ROMANIAN ACADEMY
INSTITUTE OF BIOCHEMISTRY

PhD THESIS SUMMARY

MASS SPECTROMETRIC ANALYSIS OF EYA-MODIFIED PROTEINS FROM NORMAL AND CANCEROUS CELLS

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Objectives of the study

Eyes absent proteins belong to the aspartyl protein tyrosine phosphatase group of the PTP superfamily. They were initially defined as transcriptional co-activators, members of a regulatory network of evolutionary conserved transcription factors. This network interacts with many other signaling pathways and holds important roles in the formation, development and homeostasis of various tissues and organs. Eya proteins are also capable of protein-protein interactions, playing important roles in physiological and pathological processes. Loss-of-function mutations in the Eyes absent genes lead to numerous congenital syndromes such as cardiofacial syndrome, bronchio-oto-renal syndrome, oto-facio-cervical syndrome, congenital cataract, dilated cardiomyopathy and late onset of deafness. Overexpression of the Eyes absent genes/ EYA proteins has been found in various cancer forms like breast and epithelial ovarian cancer, Wilms' tumor, malignant peripheral nerve sheath tumors, lung and esophageal adenocarcinoma. Both functions of the Eya proteins are involved in carcinogenic processes.

Eya proteins exert their functions in the nucleus as well as in the cytoplasm. Although Eya are complex proteins, only two substrates have been identified so far: histone H2AX (with implications in DNA damage repair) and ERβ (with implications in tumor growth). These substrates illustrate the role of Eya in the cell nucleus. There are many reports about the cytoplasmic roles of Eya but so far, no cytoplasmic substrates have been identified. Therefore, in order to better understand Eya's function as protein tyrosine phosphatase, the first objective of my thesis was identification of novel cytoplasmic substrates of Eya. Their identification can be a starting point in seeking new processes and signaling pathways Eya proteins are involved in.

Phosphorylation is one of the most important post-translational modifications of proteins. Protein tyrosine phosphorylation, a subcategory, although representing less than 1% of the total phosphorylation events, plays a major role in signal transduction. Several studies report phosphorylation of Eya proteins' tyrosine residues. On the other hand, Eya autodephosphorylation has also been reported in non-human Eya proteins. Characterization of the balance between the two opposing processes, phosphorylation and (auto)dephosphorylation, provides information about the signal transduction mechanism induced by phosphorylation of tyrosine residues. In the case of Eya proteins, the phosphorylation-(auto)dephosphorylation interplay is expected to ensure a phosphorylation pattern for Eya that is adequate for the cell's physiological requirements. Thus, the second objective of my thesis was to identify EYA3 tyrosine phosphosites, to characterize its phosphorylation/autodephosphorylation balance as well as to identify the cellular implications of these antagonistic post-translational modifications.

INTRODUCTION

Protein phosphorylation and dephosphorylation represent essential cellular events mediated by kinases and phosphatases, enzymes that function in complementarity and coordinate each other in signal transduction to regulate numerous cellular functions. The importance of these two processes has been outlined in growth, differentiation, proliferation, transcriptional activation, cell cycle progression, motility and adhesion. Perturbation of the phosphorylation equilibrium can lead to cancer, (auto)immune and neurodegenerative diseases, even diabetes. Notably, although kinases and phosphatases modulate other proteins when fulfilling their activity, they can also be modulated through phosphorylation.

Protein tyrosine phosphorylation is one of the major cellular signaling mechanisms implemented by the eukaryotes[1]. Amongst all the other protein phosphorylation processes, tyrosine phosphorylation is extensively utilized only by multicellular eukaryotes[2]. It is regulated by the balanced and opposing activities of protein tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs)[1]. Cells use tyrosine phosphorylation as a tool in inter- and intracellular communication, the transport of molecules, in defining their shape, in motility, when deciding between differentiation and proliferation, in regulation of gene transcription, and in messenger ribonucleic acid (mRNA) processing[2]. Tyrosine phosphorylation and dephosphorylation are also involved in correlating these events between adjacent cells, from embryogenesis to organ development and tissue homeostasis[2].

CHAPTER I PROTEIN TYROSINE PHOSPHATASES - SYNOPSIS

I.1 GENERAL CHARACTERISTICS

Protein tyrosine phosphatases are enzymes that catalyze the removal of phosphate groups from phosphorylated tyrosine residues of proteins[3]. At the beginning, PTPs were viewed as nonspecific, "house-keeping" enzymes, that functioned to reverse the action of PTKs[4,5]. In time, this simplistic assumption proved to be wrong and many evidence argued that PTPs display substrate specificity, and cooperate with the kinases to regulate signaling pathways in a well-defined manner[5–7]. Actually, PTPs form a superfamily of structurally diverse and complex enzymes. Studies have shown that, as in the case of PTKs, PTPs can have either positive or negative influence on the signaling pathways and play important roles in maintaining the cellular physiology[8,9]. Mathematical models have been implemented, with the attempt of describing how kinases and phosphatases regulate signaling pathways[10,11]. Both models describe that kinases control signal amplitude more than signal duration, whereas phosphatases have a pronounced effect on the rate and duration of the response[10,11]. Dysregulation of the PTP mediated signaling pathways has been reported in cancer as well as in cardiovascular, neurological, autoimmune and metabolic diseases[12]. Perturbations in the PTP function can be a result of protein overexpression, loss of expression[13–15] and even single amino acid polymorphism[16].

Altogether, understanding the role of tyrosine phosphorylation in physiology and disease implies that equal attention should be given to both enzymatic partners.

I.2 CLASSIFICATION

Many classifications regarding the PTP super-family have been made with the passage of time, taking into consideration the newest discoveries in the field. The latest classification of the tyrosine phosphatases was made based on the catalytic residue that initiates the nucleophilic attack. Thus, they are grouped into: Cysteine (Cys), Aspartate (Asp) and Histidine (His) phosphatases[17] (Table I.1.). This new classification is a consequence of the addition of new members to the PTP super-family. Thus, the extended human PTPome was generated[17]. The recent criteria by which a protein is included as member of the functional Tyr phosphatase family are: a) the existence of a PTP domain or b) the presence of a CxxxxxR signature motif within a non-PTP phosphatase domain or c) if it proves to have tyrosine phosphatase activity or d) it shows high sequence similarity to members with demonstrated tyrosine phosphatase activity[17]. Considering these criteria, the PTP super-family now contains up to 125 proteins, with approximately 40 of them specifically targeting phosphotyrosine-containing substrates[17].

In the PTP super-family, there is a wide range of substrate specificity, with a focus on the hydrolysis of phosphate esters: phospho-Ser or phospho-Thr containing substrates, phosphoinositide phosphates (PIPs), phosphorylated carbohydrates, mRNAs, inorganic moieties. On the other hand, the tyrosine phosphatase activity can also be performed by proteins from structurally diverse enzyme families, like haloacid dehalogenases (HAD), His phosphatases (HPs), rhodaneses or arsenate reductases[17].

Table I.1. Classification of the extended family of PTPases from the human genome (from [17])**.** Grouping of the tyrosine phosphatases has been made based on their nucleophilic catalytic residue - Cys, Asp, or His - and their topology. Different colors are attributed to each family of Tyr phosphatases that share a common nucleophilic catalytic residue (blue for Cys-based, green for Asp-based and red for His-based) and the different classes inside a family are coloured with different tones of the same colour. Abbreviations: pTyr/ pSer/ pThr – phosphotyrosine-/ phosphoserine-/ phosphothreonine-containing substrates; PIPs - phosphatidylinositol phosphates.

Family of tyrosine phosphatases sharing a common nucleophilic catalytic residue	Class	Subclass	Name of subclass	Number of members in each group	Substrate
Cys-based	Class I	Subclass I	Classic	37	pTyr, PIPs
		Subclass II	VH1-like	63	pTyr, pSer, pThr, PIPs, other (nonproteinaceous)
		Subclass III	SACs	6	PIPs
		Subclass IV	PALD1	1	unknown substrate
		Subclass V	INPP4s	2	PIPs
		Subclass VI	TMEM55s	2	PIPs
	Class II		LMW-PTP	1	pTyr
			SSU72	1	pSer
	Class III		CDC25s	3	pTyr, pThr
Asp-based	HAD		EYAs	4	pTyr, pThr
His-based	PGM		UBASH3s	2	pTyr
	Acid phosphatases		ACPs	3	pTyr, pSer, pThr, nonproteinaceous

I.3 MECHANISM OF ACTION

The tyrosine phosphatases catalyze the removal of the phosphate group from the tyrosine-phosphorylated protein (substrate) in a two-step mechanism (detailed in Fig. I-1).

The reaction is initialized by the catalytic residue (Cys, Asp or His, corresponding to each family of phosphatases) which makes a nucleophilic attack on the phosphate group of the substrate, thus forming a transient enzyme-substrate intermediate, then releases the

dephosphorylated substrate and forms a phosphoenzyme intermediate (Step 1 in Fig. I-1). In this step, an Asp residue works as a general acid, because it donates a proton to the tyrosyl group.

In the following step of the reaction (Step 2 in Fig. I-1), the phosphoenzyme intermediate is hydrolyzed and the enzyme is recovered. Here, the same Asp acid now plays the role of a general base, deprotonating a water molecule, which now acts as nucleophile and produces dephosphorylation of the enzyme.

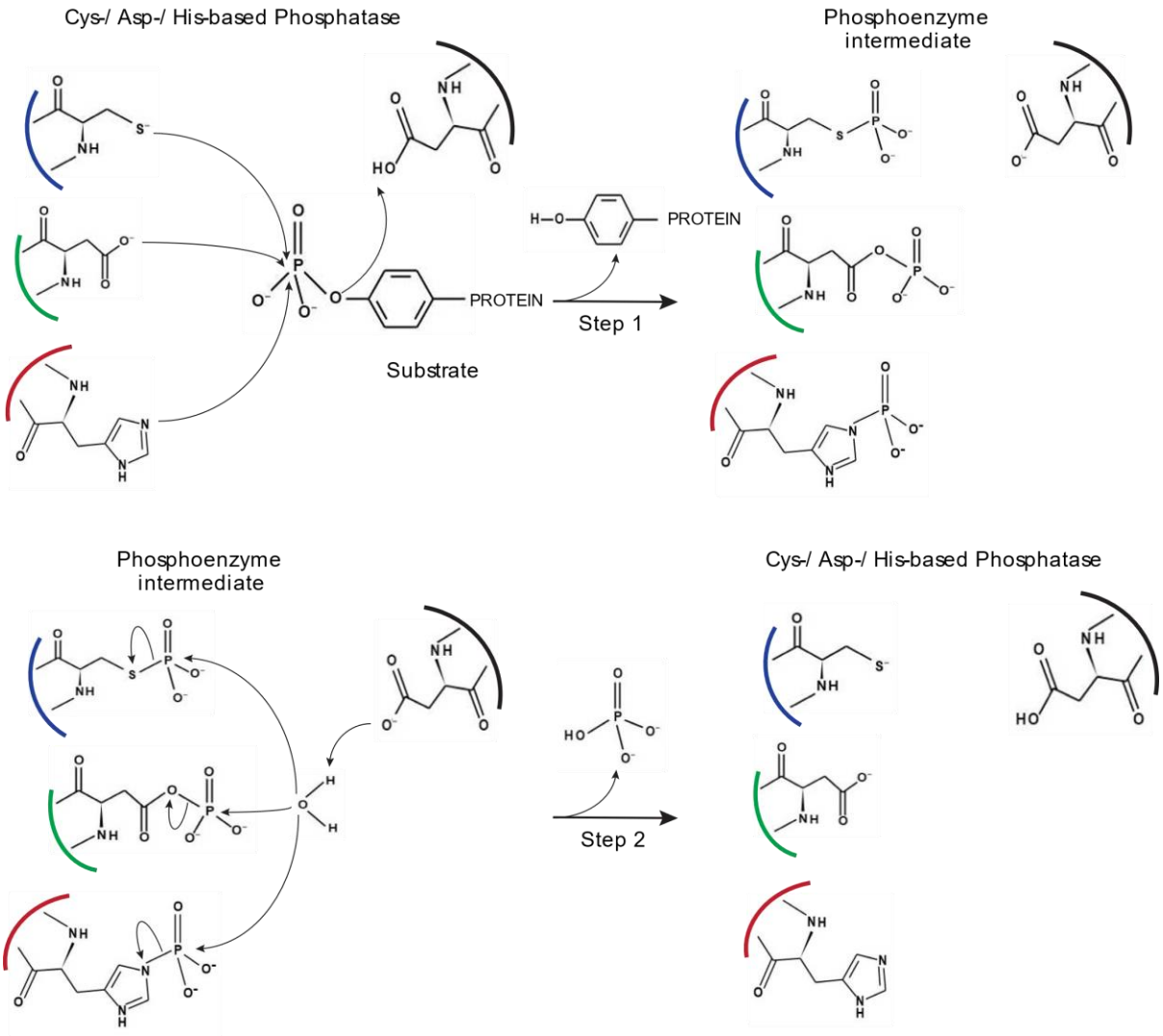


Figure I-1. Schematic reprezentation of the catalytic mechanism of the human PTP super-family (adapted from [17]).

I.4 ASP-BASED PTPs

This cathegory is represented only by the Eyes absent (EYA) proteins[17]. They were named "Eyes absent" because the first characterization, made on the *Drosophila eyes absent* (*eya*) gene product, identified the gene to be essential in the development of the normal fly eye[18]. Loss of *eya* function resulted in an eyeless adult fly phenotype and it was discovered that *eya* activity is necessary for survival of the eye progenitor cells and later, their differentiation. They were initially defined as transcriptional co-activators[19], but it was revealed they also have protein tyrosine phosphatase activity[20,21]. As transcriptional co-activator, it has been demonstrated that eyes absent (*eya*) is member of a regulatory network of evolutionary conserved transcription factors and cofactors, termed retinal determination gene network (RDGN) in *Drosophila*, along with twin of eyeless (*toy*), eyeless (*ey*), sine oculis (*so)* and dachshund (*dac*)[22–28]. From insects to humans, there are corresponding gene families - *Pax* (for *toy* and *ey*), *Six* (for *so*), *Eya* (for *eya*) and *Dach* (for *dac*) - which constitute the PSEDN (Pax-Six-Eya-Dach Network). By interaction with many other signaling pathways (TGF-β; Notch, Hedgehog, Wingless/Wnt, EGFR)[29], this network holds important roles in the formation, development and homeostasis of various tissues and organs: eyes, kidneys, nervous system, ears, heart, muscles[30]. The PSEDN is also implicated in several processes, such as limb formation[31,32], gonadogenesis[33] and neurogenesis[34]. This function resides in the N-terminal part of the protein, a region which is poorly conserved[35]. The PTP activity is located in the C-terminal part of the protein, which is a highly conserved region, also termed Eya Domain (ED). This region is also necessary in fulfilling transactivation processes, being capable of making protein-protein interactions with the DNA binding proteins[19,36,37]. Eya's PTP catalytic mechanism involves two Asp residues and a Mg^{2+} ion, as cofactor. The catalytic consensus motif, DxDx(T/V), from ED, resembles motif 1 of the HAD superfamily. Sequence alignments and structural analysis proved Eya is member of the phosphatase group of this superfamily[20,21]. The PTP activity of Eya is known to be involved in DNA damage repair[38,39], angiogenesis[40] and many carcinogenic processes[41–43].

There are four vertebrate homologs of the *Drosophila* gene *eya*, *Eya1-4*. Loss-of-function mutations in genes encoding for Eyes absent proteins (*EYA1*, *EYA4*) lead to congenital syndromes like cardiofacial syndrome[44], bronchio-oto-renal syndrome[45], oto-facio-cervical syndrome[46], congenital cataract[47], dilated cardiomyopathy[48] and late onset of deafness[49–51]. Overexpression of the Eyes absent genes/ EYA proteins has been found in breast[41,52] and epithelial ovarian cancer[53], Wilms' tumor[54], malignant peripheral nerve sheath tumors[55], lung[56] and esophageal adenocarcinoma[57]. All of Eya's functions have been linked to carcinogenic processes.

