The Nuclear Receptor Co-Activator 5 is a potential new co-regulator of the Estrogen Related Receptor α in breast cancer

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Table of content

Abstract

Nuclear receptors are transcriptional factors that are essential for a wide range of biological processes. They are partly regulated through their interaction with co-regulatory proteins. Here, we focus on the orphan nuclear receptor ERRα and its potential new co-activator protein NCOA5. Using NCOA5 knockdown or overexpression via a lentiviral system, we investigated the role of NCOA5 in ERRα regulation in Her2-positive breast cancer cells and its effect on known targets of ERRα in this context, such as the *ERBB2* amplicon transcription. We show that NCOA5 and ERRα can regulate each other, yet the precise mechanism remains to be elucidated. NCOA5 protein level affects ERRα transcription and protein level, also affecting the transcription of ERRα targets from the *ERBB2* amplicon including the Her2 receptor itself. Modulation of NCOA5 levels leads to variation in cell proliferation and metabolism, thus revealing that NCOA5 is an important factor in the ERBB2 amplicon regulation.

Résumé

Les récepteurs nucléaires sont des facteurs de transcription qui sont essentiels pour la régulation d'un grand nombre de procédés biologiques. Ils sont en partie régulés grâce à leurs interactions avec des protéines co-régulatrices. Dans ce projet, nous nous sommes intéressés plus particulièrement au récepteur nucléaire orphelin ERRα et à NCOA5, un nouveau co-activateur potentiel. En utilisant un système lentiviral pour inactiver ou surexprimer NCOA5, nous avons étudié son rôle dans la régulation de ERRα dans des cellules de cancer du sein positives pour le récepteur Her2, ainsi que son rôle dans la régulation de la transcription de cibles connues de ERRα dans ce contexte, comme les gènes de l'amplicon *ERBB2*. Nous avons observé que NCOA5 et ERRα peuvent se réguler l'un l'autre, même si le mécanisme précis reste inconnu. De plus, la modulation de l'expression de NCOA5 affecte la transcription et le niveau de protéines de ERRα, ce qui affecte aussi la transcription des gènes de l'amplicon de *ERBB2*, incluant le récepteur Her2 lui-même. La modulation du niveau de NCOA5 dans les cellules entraine des variations dans leur vitesse de prolifération et dans leur métabolisme, indiquant que NCOA5 est un facteur important dans la régulation de l'amplicon *ERBB2*.

Preface – Contributions of Authors

The research conducted and presented in this manuscript is entirely my own except for the following cases. ERRα ChIP-sequencing on breast cancer cell lines was performed by Ms Ingrid Tam, DNA samples obtained were sequenced and data processed at Genome Quebec by Dr Guillaume Bourque and final data was analysed by Ms Ingrid Tam. qChIP primers for ERRα binding sites on the *ERBB2* amplicon were designed by Dr Geneviève Deblois. Plasmids encoding for lentiviral capsid, VSV.g and Δ8.9, were provided by Dr Mathieu Vernier.

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Abbreviations

AKT	Serine Threonine Kinase/Protein kinase B
AR	Androgen Receptor
bp	Base pairs
ChIP	Chromatin Immuno-Precipitation
CIA	Co-Activator Independent of AF-2
d	Day
DBD	DNA Binding Domain
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene-bis(oxyethylenenitrilo)-Tetra-Acetic Acid
ER	Estrogen Receptor
ERE	Estrogen Receptor Response Element
ERR	Estrogen Related Receptor
ERRE	Estrogen-Related Receptor Response Element
FISH	Fluorescent in Situ Hybridization
GR	Glucocorticoid Receptor
HAT	Histone Acetyl-Transferase
HCC	Hepato-Cellular Carcinoma
HDAC	Histone Deacetylase
HER2	Human Epidermal Growth Factor Receptor 2
hr	hour
HRE	Hormone Response Element
IHC	Immuno-HistoChemistry
LBD	Ligand Binding Domain
LXR	Liver X Receptor
MAPK	Mitogen-Activated Protein Kinase
min	Minute
MR	Mineralocorticoid Receptor
NCOA5	Nuclear Receptor Co-Activator 5

NR	Nuclear Receptor
OHT	4-Hydroxytamoxifen
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffer Solution
PFA	Paraformaldehyde
PGC	Peroxisome Proliferator-Activated Receptor Gamma Coactivator
PI3K	Phospho-Inositide 3 Kinase
PPAR	Peroxisome Proliferator-Activated Receptor
PR	Progesterone Receptor
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RAR	Retinoic Acid Receptor
ROR	RAR-Related Orphan Receptor
ROS	Reactive Oxygen Species
RTKi	Receptor Tyrosine Kinase inhibitor
RVR	Rev-ErbAα-related Receptor
RXR	Retinoid X Receptor
sec	second
SRC	Steroid Receptor Co-activator
TBS-T	Tris-Buffered Saline – Tween 0,1%
TR	Thyroid hormone Receptor
VDR	Vitamin D Receptor

List of Figures

Literature Review

1. Breast Cancer

A. Statistics

In Canada, breast cancer is the most common cancer in women. According to the Canadian Cancer Society, this cancer represents 25,000 new cases per year, which account for 25% of the cancers diagnosed in women. Even though breast cancer mortality rate has decreased for the past 30 years, it is still the second leading cause of death from cancer, with 5,000 deaths per year. Breast cancer can also occur in men but it is rare and represents less than 250 new cases per year in Canada. Breast cancer has a 1 in 8 lifetime risk of occurrence for women, making it an important healthcare issue (Statistics from the Canadian Cancer Society).

B. Subtypes

Breast cancer is a heterogeneous disease that regroups diverse clinical pathological subtypes. Treatment options are numerous, with local management treatments like surgery or radiotherapy and systemic treatment options with hormonal therapies (such as Tamoxifen), targeted therapies (against human epidermal growth factor 2, (Her2)) or regular chemotherapy. One of the challenges is to choose the treatment according to each patient's tumour characteristics. To do so, tumours are staged, graded and furthermore classified based on biomarkers expression.

In the clinic, three receptors status (Estrogen Receptor (ER), Progesterone Receptor (PR) and Her2 are used to define three breast cancers subgroups: the hormone-dependant subtype, the Her2 amplified subtype and the triple negative subtype (Table 1). ER and PR status are determined by Immuno-Histo-Chemistry (IHC) and Her2 status can be determined by IHC or Fluorescent in Situ Hybridization (FISH) on tumour tissue from a biopsy. The results will help to choose which treatment to use and determine the prognostic (Eroles, 2012)(Park, 2012).

Breast Cancer Clinical subtypes

Subtype	Hormone Dependant	Her2 + or Amplified	Triple Negative
Receptors Status	ER + / PR + / Her2-	ER - / PR - / Her2 +	ER - / PR - / Her2 -
Treatment	Anti-ER therapy Tamoxifen or aromatase inhibitors	Anti-Her2 therapy Trastuzumab or other	Non- targeted chemotherapy
Prognosis	Good	Intermediate	Bad
Occurrence	Around 70% of cases	15-20% of cases	10-15% of cases

The hormone-dependant tumours rely on estrogen receptor driven proliferation pathways. Anti-ER therapies, such as Tamoxifen have been successfully used for the last 30 years for these tumours. PR is a downstream target of ER; thus PR being expressed is a sign of a functional ER pathway, indicating that anti-ER therapies are likely to be efficient (Park, 2012).

The Her2 receptor is encoded by the *ERBB2* gene. The Her2 amplified subtype displays genomic amplification of the *ERBB2* gene loci and/or high expression the Her2 receptor that is the main oncogenic driver in this subtype. Targeted therapies against Her2 signalling have been successfully developed, the most famous being the monoclonal antibody Trastuzumab (Park, 2012).

The breast tumours that have none of these three receptors expressed have a very poor prognosis, no targeted treatment exist for those tumours and the only systemic treatment available is chemotherapy (Park, 2012).

One of the main issues encountered with this method of classification is that reality is more nuanced than this three subtypes classification. Many patients having a hormone-dependant breast cancer have high ER but no PR expressed, which is often a sign that anti-ER treatments efficacy will be limited. In other cases, patients have high ER and high Her2 at the same time, those patients might benefit from a combined treatment with anti-ER and anti Her2 therapies.

More recent studies, based on "OMICs" technologies like genomics, transcriptomic analysis, copy number alterations analysis, microRNA profiling, epigenetic profiling, proteomics or metabolomics on large cohorts of patients have tried to develop new ways to classify breast tumours and tailor treatment according to each patient's tumour biology (Kristensen, 2014).

The most famous classification that has emerged from these new studies is based on gene expression analysis. According to transcriptomic analysis of a large number of tumours, breast cancers can be clustered into 5 groups based on gene expression patterns (Perou, 2000; Sorlie, 2001).

The ER-positive tumours that often display gene expression patterns close to those of luminal epithelial cells were called Luminal and were further divided into two groups: Luminal A and Luminal B. Luminal A tumours are the most common and account for 50 to 60 % of all breast cancers, they are Her2 negative with low Ki67 proliferation index and have good prognosis. Luminal B tumours are less frequent; they represent around 15 to 20% of breast cancers. They have worse prognosis than the Luminal A tumours and they display a more aggressive phenotype with a higher recurrence rate. In most cases they are ER positive, Her2 negative with high Ki67 index or ER positive and Her2 positive. The Her2 amplified group is defined by high Her2 expression and expression of genes related to the Her2 pathway, which correlates with poor prognosis and aggressiveness. They account for 15 to 20% of breast cancers. The last two groups do not express any of the three receptors ER, PR and Her2 (Yersal, 2014).

The Basal-Like tumours have high expression of genes that are specific to a basal myoepithelial cell profile hence their name basal-like. They account for 5 to 10% of breast cancers. These tumours are highly aggressive, highly proliferative, often metastasize and currently have no targeted therapies available (Yersal, 2014).

The last group is the normal-like, it represents 5 to 10% of tumours and is also negative for the three receptors. They are poorly characterized but have a better prognosis than basal-like tumours (Yersal, 2014).

Although these new molecular subtypes were appealing, they did not really change the way we treat breast cancer and how we choose therapies. The traditional sub-classification depending on ER, PR and Her2 is still the golden standard in the clinic and determines which therapy to use. The new molecular tests based on molecular classification like PAM50, Mammaprint© or Oncotype DX© only give scores indicating risk of recurrence and still need further validation before entering clinical protocols (Yersal, 2014).

This classification is based on only one type of data (gene expression), however other types of data can give information on tumour's biology and it is worth integrating them in clustering tools. Improvements of bioinformatics analysis methods lead to an effort to ingrate more datasets and combine more information in order to develop more precise classification algorithms such as PARADIGM that integrates copy number analysis, gene expression patterns and network interactions between genes and defines 10 clusters of patients with significantly different outcomes. These integrated analysis usually give better predictions for outcome and recurrence than regular gene expression classification but there are not yet ready for clinical use (Kristensen, 2014).

C. The Her2 amplified subtype

As mentioned previously, in this subtype the common feature is that the gene encoding Her2, a strong oncogene and proliferation driver, is generally amplified at the genomic level and highly transcribed. This leads to rapidly proliferating and aggressive breast cancer cells.

