches for molecular interactions within a large network to find expression activated subnetworks, for what is necessary to integrate some omics data. Such subnetworks are connected regions that comes from the final network that have significant changes in expression that distinguish them from the rest of the entities in the network. In the other hand, MCODE algorithm is based on graph-theoretical analysis, and makes clusters based on the density of the regions surrounding the protein of interest. These clusters usually correspond with molecular complexes. Thereby the principal difference between both apps is that jActiveModules considers biological processes because its clusters are based on the expression levels, or phosphorylation levels, depending on the omics data imported, whereas MCODE makes clusters based on the network structure, isolating dense regions (57, 58).

In order to know which nodes are the ones that hold the network architecture, we used the Cytoscape tool, Network Analyzer. Using this tool, we can figure out "the degree" that correspond to each node, this refers to the number of edges a node has. Usually, biological networks are held by few nodes, called hubs, that appear highly connected, with a high node degree. Most of the nodes are poorly connected, that is to say that they have a low node degree (59).

2.3 Viral transduction

INA-6 overexpressing PRL-3 and control MOCK were developed by retroviral transduction as it was described previously by other members in the group (31).

2.4. Inhibitors

-Ibrutinib (PCI-32765): It is a small molecule first designed by Celera Genomics as a selective inhibitor of Bruton's tyrosine kinase (BTK) that covalently binds to cysteine at position 481 in the kinase domain, so it blocks kinase activity so the BCR signalling pathway is interrupted (60, 61).

-Entospletinib (GS-9973): It is a second generation Syk inhibitor. As Syk is one of the most upstream proteins in the BCR signalling, it is possible to interrupt the processes activates by this pathway (62).

-PRT062607. It is a potent and highly selective small-molecule inhibitor of SYK discovered by Portola Pharmaceuticals Inc (South San Francisco, California)(63).

-Bafetinib. It is an orally active 2-phenylaminopyrimidine derivative that is able to inhibit the Src-family member Lyn tyrosine kinase, a protein with an important role in promoting and inhibiting the BCR signalling pathway, that acts as the first protein recruited by the BCR when an antigen binds to it (64).

The inhibitors Ibrutinib, Entospletinib and Bafetinib were diluted in Dimethyl Sulfoxide (DMSO), so in the experiments where these inhibitors were tested, DMSO was used as control. PRT062607 was diluted in sterile water, so water was used as control.

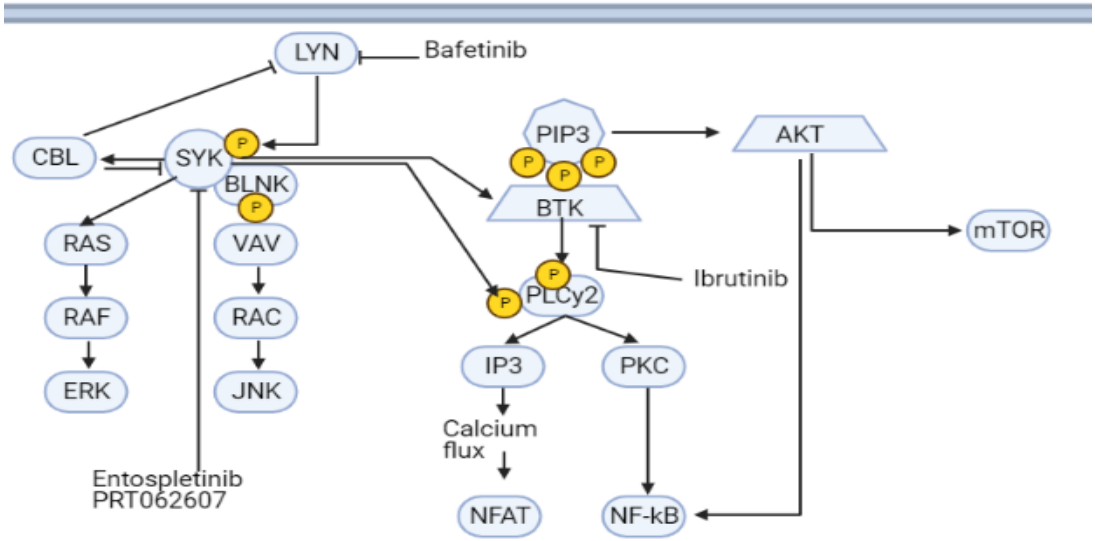


Figure 6. Simplified version of BCR signaling pathway in MM cells, where there is a lack of the immunoglobulins that form the BCR in addition to other surface molecules, such as CD19. The figure represents the targets of the inhibitors we are using in this thesis.

2.6. CellTiter- Glo (CTG)

In this assay we use the CellTiter-Glo Reagent that lyses the cells, so the ATP is released to the medium. The method is based on the mono-oxygenation of luciferin, this reaction is catalysed by luciferase in the presence of Mg^{2+}, ATP and O_2 . The result of this reaction generates light that is measured by a luminometer. The luminescence produced in this reaction is proportional to the ATP levels, that represent the live cells,

and is expressed in relative luciferase units (RLU)(65). Therefore, by this assay is possible to test how certain condition can affect to the cell proliferation and the cytotoxicity.

2.6.1. Protocol

We used 96-well opaque-walled with clear bottom plates (Corning Costar ®, NY). In each well 10.000 cells were seeded. We used different concentrations of the inhibitors using 2-fold dilutions, so we can test if there is a dose dependent effect of the inhibitor. The cells were incubated for 48 hours in a humid atmosphere of 5 % CO2 at 37° C. Once the incubation time has finished, the plate was maintained at room temperature for 30 minutes. After this time, we added 50 µL of the reagent CellTiter- Glo ®Luminiscent Cell Viability Assay (Promega, Madison, WI, USA) to each well. Following this step, the plate was shaken for 2 minutes using a plate shaker, and incubated for 10 minutes at room temperature, protected from the light. Lastly the plate was reading using the Victor Plate reader (PerkinElmer Inc. Waltman, MA).

We used this method to determine the cytotoxic effect of different inhibitor and tried to elucidate their IC50, the inhibitor concentration where the response is reduced by half. The IC50 was calculated using a graphical method. A horizontal line is drawn on the y-axis where y equals 50. This line is extended to the point where it intersects with the curve. The value which corresponds to this intersection point on the x-axis is then taken.

2.7. Annexin V-Alexa fluor 674 vs 7-amino actinomycin D apoptosis assay

This assay benefits from the versatility that flow cytometry offers to detect some of the apoptosis hallmarks. Thus, this tool allows us to do multiparameter measurements, which allows one to correlate different cellular events at a given time, single cell analysis, and rapid analysis of cell populations (66).

There are some changes in the plasma membrane that differentiate a healthy cell from a dead cell. Under normal conditions the membrane phospholipid, phosphatidylserine (PS), is located on the cytoplasmic surface of the lipid bilayer. However, during apoptosis PS is exposed in the outside part of the membrane, serving as a signal to macrophages to phagocytise the apoptotic cells. Therefore by dying the exposed PS is possible to differentiate the live cells from dead cells (66).

In this context we used an annexin V conjugate, called Alexa Fluor™ 647 annexin V conjugate. Annexin V is an anticoagulant protein that specifically binds to PS in the presence of Ca^{2+}(66). Thus, as we used Annexin V tagged it with Alexa fluor 647, so we are able to detect the PS using flow cytometry.

We also used a fluorochrome called 7-amino actinomycin D (7-AAD) to differentiate two populations, live and dead cells. This compound is a cationic dye that cannot penetrate in a cell with a healthy membrane, however the membrane makes more permeable in the late stages of the apoptotic process, so 7-ADD can penetrate in the dead cell staining them. It can intercalate into the double stranded DNA between base pairs in G-C rich regions(67).

It is important to notice that the cells become reactive to Annexin V before they acquire the permeability in the membrane that allow the 7-ADD to bind to the DNA. For

this reason, by the combined use of both compounds we can differentiate between live, apoptotic, and late apoptotic/secondary necrotic cells.

2.7.1. Protocol

To the set-up of the experiment, we used 96-clear bottom plates (Corning Costar ® NY, USA). In each well we seeded 50.000 cells and we treated with Bafetinib in concentrations that were from 20µM to 2,5 µM following series of 2-fold dilutions. As we used the same inhibitor tested in CTG, we incubated the cells in the same conditions, 5 % CO2 at 37° C for 48 hours.

After the incubation time cells were transferred to a flow tube with 2mL of 0,1% bovin serum albumin (BSA)-phosphate buffered saline (PBS), where they were spin at 1500 rpm down for 5 minutes. Cells were incubated for 20 minutes with a mix of 10 µL of Alexa Fluor™ 647 annexin V conjugate (ThermoFisher, Massachusetts, USA), and 1 µL of 7- ADD per 1 mL of Annexin Binding Buffer. Flow cytometry was performed with BD LSRII Flow Cytometer (BD, Biosciences, USA) using the filters PercpCy5.5 to detect the staining with 7-ADD and AF647 to detect Annexin V-Alexa Fluor 647. Afterwards, the results were analyzed using FlowJo v10.1 for Windows (FlowJo Software, Oregon, USA).

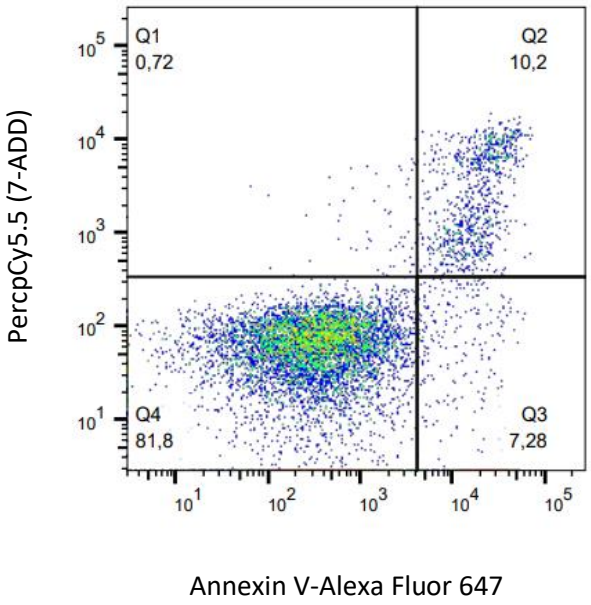


Figure 7. Example of annexin V- Alexa Fluor 647/7-ADD flow plot result. This graph represents INA-6 PRL-3 treated with DMSO used as a control to test Bafetinib. The Alexa Fluor 674 area is on the x-axis, while the PercpCy5.5 area is on the y-axis, and the dots represent the cells. Live cells occupy Q4 area, as in this area there are low levels of Annexin V and 7-ADD detection, as they do not express PS on their surface, and they are not permeable to cationic compounds. Cells in Q3 are in early apoptotic stages, so they express PS, but they are still impermeable to 7-ADD. Lastly in Q2 there are dead cells characterized by the expression of PS and the increase in membrane permeability to 7-ADD.

2.8. Western Blot

This technique is able to separate proteins according to their molecular weight by polyacrylamide gel electrophoresis (PAGE). Then the proteins in the gel are transferred to a nitrocellulose membrane thanks to the electric current. After this process the membrane represents a replica of the gel´s protein pattern, then we can target the protein of interest with a primary antibody, and later use a conjugated secondary antibody that is able to bind to the constant regions of the primary antibody, staining it. This is possible, for example by using an enzyme-conjugated secondary antibody, so the secondary antibody recognizes the primary antibody bound to the target protein and then by using a specific substrate is activated by the enzyme, it will create a detectable signal. Thus, by staining the primary antibody it is possible to identify a specific protein and know its expression level. Therefore, by this technique is possible to identify a specific protein from a complex biological sample and obtain information about its molecular weight. Moreover, as this technique is a semi-quantitative method, it also possible to use it to know the expression level of the protein of interest (68-70).

2.8.1. Protocol

Cells were stimulated and incubated with the necessary conditions and then they were harvested by centrifuging them for 5 minutes at 3400 rpm after being washed with PBS. Then cells were lysed on ice for 30 minutes using lysis buffer that contains phosphatase and protease inhibitors and detergents. The lysis buffer is a mix that contains 5mM of Tris ph 7.5, 150mM of NaCl, 10% of glycerol, 1% of IGEPAL® CA-630 (Sigma-Aldrich, USA), 1 tablet of protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Germany) and the protease inhibitors (1mM of NaF and 100 mM of Na3VO4). After 30 minutes of incubation with the lysis buffer, the samples were centrifugated at maximum speed at 4°C for 20 minutes, then the supernatant was transferred to new Eppendorf's tubes, as the pellet is a mix of nucleicacids and other elements that are not important for our purpose. The new Eppendorf´s tubes can be stored at -80°C if they are not going to be used on the same day.

At this point, it is useful to do an accurate determination of protein concentration using the Bradford assay (see point 2.7), this is to ensure there is enough protein in the sample, as the protein load capacity in polyacrylamide gels is limited(68).

After the exact concentration of protein present in each sample is known, we can decide if it is necessary to dilute the sample with lysis buffer or not. Based on the protein concentration we calculate the amount of sample buffer necessary (900 µL 4X NuPAGE ® LDS Sample buffer (Invitrogen) supplemented with 100 µL 1 M dithiothreitol (DTT)). Therefore, this mix of sample buffer with a reducing agent (DTT) makes the sample heavier than water, enabling it to sink into the well. Moreover, the sample buffer contains a negatively charged, low-molecular weight dye that will migrate at the buffer-front, allowing to monitor the progress of electrophoresis (71).

Once the samples were mixed with the sample buffer, they were heated for 10 minutes at 70 °C. Once this process has been done and the proteins have been denatured, it is possible to run the gel electrophoresis. The gels used were NuPAGE® 4-12% Bis-Tris Gels (Novex ® by Life Technologies, CA, USA). As a ladder, 2µ of SeeBlue® Plus2 Pre-Stained Protein Standard (Invitrogen) and Magic Marker were used. In addition, 20X

NuPAGE® MOPS SDS Running buffer (Invitrogen) was used. The gels were run using PowerEase® 300 W Power Supply (Invitrogen) using a two-step program, being the first step of 30 minutes at 80 V following by a second step of 90 minutes at 150V.

In this thesis two different techniques of blotting were used dry blotting and wet blotting. For dry blotting, iBlot® 2 NC Mini Stacks (Invitrogen) was used. Whereas for wet blotting it was necessary to apply electric current again during 2 hours at 80 V in 20X NuPAGE® Transfer Buffer with 10% methanol .The reason of doing these two different transfer methods was that wet blotting allows cleaner transfer, so they are usually more reliable and they are more popular to analyse larger proteins(72). On the other hand, dry blotting is an easier and faster way of transferring the proteins into a membrane.

Once the proteins were transferred to the nitrocellulose membrane, it was blocked for an hour with blocking buffer 5% BSA in TBS-T and 0,1% Tween, to avoid the unspecific binding of the antibody.

When the unspecific sites are blocked, the membranes were incubated for 1-3 days with the corresponding primary antibody. After the incubation time, the membranes were washed three times for 5 minutes using TBS-T before to incubating them with the appropriate secondary antibody for 1 hour at room temperature.

Finally, the membranes were washed 3 times for 10 minutes preceding to applying SuperSignal West Femto (Thermo Fisher Scientific, MA, USA) and visualizing them via Odyssey (LI-COR Biosciences, Lincoln, NE, USA).

With this technique we are looking for changes at protein level, so it is necessary to check the expression of a house keeping gene and have it as a loading control to ensure that the changes in the protein expression or phosphorylation are real and not caused by technical errors during the experimental setup. In this context the housekeeping genes used as loading control were β-tubulin, β-actin and Hedgehog Protein 60 (HSP60).

In this thesis this method was used as one of the main tools to detect changes on the expression and phosphorylation. Therefore, western blot allowed us to test if the overexpression of PRL-3 can cause changes in the proteins involved in the BCR signalling. In addition to the PRL-3 overexpression, we also tested the influence of growth factors and inhibitors. All the western blot experiment were developed using INA-6 cell line.

2.8.2. Relative protein quantification

Once the membrane is visualized using Odyssey, it is possible to make a relative quantification of the protein expression using Image Studio ™ (LI-COR Biosciences, Lincoln, NE, USA). Using this tool is possible to quantify the protein expression and compare it with the loading control expression, obtaining a ratio. This ratio allows one to detect differences in the expression level between the different condition.

2.8.3 Antibodies

- The primary antibodies used were:
  - From Cell Signalling Technology ®: phospho-BTK (y551); phospho-c-CBL (y674), phospho-c-CBL (Tyr700), phospho-p65 (NFKB), BTK.
  - From Biorbyt: phospho-LYN (y397)
- The primary antibodies used as loading control were from Cell Signalling Technology ®: β-actin; β-tubulin and HSP60.
- Secondary antibodies: Polyclonal Goat anti-Mouse Immunoglobulins/HRP; Polyclonal Goat anti-Rabbit Immunoglobulins/HRP (Dako ®, Denmark ApS).

The primary antibodies were diluted in the same solution used as blocking buffer, 5% BSA in TBS-T and 0,1% Tween. Whereas the secondary antibodies were diluted in TBS-T.

Table 2. Table that shows the different antibodies used in this master thesis.

Type	Company	Host production	Antibody	
Primary	Cell Signalling Technology ®	Rabbit	phospho-BTK (y551)	
	Biorbyt®	Rabbit	phospho-LYN (y397)	
	Biorbyt®	Rabbit	phospho-c-CBL (y674)	
	Cell Signalling Technology ®	Rabbit	phospho-p65 (ser536)	
	Cell Signalling Technology ®	Mouse	BTK	
	Cell Signalling Technology ®	Rabbit	c-CBL	
Primary used as Loading Control	Cell Signalling Technology ®	Rabbit	HSP60	
	Cell Signalling Technology ®	Rabbit	β-tubulin	
	Cell Signalling Technology ®	Rabbit	β-actin	
Type	Company	Host production	Directed against	Enzyme
Secondary	Dako ®	Goat	Rabbit	HRP
	Dako ®	Goat	Mouse	HRP

2.8.4 Growth factors

For these experiments, we used the cell line INA-6 overexpressing PRL-3 and INA-6 MOCK. We performed a stimulation of 15 minutes with the growth factors IL-6 and IGF-1. Considering that INA-6 cell line is IL-6 dependant, the stimulation with IL-6 is referred to an extra supplementation of this cytokine. So, cells were treated for 15 minutes using two conditions a concentration of 5 ng/mL of IL-6 and a concentration of 100 ng/mL of IGF-1.

