



# Role of NADPH Oxidase 4 in the Redox Regulation of the Sodium (Na<sup>+</sup>)/ iodide (I<sup>-</sup>) Symporter in Papillary Thyroid Cancer

Juliana Cazarin de Menezes

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Juliana Cazarin de Menezes. Role of NADPH Oxidase 4 in the Redox Regulation of the Sodium (Na<sup>+</sup>)/ iodide (I<sup>-</sup>) Symporter in Papillary Thyroid Cancer. Cancer. Université Paris Saclay (COMUE); Universidade federal do Rio de Janeiro, 2018. English. NNT : 2018SACLS068 . tel-04038560

**HAL Id: tel-04038560**

**<https://theses.hal.science/tel-04038560>**

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ROLE OF NADPH OXIDASE 4 IN THE REDOX  
REGULATION OF THE SODIUM (Na<sup>+</sup>)/ IODIDE (I<sup>-</sup>)  
SYMPORTER IN PAPILLARY THYROID CANCER

Thèse de doctorat de l'Université Paris-Saclay  
et de l'Universidade Federal do Rio de Janeiro  
préparée à Université Paris-Sud

École doctorale n°568 : Signalisation et réseaux intégratifs en  
biologie (BIOSIGNE)  
Programa de Pós-Graduação em Ciências Biológicas  
Instituto de Biofísica Carlos Chagas Filho  
Spécialité de doctorat: Aspects moléculaires et cellulaires de la biologie

Thèse présentée et soutenue à Rio de Janeiro, le 20/03/2018, à 9h par

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**Université Paris-Saclay**  
École doctorale n°568  
BIOSIGNE - Signalisations et  
réseaux intégratifs en biologie

**Universidade Federal do Rio de Janeiro**  
Instituto de Biofísica Carlos Chagas Filho  
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**JULIANA CAZARIN DE MENEZES**

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Rio de Janeiro and École Doctorale n°568 BIOSIGNE, Université Paris-Saclay  
as a requisite for the title of Doctor in Sciences.

Supervisors:

Corinne Dupuy (Université Paris-Saclay)

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Rio de Janeiro,

2018

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*“If you are lucky enough to have lived in Paris as a young man, then wherever you go  
for the rest of your life, it stays with you, for Paris is a moveable feast”*

*Ernest Hemingway, A Moveable Feast*

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Denise Pires de Carvalho and Corinne Dupuy for the effort to make this international collaboration happens although all burocratic issues. I'm also thankful for all academic support, wisdom, enthusiasm and for pushing me further then I thought I could go. You are a genuine representation of strong and competent women in science.

To all the members of Genetic Stability and Oncogenesis Unit (UMR8200 CNRS) from Gustave Roussy Cancer Campus (IGR), thank you for your kind receptivity and assistance. To my lab fellows Naima Azouzi, Camille Buffet, Raphael Corre, Rabii Ameziane, thank for your nice company, for made me feel at home and, especially, for having patience with my poor *français*. *Merci beaucoup!* It is impossible to not to mention all the help that I received from an old friend from Brazil during my stay at IGR. Thank you, Ruy Louzada, for all your support, you're an example of hard-working and competent researcher and deserve all the best wishes in your career.

I will be always grateful to all staff (students, professors and technicians) from Laboratório de Biofísica Doris Rosenthal from Instituto de Biofísica Carlos Chagas Filho, my first scientific home, that gave me all the background necessary to pursue my career as an aspiring professor and researcher. To my family and friends thank you for giving me words of encouragement and for helping me survive all the stress during the last 4 years and for not letting me give up. I hope you forgive me for my eventual absence. Obrigada!

My appreciation also extends to Universidade Federal do Rio de Janeiro, Instituto de Biofísica Carlos Chagas Filho, Université Paris-Saclay, École Doctorale BIOSIGNE and Gustave Roussy Cancer Campus. I'm very proud of to have been part of these institutions of excellence, fulfilled by so many great professionals. Thank you for accepting me and for the opportunity of writing this thesis. It's worthy to mention that research funding from both Brazilian and French government were central for this project development and I feel honored for the confidence deposited in this research.

Finally, I'm fully indebted to Fabio Hecht for sharing with me and for being by my side during all this personal and academic journey. You are my safe haven and this thesis would not exist without you.

**Title:** Role of NADPH oxidase 4 in the redox regulation of the sodium (Na<sup>+</sup>)/ iodide (I<sup>-</sup>) symporter in papillary thyroid cancer

## **ABSTRACT**

The Na<sup>+</sup>/ I<sup>-</sup> symporter (NIS) mediates iodide uptake by thyrocytes, which is a key step in hormone biosynthesis. Iodide accumulation by the thyroid gland is the basis of radioiodine therapy (RAI) that is the standard post-surgery therapeutic approach to efficiently eliminate the remaining cancer lesions and metastasis of differentiated thyroid cancer (DTC). However, 5-10% of DTC patients become RAI-refractory, which is indicative of poor prognosis. Reduced NIS expression and NIS internalization are involved in this process. BRAF<sup>V600E</sup> mutation is the most common genetic event in papillary thyroid cancers (PTCs), the most prevalent type of DTC. In rat thyrocytes, the expression of BRAF<sup>V600E</sup> induces secretion of TGFβ that activates Smad pathway resulting in NIS downregulation, and overexpression of TGFβ is associated with NIS repression in patients. NADPH oxidase NOX4, an enzyme specialized in reactive oxygen species (ROS) generation, is a key mediator of TGFβ signaling in many cell types and it has been previously demonstrated to be overexpressed in thyroid cancer. To better understand the molecular mechanisms involved in PTC loss of iodide uptake ability, the aim of this work is to evaluate whether NOX4 plays a key role in BRAF<sup>V600E</sup>-mediated NIS repression in thyroid cell lines. Using a normal rat thyroid cell line (PC-BRAF), we demonstrated that TGF-β administration or the expression of BRAF<sup>V600E</sup> reduced NIS mRNA and iodide uptake, and increased NOX4 mRNA expression. NOX4 silencing or treatment with SIS3 (inhibitor of Smad pathway) partially inhibited NIS repression, indicating the implication of both Smad pathway and NOX4. To confirm these results we used two human thyroid cancer cell lines that harbor BRAF<sup>V600E</sup> mutation (BCPAP and 8505c), and observed an increase in NIS expression followed by BRAF<sup>V600E</sup> or NOX4 downregulation. Exogenous H<sub>2</sub>O<sub>2</sub> induced DNA methyltransferase 1 (DNMT1) enrichment in tight-chromatin protein fraction, which was decreased by antioxidants or NOX4 silencing in BCPAP cells. TGFβ increased DNMT1 protein levels in chromatin-enriched cell fraction that was reversed by the NADPH oxidase inhibitor Diphenyleneiodonium (DPI). TGFβ-mediated DNA methylation and histone H3K9/K14 hypoacetylation were detected in NIS promoter, and both mechanisms correspond to repressive transcriptional markers. These data suggest that NOX4 is a mediator of BRAF<sup>V600E</sup>-TGFβ signaling and that it exerts a repressive role upon NIS expression probably through epigenetic mechanisms. These data unravel novel mechanisms involved in the regulation of NIS expression in thyroid cancer, and describe possible targets for the development of new therapeutic tools.

**Key Words:** Thyroid Cancer, NIS, NOX4, TGFβ



**Título:** O papel da NADPH oxidase 4 na regulação redox do co-transportador de sódio (Na<sup>+</sup>)/ iodeto (I<sup>-</sup>) no câncer papilífero de tireoide.

## **RESUMO**

O co-transportador Na<sup>+</sup>/I<sup>-</sup> (NIS) medeia a captação de iodeto pelos tireócitos, sendo a base da terapia com radioiodo (RAI), abordagem terapêutica pós-operatória padrão para eliminar eficientemente as lesões remanescentes de câncer diferenciado da tireóide (CDT). No entanto, 5-10% dos pacientes tornam-se refratários a RAI, o que é indicativo de um mau prognóstico. A redução da expressão e internalização do NIS são características desse processo. O oncogene BRAF<sup>V600E</sup> é a alteração genética mais frequente no câncer papilífero de tireóide (CPT), tipo mais prevalente de CDT. Em tireócitos de ratos, o BRAF<sup>V600E</sup> induz a secreção de TGFβ que ativa a via Smad, resultando na repressão de NIS. A NADPH oxidase NOX4, enzima especializada em gerar espécies reativas de oxigênio (ROS), é um mediador chave da sinalização de TGFβ em muitos tipos celulares e sua superexpressão foi previamente detectada no câncer de tireóide. O objetivo deste trabalho é avaliar se a NOX4 é um mediador da repressão de NIS induzido por BRAF<sup>V600E</sup> em linhagens celulares de tireóide. Usando uma linhagem celular de tireóide normal de rato (PC-BRAF), demonstramos que a administração de TGF-β ou a expressão de BRAF<sup>V600E</sup> resultaram na redução dos níveis de mRNA do NIS, redução da captação de iodeto e aumento da expressão de mRNA de NOX4. O silenciamento de NOX4 por SiRNA ou tratamento com SIS3, inibidor de pSmad3, inibiu a repressão de NIS mediada por BRAF<sup>V600E</sup>, indicando o envolvimento da via Smad e de NOX4. Em linhagens de célula de CPT humano portadoras do oncogene BRAF<sup>V600E</sup>, BCPAP e 8505c, também observamos aumento na expressão do mRNA do NIS induzido tanto por inibição de BRAF<sup>V600E</sup> quanto por silenciamento de NOX4. Em células BCPAP, a administração exógena de H<sub>2</sub>O<sub>2</sub> induziu aumento de expressão protéica de DNA metil-transferase 1 (DNMT1) na fração celular enriquecida em proteínas fortemente ligadas à cromatina. O inverso foi observado na presença de antioxidantes ou silenciamento de NOX4. O TGFβ aumentou os níveis da proteína DNMT1 na fração celular enriquecida em proteínas fortemente ligadas a cromatina, o que foi revertido pelo inibidor de NADPH oxidases, Diphenyleneiodonium (DPI). O aumento da metilação e hipoacetilação de histona H3K9/K14 no promotor do gene do NIS, que são marcas de repressão transcricional, foram observados após tratamento com TGFβ. Os dados obtidos sugerem que a NOX4 é um mediador da sinalização BRAF<sup>V600E</sup>-TGFβ e tem um papel inibitório sobre a expressão de NIS, provavelmente através de mecanismos epigenéticos, sugerindo novos alvos terapêuticos para o câncer de tireoide.

**Palavras chaves:** Câncer de tireóide, NIS, NOX4, TGFβ

**Titre :** Rôle de la NADPH oxydase 4 dans la régulation redox du symporteur sodium (Na<sup>+</sup>)/ iodure (I<sup>-</sup>) dans le cancer papillaire de la thyroïde

## **RESUMÉ**

Le symporteur Na<sup>+</sup>/I<sup>-</sup> (NIS) médie la capture de l'iodure par les thyrocytes et cette propriété est exploitée depuis de nombreuses années en thérapeutique pour traiter les cancers différenciés de la thyroïde à l'iode radioactif 131 (Radiothérapie métabolique ou RAI). Cependant, 5 à 10% des patients deviennent réfractaires à la RAI, ce qui indique un mauvais pronostic. La réduction de l'expression NIS et son internalisation sont caractéristiques de ce processus. La mutation activatrice BRAF<sup>V600E</sup> est la plus fréquemment identifiée au sein des cancers différenciés de la thyroïde de type papillaires (CPT), qui est le type le plus répandu. Dans les thyrocytes de souris, BRAF muté induit la sécrétion de TGFβ qui active ensuite la voie Smad ce qui entraîne la répression du NIS. La NADPH oxydase 4 (NOX4), enzyme génératrice d'espèces réactives de l'oxygène (ROS), est un médiateur clé de la signalisation du TGFβ dans de nombreux types cellulaires et sa surexpression a été détectée dans le cancer de la thyroïde. L'objectif de ce travail est d'évaluer si NOX4 est un médiateur de la répression NIS induite par BRAF<sup>V600E</sup> dans des lignées cellulaires thyroïdiennes. En utilisant une lignée cellulaire de thyroïde normal de rat (PC-BRAF), nous avons démontré que le TGF-β ou l'expression BRAF<sup>V600E</sup> promeut la diminution de l'ARNm NIS ainsi que celle du captage de l'iodure en revanche ils augmentent l'expression de l'ARNm NOX4. Le silençage de Nox4 par siRNA ou un traitement des cellules par SIS3, un inhibiteur de pSmad3, inhibe la répression du NIS médiée par BRAF<sup>V600E</sup>, indiquant l'implication de la voie Smad3 et de Nox4 dans cette répression. Dans la lignée tumorale BCPAP dérivée d'un CPT humain et porteuse de la mutation BRAF<sup>V600E</sup>, nous avons également observé une augmentation de l'expression de l'ARNm NIS lorsque BRAF<sup>V600E</sup> ou NOX4 sont inhibés. Dans les cellules BCPAP, un traitement par H<sub>2</sub>O<sub>2</sub> augmente l'expression de la protéine ADN méthyltransférase 1 (DNMT1) dans la fraction cellulaire enrichie en protéines liées à la chromatine. Un traitement par des antioxydants ou le silençage de NOX4 réduisent ce recrutement. Le TGFβ augmente le niveaux de protéines DNMT1 dans la fraction cellulaire enrichie en chromatine, lequel qui est renversé par un traitement par un inhibiteur de NADPH oxydase : le Diphenyleneiodonium (DPI). La méthylation de l'ADN induite par le TGFβ et l'hypoacétylation de l'histone H3K9/K14, qui sont des marques de répression de la transcription, sont détectées au niveau du promoteur NIS. L'ensemble de ces données suggère que Nox4 est un acteur clé de la signalisation TGF-BRAF<sup>V600E</sup> et joue un rôle répressif sur l'expression du NIS, probablement par des mécanismes épigénétiques. Cette étude apporte des données fonctionnelles pour le développement de nouveaux outils thérapeutiques.

**Mots Clés:** Cancer de la thyroïde, NIS, NOX4, TGF

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## LIST OF ABBREVIATIONS

acetylCoA - acetyl-coenzyme A  
AMP - adenosine monophosphate  
AMPK - AMP-activated kinase  
Amplex Red - 10-acetyl-3,7-dihydroxyphenoxazine  
APE/Ref-1 - Human apurinic (apyrimidinic) endonuclease/redox-factor 1  
ATC - anaplastic thyroid carcinoma  
ATP- adenosine triphosphate  
B-ZIP - basic-leucine zipper  
BMPs - bone morphogenetic proteins  
BRAF - B-Raf proto-oncogene, serine/threonine kinase  
BRAFWT – Wild Type B-Raf proto-oncogene, serine/threonine kinase  
BRCA1 – breast cancer 1  
cAMP - cyclic AMP  
CAT - catalase  
CDKN2 - cyclin dependent kinase inhibitor 2A  
CH - Congenital Hypothyroidism  
ChIP - chromatin immunoprecipitation  
CPM - counts per minute  
CRE-L - cAMP-responsive element  
CREB - cAMP-responsive element binding protein  
Da - Daltons  
DCF - 2',7'-dichlorofluorescein  
DIT - 3,5- di-iodotyrosine  
DLC1 - Rho GTPase activating protein  
DNMT - DNA methyl transferase  
DNMT1 - DNA methyl transferase 1  
DNMT3a - DNA methyl transferase 3a  
DNMT3b - DNA methyl transferase 3b  
Doxy - Doxycycline  
DPI - Diphenyleneiodonium  
DTC - differentiated thyroid carcinoma  
DTT - Dithiothreitol

DUOX1 - Dual-oxidase 1  
DUOX2 - Dual oxidase 2  
DUSP - Dual specificity protein phosphatase  
EDTA - (Ethylenedinitrilo)tetraacetic acid  
EGF1 - epidermal growth factor 1  
EGTA - Ethylene-bis(oxyethylenenitrilo)tetraacetic acid  
EMT – epithelial-to-mesenchymal-transition  
ERK - extracellular regulated MAP kinase FAD -  
FaD -flavin adenine dinucleotide  
FCS - Fetal Calf Serum  
FDA - Food and Drug Administration  
FOXO1, formerly named as FOXO-2,  
FRTL-5 -Fischer rat thyroid cell line  
FTC - Follicular thyroid carcinomas  
gp91phox - NADPH Oxidase 2  
GSH Px - glutathione peroxidase  
GSH - glutathione  
GTP - guanosine 5'-triphosphate  
H-RAS - HRas proto-oncogene, GTPase  
H2-DCF-DA - 2',7'-dichlorodihydrofluorescein diacetate  
H2A – Histone 2A  
H2B - Histone 2B  
H3 – Histone 3  
H3K9Ac – Histone 3 acetylated in lysine 9  
H3K9/14Ac - Histone 3 acetylated in lysine 9 and 14  
H4 – Histone 4  
HATs - histone acetyltransferases  
HBSS - Hank's balanced salt solution  
HDAC - histone deacetylases  
HDACi - HDAC inhibitors  
HELA – cell line derived from cervix epithelioid carcinoma  
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
hNUE - Human NIS upstream enhancer  
HRP - horseradish peroxidase



Hsp70 - 70 kilodalton heat shock protein  
IFN gamma - interferon-gamma  
IGF-1 – insulin-like growth factor 1  
IgG – Immunoglobulin G  
IL-1 alpha - interleukin-1 alpha  
ITD - Iodide transport defects  
KRAS - KRAS proto-oncogene, GTPase  
MAPK - Mitogen-activated protein kinase  
MDB - Methyl-CpG-binding domain  
MEK - extracellular signal-regulated kinase  
miRNA - MicroRNAs  
MIT - 3-mono-iodotyrosine  
mRNA - messenger RNA  
MTC - medullary thyroid cancer  
mTOR - mammalian target of rapamycin  
N-RAS - NRAS proto-oncogene, GTPase  
NAC - N-Acetyl-cysteine  
NAD<sup>+</sup> - Nicotinamide adenine dinucleotide (oxidized form)  
NADPH - Nicotinamide adenine dinucleotide phosphate  
NDE - NIS distal enhancer  
NF- κB - factor nuclear kappa B  
NIS - Na<sup>+</sup>/I<sup>-</sup> symporter  
NOX1 - NADPH Oxidase 1  
NOX2 - NADPH Oxidase 2 or gp91phox  
NOX3 - NADPH Oxidase 3  
NOX4 - NADPH Oxidase 4  
NOX4B - NADPH Oxidase 4 isoform B  
NOX4C - NADPH Oxidase 4 isoform C  
NOX4D - NADPH Oxidase 4 isoform D  
NOX5 - NADPH Oxidase 5  
NOXA1 - NADPH oxidase activator 1  
NOXO1 - NADPH oxidase organizer 1  
NSCLC - non-small cell lung cancer  
NTF-1 - NIS TSH-responsive factor 1

NThy-ORI - Normal human primary thyroid follicular epithelial cells

OS - oxidative stress

p21<sup>WAF1</sup> - cyclin-dependent kinase inhibitor 1

p22<sup>phox</sup> - human neutrophil cytochrome b light chain

p40<sup>phox</sup> - neutrophil cytosolic factor 4

p47<sup>phox</sup> - neutrophil cytosolic factor 1

p67<sup>phox</sup> - neutrophil cytosolic factor 2

PAX8 - Paired-box gene 8

PBS - Phosphate-buffered saline

PC-BRAF - PCCL3 with doxycycline-conditioned expression of BRAF<sup>V600E</sup>

PCCL3 – follicular rat thyroid cell line

PCR - Polymerase chain reaction

PDTC - poorly differentiated thyroid carcinoma

PGCR - protein G-coupled receptors

PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase

PKA - protein kinase A

PLX4032 - small-molecule B-Raf inhibitor Vemurafenib

Poldip2 - polymerase-d-interacting protein 2

PPP - pentose phosphate pathway

protein disulfide isomerase (PDI)

PTC - Papillary thyroid carcinomas

qPCR - quantitative polymerase chain reaction

RAC1 - Ras-related C3 botulinum toxin substrate 1

RAC2 - Ras-related C3 botulinum toxin substrate 2

RAI - Radioiodine therapy

RAS – Ras small GTPase

RENOX - former nomenclature for NADPH Oxidase 4

RET - rearranged during transfection

RIPA - Radioimmunoprecipitation assay buffer

RNA - Ribonucleic acid

rNUE - rat NIS upstream enhancer

ROS - Reactive oxygen species

rpm - rotations per minute

RPMI - Roswell Park Memorial Institute medium

rRNA - ribosomal RNA  
SCL5A5 – sodium/iodide symporter gene  
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
SDS - Sodium dodecyl sulfate  
Ser - Serine  
siRNA - small interfering RNA  
SIS3 - SMAD3 Inhibitor  
Smad - Signaling mother against decapentaplegic peptide  
Smad2 - Signaling mother against decapentaplegic peptide 2  
Smad3 - Signaling mother against decapentaplegic peptide 3  
Smad4 - Signaling mother against decapentaplegic peptide 4  
SOD - superoxide dismutase  
Src - Proto-oncogene tyrosine-protein kinase  
T3 - 3,5,3'-tri-iodo-L-thyronine  
T4 - 3,5,3',5'-tetra-iodo-L-thyronine or thyroxine  
TBS-T - Tris-buffered saline, 0.1% Tween 20  
TCGA - The Cancer Genome Atlas  
TERT - Telomerase reverse transcriptase  
Tg - thyroglobulin  
TGF-  $\beta$  - Transforming growth factor  $\beta$   
TGF-  $\beta$ 1 - transforming growth factor- $\beta$ 1  
TH - thyroid hormones  
Thr - Threonine  
TKR - Tyrosine kinase receptors  
TKS5 - invadopodia scaffold protein  
TNF- $\alpha$  - Tumor necrosis factor- $\alpha$   
TPO - thyroperoxidase  
TRE - TSH responsive element  
TRX - thioredoxin  
TR $\beta$ 1 - transforming growth factor- $\beta$ 1 receptor  
TSH - Thyroid-stimulating hormone  
TSHR - TSH receptor  
TTF-1 – Thyroid transcription factor 1  
TTF-2 - Thyroid transcription factor 2

TxnRd - thioredoxin reductase

T $\beta$ R1 - TGF- $\beta$  receptor type 1

T $\beta$ R2 - TGF- $\beta$  receptor type 2

VSMCs - vascular smooth muscle cells

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## 1. INTRODUCTION

### 1.1. The Importance of Iodide Uptake for Thyroid Physiology

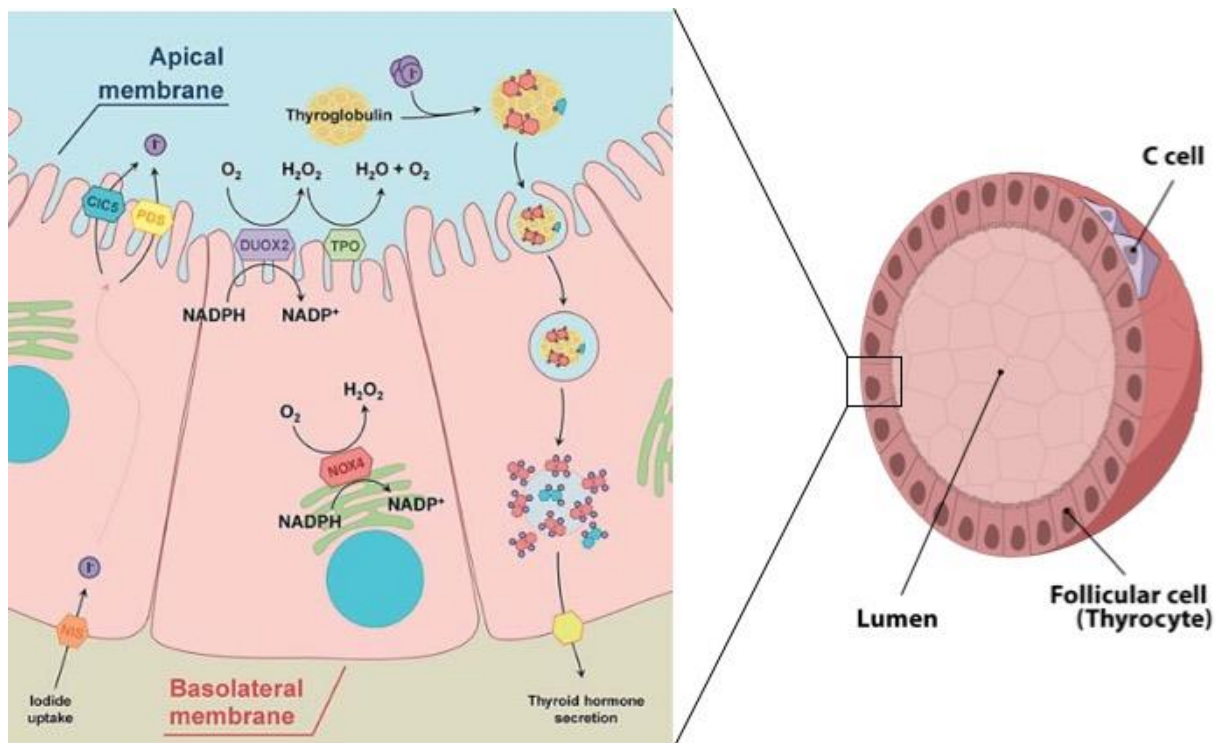
The thyroid gland functional structures are named follicles, which are composed of a monolayer of epithelial cells that surrounds an inner compartment rich in thyroglobulin, known as colloid. The follicle is the functional unit responsible for the synthesis, storage and secretion of the thyroid hormones (TH) T3 (3,5,3'-tri-iodo-L-thyronine) and T4 (3,5,3',5'-tetra-iodo-L-thyronine) that play a central role in cell differentiation, growth and metabolism in virtually all tissues during intra- and extra-uterine life (YEN, 2001).

Iodide ( $I^-$ ) is an essential component of T3 and T4 structure and is obtained through the active transport from blood stream into thyrocytes, where it is stored and used for hormonal biosynthesis. The iodide uptake and accumulation ability by the thyroid gland is known for more than a hundred years. However, only in 1996 the gene that encodes the protein responsible for  $I^-$  transport, the  $Na^+/I^-$  co-transporter (NIS), was finally cloned from rat and human cDNA libraries (DAI; LEVY; CARRASCO, 1996; SMANIK *et al.*, 1996).

Human NIS cDNA encodes a plasmatic membrane glycoprotein of 643 aminoacids that forms 13 transmembrane segments with an extracellular N-terminal extremity and an intracellular C-terminal (ESKANDARI *et al.*, 1997; SMANIK *et al.*, 1996, 1997). This transporter belongs to the family of carriers of solutes 5 (SCLA5) and is located in thyrocytes basolateral membrane (Fig 1). NIS mediates an inward electrogenic co-transport of  $Na^+$  and  $I^-$  from the blood stream into cells with a stoichiometry of  $1 I^- : 2 Na^+$ , using the  $Na^+$  transmembrane gradient generated by  $Na^+/K^+$  ATPase as driving force (DOHÁN *et al.*, 2003; ESKANDARI *et al.*, 1997).

After NIS-mediated iodide internalization, iodine is translocated across the apical membrane into the follicular lumen, where it is oxidized to iodide radical and covalently incorporated into thyroglobulin tyrosyl residues, forming the iodinated molecules 3-mono-iodotyrosine (MIT) and 3,5- di-iodotyrosine (DIT) (Fig 1). MIT and DIT are then coupled by ether bond to form T3 and T4. All these steps are catalyzed by the thyroperoxidase enzyme (TPO), located in apical membrane with its heme catalytic site facing the lumen (BIZHANOVA; KOPP, 2009). Dual oxidase 2 (DUOX2),

a protein of the NADPH oxidase family is located at the apical membrane, and was described in the late nineties as the enzymatic source of  $\text{H}_2\text{O}_2$  used by TPO for iodide oxidation (DE DEKEN *et al.*, 2000; DUPUY *et al.*, 1999). Finally, iodinated Tg is endocytosed, hydrolyzed in phagolysosome vesicles and the T3 and T4 are released in the bloodstream (BIZHANOVA; KOPP, 2009)(Fig 1).



**Figure 1:** Schematic representation of the thyroid follicle and thyroid hormone biosynthesis (Adapted from Carvalho & Dupuy, 2017).

NIS loss-of-function mutations have been implicated in iodide transport defects (ITD) that can result in dysmorphogenesis and constitute one of the causes of Congenital Hypothyroidism (CH), which is a syndrome with inadequate synthesis of TH at birth that results in deficient children growth and low intellectual development (POHLENZ; REFETTOFF, 1999). These mutations, as recently reviewed by Ravera *et cols.* (2017), impair NIS function by reducing its activity or by originating misfolded or abnormally located protein, showing that NIS-mediated iodide trapping is a key step for the hormonal biosynthesis in thyroid gland (RAVERA *et al.*, 2017).



Although first identified and characterized in thyrocytes, NIS is also found in extra-thyroidal tissues in a less-extent, where it plays tissue-specific roles (AKTURK *et al.*, 2013; ALTORJAY *et al.*, 2007; MITCHELL *et al.*, 2001; MORGENSTERN *et al.*, 2005; NICOLA *et al.*, 2009; RIESCO-EIZAGUIRRE, GARCILASO *et al.*, 2014; SPITZWEG *et al.*, 2001; TAZEBAY *et al.*, 2000; VENTURI; VENTURI, 2009). NIS mediates iodide transport from mother to fetus in placenta, iodide absorption from diet through enterocytes, and the incorporation of iodide in milk in lactating mammary gland (AKTURK *et al.*, 2013; NICOLA *et al.*, 2009; TAZEBAY *et al.*, 2000). In salivary gland, NIS is important for iodide secretion in the saliva, where it seems to play antimicrobial properties (VENTURI; VENTURI, 2009). NIS expression was also detected in stomach, lacrimal glands and breast cancer cells (ALTORJAY *et al.*, 2007; MORGENSTERN *et al.*, 2005; TAZEBAY *et al.*, 2000).

## **1.2. NIS Transcriptional and Post-Transcriptional Regulation in Thyrocytes**

### **1.2.1. Thyroid-stimulating hormone (TSH)**

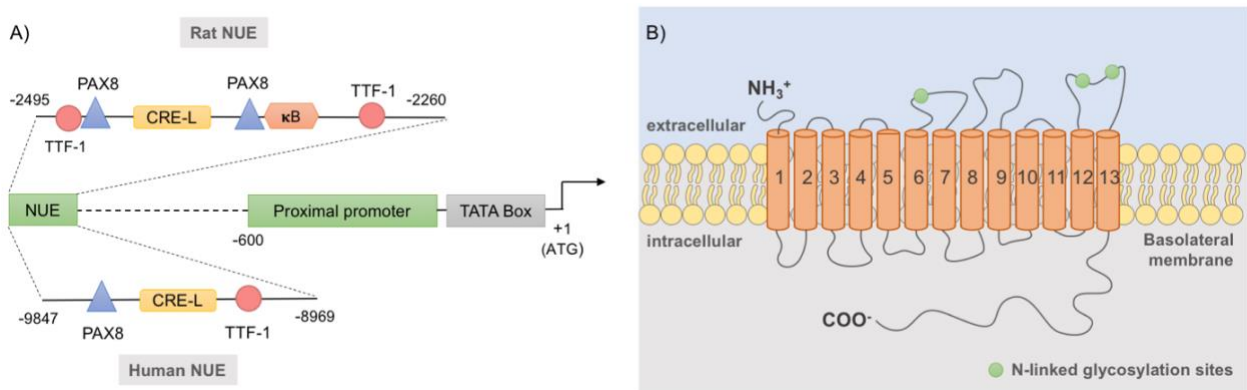
Thyroid-stimulating hormone (TSH) is the major regulator of thyrocyte proliferation and differentiation, playing a central role in the regulation of NIS expression, protein stability and proper localization by transcriptional and post-transcriptional mechanisms (KOGAI, T *et al.*, 2000; KOGAI, TAKAHIKO *et al.*, 1997; OHNO, M *et al.*, 1999; RIEDEL; LEVY; CARRASCO, 2001).

Iodine-metabolizing proteins expression, including NIS, are under the regulation of thyroid specific transcription factors: PAX8, TTF-1 (NKX2.1), TTF-2 (MISSERO *et al.*, 1998). Activation of TSH receptor (TSHR) induces protein G $\alpha$ s dissociation, adenylyl cyclase activity and, consequently, cyclic AMP (cAMP) production and PKA activation. This cascade stimulates NIS transcription by increasing the binding of the transcription factors to NIS promoter (CHUN *et al.*, 2004; OHMORI *et al.*, 1998; OHNO, MAKOTO *et al.*, 1999; PUPPIN *et al.*, 2004; TAKI *et al.*, 2002).

In the 5' regulatory region of rat NIS promoter, TSH/cAMP pathway induces binding of NIS TSH-responsive factor 1 (NTF-1) to the TSH responsive element (TRE) located between positions -420 and -385 from the initiation site, which results in around 2-fold NIS upregulation (OHMORI *et al.*, 1998). TSH/cAMP pathway also controls TTF-

1 mediated promoter activity, whose functional binding sites are located in the position -245 to -230 bp and exerts modest effect on NIS expression (ENDO *et al.*, 1997).

The most relevant responses mediated by TSH on NIS transcriptional activity are located on a more distal region of NIS promoter known as NIS upstream enhancer (NUE) (OHNO, MAKOTO *et al.*, 1999; TAKI *et al.*, 2002). In the rat genome, NUE (rNUE) is located between -2495 and -2264bp, and contains two TTF-1 binding sites that have no effect on NIS transcription, two PAX8 binding sites and a degenerate cAMP-response element (CRE-L) (CHUN *et al.*, 2004; NICOLA *et al.*, 2010; OHNO *et al.*, 1999). Binding sites for NF- $\kappa$ B and transcription factor FOXE1, formerly named as TTF-2, have been recently identified in rNUE (FERNÁNDEZ *et al.*, 2013; NICOLA *et al.*, 2010). In human genome, NUE (hNUE) is located -9242 to -9300 base pairs upstream NIS initiation site and presents PAX8 and TTF-1 binding sites and a CRE-L element (TAKI *et al.*, 2002).



**Figure 2:** Schematic representation of NIS promoter and protein structure. A) Schematic representation of rat and human NIS promoter and its main regulatory region, the NIS upstream enhancer (NUE) containing binding sites for transcription factors PAX8 and TTF-1. CRE-L is the cAMP Responsive Element; B) Schematic representation of NIS protein. The green circles represent sites of N-linked glycosylation (CHUN *et al.*, 2004; NICOLA *et al.*, 2010; OHNO, MAKOTO *et al.*, 1999; RAVERA *et al.*, 2017)

TSH/cAMP-dependent NUE transcriptional activity requires the recruitment of cAMP-responsive element binding protein (CREB) and basic-leucine zipper (B-ZIP) molecules to CRE-like element and at least one Pax8-binding site (CHUN *et al.*, 2004; OHNO, MAKOTO *et al.*, 1999; TAKI *et al.*, 2002). Recent data suggest that TSH/PKA signaling increases  $\beta$ -catenin translocation to the nucleus, where  $\beta$ -catenin physically interacts with Pax8 and increases its transcriptional activity in rNUE (SASTRE-PERONA; SANTISTEBAN, 2014). In human NUE (hNUE), it has been demonstrated

that TSH/cAMP signaling results in redox reduction of PAX8 by Ref-1, which increases its binding to hNUE and upregulates NIS transcription (PUPPIN *et al.*, 2004).

Studies also suggest that TSH-stimulated NIS iodide uptake is not restricted to its regulation at the transcriptional level. Using the FRTL-5 rat thyroid cell line, Riedel *et al.* (2001) showed that TSH deprivation reduced NIS protein half-life from 5 to 3 days, and downregulated iodide uptake. Interestingly, the transport activity decreased faster than NIS expression. This phenomenon was correlated to NIS redistribution from the plasma membrane to intracellular compartments, showing that TSH also regulates NIS subcellular localization by post-translational mechanisms (RIEDEL; LEVY; CARRASCO, 2001).

The molecular mechanisms involved in NIS post-translational regulation are not well known. Consensus sites for a variety of kinases and N-glycosylation have been identified in NIS protein sequence, raising the question whether these modifications could be involved in NIS activity, stability or trafficking to plasma membrane (LEVY *et al.*, 1998; VADYSIRISACK *et al.*, 2007).

Five different phosphorylation sites in the intracellular domain of NIS rat protein were identified. Two of them (Ser-43 and Ser-581) regulate iodide transport velocity and another residue (Thr-577) was shown to be implicated in regulating protein stability (VADYSIRISACK *et al.*, 2007). NIS phosphorylation pattern in FRTL-5 cells differs according to the presence or absence of TSH, indicating that TSH regulates this modification (RIEDEL; LEVY; CARRASCO, 2001). However, which sites are affected by TSH signaling and their functional implications were still not described.

N-linked glycosylation is a post-translational modification that regulates protein maturation and trans-localization. There are three putative sites for N-glycosylation in rat NIS protein (ESKANDARI *et al.*, 1997). Levy *et al.* (1998) showed that NIS protein is highly glycosylated in plasma membrane in rat thyroid cell line, which could be a determinant post-translational modification for NIS localization (ESKANDARI *et al.*, 1997). However, using site-directed mutagenesis, the same group showed that neither partial nor total absence of N-glycosylation interfere with NIS localization, or stability in fibroblast-like cell lines derived from monkey kidney tissue (COS cells) expressing wild-type NIS and NIS N-linked glycosylation mutants (LEVY *et al.*, 1998). Additionally, all mutants were active showing 50–90% of iodide de uptake of wild-type NIS.

A recent publication shows conflicting data with this previous study. Chung and cols. (2015) transfected the NIS gene in immortalized human cervix adenocarcinoma

cells (HELA cells) with a fusion system and a red fluorescent protein (tdTomato) to monitor glycosylation-mediated changes and NIS localization and activity (CHUNG *et al.*, 2015). Treatment of this heterologous system with cAMP resulted in increased NIS glycosylation levels, increased NIS translocation to plasma membrane and iodide trapping, which was reversed by tunicamycin, an inhibitor of N-glycosylation, showing that glycosylation is determinant for NIS subcellular location and activity. The discrepancies between these two studies might relay in specie-specific features of NIS post-transcriptional regulation. Although in both studies NIS was expressed in heterologous cell systems, Levy *et al* (1998) data were obtained from rat NIS while, in the second study, human NIS glycosylation was evaluated.

### 1.2.2. Growth Factors and Cytokines

TSH-TSH-R-cAMP–protein kinase A (PKA) signaling is the main regulator of NIS function in thyrocytes, and has been extensively studied over the years. However, it is important to highlight that physiological NIS regulation is a result of a complex interaction of signaling pathways modulated by hormones, growth factors and cytokines that synergize or antagonize TSH responses (PORTULANO; PARODERBELENITSKY; CARRASCO, 2014; RIESCO-EIZAGUIRRE; SANTISTEBAN, 2006).

Insulin/IGF-1 for example have been shown to downregulate cAMP-induced NIS expression through the PI3K-mTOR pathway in rat cells, a key signaling transducer of some tyrosine kinase receptors (RTKs) and protein G-coupled receptors (GPCRs) (DE SOUZA *et al.*, 2010; GARCÍA; SANTISTEBAN, 2002). Despite its major stimulatory effect on NIS, TSH via activation of PI3K pathway (PI3K) can reduce iodide uptake. This contradictory negative effect is mediated by protein G $\beta\gamma$  dimers dissociated from G $\alpha$  due to TSHR activation. This inhibitory TSH effect probably corresponds to an auto regulatory loop to maintain a tight control of thyroid function by TSH (ZABALLOS; GARCIA; SANTISTEBAN, 2008)

The MEK–ERK pathway is an important positive regulator of cell proliferation that mediates signal transduction in response to a variety of growth factors and hormones, including estradiol and epidermal growth factor 1 (EGF-1), well known negative regulators of NIS function (FURLANETTO; NGUYEN; JAMESON, 1999). The constitutive activation of MEK-ERK signaling has been associated to the

downregulation of both NIS mRNA and iodide uptake activity in rat and human cell lines and in *in vivo* mouse models (HOU; BOJDANI; XING, 2010; MITSUTAKE *et al.*, 2006; RIESCO-EIZAGUIRRE, G. *et al.*, 2009). This pathway seems to be involved in the loss of NIS expression during thyroid carcinogenesis, as will be discussed in the next topic.

Cytokines exert an overall negative regulatory effect on NIS. Tumor necrosis factor-alpha (TNF alpha), interferon-gamma (IFN gamma), interleukin-1 alpha (IL-1 alpha) and transforming growth factor- $\beta$ 1 (TGF-  $\beta$ 1) inhibit NIS expression and activity in rat thyroid cell lines and human primary thyroid cell culture (AJJAN *et al.*, 1998; KAWAGUCHI *et al.*, 1997; PEKARY; HERSHMAN; BERG, 1998; TATON *et al.*, 1993). It was demonstrated that TGF- $\beta$ -induced NIS downregulation is mediated by Smad (signaling mother against decapentaplegic peptide) pathway activation, which downregulates PAX8 mRNA and its binding to the NIS promoter (COSTAMAGNA, 2003).

MicroRNAs (miRNA) have been recently implicated in NIS repression. In rat thyroid cell line, PCCL3, miR-146b-3p has been demonstrated to bind to the 3'-untranslated region of NIS and PAX8, leading to mRNA degradation and impaired protein translation. Interestingly, this miRNA was downregulated by TSH and upregulated by known NIS repressors such as IGF-1 and TGF- $\beta$  (RIESCO-EIZAGUIRRE, GARCILASO *et al.*, 2015).

Over the last years, our group identified AMP-activated kinase (AMPK), a central metabolic regulator activated by increased AMP/ATP ratio or energy restriction, as another signaling pathway involved in NIS repression. AMPK activation resulted in NIS mRNA downregulation, lysosomal protein degradation and reduced iodide uptake activity in PCCL3 cells. Interestingly, AMPK phosphorylation/activation was repressed by TSH, which was reversed by PKA inhibition (ANDRADE *et al.*, 2011; CAZARIN, J M; ANDRADE; CARVALHO, 2014). These data suggest that a balance between TSH-AMPc-PKA pathway and AMPK pathway may also contribute for NIS physiological regulation.