The *ERBB2* gene, coding for the Her2 receptor, is located on chromosome 17q21, in a region referred to as the *ERBB2* amplicon. This genomic region (or amplicon) contains several genes, including *ERBB2,* and other genes involved in proliferation and aggressiveness, the most studied being *GRB7*. In this subtype, this genomic region is often amplified leading to the presence of a high number of copies of these genes, also correlating with high expression (Kao, 2006).

Her2 is a receptor tyrosine-protein kinase and a member of a family of four trans-membrane receptors containing Her1, Her2, Her3 (also called EGFR), and Her4. These receptors are found at the cellular membrane. They are composed of an extra-cellular domain (responsible for ligand binding and dimerization), a trans-membrane domain and an intra-cellular domain (responsible for trans-phosphorylation and signal transduction). Individual receptors are inactive on their own and need to form dimers so the intra-cellular domain can get phosphorylated and start the signalling cascade. Her2 have no known ligand, but it can dimerize with all the three other members of its family or with itself. In most cases, it forms dimers with Her3 upon EGF binding on Her3, or, if Her2 is present in high quantities at the cellular membrane (which happens in Her2 amplified breast cancer), it forms homo-dimers with another Her2 protein, thus activating itself. When Her2 is activated, it can in turn activate the RAS/MAPK pathway and the PI3K/Akt pathway leading to high proliferation of cancer cells (Barnes, 2004).

Several anti-Her2 strategies were developed. They can be used alone or in combination, in addition to chemotherapy. Two main classes of these anti-Her2 targeted therapies exists: (1) therapies based on monoclonal antibodies (with or without drug conjugates) that inhibits the dimerization of the receptors blocking them in an inactive form, in this category there are several available options such as Trastuzumab (monoclonal antibody targeting Her2), Pertuzumab (monoclonal targeting Her3), or Trastuzumab-emtansine (antibody-drug conjugate); (2) Receptor Tyrosine Kinase inhibitors (RTKi) that block phosphorylation of the receptor, thus inhibiting signal transduction and subsequent activation of the pathway, such as Lapatinib (RTKi that targets Her2) or Neratinib (RTKi that targets Her1, Her2 and Her4). These new therapeutic strategies have greatly improved survival in Her-2 positive breast cancer and several clinical trials are still in progress to determine which are the best therapies combinations in various settings (Loibl, 2017).

Some nuclear receptors, such as ERα and the Estrogen Receptor Related Receptor α (ERRα) are well known for their implication is diverse breast cancers development. In Her2-amplified breast cancers, ERRα was shown to regulate the expression of genes located within the *ERBB2* amplicon (Deblois, 2010).

2. Nuclear Receptors

A. Structure

The nuclear receptors are transcriptional factors that enable the cells to integrate diverse physiological stimuli, such as hormone signals or nutrients levels, by modulating gene transcription according to these signals.

Nuclear receptors share common structural features, they are composed of four distinct domains: the N-terminal domain also called modulator domain, the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD) (Giguère, 1999).

The modulator domain is highly variable in length and sequence among nuclear receptors. In most nuclear receptors, it contains a transcriptional activation function referred to as AF-1. It can interact with co-regulatory proteins that regulate the nuclear receptor activity. The DBD contains two zinc fingers domains that are widely conserved through the nuclear receptors family and that can bind directly to DNA on specific sites called hormone response elements (HRE).

The hinge region is a moving region that enables the protein to fold and unfold so that the DBD and the LDB can interact together according to the conditions. Some co-repressor proteins can bind to the hinge to block the nuclear receptor in an inactive form.

The LBD contains the ligand-binding pocket. In most cases, nuclear receptors can bind small lipophilic ligands such as hormones, however some nuclear receptors have no known ligand to date, these are called orphan nuclear receptors. The LBD also contains a highly-conserved motif required for ligand dependant transactivation called AF2. This domain also serves as an interface for interactions with other proteins, for example co-activator proteins (Gronemeyer, 2004; Zassadowski, 2012).

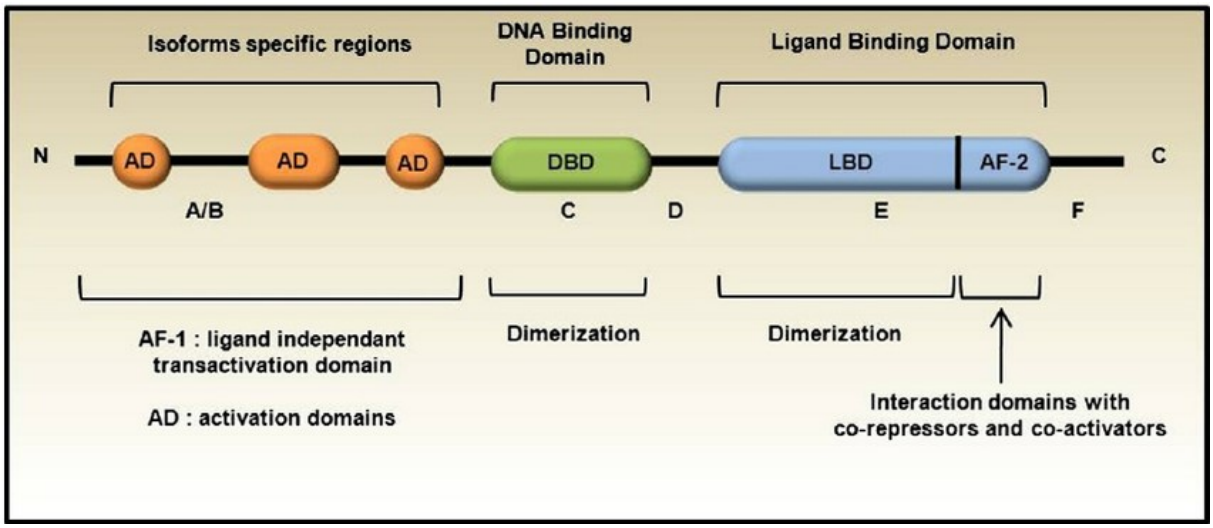

Figure 1: Structural and Functional organization of nuclear receptors.

(*figure from Zassadowski, 2012)*

B. Classification

The nuclear receptors family of proteins is a large superfamily containing 48 members. The first nuclear receptors discovered were receptors for known hormones, such as the ER, the androgen receptor (AR) and the thyroid hormone receptor (TR). Later, additional nuclear receptors were discovered and cloned without known ligand, leading to a new field of research known as reverse endocrinology, which primary objective was to find ligands that could activate these unanticipated receptors. Some of these putative receptors were soon adopted, as activating ligands were found. For example, the Liver X Receptors (LXRs) were found to interact with oxysterols. Some remained orphan and still have no ligand as of today, like the ERR subgroup (Evans, 2017).

Nuclear Receptors can be classified into four sub-families depending on their ligand (or absence of ligand). The hormone receptors can be further subdivided into two groups: the steroid hormone receptors group that comprises ER, AR, PR, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) and the thyroid hormones receptors group that comprises the TRs, the vitamin D receptor (VDR) and the retinoid acid receptors (RARs). The orphan receptors are subdivided in two groups: the adopted orphan nuclear receptors, for which a ligand was found recently such as the RAR Related Receptors (RORs), Retinoid X Receptors (RXRs), LXRs, or Peroxisome Proliferator Activated Receptors (PPARs) and the orphan nuclear receptors that still have no known ligands such as the ERRs (Evans, 2017).

C. Mechanism of action

Classic steroid hormone nuclear receptors share the same mechanism of action. When they are not activated, they remain in the cell cytoplasm in a repressive complex composed of heat shock proteins. Upon their hormone binding, the nuclear receptor detaches from the repressive complex and forms dimers that will then translocate to the nucleus where they will interact with their co-activators and bind to their DNA on HRE to activate transcription (Gronemeyer, 2004).

Most nuclear receptors act differently. In the case of orphan nuclear receptors that do not have a known ligand, their mechanism of activation relies mostly on the presence of co-regulatory complexes. In many instances, they are in complex with co-repressor proteins. When they must be activated, the co-repressor complex is replaced by an inducible co-activator complex and the nuclear receptor can then enhance transcription (Gronemeyer, 2004).

D. The ERR subfamily

1. Generalities

The ERR subfamily contains three members: ERRα, ERRβ and ERRγ (officially referred to as NR3B1, NR3B2, NR3B3, respectively). The three isoforms are close in structure to the ER but none of the three ERRs can bind estrogen (Giguère, 1988). All three ERRs can bind to co-activator proteins, such as SRC-1 a member of the steroid receptor co-activators (SRC) family (Xie, 1999) or the peroxisome proliferator activated receptor γ co-activator 1α and β (PGC-1α and β) (Deblois, 2013; Audet-Walsh, 2016). Interactions with the co-repressor complex RIP140/Nrip1 have also been reported (Castet, 2006). These interactions with co-regulatory complexes are critical for their regulation.

The ERRs are orphan receptors and their cognate ligands remain to be found to this date. However, small synthetic molecules that can inhibit their interaction with co-activators, like the inverse agonist C29, the synthetic drug XCT790 (inhibiting interaction with PGC-1 with ERRα) or 4-hydroxytamoxifen (inhibiting co-activators interaction with ERRβ and ERRγ), have been charaterized (Audet-Walsh, 2015).

The three ERRs are expressed in the entire human organism, each one at varied levels across tissues and through development. ERRβ is expressed mostly during embryogenesis while ERRγ and ERRα are ubiquitously expressed from early embryogenesis to death. ERRα is the most highly expressed and most studied of the three ERRs (Tremblay, 2007).

2. ERRα

ERRα is the most widely studied member of the ERR family. Furthermore, ERRα full body knockout (KO) mice survive and get to reach adulthood making phenotypic analysis easier. However, these mice exhibit several metabolic defects. They are smaller and have smaller fat pads than wild type mice. They gain less weight and are more resistant to obesity upon high fat diet than their wild types counterparts (Luo, 2003). They also display impaired lipids absorption in the intestine (Carrier, 2004). Gene expression profiling with microarrays showed that ERRα KO mice have altered gene expression patterns for metabolic pathways such as OXPHOS and lipid metabolism in high-energy demand tissues such as skeletal muscle (Perry, 2014), liver or white adipose tissue (Audet-Walsh, 2015). Furthermore, ChIP-seq experiments helped to discover ERRα regulatory networks (Laganière, 2003). In diverse tissues, they revealed that ERRα target genes are mostly related to metabolism (Deblois, 2008; Villena, 2008). ERRα binds to nearly all genes involved in the TCA cycle and glycolysis and over 700 genes encoding for mitochondrial proteins (Eichner, 2011), the most famous of which being *ACADM.* The *ACADM* gene encodes for the medium-chain acyl-CoA dehydrogenase (or MCAD), which is a key protein for fatty acid oxidation in the mitochondria (Sladek, 1997).