2.8.5. Bradford assay

This assay is a quick and fairly sensitive method for measuring the protein concentration in a sample (73). In the experiment we tested different conditions, that can stimulate or inhibit the cell proliferations, so we expected the protein concentration to be affected by the different treatments. In this context the Bradford assay was useful to make a relative quantification of the protein concentration within a sample.

96-clear bottom plates (Corning Costar ® NY, USA) were used. First the necessary wells were filled with 250 µL of the Bradford reagent, Quick Start Bradford 1X (Bio-Rad Laboratories, Inc., USA). It is necessary to develop a standard curve, this was made using 10 µL, 5 µL, 2.5 µL, 1.25 µL, 0.6 µL and 0 of 2mg/mL BSA solution. These BSA concentrations are selected to cover the expected range in which our samples are expected to fit. Then in other wells 1.5 µL of the cell lyses were put. It is important to make duplicates of standard and of the samples. Then the plate is read by iMarker using a wavelength single measurement filter of 595 nm.

Based on the absorbance measured by the device is possible to create a standard curve by plotting the average absorbance at 595 nm as a function of concentration of protein standard. Then we can use the standard curve to determine the relative amount of protein in the samples through the absorbance measured.

Once we know the relative protein concentration in the sample, we can calculate the volume of lysis and sample buffer to add following these equations:

- Lysis buffer to add:

$$\frac{Protein\ concentration\ in\ sample\ x}{Concentration\ in\ the\ sample\ with\ the\ lowest\ contration} \cdot Volume\ in\ the\ sample\ x - Volume\ in\ the\ sample\ \text{x}$$

- Concentrated Sample buffer (LDS+DTT) to add:

$$\frac{New\ volume\ after\ adding\ the\ Lysis\ buffer\ calculated}{2.6}$$

2.9. Statistical tests

In order to assess if the differences observed in the performed CTG assays, a two-sample paired T-test was done, using Excel. The p-value considered as significant has to be lower than 0,05. This test can quantify if the differences between means of two groups are significant taking their variance or distribution into account (74).

3. Results

3.1. Identifying relevant pathways with a system´s biology approach

In this thesis we used a phosphoproteomic dataset that shows the differences in the phosphorylation pattern in INA6 cells that overexpressed PRL3 against those that express endogenous levels of the phosphatase (MOCK) under IL-6 stimulation. Therefore, we used bioinformatic tools creating a network that connect the genes which their phosphorylation state is affected by PRL-3. This allowed us to have a better understanding of the processes that are influenced by PRL-3, and what benefits can malignant cells get from the overexpression of this phosphatase.

3.1.2. Network creation

Since we already know the genes that will build our network, we only needed to connect them. With this purpose we used different databases to import validated protein-protein interactions.

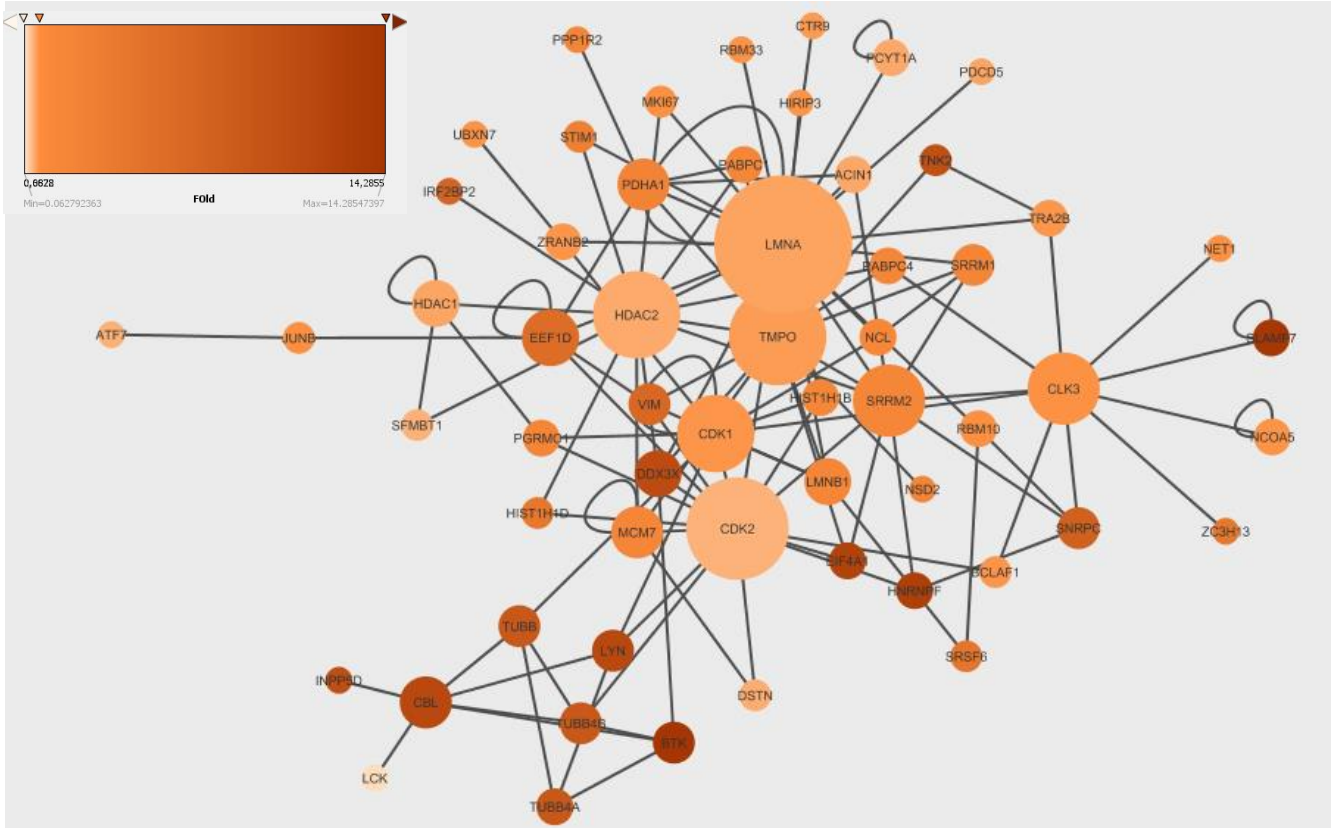


Figure 8. Network developed by importing protein-protein interactions from Biogrid. The colour scale is made in base of the fold change. This number represents if a protein is up or down-phosphorylated, if it is higher or lower than 1 respectively. Here, the colour scale that goes from 0,6628 to 14,2855 and it changes from white to orange from 0,6628 to 1, and from 1 to 14,2855 from orange to dark brown. The node size represents the node degree, this is the number of edges that the entity has.

As it was explained in the Material and Methods section, by using Biogrid we developed a network that show validated protein-protein interaction extracted from high throughput experiments. Thus, using this tool from the initial data set of 102 genes, we found that only 58 can interact directly. Therefore, we ended up with a network constituted by 58 nodes connected by 121 edges (Figure 8). This network shows the fold change, which is correlated with the phosphorylation level of the protein. Here we consider a protein to be up-phosphorylated if the fold change is greater than 1, and down-phosphorylated if the value is lower than 1. In the network this criterion is represented by a colour scale that goes from 0,6628 to 14,2855 and it changes from white to orange from 0,6628 to 1, and from 1 to 14,2855 from orange to dark brown (Figure 8). Moreover, the size of each node represents the node degree, the number of edges that each node has.

We developed new networks using tools that create their own protein-protein interaction networks, such as Signor 2.0 and String. Signor network was formed by 18 nodes and 17 edges, while the entities taken into account from String were 25 nodes connected with 32 edges. Several interactions and interactors from Biogrid, Signor and String coincide to a great extent. Thus, from all the nodes and edged that formed the String and Signor networks, only 9 nodes and 36 interactions were added to complete the network created using the information extracted from Biogrid, therefore ending the process with a network of 67 nodes and 157 edges.

In this way, by merging the three networks we can have an informative overview of the various processes, where the proteins in our list are involved. Finally, we ended up with a final network constituted by protein-protein interactions extracted from different databases.

3.1.3. The most significant pathways represented by the different networks

After the Reactome analysis of the different networks, we identified some relevant pathways to take into account.

3.1.3.1. CD28 and BCR signaling are important in the Signor 2.0 network

Signor 2.0 was able to link 18 proteins using 17 edges. This network shows the direct interactions that occur in a normal cell among the 102 proteins from our initial data set. With a later analysis of this network by Reactome we can appreciate that this network is very involved in the cytokine signaling, as different pathways related with those compounds appeared to be overrepresented. This is the case of “Signaling by Interleukins” and "Cytokine Signaling in Immune system". Other relevant pathways were “CD28 co-stimulation”, "Negative regulation of the PI3K/AKT network" and “CTLA4 inhibitory signaling”. Other important pathway in this network is the BCR signaling as it is represented by two pathways "Antigen activates B Cell Receptor (BCR) leading to generation of second messengers" and "Signaling by the B Cell Receptor (BCR)" (Tables 8 and 9 in Appendix).

In addition, a BiNGO analysis was performed, showing the importance of the proteins in regulating posttranslational protein modifications with process like phosphorylation and metabolic processes (Tables 8 and 9 in Appendix).

We can compare the relations showed by Signor, which are known to happen in a healthy cell, with the phosphorylation level observed in the data from the cell line INA-6 that overexpress PRL-3.

For example, STIM1 according to Signor should also be upregulated by MAPK1, in our data set PRL-3 makes MAPK1 to be over-phosphorylated, thus it can contribute to the later up-phosphorylation of STIM1 (Figure 9A).

The interaction between LYN and BTK is also relevant as they are up-phosphorylated in our data set and they are important entities of the BCR signaling. Signor 2.0 shows how LYN can upregulate BTK and they both are involved in a positive feedback loop (Figure 9A).

3.1.3.2 String emphasised the relevance of BCR and CD28 signaling.

String works in a similar way as Signor 2.0 as they integrate all the available information about protein-protein interactions from different public databases (51). Signor has the advantage of representing the effect that an interaction has on its target. Also, as it is a manually curated database the relations shown in this database are very trustable. Whereas String is dedicated to protein interactions at the widest scope, it shows the primary interactions from curated databases and it is also able to predict interactions and include annotated pathway knowledge or text-mining results(52).

In order to incorporate the most reliable interactions, the network was created using the interactions confirmed with experiments and from curated databases, in addition the interactions were filtered with a minimum required interaction score of 0,90. The score in String expresses the confidence, on a scale of zero to one, of the association being true, given all the available evidence (52). By analysing our protein set with String we ended up with a network of 31 entities connected by 51 edges (Figure 9B). The interactions that later were incorporated to the final network were those ones that came from both, curated databases, and experimental data, so from 51 edges we only considered 46. Moreover, String added new entities apart from what we import from the phosphoproteomic list, so after removing these entities the Signor Network was formed by 25 nodes connected by 32 edges.

We analysed the network created by String using BiNGO and Reactome to know which biological processes and pathways were overrepresented in the network.

The pathways shown as overrepresented by Reactome were very related with the cell cycle, the transitions through the mitotic phases and apoptosis, processes that are usually dysregulated in cancer diseases. In this context some pathways that are in charge of avoiding the replicative mistakes that lead to DNA damage, are also overrepresented, such as “Regulation of TP53 Activity” or "Activation of ATR in response to replication stress". Also, pathways related with the immune system were significantly overrepresented. This is the case of cytokine signaling, CD28 signaling, or BCR signaling ("Antigen activates B Cell Receptor (BCR) leading to generation of second messengers"). Other relevant pathways were “PIP3 activates AKT signaling”, "Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation" or "Constitutive Signaling by AKT1 E17K in Cancer" (Tables 8 and 9 in Appendix).

The BiNGO analysis shows that the proteins that form the network were clearly involved in the process of angiogenesis and development of the cardiovascular system, as the most significant overrepresented biological processes were related with the cardiac muscle cell and cardiac cell development (Tables 8 and 9 in Appendix). In addition, biological processes related with the cell cycle were also important. As String gives an overview of the functional enrichments in the network, we also looked the overrepresented pathways that Sting shows from other databases such as KEGG. This analysis confirmed what we showed before as it showed that the network is involved in regulating the cell cycle. It also showed the correlation of the proteins in the network with the cancer development, as pathways that are usually altered in cancer appeared overrepresented, such as p53 signaling pathway, apoptosis or microRNAs in cancer. Moreover, some of the pathways show a relation with hematological malignancies. This is the case of, B cell receptor signaling pathway, chronic myeloid leukemia or osteoclast differentiation.

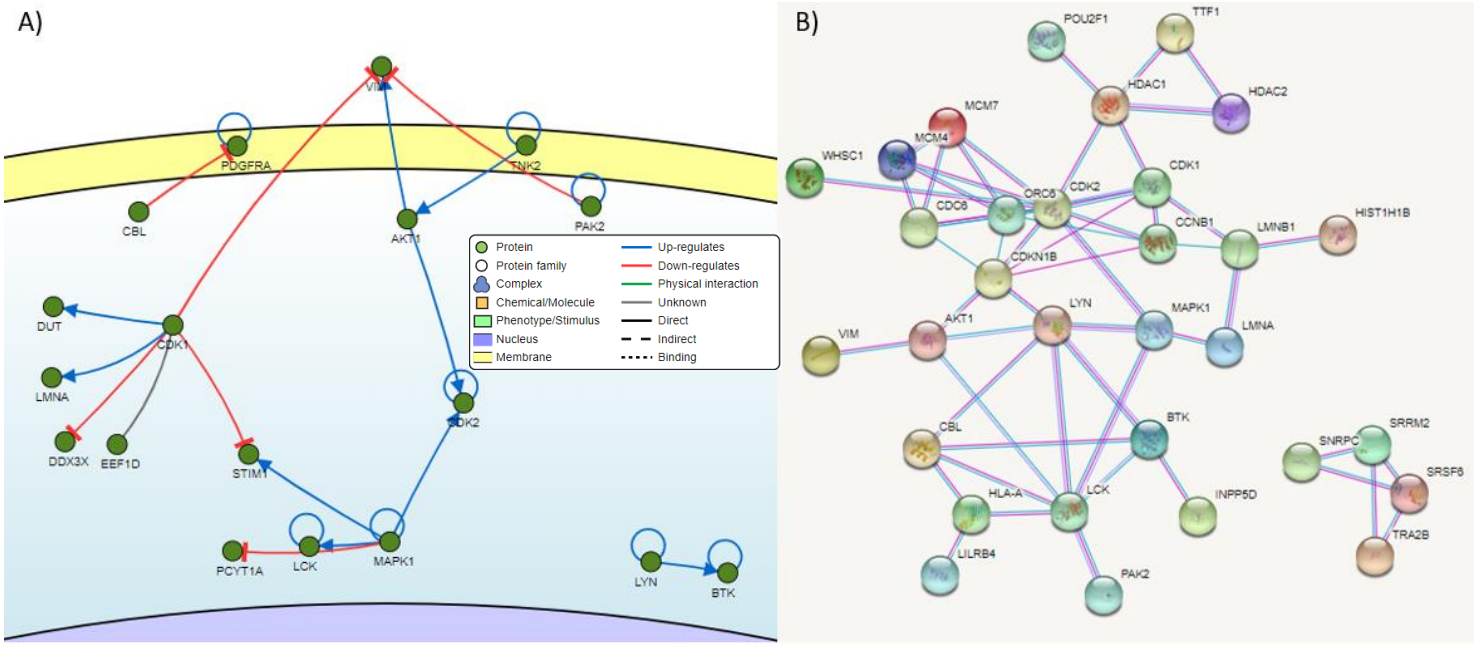


Figure 9. A) Network created by Signor 2.0. It shows how the proteins in our data set interact among each other affecting their activation and expression level, by up and downregulation processes. B) Network created by String that shows in blue the interactions imported from curated databases and in pink the interactions experimentally determined. The blue lines define the interactions imported from curated databases, while the pink lines mark the interactions experimentally determined, and the grey lines indicate that the connected proteins have some sequence similarity.

3.1.4. Final network

Once we have different networks with validated interactions but developed with different databases, we merged all of them into a final network of 67 entities connected with 157 edged. To have a first overview of the processes in which our network is involved we performed BiNGO and Reactome analysis. The BiNGO analysis showed that the overrepresented biological processes were related with the angiogenesis and cardiovascular development, as terms like "placenta blood vessel development", "labyrinthine layer blood vessel development" emerged. Terms related with the apoptosis and cell dead also appeared. Moreover, it should be noted the involvement of the proteins in the regulation of processes such as RNA splicing and post-translational process such as amino acid phosphorylation, more specifically, peptidyl-tyrosine modification (Table

8 Appendix). By observing the node degree of the network, we can see that the network architecture is composed by few nodes that are very connected, known as hubs. In our network the hubs were SRRM2, TMPO, HDAC2, CDK1, CDK2 and LMNA (Figure 25 Appendix).

Due to the extension of the network, grouping proteins into clusters (groups of highly related entities) makes it easier to perform further analyses. To achieve this goal, we used two different tools, jActive modules and MCODE.

The tool jActive modules generated two clusters. In the first one (Figure 10A), according to the results from Reactome, the pathways involved are similar to those that appeared overrepresented in the whole network, as the cell cycle and signaling by interleukins. Other pathways that have appeared before emerged again, “PIP3 activates AKT signaling", "Negative regulation of the PI3K/AKT”, “CTLA4 inhibitory signaling”, “CD28 dependent PI3K/Akt signaling” or “Regulation of TP53 Activity”. The biological processes extracted from the BiNGO analysis were related with the placenta development. In the case of the second cluster the overrepresented pathways shown by Reactome were related with the mRNA splicing and the cytokine signaling in the immune system, especially the “Interleukin-4 and Interleukin-13” signaling pathway. Moreover, the biological processes shown by BiNGO are also related to mRNA splicing and organ development, specifically placenta blood vessel development (Table 9 Appendix).