### 1.2.3. Iodide

Apart from the aforementioned regulatory factors, iodide ( $I^-$ ) itself is an important modulator of NIS function. In the late 40's Wolff & Chaikoff showed that the exposure of thyroid cells to high  $I^-$  concentrations resulted in inhibition of  $I^-$  organification and hormonal synthesis, a phenomenon known as the Wolff & Chaikoff effect. After a couple of days, a mechanism of adaptation takes place and  $I^-$  organification and hormone biosynthesis returns to normal levels (WOLFF *et al.*, 1949). This adaptation was proposed to be resultant of reduced iodide uptake mediated by NIS in thyroid tissue that reduces intra-thyroidal iodine pool and releases  $I^-$  organification inhibition (BRAVERMAN; INGBAR, 1963; SOCOLOW *et al.*, 1968).

After the NIS gene cloning, several authors provided direct evidence that NIS expression and activity are repressed by  $I^-$  overload either by transcriptional and post-transcriptional mechanisms, such as reduced mRNA stability, reduced protein translation and NIS inactivation at the plasma membrane (ENG *et al.*, 1999; LEONI *et al.*, 2011; SERRANO-NASCIMENTO *et al.*, 2014). Although the molecular mechanisms involved are not clear, PI3K pathway and increased ROS production have been associated to NIS downregulation by iodide (LEONI *et al.*, 2011; SERRANO-NASCIMENTO *et al.*, 2014).

Indeed, thyroid redox status has been shown to play a determinant role in thyroid autoregulation by  $I^-$ . High doses of  $I^-$  induce increased reactive oxygen species (ROS) production by thyrocytes from different species such as dog, sheep, pig and rat (CORVILAIN *et al.*, 2000). Leoni *et al.* (2011) showed that thioredoxin reductase (TxnRd) activity, an antioxidant enzyme, regulates NIS responses to iodide (LEONI *et al.*, 2011). Rat thyroid cell line (PCCL3) exposure to high doses of  $I^-$  inhibited iodide uptake, increased ROS production and TxnRd activity. Interestingly, when TxnRd was inhibited, NIS activity was not recovered during the escape phase, showing that reducing cell oxidative status is determinant for NIS function reestablishment (LEONI *et al.*, 2011). Corroborating these data Arriagada *et al.* (2015) demonstrated that iodide-mediated NIS inactivation at the plasma membrane was also regulated by ROS, this effect being reverted by ROS scavengers (ARRIAGADA *et al.*, 2015). However, the transcriptional and/or post-translational mechanisms by which ROS regulate NIS activity are still unknown.

### 1.3. NIS Implication in Thyroid Cancer Management

So far, the importance of NIS for hormone biosynthesis and how it is physiologically regulated in the thyroid gland were discussed. However, NIS-mediated iodide uptake is also the basis of diagnosis and treatment of certain thyroid diseases, as thyroid cancer (KOGAI, TAKAHIKO; BRENT, 2013). Thyroid cancer is a common endocrine malignancy, whose incidence is continuously increasing worldwide (ASCHEBROOK-KILFOY *et al.*, 2013; KILFOY *et al.*, 2009). Almost 57,000 new cases of thyroid cancer are predicted in the USA in 2017, being the 5<sup>th</sup> most incident cancer in women (SIEGEL; MILLER; JEMAL, 2015). Projections show that costs with management, treatment and diagnoses are estimated in almost 19 billion dollars, considering all patients of all ages diagnosed with thyroid cancer from 2010 to 2019, (ASCHEBROOK-KILFOY *et al.*, 2013). These data emphasize that thyroid cancer is not only a medical concern but also an economic issue.

Thyroid cancer may arise from two different cell populations present in thyroid tissue: the follicular thyroid cells and parafollicular cells, also known as C cells. Parafollicular cells originate medullary thyroid cancer (MTC) that accounts to around 3% of all thyroid cancers. Tumors derived from follicular thyroid cells are much more frequent and comprises: differentiated thyroid carcinoma (DTC), poorly differentiated thyroid carcinoma (PDTC) and undifferentiated or anaplastic thyroid carcinoma (ATC) (XING, M, 2013).

ATC is a rare type of thyroid malignancy accounting for 2% of the cases. These tumors usually show a rapid and dramatic evolution with low survival rates and are resistant to current therapeutic options, being the most aggressive type of thyroid cancer (KOJIC; STRUGNELL; WISEMAN, 2011). DTC comprises almost 90% of thyroid cancers and are divided in two distinct histological types: papillary thyroid carcinomas (PTC) and follicular thyroid carcinomas (FTC). Eighty per cent off all thyroid cancers are PTC, while FTC correspond to around 10% of the cases. In contrast to anaplastic carcinomas, these tumors show an indolent behavior, good prognosis and high survival rates. Poorly differentiated thyroid carcinoma show an intermediary phenotype between DTCs and ATCs (KONDO; EZZAT; ASA, 2006).

Radioiodine therapy (RAI) has been the standard therapeutic approach for DTC treatment for over 50 years. RAI consists on the administration of <sup>131</sup>I radioiodine isotope after surgical thyroidectomy. <sup>131</sup>I mainly concentrates in the thyroid tissue

thanks to its highly efficient iodide accumulation ability, and emits high energy  $\beta$ -particles that allow the ablation of remnant thyroid tissue after surgery, locoregional and destruction of residual cancer lesions and thyroid-derived metastasis (WORDEN, 2014)

Unfortunately, 5–15% of patients with DTC present tumors that do not concentrate iodide and, consequently, RAI is no longer effective (HAUGEN, B R, 1999; KOGAI, T.; TAKI; BRENT, 2006; XING, MINGZHAO; HAUGEN; SCHLUMBERGER, 2013). 10-year survival rate falls from 60% in patients with metastatic DTC that preserves iodide avidity to 10% in patients with RAI refractory metastasis, showing that radio-iodine refractoriness is associated with poor prognosis (DURANTE *et al.*, 2006). These patients represent a real therapeutic challenge since treatment options are limited. Standard chemotherapy and targeted systemic therapies are usually followed by significant secondary effects and have been proven not effective for some subset of patients in clinical studies (HO *et al.*, 2013a; ROTHENBERG *et al.*, 2015a; WORDEN, 2014).

The mechanism underlying the loss of iodide uptake avidity seems to result from a cell de-differentiation process that occurs during tumor progression, reducing the expression of NIS (ARTURI *et al.*, 1998; CAILLOU *et al.*, 1998; DURANTE *et al.*, 2007; WARD *et al.*, 2003). Reduced mRNA and protein expression, as well as increased NIS internalization have been correlated to the impairment of iodide uptake in thyroid papillary cancers (ARTURI *et al.*, 1998; CASTRO *et al.*, 2001; DOHÁN *et al.*, 2001; RIESCO-EIZAGUIRRE, G, 2006; ROMEI *et al.*, 2008). In this context, understanding the molecular background involved in thyroid cell de-differentiation is crucial to develop therapies to reverse radio-iodide refractoriness.

#### **1.4. Molecular Pathogenesis of Radioiodine Refractoriness in Papillary Thyroid Cancer**

Mitogen-activated protein kinase (MAPK) pathway is an important regulator of cell proliferation and survival. Its deregulation is reported in multiple types of cancer and well established in thyroid carcinogenesis, especially in papillary thyroid cancers. Around 70% of somatic alterations found in PTCs involve MAPK pathway defects (AGRAWAL *et al.*, 2014). The most prevalent alterations include mutually exclusive



point mutations of BRAF and RAS genes and tyrosine kinase receptor (RTK) fusions, especially involving RET gene. All these alterations induce the RAS-RAF-MEK-ERK signaling cascade constitutive activation (FAGIN; WELLS, 2016).

RET/PTC rearrangements are found in around 15% of adult PTCs (AGRAWAL *et al.*, 2014). High prevalence is found in children and in patients with a radiation-exposure history (HAMATANI *et al.*, 2008; NIKIFOROV *et al.*, 1997). RET is a transmembrane tyrosine kinase receptor (RTK) usually expressed in tissues derived from neural crest, but not in follicular thyroid cells. RET/PTC results from the fusion of the 3' terminal portion RET gene and the 5' terminal portion from a donor gene originating an intracellular fusion protein with constitutively active tyrosine kinase activity. RET/PTC activates multiple pathways including RAS-BRAF-MEK-ERK signaling and the PI3K pathway (KNAUF, JEFFREY A *et al.*, 2003; MITSUTAKE *et al.*, 2006)(Fig 3).

So far, 13 different RET/PTC rearrangements have been reported in PTC, being RET/PTC1 and RET/PTC3 being the most frequent. While RET/PTC1 is associated with slow proliferation and small tumors, RET/PTC3 is likely more aggressive, as shown in pediatric thyroid cancer after Chernobyl accident (NIKIFOROV *et al.*, 1997; ROMEI *et al.*, 2008; ROMEI; ELISEI, 2012). However, in general, PTC harboring RET/PTC translocations shows radio-iodine avidity, is not strongly associated to reduced survival and rarely progresses to undifferentiated carcinomas (TAVARES *et al.*, 2016)

RAS is a 21-kDA membrane associated protein that belongs to the family of small GTPases and participates in signal transduction from membrane receptors to intracellular targets of MAPK and PI3K pathway, regulating cell proliferation, survival and differentiation. Activating point mutations in genes of RAS superfamily (N-RAS, K-RAS, H-RAS) results in impaired RAS GTPase activity, constitutive GTP-bound state and consequently constitutive active RAS, being the most common event in tumorigenesis, found in 30% of all human cancers (FERNÁNDEZ-MEDARDE; SANTOS, 2011).

Activating point mutations in RAS gene are found in both malignant and benign thyroid tumors with mutations in N-RAS being the most prevalent. These mutations are very frequent in FTCs (40-52% of the cases), PDCs and ATCs (50% of the cases) and are associated with poor prognosis (FUKAHORI *et al.*, 2012; VOLANTE *et al.*, 2009). In a minor scale, RAS mutations are also significantly present in PTC,

accounting for 15% of the cases (AGRAWAL *et al.*, 2014; NIKIFOROVA *et al.*, 2003)(Fig 3). However, in these carcinomas, RAS mutation is associated to limited aggressive behavior, low risk and maintenance of radioiodine avidity (AGRAWAL *et al.*, 2014; MEDICI *et al.*, 2015).

BRAF point somatic mutations are the most common driver-mutation found in papillary thyroid cancer, present in around 40%–60% of the cases. The most frequent BRAF mutation is a transversion mutation in exon 15, *BRAF*<sup>T1799A</sup>, that results in a valine to glutamic acid substitution at amino acid 600. This single-nucleotide mutation encodes BRAF<sup>V600E</sup> oncogene that induces constitutive activation of RAF-MEK-ERK pathway (KIMURA *et al.*, 2003; XING, M, 2005)(Fig 3).

Mice expressing BRAF<sup>V600E</sup> exclusively in thyroid follicular cells develop invasive and poorly-differentiated thyroid tumors with a similar histology found in BRAF<sup>V600E</sup>-positive advanced human thyroid cancers (CHAKRAVARTY *et al.*, 2011). In PTC cell lines harboring this mutation, siRNA against BRAF<sup>V600E</sup> reduced cell proliferation, anchorage-independent colony formation and growth of xenograft tumor in nude mice, showing that BRAF<sup>V600E</sup> is required for thyroid cell tumorigenesis (LIU, DINGXIE; LIU; *et al.*, 2007). Indeed, a recent study performed a stable transfection of BRAF<sup>V600E</sup> in non-tumoral human thyroid cell line NThy-ORI and observed upregulation of 2.441 genes that were mostly associated with cancer-related gene ontologies and pathways, including cell migration, adhesion and MAPK cascade (KIM, BYOUNG AE *et al.*, 2017).

Even though these functional studies suggest that BRAF<sup>V600E</sup> induces aggressive behavior in PTCs, this association is not totally clear in patients. While some studies reported a correlation between this mutation and poorer prognosis including increased tumor size, recurrence after surgery, lymph node and distant metastasis, others did not confirmed the same characteristics (ELOY *et al.*, 2011; KIM, TAE HYUK *et al.*, 2012; TROVISCO *et al.*, 2005; XING, MINGZHAO, 2007; XING, MINGZHAO *et al.*, 2005). However, it cannot be stated that BRAF<sup>V600E</sup> does not contribute for PTC progression since this mutation is also found in 35% of PDTC and 45% of ATCs (AGRAWAL *et al.*, 2014). Indeed, recent studies suggest that the coexistence of BRAF<sup>V600E</sup> and *TERT* mutations are correlated to increased tumor aggressiveness in PTCs, which could explain the heterogeneity of risk stratification studies (MELO *et al.*, 2014; XING, MINGZHAO *et al.*, 2014).

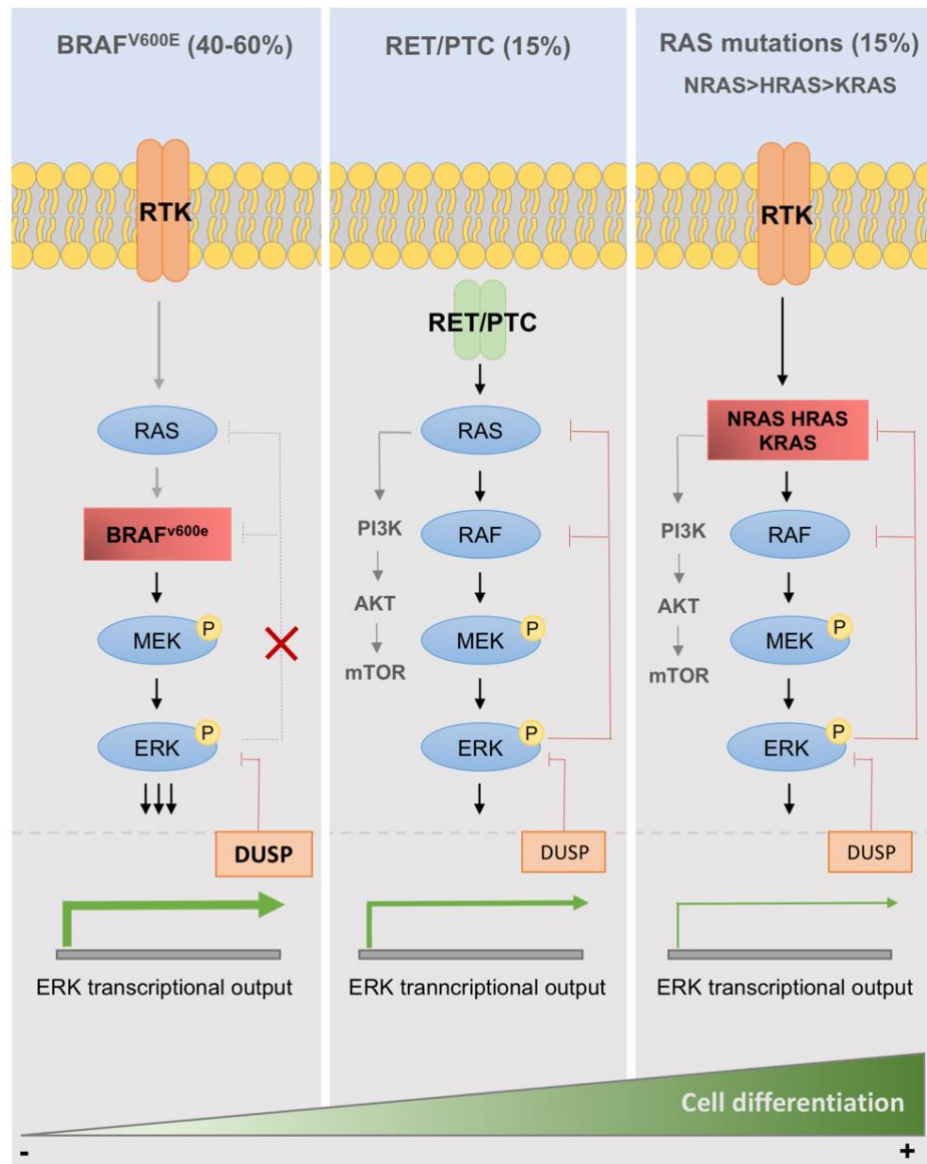
Despite the debatable correlation with prognosis, it is well documented that BRAF<sup>V600E</sup> expression in thyroid cells reduces the expression of thyroid differentiation markers, particularly NIS, *in vitro* and in mouse models (CHAKRAVARTY *et al.*, 2011; FRANCO *et al.*, 2011; KNAUF, J. A., 2005; MITSUTAKE *et al.*, 2005; RIESCO-EIZAGUIRRE, G. *et al.*, 2009; RIESCO-EIZAGUIRRE, G, 2006). Clinical studies also revealed a correlation between BRAF<sup>V600E</sup> mutation, NIS downregulation and iodine-refractory PTC in patients (RIESCO-EIZAGUIRRE, G, 2006; ROMEI *et al.*, 2008; SABRA *et al.*, 2013). Additionally, a recent multiplatform analysis of 496 PTCs from The Cancer Genome Atlas (TCGA) project showed that BRAF<sup>V600E</sup>-positive tumors are correlated to lower differentiation scores, characterized by downregulation of iodide handling genes including NIS, Tg, TPO and DUOX, when compared to PTCs harboring RAS mutations or RET/PTC translocations (AGRAWAL *et al.*, 2014).

Constitutive MEK-ERK pathway activation has been suggested by numerous studies to play a crucial role in iodide-metabolizing gene impairment during thyroid carcinogenesis. (CHAKRAVARTY *et al.*, 2011; DURANTE *et al.*, 2007; LIU, DINGXIE; HU; *et al.*, 2007; MITSUTAKE *et al.*, 2005). Interestingly, TCGA data analysis has also shed some light on the importance of MAPK activation levels in thyroid cancer cell dedifferentiation. By analyzing mRNA, protein and phosphoprotein expressions, it was demonstrated that the lowest differentiation scores are found in PTC with higher MEK-ERK pathway activation. Interestingly, BRAF<sup>V600E</sup>-positive tumors show a stronger activation of ERK transcriptional program when compared to PTCs harboring RAS mutations or RTK fusions (AGRAWAL *et al.*, 2014).

The explanation for discrepant levels of MEK-ERK activation may rely on differential signaling pathway activation. In wild-type, RAS-mutated and RTK fusion positive cells, RAF signals as a dimer through the MAPK-pathway. As an auto-regulatory mechanism, ERK induces a negative-feedback loop in upstream effectors and upregulates dual specificity protein phosphatase (DUSP), resulting in the inhibition of RAF dimerization and MAPK reduced activation (POULIKAKOS *et al.*, 2010; PRATILAS *et al.*, 2009). In contrast, BRAF<sup>V600E</sup>-positive cells signal through MAPK-pathway as monomers, which is resistant to the feed-back phenomenon. In this case, MEK-ERK pathway is highly activated, even in the presence of higher DUSP expression that is detected in BRAF<sup>V600E</sup>-positive PTC (AGRAWAL *et al.*, 2014; PRATILAS *et al.*, 2009) (Fig 3).

Due to the central role of MAPK in thyroid cell dedifferentiation, targeting this pathway with pharmacological inhibitors could be a feasible therapy to restore radioiodine incorporation in RAI-refractory metastatic thyroid cancer, especially in BRAF<sup>V600E</sup>-driven PTC. Indeed, treatment of rat thyroid cells harboring BRAF<sup>V600E</sup> oncogene with MEK inhibitors totally or partially restores NIS expression (LIU, DINGXIE; HU; *et al.*, 2007; RIESCO-EIZAGUIRRE, G, 2006). Treatment of transgenic mice with MEK and RAF pharmacological inhibitors also restored the impaired iodide uptake induced by the expression of BRAF<sup>V600E</sup> in thyroid gland (CHAKRAVARTY *et al.*, 2011).

Although *in vitro* and *in vivo* promising results, the treatment of patients with MAPK inhibitors was not correspondently effective for some cohorts, especially for BRAF<sup>V600E</sup> positive thyroid tumors. Ho *et al.* treated 20 patients with RAI-refractory metastatic thyroid cancer harboring different genetic backgrounds with Selumetinib, a MEK1/2 inhibitor, for 5 weeks (HO *et al.*, 2013a). Five of five patients with N-RAS point mutation and one of three patients harboring RET/PTC rearrangement showed increased iodide uptake that allowed radioiodine therapy. In contrast, only 1 patient from nine with BRAF<sup>V600E</sup>-positive PTC responded to Selumetinib treatment, suggesting that insufficient MAPK inactivation and/or other accessory pathways may contribute to a BRAF<sup>V600E</sup> mediated NIS repression (HO *et al.*, 2013a). Therefore, deciphering how BRAF<sup>V600E</sup> orchestrates thyroid cell dedifferentiation and consequently NIS impairment during carcinogenesis is crucial to improve iodide-refractory thyroid cancer treatment.



**Fig 3:** Driver mutations and signaling cascades in papillary thyroid cancer. Mutually exclusive activating mutations in BRAF, RAS, and RET/PTC translocations are the most common genetic alterations found in PTCs. Percentage indicates the incidence of these alterations in PTCs. Although all mutants result in constitutive activation of mitogen-activated protein kinase (MAPK) signaling, MEK-ERK transcriptional output is higher in BRAF<sup>V600E</sup>-driven tumors even in the presence of higher MEK dual specificity protein phosphatase (DUSP) expression. BRAF<sup>V600E</sup> unresponsiveness to ERK upstream negative feedback is a main cause of this phenotype. Higher ERK signaling output levels are inversely correlated to cell differentiation scores. RET/PTC and mutated RAS-driven PTCs respond to ERK feedback showing moderate to low ERK signaling output and higher differentiation scores. In these tumors PI3K-AKT-mTOR pathway is concomitantly activated (Adapted from: FAGIN; WELLS, 2016; POULIKAKOS et al., 2010)

## 1.5. Mechanisms Involved in *BRAF*<sup>V600E</sup>-Induced NIS Repression in Thyroid Cancers

### 1.5.1. Epigenetics

Multicellular organisms present different cell types that play different functions even though they have the same DNA sequence. This paradigm implies that the genotype by itself does not explain all phenotype variations. The term 'epigenetics' was coined by the developmental plasticity biologist Conrad Waddington in the early 40s to define how genes could interact with its surroundings to give rise to distinct phenotypes during development (WADDINGTON, 2012). In a more recent approach, 'Epigenetics' refers to the study of gene expression that occur in dynamic, reversible and potentially heritable way without changes in DNA code sequence (WU, 2001). Epigenetic regulation implies in changes in chromatin organization that, in turn, can limit or enable the access of protein complexes containing transcription factors that regulate gene expression (PORTELA; ESTELLER, 2010).

DNA methylation is one of the most well-described epigenetic modifications and results from covalent transfer of methyl residues to the 5 position of the cytosine pyrimidine ring (5-methyl-cytosine) (JIN; LI; ROBERTSON, 2011). In mammalian cells, the most frequent target of DNA methylation are cytosine residues from CpG dinucleotides (LISTER *et al.*, 2009). These dinucleotides are characterized by a cytosine followed by a guanine in 5'-3' direction that tend to cluster forming genomic regions with high G-C content, known as CpG islands (PORTELA; ESTELLER, 2010). CpG islands are located in the promoter region of 60% of human genes and their methylation status is regulated in a tissue-specific and time dependent manner during development and cell differentiation to control gene expression (STRAUSSMAN *et al.*, 2009).

DNA methylation is catalyzed by a class of enzymes known as DNA methyl transferases (DNMT). In mammals, three different DNMTs with methyltransferase activities have been reported: DNMT1, DNMT3a and DNMT3b. DNMT3a and DNMT3b are usually classified *de novo* DNMTs, responsible for the methylation of previous non-methylated DNA sites, showing high expression in embryonic stem cells (OKANO *et al.*, 1999). DNMT1 has a marked preference for hemi-methylated DNA and acts during DNA replication, copying DNA methylation pattern from parental DNA to the newly

synthesized strand, being classified as a maintenance DNMT (HERMANN; GOYAL; JELTSCH, 2004). However, it is important to highlight that classification in *de novo* and *maintenance* DNMTs are not very strict and that the two classes of DNMTs have overlapping functions (JONES; LIANG, 2009). DNMT1 for example has also *de novo* methyltransferase activity (PRADHAN *et al.*, 1999). Likewise, DNMT3a and DNMT3b co-localizes with methylated DNA and seems to cooperate with DNMT1 for DNA methylation maintenance (SHARMA *et al.*, 2011).

Generally, CpG island methylation is a repressive mark that results in epigenetic repression of gene transcription. The DNA methylation may physically block the recruitment of transcriptional regulators to gene promoter region (JIN; LI; ROBERTSON, 2011). Additionally, DNA methylation is recognized by a protein family that shares a methyl CpG binding domain, known as MDB family. These proteins recruit chromatin-remodeling complexes to methylated sites, resulting in chromatin condensation, attenuating gene transcription (FATEMI; WADE, 2006) .

Aberrant alterations in epigenome predispose to several diseases including cancer. Global loss of DNA methylation accompanied by hypermethylation of certain CpG islands are epigenetic hallmarks of cancer cells (FEINBERG; OHLSSON; HENIKOFF, 2006). Global hypomethylation arises mainly from loss of methylation of repetitive sequences, which results in genomic instability and increased gene rearrangements (EDEN *et al.*, 2003; QU *et al.*, 1999; RODRIGUEZ *et al.*, 2008; WEISENBERGER *et al.*, 2006). However, hypomethylation can also occur in CpG islands of some tumor promoter genes, leading to aberrant oncogene expression (WILSON, ANN S.; POWER; MOLLOY, 2007).

In contrast to hypomethylation, DNA hypermethylation is local and only occurs in the promoter of specific genes in a tissue-specific pattern in cancer cells (COSTELLO *et al.*, 2000). Several tumor-suppressor genes involved in the regulation of cell-cycle, apoptosis and DNA repair such as BRCA1, CDKN2 and are silenced by hypermethylation during tumorigenesis (EL-NAGGAR *et al.*, 1997; ESTELLER, M., 2007; ESTELLER, M. *et al.*, 2000). In thyroid cancers, aberrant hypermethylation has also been reported in genes involved in iodine metabolism such as NIS, TSHR and pendrin (GALRÃO, ANA LUIZA *et al.*, 2014; VENKATARAMAN *et al.*, 1999; XING, M *et al.*, 2003; XING, MINGZHAO *et al.*, 2003)

NIS promoter hypermethylation has been associated to reduced NIS mRNA in thyroid tumors (GALRÃO, A. L. *et al.*, 2013; GALRÃO, ANA LUIZA *et al.*, 2014;

VENKATARAMAN *et al.*, 1999). So far, two distinct CpG island have been identified in NIS promoter. CpG-island1 is located at NIS proximal promoter while CpG-island2 is set in a region with enhancer activity, a NIS distal enhancer (NDE) located 2152/1887bp from ATG. Both CpG-islands are hypermethylated in thyroid malignant and benign tumors that present low NIS expression and/or impairment of iodide uptake, suggesting that NIS gene methylation is an early event in thyroid carcinogenesis (GALRÃO, ANA LUIZA *et al.*, 2014; VENKATARAMAN *et al.*, 1999). Indeed, the treatment of a limited variety thyroid cancer cell lines with de-methylation agents was able to increase NIS mRNA, protein and iodide uptake providing a direct evidence that gene methylation is implicated in NIS expression and function regulation in cancer cells (GALRÃO, ANA LUIZA *et al.*, 2014; LI, WEI; VENKATARAMAN; AIN, 2007)

Recent studies show that BRAF<sup>V600E</sup> can regulate NIS expression and function by epigenetic mechanisms. Indeed, NIS proximal promoter was found to be highly methylated in almost 60% of BRAF<sup>V600E</sup>-positive papillary thyroid cancers, which was associated with reduced NIS expression (CHOI, YONG WON *et al.*, 2014). Interestingly, B-RafV600E also induced NIS methylation in human isolated primary thyrocytes, which was also accompanied by reduced NIS mRNA, protein and iodide uptake. This hypermethylation was attributed to a specific BRAF<sup>V600E</sup>-mediated increased expression of DNA methyltransferase (DNMT1) through unknown mechanisms, while DNA methyltransferases 3a and 3b do not seem be regulated by this oncogene (CHOI, YONG WON *et al.*, 2014). Briefly, the authors suggest that NIS repression in BRAF<sup>V600E</sup> -positive PTC could be mediated by increased DNMT1-mediated promoter DNA methylation.

Apart from DNA methylation, histone post-translational modifications play a key role in epigenetic regulation. Histones are nuclear proteins that pack DNA into structural units called nucleosomes. In nucleosomes, histones are organized in octamers containing heterodimers of core histones H2A, H2B, H3, and H4 and carry epigenetic information through covalent post-translational modifications in their N-terminal and C-terminal tails (PORTELA; ESTELLER, 2010; ROPERO; ESTELLER, 2007). Histone post-translational modifications include acetylation, phosphorylation, methylation and SUMOylation that can occur simultaneously in different sites, changing how these proteins interact with DNA and controlling transcriptional



regulation, DNA replication and repair (HUERTAS; SENDRA; MUÑOZ, 2009; LI, BING; CAREY; WORKMAN, 2007).

Lysine acetylation of histones H3 and H4 are the best characterized histone post-translational modifications. The reaction of lysine acetylation is catalyzed by histone acetyltransferases (HATs) that transfer an acetyl group from the acetyl-coenzyme A (acetylCoA) cofactor to form  $\epsilon$ -N-acetyllysine (COHEN *et al.*, 2011). Lysine acetylation neutralizes histone positive charge, reducing its affinity for the negatively charged DNA and relaxing chromatin structure, which facilitate the access of transcription factors and allows transcriptional activation. The inverse reaction, lysine deacetylation, is mediated by histone deacetylases (HDAC) and is associated to heterochromatin state and transcriptional repression (LEGUBE; TROUCHE, 2003).

A global imbalance of histone acetylation is reported in cancer cells and may contribute to the altered gene profile expression found in tumors. Several HDACs were reported to be overexpressed in a variety of human cancers including gastric, colon and prostate cancer (CHOI, J H *et al.*, 2001; HALKIDOU *et al.*, 2004; WILSON, ANDREW J *et al.*, 2006). Increased deacetylase activity may be associated to promoter hypoacetylation of some tumor-suppressor genes such as p21<sup>WAF1</sup> in myeloma cells and DLC1 in ovarian cancer (GUI *et al.*, 2004; KWONG *et al.*, 2006). Indeed, the use of HDAC inhibitors (HDACi) in cancer cells showed anticancer effects, resulting in cell cycle arrest, apoptosis and inhibition of angiogenesis, being already approved by the FDA for the treatment of hematological malignancies (BUBNA, 2015).

Anticancer effects of HDACi, including cell cycle arrest and apoptosis were also observed in anaplastic thyroid cancer cell lines (GREENBERG *et al.*, 2001). Interestingly, it has been extensively shown that several HDACi such as Butyrate, SAHA, Depsipeptide, and Valproic acid increase NIS expression and eventually iodide uptake in thyroid cancer cells, suggesting the use of these compounds as a potential strategy to improve iodine sensitivity in RAI-refractory thyroid tumors (CHENG *et al.*, 2016; FORTUNATI *et al.*, 2004; FURUYA *et al.*, 2004; PROVENZANO *et al.*, 2007; PUPPIN *et al.*, 2005; ZARNEGAR *et al.*, 2002).

A recent study showed that BRAF<sup>V600E</sup> expression in PCCL3 cells, induces global histone acetylation but reduces NIS promoter acetylation (H3K9/14 acetylation) that resulted in NIS gene repression. In the BRAF<sup>V600E</sup>-positive human thyroid cancer cell line BCPAP, inhibition of BRAF<sup>V600E</sup> with PLX4032 or the incubation with HDACi increased NIS promoter acetylation and NIS mRNA expression (ZHANG, ZONGJING

*et al.*, 2014). Indeed, simultaneous suppression of BRAF<sup>V600E</sup> and HDAC activity induced a more robust expression of NIS and iodide uptake in thyroid cancer cells *in vitro* than suppressing BRAF<sup>V600E</sup> alone (CHENG *et al.*, 2016). Therefore, BRAF<sup>V600E</sup> regulates NIS expression by both promoting NIS promoter methylation but also by promoting histone deacetylation.

### 1.5.2. Transforming growth factor $\beta$ (TGF- $\beta$ )

Transforming growth factor  $\beta$  (TGF- $\beta$ ) has been shown as an important effector of NIS repression in thyroid cells harboring BRAF<sup>V600E</sup> (RIESCO-EIZAGUIRRE, GARCILASO *et al.*, 2009). TGF- $\beta$  is a multifunctional cytokine, member of a large family of growth factors including bone morphogenetic proteins (BMPs), activins and Nodal that has a key role during mammalian cell development and differentiation. In mammals, three isoforms of TGF- $\beta$  have been described: TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, TGF- $\beta$ 1 being the most abundantly found (CHEN, JIAN; MISHRA, 2013).

The canonical TGF- $\beta$  signaling pathway relays on Smad pathway activation. TGF- $\beta$  is exported from the cell as an inactive latent polypeptide that is activated by proteolytic cleavage. Once active, TGF- $\beta$  binds to TGF- $\beta$  receptor type 2 (T $\beta$ R2) that recruits TGF- $\beta$  receptor type 1 (T $\beta$ R1), resulting in dimerization and phosphorylation of T $\beta$ R1. T $\beta$ R1 phosphorylates and activates R-Smads (Smad2 and Smad3) that associate with the co-Smad, Smad4, forming complexes that are translocated to the nucleus to regulate transcriptional activity of target genes. As an auto-regulatory mechanism, inhibitory Smads (Smad6 and Smad7) can compete with R-Smads for T $\beta$ R1-mediated phosphorylation, inhibiting the Smad pathway.

According to the cellular context, TGF- $\beta$  can act both as tumor repressor or promoter. In early stages of carcinogenesis and in normal human cells, TGF- $\beta$  induces strong pro-apoptotic and anti-proliferative responses. However, in late stages of tumor progression this molecule has a central role by inducing proliferation, invasion, angiogenesis and metastasis (PRINCIPE *et al.*, 2014). In prostate and colorectal cancer, aberrant TGF- $\beta$ 1 levels have been detected in advanced tumors, which was associated to invasiveness and increased tumor size (LANGENSKIÖLD *et al.*, 2008; SHARIAT *et al.*, 2001).

In human thyroid carcinomas harboring BRAF<sup>V600E</sup> mutation, TGF- $\beta$ 1 and central components of TGF- $\beta$  signaling, such as Smad2 and TR $\beta$ 1, are overexpressed, especially in invasive areas. Interestingly, this overexpressed pattern of TGF- $\beta$ 1 was correlated with NIS internalization and reduced expression (RIESCO-EIZAGUIRRE, GARCILASO *et al.*, 2009). This clinical data corroborates previous findings in normal epithelial thyroid cells that characterized TGF- $\beta$ 1 as a potent repressor of thyroid specific genes of the iodide metabolism, such as NIS, thyroglobulin and thyroperoxidase (KAWAGUCHI *et al.*, 1997; NICOLUSSI *et al.*, 2003; TATON *et al.*, 1993). In PCCL3 cells, NIS downregulation was at least partially associated to a reduced expression of PAX8 induced by a functional interaction between this transcriptional factor and Smad3 (COSTAMAGNA, 2003).

To demonstrate a direct connection between BRAF<sup>V600E</sup>, TGF- $\beta$  and NIS down-regulation Riesco-Eizaguirre and colleagues (2009) used a rat thyroid cell line (PCCL3) with doxycycline-conditioned expression of BRAF<sup>V600E</sup> (PC-BRAF cells). BRAF<sup>V600E</sup> expression resulted in increased TGF- $\beta$ 1 secretion, Smad pathway activation and NIS repression, which was reversed by TGF- $\beta$ -R1 inhibitor and transient expression of the inhibitory Smad, Smad7, showing that BRAF<sup>V600E</sup>-induced TGF- $\beta$ 1 autocrine loop is involved in NIS downregulation (RIESCO-EIZAGUIRRE, GARCILASO *et al.*, 2009).

Knauf et al. (2011) investigated the link between BRAF<sup>V600E</sup> and TGF- $\beta$  *in vivo*, using transgenic mice model that overexpressed BRAF<sup>V600E</sup> in thyroid cells under the control of thyroglobulin promoter (*Tg-Braf*). BRAF<sup>V600E</sup> induction resulted in the development of invasive PTC with PDTC regions and reduced expression of thyroid-specific genes (KNAUF, J. A., 2005; KNAUF, J A *et al.*, 2011). TGF- $\beta$ 1 was found overexpressed in *Tg-Braf* thyroid tissue when compared to wild-type mice, supporting the previous *in vitro* results in PC-BRAF cells. Interestingly, TGF- $\beta$ 1 expression was sharply increased in PDTC areas when compared to adjacent PTC regions. Progression from PTC to PDTC profile was associated with epithelial-to-mesenchymal transition that was secondary to concomitant MAPK and TGF- $\beta$  signaling, showing that this two pathways cooperate to thyroid cancer progression in BRAF<sup>V600E</sup> positive tumors (KNAUF, J A *et al.*, 2011). However, the molecular mechanism by which BRAF<sup>V600E</sup>-induced TGF- $\beta$ 1-loop regulates thyroid dedifferentiation and NIS downregulation is still an open question.

Reactive oxygen species (ROS) have been described as important mediators of several TGF- $\beta$  responses, such as epithelial-to-mesenchymal transition, cell motility

and fibrosis in normal and transformed cells (BOUDREAU, HOWARD E *et al.*, 2013; CHIU *et al.*, 2001; LIU, RUI MING; DESAI, 2015; RHYU *et al.*, 2005). TGF- $\beta$  induces oxidative imbalance by reducing antioxidant cell defense and by increasing mitochondrial and, especially, NADPH oxidase-dependent ROS production, that has been identified as a key effector of TGF- $\beta$  signaling (BOUDREAU, HOWARD E *et al.*, 2013; BRAVO *et al.*, 2003; CUCORANU *et al.*, 2005). However, the involvement of ROS or NADPH oxidase in TGF- $\beta$  signaling in thyrocytes has never been investigated.

### 1.6. NADPH Oxidase Family and TGF- $\beta$ Signaling

ROS comprise a group of chemically reactive molecules derived from the partial reduction of molecular oxygen ( $O_2$ ) that includes radical and non radical species, as superoxide radical ( $O_2^{\cdot-}$ ), peroxy ( $RO_2^{\cdot}$ ) alkoxy ( $RO^{\cdot}$ ), hydroxyl radical ( $OH^{\cdot}$ ), hypochlorous acid (HOCl), singlet oxygen ( $^1O_2$ ) and hydrogen peroxide ( $H_2O_2$ ), usually referred as the most biologically relevant redox-signaling molecule due to its relative stability and higher diffusion rate (HECHT *et al.*, 2016; MISHINA *et al.*, 2011).

ROS interact with various molecules such lipids, proteins, carbohydrates and nucleic acids, changing reversibly or irreversibly the structure and function of these compounds (CUCORANU *et al.*, 2005). As a second messenger, it regulates signaling pathways and several physiological cellular processes including growth, differentiation and cell death. In this context, oxidation of critical amino acid residues, such as cysteine, represent a relevant mechanism in redox signal transfer regulating protein enzymatic activity and/or structural properties that will reflect in downstream or upstream pathway regulation by ROS (HORNSVELD; DANSEN, 2016).

Physiological ROS generation occurs through different cellular mechanisms and organelle, including mitochondria, xanthine oxidase, lipoxygenases, peroxisomes and P450 oxidase as a by-product of its enzymatic activity (HECHT *et al.*, 2016). In contrast to these sources, the NADPH oxidase enzymes generate ROS as its only and specific product, and in response to a wide range of stimuli, being implicated in several physiological processes, such as host defense against pathogens, hormonal biosynthesis and fine-tuning regulation of cell signaling (BEDARD; KRAUSE, 2007).

Due to its toxicity and reactivity, cellular ROS levels must be maintained between ranges that guarantee cell survival and function. Cellular redox-state is

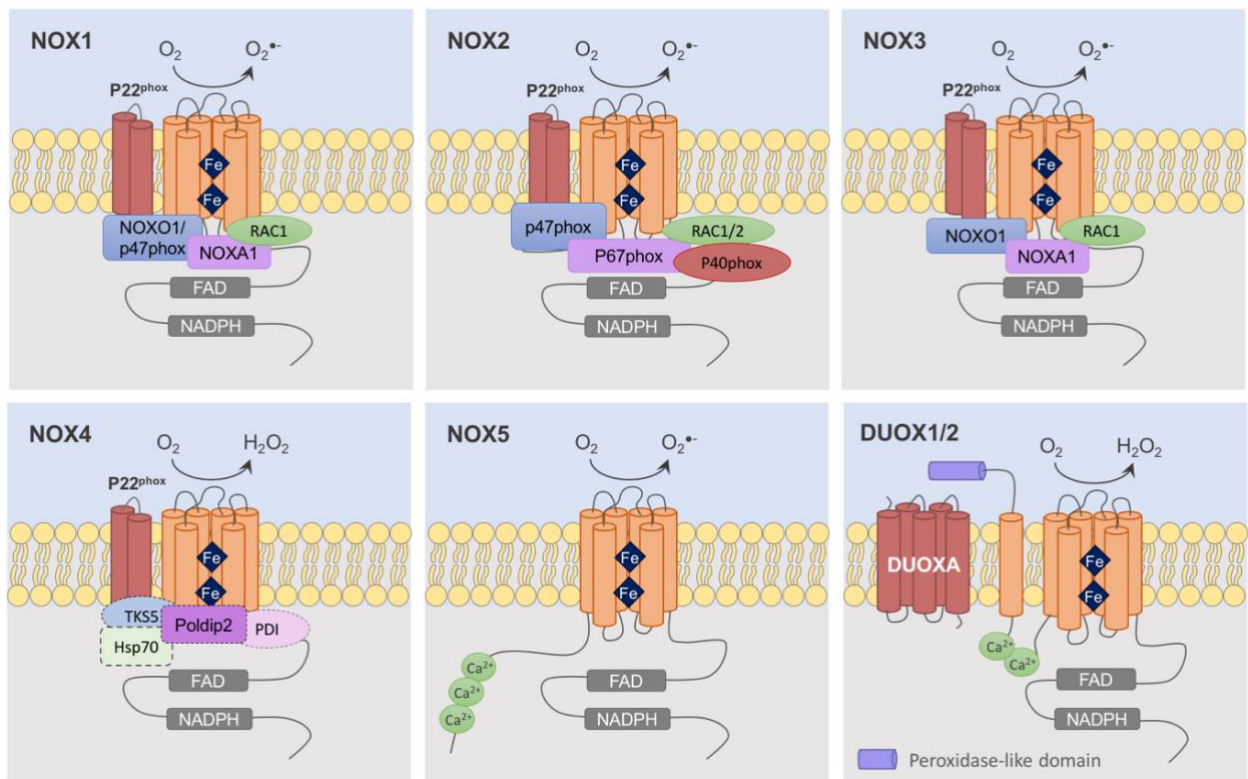
maintained by a balance between ROS production and ROS-scavenge enzymatic systems as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH Px) or antioxidant molecules, including glutathione (GSH) and thioredoxin (Trx) (SON *et al.*, 2013). Persistent imbalance between ROS production and/or detoxification by antioxidant system that result in increased ROS levels leads to oxidative stress (OS) that is implicated in the etiology of several human diseases including cancer, since ROS mediates cancer initiation, promotion and metastasis (HYBERTSON *et al.*, 2011).