ERRα is now considered as a master metabolic regulator (Giguère 2008). Even though it is not essential for basal metabolism, it is required for adaptive metabolism to face many physiological or pathological situations where the tissues must adapt to new conditions. ERRα null mice have impaired thermogenesis after exposure to cold because their brown adipose tissue has dysfunctional mitochondria due to the absence of ERRα

(Villena, 2007). ERRα is also required for macrophages activation during clearance of pathogens such as *Listeria monocytogenes* (Sonoda, 2007) and for T cells activation and proliferation (Michalek, 2011).

In cancer, cell metabolism is often rewired to meet energy and anabolic intermediates demands to sustain rapid proliferation. Each cancer type has its unique way to balance metabolic pathways to fulfil its specific needs (DeBerardinis, 2008; Pavlova, 2016). However, one of the most common metabolic adaption found in cancer is a switch from OXPHOS to aerobic glycolysis (a process where glycolysis occurs and lactate is produced even in presence of oxygen), this effect is known as the Warburg effect (Liberti, 2016). In breast cancer, ERRα regulates genes involved in maintaining high levels of aerobic glycolysis, thus providing building blocks required for cell division. ERRα is also implicated in maintenance of ROS detoxification potential (Rangwala, 2007), lipid synthesis and glutamine uptake and metabolism (McGuirk, 2013; Tam, 2016; Deblois 2016; Park, 2016).

ERRα can bind and modulate expression of some long known ERα targets genes, such as the breast cancer biomarker *pS2,* using its own response element (ERRE) (Lu, 2001). Genome-wide locations analysis of the two receptors performed with ChIP-seq later showed that ER and ERR predominantly bind to distinct sites through different DNA response elements. Notably, ERRα DNA response element (ERRE) can occasionally be found embedded in ERα DNA response element (ERE) leading to a small number of situations where ERRα and ERα can compete for gene expression regulation. *ERBB2* is the most notable example of this situation (Deblois 2010). ERRα's role in breast cancer is now studied independently of ERα and was shown to be a negative prognostic marker in breast cancer, as high expression of ERRα correlates with aggressiveness and poor prognosis (Ariazi, 2002).

ERRα was further implicated in Her2-positive breast cancer, as it was shown to be involved in the *ERBB2* amplicon regulation (Deblois, 2010) and that EGF treatment stimulates its binding to DNA (Barry, 2005). In ERα positive breast cancer cells, ERα and its co-regulator Pax2 repress the expression of Her2 and of its amplicon (Hurtado, 2008). In such conditions, the cells are sensitive to hormonal treatment with tamoxifen.

However, these cells can become resistant to treatment when ERRα and PGC1-β compete for binding with ERα and Pax2 leading to the expression of the *ERBB2* gene and its genomic loci thus increasing proliferation rates aggressiveness. In ERα-negative and Her2-positive cell lines, ERRα and PGC1-β bind to a high percentage of genes of the ERBB2 amplicon and promote their transcription (Deblois, 2010).

Recent studies also demonstrated that ERRα protein level decrease upon RTKi treatment such as Lapatinib. However, when resistance to treatment occur, ERRα protein level increase and this is required for metabolic adaption of the cancer cells. Inhibition of ERRα restores sensitivity to Lapatinib treatment (Deblois, 2016).

3. ERRβ

ERRβ is expressed during the early embryo's development and is essential for placenta formation, normal chorion development and trophoblast differentiation. ERRβ full body knockout is embryonic lethal and the mice die in utero at day 10.5 (Luo, 1997). It is worth noting though, that tissue specific knockouts of ERRβ can give birth to fertile adults (Chen, 2007).

4. ERRγ

ERRγ is ubiquitously expressed in the embryo and in the adult, and it is highly expressed in the heart. ERRγ KO mice die soon after birth from cardiac failure. ERRγ is essential for the switch between carbohydrates based metabolism during foetal development to oxidative metabolism after birth and it is also a regulator of mitochondrial function (Alaynick, 2007). However, as for ERRβ, tissue specific knockouts of ERRγ could be generated (Yoshihara, 2016).

ERRγ is also a key regulator of metabolism in other tissues such as the skeletal muscle or the liver. It is essential for muscle fitness as it maintains mitochondrial activity and promotes oxidative capacity. Recent findings in the liver showed that ERRγ is important for hepatic neoglucogenesis and that it could promote hyperglycemia in diabetic

patients. Studies in mice indicated that ERRγ pharmaceutical inhibition could help to control hepatic insulin resistance (Audet-Walsh, 2015).

ERRγ role was also investigated in various cancers. In breast cancer, it was shown that high ERRγ expression correlates with good prognosis (Ariazi, 2002). In prostate cancer, androgen mediated ERRγ inhibition induces a metabolic switch that promotes cancer cells proliferation (Audet-Walsh, 2017).

3. Nuclear Receptors Co-regulators

A. History of nuclear receptors co-regulators

Nuclear receptor co-regulatory proteins were discovered with yeast two-hybrid screens using nuclear receptor LBDs as baits, to find partner proteins that could interact and regulate said nuclear receptors. In 1995, SRC-1 (or NCoA-1) was the first nuclear receptor co-activator to be discovered, followed closely by NCoR and SMRT (also called NCoR-2) that were the first co-repressors described. They now form a broad family of proteins that comprise co-activators such as the SRCs (steroid receptor co-activator) or the chromatin remodelling complex SWI/SNF/BRG-1 and co-repressors with proteins like RIP140, SMRT or HDACs. These co-regulatory proteins perform a very wide range of functions, and work as sensors for their nuclear receptors to adapt their action to varied environmental cues. This family includes but is not limited to: Histones Acetyltransferases (HATs) and Deacetylases (HDACs), NCoA and NCoR proteins, PGC1 α and β, SIRT1, c-AMP regulated transcriptional co-activators (CRTCs), see figure 1 for examples (Mouchiroud, 2014; Perissi 2005).

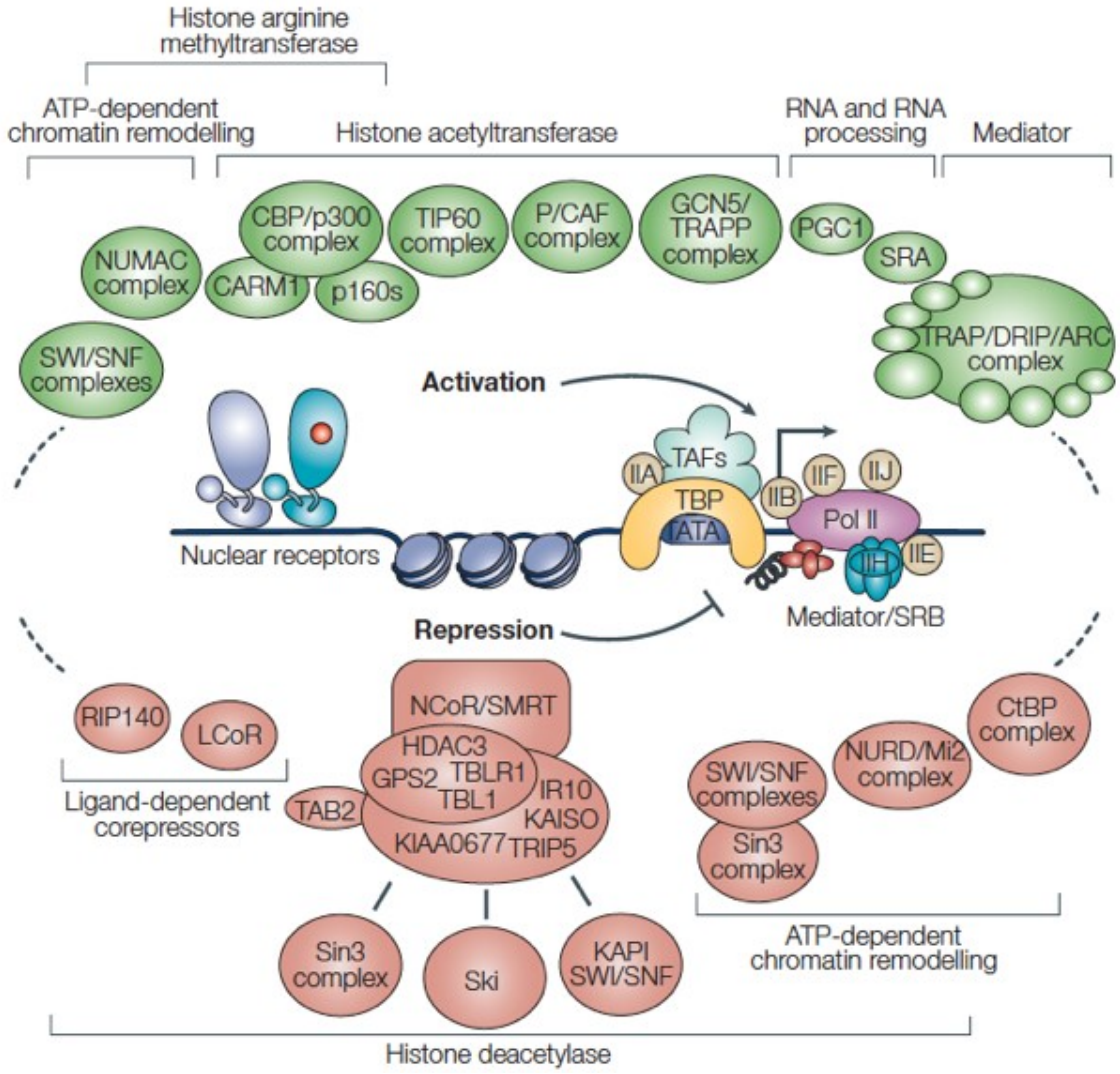

Figure 2: Examples of Coactivator and Corepressor complexes that are required for nuclear receptor mediated transcriptional regulation.
(figure from Perissi, 2005)

Many nuclear receptors co-activators and co-repressors are now known to be involved in varied diseases, and in cancers (Dasgupta, 2014). One most the most famous being NCoA-3, (also called AIB1 for amplified in breast cancer), which is a bad prognosis marker in breast cancer (Osborne, 2003). In this project, we focus on a nuclear receptor co-activator previously identified in our laboratory (Sauvé, 2001), the nuclear receptor co-activator NCOA5 (or CIA Co-activator Independent of AF-2), and its role in Her-2 positive breast as an ERR co-regulator.

B. The Nuclear Receptor Co-activator 5

NCOA5 was discovered in 2001 with a yeast two-hybrid screen using Rev-ErbAα-related receptor (RVR) LBD that lacked the AF-2 domain. Interestingly, this new co-regulatory protein contains both activating and repressing core motifs of nuclear receptors co-regulators; therefore, it can be either activating nuclear receptor activity or repressing it. At the time, it was shown to interact with RVR, Rev-ErbAα, ERα and ERβ (Sauvé, 2001). Although NCOA5 is ubiquitously expressed in the human body, only very few studies were conducted to determine its functions.

