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Overexpression of RAC1b in follicular cell derived thyroid cancers: An activator of NF-κB pathway that contributes to thyroid tumorigenesis?

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

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Setembro, 2015

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AGRADECIMENTOS

Aproveito este momento para agradecer a todos os que possibilitaram ou contribuíram para a realização desta tese de Mestrado.

Um especial agradecimento à minha orientadora Doutora Ana Luísa Silva por ter aceite orientar-me na jornada que foi a realização deste trabalho. Por todo o conhecimento e experiência transmitidos, pela autonomia concedida, pela confiança. Pelo incentivo, pelas palavras essenciais nos momentos certos que nunca me deixaram desanimar, por todo o apoio. Por ser um exemplo de competência, empenho, dedicação, esforço e por ser a prova de que a humildade e trabalho dão um sabor especial a cada vitória. Por fazer ciência porque gosta de ciência!

À Doutora Branca Cavaco, coordenadora da Unidade de Investigação em Patobiologia Molecular e do grupo de Endocrinologia Molecular (IPOLFG), por me acolher nesta unidade.

Quero agradecer também à professora Doutora Maria João Bugalho pela oportunidade, por toda a disponibilidade sempre demonstrada e pela simpatia.

A todos os meus colegas de grupo Rita Domingues, Margarida Moura, Liliana Capinha, Tiago Pinheiro, Ana Matias e Inês Marques, pelo companheirismo, disponibilidade e muito boa disposição. Foi muito importante!

Aos restantes elementos da UIPM que direta ou indiretamente contribuíram para este trabalho. Especialmente a todos aqueles que tornaram as pausas para o almoço momentos divertidos e de pura descontração. Foi essencial!

Ao Bruno Cardoso pela disponibilidade, boa disposição e pela ajuda no mundo que é a citometria de fluxo. "Sempre a 200%"! Também à Carolina e à Ana Matias pelas idas ao FACS, por toda a ajuda!

Ao Doutor Paulo Matos, por toda a ajuda neste trabalho, pela boa disposição e pela disponibilidade sempre demonstrada.

À coordenação e comissão científica do Mestrado em Genética Molecular e Biomedicina que proporcionam aos alunos todas as condições necessárias para a realização dos seus projetos.

A todos os novos amigos que acompanharam esta nova etapa da minha vida, partilhando comigo todas as emoções. À Helena e Lígia, companheiras de casa, aventuras, diversão, pela presença em todos os momentos, mesmo nos dias maus. Aos amigos de sempre, Ana, Marina, Liliana, João, Leandro e "BNTs" que apesar de não terem partilhado comigo esta nova etapa a 500km da "nossa casa", estão e estarão sempre presentes. Pela compreensão das ausências!..

O meu mais sincero agradecimento aos meus pais, Lucília e Paulo, e a toda a minha família pelos princípios incutidos, pela motivação e confiança, pela disponibilidade e pelo empenho, trabalho e esforço dedicados à minha formação. Pela compreensão da distância e das prolongadas ausências, por acreditarem que apesar disso estive sempre com eles! Pela paciência e compreensão nos momentos de maior ansiedade.

A todos, o meu mais sincero e profundo OBRIGADA!

"The beginning of all sciences is the surprise
of things being what they are."

Aristóteles

ABSTRACT

Papillary and Follicular thyroid carcinomas (PTC and FTC) are lesions derived from follicular cells. The standard therapy includes surgery and radioiodine treatment. Despite being usually associated with a good prognosis the rate of recurrence is high and a subset of patients present radioiodine refractory and non-responsive neoplasia. So, the search for new therapeutic alternatives, as well as new diagnostic/prognostic markers is highly relevant.

Our group at IPOLFG has previously shown *RAC1b* to be overexpressed in PTCs, being this overexpression significantly associated with poorer outcomes. RAC1b is a variant of the small GTPase RAC1 that was shown to have the ability to stimulate the canonical NF-κB regulatory pathway in colorectal cancer. NF-κB activation is associated with the tumorigenic process in several types of cancer, mainly due to its role in apoptosis evasion.

Here, we aimed to extend the study of the role of RAC1b to FTCs and further investigate the molecular mechanisms associated with RAC1b overexpression and downstream signalling in thyroid tumorigenesis.

Using the human papillary thyroid carcinoma derived cell line as in vitro model, we observed that both RAC1 and RAC1b were able to induce a significant increase on NF-κB and cyclin D1 reporter activity. A clear p65 nuclear localization was found in cells transfected with RAC1b-WT, confirming NF-κB canonical pathway activation. Consistently, we were able to observe a RAC1b-mediated decrease in IκBα (NF-κB inhibitor) protein levels.

These findings prompted us to further assess the cellular consequences of RAC1b-mediated NF-κB activation in a thyroid biological system. Our results indicated that RAC1b protected cells against apoptosis and stimulated G1/S progression, through a process involving the NF-κB pathway.

Summing up, presented findings suggest an important role of RAC1b in the progression of follicular cell derived thyroid carcinomas and point out NF-κB activation as one of the molecular mechanisms associated with RAC1b overexpression and downstream signalling in thyroid tumorigenesis.

Key words: Thyroid tumorigenesis; RAC1b; NF-κB canonical pathway; Cyclin D1; Apoptosis; Cell Cycle;

RESUMO

O Carcinoma Papilar (CPT) e o Carcinoma Folicular da Tiróide (CFT) são lesões derivadas de células foliculares. A terapia de referência inclui cirurgia e tratamento com iodo radioativo. Apesar de estarem normalmente associados a um bom prognóstico, o nível de recorrências é elevado dado que um subconjunto de pacientes apresentam neoplasias refratárias ou incapazes de responder ao iodo radioativo. Portanto, a busca por alternativas terapêuticas, bem como novos marcadores de diagnóstico/prognóstico é bastante relevante.

O nosso grupo no IPOLFG identificou anteriormente a sobre-expressão do *RAC1b* em CPTs, e esta sobre-expressão estava significativamente associada com um pior prognóstico. O RAC1b é uma variante da pequena GTPase RAC1, que demonstrou a capacidade de estimular a via canónica do NF-κB em cancro colo-retal. A ativação do NF-κB está associada com o processo tumorigénico em vários tipos de cancro, maioritariamente devido ao seu papel na evasão da apoptose.

O objetivo do presente estudo foi o de expandir o estudo do papel do RAC1b aos CFT, e ainda investigar os mecanismos moleculares subjacentes à sobre-expressão do RAC1b e sinalização a jusante na tumorigénese da tiróide

Usando uma linha celular derivada de CPT como modelo *in vitro*, observou-se que quer o RAC1, quer o RAC1b mostraram capacidade de induzir um aumento significativo na atividade do repórter do NF-κB e da ciclina D1. Uma clara localização nuclear da proteína p65 foi observada em células transfetadas com GFP-RAC1b-WT, confirmando a ativação da via canónica do NF-κB. Consistentemente, observou-se uma diminuição mediada pelo RAC1b dos níveis proteicos de IκBα (um inibidor do NF-κB).

Estes resultados levaram-nos a avaliar as consequências celulares da ativação do NF-κB mediada pelo RAC1b no sistema biológico da tiróide. Os nossos resultados indicam que o RAC1b protegeu as células contra a apoptose e estimulou a progressão G1/S, através de um mecanismo que envolve a via do NF-κB.

Resumindo, as presentes conclusões apontam para um importante papel do RAC1b no desenvolvimento de CFTs e indicam a ativação do NF-κB como um dos mecanismos moleculares associados com a sobre-expressão do RAC1b e sinalização a jusante na tumorigénese da tiróide.

Palavras-Chave: Tumorigénese da Tiróide; RAC1b; Via canónica do NF-κB; Ciclina D1; Apoptose; Ciclo celular;

TABLE OF CONTENTS

AGRADECIMENTOS I
ABSTRACT V
RESUMO VII
TABLE OF CONTENTS IX
LIST OF FIGURES XI
LIST OF TABLES XIII
LIST OF SUPPLEMENTARY TABLES XIII
LIST OF ABBREVIATIONS, SYMBOLS AND CONVENTIONS XV
I. INTRODUCTION ..... - 1 -
1. Tumorigenesis ..... - 1 -
2. Thyroid gland: Anatomy and physiology ..... - 2 -
3. Thyroid diseases and syndromes ..... - 3 -
3.1. Thyroid neoplasia ..... - 4 -
3.1.1. Medullary Thyroid carcinoma ..... - 5 -
3.1.2. Non-Medullary Thyroid tumours ..... - 5 -
3.1.2.1. Follicular thyroid adenoma ..... - 6 -
3.1.2.2. Follicular thyroid carcinoma ..... - 6 -
3.1.2.3. Papillary thyroid carcinoma ..... - 7 -
3.1.2.4. Poorly differentiated thyroid carcinoma ..... - 9 -
3.1.2.5. Anaplastic thyroid carcinoma ..... - 9 -
4. RAC1 and RAC1b GTPases ..... - 10 -
4.1. Organization and regulation ..... - 10 -
4.2. Biological functions ..... - 11 -
4.3. Association with cancer ..... - 13 -
5. Signalling pathways involved in cancer ..... - 13 -
5.1. NF-κB signalling pathway ..... - 14 -
5.1.1. Association with Tumorigenesis ..... - 16 -
5.1.2. Association with RAC1b ..... - 17 -
6. Follicular cell derived thyroid cancers, RAC1b and NF-κB pathway-a link to unravel ..... - 18 -
Part 1. RAC1b role in FTC Tumorigenesis ..... - 18 -
Part 2. RAC1b - An activator of NF-κB pathway that contributes to thyroid Tumorigenesis? ..... - 18 -
II. OBJECTIVES ..... - 21 -
III. MATERIALS AND METHODS ..... - 23 -
1. Biological Material – patients samples ..... - 23 -
2. DNA plasmids and constructs ..... - 23 -
3. Cell Culture and transfection ..... - 23 -
4. RNA extraction ..... - 24 -
5. cDNA synthesis and amplification ..... - 24 -
6. Quantitative reverse transcription – PCR (qRT-PCR) ..... - 26 -

7. Luciferase reporter assays - 26 -
8. Confocal immunofluorescence microscopy - 27 -
9. Cell fractionation - 27 -
10. Protein extraction with RIPA - 28 -
11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting - 28 -
12. Cell death analysis by flow cytometry (FACS) - 28 -
13. Cell Cycle analysis by flow cytometry (FACS) - 29 -
14. Statistical analysis - 30 -
IV. RESULTS - 31 -
PART 1 - 31 -
1. *RAC1b* is overexpressed in FTCs - 31 -
2. *RAC1b* overexpression is associated with presence of distant metastasis and poorer clinical outcomes - 31 -
PART 2 - 34 -
3. RAC1b stimulates NF-κB in K1 cells - 34 -
4. RAC1b stimulates Cyclin D1 in the K1 cells - 35 -
5. RAC1b stimulates p65 translocation to the nucleus - 36 -
6. RAC1b induces a decrease in total IκBα protein expression - 39 -
7. RAC1b protects cells against apoptosis induced by staurosporine - 40 -
8. RAC1b stimulates cell cycle progression - 41 -
9. RAC1b effect on Nthy cell line - 42 -
V. DISCUSSION - 45 -
VI. CONCLUSION - 49 -
VII. PERSPECTIVES - 51 -
VIII. BIBLIOGRAPHY - 53 -
APPENDICES - 63 -

LIST OF FIGURES

Figure I.1. The hallmarks of cancer. .....- 1 -
Figure I.2. Location and structure of the thyroid gland.....- 3 -
Figure I.3. Subtypes of non-meddulary thyroid carcinomas. .....- 6 -
Figure I.4. Diagram of the *RAC1b* gene. 1, 2, 3, 4, 5, 6 represents exons. .....- 10 -
Figure I.5. RAC1 and RAC1b signalling. .....- 12 -
Figure I.6. Canonical and non-canonical NF-κB activating pathways.....- 16 -
Figure IV.7. *RAC1b* overexpression and its association with distant metastasis and clinical outcome...- 32 -
Figure IV.8. Luciferase activity of NF-κB regulated reporter in transfected K1 cells.....- 35 -
Figure IV.9. Luciferase activity of cyclin D1 regulated reporter in transfected K1 cells.. .....- 36 -
Figure IV.10. RAC1b overexpression affects subcellular location of p65-NLS protein. .....- 38 -
Figure IV.10.1. RAC1b overexpression affects subcellular location of p65-NLS protein.....- 39 -
Figure IV.11. Total IκBα protein levels in K1 cells assessed by western-blotting.. .....- 40 -
Figure IV.12. Apoptosis analyzes by flow cytometry (Annexin V and PI staining).....- 41 -
Figure IV.13. Cell cycle analyzes by flow cytometric (PI staining). .....- 42 -
Figure IV.14. Luciferase activity of NF-κB and cyclin D1 regulated reporter in transfected Nthy cells. .....- 44 -

LIST OF TABLES

Table III.1. Description of plasmid amounts used in each assay, for each cell line. .....- 24 -
Table III.2. *RAC1/1b* PCR amplification conditions. .....- 25 -
Table IV.3. Clinical Data.....- 33 -

LIST OF SUPPLEMENTARY TABLES

Table 1. 10x PCR buffer.....- 64 -
Table 2. RIPA buffer. .....- 64 -
Table 3. 5x Sample buffer. .....- 64 -
Table 4. Blot buffer.....- 64 -
Table 5. Destain solution. .....- 64 -
Table 6. TBST solution.....- 65 -
Table 7. ECL solutions. .....- 65 -
Table 8. Annexin binding buffer 1x.....- 65 -
Table 9. PI solution cell cycle assay. .....- 65 -

LIST OF ABBREVIATIONS, SYMBOLS AND CONVENTIONS

ATC – Anaplastic thyroid carcinoma

BRAF – Murine sarcoma viral (v-Raf) oncogene homolog B1

Cdc42 – Cell division control protein 42 homolog

cDNA – Complementary DNA

C-terminal – Carboxyl-terminal

CTNNB1 - Catenin (Cadherin-associated protein), beta 1

DAPI – 4',6-diamidino-2-phenylindole

ddH_2O – Bidestilled water

DMEM:F12 – Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12

DNA – Deoxyribonucleic acid

dNTPs – Deoxynucleotides

DTT – Dithiothreitol

ECL – Enhanced chemiluminescence

EDTA – Ethylenediamine tetraacetic acid

EHT 1864 – RAC1 inhibitor

EMT – Epithelial-mesenchymal transition

FBS – Fetal bovine serum

FMTC – Familial MTC

FNA – Fine needle aspiration

FTA – Follicular thyroid adenoma

FTC – Follicular thyroid carcinoma

FVPTC – Follicular variant of papillary thyroid carcinoma

g – Grams

G0 phase – Quiescent phase of cell cycle

G1 phase – Gap 1 phase of cell cycle

G2 phase – Gap 2 phase of cell cycle

GAP – GTPase-activating protein

GDI – Guanine nucleotide dissociation inhibitor

GDP – Guanosine diphosphate

GEF – Guanine nucleotide exchange factor

GFP – Green fluorescent protein

GTP – Guanosine triphosphate

GTPase – Guanosine triphosphatase

h – Hours

HEPES – 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

Ig – Immunoglobulin

IPOLFG – Instituto Português de Oncologia Lisboa Francisco Gentil

IκK – IκB kinase

JNK – Jun NH_2-terminal kinase

K1 – Cell line derived from human papillary thyroid carcinoma

kDa – Kilodalton

L – Litre

L61 – Constitutively active mutant

m – Metre

M – Molar

M phase – Mitosis phase

MAPK – Mitogen-activated protein kinases

MEN 2 – Multiple endocrine neoplasia type 2

min – minutes

miRNA – MicroRNA

MMP-3 – Matrix metalloproteinase-3

MTC – Medullary thyroid carcinoma

NEMO – NF-κB essential modulator

NF-κB – Nuclear factor kappa-light-chain-gene-enhancer of activated B cells

NIK – NF-κB inducing kinase

NIS – Sodium/iodide symporter

NLS – Nuclear localization signal

NMTC – Non-medullary thyroid carcinoma

N-terminal – Amino terminal

Nthy – Nthy-ori 3-1, cell line derived from human thyroid follicular normal epithelium

OPTI-MEM – Improved minimal essential medium

p- p-value

PAK – p21-activated protein kinase

PAX8/PPARγ – paired box gene 8 (PAX8)-peroxisome proliferator-activated receptor γ (PPARγ)

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PDTC – Poorly differentiated thyroid carcinoma

PI – Propidium iodide

PI3K(CA) – phosphoinositide 3-kinase (Catalytic Alpha)

PTC – Pappilary thyroid carcinoma

PTEN – Phosphatase and tensin homolog

PVDF – Polyvinylidene difluoride

qRT-PCR – Quantitative RT-PCR

RAC1 – Ras-related C3 botulinum toxin substrate 1

RAS – Rat sarcoma vírus homolog

RET – rearrangement during transfection

Rho – Ras homologous

RIPA – Radioimmunoprecipitation assay buffer

RNA – Ribonucleic acid

RNAse – Ribonuclease

ROS – Reactive oxygen species

rpm – Rotations per minute

RPMI – Roswell Park Memorial Institute medium

RT – Room temperature

RTK – Receptor tyrosine kinases

RT-PCR – Reverse transcription-PCR

S phase – Synthesis phase of cell cycle

SB – Sample Buffer

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

T3 – Triiodothyronine

T4 – Tetraiodothyronine

TBE – Tris-borate-EDTA

TBST – Tris Buffered Saline Buffer 0,05% Triton X-100

TP53 – Tumour Protein 53

UV – Ultraviolet

WDTC – Well Differentiated Thyroid Carcinoma

WT – Wild-type

μ – micro-

% (v/v) – Concentration expressed in volume per volume

% (w/v) – Concentration expressed in mass per volume

°C – Celsius degree

I. INTRODUCTION

1. Tumorigenesis

The pursuit for an explanation about the basis of malignant changes has started many years ago with several studies being reported. For example, back to 1974, Loeb and collaborators indicated errors in DNA replication as a possible cause of malignant events. Nevertheless, in the last few years, several scientific advances took place in the cancer research field allowing the scientific community to better understand the biological mechanisms involved in the genesis of tumours – Tumorigenesis.

Modifications in the control and homeostatic balances that regulate a myriad of cellular processes, namely proliferation, differentiation and apoptosis are considered the primary sources of malignant changes (Koeffler *et al.,* 1991). The idea that genetic alterations are required to tumorigenesis is widely accepted, and evidences that tumours accumulate numerous genetic changes, such as chromosomal abnormalities, gene mutations and epigenetic alterations has been shown over the years (Loeb *et al.,* 1974; Koeffler *et al.,* 1991; Bach *et al.,* 2000; Hanahan and Weinberg, 2000; Sieber *et al.,* 2003; MacConaill and Garraway, 2010).

Despite the vast diversity of existing tumours, some intrinsic features may be shared and considered universal features of all tumours. Back to 2000, Hanahan and Weinberg proposed six essential modifications in cell physiology required in the tumorigenesis process (see Figure I.1): self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion to apoptosis, unlimited replicative potential, sustained angiogenesis and ability to invade tissues and metastasize. These features allowed tumour cells to generate their own signals and, in opposition, to escape of anti-proliferative signals. The unlimited replicative potential contributes to the immortalization of tumour cells that guarantee a continuous nutrient supply by create their own blood vessel network.

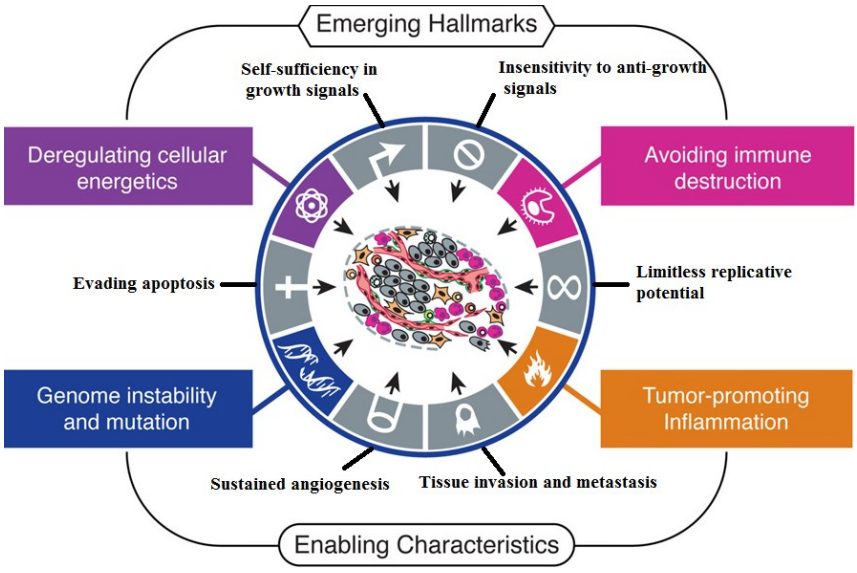


Figure I.1. The hallmarks of cancer. Adapted from Hanahan and Weinberg (2000) and Hanahan and Weinberg (2011).