Finally, MCODE gave us a cluster related with the activation of the BCR by an antigen, it was also related with the regulation of signaling by CBL (Figure 10B, Table 9 Appendix). CBL is considered as a key protein for the BCR signaling downregulation. It can avoid the excessive activation of the pathway, acting as an ubiquitin ligase, ubiquitinating proteins for their degradation (35). Other relevant pathways overrepresented were "Regulation of KIT signaling ", "Depolymerisation of the Nuclear Lamina " and "Apoptotic execution phase". The biological processes that were shown by the BiNGO analysis were very related with the cardiovascular development, positive regulation of T cell receptor signaling pathway, and regulation of calcium ion transport via store-operated calcium channel activity.

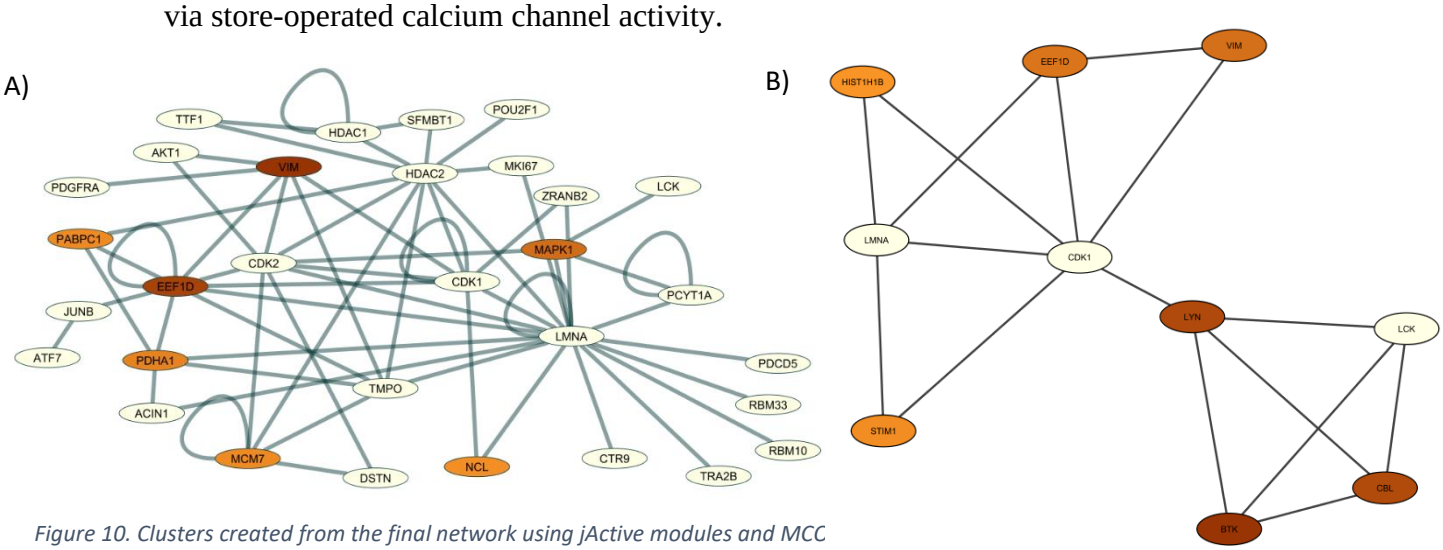


Figure 10. Clusters created from the final network using jActive modules and MCC colour scale shows in white the downregulated genes (fold change <1). The upregulated genes are classified in a colour gradient scale that ranges from a less intense orange to a more intense brown.

The pathways that appeared enriched in the Reactome analysis of the different networks are summarized in the Table 3.

Table 3. Overrepresented pathways detected by Reactome in the different networks. FDR: False discovery rate.

Overrepresented Terms Reactome	Merged Network FDR	Cluster Jactive 1 FDR	Cluster Jactive 2 FDR	MCODE FDR	Signor FDR	String FDR
CD28 co-stimulation	2,56E-04	4,04E-04	4,03E-03	3,02E-02	5,71E-06	1,39E-07
Signaling by Interleukins	2,20E-06	6,22E-04	1,10E-04	2,20E-06	2,64E-05	3,99E-08
CTLA4 inhibitory signaling	2,20E-06	1,76E-03	2,23E-03	7,24E-04	2,64E-05	3,23E-05
Depolymerizatio Nuclear Lamin	6,11E-05	1,67E-04	1,64E-04	3,31E-04	5,99E-04	7,71E-07
PI3K activates AKT	2,42E-02	1,44E-03	2,23E-03	not significant	4,13E-03	6,14E-04
KIT signaling	4,06E-04	not significant	not significant	not significant	4,14E-04	3,64E-04
Cell Cycle	2,56E-04	1,44E-03	5,44E-03	2,84E-03	1,31E-03	1,45E-11
Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	0,0861 (not significant)	not significant	not significant	4,66E-03	9,19E-03	4,73E-02
Signaling by the B Cell Receptor (BCR)	not significant	not significant	not significant	1,05E-02	2,55E-02	not significant
FLT3 signaling	2,72E-02	8,22E-03	not significant	not significant	2,56E-04	2,56E-04
mRNA splicing	2,91E-03	not significant	1,85E-03	not significant	not significant	9,87E-03
Apoptosis	1,68E-04	4,07E-03	2,85E-05	1,02E-02	8,93E-04	4,58E-05
IL-12 signaling	5,21E-04	not significant	not significant	9,37E-04	4,12E-02	7,89E-03
CD28 dependent PI3K/Akt signaling	9,47E-03	1,76E-03	7,70E-05	4,42E-02	6,50E-04	7,91E-04
Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation	2,75E-04	not significant	not significant		3,26E-02	8,12E-04
Immune System	1,60E-03	not significant	not significant	4,42E-02	1,96E-06	6,32E-07
ATR in response to stress	not significant	3,86E-02	not significant	not significant		5,67E-06
Interleukin-4 and Interleukin-13 signaling	1,28E-03	6,82E-05	1,11E-04	4,42E-04	6,58E-03	5,74E-04
Regulation of TP53 degradation	2,20E-02	4,83E-03	5,03E-03	1,02E-02	1,83E-03	2,51E-03

3.1.5. Effect of PRL-3 in BCR signalling

After all the bioinformatic analysis, despite of several pathways in which PRL-3 seemed to be relevant, we decided to focus on BCR signaling pathway, due to its representation in the MCODE cluster and the overrepresentation of the pathways activated by the BCR signaling such as PI3K-AKT, or the regulation of calcium.

Table 4. Proteins related with BCR signaling that their phosphorylation state is affected by PRL-3.

Entities related to BCR signaling influenced by PRL-3 overexpression	Entities influenced by PRL-3, related to pathways activated by BCR signaling
BTK	AKT1
CBL	MAPK1
LYN	
STIM1	
INPP5D	

3.2. In vitro assays testing relevant molecules involved in the BCR signaling.

In order to confirm the bioinformatic data, we performed different experiments to see the real effect of PRL-3 on the different entities that constitute the BCR signaling pathway.

The experiments, as we explained previously, were performed using the HMCL INA6, as the main cell line in most cases. This cell line is dependent on IL-6 for its survival (40), this cytokine is crucial for the survival of MM in the BM in vivo, so the

use of cell lines that are dependent of IL-6 represent a good model, as their characteristics are closer to primary MM cells than the IL-6 independent cell (75).

3.2.1. PRL-3 increases c-CBL expression and Y674 phosphorylation.

c-CBL is a E3 ubiquitin-protein ligase with implications in the negative regulation of BCR signaling. To know if CBL was up-phosphorylated as it was shown by the bioinformatic data, we performed a western blot assay looking for differences in the residue y674. There were differences observed between PRL-3 and MOCK in the control condition and in IL-6 condition, while the stimulation with IGF-1 seems to increase more the phosphorylation in MOCK cells (Figure 11).

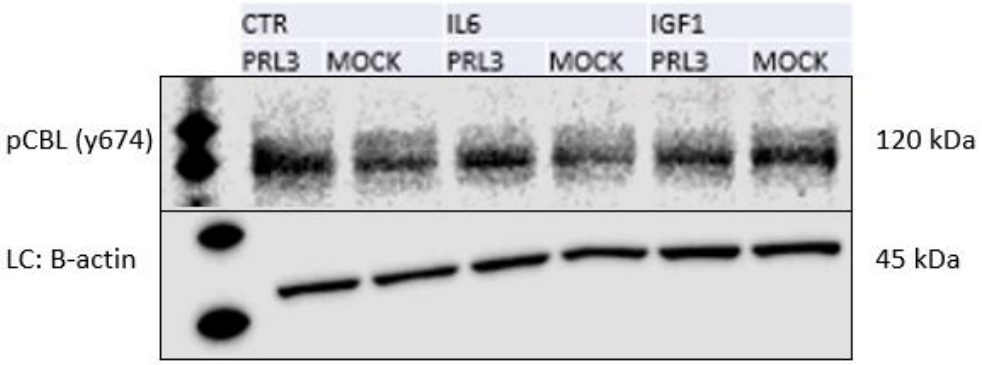


Figure 11. INA-6 PRL-3 and MOCK cells were treated with IL6 (5 ng/mL) and IGF-1 (100 ng/mL) for 15 minutes. Then they were immunoblotted with a pc-CBL antibody (y674). The result presented here is a representative result out of two biological replicates, where is possible to observe clear differences between PRL-3 and MOCK cells both in control (CTR) and IL-6 condition. However, the differences are not that clear in the IGF-1 condition, where the stimulation with this growth factor seem to affect more to MOCK cells. B- actin was used as loading control (LC).

The total expression level of c-CBL was also tested in order to see if PRL-3 overexpression affects only to the phosphorylation state of the protein, or if it also affects to the regulation of c-CBL expression. The results found were similar results to what it was observed before with p-c-CBL, as the expression was higher in PRL-3 than in MOCK in the control and IL-6 condition, however the expression in the IGF-1 condition was more balanced in PRL-3 and MOCK (Figure 12).

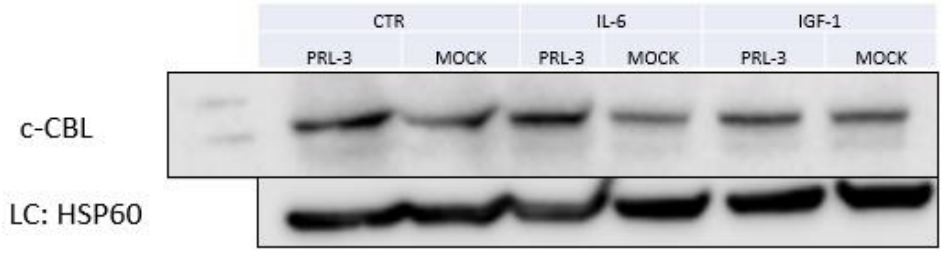


Figure 12. INA-6 PRL-3 and MOCK cells were treated with IL6 (5 ng/mL) and IGF-1 (100 ng/mL) for 15 minutes. Then they were immunoblotted with a c-CBL antibody. The result presented here is a representative result out of two biological replicates. It shows a similar result to the one shown above. It possible to observe differences between PRL-3 and MOCK cells in the control condition (CTR), where PRL-3 cells seem to over-express c-CBL. The differences observed were intensified by the IL-6 stimulation. However, the differences are less clear in the IGF-1 condition, where PRL-3 cells seem to not be affected by the IGF-1 stimulation. HSP60 was used as loading control (LC).

3.2.2. SYK

SYK is another tyrosine kinase in charge of activating BTK, so it is essential to activate downstream signaling events of the BCR signaling pathway (76). Due to this crucial role of SYK, we tested if PRL-3 can protect the cells from its inhibition.

3.2.2.1. The inhibition of SYK with PRT062607 induce cell death.

First, we tested the SYK inhibitor entospletinib. This inhibitor was tested in different HMCLs: ANBL6, U226 (Figure 13.A), in PRL3 and MOCK JJN3 (Figure 13.B) and in PRL-3 and MOCK INA-6 cells (Figure 13.C). The concentrations were from 25µM to 0,19 µM or 30µM to 0,18 µM.

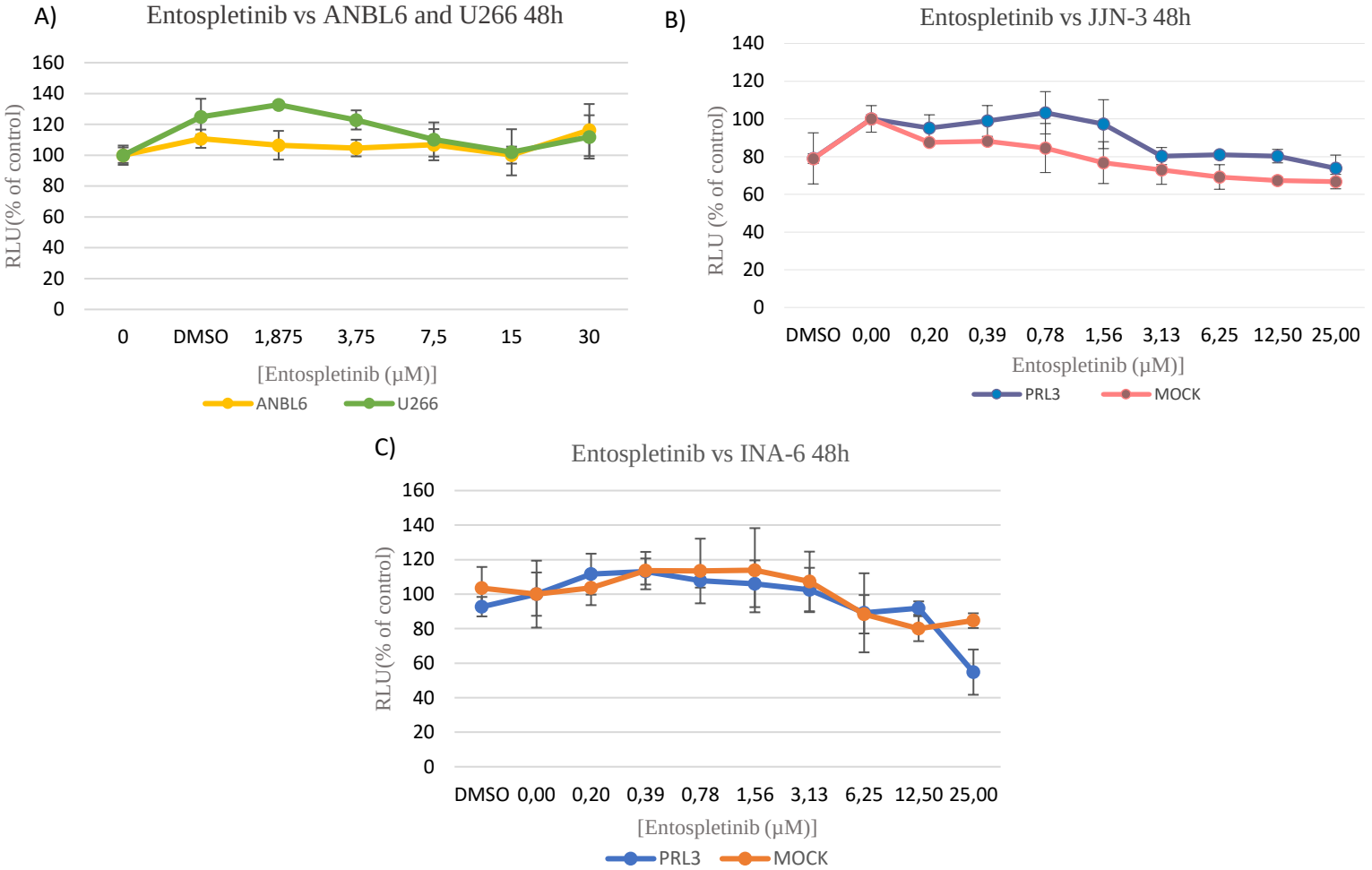


Figure 13. Entospletinib, a SYK inhibitor was tested against different cell lines. Entospletinib was not able to induce cell death, at any concentration, in neither of the HMCLs tested. A) Entospletinib vs ANBL6 and U266. ANBL6 and U266 cells were treated with entospletinib for 48h. The inhibitor concentrations selected were from 30µM to 1,875 µM following series of 2-fold dilution, using 0 µM and DMSO as controls. In this graph we can observe that entospletinib is no able to induce cell dead observed in neither of both cell lines. This result comes from one biological replicate of this experiment. B) Entospletinib vs JJN3. Entospletinib was tested against PRL-3 and MOCK JJN-3 cells. Both cell conditions were tested against different concentrations of entospletinib for 48h. The inhibitor´s concentrations were from 25 to 0,20 µM following series of 2-fold dilution, using 0 µM and DMSO as controls. There is no cell dead observed using this inhibitor against this cell line with the tested concentrations. This result comes from one biological replicate of this experiment. C) Entospletinib against INA-6. Entospletinib was tested against PRL-3 and MOCK INA-6 cells. Both cell conditions were tested against different concentrations of entospletinib for 48h. The inhibitor´s concentrations were from 25 to 0 µM following series of 2-fold dilution, using 0 µM and DMSO as controls. There is no cell dead observed using this inhibitor against this cell line with the tested concentrations. This figure is a representative result out of two biological replicates of this experiment.

The error bars shows the standard deviation of the replicates obtained in the same experiment normalized to the control (0,0 µM). The percentage of live cells is measured in Relative Luciferase Units (RLU).

The CTG assays performed shows that entospletinib is not able to induce cell death in neither of the HMCLs tested. Therefore, we used another SYK inhibitor, PRT62607, to see if the inhibition of this kinase can induce cell death. We used PRL3 and MOCK INA6 cells, against inhibitor concentrations that varied from 10 µM to 0,078 µM following series of 2-fold dilutions. As the Figure 14.A shows, PRT062607 was able to induce cell death in the INA-6 cells following a dose-dependent effect.