NADPH oxidase deregulation and aberrant activity have been reported in several types of cancer, such as prostate, melanoma and glioblastoma. Additionally, *in vitro* studies revealed that this class of enzymes is implicated in many aspects of carcinogenesis including cell transformation, tumor growth, DNA damage, genomic instability, immune-suppression, angiogenesis and metabolic alterations in cancer cells (CORZO *et al.*, 2009; GRAHAM *et al.*, 2010; MITSUSHITA; LAMBETH; KAMATA, 2004; PRATA *et al.*, 2008; USHIO-FUKAI; NAKAMURA, 2008; WEYEMI, U *et al.*, 2012). Due to their specific and regulated ROS generation capacity, NADPH oxidases emerged then as potential mediators of oxidative stress in cancer cells, which raised the interest in these proteins as possible therapeutic targets (WEYEMI, U *et al.*, 2012).

NADPH oxidase 2 (NOX2) or gp91<sup>phox</sup>, as formerly named, was the first NADPH oxidase identified and cloned in the late 1980's. This enzyme, present in phagosome membranes of macrophages, neutrophils and monocytes was identified as a critical component of the antimicrobial repertoire, generating superoxide to oxidative burst (ROYER-POKORA *et al.*, 1986; TEAHAN *et al.*, 1987). Over the following years, other six non-phagocytic NOX2 homologues have been identified in a variety of human tissues, such as NOX1, NOX3, NOX4, NOX5 and Dual-oxidases 1 (DUOX1) and 2 (DUOX2) (BEDARD; KRAUSE, 2007).

All NADPH oxidase family members are transmembrane proteins that reduce molecular oxygen by transporting electrons donated by NADPH across biological membranes, producing superoxide anion (O<sub>2</sub><sup>-</sup>) (NOX1-3 and NOX5) and/or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (NOX4, DUOX-1 and DUOX-2) as a final product. All NOX isoforms share a common core structure that includes: 1) FAD and NADPH binding sites in the cytoplasmic C-terminus; 2) six conserved transmembrane domains and 3) two conserved heme groups located in the transmembrane region (DRUMMOND *et al.*, 2011). Exceptionally, DUOX-1 and 2 possess a seventh transmembrane domain that

is in continuity with an extracellular peroxidase-like domain in their N-terminus. Calcium binding sites in cytosolic N-terminal region are also a specific feature found in Dual-oxidases and in NOX5, being determinant for their enzymatic activity (AMEZIANE-EL-HASSANI *et al.*, 2005; BÁNFI *et al.*, 2001) (Fig 4).



**Figure 4:** NADPH oxidase family and membrane-bound and/or cytosolic regulatory proteins. All NADPH oxidases isoforms are transmembrane proteins that reduce molecular oxygen producing superoxide (NOX1-3 and NOX5) and/or  $H_2O_2$  (NOX4, DUOX-1 and DUOX-2) as final products. NOX and DUOXs share common core structures: FAD and NADPH binding sites in cytoplasmic C-terminus, conserved heme groups in transmembrane region and six transmembrane segments. Exceptionally, Duox1 and 2 show a 7th transmembrane segment. Maturation and stabilization partners (DUOXA, p22<sup>phox</sup>) are shown in red. NOX1, NOX2 and NOX3 activation are dependent on sequential recruitment of a combination of cytosolic subunits (NOXA1, p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>) and small GTPases (RAC1 and RAC2). NOX5, DUOX1 and DUOX2 activity is regulated by  $Ca^{2+}$  binding to EF hand motifs (green). Although cytosolic modulators of NOX4 activity have been described (TKS5, Poldip2, PDI and Hsp70), this NADPH oxidase is constitutively active and does not require their respective recruitment for its full activation (adapted from DRUMMOND *et al.*, 2011).

Despite structural similarities, optimal enzymatic activation of different NOX isoforms relies on different mechanisms that involve the assembly of other membrane-bound and/or cytosolic regulatory proteins (Fig 4). NOX2 activation requires constitutive interaction with the membrane-bound protein p22<sup>phox</sup> that provides docking sites for p47<sup>phox</sup> recruitment in response to acute stimuli, allowing the assembly of cytosolic activating small subunits p67<sup>phox</sup>, p40<sup>phox</sup> and small GTPases RAC1 and

RAC2 (SUMIMOTO; MIYANO; TAKEYA, 2005). NOX1 and 3 also interact with p22<sup>phox</sup> and depend on the assembly of soluble subunits NOXA1, NOXO1 and RAC1 for their activation (SUMIMOTO; MIYANO; TAKEYA, 2005; TAKEYA *et al.*, 2003; UENO *et al.*, 2005). NOX4 complexes with p22<sup>phox</sup> for proper stability but, in contrast to the other NADPH oxidases, shows a constitutively active enzymatic activity (MARTYN *et al.*, 2006; SERRANDER *et al.*, 2007). DUOX-1, DUOX-2, and NOX5 also do not require cytosolic factors for activation but are sensitive to intracellular calcium levels, as mentioned before. DUOX-1 and DUOX-2 are co-expressed with maturation factors DUOXA1 and DUOXA2, respectively, that are essential to translocation of functional DUOX to the plasma membrane (GRASBERGER; REFETTOFF, 2006) (Fig 4).

NADPH oxidase family has been identified as a key source of TGF- $\beta$ - induced ROS generation (BOUDREAU, HOWARD E *et al.*, 2013; CUCORANU *et al.*, 2005). More specifically, NADPH oxidase 4 (NOX4) has been shown a central role in TGF- $\beta$  signaling. In human lung mesenchymal cells, TGF- $\beta$  increases NOX4 expression with no effect on other NOX family members (HECKER *et al.*, 2009). Corroborating these data, NOX4 was also identified as a key effector of TGF $\beta$  induced ROS generation in metastatic breast cancer cells, inducing epithelial-to-mesenchymal transition and cell migration (BOUDREAU, H E *et al.*, 2014; BOUDREAU, HOWARD E *et al.*, 2013). TGF $\beta$ -induced NOX4 is also involved in other pathological processes, such as hepatocyte oxidative stress in virus-induced hepatitis and pulmonary and kidney fibrosis (BOUDREAU, H. E. *et al.*, 2009; CARNESECCHI *et al.*, 2011; SEDEEK *et al.*, 2010).

The induction of NOX4 mediated by TGF $\beta$  involves the activation of its canonic Smad signaling pathway. Indeed, inhibition of Smad3 in human lung mesenchymal cells reversed NOX4 increased expression and ROS generation induced by TGF- $\beta$ , which was confirmed in other cell types as kidney myofibroblasts, smooth muscle cells and breast epithelial cells (BONDI *et al.*, 2010; HECKER *et al.*, 2009; MICHAELOUDES *et al.*, 2011). In a less extent, the activation of non-Smad signaling pathways, such as the MAPK and PI3K pathways, have also been implicated in TGF- $\beta$ -induced NOX4 expression in different cell contexts (ISMAIL *et al.*, 2009; TOBAR *et al.*, 2014).

### 1.7. NADPH Oxidase 4 (NOX4)

NOX4 is highly expressed in kidney where it was first identified in 2001 and named as RENOX (GEISZT *et al.*, 2000). During the following years, it was demonstrated that NOX4 is ubiquitously expressed, detected in a range of human and murine tissues, such as heart, liver, lung, vascular tissue and thyroid (BEDARD; KRAUSE, 2007; WEYEMI, U. *et al.*, 2010). This enzyme is a 66 kDa protein located in intracellular compartments including the endoplasmic reticulum, mitochondria, nucleus and focal adhesions in a cell-type specific fashion where it generates, locally, H<sub>2</sub>O<sub>2</sub> and/or O<sub>2</sub><sup>-</sup> in a constitutively active manner (CHEN, KAI *et al.*, 2008; GRAHAM *et al.*, 2010; LEE *et al.*, 2013; VON LÖHNEYSEN *et al.*, 2010).

Several NOX4 mRNA splicing variants have been identified, generating NOX4 isoforms that differ in their functional activity and subcellular localization. Alternative splicing generates NOX4 isoforms that lack the first NADPH binding site (NOX4B) or all NADPH and FAD binding sites (NOX4C) and non-membrane bound isoforms (NOX4C and NOX4D). NOX4D is probably the only active splicing isoform that maintains the same ROS generation rate than the prototype protein (GOYAL *et al.*, 2005). NOX4D generates a 28 kDa protein localized in the nucleus, whose overexpression in vascular cells leads to ROS-induced DNA damage (ANILKUMAR *et al.*, 2013). The role of these isoforms and the circumstances in which they are expressed are unclear.

The mechanisms involved in NOX4-mediated ROS production regulation are still a matter of active investigation and are not well understood. As mentioned before, in contrast with the other NADPH oxidases, NOX4 generates ROS in a constitutively active manner. NOX4 complexes to p22<sup>phox</sup>, which is important for its stabilization and maturation, but its activity does not necessarily require cytosolic regulatory subunits or respond to calcium levels (AMBASTA *et al.*, 2004; KAWAHARA *et al.*, 2005; MARTYN *et al.*, 2006). For this reason, it was postulated that NOX4-dependent ROS generation is primarily regulated by its expression level (SERRANDER *et al.*, 2007).

Several endogenous and exogenous stimuli including lung injury, hypoxia or hyperoxia, high glucose, TGF- $\beta$  and myocyte hypertrophic stimuli (angiotensin II and phenylephrine) have been described as NOX4 positive regulators, which mainly occurs at the transcriptional level (AGO *et al.*, 2010; BOUDREAU, HOWARD E *et al.*, 2013; HECKER *et al.*, 2009; MITTAL *et al.*, 2007; SEDEEK *et al.*, 2010; SZÖCS *et al.*, 2002).



On the other hand, microRNAs, particularly MIR 21a-3, MIR-25, MIR 23b, and MIR-99a-5p have been implicated in NOX4 downregulation (GORDILLO *et al.*, 2014; SHI *et al.*, 2017; VARGA *et al.*, 2013).

In contrast to the direct correlation between expression and activity proposed for NOX4, a few regulatory proteins that are able to attach NOX4/p22phox complex and regulate its enzymatic activity have been described. The polymerase-d-interacting protein 2 (Poldip2), previously described as a cell division regulator, the protein disulfide isomerase (PDI), a chaperone of the thioredoxin family and TKS5, a substrate of the protein tyrosine kinase Src, have been shown to bind and modestly increase NOX4 activity in vascular smooth muscle cells (VSMCs) (DIAZ *et al.*, 2009; JANISZEWSKI *et al.*, 2005; LYLE *et al.*, 2009). On the other hand, the chaperone Hsp70 negatively regulates NOX4/p22phox complex in the same cell model (GIL LORENZO *et al.*, 2014). The biological significance of this regulation, the cellular context in which these proteins could be recruited or whether this type of regulation could be extended to other cell types, is fairly unknown.

NOX4 physiological roles are not well defined. Indeed, whole-body NOX4 knockout mouse models are scarce in the literature and do not show marked phenotype changes or life span alterations (REZENDE *et al.*, 2017; SIROKMÁNY; DONKÓ; GEISZT, 2016). However, it was extensively demonstrated that NOX4 deregulation is implicated in the development of many diseases including, pulmonary fibrosis, diabetic nephropathy, heart disease, neurodegenerative disorders and cancer (CARNESECCHI *et al.*, 2011; GORIN; BLOCK, 2013; KLEINSCHNITZ *et al.*, 2010; VENDROV *et al.*, 2015; WEYEMI, URBAIN *et al.*, 2013).

Abundant literature suggest a close relationship between NOX4 and tumorigenesis. NOX4-derived ROS regulate many aspects of cell behavior that are usually deregulated in cancer, as cell proliferation, survival, metastasis and angiogenesis by modulating central signaling pathways including MAPK, Smad, PI3K and Fas (GUO; CHEN, 2015; WEYEMI *et al.*, 2013). Additionally, this NADPH oxidase was found to be overexpressed in a variety of human cancers including melanoma, bladder, ovarian, prostate, colorectal and papillary thyroid cancer (LIN *et al.*, 2017; MEITZLER *et al.*, 2017; WEYEMI *et al.*, 2010).

Many evidences support that NOX4 behave as an oncoprotein. NOX4 overexpression is associated with cell transformation and apoptosis resistance in epithelial breast cells (GRAHAM *et al.*, 2010). In colorectal carcinoma cell lines, NOX4

knockdown by interfering RNA reduced cell proliferation, migration and invasion (LIN *et al.*, 2017). CRISPR-Cas9 mediated NOX4 knockout also resulted in cell proliferation and invasion inhibition in Hela cells (JAFARI *et al.*, 2017). Overexpression of NOX4 in non-small cell lung cancer (NSCLC) cell lines is associated with increased tumor size, lung metastasis and lower survival rate in nude mice (ZHANG *et al.*, 2014a). Consistently, NOX4 downregulation in melanoma cells resulted in lower tumorigenesis in nude mice (YAMAURA *et al.*, 2009). Additionally, NOX4 was identified as a critical mediator in oncogenic H-Ras-induced DNA damage and senescence, and is also involved in metabolic reprogramming of cancer cells, since it increases NAD<sup>+</sup> generation, supporting active glycolysis and pentose phosphate pathway (PPP) pathway (JU *et al.*, 2017; WEYEMI, U *et al.*, 2012; ZENG *et al.*, 2016).

### 1.8. NADPH Oxidase 4 and Thyroid Tissue

Thyroid gland expresses DUOX1 and DUOX2 that are responsible for supplying hydrogen peroxide for hormone biosynthesis at the apical membrane (DE DEKEN *et al.*, 2000; DUPUY *et al.*, 1999). In 2010, Dr. Dupuy group showed that NOX4 and its partner p22<sup>phox</sup> are expressed in human thyrocytes with an intracytoplasmic staining pattern, that suggests a potential role in intracellular redox signaling (WEYEMI, U. *et al.*, 2010). In rat thyrocytes, NOX4 expression is found in the intracytoplasmic compartment, but also in the plasma membrane and is differentially expressed between genders, showing higher mRNA levels in adult female rat, which was correlated with increased ROS production (FORTUNATO *et al.*, 2013).

NOX4 is overexpressed in papillary thyroid cancer, when compared to normal tissue, suggesting a possible role in thyroid carcinogenesis (WEYEMI, U. *et al.*, 2010). Interestingly, it was demonstrated that the expression of the oncogene H-RasV12 in human non-tumoral thyroid cell line (HThy-ori3.1) results in increased ROS generation, induction of NOX4 expression and its peri-nuclear localization. NOX4 abrogation inhibited H-RasV12-induced DNA-damage and subsequent senescence, suggesting that NOX4 is a critical mediator of Ras oncogene responses in thyroid cells (WEYEMI, U *et al.*, 2012).

In a recent approach, we used the database from The Cancer Genome Atlas (TCGA) to analyze homogeneous cohort of 390 PTCs (170 BRAFWT PTCs and 220

BRAF<sup>V600E</sup> PTCs). Comparing wild type and BRAF<sup>V600E</sup>-mutated tumors, we found a higher expression of NOX4 mRNA in mutated PTC. Furthermore, BRAF<sup>V600E</sup>-positive PTCs revealed an inverse correlation between NOX4 and NIS expression. In collaboration with Dr. James Fagin team, we also detected an inverse correlation between NOX4 expression and thyroid differentiation score in genetically engineered mouse models of thyroid cancer (AZOUZI *et al.*, 2017). However, this type of study cannot provide a direct implication of NOX4 in NIS regulation or thyroid differentiation in PTCs harboring BRAFV600E.

### 1.9. Rational

It is well-known that BRAF<sup>V600E</sup> induces thyroid cell dedifferentiation and reduces NIS expression, but the mechanisms involved in these changes are not fully understood (AGRAWAL *et al.*, 2014). NIS downregulation by BRAF<sup>V600E</sup> may involve multiple pathways that includes TGF- $\beta$  secretion, Smad pathway activation and epigenetic gene regulation (CHOI, YONG WON *et al.*, 2014; RIESCO-EIZAGUIRRE, G. *et al.*, 2009; ZHANG, ZONGJING *et al.*, 2014).

ROS is an important mediator of TGF- $\beta$  responses and have recently been implicated in epigenetic gene regulation in cancer cells by modulating the recruitment of silencing complexes containing DNA methyltransferases and histone deacetylases for certain gene promoters (O'HAGAN *et al.*, 2011). Indeed, some studies suggest that the cellular redox status is determinant for NIS expression and function (LEONI *et al.*, 2015; PUPPIN *et al.*, 2005; SERRANO-NASCIMENTO *et al.*, 2014).

NOX4, a ROS generator source and a key element of TGF- $\beta$  signaling in normal and cancer cells, is overexpressed in PTCs, especially in BRAF<sup>V600E</sup>-positive tumors, where it shows inverse correlation with thyroid differentiation *status* and NIS expression (AZOUZI *et al.*, 2017). However, NOX4 involvement in NIS regulation has never been investigated in thyrocytes.

In an attempt to better understand the mechanisms involved in radioiodine (RAI)-refractory thyroid cancer development, this study involves two main aims: the first one is to address if NOX4 could be a signaling mediator of BRAF<sup>V600E</sup>/TGF- $\beta$ -induction of NIS repression in thyroid cancer; the second relies on whether epigenetic

mechanisms, including NIS promoter methylation or histone deacetylation are involved in this pathway.

In this study, we used a rat thyroid cell line (PCCL3) with inducible expression of BRAF<sup>V600E</sup> (PC-BRAF cells) as experimental model, normal human thyrocytes in primary culture and two human thyrocyte cancer cell lines harboring BRAF<sup>V600E</sup>, BCPAP and 8505c, derived from papillary and anaplastic carcinomas, respectively.

## 2. OBJECTIVES

### 2.1. General Objective

Investigate whether NOX4 is a signaling mediator of BRAF<sup>V600E</sup>/TGF- $\beta$ -induced NIS repression in thyroid cancer.

### 2.2. Specific Objectives

- Evaluate the effect of TGF $\beta$  on ROS generation, NOX4 and NIS expression and iodide uptake activity in the rat PC-BRAF cells, and in human cancer cell lines harboring BRAF<sup>V600E</sup> mutation;
- Determine the impact of BRAF<sup>V600E</sup> induction on iodide uptake, NIS expression, NOX4 expression and the Smad pathway in PC-BRAF cells;
- Investigate the effect of BRAF<sup>V600E</sup> inhibition on Smad pathway, NOX4 and NIS expression in BCPAP and 8505c cells;
- Define whether NIS expression and iodide uptake are modulated by NOX4, either by NOX4 silencing through siRNA or NOX4 overexpression, in PC-BRAF and human thyroid cancer cell lines harboring BRAF<sup>V600E</sup>;
- Evaluate the role of TGF $\beta$  and NOX4 in the regulation of DNMT1 expression and its recruitment to chromatin;
- Investigate whether TGF $\beta$  and NOX4 play a role in the NIS specific promoter methylation and histone acetylation;
- Validate the main results in primary cultures of normal human thyrocytes;

### 3. MATERIAL AND METHODS

#### 3.1. Reagents

Inhibitor of Smad3 phosphorylation (SIS3 – Sigma-Aldrich), inhibitor of BRAF<sup>V600E</sup> (PLX4032 – Selleckchem); recombinant human TGF  $\beta$ 1 (Peprotech), N-Acetyl-cysteine (Sigma-Aldrich), Diphenyleneiodonium (Sigma-Aldrich) and Doxycycline (Sigma-Aldrich) were used in this study.

#### 3.2. Cell Culture

Three different thyroid-derived cell lines were applied in this study: BCPAP, 8505c and PC-BRAF cells. BCPAP cells were originally obtained from papillary thyroid tumor with local and lymph node metastasis from a 76-year-old woman. These cells harbors BRAF<sup>V600E</sup> and p53 (Asp259Tyr) mutations (FABIEN *et al.*, 1994; SAISELET *et al.*, 2012). 8505 are derived from primary undifferentiated or anaplastic thyroid carcinoma resected from a 78-year-old-female (ITO *et al.*, 1993). These cells also harbors BRAF<sup>V600E</sup> and p53 (Arg248Gln) mutations (SAISELET *et al.*, 2012). BCPAP cells were cultured in DMEM medium (1 g/l glucose) and 8505c cells in RPMI (2g/l glucose, Life Technologies), both supplemented with 10% (vol/vol) of Fetal Calf Serum (FCS) (Life Technologies) and penicillin/streptomycin (100 mg/ml; Life Technologies).

PC-BRAF cells were kindly provided by Dr. James A. Fagin (Memorial Sloan-Kettering, New York, USA). PC-BRAF cells were derived from genetic alteration of the rat thyroid cell line PCCL3 to conditionally express BRAF<sup>V600E</sup> in response to doxycycline induction (MITSUTAKE *et al.*, 2005). PC-BRAF cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% Tetracyclin-free FCS (Life Technologies), TSH (1 mU/ml; Sigma-Aldrich), Insulin (100 ug/mL; Sigma-Aldrich), Apo-transferrin (500 ug/mL; Sigma-Aldrich), Hydrocortisone (100 nM; Sigma-Aldrich), penicillin/streptomycin (100 mg/ml; Life Technologies) and the selection antibiotics G418 (300 ug/mL) and Hygromycin (114 ug/mL). BRAF<sup>V600E</sup> induction is obtained by incubating cells in the presence of doxycycline (Sigma-Aldrich; 1ug/ml) for the indicated periods of time (MITSUTAKE *et al.*, 2005).

All cell lines were cultivated at 37°C in a humidified air atmosphere containing 5% CO<sub>2</sub>.

### 3.3. Primary human thyroid cell culture

Primary human cells were cultured, as previously described (DUTHOIT *et al.*, 2001; WEYEMI, U. *et al.*, 2010). Briefly, the thyroid tissue (2-3 g) dissected free from connective tissue was collected in Ham's F-12 medium (Gibco-Thermo Fischer) containing penicillin/streptomycin (100 mg/ml; Gibco-Thermo Fisher). The tissue was washed twice with PBS and minced into small fragments in 2 mL of sterile digestion buffer containing glucose (2 mg/mL), collagenase I (100 U/ml) and the neutral protease dispase II (2.4 U/ml) (Sigma–Aldrich), diluted in calcium- and magnesium-free PBS, pH 7.4 (Gibco-Thermo Fischer). Tissue fragments were suspended in 10 mL of digestion buffer per gram of weight and the suspension was incubated at 37°C for 45 minutes, under magnetic agitation. The digested tissue was filtered through sterile gauze swabs. PBS was added until 50 mL of final volume and the homogenate was centrifuged at 1500 rpm for 5 min. The supernatant was aspirated and the pellet was washed with PBS and centrifuged again in the same conditions. This washing step was repeated at least twice or until the supernatant was limpid. Finally, the pellet was suspended in Ham's F-12 culture medium supplemented with 5 % (v/v) FCS (Gibco-Thermo Fischer), 2 mM L-glutamine (Gibco-Thermo Fischer), 1 % nonessential amino acids (Gibco-Thermo Fischer), and six nutritional factors (6H medium): 1 U/l bovine TSH (Sigma–Aldrich); 10 mg/l human insulin (Roche Applied Science); 10 mg/l somatostatin (Sigma–Aldrich); 6 mg/l human transferrin (Sigma–Aldrich) 10<sup>-8</sup>M hydrocortisone (Sigma–Aldrich); and 10 mg/l glycyl-histidyl- lysine acetate (Sigma–Aldrich). The cell suspension was filtered in Falcon® 70µm nylon filter and plated into 75cm<sup>2</sup> culture flasks. Cells were cultivated at 37°C in a humidified air atmosphere containing 5% CO<sub>2</sub>. The primary cultures of thyroid cells formed a confluent monolayer within 7 days. Normal human thyroid tissue specimens were collected at Institut Gustave Roussy (Villejuif, France), in accordance with local and national ethical laws. Informed consent was obtained from all patients.

### 3.4. Transfection of small interfering RNAs

Cells were transfected at 50-60% confluence with specific siRNAs against rat or human NOX4 or a small interfering (si) Scramble Control (Table 1) using INTERFERIN transfection reagent (Polyplus). For each well of a six-well plate, 10 nM of siRNA duplexes was added into 200 µl of Opti-MEM® (Gibco-Thermo Fisher), followed by the addition 7 µl of transfection reagent. This mix was incubated for 10 minutes at room temperature to allow transfection complexes to form. After the incubation, the transfection mix was added drop-wise into culture wells containing 2 mL of fresh growth medium. Experiments were performed 48 or 72 h post-transfection.

**Table 1: Sequences of small interfering RNAs**

Specie	Sequence
Human siCtrl	Stealth RNAi siRNA Negative Control (Life Technologies)
Human siNOX4	Stealth RNAi duplex (Life Technologies)
	NOX4-1: 5'-GGAGAACCAGGAGAUUGUUGGAUAA-3'
	5'-UUAUCCAACAAUCUCCUGGUUCUCC-3'
	NOX4-2: 5'-AGAGUGAAGACUUUGUUGAACUGAA-3'
	5'-UUCAGUUCAACAAAGUCUUCACUGU-3'
	NOX4-3: 5'-CCUCAUGAUCACAGCCUCUACAUAU-3'
	5'-AUAUGUAGAGGCUGUGAUCAUGAGG-3'
Rat siCtrl	ON-TARGET plus non-targeting pool (Dharmacon)
Rat siNOX4	ON-TARGET plus SMART pool (Dharmacon)
	1: 5'-ACAGAAAGAUUCCGAGAUU-3'
	2: 5'-CAUAUCACCUGUGGCAUAA-3'
	3: 5'-GUGUGUGGCUGGAGGCAUU-3'
	4: 5'-CCGUUUGCAUCGAUACUAA-3'

### 3.5. Plasmid transfection

BCPAP cells or human thyrocytes from primary culture reaching 50–70% confluence were transfected with 1 µg of plasmid DNA in 6-well plates using X-tremGENE HP DNA transfection reagent (Roche), following the manufacturer's protocol. The ratio DNA/transfection reagent applied in this study was 1:3. pcDNA3.0-Nox4, and pcDNA3.0-V5-Nox4*wt* were kindly provided by Dr. Ulla G. Knaus (School of



Medicine and Medical Science, University College Dublin, Ireland) (VON LOHNEYSEN *et al.*, 2012). Experiments were performed 24 h post-transfection.

### **3.6. Measurement of intracellular ROS.**

Intracellular levels of ROS were assessed using the non-fluorescent lipophilic ester 2',7'-dichlorodihydrofluorescein diacetate (H2-DCF-DA). After crossing the plasma membrane, this compound is de-esterified to the hydrophilic alcohol H2DCF (dihydrodichlorofluorescein) that may be oxidized to the highly fluorescent molecule DCF (2',7'-dichlorofluorescein) by cellular ROS. In the appropriate time-points after treatment, cells were collected by trypsinization, washed with PBS and suspended in culture medium containing 5µM of H2-DCF-DA. Cells remained for 20 minutes at 37°C in the incubator and were then immediately transferred to ice in order to stop the reaction and minimize sample-to-sample variation. The mean fluorescence of at least 10,000 events was detected using BD Accuri™ C6 (BD Biosciences), and data were expressed as arbitrary units of fluorescence.

### **3.7. Extracellular ROS generation.**

Extracellular ROS production was measured using AmplexRed/HRP, a method that detects the accumulation of oxidized products in the reactional medium. Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) is oxidized by horseradish peroxidase (HRP), in the presence of H<sub>2</sub>O<sub>2</sub> that is produced by cells, and thus become a fluorescent molecule called resorufin (Excitation: 571nm/Emission: 585nm). For this experiment, cells were plated in multiple 6-well plates at a density of 250000 per well. In the appropriate time-point after treatment, cells were collected by trypsinization, washed with PBS and counted. Next, 10<sup>5</sup> cells were mixed with a reaction mix containing D-glucose (1 mg/mL), horseradish peroxidase (HRP) (0.5 U/mL), Amplex Red (50 µM) and PBS with calcium and magnesium in opaque 96-well plates. The accumulation of resorufin was monitored for approximately 1 hour at 37°C using a fluorescence plate-reader (Victor X4; PerkinElmer, Norwalk, CT). The slope of the curves was calculated and compared to a H<sub>2</sub>O<sub>2</sub> calibration curve. Data were

expressed as nM of H<sub>2</sub>O<sub>2</sub> produced by minute per 10<sup>5</sup> cells (nM H<sub>2</sub>O<sub>2</sub>.min<sup>-1</sup> .10<sup>5</sup> cells).

### **3.8. Whole-cell protein extraction and tight chromatin fractionation.**

To obtain whole-cell protein extract, cells were washed with cold PBS and scraped in RIPA lysis buffer (Sigma-Aldrich) supplemented with a mixture of phosphatase and protease inhibitors (Roche Applied Science). Cell lysate was sonicated for 10 seconds at an amplitude of 30% in ice bath, and then clarified by centrifugation at 10,000 rpm for 10 min at 4 °C.

To obtain a cell fraction enriched in proteins tightly associate with the chromatin, we applied tight chromatin fractioning protocol (O'HAGAN *et al.*, 2011). Briefly, 2x10<sup>6</sup> cells were washed with PBS, trypsinized and centrifuged. Supernatant was discarded and cell pellet was suspended in 100 uL of buffer A (HEPES pH 7.9 10 mM, MgCl<sub>2</sub> 1.5 mM, KCl 10 mM, Sucrose 0.117 g/mL, Glycerol 10 %, DTT 1mM, protease and phosphatase cocktail inhibitor) in an Eppendorf tube. Triton X-100 2.1% was added to the 100 uL of cell suspension and buffer A to reach a final concentration of 0.1%. The tube was vortexed and incubated for 5 min in ice and then centrifuged for 4 min at 1300 x g, 4°C. The supernatant enriched in cytoplasm soluble proteins was discarded and the pellet was washed with 300 uL of solution A and re-centrifuged. To allow nucleus lysis, the pellet was suspended in 100 uL of solution B (EDTA 3 mM, EGTA 0.2 mM, DTT 1 mM, protease and phosphatase cocktail inhibitor) vortexed and incubated 10 min in ice. The tube was centrifuged for 4 min at 1,700 x g, 4°C, the supernatant discarded, the pellet washed in 300 uL of solution B. Once again, the tube was centrifuged for 1 min at 10,000 g, 4°C, and the supernatant discarded. The pellet was washed with solution C (Tris 50 mM pH 8 + NaCl 0.45 M + IGEPAL 0.05 %, protease and phosphatase cocktail inhibitor) to solubilize proteins weakly bound to chromatin. The tube was re-centrifuged for 1 min at 10,000 g, 4°C, the supernatant discarded and the pellet suspended in RIPA lysis buffer supplemented with phosphatase and protease inhibitors. As described for whole-cell protein extract, cell lysate tightly bound to chromatin was sonicated and then clarified by centrifugation. The amount of protein present in cell lysates was measured by Pierce BCA Protein Assay Kit (Thermo), according to the manufacturer's instructions

### 3.9. Western blotting

Protein samples (20 ug) were mixed with Laemmli sample buffer supplemented with 100 mM DTT and denatured by heating (70°C for 10 min). Samples were subjected to SDS-PAGE electrophoresis at 150 V in Tris-Glycin-SDS buffer (Invitrogen) using NUPAGE Western blotting system from Invitrogen. Proteins were electro transferred to 0.2-mm nitrocellulose sheets (Amersham) for 90 min at 30 V in Tris-Glicine buffer containing 20% of isopropanol, using the same Western blotting system. To avoid unspecific antibody hybridization, membranes were incubated with 5 % non-fat milk or 5 % bovine serum albumin TBS-T solution for 1h at room temperature. Membranes were probed with the indicated primary antibodies (table 2) overnight, at 4°C, under constant agitation. Membranes were washed 3 times of 10 min with TBS-T and incubated with goat anti-rabbit IgG-HRP antibody (Southern Biotech) or goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology Inc) for 45 min at room temperature. Membranes were washed again 3 times of 10 min with TBS-T and the proteins were visualized by enhanced chemiluminescence (Amersham)

**Table 2: Primary and Secondary Antibodies used for Immunoblotting analysis**

Target	Primary Antibody			Secondary Antibody	
	Reference		Dilution	Specie	Dilution
NOX4	Abcam	ab133303	3000x milk 1,5%	rabbit HRP	10000x milk 1,5%
DNMT-1	Abcam	ab13537	1000x milk 1,5%	Mouse HRP	10000x milk 1,5%
pSmad3	Abcam	ab51451	3000x BSA 1,5%	rabbit HRP	10000x BSA 1,5%
Smad2/Smad3	Abcam	ab40854	2000x BSA 1,5%	rabbit HRP	10000x BSA 1,5%
Histone H3	Abcam	ab1791	20000x milk 1,5%	rabbit HRP	10000x milk 1,5%
H3 acetyl K9	Abcam	ab12179	2000x BSA 1,5%	mouse HRP	10000x BSA 1,5%
HDAC1	Thermo	PA1-860	20000x milk 1,5%	rabbit HRP	20000x milk 1,5%
V5	Thermo	R960-25	10000x milk 1,5%	mouse HRP	10000x milk 1,5%
Lamin AC	Sta.Cruz	A2066	1000x milk 1,5%	mouse HRP	20000x milk 1,5%
Vinculin	Abcam	ab51451	10000x milk 1,5%	mouse HRP	20000x milk 1,5%

### 3.10. Real-Time qPCR

Total RNA was extracted from cells using Nucleospin RNA plus (Macherey-Naegel) extraction kit, according to the manufacturer's instructions. The quality of RNA preparation, based on the 28S/18S rRNAs ratio, was assessed using the RNA 6000 Nano Lab-On-chip (Agilent Technologies). Total RNA (2 µg) was reverse-transcribed using Maxima Reverse Transcriptase (Thermo Fisher Scientific) and random hexamers primers in a total reaction volume of 20 µL of PCR buffer, according to the manufacturer's instructions. Reverse transcription reaction was performed in a thermocycler (Applied Biosystems) for 120 min at 50°C.

Human NOX4 and NIS mRNA qPCR were performed with TaqMan methodology using Universal Master Mix (Applied Biosystems). Reaction was performed in a total volume of 15 uL containing 9 uL of diluted cDNA sample containing around 125ng DNA, 350 nM of reverse and forward specific primers, and TaqMan specific probes. For targets of rat samples and human DNMT1, real-time qPCR was performed with SyBr Green methodology using FastStart Universal SYBR Green Master Mix (Roche). Reaction was performed in a total volume of 25 uL containing 2.5 uL of diluted cDNA samples, 300 nM of reverse and forward specific primers. All primers and Taqman probes oligonucleotide sequences for target genes or their respective controls are listed in table 3. Real-time PCR reactions were performed in Applied Biosystems 7300 machine using the following thermal cycle profile: 50°C for 2 min, 95°C for 10 min, 42 cycles of 95°C for 10 seconds, followed by 60°C for 1 min. mRNA relative expression of was determined by  $\Delta\Delta C_t$  methodology (LIVAK; SCHMITTGEN, 2001).

**Table 3: Sequences of primer pairs and TaqMan probes used for real-time PCR**

<b>Human <i>NOX4</i></b>	forward: 5'-TGTCTTCTACATGCTGCTGACGTT-3' reverse: 5'-TGAGAGCTGGTTCGGTTAAGACT-3' Probe: FAM-CAAACCTAATTTAGATACCCACCCTCCCGGC-TAMRA
<b>Human <i>NIS</i></b>	forward: 5'-CCATCCTGGATGACAACTTGG-3' reverse: 5'-AAAAACAGACGATCCTCATTG-3' Probe: FAM-AGAACTCCCCACTGGAAACAAGAAGCCC-TAMRA
<b>Human <i>PPIA</i></b>	forward: 5'- GTCAACCCCAACCGTGTTT -3' reverse: 5'-CTGCTGTCTTTGGGACCTTGT -3' Probe: FAM- AGCTCAAAGGAGACGCGGCCCA-TAMRA
<b>Human <i>DNMT1</i></b>	forward: 5'- ACCAAGCAGCGATCTCTGAC -3' reverse: 5'- GCAGGATGTTGCCCTCT -3'
<b>Human <i>GAPDH</i></b>	Human Positive Control Primer Set GAPDH-1 (Active Motif, 71004)

<b>Rat <i>DUOX1</i></b>	forward: 5'-GATACCCAAAGCTGTACCTCG-3' reverse: 5'-GTCCTTGTCACCCAGATGAAG-3'
<b>Rat <i>DUOX2</i></b>	forward: 5'-TGCTCTCAACCCCAAGTG-3' reverse: 5'-TCTCAAACCAGTAGCGATCAC-3'
<b>Rat <i>NIS</i></b>	forward: 5'-GCTCATCCTGAACCAAGTGA-3' reverse: 5'-ACGAGCATTACCACAACCTG-3'
<b>Rat <i>NOX4</i></b>	forward: 5'-TCCATCAAGCCAAGATTCTGAG-3' reverse: 5'-GGTTTCCAGTCATCCAGTAGAG-3'
<b>Rat <i>GAPDH</i></b>	forward: 5'-TGATTCTACCCACGGCAAGT-3' reverse: 5'-AGCATCACCCCATTTGATGT-3'

### 3.11. Chromatin Immunoprecipitation (ChIP)

BCPAP cells were plated at a density of  $5 \times 10^6$  cells in 175 cm<sup>2</sup> flasks. Cells were treated with TGF $\beta$  (1ng/mL) in the presence or absence of DPI (2 $\mu$ M) for 24h. Cells were trypsinized, washed and counted. For each condition, around  $10 \times 10^6$  cells were cross- linked for 10 minutes in 0.37 % formaldehyde solution and then the cross-link reaction was stopped by the addition of Glycine (125 mM). After washing cells twice with PBS, the nuclei of cells were isolated using hypotonic lysis buffer. Nuclei were then sonicated in Covaris for 10 min at 4°C and DNA was broken down to fragments ranging from 250 to 1,000 bp, but averaging 400 bp. Next, 30  $\mu$ g of shredded

DNA was precipitated using H3K9/K14Ac antibody or IgG isotype control and magnetic beads overnight (Magna ChIPTM Protein A+G Magnetic Beads).

Immunoprecipitated DNA fragments were collected with a magnetic rack and washed five times with Low and High Salt Washing Buffer, LiCl Washing Buffer and 2 times with TE Buffer. Then, cells underwent crosslink reversal to detach proteins by treatment with RNase, Proteinase K and heated at 65°C overnight. DNA was finally purified using the Active Motif's ChIP DNA Purification Kit and used in qPCR. Real-time PCR was performed as previously described, using SyBr green methodology. The sequence of primer pairs designed for different regions of NIS promoter were obtained from Zhang and collaborators (2014) and are indicated in table 4.

**Table 4: Sequences of primer pairs for different regions of human NIS promoter for ChIP-qPCR analysis**

Region/Location	Sequence
<b>P1 (-692/-370)</b>	forward: 5`-GAGTGCTGAAGCAGGCTGTGC-3` reverse: 5`-GGGAGCAGCTCGTGATTGTGG-3`
<b>P2 (-1147/-762)</b>	forward: 5`- CTGGCACAGGGCCAACTCTCA -3` reverse: 5`- TCAGGGTTTCAGGGGACCCATA -3`
<b>P3 (-1511/-1216)</b>	forward: 5`- CTGACGCTGTTTCTTTCACCC -3` reverse: 5`- GACCACCAGGGAGGTAGAGTC -3`
<b>NUE (-9525/-9287)</b>	forward: 5`- GAGCCCTCAGGCAGTTGCT -3` reverse: 5`- ACTCACGTGGAAGTCTTGA-3`

### 3.12. DNA methylation analysis

To evaluate specific DNA methylation in *NIS* promoter region the bisulfite conversion method was applied. This technique consists in treating DNA with bisulfite, which converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged by the treatment. The methylation profile of a target DNA sequence or CpG island can be determined by DNA sequencing or PCR amplification using specific primer sequences against the parental DNA sequence that corresponds to the

methyated form of this target or the bisulfite-modified sequence (cytosine to uracil exchange) that represents an unmethyated status.