In addition to these common and widely accepted features, recently new characteristics were proposed as hallmarks of cancer (see Figure I.1): cellular energy metabolism adaptation and ability to evade immune system (Hanahan and Weinberg, 2011; Serpa and Dias, 2011).

In fact, tumour cells have an altered metabolism relative to normal cells (Serpa and Dias, 2011). The increased proliferative potential of tumour cells requires higher rates of metabolic reactions in order to obtain nutrients, energy and biosynthetic activity (DeBerardinis *et al.,* 2008; Tennant *et al.,* 2009; Tennant *et al.,* 2010). This phenotype allows tumour cells to have an increased rate of glycolysis (conversion of glucose to pyruvate), either in aerobic or anaerobic conditions (Tennant *et al.,* 2009), which compensates the limited levels of oxidative phosphorylation in some regions of solid tumours (Frezza and Gottlieb, 2009).

To progress, tumour cells have to be able to evade immune system. The interaction between the malignant cells and the cells of the immune system has different stages – elimination, equilibrium and escape – and this process is known as cancer immunoediting. In the elimination or cancer surveillance phase, transformed cells are identified and killed by the innate and the adaptive immune system. These events result in an immune selection that is favourable to tumour variants with increased ability to withstand immune factors and decrease immunogenicity. The equilibrium phase is known by the persistence of the tumour cells that were not killed, but also can not expand due to the immune pressure. When the tumour is able to growth due to the immune exhaustion or inhibition, or as result of emerging tumour-cell variants able to evade immune pressure, the escape stage is ongoing (Dunn *et al.,* 2006; Kim *et al.,* 2007).

Several lines of evidence, suggest that human tumorigenesis is a multiset process (Hanahan and Weinberg, 2000) in which normal cells are progressively transformed in malignant derivatives. The progression through the many stages of this process depends on several factors, such as genomic instability, which is inherent to some cancers, or the ability of these tumours to promote inflammation that occasionally support multiple hallmark capabilities (Sieber *et al.,* 2003; Hanahan and Weinberg, 2011).

2. Thyroid gland: Anatomy and physiology

The thyroid gland, part of the endocrine system, is a butterfly-shaped organ consisting of two lobes positioned on either side of the trachea, connected by a structure named isthmus (see Figure I.2). Despite the variance in the size and appearance of the adult gland, - according to functional activity, gender, hormonal status, and iodine intake - the thyroid gland is one of the largest glands in the body, weighing approximately 15 to 25 g (Muro-Cacho and Ku, 2000; Nussey and Whitehead, 2001).

The primary role of this organ in the development of vertebrates is related with its ability to synthesize hormones that are crucial for growth, development and survival, due to their capacity to convey oxygen and nutrients into cells and convert them into energy. Two of the thyroid-synthesized hormones with higher impact are tetraiodothyronine (thyroxine or T4) and triiodothyronine (T3). The storage of thyroid hormones occurs mainly within the follicles, the functional unit of the thyroid gland (Nussey and Whitehead, 2001; Nitsch *et al.,* 2010). The synthesis of thyroid hormones requires the iodination of tyrosine molecules (Nussey and Whitehead, 2001), and their activity is mediated by thyroid hormone receptors, which exists in different isoforms (Gauthier *et al.,* 1999; Zhu *et al.,* 2010; Kim and Cheng, 2013). In fact, the iodine represents an essential element in thyroid physiology and function, and its uptake in the thyroid gland is mediated by the sodium/iodide symporter (NIS) (Filetti *et al.,* 1999; Kogai *et al.,* 2006).

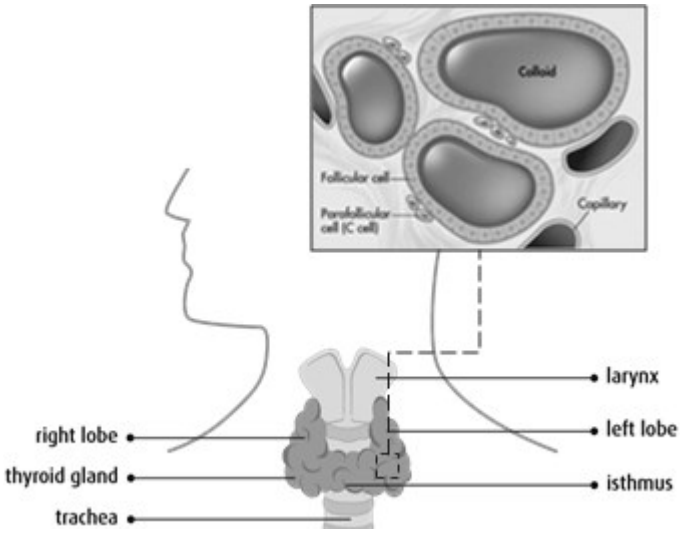


Figure I.2. Location and structure of the thyroid gland. Adapted from http://www.cancer.ca/en/cancer-information/cancer-type/thyroid/anatomy-and-physiology/?region=bc

The thyroid embryogenesis involves an initial step in which this gland develops from the anterior foregut, following a sequence of morphogenetic steps that culminate in the migration of the thyroid bud to its final anatomic position (Fagman and Nilsson 2010a). This developmental process and the final thyroid fate are influenced by the combined expression of specific transcription factors by foregut endoderm cells (Fagman and Nilsson 2010a; Fagman and Nilsson 2010b).

Several cell types, derived from the three germ layers, compose the thyroid gland (Damante and DiLauro, 1994). The most abundant cellular population are the follicular cells that are associated with the production of thyroid hormones and surround the follicles (see Figure I.2) - oval sacs filled with colloid (Ljungberg *et al.,* 1983; Damante and DiLauro, 1994; Muro-Cacho and Ku, 2000). The C cells, or parafollicular cells, are associated with calcitonin production (Ljungberg *et al.,* 1983; Muro-Cacho and Ku, 2000).

3. Thyroid diseases and syndromes

The thyroid gland is subject of a multitude of diseases and syndromes that impair the function of this gland, for example, goitres, hyper- and hypo-thyroidism, thyroiditis, Graves' disease and cancer (Muro-Cacho e Ku, 2000). Thyroid nodules are considered common and present a risk of malignancy (approximately 5%). The enormous challenge in this field is related with the management of this nodules and the evaluation of treatment needs (Gharib *et al.,* 2007). Generally, the medical approach includes

the evaluation only of nodules larger than 1 cm or nodules smaller than 1 cm in which the patient revealed suspicious ultrasound findings, a history of head and neck irradiation, or a positive family history of thyroid cancer (Cooper *et al.,* 2006). However, the controversy surrounding this subject prevails (Gharib *et al.,* 2007).

3.1. Thyroid neoplasia

Thyroid tumours account for approximately 1% of worldwide human malignant neoplasms, (Schlumberger, 1998; Biersack and Grünwald, 2005). Nevertheless, they represent the most common (almost 90%) malignancy in the endocrine system (Biersack and Grünwald, 2005; Siegel *et al*., 2013). Thyroid cancer is most prevalent among women (the incidence ratio male: female is approximately 1:3), is rare in children and the age of diagnosis varies between 20-50 years (Schlumberger, 1998; DeLellis *et al.,* 2004; Biersack and Grünwald, 2005; Vriens, 2009). Despite the higher incidence in women, some reported studies underline a worse prognostic perspective in man (Yao *et al.,* 2011).

Numerous statistical studies have described an increasing incidence of thyroid cancer (Cramer *et al.,* 2010), namely in the United States where it was estimated the appearance of approximately 45000 new cases of thyroid cancer, in 2010 (Jemal *et al.,* 2010). The debate in this field continues on whether or not this increase is only a consequence of a more efficient diagnostic surveillance and pathologic recognition (DeLellis *et al.*, 2004; Cramer *et al.,* 2010). This hypothesis is supported by the reported decrease in mortality rate that prove the impact of more sensitive and efficient diagnostic methods (DeLellis *et al.*, 2004).

The good prognosis associated with most thyroid neoplasia can be explained by its low cell proliferative cellular rate and is also probably related with the advances in the knowledge of factors that influence the malignant progression (DeLellis *et al.,* 2004; Biersack and Grünwald, 2005). The standard therapy for most thyroid carcinomas includes total thyroidectomy followed by radioiodine treatment and close follow up, which is effective for most of the cases. However, a subset of patients with advanced/dedifferentiated malignancy are unresponsive or refractory to radioiodine management, which are closely related to high recurrence and mortality levels (Sonkar *et al.,* 2010; Soares *et al.,* 2014).

The development and progression of thyroid malignancies are influenced by diverse factors, that can be environmental, hormonal or genetic as well as by the interaction between all of the above (DeLellis *et al*., 2004).

Thyroid neoplasia can be divided in different groups with remarkably different features. Firstly, it is possible to distinguish benign lesions, known as adenomas (follicular thyroid adenoma – FTA), from malignant lesions, called carcinomas (Muro-Cacho and Ku, 2000). Thyroid carcinomas comprise two groups based on cell type from which they develop: thyroid cancers that arise from follicular cells (thyroid hormone-producing) that are called non-medullary thyroid carcinomas (NMTCs) and represent approximately 95% of all tumours; carcinomas that arise from C cells (calcitonin-producing) and are known as medullary thyroid carcinomas (MTCs), representing 3–5% of all tumours (Muro-Cacho and

Ku, 2000; Capezzone *et al.,* 2008; Nikiforov and Nikiforova, 2011). The large majority of NMTCs are well-differentiated and classified as papillary (papillary thyroid carcinoma-PTC) or follicular (follicular thyroid cancinoma-FTC). The minor portion of NMTCs show a poorly differentiated (poorly differentiated thyroid carcinoma-PDTC) or undifferentiated (anaplastic thyroid carcinoma-ATC) phenotype (Liu *et al.,* 2006; Morani *et al.,* 2014).

3.1.1. Medullary Thyroid carcinoma

The medullary thyroid carcinoma arises from C cells (calcitonin-producing) and accounts for a modest percentage of all thyroid tumours (3-10%) (Dvorakova *et al.,* 2008; Nikiforov and Nikiforova, 2011). Most of MTC are sporadic (75% of all cases), however 25% of the cases can be associated with a familial predisposition (autosomal dominant). The familial forms can occur either as familial MTC (FMTC – isolated event) or as part of multiple endocrine neoplasia type 2 (MEN 2) syndromes. The MEN 2 syndromes and FMTC are usually associated with germline gain of function mutations in the *RET* proto-oncogene, which can also occur somatically, associated with the sporadic form of MTC (Dvorakova *et al.,* 2008). This form may also be associated with *RAS* point mutations that seem to work as alternative genetic events for the activating mutations of *RET* in sporadic MTC (Moura *et al.,* 2011).

3.1.2. Non-Medullary Thyroid tumours

As previously denoted, non-medullary thyroid tumours are lesions with origin in the follicular cells representing about 95% of all thyroid malignancies. The lesions can be grouped in concordance with the differentiation degree of follicular cell - Well Differentiated Thyroid Carcinoma (WDTC), Poorly Differentiated Thyroid Carcinoma (PDTC) and Anaplastic Thyroid Carcinoma (ATC). The WDTC group are composed by two different histologic types of tumours – papillary and follicular (see Figure I.3) (Muro-Cacho and Ku, 2000; Liu *et al.,* 2006; Capezzone *et al.,* 2008; Morani *et al.,* 2014).

Tumours associated with less differentiation of follicular cells are usually the most aggressive and also associated with poor prognosis due to the lack of therapies available (Kondo *et al.,* 2006). While these malignancies can arise *de novo,* some reported studies support the hypothesis that all of these tumours share an evolutionary link, in which papillary and follicular thyroid carcinomas may give rise to the poorly differentiated and anaplastic forms, through a stepwise process of dedifferentiation of papillary and follicular carcinomas (Bhaijee and Nikiforov, 2011; Caronia *et al.,* 2011; Nikiforov and Nikiforova, 2011). The majority of known genetic alterations associated with papillary and follicular thyroid carcinoma fall into one of four mutually exclusive groups (Bhaijee and Nikiforov, 2011): *BRAF* [Murine sarcoma viral (v-raf) oncogene homolog B1] and *RAS* (Rat sarcoma vírus homolog) point mutations, and *RET/PTC* (RET- rearrangement during transfection) and *PAX8/PPARγ* [paired box gene 8 (PAX8)-peroxisome proliferator-activated receptor γ (PPARγ)] rearrangements. It is known that thyroid cancer initiation and progression involve an accumulation of different types of genetic

alterations. The same probably happens in the dedifferentiation process in which some mutations that represent early events in thyroid cancer progression (such as *BRAF* and *RAS* mutation), that are found either in WDTC or in PDTC/ATC, may predispose WDTC to dedifferentiation.

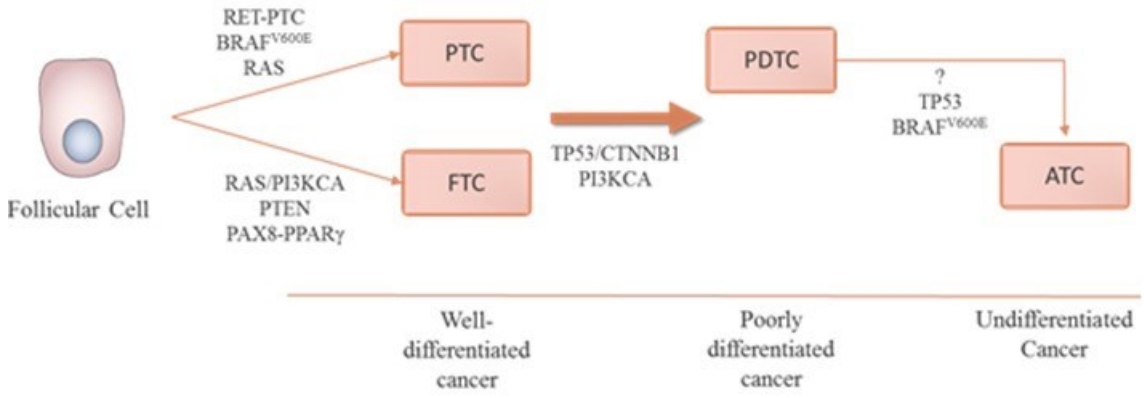


Figure I.3. Subtypes of non-meddulary thyroid carcinomas. Adapted from Nikiforov and Nikiforova (2011) and Sastre-Perona and Santisteban (2012).

3.1.2.1. Follicular thyroid adenoma

Benign tumours of thyroid gland are designated by follicular thyroid adenomas (FTA). This group of tumours is characterized by their encapsulated form (usually a fibrous capsule of variable thickness) and they are round or oval, measuring between 1 and 3 cm. This lesion can occur in normal glands or in association with thyroiditis, nodular hyperplasia or other lesions. Typically FTAs present follicular cell differentiated phenotype (Muro-Cacho and Ku, 2000; DeLellis *et al.,* 2004; Liu *et al,* 2006). It has been also proposed that FTA may serve as a precursor for some follicular carcinomas (Nikiforov and Nikiforova, 2011).

3.1.2.2. Follicular thyroid carcinoma

Follicular thyroid carcinoma is a type of malignant tumours that represents approximately 10-15% of all thyroid cancers (Bhaijee and Nikiforov, 2011; Sobrinho-Simões *et al.,* 2011). According to the WHO (World Health Organization) Classification of Tumours of Endocrine Organs, FTC can be defined as 'a malignant epithelial tumour showing follicular cell differentiation and lacking the diagnostic nuclear features of papillary thyroid carcinoma' (see DeLellis *et al.,* 2004). These are commonly solid tumours presenting as an isolated focus (unifocal), encapsulated, characterized by haematogenous spread, exhibiting less lymph node involvement (than PTCs), and with size higher than 1 cm (Schlumberger, 1998; DeLellis *et al.,* 2004; Kondo *et al.,* 2006; Romitti *et al.,* 2013). As PTCs, follicular carcinomas can also be divided in different variants, namely the conventional type and the oncocytic type (oxyphilic or Hürthle cells tumours) (Schlumberger, 1998; Bhaijee and Nikiforov, 2011; Bonora *et*

al., 2010). The invasion grade of the capsule and vessels (infiltrative growth) is a distinguishing feature between follicular carcinomas and follicular adenomas. The classification of tumours according to its pattern of invasion recognized two groups – those minimally invasive and the widely invasive carcinomas (Schlumberger, 1998; Biersack and Grünwald, 2005; Bonora *et al.,* 2010).

The most common genetic alterations associated with FTCs (70-75% of cases) are *RAS* point mutations and *PAX8/PPARγ* rearrangements which are mutually exclusive (DeLellis *et al.,* 2004; Castro *et al.,* 2006; Bhaijee and Nikiforov, 2011; Sobrinho-Simões *et al.,* 2011).

The prognosis of FTC depends on several features, namely the age of the patients at the diagnosis (usually higher comparing to PTCs), tumour size and stage, invasion rate, extensiveness of surgery and responsiveness to radioactive iodine (Muro-Cacho and Ku, 2000; DeLellis *et al.,* 2004; Kondo *et al.,* 2006).

One of the biggest challenges in this field is related with the pre-surgical discrimination between malignant follicular thyroid cancer and benign follicular thyroid adenoma. Although the gold standard for evaluating thyroid nodules is fine needle aspiration (FNA) biopsy, this method is unable to reliably distinguish FTC from FTA, since the key feature associated with this malignancy is its invasiveness rate (capsular and/or vascular penetration) (Schlumberger, 1998; Barden *et al.,* 2003; DeLellis *et al.,* 2004). Thyroid unilateral lobectomy is still used as a standard approach which allows a postsurgical diagnosis as FTC or FTA. Depending on the postsurgical conclusion, patients are managed in different ways - only cancer patients require completion total thyroidectomy, adjuvant radioiodine treatment, and long-term follow-up (Pfeifer *et al.,* 2013). The big challenge is to find a reliable method to discriminate FTC and FTA in FNA material. Over the years numerous studies proposed different immunohistochemical markers. Pfeifer and collaborators (2013) reported a panel of five genes (*ELMO1, EMCN, ITIH5, KCNAB1, SLCO2A1*) with a 72% specificity grade; 94% accuracy was found in a six gene set (*TFF3/PLAB/TG/ADM3/HGD1/LGALS3*) (Krause *et al.,* 2008). Many other studies were performed, namely studies assessing the use of miRNAs as potential distinguish markers (Stokowy *et al.,* 2014) or studies where metabolomics approach were used (Deja *et al.,* 2013). Despite all the efforts, no panel of molecular markers with confidence levels of specificity and sensitivity that allows the preoperative discrimination between FTCs and FTAs has been defined yet.

3.1.2.3. Papillary thyroid carcinoma

PTC is the most common malignant epithelial thyroid tumour presenting differentiation of follicular cell – PTC integrates the WDTC group. Papillary tumours account to nearly 80% of all thyroid cancers (DeLellis *et al.,* 2004; Caronia *et al.,* 2011). This tumour type is usually associated with good prognosis and long-term survival rates, due to a successful combination of radioiodine and levothyroxine treatment following total thyroidectomy. However, up to 10% patients would eventually die of the disease mostly due to the high levels of recurrences, since a subset of patients with advanced stage carcinomas have

radioiodine refractory and non-responsive malignancies (Sonkar *et al.,* 2010; Soares *et al.,* 2014). Older age at diagnosis, male gender, large tumour size and extra-thyroidal growth represent the most relevant poor prognostic factors (DeLellis *et al.,* 2004; LiVolsi, 2011; Soares *et al.,* 2014).

Most of papillary lesions are firm masses with the size ranging from less than 1 mm to several centimetres. The prominent papillae are a specific feature of this type of lesion, such as enlargement, oval shape, elongation and overlapping of the nuclei (DeLellis *et al*., 2004).

Papillary thyroid neoplasia is influenced by environmental, genetic and hormonal factors. Environmental factors comprise genotoxic (DNA damage due to radioactive iodine) and non-genotoxic effects (due to iodine deficiency). The thyroid dependency of iodine makes it vulnerable to radiation, being the PTC the subtype mostly linked to this factor (DeLellis *et al.,* 2004).

PTC can be divided into numerous histopathologic variants based on specific growth patterns, cell types and stromal changes (Lloyd *et al.,* 2011) – for example, follicular variant of papillary thyroid carcinoma (FVPTC) preserves nuclear features similarly to PTC, but exhibits a predominantly micro-follicular histologic growth pattern (McFadden *et al.,* 2014).