The IC50 was calculated only in the case of PRT062607, as it was the only SYK inhibitor capable of induce cell death. The IC50 calculated was the same for both PRL-3 and MOCK (1.98 µM) (Figure 14.B).

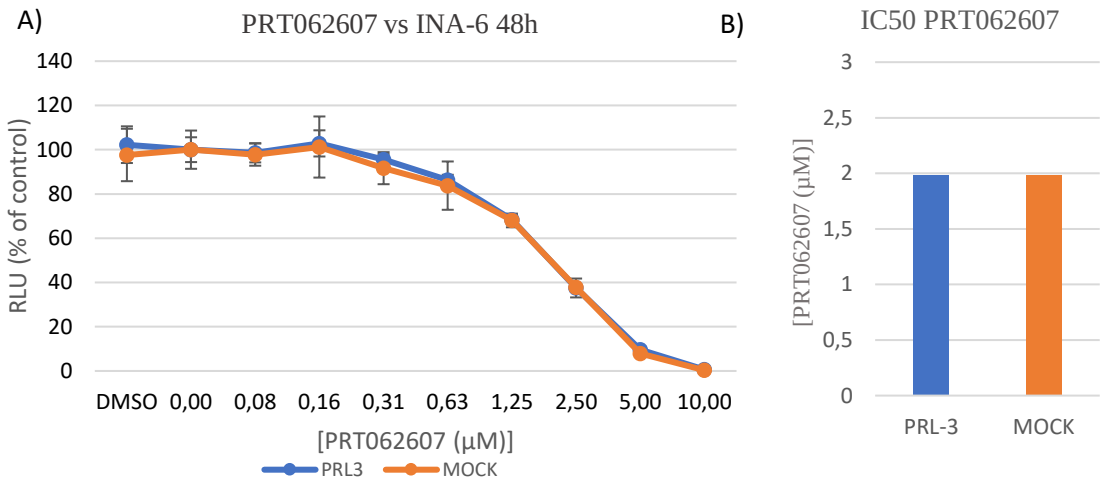


Figure 14. PRT062607 against INA-6. Entospletinib was tested against PRL-3 and MOCK INA-6 cells. Both cell conditions were tested against different concentrations of PRT06207 for 48h. The inhibitor´s concentrations were from 10 to 0,08 µM following 2-fold series, using 0 µM and DMSO as controls. This figure is a representative result out of two biological replicates showing that PRT062607 was able to induce cell death in both cell conditions, PRL-3 and MOCK. However, there are no observable differences between the responses from PRL-3 or MOCK cells against the inhibitor. The error bars shows the standard deviation of the replicates obtained in the same experiment normalized to the control (0,0 µM). The percentage of live cells is measured in Relative Luciferase Units (RLU).

B) Bar graph that shows the IC50 (half maximal inhibitory concentration) of PRT062607. It shows that the IC50 for both cell conditions was the same, therefore PRL-3 and MOCK cells response in the same way against the SYK inhibition The IC50 was calculated using the graph shown in the Figure 17.A, therefore there are no standard deviation of the mean.

3.2.3. LYN

LYN is the main Src-kinase in B cells, where it has a dual function in the BCR signaling, as it stimulates the signal initiation, but on the other hand it is considered as a critical negative regulator of B cell activation (33).

3.2.3.1. PRL-3 increases the activity of LYN.

How does PRL-3 affect to the phosphorylation state of LYN was tested by Western Blot, in different conditions. First, as the other kinases tested, we tried to show if the growth factors IL-6 and IGF-1 caused differences in the phosphorylation state of LYN. In the bioinformatic data LYN appeared up-phosphorylated at the residue Y397, the catalytic site of the kinase, so we tested this residue in the laboratory. The results show that there are differences in the phosphorylation state caused by the overexpression of PRL-3, but the action of the growth factors tested did not show relevant differences in the phosphorylation of LYN at its catalytic site (Figure 15). In addition, the same phosphosite was tested after 24h of treatment with bafetinib, to see if this LYN inhibitor is able to decrease the phosphorylation of the Y397 residue. The result was a decrease in the phosphorylation of this phosphosite after the treatment (Figure 16).

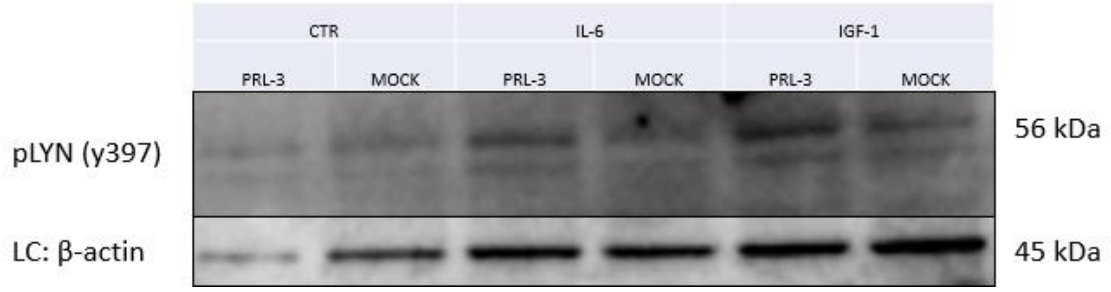


Figure 15. Gel showing the differences in phosphorylation of LYN at Y397 after 15 minutes of treatment with growth factors. INA-6 PRL-3 and MOCK cells were treated with IL6 (5 ng/mL) and IGF-1 (100 ng/mL) for 15 minutes. Then they were immunoblotted with a pLYN (y397) antibody. This gel is the only biological replicate obtained from this experiment. Despite the bad loading control observed in the control condition (CTR), it is possible to observe clear differences in the expression of pLYN between PRL-3 and MOCK cell under the stimulation with IL-6 and IGF-1. The stimulation of both growth factors is higher in PRL-3 cells. B- actin was used as loading control (LC)

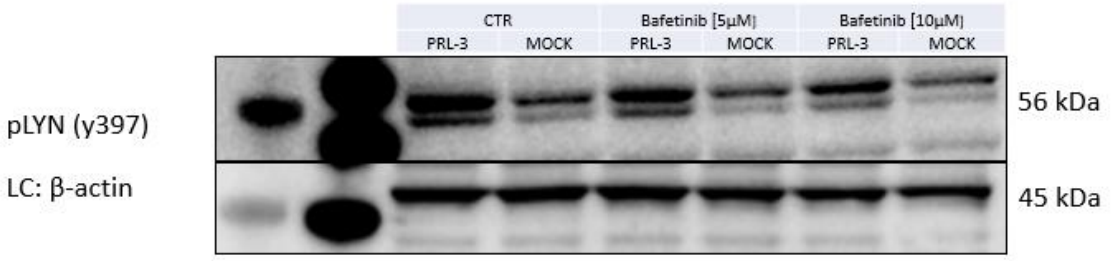


Figure 16. Gel showing the differences in the phosphorylation state between PRL-3 and MOCK in INA-6 cells of LYN, and the effect of bafetinib in the phosphorylation of the LYN´s catalytic site. INA-6 PRL-3 and MOCK cells were treated with two different concentrations of bafetinib,5 and 10 μM, for 24h. Then they were immunoblotted with a pLYN (y397) antibody. This gel is a representative result out of three biological replicates obtained from this experiment. It shows clear differences between PRL-3 and MOCK cells, being the expression of pLYN higher in PRL-3 cells in all the conditions tested. The action of the inhibitor shows a dose dependent effect of bafetinib in the LYN activation. This figure confirms that bafetinib can inhibit LYN at its catalytic site. B- actin was used as loading control (LC).

Abbreviations: CTR: Control.

3.2.3.2. PRL-3 protects against bafetinib

After the clear differences observed in the phosphorylation state of LYN at its catalytic site and the decrease in the phosphorylation caused by the treatment with bafetinib, we tested if PRL-3 can protect cells against the action of this inhibitor. Therefore, bafetinib was tested in PRL3 and MOCK INA-6 cells, using concentrations from 20 µM to 0,15 µM following series of 2-fold dilutions for 48 hours. The Figure 17 shows that PRL-3 conferred an advantage to the INA-6 cells when they face against bafetinib. By calculating the average IC50 obtained from three biological replicates, we can see that PRL-3 cells are more resistant to the LYN inhibition as the IC50 is higher (6,692 µM) than in MOCK cells (5,165 µM) (Figure 17.B). By performing a T-test is possible to see that these differences were significant (p-value=0,00459).

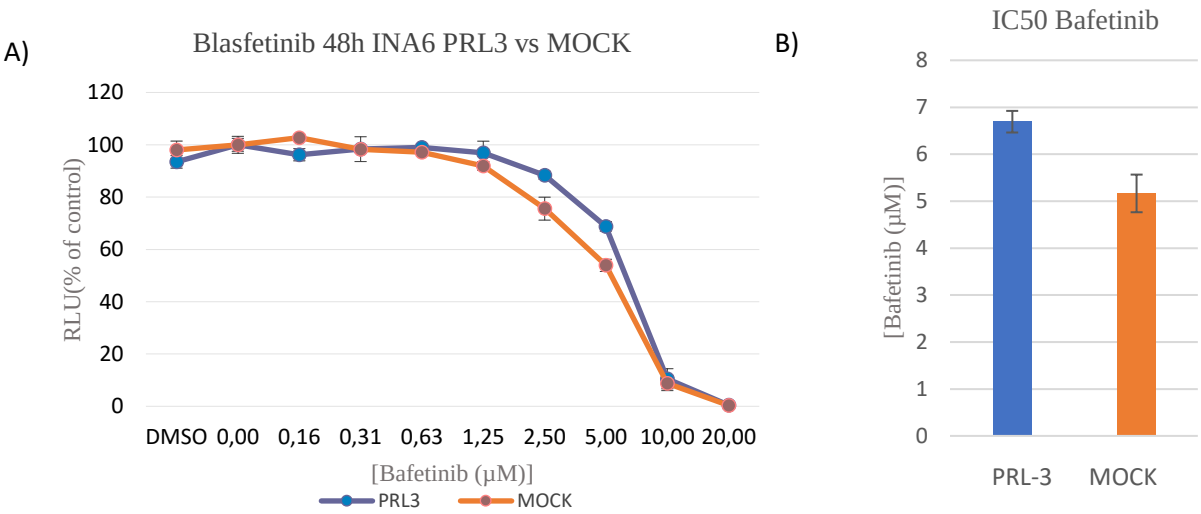


Figure 17. Bafetinib against INA-6. A) Bafetinib was tested against PRL-3 and MOCK INA-6 cells. Both cell conditions were tested against different concentrations of bafetinib for 48h. The inhibitor´s concentrations were from 20 to 0,16 µM following 2-fold series, using 0 µM and DMSO as controls. This figure is a representative result out of three biological replicates showing that bafetinib was able to induce cell death in both cell conditions, PRL-3 and MOCK. However, there PRL-3 cells seem to be more resistant than MOCK cells, at 1,25 µM; 2,50 µM and 5 µM bafetinib concentrations. The error bars shows the standard deviation of the replicates obtained in the same experiment normalized to the control (0,0 µM). The percentage of live cells is measured in Relative Luciferase Units (RLU). B) Bar graph that shows the IC50 (half maximal inhibitory concentration) of bafetinib. It shows that the IC50 for PRL-3 cells was higher than in MOCK cells, illustrating that PRL-3 can protect cells against bafetinib inhibition. The IC50 was calculated considering the avareage of three biological replicates and the error bars showed are the standard deviation of the mean.

A flow cytometry assay was performed to confirm the differences in cell viability of PRL-3 and MOCK cells after 48h of treatment with bafetinib (Figure 18, Figure 28 Appendix). The assay was consequent with the previous results. It illustrated that the percentage of live cells that overexpress PRL-3 is higher, at the same inhibitor concentration, than in MOCK cells. Therefore, we confirmed that PRL-3 confers some resistance against bafetinib inhibitor.

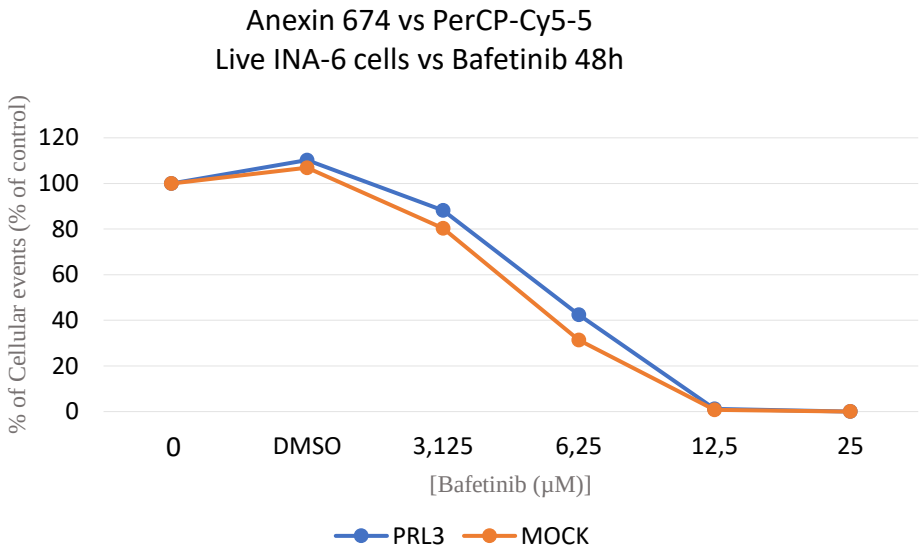
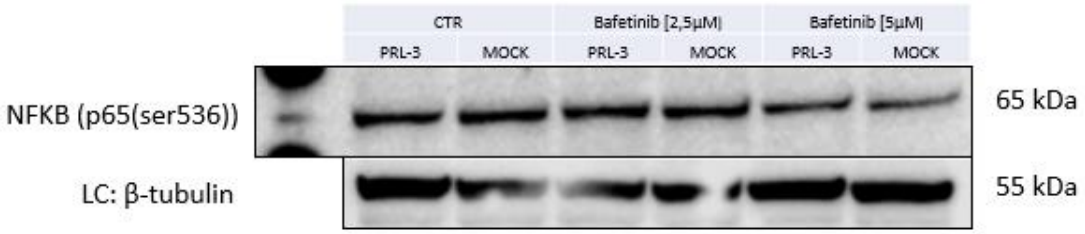


Figure 18. Graph that shows the normalized data extracted from the Q4 of a flow cytometry assay performed to test bafetinib against INA-6. Different concentrations of bafetinib were tested against PRL-3 and MOCK INA-6 cells for 48h. The inhibitor´s concentrations were from 25 to 3,125 µM following 2-fold series, using 0 µM and DMSO as controls. This figure is the only result obtained from this experiment. PRL-3 cells seem to be more resistant than MOCK cells, at 3,125 µM and 6,25 µM bafetinib concentrations. The percentage of live cells is measured as the % of cellular events detected in the Q4, where cells do not show neither annexin, nor 7-ADD, apoptosis markers.

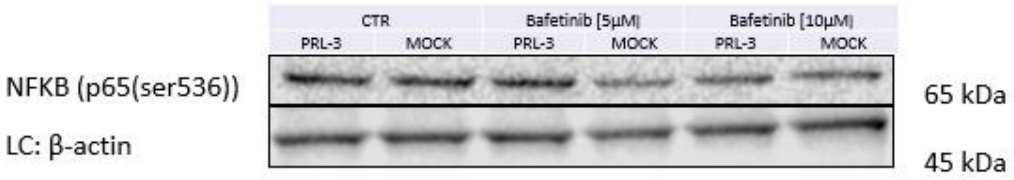
3.2.4. Bafetinib reduces NF-κB activation.

NF- κB is a family of related transcription factors that include RelA/p65, c-Rel, RelB, p50 and p52. They are important transcription factors activated by numerous pathways, with several implications in the control of key physiological processes, such as development, homeostasis, and activation of the immune system. Because of the wide range of processes in which it is involved, its dysregulation leads to the emergence of inflammatory diseases, cancer, and neurodegeneration (77). The activation of the BCR signaling triggers the downstream activation of NF- κB. Therefore, we tested if the inhibition of LYN with bafetinib can decrease the phosphorylation of p65 at the residue S536. The results obtained by Western blot show that with a concentration of 5 µM of bafetinib during 24h, the phosphorylation of p65 is more reduced in MOCK cells than in PRL-3 cells, however this difference is not observable when the inhibitor concentration reach the level of 10 µM (Figure 19).

A)



B)



C)

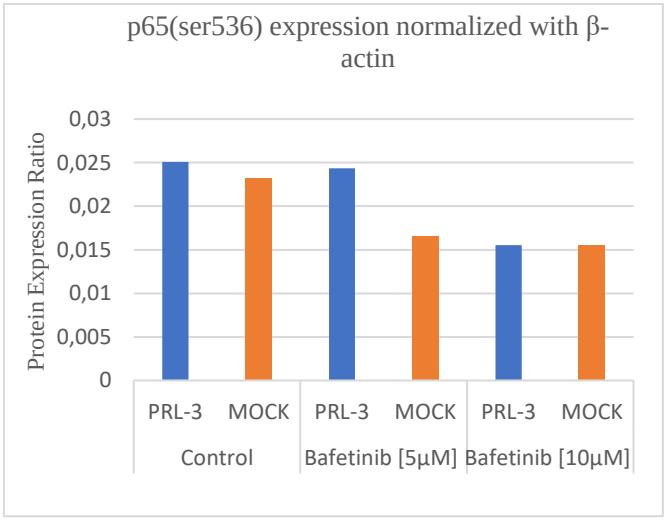


Figure 19. Effect of bafetinib in the NF-κB pathway, whit a concentration of 5 µM, cells that overexpress PRL-3 are more protected than MOCK cells from the action of the inhibitor, at the time of activating the NF-κB pathway.