BCPAP cells were treated with TGF $\beta$  (5 ng/mL) and after 24h cells were trypsinized and counted. From 20,000 cells, DNA extraction and bisulfite conversion were performed using EZ DNA Methylation-Direct Kit (Zymo Research). Next, 50 ng of bisulfite-converted DNA was submitted to PCR reaction using specific primers against unmethyated (U) or methyated (M) CpG islands of NIS proximal promoter. Primer sequences listed in table 5 where obtained from Choi et al (2014). This PCR reaction was performed using high fidelity DNA polymerase (Thermo) in a total volume of 30  $\mu$ L, containing 500 nM of reverse and forward specific primers, 200  $\mu$ M of dNTP and 1.5 mM of MgCl<sub>2</sub>. The reaction was performed using the following thermal cycle profile: 95°C for 1 min for polymerase activation, 37 cycles of 95°C for 30 seconds for denaturation, 53°C for 30 seconds for annealing and 72°C for 1 minute for extension. Final extension was performed at 72°C for 10 minutes. PCR products migrated in 1.7 % agarose gel for 45-50 min.

**Table 5: Sequences of primer pairs for NIS promoter methylation analysis by bisulfite conversion**

<b>Unmethyated NIS (U)</b>	forward: 5'-GTTGTTTTTGTAAGTTTTAGGTGA -3' reverse: 5'-TATCCCCACTATCTATCTCTACATC -3'
<b>Methyated NIS (M)</b>	forward: 5'-GAGTTGTTTTCGTAAGTTTTAAAGCC -3' reverse: 5'-TATCCCCGCTATCTATCTCTACGT -3'

### 3.13. Iodide Uptake assay

Iodide uptake assay was performed as previously described (CAZARIN, J.; ANDRADE; CARVALHO, 2014; DE SOUZA *et al.*, 2010). Briefly, 10<sup>5</sup> PC-BRAF cells grown in 24-well dishes, in the different assay conditions, were incubated for 45 min at 37°C with 1 ml Hank's balanced salt solution (HBSS) containing 0.1  $\mu$ Ci carrier-free Na<sup>125</sup>I and 10  $\mu$ M of NaI. To determine nonspecific radioiodine uptake, one well for

each experimental condition was incubated with  $\text{Na}^{125}\text{I}$  in the presence of 100  $\mu\text{M}$  of  $\text{KClO}_4$  solution, a competitive inhibitor of NIS. After the incubation, cells were washed once with ice-cold HBSS and lysed with 0.1 M NaOH, and radioactivity was measured in a gamma-counter (Automatic Gamma Counter 1470, WIZARD). An aliquot of cell lysates was taken to measure protein concentration by the BCA method. Specific radioiodine uptake was obtained by the subtraction between the amount of radioactivity expressed in counts per minute (CPM) in the absence of potassium perchlorate and on its presence, normalized by the protein content. Data was expressed as relative to control.

To investigate PC-BRAF cell response to TSH and  $\text{TGF}\beta$  (Fig 5B), cells were TSH-starved for 4 days in F12 medium containing 0.2% fetal calf serum and aforementioned hormones (Insulin, Apo-transferrin and Hydrocortisone) and antibiotics (penicillin/streptomycin, G418, Hygromycin). Then, medium was changed for F12 complete medium containing TSH and 5% of fetal calf serum that were supplemented or not with human recombinant  $\text{TGF}\beta$  (10ng/mL) Iodide uptake assay was performed 48 and 72h after.

The effect of doxycycline treatment in iodide uptake in PC-BRAF cells was evaluated in the continuous presence of TSH. Cells were trypsinized and plated in F12 complete medium 4H (TSH, Insulin, Apo-transferrin and Hydrocortisone), containing antibiotics (penicillin/streptomycin, G418, Hygromycin) and 5% of fetal calf serum. In the following day, medium was changed and cells were treated with doxycycline. Iodide uptake assay was performed 2, 3, 4 and 7 days after BRAF oncogenic stimulation.

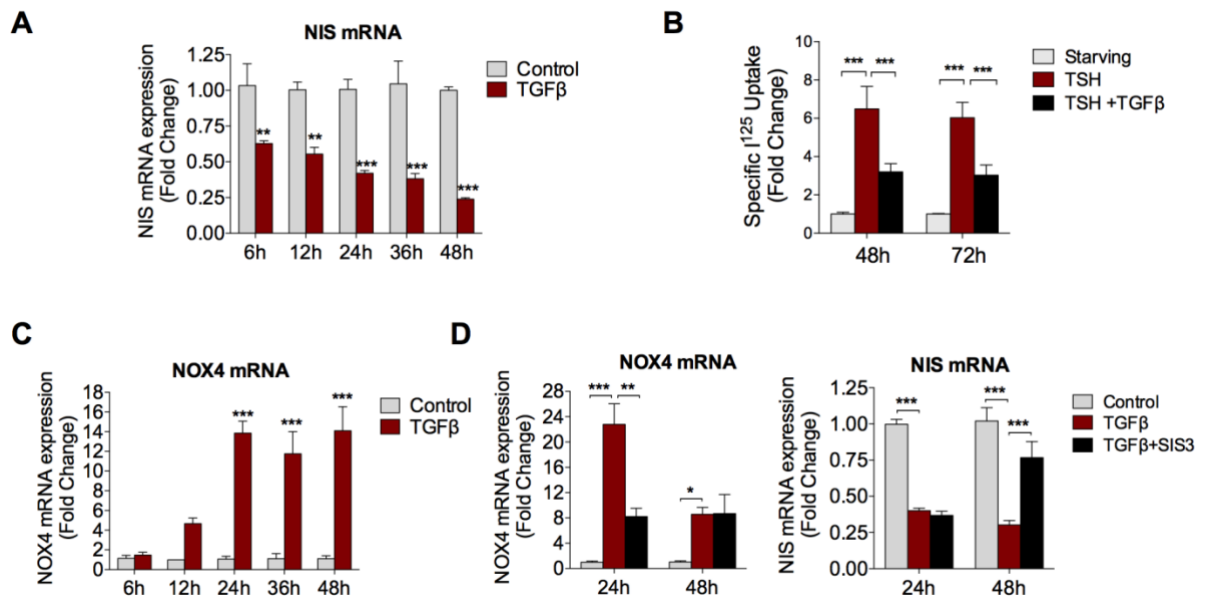
### **3.14. Statistical analyses**

Statistical analyses were performed using Graph-Pad Prism (GraphPad Software, version 6.0c). The statistical significance was evaluated by t test, one-way or two-way analysis of variance (ANOVA), depending on experimental design. Multi-comparison post-tests were eventually performed depending on experimental design and indicated in the figure legends. Error bars are SEM. Statistical significance was represented by the symbol \* as following: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and. A P-value  $< 0.05$  was considered statistically significant.



#### 4. RESULTS

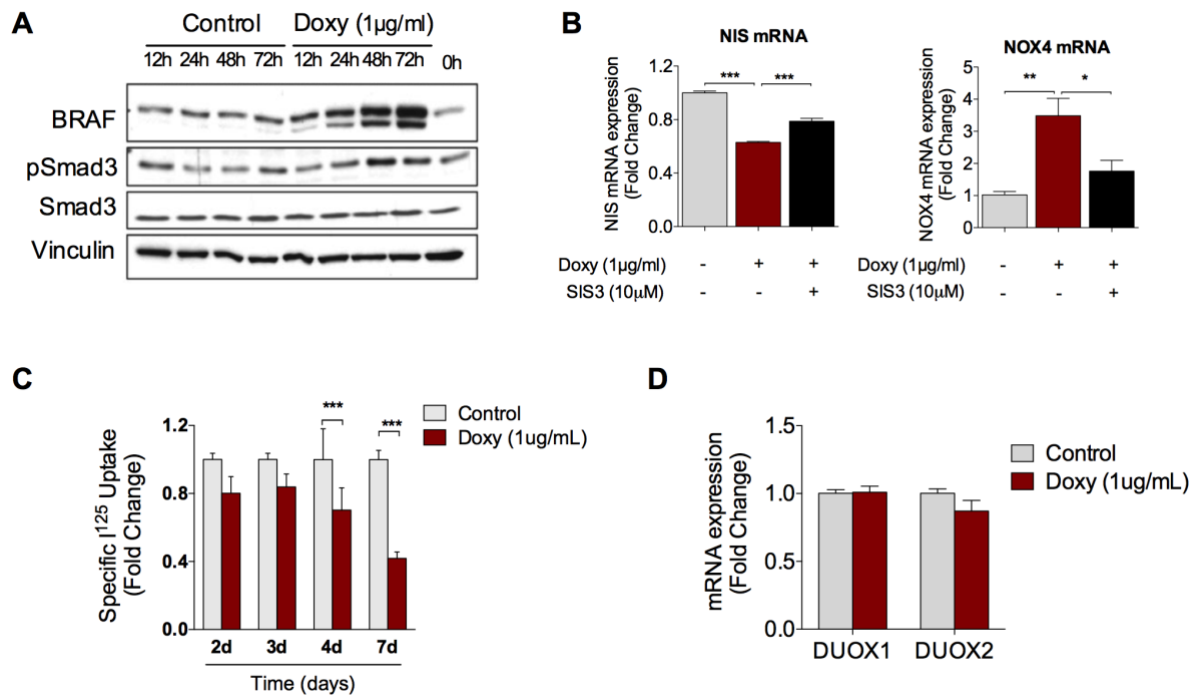
To first characterize a possible relationship between BRAF<sup>V600E</sup>, TGF $\beta$ , NOX4 and NIS regulation, we used a rat thyroid cell line (PC-BRAF) that expresses BRAF<sup>V600E</sup> in a doxycycline-inducible manner. Treatment of PC-BRAF cells (not treated with doxycycline) with TGF $\beta$  (10 ng/ml) induced time-dependent NIS mRNA downregulation (Fig 5A). Iodide uptake increased around 6-fold after TSH stimulation, which was significantly decreased by concomitant incubation with TGF $\beta$  for 48h or 72h, showing a negative role of this cytokine on NIS (Fig 5B). TGF $\beta$ -mediated also increased NOX4 mRNA expression (Fig 5C). Treatment of PC-BRAF with pSmad3 specific inhibitor, SIS3 (10  $\mu$ M), for 48h reversed NIS mRNA repression mediated by TGF $\beta$  (Fig 5D). In parallel, SIS3 repressed NOX4 mRNA upregulation mediated by TGF $\beta$  already at 24h of treatment (Fig 5D). These results show that Smad3 regulates both TGF $\beta$ -mediated NIS inhibition and NOX4 upregulation in PC-BRAF cells.



**Figure 5: TGF- $\beta$ 1 down-regulates NIS expression and activity and upregulates NOX4 expression through Smad pathway in PC-BRAF cells.** (A) Time course expression of NIS mRNAs in PC-BRAF cells in the absence or presence of human recombinant TGF- $\beta$ 1 (10 ng/ml). (B) Specific iodide uptake after 3 days of TSH withdrawn in PC-BRAF cells in the presence or absence of TSH and/or TGF- $\beta$ 1 (10 ng/ml) for 48h and 72. (C) Time course expression of NOX4 mRNA in the absence or presence of human recombinant TGF- $\beta$ 1 (10 ng/ml). (D) NOX4 and NIS mRNA levels in PC-BRAF cells treated for 24h or 48h with TGF- $\beta$ 1 (10 ng/ml) in the absence or presence of the specific inhibitor of pSmad3, SIS3 (10  $\mu$ M). Values are expressed as mean  $\pm$  SE of at least 3 independent experiments. Statistics were performed using one way Anova (B and D) or two way Anova (A and C) followed by Bonferroni post-test. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

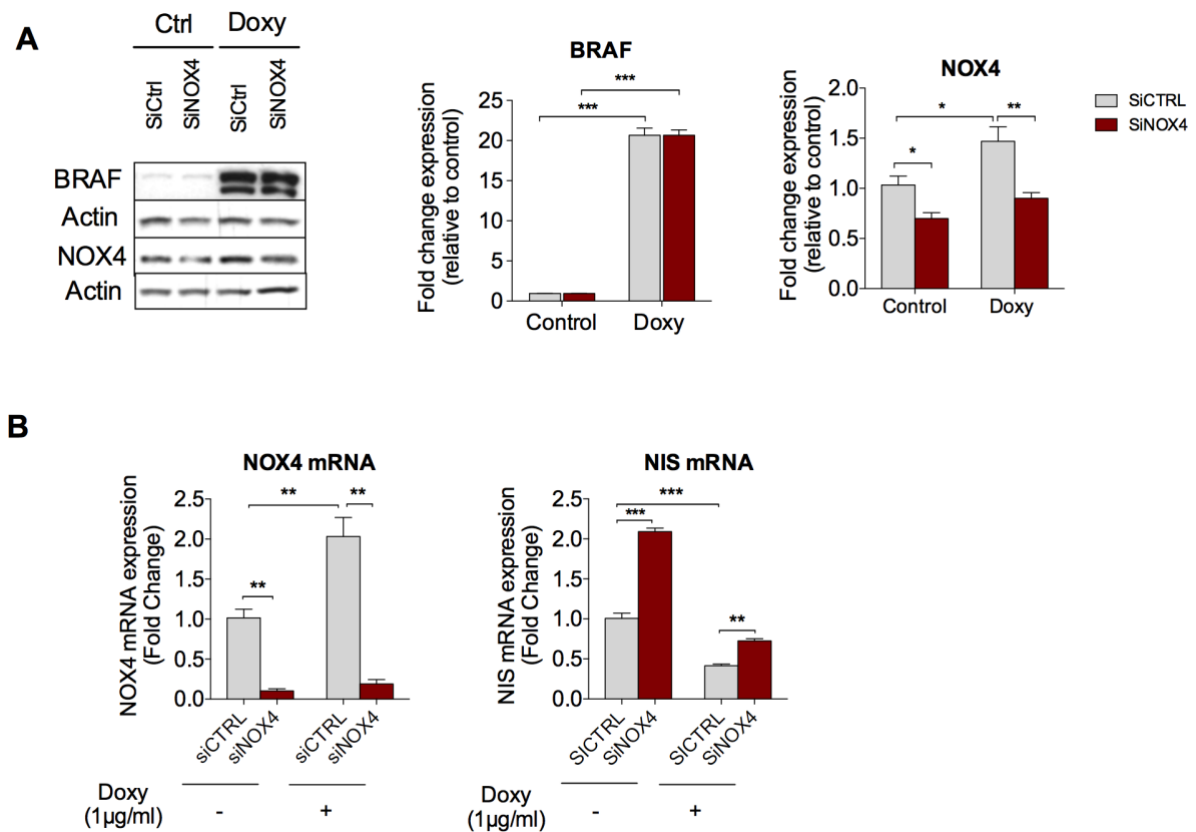
Next, PC-BRAF cells were exposed to doxycycline to induce BRAF<sup>V600E</sup> expression. Doxycycline (1 µg/mL) acutely increased BRAF protein expression already at 12h of treatment. Its effect was improved depending on the extension of the period of treatment to 48 and 72h (Fig 6A). An increase in pSmad3 expression was also detected after 48h of doxycycline treatment indicating a potential stimulating role of BRAF oncogene of Smad signaling (Fig 6A). BRAF<sup>V600E</sup> induction for 48h resulted in downregulation of NIS mRNA and upregulation of NOX4 mRNA (Fig 6B). Both responses were at least partially reversed by concomitant treatment with SIS3, which indicates involvement of Smad3 in BRAF<sup>V600E</sup> regulation of NIS and NOX4 (Fig 6B). In addition to NIS mRNA repression, doxycycline also decreased iodide uptake in PC-BRAF cells (Fig 6C). Although, NIS mRNA was downregulated at 48h of BRAF<sup>V600E</sup> induction (Fig 6B), inhibition of NIS activity occurred only after 4 days, and achieved maximal repression in 7 days of doxycycline (Fig 6C).

Since thyroid cells express two other NOX isoforms, DUOX1 and DUOX2, we evaluated whether doxycycline could also regulate the expression of these NADPH oxidases. Neither DUOX1 nor DUOX2 mRNA were regulated by 48h of BRAF<sup>V600E</sup> induction by doxycycline (Fig 6D).



**Figure 6: BRAF<sup>V600E</sup> induction in PC-BRAF cells activates Smad3 signaling, increases NOX4 expression and down-regulates NIS mRNA and activity.** (A) Time course analysis of BRAF and pSmad3 expressions in inducible PCCL3 cells treated with 1 µg/ml of doxycycline. Vinculin was used as a loading control. (B) NIS and NOX4 mRNA levels in PCCL3 cells treated for 48h with doxycycline (1 µg/ml) and/or SIS3 (10 µM). (C) Specific iodide uptake of PC-BRAF cells in the presence or absence of doxycycline (1 µg/ml) for 2, 4 or 7 days. (D) Analysis of DUOX1 and DUOX 2 mRNA expressions in PC-BRAF cells treated with 1 µg/ml of doxycycline for 48h. Values are expressed as mean ± SE of at least 3 independent experiments. Statistics were performed using one way Anova (B) or two way Anova (C and D) followed by Bonferroni post-test. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

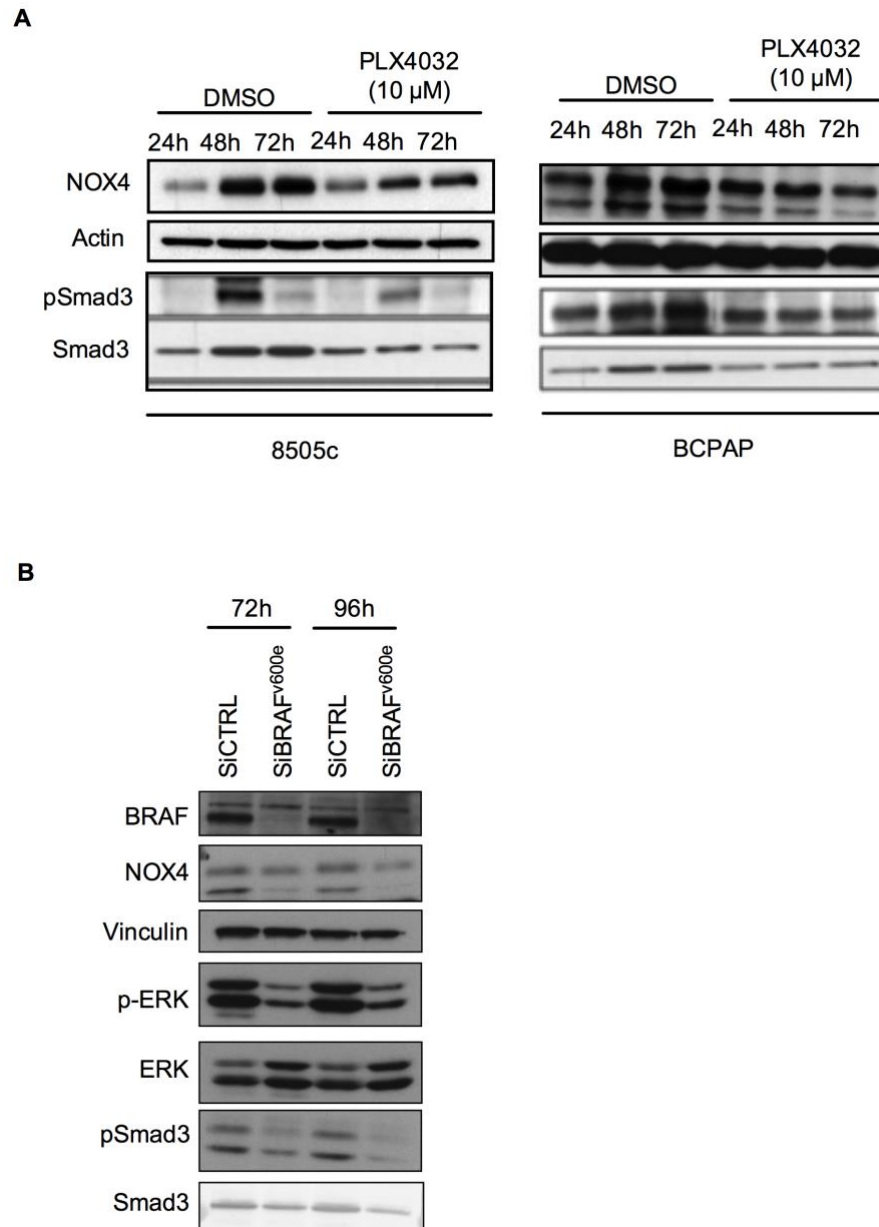
So far, we demonstrated that BRAF<sup>V600E</sup> regulates NIS and NOX4 mRNA expression. To determine the role of NOX4 in BRAF<sup>V600E</sup>-induced repression of NIS we performed interference RNA experiments to silence NOX4 (Fig 7). Treatment of PC-BRAF cells with doxycycline for 48h efficiently induced BRAF expression. Corroborating previous results regarding NOX4 mRNA levels, we also observed increased protein levels of NOX4 mediated by BRAF oncogene activation (Fig 7A). Transfection of siRNA against NOX4 for 48h induced NOX4 mRNA and protein repression in both absence or presence of doxycycline (Fig 7A and B). In basal conditions, without doxycycline, SiNOX4 resulted in increased NIS mRNA levels (Fig 7B). Doxycycline downregulated NIS mRNA, and concomitant transfection of SiNOX4 attenuated NIS repression to mRNA levels compared to doxycycline non-treated PC-BRAF cells (Fig 7B), indicating that NOX4 is significantly involved in BRAF<sup>V600E</sup>-induced NIS transcriptional repression in rat thyrocytes.



**Figure 7: BRAF<sup>V600E</sup>-mediated NIS mRNA downregulation is potentially mediated by NOX4 in PC-BRAF cells.** Analysis of BRAF and NOX4 protein expression with respective densitometric analysis (A) and NOX4 and NIS mRNA levels (B) in PC-BRAF cells treated with 1 µg/ml of doxycycline in the presence or absence of SiNOX4 (10 nM) for 48h. Values are expressed as mean ± SE of 3 independent experiments. Statistics were performed using two way Anova followed by Bonferroni post-test. \*\*P < 0.01; \*\*\*P < 0.001

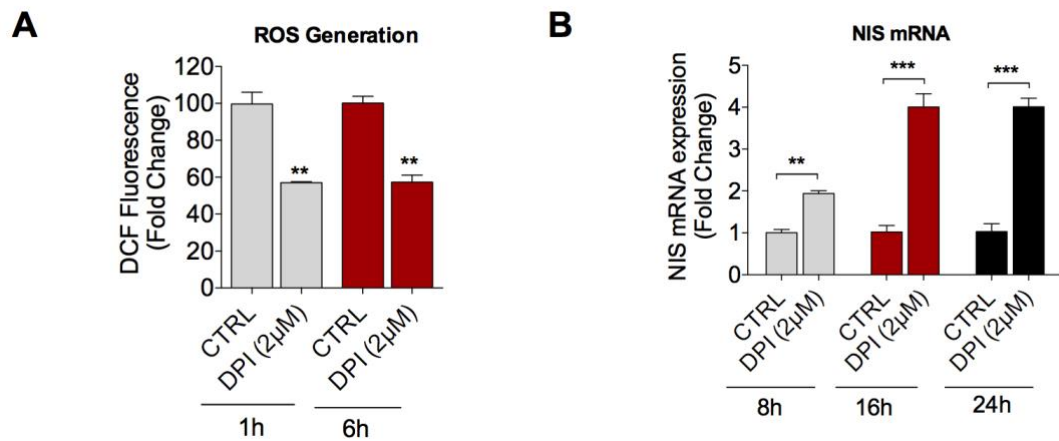
To better characterize the potential role of NOX4 in BRAF<sup>V600E</sup>/TGFβ-induced NIS repression, we extended our analysis to human thyroid cells. We used human thyroid cancer cell lines harboring BRAF<sup>V600E</sup> mutation that were originated from papillary thyroid cancer (BCPAP) or anaplastic carcinoma (8505c) as experimental models. By western blotting analysis, we observed that BRAF<sup>V600E</sup> pharmacological inhibitor PLX4032 (10 µM) treatment for 48h or 72h downregulated pSmad3 and NOX4 protein expression in BCPAP and 8505c cells (Fig 8A). We further confirmed these data using specific RNAi against BRAF<sup>V600E</sup>. Indeed, in 8505c cells, siBRAF<sup>V600E</sup> reduced protein levels of BRAF and its downstream target pERK, which is an indicative that BRAF oncogene was efficiently silenced (Fig 8B). In accordance to results obtained with PLX4032, siBRAF<sup>V600E</sup> also downregulated NOX4 and pSmad3 protein levels (Fig 8B). Corroborating previous results in rat cells, our data show that

BRAF<sup>V600E</sup> also mediates NOX4 and pSmad3 upregulation in human thyroid cancer cell lines.



**Figure 8: BRAF<sup>V600E</sup> upregulates NOX4 protein expression in human thyroid cancer cell lines.** (A) Immunoblot detection of NOX4 and pSmad3 in 8505c and BCPAP cells after pharmacological inhibition of BRAF<sup>V600E</sup> oncogene by the pharmacological inhibitor PLX4032 (10  $\mu$ M) for 24h. (B) Immunoblot detection of NOX4, BRAF, pERK, and pSmad3 in 8505c cells after knocking down BRAF by interference RNA. Immunoblottings are representative of at least two independent experiments. Vinculin and Actin were used as loading control.

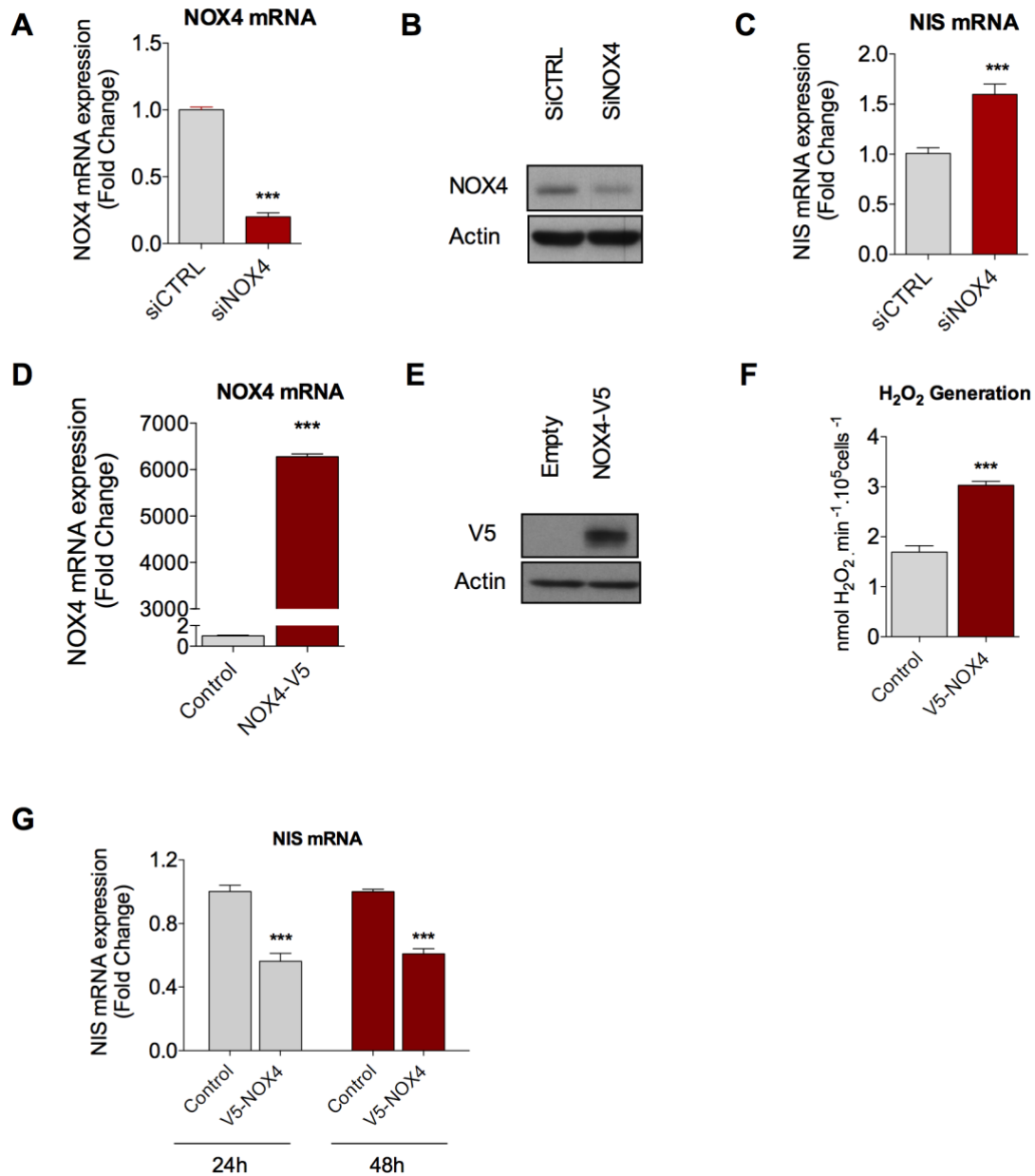
We further attempted to show whether NOX4 would also be able to regulate NIS expression in human thyroid cancer cells. First, we treated BCPAP cells with diphenyleneiodonium (DPI), a NADPH oxidase unspecific enzymatic inhibitor. DPI efficiently reduced intracellular ROS generation in early time points (1h and 6h) (Fig 9A). From 8h of DPI incubation, we observed an increase in NIS mRNA levels (Fig 9B), indicating a possible NADPH oxidase/ROS-mediated NIS regulation in this cell line.



**Figure 9: The antioxidant and NADPH inhibitor diphenyleneiodonium (DPI) decreases ROS and up-regulates NIS mRNA in BRAF<sup>V600E</sup> mutated thyroid cancer cell line.** BCPAP cells were incubated with 2 μM of NADPH oxidase unspecific inhibitor Diphenyleneiodonium (DPI) for the indicated periods of time. Intracellular ROS production (A) and NIS mRNA expression (B). Values are expressed as mean ± SE of three independent experiments. Statistics were performed using two way Anova followed by Bonferroni post-test \*\*P < 0.01; \*\*\*P < 0.001

Second, we transfected BCPAP cells with siRNA against NOX4 to address the specific role of NOX4 in NIS repression. SiNOX4 transfection for 48h efficiently reduced NOX4 mRNA and protein (Fig 10A and B), which was followed by increased NIS mRNA levels (Fig 10C). Third, we over-expressed NOX4 in BCPAP cells by transfecting NOX4 expression vectors harboring (NOX4-V5) a V5 tag in N-terminal region of NOX4 (NOX4-V5) to allow its exogenous detection. Plasmid transfection for 24h resulted in increased NOX4 mRNA expression, V5 tag detection by immunoblotting and also increased H<sub>2</sub>O<sub>2</sub> generation (Fig 10D, E and F). Finally, we observed that NOX4 over-expression further reduced NIS mRNA expression (Fig

10G). In conclusion, using different experimental approaches, our results suggest that NOX4 represses NIS mRNA levels in BCPAP cells.



**Figure 10: NOX4 increases ROS and down-regulates NIS mRNA in BRAF<sup>V600E</sup> mutated thyroid cancer cell line BCPAP.** (A, B, C) Effect of SiNOX4 transfection for 48h on NOX4 mRNA expression (A) and NIS protein (B) and mRNA (C). (D, E, F, G) - Expression of NOX4 mRNA (D), V5 tag (E) and extracellular H<sub>2</sub>O<sub>2</sub> production (F) in BCPAP cells, 48 h after NOX4 expression vector transfection. (G) Expression of NIS mRNA in normal human thyrocytes 48 h after NOX4 expression vector transfection. Values are expressed as mean  $\pm$  SE of three independent experiments. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. Statistics were performed using student *t* test in (A, C, D and F) and one way Anova followed by Bonferroni post-test (G).

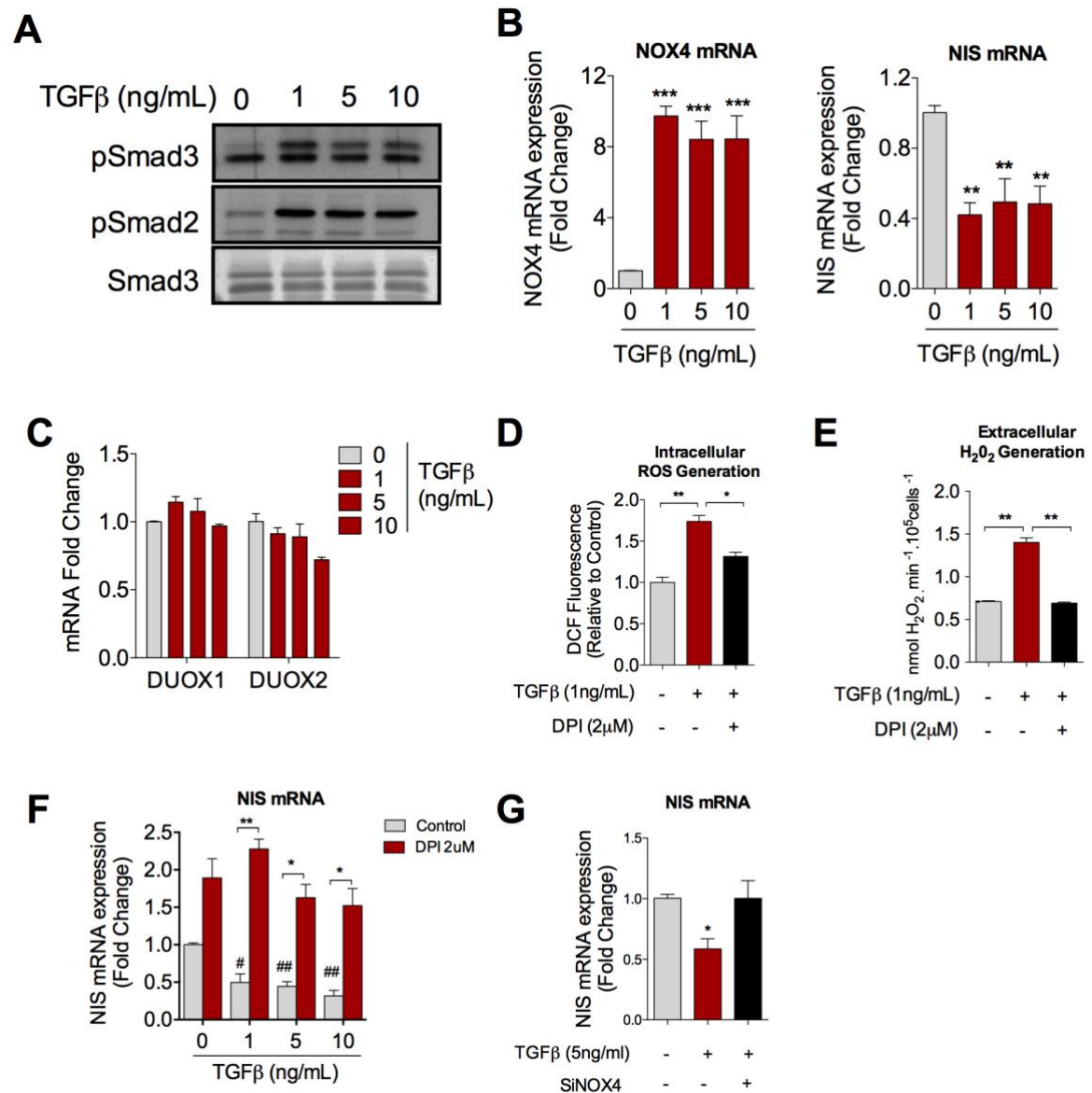
So far, our results show that BRAF<sup>V600E</sup> upregulates Smad3 and NOX4 expression in thyroid cancer cells harboring this oncogene (Fig 8). We also illustrated that NOX4 regulates NIS expression in BCPAP cells (Fig 10). Recent data from our group show that human papillary thyroid cancer cells harboring BRAF<sup>V600E</sup> mutation secrete significant amounts of TGF $\beta$  (AZOUZI *et al.*, 2017). Due to the role of TGF $\beta$  in BRAF<sup>V600E</sup>-induced NIS repression in rat thyrocytes, we characterized the effect of this cytokine on NIS expression and the potential participation of NOX4 in this response.

Treatment of BCPAP cells with the indicated concentrations of TGF $\beta$  for 24h resulted in Smad pathway activation, confirmed by the increment of both pSmad2 and pSmad 3 protein levels (Fig 11A). In parallel, TGF $\beta$  increased NOX4 mRNA levels and further reduced NIS mRNA expression (Fig 11B). TGF $\beta$  did not alter the expression of DUOX1 and DUOX2 (Fig 11C), indicating a selective effect on NOX4, at least at the mRNA levels.

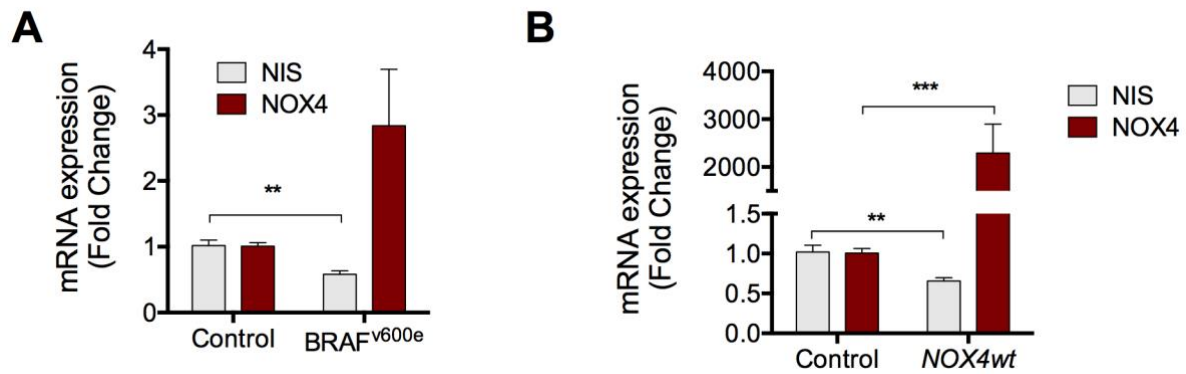
TGF $\beta$  (1 ng/ml) increased both intracellular and extracellular ROS generation in BCPAP cells, which was reversed by concomitant treatment with DPI (Fig 11D and E), indicating the participation of NADPH oxidases as a ROS source. TGF $\beta$ -mediated NIS mRNA repression was reversed by DPI treatment (Fig 11F). However, as DPI has already a basal effect on NIS mRNA levels, the implication of NADPH oxidases in TGF $\beta$  response was not so clear (Fig 9B and 11F). To clarify this point, we transfected BCPAP cells with siNOX4, and 24h after the medium was changed and cells were treated with TGF $\beta$  for 24h. Interestingly, siNOX4 reversed NIS mRNA repression induced by TGF $\beta$  (Fig 11G), indicating that NOX4 is an important mediator of TGF $\beta$  signaling.

Finally, we confirmed the relationship between BRAF<sup>V600E</sup>, NOX4 and NIS regulation in human thyrocytes primary cultures. As observed in rat and human cancer cell lines, BRAF<sup>V600E</sup> or NOX4 transfection induced NIS downregulation (Fig 12A and B). We also detect a slight upregulation of NOX4 mRNA levels in human thyrocytes transfected with BRAF<sup>V600E</sup> (Fig 12A). In conclusion, this study identifies NOX4 as a novel mediator of NIS repression in thyrocytes. Our results suggest that NOX4 is a potential player in BRAF<sup>V600E</sup>-TGF $\beta$  mediated NIS repression during thyroid carcinogenesis. However, the molecular mechanism by which NOX4 regulates NIS expression in thyrocytes is still unknown.





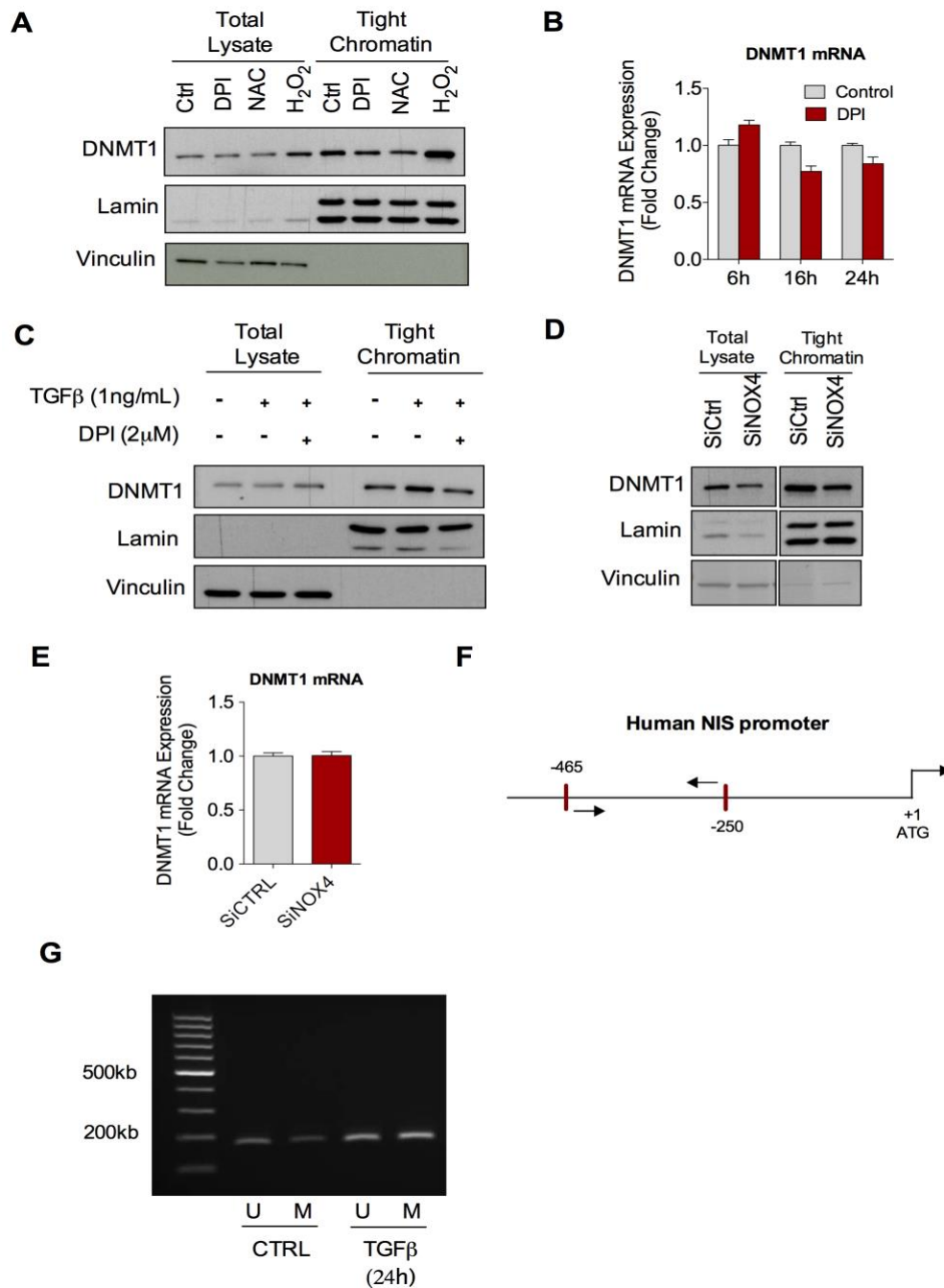
**Figure 11: TGFβ increases ROS and NOX4 mRNA and down-regulates NIS mRNA levels in BRAF<sup>V600E</sup> mutated thyroid cancer cell line.** A - pSmad3 and 2 and total Smad 3 protein levels in BCPAP cells treated with increasing doses of human recombinant TGF-β1 for 24h. B and C- Expression of NIS, NOX4 (B), DUOX 1 and 2 (C) mRNAs in BCPAP cells incubated with increasing doses of human recombinant TGF-β1 for 24h. D, E and F – Intracellular (E) and extracellular (F) ROS generation and NIS mRNA levels (F) after 24h-incubation with TGF-β1 in the presence or absence of DPI (2 μM). (G) Values are expressed as mean ± SE of at least two independent experiments with a minimum of 3 experimental replicates for each assay. Statistics were performed using one way Anova followed by Bonferroni post-test \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; In (F): #P < 0.05; ##P < 0.01 vs control without TGFβ.