Point mutations (such as mutations in the *RAS* and *BRAF* genes) and the *RET/PTC* and *PAX8/PPARγ* chromosomal rearrangements are common genetic alteration in this cancer subtype, that usually involve the effectors of the MAPK (Mitogen-activated protein kinases) and PI3K-AKT (PI3K– phosphoinositide 3-kinase; AKT – Protein Kinase B) pathways (Nikiforov and Nikiforova, 2011). Rearrangements in the *RET* proto-oncogene, known as *RET/PTC*, can be detected in PTCs with its prevalence varying between different patient cohorts – from 20% of all cases to much higher percentages (DeLellis *et al.,* 2004; Nikiforov and Nikiforova, 2011). The V600E mutation on the *BRAF* proto-oncogene is the most frequent genetic alteration in PTC, representing 40-50% of cases (Nikiforov and Nikiforova, 2011). V600E – a transversion resulting in the substitution of glutamic acid for valine - is the most common *BRAF* mutation (Lee *et al.,* 2007; Caronia *et al.,* 2011). *RAS* point mutations can also be found, but the reported prevalence is much smaller – 10-20% according to Nikiforov and Nikiforova (2011). The indicated numbers can be much different in the others variants of PTC, namely on FVPTC, where a different and less prevalent type of *BRAF* mutation (K601E) can be found. Also, *PAX8/PPARγ* rearrangements and *RAS* point mutation have a higher prevalence than in classic PTC subtype – 40% and 25% of all FVPTC cases, respectively (Castro *et al.,* 2006).

BRAF^{V600E} mutation in PTC

BRAF is a serine/threonine kinase of the RAF family of proteins and it's considered a strong activator of the MAPK pathway (involved in the regulation of several cellular responses) (Davies *et al.,* 2002; Lee *et al.,* 2007). *BRAF* mutations are not specific of thyroid tumours and are also highly prevalent in malignant melanoma, colorectal carcinoma and serous ovarian cancer (Garnett and Marais, 2004). *BRAF*^{V600E} mutation is generally accepted as the most frequent genetic alteration in PTCs. This mutation constitutively activates BRAF kinase, which works as a prolonged stimuli of the MAPK pathway, culminating in an increase of cell proliferation and defective apoptotic levels (Davies *et al.,* 2002; Soares

et al., 2003; Wan *et al.,* 2004). The association between *BRAF* mutation and morphological and functional alterations related with PTC phenotype has been reported, for example, the presence of papillary structures that are a characteristic of the classical variant of PTCs are a phenotypic feature closely associated with $BRAF^{V600E}$-positive tumours (Lee *et al.,* 2007; Rusinek *et al.,* 2011). Significant correlation between *BRAF* mutations and extra-thyroidal invasion, lymph node metastasis, and tumour stage has been also reported in PTC by Chakraborty and collaborators (2012).

3.1.2.4. Poorly differentiated thyroid carcinoma

Poorly differentiated thyroid carcinoma is a rare entity that represents less than 10% of all NMTC (Biersack and Grünwald, 2005; Bonora *et al.,* 2010). As already mentioned, this is a neoplasia originated from thyroid follicular cells, representing an intermediate morphological and biological behaviour between well-differentiated thyroid carcinomas and anaplastic thyroid carcinomas (Muro-Cacho and Ku, 2000; DeLellis *et al.,* 2004; Biersack and Grünwald, 2005). This type of malignancy affects more males than WDTC and the average age of onset is higher, at approximately 50 years old. Lymph nodes and distant metastasis are highly frequent. The survival rates are low in the first three years after the diagnosis, and lower after five years, precisely due to metastasis occurrence (30-80%) (DeLellis *et al.,* 2004; Biersack and Grünwald, 2005; Kondo *et al.,* 2006). Kondo and collaborators (2006) documented *RAS* and *TP53* (Tumour protein 53) mutations as the most frequent genetic alterations in PDTC (18-27% and 17-38% of all cases, respectively). *BRAF* point mutation are also present in some cases (0-13%) supporting the hypothesis of a WDTC to PDTC transition.

3.1.2.5. Anaplastic thyroid carcinoma

Anaplastic carcinoma is a highly malignant tumour composed by undifferentiated cells in a partial or total undifferentiated form. This type of malignancy represents less than 5% of all NMTC cases, being the least common form (Muro-Cacho and Ku, 2000; DeLellis *et al.*, 2004; Bonora *et al*., 2010). ATCs are highly aggressive and lethal tumours with a very low survival rate – the mean duration of survival is only months (DeLellis *et al.,* 2004; Biersack and Grünwald, 2005; Kondo *et al.,* 2006; Soares *et al.,* 2011). These tumours can coexist with other thyroid carcinomas or hyperplasia (30% of the cases), and are usually non-encapsulated tumours, extensively invading the perithyroid tissues. The three main morphological patterns are squamoid, pleomorphic giant cell and spindle cell (Kondo *et al.,* 2006; Soares *et al.,* 2011). The mean age at the time of initial diagnosis is about 60 years old and there is a higher frequency on females (Muro-Cacho and Ku, 2000; DeLellis *et al.*, 2004; Kondo *et al.,* 2006; Soares *et al.,* 2011). Mutations in the tumour suppressor gene *p53* are known as the most common genetic events in this type of malignancy (67-88%) (Kondo *et al.,* 2006).

4. RAC1 and RAC1b GTPases

4.1. Organization and regulation

RAC1 is a member of the Rho (Ras homologous) family of small guanosine triphosphatases (GTPases) – a subgroup of the RAS superfamily of GTP (Guanosine triphosphate)-binding proteins. This Rho GTPases family includes other members such as Rho and Cdc42 (Cell division control protein 42 homolog). RAC1 protein is encoded by *RAC1* gene and can exist in two different conformational states - an inactive GDP (Guanosine diphosphate)-bound form and an active GTP-bound form (Jordan *et al.,* 1999; Matos *et al.,* 2000; Wennerberg and Der, 2004; Jaffe and Hall, 2005).

The interconversion between the two states occurs through a cycle of guanine exchange and GTP hydrolysis, wherein GTP binding induces a conformational change that involves two important regulatory regions, termed Switch I and Switch II. Consequently, the switch regions provide a surface that, in the active state, enables their interaction with downstream effectors, allowing these GTPases to function as molecular switches (Wennerberg *et al.,* 2005). This cycling process is tightly regulated by several groups of proteins: Rho-GEFs (Guanine exchange factors) which promote exchange of GDP for GTP; Rho-GAPs (GTPase activating protein) that enhance the hydrolysis of bound GTP to GDP and inorganic phosphate, regulating in the inactivation of the GTPases; GDP dissociation inhibitors (GDI) are also regulatory proteins that sequester Rho GTPases in the cytoplasm in an inactive GDP-bound state, preventing exchange of GDP to GTP (Jordan *et al.,* 1999; Bernards and Settleman, 2004; Singh *et al.,* 2004).

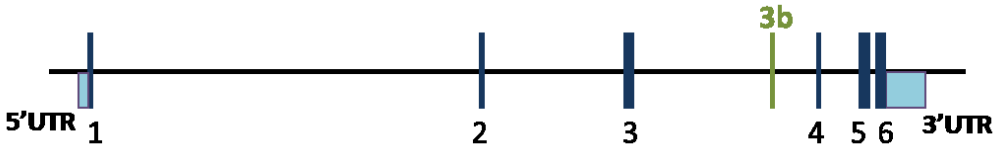


Figure I.4. Diagram of the *RAC1* gene. 1, 2, 3, 4, 5, 6 represent exons. Adapted from Matos *et al.,* 2000.

Back to 1999, Jordan and collaborators performed a RT-PCR (Reverse transcription polymerase chain reaction) based assessment of *RAC1* expression in colorectal samples (tumours and normal mucosa) that leads to the identification of a new *RAC1* splicing variant, termed *RAC1b*. This variant was also found to be expressed in breast carcinomas (Schnelzer *et al.,* 2000). The *RAC1b* isoform results from an alternative splicing event that leads to the inclusion of an additional exon (exon 3b). This additional exon is inserted between exons 3 and 4 (see Figure I.4) of *RAC1* and contains 57 additional nucleotides that result in an in-frame insertion of 19 amino acid residues between codons 75 and 76, in the vicinity of the switch II domain (Jordan *et al.,* 1999).

RAC1b is considered a highly activatable variant of RAC1: despite the lower levels of expression compared to RAC1, RAC1b exists predominantly in the active GTP-bound state. This is essentially due to RAC1b disability to interact with Rho-GDI, which keeps this GTPase constitutively membrane-bound, a location that benefits the interaction with activators, and consequently promotes the active GTP-bound state (Matos *et al.,* 2003). Moreover, RAC1b shows impaired intrinsic activity and increased GDP to GTP exchange rates, although this variant can still be downregulated by activated GAPs and it is influenced by GEFs action (Schnelzer *et al.,* 2000; Matos *et al.,* 2003; Fiegen *et al.,* 2004; Singh *et al.,* 2004). Also, RAC1b's additional amino acids seem to confer to this variant a selective downstream signalling, since several pathways activated by RAC1, are not activated by RAC1b (Matos *et al.,* 2003).

4.2. Biological functions

The Rho family of small GTPases is known to play an important role in several cellular processes, namely in actin cytoskeleton reorganization – Cdc42 regulates the formation of filopodia; RAC1 is involved in the lamellipodia formation; RhoA promotes the formation of stress fibres (Fukuda *et al.,* 2002; Matos *et al.,* 2003; Singh *et al.,* 2004).

RAC1, in particular, has the ability to interact with specific effectors, inducing activation of numerous signalling cascades that culminate in different physiological outcomes, namely cytoskeletal dynamics alteration, progression through the cell cycle and cell proliferation, apoptosis, migration and cell-cell adhesion, membrane trafficking and superoxide production (Matos *et al.,* 2000; Jaffe and Hall, 2005; Bosco *et al,* 2009). There are several downstream effectors and signalling proteins influenced by activated RAC1, namely WAVE (WASP-family verprolin-homologous protein) complex, PAK (p21 activating kinase) and LIM. For example, GTP-bound RAC1 binds to PAK1, stimulating its protein kinase activity, which allows the interaction with other proteins, leading to the modulation of several biological activities, such as the mentioned actin reorganization (Jaffe and Hall, 2005; Bosco *et al,* 2009; Nagase and Fujita, 2013).

The action of activated RAC1 also includes the stimulation of transcription factors such as the activation of the Jun NH_2-terminal kinase (JNK) cascade or the transcription factor NF-κB (Nuclear factor kappa-light-chain-gene-enhancer of activated B cells). The RAC1-related activation of the NF-κB pathway involves the production of reactive oxygen species (ROS), and initiates an anti-apoptotic transcriptional response, leading to an increased cyclin D1 expression and consequently promoting the cell cycle progression (Hinz *et al.,* 1999; Joyce *et al.,* 1999; Matos and Jordan, 2006).

As already mentioned, compared to RAC1, RAC1b, shows a selective downstream signalling. Unlike RAC1, GTP-bound RAC1b ability to induce actin cytoskeleton reorganization is impaired – RAC1b is unable to induce lamellipodia formation. RAC1b is also unable to interact with two other well-established RAC1 signalling pathways, in particular, this splice variant is incapable to activate PAK1 effector and stimulate the JNK cascade (Matos *et al.,* 2003; Fiegen *et al.,* 2004; Singh *et al.,*

2004). On the other hand, at least in the colorectal biological system, RAC1b retains the capacity to stimulate the NF-κB pathway, although its action is limited to the classical RelA-dependent NF-κB pathway, contrary to RAC1 that also stimulates the alternative pathway (RelB-dependent). The RAC1b stimulation of NF-κB classical pathway increases the cell cycle progression and cell survival, reducing the apoptotic rates (Matos and Jordan, 2005; Matos and Jordan, 2006). RAC1b was also shown to have the ability to mediate epithelial-mesenchymal transition (EMT) in breast (Radisky *et al.,* 2005).

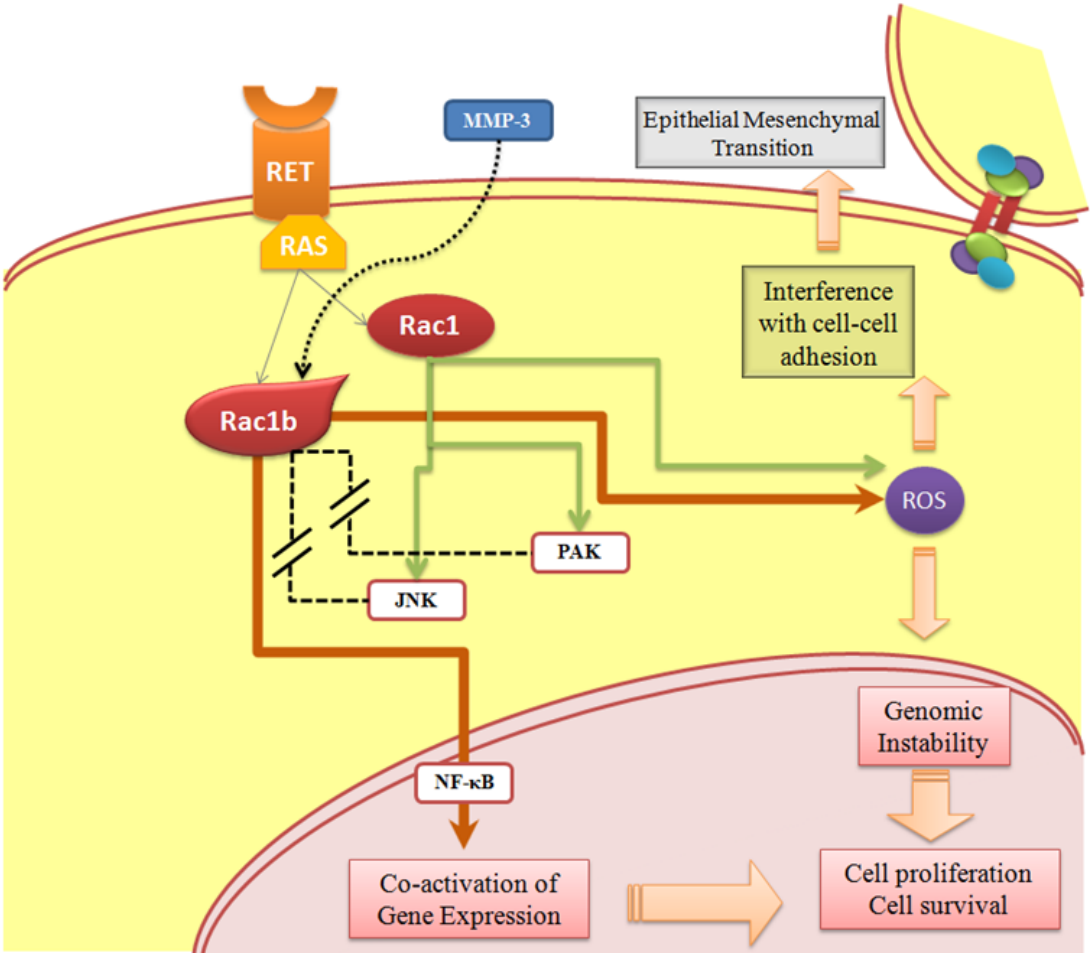


Figure I.5. **RAC1 and RAC1b signalling.**

4.3. Association with cancer

Malignant transformation is characterized by dramatic alterations in cell function and properties. This process includes the gain of migratory and invasive properties of tumour cells, increased proliferation levels and decreased apoptotic rates. RAC1 and RAC1b have been implicated in several of these cellular processes, namely in cell survival by increasing responses for apoptosis evasion and by stimulating cell cycle progression. The ability of these GTPases to interfere with these cellular functions could represent a tumorigenic property that allows the initiation of tumour formation, which, in turn, depends on the outcome of uncontrolled proliferation and anti-apoptotic mechanisms (Matos and Jordan, 2005; Fritz and Kaina, 2006; Bid *et al.,* 2013; Zhou *et al.,* 2013).

In agreement, a study using mouse mammary epithelial cells, reported that matrix metalloproteinase 3 (MMP-3) induces RAC1b expression, which promotes an increase in cellular ROS (reactive oxygen species). EMT, oxidative damage to DNA and genomic instability are some of the consequences of ROS increased levels, which are ultimately involved in tumour formation, invasion and metastasis (Radisky *et al.,* 2005).

In fact, the elevated expression of *RAC1b* has been documented in colorectal (Jordan *et al.,* 1999), breast (Schnelzer *et al.,* 2000), lung (Zhou *et al.,* 2013) and thyroid cancer (Silva *et al.,* 2013). Specifically in colorectal carcinoma, the alternative splice variant of RAC1 was shown to functionally cooperate with $BRAF^{V600E}$ mutation to sustain the proliferation and survival of tumour cells (Matos *et al.,* 2008). In a previous study, a group at IPOLFG (Silva *et al.,* 2013) reported for the first time *RAC1b* expression in thyroid tissue. Furthermore, the IPOLFG team shown that *RAC1b* is overexpressed in PTCs compared to normal thyroid tissue and that *RAC1b* overexpression is significantly associated with $BRAF^{V600E}$ mutation and poor clinical outcome in PTC. Similar to that reported in colorectal cancer, these findings point to an important role of RAC1b in PTC development, which may also be associated with a functional cooperation with $BRAF^{V600E}$ mutation.

5. Signalling pathways involved in cancer

The tumorigenesis process is often characterized by the accumulation of point mutations and changes in gene expression in pathways regulating specific aspects of cell proliferation and survival. Deregulation of some crucial pathways allows tumour cells to increase cell cycle progression, evade apoptosis and invade surrounding tissue. Many of these pathways that regulate cellular processes are controlled by specific genes. Alterations in the sequence, expression or epigenetics of these genes can alter these signalling cascades, inducing modifications in the cellular processes. For example, alteration in *TP53* gene (a gene involved in one of the major signalling pathways associated with apoptosis), induces alterations in this specific TP53 pathway, increasing tumour cells protection against apoptosis (Vogelstein and Kinzler, 2004).

In addition to TP53 pathway, there are many other signalling pathways described as being involved in the tumorigenic process of several tumours, such as JNK, PI3K, RTK (Receptor tyrosine kinases), cyclin D1 or NF-κB. NF-κB, in particular, was already implicated in several cancer types and has been studied as a potential therapeutic target (Debatin, 2004; Vogelstein and Kinzler, 2004; Dhillon *et al.,* 2007).

5.1. NF-κB signalling pathway

NF-κB (Nuclear factor kappa-light-chain-gene-enhancer of activated B cells) is a protein complex that plays a pivotal role in transcriptional control, cytokine production and in the regulation of several other cellular processes. In mammalian cells, the NF-κB family is composed of five members that form homodimers or heterodimers with each other – RelA (p65), RelB, c-Rel, p50 and p52. These proteins have an unique N-terminal Rel homology domain (approximately 300 amino acid) that allows dimer formation, binding to DNA and to NF-κB inhibitors (Karin and Ben-Neriah, 2000; Liang *et al.,* 2004; Solt and May, 2008; Madonna *et al.,* 2012). Conversely, the C-terminal domain differs between Rel (RelA, RelB and c-Rel) and NF-κB proteins (p50 and p52) – RelA, RelB and c-Rel exhibit transactivating function, while p50 and p52 contain inhibitory sequences associated to this C-terminal domain (Karin and Ben-Neriah, 2000; Madonna *et al.,* 2012). The Rel homology domain also contains a nuclear localization signal (NLS) (Huang *et al.,* 2000; Karin and Ben-Neriah, 2000; Karin *et al.,* 2002). Unlike Rel proteins, p50 and p52 proteins are produced by proteolytic removal of C-terminal sequences of two inhibitory precursor proteins – NF-κB1/p105 and NF-κB2/p100, respectively. This removal allows the final proteins to enter the nucleus, usually as heterodimers with one of the Rel proteins that bears a transactivation domain (Karin *et al.,* 2002; Bassères and Baldwin, 2006).

The NF-κB dimers remain transcriptionally unstimulated as long as they are sequestered in the cytoplasmic compartment. In this form, NF-κB proteins are usually associated with members of the IκB family (consist of an N-terminal regulatory domain followed by a series of ankyrin repeats), such as IκBα, IκBβ, IκBε or the inhibitory precursor proteins – NF-κB1/p105 and NF-κB2/p100 (Bassères and Baldwin, 2006; Solt and May, 2008). Some studies indicated that IκB proteins act by masking the nuclear localization signal, which prevents the NF-κB dimers translocation into the nuclear compartment (Huang *et al.,* 2000; Karin and Ben-Neriah, 2000).

Their activation can happen as consequence of a diversity of stimuli, such as those activating membrane B cell receptors (BCR) or tumour necrosis factor receptors (TNFR). Moreover, several extracellular stimuli can also stimulate NF-κB pathway, namely inflammatory cytokines, viral and bacterial infections, oxidative and DNA-damaging agents, UV light and osmotic shock (Chen *et al.,* 2012; Karin *et al.,* 2002).