A) Gel showing the differences in the phosphorylation state between PRL-3 and MOCK in INA-6 cells, and the effect of bafetinib in the phosphorylation of the p65 at ser536. INA-6 PRL-3 and MOCK cells were treated with two different concentrations of bafetinib,2,5 and 5 µM, for 24h. Then they were immunoblotted with a p65 (ser536) antibody. This gel represents the only result obtained from this experiment. It shows that there are no clear differences between PRL-3 and MOCK cells, neither in the control condition (CTR), nor with a bafetinib concentration of 2,5 µM. However, when the bafetinib concentration is 5 µM it is possible to observe little differences between PRL-3 and MOCK cells, being the expression of p65 higher in PRL-3 cells. Therefore, this result suggest that the inhibition of LYN affects more to MOCK cells at the time of activating the NF-κB pathway. B-tubulin was used as loading control (LC). B) Gel showing the differences in the phosphorylation state between PRL-3 and MOCK in INA-6 cells, and the effect of bafetinib in the phosphorylation of the p65 at ser536. INA-6 PRL-3 and MOCK cells were treated with two different concentrations of bafetinib, 5 and 10 µM, for 24h. Then they were immunoblotted with a p65 (ser536) antibody. This gel represents the only result obtained from this experiment. It shows that there are no clear differences between PRL-3 and MOCK cells, neither in the control condition (CTR), nor with a bafetinib concentration of 10 µM. However, when the bafetinib concentration is 5 µM it is possible to observe differences between PRL-3 and MOCK cells, being the expression of p65 higher in PRL-3 cells. Therefore, this result suggest that the inhibition of LYN affects more to MOCK cells at the time of activating the NF-κB pathway, with an inhibitor concentration of 5 µM. B- actin was used as loading control (LC). C) Bar graph that show the protein quantification from the gel shown in Figure 22B.To make the relative quantification of the protein expression Image Studio Lite was used, and the p65 expression was compared and normalized to the LC.

3.2.5. BTK

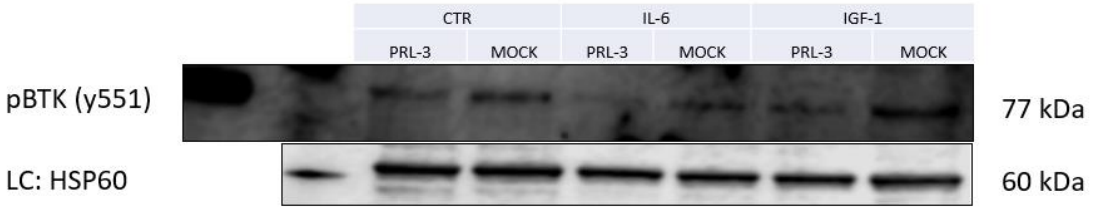
BTK is a tyrosine kinase highly expressed in $CD19^+$ B cells, $CD14^+$ monocytes and B lymphoblasts, having a crucial role in the B-cell development and plasma cell differentiation, due to its participation in the follicular maturation and plasma cell differentiation by participating in the BCR signaling (78).

3.2.5.1. PRL-3 is not related with the BTK overexpression.

The total expression level of the BTK was tested showing little differences between PRL-3 and MOCK. However, in this case, the expression seems to be higher in the MOCK cells than in the control and IL-6 condition. On the other hand, the expression in the IGF-1 condition is more similar in both type of cells (Figure 20).

Figure 20. INA-6 PRL-3 and MOCK cells were treated with IL6 (5 ng/mL) and IGF-1 (100 ng/mL) for 15 minutes. Then they were immunoblotted with a BTK antibody. It possible to observe differences between PRL-3 and MOCK cells both in control (CTR) and IL-6 condition. This result suggests that BTK is more overexpressed in the MOCK condition than in the PRL-3 condition. Again, the differences are less clear in the IGF-1 condition, where neither PRL-3 nor MOCK cells seem to be affected by the IGF-1 stimulation. B- actin was used as loading control (LC). This is the only biological replicate obtained from this experiment.

In the phosphoproteome data the residue Y368 was up-phosphorylated. As there was no available antibody directed to this phosphosite, we tested if PRL-3 affects the catalytic site of BTK, Y551. The results using this antibody were not very consistent. But at least in the IL-6 condition and IGF-1 conditions, there seem to be differences with a higher phosphorylation in the MOCK condition (Figure 21).



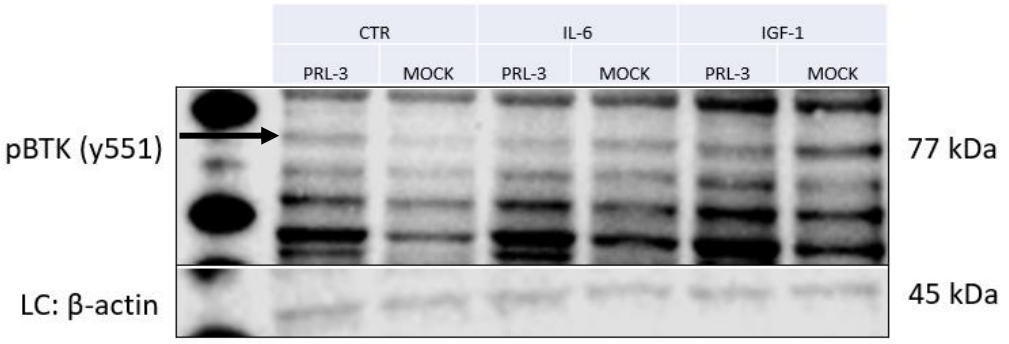


Figure 21. INA-6 PRL-3 and MOCK cells were treated with IL6 (5 ng/mL) and IGF-1 (100 ng/mL) for 15 minutes. Then they were immunoblotted with a pBTK (y551) antibody. These two gels are two biological replicates from the same experiment. However, they do not show the same result. In the first gel, the expression of pBTK seems to be higher in the MOCK cells, while in the second gel the expression seems to be higher in PRL-3 cells. However, the results showed under the stimulation with IL-6 and IGF-1 are the same. In both condition the expression of pBTK (y551) seems to be higher in MOCK cells, being the strongest differences in the IGF-1 condition. B- actin and HSP60 were used as loading controls (LC).

3.2.5.2. PRL-3 does not protect against BTK inhibition.

BTK was inhibited using ibrutinib, in order to see if the differences observed in the gels can protect any of the cell types against the BTK inhibition. We used PRL-3 and MOCK INA6 cells against different concentrations of the BTK inhibitor, that were from 25µM to 0,19 µM following series of 1,33-fold dilutions. In this case the effect of ibrutinib seemed to be very similar in both type of cells (Figure 22.A). By calculating the average IC50 obtained from the different performed experiments, we can see that PRL-3 cells are more sensitive to ibrutinib as the IC50 is lower (5,705 µM) than in MOCK cells (7,05 µM) (Figure 22.B). However, we can see after performing a T-test that these differences were not significant (p-value=0,203).

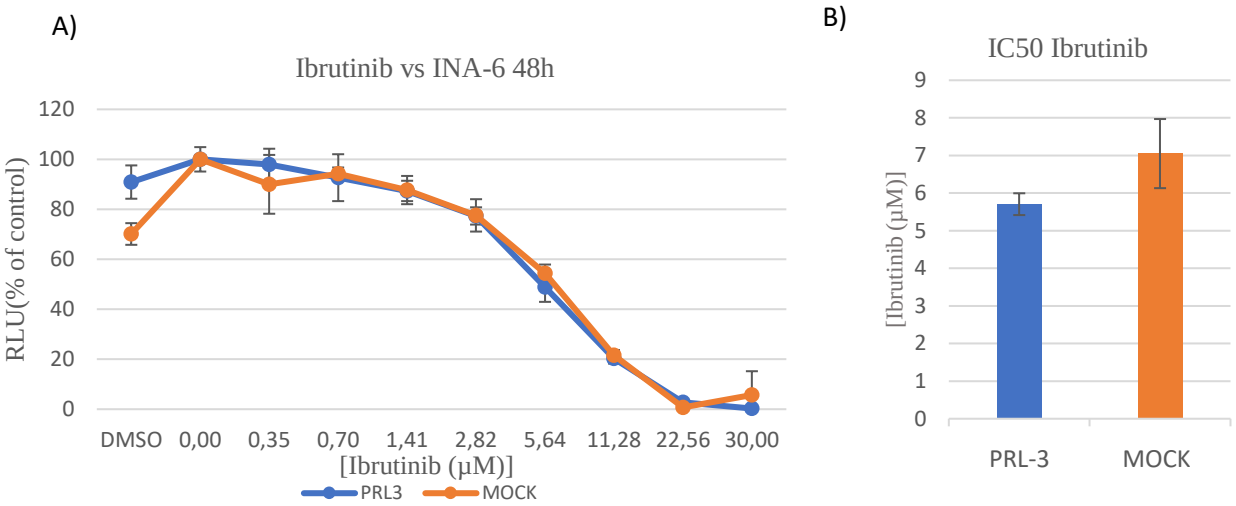


Figure 22. INA-6 vs Ibrutinib. PRL-3 and MOCK INA-6 cells were treated with ibrutinib, a BTK inhibitor, for 48h. The inhibitor concentrations selected were from 30µM to 0,35 µM following series of 1,33-fold dilutions or 2-fold dilution depending on the experiment. DMSO and 0 µM were used as controls. Here we show a representative result out of two biological replicates, where we used series of 1,33-fold dilutions A) It shows that the inhibition of BTK is able to induce the cell death in INA-6 cells, with no significant differences between PRL-3 and MOCK. The error bars shows the standard deviation of the replicates obtained in the same experiment normalized to the control (0,0 µM). The percentage of live cells is measured in Relative Luciferase Units (RLU). B) It shows the average IC50 (half maximal inhibitory concentration) of ibrutinib in PRL-3 INA-6 cells (orange) and MOCK INA-6 cells (blue). The IC50 was calculated considering the avareage of two biological replicates, we can see that MOCK cells are more tolerant to the BTK inhibition than PRL-3 cells, however this difference is not enough to be consider significant. The error bars shows the standard deviation of the mean.

3.2.5.2. Bafetinib can reduce BTK expression in INA-6 cells

Due to the differences in the cell proliferation observed when we used bafetinib, we decided to test the effect of LYN inhibition in the expression of BTK. BTK is a downstream effector of LYN in the BCR signaling pathway, as LYN can activate SYK, which in turn activates BTK. Our results showed that using 5 µM of bafetinib, the expression of BTK is reduced in both cell types, PRL-3 and MOCK (Figure 23). As it was shown before (Figure 20), BTK appeared to be more expressed in MOCK cells. Moreover, the inhibition of LYN seems to reduce more the BTK expression in PRL-3 cells.

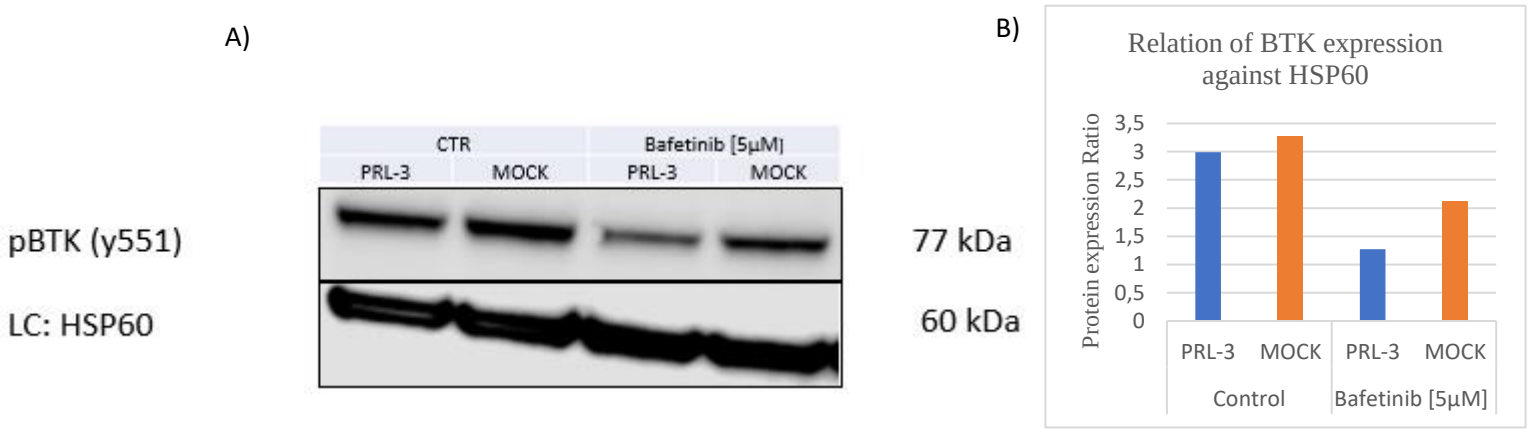


Figure 23. INA-6 PRL-3 and MOCK cells were treated with the LYN inhibitor bafetinib 5 µM for 24 hours. Then they were immunoblotted with a BTK antibody. A) This gel is the only biological replicate obtained from this experiment. The result illustrated here in the control condition (CTR) is the same as it was showed before (Figure 20), where MOCK cells shown higher expression levels of BTK. Bafetinib can reduce the levels of BTK in PRL-3 and MOCK cells. However, after the bafetinib treatment, there are still observable differences, as MOCK cells still have higher BTK levels. HSP60 was used as loading control (LC). B) Bar graph that show the protein quantification from the gel shown in Figure 23A. To make the relative quantification of the protein expression Image Studio Lite was used, and the BTK expression was compared and normalized to the LC.

Table 5. Table that summarizes the effect of the different inhibitors tested on the PRL-3 and MOCK cells.

Protein	Inhibitor	Differences in cell viability PRL-3 vs MOCK
BTK	Ibrutinib	Not significant
SYK	Entospletinib	No cell death
	PRT062607	No
LYN	Bafetinib	Yes, PRL-3 cells are more resistant

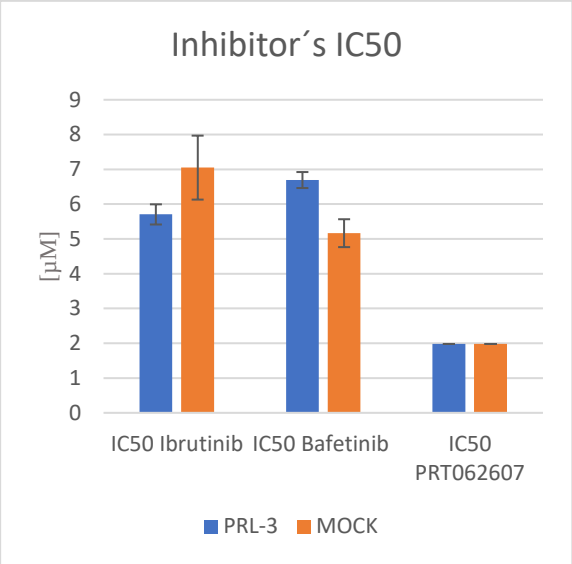


Figure 24. Graph that summarises the IC50 (half of the maximum concentration) of the different inhibitors. It compiles the graphs shown in the Figures 15B, 17B and 20B.

Table 6. Effector pathways activated downstream of the BCR signaling.

Efector pathways	Antibodies	Phosphoproteomics PRL3 vs MOCK (IL6)	Phosphosites	Expression reduced by Bafetinib
NFKB	p65 (ser536)	No	Information is focussed on S276 and S536. Phosphorylation at S536 enhanced transactivation. This process is responsible for the transcriptional activity of the subunit (77).	Yes
MAPK (MAPK1)	Not tested	Up-phosphorylated (Y187)	Its activation requires a sequential phosphorylation on activation loop residues by the MAPK kinases (MAP2Ks) MEK1/2, first on Y187 and then on T185. MAPK1 is negatively regulated by DUSP phosphatases(79).	Not tested
PI3K/AKT (AKT1)	Not tested	Down-phosporylated (S124)	Akt1 activation requires phosphorylation at Thr308; phosphorylation at Ser473 enhances the catalytic activity. AKT1 has constitutive phosphosites that are required for maximal AKT1 activity, they are S124 and S129(80).	Not tested

Table 7. Table that summarises the BCR related proteins. Abbreviation: SOCE (store-operated calcium channel)

Protein	Antibodies	Phosphoproteomics PRL3 vs MOCK (IL6)	Phosphosites	Regulated by LYN
BTK	pBTK y551	Up-phosphorilated (Y368)	- SYK phosphorylates y551 (catalytic site) - Autophosphorylation at Y233 increased the activation(78)	Yes (LYN phosphorylate BTK at Y551) (78)
SYK	Not tested	Over-expressed at protein level	- Tyr-317 (Negative regulator, Binding site for CBL phosphorilated by LYN) Tyr-342, -346 (Biding site for PLCy2 and phosphorilated by LYN)(81)	Yes (Lyn phosphorilates SYK) (81)
LYN	pLYN(Y397)	Up-phosphorylated (Y397)	- Y397, Y396 postive regulators - Y508 inhibitory site (dephosphorilated by CD45)(82)	It can be regulated by an autophosphorylation at Tyr397 resulting in an increase in protein-tyrosine kinase activity (82) .
c-CBL	pc-CBL y674	Up-phosphorylated (Y674)	- Src-family kinases primarily phosphorylate c-Cbl at Tyr-700, Tyr-731, and Tyr-774. - These sites of phosphorylation provide docking sites for downstream signaling components, such as Grb-2, PI3K, and Fyn (83)	Yes LYN phosphorylates c-CBL, activating it(84).
SHIP-1 (INPP5D)	Not tested	Up-phosphorylated (Y865)	- SHIP1 (Ser440) is phosphorylated by PKA, increasing it activation. - SHC1 and Dok- 1 can bind to Tyr 915 and 1022, Dok-3 only to Tyr1022 in human cells. (85)	Not directly
STIM1	Not tested	Up-phosphorylated (S523); (S524)	- Ser575, Ser608, and Ser621, are target sites for extracellular signal-regulated kinases 1/2 (ERK1/2). Stored Calcium depletion activates ERK1/2, which phosphorylates STIM1. - Phosphorylation of STIM1 at S486 and S668 has been shown to lead to SOCE suppression during mitosis (86).	Not directly

4. Discussion

MM is a plasma cell malignancy characterized by the accumulation and dissemination of malignant plasma cells throughout the bone marrow. Despite the new advances in the understanding of the disease and the emergence of new treatments, it remains incurable. Although there is usually an initial response to the treatment, the disease tends to emerge again leading to patients' death. Therefore, it is urgent to identify novel targets that can help patients to have a better life quality. PRL-3 has been identified as an important molecule for the MM development, as it has been identified as a key mediator of IL-6 signaling in MM cells (11). Moreover, the role of PRL-3 in MM cells migration has been suggested (15). Given the importance of IL-6 for MM cell development, and the risk that migration of the malignant cells implies for the patient, PRL-3 is postulated as a good target to treat MM.