CHAPTER II MASS SPECTROMETRY-BASED PROTEOMICS

II.1 GENERAL CONSIDERATIONS

Proteomics is defined as a branch of biotechnology which involves the large-scale characterization of all the proteins from a particular cell, tissue or organism[58,59]. Techniques such as biochemistry, cellular and molecular biology, bioinformatics and genetics are used to obtain a more global view of the structure, function and interactions of the proteins in a cell. Proteomic studies can be approached from three major perspectives: protein expression, structural and functional proteomics[60]. Structural proteomics is concerned with determining all proteins from a protein complex or from an organelle, identify their location and characterizing protein-protein interactions. The final goal in structural proteomics is building the architecture of a cell and explaining how proteins expressed at a certain moment can give the cell unique characteristics[60].

One of the most popular technologies applied in the field of structural proteomics is mass spectrometry (MS). A very sensitive analytical technique, mass spectrometry enables the determination of protein structural information such as amino acid sequences, peptide masses, the type and location of (post-translational) modifications, protein - protein interactions, protein complexes. What makes mass spectrometry a very powerful tool in the field of proteomics is that the information gained from mass spectrometric analysis of proteins can also be used in determining protein expression profiles and all information obtained can be integrated into functional data (protein signaling pathways, disease mechanisms, protein-drug interactions).

Principle and instrumentation

Mass spectrometry is an analytical technique in which the instrument measures the mass-to-charge ratio (m/z) and intensity of charged molecules or particles. The direct readouts gained from a mass spectrometric analysis are the mass spectra. The mass spectrum of an ionized compound is a representation of its signal intensity versus its m/z[61]. According to the principle described, a mass spectrometer is basically made up of three components: the ion source, the mass analyzer (measures m/z of the ionized analytes) and the detector (registers the number of ions at each m/z value). Besides these components, the instrument must also contain: a sample inlet and after the detector, a computer. The inlet is a system used to introduce a small amount of the sample to be analysed into the ion source. The computer has a special software necessary to acquire and process the data obtained from the mass spectrometer in order to generate them in the form of mass spectra[61].

Mass spectrometric techniques in proteomics

The MS-based techniques which can be approached depend on the type of sample we have and what information we want to obtain from its analysis. In proteomics MS, there are: i) two basic techniques - bottom-up and top-down MS - which offer information regarding the primary structure of the proteins (molecular weight, amino acid sequence, detection and localization of post-translational modifications - PTMs) and ii) MS-based structural techniques, which offer information regarding the secondary, tertiary and quaternary structure of the proteins, the dynamics of conformation and composition of protein complexes - native MS, cross-linking mass spectrometry, hydrogen-deuterium exchange, ion-mobility MS, covalent labeling/footprinting.

II.2 MASS SPECTROMETRY-BASED PHOSPHOPROTEOMICS

General aspects of post-translational modifications analysis through mass spectrometry

The term "post-translational modification" (PTM) refers to an enzymatic process that occurs on a polypeptide chain (protein) after its translation is complete[62]. The covalent attachment of a functional group or removal of a certain chemical moiety, a proteolytic processing of the protein termini, a folding process decisive for the functional maturation of a protein or the covalent cross-linking between protein domains, are all PTMs[62]. Through PTMs, an organism expresses an increased number of functional proteins than its genetic information would allow, thus diversifying the proteome.

In mass spectrometry, the type and even the nature of the PTM can be revealed by accurately determining the mass difference between the modified and unmodified form of a protein or the peptides which are derived from it. In order to detect which amino acid suffered the modification, tandem mass spectrometry is used to sequence the peptides or proteins. There are many characteristics of PTMs which make them more difficult to be detected and correctly assigned in MS/MS, their low abundance and substoichimetry being the most important. Their lability makes them especially challenging to be correctly assigned, some can affect peptide detection by decreasing ionization, and many increase the hydrophilicity, which can result in difficult sample handling, purification or even binding to the usual columns utilized for peptide purification and separation before MS/MS analysis[63,64]. PTMs can also affect protease cleavage efficiency, resulting in peptide products that are large and problematic when it comes to ESI and MS/MS[64].

The detection of PTMs is an opportunity to identify mechanisms by which proteins are dynamically regulated and, on a large scale (cellular level), to obtain important qualitative and quantitative information. All the same, their mass spectrometric analysis is very often entangling and can lead to incorrect conclusions regarding the data obtained.

Enrichment methods in phosphoproteomics

It is thought that in eukaryotes, at any given moment, phosphorylation occurs in one third of the total proteins[65]. Serine, threonine and tyrosine phosphorylation of proteins are key events implicated in signal transduction or amplification processes in eukaryotic cells[63]. Protein phosphorylation has a dynamic nature, involving the complementary activities of protein kinases and protein phosphatases[65]. Phosphoproteomics, which concerns the complete analysis of protein phsphorylation, implies not only the identification of phosphoproteins and phosphopeptides, but also the localization of the actual phosphorylated residues and quantification of phosphorylation. There are several facts about protein phosphorylation in general and particularities regarding each of the three major phosphorylated amino acids (S, T, Y; His phosphorylation is not considered here), which make the analysis not so easy to fulfill. Taking these facts into consideration, diverse analytical techniques have been developed and improved. Earlier strategies applied for the localization of phosphorylated sites implied Edman sequencing and ^{32}P-phosphopeptide mapping. In the case of mass spectrometry, there are diverse enrichment strategies that can be coupled to methods of detection[63]. There are three main enrichment strategies that can be applied at the protein or peptide level:

1. **Affinity-based techniques:** IMAC, MOAC, Phos-tag, hydroxyapatite;
2. **Immunoprecipitation (IP)**, using phosphospecific antibodies;
3. **Chemical (covalent) modifications:** β-elimination, carbodiimide condensation, α-diazo resin, oxidation-reduction condensation.

II.3 NATIVE MASS SPECTROMETRY

Native mass spectrometry is a technique in which the biological analyte, from a nondenaturing solvent, is introduced into the instrument using electrospray ionization[66]. Thus, the term "native" refers to the biological state of the analyte in solution, before it is subjected to ESI, because the analyte partially losses its native folded state after transition into the gas phase and until detection, being in the vacuum of a mass analyzer[67]. Native MS focuses on the investigation of the secondary, tertiary and quaternary structures of the proteins, protein complexes and also their interactions with nucleic acids (DNA, RNA, oligonucleotides), cofactors, ligands, drugs and other macromolecules such as oligosaccharides, crown ethers or

macrocyclic molecules[68,69]. Thus, in the field of structural biology, native MS is complementary with traditional methods such as NMR, electron microscopy and X-ray crystallography, because it can reveal structural as well as functional information regarding the interactions from protein complexes and dynamics of these complexes[66].

Native MS in combination with other MS methods such as hydrogen/deuterium exchange MS (HDX MS), cross-linking MS (XL-MS), covalent labeling MS, top-down and middle-down proteomics[70–73], plays a unique role in structural biology in revealing not only a detailed composition of the complexes, but also what are the structural and functional consequences of, for example, binding with cofactors, ligands, nucleic acids, macromolecules even other protein complexes.

II.4 QUANTITATION IN MASS SPECTROMETRY-BASED PROTEOMICS

By evaluating the abundance of proteins in different conditions, an extensive characterization of the biologically relevant proteins and then of the biological system can be performed. Differential protein expression can be a sign of environmental stress or even worse, of a pathological condition. Quantitative proteomics can be used to determine the expression pattern of a protein in normal versus stressed or healthy versus diseased state and this makes it a valuable tool in the field of biomarker discovery[74].

In proteomics, MS-based quantitation approaches evaluate protein abundance based on the abundance of the corresponding peptides detected. Proteolytic peptides have several physicochemical properties (ex. hydrophobicity, charge, size) which make them generate very different mass spectrometric signals; that is why, for an accurate quantitation, the comparison of each individual peptide between experiments is generally recommended[75]. Quantitation can be achieved through several approaches, taking into consideration the instrument-related (ion source parameters, efficiency of ion transmission and detection, contamination) and sample-related factors (sample preparation, signal stability and repeatability; analyte's ionization efficiency, concentration and degradation; ionization, detection and spectral interferences)[76]. These approaches are protein quantification by labeling (metabolic, chemical and enzymatic labeling); label-free quantification; selected reaction monitoring (SRM); absolute quantification.

CHAPTER III WDR1, A NOVEL EYA3 SUBSTRATE

Eyes absent proteins possess transcriptional coactivator and protein tyrosine phosphatase functions on the same polypeptide chain, which makes their study even more intriguing. It is well established that Eya proteins exert their transactivator function in the nucleus. Since the discovery of their enzymatic activity, several questions have been raised concerning the eventual connection between the two functions, particularly whether Eya's PTP activity is implicated in its transactivation function (for example, if, by dephosphorylating a specific substrate, Eya might influence its own transactivation potential) or they are independent[77]. However, Eya proteins exert their protein tyrosine phosphatase activity in both nuclear and cytoplasmic compartments. Reports show many processes in which EYA's PTP activity is involved but no substrates were identified, for example induction of transformation, migration and invasion of breast cancer cells[41], tumor angiogenesis with consequences in tumor growth[78].

In order to better understand Eya's function as protein tyrosine phosphatase, it would be of utmost importance to identify more of its substrates and determine the physiological relevance of the interaction. Here, we report human EYA1, EYA2 and EYA3 phosphorylation by protein tyrosine kinase Src, the interaction between two human EYA proteins as well as the interaction between human EYA3 and WD repeat-containing protein 1 (WDR1) protein.

III.1 MATERIALS AND METHODS

The necessary materials consisted of constructs, primers, antibodies, enzymes, kits, reagents and solutions (molecular biology grade, for mammalian and bacterial cell cultures, for protein expression and purification). The following experimental procedures and analyses were performed: mammalian cell culture, transfection, harvesting and lysis, immunoprecipitation, protein expression and purification from bacterial culture, *in vitro* kinase reactions*, in vitro* protein tyrosine phosphatase assays, siRNA mediated knockdown.

III.2 RESULTS AND DISCUSSIONS

III.2.1 EYA1, EYA2 and EYA3 phosphorylation by protein tyrosine kinase Src

EYA1, EYA2 and EYA3 are phosphorylated by Src kinase and all possess autodephosphorylation capacity

An earlier report showed that Eya proteins have autodephosphorylation capacity[20], but there were no studies concerning the human homologues. Our first objectives were: i) to find out if human Eyes absent proteins are tyrosine phosphorylated, ii) by which kinase(s), and iii) if they have the capacity of autodephosphorylation.

We found that three protein tyrosine kinases - Abl, Btk and Src - phosphorylate human EYA3 protein. Knowing that Src kinase is involved in many processes that lead to tumor development (survival, proliferation, migration, adhesion, invasion, metastasis) in various tumor types, plays roles in the EMT of cancer cells and even modulates tumor microenvironment, we chose it for further extensive studies regarding phosphorylation of EYA proteins. We detected that human EYA1 and EYA2 were also phosphorylated by Src kinase. The fact that for all three EYA protein homologues, the WT forms display a much lower phosphorylation intensity compared to the catalytically inactive ones, suggests the active PTPs have autocatalytic activity. To verify this hypothesis, we performed *in vitro* Src kinase reactions on EYA3 WT, EYA3 D309N, EYA3 ED, and EYA3 ΔED (Fig. III-1), in the presence as well as absence of BB, a specific inhibitor of Eya's PTP activity[40]. The results indicate that the reduced phosphorylation level of EYA3 WT and EYA3 ED is a consequence of EYA3 autodephosphorylation. These *in vitro* phosphorylation reactions show not only that EYA3 protein is capable of autodephosphorylation, but also that Src kinase phosphorylates both the ED and the N-terminal part of the protein and the resulted phosphoresidues can be autodephosphorylated.

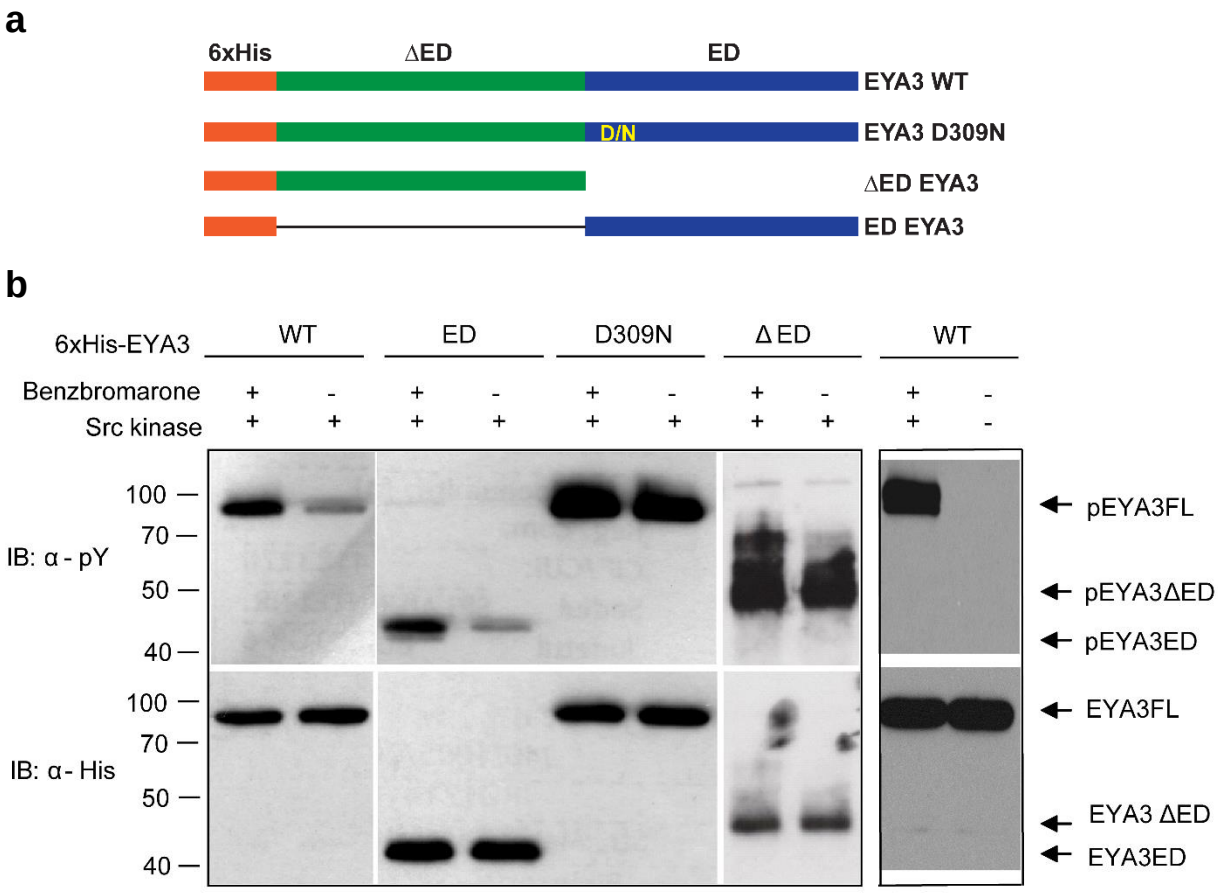


Figure III-1. *In vitro* Src-phosphorylation of EYA3 and its autodephosphorylation.
a) EYA3 constructs, bacterially expressed in *E. coli* and purified. Full-length human EYA3 WT (Uniprot number Q99504) and EYA3 D309N mutant, EYA3 ΔED and EYA3 ED, are all constructs obtained

with 6xHis tag at the N-terminal part of their sequence (adapted from [80]). **b)** *In vitro* tyrosine phosphorylation of 6xHis-EYA3 constructs by Src. The 6xHis-EYA3 proteins were incubated with active GST tagged v-Src kinase, with or without 100 μM BB. Figure from [79].