In 2013, it was shown that NCOA5 was an essential protein for planarians stem cell maintenance and that its expression is tightly controlled during mouse embryonic development (Boser, 2013). To investigate NCOA5 function in mammals, genetically engineered *Ncoa5*$^{+/-}$ mice were generated. Unfortunately, the males are sterile and no *Ncoa5*$^{-/-}$ homozygous mice can be generated. Male mice heterozygous for *Ncoa5* have a high level of liver tumour (mainly hepato-cellular carcinomas or HCC) incidence. Additional investigation showed that these mice have glucose intolerance starting six weeks of age and develop liver inflammation and steatosis before the appearance of HCC. In this setting, NCOA5 is required for ERα mediated repression of IL-6 expression (Gao, 2013). Other studies have linked NCOA5 to cancer, as it is a bad prognosis marker in oesophagus cancer (Chen, 2014) and in luminal breast cancers (Ye, 2017), or to metabolism, because it is a LXR co-repressor involved in macrophage cholesterol efflux (Gillespie, 2015).

Goal of study

This study aims to investigate the potential role of the nuclear receptor co-activator 5 (NCOA5) as an ERRα co-regulator in Her2 positive breast cancer, more precisely how it regulates breast cancer cells proliferation and metabolism.

Manuscript

1. Introduction

Orphan nuclear receptors are members of the nuclear receptor superfamily for which no natural ligand has been found (Giguère, 1999). Our laboratory is particularly interested in the NR3B subfamily of orphan nuclear receptors, which contains three members: ERR α, β and γ. The NR3B subfamily is very close in structure to the ER subfamily hence their name. It is now known that ERRα is implicated in metabolic regulation (such as glucose, glutamine and lipid metabolism) in normal tissues and in various cancers (Tam, 2016). While ERRα is known to promote mitochondria biogenesis (Eichner, 2011), oxidative phosphorylation and fatty acid oxidation (Audet-Walsh 2015), a growing body of evidence also implicates it in glycolysis and lactate production in breast cancer cells exhibiting metabolic reprogramming known as the Warburg effect (Cai, 2013; Deblois, 2016; Park, 2016).

Breast cancer is the most common cancer in women worldwide, with a 1 in 8 lifetime risk of occurrence. In the clinic, three molecular biomarkers are now widely used: ERα, PR and Her2 expression status (Yersal, 2014). Previous studies have shown that ERRα is a negative prognostic factor in breast cancer (Ariazi, 2002). It is implicated in tumour proliferation and migration potential and recent studies have shown that ERRα is also implicated in Her2 targeted therapy resistance in Her2+ breast cancer (Deblois, 2016). Thus, understanding how this factor is regulated is crucial to develop new therapeutic avenues for these poor outcome breast cancers.

NCOA5 is a nuclear receptor co-activator, which presents a bi-functional domain that can either repress or activate nuclear receptors such as ERα (Sauvé, 2001). In recent studies, NCOA5 has been shown to be implicated in hepato-carcinomas and in Type 2 Diabetes after chronic liver inflammation in both mice and humans (Gao, 2013). Moreover, NCOA5 is also expressed in breast tissue and previous work in the laboratory demonstrated that tamoxifen reduces its interaction with ERα (Sauvé, 2001).

Preliminary studies performed in our laboratory showed that NCOA5 is expressed at both mRNA and protein levels in the SkBr3 cell line, which is a Her2-positive and ERα-negative cell line. This fact is interesting as NCOA5 is a known ERα coactivator and ERα and ERRα share common structural features, NCOA5 may be able to interact with ERRα and regulate its activity. This project aims to explore the effect of the nuclear receptor coregulatory protein NCOA5 on ERRα activity and its downstream targets in Her2+ breast cancer models.

The main objective of this thesis is to identify what is the role of NCOA5 in breast cancer cell lines. To do so, NCOA5 gene expression was modulated, either via Sh RNA silencing or lentiviral overexpression. The phenotype of these cells was observed after the end of the selection process (5 days after infection) by measuring proliferation, some key metabolites levels, analysing gene expression by qRT-PCR and protein levels by western blot.

2. Material and Methods

Cell Culture

All cell lines were purchased from the ATCC and reauthenticated using the ATCC cell line authentication service in July 2016. The cells were grown in a controlled environment at 37˚C and 5% CO2 in DMEM media with phenol red (from Gibco) supplemented with 10% Fetal Bovine Serum (from Gibco), an antibiotic cocktail with penicillin and streptavidin (from Gibco) and sodium pyruvate (from Gibco).

Plasmids

PLKO.1 ShNTC, PLKO.1 Sh NCOA5 #2, PLX GFP, PLX NCOA5 were obtained from sigma libraries.

Infections with Lentiviruses

Viral solutions were prepared by transfecting 60% confluent HEK293 cells in 10cm dishes with a 1 ml transfection mix containing 6µg of lentiviral plasmid of interest and the plasmid mix expressing the viral capside proteins composed of 1 µg of Δ8.9 + 1 µg of VSV.g in HBS solution (NaCl 140mM, Hepes 25mM, 1,42mM Na2HPO4, CaCl2 0,125M). The transfected cells were incubated overnight after transfection, then treated with

sodium butyrate 500nM for 6h before replacing the culture media with 8 ml of fresh complete DMEM. After overnight incubation, the viral solution is ready to infect recipient cells. The media is filtered with 0.45µm syringe filters and added to the recipient cells plated at 60% confluence in 10cm dishes. This media was complemented with 2 ml of fresh complete DMEM + polybrene 5µM. The viral media was removed after 24h incubation and replaced by fresh complete DMEM. After 24h, puromycin 2,5 µM was added to start selection for 72h. Non-infected cells were treated with the same concentration of puromycin to ensure that all non-infected are removed from the samples.

Growth Curves

Proliferation was assessed with growth curves quantified with violet crystal staining. Cells were plated in 12 well plates (3 wells per condition) at 10x 10^3 cells/well density. They were harvested 12h after seeding for control and 48h, 4d, 6d and 8d after seeding for measurement. The cells were washed with PBS then fixed with 1% glutaraldehyde for 10min. the cells were rinsed twice in PBS and kept at 4˚C in PBS solution before crystal violet coloration. For the coloration, all liquid was removed from the plates with fixed cells and 1 ml of violet crystal coloration wad added in each well. The plates were incubated for 20min on a rotator at room temperature. The plates were washed gently in water until complete removal on non-absorbed violet crystal solution and left to dry overnight. To retrieve the violet solution for quantification the cells were incubated for 1h in acetic acid 1M. The absorbance was then measured in a spectraMax spectrometer instrument. The mean absorbance of the cells fixed at 12h after seeding constitutes the reference point for each group and the proliferation is determined as follows growth rate (at day x) = A (at day x) / A (at 12h).

Metabolism Assessment

Glucose, lactate and glutamine concentrations in the culture media were determined via a measurement with the NOVA bioprofile analyser 400 (from NOVA biomedical). Cells were seeded in 6 wells plates (3 wells per condition) at 100x10^3 cells/well density. The cells were washed with PBS and the culture media was replaced with 2 ml of fresh media 12h after seeding. After 48h, the media was collected and analysed in the NOVA instrument. The cells were trypsinized and counted to normalise results.

Protein Extraction

Proteins were extracted from whole cells lysates. The cells were washed twice with cold PBS, before being harvested (in 1.5 ml Eppendorf tubes) in buffer K (20µM of phosphate buffer pH7, NaCl 0.15M, NP40 0.1%, EDTA 5mM) supplemented with proteases inhibitors (Complete mini from ROCHE) and phosphatases inhibitors (phosphostop from ROCHE). The cells were incubated on a rotator for 1h at 4˚C and centrifuged 15min at 4˚C at 12,500 rpm. The supernatants containing proteins were then transferred to new tubes and kept frozen at -80˚C prior to western blotting. Protein concentrations were determined using a Bradford assay (Protein Assay Dye from Biorad) and absorbance of samples was read at 595 nm in a spectrometer (from SpectraMax).

Western Blot Analysis

The protein samples were separated on an 8% SDS-PAGE gel for 2h at 80V, then transferred onto PVDF membranes (Amersham Biosciences) in a semi-dry fast transfer system (from Biorad). The membranes were blocked for 1h at room temperature in TBS-T (Tris-Buffered Saline and 0,1% Tween) with 5% skimmed milk. They were incubated overnight at 4˚C with either anti-NCOA5 (1:1000), anti-ERRa (1:2000), or 1h at room temperature in anti-tubulin (1:10000), anti-Her2 (1:2000). The membranes were then washed 3 times (10min/wash) in TBS-T before a secondary staining with anti-rabbit IgG (1:3000) or anti-mouse (1:3000) diluted in TBS-Tween with 5% skimmed milk for 1h at room temperature. The membranes were then washed 5 times (10min/wash) in TBS-T before detection treatment with ECL-Prime (GE Healthcare) and development. All the primary antibodies were diluted in a preservative solution composed of PBS-Tween 0,2% + 3% BSA + 0,02% sodium azide. (See Annexe I for antibodies brands and lot numbers).

RNA extraction and Retro-transcription

For mRNA relative quantification, the treated cells were collected after the 3 days' selection. The cells were washed twice in PBS before RNA extraction with a kit (RNA easy from Qiagen) according to the manufacturer instructions. Total RNA samples were retrieved in 50 µl of non-pyrogenic sterile water. The RNA concentration was then measured using a Nanodrop instrument and 1 µg of total RNA was used to prepare cDNA samples using the retro-transcription kit (Protoscript II from NEB) according to the

manufacturer's instructions. The obtained samples were then diluted 1:10 in non-pyrogenic sterile water and kept frozen at -20˚C prior to qRT-PCR analysis.

QPCR Analysis

Quantitative Real-Time PCR was performed using a Roche 480 Light Cycler instrument with 96-wells plates and a SyBr Green (from Roche) mix (2 µl of DNA samples) using the following cycle: pre-amplification for 5min at 95˚C, 45 cycles of amplification (10sec at 95˚C, 10sec at 62˚C and 10sec at 72˚C), meting curve starting 55˚C to 97˚C at a rate of 0.11˚C/s and cooling for 30sec at 40˚C. (See annexe II from the list of primers used).