**Figure 12: NIS mRNA is regulated by BRAF<sup>V600E</sup> and NOX4 in normal human thyrocytes primary cell cultures.** Expression of NIS and NOX4 mRNA in normal human thyrocytes 48 h after BRAF<sup>V600E</sup> (A) or NOX4 (B) expression vector transfection. (B) Values are expressed as mean  $\pm$  SE of two independent experiments with a minimum of 3 experimental replicates for each assay. Statistics were performed using student *t* test. \*\**P* < 0.01, \*\*\**P* < 0.001 vs control.

Recent studies show that BRAF<sup>V600E</sup> can regulate NIS expression and function by epigenetic mechanisms. Indeed, NIS promoter is highly methylated in BRAF<sup>V600E</sup>-positive papillary thyroid cancer, which was associated with reduced NIS expression and DNA methyltransferase 1 (DNMT1) expression through unknown mechanisms. Additionally it has been demonstrated that effectors of epigenetic regulation, such as DNMT1, induces gene silencing by ROS-dependent mechanisms (O'HAGAN *et al.*, 2011)

In the light of the precedent results showed in this study, we investigated whether NOX4, via ROS production, could repress NIS expression by regulating DNMT1 expression and therefore NIS promoter methylation. First, we verified whether DNMT1 protein levels were affected by redox status in total and chromatin enriched fractions. Treatment of BCPAP cells with H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) for 6h increased DNMT1 protein levels in both the whole cell lysate and in the cell fraction enriched with tight chromatin bound proteins. Interestingly, in this last cell fraction the antioxidants DPI (2 $\mu$ M) and N-Acetyl-L-Cystein (NAC) (1 mM) decreased DNMT1 enrichment (Fig 13A), suggesting that DNMT1 protein levels and chromatin recruitment is redox-regulated.



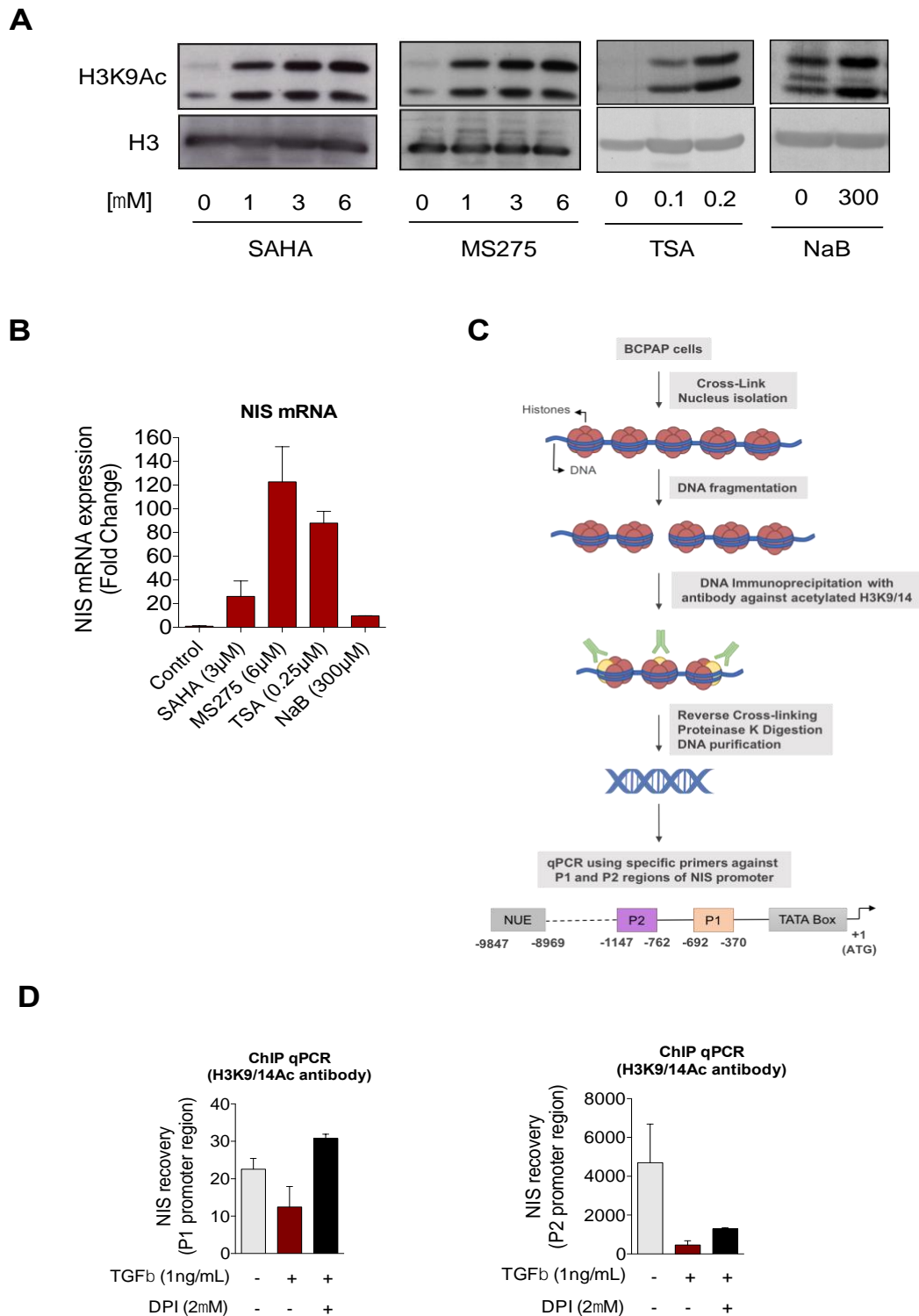
**Figure 13: DNMT1 protein levels in chromatin-enriched fraction is regulated by ROS, NOX4 and TGFβ in BCPAP cells.** (A) Effect of DPI (2 μM), NAC (1 mM) and H<sub>2</sub>O<sub>2</sub> (100 μM) incubation for 6h on DNMT1 protein levels in whole cell lysates and chromatin enriched fraction. Vinculin was used as loading control for whole lysate and Lamin was used as loading control for chromatin fraction. (B) mRNA levels of DNMT1 in BCPAP cells treated with DPI for different times. (C) Effect of SiNOX4 on DNMT1 protein levels in BCPAP cells incubated with TGF-β1 in the presence or absence of DPI for 24h. (D)(E) Effect of SiNOX4 transfection for 72h in DNMT1 Protein expression and mRNA levels. (F) Schematic representation of NIS promoter region evaluated in DNA methylation assay (G) Unmethylated (U) and methylated (M) NIS promoter in the presence of TGFβ for 24h. Values are expressed as mean ± SE of two independent experiments with a minimum of 3 experimental replicates for each assay, except for F (n=1). Statistics were performed using student t test (E) or two way anova followed by Bonferroni post-test (B).

At least in the case of DPI, the effect observed in chromatin-enriched fraction was not associated to an alteration in DNMT1 mRNA expression (Fig 13B). TGF $\beta$  (1 ng/ml for 24h) also increased DNMT1 protein levels in chromatin-enriched fraction, which was reversed by DPI in BCPAP cells (Fig 13C). Silencing of NOX4 expression by siRNA reduced DNMT1 protein levels both in the whole lysate fraction and the chromatin-enriched fraction (Fig 13D). Again, these alterations in DNMT1 protein expression were not associated with alteration in DNMT1 mRNA levels (Fig 13E).

Altogether, these results shed light on a possible ROS-dependent regulation of DNA methylation promoted by NOX4 and TGF $\beta$  in thyroid cancer cells. We used bisulfite conversion technique to evaluate whether this global alteration of DNMT1 protein expression was also associated to increased methylation of NIS promoter. Indeed, in a preliminary assay, we detected that the exposure to TGF $\beta$  for 24h increased the levels of DNA methylation in NIS proximal promoter (Fig 13G). The region of NIS promoter evaluated by PCR amplification after bisulfite conversion is indicated in figure 13F.

In addition to DNA methylation, histone acetylation is also an important epigenetic regulator of gene expression. Histone acetylation is mediated by histone acetyltransferases (HAC) and it is associated with gene transcription. Conversely, histone deacetylation is associated with gene repression and is mediated by histone deacetylases (HDAC) (ESTELLER, MANEL, 2011). In thyroid cell lines, it has been demonstrated that BRAF<sup>V600E</sup> induces hypoacetylation of NIS promoter, which is associated with NIS repression (ZHANG, ZONGJING *et al.*, 2014).

We treated BCPAP cells with different doses of multiple HDAC inhibitors (HDACi) SAHA, MS275, Thicostatin (TSA) and Sodium Butyrate (NaB). In all doses, HDACi increased the expression of the acetylated form of histone H3, which is an indicative that these drugs efficiently induced global histone acetylation (Fig 14A). Treatment of BCPAP cells with HDACi for 24h induced a strong upregulation of NIS mRNA levels, indicating that, in our model, NIS expression is also epigenetically regulated by histone acetylation (Fig 14B). To investigate NIS promoter-specific responses, we performed ChIP-qPCR experiments. TGF $\beta$  treatment for 24h slightly reduced acetylation of P1 and P2 regions in NIS promoter of BCPAP cells. DPI treatment slightly reversed P1 reduced acetylation, suggesting that histone modification in this region is potentially redox regulated.



**Figure 14: TGFβ induces global histone acetylation in BCPAP cells.** (A) Effect of 24h incubation of HDAC inhibitors SAHA, MS275, Thicostatin (TSA) and Sodium Butyrate (NaB) in Histone H3K9 acetylation. (B) Effect of HDACi incubation for 24h on NIS mRNA levels. (C) Schematic representation of ChIP-qPCR assay and NIS promoter regions P1 and P2 explored in this assay (D) Chromatin immunoprecipitation (ChIP) of DNA bound to histone 3 with acetylated lysine 9 and 14 residues (H3k9/K14) followed by qPCR using specific primers against P1 and P2 regions of human NIS promoters (n=2). Values are expressed as mean ± SE. Statistics were performed using one way Anova followed by Bonferroni post-test. \*P < 0.05; \*\*P < 0.01.

## 5. DISCUSSION

The present study identifies the ROS-generating enzyme NADPH oxidase 4 (NOX4) as a new signaling repressor of the sodium-iodide symporter in thyroid cells harboring BRAF<sup>V600E</sup> oncogene, which is the most common mutation found in papillary thyroid cancer that has previously been associated with cell dedifferentiation and loss of iodide uptake ability. We have also shown that TGF $\beta$  positively regulates NOX4 expression. TGF $\beta$  is a well-known NIS repressor that is overexpressed in PTCs and whose synthesis is stimulated by BRAF<sup>V600E</sup> oncogene. Finally, our results provide evidence that both NOX4 and TGF $\beta$  induce epigenetic modifications that contributes to NIS gene transcriptional repression.

Differentiated thyroid cancers (DTC) are usually associated to an indolent behavior and good prognosis, showing an overall 5-year survival rate greater 85%. In most of the cases, surgery followed by radioiodine (RAI) therapy are highly effective standard therapeutic approaches for DTC patients (HAUGEN, BRYAN R. *et al.*, 2016). However, 5-15% of patients become RAI refractory, which is a major cornerstone of thyroid cancer treatment since therapeutic options are scarce and prognosis is poor (WORDEN, 2014).

The development of targeted therapies for progressive advanced DTC refractory to radioactive iodine represented a drastic improvement in patient care in the last years. FDA-approved the use of multikinase inhibitors Sorafenib and Lenvatinib that resulted in increased median progression-free survival rates in phase-III trials. However, ultimately, patients still progress and face significant secondary side effects that implies in drug dose reduction or treatment interruption (BROSE *et al.*, 2014; SCHLUMBERGER *et al.*, 2015). Therefore, the identification of new molecular targets that could serve as adjuvants for improving patient treatment are of huge interest.

Loss of expression of iodine-handling genes, especially NIS, is hallmark of iodine avidity loss in thyroid cancer. Constitutive activation of MEK-ERK signaling plays a major role in thyroid cancer cell dedifferentiation (AGRAWAL *et al.*, 2014). Indeed, targeting this pathway allowed *de novo* iodine uptake in patients previously diagnosed with RAI-refractory disease, what illustrates that RAI uptake reestablishment could be used then, as a promising therapeutic approach (HO *et al.*, 2013b; HUILLARD *et al.*,

2017; ROTHENBERG *et al.*, 2015b). In this study, we focused on BRAF<sup>V600E</sup>-positive PTCs because of its significant prevalence among PTCs and its recurrent association with NIS repression and RAI-refractory disease in both mice and humans (CHAKRAVARTY *et al.*, 2011; FRANCO *et al.*, 2011; RIESCO-EIZAGUIRRE, G, 2006). PTC patients harboring BRAF<sup>V600E</sup> were particularly resistant to develop *de novo* iodine uptake followed by MEK inhibition after treatment with Selumetinib, indicating that NIS regulation in this scenario should be better explored (HO *et al.*, 2013a).

Corroborating previous data in the literature, we confirmed that TGF $\beta$  induces NIS mRNA downregulation in the rat thyrocyte cell line PC-BRAF and in human thyroid cancer cell line BCPAP (NICOLUSSI *et al.*, 2003; RIESCO-EIZAGUIRRE, GARCILASO *et al.*, 2009; TATON *et al.*, 1993). In accordance, we also observed repression of NIS activity in PC-BRAF cells. Interestingly, in parallel with NIS repression, TGF $\beta$  increased NOX4 mRNA levels in both cell lines. Although TGF $\beta$ -induced NOX4 upregulation has been already demonstrated in many tissues, such as in pulmonary mesenchymal cells, cardiac fibroblasts, endothelial cells and hepatocytes, here this regulation was reported for the first time in thyrocytes (BOUDREAU, HOWARD E *et al.*, 2009; CUCORANU *et al.*, 2005; HECKER *et al.*, 2009)(CARNESECCHI *et al.*, 2011).

Using PC-BRAF cells we demonstrated TGF $\beta$ -induced NIS downregulation was reversed by concomitant treatment with Smad3 inhibitor SIS3. These data confirm previous findings of Riesco-Eizeguirre *et al.* (2009) that demonstrated Smad pathway implication in NIS expression regulation using the same cell model. In the same conditions, Smad3 inhibition also resulted in NOX4 repression in PC-BRAF cells, which was also previously observed by our group in BCPAP and 8505c cells (AZOUZI *et al.*, 2017). Our results are supported by recent findings showing that Smad3 inhibition reduced NOX4 promoter activity in epithelial breast cells (BOUDREAU, HOWARD E *et al.*, 2013). Interestingly, it is important to highlight that SIS3 effects on NOX4 in PC-BRAF cells were observed already at 24h of treatment, prior to NIS downregulation, which suggests that Smad3-NOX4 pathway could be an upstream regulator of NIS.

Treatment of PC-BRAF cells with doxycycline from 24h properly induced BRAF<sup>V600E</sup> activation as indicated by increased BRAF expression. A concomitant pSmad3 activation was also reported after 48h of the oncogenic activation, which is

consistent with increased TGF $\beta$  secretion by these cells demonstrated by Riesco-Eizaguirre et al. (2009). Again, using concomitant treatment with SIS3, we provided direct proof that Smad pathway is involved in BRAF<sup>V600E</sup>-induced mRNA NIS repression and NOX4 mRNA and protein upregulation.

Even though NIS downregulation at mRNA levels was already observed within 48h of BRAF<sup>V600E</sup> stimulation, reduced NIS activity was only detected 4 days after doxycycline administration. Riedel et al. (2001) have demonstrated that in the presence of TSH, the major regulator of hormonal biosynthesis, NIS protein has a long half-life of approximately 5 days in FRTL-5 rat thyroid cells (RIEDEL; LEVY; CARRASCO, 2001). Since these experiments were performed in the presence of TSH, the delayed effect on iodine uptake observed in these cells may be associated to the high stability of NIS protein. However, in PC-BRAF cells treated with TGF $\beta$  the iodine uptake already reduced after 48 h of treatment. At present, we cannot explain the time course differences in iodine uptake downregulation that occur in cells treated with TGF $\beta$  or after BRAF induction. Probably, the concentration of TGF $\beta$  might exert different regulatory processes that need to be further investigated by a dose-response curve in these cells.

It is noteworthy that both NOX4 mRNA and protein levels are already stimulated within 48h of PC-BRAF doxycycline exposure that, again, could qualify NOX4 as a potential upstream regulator of NIS. Indeed, a direct implication of NOX4 in NIS transcriptional regulation was provided by siRNA experiments in which NOX4 silencing resulted in attenuation of BRAF<sup>V600E</sup>-induced NIS repression. Interestingly, siNOX4, even in the absence of doxycycline, induced NIS mRNA upregulation in PC-BRAF cells, indicating that NOX4 might also play a role in NIS physiological regulation. The role of NOX4 in thyroid physiology has never been investigated.

Our data in human thyroid cancer cell lines harboring BRAF mutation BCPAP and 8505c recapitulated the main findings in PC-BRAF cells. Treatment of these cells with PLX4032 resulted in NOX4 and pSmad3 protein downregulation. This BRAF<sup>V600E</sup> inhibitor has recently been proven to be of clinical benefit for patients harboring metastatic or unresectable RAI-refractory papillary thyroid cancer in phase 2 clinical trials (BROSE *et al.*, 2016), Decreased NOX4 and pSmad3 protein content is probably associated to the reduced TGF $\beta$  extracellular levels mediated by PLX4032 treatment found in these cells, as shown by previous data from our group (AZOUZI *et al.*, 2017).



In BCPAP cells, we reported for the first time that TGF $\beta$  increases ROS levels, which was reversed by the NADPH inhibitor DPI, corroborating previous reports in non-thyroid cell types (BOUDREAU, HOWARD E *et al.*, 2013; NAM *et al.*, 2010). DPI increased NIS mRNA, reproducing the effects of other antioxidants, such as N-acetyl cystein (NAC) indicating that NIS mRNA levels are controlled by redox-sensitive mechanisms (AZOUZI *et al.*, 2017). Silencing or overexpression of NOX4 provided the final evidence that it regulates NIS mRNA repression in human thyroid cancer cells harboring BRAF<sup>V600E</sup>.

In both PC-BRAF cells and BCPAP cells neither BRAF<sup>V600E</sup> activation nor exogenous TGF $\beta$  regulated the mRNA expression of DUOX1 and DUOX2, that are NADPH oxidases also expressed in thyrocytes (DE DEKEN *et al.*, 2000; DUPUY *et al.*, 1999). Using the normal human thyrocyte cell line NTHY-Ori, Weyemi *et al* (2012) demonstrated that conditional expression of H-RasV12 oncogene preferentially resulted in NOX4 mRNA levels upregulation when compared with DUOXs mRNA levels, showing a possible implication of NOX4 in oncogene-induced responses in thyroid cells. Thus, in the light of our results in rat and human thyroid cell lines, we propose that ROS-producing NOX4 is upregulated by BRAF<sup>V600E</sup>-TGF $\beta$ -Smad3 signaling, acting as a mediator of NIS mRNA repression.

It is well known that ROS are signaling molecules that participate in a variety of biological processes. ROS-specific cellular responses depend on subcellular location of ROS source, since these molecules are not freely permeable due to their chemically reactive nature and the existence of cell antioxidant systems (CHEN, KAI *et al.*, 2008). In H-RasV12-expressing NTHY-Ori cells, NOX4 expression was detected in perinuclear compartment and induced nuclear H<sub>2</sub>O<sub>2</sub> generation (WEYEMI, U *et al.*, 2012). In BCPAP cells, we observed that NOX4 is expressed in tight chromatin-enriched protein fraction, also suggesting NOX4 nuclear or perinuclear location. NOX4 location in nuclear membrane, for example, could be spatially compatible with ROS-mediated NIS transcriptional regulation.

BRAF<sup>V600E</sup>-positive PTCs show increased methylation of six CpG sites in the NIS promoter, which is correlated with NIS repression (CHOI, YONG WON *et al.*, 2014). Interestingly, NIS promoter hypermethylation in these tumors was correlated to increased expression of DNMT1. In both PC-BRAF and BCPAP cells, BRAF<sup>V600E</sup> has been shown to induce histone deacetylation in different sites of NIS promoter, which

was also associated to NIS repression, showing that epigenetic mechanisms are involved in BRAF<sup>V600E</sup>-mediated NIS repression (ZHANG, ZONGJING *et al.*, 2014). Both DNA demethylation genes or inhibitors of histone deacetylases (HDACi) increases NIS expression and iodide uptake in thyroid cells (LI, WEI; VENKATARAMAN; AIN, 2007; PROVENZANO *et al.*, 2007)

Oxidative stress promotes epigenetic changes that play important roles in carcinogenesis (KREUZ; FISCHLE, 2016). Relocation of silencing protein complexes in DNA has been proposed as a possible mechanism involved in promoter hypermethylation of certain genes in cancer cells. In colon cancer cell lines, ROS induced DNA damage (8-oxo-dG) once recognized by DNA repair enzymes results in DNA methyl transferases DNMT1 and DNMT3a and histone deacetylase SIRT1 recruitment to active CpG island promoters (DING *et al.*, 2016; O'HAGAN *et al.*, 2011). Interestingly, genes with a history of DNA hypermethylation in cancers were susceptible to DNMT1 enrichment followed by oxidative DNA damage, while housekeeping genes were unaffected (O'HAGAN *et al.*, 2011).

In BCPAP, we demonstrated that DNMT1 protein expression is increased in both total and tight-chromatin enriched protein fraction after exposure to exogenous H<sub>2</sub>O<sub>2</sub>. Interestingly, the inverse effect was observed in tight-chromatin enriched protein fraction when cells were treated with antioxidants NAC or DPI, showing that, in our model, DNMT1 protein levels are also redox regulated. TGFβ induced DNMT1 enrichment in tight-chromatin fraction, which was dependent on NADPH oxidase activity since it was reversed by DPI. In fact, NOX4-mediated increase in DNMT1 protein levels was confirmed by NOX4 siRNA experiments. Neither siNOX4 nor DPI altered DNMT1 mRNA levels, indicating that alterations are probably mediated by post-transcriptional mechanisms. Thus, our results suggest that NOX4 overexpression in BRAF<sup>V600E</sup>-positive PTCs might be a mechanism that contributes to increased DNMT1 protein expression previously found in these tumors (CHOI, YONG WON *et al.*, 2014).

In agreement with our results, it was demonstrated that TGFβ upregulates DNMTs expression and induces extensive DNA methylation changes that are compatible with cancer progression in ovarian cancer cells. In this *in vitro* model, well-known repressed genes during TGFβ-induced epithelial-to-mesenchymal-transition, such as E-cadherin were found to be hypermethylated in their promoter regions (CARDENAS *et al.*, 2014). Corroborating a possible methylation role, our preliminary

results revealed that TGF $\beta$  increased NIS-specific promoter methylation in BCPAP cells, indicating that alterations in DNA methylation is involved in TGF $\beta$ -mediated NIS transcriptional repression.

In our model, we confirmed previous results in the literature that demonstrated that HDACi resulted in increased NIS mRNA levels, indicating that histone acetylation participates on NIS transcriptional regulation (PROVENZANO *et al.*, 2007). We also demonstrated that TGF $\beta$  reduced NIS histone HEK9/K14 acetylation in two different promoter regions named P1 and P2. P1 hypoacetylation was reversed by DPI treatment indicating that, in this region, this epigenetic alteration could be redox-regulated. Altogether, these data show for the first time that epigenetic regulation of NIS expression is a mechanism potentially involved in NIS transcriptional repression by TGF $\beta$ -NOX4 pathway. The molecular mechanism by which NOX4 derived-ROS regulates these epigenetic changes remains undefined.

In normal thyrocytes, NOX4 overexpression by oncogenic Ras activation resulted in increased ROS and DNA damage (WEYEMI, *et al.*, 2012). Recent data have shown that the treatment of pig thyrocytes with a fungal toxin, Calicheamicin that induces DNA double-strand breaks resulted in NIS mRNA and iodide uptake inhibition. NIS repression was mediated by ataxia-telangiectasia mutated (ATM) kinase a protein involved in DNA damage response and DNA repair (LYCKESVÄRD *et al.*, 2016). Since, oxidative DNA damage and secondary DNA repair pathway activation are mechanistically implicated in epigenetic regulation of gene expression, we suggest that perturbation of genome integrity might also be implicated in NIS epigenetic changes mediated by NOX4 derived-ROS in thyroid cancers harboring BRAF<sup>V600E</sup>.

Our results revealed that epigenetic alterations mediated by BRAF<sup>V600E</sup>-TGF $\beta$ -NOX4 induces NIS mRNA downregulation. However, it is possible the other NOX4-mediated mechanisms are also involved. It was previously demonstrated that NIS mRNA downregulation induced by TGF $\beta$  in PCCL3 is mediated by a functional interaction between Smad3 and PAX8, the main thyroid transcription factor that induces NIS promoter activity. Smad3 induced PAX8 downregulation and reduced its DNA binding activity (COSTAMAGNA, 2003). PAX8 DNA binding activity is activated by the reduction of two conserved cysteine residues in its structure (KAMBE *et al.*, 1996; LEONI *et al.*, 2015). In the light of our data, it would be interesting to evaluate if NOX4-derived ROS induces PAX8 oxidation, which might also be involved in NIS

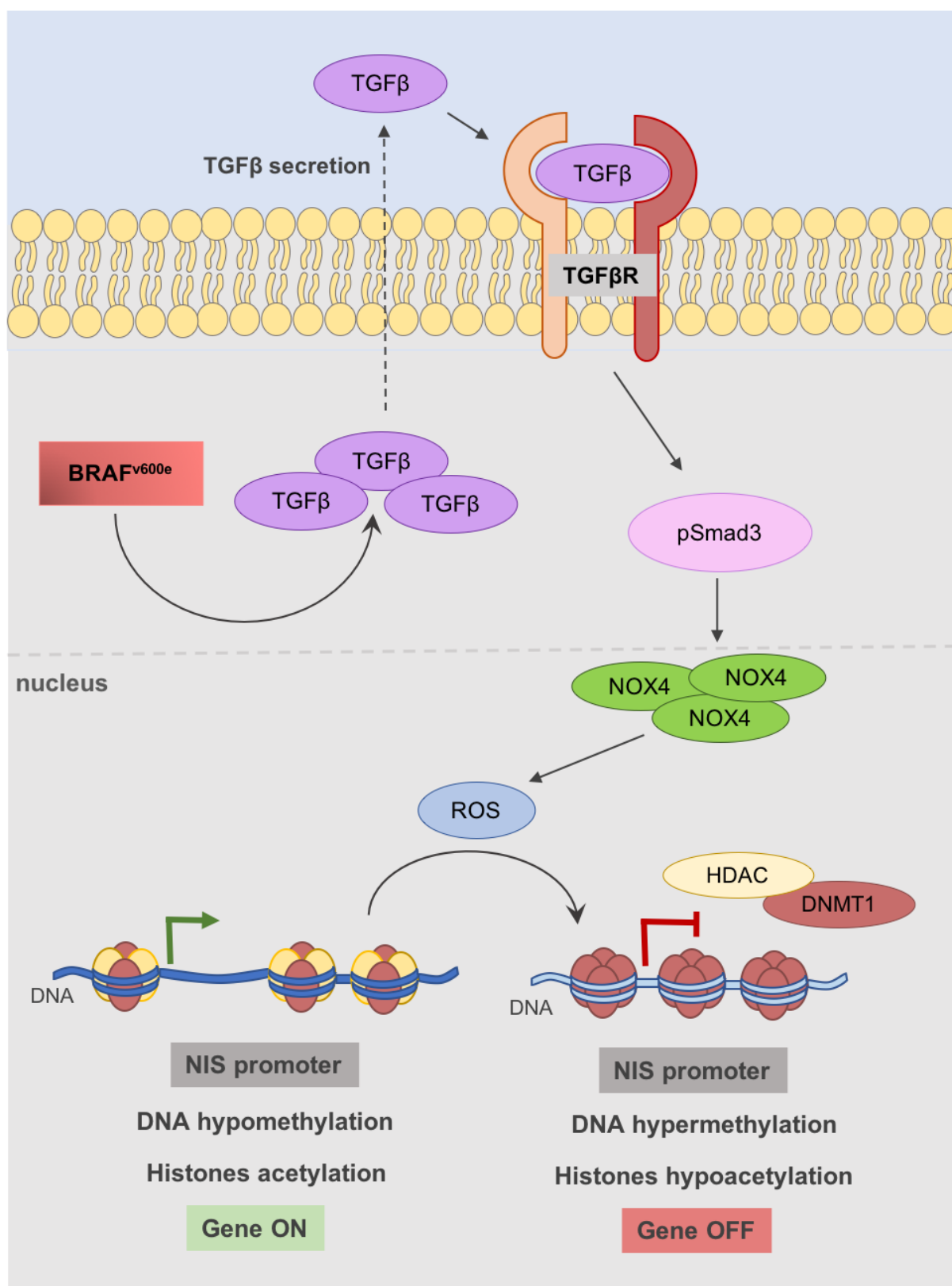
repression. Additionally, we cannot exclude that both NIS promoter DNA methylation and histone deacetylation might result in heterochromatin states that impair PAX8 DNA binding.

We provided evidences that NOX4 regulates NIS mRNA levels, but we cannot exclude the possibility that NOX4-derived ROS also regulates NIS through post-transcriptional mechanisms. In rat PCCL3 cells, iodine excess that mimics thyroid iodine autoregulation phenomenon increases cellular ROS. In this model, ROS has been suggested to inhibit NIS activity by inactivating this transporter in the plasma membrane without affecting its trafficking and/or subcellular localization (LEONI *et al.*, 2011). *In silico* analysis suggested the presence of two potentially redox-sensitive Cys residues (C272 and C276) that could be implicated in NIS inactivation, but no functional studies were performed. Interestingly, it was recently demonstrated that delta-iodolactone, an iodinated lipid synthesized by the thyroid gland, increased ROS generation by upregulating NOX4 in the thyroid cancer cell line WRO (LISA *et al.*, 2017). Indeed, NOX4 expression has been previously detected at the plasma membrane of rat thyrocytes, and might also be involved in ROS-mediated NIS post-translational regulation (FORTUNATO *et al.*, 2013). Further investigation is necessary to evaluate a possible direct implication of NOX4 in the plasma membrane on NIS inactivation.

## 6. CONCLUSION

This study leads to a better understanding of signaling pathways and molecular mechanisms involved in NIS downregulation in papillary thyroid cancers harboring BRAF<sup>V600E</sup> oncogene. We identified NOX4 as a potential therapeutic target for re-differentiation strategies in RAI-refractory thyroid cancer due to its role in NIS repression.

Based on the current data, we can propose a new model of how BRAF<sup>V600E</sup> induces NIS transcriptional repression in human thyroid cancer cells (Fig 15): BRAF<sup>V600E</sup> increases TGF- $\beta$  synthesis and secretion by thyrocytes that binds to TGF $\beta$  receptor, activating the intracellular Smad pathway. pSmad3 increases NOX4 expression, probably in the nuclear compartment, that increases local H<sub>2</sub>O<sub>2</sub> generation, leading to the recruitment of silencing complexes containing DNMT1 and HDACs to NIS promoter. These protein complexes induce epigenetic changes in NIS promoter, including DNA methylation and histone deacetylation, resulting in NIS transcriptional repression.



**Figure 15: Proposed model for BRAF<sup>V600E</sup> mediated NIS repression in thyroid cancer cells.** BRAF<sup>V600E</sup> increases TGF-β synthesis and secretion by thyrocytes. TGFβ binding to TGFβ receptor induces Smad3 phosphorylation that increases nuclear NOX4 expression. NOX4 increases local H<sub>2</sub>O<sub>2</sub> generation, leading to DNA methyl transferase 1 (DNMT1) and histone deacetylases (HDAC) recruitment to NIS promoter, which results in DNA methylation, histone deacetylation and consequently NIS transcriptional repression.

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## 8. APPENDIX

### APPENDIX 1: Thesis synthesis in french

**Titre :** Rôle de la NADPH oxydase 4 dans la régulation redox du symporteur sodium (Na<sup>+</sup>)/iodure (I<sup>-</sup>) dans le cancer papillaire de la thyroïde

#### RESUMÉ

L'absorption d'iode par la glande thyroïde est une étape clé pour la biosynthèse des hormones thyroïdiennes T3 et T4. Ce processus est médié par le co-transporteur Na<sup>+</sup> / I<sup>-</sup> (NIS), une glycoprotéine membranaire intégrale appartenant à la famille des transporteurs de soluté 5 (SCLA5) situé dans la membrane basolatérale des thyrocytes. La capacité très efficace de ce tissu d'accumuler et de capturer l'iodure est la base de la Radiothérapie métabolique ou RAI, approche thérapeutique standard utilisé pour le traitement du cancer différencié de la thyroïde (CDT), le type le plus commun de tumeur maligne de la glande thyroïde. La CDT est divisée en deux sous-types histologiques distinctes, les carcinomes papillaires (CPT) qui correspond à environ 90% des cas et des carcinomes folliculaires et, avec, en général, une évolution indolente et un bon pronostic. Chez ces patients, l'administration de doses thérapeutiques d'iode radioactif (I<sup>131</sup>) après résection chirurgicale permet l'ablation du tissu thyroïdien restant avec destruction des lésions résiduelles de la tumeur et des métastases cancéreuses. Environ 5 à 15% des patients atteints de CDT ont des tumeurs qui ne concentrent pas l'iode et sont réfractaires aux effets thérapeutiques de la Radiothérapie métabolique, ce qui est associé à un mauvais pronostic. Cette situation est associée à un processus de dédifférenciation cellulaire marqué par la réduction des taux d'ARNm et protéine du NIS et de sa translocation de la membrane plasmique à l'intérieur des cellules. Ainsi, la compréhension des mécanismes cellulaires qui régissent ce processus est essentielle au développement de thérapies visant à rendre ces tumeurs réactives à l'iode radioactif. La mutation activatrice BRAFV600E est la plus fréquemment identifiée au sein des cancers différenciés de la thyroïde de type papillaires (CPT). Cette mutation induit une forte activation de la voie MAPK (MEK-ERK) et est associée à la dédifférenciation cellulaire et à la répression du NIS dans les modèles murins et chez l'homme. Dans la lignée cellulaire de rat il a été précédemment montré que la suppression du NIS induite par BRAFV600E est dépendante de la sécrétion accrue de TGF et de l'activation de sa voie canonique : la voie de Smad. Cependant, les mécanismes moléculaires impliqués dans ce processus sont mal compris. On sait que de nombreux processus cellulaires, régulés par le TGFβ comme la transition épithéliale-mésenchymateuses et de la fibrose, sont dépendants de l'augmentation des espèces réactives de l'oxygène (ERO) induite par cette cytokine. Dans ce contexte, la NADPH oxydase 4 (NOX4) a été décrite comme une source importante de ROS impliquée dans la signalisation du TGFβ dans plusieurs types cellulaires. NOX4 appartient à la famille des NADPH oxydases qui est composée de 6 autres membres : NOX1, NOX2, NOX3, NOX5, Dual oxydase 1 (DUOX1) et 2 (DUOX2). Toutes les NADPH oxydases sont des protéines transmembranaires exprimées de manière spécifique ou ubiquitaire dans les tissus et utilisent le NADPH comme donneur d'électrons pour la réduction de l'oxygène moléculaire pour former l'anion superoxyde (NOX1-3 et NOX5) ou le peroxyde d'hydrogène (NOX4, DUOX-1 et DUOX-2). Dans la glande thyroïde, les 3 NADPH oxydases : NOX4, DUOX1 et DUOX2 sont exprimées, cette dernière étant la source d'H<sub>2</sub>O<sub>2</sub> associée à la thyroperoxydase (TPO) pour catalyser la biosynthèse hormonale. Dans les CPT, la surexpression de NOX4 par rapport au tissu normal a été mise en évidence. Utilisation de la base de données de l'Atlas du génome du cancer (TCGA) ont permis d'identifier une augmentation de l'expression de NOX4 dans les tumeurs porteuses de la mutation BRAFV600E comparées aux tumeurs ne l'exprimant pas. Dans les PTC positifs pour BRAFV600E, nous avons trouvé une corrélation inverse entre l'expression du NIS et celle



de NOX4. Cependant, le rôle direct de NOX4 dans la régulation du NIS dans les cellules thyroïdiennes normales ou tumorales n'a jamais été étudié. Ainsi, l'objectif de ce travail est d'évaluer si NOX4 est un médiateur de la répression NIS induite par la voie BRAFV600E/TGF- $\beta$  dans différentes lignées cellulaires thyroïdiennes. En utilisant une lignée cellulaire de thyroïde de rat normal (PC-BRAF), nous avons démontré que l'administration de TGF- $\beta$  ou l'expression BRAFV600E induit une diminution du taux d'ARNm du NIS, associée à une diminution de l'absorption d'iodure par les cellules et à une expression accrue de l'ARNm de NOX4. En revanche, l'induction de l'expression de BRAFV600E ne modifie pas les taux d'ARNm de DUOX1 et de DUOX2. Le silençage de NOX4 par un traitement avec des siRNAs ou un traitement pharmacologique avec un inhibiteur de Smad3 tel que SIS3, inhibe la répression du NIS médiée par BRAF<sup>V600E</sup>, indiquant l'implication de NOX4 via la voie Smad3 dans cette répression. Dans les lignées tumorales de la thyroïde humaine portant la mutation BRAFV600E, telles que les lignées BCPAP et 8505c, nous avons obtenu des résultats similaires à ceux obtenus avec les cellules de rat. Le traitement de ces cellules avec le PLX4032, un inhibiteur pharmacologique de BRAFV600E, diminue les taux protéiques de NOX4 et de pSmad3. Des résultats similaires sont obtenus avec l'inactivation de BRAFV600E par siRNA dans des cellules 8505c. Un traitement des cellules BCPAP avec le diphenylèneiodonium (DPI), un inhibiteur de NADPH oxydases, ou le silençage de Nox4 par ARN interférence conduit à une augmentation du taux d'ARNm du NIS. Inversement, la surexpression de Nox4 induit, via une augmentation de la production de ROS intracellulaire, une réduction du taux d'ARNm du NIS. Ces résultats démontrent que Nox4 joue un rôle répressif dans l'expression de ce transporteur. Le traitement des cellules BCPAP avec le TGF $\beta$  induit une augmentation de l'ARNm NOX4 et une réduction de l'ARNm NIS. Les effets du TGF $\beta$  sur le NIS sont contrecarrés lorsque le traitement est fait en présence de siNOX4, montrant que l'effet répressif de TGF $\beta$  à l'encontre du NIS est dépendant de Nox4. Le TGF $\beta$  n'affecte pas les taux d'ARNm de DUOX1 et DUOX2. Des études récentes ont montré que l'oncogène BRAFV600E régule l'expression du gène SLC5A5 codant NIS par des mécanismes épigénétiques. Les CPT qui portent cette mutation présente une hyperméthylation du promoteur du NIS par rapport au tissu normal, et cela est corrélé à une réduction de l'expression de l'ARNm du NIS. Cette hyperméthylation est attribuée à une expression accrue de l'enzyme ADN méthyltransférase (DNMT1), une enzyme catalysant la méthylation de l'ADN. De plus, chez le rat et les cellules de la thyroïde humaine, BRAFV600E réprime la transcription du NIS en induisant une hypoacétylation des histones localisés au niveau de son promoteur. Puisque les ERO régulent le recrutement des complexes de silençage contenant des DNMTs et des désacétylases de histones (HDAC) aux promoteurs de certains gènes causant des changements épigénétiques qui ont un impact sur leur expression, nous avons examiné si la répression du NIS médiée par la voie BRAFV600E-TGF- $\beta$ -NOX4, pourrait impliquer ce mécanisme épigénétique. Dans les cellules BCPAP, un traitement par H<sub>2</sub>O<sub>2</sub> augmente l'expression de la protéine ADN méthyltransférase 1 (DNMT1) dans la fraction cellulaire enrichie en protéines liées à la chromatine. Un traitement par des antioxydants ou le silençage de NOX4 réduit ce recrutement, montrant que, dans notre modèle, les niveaux de protéine DNMT1 sont régulés par des mécanismes rédox. Le TGF $\beta$  augmente les niveaux de protéines DNMT1 dans la fraction cellulaire enrichie en chromatine, lequel qui est réversé par un traitement par le DPI, ce qui suggère que cet effet est médié par une NADPH oxydase. Le TGF $\beta$  augmente la méthylation de l'ADN au niveau du promoteur du gène codant NIS. Des expériences d'immunoprécipitation de la chromatine (ChIP) montrent que le TGF $\beta$  favoriserait une hypoacétylation de l'histone H3 dans deux régions distinctes du promoteur (P1 et P2). Cette hypoacétylation est inhibée par le DPI dans la région P1. Cette étude a identifié la NADPH oxydase 4 (NOX4) en tant que médiateur de la répression du NIS induite par la voie BRAFV600E-TGF $\beta$  dans les cellules thyroïdiennes. Ainsi, NOX4 peut représenter une cible thérapeutique potentielle dans le cadre d'une stratégie de re-différenciation des cancers de la thyroïde réfractaires à la radiothérapie métabolique utilisant l'iode radioactif. Nos résultats montrent que la répression transcriptionnelle du NIS médiée par cette voie implique des mécanismes épigénétiques tels que la méthylation de l'ADN et

l'hypoacétylation des histones. Cette étude pose les bases d'une meilleure compréhension des voies de signalisation et des mécanismes moléculaires qui seraient impliqués dans la régulation négative du NIS dans les cancers papillaires thyroïdiens porteurs de la mutation BRAFV600E.