The influence of two tyrosine residues from the catalytic domain of EYA on its protein tyrosine phosphatase activity

To identify the tyrosine residues from EYA3 that are phosphorylated by Src kinase, we used prediction programs and we also performed preliminary mass spectrometry analysis. With the aim of validating tyrosine phosphorylation at these residues, several experiments involving EYA3 D309N were performed in HEK293T cells. Results show no significant contribution to the total phosphorylation of EYA3 D309N for neither one of the tyrosine residues mutated (Y96, Y105, Y208, Y237). We also tested four tyrosine residues from the ED of EYA3 - Y426, Y496, Y508 and Y532. Results indicates that neither one of these residues has a major contribution to EYA3 phosphorylation. However, the phosphorylation level of EYA3 Y508F and EYA3 Y532F was similar to the one of EYA3 D309N, the PTP inactive mutant. Knowing that EYA proteins have autodephosphorylation capacity[20], the behavior of EYA3 Y508F and EYA3 Y532F could be explained by the fact that Y→F mutation of these specific residues decreases the autodephosphorylation capacity. Thus, we performed *in vitro* PTP assays on immunoprecipitated full length EYA3 proteins as well as on EYA3 ED proteins using p-nitrophenyl phosphate (pNPP) as artificial substrate. Results show that generation of either one of the Y508F or Y532F mutations produces catalytically inactive full length EYA3 as well as ED EYA3 proteins.

From the experiments performed, we could not say whether Y496, Y508 or Y532 are Src-phosphorylation sites. A possible explanation could be that phosphorylation of the N-terminal phosphosites covers the variation in phosphorylation in the ED. Therefore, we checked for variation in phosphorylation level only in EYA3 ED. We detected that Y508 and Y496 are phosphosites of Src kinase.

Both Y508 and Y532 residues are conserved among mammalian and *Drosophila* Eyes absent proteins, so we evaluated if the catalytic activity of other EYA members is influenced by the Y→F mutation of the corresponding residues. To this aim, we used constructs encoding for EYA1 and EYA2 which contain mutations corresponding to Y508F (Y494F for EYA1 and Y473F for EYA2) and Y532F (Y518F for EYA1 and Y497F for EYA2) in EYA3. Surprisingly, Y494F and Y518F mutations do not inactivate EYA1 phosphatase. Concerning EYA2, quantified Western blot results show that Y473F mutation decreases EYA2's autodephosphorylation capacity (and thus its PTP activity), whereas Y497F does not affect it.

The direct or indirect control of these two tyrosine residues over the PTP activity of EYA seems to be specific for each human EYA protein.

EYA3 Y508F and EYA3 Y532F act as trapping mutants for EYA3 protein tyrosine phosphatase

We decided to make use of EYA's autodephosphorylation capacity, demonstrated above and also in a study involving other Eya proteins[20], to search if the two EYA3 Y→F mutants have trapping capacity, by testing if they can interact with another inactive EYA3 protein.

We determined that EYA3 Y508F and EYA3 Y532F interact with and trap EYA3 D309N with an affinity similar to that of EYA3 D311N protein (Fig. III-2a), the known trapping mutant of EYA3[82]. In a similar experiment, Y508F mutation added to EYA3 D309N mutant (EYA3 D309N Y508F construct), turned the protein, which was incapable of trapping, into an efficient trapping mutant (Fig. III-2b). EYA3 D309N trapping by EYA3 Y508F takes place only in the case of Src co-expression, indicating that the interaction between two EYA molecules depends on the induction of their tyrosine phosphorylation (Fig. III-2b). These last two experiments prove that EYA3 Y508F and EYA3 Y532F are good trapping proteins and can be used in further functional studies in the search for new EYA3 phosphatase substrates.

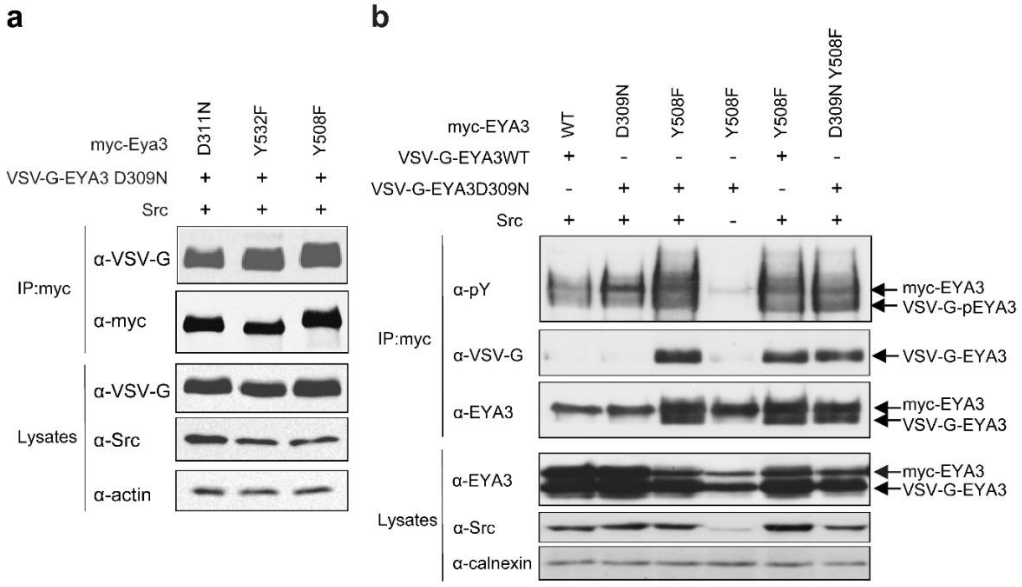


Figure III-2. EYA3 Y508F and EYA3 Y532F act as trapping mutants for EYA3 protein tyrosine phosphatase. HEK293T cells were transiently co-transfected with vectors encoding for c-Src Y527F kinase and pairs of differently tagged EYA3 proteins: **a)** VSV-G-EYA3 D309N (bait) and c-myc tagged single point mutated EYA3 proteins (D311N, Y508F or Y532F) and **b)** VSV-G-EYA3 WT and D309N as bait, with c-myc-EYA3 WT/D309N/Y508F/D309N Y508F. Figures are adapted from [79].

To determine which regions of the EYA protein participate in the autodephosphorylation reaction, we co-expressed VSV-G-EYA3 Y508F with different c-myc tagged fragments of EYA3 protein - the N-terminal (EYA3 ΔED) and ED (WT/D311N/Y508F). Results show that

the intermolecular interaction is conditioned by the presence of the ED in both molecules and the ΔED is not necessary for the interaction to take place (Fig. III-3a). Another IP experiment from HEK293T cells, in which EYA3 ED Y508F (VSV-G tagged) was co-expressed with full length c-myc-EYA3 Y508E mutant (which imitates the negative charge of the phosphate) with or without Src, demonstrated that the interaction between the ED part of EYA3 and full length EYA3 is conditioned by their phosphorylation (Fig. III-3b).

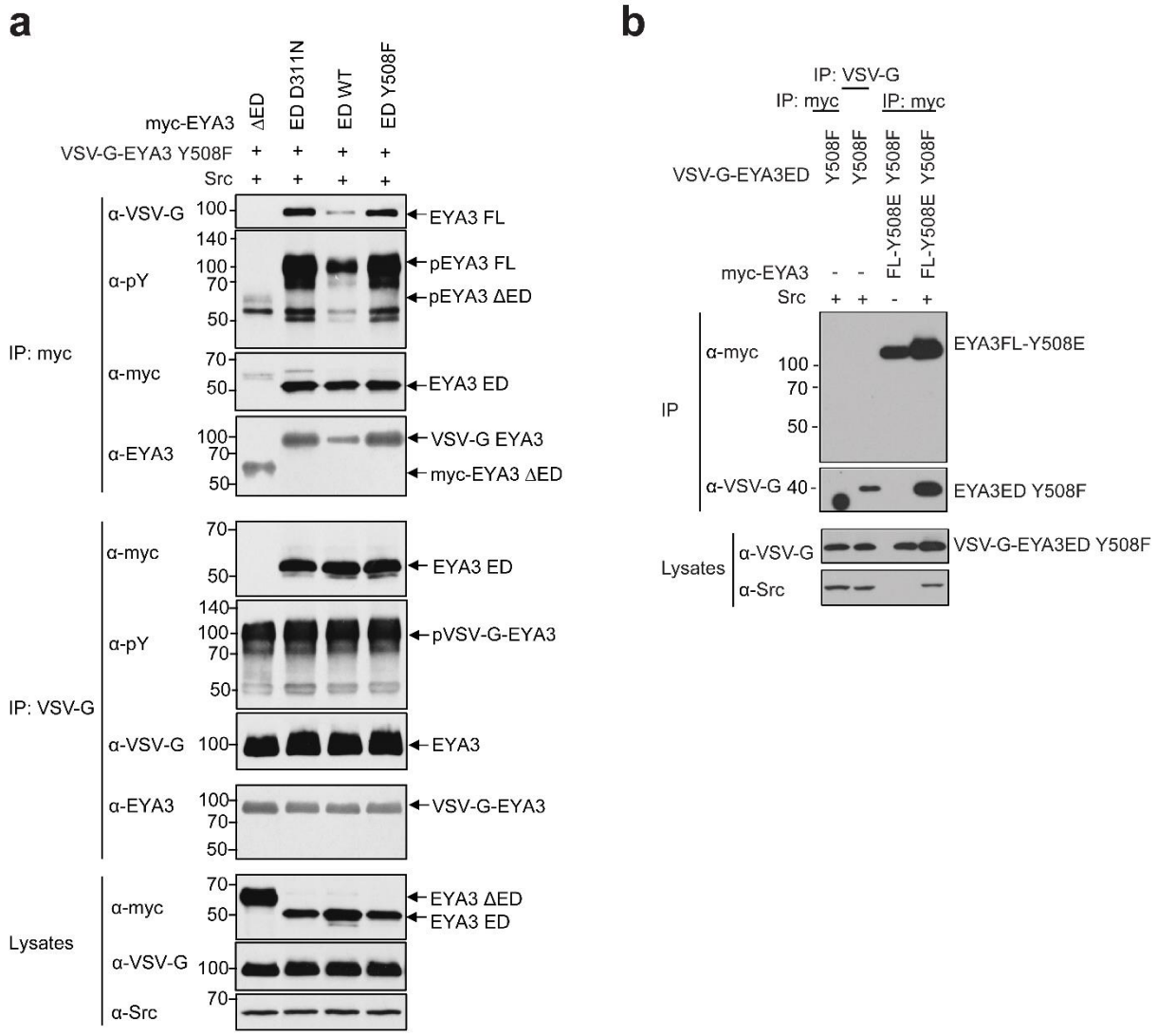


Figure III-3. The interaction between full length EYA3 and EYA3 fragments (ΔED, ED).
a) VSV-G-EYA3 Y508F was transiently co-expressed with c-Src Y527F and different c-myc tagged fragments of EYA3 protein (ΔED, ED WT, ED D311N and ED Y508F) in HEK293T cells.
b) EYA3 ED Y508F (VSV-G tag) and full length EYA3 Y508E (c-myc-tagged) were co-expressed with or without c-Src Y527F in HEK293T cells. Figure adapted from [79].

Once we determined the conditions for the EYA3-EYA3 interaction, we wanted to check if the interaction can take place between two different members of the human EYA family. We checked if EYA3 interacts with EYA1, in HEK 293T cells. Results revealed that EYA1 D297N

is not capable of trapping EYA3 Y508F. The fact that EYA1 does not interact with EYA3 indicates that EYA-EYA interaction takes place only between the same kind of paralogs.

III.2.2 EYA3 - WDR1 interaction

In order to identify new substrates for EYA3, a phosphatase activity profiling was performed on peptide microarrays by JPT Peptide Technologies, using bacterially purified His-tagged EYA3 WT - active as protein tyrosine phosphatase (described by Dr. Mihaela Mențel in her PhD Thesis and in[79]). A number of 6207 pY-containing peptides (13 amino acids long with the pY in the middle of the sequence), derived from human tyrosine phosphorylated proteins, were tested. Of these, 68 phosphopeptides were dephosphorylated more than 75%, while 7 of them were 100% dephosphorylated.

From the results obtained in the phosphatase activity profiling, we chose a candidate substrate protein based on the published data regarding the cellular implications of EYA's enzymatic activity. From the 7 phosphopeptides which were 100% dephosphorylated by EYA3, the one which corresponded to WD repeat-containing protein 1 (WDR1) was chosen for further investigations. WDR1 is a cytoskeletal protein which associates with cofilin and enhances its actin filament disassembly capacity[83]. It is implicated in regulating the dynamics of the cortical actin cytoskeleton (localizes cofilin to the cortical actin patches)[84] and directional cellular migration[85]. Its corresponding phosphopeptide from the microarray, AHDGGI-pY-AISWSP, contains Y238 as residue.

In order to validate the results obtained in the microarray experiment (in a cellular model), first we were interested in obtaining tyrosine phosphorylated WDR1 WT. To this aim, we co-expressed WDR1 and Src kinase in HEK293T cells. Western blot results indicated that WDR1 WT is phosphorylated by Src and Y238 is one of the phosphosites (Fig. III-4a). Knowing this, an experiment aimed to test whether endogenous EYA3 dephosphorylates phospho-WDR1 WT was performed in HEK293T cells. WDR1 WT and Src were co-expressed and 24 h post-transfection, either benzbromarone (specific inhibitor of EYA's PTP activity)[40], PP2 (Src kinase family inhibitor)[86] or both of them were added in the fresh media of the cells. These results indicate that EYA dephosphorylates phospho-WDR1 WT (pY-WDR1 WT) when Src is inhibited by PP2 and constitutes an important argument for the fact that tyrosine phosphorylated WDR1 WT is substrate for EYA3 PTP (Fig. III-4b).

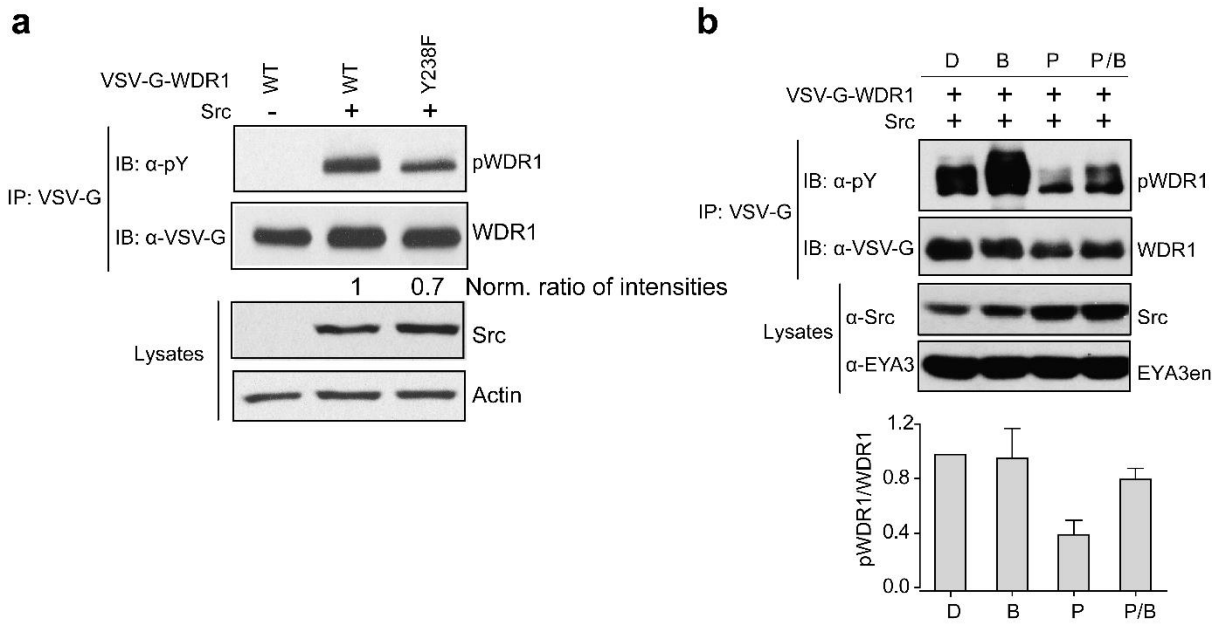


Figure III-4. WDR1 is tyrosine phosphorylated by Src kinase and dephosphorylated by endogenous EYA3.
a) VSV-G-WDR1 WT and Y238F were transiently co-expressed with or without c-Src Y527F in HEK293T cells. Tyrosine phosphorylation was quantified using ImageJ[81] software. The value obtained for WDR1 Y238F (pWDR1 Y238F/WDR1 Y238F) was normalized to the one for WDR1 WT (pWDR1WT/WDR1WT).
b) VSV-G-WDR1 WT and c-Src Y527F were transiently co-expressed in HEK293T cells and 24 h post-transfection, the cells were treated with DMSO, benzbromarone (noted here B), PP2 or both benzbromarone and PP2, for 20 h. Bands were quantified using ImageJ[81] software. The bar graph shows the relative intensities of VSV-G-WDR1 WT (pWDR1/WDR1) in each of the four samples, normalized to the relative intensity obtained in the DMSO sample. Values represent mean ± S.D. of three independent experiments. Figures are adapted from [79].