The regulation of the NF-κB dimers can be performed through two main pathways. Both signalling pathways that activate NF-κB transcriptional activity have as pivotal regulator, the IκB kinase (IκK - inhibitors of κB kinases) complex, which is composed by two catalytic subunits (IκKα and IκKβ) and a

non-catalytic regulatory subunit named NEMO (NF-κB essential modulator) or IκKγ. NEMO plays a crucial role in both pathways, whereas IκKβ is particularly important in the classical pathway and IκKα is also part of the non-canonical regulatory pathway. IκKs are involved in the phosphorylation of NF-κB inhibitor proteins at specific serine residues (Liang *et al.,* 2004; Waes, 2007; Solt and May, 2008; Sun, 2011).

The first regulatory pathway (see Figure I.6), termed canonical or classical NF-κB activation pathway, applies to dimers composed by RelA, c-Rel and p50. It is usually triggered in response to microbial and viral infections and exposure to proinflammatory cytokines. The IκKβ has a particularly important role in this classical pathway by phosphorylating IκBα, which is subsequently ubiquitinated and degraded by the 26S proteasome. The degradation of NF-κB inhibitor, culminates with the accumulation of the dimeric NF-κB transcription factor (mostly composed by p50 and p65) into the nucleus, where it is able to bind κB site and induces the transcription of different target genes, most of them implicated in inflammation, survival and proliferation responses (Karin and Ben-Neriah, 2000; Karin *et al.,* 2002).

The second regulatory pathway (see Figure I.6), named non-canonical pathway, affects preferentially NF-κB2/p100 and RelB. The TNF (Tumour necrosis factor) cytokine family provides the most important stimuli for this pathway, which selectively activates the IκKα subunit along with another protein kinase called NIK (NF-κB inducing kinase). This activation induces a phosphorylation-dependent proteolytic removal of C-terminal sequences of NF-κB2 inhibitory precursor protein, which allows RelB/p52 dimers to translocate to the nucleus and recognize specific kB sites in the promoter of target genes (Karin and Ben-Neriah, 2000; Karin *et al.,* 2002; Sun, 2011).

Each pathway have distinct regulatory functions, although some target genes are common to all NF-κB proteins and are usually related to immunoregulatory and inflammatory processes, anti-apoptotic responses and cell cycle regulation (Shishodia and Aggarwal, 2002; Liang *et al.,* 2004; Matos and Jordan, 2005).

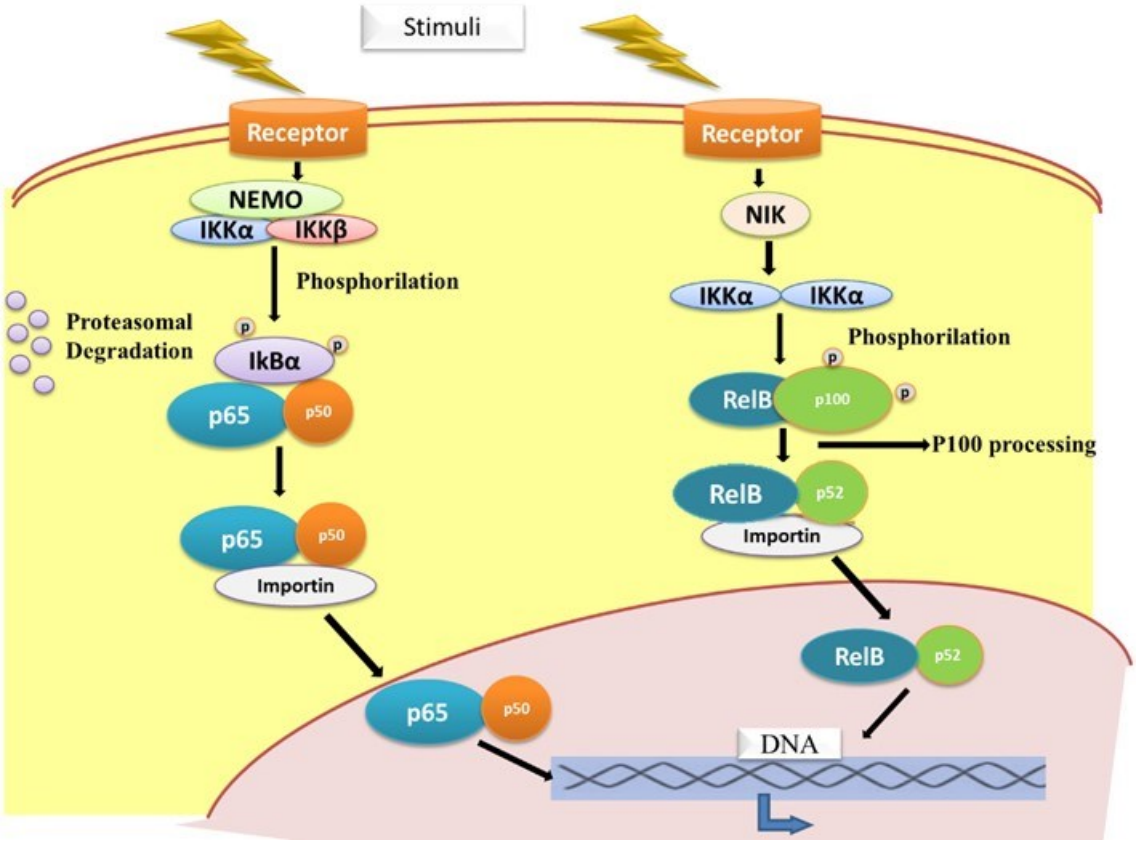


Figure I.6. **Canonical and non-canonical NF-κB activating pathways.**

5.1.1. Association with Tumorigenesis

NF-κB plays three essential roles (Madonna *et al.,* 2012). It is involved in the proinflammatory response, including immune, inflammatory and acute phase responses. It has already been established that this pathway sustains survival, maturation and recirculation of lymphocytes as well as T and B cell-mediated immune responses (Liang *et al.,* 2004). The ability to evade apoptosis is one of the most studied function of NF-κB pathway, which is known to induce the transcription of several anti-apoptotic proteins, such as Bcl-XL (B-cell lymphoma-extra-large), tumour necrosis factor receptor-associated factor 1 and 2 (TRAF1 and TRAF2, respectively), and the inhibitor-of-apoptosis (IAP) protein 1 and 2, which allows NF-κB to control the activity of the caspase family of enzymes (essential for the apoptotic cellular cascade) (Shishodia and Aggarwal, 2002; Liang *et al.,* 2004; Solt an May, 2008; Madonna *et al.,* 2012).

Ultimately, NF-κB is involved in the regulation of cell cycle progression through its ability to induce the expression of cyclin D1 (Hinz *et al.,* 1999; Joyce *et al.,* 1999; Matos and Jordan, 2005). Cyclin D1 works as a key sensor and integrator of extracellular signals of cells in G0 and early G1 phases, allowing the progression through the cell cycle, to S phase (Baldin *et al.,* 1993; Stacey, 2003; Fu *et al.,* 2004). Consequently, aberrant cyclin D1 expression is a critical event in cancer development that leads to

uncontrolled progression of cell cycle, usually induced by other cancer-related signals such as NF-κB activation or β-catenin (Hinz *et al.,* 1999; Joyce *et al.,* 1999; Fu *et al.,* 2004; Liu *et al.,* 2014).

Given the extent of biological mechanisms associated with NF-κB pathways and the overlap of these mechanisms with some hallmarks of cancer, it is expectable to find deregulation of this pathway in association with several types of malignancies. Research work in this field documented several changes in the upstream pathways that lead to NF-κB activation to become deregulated in cancer (Staudt, 2010). Moreover, some studies reported that mutations which are usually associated with cancer initiation and development, such as *RAS* mutation, can lead to IκB inactivation and consequently to the upregulation of NF-κB pathway (Mizumoto *et al.,* 2011; Chen *et al.,* 2012).

In fact, it has been reported over the years that increased activation of the NF-κB pathway is involved in some forms of cancer such as melanoma (Madonna *et al.,* 2012), lung cancer (Chen *et al.,* 2012), glioblastoma (Nogueira *et al.,* 2011), colorectal cancer (Wang *et al.,* 2009), thyroid cancer (Namba *et al.,* 2007; Bauerle *et al.,* 2010) and ovarian cancer (White *et al.,* 2011).

Given that, NF-κB pathway appears to have a crucial role in a vast diversity of tumours, the inhibition of this signalling cascade starts to be seen as a promising strategy for anti-cancer therapies.

5.1.2. Association with RAC1b

The ability of RAC1 to stimulate the NF-κB pathway is shared with its splice variant RAC1b, which was shown to be capable to promote IκB phosphorylation and nuclear translocation of RelA, in NIH 3T3 mouse embryonic fibroblast cell line (Matos *et al.,* 2003). Moreover, a study in colorectal cells demonstrated that RAC1b only stimulates the canonical NF-κB regulatory pathway, differing from RAC1 which is capable of stimulate both the classical and the alternative pathway. The activation of the non-canonical pathway by RAC1 could interfere, in a potential negative feedback regulation, with the RAC1-induced classical cascade. The selective RAC1b induction of the canonical regulatory pathway permits this GTPase to circumvent the negative feedback from the RelB pathway, which allows a continuously stimulation of the p50/RelA NF-κB-mediated transcription. Also in colorectal cells, the expression of RAC1b was shown to stimulate transcription from a luciferase reporter promoter containing consensus NF-κB binding sites, through a ROS dependent cascade (Matos and Jordan, 2006).

In opposition, Singh and collaborators (2004) reported that RAC1b failed to activate the NF-κB transcription factor or stimulate cyclin D1 expression, in NIH 3T3 cell model (fibroblasts).

Whether RAC1b contributes to NF-κB-mediated cellular processes (namely proliferation or apoptosis evasion) and to malignant progression in cell systems other than colorectal cancer remains to be clarified.

6. Follicular cell derived thyroid cancers, RAC1b and NF-κB pathway – a link to unravel

Part 1. RAC1b role in FTC Tumorigenesis

Our group has recently reported *RAC1b* expression in thyroid tissue (Silva *et al,* 2013). In fact, this variant was found to be overexpressed in PTCs compared to normal tissue, and to be associated with a subset of PTCs with a poor clinical outcome. These findings not only point to an important role of RAC1b in PTC development but also raise the possibility that RAC1b overexpression might be associated with other types of thyroid carcinoma, namely with other subtypes derived from follicular cells (non-medullary thyroid carcinomas). The second most frequent form of thyroid carcinomas is the FTC, representing about 15% of all cases. Despite the good prognosis usually associated to this type of malignancy, some patients present advanced tumours, non-responsive to radioiodine therapy.

Hence, the broadening of the study of this GTPase to other follicular cells derived neoplasia, such as the FTCs, could provide novel information about the role of RAC1b in the thyroid tumorigenesis. This might as well contribute to the definition of prognostic biomarkers for this tumoural subtype, allowing an individual case management of risk level.

On the other hand, since one major challenge in the clinical management of FTC patients is related with the inability to discriminate between benign and malignant follicular tumours at the preoperative stage, a comparative analysis of RAC1b expression between FTC and FTA may clarify the importance of this GTPase as a potential marker to be included in a broader panel of biomarkers to be used as diagnostic tool.

Part 2. RAC1b - An activator of NF-κB pathway that contributes to thyroid Tumorigenesis?

The potential association of RAC1b overexpression with PTC development, magnified the importance of investigating the molecular mechanisms associated with RAC1b overexpression and downstream signalling in thyroid tumorigenesis. In fact, the identification of precise alterations on signalling pathways associated with specific cancer processes would be pivotal for the understanding of molecular mechanisms behind tumour progression and for the development of diagnostic and prognostic biomarkers. In thyroid cancer there is, undoubtedly, the emerging need for a better understanding of the molecular events involved in the initiation and progression of malignancy as well as its association with failure in the response to iodine treatment and high recurrence rates. Understanding these mechanisms should contribute to individual case management optimization.

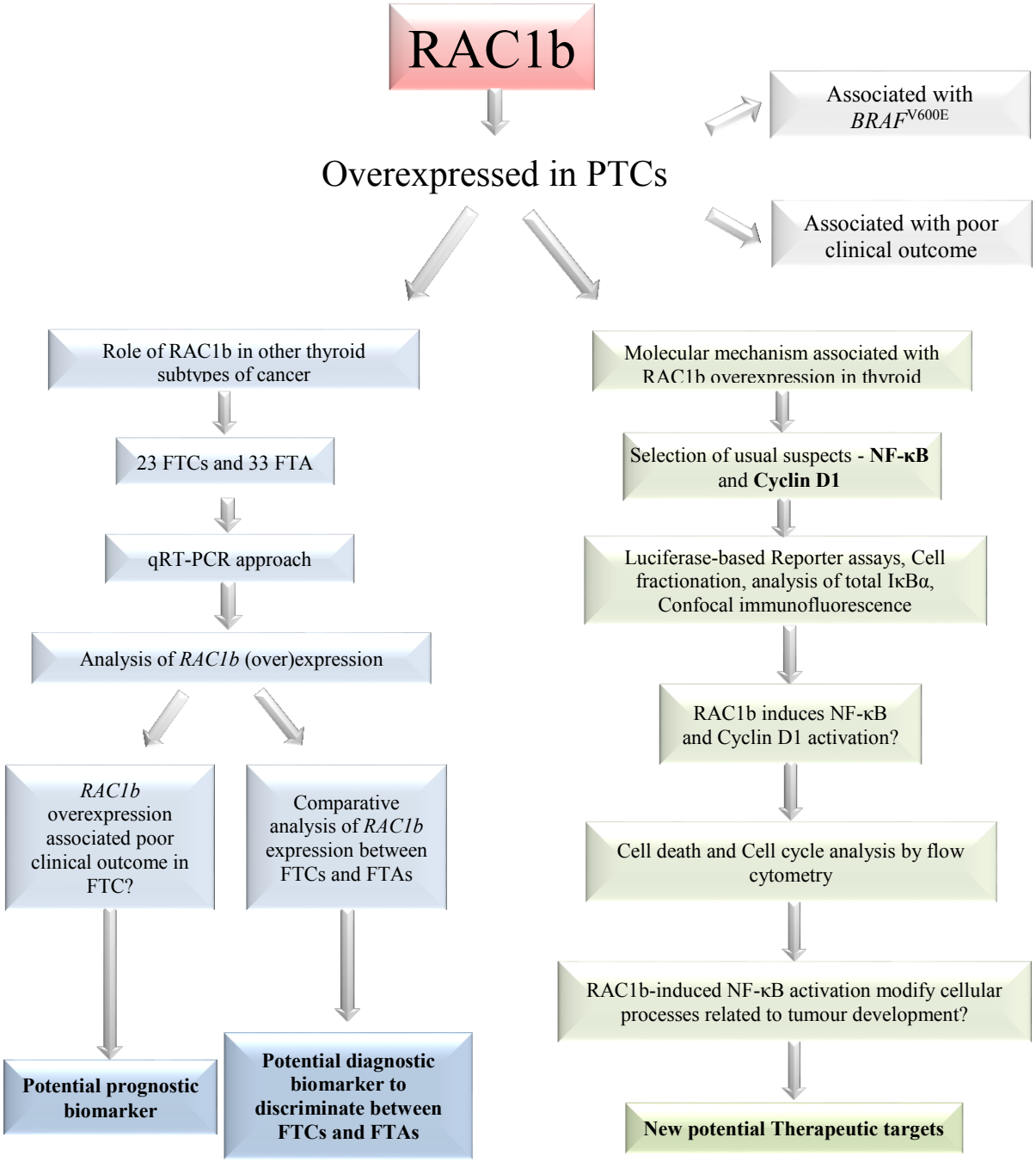
A possible rational approach to investigate the molecular mechanisms associated with RAC1b overexpression and downstream signalling in thyroid tumorigenesis is to select a signalling pathway previously implicated in the tumorigenic process as a starting point. This selection was based in two previously published facts: NF-κB activation has been also reported to play an important role in thyroid

cancer (Namba *et al.,* 2007; Bauerle *et al.,* 2010); RAC1b selectively induces the canonical regulatory NF-κB pathway in colorectal cancer (Matos and Jordan, 2006). Hence, we considered relevant to study the role of RAC1b on NF-κB activation in thyroid tissue, and to further assess the effect of this potential RAC1b-mediated NF-κB stimulation in terms of cell growth and survival. Furthermore, given the role of Cyclin D1 as a cell cycle regulator, its association with some cancer events and the capacity of NF-κB to induce cyclin D1 expression, it is reasonable to consider Cyclin D1 as another target potentially affected by RAC1b overexpression.

II. OBJECTIVES

We propose to analyse the expression of *RAC1b* in FTC and FTA, to compare the expression levels between the two groups (malignant versus benign) and correlate its expression with histopathological features and clinical outcome. This will allow us to evaluate RAC1b potential as a diagnostic/prognostic biomarker in FTC.

We also intend to assess the RAC1b effect on NF-κB in thyroid cells to further understand whether RAC1b-mediated alteration of this pathway could interfere in cellular processes, such as cell cycle and apoptosis. This will allow us to clarify the mechanisms involved in RAC1b-induced thyroid tumorigenesis.



III. MATERIALS AND METHODS

1. Biological material – patients samples

In the context of this thesis, samples representative of primary tumours from a cohort of 23 FTC patients (mean age of 54 years and Female: Male ratio 16:7) who underwent partial/total thyroidectomy at IPOLFG from 1994 to 2010, and 33 samples corresponding to FTAs were analysed. Samples were collected at surgery and immediately frozen and stored in liquid nitrogen. Tissue sample collection was carried out in accordance with protocols approved by the institutional review board and informed consent was obtained for the study together with the consent for surgery.

2. DNA plasmids and constructs

The plasmids used in this study are described in Matos and Jordan (2006) and Barros *et al.* (2009) and were a kind gift from Dr. Peter Jordan, from Instituto Nacional de Saúde Dr. Ricardo Jorge: pcDNA3-HA-IκBα(A32A36), -964-CycD1-Luc vector, 3x-kB-luc vector, pRL-TK reporter (a low level, constitutively expressed Renilla luciferase as internal control), pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT. The pEGFP-C1 (*BD Biosciences Clontech,* USA) and the pcDNA3.1 (+)-empty vector (*Invitrogen,* USA) were used as negative controls.

3. Cell Culture and transfection

Two commercially available cell lines were used in this study – the K1 cell line which is derived from a primary human papillary thyroid carcinoma, and Nthy-ori 3-1 (Nthy) cell line derived from human thyroid follicular normal epithelium.

Cells were maintained at 37°C in a humidified 5% CO_2 environment. K1 cells grew in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM: F12, 1:1) (*Gibco*, USA) containing 0,365 g/L of L-glutamine. Medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (*Biochrom*, UK), 1% (v/v) GlutaMax (*Gibco*, USA), 1,25% (v/v) sodium bicarbonate (*Gibco*, USA) and 0,5% (v/v) sodium pyruvate (*Gibco*, USA). Nthy cells were maintained in RPMI-1640 HEPES modified medium (*Sigma*, USA). Medium was supplemented with 10% (v/v) FBS and 1% (v/v) GlutaMax. When cells grow to an optimal confluence (80-100%), they were subcultured being detached by incubation with 1x trypsin-EDTA (Ethylenediamine tetraacetic acid) (*Invitrogen*, USA) at 37°C for approximately 1 minute (min), after a PBS 1x (Phosphate buffered saline) (*Gibco*, USA) washing step.

Transfections were performed using the cationic lipid-based transfection reagent Lipofectamine 2000 (*Invitrogen*, USA), according to manufacturer's instructions. Briefly, $5x10^5$ K1 cells or $2,5x10^5$ Nthy cells were seeded, 24 h before transfection, in 35 mm dishes (*Nunclon*, USA). Just before transfection, cells were fed with new fresh complete medium. Transfection was performed at 70-85% confluence. Plasmid DNA (2 μg) was diluted into 125 μL OPTI-MEM (Improved minimal essential

medium) (*Gibco*, USA); in an additional tube, 4 µL of transfection reagent was also diluted in 125 µL of OPTI-MEM and incubated 5 min at room temperature (RT). Diluted DNA and lipofectamine were then mixed gently and incubated for 20 min at RT to allow the formation of DNA-lipofectamine complexes. After incubation, 250 µL of transfection mixture was added to each culture dish.

For luciferase reporter experiments, cells were co-transfected with either RAC1, RAC1b, or Mock control expression vectors, the specific reporters (1 µg), and the Renilla luciferase expression vector (0,1 µg). In all other experiments presented in this work, cells were transfected with RAC1/1b expression vectors and a pcDNA3.1 (+)-empty vector was used to adjust the total amount of transfected DNA to 2 µg. The amount of RAC1/1b vectors used was adjusted in order to achieve appropriate expression levels (see Table III.1).

