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Le Récepteur Nucléaire de l'acide rétinoïque alpha (RARα): nouveaux effets non-génomiques et nouveaux partenaires

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Nuclear Retinoic Acid Receptor alpha (RARα): novel unconventional non-genomic effects and novel partners

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Nuclear Retinoic Acid Receptor alpha (RARα): novel unconventional non-genomic effects and novel partners

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RESUME

L'acide rétinoïque (AR), dérivé actif de la vitamine A, régule de nombreux processus biologiques comme la prolifération et la différenciation des cellules, l'embryogénèse et l'homéostasie des tissus. L'AR agit en se fixant à des récepteurs nucléaires appelés RAR pour lesquels 3 sous-types α, β et γ ont été caractérisés et qui se comportent comme des facteurs de transcription inductibles par le ligand. Selon le modèle classique, la transcription des gènes cibles induite par l'AR, nécessite la fixation des RAR au niveau de séquences spécifiques des promoteurs et met en jeu des changements conformationnels des récepteurs qui, en initiant l'association/dissociation de toute une panoplie de corégulateurs, vont permettre le recrutement de la machinerie transcriptionnelle. Cependant, en plus de ce modèle génomique et nucléaire bien établi, l'équipe du Dr Cécile Rochette-Egly a montré récemment que l'AR a aussi des effets non-génomiques et induit rapidement la voie de signalization p38MAPK/MSK1 qui ensuite cible les RAR pour des cascades de phosphorylations.

Mon travail de thèse porte sur deux nouveaux aspects de la voie de signalization de l'AR: (i) la mise en évidence d'une sous-population de RARα dans les membranes et impliquée dans les effets non-génomiques de l'AR (Piskunov and Rochette-Egly, 2011a), (ii) l'interaction de RARα dans le noyau avec un nouveau partenaire, la profiline IIA, (Piskunov et al. manuscrit en preparation) et (iii) la mise en évidence d'un nouveau rôle de RARα dans l'adhésion cellulaire.

- J'ai mis en exergue un nouveau concept, la présence de RARα dans des microdomaines membranaires, les radeaux lipiques ou "lipid rafts". J'ai montré que l'activation de la voie de la p38MAPK par l'AR résulte de l'interaction de RARα présent dans ces microdomaines avec les proteines Gαq. Pour mettre en évidence cette interaction *in vivo* des proteines endogènes j'ai mis au point une technique nouvelle et sensible, appelée « Proximity Ligation Assay » (PLA). Le principe de cette technique est très semblable a celui du FRET, mais exploite la capacité de dimérization d'oligonucléotides couplés à des anticorps lorsque ceux-ci sont assez proches, comme dans le cas de deux anticorps dirigés contre deux proteines d'un même complexe. Cette technique est tres specifique et permet une amplification importante du signal avec une meilleure sensibilité.

L'interaction RARα/Gαq, comme l'activation de la p38MAPK, ont été corrélés à l'activation des gènes cibles des RAR. De tels résultats confirment l'hypothèse du laboratoire selon laquelle les effets non-génomiques interfèrent avec les effets génomiques et sont de ce fait indispensables. De manière interessante, j'ai aussi montré que dans des cellules de cancer mammaires surexprimant le recepteur à activité tyrosine kinase erbB-2, RARα n'interagit pas avec Gαq dans les radeaux lipidiques et, par conséquent, la voie de la p38MAPK n'est pas activée. Ces résultats mettent encore en éxerque l'importance des effets non-génomiques dans le mécanisme d'action des RAR. Finalement ce travail allonge encore la liste des récepteurs nucléaires présents dans les membranes.

- J'ai aussi identifié un nouveau partenaire de RARα, la profiline IIA, en utilisant la technique du double hybride dans la levure. La profiline IIA, comme toutes les profilines, est une proteine de petite taille (14-17 kDa) aux fonctions multiples et exprimée essentiellement dans les cellules nerveuses. J'ai montré que la profiline IIA interagit spécifiquement avec RARα (et non avec les autres RAR), et que l'interaction met en jeu le motif N-terminal riche en prolines de RARα et le domaine SH3-like de la profiline IIA. J'ai aussi déterminé l'affinité de l'interaction en utilisant la technique Biacore. *In vivo*, cette interaction a été analysée en utilisant la technique PLA et les complexes RARα/profiline IIA ont été détectés dans les noyaux. Finalement la profiline IIA s'est révélée être un régulateur transcriptionnel de RARα et est recrutée avec RARα au niveau des promoteurs des gènes cibles.

- Finalement, étant donné que RARα n'est pas impliqué dans la différenciation neuronale des cellules souches embryonnaires de souris induite par l'AR (Al Tanoury *et al*, manuscript in preparation), j'ai mis en évidence une nouvelle fonction de RARα dans le contrôle de l'adhésion et de l'étalement des cellules. Des expériences sont en cours pour déterminer si RARα contrôle avec la profiline IIA l'expression des proteines d'adhésion via des effets génomiques. Cependant, de manière inattendue, j 'ai identifié une nouvelle population de RARα dans le cytoplasme de ces cellules. D'ou l'hypothèse de nouveaux effets non-génomiques via l'interaction de RARα avec des proteines d'adhésion.

En conclusion, j'ai montré que RARα peut être exprimé dans trois compartiments subcellulaires différents, avec trois fonctions différentes.

- une population membranaire avec des effets non-génomiques (activation de kinases)

- une population nucléaire majoritaire impliquée dans des effets génomiques. Dans ce contexte j'ai identifié un nouveau partenaire transcriptionnel de RARα, la profiline IIA

- une population cytosolique qui serait impliquée dans l'adhésion des cellules

SUMMARY

Retinoic Acid (RA) is the active metabolite of Vitamin A, which modulates a wide variety of biological processes such as cell proliferation and differentiation, embryogenesis and homeostasis. These effects are mediated by nuclear receptors (RARα, β and γ), which act as ligand-dependent regulators of transcription. According to the classical model, RAR-mediated transcription of cognate target genes involves the binding of the receptors to specific DNA sequences located in promoters and RA-induced conformational changes that initiate cascades of protein-protein associations/dissociations leading to communication with the transcriptional machinery. However, in addition to this well-established nuclear genomic function, recent studies from the laboratory of Dr. Cécile Rochette-Egly demonstrated that RA also has non-genomic effects and rapidly induces the p38MAPK/MSK1 pathway, which then targets RARs for phosphorylation cascades.

The work of my thesis focused on three novel and original aspects of RA and RARα signaling: (i) the characterization of a RARα pool located in membrane lipid rafts and involved in non-genomic effects (Piskunov and Rochette-Egly, 2011a), (ii) the interaction of nuclear RARα with a new partner profilin IIA (Piskunov *et al*, manuscript in preparation), and finally (iii) a novel role of RARα in cell adhesion.

- I highlighted a novel paradigm, in which a fraction of the cellular RARα pool is present in membrane lipid rafts, where it interacts with G protein alpha Q in response to RA. To explore the endogenous RARα/Gαq complexes *in vivo* and *in situ*, I set up a new technique called proximity ligation assay (PLA). The assay is similar to Fluorescence Resonance Energy Transfer (FRET), but is based on the use of two primary antibodies raised in different species that recognize the antigens of interest and on species-specific secondary antibodies attached to unique DNA strand that can hybridize when in close proximity and then be amplified. The technique is very sensitive and specific.

This interaction is the signal for the activation of p38MAPK and of the downstream kinase MSK1. Both the RARα-Gαq interaction and the p38MAPK pathway have been correlated to the activation of RA-target genes, highlighting its physiological relevance. It also corroborates the hypothesis raised by the team according to which the non-genomic effects crosstalk with the genomic ones. Remarkably, in RA-resistant breast cancer cells characterized by the overexpression of the receptor tyrosine kinase erbB-2, RARα associated to the membrane lipid rafts does not interact with Gαq and p38MAPK is not activated, outlining again the essential contribution of this non-genomic mechanism in RA signaling. Finally this work extends the long list of nuclear receptors already shown to be present in diverse membrane structures.

- I also identified a new binding partner of RARα, profilin IIA. Profilin IIA, a small (14-17 kDa) protein with multiple functions essentially in neuronal cells. However, I found that profilin IIA is also present in mouse embryonic fibroblasts (MEF cell line) and in human breast cancer cells (MCF7 cell

line). I demonstrated that profilin IIA interacts specifically with the RARα subtype (and not with the other RARs) and that the interaction involves the N-terminal proline-rich motif of RARα and the SH3-like domain of profilin IIA. I also analyzed the affinity of the interaction by using the Biacore technology. The interaction of the endogenous proteins has been analyzed by using the PLA technique and found to occur in nuclei. Remarkably, I found that profilin IIA modulates positively the expression of RA-target genes and is recruited with RARα to target genes promoters.

- Finally, in an attempt to decipher the relevance of the RARα interaction with profilin IIA, the laboratory found that RARα is not involved in RA-induced differentiation of ES cells into neurons (Al Tanoury *et al*, manuscript in preparation). However, I found that RARα controls adhesion and spreading of these cells. This might suggest a novel genomic effect of RARα, i.e the control of the expression of genes involved in adhesion. However, preliminary experiments indicate that, in these cells, as well as in fibroblasts that are well adherent cells, a pool of RARα is present in the cytosol, suggesting non-genomic effects. Whether RARα controls adhesion via its interaction with profilin IIA or other proteins will require further investigations.

In conclusion, I have highlighted that RARα can depict three different subcellular localizations with three different functions:

- A membrane pool for non-genomic effects (activation of kinase cascades)
- A nuclear pool for genomic effects. In this context I identified a new partner, profilin IIA, which acts as coregulator of RARα-mediated transcription
- A cytotosolic pool which might play a role in cell adhesion

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LIST OF ABBREVIATIONS

ABP actin-binding proteins
ACTR acetyltransferase
AD activation domains
ADA2 adaptor protein 2
ADF actin depolymerasing factor
ADH alcohol dehydrogenase
AF activation function
Akt serine/threonine protein kinase
AlB1 amplified in breast cancer I
ANCO1 ankyrin repeats cofactor-1
AP-1 activator protein-1
AR Androgen receptor
ARAT acyl-CoA retinol acyltransferase
ARNT aryl hydrocarbon receptor nuclear translocator
ArpM1 actin-related protein M1
BAF57 barrier-to-autointegration factor 57
BCMO β-carotene-monooxygenase
bHLH/PAS helix-loop-helix/Per/ARNT/Sim
CAFs carcinoma-associated fibroblasts
CAK CDK-activating kinase
CAMKII calmodulin-dependent protein kinase II
CAR constitutive androstane receptor
CARM1 coactivator-associated arginine methyltransferase1
Cdk cyclin-dependent kinase
ChIP chromatin immunoprecipitation
CoCoA coiled-coil coactivator
CoRNR corepressor nuclear receptor
CRABPII cellular retinoic acid binding protein II
CRBP cellular retinol binding protein
CREB cAMP response element-binding
c-Src a tyrosine kinase
CTE COOH-terminal extension
cycH cyclin H
CYP26 cytochrome P450
DAD deacetylase activation domain
DBD DNA binding domain
DGAT diacylglycerol O-acyltransferase
DHRS dehydrogenase/reductase SDR family member
DR direct repeats
DRIP205 vitamin D-interacting protein 205
EB embryoid body
EGF epidermal growth factor
Ena/VASP vasodilator-stimulated phosphoprotein
ER estrogen receptor
ERK extracellular signal-regulated kinase
ES embryonic stem
FliI flightless-1 homolog
FXR farnesoid x receptor
GAC63 GRIP1-associated coactivator 63
GPS2 G-protein pathway suppressor 2
GR glucocorticoid receptor
GRIP-1 glucocorticoid receptor interacting protein
GST glutathione S transferase
GTFs general transcription factors
HDAC3 histone deacetylase 3
HID histone interaction domain
HNF hepatocyte nuclear factor
Hox homeobox
IDs interactions domains
IUPAC-IUB international union of pure and applied chemistry – international union of biochemistry
JNKs c-Jun N-terminal kinases
LBD ligand binding domain
LBP ligand binding pocket
LCoR ligand-dependent corepressor
LRAT lecithin retinol acyl transferase
LXR oxysterols liver X receptor
MAPK mitogen-activated protein kinase
MAT1 cyclin-dependent kinase-activating kinase (ménage á *trois* 1)
MEF-2C myocyte-specific enhancer factor *2C*
MSK1 mitogen- and stress-activated protein kinase

NCoA nuclear receptor coactivator complex
N-CoR nuclear receptor corepressor complex
NFmB nuclear factor-mB
NMDA N-methyl-D-aspartate
NTD N-terminal domain
p300/CBP CREB-binding protein
PCAF P300/CBP-associated factor
pCIP p300/CBP-interacting protein
Per period
PI3K phosphoinositide-3-kinase
PIP2 phosphatidylinositol (4,5)-bisphosphate
PKA protein kinase A
PKC protein kinase C
PLA proximity ligation assay
PNPLA4 patatin-like phospholipase domain-containing protein 4
PPAR peroxisomal proliferator activated receptor
PR progesterone receptor
PRAME preferentially expressed antigen in melanoma
PRM proline-rich motif
PRMT1 protein arginine N-methyltransferase 1
PXR xenobiotics pregnane X receptor
RA retinoic acid
RAC3 receptor-associated coactivator
RALDH retinaldehyde dehydrogenase
RAR retinoic acid receptor
RAREs retinoic acid response elements
RBP retinol binding protein
RDH retinol dehydrogenase
RETSAT all-trans-retinol 13,14-reductase
RPE65 retinal pigment epithelium-specific 65 kDa protein
RXR retinoid x receptor
SDR short-chain dehydrogenase/reductase
SH3 Src-homology-3
sim single-minded protein
SMN survival of motor neuron
SMRT silencing mediator for retinoic acid and thyroid hormone receptors
snRNP small nuclear ribonucleoprotein
SPR surface plasmon resonance
SRC steroid receptor coactivators
SRF serum response factor
SUG-1 suppressor of Gal 1
SWI/SNF SWItch/Sucrose NonFermentable
TACC1 transforming acidic coiled coil
TBL1 transducin β-like 1
TBLR1 TBL1-related protein 1
TEF-4 transcriptional enhancer factor 4
TFIIB transcription factor IIB
THIIH transcription factor IIH
TIF1α/Trim24 transcription intermediary factor-1 α
TIF-2 transcriptional intermediary factor 2
TRAM1 thyroid hormone receptor-activator molecule I
TRAP220 thyroid hormone receptor-associated protein 220
TTR transthyretin
UGT glucuronosyltransferase
VAD vitamin A deficiency
VDR vitamin D receptor
WASP/WAVE Wiskott-Aldrich syndrome family protein/WASP family Verprolin-homologous protein
WD40 protein containing WD40 (tryptophan-aspartic acid) repeat
WW (tryptophan-tryptophan)
XPB (Xeroderma Pigmentosum B) ATP dependent DNA helicase

THIS THESIS IS BUILT ON THE FOLLOWING PUBLICATIONS:

1. **Piskunov, A.** and C. Rochette-Egly. 2011. A retinoic acid receptor RARalpha pool present in membrane lipid rafts forms complexes with G protein alphaQ to activate p38MAPK. *Oncogene*. In press.
2. **Piskunov A,** Andrimoratsiresy D, Al Tanoury Z, Rochette-Egly C. Profilin IIA: a novel coregulator of the N-terminal domain of the Retinoic acid receptor alpha (RARα). Manuscript in preparation.
3. **Piskunov, A.,** and C. Rochette-Egly. 2011b. MSK1 and Nuclear Receptors Signaling. Landes Bioscience Books, Austin, TX, USA. 2012.

OTHER PUBLICATIONS:

Ferry, C., S. Gaouar, B. Fischer, M. Boeglin, N. Paul, E. Samarut, **A. Piskunov**, G. Pankotai-Bodo, L. Brino, and C. Rochette-Egly. 2011. Cullin 3 mediates SRC-3 ubiquitination and degradation to control the retinoic acid response. *Proc Natl Acad Sci U S A*. 108:20603-8.

ORAL COMMUNICATIONS:

- Première journée des Récepteurs Nucléaires, Muséum National d'Histoire Naturelle Amphithéâtre Rouelle, 47 Rue Cuvier 75005, Paris, France, 22 April 2011. "Novel concepts of nuclear retinoic acid receptors regulation through phosphorylation".
- EMBO Workshop "Retinoids", IGBMC, Strasbourg, September 2011. "Non-genomic effects of retinoic acid receptors: crosstalk with genomic effects"

POSTER PRESENTATIONS:

- EMBO Workshop "Retinoids", IGBMC, Strasbourg, France, September 2011. "The Retinoic acid receptor RARα is present in membrane lipid rafts and activates p38MAPK through interaction with Gαq proteins"
- The 12th Hunter Meeting Australia's Premier Cellular Biology Meeting, the Sebel-Kirkton Park, Pokolbin, Hunter Valley, NSW Australia March 2012. "The Retinoic acid receptor RARα is present in membrane lipid rafts and activates p38MAPK through interaction with Gαq proteins".

Contents

INTRODUCTION

CHAPTER 1: VITAMIN A AND RETINOIDS

- HEALTH BENEFITS OF VITAMIN A

Vitamin A is very important for the life of all chordates. This vitamin has a large number of functions in vision, maintenance of epithelial surfaces, immune competence, reproduction, embryonic growth and development. Therefore, in humans, insufficient consumption of vitamin A, called Vitamin A Deficiency (VAD) is characterized by ocular features (xerophthalmia) and a generalized impaired resistance to infection (Blomhoff and Blomhoff, 2006).

For children, VAD is one of the major causes of preventable blindness and increases the risk of illness and even death from serious childhood infections such as diarrheal disease and measles.

For pregnant women, VAD causes night time blindness and builds up the risk of maternal mortality. In high-risk areas, VAD occurs particularly during the last trimester of pregnancy, when demand by both the unborn child and the mother is highest. The impact of VAD on mother-to-child HIV transmission needs additional investigation.

Thus VAD is a public health problem in more than half of all countries, especially in low-income countries (Africa and South-East Asia) (Figure 1), hard-hitting small children and pregnant women.

- An estimated 250 million preschool children are vitamin A deficient and in vitamin A deficient areas a considerable percentage of pregnant women are vitamin A deficient.
- An estimated 250 000 to 500 000 vitamin A-deficient children lose their sight every year, half of them dying within one year (http://www.who.int/en/).

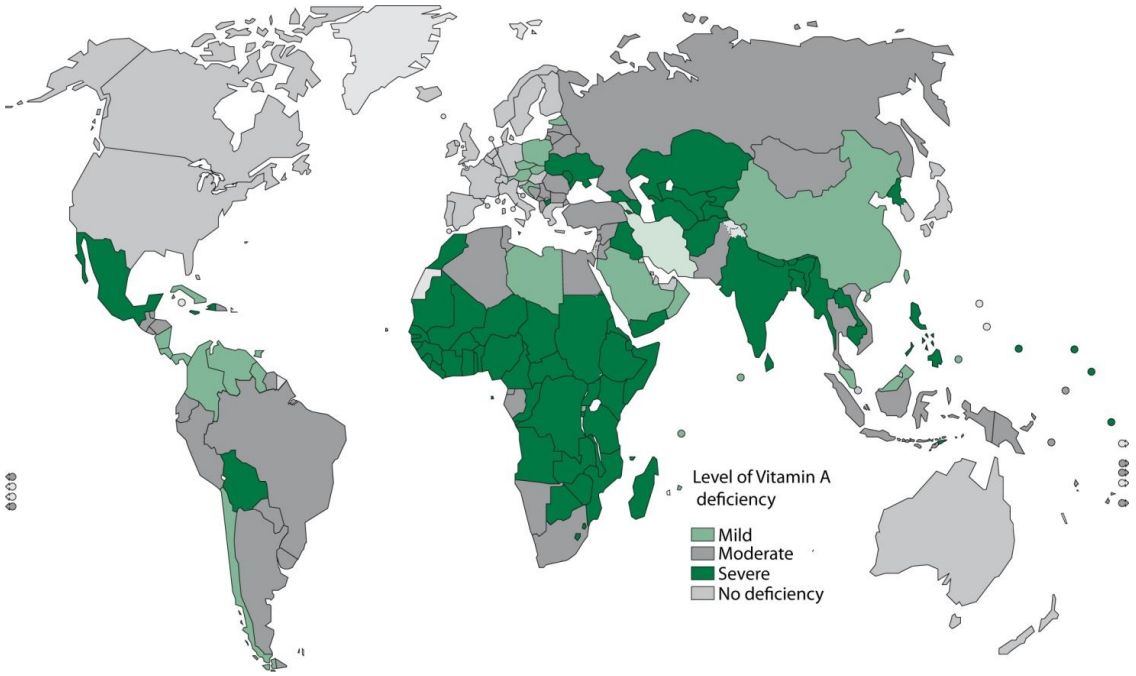

Figure 1. Map of the global prevalence of vitamin A deficiency (World Health Organization, 2009).

- ORIGIN OF VITAMIN A

No animal species have the potential for *de novo* synthesis of vitamin A (Retinol). Thus in most animals the only source is diet-derived.

Retinol is derived from carotenoids, which are present in plants and some microorganisms (bacteria and fungi) and are responsible for the yellow, orange and red colors of many vegetables, fruits, and plants (Fraser and Bramley, 2004). About 600 carotenoids are known in nature, but only 10% can be metabolized to vitamin A. Among carorenoids, β-carotene has the best biological activity (Yeum and Russell 2002).

Animals obtain vitamin A by consuming plants containing carotenoids or animal tissues that store carotenoids such as eggs, poultry and fish (Fraser and Bramley, 2004). Then they converte carotenoids to vitamin A. Alternatively, animals can also obtain vitamin A by ingesting animal tissues that have already transformed carotenoids into retinol. In general, retinol is stored as retinyl esters in fish, avian, and mammalian livers.

- NOMENCLATURE, STRUCTURE, AND CHEMICAL PROPERTIES OF RETINOIDS

The term “retinoids” was introduced by Sporn et al. in 1976 and later was designated by the International Union of Pure and Applied Chemistry – International Union of Biochemistry (IUPAC-IUB) as a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner. All retinoids may be formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional terminal group at the terminus of the acyclic portion.

Retinoids include retinol as well as its analogs and derivatives (Figure 2A). There are six biologically active isoforms of vitamin A that include all-trans, 11-cis, 13-cis, 9, 13-di-cis, 9-cis, and 11, 13-di-cis retinol, all-trans being the main physiological form. The active metabolites of retinol include all-trans Retinoic Acid (RA), 9-cis RA, 11-cis retinaldehyde, 3,4-didehydro RA, and perhaps 14-hydroxy-4, 14-retro retinol, 4-oxo RA, and 4-oxo retinol (Achkar et al., 1996; Buck et al., 1991; Napoli, 1996).

However, some synthetic compounds (Figure 2B) that do not fit with this chemical definition are much more active than retinol or retinoic acid in several assays for vitamin A or retinoid activity. Therefore it has been proposed that retinoids should be outlined as substances eliciting a particular biologic response via binding to and activating a specific receptor or set of receptors (Sporn and Roberts 1985). Today the definition of the retinoid term includes not only retinol analogues and derivatives (with or without biologic activity), but also a number of compounds that are not structurally related to retinol but elicit biological vitamin A or retinoid activity (Blomhoff and Blomhoff 2006).

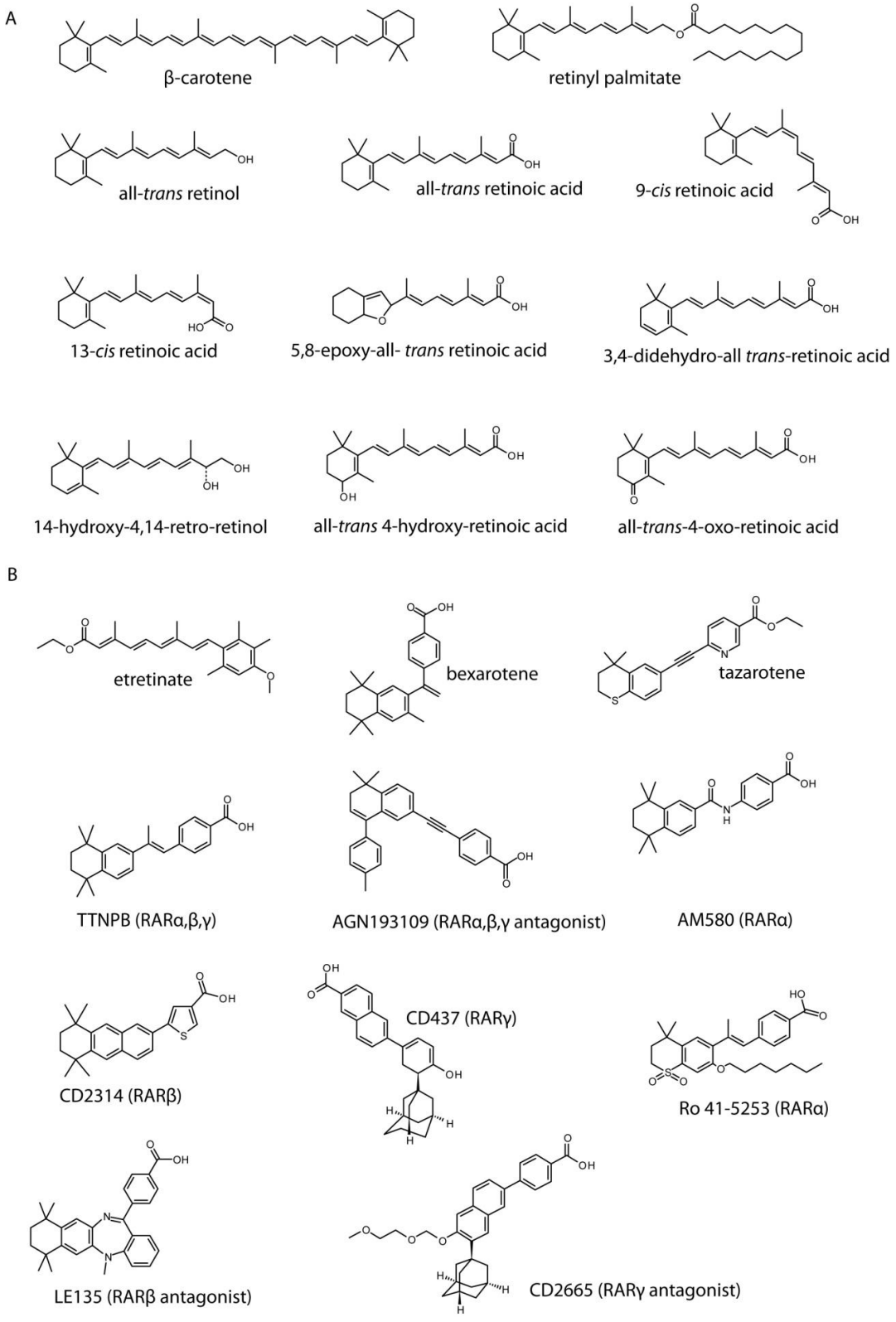

Figure 2: Chemical structure of natural (A) and synthetic (B) retinoids.

Chemically, retinoids are composed of a β-ionone ring, a polyunsaturated side chain and a polar end group (Figure 3). Such an amphipathic chemical structure makes them poorly soluble in water and provides easy transfer through membrane lipid bilayers. The polar end group can exist at several oxidation states, varying from the low oxidation state of retinol to a higher oxidation state in RA (Figure 2A).

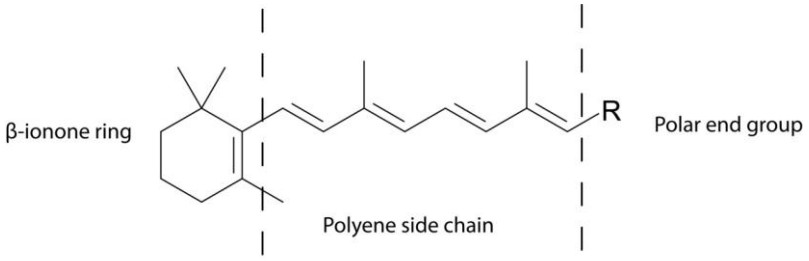

Figure 3: Structure of retinoids. Adapted from (Hong and Lotan, 1993).

- RETINOID METABOLISM

1. Enzymatic Conversion of Proretinoids (Carotenoids) to Retinoids

As early as 1930, it has been reported that β-carotene can be transformed to retinoids within the small intestine (Moore, 1930). Later on, two independent groups (Goodman and Huang, 1965; Olson and Hayaishi, 1965) showed that one molecule of β-carotene yields two molecules of retinal via a central cleavage catalyzed by an enzyme termed ββ-carotene-15, 15'-monooxygenase(BCMO-I) (Figure 4).

Another enzyme, β,β-carotene-9'10'-dioxygenase (BCMO-II) is also able to cleave *β*-carotene (Kiefer et al., 2001). However, BCMO-II catalyzes an asymmetrical cleavage of *β*-carotene at non-central double bonds of the polyene chain, yielding apocarotenals like *β*-apo-8′-, *β*-apo-10′- and *β*-apo-12′-carotenals (Figure 4). This alternative pathway is very important as it implies that, in tissues expressing BCOM-II, retinoic acid can be produced in the absence of ''classical RA synthesis pathway'' enzymes, such as alcohol dehydrogenase (ADH), short-chain dehydrogenase/reductase (SDR), retinaldehyde dehydrogenase (RALDH) (Simoes-Costa et al., 2008) or certain cytochrome P450s (Chen et al., 2000) (see below).

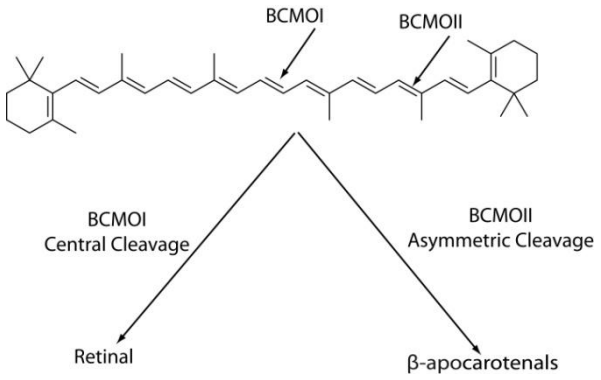

Figure 4: **Carotenoids undergo cleavage either symmetrically by BCMOI or asymmetrically by BCMOII.** Adapted from (D'Ambrosio et al., 2011).

2. Enterocyte Esterification of Retinol

Retinal obtained by carotenoid cleavage is then reduced to retinol and taken up by enterocytes. Enterocytes also take dietary consumed retinyl esters after hydrolysis into retinol within the intestinal lumen (Blomhoff and Blomhoff, 2006).

In enterocytes, retinol binds to the cellular retinol binding protein II (CRBPII), which is specifically highly expressed in the intestinal mucosa. CRBPII belongs to the greater family of fatty acid binding proteins and its role is to solubilize fat-soluble retinol (Sporn et al., 1994) (Figure 5).

Then, the majority of CRBPII-bound retinol is re-esterified via the enzyme lecithin retinol acyl transferase (LRAT). The remaining esterification activity would be assumed by two other enzymes diacylglycerol acyltransferase 1 (DGAT1) and acyl-CoA:retinol acyltransferase ARAT (D'Ambrosio et al., 2011) (Figure 5).

Afterwards retinyl esters are included into chylomicrons and secreted into general circulation (Blomhoff et al., 1982), where they are taken up by hepatocytes (Blomhoff et al., 1982), bone marrow, peripheral blood cells, spleen, adipose tissue, skeletal muscle and kidney (Paik et al., 2004). In mammals, 50-80% of the body's overall retinoids are collected in hepatic stellate cells as retinyl esters (Blomhoff and Blomhoff, 2006; Fontana and Rishi, 2002) (Figure 5).

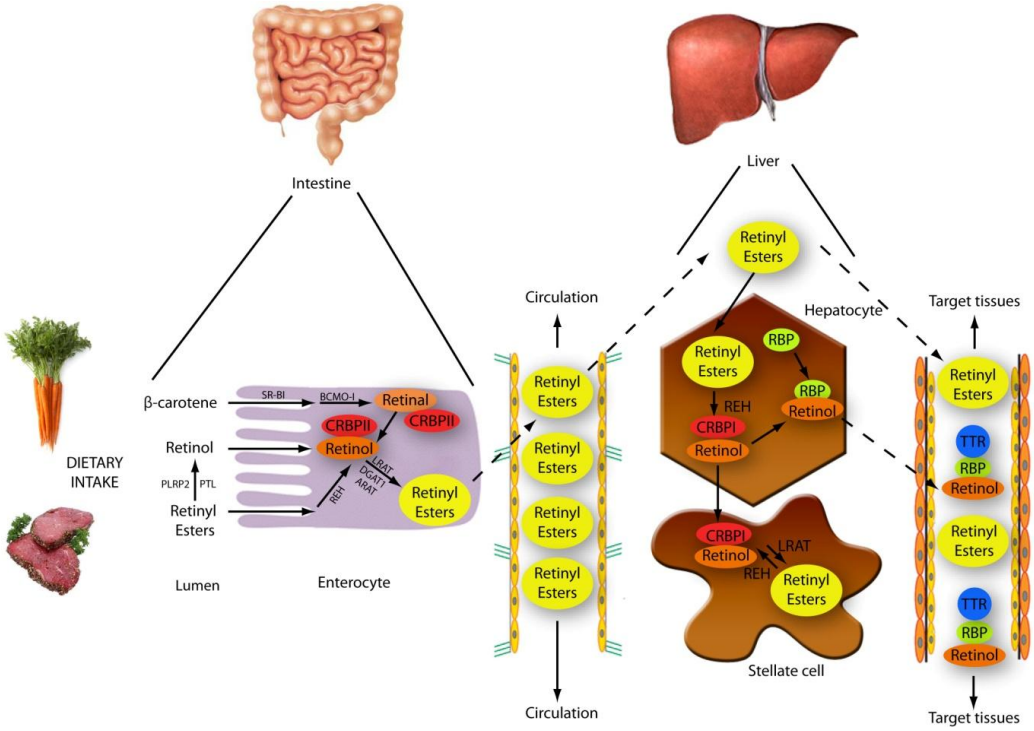

Figure 5: Metabolism of vitamin A. In the intestine, carotenoids are converted to retinol, which binds CRBP. Then retinol is transformed into retinyl esters, exported into the circulation and taken up by the liver. In hepatocytes, retinyl esters are reconverted to retinol and bind RBP for transport to target cells. In stellate cells of the liver, retinol is retransformed to retinyl esters for storage. In the bloodstream, the retinol:RBP complex is bound to TTR to avoid removing by the kidney and for guaranteed delivery to target tissues. Figure adapted from (Theodosiou et al., 2010).

3. Hepatic retinoid metabolism

In hepatocytes, retinyl esters are re-converted to retinol, which then binds retinol binding-protein (RBP) and is secreted into circulation. The majority of the retinol:RBP complexes are associated with transthyretin (TTR), which prevents elimination by the kidney and ensures delivery to target tissues. However, a big part of hepatic retinol is reesterified into retinyl esters and packed within cytoplasmic lipid droplets in stellate cells (Blomhoff and Blomhoff, 2006) (Figure 5).

4. Retinol processing

In target tissues, retinol either associates with CRBP or is transformed into active metabolites. Indeed retinol undergoes oxidation to retinaldehyde by enzymes termed retinol dehydrogenases (Figure 6) (Gottesman et al., 2001; Pares et al., 2008). These enzymes are members of the ADH (alcohol dehydrogenase) or SDR (short-chain dehydrogenase/reductase) families, which depict cytosolic and microsomal localizations respectively. Given that the next step, the irreversible oxidation of retinaldehyde to retinoic acid, takes place in cytosol, it has been suggested that ADHs would be more important than microsomal SDRs for RA synthesis (Duester et al., 2003).

Then the oxidation of retinaldehyde to RA is carried out by retinaldehyde dehydrogenases (RALDHs), such as RALDH 1, 2, 3, and 4 depending on tissue types (Niederreither et al., 2002). Finally, newly synthesized RA associates to cellular RA binding proteins types I and II (CRABP-I and CRABP-II), and then either enters nuclei for activation of transcription (autocrine) or is transported to adjacent target cells (paracrine) (Napoli, 1996).

5. Retinoic acid degradation

Catabolism is necessary to control RA levels in cells and tissues. It occurs mainly through enzymes of the Cytochrome P450 enzyme family (CYP26). There are several CYP26 enzymes and the first one to be cloned was CYP26A1, which generates several hydroxylated forms of RA, such as 4-hydroxy retinoic acid, 4-oxo retinoic acid, 18-hydroxy retinoic acid, 5,6-epoxy retinoic acid, and 5,8-epoxy retinoic acid (Swindell and Eichele, 1999). Other similar enzymes (CYP26B1 and 26C1) have been identified later and are also able to metabolize RA (Taimi et al., 2004; White et al., 2000). The expression patterns of CYP26A1, CYP26B1, and CYP26C1 normally do not overlap, suggesting specific and distinct roles for each enzyme in the catabolism of RA (Reijntjes et al., 2004).

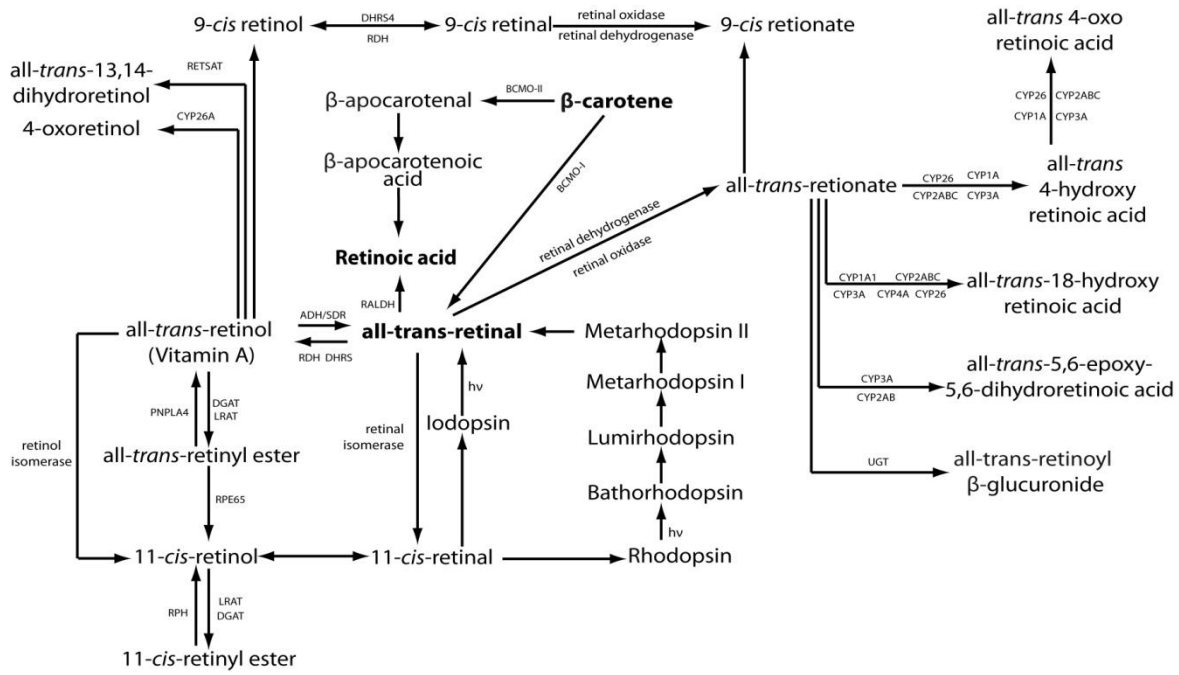

Figure 6: Biochemical pathway of retinoids. The enzymes responsible for conversion of the retinoids: ADH alcohol dehydrogenase, BCMO-I β,β-carotene-15',15'-monooxygenase, BCMO-II β,β-carotene-9',10'-dioxygenase, DGAT diacylglycerol O-acyltransferase, CYP26 cytochrome P450, DHRS dehydrogenase/reductase SDR family member, LRAT phosphatidylcholine-retinol O-acyltransferase, PNPLA4 patatin-like phospholipase domain-containing protein 4, RDH retinol dehydrogenase, RETSAT all-trans-retinol 13,14-reductase , RPE65 retinal pigment epithelium-specific 65 kDa protein, SDR short-chain dehydrogenase*/reductase,* UGT glucuronosyltransferase. Adapted from (Theodosiou et al., 2010).

CHAPTER 2: NUCLEAR RETINOIC ACID RECEPTORS

The biological effects of RA are mediated through two main families of nuclear receptors, which belong to the nuclear receptor superfamily: the retinoic acid receptors (RAR) and the retinoid x receptors (RXR). For both RARs and RXRs, there are three subtypes - α, β, and γ. They act as ligand-inducible transcription factors and usually form RAR/RXR heterodimers.

Note however that RXRs can also form homodimers and heterodimerize with other nuclear receptors. Indeed, RXRs are promiscuous receptors, which have the capacity to form heterodimers with several different nuclear receptors such as the receptors for fatty acids [peroxisomal proliferator activated receptors (PPAR)], bile acids [farnesoid x receptor (FXR)], oxysterols [liver×receptor (LXR)], xenobiotics [pregnane×receptor (PXR), androstanes [constitutive androstane receptor (CAR)], and vitamin D [vitamin D receptor (VDR)] (Germain et al., 2006b).

- RETINOIC ACID RESPONSE ELEMENTS (RARES)

RARs together with RXRs form asymmetrically oriented heterodimers, which bind to specific DNA sequences, called RA response elements (RAREs), located in the regulatory sequences of target genes.

The classical RAREs are composed of two direct repeats of a core hexameric motif, PuG(G/T)TCA, separated by 1 base pair, 2 base pairs or 5 base pairs and named DR1, DR2 and DR5 respectively (Figure 7) (Germain et al., 2003; Leid et al., 1992; Mangelsdorf and Evans, 1995). Such RAREs have been identified in the promoters of a large number of RA target genes involved in a wide variety of functions. For example, the classical DR5 elements are found in the promoters of the *RARβ2* gene itself (de The et al., 1990), of the *CYP26A1* gene (cytochrome 450, family 26, subfamily a, polypeptide 1) (Loudig et al., 2000), and of several *Homeobox (Hox)* and *hepatocyte nuclear factor (HNF)* genes (Dupe et al., 1997; Qian et al., 2000). Recently, *in silico* studies revealed new DR5 RARE-associated genes (*Meis2 and Bhlhbe40*) (Lalevee et al. 2011). DR2 elements were identified in the *CRBPI (Cellular retinol binding protein I)* and *CRABPII (Cellular retinoic acid binding protein II)* gene promoters (Durand et al., 1992; Smith et al., 1991). The only natural DR1 element has been found in the rat *CRBPII* gene promoter (Mangelsdorf et al., 1991).

Remarkably, recent ChIP-seq analysis revealed novel natural DR0 and DR8 RAREs, the latter being composed of a DR2 juxtaposed to a DR0 (I. Davidson *et al,* unpublished results).

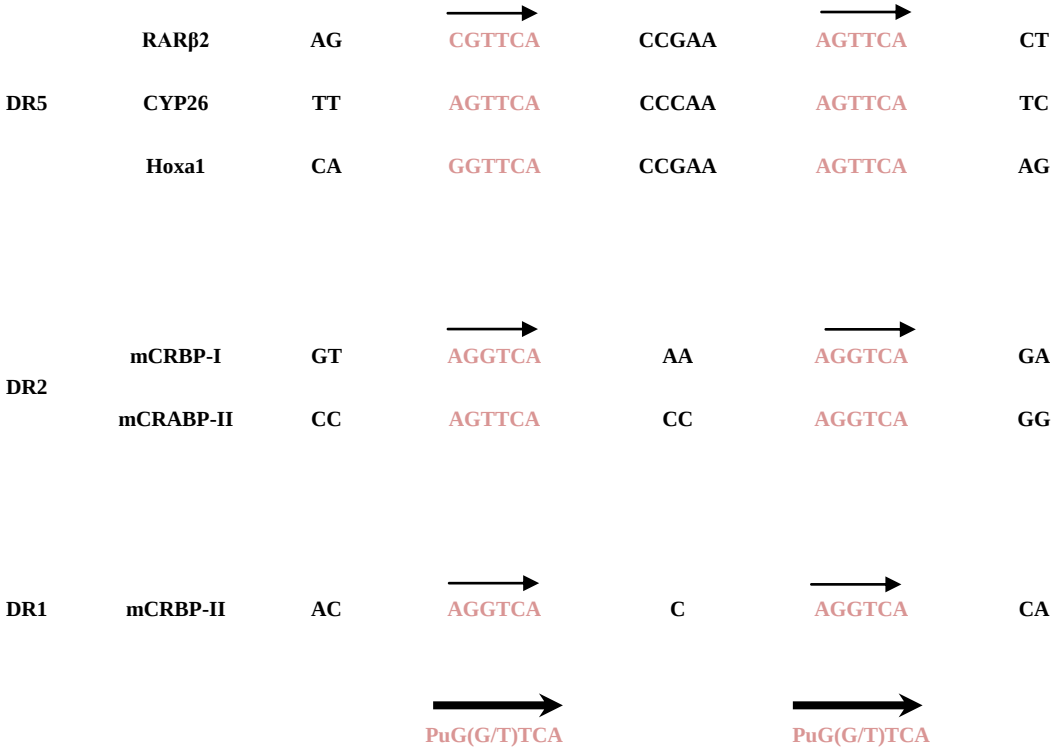

Figure 7: The classical retinoid response elements are composed of two direct repeats of the hexameric, motif 5′-PuG(G/T)TCA spaced by 1 (DR1), 2 (DR2) or 5 (DR5) base pairs. Several examples of natural retinoid response elements from the promoters of RA-target genes are shown. Adapted from (Bastien and Rochette-Egly, 2004).