To verify the hypothesis that WDR1 WT is substrate for EYA3, we next checked if WDR1 WT and EYA3 interact with each other. In Fig. III-5a, WDR1 WT is trapped by EYA3 Y508F (inactive as PTP and trapping mutant) only in the presence of Src kinase (when WDR1 WT is phosphorylated), indicating that tyrosine phosphorylation is an essential condition for the interaction to occur. Neither EYA3 WT nor the EYA3 D309N inactive mutant could trap WDR1 WT (Fig. III-5a). The protein interacted and could be immunoprecipitated with the same affinity by all of EYA3's trapping mutants (D311N, Y508F and Y532F) (Fig. III-5b).

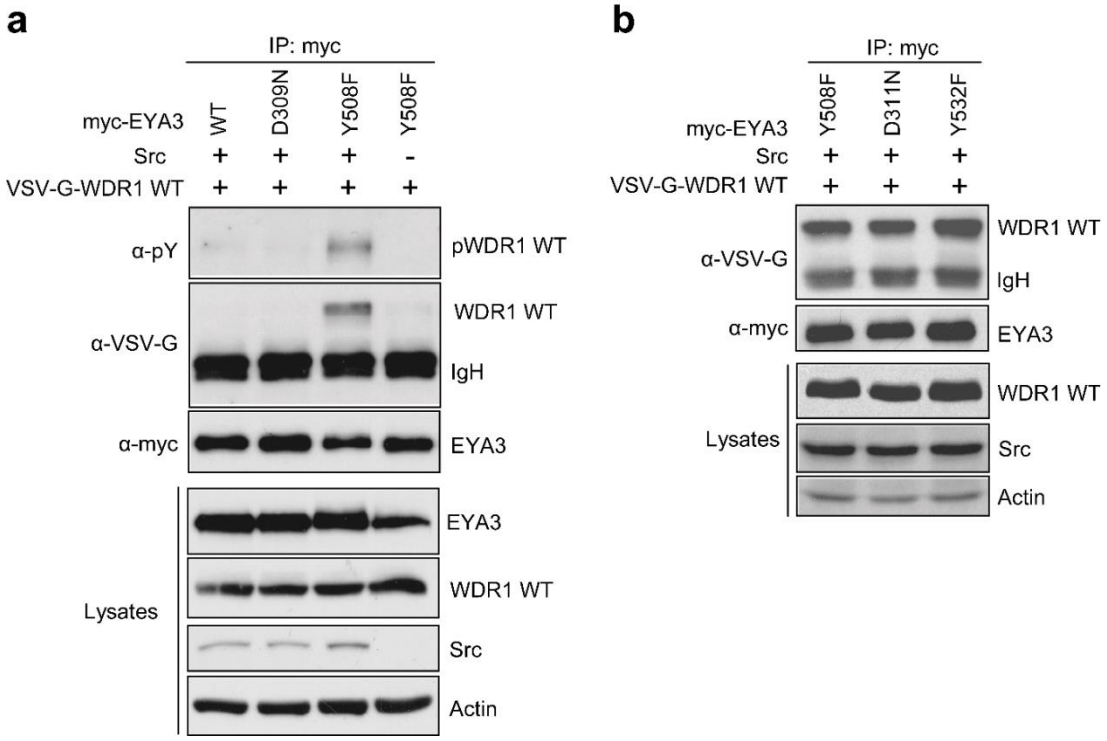


Figure III-5. Src-phosphorylated WDR1 WT is immunoprecipitated by EYA3 trapping mutants.
a) VSV-G-WDR1 WT was co-expressed in HEK293T with different c-myc-tagged EYA3 proteins (EYA3 WT, D309N and Y508F) with or without c-Src Y527F. **b)** Constructs encoding for VSV-G-WDR1 WT, c-Src Y527F and different c-myc-EYA3 proteins (EYA3 Y508F, D311N and Y532F) were transfected in HEK293T cells. Figures are from [79].

Considering EYA3's implication in tumorigenic processes, especially in breast cancer cells, we investigated whether the EYA3 - WDR1 WT interaction also takes place in this type of cells, particularly in MCF-7 cells. Western blot results show that EYA3 Y508F but not EYA3 WT can trap pY-WDR1 WT. Another type of verification performed in MCF-7 cells, showed that tyrosine phosphorylation of endogenous WDR1 was considerably increased in the cells in which EYA3 was knocked down.

Because EYA proteins (EYA1-4) have a highly conserved catalytic domain, we wanted to determine whether pY-WDR1 WT can be dephosphorylated by other EYA family members. WDR1 WT and c-Src Y527F were transiently co-expressed with EYA1, EYA2 or EYA3 proteins, in HEK293T cells. The strong interaction of WDR1 with EYA3, lack of its interaction with EYA1 and weak interaction with EYA2 suggests that EYA-WDR1 interaction is paralog-specific, favoring EYA3.

III.3 CONCLUSIONS

1. EYA3 is tyrosine phosphorylated by Src, Abl and Btk kinase.

2. EYA1, EYA2 and EYA3 are tyrosine phosphorylated by Src kinase and have autodephosphorylation capacity.

3. Src kinase phosphorylates the N-terminal part of EYA3 as well as the ED and the residues can be autodephosphorylated.

4. Single point Y→F mutations (Y96, Y105, Y208 or Y237) introduced in EYA3 D309N inactive mutant do not decrease its tyrosine phosphorylation level in the case of co-expression with c-Src Y527F in HEK293T cells.

5. Introduction of four Y→F mutations (Y426F Y496F Y508F Y532F) in EYA3 D309N does not decrease its tyrosine phosphorylation level in the case of co-expression with c-Src Y527F, in HEK293T cells.

6. Two tyrosine residues from the highly conserved C-terminal part of EYA3 (ED) influence the protein's tyrosine phosphatase activity. Mutation of either Y508 or Y532 to phenylalanine in EYA3 WT or EYA3 ED WT, abolishes their catalytic activity.

7. Y508 and Y496 are Src-phosphorylation sites of EYA3 and Y508F is also an EYA3 autodephosphorylation site.

8. Introducing the Y→F mutation corresponding to EYA3 Y508F in EYA1 (Y494F) does not abolish its PTP activity, but it decreases EYA2's autodephosphorylation capacity, thus its PTP activity (Y473F). However, neither of the EYA1 (Y518F) or EYA2 (Y497F) Y→F mutations corresponding to EYA3 Y532F inactivate their PTP enzymatic activity. The influence of the two tyrosine residues on EYA's PTP activity seems to be specific for each human EYA protein.

9. EYA3 Y508F and EYA3 Y532F interact with and trap EYA3 D309N in a manner similar to the EYA3 D311N trapping mutant, proving that both Y→F mutants can be used as EYA3 PTP trapping proteins in functional and in EYA3 substrate identification studies.

10. The interaction between two EYA3 molecules is conditioned by the presence of the ED in both EYA3 proteins and takes place only in the presence of Src-induced tyrosine phosphorylation.

11. EYA1 does not interact with EYA3. EYA-EYA interaction is paralog-specific.

12. WDR1 protein is tyrosine phosphorylated by Src kinase and Y238 is one of the phosphorylation sites.

13. WDR1's tyrosine phosphorylation is increased in cells in which EYA3 phosphatase activity is inhibited (HEK293T) or EYA3 is silenced (MCF-7).

14. EYA3 interacts with WDR1 WT only in the case of Src-induced tyrosine phosphorylation of the cytoskeletal protein.

15. WDR1 protein is a substrate of EYA3 but not of EYA1 phosphatase. The interaction between EYA2 and WDR1 is much weaker compared to EYA3.

CHAPTER IV MASS SPECTROMETRY ANALYSIS OF EYA3 TYROSINE PHOSPHORYLATION BY SRC KINASE. CELLULAR IMPLICATIONS.

Src is one of the most studied PTK, its activation being reported in more than 50% of the tumors derived from liver, pancreas, colon, breast and lung[87]. We demonstrated that Src phosphorylates human EYA1, EYA2 and EYA3 and that they are all capable of autodephosphorylation. These data indicate that Src-phosphorylation of EYA and its autodephosphorylation could be involved in regulating physiological processes or in generating and/or maintaining pathological conditions. Through autodephosphorylation, EYA proteins could be able to control and regulate their own roles in cellular processes, but there is little information (presented in the previous chapter) regarding the specific residues which are phosphorylated by Src and none regarding the extent to which they are modified or the dynamics of phosphorylation/ dephosphorylation.

We combined here two mass spectrometry techniques - native and bottom-up - to characterize tyrosine phosphorylation of EYA3 by Src kinase and EYA3 autodephosphorylation. We also evaluated the contribution to the overall EYA3 phosphorylation for some of the phosphotyrosine residues identified. Implications in the cellular proliferation of HEK293T, MCF-7 and MDA-MB-231 cells and invasion of MCF-7 cells were searched for three of the detected phosphotyrosine residues. A comparison was made between the tyrosine phosphorylation pattern of three EYA3 proteins in HEK293T and MCF-7 cells, using bottom-up mass spectrometry. Finally, a bioinformatic approach was used to assess the degree of conservation for EYA3's identified tyrosine phosphorylation sites.

IV.1 MATERIALS AND METHODS

The necessary materials consisted of constructs, primers, antibodies, enzymes, kits, reagents (molecular biology grade and liquid chromatography/mass spectrometry grade, for: mammalian and bacterial cell cultures, protein expression and purification, peptide analysis by liquid chromatography-tandem mass spectrometry - nLC-MS/MS - and protein analysis by native mass spectrometry).

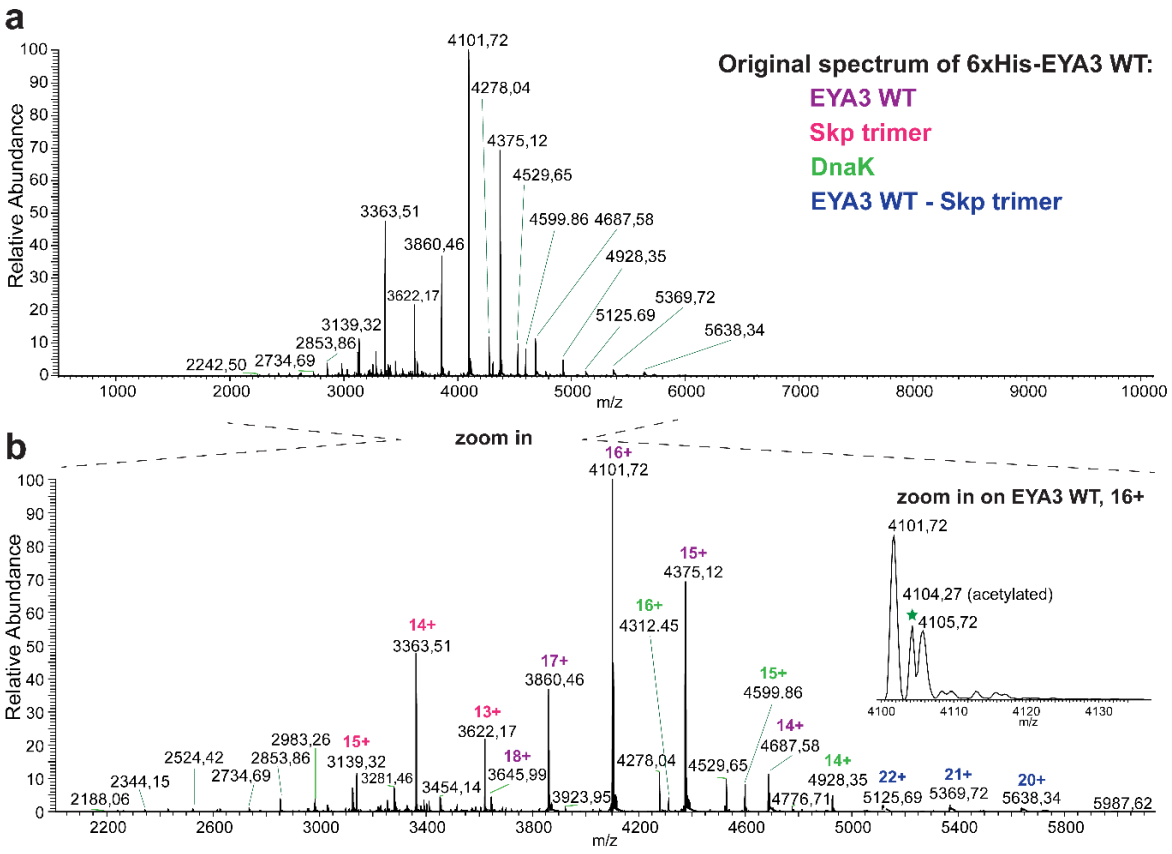
The following experimental procedures and analyses were performed: protein expression and purification from bacterial culture, mammalian cell culture, transfection, harvesting and lysis, immunoprecipitation, *in vitro* kinase reactions, kinetics assays, *in vitro* protein tyrosine phosphatase assays, native mass spectrometry (ESI-MS) and bottom-up mass spectrometry coupled with liquid chromatography, phosphopeptide enrichment using TiO_2, MTS assays (viability assays), cell cycle distribution assays and proliferation assays (which involve CFSE staining assays) - both analyzed by flow cytometry - invasion assays, sequence alignment using bioinformatic tools.

IV.2 RESULTS AND DISCUSSIONS

IV.2.1 Native mass spectrometry analysis of EYA3 and its *in vitro* phosphorylation by Src kinase

Native mass spectrometry analysis of EYA3

In the case of EYA3 phosphorylation by Src, we first used native mass spectrometry to evaluate the dynamics of the two opposing processes (tyrosine phosphorylation and autodephosphorylation) at the intact protein level. To this aim, we initially characterized the behavior in native MS of the two EYA3 proteins further used in the phosphorylation experiments - 6xHis-EYA3 WT and 6xHis-EYA3 D311N (Fig. IV-1).