Table III.1. Description of plasmid amounts used in each assay, for each cell line.

Assay / Plasmid	Luciferase reporter assay		IFs (Immunofluorescence)	Cell fractionation	Total IκBα	Cell death	Cell cycle
	K1	Nthy					
GFP-RAC1b-L61	1 µg	1 µg	-	1 µg	1 µg	1 µg	1 µg
GFP-RAC1-L61	0,3 µg	1 µg	-	-	-	-	-
pcDNA3.1 (+)-(Mock control)	1 µg	1 µg	-	1 µg	1 µg	-	-
pEGFP- C1 (Mock control)	-	-	1 µg	-	-	1 µg	1 µg
GFP-RAC1b-WT	1 µg	1 µg	1 µg	1 µg	1 µg	-	1 µg
GFP-RAC1-WT	0,3 µg	1 µg	0,3 µg	0,3 µg	-	-	-

4. RNA extraction

Total RNA extraction and purification were performed using the Rneasy Mini Kit (*QIAGEN*, Germany), according to the manufacturer's protocol. An Eppendorf centrifuge 5417C (rotor radius = 95 mm) were used during the procedures.

RNA extraction from frozen tissue was performed using RLT lysis buffer with β-mercaptoethanol 10% (v/v), after tissue fragmentation. Total RNA concentration was measured using a NanoDrop 2000 spectrophotometer (*Thermo Scientific*, USA), and frozen at -80°C.

5. cDNA synthesis and amplification

cDNA (complementary DNA) was synthetized from 2 µg of total RNA and reversely-transcribed using random primers (*Roche*, USA) and SuperScript II Reverse Transcriptase (*Invitrogen*, USA). In the first step, 2 µg RNA, 0,1 µL random primers (3 µg/µL), 0,4 µL deoxynucleotides (dNTPs, 25 µM) (*GE Healthcare,* UK) and bidestilled water (ddH_2O) to a final volume of 12,9 µL were added. This first

reaction mixture was incubated at 65°C for 10 min (denaturation step) to remove RNA secondary structures that may hamper cDNA synthesis, and then cooled to 4°C. Then a second reaction mixture (7,1 µL) consisting in 4 µL first Strand Buffer 5x (*Invitrogen*, USA), 2 µL dithiothreitol (DTT, 0.1 M) (*Invitrogen*, USA), 1 µL Superscript II (200 U/µL) (*Invitrogen*, USA) and 0,1 µL RNAse OUT (Recombinant Ribonuclease Inhibitor, 40 U/µL) (*Invitrogen*, USA), was added. cDNA synthesis was performed in a T3000 thermocycler (*Biometra*, Germany), using the following basic program: 42°C for 1 h (cDNA synthesis), 70°C for 15 min (inactivation) and 4°C (cooling) until the storage at -20°C.

Using the synthesized cDNA, RT-PCR (Reverse transcription polymerase chain reaction) amplification was performed using primers for specific amplification of *RAC1* and *RAC1b*. The primers (F-forward; R-reverse) for *RAC1/1b* amplification were RAC1/1bF (5'-atgttatggtagatggaaaaccg) and RAC1/1bR (5'-cggacattttcaaatgatgcagg), which generate PCR products of 154 and 211 base pairs, allowing the distinction between the two splice variants. The PCR reaction mix consisted of 25 µL PCR buffer 1x (home prepared by diluted 10 times a PCR buffer 10x in ddH20 and add 20 µL of each 100 mM dNTP, see Appendix 1), 0.15 µL Taq DNA Polymerase (5 U/µl) (*Invitrogen*, USA), 0,5 µL reverse and forward primer (10 µM). 23 µL of this mixture was added to 2 µL cDNA to perform a total volume of 25 µL.

Amplification reactions were performed in a T3000 thermocycler (*Biometra*, Germany), using a basic program described in Table III.2.

Table III.2. *RAC1/1b* PCR amplification conditions.

Stage	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 seconds	35
Annealing	62[1]	30 seconds	
Elongation	72	30 seconds	
Final elongation	72	10 min	1
Inactivation and cooling	4	∞	1

[1] Annealing temperature of RAC1/1b primers.

The amplified PCR products were resolved by electrophoresis, on a 2% (w/v) agarose gel in TBE (Tris-borate-EDTA) buffer 1x (diluted from TBE buffer 10x, *National diagnostics*, USA), stained with 0.05% (v/v) ethidium bromide (*Invitrogen*, USA). The visualization was performed under UV light and image acquired in BioDocAnalyse Transilluminator (*Biometra*, Germany).

6. Quantitative reverse transcription – PCR (qRT-PCR)

The *RAC1b* and total *RAC1* expression levels were quantified by qRT-PCR using TaqMan gene expression assay probes (*Applied Biosystems,* USA), accordingly to manufacturer's instructions. Two different probes were used, one of them capable of selectively amplify *RAC1b*, and the other capable to amplify both *RAC1* variants.

To each sample, a reaction mixture was prepared with 1 µL TaqMan gene expression assay, 10 µL TaqMan gene expression Mastermix (*Applied Biosystems,* USA) and 5 µL ddH_2O, in which 4 µL of diluted cDNAs (generally 1:20 in ddH_2O) were added. Amplification reactions were performed in triplicate for each sample, on ABI Prism 7900HT Sequence Detection System (*Applied Biosystems,* USA). In order to evaluate the efficiency of each reactor used, successive dilutions of the same cDNA were analysed, enabling the assessment of the reaction efficiency for each detector.

7. Luciferase reporter assays

These assays are based on the fusion of a firefly luciferase gene with a putative promoter sequence, allowing the investigation of promoter activity by measuring the light output (luminescence) from luciferase enzyme that is expressed under the control of the promoter of interest. The luciferase activity was measured in Victor3 1420 Multilabel Counter Luminoter (*Perkin Elmer*, USA), using the Dual Luciferase Assay System (*Promega*, USA), according to the manufacturer's protocol. In order to minimize errors and technical variability (distinct expression efficiencies or cell number by plate) it is recommended to use a second reporter gene constitutively active as internal control, in this case, the Renilla Luciferase. The two luciferases are measured sequentially (revised in Dual Luciferase Assay System technical manual – instructions for use of product, *Promega*, USA; Allard and Kopish, 2008).

K1 and Nthy cells were seeded in 35 mm dishes and co-transfected with 100 ng of the pRL-TK (Renilla luciferase) reporter, 1 µg of NF-κB or cyclin D1 specific reporters and an optimized amount of RAC1/1b and Mock control constructs (see Table III.1). At 24 (NF-κB experiments) or 30 h (cyclin D1 experiments) in the presence or absence of the RAC inhibitor EHT 1864, cells were scraped off and lysed with a passive lysis buffer 1x (PLB, *Promega*, USA) (specially formulated to minimize background auto-luminescence), following the manufacturer's instructions. In EHT 1864 experiments, cells were incubated with the inhibitor (final concentration 0,1 mM) by 4 h.

To 20 µL lysates, 50 µL Luciferase assay substrate (*Promega*, USA) was added and then the firefly luciferase activity was measured; the addition of 50 µL Stop&Glo reagent (*Promega*, USA) stopped the first reaction, which allows the measurement of Renilla luciferase activity. Firefly luciferase values were normalized to the internal control values and then a second normalization as -fold increase over the value of Mock control was performed.

Additionally, 20 µL lysate was collected for Western Blot analysis in order to assess protein expression levels.

8. Confocal immunofluorescence microscopy

K1 cells were grown on glass cover slips (10 by 10 mm), transfected with optimized amounts of RAC1b/RAC1 constructs and Mock control vector. Six hours after transfection, cells were subjected to starvation conditions (DMEM: F12 with 1% FBS) for 12 h. After this period, cells were stimulated with DMEM: F12 supplemented with 10% FBS for 30 min. Cells were then washed twice in PBS 1x and fixed with 4% (v/v) formaldehyde for 30 min at RT, following a permeabilization step with 0.2% (v/v) Triton X-100 in PBS for 10-15 min at RT. Cells were washed 3 times for 5 min in PBS-tween (0,05% [v/v]), and then incubated for 10 min at -20°C with methanol, following a 3 times PBS-tween wash step. Cells were incubated with the primary antibody anti-NF-κB p65 NLS (1:750) at 4°C, overnight, with shaking. After being washed 3 times in PBS-tween, cells were incubated for 1 h with Alexa Fluor 532 goat anti-Rabbit IgG (*Life Technologies,* USA) (1:500). Coverslips were then washed 3 times in PBS-tween, mounted in a mixture of VectaShield medium and VectaShield medium with DAPI (4',6-diamidino-2-phenylindole) (*Vector Laboratories*, USA) (1:1), and sealed with nail polish. Images were recorded with the 405-nm, 488-nm, and 532-nm laser lines of a Leica TCS-SPE confocal microscope and processed with Adobe Photoshop software.

9. Cell fractionation

K1 cells were transfected with optimized amounts of RAC1b/RAC1 constructs and Mock control (see Table III.1). Six hours after transfection, cells were subjected to starvation conditions (DMEM: F12 with 1% FBS) for 12 h. After that time, cells were stimulated with DMEM: F12 supplemented with 10% FBS for 30 min.

Nuclear and cytosolic proteins were prepared using a previously described procedure (Solan *et al.,* 2002). Briefly, after a PBS 1x wash step, cells were scrapped off and lysed on ice in 150 µL cytosol fractionating buffer containing 50 mM HEPES (pH 7.2), 2 mM EDTA, 10 mM NaCl, 250 mM sucrose, 2 mM DTT, 0.1% (v/v) Nonidet-P40 and a protease inhibitor cocktail (*Sigma,* USA). After 5 min on ice, the cytosolic fraction was harvested by centrifuging the lysate at 3000 g for 4 min and collecting 120 µL supernatant, in which 30 µL 5x sample buffer (5x SB, see Appendix 1) were added. The remain pellet were resuspended in 300 µL cytosol fractionating buffer before a centrifugation (3000 g, 4 min, 4°C). The nuclear proteins were extracted by resuspending the pellet in 35 µL of nuclei fractionating buffer composed by 50 mM HEPES (pH 7.2), 2 mM EDTA, 400 mM NaCl, 20% (v/v) glycerol, 2 mM DTT, and a protease inhibitor cocktail, for 20 min with occasional vortexing. After centrifugation (10000 g, 4 min, 4°C), the supernatant (nuclei proteins) was added to 8 µL 5x SB. An Eppendorf

centrifuge 5810R (rotor radius = 180 mm) were used during the procedures. Both fraction were analysed on Western Blot.

10. Protein extraction with RIPA

For the purpose of study total IκBα protein expression, K1 cells in 35 mm dishes were transfected with optimized amounts of RAC1b/RAC1 constructs and Mock control vector. At 24 h, cells were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (containing protease inhibitors, see Appendix 1). The lysates were centrifuged at 9000 rpm for 1 min in order to remove cell debris. For densitometry analysis, membranes images from three independent experiments were analysed using ImageJ software.

11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

Lysates resulting from luciferase reporter assays, cell fractionation experiments and protein extraction for total IκBα protein expression analysis, were prepared for Western Blot analysis: 5 µL of Sample buffer (5x SB, see Appendix 1) was added to each 20 µL of protein extract and these were boiled at 95°C for 10 min, in order to denature proteins. Equivalent amounts of total protein from each sample was loaded in 10 -12% SDS-PAGE gels (prepared accordingly to Sambrook and Russel, 2001; a 12% concentration were used in order to allow the distinction between RAC1 and RAC1b proteins). Electrophoresis was carried out at 0,02 Ampere per gel, for approximately 1 h 15 min. Proteins were then transferred to PVDF (polyvinylidene difluoride) membranes (*Bio-Rad,* USA) with a Mini Trans-Blot Electrophoretic Transfer Cell (*Bio-Rad,* USA), at 70 Volt, 4°C, for 1 h. A 5% (w/v) non-fat milk in Tris Buffered Saline Buffer 0,05% Triton X-100 (TBST milk) solution were used to block non-specific bindings to the membrane (1 h, RT), which were then incubated with specific primary antibodies (anti-RAC1, *Millipore;* anti-NF-κB p65 NLS, *Thermo Scientific;* anti-IκBα, *Santa Cruz Biotechnology,* USA) at appropriate dilutions (1:2000, 1:1000, 1:1000 respectively, in TBST milk), at 4°C, overnight, with shaking. Membrane were then incubated for 1 h at RT with secondary horse radish peroxidase-conjugated antibody (anti-rabbit IgG and anti-mouse IgG, *Thermo Scientific)* diluted at 1:5000 in TBST milk. Band detection was performed using the ECL substrate (see Appendix 1) protocol in a ChemiDoc XRS System (*Bio-Rad,* USA) with Image Lab software. Membranes were then reprobed with anti-RAC1 (1:2000), anti-β-actin (*Sigma;* 1:10000) and/or anti-Histone H2B (*Millipore;* 1:1000), used as loading controls.

12. Cell death analysis by flow cytometry (FACS)

The approach used in this work for cell death analysis by flow cytometry was based on the combined use of propidium iodide (PI, a fluorescent DNA intercalating dye) and Annexin V to determine if cells are viable, apoptotic (early or late stage), or necrotic. This distinction is based on differences in plasma

membrane integrity and permeability. Apoptotic stages are characterized by a loss of membrane integrity, which result in the exposure of phosphatidylserine (PS) to the extracellular environment, allowing Annexin V binding. On the other hand, PI is impermeant to live cells and early-stage apoptotic cells, while late stage apoptotic cells and necrotic cell stain positively, due to the passage of PI into the nucleus and consequent DNA binding (Annexin V Product Data Sheet, *BioLegend*, USA).

K1 cells were seeded in 35 mm dishes and then transfected with optimized amounts of RAC1b-L61 construct and Mock control vector. For IκBaa (non-degradable super-repressor) experiments, cells were co-transfected with 100 ng of pcDNA3-HA-IκBα (A32A36). 24 hours after transfection, cells were incubated in fresh medium in the absence or presence of staurosporine (*Santa Cruz Biotechnology*) at selected final concentrations (5 nM, 10 nM and 50 nM), for two more hours. After that time, cells were collected. 2 mL of the supernatant of each dish were collected into 15 mL tubes, following a PBS wash step. Cells were then trypsinized (200 µL per dish) and collected for the corresponding 15 mL tube. After centrifugation at 1200 rpm for 3 min (Eppendorf Centrifuge 5810R, rotor radius = 180 mm), supernatant were eliminated and cells were resuspended in 100 µL annexin V binding buffer (see Appendix 1). Cells were then stained with 2 µL propidium iodide (PI) (100 µg/mL) (*Sigma,* USA) and 5 µL APC annexin V (15 µg/mL) (*BioLegend*, USA), and incubated at RT in dark, for 15 min. After incubation, cells were resuspended in 100 µL of annexin V binding buffer and analysed by flow cytometry (FACScalibur – Becton Dickinson). Cell acquisition parameters were defined using three blanks (with no stain, with PI alone, with annexin V alone), using the software BD Cell Quest. The acquired data was analysed in FlowJo (*Tree Star Inc*, USA) software.

13. Cell Cycle analysis by flow cytometry (FACS)

The methodology used in this context is based on the ability of staining the cellular DNA with a fluorescent DNA intercalating dye – PI – which binds the DNA, in cells permeabilized with ethanol, in a stoichiometric manner (the amount of stain is directly proportional to the amount of DNA within the cell). This stoichiometric binding allows the distinction between three cell cycle stages – G1 phase (corresponds to a gap before S phase, in which cell is preparing for DNA synthesis), S phase (characterized by DNA synthesis and duplication), G2/M phases (this two cell cycle phases cannot be identified individually; the G2 phase correspond to a gap after the S phase and before the cellular process of mitosis that happen in M phase) (Darzynkiewicz and Zhao, 2014).

Like in cell death experiments, K1 cells were seeded in 35 mm dishes and then transfected with optimized amounts of RAC1b constructs and Mock control vector. For IκBaa (non-degradable super-repressor) experiments, cells were also transfected with 100 ng of pcDNA3-HA-IκBα (A32A36). Six hours after transfection, cells were exposed to starvation conditions (DMEM: F12 with 1%FBS) for 12h in order to allow cell cycle synchronization. After that time, cells were stimulated with DMEM: F12 supplemented with 10% FBS for another six hours. After that time, cells were collected. The supernatant

was eliminated, following a PBS wash step. Cells were then trypsinized and collected for 1,5 mL tubes. After centrifugation at 1200 rpm for 3 min (Eppendorf Centrifuge 5417C; rotor radius = 95 mm), supernatant was eliminated and cells were resuspended 1 mL 70% (v/v) ethanol (*Merck*, Germany) and placed at 4°C, overnight, in order to fix cells and permeabilize the membrane (the minimal time for fixation and permebilization is 6 h). One day after, cells were centrifuged at 1200 rpm for 5 min, the supernatant was discarded and cells were then stained with 100 µL PI solution (50 µg/mL, see Appendix 1) and incubated at 37°C in a water bath, for 40 min. After incubation, 1 mL of PBS 1x was added and cells were centrifuged at 1200 rpm, 4°C, for 10 minutes (Eppendorf Centrifuge 5810R, rotor radius = 95 mm). Ultimately, supernatant was discarded and cells were resuspended in 250 µL of 0,2% (w/v) BSA (Bovine serum albumin) in PBS 1x and analysed by flow cytometry (FACScalibur – Becton Dickinson). The acquired data was analysed in FlowJo (Tree Star Inc, USA) software and the fraction of cells in each phase of cell cycle (G1, S, and G2/M) were evaluated.

14. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 software (San Diego, USA). Whenever appropriate, values are expressed as mean ± SD (Standard deviation). The two-tailed Student's t-test or two-tailed Fisher's exact test and the paired two-tailed Student's t-test were used to perform statistical analysis and evaluate statistical significance of results (accepted as $p<0,05$).

IV. RESULTS

PART 1

1. *RAC1b* is overexpressed in FTC

RAC1b expression levels were assessed by qRT-PCR in 23 FTCs and 33 FTAs. *RAC1b* levels were firstly normalized to total *RAC1* expression levels. The mean expression levels of *RAC1b* were significantly higher in FTCs (0,76 ± 0,94) compared to FTAs (0,34 ± 0,15) (p-value= 0,0152, two-tailed Student's t-test). The mean fold increase was approximately 2,2. In order to discriminate FTCs and FTAs that overexpressed *RAC1b* from those that did not, we defined a threshold level of expression above which *RAC1b* were considered to be overexpressed. We set the threshold level at 0,76 (mean plus two standard deviations of the *RAC1b* expression level in a set of normal thyroid tissues). Based on these criteria, 7 out of 23 FTCs (30%) were found to overexpress RAC1b. Notably, in the FTAs group, no cases presenting *RAC1b* overexpression were found. Consistently, the difference in *RAC1b* overexpression between follicular adenomas and carcinomas was statistically significant (p-value= 0,001, two-tailed Fisher's exact test).

2. *RAC1b* overexpression is associated with presence of distant metastasis and poorer clinical outcomes

To investigate whether *RAC1b* overexpression was associated with histopathological parameters and clinical outcome, FTCs were grouped based on the clinical data available (see Table IV.3).

A comparison between FTC patients without distant metastasis (M0, 16 cases) versus FTC patients with distant metastasis (M1, 7 cases) disclosed a higher prevalence of *RAC1b* overexpression in M1 group (5 out of 7 M1 cases). The association between *RAC1b* overexpression and presence of distant metastasis is statistically significant (p-value= 0,011). No other statistical significant correlation was found between *RAC1b* overexpression and histopathological features such as multifocality, vascular invasion, angioinvasion, Hürthle cell subtype or presence of poorly differentiated areas.

FTCs were grouped based on the final clinical outcome. Group I (70% of patients) included patients who reached full sustained remission (no evidence of disease) and patients presenting biochemical evidence of disease. In contrast, group II (30% of patients) included patients with structural evidence of disease and those who died due to disease progression. The correlation between *RAC1b* overexpression and group II is statistically significant (p-value= 0,011), which further confirms that RAC1b is associated with poorer clinical outcomes in FTC patients.