Chromatin Immuno-Precipitation

Cells were grown in one 15cm dishes per antibody tested in the experiment. One day prior fixing the cells, the magnetic beads were prepared. 60 µl of Dynabeads A (from Invitrogen) were washed three times in 1 ml of ice-cold ChIP dilution buffer (Triton 1%, Tris (pH8) 10mM, NaCl 150mM, EDTA 2mM). Beads were then re-suspended in 0.5 ml of ChIP dilution buffer + 2 µg of antibody and rotated overnight at 4˚C. The next day, cells were fixed at 90% confluence in 1% PFA for 10min. Fixation was stopped with ice-cold PBS washing. Cells were washed twice with PBS before being scrapped and transferred to 15ml falcon tubes. After a 10min centrifugation at 4˚C and 1,400 rpm, pelleted cells were re-suspended in 1 ml of Buffer A (Triton 0.25%, Tris (pH8) 10mM, EDTA 10mM, EGTA 0.5mM with protease inhibitors (miniComplete from Roche) and phosphatases inhibitors (Phosphostop from Roche)). Cells were incubated on ice for 5min, before a 10min centrifugation at 4˚C and 1,400 rpm, pelleted cells were re-suspended in 1 ml of Buffer B (NaCl 200mM, Tris (pH8) 10mM, EDTA 1mM, EGTA 0.5mM with protease inhibitors (miniComplete from Roche) and phosphatases inhibitors (Phosphostop from Roche)). Cells were incubated on ice for 30min, before a 10min centrifugation at 4˚C and 1,400 rpm, pelleted cells were re-suspended in 0,5 ml of Sonication Buffer (SDS 0.5%, Triton 0.5%, Tris (pH8) 10mM, NaCl 140mM, EDTA 1mM, EGTA 0.5mM with protease inhibitors (miniComplete from Roche) and phosphatases inhibitors (Phosphostop from Roche)). Cells were then manually sonicated until chromatin was shredded into fragments of 200 bp to 1000 kbp of length. chromatin samples were then transferred to Eppendorfs tubes and centrifuged for 15min at 4˚C and 12,500 rpm. Meanwhile, the

beads now conjugated with antibodies, were decanted on magnet and re-suspended in 0.5 ml of fresh ChIP dilution buffer. 20 µl of chromatin samples were kept independently for input and the rest of the chromatin samples were then added to the beads and rotated overnight at 4˚C. Beads were washed 2min in buffers W1 (NP40 0.5%, KCl 150mM, Tris (pH8) 10mM, EDTA 1mM), W2 (Triton 0.5%, NaCl 100mM, Tris (pH8) 10mM), W3A (Triton 0.5%, NaCl 400mM, Tris (pH8) 10mM), W3B (Triton 0.5%, NaCl 500mM, Tris (pH8) 10mM), W4 twice (NP40 0.5%, LiCl 250mM, Tris (pH8) 10mM, EDTA 1mM), TE (Tris (pH8) 10mM, EDTA 1mM) in sequential order. Beads were then transferred to new tubes, re-suspended in 120 µl of buffer E (SDS 1%, Tris (pH8) 50mM, EDTA 10mM) and incubated overnight at 65˚C to decrosslinking proteins and DNA, from this point the input samples get the same treatments as the regular samples. Samples were treated with RNAase A for 1h at 37˚C, then with proteinase K for 1h at 55˚C. DNA was then extracted with a QiaQuick DNA extraction kit from Qiagen upon manufacturer's instructions. DNA samples were then re-suspended in Tris (pH8) 4mM and stored frozen at -20˚C prior to qPCR analysis. (See annexe III for the list of primers used).

ChIP-Sequencing

For ChIP sequencing, the obtained DNA samples were sent to Genome Quebec that created the DNA libraries, sequenced the samples and pre-analysed the data.

3. Results

NCOA5 protein level modulates ERRα transcription and protein level

I used sh-RNA-mediated knockdown to investigate NCOA5 function *in vitro* in SkBr3 cells. Cells treated with sh-RNA against NCOA5 were compared to cells treated with non-targeted sh-RNA. After treatment, qRT-PCR on mRNA samples showed a 50% decrease in *NCOA5* mRNA level compared to control cells. Interestingly, *ESRRA* mRNA levels were also decreased by 15% (Figure 3, A). Western blot analysis showed a decrease in NCOA5 protein level in treated cells relatively to control condition; ERRα protein level is also decreased (Figure 3, B). To confirm these results, I used a lentiviral overexpression system to increase NCOA5 protein level in SkBr3 cells. In this system, overexpression of

NCOA5 induces higher *ESRRA* mRNA levels and higher ERRα protein levels (Figure 3, C and D). These data suggest that NCOA5 can regulate ERRα transcription and protein levels in SkBr3 cells. Interestingly, ChIP-qPCR experiments showed that NCOA5 could bind to *ESRRA* promoter (Figure 3, E) suggesting a direct regulation of *ESRRA* transcription by NCOA5.

NCOA5 binds to ERRα targets genes on the ERBB2 amplicon

NCOA5 is a nuclear receptor co-activator and previous experiments showed a strong link between NCOA5 and ERRα. Previous work from our laboratory identified several genes of the *ERBB2* amplicon as ERRα targets and ChIP-in-Chip analysis enabled us to map ERRα precise binding sites on the amplicon. I used lentiviral overexpression of NCOA5 and performed ChIP-qPCR on known ERRα binding sites on the amplicon and I found that NCOA5 binds alongside ERRα on some important genes of the *ERBB2* amplicon, such as *ERBB2* itself, *CCR7*, *GRB*7, *NR1D1*, or *PPP1R1B* (Figure 4). Genes not bound by ERRα are also not bound by NCOA5.

NCOA5 protein level modulates ERBB2 transcription and protein level and its amplicon genes transcription

As NCOA5 can bind to ERRα binding sites on the *ERBB2* amplicon, I investigated its capacity to regulate these genes transcription using the same sh-RNA mediated knockdown and lentiviral mediated overexpression systems as previously. It appeared that a 3 days NCOA5 protein knockdown leads to decreased mRNA levels for the amplicon genes bound by NCOA5 (*ERBB2*, *CCR7*, *GRB7*, *NR1D1* and *PPP1R1B*) (Figure 5, A), NCOA5 knockdown also leads to a modest decrease in ErbB2 protein level (Figure 5, B). Conversely NCOA5 over-expression induces a robust increase in these genes transcripts levels, 2 folds for *GRB7* and *CCR7* and 6 folds for *ERBB2* and more than 10 folds for *PPP1R1B* (Figure 5, C); and a strong increase in ErbB2 protein level compared to control cells (Figure 5, D).

These data suggest that NCOA5 regulates the transcription of ERRα targets genes in the *ERBB2* amplicon either directly via its binding along ERRα on the amplicon or indirectly through regulation of ERRα protein level.

NCOA5 protein level modulate SkBr3 cells proliferation

Modulation of NCOA5 protein levels *in vitro* affects ERBB2 mRNA and protein level and the transcription of the amplicon genes, most of which being important oncogenic drivers in Her2-positive breast cancer cells; such modifications impact cell proliferation. To assess proliferation of SkBr3 cells after NCOA5 knockdown or over-expression, I performed growth curves for 8 days and quantified cell proliferation with violet crystal coloration. NCOA5 knockdown leads to decreased proliferation rates compared to control cells (Figure 6, A) whereas NCOA5 overexpression enhances proliferation (Figure 6, B), suggesting that NCOA5 is an important cell proliferation regulator in SkBr3 cells.

NCOA5 protein level affects SkBr3 cells metabolism

NCOA5 protein level has a strong impact on SkBr3 proliferation and data suggests part of its function operates through regulation of the metabolic regulator ERRα. Cell culture media was analysed with a NOVA instrument to measure glucose consumption, lactate production and glutamine consumption after NCOA5 knockdown or overexpression relatively to control cells and normalised for 100,000 cells. NCOA5 modulation is critical for glucose consumption and lactate production. NCOA5 knockdown leads to a 25% increase in glucose consumption and 30% increase in lactate production (Figure 7, A). The effect of NCOA5 overexpression is even more striking, it leads to a sharp decrease in glucose consumption and lactate production, of 40% and 50 % respectively (Figure 7, B). These findings suggest that NCOA5 can impact SkBr3 cell metabolism regulation, either through ERRα regulation or through other nuclear receptors.

ERRα binds to NCOA5 promoter in SkBr3 cells

Analysis of ERRα ChIP-seq data in different breast cancer cell lines from our laboratory (unpublished data) revealed that ERRα binds to NCOA5 promoter in SkBr3 cell line, but not in MCF7, BT474 or MDA-MB-231 cell lines (Figure 8, A). Interestingly, SkBr3 cells are Her2-positive ERα–negative and have high ERRα level. According to the ChIP-Seq data, I designed primers for ERRα binding site on NCOA5 promoter and I could validate that ERRα binds to this region with regular ChIP-qPCR (Figure 8, B). This data suggests

that NCOA5 is a transcriptional target of ERRα. Western Blot analysis of SkBr3 whole cells protein lysates showed that pharmaceutical inhibition of ERRα with the inverse agonist C29 during 24h is sufficient to decrease NCOA5 protein level (Figure 8, C). Altogether these data suggest that ERRα can, regulate NCOA5 protein level, unravelling a potential control feedback loop between the two proteins.

Summary

Figure 9 is a graphical summary of NCOA5 mechanism of action in SkBr3 cells. It is still unknown if NCOA5 and ERRα interact together, however this work showed that NCOA5 protein level can be modulated probably via transcriptional activation by ERRα and that NCOA5 can modulate ERRα mRNA and protein levels. ChIP experiments showed that NCOA5 can bind the *ERBB2* amplicon on the same sites as ERRα. Furthermore, NCOA5 modulates transcription of its targets of the *ERBB2* amplicon and induce proliferation and metabolic changes.