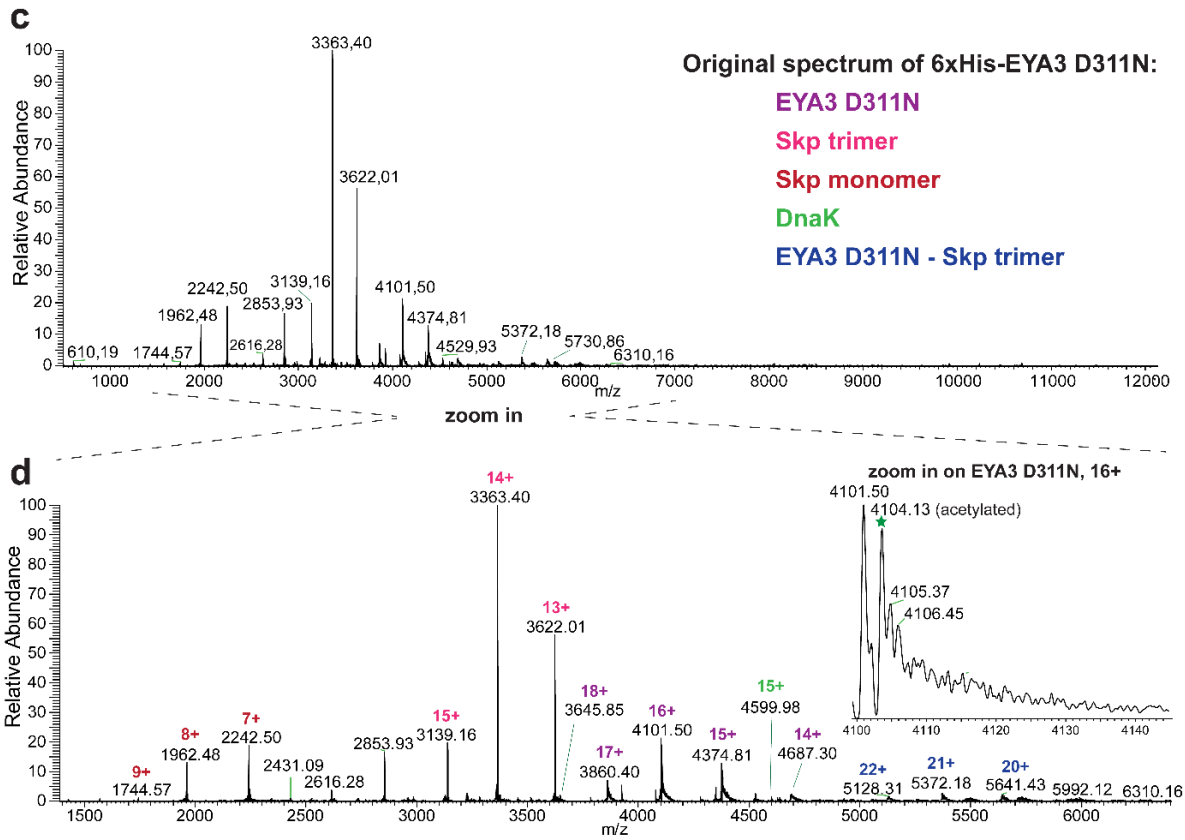


Figure IV-1. Native mass spectrometry analysis of 6xHis-EYA3 WT and 6xHis-EYA3 D311N.
Original spectrum of 6xHis-EYA3 WT (**a**) and 6xHis-EYA3 D311N (**c**) at pH 6.8, with corresponding zoom in images from **b)** and **d)**, respectively, which are annotated spectra.
The molecular weights of EYA proteins could be accurately calculated (using the m/z values of all identified charge states for that protein, shown in purple) and the obtained values correspond to the protein sequences lacking the start Met: 65611.22 ± 0.9 Da for 6xHis-EYA3 WT and 65608.09 ± 1.1 Da for 6xHis-EYA3 D311N. The theoretical MWs of the purified proteins are: 65742.1 Da (6xHis-EYA3 WT) and 65741.1 Da (6xHis-EYA3 D311N).
In the annotated spectra of 6xHis-EYA3 WT (**b**) and 6xHis-EYA3 D311N (**d**):
- the calculated MW of 47075.05 ± 0.2 Da (charges shown in pink): Skp homo-trimer in which the monomer lacks the first 20 amino acids; this monomer was better evidenced in **d)**, shown in red and its MW was calculated at 15691.49 ± 0.87 Da.
- the MW of 68983 ± 0.2 Da (charges shown in green): full lenght chaperone protein DnaK lacking the N-terminal Met.
- charge state envelope of EYA3 - Skp trimer complex (charges shown in blue) for which the MW was calculated from another spectrum (see Fig. IV-2 below).
In the narrow m/z windows from images **b)** and **d)**, corresponding to charge 16+ of each EYA3 protein, two major peaks can be observed, corresponding to two proteoforms of EYA3:
- EYA3 with the first Met cleaved: m/z value of 4101.72 in **b)** and 4101.50 in **d)**
- acetylated molecules of EYA3 with the first Met cleaved (green star above corresponding peak): m/z of 4104.27 in **b)** and 4104.13 in **d)**. The calculated MW for the acetylated 6xHis-EYA3 WT protein is 65652.45 ± 0.3 Da and 65650.82 ± 2 Da for the acetylated 6xHis-EYA3 D311N protein.

Both proteins were prokaryotically expressed and then purified to high purity. Their molecular weights were calculated based on the m/z values from the contiguous peaks of the charge state envelope (Fig. IV-1). The MW values correspond to the sequence of the 6xHis-tagged EYA3 proteins lacking the initial methionine residue. Given the high resolving power of

the mass spectrometer, the native spectra from both proteins reveal an interesting feature: there are two molecular forms of EYA3 (outlined in the zoomed-in images from Fig. IV-1b and 1d, for z = 16+). To elucidate the origin of the second molecular form (m/z of 4104.27 for WT and 4104.13 for D311N), we performed a supplementary bottom-up mass spectrometric analysis. Results show that the Ser residue immediately following the initial Met (removed), has been acetylated during prokaryotic expression.

Careful analysis of the ESI-MS spectra (Fig. IV-1) revealed two additional protein ionization patterns, indicating that two other proteins are present in the EYA3 samples. To identify these proteins, we subjected the purified protein samples to in-solution digestion followed by nLC-MS/MS. Two chaperone proteins were identified: Skp and DnaK (Fig. IV-1). The sequence coverage of Skp reveals the fact that the chaperone lacks approximatively 20 amino acids from the N-terminal part. By accurately calculating the MW from the native mass spectrometry data, we determined Skp as homo-trimer with exactly 20 amino acids missing from each of its monomers. When a higher energy is applied to the sample, Skp trimer dissociates into monomer and dimer.

Further optimization of the Orbitrap™ EMR at a higher m/z window led us to the detection of another charge state distribution. The calculated molecular weight denotes the presence of a complex between EYA3 and the Skp trimer, further denoted EYA3-$(Skp)_3$ (Fig. IV-1b and 1d). When a higher energy is applied, the complex dissociates in the monomer of Skp and EYA3-Skp dimer complex.

From the ESI-MS analysis of both types of EYA3 proteins, it can be observed that EYA3 is free in solution (as monomer) as well as in complex with Skp. The question that arises is whether Skp preferentially binds to one of the EYA3 domains, thus preventing the phosphorylation of some tyrosine residues. To elucidate this aspect, besides EYA3 WT and EYA3 D311N, the presence of Skp was also searched in other purified EYA3 proteins: EYA3 D309N, EYA3 ΔED WT and EYA3 ED WT. The presence of Skp was confirmed by nLC-MS/MS analysis after in-gel digestion of the protein band from an SDS-PAGE gel. Results from all five EYA3 proteins reveal that Skp associates with full length as well as with the N-terminal domain of EYA3. Thus, the presence of the NTD seems to be essential in the formation of the complex.

Attempts were made to express EYA3 WT and D311N in a bacterial strain which had the gene for Skp chaperone deleted (MC 4100 ΔSkp). The Western blot analysis using anti-EYA3 antibody indicated that none of the EYA3 proteins were expressed suggesting that, in absence of Skp chaperone, EYA3 is unstable and cannot be expressed as a soluble protein. Using PrDos[88]

software, we performed a disorder prediction for EYA3 and results indicated a highly disordered N-terminal region (Fig. IV-2). This finding and the fact that EYA3 could not be expressed in MC 4100 ΔSkp cells suggest that Skp plays a role in EYA3 stabilization, binding to its structurally disordered N-terminal part. The absence of Skp probably leaves the disordered regions of EYA3 exposed to proteases, leading to EYA3 degradation by proteolysis.

We could not detect any DnaK - EYA3 complex.

Figure IV-2. **Disorder profile plot of EYA3 WT**[80].
EYA3 WT disorder prediction using PrDos[88] at a 5% false positive level, mapped on the protein sequence (red/ black - above/ bellow disorder threshold).

Native mass spectrometry analysis of *in vitro* EYA3 tyrosine phosphorylation by Src

With native mass spectrometry data available for the two purified proteins, we detected how many tyrosine sites in EYA3 are phosphorylated by Src. To this end, each of the purified EYA3 proteins were *in vitro* phosphorylated and then samples were analyzed by native MS. Briefly, His-tagged EYA3 WT and EYA3 D311N were each incubated with Src (GST-v-Src) and ATP for 5 min and 2 h, respectively. In the case of EYA3 WT incubation with Src and ATP for 2 h, only molecules with one phosphorylated residue were detected (Fig. IV-3a compared to IV-3c). When EYA3 D311N, the phosphatase deficient form, was phosphorylated, molecules with up to three phosphorylated residues were detected after only 5 min. However, after 2 h, EYA3 D311N molecules with up to 12 phosphorylated residues could be observed (Fig. IV-3b compared to IV-3d). The difference between the behavior of EYA3 WT and EYA3 D311N, when phosphorylated, points out to the importance of ED activity in controlling EYA3 phosphorylation status.

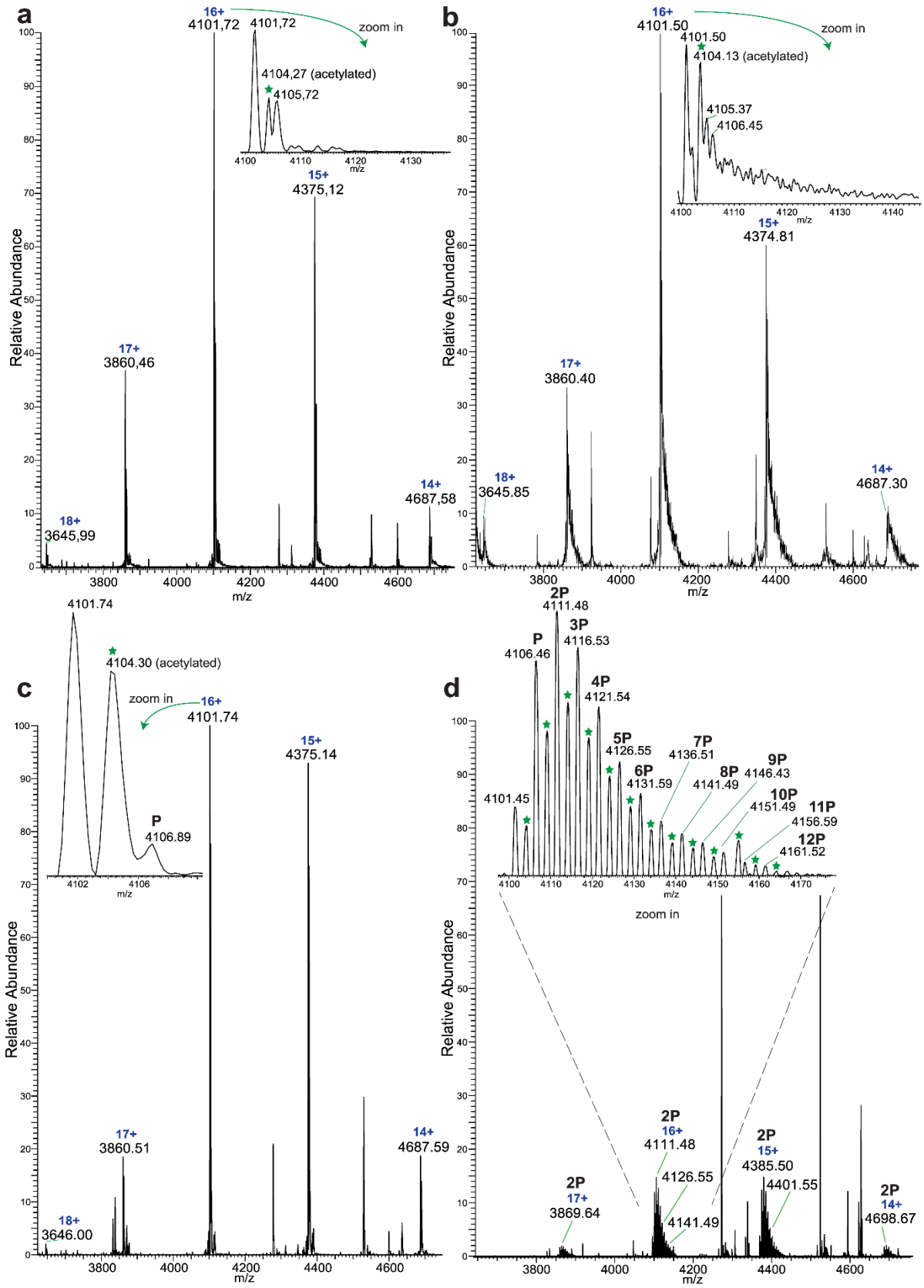


Figure IV-3. Native MS analysis of EYA3 WT and D311N proteins before and after Src phosphorylation[80].

Native mass spectrum of purified 6xHis-EYA3 WT (**a**) and 6xHis-EYA3 D311N (**b**) with zoomed-in images of charge 16+. These spectra are compared with the ones obtained in the *in vitro* phosphorylation reactions (v-Src, ATP) of 6xHis-EYA3 WT (**c**) and 6xHis-EYA3 D311N (**d**).
c) Adjoining peaks of the charge state envelope of 6xHis-EYA3 WT after 2 h of tyrosine phosphorylation. Zoom in on charge 16+: unphosphorylated molecules (m/z = 4101.74 and acetylated, at 4104.30) and molecules with only one phosphorylated residue (**P**, m/z = 4106.89) were detected.
d) Native mass spectrum of 6xHis-EYA3 D311N after 2 h of tyrosine phosphorylation. Zoom in on charge 16+: molecules which have up to twelve phosphorylated residues (**P** to **12P**) have been detected. The acetylated molecules have the same maximum number of phosphorylated residues.

Dynamics of EYA3 phosphorylation by Src and its autodephosphorylation, analyzed by native mass spectrometry

We have used the advantages given by native mass spectrometry to investigate the global dynamics of EYA3 tyrosine phosphorylation and autodephosphorylation. The experiment consisted in a set of reactions in which tyrosine phosphorylated inactive mutant of EYA3 was directly dephosphorylated by EYA3 WT (Fig. IV-4a). We quantified the native mass spectrometry raw data corresponding to 6xHis-EYA3 D311N molecules which carried charge 16+, from every reaction (Fig. IV-4b). We observed an increase in phosphorylation for EYA3 D311N from 5 min to 4 h of incubation with Src. In the sample incubated for 2 h with EYA3 WT, no signal was detected for the molecules having the highest number of phosphate moieties (7 to 9) and the signal decreased for those carrying between 6 and 2 phosphate moieties. The monophosphorylated forms were the most abundant, but very important, the intensity of the unphosphorylated molecules increased significantly, making them the second most abundant EYA3 form. Quantification of the acetylated proteoform of EYA3 in the four samples revealed a very similar behavior. This demonstrates that acetylation does not hinder phosphorylation of EYA3. Comparing results from the "2 h/Src + 2 h/EYA3 WT" sample with the ones from the "4 h/Src" sample, we can conclude that EYA3 WT directly dephosphorylated the tyrosine phosphorylated inactive EYA3 protein.

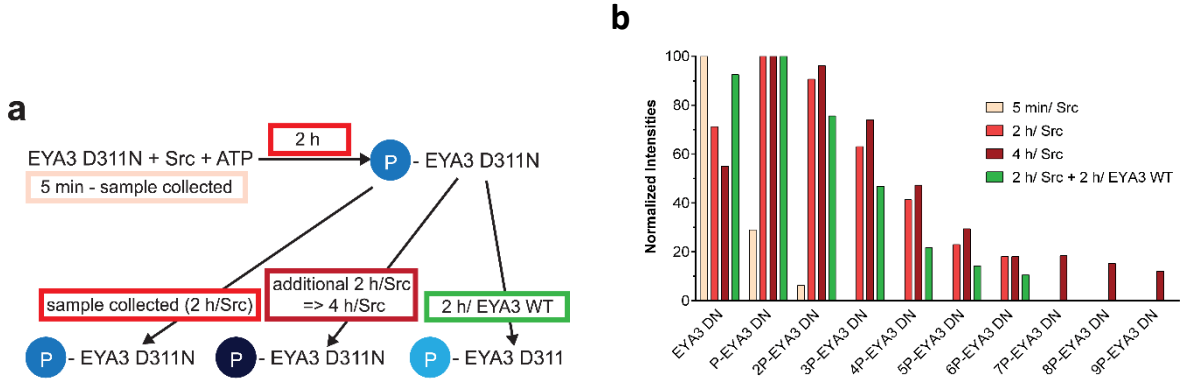


Figure IV-4. Dynamics of EYA3 tyrosine phosphorylation by Src kinase and its autodephosphorylation, studied by native mass spectrometry[80].

a) Workflow of the experiment: 6xHis-EYA3 D311N was *in vitro* phosphorylated by Src for 2 h and a sample was collected after 5 min of reaction. After the 2 h, another sample was collected, and the rest of the reaction was split in two: one half was further incubated for 2 h and in the other one we added EYA3 WT and kept it also for 2 h. All reactions were performed at 30°C. **b)** Quantification of the raw data corresponding to the 6xHis-EYA3 D311N molecules which carry charge 16+.