- STRUCTURE OF RARS

As most nuclear receptors, RARs have a modular structure consisting of 6 regions named A to F, from the N-terminal to the C-terminal end (Figure 8) (Chambon, 1996; Laudet and Gronemeyer, 2002). Some of these regions overlap with functional domains. Indeed, the C and E modules correspond to the DNA binding domain (DBD) and the Ligand Binding Domain (LBD), respectively. These domains are highly conserved between RARs and nuclear receptors and play critical roles in the classical model of RAR transcriptional activity. Oppositely, the A/B, D and F modules are less conserved.

Figure 8: Modular structure of RARs. Adapted from (Rochette-Egly and Germain, 2009).

1. DNA binding domain (DBD)

The DBD, which is responsible for sequence-specific DNA recognition, consists of two zinc-nucleated modules, two α-helices and a COOH-terminal extension (CTE) (Zechel et al., 1994). Functionally, the DBD is divided into 4 boxes: the P box within the first helix and the A box in the CTE, are responsible for discrimination between the half sites, while the D and T boxes are involved in the heterodimerization interface (Figure 9A).

According to nuclear magnetic resonance and crystallographic studies, when the DBD is complexed with DNA, helix 1 and helix 2 cross at right angles and fold into a globular conformation to form the core of the DBD (Lee et al., 1993) (Figure 9B). Indeed, helix 1 fits specifically into the major groove of the DNA through the P box, while helix 2 and the CTE cooperate to create the interface between the heterodimerization partners.

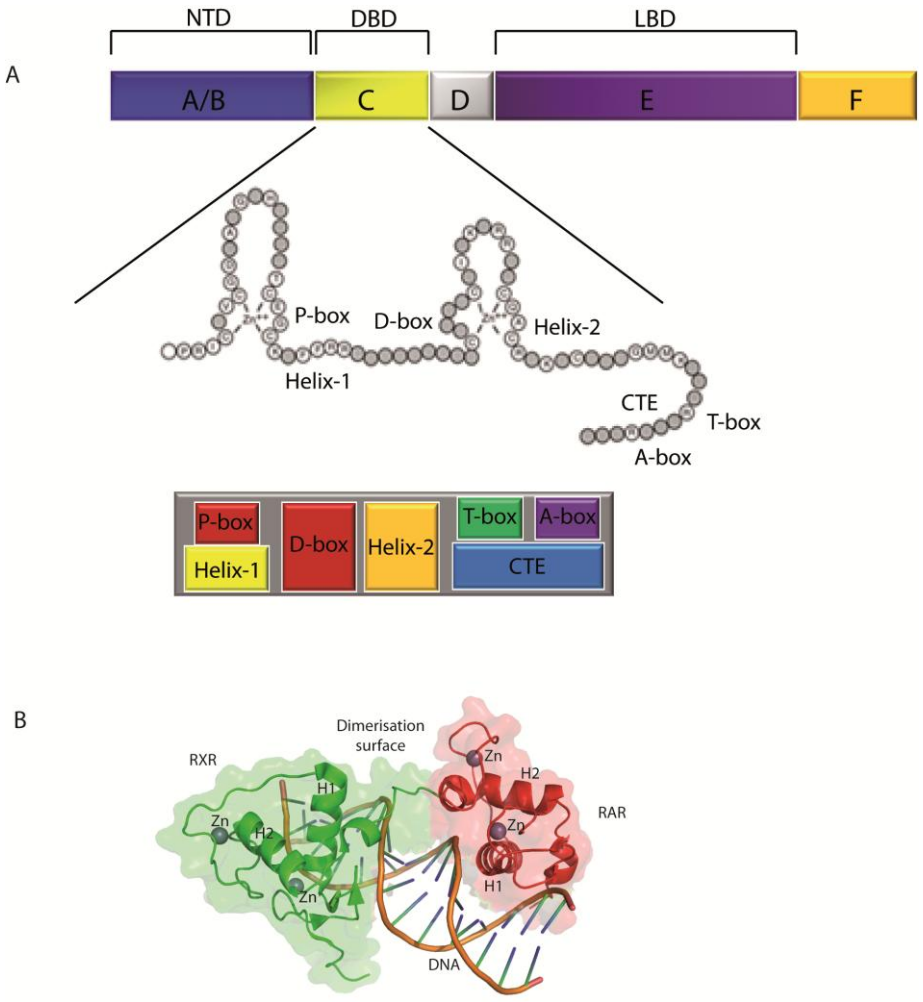

Figure 9: Structure of the DNA binding domain
A**.** Schematic view of the DBD, showing the relative locations of the P-box, D-box, T-box, A-box, Helix 1, Helix 2, and the C-terminal extension (CTE). Adapted from (Aranda and Pascual, 2001; Bain et al., 2007). B. Structure of a RAR/RXR DBD heterodimer in complex with a DR1 DNA response element. Zn = atome of zinc, H1 and H2 = helices 1 and 2. Adapted from (Rastinejad et al., 2000) pdb1dsz.

Depending on the RARE, the heterodimers are differently oriented. For example on DR2 and DR5 elements, the RXR partner occupies the 5' hexameric motif and the RAR partner the 3' motif (5'-RXR-RAR-3') (Chambon, 1996; Laudet and Gronemeyer, 2002). However, for DR1 elements, the polarity is opposite, with the RAR in 5' side and the RXR in 3' (5'-RAR-RXR-3') (Figure 10). This variation of orientation depending on the DR type, would explain why the activity of the heterodimer switches from an activator (DR5) to a repressor (DR1).

Moreover, also depending on the DR type, different regions of the DBD of each partner participate in the dimerization interface, in order to achieve the required binding to the response

elements. Indeed, the binding of RXR-RAR heterodimers to DR5 elements requires the D box of the RXR second zinc-finger, and the tip of the RAR first zinc finger. However, binding with reverse polarity to the DR1 elements involves the second zinc finger of RAR and the T box of the RXR CTE (Renaud and Moras, 2000).

Figure10: Different orientations of RXR/RAR hetodimers depending on the DR type. On DR5 elements, the 5' hexameric motif is occupied by RXR. On DR1 elements, the 5' hexameric motif is occupied by RAR. Adapted from (Bastien and Rochette-Egly, 2004).

2. Ligand- binding domain (LBD)

The structures of the RAR LBDs were demonstrated by crystallographic studies (Moras and Gronemeyer, 1998; Renaud and Moras, 2000; Wurtz et al., 1996). The LBD is composed of 12 conserved α helices and a β-turn (located between H5 and H6) (Figure 11). Helices 1-11 are folded into a three-layered, anti parallel helical sandwich where H4, H5, H8, H9 and H11 are sandwiched between H1, H2 and H3 on one side and H6, H7 and H10 on the other side. In this structure, H12 that encompasses the AF-2 activation domain, points away from the LBD core. Functionally, the LBD is divided into three main functional domains: the ligand-binding pocket (LBP), the major dimerization interface and the ligand-dependent activation function-2 (AF-2).

- Ligand-binding pocket (LBP)

The ligand-binding pocket (LBP) contains hydrophobic residues mainly from helices H3, H5, H11 and the β-sheet, which establish van der Waals interactions with the ligand (Klaholz et al., 2000). Several crystallographic studies revealed the structural basis of ligand recognition (Bourguet et al., 2000a; Li et al., 2003; Renaud et al., 1995). The carboxylic group of the retinoid molecules is buried deep inside the LBP, engaging hydrogen bonds with specific amino acids of H3 and the β-sheet. The rest of the molecule has to adapt to the overall structure of the LBP, which causes the ligand molecule to bend to accommodate the RAR LBP.

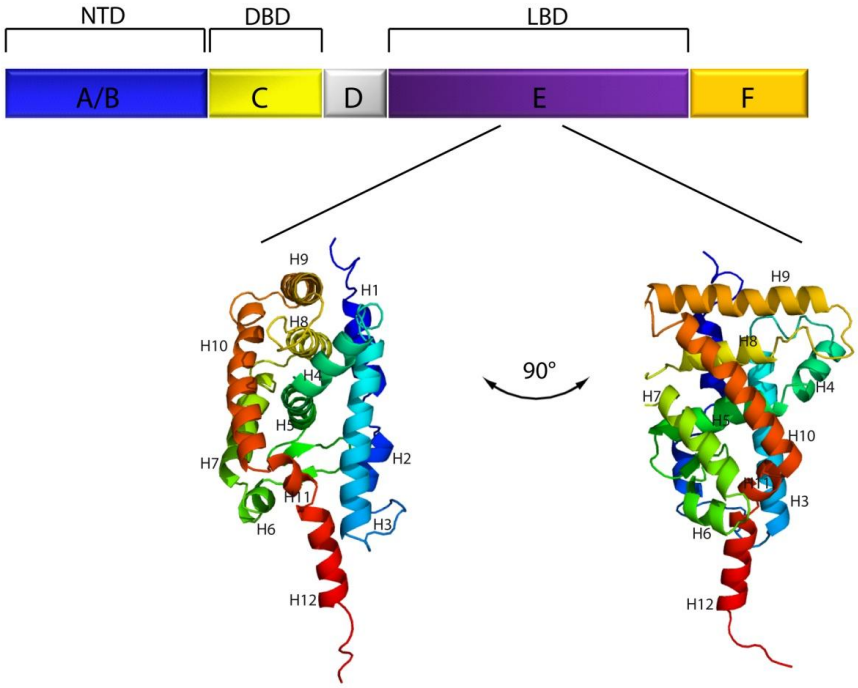

Figure 11: The 3D structure of the LBD. Helices are shown as ribbons and labelled from H1 to H12. The LBD is consisted of 12 α helices which form a three-layered, antiparallel helical sandwich (on the left side of the figure). H4, H5, H8, H9 and H11 are sandwiched between H1, H2 and H3 on one side and H6, H7 and H10 on another side. Adapted from (Bourguet et al., 1995) pdb1lbd.

The precise contacts with ligands involve three divergent residues, which are unique for each subtype receptor-cognate ligand pair and are located in H3, H5 and H11 (Table 1). Therefore, it has been possible to generate subtype-selective ligands (Germain et al., 2004). For example, the unique polar residues S232 and M272 located within LBP of RARα and RARγ respectively have been used to develop specific ligands for RARα (Am580) or RARγ (BMS270394 or CD666). Via their amino group, these ligands form hydrogen bonds with S232 of RARα or M27 of RARγ leading to increased affinity and selectivity for RARα and RARγ respectively (Table1).

Helices			
Receptor	H3	H5	H11
RARα	Ser232	Ile270	Val395
RARβ	Ala225	Ile263	Val388
RARγ	Ala234	Met272	Ala397

Table 1. Residues within the α-helices H3, H5 and H11 of the different RAR isotypes involved in ligand binding

- Heterodimerization surface

The main heterodimerization surface between the RAR and RXR partners is located in the LBD. It involves residues from helices H7, H9, H10 and H11, as well as loops L8-9 and L9-10 (Bourguet et al., 2000b; Gampe et al., 2000; Pogenberg et al., 2005). The core of the dimer is formed mostly by helices H9 and H10, which contribute to more than 75% of the total dimerization surface. In contrast to the almost perfect symmetric organization of RXR homodimers (so called butterfly shape), the heterodimer interfaces are asymmetric. Indeed, helix H7 of RXR contacts loop L8-9 of RAR, but loop L8-9 of RXR and helix H7 of RAR are not involved in the interaction (Figure 12).

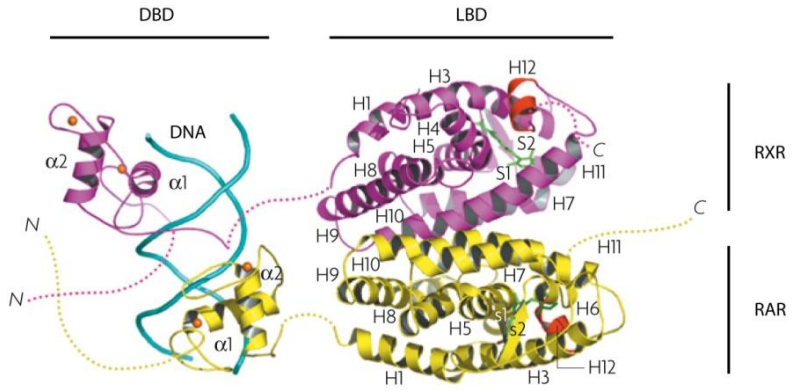

Figure 12: The three-Dimensional structure of RXR–RAR heterodimer with 9-cis-retinoic acid and DNA. Pointed lines indicate domains with unresolved structures. Helices are shown as ribbons and labelled from H1 to H12 (LBD) or α1 and α2 (DBD). Helix H12 (AF-2) is represented in red in each subunit. The short LBD β-strands are labelled S1 and S2. 9-cis-retinoic acid in RAR and RXR LBDs is shown by green sticks lines. The orange spheres in the DBD indicate atoms of zinc. Image modified from (de Lera et al., 2007).

Recently Rochel *et al* revealed new structural features of the RAR-RXR heterodimers architecture on different RAREs. They demonstrated that the RXR–RAR–DR5 complex is elongated and asymmetric, with two separate DBDs and LDBs connected by a narrow segment (Figure 13 B) (Rochel et al., 2011). The RAR–RXR–DR1 complex is similarly elongated but with a larger connecting volume between the DBDs and LBDs (Figure 13 A). Interestingly, in both cases (DR5 or DR1), the LBD dimers are always positioned at the 5′ end of the target DNA. The observed asymmetry of the overall architecture and the relative position of the domains point to the essential role played by the hinge domains in establishing and maintaining the integrity of the functional structures.

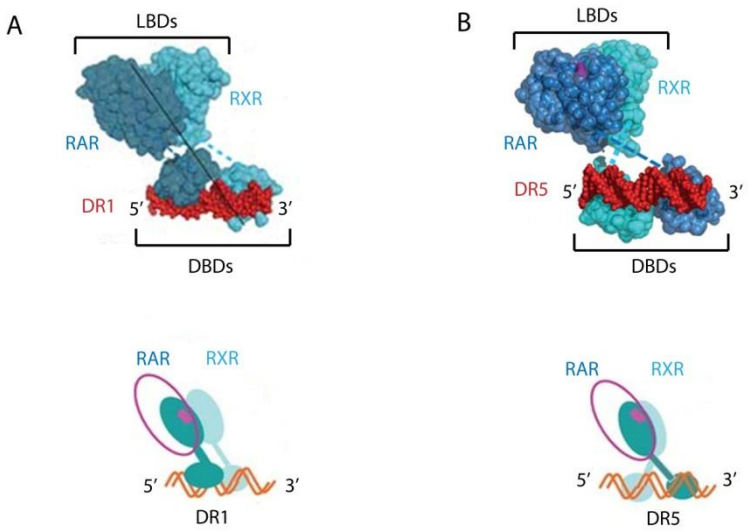

Figure 13: A. RAR-RXR-DR1 complex B. RAR-RXR-DR5 complex. Adapted from (Rochel et al., 2011).

- C-terminal helix 12, named AF-2

The C-terminal helix 12, known as AF-2, regulates the interaction of RARs with coregulators. The analysis of the crystal structures of the unliganded and ligand-bound LBDs of RXRα and RARα respectively (Bourguet et al., 1995; Renaud et al., 1995), highlighted the conformational flexibility of H12 and how AF-2 becomes transcriptionally active (Figure 14 A) (Egea et al., 2001; Steinmetz et al., 2001).

In the unliganded receptor (so called apo-conformation), H11 is almost perpendicular to H10 and points towards the ligand-binding pocket and some of the hydrophobic residues of H11 partially fill and stabilize the LBP. H12 extends away from the core LBD, pointing away from the dimer axis at an angle of about 45° (Bourguet et al., 1995).

Upon ligand binding, H11 is repositioned in the continuity of H10, causing the concomitant swinging of H12, which moves in a ‘mouse trap’ model and packs tightly against H3 and H4 (Figure 14B). Consequently the lid of the LBD is sealed and ligand binding is stabilized (Moras and Gronemeyer, 1998). Moreover, a new hydrophobic cleft between H3, H4 and H12 is formed, creating a defined surface for the interaction with transcriptional coactivators. This liganded conformation is referred as “holo” or “active” conformation (Figure 14B). Note that in the case of the RARβ and RARγ subtypes, some biochemical studies proposed that even in the absence of ligand, H12 interacts with H3 and adopts a constitutively closed conformation that approximately corresponds to the conformation of liganded RARα (Farboud et al., 2003; Farboud and Privalsky, 2004; Hauksdottir et al., 2003).

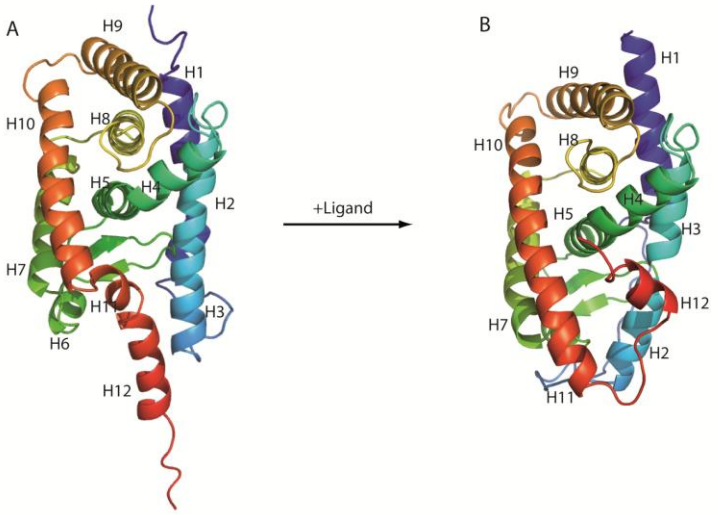

Figure 14: Three-dimensional structure of LBD and structural changements induced by ligand binding. Helices are represented as ribbons and labelled from H1 to H12. A. Structure of the LBD of RXRα in apo conformation. Adapted from (Bourguet et al., 1995) pdb1lbd. B. Structure of the LBD of RARγ with ligand (holo conformation). Adapted from (Renaud et al., 1995) pdb2lbd.

3. N-terminal AF-1 domain (NTD)

The N-terminal domain (NTD) of RARs (activation function AF-1) corresponds to the A and B regions (Nagpal et al., 1993; Nagpal et al., 1992). Within the NTD, the A regions are comparatively variable between the different RAR subtypes and isoforms (Chambon, 1996). In contrast, the B region is well conserved and holds a proline-rich motif containing phosphorylation sites (see below, Figure 26 page 42).

In contrast to the DBD and the LBD, there are still no high-resolution structures available for the NTD of RARs and most nuclear receptors. Even the relatively very short NTD of peroxisome proliferator-activated receptor-γ (PPAR-γ) failed to show any signature of structure (Chandra et al., 2008). According to several studies, the NTDs of the Estrogen Receptor (Warnmark et al., 2001), the Glucocorticoid Receptor (Warnmark et al., 2001), the Progesterone Receptor (Bain et al., 2000) and the Androgen Receptor (Reid et al., 2002) possess an intrinsically disordered (ID) conformation.

Importantly, intrinsically unstructured proteins are functional and the NTD of RARs has been shown to play an important role in the regulation of transcription (Nagpal et al., 1993). Though the mechanism of this functionality has not been elucidated yet, it has been shown that disordered domains provide the flexibility that is necessary for modifications by kinases or ubiquitin ligases (Dyson and Wright, 2005). Such modifications may change structural properties of the domain and

subsequently affect the dynamics of neighboring structural domains (Pufall et al., 2005) and therefore interactions with co-regulators and/or DNA (Dyson and Wright, 2005; Liu et al., 2006). In line with this, it is interesting to note that the NTD of RARs contains phosphorylation sites located in a proline-rich motif (PRM). As PRMs have the capacity to bind proteins with SH3 (Src-homology-3) or WW (tryptophan-tryptophan) domains (Kay et al., 2000b), one can speculate that the NTD of RARs might regulate transcription via the phospho-dependent association or dissociation of coregulators (See below chapter 5).

4. D-region

The D domain or hinge region is poorly conserved and serves as a hinge between the DBD and the LBD. It has been proposed that its flexibility would permit the adaptation of the RAR/RXR heterodimers to different types of RAREs (Glass, 1994; Rochel et al., 2011). In RARs, this domain is very small (12 amino acids) and shares 50% of identity between RARα and RARβ and 33% between RARα and RARγ. The hinge region would also harbor a nuclear localization signal (Hamy et al., 1991).

5. The F-region

The F region is the most carboxy-terminal region of RARs and is absent in RXRs. It is not conserved between RARs and its three-dimensional structure is not known. So far the precise functions of region F are not well understood. It has been proposed that in the absence of ligand this region might stabilize the H12 of RARα in open conformation, thus enhancing binding of corepressors (Farboud and Privalsky, 2004). According to recent studies, this region would be able to bind specific mRNA motifs (Poon and Chen, 2008). Interestingly, the F region is phosphorylated at multiple sites and such modificatins might change the properties of RARs (Bastien et al., 2000; Rochette-Egly et al., 1997).

CHAPTER 3: THE CLASSICAL MODEL OF RAR-MEDIATED REGULATION OF TRANSCRIPTION

The canonical mechanism of action of RARs involves the activation or repression of target-gene transcription (Figure 15).

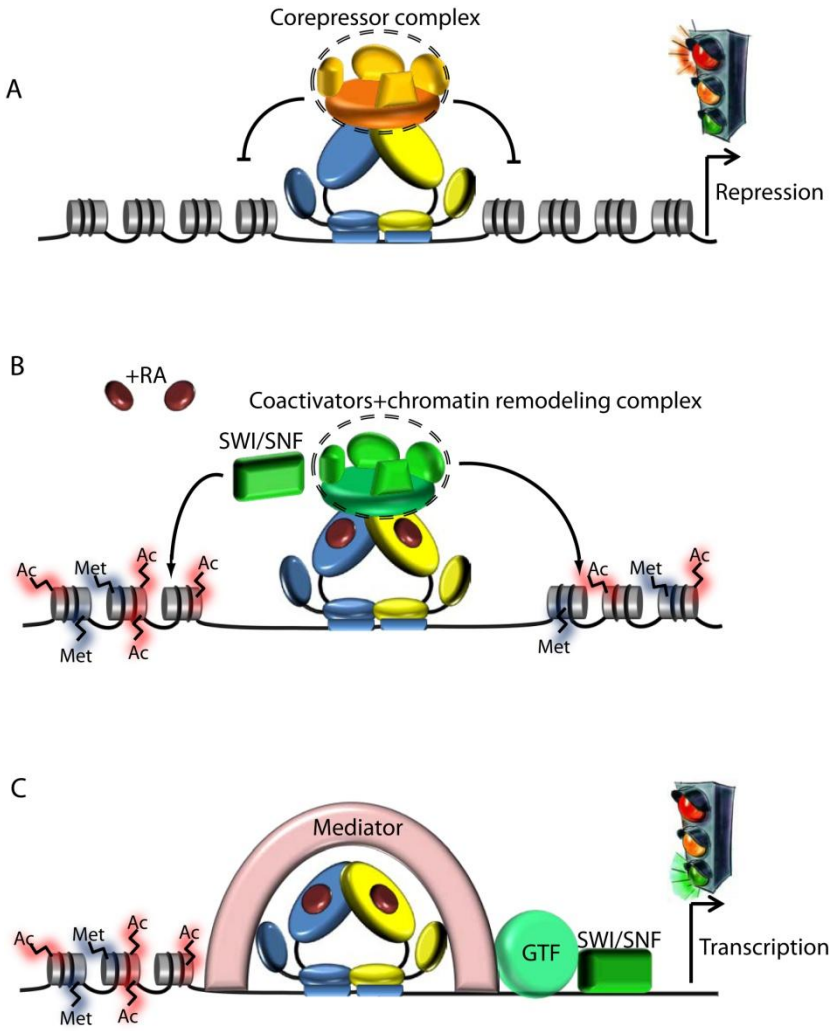

Figure 15: Classical model of activation of RA-target genes.
A. In the absence of ligand, RARα/RXR heterodimers bind DNA in association with corepressor complexes. B. Ligand binding induces the release of corepressors and the recruitment of coactivator complexes. C. Upon decompaction of chromatin, the transcriptional machinery, which consists of the Mediator, RNA polymerase II and the general transcription factors (GTFs), is recuited to the promoter, resulting in the initiation of transcription. Adapted from (Bour et al., 2007b).

This mechanism is at the basis of the regulation by RARs of gene networks involved in a wide number of functions such as homeostasis, development and reproduction. According to such a model, genes are silenced through the recruitment of corepressor-containing complexes to unliganded (apo) DNA-bound receptors. Conversely, genes are activated subsequent to corepressors release from liganded receptors (holo) and recruitment of coactivator complexes (Figure 15). At the molecular level, the discrimination between coactivators and corepressors relies in the positioning of H12 within the LBD.

- REPRESSION OF TRANSCRIPTION IN THE ABSENCE OF LIGAND

In the absence of ligand, RAR/RXR heterodimers occupy RAREs in association with large multiprotein complexes with several enzymatic activities (histone deacetylases, methylases, ubiquitin ligases etc), which maintain histones and chromatin in a compacted repressed state.

Basically, the corepressor core is composed of NCoR (nuclear receptor co-repressor) or SMRT (silencing mediator for retinoic acid and thyroid hormone receptors). NCoR and SMRT were the first identified corepressors (Chen and Evans, 1995; Lee et al., 1995; Sande and Privalsky, 1996). They bind NRs and serve as platforms for the binding of other proteins such as GPS2 (G-protein pathway suppressor 2), TBL1 (Transducin β-like 1), TBLR1 (TBL1-related protein 1), HDAC3 (Histone deacetylase 3) and Sin3 (Figure 16) (Li et al., 2000; Zhang et al., 2002).

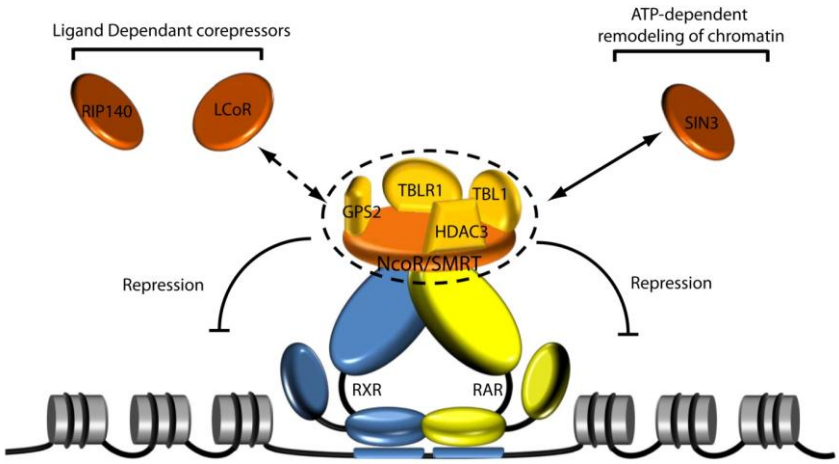

Figure 16: **Corepressors and associated complexes.** Core of the complex (pointed) and associated complexes. Adapted from (Perissi and Rosenfeld, 2005).

Of note is that SMRT is the preferential corepressor of RARs. It is an ubiquitous 270 kDa protein, which belongs to a variety of multiprotein complexes containing histone deacetylases. These complexes repress transcription by deacetylation of lysine residues located in the N-terminal tails of histones.

NCoR and SMRT are structurally and functionally similar and share about 40% amino-acid identity. Both have been shown to repress the transcriptional activity of several nuclear receptors and of a variety of unrelated transcription factors involved in several cellular processes. For example SMRT represses serum response factor (SRF), activator protein-1 (AP-1), and nuclear factor-mB (NFmB), which are all transcription factors involved in stimulation of cell proliferation (Lavinsky et al., 1998; Shibata et al., 1997; Zamir et al., 1996). SMRT as well as NCoR display specific domain structures (Figure 17).

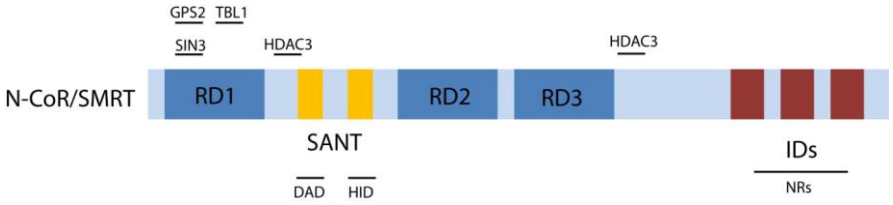

Figure 17: **Schematic N-CoR/SMRT domains with the associated proteins.** Adapted from (O'Malley and Kumar, 2008).

The C-terminus contains corepressor nuclear receptor (CoRNR) boxes, also called NR interactions domains (IDs), which interact with the LBD of NRs (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). The ID contains the sequence (L/I)XX(I/V)I or LXXX(I/L)XXX(I/L) (where X is any amino acid), and forms an extended α helix that interacts with the hydrophobic groove generated by H3, L3-4 and H4 of RARs, the N-terminal extension of the motif masking the H12 interaction interface (Figure 18). As this surface is topologically related to that involved in coactivator interaction, but without H12; this may explain why the binding of corepressors and coactivators is mutually exclusive (Hu and Lazar, 1999).

In contrast, the amino terminus of N-CoR and SMRT contains the domains responsible for transcriptional repression (Figure 17). Three repressive regions (RD1, RD2, and RD3) were originally described due to their ability to act as autonomous repression domains when assosiated to DNA binding proteins (Horlein et al., 1995). RD1 interacts with GPS2 but little is known about the role of GPS2 in NR repression (Zhang et al., 2002). RD1 also interacts with TBL1 and TBLR1, which are members of the WD40 family.

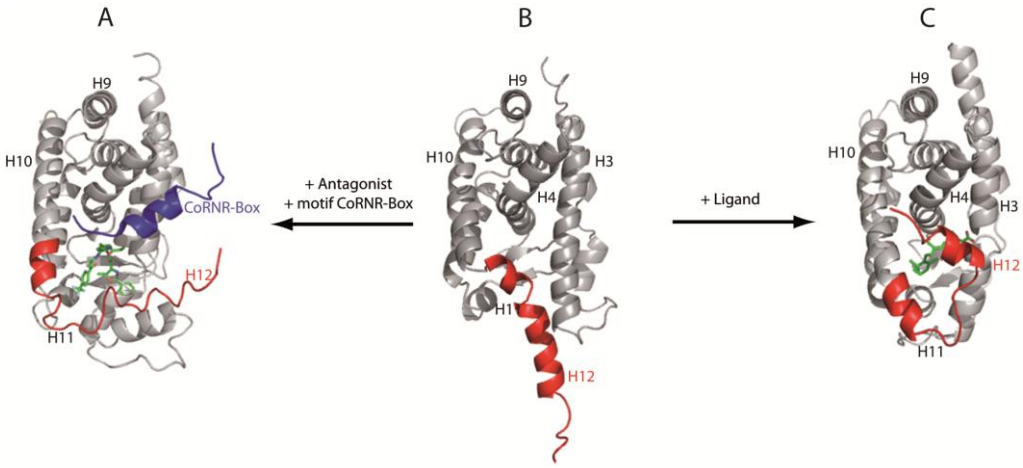

Figure 18: Interaction of NR LBDs with corepressors.
A. Structure of the LBD of PPARα complexed with an antagonist and a CoRNR box. Adapted from (Xu et al., 2002) pdb1kkq. B. Structure of apo RXR LBD. Adapted from (Bourguet et al., 1995) pdb1lbd. C. Structure of holo RARγ LBD (with an agonist). Adapted from (Renaud et al., 1995) pdb2lbd

TBL1 and TBLR1 interact simultaneously with deacetylated histone H4 (Yoon et al., 2003), thereby stabilizing the corepressor complex on chromatin and facilitating repression. The other repressive domains interact with HDAC3 and with SIN3, a component of the Sin3A corepressor complex (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). Remarkably, SIN3 can recruit additional enzymes with repressive activity such as the histone H3K9 methyltransferase ESET/SETDB1 and the ATP-dependent chromatin remodeling complex SWI/SNF (Underhill et al., 2000; Yang et al., 2003).

Between RD1 and RD2 there are two SANT (SWI3, ADA2, N-CoR, and TFIIB) motifs, which are also important for corepressor function. The first SANT motif forms a deacetylase activation domain (DAD), which stably associates with and activates histone deacetylase 3 (HDAC3) (Danielian et al., 1992; Umesono et al., 1991). The second one named histone interaction domain (HID), interacts directly with unacetylated histone H4 N-terminus tails (Yu et al., 2003).

- ACTIVATION OF TRANSCRIPTION UPON LIGAND BINDING

Ligand binding induces conformational changes of RARα, with reorientation of H12 (Figure 19), resulting in the formation of a charge clamp between a conserved glutamate residue in H12 and a lysine residue in H3. Such a charge clamp can form hydrogen bonds with the LxxLL motif of coactivators. However it does not fit with the extended LxxI/HIxxxI/L motif of corepressors. Thus, it has been proposed that the length difference of the interacting motifs might be at the origin of the alternative interactions of the hydrophobic cleft in the apo or holo conformations, with corepressors and coactivators (Germain et al., 2006b; Perissi et al., 1999).

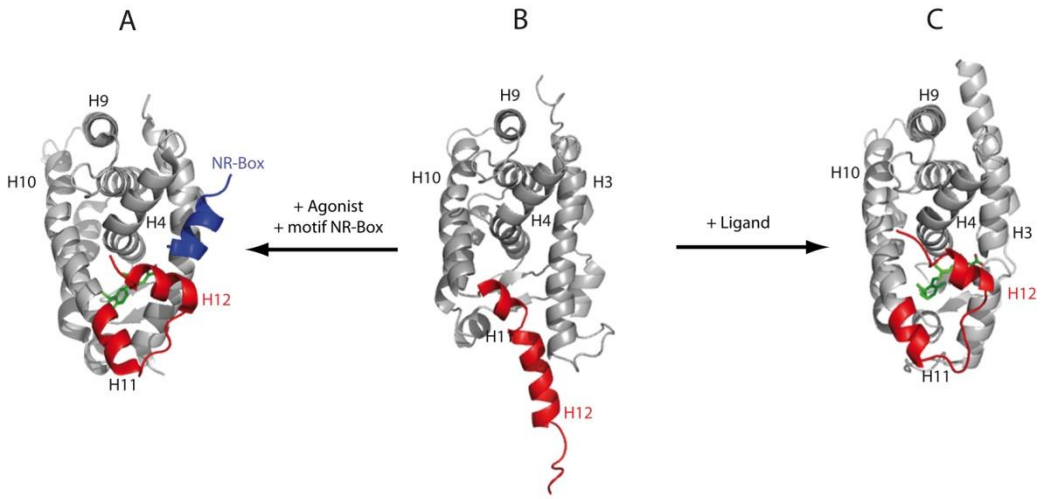

Figure 19: **Conformational changes in LBD and interaction with coactivators.**
A. Structure of the LBD of RXR bound to an agonist and to the NR box of coactivators. Adapted from (Lippert et al., 2009) pdb2zxz. B. Structure of apo RXR LBD. Adapted from (Bourguet et al., 1995) pdb1lbd. C. Structure of holo RARγ LBD complexed with a ligand. Adapted from (Renaud et al., 1995) pdb2lbd.

According to the classical model of transcriptional activation, ligand binding releases bound corepressors and promotes the recruitment of coactivators that serve as a platform for larger complexes with chromatin modifying and remodeling activities (Figure 20).

The coactivators of the p160/SRC (Steroid Receptor Coactivators) family have been the most extensively studied among the large spectrum of identified coactivators. This family comprises three members: SRC-1 (also referred to as NCoA-1), SRC-2 [NCoA2, TIF-2 (transcriptional intermediary factor 2), GRIP-1 (glucocorticoid receptor interacting protein 1)] and SRC-3 [pCIP (p300/CBP-interacting protein), ACTR (Acetyltransferase), AlB1 (amplified in breast cancer I), TRAM1 (thyroid

hormone receptor-activator molecule I), RAC3 (Receptor-Associated Coactivator 3)] (Chatterjee and Kashfi, 2011).

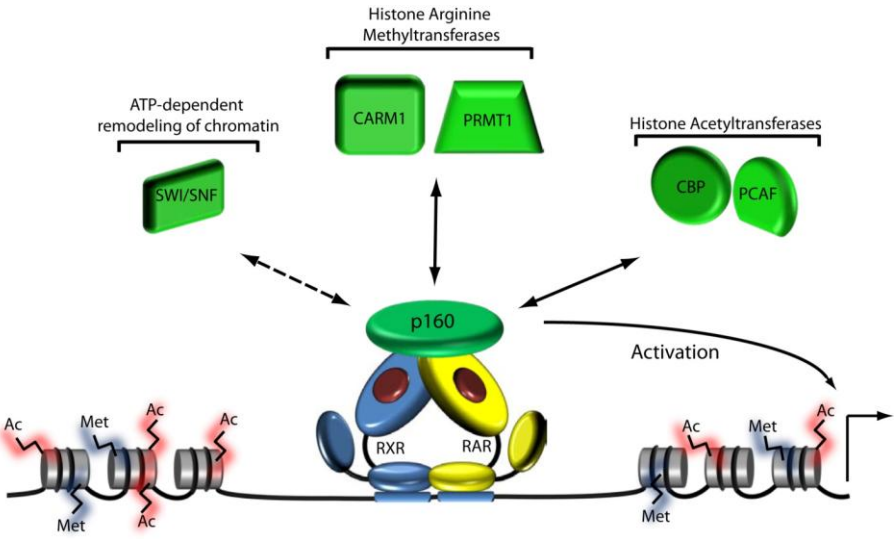

Figure 20: **Coactivators and associated complexes.** The RAR/RXR heterodimers in holo conformation recruit the p160 coactivators, which serve as a platform for chromatin modifying and remodeling complexes. Adapted from (Perissi and Rosenfeld, 2005).

The p160 SRCs are approximately 160 kDa in size and share an overall of 50-55% sequence similarity and 43-48% of sequence identity. Structurally p160/SRCs are composed of several domains (Figure 21). The central Receptor-Interacting Domain (RID) contains three LXXLL motifs or NR boxes and is responsible for interaction with the hydrophobic cleft of ligand-bound RARs. The C-terminal transcriptional activation domains (AD1 and AD2) recruit proteins that contribute to chromatin remodelling. AD1 is responsible for the recruitment of histone acetyltransferases such as p300/CBP (CREB-binding protein) and PCAF (P300/CBP-associated factor) (Chen et al., 1997; Stallcup et al., 2003; Torchia et al., 1997). AD2 usually recruits histone methyltransferases such as CARM1 (coactivator-associated arginine methyltransferase 1) and PRMT1 (Protein arginine N-methyltransferase 1) (Chen et al., 1999; Lee et al., 2005; Stallcup et al., 2003). Moreover, the C-terminal domain of SRCs itself shows a weak HAT activites (Chen et al., 1997; Spencer et al., 1997).

The N-terminal domain called bHLH/PAS (helix-loop-helix/Per/ARNT/Sim) is highly conserved and functions as a third AD (AD3) domain. It serves as a binding site for DNA-binding transcription factors such as TEF-4, MEF-2C, p53 and myogenin. Additionally, bHLH/PAS can recruit other coactivators including GAC63, CoCoA, FliI, G9a,BAF57 and ANCO1 (Kim et al., 2003; Lee et al., 2006).

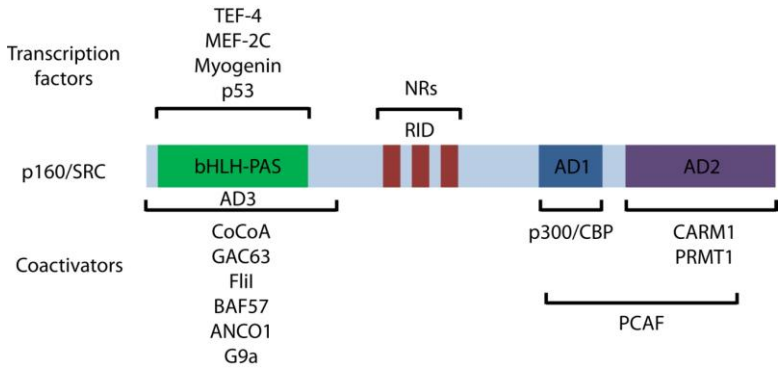

Figure 21: **Schematic representation of functional domains and interacting proteins of p160/SRC family.** Adapted from (O'Malley and Kumar, 2008).

The complexes with enzymatic activities associated to coactivators alter the chromatin structure around the promoter of target genes and create modifications of histone tails according to a "histone code". Indeed these modifications create new sites for the recruitment of other complexes such as SWI/SNF (SWItch/Sucrose NonFermentable) that also contribute to chromatin remodeling using the energy of ATP hydrolysis (Huang et al., 2003; Sims and Reinberg, 2008).

Finally, chromatin remodeling and modifications pave the way for the recruitment of the transcriptional machinery that includes the multisubunit Mediator complex DRIP205/TRAP220, RNA polymerase II and the general transcription factors (Bastien and Rochette-Egly, 2004; Dilworth and Chambon, 2001; Rochette-Egly, 2005; Rosenfeld et al., 2006). Note that the recruitment of the transcription machinery involves interaction of RARs with a specific subunit of the Mediator complex, DRIP205/TRAP220, which contains two LxxLL motifs (Lefebvre et al., 2005).

It must be noted that depending on the target gene's promoter context, RARs can carry out different programs for gene activation. Indeed, recent chromatin immunoprecipitation experiments showed that even in the absence of RA, RARα can occupy the promoters of some genes in association with the Mediator complex and RNA PolII (Flajollet et al., 2006; Mendoza-Parra et al., 2011; Pavri et al., 2005; Perissi et al., 2004). In this case, initiation of transcription relies on the dissociation of cdk8 (cyclin-dependent kinase 8 inhibitory subunit) from the Mediator complex (Andrau et al., 2006; Elmlund et al., 2006) and on the subsequent recruitment of the general transcription factors such as TFIIH.

- UNCONVENTIONAL COREGULATORS OF RARS

In addition to the above classical corepressors and coactivators several other unconventional coregulators have been characterized and divided in two groups: (i) corepressors with LXXLL motifs and (ii) coregulators devoid of LXXLL motifs.

1. Corepressors with LXXLL motifs

This group includes LCoR (Ligand-dependent CoRepressor), RIP140/NRIP1 (receptor interacting protein of 140kDa), PRAME (preferentially expressed antigen in melanoma) and TIF1α/Trim24 (transcription intermediary factor-1 α) (Augereau et al., 2006; Epping et al., 2005; Fernandes et al., 2003; Hu et al., 2004; Le Douarin et al., 1995). These proteins interact with liganded RARs via LXXLL motifs (one in TIF1α, seven in PRAME and nine plus a modified LxxML motif in RIP140), but in contrast to the classical coactivators, they attenuate the activity of RARs (Farooqui et al., 2003; Heery et al., 1997) via the recruitment of complexes with repressive activity.

2. Coregulators devoid of LXXLL motifs

This group includes:

SUG-1 (Suppressor of Gal 1) interacts with RARs and participates to chromatin remodeling via its ATPases activity. It is also a subunit of the 19S regulatory complex of the 26S proteasome and as such it promotes the degradation of RARs and SRC-3 (Ferry et al., 2009; Gianni et al., 2002).

CRABPII (Cellular Retinoic Acid Binding Protein II) together with cyclin D3 interacts with RARα (Bastie et al., 2001; Delva et al., 1999; Despouy et al., 2003), and serves for channeling of RA to the receptor (Budhu and Noy, 2002; Dong et al., 1999).

Cyclin H binds RARα at a specific docking site located in loop L8–9 of the LBD and allows the well-positioning of its-associated kinase cdk7 which then can phosphorylate the NTD of the receptor (Bour et al., 2005a) (see chapter 4).

TACC1 (transforming acidic coiled coil 1) serves as a scaffold protein and builds up a transcriptional complex around RARα (Guyot et al., 2010).