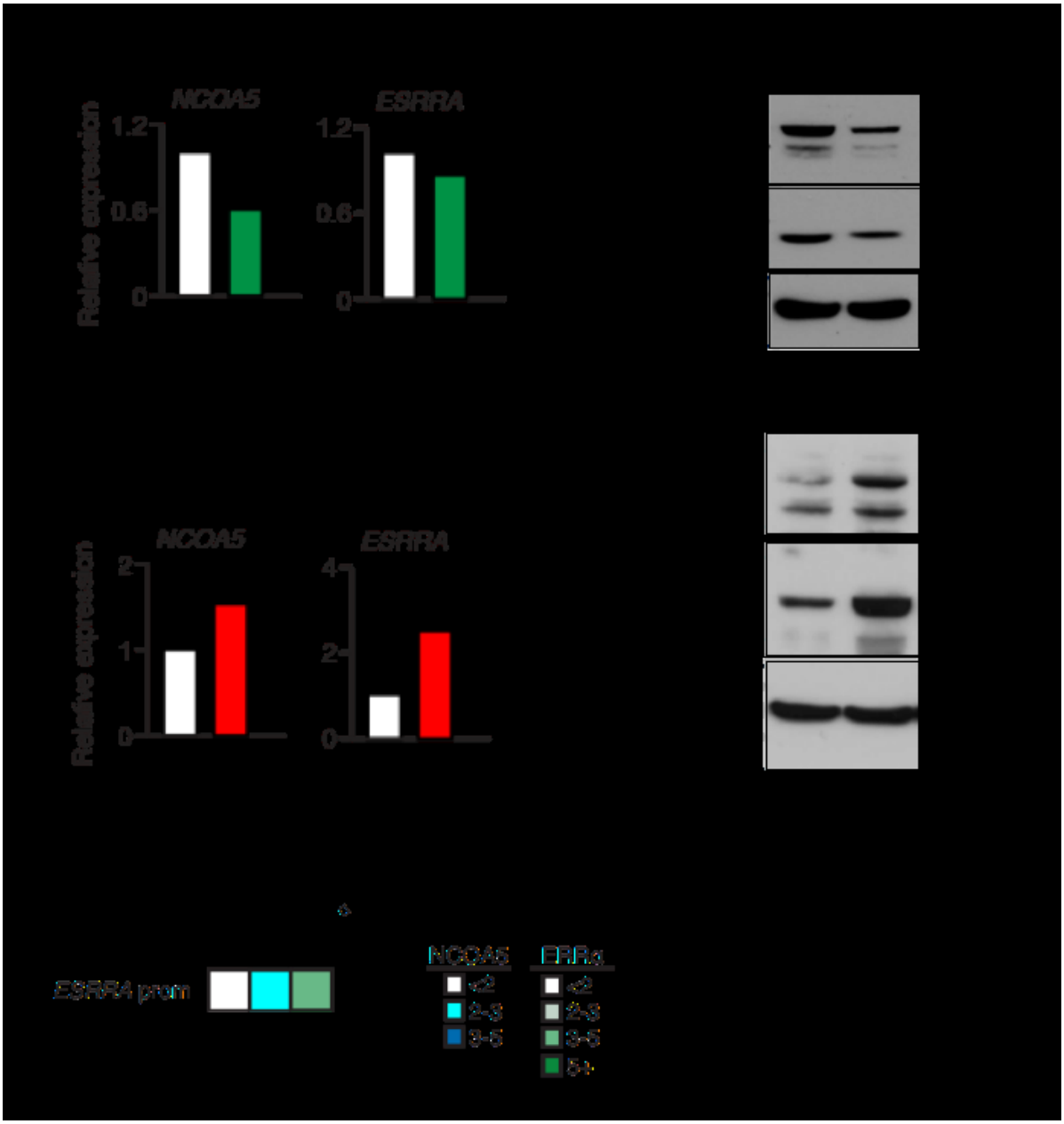

Figure 3: NCOA5 protein level modulates ERRα transcription and protein level

(A) qRT-PCR Analysis of NCOA5 and ERRα mRNA levels after 3 days of Sh-RNA mediated NCOA5 knockdown. (* p<0,5 , ** p<0,05 , ***p<0,005)

(B) Western Blot Analysis of NCOA5 and ERRα after 3 days of Sh-RNA mediated NCOA5 knockdown with tubulin as a loading control.

(C) qRT-PCR Analysis of NCOA5 and ERRα mRNA levels after 3 days of of lentiviral-mediated over-expression of NCOA5. (* p<0,5 , ** p<0,05 , ***p<0,005)

(D) Western Blot Analysis of NCOA5 and ERRα after 3 days of lentiviral-mediated over-expression of NCOA5 with tubulin as a loading control.

(E) qChIP of NCOA5 and ERRα on *ESRRA* promoter

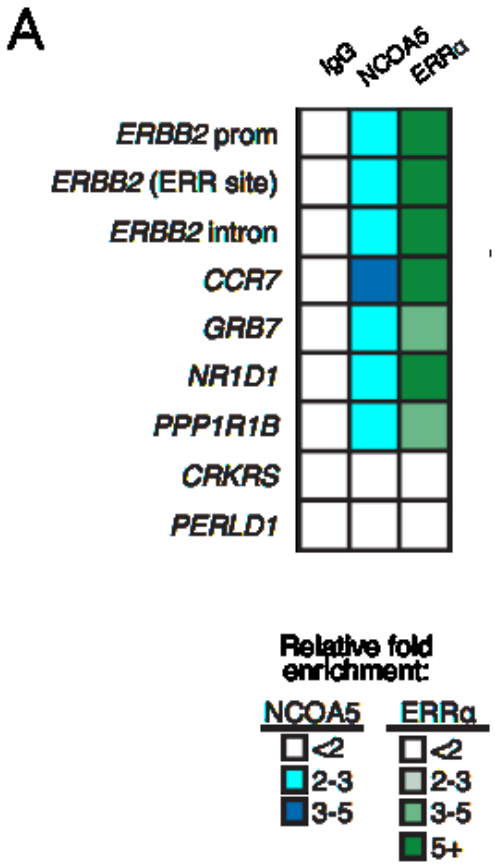

Figure 4: NCOA5 binds to ERRα targets on the *ERBB2* amplicon

(A) ChIP-qPCR analysis of NCOA5 and ERRα binding on genes from the *ERBB2* amplicon.

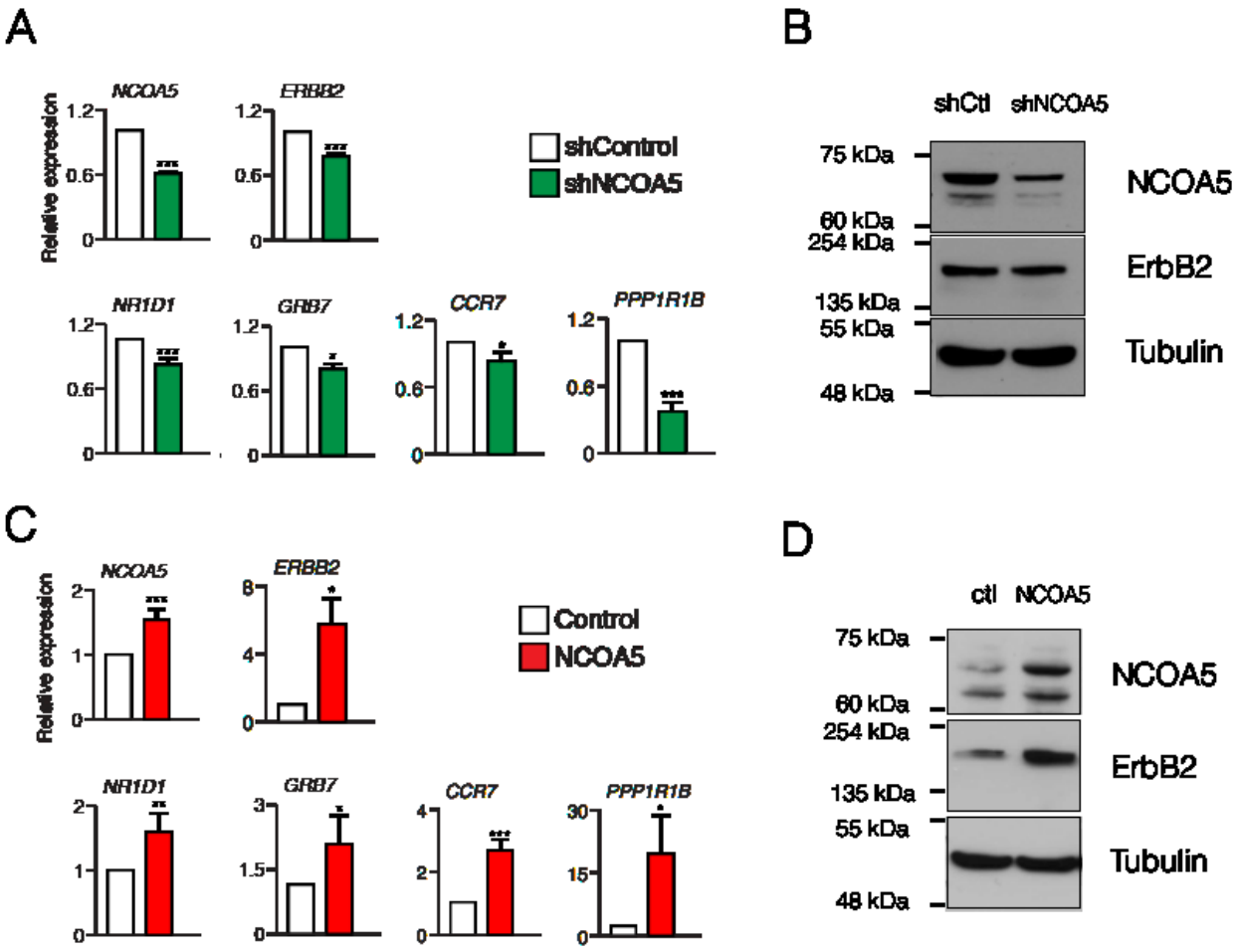

Figure 5: NCOA5 protein level modulates ERBB2 transcription and protein level and its amplicon genes transcription

(A) qRT-PCR Analysis of NCOA5 and ErbB2 and genes from the ErbB2 amplicon mRNA levels after 3 days of Sh-RNA mediated NCOA5 knockdown. (* p<0,5, ** p<0,05, ***p<0,005)

(B) Western Blot Analysis of NCOA5 and ErbB2 after 3 days of Sh-RNA mediated NCOA5 knockdown with tubulin as a loading control. (NCOA5 and tubulin immunoblot are the same as in figure 4, they are re-shown as controls)

(C) qRT-PCR Analysis of NCOA5, ErbB2 and genes from the ErbB2 amplicon mRNA levels after 3 days of of lentiviral-mediated over-expression of NCOA5. (* p<0,5 , ** p<0,05, ***p<0,005)

(D) Western Blot Analysis of NCOA5 and ErbB2 after 3 days of lentiviral-mediated over-expression of NCOA5 with tubulin as a loading control. (NCOA5 and tubulin immunoblot are the same as in figure 4, they are re-shown as controls)

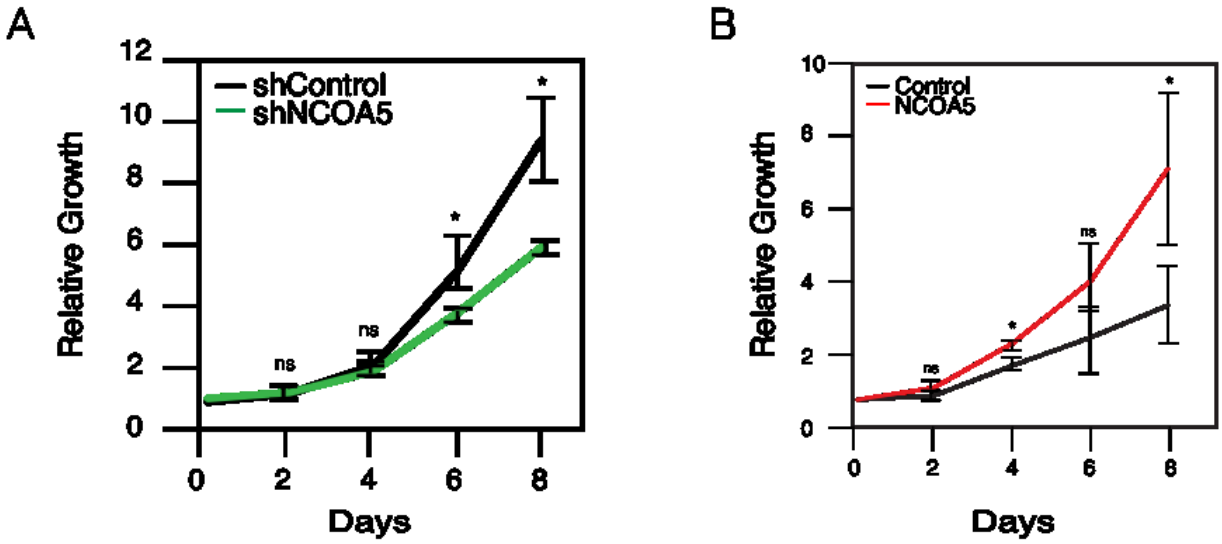

Figure 6: NCOA5 protein level modulates SkBr3 cells proliferation

(A) Growth Curve of SkBr3 cells with Sh-RNA mediated NCOA5 knockdown compared to control cells. (* p<0,5)