## APPENDIX 2: Thesis synthesis in portuguese

**Título:** O papel da NADPH oxidase 4 na regulação redox do co-transportador de sódio ( $\text{Na}^+$ )/iodeto ( $\text{I}^-$ ) no câncer papilífero de tireoide.

### RESUMO EXPANDIDO

A captação de iodeto pela glândula tireoide é uma etapa chave para biossíntese dos hormônios tireoidianos T3 e T4. Esse processo é mediado pelo co-transportador de  $\text{Na}^+/\text{I}^-$  (NIS), glicoproteína integral de membrana pertencente à família dos carreadores de solutos 5 (SCLA5), localizada na membrana basolateral dos tireócitos. A capacidade desse tecido captar e acumular iodeto altamente eficiente é a base da terapia com radioiodo, abordagem terapêutica padrão empregada para o tratamento de câncer diferenciado de tireoide (CDT), tipo mais frequente de tumor maligno da glândula tireoide.

O CDT é subdividido em dois subtipos histológicos distintos, os carcinomas papilíferos que corresponde a cerca de 90% dos CDTs e os carcinomas foliculares, apresentando, de maneira geral, um curso indolente e bom prognóstico. Nesses pacientes administração de doses terapêuticas de iodo radiativo ( $\text{I}^{131}$ ) pós-resssecção cirúrgica do tumor permitem a ablação do tecido remanescente da tireoide, destruição de lesões residuais de câncer e metástases de maneira bastante eficaz e direcionada. Cerca de 5 a 15% dos pacientes portadores de CDT apresentam tumores que não concentram iodo e são refratários aos efeitos terapêuticos da radioiodo o que está associado a mau prognóstico. Esse quadro está associado a um processo de desdiferenciação celular que tem como característica marcante a redução dos níveis de mRNA e expressão proteica do NIS bem como sua translocação da membrana para o interior celular. Assim, a compreensão de mecanismos celulares que governam esse processo é central para o desenvolvimento de terapias que visam tornar esses tumores novamente responsivos ao radioiodo. Em carcinomas papilíferos de tireoide (CPT) a mutação no gene BRAF (V600E) é a alteração genética mais frequente, sendo encontrada em cerca de 40%-60% dos casos. Essa mutação induz forte ativação da via de MAPK (MEK-ERK) e está associada a desdiferenciação celular e repressão do NIS em modelos murinos e em humanos. Em linhagem celular de rato foi previamente demonstrado que a repressão do NIS mediada pelo oncogene BRAF<sup>V600E</sup> é dependente do aumento da secreção de TGF $\beta$  e da ativação da sua via canônica, a via de Smads. No entanto, os mecanismos moleculares envolvidos nesse processo são pouco compreendidos. Sabe-se que vários dos processos celulares regulados por TGF $\beta$  como a transição epitélio-mesênquima e fibrose são dependentes do aumento de espécies reativas de oxigênio (ERO) induzido por essa citocina. Nesse contexto, a NADPH oxidase 4 (NOX4) foi descrita como uma importante fonte geradora de ERO envolvida na sinalização de TGF $\beta$  em diversos tipos celulares. A NOX4 faz parte da família das NADPH oxidases que é composta ainda por outros 6 membros, NOX1, NOX3, NOX5 e Dual-oxidases 1 (DUOX1) e 2 (DUOX2). Todas as NADPH oxidases são proteínas transmembrana expressas de maneira tecido-específica que utilizam o NADPH como doador de elétrons para redução do oxigênio molecular ao ânion superóxido (NOX1-3 e NOX5) ou ao peróxido de hidrogênio (NOX4, DUOX-1 e DUOX-2). Na glândula tireoide são expressas a NOX4, DUOX1 e DUOX2, sendo esta última a fonte de  $\text{H}_2\text{O}_2$  para a biossíntese hormonal.

Em CPTs foi detectado superexpressão de NOX4 quando comparado ao tecido não-tumoral. Utilizando a base de dados do *The Cancer Genome Atlas* (TCGA) observamos maior expressão de NOX4 em CPTs portando a mutação BRAF<sup>V600E</sup> quando comparados a tumores que expressam BRAF não-mutado. Além disso, em PTCs positivos para BRAF<sup>V600E</sup>, encontramos uma correlação inversa entre a expressão de NIS e NOX4. No entanto, o papel direto da NOX4 na regulação do NIS em células tireoidianas nunca foi estudado. Dessa forma, objetivo deste trabalho é avaliar se a NOX4 é um mediador da repressão NIS induzido por BRAF<sup>V600E</sup>/TGF $\beta$  em linhas celulares tireoidianas.

Usando uma linhagem celular de tireoide normal de rato (PC-BRAF), demonstramos que a administração de TGF- $\beta$  ou a expressão de BRAF<sup>V600E</sup> resultaram na redução dos níveis de

mRNA do NIS, redução da captação de iodeto e aumento da expressão de mRNA de NOX4. A indução da expressão de BRAF<sup>V600E</sup> não alterou os níveis de RNAm de DUOX1 e DUOX2. O silenciamento de NOX4 por siRNA ou tratamento com SIS3, inibidor de pSmad3, reverteu a repressão NIS mediada por BRAF<sup>V600E</sup>, indicando a implicação da via Smad e de NOX4 nessa regulação. Nas linhagens de câncer de tireóide humanas que portam a mutação BRAF<sup>V600E</sup>, BCPAP e 8505c, obtivemos resultados semelhantes aos obtidos em células de rato. O tratamento dessas células com PLX4032, inibidor farmacológico de BRAF<sup>V600E</sup>, reduziu os níveis protéicos de NOX4 e pSmad3. Resultados semelhantes foram obtidos com o silenciamento BRAF<sup>V600E</sup> por siRNA em células 8505c. Tanto o tratamento de células BCPAP com Diphenyleneiodonium (DPI), inibidor de NADPH oxidases, quanto o silenciamento de NOX4 por siRNA resultaram no aumento dos níveis de RNAm do NIS. A superexpressão da NOX4, por outro lado, aumentou a geração celular de ERO e reduziu os níveis de RNAm do NIS, demonstrando que a NOX4 exerce um papel repressor sobre a expressão desse transportador. O tratamento de células BCPAP com TGF $\beta$  induziu aumento do RNAm de NOX4 e redução do RNAm de NIS. Os efeitos sobre o NIS foram revertidos quando o tratamento com TGF $\beta$  foi feito na presença de siNOX4, demonstrando que o efeito repressivo de TGF $\beta$  sobre o NIS é dependente de NOX4. TGF $\beta$  não exerceu efeitos sobre os níveis de RNAm de DUOX1 e DUOX2. Estudos recentes demonstram que o oncogene BRAF<sup>V600E</sup> regula a expressão NIS por mecanismos epigenéticos. Visto que ERO regula recrutamento de complexos de silenciamento contendo enzimas DNA metiltransferase (DNMTs) e histonas deacetilases (HDACs) para o promotor de certos genes causando alterações epigenéticas que impactam em sua expressão, investigamos se a repressão de NIS mediada por pela via BRAF<sup>V600E</sup>-TGF $\beta$ -NOX4, poderia envolver mecanismos epigenéticos. CPTs portanto essa mutação apresentam o promotor NIS hipermetilado quando comparado ao tecido normal, o que foi correlacionado à redução da expressão de NIS. Esta hipermetilação foi atribuída a um aumento da expressão da DNMT1, enzima catalisadora da metilação de DNA. Em células tireoidianas de rato e humanas, BRAF<sup>V600E</sup> também foi capaz de reprimir a transcrição no NIS ao induzir hipoacetilação de histonas em seu promotor. Em células BCPAP demonstramos que o tratamento com H<sub>2</sub>O<sub>2</sub> exógeno aumentou a expressão da proteína DNMT1 tanto na fração celular total quanto na fração celular enriquecida em proteínas fortemente ligadas a cromatina. Curiosamente, o efeito inverso foi observado nesta última fração quando as células foram tratadas com antioxidantes N-Acetil-L-Cisteína (NAC) ou DPI, mostrando que, em nosso modelo, os níveis de proteína DNMT1 são regulados por mecanismos redox. O TGF $\beta$  induziu o enriquecimento de DNMT1 na fração celular enriquecida em cromatina o que foi revertido pela DPI, demonstrando que esse efeito é mediado por NADPH oxidases. De fato, o silenciamento de NOX4 inibiu a expressão protéica de DNMT1. Finalmente, demonstramos que TGF $\beta$  aumentou a metilação de DNA, especificamente no promotor do gene NIS. Experimentos de imunoprecipitação de cromatina (ChIP) também revelaram uma tendência do TGF $\beta$  induzir hipoacetilação de histona H3 em duas regiões distintas do promotor do NIS (P1 e P2), o que foi atenuado por DPI na região P1. Tanto a metilação de DNA quanto a hipoacetilação de histonas são marcadores de repressão transcricional, o que corrobora o papel repressivo da NOX4 sobre o NIS. Este estudo identificou a NOX4 como mediador da repressão do NIS mediada pela via BRAF<sup>V600E</sup>-TGF $\beta$  em células tireoidianas. Assim, NOX4 pode representar um potencial alvo terapêutico para estratégias de re-diferenciação em câncer de tireóide refratário ao radio-iodo. Nossos resultados fornecem evidências de que a repressão transcricional de NIS mediada por essa via envolve mecanismos epigenéticos como a metilação de DNA e hipoacetilação de histonas. Este estudo leva a uma melhor compreensão das vias de sinalização e dos mecanismos moleculares envolvidos na regulação negativa de NIS nos cânceres papilíferos da tireóide BRAF<sup>V600E</sup> positivos.

**APPENDIX 3: Published paper related to thesis subject**

## Original Research Communication

***NADPH oxidase NOX4 is a critical mediator of BRAF<sup>V600E</sup>-induced down regulation of the sodium iodide symporter in papillary thyroid carcinomas***

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**Abbreviated title:** NOX4 is involved in BRAF<sup>V600E</sup>-induced NIS repression

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Word count: 5082

Reference numbers: 53

Greyscale illustrations: 8

## Abstract

**Aims:** The BRAF<sup>V600E</sup> oncogene, reported in 40-60% of papillary thyroid carcinoma (PTC), has an important role in the pathogenesis of PTC. It is associated with the loss of thyroid iodide-metabolizing genes, such as sodium/iodide symporter (NIS) and therefore with radioiodide-refractoriness. Inhibition of MAPK pathway, constitutively activated by BRAF<sup>V600E</sup>, is not always efficient in resistant tumors suggesting that other compensatory mechanisms contribute to a BRAF<sup>V600E</sup> adaptive resistance. Recent studies pointed to a key role of TGF- $\beta$  in BRAF<sup>V600E</sup>-induced effects. The reactive oxygen species (ROS)-generating NADPH oxidase NOX4, which is increased in PTC, has been identified as a new key effector of TGF- $\beta$  in cancer, suggestive of a potential role in BRAF<sup>V600E</sup>-induced thyroid tumors. **Results:** Here, using two human BRAF<sup>V600E</sup> mutated thyroid cell lines and a rat thyroid cell line expressing BRAF<sup>V600E</sup> in a conditional manner we show that NOX4 up-regulation is controlled at the transcriptional level by the oncogene *via* the TGF- $\beta$ /Smad3 signaling pathway. Importantly, treatment of cells with NOX4-targeted siRNA downregulates BRAF<sup>V600E</sup>-induced NIS repression. **Innovation and Conclusion:** Our results establish a link between BRAF<sup>V600E</sup> and NOX4, which is confirmed by a comparative analysis of NOX4 expression in human (TCGA) and mouse thyroid cancers. Remarkably, analysis of human and murine BRAF<sup>V600E</sup>-mutated thyroid tumors highlights that the level of NOX4 expression is inversely correlated to thyroid differentiation suggesting that other genes involved in thyroid differentiation in addition to NIS might be silenced by a mechanism controlled by NOX4-derived ROS. This study opens a new opportunity to optimize thyroid cancer therapy.

## Introduction

Papillary thyroid cancer (PTC) is the most frequent histologic type of human thyroid carcinoma (26). *BRAF* mutations are found in 40-60% of the cases (7) and the most frequent is the *BRAF*<sup>T1799A</sup> point mutation that encodes the *BRAF*<sup>V600E</sup> oncogenic constitutively active protein kinase (50). This mutation is associated with more extensive disease, a higher rate of recurrence and decreased survival (51). It is also associated with thyroid cell dedifferentiation with a decreased expression of thyroid functional genes (7).

The property of thyroid cells to accumulate iodide is mediated by the sodium/iodide symporter (NIS) (13). This is clinically highly relevant because it enables treatment of thyroid cancers with radioiodine. However, *BRAF*<sup>V600E</sup> positive tumors are often associated with a significant decrease or a complete loss of NIS expression.

*BRAF* mediates the signal transduction of the MAP/extracellular signal-regulated kinase (MEK)-ERK pathway and the presence of a *BRAF* mutation predicts for a high sensitivity to MEK inhibitors in human cancers (28). Thus, *BRAF*<sup>V600E</sup>-positive thyroid cancer cell lines are sensitive to the growth suppressive effects of MAPK pathway inhibitors (28, 38) and small-molecule *BRAF* or MEK inhibitors restores radioiodine incorporation in the thyroid tumors of transgenic mice with doxycycline-inducible expression of *BRAF*<sup>V600E</sup> (8). However, it has been also observed that MEK inhibition did not rescue *BRAF*<sup>V600E</sup>-induced functional NIS repression in a rat thyroid cell line (41) and in some patients harbouring thyroid cancer with *BRAF* mutation indicating that, the MAPK pathway inhibition did not always fully revert cancer progression and *BRAF*<sup>V600E</sup>-induced effects (19). A possible explanation is that MAPK signalling is incompletely inhibited in some *BRAF*-mutant tumors because of relief of negative feedback effects on upstream components of the pathway (35, 30). The strong activation of the MAP kinase pathway also activates additional pathway(s) contributing to dedifferentiation and epithelio-mesenchymal transition (EMT) in tumors harbouring oncogenic *BRAF*.



Several studies have shown an important role of Transforming Growth Factor  $\beta$ 1 (TGF- $\beta$ ) in this process. TGF- $\beta$  is overexpressed in human tumors (40), including thyroid malignancies (31, 47), and is a potent pro-oncogenic and prometastatic factor. Its role in cancer biology and in cell signaling is complex, and the cellular context appears to be a crucial determinant of the ultimate outcome of TGF- $\beta$  signaling in normal and tumor cells. TGF- $\beta$  was first shown to play a major role as a local modulator of thyroid by inhibiting both growth and differentiation in several species (17, 44, 46). It can be a potent repressor of the expression of thyroid specific functional genes, such as the NIS, thyroglobulin (Tg) and thyroperoxydase (TPO) (24, 37, 11). BRAF<sup>V600E</sup> expression induces the production of functional TGF- $\beta$ 1, which leads to a TGF- $\beta$ -driven autocrine loop that mediates, at least in part, the effects of the BRAF<sup>V600E</sup> oncoprotein, in particular the decreased expression of NIS (41) and the promotion of cell migration, invasiveness and epithelial to mesenchymal transition (EMT) (25). However, the molecular basis of the BRAF<sup>V600E</sup>-induced TGF- $\beta$ 1-loop in the development of thyroid cancers remains unclear.

Recent observations suggest that reactive oxygen species (ROS) play an important role in the TGF- $\beta$ 1-induced EMT and cell mobility of many cell types. The NADPH oxidases (NOXs and DUOXs) generate ROS in a wide variety of tissues (4). Cancer cells produce high levels of ROS, and in some cases, the source of these ROS has been linked to NOXs/DUOXs deregulation as reported for prostate cancer (4), melanoma (52) and glioblastoma (20). Among the NADPH oxidases, NOX4 is prominently expressed in a number of tumors such as papillary thyroid cancers (48) and recent studies underscored NOX4 as a new key effector of TGF- $\beta$  in cancers (6). This led us to consider NOX4 as a potential candidate for a ROS producing system regulated by the BRAF<sup>V600E</sup>-induced TGF- $\beta$ -loop.

In this report, we show that NADPH oxidase NOX4 is regulated by BRAF<sup>V600E</sup> in a TGF $\beta$ -Smad3-dependent pathway in thyroid cancer cells, and that NOX4-dependent ROS generation has a critical role in NIS repression induced by mutated BRAF. We confirmed our findings in both patient tissue samples from thyroid cancers harbouring the BRAF<sup>V600E</sup> mutation and in mouse BRAF<sup>V600E</sup>-induced thyroid tumors by showing a positive correlation

with enhanced NOX4 expression. Strikingly, BRAF-mutated tumors have a thyroid differentiation score inversely correlated to NOX4 expression level supporting a model where NOX4-derived ROS silences the transcription of genes involved in thyroid differentiation during tumorigenesis.

## Results

### *Activated BRAF oncogene promotes ROS generation via the NADPH oxidase NOX4*

p22<sup>phox</sup> forms a functional heterodimeric complex with NOX4, and accordingly we evaluated by real-time QPCR the time course changes in the levels of both mRNAs in two human thyroid cancer cell lines harbouring the BRAF<sup>V600E</sup> mutation (BCPAP and 8505C). Fig 1A and supplementary Fig S1A show that the expression of NOX4 and p22<sup>phox</sup> mRNAs increased with time in culture in the two cell lines. This was associated with an increased expression of both NOX4 and p22<sup>phox</sup> proteins in both cell lines (Fig 1B and supplementary S1B). Thyroid cells also express two other NADPH oxidases generating H<sub>2</sub>O<sub>2</sub> at the apical plasma membrane: DUOX2, which is involved in thyroid hormone biosynthesis, and DUOX1, whose physiologic role in this tissue is still unknown. The mRNA levels of two DUOXs were not increased in these experimental conditions (Supplementary Fig. S1C). Knocking down NOX4 or p22<sup>phox</sup> with specific siRNA reduced the intracellular level of ROS in both cell lines (Fig. 1C and supplementary Fig. S1D and Fig. S1E). Treatment of cells for 72 hours with the BRAF<sup>V600E</sup> kinase inhibitor PLX4032 led to down-regulation of NOX4 and p22<sup>phox</sup> protein levels, indicating that mutated BRAF regulated both protein expressions in these two tumour cell lines (Fig. 1D and supplementary Fig. S2A).

### *BRAF<sup>V600E</sup> induced-TGF-β secretion*

We analyzed at different time points the cell culture supernatants for the presence of TGF-β1 (Fig. 2A). BCPAP cells produced TGF-β1 and this was associated with an increase in both total and phosphorylated form of Smad3, consistent with activation of the TGF-β1/Smad signalling pathway (Fig 2B). Treatment of cells with a TGF-β receptor inhibitor (SB431542) strongly decreased mRNA expression of NOX4 indicating that TGF-β1 regulates the protein at the transcriptional level (Supplementary Fig. S2B). Treatment of BCPAP cells with PLX4032 inhibited TGF-β1 secretion, total and phosphorylation of Smad3 (Supplementary Fig. S2C and S2D). Therefore, we conclude that mutated BRAF controls TGF-β secretion.

Down regulation of BRAF by RNA interference confirmed the results (supplementary Fig. S3A). In 8505C cells phospho-Smad3 level, which transiently increased during the 72 hours incubation, and total Smad3 protein level were also inhibited by PLX4032 (Supplementary Fig. S2E).

#### *TGF- $\beta$ regulates NOX4 via Smad3 activation*

To determine the role of phospho-Smad3 on NOX4, we treated BCPAP and 8505C cells with SIS3, a specific inhibitor of phospho-Smad3 (21). Immunoblot analysis showed that SIS3 decreased both total and phosphorylated form of Smad3 (Fig. 2C and supplementary Fig. S3B) and down-regulated NOX4 mRNA and protein levels in both cell lines (Fig. 2C, supplementary Fig S2B, S3B and S3C). As expected, the level of ROS was also reduced by treatment with both compounds that inhibited TGF- $\beta$ 1 signaling (supplementary Fig. S3D). Taken together, these results show that the TGF- $\beta$ 1 signaling pathway up-regulates NOX4 in BRAF<sup>V600E</sup>-mutated thyroid cells at the transcriptional level in a Smad3-dependent manner. As shown in figure 2D and supplementary Fig. S4A, the MEK inhibitor U0126 impaired both phosphorylation and expression of Smad3 as expressions of NOX4 and p22<sup>phox</sup> in two BRAF-mutated thyroid cancer cell lines. This was associated with a decreased intracellular level of ROS (Supplementary Fig. S4B). Therefore, Smad3 activation and BRAF<sup>V600E</sup>-induced NADPH oxidase expression were both MEK dependent.

#### *NOX4-dependent ROS generation down-regulates NIS expression in BRAF-mutated cell lines*

Based on these results, we postulated that NOX4-dependent ROS generation might play a role in regulating NIS expression in BRAF-mutated thyroid cells. We first analyzed the effect of the ROS scavenger N-acetyl cysteine (NAC) on NIS expression in BCPAP cells. As shown in figure 3A the ROS scavenger increased the NIS at both mRNA and protein levels indicating that ROS are involved in NIS repression at the transcriptional level. DPI, an inhibitor of flavoproteins such as NADPH oxidases also induced NIS mRNA expression (Fig.

3B). As expected, knocking down NOX4 with specific siRNA reinduced NIS mRNA and protein levels (Fig. 3C). The same results were obtained in 8505C cells (supplementary Fig. S4C). We next analyzed the cell-surface expression of NIS, as membrane localization of the symporter is essential to its function. Cell-surface biotinylation experiments were carried out with a nonpermeable cross-linker on both cell lines. Sulfo-NHS-biotin treatment revealed that a fraction of NIS protein, detected as two bands corresponding to the hypo- and hyperglycosylated forms (29), was increased at the cell surface after NOX4 and p22<sup>phox</sup> inactivation, respectively (Fig. 3D and supplementary Fig. S4D). Collectively, these data show for the first time that a NOX4-dependent ROS generation is involved in BRAF<sup>V600E</sup>-induced NIS repression via the TGF  $\beta$  /Smad3 signaling pathway.

#### *BRAF<sup>V600E</sup> induced NOX4 expression through Smad signalling in thyroid PCCL3 cells*

We next investigated the effect of acute BRAF<sup>V600E</sup> expression on NOX4 expression in thyroid cells. To this end, we used the doxycycline-inducible BRAF<sup>V600E</sup> thyroid PCCL3 cells where mutated BRAF was previously shown to decrease NIS expression through the TGF- $\beta$ /Smad signalling pathway (41). Importantly, induction of BRAF<sup>V600E</sup> by doxycycline, which led to activation of Smad3 phosphorylation (supplementary Fig. S5A) upregulated NOX4 protein and mRNA levels (Fig. 4A and 4C). Treatment with the Smad3-specific inhibitor (SIS3) abolished the BRAF induction of NOX4 mRNA induction in these cells, which counteracted repression of NIS mRNA level by doxycycline (Fig. 4B). This underscored that acute BRAF<sup>V600E</sup> activation up-regulated NOX4 at the transcriptional level in a Smad3-dependent manner. To determine the role of NOX4 in BRAF<sup>V600E</sup>-induced repression of NIS we performed interference RNA experiments. As shown in figure 4C, NOX4 knockdown dampened the doxycycline-induced repression of TSH-induced NIS expression. Of note, in the absence of doxycycline, NOX4-inactivated PCCL3 cells displayed a significant increase of NIS mRNA expression after TSH stimulation, indicating that NOX4 may also participate in the regulation of NIS mRNA level expression in normal thyroid cells. Expression of DUOX1 and DUOX2 mRNA levels was not affected by doxycycline treatment (Supplementary Fig.

S5B).

*TGF- $\beta$  downregulates Na<sup>+</sup>/I<sup>-</sup> symporter (NIS) via NOX4 in human thyrocytes*

To investigate whether NOX4 and p22<sup>phox</sup> are regulated by TGF- $\beta$ 1 in normal human primary thyroid follicular epithelial cells we analyzed the time course effect of TGF- $\beta$ 1 on both NOX4 and p22<sup>phox</sup> mRNA by real-time (RT)-qPCR. Figure 5A shows that TGF- $\beta$ 1 increased NOX4 mRNA in a time-dependent manner. This was associated with increased phospho-Smad3 and NOX4 protein, and inhibited by the Smad3-specific inhibitor (SIS3) (Fig. 5B). NIS mRNA was downregulated by TGF- $\beta$ 1 (Fig. 5C) and SIS3 counteracted this effect (supplementary Fig S6A and S6B). Of note, TGF- $\beta$ 1 did not increase DUOX1 and DUOX2 mRNA levels in these cells (Supplementary Fig. S6C). These data indicate that TGF- $\beta$ 1 regulates NOX4 expression *via* a Smad3-dependent pathway in human thyrocytes. We next analyzed the effect of NOX4 inactivation on NIS mRNA level in both control and TGF- $\beta$ 1-treated thyrocytes. Figure 5D shows that NOX4 inactivation counteracted the inhibitory effect of TGF- $\beta$ 1 on NIS mRNA in the presence of TSH. This was related to a significant decrease in intracellular ROS level (Supplementary Fig. S6D). Taken together, these data show that NOX4 plays a key role in TGF- $\beta$ 1-induced downregulation of NIS in normal human thyrocytes.

*NOX4 is increased in BRAF-mutated papillary thyroid carcinomas (PTCs)*

From the Cancer Genome Atlas (TCGA) it is now possible to perform combined analysis of gene expression as a function of different driver mutation groups that lead to different pathologies with distinct signalling and differentiation characteristics (7). BRAF<sup>V600E</sup> status has been characterized in 390 of the 496 PTC and we compared NOX4 mRNA expression levels in BRAF<sup>WT</sup>-PTCs (n=170) and BRAF<sup>MUT</sup>-PTCs (n=220). NOX4 was significantly increased in PTCs harbouring BRAF mutation (Fig 6A) and this was associated with a significant decrease of NIS mRNA expression level (Fig. 6B). In BRAF-mutated PTCs, high NOX4 mRNA level mostly correlated with low NIS mRNA level (Fig 6C). In addition, an inverse and highly significant correlation was found between NOX4 expression and the

thyroid differentiation score (7) (Fig 6D). Importantly, both TGF- $\beta$ 1 and Smad3 mRNA were found to be significantly increased in BRAF-mutated PTCs (Fig 6E and 6F). A *BRAF*<sup>V600E</sup>-RAS score (BRS) has been also previously developed to quantify the extent to which the gene expression profile of a given tumor resembles either the *BRAF*<sup>V600E</sup> or RAS mutant profiles (7). We found that a higher level of NOX4 expression was associated with a negative score that characterizes the *BRAF*<sup>V600E</sup>-like samples (Supplementary Fig. S6E). In addition, ERK activation level, which is highly correlated to BRS (7), was associated with an increased expression of NOX4 gene (Supplementary Fig. S6F). Overall, these data support a role of NOX4 in thyroid tumors driven by *BRAF*<sup>V600E</sup>, in particular, a role in mechanisms leading to thyroid dedifferentiation. A schematic model summarizing all the steps involved in NOX4 regulation is presented in Figure 7A.

Since dedifferentiation during tumorigenesis might follow the inverse gene expression pattern observed during normal differentiation, we wondered whether ontogenic changes of mRNA expression of thyroid differentiation markers, such as NIS and TPO could be associated with a modulation of the expression level of *NOX4* gene. To this end, we analysed by quantitative PCR mRNA expression for *NOX4*, *TPO* and *NIS* genes in thyroid tissues during human development between 8 and 12 gestational weeks (GW). While a significant increase in NIS and TPO mRNA levels was detected at 10 GW in accordance with the onset of thyroid function (43) no modulation of the expression of NOX4 mRNA level was observed in these conditions (Fig. 7B), meaning that only an increase of NOX4 may impact their expression.

*NOX4 mRNA level is inversely correlated to the Thyroid Differentiation Score in genetically engineered mouse models of thyroid cancer.*

The profiles of gene expression have been previously established for different mouse PTCs models induced by knock-in mutations of drivers such as *BRAF*<sup>V600E</sup> (14) and H-RAS<sup>G12V</sup> in the context of NF2, PTEN or p53 loss (15). These tumors present phenotypic differences ranging from differentiated (PTC) to poorly differentiated (PDTC) and undifferentiated (anaplastic carcinoma ATC). Analysis of the gene expression data clearly show that NOX4

mRNA level was increased mostly in BRAF-mutated tumors (Fig. 8A), which significantly correlated with increased expression of TGF- $\beta$ 1 mRNA and with activation of TGF-  $\beta$ /Smad3 signaling pathway (Fig 8B and supplementary Fig. S7A, S7B and S7C). Strikingly, BRAF-mutated tumors had a very low thyroid differentiation score, which, as observed in their human counterparts, was strongly inversely associated with NOX4 mRNA levels (Fig 8C and 8D).



## Discussion

### *NOX4 as a new effector of BRAF<sup>V600E</sup> and TGF-β1 oncogenic signaling in PTC*

The BRAF<sup>T1799A</sup> mutation, which encodes BRAF<sup>V600E</sup> is the most common genetic event in human papillary thyroid cancers (7). BRAF is a potent activator of the MAPK/ERK pathway and, as such, a critical regulator of normal growth, differentiation and oncogenic transformation. TGF-β pathway activation is strongly associated with thyroid cancer progression in mice (25). Overexpression of TGF-β in human thyroid tumors is consistent with this role, and led us to explore downstream mechanisms that may account for these effects in thyroid malignancies. ROS are known to be important effectors of the TGF-β pathway at the molecular level and to induce EMT and cell mobility in many cell types (53, 36). NOX4-dependent ROS production mediates TGF-β-induced effects such as fibrosis (3) and EMT (5). We have previously shown that NOX4 is over-expressed in human papillary thyroid cancers, but its functional consequences have not been previously explored (48).

The present study shows that NOX4 expression is significantly higher in human and mouse papillary thyroid cancers harbouring the BRAF<sup>V600E</sup> mutation (Fig. 6 and 8). Using two human BRAF<sup>V600E</sup> thyroid cancer cell lines and a rat thyroid cell line expressing BRAF<sup>V600E</sup> in a conditional manner, we show that NOX4 up-regulation is controlled at the transcriptional level by the BRAF<sup>V600E</sup> oncogenic protein *via* the TGF-β/Smad3 signaling pathway. Inhibitors targeting specifically this pathway impaired NOX4 expression and consequently the intracellular ROS production in all thyroid cells tested including normal human thyrocytes. Smad3 is a transcription factor known as an effector of TGF-β1. Recent data describe the identification of a Smad-binding element involved in TGF-β1 regulation of the NOX4 gene promoter (5). Thus, NOX4 transcriptional regulation by TGF-β1 in thyroid cells is likely due to an increase of NOX4 promoter activity by the binding of Smad3. NOX4 heterodimerization with p22<sup>phox</sup> is sufficient to enhance its ROS-generating activity and does not require cytosolic subunits that are essential for other NOX isoforms (4). NOX4 is the only NOX with a constitutive ROS generating activity that depends directly on its gene expression. The protein p22<sup>phox</sup> tightly interacts with NOX4 (1). Except for a few cases and what was observed in

BCPAP and 8505C cells, p22<sup>phox</sup> is not consistently regulated by TGF- $\beta$ 1 at the transcriptional level. NOX4 has the capacity to stabilize p22<sup>phox</sup> and therefore to increase its intra-cellular protein level (1). Thus, unlike NOX4, p22<sup>phox</sup> mRNA expression is not the limiting factor for both p22<sup>phox</sup> protein expression and formation of the active NADPH oxidase.

The MAP kinase pathway contributes to the progression of BRAF<sup>V600E</sup>-induced thyroid cancer. Constitutive activation of the MAPK pathway is required for optimal TGF- $\beta$  signaling in mouse thyroid cancer cells induced by BRAF<sup>V600E</sup> (25). However, in rat thyroid cell line expressing conditionally BRAF<sup>V600E</sup>, the involvement of the MEK-ERK pathway in the mutant BRAF-induced TGF- $\beta$  effects appeared to be selective. Indeed, unlike BRAF<sup>V600E</sup>-induced EMT, mutant BRAF-induced NIS repression was a MEK-ERK independent process (41). In the present study, MEK inhibition impaired Smad3 activation and NADPH oxidase expression in both BRAF<sup>V600E</sup> thyroid cancer cell lines indicating that BRAF<sup>V600E</sup>-induced NOX4 expression relied on the autocrine TGF- $\beta$  loop is MEK-ERK dependent.

#### *Regulation of NIS mRNA transcription*

Since BRAF<sup>V600E</sup>-induced repression of NIS was previously shown to be dependent of TGF- $\beta$  we wondered whether NOX4-dependent ROS generation, regulated by TGF- $\beta$ 1, could be involved in this mechanism. Using antioxidant (NAC) and RNA interference-mediated NOX4 knockdown we show for the first time that NIS gene expression could be controlled at the transcriptional level by a potential redox-sensitive mechanism in thyroid cancer cells. Our results highlight that BRAF-induced TGF- $\beta$ 1 represses NIS gene expression through a NOX4-dependent pathway downstream of Smad3. An alternative mechanism to explain NIS repression by TGF- $\beta$ 1 has been previously proposed. This involved a functional interaction between the transcription factor PAX8, a critical mediator of NIS gene transcription, and Smad3, resulting in inhibition of PAX8 transcriptional activity (11). However, in light of these new data, Smad3 might also contribute to the inhibition of the action of PAX8 by an oxidation process through an upregulation of NOX4-derived ROS. Moreover, a redox regulation of PAX8, involved in its DNA-binding activity, has been previously described in rat thyroid FRTL-

5 cells (23). Furthermore, analysis of results obtained with human and murine tumor tissues underscore that the level of NOX4 expression is inversely correlated to thyroid differentiation suggesting that, beyond NIS, other genes involved in thyroid differentiation might be silenced by a general mechanism finely controlled by NOX4-derived ROS. This mechanism could involve redox-sensitive epigenetic modifications, such as DNA methylation and histone acetylation (18). Hypermethylation of the NIS promoter is observed in BRAF<sup>V600E</sup> harbouring PTC (10, 42) and the use of demethylating agents has been shown to restore decreased iodine uptake to cells with methylated promoter regions *in vivo*. ROS have been shown to provide an oxidative stress-mediated epigenetic imbalance, which perturbed genomic integrity in numerous oxygen radical-induced injuries, disturbing the DNA demethylation mechanisms (33). It was in particular observed that oxidative damage could contribute to the relocalization of a silencing complex containing DNA methyl transferases (DNMTs) (39). The molecular mechanisms of a potential redox-sensitive control by NOX4-derived ROS of genes involved in thyroid differentiation have to be further investigated. Interestingly, the increases in NIS and TPO mRNA expressions during human thyroid differentiation were not associated with a modulation of NOX4 mRNA suggesting that the onset of human fetal thyroid function is not dependent on the level of NOX4.

#### *NOX4 as a potential therapeutic target?*

A clinical study using the MAPK kinase inhibitor selumetinib to reverse refractoriness to radio-iodine in patients with radioiodine refractory metastatic thyroid cancer partially restored RAI incorporation in patients with BRAF mutated tumors, but yielded much better results in patients with RAS-mutated tumors (10). Analysis of human PTC (TCGA) shows that NOX4 expression level is correlated to ERK activation. Interestingly, we have recently shown that overexpression of oncogenic H-RAS<sup>V12</sup> upregulates NOX4 in thyroid cells (49). This underscores that NOX4 expression in thyroid tumors may depend on the degree of MAPK activation. It was recently shown that BRAF<sup>V600E</sup> PTCs are a heterogeneous group of tumors with variable degrees of thyroid differentiation (7). Since the level of NOX4 appears to be

correlated with thyroid dedifferentiation in BRAF<sup>V600E</sup> PTC, it would be of importance to evaluate the potential role of NOX4 as a RAI therapy response predictive marker in patients with metastatic thyroid cancers. Moreover, this study identifies NOX4 as a potential therapeutic target through its role in NIS repression. Specific inhibitors might be used in association with MAPK kinase inhibitors in order to potentiate their effect on RAI therapy.

## Innovation

Our results establish a link between BRAF<sup>V600E</sup> and NOX4, which is confirmed by a comparative analysis of NOX4 expression in human (TCGA) and mouse thyroid cancers. Remarkably, analysis of human and murine BRAF<sup>V600E</sup>-mutated thyroid tumors highlights that the level of NOX4 expression is inversely correlated to thyroid differentiation suggesting that other genes involved in thyroid differentiation in addition to NIS might be silenced by a mechanism controlled by NOX4-derived ROS

## Materials & Methods

**Cell Culture and reagents.** Human thyroid cancer cell lines harboring the BRAF<sup>V600E</sup> mutation (BCPAP and 8505C cells) were cultured in DMEM medium (4.5 g/l glucose) (Life Technologies) supplemented with 10% (vol/vol) of FCS (Life Technologies) and penicillin/streptomycin (100 mg/ml; Life Technologies). Primary human thyroid cells were cultured as previously described (2).

PC-BRAF<sup>V600E</sup> cells were derived from the rat thyroid cell line PCCL3 to obtain doxycycline-inducible expression of BRAF<sup>V600E</sup> (34). PC-BRAF<sup>V600E</sup> cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% Tet-free FCS (Life Technologies) and a six-hormone mixture including TSH and insulin. Inhibitor of TGF  $\beta$ 1 pathway (SB431542 - Calbiochem), inhibitor of Smad3 phosphorylation (SIS3 – Sigma-Aldrich), inhibitor of MAPK pathway (U0126 - Calbiochem), inhibitor of BRAF<sup>V600E</sup> (PLX4032 – Selleckchem); recombinant human TGF  $\beta$ 1 (Peprotech), N-Acetyl-cysteine (Sigma-Aldrich), and Doxycycline (Sigma-Aldrich) were used in this study.

**Transfection of small interfering RNAs.** Cells were transfected at 50-60% confluence with specific human siRNA against NOX4 (stealth RNAi duplex, Invitrogen), scrambled siRNA control (Invitrogen), or siRNA against p22<sup>phox</sup>, siRNA against BRAF and Rat siRNA against NOX4 (smart pool from Dharmacon) using INTERFERIN transfection reagent (Polyplus-Transfection) according to the manufacturer's protocol.

**Measurement of intracellular ROS.** The peroxide-sensitive fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA; Molecular Probes, Life Technologies) was used to assess the generation of intracellular reactive oxygen species (ROS).

**Western blot analysis and ELISA test.** Cells were solubilized in RIPA lysis buffer (Sigma-Aldrich) supplemented with a mixture of phosphatase and protease inhibitors (Roche Applied Science). Immunodetection was performed as described previously (48) using as primary antibody either anti-NOX4 (OriGene); anti- pSmad3 (ser 423) (Abcam); anti-Smad3 (Abcam); anti-p22<sup>phox</sup> (Santa Cruz Biotechnology Inc.), anti-NIS (Thermoscientific); anti-BRAF<sup>V600E</sup>

(Spring); anti- $\beta$ -actin (Sigma-Aldrich) and anti-Vinculin (Abcam). The proteins were visualized by enhanced chemiluminescence (Amersham) using secondary antibodies conjugated to HRP: goat anti-rabbit IgG-HRP antibody (Southern Biotech), and goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology Inc). Secreted TGF- $\beta$  was measured by ELISA test according to the manufacturer's protocol (Promega).

**Cell surface biotinylation.** Membrane-associated proteins were prepared using Cell Surface Protein Isolation Kit (Pierce, Life Technologies), and avidin-agarose (Sigma-Aldrich) according to the manufacturer's protocol.