4.1 Bioinformatic phosphoproteome analysis

Nowadays the advances in technology allow us to use bioinformatic tools to make *in-silico* assays that save a considerably amount of time doing *in-vitro* assays. However, they did not totally replace the laboratory work as it is still necessary to confirm and extend the knowledge provided by bioinformatics.

Therefore, we performed some bioinformatic analysis to have an overview of the processes altered by the overexpression of PRL-3. In this context we built a network importing validated protein-protein interaction from different curated databases. Thus, we ended up with a network that show how the different proteins, which phosphorylation state has been affected by the overexpression of PRL-3, interact among each other. Then, this network was analysed using different tools in order to know which pathways and biological processes that were most involved.

4.1.2 BiNGO analysis

The BiNGO analysis was performed to know the most overrepresented Biological Processes in the different networks. The results are shown in the Appendix Tables 8 and 9, where we can observe that the phosphorylation and protein modification are relevant biological processes in the network. This goes in consonancy with the importance of the dysregulations described in the phosphoproteome that lead to the emergence of malignancies(19), that constitute one of the reasons of why are we investigating the role of PRL-3 in MM. Moreover, the involvement of PRL-3 in the vascular system development comes to light when we notice that several overrepresented Biological Processes were related with the placenta development and cardiac development. Also, PRL-3 is expressed in skeletal muscle and heart (22), therefore it makes sense that the muscle cell development and the cardiac muscle cell development appear overrepresented. In addition, mRNA processing and mRNA splicing appeared overrepresented in the Final Network, which is consistent with the fact that mRNA splicing is the most well-studied mRNA processing event in relation with cancer (87). This process allows identical pre-mRNA molecules to be processed in different ways and, thus, generating the vast protein diversity we find in complex organisms (88). Several studies have shown that alternative splicing plays a significant role in cancer, as it can create alternatively spliced products that stimulate the tumor progression (87-89).

4.1.3. Reactome analysis

By looking at Table 3 we can notice that there are some overrepresented pathways shared by all networks analysed. These pathways are “CD28 co-stimulation”, “Signaling by Interleukins”, “CTLA4 inhibitory signaling”, “Depolymerization of Nuclear Lamin, Cell Cycle”, “CD28 dependent PI3K/Akt signaling", "Interleukin-4 and Interleukin-13 signaling", "Regulation of TP53 degradation" and “Apoptosis”.

4.1.3.1. CD28 related pathways

Draws attention that pathways related with CD28 seem to be very important in the network, despite of CD28 is a T cell specific homodimeric receptor(90). This can be explained because CD28 is expressed in differentiated plasma cells, being essential for long-lived plasma cells(91). In addition, it has been described that myeloma cells expressed similar levels of CD28 to that of normal T cells, but only in T-cells its activation upregulate IL-2 production (92). In MM cells CD28 is able to bind its counter receptors, the B7 molecules, and this binding induces activation of PI-3 kinase. CD28 is functional in T cells where it has a role in apoptosis prevention. MM cells can benefit from this characteristic of CD28, dysregulating the pathway to increase their proliferation, survival, or differentiation (92, 93). Moreover, another pathway that appears overrepresented in every network also related with CD28 is “CTLA4 inhibitory signaling”. CTLA4 is a receptor of CD28 family that can bind to the same ligands as CD28 but with a higher affinity. However, CTLA4 has antagonist functions compared with CD28, as when it is activated it inhibit T cell activation. Despite CTL-4 is supposed not to be expressed by MM cells, but by B-cells activated by T-cells. Therefore, the proteins implicated on this pathway can be altered by PRL-3, so it cannot inhibit the proliferation stimuli enhanced by CD28 (93, 94).

4.1.3.2. Cell cycle related pathways

Other pathways that seem to be relevant in the networks, are related with the cell cycle, such as “Cell Cycle”, “Depolymerization of Nuclear Lamin”, “Apoptosis” or terms related with TP53 such as “Regulation of TP53 degradation”. It is also remarkable that BiNGO also identified as overexpressed Biological Processes: “Positive regulation of cell death”, “Positive regulation of apoptosis” (Final network), “Regulation of cell proliferation”, “Regulation of cell cycle” (String Network).

Defects in cell cycle are very common in cancer cells, as they benefit from alterations in the proteins that regulate this process to become more proliferative and avoid cell death. These alterations cause unscheduled proliferation and genomic instability leading to a malignant transformation of the normal cells. One of the MM hallmarks is the dysregulation of cyclin D, a protein which upregulation is considered as an early oncogenic event in MGUS and MM (95). Moreover, TP53 is a gene that codes for p53 tumor suppressor protein. The p53 signaling is downregulated in normal physiological conditions, but it is activated by different stimulus that cause cellular stress, such as hypoxia or DNA damage. The activation of this pathway promotes the action of tumor suppressive mechanisms such as cycle arrest or apoptosis (96, 97). This pathway is dysregulated in 50% of all human cancers (98), and MM is not an exception, as it has been shown that TP53 alterations increase with the disease´s progression and it is involved in the acquisition of drug resistance (99). Our data supports the idea that PRL-3 can influence the TP53 signaling pathway, by adding post-translational modifications,

into the proteins CDK1, CDK2 and AKT1, leading to the dysregulation of TP53 gene product. This goes in line with the results showed by previous studies that defined the association of PRL-3 and p53. It has been shown that Prl-3 Is a p53 Target Involved in cell-cycle Regulation. It has been shown that PRL-3 overexpression can be involved in the activation of a PI3K-AKT negative feedback loop that induce the cell cycle arrest in $G_{1(100)}$.

Important proteins in the cell cycle are LMNA, CDK1, TMPO and LMNB1. These proteins are corresponded with the network´s nodes, thus, they are interacting with several entities, holding the network´s architecture (Figure 24 Appendix). CDK1 is down-phoshorylated in our data at the threonine and tyrosine residues T14 and Y15, which are inhibitory phosphosites. When a cell is going to enter in mitosis, these residues are dephosphorylated by CDC25 phosphatases. Therefore, the down-phosphorylation of these residues can promote a constant mitotic state (101).

4.1.3.3. Interleukins related pathways:

The cytokines and growth factors secreted by the BM elements are crucial for the MM cells (102). We can see this fact as "Signaling by Interleukins" "Interleukin-4 and Interleukin-13" are overrepresented in all networks. In MM, some cytokines have been described to be crucial for malignant cell´s development. This is the case of IL-6, IL-7, IL-8, and TGFβ, cytokines produced by the BM cells that can influence in the growth and survival of malignant cells. MM cells can produce in an autocrine way IL-15 contributing to their survival (103). On the other hand, IL-4 and IL-13 are very similar cytokines, as they share both functional properties and a receptor subunit. They are anti-inflammatory cytokines that control the immune response homeostasis(104, 105). As the development of MM disease led to suppression of the immune responses due to the overproduction of monoclonal antibodies, so an anti-inflammatory environment is promoted to evade the recognition by the immune system(103). Moreover, in Hodgkin lymphoma cells in the presence of PRL-3, an autocrine stimulation by IL-13 activates STAT6, enhancing the cell proliferation (106). Therefore PRL-3 can affect to the interleukin signaling enhancing an anti-inflammatory response and a pro-tumorigenic signals.

4.1.3.4. B-Cell Receptor signaling pathway:

Other relevant pathway where PRL-3 has a strong influence is the BCR-signaling pathway. Despite of the fact that this pathway does not appear overrepresented in the final network, nor in the clusters created by jActive modules, it does in the MCODE cluster and in the String and Signor networks. MCODE cluster shows the most interconnected nodes between themselves, highlighting the protein complexes, inside the final network. In addition, the interactions shown by Signor are manually curated, and the interactions shown by String were filtered with a minimum required interaction score of 0,90, confirmed with experiments and validated by curated databases, thus, the pathways that appear overrepresented in these networks are very reliable.

During B cell development, when the BCR recognizes an antigen, the triggered signaling leads to the activation of the B-cell, contributing to its differentiation into an antibody-secreting plasma cell (32). The positive signals will induce the proliferation and differentiation of the cell, but they are controlled by negative signals that induce the

dephosphorylation of the proteins previously activated by phosphorylation. Therefore, in healthy conditions, once the BCR activated upon antigen binding, and proliferative signals are trigger, an inhibitory pathway is activated to down-regulate BCR signaling, which leads to the loss of the membrane immunoglobulins that constitute the receptor (32, 36, 107). Thus, it appeared interesting that this pathway emerged overrepresented in the networks, when it is not supposed to be functional in plasma cells. This makes BCR signaling a not common target for MM treatment. Furthermore, it was shown that the BCR signaling is related with the appearance of lymphoid malignancies, when it is chronically activated by foreign microorganisms or viral antigens, or when mutations in the proteins involved in the pathway emerge (108).

In conclusion, the unexpected finding of BCR signaling as a relevant pathway in MM cells, in addition to the lack of studies related with the BCR signaling in MM, took us to perform experimental assays in the laboratory to observe if results from the exploration with computational resources were coherent, and BCR was indeed influenced by PRL-3 in MM cells.

The entities affected by PRL-3 involved in the BCR signaling pathway appear summarized in the Table 4.

4.2. BCR signaling in MM

Once we knew the pathway that would be the main topic of this thesis, we tried to confirm the differences in the phosphorylation state that were observed in the phosphoproteomic dataset. In addition, we screened if the proteins involved in triggering the pathway can be considered as drug targets. Therefore, we tested different inhibitors against some relevant proteins that participate at the beginning of the pathway amplifying the downstream signaling. The proteins identified as relevant are summarized in the Table 6 and 7.

The BCR signaling in a healthy B-cell starts when the BCR recognizes an antigen, and it binds to it. This binding promotes the aggregation of the BCR membrane complex that will bring closer the LYN kinase that will be in charge of the phosphorylation of ITAMs in the cytoplasmic tails of CD79a and CD79b, activating them. The activated ITAMs will recruit SYK to the receptor, leading to its phosphorylation and activation. SYK phosphorylate the adaptor protein BLNK that will recruit BTK facilitating its phosphorylation by SYK. This process leads to the formation of the signalosome, a protein complex formed by SYK, LYN, adaptor proteins like CD19 and BLNK, and signaling enzymes like PLCγ2, PI3K, and Vav. The signalosome will emit signals that will activate the downstream signaling pathways such, NF-κB, PI3K-AKT and RAS/RAF/MEK/ERK pathway, which are crucial for B-cell fate decisions such as proliferation, survival, differentiation and cell death (32, 36).

In the activated plasma cell, there is a lack of membrane immunoglobulins, so in the case that this pathway is still active in the MM cells, it might be directly initiated by a dysregulated LYN kinase.

LYN is a member of the Src kinase family, involved in maintaining the homeostasis of the BCR signaling, being the predominant Src kinase in B-cells. Its regulatory ability makes that when its activity or expression is dysregulated the immune

system does not function properly, favouring emergence of autoimmune diseases and malignancies. LYN has been shown to bind PI 3-kinase upregulating its activity, moreover LYN has a strong influence in the AKT activation, a molecule involved in the degradation of the apoptotic protein p53. Therefore MM cells that overexpress LYN have reduced protein levels of p53, which may promote the cell proliferation (109). Previous studies have shown the relation between PRL-3 and Src kinases, showing that PRL-3 can activate Src kinases MM cells to avoid apoptosis (31). In the phosphoproteomic data LYN appeared as up-phosphorylated at the residue Y397. This phosphosite acts as a positive regulator of LYN (82) , so this modification could imply a more active protein that constitutively trigger the activation of the downstream BCR signaling. All this previous knowledge was confirmed by our result as Figure 15 and 16 show. These figures illustrate that PRL-3 increases the phosphorylation of LYN at Y397 generating an overactivation of the kinase.

As PRL-3 increase the activation of LYN we expected that its inhibition would be less harmful to PRL-3 cells. This was confirmed after observing the cytotoxic effect of bafetinib in PRL-3 and MOCK cells. Our results suggest that when LYN is inhibited, the overactivation of this protein by PRL-3 confers, to those cell that overexpress the phosphatase, protection against cell death (Figure 17). Previous studies have described that bafetinib can induce programmed cell death through both Caspase-mediated and Caspase-independent pathways in CML cell lines(64, 110). Therefore, it is possible that PRL-3 can dysregulate the apoptosis mechanisms through the overexpression of LYN to avoid the cell death. In addition, we tested how LYN inhibition affected the NF-κB pathway. As it was shown in the Figure 19, we suggest that PRL-3 can maintain the activation of NF-κB pathway at moderate bafetinib concentrations (5μM). This probably help PRL-3 cells to resist the inhibition of the cell proliferation, when they are treated with bafetinib.

Once we further confirmed that PRL-3 can over-activate LYN, we expect that if the BCR signaling is affected by PRL-3, the tyrosine kinases activated downstream of LYN, would be overactivated. Therefore, we tested if PRL-3 was able to protect MM cells against the inhibition of SYK, in the same way as it happened when LYN was inhibited.

The spleen tyrosine kinase, SYK, is a cytosolic non-receptor protein tyrosine kinase is mainly expressed in hematopoietic cells (62). As it was explained before, this kinase is an essential protein for the downstream signal transduction from the BCR, but in the recent years it has been shown that SYK can be involved in more and more roles, such us osteoclast maturation, vascular development and cytokine signal transduction(111), processes that are important in the MM development. SYK did not emerge in the phosphoproteomic data set, so there are not expected differences in the phosphorylation state of this protein. However, due to its importance for the BCR signaling and in the other aforementioned processes, we considered relevant to observe the effects of the SYK inhibition.

SYK has been considered as a target to treat non-Hodgkin lymphoma and chronic lymphocytic leukemia (CLL). In this context, entospletinib has been proved to be an ideal drug to treat refractory CLL and B cell non-Hodgkin lymphoma(62). This background

has made that in MM, SYK has been proposed as a target for new treatments. SYK inhibitors R406, Piceatannol and Bay61-3606 are able to block the proliferation, migration and survival of MM cells by the inhibition of the MAPK and NF-κB signaling pathways (112). Our results show that entospletinib is not able to cause cell death in any of the cell lines tested (Figure 13). Therefore, we decided to use another SYK inhibitor, PRT062607. This compound showed a dose-dependent effect, able to cause the cell death of MM cells (Figure 14). However, there were no differences between PRL-3 and MOCK cells. This result suggests that, SYK is an important protein for the development of MM cells, although PRL-3 does not have a strong influence on the kinase activity. Despite of the fact that SYK inhibitors are able to decrease cell proliferation and migration, some authors have discarded SYK as a relevant target for MM, as its expression level is increases in some HMCLs but no in primary bone marrow samples (113).

The Bruton´s tyrosine kinase (BTK) has crucial implications for the normal B-cell development due to its central role in the BCR signaling pathway. Elevated levels of BTK in MM patients have been described as a poor prognosis marker. BTK has also been described as an important regulator of the osteoclast differentiation (114). Therefore, its role in B cell development, in addition to its ability to regulate the osteoclast maturation, has caused scientists to use this protein as target to treat MM (114). Thus, it has been shown that the BTK inhibitor, ibrutinib, is able to block the osteoclastogenic signaling pathway mediated by BTK, reducing bone resorption activity (114). Moreover, ibrutinib has described to be cytotoxic to MM cell by inhibiting the activation of the NF-κB pathway (115).

Our results show that ibrutinib is able to induce cell death in INA-6 cells, but the overexpression of PRL-3 does not seem to benefit the cell against the inhibitor. According to IC50, MOCK cells survive better than PRL-3 cells (Figure 22.B), however in the rest of the concentrations there are no visible differences (Figure 22.A). Moreover, IC50 differences are not significant. Therefore, with this data we cannot say that PRL-3 confers an advantage nor a disadvantage against BTK inhibition.

However, MOCK cells seem to express higher levels of total BTK (Figure 20, Figure 23). In addition, MOCK cells have higher levels activated BTK (y551) (Figure 21). These results go in the opposite direction to what was expected, as due to the relevance of BTK in the transmission of the BCR signaling an its implications in proliferation, we expected this protein to be more expressed and phosphorylated in PRL-3 cells, as it was shown in the phosphoproteome. It is important to notice that BTK appeared up-phosphorylated in the bioinformatic data in the residue Y368, which its function has not been described and there are no antibodies available in the market directed towards this phosphosite. Maybe PRL-3 can phosphorylate Y368 triggering in some process that benefit the cell survive and can compensate the lower expression levels of BTK.

BTK is a very important link between the surface receptor and the downstream pathways involved in cell survival and proliferation such as AKT and NF-κB pathways. It is known that BTK helps the cell to avoid senescence through activating AKT/P27/Rb signaling. AKT signaling is an important regulator of cell homeostasis and its dysregulation is associated with solid tumor and hematological cancers (116). AKT1

appeared down-phosphorylated at S124, when PRL-3 is overexpressed, in the protein set. This residue is a constitutive phosphosite necessary for the complete activation of the protein. BTK can directly phosphorylate AKT at the catalytic site S473, thus activating it (116). Despite of the fact that we did not analyse AKT phosphorylation in the laboratory, it seems like PRL-3 cannot influence BTK to dysregulate the AKT pathway to induce cell proliferation.