A similar behavior to the unbound EYA3 D311N protein was observed in the case of EYA3 D311N-$(Skp)_3$ complex. We point out that, although during the mentioned 2 h of incubation with EYA3 WT, Src was still active, so both opposing reactions were simultaneously taking place, the dephosphorylation of EYA3 D311N was evident.

IV.2.2 Detection of Src-phosphorylated tyrosine residues of EYA3 and identification of EYA3 autodephosphorylation sites

EYA3 phosphorylation by Src and EYA3 autodephosphorylation are two opposing processes which, at least *in vitro*, happen simultaneously. Therefore, their detailed analysis requires their separation. First, we investigated tyrosine phosphorylation by Src using a catalytically inactive form, EYA3 D311N. Then, to study autodephosphorylation, we used the WT form of EYA3. Because it is active as PTP, when incubated with Src and ATP, we could observe which residues were dephosphorylated and the time dependence of the phosphorylation level for the residues which were still phosphorylated. For our purpose, we perfomed a set of *in vitro* reactions and the samples were analyzed by bottom-up mass spectrometry (nLS-MS/MS) using HCD as fragmentation method.

Time course phosphorylation of EYA3 D311N revealed five phosphorylated tyrosine residues (Y72, Y77, Y96, Y105, Y237) after 5 min of reaction and eleven residues after 6 h (Y67, Y72, Y77, Y90, Y96, Y105, Y108, Y208, Y237, Y426, Y532) (Fig. IV-5a). All detected tyrosine residues were phosphorylated within 30 min of incubation with Src, but the evolution of phosphorylation was different for each of these residues.

Time course phosphorylation analysis of EYA3 WT by Src (Fig. IV-5b) identified four tyrosine residues after 5 min (Y77, Y96, Y105 and Y237). The same residues were detected after 6 h. At 30 min and even at 2 h time point, Y72 was also phosphorylated, but this residue was eventually dephosphorylated. The seven tyrosine residues - Y67, Y72, Y90, Y108, Y208, Y426, Y532 - which were not detected here, but were found in the case of EYA3 D311N phosphorylation by Src could be considered the main autodephosphorylation sites.

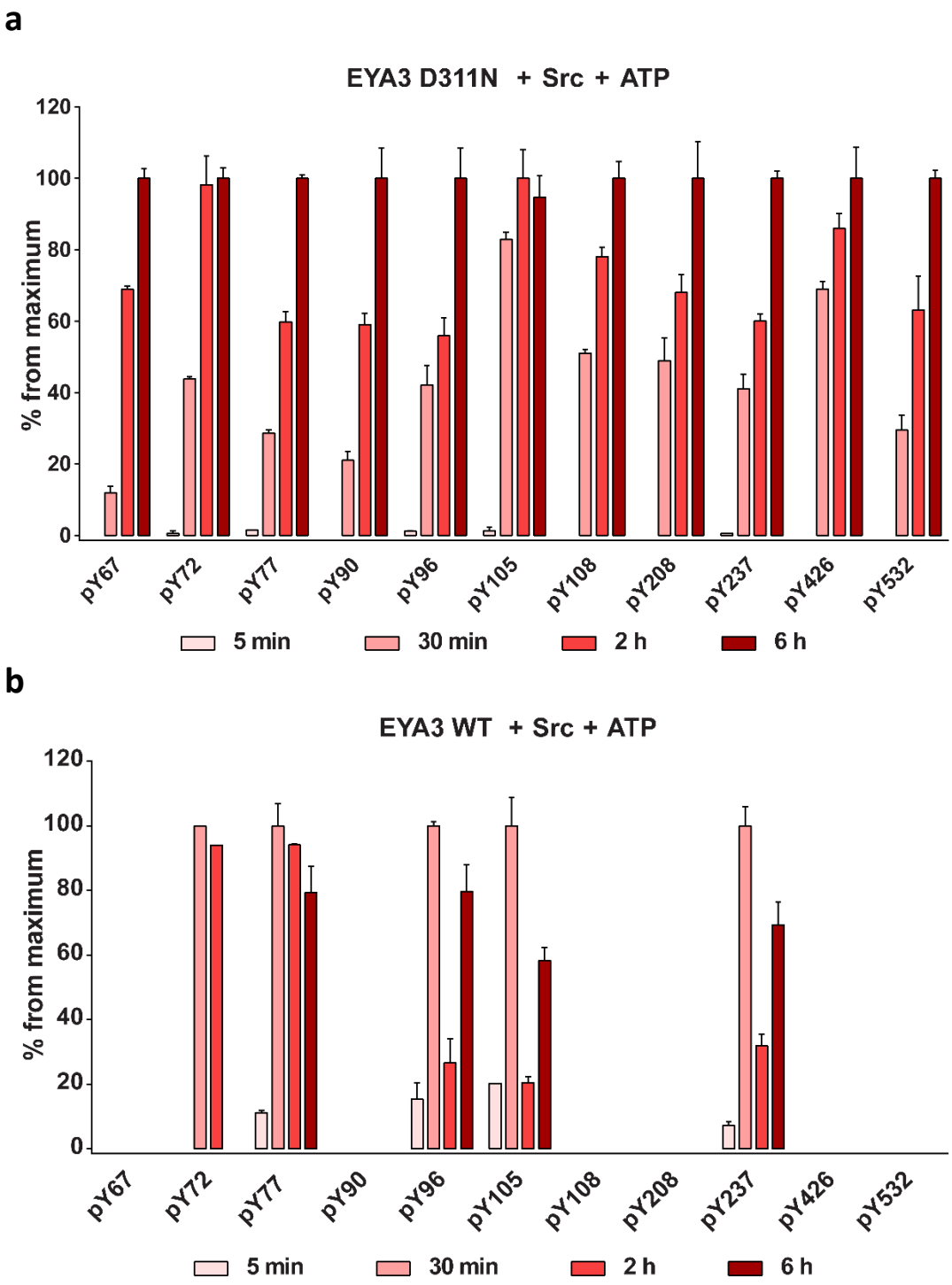


Figure IV-5. Src-phosphorylated tyrosine residues of EYA3 and autodephosphorylation residues[80].

a) and **b)** Results obtained after nLC-MS/MS analysis followed by data interpretation, relative quantification and normalization: graphical representation of the evolution of phosphorylation for every tyrosine residue which was found to be phosphorylated in the *in vitro* reactions EYA3 D311N + Src + ATP (**a**) and EYA3 WT + Src + ATP (**b**).

A characterization has been made for most of these residues, regarding their time-dependent kinetics and resistance to autodephosphorylation. These results allow us to assume that, compared to the unphosphorylated form, Src-phosphorylated EYA3 WT has a different

protein tyrosine phosphatase activity, and thus a different autodephosphorylation capacity. To verify this hypothesis, an initial *in vitro* kinase reaction was performed (N-terminally His-tagged EYA3 WT was phosphorylated with v-Src kinase (in parallel with non-phosphorylated control sample), then the enzymatic activity of 6xHis-EYA3 WT was assessed using pNPP as substrate. The results obtained reveal that the Src-phosphorylated form of EYA3 has a slightly increased protein tyrosine phosphatase activity.

Thus, the phosphorylation trend corresponding to residues Y72, Y77, Y96, Y105, and Y237 in EYA3 WT phosphorylated by v-Src kinase (Fig. IV-5b) can be explained considering this new information. In the first 30 min, Src phosphorylates EYA3 WT and many tyrosine phosphorylated EYA3 WT molecules, with higher PTP activity, are generated. These molecules intensely dephosphorylate phospho-Y96, Y105, and Y237 residues and manage to dephosphorylate phospho-Y72 and Y77, but to a much lesser extent. The number of unphosphorylated EYA3 WT molecules increases and the initial state is restored. In time, the process develops toward a steady state.

The four tyrosine residues that remained phosphorylated even when EYA3 WT was incubated with Src for 6 h, could be considered residues with increased resistance to autodephosphorylation.

IV.2.3 Dynamics of *in vitro* EYA3 D311N tyrosine phosphorylation and dephosphorylation

Our next goal was to find the order in which the EYA3 D311N phosphorylated residues are dephosphorylated by the active PTP. In depth study of the time dependence of EYA3 phosphorylation/ dephosphorylation could provide useful information in determining the *in vivo* relevance of this PTM, concerning EYA3 and its interacting partners.

The difference between this experiment and those described earlier in this chapter was that here Src was inactivated, so that the process of phosphorylation would not interfere with EYA3 D311N dephosphorylation. The set of *in vitro* reactions performed in order to achieve our goal consisted in tyrosine phosphorylation of EYA3 D311N by Src, inactivation of the kinase (with PP2) and finally, dephosphorylation of the inactive mutant by a catalytically active form of EYA3 (ED EYA3 WT). Samples were first verified by Western blotting with anti-pY and anti-His antibodies and the 6xHis-EYA3 D311N bands were in-gel digested with sequencing grade chymotrypsin. Extracted peptides were analyzed by nLC-MS/MS using CID and ETD fragmentation methods. Assessment of phosphorylation variation for each of the detected phosphotyrosine residues was done by relative quantification (Fig. IV-6).

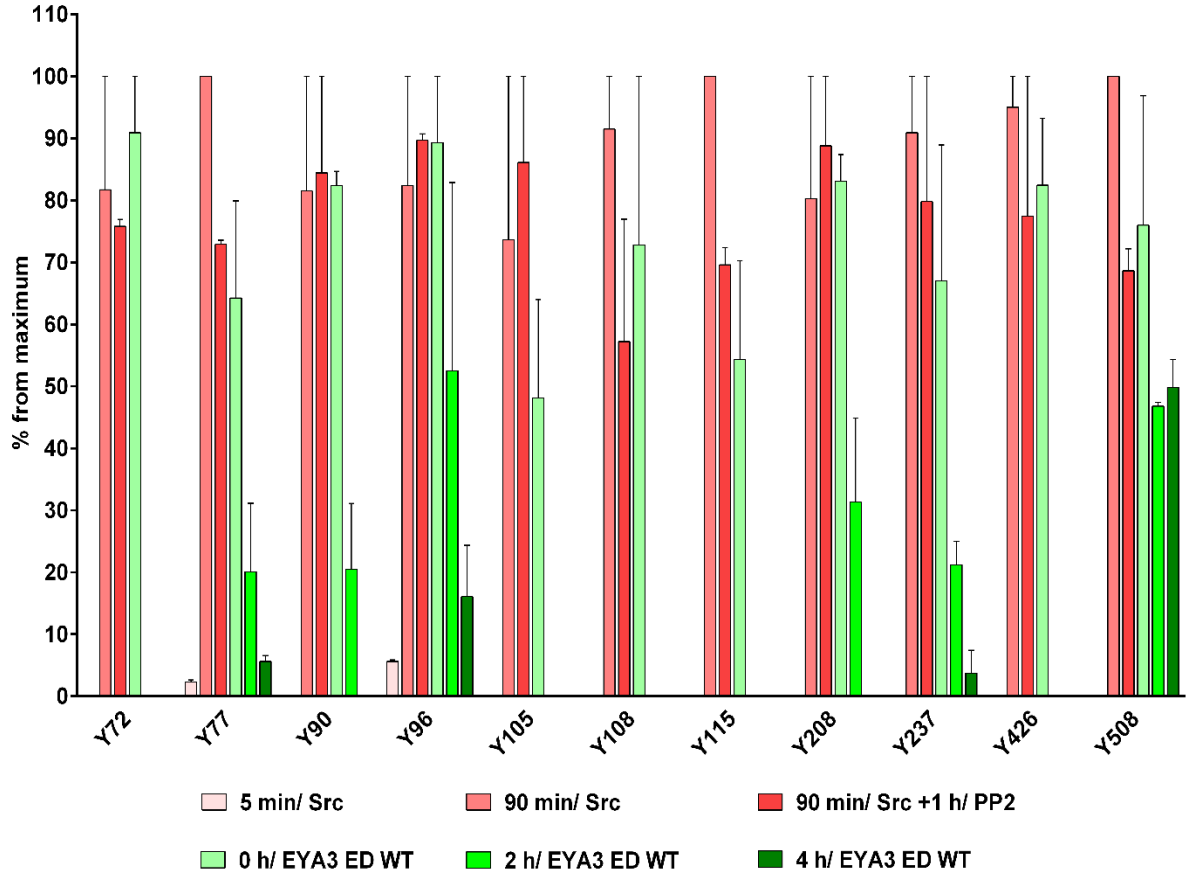


Figure IV-6. The dynamics of *in vitro* EYA3 D311N tyrosine phosphorylation (by v-Src) and dephosphorylation (by ED EYA3 WT), analyzed by nLC-MS/MS[80].
Site-specific relative quantification of the changes in phosphorylation status of the tyrosine residues detected on EYA3 D311N. The bar chart shows the mean ± SEM of two independent experiments, each analyzed in two technical replicates (n = 4).

Eleven phosphotyrosine residues were identified (Fig. IV-6). Only six residues Y77, Y90, Y96, Y208, Y237 and Y508 were still phosphorylated after the 2 h period of incubation with the catalytic domain of EYA3 and after 4 h, only four of them remained phosphorylated (Y77, Y96, Y237 and Y508). Phospho-Y77, -Y96 and -Y237 have similar dephosphorylation kinetics from 0 h to 4 h incubation with EYA3 ED WT, but phospho-Y508 is at the same phosphorylation level after 2 h and 4 h of ED WT activity. This makes phospho-Y508 another residue with high resistance to autodephosporylation and the only one from the C-terminal part of EYA3 that showed resistance to this process.

Based on all the data obtained from the bottom-up mass spectrometry experiments described in this chapter, we can conclude that *in vitro*, Src kinase can phosphorylate thirteen tyrosine residues from EYA3: Y67, Y72, Y77, Y90, Y96, Y105, Y108, Y115, Y208, Y237, Y426, Y508, Y532. The fact that Y77, Y96 and Y237 were not completely dephosphorylated after 4 (Fig. IV-6) or even 6 h (Fig. IV-5b) of incubation with a catalytically active form of EYA3 (full length WT or ED WT) supports the idea that these three residues have increased resistance to

autodephosphorylation. Although Y508 was not detected in the time-course *in vitro* reaction, because it was still found to be phosphorylated after 4 h of incubating phospho-EYA3 D311N with ED WT, this residue can also be considered a site with increased resistance to autodephosphorylation.

IV.2.4 Contribution of the identified Src-phosphotyrosine sites to the overall EYA3 phosphorylation

Thirteen tyrosine sites from EYA3 have been detected as phosphorylated by Src in the *in vitro* kinase reactions. Validation of these tyrosine sites is necessary for better understanding the impact of Src-induced EYA3 tyrosine phosphorylation in mammalian cells. To approach this issue, succesive tyrosine to phenylalanine (Y→F) mutations of the previously detected phosphotyrosine residues were introduced in the sequence of EYA3 D309N through site-directed mutagenesis.

Transient co-transfections of vectors encoding for EYA3 WT, EYA3 D309N (DN) or its Y→F mutants (termend T0 to T11) and c-Src (Uniprot accession number: P00523) with Y527F mutation (strongly enhances c-Src protein tyrosine kinase activity[89,90]), were performed in HEK293T cells. Following IP of EYA3, Western blot experiments were quantified to detect variation in the phosphorylation level of the mutants. The first mutants, T0 to T3, contain Y→F mutations at the residues which were found to have increased resistance to autodephosphorylation (Y77, Y96, Y105 and Y237, see Fig. IV-5, 6).

A gradual decrease in phosphorylation can be observed between DN and its first six consecutive Y→F mutants (Fig. IV-7a). These results show that phosphorylation of residues Y96, Y77, Y105, Y90 and Y115 has a measurable contribution to the overall EYA3 phosphorylation. Quantification and normalization of data from all other mutants (T6 to T11) which were co-expressed with Src, revealed inconsistent variations of the phosphorylation level and we could not notice any trend. It is possible that the relatively large number of mutations induced conformational changes, eventually leading to aberrant tyrosine phosphorylation.