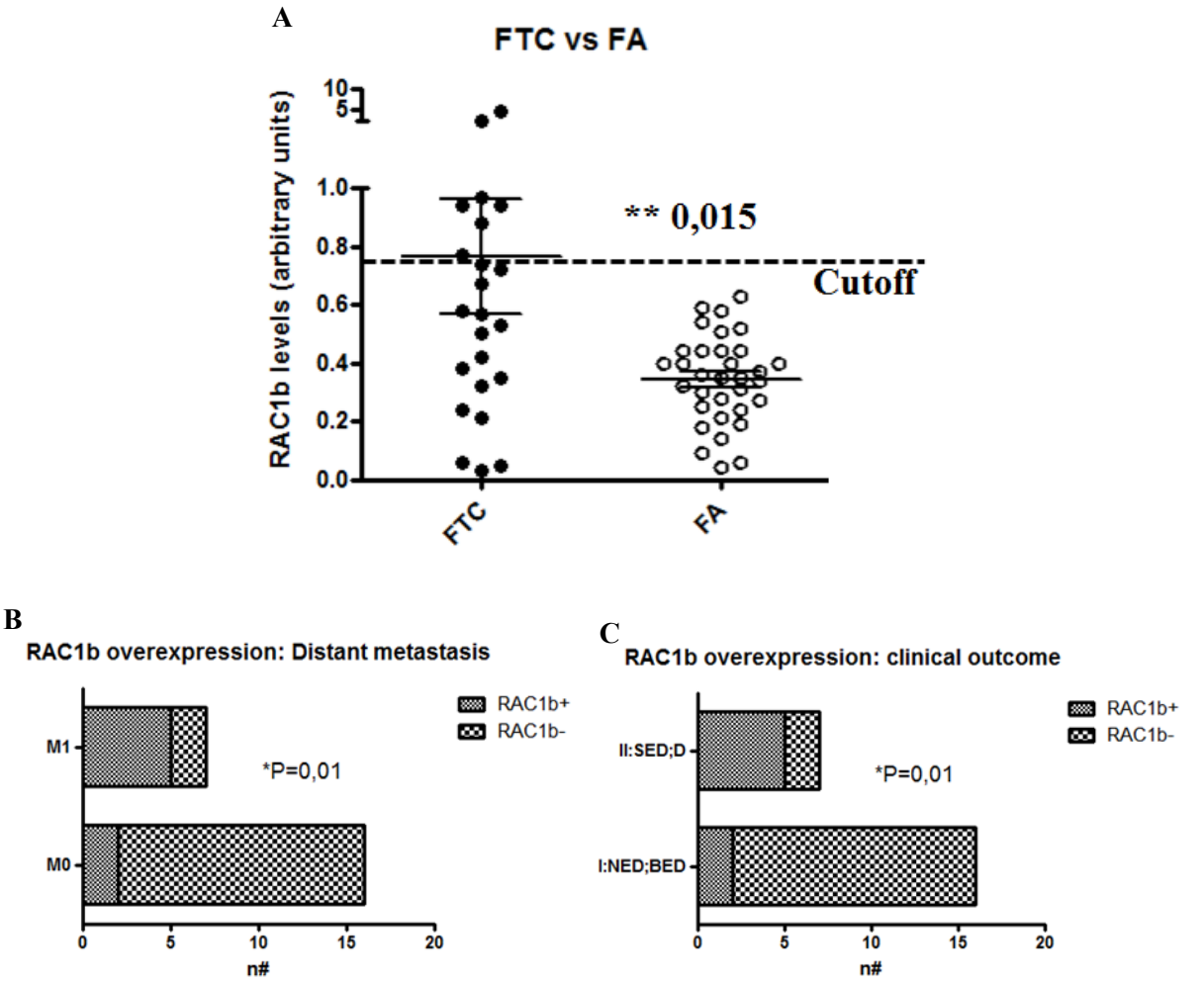


Figure IV.7. ***RAC1b*** **overexpression and its association with distant metastasis and clinical outcome.** (A) 7 out of 23 FTCs overexpressed *RAC1b* in opposition to none of the 33 FTA (p-value= 0,001). (B) The 23 FTCs were grouped according to presence of distant mestatasis: M0 – absecence of distant metastasis; M1 – presence of distant metastasis. The association between *RAC1b* overexpression and M1 group is statistically significant (p-value= 0,011). (C) The 23 FTCs were grouped according to final outcome: group I –No evidence of disease (NED) and Biochemical evidence of disease (BED); group II - Structural evidence of disease (SED) and Death due to disease (D). The association between *RAC1b* overexpression and group II is statistically significant (p-value= 0,0107). A two-tailed Fisher's exact test were used to evaluate statistical significance of the associations. *p≤0.05 **p ≤0.01 ***p≤0.0001.

Table IV.3. Clinical Data.

Patient			Histopathology			Treatment	Follow up				Clinical outcome					Molecular analysis	
ID	Gender	Age at diagnosis (years)	TNM	Pattern	WI/EI/M/A/PD	Surgery/^{131}I	years	last TG (ng/mL)	anti TG	M1	NED	BED	SED	D	Group	RAS (K/N/H)	RAC1b over-expression
1	F	68	T2NxMx	HCC	(-/-/-/+/-)	TT/1	5	<0,2	0	-	√				**I**	(-/-/-)	-
2	F	45	T3NxMx	HCC	(-/-/-/+/-)	HT/0	8	1,7	nt	-	√				**I**	(-/-/-)	-
3	F	40	T3NxMx	FTC	(-/-/-/+/-)	HT/0	7	1,9	nt	-	√				**I**	(-/Q61R/-)	-
4	M	38	T3NxMx	FTC	(-/-/-/-/-)	TT/1	13	<0,2	0	-	√				**I**	(-/-/-)	-
5	F	70	T3NxMx	FTC	(-/-/-/+/-)	TT/2	12	<0,2	0	-	√				**I**	(-/-/-)	-
6	F	63	T3NxMx	FTC	(-/-/-/+/-)	TT/1	7	<0,2	0	-	√				**I**	(-/-/-)	-
7	F	57	T2NxMx	HCC	(-/-/-/-/-)	HT/0	16	8,7	nt	-	√				**I**	(-/-/-)	+
8	F	59	T2NxMx	FTC	(-/-/-/-/-)	TT/1	15	<0,2	0	-	√				**I**	(-/-/-)	+
9	M	38	T2NxMx	FTC	(-/-/-/-/-)	TT/1	11	<0,2	0	-	√				**I**	(-/-/-)	-
10	F	39	T3NxMx	FTC	(+/-/-/+/+)	TT/2	9	<0,2	0	-	√				**I**	(-/-/-)	-
11	F	34	T2NxMx	HCC	(-/-/-/-/-)	HT/0	7	11,1	nt	-	√				**I**	(-/-/-)	-
12	F	39	T2NxMx	FTC	(-/-/-/-/-)	TT/2	7	2,7	nt	-		√			**I**	(-/-/-)	-
13	F	81	T3NxMx	HCC	(-/+/-/-/-)	TT/1	4	<0,2	0	-	√				**I**	(-/-/nt)	-
14	M	39	T3NxMx	FTC	(-/-/-/-/-)	TT/1	7	<0,2	0	-	√				**I**	(-/-/-)	-
15	F	49	TxNxMx	FTC	(-/-/-/+/-)	HT/0	23	<0,2	0	-	√				**I**	nt	-
16	F	65	T2NxMx	FTC	(-/-/-/+/-)	HT/0	6	<0,2	0	-	√				**I**	(-/-/-)	-
17	F	71	T1NxM1	HCC	(+/+/+/+/-)	TT/2	4	300000	0	lung adrenal gland				√	**II**	nt	-
18	F	42	T3NxMx	HCC	(-/-/-/+/+)	TT/6	10	2108	0	lung				√	**II**	(-/-/-)	+
19	M	58	T3NxM1	FTC	(-/-/-/+/-)	TT/4	0	376	0	bone			√		**II**	(-/Q61R/-)	+
20	M	53	T2NxMx	HCC	(+/-/+/+/-)	TT/4	7	632	0	lung				√	**II**	(-/-/-)	+
21	M	69	T2NxM1	FTC	(-/-/-/+/-)	TT/5	2	4668	0	bone, inguinal node				√	**II**	(-/-/-)	+
22	M	64	T4aN1bMx	FTC	(+/+/-/+/+)	TTC/3	2	28,6	0	lung			√		**II**	(-/-/-)	+
23	F	87	T3NxMx	FTC	(+/+/-/+/-)	TT/5	10	43500	nt	soft tissues			√		**II**	(-/Q61L/-)	-

ID, patient identification; **M**, male; **F**, female; **HCC**, Hürthle cells carcinoma; **FTC**, Follicular thyroid carcinoma; **TNM**, Tumour, node, metastasis staging; **WI**, widely invasive; **EI**, extrathyroidal invasion; **M**, Multifocal; **A**, angioinvasion; **PD**, poorly differentiated areas; **^{131}I**, number of ^{131}I treatments; **TT**, Total thyroidectomy; **HT**, Hemithyroidectomy; **TTC**, Total thyroidectomy with cervical dissection; **TG**. Thyroglobulin; **M1**, Distant Metastasis; **NED**, No evidence of disease; **BED**, Biochemical evidence of disease; **SED**, Structural evidence of disease; **D**; Death due to disease;

PART 2

3. RAC1b stimulates NF-κB in K1 cells

In order to evaluate whether RAC1/1b overexpression influences the activation of the transcription factor NF-κB in thyroid biological systems, a luciferase reporter regulated by the canonical NF-κB-consensus motif was tested in the human PTC derived cell line, K1, which does not present measurable RAC1b expression.

Cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or pcDNA3.1 (+)-empty vector (Mock control) in the presence of the NF-κB reporter. The effect of both RAC1/1b wild type forms and constitutively active mutants (L61) were measured and normalize to the Mock control. Differences in NF-κB reporter levels induced by the RAC1 variants were observed: whereas RAC1b-L61 increased luciferase expression by 3,0-fold, RAC1-L61 only induced a 1,6-fold increase. In agreement, also RAC1b-WT and RAC1-WT show different reporter induction capacities, being the first more effective (2,5- versus 1,9-fold increase, respectively). At least three independent experiments were performed and this differences were statistically significant (two-tailed Student's t-test; see Figure IV.8, -A). To further confirm that RAC1/1b were responsible for the stimulation of NF-κB reporter, a RAC1/1b inhibitor was used (EHT 1864), to test whether a reversion on reporter activity was observed. In fact, we observed a decrease in NF-κB reporter activation when RAC1/1b activity was repressed. This decrease was statistically significant in three independent experiments (paired two-tailed Student's t-test), for both RAC1b-L61/RAC1b-WT and RAC1-L61/RAC1-WT (see Figure IV.8, -A).

Transfection efficiency and overexpression of RAC1 variants was monitored by Western Blot (see Figure IV.8, -B) being the GFP-RAC1 and GFP-RAC1b proteins immunodetected as bands of approximately 48 KiloDalton (KDa) and 50 KDa, respectively. Detection of endogenous RAC1 was used as a loading control (approximately 21 KDa).

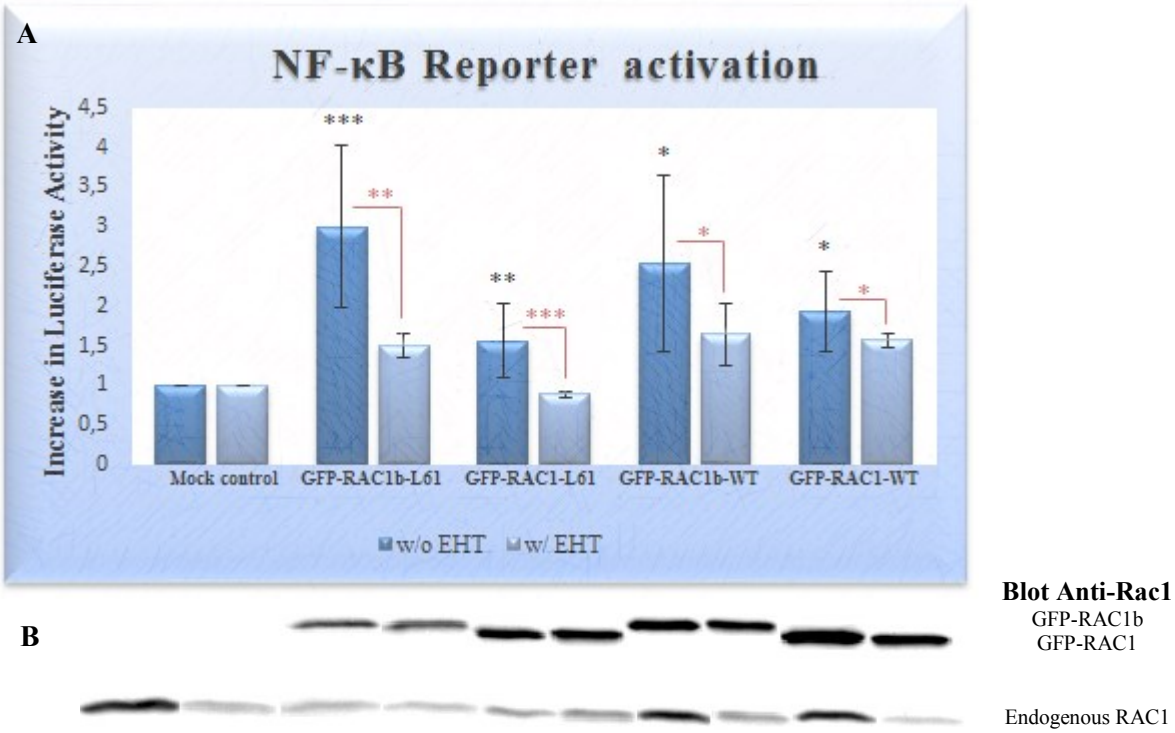


Figure IV.8. Luciferase activity of NF-κB regulated reporter in transfected K1 cells. (A) K1 cells were co-transfected with a luciferase reporter construct driven by the NF-κB consensus motif and with pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or Mock control. (B) A Western Blot analysis was performed to monitor the expression levels of transfected GFP-RAC1/1b. Detection of endogenous RAC1 served as a loading control. Data are mean ± error bars (SD) of at least three independent experiments. p-values were calculated comparing the effect of RAC1/1b with the empty vector, using a two-tailed Student's t-test. *p≤0.05 **p ≤0.01 ***p ≤0.001. A paired two-tailed Student's t-test were used to evaluate the effect of EHT 1864 incubation compared to not treated cells. *p≤0.05 **p ≤0.01 ***p ≤0.001.

4. RAC1b stimulates Cyclin D1 in the K1 cells

We further studied the effect of RAC1b overexpression on Cyclin D1 (a downstream target for NF-κB, which is involved in cell cycle regulation and associated with cancer development under specific conditions). For that, we measured the activity of a luciferase reporter driven by the cyclin D1 promoter in the PTC derived K1 cell line. Similarly to that described in NF-κB reporter assays, cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or pcDNA3.1 (+)-empty vector (Mock control), together with the luciferase reporters. As shown in Figure IV.9, -A, the cyclin D1 promoter was activated by the expression of both RAC1 and RAC1b, compared to Mock control, with the exception of RAC1-WT. Both RAC1-L61 and RAC1b-WT activate cyclin D1 promoter to the same extent (1,2- fold increase), whereas RAC1b-L61-induced activation was much higher (1,8-fold increase), with a statistically highly significant difference in at least three independent experiments. Using, the RAC1 inhibitor EHT 1864, it was possible to observe a reversion on luciferase activity, indicating that cyclin D1 promoter activity decreases when RAC1/1b action is repressed and confirming RAC1/1b regulation of cyclin D1 pathway. Although the obvious reduction on reporter activity levels in cells incubated with EHT 1864, compared to cell not subjected to EHT 1864 action (3,0-; 1,4-; 1,1- and 1,4-fold decrease for RAC1b-L61, RAC1b WT, RAC1-L61 and RAC1-WT,

respectively), just one experiment was performed and, consequently, we were not able to evaluate the statistical significance of this results (see Figure IV.9, -A).

The immunodetection by Western Blot allowed us to confirm the overexpression of all the transfected proteins. Detection of endogenous RAC1 was used as a loading control (see Figure IV.9, -B).

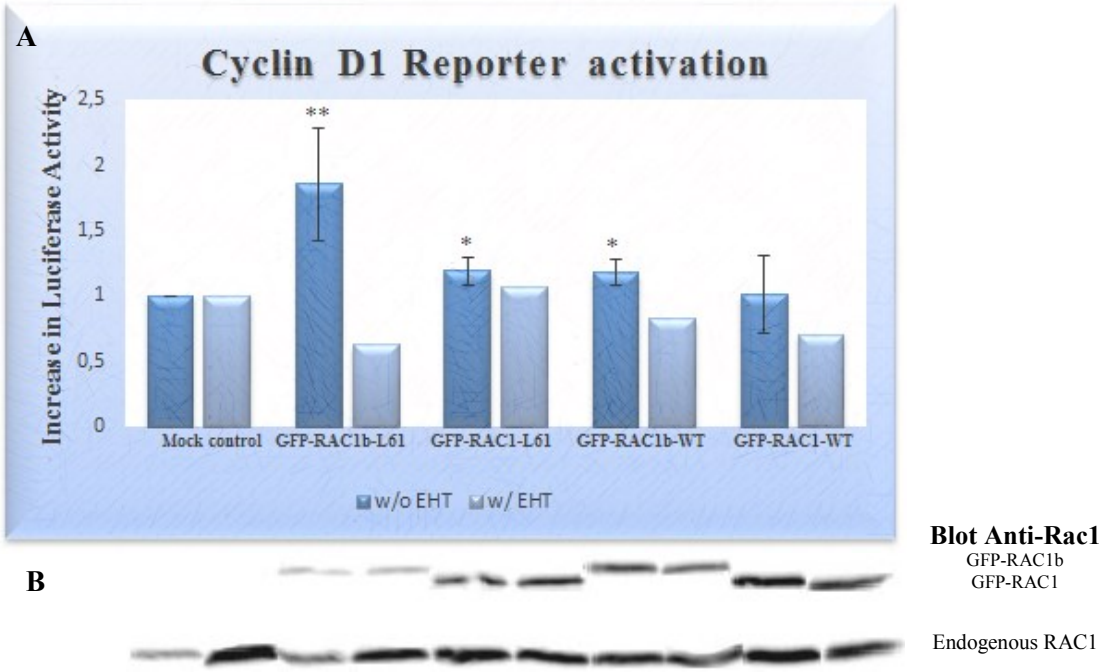


Figure IV.9. Luciferase activity of cyclin D1 regulated reporter in transfected K1 cells. (A) K1 cells were co-transfected with a luciferase reporter construct driven by the cyclin D1 promoter and with pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or Mock control. (B) The immunodetection by Western Blot confirmed the expression of transfected GFP-RAC1/1b. Detection of endogenous RAC1 served as a loading control. Data are mean ± error bars (SD) of at least three independent experiments, with the exception of EHT 1864 experiment, which just one was performed. p-values were calculated comparing the effect of RAC1/1b with the Mock control, using a two-tailed Student's t-test. *p≤0.05 **p ≤0.01 ***p ≤0.001.