Actin and actin-binding proteins (ABP). In recent years, more and more attention has focused on the role of actin and ABPs in transcriptional regulation (Zheng et al., 2009a). Recently, the group of Dr. Rochette-Egly identified vinexin β as a new partner of the RARγ subtype (Bour et al., 2005b). In fact

vinexin β, which contains an SH3 domain, interacts with the NTD of RARγ and functions as a repressor via sequestering RARγ out of chromatin (Lalevee et al., 2010a) (see chapter 4, pages 43-44).

CHAPTER 4: NEW PICTURES OF RARS: RARS HAVE NON-GENOMIC EFFECTS AND ARE PHOSPHOPROTEINS

- THE NON-GENOMIC EFFECTS OF RARS: ACTIVATION OF KINASE PATHWAYS

Today, it is becoming increasingly evident that, in addition to their genomic effects, RARs also have a number of non-genomic effects. Indeed, recent studies from several laboratories, including ours, demonstrated that RA activates rapidly and transiently several kinase cascades.

RA has been shown to activate Protein Kinase C (PKC) (Kambhampati et al., 2003) and the PI3K/Akt pathway (Álvarez et al., 2007; Bastien et al., 2006) in several cell lines. RA also activates the MAPK/ERK signaling pathways in several neuronal cell lines such as neuroblastoma cell lines (Miloso et al., 2004), hippocampal cells (Chen and Napoli, 2008) and mouse embryonic stem cells committed to differentiate into neurons by RA (Stavridis et al., 2010). RA also induces this pathway in embryocarcimoma cells (Gupta et al., 2008).

RA was also found to activate p38MAPK and the downstream mitogen and stress-activated kinase MSK1 in various cell lines such as rat cardiac myoblasts, mouse embryonic fibroblasts, mouse embryocarcinoma cells, mammary breast tumor cells and leukemic cells (Alsayed et al., 2001; Bruck et al., 2009; Ren et al., 2007).

According to recent results from the laboratory of C. Rochette-Egly, these effects of retinoic acid are transcriptionally independent but involve RARs and more precisely the RARα subtype. Indeed no p38MAPK activation was observed in cells knockout for RARα (Bruck et al., 2009). However the molecular mechanism of p38MAPK activation by RARs was still unknown at the time of my arrival to the laboratory.

Remarkably, the other nuclear receptors and their cognate hormones are also able to activate the MAPK pathways (Piskunov and Rochétte-Egly 2012) (Figure 22). Indeed, most of the steroid hormones, estrogens, progestins, and androgens activate the ERK pathway while glucocorticoids and vitamin D activate p38MAPK like RA. The interesting point is that the non-genomic effects of these hormones were recently found to be mediated by a subpopulation of their cognate receptor present in specialized plasma membrane structures such as caveolae and lipid rafts (Marquez et al., 2006; Matthews et al., 2008; Norman et al., 2004), that contain lipids, structural proteins like flotillin and caveolin, and several proteins involved in signal transduction including heterodimeric G proteins, c-Src, Rho and RAC GTPases and Phosphoinositide 3-kinase (PI3K) (de Laurentiis et al., 2007; Luoma et al., 2008).

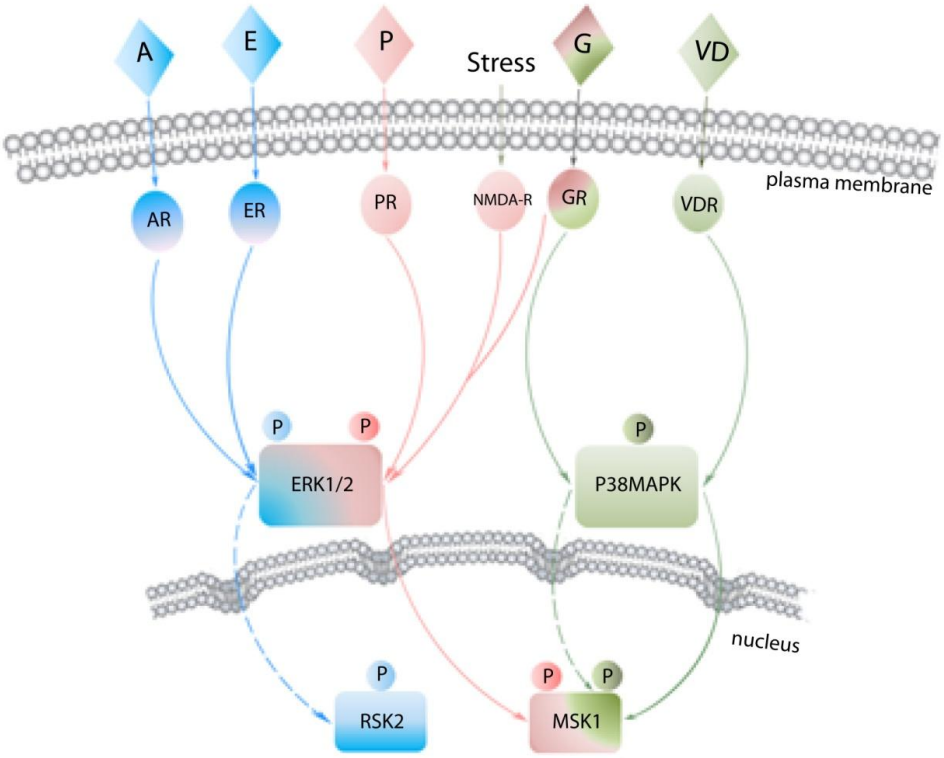

Figure 22: **Non-genomic effects of nuclear receptors.** A subpopulation of the classical steroid receptors (ER, PR, GR, AR) and non-steroid receptors VDR is associated to cell membranes and initiates cascades of kinase activations upon binding of their cognate ligands. Accordingly, liganded non-steroid receptor VDR activates p38MAPK and the downstream MSK1 kinase. Among the steroid receptors, GR bound to glucocorticoids also activates p38MAPK but there is no data indicating whether MSK1 is activated or not. However, upon the concomitant activation of NMDA-R under stress conditions, liganded GR activates MSK1 but through Erks. The other steroid receptors PR, ER and AR also activate Erks but only PR was found to activate the downstream MSK1. In the case of ER and AR another downstream effector of Erks, RSK2 would be an interesting candidate. Adapted from (Piskunov and Rochette-Egly, 2011b).

In these rafts, steroid NRs are part of protein complexes and activate MAPK signaling through interaction with specific signaling proteins. As an exemple, in response to the hormone, ERα rapidly activates the Src/p21ras/Erk pathway via direct interaction with the SH2 domain of c-Src. Progestins and androgens also activate this signaling cascade via direct interaction of the cognate receptor with the SH3 domain of c-Src or with ERα which itself activates c-Src (Castoria et al., 2003; Hagan et al., 2009; Migliaccio et al., 2000; Migliaccio et al., 1998) (Figure 22).

- RARS ARE PHOSPHOPROTEINS

A number of studies demonstrated that RARs, like most nuclear receptors, are targets for phosphorylation processes (Rochette-Egly, 2003; Rochette-Egly, 2005). Several phosphorylation sites and associated kinases have been identified for RARs and RXRs (Table 2).

Receptor	Domain/Region	Sesidue	Kinese	Referense
RARα	NTD	S77	cdk7/cycH(TFIIH)	(Rochette-Egly et al., 1997)
	DBD	S96	Akt	(Srinivas et al., 2006)
		S115	PKC	(Sun et al., 2007)
		S157	PKC	(Delmotte et al., 1999)
		T181	JNK	(Srinivas et al., 2005)
	Hinge		CAMKIIγ	(Si et al., 2007)
	LBD	S369	PKA/MSK1	(Bruck et al., 2009; Rochette-Egly et al., 1995)
	F region	S445, S461	JNK,cdk7	(Rochette-Egly et al., 1997; Srinivas et al., 2005)
RARβ2	NTD			(Rochette-Egly and Germain, 2009)
RARγ2	NTD	S66	p38MAPK	(Bastien et al., 2000)
		S68	cdk7/cycH(TFIIH)	(Gianni et al., 2002)
	LBD	S360	PKA/MSK1	(Rochette-Egly et al., 1995)
RXRα1	NTD	S22	cdk/cyc	(Bastien et al., 2002)
		S32	JNK	(Mann et al., 2005)
		S61, S75, T87	JNK	(Adam-Stitah et al., 1999; Bruck et al., 2005)
	DBD	T162	PKC	(Sun et al., 2007)
	LBD	S265	JNK	(Adam-Stitah et al., 1999; Bruck et al., 2005; Li et al., 2002)

Table 2: RAR and RXR phosphorylation sites and associated kinases.

However the most important and functional phosphorylated residues lie within the LBD and the N-terminal domain (NTD) (Figure 23). The LBD of RARs depicts one main phosphorylation site, serine 369 in RARα, which is located in the loop between helices 9 and 10 and belongs to an arginine-lysine-rich motif. This motif corresponds to a consensus phosphorylation motif for several kinases such as cyclic AMP-dependent protein kinase (PKA) and MSK1 and thus may integrate several signaling pathways (Gaillard et al., 2006).

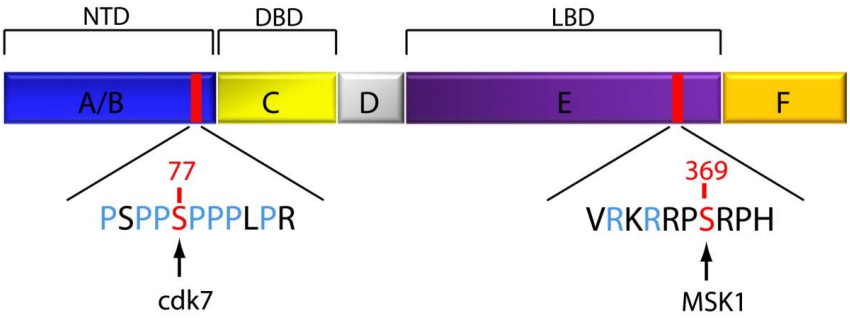

Figure 23: Main phosphorylation sites of RARα. The LBD contains a phosphorylation site for the MSK1 kinase. The N-terminal domain contains a proline-rich motif with a phosphorylation site for Cdk7. Adapted from (Rochette-Egly and Germain, 2009).

The NTD also contains a phosphorylation site, serine 77 in RARα, which is located at the C-terminal end of the NTD, in proximity of the DBD. This serine belongs to a proline-rich motif (PRM) and therefore corresponds to a consensus site for cyclin-dependent protein kinases (cdks) and Mitogen-Activated Protein Kinases (MAPKs). The kinase involved in the phosphorylation of this residue has been identified in the laboratory (Rochette-Egly et al., 1997). It is the cdk7 kinase, which belongs to the cyclin-dependent kinase (CDK)-activating kinase (CAK) subcomplex of TFIIH (general transcription factor composed of 10 subunits). Interestingly, the activity of cdk7 depends on the association of the kinase with other components of the CAK complex, cyclin H and MAT1 (Giglia-Mari et al., 2004). Recently, it has been shown in the laboratory that the accurate positioning of the cdk7 kinase and thus the phosphorylation of the NTD by cdk7 depends on the docking of cyclin H at a specific domain of the LBD, encompassing loop L8-9 and the N-terminal part of H9 (Bour et al., 2005a; Gaillard et al., 2006).

Remarkably, the two phosphorylation sites are conserved between the different RAR subtypes in mammals (Samarut et al., 2012). However the phosphorylation site located in the LBD is not conserved in the other nuclear receptors (Rochette-Egly, 2003). The phosphorylation site of the NTD is not conserved either as none of them depict a stretch of proline residues around the serine residue similar to that of RARs. Nethertheless all nuclear receptors (except VDR due to its very short NTD), contain other phosphorylation sites that are substrates for several types of kinases such as cdks, p42/p44MAPK, p38MAPK, and c-Jun N-terminal kinases (JNKs) (Chen et al., 2008b; Lannigan, 2003; Rochette-Egly, 2003; Wang et al., 2007; Weigel and Moore, 2007a; Weigel and Moore, 2007b; Weigel and Moore, 2007c).

- RARS PHOSPHORYLATION INVOLVES A CASCADE OF KINASES

Remarkably, the two main serine residues of RARs are rapidly phosphorylated in response to RA (Bruck et al., 2009). Recent studies demonstrated that this RA-induced phosphorylation of RARα results from a coordinated phosphorylation cascade starting with the phosphorylation of serine 369 by MSK1 (Bruck et al., 2009). Interestingly, phosphorylation of this serine increases the flexibility of loop L8–9 (Samarut et al., 2012), which is in close proximity and corresponds to the cyclin H binding domain (Figure 24). Consequently, the binding of cyclin H and the associated cdk7 to this domain is facilitated and the serine located in the NTD can be phosphorylated by cdk7 (Gaillard et al., 2006).

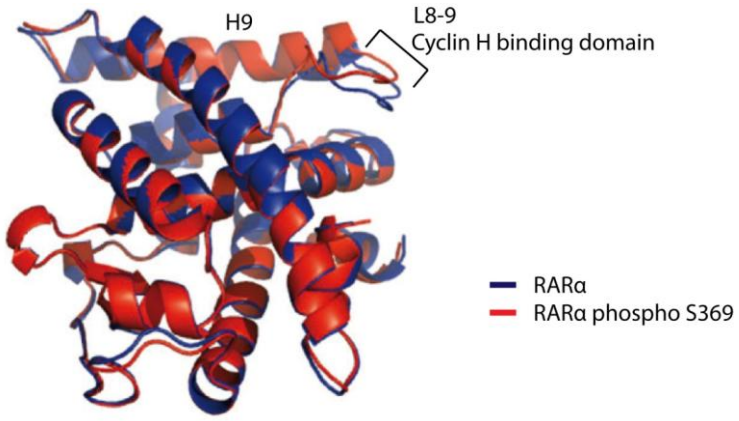

Figure 24: Superposition of the average structures of the RARα LBD unphosphorylated (blue) and phosphorylated (red). Adapted from (Samarut et al., 2012).

This is a unique example of cooperation between the N- and C-terminal domains of RARs through a kinase complex. The docking site of cyclin H and the two phosphorylation sites are conserved between RARs, but whether the above cascade is common for all RARs needs to be further investigated.

Remarkably, the cyclin H docking site as well as the serine located in loop L9-10 are not conserved in other nuclear receptors and other cdk7 targets, suggesting that the cascade and the phosphorylation by cdk7 might be specific for RARs.

- CONSEQUENCES OF PHOSPHORYLATION ON RARS ACTIVITY

1. RAR phosphorylation and the transcription of RA target genes

Recently, Bruck *et al.* highlighted a new paradigm in which, *in vivo*, the RA-induced phosphorylation cascade, starting with the rapid activation of p38MAPK and ending with the phosphorylation of RARα, is crucial for RARα-mediated transcription (Bruck et al., 2009) (Figure 25). Indeed, they demonstrated that the expression of several RA target genes is abolished upon silencing of p38MAPK or MSK1 and upon mutation of RAR phosphorylation sites. At the molecular level, the MSK1-dependent phosphorylation of S369 located in the LBD of RARα is critical for the binding of TFIIH. Moreover, phosphorylation of S77, which is a consequence of S369 phosphorylation and the last step of all phosphorylation cascades, promotes the recruitment of RARα to the target genes promoters. These results corroborate previous observations in fibroblasts from patients suffering from Xeroderma pigmentosum (Keriel et al., 2002). In these cells, which are characterized by mutations of a TFIIH subunit, RARα is hypophosphorylated at serine 77 and the RA response is deficient.

In parallel, activated MSK1 is also recruited to RARα target promoters where it phosphorylates histones H3 at serine 10 (Bruck et al., 2009). This phosphorylation process, which is coupled to the acetylation of nearby lysine residues (K9 or K14) (Cheung et al., 2000; Clayton et al., 2000; Lo et al., 2000), and to the recruitment of the chromatin remodeling complex SWI/SNF (Vicent et al., 2006) paves the way for the recruitment of RARs and the transcriptional machinery.

Altogether, these data highlight that there is cooperation between the phosphorylation of RARα and that of histones for the recruitment of RARα to RAREs and the initiation of transcription of the target genes (Figure 25).

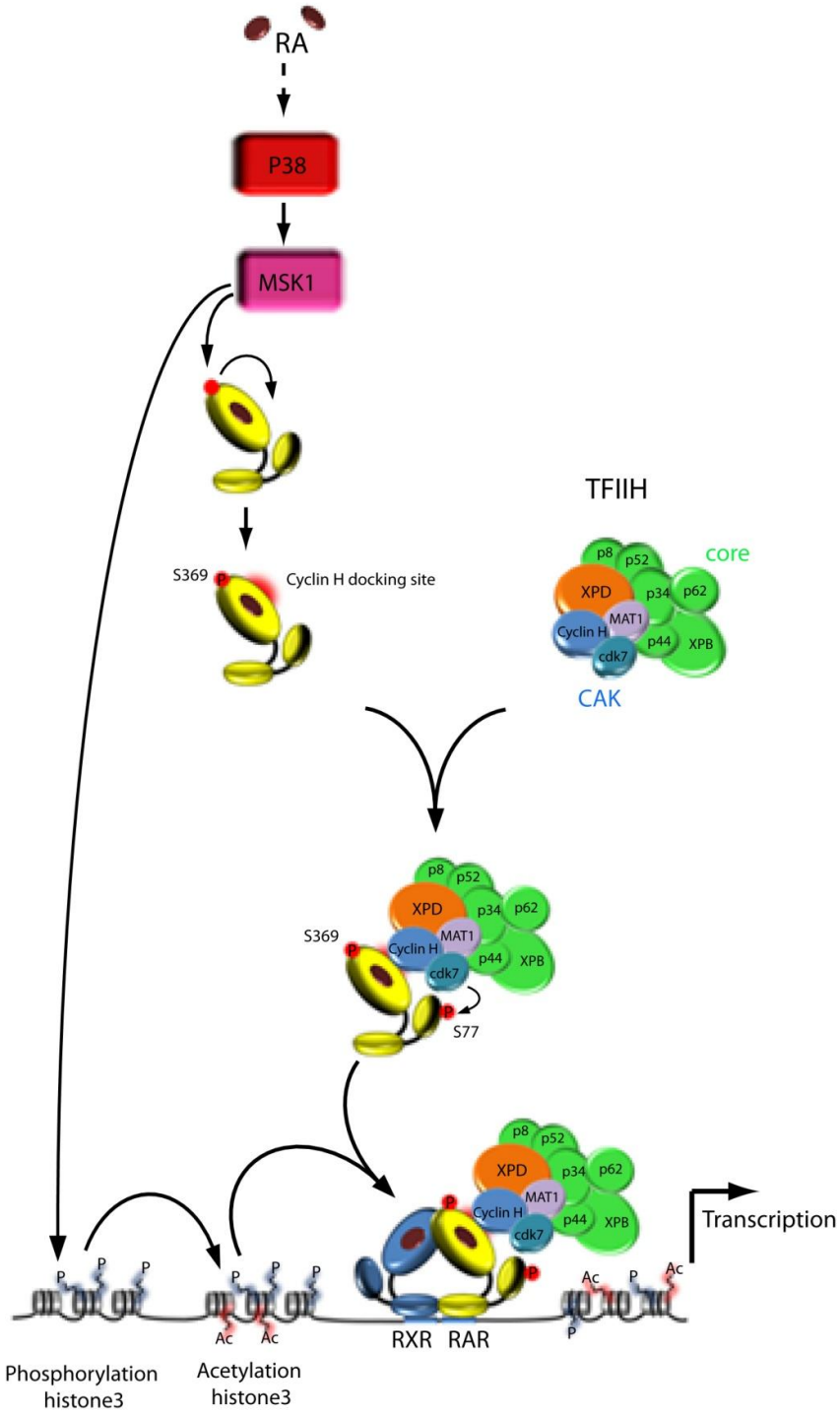

Figure 25: **Cooperation of genomic and non-genomic effects in the initiation of transcription of RARα target genes in response to RA**. Adapted from (Bruck et al., 2009).

2. RARs phosphorylation and their subcellular localization

Generaly, nuclear receptors display a nuclear localization. However, some of them such as AR, GR, and MR are cytoplasmic and translocate to a nuclei upon ligand induction.

Several processes control the nuclear localization of nuclear receptors among which phosphorylation processes. Indeed, recent results indicated that phosphorylation of specific sites enhances nuclear localization while phosphorylation of others increases nuclear export (Lombardi et al., 2008; Sun et al., 2007; Weigel and Moore, 2007a). Han *et al.* recently demonstrated that phosphorylation of the RARγ NTD by p38MAPK induces the cytoplasmic localization of receptor (Han et al., 2009).

3. RARs phosphorylation and binding proteins with WW or SH3 domains

The phosphorylation site located in the NTD of RARs belongs to a proline-rich motif (PRM) (Figure 26).

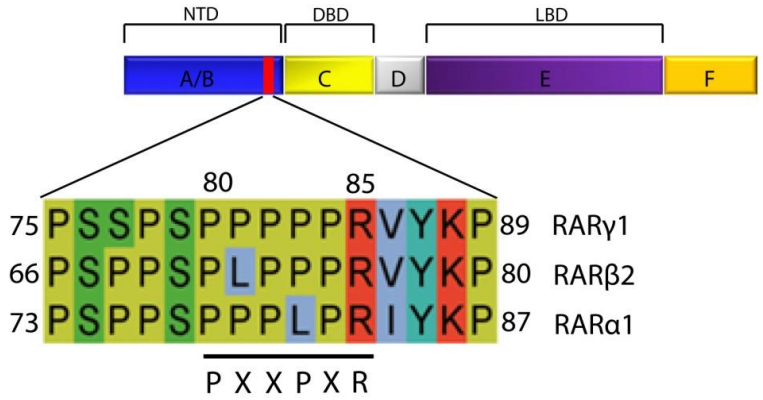

Figure 26: Amino acid sequence of the PRM of RARγ1, RARβ2 and RARα1. Adapted from (Lalevee et al., 2010a).

Such motifs are well known to bind proteins with SH3 or WW domains. Moreover, phosphorylation of a serine residue located in PRMs has been shown to prevent or favorize interactions (Kay et al., 2000b; Macias et al., 2002; Sudol et al., 2001; Zarrinpar and Lim, 2000). Indeed, phosphorylation may involve cis-trans isomerization of the proline residues surrounding the phosphorylated serines, creating new specific recognition sites for interacting factors (Bao et al.,

2004). In this context the peptidyl-prolylisomerase Pin1, an important enzyme with a WW domain and involved in the regulation of signaling pathways, transcription and cell cycle progression (Nelson et al., 2006) has been shown to interact with the phosphorylated form of the NTD of RARα (Brondani et al., 2005).

Recently, the group of Dr. Rochette-Egly identified and characterized vinexin β as a novel co-regulator interacting directly with the PRM of RARγ (Bour et al., 2005b). Vinexin β is an actin-binding protein which plays a role in cytoskeleton organization, cell spreading and intracellular signaling. It is an adaptor composed of three SH3 domains without any enzymatic activity (Kioka, 2002). Only the third C-terminal domain of vinexin β (SH3-3) interacts with a consensus PxxPxR motif located in the PRM of RARγ (Lalevee et al., 2010a) (Figure 26). Most interestingly, substitution of the second proline of the motif with a leucine, as in RARα, abolishes the interaction and explains why vinexin β interacts specifically with the RARγ subtype.

Vinexin β is present not only in the cytosol but alo in the nucleus where it interacts with the non-phosphorylated form of RARγ (Bour et al., 2005b). Then in response to RA, phosphorylation of serine 79 (the equivalent of S77 in RARα), which is in the vicinity of the PxxPxR motif, induces the dissociation of vinexin β. According to recent unpublished results from the laboratory, phosphorylation of this residue would induce subtle changes in the conformation of the polyproline helix, decreasing its propensity to interact with the SH3 domain of vinexin β.

In fact, vinexin β is a repressor of RARγ-mediated transcription. As vinexin β is undetectable in chromatin, Lalevée *et al.* proposed that it would sequester the non-phosphorylated RARγ in an inactive state out of chromatin. The dissociation of vinexin β subsequent to the phosphorylation of the PRM would allow the recruitment of RARγ to chromatin and thereby the activation of the target genes (Lalevee et al., 2010a).

Knowing that vinexin β is a scaffolding protein with three SH3 domains, which can interact with several proteins at the same time, the following model was proposed. Vinexin β and RARγ belong to a multiprotein complex in which vinexin β interacts directly with the NTD of RARγ via its third SH3 domain and indirectly with the LBD of RARγ through two other SH3 domains and intermediary proteins (Lalevee et al., 2010a) (Figure 27).

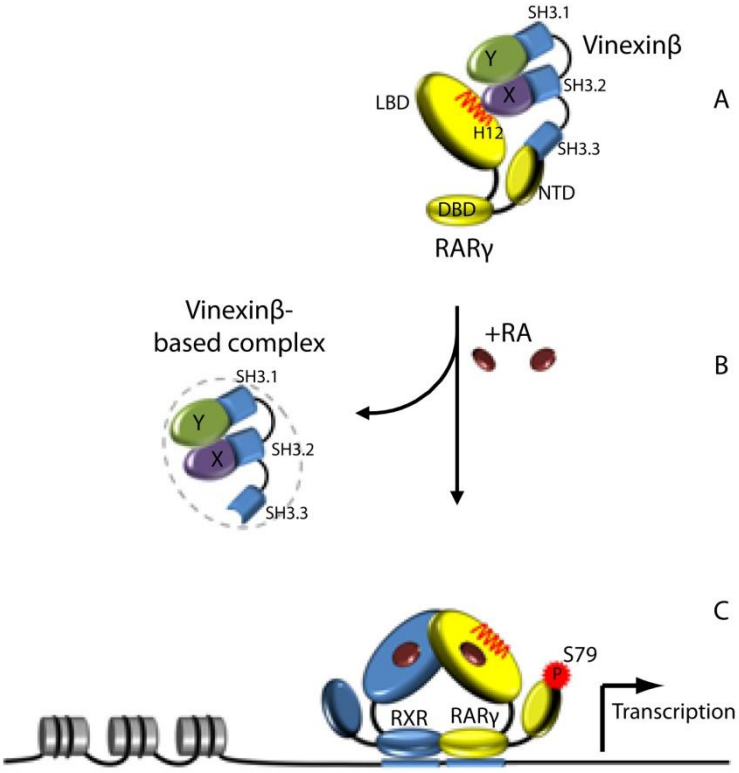

Figure 27: Model for the role of vinexin β in the control of RARγ transcriptional activity.
A. In the absence of RA, the third SH3 domain of vinexin β (SH3.3) interacts with the non-phosphorylated N-terminal PRM of RARγ. Through other two SH3 domains, vinexin β might also act as a scaffold linking the NTD to the LBD of RARγ *via* protein complexes (exemplified as X and Y). Such a complex sequesters RARγ out of chromatin, thereby impeding transcription. B. Upon RA addition, phosphorylation of the RARγ PRM and the conformational changes of the LBD cooperate to induce the dissociation of the vinexin β-based complex. C. Once separated from vinexin β-based complex, phosphorylated RARγ can dimerize with RXR and occupy the promoters of the target genes to initiate the transcription of RA-target genes. Adapted from (Lalevee et al., 2010a).

CHAPTER 5: IMPORTANCE OF ACTIN-BINDING PROTEINS WITH SH3 DOMAINS

The discovery that RARs can interact with actin-binding proteins such as vinexin β was new and rather unexpected. In fact, it is not so surprising as other proteins of the same vinexin family, and several other actin-binding proteins such as gelsolin have been shown recently to interact with several activators of transcription including nuclear receptors (Table 3) (Zheng et al., 2009a). Moreover, there is a growing evidence that actin and actin-binding proteins (ABPs) are present in nuclei and play an important role in key nuclear processes: chromatin remodeling, transcription, DNA repair, DNA replication and formation and maintenance of nuclear structure (Figure 28) (For review see (Castano et al., 2010)).

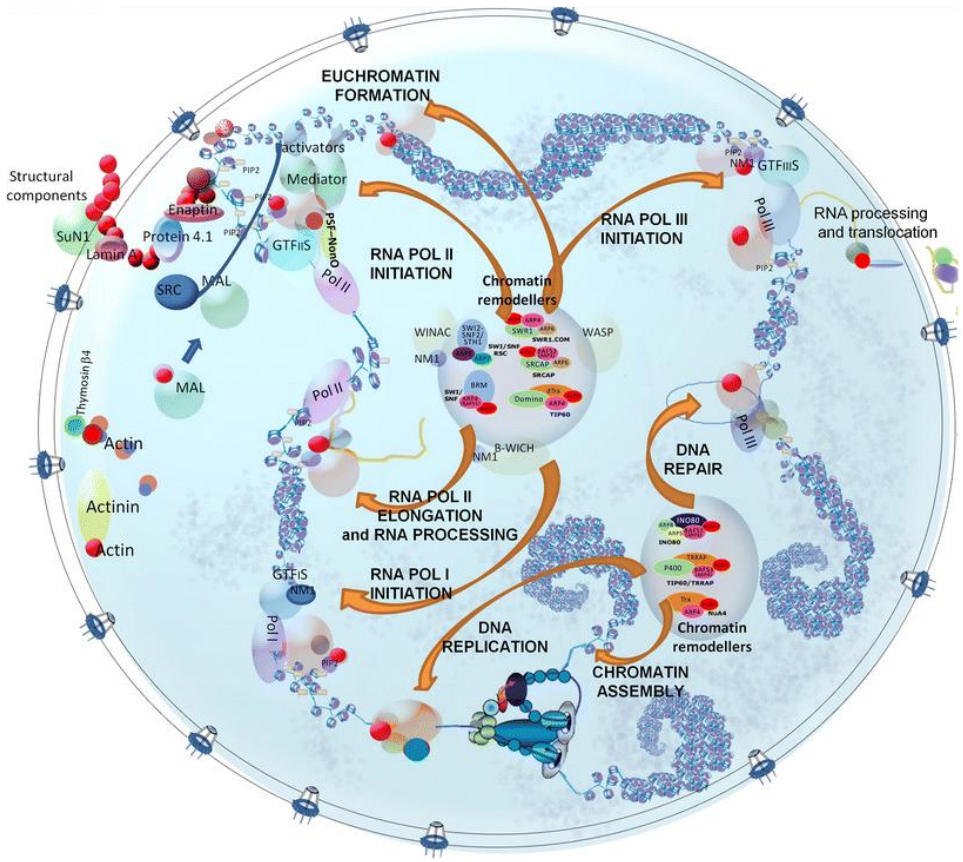

Figure 28: Model showing the actin, actin-binding proteins and actin complexes involved in chromatin remodeling, transcription initiation and elongation, DNA repair and replication. Actin and actin-binding proteins may function as key proteins bringing together various factors to form a network that can be used in several processes in the nucleus. Adapted from (Castano et al., 2010).

How ABPs regulate nuclear processes is not fully understood but they may function as adaptors recruiting actin, which is now known to have a structural and/or regulatory role with chromatin-remodeling complexes and the RNA polymerase machinery.

Actin-binding protein	Classes	Role in the cytoplasm	NR type assosiation	NR effect	Direct/ indirect association with the AR	Region
Gelsolin	Actin filament severing and capping protein	Involved in gel-to-sol transformations;severs and caps polymeric actin filaments; acts in the actin-scavenging system; inhibits actin polymerization	AR	Co-activator	Direct	LBD
Flightless I	Actin-remodeling proteins	Possess F-actin-serving activity	AR	Co-activator	Direct	
α-actinin 2	Bundling proteins	Functions as scaffolds for signaling intermediates that stimulate actin elongation; binding partners for ICAM-1	AR	Co-activator	Indirect	
α-actinin 4	Bundling proteins	Functions as scaffolds for signaling intermediates that stimulate actin elongation; binding partners for ICAM-1	ER VDR	Co-activator	Direct	
Vinexin α	adaptor protein	Promotes up-regulation of actin stress fiber formation	AR, ERα , ERβ, GR	Co-activator		
Vinexin β	adaptor protein	Regulates cell spreading	RARγ	Co-repressor	Direct	NH2-terminal
Supervillin	F-actin- and membrane-associated scaffolding protein	Regulates cell-substrate adhesion; organization of muscle co-stameres; stimulus-mediated contractility of smooth muscle and myogenic differentiation	AR	Co-activator	Direct	NH2-and COOH-terminal
Filamin	Cross-linking proteins	Cytoplasmic transport; membrane integrity; cellular adhesion	AR	Co-activator	Direct	Hinge
Filamin A	Cross-linking proteins	Cross-links actin filaments; recruits F-actin into extended networks	AR	Co-repressor	Direct	Hinge
Transgelin	Cross-linking proteins	Organizes actin filaments into dense meshworks	AR	Co-repressor	Indirect	LBD

Table 3: Actin-binding proteins interacting with Nuclear Receptors.
Adapted from (Zheng et al., 2009a).

According to recent studies NRs also interact with cytoskeleton proteins out of the nucleus and participate to their non-genomic effets. For example the androgen receptor has been shown to interact with filamin out of nuclei. As such, AR belongs to a multiprotein complex which is involveved in regulation of cell migration (Castoria et al., 2011).

As all RARs contain a PRM in their NTD, the team speculated that they might interact not only with vinexin β but also with other actin-binding proteins with SH3 domains. As such proteins are generally adaptors, they might link RARs either to big nuclear complexes controlling transcription or to extranuclear signaling complexes.

Recently, the group identified in a yeast two-hybrid screening, a new binding partner of the NTD of RARα, profilin IIA, which is also an actin-binding protein with an SH3-like domain. Therefore another aspect of my work was to focus on this new unconventional partner.

- PROFILIN

Profilin is a small (MW = 12-16 kDa) ubiquitous actin-binding protein, which was originally identified in calf spleen. Its principle role is to form a complex with actin monomers and to inhibit their polymerization (Carlsson et al., 1977). Up to date, profilins have been found in all studied eukaryotes and their cells, indicating that it is a fundamental actin-binding protein (Witke, 2004).

1. *Profilin Isoforms*

In mammals, five isoforms of profilin are known: profilin I, IIA, IIB, III and IV (Table 4) (Carlsson et al., 1977; Di Nardo et al., 2000; Honore et al., 1993; Hu et al., 2001; Obermann et al., 2005). While all isoforms of profilin are conserved in terms of function, overall fold and crystallized structures, their sequence identity is variable. For example, profilin 1 shares only 37 and 30% amino acid homology with profilins III and IV, respectively (Obermann et al., 2005; Witke, 2004).

However, there are several examples showing that in some species, profilin-deficiency can be rescued by reintroducing profilins from another species. Indeed, Rothkegel et al. (Rothkegel et al., 1996) demonstrated that plants profilin, which share only 22% sequence identity with bovine thymus profilin, can functionally substitute the endogenous mammalian profilin. Another study demonstrated that defects in cell shape, cytokinesis, and development of *Dictyostelium discoideum* profilin-deficient cells can be rescued by profilins I or II from maize (Karakesisoglou et al., 1996). However, isoforms within the same organism differ sufficiently and cannot complement each other. As an example, deletion of profilin II isoforms in mice, cause severe neurological abnormalities, despite the presence of profilin I (Witke, 2004).

Profilin 1 is expressed in various tissues except skeletal muscle (Witke et al., 1998). It shows diffuse cytoplasmic distribution and dot-like nuclear localization (Giesemann et al., 1999). Profilin I is actively involved in the regulation and re/organization of the cytoskeleton and has been involved in the regulation of nonmuscle cell motility.

Profilin II is almost exclusively expressed in the central nervous system, but mRNAs encoding this isoform has been detected in kidney and muscle (Lappalainen, 2007). Profilin II can be alternatively spliced in two isoforms: profilin IIA and IIB. They share the first 107 amino acids but have different C-terminal domains. It was shown in mice that profilin IIA is dominantly expressed during embryogenesis at stages of rapid brain development (Di Nardo et al., 2000; Lambrechts et al., 2000), and mice deficient for this isoform have neurological defects (Witke et al., 2001). The minor IIB-isoform, similarly to the profilin encoded by Vaccinia virus (Machesky et al., 1994), is unique among profilins because it does not show any binding affinity to poly-L-proline. Interestingly, this particular profilin also has low affinity for actin, but exceptionaly appears to bind tubulin (Di Nardo et al., 2000).

Profilins III and **IV** are both testis-specific (Braun et al., 2002; Hu et al., 2001; Obermann et al., 2005). They are expressed at various stages of spermatogenesis and are differentially regulated during postnatal testicular development. They interact with the actin cytoskeleton of developing male germs cells at distinct point locations. Their deficiency causes morphological abnormalities in the head of sperm and functional defects of acrosome in infertile male (Obermann et al., 2005).

2. Profilin structure

The structure of profilins has been solved by x-ray crystallography either complexed with β-actin (Schutt et al., 1993) or separately (Cedergren-Zeppezauer et al., 1994). It consists of a central seven-stranded anti-parallel β-pleated sheet and of 4 alpha helices: the N- and C-terminal α-helices (named helix 1 and helix 4, respectively) are closely packed on one side while the two short α-helices (helix 2 and helix 3) are on the opposite side (Cedergren-Zeppezauer et al., 1994; Schutt et al., 1993) (Figure 29).

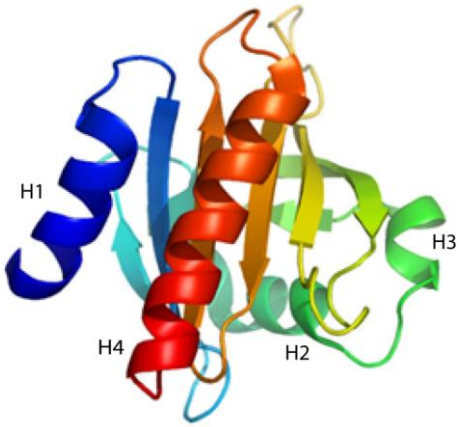

Figure 29: The three-dimensional structure of mouse profilin IIA. Helices are shown as ribbons and labelled from H1 to H4**.** Adapted from (Kursula et al., 2008) pdb 2v8c.

In mammalian profilin, strands 5 and 6 (K90-T97) are connected with a loop, which protrudes from the surface of the protein (Schutt et al., 1993) and is positioned close to an actin-binding site. Remarkably, profilins also depict an SH3-like domain, which is composed of the N- and C-terminal α-helices.

3. Profilin interaction domains

In general, profilins were found to interact with three classes of ligands: (i) actins (Schutt et al., 1993) including actin-related proteins (ARPs) (Machesky et al., 1994), (ii) poly-L-proline (PLP) stretches (Mahoney et al., 1997) and (iii) phosphatidyl- inositol lipids (Lassing and Lindberg, 1988) (Figure 30).

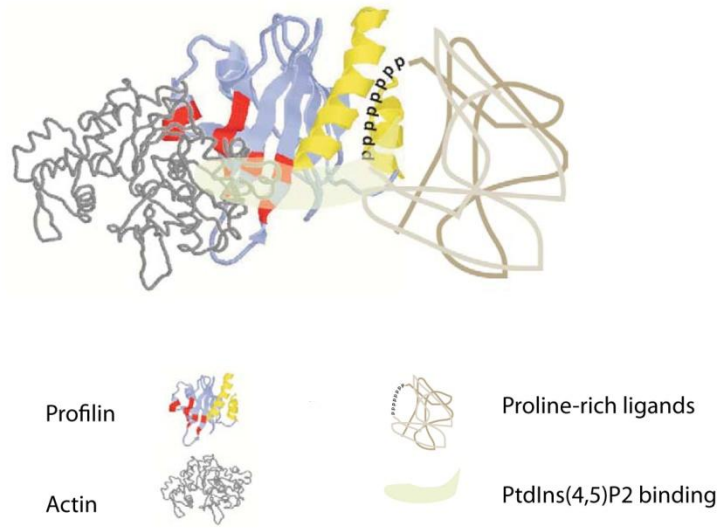

Figure 30: Structure of profilin complexes. Profilins form complexes with actin, proline-rich ligands and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2]. The actin-binding sites (red) and poly-L-proline (yellow) are distinct, while the PtdIns(4,5)P2-binding surface (light green) is more spread out over the surface of the molecule and overlaps with both actin-binding and poly-L-proline binding. Adapted from (Witke, 2004).

- Actin-binding site

The actin-binding site of profilin consists of amino acid residues located in α-helices 3 and 4, β-strands 4, 5, 6 (Figure 31). It also consists of the extended loop regions between β-strands 5 and 6, as a double-deletion of two residues (P96 and T97) within this loop, lowers the interaction with actin (the Kd value changed from 0.37 μM to 1.15 μM (Hajkova et al., 1997).

All these residues make contacts with residues in subdomains 1 and 3 at the barbed end of actin monomers (Cedergren-Zeppezauer et al., 1994; Schutt et al., 1993). However they can also bind

the most distal subunit of the fast-growing end of filamentous actin (Gutsche-Perelroizen et al., 1999) as well as actin related proteins (ARPs) (Mullins et al., 1998) and postsynaptic scaffolding protein gephyrin (Giesemann et al., 2003).

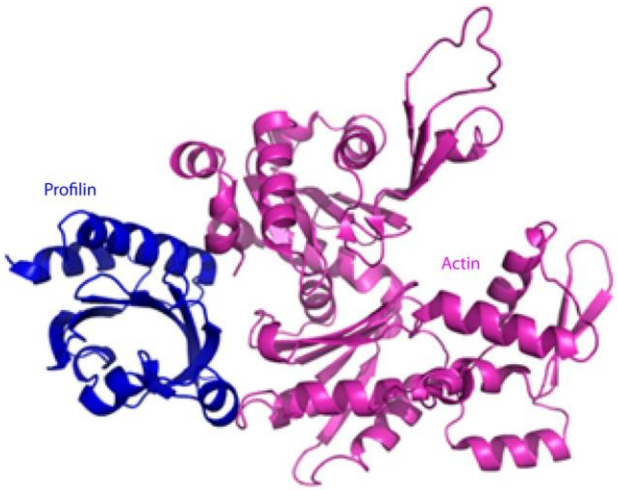

Figure 31: Structure of bovine beta-actin(pink)-profilin(blue) complex. Adapted from (Chik et al., 1996) pdb 1hlu.

- Polyphosphoinositide lipid binding site and association with plasma membrane

Profilin has two phosphatidylinositol-4,5-bisphosphate (PIP2) interaction sites: one partially overlaping with the poly-L-proline binding surface and the other one with the actin-binding surface (Jockusch et al., 2007; Lambrechts et al., 2002; Lappalainen et al., 2007; Skare and Karlsson, 2002) (Figure 30). The affinity for PIP2 is variable between the profilin isoforms, with profilin I displaying a higher affinity than profilins IIA and IIB (Lambrechts et al., 2000).

The interaction of profilin with PIP2 mediates its association to the plasma membrane. Indeed, depletion of membrane PIP2 levels results in a translocation of profilin from the membrane to the cytosol. Remarkably, upon restoration of PIP2 plasma membrane levels, profilin translocates back to the membrane fraction, suggesting that phosphoinositide metabolism plays a role in the localization of profilin (Ostrander et al., 1995).

- Poly-L-Proline binding site

Binding of poly-L-proline involves the N- and C-terminal α-helices of profilins (Figure 32), which form a hydrophobic and aromatic core (SH3-like domain). In profilin I, this domain consists of the side chains of residues W3, Y6, W31, H133, L134 and Y139 (Bjorkegren et al., 1993; Cedergren-Zeppezauer et al., 1994; Mahoney et al., 1997; Mahoney et al., 1999; Schutt et al., 1993). In profilin IIA it consists of residues Y7, N10, Y134 and F140 (Haikarainen et al., 2009). It has been suggested that this difference in amino acid composition would be at the basis of the higher affinity of profilin IIA for poly-L-proline ligands (Lambrechts et al., 1995; Lambrechts et al., 1997).

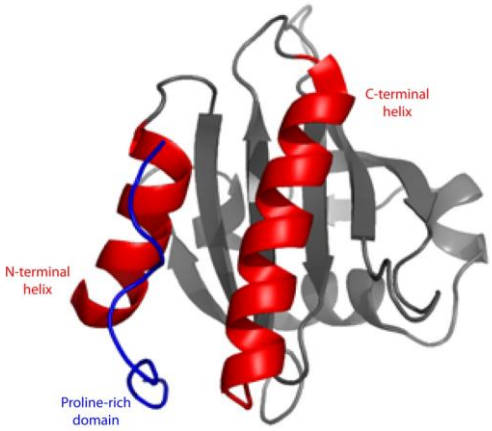

Figure 32: Mouse profilin IIA in complex with proline-rich domain (blue). Binding to proline-rich domain involves the N- and C-terminal α-helices (red) of profilins. Adapted from (Kursula et al., 2008) pdb 2v8c.