(B) Growth Curve of SkBr3 cells over-expressing NCOA5 via lentiviral infection compared to control cells. (* p<0,5)

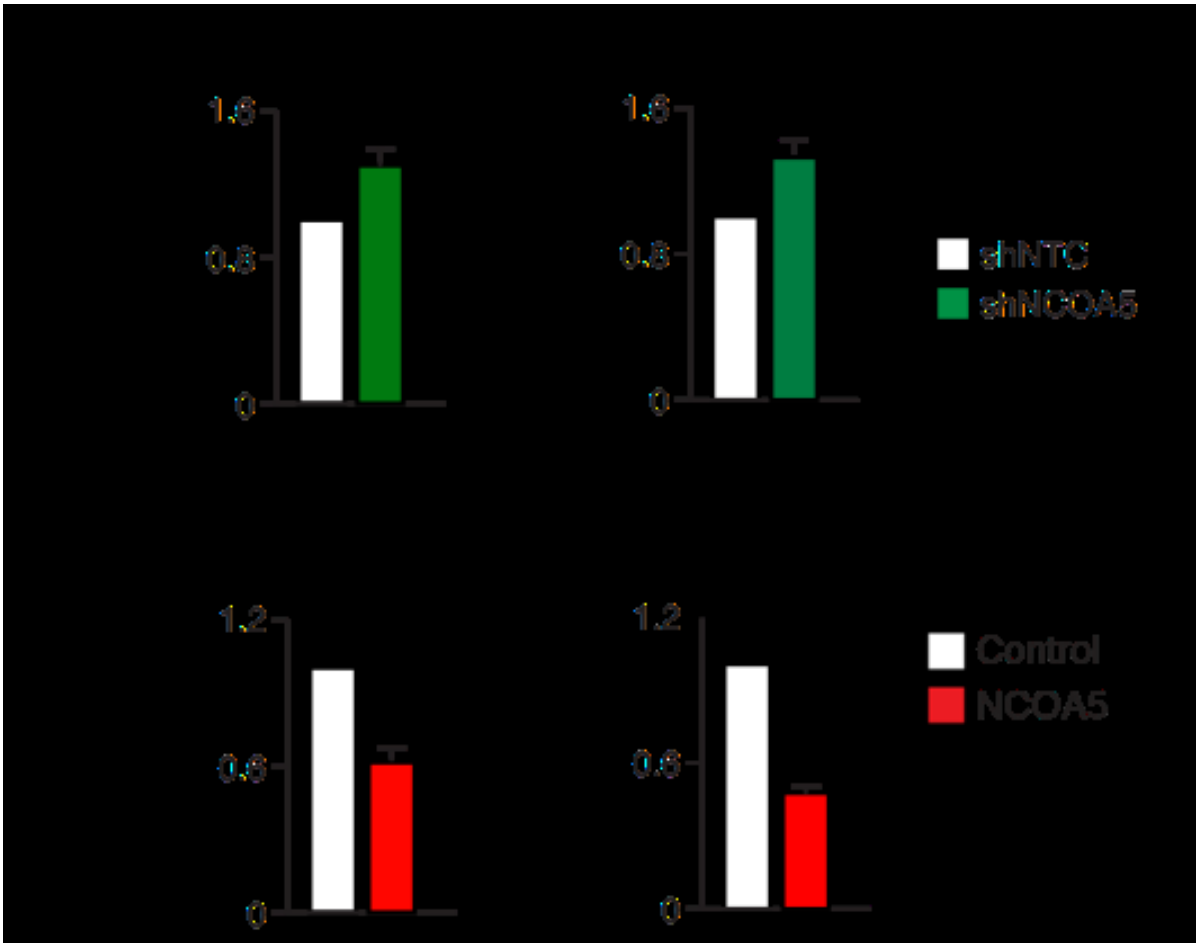

Figure 7: NCOA5 protein level affects SkBr3 cells metabolism

(A) Relative glucose consumption, lactate production in SkBr3 cells with Sh-RNA mediated NCOA5 knockdown compared to control cells. (* p<0,5)

(B) Relative glucose consumption, lactate production in SkBr3 cells over-expressing NCOA5 via lentiviral infection compared to control cells. (* p<0,5)

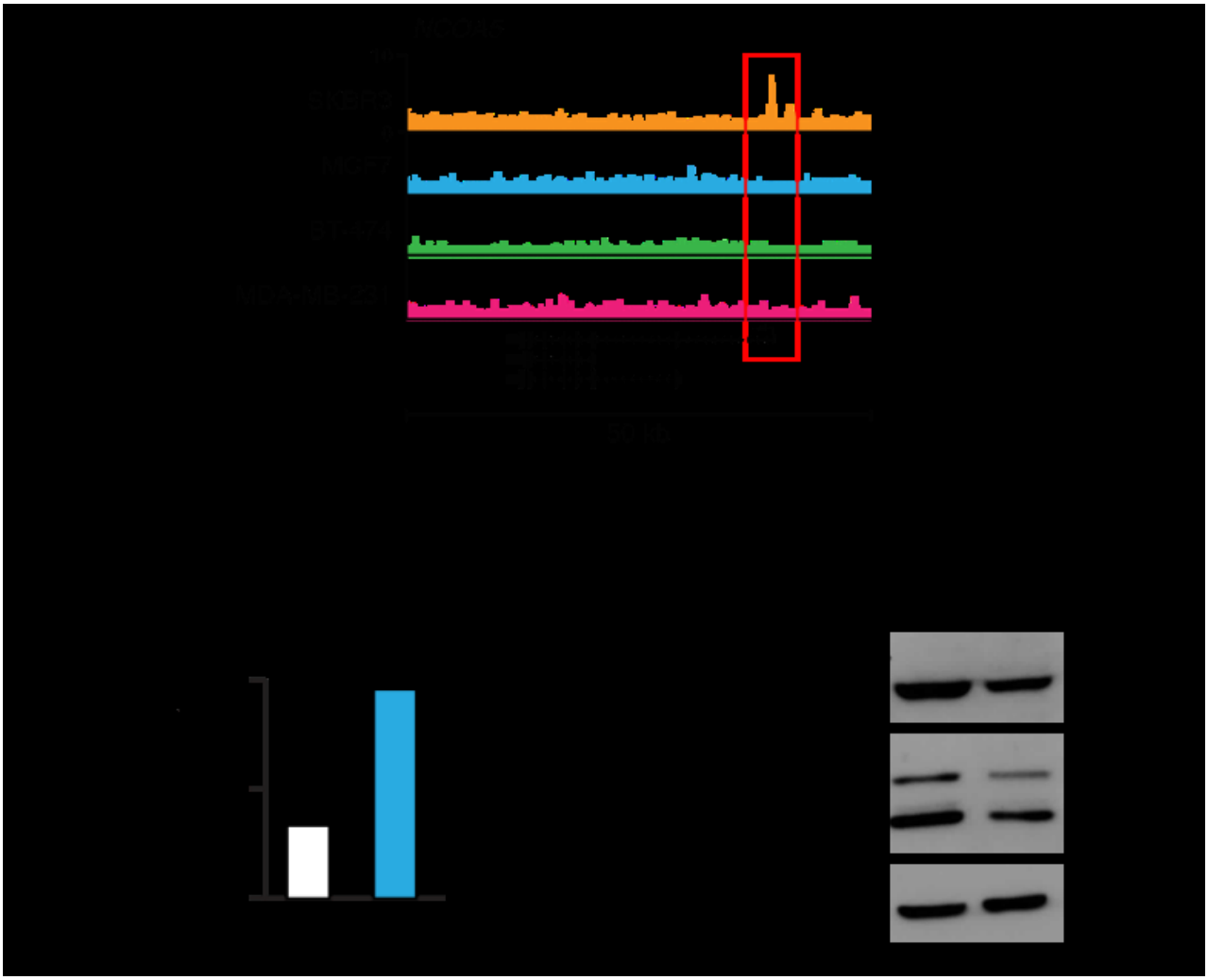

Figure 8: ERRα binds to NCOA5 promoter in SkBr3 cell line

(A) ERRα ChIP-seq data on NCOA5 promoter in diverse breast cancer cell lines.

(B) ChIP-qPCR of ERRα on NCOA5 promoter. (* p<0,5)

(C) Western Blot of ERRα and NCOA5 in SkBr3 cells after 24h treatment with the ERRα inverse agonist C29.

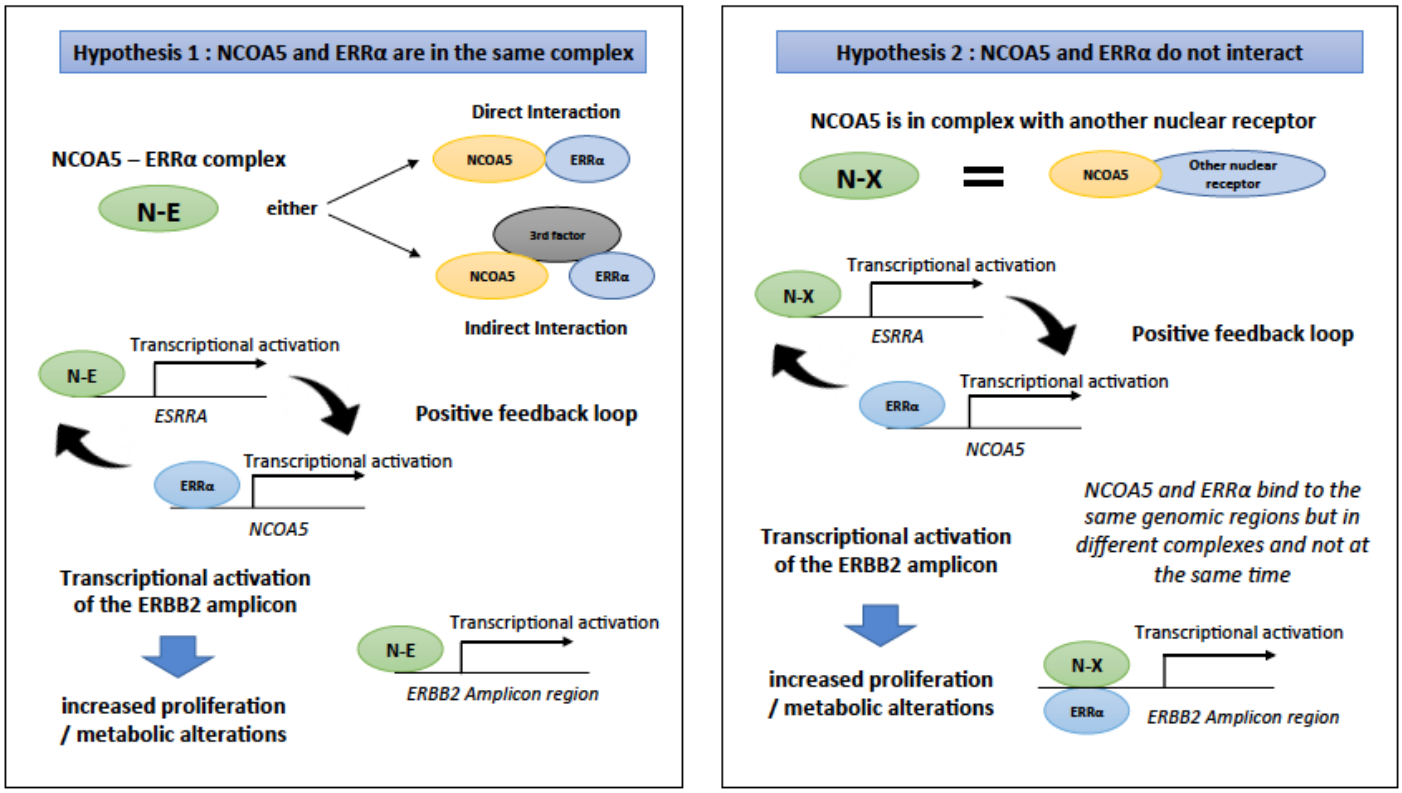

Figure 9 : Schematic summary of NCOA5 action in SkBr3 Cells

4. Discussion

As of today, there are a very limited number of studies on NCOA5 functions. NCOA5 is known to be a conserved protein in stem cells from planarians to mammals; it is an important factor in Type 2 diabetes and HCC development, and it has been involved in macrophage cholesterol efflux. In an *in-silico* study, NCOA5 was linked to luminal breast cancer progression, however no functional studies were pursued.