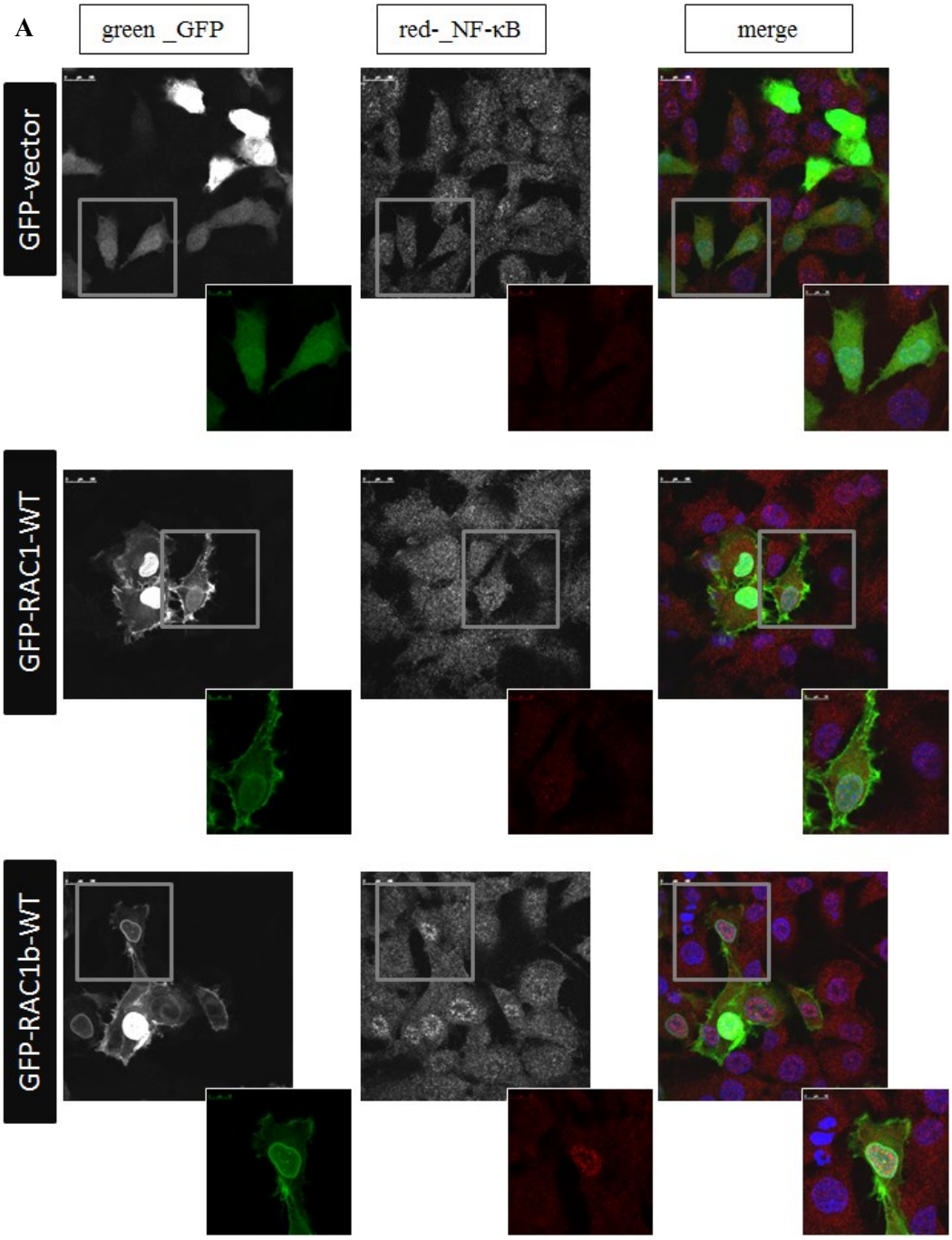
5. RAC1b stimulates p65 translocation to the nucleus

In order to address RAC1b mediated activation of the transcription factor NF-κB *in situ*, we first study the effect of RAC1b overexpression on the subcellular localization of p65 protein (RelA) in our PTC derived cell model. For that, we used an antibody that specifically binds the NLS (nuclear localization signal) region on N-terminal Rel homology domain of p65 protein (approximately 60 KDa). This protein is involved in the canonical NF-κB regulatory pathway and it is able to form p50/p65-heterodimers. IκBα is capable to interact with p65 dimers and inactivates their transcriptional potential by masking the NLS, trapping these dimers into the cytoplasmic compartment. When the NF-κB dimers are activated, the IκBα is marked to degradation which exposes the NLS region, allowing the nuclear translocation and DNA binding. To assess p65 nuclear translocation, we used an antibody that binds the

uncovered NLS region of p65, i.e. the active p65 dimers. We addressed p65 subcellular localization by two methodologies– immunofluorescence microscopy and cell fractionation.

For immunofluorescence experiments, K1 cells were transfected with pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or pEGFP- C1 (Mock control). The discrimination between transfected and non-transfected cells was possible due GFP fluorescence signal (green). Nuclei were counterstained with DAPI (blue). Additionally, cells were immunostained with Anti-p65 NLS (red). As shown in Figure IV.10, -A, red signal was more intense on cells transfected with RAC1b compared to that transfected with only GFP, indicating higher levels of active p65. Moreover, cells transfected with RAC1b-WT revealed a clear nuclear localization of p65, compared to non-transfected or to GFP vector transfected cells. In contrast, RAC1-WT had little effect on the accumulation of p65 in the nucleus, and the red signal intensity was slightly higher than that observed in GFP vector transfected cells, suggesting a modest effect of RAC1-WT on p65 dimers activation. These results are in agreement with the higher stimulation of the NF-κB controlled reporter that we have previously observed for RAC1b.

An additional set of assays with the NF-κB luciferase reporter was performed in parallel with immunofluorescence experiments, in which cells were submitted to the exact same experimental conditions, using a pcDNA3.1 (+)-empty vector as negative control (Mock). As we can see in Figure IV.10, -B, an activation of NF-κB promoter was observed in cells transfected with RAC1b-WT (statistically significant difference compared to Mock control), in opposition with RAC1-WT which showed no effect. These results are consistent with the immufluorescence data.



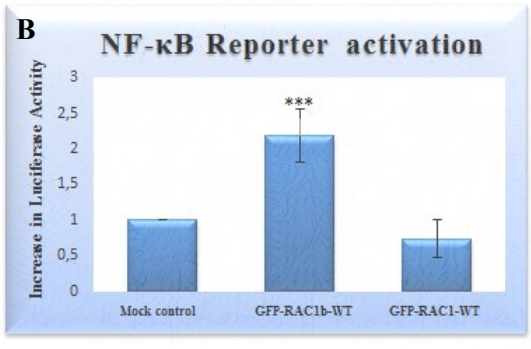


Figure IV.10. RAC1b overexpression affects subcellular location of p65-NLS protein. (A) K1 cells were transfected with pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or Mock control. Cells were submitted to starvation conditions, and stimulated with higher FBS levels before fixation. Nuclei were counterstained with DAPI, and fluorescent signals were recorded by confocal microscopy. The overlay image of the DAPI, GFP and Red channels is shown. (B) Luciferase activity of NF-κB regulated reporter was measured in K1 cells submitted to the exact same conditions that cells analyse by immunofluorescence. Data are mean ± error bars (SD) of four independent experiments. p-values were calculated comparing the effect of RAC1/1b with the Mock control, using a two-tailed Student's t-test. *p≤0.05 **p ≤0.01 ***p ≤0.001.

To further confirm the data obtained with the immufluorescence microscopy approach, we assessed the effect of RAC1b on the subcellular localization of p65 protein by subcellular fractionation experiments. K1 cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or pcDNA3.1 (+)-empty vector (Mock control). As we can see on Figure IV.10.1, in cells transfected with RAC1b-L61 and RAC1b-WT we observed an increase on p65-NLS protein expression in the nuclear fraction, compared to Mock control. These data indicate that RAC1b expression activates p65 dimers in higher extent than RAC1-WT or Mock control, inducing nuclear translocation. Transfection of RAC1/1b variants was monitored by Western Blot. Histone H2B (approximately 15KDa) and β-Actin were used as a markers of nuclear and cytoplasmic fractions, respectively. Although this analysis revealed some cross-contamination between nuclear and cytoplasmic fractions, marker's expression was similar in all samples. This allows us to consider these results as an indicative that RAC1b induces p65 activation and nuclear translocation, which is in agreement with the results of NF-κB reporter experiments. Altogether, these data reinforce the ability of RAC1b to activate the NF-κB canonical pathway, inducing the translocation of p65 dimers into the nucleus.

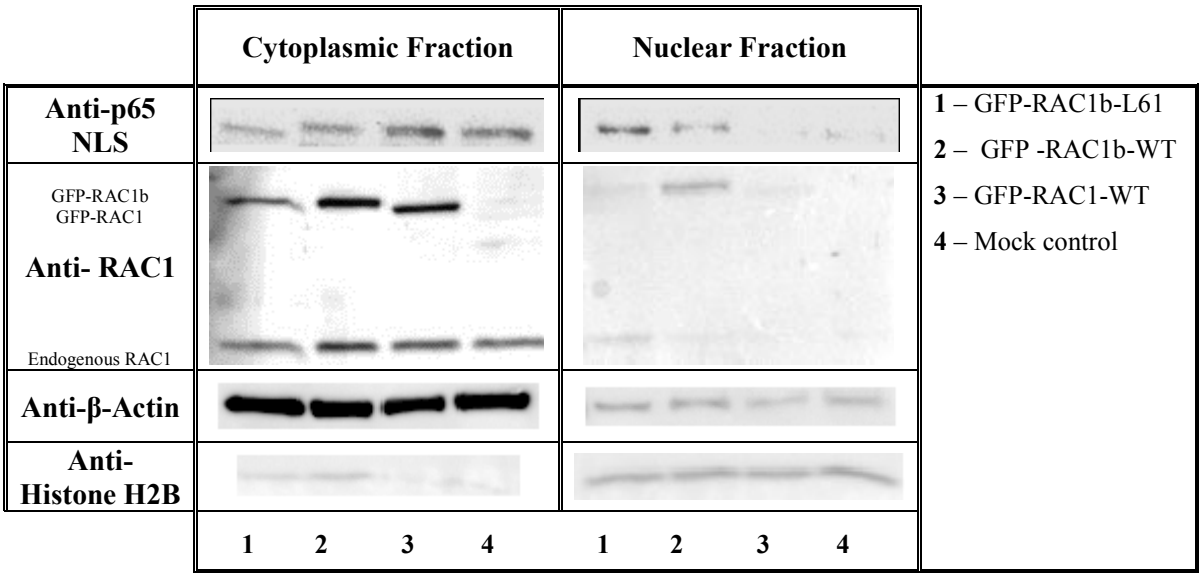


Figure IV.10.1. RAC1b overexpression affects subcellular location of p65-NLS protein. K1 cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or Mock control. Cells were submitted to starvation conditions, and stimulated with higher FBS levels before the lysis process. A cell fractionation protocol were use so that a nuclear and a cytoplasmic fraction was obtained. A Western Blot analysis of these fractions were performed. Histone H2B was detected as a marker for nuclear proteins fraction, and β-Actin was detected as a marker for cytoplasmic fraction.

6. RAC1b induces a decrease in total IκBα protein expression

To further confirm the RAC1b impact on the activation of the canonical NF-κB pathway, we study the influence of RAC1b on total IκBα protein expression (a negative regulator in the canonical NF-κB pathway). K1 cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1b-WT, or pcDNA3.1 (+)-empty vector (Mock control). Levels of total IκBα were assessed by Western Blot (see Figure IV.11, -A) and posteriorly analyse by densiometry using ImageJ software (see Figure IV.11, -B). These levels

were first normalize to β-actin (used as endogenous control) and then normalized to Mock control. As shown in Figure IV.11, both RAC1b-L61 and RAC1b-WT induced a decreased in total IκBα levels, compared to the Mock control. These decreases were statistically significant.

Since IκBα is a potent inhibitor of p50-p65 dimers, the decreased levels of this protein in the cell is likely related to higher activation levels of NF-κB transcription factor. These data corroborate the effect of RAC1b overexpression in the stimulation of the canonical NF-κB pathway.

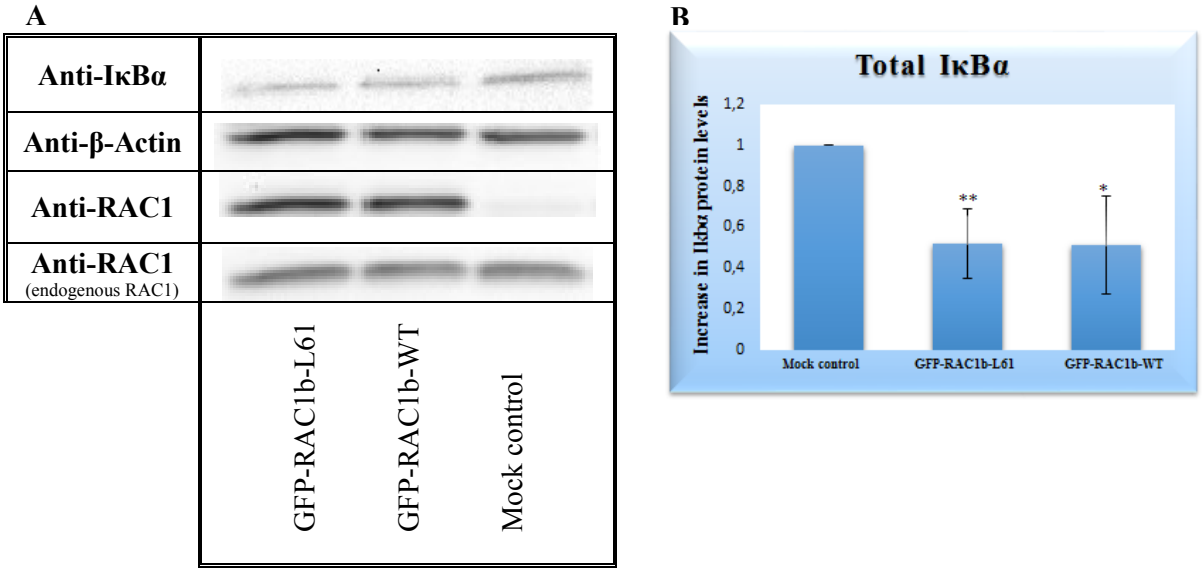


Figure IV.11. Total IκBα protein levels in K1 cells assessed by western-blotting. (A) K1 cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1b-WT, or Mock control. IκBα protein levels were analysed by Western Blot. RAC1 analysis by Western Blot were performed to monitor expression levels of transfected GFP-RAC1b. Detection of endogenous RAC1 can also serve as a loading control. (B) Protein levels were analysed by densiometry using ImageJ software. These levels were first normalized to β-actin (≈ 42KDa), and then normalized to Mock control. p-values were calculated comparing the effect of RAC1b with the Mock control, using a two-tailed Student's t-test. *p≤0.05 **p ≤0.01 ***p ≤0.001.

7. RAC1b protects cells against apoptosis induced by staurosporine

In this work, we were also able to gain some insights into how RAC1b-induced NF-κB activation in thyroid cells modify cellular processes related to tumour development such as apoptosis. K1 cells were transfected with pEGFP-RAC1b-L61 or pEGFP-C1 (Mock control). Then, cells were subjected to different concentrations of staurosporine, a compound that induces apoptotic cell death (Belmokhtar *et al.,* 2001).

The data acquired in FACScalibur was processed using the FlowJo software so that only transfected cells were considered for analysis and quantification of the levels of apoptosis – the distinction between transfected and non-transfected cells was possible due GFP signal also detected by FACScalibur. The results were normalized to Mock, RAC1b-L61 and RAC1b-L61 plus IκBaa controls without staurosporine, respectively. As shown on Figure IV.12, we observed an increase in apoptosis when cells were treated with increasing amounts of staurosporine, with the exception of cells transfected with

RAC1b-L61, for which staurosporine was not able to induce apoptosis. It is important to highlight the decrease on apoptosis observed in cells transfected with RAC1b, compared to Mock control (1,9-fold decrease, for 5 nM staurosporine), for the same staurosporine concentrations. Nevertheless, this decrease was only statistically significant for the lowest concentration of staurosporine (5 nM) (see Figure IV.12). Moreover, in cells expressing the IκBaa super-repressor (the mutated non-degradable IκBαA32A36) which traps the p50/p65 dimers on the inactive form, we were able to observe a reversion of the effect of RAC1b on apoptosis inhibition, indicating that this effect is NF-κB-dependent.

Altogether, these data suggest that RAC1b is able to protect cells against apoptosis induced by staurosporine, in a process involving the NF-κB pathway. Nevertheless, further assays are needed to confirm and increase the statistical significance of these results.

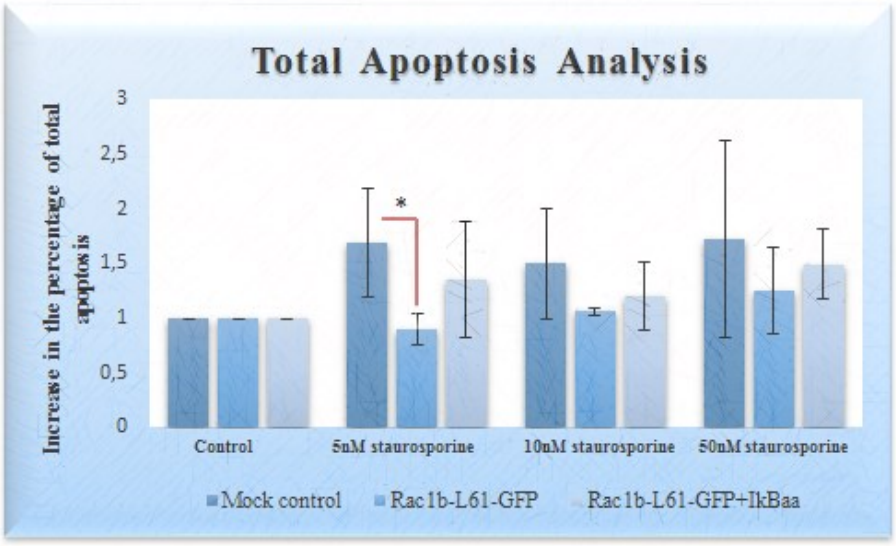


Figure IV.12. Apoptosis analyzes by flow cytometry (Annexin V and PI staining). K1 cells were transfected with pEGFP-RAC1b-L61, or Mock control. For study if RAC1b effect was influenced by the NF-κB canonical pathway, cells were also transfected with pcDNA3-HA-IκBα (A32A36). The percentage of total apoptosis (early and late stages) was normalize to Mock, RAC1b-L61 and RAC1b-L61+IκBaa controls, without staurosporine. Data are means ± error bars (SD) of four individual experiments. p-values were calculated comparing the effect of RAC1b with the Mock control, using a two-tailed Student's t-test. *p≤0.05 **p ≤0.01 ***p ≤0.001.

8. RAC1b stimulates cell cycle progression

In view of the effect of RAC1b overexpression on cyclin D1 promoter observed in luciferase reporter assays, we decided to analyse RAC1b effect on cell cycle progression, by flow cytometry. K1 cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1b-WT or pEGFP-C1 vector (Mock control). Only transfected cells were considered in the present analysis. As shown in Figure IV.13, RAC1b-L61 was able to increase the proportion of cells in the S and G2 phases, compared to the Mock control (phase S: 12,0% and 9,9%, respectively; phase G2: 13,6% and 10,2%, respectively), indicating a progression in cell cycle. Also RAC1b-WT was able to increase the proportion of cells in the S phase,

compared to the Mock control (13,3% and 9,9%, respectively). Lower percentage of cells transfected with RAC1b-WT were found in G2 phase, compared to RAC1b-L61 (7,2% and 13,6% respectively). Moreover, in cells co-transfected with the IκBaa super-repressor we observed a reversion on the RAC1b-L61 effect. These data led us to hypothesize that RAC1b-L61 is able to induce cell cycle progression and increase proliferative activity, in a process involving the NF-κB pathway. Wild type RAC1b also induced cell progression from G1 phase to S phase although its effect on cell cycle progression (S and G2/M phases) was not obviously reversed by the IκBaa super-repressor. Only two experiments were performed, so further assays are needed to confirm these results and increase its statistical significance.

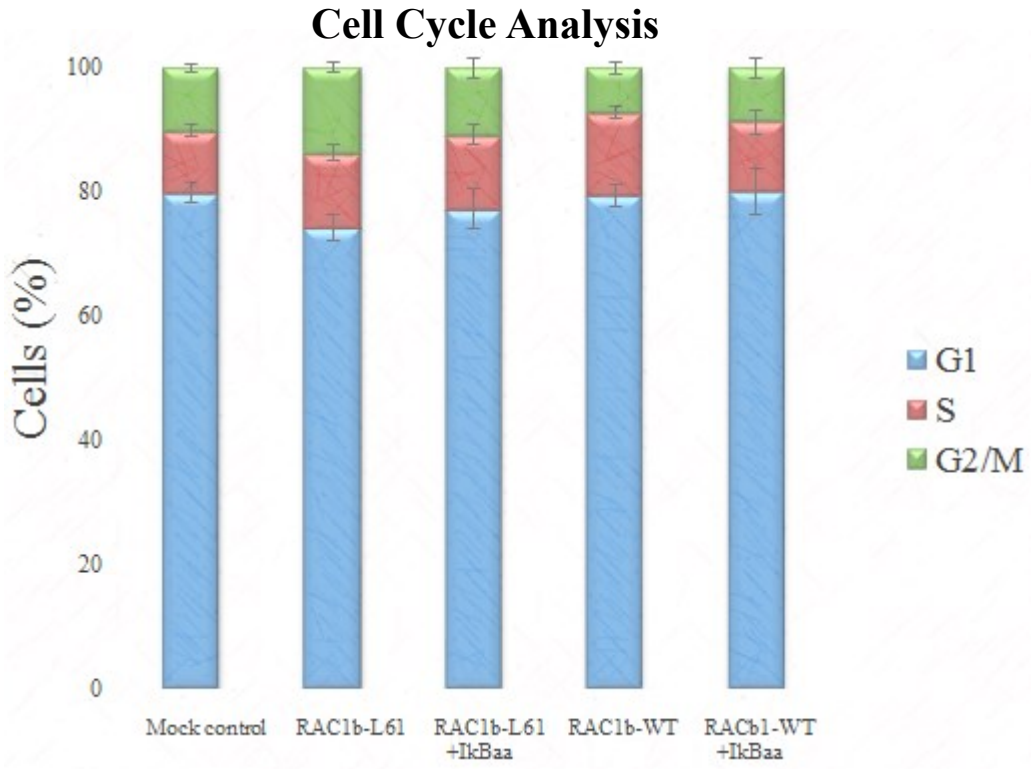


Figure 13. Cell cycle analyzes by flow cytometric (PI staining). K1 cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1b-WT, or Mock control. To study whether RAC1b effect was related to NF-κB, cells were co-transfected with pcDNA3-HA-IκBα (A32A36). The percentage of cell in each phase of cell cycle is represented. Data are means ± error bars (SD) of two individual experiments.