Initially, the ability of profilin to binds poly-L-proline was widely used for the purification of profilin and profilin:actin-complex by affinity chromatography on poly-L-proline (Celis, 2006; Lindberg et al., 1988). Now, the poly-L-proline binding activity is considered as an important feature of profilin and several proline-rich ligands have been identified. The first one to be identified was the vasodilator-stimulated phosphoprotein (VASP) (Reinhard et al., 1995), which is a component of focal adhesions and is thought to regulate actin polymerization (Krause et al., 2003; Walders-Harbeck et al., 2002). Several other proline-rich ligands have been identified (Jockusch et al., 2007; Witke, 2004), with a variety of proline-rich sequence-motifs with eight to ten prolines, either in continuous sequence or interrupted by single glycines (Schlüter et al., 1997). It is also worth to note that the binding of

poly-L-proline by profilin might occur in either polypeptide backbone orientation (Mahoney et al., 1997; Mahoney et al., 1999).

4. Cellular localization of profilin

Basically, profilins are enriched in cellular areas characterized by high actin dynamics such as ruffles, lamellipodia, stress fibers and focal adhesions. Profilins were also found at the surface of *Listeria monocytogenes* after infection of host cells cytoplasm (Buss et al., 1992; Geese et al., 2000; Grenklo et al., 2004; Mayboroda et al., 1997; Skare et al., 2003).

Because of their ability to interact with membrane-bound phospholipids, profilins have also been observed in close association with a variety of membrane organelles such as Golgi-derived vesicles (Dong et al., 2000; Finger and Novick, 1997) and endocytotic machinery (Gareus et al., 2006).

Surprisingly, profilins have been reported to be also present in the nucleus. Indeed, profilin I has been detected in nuclei of human fibroblasts (Skare et al., 2003), rat kidney epithelial cells (Lederer et al., 2005a), bovine oocyte germinal vesicles and early embryos (Rawe et al., 2006). In addition, Birbach *et al* showed that profilin II accumulates in the nucleus of hippocampal neurons in response to NMDA receptor signaling (Birbach et al., 2006). Finally, the testis-specific profilin III was found in the nucleus of testicular germ cells, complexed with ArpM1 (actin-related protein M1).

5. Profilin functions

- Role in actin dynamics and cell signaling

The main function of profilins is to regulate the dynamics of actin assembly and organization. Indeed, they are key factors for actin filaments treadmilling, which is a dynamic process of actin turn over *in vivo*, primarily by monomer addition and growth at the fast-growing (barbed) ends and by monomer loss and shortening from the slow-growing (pointed) ends (Littlefield et al., 2001).

Profilins sequester actin monomers in the absence of free actin filament barbed ends and promote the assembly of ATP-actin monomers to filaments when barbed ends are available (Pantaloni and Carlier, 1993) (Figure 33). Then via interactions with polyproline-rich proteins (formins, Ena/VASP, WASP/WAVE), profilins localize monomers of ATP-actin to the sites of rapid actin filament assembly in cells (Reinhard et al., 1995; Evangelista et al., 1997).

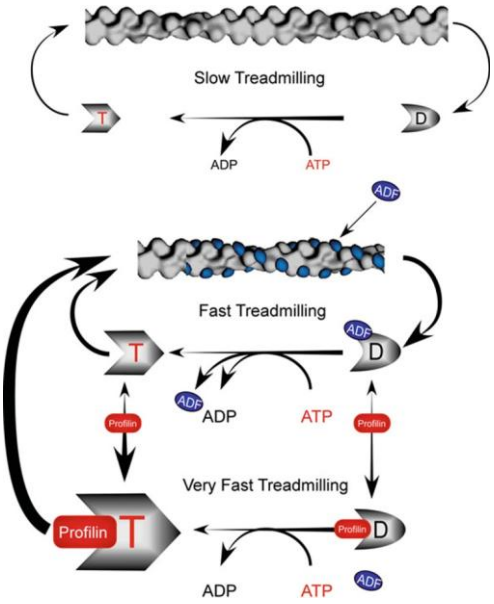

Figure 33: Regulation of actin filament treadmilling by ADF and profilin. *Top panel:* basic slow treadmilling of pure actin assembled at a steady state in ATP. Monomer-polymer exchange occurs at both ends of the filament (ADP-actin and ATP-actin labaled as D and T respectively); nucleotide exchange takes place on monomeric actin only. *Bottom panel:* enhancement of treadmilling by a synergistic action of ADF/cofilin and profilin. ADF (shown in *blue*) binds to ADP-F-actin and causes a structural destabilization of the actin-actin interactions in the filament, which results, at steady state, in a large increase in the rate of dissociation of ADP-actin from the pointed ends. Profilin binds preferentially ATP-G-actin, accelerates nucleotide exchange on G-actin and forms a complex with ATP-G-actin that associates to barbed ends. Addition of profilin to F-actin at steady state in the presence of ADF thus shifts all binding equilibrium toward the predominant profilin-ATP-G-actin, thus facilitating the vectorial turnover of actin filaments. Adapted from (Carlier, 2010).

In addition, through their ability to bind PIP2, profilins are coupled to ligand-induced transmembrane-receptor signaling (Lindberg et al., 2008; Sohn and Goldschmidtclermont, 1994) and thus connect closely signaling pathways and actin organization (Ridley et al., 2003). Indeed, activation of transmembrane receptors cause dramatic remodeling of cytoskeleton with rapid formation of actin filaments (Chinkers et al., 1979). It has been proposed that fluctuations in the concentration of PIP2 at the membrane might cause profilin shuttling between membranes and cytosol (Figure 34). Therefore, profilin could be a potent mediator of external signals to microfilaments (Machesky and Poland, 1993; van Rheenen et al., 2007). Thus, through its participation to the formation of actin filaments, profilin is involved in membrane associated processes such as lamellipodial protrusion during locomotion, cell adhesion and spreading cytokinesis and morphogenesis (Jockusch et al., 2007).

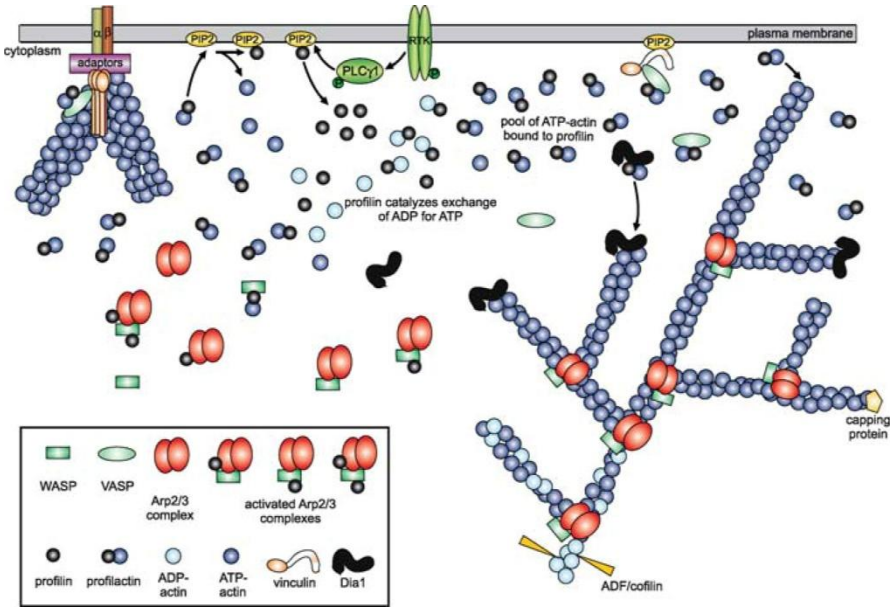

Figure 34: Multiple functional connections of plasma membrane assosiated and cytoplasmic profilin. The release of profilin from membrane is regulated by the level of PIP2 and determines the formation of profilin-G-actin complex. Released from the membrane, profilin (*center*) bind ATP-G-actin and adds G-actin to nascent actin filaments and participates in the generation of actin filaments as needed for adhesion complexes (*left*) and lamellipodial actin networks (*right*). Adapted from (Jockusch et al., 2007).

Finally, several molecular genetic studies demonstrated that profilin is important for proper cell function and viability in unicellular and multicellular eukaryotes. Indeed, knockout of profilin drastically impaired cell growth in *Saccharomyces cerevisiae* (Haarer et al., 1990) and led to cell death in *Schizosaccharomyces pombe*, *Dictyostelium discoideum* and *Drosophila melanogaster* (Balasubramanian et al., 1994; Haugwitz et al., 1994; Verheyen and Cooley, 1994). Profilin is also crucial for embryonic development as mouse embryos homozygous for profilin I disrupted gene (profilin (-/-)) die even before the development of the blastula at the two-cell stage. Concerning heterozygous embryos, (profilin (-/+)), they survive but display a reduced viability (Witke et al., 2001).

- Profilin in the nucleus

In addition to its well established cytoplasmic localization, profilin has been found in the nucleus and reported to be involved in regulation of transcription (Lederer et al., 2005a). Indeed profilin reduces repressive transcription activity of p42*POP*-Myb-related transcription factor.

Moreover, profilin was found to accumulate in subnuclear structures such as gems, Cajal bodies and Speckles (Birbach et al., 2006; Giesemann et al., 1999; Rawe et al., 2006; Skare et al., 2003). This localization of profilin in pre-mRNA processing components, in addition to its interaction with the survival of motor neuron (SMN) protein, which is important in the formation of small nuclear ribonucleoproteins (snRNPs), suggests a role for profilin in the maturation of mRNA.

OBJECTIVES

The main focus of the group of Dr. Cécile Rochette-Egly over the last several years is to understand the role of post translational modifications, mainly phosphorylations, on the activity of nuclear retinoic acid receptors. In that context, the group

- highlighted that RARs become rapidly phosphorylated in response to RA
- identified the phosphorylated residue
- demonstrated that the RA-induced phosphorylation of RARs involves the p38MAPK pathway
- Evidenced that phosphorylation is crucial for RAR transcriptional activity (see Figure 35)

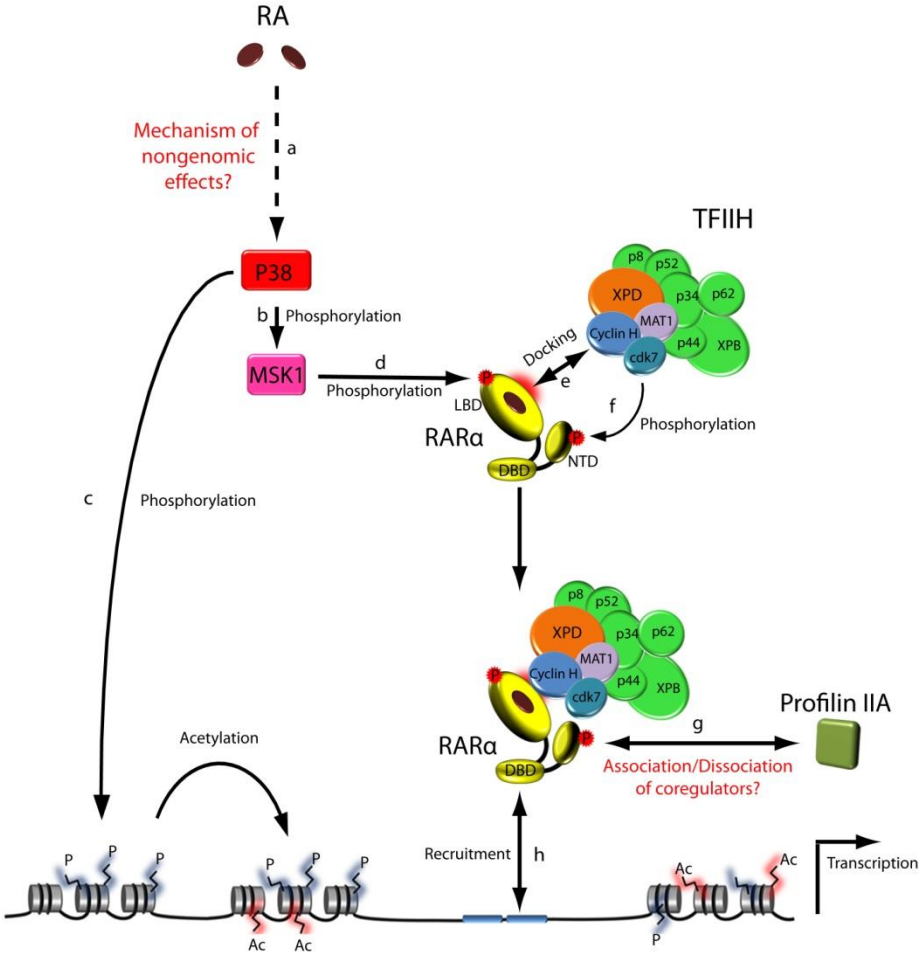

Figure 35: In response to RA, p38MAPK (a) and MSK1 (b) are activated. MSK1 phosphorylates histones at H3S10 (c) and RARα at a serine located in the LBD (d). Subsequently, the cyclin H subunit of the CAK subcomplex of TFIIH is recruited to an adjacent domain (e), allowing the formation of a RARα/TFIIH complex and the phosphorylation of the NTD by the cdk7 kinase (f). Phosphorylation of NTD might promote association/dissociation of coregulators (g). Finally, RARα phosphorylated and associated with TFIIH and is recruited to response elements located in the promoter of target genes (h).

Such results raised several questions:

- How RA activates the p38MAPK pathway? As the RA-induced activation of p38MAPK is rapid and transcriptionnally independent, the hypothesis was that this effect is non-genomic, as already described for other nuclear receptors (Marquez et al., 2006; Matthews et al., 2008; Norman et al., 2004)

- How phosphorylation controls RAR-mediated transcription? As the last phosphorylation event of the cascade concerns a serine residue located in a proline rich motif of the NTD, one could speculate that phosphorylation of this residue controls the association/dissociation of coregulators with SH3 domains.

Recently, the group identified in a yeast two-hybrid screening, profilin IIA as a new binding partner of the NTD of RARα. Profilin IIA is an actin-binding protein, which as several other proteins with SH3 domains, interacts with proline-rich motifs (PRMs) of a huge variety of proteins (Gareus et al., 2006; Mahoney et al., 1999). Most importantly, profilin IIA has been identified in the nucleus of several cell types and shown to interact with transcription factors (Lederer et al., 2005a). The precise mechanism by which profilin IIA regulates transcription is still ill-defined. However, one can suggest that, similarly to vinexin β (Lalevee et al., 2010a), profilin IIA functions as an adaptor, recruiting actin and/or other nuclear proteins with a structural and/or regulatory role within chromatin and/or the transcriptional machinery (Bettinger et al., 2004; Wang et al., 2006).

In this context, the objectives of my thesis have been to attempt to answer these two questions, i.e. decipher at the molecular level:

- **How RARs activates the p38MAPK pathway through non-genomic effects**
- **How profilin IIA interacts with RARα and controls its transcriptional properties**

RESULTS

1. NOVEL NON-GENOMIC EFFECTS OF RA: ACTIVATION OF THE P38MAPK PATHWAY VIA A MEMBRANE-ASSOCIATED POOL OF RARα (PUBLICATION 1)

Recent results from the group demonstrated that RA rapidly and transiently activates the p38MAPK pathway in several cell lines such as MCF7, Hela, MEF and F9 cells (Bruck et al., 2009). By analogy with other nuclear receptors we hypothesized that it occurs through a non-genomic effect.

- RA ACTIVATES P38MAPK VIA GαQ PROTEINS AND A POOL OF RARα PRESENT IN MEMBRANE LIPID RAFTS

The first question was what is upstream of p38MAPK. First of all, we found that Rho-GTPases such as Rac and Rock, which are upstream of p38MAPK, were also activated in response to RA (Publication1, Figures 1e and 1f). Then, as Rho-GTPases activation involves upstream G proteins alpha Q (Gαq) that interact with scaffolding proteins in membranes (Mizuno and Itoh, 2009; Sugawara et al., 2007), we analyzed whether Gαq proteins are involved in the rapid RA-induced activation of the p38MAPK pathway by using the siRNA strategy. No activation of p38MAPK was observed after knockdown of Gαq (Publication 1, Figures 2a and 2c), indicating that the rapid activation of the p38MAPK pathway by RA involves Gαq.

Then we asked whether the RA-induced activation of p38MAPK is RAR-dependent. Taking advantage of different cell lines invalidated or knockdown for one specific RAR subtype we demonstrated that the activation of p38MAPK in response to RA, involves specifically the RARα subtype (Publication1, Figures 1b, 2b and 2c). Moreover, in MEFs knockout for the three RARs, p38MAPK was restored only upon re-expression of RARα WT (Publication1 Figures 1c and 1d). Thus the rapid activation of p38MAPK by RA appears to involve RARα, in addition to Gαq.

Remarkably, Gαq proteins as well as most signal components are concentrated in specific highly ordered membrane micro domains termed “lipid rafts” (Pike, 2003; Simons and Toomre, 2000), which orchestrate intracellular signaling pathways and thus are considered as signaling centers. Therefore, we investigated whether these membrane domains contain RARα. We isolated lipid rafts from MCF-7 cells and found that they do contain RARα (but not RARγ or RARβ) in addition to flotillin-2, a marker of lipid rafts, and signaling proteins such as Rock-2, PKCσ and Gαq (Publication 1 Figure 3b). However, the amount of RARα present in lipid rafts was very low compared with the amount of RARα present in nuclei as assessed in immunofluorescence experiments, (Publication 1, Figure 3d). Indeed, only low amounts of RARα were found to colocalize with Flotillin-2 out of nuclei

(Publication 1, Figure 3f and 3g). Collectively, these results converge to a novelty in the field, i.e. a small population of RARα is present out of nuclei in membrane lipid rafts.

Then, the next question was which domain/region/sequence is responsible for targeting RARα to membranes. Given that the three RAR subtypes differ essentially in their NTD, one can speculate that this domain might specifically target RARα at the membrane. To verify this hypothesis we took advantage of F9 cells reexpressing RARα WT or deleted of the NTD (RARαΔNTD) in a RARα null background (Rochette-Egly et al., 2000). Remarkably, RARαΔNTD could not be detected in lipid rafts, indicating that the NTD would be involved in targeting RARα to membranes (Publication 1, Figure 3h).

- RARα INTERACTS WITH GαQ *IN VITRO* AND *IN VIVO*

Given that Gαq proteins colocalize with RARα in lipid rafts, we investigated whether the two proteins can interact with each other. We found that, *in vitro*, recombinant RARα and Gαq interact with each other in GST pull down and coimmunoprecipitation experiments. Remarkably, the interaction was increased in response to RA. Endogenous RARα and Gαq also interacted *in vivo* in immunoprecipitation experiments performed with lipid rafts (Publication1, Figures 3a-c).

However, due to the low amounts of RARα in lipid rafts, the RARα/Gαq complexes were rather difficult to detect. Therefore we set up a "Proximity Ligation Assay" (PLA), which can reveal transient interactions between endogenous proteins even when present at very low levels (Soderberg et al., 2006). Rabbit anti-RARα and mouse anti-Gαq antibodies were used, followed by species-specific secondary antibodies, called PLA probes, each attached with a unique short DNA strand. When the PLA probes are in close proximity, the DNA strands can be joined through the addition of a circle-forming DNA oligonucleotide that can be amplified using a polymerase. Then the amplified products are revealed with labeled complementary oligonucleotide probes and are easily visible as bright red spots under a fluorescence microscope. This technique allowed us to visualize *in situ* a rapid and transient increase (10-15 min after RA addition) in the number of RARα/Gαq complexes. Remarkably, the complexes were found out of the nuclei, confirming that the non-genomic effects of RA involve a pool of extra nuclear RARα (Publication1, Figure 5). It is important to note that the increased interaction between RARα and Gαq occurred specifically in response to RA. Indeed, in response to EGF, which also activates p38MAPK through Gαq, no interaction between RARα and Gαq could be detected (Publication1, Figure 7a-g).

- RARα INTERACTS WITH GαQ ONLY IN CELLS THAT RESPOND TO RA VIA THE ACTIVATION OF P38MAPK

Then the question was whether the RA-induced interaction between RARα and Gαq was a general phenomenom.

In fact, the formation of RARα-Gαq complexes was observed in epithelial and fibroblastic cells, which respond to RA by the activation of p38MAPK. However, in other cells, such as neuronal cells (human neuroblastoma SH-SY5Y cell line), RARα, though present in lipid rafts, doesn't interact with Gαq in response to RA and p38MAPK is not activated (Figure 36). Of note is that in neuronal cells RA rather activates the Erk pathway through Src and PI3K (Chen and Napoli, 2008; Dey et al., 2007; Masia et al., 2007; Pan et al., 2005; Zanotto-Filho et al., 2008). Thus, depending on the cell type, it appears that RA can activate various MAPK pathways via different membrane-associated complexes.

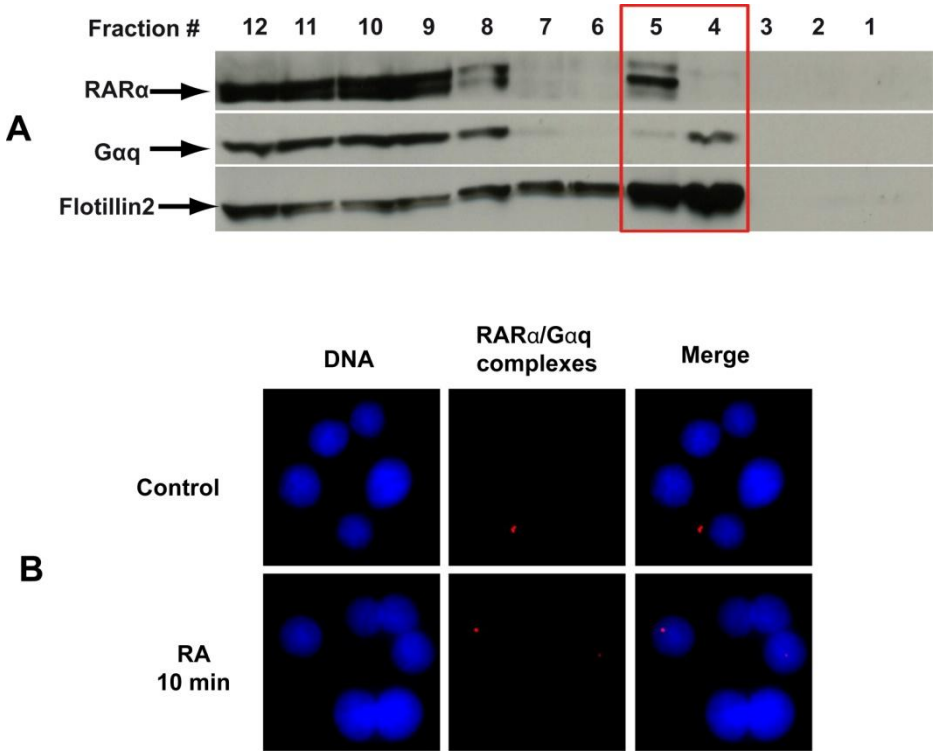

Figure 36: A. Immunoblotting analysis of the different gradient fractions from SH-SY5Y cells, showing that RARα is present with Flotillin-2 and Gαq in lipid rafts. B. Analysis of RARα/Gαq complexes in SH-SY5Y cells by immunofluorescence microscopy in combination with in situ proximity ligation assay (PLA).

2. THE NON-GENOMIC EFFECTS OF RA CONTROL THE GENOMIC ONES (Publication 1)

Given that RARs are basically ligand-dependent regulators of gene transcription, we asked whether the actors of the non-genomic effects, i.e. Gαq and the downstream p38MAPK/MSK1 pathway, cross-talk with the genomic ones.

Remarkably, knockdown of Gαq, p38MAPK and MSK1 decreased the RA-induced expression of several RA-target genes, as well as the antiproliferative action of RA (Publication1, Figure 9). Such results highlight the importance of the Gαq/p38MAPK/MSK1 pathway and thus of the non-genomic effects of RA for the genomic effects, i.e., RAR-target genes expression and growth arrest. Corroborating this conclusion, we found that in cells that are resistant to the antiproliferative effect of RA and exemplified by the human erbB-2 positive breast cancer cells, BT474, SKBR3, MDA-MB453 and MDA-MB361 cells no interaction could be detected between RARα and Gαq either in the absence or presence of RA and p38MAPK was not activated (Publication1, Figure 8).

Conclusion

We highlighted a novel unconventional localization of RARα in membrane lipid rafts. We also demonstrated that this membrane pool interacts with Gαq proteins in response to RA and is responsible for the non-genomic effects of RA, i.e. the activation of the p38MAPK pathway. The other novelty is that these non-genomic effects are required for genomic ones.

3. PROFILIN IIA, A NOVEL COREGULATOR OF RARα (publication 2)

The main consequence of the non-genomic effects of RA, i.e. the activation of the p38MAPK/MSK1 pathway, is a phosphorylation cascade targeting RARs. This cascade starts with the phosphorylation of the LBD by MSK1 (downstream of p38MAPK), and ends with the phosphorylation by cdk7/cyclin H of another serine residue, located in a proline-rich motif (PRM) of the NTD. Phosphorylation of this second residue proved to be crucial for the transcriptional activity of RARs, but the question is how.

Now it is increasingly evident that phosphorylations induce subtle conformation changes of adjacent domains (Bao et al., 2004) or the cis-trans isomerization of adjacent proline residues. The result is the creation of new recognition sites for interacting factors (Bao et al., 2004). Thus a significant effort in the laboratory has been made to identify and characterize co-regulators that interact directly with the PRM of RARs in a phosphorylation-dependent manner. Such an effort led to the discovery, in yeast two-hybrid screenings, of new coregulators with SH3 domains: Vinexin β for the RARγ subtype (Lalevee et al., 2010a), and profilin IIA for RARα. During my thesis I deciphered the mechanism and the relevance of the RARα/profilin IIA interaction.

- PROFILIN IIA INTERACTS WITH THE N-TERMINAL PRM OF RARα BUT INDEPENDENTLY OF ITS PHOSPHORYLATION

In a first step, the data obtained by yeast two-hybrid screening were corroborated in GST pull down and coimmunoprecipitation experiments using recombinant WT or mutated proteins (Publication 2, Figure 2A). These experiments confirmed that profilin IIA interacts specifically with the RARα subtype and not with RARβ or RARγ. We also deciphered further the mechanism of the interaction and demonstrated that it involves the PRM of RARα located in the NTD and the SH3-like domain of profilin IIA (Publication 2, Figure 2B-D).

The PRM of RARα involved in the interaction with profilin IIA contains a serine residue (S77), which can be phosphorylated *in vitro* and *in vivo*. Therefore we aimed at investigating whether phosphorylation of this residue modulates the interaction as previously described for RARγ/vinexin β. In fact, coimmunoprecipitation experiments revealed that profilin IIA interacts as efficiently with RARα, whatever S77 is substituted with a glutamic acid (RARαS77E) or an alanine (RARαS77A), which mimic the phosphorylated and non-phosphorylated forms respectively (Publication 2, Figure

3A). Moreover, the interaction was not affected upon RA addition, which induces RARα phosphorylation (Publication 2, Figure 3B-C).

Then by using Surface Plasmon Resonance (SPR) we measured the equilibrium affinity of the interaction, using GST-profilin IIA and synthetic peptides corresponding to the proline-rich motif of RARα. The use of peptides in which S77 is phosphorylated confirmed that phosphorylation does not affect the affinity (Publication 2, Table1).

- PROFILIN IIA INTERACTS WITH RARα IN NUCLEI

Profilin IIA is an actin-binding protein, which is not expressed in all cell types. Indeed, profilin IIA is well known to be expressed mainly in brain and neuronal cells (Birbach, 2008; Michaelsen et al., 2010). Accordingly, in immunoblotting experiments, we detected significant levels of profilin IIA in human neuroblastoma cells (SH-SY5Y cell line) and mouse hippocampus cells (HT22 cell line) (Publication 2, Figure 4A). Interestingly, significant levels of profilin IIA were also detected in mouse embryonic fibroblasts and human breast cancer cell lines (MCF7 and SKBR3 cell lines) (Publication 2, Figure 4A). In contrast, profilin IIA was hardly detected in mouse embryocarcinoma cells (F9 and P19 cell lines) and no profilin IIA was found in human acute promyelocytic cells (NB4 cell line), (Publication 2, Figure 4A).

In immunofluorescence experiments, profilin IIA was detected mainly in the nucleus (Publication 2, Figure 4B) where it colocalized with RARα (Figure 5A). Therefore we investigated whether endogenous profilin IIA and RARα interact with each other in nuclei, using the proximity ligation assays (PLA) described above, which allows the detection *in situ* of interacting endogenous proteins. RARα/profilin IIA complexes were seen in the nuclei of MCF7 cells and MEFs (Publication 2, Figure 5B). The number of these complexes did not change after RA addition up to 1 hour, in line with the absence of regulation of the interaction upon RARα phosphorylation.

Altogether these results indicate that profilin IIA interacts with RARα in nuclei, independently of RARα phosphorylation.

- PROFILIN IIA AND RARα: ROLE IN THE TRANSCRIPTION OF RARα TARGET GENES

Given that profilin IIA interacts with RARα in the nuclei of MCF7 cells and MEFs, one could speculate that this actin-binding protein is involved in the transcription of RA target genes. Corroborating this hypothesis, we found that knockdown of profilin IIA decreases the RA-induced

expression of several genes as assessed by qRTR-PCR (Publication 2, Figure 6). Moreover, in ChIP experiments, profilin IIA was recruited with RARα to the promoter of these genes. Finally, ChIP western experiments revealed that profilin IIA coimmunoprecipitates with RARα in chromatin (Publication 2, Figure 7).

Conclusion

We characterized a new coregulator of RARα, profilin IIA, which is an actin-binding protein. Profilin IIA interacts with the N-terminal proline-rich motif of RARα but independently of its phosphorylation. The interesting point is that profilin IIA is present in chromatin and is involved in the transcription of some RA-target genes. However the *in vivo* relevance of the RARα/profilin IIA interaction (in cell differentiation/proliferation) remains to be investigated.

4. NEW UNCONVENTIONAL ROLE OF RARα IN CELL ADHESION (UNPUBLISHED RESULTS)

- RARα KNOCKOUT CELLS ARE DEFICIENT IN ADHESION

Then the challenge was to investigate the role of RARα (with profilin IIA) in the RA response. As mammal models are quite complex, we selected mouse embryonic stem (ES) cells, which differentiate into neurons after RA addition, recapitulating early stages of mouse embryogenesis (Bibel et al., 2004b). These cells are an experimental paradigm and arer extensively used as a model to investigate RA signaling *in vitro.* ES cells express the different RAR subtypes and ES cells either WT or invalidated for the different RARs are available in the laboratory (Al Tanoury *et al*, manuscript in preparation). Most interestingly, disruption of the whole RARα gene (RARα-/- cells) did not affect the ability of ES cells to commit into neuronal lineages after RA treatment. Indeed, RARα-/- cells have kept their ability to become neural progenitors characterized by β-tubulinIII (Figure 37f). They have also kept their ability to develop a dense neuritic network within 2-3 days after dissociation and plating of embryonic bodies (Figure 37 c and f). This is in contrast to the targeted disruption of the whole RARγ gene (RARγ-/- cells) that severely blunted the RA response so that neurons with fine and long processes were almost absent (Figure 37b,e) (Al Tanoury *et al*, manuscript in preparation).

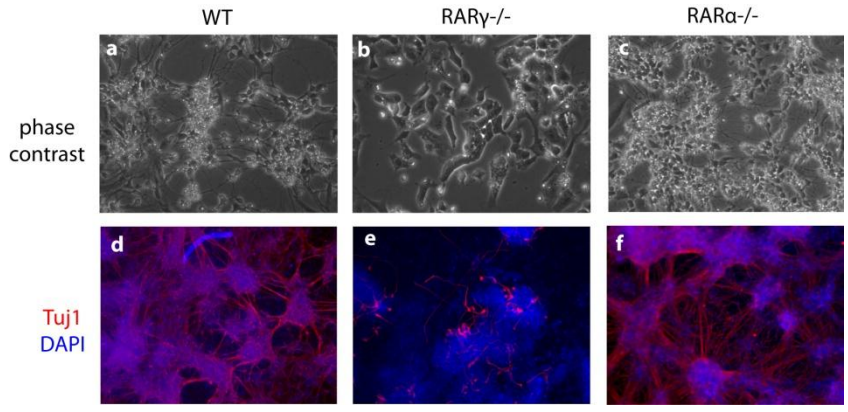

Figure 37: WT, RARγ-/- and RARα-/- mouse ES cells were compared for their ability to differentiate into neuronal cells in response to RA as assessed by immunofluorescence analysis of β tubulin III (Tuj1).

However we found that RARα-/- cells (and not RARγ-/- cells) were severely impaired in adhesion. Indeed differentiated neurons were elongated but did not adhere efficiently to the substrtae. Moreover, the undifferentiated cells did not adhere and did not spread on laminin-coated coverslips as assessed by spreading-adhesion assay (Figure 38). Indeed, while WT ES cells are fast well-spreading cells, RARα-/- cells remain compact and round, without substantial spreading.

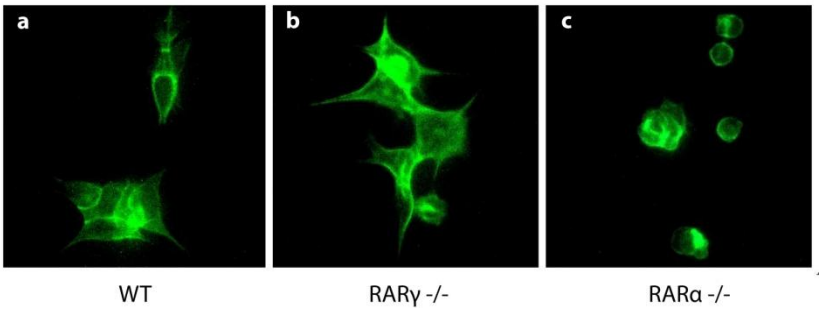

Figure 38. Spreading-adhesion assay. Mouse ES cells (WT, RARα-/- or RARγ-/-) were allowed to adhere for 3 hours onto laminin-coated coverslips and then fixed and stained for F-actin.

- A NOVEL UNCONVENTIONAL LOCALIZATION OF RARα IN THE CYTOSOL OF NEURONAL AND FIBROBLASTIC CELLS

Unexpectedly, in immunofluorescence experiments performed with our highly specific purified rabbit polyclonal antibodies (Buchanan et al., 2011), we observed that RARα is present in the cytosol of ES cells either pluripotent (Figure 39a-c) or differentiated into neurons (Figure 39d-f).

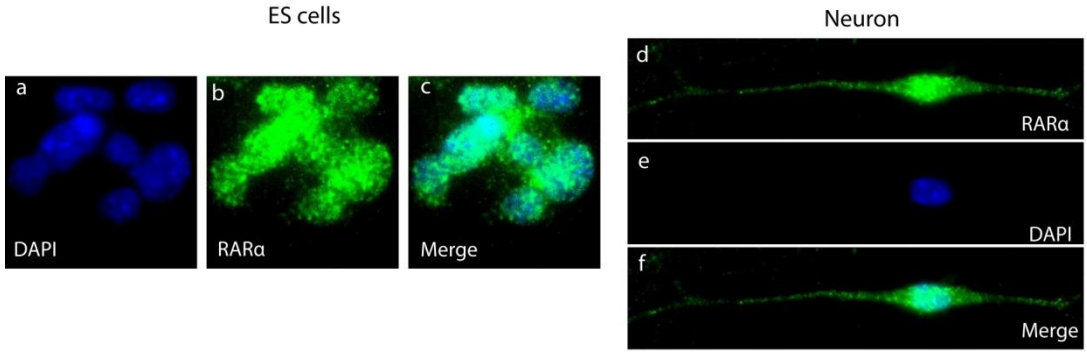

Figure 39: Nuclear and cytosolic localization of RARα. Immunofluorescence experiments showing that RARα is present in nuclei and also in the cytosol of mouse ES cells either undifferentiated or differentiated into neurons.

Remarkably, RARα was also detected in the cytosol of other neuronal cells such as neuroblastoma cells (SH-SY5Y cell line) and hippocampus cells (HT22 cell line) (Figure 40 a-h). Moreover RARα was also detected in the cytosol of MEFs (Figure 40 i-l). In contrast, in these cells, RARγ was strictly nuclear (Figure 40 compare pannels l and t). The specificity of our antibodies was confirmed by using MEF RAR (αβγ) -/- (Figure 40 m-p and u-x). Note that these KO cells were more round than the WT ones, corroborating the role of RARs in cell adhesion and spreading.

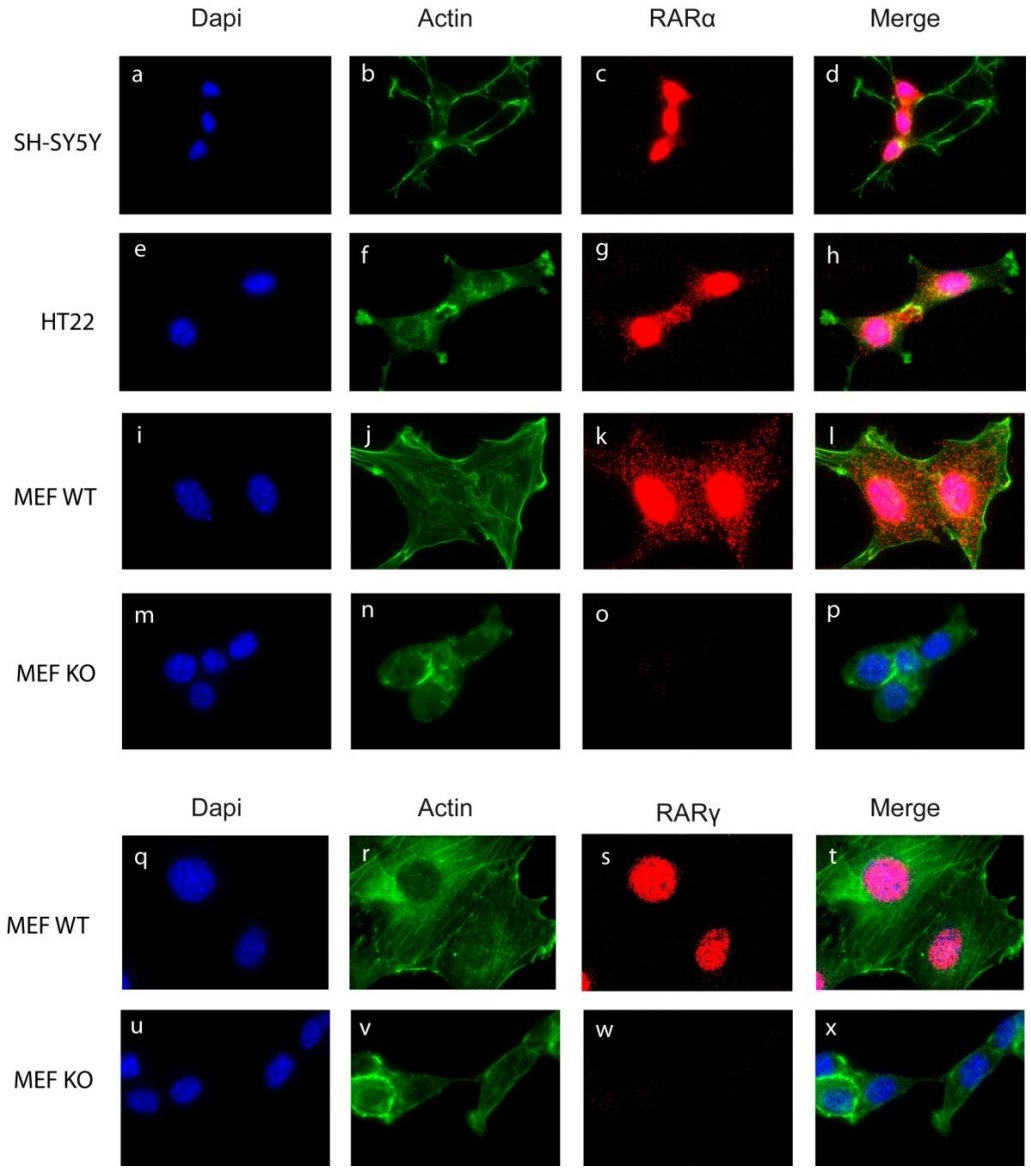

Figure 40: Nuclear and cytosolic localization of RARα. Immunofluorescence experiments showing mainly nuclear and additional cytosolic RARα in MEFWT, HT-22 and SH-SY5Y cells. In contrast RARγ in MEFWT cells is strictly nuclear. Negative MEFKO cells are depleted in all RARs.

- THE POOL OF CYTOSOLIC RARα IS INCREASED IN CARCINOMA-ASSOCIATED FIBROBLASTS (CAFS)

In the context of the study of the consequences of aberrant kinases activity on RARs phosphorylation in tumors, the laboratory succeeded to generate primary cultures of carcinoma-assosiated fibroblasts (CAFs) from erbB-2 positive breast cancer surgery sections (obtained in collaboration with the hospital) and to keep these cells viable after several passages. The interesting point is that these CAFs express higher amounts of cytosolic RARα than normal fibroblasts (Figure 41). Moreover the localization of RARα in CAFs was rather speckled than diffused.

As CAFs play a key role in tumor invasion and metastasis, one can be suggested that increased amounts of cytosolic RARα might be linked to these processes.

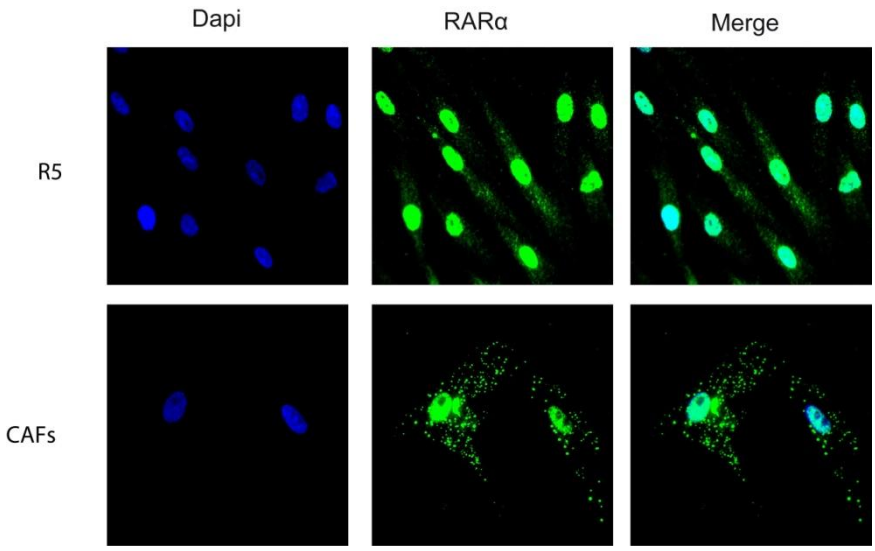

Figure 41: Nuclear and cytosolic localization of RARα. Immunofluorescence experiments showing mainly nuclear and additional cytosolic RARα in normal human fibroblasts (R5) and carcinoma-associated fibroblasts (CAFs) isolated from the stroma of highly invasive erbB-2 positive breast cancer tumors.

Conclusion

Here, we highlighted a novelty in the field of RARα, i.e. an unconventional localization in the cytosol and a role in cell adhesion.

- MATERIALS AND METHODS (UNPUBLISHED)

Antibodies and reagents

Mouse monoclonal antibodies recognizing neuronal class III β-tubulin (Tuj1) were from Covance (Eurogentec France). Purified rabbit polyclonal antibodies raised against the F region of RARα (RPα(F)) are described in publication 2. Purified rabbit polyclonal antibodies against RARγ were described earlier (Lalevee et al., 2010a). Fluorescent green 488 phalloidin was from Biotium, Inc. Hayward USA.

Mouse embryonic stem cells

Wild type mouse ES cells were derived from the 129 sub-strain. RARα-/- and RARγ-/- knockout ES cell lines were previously described (Lohnes et al., 1993; Lufkin et al., 1993).

ES cell lines were cultivated and differentiated as described (Bibel et al., 2007; Bibel et al., 2004a). Briefly, they were kept undifferentiated by repeated splitting on feeders (inactivated mouse embryonic fibroblasts) in ES cell culture medium (DMEM supplemented with GLUTAMAX-I, 15% FCS, 700 U leukemia Inhibitory Factor (LIF), gentamicin and β-mercaptoethanol at 37 °C and 5.5 % CO2). Then after deprivation of feeders, embryoid bodies (EB) were formed by plating $4x10^6$ cells onto non adherent bacterialogical petri dishes (Greiner, cat.no. 633102) in 15 ml EB medium (DMEM supplemented with GLUTAMAX-I, 10% FCS, non essential amino acids, gentamicin and β-mercaptoethanol). Medium was changed every 2 days and RA (2-5μM) was added after 4 days. Then after another 4 days, EB were dissociated and plated into laminin-precoated culture dishes in N2 medium [DMEM/ Ham-F12 (1:1), supplemented with N2 (Fisher Scientific), BSA (50 mg/ml) and penicillin/streptomycin]. N2 medium was changed after 1 day and replaced by neurobasal medium supplemented with B27 (Fisher Scientific) after 2 additional days.