**Real-Time qRT-PCR.** Total RNA was extracted from cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The quality of RNA preparation, based on the 28S/18S rRNAs ratio, was assessed using the RNA 6000 Nano Lab-On-chip (Agilent Technologies). Total RNA (1  $\mu$ g) was reverse-transcribed using Maxima Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primers in a total reaction volume of 20  $\mu$ L of PCR buffer according to the manufacturer's protocol for 60 min at 60 °C. Real-time qRT-PCR was performed as described previously (48). Sequences of primer pairs and TaqMan probes were as follows: Human DUOX1 primer: sens, 5'-CCAGCAATCATCTATGGGGGC-3'; antisens, 5'-TGGGGCCGCTGGAACC-3'; DUOX2 primer : sens, 5'-CCGGCAATCATC TATGGAGGT-3'; antisens, 5'-CCTTGGGGCCTCTGGAATT-3'; the same TaqMan fluorescent probe for DUOX1 and DUOX2: 5' FAM-AGCAGCGTGGTGAAGGCGGAGC-3' TAMRA; NOX4 primer: sens, 5'-TGTCTTCTACATGCTGCTGACGTT-3'; antisens, 5'-CTGAGAGCTGGTTCGGTTAAGACT-3'; TaqMan fluorescent probe: 5'-FAM-CAAACCTAATTTAGATACCCACCCTCCCGGC-TAMRA; Human *p22<sup>phox</sup>* primer : sens, 5'-GCGCTTCACCCAGTGGTACTT-3'; antisens, 5'-GGTGGAGCCCTTCTTCCTCTT-3'; TaqMan fluorescent probe : 5'-FAM-TTGTGGCGGGCGTGTTTGTGTG-TAMRA; human NIS primer : sens, 5'-CCATCCTGGATGACAACTTGG-3'; antisens, 5'-AAAAACAGACGATCCTCATTG-3'; TaqMan fluorescent probe : 5'-FAM-AGAACTCCCCACTGGAAACAAGAAGCCC-TAMRA;

human TBBP primer : sens, 5'-GAATATAATCCCAAGCGGTTTGC-3'; antisens, 5'-CTTAGCTGGAAAACCCAACTTCTG-3'; TaqMan fluorescent probe : 5'-FAM-AGGATAAGAGAGCCACGAACCACGGC-TAMRA, housekeeping gene TBBP primer: sense, 5'-GAATATAATCCCAAGCGGTTTGC-3'; antisense, 5'-CTTAGCTGGAAAACCCAACTTCTG-3'; TaqMan fluorescent probe: 5'-FAM-AGGATAAGAGAGCCACGAACCACGGC-TAMRA. Oligonucleotides were purchased from Eurofins Genomics.

For PC-BRAF<sup>V600E</sup> cells real-time qRT-PCR was performed with FastStart Universal SYBR Green Master (Roche). Sequence of primer pairs were as follow: rat DUOX1: sens, 5'-GATACCCAAAGCTGTACCTCG-3', antisens, 5'-GTCCTTGTCACCCAGATGAAG-3'; rat DUOX2 : sens, 5'-TGCTCTCAACCCCAAAGTG-3'; antisens, 5'-TCTCAAACCAGTAGCGATCAC-3'; rat NIS : sens, 5'-GCTCATCCTGAACCAAGTGA-3'; antisens, 5'-ACGAGCATTACCACAACCTG-3'; rat NOX4 ; sens, 5'-TCCATCAAGCCAAGATTCTGAG-3'; antisens, 5'-GGTTTCCAGTCATCCAGTAGAG-3'; rat GAPDH : sens, 5'-TGATTCTACCCACGGCAAGT-3'; antisens, 5'-AGCATCACCCCATTGTGATGT-3'.

**Expression arrays :** Samples were processed and hybridized on Affymetrix MOE 430A 2.0 arrays by the MSKCC Genomic core facility. Gene expression data of all samples were log2-transformed and quantile-normalized with Partek GS software. Fold change was calculated compared to the average expression of normal thyroid tissues (TPO-Cre negative or wild-type animals). We determined the thyroid differentiation (TDS) and TGF $\beta$  transcriptional output scores from thyroid specimens of the different mouse models by measuring expression of genes involved in thyroid hormone biosynthesis or that are transcriptionally regulated by the TGF $\beta$  pathway (Supplementary Table S1). Score= Average of all genes (Invlog10), where each gene= log2(gene)/(log2(controls). Genes used for the TDS were the mouse homologues of the human gene list used to develop the TDS in the TCGA paper on papillary thyroid carcinoma (7). The TGF $\beta$  score was based on genes upregulated by TGF $\beta$  in mouse primary hepatocytes (12) that were concomitantly upregulated in LSL-Braf/TPO-



Cre PTCs relative to normal thyroid tissue. Of note, we confirmed that these murine Braf-induced PTC have increased activation of the TGF $\beta$  pathway (Supplementary Figure S7A and S7B). For genes with multiple probes in the expression array we used the probe with the highest standard deviation between samples of different characteristics to calculate scores. Correlations between Nox4 expression and TGF $\beta$  output and TDS were calculated in Graphpad using Pearson correlation.

**Human embryonic tissue samples.** Human embryonic and fetal thyroid were extracted from tissue fragments that were obtained immediately after elective termination of pregnancy between 7 and 12 weeks of gestation in compliance with the French bioethics legislation. Approval was obtained from Agence de Biomedecine, the French competent authority along with maternal written consent. The number of sampled thyroid tissues per GW was as follows: 2 at 8 GW; 2 at 10 GW; 2 at 12 GW. Tissue samples were snap-frozen and stored at -80°C before RNA analysis. Total RNA was purified from frozen thyroid tissues using RNeasy extraction, according to the manufacturer's instruction (QIAGEN, Hilden, Germany).

**Mouse genetic models:** *TPO-Cre* mice express Cre recombinase under the control of the thyroid peroxidase gene promoter, which is active only in thyroid follicular cells beginning at E14.5 (27). *FR-Hras*<sup>G12V</sup> mice conditionally express a latent *Hras*<sup>G12V</sup> allele under the regulatory control of its endogenous gene promoter (9). To generate triple transgenic mice, *TPO-Cre/FR-Hras* mice were bred with *Nf2*<sup>fllox2</sup> (16), *Trp53*<sup>fllox2</sup> (22), or *Pten*<sup>fllox2</sup> mice (45). Mice with homozygous loss of Nf2 develop thyroid hyperplasia, whereas *Hras*<sup>G12V</sup>/Nf2-null and *Hras*<sup>G12V</sup>/Pten-null develop poorly differentiated thyroid cancers (PDTC). *Hras*<sup>G12V</sup>/p53 null develop either PDTC or anaplastic thyroid cancer (15). *TPO-Cre* mice crossed with *LSL-Braf*<sup>V600E</sup> (32), which harbor conditional knock-in allele of Braf<sup>V600E</sup>, result in development of PTC with short latency (14). Both male and female mice were used. Animal care and all procedures were approved by the MSKCC Institutional Animal Care and Use Committee.

**Statistical analysis.** Statistical analyses were performed using GraphPad InStat software for ANOVA and the Student t test, with the level of significance set at  $P < 0.05$ .

**Acknowledgments:** This work was supported by grants from Electricité de France (EDF), Ligue Contre le Cancer (comité du Val-de Marne), Programmes Internationaux de Coopération Scientifique (PICS): CNRS-France/CNRST-Maroc, PHC Volubilis/Toubkal, International Atomic Energy Agency (IAEA), NIH grants RO1-CA50706, P50-CA172012, P30-CA008748 and Programme d'appui à la recherche sectorielle (CNRST/Maroc). Naima AZOUZI was supported by University Paris-Saclay, Jérémy Cailloux was the recipient of a doctoral fellowship of the French Ministry of Research and Technology and Juliana M. Cazarin was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

**Author Disclosure Statement:** The authors declare no conflict of interest.

### Abbreviations List

BSA	bovine serum albumin
DUOX	dual oxidase
DUOXA	DUOX activator
EMT	Epithelio-mesenchymal transition
ERK	Extracellular signal-regulated kinases
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
NAC	N-Acetyl-cysteine
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NIS	Sodium Iodide Symporter
NOX	NADPH oxidase
PTC	Papillary Thyroid Carcinoma
ROS	Reactive oxygen species
RT	Reverse Transcription
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA

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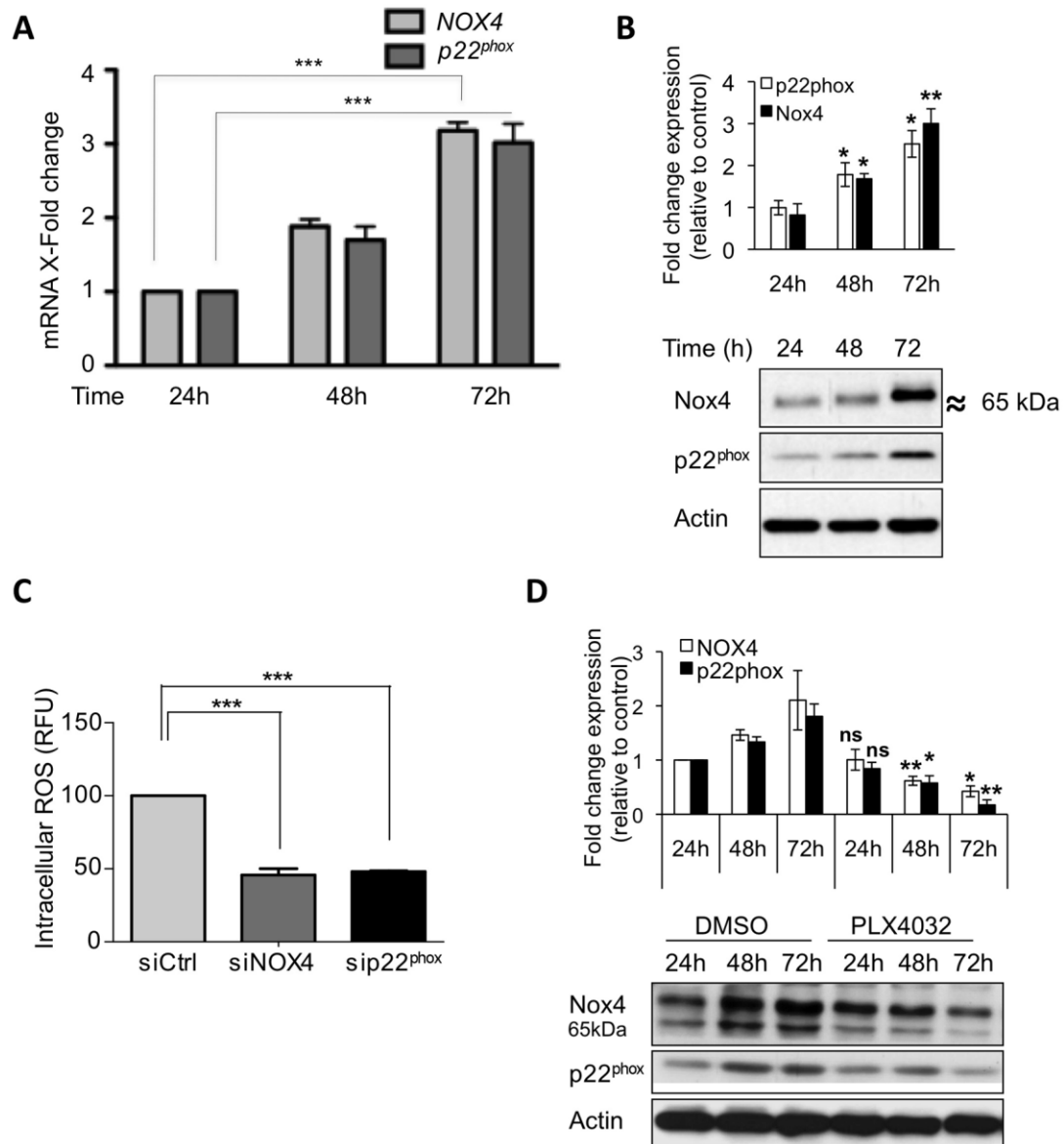
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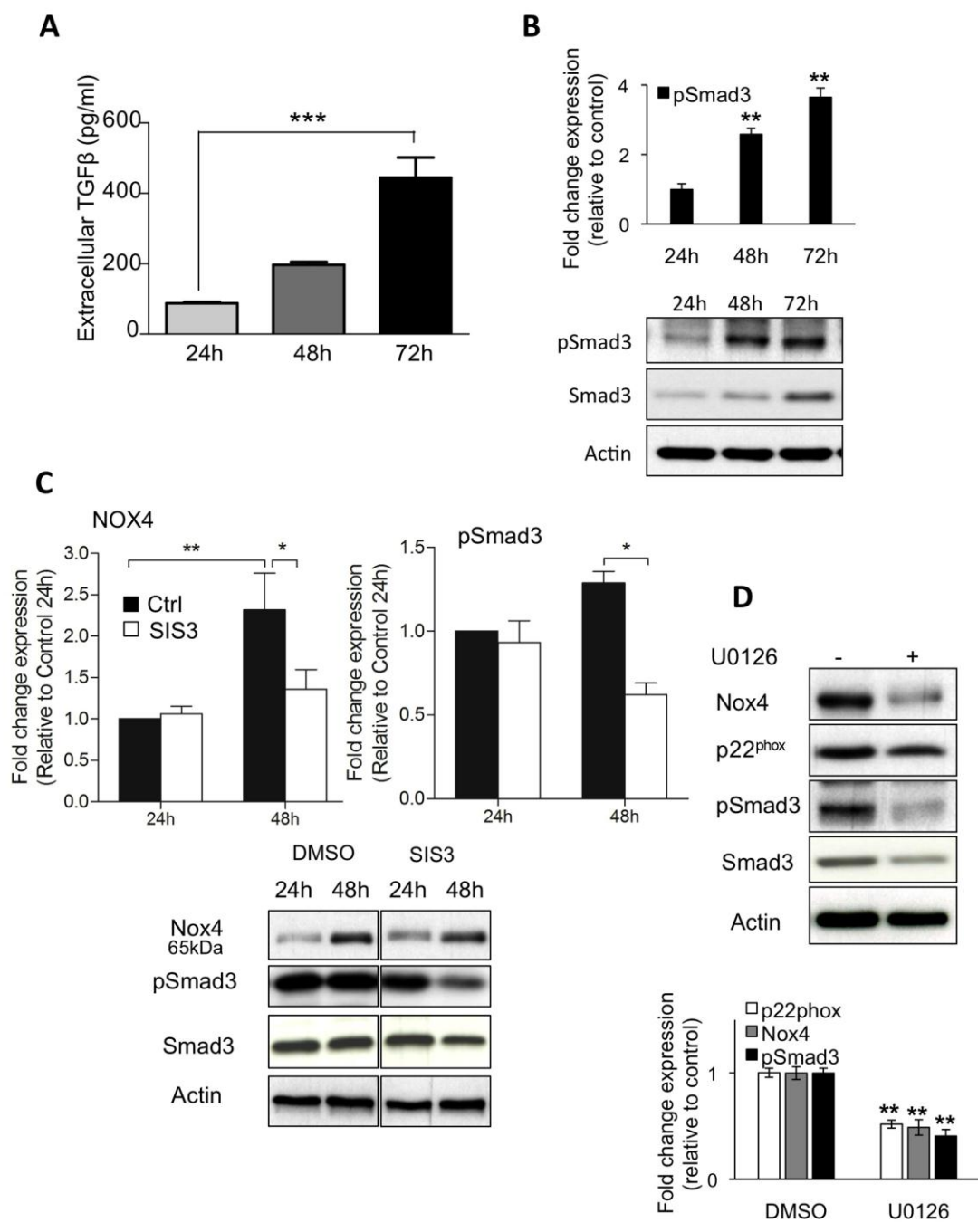
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## Figures legends



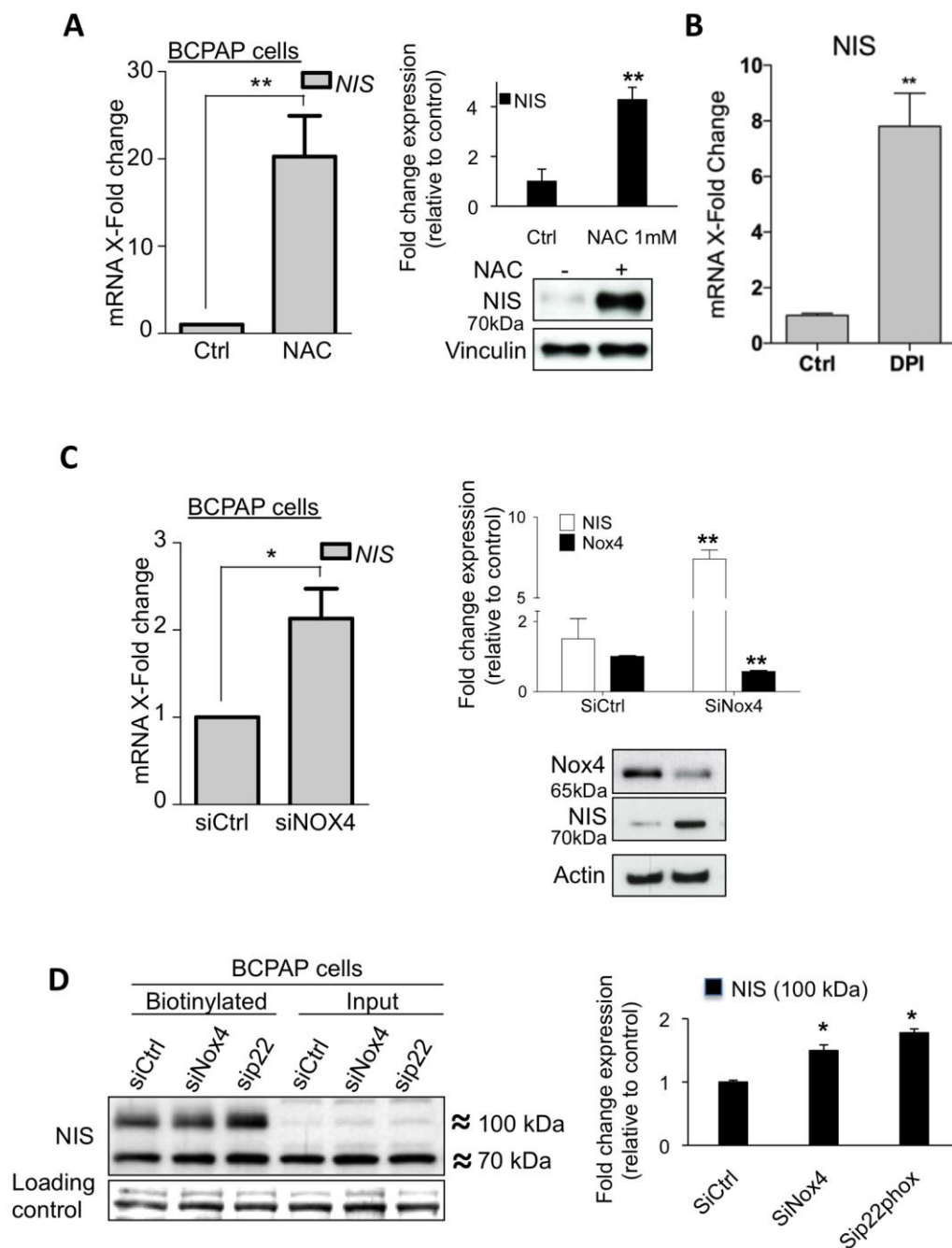
**Figure 1: Expression of NADPH oxidase NOX4 and its functional partner p22<sup>phox</sup> under the control of BRAF<sup>V600E</sup> oncogene.** Time course expression of NOX4 and p22<sup>phox</sup> in BCPAP. A- NOX4 and p22<sup>phox</sup> mRNA were quantified by qRT-PCR. B- NOX4 and p22<sup>phox</sup> proteins were analyzed by Western-blot as described in Materials and method. Densitometric

quantification of Nox4 and p22<sup>phox</sup> levels normalized to actin levels and presented as fold change compared with the basal level (24h). C- Intracellular ROS detection in BCPAP cells by DCF fluorescence using flow cytometry 48h after knocking down of NOX4 or p22<sup>phox</sup> with specific siRNA (10 nM). The relative fluorescence units (RFU) are normalized to 100 in the control. D- Immunoblot detection of NOX4 and p22<sup>phox</sup> in BCPAP cells after pharmacological inhibition of BRAF<sup>V600E</sup> oncogene by PLX4032 (10  $\mu$ M). Densitometric quantification of Nox4 and p22<sup>phox</sup> levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 24h. Student t test is realized by comparing PLX vs DMSO for each corresponding time of the kinetic. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01 and \*\*\*P < 0.001.



**Figure 2: BRAF<sup>V600E</sup> oncogene up-regulates NOX4 via TGF-β1 signalling pathway in papillary thyroid cancer cell line.** A- Quantitative detection of secreted and accumulated TGF-β1 in the extracellular medium of BCPAP cells using ELISA test. B- Western blot analysis of total and phospho Smad3 proteins in BCPAP cells at indicated time of culture.

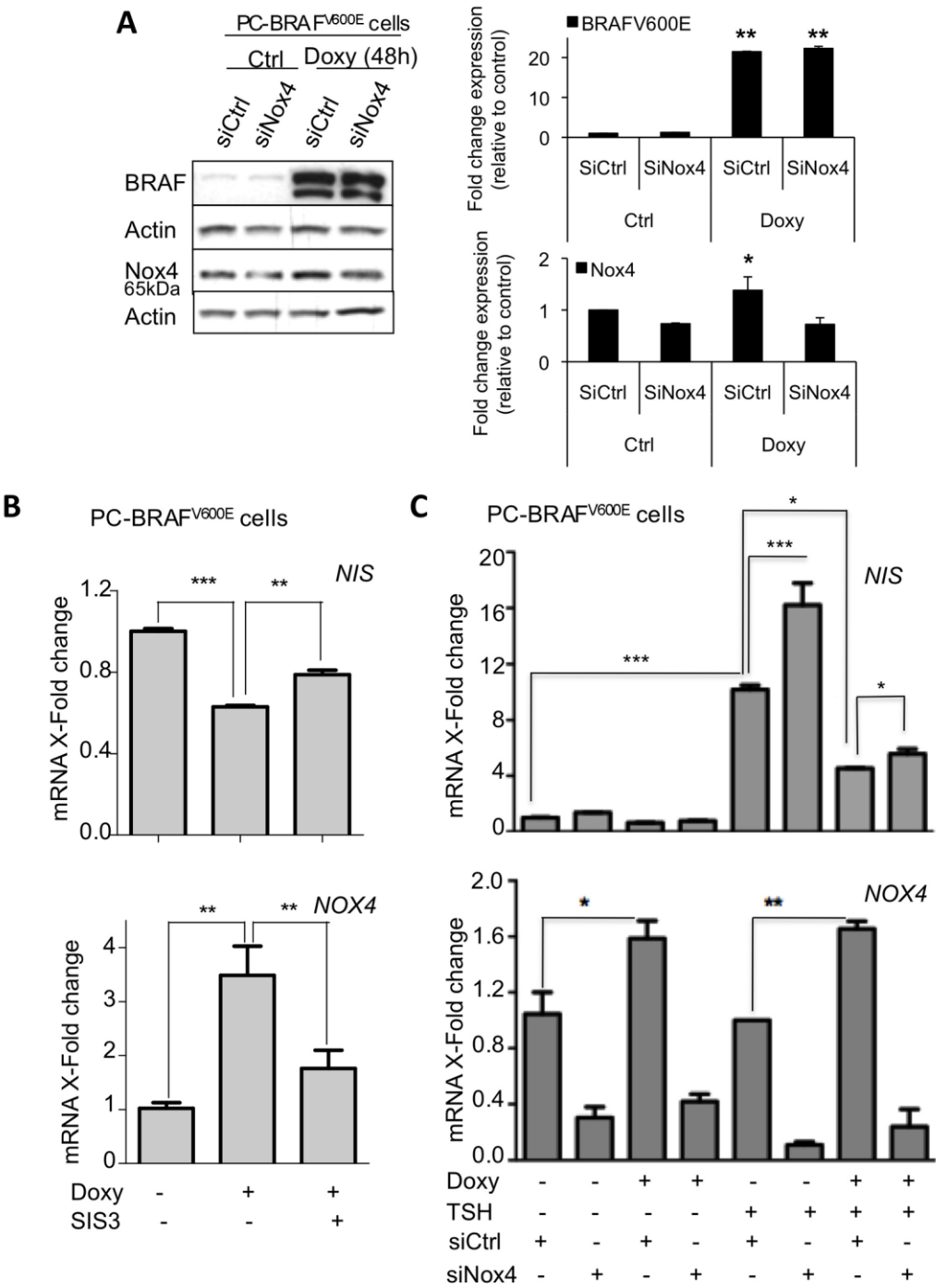
Densitometric quantification of phospho Smad3 levels normalized to actin levels and presented as fold change compared with the basal level (24h). C- Immunoblot detection of Nox4, Smad3 and phospho-Smad3 proteins in BCPAP cells treated with SIS3 (10  $\mu$ M). Densitometric quantification of Nox4 and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells or inhibitor-treated cells for 24h. Student t test is realized by comparing SiS3 vs DMSO for each corresponding time of the kinetic. D- Inhibition of NOX4, p22<sup>phox</sup>, Smad3 and phospho-Smad3 protein expressions by the specific inhibitor of MAPK pathway U0126 (10  $\mu$ M). Proteins were visualized by western-blot in BCPAP cells treated with U0126 for 48h. Densitometric quantification of Nox4, p22<sup>phox</sup> and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 48h. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01 and \*\*\*P < 0.001.



**Figure 3: ROS-generating NOX4 down-regulates the sodium/iodide symporter NIS in both BRAF<sup>V600E</sup> mutated thyroid cell lines.** A- Expression of NIS (mRNA and protein) 24h after treatment of BCPAP cells with 1 mM of N-Acetyl-cysteine (NAC). Densitometric quantification of NIS protein level normalized to vinculin level and presented as fold change

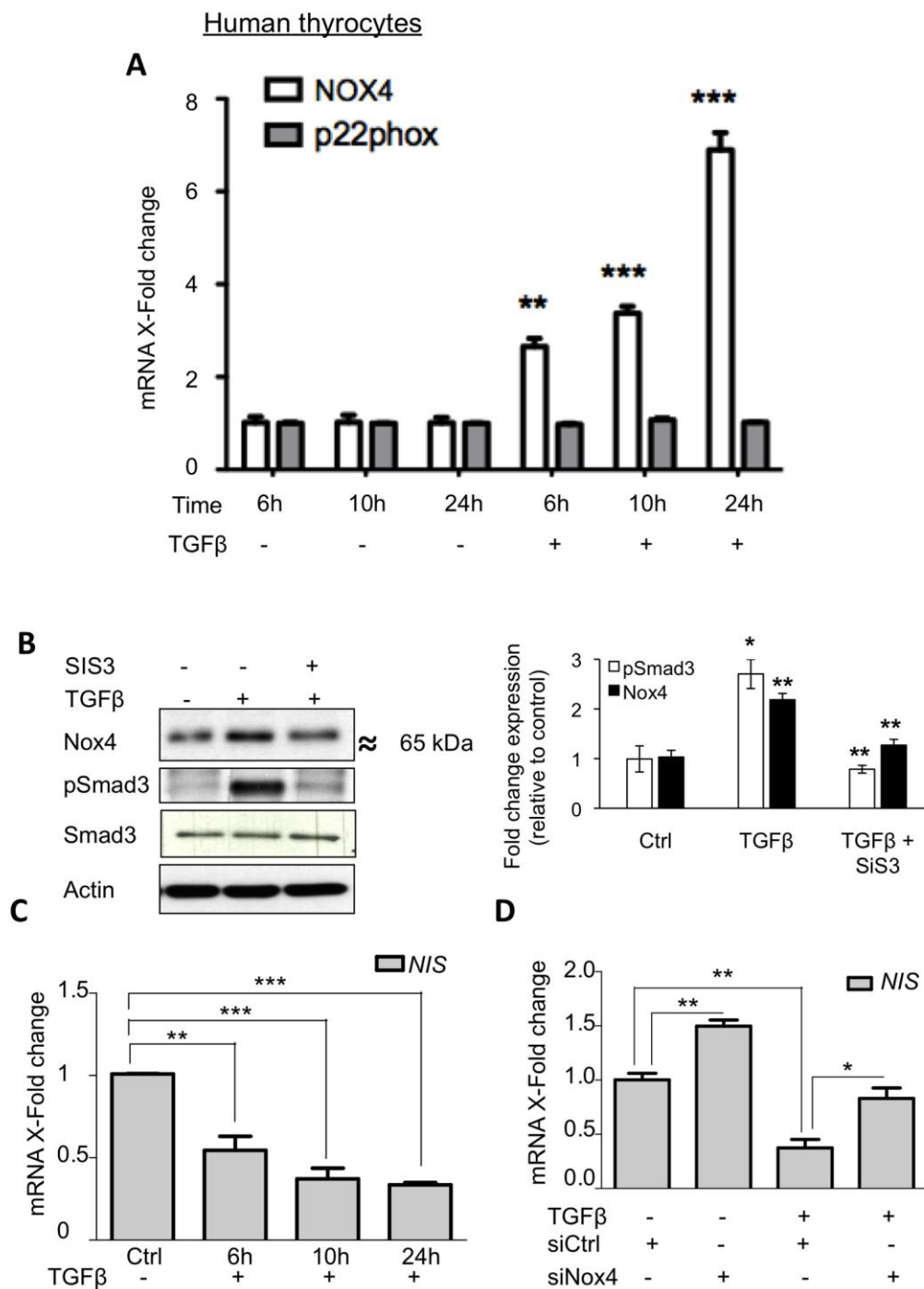


compared with vehicle-treated cells for 24h. B- Expression of NIS (mRNA) 24h after treatment of BCPAP cells with 2  $\mu$ M of Diphenyleneiodonium (DPI). C- Expression of NIS (mRNA and protein) 48h after knocking down of NOX4 in BCPAP cells. Densitometric quantification of Nox4 and NIS levels normalized to actin levels and presented as fold change compared with siRNA control-treated cells for 48h D- Western blot analysis of cell surface NIS expression in BCPAP cells, 48h after knocking down of NOX4 or p22<sup>phox</sup> by RNA interference. Red Ponceau staining at a corresponding molecular weight area of NIS protein was used as loading control. Densitometric quantification of NIS (hyperglycosylated form of 100 kDa) levels normalized to loading control levels and presented as fold change compared with siRNA control-treated cells for 48h. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01.



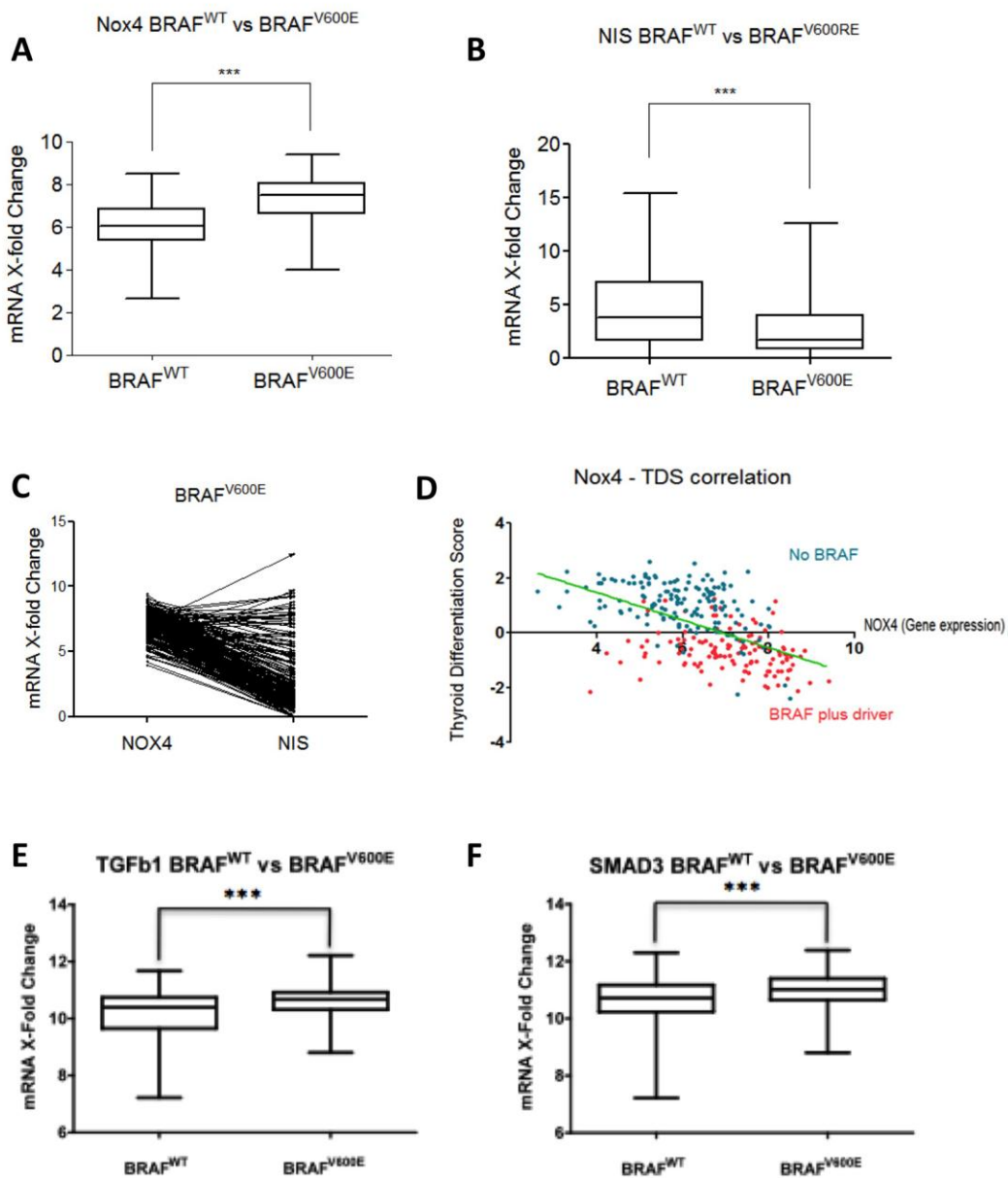
**Figure 4: NOX4 induced by BRAF<sup>V600E</sup> via Smad3 signalling pathway down-regulates NIS in thyroid PCC13 cells.** A- Western blot analysis of BRAF<sup>V600E</sup> and NOX4 protein expressions in PC-BRAF<sup>V600E</sup> cells treated for 48h with or without doxycycline (1µg/ml). Densitometric quantification of BRAF<sup>V600E</sup> levels normalized to actin levels and presented as

fold change compared with vehicle-treated cells for 48h. Densitometric quantification of Nox4 levels normalized to actin levels and presented as fold change compared with siRNA control-treated cells for 48h in the absence of doxycycline. B- QRT-PCR analysis of NIS and NOX4 mRNA levels in PC-BRAF<sup>V600E</sup> cells treated for 48h with or without doxycycline (1µg/ml) and SIS3 (10 µM). C- QRT-PCR analysis of NIS and NOX4 mRNA levels in PC-BRAF<sup>V600E</sup> cells treated for 48h in the absence or presence of TSH (1 mU/L, doxycycline (1µg/ml) and specific siRNAs (10 nM). Values are mean ± SE. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.



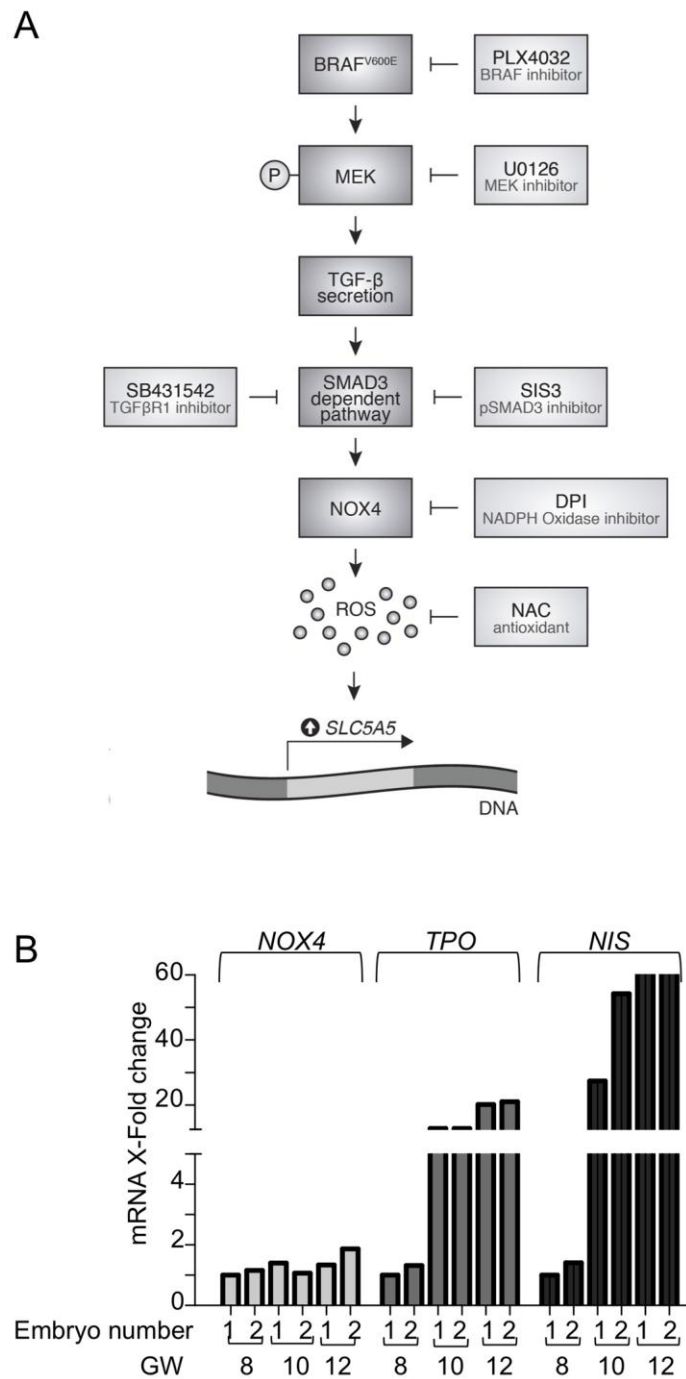
**Figure 5: NOX4 is involved in TGF- $\beta$ -induced down-regulation of NIS in human thyrocytes.** A- Time course expression of NOX4 and p22<sup>phox</sup> mRNAs (qRT-PCR) in human thyrocytes treated with human recombinant TGF- $\beta$  (10 ng/ml). B- Western blot analysis of NOX4, Smad3 and phospho-Smad3 in human thyrocytes treated for 12h in the absence or

presence of human recombinant TGF- $\beta$  (10 ng/ml) and SIS3 (10  $\mu$ M). Densitometric quantification of Nox4 and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with untreated cells. Student t test is realized by comparing TGF $\beta$  with Ctrl and TGF $\beta$  + SiS3 with TGF $\beta$ . C- Time course analysis of expression of NIS mRNA (qRT-PCR) in human thyrocytes treated with human recombinant TGF- $\beta$  (10 ng/ml). D- QRT-PCR analysis of NIS mRNA expression in human thyrocytes treated or not by human recombinant TGF- $\beta$  (10 ng/ml for 12h) in the presence of specific siRNAs (10 nM for 48h). Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.



**Figure 6: High level of NOX4 mRNA is correlated with low level of NIS mRNA and low Thyroid Differentiation Score (TDS) in papillary thyroid carcinoma (PTC) harboring BRAF<sup>V600E</sup> mutation.** A homogeneous cohort of 390 PTCs (170 BRAF<sup>WT</sup> PTCs and 220 BRAF<sup>V600E</sup> PTCs) were included in this analysis. Correlative analysis between NOX4 mRNA

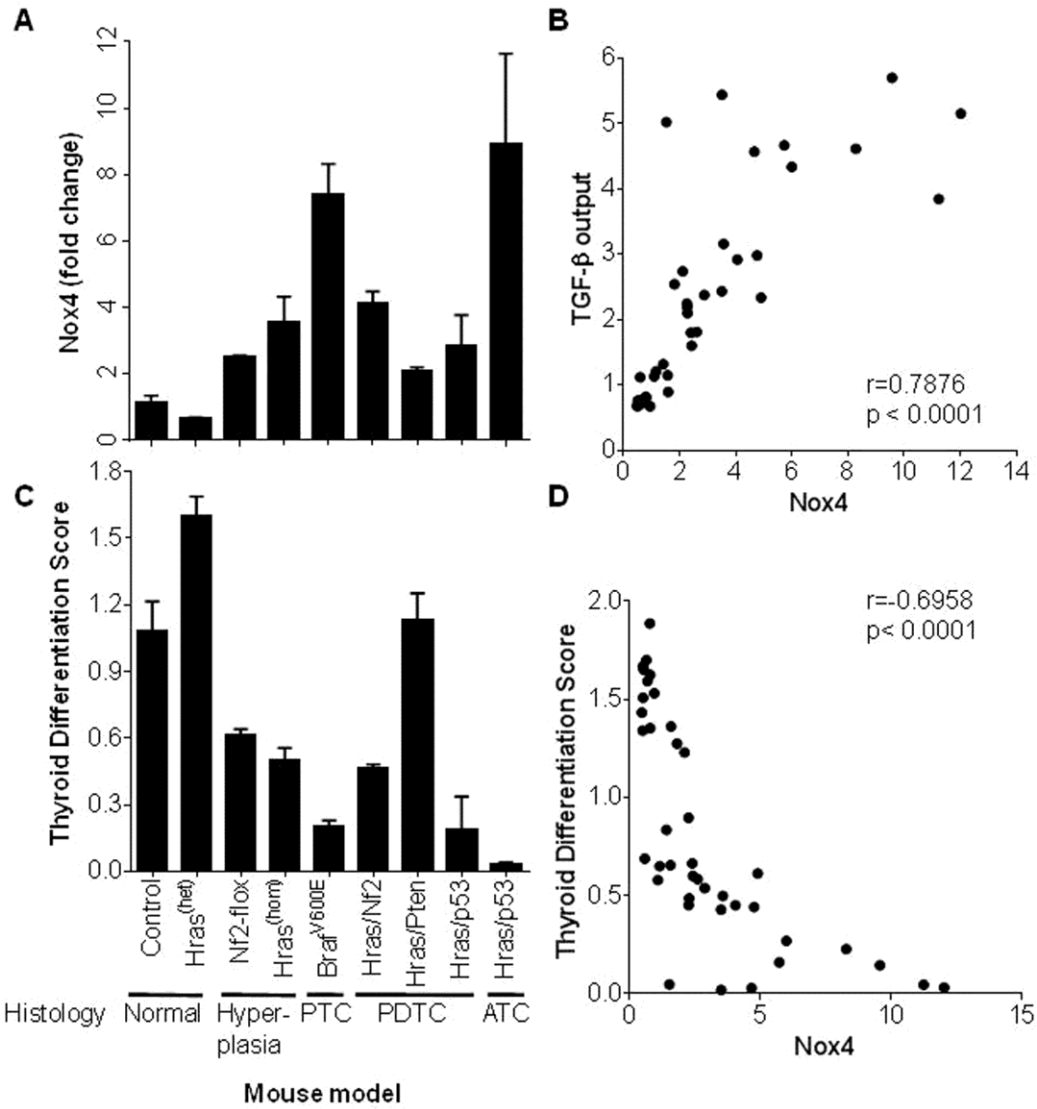
(A) or NIS mRNA levels (B) and BRAF<sup>V600E</sup> mutation in 390 PTCs. Correlation of NOX4 mRNA level and NIS mRNA level in 220 PTCs harboring BRAF<sup>V600E</sup> mutation (C). Correlative analysis between BRAF<sup>V600E</sup> mutation and Thyroid Differentiation Score (TDS) in 390 PTCs (D). Correlative analysis between TGF- $\beta$ 1 mRNA (E) or Smad3 mRNA levels (F) and BRAF<sup>V600E</sup> mutation in 390 PTCs



**Figure 7: A-** A schematic model of all the steps involved in the oncogenic BRAF-dependent regulation of NOX4. Our data show that BRAF<sup>V600E</sup> up-regulates NOX4



expression via the TGF-B1/Smad3 signaling pathway in thyroid cancer cells. The NOX4-dependent ROS production plays a key role in SLC5A5 gene repression. Consequently, it might be involved in the absence of radioiodide uptake by BRAF-mutated cancer cells. The targets of different inhibitors used in this study are indicated. **B- Ontogenic changes of mRNA expression of NIS, TPO and NOX4.** QRT-PCR analysis of NOX4, TPO and NIS mRNA expression in 6 human thyroids at 8, 10 or 12 weeks of development. GW: gestational week.