In this study, it became evident that NCOA5 and ERRα are involved in a feedback regulatory loop where NCOA5 is a target of ERRα and vice versa. However, the exact role of ERRα in NCOA5 transcriptional regulation is still unknown and more characterization of this mechanism is required. Luciferase reporter assays with NCOA5 promoter in presence or absence of ERRα and monitoring NCOA5 mRNA and protein levels upon silencing of ERRα either via si-RNA or pharmaceutical inhibition should tell us if this regulation is direct or not.

NCOA5 and ERRα are both implicated in the transcriptional regulation of the ErbB2 amplicon, however it is still not clear if NCOA5 effect is direct through ERRα binding and modulation of its activity or it is indirect via regulation of ERRα level. An elegant way to address this problematic would be to set up rescue experiments by monitoring amplicon genes' transcription after Sh-RNA mediated knockdown of NCOA5 followed by re-expression of ERRα or lentiviral overexpression of NCOA5 followed by silencing of ERRα, and add ChIP-on-ChIP experiments to find out if NCOA5 and ERRα bind together on DNA.

It is also important to discover all the cellular processes regulated by NCOA5. A microarray analysis of cells with NCOA5 knockdown compared to controls and/or cells overexpressing NCOA5 compared to controls should give us a good overview of transcriptional pathways regulated by NCOA5 and potentially give us information about which nuclear receptors are partners of NCOA5, aside ERRα in this context.

This study unravelled a role for NCOA5 in metabolism regulation, as it is important for glucose uptake and lactate production. Metabolomics studies combined with the microarray data would help to determine which metabolic pathways are regulated by NCOA5 and how it affects the balance between OXPHOS and glycolysis. Furthermore, ERRα is now known to be a master regulator of mitochondrial function, respiratory assays in the presence or absence of NCOA5 will be important to determine if NCOA5 affects cellular respiration.

Although encouraging, the results of this study were obtained *in vitro* in only one model of Her2-positive ERα–negative cell line. It is important to confirm these results in other cell lines with similar characteristics, such as HCC 1569 (ER/PR negative, Her2 positive, high potential for xenografts). Creating stable cell lines having a permanent deletion of NCOA5 using the CRISPR-Cas9 system would be of great interest to perform more studies on this co-activator. It would enable us to investigate the role of NCOA5 *in vivo* with *NCOA5*$^{-/-}$ xenografted tumours in mice.

Previous studies from our laboratory demonstrated that ERRα protein level go down upon Her2 targeted therapies such as Lapatinib treatment, but this level goes up again when resistance occur and that this resistance mechanism can be overcome by inhibiting ERRα. Verifying if NCOA5 is required for this reactivation of ERRα and if NCOA5 inhibition can overcome resistance to Her2 targeted therapies such as Lapatinib would also be interesting.

This work uncovered a previously unsuspected role for NCOA5 in Her2-positive breast cancer cells. It is now clear that there is a link between ERRα and NCOA5, however a lot of questions remain unanswered. Indeed, there is no evidence of physical interaction between ERRα and NCOA5. A validated antibody for mice NCOA5 is available for IHC but there is a lack of validated tools for human NCOA5 and for immunoprecipitation in general, thus traditional co-immunoprecipitation has failed, but it might be interesting to go further and use an immunoprecipitation followed by mass spectrometry analysis (such as the RIME method (Mohammed, 2016)) or BioID labelling (Kim, 2016) to identify protein partners of NCOA5.

Conclusion

This study unveiled a new role for NCOA5 in Her2-positive breast cancer. NCOA5 is linked to ERRα and regulates its expression at both mRNA and protein level in SkBr3 cells. It is an important nuclear receptor co-activator that can modulate SkBr3 cell proliferation and metabolism *in vitro,* making it interesting as a potential therapeutic target and/or biomarker. More *in vitro* and *in vivo* studies with genetically engineered mice will be required to fully characterize its actions. Discovering the precise mechanism of action of NCOA5 and all its targeted pathways will lead to a better understanding of how the Her2-positive breast cancer cells regulate their metabolism and sustain their proliferation.

NCOA5 is a nuclear receptor co-activator expressed in nearly all tissues, according to the protein atlas project. It is very likely that it has many other biological functions and it would be interesting to investigate its roles in other organs where it is highly expressed.

NCOA5 protein is highly expressed in the brain and the heart, organs with strong energy demands and tight metabolism control. NCOA5 was already shown to be involved in diverse metabolism regulation processes in the liver (Gao, 2013) or in macrophages (Gillespie, 2015) so it is likely that it can regulate metabolism in other tissues.

NCOA5 was shown to interact with the famous hormone receptor ERα (Sauvé, 2001; Gao 2013) and it is also present in diverse hormone producing organs such as the thyroid gland, the adrenal glands or the pancreas, possibly indicating a broad role in the regulation of hormone nuclear receptors.

Ncoa5 -/+ mice were generated but the males were sterile (Gao, 2013) indicating that a functional NCAO5 is required to generate functional spermatozoids. Furthermore, NCOA5 is also found at varied levels in all male and female reproductive organs potentially indicating a role for NCOA5 in reproduction.

Stem cell fate and divisions are very tightly controlled by a variety of factors, including nuclear receptors. ERRα was shown to be involved in the metabolic adaption required to transform somatic cells into pluripotent stem cells (Kida, 2015) and NCOA5 was also shown to be a conserved protein in stem cells from planarians to mice (Boser, 2013), it is then possible that an NCOA5 – ERRα axis plays a role in iPSC reprogramming or in stem cells metabolism regulation.

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Annexes

1. Antibodies

Antibody	Brand	Reference	Lot number
Anti-NCOA5 (rb)	NOVUS	NB-100-2771	A1
Anti-ERR (rb)	ABCAM	AB 76228	GR150069-2
Anti-ErbB2 (mouse)	Santa Cruz	SC-284	K0711
Anti-Tubulin (mouse)	Cedarlane	CLT 9002	-
Anti-Rabbit linked to HRP (Donkey)	GE Healthcare	NA-934	-
Anti-Mouse linked to HRP (sheep)	GE Healthcare	NX-A931	-
Rabbit IgG	Invitrogen	-	-

2. qRT-PCR Primers

ERRa F	GATGAATCTGCAGGGAGAGGAG
ERRa R	GTTACTCATGTGCCTGATGTGG
NCOA5 F	TCTCTGCCTGGCCCGATTTCCCCG
NCOA5 R	CTGGCTGTTTGCTGCTGTGGA
ERBB2 F	GAAGTACACGATGCGGAGACTG
ERBB2 R	CAGATGCCCTTGTAGACTGTGC
c17orf37 F	GCACAGGTGCCTTTGAGATAGAGA
c17orf37 R	GATGGCCTCAATGAGATCTTTCTC
CCR7 F	GCGTCATGGACCTGGGGAAACCAA
CCR7 R	TCCACTGTGGTGTTGTCTCCGATG
CRKRS F	GATCAGCCGACTTTGTGGTAGC
CRKRS R	TCAGCATGTGGTCCAATAAATC
GRB7 F	CCATCCACCGCACCCAACTCTG
GRB7 R	CGCTCGGCAGGATGAGATAATG
NR1D1 F	CATAACGAGGCCCTAAATGGTC
NR1D1 R	CATGCCAGCAGAACATTCTTTG
PerlD1 F	CCGCTTCGACTATGGCTACAAC
PerlD1 R	CAGAAAGCTGAAAAAGAGGACG
PPP1R1B F	TCCTCCTCCTCTCGCCGCACAG
PPP1R1B R	CTCTCCTGAGGCTCTCTGGTGG

3. qChIP primers

CTRL Neg c17 1 F	TCACACAGTTCCACCCGCTCCAGAAA
CTRL Neg c17 1 R	GGTGTGGTATTGACAAGCCCTCAGGT
CTRL Neg c17 2 F	ACAAAGGGAATAAGCAATCTGGCATA
CTRL Neg c17 2 R	TCGGCTCCAACTCAACTCGTCTATGT
NCOA5 F	GAGTGGAGATGGGGGCAGA
NCOA5 R	ATTGCCAAGGAGATCGACGC
ERRa promoter	CAGACGACAGGCCCAGTGAAT
ERRa promoter	CGGGCAAGGCATAGAGCTT
ERBB2 common site F	GGCACTTTAATCTCCCCTCACTGGCA
ERBB2 common site R	ACTTGGACCCCAGCCTGGTATCCCTG
ERBB2 promF	GCGAAGAGAGGGAGAAAGTG
ERBB2 prom R	GGGGAATCTCAGCTTCACAA
ERBB2 intronic F	CTCCGCCACTGCTACGTTTTGACCTC
ERBB2 intronic R	GAGTAAATGAAGCCATGAAGTCCAGC
CRKRS F	TGTCACGGAGCCTGGGAGTGTTTGTT
CRKRS R	TGCTTCGCTTTCACCTCATTCCCCTG
CCR7 F	CACAGGAACAAGAGGACTTCAGGGCA
CCR7 R	AAATAAACATCTCCGCACAGGCTTGG
GRB7F	TCTGGCCTGGAGGGTTGTCCTGAGGT
GRB7 R	CAGCCTGGAGTTAGCCAAGGATGGGG
NR1D1 F	AGAGGTGGGGAGTGAGTTCTCAGTGC
NR1D1 R	CTGCGTAAGGAGGGAGATTGAGTTAA
Perld1 F	TCCATAGTAGGTGCCCATCAATGTCA
Perld1 R	CTATAGCTGAGGTGTCTCCGTTCTGG
PPP1R1B F	CCCCAGGACAGGAAAATGAAGGGTCA
PPP1R1B R	GGTGGGAGGGAGAGGGATTTCTTAAA