We also tested how LYN inhibition affects the expression level of BTK. As it was discussed previously, the expression level of BTK was higher in MOCK cells (Figure 20, Figure 23). On the other hand, MOCK cells are more affected by bafetinib (Figure 17, Figure 18). Therefore, we expected that the resistance of PRL-3 cells to bafetinib compensates the overexpression of BTK by MOCK cells. Our results showed that LYN inhibition reduce BTK expression. However, after the treatment BTK expression was still higher in MOCK cells. These results suggest that the relation between LYN and BTK is occurring in MM cells. However, we propose that PRL-3 is not related with the dysregulation of this interaction. BTK is a key protein in the BCR signaling, so the low levels of BTK expression in PRL-3 cells compared to MOCK, in both conditions, the control and after bafetinib treatment (Figure 23), led us to think that PRL-3 might not be related to BCR signaling. Probably, PRL-3 can over-activate LYN, and this can lead to an increase of the proliferation, by activating different pathways that are not related to BCR signaling.

NF-κB is crucial for myeloma survival (115), and it is another effector pathway activated downstream of BTK, LYN and SYK. As we can see in the Figure 19, the expression level of the subunit p65 in the control condition is the same in PRL-3 and MOCK Therefore, the differences observed in the expression or phosphorylation of the upstream proteins do not affect the NF-κB pathway, so with this data we cannot say that PRL-3 can increase the cell survival or proliferation by modifying BTK to take advantage of its downstream events.

At this point we have looked at kinases in charge of give positive signals that activate cell proliferation through the BCR activation as their activation leads to the activation of downstream effector pathways. However, for the dysregulation of the pathway, probably it is not enough with a strong positive signaling, a dysregulation of the negative signals that control the activation of the pathway is also needed. In this context some essential proteins are c-CBL and SHIP-1, in addition to the already mentioned, LYN.

CBL proteins are ubiquitin ligase enzymes that are involved in the degradation of, activated tyrosine kinases, such as LYN or SYK, therefore mediating their downregulation. CBL function as E3 ubiquitin ligases, it can recruit the ubiquitin-bound enzyme E2, this protein can transfer a ubiquitin to the targeted tyrosine kinase, resulting in its ubiquitination and subsequent down-regulation. When the BCR recognises an antigen CBL is rapidly phosphorylated by LYN and SYK. Therefore, when the BCR signaling pathway is activated, the cell prepares rapidly the downregulation of the pathway to avoid excessive proliferations signals.

In the phosphoproteomic data set, CBL appeared over-phosphorylated in PRL-3 cells at the residue Y674, a phosphosite with a no defined function. This fact was

confirmed in the laboratory as CBL appeared over-phosphorylated and over-expressed in the PRL-3 condition (Figure 11). CBL is in charge of regulating the BCR signaling via targeting the ubiquitination of LYN and SYK. However, it has been defined that the overexpression of CBL leads to an inhibition of SYK kinase activity, without affecting the LYN function (35, 117). This can explain why SYK did not appear as a dysregulated protein in the phosphoproteomic data set, and why there were no differences in the inhibition of SYK between PRL-3 and MOCK; and in the other hand, we observed significant differences in the LYN activation state.

This can be explained by the overexpression at a protein level, however as the phosphosite has not been functionally described, more experiments are needed to define the consequences of the act of PRL-3 in the activity of c-CBL.

With these results we cannot say that PRL-3 influences the BCR signaling. However, when targeting the relevant proteins for the BCR signaling pathway we observed a decrease in cell proliferation. This could be indicating that, probably, this pathway is relevant for MM cells, although it could not be related to PRL-3.

4.2.1. IL-6 stimulates PRL-3 but not IGF-1

The use of IL-6 and IGF1 growth factors was a trial for increasing the possible differences in the protein expression or phosphorylation between PRL-3 and MOCK. However, the obtained results were different, dependent on the growth factor used.

IL-6 is considered as a crucial cytokine for MM development. IL6 activates STAT3, which acts as a direct transcriptional regulator of PRL-3, therefore IL-6 is involved in the upregulation of PRL-3 in MM. Moreover, it has been described that IL-6 stimulation is involved in LYN activation, as this process is dependent on association of LYN with CD45 tyrosine phosphatase (31). Therefore, we expect that after a stimulation with IL-6, a greater activation of PRL-3 that ends up in a greater activation of LYN. Therefore, this will lead to a stronger downstream signaling, that should increase the phosphorylation state of the BCR signaling elements. Our results shows that the IL-6 stimulation is able to increase the phosphorylation state of c-CBL and LYN. However, in the case of BTK the effect was the opposite. In this case, after IL-6 stimulation, BTK phosphorylation at Y511 seemed to decrease in PRL-3 cells. This can be related with what we explained before, that CBL overexpression reduce the activity of SYK, but it does not affect to LYN. Therefore, a possible explanation could be that IL-6 stimulates PRL-3 activity, the over-active PRL-3 acts increasing the activation state of LYN and CBL; then CBL downregulates SYK activity, thus downregulating BTK.

The other growth factor used was IGF-1. This growth factor increases MM cell survival by activating signal transduction pathways independent from IL-6. It is known that IGF-I cannot activate STAT1 nor STAT3 in multiple myeloma cells (118). It has been shown that stimulation with IGF-1 increased PRL-3 expression in B-cells of acute lymphoblastic leukemia. Our results suggest that IGF-1 is not able to increase the activity of PRL-3 in MM cells. This could be the explanation of what we observed in the case of CBL and BTK.

The Figure 13 shows that the expression level of CBL in the PRL-3 condition is decreased, balancing the expression level to the MOCK condition. When we observe how

IGF-1 affects to the phosphorylation state of CBL, it follows the same pattern (Figure 12), but in this case the phosphorylated CBL in the MOCK cells seems to be higher.

The BTK protein follows the same pattern, as PRL-3 decreased its expression, thus, PRL-3 and MOCK has a similar expression level under IGF-1 stimulation (Figure 21). In the case of phosphorylated BTK, despite the blots are not very clear, it seems that IGF-1 increased more the activation of BTK in the MOCK condition.

However, the effect of IGF-1 in the MOCK cells does not seem to affect the phosphorylation of LYN, as the level of phosphorylation seem to be the same under the IL-6 and IGF-1 condition.

4.3. Methodological Challenges

During the development of this thesis, we had to deal with various challenges both methodological and logistic. The hardest challenges were related with the WB technique. The process that involves the use of this method includes going through a succession of multiple steps that increase the chance of errors. Moreover, there are no available antibodies in the market against several of the phosphosites detected by in the phosphoproteomic dataset. In addition, some antibodies used to identify phosphorylations were not that good as those ones directed against the total protein, as is the case of c-CBL, BTK or SYK (blots not shown).

4.4. Further steps

Compiling all the results, we suggest that PRL-3 has not a strong influence in the BCR signaling, although it affects to the regulation of some proteins related with this pathway. Therefore, more experiments are needed in other to confirm if there is a real effect of the phosphatase in the BCR signaling or not.

The next steps should consolidate the results of this report, researching the effect of other BTK´s phosphosites. It is also needed to figure out how PRL-3 affects to the LYN´s negative domains, as it is an important way of downregulating the signaling.

In addition, after observing the effect of IGF-1 in the PRL-3 cells, other studies can go in line with a better description of how IGF-1 stimulation affects the PRL-3 activation state.

Moreover, to discover if PRL-3 has a real effect in the BCR signaling or not, we can investigate further how PRL-3 affect to the effector pathways activated by the BCR signaling. Therefore, pathways like PI3K/AKT, MAPK1 and the calcium efflux, that appeared overrepresented in the bioinformatic analysis, and they are strongly related with cancer development.

An interesting protein that emerges from the bioinformatic analysis is STIM-1. It is the stromal interaction molecule and is involve in the regulation of the calcium efflux needed for the correct activation of downstream proteins involved in the BCR signaling, such as PKC, calmodulin and JNK 1/2. The activation of the tyrosine kinases at the beginning of the pathway lead to the release of inositol triphosphate (IP3) that triggers the release of $Ca^{2+}$from the endoplasmic reticulum. The decrease in the stored calcium leads to the phosphorylation of STIM-1 activating it. This activates a protein called ORA-1, that form a calcium channel in the membrane, so its activation by STIM-1 leads to the

channel opening, so the calcium from the extracellular media enters into the cell, where it can trigger the activation of several processes (119). It has been previously described that dysregulations in the calcium efflux mediated by Ora1 and STIM1 are related with a poor prognosis in MM (120). It appeared over-phosphorylated in the phosphoproteomic data set at the residue S523/S524. This residue is not well defined, beyond that STIM-1 amino acids from 448 to 530 are part of an inhibitory domain that regulates the slow Ca^{2+}-dependent inactivation of Orai1(86). All the aforementioned information, in addition with the fact that PRL-3 has already been described to mediate calcium release acute in lymphoblastic leukemia (121), makes STIM-1 a good target for further studies.

SHIP1 is a phosphatase found in hematopoietic lineage and bone-forming osteo-lineage cells. It forms a loop with Lyn and CD22 that end up with the BCR internalization in healthy B-cells. It has been shown that SHIP-1 can dephosphorylate PIP3 into PIP2, this is supposed to downregulate the axis AKT/PI3K. However, PIP2 can bind with more affinity to AKT leading to a more potent activation of Akt than PIP3, leading to an increase in cell proliferation (122). Moreover, PIP3 is necessary for BTK activation (32), so if the over-activity of SHIP-1 is confirmed it can explain why we observed lower activity by the tyrosine kinase in the PRL-3 cells. SHIP1 inhibition has been described to reduce the activation of AKT pathway, and that moreover SHIP1 has been proposed as an important tumorigenic factor for hematopoietic cells. The phosphoproteomic data set shows that SHIP-1 (INPDD5) is over-phosphorylated at Y865, a phosphosite with a not defined function. Therefore, could be a smart strategy to try to assess how PRL-3 affects SHIP-1, and, and if this protein is certainly dysregulated:

- What does the cell get from the dysregulation of this protein?
- Is the dysregulation of SHIP-1 by PRL-3 affecting other proteins related with the BCR signaling?

On the other hand, and out of the context of the BCR signaling, maybe is useful to research about the other pathways that emerged from the bioinformatic analysis, such as CD28, the apoptosis or cytokines that not that well defined in MM, like IL-4 and IL-13.

5. Conclusion

In this thesis we tried to figure out the processes that PRL-3 overexpression is able to modify, finding the BCR signaling as a relevant pathway, among others.

The screening of the BCR signaling proteins resulted in the confirmation of previous studies that described the influence of PRL-3 in the overactivity of Src kinases, placing bafetinib as a good inhibitor to induce apoptosis in MM cells, despite of PRL-3 cells being more resistant. The SYK inhibitor (PRT062607) and the BTK inhibitor (ibrutinib) were also effective as they also induced apoptosis in MM cells, however the overexpression of PRL-3 did not seem to give INA-6 any advantage against these compounds.

BTK, contrary to expectations, was more expressed in MOCK cells, therefore more experiments are needed in order to define the reason of this situation. In addition, we suggested that the regulation of BTK by LYN is happening in the INA-6 cells, as bafetinib reduced BTK expression in PRL-3 and MOCK cells.

The findings with respect to c-CBL were in line with the expectations as its expression and phosphorylation was higher in PRL-3 cells, which could give an advantage by the over-activation of LYN and the inhibition of SYK, but this should be confirmed by further research.

Regarding growth factors, it is known that MM cells are strongly dependant of the microenvironment signaling for their development, being IL-6 one of the more important cytokines in this context. Moreover, it is known that PRL-3 activation is a downstream process triggered by IL-6 stimulation. Thus, there were no surprise as our results show that IL-6 stimulation increases the phosphorylation of c-CBL and LYN, with a more marked effect in the PRL-3 condition. However, IGF-1, in spite of being an important growth factor for MM cell development, its stimulation did not change neither the protein expression, nor its phosphorylation state. However, it balanced the differences observed in protein phosphorylation observed between PRL-3 and MOCK cells, suggesting that IGF-1 stimulation is not involved in the PRL-3 activation.

Our results suggest that PRL-3 probably has not a strong influence in the tyrosine kinases involved in the activation of the BCR signaling pathway. In the other hand, PRL-3 can have a role affecting the negative regulators that control the activation of the effector pathways triggered by the BCR signaling pathway. This is shown as LYN, SHIP-1 and CBL, proteins in charge of creating negative feedback loops and related with the BCR degradation are more phosphorylated in the PRL-3 condition.

Other possible explanation could be that PRL-3 can dysregulate proteins involved in the BCR signaling, altering other pathways not related with it, but with implications in processes related with MM development. For example, CBL is required for functions associated with bone resorption, BTK and LYN have implications in other processes that can benefit the myeloma development. However, further research is needed in other to confirm or discard if there is a real effect of PRL-3 in the BCR signaling in MM.

6. Appendix

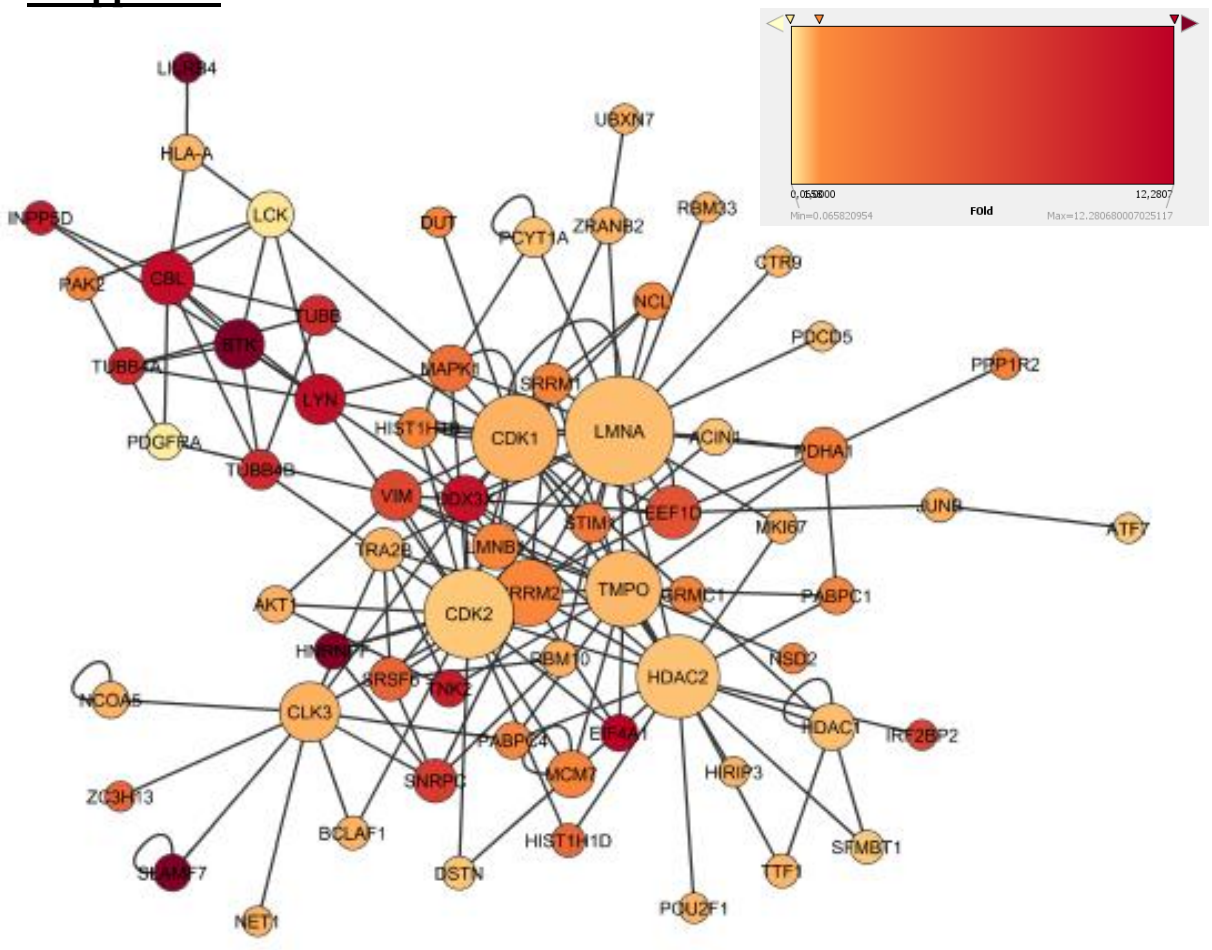


Figure 25. Final network that shows protein-protein interactions extracted from Biogrid, String and Signor. The colour scale is made in base of the fold change. This number represents if a protein is up or down-phosphorylated, if it is higher or lower than 1 respectively. Here, the colour scale that goes from 0,6628 to 12,2807 and it changes from yellow to orange from 0,6628 to 1, and from 1 to 12,2807 from orange to dark brown. The node size represents the node degree, this is the number of edges that the entity has.

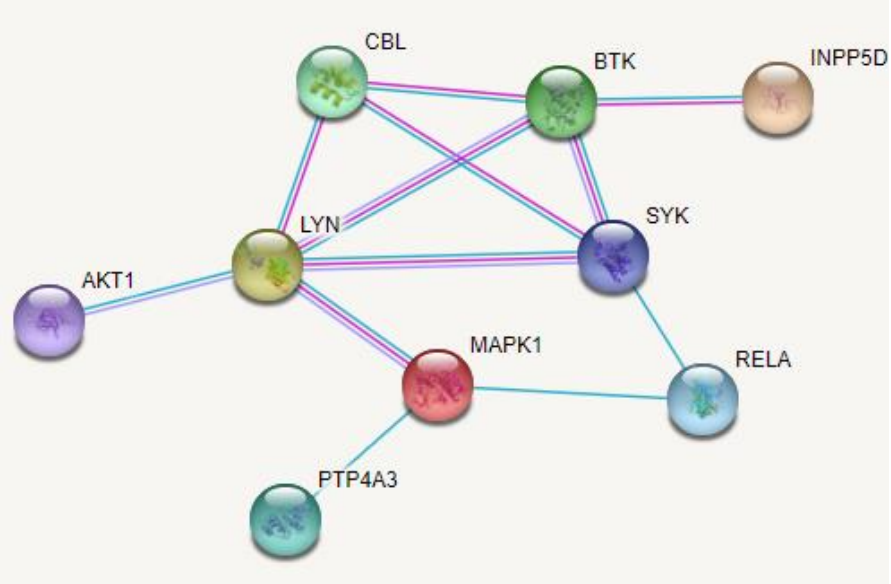


Figure 26. Network imported from String that shows the relations between the relevant proteins for the BCR signaling discussed in this thesis, and their direct relation with PRL-3 (PTP4A3). The blue lines define the interactions imported from curated databases, while the pink lines mark the interactions experimentally determined, and the grey lines indicate that the connected proteins have some sequence similarity.