MEFs were cultured as described before (Piskunov and Rochette-Egly, 2011a). HT22 and SH-SY5Y cells were cultured according to standard procedures (Ha et al., 2010).

Immunofluorescense experiments were performed as described earlier (Piskunov and Rochette-Egly, 2011a)

Spreading and motility assays

Adhesion and spreading of mouse ES cells were analyzed by plating cells onto laminin-coated coverslips. Cells were allowed to adhere for 3 hours and washed three times in PBS. Then cells were fixed in 4% paraformaldehyde, permeabilized in 0,5% Triton X-100 and stained with 488 phalloidin (Biotium, Inc. Hayward USA).

DISCUSSION AND PERSPECTIVES

My work revealed three novelties in the field of the RARα subtype:

- A pool of RARα is present in membrane lipid rafts. This membrane fraction of RARα is involved in non-genomic effects, i.e. the activation of the p38MAPK pathway, which cross talk with the genomic ones.

- Profilin IIA an actin-binding protein, has been identified as a new partner of nuclear RARα. It positively modulates the genomic effects of RARα.

- RARα plays a novel unconventional role in cell adhesion. The function of another unconventional pool of RARα in the cytosol is proposed.

- A POOL OF RARα IS PRESENT IN LIPID RAFTS FOR NON-GENOMIC EFFECTS

In this study we discovered a novel unconventional localization of RARα in membrane lipid rafts, which are signaling centers. This was new in the field of RARs and added a new element to the cohort of nuclear receptors (ER, GR, PR, AR, VDR), which were already shown to be localized in lipid rafts (Figure 42) (Huhtakangas et al., 2004; Luoma et al., 2008; Marquez et al., 2006; Matthews et al., 2008; Pedram et al., 2007; Piskunov and Rochette-Egly, 2011b) .

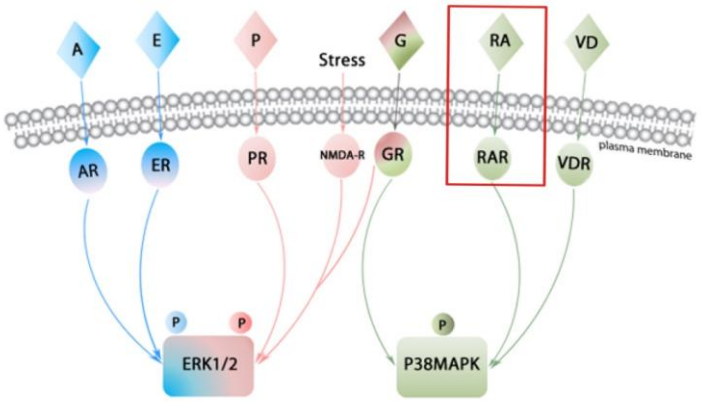

Figure 42: **Non-genomic effects of nuclear receptors.** A subpopulation RARα as well as the other nuclear receptors (ER, PR, GR, AR VDR) is associated to cell membranes and initiates cascades of kinases upon binding of their cognate ligands. Adapted from (Piskunov and Rochette-Egly, 2011b).

The important point is that RARα is recruited to the plasma membrane via its NTD, but the mechanism of this recruitment is still undefined and would require further investigation.

One possibility might be that membrane anchoring involves posttranslational modifications of the NTD, such as palmitoylation and myristoylation, a covalent attachment of fatty acids, such as palmitic acid or a myristoyl group (derived from myristic acid) (Krauss, 2008), as described for the steroid receptors (Pedram et al., 2007). Unfortunately the NTD of RARα does not depict any motif for these modifications (Pedram et al., 2007), eliminating such a mechanism.

Another possibility would be that membrane recruitment involves the interaction of the N-terminal PRM of RARα with profilin IIA, which belongs to the actin cytoskeleton associated to membranes and which also interacts with phosphatidyl-inositol lipids (see introduction). However, according to our results, no profilin IIA could be detected in lipid rafts.

The last possibility might be that RARα, like the other nuclear receptors, is targeted to membranes via the binding of its proline-rich motif to other SH3-domain containing membrane-associated proteins such as c-Src tyrosine kinase (Le Romancer et al., 2011; Migliaccio et al., 2000; Migliaccio et al., 1998). The other interesting point is that in response to its ligand, RARα localized in lipid rafts interacts with Gαq proteins and then activate Rho-GTPases and the p38MAPK pathway (Figure 43).

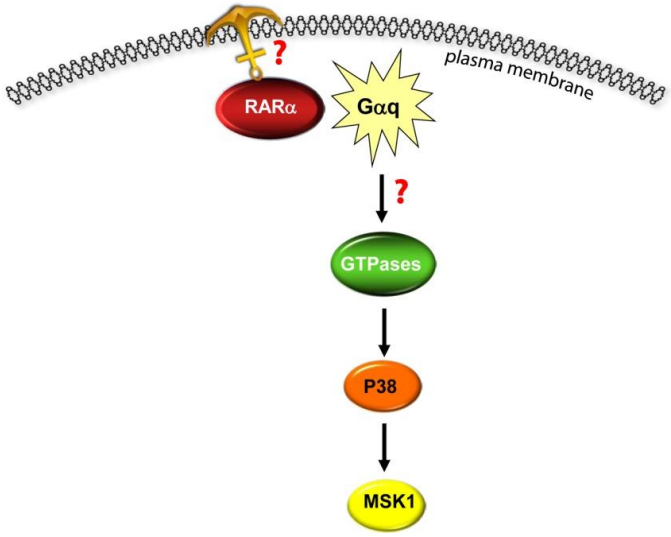

Figure 43: Model of the activation of the p38MAPK pathway through RARα and Gαq located in membranes. Question marks show that it is still unclear how RARα is anchored to the plasma membrane and what is the mechanism of GTPases activation via Gαq. Adapted from (Piskunov and Rochette-Egly, 2011a).

However, the steps between Gαq and Rho-GTPases have not been elucidated yet. Further investigations should be required to determine whether, similarly to the steroid receptors, there is a rapid increase in intracellular calcium concentration [Ca2+] and the activation of effector molecules such as PKC, PKA, PI3K/AKT, Phospholipase C, c-Src, Raf etc (Mizuno and Itoh, 2009).

Nevertheless, according to our results, the integrity of lipid rafts composition is required for the RA-induced interaction between RARα and Gαq, and thus for the activation of p38MAPK. As an example, no interaction was observed in erbB-2 positive cells (Figure 44). One can suggest that high levels of erbB-2 in membranes alter the organization of rafts and therefore affect the formation of the multiprotein complexes which include RARα and Gαq. In line with such a hypothesis, the EGF pathway, which also involves Gαq proteins, was also affected. Note that in these cells, caveolin-1, a component of lipid rafts is frequently down regulated (Park et al., 2005) corroborating that the integrity of the rafts is required for the activation of signaling pathways.

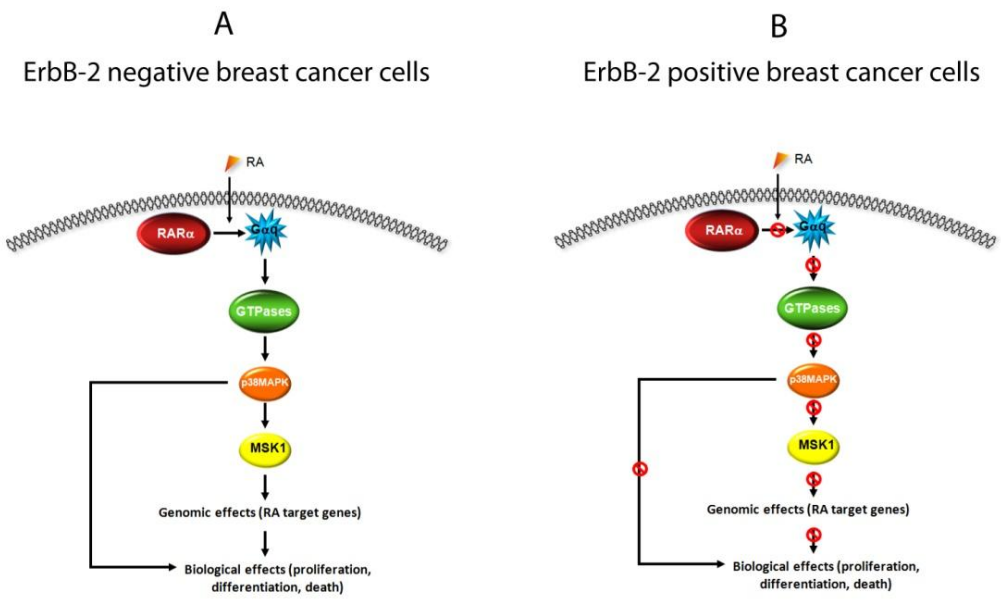

Figure 44: Models of RA signaling in erbB2 positive and negative breast cencer cells. A. In erbB2 negative cells, RA induces formation of RARα/Gαq complexes and subsequently p38MAPK/MSK1 pathway which is resulting in various biological activities. B. In erbB2 positive cells, RA does not induce formation of RARα/Gαq complexes therefore p38MAPK/MSK1 pathway is blocked.

Remarkably, the RA-induced activation of p38MAPK via RARα and Gαq protein was observed only in epithelial and fibroblastic cells and not in neuronal cells where RA activates erks (Chen and Napoli, 2008; Dey et al., 2007; Masia et al., 2007; Pan et al., 2005; Zanotto-Filho et al., 2008). This raises the hypothesis that as for the other nuclear receptors, different kinases can be activated by a same ligand via different membrane molecular complexes, depending on their functional significance (Losel and Wehling, 2003; Norman et al., 2004). As an example, in breast cancer cells and in response to its cognate ligand, the estrogen receptor ERα, activates the Erk pathway through complexes containing c-Src and the regulatory subunit of PI3K (p85α) (Le Romancer et al., 2008; Migliaccio et al., 1998). In contrast, in neuronal cells, ER rather activates Protein Kinase C through Gαq and phospholipase C (Qiu et al., 2003). In the same line of idea, depending on the cell type, VDR activates either the RhoA-Rock-p38MAPK pathway (Ordonez-Moran et al., 2008) or the Raf/Erk pathway (Losel and Wehling, 2003; Norman et al., 2001).

- RELEVANCE OF THE NON-GENOMIC EFFECTS OF RARα: CROSS TALK WITH THE GENOMIC EFFECTS

According to our data, Gαq and the downstream effectors, p38MAPK and MSK1 have an important role in the transcription of RA-target genes and in the antiproliferative effects of RA. This is in line with a general concept that Gαq proteins as well as several components of lipid rafts control not only the signaling pathways but also transcription and cell growth (Lai et al., 2008; Staubach and Hanisch, 2011; White et al., 2008). Moreover MAPKs are now considered as integrators dispensing decisions to the downstream cellular and transcrriptional machineries that coordinately manage major cellular fates, including cell proliferation (or inappropriate proliferation/malignant transformation), differentiation, or death (Figure 45). In line with this concept and according to other work from the laboratory, RA-activated p38MAPK targets several transcriptional actors. Indeed p38MAPK phosphorylates RARs coactivators such as SRC-3 and influences the dynamics of their association/dissociation (Ferry et al., 2011). Moreover, p38MAPK also activates MSK1, which initiates coordinated phosphorylation cascades that target RARs and histones (Bruck et al., 2009). *In fin*e these phosphorylations cooperate for RARs recruitment to DNA via modifications of the chromatin environment according to the histone code and via changes in the affinity of RARs for their response elements (Figure 46). According to these data, it is tempting to speculate that, in erbB-2 positive cells, the absence of activation of the p38MAPK pathway might be one of the reasons among several others, of the resistance of these cells to RA.

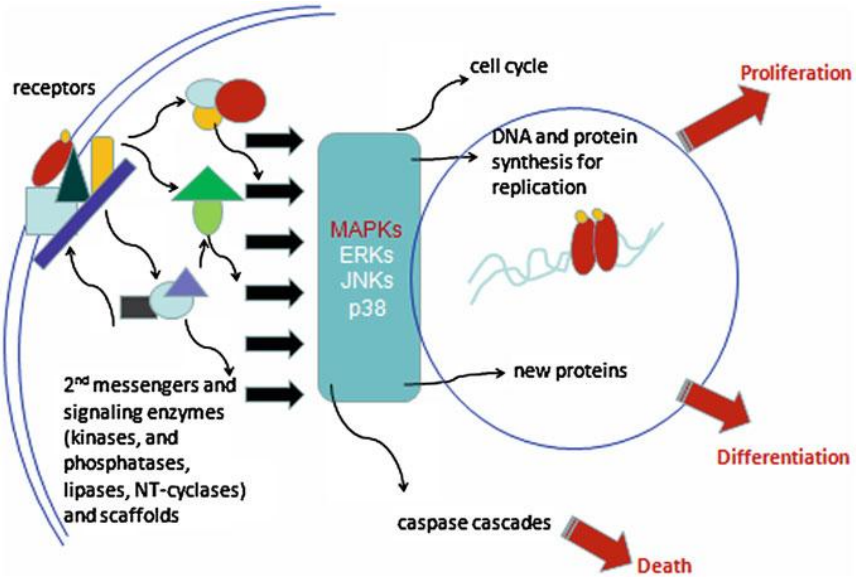

Figure 45: Biological relevance on non-genomic effects of NRs. Nuclear receptors (yellow liganded red oval at the membrane) interact with a variety of signaling proteins (various colored shapes) resulting in activation of MAPKs, which then can regulate cell proliferation (altering activation of cell cycle proteins and causing production of new DNA and proteins), differentiation (the production of new proteins for an altered function), or cell death (by initiating caspase cascades or other forms of active cell death mechanisms). Adapted from

Of note is that other nuclear receptors such as ER, AR, GR and PR, are also phosphorylated by MAPKs, in response to steroid hormones (Chen et al., 2008a; Faus and Haendler, 2006; Lannigan, 2003) and that MSK1 also contributes to histone phosphorylation and chromatin remodeling (Vicent et al., 2006; Vicent et al., 2010; Vicent et al., 2009) with characteristic downstream consequences on target genes expression (Weigel and Moore, 2007a) (Figure 46).

Altogether these data corroborate our results and converge towards the concept that the non-genomic effects of RARs are required for the genomic ones.

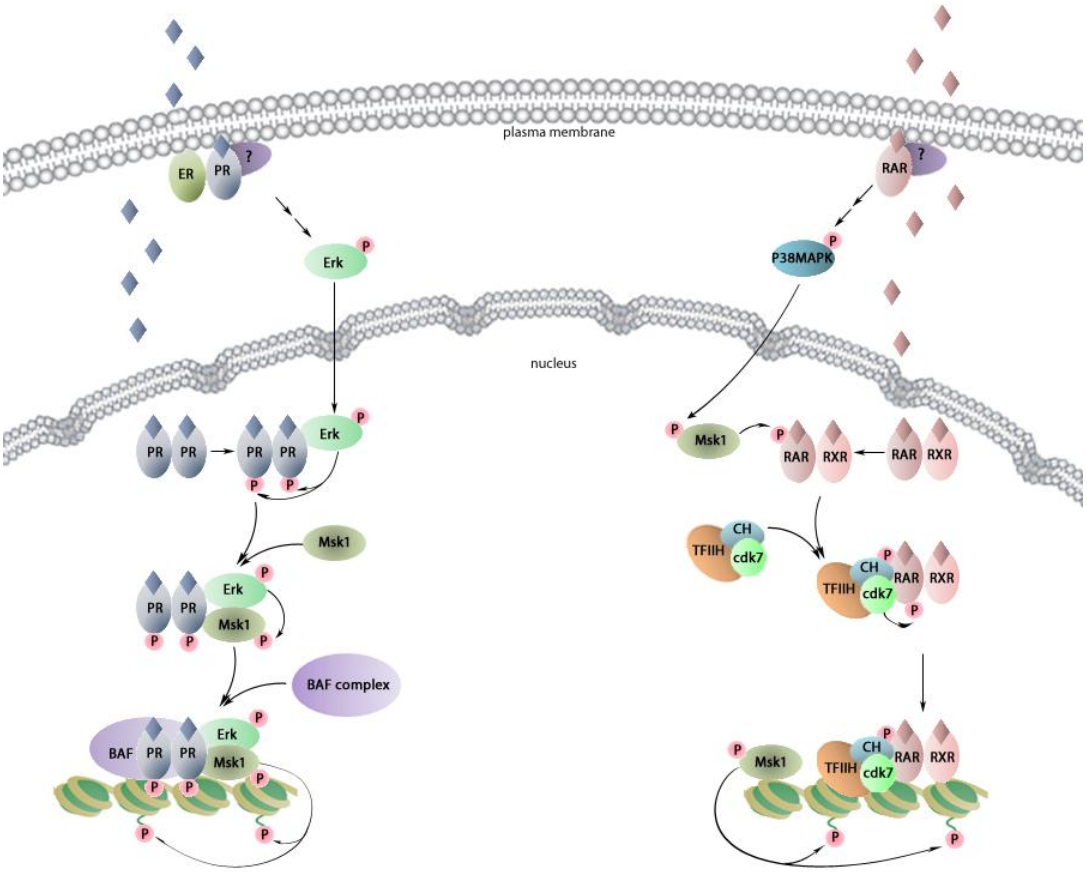

Figure 46: Convergence of genomic and non-genomic effects of nuclear receptors. Progestins activate the Src/Ras/Erk pathway via membrane associated PR, leading to accumulation of activated Erk in the nucleus. Then a nuclear population of PR becomes phosphorylated by activated pErk, which also phosphorylates MSK1. A "PR‐activated complex" composed of pPR, pErk and pMSK1 is recruited to the promoter, followed by histone H3 phosphorylation and acetylation. The BAF complex is also recruited through direct interaction with PR and is anchored to chromatin through the histone marks. Due to a lack of kinetic experiments, a precise order of events cannot be proposed. Right). Upon RA binding, a subpopulation of membrane RAR activates the p38MAPK/MSK1 pathway. Activated MSK1 phosphorylates RARα at S369 located in the LBD, subsequently facilitating the docking of cyclin H, which forms with cdk7 and MAT1 the CAK subcomplex of the general transcription factor TFIIH. Within the RARα‐TFIIH complex, cdk7 phosphorylates RARα at S77 located in the N‐terminal domain. Finally, the phosphorylated RARα/TFIIH complex is recruited to response elements located in the promoter of target genes. MSK1 is also recruited, but separately of the RARα–TFIIH complex, and phosphorylates histones H3.

- NUCLEAR RARα INTERACTS WITH AN ACTIN-BINDING PROTEIN, PROFILIN IIA

The fact that phosphorylation of the NTD is crucial for the transcriptional activity of RARα led us to find phospho-dependent partners of this domain. Up to now, only a few proteins have been reported to interact with the N-terminal domain of nuclear receptors including RARs (Boonyaratanakornkit et al., 2001; Bour et al., 2007a; Bour et al., 2005; Zhao et al., 2009).

With that aim, we isolated profilin IIA, an actin-binding protein with an SH3-like domain. However, we found that the interaction of profilin IIA with RARα is not affected by the phosphorylation of the serine residue flanking the proline motif. This is in contrast to the traditional model in which phosphorylation of serine residues flanking the proline motifs have the ability to positively or negatively regulate the binding of SH3 domains (Kay et al., 2000a; Lalevee et al., 2010b). It is also in contrast to our previous report where we demonstrated that phosphorylation of the serine residue flanking the proline stretch of RARγ induces the dissociation of vinexin β.

- PROFILIN IIA MODULATES THE TRANSCRIPTIONAL ACTIVITY OF RARα

In fact, our results challenged the hypothesis that profilin IIA would control the activity of RARα via a mechanism different from that which was described for vinexin β and RARγ. Indeed, we found that, in contrast to vinexin β, profilin IIA participates positively to the transcription of RARα target genes. Such results are in line with another study showing that nuclear profilin also modulates the activity of other transcription factors (Lederer et al., 2005b). Moreover, profilin IIA is present in chromatin and is recruited with RARα to the promoters of target genes in response to RA.

Most importantly, our results also corroborate the evergrowing evidence that actin, actin-related proteins and actin-binding proteins are constituents of nuclear protein complexes and play a role in transcription (Bettinger et al., 2004; Jockusch et al., 2006; Zheng et al., 2009b). Indeed, a number of actin-binding proteins have been shown to regulate the activity of several transcription factors including nuclear receptors through the recruitment of multiple components of transcription complexes such as chromatin-remodelling, histone acetyl transferase complexes (Archer et al., 2005; Blessing et al., 2004; Gettemans et al., 2005). Such a role of actin-binding proteins in transcription complexes emerged only recently but up to now there are no data showing that profilin IIA belongs to nuclear complexes.

However, on can propose that, profilin IIA might have a role in regulating the properties of nuclear actin through its ability to promote ADP to ATP exchange in G-actin (Fenn et al., 2011; Kast and Dominguez, 2011). Such a role might explain why nuclear forms of actin are prominently found in monomeric states within transcription complexes. Nevertheless, it will require further experiments to test whether profilin IIA has such a role in maintaining the pool of monomeric actin in the nucleus,

when complexed with RARα at RA-target genes promoters. Moreover, it would be interesting to address whether profilin IIA also modulates the activity of the coregulatory complexes of RARα through nucleotide-dependent conformational transitions. Finally, whether the interaction of RARα with profilin IIA can be modulated by other processes than RA signaling or RARα phosphorylation would provide important insights into the role of this adaptor in RA signaling.

In conclusion this study opened new concepts and avenues in the regulation of RA-target genes transcription via an actin-binding protein interacting with the NTD of the RARα subtype. However the biological relevance of profilin IIA remains unclear and requires further investigations.

- NOVEL UNCONVENTIONAL ROLE OF RARα IN CELL ADHESION

Then in order to find out the *in vivo* biological relevance of the RARα interaction with profilinIIA, we looked for a biological model. As mammal biological models are rather complex, we selected mouse ES cells, which are well known to undergo neuronal differentiation in response to RA. WT ES cells are available at the IGBMC and express RARα. However, according to recent studies from the laboratory, RARα is not involved in RA-induced neuronal differentiation of ES cells. Indeed, invalidation of RARα did not affect the ability of these cells to form neurons (Al Tanoury *et al*, manuscript in preparation). Surprisingly, we found that these RARα-/- ES cells were severely impaired in adhesion and spreading. This result brought us to the conclusion that RARα may have a role in cell adhesion and spreading. Two main molecular mechanisms can be proposed for this RARα-dependent process:

- RARα might control the expression of proteins involved in adhesion via genomic effects. Therefore, recently we lunched RNA-seq experiments using WT and RARα-/- cells that will give some indications soon. Whether profilin IIA is involved in such genomic process as a corregulator of RARα is still unknown and will require further profilin IIA gain and loss of function experiments.

- By analogy with the androgen receptor, which was shown to be involved in cell migration via its association with filamin A (Castoria et al., 2011), RARα might also be involved in adhesion and cell spreading through non-genomic effects via its interaction with cytoskeleton actin-binding proteins. One candidate could be profilin IIA, which is known to be involved in cell adhesion (Murk et al., 2009). In this line of idea, we found that a pool of RARα is present outside of nuclei, in the cytosol of ES cells as well as of several neuronal cells and fibroblasts. Unfortunately, profilin IIA was hardly detectable in the cytosol of these cells with our antibodies in immunofluorescense experiments. RARα/profilin IIA complexes were also hardly detectable in the cytosol, eliminating such a possibility. Nevertheless, one cannot exclude that RARα interacts with other actin-binding proteins and/or proteins involved in matrix adhesion (integrin, talin, α-actinin, filamin, vinculin etc.) through

adaptors with SH3 or WW domains. Such a hypothesis will require further experiments.

Finally, the striking point of this study is that CAFs depict higher amounts of cytosolic RARα than normal fibroblasts. As these cells are highly invasive, it strengthens our hypothesis concerning a role for RARα in cell adhesion and migration.

Now the challenge will be to investigate how RARα controls cell adhesion and to discriminate between the genomic and/or non-genomic effects. This will implicate the identification of potential adhesion genes by RNA-seq and or new partners in the cytosol. Finally, it opens new avenues in tumor invasion with RARα as a novel potential marker for prognosis.

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ORIGINAL ARTICLE

A retinoic acid receptor RARα pool present in membrane lipid rafts forms complexes with G protein αQ to activate p38MAPK

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Retinoic acid (RA) regulates several gene programs by nuclear RA receptors (RARs) that are ligand-dependent transcriptional transregulators. The basic mechanism for switching on transcription of cognate-target genes involves RAR binding at specific response elements and a network of interactions with coregulatory protein complexes. In addition to these classical genomic effects, we recently demonstrated that RA also induces the rapid activation of the p38MAPK/MSK1 pathway, with characteristic downstream consequences on the phosphorylation of RARs and the expression of their target genes. Here, we aimed at deciphering the underlying mechanism of the rapid non-genomic effects of RA. We highlighted a novel paradigm in which a fraction of the cellular RARα pool is present in membrane lipid rafts, where it forms complexes with G protein alpha Q (Gαq) in response to RA. This rapid RA-induced formation of RARα/Gαq complexes in lipid rafts is required for the activation of p38MAPK that occurs in response to RA. Accordingly, in RA-resistant cancer cells, characterized by the absence of p38MAPK activation, RARα present in membrane lipid rafts does not associate with Gαq, pointing out the essential contribution of RARα/Gαq complexes in RA signaling.
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Keywords: retinoic acid; RAR; p38MAPK; G alpha Q; lipid rafts

Introduction

Retinoic acid (RA), a pleiotropic signaling molecule derived from vitamin A, regulates critical genetic programs that control development and homeostasis, cell proliferation and differentiation, as well as cell death or survival (Clagett-Dame and Knutson, 2011). This is the basis for the use of RA in cancer therapy (Altucci *et al*., 2007). These effects of RA are mediated by specific nuclear receptors, RA receptors (RARs), which consist of three subtypes, α (NR1B1), β (NR1B2) and γ (NR1B3) (Germain *et al*., 2006a, b). The basics of RARs' structure and function have been recapitulated in several reviews (Laudet and Gronemeyer, 2001; Bastien and Rochette-Egly, 2004; Rochette-Egly and Germain, 2009). Briefly, RARs are multidomain proteins with a central DNA-binding domain linked to an N-terminal domain (NTD) and a C-terminal ligand-binding domain. Classically, RARs function as ligand-inducible transcriptional regulators, heterodimerized with retinoid X receptors (RXRs). As such they regulate the expression of subsets of target genes involved in cell proliferation and differentiation (Duong and Rochette-Egly, 2011; Samarut and Rochette-Egly, 2011). The basic mechanism for switching on gene transcription by RA relies on the binding of RAR/RXR heterodimers to specific sequence elements located in the promoters of target genes and on ligand-induced conformational changes, which cause the association/dissociation of a complex and ever-growing network of coregulatory proteins (Lefebvre *et al*., 2005; Rochette-Egly and Germain, 2009).

In addition to this scenario, recent studies highlighted a novel paradigm in which, *in vivo*, RA also induces the rapid activation of p38MAPK and of the downstream MSK1 (Alsayed *et al*., 2001; Gianni *et al*., 2002; Bruck *et al*., 2009). Most importantly, the activation of the p38MAPK/MSK1 pathway proved to be crucial for fine-tuning the expression of RAR-target genes through the phosphorylation of RARs and their coregulators (Gianni *et al*., 2002, 2006; Bruck *et al*., 2009). Most interestingly, the activation of p38MAPK by RA occurs very rapidly (within minutes), suggesting a non-genomic action of RA and RARs, as described for steroid hormone receptors (Losel and Wehling, 2003; Vasudevan and Pfaff, 2008). Therefore, we aimed at deciphering the underlying mechanism of these rapid non-genomic effects. Here, we showed that a fraction of the cellular RARα pool is present in membrane lipid rafts, which are microdomains of cell membranes enriched not only in cholesterol and sphingolipids but also in signal-transducing molecules, such as flotillins, G proteins-coupled receptors, heterotrimeric G proteins, Rho and Rac GTPases, and their effectors (Pike, 2003; de Laurentiis *et al*., 2007; Sugawara *et al*., 2007; Yao *et al*., 2009). In lipid rafts, G proteins consist of three subunits α, β and γ, and transmit signals to downstream effector molecules such as phospholipase C, protein-kinase C and p38MAPK (Mizuno and Itoh, 2009).

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We identified G protein alpha Q (Gαq), a member of the Gα group, as a new RARα interacting protein in lipid rafts. Finally, both RARα and Gαq were found to mediate the activation of p38MAPK, with characteristic downstream consequences on the expression of the RA-target genes and on cell growth.

Results

RA activates p38MAPK through RARα and Gαq

P38MAPK was rapidly and transiently activated following RA treatment of several cell lines such as human mammary cancer cells (MCF7 cells), HeLa cells, mouse embryocarcinoma cell lines (F9 cells) and mouse embryonic fibroblasts (MEFs) (Figures 1a–d). Simultaneously, the upstream Rho-GTPases Rac and Rock were also strongly activated in response to RA (Figures 1e and f).

Now considerable evidence indicates that Rho-GTPases activation involves upstream Gαq that interacts with scaffolding proteins in membranes (Sugawara *et al*., 2007; Mizuno and Itoh, 2009). Therefore, we analyzed whether Gαq is involved in the rapid RA-induced activation of the p38MAPK pathway. With this aim, Gαq was knocked down with specific small interfering RNAs (siRNAs) in MCF7 and HeLa cells. No activation of p38MAPK was observed in both cell lines (Figures 2a and c), suggesting that the rapid activation of the p38MAPK pathway may be mediated by Gαq. Most interestingly, the activation of p38MAPK was also inhibited upon RARα knockdown in these two cell lines (Figures 2b and c), suggesting that the rapid activation of the p38MAPK pathway may be mediated by both Gαq and RARα. In line with this, p38MAPK was abrogated in F9 cells knockout for RARα (F9 RARα−/−) but not in F9 RARγ−/− and RARβ−/− cells (Figure 1b). Finally, in MEF knockout for the three RARs, MEF (RARα, β, γ)−/− p38MAPK was restored upon re-expression of RARα WT, but not of RARγ WT (Figures 1c and d).

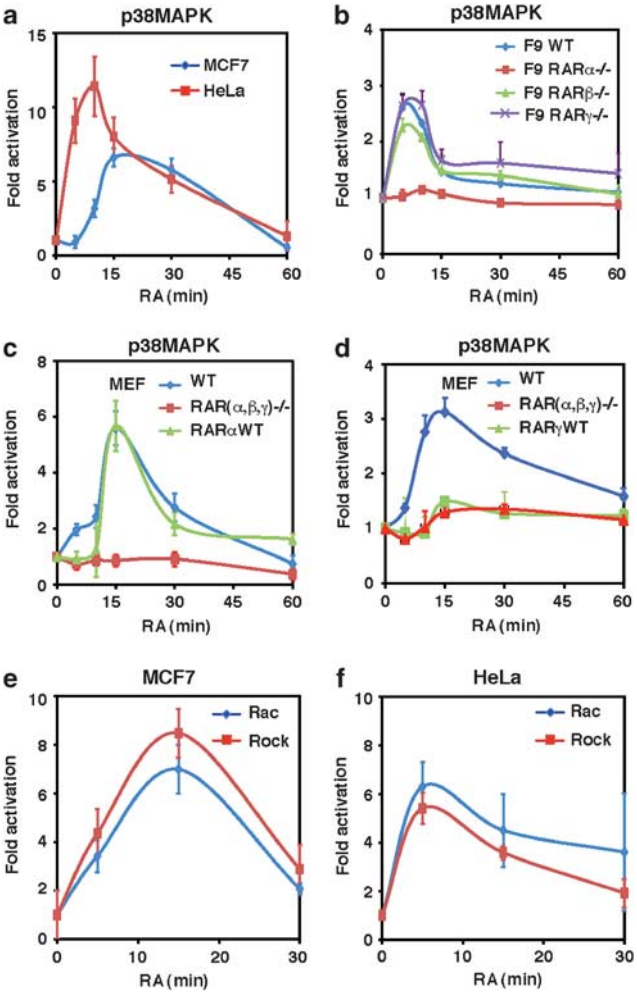


Figure 1 p38MAPK is activated in response to RA. (**a**–**d**) Analysis of p38MAPK activation (phospho p38MAPK ELISA (enzyme-linked immunosorbent assay)) in RA-treated MCF7 cells, HeLa cells, F9 cells (either WT, RARα−/−, RARγ−/− and RARβ−/−) and mouse embryonic fibroblasts (MEFs) [WT, RAR (α, β, γ)−/−, or re-expressing RARα WT or RARγ WT in the RAR (α, β, γ) null background). (**e**, **f**) Analysis of Rac-1 and Rock-2 activities in MCF7 cells (**e**) and HeLa cells (**f**) treated with RA for the indicated times. All results are the mean ± s.d. of duplicates from three distinct experiments.

RARα is present with Gαq in membrane lipid rafts

There is increasing evidence that intracellular signaling pathways are orchestrated by specific, highly ordered membrane microdomains termed as 'lipid rafts' (Simons and Toomre, 2000; Pike, 2003). Indeed, lipid rafts are enriched in a large array of signal transduction components, suggesting that they would be 'signaling centers'. As the rapid activation of p38MAPK by RA involves RARα, we isolated these membrane subfractions in order to investigate whether they contain RARα in addition to the signaling molecules.

MCF7 cells were disrupted and lipid rafts were extracted using a unique technique based on their relative insolubility in certain detergent conditions. Then lipid rafts were isolated by virtue of their high buoyancy when centrifuged on a discontinuous sucrose density gradient (Ostrom and Insel, 2006; Waugh and Hsuan, 2009) (Figure 3a). Such a technique has the advantage of isolating buoyant rafts with their natural composition. Moreover, protein interactions and functions are maintained. As expected, after centrifugation, a faint light-scattering band, which consists of the buoyant lipid rafts material was visible at the 35% sucrose–5% sucrose interface (Ostrom and Insel, 2006). Then all sucrose gradient fractions were collected, resolved by SDS–polyacrylamide gel electrophoresis and analyzed by immunoblotting for lipid raft-associated proteins. Fractions 4 and 5, which correspond to buoyant rafts (Ostrom and Insel, 2006) contained flotillin-2, a marker of lipid rafts (Stuermer, 2011) (Figure 3b). They also contained other proteins that are well known to participate in signaling events, such as Rock-2, PKCδ and Gαq, (Figure 3b), corroborating that these fractions correspond to lipid rafts and are signaling centers.

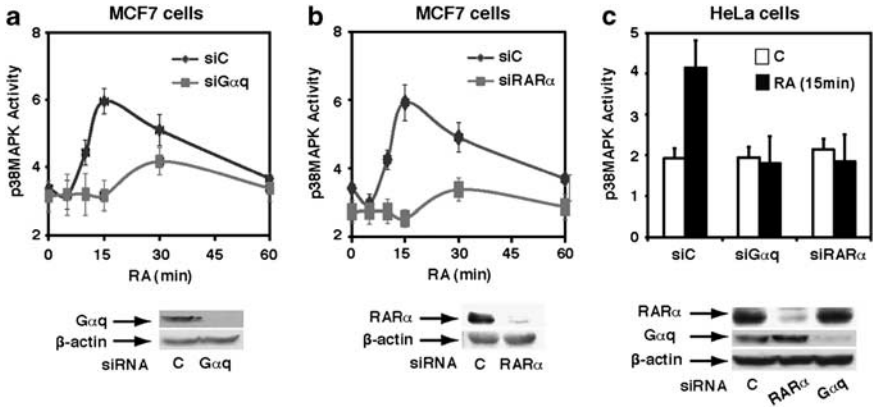


Figure 2 G protein alpha Q and RARα are required for the RA-induced activation of p38MAPK (mitogen-activated protein kinase). Knockdown of Gαq or RARα with specific small interfering RNAs (siRNAs) in MCF7 cells (**a**, **b**) and HeLa cells (**c**) inhibit the activation of p38MAPK that occurs in response to RA. The results are the mean ± s.d. of three experiments. The efficiency of the knockdown was checked by immunoblotting.

Most interestingly, RARα (and not RARγ and RARβ) could be detected in the same fractions corresponding to lipid rafts (Figure 3b). Of note is that the amount of RARα present in lipid rafts was very low compared with the amount of RARα present in the bottom fractions (fractions 8–12), which contain the rest of the cellular material, including the nuclear components. Indeed, in immunofluorescence experiments performed with MCF7 cells, RARα was detected essentially in nuclei (Figure 3d). However, low levels of RARα were also detected out of nuclei and colocalized with flotillin-2 (Figures 3f and g). Collectively, these results converge to the conclusion that a small population of RARα is present out of nuclei in membrane lipid rafts.

Then the question is, how RARα (and not RARβ nor RARγ) is targeted to membranes. Given that the three RAR subtypes depict a very high degree of identity in the sequence of their DNA-binding domains and ligand-binding domains, one can speculate that the NTD might specifically target RARα at the membrane. Therefore, we analyzed whether RARα deleted for this domain (RARαΔNTD) was present or not in lipid rafts, taking advantage of the F9 cells re-expressing RARα WT or ΔNTD in a RARα null background (Rochette-Egly *et al*., 2000). Remarkably, in contrast to RARα WT, RARαΔNTD could not be detected in lipid rafts isolated from the corresponding rescue F9 cell line, indicating that the NTD would be involved in targeting RARα to membranes. In contrast, RARα deleted from the AF-2 domain (RARαΔH12) could be detected in rafts isolated from F9 cells expressing this mutant (Rochette-Egly *et al*., 2000) (Figure 3h), validating our conclusions.

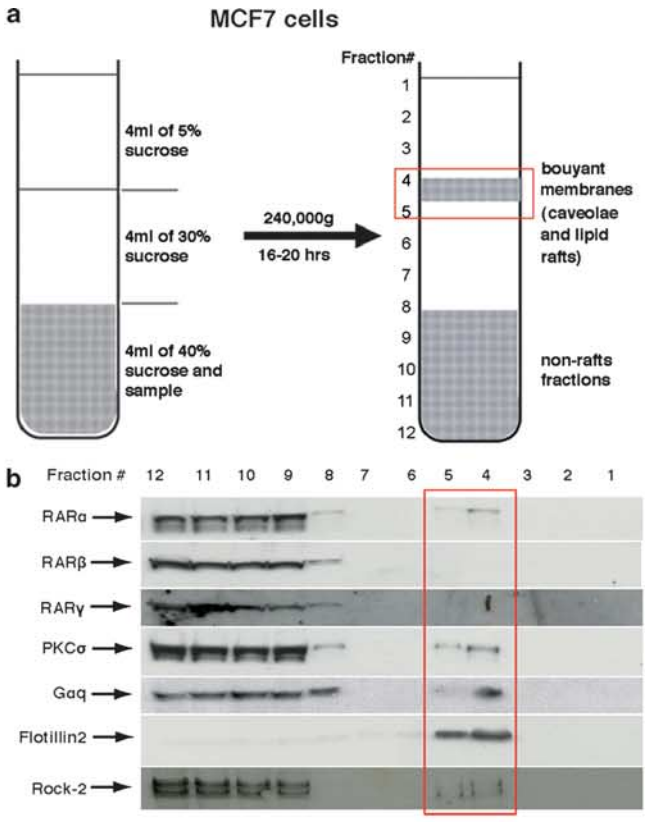
RARα interacts with Gαq in vitro *and* in vivo

Given that Gαq proteins colocalize with RARα in lipid rafts and are involved in the activation of the p38MAPK pathway, we investigated whether both proteins can interact with each other. First, the ability of RARα to interact with Gαq was analyzed with the recombinant proteins in *in vitro* protein–protein interaction and coimmunoprecipitation experiments. Recombinant GST (glutathione S-transferase)-RARα expressed in *E.coli* and bound to glutathione-sepharose beads interacted with *in vitro*-translated Gαq (Figure 4a, lane 5). A similar interaction was observed with a constitutively active form of Gαq (Figure 4a, lane 6). These results were confirmed in coimmunoprecipitation experiments performed with extracts from COS-1 cells over expressing RARα and Gαq, and treated or not with RA. Indeed, some Gαq was detected in the RARα immunoprecipitates in the absence of RA (Figure 4b, lane 4). Most interestingly, the efficiency of the interaction increased markedly after addition of RA (Figure 4b, lanes 5–8). Altogether, these results indicate that RARα can form complexes with Gαq and that these complexes are increased in the presence of RA.

Finally, whether endogenous RARα and Gαq form complexes *in vivo* in lipid rafts was investigated. High amounts of lipid rafts were prepared from RA-treated MCF7 cells and immunoprecipitated with RARα antibodies. As shown in Figure 4c, Gαq could be detected in RARα immunoprecipitates corroborating that RARα and Gαq belong to a same complex in membrane lipid rafts.

Visualization of endogenous RARα/Gαq complexes, in situ*, by proximity ligation*

Next*, in situ* proximity ligation assay (PLA) (Soderberg *et al*., 2006) was used to explore further the endogenous RARα/Gαq complexes. The assay is similar to fluorescence resonance energy transfer and allows the detection of proteins in sufficient proximity (<40 nm). It is based on the use of two primary antibodies raised in different species that recognize the antigens of interest. Then species-specific secondary antibodies, called PLA probes, each with a unique short DNA strand attached to it, are added. When the PLA probes are in close proximity, the DNA strands can be joined through the subsequent addition of a circle-forming DNA oligonucleotide that is amplified by rolling circle amplification using a polymerase. After the amplification reaction, several-hundred fold replication of the DNA circle has occurred, and labeled complementary oligonucleotide



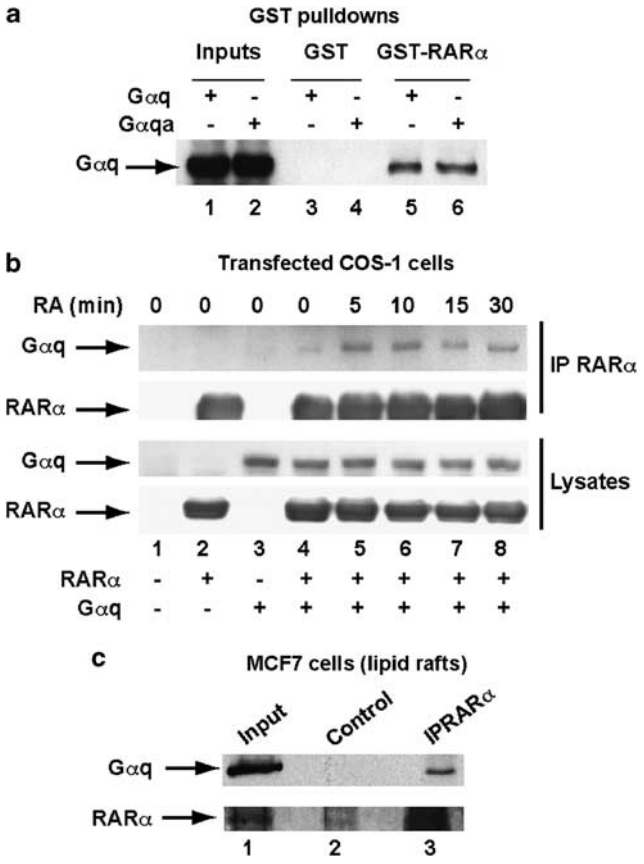


Figure 4 G protein alpha Q interacts with RARα both *in vitro* and *in vivo*. (**a**) *In vitro* translated Gαq and a constitutively active form of Gαq (Gαqa) were incubated with glutathione S-transferase (GST) or GST-RARα immobilized on glutathione-sepharose beads. Bound Gαq was analyzed by immunoblotting. (**b**) COS-1 cells were transfected with the RARα vector along with Gαq and treated with RA for the indicated times. Extracts were incubated with mouse monoclonal RARα antibodies and protein G-sepharose beads. Immunocomplexes were resolved by SDS–polyacrylamide gel electrophoresis and immunoblotted with RPα (F) and Gαq antibodies. The two bottom panels correspond to 5% of the amount of immunoprecipitated extracts. Lanes 1 to 3 correspond to the immunoprecipitation controls. (**c**) Lipid rafts were prepared from 2×10^8 MCF7 cells treated with RA for 10 min. The light scattering bands, which consist of the buoyant lipid rafts material were collected and immunoprecipitated with monoclonal RARα antibodies. Bound Gαq was analyzed by immunoblotting.