In order to obtain a more accurate assessment of the contribution of each phosphorylated residue to the total EYA3 phosphorylation, we obtained additional mutants derived from DN, T0 and T1 and performed similar experiments to determine their relative phosphorylation (Fig. IV-7b, c). Quantification results showed that Y77 and Y96 had the highest contribution to phosphorylation. Y237 does not seem to have any contribution to the phosphorylation. When Y90F or Y105F were introduced in T0 mutant, no significant decrease in intensity was detected.

The contribution of Y90 and Y105 was observed only when those residues such as Y77 and Y96 (with high contribution to phosphorylation) were mutated to phenylalanine (Fig. IV-7a).

Of these mutagenesis experiments coupled with IP and Western blot, Y77 and Y96 are noted to have the highest contribution to EYA3 phosphorylation. This result will be useful in future research involving EYA3 protein, for example in signal transduction pathway determination.

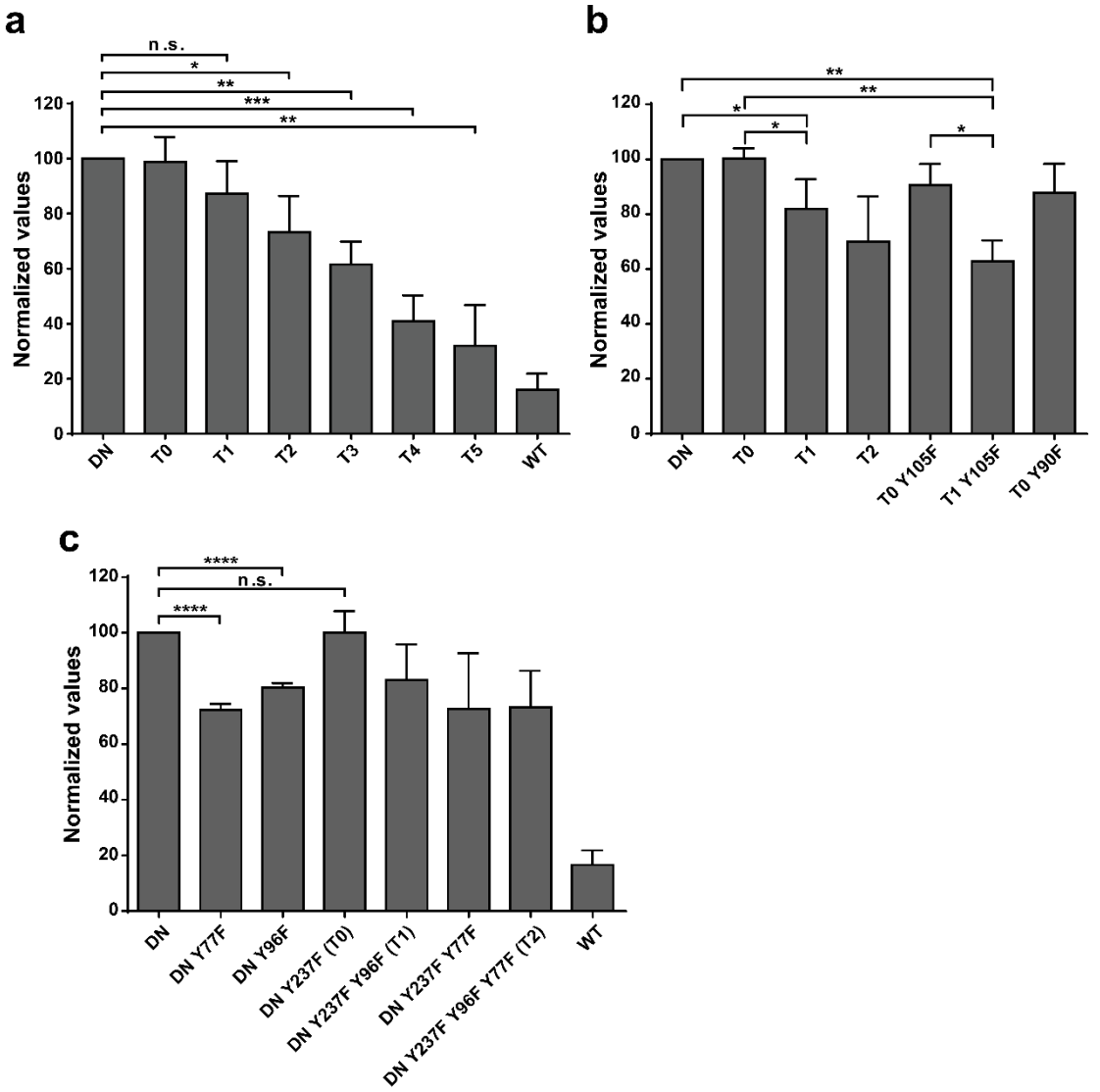


Figure IV-7. Tyrosine phosphorylation of various EYA3 mutants, in HEK293T cells[80].
For each immunoprecipitated EYA3 protein, the intensity of the band obtained from anti-pY Western blot was divided by the one from the anti-c-Myc Western blot. Bar graphs represent values of the quantified phosphorylation of the samples normalized to the quantified phosphorylation of EYA3 D309N (n.s. for not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Quantification of the Western blot bands was achieved using ImageJ[81] software. For each of the experiments described (**a**, **b**, **c**), the values represent the mean ± SD of three independent experiments ($n = 3$) and statistics (unpaired t-test, two-tailed) were performed using GraphPad Prism6.

IV.2.5 Cellular implications for some of EYA3's tyrosine sites phosphorylated by Src kinase

Knowing EYA proteins are involved in many carcinogenic processes[41,78], we investigated whether certain Src-phosphorylated tyrosine residues could be implicated in cellular proliferation and invasion. First, we investigated whether endogenous EYA3 was tyrosine phosphorylated in HEK293T cells transiently expressing c-Src Y527F. EYA3 was tyrosine phosphorylated only in HEK293T cells that overexpressed c-Src Y527F. Benzbromarone treatment induced an increase in endogenous EYA3's tyrosine phosphorylation compared to the cells treated only with DMSO, the control cells. This demonstrates that endogenous EYA3 has autodephosphorylation capacity.

Thus, we chose to look for the mentioned cellular effects among the Src-phosphorylated tyrosine sites from the N-terminal part as well as among those which displayed increased resistance to EYA3 autodephosphorylation. We reasoned that especially the tyrosine residues of EYA3 that remain phosphorylated for a longer period could be part of a signaling cascade activated by tyrosine phosphorylation. Two EYA3 WT Y→F mutants were chosen: EYA3 Y237F Y96F Y77F (EYA3 WT T2) and a mutant which had all Src-phosphorylated Y residues from the N-terminal part mutated to F (EYA3 WT T9). We initially measured their protein tyrosine-phosphatase activity using pNNP as substrate and compared it to the activity of EYA3 WT. Only EYA3 WT T2 was chosen for further investigations because it had retained most of the PTP activity (90%).

The MTS assay, by which the viability of the cells can be assessed, was first performed on HEK293T cells. The results showed that in the absence of c-Src Y527F co-expression, cells which overexpressed EYA3 WT T2 mutant had lower absorbance values compared to those that overexpressed EYA3 WT or control (empty vector) (*** $p<0.001$, Fig. IV-8a). This result suggests that one or more of the three tyrosine residues could be implicated in EYA3 induced proliferation events. We also observed that EYA3 WT overexpression increased the absorbance value of the respective HEK293T cells (Fig. IV-8a). However, this effect was abolished when c-Src Y527F was co-expressed with EYA3 WT (Fig. IV-8a).

As the results of the MTS assay could also be explained by increased cellular survival or metabolism in addition to increased proliferation rates, we performed a cell cycle distribution analysis to find out whether EYA3 has a role in HEK293T cell proliferation. Cell cycle flow cytometry analysis (Fig. IV-8b) was performed 24 h after the cells were transfected. The results revealed that the highest proportion of cells in the S+G2/M phases was found in cases where the dominant negative form of the kinase was transiently expressed. The samples in which no c-

Src forms were overexpressed had the smallest proportion of cells in the S+G2/M phases. When c-Src Y527F K295R was present, overexpression of EYA3 WT generated a higher proportion of cells in the S+G2/M phases compared to EYA3 WT T2. Interestingly, the accumulation in G0/G1, and thus a reduced proportion of cells in S+G2/M phases, generated by the WT T2 mutant of EYA3, was not observed when the constitutively active form of c-Src was overexpressed.

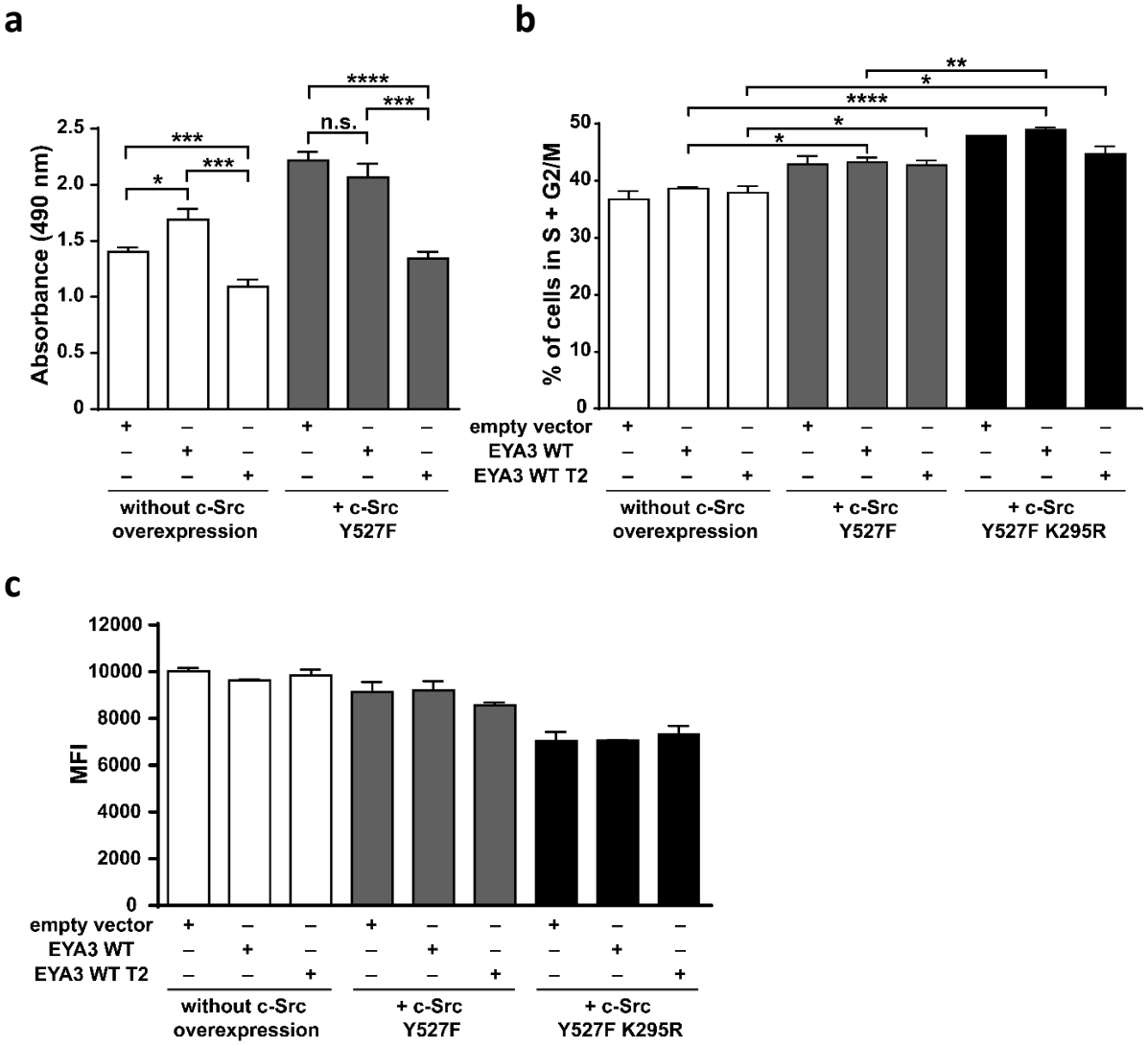


Figure IV-8. Cellular implications of tyrosine to phenylalanine mutation of EYA3 phosphotyrosine sites, in HEK293T cells[80].
a) MTS assay of HEK293T cells at 72 h after constructs transfection. The results represent mean ± SEM of three independent experiments, each having three replicates (n = 9). Statistical analysis was achieved by applying unpaired Student's t-test (two-tailed; * p< 0.05; *** p< 0.001; **** p<0.0001).
b) Flow cytometry analysis of cell cycle using HEK293T cells transiently expressing EYA3 WT or EYA3 WT T2 with or without co-expression of c-Src Y527F or c-Src Y527F K295R, at 24 h post-transfection. The values represent the cumulated value of cell percentages in the S and G2/M phases. The results represent mean ± SEM of two independent experiments (n = 3). Statistical analysis was achieved by applying unpaired Student's t-test (two-tailed; * p< 0.05; ** p< 0.01; **** p< 0.0001).

c) Flow cytometry analysis of HEK293T cells stained with CFSE. Bar graph shows the median value of the CFSE intensity (MFI), at 48 h post-transfection. Values represent the mean ± SD of one representative experiment out of two performed (n = 2).

Next, we wanted to check whether the detected increase in the proportion of cells in the S+G2/M phases was caused by a higher proliferation rate or it was a consequence of cellular arrest in the G2/M phases. To this end, we stained the cells with CFSE and kept track of their proliferation by measuring the dilution of this dye with time. The flow cytometry analysis (Fig. IV-8c) revealed that cells had the lowest proliferation rates (translated from highest MFI values) in the absence of c-Src overexpression, while in the cases where the dominant negative form of the kinase was overexpressed cells had the highest proliferation rates. The MFI values (measurements performed 48 h post-transfection) reflect the cell cycle distribution (measurements performed 24 h post-transfection). Therefore, the cellular proliferation profiles obtained in the MTS assay (illustrated in Fig. IV-8a) reflect a true modification of the cell cycle distribution. In conclusion, the Y→F mutation of the three residues - Y77, Y96 and Y237 - abolishes the pro-proliferative effect of EYA3 overexpression.

We performed MTS as well as invasion experiments on MCF-7, a breast cancer cell line. No significant differences in proliferation or invasion were found between cells that overexpressed EYA3 WT and EYA3 WT T2, irrespective of c-Src Y527F overexpression. This suggests a cellular specificity regarding EYA3's proliferative effect.

The influence of Src-induced phosphorylation of EYA3 WT and EYA3 WT T2 on proliferation was also tested in MDA-MB-231, a highly aggressive, triple-negative breast cancer cell line. MTS experiments showed that MDA-MB-231 cells over-expressing EYA3 WT have increased proliferation compared with those which overexpressed EYA3 WT T2 and the control sample (Fig. IV-9a). Western blot experiments on the samples collected 24 h and 48 h post-transfection show that c-Src Y527F drastically diminishes the expression of EYA3 WT and EYA3 WT T2 (Fig. IV-9b). Notably, in HEK293T and MCF-7 cells we did not detect any decrease in EYA3 when it was co-expressed with c-Src Y527F, neither 48 nor 72 h post-transfection. All these results indicate cellular specificity concerning the consequences of the EYA3 - Src interaction.

We also wanted to investigate whether c-Src Y527F influences the expression of endogenous EYA3 protein. Endogenous EYA3 expression decreased in the cells that overexpressed the highest quantity of c-Src Y527F and in this cathegory, the lowest expression of EYA3 was detected in the cells harvested 48 h post-transfection. These results indicate that overexpression of constitutively active c-Src decreases EYA3 expression.