Table 8. Adjusted P-values and False Discovery Rate (FDR) from the most relevant pathways given by BiNGO and Reactome (and KEGG) analysis of the different networks.

Signor 2.0				Merged Network				String					
Reactome		BiNGO		Reactome		BiNGO		KEGG		BiNGO		Reactome	
Overrepresented Pathways	FDR	Overrepresented GO: Biological Process	adjusted p-value	Overrepresented Pathways	FDR	Overrepresented GO: Biological Process	adjusted p-value	Overrepresented Pathways	FDR	Overrepresented GO: Biological Process	djusted p-valu	verrepresented Pathways	FDR
CD28 co-stimulation	1,07E-08	positive regulation of biological process	1,37E-04	Signaling by Interleukins	2,20E-06	protein amino acid phosphorylation	4,28E-03	Cell cycle	8,49E-13	cellular macromolecule metabolic process	2,05E-04	Mitotic G1 phase and G1/S transition	2,14E-12
ignaling by Interleukins	1,22E-07	phosphorylation	4,42E-04	Apoptotic execution phase	1,12E-05	placenta blood vessel development	3,53E-03	Epstein-Barr virus infection	1,40E-09	ventricular cardiac muscle cell development	2,49E-04	Cell Cycle, Mitotic	8,02E-12
TLA4 inhibitory signalir	1,49E-07	post-translational protein modification	4,24E-04	Depolymerisation of the Nuclear Lamina	6,11E-05	peptidyl-tyrosine modification	1,98E-03	Viral carcinogenesis	2,24E-08	ventricular cardiac muscle cell differentiation	3,21E-04	Signaling by Interleukins	3,99E-08
Costimulation by the CD28 family	9,50E-07	regulation of localization	3,05E-04	Cell Cycle, Mitotic	1,58E-04	labyrinthine layer blood vessel development	1,73E-03	Chronic myeloid leukemia	1,11E-07	cardiac cell development	3,11E-04	Cytokine Signaling in Immune system	6,32E-07
Diseases of signal transduction by growth factor receptors and second messengers	1,17E-06	interspecies interaction between organisms	9,94E-05	Apoptosis	1,68E-04	peptidyl-tyrosine phosphorylation	1,73E-03	Fc epsilon RI signaling pathway	2,70E-06	terphase of mitotic cell cyc	3,21E-04	Depolymerisation of the Nuclear Lamina	7,71E-07
Negative regulation of the PI3K/AKT network	5,07E-06	protein amino acid phosphorylation	9,91E-05	Cytokine Signaling in Immune system	2,27E-04	RNA processing	1,73E-03	B cell receptor signaling pathway	2,95E-06	positive regulation of cellular process	3,21E-04	Activation of the pre-replicative complex	4,69E-06
egulation of KIT signalir	5,45E-06	protein import into nucleus, translocation	9,35E-05	CD28 co-stimulation	2,56E-04	positive regulation of cell death	1,66E-03	Cellular senescence	3,79E-06	cromolecule metabolic proc	3,21E-04	CD28 co-stimulation	5,67E-06
Signaling by Receptor Tyrosine Kinases	8,53E-06	protein modification process	5,56E-05	Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation	2,75E-04	positive regulation of apoptosis	1,66E-03	ErbB signaling pathway	4,63E-06	regulation of cell cycle	3,21E-04	Activation of ATR in response to replication stress	5,67E-06
Cell Cycle, Mitotic	8,85E-06	phosphorus metabolic process	3,81E-05	Interleukin-12 signaling	5,21E-04	positive regulation of programmed cell death	1,66E-03	Osteoclast differentiation	2,26E-05	diac muscle cell developme	3,21E-04	FLT3 Signaling	1,35E-05
Depolymerisation of the Nuclear Lamina	1,13E-05	macromolecule modification	6,60E-06	CTLA4 inhibitory signaling	7,24E-04	mRNA metabolic process	6,07E-04	FoxO signaling pathway	2,50E-05	interphase	3,21E-04	Apoptotic execution phase	2,26E-05
Cytokine Signaling in Immune system	1,47E-05	protein metabolic process	6,60E-06	Interleukin-4 and Interleukin-13 signaling	0,001	RNA splicing	2,11E-04	Fc gamma R-mediated phagocytosis	1,50E-04	interspecies interaction between organisms	3,88E-04	CTLA4 inhibitory signaling	3,23E-05
Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	9,19E-03	cellular protein metabolic process	6,60E-06	Immune System	0,002			Pathways in cancer	1,50E-04			Immune System	1,71E-04
		positive regulation of celular process	2,57E-06	Mitotic G1 phase and G1/S transition	0,002			Apoptosis	4,80E-04			PI3 activates AKT signaling	6,14E-04
								MicroRNAs in cancer	6,10E-04			TP53 Regulates Transcription of Cell Cycle Genes	6,14E-04
Signaling by the B Cell Receptor (BCR)	2,55E-02	phosphate metabolic process	9,04E-07	mRNA Splicing - Major Pathway	0.003	mRNA processing	2,11E-04	p53 signaling pathway	1,10E-03	egulation of cell proliferatio	4,62E-04	CD28 dependent PI3K/Akt signaling	7,91E-04
								NF-kappa B signaling pathway	2,20E-03			Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation	8,12E-04
												Constitutive Signaling by AKT1 E17K in Cancer	9,92E-04
												Regulation of TP53 Activity	1,82E-03
												Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	4,73E-02

Table 9. Adjusted P-value and False Discovery Rate (FDR) extracted from BiNGO and Reactome analyses from the clusters obtained, using MCODE and jActive modules, from the final network.

jActive Cluster 1				jActive Cluster 2				MCODE			
Reactome		BiNGO		Reactome		BiNGO		Reactome		BiNGO	
Overrepresented Pathways	FDR	Overrepresented GO: Biological Process	adjusted p-value	Overrepresented Pathways	FDR	Overrepresented GO: Biological Process	p-value	Overrepresented Pathways	FDR	Overrepresented GO: Biological Process	p-value
Interleukin-4 and Interleukin-13 signaling	6,82E-05	positive regulation of macromolecule metabolic process	1,56E-03	Interleukin-4 and Interleukin-13 signaling	1,11E-04	RNA splicing	5,91E-04	Regulation of KIT signaling	3,19E-04	ventricular cardiac muscle cell development	4,32E-03
Depolymerisation of the Nuclear Lamina	1,67E-04	positive regulation of receptor biosynthetic process	2,67E-03	Signaling by Interleukins	1,11E-04	labyrinthine layer blood vessel development	5,91E-04	Depolymerisation of the Nuclear Lamina	3,31E-04	ventricular cardiac muscle cell diferentiation	4,32E-03
Initiation of Nuclear Envelope (NE) Reformation	2,10E-04	placenta blood vessel development	9,60E-04	Depolymerisation of the Nuclear Lamina	1,59E-04	labyrinthine layer development	2,39E-03	Apoptotic execution phase	1,00E-03	cardiac cell development	4,32E-03
G1/S Transition	4,28E-04	organelle organization	3,56E-03	Initiation of Nuclear Envelope (NE) Reformation	2,24E-04	mRNA metabolic process	1,40E-03	Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	5,00E-03	muscle cell development	1,55E-02
Signaling by Interleukins	6,22E-04	positive regulation of gene expression	1,98E-03	mRNA Splicing - Major Pathway	6,86E-04	mRNA processing	7,50E-04	Regulation of signaling by CBL	7,00E-03	cardiac muscle tissue development	1,79E-02
PIP3 activates AKT signaling	1,00E-03	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	8,49E-04	Apoptotic execution phase	2,00E-03	organ development	3,70E-03	Signaling by KIT in disease	7,00E-03	protein acid amino acid phosphorylation	1,79E-02
Diseases of signal transduction by growth factor receptors and second messengers	1,00E-03	labyrinthine layer blood vessel development	4,74E-04	Apoptosis	2,00E-03	organelle organization	2,04E-03	IL6 signaling	4,42E-02	positive regulation of T cell receptor signaling pathway	3,50E-02
Negative regulation of the PI3K/AKT network	2,00E-03	positive regulation of cellular biosynthetic process	3,37E-04	mRNA Splicing	2,00E-03	placenta blood vessel development	1,11E-03	BCR signaling	1,05E-02	regulation of calcium ion transport via store-operated calcium channel activity	3,60E-02
CTLA4 inhibitory signaling	2,00E-03	positive regulation of cellular process	1,56E-03	PIP3 activates AKT signaling	2,00E-03						
CD28 dependent PI3K/Akt signaling	2,20E-03	positive regulation of nitrogen compound metabolic process	3,34E-04	Mitotic Prophase	2,00E-03						
Regulation of TP53 Activity	2,20E-03	positive regulation of cellular metabolic process	1,56E-03	Negative regulation of the PI3K/AKT network	2,00E-03						
		labyrinthine layer development	2,67E-03	CTLA4 inhibitory signaling	2,00E-03						
		positive regulation of macromolecule biosynthetic process	3,34E-04	Diseases of signal transduction by growth factor receptors and second messengers	2,00E-03						
		positive regulation of biosynthetic process	3,37E-04	CD28 dependent PI3K/Akt signaling	2,00E-03						
		protein import into nucleus, translocation	2,60E-03	Cytokine Signaling in Immune system	2,00E-03						
		positive regulation of metabolic process	1,98E-03								
		positive regulation of biological process	3,10E-03								
		regulation of DNA replication	1,56E-03								
				CD28 co-stimulation	4,04E-03						
				Regulation of TP53 Activity	4,04E-03						

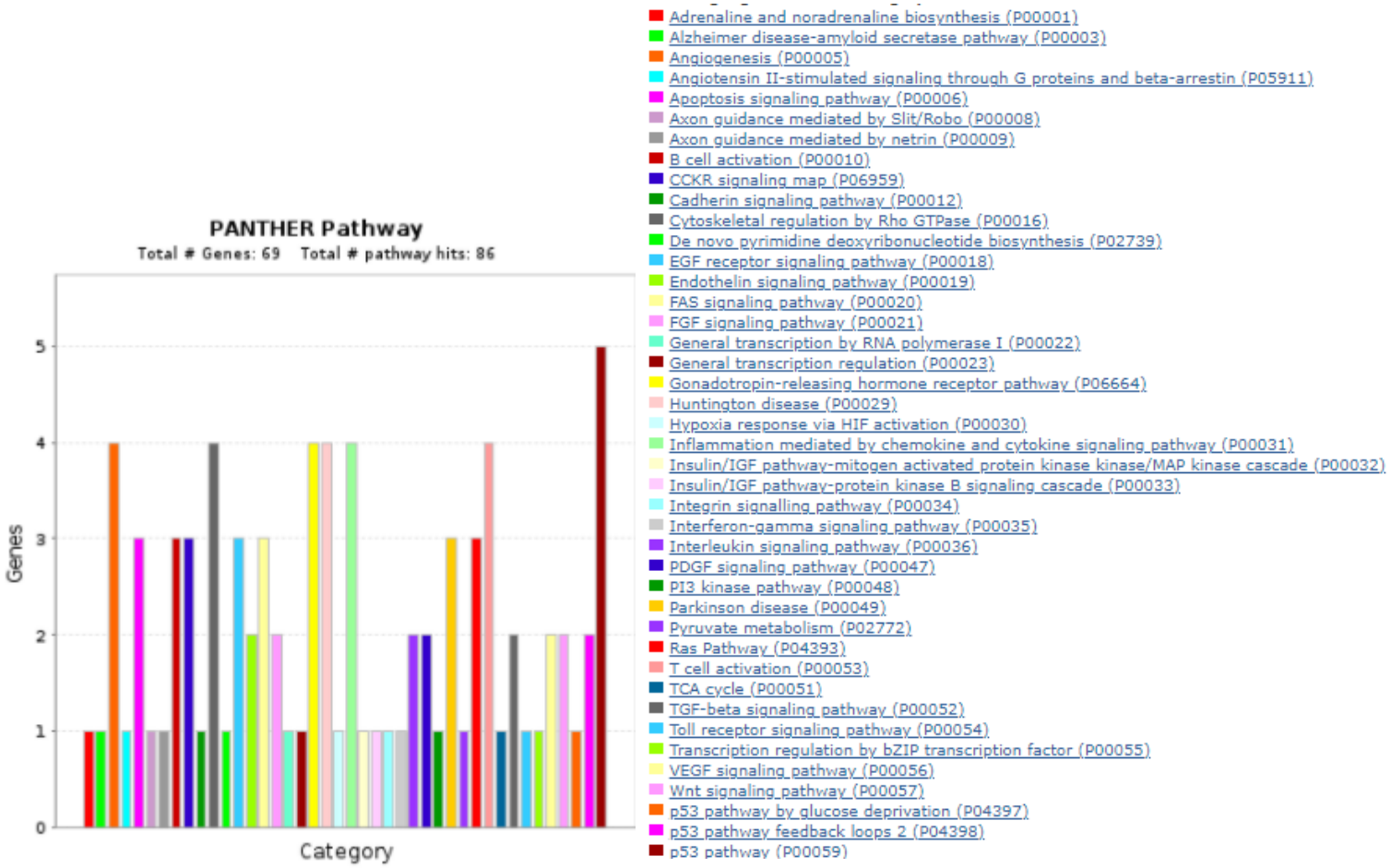


Figure 27. Panther analysis. Another analysis of the overrepresented pathways of the final network. It shows that a relevant part of the genes that form the network are involved in the BCR signaling. Data not discussed in the text. PANTHER (Protein Analysis Through Evolutionary Relationships is an integrated knowledgebase that can be used to analyse large-scale genomics data. It has a module that allows one to know which pathways are overrepresented in a protein dataset. It assigns protein clades to pathway components; in addition, it shows a pathway model generated using CellDesigner. It also integrates annotations from other databases. It annotates individual proteins to functional classes, importing Gene Onotolgies from the GO resource, or the Reactome pathways (1).

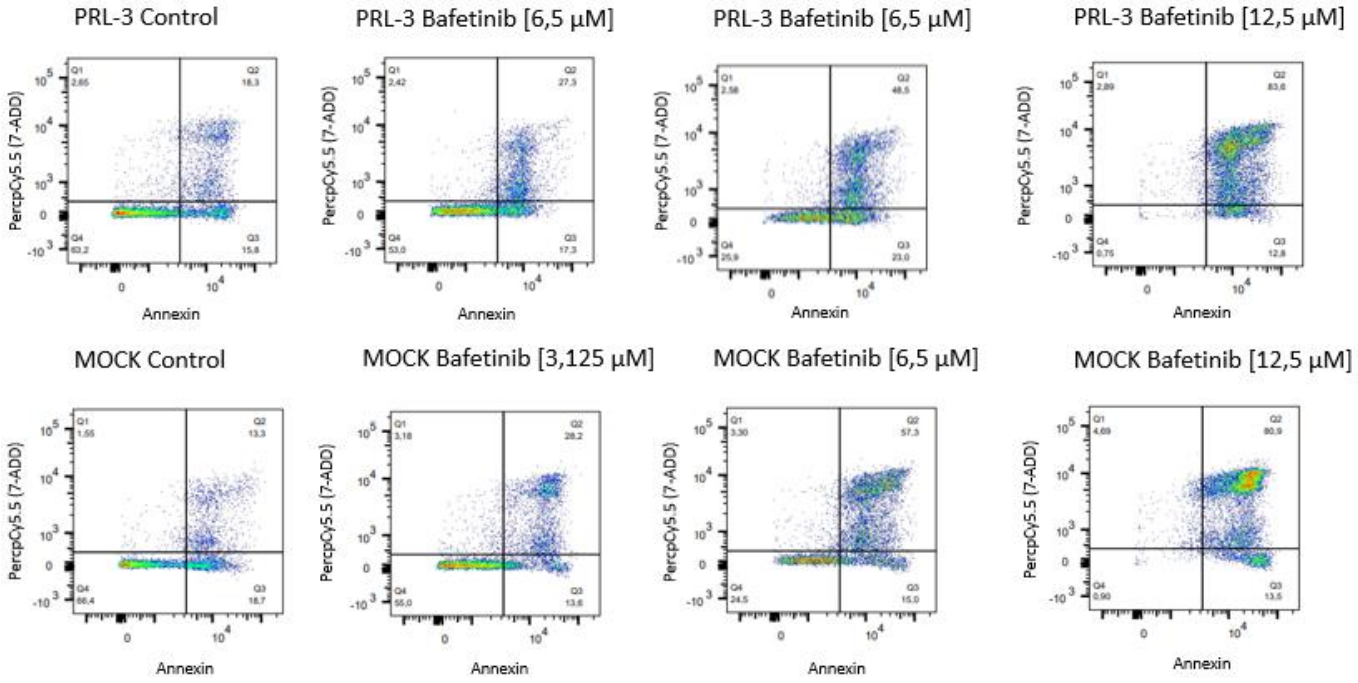


Figure 28. Flow cytometry assay that shows the differences in the light scatter pattern. This graph represents INA-6 cells treated with different concentrations of bafetinib, showing a dose dependent effect of the inhibitor with a higher effect on MOCK cells than in PRL-3 cells. Annexin was marked Alexa Fluor 674 area, the number of events detected by this fluorophore are represented on the x-axis. The 7ADD is detected by PercpCy5.5, and the number of events detected is represented in y-axis. In order to assess if there are differences in the bafetinib effect between PRL-3 and MOCK cells we observed the Q4, that represent the live cells. Live cells occupy Q4 area, as in this area there are low levels of Annexin V and 7-ADD detection, as they do not express PS on their surface, and they are not permeable to cationic compounds.

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