Figure 3 RARα is present in membrane lipid rafts. (**a**) Schematic representation of the rafts isolation procedure by sucrose gradient centrifugation. (**b**) Immunoblotting analysis of the different gradient fractions showing that RARα is present with Flotillin-2, Gαq and other signaling components in lipid rafts. (**c**–**g**) Confocal microscopy analysis of MCF7 cells fixed and triple stained with 4,6-diamidino-2-phenyl indole (DAPI) (blue, **c**), anti-RARα antibody (green, **d**), and anti-Flotillin-2 antibody (red, **e**). The merge images overlapping the red, green and blue fluorescence (**f**, **g**) show that though mainly nuclear, RARα can be detected out of nuclei and colocalizes with flotillin-2. (**h**) Immunoblotting analysis of lipid rafts (fractions 4 and 5) isolated from F9 cells expressing RARα, that is, WT, ΔNTD or ΔAF-2 in a RARα null background, and showing that RARαΔNTD cannot be detected in rafts.

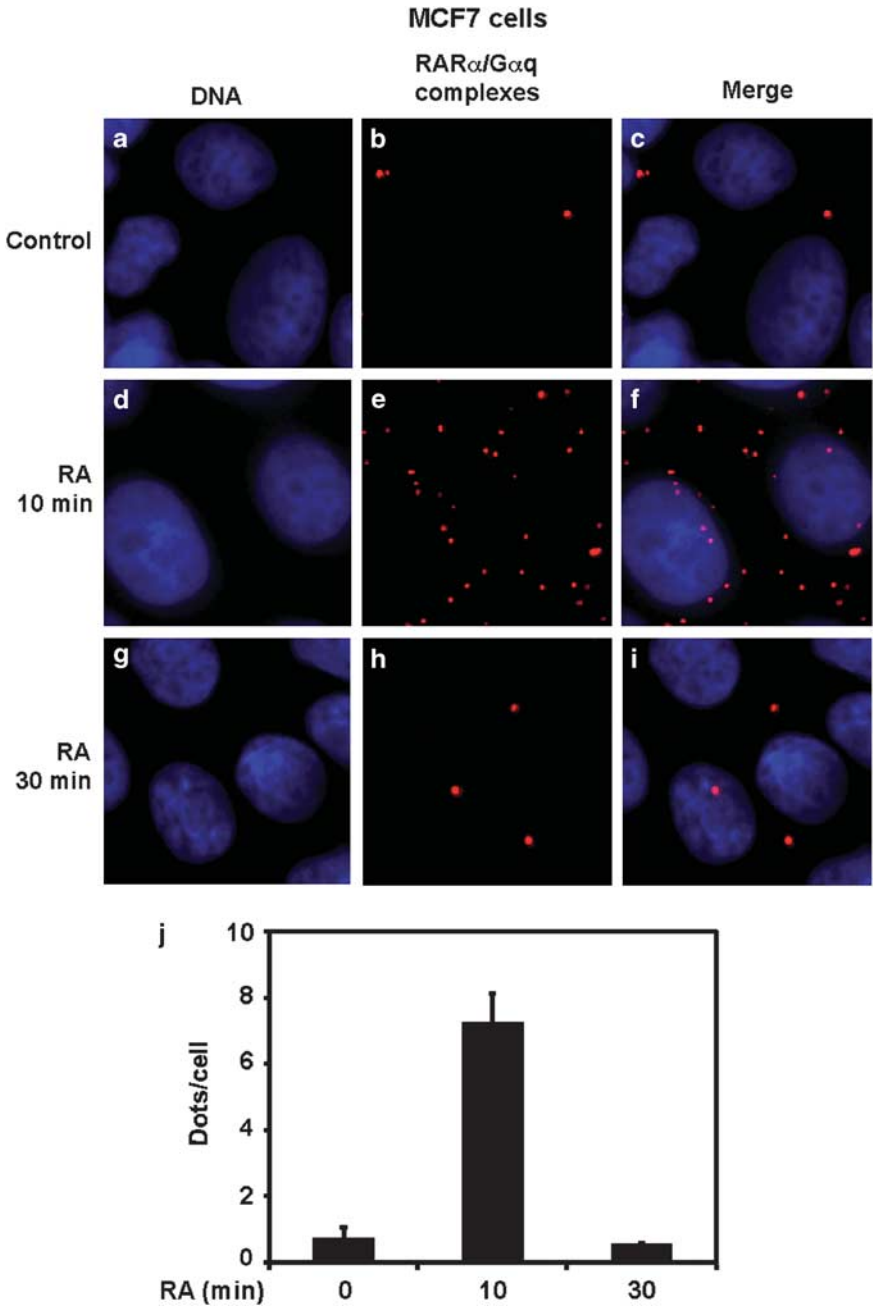


Figure 5 Analysis of RARα/Gαq complexes in MCF7 cells by immunofluorescence microscopy in combination with *in situ* proximity ligation assay (PLA). (**a**–**i**) PLA is highly specific for detecting physically interacting protein–protein complexes (red, panels **b**, **e**, **h**). DNA was counterstained with 4,6-diamidino-2-phenyl indole (DAPI) (blue, panels **a**, **d**, **g**). The merge between blue and red is also shown (panels **c**, **f**, **i**). A few RARα/Gαq complexes are shown in control MCF7 cells (**b**, **c**). The amount of complexes increased markedly out of nuclei in MCF7 cells treated with RA for 10 min (**e**, **f**) and returned to control values at 30 min (**h**, **i**). (**j**) Statistical analysis of the signals using the Blobfinder V3.2 software.

probes highlight the product. The resulting high concentration of fluorescence in each single-molecule amplification product is easily visible as a distinct bright spot when viewed under a fluorescence microscope.

Here, rabbit anti-RARα and mouse anti-Gαq antibodies were used under the PLA conditions to explore, *in situ*, endogenous RARα/Gαq complexes, in MCF7 cells treated with RA for different times. Quite few RARα/Gαq complexes were detected in the control untreated cells (Figures 5b and c). Considerable higher amounts of complexes were seen in the RA-treated cells with a peak around 10 min (Figures 5e and f), which corresponds to the peak of p38 MAPK activation. Most interestingly, the signals were out of nuclei. Then the number of spots returned to control values at 30 min (Figures 5h and i). Spots number was quantified using the Blobfinder V3.2 software (Centre for Image Analysis, Uppsala University, Sweden, http://www.cb.uu.se/~amin/BlobFinder/) and the average values ± s.d. from at least two experiments are shown (Figure 5j). No signal was seen

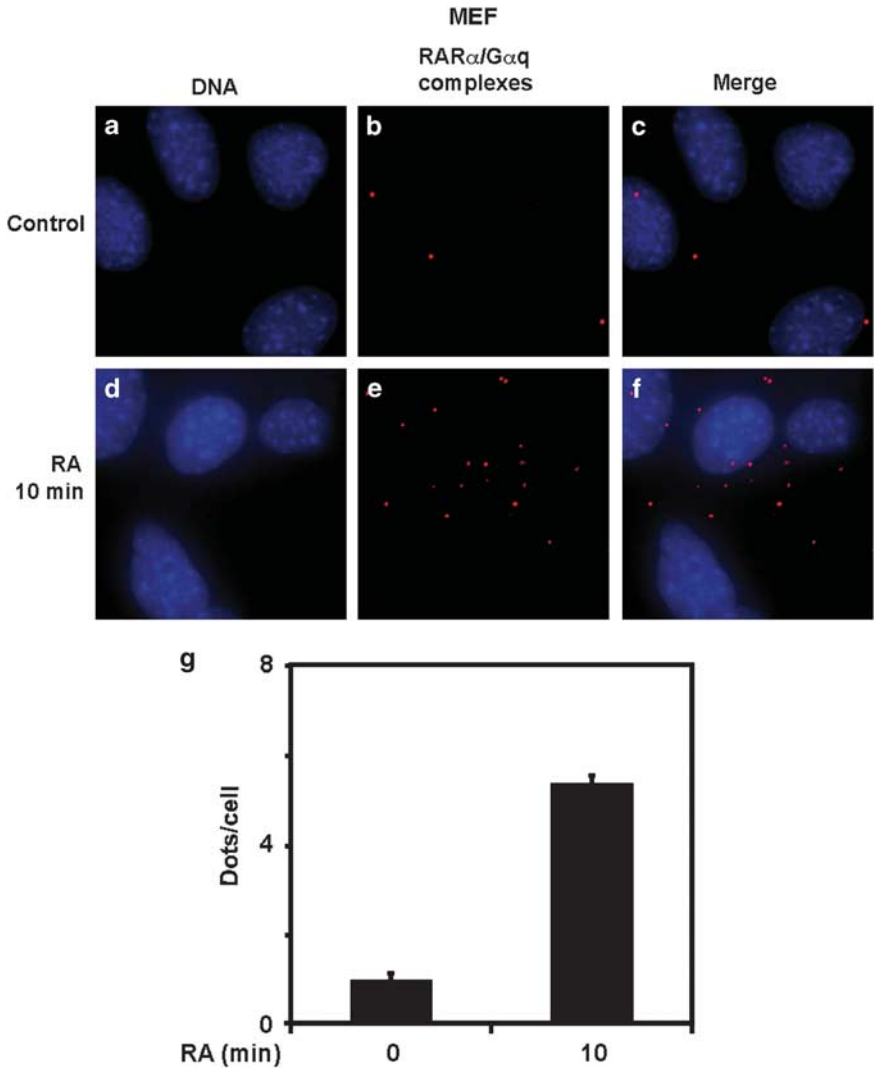


Figure 6 Analysis of RARα/Gαq complexes in mouse embryonic fibroblasts (MEFs) by immunofluorescence microscopy in combination with *in situ* proximity ligation assay (PLA). (**a**–**f**) The number of red dots corresponding to the RARα/Gαq complexes increases in MEFs treated with RA for 10 min as in Figure 5. (**g**) Statistical analysis as in Figure 5j.

when each primary antibody was used individually, validating the specificity of the technique. Similar results were obtained with several other RA-treated cells such as HeLa cells and MEF (Figure 6). Collectively, these results confirm that endogenous RARα is present in a complex with endogenous Gαq out of nuclei, in response to RA.

Note, however, that the increased number of RARα/Gαq complexes was observed specifically in response to RA. Indeed, the activation of the p38MAPK pathway that occurs in response to epidermal growth factor (EGF) through Gαq proteins (Figure 7a), does not involve RARα (Figure 7a). Accordingly, no increase in RARα/Gαq complexes could be observed in response to EGF as assessed in a PLA (Figure 7b).

In erbB-2 positive breast cancer cells, p38MAPK is not activated in response to RA and RARα does not form complexes with Gαq

Then, we asked whether RA-resistant cells exemplified by the human erbB-2 positive breast cancer cells also depict an activation of p38MAPK and the formation of RARα/Gαq complexes in response to RA. First, the erbB-2 positive cells, BT474, SKBR3, MDA-MB453 and MDA-MB361 cells (Tari *et al*., 2002) (Figure 8a) were compared with MCF7 cells for p38MAPK activation in response to RA. Remarkably, in these cell lines p38MAPK activation was decreased or abrogated up to 60 min after RA addition (Figure 8b).

Then, we concentrated on BT474 cells, which depict the highest levels of erbB-2 and are completely defective in p38MAPK activation. Membrane lipid rafts were isolated from these cells and were analyzed by immunoblotting. Both RARα and Gαq could be detected in lipid rafts from these cells (Figure 8c). However, no RARα/Gαq complexes could be visualized *in situ* by proximity ligation (Figures 8e–j), whereas a high amount of complexes were seen in MCF7 cells under the same conditions (Figures 8k–p). Note that the ability of EGF to activate p38MAPK was also abrogated in BT474 cells (Figure 8d), though this

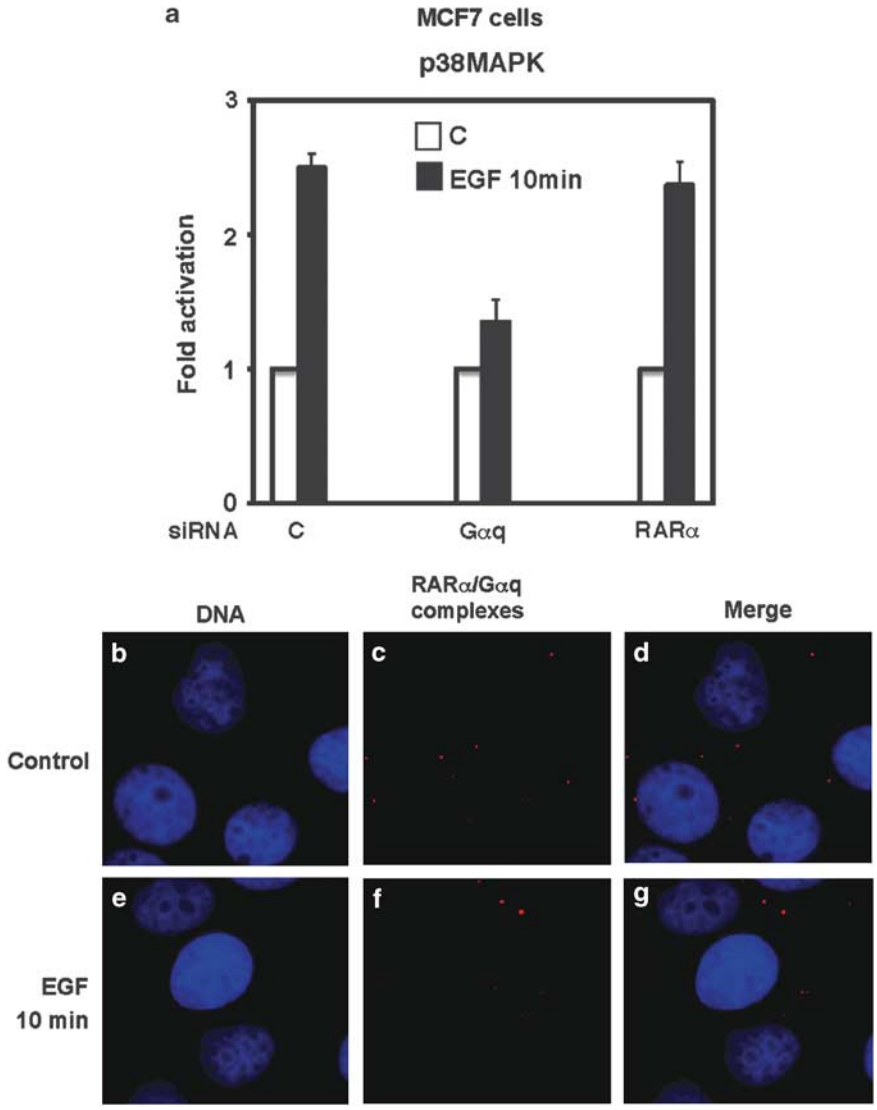


Figure 7 P38MAPK (mitogen-activated protein kinase) activation that occurs in response to epidermal growth factor (EGF) does not involve RARα. (**a**) Analysis of the EGF-induced activation of p38MAPK in MCF7 cells knockdown for Gαq or RARα with specific small interfering RNA (siRNAs). (**b**–**g**) EGF does not induce the formation of RARα/Gαq complexes in MCF7 cells, as assessed by immunofluorescence microscopy in combination with *in situ* proximity ligation assay (PLA).

pathway does not involve the formation of RARα/Gαq complexes (see Figure 7). Collectively, these data highlight the importance of the initial formation of RARα/Gαq complexes in lipid rafts for p38MAPK activation in response to RA. They also suggest that the signaling pathways, involving Gαq proteins, are affected in erbB-2 positive cells (see Discussion).

The integrity of the Gαq/p38MAPK pathway is required for the full RA-induction of RARα-target genes and the antiproliferative action of RA

According to our previous work, activation of the p38MAPK/MSK1 pathway is significant for the activation of RARα-target genes in several cell lines, including MCF7 cells and MEF (Bruck *et al*., 2009). Then, we asked whether upstream Gαq is required for the induction of RA-target genes. In MCF7 cells, RA treatment enhances the expression of the *Hoxa-1* and *Btg2* genes, which are the paradigm of the RA-target genes as assessed by quantitative real time–PCR. Knockdown of Gαq decreased the RA-induced expression of both genes (Figures 9a and b). Most interestingly, it also reduced the antiproliferative action of RA (Figure 9c) in line with other studies (Lai *et al*., 2008; White *et al*., 2008). Of note is that knockdown of the downstream effectors p38MAPK and MSK1 also decreased the RA-induced expression of RA-target genes, though less efficiently than the knockdown of RARα (Figure 8c), corroborating our previous studies (Bruck *et al*., 2009). Collectively, these results highlight the importance of the Gαq/p38MAPK/MSK1 pathway for RAR-target gene expression and growth arrest that occur in response to RA.

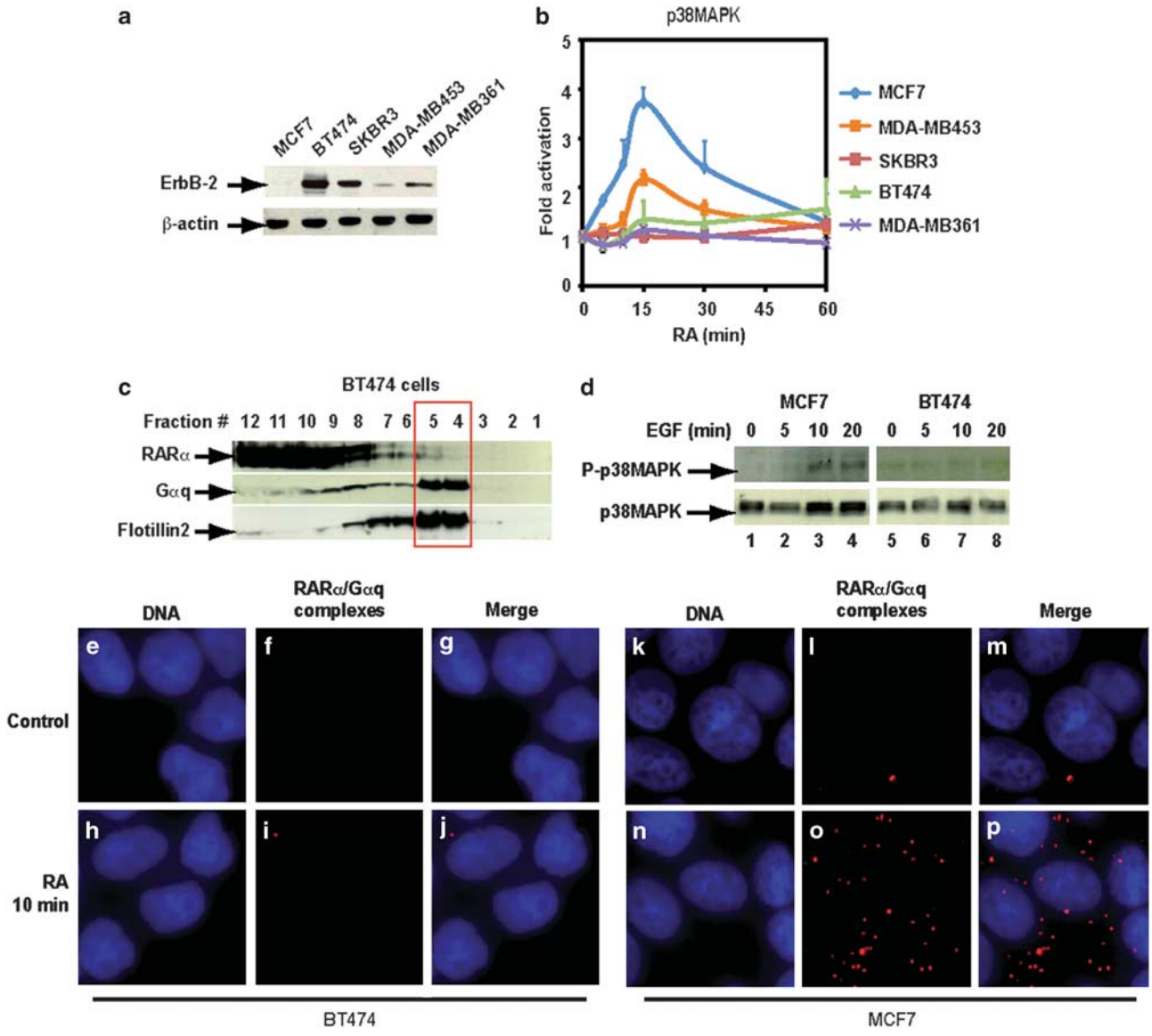


Figure 8 In erbB-2 positive breast cancer cells, p38MAPK (mitogen-activated protein kinase) is not activated in response to RA and RARα does not form complexes with Gαq. (**a**) ErbB-2 expression in MCF7, SKBR3, BT474, MDA-MD453 and MDA-MB361 human breast cancer cells. (**b**) Comparison of p38MAPK activation in RA-treated MCF7 and erbB-2 positive cells. (**c**) Lipid rafts prepared from BT474 cells contain RARα and Gαq. (**d**) In BT474 cells, p38MAPK is not activated in response to epidermal growth factor (EGF), as assessed by immunoblotting analysis of active phospho p38MAPK. (**e–j**) In BT474 cells, RARα does not form complexes with Gαq in response to RA, as assessed by immunofluorescence microscopy in combination with *in situ* proximity ligation assay (PLA). (**k–p**) RARα/Gαq complexes in RA-treated MCF7 cells as assessed by *in situ* PLA.

Discussion

Rapid responses, such as the activation of signaling pathways, are initiated at the plasma membrane in lipid rafts and then amplified by cascades of activation of downstream effectors including G proteins, Rho family small GTPases, phospholipase C, the Src family tyrosine kinases and p38MAPK. As RA activates very rapidly the p38MAPK pathway, we speculated that this effect would be non-genomic and mediated by a membrane-associated RAR. Here, we provide evidence that in various mammalian epithelial and fibroblastic cells, the RARα subtype, which is normally found in the nucleus, is also present in plasma membranes. Moreover, membrane-associated RARα forms complexes with Gαq to generate rapid p38MAPkinase activation in response to RA (see Figure 9e).

First of all, to find out the mechanism of activation of the p38MAPK pathway by RA, we investigated, which RAR subtype is involved and whether this RAR could be detected in membrane lipid rafts that are 'signaling centers'. Taking advantage of cell lines invalidated for one specific RAR subtype or re-expressing a specific RAR in a triple RAR (α, β, γ) null background, we demonstrated that the activation of p38MAPK in response to RA, involves specifically the RARα subtype.

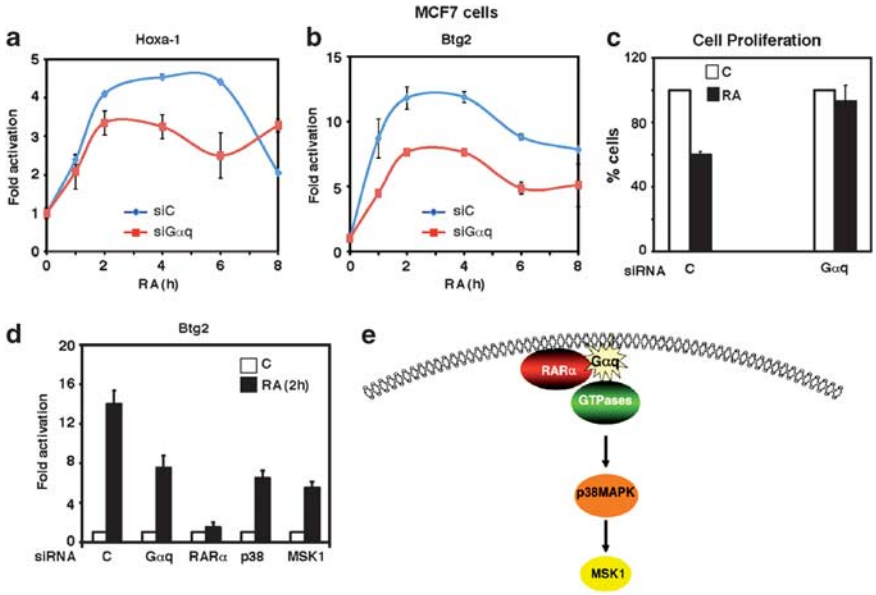


Figure 9 Gαq is required for the RA-induction of the *Hoxa1* and *Btg2* genes and for the antiproliferative action of RA. In MCF7 cells, the RA-induced expression of the *Hoxa-1* (**a**) and *Btg2* (**b**) genes is decreased upon knockdown of Gαq, as assessed by quantitative real time–PCR. (**c**) Knockdown of Gαq also decreases the antiproliferative action of RA. (**d**) Knockdown of the downstream effectors p38MAPK (mitogen-activated protein kinase) and MSK1 also decreases the RA-induced expression of the *Btg2* gene. The results are the mean ± s.d. of three individual experiments. (**e**) Model of the activation of the p38MAPK pathway through RARα and Gαq located in membranes.

However, in immunofluorescence confocal studies, RARα was present essentially in nuclei and was hardly detected in plasma membranes, despite some spots suggesting a colocalization of RARα with flotillin-2, a marker of lipid rafts. In fact, RARα could be detected in detergent-insoluble membrane lipid rafts that are concentrated by sucrose equilibrium density gradients and that are enriched in components of signal transduction pathways (Pike, 2003; de Laurentiis *et al*., 2007). RARα was detected in the same low-density buoyant fractions as flotillin and Gαq. The fact that RARα colocalizes with flotillin-2 in lipid rafts is a novelty in the field. It is also in line with the new concept in which most of the classical so-called nuclear steroid and non-steroid receptors (ER, GR, PR, AR, VDR) can be found in specialized plasma membrane structures such as caveolae and lipid rafts (Huhtakangas *et al*., 2004; Marquez *et al*., 2006; Pedram *et al*., 2007; Luoma *et al*., 2008; Matthews *et al*., 2008).

How RARα is recruited to the plasma membrane is still ill-defined. Indeed, in contrast to the other steroid receptors, RARα does not depict any palmitoylation motif that would facilitate its membrane localization (Pedram *et al*., 2007). However, according to our results, the N-terminal domain that is not conserved between RARs, appears to be necessary for targetting specifically RARα to lipid rafts. In addition, RARα present in lipid rafts forms complexes with Gαq proteins and the number of these complexes is increased in response to RA. These complexes were seen *in vitro* with the recombinant proteins by GST pull down and coimmunoprecipitation and were corroborated *in vivo*, with the endogenous proteins in coimmunoprecipitation experiments performed with enriched lipid rafts. In fact, the originality of the present study resides in the use of a 'PLA', which can reveal transient endogenous protein complexes even when present at very low levels (Soderberg *et al*., 2006). Such a technique allowed us to visualize, *in situ*, a rapid and transient increase (10–15 min after RA addition) in RARα/Gαq complexes. It also revealed that the complexes are out of the nuclei, confirming the hypothesis that the non-genomic effects of RA involve a pool of extra nuclear RARα.

The interesting point is that Gαq is involved with RARα in the activation of p38MAPK (Figure 8d). This is a novelty in the field of the non-genomic effects of RA and is in agreement with the well-known role of Gαq in the activation of the p38MAPK pathway (Sugawara *et al*., 2007; White *et al*., 2008; Mizuno and Itoh, 2009). It is worth noting that this pathway was activated by RA in epithelial and fibroblastic cells but not in neuronal and sertoli cells, where RA rather activates the p42/p44MAPK (Erk) pathway through Src and PI3K (Pan *et al*., 2005; Dey *et al*., 2007; Masia *et al*., 2007; Chen and Napoli, 2008; Zanotto-Filho *et al*., 2008). Thus, the mechanism of the non-genomic effects of RA appears to involve different membrane-associated complexes, depending on the MAPK pathway that is RA-activated and on the cell type. Similarly, the non-genomic effects of the other nuclear receptors involve different kinases and different membrane molecular complexes, depending on their functional significance (Losel and Wehling, 2003; Norman *et al*., 2004). As an example, in breast cancer cells and in response to its cognate ligand, the estrogen receptor ERα activates the Erk pathway through complexes containing c-Src and the regulatory subunit of PI3K (p85α) (Migliaccio *et al*., 1998; Le Romancer *et al*., 2008). In contrast, in neuronal cells, ER rather activates protein kinase C through Gαq

and phospholipase C (Qiu *et al*., 2003). In the same line of idea, depending on the cell type, VDR activates either the RhoA-Rock-p38MAPK pathway (Ordonez-Moran *et al*., 2008) or the Raf/Erk pathway (Norman *et al*., 2001; Losel and Wehling, 2003).

The present study also corroborates the new concept, according to which the non-genomic effects of RA influence the genomic effects (Rochette-Egly and Germain, 2009; Piskunov and Rochette-Egly, 2011). Indeed, our results indicate that the knockdown of Gαq and its downstream effectors, p38MAPK and MSK1, decreases the expression of RAR-target genes. The modes of interaction between the non-genomic and genomic effects of RA are complex, but according to other studies from our laboratory, the downstream effectors of the non-genomic effects, p38MAPK and MSK1, phosphorylate the actors of the genomic action of RA, that is, histones, RARs and their coregulators such as SRC-3 (Gianni *et al*., 2002, 2006; Bruck *et al*., 2009). As such, the RA-induced phosphorylation cascades control protein–protein and DNA–protein interactions, and the dynamics of transcription. Thus, one can conclude that the non-genomic effects of RA cross talk with the genomic processes for assuming the specificity of RAR-target genes expression.

Finally, our results highlight that in RA-resistant breast cancer cells characterized by aberrant receptor tyrosine kinase expression and/or activity (exemplified by erbB-2 breast cancers), the formation of RARα/Gαq complexes is suppressed, resulting in the abrogation of the non-genomic effects of RA, that is, the activation of p38MAPK. Remarkably, other signaling pathways involving Gαq proteins, such as the EGF pathway, are also affected in such cells. It is important to note that most signaling pathways are controlled by essential scaffolding proteins of lipid rafts such as caveolin-1 (Sugawara *et al*., 2007; Staubach and Hanisch, 2011). Moreover, caveolin-1 is frequently downregulated in erbB-2 positive cells (Park *et al*., 2005). Therefore, one can speculate that the integrity of lipid rafts composition would be required for the formation of Gαq-based complexes including RARα/Gαq complexes, and thereby for the activation of the signaling pathways. Whether there is a correlation between these observations and the resistance of such cancer cells to the antiproliferative action of RA would require further investigations, but is out of the scope of this study. Nevertheless, our results and others (Lai *et al*., 2008; White *et al*., 2008; Staubach and Hanisch, 2011) suggest that Gαq proteins, as well as several components of lipid rafts, would have an important role in the control of not only gene expression but also cell growth.

Materials and methods

Plasmids, antibodies and reagents

The pSG5-based expression vector for RARα and the prokaryotic pGEX-2T vector encoding RARα fused to GST were previously described (Bour *et al*., 2005). The pcDNA3.1 Gαq vectors (WT and constitutively active) were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA).

Mouse monoclonal antibodies (Ab10α (A) and Ab9α(F)) as well as rabbit polyclonal antibodies (RPα(F)) raised against RARα were described earlier (Gaub *et al*., 1992). Rabbit polyclonal antibodies against PKCδ (C-17), goat polyclonal antibodies against β-actin (C-11) and Rock-2 as well as mouse monoclonal antibodies against Gαq and Flotillin-2 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The rabbit polyclonal antibodies against RARα (C-20), used in the immunofluorescence experiments were also from Santa Cruz. Antibodies against p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182) and HER2/ErbB2 antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies against GAPDH were from Sigma Aldrich Chemie (Saint Quentin Fallavier, France), as well as all trans RA and epidermal growth factor (EGF).

Cell culture, proliferation, transfections and immunoprecipitations

MCF7, BT474, SKBR3, MDA-MB453, MDA-MB361 and HeLa cells were cultured under standard conditions. MEFs with all three RARs deleted and re-expressing RARα WT or RARγ WT were previously described (Bruck *et al*., 2009). Mouse embryocarcinoma cells (F9 cells), WT, RARα−/−, RARγ−/− and RARβ−/−, were previously described (Taneja *et al*., 1997; Faria *et al*., 1999), as well as F9 cells re-expressing RARα, WT, ΔNTD and ΔAF-2 in a RARα null background (Rochette-Egly *et al*., 2000). COS-1 cells were transiently transfected using the FuGENE 6 reagent (Roche, Meylan, France) according to the manufacturer's protocol. When they have reached 80–90% confluency, cells were treated with RA (10^{-7} M) or EGF (100 ng/ml), after 24 h in a medium containing 1% fetal calf serum (Bruck *et al*., 2009). Extracts were prepared, immunoblotted or immunoprecipitated as described (Bruck *et al*., 2009).

Cell proliferation was analyzed by using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay kit according to the manufacturer's instructions (Roche Diagnostics).

Detection of active p38MAPK, Rac1 and Rock2

Phosphorylated p38MAPK was detected by using a phospho p38MAPK (Thr (P)-180/Tyr (P)-182) ELISA kit (Biosource Invitrogen Corporation). Where indicated it was also analyzed by immunoblotting with antibodies recognizing specifically the active phosphorylated form of p38MAPK. Activation of Rho family GTPases was analyzed using the Rac1,Rac 3 and 3 G-LISA Rac activation assay Biochem Kit (Cytoskeleton, Denver, CO, USA), and the Rock activity assay kit (Cell Biolabs, San Diego, CA, USA).

GST pull-down assays

Equimolar amounts of GST and GST-fusion proteins expressed in *Escherichia coli* were purified on glutathione-sepharose 4B beads (Amersham Biosciences, GE Healthcare Europe GmbH, Branch France, Velizy-Villacoublay, France) and incubated as previously described (Bour *et al*., 2005), with *in vitro*-translated Gαq produced in Quick Coupled Transcription/Translation System (Promega, Charbonnières les Bains, France).

Small interfering RNA

The ON-TARGET plus SMART pool siRNA against human Gαq (M-008562-00-0005), human RARα (L-003437-00-0005), human p38MAPKα (L-003512-00), human MSK1 (M-004665-01) and the control non-targeting siRNA pool (D-001206-13) were purchased from Dharmacon (Thermo Fisher Scientific, Illkirch, France). Cells were transfected with siRNAs (50 nM) according to

the manufacturer's protocol and treated with RA (10^{-7} M) 48 h post-transfection. Then at the indicated times, the cells were harvested and subjected to RNA and protein analysis.

Rafts Isolation

Membrane lipid rafts were isolated using the procedure described in Ostrom and Insel (2006) and Waugh and Hsuan (2009). Briefly, 6×10^7 cells grown in petri dishes were washed twice in phosphate-buffered saline and then lyzed for 1 min on ice in 1 ml of 10 mM Tris–HCl (pH 7.4) containing 1% (v/v) Triton X-100, 1 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid and protease/phosphatase inhibitors. Then the cell lysate was scrapped, transferred to a 2-ml Dounce homogenizer and homogenized with 10 strokes on ice. The lysate was adjusted to 2 ml and an equal volume of 80% sucrose was added. The two solutions were then mixed thoroughly by pipetting up and down several times to give a final concentration of 40% sucrose in a 4-ml volume. Then 4 ml of 30% sucrose and 4 ml of 5% sucrose were layered carefully. After centrifugation at $240\,000 \times g$ for 16 h at 4 °C, samples were collected from the top of the tube as 1-ml fractions (12 fractions in total). Fractions were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting. A faint light-scattering band, which consists of the buoyant lipid rafts material, was often visible at the 35%-5% sucrose interface (fractions 4 and 5).

For further immunoprecipitation of rafts proteins, rafts were prepared from 2×10^8 cells. After sucrose gradient centrifugation, fractions corresponding to rafts were collected, centrifuged ($100\,000 \times g$ for 1 h at 4 °C) and the final pellet was resuspended in immunoprecipitation buffer.

Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde –phosphate-buffered saline for 20 min, permeabilized with 0.5% Triton X-100 and blocked with 1% bovine serum albumin in phosphate-buffered saline for 30 min. Then the cells were incubated with the primary antibodies, followed by ALEXAFluor 448 or 555 conjugated secondary antibodies (Invitrogen, Villebon sur Yvette, France). Nuclei were counterstained with 4,6-diamidino-2-phenyl indole (Sigma-Aldrich Chimie). Cells were analyzed by fluorescence microscopy using a LEICA DMRX microscope (LEICA Microsystems, Rueil Malmaison, France) equipped with a LEICA True Confocal Scanner TCS SP. The used objective was Leica HCX PL APO 63×1.40 LBL.

Proximity ligation assay (PLA)

Cells were grown on coverslips, fixed, permeabilized, blocked and incubated with primary antibodies (anti-RARα and anti Gαq), as described for immunofluorescence experiments. Duolink II (Eurogentec, Angers, France) *in situ* PLA was performed according to the manufacturer's protocol. PLA probes were incubated for 1 h at 37 °C, followed by hybridization, ligation, amplification and detection. Nuclei were counterstained with 4,6-diamidino-2-phenyl indole. Slides were analyzed by fluorescence microscopy using a LEICA DM4000B microscope (LEICA Microsystems) equipped with a Cool SNAP photometric camera. The used objective was Leica HCX PLAN APO 40x0, 75 PH 2. The number of fluorescent signals and nuclei in an image were counted, and were statistically analyzed using the Blobfinder V3.2 software from the Centre for Image Analysis at Uppsala University (http://www.cb.uu.se/~amin/BlobFinder/). Statistical significance was accepted when $P<0.05$ using one-tailed Student t test. Values are presented as means ± s.d.

RNA isolation and quantitative real time–PCR

Total RNAs were isolated and subjected to quantitative real time–PCR as described (Bruck *et al*., 2009). All primers are QuantiTect Primer Assays from Qiagen (Courtaboeuf, France, Hs_BTG2_1_SG QuantiTect Primer Assay QT00240247; Hs_HOXA1_1_SG QuantiTect Primer Assay QT00011963). Transcript levels were normalized according to β-actin transcripts, which are unresponsive to RA.

Conflict of interest

The authors declare no conflict of interest.

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PUBLICATION 2

Profilin IIA: a novel coregulator of the N-terminal domain of the Retinoic acid receptor alpha (RARα)

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ABSTRACT

Nuclear retinoic acid (RA) receptors (RARs) are ligand-dependent regulators of transcription. Their transcriptional activity relies mainly on their recruitment to specific DNA response elements and on their interactions with several coregulators at the ligand-binding domain. However, the N-terminal domain (NTD) also plays a role through its phosphorylation. Here, using a yeast two-hybrid system, we isolated profilin IIA as a novel partner of the NTD of the RARα subtype. Profilin IIA is a small ubiquitous actin-binding protein with an SH3-like domain. We demonstrated that the SH3-like domain of profilin IIA interacts with the proline-rich motif (PRM) located in RARα NTD. The interaction was not affected by the phosphorylation of the serine residue located in the PRM or by RA addition. *In vivo*, profilin IIA modulates positively RARα-mediated transcription of several target genes. In the absence of RA, profilin IIA interacts with RARα in nuclei. In response to RA, profilin IIA is corecruited with RARα to the promoters of RA-target genes and modulates their expression.

INTRODUCTION

Retinoic Acid (RA) influences the differentiation, proliferation and apoptosis of a variety of cell types through modifications in the expression of target genes. The effects of RA are mediated by specific nuclear receptors, RA receptors (RARs), which consist of three subtypes α, β and γ and function as ligand-dependent regulators of transcription heterodimerized with other nuclear receptors, the Retinoid X receptors (RXRs) (for reviews see (Bastien & Rochette-Egly, 2004; Rochette-Egly & Germain, 2009; Samarut & Rochette-Egly, 2012)).

The basic mechanism for switching on gene transcription by RAR/RXR heterodimers relies on binding to specific elements located in the promoters of target genes and on ligand-induced conformational changes that cause the association/dissociation of a complex network of coregulatory proteins (Rochette-Egly & Germain, 2009). At the end, these events alter the chromatin structure surrounding the promoter of target genes and pave the way for the recruitment of the transcriptional machinery.

However, in addition to this classical scenario, which is directed by the LBD, it is now evident that, the N-terminal domain (NTD), although of naturally disordered structure, also plays a role, increasing the complexity of RAR-mediated transcription mechanisms (Ang et al, 2005; Bour et al, 2007; Liu et al, 2006). In this context, we recently highlighted that the NTD of RARs depicts a proline-rich motif (PRM) containing a serine residue, which becomes rapidly phosphorylated subsequently to non-genomic effects of RA, i.e. the activation of kinase cascades (Bruck et al, 2009; Lalevee et al, 2010; Piskunov & Rochette-Egly, 2011). *In fine*, phosphorylation of this residue drives the recruitment of RARs to promoters (Bruck et al, 2009; Lalevee et al, 2010).

The interesting point is that PRMs are well known to bind proteins with SH3 or WW domains (Ball et al, 2005; Freund et al, 2008; Kay et al, 2000). In that context we found that the PRM of the RARγ subtype interacts with vinexinβ (Bour et al, 2005b; Lalevee et al, 2010), an adaptor protein with SH3 domains, which is generally associated as a scaffold to complexes involved in cytoskeleton organization and signal transduction. However it is also one of the growing number of actin-binding that are nuclear and modulate transcription (Kast & Dominguez, 2011; Zheng et al, 2009) and we found that vinexinβ represses the transcriptional

activity of RARγ through sequestering the non phosphorylated form of the receptor out of gene promoters (Lalevee et al, 2010). Remarkably, vinexinβ dissociates in response to RA subsequently to RARγ phosphorylation, making the phosphorylated form of RARγ able to occupy promoters and to initiate transcription. This was the first report of a RAR corepressor association/dissociation out of promoters and regulated by phosphorylation.

Here, we aimed at identifying new partners of the NTD of the RARα subtype, which like RARγ, depicts a N-terminal PRM with a phosphorylation site (Samarut et al, 2011). Yeast two-hybrid screenings led to the identification of profilin IIA as a novel partner of the NTD of RARα. Profilin IIA is an other cytoskeleton actin-binding protein with an SH3-like domain, and is a regulator of the actin microfilament system (Haikarainen et al, 2009; Jockusch et al, 2007). We demonstrated that the interaction involves the SH3-like domain of profilin IIA and the PRM of RARα and we determined the affinity of the interaction. However, the role of profilin IIA in transcription proved to be different from that of vinexinβ and the classical coregulators. Indeed profilin IIA is present in the nucleus, is recruited with RARα to the promoters of target genes and modulates positively RARα-mediated transcription. However the interaction of profilin IIA is not modulated by the phosphorylation of RARα or by RA, suggesting a novel regulation mechanism.

MATERIALS AND METHODS

Plasmids and reagents

The pSG5-based expression vectors for mouse RARα1 (WT, S77A, S77E, ΔA and ΔAB) and RARγ1 have been previously described as well as the prokaryotic pGEX-2T vectors encoding RARα1 (WT, DEF and ABC) RARβ2 and RARγ1 fused to glutathione S-transferase (GST) (Bour et al, 2005a; Nagpal et al, 1992). The yeast pBTM116mod plasmid encoding N-terminal domain of RARαS77A fused to the LexA-DNA-binding domain was described in (Bour et al, 2005b).

Drs M. Noda and H. Kitayama (Xu et al, 2007) provided the pcDNA3.1-profilin IIA vector. The cDNA of profilin IIA was amplified by PCR from the pCDNA3.1 vector and inserted into XhoI/BamHI-digested pSG5 containing the epitope B of human estrogen receptor as a tag (a gift from T. Lerouge). It was also inserted into EcoRI/BamHI-digested pGEX-2T. Profilin IIA (Y7A/N10D) and profilin IIA (Y134S/F140A) in pSG5-B10 were constructed by double PCR amplification. All constructs were generated using standard cloning procedures and were verified by restriction enzyme analysis and automated DNA sequencing.

Mouse GIPZ lentiviral shRNAmir control (RHS4346) or targeting profilin IIA (RMM4532-NM_019410), as well as On-target plus SMART Pool human PFN2 (L-063038-01) and non targeting siRNA (D-001210-01-05) were from Thermofisher Scientific. All-trans Retinoic Acid was from Sigma-Aldrich.

Antibodies

Rabbit polyclonal antibodies raised against the F region of RARα (RPα(F)) were described earlier (Bruck et al, 2009) and were purified by application onto sulfolink gel columns (Thermo Scientific Pierce) coupled with the corresponding synthetic peptides (Buchanan et al, 2011; Vernet et al, 2006). Mouse monoclonal antibodies recognizing specifically RARα phosphorylated at position S77 (MAb 27α) have been described previously (Bruck et al, 2009; Gaillard et al, 2006), as well as antibodies against the epitope B of the N-terminal domain of the estrogen receptor (B10) (Ali et al, 1993). Mouse monoclonal antibodies against profilin IIA (sc-1000955), goat antibodies against β-actin (sc-1615) and rabbit polyclonal antibodies against RARα for ChIP experiments (sc-551-X) were from Santa Cruz

Biotechnology Inc. (USA). Mouse monoclonal antibodies recognizing GAPDH (MAB374) were from Merk-Millipore.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed as previously described (Bour et al, 2005b), using the L40 reporter strain harboring the HIS3 and LacZ reporter genes, both under the control of LexA binding sites. The mouse embryo (12.5 days pc) cDNA library in yeast VP16-AAD fusion vector pASV3 was introduced by lithium acetate transformation into the reporter strain expressing the LexA-RARα1(A/B)(S77A) fusion protein from the pBTM116mod vector. Approximately 2.10^6 yeast transformants were screened for their ability to grow on medium lacking histidine and containing 30 mM 3 amino-1,2,4 triazole (3-AT) (ICN Pharmaceuticals, France) and to express β-galactosidase. After several rounds of replica plating on selective medium, library plasmids were recovered from the positive clones, amplified, subjected to restriction analysis and sequenced.