9. RAC1b effect on Nthy cell line

In order to address whether the effect of RAC1b overexpression on NF-κB pathway is present in thyroid biological systems other than the K1 PTC derived cell line, we evaluated this effect on the human normal follicular thyroid epithelium derived cell line, Nthy. Cells were transfected with pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, pcDNA3.1 (+)-empty vector (Mock control). We use the luciferase reporter systems for NF-κB and cyclin D1 as described above for K1 cells.

The effect of RAC1 and RAC1b was measured and normalized to the Mock control. Both RAC1 and RAC1b, either the WT or the active dominant L61, induced a statistically significant increase in the activity of NF-κB reporter (results shown in Figure IV.14, -A). Notably, in this system, the effect of RAC1 was remarkably more pronounced compared to its splice variant RAC1b: 10,0- versus 2,3- fold increase for active dominants; 2,3- versus 1,8- fold increase for WT forms, respectively. Results obtained in Nthy confirm the ability of RAC1b to induce NF-κB reporter activity, similar to that found in K1 cells. Nevertheless in this cell type, RAC1 has found to have a considerable more intense effect on NF-κB reporter activation than that observed for RAC1b.

We also evaluate the effect of both variants in the activation of cyclin D1 promoter activity. Both RAC1 and RAC1b were shown to increase the promoter activity, compared to the Mock control (see Figure IV.14, -C). Compared to K1, the extent of this effect was lower and only RAC1 was able to induce a statistically significant increase (2,8- and 1,6- fold increase for L61 and WT form of RAC1, respectively). RAC1b induced a 1,4- (L61) and 1,3- (WT) fold increase in cyclin D1 promoter activity.

A Western Blot analysis was performed to monitor the expression levels of transfected GFP-RAC1/1b proteins (Figure IV.14, -B-D).

Unfortunately we were unable to go further on this study due to time constraints. Nevertheless the data obtained are consistent with the effect of RAC1b overexpression on the stimulation of NF-κB pathway, being this effect also able to occur in a thyroid cell system derived from normal tissue.

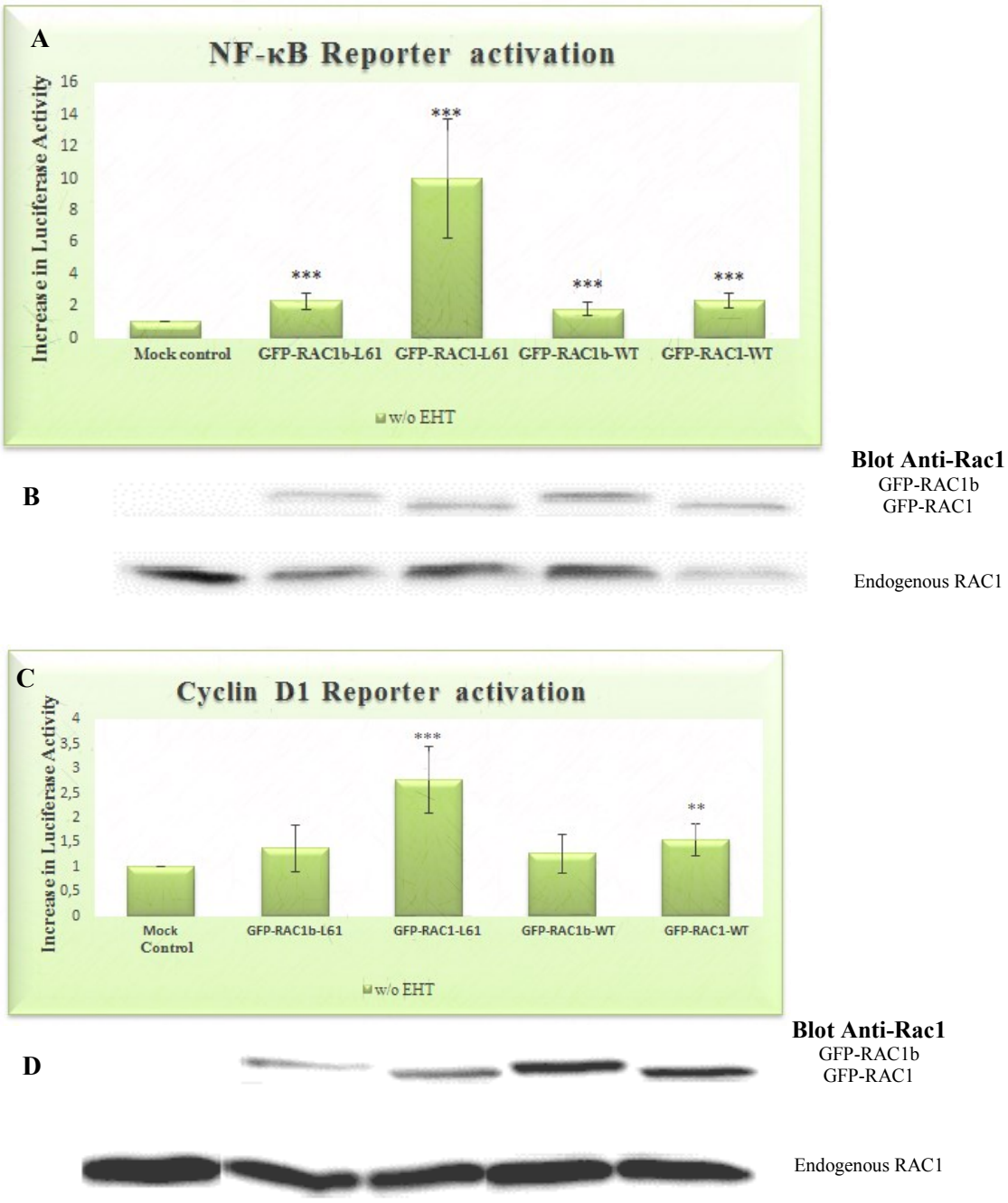


Figure IV.14. Luciferase activity of NF-κB and cyclin D1 regulated reporter in transfected Nthy cells. Nthy cells were co-transfected with a luciferase reporter construct driven by the NF-κB consensus motif (A) or driven by the cyclin D1 promoter (B) and with pEGFP-RAC1b-L61, pEGFP-RAC1-L61, pEGFP-RAC1b-WT, pEGFP-RAC1-WT, or Mock control. (B, D) A Western Blot analysis were performed to monitor expression levels of transfected GFP-RAC1/1b. Detection of endogenous RAC1 served as a loading control. Data are mean ± error bars (SD) of at least three independent experiments. p-values were calculated comparing the effect of RAC1/1b with the Mock control, using a two-tailed Student's t-test. *p≤0.05 **p ≤0.01 ***p ≤0.001.

V. DISCUSSION

Cancer is a disease that may arise from all different types of cell in the body, resulting from abnormal proliferation of these cells (Cooper, G., 2000). After years of rapid advances, cancer research has defined a group of underlying principles that are shared by all cancers – hallmarks of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Nevertheless, there are colossal differences between different types of cancer in terms of biological and molecular behaviour. Moreover, phenotypic and functional heterogeneity can arise among cancer cells within the same tumour (Cooper, G., 2000; Meacham and Morrison; 2013). During many years, the clinical behaviour of human cancer has been predicted based on histological features. However, different biological evolution and clinical responses were observed for the same histological type of tumours (Liotta and Petricoin, 2000).

The pursuit for new ways to characterize each tumour as a particular entity, tailoring case management to individual risk level, has been persistent. One widely used approach is related to genetic alterations linked to several types of cancer. Genetic analysis has been performed in order to study how mutations identified in cancer-critical genes can affected crucial pathways, and consequently modify tumour cell properties and behaviour (Alberts *et al.,* 2002). Several proteins have also been associated with cancer development, namely Rho-family proteins, interfering with cancer-related pathways and cellular features (Sahai and Marshall, 2002).

Recent findings of our group point to an important role of RAC1b, a small GTPase belonging to Rho-family, in PTC development and definition of poor clinical outcomes (Silva *et al.,* 2013). This finding made us to consider the hypothesis that RAC1b may be involved in tumorigenesis of other thyroid tumour subtypes, namely in other tumours derived from follicular cells besides PTCs. One major finding in this thesis work is that *RAC1b* is overexpressed in FTCs (30%) compared to FTAs (0%), being this difference statistically significant. We also found a striking correlation between poorer clinical outcome or the presence of distant metastasis and *RAC1b* overexpression (p= 0,01 for both associations). Altogether, these data suggest that RAC1b is involved in FTC development, similar to that previously found in PTCs. Further validation is needed to confirm the potential of this GTPase as a biomarker of clinical outcome for FTC patients.

Moreover, the dissimilarity found in *RAC1b* overexpression between FTCs and FTAs strengthens the potential of RAC1b as a marker for discrimination between benign and malignant follicular lesions. In fact, the distinction between benign and malignant tumours continues to be an important issue in cancer pathology (Cooper, 2000). In thyroid follicular neoplasia, this problem earns bigger proportions since surgery is the only reliable way to distinguish FTCs from FTAs. Many studies have been performed in order to find pre-surgical discrimination markers. *HMGA2* and *hTETR*, for instance, despite being differential expressed between FTCs and FTAs, were shown not to be consistently able to differentiate benign from malignant lesions (Nagar *et al.,* 2014.) Also, RAC1b may not be used as a single marker of discrimination, because of its low sensitivity levels (only 30% of FTCs were found to

overexpress *RAC1b*). Nevertheless, we can conceive that it might potentially incorporate a more extensive panel of biomarkers, allowing the distinction from benign vs. malignant thyroid lesions with reliable sensitivity and specificity levels, in a preoperative stage. The optimal outcome would be a long term adaptation in standard therapy, in which surgery would only be necessary for confirmed FTC cases.

In a previous study of our group, it was shown that *RAC1b* is expressed in thyroid tissue and that is overexpressed in a subset of PTCs associated with poorer outcomes. Nevertheless, the molecular pathways through which this GTPase participates in thyroid tumorigenesis requires further investigation. In this thesis project, we proposed the investigation of the molecular mechanisms associated with RAC1b overexpression and downstream signalling in thyroid tumorigenesis. High-throughput screening methodologies are presently the state of the art approaches for providing starting points about gene expression changes that could be associated with cancer development, as well as the signalling pathways implicated (Alberts *et al.,* 2002; Gombos *et al.,* 2007). However, there are some signalling pathways reported to be involved in several types of tumours, such as p53, JNK, PI3K, RTK, cyclin D1 or NF-κB – the "usual suspects" to be involved in cancer development – that may be worth considering (Debatin, 2004; Dhillon *et al.,* 2007).

In this project, we choose to study the transcription factor NF-κB, which seems to be implicated in several types of cancer. The activation of this transcription factor by RAC1 signalling has been documented over the years and this activation process seems to involve ROS production, and promote tumorigenesis by increasing the expression of cyclin D1 (Hinz *et al.,* 1999; Joyce *et al.,* 1999; Matos and Jordan, 2006), matrix metalloproteinases (Kheradmand *et al.,* 1998) and anti-apoptotic genes (Karin *et al.,* 2002; Shishodia and Aggarwal, 2002). Back to 2006, RAC1b was also shown to be a potent activator of NF-κB canonical pathway in colorectal cancer biological system (Matos and Jordan, 2006). However, whether the RAC1b-mediated activation of NF-κB is relevant in thyroid cancer remained to be determined.

Another important finding in this work is that RAC1b overexpression is able to induce the activation of the canonical NF-κB activating pathway and cyclin D1 in thyroid biological system. Also, the activation of this pathway in thyroid seems to have implications on the regulation of crucial cellular processes such as apoptosis and cell cycle progression. The effect, described here in thyroid, of RAC1/1b on the activation of the NF-κB canonical pathway and cyclin D1 transcription is consistent with data reported by Matos and Jordan (2006) in colorectal cancer, demonstrating the role of RAC1b as a potent activator of NF-κB pathway. However, while in colorectal cancer both RAC1 and RAC1b stimulate the cyclin D1 reporter to an equivalent extent and RAC1 appeared to be more effective than RAC1b in activating the NF-κB reporter, in the cell line derived from a human papillary thyroid carcinoma (K1) RAC1b was shown to induce a substantially greater effect than RAC1. These results point to differences in the RAC1 and RAC1b mechanism of regulation between thyroid cancer cells and colorectal cancer cells. Likewise, differences in these same mechanisms were also observed between thyroid cancer cells and cells derived from human normal follicular thyroid epithelium (Nthy).

In order to further confirm RAC1/1b-mediated activation of NF-κB transcription factor, considering the canonical regulatory pathway in particular, we evaluated the subcellular localization of p65 – a Rel protein involved in the canonical pathway. We detected higher levels of active p65 and a clear nuclear localization of this protein in cells transfected with RAC1b-WT compared to the Mock control. These data corroborate the effect of RAC1b on the activation of NF-κB canonical pathway, since P50-p65 dimers, are only capable to enter the nucleus when they are on the active state (Karin and Ben-Neriah, 2000; Karin *et al.,* 2002). Additionally, we evaluated the effect of RAC1b expression on the levels of endogenous IκBα, a NF-κB inhibitor with a crucial role in the canonical pathway regulation. Our results show that the endogenous IκBα protein levels were halved by both wild type and constitutively active RAC1b. These data are in agreement with Matos and Jordan (2006) results that showed a RAC1b-induced decrease on endogenous IκBα levels on colorectal cancer.

Then, we aimed to address the consequences of the RAC1b-induced NF-κB activation on thyroid cellular processes. The capacity of NF-κB to block TNF-induced apoptosis has been shown many years ago (Liu *et al.,* 1996). Actually, the anti-apoptotic effect of NF-κB is well accepted, which could protect cells from various apoptotic agents. The NF-κB transcription factor promotes cell survival by either inducing the transcription of anti-apoptotic genes or downregulating the activity of apoptotic genes (Karin *et al.,* 2002; Shishodia and Aggarwal, 2002). This supports our data showing a RAC1b-induced protection against the effect of staurosporine, a strong inducer of apoptosis in many different cell types, through a mechanism mediated by NF-κB transcription factor.

In this thesis work we demonstrated the ability of RAC1/1b to activate cyclin D1 reporter activity. Given the role of cyclin D1 as a key regulator of cell cycle (Baldin *et al.,* 1993; Stacey, 2003; Fu *et al.,* 2004), we decided to evaluate the effect of RAC1b in cell cycle progression in thyroid cells. Our results indicated that RAC1b-L61 induced a cell cycle progression through a mechanism influenced by NF-κB transcription factor, which is supported by evidences that cyclin D1 can be induced by NF-κB activation (Hinz *et al.,* 1999; Joyce *et al.,* 1999; Fu *et al.,* 2004). Also RAC1b-WT seems to stimulate NF-κB-mediated G1/S progression, which enhances the RAC1b ability to act as a pro-proliferative signal. The ability of RAC1b, both the wild type and the constitutively active mutant, induce G1/S progression is in agreement with Matos and Jordan (2005) findings that show RAC1b expression to be sufficient to stimulate G1/S progression via the activation of NF-κB. Furthermore, the lower percentage of cells transfected with RAC1b-WT found in G2 phase, compared to RAC1b-L61, is in agreement with the data obtained in reporter assays, in which RAC1b-L61 revealed to induce a higher increase in cyclin D1 promoter activity, compared to RAC1b-WT, since at G2 phase cyclin D1 levels are usually elevated (Stacey, 2003; Yang, 2006).

VI. CONCLUSION

In this thesis work, we tried to clarify the role of RAC1b in follicular cell derived thyroid tumorigenesis and progression. Our finding about the association of *RAC1b* overexpression with a worse prognosis and presence of distant metastases in FTC patients emphasizes the role of this GTPase as a prognostic marker for follicular cell derived thyroid cancers.

Our data supports the potential of RAC1b to be incorporated into a wide panel of biomarkers, which could have important implications in the diagnosis of follicular thyroid carcinoma, and help to circumvent the problem of discrimination between FTCs and FTAs.

Given the particular features of each tumour, the clarification of the molecular mechanisms behind neoplasia development and progression can be pivotal. Many genetic alterations and altered signalling pathways are known to be recurrently associated with several types of tumours.

The role of NF-κB signalling in thyroid cancer cell proliferation, invasion, and apoptosis has been reported. Our data provide a link between RAC1b overexpression and NF-κB signalling in thyroid cancer and address the consequences of this RAC1b-induced NF-κB stimulation in thyroid cellular processes, highlighting the RAC1b ability to interfere with apoptosis and cell cycle progression, via NF-κB activation.

In sum, this work contributed to the characterization of a pathway apparently involved in thyroid tumorigenesis, which may constitute a progress in the search for new therapeutic targets in thyroid carcinomas.

VII. PERSPECTIVES

Future studies are needed to clarify the oncogenic potential of RAC1b in follicular cell derived thyroid carcinoma tumorigenesis, as well as in the assessment of differentially altered pathways between FTCs and FTAs, due to overexpression of *Rac1b* found in the malignant lesions.

To better understand the cellular alterations caused by RAC1b-induced NF-κB activation, in the future, it would be important to further analyse cell cycle progression by flow cytometry in order to confirm the statistical significance of our preliminary data. Moreover, other cellular functions, such as migration, could be evaluated to better elucidate the cellular processes affected by RAC1b overexpression in thyroid biological systems. In fact, testing different *in vitro* thyroid models could help us to confirm and better understand the differences in the mechanisms behind RAC1/1b role in thyroid cancer cells compared to thyroid normal cells.

The evaluation of the levels of activation of the NF-κB transcription factor in patient tumour samples with *Rac1b* overexpression would be helpful to corroborate the ability of this GTPase to stimulate the NF-κB pathway in thyroid tissue, in an *in vivo* setting. An immunohistochemistry approach can enable us to assess, in paraffin preserved tissues, the differences on subcellular localization of p65 protein on tumour vs normal cells, as well as between tumour cells with and without *RAC1b* overexpression.

Recently acquired knowledge in the cancer field revealed that each specific type of neoplasia may be dependent on more than one altered signalling pathway. On that basis, the search for new pathways that might be affected by Rac1b expression and cooperate with the NF-κB altered pathway to modify cellular processes associated with tumour progression, may be decisive.

The use of high-throughput screening methodologies to identify new potential targets of RAC1b is a viable possibility. This approach can also be used in order to identify pathways which can be affected by the potential synergy between RAC1b and $BRAF^{V600E}$ mutation, an association suggested by previous studies in colorectal cancer and also by our group in thyroid (Matos *et al.,* 2008; Silva *et al.,* 2013).

Overall, we hope that this work could contribute to the development of new treatments that target multiple pathways, for patients with follicular cell derived thyroid cancers.

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APPENDICES

APPENDIX 1 – Solutions prepared for the experimental work:

Table 4. 10x PCR buffer.

Reagents	Final Concentration
Tris-HCl	100 mM
KCl	500 mM
$MgCl_2$	15 mM
Gelatin	0,1% (w/v)

Table 5. RIPA buffer.

Reagents	Final Concentration
Tris-HCl	20 mM
NaCl	150 mM
KCl	5 mM
$MgCl_2$	5 mM
Triton X-100	1% (v/v)
Complete, Mini, EDTA-free Protease Inhibitor Cocktail	1 tablet per 10 mL solution
pH 7,5	

Table 6. 5x Sample buffer.

Reagents	Final Concentration
Tris-HCl	200 mM
SDS	10% (w/v)
DTT	325 mM
Glycerol	25% (v/v)
Bromophenol blue	0,05% (w/v)
pH 6,8	

Table 7. Blot buffer.

Reagents	Final Concentration
Tris	25 mM
Glycine	192 mM
SDS	0,03% (w/v)
Methanol	5% (v/v)
pH 7,6	

Table 8. Destain solution.

Reagents	Final Concentration
Acetic acid	10% (v/v)
Methanol	45% (v/v)

Table 9. TBST solution.

Reagents	Final Concentration
Tris-HCl	50 mM
NaCl	150 mM
Triton X-100	15 mM
pH 7,6	

Table 10. ECL solutions.

Reagents (Solution 1)	**Final Concentration**	**Reagents** (Solution 2)	**Final Concentration**
Tris pH 8,8	100 mM	**Tris pH 8,8**	100 mM
Luminol	3,75 mM	**Hydrogen Peroxide**	0.1% (v/v)
Cumaric acid	450 µM	Solution 1 and Solution 2 are mixed immediately before use.	

Table 11. Annexin binding buffer 1x.

Reagents	Final Concentration
HEPES	0,01 M
NaCl	0,14 M
$CaCl_2$	2,5 mM

Table 12. PI solution cell cycle assay.

Reagents	Final Concentration
PI	50 µg/mL
RNase	0,1 mg/mL
Triton X-100	0,05% (v/v)