**Figure 8: High level of NOX4 mRNA is correlated with low thyroid differentiation score in genetically engineered mouse models of thyroid cancer. Gene expression profiling of GEM models of thyroid hyperplasia or the indicated thyroid cancer phenotypes. A-** Fold change in NOX4 expression compared to normal thyroid controls calculated from

Affymetrix expression array data. B- Association of NOX4 expression levels with the TGF $\beta$  transcriptional output score. Pearson correlation was used to calculate r- and p-values. C- Thyroid differentiation score for each mouse model. D- Correlation between thyroid differentiation score and NOX4 expression. Pearson correlation was used to calculate r- and p-values.

## Supporting Informations

## Supplementary Figures

**Supplementary Table S2:** Genes used in calculation of TGF $\beta$  output and thyroid differentiation (TDS) scores.

TGF $\beta$	TDS
Abcb1b	Dio1
Acvr1	Dio2
Acvr1b	Nkx2-1
Bmpr2	Pax8
Btg1	Slc26a4
Cda	Slc5a5
Cldn4	Slc5a8
Ddi2 /// Rsc1a1	Tg
Ddit4	Thra
Dyrk3	Thrb
Foxq1	Tpo
Gadd45a	Tshr
Gadd45g	
Gcnt2	
Hmga2	
Id2	
Ier3	
Jag1	
Lgmn	
Lpgat1	
Lrp1	
Ndel1	
Nrp1	
Pcdh1	
Pdcd10	
Pdlim7	
Prg4	
Rdh10	
Relb	
Serpine1	
Sh3bp2	
Skil	
Slc25a37	
Smad7	
Snx30	
Sox4	
Svil	
Tex10	
Tex10	
Tfpi2	
Tmsb4x	
Tnfrsf1b	
Trib3	
Ubr2	
Zcchc10	
Zfp125	
Zfp36l2	

**Table S1:** Sequences of selected siRNAs

**Table S2 : Genes used in calculation of TGF $\beta$  output and thyroid differentiation (TDS) scores**

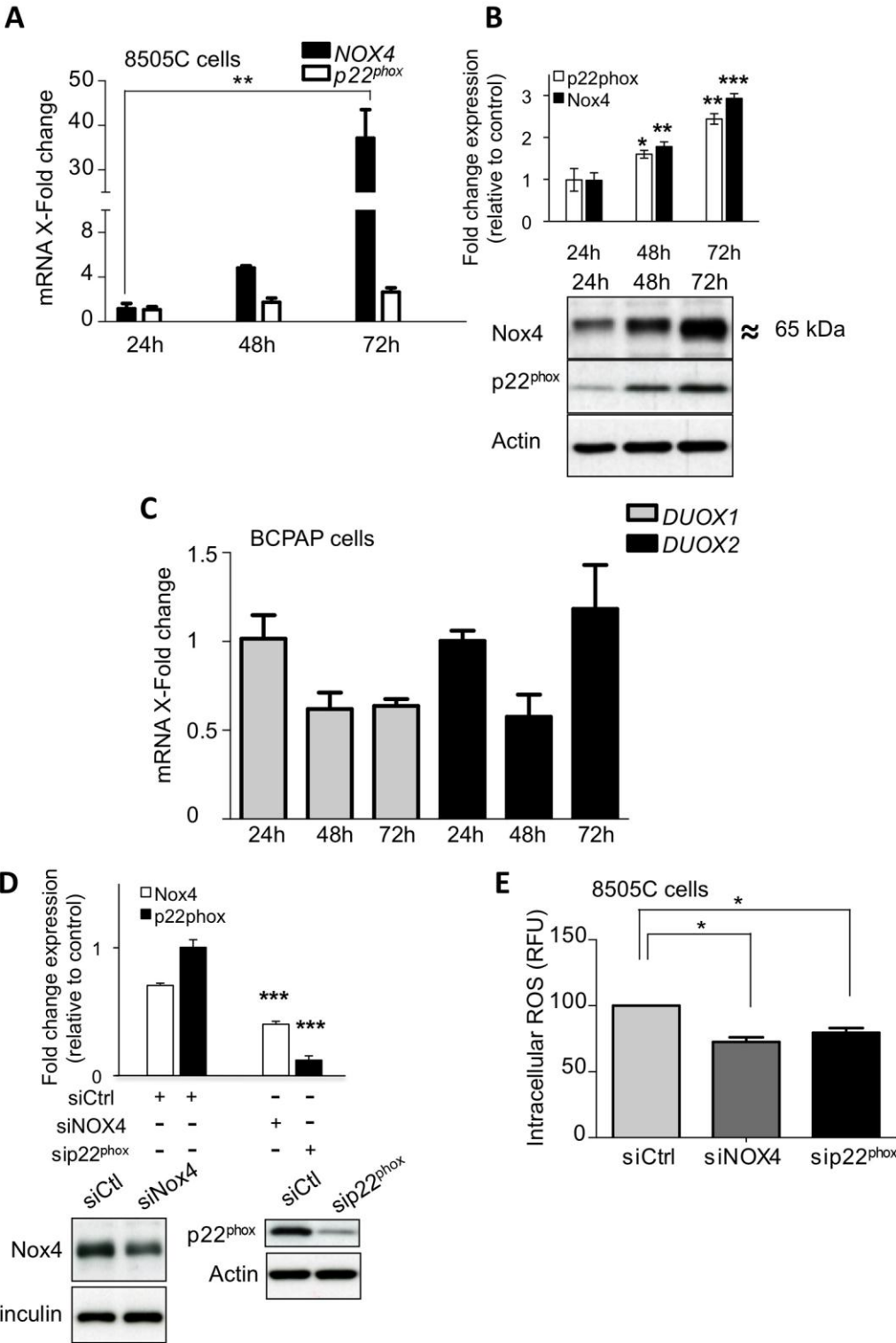


Figure S1  
**Figure S1: Expression of NADPH oxidase NOX4 and its functional partner p22<sup>phox</sup> under the control of BRAF<sup>V600E</sup> oncogene.** Time course expression of

NOX4 and p22<sup>phox</sup> in 8505C cells. A- NOX4 and p22<sup>phox</sup> mRNA were quantified by qRT-PCR. B- NOX4 and p22<sup>phox</sup> proteins were analyzed by Western-blot as described in Materials and method. Densitometric quantification of Nox4 and p22<sup>phox</sup> levels normalized to actin levels and presented as fold change compared with the basal level (24h). C- Time course analysis of DUOX1 and DUOX2 mRNA expressions by qRT-PCR in BCPAP cells at indicated time culture. D- Nox4 and p22<sup>phox</sup> expressions after treatment of cells with siRNA directed against Nox4 or p22<sup>phox</sup> respectively. Densitometric quantification of Nox4 and p22<sup>phox</sup> levels normalized to vinculin or actin levels and presented as fold change compared with siRNA control-treated cells. E- Intracellular ROS detection in 8505C cells by DCF fluorescence using flow cytometry 48h after knocking down of NOX4 or p22<sup>phox</sup> with specific siRNA (10 nM). The relative fluorescence units (RFU) are normalized to 100 in the control. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01 and \*\*\*P < 0.001.

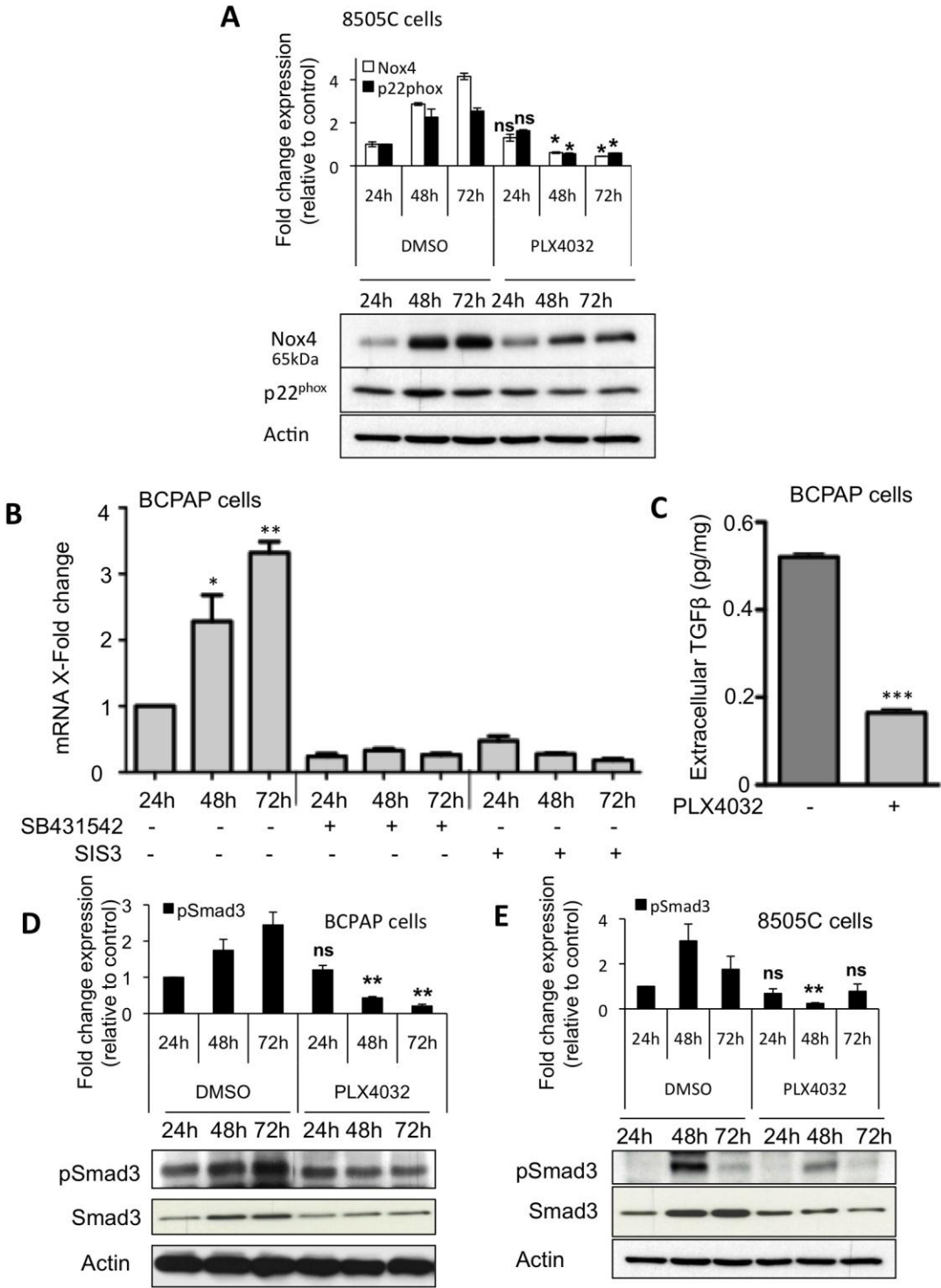


Figure S2

**Figure S2: BRAF<sup>V600E</sup> oncogene up-regulates NOX4 via TGF-β1 signalling pathway in papillary thyroid cancer cell line.** A- Immunoblot detection of NOX4



and p22<sup>phox</sup> in 8505C cells after pharmacological inhibition of BRAF<sup>V600E</sup> oncogene by PLX4032 (10  $\mu$ M). Densitometric quantification of Nox4 and p22<sup>phox</sup> levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 24h. Student t test is realized by comparing PLX vs DMSO for each corresponding time of the kinetic. B- Time course expression of NOX4 mRNAs in BCPAP cells at indicated time culture in the absence or presence of specific inhibitors: of TGF  $\beta$ 1 pathway SB431542 (10  $\mu$ M) or of Smad3 (SIS3 10  $\mu$ M). C- Quantitative detection of secreted TGF- $\beta$ 1 in the extracellular medium of BCPAP cells in the absence or presence of PLX4032 (10  $\mu$ M) for 72h. D- Western blot analysis of Smad3 and phospho Smad3 at indicated time of culture of BCPAP cells treated with PLX4032 (10  $\mu$ M). Densitometric quantification of phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 24h. Student t test is realized by comparing PLX vs DMSO for each corresponding time of the kinetic . E- Time-dependent expression of Smad3 and phospho Smad3 in 8505C cells treated or not with PLX4032 (10  $\mu$ M). Densitometric quantification of phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 24h. Student t test is realized by comparing PLX vs DMSO for each corresponding time of the kinetic. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01 and \*\*\*P < 0.001.

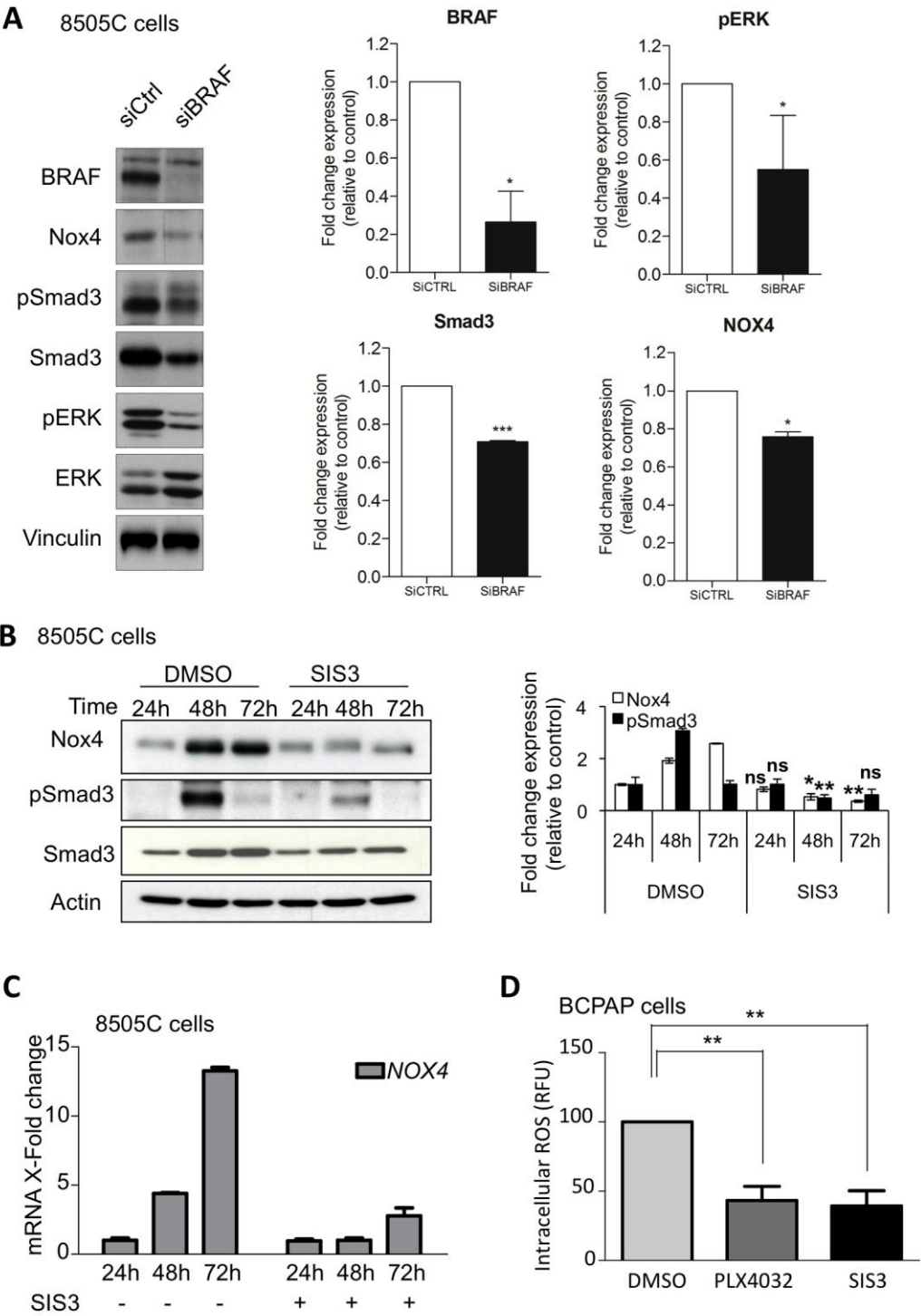


Figure S3

**Fig S3:** A- Western blot analysis of BRAF, Nox4, Smad3, phospho-Smad3, phospho-ERK and ERK expressions in 8505C cells 48h after knocking down of BRAF by RNA

interference. Densitometric quantification of protein levels normalized to vinculin levels and presented as fold change compared with siRNA control-treated cells for 48h B- Time-dependent expressions of Nox4, Smad3 and phospho Smad3 in 8505C cells treated or not with SIS3 (10  $\mu$ M). Densitometric quantification of Nox4 and phospho-Smad3 levels normalized to actin levels and presented as fold change compared with vehicle-treated cells for 24h. Student t test is realized by comparing SiS3 vs DMSO for each corresponding time of the kinetic. C- Time course expression of NOX4 mRNA analyzed by qRT-PCR in 8505C cells treated or not with SIS3 (10  $\mu$ M). D- Intracellular ROS detection by DCF fluorescence using flow cytometry in BCPAP cells treated for 48h with PLX4032 (10  $\mu$ M) or SIS3 (10  $\mu$ M). The relative fluorescence units (RFU) are normalized to 100 in the control. Values are mean  $\pm$  SE.

\*P < 0.05; \*\*P < 0.01.

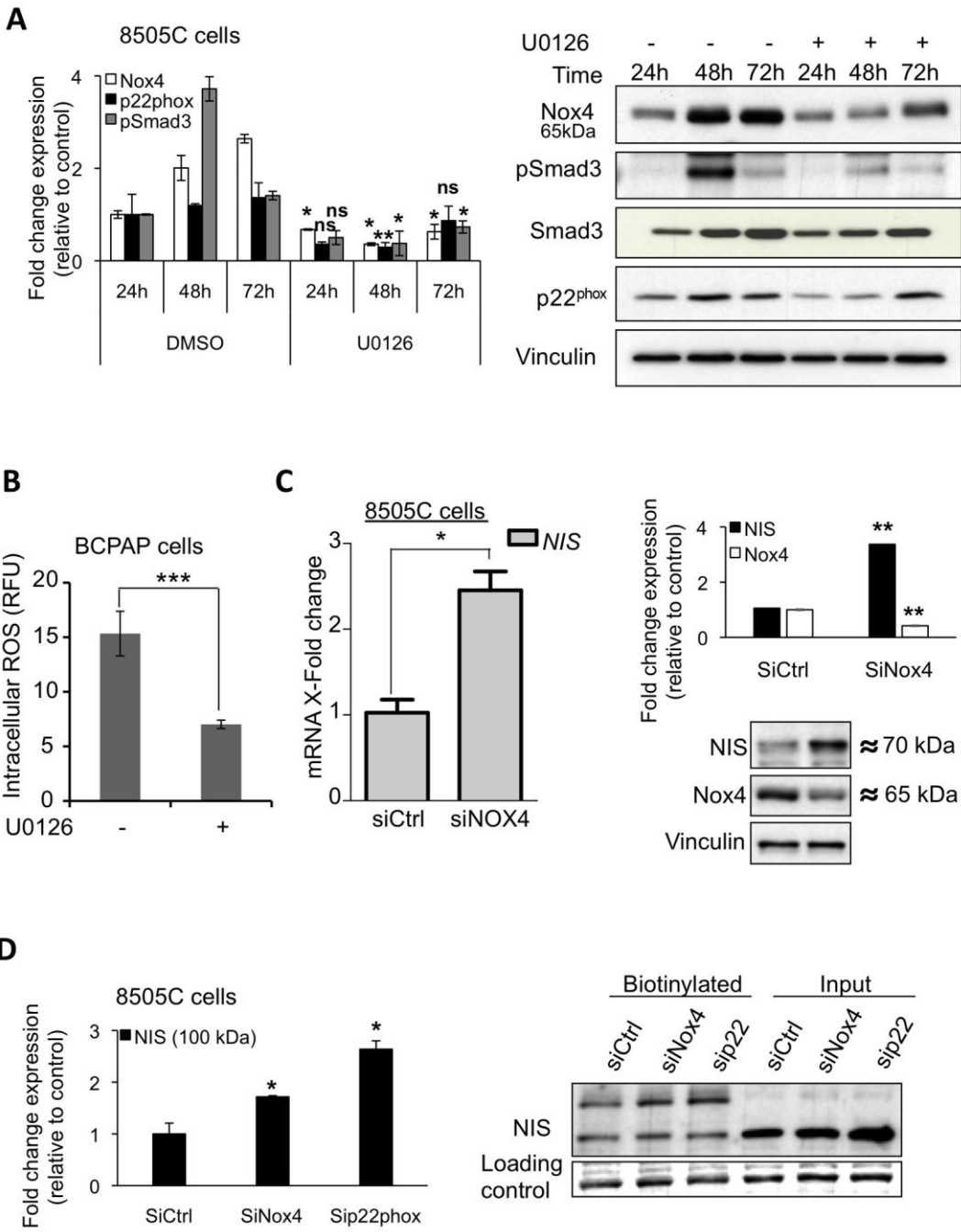


Figure S4

**Fig S4** : A- Time-dependent expressions of NOX4, p22<sup>phox</sup>, Smad3 and phospho-Smad3 in 8505C cells treated or not with U0126 (10 μM). Densitometric

quantification of Nox4, p22<sup>phox</sup> and phospho-Smad3 levels normalized to vinculin levels and presented as fold change compared with vehicle-treated cells for 24h. Student t test is realized by comparing U0126 vs DMSO for each corresponding time of the kinetic. . B- Intracellular level of ROS measured at 48h in BCPAP cells treated or not with U0126 (10  $\mu$ M). The detection was done by DCF fluorescence using flow cytometry. C- Expression of NIS (mRNA and protein) 48h after knocking down of Nox4 in 8505C cells. Densitometric quantification of Nox4 and NIS levels normalized to vinculin levels and presented as fold change compared with siRNA control-treated cells for 48h. D- Western blot analysis of cell surface NIS expression in 8505C cells 48h after knocking down of NOX4 or p22<sup>phox</sup> by RNA interference. Red Ponceau staining at a corresponding molecular weight area of NIS protein was used as loading control. Densitometric quantification of NIS (hyperglycosylated form of 100 kDa) levels normalized to loading control levels and presented as fold change compared with siRNA control-treated cells for 48h.. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01 and \*\*\*P < 0.001.

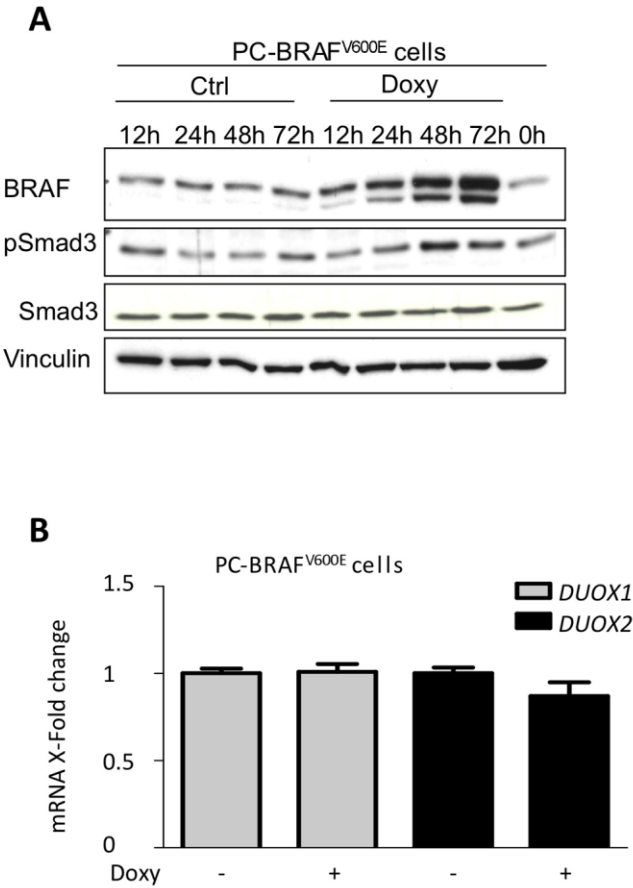
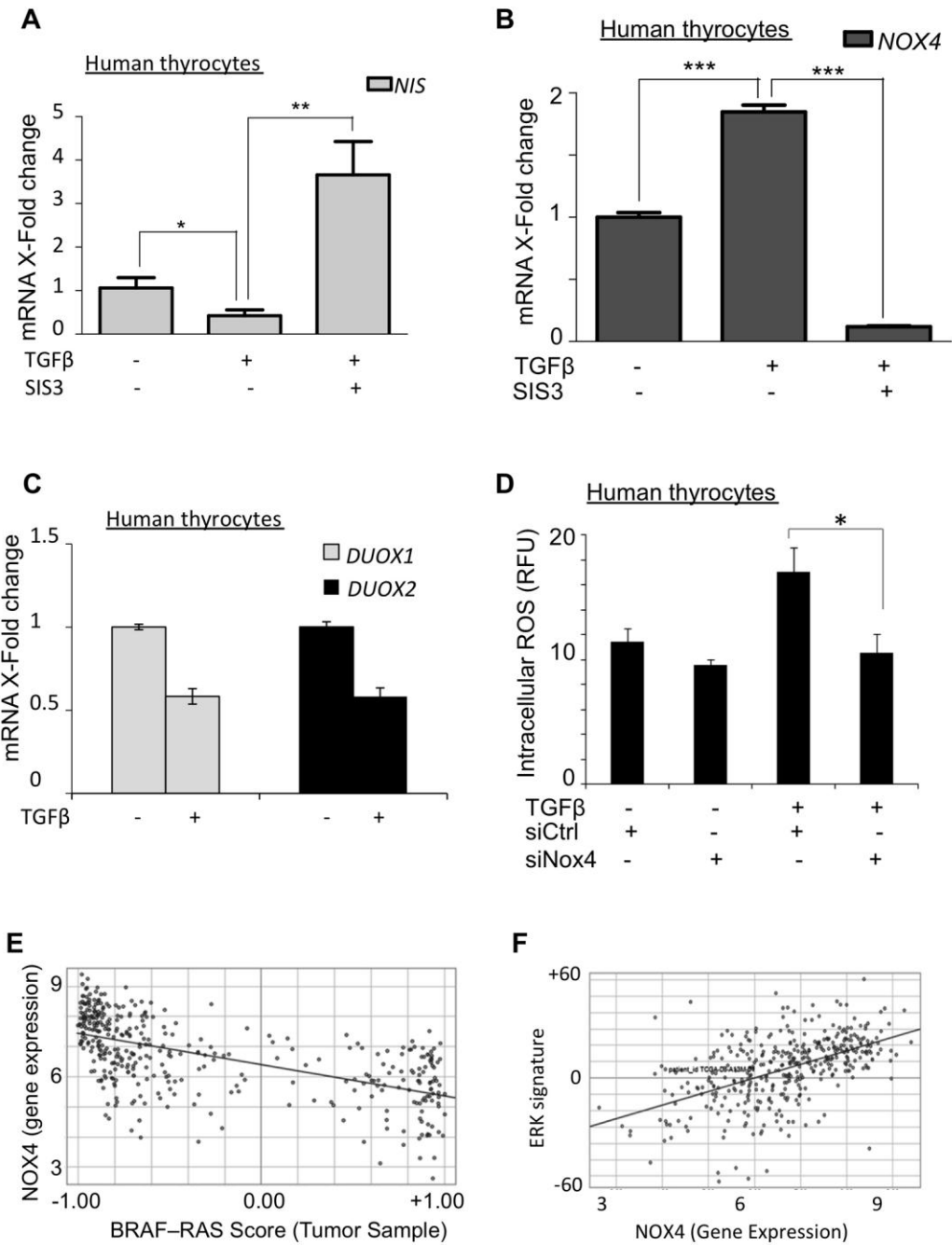


Figure S5

**Fig S5:** A- Time course analysis of BRAF<sup>V600E</sup>, Smad3 and phospho-Smad3 expressions in PC BRAF<sup>V600E</sup> cells treated with 1µg/ml of doxycycline. Vinculin was

used as a loading control. B- QRT-PCR analysis of DUOX1 and DUOX2 mRNA levels in PC-BRAF<sup>V600E</sup> cells in the absence or presence of doxycycline (1µg/ml) for 48h

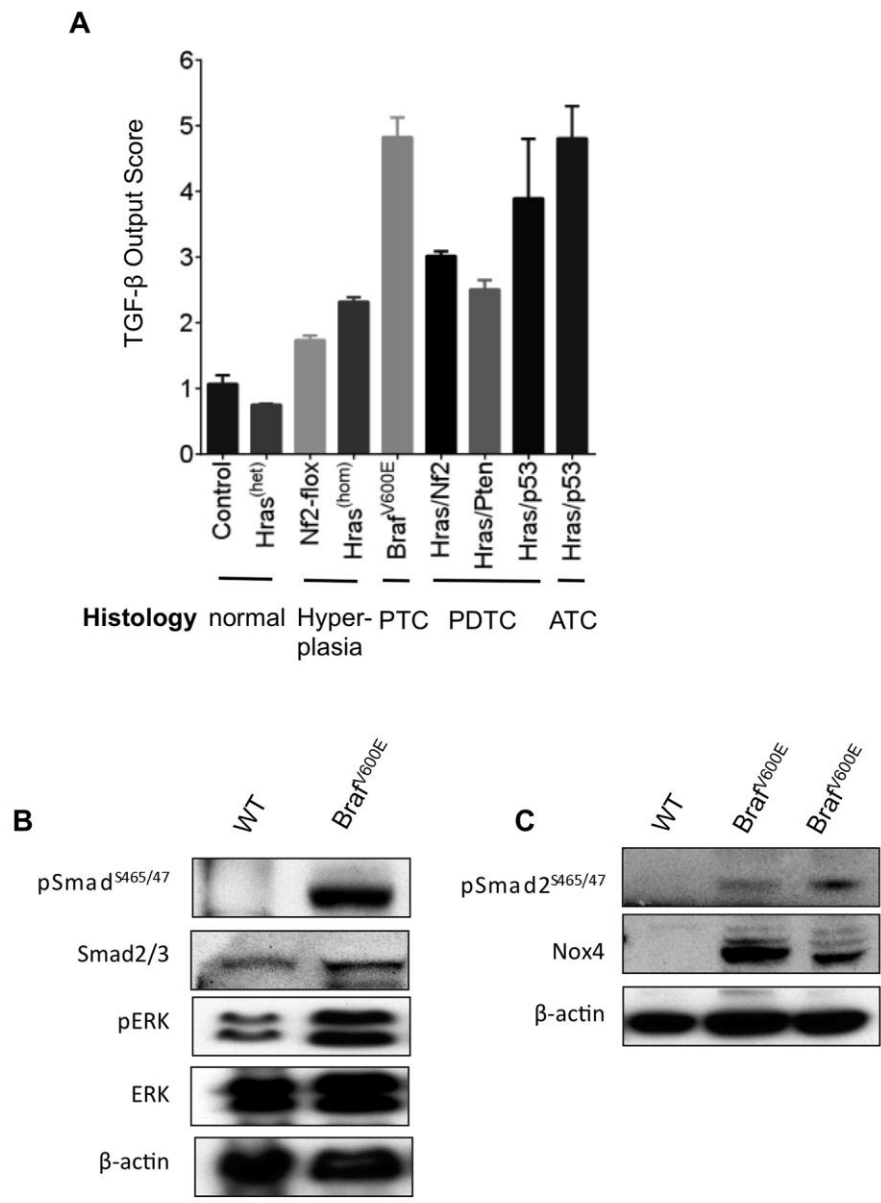


**Fig S6 : NOX4 is involved in TGF-β-induced down-regulation of NIS in human thyrocytes.** A- Expression of NIS mRNA evaluated by qRT-PCR in human



thyrocytes treated or not for 12h with human recombinant TGF- $\beta$  (10 ng/ml) and SIS3 (10 $\mu$ M). B- QRT-PCR analysis of NOX4 mRNA level in human thyrocytes treated in the absence or presence of human recombinant TGF- $\beta$ 1 (10 ng/ml for 12h) with or without SIS3 (10 $\mu$ M). C- QRT-PCR analysis of DUOX1 and DUOX2 mRNA levels in human thyrocytes treated or not for 12h with human recombinant TGF $\beta$ 1 (10 ng/ml). D- Intracellular level of ROS measured at 48h in human thyrocytes treated with control or specific siRNAs in the absence or presence of human recombinant TGF- $\beta$ 1. Values are mean  $\pm$  SE. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

**Analysis of TCGA data.** Correlative analysis between NOX4 gene expression and BRAF-RAS score (BRS) in human PTC (E). Correlative analysis between NOX4 gene expression and ERK activation (F).



**Fig S7 : High level of NOX4 mRNA is correlated with high level of TGF-β mRNA in genetically engineered mouse models of thyroid cancer. A- TGF-β output**

score in different thyroid cancer mouse models. B-C Activation of TGF $\beta$  pathway in papillary thyroid cancers of 13 week old LSL-Braf<sup>V600E</sup>/TPO-Cre (Braf<sup>V600E</sup>) Mice. Protein lysates were prepared from thyroids of wild-type and LSL-Braf<sup>V600E</sup>/TPO-Cre mice and subjected to Western blotting for the indicated proteins. B- Increased phosphorylation of Smad2 in Braf<sup>V600E</sup>-induced PTCs as compared to wild type thyroids. C- Increased phospho-Smad in Braf<sup>V600E</sup>-induced PTC is associated with higher NOX4 protein levels.

**Titre :** Rôle de la NADPH oxydase 4 dans la régulation redox du symporteur sodium (Na<sup>+</sup>)/ iodure (I<sup>-</sup>) dans le cancer papillaire de la thyroïde

**Mots clés :** Cancer de la thyroïde, NIS, NOX4, TGFβ

**Résumé :** Le symporteur Na<sup>+</sup>/I<sup>-</sup> (NIS) médie la capture de l'iodure par les thyrocytes et cette propriété est exploitée depuis de nombreuses années en thérapeutique pour traiter les cancers différenciés de la thyroïde à l'iode radioactif I<sup>131</sup> (Radiothérapie métabolique ou RAI). Cependant, 5 à 10% des patients deviennent réfractaires à la RAI, ce qui indique un mauvais pronostic. La réduction de l'expression NIS et son internalisation sont caractéristiques de ce processus. La mutation activatrice BRAF<sup>V600E</sup> est la plus fréquemment identifiée au sein des cancers différenciés de la thyroïde de type papillaires (CPT), qui est le type le plus répandu. Dans les thyrocytes de souris, BRAF muté induit la sécrétion de TGFβ qui active ensuite la voie Smad ce qui entraîne la répression du NIS. La NADPH oxydase 4 (NOX4), enzyme génératrice d'espèces réactives de l'oxygène (ROS), est un médiateur clé de la signalisation du TGFβ dans de nombreux types cellulaires et sa surexpression a été détectée dans le cancer de la thyroïde. L'objectif de ce travail est d'évaluer si NOX4 est un médiateur de la répression NIS induite par BRAF<sup>V600E</sup> dans des lignées cellulaires thyroïdiennes. En utilisant une lignée cellulaire de thyroïde normale de rat (PC-BRAF), nous avons démontré que le TGF-β ou l'expression BRAF<sup>V600E</sup> promeut la diminution de l'ARNm NIS ainsi que celle du captage de l'iodure en revanche ils augmentent l'expression de l'ARNm NOX4.

Le silençage de Nox4 par siRNA ou un traitement des cellules par SIS3, un inhibiteur de pSmad3, inhibe la répression du NIS médiée par BRAF<sup>V600E</sup>, indiquant l'implication de la voie Smad3 et de Nox4 dans cette répression. Dans la lignée tumorale BCPAP dérivée d'un CPT humain et porteuse de la mutation BRAF<sup>V600E</sup>, nous avons également observé une augmentation de l'expression de l'ARNm NIS lorsque BRAF<sup>V600E</sup> ou NOX4 sont inhibés. Dans les cellules BCPAP, un traitement par H<sub>2</sub>O<sub>2</sub> augmente l'expression de la protéine ADN méthyltransférase 1 (DNMT1) dans la fraction cellulaire enrichie en protéines liées à la chromatine. Un traitement par des antioxydants ou le silençage de NOX4 réduit ce recrutement. Le TGFβ augmente le niveau de protéines DNMT1 dans la fraction cellulaire enrichie en chromatine, lequel est renversé par un traitement par un inhibiteur de NADPH oxydase : le Diphenyleiodonium (DPI). La méthylation de l'ADN induite par le TGFβ et l'hypoacétylation de l'histone H3K9/K14, qui sont des marques de répression de la transcription, sont détectées au niveau du promoteur NIS. L'ensemble de ces données suggère que Nox4 est un acteur clé de la signalisation TGF-β et joue un rôle répressif sur l'expression du NIS, probablement par des mécanismes épigénétiques. Cette étude apporte des données fonctionnelles pour le développement de nouveaux outils thérapeutiques.

**Title :** Role of NADPH oxidase 4 in the redox regulation of the sodium (Na<sup>+</sup>)/ iodide (I<sup>-</sup>) symporter in papillary thyroid cancer

**Keywords :** Thyroid Cancer, NIS, NOX4, TGFβ

**Abstract :** The Na<sup>+</sup>/I<sup>-</sup> symporter (NIS) mediates iodide uptake by thyrocytes, which is a key step in hormone biosynthesis. Iodide accumulation by the thyroid gland is the basis of radioiodine therapy (RAI) that is the standard post-surgery therapeutic approach to efficiently eliminate the remaining cancer lesions and metastasis of differentiated thyroid cancer (DTC). However, 5-10% of DTC patients become RAI-refractory, which is indicative of poor prognosis. Reduced NIS expression and NIS internalization are involved in this process. BRAFV600E mutation is the most common genetic event in papillary thyroid cancers (PTCs), the most prevalent type of DTC. In rat thyrocytes, the expression of BRAFV600E induces secretion of TGFβ that activates Smad pathway resulting in NIS downregulation and overexpression of TGFβ is associated with NIS repression in patients. NADPH oxidase NOX4, an enzyme specialized in reactive oxygen species (ROS) generation, is a key mediator of TGFβ signaling in many cell types and has been previously demonstrated to be overexpressed in thyroid cancers. To better understand the molecular mechanisms involved in PTC loss of iodide uptake ability, the aim of this work is to evaluate whether NOX4 plays a key role in BRAFV600E-mediated NIS repression in thyroid cell lines. Using a normal rat thyroid cell line (PC-BRAF), we demonstrated that TGF-β administration or the expression of

BRAFV600E resulted in reduced NIS mRNA, reduced iodide uptake and increased NOX4 mRNA expression. NOX4 silencing or treatment with SIS3 an inhibitor of Smad pathway partially inhibited NIS repression indicating the implication of both Smad pathway and NOX4. To confirm these results we used two human thyroid cancer cell lines that harbor BRAF<sup>V600E</sup> mutation (BCPAP and 8505c) and observed an increase in NIS expression followed by BRAF<sup>V600E</sup> or NOX4 downregulation. Exogenous H<sub>2</sub>O<sub>2</sub> induced DNA methyltransferase 1 (DNMT1) enrichment in tight-chromatin protein fraction, which was decreased by antioxidants or NOX4 silencing in BCPAP cells. TGFβ increased DNMT1 protein levels in chromatin-enriched cell fraction that was reversed by the NADPH oxidase inhibitor Diphenyleiodonium (DPI). TGFβ-mediated DNA methylation and histone H3K9/K14 hypoacetylation were detected in NIS promoter, and both mechanisms correspond to repressive transcriptional markers. The data obtained suggest that NOX4 is a mediator of BRAF<sup>V600E</sup>-TGFβ signaling and that it exerts a repressive role upon NIS expression probably through epigenetic mechanisms. These data unravel novel mechanisms involved in the regulation of NIS expression in thyroid cancer, and describe possible targets for the development of new therapeutic tools.