GST Pull-down Assays

Equimolar amounts of GST and GST fusion proteins expressed in Escherichia coli were purified on glutathione-Sepharose 4B beads (Amersham Biosciences) and incubated with COS-1 cell extracts expressing B-tagged profilin IIA protein as described (Bour et al, 2005b).

Surface Plasmon resonance measurements.

SPR experiments were carried out with a BIAcore T100 instrument and research grade CM5 sensor chips (Altschuh et al, 2006; Lalevee et al, 2010). The ligand (GST-profilin IIA) was captured via anti-GST antibodies that were immobilized on the sensor surface using standard amine coupling procedures and following the manufacturer's instructions. Synthetic peptides corresponding to the proline-rich motif of RARγ or RARα (with S77 phosphorylated or not) (Table 1) were diluted in running buffer (HBS EP: 10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20) and injected over the surface in a continuous flow at 25°C.

Cells, Transfections and Immunoprecipitations

COS-1 cells, human neuroblastoma SH-SY-5Y cells, human acute promyelocytic leukemia (NB4) cells, mouse hippocampus HT22 cells, human breast cancer cells (MCF7 and SKBR3 cell lines), mouse embryocarcima cells (F9 and P19 cell lines) and mouse embryonic fibroblasts (MEF WT and MEF RAR($\alpha\beta\gamma$)-/-) (Bruck et al, 2009) were cultured according to standard procedures.

COS-1 cells, MCF7 cells and MEFs were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. When 80–90% confluent, cells were treated with RA (10-7 M) after 24 h in a medium containing 1% fetal calf serum. Extracts were prepared, immunoblotted or immunoprecipitated as described (Bruck et al 2009).

Immunofluorescence

Cells grown on coverslips were fixed in 4% formaldehyde (PFA)-PBS for 20 minutes, permeabilized with 0,1% Triton X-100 and blocked with 1% BSA in PBS for 30 minutes. Then the cells were incubated with the primary antibodies, followed by ALEXAFluor™ 448 or 555 conjugated secondary antibodies (Invitrogen). Cells were also incubated with fluorescent green 488 phalloidin (Biotium, Inc. Hayward USA). Nuclei were counterstained with DAPI (Sigma-Aldrich Chimie, France). Cells were analyzed by fluorescence microscopy using a LEICA DMRX microscope equipped with a LEICA True Confocal Scanner TCS SP. The used objective was Leica HCX PL APO 63x1.40 LBL.

Proximity Ligation Assay (PLA)

Duo link II (Eurogentec, France) *in situ* proximity ligation assay (PLA) was performed according to the manufacturer's protocol and as previously described (Ferry et al, 2011; Piskunov & Rochette-Egly, 2011). Cells were grown on coverslips, fixed, permeabilized, blocked and incubated with primary antibodies (rabbit anti-RARα and mouse anti profilin IIA) as described for immunofluorescence experiments then PLA probes were incubated for 1 h at 37°C, followed by hybridization, ligation, amplification and detection. Nuclei were counterstained with Dapi. Slides were analyzed by fluorescence microscopy using a LEICA DM4000B microscope equipped with a Cool SNAP Photometric camera. The used objective was

Leica HCX PLAN APO 40x0, 75 PH 2.

RNA isolation and quantitative RT-PCR was performed as described (Ferry et al, 2009). Primer sequences are as follows: Mouse RARβ2: 5'-TTTGGCACGTAGGCTGTTGG-3'and 5'-GAGCGAGCCTGGAAAATGGT-3'; Mouse Cyp26A1: 5′-GGGCTTACTTTGCAAGAGCA-3′ and 5′-GAAGGCCTCCTCCAAATGGA-3′; Mouse GAPDH: 5′-GTCTTCTGGGTGGCAGTGAT -3′ and 5′-CTGCACCACCAACTGCTTAG -3′. Human primers were from QIAGEN SA: BTG2 QUANTITECT PRIMER ASSAY QT00240247, CYP26A1 QUANTITECT PRIMER ASSAY QT00026817, and ACTIN QUANTITECT PRIMER ASSAY QT00095431.

Chromatin immunoprecipitation (ChIP) and ChIP western experiments

ChIPs were performed with sub confluent MCF7 cells and MEFs as previously described (Bruck et al, 2009; Ferry et al, 2011; Ferry et al, 2009). The primers pairs used for qPCR amplification are as follows: Mouse 36B4 5'-TTTGCTGTACT GACTCGGTGA-3' and 5'-CCTCCCACAACAAAACAACC-3'; Human 36B4, 5'-AGGACTCCATGTTCCCAAAG-3' and 5'-CGCAGCCAATAGACAGGAG-3'; Mouse Cyp26A1: R2, 5′-AAACAGGAGCAGGCTGAACT-3′ and 5′-CGCTGCCACTGTCATATCTT-3′; R1, 5′-GGTAACTCGGAGCTCTGCAC-3′ and 5′-CCAGGTTACTGCCCACGTTA-3′; Human Cyp26A1: R1, 5′-GCGGAACAAACGGTTAAAGA-3′ and 5′-GCAGTACAGGTCCCAGAGCTT-3′; R2, 5′-GAGTTCACTCGATGTCACG-3′ and 5′-ATCGCGCTGGAGGTAATTCT-3′; Mouse RARβ2: 5'-CGATCCCAAGT TCTCCCTTC-3' and 5'-CAGACTGGT TGGGTCATTTG-3'; Human BTG2: 5′-CCCGGCTACACTGTATATTGACTTGG -3′ and 5′- GGGTTTCATCACGTTGGTCAGGAT -3′.

Occupancy of the promoters was calculated by normalizing the PCR signals from the immunoprecipitated samples to the signals obtained from the input DNA.

For ChIP-Western experiments, the precipitated chromatin complexes were proceeded as described (Bruck et al, 2009) and bound proteins were revealed by immunoblotting.

RESULTS

Profilin IIA, a new partner of the N-terminal domain of RARα

In order to identify proteins that interact with the N-terminal domain (NTD) of RARα, a yeast two-hybrid screen of a mouse embryo cDNA library was performed as previously described (Bour et al, 2005b), using as a bait the NTD (A and B regions) of mouse (m) RARα in which the phosphorylatable serine residue (S77) was substituted with an alanine (RARα(AB)(S77A) (Fig. 1B). Among the positive clones we isolated, one contained a 430 kb cDNA insert displaying 96% sequence identity to human profilin IIA (NM_053024.3). This DNA fragment encoded a 140 amino acids protein that corresponds to full length profilin IIA (Fig. 1C). The encoded protein contained the N- and C-terminal sequence regions that form an SH3-like domain involved in the binding of poly-L-proline stretches.

The SH3-like domain of profilin IIA interacts specifically with the N-terminal PRM of RARα.

The data obtained by yeast two-hybrid screening were further investigated in *in vitro* protein-protein interaction assays using recombinant GST-RARα expressed in E-Coli and bound to glutathione-sepharose beads. When expressed in E. Coli, GST-RARαWT is not phosphorylated at S77 within the NTD. After incubation with extracts from COS-1 cells over expressing B-tagged profilin IIA, GST-RARαWT interacted with profilin IIA (Fig. 2A). We also assessed whether profilin IIA could interact with the other RAR subtypes. Interestingly we did not observe any significant binding of profilin IIA with RARγ1 and RARβ2 in GST pull down experiments (Fig. 2A).

Profilin IIA being characterized by the presence of an SH3-like domain known to interact with PRMs (Kursula et al, 2008), we investigated whether the PRM of RARα (located in the B region of the NTD) is indeed involved in the interaction. First we corroborated the yeast two-hybrid data by showing that profilin IIA interacts with the isolated N-terminal (A to C) regions and not with the C-terminal (D-F) ones of RARα fused to GST (Fig. 2B). Then a synthetic peptide corresponding to the PRM of RARα, PI 80 (Table 1) was generated and assayed for its ability to prevent the interaction between RARα and profilin IIA. PI 80 efficiently

disrupted the interaction (Fig. 2C), indicating that profilin IIA interacts with RARα via the PRM located in the B region of the receptor. These results were confirmed in coimmunoprecipitation experiments performed with extract from COS-1 cells over expressing B-10 tagged profilin IIA and RARα deletion mutants. Indeed deletion of the whole NTD (A and B regions) and not of the A region abrogated the interaction of RARα with profilin IIA (Fig. 2D)

Finally we investigated whether the SH3-like domain of profilin IIA is indeed involved in the interaction. The SH3-like domain of profilin IIA encompasses the N and C terminal helices (Haikarainen et al, 2009). Given that two residues in the C-terminal helix (Tyr 134 and Phe140) and two in the N-terminal one (Tyr 7 and Asn 10) have been shown to be important for the interaction with proline-rich ligands (Haikarainen et al, 2009) (Fig. 2E), we investigated whether mutation of these residues abrogates the interaction with RARα. We found that profilin IIA mutated at the two residues located in the N-terminal helix [Profilin IIA(Y7S/N10D)] as well as profilin IIA mutated at the C-terminal ones [Profilin IIA(Y134S/F140A)] have lost their ability to interact with RARα in GST-pull down experiments (Fig. 2F).

Collectively all these results confirm that the SH3-like domain of profilin IIA interacts specifically with the PRM of RARα, located in the NTD.

Profilin IIA interaction is not affected by RARα phosphorylation.

The PRM of RARα involved in the interaction with profilin IIA contains a serine residue (S77), which can be phosphorylated *in vitro* and *in vivo* (Bruck et al, 2009; Rochette-Egly et al, 1997). Therefore, we aimed at investigating whether phosphorylation of S77 modulates the interaction of RARα with profilin IIA.

First, coimmunoprecipitation experiments were performed with COS-1 cells over expressing B-tagged profilin IIA in combination with RARα WT or RARα mutants with S77 substituted with a glutamic acid (RARαS77E) or an alanine (RARαS77A), which mimic the phosphorylated and non-phosphorylated forms respectively (Fig.1A). After immunoprecipitation of the extracts with B10 antibodies, immunoblotting of RARα showed that the RARα mutants interacted as efficiently as RARαWT with profilin IIA (Fig.3A, lanes 10-12).

In transfected COS-1 cells, RA induced the rapid phosphorylation of RARα at S77 as assessed by immunoblotting after immunoprecipitation of RARα with a monoclonal antibody recognizing specifically RARα phosphorylated at this residue (Fig. 3B). However the interaction of RARα with profilin IIA was not affected upon RA addition (Fig.3C, lanes 12-16). Altogether these results converge towards the conclusion that the interaction is not modulated by the phosphorylation of RARα at S77.

Affinity of the interaction between profilin IIA and the PRM of RARα.

We used Surface Plasmon Resonance (SPR) to measure the equilibrium affinity and kinetic parameters of interaction of profilin IIA with synthetic peptides corresponding to the PRM of RARα (Table 1) (Lalevee et al, 2010). Affinity between profilin IIA and peptide PI 80 that corresponds to the non-phosphorylated form was calculated with the simple Langmuir 1:1 model and the Biacore T100 evaluation software (v 1.1.1), and found to be 0,1 mM (Table 1). Affinity for peptide PI 81 in which S77 is phosphorylated was similar, in line with the in vivo results. A 10 times lower affinity was found for peptide PI121, which corresponds to the PRM of RARγ, corroborating the specificity of the interaction.

Endogenous Profilin IIA and RARα interact in the nuclei of neuronal and fibroblastic cells.

Then we aimed at determining whether profilin IIA interacts with RARα *in vivo*. Profilin IIA is well known to be expressed mainly in brain and neuronal cells (Birbach, 2008; Michaelsen et al, 2010). Accordingly, significant levels of profilin IIA were detected by immunoblotting in human SH-SY-5Y neuroblastoma cells line and mouse hippocampus HT22 cells (Fig. 4A lanes 2 and 3). Profilin IIA was also present in mouse embryonic fibroblasts (MEFs) (Fig.4A, lanes 4 and 5) and in human breast cancer cells (MCF7 and SKBR3 cell lines) (Fig.4A, lanes 8 and 9). In contrast, profilin IIA was hardly detectable in mouse embryo carcinoma cells (F9 and P19 cell lines) (Fig. 4A, lanes 1 and 6) and no profilin IIA could be detected in human acute promyelocytic leukemia (APL) cells (NB4 cell line, Fig. 4A, lane 7).

The presence of profilin IIA in SH-SY-5Y, HT22, MCF7 cells and MEFs was corroborated in immunofluorescence experiments (Fig. 4B). Most interestingly, in

all these cells, profilin IIA was nuclear (Fig.4B), like RARα (Fig. 5A), raising the question whether profilin IIA interacts with RARα in the nuclear compartment.

With that aim we performed proximity ligation assays (PLA), which allow the detection *in situ* of interacting endogenous proteins (Ferry et al, 2011; Piskunov & Rochette-Egly, 2011; Soderberg et al, 2006). Rabbit anti-RARα and mouse anti-profilin IIA antibodies were used followed by species-specific secondary antibodies, called PLA probes, each attached with a unique short DNA strand. When the PLA probes are in close proximity, the DNA strands can be joined through the addition of a circle-forming DNA oligonucleotide that can be amplified using a polymerase. Then the amplified products are revealed with labeled complementary oligonucleotide probes and are easily visible as bright red spots under a fluorescence microscope.

RARα/profilin IIA complexes were seen in the nuclei of MCF7 cells and MEFs (Fig.5B). The number of complexes did not change after RA addition up to 1 hour (Fig. 5B, compare panels f and I and panels l and o), in line with the absence of regulation of the interaction in response to RA and upon RARα phosphorylation (see Figure 3). No signal was seen when each primary antibody was used individually and with MEF invalidated for RARs (Fig.5B, panels a-c), validating the specificity of the technique.

Altogether these results indicate that profilin IIA interacts with RARα in the nuclei of fibroblastic and breast cancer cells.

Profilin IIA modulates the transcriptional activity of RARα

Then the question is the functional role of the nuclear RARα-profilin IIA interaction. With that aim we analyzed the consequences of profilin IIA knockdown on the expression of RA-target genes.

MEFs respond to RA through the expression of several target genes such as the *Cyp26A1* and *RARβ2* genes as assessed by quantitative RT-PCR (Fig. 6A). The RA-induced expression of these genes was previously shown to depend on RARα (Bruck et al, 2009). Transfection of MEFs cells with an shRNA targeting profilin IIA reduced significantly the protein levels of profilin IIA, without affecting RARα levels, as shown by immunoblotting (Fig. 6A). Remarkably, when profilin IIA was reduced, the

RA-induced expression of both *Cyp26A1* and *RARβ2* genes was significantly down-regulated (Fig. 6A), indicating that profilin IIA plays a positive role in the expression of these target genes.

Similar results were obtained with MCF7 cells, which also respond to RA through RARα-mediated expression of several genes such as *Cyp26A1* and *Btg2* (Bruck et al, 2009; Ferry et al, 2011; Piskunov & Rochette-Egly, 2011). Knockdown of profilin IIA with specific siRNA significantly reduced the RA-induced expression of *Btg2* (Fig. 6B). However the expression of *Cyp26A1* was not affected (Fig. 6B). Altogether, these results indicate that profilin IIA plays a positive role in the expression of RARα–target genes but that this effect can differ depending on the cell type.

Profilin IIA is recruited with RARα to the promoters of RA-target genes

Given that profilin IIA modulates positively the expression of RARα target genes, one can hypothesize that profilin IIA is recruited with RARα at the promoters of these genes. Therefore ChiP experiments were performed with MEF and MCF7 cells to assess the occupancy of the promoters. Antibodies directed against RARα or profilin IIA were used to immunoprecipitate RARα and/or profilin IIA-bound DNA fragments that were further analyzed by quantitative PCR using specific pairs of primers spanning the RAREs (Fig. 7A). The specificity of the experimental conditions was checked in the absence of antibodies and with the promoter of the control 36B4 gene, which does not contain any RARE.

In MEFs, at 1 hour after RA addition, there was an enrichment of RARα bound at the RARβ2 promoter region (Fig. 7B), in line with previous studies (Bruck et al, 2009). Concerning the *cyp26A1* gene promoter, which contains two functional DR5 RAREs, a proximal one (R1) and a distal one (R2) (Fig. 7A), the R2 region was also specifically enriched after RA addition (Fig. 7B) (Bruck et al, 2009). Interestingly, after RA addition, there was also an enrichment of profilin IIA bound at the *RARβ2* promoter and the R2 promoter region of *Cyp26A1* (Fig. 7C).

ChIP experiments performed with MCF7 cells also showed that both RARα and profilin IIA are recruited to the promoter region of the *Btg2* gene (Fig. 7D and 7E). However, in the case of the *Cyp26A1* gene promoter, RARα only was recruited

and not profilin IIA, in line with the inability of the latter to modulate the expression of this gene in this cell line (Fig. 7D and 7E).

Altogether these results indicate that profilin II can be recruited with RARα to the promoters of some target genes

Finally profilin IIA interaction with RARα in chromatin was analyzed in ChIP western experiments. As shown in Figure 7F, immunoprecipitated profilin IIA interacted with RARα. This interaction was not affected after RA addition, corroborating the *in vitro* coimmunoprecipitation (see Figure 3) and PLA results (see Figure 5).

DISCUSSION

Up to now, only a few proteins have been reported to interact with the N-terminal domain of nuclear receptors including RARs (Boonyaratanakornkit et al, 2001; Bour et al, 2007; Bour et al, 2005b; Zhao et al, 2009). The novelty of the present study is the isolation of profilin IIA as a novel coregulator of the RAR subtype. Profilin IIA is a small (MW = 12-16 kDa) actin-binding protein that was originally identified as a key regulator of the actin microfilament system, especially in the cytoplasm of neuronal cells (Haikarainen et al, 2009; Jockusch et al, 2007). Here we present evidence that profilin IIA is also present in nuclei of several non-neuronal cells, where it interacts with the NTD of RARα and controls positively its transcriptional activity.

The main characteristic of the profilin IIA protein is the presence of an SH3-like domain, which is known to interact with PRMs (Ball et al, 2005; Kursula et al, 2008). RARs contain such a PRM in their NTD (Lalevee et al, 2010). Although this PRM is well conserved between RARs (Samarut et al, 2011), according to our data, the interaction with profilin IIA appears to concern only RARα and not the other RAR subtypes. Similarly the interaction of vinexinβ, another protein with an SH3 domain, was specific for the RARγ subtype and did not concern RARα. It must be noted that PRMs adopt a secondary structure known as poly proline II helix (PPII), which is determined by the pattern and number of the consecutive proline residues and by the presence of other amino acids interleaved with the proline residues. The PRM of RARα differs from that of RARγ by the substitution of one of the 5 consecutive prolines by a leucine. This may be at the basis of the specific interaction of each RAR with a particular SH3 domain.

According to our data, the interaction of profilin IIA with RARα is not affected by the phosphorylation of the serine residue flanking the PRM. This was in contrast to the traditional model in which phosphorylation of serine residues flanking the proline motifs have the ability to positively or negatively regulate the binding of SH3 domains (Kay et al, 2000; Lalevee et al, 2010). It is also in contrast to our previous report where we demonstrated that phosphorylation of the serine residue flanking the proline stretch of RARγ induces the dissociation of vinexinβ. Nevertheless, such

results challenge the hypothesis that profilin IIA would control the activity of RARα via a mechanism different from that, which was described for vinexinβ and RARγ.

An important clue in the present study is that profilin IIA participates positively to the transcription of RARα-target genes. Moreover profilin IIA is present in chromatin and is recruited with RARα to the promoters of target genes in response to RA. Such results are in line with another study showing that nuclear profilin plays a role in transcription (Lederer et al, 2005). Most importantly, they also corroborate the ever-growing evidence that actin, actin-related proteins and actin-binding proteins are constituents of nuclear protein complexes and play a role in transcription (Bettinger et al, 2004; Jockusch et al, 2006; Zheng et al, 2009). Indeed a number of actin-binding proteins have been shown to regulate the activity of several transcription factors including nuclear receptors through the recruitment of multiple components of transcription complexes such as chromatin-remodeling, histone acetyl transferase complexes (Archer et al, 2005; Blessing et al, 2004; Gettemans et al, 2005). Such a role of actin-binding proteins in transcription complexes emerged only recently and up to now there are no data showing that profilin IIA belongs to nuclear complexes. However on can propose that, as in the cytoplasm, profilin IIA might have a role in regulating the properties of nuclear actin through its ability to promote ADP to ATP exchange in G-actin (Fenn et al, 2011; Kast & Dominguez, 2011). Such a role might explain why nuclear forms of actin are prominently found in monomer states within transcription complexes.

Nevertheless it will require further experiments to test whether profilin IIA has such a role in maintaining the pool of monomer actin in the nucleus, when complexes with RARα at RA-target genes promoters. Moreover, it would be interesting to address whether profilin IIA also modulates the activity of the coregulatory complexes of RARα through nucleotide-dependent conformational transitions. Finally whether other processes than RA signaling can modulate the interaction of RARa with profilin IIA or RARα phosphorylation would provide important insights into the role of this adaptor in RA signaling.

In conclusion this study opened new concepts and avenues in the regulation of RA-target genes transcription via an actin-binding protein interacting with the NTD of the RARα subtype.

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LEGENDS TO FIGURES

Figure 1: Profilin IIA a new partner of the N-terminal domain of RARα

A. Schematic representation (not to scale) of the RARα1 protein with the known functional domains and the N-terminal phosphorylation site. The RARα PRM with S77 substituted with alanine or glutamic acid residues is also shown. B. Schematic representation of the LexA-RARα(A/B) proteins used as a bait in the yeast two-hybrid experiments. C. Alignment of the protein sequence deduced from our isolated mouse clone with those of human profilin IIA and IIB.

Figure 2: The SH3-like domain of Profilin IIA interacts with the N-terminal PRM of RARα

A. *In vitro* pull down experiments showing that B10-profilin IIA over expressed in COS-1 cells, interacts with RARα but not with the other RARβ and RARγ subtypes. B. *In vitro*, B10-profilin IIA interacts with the N-terminal A-C regions and not with the C-terminal DEF regions of RARα fused to GST. C. Immobilized GST-RARα proteins were incubated with B10-profilin IIA in the absence or presence of increasing amounts of the synthetic peptide PI80 corresponding to the PRM of RARα. D. COS-1 cells were transfected with the B10-profilin IIA vector along with RARα either WT, ΔA or ΔAB and whole cell extracts were incubated with B10 antibodies. Immunocomplexes were resolved by SDS-PAGE and incubated with RPα(F) and B10 antibodies. Lanes 1-6 correspond to 5% of the amount of immunoprecipitated extracts. E. Amino acid sequence of the N-terminal and C-terminal ends of profilin IIA. The mutations affecting the interaction with proline rich ligands are shown. F. *In vitro* pulldown experiments showing that the profilin IIA mutants (Y7S/N10D) and (Y134S/F140A) do not interact with RARα fused to GST.

Figure 3: Phosphorylation of the serine residue located in the PRM of RARα does not affect the interaction with profilin IIA

A. Coimmunoprecipitation experiments showing that in transfected COS-1 cells, substitution of serine 77 with a glutamic acid or an alanine does not affect the interaction of RARα with profilin IIA. B. In transfected COS-1 cells RA induces the

rapid phosphorylation of RARα at S77. Phosphorylated RARα was immunoprecipitated with monoclonal antibodies recognizing specifically the receptor phosphorylated at S77 and immunoblotted with RPα(F). Bottom panel corresponds to the inputs. C. Coimmunoprecipitation experiments showing that after RA addition, the interaction between RARα and profilin IIA is not affected.

Figure 4. Profilin IIA is expressed in the nuclei of neuronal cells, fibroblasts and breast cancer cell lines

A. immunoblotting experiments showing that profilin IIA is expressed in human SH-YH-5H neuroblastoma cells, mouse HT22 hippocampus cells, MEF, MCF7 and SKBR3 breast cancer cells, but not in mouse embryo carcinoma cells (P19 and F9 cell lines) and acute promyelocytic leukemia cells (NB4 cell line).

B. Immunofluorescence experiments showing that profilin IIA is present mainly in the nuclear compartments of SH-YH-5H, HT22, MEF and MCF7 cells. Cells were double stained with DAPI (blue) and profilin IIA antibodies (red). The merge images overlapping the blue and red are shown.

Figure 5: Nuclear Profilin IIA interacts with RARα

A. immunofluorescence experiments showing the nuclear localization of both RARα and profilin IIA in MEFs (panels a-d) and MCF7 cells (e-h). Cells were triple stained with DAPI (blue), profilin IIA (red) and RARα (green) antibodies. The merge images overlapping the red, green and blue fluorescence are shown (panels d and h).

B. PLA (Proximity Ligation Assay) showing the profilin IIA/RARα complexes (red) in MEFs WT (panels d-i), MEF RAR KO (panels a-c) and MCF7 cells (panels j-o) treated or not with RA for 1 hour. DNA was counterstained with Dapi (blue). The merge between blue, green and red is also shown (panels c, f, I, l, o).

Figure 6: Profilin IIA participates in the transcription of RA-target genes

A. Silencing of profilin IIA decreases the RA-induced expression of the *cyp26A1* and *RARb2* genes in MEFs as monitored by quantitative qRT-PCR. B. Silencing of profilin IIA decreases the RA-induced expression of the *Btg2* gene but not of the *cyp26A1* one in MCF7 cells. In all cases, values are the mean ±SD of three different experiments. Knockdown efficiency was controlled by immunoblotting.

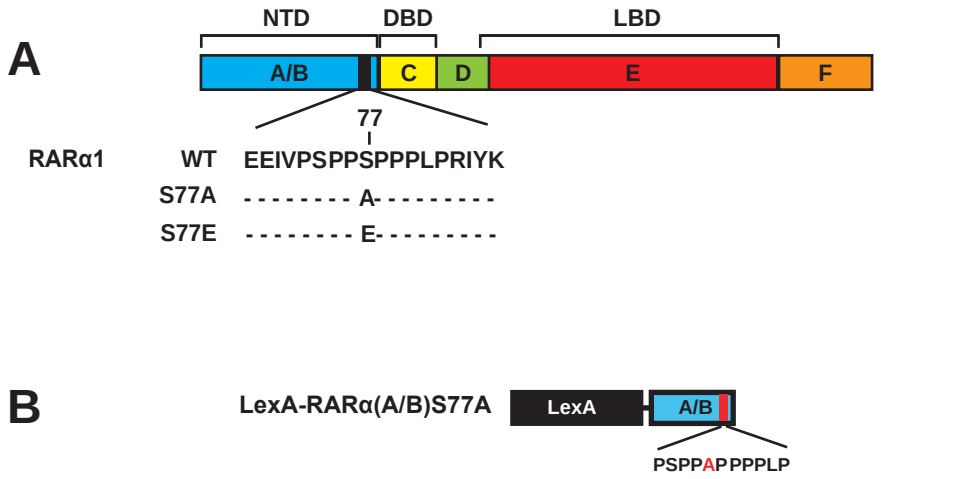
Figure 7: Profilin IIA occupies with RARα the promoters of target genes

A. Schematic representation of the promoter regions of the *Cyp26A1*, *RARβ2* and *Btg2* genes with the primer pairs used for qPCR amplification. B and C. Kinetic ChIP experiments performed with RA-treated MEFs and showing the recruitment of RARα (B) and profilin IIA (C) to the R1 and R2 regions of the *cyp26A1* gene promoter, to the *RARβ2* gene promoter and to the control *36B4* gene. Values (% of the inputs) correspond to a representative experiment among 3.

D and E. Same kinetic ChIP experiments, showing that profilin IIA is recruited with RARα to the *Btg2* promoter but not to the *Cyp26A1* one in RA-treated MCF7 cells.

F. ChIP western experiments performed with MEFs treated or not with RA for 1 hour. The complexes immunoprecipitated with profilin IIA or RARα antibodies were immunoblotted with RARα and profilin IIA antibodies.

Figure 1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NTD
DBD
LBD
A/B
C
D
E
F
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RARα1
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S77E - - - - - - - - E- - - - - - - - -
B
LexA-RARα(A/B)S77A
LexA
A/B
PSPPAPPPPLP
C
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RARα(A/B)-BP
h_profilin_2_isoform_b
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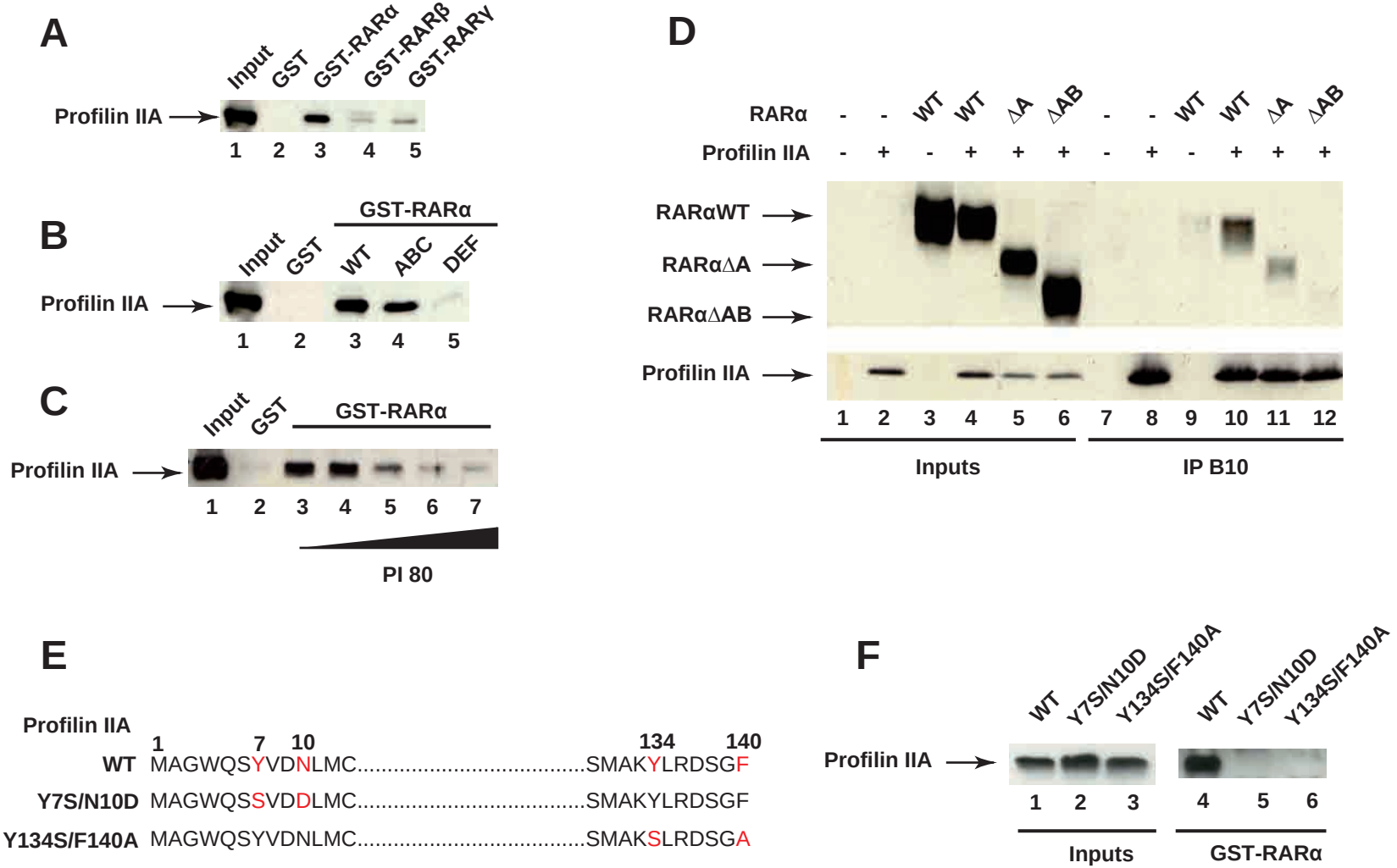
Figure 2


A
Input
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GST-RARα
GST-RARβ
GST-RARγ
Profilin IIA
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B
GST-RARα
Input
GST
WT
ABC
DEF
Profilin IIA
1 2 3 4 5
C
Input
GST
GST-RARα
Profilin IIA
1 2 3 4 5 6 7
PI 80
D
RARα - - WT WT ΔA ΔAB - - WT WT ΔA ΔAB
Profilin IIA - + - + + + - + - + + +
RARαWT
RARαΔA
RARαΔAB
Profilin IIA
1 2 3 4 5 6 7 8 9 10 11 12
Inputs
IP B10
E
Profilin IIA
1 7 10 134 140
WT MAGWQSYVDNLMC.....SMAKYLRDSGF
Y7S/N10D MAGWQSSVDDLMC.....SMAKYLRDSGF
Y134S/F140A MAGWQSYVDNLMC.....SMAKSLRDSGA
F
WT
Y7S/N10D
Y134S/F140A
WT
Y7S/N10D
Y134S/F140A
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1 2 3 4 5 6
Inputs
GST-RARα

Figure 3

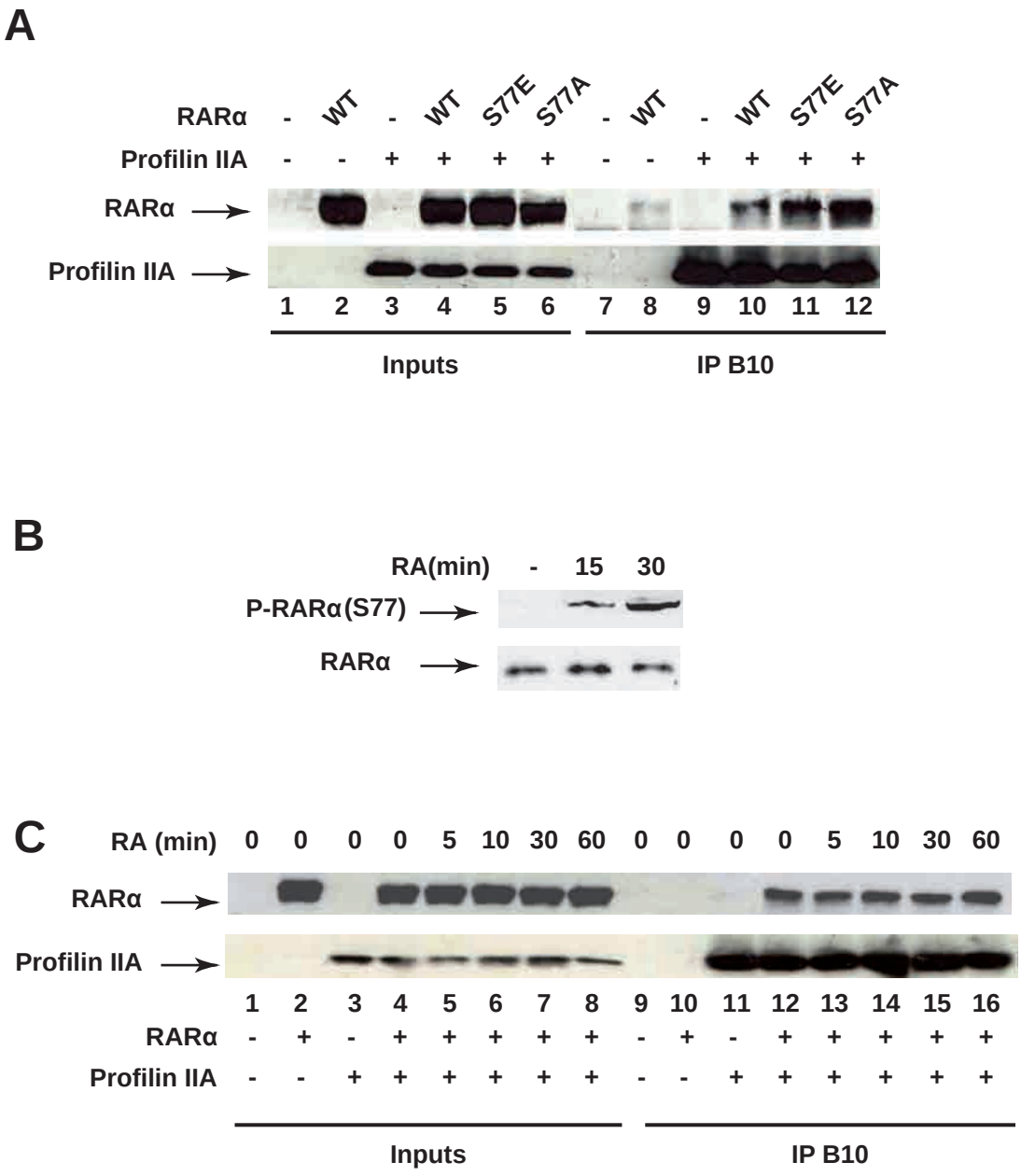
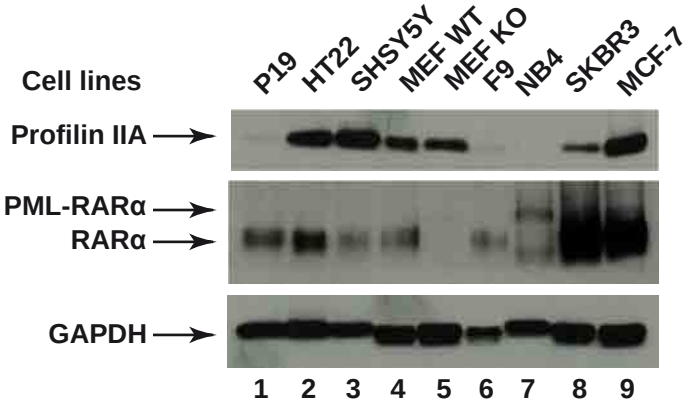

A
RARα - WT - WT S77E S77A - WT - WT S77E S77A
Profilin IIA - - + + + + - - + + + +
RARα
Profilin IIA
1 2 3 4 5 6 7 8 9 10 11 12
Inputs
IP B10
B
RA(min) - 15 30
P-RARα(S77)
RARα
C
RA (min) 0 0 0 0 5 10 30 60 0 0 0 0 5 10 30 60
RARα
Profilin IIA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
RARα - + - + + + + + - + - + + + + +
Profilin IIA - - + + + + + + - - + + + + + +
Inputs
IP B10

Figure 4

A


Cell lines
P19
HT22
SHSY5Y
MEF WT
MEF KO
F9
NB4
SKBR3
MCF-7
Profilin IIA
PML-RARα
RARα
GAPDH
1 2 3 4 5 6 7 8 9

B

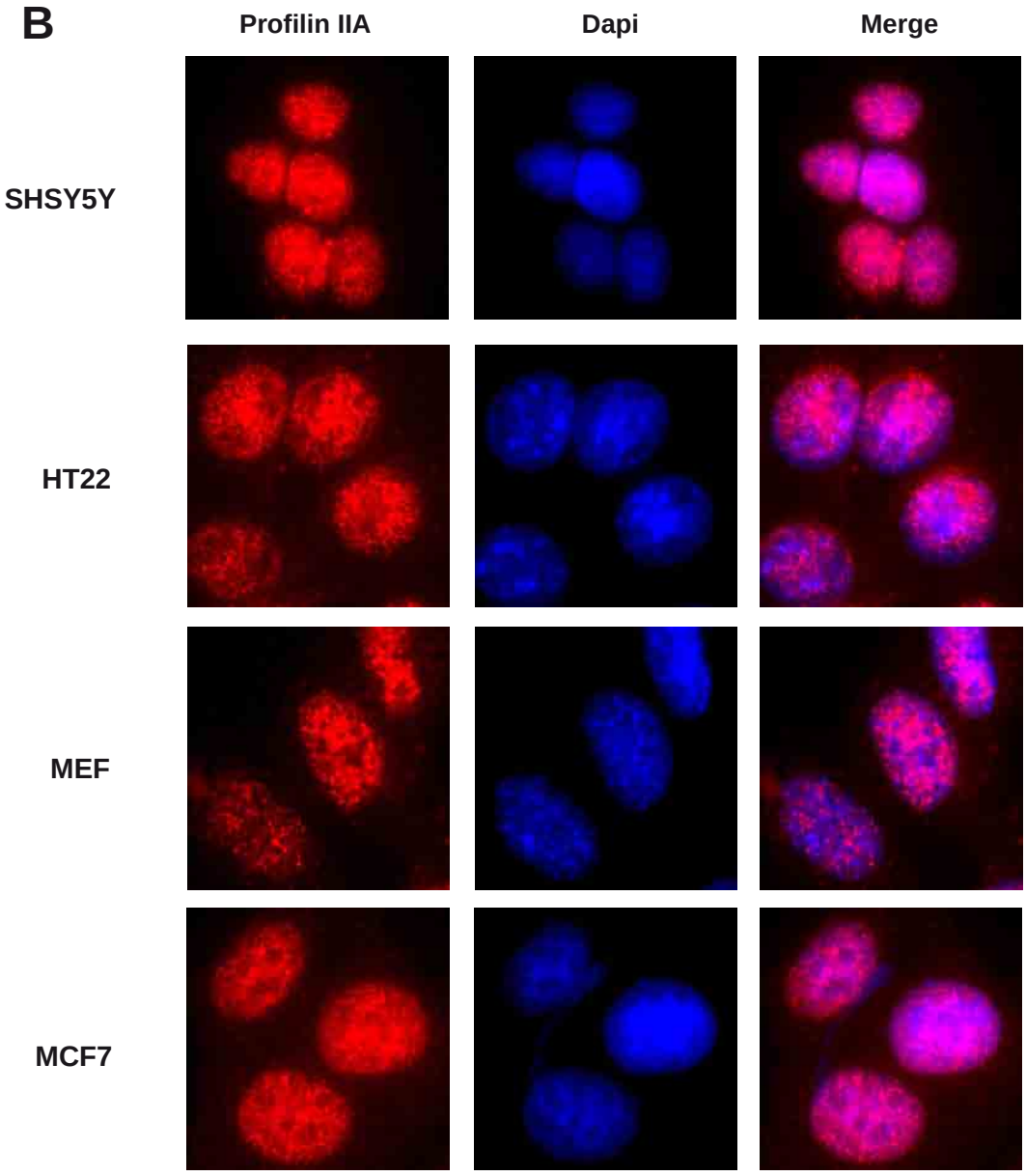
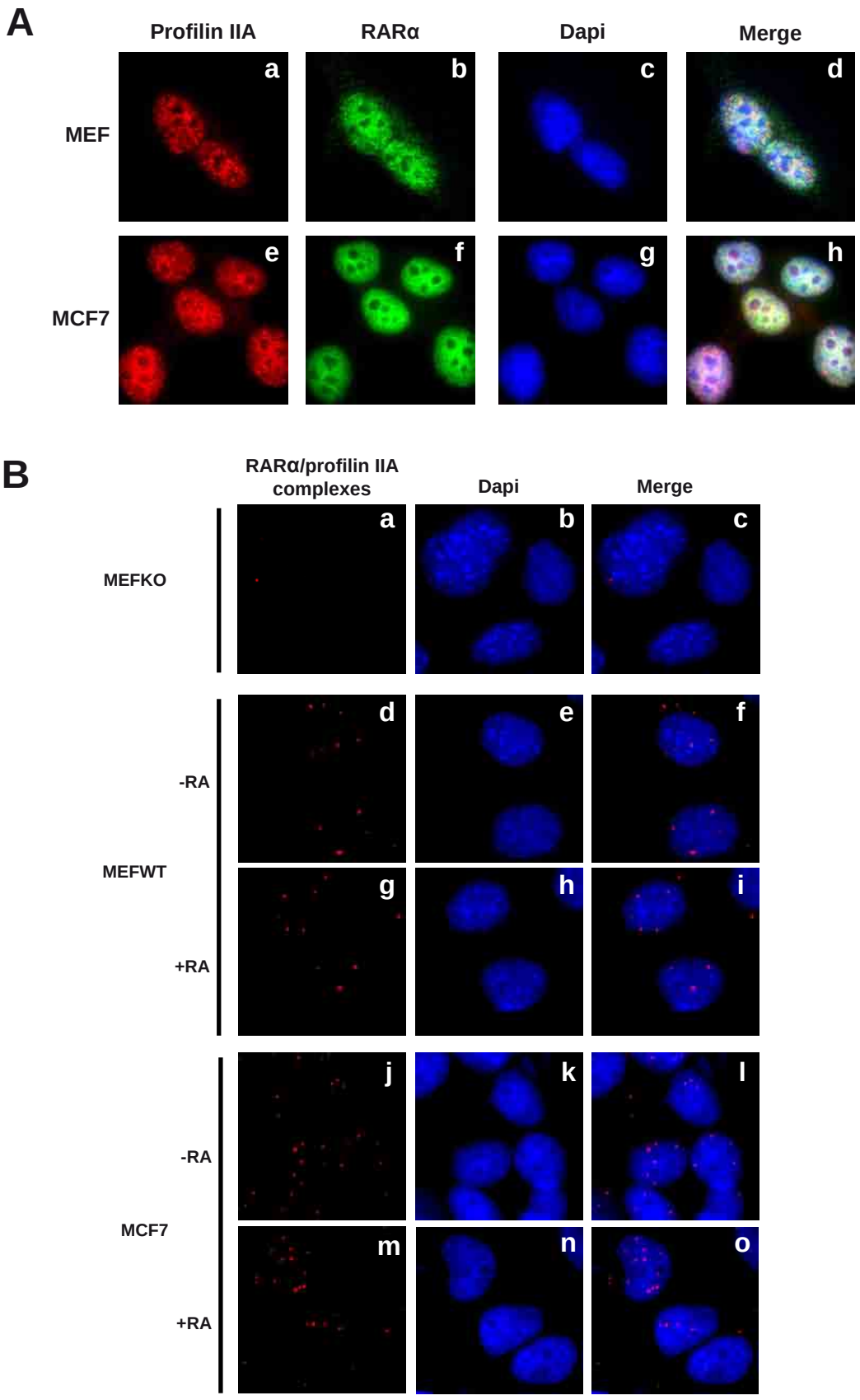