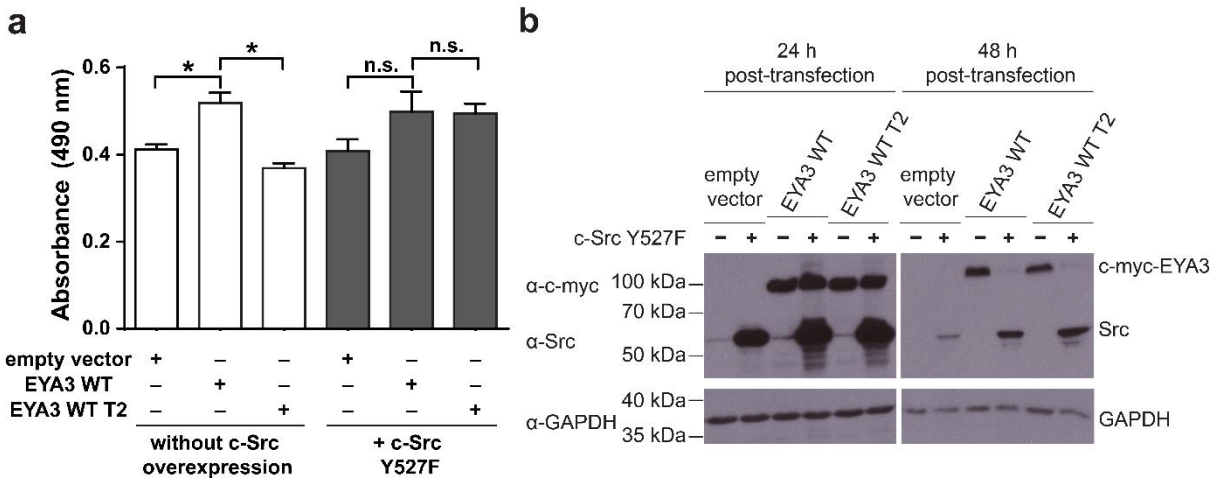


Figure IV-9. Proliferation experiment in MDA-MB-231 cells. Influence of c-Src Y527F overexpression on endogenous EYA3.
a) Results obtained from proliferation experiments performed on MDA-MB-231 cells using MTS assay. The results represent mean ± SEM of three independent experiments, each having three replicates (n = 9). Statistic analysis was achieved by applying unpaired Student's t-test (two-tailed; n.s. - not significant; * $p < 0.05$), using GraphPad Prism6.
b) Representative Western blot result obtained from analyzing the MDA-MB-231 cells subjected to proliferation assay. Samples were collected 24 h post-transfection as well as after the absorbance measurements (48 h post-transfection).

IV.2.6 Src-induced phosphorylation pattern of EYA3 in HEK293T and MCF-7 cells

We found that in the same experimental conditions (EYA3 WT or WT T2 overexpressed alone or with c-Src Y527F), EYA3 has different proliferative effects in HEK293T and MCF-7 cells. Given these results, we wanted to identify the Src-induced phosphorylation pattern of EYA3 in the two cell types, so we transiently expressed the EYA3 proteins (EYA3 WT, EYA3 D309N and EYA3 D309N Y77F) with and without c-Src Y527F. Then, we identified the phosphorylated residues of EYA3 proteins by nLC-MS/MS (applying CID and ETD fragmentation), after enrichment of the phosphorylated peptides using TiO_2. EYA3 D309N Y77F contains Y→F mutation at the residue which displayed the highest contribution to EYA3 phosphorylation.

The majority of the phosphoresidues detected are the same with the ones found in the *in vitro* EYA3 D311N phosphorylation reactions. Y325 and Y329 are new phosphosites. Phosphoserine residues were also identified due to the application of the TiO_2 enrichment strategy. The different Src-induced phosphorylation pattern of EYA3 in the two cell lines leads us to assume that Src-induced phosphorylation of EYA3 triggers different processes depending on the cell type in which it occurs. Also, the obtained results suggest there is a complex interplay between Src-induced phosphorylation and EYA3 autodephosphorylation, with potential

implications in cellular physiology and pathology, and the identification of interactors for EYA3 would be very useful in signal transduction pathway determination.

IV.2.7 Assessment of the variability of EYA3's tyrosine residues using bioinformatic tools

The *in vitro* identification of a phosphorylated residue does not necessarily mean this residue plays any functional role; the result could be an experimental artifact. In many reports, it has been proven that phosphosites with functional role have a significantly higher degree of conservation compared to the phosphosites with unknown function[91]. This makes sequence conservation analysis a useful approach to predict which phosphosites have functional roles. That is why, we performed sequence conservation analysis for EYA3, seeking to determine the degree of conservation for all the Src-phosphosites identified in this chapter (a total of fifteen: Y67, Y72, Y77, Y90, Y96, Y105, Y108, Y115, Y208, Y237, Y325, Y329, Y426, Y508, Y532).

The variability analysis was carried out on a set of 721 homologues of EYA3, which were selected from the Representative Proteomes RP55 database[92]. We could estimate the degree of conservation for EYA3 tyrosine residues as well as see the how conserved are the rest of EYA3's residues amongst the 721 homologues. Tyrosine residues from the N-terminal part as well as from the ED have a high degree of conservation. This suggests they could have physiological implications and argument in favor of further functional studies. Tyrosine residues Y72, Y77, Y90, Y105, Y108 and Y208 are all highly conserved. These residues were detected as phosphosites in both HEK293T and MCF-7 cells, which suggests they should be taken into consideration in future functional studies. Y237 and Y426, phosphoresidues detected only in MCF-7 cells, are also highly conserved and should be considered when investigating implications of EYA3 phosphorylation in this cell line. EYA3 could be involved in signal transduction pathways also through the other tyrosine residues which displayed a high degree of conservation, so these residues should also not be ignored in future studies concerning this protein.

IV.3 CONCLUSIONS

1. Mass spectrometry analysis, native and bottom-up, reveal that prokaryotically expressed EYA3 WT and EYA3 D311N lack the initial methionine residue and the following serine residue has been acetylated during prokaryotic expression.

2. Prokaryotically expressed and purified EYA3 proteins form a complex with Skp trimer. Skp may stabilize EYA3, especially the N-terminal part of the protein, which is highly disordered.

3. Native MS analysis after *in vitro* phosphorylation of EYA3 D311N with Src for 2 h revealed 12 phosphorylated residues. The same experiment performed on EYA3 WT revealed only one phosphorylated residue, demonstrating the enzyme's autodephosphorylation capacity exceeds Src phosphorylation and the importance of the ED in controlling EYA3 phosphorylation status.

4. Native MS analysis showed that EYA3 WT directly dephosphorylates the Src-phosphorylated EYA3 D311N protein. The acetylated form of phospho-EYA3 D311N was also dephosphorylated. For EYA3 D311N in complex with Skp, there was a slight hindrance induced by the chaperone protein concerning phosphorylation by Src.

5. Eleven tyrosine residues (Y67, Y72, Y77, Y90, Y96, Y105, Y108, Y208, Y237, Y426, Y532) were detected as phosphorylated in EYA3 D311N, by bottom-up mass spectrometry analysis of the time course *in vitro* phosphorylation of EYA3 D311N by Src kinase. In the case of EYA3 WT, in the same type of experiment, four tyrosine residues (Y77, Y96, Y105 and Y237) were detected as phosphorylated and could be considered residues with increased resistance to autodephosphorylation. The seven tyrosine residues - Y67, Y72, Y90, Y108, Y208, Y426, Y532 - could be considered the main autodephosphorylation sites.

6. Bottom-up MS analysis of EYA3 D311N phosphorylation by Src kinase and its dephosphorylation by EYA3 ED WT revealed not only two new phosphotyrosine residues (Y115 and Y508) but also the dynamics of phosphorylation/ dephosphorylation for each of the detected phosphotyrosine residues. In this experiment, only Y77, Y96, Y237 and Y508 were found to be phosphorylated after 4 h of incubation with EYA3 ED WT. Y508 was detected as another residue with high resistance to autodephosphorylation and the only residue from the ED that had this feature.

7. *In vitro*, Src kinase could phosphorylate thirteen tyrosine residues from EYA3: Y67, Y72, Y77, Y90, Y96, Y105, Y108, Y115, Y208, Y237, Y426, Y508 and Y532.

8. Phosphorylation of Y96, Y77, Y105, Y90 and Y115 proved to have a measurable contribution to the overall EYA3 phosphorylation. Y77 and Y96 had the highest contribution to overall phosphorylation. The contribution of Y90 and Y105 could be observed only when the residues with high contribution to the total phosphorylation (Y77 and Y96) were mutated to phenylalanine.

9. Endogenous EYA3 has autodephosphorylation capacity and is tyrosine phosphorylated in HEK293T cells that transiently overexpressed c-Src Y527F.

10. Proliferation experiments performed in HEK293T cells suggest that one or more of the three tyrosine residues mutated to phenylalanine in EYA3 WT T2 (Y77, Y96 and Y237) could be

involved in proliferation events. In MTS assays and cell cycle distribution analyses, the mentioned triple mutation abolished the pro-proliferative effect of EYA3 overexpression. Proliferation assays showed that overexpression of EYA3 WT increased proliferation of HEK293T cells, but the effect was masked when c-Src Y527F was co-expressed.

11. No significant differences in proliferation were found between MCF-7 cells which transiently overexpressed EYA3 WT and EYA3 WT T2, irrespective of c-Src Y527F overexpression, suggesting EYA3's proliferative effect is cell specific.

12. Concerning EYA3's capacity to influence invasion of breast cancer cells, in MCF-7 cells, no significant differences were detected following EYA3 WT or EYA3 WT T2 overexpression, neither with nor without c-Src Y527F co-expression.

13. Proliferation experiments performed on MDA-MB-231 breast cancer cells show that transient expression of EYA3 WT induces an increase in proliferation compared to EYA3 WT T2 overexpression and control samples. These results are similar to those detected in HEK293T cells and outline a potential role in MDA-MB-231 proliferation for one or more of the three tyrosine residues mutated to phenylalanine in EYA3 WT T2 (Y77, Y96, Y237).

14. In MDA-MB-231 cells, EYA3 expression drastically decreased 48 h post-transfection when it was co-expressed with c-Src Y527F. Further studies performed on the same cell line showed that expression of endogenous EYA3 decreased when constitutively active c-Src was transiently expressed. These results suggest that the expression of EYA3 protein decreases when Src kinase is maintained in an active state.

15. Bottom-up MS analysis of the Src-induced phosphorylation pattern of EYA3 revealed two new phosphotyrosine sites: Y325 in HEK293T cells and Y329 in MCF-7 cells. The phosphotyrosine residues which displayed increased resistance to autodephosphorylation in both cell lines were Y77, Y108 and Y208. Phosphoserine residues were also detected on EYA3 proteins, in both cell lines, highlighting the complexity of Src-induced phosphorylation.

16. Results from the sequence conservation analysis performed using bioinformatic tools showed that tyrosine residues from the N-terminal region as well as from the ED are highly conserved among the 721 EYA3 homologues, whether they were Src-induced phosphoresidues or not. This suggests they could have functional roles and should be taken into consideration when searching for EYA3's implications in signal transduction or other cellular processes.

FINAL CONCLUSIONS

Identification of WDR1 as the first cytoplasmic substrate of EYA3 opens new avenues in the search of novel cytoplasmic processes involving EYA3. Given the autodephosphorylation

capacity of the human paralogs, tyrosine phosphorylated EYA is its own substrate. The fact that EYA-WDR1 and EYA-EYA interactions are paralog-specific is another important finding. In other words, at least in these situations, EYA proteins do not substitute each other. Although the ED is highly conserved, it can be assumed that interaction peculiarities and substrate specificity of the paralogs are imbedded in their non-conserved sequence regions. Presumably, the weakly conserved N-terminal region provides specificity to the corresponding EYA protein. The new EYA3 trapping mutants identified in this thesis will be useful for the identification of other EYA3 substrates and implicitly, new physiological roles of EYA3.

Detailed characterization of Src-induced EYA3 phosphorylation/(auto)dephosphorylation revealed that a number of tyrosine residues with increased resistance to autodephosphorylation are involved in EYA3 dependent cellular proliferation. Interestingly, experiments performed in cancer cell lines showed these effects are cell specific.

Taken together, the results obtained in this thesis highlight the necessity of future in-depth studies seeking to unravel the physiological and pathological significance of EYA3 phosphorylation/autodephosphorylation balance.

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The Institute is not just a working place where you have the opportunity to learn and advance in your career, it is a place where you meet some intelligent, but also serious, hard-working, dedicated, but most importantly honest, modest, discrete and worm-hearted people.

I will mention some with whom I have collaborated and discussed some "science stuff" along the years, but I thank them all. They have all impressed me in a certain way and I tried to take the best part out of their personality, hoping to be a better person if not a good scientist!

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LIST OF PUBLICATIONS AND POSTER PRESENTATIONS

Publications:

1. **Analysis of EYA3 Phosphorylation by Src Kinase Identifies Residues Involved in Cell Proliferation**

   Aura E. Ionescu; Mihaela Mentel; Cristian V.A. Munteanu; Livia E. Sima; Eliza C. Martin; Georgiana Necula-Petrareanu; Stefan E. Szedlacsek

   International Journal of Molecular Sciences 2019, Volume 20, Issue 24, 6307.

   https://doi.org/10.3390/ijms20246307

2. **Regulation of TRPM8 Channel Activity by Src-mediated Tyrosine Phosphorylation**

   Alexandra Manolache, Tudor Selescu, Georgiana Maier, Mihaela Mentel, Aura Ionescu, Cristian Neacsu, Alexandru Babes, Stefan Szedlacsek

   Journal of Cellular Physiology, 2019 Nov 14. doi: 10.1002/jcp.29397.

3. **WDR1 is a novel EYA3 substrate and its dephosphorylation induces modifcations of the cellular actin cytoskeleton**

   Mihaela Mentel, Aura E. Ionescu, Ioana Puscalau-Girtu, Martin S. Helm, Rodica A. Badea, Silvio O. Rizzoli & Stefan E. Szedlacsek

Scientific Reports, 2018 Feb 13;8(1):2910. doi: 10.1038/s41598-018-21155-w.

4. **Expression, Purification, and Kinetic Analysis of PTP Domains**

   Mihaela Menţel, Rodica A. Badea, Georgiana Petrăreanu, Sujay T. Mallikarjuna, Aura E. Ionescu, Ştefan E. Szedlacsek

   Methods in Molecular Biology, Vol. 1447, Protein Tyrosine Phosphatases, 2016.

5. **Phosphoketolases from Lactococcus lactis, Leuconostoc mesenteroides and Pseudomonas aeruginosa: dissimilar sequences, similar substrates but distinct enzymatic characteristics**

   Georgiana Petrăreanu, Mihaela C Balaşu, Andrei M Vacaru, Cristian V A Munteanu, Aura E Ionescu, Iulia Matei, Ştefan E Szedlacsek

   Applied Microbiology and Biotechnology. 04/2014; DOI:10.1007/s00253-014-5723-6.

6. **Synthesis and Biological Evaluation of Some New N^1-[4-(4-Chlorophenylsulfonyl) benzoyl]-N^4-(aryl)-thiosemicarbazides and Products of Their Cyclization**

   Ştefania-Felicia Bărbuceanu, Gabriela Băncescu, Gabriel Şaramet, Florica Bărbuceanu, Constantin Drăghici, Flavian Ştefan Rădulescu, Aura Ionescu and Simona Negreş

   Heteroatom Chemistry, Volume 24, Issue 4, pages 309-321, July 2013.

Poster presentations:

1. Aura-Elena Ionescu, Mihaela Menţel, Aneika C. Leney, Cristian V.A. Munteanu, Albert J. Heck, Ştefan E. Szedlacsek, "EYA3 tyrosine phosphorylation by Src kinase. From mass spectrometry to implications in proliferation." The 12th Central and Eastern European Proteomic Conference "Advances in Proteomics and Progress in Precision Medicine", 24th-26th of October 2018, Bucharest, Romania. Third Prize at the "Best poster" competition.
2. Aura-Elena Ionescu, Mihaela Menţel, Rodica Aura Badea, Ştefan Eugen Szedlacsek, "Evidence for intermolecular autodephosphorylation of human Eya3", Europhosphatase 2017 "Phosphatases in cell fates and decisions", 23rd - 28th of July, Paris, France.
3. Aura Elena Ionescu, Mihaela Menţel, Cristian V. A. Munteanu, Cristian Marian Butnaru, Rodica Aura Badea, Ioana Iancu, Ştefan Eugen Szedlacsek, "Mass Spectrometric investigations regarding phosphorylation of human Eya3 by Src kinase", Europhosphatase 2015 "Phosphorylation switches and cellular homeostasis", 24th - 29th of June, Turku, Finland.

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