Profilin IIA
Dapi
Merge
SHSY5Y
HT22
MEF
MCF7

Figure 5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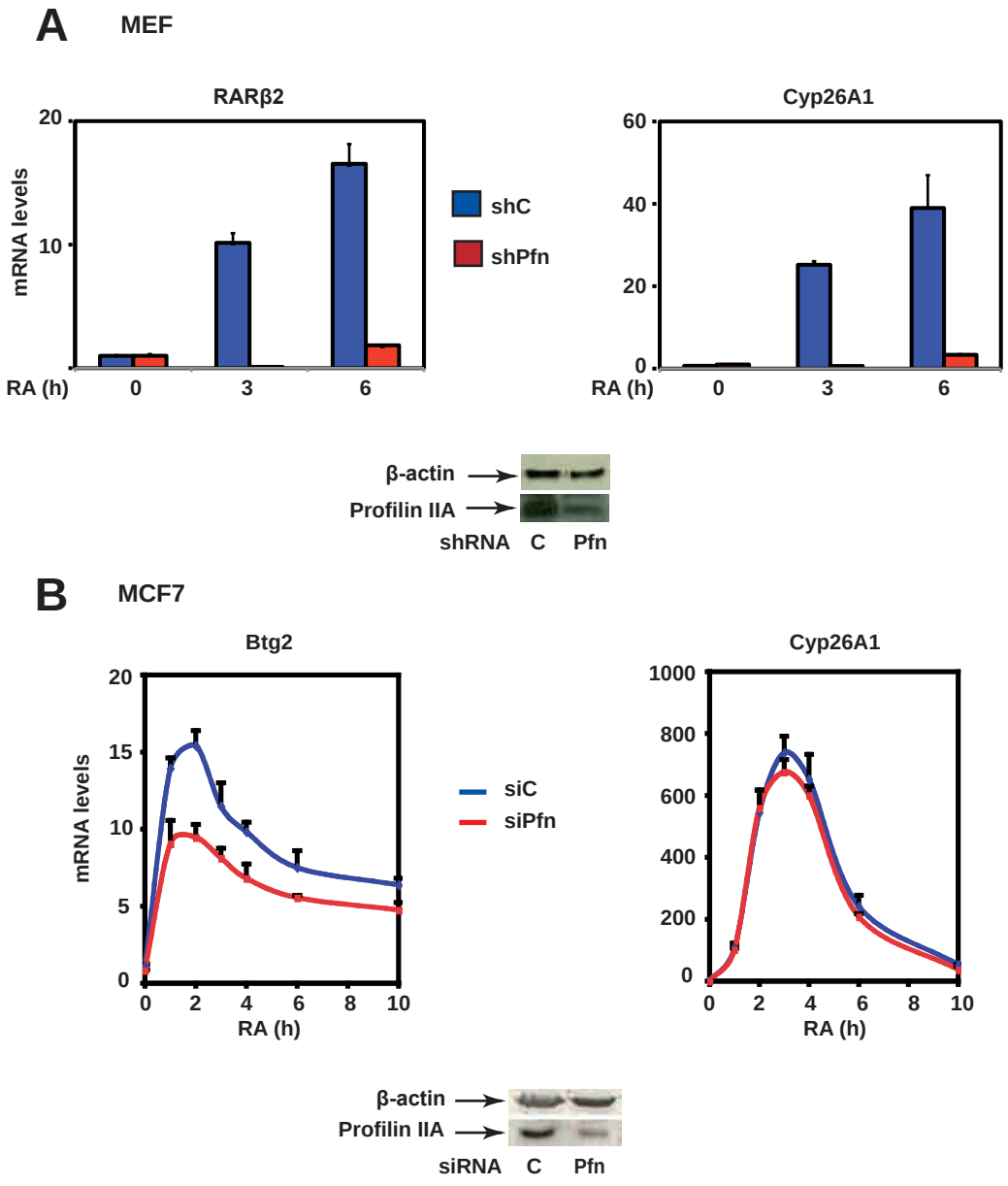
Profilin IIA
RARα
Dapi
Merge
MEF
MCF7
a
b
c
d
e
f
g
h
B
RARα/profilin IIA complexes
Dapi
Merge
MEFKO
MEFWT
-RA
+RA
MCF7
-RA
+RA
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

Figure 6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Cyp26A1
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shPfn
RA (h)
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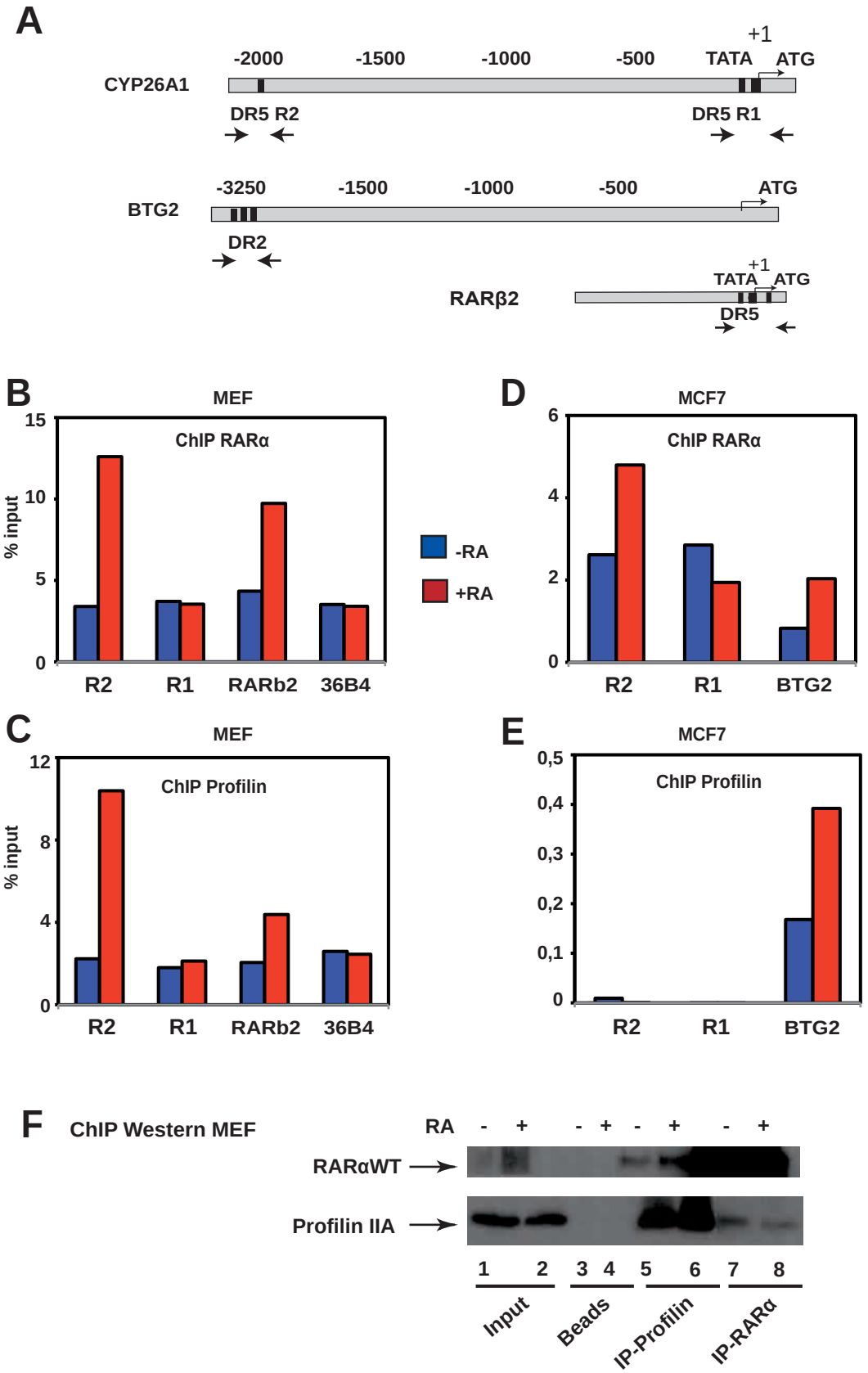
3
6
β-actin
Profilin IIA
shRNA C Pfn
B
MCF7
Btg2
Cyp26A1
siC
siPfn
RA (h)
β-actin
Profilin IIA
siRNA C Pfn

Figure 7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-1500
-1000
-500
TATA
ATG
CYP26A1
DR5 R2
DR5 R1
-3250
-1500
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-500
ATG
BTG2
DR2
+1
TATA
ATG
RARβ2
DR5
B
MEF
ChIP RARα
% input
15
10
5
0
R2
R1
RARb2
36B4
-RA
+RA
D
MCF7
ChIP RARα
6
4
2
0
R2
R1
BTG2
C
MEF
ChIP Profilin
% input
12
8
4
0
R2
R1
RARb2
36B4
E
MCF7
ChIP Profilin
0,5
0,4
0,3
0,2
0,1
0
R2
R1
BTG2
F
ChIP Western MEF
RA - + - + - + - +
RARαWT
Profilin IIA
1 2 3 4 5 6 7 8
Input
Beads
IP-Profilin
IP-RARα

Peptide	Amino Acid sequence	Description	Affinity for Profilin IIA
PI 80	EEIVPSPPPLPRIYK	Non phosphorylated PRD of RARα	0,1 mM
PI 81	EEIVPSPPPLPRIYK P	PRD of RARα phosphorylated at S77	0,1 mM
PI 121	EEMVPSSPSPPPPPRVYK	Non phosphorylated PRD of RARγ	1 mM

Table 1: SPR analysis of the interaction between GST-profilin IIA and synthetic peptides corresponding to the phosphorylated and non phosphorylated PRM of RARα and RARγ. GST and GST-profilin IIA were immobilized on a sensor chip. Peptides were injected at different concentrations (0,5-5x10^{-4} M) for 60 s. GST signal [relative units (RU)] was substracted from the GST-profilin IIA signals. Nonlinear curve fitting of the equilibrium responses *vs* peptide concenration was used to derive equilibrium K_d values.

PUBLICATION 3

Chapter

MSK1 and Nuclear Receptors Signaling

Aleksandr Piskunov and Cécile Rochette-Egly*

Abstract

Nuclear receptors for steroid and non-steroid hormones act through both genomic and non-genomic mechanisms. Genomic events involve binding to cognate specific DNA sequences and subsequent recruitment of a battery of coregulators at the promoter. Non-genomic events involve the rapid activation of kinase cascades and steroid as well as non-steroid hormones trigger NR-mediated activation of the MAPK/MSK1 pathway. Here we review the recent insights concerning the crosstalk between the genomic and non-genomic actions of NRs, focusing on the mechanisms of MSK1 activation and influence on NR-target genes transcriptional regulation.

Introduction

Nuclear receptors (NRs) form a super family of ligand-regulated transcription factors, which regulate various physiological functions from development and reproduction to homeostasis and metabolism. This super family includes receptors for steroid hormones [Estrogen receptors (ER), Progesterone receptors (PR), Androgen receptors (AR) and Glucocorticoid receptors (GR)] and for non-steroid ligands [Vitamin D receptor (VDR), Retinoic Acid receptors (RAR), Retinoic X receptors (RXR) and the Peroxisome Proliferator Activated Receptors (PPAR)]. They also include a large number of so-called orphan receptors for which ligands do not exist or have not been identified yet.[1-3]

Classically NRs are known to act through genomic events, which involve binding of liganded homo or heterodimerized NRs to cognate specific DNA sequences followed by recruitment of coactivators and remodeling of chromatin at the promoter sequences of the activated genes.[1,4]

In addition to these so-called genomic effects, steroid and non-steroid hormones and their cognate NRs crosstalk with kinase cascades activated by signals impinging on membrane receptors. As an example, progestins and retinoic acid (RA) have been shown to activate the Mitogen-Activated Protein Kinase (MAPK) pathways and the downstream Mitogen- and Stress-activated protein Kinase 1 (MSK1).[5,6] Traditionally, the non-genomic and genomic effects of steroid and non-steroid hormones have been considered as independent pathways. However it appeared that hormone-activated MSK1 is involved in the regulation of NR-target genes by phosphorylating NRs themselves and/or chromatin associated proteins. Here we will review the current knowledge on MSK1 activation by steroid and non-steroid hormones and on MSK1 molecular targets, focusing on recent insights into the role of MSK1 in transcriptional regulation of NR-target genes.

The Classical Picture of NRs: Ligand-Dependent Regulators of Transcription

NRs have a well-defined domain organization and structure, consisting mainly of two highly conserved and structured domains: the central DNA-binding domain (DBD) linked to the C-terminal Ligand-Binding Domain (LBD). The structure of the DBDs and LBDs has been

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MSKs, edited by Linda Vermeulen and J.S.C. Arthur.

determined by nuclear magnetic resonance and crystallographic studies[1,2,7] (Fig. 1). Briefly, the DBD contains two typical cysteine-rich zinc-binding motifs and two α-helices, which cross at right angles and fold into a globular conformation to form the core of the DBD. In contrast, the LBD is more complex because it contains not only the ligand-binding pocket, but also the main dimerization domain and a hydrophobic cleft involved in coregulators binding. The LBD shows a common fold comprising 12 conserved α-helices and a short β-turn, separated by exposed and flexible loops and arranged in three layers to form an antiparallel alpha-helical sandwich.

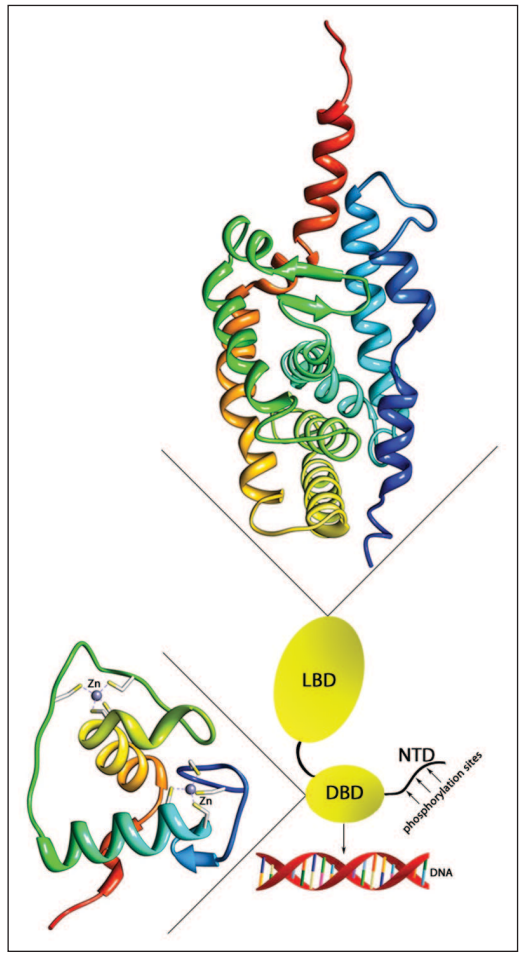

Figure 1. Structural organization of nuclear receptors. Nuclear receptors consist mainly of a central DNA-binding domain (DBD) linked to a C-terminal ligand-binding domain (LBD) and an N-terminal domain (NTD). The DBD (PDB1DSZ)[77] is composed of two zinc-nucleated modules, two α-helices and a COOH-terminal extension. Helices cross at right angles to form the core of the DBD. This structure provides sequence-specific DNA recognition. The LBD (PDB1LBD)[78] is composed of 12 conserved α-helices and a β-turn separared by exposed and flexible loops. In RARs, loop L9–10 contains a serine residue, which can be phosphorylated by MSK1. Phosphorylation of this residue increases the flexibility of the nearby loop L8–9 and the N-terminal part of H9 and thereby the docking of cyclin H to this domain. The N-terminal domain (NTD) is natively disordered and contains several serine residues within proline-rich motifs, which can be phosphorylated by cdks or MAPKs.

Gene induction by NRs is based on a complex network of NRs conformational changes and dynamic interactions with coregulatory proteins.[2,7,8] Ligand binding is the first and crucial molecular event that switches NRs from inactive to active state by inducing conformational changes in the LBD. These changes favor the dimerization of NRs and increase their DNA affinity. They also create a new surface for coactivators binding, which initiates an ordered and coordinated dissociation and/or recruitment of a series of coregulator complexes with different enzymatic activities including Histone Acetyl- and Methyl-Transferases, and DNA-dependent ATPases.[9] In fine, these events alter the chromatin structure surrounding the promoter of target genes and pave the way for the recruitment of the transcription machinery including RNA Polymerase II and general transcription factors.

NRs Are Phosphoproteins

In addition to this scenario NRs are targets for phosphorylation processes, which modulate their transcriptional activity.[8,10] A number of studies demonstrated that the majority of the phosphorylated residues lie within the N-terminal domain (NTD) (Fig. 1). The phosphorylation sites located in the NTD of NRs are serine residues surrounded by prolines and therefore correspond to consensus sites for cyclin-dependent protein kinases (cdks) and Mitogen-Activated Protein Kinases (MAPKs). Accordingly, the NTD of PR, ER, AR, GR, RARs and PPARs were reported to be substrates for cdks, p42/p44MAPK (also called Erk1/2), p38MAPK, and c-Jun N-terminal kinases (JNKs).[10-16] Note that VDR is an exception, probably due to its very short NTD.

It must be noted that, in contrast to the DBDs and LBDs, the NTDs of NRs are not conserved and there are still no high-resolution structures available. Several biochemical studies coupled to structure prediction algorithms suggested that the NTDs of any member of the NR family are naturally disordered,[17,18] providing the flexibility that is needed for modifications by enzymes such as kinases.[19] Such modifications would induce changes in the structural properties of the domain with profound impacts on its interactions with coregulators and/or on the dynamics of adjacent structural domains.[5,20]

Interestingly, loops between the α-helices of the LBDs are exposed and flexible and thus are accessible for phosphorylation processes. As an example, in the LBD of RARs, loop L9–10 contains a serine residue located within an arginine/lysine rich motif, which corresponds to a consensus phosphorylation site for several kinases including MSK1[5,21] (Fig. 1).

A New Picture of NRs: NRs Are Associated to the Cell Membrane and Activate MAPK Signaling Pathways

It is becoming increasingly evident that NRs induce rapid non-genomic responses in addition to their classical genomic effects. These non-genomic effects involve the rapid and transient activation of several kinase cascades mediated by a subpopulation of NRs anchored at the cytoplasmic side of the cell membrane.

Indeed, most of the classical steroid receptors (ER, PR, GR, AR) have been found in specialized plasma membrane structures such as caveolae and lipid rafts[22-24] that contain lipids, structural proteins like flotillin and caveolin, and several proteins involved in signal transduction including heterodimeric G proteins, c-Src, Rho and RAC GTPases and Phosphoinositide 3-kinase (PI3K).[25,26] The membrane localization of steroid NRs depends on post-translational modifications and it has been shown that palmitoylation of a highly conserved nine-amino acids motif in the LBD is critical for membrane localization of ER and PR via caveolin-1 association.[27] In addition, steroid NRs are part of membrane molecular complexes, which differ depending on the cell type and context and contain c-Src, the regulatory subunit of PI3K (p85α) and heterodimeric Gα proteins.[25,28,29] In response to the hormone, ERα rapidly activates the Src/p21ras/Erk pathway via direct interaction with the SH2 domain of c-Src (Fig. 2). Progestins and androgens also activate this signaling cascade via direct interaction of the cognate receptor with the SH3 domain of c-Src or with ERα which itself activates c-Src[30-33] (Fig. 2). Note however that, in response to glucocorticoids, GR does not activate Erk but p38MAPK and JNKs[11,34] (Fig. 2).

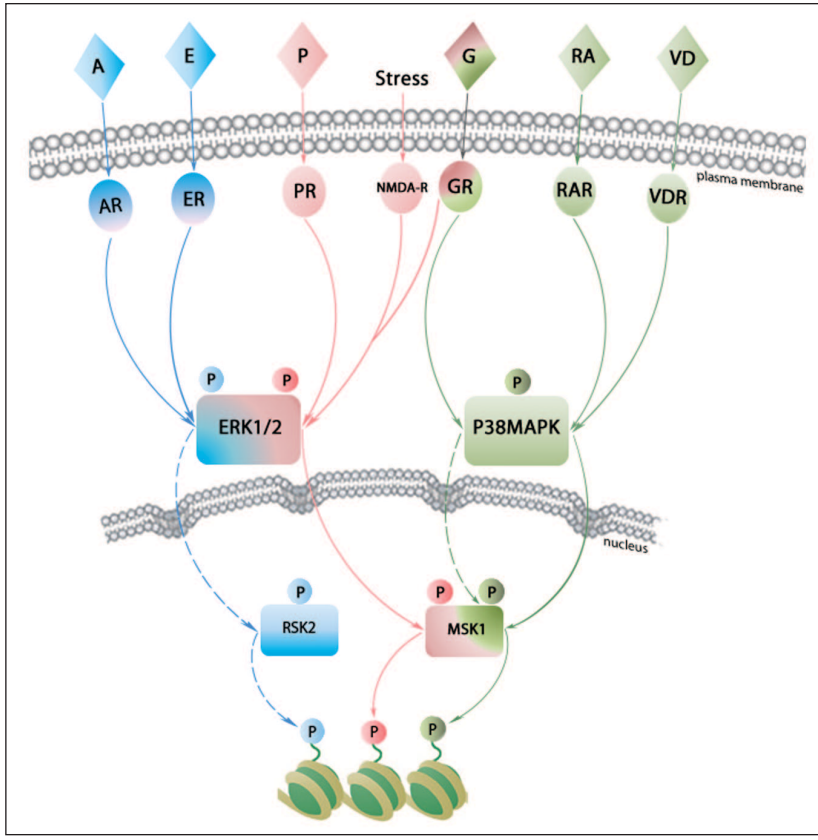

Figure 2. A subpopulation of the classical steroid receptors (ER, PR, GR, AR) and non-steroid receptors (VDR and RARs) is associated to cell membranes and initiates cascades of kinase activations upon binding of their cognate ligands. Accordingly, liganded non-steroid receptors RAR and VDR activate p38MAPK and the downstream MSK1 kinase. Among the steroid receptors, GR bound to glucocorticoids also activates p38MAPK but there is no data indicating whether MSK1 is activated or not. However, upon the concomitant activation of NMDA-R under stress conditions, liganded GR activates MSK1 but through Erks. The other steroid receptors PR, ER and AR also activate Erks but only PR was found to activate the downstream MSK1. In the case of ER and AR another downstream effector of Erks, RSK2 would be an interesting candidate.

Non-steroid receptors such as VDR and RARs have been also found associated to cell membrane fractions, in association with caveolin-1,[35] PI3K,[36] c-Src[37] or G alpha Q proteins.[38] However, vitamin D[39] and RA[5,40-42] rather activate p38MAPK (Fig. 2). Activation of this pathway occurs very rapidly through the transient activation of small GTPases such as RhoA and its immediate effector ROCK[43] or RAC-1[37,40] suggesting a non-genomic activation event similar to that described for steroid receptors. Of note is that this process appears to be cell specific as RA has been shown to activate Erks in neuronal and Sertoli cells.[36,44-46]

NRs Activate MSK1, Downstream of Erk and p38MAPK

Among the targets of NRs-activated Erk and p38MAPK, there is MSK1 (Mitogen- and stress-activated protein kinase), which presents a large structural analogy with the N-terminal ribosomal S6 kinase (RSK) domain.[47-49] MSK1 is predominantly localized in the nucleus and is composed of

two kinase domains connected with a linker region. A C-terminal docking domain assures binding of activating Erk or p38MAPK, which then phosphorylates a threonine residue located in the C-terminal kinase domain. Then this activated C-terminal kinase domain phosphorylates a serine in the N-terminal kinase domain, which at the end is responsible for the phosphorylation of MSK1 substrates.

After activation by the steroid hormone progestin, phosphorylated Erk translocates to the nucleus and forms a complex with nuclear PR homodimers. Then Erk binds and phosphorylates MSK1[50] leading to its activation[6] (Fig. 2 and Fig. 3, left). Non-steroid hormones such as RA and Vitamin D also activate MSK1 downstream of p38MAPK [5,39,43] (Fig. 2), in line with the fact that MSK1 can be activated through both Erks and p38MAPK. Whether VDR and RAR form with p38MAPK and MSK-1 trimeric complexes has not been elucidated yet and will require further investigations.

In conclusion, it appears that steroid and non-steroid NRs are able to activate the MAPK/MSK1 pathway in response to their cognate hormone. However whether this mechanism is general has not been demonstrated yet. Indeed, ER and estrogens do not activate[51] or rather inactivate[52] MSK1. It is interesting to note that p90 ribosomal S6 kinase (RSK), which is another member of the subfamily of MAPK-activated protein kinases downstream of Erk[47] and which is overexpressed in several cancers including breast cancers,[53] has been shown to bind and phosphorylate ER.[12,54] Whether ER and estrogens activate RSK instead of MSK1, will require further investigations (Fig. 2).

Concerning AR and androgens, there are still no data concerning MSK1. However, as for ER, RSK would be an interesting candidate[53,55,56] (Fig. 2). Similarly, whether GR and glucocorticoids activate MSKs or not has not been reported yet. Note however that an activation of the Erk/MSK1 pathway has been observed in neurons upon concomitant activation of GR and the N-methyl-D-aspartate receptor (NMDA-R) in stress processes[57,58] (Fig. 2).

RARα Is Phosphorylated by MSK1 While the Other NRs Are Targets for the Upstream MAPK

MSK1 was originally shown to phosphorylate several transcription factors including CREB, ATF1 and Nuclear Factor-κB p65 (NF-κB).[48,49] Similarly, MSK1 phosphorylates rapidly RARα at a serine residue (S369) located in loop L9–10 within the LBD[5,7] (Fig. 1). This serine is an exposed residue located in a flexible loop and belongs to an Arginine-Lysine-rich motif that corresponds to a consensus phosphorylation motif for several kinases including MSK1. The interesting point is that phosphorylation of this serine initiates a coordinated phophorylation cascade (Fig. 3, right). Indeed, our laboratory recently demonstrated that phosphorylation of S369 by MSK1 increases the dynamics/flexibility of the nearby loop L8–9,[59] which corresponds to the docking site of cyclin H[60] (Fig. 1) that forms with cdk7 and MAT1, the CAK subcomplex of the general transcription factor TFIIH. Consequently, the binding efficiency of cyclin H is increased, allowing the right positioning of the cdk7 kinase and the phopshorylation of serine 77 located in the NTD by this kinase.[21] To our knowledge, it was the first example of cooperation between the N-and C-terminal domains of RARs through a kinase complex. The serine in L9–10 and the docking site of cyclin H are conserved between RARs but not in other NRs indicating that this kinase cascade would be RAR specific.[59]

Note that in contrast to RAR, PR within the trimeric PR/Erk/MSK1 complex is phosphorylated by Erk and not by MSK1.[6] However, concerning VDR, there are no data showing whether it is phosphorylated by MSK1 or by the upstream p38MAPK. Finally, concerning the other NRs such as ER, AR and GR, which were not found to activate MSK1 in response to the hormone, they are phosphorylated by MAPKs.[11,12,61]

MSK1 Participates in the Activation of NR-Target Genes and Is Recruited at the Promoters of NR-Target Genes

Steroid and non-steroid hormones are well known to activate the expression of several target genes.[1,3,4,62,63] Traditionally, this genomic action has been considered as independent of the non-genomic effects. However, it now emerges that the two pathways converge in the modification

of structural components of the chromatin. Indeed MSKs are well known to facilitate gene relaxation[47,49] and a number of studies indicated that inhibition of MSK1 abolished the induction of progestin-, RA- or Vitamin D-target genes.[5,6,43]

One of the well characterized example of transcriptional control by progestins is the promoter of the mouse mammary tumor virus (MMTV), which is organized into positioned nucleosomes with one nucleosome (nucleosome B) covering the hormone response element (HRE) and which is induced upon hormone treatment.[50,64,65] Though progestin activation of the MMTV promoter is sensitive to Erk and MSK1 inhibition, this behavior does not represent an exception, as about 25% of the hormonally-regulated genes are also sensitive to Erk inhibition in microarrays analysis.[65] Series of chromatin immunoprecipitation experiments indicated that phosphorylated PR complexed with Erk and MSK1 is rapidly recruited to the MMTV promoter nucleosome containing the HRE[6] (Fig. 3, left).

A similar strategy has been followed for RA-target genes and it has been demonstrated that phosphorylated RARα and MSK1 are also rapidly recruited to RA response elements[5] (Fig. 3, right). However, MSK1 was not associated with RARα, in contrast to what was reported for PR. In fact, TFIIH, the second kinase complex of the MSK-1 initiated kinase cascade, was found associated with RARα at the promoters.[5] Unfortunately, there is no data indicating whether MSK1 is also recruited to the promoters of VDR-target genes.

MSK 1 Regulates the Chromatin Environment of NR-Target Promoters

Once recruited to target promoters, liganded NRs are known to induce an ordered and cyclical recruitment of coactivator complexes with enzymatic activities, which modify histones and remodel chromatin in an ATP-dependent manner.[66,67] Given that MSK1 is also recruited to NRs-target promoters, the question was whether the kinase also contributes to histone phosphorylation and chromatin remodeling at these promoters, as previously described for genes implicated in cell transformation.[49,68]

It has been found that, concomitantly to the recruitment of the ternary PR/Erk/MSK1 complex to nucleosome B of the MMTV promoter, histone H3 becomes phosphorylated at serine 10 by MSK1 (Fig. 3, left), an event coupled to acetylation of lysine 14 by pCAF and displacement of the repressive complex containing HP1γ.[6,64,69] BAF complexes (SWI/SNF ATP-dependent chromatin remodeling complexes) are also recruited to the promoter (Fig. 3, left) through a direct interaction with the activated PR and H3K14 acetylation participate in anchoring the complex to the promoter.[69] Unfortunately no precise order of the different events can be proposed up to now.[69] Nevertheless, BAF uses the energy of ATP hydrolysis to initiate nucleosome remodeling and to remove H2A/H2B dimers from nucleosome B, allowing binding of further PR molecules, coactivators and the transcriptional machinery including RNA polymerase II.[64]

Figure 3, viewed on following page. Left) Progestins bind a subpopulation of membrane-anchored PR complexes, and activate the Src/Ras/Erk pathway, leading to accumulation of activated Erk in the nucleus. Then a nuclear population of PR becomes phosphorylated by activated pErk, which also phosphorylates MSK1. A "PR-activated complex" composed of pPR, pErk and pMSK1 is recruited to the promoter, followed by histone H3 phosphorylation and acetylation. The BAF complex is also recruited through direct interaction with PR and is anchored to chromatin through the histone marks. Due to a lack of kinetic experiments, a precise order of events cannot be proposed. Right) Upon RA binding, a subpopulation of membrane RAR activates the p38MAPK/MSK1 pathway. Activated MSK1 phosphorylates RARα at S369 located in the LBD, subsequently facilitating the docking of cyclin H, which forms with cdk7 and MAT1 the CAK subcomplex of the general transcription factor TFIIH. Within the RARα-TFIIH complex, cdk7 phosphorylates RARα at S77 located in the N-terminal domain. Finally, the phosphorylated RARα/TFIIH complex is recruited to response elements located in the promoter of target genes. MSK1 is also recruited, but separately of the RARα–TFIIH complex, and phosphorylates histones H3.

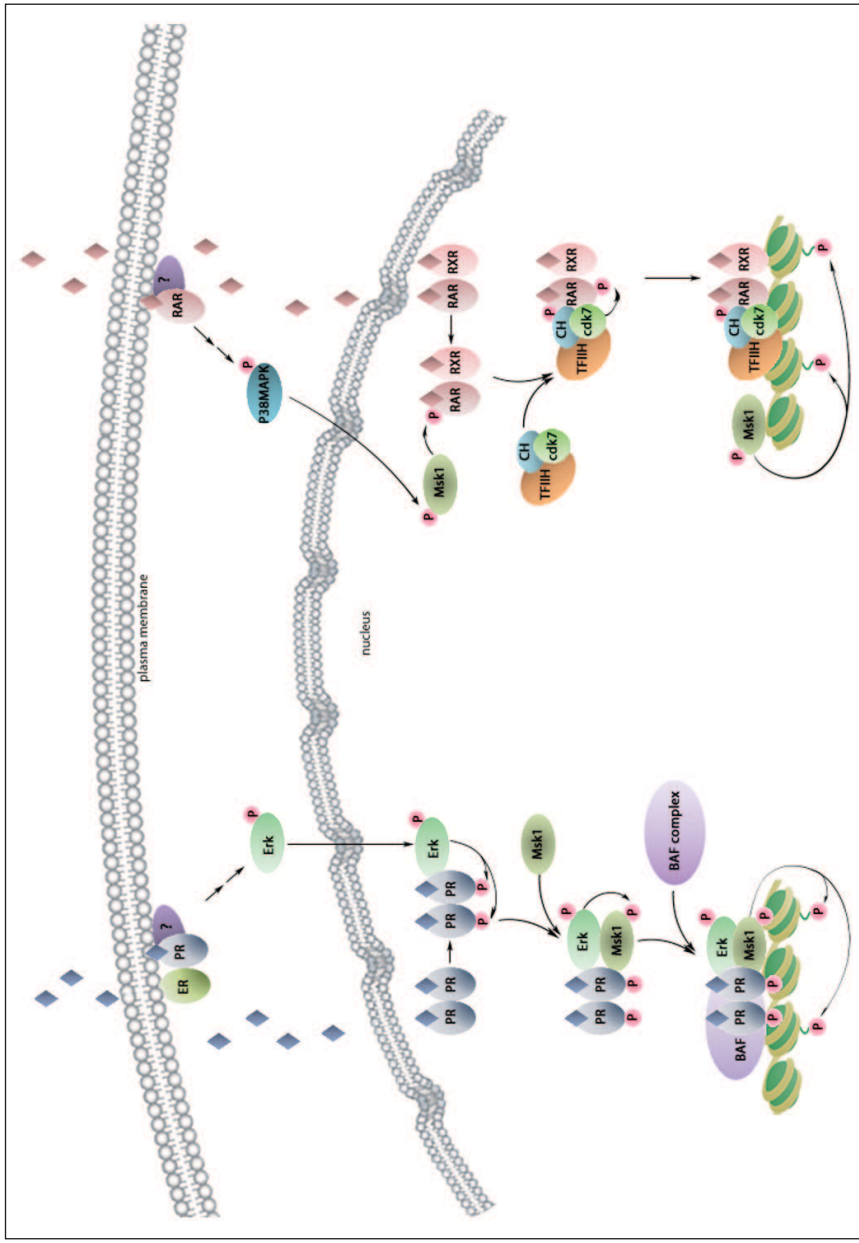

Figure 3. Please see legend on previous page.

Most interestingly, there are similarities between PR and RARα. Indeed the recruitment of RARα to target promoters was also concomitant with histone phosphorylation and acetylation and the subsequent recruitment of the transcriptional machinery[5] (Fig. 3, right). However, whether MSK1 also controls the recruitment/dissociation of other complexes will require further investigations. Of note is that histone H3 phosphorylation-acetylation was also observed in response to concomitant activation of GR and NMDA-R.[57]

MSK1: a Novel Regulator of NR-Target Genes?

MSK1 is well known to be activated by many physiological and pathological stimuli and to regulate gene transcription at multiple levels.[49] Indeed, MSK1 targets directly several transcription factors such as CREB, ATF-1 and NF-κB and induces histone phosphorylation, chromatin relaxation and facilitated recruitment of other coregulators at the cognate target promoters.[70]

The present review points out that MSK1 is also activated by several steroid and non-steroid hormones and regulates the expression of the cognate NR-target genes by regulating the transcriptional activity of NRs and by interfering with chromatin environment. Indeed MSK1-mediated phosphorylation of H3 contributes to several NR-target genes induction, very probably as a chromatin mark accounting in cooperation with other histones modifications for the dissociation of repressive complexes and/or the recruitment of chromatin-remodeling complexes.[5,6,69] Thus one can propose that MSK1-mediated phosphorylation events might act as a "transcriptional clock" fine-tuning the dynamics of chromatin so that at the end the correct proteins are present with the right activity, at the right place and at the right time.

Then the question is whether all NR-target genes promoters are modified and remodeled through H3 phosphorylation by MSK1. Another question is whether MSK1 is also involved in the phosphorylation of NRs coregulators, given that most coactivators and corepressors are known to be also targets for phosphorylation processes.[7]

Future Applications

NRs and Cancer: a Versatile Role of MSK1

Steroid hormone receptors are well established in the etiology of many cancers including classical hormone-dependent cancers like breast and prostate cancer. MSKs are also well known to play a role in cell proliferation and malignant transformation through transcription regulation of the immediate early genes c-fos and junB,[68,70] ER81[71] and the Nur77, Nurr1 and Nor1 orphan nuclear receptors,[72] coupled with histone H3 phosphorylation at specific loci and AP-1 activation.

In line with this, the non-genomic progestin signaling to MSK1 has been correlated with tumor proliferation.[6] In addition, given that MAPK signaling is hyperactivated in several cancers such as breast and prostate cancer,[47,73] one can speculate that signaling to MSK is also increased, potentiating the proliferative effects of ER, PR and AR. Knowing this, MSK-specific drugs would have therapeutic effect in such cancers where MSK deregulation is clearly involved. Note however, that in other cancer types such as colon cancer, the p38MAPK/MSK1 pathway rather cooperates with vitamin D and VDR for the expression of genes involved in tumor suppression.[39] Thus, depending on the type of cancer and on the NR, the MAPK/MSK1 pathway can have opposite effects on cell growth, restricting the use of MSK specific drugs in cancer therapy.

NRs and MSK1 Inhibition

Evidence has accumulated over the past few years that the action of NRs is not restricted to the regulation of cognate target gene expression, but also concerns several other gene programs by interfering with other transcription factors. A well-known example is the anti-inflammatory action of GR and glucocorticoids of which the action mechanism is mainly based on interference with the activity of the transcription factor NF-κB. Indeed, in response to inflammatory cytokines, the classical NF-κB activation pathway determines the expression of various pro-inflammatory genes. The transactivation of NF-κB is fine-tuned by MSK1, which phosphorylates NF-κB p65 and histone H3 S10 at inflammatory genes promoters.[49,74] All these events participate in

the establishment of a transcription-prone chromatin environment. Recent research by Beck et al.[75] demonstrated a completely new aspect of the anti-inflammatory action of glucocorticoids. Indeed, they found that glucocorticoids counteract MSK1 recruitment at inflammatory gene promoters, through the formation of a complex between activated MSK1 and GR, followed by a subcellular relocalization of activated MSK1 to the cytoplasm. The subsequent absence of MSK1 at inflammatory gene promoters leads to impaired phosphorylation of histone and transcription factor components, resulting in a lack of activation of MSK1-dependent NF-κB-driven promoters.

Another well known example is the inhibition of AP-1 by certain NRs such as GR, ER and RARs.[1,7] AP-1 complexes (heterodimers of the proto-oncogene products c-Fos and c-Jun) regulate the expression of several genes involved in oncogenesis and cell transformation. Most interestingly, a network of phosphorylation processes involving MSK1 controls the activity of AP-1 complexes.[68] Though the molecular basis of the anti-AP-1 activity of GR and RARs has remained elusive,[1,7] one cannot exclude a nucleocytoplasmic shuttling of MSK1 as above. Nevertheless, nucleocytoplasmic shuttling of MSK appears to be another new action mechanism for certain NRs, opening perspectives for novel therapeutic strategies.[76]

Conclusion

It is clear that MSK-mediated chromatin remodeling plays a role in many physiological and pathological processes and the list of the agents that can activate the MAPK/MSK signaling pathway is still growing. In this context, hormones and their cognate NRs can activate this pathway, which then contributes to the activation of target genes. However, depending on the NR, MSK1 activation has been related to cell growth[6] or differentiation.[39] Moreover, under certain conditions, NRs do not activate but inhibit MSK1 and thereby the activity of several genes regulated by this kinase. Therefore it does not appear to be a general rule for the cross talk between NRs and MSKs and MSK-specific drugs should have therapeutic benefits only in specific NR-related cancers or diseases.

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RESUME

Les récepteurs nucléaires de l'acide rétinoïque (AR) appelés RAR, se comportent comme des facteurs de transcription inductibles par le ligand. La transcription des gènes cibles induite par l'AR, nécessite la fixation des RAR au niveau de séquences spécifiques des promoteurs et met en jeu des changements conformationnels des récepteurs qui contrôlent l'association/dissociation de toute une panoplie de corégulateurs. Cependant, en plus de ce modèle génomique et nucléaire bien établi, l'équipe du Dr Cécile Rochette-Egly a montré récemment que l'AR a aussi des effets non-génomiques et induit rapidement la voie de signalisation p38MAPK/MSK1 qui ensuite cible les RAR pour des cascades de phosphorylations et module la transcription des gènes cibles.

Pendant mon travail de thèse, j'ai mis en exergue trois nouveaux concepts originaux du mécanisme d'action du sous-type RARα.

J'ai montré qu'une sous-population de RARα est présente dans des microdomaines membranaires, les radeaux lipiques ou "lipid rafts"où elle interagit avec les proteines Gαq. Cette interaction est le signal des effets non génomiques de l'AR, l'activation de la voie de la p38MAPK. Ces effets ont été corrélés à l'activité des gènes cibles de l'AR, prouvant ainsi leur necessité.

J'ai identifié un nouveau partenaire de RARα, la profiline IIA. J'ai analysé le mécanisme moléculaire de l'interaction et démontré qu'elle a lieu dans le noyau. La profiline IIA s'est révélée être un régulateur des effects génomiques de RARα et est recrutée avec RARα au niveau des promoteurs des gènes cibles.

Finalement j'ai mis en évidence une nouvelle fonction de RARα dans le contrôle de l'adhésion et de l'étalement des cellules. D'où l'hypothèse de nouveaux effets génomiques de RARα avec la profiline IIA dans le contrôle de l'expression des proteines d'adhésion. Cependant, de manière inattendue, j 'ai identifié une nouvelle population de RARα dans le cytoplasme de ces cellules. D'où l'hypothèse de nouveaux effets non génomiques dans le cytoplasme, via l'interaction de RARα avec des proteines d'adhésion.

SUMMARY

Nuclear retinoic acid (RA) receptors (RARs) are ligand-dependent regulators of transcription. Their transcriptional activity relies mainly on their recruitment to specific DNA response elements and on their interactions with several coregulators at the ligand-binding domain. In addition to these classical genomic effects, the team of C. Rochette-Egly demonstrated that RA also induces the rapid activation of the p38MAPK/MSK1 pathway with characteristic downstream consequences on the phosphorylation of RARs and the expression of their target genes.

Here I highlighted three novel paradigms in the field of the RARα subtype.

I found that a fraction of the cellular RARα pool is present in membrane lipid rafts, where it interacts with G protein alpha Q in response to RA. This interaction is the signal for nongenomic effects, i.e. the activation of p38MAPK and of the downstream kinase MSK1. These effects have been correlated to the activation of RA-target genes, highlighting its physiological relevance.

I identified a new binding partner of RARα, profilin IIA. I deciphered the mechanism of the interaction and found that it occurs in nuclei. Remarkably, profilin IIA modulates positively the genomic effects of RARα and is recruited with RARα to target genes promoters.

Finally, in an attempt to decipher the relevance of the RARα interaction with profilin IIA, I found that RARα controls cell adhesion and spreading. This might suggest a novel genomic function of RARα and profiling in the control of the expression of genes involved in adhesion. However, preliminary experiments indicate that a pool of RARα is present in the cytosol, suggesting also novel nongenomic effects. Whether RARα controls adhesion via its interaction in the cytosol proteins involved in adhesion will require further investigations.